University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS)

Role of proteinase-activated receptor-2 in central mediated behaviour

Roua Abulkassim

A thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy

March, 2014

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: Date:

Dedication

I would like to dedicate my thesis to my husband

Mr Reza Shiehzadeh

Acknowledgements

I also would like to dedicate my thesis to the soul of my father and youngest brother 'Mohamed Hussan'. I am indebted to my mum, sisters and brothers for their love and affection and constant encouragement.

I owe my deepest gratitude to my supervisors T. Bushell and R. Brett for their support during my entire course of the present research program. I am indebted to them for their immense co-operation and guidance.

I would also like to thank Prof. R. Plevin, very especially, Roth Tate for his support in cell biology lab.

Thanks To Mark Thompson for demonstrating the prepulse inhibition and T-maze continuous alternation tests behavioural apparatus and advising on data analysis. Carol and all other members of BPU for taking care of the animals and for being immensely helpful.

Thank my writing up-room Sukut, Sajjad Ali Khan, Zaihana, Majed, Molly and Dr.Ibrahim Khadra for their help and co-operation, as well as making my time at Strathclyde enjoyable and memorable.

I would like to acknowledge, with great pleasure, the help of Sajjad Ali Khan, Farzana Khaliq, Mark Thompson and Jennifer Shearer with proof reading.

Special thanks to my close friends Jehan Alestad, Marwah Ismael, Salha Altowati, Ruwida, Shetha A-Ostad, Ikram Alsead, Ahlam Altowati, Eman Santali, Ghazzwa Almozany and Dima Semaan, for their constant support and help throughout my stay here in Glasgow.

Special thanks to Iraqi government for fund support for this study special thank for for Zahair for his support.

Thank you all.

Roua Abulkassim, February, 2014

Abstract

Proteinase-activated receptors (PARs) are a family of novel G protein-coupled receptors, which are widely expressed in the brain. It has been recently shown that PAR2 activation indirectly modulates hippocampal neuronal excitability and synaptic transmission *in vitro*, and treatment with the PAR2 agonist SLIGRL-NH_2 induced deficits in tests of motivational learning in rats. Hence, in this study we have investigated whether PAR2 deletion affects mouse behaviour in tests of learning and emotional behaviours under physiological and pathological conditions.

Deletion of PAR2 had no effect on locomotor activity in the open field test. Heterozygous males appeared more anxious in the open field test but knockout females exhibited less anxiety in the elevated plus maze. PAR2 deletion had no effect on spatial reference memory in the Morris water maze but reduced mean percent alternation to chance level in the T-maze continuous alternation test and produced deficits in the male mice in the novel object recognition test. Deletion of PAR2 did not affect general startle reactivity and sensorimotor gating but it decreased the startle response at the highest stimuli in females.

In a sickness behaviour model, deletion of PAR2 delayed induction of anhedonia as measured in the sucrose preference test after injection of lipopolysaccharide. It also induced a more rapid recovery from other symptoms of sickness behaviour as shown by increased locomotor activity 24 and 48 h post injection, increased body weight at 48 and 72 h post injection, increased food intake during the second day post injection and reduced anxiety-like behaviour 24 h post injection.

The novel PAR2 agonist AC-264613 penetrated into mouse brain. AC-264613 had no effects on locomotor activity and anxiety-like behaviour but showed a tendency to induce anhedonia.

In conclusion, these data indicate that proteinase-activated receptor-2 may be involved in mouse behaviour under normal and pathological conditions.

Table of contents

List of figures

List of tables

List of abbreviations

AC	Adenylate cyclase
ACh	Acetylcholine
AIC	Anti-inflammatory cytokines
AP	Activating peptide
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BPU	Biological Procedures Unit
Ca^{2+}	Calcium
cAMP	Cyclic adenosine 3',5'-monophosphate
CG	Control group
CNS	Central nervous system
DAG	Diacylglycerol
dB	Decibel
EPM	Elevated plus maze
ER	Endoplasmic reticulum
ERK	Extracellular-regulated kinase
GABA	γ-aminobutyric acid
GAERS	Genetic absence epilepsy rat from Strasbourg
GM	Genetic modified
GPCR	G protein-coupled receptor
h	Hour
HT	Heterozygous
i.p.	Intraperitoneal
IL	Interleukin
IP_3	Inositol (1,4,5) triphosphate
JNKs	c-Jun N-terminal kinases
KO	Knockout
LC-MS	Liquid chromatography- mass spectrometry
LPS	Lipopolysaccharide
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
min	Minutes
MS	Mass spectrometry
MWM	Morris water maze
n.s.	Non-significant

NOR	Novel object recognition
OF	Open field
OFT	Open field test
PAR	Proteinase-activated receptor
PCR	Polymerase chain reaction
PIC	Pro-inflammatory cytokines
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPI	Prepulse inhibition
RhoGEFs	Rho guanine nucleotide exchange factors
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
s.c.	Subcutanouse injection
SPT	Sucrose preference test
SR	Startle reactivity
T-CAT	T-Maze continuous alternation test
TLR-4	Toll-like receptor-4
TNF-α	Tumour necrosis factor-α
VG	Vehicle group
WT	Wildtype
%	Percent

General introduction

1.1 G protein-coupled receptors

A receptor is a cellular macromolecule found either on the cell surface or within a cell that recognizes and binds with specific molecules, producing a specific effect in the cell (Congreve *et al.,* 2010). Receptors are distributed over all the body with cell surface receptor families being classified into three groups, ligand-gated ion channel receptors, G protein-coupled receptors (GPCRs), and enzyme linked receptors (Alberts *et al.,* 2002).

GPCRs constitute the largest family of plasma membrane receptors with well over 1000 human genes identified (Kawasawa *et al.,* 2003). GPCRs are made up of a single polypeptide chain of about 1100 residues and they have been shown to play a key role in physiological processes and pathological conditions (Seifert and Wenzel-Seifert, 2002). They have been shown to be involved in a diverse range of physiological processes including perception of light, pain, taste, smell, digestion, cardiovascular regulation, and are regulators of the immune system (Lattin *et al.,*2007). In addition, GPCRs are associated with pathological conditions including diabetes, cancer, neurodegenerative diseases and inflammatory diseases (Sun *et al.,* 2011). The importance of GPCR function has made them key targets in modern drug discovery with GPCRs representing the largest and most important family of drug targets with more than 50% of the drugs in the market today being based on GPCRs targets (Klabunde and Hessler 2002; Congreve and Marshall, 2010).

1.1.1 Mechanism of activation and signalling pathways of GPCRs

GPCRs are so named as they interact with the intracellular proteins known as G-proteins, G-proteins normally bind either to guanosine diphosphate (GDP) or triphosphate (GTP) (Wettschureck and Offermanns, 2005). GPCRs are also known as seven trans-membrane receptors and the characteristic structure of these receptors consists of seven trans-membrane α helices, with an extracellular N-terminal domain and an intracellular C-terminal domain (Tuteja, 2009). Receptor occupation by its ligand leads to a change in conformation leading to G-protein activation (Damian *et*

al., 2008). The G-protein is a membrane protein consisting of 3 subunits (α, β, γ). The α subunit binds the guanosine diphosphate (GDP), which leads to the activation of the G-protein so that guanosine triphoshate (GTP) replaces GDP on the α subunit. This 'activated' G-protein dissociates into α-GTP and βγ where both the α-GTP and the βγ subunits can then activate second messengers, which are responsible for the intracellular signalling cascade associated with numerous physiological and pathophysiological processes (Pierce *et al.*, 2002; Tuteja, 2009; figure 1.1).

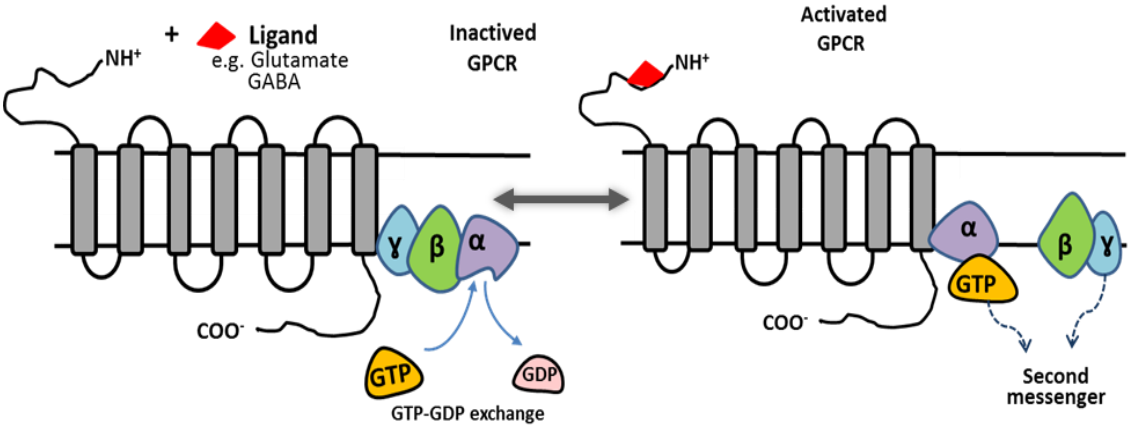


Figure 1.1: Activation of GPCRs. The ligand binds to the GPCR which causes GDP to be replaced by GTP initiating G-protein activation. The Gα and βγ subunits then initiate activation of intracellular second messenger cascades.

The actual signalling cascade that is regulated depends on which α-GTP subunit is activated (Hur and Kim, 2002). Four functional subunits of α-GTP have been identified thus far: $G\alpha_{12/13}$, $G\alpha_{s}$, $G\alpha_{i}$, and $G\alpha_{q/11}$. $G\alpha_{12/13}$ regulates cell processes through the use of guanine nucleotide exchange factors (GEFs) (Chan *et al.*, 2013). These activate the small GTPase superfamily including Rho which regulate a variety of processes including proliferation, differentiation, vesicle trafficking, gene expression, adhesion, and mobility (Radhika and Dhanasekaran 2001).

The $G\alpha_{s}$ subunit stimulates adenylate cyclase (AC), which catalyses the conversion of adenosine triphosphate (ATP) into Cyclic adenosine 3',5'-monophosphate (cAMP; Kozasa *et al.*, 2011). Activation of AC leads to increased levels of cytosolic cAMP, which acts as a second messenger by interacting with and regulating other proteins such as protein kinase A (PKA) and ion channels (Cooper, 2000). In contrast, $G\alpha_{i}$ inhibits AC resulting in the inhibition of cAMP production, which down regulates

PKA activity (Sanborn *et al.,* 1998). The $G\alpha_{q/11}$ subunit, which activates phospholipase Cβ, catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG; Bansal *et al.,* 2007). IP_3 binds to IP_3 receptors in the membrane of the endoplasmic reticulum (ER) leading to release of calcium (Ca^{2+}) and an increase in its cytosolic concentration (Majerus, 1992). Both Ca^{2+} and the other product of PIP_2 hydrolysis, DAG, have been shown to activate protein kinase C (PKC), which modulates the activity of numerous downstream molecules via phosphorylation (Tanak & Nishizuka, 1994).

The βγ subunits couple to GPCRs to form the GPCR-βγ complex (Smrcka, 2008). Subsequently, this complex can activate K^+ channels, PKC, mitogen-activated protein kinases (MAPKs) and increases cytosolic Ca^{2+} which can produce activation of different enzyme pathways (Cooper *et al.,* 2000; figure 1.2). Additionally, GPCR-βγ complex modulate the release of neurotransmitter through direct binding to and inhibition of voltage-gated Ca^{2+} channels (Yoon *et al.,* 2008).

1.1.2 Regulation of GPCR desensitization and internalization

After prolonged activation, GPCRs become inactivated and are resistant to further activation by ligands (Tuteja, 2009). Inactivation is usually mediated initially by desensitization of the receptor with this being followed by internalization (Perez and Karnik, 2005). Desensitization is mediated via agonist-induced phosphorylation of the GPCR C-terminus by GPCR kinases (GRKs) (Tobin, 2008). GRK C-terminal phosphorylation prepares the activated receptor for β-arrestin binding and this prevents further G-protein binding and G-protein-mediated signalling (Moore *et al.,* 2007). The GPCR-β-arrestin complex is targeted for endocytosis via clathrin–coated pits, where following internalization the receptors are sorted for recycling or degradation depending on the receptor in question (Soh *et al.,* 2010). Furthermore, β-arrestin-mediated internalization is also involved in GPCR signalling with β-arrestin-GPCR complexes being known to interact with other signalling proteins including MAPKs (Luttrell and Lefkowitz, 2002; figure 1.3). β-arrestin has multiple functions in the cell, including activation of G-protein independent signalling which activates different pathways (Pearson *et al* 2001).

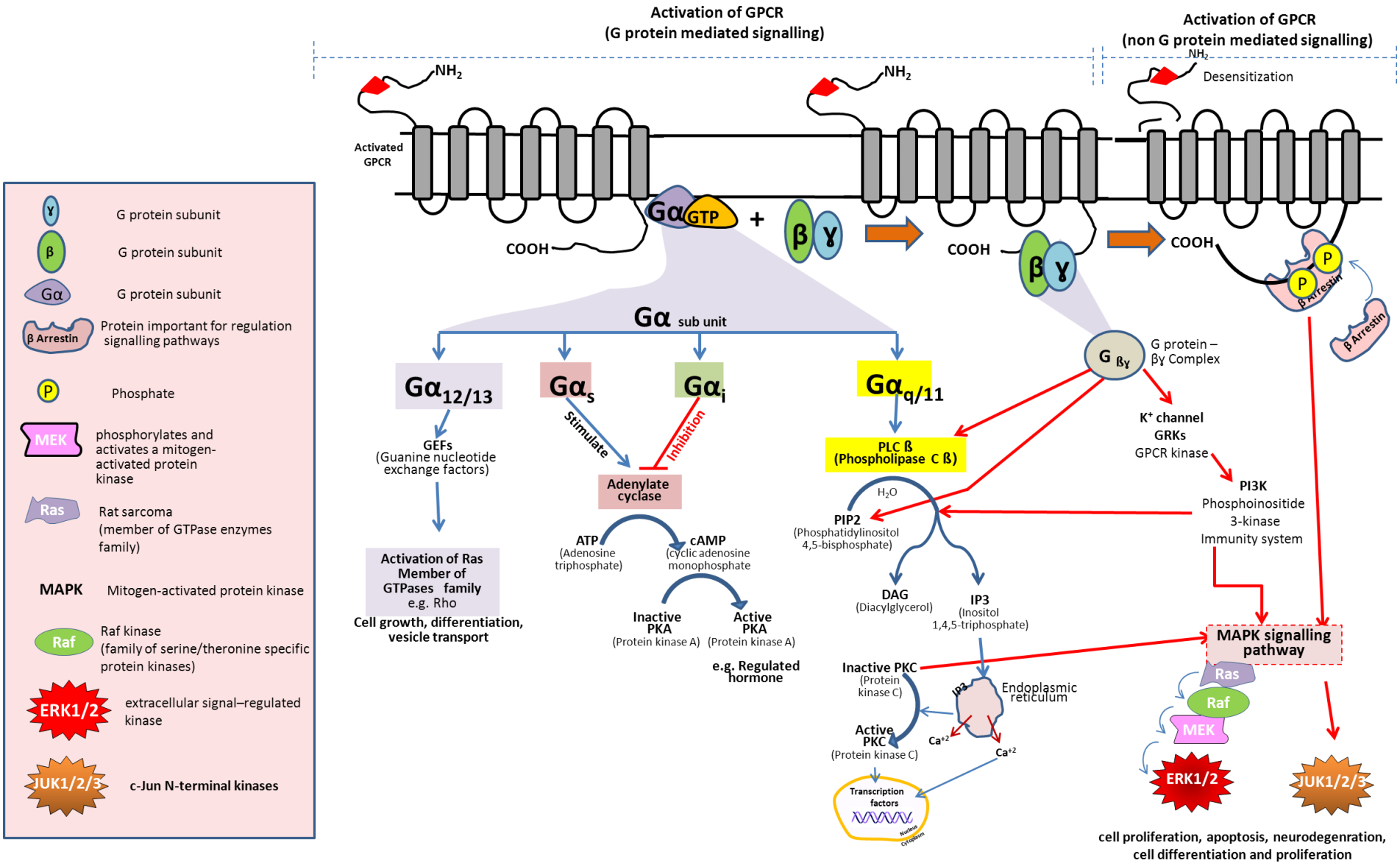


Figure 1.2: Gα subunits and intracellular signalling activation following GPCR sactivation.

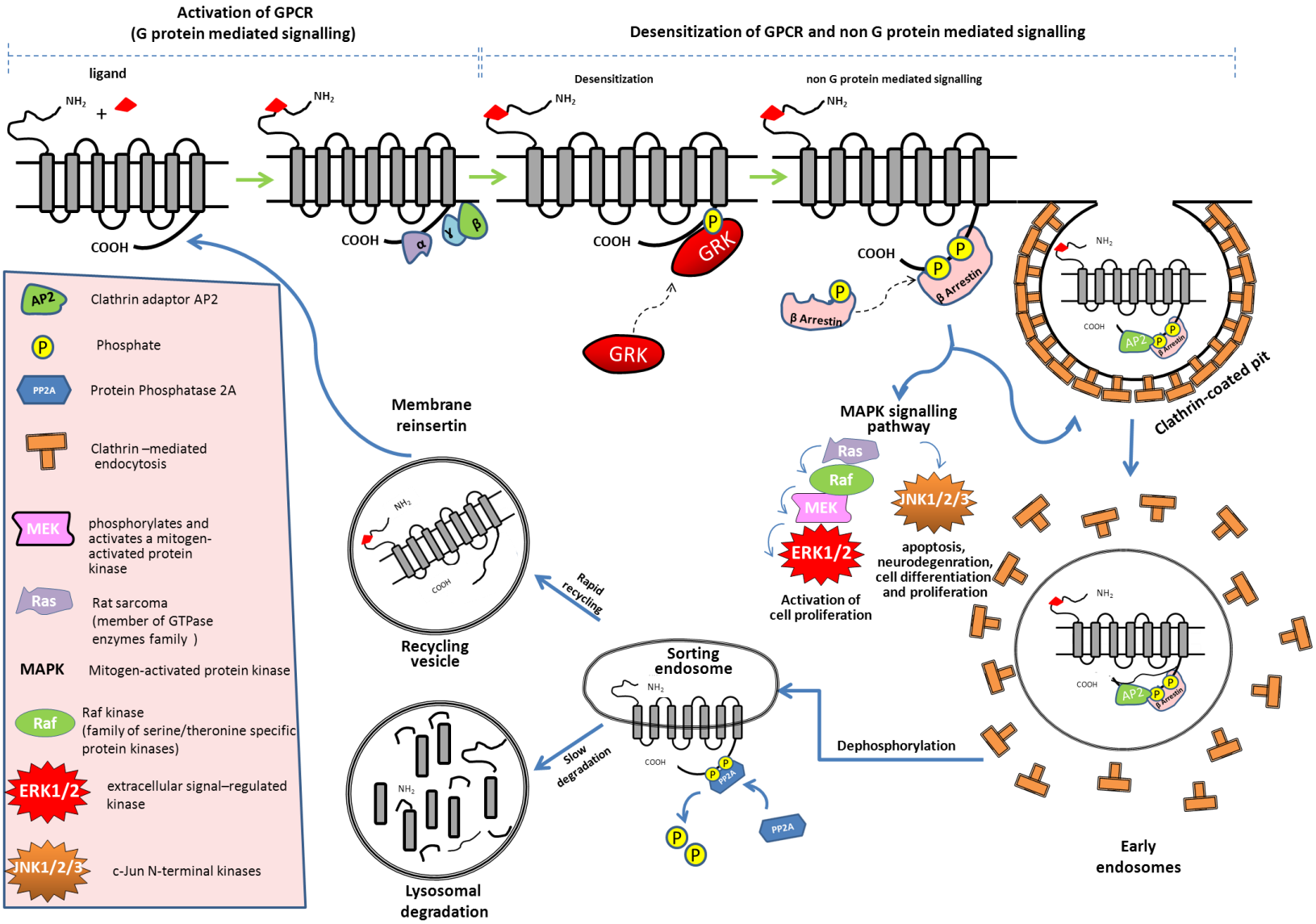


Figure 1.3: Mechanisms that regulate desensitization, internalization and non-G-protein mediated signalling of GPCR.

For example, activation of GPCR-β-arrestin complex can activate MAPKs including extracellular-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) enzymes to modulate cell proliferation, differentiation and apoptosis (Pearson *et al* 2001; Lefkowtiz and Shenoy, 2005; Moore *et al*., 2007; figure 1.3).

1.2 PARs

Classically, GPCRs are activated by soluble ligands, for example glutamate, Ca^{2+}, γ-aminobutyric acid-ß (GABA-ß), and acetylcholine (Ach) (Holz and Fisher (1999). However, in the early 1990s, a unique subtype of GPCR was discovered that is activated by endogenous proteinases rather than by soluble ligands and these were named proteinase-activated receptors (PARs; Vu *et al.,* 1991). PARs were recently described as a novel sub-class of GPCR (Eglen and Reisine, 2011). Activation of PARs occurs by a unique mechanism and PARs can be defined by their mechanisms of activation (Ossovskaya and Bunnett, 2004).

1.2.1 Mechanism of activation PARs by proteinases

Unlike other GPCRs, PARs are activated by endogenous proteinases (Ossovskaya and Bunnett, 2004). Endogenous proteinases bind to and cleave the N-terminal of PARs at a specific site of amino acid residues to unmask a new terminal (Soh *et al.,* 2010). Consequently, the new terminus functions as an intra-molecular ligand to activate the receptor and triggers intracellular signalling (Seatter *et al.,*2004). This is known as the tethered ligand mode of activation (Macfarlane *et al.,* 2001). The PAR activator unmasks a 'tethered ligand' which binds to the second extracellular loop of the receptor leading to activation of signalling pathways (Macfarlane *et al.,* 2001; Seatter *et al.,* 2004; figure 1.4 A).

1.2.2 Mechanism of activation of PARs by synthetic peptides

Synthetic PAR-activating peptides (PAR-APs) can be generated that mimic the tethered ligand sequences and activate PARs without the need for enzymatic cleavage of the receptor leading to PAR activation of signalling pathways (Hollenberg and Compton, 2002; figure 1.4 B). For example, it has been shown that

PAR2-AP such as SLIGKV-NH_2 activate intracellular signalling pathways via human PAR2 (Kanke *et al.*, 2009).

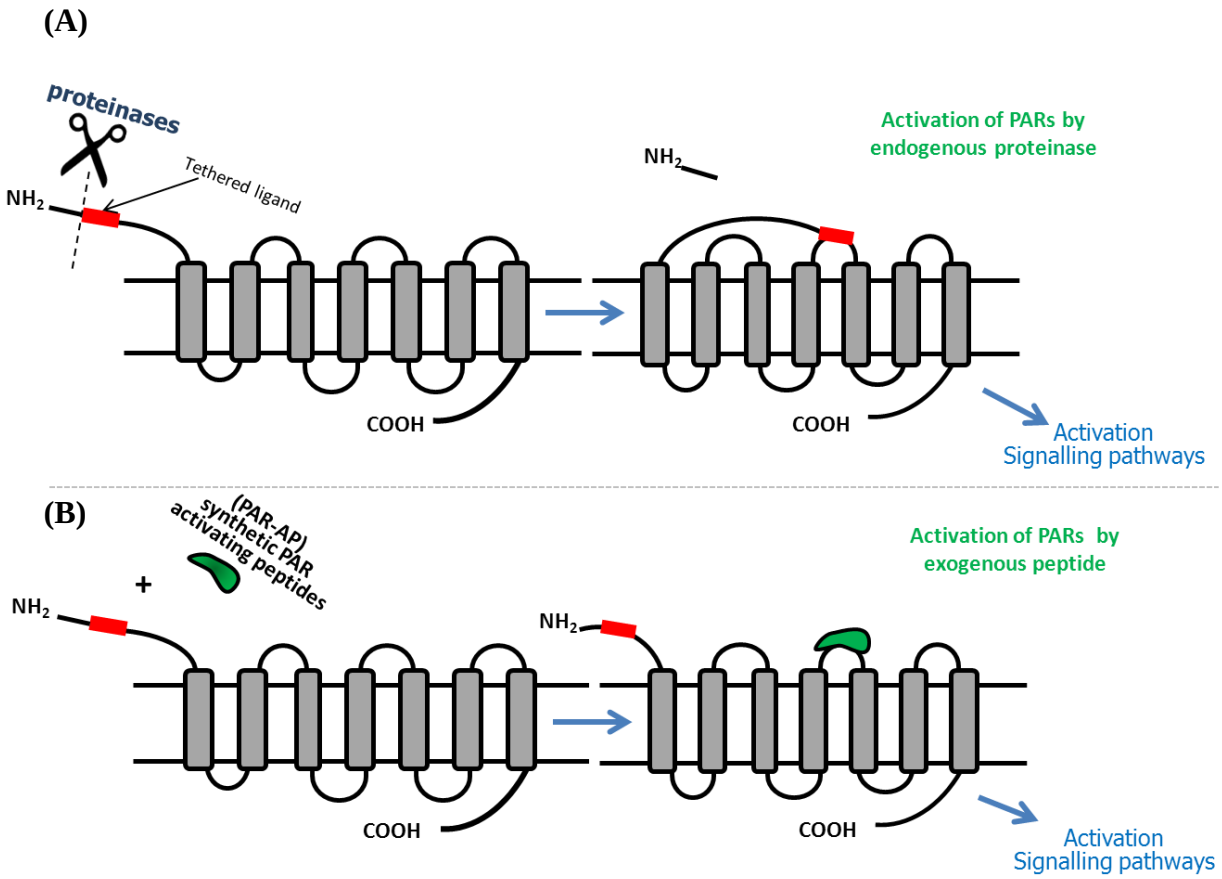


Figure 1.4: Mechanism of activation of PARs by endogenous proteinase or PAR-APs. (A) The endogenous activation of PARs by endogenous serine proteinases. The cleavage of PAR by proteinase exposes a 'tethered ligand' that binds to the second extra loop of the same receptor leading to activate signalling pathways. **(**B) The exogenous activation of PARs by synthetic PAR-APs. The synthetic peptide binds to the second loop leading to activate signalling pathways.

1.2.3 Disarming of PARs

If endogenous proteinases cleave the N-terminus at the site downstream of the tethered ligand sequence, the receptor is no longer available for activation by its targeting endogenous proteinases (Ramachandran and Hollenberg, 2008). This negatively regulates functioning through ''disarming'' by removing the tethered ligand sequences (Ramachandran and Hollenberg, 2008; figure 1.5A). These truncated receptors cannot be activated subsequently by a second endogenous

proteinase and may sometimes be retained on the cell surface and be available for activation by PAR-APs (Ramachandran *et al*., 2007; figure 1.5 B).

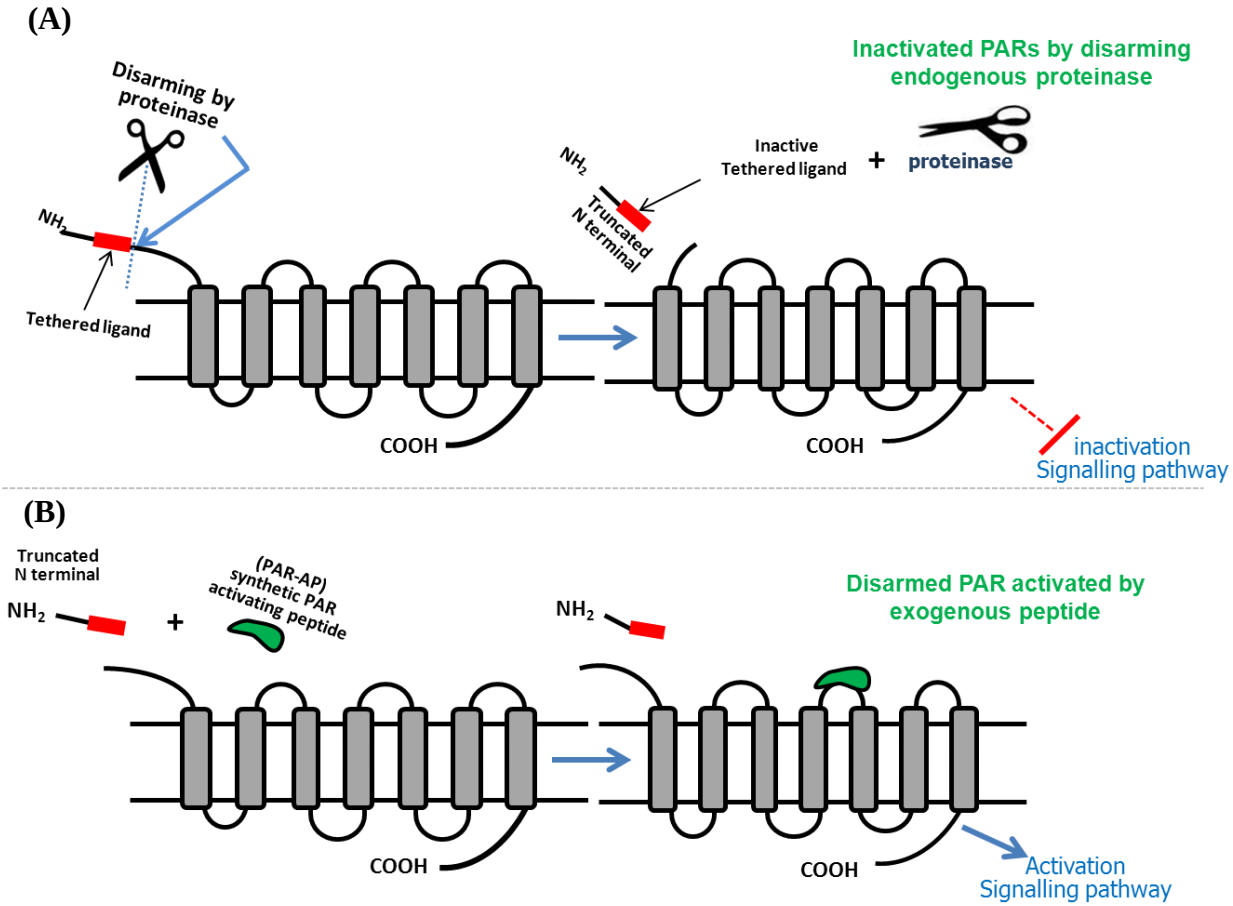


Figure 1.5: Schematic demonstration of the inactivation of PARs by a disarming proteinase.(A) Disarming proteinase cleaves the N terminus at a site downstream of the tethered ligand sequence which leads to inactivation of signalling pathways. (B) Truncated N-terminal cannot be activated by endogenous proteinase but it can be activated by synthetic peptide which leads to activation of signalling pathways.

1.2.4 Biased agonism of PARs

The observation that different ligands can lead to the activation of different signalling pathways via the same receptor is a process called functional selectivity or biased agonism (Mailman, 2007). At the molecular level, a ligand induces stabilization of distinct active receptor conformations and this directs the receptor to couple to specific signalling effectors (Zheng *et al.*, 2008). Functional selectivity has been identified as a route to develop drugs that are selective for specific receptor

conformations and therefore elicit GPCR specific signalling that may lead to reduced side effects of the drugs (Hoffmann *et al.,* 2008). The unique activation mechanism of PARs suggested that PARs would not be able to display functional selectivity (Russo *et al.,* 2009). However, recent studies have indicated, at least for PAR1, that different PAR1 activators can produce different signalling responses (Russo *et al.,* 2009; Mosnier *et al.,* 2012). Thus, depending on the proteinase activating the receptor, PAR1 have the ability to display biased agonism. The concept of functional selectivity may be particularly important as PARs are proposed to be activated by a number of circulating endogenous proteinases thus indicating that PAR activation may lead to a number of differing physiological outcomes depending on the activator and the signalling pathway activated (Kenakin, 2011).

1.3 Discovery and structural features of PARs

In 1991, the first member of the PAR family was successfully cloned (Rasmussen *et al.,* 1991; Vu *et al.,* 1991). This receptor was initially called coagulation factor II (thrombin) receptor and is currently known as PAR1 (Schmidt *et al.,* 1997; Kahn *et al.,* 1998). In 2003, a full sequence of the human gene was published and four gene sequences were identified for the PAR family (Everts-van *et al.,* 2004; Suen *et al.,* 2010). The PAR family consists of PAR1, PAR2, PAR3 and PAR4 (Komatsu *et al.,* 2013). The official symbol for the PAR1 human gene is (*F2R*), for the PAR2 human gene is (*F2RL1*), for the PAR3 human gene is (*F2RL2*) and for the PAR4 human gene is (*F2RL3*). PAR genes are located on chromosome 5 and 19. PAR1, PAR2 and PAR3 are clustered on the longer arm of chromosome 5 at the 5q13 region, whereas PAR4 is located on the chromosome 19 on shorter arm at the 19P12 region (Schmidt *et al*., 1997 Kahn *et al*., 1998; figure 1.6).

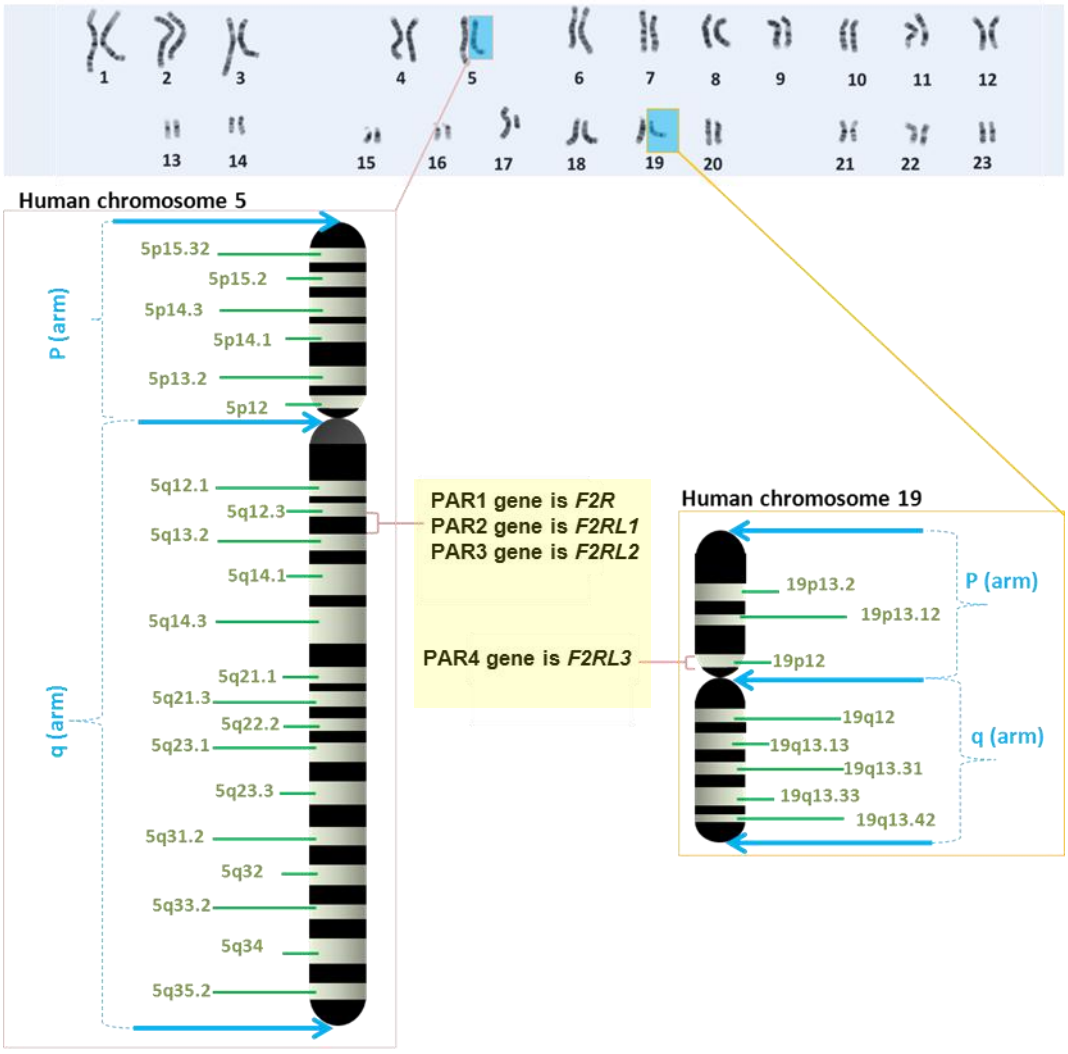


Figure 1.6: Location of PAR genes on human chromosomes. PAR1, PAR2 and PAR3 are clustered on the longer arm of chromosome 5 at 5q13 and PAR4 is located on the short arm of chromosome 19 in the 19P12 region.

1.3.1 PAR1

The discovery of the first member of PAR was mainly based on research into the regulation of blood clot formation and platelet aggregation in the cardiovascular system by an endogenous proteinase like thrombin (Traupe and Barton, 2004). Several thrombin binding proteins were identified which helped to understand the role of thrombin (Saneyoshi *et al.*, 2009). It was found that thrombin could act as either a platelet agonist or antagonist which gave an indication of the possible mechanism of the thrombin binding (Macfarlane *et al.*, 2001). Further work was performed to identify the mechanism of thrombin binding, whether as a classical

GPCR or through a novel mechanism (Shuman, 1986). It was observed that thrombin signals were found in different cell types other than blood cells, including endothelial cells, vascular cells, vascular smooth muscle cells, monocytes and lymphocytes through cleaving PAR, which belong to the GPCR family (Macfarlane *et al.*, 2001).

In 1991, two laboratories cloned the hamster and the human thrombin receptor separately and later the receptor was designated PAR1 (Rasmussen *et al.*, 1991; Vu *et al.*, 1991). PAR1 cDNA was isolated and subsequently studied in Xenopus oocytes (Vu *et al.*, 1991). Analysis of the amino acid sequence discovered that this receptor was a novel member of the seven trans-membrane receptor family (Vu *et al.*, 1991).

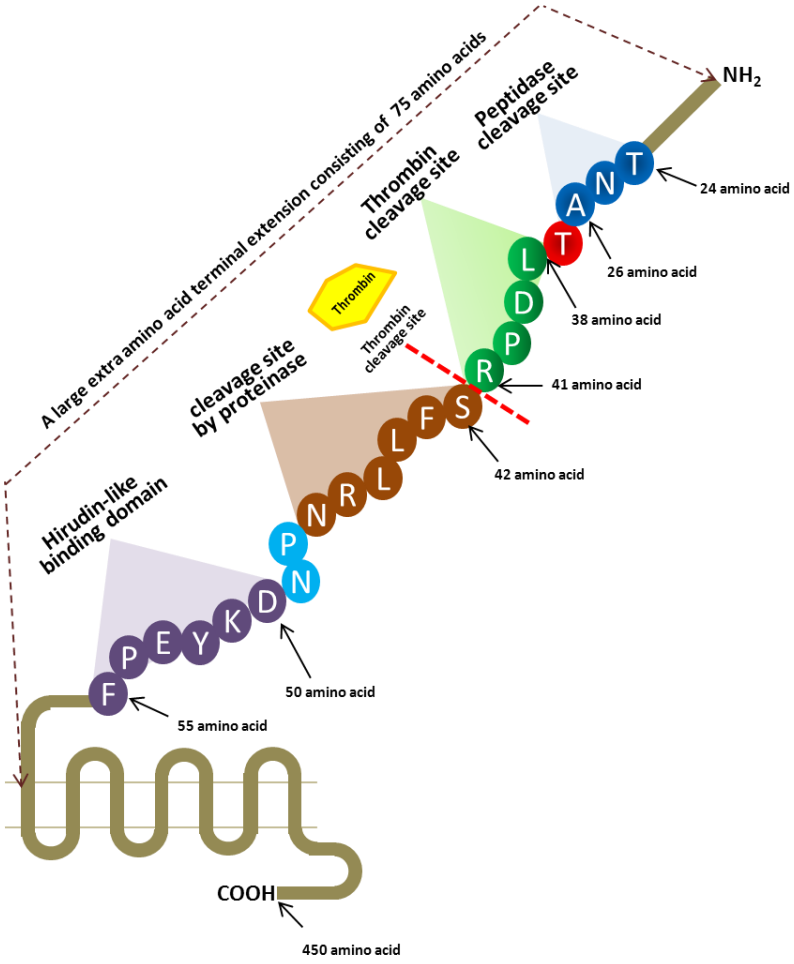


Figure 1.7: Diagram illustrating the human thrombin receptor (PAR1). The human thrombin receptor is characterized by seven trans-membrane domains with large extracellular amino terminal that contains different active sites such as hirudin-like binding domains, thrombin and peptidase cleavage sites. The receptor consists of 450 amino acids and its extracellular domain consists of 75 amino acids.

A remarkable feature of this new receptor was a long terminal extracellular amino acid extension (Vu *et al.,* 1991). This extracellular extension consists of 75 amino acids and includes many active sites, including a hirudin-like binding domain, and thrombin and peptidase cleavage sites (Vu *et al.,* 1991). The proposed thrombin cleavage site is located between the R41 and S42 amino acids and cleavage unveils a new amino terminal SFLLR-NH_2 (Norton *et al.,* 1993). This new amino terminal functions as a tethered ligand binds to an extracellular second loop of PAR1 and activates the signalling pathway (Vu *et al.,* 1991). A peptidase sequence is located at T24 and A26 and acts as a site for degradation of the receptor by proteolysis. In addition, an acidic region, the hirudin-like site DKYEPF-NH_2, is important in enhancing the affinity of the receptor binding to thrombin (Vu *et al.,* 1991; Ishii *et al.,* 1995; figure 1.7).

The synthetic peptide, SFLLR-NH_2, is based on the tethered ligand sequence (Norton *et al.,* 1993). This synthetic peptide directly binds to the extracellular loop of PAR1 without cleaving the terminal amino acid thus mimicking the effect of thrombin on the PAR1 (Ossovskaya and Bunnett, 2004; figure 1.8). SFLLR-NH_2 acts as a potent agonist for the thrombin receptor and promotes platelet aggregation (Hollenberg and Saifeddine, 2001).

1.3.2 PAR2

Further studies on the effect of thrombin in haemostasis showed that not all of the actions of thrombin can be explained through activation of PAR1 (Vouret-Craviari *et al.,* 1992). It was widely believed that a second thrombin receptor must exist. This proposed receptor was discovered during the study of genomic cDNA from the bovine substance K (neurokinin) receptor (Masu *et al.,* 1987). Furthermore, it was reported the cloning a DNA sequence encoding a protein 395 residues with seven trans-membrane domain structures that identified as a second thrombin receptor (Nystedt *et al.,* 1994; Nystedt *et al.,* 1995).

It was noticed that this receptor shared 30% amino acid homology with human PAR1 receptor and 28% amino acid with mouse PAR1 (Nystedt *et al.,* 1994). Surprisingly, this receptor was insensitive to thrombin, but was shown to be highly

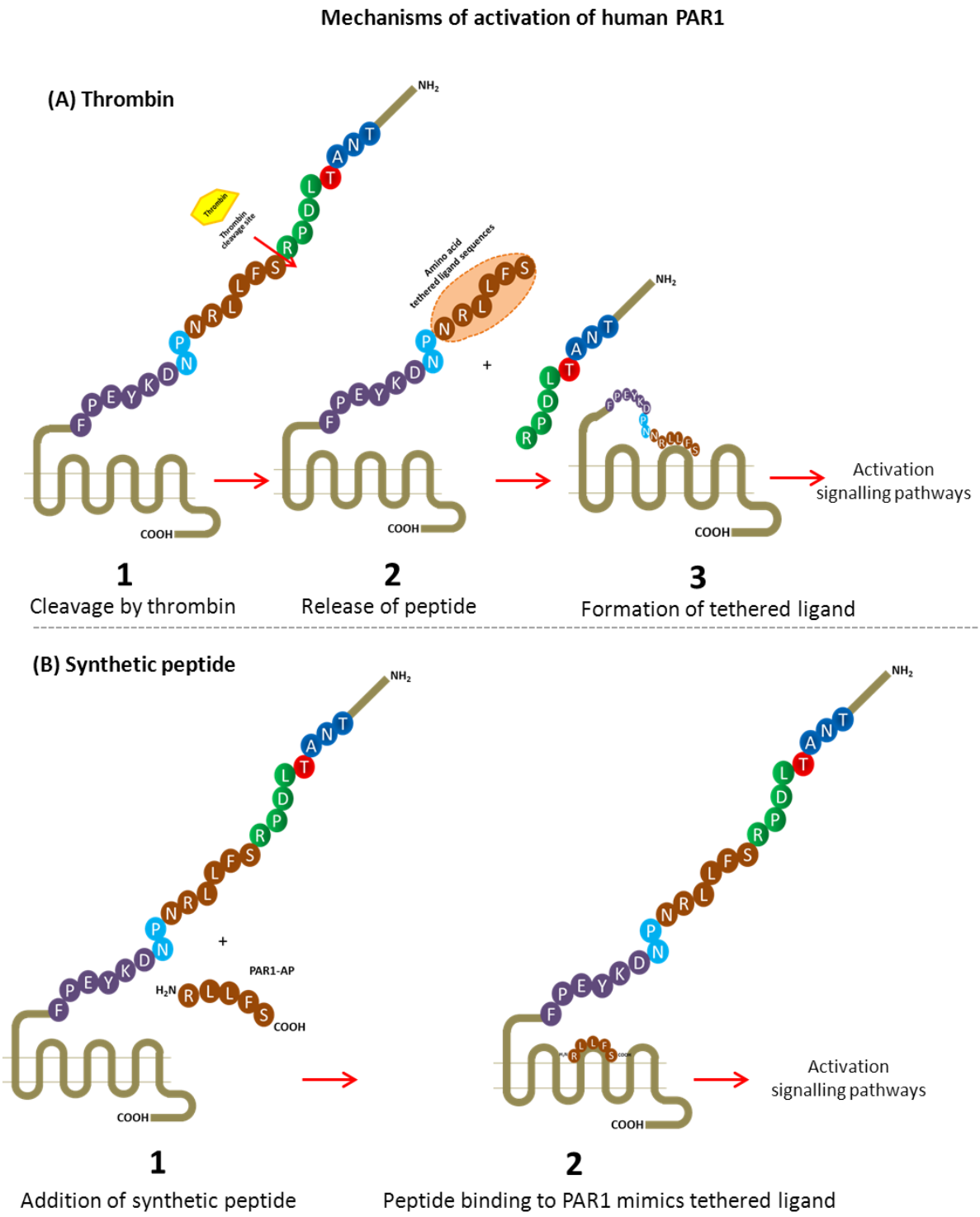


Figure1.8: Activation mechanism of human PAR1 by both thrombin and PAR1 synthetic peptide. (A) Thrombin cleaves amino acid terminal at a specific site, unveiling a new terminal peptide which acts as tethered ligand. The tethered ligand binds to the second extracellular loop and activates the receptor signalling pathway. (B) PAR1 synthetic peptide (SFLLR-NH_2) binds directly to PAR1 without cleaving the peptide and mimics the effect of PAR1 signalling pathway.

sensitive to low levels of trypsin (Nystedt *et al.*, 1995).

Similar to PAR1, cleavage by trypsin in the N-terminus was shown to be required for the receptor activation (Nystedt et *al.*, 1994). This receptor was identified to be a second member of the PAR family, known as PAR2 (Schmidt *et al.*, 1997; Kahn *et al.*, 1998). Subsequently, the human PAR2 was cloned in the following next year (Nystedt *et al.* 1995). The trypsin cleavage site in the N terminus was shown to have differences between human and mouse. In human PAR2, the cleavage site of a trypsin is located between R 36 and S 37 amino acid in the N terminus while in mouse it is located between the R 34 and S 35 amino acids in the N terminus (Al-Ani *et al.*, 2004; Ossovskaya and Bunnett, 2004; figure 1.9).

As in the PAR1 receptor, trypsin cleaves the extracellular amino acid terminal and unveils a new amino terminal (SLIGRL-NH_2 in the mouse and SLIGKV-NH_2 in humans) functions as a tethered ligand that binds to the extracellular second loop of PAR2 to activate the signalling pathway (Ricciardolo *et al.*, 2000; figure1.10).

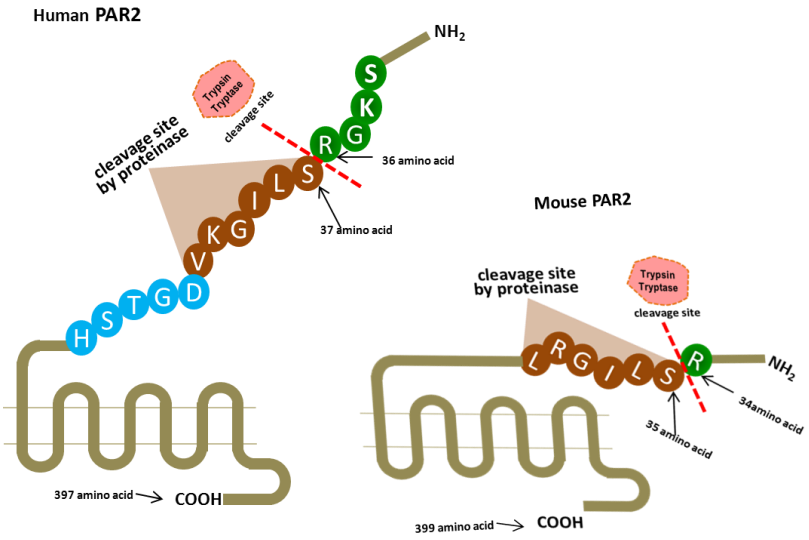


Figure 1.9: Diagram illustrating the human and mouse PAR2. The receptor is characterized by seven trans-membrane domains with a large extracellular amino acid terminal which contains the proteinase (trypsin or tryptase) cleavage site. The human PAR2 receptor consists of 397 amino acids while the mouse PAR2 consists of 399 amino acids.

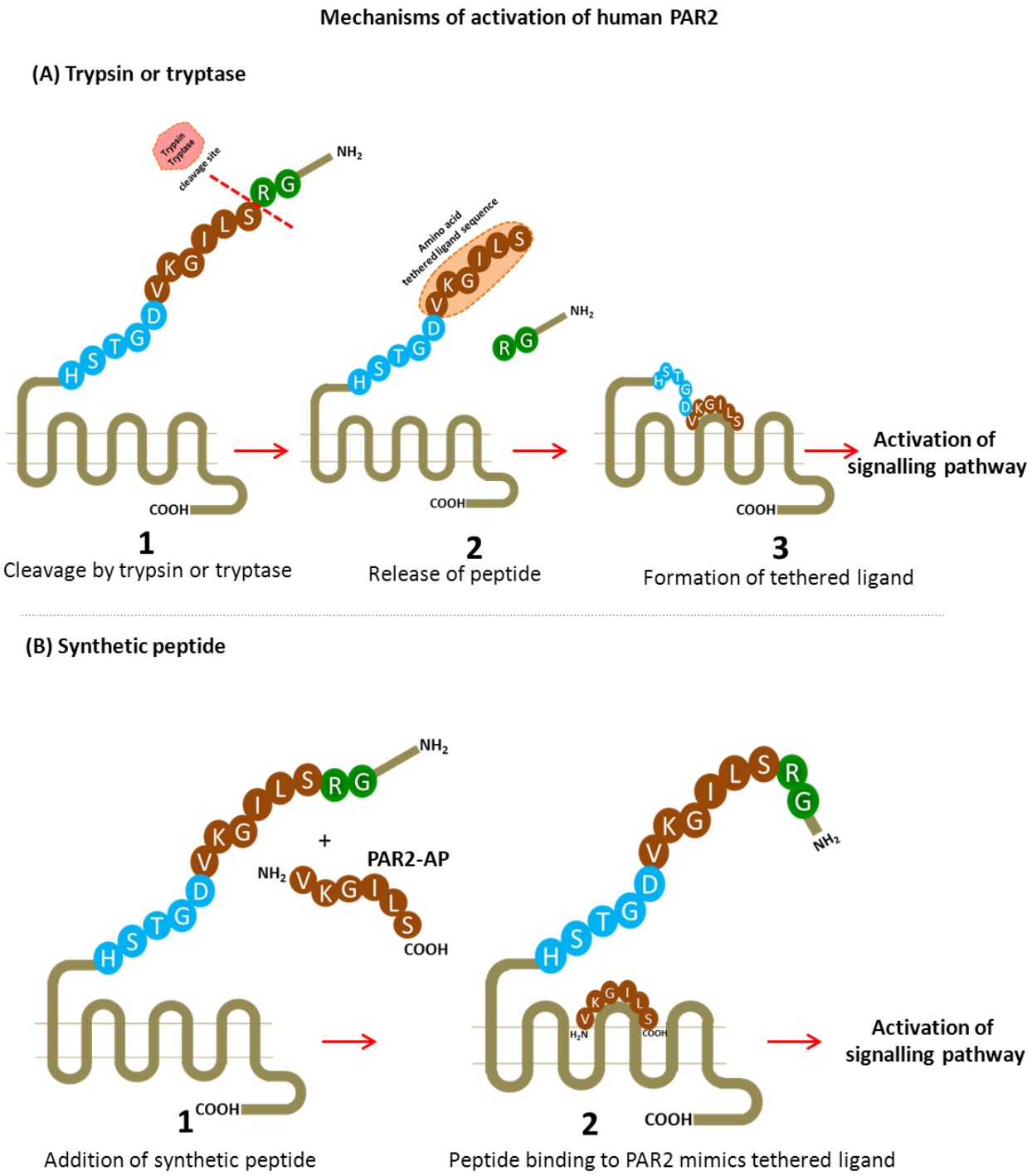


Figure 1.10: Activation mechanism of human PAR2 by both trypsin or tryptase and synthetic peptide. (A) Proteinases such as trypsin or tryptase cleave the amino acid terminal at a specific site, unveiling a new terminal peptide which acts as tethered ligand. The tethered ligand binds to the second extracellular loop and activates the receptor signalling pathway. (B) PAR2 synthetic peptide (SLIGKV-NH_2) binds directly to PAR2 without cleaving the peptide and mimics the effects of PAR2 signalling pathway.

The synthetic PAR2 peptide is designed based on the tethered ligand sequence (for example in mouse is SLIGRL-NH_2 and in humans is SLIGKV-NH_2) and it directly binds to the extracellular loop of PAR2 without cleaving the terminal amino acid thus mimicking the function of PAR2 (Ricciardolo *et al.,* 2000; Macfarlane *et al.,* 2001).

1.3.3 PAR3

The cloning of PAR2 opened the door for determining more members of the PAR family (Connolly *et al.,* 1996). In further studies examining the effect of thrombin in platelets, it was observed that PAR1 knockout (KO) mice responded normally to thrombin but were unaffected by a PAR1 synthetic peptide (Connolly *et al.,* 1996). This finding suggested the presence of another receptor type. A year later, a third member of the PAR family was discovered and is currently named PAR3 (Ishihara *et al.,* 1997; Schmidt *et al*., 1997; Kahn *et al*., 1998).

PAR3 has been cloned in both human and mouse tissue (Ishihara *et al.,* 1997). Analysis identified that the amino acid sequence for PAR3 shows 27% homology to both human PAR1 and 28% homology to human PAR2 (Taylor *et al.,* 2001). In addition, the structure of PAR3 is similar to PAR1 which both selectively bind thrombin among other proteinases due to the presence of a hirudin-like site (Vu *et al*., 1991; Ishihara *et al.,* 1997).

The thrombin cleavage site in human PAR3 is located between the amino acids K38 and T39 (figure 1.11). As with the other receptors in this family, thrombin unveils a new amino terminal (TFRGAP-NH_2) which functions as a tethered ligand and binds to an extracellular second loop of the receptor but does not activate signalling pathways like the other PARs (Hansen *et al.,* 2004). The synthetic peptide for PAR3 is based on the amino acid sequence of its tethered ligand (TFRGAP-NH_2) and binds to the second extracellular loop of PAR3 (Hansen *et al.,* 2004; figure 1.12). However, this peptide does not activate the PAR3 receptor.

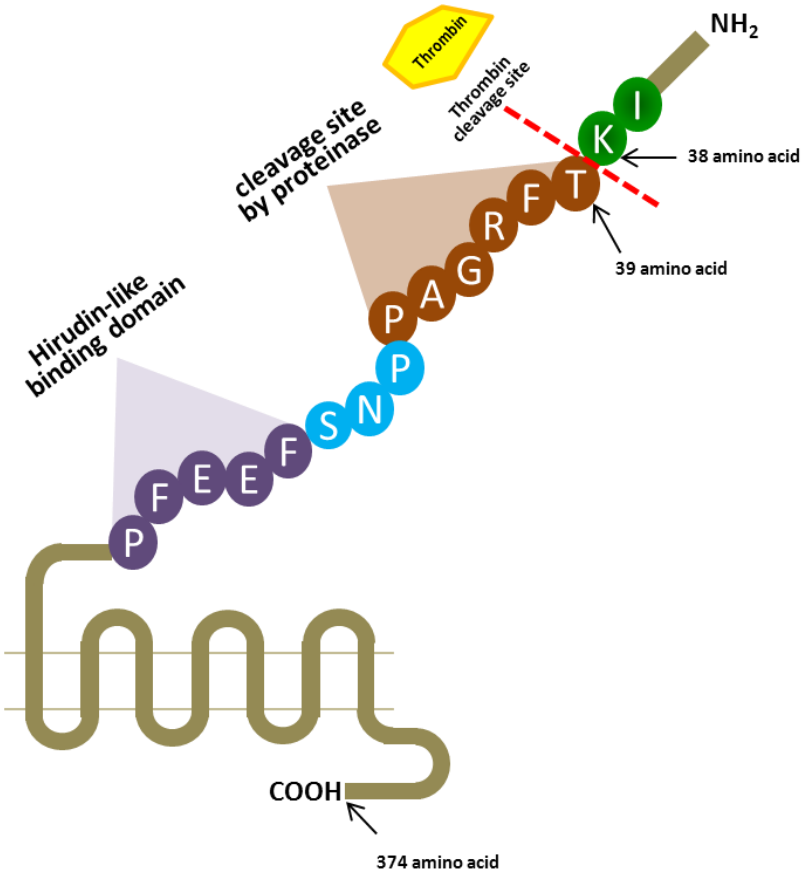


Figure 1.11: Diagram illustrating the human PAR3. The receptor is characterized by seven trans-membrane domains with a large extracellular amino acid terminal that contains different sites such as hirudin-like binding domain, thrombin and peptidase cleavage sites. PAR3 receptor consists of 374 amino acids.

1.3.4 PAR4

It was noticed that thrombin responses in platelet from PAR3 KO mice were extensively delayed, but not absent (Kahn *et al.*, 1998). As PAR1 has no function in mouse platelet aggregation (Connolly *et al.*, 1994; Connolly *et al.*, 1996), this result suggests that another receptor is involved in mediating the response to thrombin. In 1998, a fourth member of the PAR family was cloned and termed as PAR4 (Schmidt *et al.*, 1997; Kahn *et al.*, 1998).

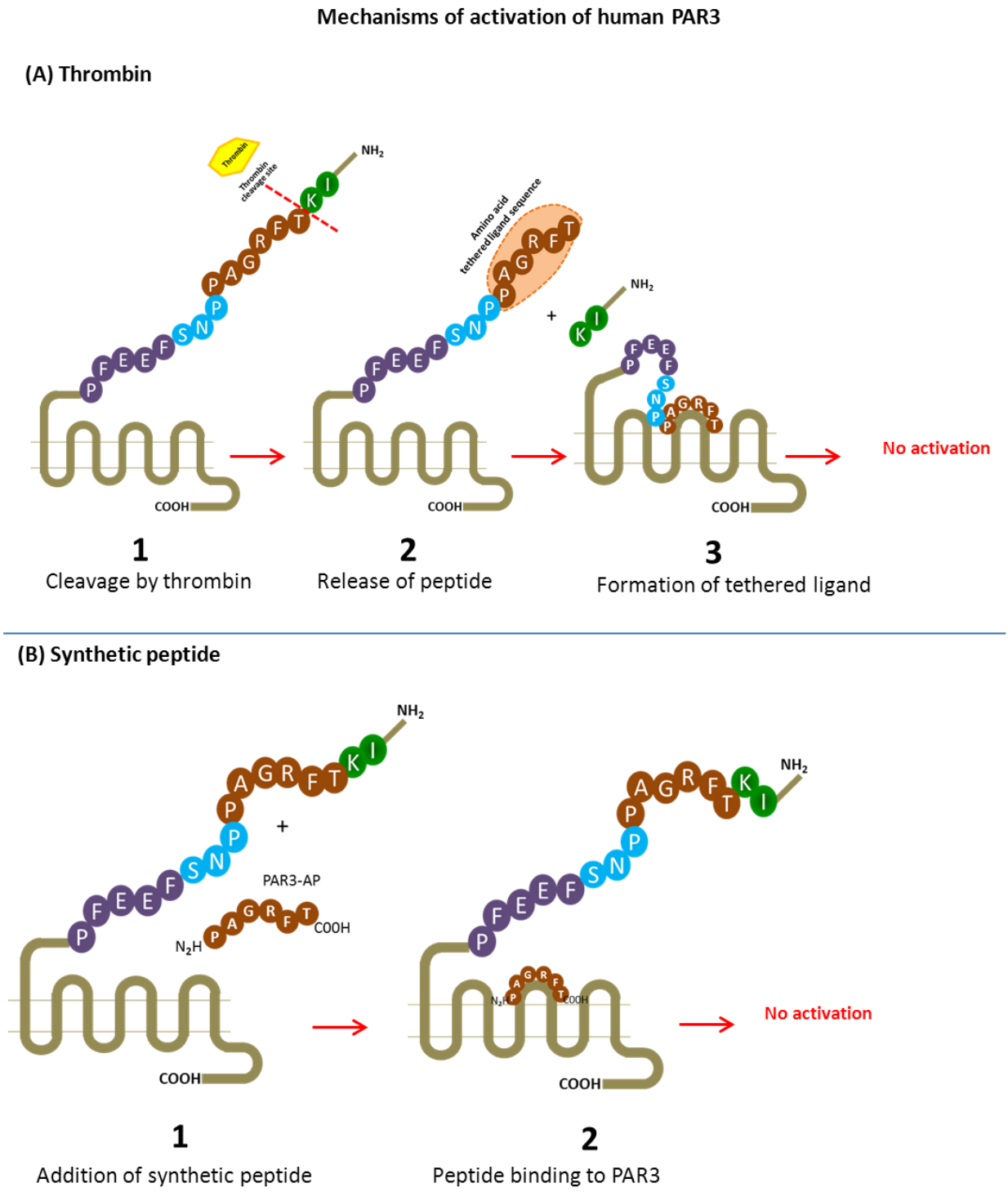


Figure 1.12: Mechanism of human PAR3 by both thrombin and synthetic peptide. (A) Thrombin cleaves amino terminal at a specific site unveiling the new terminal peptide which acts as a tethered ligand and binds to the second extracellular loop but without activating the signalling pathway. (B) Synthetic peptide (NH_2-TFRGAP) binds to the second extra loop of PAR3 without cleaving the peptide and this binding does not activate the signalling pathway.

The thrombin cleavage site of human PAR4 is located between the amino acids R 47 and G 48 and cleavage unveils a new amino terminal GYPGQV-NH_2 (Asfaha *et al.*, 2007).

This new amino acid terminal is a tethered ligand, which interacts with the receptor and activates the cell-signalling pathway (figure1.13). PAR1 and PAR4 exhibit different sensitivities to thrombin. PAR1 can be activated by low concentrations of thrombin, whereas, PAR4 only responds to higher concentrations. Similar to other PARs, PAR4 can be activated by a synthetic peptide (GYPGKF-NH_2 in the mouse and GYPGQV-NH_2 in humans) through a similar mechanism to the other PARs (Hoogerwerf *et al.*, 2002; figure1.14).

PAR4 can also play a role in mouse platelet aggregation (Wu *et al.*, 2010). A further study demonstrated that PAR4 could be equally activated by both thrombin and trypsin (Asfaha *et al.*, 2007).

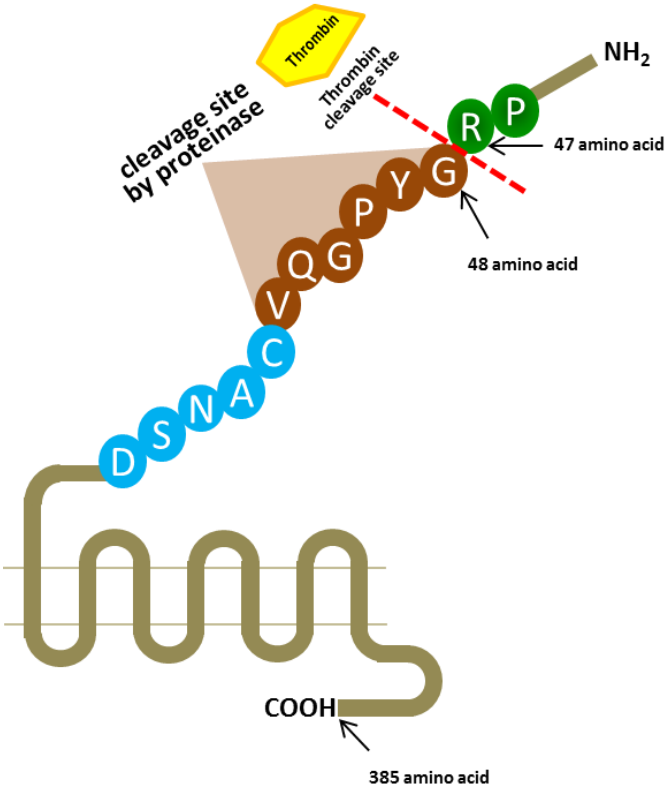


Figure 1.13: Diagram illustrating the human PAR4. The PAR4 receptor is characterized by seven transmembrane domains with a large extracellular amino terminal that contains active sites including the proteinase cleavage site. The receptor consists of 385 amino acids.

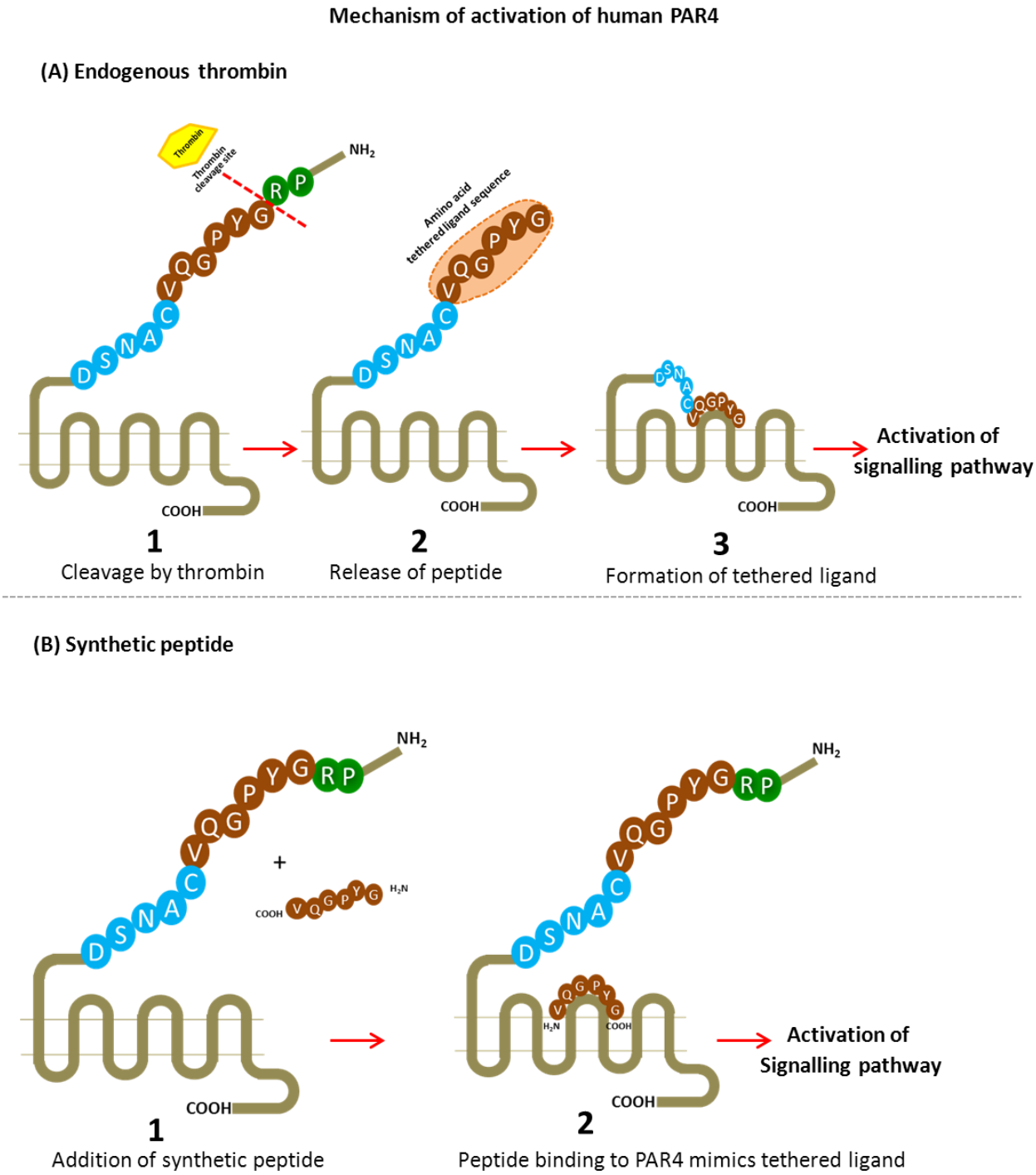


Figure 1.14: Activation mechanism of human PAR4 by both endogenous thrombin and exogenous synthetic peptide. (A) Thrombin cleaves amino terminal at a specific site. The new amino terminal of PAR4 acts as a tethered ligand and binds to the second extracellular loop that activates the receptor signalling pathway. (B) Synthetic peptide (NH_2-GYPGQV) binds directly to PAR4 without cleaving the peptide and mimics the effect of PAR4 signalling pathway in the same way as PAR1 and PAR2.

Each PAR is characterised by a preferred activating enzyme, its cleavage and binding sites for the interaction with different signalling pathways and its internalization and desensitization pathways (Ossovskaya and Bunnett, 2004; figure 1.15)

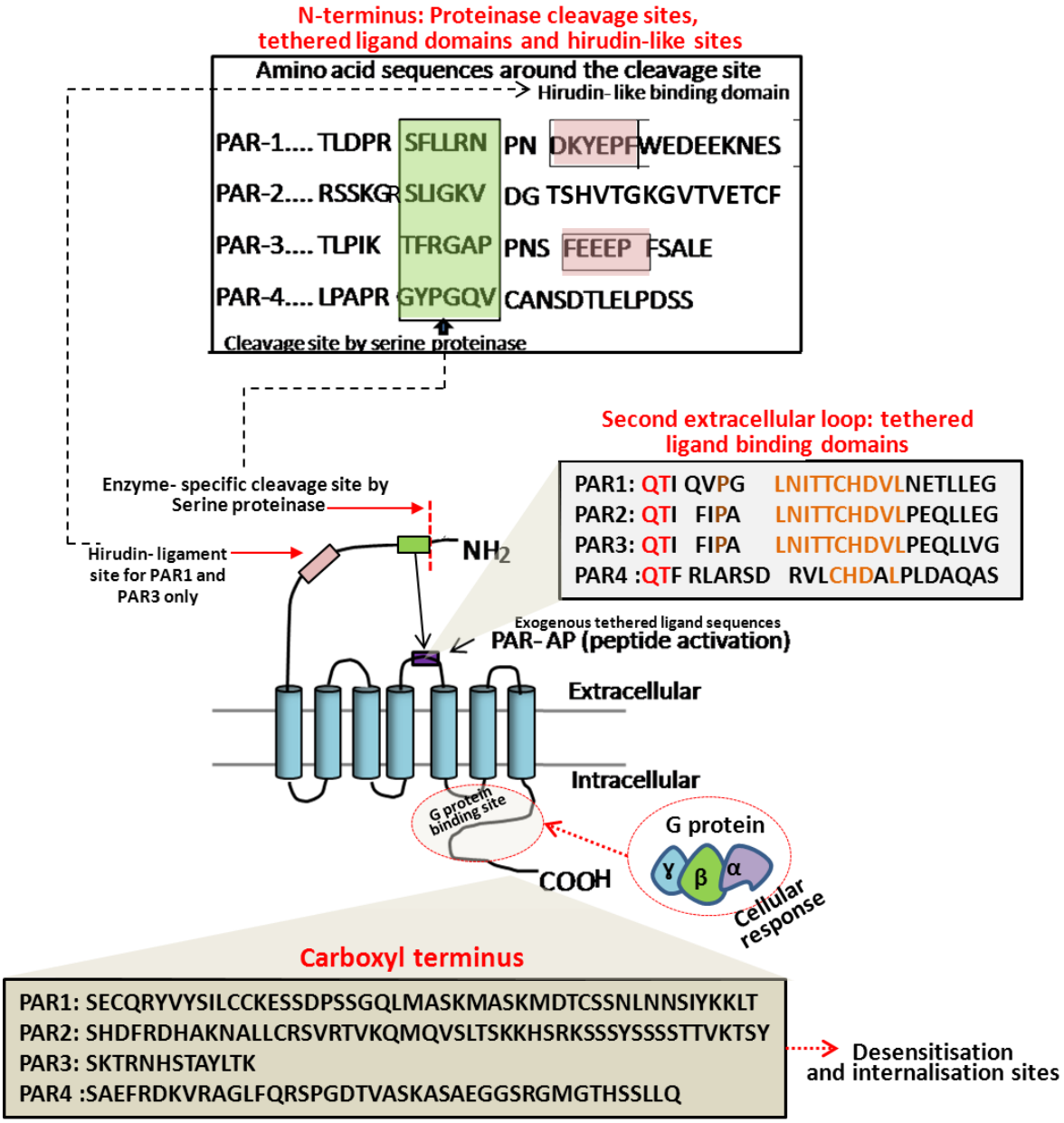


Figure 1.15: Summary of the structural features of PARs. Similarities and differences within in the PAR family in structural features and the amino acid sequences. Each PAR has a different amino acid sequence of proteinase cleavage site within its amino terminal, tethered ligament and sequence, hirudin–like binding site sequence and G-protein binding site of carboxyl terminal sequence. The scheme represents human PARs.

1.4 PAR Signalling

PARs can be activated through proteinase-induced formation of a tethered ligand or PAR agonists (synthetic peptides) which mimic this ligand leading to stimulation of both G-proteins dependent and independent signalling pathways. PAR1, PAR2 and PAR4 couple with G-proteins to stimulate signalling pathways, whereas PAR3 couples with Gα protein without signalling pathway (Kenakin, 2011).

1.4.1 PAR1 Signalling

It was confirmed that activation of PAR1 couples to G-protein subunits, including Gα which consists of $G\alpha_{12/13}$, $G\alpha_{q/11}$ and $G\alpha_{i}$ subunits. G-proteins activate different signalling pathways as discussed below (Coughlin, 2005; Russo *et al* 2009). The main pathway for PAR1 signalling is via $G\alpha_{q/11}$, which stimulates PLC-β. PLC-β generates IP_3 mobilizes Ca^{2+} release from intracellular stores and Ca^{2+} induced activation of PKC. Additionally, PLC-β generates DAG that activates PKC. Moreover, $G\alpha_{q/11}$ regulates MAPK mediated ERK1 and ERK2 signalling which activates transcription factors (Trejo *et al.,* 1996). PAR1 coupling to $G\alpha_{q/11}$ has been identified in many tissues, including fibroblast cells and astrocytes (LaMorte *et al.*, 1993; Baffy *et al.,* 1996; Wang *et al.,* 2002). Another major signalling pathway of PAR1 is through coupling of the receptors to $G\alpha_{12/13}$. In both platelets and astrocytes, PAR1 couples to $G\alpha_{12/13}$ that interacts with Rho guanine nucleotide exchange factors (RhoGEFs) which control migration and cell shape through activation Rho-Kinases (Offermanns *et al*., 1994). The signalling through $G\alpha_{i}$ pathway which inhibits AC and prevents formation of cAMP, was shown in several cell types including human erythroleukaemia cells, vascular smooth muscle cells, osteosarcoma cells, fibroblasts, platelets, endothelial cells and astrocytes (Babich *et al.,* 1990; Brass *et al.,* 1991; Hung *et al.,* 1992; Kanthou *et al.,* 1996; Kim *et al.,* 2002; Vanhauwe *et al.,* 2002; Wang *et al.,* 2002; figure 1.17).

The Gβγ subunits released from coupling PAR1 to heterogeneous G-protein also initiate signalling transduction. Coupling of Gβγ to PAR1 activates phosphatidylinositol (PI) 3-kinase which leads to changes in cell skeleton structure,

motility, survival and mitogenesis. In addition, G-proteins βγ has been postulated that activates PLC-ß, Rac 1, GRKs and K^+ channels (Wang *et al*., 2002; figure 1.16).

Apart from classical GPCR signalling, PAR1 activates other multiple intracellular kinase cascades in a G-protein dependent or G-protein independent. The activation of G-protein independent signalling leads to activation of MAPK signalling molecules including ERK1/2, c-Jun N-terminal kinases (JNKs) and p38 MAPK (Widmann *et al.,* 1999). Activation of MAPK signalling modulates the cytoskeleton and promotes mitogenesis and cell survival. ERK signalling stimulated by activation of PAR1 has been shown in astrocytes, intestine epithelial cells and fibroblasts (Vouret-Craviari *et al.,* 1993; Buresi *et al.,* 2002; Wang, *et al.,* 2002). In addition, activation of PAR1 stimulates p38 MAPK (Sabri *et al.,* 2002) and JNK signalling (Wang *et al*., 2006) in fibroblasts and astrocytes, respectively.

1.4.1.1 Location and biased agonism of PAR1 signalling

Recent studies have shown that PAR1 signalling can have multiple functions depending on the biased reaction and location of the receptor, however, the mechanisms involved remain poorly understood (Urban *et al.,* 2007). For example, thrombin activates PAR1 in human endothelial cells and preferentially couples with $G\alpha_{12/13}$ over $G\alpha_{q/11}$ and $G\alpha_i$ with $G\alpha_{12/13}$ to facilitate RhoA-mediated endothelial permeability (McLaughlin *et al.,* 2005). In contrast, PAR1 synthetic peptide (SFLLRN-NH_2) produces activation of PAR1 that preferentially couples with $G\alpha_{q/11}$ and increases mobilization of Ca^{2+} (McLaughlin *et al.,* 2005; figure 1.17). In addition, the location of the PAR1 also affects the selectivity of the receptor for a specific signalling pathway. For example, PAR1 in the endothelium is preferentially activated by thrombin and couples to $G\alpha_{12/13}$ which facilities RhoA signalling and results in endothelial barrier disruption. In contrast, PAR1, in a membrane microdomain, is preferentially activated through activated protein C (APC) and actives small GTPase protein Rac1 rather than RhoA which results in endothelial cell protection (Russo *et al*., 2009).

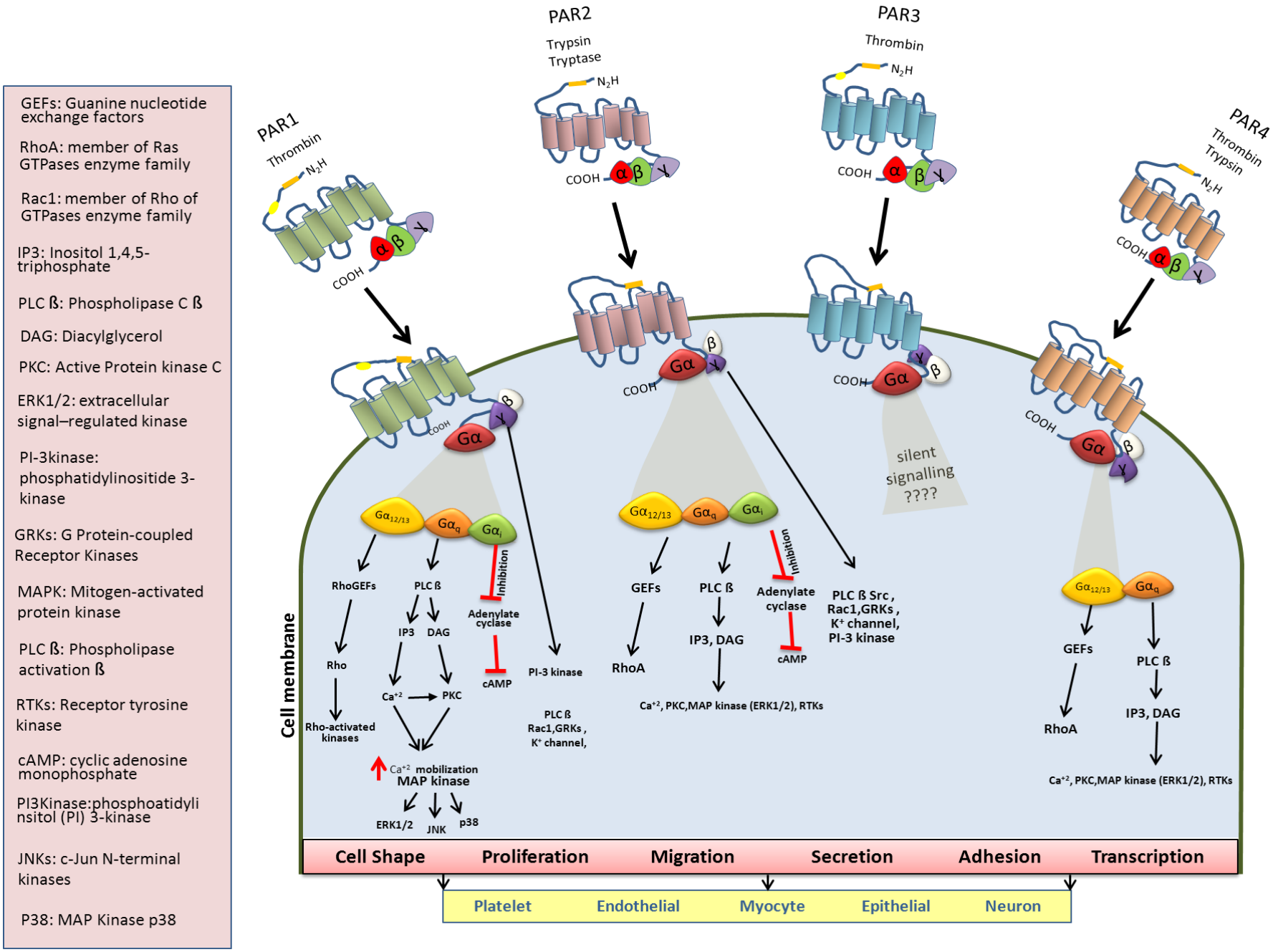


Figure 1.16: G-protein signalling pathways for PAR members.

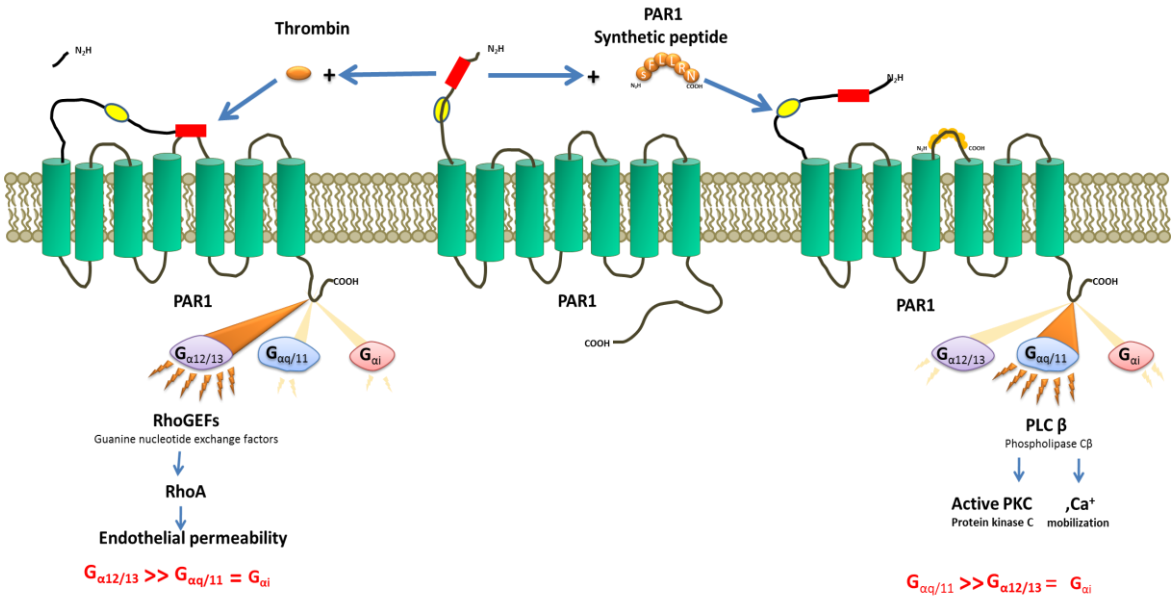


Figure 1.17: Biased agonism of PAR1. Thrombin preferentially activates $G\alpha_{12/13}$ signalling pathways, whereas a synthetic peptide for PAR1 preferentially activates $G\alpha_{q/11}$.

1.4.2 PAR2 signalling

PAR2 signalling has been less investigated compared to wide studies into PAR1 mediated signalling. Similar to PAR1, it has been reported that PAR2 couples to $G\alpha_{q/11}$, $G\alpha_i$, and $G\alpha_{12/13}$ subunits to stimulate cellular responses in several cell types (figure 1.16). Following activation by trypsin or PAR2-AP, PAR2 couples to $G\alpha_{q/11}$ and increases intracellular Ca^{2+}, DAG and IP_3 in several cell types including tumuor cells, ovary cells, neurons and astrocytes (Nysted *et al.*, 1995b; Bohm *et al.*, 1996; Ubl *et al.*, 1998; Bushell *et al.*, 2006). The PAR2 coupling to $G\alpha_i$ is unclear. One study has reported that in enterocytes, PAR2 signalling was not affected when treated with pertussis toxin which is $G\alpha_i$ blocker, suggesting PAR2 did not signal via $G\alpha_i$ pathway (DeFea *et al.*, 2000). On the other hand, another study has shown that in *Xenopus* oocytes, Ca^{2+} signalling in response to trypsin was pertussis toxin sensitive (Schultheiss *et al.*, 1997). The coupling of PAR2 with $G_{12/13}$ has been also reported (Soh *et al.*, 2011).

PAR2 couples with β-arrestin and activates G-protein independent signal transduction. Following receptor activation, β-arrestin will form a complex with

PAR2 to desensitize the receptor. PAR2 differs from other PARs as the complex either promotes internalization of the receptor and facilitates activation of MAPK mediated signalling (figure 1.19). In contrast, the other PARs bind with β-arrestin and produce complexes which promote receptor internalization only. Similar to other GPCRs, PAR2 and β-arrestin complex strongly activates ERK1/2 but only moderately activates p38 MAPK to modulate changes in the actin cytoskeleton (CSK) and cell movement (Staiheim *et al.*, 2005; Belham *et al.*, 1996). However, a study has shown that PAR2 binds with Jun activating binding protein 1(Jab 1) to activate c-Jun which transfer to the nucleus and binds to activator protein 1 (AP1) to initiate gene expression. This signalling pathway may mediate the release of proinflammatory factors and regulates signalling of extracellular proteinases, including trypsin and tryptase, to the nucleus (Luo *et al.*, 2006).

1.4.2.1 Location and biased agonism of PAR2 signalling

Similar to PAR1, PAR2 shows biased signalling and the selectivity depends on location of the receptor. For example, PAR2 in a membrane lipid raft is selectively activated by the tissue factor/factor VII complex rather than other proteinases (Awasthi *et al.*, 2007).

1.4.3 PAR3 and PAR4 signalling

PAR3 only couples with Gα but does not appear to activate signalling pathways on its own. However, it plays a role as co-activator of PAR4 activation. *In vitro* study has shown that thrombin promotes release of ATP from human lung epithelial (A549) cells that do not express PAR1 and PAR4 (Seminor-Vidal *et al.*, 2009). In addition, activation of PAR3 leads to release of ATP through activation of Rho and Ca^{+2} dependent signalling pathways (Seminor-Vidal *et al.*, 2009). To date, there is no evidence for G-protein independent intracellular signalling pathways for PAR3 (Soh *et al.*, 2010).

PAR4 activation leads to the aggregation of platelets in both human and mouse (Kahn *et al.*, 1998). PAR4 couples to $G\alpha_{q/11}$ and $G\alpha_{12/13}$ and can activate different signalling pathways (Nakanishi-Matsui *et al.*, 2000). Activation of PAR4 via

coupling to $G\alpha_{q/11}$ promotes formation of IP_3 (Xu *et al.,* 1998). PAR4 signalling via ERK1/2 was shown in smooth muscle cells (Bretschneider *et al.,* 2001). PAR4 activation by PAR4-AP in microglial cells can induce increasing of the receptor binding to GRK5 and GRK2 (Suo *et al.,* 2003). Activation of PAR4 signalling through p38 MAPK Phosphorylation pathways in human lung endothelial (Fujiwara *et al.,* 2005).

In summary, activation of PARs by either endogenous proteinases or agonists leads to activation of G-protein dependent and independent signalling pathways. Both signalling pathways are crucial for producing specific types of responses and irregularity in those pathways can cause various diseases, including cancer. Therefore, understanding the mechansim of biased agonism of PARs could be useful to develop drug target the PAR signalling selectivity (Russo *et al.,* 2009).

1.5 Desensitization and internalization of PARs

These mechanisms are important to regulate signalling pathways and endocytosis of PARs. Unlike classical GPCRs, PARs are activated irreversibly and the signal is terminated quickly (Soh *et al.,* 2010). Activation of PARs is followed by rapid phosphorylation and desensitization within a few seconds while the phosphorylation of GPCR occurs within minutes (min) (Billington and Penn, 2003). PAR couples with β-arrestin which either activates MAPK signalling pathways or desensitizes and internalizes PARs (Lohse *et al.,* 1990).

1.5.1 PAR1 internalization

Recent studies have shown that the internalization of PAR1 may involve β-arrestin binding (Paing *et al.* 2002). Once PAR1 is activated by endogenous proteinase, the C terminal of the receptor is phosphorylated by a number of GPCR Kinase (GRKs), which include GRK1 and GRK3 (Chen *et al.,* 1994). Then the receptor is rapidly phosphorylated and couples with β-arrestin (Roy *et al.,* 1998). The phosphorylation of the receptor has been found not to be involved in desensitization and degradation of the receptor nor in receptor trafficking (Rickes *et al.,* 2009).

As an alternative to phosphorylation and degradation pathways of PAR1, it may be degraded via deubiquitination (DUB) of the receptor (Chen *et al.,* 2002). Once small protein ubiquitinating enzyme (Ub) combines with the complex to activate PAR1 coupling with clathrin adaptor AP2 and epsin 1, the receptor- AP2-epsin1 complex results in clathrin dependent endocytosis of the activated PAR1 receptor (*Paing et al.,* 2006). The structure specificity of the amino acid sequence of the C terminal is essential for the degradation process. This structure causes high affinity PAR1 binding with the lysosome binding sorting nexin 1 (SNX1) and bicaudal D homolog 1 (BICD1; Ramachandran *et al.,* 2012). These two proteins are important in transporting activated PAR1 from the plasma membrane to endocytic vesicles. In summary, internalized activated PAR1 is directly sorted to lysosome degradation and this process is independent of β-arrestin. Lysosome degradation prevents PAR1 from returning to the cell surface and continuing to signal, therefore, the PAR1 signalling is terminated (Trejo *et al.,* 1998; figure 1.18).

1.5.2 PAR2 internalization

In contrast to PAR1, the desensitization and internalization of activated PAR2 is mediated through coupling of the phosphorylated receptor with β-arrestin (Kumar *et al.,* 2007). In addition, PAR2 is uncoupled from the G-protein and then couples with β-arrestin which leads to stimulation of ERK1/2 signalling and this signalling is sustained due to activated PAR2 in the cytoplasm (Stalheim *et al*., 2005).

The first step of PAR2 endocytosis is phosphorylation of the receptor by GRK enzyme to initiate desensitization and internalization of the receptor (Soh *et al.,* 2010). The phosphorylated receptor produced has an affinity site that interacts with β-arrestin (either β-arrestin 1, β-arrestin 2 or β-arrestin 4; Stalheim *et al*., 2005). When the ß-arrestin is coupled to the activated receptor, this protein can act as an adaptor molecule and interact with several other proteins (Aubry *et al.,* 2009).

β-arrestin interacts with clathrin adaptor protein AP2 and clathrin to regulate formation of clathrin-coated pits and vesicles (Aubry *et al.,* 2009). Finally, the receptor complex is kept in RAB5A positive early endosomes and is either targeted for degradation or recycled back in active form to the cell surface (Soh *et al.,* 2010; figure 1.19).

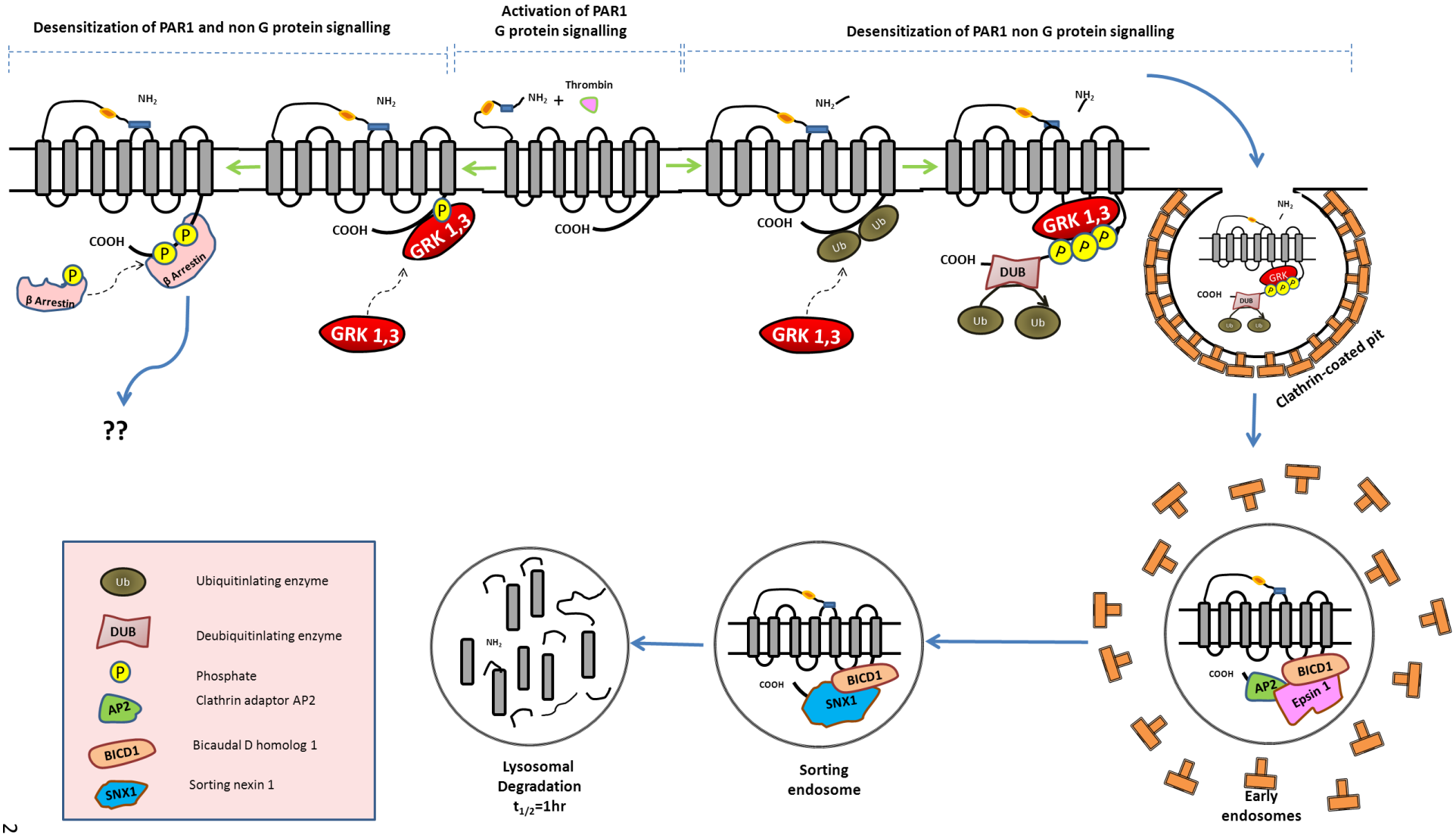


Figure 1.18: PAR1 internalization and desensitization mechanism. (Adapted from Ramachandran *et al.*, 2012)

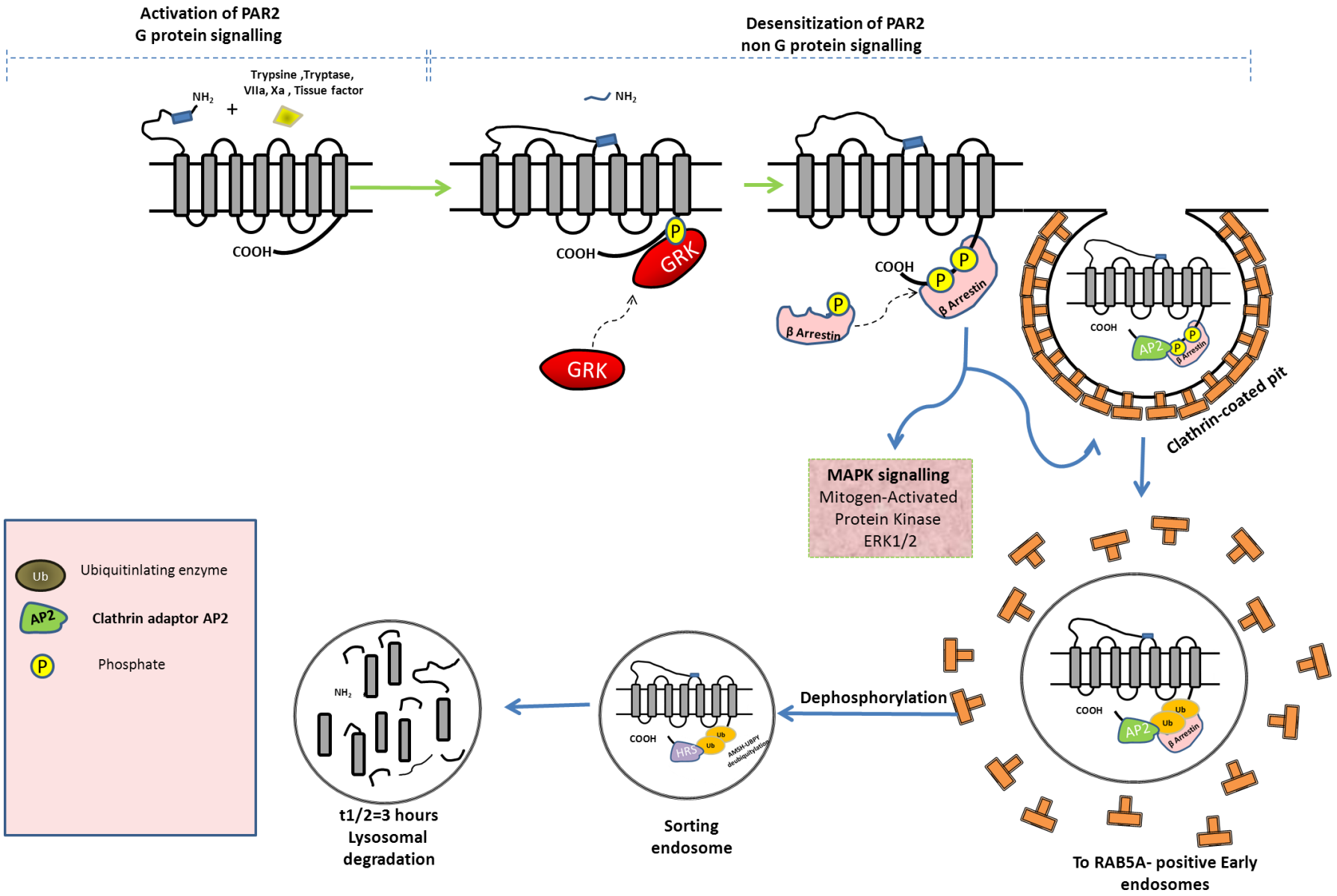


Figure 1.19: PAR2 internalization and desensitization mechanism. (Adapted from Ramachandran *et al*., 2012).

