Investigating the neuroprotective properties of PAR2.

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Declaration of Authenticity and Author's Rights

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Joy and love only become real when shared. Martin Werno.

Previously Published Work

1. **Moudio Serge**, Trevor Bushell, Hugh Nuthall and Jim McCarty (2016) Investigating the neuroprotective properties of PAR2 in a kainic acid induced model of neurotoxicity. Instituto de Neurociencias meeting, Alicante, Spain (poster and keynote symposium).

2. **Moudio Serge**, Trevor Bushell, Hugh Nuthall and Jim McCarty (2016) Investigating the neuroprotective properties of PAR2 in a kainic acid induced model of neurotoxicity. London's students Neuroscience network, London, UK (poster).

3. **Moudio Serge**, Trevor Bushell, Hugh Nuthall and Jim McCarty (2015) Investigating the neuroprotective properties of PAR2 in a kainic acid induced model of neurotoxicity. Society for Neuroscience, Chicago, USA (poster).

4. **Moudio Serge**, Trevor Bushell (2015) Characterising PAR2 in the central nervous. British Neuroscience Association, Edinburgh, Scotland (poster).

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LIST OF ABBREVIATIONS

µg Microgram

µM Micro molar

µl Microliter

µm Micrometre

2-f 2-furoyl-LIGRLO-NH_2

7-TM 7-Transmembrane receptors

AC AC-264163

AC Adenylate cyclase

Ach Acetylcholine

AD Alzheimer's disease

ADP Adenosine diphosphate

AMP Adenosine monophosphate

ANOVA Analysis of variance

ATP Adenosine triphosphate

BBB Blood brain barrier

BMK Big MAPK

BSA Bovine serum albumin

CA1 Cornu ammonis area 1

CA2 Cornu ammonis area 2

Ca^{2+} Calcium

CA3 Cornu ammonis area 3

CaMKII Ca^{2+}/calmodulin-dependent protein kinase II

$CaCl_2$ Calcium chloride

CNS Central nervous system

cAMP Cyclic adenosine monophosphate

CGRP Calcitonin gene-elated peptide

DAG Diacylglycerol

DIV Days in vitro

DRG Dorsal root ganglion

DG Dentate gyrus

DNA Deoxyribonucleic acid

EAE Experimental autoimmune encephalomyelitis

EDTA Ethylene-di-amine tetra-acetic acid

EPAC Guanine nucleotide exchange protein

ER Endoplasmic reticulum

ERK Extracellular signal-regulated kinases

FBS Foetal bovine serum

G-protein Guanine nucleotide-binding protein

Gα G-protein alpha subunit

Gβγ G-protein beta-gamma subunit

GEF Guanine nucleotide exchange factor

GPCR G-protein coupled receptors

GABA Gamma-Aminobutyric acid

GFAP Glial fibrillary acidic protein

GFP Green fluorescent protein

GPCR G-protein coupled receptors

GRK G-protein receptor kinase

GTP Guanosine-5'-triphosphate

HCl Hydrochloric acid

HEK Human Embryonic Kidney

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV Human Immunodeficiency Virus

IL Interleukin

IP_3 Inositol 1,4,5-triphosphate

JNK c-Jun NH2-terminal Kinases

K^+ Potassium

KA Kainic acid

KCl Potassium chloride

$KMeSO_2$ Potassium methyl sulphate

KO Knock-out

KYN Kynurenic acid

L Litre

M Molar

MAPK Mitogen-Activated Protein Kinase

MEM Minimum essential medium

mGluR Metabotropic glutamate receptor

$MgSO_4$ Magnesium sulphate

ml Millilitre

mm Millimetre

mM Mill molar

ms Millisecond

MS Multiple sclerosis

Na^+ Sodium

NaCl Sodium chloride

$NaHCO_3$ Sodium hydrogen carbonate

NaH_2PO_4 Sodium dihydrogen phosphate

NMDAR N-methyl-D-aspartic acid receptor

NO Nitric oxide

PAR Proteinase-activated receptor

PBS Phosphate buffered saline

PD Parkinson's disease

PDE Cyclic nucleotide phosphodiesterases

PI Propidium Iodide

PIP_2 Phosphatidylinositol 4,5-bisphosphate

PKC Protein kinase C

PLC Phospholipase C

PKA Protein kinase A

PNS Peripheral nervous system

Poly I:C Polyinosinic:polycytidylic acid

RGC Retinal ganglion cells

ROI Regions of interest

ROS Reactive oxygen species

RT Room temperature

SEM Standard error of mean

SLIGRL	Ser-Leu-Ile-Gly-Arg-Leu-NH_2
TNF-α	Tumour necrosis factor alpha
TRPV	Transient receptors potential vanilloid
UN	United Nations
UK	United Kingdom
US $	United States Dollar
WT	Wildtype
WHO	World Health Organisation
YFP	Yellow fluorescent protein

ABSTRACT

Aims: Proteinase-activated receptor 2 (PAR2), a GPCR subtype with a novel activation mechanism, has recently received increasing interest due to its potential neuroprotective role in CNS diseases. Investigating the role and properties of PAR2 in CNS was previously made difficult by the limited selectivity and potency of available activators. Recently however, novel PAR2 activators have been developed allowing further characterisation and investigation.

Methods: Ca^{2+} imaging was performed on rat primary hippocampal cultures used at 10-14 DIV. Internalisation studies were performed using PAR2 transfected tsA-201 cells. Mice pup organotypic hippocampal slices were prepared with protection against kainite (KA)-induced neurotoxicity investigated from 15 DIV. Finally, effects of PAR2 activation were assessed *in vivo* on cardiac function, on behaviour such as locomotion and anxiety and in the EAE mouse model of MS. Statistical analysis were performed using one or two-way ANOVA and Dunnett's or Bonferroni post hoc tests for multiple comparisons, with $p < 0.05$ considered as significant.

Results: In this report we have been able to study the effects of PAR2 activation in CNS preparations where we monitored comparable increases in Ca^{2+} concentration to those seen in previous studies following application of peptide-based activators. Furthermore, the proposed PAR2 antagonist GB88, while not inducing changes in Ca^{2+} concentrations, led to PAR2 internalisation, therefore suggesting it is a biased agonist. Additionally, we have shown in an *ex vivo* model of excito-toxicity, consistent and long lasting beneficial effects of PAR2 activation. Finally, in the EAE model of CNS inflammation, we have detected beneficial effects induced by PAR2 activation including decreases clinical signs intensity as well as in the relapses occurrence and intensity furthermore, looking at the cytokine profiles, we were able to identify correlations between PAR2 activation and IL-6 production.

Conclusions: The results presented in this thesis establish PAR2 activation as neuroprotective *in vitro* and *in vivo* in the context of neurodegeneration, thus constituting a solid foundation to establish PAR2 as an intriguing potential target for CNS drug discovery and suggest that modulation of its expression or function may be a viable strategy in the treatment of a large range of CNS diseases.

Keywords: GPCR, PAR2, Hippocampal organotypic culture, Cell death, Neuroprotection.

Chapter I. INTRODUCTION.

1.1. Context.

1.1.1. CNS diseases.

The nervous system corresponds to a network of nerves allowing the body to send messages to and from its organs in order to respond and adapt to signals from the surrounding environment. It is divided into two compartments; the central nervous system (CNS) consisting of the brain and spinal cord enclosed within the skull and spine and the peripheral nervous system (PNS) consisting of all other nerves used to connect the CNS to the rest of the body. The CNS receives processes and stores information from the PNS and sends out messages directing the response. It is vulnerable to various disorders affecting either the brain and/or the spinal cord and resulting in neurological or psychiatric disorders with the causes of these diseases being numerous including trauma, infections, autoimmune reactions, structural defects, tumours, degeneration or blood flow disruptions (Barberger-Gateau & Fabrigoule, 1997).

1.1.1.1. Dementia

The term dementia refers to a group of syndromes, usually of a chronic or progressive nature, in which higher cortical functions, including memory, orientation, learning capacity and language are altered (Tarawneh & Holtzman, 2012). It is characterised by a decline in cognitive abilities of sufficient amplitude to interfere with normal social behaviour (Plassman et al., 2007). Dementia is mainly caused by disease or trauma; it is also often associated with increasing age, to date over 100 subtypes of dementia have been described, each characterised by differences in course and variations in pattern of expression and neuropathology (Brunnström et al., 2009). The

main subtypes include Alzheimer's disease (AD), vascular dementia, alcohol-related dementia and dementia with Lewy bodies but dementia is part of the symptomatology of other CNS diseases including multiple sclerosis (MS), Parkinson's disease (PD) and Huntington's disease (Gupta et al., 2009; table 1.1). The pathological origin and aetiology of dementia remains mostly unknown, while treatments and prevention largely depend on the level of understanding of the underlying biological and environmental factors associated with increased risks.

Table 1.1 - Classification of CNS disorders.

Vascular disorders	Infections	Structural disorders	Functional disorders	Degenerative disorders
Stroke, Ischemia, vascular dementia.	Meningitis, Encephalitis, Guillain-Barré syndrome…	Brain or Spinal cord injuries, Tumours, alcohol-related dementia …	Epilepsy, Depression, Schizophrenia…	Parkinson disease, Multiple sclerosis, Alzheimer disease, dementia with Lewy bodies…
Cognitive impairment, language dysfunctions, paralysis, muscle weakness.	Hallucinations, seizures, involuntary nervous system dysfunctions.	Coordination problems language dysfunctions.	Mood disorders, abnormal social behaviour, seizures.	Memory, language and cognitive impairment, mood disorders, motor system dysfunctions.

One frequent feature of dementia is neurodegeneration where degeneration of a specific group of neurons or brain regions causes characteristic clinical syndromes (structural, then functional loss). AD is the most prevalent dementia subtype, accounting for approximately 70% of all cases (Ballard et al., 2011), and can be defined by a combination of steady and progressive loss of memory and cognitive faculties including difficulties in producing language, impaired visuospatial skills and

altered judgement (Braak & Braak, 1991; Hardy & Selkoe, 2002). At a cellular level, AD exhibits a distinct neuropathological physiology of amyloid plaques and neurofibrillary tangles primarily in the neocortex, which spread with disease progression (Nelson et al., 2016).

1.1.1.2. Epidemiology of dementia.

Globally, the total number of people affected by dementia and neurodegenerative diseases is estimated to be around 47.5 million in 2015, representing approximately 7.7 million new cases of dementia each year which equates to 15 new cases every minute, explaining why the number of people with dementia is expected to reach 75.6 million in 2030 and 135.5 million in 2050 (World Health Organisation -WHO- report, 2015). Additionally, among these patients, 58% live in low and middle-income countries, parts of the world where the demographic growth is the most important; therefore, this proportion is projected to rise significantly in the future (see table 1.2). The strongest risk factor identified for dementia so far is age as dementia affects 1 in 20 people aged over 65 (WHO report, 2015), at the same time people over 65 years old represent the most rapidly expanding segment of the population worldwide, their prevalence estimated at just over 10% of the population in 2000 is expected to rise to 22% of the world population by 2050 representing nearly two billion people worldwide (United Nations report, 2015). In the United Kingdom alone, the percentage of people aged 65 and over increased from 13% of the total population in 1971, to 18% in 2014 and of those individuals, it is estimated that 6% are suffering from dementia, with those over 75 being about 15% (Office for National Statistics, 2015). The change in population age structures will influence the prevalence for age related conditions such as dementia, already, the incidence rate of dementia is the highest for people aged over 60 years old and it will rise even more exponentially with age increasing.

1.1.1.3. The economic impact of CNS diseases.

Considering the ageing population, the prevalence of neurodegenerative diseases is bound to increase progressively and dramatically along with life expectancy, so will the social and economic costs both direct and indirect (comprising elements such as nursing homes, absenteeism from work, benefits, early retirement, pensions, etc...). Globally, WHO recently estimated that brain disorders represent a third of the global costs associated with all existing diseases, confirming that no group of diseases is costlier than mental disorders (World Health Organisation report, 2015).

Table 1.2 - Global cost of mental health conditions in 2010 and estimated cost for 2030 in billions of US $ (source World Health Organisation report, 2011).

	Low and middle income countries			High income countries			World		
	Direct Cost	**Indirect Costs**	**Total cost**	**Direct Cost**	**Indirect Costs**	**Total cost**	**Direct Cost**	**Indirect Costs**	**Total cost**
2010	287	583	870	536	1,088	1,624	823	1,671	2,493
2030	697	1,416	2,113	1,298	2,635	3,933	1,995	4,651	6,046

Until now, only a few countries have investigated that economic burden in more details. In the UK, disorders of the brain amounted to an estimated £115 billion (Fineberg et al., 2012) of which 26 billion were devoted to the treatment of dementia's direct costs (figure 1.1, UK annual public health report, 2015), more than the combined cost of cancer, heart disease and stroke (estimated respectively at 12, 8 and 5 billion). These numbers are likely to be similar for most of the others developed countries, highlighting once more why dementia research is a global

critical public health priority, which is set to become even more important in the near future. However, although brain disorders affect more people and cost more than cancers and cardiovascular diseases, they traditionally receive significantly less interest from the general public resulting in less funding for public health programs as well as for research (Fineberg et al., 2012).

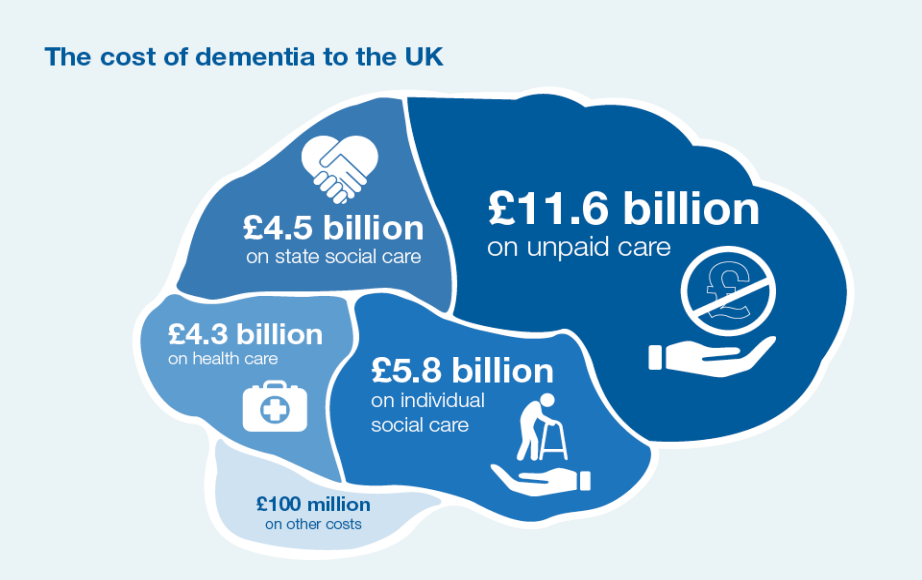


Figure 1.1 - The cost of dementia in the UK in 2015. (Source: the UK annual public health report 2016).