1.5.3 PAR3 and PAR4 internalization

The internalization mechanism of PAR3 is not yet established but a little is known about the mechanism of internalization of PAR4 (Ramachandran *et al.,* 2012). Similar to PAR1, PAR4 internalization also leads to termination of signalling. The phosphorylation of the receptor may not be involved in this mechanism or it has not been clarified yet (Shapiro *et al.,* 2000). Unlike PAR1, the internalization rate of PAR4 is slow or it may be that the absence of phosphorylation of activated receptor leads to sustained signalling which is important for stimulating platelet aggregation (Covic *et al*., 2000). This delay may be due to the absence or delay of the phosphorylation of activated receptor. Current studies have shown that activated PAR4 combines with β-arrestin 2 in platelets but say little about the importance of this combination in regulating receptor transduction (Ramachandran *et al.,* 2012; figure 1.20).

1.6 Crosstalk between different PARs and between PAR and other receptors

In addition to previously discussed mechanisms, there is a possible crosstalk or functional interaction either between different PARs or between PARs and other molecules. Several PARs can be expressed in one cell type supporting crosstalk between receptors, for example, PAR1 and PAR2 are expressed in the cardiovascular system (Coughlin, 2005; Coughlin, 2000). Damiano *et al* (1999) studied the interaction between PAR1 and PAR2 in the cardiovascular system and it was reported that *in vitro* both PAR1-AP (TFLLRN-NH_2) and PAR2-AP (SLIGRL-NH_2) modulate heart rate and blood pressure (Damiano *et al.,* 1999). The same study has shown that some PAR1 effects on heart rate and blood pressure were observed in PAR2 KO mice (Damiano *et al.,* 1999), while in dual PAR1 and PAR2 KO mice no effect on heart rate and blood pressure is seen.

The possible mechansim of crosstalk between these receptors though cleavage of the N-terminal sequence of PAR1 by thrombin to unveil a new sequence which then binds to a second extra loop of PAR2 and activates the signalling pathway of PAR2 (Damiano *et al.,* 1999; figure 1.21).

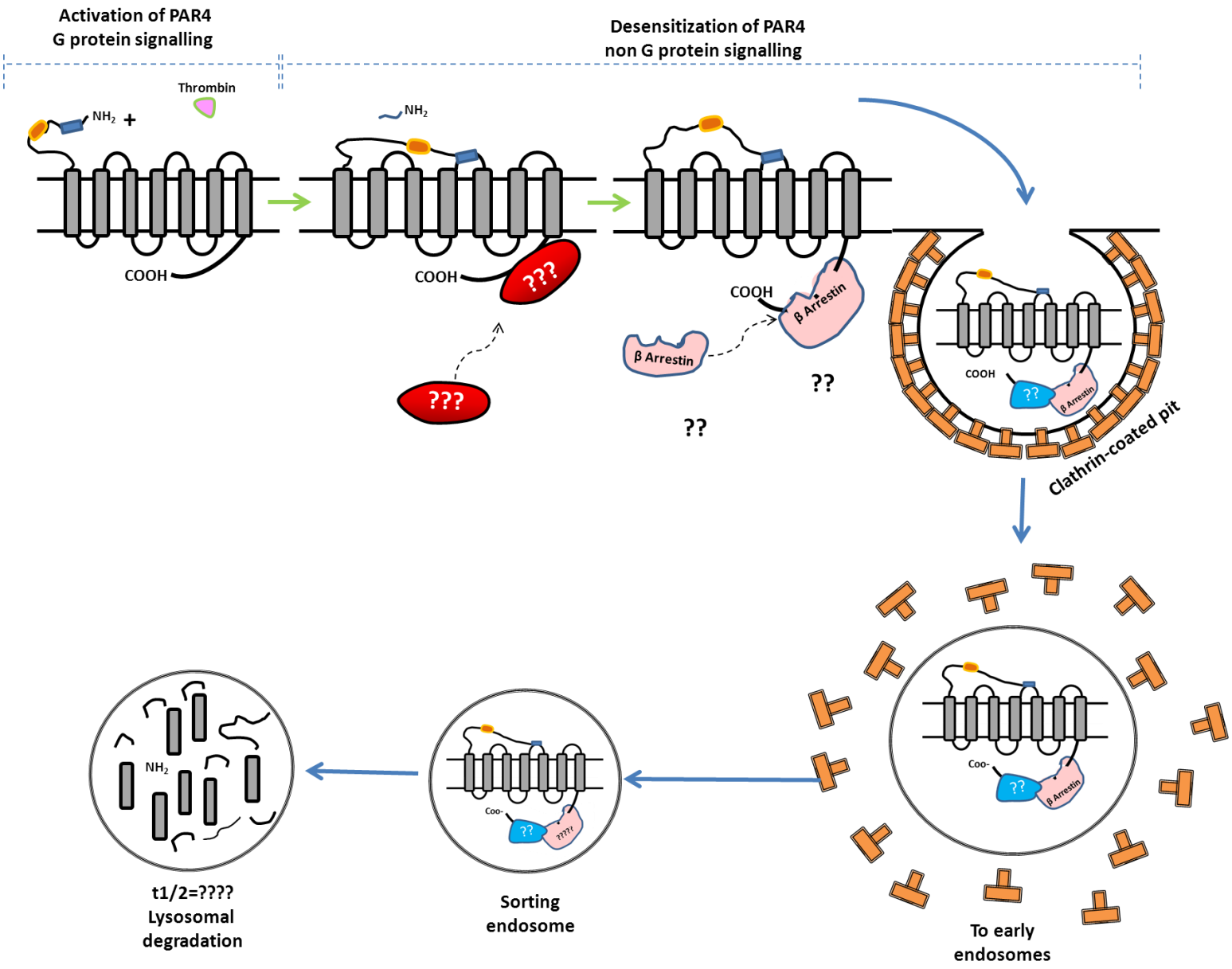


Figure 1.20: PAR4 internalization and desensitization mechanism. (Adapted from Ramachandran *et al*., 2012).

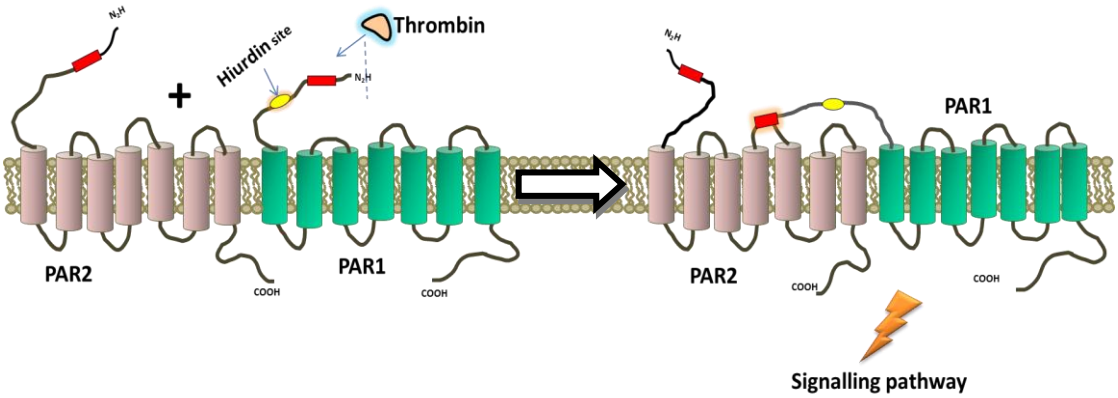


Figure 1.21: Crosstalk between PAR1 and PAR2. Thrombin cleaves N-terminal sequence of PAR1 and unveils a new sequence which then binds to the second extra loop of PAR2 and activates signalling pathway.

Additionally, a novel study by Plevin's laboratory has identified that during inflammation, there is crosstalk between PAR2 and PAR4, PAR2 enhanced glycosylation of PAR4 and activation of PAR4 signalling mediated proinflammatory response (Cunningham *et al.*, 2012).

A recent study has shown that PAR2 signalling interacts synergistically with Toll-like receptor 4 (TLR-4) in the inflammatory response. TLR-4 detects lipopolysaccharides (LPS) from gram-negative bacteria and plays an important role in activation of the innate immune system. Co-expression of PAR2 and TLR-4 complex causes synergy of signalling pathways compared to signalling pathways for PAR2 and TLR-4 individually as shown in chapter 4, figure 4.16 (Rallabhandi *et al.*, 2008).

1.7 Endogenous activators and inhibitors of PARs

Endogenous proteinases also play an important role in regulation of PAR signalling. Proteinases regulate signalling pathways either by activation of PARs through formation of a tethered ligand or inhibition of PARs through disarming of the amino terminal (Coughlin, 2005). As has been explained previously thrombin preferentially activates both PAR1and PAR3 which contain high affinity binding sites for thrombin called hirudin sites (figure 1.87 and 1.11). PAR4 does not contain such a site and, therefore, is less selective for thrombin (Kawabata, 2002). PAR2 is distinct from

other PARs as it is preferentially activated by trypsin over thrombin and other proteinases (Bucci *et al.*, 2005; figure 1.22). For each individual PAR the endogenous PAR activators are explained in detail in table 1.1.

In addition to thrombin other PAR1 activators have been proposed such as trypsin, plasmin, granzyme A, and Cathepsin G (Parry *et al.*, 1996). However, cathepsin G, proteinase and human leucocyte elastase inactivate PAR1 by destroying the tethered ligand (Ossovskaya and Bunnett, 2004; figure 1.22). PAR2 is activated by tryptase similarly to trypsin, however, tryptase can also inhibit PAR2 via destruction or removal of the tethered ligand (Ossovskaya and Bunnett, 2004; figure 1.22). Thus, in comparison to other PARs, PAR2 has a potentially complex mode of activation depending upon the tissue or cellular expression of the relevant activators (Valeriani *et al.*, 2004). More than one activator could activate one PAR and one proteinase could activate more than one PAR, therefore, it is difficult to target proteinase activator or inhibitor for development of drug either activates or inhibits PARs.

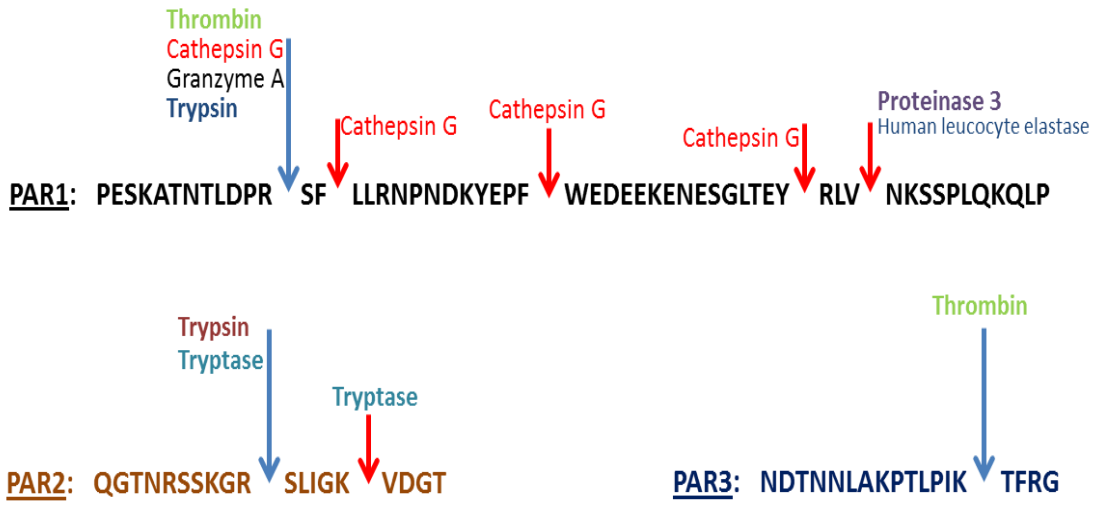


Figure 1.22: Principal proteinase cleavage site for PAR1, PAR2 and PAR3. Proteinase activates the PAR receptor if it unveils the tethered ligand (blue arrow) but may inactivate the receptor if the proteinase destroys or removes the tethered ligand (red arrow). Adapted and modified from Dery *et al.*, 1998.

1.7.1 Proteinases in the central nervous system (CNS)

Many studies have reported that PARs and their proteinases are expressed in both peripheral and central nervous system (Striggow *et al.*, 2001). For example,

thrombin and the substances required for production and regulation of thrombin, such as prothrombin and its activator (Factor X), are also expressed and have been detected in rat and human neuronal cell lines (Dihanich, *et al.,* 1991; Deschepper *et al.,* 1991). In addition, thrombin inhibitors such as proteinase nexin-1 (PN1) and anti-thrombin 3 (AT3) have also been detected in astroglia (Weinstein *et al.,* 1995). In the peripheral nervous system following injury to blood vessels or tissues, proteinases, such as thrombin and cathepsin G, are released (Vergnolle, 2003). Thrombin and cathepsin G regulate PAR1 and PAR4 function in the vicinity of the nerve through activation or inhibition of PARs (Asfaha *et al.,* 2007). Thrombin activates PAR1 and PAR2 while cathepsin G inhibits PAR1 and activates PAR4 (Noorbakhsh *et al.,* 2003).

Trypsin and tryptase are known to activate PAR2; however, trypsin is not present in all tissues. Tryptase is released from mast cells in the CNS in the choroid plexus, parenchymal and perivascular areas and adjacent to peripheral nerves (Stead, *et al.,* 1978; Theoharides *et al.,* 1990). Other PAR2 proteinases have been detected in the brain, including trypsin like proteinase, activated factor X and trypsinogen-IV (Sawada *et al.,* 2000); however, their function is still not well understood. Additionally, proteinases that regulate PAR functions in the CNS include thrombin, plasmin, and tissue kallikreins but it is difficult to identify the proteinases that regulate PAR2 function in the CNS (Oikonomopoulou *et al.,* 2006; Nagai *et al.,* 2006).

CNS diseases states have shown that the levels of proteinases that regulate PAR function are modulated in CNS, for example, upregulation of thrombin and downregulation of plasmin and neuropsin in Alzheimer's Disease (Ogawa *et al.,* 2000; Boven *et al.,* 2003; Dotti *et al.,* 2004). Additionally, there is upregulation of neurosin and neuropsin in experimental autoimmune encephalomyelitis and spinal cord injury is proposed to lead to an increase PAR2 stimulation (Terayama *et al.,* 2004).

1.8 Physiological and pathophysiological role of PARs

1.8.1 PARs in the cardiovascular and circulatory system

PARs are expressed in the cardiovascular and circulatory system on endothelial cells and vascular smooth muscle cells (Molino *et al.,* 1998). Activation of endothelial PAR1 causes vascular relaxation in both the human and rodent under physiological conditions, while following endothelial damage it causes muscle contraction (Ku and Dai, 1997). In vascular smooth muscle, PAR1 activation causes proliferation (Leger *et al.,* 2006). Similar to PAR1, activation of endothelial PAR2 causes vascular relaxation in both humans and rodents (Hamilton *et al.,* 2002). Moreover, activation of PAR1 increases human platelet aggregation and, in animal models, inhibition of PAR1 reduces thrombosis formation (Cook *et al.,* 1995). In addition, PAR2 activation can cause proliferation of vascular smooth muscle and endothelial cells and stimulation of angiogenesis (Milia *et al.,* 2002). Additionally, inflammatory agents like LPS and tissue damage are found to upregulate PAR2 in the vasculature (Cicala *et al.,* 1999). In contrast, PAR3 has no effect on vascular tone but it works as a cofactor in the regulation of platelet functions in mice. In normal conditions, activation of PAR4 in coronary arteries has no effect on vascular tone, however, during inflammation PAR4 activation leads to relaxation of these vessels (Hamilton *et al.,* 2001). As well, activation of PAR1and PAR4 regulates human platelet function (Cook *et al.*, 1995).

1.8.2 PARs in the Respiratory System

All PARs are expressed in the respiratory system, including pulmonary fibroblasts, endothelial and epithelial cells and smooth muscle (Walker *et al.* 2005). In addition to PARs, several proteinases such as tryptase, trypsin and neutrophil proteinase are expressed in the respiratory system and regulate signalling pathways (Asokananthan *et al.,* 2005). The most important role of PARs in respiratory system is modulation of vascular tone by either causing contraction or relaxation of smooth muscles (Lan *et al*., 2002). They are also involved in remodelling and regulation of the inflammatory response through recruitment of inflammatory cells (Lan *et al*., 2002).

Some studies indicate that activation of PAR1 in the airway can modulate cell mitogenesis (Ramachandran *et al.,* 2007). However, upregulation of PAR1 induces pulmonary fibrosis, therefore, PAR1 antagonists could play a key role in the treatment of fibrotic lung diseases (Yasui *et al.,* 2001).

PAR2 is expressed on epithelial cells and fibroblasts in the lungs (Ramachandran *et al.,* 2006). Activation of PAR2 produces a protective response either as a bronchodilator or as a bronchoconstrictor under normal physiological conditions. However, PAR2 activation leads to an increase in the severity of pulmonary constriction in diseases such as asthma (Berger *et al.,* 2001). Therefore, inhibition of PAR2 may be helpful in the treatment of asthma. Additionally, PAR2 may also play a crucial role in respiratory infections caused by bacteria (Moraes *et al*., 2008).

In endothelial cells, activation of PAR4 is involved in development of fibrosis through stimulation of collagen production (Ramachandran *et al*., 2007). However, to date, the role of PAR4 in the respiratory system is unclear and further information is required to understand its role in inflammation (Ramachandran *et al*., 2012).

1.8.3 PARs in the gastrointestinal system

The gastrointestinal system is the system of all the body systems that is most exposed to proteinases. Firstly, from physiologically related processes such as digestion and secondly, from exposure to the bacterial flora of the gut. All of the PARs have been found to be expressed in the gastrointestinal tract and PAR activation is involved in permeability, ion transport, motility and inflammation (Macfarlane *et al*, 2001). PAR1 is also involved in cell membrane permeability and regulates the passage of fluids and microorganisms across gut mucosa; for that reason activation of PAR1 has a protective effect against pathogens and toxins (Cenac *et al.,* 2004). Other studies have shown that PAR1 coordinates both contraction and relaxation within the gastrointestinal system to enhance gastrointestinal transit (Kawabata *et al.,* 2004).

PAR2 expression is detected on enterocytes, epithelial cells, smooth muscle cells, endothelial cells, colonic myocytes, myofibroblasts and neurons (Vergnolle, 2004). The functions of PAR2 are similar to that of PAR1, that is, a protective effect against

pathogens and toxins (Ramachandran *et al.,* 2012). In addition, activation of PAR2 mediates inflammation via activation of enteric nerves and the release of neuropeptides (Vergnolle, 2004). It is widely believed that PAR2 may also play an important role in ulcerative colitis and it plays a role in gastric mucosal cytoprotection through activation of sensory neurons. Additionally, PAR2 regulates gastrointestinal smooth muscle motility and activates exocrine secretion by the salivary glands and pancreas (Kawabata *et al.,* 2004).

There has been little study into the role of PAR3 in the gastrointestinal system. However, an *in vivo* study has reported that activation of PAR4 causes contraction of colonic longitudinal muscle (Mule *et al.,* 2002).

1.8.4 PARs in the urinary system

PAR studies showed that PAR1, PAR2 and PAR4 are widely expressed in kidney cells (Gui *et al.,* 2003). Some studies have shown a relationship between renal inflammation and PAR1 (Chen *et al.,* 2008). *In vivo,* treatment with PAR1 activating peptide (SFLLRN-NH_2) caused a greater inflammatory response in wildtype (WT) mice compared to PAR1 KO mice (Cunningham *et al.,* 2000). However, PAR2 activation has been found to cause vasodilatation in a perfused rat kidney model and mediates mesangial cell proliferation in normal physiological condition (Tanaka *et al.,* 2005). Moreover, activation of PAR2 during acute renal inflammation has a cytoprotective effects (McHowat and Creer, 2001).

1.8.4.1 PARs and peripheral inflammatory diseases

Some studies showed PAR2 are involved in inflammatory disease such as ulcerative colitis and Rheumatoid Arthritis**.**

1.8.4.1.1 PAR2 and ulcerative colitis

Ulcerative colitis is a form of inflammatory bowel disease which is characterized by swelling and ulceration of the large intestine (Travis *et al.,* 2011). The relationship between PAR2, mast cells, trypsin and tumour necrosis factor-α (TNF-α) were studied in this disease. Treatment of human mast cells with a PAR2 agonist leads to

increased secretion of TNF-α in the tissue culture (Kleij and Bienenstock, 2005). Both PAR2 and TNF-α are also detected in normal and ulcerative colitis tissue. However, in ulcerative colitis tissue tryptase was also detected in addition to PAR2, TNF-α, and mast cells (Wallace and Chin, 1997). This study suggests that mast cells release tryptase which activates PAR2 which could play a key role in the pathogenesis of ulcerative colitis and thus could be useful in the development of drugs for the treatment of this condition (Kim *et al.,* 2003). Recently, it was suggested that PAR2 antagonism is a potential new therapy for the treatment of human chronic inflammatory bowel diseases (Lohman *et al.,* 2012).

1.8.4.1.2 PAR2 and Rheumatoid Arthritis

Several studies have observed the essential role of PAR2 in chronic inflammation (Suen *et al.,* 2010; Sevigny *et al.,* 2011; Lohman *et al.,* 2012). Further investigation has also noted the role of PAR2 in the treatment of chronic inflammation, such as rheumatoid arthritis (Fiorucci and Distrutti, 2002; Crilly *et al.,* 2012; Lohman *et al.,* 2012). This disease is characterized by joint swelling which is caused by an elevated level of inflammatory cytokines, including interleukins (IL) and TNF-α (Gordon and Urowitz, 1982). It was shown that the disease is also associated with upregulation of PAR2 in synovial joints (Kelso *et al.,* 2006; Kelso *et al.,* 2007; Crilly *et al.,* 2012). PAR2 KO mice were studied to investigate the role of PAR2 in arthritis and it was found that there was a four-fold reduction in joint damage compared to WT mice. In addition, injecting WT mice with a PAR2 agonist induced prolonged joint swelling and synovial hyperthermia (Ferrell *et al.,* 2003). These studies suggest that PAR2 antagonism could help to reduce swelling in joint inflammation. In recent years, there has been an increase in interest into the use of PAR2 antagonists in the treatment of rheumatoid arthritis in patients who do not respond to TNF-α inhibitors which is the classic treatment for artherities (Kelso *et al.,* 2006; Kelso *et al.,* 2007; Crilly *et al.,* 2012). Therefore, PAR2 could be a useful target in the development of antirheumatic drugs.

1.9 PAR1 in the CNS

1.9.1 Localization and normal physiological role of PAR1 in the brain

The four PAR subtypes are expressed and regulated differently in the CNS rodents (Ubl *et al.,* 1988; Grishina *et al.,* 2005; Kaufmann *et al.,* 2000; D' Andera *et al.,* 1998). PAR1 is present in the CA2 and CA3 of the pyramidal layer of the hippocampus, but it is also expressed in neurons of the thalamus, amygdala and cortex (Striggow *et al.,* 2001). PAR1 has been found on astrocytes, microglia and neurons (Striggow *et al.,* 2001; Ishida *et al.,* 2006). In normal conditions, activation of PAR1 by thrombin is neuroprotective and reduces cell death in neurons and astrocytes in response to hypoglycaemia or oxidative stress (Vaughan *et al.,* 1995). Additionally, activation of PAR1 has a crucial role in the regulation of astrocyte morphology and proliferation (Boven *et al.,* 2003). The concentration of PAR1 agonists, such as thrombin, can influence the effect of PAR1 on astrocyte morphology and proliferation. Low thrombin concentrations resulting in lower PAR1 activation that can affect astrocyte morphology and lead to abnormal blood-brain barrier (BBB) function (Noorbakhsh *et al.,* 2003). However, high concentrations of thrombin cause over activation of PAR1 which stimulates cell proliferation of astrocytes resulting in astrogliosis (Nicole *et al.,* 2005). Furthermore, in the CNS PAR1 is also involved in synaptic plasticity (Gingrich *et al.,* 2000; Suo *et al.,* 2002). Moreover, *in vitro* study identified a protective role for PAR1, as treatment of neuronal cultures with low concentrations of thrombin or PAR1-AP can reduce β-amyloid neurotoxicity measured as a decrease in growth factor release (Pike *et al.,* 1996).

1.9.2 Role of PAR1 in Synaptic Plasticity

In astrocytes, activation of PAR1 stimulated the release of glutamate (Lee *et al.,* 2007; Shigetomi *et al.,* 2008). Increase in extracellular glutamate levels sequentially stimulates *N*-methyl *D*-aspartate (NMDA) receptors on the neighbouring neurons (Lee *et al.,* 2007). In addition, it has been established that activation of PAR1 has been shown to enhance the function of the NMDA receptor; this receptor is proposed to play an important in learning and memory (Hamill *et al.,* 2009). Activation of

NMDA receptors allows influx of extracellular Ca^{2+} through it. It is thought that the influx of Ca^{2+} through these receptors plays a critical role in synaptic plasticity, a cellular mechanism involved in learning and memory (Lee *et al.,* 2007). Additionally, activation of PAR1 by thrombin potentates the function of NMDA receptors in hippocampal slices (Lee *et al.,* 2007) and enhances NMDA receptor-mediated long term potentiation (LTP) which leads to improvements in both learning and memory (Almonte *et al.,* 2007; Wang *et al*., 2008). Almonte *et al* (2007) observed that PAR1 KO mice, have deficits in learning and memory in two tasks, passive avoidance and cued fear conditioning, compared to WT mice (Almonte *et al.,* 2007). This finding suggests that PAR1 may play an important role in emotionally motivated learning. Recently, further work of Almonte *et al* (2012) observed that activation of PAR1 leads to increased levels of NMDA-dependent LTP in mice hippocampus slices compared with PAR1 knockout mice. This result suggests an improtant function of PAR1 in NMDA-dependent memory formation and synaptic plasticity in the hippocampus (Almonte *et al.,* 2012).

1.9.3 PAR1 and Alzheimer's Disease

PAR1 plays a role in Alzheimer's disease (Pompili *et al.,* 2011). Some studies have shown decreased levels of the thrombin inhibitor and protein nexin-1 (SNX1), which is a sorting protein involved in regulating the trafficking and internalization of PAR1 from the early endosome to the lysosome (Choi *et al.,* 1995). Decreased levels of SNX1 can cause irregular PAR1 signalling and over activation of the receptor. Additionally, Alzheimer's Disease is associated with increased levels of thrombin and expression of PAR1 which causes neurotoxicity, thus, these findings suggest that PAR1 has a detrimental role in Alzheimer's Disease (Yin *et al.,* 2010). Furthermore, another study using an *in vitro* model of Alzheimer's Disease showed that PAR1 expression levels increased significantly in hippocampal astrocytes (Pompili *et al.*, 2004).

1.9.4 PAR1 and Parkinson's disease

A study has reported upregulation of PAR1 in astrocytes in human Parkinson's disease brain (Ishida *et al.,* 2006). This study has observed increased PAR1

expression in the substantia nigra pars compacta of these patients. PAR1 is widely expressed in astrocytes and the numbers of astrocytes expressing PAR1 is increased in brain from Parkinson's disease patients compared with normal controls (Ishida *et al.*, 2006).

1.9.5 PAR1 and human immunodeficiency virus (HIV) infection

PAR1 has also been suggested to have a detrimental role in acute brain inflammation and may contribute to the inflammatory response. Acute brain inflammation in HIV and encephalitis has been associated with increased PAR1 expression and activation of signalling pathways (Boven *et al.,* 2003). In human astrocyte cultures, treatment with thrombin or a PAR1 agonist has been shown to increase interleukin-1β (IL-1β) and nitric oxide and upregulate expression of PAR1 mRNA which can promote apoptosis. These studies suggest that activation and upregulation of PAR1 possibly participate to both brain inflammation and neuronal damage in HIV infection (Boven *et al.,* 2003).

1.9.6 Role of PAR1 in CNS injury

In CNS disorders PAR1 activation by either its endogenous proteinase or synthetic agonist exerts a cytotoxic effect and induces morphological changes in neuronal cells (Debeir *et al.,* 1998). Several studies have shown that activation of PAR1 by thrombin has an important role in CNS injury. For example, in patients with cerebrovascular injury levels of thrombin are increased at the site of injury resulting in activation of microglia and astrocytes (Akiyama *et al.,* 2000). Additionally, it is widely believed that thrombin is released in the brain tissue after the damage of BBB that is a characteristic feature of brain ischaemia, for example stroke (Chodobski *et al.,* 2011). *In vitro* studies showed that thrombin is able to kill cultured hippocampal neurons and astrocytes mediated by PAR1 activation (Donovan *et al.*, 1997). It has been shown that thrombin activity is elevated in the ischaemic brain areas (Xi *et al.,* 2003). *In vivo* studies, it was noticed that PAR1 contributed to the infarct volume in the brain after transient focal cerebral ischaemia that was significantly decreased in PAR1 KO mice (Junge *et al.,* 2003). This effect was not observed in PAR1 KO mice after unilateral cerebral hypoxia/ischaemia (Olson *et al.,* 2004). Furthermore,

activation of PAR1 in the CNS by another proteinase, known as metalloproteinase 9, caused increased neuronal toxicity in a mouse model of intercerebral haemmorhage (Xue *et al.,* 2006)

Briefly, in normal physiological conditions activation of PAR1 by thrombin plays a crucial role in morphology and proliferation of astrocytes and can exert a neuroprotective effect. In Alzheimer's disease activation of PAR1 can exacerbate neurotoxicity and upregulation of PAR1 expression is also observed in HIV encephalitis.

1.10 PAR2 in the CNS

1.10.1 Localization and normal physiological function of PAR2 in the brain

PAR2 is expressed on neurons in the CA1, CA2, CA3 regions of the hippocampus (Smith-Swintosky *et al.,* 1997; Bushell et al., 2006), but also in all the cortical layers of the amygdala, thalamus, hypothalamus and the striatum (Striggow *et al.,* 2001; Reed *et al.,* 2003). Similar to thrombin, the existence of trypsin, which is a major activator of PAR2, suggests intrinsic regulation and receptor interaction of this potent proteinase in the brain. However, *in vivo,* PAR2 has been shown to activate peripheral sensory neurons (Amadesi *et al.,* 2006). However, an *in vitro* study has shown that the strong response in neurons in culture to both trypsin and PAR2 peptides like SLIGRL-NH_2 is an IP_3-mediated increase in intracellular Ca^{2+} (Hong *et al.,* 2008). Smith-Swintowsky *et al* (1997) showed that SLIGRL-NH_2 is neurotoxic to cultured pyramidal neurons in a concentration-dependent manner (Smith-Swintosky *et al.,* 1997). In astrocytes, activation of PAR2 with trypsin or SLIGRL-NH_2 has been shown to prompt reversal of stellation of cultured astrocytes (Park *et al.,* 2006). In a recent study *in vitro* exposure of hippocampal slice cultures to the PAR2 agonist SLIGRL-NH_2 did not induce cell death. Kainate agonists which bind to AMPA/kainate receptors (subtypes of glutmate receptor) induced neurotoxicity and cell death which was reduced by exposure to the PAR2 agonist SLIGRL-NH_2. This experiment suggests that PAR2 agonists can protect neurons against kainate-induced neurotoxicity (Greenwood and Bushell, 2010). Studies on PAR2 function in the peripheral nervous system have noted a role for PAR2 receptors in neurogenic

inflammation in peripheral nervous system, hyperalgesia, analgesia and itching, as well as playing roles in nerve regeneration, gastric epithelial ion secretion and mucous secretion function (Poole *et al.,* 2013). Recently, PAR2 has been implicated in the stimulation of the ion channels, transient receptor potential channels vanilloid-1 (TRPV-1) and -4 (TRPV-4), which are essential for neurogenic inflammation and pain (Poole *et al.,* 2013).

1.10.2 Role of PAR2 in synaptic plasticity

PAR2 is expressed in astrocytes and microglia cells, activation of this receptor will mediate cell signalling through the $G_q/_{11}$ PLC pathway leading to influx of Ca^{2+} from the extracellular space or release from internal stores (Bushell *et al.,* 2006). This highlights the important role of Ca^{2+} signalling in hippocampal synaptic plasticity. Additionally, endogenous proteinases, including tissue plasminogen activator, neuropsin, motopsin, trypsin and thrombin, are associated with neuronal plasticity and matrix remodelling related to synaptogenesis, LTP, long term depression (LTD) and memory (Pawlak and Strickland, 2002).

PAR2 may also have an important role in learning and memory. PAR2 may influence learning and memory as it is highly expressed in the hippocampus and can activate NMDA receptors, which are associated with hippocampal neuronal plasticity, and potentiate LTP (Lohman *et al.,* 2008). Recently, Lohman *et al* (2009) studied the effect of administration of the PAR2 agonist SLIGRL-NH_2 (1.5 mg/kg) on learning and memory in the rats. Administration of SLGRL-NH_2 induced memory deficits in two memory tasks, the Morris water maze (MWM) test and fear avoidance learning produced by repeated exposure to the elevated plus maze (EPM) test. In addition, these results may have implications for the understanding of experience-dependent learning processes and suggest new roles for PAR2 the in neuroplasticity of limbic structures in the brain.

Recent study indicates that activation of PAR2 can cause cell depolarization of hippocampal neurons and subsequent decrease in action potential frequency. PAR2-induced hippocampal neuronal depolarization is inhibited either after inhibition of astrocyte function or after antagonism of glutamate receptors. At the same time,

inhibition of astrocyte cell function reduces PAR2-induced reduction in action potential frequency. On the other hand, when studied in acute hippocampal slices, activation of PAR2 induced a profound LTD of synaptic transmission. This effect is dependent on NMDA receptor activation and is sensitive to disorders of astrocytes function. This finding suggests that PAR2 activation indirectly inhibits hippocampal synaptic activity (Gan *et al.*, 2011).

1.10.3 PAR2 and HIV infection

HIV-associated dementia is a neurodegenerative disease that occurs in patients with acquired immunodeficiency syndrome (AIDS) which is characterized by gradual failure of the immune system (Lindl *et al.*, 2010). Similar to PAR1, PAR2 also plays an important role in CNS disorders such as HIV infection of the brain. It was observed that patients with HIV-associated dementia have an upregulation of PAR2 on neurons in conjunction with neuro-inflammation in brain tissue compared with normal patients (Noorbakhsh *et al.*, 2005). This result suggests that PAR2 has a neurodegenerative role in the CNS. In patients with HIV-associated dementia, over expression of PAR2 and increased levels of proinflammatory cytokines (PICs), including TNF-α and interlukein-1β, in the brain have been detected. Activation of PAR2 and upregulation of TNF-α can protect neurons from toxicity induced by the HIV encoded protein, trans-activator of transcription (tat). Additionally, studies in an animal model of HIV demonstrated that there was an increase in neuroinflammation and neuronal damage in PAR2 KO mice compared with WT mice. As such, activation of PAR2 can be considered to be neuroprotective in HIV (D'Andrea *et al*., 1998; Noorbakhsh *et al*., 2005).

1.10.4 PAR2 and multiple sclerosis

Multiple sclerosis is neurodegenerative disease that is caused by inflammatory demyelinating of certain nerve fibers and results in disability in coordination movement (Hafler *et al.*, 2005). Similar to PAR1, PAR2 also plays an important role in CNS disorders, including multiple sclerosis. It is observed that the inflammatory cytokines such as TNF-α, TNF-β, IL-1β, and IL-6 are upregulated in this disease (Campbell, 2004). A study by Noorbakhsh (2006) observed that PAR2 modulates

neuroinflammation in multiple sclerosis disease (Noorbakhsh *et al.*, 2006). It was shown that over expression of PAR2 have been shown increased on astrocytes and infiltrating macrophages in brain white matter from human multiple sclerosis samples (Noorbakhsh *et al.*, 2006). In patients with multiple sclerosis, a high number of mast cells have been identified in brain tissue and cerebral spinal fluid (Noorbakhsh *et al.*, 2006). Additionally, PAR2 KO mice showed a significant decrease in microglial activation and T lymphocyte infiltration accompanied by enhanced demyelination and axonal injury in the CNS compared with WT mice (Noorbakhsh *et al.*, 2006). A similar observation is reported in experimental autoimmune encephalomyelitis in the mouse (Noorbakhsh *et al.*, 2006).

1.10.5 PAR2 and radiation damage

It was shown that expression of PAR2 is upregulated after experimental CNS radiation damage. This upregulation of PAR2 is observed for up to 40 days post-radiation exposure (Olejar *et al.*, 2002). Radiation treatment can induce radiation injury and activate chronic inflammation pathways (Monje *et al.*, 2003) which can cause abnormalities in hippocampal neurogenesis (Abayomi, 2002). As a result, upregulation of PAR2 during radiation injury may cause inhibition of neurogenesis that may contribute to cognitive deficits associated with radiation therapy (Ekdahl *et al.*, 2003).

1.10.6 PAR2 and ischaemic brain injury

PAR2 is also involved in ischaemic brain injury. Acute focal cerebral ischaemia produced by transient occlusion of the middle cerebral artery (tMCAO) caused an upregulation of PAR2. It is proposed that PAR2 exerts a neuroprotective effect in focal cerebral ischaemia as infarct volume was increased in PAR2 KO mice compared to WT mice. This result suggests that PAR2 has a neuroprotective role in the CNS (Striggow *et al.*, 2001; Jin *et al.*, 2005).

1.10.7 Upregulation of PAR2 has a dual function in inflammatory disease and conditions

In further investigation of increased expression of PAR2, it has been observed that upregulation of pro-inflammatory cytokines (PIC) is associated with increased neurological diseases and in experimental models (Bushell, 2007). Upregulation of PAR2 can have a dual effect, either improving neuroprotection or worsening neurodegenerative effects. The effect of PAR2 upregulation depends on the cell type and extent of receptor expression. It has been suggested that low expression of PAR2 is neuroprotective whereas over expression of PAR2 can cause neurodegeneration (Bushell, 2007).

1.11 PAR3 in the CNS

Similarly, PAR3 is highly expressed in the pyramidal cells of the CA1, CA2, CA3, dentate hilus and granule cells of the dentate gyrus of the hippocampus (Striggow *et al.*, 2001), also is located in cortical neurons, microglia and astrocytes (Wang *et al.*, 2002). The expression of PAR3 in neurons is generally restricted to the soma (Striggow *et al.*, 2001).

1.12 PAR4 in the CNS

It was observed that PAR4 is expressed in neurons in all regions of the hippocampus, and in all cortical layers, as well as the thalamus, hypothalamus and amygdala (Striggow et al., 2001). In Alzheimer's disease activation of PAR4 appears to mediate the formation of tau protein aggregates resulting in delayed hippocampal neuronal death (Suo *et al.*, 2003). These findings suggest that thrombin, through activation of PAR4, is involved in the pathophysiology of Alzheimer disease. PAR4 activation also mediates thrombin-induced microglia activation (Suo *et al.*, 2003).

1.13 PAR2 as a target for drug discovery

1.13.1 Challenges in PAR Research

PARs activate a variety of second messengers that initiate signalling cascades in different tissues and cells, therefore, it is not surprising that PARs have a role in physiological functions and pathophysiological conditions. However, quantitative and specific analysis of PAR involvement in several processes has been

compromised by many restrictions. Firstly, PARs functions in a complex system. PAR signalling requires a communication among many proteinases, proteinase inhibitors and cofactors in a timing dependent manner. In several situations, for example in CNS, the full components of this system are still not fully understood. Secondly, although much progress and effort has been made for development of PARs antagonist, selective and potent antagonists for all PARs are not available (table 1.1). Thirdly, there is difficulty in development of selective serine proteinase inhibitors or activator because, in most cases, more than one proteinase activator could activate one member of PAR or one proteinase could activate more than one PAR (figure 1.23). Finally, the endogenous activators for PARs in the CNS are not fully understood. For example, trypsin modulates many physiological functions in the CNS although the target of endogenous trypsin activates PAR2 in the brain is under investigation.

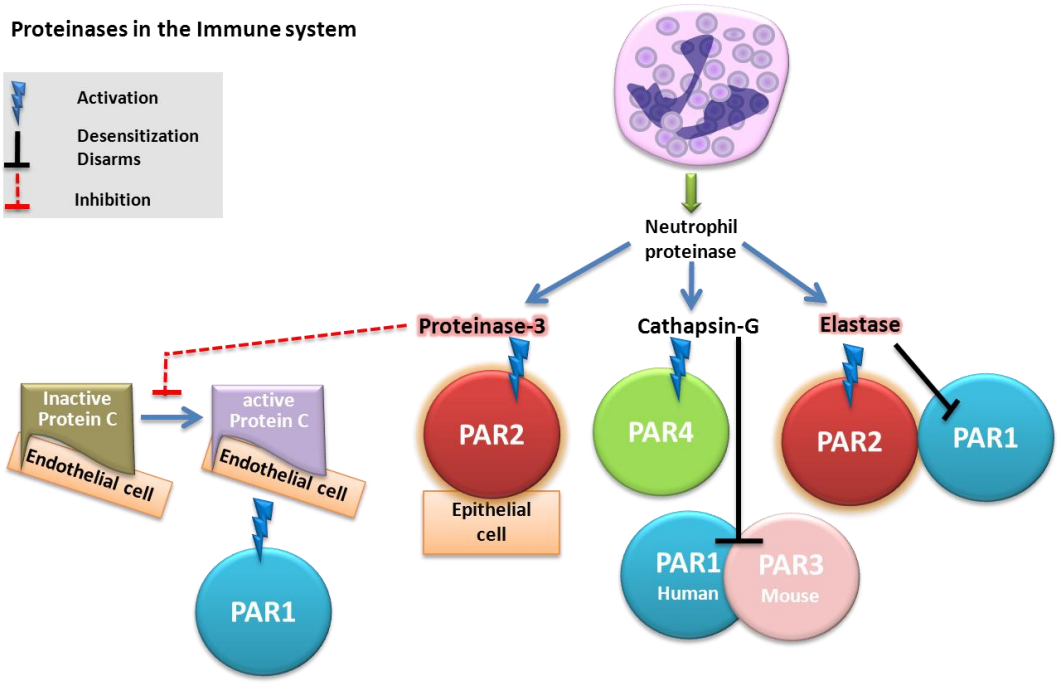


Figure 1.23: Proteinase regulates haemostasis. Some proteinases can activate more than one PAR, for example, thrombin activates PAR1, PAR3 and PAR4, however, one proteinase can either activate or disarm the same PAR such as plasmin which activates and disarms PAR1. Other proteinases can activate one PAR and disarm another PAR such as activated protein C which can activate PAR1 and PAR4 but desensitize PAR2.

1.13.2 PAR Agonists and antagonists

Cloning studies have identified species differences between the tethered ligand in PARs which have been shown to differ between human and mouse receptors (Macfarlane *et al*, 2001; figure 1.9). PAR-APs, which mimic the tethered ligands, have been utilised extensively to investigate PAR function (Steinberg, 2005; section 1.3.1, 1.3.2, 1.3.3 and 1.3.4). Recent studies have identified potential small molecule agonists and peptoids that may prove useful for investigating PAR2 functions (Ramachandran *et al.,* 2012; figure 1.24). Identification of the tethered ligand amino acid sequence required for PAR activation has led to the development of synthetic PAR activators, known as PAR-APs (Ramachandran and Hollenberg, 2008). PAR-APs are based on the proteolytically revealed tethered ligand sequence and have been shown to work as selective PAR agonists (Macfarlane *et al*, 2001). However, care is required when interpreting these results as in certain preparations PAR-APs have been shown to elicit non-PAR mediated responses (Choi *et al.,* 2012). Additionally, some PAR-APs can activate more than one PAR at low concentrations; for example, the PAR1-AP like SFLLRN-NH_2 can activate both PAR1 and PAR2, whereas, the PAR 2-AP like SLIGRL-NH_2 selectively activates PAR2 and is unable to activate any other PARs (Boitano *et al.,* 2011). PAR2 APs are designed to mimic the natural tethered ligand sequences from human SLIGKV-NH_2 and mouse SLIGRL-NH_2 PAR2 receptors as well as for both mouse and rat 2-furoyl-LIGRLO-NH_2 (Macfarlane *et al*, 2001).

There is a limitation in the use of PAR-AP in bioassays due to their relatively low potency and high susceptibility to aminopeptidase (Steinhoff *et al.,* 2005). Novel PAR agonists have been developed by modifying synthetic peptides to non-peptides to produce compounds with higher potency and increased resistance to aminopeptidase (Wang *et al.,* 2010). These selective PAR2 agonists have been used to study the importance of PAR activation *in vivo* (Steinhoff *et al.,* 2003; Wang *et al.,* 2010; Flynn et al., 2013).

SLIGRL-NH_2 is a selective agonist for mouse PAR2 made from synthetic small peptides (Serine-Leucine-Isoleucine-Glycine-Argenine-Leucine-NH_2) and designed

based on the peptide sequences that mimic the natural tethered ligand sequences in this species (Maul *et al.,* 2003). SLIGRL-NH_2 is selective agonist for the activation of PAR2, however, this PAR2 agonist has low potency and poor bioavailability which limits its use *in vivo* (Barry *et al.,* 2010).

By studying structure-activity relationships, a new PAR2 agonist containing a non-peptidic functional group was developed. It was demonstrated that the first five amino acids of PAR2-AP were important for determining selectivity of the compound, however, the last amino acid, Leucine, is less important for selectivity and is involved in enhancing potency (Maryanoff *et al.,* 2001). As a result of this finding a more potent PAR2 agonist, compared to SLIGRL-NH_2, has been developed. A potent non-peptide PAR2 agonist has been developed by replacement of the amino acid Leucine with an aromatic group to further increase potency (Barry *et al.,* 2006). For example, in 2008 the non-peptide PAR2 agonist AC-264613 was identified from a screen of 250,000 drug-like compounds based on structure activity studies which indicated that metasubstitution of bromide for leucine is preferable (Gardell *et al.,* 2008). AC-264613 is a more potent agonist than SLIGRL-NH_2 which is selective for PAR2 and is relatively stable, however, it also has poor solubility (Gardell *et al.,* 2008). To progress the drug-like properties of these agonists, a new class of non- peptidic PAR2 agonist has been developed from SLIGRIL-NH_2 via a heterocyclic replacement of the N-terminal serine shorten ion from the C-terminus and by adding a C-terminal non peptidic region (Barry *et al.,* 2010). This compound, known as GB110, is selective for PAR2 with no effect on PAR1 and is the most potent synthetic peptide for PAR2 reported to date (Adams and Ramachandran *et al.,* 2011). In addition, a recent study has demonstrated that GB110 is stable in serum and, as such, is suitable for use in humans (Barry *et al.,* 2010). GB83 is a novel PAR2 antagonist derived from GB110 which has the same modified N –terminus also contains a spiroindenpiperiden molecule (Barry *et al.,* 2010; Suen *et al.,* 2010). This is the first reversible PAR2 antagonist and inhibits activation of PAR2 by endogenous proteinases and synthetic PAR2 agonists (Barry et al., 2010; figure 1.24).

As for PAR agonists, PAR antagonists have been designed based on the tethered ligand sequences that bind extracellularly to inhibit PARs (Kanke *et al.,* 2009). Recently a new class of intracellular PAR antagonists called pepducins have been identified which block the effects of PAR activation by interfering with cell signalling pathways (Cisowski *et al.,* 2011). Summary of the pharmacology of PAR1, 2, 3, and 4 it is shown in table 1.1.

1.14 Animal use in research

In the UK, animal use in research is under regulations and the institution's work must be licensed by the Home Office. Animal use in research is governed by the principles of the 3Rs which means reduction, replacement and refinement of animals used in research. The first R is 'Reduction' which means reducing the number of animals used in experiments. For example, by improving the experimental design and statistical analysis it is possible to reduce the number of animals used while getting the same levels of information, or use the same number of animals and get more information. The second R is 'Replacement' means preferring using non-animal methods instead of animal methods in the research if it provides results as accurate as the animal methods, for example, replacing animals by using cell culture, human volunteers, computer simulations and new imaging techniques. In addition, replacement also means using a less developed animal species instead of a more developed animal species, for instance, replacing a mouse with a fish. The last R is refinement, which means improving animal welfare and reducing any possible pain or suffering as well as relating to the design of experiments and protocols such that animal usage is reduced. The refinement not only improves the quality of animal life used in research but it also improves the quality of research. For example, improving animal life by improving the housing conditions of the laboratory animal in and care through 'environmental enrichment' means animals live better lives with less stress. Another example is improving the quality of research by a study of GM mice with similar mutations to those found in patients with Huntington's disease. This disease causes difficulties in movement and memory. A study has shown that in mice with Huntington's disease kept in barren cages the disease progresses quicker than in the mice kept in complex cages (Olsson *et al.,* 2008). Therefore, keeping mice in suitable

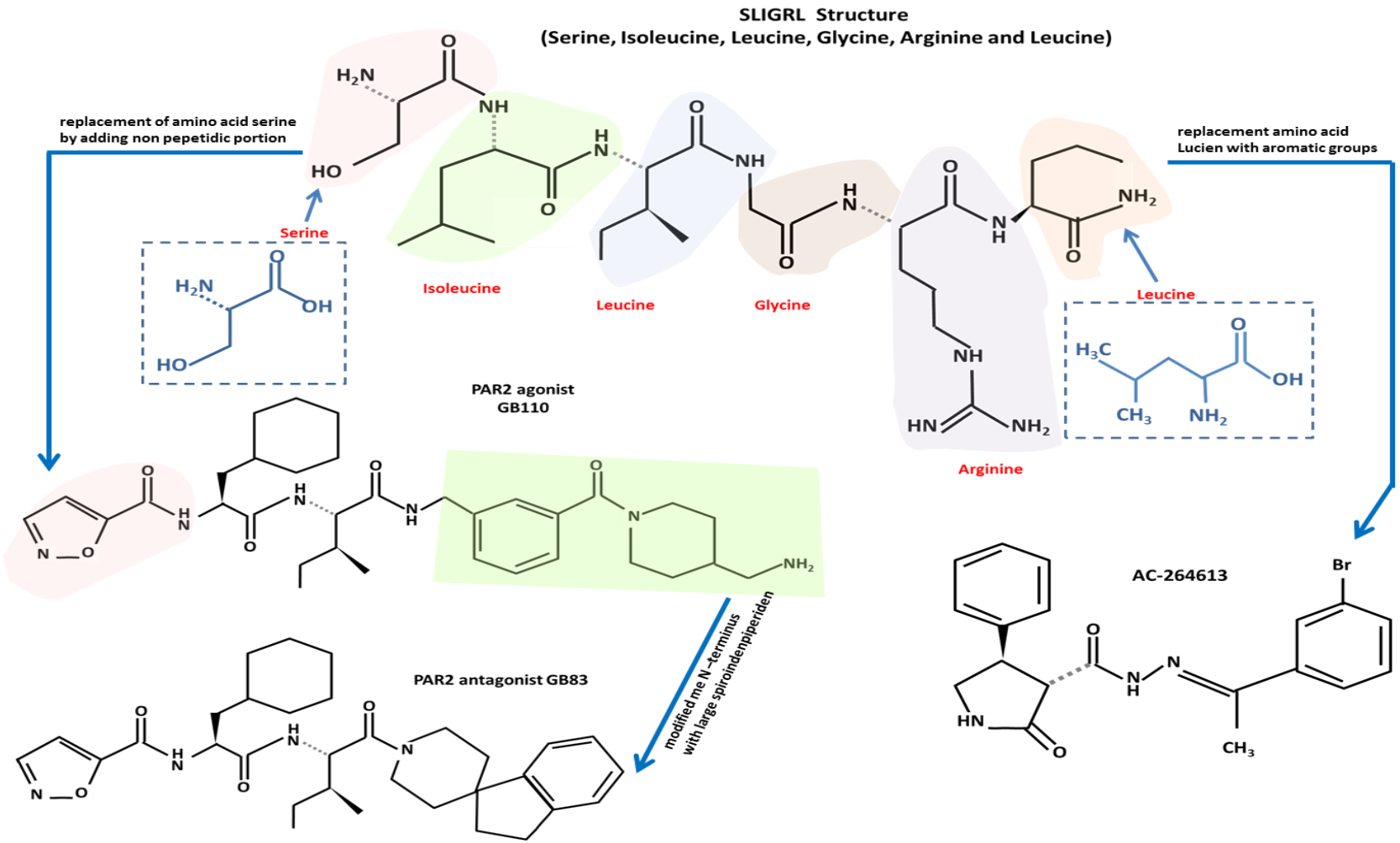


Figure 1.24: Structure of PAR2 synthetic peptide agonist (SLIGRL-NH_2) and the development of non-peptide (GB110- AC-264613) PAR2 agonists and the PAR2 antagonist GB83.

Table 1.1: Summary of the pharmacology of PAR family members, tethered ligand sequences, PAR agonists and antagonists, activating and disarming proteinases. Adapted from (Russo et al., 2009; Soh *et al.*, 2010; Adams *et al.*, 2011).

	PAR1	**PAR2**	**PAR3**	**PAR4**
Encoding Gene	**-Coagulation factor II (thrombin) receptor (F2R)**	**-Coagulation factor II (thrombin) receptor-like 1 (F2RL1)**	**-Coagulation factor II (thrombin) receptor-like 2 (F2R)L2**	**-Coagulation factor II (thrombin) receptor-like 3 (F2RL3)**
Tethered ligand sequence	**-SFLLRN** (human) **-SFFLRN** (rat and mouse)	**-SLIGKV** (human) **-SLIGRL** (rat and mouse)	**-TFRGAP** (human) **-SFNGGP** (mouse)	**-GYPGQV** (human) **-GFPGKP** (rat) **-AYPGKF** (mouse)
PARs agonists (Selective peptide) agonist	**-TFLLR-NH_2** (human) **-SFFLR-NH_2** (rat and mouse)	**-SLIGKV-NH_2** (human) **-SLIGRL-NH_2** (rat and mouse) **-2-furoyle-LIGRLO-NH_2** (rat and mouse)	-----	**-GYPGKF-NH_2** (human) **-AYPGKF-NH_2** (rat and mouse)
Other PARs agonist (Selective non-peptide agonist)		**-GB110** **-AC-264613** **-AC-55541**		
PARs antagonists	**-RWJ-56110** **-RWJ-58259** **-SCH-205831** **-SCH530348** **-BMS-200261** **-Pepducin P1pal- 12** **-Pepducin P1pal- 7**	**-FSLLRY-NH_2** (amino acid peptide antagonist) **-LSIGRL-NH_2** (amino acid peptide antagonist) **-*N*1-3-methylbutyryl-*N*4-6-aminohexanoyl-piperazine (ENMD-1068)** **-GB83** (non-peptide antagonist)	-----	**-Trans-cinnamoyl-YPGKF-NH_2** **- Pepducin P4pal- 10** (also inhibit PAR1)
PARs KO	**PAR1 KO mouse**	**PAR2 KO mouse were used in this study**	**PAR3 KO mouse**	**PAR4 KO mouse**
High-affinity activating proteinase	**-Thrombin**	**-Trypsin** **-Tryptase**	**-Thrombin**	**-Thrombin,** **-Trypsin**
Activating proteinase	**-Thrombin** **-Trypsin IV** **-Plasmin** **-Factor VIIa** **-Factor Xa** **-Granzyme A** **- Kallikrein 4, 5, 6, 14** **-Pen C 13** **-Cathepsin G** **-Proatherocytin** **-Matrix metalloproteinase-(1MMP-1)**	**-Trypsin** **-Mast cell tryptase** **-Trypsin IV** **-Matriptase** **-MT-serine protease-1 (MT-SP1)** **-Der P1, Der P2, Der P3, Der p9** **-Factor VIIa** **-Factor FXa** **-Cockroach** **-Proteinase-3** **-Granzyme A** **-Kallikrein 2, 4, 5, 6, 14** **- Pen C 13** **-Acrosin**	**-Trypsin** **-Factor Xa** **-**	**-Cathepsin G** **-Factor VIIa** **-Factor X** **-Trypsin IV** **-Plasmin** **-Factor Xa** **- Kallikrein 1** **-Mannose-associated serine protease 1 (MASP-1)**
Disarming proteinase	**-Cathepsin G** **-Trypsin** **-Plasmin** **-Kallikrein 1, 14** **-Proteinase 3** **-Metallopeptidase domain (ADAM)** **-Elastase**	**-Cathepsin G** **-Plasmin** **-Proteinase 3** **-Elastase**	**-Cathepsin G**	**-Kallikrein 14**

cage condition gives a more realistic 'model' of Huntington's disease that can be used to try to understand the disease.

Behavioural tests

Behavioural tests have been developed in rodents to measure locomotor activity, anxiety-like behaviour, anhedonia, spatial memory, working memory, sensorimotor gating and startle response and these have been used extensively as an initial pharmacological screen for predicting the therapeutic efficacy of a drug in humans as well as identifying the role of a large number of proteins including enzymes and receptors in genetically modified animals.

1.14.1 Behavioural tests used for assessing locomotor activity and exploratory behaviour

1.14.1.1 Locomotor activity and exploratory behaviour

Locomotor activity can be defined as movement from one location to another in order to explore the environment which is necessary for survival (Gilchrist *et al.,* 1997; Jordan *et al.,* 2006; Brown, 2008). When the rodent faces an unfamiliar environment, the rodent is motivated to explore this novel environment and its exploratory behaviour presents as locomotion around the environment (Hill,1960; Greenberg, 2003). The aim of this exploration in rodents is to provide new information about food sources, shelters, predators or mating opportunities (Montgomery, 1954). Exploratory behaviour in rodents has been of interest within a number of areas of behavioural pharmacology and this may be measured using the open field test (OFT).

1.14.1.1 OFT

The OFT is a well-established assay for assessing general locomotor activity and exploratory behaviour in both rats and mice (Christmas and Maxwell 1970; Prut and Belzung, 2003). Additionally, it is also used for assessing habituation in rodents (Barbosa *et al.,* 2008; Siemiatkowski *et al.,* 2000). It is a simple and sensitive test and therefore it is widely used to detect the effects of drugs and genetic manipulation

on locomotor function (Walsh and Cummins, 1976; Bailey and Crawely, 2009). For example, epilepsy induces neurodegeneration in the brain of rats and this damage can cause increase locomotor activity (Matos *et al.,* 2012); as another example, administration of baclofen a $GABA_B$ agonist to rats can cause stimulation of locomotor activity (Liljequist, 1993). Furthermore, pyscho-stimulant drugs, for example amphetamine and cocaine, can also stimulate locomotor activity (Narayanan *et al.,* 1996).

Many brain areas and neurotransmitters are involved in locomotion, but the hippocampus and dopamine have been shown to play an important role. One study has shown that the hippocampus is a brain region important for generation and maintenance of locomotor activity (Bardgett and Henry, 1999). Other studies have shown that dopamine agonists increase locomotor activity whereas dopamine antagonists decrease locomotor activity (Beninger, 1983). The OF box and experimental protocol used in our laboratory are explained in detail in chapter 2, section 2.4.1, figure 2.2.

1.15 Behaviour tests used for assessing anxiety-like behaviour

1.15.1 Anxiety

Anxiety in humans can be defined as a psychological and physiological state characterized by cognitive, somatic, emotional, and behavioural components (American Psychiatric Association, 2000). Anxiety can create an unpleasant feeling associated with uneasiness, fear, or worry in the presence and absence of psychological stress (Bouras, 2007). Anxiety can be distinguished from fear as fear happens in the presence of an external threat (Davison, 2008). Anxiety is a normal reaction to a stressor and it may help the individual to deal with a difficult situation, for example at work or at school, by prompting one to cope with it (American Psychiatric Association, 2000).

Animal models for anxiety-like behaviour disorders have shown that the brain areas involved and playing an important role in anxiety-like behaviour are prefrontal cortex, hippocampus and amygdala (Bremner, 2004). It is widely believed that the

amygdala and the prefrontal cortex play a crucial role in anxiety disorders and fear (Etkin *et al.,* 2009; Lacroix *et al.,* 2000), while the hippocampus plays a role in storage of the old memory of painful experiences (Engin, and Treit, 2007). Other brain areas involved in anxiety-like behaviour include the brain stem, the hypothalamus and the cerebellum (Etkin *et al.*, 2009). Neurotransmitters proposed to be involved in anxiety-like behaviour are GABA, serotonin (5-hydroxytryptamine), noradrenaline and corticotropin-releasing hormone (Holsboer and Ising, 2008; Durant *et al.,* 2010). A reduction in serotonin and GABA can cause anxiety-like behaviour whereas in contrast increased noradrenaline and corticotropin-releasing hormone can cause anxiety-like behaviour (Holsboer and Ising, 2008; Dell'Osso *et al.,* 2010).

Both in neuroscience laboratories and the pharmaceutical industry, anxiety-like behaviour in rats and mice are measured either by non-conditioned behaviour tests, such as the OFT, EPM, elevated zero-maze, light-dark exploration test, or conditioned behaviour tests, for example, the four plate test, fear potentiated startle and the Vogel water-lick conflict test (Bourin *et al.,* 2007).

1.15.1.1 OFT

This test was originated by Calvin Hall (1934), who used the urination and defecation in this test as an index of anxiety-like behaviour in rodents (Hall, 1934; Walsh and Cummins, 1976). The OFT is a very frequently used test in psychology and it is also used to assess anxiety-like behaviour in rats and mice (Russell, 1973; Belzung and Gerlai 1999; Prut and Belzung, 2003). Measurement of anxiety-like behaviour in the OFT is based on the conflict between the tendency of the rodent to prefer a dark place and a natural curiosity of the rodent to explore a novel environment (Christmas and Maxwell 1970; File, 1980; Prut and Belzung, 2003). For this reason, normal rodents spend much of the time close to the wall with little time spent entering the centre (Bailey and Crawley 2009). This behaviour is exaggerated in rodents with a high level of anxiety-like behaviour who spend the majority of time close to the wall without entering the centre (Prut and Belzung, 2003).

This test is also used for the evaluation of the effects of anxiolytic (anxiety decreasing) and anxiogenic (anxiety-increasing) compounds. Prut and Belzung (2003) studied the effects of anxiolytic and anxiogenic compounds in the OFT. Anxiolytic compounds are able to reduce anxiety-like behaviour in rodents and the effect of these compounds in the OFT is to cause an increase in central locomotion or in time spent in the central part of the arena without changes to total locomotion and exploration. In contrast, anxiogenic compounds are able to cause rodent anxiety-like behaviour and therefore, the effects of these compounds in the OFT is to cause a decrease in central locomotion or in time spent in the central part of the arena without change of total locomotion and exploration (Prut and Belzung, 2003). In addition, the OFT is not only used to measure exploratory behaviour and anxiety-like behaviour in rodent, but it is also used to measure rearing, grooming, freezing, urination and defecation in rodents (Walsh and Cummins, 1976; Brown, 1999).

1.15.1.2 EPM test

This is a commonly used model of anxiety-like behaviour for drug discovery in pharmaceutical companies (Dawson and Tricklebank 1995; Crawley, 2000). The EPM is a well-established assay for measuring the levels of anxiety-like behaviour in the rodent (Pellow *et al.*, 1985a). It has been validated to assess anxiety-like behaviour in rats and mice (Pellow *et al.*, 1985b; Lister,1987). The advantages of the EPM test are that it is a simple and quick test, requires only inexpensive equipment, it is a more accurate test than other anxiety-like behaviour measures, for example, OFT and currently EPM test is the most popular test for anxiety (Dawson and Tricklebank, 1995; Holmes *et al.*, 2000; Prut, 2003; Ramos, 2008)

Pellow *et al* (1985b) validated this test for assessing anxiety-like behaviour developed from the work of Montgomery (1955) and Handley and Mithani (1984) (Montgomery, 1955; Handley and Mithani, 1984; Pellow *et al.*, 1985b). Montgomery (1955) observed that rats spent less time exploring the open arms of a novel elevated Y maze (in which the number of open arms varied from zero to three) than the closed arms (Montgomery, 1955). He also noticed that rats obviously preferred the enclosed arm in all cases and he believed that rats when exposed to a novel stimulus (elevated

Y maze) have a conflict between the tendency of the rat to explore the open arms and a fear of the high places of the open arms (Montgomery, 1955; Pellow *et al.,* 1985a; Dawson and Tricklebank, 1995). Then Handley and Mithani (1984) modified the elevated Y maze to what they called an elevated X-maze with 2 open arms and 2 closed arms as shown in chapter 2, figure 2.4. These authors defined anxiety-like behaviour of rodents by using the ratio of time spent on the open arms to the time spent on the closed arms (Handley and Mithani, 1984; Walf and Frye, 2007).

The EPM test is based on the conflict between the tendency of rodents to explore novel environments and a natural aversion of rodents to open spaces (Lister, 1987; Crawley, 2000). Therefore, a mouse with normal level of anxiety-like behaviour prefers the closed arms to open arms. The EPM test also helps to evaluate effects on anxiety-like behaviour of newly developed drugs in the pharmaceutical industry (Walf and Frye, 2007). For example, normal rodents spent significantly greater amount of time and numbers of entries in the closed arms than open arms (Pellow *et al.,* 1985a). However, administration of anxiolytic drugs such as an α-$_2$ adrenoreceptor selective agonist for example Yohimbine in rats has shown increased exploration of the open arms. In contrast, administration of anxiogenic drugs such as an α-$_2$ adrenoreceptor selective antagonist for example delequamine (RS-15385-197) in rats has shown decreased exploration of the open arms (Tallentire *et al.,* 1996). Furthermore, it has been shown that drugs clinically used as anxiolytics in humans can be evaluated using the EPM test. It was shown in the original validation that a significant increase in the % of time spent in open arms and the number of entries into open arms was observed only with drugs that were clinically effective anxiolytics, for example, chlordiazepoxide and diazepam. Moreover, compounds that cause anxiety in humans such as yohimbine, phenylenetetrazole, caffeine and amphetamine, significantly reduced the % of time spent and the number of entries into the open arms (Pellow *et al.,* 1985a; Pellow *et al.,* 1985b; Pellow and File, 1986; Lister, 1987). During evaluation of anxiolytic compounds by EPM test, inconsistent results were noted with some of these compounds and a need for new targeted therapeutic treatments for anxiety suggested that scoring other ethologically related behavioural indicators, for example, head dipping, stretch-attend postures may possibly provide more sensitive procedures of the effects of new anxiolytic

compounds (Rodgers *et al.,* 1997; Borsini *et al.,* 2002; Bailey and Crawley, 2009). The EPM apparatus and experimental protocol are explained in detail in chapter 2, section 2.4.2.

1.16 Behavioural tests used for assessing hedonic state

1.16.1 Anhedonia

Depression is a clinical illness which is characterized by significant and long-lasting disorders in mood and cognitive function (Klein, 1974). One of the most common symptoms of clinical depression is the inability of a person to obtain pleasure from things that previously brought enjoyment; this symptom is called anhedonia (Klein, 1974; Hamilton, 1967; Koob *et al.,* 1989).

As with anxiety, there are several brain areas and neurotransmitters involved in depression (Maletic *et al.,* 2007). The brain areas that affect depression-like behaviour are the prefrontal cortex, the amygdala, the hypothalamus, the striatum and the hippocampus (Drevets, 2001; Nestler *et al.,* 2002; Drevets *et al.,* 2008). These brain areas produce several neurotransmitters including serotonin, ACh, adrenaline, dopamine, glutamate and GABA (Nestler *et al.,* 2002).

Many researchers believe that serotonin level in brain influences mood, for example, serotonin level imbalance causes depression (McHenry, 2006). Most antidepressant drugs work as serotonin reuptake inhibitors. Adrenaline is another neurotransmitter that is involved in depression. Treatment with adrenergic antagonists leads to a more calm and relaxed mood because a reduced level of adrenaline decrease heart rate and blood pressure (Lechine *et al.,* 1995). In contrast, an increased dopamine level has positive effects on mood and is responsible for pleasure and desire. It also has a role in regulating memory (D'Aquila *et al.,* 2000). A good antidepressant treatment suggests the combination of these three neurotransmitters in one dosage form, for example, triple reuptake inhibitors (Marks, 2008).

The amygdala is involved with emotions such as pleasure or sadness (Nestler *et al.,* 2002). However, the hippocampus is involved in storage and recall of memory

(Lisman *et al.*, 2001). It is believed that an interaction between the hippocampus and the amygdala might be involved in depression (Nestler *et al.*, 2002).

Several animal models of depression-like behaviour have been developed as screening tests to assess the efficacy of antidepressant drugs. There are a number of behaviour tests used to assess depression-like behaviour in rodents including the sucrose preference test (SPT), sucrose consumption, forced-swim and tail suspension tests (Sarkisova *et al.*, 2003; Cryan *et al.*, 2005; Petit-Demouliere *et al.*, 2005). In this study, SPT was used to evaluate anhedonia in mice.

1.16.1.1 SPT

Simillar to humans, rodents also prefered to drink the sweet solution (Keskitalo *et al.*, 2007). So, the amount of sucrose drunk can be used as an index to measuer the hedonic state in rodents. The SPT is used as a measure of the hedonic state of rodents (Sarkisova *et al.*, 2003). The first chronic mild stress model of depression was introduced by Katz *et al* (1981) and developed by Willner (Willner, 1997; Katz *et al.*, 1981). These studies provide the basis of the present models (Yan *et al.*, 2010). An earlier study by Katz demonstrated that rats exposed to a 3 week stress protocol, including electric shocks, immobilization, swimming in cold water, and other strong stimuli, caused a reduction of sucrose drinking that was explained as a symptom of anhedonia (Katz *et al.*, 1981). The series of stressors causes an elevation of plasma corticosteroid levels and a reduction in sucrose drinking (Katz, 1981). This result suggests that chronic stress probably can cause anhedonia (Katz *et al.*, 1981; Katz, 1981).

In order to obtain closer similarity to the human situation, Willner (1997) studied the effects of stress on a sucrose consumption test in rats (Willner, 1997). Stress was induced by using stressors such as solid bottom cage, wire-bottomed cages, presence of novel objects and the stress exposure was extended up to three months (Willner, 1997). This study found that long exposure to stressors could cause anhedonia (Willner *et al.*, 1987). This author also studied the effect of tricyclic antidepressant drugs in the sucrose consumption test. There was no effect in the sucrose consumption test after treating the stressed rats with the tricyclic antidepressant

desmethylimipramine for one week. However, the sucrose consumption was returned to its normal levels after the animals had been treated with the same drug for 2-4 weeks (Willner *et al.*, 1987; Willner, 1997). The SPT experimental protocol is explained in detail in chapter 2, section 2.4.3.

1.17 Learning and memory

Learning and memory are continuous processes (Kandel *et al.*, 2000). According to Kimble *et al* (1961) learning is defined as a relatively permanent change in behaviour because of practice (Kimble *et al.*, 1961). However, memory can be defined as the process by which information is encoded, stored, and retrieved (Nadel and Hardt, 2011). The memory process occurs in 3 stages; loss of one these stages when information is not transferred to the next step leads to forgetting. The stages are encoding, storage and retrieval (Nadel and Hardt, 2011). Encoding is the first step in creating a new memory and it can be defined as forming a memory code. In order to get this information into the memory the code is then stored in various different areas of the brain (Okado and Stark, 2005). The new memory code is stored and consolidates within the brain (McGaugh *et al.*, 1966). Then this stored information is recalled from short- or long-term memory when it is needed and this last stage is called retrieval (Jones *et al.*, 2012). Short-term memory has a limited capacity of information and keeps this information for a few seconds (s) to min but the long-term memory can store larger quantities of information for an unlimited period (Sharm *et al.*, 2010; figure 24).

Failure in one of the these memory process leads to loss of memory; for example, patients brain-damaged during the 2nd World War suffered from retrograde amnesia and loss of access to memories formed before the 2nd World War (Nadel and Hardt, 2011). One of the possibilities is that the problem might be one of retrieval, where the memories are available but inaccessible, and another possibility is the problem might be one of storage in that memory was being stored at the time of the brain injury and these memories had not yet been consolidated and could be lost forever (Russell and Nathan, 1946). The memory system in humans is one but types of memory are different. Richard Atkinson and Richard Shiffrin developed a model of

human memory in 1968 and they described memory as multi-storage model (Atkinson and Shiffrin, 1968).

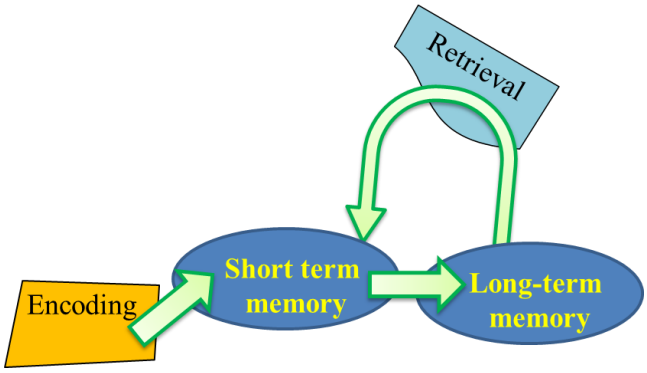


Figure 1.24: Memory processes steps are encoding, storage memory and retrieval.

This model consists of three stages, which are, first sensory memory then short-memory and finally long-term memory (Atkinson and Shiffrin, 1968). Sensory memory is a very short memory and can be defined as the ability to remember impressions of sensory information after the sensory stimuli have ended (Galan *et al.*, 2006). Sensory stimulus can be received through any of the five senses which are sight, hearing, smell, taste and touch (Wicher, 2010). An example for sensory memory is the ability to look at something and remember what it looked like by observing that for just a second (Wicher, 2010). However, short-term memory is ability to remember a small amount of information and processe this information at the same time (Jones *et al.*, 2012). In humans, a short-memory example is a person reading the beginning of the sentence and in order to understand this sentence, the beginning of the sentence needs to be held in mind while the rest is read (Nadel and Hardt, 2011). Short-term memory is a necessary step to long-term memory in which the information is transferred to a more permanent storage, for example, facilitation and improving the long term- memory by mental repetition of information (Spitzer, 2008).

Long-term memory usually is divided into two sub-types, explicit (or declarative) and implicit (procedural) memories (Anderson, 1976). Explicit memory is defined as the conscious memory of facts and events and is used every day, for example, remembering the list of dates of history (Ullman, 2004). Explicit memory can be further sub-divided into episodic and semantic memories. Episodic memory is the

memory that is involved with recollection of specific events, situations or experiences, for example, you remember the date and the place of events like your first day in the school or your brother's graduation day (Miller, 1956; Tulving, 1972; Sharma and Raloczy, 2010).

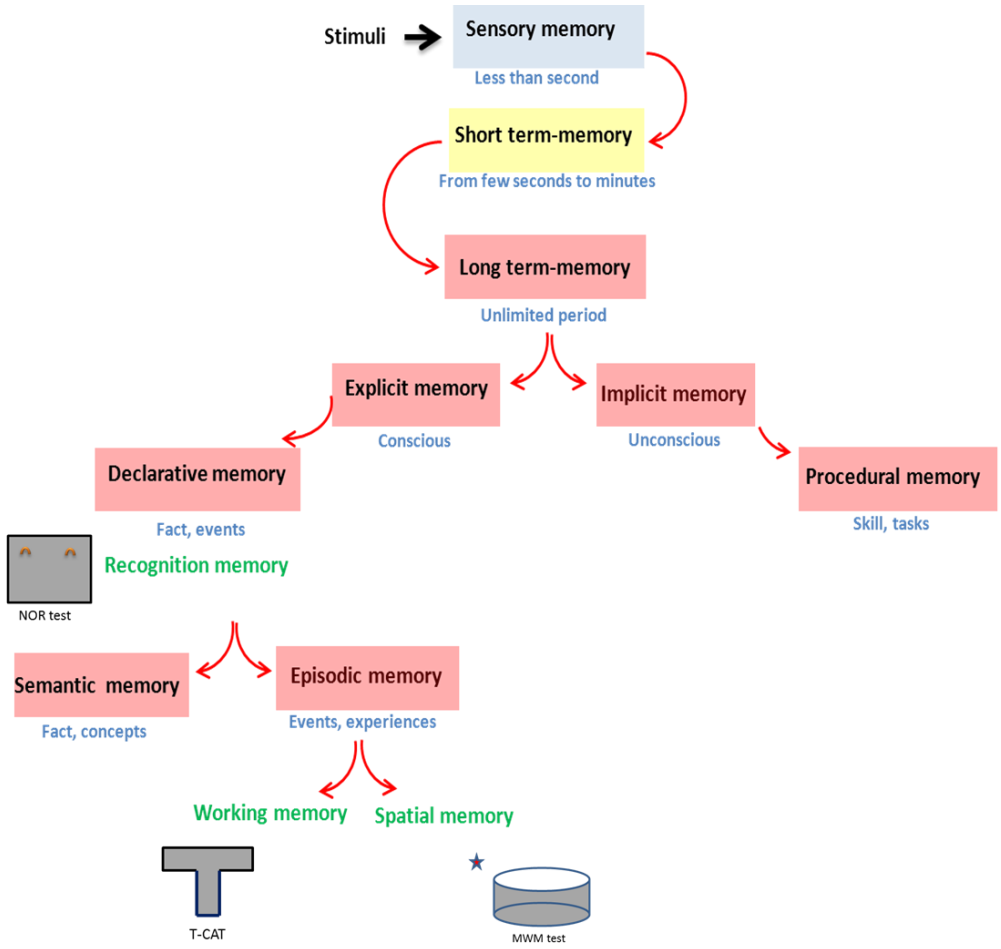


Figure 1.25: Types of memory.

Semantic memory is involved in knowledge and it uses a store for general world knowledge but not related to specific events, for example, people can answer questions like "What is the capital city of England?", London is the answer, without remembering any specific event (Miller, 1956; Tulving, 1972; Sharma and Raloczy, 2010). The second type of long-term memory is implicit memory, which is unconscious memory of motor skills and habits. It is memory of how to do things, for example, previous experience aids learning how to type without looking at the keyboard (Bechara, 1995).

The hippocampus, along with other medial temporal lobe structures, constitutes the main brain areas involved in declarative memory (Squire, 1996). Patients with bilateral medial temporal lobe damage show highly impaired episodic memory but little impaired semantic memory (Bayley, 2008). These patients can perform well on a procedural skill, habits, vocabulary, fact and knowledge learnt in the past (Bayley, 2008). Additionally, functional brain imaging shows hippocampus to be the brain area that is highly active during the navigation process in both human and animals (Bohbot, 2004). However, implicit memory does not depend on hippocampus but it is mediated by neostriatum and cerebellum (Knowlton, 1996).

Memory is a complex system and many brain areas and neurotransmitters are involved in this process. The brain areas involved include the hippocampus, cerebral cortex, amygdala and cerebellum (Huang *et al.,* 1989). The hippocampus is part of the limbic system and is involved in complex memory processes such as forming and organizing the storage of memory; it plays an important role in consolidating information from short-term memory to long-term memory specially (Riedel and Micheau 2001; Rossato *et al.,* 2007). Studies of the role of the hippocampus in memory processes have shown that it has an important role particularly in spatial memory and spatial memory is impaired in patients with right hippocampal damage (Abrahams *et al.,* 1997; Banta and Lavenex, 2009). Another brain area which also plays a key role in the memory process is the cerebral cortex which is thought to be the final storage place for various types of memories for example visual and auditory memories (Eichenbaum, 2000; Dobbins *et al.,* 2002). Additionally, the amygdala is believed to be involved in the encoding and retrieval of emotional memories (McGaugh, 2004; Sharot *et al.,* 2007). However, the cerebellum is the site of memory formation for learned responses; it is important in procedural memory (for example, motor skills) and conditioned memory (the formation of a learned response to the previously neutral stimulus); for example, damage to the cerebellum causes rabbits to be unable to learn conditional eye-blind responses (Krupa *et al.,* 1993; Finn *et al.,* 1999).

Several neurotransmitters are involved in this process of memory formation and retrieval, including Ach, glutamate, GABA and catecholamines (Miranda, 2007). For

example, Ach is produced from the cerebral cortex, the hippocampus and the striatum during the consolidation process (Power, 2004). However, the same neurotransmitter is produced from the hippocampus and the frontal cortex during the retrieval process (Pepeu and Giovannini, 2004).

Although there is one basic memory process there are different types of memories; in this study we examine the role of PAR2 in spatial reference memory, working memory and recognition memory. In neuroscience laboratories, various animal models are used to assess each type of memory.

1.17.1 Behaviour tests used for assessing memory

1.17.2 Spatial reference memory

The hippocampus appears to be important for long-term episodic memory (memory of specific situations, places, events and experiences) and spatial memory. Spatial memory is defined as a subtype of episodic memory because it stores information within a spatiotemporal frame (O'Keefe and Nadel, 1978). The most important theory of hippocampal function is the cognitive mapping theory (O'Keefe and Conway, 1978). O'Keefe and Conway's cognitive map theory proposed that spatial memory depend on hippocampus (O'Keefe and Conway, 1978). O'Keefe and Conway (1978) suggested that learning and memory was supported by two learning systems. The first system is dependent on hippocampus and supports cognitive theory and it refers to learning about the spatial relationships between cues. The second learning system is independent of the hippocampus and it refers to stimulus response learning or habit learning (O'Keefe and Conway, 1978). The first system represents declarative memory and depends on hippocampus while the second system represents procedural memory and is independent of hippocampus and is mediated by neostriatum and cerebellum (Knowlton, 1996; figure 1.25).

Discovery of "place cells" also supports mapping theory. Place cells are neurons that fire, depending on animal's place in the environment but independent of any specific stimulus or current behaviour (Eichenbaum, 1996). Recently, cognitive mapping has

developed our knowledge about the role of hippocampus in the construction of mental images (Sharma and Rakoczy, 2010).

Several studies have shown that hippocampus is important in learning and memorising as well as age-related cognitive deficits (Corkin, 2002; Gadian, 2000). In humans, damage of the hippocampus has shown evidence that this brain area plays important role in spatial memory because this brain area has a crucial role in construction of mental images (Astur, 2002; Bird and Burgess, 2008). Several studies have supported the cognitive mapping theory in that animals with hippocampal damage have shown deficits in spatial learning memory (O'Keefe and Conway, 1978). For example, rats with hippocampal lesions are impaired in spatial memory tests such as the radial arm maze and the MWM tests (Jarrard, 1983; Morris *et al.,* 1986).

Spatial reference memory has been evaluated by several behaviour tests. Firstly, these tests are designed and established in rats and then adapted for use in the mouse (Sharma *et al.,* 2010). Various studies have used the multiple T-maze, radial arm maze, and MWM tests for assessing spatial memory in mice (Morris, 1984; Dubreuil *et al.,* 2003; Kunesova *et al.,* 2008; Patil *et al.,* 2009). In our study the MWM test was used to assess spatial memory in mouse.

1.17.2.1 MWM test

The test was developed by Richard Morris (1981) as an alternative to the multiple T-maze test (Morris, 1981). It is a well-known and widely used assay for determining spatial memory in rodents and it is commonly used today to explore the role of the hippocampus in the formation of spatial memories (Dubreuil *et al.,* 2003; Vorhees and Williams, 2006). The test is based on the rat learning to locate a platform hidden underneath the water in a pool from four release points (Morris, 1981). Through a series of trials, the mouse learns the location of the hidden platform as measured by the decreases in the time (latency) and distance (path) taken to reach the platform (Morris, 1981). These two parameters are indicators for the spatial learning and memory abilities of the mouse.

At the end of the learning, the spatial memory is also assessed by the probe test (Vorhees and Williams 2006), in which the mouse is released from one of four release sites. The mouse spends more time and travels more distance in the previous position of the hidden platform, which is removed before this test (Morris, 1981). Therefore, the probe test is used to assess the ability of the rodent to recall information learnt about the previous location of the previous hidden platform (D'Hooge and De Deyn, 2001). These authors have studied the effect of brain area lesions on the performance of spatial memory and shown that lesions in hippocampus, striatum, basal forebrain, cerebellum and cerebral cortex impaired MWM performance (D'Hooge and De Deyn, 2001). However, the performance of the MWM test depends not only on these areas but it depends on the coordinated action between these brain areas and neurotransmitter systems constituting a functionally integrated neural network (D'Hooge and De Deyn, 2001).

Focusing on the hippocampus, many studies shown that the integrity of the hippocampal formation is essential for spatial memory and the MWM test has been shown to be highly sensitive in the evaluation of the damage to the hippocampus (Morris *et al.*, 1982; Bannerman *et al.*, 1999; D'Hooge *et al* (2001). Studies have shown that rats' learning in MWM test is impaired by a hippocampal lesion (D'Hooge *et al.*, 2001). The earlier study of Morris (1982) showed by testing the directional heading of hippocampal lesioned rats compared to the normal rat that hippocampal lesions in rats affect the swimming pathways in MWM learning. The author noticed that on trial 28, normal rats adapted a good strategy to find the platform with very little movement in other directions. However, rats with hippocampal lesion go astray rather than going straight in the direction of the platform (Morris *et al*., 1982; see figure 1.26).

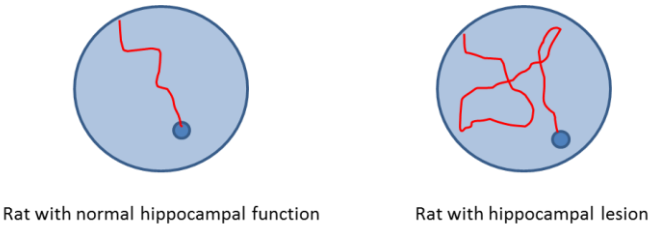


Figure 1.26: Comparison between the swimming pathways of hippocampal lesion rat and normal hippocampal function in rat. The small circle is hidden platform. The red line is swimming pathways.

The MWM test is also used to evaluate aged-related reduction in spatial memory in mice (Pan *et al.,* 2008). Some studies have been shown that aged rats displayed impairment in learning the location of the hidden platform in the MWM test (Gallagher *et al.,* 1990; Foster *et al.,* 1991). The MWM pool and experimental protocol are explained in detail in chapter 2, section 2.4.4, figures 2.6, 2.7 and 2.8.

1.17.3 Recognition memory

Declarative memory is a type of long-term memory that stores knowledge of facts such as places, things and people, and the meaning of these facts (Ullman, 2004). Recognition memory is a subtype of declarative memory that can be defined as the ability to recognize events, objects, or people previously encountered (Medina, 2009). The human infant is able to recognition its mother's face within a short time after birth (Pascalis, *et al.,* 1995). Interaction between different brain areas is involved in the process of recognition (Antunes and Biala, 2012). The most important areas in the brain involved in recognition memory are the CA1 region in the hippocampus and an adjacent region to hippocampus, the perirhinal cortex (Brown and Aggleton, 2001; Medina, 2009). The perirhinal cortex is responsible for identification of novel items, for example, an object that has not been seen previously (Wan *et al.,* 1999). A study has shown that disruption of perirhinal cortex function results in an impaired ability of the animal to recognise the novel objects in an object in place test (Barker *et al.,* 2007). Additionally, the medial prefrontal cortex is also involved in process of recognition memory (Cross *et al.,* 2012). Recently, it has been suggested that both perirhinal cortices and medial prefrontal cortex are involved in information storage; the perirhinal cortex's function is involved in storage of the information necessary for recognition memory but the medial prefrontal cortex is acting as a store for this information (Banks *et al.,* 2012). Furthermore, several neurotransmitter systems involved in storage and recall memory are also involved in recognition memory including glutamate, GABA, Ach, serotonin, dopamine and noradrenaline (Steckler *et al.,* 1998). For example, blockade of cholinergic receptors with scopolamine before the sample phase (exploration of two identical objects) results in impaired discrimination by rats during choice phase (exposure to a familiar

and a novel object) in the novel object recognition (NOR) test (Warburton *et al.*, 2003).

In neuroscience, recognition memory has been studied for the development of pharmacological compounds for treatment of memory deficit disorders. NOR is used to evaluate of recognition memory but there are other behavioural tests are also used to assess recognition memory, for example, Y-maze object recognition and delayed-non-matching-to-sample (Aggleton *et al.,* 1986; Ennaceur and Delacour, 1988; Dudchenko, 2004; Chambon, 2011). In this study, the NOR test is used to evaluate recognition memory.

1.17.3.1 NOR test

This simple and quick test is widely used as a standard test for evaluating recognition memory in both human and rodents (Broadbent *et al.,* 2010; Antunes and Biala 2012). The test is a useful model for assessing pure recognition memory, cognitive impairment, cognitive ability in genetically modified strains of mice and in developing pharmacological treatment of brain damage (Antunes and Biala 2012).

The test was developed by Ennaceur and Delacour in 1988 and it can regarded as a spontaneous delayed-non-matching-to-sample (Aggleton *et al.,* 1986; Ennaceur and Delacour, 1988). The NOR test is based on the natural tendency of rodents to spend more time exploring a novel object than a familiar one (Albasser *et al.,* 2010). Ennaceur and Delacour (1988) observed that most normal rats distinguish between the two objects in the choice phase and they spend more time in exploring the new object than the familiar one (Ennaceur and Delacour, 1988). The discrimination ratio (ratio of time spent in exploring the novel object) is measured as an index of recognition memory in this test. In a study of humans and monkeys with hippocampal damage this damage caused impaired recognition memory in the NOR test (Broadbent *et al.,* 2010). The experimental protocol is explained in detail in chapter 2, section 2.4.6.

1.17.4 Working memory

In humans, working memory is more described as explicit memory (Dudchenko, 2004). It is defined as the brain system responsible for maintaining and storing information for a short time and reusing this information for complex cognitive tasks, for example, language comprehension, learning, thought, and reasoning. Working memory has been found to require the simultaneous storage and quick processing of information (Baddeley, 1992; Baddeley, 2003). The term working memory is frequently used interchangeably with short-term memory but working memory differs from short-term memory (Aben *et al.,* 2012). Working memory refers more to the processes that are used to temporarily store, organize and manipulate information but the short-term memory refers only to the short-term storage of information in the memory (Cowan, 2008).

The central executive, which is the part of the prefrontal cortex at the front of the brain, plays an important role in working and short-term memory (Rodriguez and Paule, 2009). The function of central executive is temporary storage of short-term memory, where information is available when it is needed for current reasoning processes, but it also "calls up" information from other brain areas (Mizuhara and Yamaguchi, 2007). In humans, the central executive controls consist of two neural loops, one for visual data, and another for language (Baddeley, 1992). These two neuronal loops temporarily hold information until it is deleted by the next job (Baddeley, 1992). Besides the prefrontal cortex, other areas of cortex are also involved in working memory (Rowe *et al.,* 2000). Prefrontal cortex cooperates with other parts of the cortex from which it extracts information for brief periods (Kane, 2002). It is important to note that damage to the prefrontal cortex in primates caused deficits in short-term memory (Umeda *et al.,* 2011).

One of the classical tests assesses working memory in rodents is the eight radical maze (Dudchenko, 2004). The maze consists of eight arms radiating from central platform. The originators of this test observed that rats retrieve the food from each arm and they quickly learned to visit eight arms without re-entering into a visited arm (Dudchenko, 2004). This result suggests that in a single session, the rats were

able to remember which arm they had visited. This is working memory that allows the rats to remember which arm has been visited in a session (Dudchenko, 2004).

Working memory can be distinguished from reference memory. In MWM test, working memory refers to the memory within a trial that must be forgotten in subsequent trial (Vorhees and Williams 2006). However, refernce memory refers to the memory between trials. This is a long-term association between stimuli and response, for example, the cue is represented over a delay period and this cue makes a subsequent response (Dudchenko, 2004; Sharma 2010).

There are different behaviour tests that measure working memory in rodents, for example the Y-maze, which measures the alternation of rat between arms (Bryan *et al.,* 2009; Deacon and Rawlins, 2006). The MWM test with the hidden platform in a different location on each testing day is also used for assessing working memory in rodents (Dudchenko, 2004). In addition, Olton and Samuelson also used radial arm maze for measuring working memory in rodents (Olton and Samuelson, 1976). T-maze continuous alternation test (T-CAT) is another test that measures working memory in rodents (Bryan *et al.,* 2009; Deacon and Rawlins, 2006). In this study we used T-CAT.

1.17.4.1 T-CAT

In this study, the T-CAT is used to assess working memory by evaluating spontaneous alternation of the mouse between two arms of a T-maze (Deacon and Rawlins, 2006). In this test the rodents are started at the base of the T-maze apparatus and allowed to freely choose one of the two goal arms (Bryan *et al.,* 2009). If the next trials are given in quick session, on the next trial the rodents have a tendency to choose the arm not visited immediately before, reflecting memory of the first choice, and this behaviour is called spontaneous alternation (Olton *et al.,* 1979; Spowart-Manning and der Staay, 2004). The information about the previous goal arm visit is stored in working memory (Bartus *et al.,* 1982).

The T-CAT is used as screen for a pharmacological compounds which produce a cognitive deficit; for example, administration of scopolamine induces memory deficit

resulting in reduction in the number of alternations (Deacon and Rawlins, 2006; Bryan et al., 2009). The T-CAT is also used for evaluation of rodent models for CNS disorders in humans, for example, Alzheimer's Disease (Bryan et al., 2009). Furthermore, the test is also used to measure cognitive deficits in genetically modified strains of mice (Rodriguiz and Wetsel, 2006).

In spontaneous alternation, again several brain areas and neurotransmitters are involved; the brain areas include the hippocampus, septum, basal forebrain, and prefrontal cortex and thalamus, and the neurotransmitters include Ach, GABA and dopamine (Lalonde, 2002). The hippocampus has been implicated in the exploration of novel maze arms therefore T-CAT test can test hippocampal dysfunction (Deacon, 2006).

1.18 Behaviour tests used for assessing sensorimotor gating

The startle response is an unconditioned reflexive response to a sudden environmental stimulus and is commonly classified as a defence mechanism (Swerdlow and Geyer, 1998; Geyer *et al.,* 2002). The startle response has been used in both human and rodents (Davis, 1980). In rodents, the startle response is usually induced either using acoustic stimuli or tactile stimuli (for example via air puffs onto the skin) and is characterised by contractions of most of the muscles of the body (Curzon *et al.,* 2009). Usually startle response leads to an extension of both forepaws and hid paws followed by muscle contraction into an arched position (Koch and Schnitzler, 1997; figure 1.27). One study has demonstrated that the acoustic startle reflex is mediated by auditory stimuli that generate auditory information, entering the CNS via auditory nerve input to the cochlear nucleus which projects to the reticular pontine nucleus (Nodal *et al.,* 2003). Motor outputs are created in the reticular pontine nucleus which projects to the spinal cord and stimulates the muscle contraction (Li *et al.,* 2001). However, the reduction in startle response produced by a weak stimulus preceding the stimulus by a very short duration is known as prepulse inhibition (PPI) of the startle response (Graham, 1975; Ison *et al.,* 1973). The attenuation of the amplitude of startle response indicates the ability of the nervous

system to temporarily adapt to a strong sensory stimulus when a prior weaker signal is given to the rodent (Geyer, 1999; Ouagazzal *et al.*, 2001).

Figure 1. 27: Startle response in the rat. Adapted from Yeomans *et al.*, 2002.

PPI of the startle reflex is identified in many species including humans and mice (Geyer, 1999; Ouagazzal *et al.*, 2001). Although the extent of the adaptation affects various systems in the body, those most commonly measured are the muscular reactions which normally are reduced as a result of the nervous inhibition (Bauerle *et al.*, 2011).

Deficits of PPI of the startle response have been demonstrated in many neurological and psychological disorders (Ludewig, 2003). Such deficits give an indication of the inability of the brain to filter out unnecessary information, that is, they are due to abnormalities of sensorimotor gating. Such deficits are common in patients suffering from schizophrenia and Alzheimer's Disease (Swerdlow *et al.*, 2001; Swerdlow *et al.*, 2008). Additionally, decreased PPI is observed in mice lacking mGlu5 receptors (Brody *et al.*, 2004). Many studies have shown that patients with schizophrenia have deficits in PPI and fail to habituation to the startle response because these patients have problems with inhibitory mechanisms in the neuronal circuitry used for sensorimotor gating (Braff *et al.*, 1978; Braff and Geyer, 1990). Interestingly, PPI of startle response is sensitive to sex differences in both human and rodents. Some studies have observed that PPI of the startle response is weaker in females than males and this difference is due to the effect of reproductive hormones for both humans and rodents (Ison, 2007; Neill and Kulkarni, 2011)

The pharmacology of PPI of the startle response is quite complex and several brain areas are involved in modulating sensorimotor gating including limbic cortex, ventral striatum, the pallidum, pontine tegmentum, the hippocampus and prefrontal cortex (Geyer, 1996; Swerdlow and Geyer, 1998; Geyer *et al.*, 2002). Systemic administration of dopamine agonists, serotonin (5-hydroxytryptamine) agonists and glutamate antagonists disrupt sensorimotor gating in rats, suggesting the involvement of these three neurotransmitters in control of PPI (Fletcher *et al.*, 2001).

1.18.1 PPI of startle response

The PPI test is the test of choice for development of rodent models that measure sensorimotor gating deficits, which helps in the investigation of the role of genes that regulate sensorimotor gating and the physiology and pathology of sensorimotor gating (Swerdlow *et al.*, 2001). Tests of PPI are based on measuring the transmission of information from example, auditory stimuli to the CNS (Curzon *et al.*, 2009).

However, a parallel procedure can be used for measuring the startle response *per se*. The startle response is a reflex and it can be modulated by several different stimuli. For example, the startle response is higher in the presence of threat, fear, and pain and less in the absence of these stimuli (Davis, 1989; Davis *et al.*, 1997). Similar to the PPI test, startle response test is based on measuring the transmission of information to the CNS (Curzon *et al.*, 2009). In humans, startle response is mostly measured by the movement of oculomotor muscles but in rodents the startle response is measured by the movement of whole body muscles (Caeser, 1989; Hoffman *et al.*,1969).

1.19 Conclusion and aims

PAR2 is extensively expressed in brain in normal and pathological conditions, including the hippocampus, cortex, the amygdala, thalamic, nuclei, both astrocytes and neurons. During pathological conditions, an over expression of PAR2 has been observed in experimental CNS disease models and from the tissue of patients suffering from CNS neurological diseases such as ischaemia brain injury, Parkinson's disease, multiple sclerosis, Alzheimer's Disease, and HIV-associated dementia, suggesting that PAR2 plays key roles in pathophysiological processes in the brain. Depending on the cell type in which over expression of PAR2 is detected, PAR2 has been proposed to play dual function, either degenerative as in the case of glial cells, or neuroprotective, as in neuronal cells. PAR2 receptors have a functional role in neurogenic inflammation, either neuroprotective or neurodegenerative. The hippocampus is the area in the brain with highly organized neuronal circuits. *In vitro,* the well-known experimental models that are acute slice preparation and primary hippocampal are used to study in studying neuronal and synaptic functions in the hippocampus. Recently it was shown that activation of PAR2 in hippocampus indirectly modulates neuronal excitability and synaptic transmission that suggests that PAR2 could be involved in learning and memory.

Therefore, the hypothesis of this study is that is PAR2 plays a critical role in CNS function that underlies mouse behaviour. The aim of this study was to investigate whether PAR2 plays a role in mouse behaviour, parcticularly in emotion and cognition.

Therefore, the specific aims of my PhD research is to investigate:

1. Does PAR2 affect mouse congnitive and emotional behaviour under normal physiological conditions?
2. Does PAR2 play a neuroprotective or neurodegenerative role in sickness behaviour?
3. How do PAR2 agonists affect mouse behaviour particularly in emotion and cognition?

Chapter 2

Materials and Methods

2.1 Introduction

The aim of the study was to measure the role of PAR2 in mouse behaviour, specifically on locomotor activity, anxiety-like behaviour, anhedonia, spatial memory, working memory, recognition memory, sensorimotor gating and startle response. The evaluation of locomotor activity was measured using the OFT whereas anxiety-like behaviour was examined using both the OFT and EPM test. The SPT was used to determine the expression of anhedonia. A number of tests were used to investigate memory: the MWM test for spatial memory, the T-CAT for working memory and the NOR test for recognition memory. In this study, the startle response and sensorimotor gating were also investigated by using the startle response (SR) test and the preppulse inhibiton (PPI) test respectively.

2.2 Experiments

2.2.1 Chemicals

Lipopolysaccharide (LPS) was purchased from Sigma Aldrich, UK and it was extracted from *Salmonella enteritidis*. The LPS used was made up in phosphate buffered saline (PBS, Sigma Aldrich, UK). The PAR2 agonists used in this study were SLIGRL-NH_2, AC-264613 and GB110. SLIGRL-NH_2 peptide (Peptide Protein Research Ltd, UK) was diluted in 0.9% saline (sodium chloride, Honeywell Riedel-de-Haën, UK). PAR2 agonists AC-264613 (made by Dr. O. Sutcliffe, University of Strathclyde, UK) and GB110 (gift from Professor D. Fairlie, University of Queensland, Australia) were suspended in 1% Tween 80 (polyethylene glycol sorbitan monooleate, Sigma-Aldrich, UK) in 0.9% saline. Sucrose (Fisher, Scientific UK) was diluted with tap water to give a 1% sucrose solution.

The chemicals used for Liquid chromatography- mass spectrometry (LC-MS) were purchased from Fisher Scientific, Loughborough, UK. They were ammonium acetate CH3COO(NH_4), ammonium formate, $HCOONH_4$, ammonium carbonate (NH_4)2CO3, formic acid (HCOOH). Methanol and acetonitrile solvents were of HPLC grade and were also obtained from Fischer Scientific. HPLC water was prepared using a Milli Q purification system.

2.2.2 Animals

All animal experiments were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Male and female C57BL/6J mice (12 weeks old; weight (g): males 27.3 ± 0.35, females 20.6 ± 0.21) were obtained from an in-house breeding colony in the Biological Procedures Unit (BPU) of the University of Strathclyde. PAR2 genetically modified mice were obtained by cross-breeding 14 pairs of PAR2 heterozygous (HT) mice (*F2rl1+/-*, figure 2.1). These mice were generously supplied by Professor R. Plevin at the University of Strathclyde. After weaning, the WT, HT and PAR2 KO mice (*F2rl1-/-*) were separated based on gender and group housed (3-6 mice per cage), except where required to be singly housed for the purposes of the experiment, for example, sucrose preference test experiment. All mice were housed in the BPU and had free access to food pellets (CRM (P), Special Diets Services, UK) and water. Room temperature and humidity were held at 21 ± 2 °C and 45 - 55% respectively with light and dark cycles rotated every twelve h (6 am to 6 pm). Mice were housed in either MB1 cages (45 x 28 x 13cm) or M3 cages (48 x 15 x 13cm; North Kent Plastics, UK) lined with beta bed Grade 6 wood bedding (Datesend Ltd, UK) and provided with environmental enrichment in the form of plastic huts (North Kent Plastics, UK) and nesting material (Sizzlenest; Datesend Ltd, UK). All behavioural testing was also conducted in a temperature and humidity regulated environment, 21 ± 2 ºC and 45 - 55% humidity, respectively.

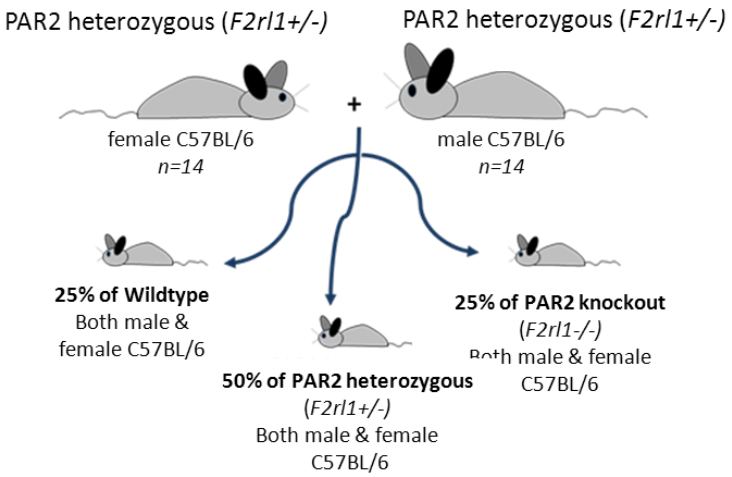


Figure 2.1: Protocol used to produce mice with different PAR2 genotypes.

2.3 Genotyping of PAR2 receptor KO mice

2.3.1 Breeding and maintenance of the PAR2 KO colony

PAR2 KO mice were generated using homologous recombination methods previously described (Ferrell *et al.,* 2003). The null allele of the PAR2 gene (non-functional PAR2 gene) was created using embryonic stem cells (E14Tg2a) that were then injected into C57BL/6 blastocysts to generate chimeras. Male chimeras were subsequently crossed with C57BL/6 females. The offspring were tested using Southern blot analysis to identify the PAR2 gene. HT PAR2 mice were then crossbred to generate PAR2 KO mice which were phenotypically similar to PAR2 HT and WT littermates (Ferrell *et al.*, 2003). These mice have been used extensively in previous studies (Ferrell *et al.*, 2003).

2.3.2 DNA extraction

Tissue biopsies were collected from the tail (0.5cm of tail tip) or ear of PAR2 genetically modified mice after birth and during weaning (aged 2-3 weeks). DNA was extracted from these biopsies and PCR was performed to identify the PAR2 genotype of these mice.

2.3.3 Polymerase chain reaction (PCR)

Three PAR2 primers (Invitrogen, UK) designed at the University of Strathclyde (Professor R. Plevin) were used to initiate PAR2 DNA synthesis: PAR2 primer 1 (5‟-GCC ATT GGA GTC TTC CTG TT-3‟), PAR2 primer 2 (5‟-AGC GAG ATT TGC ATG CGA TT 3‟), and PAR2 primer 3 (5'-TGC CGA GAA AGT ATC CAT CA-3'). PCR products were separated based on their size using agarose gel electrophoresis. Agarose gel was prepared from agarose (Bioline, UK) and 10% Tris Acetate-EDTA (TAE) buffer (Sigma-Aldrich, UK). Ethidium bromide solution (10mg/ml; Sigma-Aldrich, UK) was added to stain the DNA bands and make them visible under UV illumination. A DNA size ladder (Hyper Ladder II; Bioline, UK) was used as a reference to estimate the size of the DNA products.

DNA preparation

Following biopsies, the tissue was placed in a microcentrifuge tube. The DNA extraction was carried out as described in the Extraction–N-Amp tissue kit manual (Sigma-Aldrich, UK). Briefly, the extraction solution (100 µl) was added to the tissue followed by 25 µl of tissue preparation solution. The mixture was incubated at room temperature (20 °C) for 10 min to initiate digestion of the tissue and then incubated at 95 ºC for 3 min to complete the digestion process. The digestion process was stopped by adding neutralization solution B (100 µl) and the mixture was vortexed thoroughly. The resulting DNA solution was stored at 4 ºC until DNA amplification and gel electrophoresis were performed.

DNA amplification

PCR was carried out by placing the samples in sterile PCR microcentrifuge tubes, and then the following reagents were added in sequence: 0.5 µl of 10 pmol/ ml PAR2-primer 1, 0.5 µl of 10% PAR2-primer 2, 0.5 µl of 10% PAR2-primer 3, 10 µl Ex-tract-N-Amp PCR reaction mix, 4 µl of DNA extract, 4.5 µl of ultra pure water, to give a 20 µl PCR reaction solution. The PCR solution was then placed in a PCR machine (MWG AG Biotech, Germany) to initiate amplification. Copies of PAR2 DNA underwent thermal cycling that consisted of an initial denaturation of 5 min at 95ºC, followed by 30 cycles of 30 s at 95ºC, 30 s at 60ºC and 1 min at 72ºC. A final extension of 5 min at 72ºC followed, with the reaction stopped by an extended incubation and cool down to 4ºC.

Agarose gel electrophoresis

The PCR product was analysed by agarose gel electrophoresis. For this experiment a 2% w/v agarose gel, 10% TAE running buffer and 2µl of 10mg/ml ethidium bromide solution were prepared. Hyper Ladder II (5 µl) was added to the first well of the gel and 15 µl of the PCR sample was added into each of the remaining wells. The gel was then run at 45 volts for 45 min. Finally, the gel was visualised under UV light using a Bio-vision UV illuminator (BioGene Ltd, UK) to identify the DNA bands.

2.4 Behaviour test procedures

For behavioural experiments, mice were initially handled by the experimenter on the day before the first day of testing to minimise the stress induced by handling. Normally, the mice remained in their home cage when they were transferred from their home environment (procedure room) to the testing area and then were placed in the testing apparatus by hand. All apparatus was thoroughly cleaned with 10% Decon (Decon Laboratories Limited, UK) between animals.

2.4.1 OFT

Open field (OF) apparatus

The OF apparatus consisted of a black square box 40cm x 40cm x 40cm high made from infrared light (IR)-translucent Perspex and was placed on an IR light box (Tracksys, Nottingham, UK, figure 2.2).

OF procedure

The OF apparatus was placed in the centre of the room. Lighting was set between 40-45 lux at the centre of the OF maze. The day before testing began, mice were handled to reduce the stress during the test. To control for habituation to the OF (open field) environment, a habituation session was carried out on the first day. Mice were taken to the testing room in their home cages. Before starting the test, the apparatus was cleaned with 10% Decon. The mouse was then placed in the centre of the box and allowed to freely explore the box for 10 min in the experimental room.

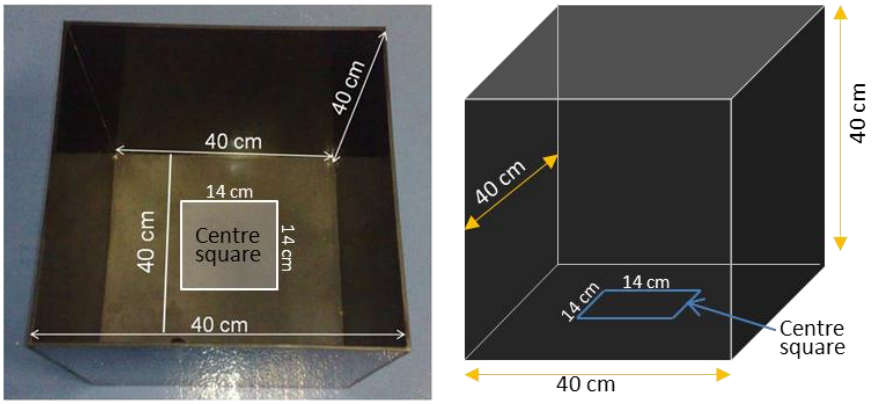


Figure 2.2: OF apparatus for mouse.

Following this, the same procedure and experimental conditions were followed on day 2 and the parameters measured using the tracking software (Ethovision V3.1.1.6 Noldus Information Technology, Netherlands). The parameters were total distance moved (cm), number of entries into the centre square and the time spent in centre square (s). The centre square size, defined on the tracking software but not physically marked on the OF floor, was 14cm x 14 cm (figure 2.3).

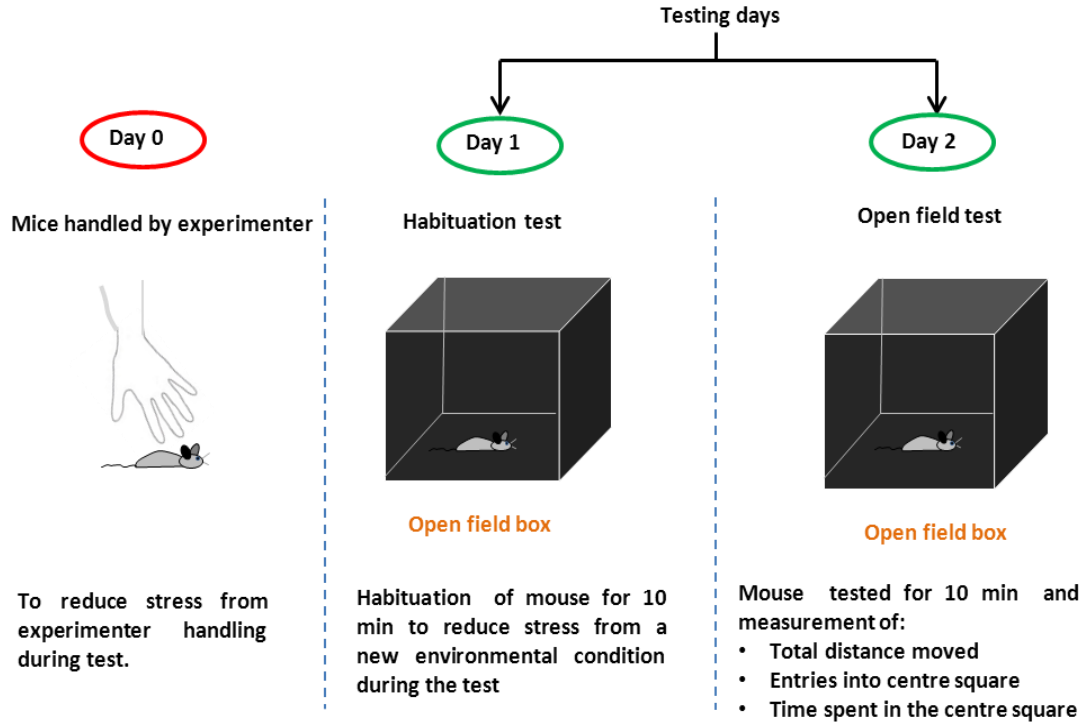


Figure 2.3: Schematic representation of the OFT procedure.

2.4.2 EPM

EPM apparatus

The apparatus consisted of four arms, two open arms and two closed arms arranged in a plus shape, with arms of the same type opposite to each other. The open arm dimensions were 30cm x 5cm, the closed arm dimensions were 30cm x 5cm with a wall of height 15 cm. The apparatus was elevated 70 cm from the floor and similar to OF box, it was also made from IR-translucent Perspex with integral IR light sources (Tracksys, Nottingham, UK, figure 2.4).

EPM procedure

The light in the testing room was regulated to give a light intensity of ~ 40-45 lux measured in the centre of the maze, 60 lux in the open arms and 6 lux in the closed arms. Before starting testing each mouse, the EPM was cleaned with 10% Decon. Each mouse was placed in the centre of the EPM facing a closed arm and had free access to any arm of the maze. The mouse was allowed to explore the maze for a 10 min period. The movement of the mouse during this time was recorded using the tracking software (Ethovision V3.1.1.6 Noldus Information Technology, Netherlands) and the % number of entries into the open arms, the % time spent in the open arm and the total number of entries into the open and closed arms were calculated as follows:

$$\% \text{ Open entries} = \frac{\text{Number of entries into open arm}}{\text{Number of entries into open arm} + \text{Number of entries into closed arm}} \times 100$$

$$\% \text{ Time in open arms} = \frac{\text{Time spent in open arm}}{\text{Time spent in open arm} + \text{Time spent in closed arm}} \times 100$$

$$\text{Total entries} = \text{Number of entries into open arm} + \text{Number of entries into closed arm}$$

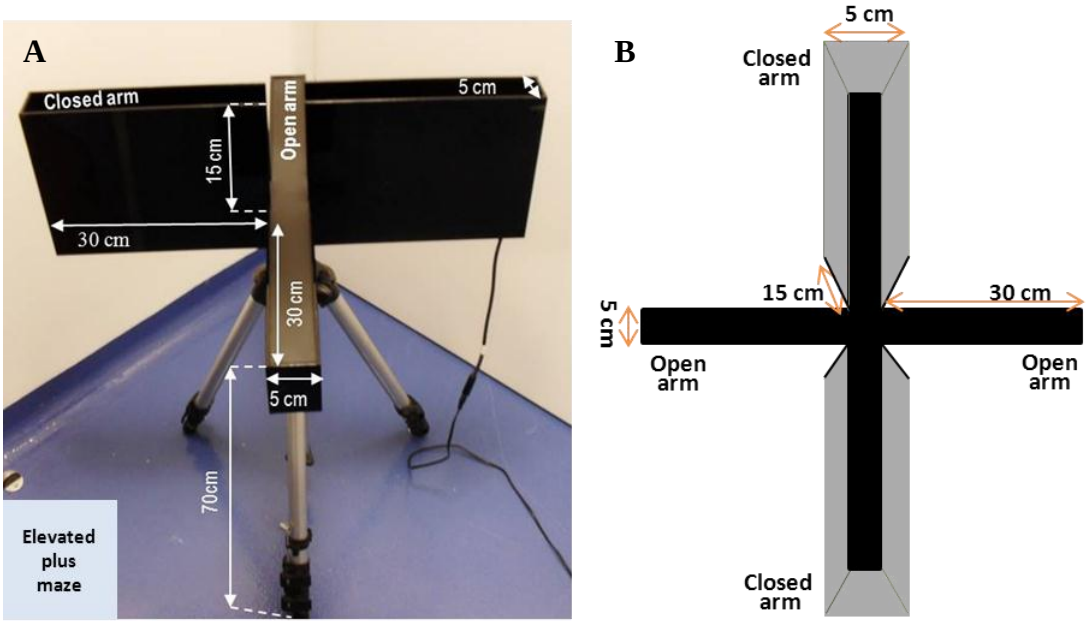


Figure 2.4: The EPM apparatus used for mouse experiments. (A) Represents the EPM apparatus (B) Represents schematic drawing of the same EPM.

2.4.3 SPT

The SPT was performed over seven consecutive days. On the first two days the preferred position of the water bottle (left or right) was identified and the SPT was performed on the five following days (figure 2.5).

Prior to the test, each mouse was singly housed in an MB1 cage provided with environmental enrichment in the form of plastic huts and nesting material (section 2.1.1.). Each cage was supplied with two bottles (similar to the normal tap water bottles in the home cages) which were filled with tap water, weighed and placed in the same position as the water bottles in the home cage.

The amount of water drunk was measured daily by weight for two consecutive days in order to determine which bottle position, either left or right, was preferred. On the next day (3rd day), the SPT was started by replacing the water bottle in the non-preferred position with an identical bottle containing 1 % sucrose. The 1% sucrose solution was freshly prepared by dilution of sucrose (Fisher, Scientific UK) with tap water, and the amount of water and sucrose consumed was measured over the time period.

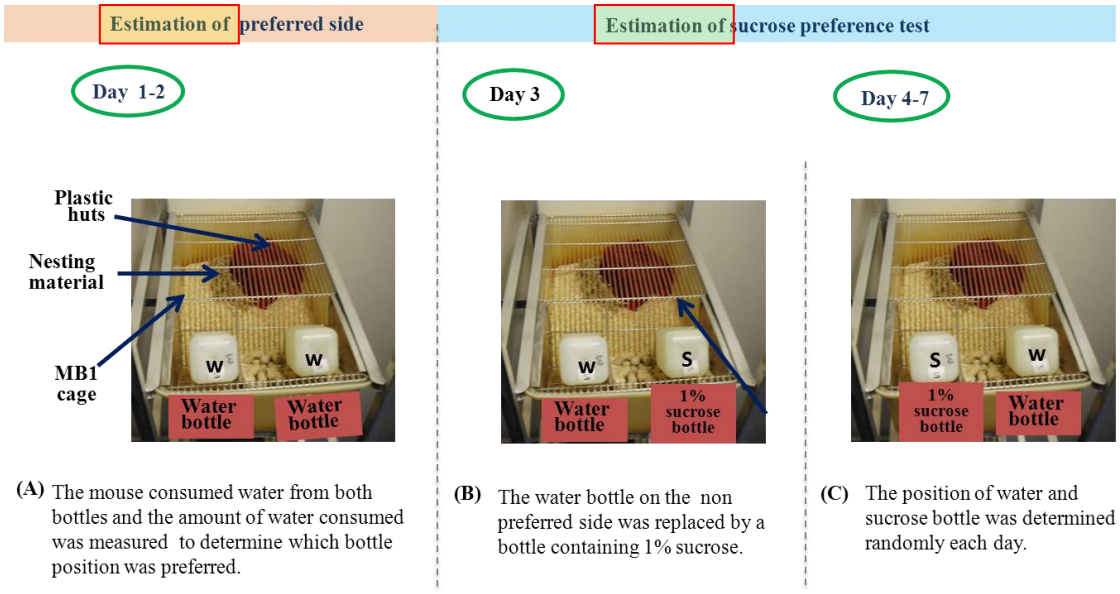


Figure 2.5: SPT procedure. (A) The mouse was housed indivitually with 2 bottles of water. (B) The mouse was housed with a bottle of water and a bottle of 1% sucrose. (C) The position of water and sucrose bottles was randomally alocated daily.

On subsequent days (fourth to seventh testing days), the position of the two bottles (water and 1% sucrose solution) was randomly determined to avoid a place preference. Similar to previous days the amount of sucrose solution and water consumed was measured daily during consecutive days. The time of measuring the weight of these two bottles was the same time during all testing days, usually between 9:00 am and 10:00 am. The amount of water and sucrose drunk was measured daily in order to determine which solution (either water or sucrose solution) was preferred (figure 2.5). The parameters measured were % of sucrose drunk and total drunk; the parameters were calculated by the following equations:

$$\% \text{ sucrose drunk} = \frac{\text{Amount of sucrose solution drunk (g)}}{\text{Amount of sucrose solution drunk (g)} + \text{Amount of water solution drunk (g)}} \times 100$$

$$\text{Total drunk} = \text{Amount of sucrose solution drunk (g)} + \text{Amount of water solution drunk (g)}$$

2.4.4 MWM test

MWM apparatus

The MWM apparatus consisted of a large black circular pool (diameter 98 cm and height 60.5 cm) made from IR-translucent Perspex and placed on an IR light box (figure 2.6). The pool was filled up to a depth of 31.5 – 32.0 cm with water (maintained at 21 ºC)

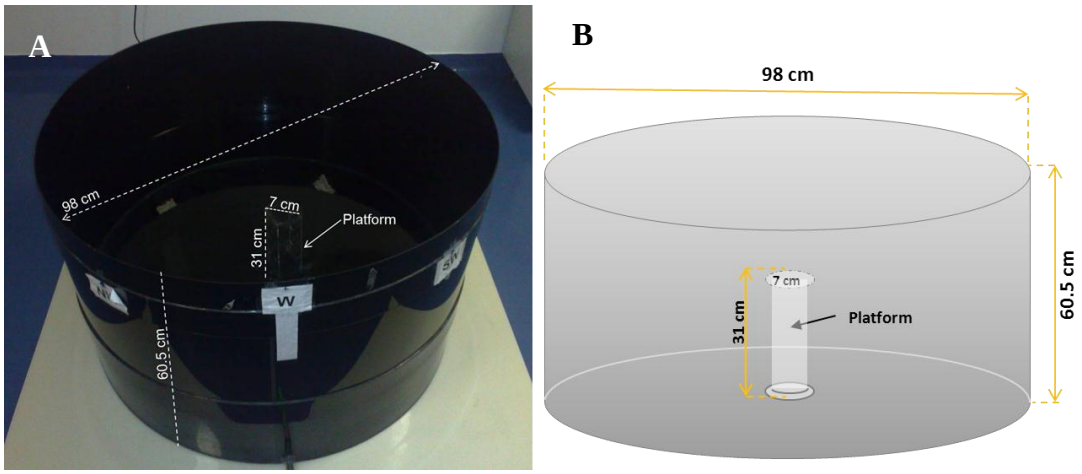


Figure 2.6: Black water maze pool used for mouse. (A) Picture represents the water maze pool used for the MWM test. (B) Picture represents schematic drawing of the same pool.

to cover an invisible (transparent) 7 cm diameter circular platform (figure 2.6). The platform was submerged ~ 0.5 - 1.0 cm below the surface of the water and placed in the pool according to the experimental protocol required.

MWM procedure

The pool was located in the testing room with a number of extra visual cues, which were geometric images, for example squares and triangles, hung on the wall (figure 2.7). In the testing room, the light level was kept constant during the training days at between ~ 40-45 lux.

Each mouse was given four trials per day for five consecutive days (figure 2.8). On the first day, the first trial was a habituation trial, followed by three subsequent acquisition trials. For each trial, the mouse saw the visual cues. The time between each trial was 15 min and trials lasted for a maximum 60 s, in order to prevent hypothermia (Iivonen *et al.*, 2003). In the habituation trials, the platform was placed in the centre of the pool; this position was chosen for habituation because it is never used in the acquisition phase (figure 2.7A). The trial was initiated by placing the mouse on the platform and allowing it to jump freely into the water. If the mouse did not go freely into water within 5 s, it was gently encouraged to jump into the water and allowed to swim until it returned to the platform, where it was allowed to remain for a few s before removal from the platform. If it did not reach the platform within 60 s, it was removed from the water and put onto the platform for 5 s. At the end of the trial, the mouse was removed from the pool, dried with a towel and placed in a cage under a ceramic heater.

The pool was notionally divided equally into four quadrants (north, south, east, and west). In the acquisition trials, the platform was placed approximately 20 cm from the pool wall in one of four different locations (N, S, E, and W) which remained constant for each individual mouse over the training days (figure 2.7 B) while the release site was changed randomly. Each trial was initiated by placing the mouse in the water facing the pool wall at one of the release points (SE, SW, NE, NW) and allowing it to swim for a maximum for 60 s and attempt to locate the hidden platform.

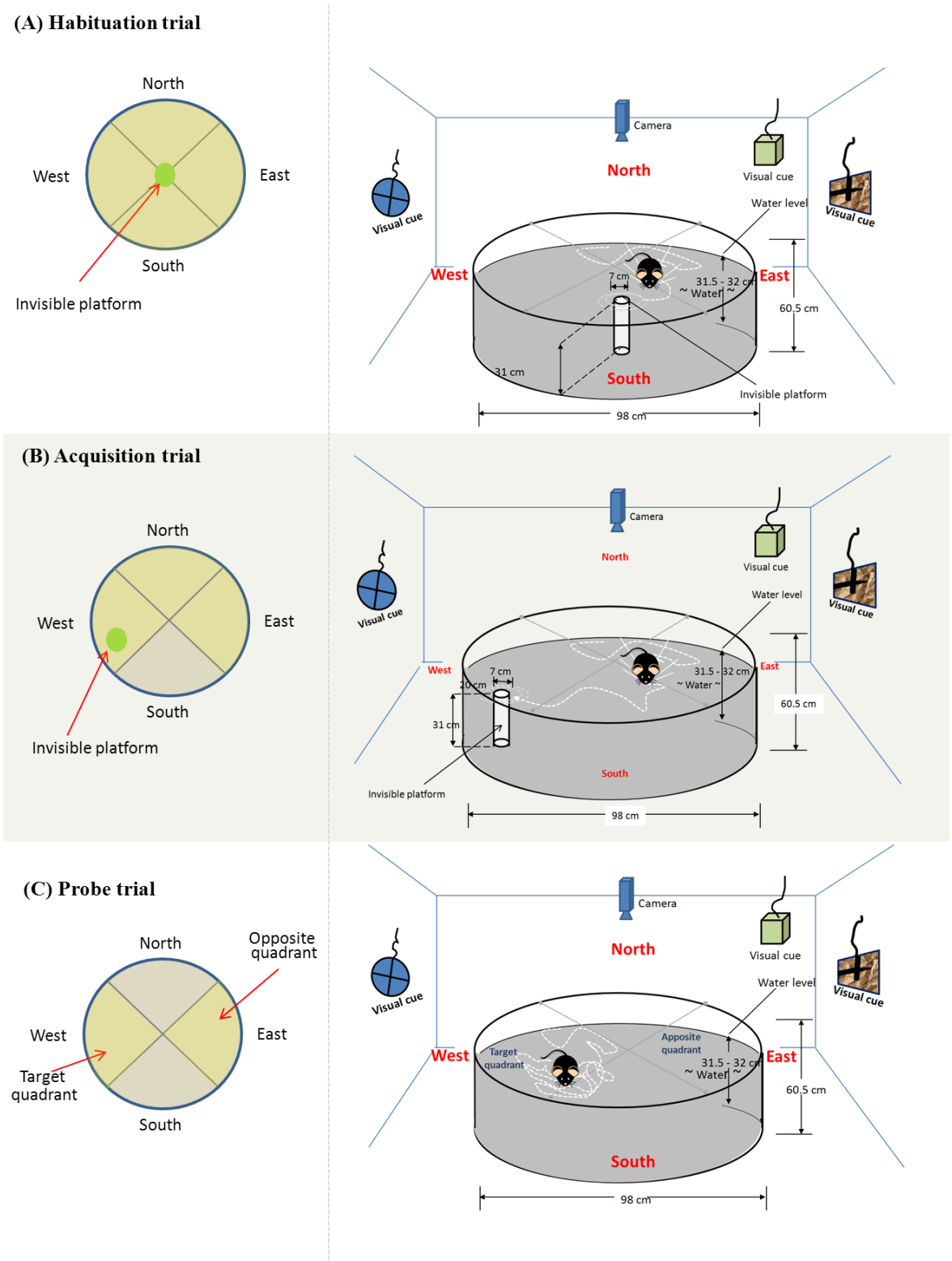


Figure 2.7: Set-up of apparatus for the MWM during habituation, acquisition and probe trials. (A) During the habituation trial the platform was in the centre, (B) During the acquisition trial the hidden platform was in the same position (for each mouse) during all testing days, (C) Probe trial: during this trial the mouse swam in the pool in the absence of the platform.

If successful, the mouse was allowed to remain on the platform for a few s before being removed and dried as described previously. If the mouse did not locate the platform within 60 s of being released then it was guided to the platform by the experimenter and allowed to remain there for 5 s. The mouse was then removed from the pool and dried.

On the subsequent days (2nd to 4th testing days), 4 acquisition trials were performed each day in the same way (figure 2.8). On the final day, 3 acquisition trials were performed exactly as before. Following this, the mouse underwent a probe trial, in which the platform was removed (figure 2.7 C) and the mice were tested whether they had learnt the position of the hidden platform during the training trials. The probe trial was initiated by releasing the mouse and allowing it to swim in the pool freely without the platform for 60 s (figure 2.7 C).

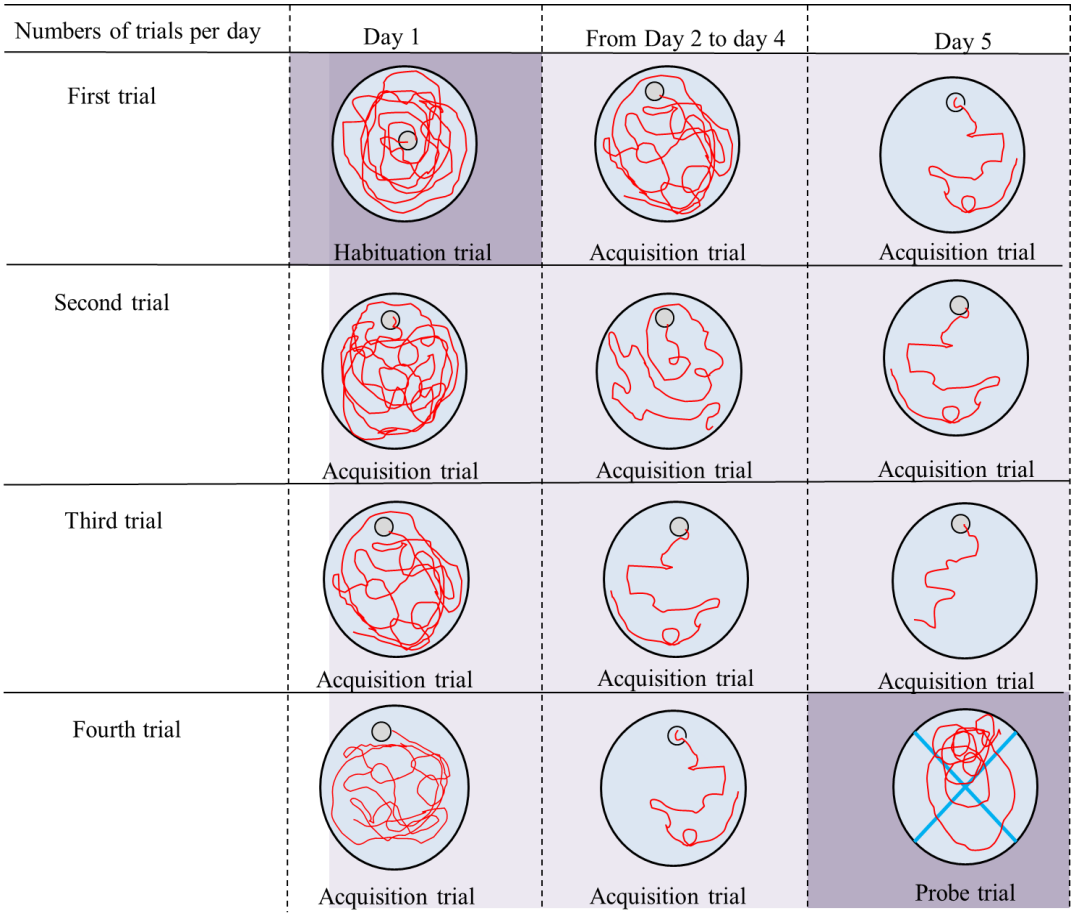


Figure 2.8: Schematic of the MWM test procedure. The big blue circle represents the pool, the red line represents the swimming path of the mouse, the small grey circular shape represents the position of the platform and the two blue lines represent the four quadrants of the maze.

The mouse then was removed and dried. Ethovision software (V31.1.6 Noldus Information Technology, Netherlands) was used to track the mouse's movements. For the acquisition trial, the distance travelled and duration taken to reach the hidden platform was recorded (figure 2.8). For the probe trial, the time spent in the training quadrant where the platform was previously located was compared to the time spent in the opposite quadrant to determine whether the mouse had remember the location of the hidden platform.

2.4.5 T-CAT test

T-maze apparatus

The T-maze consisted of a horizontal T-shaped apparatus made from black plastic. The length of the start and goal arms was 30 cm, the width 10 cm and the height of the wall 12 cm. The T-maze was equipped with three removable guillotine doors. One of these doors was located at the beginning of the start arm and separated by a 20 cm compartment at the beginning of the start arm, while the other two were located at the entrance of each goal arm (figures 2.9).

T-CAT procedure

The T-CAT procedure consisted of a single session which included a forced-choice trial followed by 14 free-choice trials (figure 2.10). As with the OF and EPM tests, the apparatus was cleaned with 10% Decon between individual mice. The mouse was placed in the start arm of the T-maze under low light conditions of ~ 45-50 lux and the first trial (forced-choice) was started. In the forced choice trial, the start arm door was lifted and the mouse was allowed to explore the start arm and one of the goal arms. In the forced choice trial, the entry to the other goal arm was blocked manually by lowering the guillotine door (figure 2.9). The first blocked arm (either left or right guillotine doors) was chosen randomly by the experimenter. When the mouse was returned to the start compartment, it was confined there for 5 s by lowering the start arm door. In the free-choice trials, the start door was lifted and mouse was allowed to explore all three arms. After the mouse had chosen and entered a goal arm, the other arm was blocked by the experimenter using the manual guillotine door. When the

mouse returned to the start compartment, it was confined there for 5 s before the next free choice trial (figure 2.10). A total of 14 such free choice trials were carried out during a session. At the end of each session, the mouse was removed from the T-maze and returned to the home cage. The % spontaneous alternation was measured 50% spontaneous alternation represents the chance level.

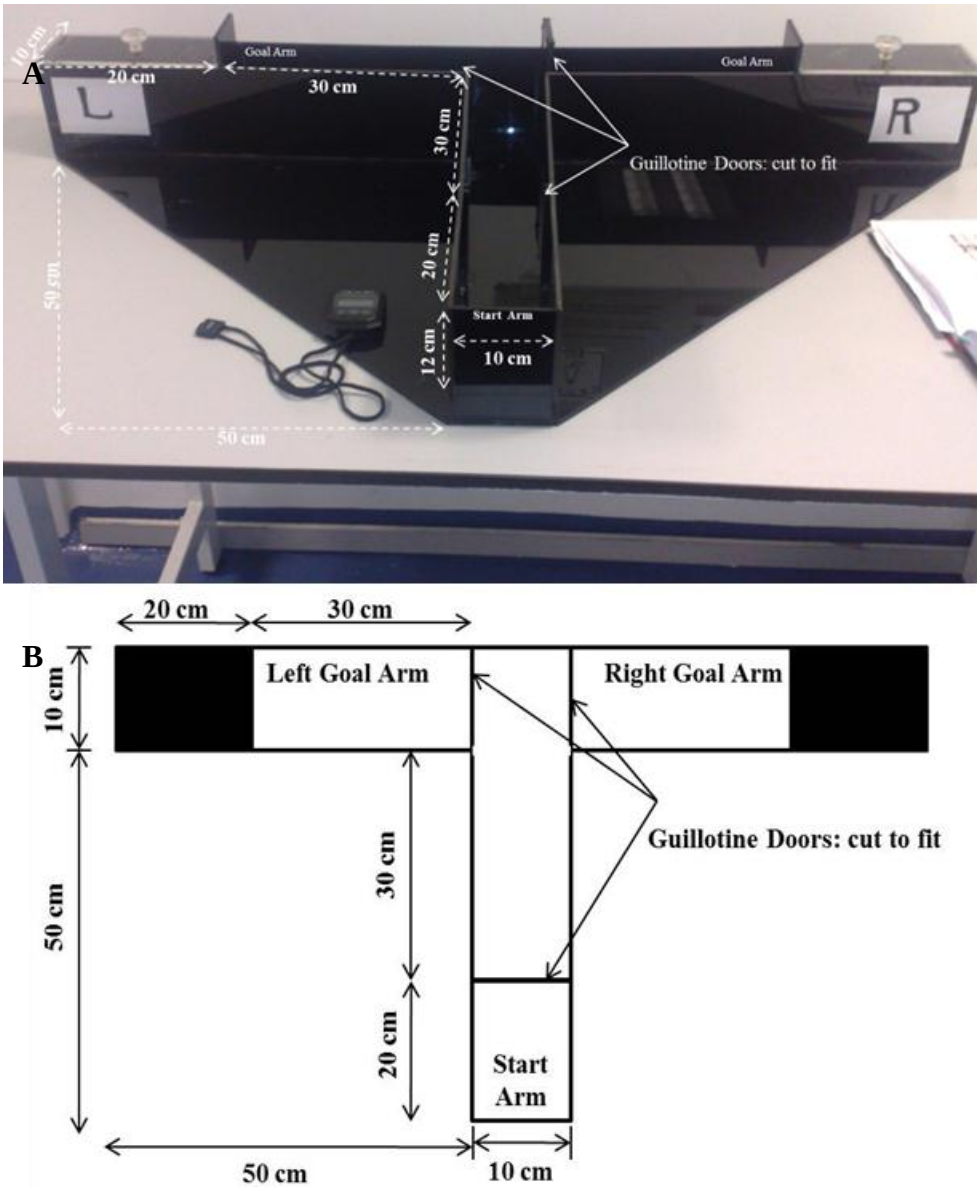


Figure 2.9: T-maze apparatus for mice. (A) Picture represents the T-maze (B) Picture represents schematic drawing of the same T-maze. The black areas at the end of the goal arms were never accessible to the mouse.

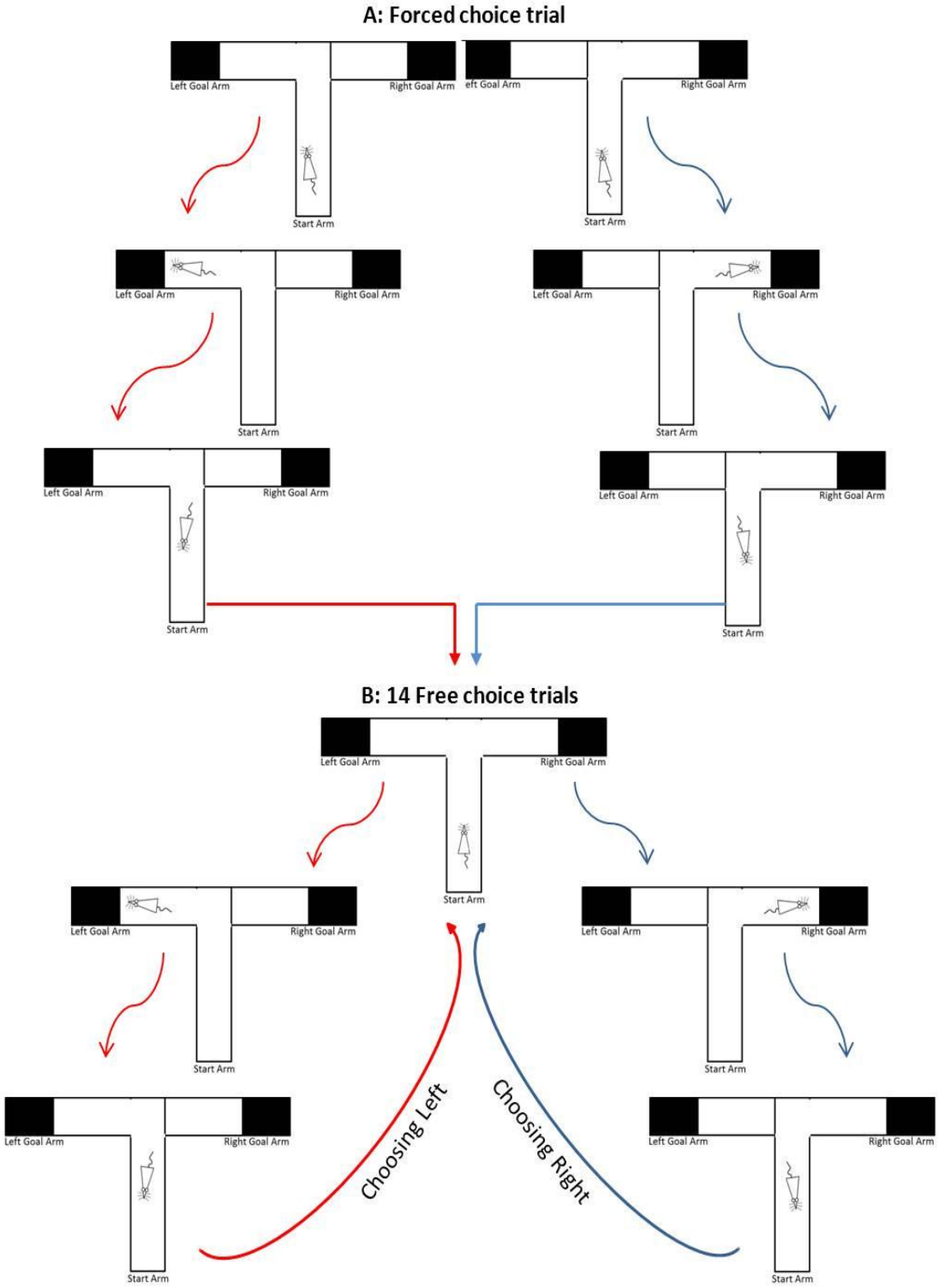


Figure 2.10: The T-CAT procedure for mice. A single session consisted of a forced-choice trial which was then followed by 14 free-choice trials.

2.4.6 NOR test

NOR apparatus

The NOR test used the same box which was used for OF test as described earlier (see section 2.4.1).

NOR procedure

The NOR test was performed over two consecutive days. As with the other tests the apparatus was cleaned with 10% Decon between mice. On the first day, the mouse was habituated to the testing environment. On the second day, the mouse was initially allowed to explore two identical objects and then 1 h later one of the objects was replaced by a novel object which the mouse had not seen previously.

A day before the NOR test, the mouse was placed in the OF box and allowed to explore for 15 min to habituate the animal to the environment and reduce general environmental exploration during the test. The following day the mouse underwent the NOR test. The OF box and familiar and novel objects (described in figure 2.11) were cleaned with 10% Decon prior to each test to remove possible odor. During the sample phase, two identical objects were placed symmetrically in the OF box 10cm away from each other and the mouse was placed in the centre of the box and allowed to explore the objects for 10 min. The time spent exploring the two identical objects was scored by hand. The object exploration was defined as when the head of the mouse was facing and proximal (within 1cm) to the object. The mice were returned to their home cages immediately after the sample phase (figure 2.11).

The choice phase of the test was performed 1 h after the end of the sample phase. In this phase, one of the familiar objects used during training was replaced by a novel object (figure 2.11). The mouse was placed in the OF box and allowed to explore the familiar and novel objects for a period of 10 min. The time spent exploring the familiar and novel objects was measured in a similar manner to that used in the sample phase (figure 2.11). The time ratio (during the sample phase) and discrimination ratio (during the choice phase) were calculated as follows:

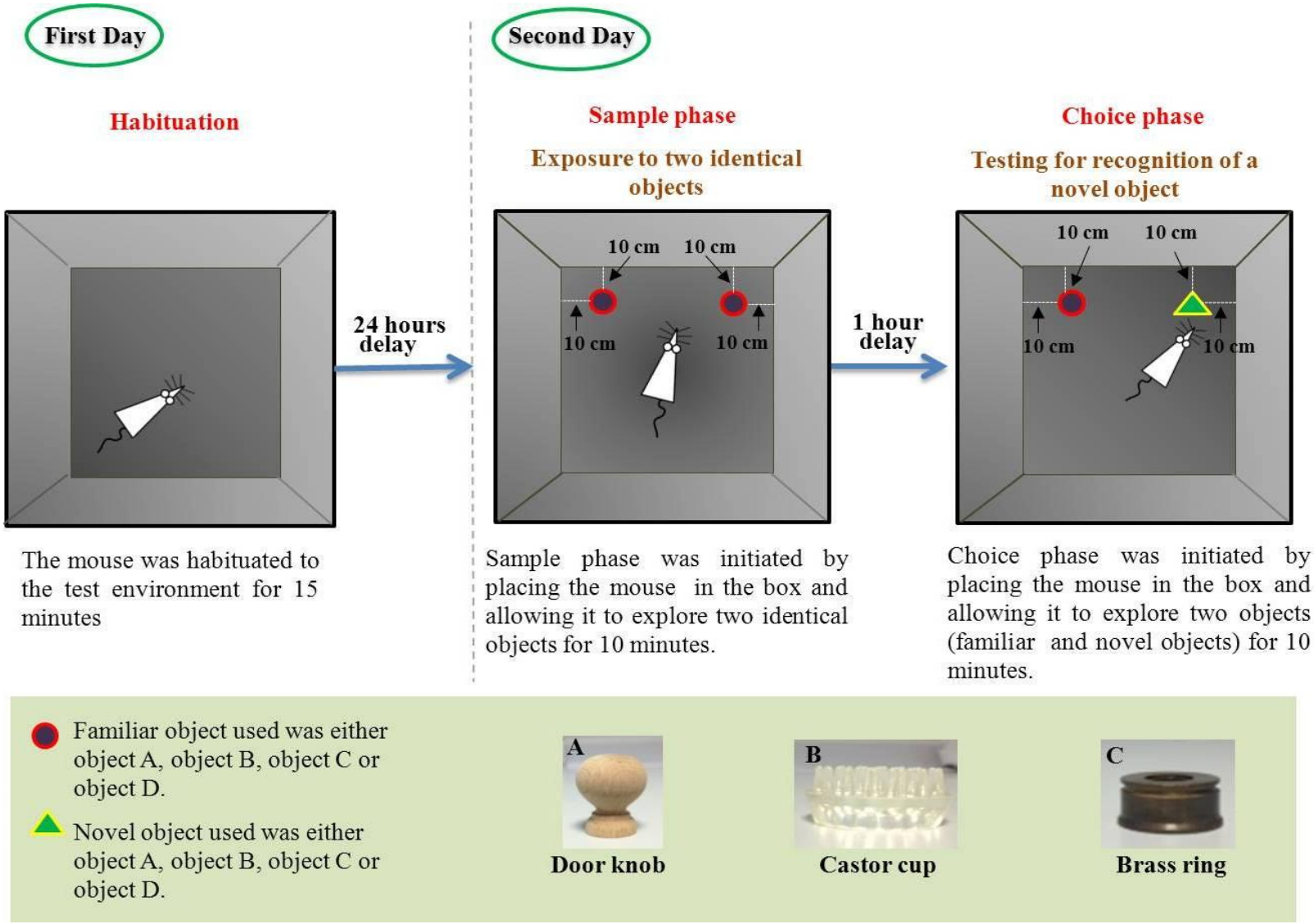


Figure 2.11: The NOR test protocol. The familiar and novel objects were used either object A (door knob), object B (castor cup) or object C (brass ring).

$$\text{Time (ratio)} = \frac{\text{Time spent in exploration of either left or right identical object}}{\text{Time spent in exploration of left object} + \text{Time spent in exploration of right object}}$$

$$\text{Discrimination ratio} = \frac{\text{Time spent in exploration of novel object}}{\text{Time spent in exploration of familiar object} + \text{Time spent in exploration of novel object}}$$

2.4.7 SR and PPI tests

SR and PPI apparatus

All SR and PPI experiments were performed in 3 sound-attenuated startle chambers (MED Associates Inc, USA). Briefly, mice were placed in a clear, ventilated and illuminated Plexiglas cylinder (12.5cm long and 5.5cm in diameter) mounted on a platform within the chamber. A high-frequency loudspeaker was mounted above the mouse which produced both continuous background noise and a number of acoustic stimuli. A whole-body startle response caused displacement of the plexiglas cylinder which was measured by a piezoelectric accelerometer mounted under each platform. The startle response results were digitised and stored by an interfacing computer assembly. Stimuli presentation and data acquisition were controlled by a computer using MED Associates software (figure 2.12). The peak response to stimuli is presented in arbitrary units.

SR and PPI procedures

The tests consisted of three consecutive days with one session a day with a duration of 30 min for each session. Within the first day, mice were habituated to the environment and auditory stimuli. The mice were then tested on the next 2 days, firstly the mice were tested for SR and then were tested for PPI. Both tests followed the same procedure with different stimulus presentation design (figure 2.12).

Startle response paradigm

The startle paradigm was divided into 2 blocks. Block 1 contained 6 trials of the startle stimulus alone 120 decibel (dB).

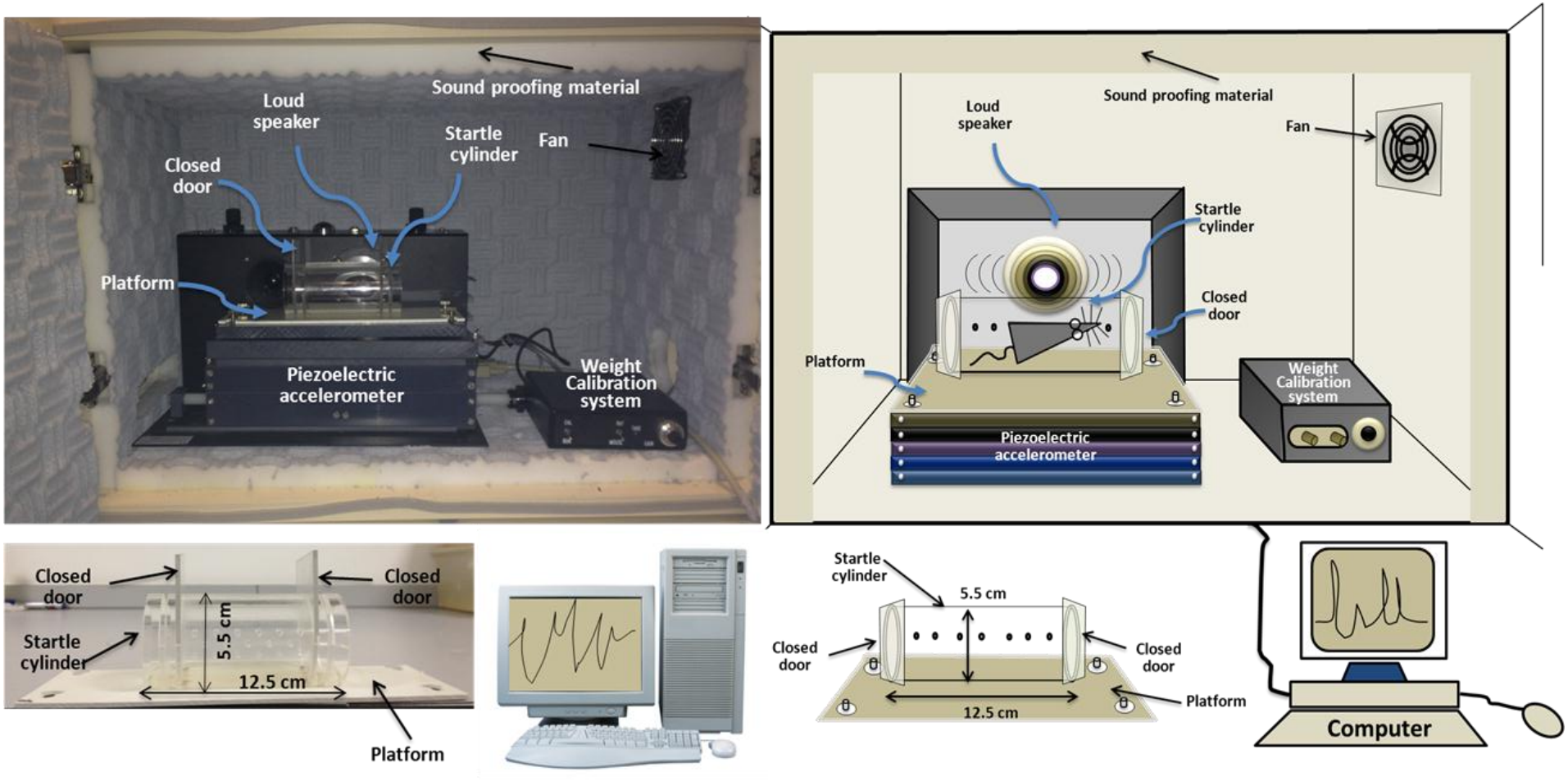


Figure 2.12: The apparatus used to evaluate the SR and PPI of startle response.

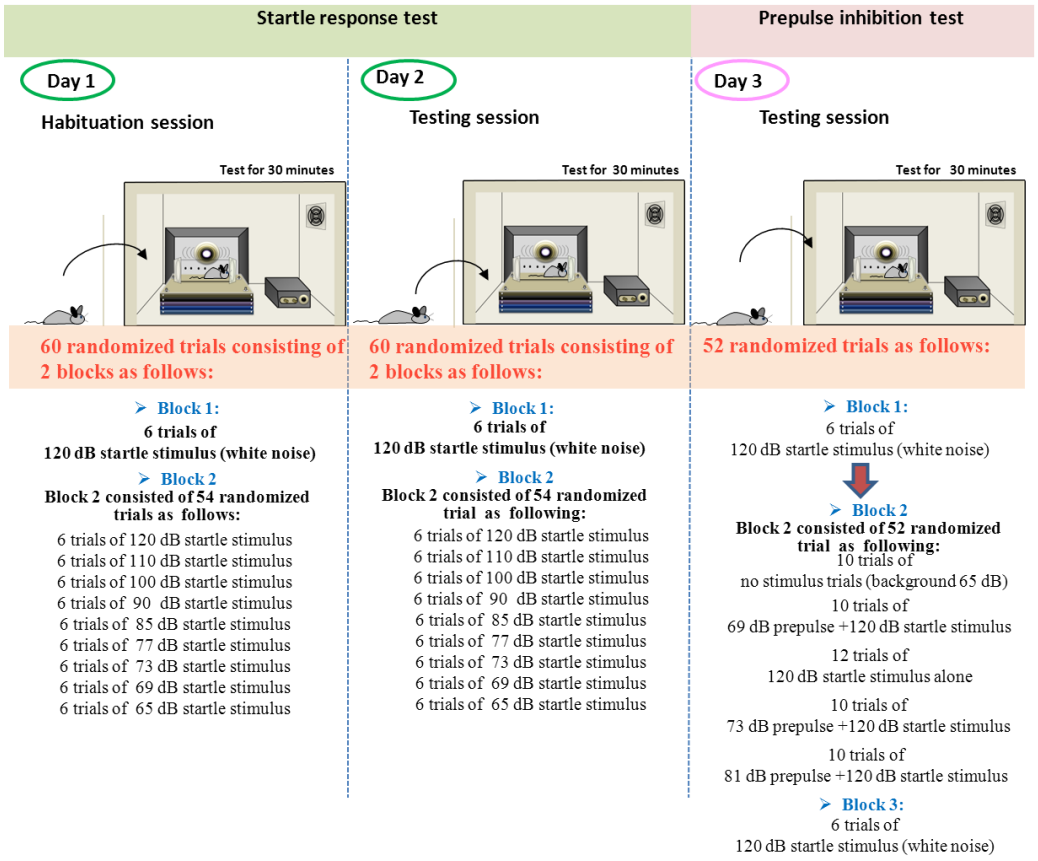


Figure 2.13: SR and PPI procedure. Trial sessions of the three testing days.

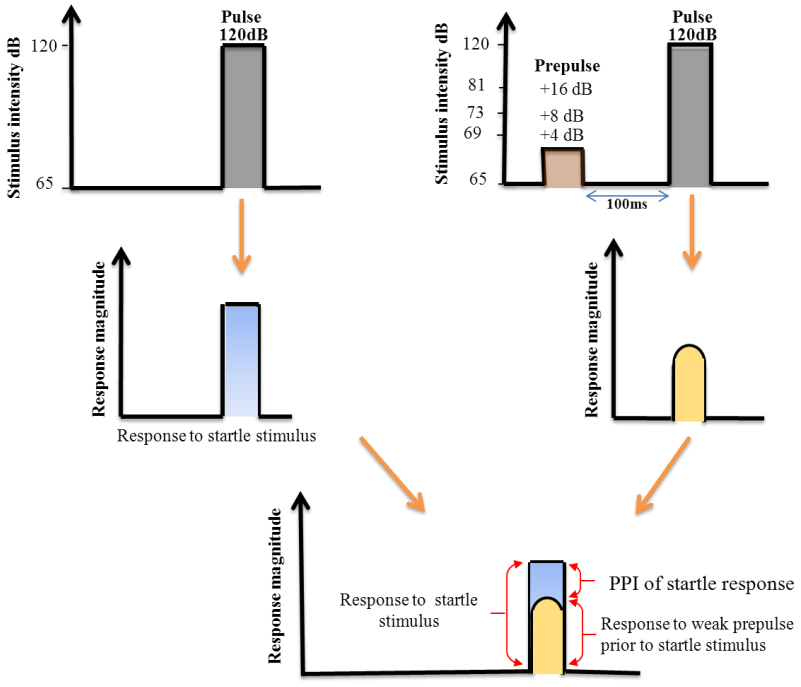


Figure 2.14: Experimental detail for measuring of PPI. The parameter measured is the percentage PPI induced by each prepulse intensity (4, 8 and 16 dB).

Block 2 consisted of 54 randomized trials, which were 6 trials of each of the following: 120dB, 110dB, 100dB, 90dB, 85dB, 77dB, 73dB, 69dB and 65dB (figure 2.13).

PPI test session

The PPI paradigm was divided into 3 blocks, block 1 contained 6 trials of the startle stimulus alone 120 decibel (dB) while in block 2, the startle stimulus was a 120 dB white noise, occurring 100 milliseconds after the prepulse. The recording duration was 65 milliseconds following the offset of the startle stimulus. A 5 min acclimation period with 65 dB proceded the test session. The block 2 consisted of 52 randomized trials (figure 2.13). 10 trials each of the following: no stimulus trials (background 65dB only), 69dB prepulse + 120dB, 73dB prepulse + 120dB and 81dB prepulse + 120dB and 12 trials of the startle stimulus alone (120dB white noise). We used white noise prepulse and startle stimuli in order to avoid any effects of mild hearing deficits at specific frequencies. Finally, block 3 contained 6 trials of the startle stimulus alone 120 dB. The percentage PPI induced by each prepulse intensity was calculated as follows (figure 2.14):

$$\%\ \text{PPI} = \frac{\text{Response to startle stimulus} - \text{Response to prepulse and startle stimulus}}{\text{Response to startle stimulus}} \times 100$$

2.5 Determination of PAR2 agonist content in mouse brain after peripheral injection

2.5.1 Isolation of mouse brains

Mice injected intraperitoneally (i.p.) with the PAR2 agonists (SLIGRL-NH_2, AC-264613 and GB110) or the vehicle of PAR2 agonists (0.9% sanline for SLIGRL-NH_2 and 1% Tween 80 in 0.9% sanline for both AC-264613 and GB110). The mice were killed by cervical dislocation. The mouse brains were removed and immediately frozen by immersion in -40 $^{\circ}$C isopentane (2-methyl butane; Fisher, Scientific UK). The brains were transported in dry ice prior to long-term storage at -80 $^{\circ}$C. The time between injection of the PAR2 agonists and removal of the brain depended upon the

experimental design (see chapter 5, figure 5.13). The doses of SLIGRL-NH_2 and GB110 was used were 1.5 mg/kg and 5 mg/kg. However, the doses of AC-264613 were 3 mg/kg and 10 mg/kg because the compound was made as racemic mixture of the stereoisomers.

Homogenisation of mouse brain tissue

Brain tissue (100 mg) was weighed and mixed with 200µl of 0.1% formic acid and 800µl of methanol using a Ultra-Turrax homogenizer. The mixture was transferred to a microcentrifuge tube and centrifuged for 10 min at 5000 RPM at room temperature (20 °C). The supernatant was removed and transferred to a glass vial using a glass pipette. The samples were then loaded into a LC-MS (Thermo Fisher Scientific, UK) which measured the mass-to-charge-ratio of the PAR2 agonists in order to detect and determine the concentration of PAR2 agonists in the brain tissue. The LC-MS analysis was conducted by Lynsey Macintyre and T. Zahang in the Pharmaceutical Analysis lab at the University of Strathclyde.

2.5.2 LC-MS

A MS is an instrument consisting of an ionisation chamber, an analyser and a detector which is used to measure the molecular mass of compounds. A sample is usually introduced into the ionisation chamber where ions can be generated and focused towards the analyser where they are separated based on their molecular weights, expressed as the mass (m)-to-charge (z) ratio (m/z). The separated ions are identified and the signals forwarded to an electronic system where the m/z ratios are kept collectively with their relative abundance and displayed in the form of an m/z spectrum. The MS operates under a high vacuum to enable ions to travel without any effect of air.

LC-MS instrumentation

An Orbitrap Exactive MS (ThermoFisher, Hemel Hempstead, UK) was coupled to a Dionex HPLC system (Dionex, Camberly, UK). The gas flow in the ESI source was adjusted to sheath gas 60 (arbitrary units) and auxiliary gas 25 (arbitrary units) to give a flow rate was 0.3 ml/min. The capillary temperature was 275° C and the needle voltage was 4.5 kV. The instrument was calibrated according to the

manufacturer’s instructions prior to use and was internally calibrated by lock masses (positive ion mode m/z 83.06037 due to a acetonitrile dimer and negative ion mode 91.00368 due to a formic acid dimer).

Chapter 3

Investigation into the role of PAR2 in normal mouse behaviour

3.1 Introduction

PAR2 receptors are widely expressed throughout the body including the CNS. Much of the knowledge regarding the physiological and pathophysiological roles of PAR2 has been gained from studies within the periphery with PAR2 linked to blood clotting (Alm *et al.,* 1999; Coughlin, 2005; McEachron *et al.,* 2010) and inflammation, as well as playing a role in diseases such as rheumatoid arthritis (Ferrell *et al.,* 2003; Kelso *et al.,* 2007). However, despite this knowledge in peripheral systems, our knowledge of the function of PAR2 in the CNS remains limited.

Within the CNS, PAR2 is expressed within the hippocampus, cortical layers, the amygdala, thalamic nuclei, the hypothalamus and striatum (Smith *et al.,* 1997; Striggow *et al.,* 2001; Noorbakhsh *et al.,* 2005; Steinhoff *et al.,* 2005; Bushell *et al.,* 2006; Noorbakhsh *et al.,* 2006). PAR2 has been shown to be expressed in neurons, astrocytes (Bushell *et al.,* 2006; Wang *et al.,* 2002; Ubl *et al.,* 1998) and microglia (Balcaitis *et al.,* 2003; Park *et al.,* 2010). In addition, proposed PAR2 activators are also expressed in the brain, for example, trypsinogen IV, neurotrypsin and kallikreins, thus suggesting that activation of PAR2 receptors may have an important role in the CNS (Gschwend *et al.,* 1997; Toth *et al.,* 2007; Yousef *et al.,* 2003; Wang *et al.,* 2008).

Many studies have found that PAR2 plays an important role in activation of peripheral sensory neurons *in vivo* (Vergnolle *et al.,* 1999; Vergnolle *et al.,* 2001; Amadesi *et al.,* 2004; Vergnolle, 2005; Amadesi *et al.* 2006; Bunnett, 2006; Alier *et al.,* 2008). PAR2 activation on central neurons results in an IP_3-mediated increase in intracellular Ca^{2+} (Smith-Swintosky *et al.,* 1997; Bushell *et al.,* 2006; Gorbacheva *et al.,* 2006). In astrocytes, activation of PAR2 with trypsin leads to a prolonged desensitization of PAR2 receptors and inhibits further stimulation of the receptor (Bushell *et al.,* 2006). In addition to prolonged desensitization, activation of PAR2 with trypsin or SLIGRL-NH_2 induced reversal of stellation (morphological change into a star-shaped cell) of cultured astrocytes (Park *et al.,* 2006). Trypsin and PAR2 activation increases nitric oxide (NO) production from astrocytes by NF-κB

mediated nitric oxide synthase (iNOS) in addition to increasing production of several PIC (Park *et al.,* 2009). However, PAR2 activation may induce microglia activation, which contributes to neuronal cell death through activation of PKC-MAPK-NF-κB dependent pathways resulting in increased nitric oxide (NO), and PIC (Park *et al.,* 2010).

PAR2 has been proposed to play either a neuroprotective or neurodegenerative role in the CNS and potentially has dual effects in some neurological diseases. A number of studies have found that activation of PAR2 has neuroprotective properties under certain conditions (Noorbakhsh *et al.,* 2005; Jin *et al,* 2005; Wang *et al.,* 2007) Greenwood and Bushell, 2010). One of these studies indicated that PAR2 activation protects the brain from acute focal ischaemic injury, as PAR2 KO mice show elevated infarct volumes in a transient ischaemia/reperfusion model. This result suggests a neuroprotective role for PAR2 function (Jin *et al.,* 2005). Similarly, PAR2 activation by the PAR2 agonist SLIGRL-NH_2 was protective against seizures in an electrical amygdala-kindling seizure model (Lohman *et al.,* 2008). Furthermore, PAR2 activation was shown to be neuroprotective in an *in vitro* model of neurotoxicity (Greenwood and Bushell, 2010). In brain tissue from human HIV-1, it has been noticed that increased PAR2 expression and subsequent activation prevent neuronal cell death and protect neurons from HIV-induced neuroinflammation (Noorbakhsh *et al.,* 2005).

In addition to its neuroprotective properties, PAR2 has been linked with neurodegenerative disorders including Alzheimer's Disease. In a murine model of Alzheimer's Disease, the dual role of PAR2 activation has also been suggested (Afkhami-Goli *et al.,* 2007). In another neurological disease, multiple sclerosis, PAR2 has been proposed to play a contributory role due to modulation of neuroinflammation (Noorbakhsh *et al.,* 2006). According to previous studies, PAR2 plays an important role in pathophysiological conditions but whether PAR2 plays a key physiological role in the CNS when investigated *in vivo* remains unclear despite its widespread expression. A recent study indicated that the peripheral injection of a PAR2 agonist in rats produced deficits in motivational learning in the MWM and the test-retest paradigm of the EPM (Lohman *et al.,* 2009). In addition,

electrophysiology experiments indicated that activation of PAR2 by SLIGRL-NH_2 indirectly modulates neuronal excitability and synaptic transmission in the hippocampus (Gan *et al.*, 2011).

According to these previous studies, PAR2 may play an important role in the CNS under physiological and pathophysiological conditions. In addition, in our laboratory as PAR2 is expressed in brain areas which are involved in emotional behaviour and cognition, PAR2 may play an important role in mouse behaviour such as anxiety-like behaviour and learning and memory.

3.1.1 Aim

The aim of this study was to investigate the role of PAR2 in locomotor activity, anxiety-like behaviour, spatial memory, recognition memory, working memory, SR and PPI in male and female mice from three genotypes (WT, PAR2 HT and PAR2 KO mice).

3.2 Material

3.2.1 Breeding of the PAR2 colony

Experiments were performed on PAR2 genetic modified (GM) mice obtained by crossing 14 pairs of HT mice to obtain different genotypes as explained in chapter 2, section 2.2.2 (figure 2.1). The mice were housed as described in chapter 2, section 2.2.2. The WT, HT and KO mice were separated based on gender and group housed, except where required to be singly housed for the purposes of the experiment as described in chapter 2, section 2.2.2.

3.3 Effect of PAR2 deletion on locomotor activity, anxiety-like behaviour and spatial reference memory

3.3.1 Animals

This experiment was performed on 95 adult mice of both genders obtained as described above. The mice were 12 weeks old males (26.0 ± 0.4g) or females (19.6

± 0.4g) of different genotypes (WT, HT and KO). The behavioural tests used in this study were the OFT, the EPM and the MWM (figure 3.1) and were performed on the same mice. The number of mice per genotype used for OF, EPM and MWM tests were as follows: (males: WT *n*=16, HT *n*=16, KO *n*=16; females: WT *n*=16, HT *n*=16, KO *n*=15).

3.3.2 Protocol

The experiments were carried out according to the availability of the KO mice. Thus more than one batch of mice was used and the data pooled. Male and female mice were tested separately and mice were tested in an order randomised for genotype. The behavioural testing protocol was designed to start with the least stressful test followed by a more stressful test. Thus, the initial experiment in this study was the OFT, followed by the EPM and finally the MWM. As illustrated in figure 2.3, on day 1, the mice were handled by the experimenter to reduce stress during the test.

OF protocol

On day 2, the mice were habituated to the OF box for 10 min to reduce the stress of the new environment. On day 3, the mice were divided into 6 groups (3 male groups: WT *n*=16, HT *n*=16, KO *n*=16; 3 female groups: WT *n*=16, HT *n*=16, KO *n*=15). Data was lost from one male KO mouse because it was not recorded correctly. Therefore in OFT the number of male KO mice were used was 15 mice instead of 16 mice. In this experiment and in all behavioural experiments, the male and female mice were tested in separate sessions to prevent any involvement of sexual attraction during the experiment (Shaw and Parsons, 2002). The mice were tested in the OF box for 10 mins to investigate the effect of PAR2 deletion on general locomotor activity and anxiety-like behaviour (figure 3.1). The OF apparatus, its experimental condition, and parameters measured were identical to those described in chapter 2, section 2.4.1.

EPM protocol

On day 4, the mice were tested in the EPM to determine the effect of PAR2 deletion on anxiety-like behaviour. In this test, the animal groups were as used in the OFT and male and female mice were again tested separately. In each session a mouse was

placed in the centre of the maze and allowed to explore freely for 10 mins. The EPM apparatus, experimental procedures, conditions and the parameters measured are detailed in chapter 2, section 2.4.2.

MWM protocol

On days 5-9, the MWM test was carried out. For this test, each mouse was given four trials per day for five consecutive days as shown in figure 3.1 . Groups were the same as those in the OFT and EPM tests and male and female mice were tested separately. On the first day of the MWM test (day 5), a trial was performed to familiarise the animals to the test. This trial was followed by three consecutive acquisition trials with each trial lasting for a maximum of 60 s with a 15 min interval between consecutive trials in a single animal. On subsequent days (days 6-8) mice underwent 4 acquisition trials per day. On the last day of testing (day 9), mice underwent 3 acquisition trials followed by a final probe trial.

The habituation trial, acquisition and probe trials have been explained in detail earlier in chapter 2, section 2.4.4. Additionally, the swimming pool, experimental procedures and conditions and the parameters measured were as explained in the same chapter and section.

3.4 Effect of deletion of PAR2 on working memory and recognition memory

3.4.1 Animals

A total of 70 GM mice were used in this experiment and were housed as described previously. These mice were obtained in several batches. The behavioural experiment was carried out on both genders with three genotypes (WT, HT and KO). The behavioural tests T-CAT and NOR test were performed on adult mice, 12 weeks old and weighing 26.0 ± 0.8 g (males) and 20.6 ± 0.3 g (females). The number of male mice per genotype used for NOR were: WT *n*=14, HT *n*=14, KO *n*=8.

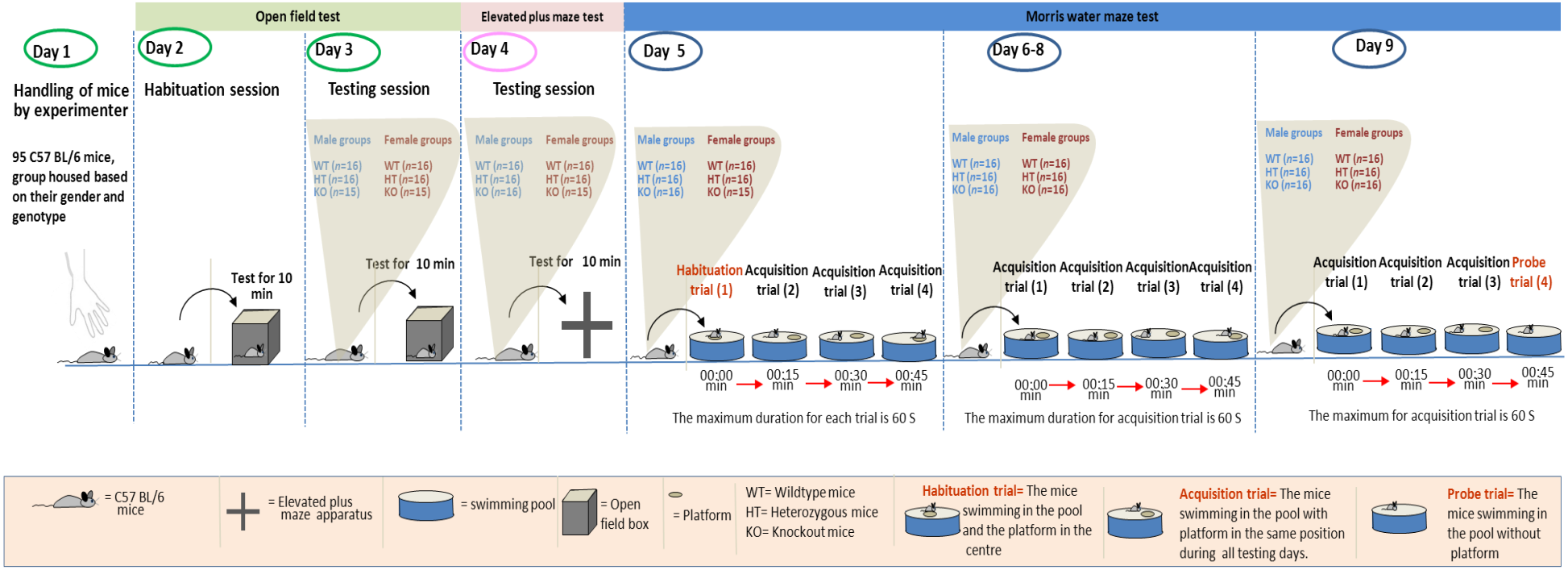


Figure 3.1: Experimental protocol for the effect of PAR2 deletion on mouse locomotor activity, anxiety-like behaviour and spatial reference memory.

The same male mice were used for T-CAT but the data from 4 male WT mice were excluded because they did not complete 14 trials of the T-CAT, thus the number per genotype were: WT *n*=10, HT *n*=14, KO *n*=8).

The number of female mice per genotype used for the NOR test were: WT *n*=12, HT *n*=15, KO *n*=4. The female mice used for the NOR test were also used for T-CAT with more female mice added (T-CAT was carried out on 3 female batches of mice whereas NOR was carried out on 2 female batches). Some of the female mice did not complete the 14 trials of T-CAT test so the data from 7 female mice (3 WT, 4 HT) were excluded. For these reasons the number of female mice used for T-CAT were: WT *n*=13, HT *n*=17, KO *n*=8. According to the protocol the T-CAT was carried out first followed by NOR. The mice were randomly tested according to their genotypes. Similar to other experiments the male groups were tested separately from female groups.

3.4.2 Protocol

As previously mentioned, the mice were handled a day before starting the experiment. On the second day, the mice were tested in the T-CAT (figure 3.2). The test consisted of one single session, which started with a forced-choice trial, followed by 14 free-choice trials; each trial was completed between 30 s to 3 min (chapter 2, figure 2.10). The time between trials was measured by the technical support staff working within the Institute's BPU. Apparatus, experimental procedures and conditions for the T-CAT, and the parameters measured, are as explained in chapter 2, section 2.4.5. On the third and fourth day, the mice were tested in the NOR test (figure 3.2). Firstly, the mice were placed for 15 min in the OF box in order to habituate to a new environment. On the next day, the mice were firstly exposed to two identical objects and then after 1 h the mice were tested for recognition of a novel object (chapter 2, figure 2.11). Apparatus, experimental procedures and conditions for the NOR, and the parameters measured, are as explained in chapter 2, section 2.4.6.

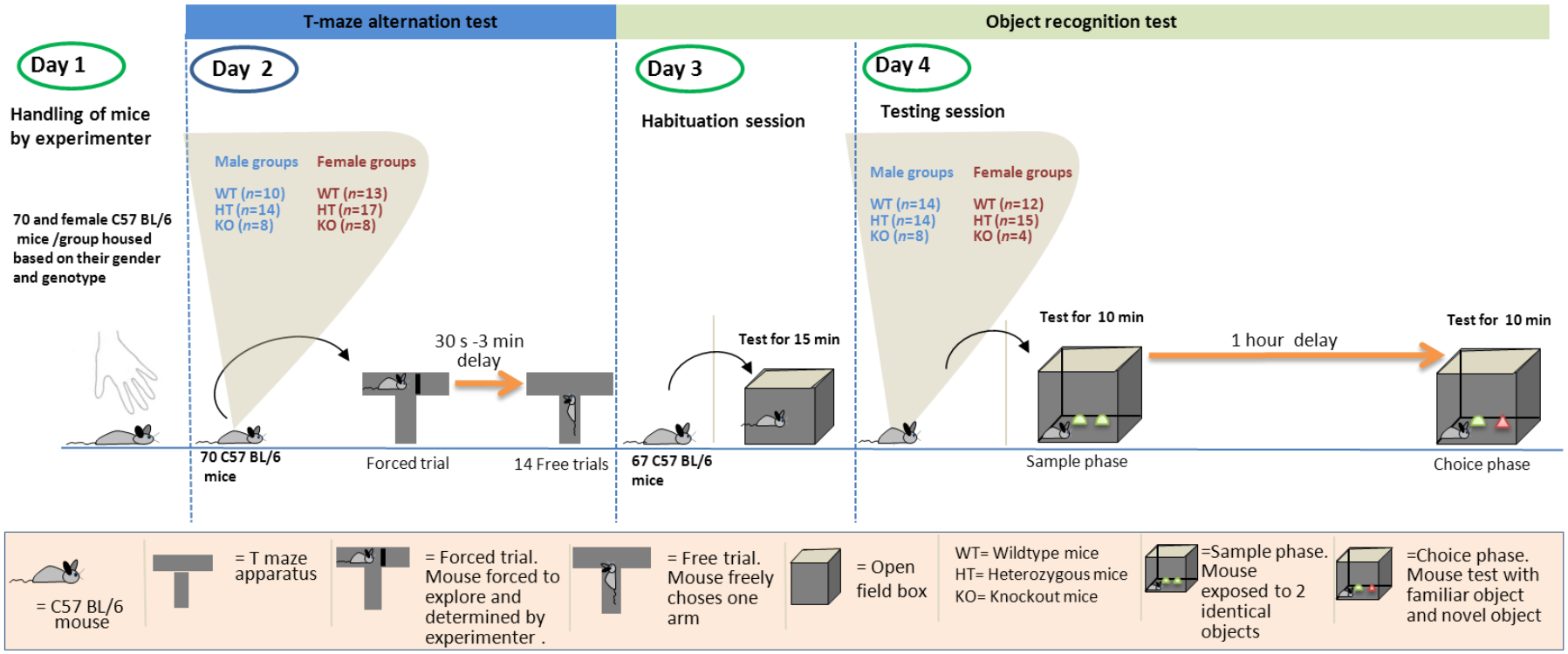


Figure 3.2: Experimental protocol for the effect of PAR2 deletion on mouse spontaneous alternation and recognition memory.

3.5 Effect of PAR2 deletion on the SR and PPI tests

3.5.1 Animals

The behavioural tests (SR and PPI) were performed on 71 adult mice approximately 12 weeks old, of all three genotypes and of both genders weighing 29.1 ± 0.5g (males) and 21.5 ± 0.2g (females), males: WT *n*=12, HT *n*=9, KO *n*=12; females: WT *n*=14, HT *n*=10, KO *n*=12.

3.5.2 Protocol

Similar to other experiments, the male groups were tested separately from the female groups. WT, HT and KO mice were tested in a random order. As described before, a day before the experiment, the mice were handled by experimenter.

On the second day, mice were habituated to the new environment and to the auditory stimuli in order to avoid any excessive stress effect during the SR testing. An illustration of the experimental procedure is shown in figure 3.3. On the third day, the mice were tested for SR. The SR session consisted of 60 trials over a 30 min period as shown in chapter 2, figure 2.4.7. On the fourth day, the mice were tested for PPI and exposed to the 64 random auditory stimuli trials (chapter 2, figure 2.13). The apparatus, procedures, conditions, auditory stimuli and parameters measured were as explained in chapter 2, section 2.4.7.

3.6 Statistical analysis

All values shown are mean ± standard error of the mean (*S.E.M.).* Data was analysed using Prism, SPSS and Minitab 15 software with differences considered significant if $P<0.05$. A chi-square statistical test was used to compare the numbers of the three genotypes of mice obtained and expected. Data from male and female mice are presented separately in figures because the two way ANOVA (general linear model) analysis did not identify any interaction between gender and genotype in most of the tests performed.

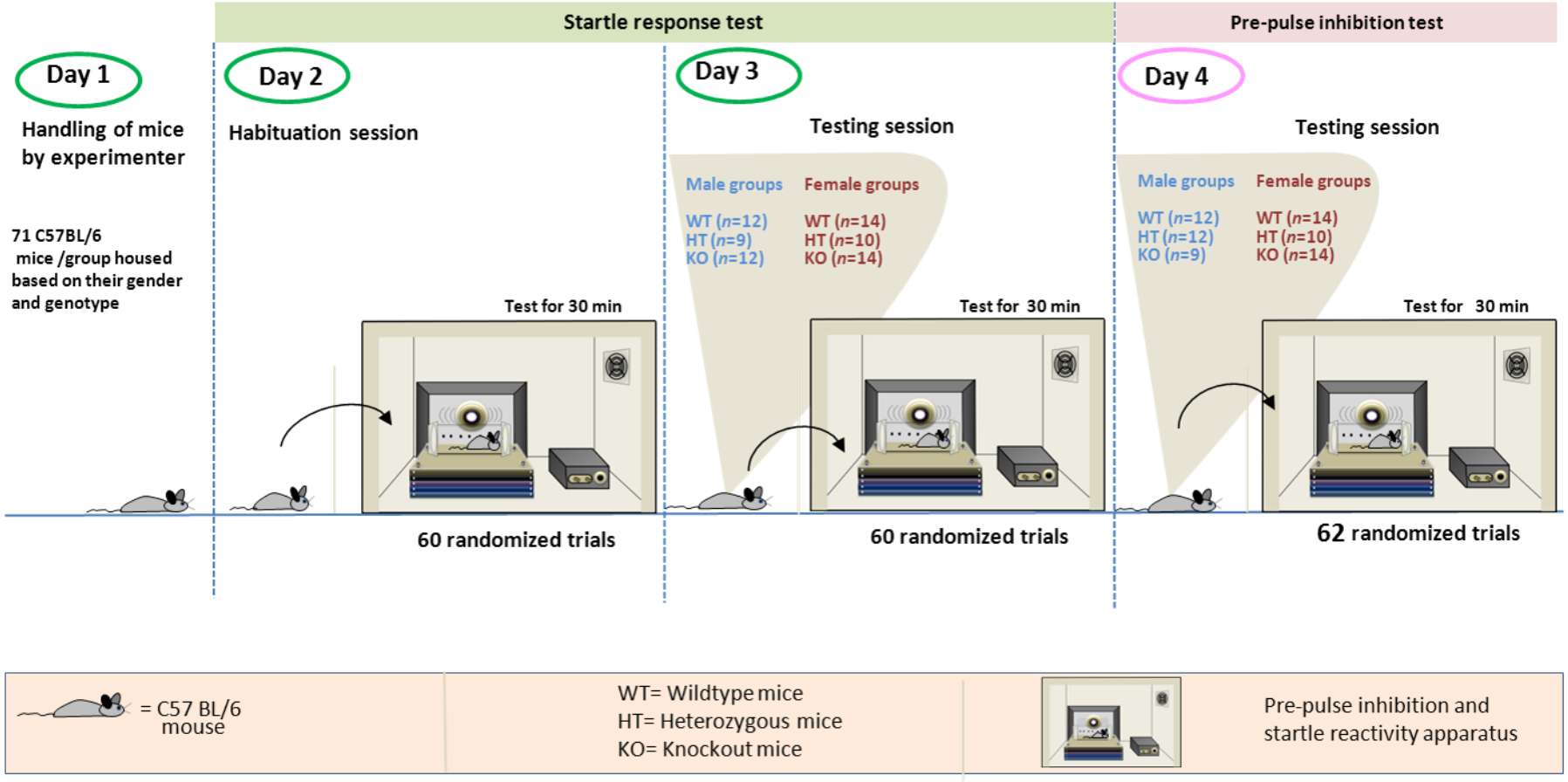


Figure 3.3: Experimental protocol for the effect of deletion of PAR2 on SR and PPI tests.

In OFT and EPM tests the data was analysed by using two way ANOVA with Tukey's post hoc test to examine the effects of gender and genotype as well to assess the interaction between these factors. However, one way-ANOVA was used to compare the three genotypes for each gender. The data for the MWM was analysed for each gender by using a repeated measures two way ANOVA with Tukey's post hoc test, which provides more detail about the difference between groups on a single testing day in time and distance moved to the platform. However, in the probe trial, a paired t-test was used to compare between the time spent in platform quadrant and opposite quadrant for each group.

Data of the T-CAT test was analysed using one way-ANOVA to compare between the three genotypes for each gender. A two-way ANOVA examined the effects of both gender and genotype and assessed the interaction between these factors. Subsequently, the same data was also analysed by a one-sample t-test to provide more detail about the difference between the observation mean of each genotype for both genders with the expected chance level value. In the NOR test, the data was analysed by a paired t-test to compare the difference between the time spent on the left and right objects during the sample phase for each genotype, while the one sample t-test was used to determine whether the discrimination ratio (time spent in exploration of the novel object/total exploration time) differed from the chance value.

Finally, the data for both SR and PPI tests were analysed for each gender separately using repeated measures two-way ANOVA followed by Tukey's post hoc test to provide details about the differences between the three groups on each pulse intensity or prepulse intensity in startle response and % PPI of startle response.

3.7 Results

3.7.1 PAR2 offspring do not display a Mendelian ratio

Genotyping was performed in each GM mouse to allow the mice to be placed in the appropriate group for behavioural testing. Based on the design of the PAR2 primers, each genotype can be identified by the number and size of specific DNA bands. For example, the PAR2 WT sample has a band at 383 base pairs (bp), the PAR2 KO sample has a band at 650 bp and the PAR2 HT sample has 2 bands at 383 bp and 650 bp, respectively (figure 3.4). The number of mice obtained for each genotype using this breeding protocol is shown in table 2.1.

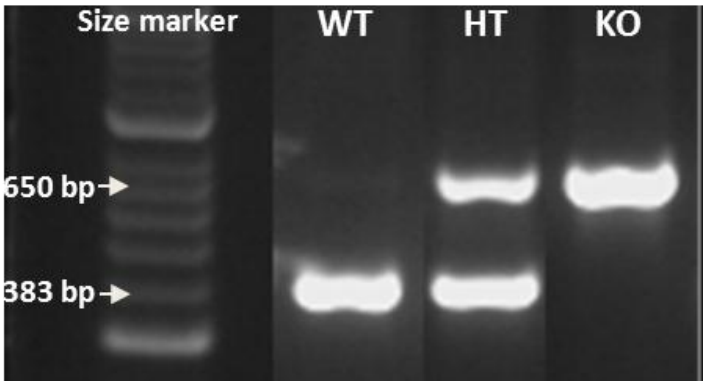


Figure 3.4: Representative example of the DNA bands produced by genotyping the PAR2 GM mice. The DNA band at position 383 bp represents the WT mouse, the two bands at position 650 bp and 383 bp represent the PAR2 HT mouse and the band at position 650 bp represents the PAR2 KO mouse.

There were significant differences between the number of mice (for both genders) obtained using this breeding protocol and the expected number of genotypes based on Mendelian ratio ($^1/_4$ WT, $^1/_2$ HT and $^1/_4$ KO) (df=2, chi-square value x^2= 13.429, p< 0.01). The number of WT mice (261 mice) that were obtained for both genders was greater than the expected number based on Mendelian ratio (210 mice). However, the number of PAR2 HT (411 mice) that were obtained for both genders was approximately close expected numbers based on Mendelian ratio (421 mice). In contrast to WT mice, PAR2 KO mice (169 mice) obtained from crossing the same mice was less than the expected Mendelian ratio of HT (421mice) and KO (210 mice).

Table 3.1: Number of mice of each genotype produced by breeding 14 pairs of PAR2 HT mice.

Genotype	Male	Female	Total
WT	109	152	261
HT	228	183	411
KO	76	93	169
Total	413	428	841

3.8 Effect of PAR2 deletion on the OFT

3.8.1 OFT: 10 min testing period

During the test, data from one male KO mouse was not recorded, therefore, the number of male KO mice used was 15 mice instead of 16 mice (figure 3.1). There was no significant effect of genotype or gender and no interaction between genotype and gender on the total distance moved (genotypes: $F_{(2,88)}= 0.02$, $p= 0.981$, genders: $F_{(1,88)}= 0.14$, $p= 0.744$, interaction: $F_{(2,88)}= 0.899$, $p= 0.411$) and entries into centre square (genotypes: $F_{(2,88)}= 0.415$, $p=0.707$, genders: $F_{(1,88)}= 0.07$, $p= 0.823$, interaction: $F_{(2,88)}= 2.14$, $p= 0.124$). However, there was a significant effect of genotypes but no significant effect of gender on time spent in centre square and there was a significant interaction between gender and genotypes (genotypes: $F_{(2,88)}= 0.90$, $p= 0.526$, gender: $F_{(1,88)}= 0.00$, $p= 0.960$; interaction: $F_{(2,88)}= 5.01$, $p= 0.009$).

Further analysis was carried out to compare the three genotypes for each gender; therefore the same data was re-analysed using a one way ANOVA followed by Tukey's post hoc test. Deletion of PAR2 did not affect locomotor activity in male mice, measured using the OFT over a 10 min period as there was no significant difference between male groups in general locomotor activity measured by distance moved (cm) over the testing period ($F_{(2,46)}= 0.52$, $p= 5.970$, figure 3.5 A). No significant difference was found between male groups in the number of entries into the centre square ($F_{(2,46)}= 1.69$, $p= 0.196$, figure 3.5 C) whereas HT male mice spent more time in the centre square compared to both WT and KO mice ($F_{(2,46)}= 8.72$, $p=$

0.0006, HT versus WT $p<0.01$, HT versus KO $p< 0.01$, KO non-significant (n.s.) compared with WT, figure 3.5 E).

However, in the females PAR2 deletion did not affect any of the parameters measured in the OFT. There was no significant difference between genotypes in total distance moved ($F_{(2,46)}= 0.38$, $p= 0.688$, figure 3.5 B), entries into the centre square ($F_{(2,46)}= 0.99$, $p= 0.381$, figure 3.5 D) and time spent in the centre square ($F_{(2,46)}= 1.01$, $p= 0.373$, figure 3.5 F).

3.8.2 OFT: Breakdown of the 10 min testing period

The effect of PAR2 deletion on locomotor activity and anxiety-like behaviour was initially measured in the OFT over 10 min, as is standard in our laboratory (figure 3.5). However, other groups using the OFT measure activity over shorter durations, therefore the effect of PAR2 deletion on locomotor activity and anxiety-like behaviour was also separately analysed for the first 5 min (Bailey and Crawley, 2009).