1.1.1.4. Medical advances have benefited human health globally.

The understanding of human physiology has greatly increased in the XXth century, as a consequence, people around the world have benefited from large improvements in various areas of healthcare treatments leading to massive increases in life expectancy. It is estimated that from 1900 to 2015, longevity has more than doubled and is now close to 70 years (see figure 1.2).

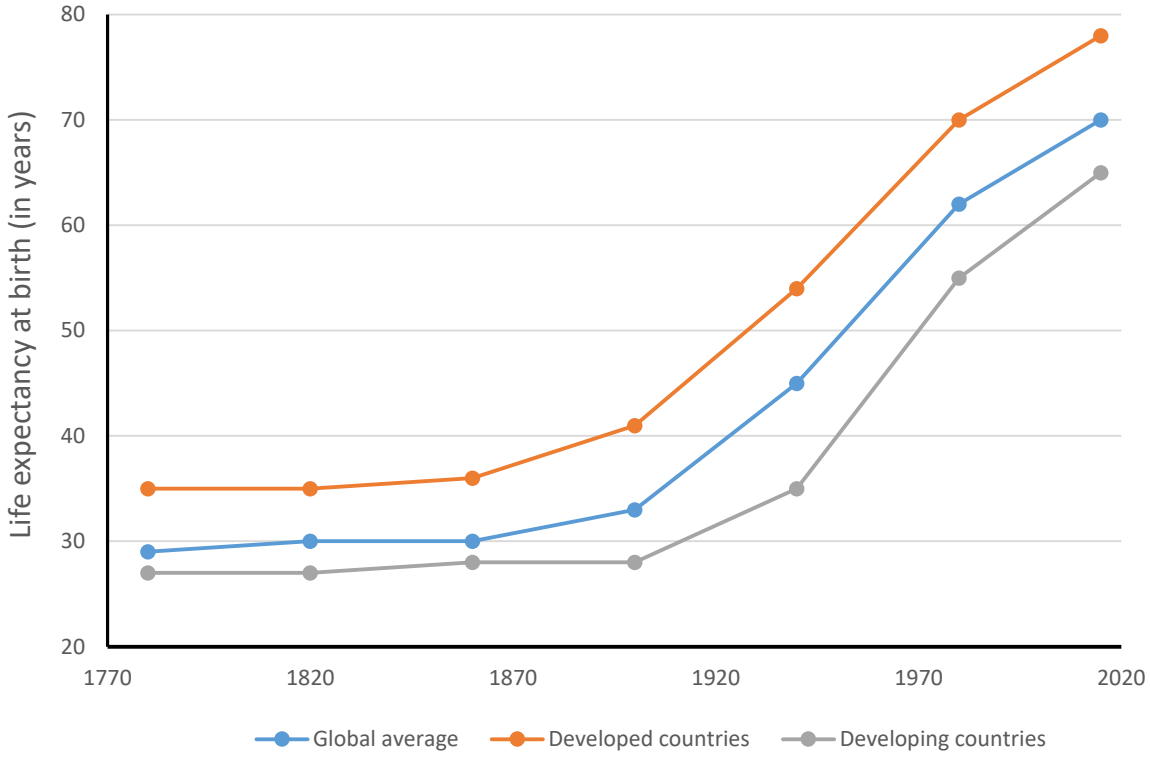


Figure 1.2 - Human Life expectancy at birth measured by areas from 1780 to 2015. (Source: UN world population prospect 2015).

Discoveries such as antibiotics and immunisation have allowed a drastic reduction in the prevalence of infectious diseases, globally the largest cause of death at the beginning of the century (Jones et al., 2012). Additionally, following World War II, intensive investigations aimed at understanding cardiovascular diseases, cancers, and other chronic conditions together with sustained prevention efforts and improvements in risk factors' identification, early detection and treatment have resulted in considerable amelioration in global healthcare (Bunker, 2001). Compared to these major advances, and despite the urgency, little has happened with regards to CNS related disorders, even though with the increasing life-expectancy, CNS disorders are an ever heavier burden on public health services. A much deeper understanding of the brain is necessary to address the growing cost of brain diseases; with this objective, the identification of modifiable risk factors allowing prevention, delay and treatment of dementia should be a public health

priority. The development of efficient pharmacological therapeutics to cure neurodegenerative diseases has so far been obstructed by their complex physiopathology as well as by the complexity of the CNS itself, still a better understanding may soon help characterise new therapeutic approaches.

1.1.2. Neurodegenerative diseases.

Neurodegenerative diseases are defined as hereditary and/or sporadic conditions characterised by a progressive nervous system dysfunction resulting in both losses of structures and function with a direct impact on cognitive and motor abilities (Thompson, 1995; Vajda, 2002). In these diseases with a neurodegenerative component, cell death is induced by a complex conjunction between necrosis and apoptosis (Thompson, 1995). Necrosis results from a reaction to direct, extensive tissue damages, typically observed after a stroke or trauma and is a process characterised by inflammation with adverse consequences on the viability of surrounding cells thus leading to subsequent widespread tissue damage (Majno & Joris, 1995). Differently, apoptosis is a cell death mechanism, highly conserved between species, and characterised by a programmed nuclear membrane fragmentation eventually leading to cell disintegration and degradation (Jacobson et al., 1997; Majno & Joris, 1995). In neurodegenerative diseases, cellular death leads to progressive brain damage gradually causing a loss of cognitive and motor abilities. Although a variety of different clinical features have been described, the processes at the cellular level appear to be comparable in most cases. For example, in Alzheimer's disease, the commonest cause of dementia, plaques and tangles are thought to cause cell death and tissue loss. Although subtleties exist, it is generally accepted that plaques are made of aggregated β-amyloid building up between nerve cells, thereby interfering with cell-to-cell signalling (Oakley, 2006; Killick et al., 2014) thus inducing apoptosis. Tangles are twisted fibres of the aggregating tau protein forming inside neuronal cells, under normal conditions, tau plays a role in the

microtubule stability (Ahmed et al., 2014). In disease however, tau tangles essentially disintegrate the transport system in areas where they are forming, so that nutrients and other essential supplies can no longer move through the cells, eventually leading to cell death (Avila, 2004; Ahmed et al., 2014), this progressive loss of neuronal cells explains the gradual appearance of Alzheimer's related symptoms (Castellani et al., 2014). On the other hand, in Parkinson's the disease affects the basal ganglia of the brain, depleting it of its dopaminergic neurons, thus leading to increasing stiffness and tremors, which are typical features of the disease (Sweet, 2014). Still, because our understanding of CNS diseases remains limited, the design of specific therapies to address underlying causes of dementia is currently out of reach and in most cases, available pharmacological therapies can only relieve symptoms with the objective to improve patient's quality of life. For example, in Alzheimer's disease, galantamine, an Acetylcholinesterase (AchE) inhibitors (Cummings, 2014) and memantine an NMDA antagonist (Sonkusare et al., 2005), can potentially slow down the progression of the symptoms by respectively increasing acetylcholine levels and reducing glutamate induced excitotoxicity, hence reducing the symptoms while Levodopa can increase the brain's dopamine level to help relieve some of the main symptoms of Parkinson's disease (Sweet, 2014). These examples however, demonstrate that it is possible to slow down diseases progression using current knowledge of cell physiology in order to target specific identified mechanisms. Neurodegeneration is a central feature of a large number of diseases, in both acute conditions, like stroke or traumatic injury, and chronic disorders such as Alzheimer, Parkinson and Huntington's diseases, therefore it has become one of the main therapeutic target for dementia. With that idea in mind, a number of studies have focused in attempts to interfere with cell pathophysiology so that it would be possible to rescue cells from early death or at least to delay and decrease dysfunctions.

1.1.3. Neurodegenerative strategies.

Neuroprotection refers to the mechanisms and strategies allowing protection against neuronal injury or degeneration in the CNS, it can be achieved using pharmaceutical or physiological therapies able to inhibit biochemical, metabolic or cellular consequences of degeneration. Neuroprotection is a source of considerable interest and the potential ability to preserve brain tissue thus improving overall disease outcome makes it one of the major goal of both academia and industry (Vajda, 2002; Stocchetti et al., 2015). It has been described that neuroprotective mechanisms can be initiated endogenously as the body tries to compensate for an insult, strategies used includes promoting synaptic plasticity, neurogenesis, gliogenesis and axonal sprouting (Yenari, 2012). These processes occur on a regular basis they may persist for months to even years in an attempt to facilitate functional and structural recovery and are stimulated by endogenous factors, including neurotrophic factors (Barbacid, 1995). Unfortunately, these endogenous neuroprotective strategies are mostly ineffective in the long term and for damages of the type and severity observed in neurodegenerative diseases. Nonetheless, inspired by some these endogenous strategies, scientists have tried to understand, mimic and use molecules and chemicals in order to initiate and improve neuro-restoration and protection in the CNS. There are currently a few strategies used or under investigation for neuroprotection that can be divided in the following categories: apoptosis inhibitors, neurogenesis factors, anti-inflammatory agents and neurotrophic factors (figure 1.3), but also anti-excitotoxic agents, metal ion chelators, ion channel modulators, free radical scavengers or even gene therapy (Jain, 2000; Yenari, 2012). Considering the fact that mechanisms underlying damage to neuronal tissue seem to have similar modes of action, often mediated at the receptor level including at G-protein coupled receptors, some of these neuroprotective strategies offer the potential to be useful for several disorders at the same time. However, although they have been the subject of extensive efforts and studies, the mechanisms and strategies allowing the brain to compensate or to be protected from neuronal death are yet to be fully understood.

Additionally, considering the current lack of understanding of the physiopathology of most CNS pathologies, strategies able to promote neuroprotection could be of critical importance.

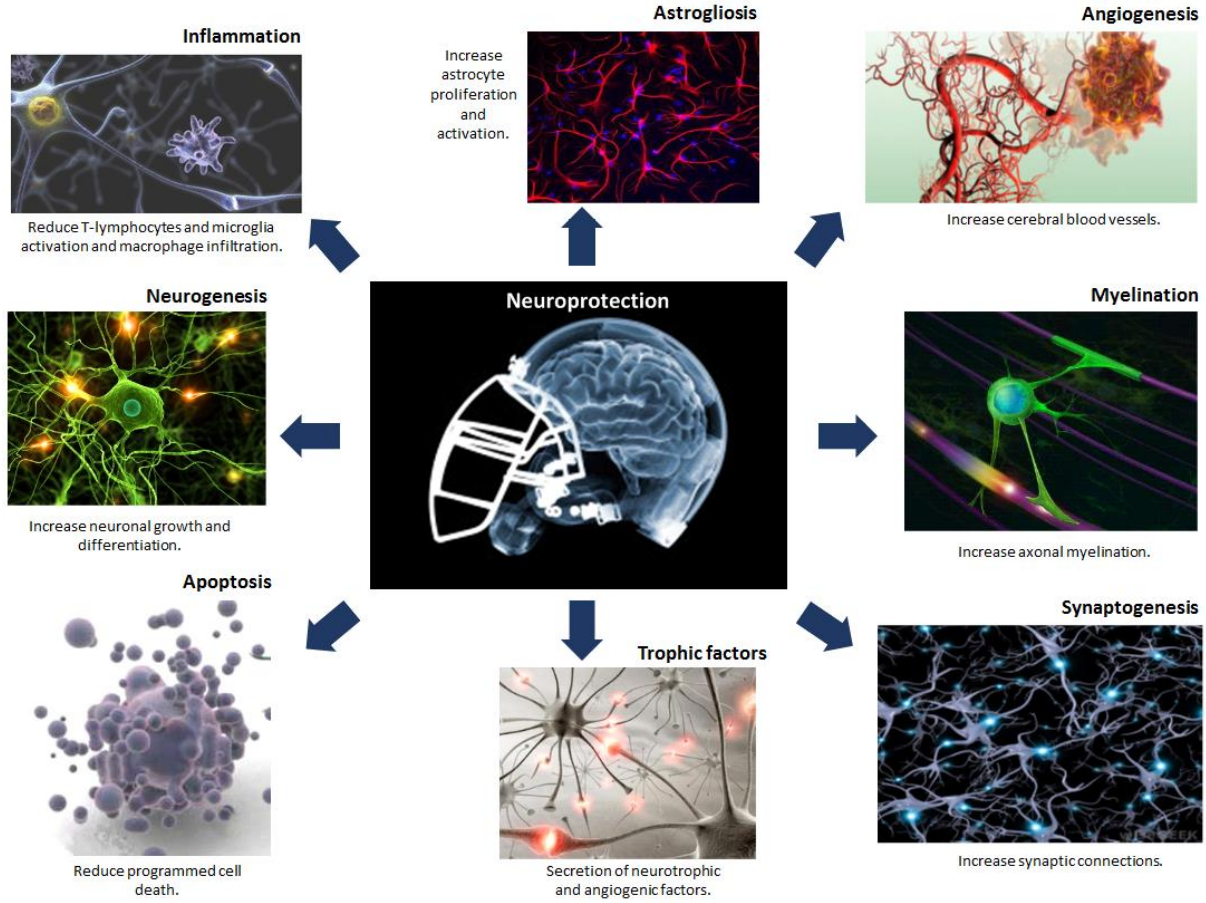


Figure 1.3 - Main current areas of interest in neuroprotection research.