During the first 5 min of the OFT, a similar effect on anxiety-like behaviour was observed to that over the 10 min test period. There was no significant effect of genotype, gender and there was no interaction between genotypes and gender during the first 5 min test in total distance moved (genotype: $F_{(2,88)}= 0.03$, $p= 0.972$, gender: $F_{(1,88)}= 0.80$, $p= 0.465$, interaction: $F_{(2,88)}= 1.30$, $p= 0.277$) and entries into centre square (genotype: $F_{(2,88)}= 0.43$, $p= 0.702$, gender: $F_{(1,88)}= 0.30$, $p= 0.641$, interaction: $F_{(2,88)}= 1.75$, $p= 0.180$). Although there was no effect of genotypes and gender there was significant effect of interaction between genotypes and genders during first 5 min in time spent in the centre square (genotype: $F_{(2,88)}= 0.42$, $p= 0.707$, gender: $F_{(1,88)}= 0.00$, $p= 0.997$, interaction: $F_{(2,88)}= 4.50$, $p= 0.014$). To determine changes at individual time-points, the data were re-analysed separately for each gender using a one way ANOVA followed by Tukey's post hoc test. From this analysis, in males, there was no significant differences between genotypes on the total distance moved ($F_{(2,46)}= 0. 61$, $p= 0.500$, figure 3.6 A) and number of entries into the centre square during the first 5 min of the test ($F_{(2,46)}= 1. 49$, $p= 0.236$, figure 3.6 C).

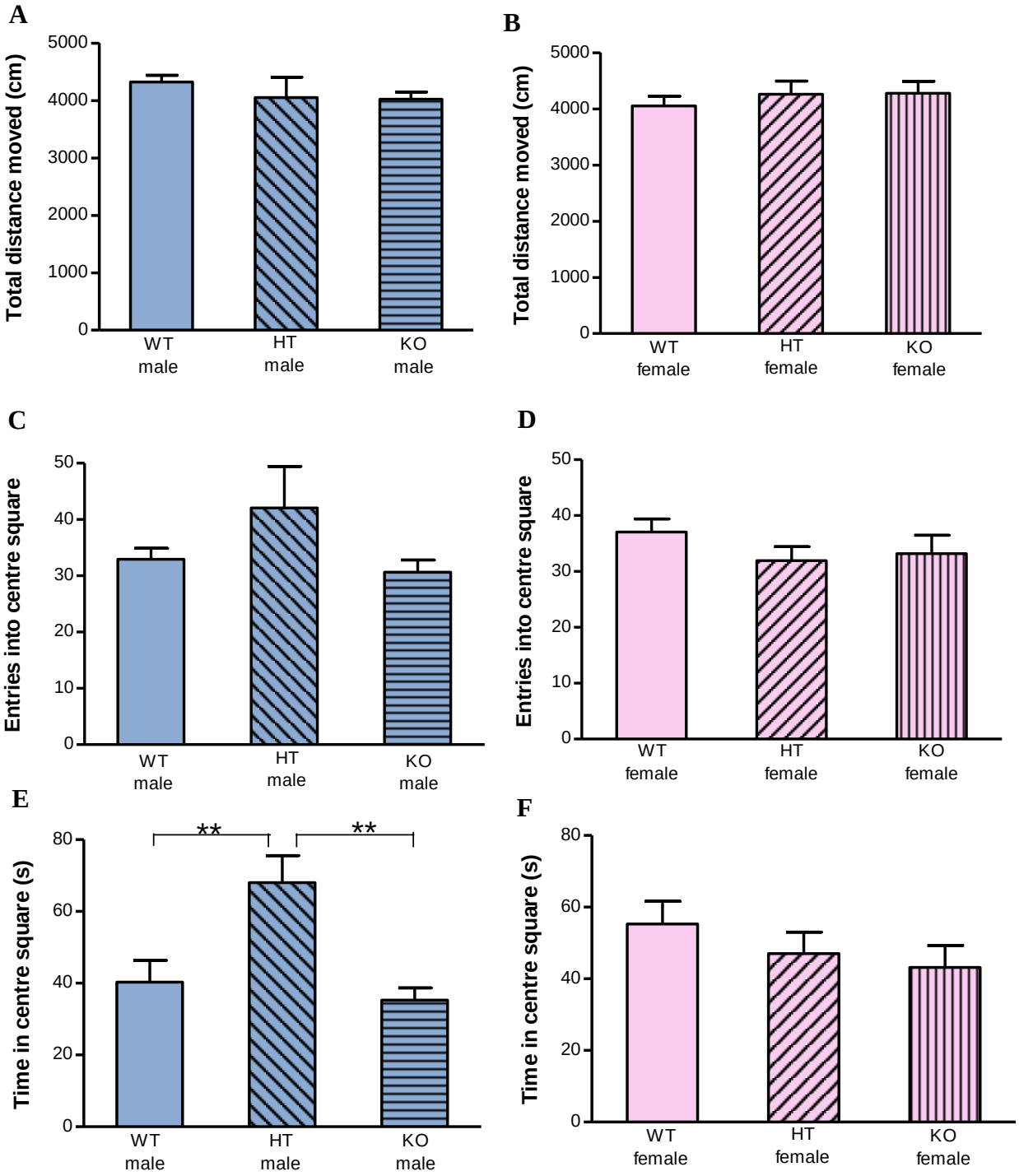


Figure 3.5: Effect of PAR2 deletion on performance in the OFT over 10 min in male and female mice. (A + B): No significant difference was observed between genotypes in either male or female groups in distance moved, (C + D): No significant difference was observed between genotypes in either male or female groups in entries into the centre square, (E) PAR2 HT males differ significantly from WT (p<0.01) and PAR2 KO males (p<0.01) in time spent in the centre square, (F): No significant difference observed between female groups in time spent in the centre square. Males and female n= 15-16 per group. Data is presented as mean ± *S.E.M.* and the data was analysed using a one way ANOVA with Tukey's post hoc test between genotypes where appropriate, ** = p<0.001.

However, during the first 5 min, HT males spent more time in the centre compared with WT and KO ($F_{(2,46)}$= 5.61, p= 0.007, WT versus HT p<0.05, HT versus KO p<0.05, KO n.s. compared with WT, figure 3.6 E). Similar to 10 min testing, in the female mice no significant differences were observed between genotypes for any of the parameters measured during the first 5 min in the OFT; total distance moved ($F_{(2,46)}$= 0.81, p= 0.453, figure 3.6 B), entries into centre square ($F_{(2,46)}$= 0.28, p= 0.755, figure 3.6 D), and duration in the centre square ($F_{(2,46)}$= 0.92, p= 0.408, figure 3.6 F).

3.9 Effect of PAR2 deletion on the EPM test

3.9.1 EPM: 10 min testing period

PAR2 deletion did not affect anxiety-like behaviour in the EPM test in either male or female mice over 10 min testing period. There was no significant effect of genotypes, gender and no interaction between genotypes and genders on all of the parameters measured in the EPM: % time in open arms (genotype: $F_{(2,89)}$= 6.26, p= 0.138; genders: $F_{(1,89)}$= 0.23, p= 0.682; interaction: $F_{(2,89)}$= 0.19, p= 0.824), % open entries (genotype: $F_{(2,89)}$= 0.64, p= 0.611; genders: $F_{(1,89)}$= 0.02, p= 0.890; interaction: $F_{(2,89)}$= 1.49, p= 0.232) and total entries (genotype: $F_{(2,89)}$= 0.04, p= 0.961; genders: $F_{(1,89)}$= 2.82, p= 0.235; interaction $F_{(2,89)}$=1.02, p= 0.366).

Further analyses were performed the same data was re-analysed separately for each gender using a one way ANOVA followed by Tukey's post hoc test.

Analysis of the male groups showed there were no significant differences between genotypes in the % time spent in open arms ($F_{(2,47)}$= 0.26, p= 0.771, figure 3.7 A), % open arm entries ($F_{(2,47)}$= 0.11, p= 0.893, figure 3.7 C) and total entries ($F_{(2,47)}$= 0.76, p= 0.472, figure 3.7 E) over the 10 min test period. Similarly, in female mice there was no significant difference between genotypes in the % time in open arms ($F_{(2,46)}$= 1.01, p= 0.371, figure 3.7 B) , % open arm entries ($F_{(2,46)}$= 2.35, p= 0.107, figure 3.7 D) and total entries ($F_{(2,46)}$= 0.34, p= 0.717, figure 3.7 F) during the 10 min test period.

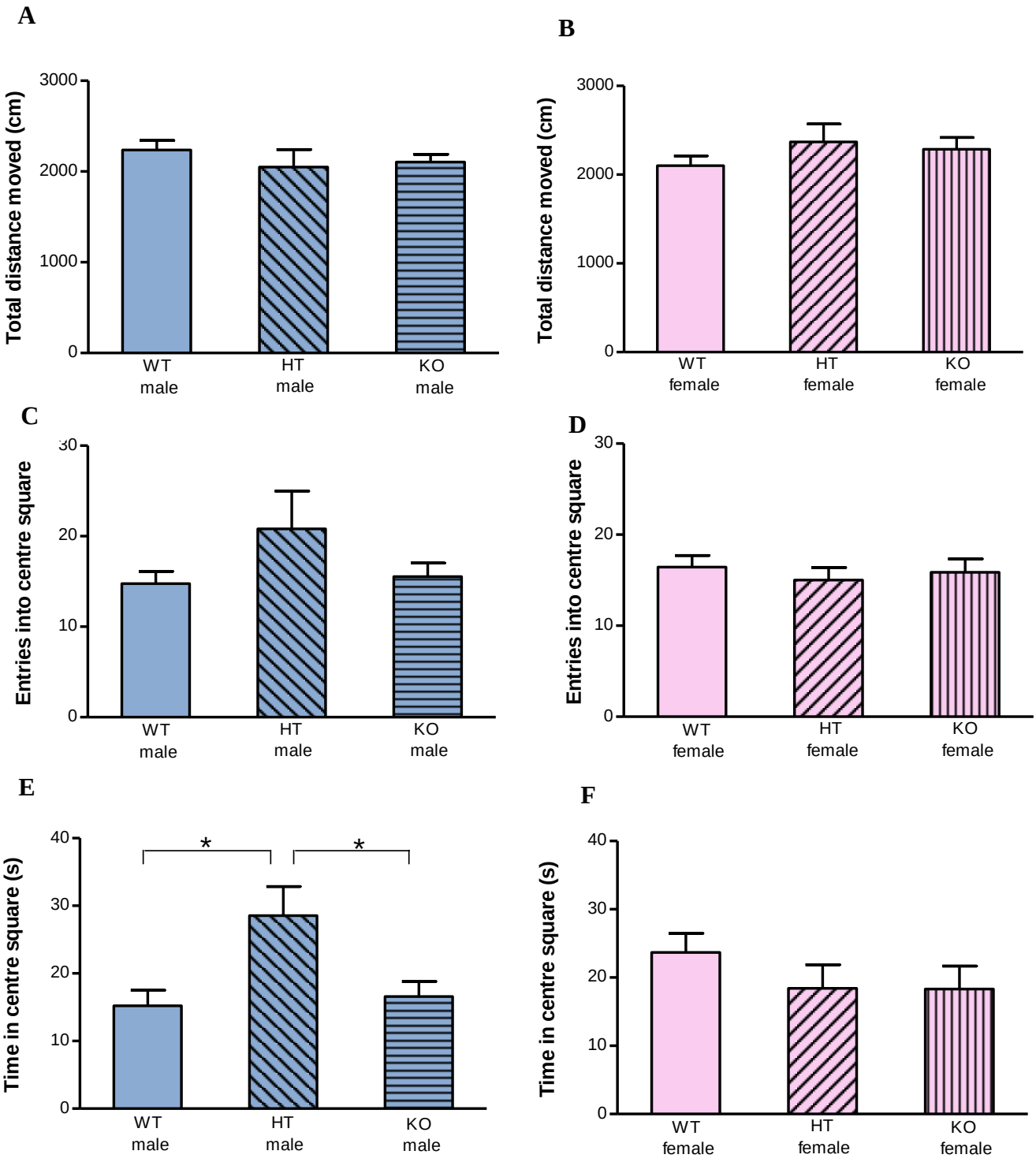


Figure 3.6: Effect of PAR2 deletion on performance in the OFT in the first 5 min in male and female mice. (A + B): No significant difference was observed between male groups or female groups in distance moved. (C + D): No significant difference was observed between male groups or female groups in entries into the centre square (E): PAR2 HT males differ significantly (p<0.05) from WT and PAR2 KO males in the time spent in the centre square, (F): No significant difference was observed between female groups in the entries into centre square. Males and females n= 15-16 per group. Data are presented as mean ± *S.E.M.* and the data was analysed using a one way ANOVA with Tukey's post hoc test between genotypes where appropriate. ∗ = p<0.05.

3.9.2 EPM during first 5 min

There was no significant effect of genotypes and genders nor any interaction between genotypes and genders in the % time spent in the open arms in the first 5 min of the test (genotypes: $F_{(2,89)}$= 3.68, p= 0.214, genders: $F_{(1,89)}$= 1.163, p= 0.394; interaction: $F_{(2,89)}$= 1.38, p= 0.256). There was no significant effect of genotypes and gender but there was interaction between gender and genotype in % open entries in the first 5 min of the test (genotypes: $F_{(1,89)}$= 0.83, p= 0.547, genders: $F_{(1,89)}$= 0.36, p= 0.611, interaction: $F_{(2,89)}$= 3.17, p= 0.047). Additionally, there was no significant effect of genotypes but significant effect of genders in total entries during the first 5 min of the test, however, there was interaction between genders and genotypes in total entries during the first 5 min of the test (genotypes: $F_{(1,89)}$= 1.91, p= 0.344, genders: $F_{(2,89)}$= 22.80, p= 0.041; interaction: $F_{(2,89)}$= 0.380, p= 0.69).

The same data was re-analysed separately for each gender using a one way ANOVA followed by Tukey's post hoc test. In males there was no significant difference between genotypes in % time spent in the open arms ($F_{(2,47)}$= 1.15, p= 0.325), % open entries ($F_{(2,47)}$= 0.32, p= 0.731) and total entries ($F_{(2,47)}$= 1.13, p= 0.334) during first 5 min of the test.

Interestingly, a significant difference was evident between genotypes in female mice in % time spent in open arms during the first 5 min of the test period revealing that PAR2 KO female mice spent significantly less time in the open arms than WT mice ($F_{(2,46)}$= 3.98, p= 0.026, KO versus WT p<0.05, both KO and WT n.s. compared with HT, figure 3.8 B). Additionally, there were significant differences between female genotypes in the % entries into the open arms. Female KO mice made a reduced number of entries into the open arms compared to WT and HT during first 5 min of the test ($F_{(2,46)}$= 0.02, p= 0.015; KO versus WT p< 0.05, HT versus KO p< 0.05, figure 3.8 D). However, there was no significant difference between genotypes in the total number of entries ($F_{(2,46)}$= 0.03, p= 0.973, figure 3.8 D).

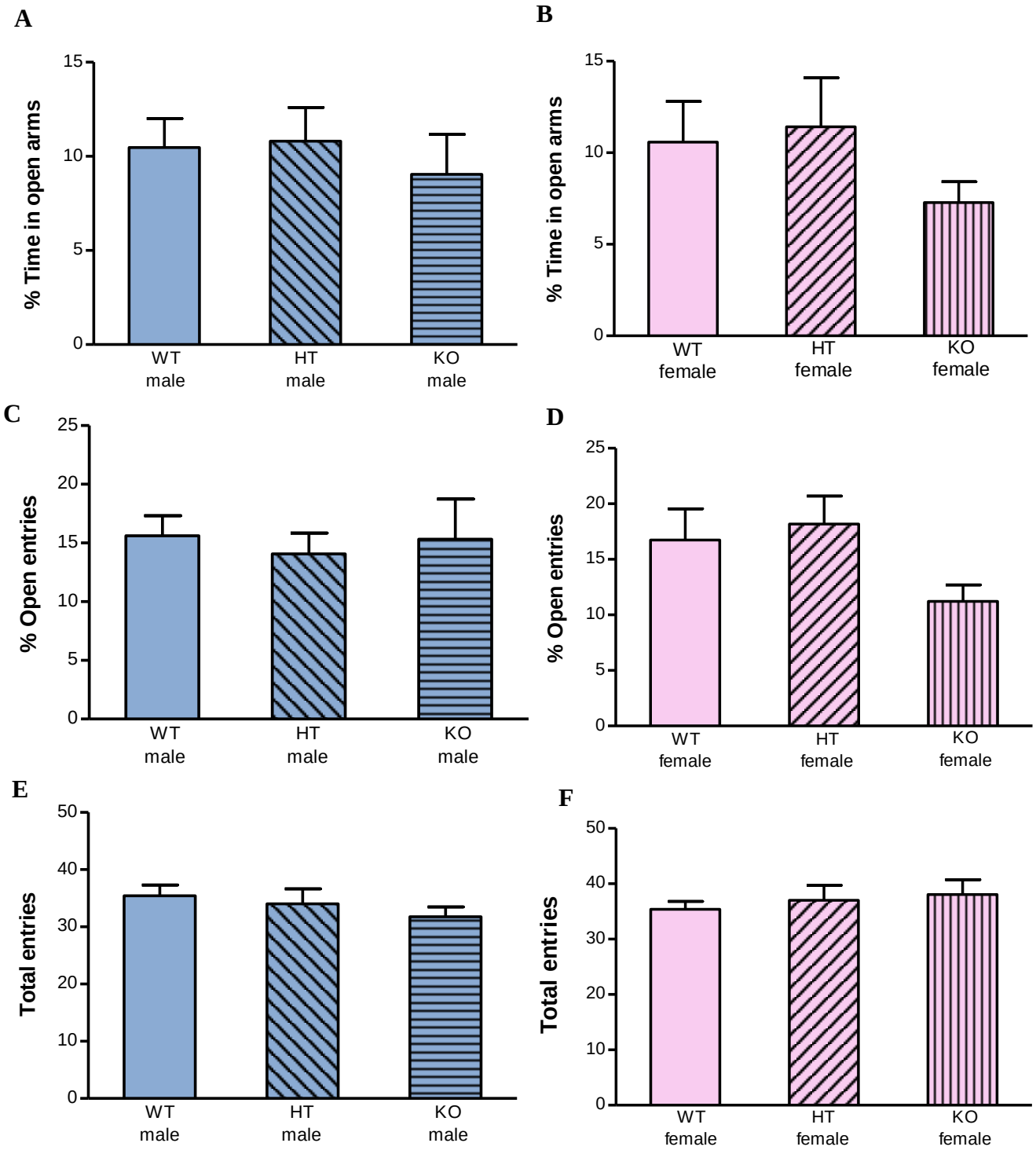


Figure 3.7: Effect of PAR2 deletion on performance in the EPM in male and female mice over 10 min testing period. (A + B): No significant difference was observed between male or female groups in % time spent in the open arms, (C + D): No significant difference was seen between male or female groups % entries into the open arms, (E + F): No significant difference was observed between male or female groups in the total number of arm entries. Males *n*= 16 per group, females *n*= 15-16 per group. Data are presented as mean ± *S.E.M.* the data was analysed using a one way ANOVA with Tukey's post hoc test between genotypes where appropriate.

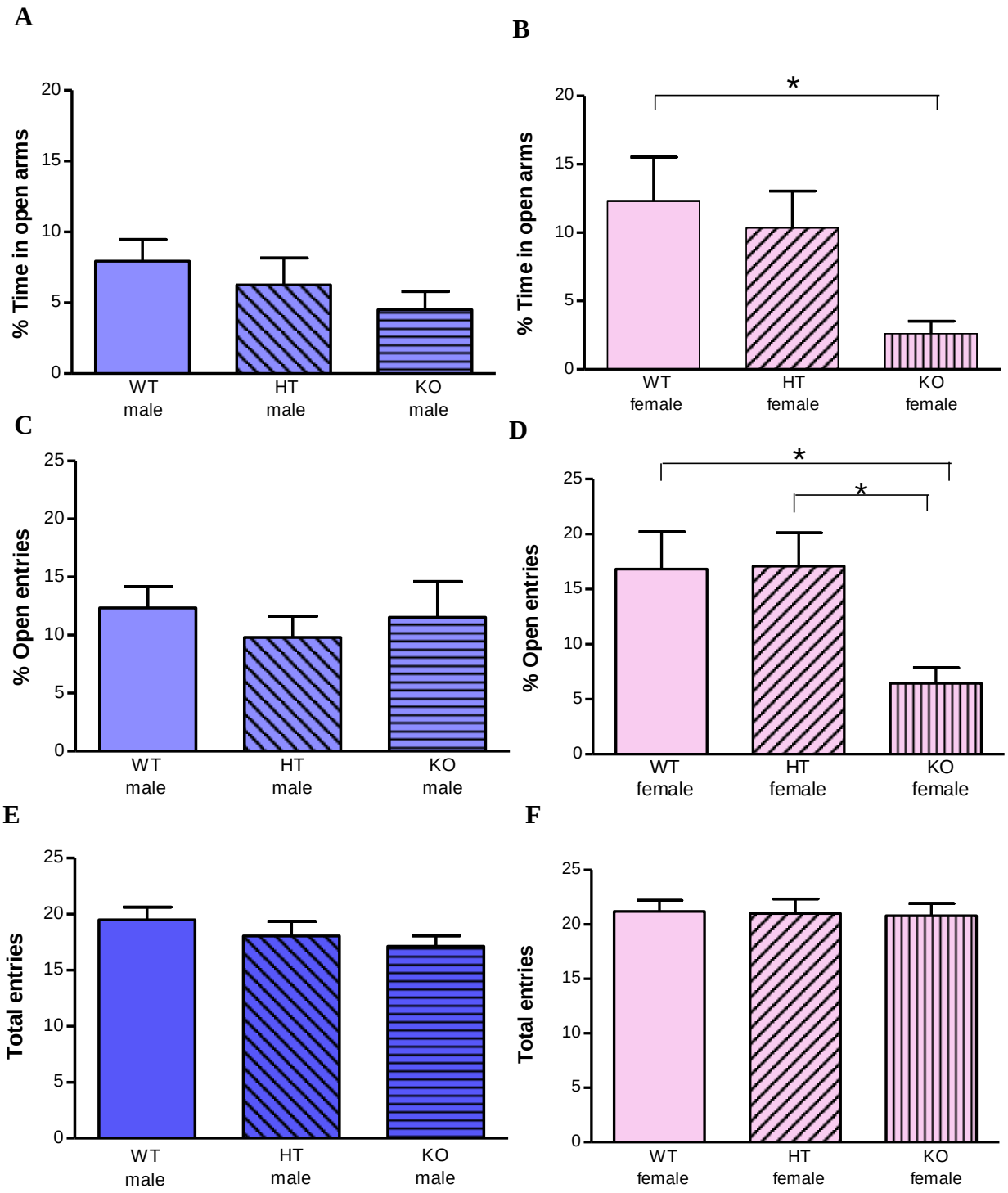


Figure 3.8: Effect of PAR2 deletion on performance in the EPM in the first 5 min in male and female mice. (A) No significant difference was seen between male groups in % time spent on the open arms for males. (B) Female PAR2 KO mice have significantly reduced time spent in the open arms when compared to WT mice (p<0.05). (C) No significant difference was observed between male groups in entries into the open arms (D) KO female mice have significantly reduced in % entries into the open arms compared to female WT and HT mice (p<0.05). (E + F) No significant difference was seen between male or female groups in total number of arm entries. Males n= 16 per group, females n= 15-16 per group. Data are presented as mean ± *S.E.M.* and the data was analysed using a one way ANOVA with Tukey's post hoc test between genotypes where appropriate. * = p<0.05.

3.10 Effect of PAR2 deletion on the MWM test

As PAR2 activation has previously been shown to induce a proposed molecular correlate of memory, i.e. long term depression, *in vitro* (Gan *et al.*, 2011), mice with genetic modification of PAR2 were then examined to investigate whether PAR2 plays a role in spatial memory formation using the MWM test.Deletion of PAR2 had no effect on latency to location of the platform or distance travelled to the platform position over the 5 test days for either male or female mice.

In male groups, there was a significant effect of time in both latency to locate platform ($F_{(4,180)}$= 50.04, p= 0.0005, figure 3.9 A) and distance travelled to the platform ($F_{(4,180)}$= 50.10, p= 0.0005, figure 3.9 C) over the 5 consecutive testing days.

However, in males, there was no significant effect of genotypes and no interaction between days and genotypes in both latency to locate platform (genotypes: $F_{(2,45)}$= 0.72, p= 0.494, interaction: $F_{(8,180)}$= 0.178, p= 0.994, figure 3.9 A) and distance travelled to the platform (genotypes: $F_{(2,45)}$= 0.37, p= 0.692, interaction: $F_{(8,180)}$= 0.33, p= 0.954, figure 3.9 C).

Similarly, in females, there was a significant effect of day in latency to locate platform (time in s: $F_{(4,176)}$= 38.92, p= 0.0005, figure 3.9 B) and distance travelled to the platform over the 5 consecutive testing days ($F_{(4,176)}$= 59.04, p= 0.0005, figure 3.9 D). However, there was no significant effect of genotypes and no interaction between days and genotypes in latency to locate platform (genotypes: $F_{(2,42)}$= 1.01, p= 0.371, interaction: $F_{(8,176)}$= 0.52, p= 0.842, figure 3.9 B) and distance travelled over 5 testing days (genotypes: $F_{(2,44)}$= 0.79, p= 0.462, interaction: $F_{(8,176)}$= 0.77, p= 0.634, figure 3.9 D).

3.10.1 Effect of PAR2 deletion on the probe test

In the last trial of MWM test as shown in chapter 2, figures 2.7 C and 2.8 the mice were made to swim in the pool without a platform in order to examine their ability to remember where the hidden platform was placed (the ‘target’ quadrant) during the 5 testing days.

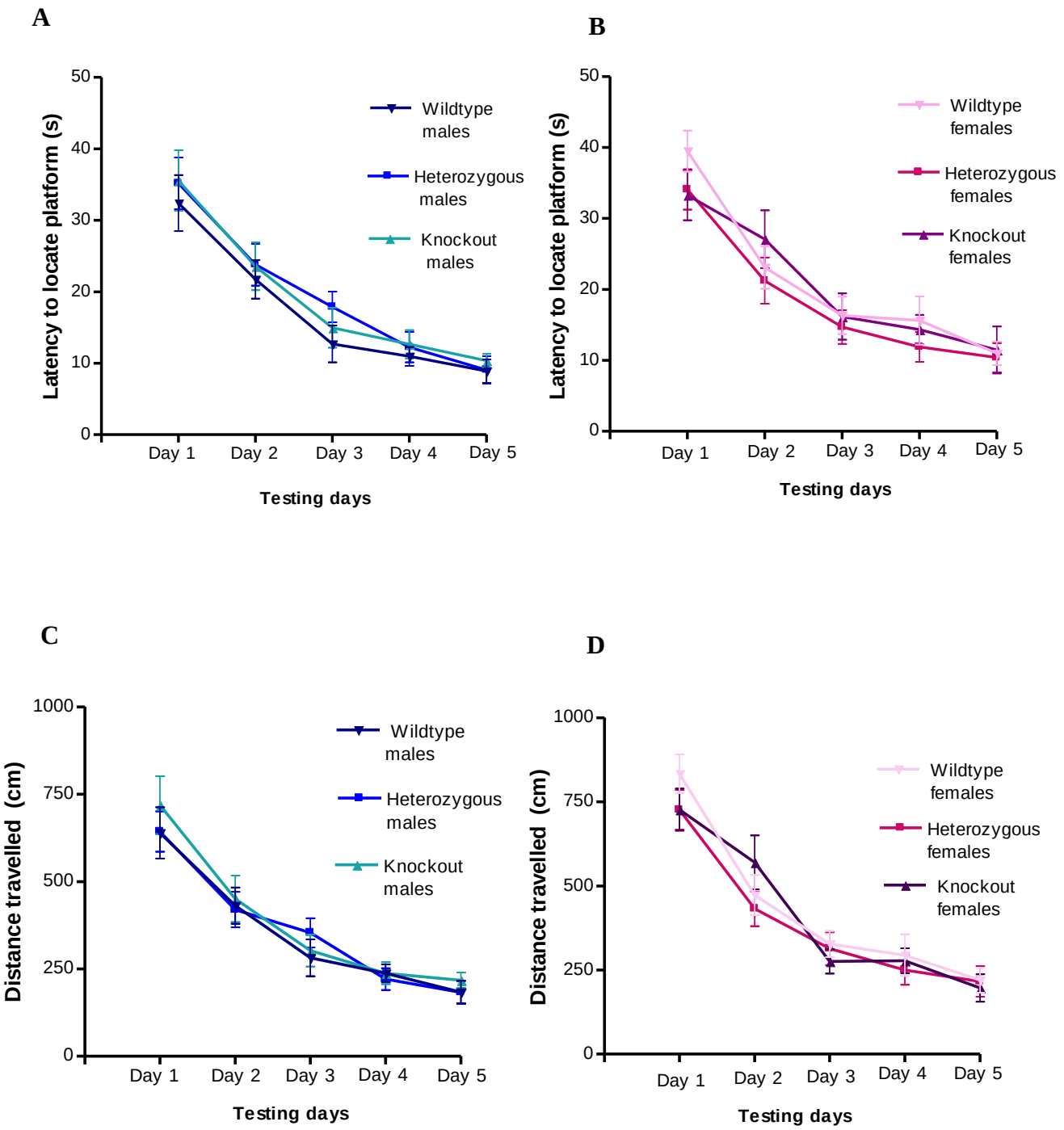


Figure 3.9: Effect of PAR2 deletion on performance in the MWM test in male and female mice. (A + B) No significant difference was observed between male or female groups in latency to locate the hidden platform. (C + D) No significant difference was seen between male or female groups in the distance travelled to hidden platform. Males *n*=16 per group, females *n*= 15-16 per group. Data are presented as mean ± *S.E.M.* and analysed using repeated measures two way ANOVA with Tukey's post hoc test the between genotypes where appropriate.

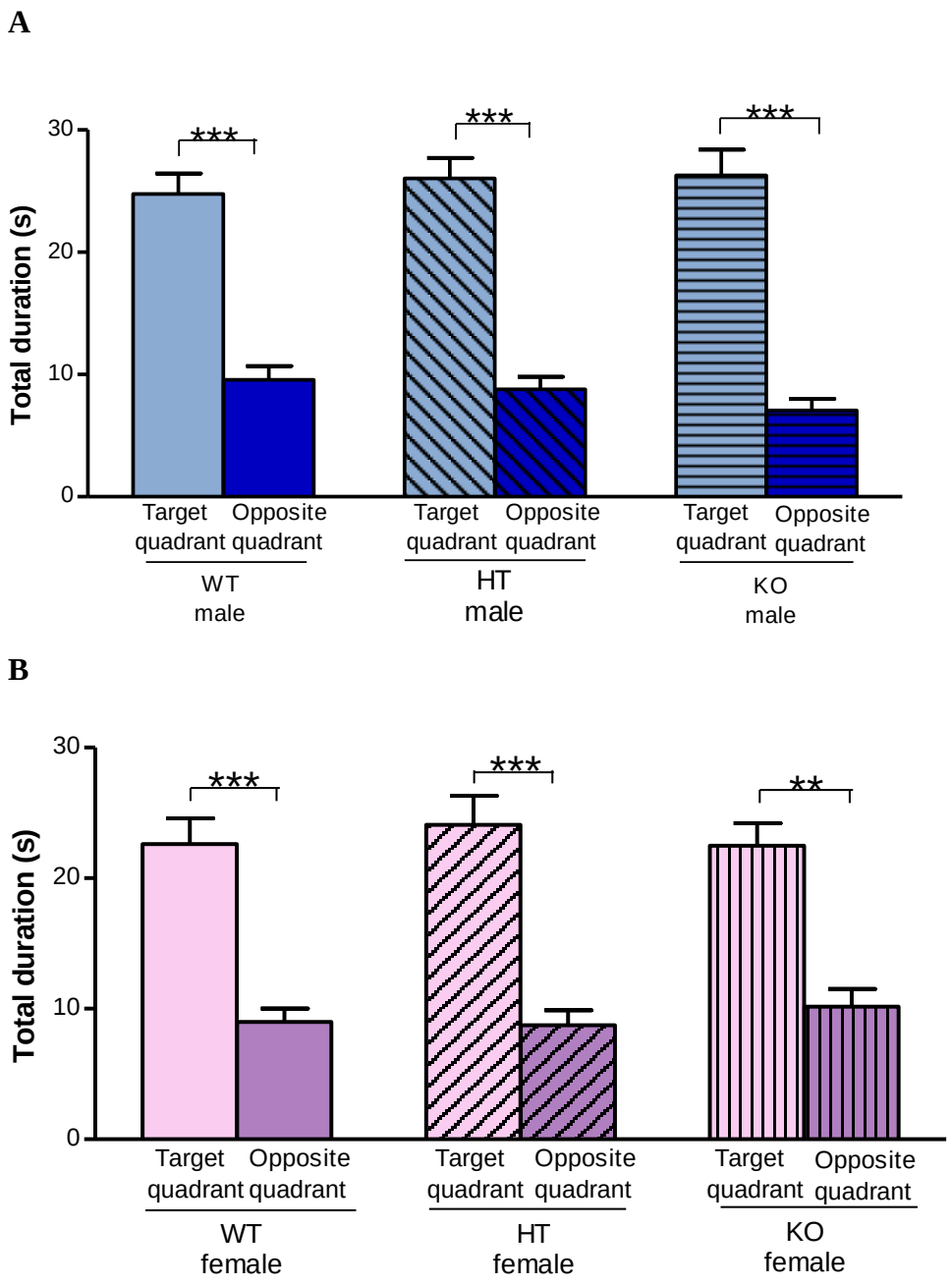


Figure 3.10: Effect of PAR2 deletion on performance in the probe trial of the MWM for male and female mice. (A + B): A significant difference between time spent in the target quadrant and opposite quadrant was observed in the absence of the platform for all male and female groups. Males *n*= 16 per group, females *n*= 15-16 per group. Data are presented as mean ± *S.E.M.* and analysed using a one way-ANOVA to compare between genotype groups of the same gender and using paired t-tests to compare between time spent in the target quadrant vs. the opposite quadrant. ***= $p<0.0001$, **= $p<0.001$.

The results show that PAR2 deletion did not affect the spatial memory of these mice. Males and females spent significantly more time in target quadrant compared with the time spent in the opposite quadrant during the testing period (time in s: males: WT: target quadrant vs opposite quadrant, p= 0.0001; HT males: target quadrant vs opposite quadrant , p=0.0001; KO males: target quadrant vs opposite quadrant, p= 0.0001, figure 3.10 A; WT females: target quadrant vs opposite quadrant, p= 0.0003; HT females: target quadrant vs opposite quadrant, p= 0.0003; KO females: target quadrant vs opposite quadrant, p= 0.0008, figure 3.10 B). Additionally, there was no significant difference between genotypes in both males and females (genotype males: $F_{(2,44)}$= 1.52; p= 0.230, 3.10 A; genotype females: $F_{(2,44)}$= 0.20; p= 0.822, in figure 3.10 B) in time spent in the target quadrant.

3.11.2. Swim pathways

It was observed during testing that certain mice had a different swim pattern whilst undertaking the MWM. Therefore, as the swim track of each animal was recorded during the MWM, the swim track was investigated to examine whether this was the case. Male and female HT and PAR2 KO mice exhibited a different swim pathway compared to WT mice in that these mice appeared to swim in spiralling pathways in contrast to WT mice which travelled in straighter pathways when searching for the platform. The altered swim pathways observed were not investigated further.

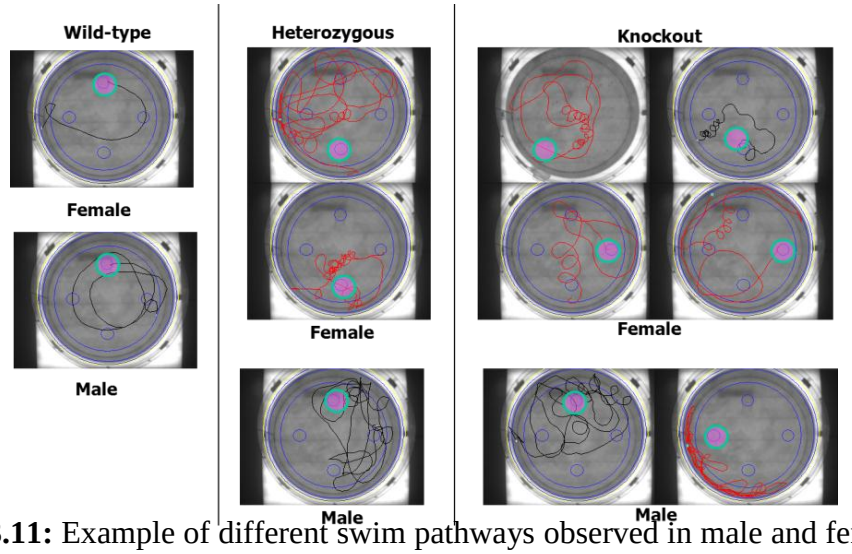


Figure 3.11: Example of different swim pathways observed in male and female mice with different PAR2 genotypes; WT, HT and KO. The swim tracks were recorded using Ethovision software. The location of the hidden platform is indicated by the pink circle.

3.11 Effect of PAR2 deletion on the T- CAT

As explained in section 3.4.1, 11 mice were excluded from the T-CAT data because these mice did not complete the 14 trials. Therefore, the number of mice included in this experiment was (males: WT *n*= 10, HT *n*=14, KO *n*=8; females: WT *n*= 13, HT *n*=17, KO *n*=8). There was no significant effect of genotypes ($F_{(2,26)}$= 2.42, *p*= 0.097), genders ($F_{(1,64)}$= 0.97, *p*= 0.329) and no interaction between genotypes and genders ($F_{(2,64)}$= 0.03, *p*= 0.966) in mean % alternation.

The same data were re-analysed using one sample t-test to compare individual genotypes with the chance level (50 % of mean % alternation). Both the WT and HT mice for both genders were significantly different from chance in the mean % alternation. However, KO mice for both genders exhibited no significant difference from chance in the mean % alternation. (WT males , *p*= 0.0015, HT males *p*= 0.0007; KO males, *p*= 0.955; WT females *p*= 0.0027; HT females, *p*= 0.0002; KO female, *p*=0.138; figure 3.12 A& B).

3.12 Effect of PAR2 deletion on the NOR test

In the sample phase, both WT and HT male mice spent similar time exploring the left and right objects, while KO male mice spent significantly more time exploring the right object than the left object expressed either as ratio (WT left side vs WT right side, *p*= 0.230; HT left side vs HT right side, *p*= 0.537; KO left side vs KO right side, *p*= 0.017; figure 3.13 A) or as exploration time in s (WT left side vs WT right side, *p*= 0.642, HT left side vs HT right side , *p*= 332, KO left side vs KO right side, *p*= 0.0213; figure 3.13 B). Total time investigating both objects in s (figure 3.13 B).

Moreover, in the sample phase of females, WT, HT and KO female mice spent the same time exploring left and right objects measured either as a ratio (WT: left side vs WT right side, *p*= 0.136; HT left side vs right side, *p*= 0.121; left side vs KO right side, *p*= 0.551; figure 3.13 C) or as exploration time in s (WT left side vs WT right side, *p*= 0.120, HT left side vs HT right side, *p*= 0.157, KO left side vs KO right side, *p*= 0.420; figure 3.13 D).

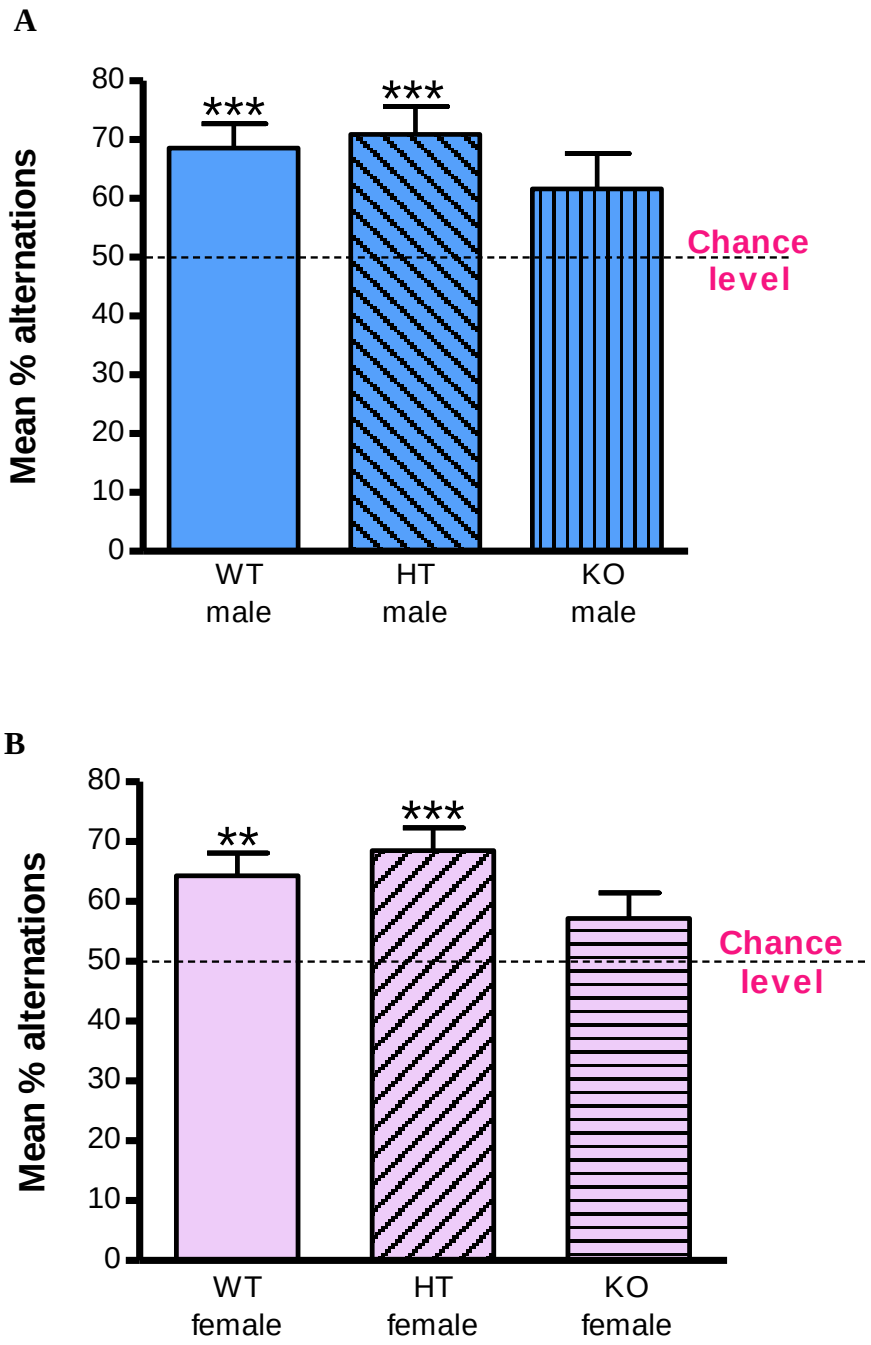


Figure 3.12: Effect of PAR2 deletion on T-CAT test in male and female mice. (A): Male WT and HT mice differed significantly from chance in mean % alternation but male KO mice did not. (B): female WT and HT mice differed significantly from chance in mean % alternation but female KO mice did not. Data are presented as mean ± *SEM* and analysed using one-sample t-tests. Males: WT *n*= 10, HT *n*=14, KO *n*=8; females: WT *n*= 13, HT *n*=17, KO *n*=8. **=p<0.01, ***=p<0.001.

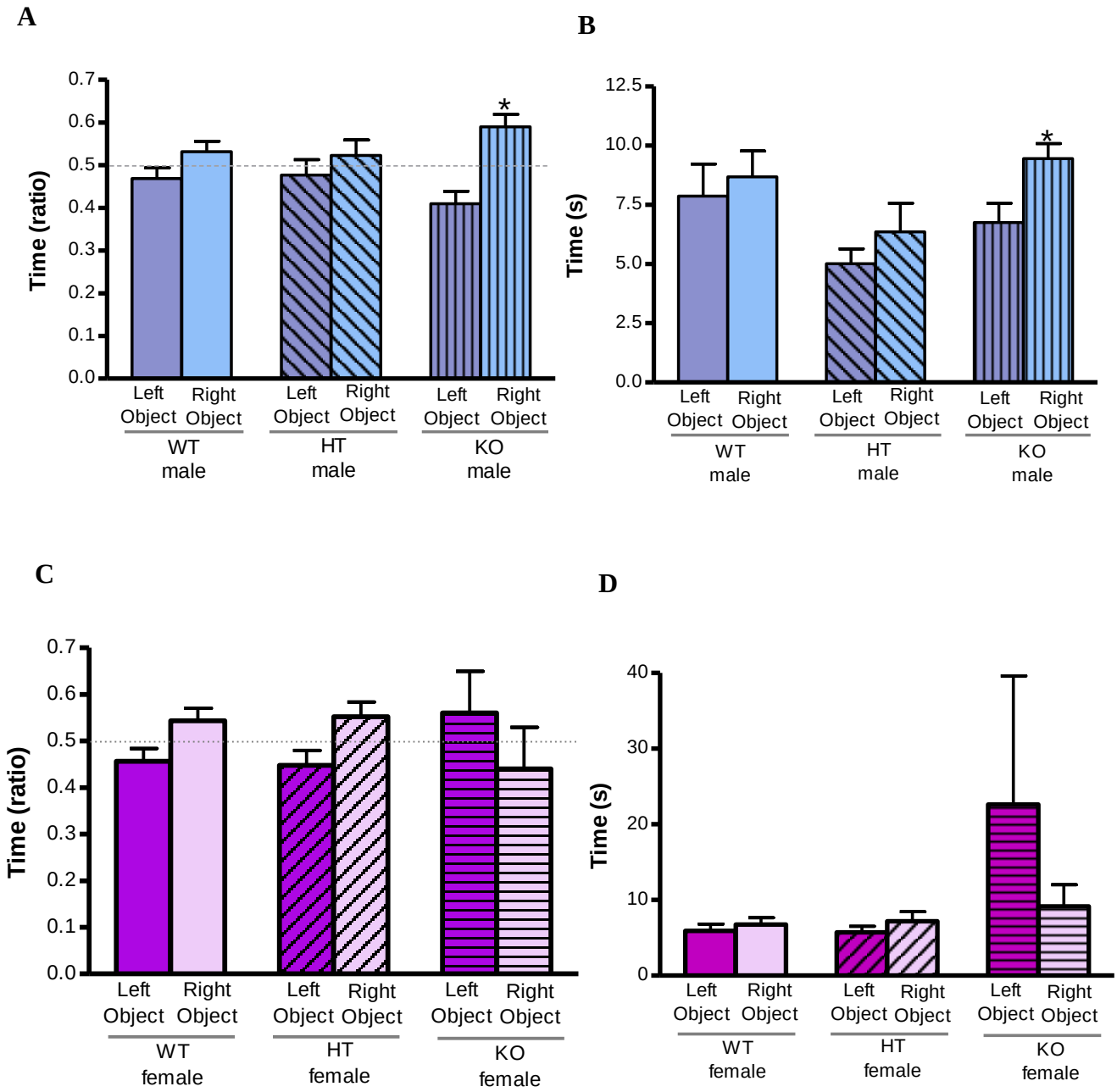


Figure 3.13: Effect of deletion of PAR2 on NOR test (sample phase) in male and female mice. (A + B): Both WT and HT male mice spent similar time exploring left and right objects whereas KO male mice significantly spent more time exploring right object more than left object. (C + D): All three genotypes of female mice spent similar time exploring left and right objects. Data are presented as mean ± *SEM* and the data were analysed by paired t–test. Males: WT *n*= 14, HT *n*=14, KO *n*=8; females: WT *n*= 12, HT *n*=15, KO *n*=4. *=$p<0.05$.

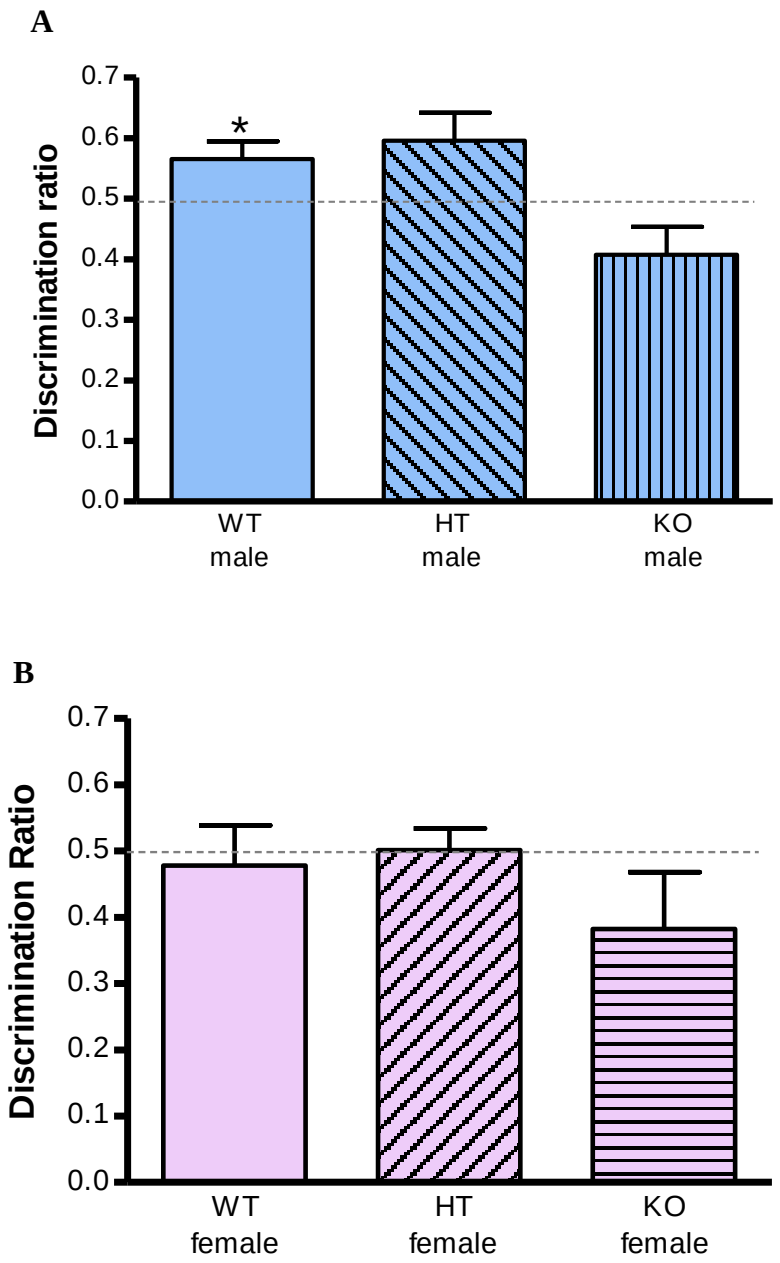


Figure 3.14: Effect of deletion of PAR2 on NOR test (choice phase) in male and female mice. (A): WT male mice spent significantly more time exploring the novel object than the familiar object, while the HT and KO mice spent similar time exploring both objects. (B): All female mice spent a similar time exploring novel and familiar objects. Data are presented as mean ± *SEM* and analysed by one sample t-test. Males: WT *n*= 14, HT *n*=14, KO *n*=8; females: WT *n*= 12, HT *n*=15, KO *n*=4. *=$p<0.05$.

In the choice phase, the data was analysed using a one sample t-test to compare the discrimination ratio for individual genotypes with the chance discrimination ratio (0.5 discrimination ratio means the mouse spent the same time exploring novel and familiar objects). WT male mice spent significantly more time exploring the novel object than the familiar object. There was a significant difference from chance in WT male mice but not in HT and KO male mice (WT p= 0.041; HT p= 0.058; KO p= 0.084, figure 3.14 A).

In the choice phase, all female mice spent the same time exploring both novel and familiar objects. There was no significant difference from chance discrimination ratio in any genotypes (WT p= 0.727; HT p= 0.968; KO p= 0.263, figure 3.14 B).

3.13 Effect of PAR2 deletion on the SR test

In male mice, there was a significant effect of pulse intensity (dB) on amplitude of the startle response while there were no significant effect of genotypes and no interaction between pulse intensity and genotypes on the startle response pulse intensity: $F_{(8,240)}$= 29.40, p= 0.005; genotypes: $F_{(2,30)}$= 0.37, p= 0.963; interaction: $F_{(16,240)}$= 0.69, p= 0.806; figure 3.15 A). In this study, the arbitrary unit is used to measure the startle response values.

Similarly, in female mice, there was a significant increase of pulse intensity (dB) on startle response but no significant effect of genotypes and no interaction between pulse intensities and genotypes on startle response (pulse intensity: $F_{(8,280)}$=36.02, p= 0.005; genotypes: $F_{(2,35)}$=2.61, p= 0.088; interaction: $F_{(16,280)}$=1.42, p= 0.132; figure 3.15 B).

The data was also analysed separately for each gender using one way ANOVA followed by Tukey's post hoc test at each pulse intensity which revealed that KO female mice had a reduced startle response at the highest pulse intensity (120 dB) only ($F_{(2,37)}$= 3.39, p= 0.045, KO p<0.05 compared with WT, both WT and KO n.s. compared with HT; figure 3.17 C). However, no significant difference in the startle response was observed between males at the pulse intensity of 120 dB ($F_{(2,32)}$= 0.30, p= 0.743; figure 3.17 A).

3.14 Effect of PAR2 deletion on the PPI test

In male mice, there was a significant increase of prepulse intensity on % PPI while there was no effect of genotypes and no interaction between prepulse intensity and genotypes on % PPI (prepulse intensity: $F_{(2,60)}$=3.80, p= 0.029; genotypes: $F_{(2,30)}$=1.94, p= 0.162, interaction: $F_{(4,60)}$= 0.69, p= 0.606 figure 3.16 A).

Similar to male mice, there was a significant increase of prepulse intensity on % PPI in female mice while there was no significant effect of genotypes and no interaction between prepulse intensity and genotypes (prepulse intensity: $F_{(2,70)}$=5.39, p=0.007; genotypes: $F_{(2,35)}$= 0.50, p= 0.609; interaction: $F_{(4,70)}$= 1.56, p=0.196; figure 3.16 B).

Additionally, in the PPI test, the mice were exposed firstly at highest pulse intensity (120 dB) alone before exposure to the pulse preceded by the prepulse and after the prepulse as shown in chapter2, figure 2.13. Examining the startle response at pulse intensity 120 dB alone without prepulse revealed that KO female mice had a reduced startle response at this pulse intensity ($F_{(2,37)}$= 4.72, p=0.012, KO p<0.05 compared with WT, both WT and KO n.s. compared with HT; figure 3.17 D). However, no significant differences in the startle response was observed between males in the 120dB alone trials ($F_{(2,32)}$= 0.09, p= 0.919; figure 3.17 B).

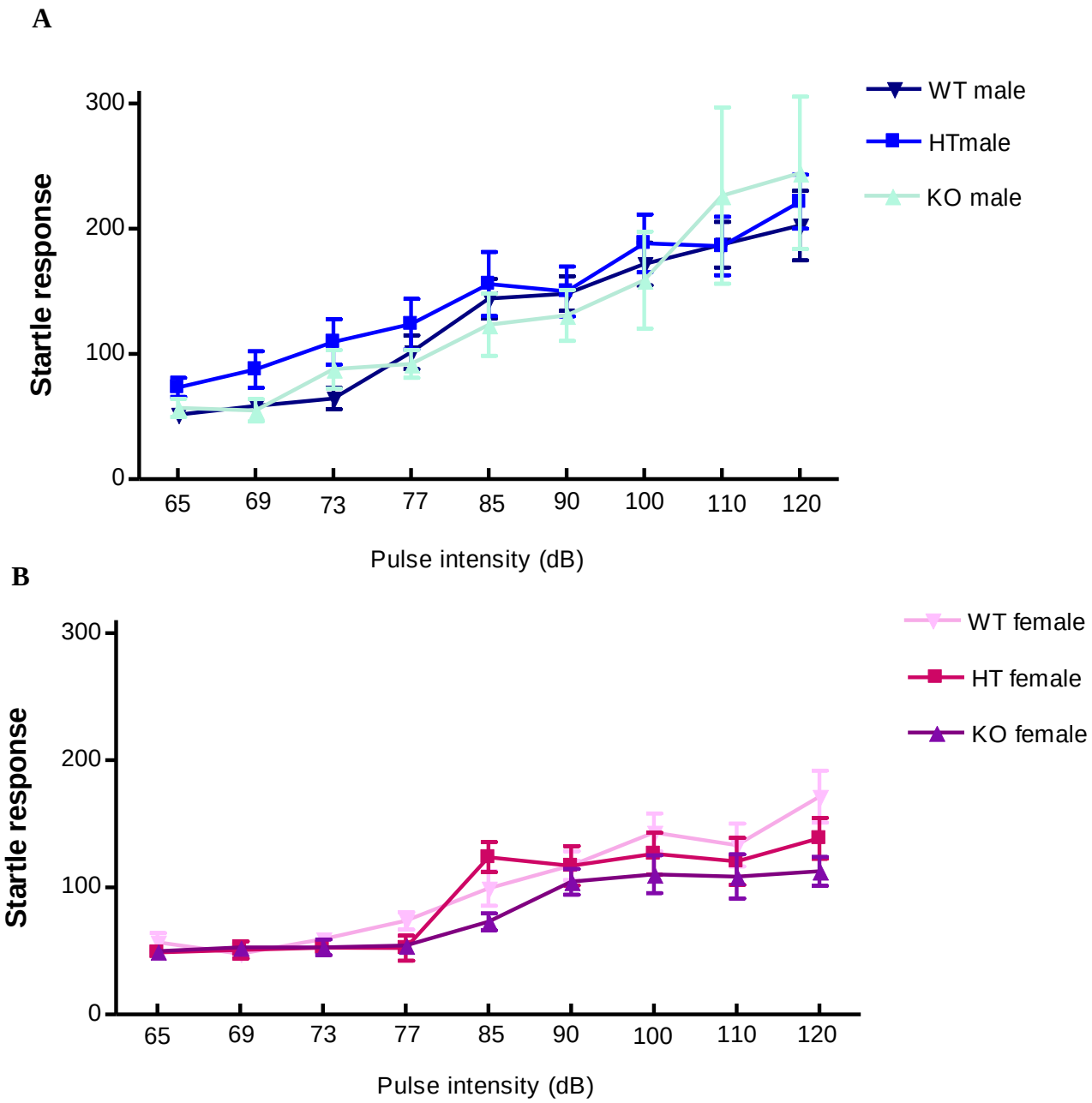


Figure 3.15: Effect of deletion of PAR2 on startle response using a range of stimulus intensities (65 dB – 120 dB) in both genders of mice. (A + B): No significant differences was observed between three different PAR2 male or female genotype groups in SR to different pulse intensities (P>0.05). Data are presented as mean startle response (arbitrary units) ± *SEM* and analysed using repeated measures two way ANOVA with Tukey's post hoc test the between genotypes where appropriate. Males: WT n= 12, HT n=12, KO n=9; females: WT n= 14, HT n=10, KO n=14.

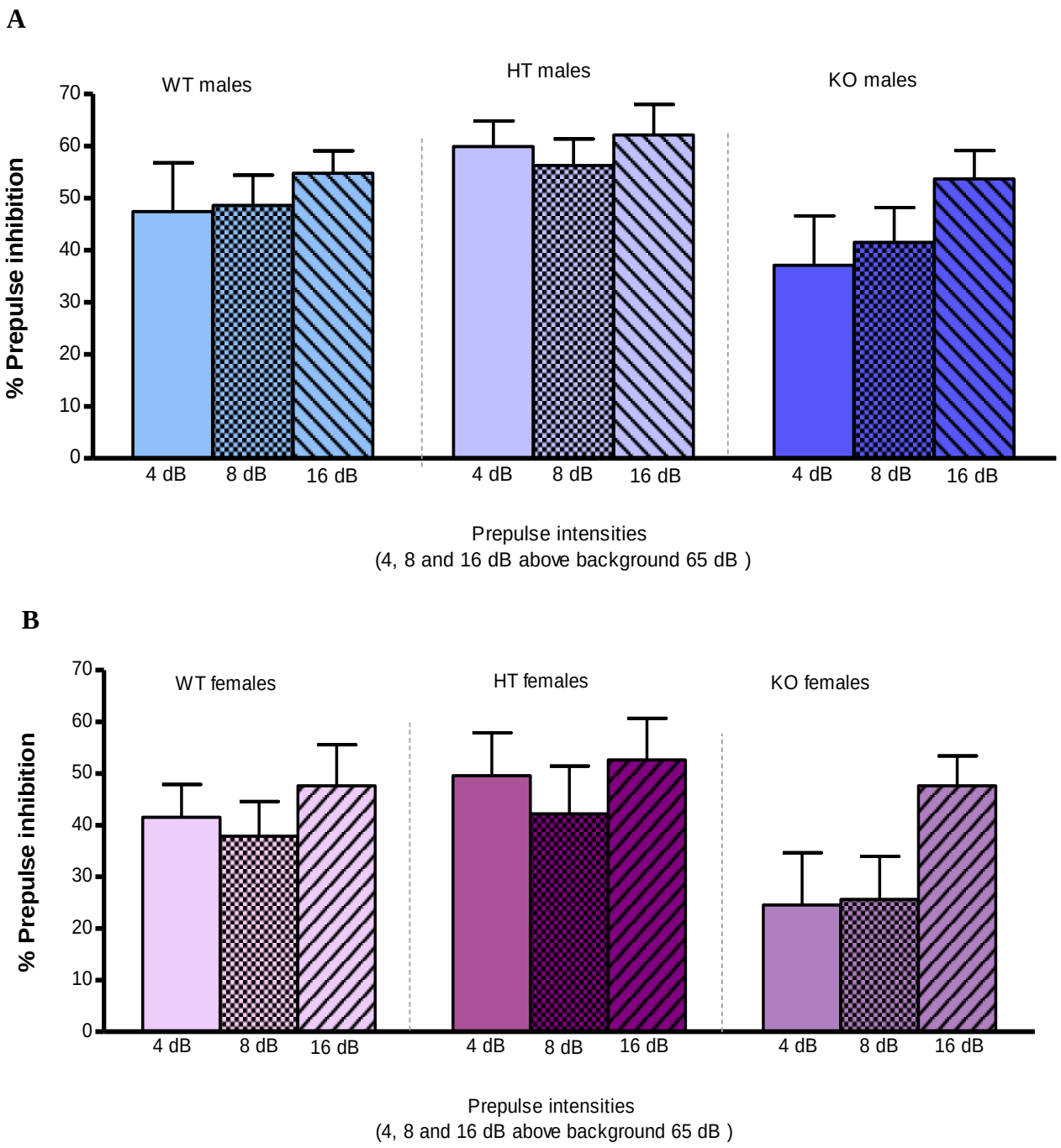


Figure 3.16: Effect of deletion of PAR2 on % PPI in mice for both male and female mice. (A +B): No significant differences were observed among three different PAR2 male or female genotype groups in % PPI. Data are presented as mean (arbitrary units) ± *SEM* and analysed using repeated measures two way ANOVA with Tukey post hoc test the between genotypes where appropriate. Males: WT *n*= 12, HT *n*=12, KO *n*=9; females: WT *n*= 14, HT *n*=10, KO *n*=14.

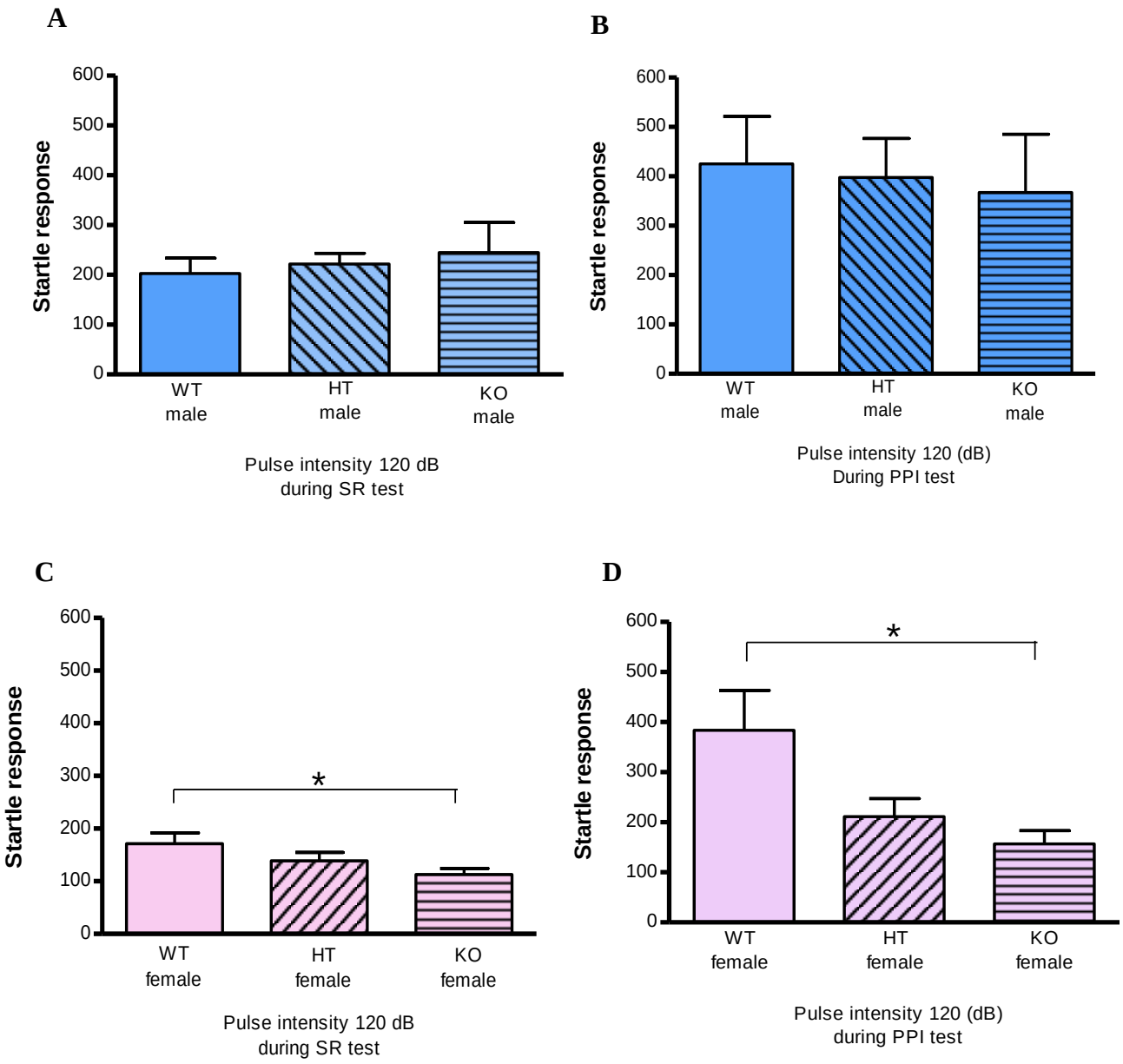


Figure 3.17: Effect of deletion of PAR2 on startle response to highest pulse intensity 120 dB during PPI test in male and female mice. (A+B): No significant difference was observed on startle response among three different PAR2 male genotype groups during SR and PPI test. (C+D): Startle response was reduced significantly in PAR2 KO female mice compared with WT during SR and PPI test. Data are presented as mean ± *SEM* and analysed using one way ANOVA with Tukey's post hoc test between genotypes where appropriate. Males: WT *n*= 12, HT *n*=12, KO *n*=9; females: WT *n*= 14, HT *n*=10, KO *n*=14.*= $p<0.05$.

3.15 Discussion

3.15.1 Deletion of functional PAR2 allele reduces survival of KO mice

All experiments in this chapter were carried out on GM mice. The breeding of GM mice by crossing HT PAR2 mice produced offspring with the following genotypes: 31% WT, 49% PAR2 HT and 20% PAR2 KO. The genotyping results (table 3.1) do not demonstrate a Mendelian ratio ($^1/_4$ WT, $^1/_2$ HT and $^1/_4$ KO) which suggests that deletion of the functional PAR2 allele reduces survival as the number of KO mice is reduced, whereas, the number of WT mice is relatively increased. As cell survival is promoted by several signalling pathways and associated with PAR2 activation (Bonni *et al.,* 1999; Jin *et al,.* 2005; Ramachandran *et al.,* 2009; Ramachandran, *et al.,* 2011; Dutra-Oliveira *et al.,* 2012), then it may be argued that a reduction in this signalling could contribute to a reduced cell survival in PAR2 KO mice which in turn leads to a reduced survival of KO offspring. However, this is only speculation and the exact nature of this reduced KO survival, which is not uncommon in GM mice, requires further investigation (Fiorucci *et al.,* 2001; Arora *et al.,* 2007).

3.15.2 Effect of deletion of PAR2 on locomotor activity

Locomotor activity can be defined as the movement from place to place; the movement of rodents is due to a variety of reasons, for example looking for food or a mate or to escape from predators (Gilchrist *et al.,* 1997; Jordan *et al.,* 2006), and is essential for survival (Jordan *et al.,* 2006). In other words, locomotor activity in rodents is due to a motivation to explore the environment which is necessary for survival. In this study, the effect of PAR2 on general locomotor activity in mice was examined. The results obtained from the OFT provided evidence that PAR2 does not alter general locomotor activity and all groups of mice moved similar distances. This result suggests that PAR2 does not have any effect on motor system function in either gender and all mice have normal exploratory and locomotor activity. This result is consistent with the recent finding of Lohman *et al* (2009) who demonstrated that administration of 1.5 mg/kg subcutaneous (s.c.) of the PAR2 agonist SLIGRL-NH_2 to adult male genetic absence epilepsy rat from Strasbourg (GAERS) rats did not alter the distance moved. The GAERS rat is a model of epilepsy in rats with high

level of anxiety (Scarisbrick *et al.,* 2001; Lakaye *et al.,* 2002). This is similar to the related receptor PAR1; it was shown by Almonte *et al* (2007) that in adult PAR1 KO mice normal exploratory and locomotor activity was exhibited. However, in an another study the proteinase inhibitor neuroserpin has been proposed to play a role in locomotor activity as neuroserpin KO mice display reduced locomotor activity and exploratory behaviour in a novel environment (Madani *et al.,* 2003). This result suggests a role of this proteinase inhibitor in mood regulation (Madani *et al.,* 2003) but this is unlikely to involve PAR1 or PAR2 given these results of our own studies and that of others. In conclusion, PAR2 appears not to be involved in locomotor activity in either gender.

3.15.3 Effect of deletion PAR2 on anxiety-like behaviour

Besides locomotor activity, the OFT is a useful model for measuring levels of anxiety-like behaviour in mice (Christmas and Maxwell 1970; Prut and Belzung, 2003). Measuring anxiety-like behaviour is based on conflict between the tendency of the rodent to prefer a dark place and a natural curiosity of the rodent to explore a novel environment (Prut and Belzung 2003; File, 1980). In the OF box, the darker environment is close to the wall whereas the brighter environment is the centre of the box (see chapter 2, section 2.4.1). Therefore, measurement of anxiety-like behaviour levels was expressed by the number of entries into and the length of time spent in the central square: the longer spent in the exploration of the bright central square, the less anxious the mouse. The result obtained from the OFT suggests that PAR2 deletion reduced anxiety-like behaviour in HT male mice as demonstrated by an increase in the time spent in the centre square of the OF. The present data suggests that HT male mice are less anxious than WT male mice in OFT. In contrast, the result in the female groups suggests that PAR2 deletion did not affect anxiety-like behaviour level in female mice. This result indicats that deletion of PAR2 alters the anxiety-like behaviour level in male mice but not in female mice in the OFT.

The same mice from our OFT study were re-examined for anxiety-like behaviour in a more specific test. The EPM (Wall and Messier, 2001) is based on a result of conflict between the tendencies of a mouse to avoid open arms which may represent danger

and the natural curiosity to explore a novel environment (Rodgers and Dalvi, 1997; Borsini *et al.,* 2002). The longer the time spent exploring the open arms, the less anxious the animal. The percentage of entries and time spent into open arm was measured to assess the anxiety-like behaviour level (Pellow *et al.,* 1985).

The result obtained from the EPM suggested that the deletion of PAR2 alters anxiety-like behaviour in female mice as shown by the reduction in percentage of open arm entries and time spent in the open arms during the first 5 min of the EPM test compared with WT mice. This suggests that female PAR2 KO mice are more anxious than WT female mice in EPM test. This data indicates that PAR2 may play a role in anxiety-like behaviour in female mice. Suprisingly, this result did not support our previous result in OFT. Therefore, these results suggest that PAR2 deletion reduces anxiety-like behaviour in male mice, whereas it increases anxiety-like behaviour in female mice depending on the test used. So deletion of PAR2 alters the anxiety-like behaviour and the effect is gender specific. Our result did not agree with the previous finding of Lohman *et al.,* (2009) that peripheral injection of GAERS rat, a strain of Wister rat showing high levels of anxiety-like behaviour (Jones *et al.,* 2008), with SLIGRL-NH_2 did not alter anxiety-like behaviour in these rats (Lohman *et al.,* 2009). These latter results support the idea that the PAR2 agonist did not affect anxiety-like behaviour in rats. Furthermore, the findings obtained are not consistent with the role of the related receptor PAR1, as shown by a study where PAR1 deletion did not change anxiety-like behaviour in male mice when compared with both the OFT and EPM (Almonte *et al***.,** 2007).

There are controversies with regard to the role of gender in the behaviour of laboratory mice and rats. Many studies provide evidence of gender specific differences in behaviour (Rodgers and Cole 1993; Frick *et al.,* 1999; Voikar *et al.,* 2001). Male C57BL/6 mice had higher levels of anxiety-like behaviour than C57/BL6 female mice in the EPM test (Voikar *et al.,* 2001). Adult female C57/BL6 mice at 17 months old showed increased anxiety-like behaviour compared to the same age of male C57/BL6 mice in the EPM test while 25 month old females had similar anxiety-like behaviour level compared to 25 month old males in the same test. This result suggests the differences in anxiety level between genders arose

because 80% of 17-month-old females displayed either irregular or absent oestrous cycling while all 25-month-old females had ceased cycling (Frick *et al.*, 1999).

Many studies support the idea that there are differences in anxiety-like behaviour levels between males and females due to the difference in sex hormones (Altemus, 2006). This is in agreement with the study of Frye *et al.*, (2000), which showed increased anxiety-like behaviour of pre-oestrous rats, compared with rats in oestrus and dioestrus and with males, as demonstrated by significantly more open arm entries and time in the elevated plus-maze (Frye, *et al.*, 2000).

Administration of the ovarian steroid 17 beta-estradiol 2 (0.09 mg/kg, s.c.) to ovariectomized rats was associated with significant increases in anxiety-like behaviour in the light-dark transition task compared with ovariectomized rats injected with vehicle. This result suggests that 17 beta-estradiol increases anxiety-like behaviour in female rats (Walf and Frye, 2009).

Adult female Long-Evans rats were found to be less anxious compared to adult males in the EPM test indicates the role of sex differences in anxiety-like behaviour. Further investigation with male and female rats treated with tamoxifen (an antagonist of the oestrogen receptor), showed that the females were more anxious than males in EPM test indicates the role of oestrogen receptors in anxiety-like behaviour. These experiments suggest the role of both sex and sex hormones in anxiety- like behaviour in rats (Zimmerberg and Farley, 1993).

In conclusion, deletion of PAR2 decreases anxiety-like behaviour in males but it increases anxiety-like behaviour in females.

3.15.4 Effect of deletion of PAR2 on spatial reference memory

Cognitive maps can be defined as mental representations of physical locations in both humans and animals (Tolman, 1948). The cognitive map helps rodents to show where to go and the routes used (Eichenbaum *et al.*, 1999). It is widely believed cognitive mapping is a function of the hippocampus (Burgess *et al.*, 2002). The hippocampus is a major part of the brain, which belongs to the limbic system and

plays crucial roles in the consolidation of information from short-term memory to long-term memory and spatial reference memory (Kogan *et al.,* 2000; Pearce, 2001; Broadbent *et al.,* 2004; Kumaran, 2008). Spatial reference memory is defined as how the brain stores and remembers information about the location of a physical object and its environment (Gaskin *et al.,* 2011). Spatial memory is necessary for the survival of many species (Nairne *et al*., 2007), for example, spatial memory allows mice to learn the location of food in the wild.

In neuroscience laboratories, the MWM test is designed to measure spatial learning memory in rodents (Morris *et al.,* 1982; Morris, 1984; Terry, 2009). Neuroscientist Richard G. Morris established this test firstly in 1981 in order to test hippocampal-dependent learning including acquisition of spatial memory and long-term spatial memory (Morris, 1981). In this test, the mouse is placed in a pool of water where it must use and remember visual cues located in the room to find a platform hidden below the surface of the water. The test was carried out for 5 consecutive days (4 trials/day) to determine learning (chapter 2, figure 2.7 B). All genotypes of mice examined in this study learned the location of the platform in the MWM over the 5 day test period. The results of the MWM test clearly show all genotypes of mice decreased the distance moved and time spent to reach the hidden platform during acquisition trials within 5 testing days.

The capacity of the mouse to retrieve and retain information learned is measured by a probe trial (Morris, 1981). In the probe trial the platform is removed from the swimming pool and the mouse is allowed to swim in the pool. If the location is remembered, the mouse spends more time in the place where the platform was contained before, compared with the time spent in the opposite side of the pool (chapter 2, figure 2.7 C). In the probe trial, all mice remembered the location of the hidden platform during testing days. This result clearly shows a normal learning behaviour of PAR2 deleted mice in relation to the location of the platform. Deletion of PAR2 did not affect the formation of memory of the platform location and the recall of the memory of it on the second day. Thus, deletion of PAR2 does not alter the spatial memory (within the day) and long term memory (between days). Therefore, these data suggest that PAR2 is not necessary for spatial reference

memory. Despite the proposed connection between synaptic plasticity and learning and memory (Martin and Grimwood *et al.,* 2000) and the recent finding that PAR2 activation induces a LTD of synaptic transmission when investigated in hippocampal slices (Gan *et al.,* 2011), our result indicates that PAR2 does not play a role in memory formation under normal physiological conditions. Despite these findings, studies have described effects of proteinases in learning and memory (Qian *et al.,* 1993; Nakagami *et al.,* 2000) and indeed it may be that under conditions where PAR2 is activated that its effects on learning and memory may be unmasked.

This result is consistent with the finding of Lohman (2009) that peripheral injection of GAERS rats with SLIGRL-NH_2 (1.5 mg/kg, s.c.) induced deficits in recall memory but not in spatial memory in MWM test. SLIGRL-NH_2 treatment inhibited either the formation of a memory of the platform location from the previous day or prevented recall of the memory of it on the second day. SLIGRL-NH_2 rats still learned the platform location within a day, as observed within all four days of trials, but had a between-day deficit (Lohman *et al.*, 2009). This result supports a role for PAR2 in the formation of intermediate-term (between days) hippocampal-dependent memories, but not acute, or short-term (within days) memories (Lohman *et a.l,* 2009).

The lack of any effect of PAR2 deletion on memory in this study may be due to only partial involvement of PAR2 in learning and memory processes with an additional role for PAR1 in these pathways. There is evidence for this in the findings of Almonte *et al* (2007) who investigated the role of PAR1 deletion in learning and memory in two tests of behavioural learning, the passive avoidance test and cued fear conditioning. PAR1 KO mice showed significant deficits in passive avoidance and cued fear conditioning tests. These data suggest that PAR1 may play an important role in emotionally motivated learning. Additionally, this finding is supported by the fact that PAR1 is highly expressed in brain regions involved with emotional learning including hippocampus and amygdala (Striggow *et al.,* 2001). In addition, PAR1 activation can enhance the function of NMDA receptors (Gingrich *et al.,* 2000; Lee *et al.,* 2007; Mannaioni *et al.,* 2008) and the thrombin-induced potentiation of NMDA receptor function is greatly reduced in PAR1 KO mice (Gingrich *et al.,*

2000). Significantly, a recent study indicates that PAR1 plays a significant role in hippocampal memory formation (Almonte *et al.,* 2013). Taken together these studies indicate that PAR1 modulates hippocampal memory formation. Furthermore, all PAR members are expressed in the mouse brain (our laboratory but not included in this thesis) and therefore it is feasible that PAR2 deletion may lead to a compensatory increase in the expression of PAR1. Based on this finding, future work should include investigation of the level of expression of different PARs in brain samples from the different genotypes of adult mice of both genders used in this study.

In conclusion, deletion of PAR2 is not nessary for spatial reference memory in either gender.

3.15.5 Effect of deletion PAR2 on swimming pathway

The British neuroscientist who developed the MWM test noticed that both male and female rats probably escape from the water either by swimming randomly or in unsystematic search pathways in the pool (Morris, 1984). In addition, he noticed that normal rats learn quickly swiming directly in the direction of the hidden platform from any starting position in the pool when the platform in the same position during the testing days (Morris, 1984). Furthermore, the mice learn how to find hidden platform in MWM test, but there are different strategies between species and even in between the same species (Vorhees and Williams, 2006).

Interestingly, there was an intriguing suggestion that PAR2 deletion induced a ‘spiralling’ form of swim-path in the MWM test which was not found in WT mice, although there was no significant difference observed in the latency to locate the hidden platform or path-length. This suggests that PAR2 is not essential for spatial memory formation but may affect the directional pathways of the mice or strategies of these mice to find the hidden platform. This finding needs to be investigated further. This type of movement has been noticed with adult PDAPP mice, a model of Alzheimer's disease-like lesions, in the MWM test in that the adult PDAPP mice change the direction of pathways to a less efficient strategy compared with WT C57BL/6, for example, WT C57BL/6 mice use spatial strategies and swim directly

whereas the mice with Alzheimer's disease use chaining, circulating and focal incorrect strategies rather than directly swimming to the hidden platform (Brody and Holtzman, 2006; see figure 3.18)

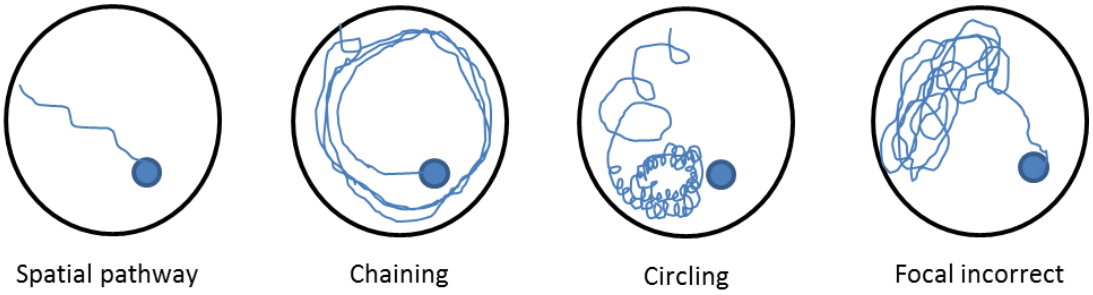


Figure 3.18: Different strategies of swimming pathways.

In conclusion, deletion of PAR2 may affect the directional pathways of the mice or strategies of these mice to find the hidden platform but it did not affect the performance of the mice to find the hidden platform.

3.15.6 Effect of deletion of PAR2 on working memory

Mice continuously explore environments for many reasons including looking for the location of food and water, shelter and mates (Crusio and Abeelen, 1986; Deacon and Rawlins, 2006; Brown and Nemes, 2008). Exploration behaviour is an essential part of learning, allowing the mice to get information about novel places and things, which is crucial for them to survive (Gerlai, 1993). Exploration behaviour helps mice find resources such as food and water and it allows them to find probable ways to escape from predators (Gerlai, 1993; Gerlai, 1998).

The T-CAT test exploits the natural tendency of rodents to fully explore a test environment. Spontaneous alternation is defined as the visit of the rodent to the arm goal of the T-maze apparatus that was not visited in the immediately previous trial (Spowart-Manning and van der Staay, 2004, Robert and Rawlins, 2006). The mice must remember which arm they had entered on a previous trial to enable it to alternate its choice on a subsequent trial (Hughes, 2004). A number of brain areas and neurotransmitters are involved in the spontaneous alternation processes in mice

as shown in chapter 1, section 1.6.3.1. (Lalonde, 2002). Due to this behaviour, this test has become a useful model for the evaluation of working memory (Olton *et al.*, 1979; Robert and Rawlins, 2006).

In our laboratory, each mouse was first put through a forced trial followed by 14 subsequent free trials. During the forced-choice trial, (see chapter 2, section 2.4.5 and figure 2.10) the mouse visited the arm chosen by the experimenter. The mouse acquired information about that visited arm. In the next trial, which was a free trial, the mouse chose which arm to visit. If the mouse remembered the arm previously visited the un-visited arm would be chosen otherwise the same arm would be visited again. The normal mouse must learn fast to visit another arm without re-entering the previous arm after 14 free trials. This refers to working memory that allows the mouse to remember which arm has been visited in a trial.

The index for measuring the working memory in the T-CAT test is mean % continuous alternation. Mice normally alternate at levels significantly above chance (Dember and Fowler, 1959) indicating the mice are willing normally to explore a novel environment (Lalonde, 2002). The alternation rate of C57BL/6 mice is usually found to be between 60% and 70% in the T-maze (Hughes, 1990). All mice were examined for working memory using T-CAT. Deletion of PAR2 reduced mean % continuous alternation to chance level in both genders. This result suggests that deletion of PAR2 may impair working memory. This may be due to deletion of PAR2 reducing the ability of the mouse to aquire the information while visiting the arm and/or reducing recall of the acquired information when making the next arm choice.

The study of Gerlai and co-workers (1994) showed that mice overexpressing human S100β (a Ca^{2+} binding brain specific protein which can affect brain development and hippocampal LTP) showed less preference to explore novel places than control mice. Therefore, in our study decreased spontaneous alternation rates may be the PAR2 deletion reducing the exploration of the novel environment.

T-CAT spontaneous alternation is a measure of working memory which involves different areas of the brain, including the hippocampus. Some researchers believe

that spontaneous alternation detects hippocampal dysfunction better than the MWM test (Rawlins and Olton 1982; Reisel *et al.,* 2002), as animals with hippocampal removal or damage can still solve very difficult reference memory problems in the MWM test (Deacon *et al.,* 2001). Both the T-CAT and MWM tests detect full lesions of the hippocampus (Olton *et al.,* 1979; Lalonde, 2002; Deacon and Rawlins, 2005). However, the T-CAT test may not successfully detect hippocampal damage, as for example, hippocampectomized rodents showed side preferences causing them to prefer to turn right or left on a T- maze. As presented in this chapter, our data shows that deletion of PAR2 did not alter reference memory but may have affected swimming pathways in the MWM; however, deletion of PAR2 reduced alternation memory to within chance level in the T-CAT. These results suggest that deletion of PAR2 may affect hippocampal function and thus cause reduction in spontaneous alternation rates in T-CAT.

The prefrontal cortex may also be involved in spontanteous alternation. This area influences learning of alternation behaviour in rats (Lalonde, 2002). Male albino rats with lesions of medial prefrontal cortex have a reduced spontaneous alternation rate compared with sham operated controls (Divac *et al.,* 1975). One possibility for the explanation of reduction of the spontanous alternation rates is a medial prefrontal cortex lesion deficit in working memory (Roozendaal *et al.,* 2009) which can cause impairment in learning the left-right alternation in the T- maze (Brito and Brito, 1990). The same deficit in working memory was also shown in rats with a prelimbic cortex lesion (Delatour and Gisquet-Verrier, 1996). The other possibility is that the lesion in prefrontal cortex can cause deficits in spontaneous alternation because of increased ‘‘neophobia’’. Neophobia can be defined as the aversion that the rodent displays towards approaching a novel object or place (Brown and Nemes, 2008). Lower rates of alternation have been demonstrated with increased fear (Bats *et al.,* 2001). From these previous studies we can conclude that prefrontal cortex plays an role in spontaneous alternation. Our result in this test suggests that deletion of PAR2 reduces spontaneous alternation and it may be that deletion of PAR2 causes a deficit in working memory or induces neophobia. On the other hand this result supports the finding of Gan (2011) that activation of PAR2 receptors by SLIGRL-NH_2 can cause the induction of synaptic plasticity i.e. LTD (Gan *et al.,* 2011). These results suggest

PAR2 can play a role in learning and memory. This result needs further investigation. In conclusion, deletion of PAR2 did not alter spatial reference memory in MWM test but reduced working memory in T-CAT.

3.15.7 Effect of deletion of PAR2 on recognition memory

Recognition memory was examined in the three genotypes using the NOR test. This test is used to evaluate recognition memory in rodents (Ennaceur *et al*., 1997; Puma *et al.,* 1999; Bizot *et al.,* 2005). Within this test, memory is assessed by measuring the preferential exploration of a novel object, which depends on the ability of the rodent to remember an object that it has previously explored (Ennaceur, 2010). This allows information to be obtained regarding the short-term memory under investigation and/or examination of exploration behaviour which is related to attention (Taglialatela *et al.,* 2009; Antunes and Biala, 2012). The discrimination index is the proportion of the time spent in exploring a novel object presented along with a familiar object during a choice trial. A discrimination index higher than 0.5 (chance level) indicates that the mice remember the familiar object. The exploration index is the time spent in exploring objects during the sample trial. Surprisingly, the result obtained from this test showed a gender difference in recognition memory in mice. In the sample phase, WT male mice spent similar amounts of time in exploration of two identical objects. After 1 h, WT mice preferentially explored the novel object during the choice phase, presumably because they recognized it as novel using the information collected during the sample phase (recognition memory). However, the HT and KO male mice did not preferentially explore the novel object (and therefore presumably failed to recognize it as novel) during the choice phase. This suggests that deletion of PAR2 affects the acquisition of the information during exploration of the identical objects or it affects the recall memory to recognize the novel object. Interestingly, a significant right side bias was observed in male KO, but not WT or HT mice during the sample phase. There is a possibility that this was a confound in the exploration of objects in the choice phase; however, as the allocation of objects and object placings was according to a balanced design, this is unlikely to have caused a consistent error. We can therefore conclude that the results in the KO male mice support the idea that PAR2 deletion altered recognition memory in mice.

This result indicates that PAR2 is necessary for recognition memory in male mice. The result is supported by several studies that show activation of PAR2 may play a role in learning and memory (Lohman *et al.,* 2008; Lohman *et al.,* 2009; Gan *et al.,* 2011).

Recognition memory involves different aspects of the memory process including acquisition, consolidation and recall. The most important brain areas which play a crucial role in recognition memory are rhinal cortex (perirhinal and entorhinal) and hippocampus (Steckler *et al*., 1998; Aggleton *et al*., 2010).). The function of the perirhinal cortex in object recognition is to represent basic information about familiar objects or novel objects, thus brain lesions in perirhinal cortex area can cause deficits in recognition memory in rats (Albasser *et al.,* 2009). The hippocampus collects several types of information from the perirhinal cortex (Clarke *et al.,* 2010). The hippocampus plays a role in memorization of the object by acquiring information about the experience of object and is also involved in consolidation memory (Antunes, 2012). The perirhinal cortex is involved in object recognition after short retention intervals, while hippocampus is responsible for long-term object recognition (Reger *et al.,* 2009). Some studies have shown that the hippocampus is the important part in recognition memory and that hippocampal lesions can cause impairment in object recognition memory (Burke *et al.,* 2010). PAR2 are highly expressed in the different brain areas that are involved in recognition memory (Smith *et al.,* 1997; Striggow *et al.,* 2001; Noorbakhsh *et al.,* 2005; Steinhoff *et al.,* 2005; Bushell *et al.,* 2006; Noorbakhsh *et al.,* 2006). Deletion of PAR2 may affect the function of these areas and cause deficits in recognition memory of male KO mice.

Surprisingly, WT female mice did not preferentially explore the novel object, suggesting that the gender difference may affect experimental performance in this test. This suggests the need either to re-evaluate the NOR test in female mice or re-design the test before testing female mice. For example, the protocol of the experiment could be re-designed either by increasing the time spent in exploring the 2 identical objects in the sample phase or decreasing the delay time between sample and choice phases.

There is a gender difference in the NOR test. The differences between male WT mice and female WT mice in recognition memory may because of the effect of the oestrous cycle on recognition memory in female mice. The hippocampus is one of the brain areas which is important for both spatial memory and recognition memory. However, spatial memory is more hippocampus dependent than recognition memory (Broadbent *et al.,* 2004). Many studies have demonstrated that there is an effect of female sex hormones on hippocampal function. Oestrogen receptors are expressed in several areas of the brain including amygdala, cortex, cerebellum and hippocampus (Maggi *et al.,* 2004). Therefore, oestrogen may play an important role in emotion and memory (Fink *et al.,* 1998). Oestrogen and progesterone have different effects on the hippocampus; oestrogen has an excitatory effect (Behl, 2002) but progesterone has an inhibitory effect (Tolmacheva and Luijtelaar, 2007). In addition, other studies have showed that hippocampal plasticity is modulated across the oestrous cycle in rats (Warren and Juraska 1997). The results obtained are not supported by the study of Sutcliffe *et al* (2007) that noticed female rats with regular oestrous cycles performed better than male rats in the NOR, although male rats showed improved memory in the spatial version of the NOR compared with female rats (Sutcliffe *et al.,* 2007).

In conclusion, deletion of PAR2 is not necessary for spatial memory in MWM but it reduces spontaneous alternation rates in T-CAT in both genders. Additionally, deletion of PAR2 reduces the recognition memory in NOR test in males and male KO mice display a side bias. However, in females the test needs more investigation before any conclusions can be drawn about the effect of PAR2 deletion on recognition memory.

3.15.8 Effect of deletion PAR2 on startle response and sensorimotor gating

The PPI of acoustic startle test is used to measure sensorimotor gating, which refers to the ability of a pre-stimulus to inhibit the response to a startling stimulus (Geyer *et al.,* 2001; Swerdlow *et al.,* 2001). Several brain regions including the hippocampus, the prefrontal cortex and the amygdala are involved in modulation of the PPI to acoustic startle (Geyer, 1996; Geyer *et al.,* 2002). The startle response itself is a protection response to sudden stimuli for example auditory stimuli, and is a

brainstem reflex that serves to protect the whole body and facilitates escape from sudden potentially threatening stimuli (Davis, 1980; Geyer and Swerdlow, 2001; Zhang *et al.,* 2011). Startle response is regulated by several brain regions including the amygdala, prefrontal cortex, hippocampus and thalamus (Zhang *et al.,* 2011). The result of the SR test showed normal startle response of mice at different startle intensities (65 -120 dB) in both genders. All mice showed increases in startle response with increasing pulse intensities. Additionally, all mice startled normally, which indicates all these mice had normal hearing. There were no significant differences between genotypes in startle response at any of the startle intensities, indicating that deletion of PAR2 did not change startle responses in mice. However, there was an observable trend to a reduction in startle response at the higher intensities in KO females compared with WT, with HT females at intermediate levels.

There was normal PPI at all prepulse intensities in all groups, indicating that deletion of PAR2 did not change PPI in these mice. While there has been no previous study of the role of PAR2 in PPI, one study has investigated the role of PAR1 on sensorimotor gating and has shown there was no difference between PAR1 KO male mice and WT mice in PPI at any different prepulse intensity, nor in baseline startle (Almonte *et al.,* 2007). This indicates that the deletion of PAR1 did not affect the function of brain areas which are responsible for startle response, and the PPI of startle response. The present study contributes the additional information that deletion of PAR2 does not alter the function of brain areas responsible for startle response and PPI of startle response either. The result obtained from the PPI test supports the idea that PAR2 does not have a role in sensorimotor gating.

Both at 120 dB during the SR test and in 120 dB pulse alone trials in the PPI paradigm, KO female mice showed a reduction in startle response compared with WT, suggesting deletion of PAR2 may affect the startle response of female mice at the highest auditory stimuli. This result suggests that female KO mice are less anxious when exposed to the highest auditory stimuli. However, male KO mice did not differ in startle response at 120 dB pulse alone during the PPI test, again suggesting a gender difference in this test.

The gender differences might be due to the effect of the oestrous cycle on stress and anxiety-like behaviour in female mice. In the menstrual cycle in women and the oestrous cycle in animals, the female sex hormones (oestrogen and progesterone) produced in the periphery are highly lipophilic and pass the BBB quickly from the plasma to the brain where they can affect neuronal function (Lovick, 2012). Besides oestrogen and progesterone being important in the reproductive system, these hormones are also important during brain injury. Oestrogen appears to be more prophylactically effective in the treatment of females at risk for ischaemic brain injury, while progesterone seems to be more helpful in the post-injury treatment of both genders with acute traumatic brain damage (Stein, 2001). These studies suggest these hormones may have an important role in brain function. *In vivo,* it has been shown that female rats were treated for 23 days with oestrogen and progesterone by s.c implanted tubes filled with hormones, rapid withdrawal from these hormones by removal of the tubes increased startle response and anxiety-like behaviour in OFT (Saavedra *et al.,* 2006; Doornbos *et al.,* 2009). From these studies, we can conclude that sex hormones seem to affect emotional brain function in females, and therefore the difference between male and females in behavioural tests in which there is an emotional component is not surprising and PAR2 may play a role in modulating the behavioural effects of sex hormones.

Some studies have been shown exaggerated startle response in posttraumatic stress disorders (Landis, 1939; Cook, 1999), generalized anxiety disorders (Ray *et al.,* 2009) and fear-like behaviour (Davis, 1992; Grillon *et al.,* 1994). It has been shown that startle response is increased in both anxiety-like behaviour or fear-like behaviour in rats (Davis, 1992). In addition, fear can change the startle response (Grillon *et al.,* 1997). Anxiolytic drugs can modulate the fear effect, for example, benzodiazepines block the effect of fear potentiated startle in both humans and rodents (Patrick *et al.,* 1996; Davis, 1979). These studies suggest that either anxiety or fear may modulate the startle response. Therefore, decreased startle response of female mice at pulse intensity the 120 dB may be because of the effect of deletion PAR2 modulating anxiety or fear at highest pulse intensity.

In conclusion, deletion of PAR2 did not affect SR and PPI in either genders but it decreased startle response at highest startle stimuli in females.

3.15.9 Summary and conclusion

The results of current study suggests that deletion of PAR2 under normal conditions reduces anxiety-like behaviour in males but increase anxiety-like behaviour in female mice, although in females it seemed to reduce anxiety-like behaviour and startle at highest pulse intensity 120 dB in both SR and PPI tests. It also reduced working memory in both genders and recognition memory in males. However, deletion of PAR2 did not alter locomotor activity, spatial reference memory, startle response and sensorimotor gating under the same physiological conditions. In conclusion, PAR2 receptors may have a limited role in emotion and memory under normal conditions in mice.

Chapter 4

Does PAR2 play a role in sickness behaviour?

4.1 Introduction

From my investigation of the consequences of PAR2 deletion on mouse behaviour (chapter 3), it can be concluded that PAR2 contributes minimally to mouse behaviour under normal physiological conditions. To further investigate the potential role of PAR2 in the CNS, I examined the effects of PAR2 deletion under a pathological condition where there is an increased likelihood that endogenous activation of PAR2 is taking place that is, sickness behaviour induced by injection of a bacterial endotoxin.

Sickness behaviour in both humans and animals can be defined as a group of non-specific symptoms including fever, increased need to sleep, hyperalgesia, anorexia, a loss of interest in everyday activities, a decreased social interaction and attention to body care, depression and impaired concentration, which accompany an acute infective illness (Dantzer *et al.,*1996). Sickness behaviour is caused by the production of PIC, which include tumour necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6), by activated immune cells which are an important part of the host response to infection (Papanicolaou, 1998). Strikingly, administration of cytokines in the treatment of chronic infections such as hepatitis B and C produce side effects and symptoms that are similar to those seen in sickness behaviour (Dinarello, 1997). In the present study examining the role of PAR2 in sickness behaviour, the mouse model of sickness behaviour was induced by injecting mice with the bacterial endotoxin (LPS, 1 mg/kg i.p.). LPS is a cell wall component of Gram negative bacteria, which activates immune cells to induce production of PICs. These cytokines cross the BBB and act on different regions of the brain, which leads to the production of the symptoms described above (Burton *et al.,* 2011).

Certain members of the PAR family have been shown to be associated with inflammation (Macfarlane *et al.,* 2001; Vergnolle *et al.,* 2004). Indeed, PAR2 has been shown to play an important role in rheumatoid arthritis and osteoarthritis (Kelso *et al.,* 2007a; Boileau, *et al.,* 2008). Furthermore, in PAR2 KO mice the inflammation process was delayed in a mouse arthritis model (Kelso *et al.*, 2006). Additionally, PAR2 has been found to be involved in multiple cellular responses

related to tissue injury and repair, angiogenesis, leukocyte infiltration, nociception and neurogenic inflammation (Langer *et al.*, 1999; Vergnolle *et al.*, 1999; Poole *et al.*, 2013). Additionally, it was reported that PAR2 could be an upstream regulator of PIC in the synovium and may be responsible for their regulation (Kelso *et al.*, 2007b).

From these previous studies, a clear relationship was shown between PAR2 and inflammation. Surprisingly however, there are no documented studies about the role of PAR2 in the induction and recovery from sickness behaviour. Therefore, the first experiment in this study was to determine the appearance of sickness behaviour in response to LPS in our laboratory as this model had not previously been used. Markers of induction and the recovery from sickness behaviour were measured in male adult C57/BL6 mice. The markers for sickness behaviour were locomotor activity, anxiety-like behaviour, anhedonia, fluid intake, food intake and change in body weight.

Having established the mouse model, I then determined the effect of PAR2 deletion on the induction of and recovery from sickness behaviour in different PAR2 genotypes with the markers of sickness behaviour being identical to those described above being investigated

4.2 Materials

Animals

The mice used in this chapter were either WT C57/BL6 mice obtained from Strathclyde colonies or PAR2 genetically modified mice and were housed in the BPU at Strathclyde University as explained previously (see chapter 2, section 2.2.2). Mice were group housed (2-3 per cage) in MB1 cages before starting the experiments and transferred to individual housing in the same size cage prior to the OFT and SPT and provided with environmental enrichment as described earlier (see chapter 2, section 2.2.2).

The pilot study of sickness behaviour in response to LPS was carried out in 3 adult C57BL/6J mice (weight 28.2 ± 0.8 g, 12 weeks old, males *n*=2 mice, female *n*=1 mouse) obtained from Strathclyde colonies. The effect of PAR2 deletion on the induction and recovery from sickness behaviour was carried out on 32 adult male genetically modified mice (12 weeks old, weight 27.7 ± 0.6g) of different genotypes, WT *n*=12, HT *n*=11 and KO *n*=9). These PAR2 genetically modified mice were obtained by crossing HT pairs as described in chapter 2, section 2.9. The experiment investigating the effect of habituation and vehicle injection on parameters of sickness behaviour was carried out on 14 WT adult male C57BL/6J mice (weight 27.6 ± 0.9 g, 12 weeks old) obtained from Strathclyde colonies.

4.3 Behavioural test protocols

These experiments were designed based on the results obtained from the pilot study of LPS-induced sickness behaviour. Therefore, this experiment was performed initially to validate the LPS induction of sickness behaviour and then followed by studying the effect of PAR2 deletion on the induction of and recovery from sickness behaviour.

4.3.1 Experimental protocol for the pilot study of LPS-induced sickness behaviour

This experiment was performed on a small number of mice (*n*=3 mice; male *n*=2, females *n*=1), as LPS-induced sickness behaviour had not previously been investigated in our laboratory. The aim was to determine the time of induction and the time of recovery from sickness behaviour in response to LPS injection (1 mg/kg i.p.) in order to design the PAR2 experiments.

On day 1, the day prior to the experiment, the mice were handled to reduce the stress by the experimenter during the test as described earlier (chapter 2, section 2.4.1). On day 2, the mice were single housed in individual MB1 cages and provided with environmental enrichment in the form of plastic huts and nesting material (cahpter 2, section 2.2.2**)**. One bottle of water and food were provided ad libitum in order to measure the amount of food and fluid intake at each time-point. Measurements were taken at several time-points with baseline = prior to testing, then immediately following injection 0 h, 2 h, 4 h, 8 h, 24 h and 48 h post injection with LPS (figure 4.1).

4.3.2 Experimental protocol for the effect of PAR2 deletion on the induction of and recovery from sickness behaviour

This experiment was designed according to the results obtained from the LPS validation experiment. The 32 male mice were mice injected with LPS (1 mg/kg i.p.; WT (*n*=12); HT (*n*=11) and KO (*n*=9)), the mice were tested in random order. On day 1, the day prior to the experiment, the mice were handled to reduce the stress by the experimenter during the test as described earlier (see chapter 2, section 2.4.1). On the second and third days, the SPT was initiated to determine the hedonic state of the mice (see chapter 2, section 2.4.3). Firstly, the mice were single housed in individual MB1 cages and provided with environmental enrichment in the form of plastic huts and nesting material (section 2.1.1**)**. Two bottles of water were provided in order to determine the preferred bottle position. On day 4, one of the water bottles was replaced with a bottle containing 1% sucrose in the non-preferred bottle position, with the placement of the sucrose bottle being randomised during days 4-11. The

amount of water and sucrose drunk were measured over days 4-11. During the testing days, the water and sucrose solution levels were measured daily at the same time (10:00 am, figure 4.1). On day 8, before LPS injection, baseline parameters of the OFT and SPT were measured; the mice were tested in the OF box for 10 min followed by measuring of SPT parameters (water drunk, sucrose drunk, food intake, and mouse weight). Then, similarly to the first experiment, measures were made over several time-points (baseline = prior to testing, 0 h, 2 h, 4 h, 8 h, 24 h and 48 h post injection with LPS; figure 4.2).

4.3.3 Experimental protocol for the effect of habituation and injection of LPS-induction of sickness behaviour

For this experiment, 14 mice were randomly allocated to 2 groups, a control group (CG) and a vehicle group (VG). The CG (n=7) remained uninjected on day 8, whereas the VG were injected with PBS (the vehicle for LPS) on day 8. On days 1 to 11, the experimental protocol was identical to that for investigating PAR2 deletion on the induction and recovery from sickness behaviour (figure 4.3).

For all experiments, the amount of food intake was measured by comparing the amount of food remaining in the hopper at each time-point with the amount of food remaining at the previous reading. Similar to food intake, the amount of fluid intake was measured by comparing the amount of fluid remaining in the bottle at each time-point with the amount of fluid remaining at the previous reading. However, the % change in body weight was measured by comparing body weight with the baseline body weight.

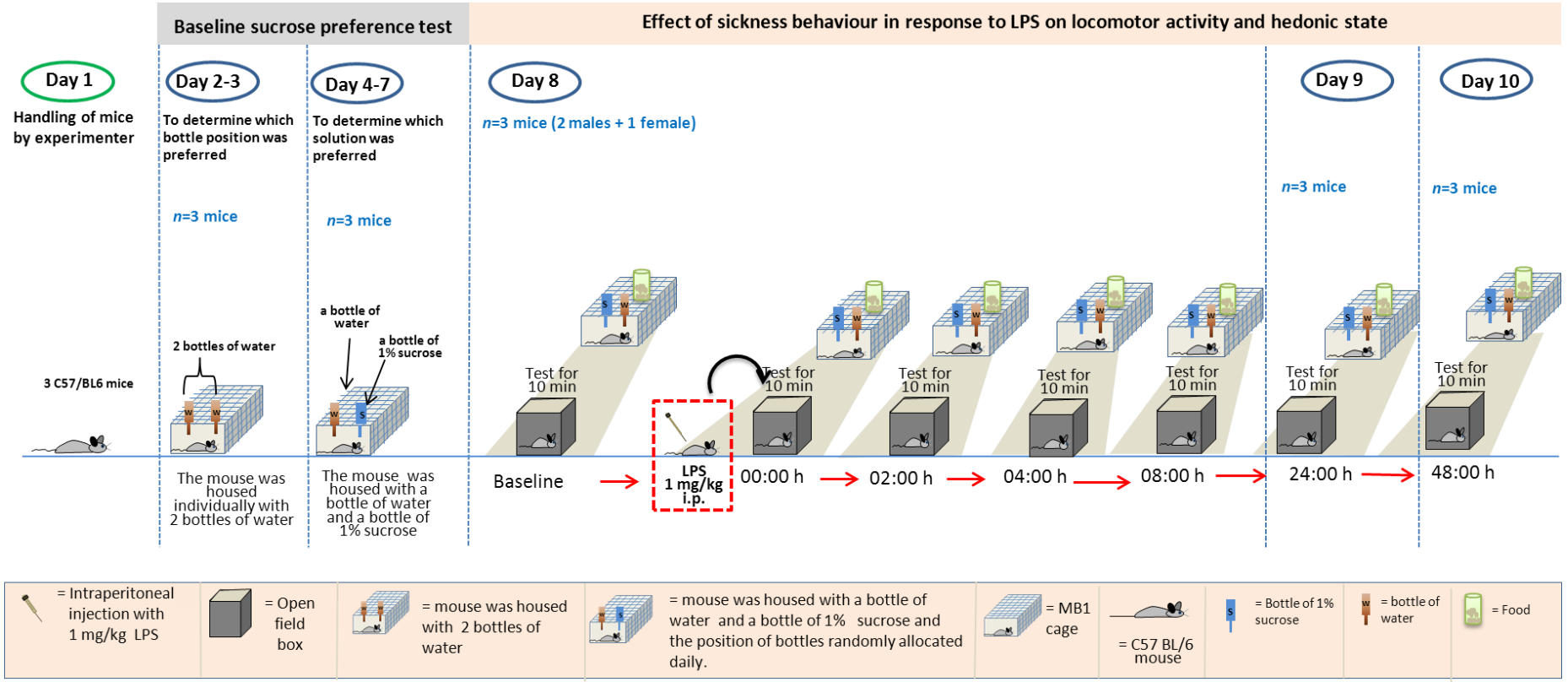


Figure 4.1: Experimental protocol for the pilot study of sickness behaviour in response to LPS on locomotor activity, anxiety-like behaviour, food intake, fluid intake and body weight.

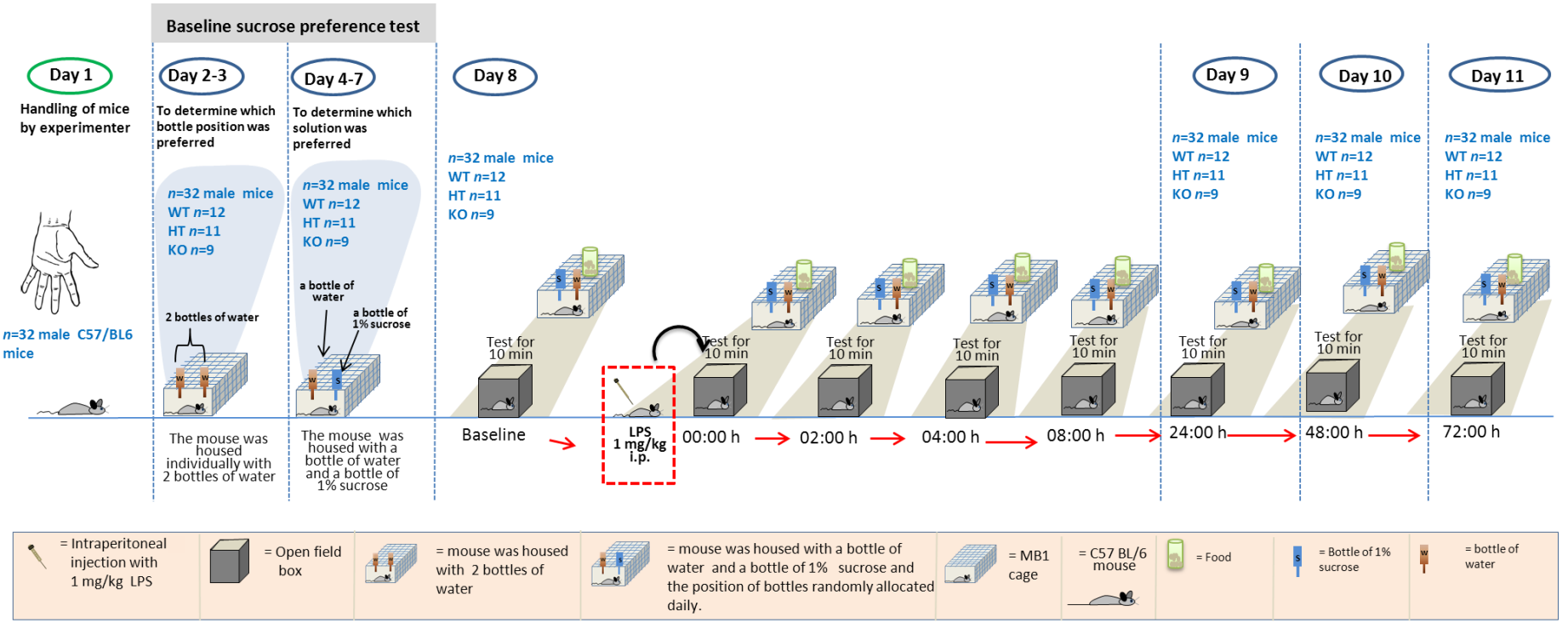


Figure 4.2: Experimental protocol for the effect of PAR2 deletion on the induction of and recovery from LPS-induced sickness behaviour.

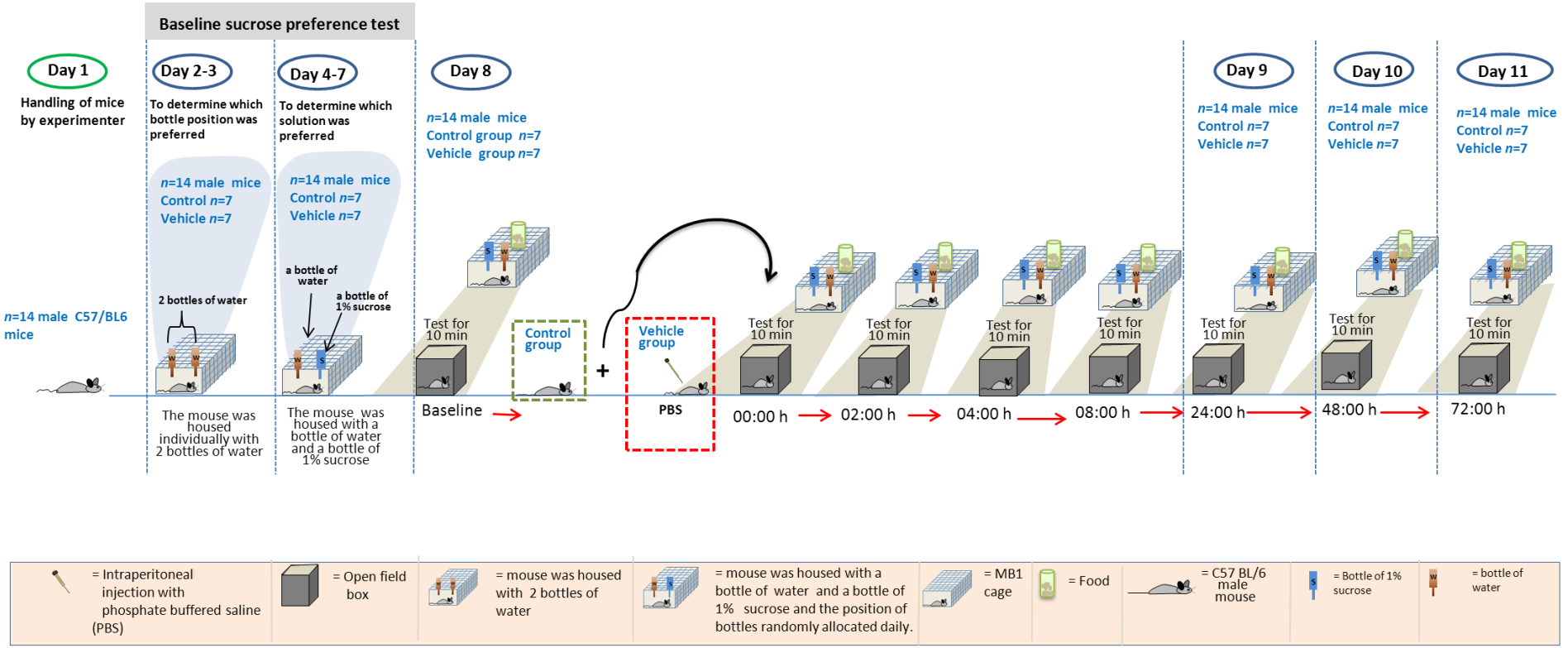


Figure 4.3: Experimental protocol for the effect of habituation and injection on the parameters of sickness behaviour.

4.4 Statistical analysis

All values shown are mean ± standard error of the mean (*S.E.M.)*. The level of significance was set at $p < 0.05$ for all tests. The data were analysed using Prism software for one-way ANOVA and SPSS software for two-way ANOVA with the appropriate post hoc test.

In the pilot study for LPS-induced sickness behaviour, data were analysed using repeated measures one-way ANOVA followed by a Dunnett's test to compare the different time-point measures for the same group of mice with 0 h. The data were compared with 0h instead of baseline because the baseline time was shown the effect of habituation to the OF enviroment.

However, the effect of PAR2 deletion on the induction of and recovery from sickness behaviour data was analysed using a general linear model repeated measures two-way ANOVA followed by a Tukey test to analyse the effect of time and of genotypes. Following the two-way ANOVA, a one-way ANOVA was used to further investigate the different between genotype groups at each individual time-point.

For the experiment to study the effect of habituation and injection on parameters of sickness behaviour, the data was analysed using a general linear model repeated measures two-way ANOVA.

4.5 LPS-induces sickness behaviour under our experimental conditions

4.5.1 Effect of LPS injection in the OFT

There was a significant effect of LPS injection (1 mg/kg i.p.) on the total distance moved (cm) in the OFT over 10 min ($F_{(6,20)}$= 15.32, p= 0.0001, figure 4.4 A). The distance moved reduced significantly from 0 h to 8 h post injection, after which point it started returning back to normal (2 h p<0.05, 4 h p<0.05, 8 h p<0.05, 24 h and 48 h n.s. compared with 0 h, figure 4.4 A). In addition, the mice moved significantly more before injection with LPS (baseline time) compared with the 0 h (p<0.01, figure 4.4 A).

There was a significant effect of time on the number of entries into centre square ($F_{(6,20)}$= 8.69, p= 0.0008). However, while there was a significant decrease at 0 h compared with baseline (p<0.05), there were no significant difference at 2, 4, 8, 24 and 48 h post injection compared with 0 h (p>0.05, figure 4.4 B).

Furthermore, no significant reduction was observed in the time spent in the centre square (s) ($F_{(5,17)}$= 1.66 , p= 0.231, figure 4.4 C). No overall significant change was seen in time spent in centre square from baseline time to 48 h post-injection (baseline time, 2, 4, 8, 24, 48 h n.s. compared to 0 h, figure 4.4 C).

4.5.4 Effect of LPS injection on food intake, fluid intake and body weight

Injection with LPS did not change the amount of food intake ($F_{(2,8)}$= 6.00 , p= 0.063, figure 4.5 A). No significant reductions in food intake being observed 24 and 48 h post LPS injection (both 24 and 48 h n.s. compared to baseline, figure 4.5 A). However, the p value was close to be significant but not significant. Additionally, LPS injection did not change fluid intake ($F_{(2,8)}$= 4.76, p= 0.088, figure 4.5 B) and no significant changes in fluid intake were observed 24 and 48 h post LPS injection (both 24 and 48 h n.s. compared to baseline, figure 4.5 B). However, the p value was close to significant.

Unlike food intake and fluid intake, % change in body weight significantly was change following LPS injection $F_{(6, 20)}$= 4.21 , p= 0.016). Following LPS injection

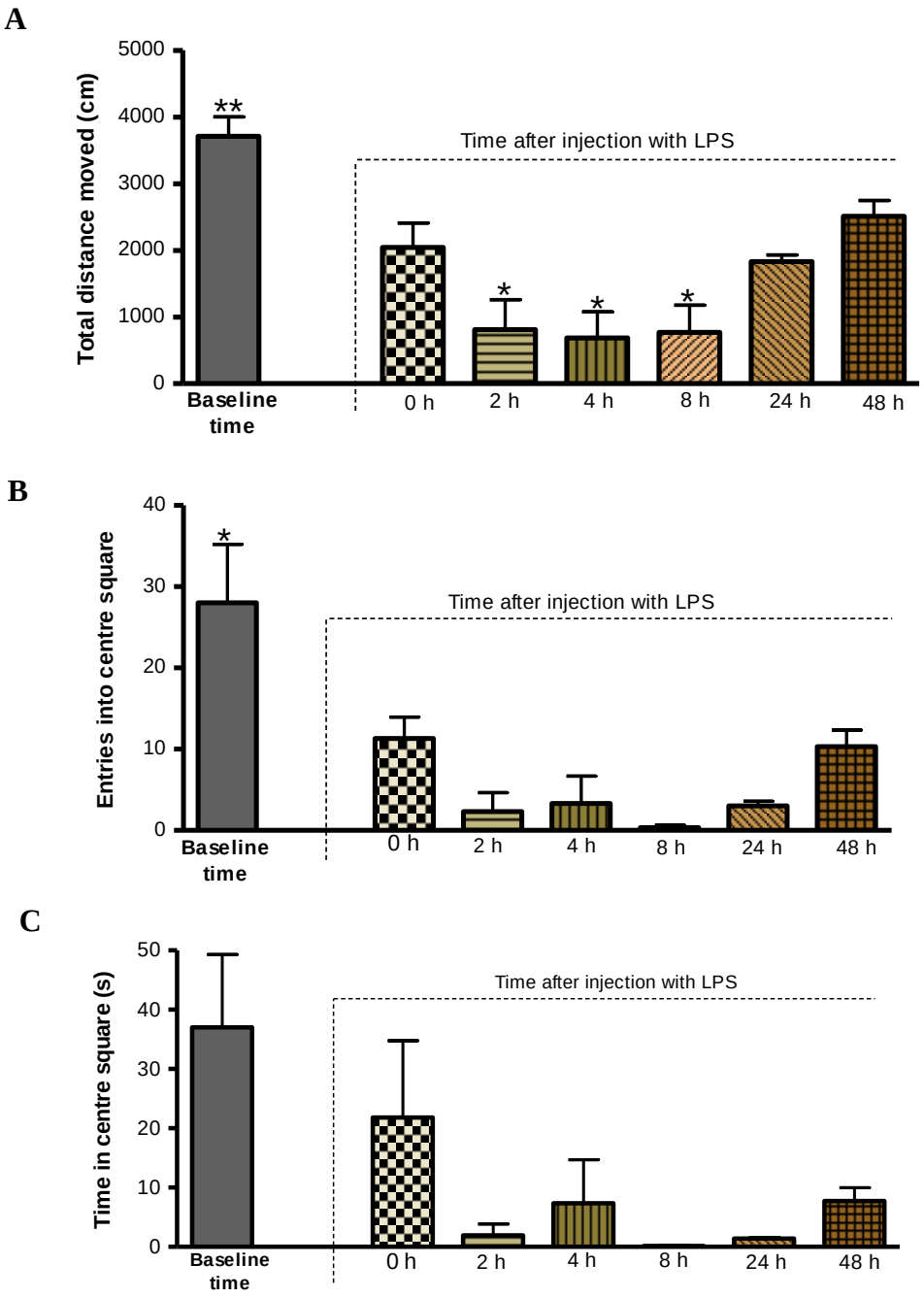


Figure 4.4: LPS effects on the OFT parameters in C57BL/6J mice. (A) Significant differences were observed in the total distance moved at time intervals 2, 4 and 8 h after LPS injection compared to 0 h (p<0.05). In addition, there was a significant difference between baseline time and 0 h (p<0.01). (B) No significant differences at time intervals 2, 4, 8, 24 and 48 h post injection with LPS compared to 0 h in entries into centre square, while there was significant difference between baseline and 0 h (p<0.05). (C) LPS injection did not induce changes in the time spent in the centre square. There were no significant differences observed at baseline 2, 8, 24 and 48 h post injection compared with 0h. n= 3 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measure one-way ANOVA and Dunnett's post hoc test where appropriate. *=p<0.05, **=p<0.01 versus 0 h.

the % change in body weight incresed significantly at 24 h period post LPS injection but returned to normal at 48 h post injection (24 h $p<0.05$, baseline time, 2, 4, 8 h, 48 h n.s. compared to 0 h, figure 4.5 C).

4.6 Does PAR2 deletion alter the induction of and recovery from LPS-induced sickness behaviour?

4.6.1 Effect of PAR2 deletion on sickness behaviour assessed in the OFT

There was a significant effect of time on total distance moved (cm) ($F_{(7,203)}= 113.42$, $p= 0.0005$, figure 4.6 A).

However, there was no significant differences in effect of genotypes and there was no interaction between time and genotypes in the total distance moved (genotypes: $F_{(2,29)}= 2.59$, $p= 0.093$; interaction: $F_{(14,203)}= 1.26$, $p= 0.238$, figure 4.6 A). To determine changes at individual time-points, the data were reanalysed using a one-way ANOVA followed by Tukey's post hoc test. From this analysis, KO mice significantly increased in total distance moved at 24 h and 48 h post injection compared with WT (after 24 h: $F_{(2,31)}= 4.27$, $p= 0.024$, WT versus KO $p<0.05$, both WT and KO n.s. compared with HT; after 48 h: $F_{(2,31)}= 3.96$, $p= 0.030$, WT versus KO $p<0.05$, both WT and KO n.s. compared with HT, figure 4.6 A).

Similar to total distance moved, there was a significant effect of time on entries into centre square (times: $F_{(7,203)}= 65.91$, $p= 0.0005$, figure 4.8 B). However, there was no effect of genotypes and no interaction between time and genotypes on the same parameter (genotypes: $F_{(2,29)}= 1.82$, $p=0.179$; interaction: $F_{(14,203)}= 1.61$, $p= 0.078$, figure 4.8 B).

Further analysis was done to determine changes at individual time-points, so the same data were reanalysed using a one-way ANOVA followed by Tukey's post hoc test. There was a significant increase in the number of entries of KO mice into the centre square at 24 h post injection compared with WT (after 24 h genotypes: $F_{(2, 31)}= 3.73$, $p= 0.036$, WT versus KO $p<0.05$, both WT and KO n.s. compared with HT,

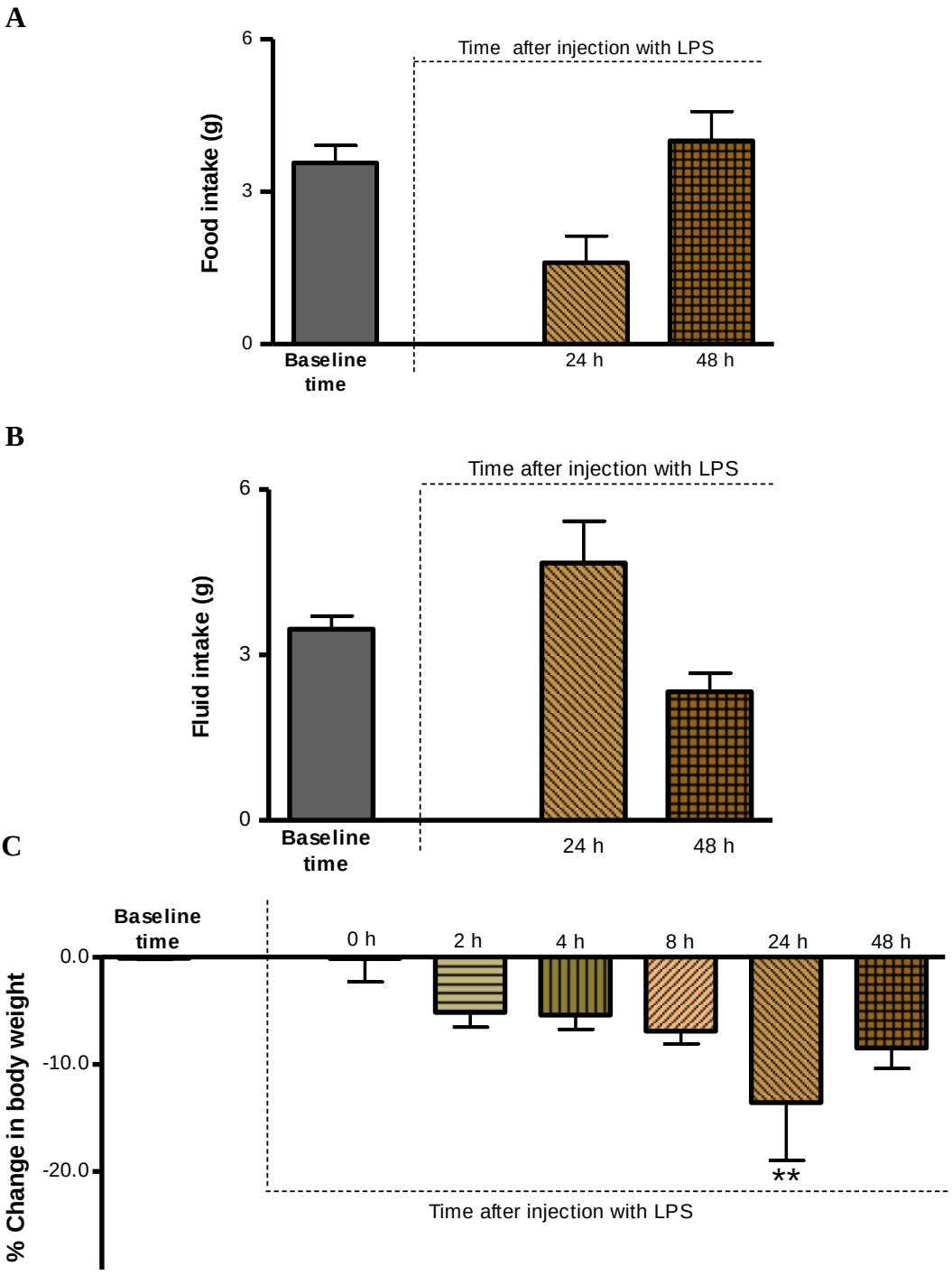


Figure 4.5: Effect of LPS injection on food intake, fluid intake and % change in body weight in C57BL/6J mice. (A) No significant differences were observed in food intake at time interval 24 and 48 h after LPS injection compared to baseline time. (B) No significant differences were observed in the fluid intake at time interval 24 and 48 h after injection with LPS compared to baseline time. (C) LPS injection induced change in % change in body weight with a significant decrease observed at 24 h post LPS injection compared to the 0 h. *n*= 3 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measure one-way ANOVA and Dunnett's post hoc test where appropriate. **=$p<0.01$ versus 0 h.

figure 4.6 B). In addition, there was a significant effect of time on time spent in the centre square (s) (times: $F_{(7,203)}$= 12.20, *p*= 0.0005, figure 4.6 C).

However, there was no effect of genotypes and no interaction between time and genotypes in time spent in the centre square (genotypes: $F_{(2,29)}$= 1.01, *p*= 0.377; interaction: $F_{(14,203)}$= 1.36, *p*= 0.174, figure 4.6 C). Similar to the other parameters, data of time spent in the centre square was also reanalysed using one-way ANOVA between each time-point. The result of this data showed that there was no significant difference between genotypes.

4.6.2 Effect of PAR2 deletion on baseline sucrose preference

PAR2 deletion did not change baseline SPT parameters between 3 consecutive testing days prior to LPS injection (figure 4.7). There was no effect of days, genotypes and no interaction between days and genotypes on % sucrose drunk on baseline level (days: $F_{(2,60)}$= 0.33, *p*= 0.723; genotypes: $F_{(2,30)}$= 0.07, *p*= 0.931; interaction: $F_{(4,60)}$= 0.41, *p*= 0.799, figure 4.7 A).

Further analysis was done to determine changes at individual testing days, so the same data were reanalysed using one-way ANOVA followed by Tukey's test. There was no differences between genotypes on % sucrose drunk on baseline level (day 4-5: $F_{(2,32)}$= 0.52, *p*= 0.668; day 5-6: $F_{(2,32)}$= 0.95, *p*= 0.995; day 6-7: $F_{(2,32)}$= 0.0460, *p*= 0.995).

Regarding the total fluid drunk (g), there was no effect of days, genotypes and no interaction between days and genotypes (days: $F_{(2,58)}$= 0.10, *p*=0.908; genotypes: $F_{(2,29)}$= 1.95, *p*= 0.161; interaction $F_{(4,58)}$= 1.89, *p*= 0.125, figure 4.7 B).

Further analysis was done to determine changes at individual testing days, so the same data were reanalysed using one-way ANOVA Followed by Tukey's test. There was no differences between genotypes on total fluid drunk on baseline level (day 4-5: $F_{(2,32)}$= 1.54, *p*= 0.230; day 5-6: $F_{(2,32)}$= 0.47, *p*= 0.765; day 6-7: $F_{(2,32)}$= 3.236, *p*= 0.0534).

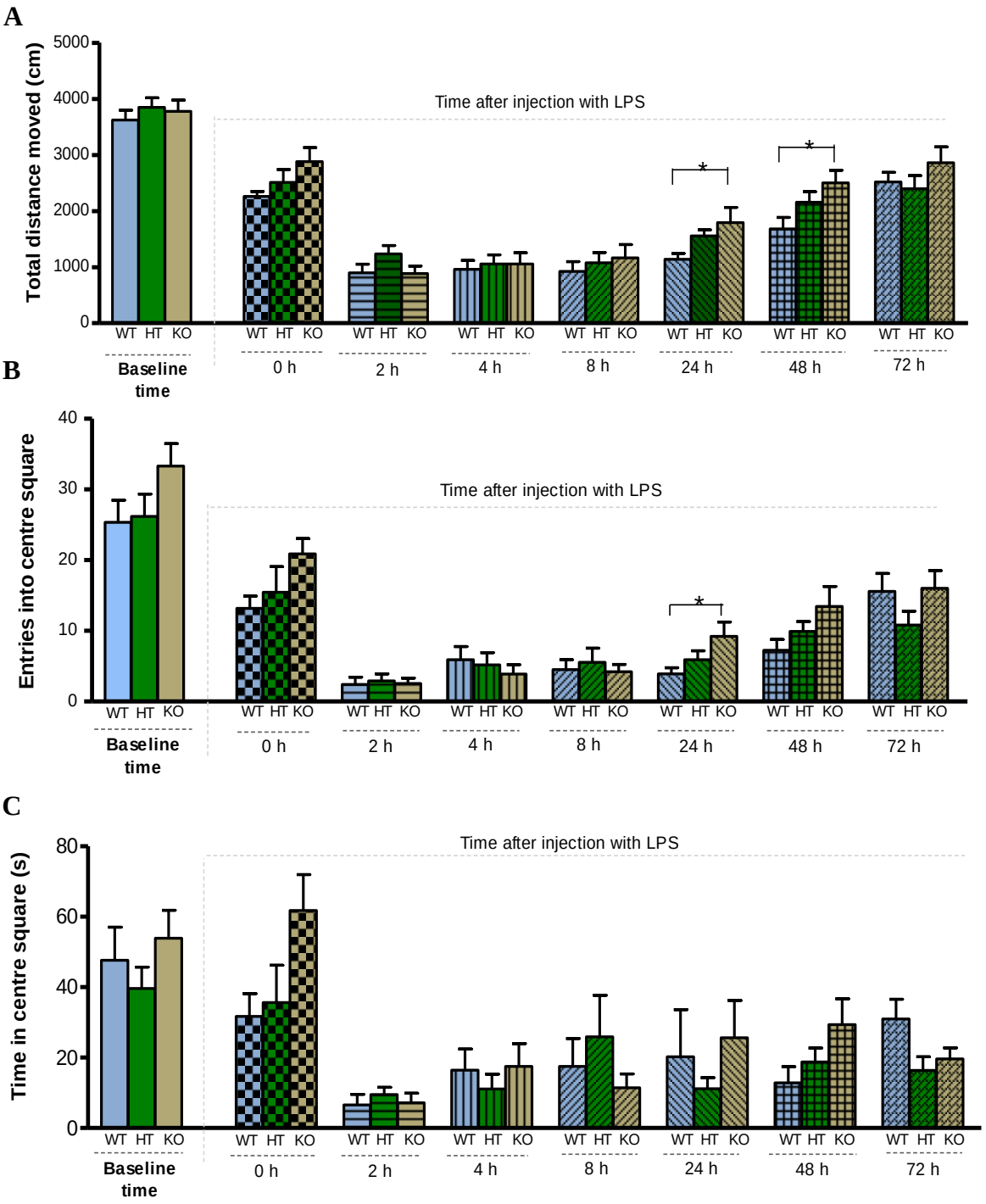
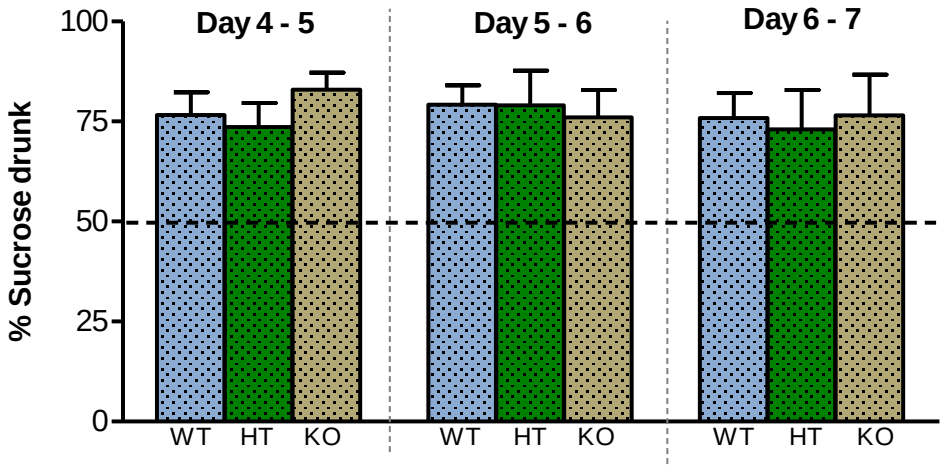


Figure 4.6: Effect of PAR2 deletion on the induction of and recovery from sickness behaviour using the OFT. (A) A significant difference is seen between WT and KO mice 24 h and 48 h post injection with LPS in total distance moved over the test period. (B) A significant difference is observed between WT and KO 24 h post injection in entries into centre square over the test period. (C) No significant differences between genotypes in all time-points in time spent into centre square over the test period. WT *n*=12 mice, HT *n*=11, KO *n*=9. Data are presented as mean ± *S.E.M* and analysed using one-way ANOVA with Tukey's post hoc test between genotypes where appropriate. **p*= <0.05.

A



B

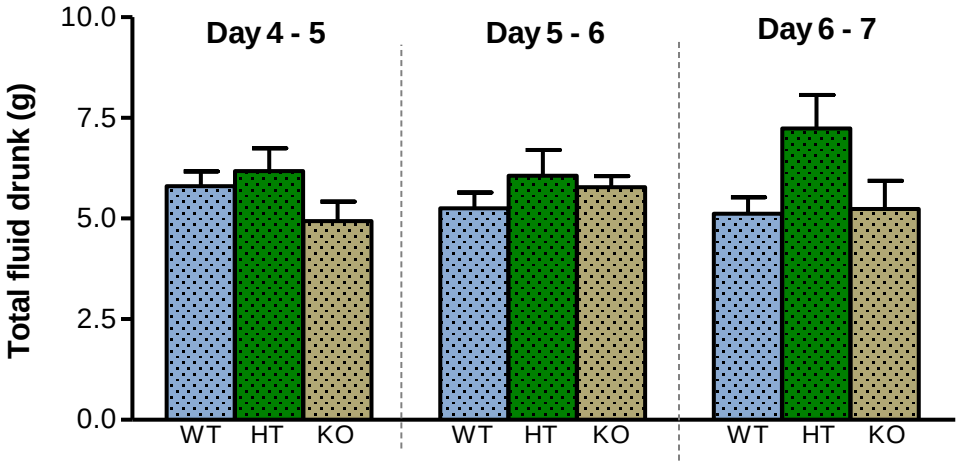


Figure 4.7: PAR2 deletion does not alter the SPT baseline parameters. (A) No significant differences between genotypes in % sucrose drunk from day 4 to 6. (B) No significant differences between genotypes in total fluid drunk. WT *n*=12 mice, HT *n*=11, KO *n*=9. Data are presented as mean ± *S.E.M.* and analysed using one ANOVA with Tukey's post hoc test between genotypes where appropriate.

4.6.3 Effect of LPS on sucrose preference in different PAR2 genotypes

There was a significant effect of time post LPS injection on % sucrose drunk but there was no effect of genotypes and no interaction between time and genotypes (times $F_{(6,174)}$= 8.68, p= 0.0005; genotypes $F_{(2,29)}$= 0.01, p= 0.991, interaction $F_{(12,174)}$= 1.41, p= 0.163, figure 4.8 A).

Similar to OFT, the data was also re-analysed by one-way ANOVA followed by Tukey's post hoc test at each time-point and it was observed that there were significant increases between HT and KO mice in % sucrose drunk in the first 2 h post injection ($F_{(2,31)}$= 3.75, p= 0.036, KO p<0.05 compared to HT, both KO and HT n.s. compared to WT, figure 4.8 A).

Additionally, there was no significant effect of genotypes in the total amount of fluid drunk ($F_{(2,29)}$= 0.64, p= 0.533, figure 4.8 B). However, the data was also analysed by one-way ANOVA at each time-point and it was observed that there were significant differences between HT and KO in total fluid drunk before injection with LPS ($F_{(2,31)}$= 4.66, p= 0.017, both HT and KO n.s. compared to WT, figure 4.8 B). Both WT and HT drank significantly less fluid (sucrose and water) than KO at 24 h post injection ($F_{(2,31)}$= 0.01, p= 0.0009, WT p< 0.01 compared to KO, HT p< 0.01 compared to KO, WT n.s. compared to HT, figure 4.8 B).

4.6.4 Effect of PAR2 deletion on baseline food intake and % change in body weight

PAR2 deletion did not change baseline food intake and % change in body weight between 3 consecutive testing days (figure 4.8). There was a significant effect of time (testing days) on food intake on baseline level (days: $F_{(2,60)}$= 3.79, p=0.028; figure 4.8 A). However, there was no effect of genotypes and interaction between time and genotypes on food intake on baseline level (genotypes: $F_{(2,30)}$= 1.93, p= 0.163; interaction $F_{(4,60)}$= 0.75, p= 0.559, figure 4.9 A).

Additionally, there was no significant change of time (testing days) on % change in body weight Moreover, there was no significant effect of genotypes and no

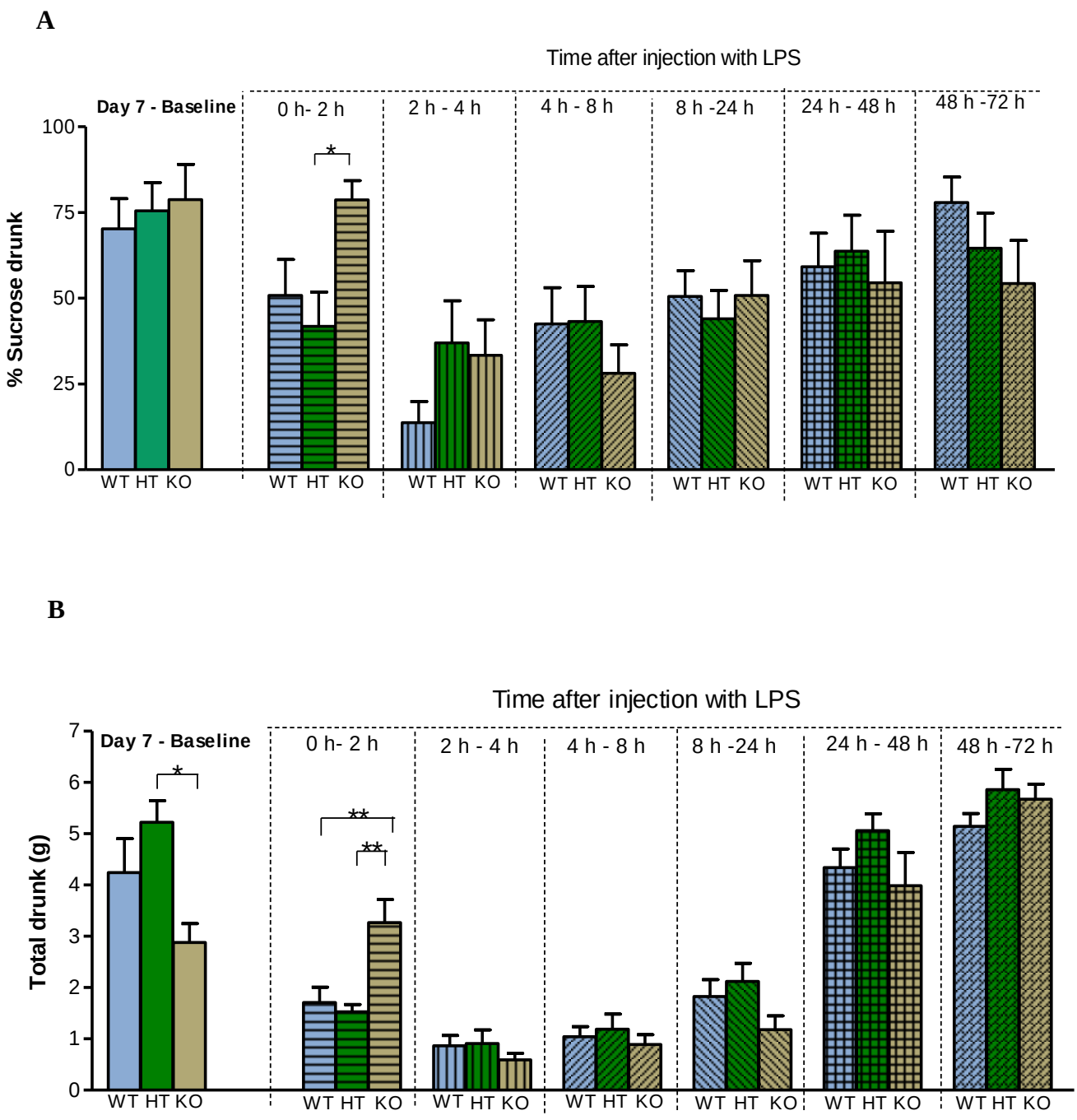


Figure 4.8: : Effect of PAR2 deletion on the induction and recovery from sickness behaviour: SPT. (A) No significant difference was observed between genotypes before injection but significant differences were seen between HT and KO 2 h post injection in % sucrose drunk. (B) Significant differences were observed between HT and KO mice before injection with LPS and significant difference between KO and both HT and WT mice were seen 2 after LPS injection on total drunk. WT *n*=12 mice, HT *n*=11, KO *n*=9. Data are presented as mean ± *S.E.M*, and analysed using one-way ANOVA with Tukey's post hoc test between genotypes where appropriate. *=$p<0.05$, **=$p<0.01$.

interaction between time and genotypes on % change in body weight (time: $F_{(2,60)}$= 2.230, p=0.12; genotypes: $F_{(2,30)}$= 0.86, p= 0.430; interaction $F_{(4,60)}$= 0.33, p= 0.856, figure 4.9 B).

4.6.5 Effect of LPS on food intake and the % change in body weight

There was a significant effect of genotypes post LPS injection on amount of food intake (genotypes $F_{(2,29)}$= 3.67, p= 0.038; figure 4.10 A). The data was also analysed using one-way ANOVA followed by Tukey's post hoc test at each time-point which revealed that HT mice had eaten significantly more food than WT mice at 48 h post injection ($F_{(2,31)}$= 5.48, p= 0.009, HT p< 0.01 compared to WT, both WT and HT n.s. compared to KO, figure 4.10 A).

In addition, there was a significant effect of time on the % change in body weight (times $F_{(6,174)}$= 61.14, p= 0.0005, figure 4.10 B).

However, there was no effect of genotypes but there was a significant interaction between time and genotypes on % change in body weight (genotypes $F_{(2,29)}$= 1.39, p= 0.226, interaction $F_{(12,174)}$= 2.29, p= 0.010, figure 4.10 B). The data were reanalysed using one-way ANOVA followed by Tukey's post hoc test showed that KO mice gained weight back faster than WT mice. This increse in weight was measured by a significant reduction of % of change in body weight of KO mice compared to WT in two time points at 48 h and 72 h post injection (at 48 h post injection: $F_{(2,31)}$= 3.31, p= 0.050, KO p<0.05 compared to WT, both WT and KO n.s. compared to HT; at 72 h post injection: $F_{(2,31)}$= 3.81, p= 0.0340, KO p<0.05 compared to WT, both WT and KO n.s. compared to HT, figure 4.10 B).

4.7 Investigation into the effect of habituation and vehicle injection on parameters of sickness behaviour

A control experiment was conducted to identify the effect of injection *per se* on the parameters measured.

4.7.1 Effect of injection of vehicle of LPS on OFT over 10 min test

Injection with PBS (vehicle of LPS) did not have any effect on total distance moved, entries into centre square and time into centre square at any time-point investigated. There was a significant effect of time from zero time to 72 h in total distance moved (cm) but no significant effect of injection and no interaction between time and injection from zero time to 72 h in total distance moved (cm) (CG: baseline time: time $F_{(7,84)}$= 10.94, p= 0.0005; groups $F_{(1,12)}$= 0.095, p= 0.763; interaction $F_{(7,84)}$= 1.56, p= 0.159, figure 4.11 A), entries into centre square (time $F_{(7,84)}$= 8.725, p= 0.0005; groups $F_{(1,12)}$= 0.63, p= 0.443; interaction $F_{(7,84)}$= 1.19, p= 0.315, figure 4.11 B) and time in centre square (s) (time $F_{(7,84)}$= 2.275, p= 0.004; groups $F_{(1,12)}$= 0.67, p= 0.430; interaction $F_{(7,84)}$= 0.94, p= 0.482 , figure 4.11 C).

4.7.2 Baseline sucrose preference, food intake and % change in body weight

Baseline sucrose preference

There was no significant effect of time and groups nor any no interaction between time and groups on % sucrose drunk observed over 3 consecutive testing days (time $F_{(2, 24)}$= 1.418, p= 0.262; groups $F_{(1,12)}$= 2.82, p= 0.119; interaction ($F_{(2, 24)}$= 1.06, p= 0.363, figure 4.12 A).

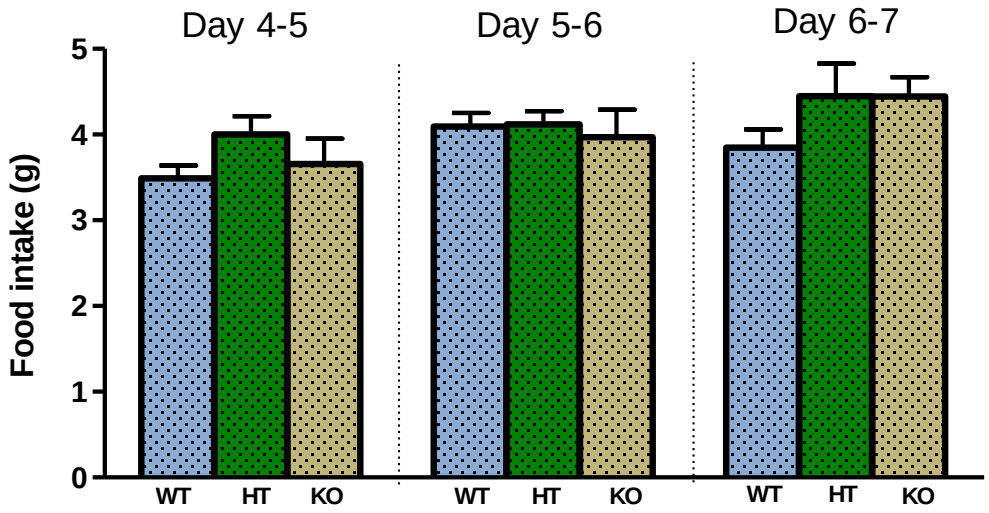
However, there was significant effect of time which was increasing the total volume drunk over the 3 consecutive testing days ($F_{(2, 24)}$= 4.50, p= 0.022, figure 4.12 B). However, there were no effect of groups on total drunk ($F_{(1,12)}$= 0.64, p= 0.439, figure 4.12 B). But there was a significant interaction between time and groups on total volume drunk ($F_{(2, 24)}$= 5.24, p= 0.013, figure 4.12 B).

4.7.3 Effect of vehicle injection on sucrose preference, food intake and % change in body weight

Effect of vehicle injection of LPS on sucrose preference

There were no differences observed at any time-point post vehicle injection in % sucrose drunk and total fluid drunk. There was a significant effect of time ($F_{(6,72)}$= 3.58, p= 0.004, figure 4.14 A) but no significant effect of groups and no interaction

A



B

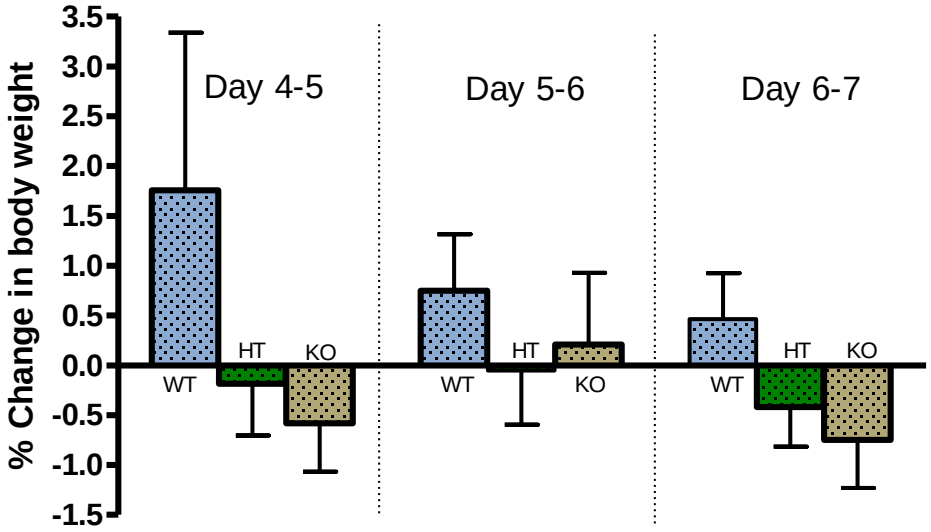


Figure 4.9: Effect of PAR2 deletion on food intake and % change in body weight in mice from day 5 to day 6. (A) No significant differences between genotypes in food intake from day 4 to 6. (B) No significant differences between genotypes % change in body weight. WT *n*=12 mice, HT *n*=11, KO *n*=9. Data are presented as mean ± *S.E.M.* and analysed using one ANOVA with Tukey's post hoc test between genotypes where appropriate.

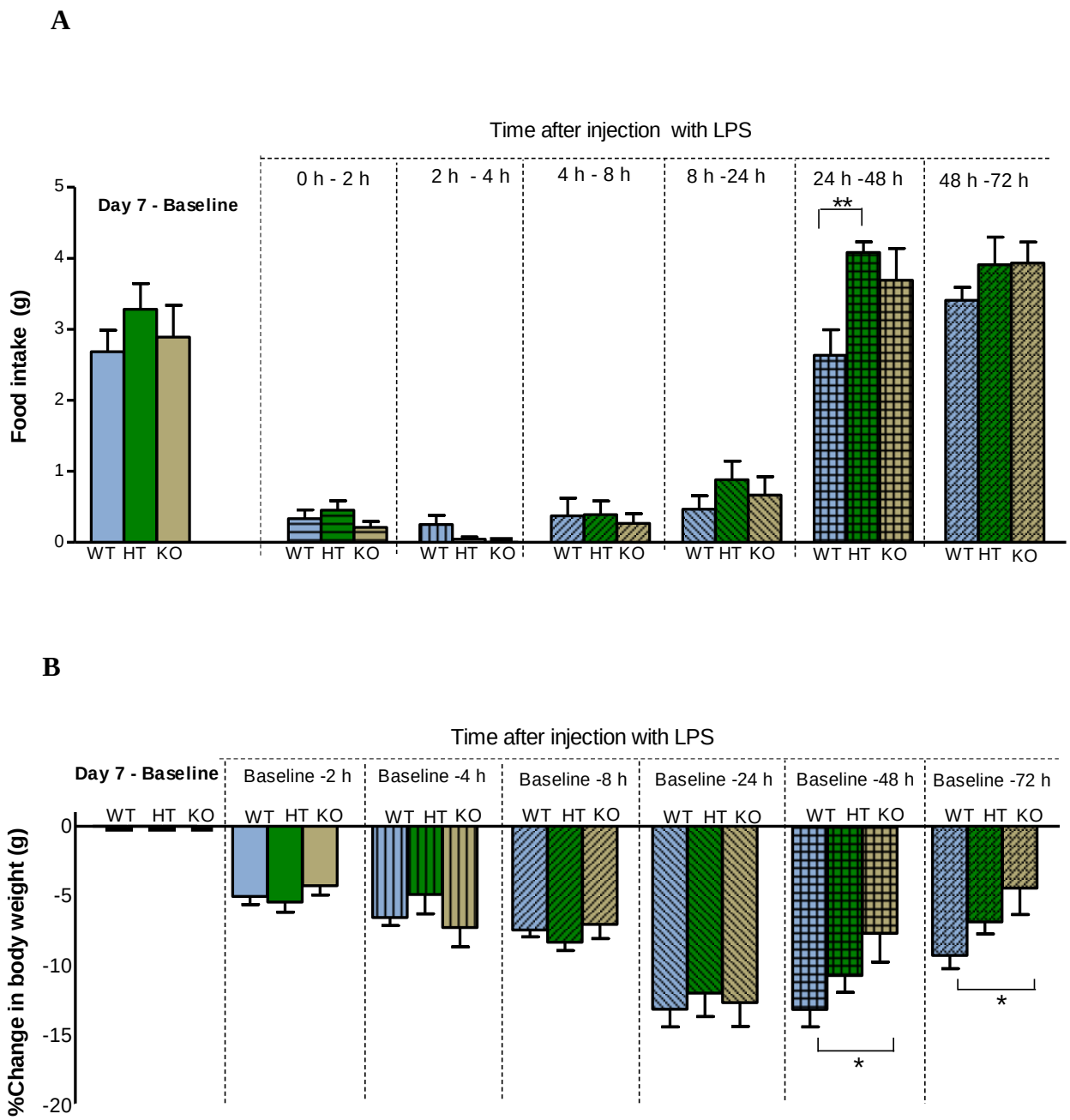


Figure 4.10: Effect of PAR2 deletion on recovery from sickness behaviour: food intake and % change in body weight. (A) No significant differences were seen between genotypes before injection but significant difference between WT and HT was observed 48 h post injection in the amount of food intake. (B) No significant differences were seen between genotypes before injection but significant differences were observed between WT and KO at 48 h and 72 h post LPS injection in % change in body weight. WT *n*=12 mice, HT *n*=11, KO *n*=9. Data are presented as mean ± *S.E.M* and analysed using one-way ANOVA with Tukey's post hoc test between genotypes where appropriate. *=$p<0.05$, **=$p<0.0$.

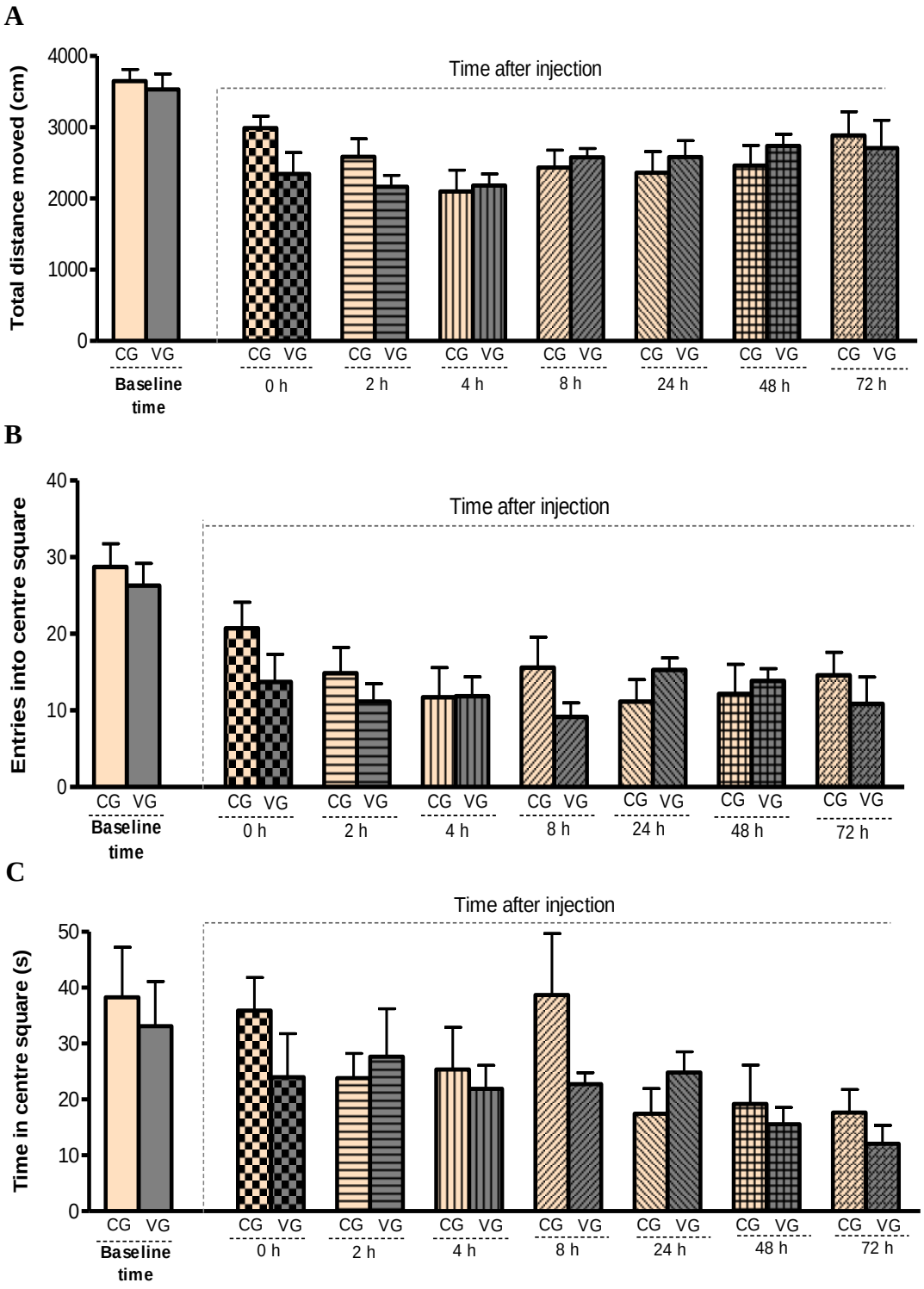


Figure 4.11: Vehicle injection had no effect on the OFT parameters compared with control. (A) No significant differences were seen between groups at any time-point investigated in total distance moved over the test period. (B) No significant differences were seen between groups at any time-point for entries into centre square over the test period. (C) No significant differences between CG and VG at any time-point in entries in centre square over the test period. CG *n*= 7 mice and VG *n*=7 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measures two-way ANOVA.

between time and groups (groups $F_{(1,12)}$= 0.20, p= 0.664; interaction $F_{(6,72)}$= 0.92, p= 0.489, figure 4.13 A) in the % sucrose drunk. Similarly, there was no significant effect of groups in total volume drunk (groups $F_{(1,12)}$= 0.14, p= 0.715; figure 4.13 B).

4.7.4 Baseline food intake and % change in body weight

There was no significant effect of time (days $F_{(2, 24)}$= 1.66, p= 0.211), groups ($F_{(1,12)}$= 0.14, p= 0.718, figure 4.13 A) and no interaction between time and groups ($F_{(2, 24)}$= 0.69, p= 0.510, figure 4.14 A) on food intake (g).

Similarly, there was no difference in % change in body weight over the 3 consecutive testing days and between groups. There was no significant difference between time (days $F_{(2, 24)}$= 0.051, p= 0.951, figure 4.14 B), groups ($F_{(1,12)}$= 0.22, p= 0.650, figure 4.13 B) and no interaction between time and groups ($F_{(2, 24)}$= 1.85, p= 0.178 , figure 4.14 B) in % change in body weight.

4.7.5 Effect of vehicle injection on food intake and % change in body weight

There was a significant effect of groups on food intake (groups: $F_{(1,12)}$= 6.46, p= 0.026; figure 4.15 A). Furthermore, the data was also analysed by unpaired *t-test* at each time-point and it was observed that there were significant differences between CG and VG in food intake before injection with vehicle of LPS ($F_{(12)}$= 3.59, p=0.0037; figure 4.15 A).

There were no differences observed at any time-point post vehicle injection in the % change in body weight. There was a significant effect of time (time $F_{(6,72)}$= 2.89, p= 0.014, figure 15 B) but no significant effect of groups and no interaction between groups and time in % change in body weight at all time-points after i.p. injection (groups $F_{(1,12)}$= 2.03, p= 0.180; interaction $F_{(6,72)}$= 1.88, p= 0.096, figure 4.15 B).

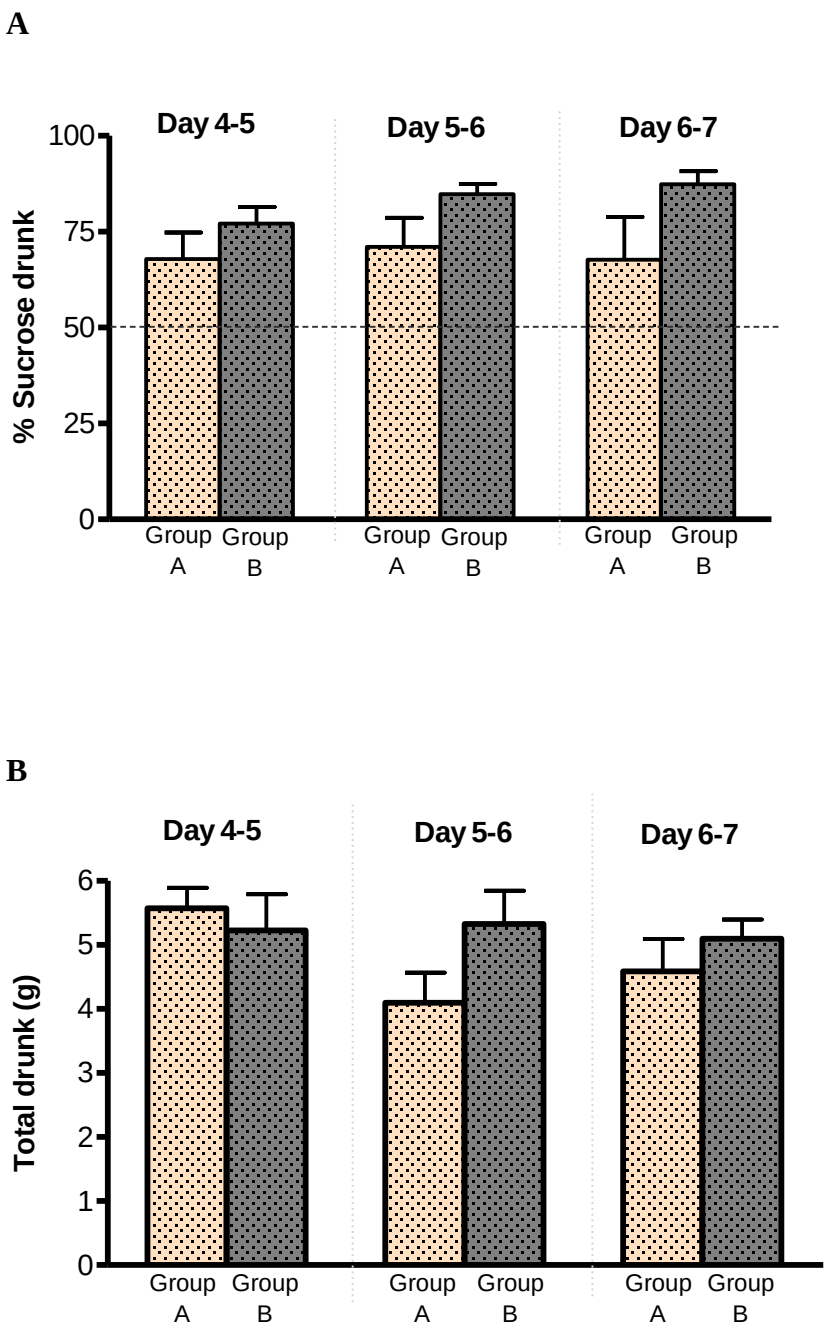


Figure 4.12: Baseline sucrose preference. (A) No significant differences were seen between allocated groups in % sucrose drunk from day 4 to 7. (B) No significant differences were observed between allocated groups in total fluid drunk. Group A *n*= 7 mice and group B *n*=7 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measure 2 way-ANOVA. Group A: this group of mice were allocated to CG, Group B: this group of mice were allocated to VG.

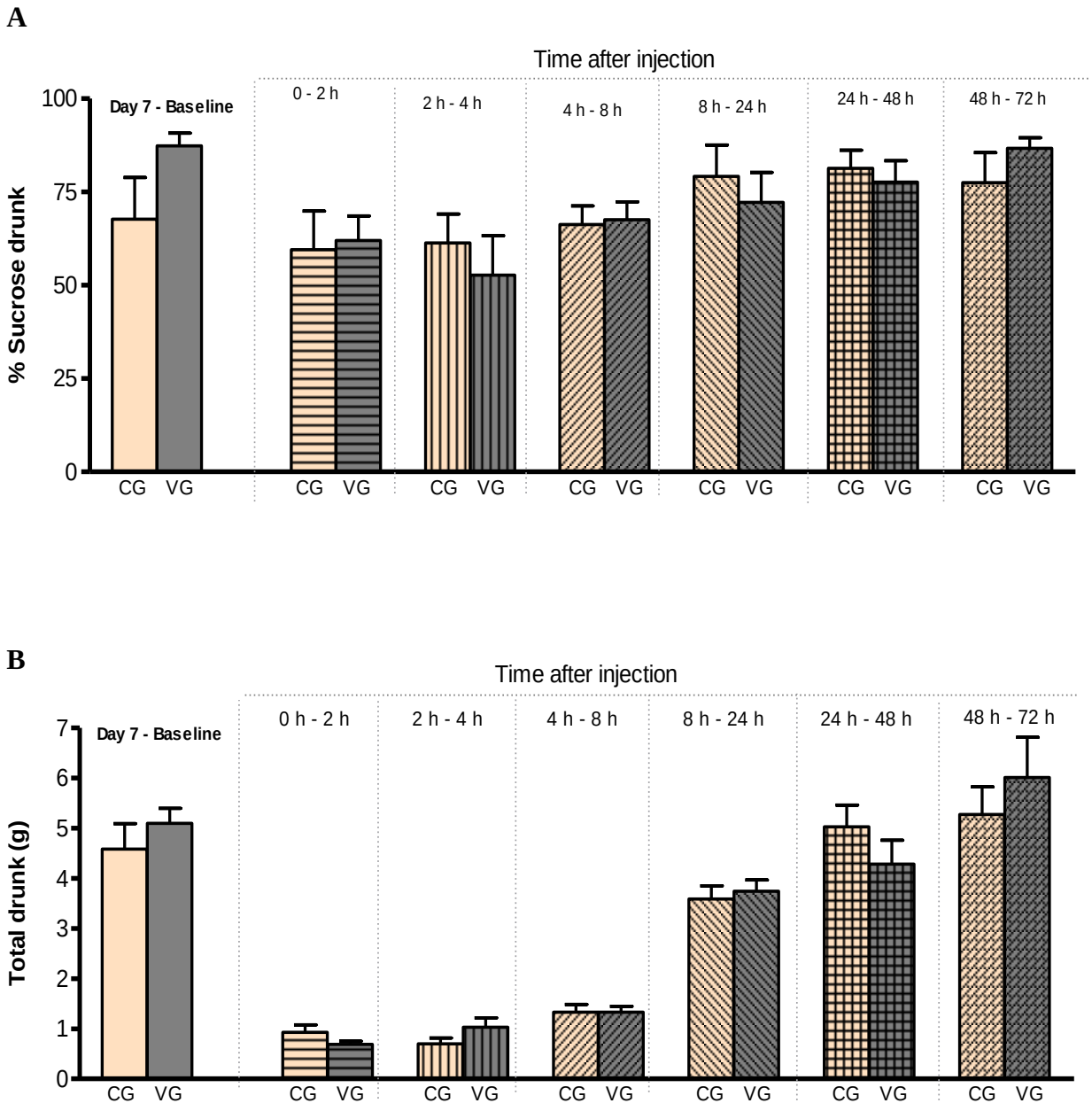


Figure 4.13: Vehicle injection had no effect on the SPT parameters compared with control. (A) No significant differences were observed between groups and at any time-point investigated in % sucrose drunk. (B) No significant difference was seen between groups at any time-point investigated in total fluid drunk. CG *n*= 7 mice and VG *n*=7 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measures way ANOVA.

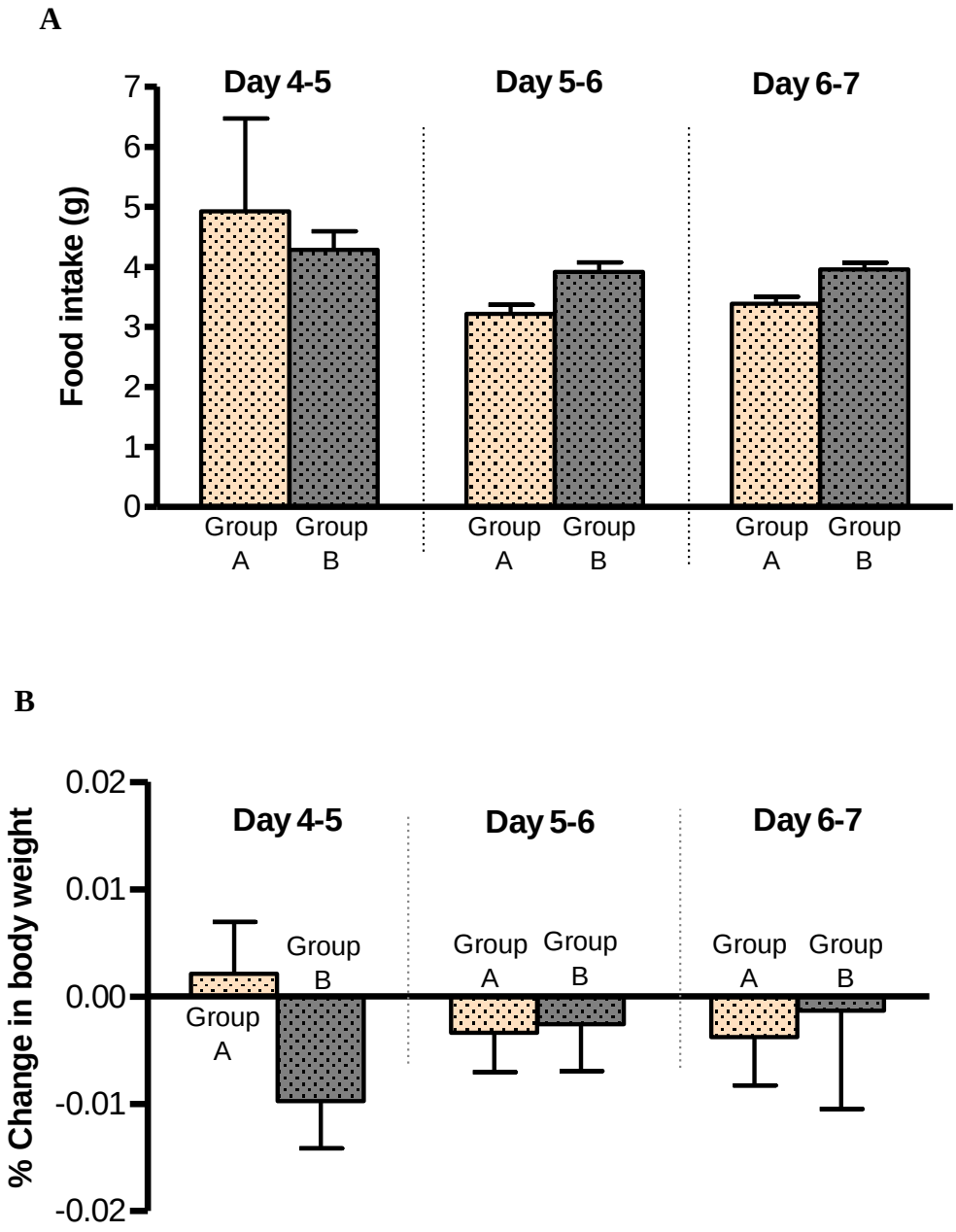


Figure 4.14: Vehicle injection had no effect on baseline food intake and % change in body weight. (A) No significant differences were observed between allocated groups in food intake from day 4 to7. (B) No significant difference was seen between group A and group B in % change in body weight from day 4 to7. Group A *n*= 7 mice and group B *n*=7 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measure two-way ANOVA. Group A were allocated CG, Group B were allocated to VG.

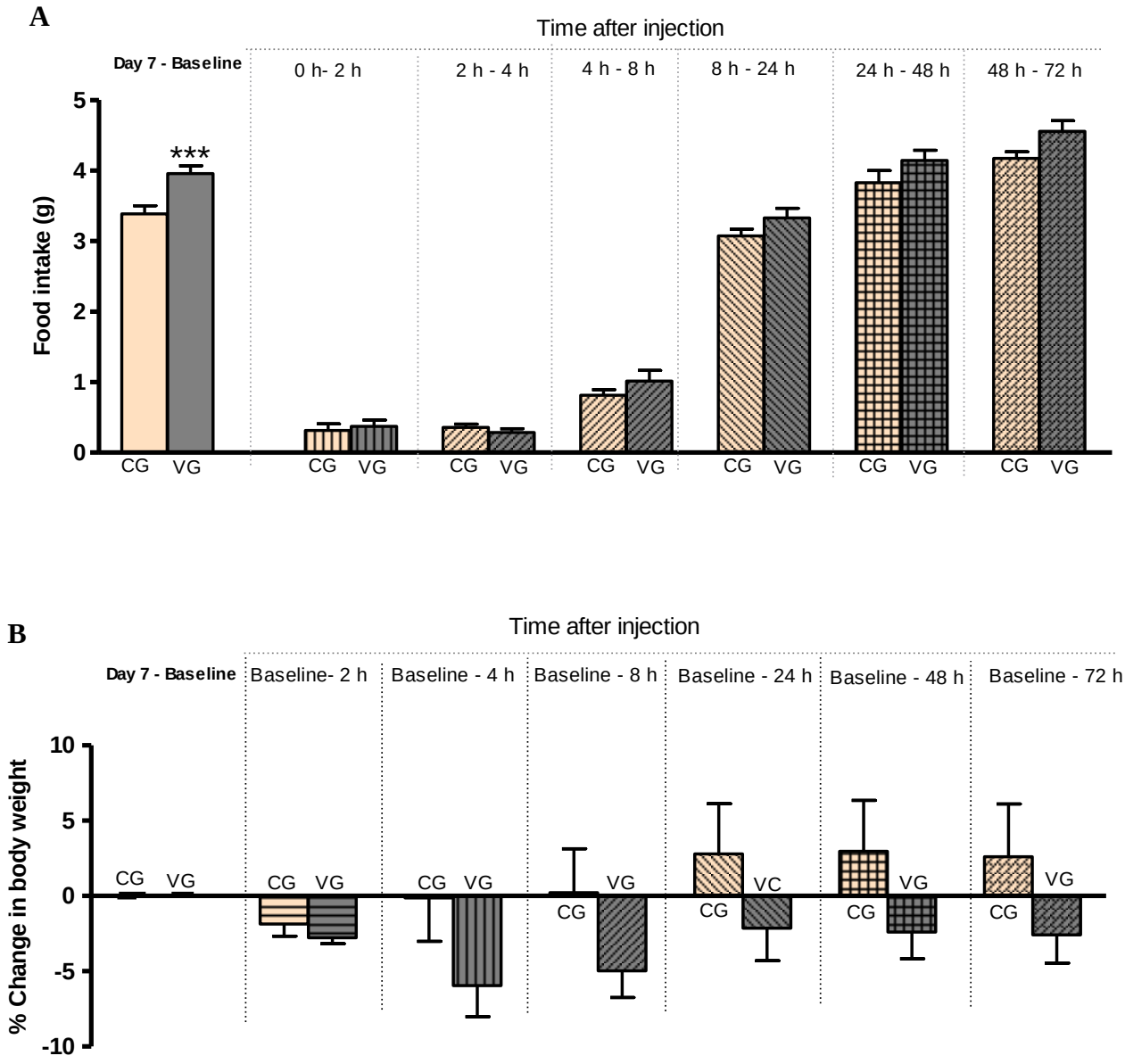


Figure 4.15: Vehicle injection had no effect on the SPT parameters compared with control. (A) There was significant difference between CG and VG before injection whereas no significant differences were seen between groups at any time-point investigated after injection with vehicle of LPS in food intake. (B) No significant differences were observed between groups at any time-point investigated in % change in body weight. CG *n*= 7 mice and VG *n*=7 mice. Data are presented as mean ± *S.E.M.* and analysed using repeated measures two-way ANOVA and unpaired *t-test* between groups at each time point.

4.8 Discussion

4.8.1 Mouse model of sickness behaviour

Systemic inflammation is associated with an increased frequency of behavioural and cognitive disorders, especially in the elderly (Evans *et al.,* 2005; Penninx *et al.,* 2003). Inflammation causes stimulation of the peripheral immune system by inflammatory inducers such as bacteria and viruses. The inflammatory inducers are detected by receptors in the innate immune system leading to the release of PICs in the periphery including IL-1α, IL-1ß, TNF-α and IL-6 (Godbout *et al.,* 2005; Tizard, 2008; Dantzer, 2009; Medzhitov *et al,* 2010).

Under normal conditions, PICs are actively transported into the brain by endothelial cell transporters whereas during inflammation these cytokines gain entry by diffusing through BBB dysfunctional areas (Stolp and Dziegielewska, 2009*;* Abbott, 2000; Maes *et al.,* 2011). At the same time, the brain also contains immune cells including microglia, which are expressed in the choroid plexus and meninges, that can respond to inflammatory stimuli by inducing exaggerated cytokine production (Godbout et al. 2005; Dantzer *et al.,* 2008). In the brain PICs are known to play a role in the induction of sickness behaviour (Kent *et al.,* 1992a; Dantzer, 2001a; Kelley *et al.,* 2003; Dantzer, 2009; Burton *et al.,* 2011) and they play an important role in the resolution of inflammation and increased energy needs during inflammation (Kelley *et al.,* 2003; Kobayashi, 2010; Maskrey *et al.,* 2011).

The intensity and duration of sickness behaviour is regulated by anti-inflammatory cytokines (AIC), by either inhibiting PIC production or attenuating PIC signalling (Heyen, *et al.,* 2000; Strle *et al.,* 2007). The balance between PICs and AICs regulates the intensity and duration of the response to immune stimuli (Keane and Strieter, 2002; Dantzer *et al.,* 2008; Sydora *et al.,* 2010). In the CNS, the AICs probably are IL-10 (Bluthe *et al.,* 1999; Gao, 2012) and insulin-like growth factor-1 (Dantzer *et al.,* 1999; Bluthe *et al.,* 2006) that attenuate the behavioural symptoms of sickness behaviour.

In both humans and rodents, sickness behaviour is induced by infections (Cunningham *et al.*, 2007). Individuals having sickness behaviour are characterized by little motivation to eat, restlessness, fatigue, no interest in social activities and significant changes in sleep patterns (Kelley *et al.*, 2003). Similarly, in rodents, sickness behaviour is a behavioural complex and is characterized by several symptoms including reduction of locomotor activity, food intake and fluid intake, body weight and induction both of anxiety-like behaviour and anhedonia (Godbout *et al.*, 2005; Dantzer *et al.*, 2008; Dantzer, 2009; Maes *et al*,. 2012). Therefore, in the current study, a number of parameters were measured as markers of the induction of and recovery from these sickness behaviour symptoms. These markers were locomotor activity, anxiety-like behaviour, anhedonia, food intake, fluid intake and % change in body weight.

The results demonstrate that peripheral administration of LPS induced symptoms similar to the symptoms of sickness behaviour. In this model of sickness behaviour, mice treated with LPS (1 mg/kg, i.p.) had decreased locomotor activity and body weight in the first 8 h post injection but returned back to normal within 24 - 48 h post injection. These results are consistent with previous studies where LPS-induced sickness behaviour has been investigated (Nava *et al.*, 2000; Godbout *et al.*, 2005; Spulber *et al.*, 2012) and therefore confirms that sickness behaviour is induced under our experimental conditions.

4.8.2 PAR2 and sickness behaviour

At the molecular level, toll-like receptor-4 (TLR-4) is a member of TLR family which has a role in recognition of pathogen and activation of the innate immune system (Janssens and Beyaert, 2003). TLR-4 are expressed in macrophages and dendritic cells (Hansson and Edfeldt, 2005). The interaction between TLR-4 and pathogen stimulates production of necessary cytokines for the development of an infection (Mogensen, 2009). In several fungi, viruses and bacteria, there are components, known as pathogen-associated molecular patterns, that can activate TLR-4 (Lu *et al.*, 2008). One of these components is LPS, which is the main component of cell wall of Gram-negative bacteria; this acts as endotoxin and induces

strong response to the innate immune system (Peterson, 1996). LPS binds with TLR-4 and the formation of DY88-TLR-4 complex can cause activation of NF-$_K$B signalling pathways that stimulate release of PICs in several cell types especially in macrophages (Arroyo-Espliguero and Avanzas *et al.*, 2004). PICs penetrate into brain and induce sickness behaviour (Dantzer, 2009; Burton *et al.*, 2011). Beside activation of NF-$_K$B, the DY88-TLR-4 complex can cause activation of mast cells which are involved both in immune system and release different molecules that are involved in immune function, such as serine proteinases, serotonin, cytokines, prostaglandin, histamines, heparin. One of the serine proteinases released form mast cell is trypsin (Theoharides *et al.*, 2012).

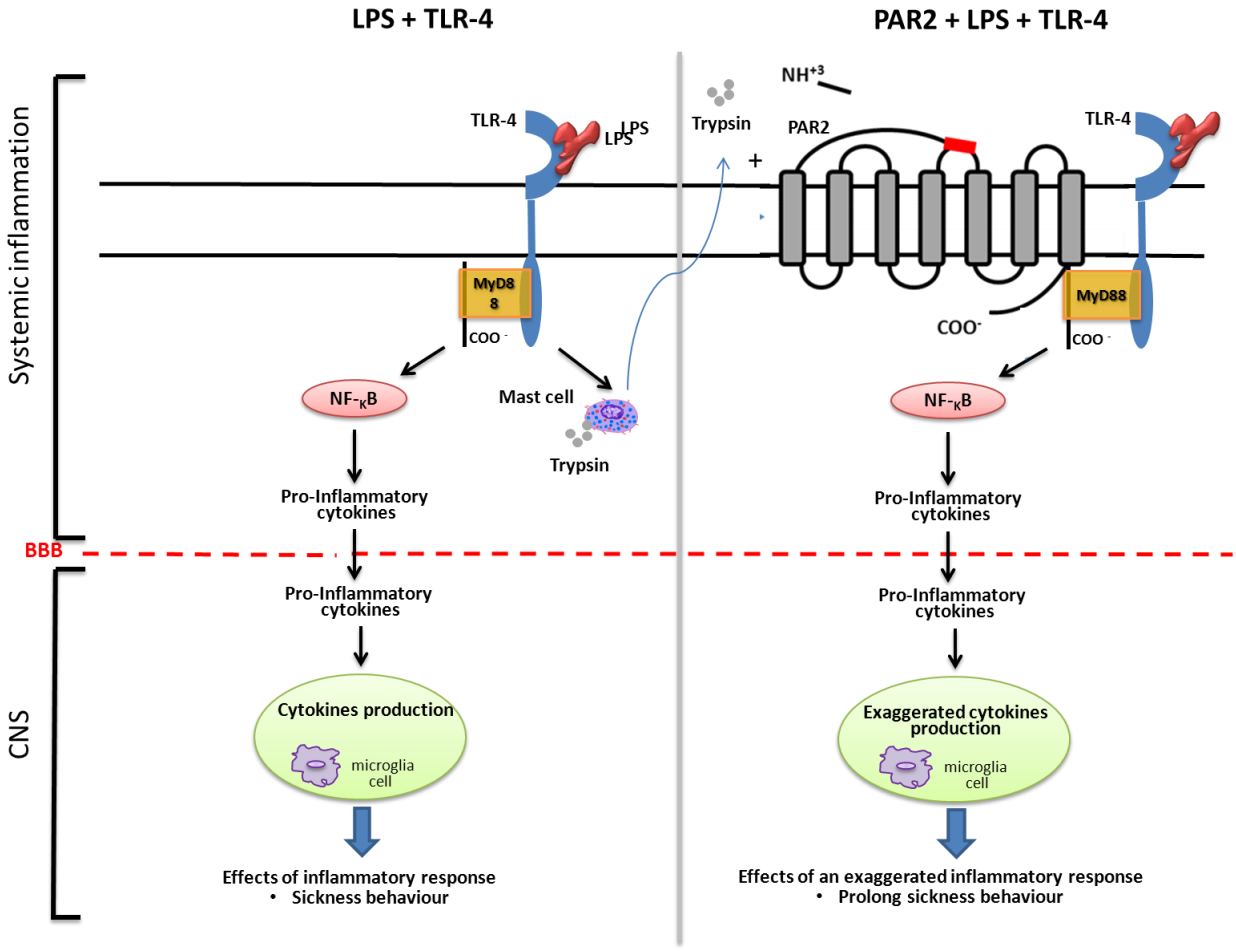


Figure 4.16: Crosstalk between PAR2 receptors and TLR-4. PAR2 receptors interact with protein activator MYD88 and then bind to TLR-4 and formation of PAR2-DY88-TLR-4 complex can cause activation of NF-$_K$B signalling pathways which stimulate release of extra cytokines.

Injecting mice with LPS causes activation of TLR-4 and release PICs (Dantzer, 2009). During inflammation, excessive release of trypsin in the body can cause activation of PAR2 and leads to crosstalk between PAR2 and TLR-4 receptors (Rallabhandi *et al.,* 2008). PAR2 receptors interact with the protein activator MYD88 and then bind to TLR-4 and the formation of PAR2-DY88-TLR-4 complex can cause activation of NF-$_K$B signalling pathways which stimulate release of cytokines (Dunne *et al.,* 2003). Formation of this complex may change trafficking and internalization mechanisms and may affect the induction of and recovery from sickness behaviour.

Under normal physiological conditions, there is a possibility of crosstalk between PAR2 and TLR-4 receptor. The cooperation between these receptors exaggerates production of cytokines in the CNS (Rallabhandi *et al*., 2008). Additionally, under inflammatory condition, LPS induced overexpression of PAR2 that also can cause exaggeration of production of CNS via the crosstalk between PAR2 and TLR-4 receptors (Bucci *et al.,* 2012). Moreover, during inflammation, the PAR2 activator trypsin releases from mast cell and pentrates into the CNS due to the damage of BBB. Trypsin activates PAR2 receptor and encourages the crosstalk between PAR2 and TLR-4, hence, it is possible that PAR2 is involved in LPS induced sickness behaviour.

4.8.3 Effect of deletion of PAR2 on locomotor activity

Decreased locomotor activity is a classic symptom of sickness behaviour (Dantzer, 2001b; Godbout *et al.,* 2005; Dantzer *et al.,* 2008; Dantzer, 2009; Maes *et al.,* 2012). One possibility for decreased locomotor activity is the reduction in the availability of body energy during inflammation as the body fights infection by using different strategies (Hart, 1988). One of these strategies is an elevation in set point temperature regulation in response to infection (Kluger, 1991). The increase in body temperature causes fever by increased heat production and decreased heat loss (Cranston, 1965). An increase in body temperature is required for energy and the amount of energy required is high. For example, in humans, increasing the body temperature by 1°C increases the body's metabolic rate 13% (Leonov, 1963). At the

same time, increasing body temperature stimulates proliferation of immune cells and it may help the body to prevent the growth of bacteria or viruses by release of cytokines (Shen *et al.,* 1994; Kelley *et al.,* 2003).

Additionally, an infection was mimicked in our model by injecting the mice with the bacterial endotoxin, LPS and in response to the LPS injection, PICs are produced (Burton *et al.,* 2011). In the periphery, PIC signalling modulates the balance between increased energy demand and supply, controls energy spending and food intake and substrate use (Ye and Keller, 2010; Steinberg *et al.,* 2009; Roubenoff *et al.,* 1994; Maes *et al.,* 2012). Increased energy demand is characterised by gluconeogenesis, increased lipolysis, lower muscle protein synthesis, loss of tissue protein and decrease in energy utilized (Prters, 2006; Mase *et al.,* 2012). However, in our sickness behaviour model a decrease in locomotor activity was not associated with decreased food intake. The CNS receives neuronal and humoral signals about the peripheral inflammatory response via PICs and activation of the afferent vagal nerves. These PICs, which include IL-1, IL-6 and TNF-α, penetrate the brain by different pathways and affect sensory nuclei of the solitary tract which leads to a reduction in energy consumption by shutting off the energy consuming processes, such as decreasing locomotor, neurocognitive and reproductive activity (Maes *et al.,* 2011; Peters, 2006). This metabolic energy is saved from the brain and peripheral organs to be used for helping the immune system to fight bacteria. Therefore, immune responses are highly calorie dependent associated with an increased energy demand and decreased energy expenditure, therefore sickness behaviour symptoms, for example motor inhibition, may save critical energy which can be used to fight the infection (Cunningham-Rundles *et al.,* 2005).

The marker for energy used in our study was locomotor activity in the OFT (total distance moved). The OFT is widely used in neuroscience laboratories for testing general locomotor activity and exploratory behaviour in addition to anxiety in rodents (Stanford , 2007; Barbosa *et al.,* 2008; Carey *et al.,* 2008; Siemiatkowski *et al.,* 2000). In the sickness behaviour model, a reduction in locomotor activity was observed from 2 to 8 h post LPS injection compared with locomotor activity 0 h post injection. This result suggests that sickness behaviour symptoms start to appear

between 2 h to 8 h post injection. However, locomotor activity returned back to normal levels 24 h and 48 h post injection indicating that the mice recover from sickness behaviour within this period. The activity of the mice was compared with 0 h because the baseline time measurements would involve habituation with the environment whereas the 0 h measurement represents the effects immediately following LPS injection.

During habituation to a novel place, the mice were exposed to OF box for 10 min before injection with LPS. The first trial is a habituation trial (baseline time) where the mice are exposed to a novel place before the test trial (0 h). In habituation, the mice move more distance exploring the novel environment of the OF box because of the neophobic reaction to the novel environment for these tested mice. During the second trial at 0 h, the mice moved less distance exploring the same environment (OF box) because these mice may have had an adaptive reaction to the first trial aversive experience (neophobia). Additionally, there were an increased number of entries into the centre square during the first trial (the habitation trial at base line time) compared with 0 h. This result is in support of previous results (Siemiatkowski *et al.,* 2000; Carey *et al.,* 2008) and suggests that there were a decrease the number of entries into the centre square of OFT after hibituation trial in rats.