The theme of neuroprotection is a complex biomedical challenge, which can be easily illustrated by the large variety of approaches being currently investigated. Nevertheless, although it is the focus of intense research effort, both in academia and in industry, it is important to note that the vast majority of neuroprotective therapies designed and tested in animal models so far, have failed to produce significant results when translated to human trials, consequently treatment options remain extremely limited (Suchowersky, 2006; Marcoux & Choi, 2013; Yildiz-Unal, 2015). Realistically,

it is difficult to imagine that a therapy or even combination of therapies would be able to compensate for the totality of damage occurring in the CNS during pathology. Still, the identification of a few neuroprotective agents such as vitamin E, have proved promising (Suchowersky, 2006), moreover, the recent emergence of a number of neuroprotective strategies such as deep hypothermic circulatory arrest (Ziganshin & Elefteriades, 2013), increases the prospect that one or more neuroprotective therapies will ultimately have a positive impact for the treatment of neurodegenerative diseases in the near future.

The ability to understand and instigate neuroprotection would be pivotal and fascinating, but in this moment of time it remains particularly difficult to therapeutically modulate neuroprotection in the context of CNS diseases where there is a lot of variability in the expression, intensity and nature of cellular imbalances and their related translation in clinical signs. Therefore, being able to identify and target common neuroprotective mechanisms and pathways looks a rational option to explore. In this regards, G-protein coupled receptors known to be central modulators of cellular and molecular activity in the CNS, constitute an obvious target for neuroprotective therapeutic strategies.

1.2. G-protein Coupled Receptors.

1.2.1. Introduction.

G-protein coupled receptors (GPCRs) constitute the largest family of membrane proteins in the human genome (Gilman, 1987) and the richest source of targets for the pharmaceutical industry with more than 60% of the available prescription drugs targeting GPCRs, even more when only considering drugs targeting the CNS (Lundstrom, 2009). They are macromolecules found either on the surface or within a cell and are essential for cell communication; as such they are associated with a multitude of diseases (Kroeze et al., 2003). In living organisms, GPCRs mediate many important physiological processes, from basic to more sophisticated functions such

as the immune system functions, fight-or-flight responses, or even taste and smell (Kobilka, 2007). They are made of a single chain of more than 1100 polypeptides running seven times back and forth across the cellular lipid bilayer to create a seven transmembrane receptor with three intracellular and three extracellular loops, an extracellular N-terminus and an intracellular C-terminal tail within the cell (figure 1.4). The extracellular region contains the ligand binding region in the majority of GPCR and is a mean of transmitting extracellular signals into the cell, while the cytoplasmic regions interact with a multi-subunit G-protein (Schenk & Snaar-Jagalska, 1999).

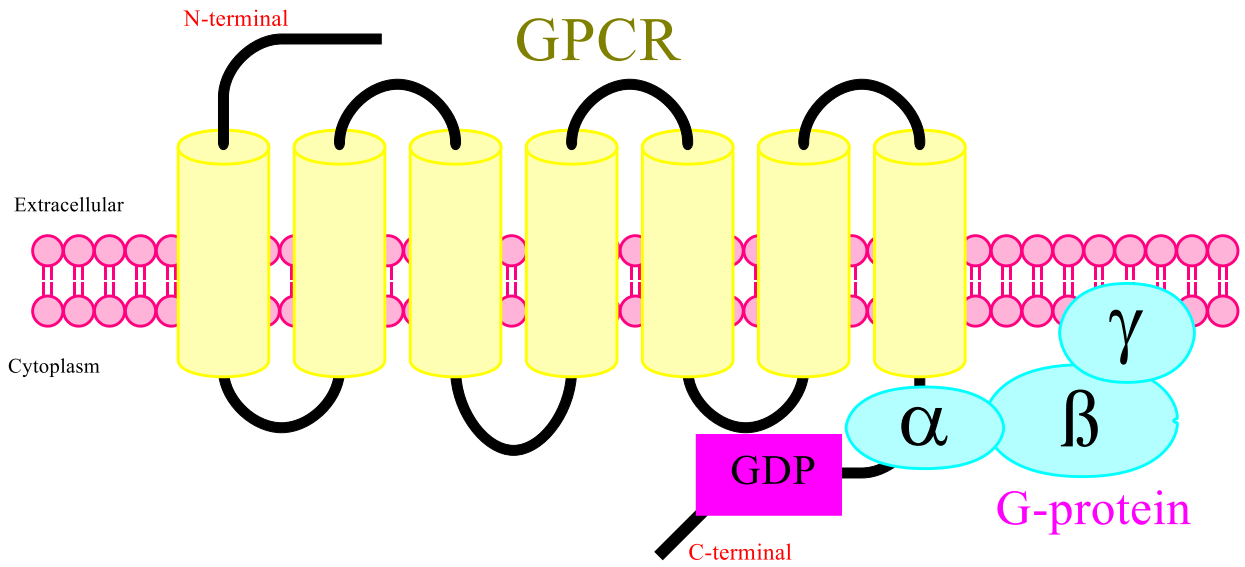


Figure 1.4 - Schematic representation of a G-proteins coupled receptor. GPCRs are characterised by an extracellular N-terminal, followed by seven transmembrane α-helices connected by three intracellular (ICL1, ICL2 and ICL3) and three extracellular loops (ECL1, ECL2 and ECL3), and finally an intracellular C-terminal. The G-proteins consist of 3 subunits, the Gα and the associated Gβγ subunits.

To date, more than 800 GPCRs have been identified, their diversity is illustrated by the multiplicity of stimuli to which they respond, including sight, smell, and taste (Deupi & Kobilka, 2007), as well as by the variety of intracellular signalling pathways they activate, including hormones, neurotransmitters, lipids, proteins, chemokines

and possibly many more (Kroeze et al., 2003). Based on their sequence similarities, they have been divided into 6 main families: rhodopsin-like, the largest with 683 members (Class A), Secretin receptor family (Class B), metabotropic glutamate (Class C), fungal mating pheromone receptors (Class D), cyclic AMP receptors (Class E) and frizzled /smoothened (Class F) (Krishnan et al., 2012). GPCRs share the greatest homology within the transmembrane segments (hence, the name often used in the literature, seven transmembrane or 7-TM). The greatest structural heterogeneity is observed in the amino terminus with this sequence being relatively short (10 –50 amino acids) for the secretin and frizzled family receptors, whilst it is much larger (350–600 amino acids) for the metabotropic glutamate family receptors (Lagerström & Schiöth, 2008). A few additional receptors (called orphan GPCRs) have also been identified whose physiological function is still unknown, some GPCRs are also said to be pleiotropic with the ability to interact with more than one pathway (Kobilka, 2013).

1.2.2. Activation of GPCRs.

GPCR ligands are highly variable and range from hormones to neurotransmitters (Kroeze et al., 2003). During their activation, the intracellular C terminus interacts with a subtype of protein known as G-proteins, located at the intracellular side of the plasma membrane. These proteins are made of three subunits (α, β, γ) which are tethered to the membrane surface by lipid molecules (Oldham & Hamm, 2001; Deupi & Kobilka, 2007). When associated with the GPCR, G-proteins bind to GDP however when a ligand binds to it, and because GPCRs are guanine nucleotide exchange factors (GEF) they exchange their GDP for GTP, therefore stimulating the attached G-protein (figure 1.5 B). This acts as a switch for the signalling activity of the protein, and the now activated G-protein dissociates into an alpha (Gα) and a beta-gamma (Gβγ) subunits (figure 1.5 C). Typically, the Gα subunit will diffuse along the membrane surface to activate specific proteins that will generate second messengers and carries out signal transduction while the Gβγ subunit will more likely directly

modulate proteins including ion channels but it also inhibits the Gα subunit (Kobilka, 2007; Kimple, 2011). Activation of either one of these two subunits will then result in a signalling cascade, responsible for various physiological and pathophysiological processes. Subtleties exist within these units and represent the main determinant in the signalling activity and pathways they are involved in, several subtypes have been documented and are prominently those targeted by drug research (Lappano, 2011). Four different isoforms of Gα units have been identified which include the $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ families (Strathmann, 1990; figure 1.6).

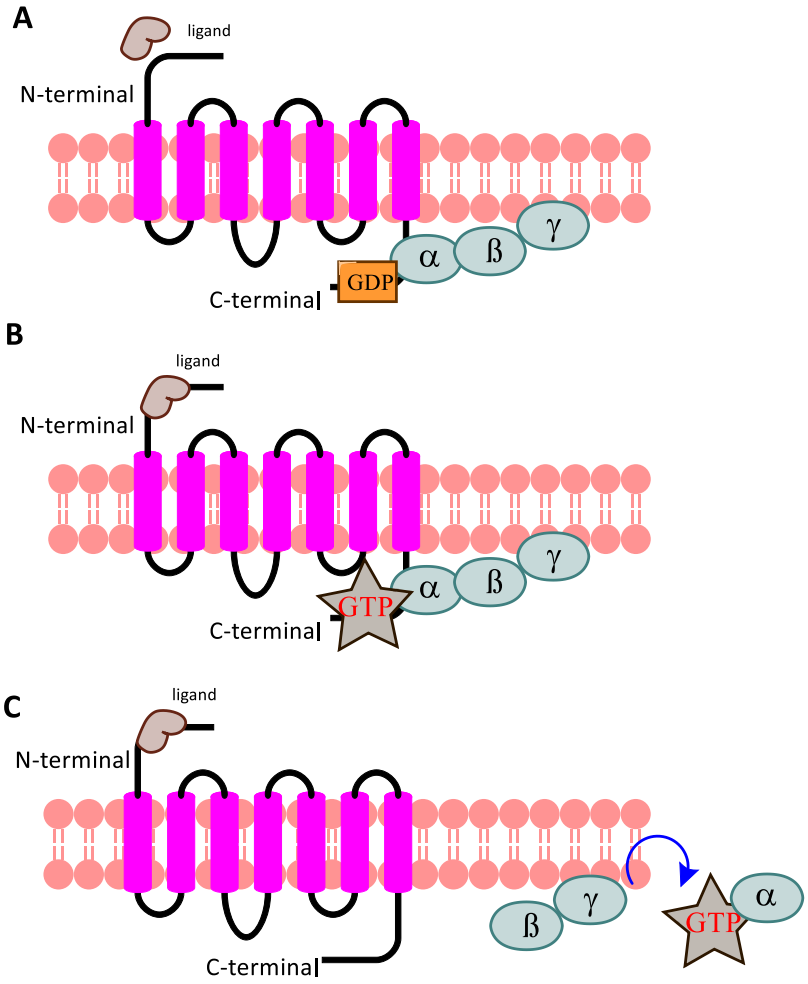


Figure 1.5 - Schematic representation of the GPCR activation process. (A) GPCR leads to conformational change allowing the α subunit of the G-protein to bind to the receptor. In reaction, (B) this binding induces a conformational change that promotes the exchange of GTP for GDP at the level of the α subunit. (C) The activated G-protein dissociates into α and βγ subunits which have the ability to interact with effector molecules, enzymes or intracellular proteins in the cytoplasm for the transmission of the external stimulus inside the cell.

1.2.3. The four families of Gα units.

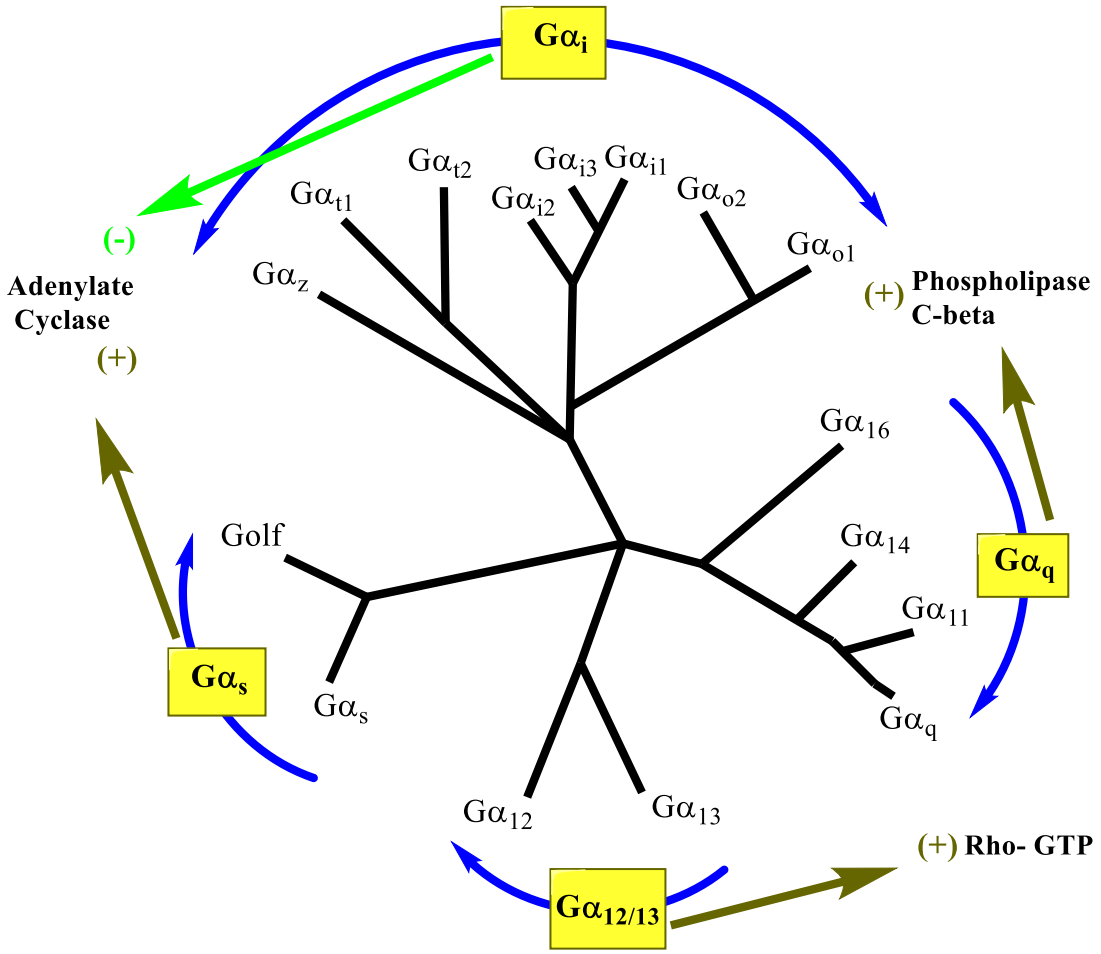


Figure 1.6 - Representation of the four families of G-proteins subunits (Inspired from Simon et al., 1991).