It was explained in detail in section 4.8.2 that LPS is recognised by TLR-4 which are expressed in phagocytes. Binding of LPS with TLR-4 results in production of PICs and AICs. The induction and recovery from sickness behaviour depends on the release and penetration of PICs into the brain. In contrast, the time of recovery from these symptoms depends on the reduction of PIC levels and the recovery from their actions within the brain. The role of PICS has been established for many years with studies revealing that systemic and intracranial injection with PICs, for example IL-1β (Dinarello *et al.,* 1977), produces symptoms similar to sickness behaviour. In addition, peripheral and central administration of rats with IL-1 induces sickness behaviour and fever. Injecting the same rats with an IL-1 antagonist (IL-IRa) inhibits many of these central effects including sickness behaviour symptoms but not fever (Rothwell and Hopkins, 1995; Kent *et al.,* 1992). Another cytokine strongly linked to sickness behaviour is TNF-α (Feuerstein *et al.,* 1994; Palin *et al.,* 2007; Palin *et al.,*

2008). It was found that inflammatory response caused by injecting mice with LPS, TNF-α increased in the plasma within 2 h post injection and it returned back to normal level after 4-6 h post injection (Copeland *et al.,* 2005). Other studies have shown that in response to brain inflammation or ischaemia, TNF-α mRNA in the brain is produced rapidly within 1 h, reaching a peak at 6-12 h post brain ischaemia and returns to normal levels 1-2 days later (Feuerstein *et al.,* 1994).

The timing of the appearance and recovery from the symptoms associated with sickness behaviour in our findings are in agreement with those from previous studies and as such confirm that the symptoms of sickness behaviour involves the effects of cytokines in the brain. Accordingly, our findings agree with previous studies that show peripheral administration of LPS decreases locomotor activity (Nava *et al*., 1997, 1999). Additionally, other studies have shown that intracerebroventricular administration of IL-1β induces sickness behaviour as indicated by reduced locomotor activity (Song *et al.,* 2003) and that peripheral administration of LPS or IL-1β in mice significantly reduces locomotor activity in the OFT (Dunn *et al.,* 2005). These studies agree with our findings that LPS induces sickness behaviour and reduces locomotor activity.

Having established the sickness behaviour model, this was used to examine the effect of PAR2 deletion on sickness behaviour. The first parameter studied was locomotor activity. The result clearly shows no effect of PAR2 deletion on onset of appearance of sickness behaviour as represented by the reduction in locomotor activity from 0 h to 8 h post injection. At the same time, the result clearly shows an increased recovery as shown by the increase in locomotor activity of PAR2 KO mice 24 and 48 h post injection with LPS compared with WT mice. This result suggests a pro-inflammatory role of PAR2 in sickness behaviour.

4.8.4 Effect of deletion PAR2 on anxiety- like behaviour

The second marker measured was anxiety-like behaviour. Previous research confirms that systemic LPS administration induces central effects (Hopkins *et al*., 1995; Nava *et al.,* 2000; Yirmiya *et al.,* 1996) with the systemic administration of LPS (1 mg/kg) increasing serum level of cytokines (Nava *et al.,* 1997b). Several studies suggest that

the primary cerebral actions of LPS, including anxiety-like behaviour, may be mediated by an enhancement of cytokines both in the periphery and in the brain (Nava *et al.,* 1996; Nava *et al.,* 1997b; Hopkins *et al.,* 1995; Rothwell and Hopkins, 1995; Maes *et al.,* 2011). Administration of LPS increases cytokine levels and these may remain elevated for months and are associated with the onset of inflammation (Qin *et al.,* 2007; Maes *et al.,* 2012). However, an exaggerated and prolonged production of cytokines in the body can damage the body's tissues including the brain tissues, which allow the cytokines pass into the brain and it may cause behavioural deficits such as anxiety (Dantzer, 2004; Bassi *et al.,* 2012).

Anxiety-like behaviour was measured as the number of entries into the centre square and the time spent in the centre square. In the initial experiments, LPS injection did not significantly reduce the number of entries into the centre square or the time spent in the centre square at all time-points investigated, thus suggesting that under our conditions, the anxiety-like behaviour does not change significantly during sickness behaviour. The reason for that firstly may be the use of a small number of mice for this experiment (n=3) or secondly, that OFT is less sensitive to anxiety-like behaviour than other tests for anxiety-like behaviour, for example, EPM test (Prut, 2003).

Other study has shown that injection of adult male rats with different doses of LPS (0.025, 0.5 and 1 mg/kg), led to a dose dependent increase in anxiety-like behaviour as measured by increased numbers of entries and time spent in open arms of EPM (Nava and Carta, 2001). Furthermore, intracerebroventricular administration of IL-1β in rats induced a sickness behaviour response as indicated by anxiety-like behaviour in EPM 20 min post injection (Montkowski *et al.,* 1997). Administration of different doses of LPS (10, 20, 50 and 200 µg/kg) in Wister rats also induced sickness behaviour which was characterized by increased anxiety-like behaviour (Bassi *et al.,* 2012). This suggests that LPS induces anxiety-like behaviour. Examining of the effect of deletion of PAR2 on anxiety-like behaviour in the sickness behaviour model showed that similar to the results of locomotor activity, PAR2 deletion has no effect on the onset of appearance of anxiety-like behaviour but there was an improved recovery from the symptoms. As such, LPS injection did not show a significant

reduction in anxiety like behaviour measured by the number of entries into the centre square or the time spent in the centre square from 0h to 8 h. However, there was a significant recovery from anxiety-like behaviour 24 and 48 h post injection measured by an increase in the number of entries into the centre square by KO mice compared with WT mice, thus suggesting KO mice recover from LPS-induced anxiety-like behaviour faster than WT. Both results support the suggestion that PAR2 has a pro-inflammatory effect during the recovery from sickness behaviour. In addition, injection alone did not alter anxiety-like behaviour and therefore could not account for any observation in these experiments.

4.8.5 Effect of deletion of PAR2 on anhedonia

The third marker measured was anhedonia. Anhedonia is a reduction in the ability to experience pleasure and it is a symptom of many psychiatric disorders such as depression (Hamilton, 1967; Kessler et al., 1994; Juckel *et al.,* 2003). One of the animal models used to assess anhedonia in rodents is SPT (Katz, 1981). Some evidence comes from depressed human patients that increased level of cytokines causes depression (Irwin and Miller 2007). Depressed patients had elevated IL-6, IL-1ß and TNF-α and there is a positive correlation between the cytokine level and the severity of depression (Maes *et al.,* 1995; Maes, 1995; Dantzer *et al.,* 1999; Charlton, *2000).* Cytokines also induce changes in the metabolism of neurotransmitters including serotonin (Maes, 1995; Miller *et al.,* 2009). IL-1 can cause sickness behaviour (Kent *et al.,* 1992) and it is also involved in depression (Maes *et al.,* 1995; Maes, 1995; Charlton, 2000). It is thought that IL-1 can induce production of other cytokines during inflammation which causes sickness behaviour (Dinarello *et al.,* 1988). Several lines of evidence have shown that there is increase in the level of IL-1 in brain of patients suffering from depression (Dantzer *et al.,* 1999*;* Dunn *et al.,* 2005).

At a behavioural level, administration of IL-1β or LPS (i.p.) to mice can cause a deficit in depression-like behaviour test as measured by increased time spent immobile in the tail suspension test and in the time spent floating in the forced swim test. This result suggests that both LPS and IL-1β induce depression-like symptoms (Dunn *et al.,* 2005). Moreover, administration of cytokines to non-psychiatric

patients induces the development of depression in most patients (Meyers, 1999). Patients treated with IL-2 or interferons (INF-α) become clinically depressed after treatment (Capuron and Ravaud, 1999) and a number of studies provide evidence that cytokines play a role in animal models of depression with cytokines being found to induce anhedonia in rats (Anisman and Merali, 1999).

It has been suggested that cytokines may play a role in both inflammation and depression (Dantzer, 2001b). Cytokines produce fatigue and neurasthenia that are noticed in patients suffering from viral infection. Fatigue symptoms such as lack of energy and loss of interest occur very frequently in depressed patients. Depressed patients also suffer from a lowering of mood, reduction of energy and decreased activity, impaired ability to enjoy pleasurable activities, impaired interest and concentration (Marvel and Paradiso, 2004).

The SPT is commonly used to assess hedonic state in rodents (Katz, 1981) and it is measured as a decrease in the amount of sucrose solution drunk. In this study, this test was used to determine the effect of PAR2 deletion in the sickness behaviour model. The index for anhedonia is a reduction in preference for a sucrose solution over that of plain water. Our data indicates that at 2 h post LPS injection, the sucrose preference is maintained in PAR2 KO mice whereas it is reduced in both WT and HT mice. This result suggests that PAR2 deletion delays the onset of anhedonia following LPS injection. It is possible that hedonic state is more sensitive to infection-like conditions/cytokine increases compared with other markers we measured such as locomotor activity and anxiety. Both locomotor activity and anxiety showed no change in onset of induction sickness behaviour but improved recovery from sickness behaviour. This result suggests that PAR2 is involved partially in the delay of the induction of sickness behaviour and recovery from sickness behaviour. Also this result confirms that deletion of PAR2 may have pro-inflammatory properties leading to sickness behaviour.

4.8.6 Effect of PAR2 on food intake

The fourth marker measured was food intake. It was introduced earlier that the body has different strategies to fight infection; one of these strategies is reduced food

intake. However, prolonged reduction of food intake and increased metabolic rate lead to reduction of body fat and protein reserves (Konsman and Dantzer, 2001). The reduction in food intake leads to reduced plasma levels of iron and zinc and decreases the availability of these minerals for the growth and proliferation of bacteria and viruses (Kluger, 1986; MacDonald, 2000; Kelley *et al.,* 2003). Therefore, reduced food intake inhibits bacterial and viral growth. Since the CNS controls food intake, cytokines secreted by the innate immune system may act on the brain to reduce food intake during inflammation (Buchanan and Johnson, 2007). The vagus nerve, which is involved in the control of food intake and body weight, is a quick route for communication from the immune system to the brain (Pavlov *et al.,* 2003; Sobocki *et al.,* 2005). Cytokines are released in response to peripheral administration of LPS, cytokines penetrate the brain and act on the neurons in the hypothalamus, which regulates food intake and as a result, causes a reduction in food intake (Dantzer *et al.,* 2008).

Similar to inflammation, sickness behaviour is mediated also by release of cytokines which produce a reduction of food intake (Dantzer *et al.,* 2008; Tizared, 2008; Maes *et al.,* 2012). Therefore, food intake was also measured as a marker for sickness behaviour. Similar to the locomotor activity, the amount of food intake in our initial experiments was reduced 24 h post LPS injection; however, the amount of food eaten by the mice returned back to normal levels 48 h post injection. However, the changes in food intake was non significant p=0.063, but close to be significant as small numbers of mice were used (see section 4.5.4). This result suggests that similar to the change in locomotor activity, the mice may start to recover from sickness behaviour 48 h post injection. This result is consistent with other findings that LPS-induced reduction in food intake after daily injection of 100 or 400 µg for 10 days to male and females chicks led to decreased food intake and body weight (Webel, *et al.,* 1998). Recently, injecting the mice with LPS (100 µg/ kg) produced a reduction of food intake and body weight a day after injection compared to control mice (Lawrence *et al.,* 2012). All C57/BL6 mice injected with 0.33 mg/kg LPS showed reduced food intake and body weight compared to mice injected with vehicle of LPS after 24, 48, 96 h post injection (Martin *et al.,* 2012).

The relation between the deletion of PAR2 and reduced food intake due to sickness behaviour was studied in our laboratory. The results show an increased amount of food intake for HT mice compared with WT 48 h post injection. These results suggest that HT mice were quick recovery from sickness behaviour. Additionally, KO mice also displayed non significant increase in the amount of food intake 48 h post injection.

4.8.7 Effect of deletion of PAR2 on body weight

The fifth marker measured was body weight**.** A reduction in body weight is associated with inflammation as a result of the reduction of food intake (Lennie, 1998). As a result of reduction in and food intake can lead to a lean body mass, increased protein catabolism, loss of body protein and loss of fat mass these will all lead to a loss in body weight (Maes *et al*., 2012). Similar to the reduction of food intake, sickness behaviour is associated with a reduction in body weight (Tizared, 2008; Dantzer *et al.*, 2008; Dantzer, 2012; Mase *et al*., 2012). Therefore, the body weight was also measured as a marker of sickness behaviour. Similar to locomotor activity and food intake, % change in body weight was significantly reduced 24 h post injection and the mice returned back to normal 48 h post injection. This result in consisted with other findings that reduction in food intake cause weight loss.

The relation between deletion of PAR2 and the change in body weight due to sickness behaviour was also studied. The result has shown KO mice were increased body weight 48 and 72 h post injection compared with WT mice. As explained before both reduced locomotor activity and amount of food intake cause decrease body weight. Deletion of PAR2 might improve locomotor activity and increase amount of food intake, which then lead to an improvement in body weight.

4.8.8 Effect of deletion of PAR2 on fluid intake

The last marker measured was fluid intake**.** In humans, during inflammation, body temperature increases while the body energy reduces (Van *et al.,* 1996). To reduce internal body temperature the body increases sweating during inflammation and thus, increased sweating leads to the loss of salt, water and water-soluble vitamins (Diaz *et*

al., 2010). Therefore, the amount of fluid intake is increased to replace the water loss during sweating (Maughan, 1991). The body energy and minerals are reduced and unavailable for bacteria to grow and survive (Rodriguez, 2011).

However, in rodents, the amount of water intake is decreased during sickness behaviour (Chance and Fischer 1991; Kent *et al.,* 1992). However, the decrease in fluid intake was non significant p=0.088, but close to significant because small numbers of mice were used (see section 4.5.4). Several studies have found that LPS induces an inhibition of water intake (Nava *et al.,* 1996; Nava *et al.,* 1997b ; Nava and Caputi, 1999). Injection of LPS (1.6 mg/kg, i.p.) in rats produces a reduction in the amount of water intake and it is associated with reducing the amount of food intake (Langhans *et al.,* 1990). Mice with acute inflammation have decreased locomotor activity, food intake, water intake and body weight because of the change in metabolic rate during inflammation (Melgar *et al.,* 2007).

Other studies have shown that injecting rats with different doses of LPS (0.25, 0.50 and 1 mg/kg, i.p.) induces an inhibition of water intake, food intake and reduced locmotor activity as well increased levels of anxiety-like behaviour (Nava and Caputi, 1999). All LPS effects disappeared on intracerebroventricular injection of the rats with cromoglycate sodium salt which is the mast cell stabilizer, before injection with LPS. This result suggests that peripheral administration of LPS may activate brain mast cells and indicates association of these cells in brain pathophysiology (Nava and Caputi, 1999). Further studies have shown that LPS-induced release of PICs, prostaglandins and nitric oxide play an important role in inhibition of water intake and food intake as well as fever induced by LPS (Calapai *et al.,* 1992; Calapai *et al.,*1994; Nava *et al.,* 1996; Nava *et al.,* 1997b; Nava and Caputi, 1999). The central effect was seen 3 h post treatment with LPS while the increase in cytokines and prostaglandins levels appeared 1 h after treatment and continued for up to 3 h (Nava *et al.,* 1996; Nava *et al.,* 1997b; Nava and Caputi, 1999). Injection of LPS induced inhibition of water intake 3 h after treatment with LPS (0.25. 0.5 and 1 mg/kg) in a dose-dependent manner in rats (Nava and Carta, 2000). Inhibition of water intake may be due to the central effect of neurotransmitter nitric oxide in the brain and its role in the brain is still controversial (Nava *et al.,* 1996; Nava *et al.,*

1997b; Galapai *et al.,* 1992; Galapai *et al.,* 1994). Another study also suggests that nitric oxide may be involved in increasing body temperature after LPS injection that leads to decreased fluid intake (Almeida *et al.,* 1999). In our experiment, the results have shown that injecting male mice with LPS (1mg/kg, i.p.) did not change the amount of fluid intake 24 and 48 h post injection. The result suggests that the mice appear to drink normal amounts of water 24 h and 48 h post injection with LPS. This result disagrees with other studies that show that LPS (i.p.) injection induces a reduction in fluid intake in rats (Nava *et al.,* 1996; Nava *et al*., 1997b).

The relation between deletion of PAR2 and the amount of fluid intake post injection with LPS was also studied. After the baseline of SPT (day 7) and prior injection the mice with LPS, HT mice drank more fluid compared with KO. 2 h post injection with LPS, KO mice drunk more fluid compared with WT and HT. Our result has shown that deletion of PAR2 increases the total amount of fluid intake (water and sucrose solution) 2 h post injection with LPS. This increasing in fluid intake was because of increase in the amount of sucrose drunk. This suggests that deletion of PAR2 delay the onset of appearance of sickness behaviour symptoms where previous studies showed that LPS injection can cause a decrease in the amount of sweetened fluid intake in the form of chocolate solution, chocolate milk and saccharin that can reduce the amount of fluid intake (Larson and Adrian *et al.,* 2001*;* Anisman *et al.,* 1998).

I explained in section 4.8.2 that crosstalk between PAR2 and TLR-4 leads to formation of the PAR2-DY88-TLR4 complex which can stimulate release of cytokines. Formation of this complex may change trafficking and internalization mechanisms. Therefore, further studies are required to show the trafficking mechanisms involved in PAR2 and TLR-4 receptor interaction (figure 4.16). An explanation for our result may be that PAR2 acts as a pro-inflammatory mediator and induces the release of PICs during the latter stages of the sickness behaviour period. Therefore, removal of the receptor may account partially for quicker reversibility whereas less effect is seen on the induction.

As stated previously, there is a strong link between PAR2 and inflammation. It is well established that PAR2 is expressed in epithelial and endothelial cells throughout the body, which suggests that PAR2 may be involved in defence of cell barrier (Cocks and Moffatt, 2001). Indeed, PAR2 are located in plasma membrane of these cell types which allows the active detection of circulating trypsin (Cocks and Moffatt, 2000) with their activation potentially leading to prostaglandin (Cocks *et al*., 1999; Chan *et al*., 2004), and NO release (Cocks *et al*., 1999; Vergnolle *et al.*, 2001). These released substances induce several effects when activated during inflammation such as increased blood vesicle permeability and odema; however, whether PAR2 activation under these conditions is either pro-inflammatory or anti-inflammatory remains unclear.

Some studies have shown that PAR2 function during central and peripheral inflammation is pro-inflammatory whilst other studies have shown that its function is anti-inflammatory (Seeliger *et al.,* 2003; Bushell, 2007; Ishikawa *et al.,* 2009; Kelso *et al.,* 2007b; Chen *et al.,* 2011). In this study, the induction and recovery from the symptoms associated with sickness behaviour is an index for the central role of PAR2. If the central role of PAR2 is pro-inflammatory, then it would be expected to contribute to the appearance of symptoms and a slow recovery from these symptoms would be expected. In contrast, if the central role of PAR2 is anti-inflammatory, then PAR2 may delay the appearance of sickness behaviour symptoms and increase the recovery from these symptoms. As locomotor activity is increased in KO mice 24 and 48 h post injection, this result suggests PAR2 deletion hastened the recovery from sickness behaviour but did not delay the onset of these symptoms. This result partially supports the idea that PAR2's role is pro-inflammatory. Many studies have supported a pro-inflammatory role for PAR2. As such, PAR2 are upregulated in many inflammatory diseases including multiple sclerosis, autoimmune encephalomyelitis, Alzheimer's Disease, asthma, chronic obstructive pulmonary disease (Noorbakhsh et al,. 2006; Afkhami-Goli et al,. 2007; Kirkup et al,. 2003; Gatti et al., 2006). Additionally, PAR2 promote the rolling, adhesion and extravasation of leucocytes (Vergnolle, 1999) and induce the secretion of numerous inflammatory mediators, for example; prostaglandin 2, TNF-α, IL-6,1L-12, IL-1β (Dube et al.,2005; Luo et al., 2007), stimulating eosinophil infiltration (Schmidlin et

al. 2002), granulocyte influx and sensory nerve dependent oedema (Vergnolle et al., 1999; Vergnolle, 1999; Macfarlane et al., 2001; Steinhoff et al., 2005). Furthermore, peripheral administration of the PAR2 agonist SLIGRL-NH2 to the rat induces cough, allergic reaction and hyperlgesia via ctivation of sensory neurons (Vergnolle 1999; Vergnolle *et al*., 2001; Amadesi *et al.,* 2004; Bunnett, 2006; Vergnolle, 2005; Gatti, *et al*., 2006; Alier *et al.,* 2008; Amadesi *et al.,* 2006). These studies suggest that PAR2 promotes inflammation and it is widely believed that in many cases PAR2 is pro-inflammatory and can worsen chronic inflammatory diseases (Noorbakhsh *et al.,* 2006; Hyun *et al.,* 2008; Cirino *et al.,* 2000). Our data would in part support these findings but it should be noted that under other conditions, PAR2, as outlined earlier, is proposed to have anti-inflammatory actions. Therefore, as PAR2 deletion only affects recovery from and delay the induction of sickness behaviour, this then may indicate that PAR2 plays a role in the sustained release of cytokines but does not contribute in the initial stages of sickness behaviour. However, it is unknown what the consequence of PAR2 activation is on cytokine levels within the CNS and this requires further investigation.

A control experiment was conducted to identify the effect of injection *per se* on locomotor activity. The result clearly shows no effect of vehicle injection (phosphate buffered saline) on locomotor activity, anxiety-like behaviour, food intake and fluid intake, body weight at all time-points post injection compared with un-injected mice. Therefore, the result suggests that injection alone does not affect the onset of and recovery from these sickness behaviour symptoms.

In summary, the pilot study of sickness behaviour suggests that symptoms of sickness behaviour appear between 2h and 8 h post injection and recovery occurs between 24 and 48 h. However, the deletion of PAR2 receptors has little effect on the induction of sickness behaviour and their effect is based more on recovery from sickness behaviour.

Chapter 5

The effects of PAR2 agonists on mouse behaviour

5.1 Introduction

From investigation of the consequence of PAR2 deletion on mouse behaviour (chapter 3 and 4), it can be concluded that PAR2 contributes minimally to mouse behaviour under normal physiological conditions but is involved in sickness behaviour when investigated in mouse models. To further understand the observed effects, it was necessary to examine mouse behaviour following PAR2 activation using PAR2 agonists. Many previous studies performed to examine the role of PAR2 in CNS have used the peptide agonist, SLIGRL-NH_2 (Maryanoff *et al.*, 2001). For example, PAR2 activation in rat primary hippocampal cultures by both trypsin and SLIGRL-NH_2 induced transient increases in intracellular Ca^{2+} in both neurons and astrocytes through the $G_{\alpha q/11}$ and PLC signalling pathway (Smith-Swintosky *et al*., 1997; Steinhoff *et al*., 2005; Noorbakish *et al.*, 2005; Bushell *et al.*, 2006). A further investigation showed that activation of PAR2 by SLIGRL-NH_2 in both primary cultures and rat hippocampal slices leads to indirect inhibition of neuronal excitability and synaptic transmission (Gan *et al.*, 2011). These *in vitro* results suggest that PAR2 activation may modulate mouse behaviour as well as indicating that it might become target for treatment of neurological disorders.

In addition to the *in vitro* effects of PAR2 activation, the effect of the PAR2 agonist SLIGRL-NH_2 has been studied *in vivo* (Lohman *et al*., 2009). This study showed that SLIGRL-NH_2 was detected in Wistar rat brain 10 min following subcutaneous administration of 1.5 mg/kg, with a half-life of 25 min, which suggests that SLIGRL-NH_2 crosses the BBB under these conditions. This finding supported the previous findings of Lohman *et al* (2008) that SLIGRL-NH_2 has centrally mediated effects when injected directly into the cerebrospinal fluid (0.15 mg/kg, intracerebroventricular) of the Wistar rat. Furthermore, SLIGRL-NH_2 was shown to induce behavioural changes in a dose-dependent manner: in high doses SLIGRL-NH_2 induced catatonia and seizure. In the present study, two doses of SLIGRL-NH_2 (1.5 mg/kg and 5 mg/kg, i.p.) were examined. The choice of first dose, 1.5 mg/kg, was based on previous study in rat (Lohman *et al.*, 2009) whereas the higher dose (5 mg/kg, i.p.) was chosen in order to investigate the central effects of peripherally

administered SLIGRL-NH_2 in higher doses, which has never been studied before *in vivo*.

The majority of previous investigations of effects of peripheral administration of SLIGRL-NH_2 on behaviour have been performed only on rats. Surprisingly, few studies have examined central effects and doses of PAR2 agonist in mice. Thus, there is a need to investigate this approach in other rodents, for example, the C57BL/6 mouse. So, the aim of present experiments in this chapter was to investigate the role of PAR2 agonists, SLIGRL-NH_2, AC-264613 and GB110, in various mouse behaviours: locomotor activity, anxiety-like behaviour and anhedonia. The locomotor activity was tested by using OFT, anxiety-like behaviour was tested by using both OF and plus maze tests, whereas anhedonia was measured using the SPT. Further investigations were carried out to establish whether PAR2 agonists (SLIGRL-NH_2, GB110 and AC-264613) entered the brain of the mice or not.

Most PAR2 researchers have made efforts to define physiological and pathophysiological roles for this receptor using genetically modified animals, PAR2 agonists and antagonists as these become available. The known PAR2- AP for mouse is SLIGRL-NH_2 but its use in research is limited especially in *in vivo* studies because it has low potency when administered in low concentration. Therefore, it should be administratered in high concentration to produce required responses (Grand *et al.,* 1996). Additionally, SLIGRL-NH_2 is selective to PAR2 over PAR1 but only at high concentration, therefore, it is not selective and can interact with other receptors (Vergnolle *et al.,* 2001; Abey *et al.,* 2006). Furthermore, SLIGRL-NH_2 is also poorly bioavailable because it is susceptible to proteolytic degradation which limits its use in *in vivo* study. Because of these disadvantages of the peptide PAR2 agonist (SLIGRL-NH_2) novel non-peptide PAR2 agonists have been developed, as discussed in a previous section 1.2.14.

Based on SLIGRL-NH_2 the novel PAR2 agonists AC-264613 and GB110 have been developed. According to previous studies, the novel PAR2 agonists are more selective, potent and metabolically stable. Therefore, it may be suitable for *in vivo*

studies in order to further understanding of the physiology and pathophysiology of PAR2 receptor.

Recently, it has been reported that GB110 mobilizes intracellular Ca^{2+} in six human cell lines, including adenocarcinoma of human alveolar basal epithelial cells (A549), human pancreatic carcinoma, epithelial-like **(**Panc-1) and it behaves similarly to trypsin and 2f-LIGRLO-NH_2,which is the most potent known PAR2 agonist (Suen *et al.*, 2012). GB110 is selective to PAR2 over PAR1, as evaluated by a desensitization assay. The desensitization assay for PAR2 was assessed by addition of 2f-LIGRLO-NH_2 to the human colorectal carcinoma cells (HT29) which leads to generation of Ca^{2+}. Then, 2f-LIGRLO-NH_2 was re-added to the same cells. The second addition of 2f-LIGRLO-NH_2 failed to generate Ca^{2+} which indicated the cell was desensitized to the PAR2 activation. So, assessment of GB110 selectivity to cells desensitization to PAR2 activation has shown that GB110 has no effect on the intracellular Ca^{2+} release in these cells (Barry *et al.*, 2010). This result indicts that GB110 is highly selective to PAR2. Additionally, potency of activation of PAR2 with GB110 was assessed using the mediated intracellular Ca^{2+} release method; it has shown that GB110 is more potent than 2f-LIGRLO-NH_2 in activation of PAR2 in HT29 cells. So this result suggests GB110 is a potent PAR2 agonist (Kawabata *et al.,* 1999; Barry *et al.*, 2010).

Comparing the potency of GB110 with SLIGRL-NH_2 and trypsin, it is 10 times more potent than SLIGRL-NH_2 but 35 times less potent than trypsin. This improvement of potency in GB110 compared to 2f-LIGRLO-NH_2 may be due to a difference in internalization potency of PAR2 receptor after activation with PAR2 agonist. The internalization potency of GB110 is less than 2f-LIGRLO-NH_2. This is because 2f-LIGRLO-NH_2 stabilizes the receptor more than GB110 when it is coupled with β-arrestin (Suen *et al.,* 2012). Furthermore, GB110 is serum stable as evaluated by measuring the resistance to the proteolysis compared to SLIGRL-NH_2. In an HPLC assay, it was shown that over 95% of GB110 was detected in fetal calf serum after 4 h compared with 57% of SLIGRL-NH_2. This result suggests GB110 is serum stable and it is suitable for studying in experimental pharmacology with animal models (Barry *et al.*, 2010).

The other novel PAR2 agonist is the small molecule AC-264613 with a molecular weight smaller than other PAR2 agonists (figure 1.32). It has been shown to stimulate phosphatidylinositol hydrolysis, mobilization of intracellular Ca^{2+} and cellular proliferation in cell cultures (Gardell *et al.,* 2008). AC-264613 is highly potent at PAR2 receptors and its potency is 30-300 times more than SLIGRL-NH_2 but it has a similar potency to 2f-LIGRLO-NH_2 (Gardell *et al*., 2008; Seitzberg *et al.,* 2008). Additionally, AC-264613 is highly selective and it is has no activity towards other PARs or at other receptors, for example, the receptors implicated in nociception and inflammation (Seitzberg *et al.,* 2008). Moreover, AC-264613 is well absorbed and metabolically stable to liver microsomes when administered i.p. to rats. Its elimination half-life is 2.5 h (Gardell *et al.,* 2008). Therefore, AC-264613 is suitable for *vivo* study (Seitzberg *et al.,* 2008).

5.1.1 Aim

Therefore, the first aim of this study was to determine which PAR2 agonists (SLIGRL-NH_2, AC-264613 and GB110) are suitable for *in vivo* study by examining penetration of these agonists into mouse brain using LC-MS. The second aim was to determine the effect of these PAR2 agonists in mouse on locomotor activity, anxiety-like behaviour and anhedonia.

5.2 Effects of SLIGRL-NH_2 on locomotor activity, anxiety-like behaviour and anhedonia in mice

5.2 .1 Animals

The effect of SLIGRL-NH_2 was tested in 23 WT adult male C57BL/6 mice (weight 26.1 ± 0.4g , 12 weeks old) obtained from Strathclyde colonies and they were housed in the BPU at Strathclyde University as explained previously in section 2.1. Mice were housed (8 per cage) in MB1 cages during OF and EPM testing and transferred to individual housing in the same size cage prior to the SPT and provided with environmental enrichment as described earlier in chapter 2, section 2.4.3.

5.2.2 Behavioural test protocols

The design of behavioural experiments in this study was based on which tests are chosen in chapter 3 and 4. Thus, the OF experiments were performed initially, and were then followed with the EPM test and finally the SPT.

OFT protocol

On day 1, the day before the experiment, the mice were handled to reduce the stress by the experimenter during the test as described earlier in chapter 2, section 2.4.1 (figure 5.1). On the second day, mice were injected with 0.9% saline and after 20 min habituated to the OF box for 10 min to reduce the stress from a new environment as explained previously in chapter 2, section 2.4.1 (figure 5.1). After 24 h (day three), the 23 mice were randomly allocated to 3 groups: control (0.9% saline i.p, *n*=7); SLIGRL-NH_2 1.5 mg/kg i.p. (*n*=8); SLIGRL-NH_2 5mg/kg i.p (*n*=8). Following i.p. injection, the mice were returned to the home cage for 20 min. After 20 min, the mice were tested for 10 min in an OF box to investigate the effect of SLIGRL-NH_2 on general locomotor activity and anxiety-like behaviours (figure 5.1). The OF apparatus, its experimental procedures and conditions as well as the parameters measured were identical to those in section 2.2.

EPM test protocol

Mice were tested in the EPM on the fourth day to determine the effect of SLIGRL-NH_2 on anxiety-like behaviours. Groups and treatment were as for the OFT. The

session consisted of a period of 10 min when each mouse was placed in the centre of the maze and was allowed to explore the maze freely. The EPM apparatus, experimental procedures and conditions and the parameters measured were as explained in section 2.3.

SPT protocol

On the fifth and sixth days, the SPT was initiated to determine the hedonic state of the mice as mentioned in section 2.6. Firstly, the mice were single housed in individual MB1 cages and provided with environmental enrichment in the form of plastic huts and nesting material (section 2.1.1**)**. Two bottles of water were provided in order to determine the preferred bottle position. On day 7, one of the water bottles was replaced with a 1% sucrose bottle in the non-preferred bottle position. The amount of water and sucrose drunk were measured over days 7-10, with the placement of the sucrose bottle being randomised during days 8-10. During the testing days, the water and sucrose solution levels were measured daily at the same time (10:00 am, see Figure 5.1). The effect of SLIGRL-NH_2 on hedonic state of the mice was tested on day 11 and 12. On day 11, drug or vehicle injection was repeated as before and the consumption of sucrose solution and water was measured 24 h later. Finally, on day 12, the mice were injected with SLIGRL-NH_2 or vehicle as before and the consumption of sucrose solution and water was measured again after 2 h.

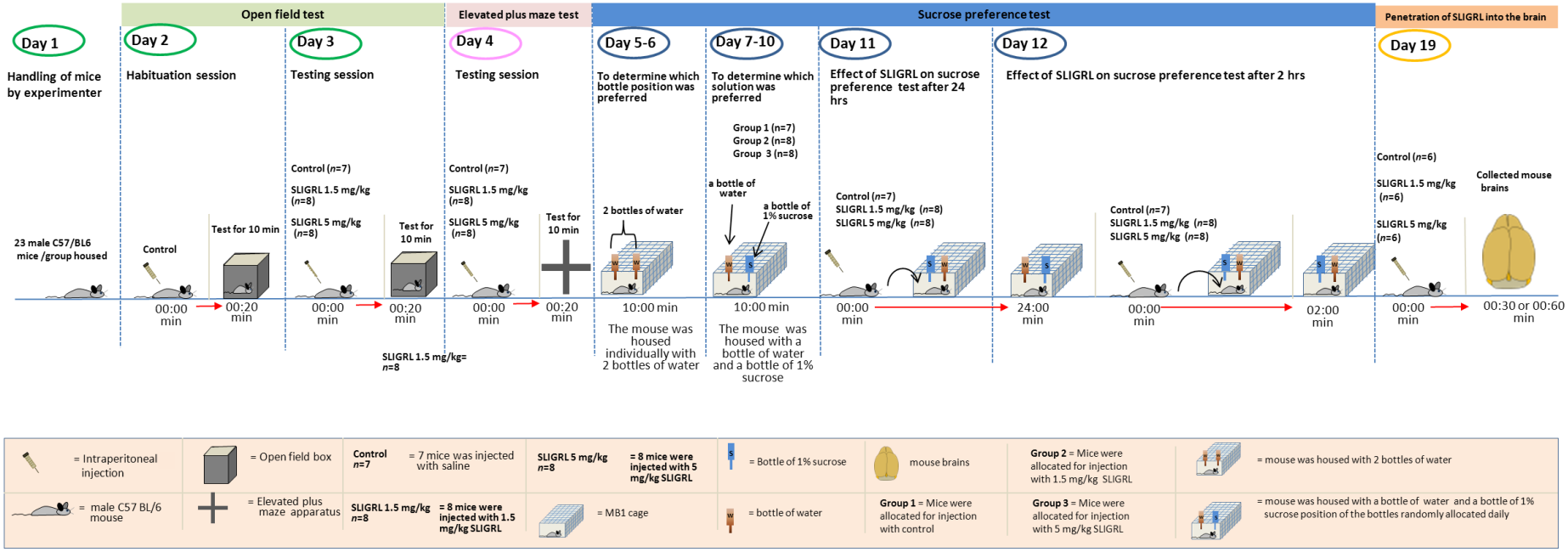


Figure 5.1: Experimental protocol for the effect of peripheral administration of SLIGRL-NH_2 on OFT, EPM test and SPT.

5.2.3 Statistical analysis

All values shown are mean ± standard error of the mean (*S.E.M.)*. The level of significance was set at $p < 0.05$ for all tests. The data and the statistics were analysed by using Prism software for one-way ANOVA and SPSS software for two-way ANOVA.

The data OF and EPM tests were analysed using one-way ANOVA followed by a Tukey test to compare between the 3 treatment groups. For the SPT baseline measures, a two-way ANOVA followed by a Tukey test was used to investigate the effect of the allocated treatment groups and of the day, while for the SPT drug day measures, one-way ANOVA was followed by a Tukey test to compare among the means of 3 treatment groups (control, dose 1, dose 2). The amount of AC-264613 pentrate into mouse brain was compared between the groups after two time intervals using a two-way ANOVA. Then it was followed by one way ANOVA Tukey test in order to compare the means of the 3 treatment groups in one interval times. In repeated measures two-way ANOVA the Mauchly's test of sphericity was used to assess homogeneity of variance in between subjects, if the *p* value of Mauchly's test was significant ($p<0.05$) the Greenhouse-Geisser test was used to calculate the adjusted *df* of F value which is called *df*-distribution instead of *df*-error (Paul and Gray, 2001). Therefore, in some tests the *df*-error was used instead of F value when the *p* value of Mauchly's test was significant ($p<0.05$).

5.2.4 Results

5.2.4.1 Effect of SLIGRL-NH_2 in the OFT

20 min after injection of SLIGRL-NH_2 (i.p.), no effect of the drug was observed in the 10 min OFT at any of the doses used. No significant differences were observed between groups in total distance moved (cm) over the 10 min test period ($F_{(2,19)}$ = 0.02; *p*= 0.976, figure 5.2A), and numbers of entries into the centre square of the OF box ($F_{(2,19)}$ = 0.33; *p*= 0.724, figure 5.2 B), time spent (s) in centre square of the arena, ($F_{(2,19)}$ =0.03; *p*=0.973, figure 5.2 C).

5.2.4.2 Effect of SLIGRL-NH_2 in the EPM

Similar to the effect of SLIGRL-NH_2 in the OFT, there was no effect of SLIGRL-NH_2 in the EPM test (figure 5.3). The 3 groups of mice spent a similar % time in the open arms of EPM ($F_{(2,20)}$ =0.80; *p*=0.463, figure 5.3A) and made a similar percentage of entries into open arms of the maze ($F_{(2,20)}$ = 0.06; *p*= 0.938, figure 5.3B) during the 10 min test session. In addition, there was no significant difference between the 3 groups in total number of arm entries ($F_{(2,20)}$ = 0.92; *p*= 0.414, figure 5.3 C).

5.2.4.3 SPT

Baseline sucrose preference

No significant effect of % sucrose drunk was observed over 3 consecutive testing days or between allocated groups (days: $F_{(1.319,40)}$ = 2.503, *p*= 0.118, allocated groups: $F_{(2,20)}$ = 0.81, *p*= 0.460; figure 5.4 A) as well as there being no interaction between days and allocated groups ($F_{(4,40)}$ = 1.00, *p*= 0.419, figure 5.4 A). All mice drank more than 50% sucrose solution. This result suggests that mice did not show anhedonia during the baseline period (before injection with SLIGRL-NH_2), figure 5.4 A.

In addition, there was no significant effect of total volume drunk (water plus sucrose solution) measured in g over the 3 consecutive testing days and between allocated groups (days: $F_{(2,40)}$ = 1.20, *p*= 0.343; allocated groups: $F_{(2,20)}$ = 1.07 *p*= 0.364;

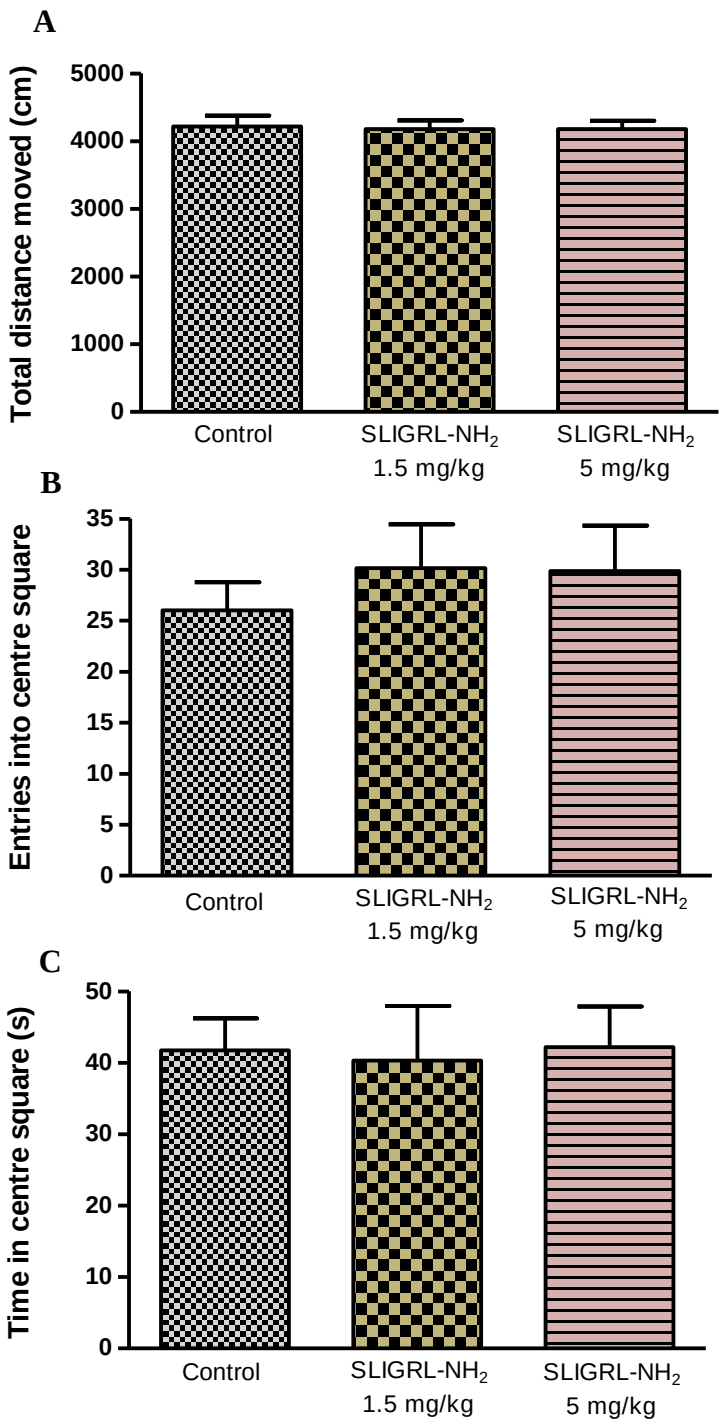


Figure 5.2: SLIGRL-NH_2 has no effect on the OFT parameters in male C57BL/6 mice. (A) No significant differences between groups (p> 0.05) in total distance moved during 10 min trial, (B) No significant differences between groups (p> 0.05) in entries into centre square of the arena during the 10 min trial, (C) No significant differences between groups (p> 0.05) in time in centre square of the arena over the test period. Data are presented as mean ± *SEM.* The data were analysed using one-way ANOVA with Tukey's post hoc test between groups where appropriate. Control n=7; SLIGRL-NH_2 (1.5 mg/kg) n=8; SLIGRL-NH_2 (5 mg/kg) n=8.

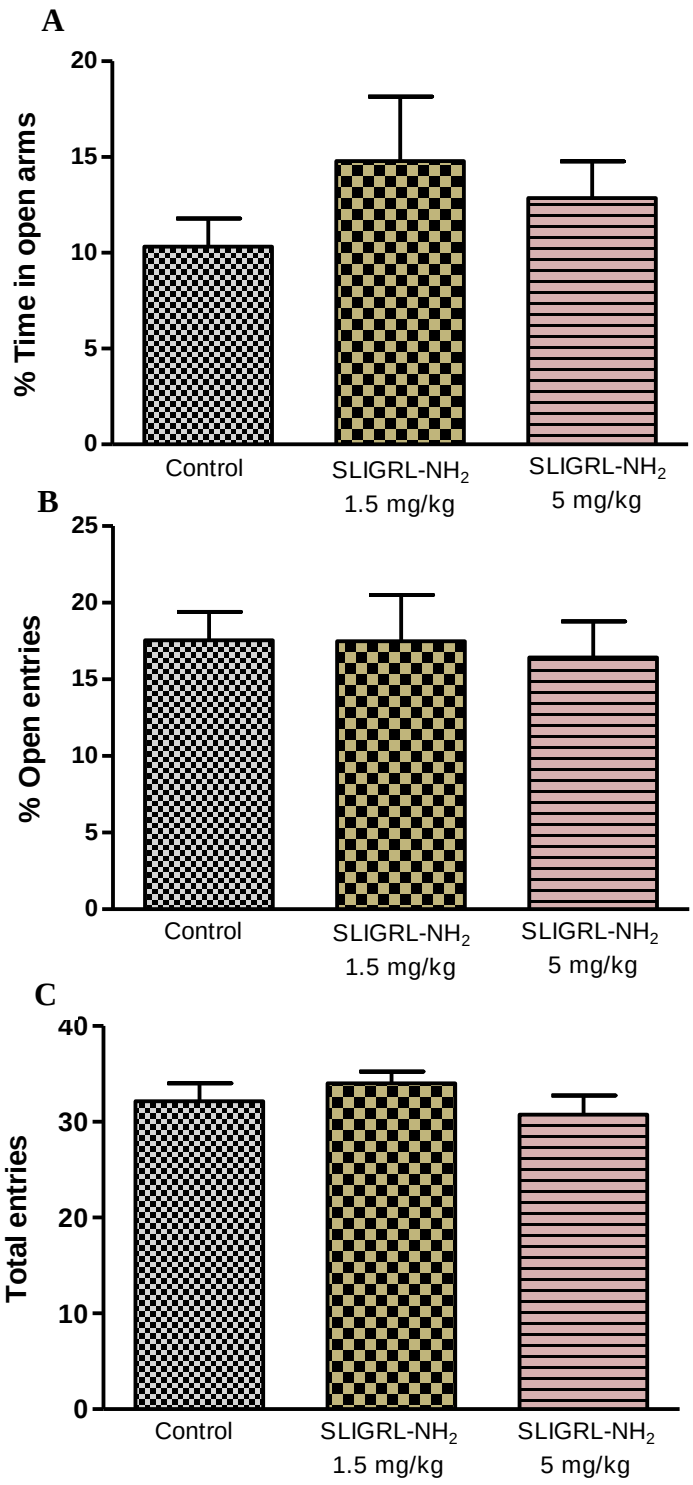


Figure 5.3: SLIGRL-NH_2 does not affect the parameters measured in the EPM test in male C57BL/6 mice. (A) No significant differences between groups (p> 0.05) in percentage of the time spent on the open arms of the EPM, (B) No significant differences between groups (p> 0.05) in percentage of the number of entries into the open arms of EPM, and (C) No significant differences between groups (p> 0.05) in total number of arm entries. Data are presented as mean ± *SEM*. The data were analysed using one-way ANOVA with Tukey's post hoc test between groups where appropriate. Control n=7; SLIGRL-NH_2 (1.5 mg/kg) n=8; SLIGRL-NH_2 (5 mg/kg) n=8**.**

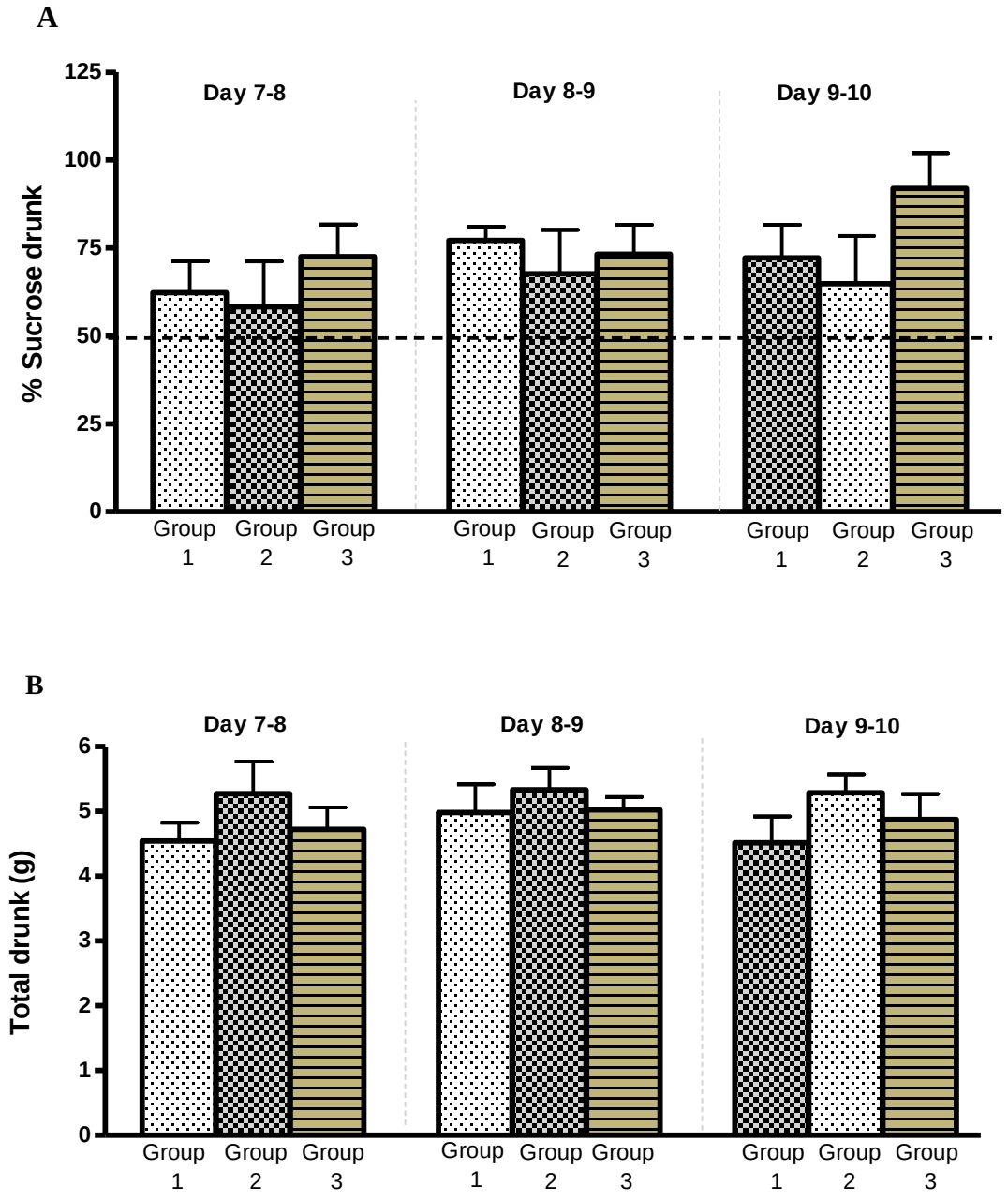


Figure 5.4: All mice show clear sucrose preference in the baseline period from day 7 to day 10 in male C57BL/6 mice. (A) No significant differences were observed between groups ($p > 0.05$) in percentage of sucrose drunk from day 7 to day 10. (B) No significant differences were observed between groups ($p > 0.05$) in total volume drunk. Data are presented as mean ± *SEM*. The data were analysed using repeated measure two-way ANOVA with Tukey's post hoc test between groups. Mice were allocated into three groups: group 1 (mice allocated for injection with vehicle on day 11, n=7), group 2 (mice allocated for injection with (1.5 mg/kg) SLIGRL-NH_2 on day 11, n=8) and group 3 (mice allocated for injection with (5 mg/kg) SLIGRL-NH_2 on day 11, n=8).

figure 5.4B) with no interaction seen between testing days and allocated groups ($F_{(4,40)}$ = 0.27, *p*= 0.895, figure 5.4 B).

5.2.4.4 Effect of SLIGRL-NH_2 in the SPT

24 h after SLIGRL-NH_2 injection

Initial experiments were performed in which the sucrose preference was tested over the 24 h following injection of SLIGRL-NH_2 (i.p.). No significant differences were observed between groups ($F_{(2,20)}$ = 0.34; *p*= 0.718; figure 5.5A) in the percentage of sucrose drunk in the 24 h after injection. Similarly, there were no significant differences between groups ($F_{(2,20)}$ = 1.43; *p*= 0.262; figure 5.6 A) in the total volume drunk (g) in the 24 h treatment following SLIGRL-NH_2 injection.

2 h after SLIGRL-NH_2 injection

As there were no differences observed 24 h post injection, I then looked to see if any changes occurred within 2 h of injection in case a short term effect of SLIGRL-NH_2 had been missed. No significant difference was seen between groups ($F_{(2,20)}$ = 0.05, *p*= 0.948; figure 5.6A) in the percentage of sucrose drunk over 2 h after injection. Similarly, there was no significant difference in the total volume drunk (g) in the 2 h after i.p. injection. ($F_{(2,20)}$ =0.87, *p*=0.433, figure 5.6 B).

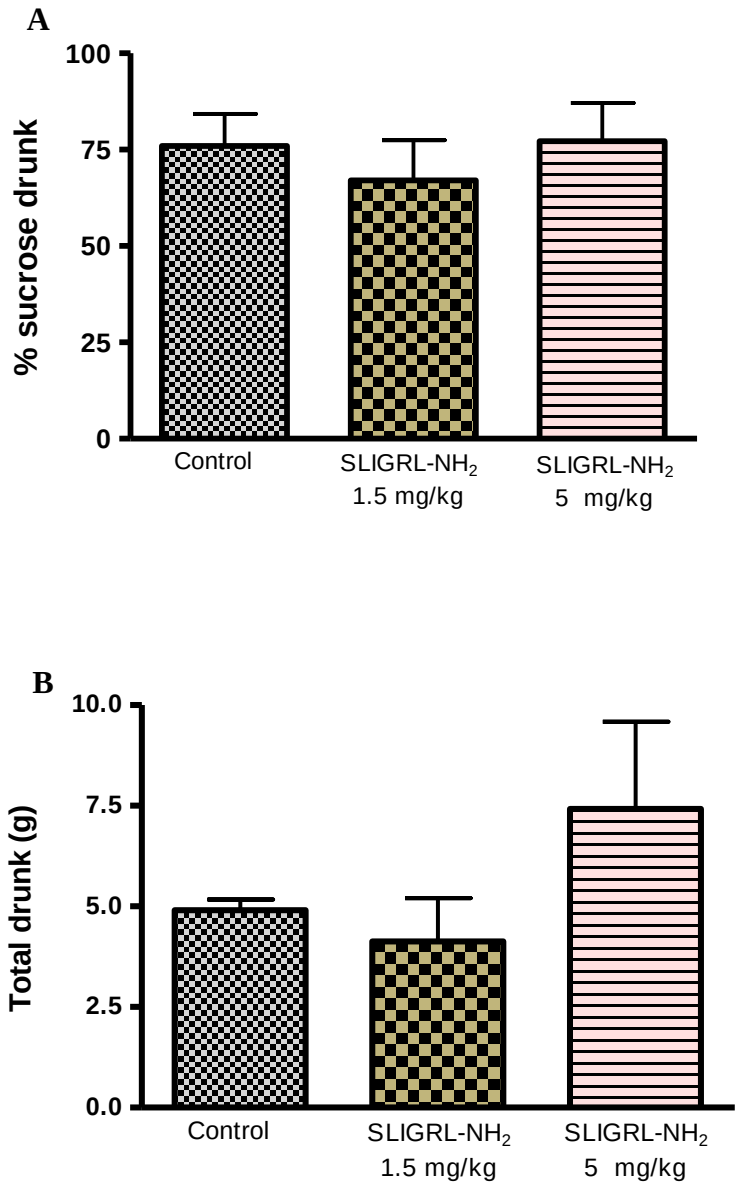


Figure 5.5: SLIGRL-NH_2 has no effect in the SPT in male C57BL/6 mice over the 24 h following i.p. injection. (A) No differences were observed between groups in the % sucrose drunk in the 24 h post injection. (B) SLIGRL-NH_2 does not affect the total drunk during the 24 h post injection ($p > 0.05$). Data are presented as mean ± *SEM*. The data were analysed using one-way ANOVA with Tukey's post hoc test between groups where appropriate. Control $n=7$, SLIGRL-NH_2 (1.5 mg/kg) $n=8$ and SLIGRL-NH_2 (5 mg/kg) $n=8$.

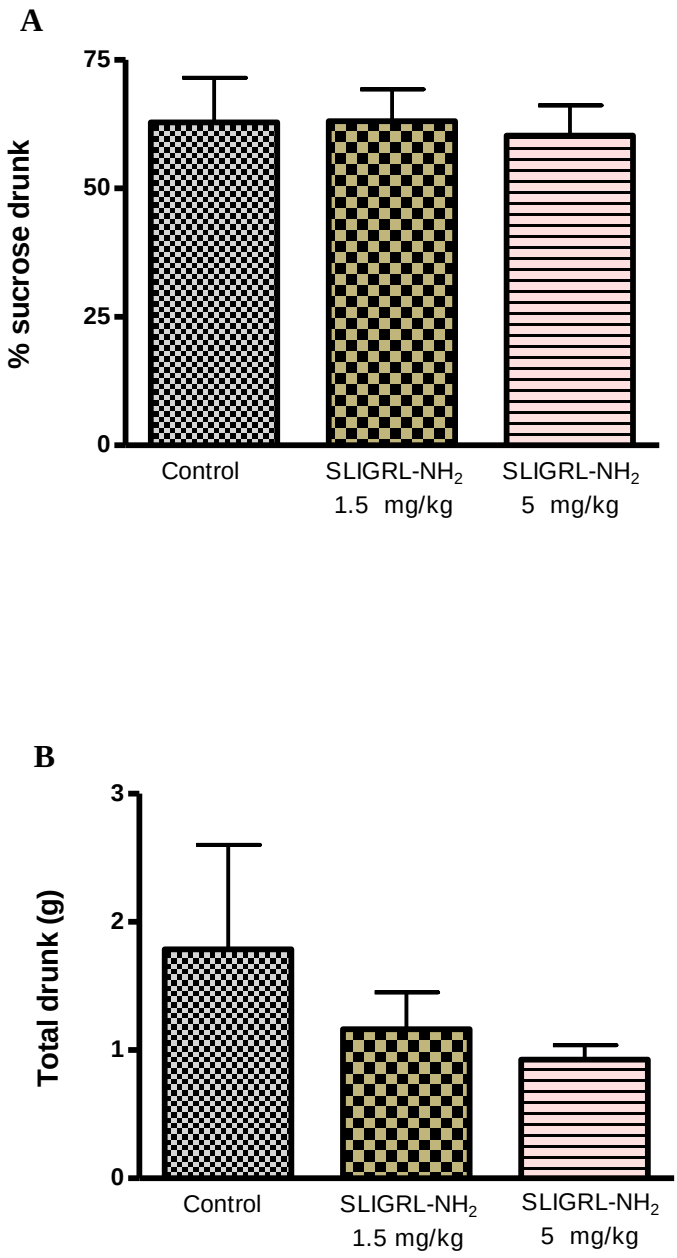


Figure 5.6: SLIGRL-NH_2 has no effect in the SPT in male C57BL/6 mice over the 2 h post i.p. injection. (A) % Sucrose drunk showed no significant difference ($p > 0.05$) in percentage between groups. (B) Total drunk during 2 h post treatment with SLIGRL-NH_2. Data are presented as mean ± *SEM*, the data were analysed using one-way ANOVA with Tukey's post hoc test between groups. Control n=7; SLIGRL-NH_2 (1.5 mg/kg) n=8; SLIGRL-NH_2 (5 mg/kg) n=8.

5.2.4 Discussion

5.2.4.1 Effect of SLIGRL-NH_2 on locomotor activity

As previously explained in chapter 3, section 3.15.2, locomotor activity in rodents is due to a motivation to explore the environment which is necessary for survival (Gilchrist *et al.,* 1997; Jordan *et al.,* 2006). The results demonstrate the peripheral administration of PAR2 agonist did not alter general locomotor activity. SLIGRL-NH_2 (either 1.5 mg/kg or 5 mg/kg) i.p. did not induce any change in general locomotor activity in the OFT as measured by the total distance moved in OF box. This result is consistent with our finding (chapter 3, section 3.2.4) that deletion of PAR2 has no effect on general locomotor activity in the OFT. Additionally, the findings of the current experiment are similar to the previous research of Lohman *et al* (2009) that SLIGRL-NH_2 did not induce hyperactivity in the OF in Genetic Absence Epilepsy Rats of Strasbourg (GAERS) (Lohman *et al.,* 2009).

5.2.4.2 Effect of SLIGRL-NH_2 on anxiety-like behaviour

It was explained in chapter 3, section 3.15.3, that both OFT and EPM test are useful models for measuring anxiety-like behaviour in rodents. Peripheral administration of PAR2 agonist did not alter anxiety-like behaviour in mouse. The OF and EPM tests are good models for evaluation of anxiety-like behaviour. As discussed previously in chapter 3 section 3.15.3 these tests evaluate anxiety-like behaviour based on the conflict in the rodent between the innate fear of the central area of a brightly lit OF box or the open arms of the EPM and their desire to explore new environments (Christmas and Maxwell 1970; File, 1980; Wall and Messier, 2001; Prut and Belzung, 2003). Neither dose of SLIGRL-NH_2 had any effect on anxiety-like behaviour, as measured by entries into and time spent in the central square of the OF and percentage time spent in and entries into the open arms of the EPM. However, Lehman *et al* (2009) also found that SLIGRL-NH_2 did not alter anxiety like behaviour in GAERS rats which have higher level of anxiety-like behaviour (Lohman *et al.,* 2009).

5.2.4.1 Effect of SLIGRL-NH_2 on anhedonia

It was explained previously that SPT is used for measuring anhedonia based on the rodents' inherent interest in sweet foods or solutions (Nielsen *et al.,* 2000). Anhedonia is measured by a reduction of the amount of sucrose drunk. Our results show that peripheral administration of PAR2 agonist did not alter the amount of sucrose solution drunk by mice. Most mice developed a preference for sucrose before injection with SLIGRL-NH_2. However, SLIGRL-NH_2 did not have any effects on the amount of sucrose drunk over the full 24 h after injection or in the 2 h immediately post treatment and therefore it is unlikely that SLIGRL-NH_2 has any effect on anhedonia. This result is consistent with our previous result that deletion of PAR2 did not change amount of sucrose drunk under normal conditions. Moreover, the effect of SLIGRL on anhedonia is similar to the result of Lohman *et al*. (2009) that, in GAERS rats, peripheral injection of SLIGRL-NH_2 (1.5 mg/kg, s.c.) did not induce anhedonia. In conclusion, SLIGRL-NH_2 has no effect on sucrose preference measured over 24 h or 2 h post injections.

In summary, peripheral administration of PAR2 agonist did not alter general locomotor activity, anxiety-like behaviour and hedonic response in mouse. These experiments were based on the study of Lohman *et al.,* 2009 which found that SLIGRL-NH_2 penetrates quickly into rat brains after peripheral injection. The similarity between mice and rats suggests that peripheral injection would allow SLIGRL to penetrate easily into mouse brain. Interestingly, our result using SLIGRL did not show the expected effect on locomotor activity, anxiety-like behaviour and anhedonia. In addition, the lack of behavioural effects of activation of PAR2 receptors by SLIGRL did not support our previous finding using PAR2 KO mice (chapter 3 and chapter 4). Therefore, direct investigation of penetration of SLIGRL-NH_2 and other PAR2 agonists into mouse brain were next carried out.

5.3 Penetration of PAR2 agonists into the brain after peripheral injection

5.3.1 Introduction

SLIGRL-NH_2 has been previously shown to cross the BBB into the brain of the GAERS rats (Lohman *et al.,* 2008, 2009). With this in mind and with it being accepted that the there is a similarity between the permeability of the BBB in mice and rats, the experiments described above were designed without checking whether SLIGRL-NH_2 enters into the mouse brain (Murakami *et al.,* 2000). Having found that SLIGRL-NH_2 injection (i.p.) has no effect in the behavioural tests used, it was necessary to examine whether SLIGRL-NH_2 enters the mouse brain as has been previously reported in the rats.

The mass spectrometry (MS) model is used due to high sensitivity and specificity for measuring molecular weights of a sample and also the relative amounts and probably structure of specific fragments for component with small molecular weight can be determined (Pitt, 2009). For these reasons, LC-MS is commonly used for pharmacokinetic studies of drugs and peptides, given an indication of relative concentration and of the component in the biological fluid (Tai and Welch, 2004). The PAR2 agonists (SLIGRL-NH_2, AC-264613and GB110) with molecular weights 657.9 dalton, 400.1 dalton and 609.4 dalton are good candidate for LC-MS analysis and detection of levels in mouse brains post peripheral administration.

As explained earlier in details in general discussion (chapter 1, section 1. 1.13.2, figure 1.24) that SLIGRL-NH_2 is mouse a PAR2 agonist and it is designed based on the peptide sequences to mimic the natural tethered ligand sequences for mouse. Novel PAR2 agonists (AC-264613 and GB110) have been developed by modifying synthetic peptides to produce compounds with higher potency and selectivity compared with SLIGRL. Therefore, the aim of current experiment that was to examine the penetration of 3 PAR2 agonists into mouse brains following peripheral administration.

5.3.2 Animals

Penetration of SLIGRL-NH_2 into the brain after peripheral injection was tested on the same mice used to examine the effect of SLIGRL-NH_2 on mouse behaviour (chapter 5, section 5.2.1).

Six HT adult male C57BL/6 mice (weighing 22.1 ± 4.0, age 12 weeks, *n*=2/group) were used to examine penetration of AC-263613 into the brain after peripheral injection before designing the behaviour experiment with AC-263613. In this experiment, HT mice were used instead of WT mice because of availability of WT mice only in BPU at the time of experiment. These mice were obtained from crossing the PAR2 genetically modified mice and were housed as explained in chapter 2, section 2.2.2. Similarly to the SLIGRL-NH_2 experiment, comparison of AC-264613 penetration 30 and 60 min after i.p. injection was tested on the same mice (chapter 5, section 5.3.1) which were used to examine the effect of AC-264613 on mouse behaviour (chapter 5, section 5.3.2).

Twelve WT adult female C57BL/6 mice (weighing 26.0 ± 0.2 g, aged 12 weeks, *n*=4) that were obtained from crossing the PAR2 genetically modified mice were housed as explained in chapter 2, section 2.1. These female mice were used to examine the penetration of GB110 into the brain after peripheral injection. In this experiment the female mice were used because it was available in the laboratory by that time.

5.3.3 Protocol

SLIGRL-NH_2

A week after the end of the behavioural testing of the effects of SLIGRL-NH_2, the same mice (section 5.1.2) were tested to determine whether SLIGRL-NH_2 enters the mouse brain after i.p. injection or not. Mice were injected either with vehicle (0.9% saline) or SLIGRL-NH_2 (1.5 mg/kg or 5 mg/kg, *n*=3/group). The mice were killed either 20 min or 60 min post injection, the time points were chosen according to behaviour experimental protocols, for example the mice were injected with SLIGRL-

NH_2 and tested for OFT and EPM test after 20 min and also test the mice after 2 hr in SPT. The whole mouse brains were dissected out (figure 5.7). The brain samples were homogenised (section chapter 2, 2.5.1) and analysed using MS to detect the SLIGRL-NH_2. The MS was carried out by Lynsey Macintyre (Pharmaceutical Analysis Lab at Strathclyde University).

AC-264613

From the results was obtained from MS, it was seen that SLIGRL-NH_2 does not enter the mouse brain. Therefore, before starting to design protocol to examine the effect of AC-264613 on mouse behaviour, its entry to the mouse brain was studied on a small number of mice (*n*=6). These mice were injected (i.p.) either with vehicle (1% Tween 80 in 0.9% saline, *n*=2) or AC-264613 (3 mg/kg or 10 mg/kg, *n*=2/group). After 30 and 60 min, the mouse brains were collected and homogenised individually for each mouse (chapter 2, section 2.5.1). Then the samples were analysed by MS (figure 5.8 and 5.9). The results obtained showed that after 30 and 60 min both doses of AC-264613 had crossed the BBB. This finding suggested first designing an experiment to investigate the effect of AC-264613 on mouse behaviour (chapter 5, section 5.7) and then further investigation of the amount of the AC-264613 penetrating into the brain after i.p. injection for each dose.

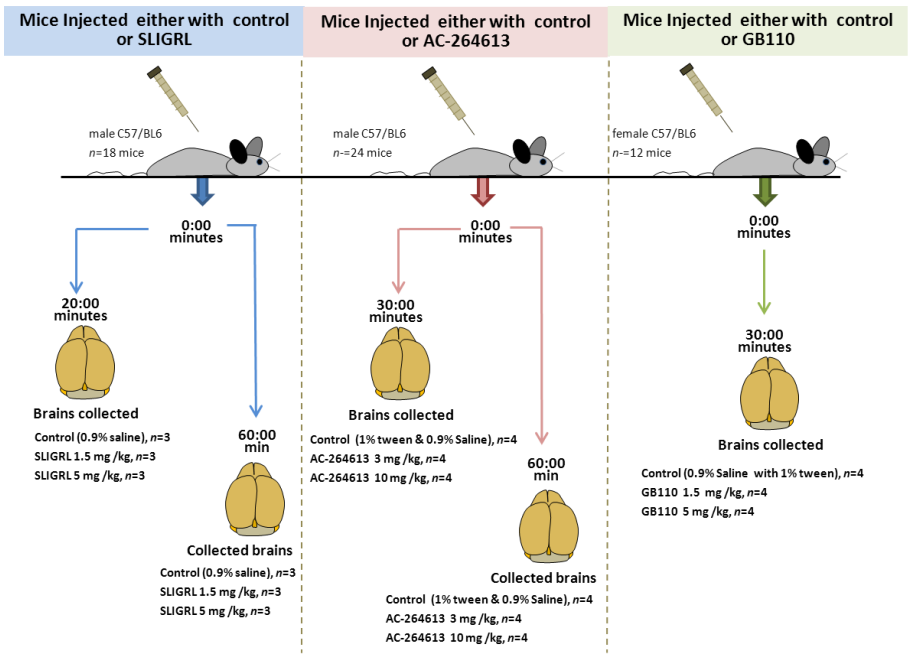


Figure 5.7: Protocol for experiment to determine penetration of PAR2 agonists into the brain after i.p. injection (A) SLIGRL-NH_2 (B) AC-264613 (C) GB110.

Similarly to the SLIGRL-NH_2 experiment, one week after the end of the behavioural testing with AC-264613 (chapter 5, section 5.3.1), the same mice were used (chapter 5, section 5.3.2) to verify AC-264613 penetration in to the brain. They were injected with vehicle (1% Tween 80 in 0.9% saline), AC-264613 3mg/kg or 10 mg/kg. Brains were collected after 30 and 60 min (*n*=4/group), figure 5.13. The brain samples were homogenised (chapter 2, section 2.5.1) and analysed using MS to determine the amount of AC-264613 in mouse brains after 30 and 60 min.

GB110

Twelve adult WT C57BL/6 female mice were allocated to 3 groups and injected with vehicle (1% Tween 80 in 0.9% saline *n*=4), GB110 (1.5 mg/kg or 5 mg/kg, *n*=4/group, figure 5.7). The brains were collected 30 min post injection (*n*=4/group) and the samples were homogenised (section 2.9) and analysed using MS to detect the GB110.

5.3.4 Result

5.3.4.1 Penetration of PAR2 agonists into brain after peripheral injection

MS results were obtained from the Metabolomics Facility at Strathclyde University. The parameters measured to detect the entry of PAR2 agonist into brain were retention peak time and the molecular weight of the PAR2 agonists. Firstly, the parameters were measured for the standard and then compared with results of the samples to confirm crossing of PAR2 agonists into the brain. The first PAR2 agonist tested was SLIGRL-NH_2 which showed a retention peak time at 16.32 min and the molecular weight 609.3755 dalton in the standard but was not detected in the brain sample, which indicates that this agonist did not cross BBB (figure 5.8 A and B and figure 5.9 A and B). AC-264613 showed a retention peak time at 16.2 min in both standard and brain samples (figure 5.8 C and D) and a molecular weight of 401.000 dalton in both the standard and brain samples which demonstrated that AC-264613 crossed the BBB and was detected in the brain both 30 and 60 min following injection (figures 5.9 C and D). The same experiment repeated for GB110 showed the same result as SLIGRL-NH_2 which indicated that this agonist did not cross BBB

(GB110 standard retention peak time 11.57 min, GB110 molecular weight 610.000 dalton; figure 5.8 E and F and figure 5.9 E and F).

5.3.4.2 Comparison of AC-262613 penetration into brain 30 and 60 min after i.p. injection

The relative area under the curve (AUC) was measured to compare the amount of AC-264613 penetration into the mouse brain. The relative AUC was calculated by dividing the AUC of the sample by the AUC of the standard. There was no significant effect of the time of injection of AC-264613 and no interaction between the injection time and doses of AC-264613 (time: $F_{(1,9)}$= 0.03, p= 0.865; interaction $F_{(2,9)}$= 0.72, p= 0.515). However, there were significant difference between doses in the amount of AC-264613 entering the brain ($F_{(2,9)}$= 175.43, p= 0.0005). Further analysis was done to determine change in amount of AC-264613 entering the brain at each individual time point, so the data were reanalysed using one-way ANOVA followed by Tukey's post hoc test. There was a statistically non-significant difference between AC-264613 (3 mg/kg) at 30 min and 60 min post injection and control. However, there was significant differences seen between AC-264613 (10 mg/kg) and control and AC-264613 (3 mg/kg) at 30 min and 60 min post injection (30 min post injection: $F_{(2,111)}$=14.71, p=0.0015, AC-264613 (10 mg/kg) vs control p<0.01, AC-264613 (10 mg/kg) vs AC-264613 (3 mg/kg) p<0.05, AC-264613 (3 mg/kg) n.s. compared with control); 60 min post injection: ($F_{(2,111)}$=25.90, p=0.0002, AC-264613 (10 mg/kg) vs control p<0.001, AC-264613 (10 mg/kg) vs AC-264613 (3 mg/kg) p<0.01, AC-264613 (3 mg/kg) n.s. compared with control; figure 5.10).

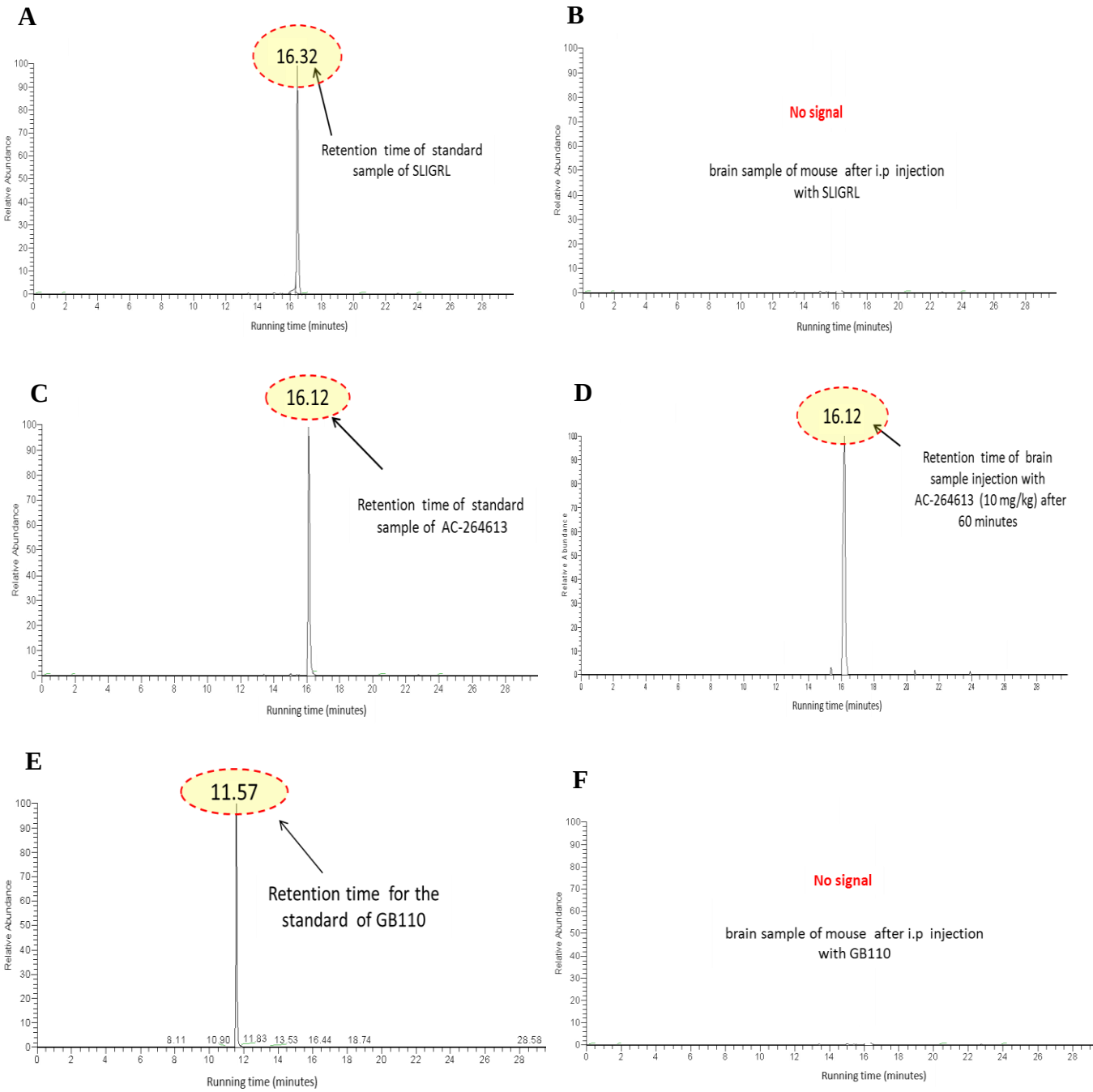


Figure 5.8: Retention time for PAR2 agonists into C57BL/6 mouse brain after i.p. injection compared to standard. (A) Retention time peak of SLIGRL-NH_2 standard sample was detected at 16.32 min, (B) No signal of retention time peak was detected mouse brain sample after injection with SLIGRL-NH_2, (C) Retention time peak of AC-2646-13 standard sample was detected at 16.12 min, (D) AC-2646-13 was also detected in the brain sample after i.p. injection and its retention peak was detected at 16.32 min, (E) Retention time peak of GB110 standard sample was detected at 11.57 min, (F) No signal of retention peak time was detected for mouse brain sample after injection with GB110. The data were produced by T.Zhang in the Metabolomics Facility at Strathclyde University.

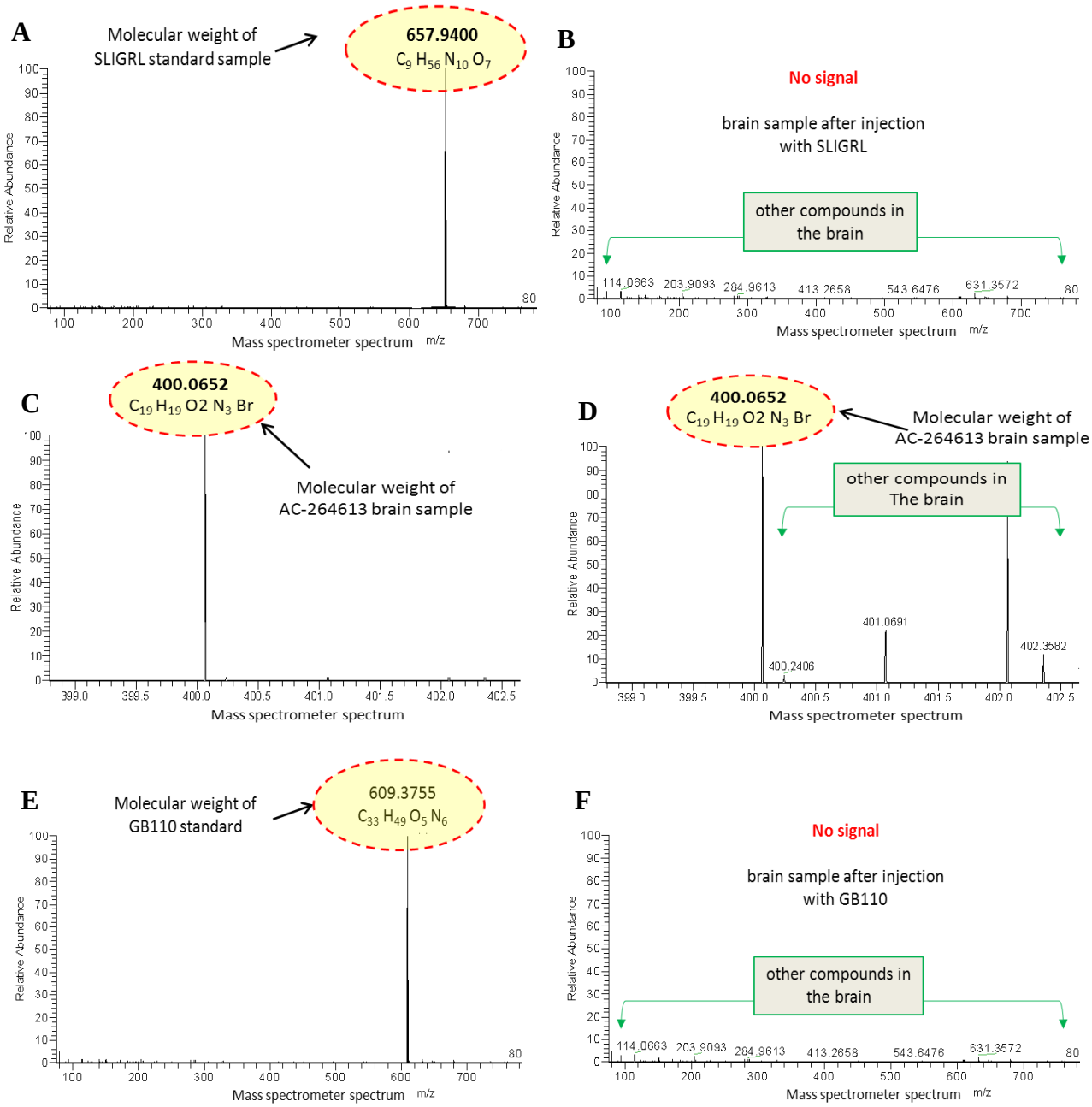


Figure 5.9: Molecular weight of PAR2 agonists in brain after i.p. injection compared with molecular weight for the standard. (A) Molecular weight of SLIGRL-NH_2 standard sample was detected at 657.9700 MS, (B) No signal of molecular weight peak was detected for brain sample of mouse after injection with SLIGRL-NH_2, (C) Molecular weight of AC-264613 standard sample was detected at 400.0652 MS, (D) Molecular weight of AC-264613 in the brain sample after i.p. injection was detected at 400.0652 MS which indicates AC-264613 crossed the BBB, (E) Molecular weight of GB110 standard sample was detected at 607.3755 MS, (F) No signal of molecular weight peak was detected for brain sample of mouse after injection with GB110. The data were supplied by T.Zhang of the Metabolomis Facility, Strathclyde University.

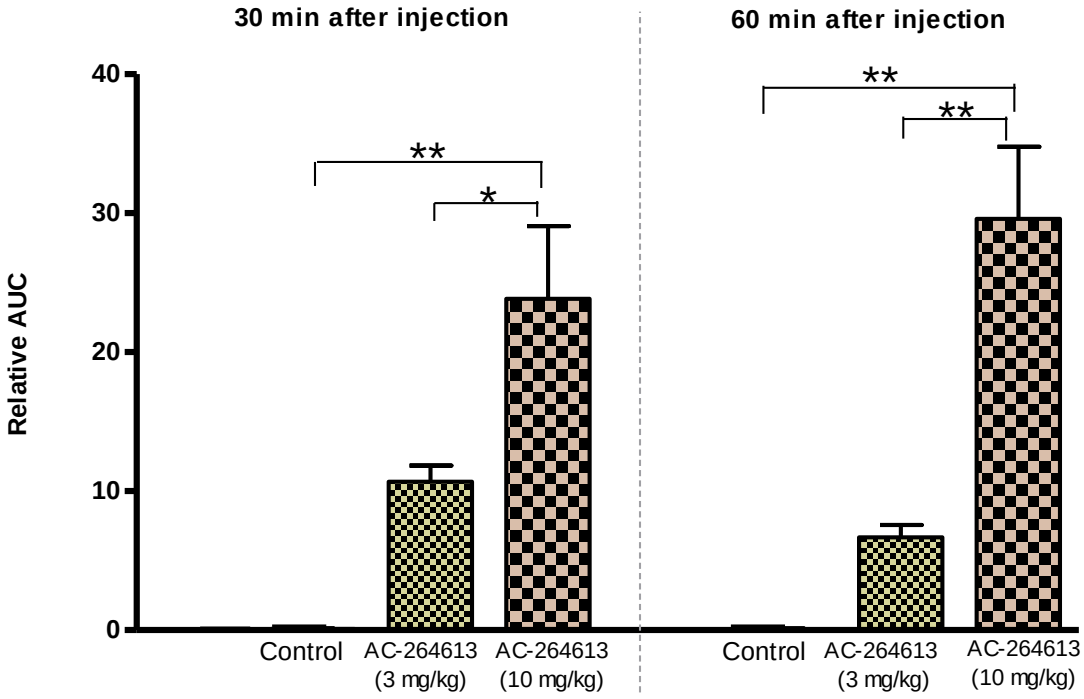


Figure 5.10: AC-264613 enters the brain in a dose-dependent manner. Significantly higher amounts of AC-264613 were detected in the brain after 10 mg/kg injection compared to both AC-264613 (3mg/kg) and control at both 30 and 60 min post injection. Data are presented as mean ± *SEM* and analysed using one-way ANOVA with Tukey's post hoc test between groups where appropriate. Control *n*=4; AC-264613 (3 mg/kg) *n*=4; AC-264613 (10 mg/kg) *n*=4.

5.3.5 Discussion

As PAR2 is proposed to have a role in many neurological and pathological processes, it is necessary to identify whether PAR2 agonists cross the BBB and cause activation of PAR2 in the brain as these PAR2 agonists may be useful for treatment of neurological diseases in the future. Therefore, in this experiment we looked for PAR2 agonists which could cross the BBB and thus potentially activate PAR2s in the brain. Three PAR2 agonists were examined for their brain penetration. These PAR2 agonists were SLIGRL, GB110 and AC-242613.

The BBB is one of the main factors limiting the future development of neurotherapeutics (Pardridge, 2005; Jian, 2012). There are two barrier systems in the brain which control the transport of the drugs from blood to brain interstitial fluid after peripheral administration (Pardridge, 2011). These barrier systems are the BBB and the blood cerebrospinal fluid barrier. For central effects, the drug is firstly required to cross the BBB and then distribute to the brain to produce its effects (Alavijeh *et al.,* 2005; Pardridge, 2011). The BBB consists of continuous layers of endothelial cells separated by tight junctions that restrict diffusion of bacteria and hydrophobic drugs and support diffusion of lipophilic drugs (Dehouck *et al.,* 1995; Muldoon *et al.,* 1999). However, some drugs show special diffusion methods such as specific uptake transporter mechanisms or active transport mechanisms; for example, glucose and amino acids bind with specific proteins to be transported to the brain (Laterra *et al.,* 1999; Mann *et al.,* 2003).

LC-MS is a highly sensitive and specific method widely used for detecting small molecular weight compounds in brain samples (Pitt, 2009). In addition it was also used for indicating the relative concentration of these compounds in the samples (Tai and Welch, 2004). For these reasons, LC-MS was used to detect PAR2 agonists (SLIGRL, AC-242613 and GB110) in mouse brains after peripheral administration.

5.3.5.1 Penetration of PAR2 agonist SLIGRL-NH_2 into mouse brain

LC-MS was used to detect SLIGRL-NH_2 in mouse brain samples 20 and 60 min post injection by comparing the molecular weight and retention time of SLIGRL in these

samples with the molecular weight and retention time of SLIGRL standards. Detection of SLIGRL-NH_2 with a molecular weight of 657.9 dalton was evident at a retention time of 16.3. Comparison of the molecular weight and retention time of SLIGRL-NH_2 standard with the molecular weights and retention times of the compounds in the brain samples showed that SLIGRL-NH_2 could not be detected in mouse brain injected with either dose (1.5 mg/kg or 5 mg/kg) at 20 or 60 min post peripheral injection. The result suggests that the PAR2 agonist SLIGRL-NH_2 does not cross the BBB.

After demonstration that SLIGRL-NH_2 did not affect mouse behaviour, this subsequent investigation suggested that SLIGRL-NH_2 does not pass the BBB and enter the brain. This is in contrast to the finding of Lohman *et al*., (2009) where they showed that in rats, SLIGRL-NH_2 (1.5 mg/kg, s.c.) rapidly crossed the BBB and was detected in cerebrospinal fluid 10 min following peripheral administration (Lohman *et al.,* 2009). Thus, this finding that SLIGRL-NH_2 (i.p.) did not alter behaviour in mice is likely to be due to inadequate penetration into the brain of the peripherally administrated peptide. Interestingly, Lohman and co-workers later stated that they had also tried SLIGRL-NH_2 (i.p.) in mice and did not detect it in the brain; hence, they only perform these experiments in rats (personal communication).

SLIGRL did not cross the mouse BBB but crossed the BBB of rats. These results support the idea that there are species differences in transport of drug molecules through the BBB (Egleton, 2005; Syvanen *et al.,* 2009). The reason for this difference between mice and rats in terms of SLIGRL-NH_2 crossing the BBB is not yet established and needs further investigation.

In conclusion, these result demonstrate that SLIGRL-NH_2 does not affect mouse behaviour; however, our subsequent investigation suggested that SLIGRL-NH_2 (1.5 and 5 mg/kg, i.p.) does not pass the BBB and enter the brain. Although the SLIGRL did not enter the mouse brain and thus did not have any effects in behavioural tests of central nervous system function, the important fact was clarified that i.p. administration of SLIGRL-NH_2 does not affect these behaviour tests in any way, indicating that any peripheral actions SLIGRL-NH_2 do not have an effect mouse

behaviour. This is important as PAR2 has been shown to have many peripheral actions including modulation of smooth muscle mobility in the gastrointestinal system, vascular relaxation in the cardiovascular system and modulation of smooth muscle activity in the respiratory system but if we accept that these occur under our experiemental conditons, these do not elicit behavioural changes (Moffatt and Cocks, 1999; Kawabata *et al*., 2001; Hamilton *et al.*, 2002; Lan *et al.*, 2002).

5.3.5.2 Penetration of PAR2 agonist GB110 into mouse brain

In general, SLIGRL-NH_2 is widely used for investigation of PAR2 function, but it has low potency and poor bioavailability that limit its use *in vivo* (Barry, 2010). SLIGRL-NH_2 has been modified to produce a non-peptide PAR2 agonist (GB110) with higher potency, stable bioavailability and highly selective to PAR2 over PAR1 (Barry, 2010), therefore GB110 is proposed to be suitable for *in vivo* studies (see chapter 1, section 1.13.2, and figure 1.24). Similar to trypsin, GB110 also stimulates the release of intracellular Ca^{2+} in seven human cell lines (Suen *et al.*, 2012). Further investigation has shown that GB110 is less potent than another PAR2 agonist 2f-LIGLO-NH_2 in PAR2 internalization (ß-arrestin dependent), as the cells treated with GB110 re-expressed PAR2 on the cell surface thus indicating post-activation recovery of receptor (Suen *et al.*, 2012). However, a recent study has shown that GB110 induces acute paw oedema in rodents in similar potency to SLIGRL-NH_2 and 2f-LIGLO-NH_2 (Vergnolle *et al.*, 1999; Kelso *et al.*, 2006; Suen *et al.*, 2012).

As SLIGRL-NH_2 unexpectedly did not penetrate into mouse brain, and although GB110 has higher potency, stable bioavailability and high selectivity to PAR2, it was necessary to examine its penetration into mouse brain before starting to design behavioural experiments including its use.

The LC-MS technique was also used to examine the penetration of agonist GB110 in mouse brain. Comparison of the molecular weight and retention time of GB110 standard (molecular weight; 609.4 dalton; retention time 11.6 min) with the molecular weights and retention times of the compounds in the brain samples showed that GB110 was not detected in mouse brain post 30 min of peripheral

injection (dose: 1.5/kg or 5mg/kg). Our data suggest that novel PAR2 agonist GB110 does not cross the BBB.

GB110 was not detected in mouse brain. This was not entirely unsurprising as discussions with Professor David Fairlie (University of Queensland, Australia) indicated that the compound was designed to activate PAR2-mediated ERK-phosphorylation and not specifically to cross the BBB (personal communication). Barry *et al*. (2010) found that GB110 is selective to PAR2 over PAR1, serum stable and it was designed to be suitable for oral administration thus allowing pharmacological research of the effects of PAR2 regulation in animal models of human disease (Barry *et al.,* 2010). However, as GB110 did not enter the brain, no further experiment was carried out with this compound in this study, but it may prove useful for experiments investigating the effect of PAR2 activation in the periphery or in *in vitro* CNS preparations.

5.3.5.3Penetration of PAR2 agonist AC-264613 into mouse brain

The second PAR2 agonist tested is also derived from SLIGRL-NH_2 and modified to a non-peptide PAR2 agonist AC-264613 (see chapter 1, section 1.13.2, and figure 1.24**).** AC-264613 is more potent than SLIGRL-NH_2 (EC_{50} of SLIGRL-NH_2: 5.0 ± 0.1; EC_{50} of AC-264613 7.0 ± 0.2), highly selective to PAR2 over other PARs and metabolically stable (Gardell, *et al.* 2008). Similar to SLIGRL-NH_2, AC-264613 stimulates the mobilization of intracellular Ca^{2+} in cultured cell lines (Gardell, *et al.* 2008). PAR2 receptors have been implicated in nociception and inflammatory processes (Vergnolle, 2009) and similar to SLIGRL-NH_2 and trypsin, systemic administration of AC-264613 into Sprague-Dawley rats can cause oedema and thermal hyperalgesia (increased sensativity to painful thermal stimuli) (Vergnolle, *et al.,* 1999; Steinhoff, *et al.,* 2000; Vergnolle, 2009; Gardell *et al.,* 2008). Additionally, PAR2 have been documented to cause pain and inflammation via a neurogenic mechanism of action (Steinhoff, *et al.,* 2000; Vergnolle *et al.,* 2001). Intrapaw administration of AC-264613 into rats causes pain and inflammation via the mechanism of PAR2 receptors, which is the same as neurogenic mechanism (Gardell, *et al.,* 2008). Based on previous studies AC-264613 may be suitable for

PAR2 *in vivo* studies. Therefore, it was necessary to examine whether AC-264613 enters the mouse brain.

Firstly, the penetration of AC-264613 compound in mouse brain was examined using the LC-MS technique. The comparison of molecular weight (400.1 dalton) and retention time (16.2 min) of AC-264613 compound observed 30 and 60 min post injection with AC-264613 standard) has shown that both doses, 3 mg/kg and 10 mg/kg of AC-264613 compounds, crossed BBB and were detected in the mouse brain at 30 and 60 min post injection.

Secondly, increasing the injected dose of AC-264613 led to an increased amount of AC-264613 detected in the brain. The amount of AC-264613 penetrating to the brain is measured as relative AUC 30 and 60 min post injection, the relative AUC of AC-264613 (10 mg/kg) was higher than the relative AUC of AC-264613 (3 mg/kg) and control. This suggests that AC-264613 penetrates into mouse brain in a dose dependent manner.

The novel finding in this study is that AC-264613 is the only PAR2 agonist of those examined which crosses the BBB in mice. There are no previous pharmacokinetic studies of AC-264613 in mouse brain. As discussed earlier in chapter 1, sections 1.13.2, the mechanisms regulating PAR2 signalling are complex and incompletely understood. AC-264613 induces internalization and clathrin vesicle formation is faster with AC-264613 than with SLIGRL-NH_2 (Gardell *et al.,* 2008). Further investigation is needed to understand the role of AC-264613 in desensitization and internalization of PAR2 receptors. Furthermore, the effect of AC-264613 on regulation of endogenous proteinases in the brain is not known. Further investigation is also required for a better understanding of the pharmacokinetics of AC-264613 is entry into the brain and its effect on regulation of endogenous proteinase activators in the brain, for example trypsin.

In conclusion, AC-264613 was the only PAR2 agonist tested that penetrated into the mouse brain. This result suggested testing the effect of a PAR2 agonist on mouse behaviour using AC-264613.

5.4 Effect of AC-264613 on locomotor activity, anxiety-like behaviour and anhedonia in mice

5.4.1 Introduction

As introduced earlier, *in vivo* and *in vitro* studies have shown that PAR2 potentially plays an important role in CNS function and may be a novel therapeutic target for the treatment of neurological disorders (Lohman 2008, 2009; Greenwood and Bushell, 2010; Gan *et al*, 2011;). As shown in the previous section, the small molecule AC-264613 is the only PAR2 agonist tested that crossed the BBB. Therefore, an identical experimental plan to that used for the SLIGRL-NH_2 investigation was used for AC-264613 to investigate the role of the PAR2 agonist on locomotor activity, anxiety-like behaviour and anhedonia in mice.

5.4.2 Animals

The effect of AC-264613 was tested in 40 adult male C57BL/6 mice weighing 27.0 ± 0.3 g and aged 12 weeks that were obtained from Strathclyde colonies and were housed in BPU at Strathclyde University (section 2.1). The mice were housed as per the SLIGRL-NH_2 experiments (section 5.2.1).

5.4.3 Behavioural test protocols

The protocol used to investigate the effect of AC-264613 on behaviour in mice was identical to that described for SLIGRL-NH_2. The mice were randomly allocated into 3 groups: control (1% Tween 80 in 0.9% saline i.p., n=14); AC-264613 3 mg/kg i.p. (n=13); AC-264613 10 mg/kg i.p. (n=13). The only difference between the two protocols was that the mice were tested 30 min after injection of AC-264613 based on the result of the brain penetration assay.