1.2.3.1. Gαs family

Upon ligand binding to the G-protein coupled receptor, $G\alpha_s$ exchanges GDP for GTP, which allows its dissociation from the cytoplasm and the activation an enzyme known as adenylate cyclase (AC). In turn, AC once activated, catalyses the conversion of ATP into cyclic adenosine monophosphate (cAMP), second messenger of the Gαs pathway (Hanoune & Defer, 2001). The main duty of cAMP in the cytoplasm is to bind to and activate cAMP-dependent kinase (or protein kinase A; PKA) and a group of guanine nucleotide exchange proteins, EPAC (de Rooij et al., 1998). These are involved in

regulating cell proliferation, differentiation and survival but also apoptosis, through the production of reactive oxygen species, in the regulation of gene transcription and in the activity of various kinases and cellular metabolisms also including calcium ion channels (Schmidt, et al., 2013). Dysfunctions of the signalling properties of EPAC have been linked to the development of diseases including cardiac hypertrophy, heart failure and Alzheimer's disease (de Rooij et al., 1998; Schmidt et al., 2013). cAMP also directly regulates calcium ion channels with an impact on a broad spectrum of targets, while its PKA's activation leads to the phosphorylation of a variety of subunits, mostly involved in the regulation of energy utilisation in cells (Holz, 2004; Cheng et al., 2008). Finally, cAMP signalling is stopped by being broken down by specific phosphodiesterases, including by cyclic nucleotide phosphodiesterases (PDE) who dephosphorylates cAMP into AMP (de Rooij et al., 1998). Depending on their specific cellular environments, as well as concentration and localisation, EPAC and PKA may act independently, in synergy or oppose each other (Cheng et al., 2008).

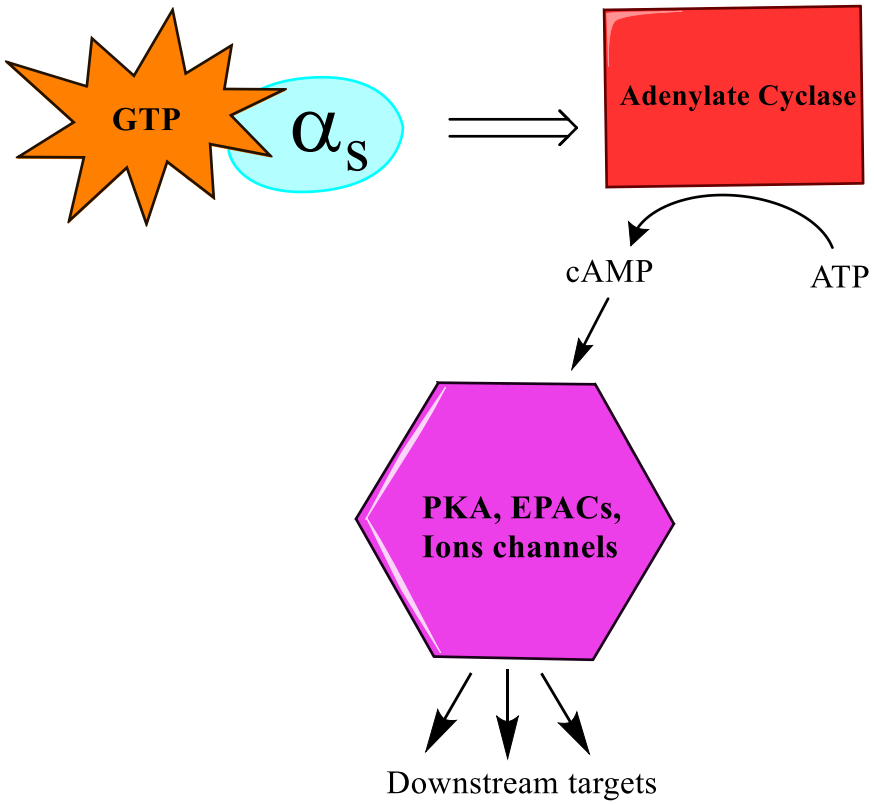


Figure 1.7 - Schematic representation of the Gα_s family's activation.

1.2.3.2. Gαi family

It was originally considered that the $G\alpha_i$ pathway antagonises $G\alpha_s$ through the inhibition of adenylate cyclase leading to intracellular cAMP reduction (Zhang et al., 2006). Hence, upon ligand binding, $G\alpha_i$ dissociates from Gβγ and inhibits AC consequently preventing PKA activation, therefore also antagonising Gαs and the cAMP dependent pathways (Thatcher, 2010). It is now considered that there might be positive feedback mechanisms at play between Gαi and Gαs to control intracellular cAMP (Stefan et al., 2011). $G\alpha_i$ also directly controls ion channels for example it leads to the opening of potassium and closing of Ca^{2+} channels mediated by Gβγ (Birnbaumer, 2007), and also plays a minor role in activation of the phospholipase C pathway (Blaukat et al., 2000). Some GPCRs are pleiotropic and will activate both $G\alpha_i$ and $G\alpha_s$ as a way of modulating the signal (Della Rocca, 1999).

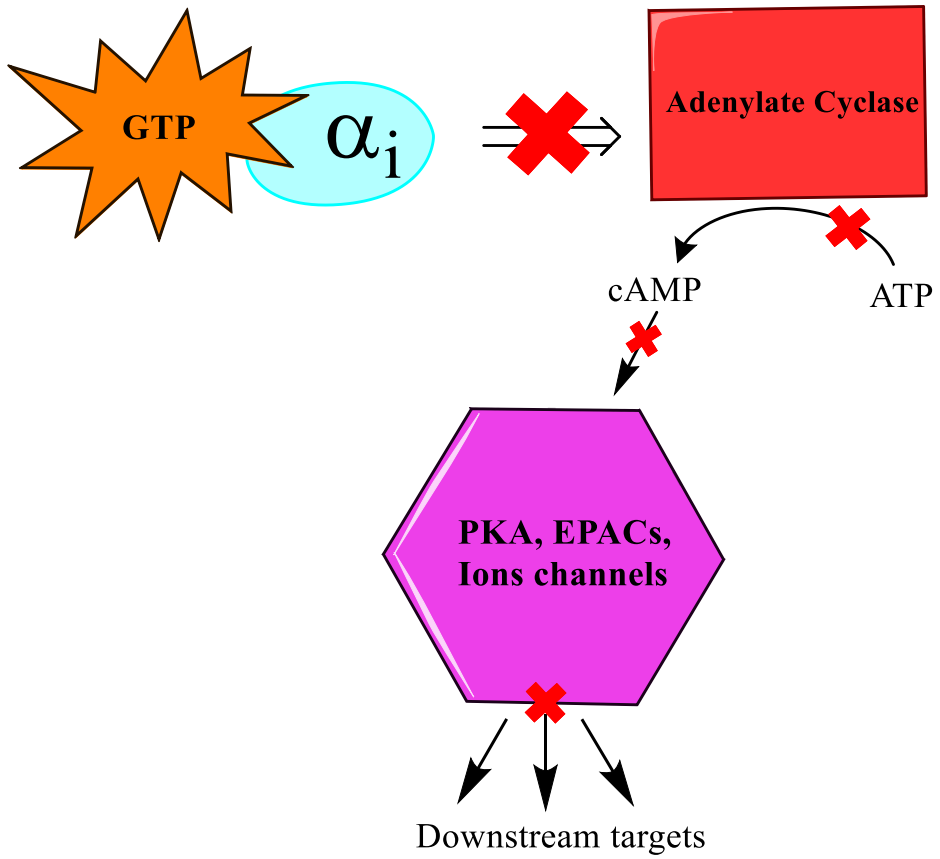


Figure 1.8 - Schematic representation of Gαi family's activation.

1.2.3.3. Gαq family

This G-protein subunit signalling involve a two-step mechanism, it starts with the activation of the enzyme phospholipase C (PLC) attached to the membrane which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2), a membrane phospholipid, to produce two second messengers, Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Blaukat et al., 2000). Diacylglycerol (DAG) remains bound to the membrane from where it activates PKC, while IP_3 is released into the cytosol via the plasma membrane (Dellis et al., 2006). IP_3, produced through phosphoinositide turnover in response to several extracellular stimuli (including neurotransmitters, light and growth factors) is involved in the control of several Ca^{2+} dependent cell functions (Dellis et al., 2006). In the $G\alpha_q$ pathway, IP_3 along the plasma membrane bind to and open its IP_3 receptor, a ligand-gated calcium ion channel located in the sarcoplasmic reticulum that is involved in the cross talk between calcium release and phosphorylation (Mikoshiba, 2007). IP_3 receptor activation results in the release of Ca^{2+} from the sarcoplasmic reticulum, leading to an increase in cytoplasmic Ca^{2+} levels. That increase in Ca^{2+} concentration brings about the activation or inhibition of numerous targets regulating a variety of cellular processes including gene regulation and contraction (Mikoshiba, 2007). DAG, on the other hand, activates protein kinase C, which like PKA phosphorylates specific target proteins involved in a variety of cellular functions, including but not limited to gene transcription and translation, cell migration, proliferation and cell death and the regulation of ion channels (Mochly-Rosen, 2012).

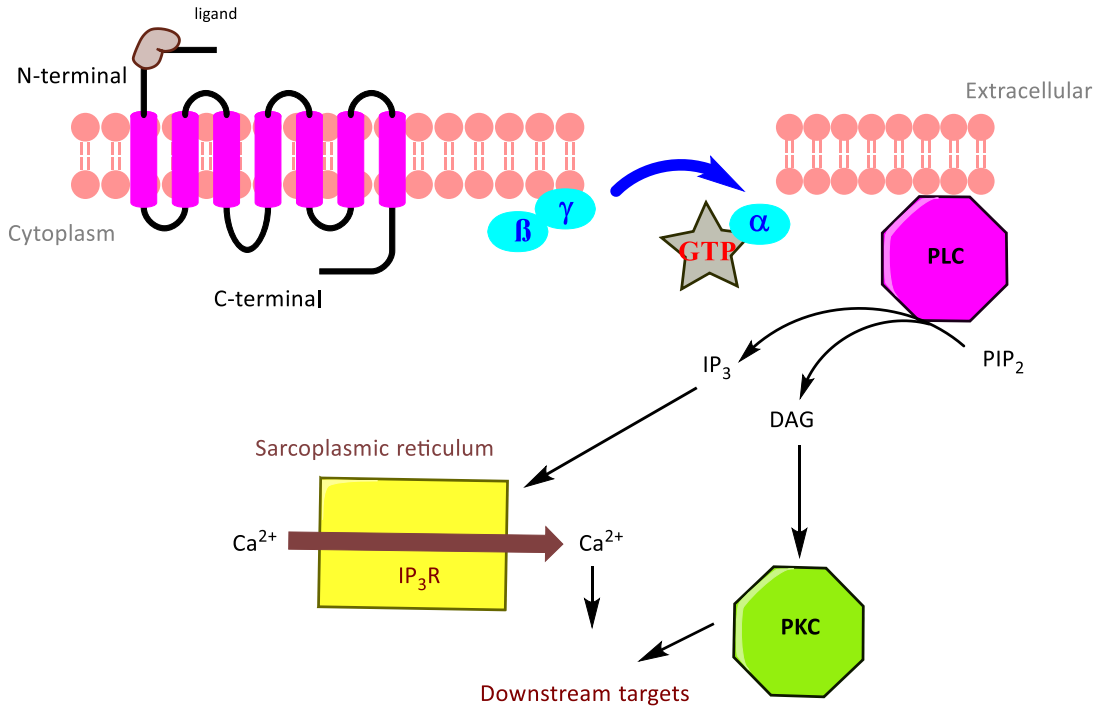


Figure 1.9 - Schematic representation of the Gα$_q$ family's activation.

1.2.3.4. Gα12/13 family

Finally, the Gα12/13 α-subunits signal through the Rho family, they also target cytoskeletal contraction and gene regulation though a protein called ROCK. In the nervous system, this pathway plays an important role in neuronal migration, axonal guidance, formation of cortices, and neurotransmitter release (Hiley et al., 2006). Moreover, deregulation of this pathway has been observed in disease conditions such as leukaemia, tumour cell invasion and metastasis (Suzuki et al., 2009).

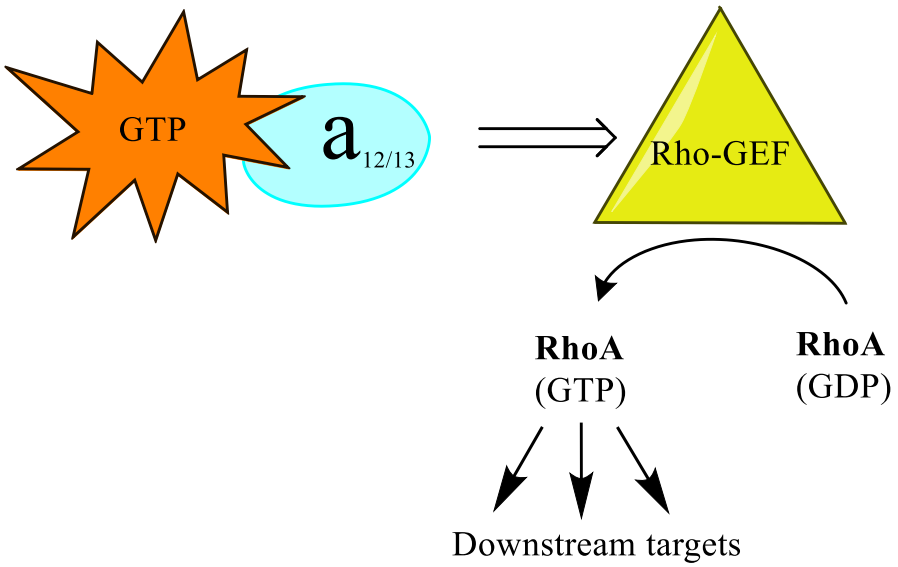


Figure 1.10 - Schematic representation of the G12/13 family's activation.