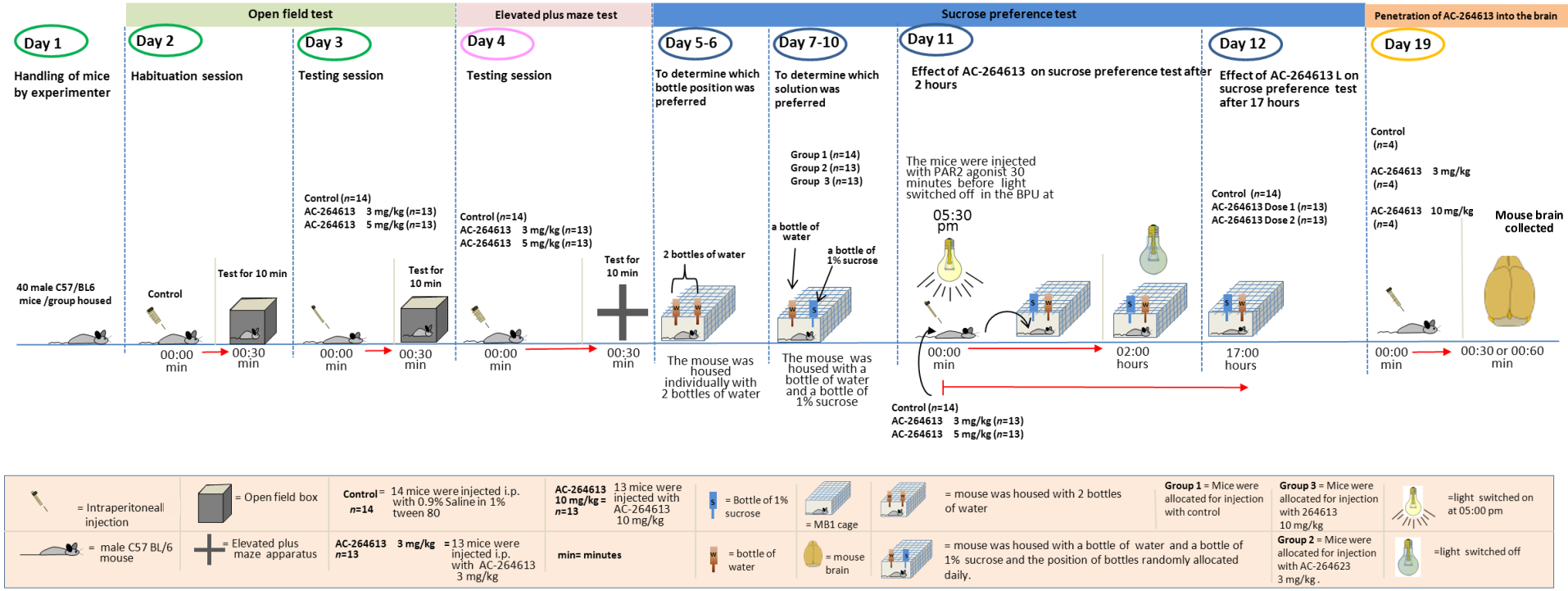


Figure 5.11: Experimental protocol for the effect of peripheral administration of AC-264613 on OFT, EPM and SPT.

OF and EPM tests

From day 1 to day 4, the protocol of both tests (OF and EPM) was identical to the protocol of the previous experiment except that the mice were injected with AC-264613 and tested after 30 min (figure 5.11).

SPT

The protocol investigating baseline sucrose preference was similar to the previous experiment (from day 7 to day 10). However, there was a difference in the protocols on days 11 and 12. On day 11, the mice were injected with AC-264613 30 min before the beginning of the dark period (section 2.1).

This time was chosen for injection because during the dark cycle mice are more active and eat and drink more than in the light period. The parameters were measured 2 h (day 11) and 17 h (day 12) after injection (figure 5.11).

5.4.4 Results

5.4.4.1 Effect of AC-264613 in the OFT

The PAR2 agonist AC-264613 (i.p.) had no effect in the 10 min OFT at any of the doses investigated. No significant differences were observed between groups in total distance moved (cm) over the test period ($F_{(2,19)}$= 0.03, *p*= 0.976, figure 5.12 A), numbers of entries into centre square of the OF (control: 25.4±3.5, AC-264613 (3 mg/kg): 28.6 ± 3.6, AC-264613 (10 mg/kg): 25.7 ± 2.9, $F_{(2,37)}$ = 0.282, *p*= 7560, figure 5.12 B), and time spent (s) in centre square of the OF box ($F_{(2,37)}$ = 0.05, *p*= 0.947, figure 5.12 C).

5.4.4.2 Effect of AC-264613 in the EPM test

Similar to the effect of AC-264613 in the OFT, there was no effect of AC-264613 in the EPM parameters during a 10 min test (figure 5.13). The 3 groups of mice spent an equal percentage of time (s) in the open arms of the maze ($F_{(2,37)}$= 0.37; *p*= 0.692, figure 5.13 A) and made a similar percentage of entries into the open arms of the maze ($F_{(2,37)}$=0.08, *p*= 0.992, figure 5.13 B) during the 10 min test session.

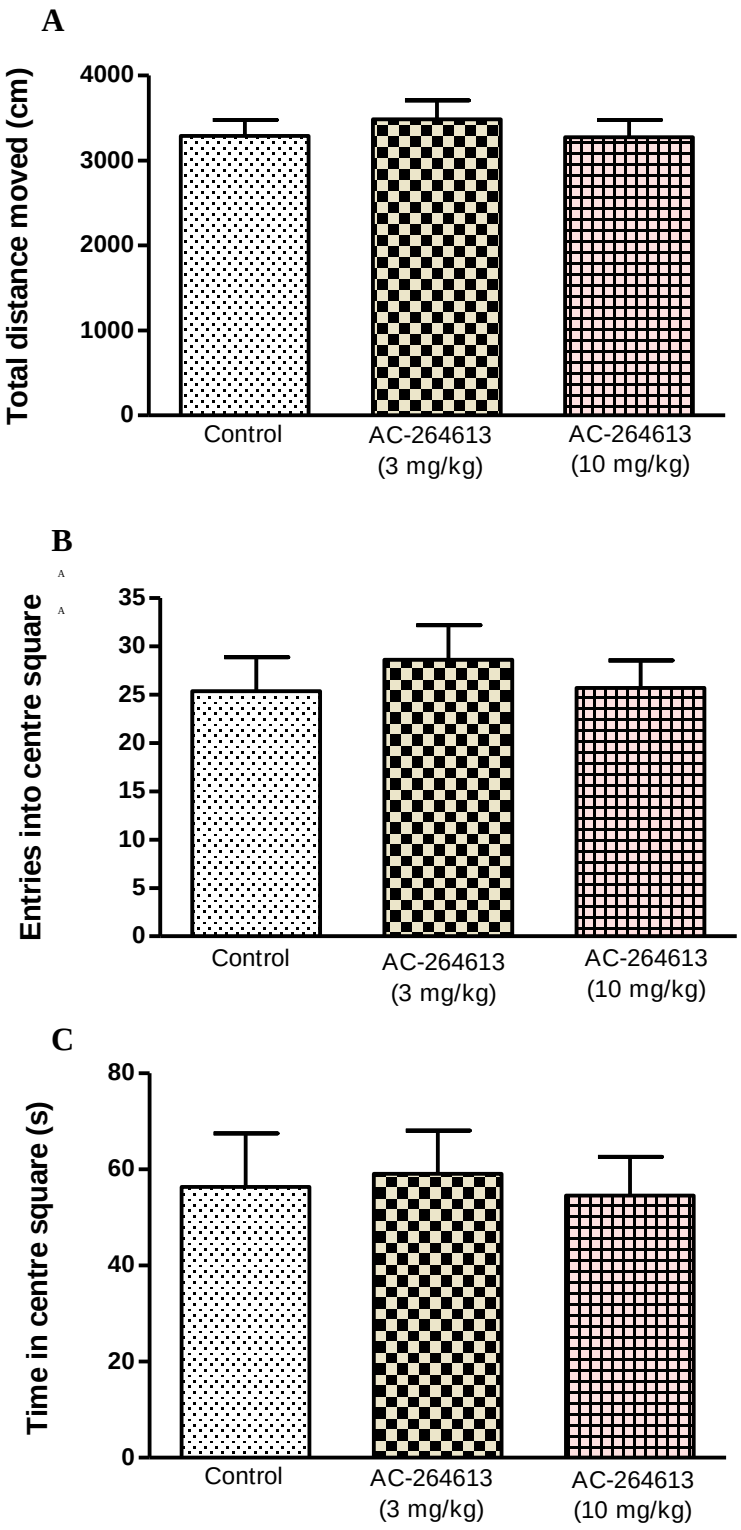


Figure 5.12: AC-264613 does not affect locomotor activity in the OFT in male C57BL/6 mice. AC-264613 does not affect A) the distance moved during the 10 min trial, B) entries into centre square of the arena during the 10 min trial and C) time in centre square of the arena over the test period. Data are presented as mean ± *SEM*. the data were analysed using one-way ANOVA with Tukey's post hoc test between groups. Control *n*=14; AC-264613 (3 mg/kg) *n*=13; AC-264613 (10 mg/kg) *n*=13.

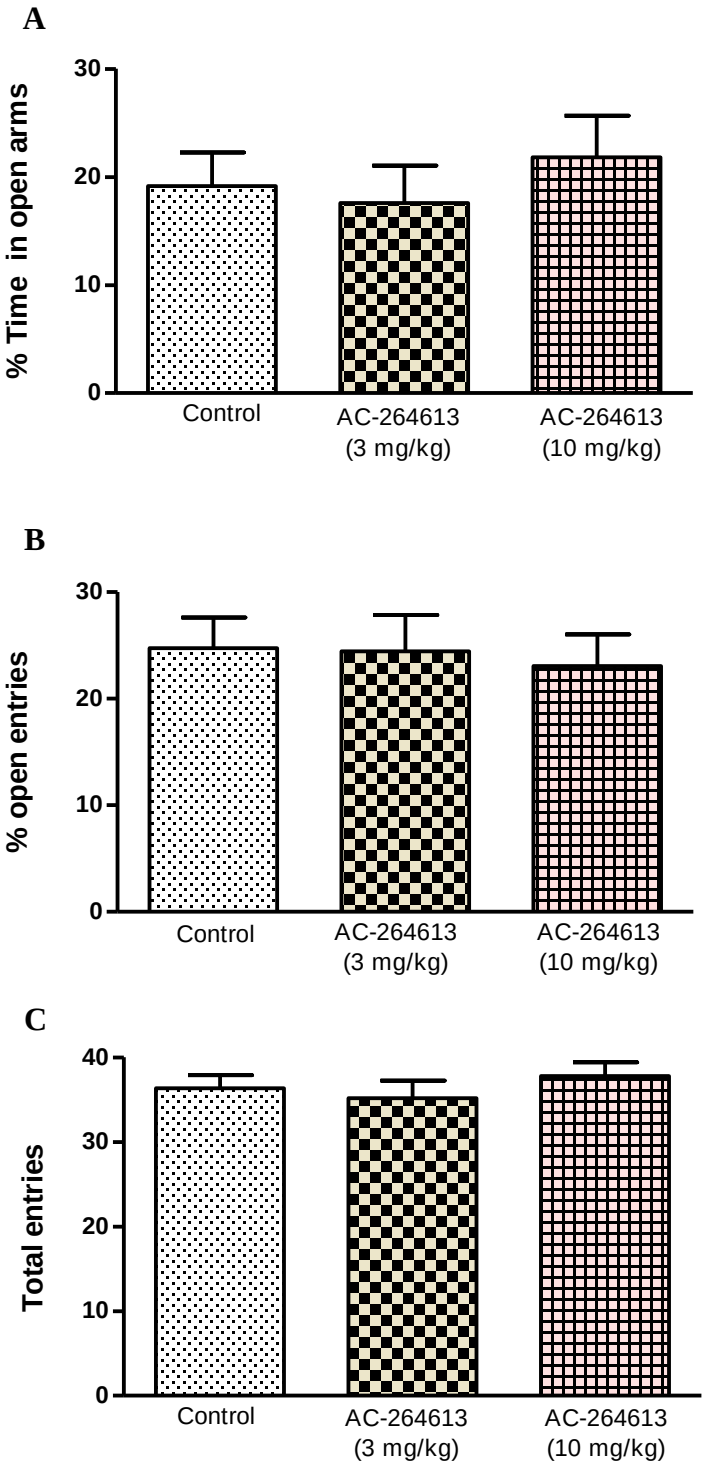


Figure 5.13: AC-264613 does not alter behaviour in the EPM test in male C57BL/6 mice. AC-264613 does not alter A) the percentage of the time spent on the open arms of the EPM, B) the percentage of entries into the open arms of EPM and C) the total number of arm entries. Data are presented as mean ± *SEM*, and analysed using one-way ANOVA with Tukey's post hoc test between groups. Control *n*=14; AC-264613 (3 mg/kg) *n*=13; AC-264613 (10 mg/kg) *n*=13.

There were no significant differences between the 3 groups in total number of arm entries ($F_{(2,37)}$= 0.53; *p*= 0.592, figure 5.13 C).

5.4.4.3 Mice show a baseline sucrose preference before AC-264613 injection

All the mice had drunk more than 50% sucrose solution more than water during 3 consuctive testing days. No significant change were detected in the % sucrose drunk over 3 consecutive testing days and between the allocated groups ($F_{(1,74)}$ = 1.71, *p*= 0.105; allocated groups: $F_{(2,37)}$ = 0.54, *p*= 0.588; figure 5.4A). Similarly, there was no significant interaction between days and allocated groups (allocated group: $F_{(4,74)}$ =1.75, *p*= 0.147, figure 5.14 A). There was also no significant change in the total volume drunk (water plus sucrose solution) measured in g over 3 consecutive testing days, between the allocated groups (allocated groups: $F_{(2,37)}$ = 1.12, *p*=0.338; figure 5.21B) and no interaction was shown between testing days and allocated groups ($F_{(4,74)}$ = 0.56, *p*= 0.694, figure 5.14 B).

5.4.4.4 Effect of AC-264613 in the SPT

2 h post AC-264613 injection

Sucrose preference appeared to be reduced in the 2 h post injection in the two groups treated with AC-264613; however this just failed to reach significance ($F_{(2,37)}$= 3.03; *p*= 0.060, figure 5.15 A). There was no significant difference between the groups in total drunk (g) in the 2 h treatment post AC-264613 injection ($F_{(2,37)}$= 0.490, *p*= 0.616, figure 5.15 B).

17 h after injection with AC-264613

The parameters were also measured 17 h from the time of injection of AC-264613. There was no significant difference between these groups in the percentage of sucrose drunk during the 17 h after injection with AC-264613 ($F_{(2,37)}$= 0.15, *p*= 0.861, figure 5.16 A). Similarly, there was no significant difference between the groups in total drunk (g) in the 17 h following treatment with AC-264613 ($F_{(2,37)}$=0.47, *p*= 0.630, figure 5.16 B) .

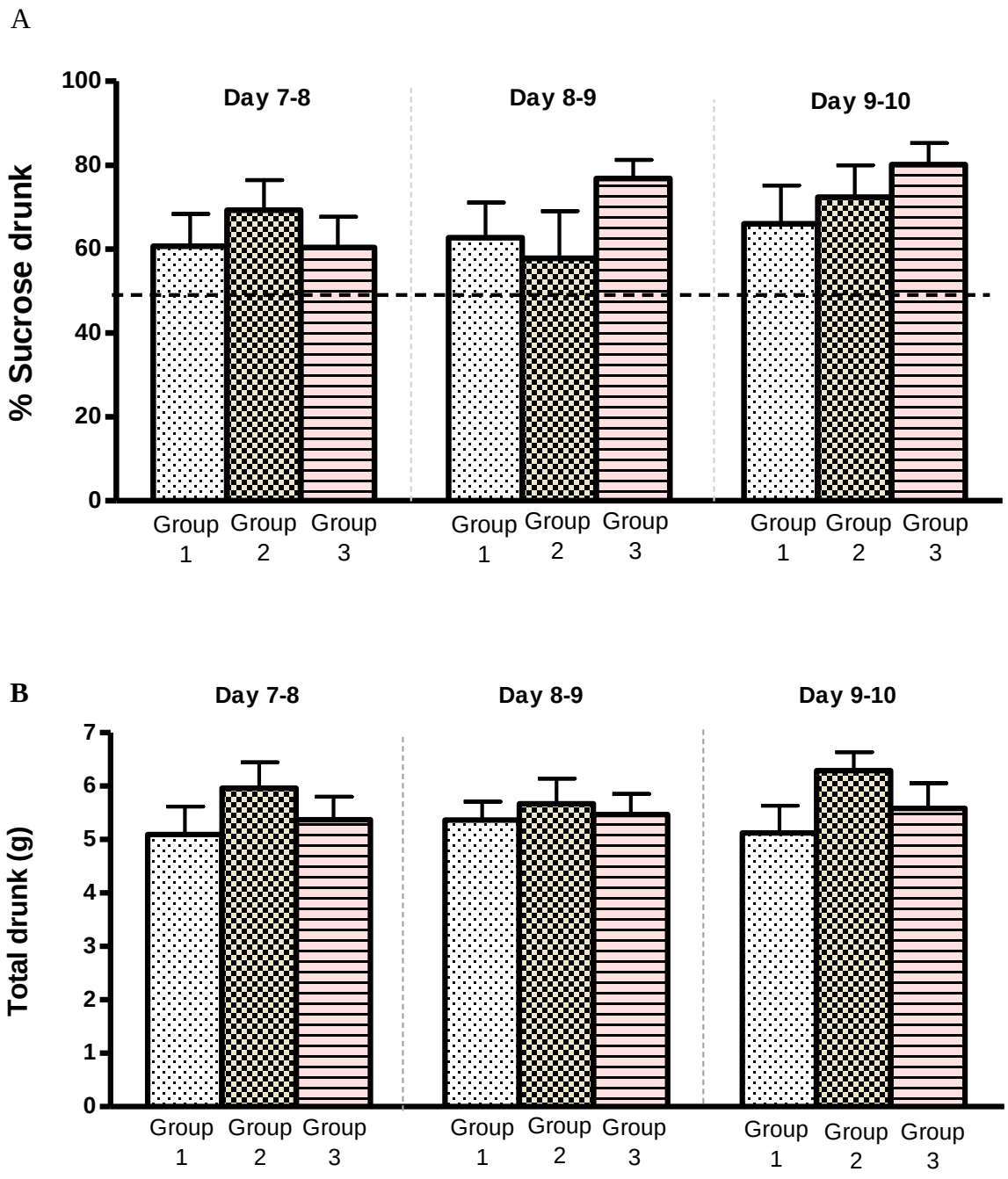


Figure 5.14: Male C57BL/6 mice show clear baseline sucrose preference from day 7 to day 10. (A) No significant differences were observed between groups ($p > 0.05$) in percentage of sucrose drunk from day 7 to day 10. (B) No significant differences were observed between groups ($p > 0.05$) in total volume drunk from day 7 to day 10. Data are presented as mean ± *SEM*. The data were analysed using repeated measure two-way ANOVA with Tukey's post hoc test. Group 1 n=14; group 2 n=13; group 3 n=13.

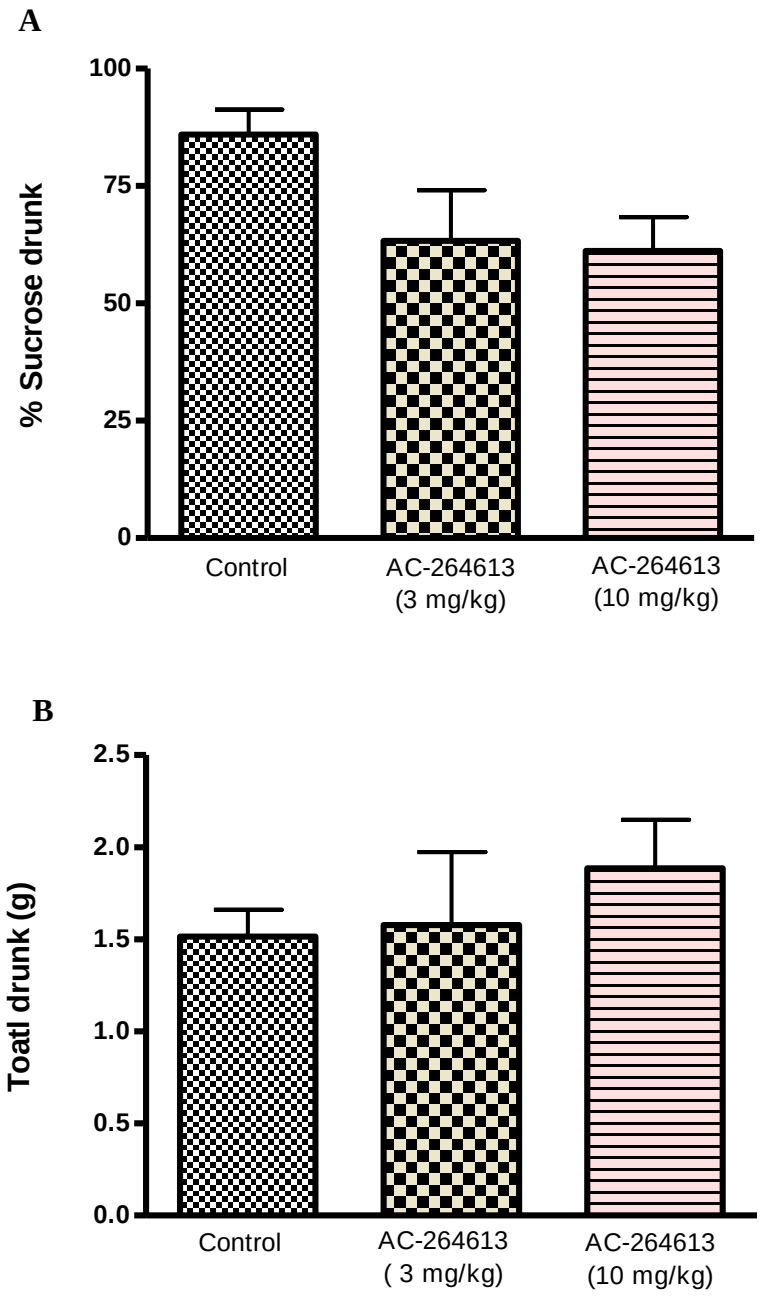


Figure 5.15: AC-264613 shows a trend to reduce sucrose preference in male C57BL/6 mice over the 2 h post i.p. injection. (A) Effect on % sucrose drunk just missed significance (p=0.06, 2-way ANOVA). (B) Total drunk during the 2 h after treatment with AC-264613. Data are presented as mean ± *SEM*, the data were analysed using one-way ANOVA with Tukey's post hoc test between groups. Control n=14; AC-264613 (3 mg/kg) n=13; AC-264613 (10 mg/kg) n=13.

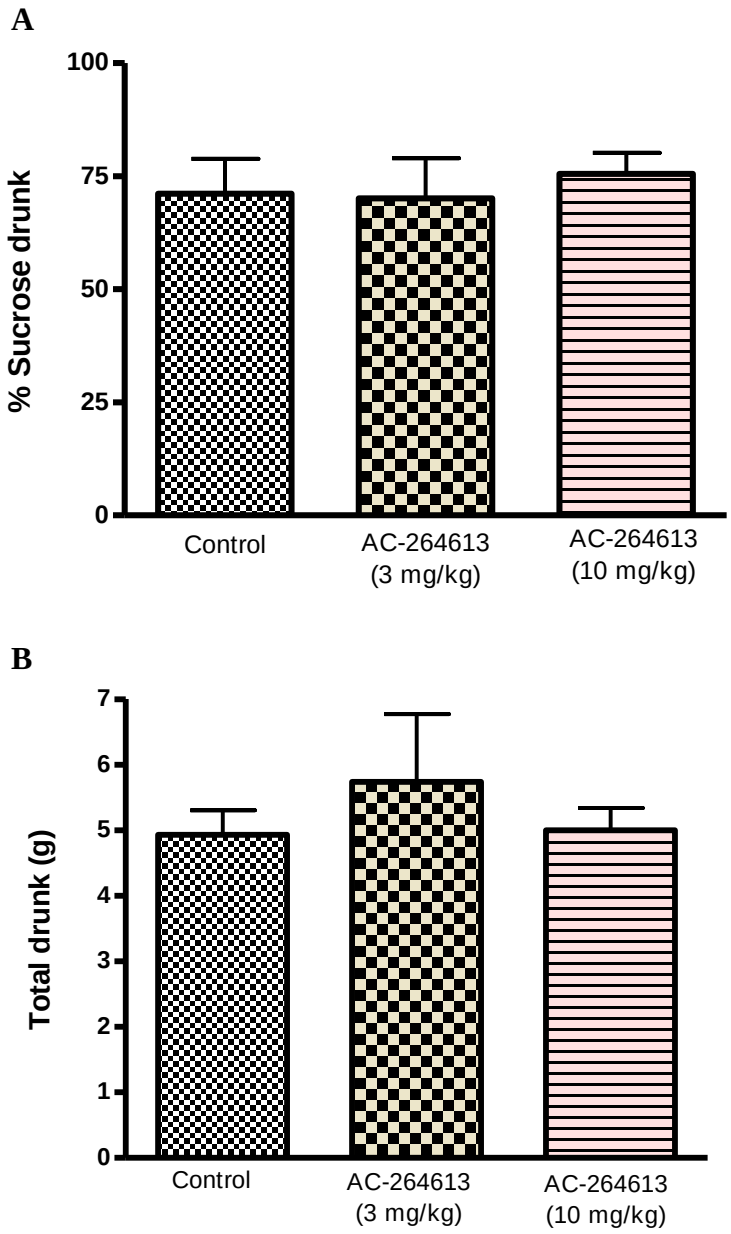


Figure 5.22: AC-264613 does not alter sucrose preference in male C57BL/6 mice over 17 h post i.p. injection. (A) % Sucrose drunk showed no significant difference between groups (p0.05) (B) Total drunk over 17 h post treatment with AC-264613. Data are presented as mean ± *SEM*, the data were analysed using one-way ANOVA with Tukey's post hoc test between groups. Control n=14; AC-264613 (3 mg/kg) n=13; AC-264613 (10 mg/kg) n=13).

5.5 Discussion

AC-264613 is a novel PAR2 agonist that enters mouse brain in a dose-dependent manner (section 5.2.5.3). As explained in detail in chapter 3, the results achieved from PAR2 KO mice experiments suggest that PAR2 plays a minor role in anxiety-like behaviour and memory under normal physiological conditions. For these reasons, it was clear that the development of a brain-penetrant PAR2 agonist would help increase our understanding of the function of PAR2 in the brain. Therefore, following my initial experiments with KO mice, I performed experiments with the novel PAR2 agonist, AC-264613. The doses of AC-264613 were chosen to be equivalent to the SLIGRL-NH_2 doses because very little was known about PAR2 agonists in the mouse brain as explained before.

5.5.1 Effect of AC-264613 on locomotor activity

It was explained before in chapter 3, section 3.15.2, that exploratory behaviour in rodents is necessary for survival (Gilchrist *et al.,* 1997; Jordan *et al.,* 2006). The results of current experiment showed that peripheral administration of PAR2 agonist AC-264613 did not change general locomotor activity. Both i.p. doses of AC-264613 compound (either 3 mg/kg or 10 mg/kg) did not prompt any change in general locomotor activity in the OFT as measured by total distance moved in OF box. This result is consistent with our finding (chapter 3, section 3.8) that deletion of PAR2 has no effect on general locomotor activity using the same test. Additionally, our result of this test is similar to previous finding of Lohman *et al* (2009) who demonstrated that s.c. administration of 1.5 mg/kg of the PAR2 agonist SLIGRL-NH_2 to adult male GAERS rats did not change the distance moved in OFT (Lohman *et al*., 2009). Our result indicats that peripherial effects of AC-264613 injection do not affect lcomotor activity.

5.5.2 Effect of AC-264613 on anxiety-like behaviour

It was explained previously in chapter 3, section 3.15.3, that both OFT and EPM test are useful models for measuring anxiety-like behaviour in rodents. In this study, peripheral administration of both doses of AC-264613 compound had no effect on

anxiety-like behaviour, as measured by number of entries into and time spent into centre square of the OF box and % time spent into the centre and number of entries into the open arm of EPM apparatus. These results are similar to the finding of Lohman *et al* (2009) that peripheral administration of 1.5 mg/kg of the PAR2 agonist SLIGRL-NH_2 to adult male GAERS rats did not alter anxiety-like behaviour in different tests like OFT, EPM test and the light-dark box test. On the other hand, previous results in chapter 3, section 2.8.3 shown that HT male spent more time in centre square of OF box compared with WT and KO males. This result shows that there was a difference in the effect of the receptors on the anxiety-like behaviour when PAR2 was activated and when PAR2 was deleted.

5.5.3 Effect of AC-264613 on anhedonia

It was explained previously in chapter 1, section 1.16.1 that SPT is used for measuring anhedonia, which in humans is a common symptom of depression and can be used in animals as a measure of depression-like behaviour. The test is based on rodents' inherent liking of sweet foods or solutions (Nielsen *et al.,* 2000). Anhedonia is measured by reduction in amount of sucrose drinking. The results obtained from this test demonstrated that most mice developed a preference for sucrose before injection with AC-262613; however, 3 mg/kg or 10 mg/kg AC-264613 did not significantly alter this preference either over 17 h after treatment or in the 2 h after treatment. However, there was a trend for AC-264613 to reduce % sucrose drunk over the 2 hr period following administration (p= 0.060), which merits further investigation, particularly in the light of the results in chapter 4, section 4.5.3 which indicate that PAR2 deletion mitigates the effect of LPS on sucrose preference. Thus, these results suggest that activation of PAR2 receptors with an agonist reduces sucrose preference and may indicate induction of anhedonia, while deletion of PAR2 may delay the onset of anhedonia following LPS injection. Therefore, PAR2 receptors may play some role in hedonic responses.

Anhedonia is a core symptom of depression (Moreau, 1997; Moreau, 2002) and our results have shown that PAR2 receptors may have some effect on anhedonia. Therefore an alternative test for depression-like behaviour, the forced swim test, was

performed by another researcher in our laboratory (data not shown). There was no effect of peripheral administration of either dose of AC-264613 (3 mg/kg or 10 mg/kg) on immobility time.

Peripheral administration of the PAR2 agonist AC-264613 did not alter depression-like behaviour in forced swim test but it induced anhedonia as measured by a decrease in the amount of sucrose intake in SPT. Thus the PAR2 agonist may not be generally inducing depression-like behaviour, but may be interacting with other factors related to anhedonia.

The first possible factor is decreased sensitivity to reward. Sucrose preference is a widely used index of reward (Cannon and Palmiter, 2003). The brain reward process may have evolved in response to natural reward, for example, a preference for the sweet taste of sucrose (White and Carr, 1985). Solutions of sucrose stimulate a hedonic response in both human and rodent which is established in an early stage of life. In humans, the reward stimulates this response in new born infants whereas the reward stimulates this response in 3 day old rat pups (Steiner, 1973). A study has shown a reduction in amount of sucrose solution drunk in reward deficit rats (McArthur and Borsini, 2008). These results suggest that a decrease in amount of sucrose drunk may be because of a decrease in sensitivity to reward. Thus, our finding that peripheral administration of PAR2 reduces the amount of sucrose drinking may possibly be due to decreased sensitivity to reward. This result needs more investigation by examining the effect of peripheral administration of PAR2 agonist in tests of sensitivity to reward, for example, novel object place conditioning, conditioned place preference and progressive-ratio responding tests (Bevins *et al.*, 2002; Tzschentke, 2007; Finger *et al.*, 2010).

The other factor possibly affecting sucrose preference is sensitivity of the mice to the sweet taste. A study has shown that mice with deficiency of sweet taste receptors trypanosome infection response 3 (Tir3) have a significant preference for sucrose solution versus water. This result suggests that the taste is not an essential factor in preferring sucrose to water. At the same time, olfactory bulbectomy mice (a mouse model of depression and the mice also have deficit in smell) have a lower sucrose

preference (Zukerman *et al.,* 2009). These results suggest the importance of odour of the sweetened solution rather than the taste of the solution in preference for sucrose solution over water in SPT. This result suggests that an effect of AC-264613 on either taste or ability to smell – which affects taste, in humans at least – could explain these results.

In conclusion, AC-264613 is the only PAR2 agonist examined which penetrates into the mouse brain whereas the other PAR2 agonists tested, SLIGRL and GB110 did not penetrate into the mouse brain, therefore, the GB110 was not tested . Peripheral administration of AC-264613 did not alter general locomotor activity, anxiety-like behaviour had effect on anhedonia but non-significant.

Chapter 6

General discussion

6.1 General discussion

The main purpose of this thesis was to investigate whether PAR2 plays a role in mouse behaviour, particularly in emotional behaviour and cognition. The central hypothesis of this investigation was that as PAR2 is expressed in the brain areas that are involved in emotional behaviour and cognition (Striggow *et al.,* 2001; Noorbakhsh *et al.,* 2005; Bushell *et al.,* 2006), that PAR2 plays a crucial role in at least some aspects of emotion and cognition. Additionally, PAR2 receptors have a functional role in neurogenic inflammation as well as these receptors playing either a neuroprotective or a neurodegenerative role in some neurological diseases (Vergnolle *et al.,* 1999; Vergnolle *et al.,* 2001; Amadesi *et al.,* 2004; Vergnolle, 2005; Amadesi *et al.* 2006; Bunnett, 2006; Alier *et al.,* 2008; (Noorbakhsh *et al.,* 2005; Jin *et al,* 2005; Wang *et al.,* 2007). In addition, a recent study has shown that activation of these receptors by a PAR2 agonist indirectly modulates neuronal excitability and synaptic transmission in the hippocampus (Gan *et al.,* 2011). Other studies have indicated that peripheral injections of the PAR2 agonist SLIGRL in rats produce deficits in motivation learning in the MWM and the test-retest model of EPM tests (Lohman *et al.,* 2009). Furthermore, many studies have shown a clear relationship between PAR2 and inflammation (Wallace and Chin, 19997; Ferrell *et al.,* 2003; Kim *et al.,* 2003; Lohman *et al.,* 2012). Under normal physiological conditions, there is a possibility of crosstalk between PAR2 and the TLR-4 receptor that stimulates release of extra PICs and exaggerates inflammatory responses thus prolonging the symptoms of sickness behaviour (Rallabhandi *et al.,* 2008*;* Bucci *et al.,* 2012). According to these previous studies, we hypothesise that PAR2 may have a physiological role in the CNS in emotion and cognition, as well as probably having a role in sickness behaviour induced by infection. It was expected that the experiments described in this thesis might finally provide experimental evidence as to whether PAR2 is a possible candidate for therapeutic treatment for CNS diseases. Therefore, in this final chapter, I will summarise the main findings of this investigation with novel results highlighted. I will discuss the significance of this investigation for the role of PAR2 in behaviour under normal physiological conditions. Following from this, I will discuss the role of PAR2 in sickness behaviour. I will then discuss the

effect of PAR2 agonists on behaviour. Finally, I will make suggestions for future studies based on the data of this thesis.

6.2 Main findings

The main findings reported in this thesis are as follows:

Firstly, from investigation of the role of PAR2 in normal mouse behaviour

1. PAR2 is not necessary for normal locomotor activity in both genders.
2. Deletion of PAR2 decreases anxiety-like behaviour in male in the OFT, but it increases anxiety like behaviour in females in the EPM test.
3. PAR2 is not necessary for spatial memory, but deletion may affect the directional pathways of the mice or strategies used by these mice to find the hidden platform in MWM test in both genders.
4. Deletion of PAR2 reduces working memory in the T-CAT in both genders.
5. Deletion of PAR2 decreases recognition memory in the NOR test in male mice.
6. Deletion of PAR2 did not affect startle response and sensorimotor gating in either gender but it decreased startle response at the highest startle stimuli in females in SR and PPI tests.

Secondly, from study of the role of PAR2 in sickness behaviour

1. Peripheral injection with 1 mg/kg LPS induced symptoms similar to the symptoms of sickness behaviour: locomotor activity and body weight were decreased in the first 8 h after injection but returned to normal within 24-48 h post injection.
2. Deletion of PAR2 has no effect on hedonic state under normal physiological conditions.
3. In the sickness behaviour model, deletion of PAR2 delays the onset of the appearance of anhedonia.
4. In the sickness behaviour model, deletion of PAR2 mediated faster recovery from sickness behaviour symptoms.

Finally, from the experiments on effects of PAR2 agonist on mouse behaviour

1. PAR2 agonists SLIGRL-NH_2 and GB110 did not penetrate into the mouse brain post peripheral administration.
2. PAR2 agonist AC-264613 was the only PAR2 agonist that penetrated into the mouse brain post peripheral injection in a dose dependent manner.
3. Peripheral administration of AC-264613 did not change locomotor activity and anxiety-like behaviour in the OFT.
4. Peripheral administration of PAR2 agonist AC-264613 showed a tendency to induce anhedonia as measured in the SPT.

6.3 PAR2 involvement in emotion and cognition under normal physiological conditions

In this thesis, the involvement of PAR2 in emotion and cognition under normal conditions has been indicated by experimental data from deletion of PAR2 and activation of the receptors with PAR2 agonist AC-264613. This may suggest that PAR2 is involved in emotion as well as learning and memory. In the OFT, data suggest that deletion of PAR2 decreases anxiety-like behaviour in male mice. In contrast, in EPM test anxiety-like behaviour increases in female mice (chapter 3, section 3.15.3). Additionally, pathological condition our result in chapter 4, section 4.8.4 suggets that deletion of PAR2 reduces anxiety-like behaviour 24 h post injection with LPS (chapter 4, section 4.8.4). However, these results do not support the finding of other studies that peripheral administration of PAR2 agonist SLIGRL-NH_2 did not alter anxiety-like behaviour in high level of anxiety GAERS rats (Lohman *et al.,* 2009). Our results suggest that PAR2 can modulate anxiety-like behaviour under normal physiological condition and pathological conditions.

Data in chapter 3, section 3.15.6 and 3.15.7 suggests that deletion of PAR2 reduces working memory in T-CAT in both genders and it reduces the recognition memory in NOR test in males, suggesting a role for PAR2 in learning and memory. This finding supported by finding of Gan *et al* (2011) that activation of PAR2 receptor by SLIGRL-NH_2 indirect modulates neuronal excitability and synaptic transmission in the hippocampus (Gan *et al.,* 2011). The current results suggest PAR2 can play a role in learning and memory.

Furthermore, the data obtained from SR and PPI tests (chapter 3, section 3.15.8) suggest that deletion of PAR2 reduces the startle response at pulse intensity 120 dB in female mice. This result did not support the finding of Almonate *et al* (2007) also similar receptor deletion of PAR1 did not alter startle responses in the PPI test (Almonate *et al*., 2007).

The importance of investigation the physiological function of PAR2 in emotion and cognition under normal conditions, my thesis suggests that PAR2 can modulate anxiety-like behaviour in a gender specific manner. Deletion reduced working memory in both genders and deficits in recognition memory in males, suggesting a role for PAR2 in at least some cognitive processes. These findings provide more evidence which is needed to fully understand the physiological function of PAR2 in the CNS which at present is limited.

6.4 PAR2 involve in sickness behaviour

In this thesis, the involvement of PAR2 in sickness behaviour has been indicated by experiment data from the effect of deletion of PAR2 on the sickness behaviour model. This may suggest that PAR2 is involved in induction and recovery from sickness behaviour. My data suggest that deletion of PAR2 delays onset appearance of anhedonia and total drunk 2 h post injection with LPS measured by increase in amount of sucrose drunk in SPT (chapter 4, section 4.8.5). This result supports our finding that peripherial adminstration of 10 mg/kg of AC-264613 increases anhedonia measured by decrease in amount of sucrose drunk in SPT (chapter 5. section 5.5.3). These results suggest that PAR2 may paly a role in anhedonic state.

Additionally, deletion of PAR2 clearly shows an increased locomotor activity 24 h and 48 h post injection with LPS (chapter 4, section 4.8.3). Furthermore, deletion of PAR2 reduces anxiety-like behaviour 24 h post injection with LPS (chapter 4, section 4.8.4). Finally, deletion of PAR2 increases the amount of food intake 48 h post injection and reduce % of change in body weight 48 and 72 h post injection. These results suggest that deletion of PAR2 receptors has little effect on the induction of sickness behaviour but has obvious effects on the recovery from sickness behaviour. Our finding provides novel evidence that PAR2 has a pro-

inflammatory role in sickness behaviour and this finding supports other studies that PAR2 has pro-inflammatory role in some neurological disease as was explained in details in chapter 4, section 4.8.8 (Noorbakhsh *et al.,* 2006; Gatti *et al.,* 2006; Afkhami-Goli *et al,* 2007; Kirkup *et al.,* 2003; Hyun *et al.,* 2008; Cirino *et al.,* 2000).

The importance of this investigation provide clear and novel evidence to address what is the functional role of PAR2 in the CNS during sickness behaviour *in vivo*, which is needed to clarify the functional role of PAR2 in the CNS during inflammatory challenge.

6.5 Penetration of PAR2 agonist in the mouse brain

In my thesis, the involvement of PAR2 agonist penetrates in mouse brain has been indicated by experiment data from the effect of PAR2 agonist on mouse behaviour. Current data provides strong evidence that PAR2 agonist AC-264613 penetrates mouse brain in dose dependent manner (chapter 5, section 5.5). This thesis provides a novel finding that the PAR2 agonist AC-264613 penetrates the mouse brain post peripheral administration, helpful for further investigation of the physiological and pathological function of PAR2 in CNS in *in vivo* studies.

6.6 Effect of PAR2 agonist AC-264613 on mouse behaviour

In this thesis, the involvement of PAR2 agonist AC-264613 in mouse behaviour has been indicated by experiment data from the effect of PAR2 agonists on mouse behaviour. This may suggest that PAR2 agonist AC-264613 showed a tendency to induce anhedonia as measured in the SPT. My data suggest that peripheral administration of AC-264613 (10 mg/kg, s.c.) reduced amount of sucrose intake in SPT (chapter 5. section 5.5.3). Our finding provides novel evidence that PAR2 AC-264613 changed sucrose preference in SPT and this finding does not support other studies that peripheral administration of PAR2 agonist SLIGRL-NH_2 has no effect on anhedonia in GAERS rats in similar test of measuring anhedonia which is sucrose consumption test (Lohman *et al.,* 2009). It was explined previously this result support our previous finding that that deletion of PAR2 delays onset appearance of

anhedonia and total drunk 2 h post injection with LPS measured by increase in amount of sucrose drunk in SPT (chapter 4, section 4.8.5).

This thesis provides a novel finding that the peripheral administration of PAR2 agonist AC-264613 alter sucrose preference in SPT, helpful for further investigation of the physiological function of PAR2 in CNS in *in vivo* studies in field of treatment of psychiatric disease such as depression.

6.7 The relationship between PAR2 and anhedonia

It was found from preclinical and clinical studies that major depressive disorders are associated with inflammatory processes suggesting that inflammation may play a role in the pathophysiology of depression (Krishnadas and Cavanagh, 2012). Most of the evidence that links inflammation to depression comes from the observation that elevated levels of PICs are present in depressed individuals (Miller *et al.,* 2009) and the elevation of PICs has been shown to induce changes in brain structure and function that leads to the development of depression (Dantzer, 2009). Cancer patients treated with inflammatory cytokines (are at a high risk of developing depression (O'Connor *et al.,* 2007), it is proposed that the mechanism that underlies this may involve an effect of cytokines on either the hypothalamic-pituitary-adrenal axis,on neurotransmission or a direct action on hippocampal neurogenesis (Zunszain *et al.,* 2011).

In response to a peripheral infection, innate immune cells produce PICs that act on the brain to cause sickness behavior (Dinarello, 2000). When the peripheral immune system is activated continuously, as in inflammatory autoimmune diseases, the consequent immune signalling pathways to the brain lead to an exacerbation of sickness symptoms and development of depression in such people (Dantzer, 2009). Inflammation is therefore an important biological factor that may increase the possibility of developing depressive disorders (Dantzer *et al.,* 2008).

Inflammation has been linked to depression in a number of peripheral and neurological diseases. Patients with inflammatory diseases are associated with high rate of depression (Shelton and Miller, 2011). So treating depression can improve the

quality of life of those patients and may reduce pain and inflammation associated with the disease, for example, people with an autoimmune chronic systemic inflammatory disease such as rheumatoid arthritis, (Eccleston, 2001). Many studies have shown that people with rheumatoid arthritis are highly susceptible to developing depression as compared with healthy controls (Covic *et al.,* 2012). Therefore, inflammation may play a role in causing or contributing to depression in people with rheumatoid arthritis (Shelton, 2011; Haussleiter *et al.,* 2009).

Multiple sclerosis is a disorder of brain and spinal cord where areas of chronic inflammation and demyelination occur in parts of CNS (Love, 2006). This can cause damage to parts of the brain leading to various symptoms, some of which may cause disability (Frohman *et al.,* 2011). There is a link between brain abnormalities and depression in people with multiple sclerosis (Feinstein *et al.,* 2004). Several studies have reported high rates of depression in patients of multiple sclerosis compared with healthy control, causing suicidal feelings in such patients (Siegert and Abernethy, 2005). Therefore, inflammation may also add to depression in patients of multiple sclerosis.

Similarly, acute brain ischemia is also associated with an inflammatory response that contributes to ischemic damage (Zunszain *et al.,* 2012). Around 30% of people with acute brain ischemia have depression (Hackett and Anderson, 2005). This also suggests a link between inflammation and depression disorders.

Many studies have reported that PAR2 has an important role in chronic inflammation (Suen *et al*., 2010; Sevigny *et al.*, 2011; Lohman *et al.*, 2012). Some studies suggest a role of PAR2 in the treatment of chronic inflammation, for example, rheumatoid arthritis (Fiorucci and Distrutti, 2002; Crilly *et al.*, 2012; Lohman *et al.*, 2012). That study shown that PAR2 KO mice with arthritis have four-time less inflammation in joints as compared with WT mice (Ferrell *et al.,* 2003). In addition, injecting WT mice with a PAR2 agonist induced prolonged joint swelling and synovial hyperthermia (Ferrell *et al.,* 2003). Other studies have shown that this disease is associated with elevation of PICs level and upregulation of PAR2 in synovial joints

(Gordon and Urowitz, 1982; Kelso *et al.,* 2006; Kelso *et al.,* 2007; Crilly *et al.,* 2012).

In my work, I investigated anhedonia in the PAR2 KO model that was same as that used in the previous studies to determine the relationship between PAR2 and rheumatoid arthritis with anhedonia induction being evaluated by measuring the reduction in % sucrose drunk. Our result shows that there was no effect on % sucrose drunk after 2 hr of LPS injection in PAR2 KO mice, whereas it anhedonia was induced in both WT and HT. Therefore, my data suggests that deletion of PAR2 delays onset of appearance of anhedonia. Our result provides novel evidence that PAR2 has a pro-inflammatory role in sickness behaviour. The link between PAR2 and anhedonia was further supported by the finding that peripheral administration of PAR2 agonist (AC-264613) to adult WT mice at a dose of 10 mg/kg induced anhedonia 2 hr post injection. Thus a clear link between PAR2 and anhedonia in my studies may also be relevant for the induction of depression associated with many inflammatory related diseases.

6.8 Future studies

The studies detailed in this thesis have opened the window for a full investigation of PARs involvement by determining the functional role of PAR2 in behaviour in emotion and cognition under normal and pathological conditions. These results have opened a new field for research and placed the groundwork for future more detailed studies. From the previous discussion of this thesis, it has been found that further experiments are needed. The following are some of the unresolved questions which may be worth to identify:

1. Is PAR2 involved in anxiety-like behaviour? Strategies to address this would be either by examining the effect of deletion of PAR2 on anxiogenic and anxiolytic compounds *in vivo* or by examining the effect of PAR2 agonist on the other anxiety-like behaviour tests, for example, elevated zero-maze and light dark exploration test.
2. Is the level of expression of other PARs in mouse brain changed after deletion of PAR2? The plan to achieve this would be by measuring the level of expression of

PAR1, PAR2, PAR3 and PAR4 using the reverse transcription polymerase chain reaction (RT-PCR) technique in the brain of KO mice and comparing with the WT.

3. Is the level of expression of endogenous PAR2 activator trypsin changed in the brain after deletion of PAR2? Using RT-PCR, the level of expression of trypsin could be measured in the brain of both KO mice and WT.
4. Deletion of PAR2 or administration of PAR2 agonist induces behaviour changes, therefore, pre-clinical pharmacological studies require to be done at the time of the behaviour test to provide information about the neurotransmitters which are involved in these behaviours. Additionally, neuro-imaging such as functional magnetic resonance imaging (MRI) is also useful and provides information about the brain areas which are involved in emotion and cognition. The information collected from the pre-clinical pharmacological and brain imaging studies may be helpful in designing of a new drug for treatment of CNS diseases.
5. Does deletion of PAR2 change the swim pathway in the MWM? To understand this it will necessary to examine the extent and direction of circling movement in the MWM and OFT tests.
6. Does sickness behaviour affect the level of expression of PARs in the brain? RT-PCR might be used to measure the level of PARs in the brain in the sickness behaviour model.
7. Does PAR2 affect the level of expression of PICs and AICs in the brain during sickness behaviour? RT-PCR or western blotting might be used to measure the level of expression of PICs and AICs in the brain.
8. Dose PAR2 affect the level of expression of TLR-4 in the brain? Again, RT-PCR might be used to measure the level of expression of TLR-4 in the brain.
9. What is the pharmacokinetics of the PAR2 agonist AC-264613 in the brain? The concentration of AC-264613 as well as the metabolites could be measured by LC-MS.
10. Does activation of PAR2 by the PAR2 agonist AC-264613 alter the level of expression of trypsin in the brain? The level of expression of trypsin post peripheral administration of PAR2 agonist might be measured by RT-PCR.

11. Does activation of PAR2 by the PAR2 agonist AC-264613 alter spatial reference memory, working memory, recognition memory and sensorimotor gating? These results could be achieved by examining the effect of peripheral administration of AC-264613 on spatial memory using MWM test, on working memory by T-CAT, on recognition memory by NOR test and on sensorimotor gating by PPI test.

In summary, it is clear that the current study has opened up a new area of research in that PAR2 receptors may have a limited role in emotion and memory under normal conditions but that PAR2 has a pro-inflammatory role during sickness behaviour. Furthermore, AC-264613, as the only PAR2 agonist investigated which penetrates into the mouse brain, is a useful compound to study the role of PARs. Clearly, this research has produced a number of novel results, but the other important questions will need to be investigated in the coming years if we want to full understand the role of PAR2 in the physiology of the brain and disease conditions. Indeed, PAR2 could be a target for possible therapeutic applications in the treatment of CNS diseases.

Publication

Paper under process

Roua Abulkassim, Ros Brett, Tong Zang, David G. Watson, Oliver Sutcliffe and Trevor J. Bushell (2013). Proteinase-activated receptor-2 is involved in the behavioural changes associated with sickness behaviour. Pyschopharmacology

Abstract

Abulkassim, R., Bushell, T. and Brett, R (2011). Deletion of the proteinase-activated receptor-2 has limitated effects on anxiety behaviour and spares reference and working memory. British association for psychopharmacology (**BAP**), volume 25, page A51.

Posters and abstract for confernces

Abulkassim, R., Bushell, T. and Brett, R (2012). Determining the role of proteinase-activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory. **8** Federation of European neurosciences (**FENS)** forum of neurosience, Barcelona, Spain, July 2012

Abulkassim, R., Bushell, T. and Brett, R (2012). Determining the role of proteinase-activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory. **CENSUS** and health technologies neuroscience conference. University of Strathclyde, Glasgow, UK, May 2012.

Abulkassim, R., Bushell, T. and Brett, R (2012). Determining the role of proteinase-activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory. Glasgow neuroscience day. Glasgow, UK, January 2012

Abulkassim, R., Bushell, T. and Brett, R (2011). Is proteinase-activated receptor-2 involved in mouse behavior?. Annual research for University of Strathclyde, University of Strathclyde, Glasgow, UK, June 2011

Abulkassim, R., Bushell, T. and Brett, R (2011). Determining the role of proteinase-activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory. 8 th Scottish Neuroscience Group **(SNG)** 2011. University of Aberdeen Aberdeen, UK, Augest 2012

Abulkassim, R., Bushell, T. and Brett, R (2011). Deletion of the proteinase-activated receptor-2 has limitated effects on anxiety behaviour and spares reference and working memory. BNA symposium Neuro-developmental disorders a cross the life span. University of Edinburgh, Edinburgh, UK. September,2011

Abulkassim, R., Bushell, T. and Brett, R (2010). Determining the role of proteinase-activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory. SIPIBS research day of Strathclyde University. University of Strathclyde, Glasgow, UK, May 2010

Abulkassim, R., Bushell, T. and Brett, R (2010). Determining the role of proteinase-activated receptor-2 in mouse behaviour: locomotor activity, anxiety and learning and memory. The British Neuroscience Association (**BNA**) meeting 2011. Harrogate, UK. April, 2011

References

Abayomi, OK (2002) Pathogenesis of cognitive decline following therapeutic irradiation for head and neck tumors. Acta Oncol 41(4): 346-351.

Aben, B, Stapert, S, Blokland, A (2012) About the Distinction between Working Memory and Short-Term Memory. Front Psychol 3: 301.

Abey, HT, Fairlie, DP, Moffatt, JD, Balzary, RW, Cocks, TM (2006) Protease-activated receptor-2 peptides activate neurokinin-1 receptors in the mouse isolated trachea. The Journal of pharmacology and experimental therapeutics 317(2): 598-605.

Abrahams, S, Pickering, A, Polkey, CE, Morris, RG (1997) Spatial memory deficits in patients with unilateral damage to the right hippocampal formation. Neuropsychologia 35(1): 11-24.

Adams, MN, Ramachandran, R, Yau, MK, Suen, JY, Fairlie, DP, Hollenberg, MD, Hooper, JD (2011) Structure, function and pathophysiology of protease activated receptors. Pharmacol Ther 130(3): 248-282.

Afkhami-Goli, A, Noorbakhsh, F, Keller, AJ, Vergnolle, N, Westaway, D, Jhamandas, JH, Andrade-Gordon, P, Hollenberg, MD, Arab, H, Dyck, RH, Power, C (2007) Proteinase-activated receptor-2 exerts protective and pathogenic cell type-specific effects in Alzheimer's disease. J Immunol 179(8): 5493-5503.

Aggleton, JP, Albasser, MM, Aggleton, DJ, Poirier, GL, Pearce, JM (2010) Lesions of the rat perirhinal cortex spare the acquisition of a complex configural visual discrimination yet impair object recognition. Behav Neurosci 124(1): 55-68.

Aggleton, JP, Hunt, PR, Rawlins, JN (1986) The effects of hippocampal lesions upon spatial and non-spatial tests of working memory. Behav Brain Res 19(2): 133-146.

Akiyama, H, Barger, S, Barnum, S, Bradt, B, Bauer, J, Cole, GM, Cooper, NR, Eikelenboom, P, Emmerling, M, Fiebich, BL, Finch, CE, Frautschy, S, Griffin, WS, Hampel, H, Hull, M, (2000) Inflammation and Alzheimer's disease. Neurobiol Aging 21(3): 383-421.

Al-Ani, B, Hansen, KK, Hollenberg, MD (2004) Proteinase-activated receptor-2: key role of amino-terminal dipeptide residues of the tethered ligand for receptor activation. Mol Pharmacol 65(1): 149-156.

Alavijeh, MS, Chishty, M, Qaiser, MZ, Palmer, AM (2005) Drug metabolism and pharmacokinetics, the blood-brain barrier, and central nervous system drug discovery. NeuroRx 2(4): 554-571.

Albasser, MM, Chapman, RJ, Amin, E, Iordanova, MD, Vann, SD, Aggleton, JP (2010) New behavioral protocols to extend our knowledge of rodent object recognition memory. Learn Mem 17(8): 407-419.

Albasser, MM, Davies, M, Futter, JE, Aggleton, JP (2009) Magnitude of the object recognition deficit associated with perirhinal cortex damage in rats: Effects of varying the lesion extent and the duration of the sample period. Behav Neurosci 123(1): 115-124.

Alberts, B, Johnson, A, Lewis, J, al., e (2002) Signaling through G-Protein-Linked Cell-Surface Receptors. In: Molecular Biology of the Cell, 4th edition edn. New York: Garland Science.

Alier, KA, Endicott, JA, Stemkowski, PL, Cenac, N, Cellars, L, Chapman, K, Andrade-Gordon, P, Vergnolle, N, Smith, PA (2008) Intrathecal administration of proteinase-activated receptor-2 agonists produces hyperalgesia by exciting the cell bodies of primary sensory neurons. The Journal of pharmacology and experimental therapeutics 324(1): 224-233.

Alm, AK, Norstrom, E, Sundelin, J, Nystedt, S (1999) Stimulation of proteinase-activated receptor-2 causes endothelial cells to promote blood coagulation in vitro. Thromb Haemost 81(6): 984-988.

Almeida, MC, Trevisan, FN, Barros, RC, Carnio, EC, Branco, LG (1999) Tolerance to lipopolysaccharide is related to the nitric oxide pathway. Neuroreport 10(14): 3061-3065.

Almonte, AG, Hamill, CE, Chhatwal, JP, Wingo, TS, Barber, JA, Lyuboslavsky, PN, David Sweatt, J, Ressler, KJ, White, DA, Traynelis, SF (2007) Learning and memory deficits in mice lacking protease activated receptor-1. Neurobiol Learn Mem 88(3): 295-304.

Almonte, AG, Qadri, LH, Sultan, FA, Watson, JA, Mount, DJ, Rumbaugh, G, Sweatt, JD (2013) Protease-activated receptor-1 modulates hippocampal memory formation and synaptic plasticity. J Neurochem 124(1): 109-122.

Almonte, AG, Qadri, LH, Sultan, FA, Watson, JA, Mount, DJ, Rumbaugh, G, Sweatt, JD (2012) Protease-activated receptor-1 modulates hippocampal memory formation and synaptic plasticity. J Neurochem 124(1): 109-122.

Altemus, M (2006) Sex differences in depression and anxiety disorders: potential biological determinants. Horm Behav 50(4): 534-538.

Amadesi, S, Cottrell, GS, Divino, L, Chapman, K, Grady, EF, Bautista, F, Karanjia, R, Barajas-Lopez, C, Vanner, S, Vergnolle, N, Bunnett, NW (2006) Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. The Journal of physiology 575(Pt 2): 555-571.

Amadesi, S, Nie, J, Vergnolle, N, Cottrell, GS, Grady, EF, Trevisani, M, Manni, C, Geppetti, P, McRoberts, JA, Ennes, H, Davis, JB, Mayer, EA, Bunnett, NW (2004) Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. J Neurosci 24(18): 4300-4312.

American-Psychiatric-Association (2000) Diagnostic and Statistical Manual of Mental Disorders, Fourth edn. Washington DC: American Psychiatric Association.

Anderson, J (1976) Learning and Retention. In: Language, Memory and Thought. Lawrence, Erlbaum (eds), pp 378–437. New Jersey.

Anisman, H, Kokkinidis, L, Borowski, T, Merali, Z (1998) Differential effects of interleukin (IL)-1beta, IL-2 and IL-6 on responding for rewarding lateral hypothalamic stimulation. Brain research 779(1-2): 177-187.

Anisman, H, Merali, Z (1999) Anhedonic and anxiogenic effects of cytokine exposure. Advances in experimental medicine and biology 461: 199-233.

Antunes, M, Biala, G (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process 13(2): 93-110.

Arora, P, Ricks, TK, Trejo, J (2007) Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. J Cell Sci 120(Pt 6): 921-928.

Arroyo-Espliguero, R, Avanzas, P, Jeffery, S, Kaski, JC (2004) CD14 and toll-like receptor 4: a link between infection and acute coronary events? Heart 90(9): 983-988.

Asfaha, S, Cenac, N, Houle, S, Altier, C, Papez, MD, Nguyen, C, Steinhoff, M, Chapman, K, Zamponi, GW, Vergnolle, N (2007) Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation. Br J Pharmacol 150(2): 176-185.

Asokananthan, N, Graham, PT, Fink, J, Knight, DA, Bakker, AJ, McWilliam, AS, Thompson, PJ, Stewart, GA (2002) Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. J Immunol 168(7): 3577-3585.

Astur, RS, Taylor, LB, Mamelak, AN, Philpott, L, Sutherland, RJ (2002) Humans with hippocampus damage display severe spatial memory impairments in a virtual Morris water task. Behav Brain Res 132(1): 77-84.

Atkinson, RC, Shiffrin, RM (1968) Human memory: A proposed system and its control processes. In: The psychology of learning and motivation Spence, KW, Spence, JT (eds), pp 89–195. New York: Academic

Aubry, L, Guetta, D, Klein, G (2009) The arrestin fold: variations on a theme. Curr Genomics 10(2): 133-142.

Awasthi, V, Mandal, SK, Papanna, V, Rao, LV, Pendurthi, UR (2007) Modulation of tissue factor-factor VIIa signaling by lipid rafts and caveolae. Arterioscler Thromb Vasc Biol 27(6): 1447-1455.

Babich, M, King, KL, Nissenson, RA (1990) Thrombin stimulates inositol phosphate production and intracellular free calcium by a pertussis toxin-insensitive mechanism in osteosarcoma cells. Endocrinology 126(2): 948-954.

Baddeley, A (1992) Working memory. Science 255(5044): 556-559.

Baddeley, A (2003) Working memory: looking back and looking forward. Nat Rev Neurosci 4(10): 829-839.

Baffy, G, Yang, L, Raj, S, Manning, DR, Williamson, JR (1994) G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. J Biol Chem 269(11): 8483-8487.

Bailey, KR, Crawley, JN (2009) Anxiety-Related Behaviors in Mice.

Balcaitis, S, Xie, Y, Weinstein, JR, Andersen, H, Hanisch, UK, Ransom, BR, Moller, T (2003) Expression of proteinase-activated receptors in mouse microglial cells. Neuroreport 14(18): 2373-2377.

Banks, PJ, Bashir, ZI, Brown, MW (2012) Recognition memory and synaptic plasticity in the perirhinal and prefrontal cortices. Hippocampus 22(10): 2012-2031.

Bannerman, DM, Yee, BK, Good, MA, Heupel, MJ, Iversen, SD, Rawlins, JN (1999) Double dissociation of function within the hippocampus: a comparison of dorsal, ventral, and complete hippocampal cytotoxic lesions. Behav Neurosci 113(6): 1170-1188.

Bansal, G, Druey, KM, Xie, Z (2007) R4 RGS proteins: Regulation of G-protein signaling and beyond. Pharmacology & Therapeutics 116(3): 473-495.

Banta Lavenex, P, Lavenex, P (2009) Spatial memory and the monkey hippocampus: not all space is created equal. Hippocampus 19(1): 8-19.

Barbosa, PR, Valvassori, SS, Bordignon, CL, Jr., Kappel, VD, Martins, MR, Gavioli, EC, Quevedo, J, Reginatto, FH (2008) The aqueous extracts of Passiflora alata and Passiflora edulis reduce anxiety-related behaviors without affecting memory process in rats. J Med Food 11(2): 282-288.

Bardgett, ME, Henry, JD (1999) Locomotor activity and accumbens Fos expression driven by ventral hippocampal stimulation require D1 and D2 receptors. Neuroscience 94(1): 59-70.

Barker, GR, Bird, F, Alexander, V, Warburton, EC (2007) Recognition memory for objects, place, and temporal order: a disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. J Neurosci 27(11): 2948-2957.

Barry, GD, Le, GT, Fairlie, DP (2006) Agonists and antagonists of protease activated receptors (PARs). Curr Med Chem 13(3): 243-265.

Barry, GD, Suen, JY, Le, GT, Cotterell, A, Reid, RC, Fairlie, DP (2010) Novel agonists and antagonists for human protease activated receptor 2. J Med Chem 53(20): 7428-7440.

Bartus, RT, Dean, RL, 3rd, Beer, B, Lippa, AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217(4558): 408-414.

Bassi, GS, Kanashiro, A, Santin, FM, de Souza, GE, Nobre, MJ, Coimbra, NC (2012) Lipopolysaccharide-induced sickness behaviour evaluated in different models of anxiety and innate fear in rats. Basic Clin Pharmacol Toxicol 110(4): 359-369.

Bats, S, Thoumas, JL, Lordi, B, Tonon, MC, Lalonde, R, Caston, J (2001) The effects of a mild stressor on spontaneous alternation in mice. Behav Brain Res 118(1): 11-15.

Bauerle, P, von der Behrens, W, Kossl, M, Gaese, BH (2011) Stimulus-specific adaptation in the gerbil primary auditory thalamus is the result of a fast frequency-specific habituation and is regulated by the corticofugal system. J Neurosci 31(26): 9708-9722.

Bayley, PJ, O'Reilly, RC, Curran, T, Squire, LR (2008) New semantic learning in patients with large medial temporal lobe lesions. Hippocampus 18(6): 575-583.

Bechara, A, Tranel, D, Damasio, H, Adolphs, R, Rockland, C, Damasio, AR (1995) Double dissociation of conditioning and declarative knowledge relative to the amygdala and hippocampus in humans. Science 269(5227): 1115-1118.

Behl, C (2002) Oestrogen as a neuroprotective hormone. Nat Rev Neurosci 3(6): 433-442.

Belham, CM, Tate, RJ, Scott, PH, Pemberton, AD, Miller, HR, Wadsworth, RM, Gould, GW, Plevin, R (1996) Trypsin stimulates proteinase-activated receptor-2-dependent and -independent activation of mitogen-activated protein kinases. Biochem J 320 (Pt 3): 939-946.

Belzung, C, Gerlai, WECaRT (1999) Chapter 4.11 Measuring rodent exploratory behavior. In: Techniques in the Behavioral and Neural Sciences Vol. Volume 13, pp 738-749: Elsevier.

Beninger, RJ (1983) The role of dopamine in locomotor activity and learning. Brain research 287(2): 173-196.

Berger, P, Perng, DW, Thabrew, H, Compton, SJ, Cairns, JA, McEuen, AR, Marthan, R, Tunon De Lara, JM, Walls, AF (2001) Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells. J Appl Physiol 91(3): 1372-1379.

Bevins, RA, Besheer, J, Palmatier, MI, Jensen, HC, Pickett, KS, Eurek, S (2002) Novel-object place conditioning: behavioral and dopaminergic processes in expression of novelty reward. Behav Brain Res 129(1-2): 41-50.

Billington, CK, Penn, RB (2003) Signaling and regulation of G protein-coupled receptors in airway smooth muscle. Respir Res 4: 2.

Bird, CM, Burgess, N (2008) The hippocampus and memory: insights from spatial processing. Nat Rev Neurosci 9(3): 182-194.

Bizot, JC, Herpin, A, Pothion, S, Pirot, S, Trovero, F, Ollat, H (2005) Chronic treatment with sulbutiamine improves memory in an object recognition task and reduces some amnesic effects of dizocilpine in a spatial delayed-non-match-to-sample task. Prog Neuropsychopharmacol Biol Psychiatry 29(6): 928-935.

Bluthe, RM, Castanon, N, Pousset, F, Bristow, A, Ball, C, Lestage, J, Michaud, B, Kelley, KW, Dantzer, R (1999) Central injection of IL-10 antagonizes the behavioural effects of lipopolysaccharide in rats. Psychoneuroendocrinology 24(3): 301-311.luthe, RM, Kelley, KW, Dantzer, R (2006) Effects of insulin-like growth factor-I on cytokine-induced sickness behavior in mice. Brain, behavior, and immunity 20(1): 57-63.

Bohbot, VD, Iaria, G, Petrides, M (2004) Hippocampal function and spatial memory: evidence from functional neuroimaging in healthy participants and performance of patients with medial temporal lobe resections. Neuropsychology 18(3): 418-425.

Bohm, SK, Kong, W, Bromme, D, Smeekens, SP, Anderson, DC, Connolly, A, Kahn, M, Nelken, NA, Coughlin, SR, Payan, DG, Bunnett, NW (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochem J 314 (Pt 3): 1009-1016.

Boileau, C, Nathalie Amiable, Jean-Pierre, Pelletier, and Martel- pelletier (2008) Proteinase-activated receptor-2 A new and attractive therapeutic target for osteoarthritis. Musculoskeletal review 43-44.

Boitano, S, Flynn, AN, Schulz, SM, Hoffman, J, Price, TJ, Vagner, J (2011) Potent agonists of the protease activated receptor 2 (PAR2). J Med Chem 54(5): 1308-1313.

Bonni, A, Brunet, A, West, AE, Datta, SR, Takasu, MA, Greenberg, ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286(5443): 1358-1362.

Borsini, F, Podhorna, J, Marazziti, D (2002) Do animal models of anxiety predict anxiolytic-like effects of antidepressants? Psychopharmacology (Berl) 163(2): 121-141.

Bouras, naH, G. (2007) Psychiatric and Behavioural Disorders in Intellectual and Developmental Disabilities 2nd edn: Cambridge University Press: UK.

Bourin, M, Petit-Demouliere, B, Dhonnchadha, BN, Hascoet, M (2007) Animal models of anxiety in mice. Fundam Clin Pharmacol 21(6): 567-574.

Boven, LA, Vergnolle, N, Henry, SD, Silva, C, Imai, Y, Holden, J, Warren, K, Hollenberg, MD, Power, C (2003) Up-regulation of proteinase-activated receptor 1 expression in astrocytes during HIV encephalitis. J Immunol 170(5): 2638-2646.

Braff, D, Stone, C, Callaway, E, Geyer, M, Glick, I, Bali, L (1978) Prestimulus Effects on Human Startle Reflex in Normals and Schizophrenics. Psychophysiology 15(4): 339-343.

Braff, DL, Geyer, MA (1990) Sensorimotor gating and schizophrenia. Human and animal model studies. Arch Gen Psychiatry 47(2): 181-188.

Brass, LF, Manning, DR, Williams, AG, Woolkalis, MJ, Poncz, M (1991) Receptor and G protein-mediated responses to thrombin in HEL cells. J Biol Chem 266(2): 958-965.

Bremner, JD (2004) Brain imaging in anxiety disorders. Expert Rev Neurother 4(2): 275-284.

Bretschneider, E, Kaufmann, R, Braun, M, Nowak, G, Glusa, E, Schror, K (2001) Evidence for functionally active protease-activated receptor-4 (PAR-4) in human vascular smooth muscle cells. Br J Pharmacol 132(7): 1441-1446.

Brito, GN, Brito, LS (1990) Septohippocampal system and the prelimbic sector of frontal cortex: a neuropsychological battery analysis in the rat. Behav Brain Res 36(1-2): 127-146.

Broadbent, NJ, Gaskin, S, Squire, LR, Clark, RE (2010) Object recognition memory and the rodent hippocampus. Learn Mem 17(1): 5-11.

Broadbent, NJ, Squire, LR, Clark, RE (2004) Spatial memory, recognition memory, and the hippocampus. Proceedings of the National Academy of Sciences of the United States of America 101(40): 14515-14520.

Brody, DL, Holtzman, DM (2006) Morris water maze search strategy analysis in PDAPP mice before and after experimental traumatic brain injury. Exp Neurol 197(2): 330-340.

Brody, SA, Dulawa, SC, Conquet, F, Geyer, MA (2004) Assessment of a prepulse inhibition deficit in a mutant mouse lacking mGlu5 receptors. Mol Psychiatry 9(1): 35-41.

Brown, GR, Nemes, C (2008) The exploratory behaviour of rats in the hole-board apparatus: is head-dipping a valid measure of neophilia? Behav Processes 78(3): 442-448.

Brown, MW, Aggleton, JP (2001) Recognition memory: What are the roles of the perirhinal cortex and hippocampus? Nat Rev Neurosci 2(1): 51-61.

Brown, R, Corey, S, Moore, A (1999) Differences in Measures of Exploration and Fear in MHC-Congenic C57BL/6J and B6-H-2K Mice. Behavior Genetics 29(4): 263-271.

Bryan, K, Lee, H, Perry, G, (2009) Transgenic Mouse Models of Alzheimer's Disease: Behavioral Testing and Considerations. In: Methods of Behavior Analysis in Neuroscience., Buccafusco, J (ed), 2nd Edirion edn. Boca Raton (FL): CRC Press.

Bucci, M, Roviezzo, F, Cirino, G (2005) Protease-activated receptor-2 (PAR2) in cardiovascular system. Vascular Pharmacology 43(4): 247-253.

Bucci, M, Vellecco, V, Harrington, L, Brancaleone, V, Roviezzo, F, Mattace Raso, G, Ianaro, A, Lungarella, G, De Palma, R, Meli, R, Cirino, G (2012) Cross-talk between toll-like receptor 4 (TLR4) and proteinase-activated receptor 2 (PAR2) is involved in vascular function. British journal of pharmacology 168(2): 411-420.

Buchanan, JB, Johnson, RW (2007) Regulation of food intake by inflammatory cytokines in the brain. Neuroendocrinology 86(3): 183-190.

Bunnett, NW (2006) Protease-activated receptors: how proteases signal to cells to cause inflammation and pain. Seminars in thrombosis and hemostasis 32 Suppl 1: 39-48.

Buresi, MC, Buret, AG, Hollenberg, MD, MacNaughton, WK (2002) Activation of proteinase-activated receptor 1 stimulates epithelial chloride secretion through a unique MAP kinase- and cyclo-oxygenase-dependent pathway. Faseb J 16(12): 1515-1525.

Burgess, N, Maguire, EA, O'Keefe, J (2002) The human hippocampus and spatial and episodic memory. Neuron 35(4): 625-641.

Burke, SN, Wallace, JL, Nematollahi, S, Uprety, AR, Barnes, CA (2010) Pattern separation deficits may contribute to age-associated recognition impairments. Behav Neurosci 124(5): 559-573.

Burton, MD, Sparkman, NL, Johnson, RW (2011) Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. J Neuroinflammation 8: 54.

Bushell, T (2007) The emergence of proteinase-activated receptor-2 as a novel target for the treatment of inflammation-related CNS disorders. The Journal of physiology 581(Pt 1): 7-16.

Bushell, TJ, Plevin, R, Cobb, S, Irving, AJ (2006) Characterization of proteinase-activated receptor 2 signalling and expression in rat hippocampal neurons and astrocytes. Neuropharmacology 50(6): 714-725.

C. Neill, Kulkarni, J (2011) Biological Basis of Sex Differences in Psychopharmacology.

Caeser, M, Ostwald, J, Pilz, PK (1989) Startle responses measured in muscles innervated by facial and trigeminal nerves show common modulation. Behav Neurosci 103(5): 1075-1081.

Calapai, G, Mazzaglia, G, Cilia, M, Zingarelli, B, Squadrito, F, Caputi, AP (1994) Mediation by nitric oxide formation in the preoptic area of endotoxin and tumour necrosis factor-induced inhibition of water intake in the rat. British journal of pharmacology 111(4): 1328-1332.

Calapai, G, Squadrito, F, Altavilla, D, Zingarelli, B, Campo, GM, Cilia, M, Caputi, AP (1992) Evidence that nitric oxide modulates drinking behaviour. Neuropharmacology 31(8): 761-764.

Campbell, A (2004) Inflammation, neurodegenerative diseases, and environmental exposures. Ann N Y Acad Sci 1035: 117-132.

Cannon, CM, Palmiter, RD (2003) Reward without dopamine. J Neurosci 23(34): 10827-10831.

Capuron, L, Ravaud, A (1999) Prediction of the depressive effects of interferon alfa therapy by the patient's initial affective state. The New England journal of medicine 340(17): 1370.

Carey, RJ, Damianopoulos, EN, Shanahan, AB (2008) Cocaine conditioned behavior: a cocaine memory trace or an anti-habituation effect. Pharmacol Biochem Behav 90(4): 625-631.

Cenac, N, Chin, AC, Garcia-Villar, R, Salvador-Cartier, C, Ferrier, L, Vergnolle, N, Buret, AG, Fioramonti, J, Bueno, L (2004) PAR2 activation alters colonic paracellular permeability in mice via IFN-gamma-dependent and -independent pathways. J Physiol 558(Pt 3): 913-925.

Chambon, C, Wegener, N, Gravius, A, Danysz, W (2011) A new automated method to assess the rat recognition memory: Validation of the method. Behavioural Brain Research 222(1): 151-157.

Chan, AK, Vergnolle, N, Hollenberg, MD, von der Weid, PY (2004) Proteinase-activated receptor 2 activation modulates guinea-pig mesenteric lymphatic vessel pacemaker potential and contractile activity. The Journal of physiology 560(Pt 2): 563-576.

Chan, P, Thomas, CJ, Sprang, SR, Tall, GG (2013) Molecular chaperoning function of Ric-8 is to fold nascent heterotrimeric G protein Î± subunits.

Chance, WT, Fischer, JE (1991) Aphagic and adipsic effects of interleukin-1. Brain research 568(1-2): 261-264.

Charlton, BG (2000) The malaise theory of depression: major depressive disorder is sickness behavior and antidepressants are analgesic. Med Hypotheses 54(1): 126-130.

Chen, D., Carpenter, A., Abrahams, J., Chambers, R.C., Lechler, R.I., Mcvey, J.H. & Dorling, A. (2008) Protease-activated receptor 1 activation is necessary for monocyte chemoattractant protein 1-dependent leukocyte recruitment in vivo. J Exp Med, 205, 1739-46.

Chen, J, Ishii, M, Wang, L, Ishii, K, Coughlin, SR (1994) Thrombin receptor activation. Confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. J Biol Chem 269(23): 16041-16045.

Chen, TL, Lin, YF, Cheng, CW, Chen, SY, Sheu, MT, Leung, TK, Qin, CH, Chen, CH (2011) Anti-Inflammatory mechanisms of the proteinase-activated receptor 2-inhibiting peptide in human synovial cells. J Biomed Sci 18: 43.

Chen, X, Zhang, B, Fischer, JA (2002) A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev 16(3): 289-294.

Chodobski, A, Zink, BJ, Szmydynger-Chodobska, J (2011) Blood-brain barrier pathophysiology in traumatic brain injury. Transl Stroke Res 2(4): 492-516.

Choi, BH, Kim, RC, Vaughan, PJ, Lau, A, Van Nostrand, WE, Cotman, CW, Cunningham, DD (1995) Decreases in protease nexins in Alzheimer's disease brain. Neurobiol Aging 16(4): 557-562.

Choi, KY, Swierczewska, M, Lee, S, Chen, X (2012) Protease-activated drug development. Theranostics 2(2): 156-178.

Christmas, AJ, Maxwell, DR (1970) A comparison of the effects of some benzodiazepines and other drugs on aggressive and exploratory behaviour in mice and rats. Neuropharmacology 9(1): 17-29.

Cicala, C, Pinto, A, Bucci, M, Sorrentino, R, Walker, B, Harriot, P, Cruchley, A, Kapas, S, Howells, GL, Cirino, G (1999) Protease-activated receptor-2 involvement in hypotension in normal and endotoxemic rats in vivo. Circulation 99(19): 2590-2597.

Cirino, G, Napoli, C, Bucci, M, Cicala, C (2000) Inflammation-coagulation network: are serine protease receptors the knot? Trends in pharmacological sciences 21(5): 170-172.

Cisowski, J, O'Callaghan, K, Kuliopulos, A, Yang, J, Nguyen, N, Deng, Q, Yang, E, Fogel, M, Tressel, S, Foley, C, Agarwal, A, Hunt, SW, 3rd, McMurry, T, Brinckerhoff, L, Covic, L (2011) Targeting protease-activated receptor-1 with cell-penetrating pepducins in lung cancer. Am J Pathol 179(1): 513-523.

Clarke, JR, Cammarota, M, Gruart, A, Izquierdo, I, Delgado-Garcia, JM (2010) Plastic modifications induced by object recognition memory processing. Proceedings of the National Academy of Sciences of the United States of America 107(6): 2652-2657.

Cocks, TM, Fong, B, Chow, JM, Anderson, GP, Frauman, AG, Goldie, RG, Henry, PJ, Carr, MJ, Hamilton, JR, Moffatt, JD (1999) A protective role for protease-activated receptors in the airways. Nature 398(6723): 156-160.

Cocks, TM, Moffatt, JD (2000) Protease-activated receptors: sentries for inflammation? Trends in pharmacological sciences 21(3): 103-108.

Cocks, TM, Moffatt, JD (2001) Protease-activated receptor-2 (PAR2) in the airways. Pulmonary pharmacology & therapeutics 14(3): 183-191.

Congreve, M, Marshall, F (2010) The impact of GPCR structures on pharmacology and structure-based drug design. British Journal of Pharmacology 159(5): 986-996.

Connolly, AJ, Ishihara, H, Kahn, ML, Farese, RV, Jr., Coughlin, SR (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381(6582): 516-519.

Connolly, TM, Condra, C, Feng, DM, Cook, JJ, Stranieri, MT, Reilly, CF, Nutt, RF, Gould, RJ (1994) Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. Thromb Haemost 72(4): 627-633.

Cook, JJ, Sitko, GR, Bednar, B, Condra, C, Mellott, MJ, Feng, DM, Nutt, RF, Shafer, JA, Gould, RJ, Connolly, TM (1995) An antibody against the exosite of the cloned thrombin receptor inhibits experimental arterial thrombosis in the African green monkey. Circulation 91(12): 2961-2971.

Cooper, G (2000) The Cell: A Molecular Approach. In: Pathways of Intracellular Signal Transduction, (MA), S (ed), 2nd edition edn. Sinauer Associates.

Copeland, S, Warren, HS, Lowry, SF, Calvano, SE, Remick, D (2005) Acute inflammatory response to endotoxin in mice and humans. Clin Diagn Lab Immunol 12(1): 60-67.

Corkin, S (2002) What's new with the amnesic patient H.M.? Nat Rev Neurosci 3(2): 153-160.

Coughlin, SR (2000) Thrombin signalling and protease-activated receptors. Nature 407(6801): 258-264.

Coughlin, SR (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost 3(8): 1800-1814.

Covic, L, Gresser, AL, Kuliopulos, A (2000) Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. Biochemistry 39(18): 5458-5467.

Covic, T, Cumming, SR, Pallant, JF, Manolios, N, Emery, P, Conaghan, PG, Tennant, A (2012) Depression and anxiety in patients with rheumatoid arthritis: prevalence rates based on a comparison of the Depression, Anxiety and Stress Scale (DASS) and the hospital, Anxiety and Depression Scale (HADS). BMC Psychiatry 12: 6.

Cowan, N (2008) What are the differences between long-term, short-term, and working memory? Prog Brain Res 169: 323-338.

Cranston, WI (1965) The regulation of body temperature in fever. Proc R Soc Med 58(9): 739-740.

Crawley, JN (2000) What's wrong with my mouse?: behavioral phenotyping of transgenic and knockout mice. Wiley-Liss.

Crilly, A., Burns, E., Nickdel, M.B., Lockhart, J.C., Perry, M.E., Ferrell, P.W., Baxter, D., Dale, J., Dunning, L., Wilson, H., Nijjar, J.S., Gracie, J.A., Ferrell,

W.R. & Mcinnes, I.B. (2012) PAR(2) expression in peripheral blood monocytes of patients with rheumatoid arthritis. Ann Rheum Dis, 71, 1049-54.

Cross, L, Brown, MW, Aggleton, JP, Warburton, EC (2012) The medial dorsal thalamic nucleus and the medial prefrontal cortex of the rat function together to support associative recognition and recency but not item recognition. Learn Mem 20(1): 41-50.

Crusio, WE, van Abeelen, JH (1986) The genetic architecture of behavioural responses to novelty in mice. Heredity (Edinb) 56 (Pt 1): 55-63.

Cryan, JF, Mombereau, C, Vassout, A (2005) The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 29(4-5): 571-625.

Cunningham, C, Campion, S, Teeling, J, Felton, L, Perry, VH (2007) The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C). Brain, behavior, and immunity 21(4): 490-502.

Cunningham, MA, Rondeau, E, Chen, X, Coughlin, SR, Holdsworth, SR, Tipping, PG (2000) Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. J Exp Med 191(3): 455-462.

Cunningham, MR, McIntosh, KA, Pediani, JD, Robben, J, Cooke, AE, Nilsson, M, Gould, GW, Mundell, S, Milligan, G, Plevin, R (2012) Novel role for proteinase-activated receptor 2 (PAR2) in membrane trafficking of proteinase-activated receptor 4 (PAR4). J Biol Chem 287(20): 16656-16669.

Cunningham-Rundles, S, McNeeley, DF, Moon, A (2005) Mechanisms of nutrient modulation of the immune response. J Allergy Clin Immunol 115(6): 1119-1128; quiz 1129.

Curzon, P, Zhang, M, Radek, RJ, Fox, GB (2009) The Behavioral Assessment of Sensorimotor Processes in the Mouse: Acoustic Startle, Sensory Gating, Locomotor Activity, Rotarod, and Beam Walking.

Damian, M, Mary, S, Martin, A, Pin, JP, Baneres, JL (2008) G protein activation by the leukotriene B4 receptor dimer. Evidence for an absence of trans-activation. J Biol Chem 283(30): 21084-21092.

Damiano, BP, Cheung, WM, Santulli, RJ, Fung-Leung, WP, Ngo, K, Ye, RD, Darrow, AL, Derian, CK, de Garavilla, L, Andrade-Gordon, P (1999) Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. J Pharmacol Exp Ther 288(2): 671-678.

D'Andrea, MR, Derian, CK, Leturcq, D, Baker, SM, Brunmark, A, Ling, P, Darrow, AL, Santulli, RJ, Brass, LF, Andrade-Gordon, P (1998) Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. J Histochem Cytochem 46(2): 157-164.

Dantzer, R (2001a) Cytokine-induced sickness behavior: mechanisms and implications. Ann N Y Acad Sci 933: 222-234.

Dantzer, R (2001b) Cytokine-induced sickness behavior: where do we stand? Brain, behavior, and immunity 15(1): 7-24.

Dantzer, R (2004) Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. Eur J Pharmacol 500(1-3): 399-411.

Dantzer, R (2009) Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am 29(2): 247-264.

Dantzer, R, O'Connor, JC, Freund, GG, Johnson, RW, Kelley, KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9(1): 46-56.

Dantzer, R, R. M. Bluthé, A. Aubert, G. Goodall, J. L. Bret-Dibat, S. Kent, E. Goujon, S. Layé, P. Parnet & K. W. Kelley (1996) Cytokine actions on behavior. In Cytokines in the Nervous System. N. J. Rothwell, Ed edn: Landes. Georgetown, MD.

Dantzer, R, Wollman, EE, Vitkovic, L, Yirmiya, R (1999) Cytokines, stress, and depression. Conclusions and perspectives. Advances in experimental medicine and biology 461: 317-329.

D'Aquila, PS, Collu, M, Gessa, GL, Serra, G (2000) The role of dopamine in the mechanism of action of antidepressant drugs. Eur J Pharmacol 405(1-3): 365-373.

Darmoul, D, Gratio, V, Devaud, H, Lehy, T, Laburthe, M (2003) Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. Am J Pathol 162(5): 1503-1513.

Davis, M (1979) Diazepam and flurazepam: effects on conditioned fear as measured with the potentiated startle paradigm. Psychopharmacology (Berl) 62(1): 1-7.

Davis, M (1980) Neurochemical modulation of sensory-motor reactivity: acoustic and tactile startle reflexes. Neurosci Biobehav Rev 4(2): 241-263.

Davis, M (1989) Sensitization of the acoustic startle reflex by footshock. Behav Neurosci 103(3): 495-503.

Davis, M (1992) The role of the amygdala in fear and anxiety. Annu Rev Neurosci 15: 353-375.

Davis, M, Walker, DL, Lee, Y (1997) Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex. Philos Trans R Soc Lond B Biol Sci 352(1362): 1675-1687.

Davison, GC (2008) Abnormal Psychology. Toronto:Veronica Visentin.

Dawson, GR, Tricklebank, MD (1995) Use of the elevated plus maze in the search for novel anxiolytic agents. Trends in pharmacological sciences 16(2): 33-36.

Deacon, RM, Bannerman, DM, Rawlins, NP (2001) Conditional discriminations based on external and internal cues in rats with cytotoxic hippocampal lesions. Behav Neurosci 115(1): 43-57.

Deacon, RM, Rawlins, JN (2006) T-maze alternation in the rodent. Nat Protoc 1(1): 7-12.

Deacon, RMJ, Rawlins, JNP (2005) Hippocampal lesions, species-typical behaviours and anxiety in mice. Behavioural Brain Research 156(2): 241-249.

Debeir, T, Benavides, J, Vige, X (1998) Involvement of protease-activated receptor-1 in the in vitro development of mesencephalic dopaminergic neurons. Neuroscience 82(3): 739-752.

DeFea, KA, Zalevsky, J, Thoma, MS, Dery, O, Mullins, RD, Bunnett, NW (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. J Cell Biol 148(6): 1267-1281.

Dehouck, MP, Dehouck, B, et al (1995) Drug transport to the brain: comparison between in vitro and in vivo models of the blood-brain barrier. European Journal of Pharmaceutical Sciences. 3(6): 357-365.

Delatour, B, Gisquet-Verrier, P (1996) Prelimbic cortex specific lesions disrupt delayed-variable response tasks in the rat. Behav Neurosci 110(6): 1282-1298.

Dell'Osso, B, Buoli, M, Baldwin, DS, Altamura, AC (2010) Serotonin norepinephrine reuptake inhibitors (SNRIs) in anxiety disorders: a comprehensive review of their clinical efficacy. Hum Psychopharmacol 25(1): 17-29.

Dember, WN, Fowler, H (1959) Spontaneous alteration after free and forced trials. Can J Psychol 13: 151-154.

Dery, O, Corvera, CU, Steinhoff, M, Bunnett, NW (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Physiol 274(6 Pt 1): C1429-1452.

Deschepper, CF, Bigornia, V, Berens, ME, Lapointe, MC (1991) Production of thrombin and antithrombin III by brain and astroglial cell cultures. Brain Res Mol Brain Res 11(3-4): 355-358.

D'Hooge, R, De Deyn, PP (2001) Applications of the Morris water maze in the study of learning and memory. Brain Res Brain Res Rev 36(1): 60-90.

Diaz, M, Becker, DE (2010) Thermoregulation: physiological and clinical considerations during sedation and general anesthesia. Anesth Prog 57(1): 25-32; quiz 33-24.

Dihanich, M, Kaser, M, Reinhard, E, Cunningham, D, Monard, D (1991) Prothrombin mRNA is expressed by cells of the nervous system. Neuron 6(4): 575-581.

Dinarello, CA (1988) Biology of interleukin 1. FASEB J 2(2): 108-115.

Dinarello, CA (1997) Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. Chest. 1997 Dec;112(6 Suppl):321S-329S.

Dinarello, CA (2000) Proinflammatory cytokines. Chest 118(2): 503-508.

Dinarello, CA, Renfer, L, Wolff, SM (1977) Human leukocytic pyrogen: purification and development of a radioimmunoassay. Proceedings of the National Academy of Sciences of the United States of America 74(10): 4624-4627.

Divac, I, Wikmark, R, Gade, A (1975) Spontaneous alternation in rats with lesions in the frontal lobes: An extension of the frontal lobe syndrome. Physiological Psychology 3(1): 39-42.

Dobbins, IG, Foley, H, Schacter, DL, Wagner, AD (2002) Executive control during episodic retrieval: multiple prefrontal processes subserve source memory. Neuron 35(5): 989-996.

Donovan, FM, Pike, CJ, Cotman, CW, Cunningham, DD (1997) Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and RhoA activities. J Neurosci 17(14): 5316-5326

Doornbos, B, Fokkema, DS, Molhoek, M, Tanke, MA, Postema, F, Korf, J (2009) Abrupt rather than gradual hormonal changes induce postpartum blues-like behavior in rats. Life sciences 84(3-4): 69-74.

Dotti, CG, Galvan, C, Ledesma, MD (2004) Plasmin deficiency in Alzheimer's disease brains: causal or casual? Neurodegener Dis 1(4-5): 205-212.

Drevets, WC (2001) Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. Curr Opin Neurobiol 11(2): 240-249.

Drevets, WC, Price, JL, Furey, ML (2008) Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. Brain Struct Funct 213(1-2): 93-118.

Dube, C, Vezzani, A, Behrens, M, Bartfai, T, Baram, TZ (2005) Interleukin-1beta contributes to the generation of experimental febrile seizures. Annals of neurology 57(1): 152-155.

Dubreuil, D, Tixier, C, Dutrieux, G, Edeline, JM (2003) Does the radial arm maze necessarily test spatial memory? Neurobiol Learn Mem 79(1): 109-117.

Dudchenko, PA (2004) An overview of the tasks used to test working memory in rodents. Neurosci Biobehav Rev 28(7): 699-709.

Dunn, AJ, Swiergiel, AH (2005) Effects of interleukin-1 and endotoxin in the forced swim and tail suspension tests in mice. Pharmacol Biochem Behav 81(3): 688-693.

Dunn, AJ, Swiergiel, AH, de Beaurepaire, R (2005) Cytokines as mediators of depression: what can we learn from animal studies? Neurosci Biobehav Rev 29(4-5): 891-909.

Dunne, A, Ejdeback, M, Ludidi, PL, O'Neill, LA, Gay, NJ (2003) Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. J Biol Chem 278(42): 41443-41451.

Durant, C, Christmas, D, Nutt, D (2010) The pharmacology of anxiety. Curr Top Behav Neurosci 2: 303-330.

Dutra-Oliveira, A, Monteiro, RQ, Mariano-Oliveira, A (2012) Protease-activated receptor-2 (PAR2) mediates VEGF production through the ERK1/2 pathway in human glioblastoma cell lines. Biochem Biophys Res Commun 421(2): 221-227.

Eccleston, C (2001) Role of psychology in pain management. Br J Anaesth 87(1): 144-152.

Eglen, R, Reisine, T (2011) GPCRs Revisited: New Insights Lead to Novel Drugs. Pharmaceuticals 4(2): 244-272.

Egleton, RD, Davis, TP (2005) Development of neuropeptide drugs that cross the blood-brain barrier. NeuroRx 2(1): 44-53.

Eichenbaum, H (1996) Is the rodent hippocampus just for â€˜placeâ€™? Current Opinion in Neurobiology 6(2): 187-195.

Eichenbaum, H (2000) A cortical-hippocampal system for declarative memory. Nat Rev Neurosci 1(1): 41-50.

Eichenbaum, H, Dudchenko, P, Wood, E, Shapiro, M, Tanila, H (1999) The hippocampus, memory, and place cells: is it spatial memory or a memory space? Neuron 23(2): 209-226.

Ekdahl, CT, Claasen, J-H, Bonde, S, Kokaia, Z, Lindvall, O (2003) Inflammation is detrimental for neurogenesis in adult brain Vol. 100, pp 13632-13637.

Engin, E, Treit, D (2007) The role of hippocampus in anxiety: intracerebral infusion studies. Behav Pharmacol 18(5-6): 365-374.

Ennaceur, A (2010) One-trial object recognition in rats and mice: methodological and theoretical issues. Behav Brain Res 215(2): 244-254.

Ennaceur, A, Delacour, J (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31(1): 47-59.

Ennaceur, A, Neave, N, Aggleton, JP (1997) Spontaneous object recognition and object location memory in rats: the effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. Exp Brain Res 113(3): 509-519.

Etkin, A, Prater, KE, Schatzberg, AF, Menon, V, Greicius, MD (2009) Disrupted amygdalar subregion functional connectivity and evidence of a compensatory network in generalized anxiety disorder. Arch Gen Psychiatry 66(12): 1361-1372.

Evans, DL, Charney, DS, Lewis, L, Golden, RN, Gorman, JM, Krishnan, KR, Nemeroff, CB, Bremner, JD, Carney, RM, Coyne, JC, et al (2005) Mood disorders in the medically ill: scientific review and recommendations. Biol Psychiatry 58(3): 175-189.

Everts-van der Wind, A, Kata, SR, Band, MR, Rebeiz, M, Larkin, DM, Everts, RE, Green, CA, Liu, L, Natarajan, S, Goldammer, T, Lee, JH, McKay, S, Womack, JE, Lewin, HA (2004) A 1463 gene cattle-human comparative map with anchor points defined by human genome sequence coordinates. Genome Res 14(7): 1424-1437.

Feinstein, A, Roy, P, Lobaugh, N, Feinstein, K, O'Connor, P, Black, S (2004) Structural brain abnormalities in multiple sclerosis patients with major depression. Neurology 62(4): 586-590.

Fernstrom, JD, Munger, SD, Sclafani, A, de Araujo, IE, Roberts, A, Molinary, S (2012) Mechanisms for sweetness. J Nutr 142(6): 1134S-1141S.

Ferrell, WR, Lockhart, JC, Kelso, EB, Dunning, L, Plevin, R, Meek, SE, Smith, AJ, Hunter, GD, McLean, JS, McGarry, F, Ramage, R, Jiang, L, Kanke, T,

Kawagoe, J (2003) Essential role for proteinase-activated receptor-2 in arthritis. J Clin Invest 111(1): 35-41.

Ferrell, WR, Lockhart, JC, Kelso, EB, Dunning, L, Plevin, R, Meek, SE, Smith, AJ, Hunter, GD, McLean, JS, McGarry, F, Ramage, R, Jiang, L, Kanke, T, Kawagoe, J (2003) Essential role for proteinase-activated receptor-2 in arthritis. J Clin Invest 111(1): 35-41.

Feuerstein, GZ, Liu, T, Barone, FC (1994) Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha. Cerebrovasc Brain Metab Rev 6(4): 341-360.

File, SE (1980) The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. J Neurosci Methods 2(3): 219-238.

Finger, BC, Dinan, TG, Cryan, JF (2010) Progressive ratio responding in an obese mouse model: Effects of fenfluramine. Neuropharmacology 59(7-8): 619-626.

Fink, G, Sumner, BE, McQueen, JK, Wilson, H, Rosie, R (1998) Sex steroid control of mood, mental state and memory. Clin Exp Pharmacol Physiol 25(10): 764-775.

Finn, PR, Justus, A, Mazas, C, Steinmetz, JE (1999) Working memory, executive processes and the effects of alcohol on Go/No-Go learning: testing a model of behavioral regulation and impulsivity. Psychopharmacology (Berl) 146(4): 465-472.