1.2.4. Signalling pathways.

To summarise, it has been established over the last 50 years that GPCRs signalling can be modulated at multiple levels. First at the intracellular level, receptors recruit or interact with various different cellular effectors, additionally at the receptor level, they can aggregate with other cell surface receptors and form heteromers and homomers, finally at the ligand level agonists, and in some case antagonists (Pang et al., 2013) can induce specific conformations by binding to receptors. This variety of possible interactions offer a large range of targets for drug discovery as well as it illustrates how challenging it is to fully address the complexity of GPCR signalling mechanisms (Liu et al., 2012). Multiple signalling pathways activated by GPCRs have been well characterised over time, these include the cAMP/PKA pathway, the Ca2+/PKC pathway, the IP3/PLC pathway and the Rho pathway, as briefly described earlier but also the β-arrestin, the protein tyrosine kinase (PTK), the PI-3 Kinase/AKT, or the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways. Moreover, it is established that GPCRs can also signal through direct activation of G-protein gated ion channels (Eishingdrelo & Kongsamut, 2013). Most of these signalling pathways function with a high degree of interconnection

with each other, nonetheless they can also be independently regulated and therefore individually represent targets for drug discovery.

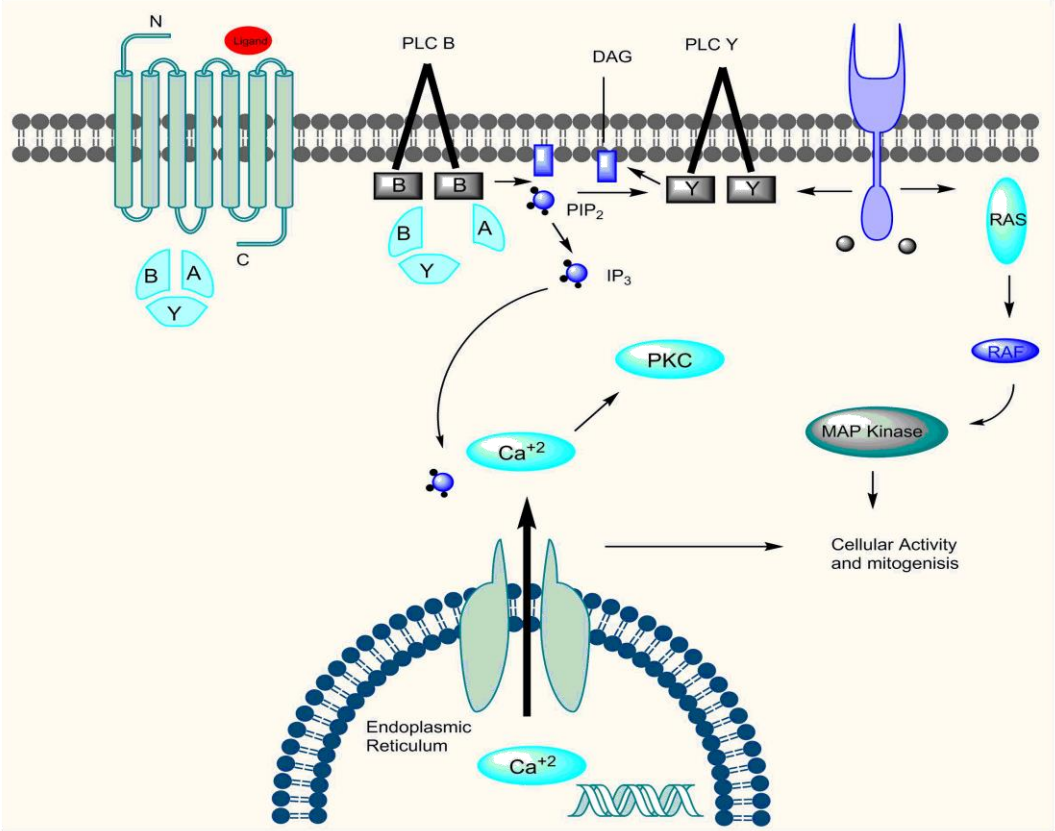


Figure 1.12 - Schematic representation of GPCR pathways (adapted from ChemBioDraw template).

1.2.4.1. MAPK and ERK signalling pathways.

As previously described, several intracellular signalling cascades have been clarified over the years, including those belonging to the MAPK (Mitogen-Activated Protein Kinase) signalling cascades. The MAPKs are a class of protein serine/threonine kinases activated in response to a variety of extracellular stimuli, mainly, they mediate signal transduction from the cell surface to the nucleus (Seger, 1995). MAPKs are also involved in the phosphorylation of numerous proteins, including transcription factors, cytoskeletal proteins, kinases and other enzymes, and have a central influence on metabolism, gene expression, cell division and survival (Cuenda &

Rousseau, 2007). Furthermore, alterations in the normal functioning of these enzymes or of the signalling cascades regulating them have been linked to a variety of human diseases including cancers, inflammatory diseases and cardiovascular diseases (Chang, 2001). In mammalian cells, four major groups of MAPKs have been described so far; the ERKs (Extracellular signal-Regulated Kinases), the p38 MAPKs, the JNKs (c-Jun NH_2-terminal Kinases) and the ERK5 or BMK cascades (Cargnello & Roux, 2011). The extracellular signal regulated kinase is one of the major cellular effectors activated by GPCRs as such, the ERK cascade is a crucial signalling pathway regulating a wide range of cellular processes including differentiation, migration, proliferation, growth, survival and apoptosis (Karmarkar, 2011). Furthermore, the ability of ERKs to signal in the cytoplasm as well as in the nucleus, combined to the fact that they have been associated with various cellular and physiological functions, makes their pathways and cascades particularly interesting for drug discovery (Eishingdrelo, 2013). It is especially true for ERK1 (MAPK3) and ERK2 (MAPK1) which have been recently linked with endogenous neuroprotection (Karmarkar, 2011) and as such have attracted a recent surge of interest.

1.2.4.2. Biased agonism.

Gα subunits were separated in 4 families according to the 4 isoforms we have just described based on the effect of ligand-binding on the activity of only the primary effector pathway therefore, on receptor specificity and intrinsic activity (Preininger & Hamm, 2004). Originally, it was considered that GPCRs activation via these heterotrimeric G-proteins resulted in propagating specific intracellular signalling pathways through β-arrestins and G-protein pathways (Lefkowitz, 2005). However, until recently, the underlying mechanisms were poorly understood; with the recent development of GPCRs modulators, more light has been shed on this concept with critical impact on the understanding of pharmacology. The concept of ligand-directed trafficking suggest that binding of a ligand can result in a response mediated by several subunits at the same time thus adopting multiple conformations upon agonist

binding (Denis et al., 2012; Liu, 2012; Koblika, 2013). Consequently, it is now understood that GPCRs, rather than functioning as classical on/off switches, can act as filters able to selectively activate specific pathways or subsets of pathways from different subunits if required, thereby producing adaptive responses upon stimulation (Rajagopal et al., 2010). Hence, this new concept also imply that a single receptor might have pleiotropic signalling properties, with a variety of crosstalk and feedback at different levels. Biased agonists selectively activate part of the subset of receptor conformations induced by the natural ligand without activating all of them, therefore preferentially stimulating some signalling pathways over others with an increased specificity. This concept extends our understanding of GPCR and may allow the identification of new classes of therapeutic agents able to prompt receptors to signal via specific and potentially beneficial pathways (Zhao et al., 2014, figure 1.12).

1.2.5. Inactivation and desensitisation of GPCRs.

Prolonged GPCRs activation leads to their desensitisation, the mechanisms involved have been largely documented (Lappano, 2011). A protein known as a G-protein receptor kinase (GRK) phosphorylates the receptor on specific residues, by doing that it also increases its affinity for β-arrestin. β-arrestin binds to the receptor and prevents it from associating with a trimeric G-protein, it also targets the receptor for endocytosis, leading to a down regulation of the receptor's activity (Gainetdinov & Lefkowitz, 2004). The Gα unit is able, after GTP hydrolysis, to bind to GDP again and to reunify with a Gβγ unit to form an inactivated trimeric G-protein that can again associate with a receptor (figure 1.11).

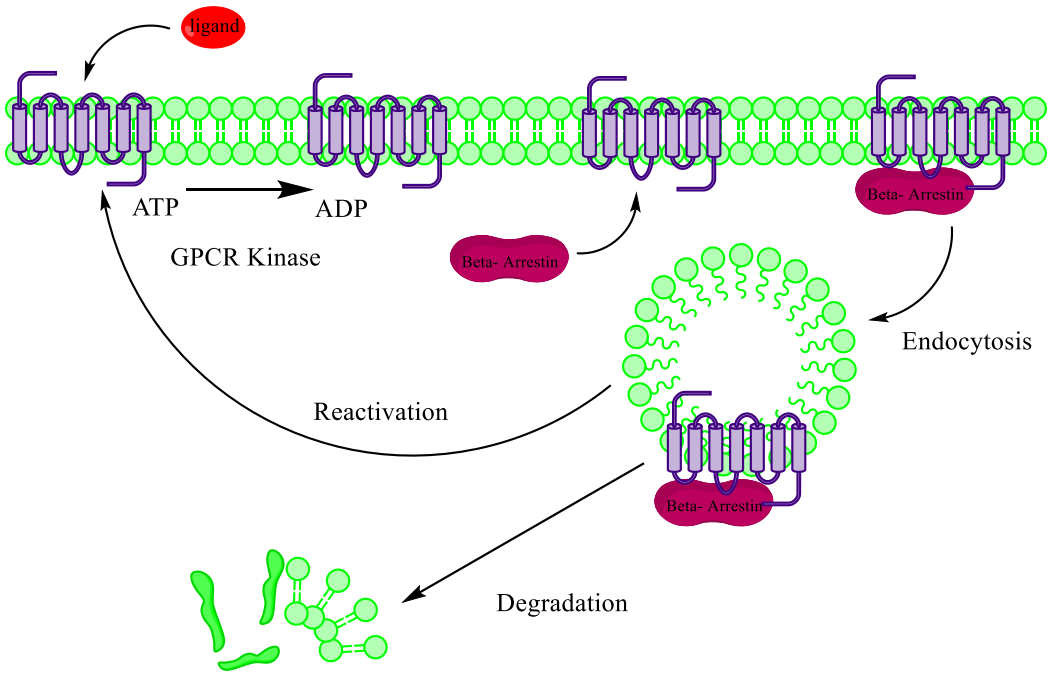


Figure 1.11 - Schematic representation of GPCR inactivation.

It was previously considered that most GPCRs can only be activated by soluble ligands including calcium (Ca^{2+}), acetylcholine (Ach) glutamate or GABA (Venkatakrishnan et al., 2013) however in the late 90s a novel subtype of GPCR found throughout the mammalian body and activated by endogenous proteinases was identified and named protease-activated receptors (PARs).

1.3. Proteinase-activated receptors (PARs) - a unique family of G-protein coupled receptors.

1.3.1. Introduction.

From the abundant literature on the topic, we have previously been able to describe that for most GPCRs activation depends on soluble ligands binding to the N-terminus, including calcium (Ca^{2+}), acetylcholine (Ach), glutamate or GABA (Venkatakrishnan et al., 2013). This was the accepted understanding until the early 90's when a novel

subtype of GPCRs was identified in studies examining the mediator for the cellular actions of thrombin, a coagulation serine proteinase (Vu et al., 1991; Rasmussen et al., 1991). Accordingly, that family, and those whose activation is mediated by endogenous proteinases, was named protease-activated receptors (PARs). PARs are expressed extensively throughout the mammalian body, in platelets but also on endothelial cells, myocytes and neurons and so far 4 different subtypes have been identified, namely PAR1 to PAR4. PAR1 was the first member of the PAR family to be discovered and cloned (Vu et al., 1991; Rasmussen et al., 1991), soon after PAR2 was identified (Nystedt et al., 1994), quickly followed by two other receptors; PAR3 and PAR4 (Ishihara et al., 1997; Xu et al., 1998). PAR1, PAR3 and PAR4 are preferentially cleaved by thrombin and as such are classified as thrombin receptors, while PAR2 is cleaved preferentially by trypsin and tryptase, PAR4 can also be cleaved by cathepsin-G (Macfarlane et al., 2001; Zhao 2014, table 1.3). PARs unique proteolytic activation mechanism is the hallmark that singles them out from other G protein-coupled receptors, their discovery prompted intensive investigations to identify the physiological and pathophysiological functions of these receptors as well as their distribution at both tissue and cellular levels (table 1.3). The data gathered until now suggest a broad spectrum of possible roles for PARs in both normal and pathological tissue, explaining why these receptors are emerging attractive new targets from a drug discovery perspective for several disease states including arthritis, colitis, asthma, neurodegenerative conditions, tumour invasion, cardiovascular diseases but also inflammation, pain and healing (Cisowski et al., 2011; Ramachandran et al., 2012; Gieseler, 2013). Therefore, considerable efforts have been made to identify, understand and develop molecules that modulate PARs function, however, their uniqueness and unusual mechanism of activation and signalling has presented significant challenges (Barry et al., 2006).

Table 1.3 - Classification physiological and functional properties of PARs.

Accepted name	PAR1 Thrombin Receptor	PAR2	PAR3 Thrombin Receptor	PAR4 Thrombin Receptor
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).
Activating proteinases	Thrombin, Trypsin IV, Plasmin, Factor VIIa, Factor Xa,Cathepsin G, Pen C 13, Cysteine proteinase, 1MMP-1, Gingipains.	Trypsin, Triptase, Mast cell tryptase, Trypsin IV, Matriptase, Factor VIIa, Factor FXa, Proteinase-3, GranzymeA.	Trypsin, Factor Xa	Cathepsin G, Factor VIIa, Factor X, Trypsin IV ,Plasmin, Factor Xa, MASP-1.
Putative antagonist	BMS-200261,RWJ-56110, RWJ-58259, SCH-205831, SCH530348, Pepducin P1pal-7, Pepducin P1pal- 12.	ENMD-1068, GB83, GB88.	Unknown	Trans-cinnamoyl, YPGKF-NH_2, Pepducin,P4pal-10 (also inhibit PAR1).
Selective agonists	TFLLR-NH_2, TF1FDb	SLIGKV-NH_2 (human), SLIGRL-NH_2 (rat and mouse),2-furoyl-LIGRLO-NH_2 (rat and mouse), GB110, AC-264613, AC-55541.	Thrombin cleaves but doesn't activate.	GYPGKF-NH_2 (human), AYPGKF-NH_2(rat and mouse).
Signal transduction Mechanisms	Gq/11 (increases IP_3/DAG), GI (cAMP modulation), G12/13 (actin rearrangement).	Gq/11 (increases IP_3/DAG), GI (cAMP modulation).	Unknown	Gq/11 (increases IP_3/DAG).
Tissue expression	Platelet, endothelium and smooth muscle, G.I. tract, lung, kidney, liver, heart, leucocytes, neurons and astrocytes.	Endothelium, G.I. tract lung, kidney, liver, heart, leucocytes, neurons and astrocytes.	Platelet, endothelium, liver, leucocytes.	Platelet, endothelium G.I. tract leucocytes, lung, heart.
Physiological Functions	Platelet aggregation and secretion, vaso-regulation, gastric mobility,inflammation, nociception, neuronal regulation.	Vaso-regulation, gastric mobility, inflammation, nociception,neuronal regulation.	Unknown	Platelet aggregation and secretion, inflammation.
Disease Relevance	Coronary thrombosis, inflammatory bowel disease, cancer.	Arthritis, inflammatory bowel disease, cancer, infectious colitis.	Unknown	Coronary thrombosis, inflammatory bowel disease, cancer.

1.3.2. PARs exhibit an unusual activation mechanism.

Although the PAR family of receptors shares the main basic structural features with all GPCRs, including a central domain composed of seven transmembrane segments, including three intracellular and three extracellular termini, they also display a unique mechanism of activation. The receptor is activated when a protease cleaves this central domain and by doing so, exposes an innate receptor ligand, which then translocates to a binding site located in the transmembrane domain located in the 2nd extracellular loop (ECL2) of the same receptor molecule (Macfarlane et al., 2001; Coughlin, 2003). The N-terminal sequence acts as a tethered ligand activating the receptor and binding to it in order to induce conformational changes and signalling. Interestingly, pharmacological studies have shown that there is a possibility to activate the receptor by directly targeting ECL2 of PARs, hence triggering signalling cascades (Macfarlane et al., 2001; Noorbakhsh et al., 2012; figure 1.12). Moreover, biased agonism (see section 1.2.6.2.) has been demonstrated for PAR1 with indications it might be relevant for PAR2 as well (Mosnier et al., 2012).

1.3.3. What roles for PARs?

Protease-activated receptors were initially identified as mediators of the cellular effects of thrombin (Macfarlane et al., 2001), the main effector protease of the coagulation cascade (Coughlin, 2003). Therefore, PARs play important roles in normal blood vessel biology and as such are predominantly expressed around blood vessels by a variety of cell types, including endothelial cells (Coughlin, 2000). Previous studies have described that PARs activation participates to thrombin signalling in platelets, thereby contributing to homeostasis (Coughlin, 2000; Leger et al., 2006). Additionally, in the cardiovascular system, PARs mediate the regulation of vascular tone and permeability (Coughlin, 2000), while in vascular smooth muscles they play a role in the regulation of contraction, proliferation, and hypertrophy (Leger et al., 2006). Finally, PARs have been linked to muscle growth and bone cell differentiation and

proliferation (Coughlin, 2000), as well as in the pro-inflammatory response observed in disease state including thrombosis and atherosclerosis (Leger et al., 2006).

1.3.4. PARs in the CNS.

The many singularities of PARs compared to other GPCRs added to the fact that there is extensive evidence showing connections between their activation and processes such as inflammation, have quickly prompted interest in links with CNS diseases.

Localisation studies using in situ hybridisation and immunohistochemistry have confirmed the presence of all four types of PARs in the mammalian brains of both rodent and human (Noorbakhsh et al., 2003; Bunnett, 2005; Bushell, 2007), but with differences suggesting distinct roles for each subfamily of these receptors. PAR1 and PAR3 have been observed more abundantly in the pyramidal layers of the hippocampus, in neurons of thalamic nuclei and in the cortex, while PAR4 expression was observed in most regions of the hippocampus, and less abundantly in cortical layers, thalamus, hypothalamus and amygdala (Gieseler, 2013). Although it is found on all cortical layers, PAR2 is preferentially expressed in the amygdala, thalamic nuclei, the hypothalamus and the striatum (Noorbakhsh et al., 2003; Bushell, 2006). At the cellular level, PAR1 was primarily identified in astrocytes, microglia and also in the cytoplasmic membrane of the neuron (Kauffmann et al., 1998; Noorbakhsh et al., 2003), PAR2 on the other hand, was identified on neurons (proximal processes and soma) and astrocytes in the CA1, CA2, CA3 regions of the hippocampus and on sensory neurons (D'Andrea et al., 1998). Finally, PAR3 and PAR4 were found in neurons, axons and dendrites (Noorbakhsh et al., 2003).

Indications supporting widespread PARs localisation is in the brain, could indicate a potential implication in a variety of CNS processes with roles depending on the biological context, level of expression and tissue where they are observed. Indeed, studies showing that PAR2 is up-regulated in several inflammatory diseases imply a detrimental pro-inflammatory role (Festoff, 2003; Noorbakhsh et al., 2006; Yoshida, 2011; Cattaruzza, 2014), while others studies reporting PAR2 down-regulation in

disease conditions with an inflammatory component rather infer a beneficial anti-inflammatory role (see Cottrell, 2003; Mahajan, 2011). That potential dual role combined to observations that its activation can have neuroprotective effects explains why as a potential target for therapeutics, PAR2 have been increasingly investigated over the last few years, (Greenwood & Bushell, 2010; Ramachandran et al., 2012; Zeng et al., 2013).

1.4. Proteinase activated receptor 2 (PAR2).

1.4.1. Introduction.

Considering the variety of possible roles for PAR2 in critical processes including its potential neuroprotective properties, this research was primarily focused on this specific PAR. One of the objective was trying to advance the global understanding of the mechanisms underlying PAR2 activation and pathways and describe how these could potentially open new paths, relevant to the treatment of CNS disorders.

Of all the members of the PAR family, PAR2 is seen as the most distinct, it responds to several serine proteases, including tryptase and matriptase which are not believed to cleave any other PARs, it is also the only PAR not to be directly activated by thrombin (Geiseler, 2013). First identified in 1994 (Nystedt et al., 1994), PAR2 is closely related to PAR1 but has a slightly different sequence (Nystedt et al., 1995). It is widely distributed in the human body and throughout the CNS and is located in a variety of cells types including epithelial and endothelial cells, myocytes, fibroblasts, immune cells, neurons and glial cells (Cottrell et al., 2003; Kanke et al., 2005; Gieseler, 2013). Additionally, in the spinal cord, PAR2 was localised around primary spinal afferent neurons, (Noorbakhsh et al., 2003), where it was co-expressed with transient receptors potential from the vanilloid family (TRPV) and with substance P, both major actors of neurogenic inflammation (Pedersen et al., 2005) and nociceptive signalling (Vergnolle et al., 2001) suggesting PAR2's involvement in inflammation and regulation of the immune system. Finally, in addition to the above, studies have

demonstrated that many of proteases known to activate PAR2 are normally generated and released during inflammation (Grant et al., 2007). Altogether, these data tend to suggest a role for PAR2 in inflammation-related processes, therefore highlighting the need and interest to further advance the understanding of PAR2 which might allow the development of a range of novel therapeutic tools involving distinct cellular pathways in the future.

1.4.2. PAR2 mechanisms of activation and deactivation.

Knowledge of the mechanisms inherent to PAR2 activation in the CNS are largely based on data obtained from investigations of PAR1 and other thrombin activated PARs (Macfarlane et al., 2001; Kanke et al., 2004), and these observations suggest that PAR2 might have a very distinct activity.

It has been established that PAR2 initiates downstream pathways through coupling to G proteins Gαq /11, Gαs, Gαi/o and Gα12/13 (Adams et al., 2011), resulting in activation of phospholipase C and formation of IP3 and DAG (Macfarlane et al., 2001; DeFea, 2000; Cottrell et al., 2003; Bushell, 2006), which mobilise intracellular Ca2+ ions and activate PKC. For example, activation of PAR2 by trypsin involves N-terminal proteolytic cleavage, which reveals the tethered ligand SLIGKV (human) or SLIGRL (mouse) (figure 1.13).

This newly unmasked ligand then interacts with the second extracellular domain of the receptor to classically initiate various G protein-dependent pathways (Hollenberg et al., 1999) including Gαq mediated Ca2+ mobilisation and formation of cAMP (Bunnett et al., 1996), Gα12/13 mediated increase in Rho Kinase activity (Scott et al., 2003), but also recruitment of β-arrestin 1 and 2 (DeFea, 2000; Bunnett et al., 2000), ERK1/2 and p38 MAPK phosphorylation (Macfarlane et al., 2001), both interestingly identified as important modulators of inflammation, neuroprotection and astrocytic function (Zeng et al., 2013). The activation of these pathways following PAR2 cleavage lead to the generation of second messengers, of which IP3 and DAG are

essential to trigger activation of protein kinase C (PKC) and Ca^{2+} dependent signalling pathways, crucial regulators of metabolism (Lappano, 2011). As PAR2 proteolytic activation is not reversible, the most common way to terminate its signalling is by degradation of the receptor itself (Stalheim et al., 2005). Following stimulation, G-protein-coupled receptor kinases (GRKs) mediates phosphorylation of activated PAR2 and increases receptors affinity for β-arrestin (Ribas et al., 2007; Soh et al., 2010), which recruitment to the carboxyl-terminal tail causes it to interact with the phosphorylated GPCR and to disrupt further association with heterotrimeric G proteins (Lin, 2013), thereby terminating signal transduction to deactivate the receptor (Krishnan et al., 2012). Additionally, arrestins including β-arrestin 1 and 2 (Lefkowitz, 2005), serve as MAPKs scaffolds, holding together components of the MAPK signalling modules (DeWire et al., 2007) which activate ERK1/2 JNK3and p38 (Song et al., 2009) thus acting as signal transducers to guide signals from the cell membrane (Ma & Pei, 2007). Once the receptor is no longer stimulated, PAR2 migrates to lysosomes for degradation and recycling. Initial re-sensitisation depends upon the existence of reserve receptor stores located into the membrane (Soh et al., 2010), however, repeated and/or prolonged stimulation of PAR2 makes the cell unresponsive to further stimulation until more receptors have been synthesised (Cottrell et al., 2003).

To be fully re-sensitised and activated again, and in contrast to other PARs, PAR2 requires not only a de novo receptor synthesis (Bohm et al., 1996), but also the mobilisation of p24a, a specific type of receptors from the Golgi-network involved in protein transport (Luo et al., 2007). P24a is a member of the p24 family of proteins involved in protein transport through the Golgi network (Blum et al., 1996) and was identified as a pivotal mediator of PAR2 transport. Indeed, it has been described that it interacts with the second extracellular loop of PAR2, thereby maintaining it at the Golgi level in unstimulated cells (Luo et al., 2007). However, upon PAR2 activation, the PAR2/p24a complex dissociates, allowing exocytic transport and renewal of the receptor in the plasma membrane (Luo et al., 2011), thus prolonged PAR2 activation leads to the depletion of these receptors.

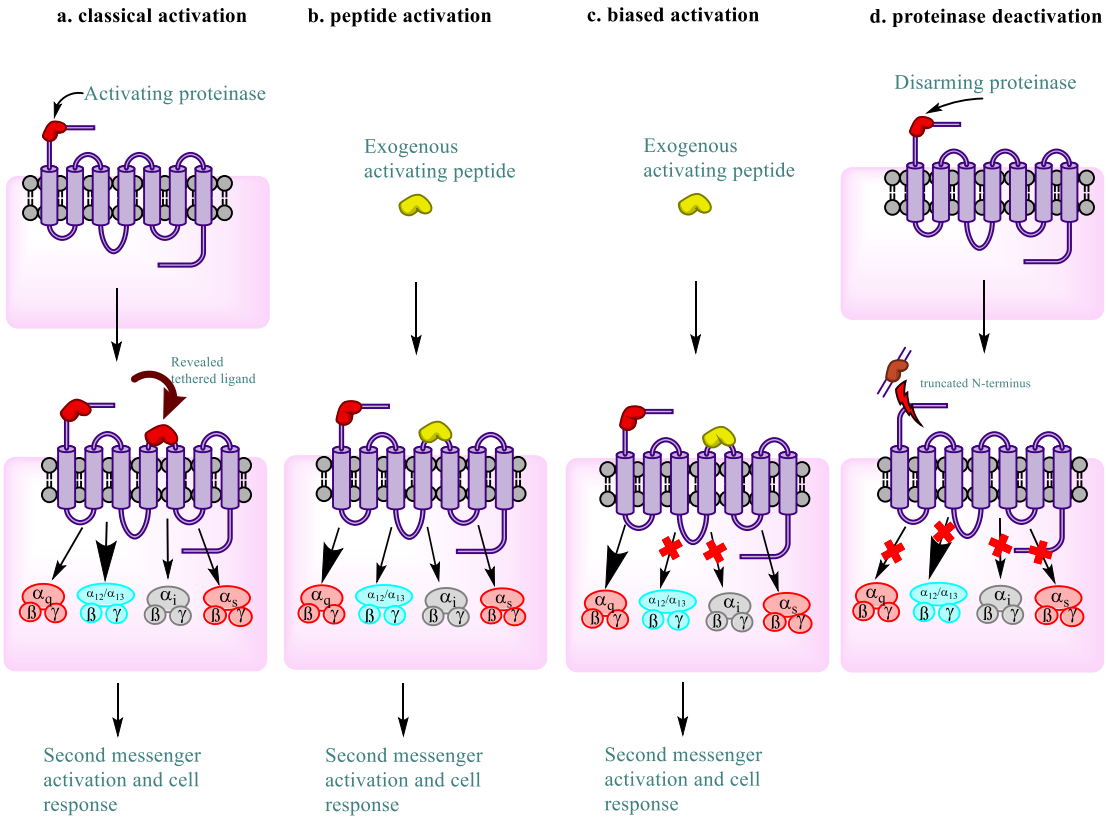


Figure 1.13 - Schematic representation of PAR2 activation. The cleavage of PAR2 exposes a 'tethered ligand' that binds to the second extra loop of the receptor leading to activation of specific signalling pathways. Mechanism of PAR2 activation via (a) an endogenous proteinase (b) an exogenous agonist (c) biased activation and (d) deactivation via a disarming antagonist.