Fiorucci, S, Distrutti, E (2002) Role of PAR2 in pain and inflammation. Trends Pharmacol Sci 23(4): 153-155.

Fiorucci, S, Mencarelli, A, Palazzetti, B, Distrutti, E, Vergnolle, N, Hollenberg, MD, Wallace, JL, Morelli, A, Cirino, G (2001) Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. Proceedings of the National Academy of Sciences of the United States of America 98(24): 13936-13941.

Fletcher, PJ, Selhi, ZF, Azampanah, A, Sills, TL (2001) Reduced Brain Serotonin Activity Disrupts Prepulse Inhibition of the Acoustic Startle Reflex: Effects of 5,7-dihydroxytryptamine and p-chlorophenylalanine. Neuropsychopharmacology 24(4): 399-409.

Flynn, AN, Hoffman, J, Tillu, DV, Sherwood, CL, Zhang, Z, Patek, R, Asiedu, MN, Vagner, J, Price, TJ, Boitano, S (2013) Development of highly potent protease-activated receptor 2 agonists via synthetic lipid tethering. FASEB J 27(4): 1498-1510.

Foster, TC, Barnes, CA, Rao, G, McNaughton, BL (1991) Increase in perforant path quantal size in aged F-344 rats. Neurobiology of aging 12(5): 441-448.

Frick, KM, Burlingame, LA, Arters, JA, Berger-Sweeney, J (1999) Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. Neuroscience 95(1): 293-307.

Frohman, TC, Castro, W, Shah, A, Courtney, A, Ortstadt, J, Davis, SL, Logan, D, Abraham, T, Abraham, J, Remington, G, Treadaway, K, Graves, D, Hart, J, Stuve, O, Lemack, G, Greenberg, B, Frohman, EM (2011) Symptomatic therapy in multiple sclerosis. Ther Adv Neurol Disord 4(2): 83-98.

Frye, CA, Petralia, SM, Rhodes, ME (2000) Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3alpha,5alpha-THP. Pharmacol Biochem Behav 67(3): 587-596.

Fujiwara, M., Jin, E., Ghazizadeh, M. & Kawanami, O. (2005) Activation of PAR4 induces a distinct actin fiber formation via p38 MAPK in human lung endothelial cells. J Histochem Cytochem, 53, 1121-9.

Gadian, DG, Aicardi, J, Watkins, KE, Porter, DA, Mishkin, M, Vargha-Khadem, F (2000) Developmental amnesia associated with early hypoxic-ischaemic injury. Brain 123 Pt 3: 499-507.

Galan, RF, Weidert, M, Menzel, R, Herz, AV, Galizia, CG (2006) Sensory memory for odors is encoded in spontaneous correlated activity between olfactory glomeruli. Neural Comput 18(1): 10-25.

Gallagher, M, Burwell, RD, Kodsi, MH, McKinney, M, Southerland, S, Vella-Rountree, L, Lewis, MH (1990) Markers for biogenic amines in the aged rat brain: relationship to decline in spatial learning ability. Neurobiol Aging 11(5): 507-514.

Gan, J, Greenwood, SM, Cobb, SR, Bushell, TJ (2011) Indirect modulation of neuronal excitability and synaptic transmission in the hippocampus by activation of proteinase-activated receptor-2. British journal of pharmacology 163(5): 984-994.

Gao, B (2012) Hepatoprotective and anti-inflammatory cytokines in alcoholic liver disease. J Gastroenterol Hepatol 27 Suppl 2: 89-93.

Gardell, LR, Ma, J-N, Seitzberg, JG, Knapp, AE, Schiffer, HH, Tabatabaei, A, Davis, CN, Owens, M, Clemons, B, Wong, KK, Lund, B, Nash, NR, Gao, Y, Lameh, J, Schmelzer, K, Olsson, R, Burstein, ES (2008) Identification and characterization of novel small-molecule protease-activated receptor 2 agonists. J. Pharmacol. Exp. Ther. 327(3): 799-808.

Gaskin, S, Tardif, M, Mumby, DG (2011) Prolonged inactivation of the hippocampus reveals temporally graded retrograde amnesia for unreinforced spatial learning in rats. Neurobiol Learn Mem 96(2): 288-296.

Gatti, R, Andre, E, Amadesi, S, Dinh, TQ, Fischer, A, Bunnett, NW, Harrison, S, Geppetti, P, Trevisani, M (2006) Protease-activated receptor-2 activation exaggerates TRPV1-mediated cough in guinea pigs. J Appl Physiol 101(2): 506-511.

Gerlai, R (1993) Can Paradise Fish (Macropodus opercularis, Anabantidae) Recognize a Natural Predator? An Ethological Analysis. Ethology 94(2): 127-136.

Gerlai, R (1998) A new continuous alternation task in T-maze detects hippocampal dysfunction in mice. A strain comparison and lesion study. Behav Brain Res 95(1): 91-101.

Geyer, MA (1996) Serotonergic functions in arousal and motor activity. Behav Brain Res 73(1-2): 31-35.

Geyer, MA (1999) Assessing prepulse inhibition of startle in wild-type and knockout mice. Psychopharmacology (Berl) 147(1): 11-13.

Geyer, MA, Krebs-Thomson, K, Braff, DL, Swerdlow, NR (2001) Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. Psychopharmacology (Berl) 156(2-3): 117-154.

Geyer, MA, McIlwain, KL, Paylor, R (2002) Mouse genetic models for prepulse inhibition: an early review. Mol Psychiatry 7(10): 1039-1053.

Geyer, MA, Swerdlow, NR (2001) Measurement of startle response, prepulse inhibition, and habituation. Curr Protoc Neurosci Chapter 8: Unit 8 7.

Gilchrist, GW, Huey, RB, Partridge, L (1997) Thermal sensitivity of Drosophila melanogaster: evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. Physiol Zool 70(4): 403-414.

Gingrich, MB, Junge, CE, Lyuboslavsky, P, Traynelis, SF (2000) Potentiation of NMDA receptor function by the serine protease thrombin. J Neurosci 20(12): 4582-4595.

Godbout, JP, Chen, J, Abraham, J, Richwine, AF, Berg, BM, Kelley, KW, Johnson, RW (2005) Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J 19(10): 1329-1331.

Gorbacheva, LR, Storozhevykh, TP, Pinelis, VG, Ishiwata, S, Strukova, SM (2006) Modulation of hippocampal neuron survival by thrombin and factor Xa. Biochemistry (Mosc) 71(10): 1082-1089.

Gordon, DA, Urowitz, MB (1982) Use of azathioprine for rheumatoid arthritis. Arthritis & Rheumatism 25(10): 1269-1270.

Graham, FK (1975) The More or Less Startling Effects of Weak Prestimulation. Psychophysiology 12(3): 238-248.

Grand, RJ, Turnell, AS, Grabham, PW (1996) Cellular consequences of thrombin-receptor activation. Biochem J 313 (Pt 2): 353-368.

Greenberg, R (2003) The role of neophobia and neophilia in the development of innovative behaviour of birds. In: Animal Innovation, Reader S.M., LKN (ed), pp 175–196. Cambridge University Press; Cambridge.

Greenwood, SM, Bushell, TJ (2010) Astrocytic activation and an inhibition of MAP kinases are required for proteinase-activated receptor-2-mediated protection from neurotoxicity. J Neurochem 113(6): 1471-1480.

Grillon, C, Ameli, R, Goddard, A, Woods, SW, Davis, M (1994) Baseline and fear-potentiated startle in panic disorder patients. Biological Psychiatry 35(7): 431-439.

Grillon, C, Pellowski, M, Merikangas, KR, Davis, M (1997) Darkness facilitates the acoustic startle reflex in humans. Biol Psychiatry 42(6): 453-460.

Grishina, Z, Ostrowska, E, Halangk, W, Sahin-Toth, M, Reiser, G (2005) Activity of recombinant trypsin isoforms on human proteinase-activated receptors (PAR): mesotrypsin cannot activate epithelial PAR-1, -2, but weakly activates brain PAR-1. Br J Pharmacol 146(7): 990-999.

Gschwend, TP, Krueger, SR, Kozlov, SV, Wolfer, DP, Sonderegger, P (1997) Neurotrypsin, a novel multidomain serine protease expressed in the nervous system. Mol Cell Neurosci 9(3): 207-219.

Gui, Y, Loutzenhiser, R, Hollenberg, MD (2003) Bidirectional regulation of renal hemodynamics by activation of PAR1 and PAR2 in isolated perfused rat kidney. Am J Physiol Renal Physiol 285(1): F95-104.

Hackett, ML, Anderson, CS (2005) Predictors of depression after stroke: a systematic review of observational studies. Stroke 36(10): 2296-2301.

Hafler, DA, Slavik, JM, Anderson, DE, O'Connor, KC, De Jager, P, Baecher-Allan, C (2005) Multiple sclerosis. Immunol Rev 204: 208-231.

Hall, CS (1934) Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J. Comp. Psychol. 18: 385–403.

Hamill, CE, Mannaioni, G, Lyuboslavsky, P, Sastre, AA, Traynelis, SF (2009) Protease-activated receptor 1-dependent neuronal damage involves NMDA receptor function. Exp Neurol 217(1): 136-146.

Hamilton, JR, Frauman, AG, Cocks, TM (2001) Increased expression of protease-activated receptor-2 (PAR2) and PAR4 in human coronary artery by inflammatory stimuli unveils endothelium-dependent relaxations to PAR2 and PAR4 agonists. Circ Res 89(1): 92-98.

Hamilton, JR, Moffatt, JD, Tatoulis, J, Cocks, TM (2002) Enzymatic activation of endothelial protease-activated receptors is dependent on artery diameter in human and porcine isolated coronary arteries. British journal of pharmacology 136(4): 492-501.

Hamilton, M (1967) Development of a rating scale for primary depressive illness. Br J Soc Clin Psychol 6(4): 278-296.

Handley, SL, Mithani, S (1984) Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn Schmiedebergs Arch Pharmacol 327(1): 1-5.

Hansen, KK, Saifeddine, M, Hollenberg, MD (2004) Tethered ligand-derived peptides of proteinase-activated receptor 3 (PAR3) activate PAR1 and PAR2 in Jurkat T cells. Immunology 112(2): 183-190.

Hansson, GK, Edfeldt, K (2005) Toll to be paid at the gateway to the vessel wall. Arterioscler Thromb Vasc Biol 25(6): 1085-1087.

Hart, BL (1988) Biological basis of the behavior of sick animals. Neurosci Biobehav Rev 12(2): 123-137.

Haussleiter, IS, Brune, M, Juckel, G (2009) Psychopathology in multiple sclerosis: diagnosis, prevalence and treatment. Ther Adv Neurol Disord 2(1): 13-29.

Heyen, JR, Ye, S, Finck, BN, Johnson, RW (2000) Interleukin (IL)-10 inhibits IL-6 production in microglia by preventing activation of NF-kappaB. Brain Res Mol Brain Res 77(1): 138-147.

Hill., BDEM (1960) Conflict, Arousal and Curiosity. New York.

Hoffman, HS, Marsh, RR, Stein, N (1969) Persistence of background acoustic stimulation in controlling startle. J Comp Physiol Psychol 68(2): 280-283.

Hoffmann, C, Zurn, A, Bunemann, M, Lohse, MJ (2008) Conformational changes in G-protein-coupled receptors-the quest for functionally selective conformations is open. Br J Pharmacol 153 Suppl 1: S358-366.

Hollenberg, MD, Compton, SJ (2002) International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol Rev 54(2): 203-217.

Hollenberg, MD, Saifeddine, M (2001) Proteinase-activated receptor 4 (PAR4): activation and inhibition of rat platelet aggregation by PAR4-derived peptides. Can J Physiol Pharmacol 79(5): 439-442.

Holmes, A, Parmigiani, S, Ferrari, PF, Palanza, P, Rodgers, RJ (2000) Behavioral profile of wild mice in the elevated plus-maze test for anxiety. Physiology & Behavior 71(5): 509-516.

Holsboer, F, Ising, M (2008) Central CRH system in depression and anxiety--evidence from clinical studies with CRH1 receptor antagonists. Eur J Pharmacol 583(2-3): 350-357.

Holz, R, Fisher, S (1999) Cellular Signaling Mechanisms. In: Basic Neurochemistry: Molecular, Cellular and Medical Aspects, Siegel, G, Agranoff, B, Albers, R, al., e (eds), 6th edition. edn. Philadelphia: Lippincott-Raven.

Hong, JH, Hong, JY, Park, B, Lee, SI, Seo, JT, Kim, KE, Sohn, MH, Shin, DM (2008) Chitinase activates protease-activated receptor-2 in human airway epithelial cells. Am J Respir Cell Mol Biol 39(5): 530-535.

Hoogerwerf, WA, Hellmich, HL, Micci, MA, Winston, JH, Zou, L, Pasricha, PJ (2002) Molecular cloning of the rat proteinase-activated receptor 4 (PAR4). BMC Mol Biol 3: 2.

Hopkins, SJ, Rothwell, NJ (1995) Cytokines and the nervous system. I: Expression and recognition. Trends Neurosci 18(2): 83-88.

Huang, FL, Yoshida, Y, Nakabayashi, H, Friedman, DP, Ungerleider, LG, Young, WS, 3rd, Huang, KP (1989) Type I protein kinase C isozyme in the visual-information-processing pathway of monkey brain. J Cell Biochem 39(4): 401-410.

Hughes, RN (1990) Spontaneouse alternation behavior In: Phylogenetic comparisons Richman, WNDLL (ed), pp 39-59. New York: Springer -Verlag.

Hughes, RN (2004) The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. Neuroscience & Biobehavioral Reviews 28(5): 497-505.

Hung, DT, Vu, TH, Nelken, NA, Coughlin, SR (1992) Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. J Cell Biol 116(3): 827-832.

Hur, E-M, Kim, K-T (2002) G protein-coupled receptor signalling and cross-talk: Achieving rapidity and specificity. Cellular Signalling 14(5): 397-405.

Hyun, E, Andrade-Gordon, P, Steinhoff, M, Vergnolle, N (2008) Protease-activated receptor-2 activation: a major actor in intestinal inflammation. Gut 57(9): 1222-1229.

Iivonen, H, Nurminen, L, Harri, M, Tanila, H, Puolivali, J (2003) Hypothermia in mice tested in Morris water maze. Behav Brain Res. 2003 May 15;141(2):207-13.

Irwin, MR, Miller, AH (2007) Depressive disorders and immunity: 20 years of progress and discovery. Brain, behavior, and immunity 21(4): 374-383.

Ishida, Y, Nagai, A, Kobayashi, S, Kim, SU (2006) Upregulation of protease-activated receptor-1 in astrocytes in Parkinson disease: astrocyte-mediated neuroprotection through increased levels of glutathione peroxidase. J Neuropathol Exp Neurol 65(1): 66-77.

Ishihara, H, Connolly, AJ, Zeng, D, Kahn, ML, Zheng, YW, Timmons, C, Tram, T, Coughlin, SR (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. Nature 386(6624): 502-506.

Ishii, K, Gerszten, R, Zheng, YW, Welsh, JB, Turck, CW, Coughlin, SR (1995) Determinants of thrombin receptor cleavage. Receptor domains involved, specificity, and role of the P3 aspartate. J Biol Chem 270(27): 16435-16440.

Ishikawa, C, Tsuda, T, Konishi, H, Nakagawa, N, Yamanishi, K (2009) Tetracyclines modulate protease-activated receptor 2-mediated proinflammatory reactions in epidermal keratinocytes. Antimicrob Agents Chemother 53(5): 1760-1765.

Ison, JR, Allen, PD (2007) Pre- but not post-menopausal female CBA/CaJ mice show less prepulse inhibition than male mice of the same age. Behav Brain Res 185(2): 76-81.

Ison, JR, McAdam, DW, Hammond, GR (1973) Latency and amplitude changes in the acoustic startle reflex of the rat produced by variation in auditory prestimulation. Physiology & Behavior 10(6): 1035-1039.

Janssens, S, Beyaert, R (2003) Role of Toll-like receptors in pathogen recognition. Clin Microbiol Rev 16(4): 637-646.

Jarrard, LE (1983) Selective hippocampal lesions and behavior: effects of kainic acid lesions on performance of place and cue tasks. Behav Neurosci 97(6): 873-889.

Jian, L (2012) A novel hypothesis of blood-brain barrier (BBB) development and in vitro BBB model: neural stem cell is the driver of BBB formation and maintenance. J Exp Integr Med 2(1): 39-43.

Jin, G, Hayashi, T, Kawagoe, J, Takizawa, T, Nagata, T, Nagano, I, Syoji, M, Abe, K (2005) Deficiency of PAR-2 gene increases acute focal ischemic brain injury. J Cereb Blood Flow Metab 25(3): 302-313.

Jones, B, Bukoski, E, Nadel, L, Fellous, JM (2012) Remaking memories: reconsolidation updates positively motivated spatial memory in rats. Learn Mem 19(3): 91-98.

Jones, NC, Salzberg, MR, Kumar, G, Couper, A, Morris, MJ, O'Brien, TJ (2008) Elevated anxiety and depressive-like behavior in a rat model of genetic generalized epilepsy suggesting common causation. Exp Neurol 209(1): 254-260.

Jordan, KW, Morgan, TJ, Mackay, TF (2006) Quantitative trait loci for locomotor behavior in Drosophila melanogaster. Genetics 174(1): 271-284.

Juckel, G, Sass, L, Heinz, A (2003) Anhedonia, self-experience in schizophrenia, and implications for treatment. Pharmacopsychiatry 36 Suppl 3: S176-180.

Junge, CE, Sugawara, T, Mannaioni, G, Alagarsamy, S, Conn, PJ, Brat, DJ, Chan, PH, Traynelis, SF (2003) The contribution of protease-activated receptor 1 to neuronal damage caused by transient focal cerebral ischemia. Proceedings of the National Academy of Sciences 100(22): 13019-13024.

Kahn, ML, Hammes, SR, Botka, C, Coughlin, SR (1998) Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. J Biol Chem 273(36): 23290-23296.

Kandel, ER, Schwartz, J, H., Jessell, TM (2000) Principles of Neural Science. 4 edn: McGraw-Hill Companies, New York

Kane, MJ, Engle, RW (2002) The role of prefrontal cortex in working-memory capacity, executive attention, and general fluid intelligence: an individual-differences perspective. Psychon Bull Rev 9(4): 637-671.

Kanke, T, Kabeya, M, Kubo, S, Kondo, S, Yasuoka, K, Tagashira, J, Ishiwata, H, Saka, M, Furuyama, T, Nishiyama, T, Doi, T, Hattori, Y, Kawabata, A, Cunningham, MR, Plevin, R (2009) Novel antagonists for proteinase-activated receptor 2: inhibition of cellular and vascular responses in vitro and in vivo. Br J Pharmacol 158(1): 361-371.

Kanthou, C, Kanse, SM, Kakkar, VV, Benzakour, O (1996) Involvement of pertussis toxin-sensitive and -insensitive G proteins in alpha-thrombin signalling on cultured human vascular smooth muscle cells. Cell Signal 8(1): 59-66.

Katz, RJ (1981) Animal models and human depressive disorders. Neurosci Biobehav Rev 5(2): 231-246.

Katz, RJ, Roth, KA, Carroll, BJ (1981) Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. Neurosci Biobehav Rev 5(2): 247-251.

Kaufmann, R, Patt, S, Zieger, M, Kraft, R, Tausch, S, Henklein, P, Nowak, G (2000) The two-receptor system PAR-1/PAR-4 mediates alpha-thrombin-induced [Ca(2+)](i) mobilization in human astrocytoma cells. J Cancer Res Clin Oncol 126(2): 91-94.

Kawabata, A (2002) PAR-2: structure, function and relevance to human diseases of the gastric mucosa. Expert Rev Mol Med 4(16): 1-17.

Kawabata, A, Kuroda, R, Nagata, N, Kawao, N, Masuko, T, Nishikawa, H, Kawai, K (2001) In vivo evidence that protease-activated receptors 1 and 2 modulate gastrointestinal transit in the mouse. British journal of pharmacology 133(8): 1213-1218.

Kawabata, A, Nishikawa, H, Saitoh, H, Nakaya, Y, Hiramatsu, K, Kubo, S, Nishida, M, Kawao, N, Kuroda, R, Sekiguchi, F, Kinoshita, M, Kakehi, K, Arizono, N, Yamagishi, H, Kawai, K (2004) A protective role of protease-activated receptor 1 in rat gastric mucosa. Gastroenterology 126(1): 208-219.

Kawabata, A, Saifeddine, M, Al-Ani, B, Leblond, L, Hollenberg, MD (1999) Evaluation of proteinase-activated receptor-1 (PAR1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR2 by PAR1-targeted ligands. The Journal of pharmacology and experimental therapeutics 288(1): 358-370.

Kawasawa, Y, McKenzie, LM, Hill, DP, Bono, H, Yanagisawa, M (2003) G protein-coupled receptor genes in the FANTOM2 database. Genome Res 13(6B): 1466-1477.

Keane, MP, Strieter, RM (2002) The importance of balanced pro-inflammatory and anti-inflammatory mechanisms in diffuse lung disease. Respir Res 3: 5.

Kelley, KW, Bluthe, RM, Dantzer, R, Zhou, JH, Shen, WH, Johnson, RW, Broussard, SR (2003) Cytokine-induced sickness behavior. Brain, behavior, and immunity 17 Suppl 1: S112-118.

Kelso, EB, Ferrell, WR, Lockhart, JC, Elias-Jones, I, Hembrough, T, Dunning, L, Gracie, JA, McInnes, IB (2007) Expression and proinflammatory role of

proteinase-activated receptor 2 in rheumatoid synovium: ex vivo studies using a novel proteinase-activated receptor 2 antagonist. Arthritis Rheum 56(3): 765-771.

Kelso, EB, Lockhart, JC, Hembrough, T, Dunning, L, Plevin, R, Hollenberg, MD, Sommerhoff, CP, McLean, JS, Ferrell, WR (2006) Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. J Pharmacol Exp Ther 316(3):1017-24.

Kelso, EB, Lockhart, JC, Hembrough, T, Dunning, L, Plevin, R, Hollenberg, MD, Sommerhoff, CP, McLean, JS, Ferrell, WR (2006) Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. J Pharmacol Exp Ther 316(3): 1017-1024.

Kenakin, T (2011) Functional selectivity and biased receptor signaling. J Pharmacol Exp Ther 336(2): 296-302.

Kent, S, Bluthe, RM, Dantzer, R, Hardwick, AJ, Kelley, KW, Rothwell, NJ, Vannice, JL (1992a) Different receptor mechanisms mediate the pyrogenic and behavioral effects of interleukin 1. Proceedings of the National Academy of Sciences of the United States of America 89(19): 9117-9120.

Kent, S, Bluthe, RM, Kelley, KW, Dantzer, R (1992b) Sickness behavior as a new target for drug development. Trends in pharmacological sciences 13(1): 24-28.

Keskitalo, K, Knaapila, A, Kallela, M, Palotie, A, Wessman, M, Sammalisto, S, Peltonen, L, Tuorila, H, Perola, M (2007) Sweet taste preferences are partly genetically determined: identification of a trait locus on chromosome 16. The American Journal of Clinical Nutrition 86(1): 55-63.

Kessler, RC, McGonagle, KA, Zhao, S, Nelson, CB, Hughes, M, Eshleman, S, Wittchen, HU, Kendler, KS (1994) Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Arch Gen Psychiatry 51(1): 8-19.

Kim, JA, Choi, SC, Yun, KJ, Kim, DK, Han, MK, Seo, GS, Yeom, JJ, Kim, TH, Nah, YH, Lee, YM (2003) Expression of protease-activated receptor 2 in ulcerative colitis. Inflamm Bowel Dis 9(4): 224-229.

Kim, S, Foster, C, Lecchi, A, Quinton, TM, Prosser, DM, Jin, J, Cattaneo, M, Kunapuli, SP (2002) Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. Blood 99(10): 3629-3636.

Kimble, G, Hilgard, E, Marquis, D (1961) The defination of learning In: Conditining and learning In: Kimble, G (ed), pp 1-3. Appleton-Century-Crofts, New York

Kirkup, AJ, Jiang, W, Bunnett, NW, Grundy, D (2003) Stimulation of proteinase-activated receptor 2 excites jejunal afferent nerves in anaesthetised rats. The Journal of physiology 552(Pt 2): 589-601.

Klabunde, T, Hessler, G (2002) Drug design strategies for targeting G-protein-coupled receptors. Chembiochem 3(10): 928-944.

Kleij, HP, Bienenstock, J (2005) Significance of Conversation between Mast Cells and Nerves. Allergy Asthma Clin Immunol 1(2): 65-80.

Klein, DF (1974) Endogenomorphic depression: A conceptual and terminological revision. Archives of General Psychiatry 31(4): 447-454.

Kluger, MJ (1986) Is fever beneficial? Yale J Biol Med 59(2): 89-95.

Kluger, MJ (1991) Fever: role of pyrogens and cryogens. Physiological reviews 71(1): 93-127.

Knowlton, BJ, Mangels, JA, Squire, LR (1996) A neostriatal habit learning system in humans. Science 273(5280): 1399-1402.

Kobayashi, Y (2010) The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. J Leukoc Biol 88(6): 1157-1162.

Koch, M, Schnitzler, HU (1997) The acoustic startle response in rats--circuits mediating evocation, inhibition and potentiation. Behav Brain Res 89(1-2): 35-49.

Kogan, JH, Frankland, PW, Silva, AJ (2000) Long-term memory underlying hippocampus-dependent social recognition in mice. Hippocampus 10(1): 47-56.

Komatsu, H, Enjouji, S, Ito, A, Ohama, T, Sato, K (2013) Prostaglandin E2 Inhibits Proteinase-Activated Receptor 2-Signal Transduction through Regulation of Receptor Internalization. J Vet Med Sci 75(3): 255-261.

Konsman, JP, Dantzer, R (2001) How the immune and nervous systems interact during disease-associated anorexia. Nutrition 17(7-8): 664-668.

Koob, G, Ehlers, C, Kupfer, D (1989) Anhedonia as an Animal Model of Depression. In: Animal Models of Depression, pp 162-183: BirkhÃ¤user Boston.

Kozasa, T, Hajicek, N, Chow, CR, Suzuki, N (2011) Signalling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13. J Biochem 150(4): 357-369.

Krishnadas, R, Cavanagh, J (2012) Depression: an inflammatory illness? J Neurol Neurosurg Psychiatry 83(5): 495-502.

Krupa, DJ, Thompson, JK, Thompson, RF (1993) Localization of a memory trace in the mammalian brain. Science 260(5110): 989-991.

Ku, DD, Dai, J (1997) Expression of thrombin receptors in human atherosclerotic coronary arteries leads to an exaggerated vasoconstrictory response in vitro. J Cardiovasc Pharmacol 30(5): 649-657.

Kumar, P, Lau, CS, Mathur, M, Wang, P, DeFea, KA (2007) Differential effects of beta-arrestins on the internalization, desensitization and ERK1/2 activation downstream of protease activated receptor-2. Am J Physiol Cell Physiol 293(1): C346-357.

Kumaran, D (2008) Short-term memory and the human hippocampus. J Neurosci 28(15): 3837-3838.

Kunesova, G, Hlavacek, J, Patocka, J, Evangelou, A, Zikos, C, Benaki, D, Paravatou-Petsotas, M, Pelecanou, M, Livaniou, E, Slaninova, J (2008) The multiple T-maze in vivo testing of the neuroprotective effect of humanin analogues. Peptides 29(11): 1982-1987.

Lacroix, L, Spinelli, S, Heidbreder, CA, Feldon, J (2000) Differential role of the medial and lateral prefrontal cortices in fear and anxiety. Behav Neurosci 114(6): 1119-1130.

Lakaye, B, Thomas, E, Minet, A, Grisar, T (2002) The genetic absence epilepsy rat from Strasbourg (GAERS), a rat model of absence epilepsy: computer modeling and differential gene expression. Epilepsia 43 Suppl 5: 123-129.

Lalonde, R (2002) The neurobiological basis of spontaneous alternation. Neurosci Biobehav Rev 26(1): 91-104.

LaMorte, VJ, Kennedy, ED, Collins, LR, Goldstein, D, Harootunian, AT, Brown, JH, Feramisco, JR (1993) A requirement for Ras protein function in thrombin-stimulated mitogenesis in astrocytoma cells. J Biol Chem 268(26): 19411-19415.

Lan, RS, Stewart, GA, Henry, PJ (2002) Role of protease-activated receptors in airway function: a target for therapeutic intervention? Pharmacol Ther 95(3): 239-257.

Landis, C (1939) The startle pattern New York

Langer, F, Morys-Wortmann, C, Kusters, B, Storck, J (1999) Endothelial protease-activated receptor-2 induces tissue factor expression and von Willebrand factor release. Br J Haematol 105(2): 542-550.

Langhans, W, Harlacher, R, Balkowski, G, Scharrer, E (1990) Comparison of the effects of bacterial lipopolysaccharide and muramyl dipeptide on food intake. Physiol Behav 47(5): 805-813.

Larson, SJ, Dunn, AJ (2001) Behavioral effects of cytokines. Brain, behavior, and immunity 15(4): 371-387.

Laterra, J, Keep, R, Betz, L, al., e (1999) Blood—Brain Barrier. In: Basic Neurochemistry: Molecular, Cellular and Medical Aspects. , Siegel, GJ, Agranoff, BW, Albers, R, al., e (eds), 6th edition edn. Philadelphia: Lippincott-Raven.

Lattin, J, Zidar, DA, Schroder, K, Kellie, S, Hume, DA, Sweet, MJ (2007) G-protein-coupled receptor expression, function, and signaling in macrophages. J Leukoc Biol 82(1): 16-32.

Lawrence, CB, Brough, D, Knight, EM (2012) Obese mice exhibit an altered behavioural and inflammatory response to lipopolysaccharide. Dis Model Mech 5(5): 649-659.

Lechin, F, van der Dijs, B, Orozco, B, Lechin, ME, Baez, S, Lechin, AE, Rada, I, Acosta, E, Arocha, L, Jimenez, V, et al. (1995) Plasma neurotransmitters, blood pressure, and heart rate during supine-resting, orthostasis, and moderate exercise conditions in major depressed patients. Biol Psychiatry 38(3): 166-173.

Lee, CJ, Mannaioni, G, Yuan, H, Woo, DH, Gingrich, MB, Traynelis, SF (2007) Astrocytic control of synaptic NMDA receptors. The Journal of physiology 581(Pt 3): 1057-1081.

Lefkowitz, RJ, Shenoy, SK (2005) Transduction of receptor signals by beta-arrestins. Science 308(5721): 512-517.

Leger, A.J., Covic, L. & Kuliopulos, A. (2006) Protease-activated receptors in cardiovascular diseases. Circulation, 114, 1070-7.

Lennie, TA (1998) Relationship of body energy status to inflammation-induced anorexia and weight loss. Physiol Behav 64(4): 475-481.

Leonov, VM (1963) [Basal Metabolism and Fever in Typhoid-Paratyphoid Patients]. Arkh Patol 25: 75-81.

Li, L, Steidl, S, Yeomans, JS (2001) Contributions of the vestibular nucleus and vestibulospinal tract to the startle reflex. Neuroscience 106(4): 811-821.

Liljequist, R (1993) Interaction of taurine and related compounds with GABAergic neurones in the nucleus raphe dorsalis. Pharmacol Biochem Behav 44(1): 107-112.

Lindl, KA, Marks, DR, Kolson, DL, Jordan-Sciutto, KL (2010) HIV-associated neurocognitive disorder: pathogenesis and therapeutic opportunities. J Neuroimmune Pharmacol 5(3): 294-309.

Lisman, JE, Otmakhova, NA (2001) Storage, recall, and novelty detection of sequences by the hippocampus: elaborating on the SOCRATIC model to account for normal and aberrant effects of dopamine. Hippocampus 11(5): 551-568.

Lister, RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92(2): 180-185.

Lohman, R.J., Cotterell, A.J., Barry, G.D., Liu, L., Suen, J.Y., Vesey, D.A. & Fairlie, D.P. (2012) An antagonist of human protease activated receptor-2 attenuates PAR2 signaling, macrophage activation, mast cell degranulation, and collagen-induced arthritis in rats. Faseb J, 26, 2877-87.

Lohman, RJ, Cotterell, AJ, Suen, J, Liu, L, Do, AT, Vesey, DA, Fairlie, DP (2012) Antagonism of protease-activated receptor 2 protects against experimental colitis. J Pharmacol Exp Ther 340(2): 256-265.

Lohman, RJ, Jones, NC, O'Brien, TJ, Cocks, TM (2009) A regulatory role for protease-activated receptor-2 in motivational learning in rats. Neurobiol Learn Mem 92(3): 301-309.

Lohman, RJ, O'Brien, TJ, Cocks, TM (2008) Protease-activated receptor-2 regulates trypsin expression in the brain and protects against seizures and epileptogenesis. Neurobiol Dis 30(1): 84-93.

Lohse, MJ, Benovic, JL, Codina, J, Caron, MG, Lefkowitz, RJ (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 248(4962): 1547-1550.

Love, S (2006) Demyelinating diseases. J Clin Pathol 59(11): 1151-1159.

Lovick, TA (2012) Estrous cycle and stress: influence of progesterone on the female brain. Braz J Med Biol Res 45(4): 314-320.

Lu, Y-C, Yeh, W-C, Ohashi, PS (2008) LPS/TLR4 signal transduction pathway. Cytokine 42(2): 145-151.

Ludewig, S, Ludewig, K (2003) No prepulse inhibition deficits in patients with unipolar depression. Depress Anxiety 17(4): 224-225.

Luo, W, Wang, Y, Hanck, T, Stricker, R, Reiser, G (2006) Jab1, a novel protease-activated receptor-2 (PAR-2)-interacting protein, is involved in PAR-2-induced activation of activator protein-1. J Biol Chem 281(12): 7927-7936.

Luo, W, Wang, Y, Reiser, G (2007) Protease-activated receptors in the brain: receptor expression, activation, and functions in neurodegeneration and neuroprotection. Brain research reviews 56(2): 331-345.

Luttrell, LM, Lefkowitz, RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115(Pt 3): 455-465.

MacDonald, RS (2000) The role of zinc in growth and cell proliferation. J Nutr 130(5S Suppl): 1500S-1508S.

Macfarlane, SR, Seatter, MJ, Kanke, T, Hunter, GD, Plevin, R (2001) Proteinase-activated receptors. Pharmacol Rev 53(2): 245-282.

Madani, R, Kozlov, S, Akhmedov, A, Cinelli, P, Kinter, J, Lipp, HP, Sonderegger, P, Wolfer, DP (2003) Impaired explorative behavior and neophobia in genetically modified mice lacking or overexpressing the extracellular serine protease inhibitor neuroserpin. Mol Cell Neurosci 23(3): 473-494.

Maes, M (1995) Evidence for an immune response in major depression: a review and hypothesis. Prog Neuropsychopharmacol Biol Psychiatry 19(1): 11-38.

Maes, M, Berk, M, Goehler, L, Song, C, Anderson, G, GaÅ‚ecki, P, Leonard, B (2012) Depression and sickness behavior are Janus-faced responses to shared inflammatory pathways. BMC Medicine C7 - 66 10(1): 1-19.

Maes, M, Kubera, M, Obuchowiczwa, E, Goehler, L, Brzeszcz, J (2011) Depression's multiple comorbidities explained by (neuro)inflammatory and oxidative & nitrosative stress pathways. Neuro endocrinology letters 32(1): 7-24.

Maes, M, Meltzer, HY, Bosmans, E, Bergmans, R, Vandoolaeghe, E, Ranjan, R, Desnyder, R (1995) Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression. J Affect Disord 34(4): 301-309.

Maggi, A, Ciana, P, Belcredito, S, Vegeto, E (2004) Estrogens in the nervous system: mechanisms and nonreproductive functions. Annu Rev Physiol 66: 291-313.

Mailman, RB (2007) GPCR functional selectivity has therapeutic impact. Trends Pharmacol Sci 28(8): 390-396.

Majerus, PW (1992) Inositol phosphate biochemistry. Annu Rev Biochem 61: 225-250.

Maletic, V, Robinson, M, Oakes, T, Iyengar, S, Ball, SG, Russell, J (2007) Neurobiology of depression: an integrated view of key findings. International Journal of Clinical Practice 61(12): 2030-2040.

Mann, GE, Yudilevich, DL, Sobrevia, L (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiological reviews 83(1): 183-252.

Mannaioni, G, Orr, AG, Hamill, CE, Yuan, H, Pedone, KH, McCoy, KL, Berlinguer Palmini, R, Junge, CE, Lee, CJ, Yepes, M, Hepler, JR, Traynelis, SF (2008) Plasmin potentiates synaptic N-methyl-D-aspartate receptor function in hippocampal neurons through activation of protease-activated receptor-1. J Biol Chem 283(29): 20600-20611.

Marks, DM, Pae, CU, Patkar, AA (2008) Triple reuptake inhibitors: the next generation of antidepressants. Curr Neuropharmacol 6(4): 338-343.

Martin, SA, Pence, BD, Greene, RM, Johnson, SJ, Dantzer, R, Kelley, KW, Woods, JA (2012) Effects of voluntary wheel running on LPS-induced sickness behavior in aged mice. Brain, behavior, and immunity 29(0): 113-123.

Martin, SJ, Grimwood, PD, Morris, RG (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci 23: 649-711.

Marvel, CL, Paradiso, S (2004) Cognitive and neurological impairment in mood disorders. Psychiatric Clinics of North America 27(1): 19-36.

Maryanoff, BE, Santulli, RJ, McComsey, DF, Hoekstra, WJ, Hoey, K, Smith, CE, Addo, M, Darrow, AL, Andrade-Gordon, P (2001) Protease-activated receptor-2 (PAR-2): structure-function study of receptor activation by diverse peptides related to tethered-ligand epitopes. Arch Biochem Biophys 386(2): 195-204.

Maryanoff, BE, Santulli, RJ, McComsey, DF, Hoekstra, WJ, Hoey, K, Smith, CE, Addo, M, Darrow, AL, Andrade-Gordon, P (2001) Protease-activated receptor-2 (PAR-2): structure-function study of receptor activation by diverse peptides related to tethered-ligand epitopes. Arch Biochem Biophys 386(2): 195-204.

Maskrey, BH, Megson, IL, Whitfield, PD, Rossi, AG (2011) Mechanisms of resolution of inflammation: a focus on cardiovascular disease. Arterioscler Thromb Vasc Biol 31(5): 1001-1006.

Masu, Y, Nakayama, K, Tamaki, H, Harada, Y, Kuno, M, Nakanishi, S (1987) cDNA cloning of bovine substance-K receptor through oocyte expression system. Nature 329(6142): 836-838.

Matos, G, Ribeiro, DA, Alvarenga, TA, Hirotsu, C, Scorza, FA, Le Sueur-Maluf, L, Noguti, J, Cavalheiro, EA, Tufik, S, Andersen, ML (2012) Behavioral and genetic effects promoted by sleep deprivation in rats submitted to pilocarpine-induced status epilepticus. Neurosci Lett 515(2): 137-140.

Maughan, RJ (1991) Fluid and electrolyte loss and replacement in exercise. J Sports Sci 9 Spec No: 117-142.

Maul, H, Bytautiene, E, Vedernikov, Y, Garfield, RE, Saade, GR (2003) Proteinase-activated receptor-2 activation induces uterine contractility in term pregnant rats that is not dependent on mast cell activation and cyclo-oxygenase products. Am J Obstet Gynecol 188(6): 1498-1502; discussion 1502-1493.

McArthur, RA, Borsini, F (2008) Animal and translational models for CNS drug discovery. Elsevier/Academic Press: Amsterdam; Boston.

McEachron, TA, Pawlinski, R, Richards, KL, Church, FC, Mackman, N (2010) Protease-activated receptors mediate crosstalk between coagulation and fibrinolysis. Blood 116(23): 5037-5044.

McGaugh, JL (1966) Time-dependent processes in memory storage. Science 153(3742): 1351-1358.

McGaugh, JL (2004) The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci 27: 1-28.

McHenry, L (2006) Ethical issues in psychopharmacology. J Med Ethics 32(7): 405-410.

McHowat, J, Creer, MH, Rickard, A (2001) Stimulation of protease activated receptors on RT4 cells mediates arachidonic acid release via Ca2+ independent phospholipase A2. J Urol 165(6 Pt 1): 2063-2067.

McLaughlin, JN, Shen, L, Holinstat, M, Brooks, JD, Dibenedetto, E, Hamm, HE (2005) Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. J Biol Chem 280(26): 25048-25059.

Medina, JJ (2009) The biology of recognition memory. Psychiatric Times: 13.

Medzhitov, R (2010) Inflammation 2010: new adventures of an old flame. Cell 140(6): 771-776.

Melgar, S, Bjursell, M, Gerdin, AK, Svensson, L, Michaelsson, E, Bohlooly, YM (2007) Mice with experimental colitis show an altered metabolism with decreased metabolic rate. Am J Physiol Gastrointest Liver Physiol 292(1): G165-172.

Meyers, CA (1999) Mood and cognitive disorders in cancer patients receiving cytokine therapy. Advances in experimental medicine and biology 461: 75-81.

Milia, AF, Salis, MB, Stacca, T, Pinna, A, Madeddu, P, Trevisani, M, Geppetti, P, Emanueli, C (2002) Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia. Circ Res 91(4): 346-352.

Miller, AH, Maletic, V, Raison, CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65(9): 732-741.

Miller, GA (1956) The magical number seven plus or minus two: some limits on our capacity for processing information. Psychol Rev 63(2): 81-97.

Miranda, MI (ed) (2007) Changes in neurotransmitter extracellular levels during memory formation.

Mizuhara, H, Yamaguchi, Y (2007) Human cortical circuits for central executive function emerge by theta phase synchronization. NeuroImage 36(1): 232-244.

Moffatt, JD, Cocks, TM (1999) The role of protease-activated receptor-2 (PAR2) in the modulation of beating of the mouse isolated ureter: lack of involvement of mast cells or sensory nerves. British journal of pharmacology 128(4): 860-864.

Mogensen, TH (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 22(2): 240-273, Table of Contents.

Molino, M, Raghunath, PN, Kuo, A, Ahuja, M, Hoxie, JA, Brass, LF, Barnathan, ES (1998) Differential expression of functional protease-activated receptor-2 (PAR-2) in human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 18(5): 825-832.

Monje, ML, Toda, H, Palmer, TD (2003) Inflammatory blockade restores adult hippocampal neurogenesis. Science 302(5651): 1760-1765.

Montgomery, KC (1954) The role of the exploratory drive in learning. J Comp Physiol Psychol 47(1): 60-64.

Montgomery, KC (1955) The relation between fear induced by novel stimulation and exploratory behavior. J Comp Physiol Psychol 48(4): 254-260.

Montkowski, A, Landgraf, R, Yassouridis, A, Holsboer, F, Schobitz, B (1997) Central administration of IL-1 reduces anxiety and induces sickness behaviour in rats. Pharmacol Biochem Behav 58(2): 329-336.

Moore, CA, Milano, SK, Benovic, JL (2007) Regulation of receptor trafficking by GRKs and arrestins. Annu Rev Physiol 69: 451-482.

Moraes, TJ, Martin, R, Plumb, JD, Vachon, E, Cameron, CM, Danesh, A, Kelvin, DJ, Ruf, W, Downey, GP (2008) Role of PAR2 in murine pulmonary pseudomonal infection. Am J Physiol Lung Cell Mol Physiol 294(2): L368-377.

Moreau, JL (1997) Validation of an animal model of anhedonia, a major symptom of depression]. Encephale 23(4): 280-289.

Moreau, JL (2002) Simulating the anhedonia symptom of depression in animals. Dialogues Clin Neurosci 4(4): 351-360.

Morris, R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11(1): 47-60.

Morris, RG, Anderson, E, Lynch, GS, Baudry, M (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature 319(6056): 774-776.

Morris, RGM (1981) Spatial localization does not require the presence of local cues. Learning and Motivation 12(2): 239-260.

Morris, RGM, Garrud, P, Rawlins, JNP, O'Keefe, J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297(5868): 681-683.

Mosnier, LO, Sinha, RK, Burnier, L, Bouwens, EA, Griffin, JH (2012) Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. Blood 120(26): 5237-5246.

Muldoon, LL, Pagel, MA, Kroll, RA, Roman-Goldstein, S, Jones, RS, Neuwelt, EA (1999) A physiological barrier distal to the anatomic blood-brain barrier in a model of transvascular delivery. AJNR Am J Neuroradiol 20(2): 217-222.

Mule, F, Baffi, MC, Cerra, MC (2002) Dual effect mediated by protease-activated receptors on the mechanical activity of rat colon. Br J Pharmacol 136(3): 367-374.

Murakami, H, Takanaga, H, Matsuo, H, Ohtani, H, Sawada, Y (2000) Comparison of blood-brain barrier permeability in mice and rats using in situ brain perfusion technique. Am J Physiol Heart Circ Physiol 279(3): H1022-1028.

Nadel, L, Hardt, O (2011) Update on memory systems and processes. Neuropsychopharmacology 36(1): 251-273.

Nagai, T, Ito, M, Nakamichi, N, Mizoguchi, H, Kamei, H, Fukakusa, A, Nabeshima, T, Takuma, K, Yamada, K (2006) The rewards of nicotine: regulation by tissue plasminogen activator-plasmin system through protease activated receptor-1. J Neurosci 26(47): 12374-12383.

Nairne, JS, Thompson, SR, Pandeirada, JN (2007) Adaptive memory: survival processing enhances retention. J Exp Psychol Learn Mem Cogn 33(2): 263-273.

Nakagami, Y, Abe, K, Nishiyama, N, Matsuki, N (2000) Laminin degradation by plasmin regulates long-term potentiation. J Neurosci 20(5): 2003-2010.

Nakanishi-Matsui, M, Zheng, YW, Sulciner, DJ, Weiss, EJ, Ludeman, MJ, Coughlin, SR (2000) PAR3 is a cofactor for PAR4 activation by thrombin. Nature 404(6778): 609-613.

Narayanan, S, Wallace, L, Uretsky, N (1996) Spontaneous and drug-stimulated locomotor activity after the administration of pertussis toxin into the ventral tegmental area. J Psychiatry Neurosci 21(3): 172-180.

Nava, F, Calapai, G, De Sarro, A, Caputi, AP (1996) Interleukin-1 receptor antagonist does not reverse lipopolysaccharide-induced inhibition of water intake in rat. Eur J Pharmacol 309(3): 223-227.

Nava, F, Calapai, G, Facciola, G, Cuzzocrea, S, Giuliani, G, De Sarro, A, Caputi, AP (1997b) Melatonin effects on inhibition of thirst and fever induced by lipopolysaccharide in rat. Eur J Pharmacol 331(2-3): 267-274.

Nava, F, Calapai, G, Facciola, G, Cuzzocrea, S, Marciano, MC, De Sarro, A, Caputi, AP (1997a) Effects of interleukin-10 on water intake, locomotory activity, and rectal temperature in rat treated with endotoxin. Int J Immunopharmacol 19(1): 31-38.

Nava, F, Caputi, AP (1999) Central effects of cromoglycate sodium salt in rats treated with lipopolysaccharide. Eur J Pharmacol 367(2-3): 351-359.

Nava, F, Carta, G (2000) Repeated lipopolysaccharide administration produces tolerance to anorexia and fever but not to inhibition of thirst in rat. International Journal of Immunopharmacology 22(11): 943-953.

Nava, F, Carta, G (2001) Melatonin reduces anxiety induced by lipopolysaccharide in the rat. Neurosci Lett 307(1): 57-60.

Nava, F, Carta, G, Haynes, LW (2000) Lipopolysaccharide increases arginine-vasopressin release from rat suprachiasmatic nucleus slice cultures. Neurosci Lett 288(3): 228-230.

Nestler, EJ, Barrot, M, DiLeone, RJ, Eisch, AJ, Gold, SJ, Monteggia, LM (2002) Neurobiology of depression. Neuron 34(1): 13-25.

Nicole, O, Docagne, F, Ali, C, Margaill, I, Carmeliet, P, MacKenzie, ET, Vivien, D, Buisson, A (2001) The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nature medicine 7(1): 59-64.

Nicole, O, Goldshmidt, A, Hamill, CE, Sorensen, SD, Sastre, A, Lyuboslavsky, P, Hepler, JR, McKeon, RJ, Traynelis, SF (2005) Activation of protease-activated receptor-1 triggers astrogliosis after brain injury. J Neurosci 25(17): 4319-4329.

Nielsen, CK, Arnt, J, Sanchez, C (2000) Intracranial self-stimulation and sucrose intake differ as hedonic measures following chronic mild stress: interstrain and interindividual differences. Behav Brain Res 107(1-2): 21-33.

Nodal, FR, Lopez, DE (2003) Direct input from cochlear root neurons to pontine reticulospinal neurons in albino rat. J Comp Neurol 460(1): 80-93.

Noorbakhsh, F, Tsutsui, S, Vergnolle, N, Boven, LA, Shariat, N, Vodjgani, M, Warren, KG, Andrade-Gordon, P, Hollenberg, MD, Power, C (2006) Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. J Exp Med 203(2): 425-435.

Noorbakhsh, F, Vergnolle, N, Hollenberg, MD, Power, C (2003) Proteinase-activated receptors in the nervous system. Nat Rev Neurosci 4(12): 981-990.

Noorbakhsh, F, Vergnolle, N, McArthur, JC, Silva, C, Vodjgani, M, Andrade-Gordon, P, Hollenberg, MD, Power, C (2005) Proteinase-activated receptor-2 induction by neuroinflammation prevents neuronal death during HIV infection. J Immunol 174(11): 7320-7329.

Norton, KJ, Scarborough, RM, Kutok, JL, Escobedo, MA, Nannizzi, L, Coller, BS (1993) Immunologic analysis of the cloned platelet thrombin receptor activation mechanism: evidence supporting receptor cleavage, release of the N-terminal peptide, and insertion of the tethered ligand into a protected environment. Blood 82(7): 2125-2136.

Nystedt, S, Emilsson, K, Wahlestedt, C, Sundelin, J (1994) Molecular cloning of a potential proteinase activated receptor. Proc Natl Acad Sci U S A 91(20): 9208-9212.

Nystedt, S, Larsson, AK, Aberg, H, Sundelin, J (1995) The mouse proteinase-activated receptor-2 cDNA and gene. Molecular cloning and functional expression. J Biol Chem 270(11): 5950-5955.

O'Connor, MF, Irwin, MR, Seldon, J, Kwan, L, Ganz, PA (2007) Pro-inflammatory cytokines and depression in a familial cancer registry. Psychooncology 16(5): 499-501.

Offermanns, S, Laugwitz, KL, Spicher, K, Schultz, G (1994) G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. Proc Natl Acad Sci U S A 91(2): 504-508.

Ogawa, K, Yamada, T, Tsujioka, Y, Taguchi, J, Takahashi, M, Tsuboi, Y, Fujino, Y, Nakajima, M, Yamamoto, T, Akatsu, H, Mitsui, S, Yamaguchi, N (2000) Localization of a novel type trypsin-like serine protease, neurosin, in brain tissues of Alzheimer's disease and Parkinson's disease. Psychiatry Clin Neurosci 54(4): 419-426.

Oikonomopoulou, K, Hansen, KK, Saifeddine, M, Tea, I, Blaber, M, Blaber, SI, Scarisbrick, I, Andrade-Gordon, P, Cottrell, GS, Bunnett, NW, Diamandis, EP, Hollenberg, MD (2006) Proteinase-activated receptors, targets for kallikrein signaling. J Biol Chem 281(43): 32095-32112.

Okado, Y, Stark, CE (2005) Neural activity during encoding predicts false memories created by misinformation. Learn Mem 12(1): 3-11.

O'Keefe, J, Conway, DH (1978) Hippocampal place units in the freely moving rat: why they fire where they fire. Exp Brain Res 31(4): 573-590.

O'Keefe, J, Lynn, N (eds) (1978) The Hippocampus as a Cognitive Map Oxford University Press.

Olejar, T, Matej, R, Zadinova, M, Pouckova, P (2002) Proteinase-activated receptor-2 expression on cerebral neurones after radiation damage: immunohistochemical observation in Wistar rats. Int J Tissue React 24(3): 81-88.

Olson, EE, Lyuboslavsky, P, Traynelis, SF, McKeon, RJ (2004) PAR-1 deficiency protects against neuronal damage and neurologic deficits after unilateral cerebral hypoxia/ischemia. J Cereb Blood Flow Metab 24(9): 964-971.

Olsson, IA, Hansen, AK, Sandoe, P (2008) Animal welfare and the refinement of neuroscience research methods--a case study of Huntington's disease models. Lab Anim 42(3): 277-283.

Olton, D, Samuelson, R (1976) Remembrance of places passed: spatial memory in rats. J Exp Psychol: Anim Behav Process 2: 97–116.

Olton, DS, Becker, JT, Handelmann, GE (1979) Hippocampus, space, and memory. Behavioral and Brain Sciences 2(03): 313-322.

Ossovskaya, VS, Bunnett, NW (2004) Protease-activated receptors: contribution to physiology and disease. Physiol Rev 84(2): 579-621.

Ouagazzal, AM, Jenck, F, Moreau, JL (2001) Drug-induced potentiation of prepulse inhibition of acoustic startle reflex in mice: a model for detecting antipsychotic activity? Psychopharmacology (Berl) 156(2-3): 273-283.

Paing, MM, Johnston, CA, Siderovski, DP, Trejo, J (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. Mol Cell Biol 26(8): 3231-3242.

Paing, MM, Stutts, AB, Kohout, TA, Lefkowitz, RJ, Trejo, J (2002) beta -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. J Biol Chem 277(2): 1292-1300.

Palin, K, Bluthe, RM, McCusker, RH, Moos, F, Dantzer, R, Kelley, KW (2007) TNFalpha-induced sickness behavior in mice with functional 55 kD TNF receptors is blocked by central IGF-I. J Neuroimmunol 187(1-2): 55-60.

Palin, K, McCusker, RH, Strle, K, Moos, F, Dantzer, R, Kelley, KW (2008) Tumor necrosis factor-alpha-induced sickness behavior is impaired by central administration of an inhibitor of c-jun N-terminal kinase. Psychopharmacology (Berl) 197(4): 629-635.

Pan, D, Sciascia, A, 2nd, Vorhees, CV, Williams, MT (2008) Progression of multiple behavioral deficits with various ages of onset in a murine model of Hurler syndrome. Brain research 1188: 241-253.

Papanicolaou, DA, Wilder, RL, Manolagas, SC, Chrousos, GP (1998) The pathophysiologic roles of interleukin-6 in human disease. Ann Intern Med 128(2): 127-137.

Pardridge, WM (2005) The blood-brain barrier: bottleneck in brain drug development. NeuroRx 2(1): 3-14.

Pardridge, WM (2012) Drug transport in brain via the cerebrospinal fluid. Fluids Barriers CNS 8(1): 7.

Park, GH, Jeon, SJ, Ko, HM, Ryu, JR, Lee, JM, Kim, H-Y, Han, S-H, Kang, YS, Park, SH, Shin, CY, Ko, KH (2010) Activation of microglial cells via protease-activated receptor 2 mediates neuronal cell death in cultured rat primary neuron. Nitric Oxide 22(1): 18-29.

Park, GH, Jeon, SJ, Ryu, JR, Choi, MS, Han, SH, Yang, SI, Ryu, JH, Cheong, JH, Shin, CY, Ko, KH (2009) Essential role of mitogen-activated protein kinase pathways in protease activated receptor 2-mediated nitric-oxide production from rat primary astrocytes. Nitric Oxide 21(2): 110-119.

Park, GH, Ryu, JR, Shin, CY, Choi, MS, Han, BH, Kim, WK, Kim, HC, Ko, KH (2006) Evidence that protease-activated receptor-2 mediates trypsin-induced reversal of stellation in cultured rat astrocytes. Neurosci Res 54(1): 15-23.

Parry, MA, Myles, T, Tschopp, J, Stone, SR (1996) Cleavage of the thrombin receptor: identification of potential activators and inactivators. Biochem J 320 (Pt 1): 335-341.

Pascalis, O, de Schonen, S, Morton, J, Deruelle, C, Fabre-Grenet, M (1995) Mother's face recognition by neonates: A replication and an extension. Infant Behavior and Development 18(1): 79-85.

Passingham, D, Sakai, K (2004) The prefrontal cortex and working memory: physiology and brain imaging. Curr Opin Neurobiol 14(2): 163-168.

Patil, SS, Sunyer, B, Hoger, H, Lubec, G (2009) Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze. Behav Brain Res 198(1): 58-68.

Patrick, CJ, Berthot, BD, Moore, JD (1996) Diazepam blocks fear-potentiated startle in humans. J Abnorm Psychol 105(1): 89-96.

Paul, RKaC, D. Gray (2001) SPSS for Windows Made Simple Release 10 UK.

Pavlov, VA, Wang, H, Czura, CJ, Friedman, SG, Tracey, KJ (2003) The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol Med 9(5-8): 125-134.

Pawlak, R, Strickland, S (2002) Tissue plasminogen activator and seizures: a clot-buster's secret life. J Clin Invest 109(12): 1529-1531.

Pearce, JM (2001) Ammon's horn and the hippocampus. J Neurol Neurosurg Psychiatry 71(3): 351.

Pearson, G, Robinson, F, Beers Gibson, T, Xu, BE, Karandikar, M, Berman, K, Cobb, MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22(2): 153-183.

Pellow, S, Chopin, P, File, SE (1985a) Are the anxiogenic effects of yohimbine mediated by its action at benzodiazepine receptors? Neurosci Lett 55(1): 5-9.

Pellow, S, Chopin, P, File, SE, Briley, M (1985b) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14(3): 149-167.

Pellow, S, File, SE (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav 24(3): 525-529.

Penninx, BW, Kritchevsky, SB, Yaffe, K, Newman, AB, Simonsick, EM, Rubin, S, Ferrucci, L, Harris, T, Pahor, M (2003) Inflammatory markers and depressed mood in older persons: results from the Health, Aging and Body Composition study. Biol Psychiatry 54(5): 566-572.

Pepeu, G, Giovannini, MG (2004) Changes in acetylcholine extracellular levels during cognitive processes. Learn Mem 11(1): 21-27.

Perez, DM, Karnik, SS (2005) Multiple signaling states of G-protein-coupled receptors. Pharmacol Rev 57(2): 147-161.

Peters, A (2006) The energy request of inflammation. Endocrinology 147(10): 4550-4552.

Peters, T, Henry, PJ (2009) Protease-activated receptors and prostaglandins in inflammatory lung disease. Br. J. Pharmacol. 158(4): 1017-1033.

Peterson, JW (1996) Bacterial Pathogenesis. In: Medical Microbiology, (TX), G (ed). University of Texas Medical Branch at Galveston.

Petit-Demouliere, B, Chenu, F, Bourin, M (2005) Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl) 177(3): 245-255.

Pierce, KL, Premont, RT, Lefkowitz, RJ (2002) Seven-transmembrane receptors. Nat Rev Mol Cell Biol 3(9): 639-650.

Pike, CJ, Vaughan, PJ, Cunningham, DD, Cotman, CW (1996) Thrombin attenuates neuronal cell death and modulates astrocyte reactivity induced by beta-amyloid in vitro. J Neurochem 66(4): 1374-1382.

Pitt, JJ (2009) Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30(1): 19-34.

Pompili, E, Fabrizi, C, Nori, SL, Panetta, B, Geloso, MC, Corvino, V, Michetti, F, Fumagalli, L (2011) Protease-activated receptor-1 expression in rat microglia after trimethyltin treatment. J Histochem Cytochem 59(3): 302-311.

Pompili, E, Nori, SL, Geloso, MC, Guadagni, E, Corvino, V, Michetti, F, Fumagalli, L (2004) Trimethyltin-induced differential expression of PAR subtypes in reactive astrocytes of the rat hippocampus. Molecular Brain Research 122(1): 93-98.

Poole, DP, Amadesi, S, Veldhuis, NA, Abogadie, FC, Lieu, T, Darby, W, Liedtke, W, Lew, MJ, McIntyre, P, Bunnett, NW (2013) Protease-activated receptor 2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. J Biol Chem 288(8): 5790-5802.

Power, AE (2004) Muscarinic cholinergic contribution to memory consolidation: with attention to Involvement of the basolateral amygdala. Current Medicinal Chemistry 11(8): 987-996.

Prut, L, Belzung, C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463(1-3): 3-33.

Puma, C, Deschaux, O, Molimard, R, Bizot, J-C (1999) Nicotine improves memory in an object recognition task in rats. European Neuropsychopharmacology 9(4): 323-327.

Qian, Z, Gilbert, ME, Colicos, MA, Kandel, ER, Kuhl, D (1993) Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature 361(6411): 453-457.

Qin, L, Wu, X, Block, ML, Liu, Y, Breese, GR, Hong, JS, Knapp, DJ, Crews, FT (2007) Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55(5): 453-462.

Radhika, V, Dhanasekaran, N (2001) Transforming G proteins. Oncogene 20(13): 1607-1614.

Rallabhandi, P, Nhu, QM, Toshchakov, VY, Piao, W, Medvedev, AE, Hollenberg, MD, Fasano, A, Vogel, SN (2008) Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. J Biol Chem 283(36): 24314-24325.

Ramachandran, R, Hollenberg, MD (2008) Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. Br J Pharmacol 153 Suppl 1: S263-282.

Ramachandran, R, Mihara, K, Chung, H, Renaux, B, Lau, CS, Muruve, DA, DeFea, KA, Bouvier, M, Hollenberg, MD (2011) Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2). J Biol Chem 286(28): 24638-24648.

Ramachandran, R, Mihara, K, Mathur, M, Rochdi, MD, Bouvier, M, Defea, K, Hollenberg, MD (2009) Agonist-biased signaling via proteinase activated receptor-2: differential activation of calcium and mitogen-activated protein kinase pathways. Mol Pharmacol 76(4): 791-801.

Ramachandran, R, Morice, AH, Compton, SJ (2006) Proteinase-activated receptor2 agonists upregulate granulocyte colony-stimulating factor, IL-8, and VCAM-1 expression in human bronchial fibroblasts. Am J Respir Cell Mol Biol 35(1): 133-141.

Ramachandran, R, Noorbakhsh, F, DeFea, K, Hollenberg, MD (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. Nature Reviews Drug Discovery 11(1): 69-86.

Ramachandran, R, Sadofsky, LR, Xiao, Y, Botham, A, Cowen, M, Morice, AH, Compton, SJ (2007) Inflammatory mediators modulate thrombin and cathepsin-G signaling in human bronchial fibroblasts by inducing expression of proteinase-activated receptor-4. Am J Physiol Lung Cell Mol Physiol 292(3): L788-798.

Ramos, A (2008) Animal models of anxiety: do I need multiple tests? Trends in pharmacological sciences 29(10): 493-498.

Rasmussen, UB, Vouret-Craviari, V, Jallat, S, Schlesinger, Y, Pages, G, Pavirani, A, Lecocq, JP, Pouyssegur, J, Van Obberghen-Schilling, E (1991) cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca2+ mobilization. FEBS Lett 288(1-2): 123-128.

Rawlins, JNP, Olton, DS (1982) The septo-hippocampal system and cognitive mapping. Behavioural Brain Research 5(4): 331-358.

Ray, WJ, Molnar, C, Aikins, D, Yamasaki, A, Newman, MG, Castonguay, L, Borkovec, TD (2009) Startle response in generalized anxiety disorder. Depress Anxiety 26(2): 147-154.

Reed, DE, Barajas-Lopez, C, Cottrell, G, Velazquez-Rocha, S, Dery, O, Grady, EF, Bunnett, NW, Vanner, SJ (2003) Mast cell tryptase and proteinase-activated receptor 2 induce hyperexcitability of guinea-pig submucosal neurons. J Physiol 547(Pt 2): 531-542.

Reger, ML, Hovda, DA, Giza, CC (2009) Ontogeny of Rat Recognition Memory measured by the novel object recognition task. Developmental psychobiology 51(8): 672-678.

Reisel, D, Bannerman, DM, Schmitt, WB, Deacon, RM, Flint, J, Borchardt, T, Seeburg, PH, Rawlins, JN (2002) Spatial memory dissociations in mice lacking GluR1. Nat Neurosci 5(9): 868-873.

RGM, M (1993) An attempt to dissociate 'spatial-mapping' and 'working-memory' In: theories of hippocampal function. Academic Press; New York

Ricciardolo, FL, Steinhoff, M, Amadesi, S, Guerrini, R, Tognetto, M, Trevisani, M, Creminon, C, Bertrand, C, Bunnett, NW, Fabbri, LM, Salvadori, S, Geppetti, P (2000) Presence and bronchomotor activity of protease-activated receptor-2 in guinea pig airways. Am J Respir Crit Care Med 161(5): 1672-1680.

Rickles, FR, Edwards, RL (1983) Activation of blood coagulation in cancer: Trousseau's syndrome revisited. Blood 62(1): 14-31.

Riedel, G, Micheau, J (2001) Function of the hippocampus in memory formation: desperately seeking resolution. Prog Neuropsychopharmacol Biol Psychiatry 25(4): 835-853.

Rodgers, RJ, Cao, B-J, Dalvi, A, Holmes, A (1997) Animal models of anxiety: an ethological perspective. Brazilian Journal of Medical and Biological Research 30: 289-304.

Rodgers, RJ, Cole, JC (1993) Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. Physiology & Behavior 54(4): 729-736.

Rodgers, RJ, Dalvi, A (1997) Anxiety, defence and the elevated plus-maze. Neuroscience & Biobehavioral Reviews 21(6): 801-810.

Rodriguez, JS, Paule, MG (2009) Working Memory Delayed Response Tasks in Monkeys.

Rodriguez, L, Cervantes, E, Ortiz, R (2011) Malnutrition and gastrointestinal and respiratory infections in children: a public health problem. Int J Environ Res Public Health 8(4): 1174-1205.

Rodriguiz, RM, Wetsel, WC (2006) Assessments of Cognitive Deficits in Mutant Mice.

Roozendaal, B, McEwen, BS, Chattarji, S (2009) Stress, memory and the amygdala. Nat Rev Neurosci 10(6): 423-433.

Rossato, JI, Bevilaqua, LR, Myskiw, JC, Medina, JH, Izquierdo, I, Cammarota, M (2007) On the role of hippocampal protein synthesis in the consolidation and reconsolidation of object recognition memory. Learn Mem 14(1): 36-46.

Rothwell, NJ, SJ (1995) Cytokines and the nervous system II: Actions and mechanisms of action. Trends Neurosci. 1995 Mar;18(3):130-6.

Roubenoff, R, Roubenoff, RA, Cannon, JG, Kehayias, JJ, Zhuang, H, Dawson-Hughes, B, Dinarello, CA, Rosenberg, IH (1994) Rheumatoid cachexia: cytokine-driven hypermetabolism accompanying reduced body cell mass in chronic inflammation. J Clin Invest 93(6): 2379-2386.

Rowe, JB, Toni, I, Josephs, O, Frackowiak, RS, Passingham, RE (2000) The prefrontal cortex: response selection or maintenance within working memory? Science 288(5471): 1656-1660.

Roy, SS, Saifeddine, M, Loutzenhiser, R, Triggle, CR, Hollenberg, MD (1998) Dual endothelium-dependent vascular activities of proteinase-activated receptor-2-activating peptides: evidence for receptor heterogeneity. Br J Pharmacol 123(7): 1434-1440.

Russell, PA (1973) Relationships between exploratory behaviour and fear: a review. British Journal of Psychology 64(3): 417-433.

Russell, WR, Nathan, PW (1946) Traumatic amnesia. Brain 69(4): 280-300.

Russo, A, Soh, UJ, Trejo, J (2009) Proteases display biased agonism at protease-activated receptors: location matters! Mol Interv 9(2): 87-96.

Saavedra, M, Contreras, CM, Azamar-Arizmendi, G, Hernandez-Lozano, M (2006) Differential progesterone effects on defensive burying and forced swimming tests depending upon a gradual decrease or an abrupt suppression schedules. Pharmacol Biochem Behav 83(1): 130-135.

Sabri, A, Short, J, Guo, J, Steinberg, SF (2002) Protease-activated receptor-1-mediated DNA synthesis in cardiac fibroblast is via epidermal growth factor receptor transactivation: distinct PAR-1 signaling pathways in cardiac fibroblasts and cardiomyocytes. Circ Res 91(6): 532-539.

Sanborn, BM, Yue, C, Wang, W, Dodge, KL (1998) G protein signalling pathways in myometrium: affecting the balance between contraction and relaxation. Rev Reprod 3(3): 196-205.

Saneyoshi, H, Mazzini, S, Avino, A, Portella, G, Gonzalez, C, Orozco, M, Marquez, VE, Eritja, R (2009) Conformationally rigid nucleoside probes help understand the role of sugar pucker and nucleobase orientation in the thrombin-binding aptamer. Nucleic Acids Res 37(17): 5589-5601.

Sarkisova, KY, Midzianovskaia, IS, Kulikov, MA (2003) Depressive-like behavioral alterations and c-fos expression in the dopaminergic brain regions in WAG/Rij rats with genetic absence epilepsy. Behav Brain Res 144(1-2): 211-226.

Sawada, K, Nishibori, M, Nakaya, N, Wang, Z, Saeki, K (2000) Purification and characterization of a trypsin-like serine proteinase from rat brain slices that degrades laminin and type IV collagen and stimulates protease-activated receptor-2. J Neurochem 74(4): 1731-1738.

Scarisbrick, IA, Isackson, PJ, Ciric, B, Windebank, AJ, Rodriguez, M (2001) MSP, a trypsin-like serine protease, is abundantly expressed in the human nervous system. J Comp Neurol 431(3): 347-361.

Schmidlin, F, Amadesi, S, Dabbagh, K, Lewis, DE, Knott, P, Bunnett, NW, Gater, PR, Geppetti, P, Bertrand, C, Stevens, ME (2002) Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J Immunol 169(9): 5315-5321.

Schmidt, VA, Nierman, WC, Feldblyum, TV, Maglott, DR, Bahou, WF (1997) The human thrombin receptor and proteinase activated receptor-2 genes are tightly linked on chromosome 5q13. Br J Haematol 97(3): 523-529.

Schultheiss, M, Neumcke, B, Richter, HP (1997) Endogenous trypsin receptors in Xenopus oocytes: linkage to internal calcium stores. Cell Mol Life Sci 53(10): 842-849.

Seatter, MJ, Drummond, R, Kanke, T, Macfarlane, SR, Hollenberg, MD, Plevin, R (2004) The role of the C-terminal tail in protease-activated receptor-2-mediated Ca2+ signalling, proline-rich tyrosine kinase-2 activation, and mitogen-activated protein kinase activity. Cell Signal 16(1): 21-29.

Seeliger, S, Derian, CK, Vergnolle, N, Bunnett, NW, Nawroth, R, Schmelz, M, Von Der Weid, PY, Buddenkotte, J, Sunderkotter, C, Metze, D, Andrade-Gordon, P, Harms, E, Vestweber, D, Luger, TA, Steinhoff, M (2003) Proinflammatory

role of proteinase-activated receptor-2 in humans and mice during cutaneous inflammation in vivo. FASEB J 17(13): 1871-1885.

Seifert, R, Wenzel-Seifert, K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. Naunyn Schmiedebergs Arch Pharmacol 366(5): 381-416.

Seitzberg, JG, Knapp, AE, Lund, BW, Mandrup Bertozzi, S, Currier, EA, Ma, JN, Sherbukhin, V, Burstein, ES, Olsson, R (2008) Discovery of potent and selective small-molecule PAR-2 agonists. J Med Chem 51(18): 5490-5493.

Seminario-Vidal, L, Kreda, S, Jones, L, O'Neal, W, Trejo, J, Boucher, RC, Lazarowski, ER (2009) Thrombin promotes release of ATP from lung epithelial cells through coordinated activation of rho- and Ca2+-dependent signaling pathways. J Biol Chem 284(31): 20638-20648.

Sevigny, L.M., Zhang, P., Bohm, A., Lazarides, K., Perides, G., Covic, L. & Kuliopulos, A. (2011) Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins. PNAS ,108 (20), 8491-8496.

Shapiro, MJ, Weiss, EJ, Faruqi, TR, Coughlin, SR (2000) Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. J Biol Chem 275(33): 25216-25221.

Sharma, S, Rakoczy, S, Brown-Borg, H (2010) Assessment of spatial memory in mice. Life Sci 87(17-18): 521-536.

Sharot, T, Martorella, EA, Delgado, MR, Phelps, EA (2007) How personal experience modulates the neural circuitry of memories of September 11. Proceedings of the National Academy of Sciences of the United States of America 104(1): 389-394.

Shaw, KL, Parsons, YM (2002) Divergence of mate recognition behavior and its consequences for genetic architectures of speciation. Am Nat 159 Suppl 3: S61-75.

Shelton, RC, Miller, AH (2011) Inflammation in depression: is adiposity a cause? Dialogues Clin Neurosci 13(1): 41-53.

Shen, R-N, Lu, L, Young, P, Shidnia, H, Hornback, NB, Broxmeyer, HE (1994) Influence of elevated temperature on natural killer cell activity, lymphokine-activated killer cell activity and lectin-dependent cytotoxicity of human umbilical cord blood and adult blood cells. International Journal of Radiation Oncology*Biology*Physics 29(4): 821-826.

Shigetomi, E, Bowser, DN, Sofroniew, MV, Khakh, BS (2008) Two forms of astrocyte calcium excitability have distinct effects on NMDA receptor-mediated slow inward currents in pyramidal neurons. J Neurosci 28(26): 6659-6663.

Shuman, MA (1986) Thrombin-cellular interactions. Ann N Y Acad Sci 485: 228-239.

Siegert, RJ, Abernethy, DA (2005) Depression in multiple sclerosis: a review. J Neurol Neurosurg Psychiatry 76(4): 469-475.

Siemiatkowski, M, Sienkiewicz-Jarosz, H, Czlonkowska, AI, Bidzinski, A, Plaznik, A (2000) Effects of buspirone, diazepam, and zolpidem on open field behavior, and brain [3H]muscimol binding after buspirone pretreatment. Pharmacol Biochem Behav 66(3): 645-651.

Smith-Swintosky, VL, Cheo-Isaacs, CT, D'Andrea, MR, Santulli, RJ, Darrow, AL, Andrade-Gordon, P (1997) Protease-activated receptor-2 (PAR-2) is present in the rat hippocampus and is associated with neurodegeneration. J Neurochem 69(5): 1890-1896.

Smrcka, AV (2008) G protein betagamma subunits: central mediators of G protein-coupled receptor signaling. Cell Mol Life Sci 65(14): 2191-2214.

Sobocki, J, Krolczyk, G, Herman, RM, Matyja, A, Thor, PJ (2005) Influence of vagal nerve stimulation on food intake and body weight--results of experimental studies. J Physiol Pharmacol 56 Suppl 6: 27-33.

Soh, UJ, Dores, MR, Chen, B, Trejo, J (2010) Signal transduction by protease-activated receptors. Br J Pharmacol 160(2): 191-203.

Song, C, Phillips, AG, Leonard, B (2003) Interleukin 1 beta enhances conditioned fear memory in rats: possible involvement of glucocorticoids. Eur J Neurosci 18(7): 1739-1743.

Spitzer, M (2008) Decade of the mind. Philos Ethics Humanit Med 3: 7.

Spowart-Manning, L, van der Staay, FJ (2004) The T-maze continuous alternation task for assessing the effects of putative cognition enhancers in the mouse. Behav Brain Res 151(1-2): 37-46.

Spulber, S, Edoff, K, Hong, L, Morisawa, S, Shirahata, S, Ceccatelli, S (2012) Molecular hydrogen reduces LPS-induced neuroinflammation and promotes recovery from sickness behaviour in mice. PloS one 7(7): e42078.

Squire, LR, Zola, SM (1996) Structure and function of declarative and nondeclarative memory systems. Proceedings of the National Academy of Sciences of the United States of America 93(24): 13515-13522.

Stalheim, L, Ding, Y, Gullapalli, A, Paing, MM, Wolfe, BL, Morris, DR, Trejo, J (2005) Multiple independent functions of arrestins in the regulation of protease-activated receptor-2 signaling and trafficking. Mol Pharmacol 67(1): 78-87.

Stanford, SC (2007) The open field test: reinventing the wheel. Journal of psychopharmacology (Oxford, England) 21(2): 134-135.

Stead, RH, Tomioka, M, Quinonez, G, Simon, GT, Felten, SY, Bienenstock, J (1987) Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proc Natl Acad Sci U S A 84(9): 2975-2979.

Steckler, T, Drinkenburg, WH, Sahgal, A, Aggleton, JP (1998) Recognition memory in rats--II. Neuroanatomical substrates. Prog Neurobiol 54(3): 313-332.

Stein, DG (2001) Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? Trends Neurosci 24(7): 386-391.

Steinberg, GR, Watt, MJ, Febbraio, MA (2009) Cytokine regulation of AMPK signalling. Front Biosci 14: 1902-1916.

Steinberg, SF (2005) The cardiovascular actions of protease-activated receptors. Mol Pharmacol 67(1): 2-11.

Steiner, JE (1973) The gustofacial response: observation on normal and anencephalic newborn infants. Symp Oral Sens Percept(4): 254-278.

Steinhoff, M, Buddenkotte, J, Shpacovitch, V, Rattenholl, A, Moormann, C, Vergnolle, N, Luger, TA, Hollenberg, MD (2005) Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocrine reviews 26(1): 1-43.

Steinhoff, M, Neisius, U, Ikoma, A, Fartasch, M, Heyer, G, Skov, PS, Luger, TA, Schmelz, M (2003) Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 23(15): 6176-6180.

Steinhoff, M, Vergnolle, N, Young, SH, Tognetto, M, Amadesi, S, Ennes, HS, Trevisani, M, Hollenberg, MD, Wallace, JL, Caughey, GH, Mitchell, SE, Williams, LM, Geppetti, P, Mayer, EA, Bunnett, NW (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nature medicine 6(2): 151-158.

Stolp, HB, Dziegielewska, KM (2009) Review: Role of developmental inflammation and blood-brain barrier dysfunction in neurodevelopmental and neurodegenerative diseases. Neuropathol Appl Neurobiol 35(2): 132-146.

Striggow, F, Riek-Burchardt, M, Kiesel, A, Schmidt, W, Henrich-Noack, P, Breder, J, Krug, M, Reymann, KG, Reiser, G (2001) Four different types of protease-

activated receptors are widely expressed in the brain and are up-regulated in hippocampus by severe ischemia. Eur J Neurosci 14(4): 595-608.

Strle, K, McCusker, RH, Tran, L, King, A, Johnson, RW, Freund, GG, Dantzer, R, Kelley, KW (2007) Novel activity of an anti-inflammatory cytokine: IL-10 prevents TNFalpha-induced resistance to IGF-I in myoblasts. J Neuroimmunol 188(1-2): 48-55.

Suen, JY, Barry, GD, Lohman, RJ, Halili, MA, Cotterell, AJ, Le, GT, Fairlie, DP (2012) Modulating human proteinase activated receptor 2 with a novel antagonist (GB88) and agonist (GB110). British journal of pharmacology 165(5): 1413-1423.

Suen, JY, Gardiner, B, Grimmond, S, Fairlie, DP (2010) Profiling gene expression induced by protease-activated receptor 2 (PAR2) activation in human kidney cells. PLoS One 5(11): e13809.

Sun, J, Singh, V, Kajino-Sakamoto, R, Aballay, A (2011) Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. Science 332(6030): 729-732.

Suo, Z, Wu, M, Ameenuddin, S, Anderson, HE, Zoloty, JE, Citron, BA, Andrade-Gordon, P, Festoff, BW (2002) Participation of protease-activated receptor-1 in thrombin-induced microglial activation. J Neurochem 80(4): 655-666.

Suo, Z, Wu, M, Citron, BA, Gao, C, Festoff, BW (2003) Persistent protease-activated receptor 4 signaling mediates thrombin-induced microglial activation. J Biol Chem 278(33): 31177-31183.

Suo, Z., Wu, M., Citron, B.A., Gao, C. & Festoff, B.W. (2003) Persistent protease-activated receptor 4 signaling mediates thrombin-induced microglial activation. J Biol Chem, 278, 31177-83.

Sutcliffe, JS, Marshall, KM, Neill, JC (2007) Influence of gender on working and spatial memory in the novel object recognition task in the rat. Behav Brain Res 177(1): 117-125.

Swerdlow, NR, Geyer, MA (1998) Using an animal model of deficient sensorimotor gating to study the pathophysiology and new treatments of schizophrenia. Schizophr Bull 24(2): 285-301.

Swerdlow, NR, Geyer, MA, Braff, DL (2001) Neural circuit regulation of prepulse inhibition of startle in the rat: current knowledge and future challenges. Psychopharmacology (Berl) 156(2-3): 194-215.

Swerdlow, NR, Weber, M, Qu, Y, Light, GA, Braff, DL (2008) Realistic expectations of prepulse inhibition in translational models for schizophrenia research. Psychopharmacology (Berl) 199(3): 331-388.

Sydora, BC, MacFarlane, SM, Lupicki, M, Dmytrash, AL, Dieleman, LA, Fedorak, RN (2010) An imbalance in mucosal cytokine profile causes transient intestinal inflammation following an animal's first exposure to faecal bacteria and antigens. Clin Exp Immunol 161(1): 187-196.

Syvanen, S, Lindhe, O, Palner, M, Kornum, BR, Rahman, O, Langstrom, B, Knudsen, GM, Hammarlund-Udenaes, M (2009) Species differences in blood-brain barrier transport of three positron emission tomography radioligands with emphasis on P-glycoprotein transport. Drug Metab Dispos 37(3): 635-643.

Taglialatela, G, Hogan, D, Zhang, WR, Dineley, KT (2009) Intermediate- and long-term recognition memory deficits in Tg2576 mice are reversed with acute calcineurin inhibition. Behav Brain Res 200(1): 95-99.

Tai, SS, Welch, MJ (2004) Development and evaluation of a candidate reference method for the determination of total cortisol in human serum using isotope dilution liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. Anal Chem 76(4): 1008-1014.

Tallentire, D, McRae, G, Spedding, M, Clark, R, Vickery, B (1996) Modulation of sexual behaviour in the rat by a potent and selective alpha 2-adrenoceptor antagonist, delequamine (RS-15385-197). British journal of pharmacology 118(1): 63-72.

Tanaka, C, Nishizuka, Y (1994) The protein kinase C family for neuronal signaling. Annu Rev Neurosci 17: 551-567.

Tanaka, M, Arai, H, Liu, N, Nogaki, F, Nomura, K, Kasuno, K, Oida, E, Kita, T, Ono, T (2005) Role of coagulation factor Xa and protease-activated receptor 2 in human mesangial cell proliferation. Kidney Int 67(6): 2123-2133.

Taylor, EM, Moghraby, JS, Lees, JH, Smit, B, Moens, PB, Lehmann, AR (2001) Characterization of a novel human SMC heterodimer homologous to the Schizosaccharomyces pombe Rad18/Spr18 complex. Mol Biol Cell 12(6): 1583-1594.

Terayama, R, Bando, Y, Takahashi, T, Yoshida, S (2004) Differential expression of neuropsin and protease M/neurosin in oligodendrocytes after injury to the spinal cord. Glia 48(2): 91-101.

Terry, AV, Jr. (2009) Spatial Navigation (Water Maze) Tasks. In: Methods of Behavior Analysis in Neuroscience., Buccafusco, J (ed), 2011/01/05 edn. Boca Raton (FL): CRC Press.

Theoharides, TC (1990) Mast cells: the immune gate to the brain. Life Sci 46(9): 607-617.

Theoharides, TC, Alysandratos, KD, Angelidou, A, Delivanis, DA, Sismanopoulos, N, Zhang, B, Asadi, S, Vasiadi, M, Weng, Z, Miniati, A, Kalogeromitros, D (2012) Mast cells and inflammation. Biochim Biophys Acta 1822(1): 21-33.

Tizard, I (2008) Sickness behavior, its mechanisms and significance. Anim Health Res Rev 9(1): 87-99.

Tobin, AB (2008) G-protein-coupled receptor phosphorylation: where, when and by whom. Br J Pharmacol 153 Suppl 1: S167-176.

Tolmacheva, EA, van Luijtelaar, G (2007) Absence seizures are reduced by the enhancement of GABA-ergic inhibition in the hippocampus in WAG/Rij rats. Neurosci Lett 416(1): 17-21.

Tolman, EC (1948) Cognitive maps in rats and men. Psychol Rev 55(4): 189-208.

Toth, J, Siklodi, E, Medveczky, P, Gallatz, K, Nemeth, P, Szilagyi, L, Graf, L, Palkovits, M (2007) Regional distribution of human trypsinogen 4 in human brain at mRNA and protein level. Neurochem Res 32(9): 1423-1433.

Traupe, T, Barton, M (2004) Protease-activated receptors and EDHF: the icing on the cake? Cardiovasc Res 61(4): 645-647.

Travis, SP, Higgins, PD, Orchard, T, Van Der Woude, CJ, Panaccione, R, Bitton, A, O'Morain, C, Panes, J, Sturm, A, Reinisch, W, Kamm, MA, D'Haens, G (2011) Review article: defining remission in ulcerative colitis. Aliment Pharmacol Ther 34(2): 113-124.

Trejo, J, Connolly, AJ, Coughlin, SR (1996) The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. J Biol Chem 271(35): 21536-21541.

Trejo, J, Hammes, SR, Coughlin, SR (1998) Termination of signaling by protease-activated receptor-1 is linked to lysosomal sorting Vol. 95, pp 13698-13702.

Tulving, E (1972) Organization of Memory. In: Episodic and semantic memory, Tulving, E, Donaldson, W (eds), pp 381–403. Academic Press, New York.

Tuteja, N (2009) Signaling through G protein coupled receptors. Plant Signal Behav 4(10): 942-947.

Tzschentke, TM (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol 12(3-4): 227-462.

Ubl, JJ, Vohringer, C, Reiser, G (1998) Co-existence of two types of [Ca2+]i-inducing protease-activated receptors (PAR-1 and PAR-2) in rat astrocytes and C6 glioma cells. Neuroscience 86(2): 597-609.

Ullman, MT (2004) Contributions of memory circuits to language: the declarative/procedural model. Cognition 92(1-2): 231-270.

Umeda, S, Kurosaki, Y, Terasawa, Y, Kato, M, Miyahara, Y (2011) Deficits in prospective memory following damage to the prefrontal cortex. Neuropsychologia 49(8): 2178-2184.

Urban, JD, Clarke, WP, von Zastrow, M, Nichols, DE, Kobilka, B, Weinstein, H, Javitch, JA, Roth, BL, Christopoulos, A, Sexton, PM, Miller, KJ, Spedding, M, Mailman, RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther 320(1): 1-13.

Valeriani, M, Mariotti, P, Le Pera, D, Restuccia, D, De Armas, L, Maiese, T, Vigevano, F, Antuzzi, D, Zampino, G, Ricci, R, Tonali, P (2004) Functional assessment of A delta and C fibers in patients with Fabry's disease. Muscle Nerve 30(6): 708-713.

Van Dam, JT, Schrama, JW, van der Hel, W, Verstegen, MW, Zwart, D (1996) Heat production, body temperature, and body posture in west African dwarf goats infected with Trypanosoma vivax. Vet Q 18(2): 55-59.

Vanhauwe, JF, Thomas, TO, Minshall, RD, Tiruppathi, C, Li, A, Gilchrist, A, Yoon, EJ, Malik, AB, Hamm, HE (2002) Thrombin receptors activate G(o) proteins in endothelial cells to regulate intracellular calcium and cell shape changes. J Biol Chem 277(37): 34143-34149.

Vaughan, PJ, Pike, CJ, Cotman, CW, Cunningham, DD (1995) Thrombin receptor activation protects neurons and astrocytes from cell death produced by environmental insults. J Neurosci 15(7 Pt 2): 5389-5401.

Vergnolle, N (1999) Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. J Immunol 163(9): 5064-5069.

Vergnolle, N (2003) Proteinase-activated receptors and nociceptive pathways. Drug Development Research 59(4): 382-385.

Vergnolle, N (2004) Modulation of visceral pain and inflammation by protease-activated receptors. Br J Pharmacol 141(8): 1264-1274.

Vergnolle, N (2005) Protease-activated receptors and inflammatory hyperalgesia. Memorias do Instituto Oswaldo Cruz 100 Suppl 1: 173-176.

Vergnolle, N, Bunnett, NW, Sharkey, KA, Brussee, V, Compton, SJ, Grady, EF, Cirino, G, Gerard, N, Basbaum, AI, Andrade-Gordon, P, Hollenberg, MD, Wallace, JL (2001) Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway. Nature medicine 7(7): 821-826.

Vergnolle, N, Cellars, L, Mencarelli, A, Rizzo, G, Swaminathan, S, Beck, P, Steinhoff, M, Andrade-Gordon, P, Bunnett, NW, Hollenberg, MD, Wallace, JL, Cirino, G, Fiorucci, S, et al (2004) A role for proteinase-activated receptor-1 in inflammatory bowel diseases. J Clin Invest. 2004 Nov;114(10):1444-56.

Vergnolle, N, Hollenberg, MD, Sharkey, KA, Wallace, JL (1999) Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. British journal of pharmacology 127(5): 1083-1090.

Voikar, V, Koks, S, Vasar, E, Rauvala, H (2001) Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies. Physiol Behav 72(1-2): 271-281.

Vorhees, CV, Williams, MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 1(2): 848-858.

Vouret-Craviari, V, Van Obberghen-Schilling, E, Rasmussen, UB, Pavirani, A, Lecocq, JP, Pouyssegur, J (1992) Synthetic alpha-thrombin receptor peptides activate G protein-coupled signaling pathways but are unable to induce mitogenesis. Mol Biol Cell 3(1): 95-102.

Vouret-Craviari, V, Van Obberghen-Schilling, E, Scimeca, JC, Van Obberghen, E, Pouyssegur, J (1993) Differential activation of p44mapk (ERK1) by alpha-thrombin and thrombin-receptor peptide agonist. Biochem J 289 (Pt 1): 209-214.

Vu, TK, Hung, DT, Wheaton, VI, Coughlin, SR (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64(6): 1057-1068.

Walf, AA, Frye, CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protocols 2(2): 322-328.

Walf, AA, Frye, CA (2009) Effects of two estradiol regimens on anxiety and depressive behaviors and trophic effects in peripheral tissues in a rodent model. Gend Med 6(1): 300-311.

Walker, TR, Cadwallader, KA, MacKinnon, A, Chilvers, ER (2005) Thrombin induces DNA synthesis and phosphoinositide hydrolysis in airway smooth muscle by activation of distinct receptors. Biochem Pharmacol 70(6): 959-967.

Wall, PM, Messier, C (2001) Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. Neurosci Biobehav. Rev 25(3):275-86.

Wallace, JL, Chin, BC (1997) Inflammatory mediators in gastrointestinal defense and injury. Proc Soc Exp Biol Med 214(3): 192-203.

Walsh, RN, Cummins, RA (1976) The Open-Field Test: a critical review. Psychol Bull 83(3): 482-504.

Wan, H, Aggleton, JP, Brown, MW (1999) Different contributions of the hippocampus and perirhinal cortex to recognition memory. J Neurosci 19(3): 1142-1148.

Wang, H, Ubl, JJ, Reiser, G (2002) Four subtypes of protease-activated receptors, co-expressed in rat astrocytes, evoke different physiological signaling. Glia 37(1): 53-63.

Wang, H, Ubl, JJ, Stricker, R, Reiser, G (2002) Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. Am J Physiol Cell Physiol 283(5): C1351-1364.

Wang, J, Boerma, M, Kulkarni, A, Hollenberg, MD, Hauer-Jensen, M (2010) Activation of protease activated receptor 2 by exogenous agonist exacerbates early radiation injury in rat intestine. Int J Radiat Oncol Biol Phys 77(4): 1206-1212.

Wang, L, Luo, J, Fu, Y, He, S (2006) Induction of interleukin-8 secretion and activation of ERK1/2, p38 MAPK signaling pathways by thrombin in dermal fibroblasts. Int J Biochem Cell Biol 38(9): 1571-1583.

Wang, Y, Luo, W, Reiser, G (2007) Proteinase-activated receptor-1 and -2 induce the release of chemokine GRO/CINC-1 from rat astrocytes via differential activation of JNK isoforms, evoking multiple protective pathways in brain. Biochem J 401(1): 65-78.

Wang, Y, Luo, W, Reiser, G (2008) Trypsin and trypsin-like proteases in the brain: proteolysis and cellular functions. Cell Mol Life Sci 65(2): 237-252.

Warburton, EC, Koder, T, Cho, K, Massey, PV, Duguid, G, Barker, GR, Aggleton, JP, Bashir, ZI, Brown, MW (2003) Cholinergic neurotransmission is essential for perirhinal cortical plasticity and recognition memory. Neuron 38(6): 987-996.

Warren, SG, Juraska, JM (1997) Spatial and nonspatial learning across the rat estrous cycle. Behav Neurosci 111(2): 259-266.

Webel, DM, Johnson, RW, Baker, DH (1998) Lipopolysaccharide-induced reductions in food intake do not decrease the efficiency of lysine and threonine utilization for protein accretion in chickens. J Nutr 128(10): 1760-1766.

Weinstein, JR, Gold, SJ, Cunningham, DD, Gall, CM (1995) Cellular localization of thrombin receptor mRNA in rat brain: expression by mesencephalic dopaminergic neurons and codistribution with prothrombin mRNA. J Neurosci 15(4): 2906-2919.

Wettschureck, N, Offermanns, S (2005) Mammalian G proteins and their cell type specific functions. Physiol Rev 85(4): 1159-1204.

White, NM, Carr, GD (1985) The conditioned place preference is affected by two independent reinforcement processes. Pharmacol Biochem Behav 23(1): 37-42.

Wicher, D (2010) Design principles of sensory receptors. Front Cell Neurosci 4.

Widmann, C, Gibson, S, Jarpe, MB, Johnson, GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79(1): 143-180.

Willner, P (1997) Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology (Berl) 134(4): 319-329.

Willner, P, Towell, A, Sampson, D, Sophokleous, S, Muscat, R (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology (Berl) 93(3): 358-364.

Wu, CC, Wu, SY, Liao, CY, Teng, CM, Wu, YC, Kuo, SC (2010) The roles and mechanisms of PAR4 and P2Y12/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation. Br J Pharmacol 161(3): 643-658.

Xi, G, Reiser, G, Keep, RF (2003) The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? J Neurochem 84(1): 3-9.

Xu, WF, Andersen, H, Whitmore, TE, Presnell, SR, Yee, DP, Ching, A, Gilbert, T, Davie, EW, Foster, DC (1998) Cloning and characterization of human protease-activated receptor 4. Proc Natl Acad Sci U S A 95(12): 6642-6646.

Xue, M, Hollenberg, MD, Yong, VW (2006) Combination of thrombin and matrix metalloproteinase-9 exacerbates neurotoxicity in cell culture and intracerebral hemorrhage in mice. J Neurosci 26(40): 10281-10291.

Yan, HC, Cao, X, Das, M, Zhu, XH, Gao, TM (2010) Behavioral animal models of depression. Neurosci Bull 26(4): 327-337.

Yasui, H, Gabazza, EC, Tamaki, S, Kobayashi, T, Hataji, O, Yuda, H, Shimizu, S, Suzuki, K, Adachi, Y, Taguchi, O (2001) Intratracheal administration of activated protein C inhibits bleomycin-induced lung fibrosis in the mouse. Am J Respir Crit Care Med 163(7): 1660-1668.

Ye, J, Keller, JN (2010) Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction. Aging (Albany NY) 2(6): 361-368.

Yeomans, JS, Li, L, Scott, BW, Frankland, PW (2002) Tactile, acoustic and vestibular systems sum to elicit the startle reflex. Neurosci Biobehav Rev 26(1): 1-11.

Yin, X, Wright, J, Wall, T, Grammas, P (2010) Brain endothelial cells synthesize neurotoxic thrombin in Alzheimer's disease. Am J Pathol 176(4): 1600-1606.

Yirmiya, R (1996) Endotoxin produces a depressive-like episode in rats. Brain research 711(1-2): 163-174.

Yoon, E.J., Hamm, H.E.& Currie, K.P. (2008) G protein betagamma subunits modulate the number and nature of exocytotic fusion events in adrenal chromaffin cells independent of calcium entry. J Neurophysiol, 100, 2929-39.

Yousef, GM, Kishi, T, Diamandis, EP (2003) Role of kallikrein enzymes in the central nervous system. Clin Chim Acta 329(1-2): 1-8.

Zhang, L, Hu, XZ, Li, H, Li, X, Smerin, S, Benedek, DM, Ursano, R (2011) Startle response related genes. Med Hypotheses 77(4): 685-691.

Zheng, H, Chu, J, Qiu, Y, Loh, HH, Law, PY (2008) Agonist-selective signaling is determined by the receptor location within the membrane domains. Proc Natl Acad Sci U S A 105(27): 9421-9426.

Zimmerberg, B, Farley, MJ (1993) Sex differences in anxiety behavior in rats: Role of gonadal hormones. Physiology & Behavior 54(6): 1119-1124.

Zukerman, S, Touzani, K, Margolskee, RF, Sclafani, A (2009) Role of olfaction in the conditioned sucrose preference of sweet-ageusic T1R3 knockout mice. Chem Senses 34(8): 685-694.

Zunszain, PA, Anacker, C, Cattaneo, A, Carvalho, LA, Pariante, CM (2011) Glucocorticoids, cytokines and brain abnormalities in depression. Prog Neuropsychopharmacol Biol Psychiatry 35(3): 722-729.

Zunszain, PA, Hepgul, N, Pariante, CM (2012) Inflammation and depression. Curr Top Behav Neurosci 14: 135-151.