1.4.3. What roles for PAR2 in the human body?

Following identification of PAR2 (Nystedt et al., 1994), studies were conducted to investigate its potential physiological roles as well as identify ways to modify or control its related pathways. PAR2 was initially studied through the effects of PAR2 gene deletion (Kanke et al., 2005) with these experiments conducted in the vascular system demonstrating that activation of PAR2 mediates relaxation of isolated rat aorta in an endothelium-dependent pathway *in vivo* therefore highlighting a role for PAR2 in regulating vascular tone (Compton et al., 2002). In addition to that regulatory

role, PAR2 was linked to vascular inflammation, as the observation of a PAR2-gene-deficient mouse, showed that in the endothelium the expression of leukocyte adhesion molecule P-selectin, is down regulated (Cleator et al., 2006), suggesting that PAR2 activation could have an impact on leukocyte accumulation or rolling, and on its related chronic inflammation. Furthermore, up-regulation of PAR2 in inflamed tissues was also observed in arthritis (Cottrell, 2003; Ferrell et al., 2003; Sevigny et al., 2011), suggesting the pro-inflammatory effects of PAR2 might also affect local tissues with involvement in allergic inflammation and skin disorders (Mcfarlane, 2005; Kanke, 2009; Gieseler et al., 2013). In the peripheral nervous system, it is thought that PAR2 mediated secretion of prostanoids and nitric oxide may contribute to enhance inflammatory responses, while activation of PAR2 in nerve endings induces the release of calcitonin gene-elated peptide (CGRP) and substance P (both involved in pain signalling) suggesting once more PAR2's involvement in immune responses and inflammation-related pain including hyperalgesia (Grant et al., 2007). Moreover, PAR2 deficient or knockout mice show reduced dendritic cells maturation, differentiated antigen transport to draining lymph nodes and reduced T-cell activation, thus suggesting a role for PAR2 in enhancing immune activation (Ramelli et al., 2010). At the genetic level, PAR2 activation was described as influencing the levels of expression of about 2500 genes implicated mostly in cell metabolism but also of genes involved in the initiation of MAPK pathways and inflammatory cytokines (Suen et al., 2010). The role of PAR2 although initially described as detrimental seems now a bit more ambivalent as contrasting studies have also reported a protective role of PAR2 in airway inflammation, and influenza virus infection (Kanke et al., 2005).

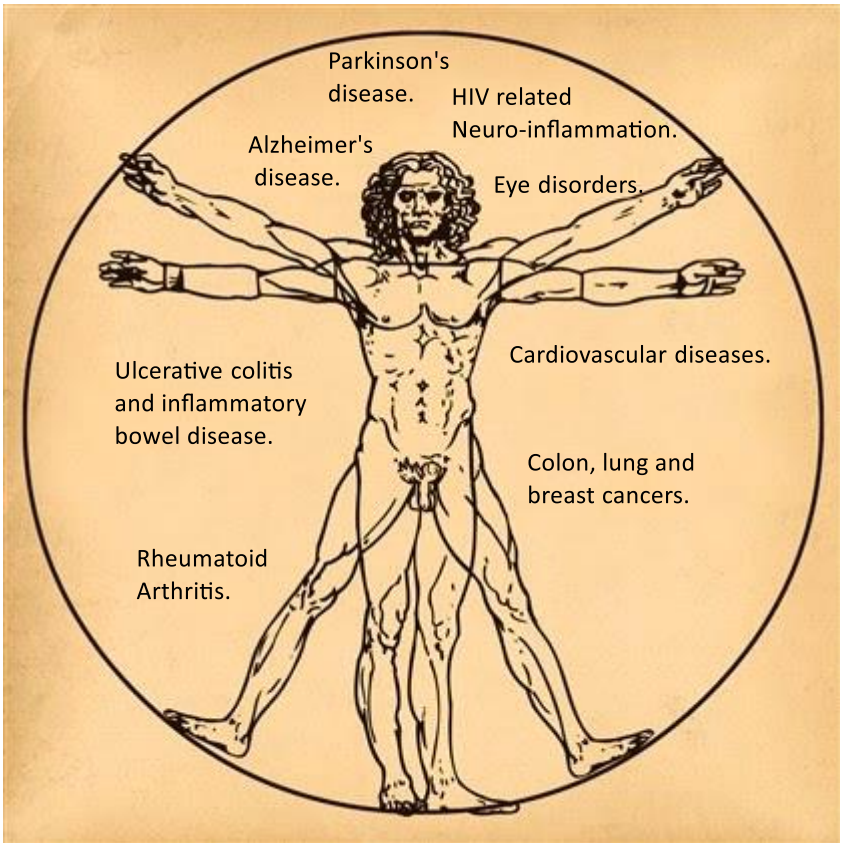


Figure 1.14 - Representation of diseases with an inflammatory component linked with PAR2 expression.

The role of PAR2 in diseases with an inflammatory component (figure 1.14) has led to an increased focus on these receptors, including for disorders of the CNS, however due to the lack of PAR2 specific experimental protocols within the CNS, only a limited amount of data is available to date, mostly using the knowledge gathered from experiments on other PAR receptors but also on PAR2 in the peripheral system.

1.4.4. What role for PAR2 in the CNS?

The physiological role of PAR2 in the CNS remains unclear, localisation studies have demonstrated that PAR2 is co-localised with transient receptor potential channels vanilloid-1 (TRPV-1) and 4 (TRPV-4), including on nociceptive DRG neurons (Steinhoff et al., 2000) similarly to what was previously reported in the peripheral nervous

system (Poole et al., 2013; Zhao, 2015). Furthermore, it has been established that at these locations PAR2 potentiate TRPV-1 and TRPV-4 activation (Amadesi et al., 2006), knowing the role of these receptors in inflammation and pain signalling in mammalian physiology, it suggests that even at the CNS level PAR2 might be an important mediator of inflammation and pain perception. In the hippocampus, PAR2 receptors have been shown to be expressed in both astrocytes and neurons (Bushell, 2006). Furthermore, at these locations PAR2 activation has been shown to increase intracellular Ca^{2+} levels in both cell types (Ubl et al., 2002; Bushell, 2006; Zhao, 2015; Jairaman, 2015). The increase in intracellular Ca^{2+} concentration induces a diverse range of cellular responses, including cell proliferation, differentiation, initiation of several additional kinase cascades and production of proteins such as interleukin-6 and eotaxin, some of these processes are thought to be central regulators of inflammatory responses (Macfarlane et al., 2001, Bushell, 2007, Matej, 2012). Recent studies also established that PAR2 activation can cause depolarisation of hippocampal neurons, possibly mediated by astrocytic activity (Gan et al., 2011), with subsequent inhibition of evoked glutamatergic (excitatory) synaptic transmission (Gan et al., 2011) and alteration of network dynamics (Lohman et al., 2009). The potential ability to down regulate glutamatergic synaptic transmission and pathways is very interesting because it has been shown in diseases conditions such as Alzheimer that glutamate-induced toxicity is a potential cause of neurodegeneration (Strukova et al., 2006). Taken together, these studies tend to indicate that PAR2 seems to be acting as a fine tuning mediator of different processes inducing detrimental or protective effects depending on the cellular context and environment (figure 1.15). For example, in a mouse model of Alzheimer disease, PAR2 protects neurons from amyloid–induced toxicity (Afkhami-Goli et al., 2007), while its activation in microglia cells lead to neurotoxicity (Pompili et al., 2004).

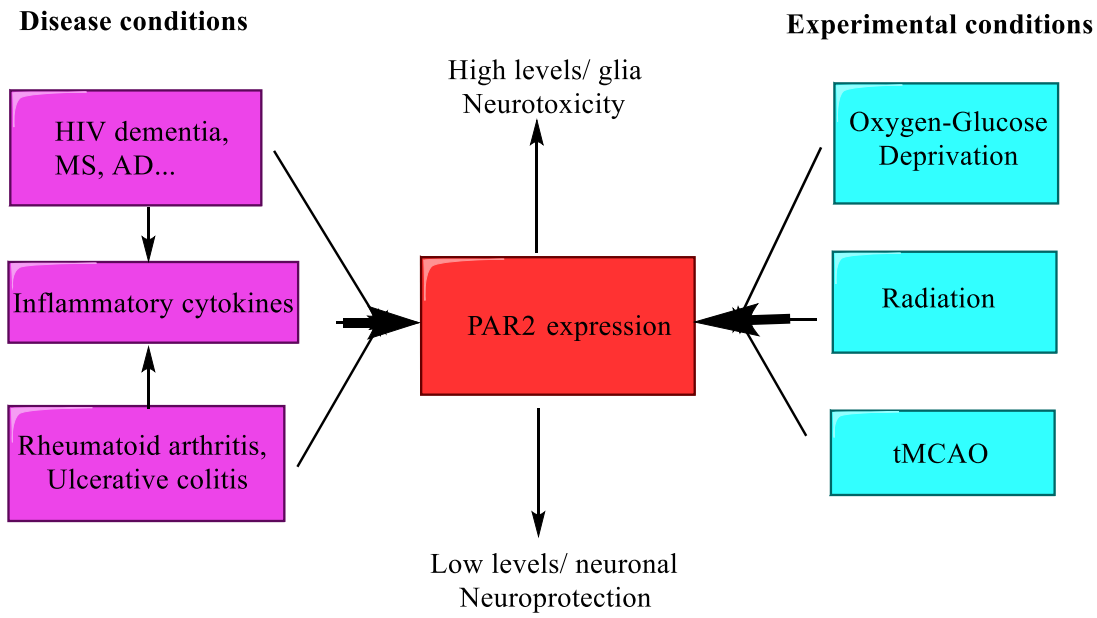


Figure 1.15 - Summary of the potential roles of PAR2 in the CNS (Adapted from Bushell, 2007).

1.5. PAR2 in CNS disease, a context-specific mediator.

Many of the data from previous studies tend to suggest that changes in PAR2 expression might bi-directionally, possibly in a concentration-dependent manner, play a role in both neurodegeneration and neuroprotection, making it an intriguing potential valuable therapeutic target. However, the pathways underlying these diverse cellular responses remain only partially characterised.

1.5.1. PAR2 is a mediator of neurodegeneration in the CNS.

It was first suggested that the PAR2 activation via peptide SLIGRL-NH_2 could be neurotoxic to pyramidal neurons *in vitro* depending on the context and concentration (Smith-Swintowsky et al., 1997). Since then a number of studies both *in vitro* and *in vivo* have described PAR2 activation as mediating inflammatory responses in the CNS, with neurotoxic effects. In patients with multiple sclerosis and also in the experimental autoimmune encephalomyelitis (EAE) mouse model, where the inflammatory component is central, it was observed that PAR2 KO mice exhibited milder clinical signs, inflammation and T cell proliferation (Noorbakhsh et al., 2006). Additionally, using the same mouse model of MS, studies described that PAR2 expression is up-regulated on monocytoid cells of the white matter particularly on astrocytes and macrophages (Noorbakhsh et al., 2006). That activation leads to the production of pro-inflammatory cytokines and chemokines able to regulate trafficking toward the CNS (Stein & Nombela-Arrieta, 2005), where they are thought to disturb oligodendrocyte viability (Buschmann et al., 2012) thereby, raising the possibility that blocking PAR2 could reduce neuro-inflammation, in MS and EAE. Also, recent studies have documented the role of α-synuclein pathologic accumulation as mediating apoptosis in a number of neurodegenerative diseases, including PD (Polymeropoulos et al., 1997; Jellinger, 2003; Stefanis, 2012; Liu et al., 2014), AD (Jellinger, 2003; Moussaud et al., 2014) and MS (Papadopoulos et al., 2006). The

function of α-synuclein is not very well defined, but studies have suggested a role in synaptic vesicles recycling and transport and as such in the regulation of neurotransmitters release including dopamine. Interestingly recent studies have identified PAR2 as an important modulator of α-synuclein (Liu et al., 2014) with PAR2 inhibition shown to increase α-synuclein levels thereby potentially contributing to subsequent motor dysfunction in a rodent model of PD, hence suggesting that the ability to antagonise PAR2 expression might be a way to achieve neuroprotection.

1.5.2. PAR2 activation is neuroprotective in CNS diseases.

In contrast to this detrimental role for PAR2, various studies have also documented an opposing neuroprotective role for its activation in the CNS. At the cellular level, it was reported that PAR2 activation can prevent ceramide-induced apoptosis in cultured primary rat astrocytes (Luo et al., 2007). Additionally, PAR2 activation on neurons was shown to prevent p53-induced neurotoxicity and subsequent cell death, while also reducing cell death against Aβ toxicity (Andrade-Gordon et al., 2005). Furthermore, additional studies have established *in vitro*, that PAR2 activation induces a significant reduction in kainate-induced neuronal toxicity (Greenwood & Bushell, 2010). Finally, more recently investigations have again highlighted PAR2 neuroprotective properties against hypoxia-induced apoptosis of RGC-5 cells (Peng et al., 2013). Moreover, *in vivo* using animals injected with Human Immunodeficiency Virus (HIV), PAR2 upregulation on neurons was correlated with an increase in TNF-α and interleukin-1β concentrations and was shown to induce neuroprotection, with PAR2 KO animals showing more sensitivity to the neurotoxic effects of the protein Tat (Andrade-Gordon et al., 2005), released from HIV-1-infected cells (Kruman et al., 1998) and involved in the development of HIV dementia (Mattson et al., 2005). Likewise, other studies have suggested a neuroprotective role of PAR2 activation in ischaemic stroke with PAR2 KO animals exhibiting more apoptotic cells and larger infarcts in a model of focal ischaemia (Jin et al., 2005). Ultimately, in PD and AD recent

studies have described how PAR2 expression patterns and PAR2 mRNA levels are correlated to neurodegeneration in post mortem studies of patient brains, suggesting once more a possible neuroprotective role for PAR2 activation (Afkhami-Goli et al., 2007; Hurley et al., 2015). No clear explanation has yet been advanced regarding what mechanisms and processes are involved in PAR2 mediated neuroprotection, although it was suggested that MAPKs including ERK1/2, might play a critical role (Jin et al., 2005; Zeng, 2013). Interestingly, these observations show how the role of PAR2 in different CNS pathologies represents a good example of cell or contextual specific effect, as illustrated in the physiopathology of AD where controlling PAR2 it is possible to induce both degenerative and neuroprotective effects, with PAR2 activation protecting against β-amyloid mediated neuronal toxicity, while on the other hand PAR2 stimulation on microglia was shown to enhance the generation of inflammatory mediators (Ramachandran et al., 2012). The effect of PAR2 on cell death and cell survival could share common initial signalling patterns but variations in the duration or in the amplitude of the signal may produce different outcomes. Understanding the subtleties and role of PAR2 activation is and will be a challenge due to an incomplete understanding of the underlying mechanisms, but could lead to important advances in the understanding of neuronal physiology as well as in the treatment of various CNS disorders.

1.6. Today's Challenges in PAR2 Research.

As previously described PAR2 is proposed to be involved in a variety of processes in normal and pathological conditions, which explains both the increased interest for this receptor as well as the difficulties faced in identifying precise therapeutic areas. Moreover, and considering the lack of consistency of the currently available PAR2 antibodies (Adams et al., 2012), current research almost solely relies on PAR2 activators.

1.6.1. PAR2 endogenous activation.

The main endogenous activator for PAR2 in the mammalian body is trypsin, however a few more endogenous proteases have been described as able to activate PAR2, including mast cell tryptase, P22, acrosein, proteinase 3, matriptase or the coagulation proteases FVIIa and FXa. (See table 1.4, Cottrell et al., 2003; Lohman et al., 2009). It is important to note that although these can activate PAR2, they are not ligands for PAR2 (Suen et al., 2014), it is considered that it is by cleaving the receptor N-terminus thus exposing the tethered ligand which they interact with that these proteases activate PAR2 (Suen et al., 2014). In the peripheral system, it was described that endogenous activation of PAR2 leads to an increase of intracellular Ca^{2+} concentration and subsequent stimulation of multiple signalling pathways (Macfarlane et al., 2001; Bushell, 2007). However, the exact nature of the PAR2 endogenous activation within the CNS remain to be detailed, one of the main reasons behind the difficulties to define or even clarify PAR2's distinct role in the brain can be the fact that these endogenous activators also interact with other receptors, including PAR1 and PAR4 (Compton et al., 2000; Sidhu et al., 2014), due to a limited selectivity for PAR2, thus complicating throughout investigations. Therefore, there was a crucial need for the development of activators specific enough to allow studies of PAR2 specific mechanisms and pathways.

1.6.2. Synthetic peptides for PAR2 activation.

Once the tethered ligand amino acid sequence required for PAR2 activation was identified, researchers have been able to engineer different synthetic activators, matching or approximating the sequence of the tethered ligand. Therefore, a series of potent PAR2 synthetic activating peptides have been described acting as selective PAR2 agonists thus mimicking the effects of endogenous activating proteases in a more specific manner (al-Ani et al., 1995; Macfarlane et al., 2001; Hollenberg et al., 2008; Ramachandran et al., 2011; Lohman et al., 2012). Ser-Leu-Ile-Gly- Lys-Val (SLIGKV-OH) (human sequence) and SLIGRL (murine variant) were identified as able to interact with the activation domain of the receptor and initiate intracellular signalling pathways and functional responses (Scarborough, 2003; Al-Ani et al., 2004). In order to increase their potency, analogues of these synthetic peptides have been designed whereby a serine was replaced by 2-furoyl (such as 2-furoyl-LIGRL-NH_2), a residue was added to the seventh residue (such as in SLIGRLI-NH_2), or combinations of both these changes (Kanke et al., 2009). It was reported that these synthetic peptides have an agonist potency of 5 to 30 times more than SLIGRL-NH_2 with 2-furoyl-LIGRL-NH_2 exhibiting the highest agonist properties (Barry et al., 2010). On the functional side, SLIGRL-NH_2 (and SLIGKV-NH_2 its human equivalent) has been shown to induce a Gαq-dependent increase in Ca^{2+} (Bunnett et al., 1996; Ramachandran et al., 2009), ERK1/2 activation (Stalheim et al., 2005), and also β-arrestin recruitment (Gardell et al., 2008). Nevertheless, these synthetic peptides have been suggested to be insufficiently specific for PAR2, moreover due to their still limited potency, they require relatively high concentrations to induce responses, at which concentrations they also interact with other receptors, sometimes even inducing toxic effects (Steinhoff et al., 2005; Gardell et al., 2008; Seitzberg, 2008).

1.6.3. Non-peptide activators for PAR2.

Recent advances in the understanding of PAR2 structure have facilitated the development of smaller, non-peptide molecule agonists and antagonists even though the way these molecules affect PAR2 signalling pathways requires further investigation. Short activating peptides were designed by replacing amino acids with non-peptidic fragments taken from other GPCR ligands (Yau et al., 2013). Indeed, GB110, a non-peptide PAR2 agonist was developed from hexo-peptides. Additionally, a replacement of the N-terminal serine shorten ion from the C-terminus for a C-terminal non-peptidic region was performed, thus conferring PAR2 agonism at low or limited concentrations (Seitzberg, 2008). The thereby designed PAR2 activator GB110 is considered as a very specific agonist as it did not induce any reaction in PAR2 negative cell lines (Barry et al., 2010). It is reported to be as potent as 2-f-LIGRLO-NH_2, previously identified as the most potent PAR2 agonist (Kanke et al., 2005) although it induces a reduced PAR2 internalisation hence suggesting different mechanisms for PAR2 activation and receptor internalisation (Barry et al., 2010), these findings also suggest that all these different PAR2 activators might be activating PAR2 receptors via different mechanisms. Finally, this non-peptide compound has a low molecular weight, is stable in serum solutions and suitable for use in human based studies, hence making it a good tool for research (Barry et al., 2010).

1.6.4. PAR2 antagonists.

Considering the complex and multiple mechanisms of PAR2 activation the ability to reduce or block its expression might be crucially important for the development of PAR2 specific therapeutic strategies in CNS diseases, however the development of pure antagonists has proved challenging. GB88, derived from GB110 with the same modified N–terminus is the first reversible PAR2 antagonist documented so far (Barry et al., 2010; Suen et al., 2010). It was described as able to inhibit activation by mechanistically and structurally different PAR2 agonists such as endogenous

proteinase trypsin, and synthetic PAR2 agonists 2-f-LIGRLO-NH_2 and GB110 with comparable potency, this even at low molecular concentrations (Barry et al., 2010). Nonetheless, studies have inferred that the way GB88 acts is highly dependent on the agonist it is used against. Indeed, used against 2-f-LIGRLO-NH_2 it is completely reversible causing no significant reduction in the maximum response produced, however, it causes a reduction in SLIGRL and GB110 maximum induced response (Suen et al., 2010). Additionally, it has been shown that GB88 can activate several PAR2 dependent pathways, including cAMP formation, Rho-kinase stimulation, and ERK1/2 phosphorylation (Yau et al., 2013), therefore rather than acting as a classic antagonist for PAR2, GB88 may in fact act as a biased agonist for this receptor. Other PAR2 activators described as antagonists, including ENMD-1068 and K-14585 have been investigated so far with results suggesting that they might be biased activators too (Ramachandran et al., 2012). More recently, pepducins have also been investigated for their potential PAR2 agonistic and antagonistic properties conferred by interfering with cell signalling pathways although with a questionable selectivity (Cisowski et al., 2011; Sevigny et al., 2011; Yau et al., 2013). Based on their study of GB88 and using the same basis, Craig Jamieson's lab (University of Strathclyde) has managed to isolate some of GB88 keys chemical characteristics, to improve specific areas such as selectivity for PAR2 and molecular weight, in order to develop 2 novel PAR2 antagonists DM/8/36 and Jami1066a (Jamieson, unpublished work). Both these compounds have been designed to antagonise PAR2 in a similar way to GB88 but haven't been investigated in live CNS cells yet.

1.6.5. Small molecules agonists may allow the development of new drugs targeting PAR2 in the CNS.

Synthetic peptides and non-peptides activators are good investigative tools for PAR2 studies, however, they still pose a few challenges as previously highlighted. Considering the fact that being large molecule compounds they have a limited penetration capacity of the blood–brain barrier (Banks, 2009), there was a need for

the design of more refined tools in order to investigate PAR2 in the CNS. In collaboration with Steinhoff's group, Gardell et al., (2008) reported the characterisation of a novel small-molecule PAR2 agonists AC-264613. AC stimulated internalisation of PAR2 receptors and elicited hyperalgesia, and pro-inflammatory effects *in vivo* (Gardell et al., 2008). Also, and despite a lower solubility, it displayed higher potency and selectivity for PAR2 (Ma & Burstein, 2013), with no activity observed against other PAR subtypes, compared to synthetic and non-peptide PAR2 activators in a variety of functional assays including cellular proliferation, Ca^{2+} mobilisation and receptor internalisation assays, additionally, it has recently been described how following an intraperitoneal injection, AC-264613 was found in the brain (Abulkassim, unpublished work, 2013) suggesting that it can cross the blood brain barrier, therefore providing a useful alternative activator to investigate PAR2 function in the CNS (Gardell et al., 2008; Seitzberg et al., 2008).

Table 1.4 - Currently available pharmacological data on PAR2 activators used in this study.

Name	Proposed activity	Molecular Formula	Molecular Weight	Solubility	IC/EC_{50}
SLIGRL-NH_2	agonist	$C_{29}H_{56}N_{10}O_7$	656.82	Soluble in water (1 mg/ml)	EC_{50}50 = ~5 µM
2-f-LIGRLO-NH_2	agonist	$C_{36}H_{63}N_{11}O_8$	777.95	Soluble in water (>2 mg/ml)	EC_{50} = 340 nM
GB110	agonist	N/A	608.37	N/A	IC_{50}~1 µM
AC-264613	agonist	$C_{19}H_{18}BrN_3O_2$	400.27	Soluble in DMSO > 10 mM-100mM	EC_{50}50 = 31.62 nM
GB88	antagonist	N/A	546.32	N/A	IC_{50} 2–9 µM
Jami1066a	antagonist	$C_{31}H_{44}N_4O_5$	552.70	N/A	N/A
DM/8/36	antagonist	$C_{27}H_{38}N_4O_5$	498.61	N/A	N/A

Although recent advances have provided more efficient tools for PAR2 investigation including in the CNS, a few challenges remain. Despite a good selectivity and potency, details of the mechanisms of action, of the pathways activated and on the ways to control PAR2 activation need to be specified and so far very few data are available. A better general understanding of PAR2 will also allow experimental designs able to advance the understanding of whether PAR2 is a viable CNS therapeutic target. Recently, extensive progress has been made in characterising the specific mechanisms allowing proteases, synthetic agonists, non-peptide and small molecules activators to activate PAR2. Data gathered from these studies together with future work may provide insights into the signalling pathways that are responsible for certain pathologies and the way to interfere using PAR2 activation. Also, in disease states and pathological processes where changes in PAR2 expression patterns is observed, potent and stable PAR2 activators might prove to be powerful tools.

A better understanding of the mechanisms underlying PAR2 signalling, its initiation, regulation, and termination will have a deep impact in developing new drugs for many critical conditions. In the same way the future development of PAR2 antagonists will also potentially be therapeutically beneficial for treatment of a number of inflammatory, or neurodegenerative diseases but also for neurogenic and chronic pain. However, although considerable efforts have been made toward the identification and engineering of potent and selective agonist there is still a lot of progress to be made in that regard.

1.7. Hypothesis and Aims.

Given previous findings within our lab and available evidence from the literature, our central hypothesis is that PAR2 activation is neuroprotective in the CNS. Collectively, this study will provide more detailed information on the role of PAR2 in the CNS, on its pathways, and on its impact in inducing neuroprotection. It is hoped that this research will advance the understanding of PAR2, thus allowing future identification of new points of intervention in CNS diseases. In order to test the central hypothesis, we will in this project investigate individual aims as follows:

- 1. Given the limited data available regarding exogenous PAR2 activation, including in the CNS, we will investigate a selected range of PAR2 activators including newly developed non-peptide activators, small molecule activator and PAR2 antagonists *in vitro* using CNS preparation to study their effect on activation of Ca^{2+} pathways. Using the same PAR2 activators, we will also study another well characterised GPCR's pathways, receptor internalisation, in cell line, in order to **further characterise PAR2 and its mechanisms of activation.**

- 2. Glutamate excitotoxicity is a well-established mechanism involved in the physiopathology of a variety of CNS diseases. Given that previous studies, including in our lab, have identified neuroprotective properties of PAR2 activation, it is reasonable to **investigate PAR2 potential neuroprotective properties in an *ex vivo* model of excitotoxicity**.

- 3. Knowing that there is a wide gap between *in vitro* experiments and applications in a living subject, we will **study the effect of PAR2 activation on locomotor activity and on anxiety-like behaviour,** before ensuring that PAR2 activation doesn't cause majors physiologic changes by **investigating**

the effects of PAR2 activation on vitals measurements (heart rate and fractional shortening).

- 4. Finally we will **investigate PAR2 potential neuroprotective properties in an *in vivo* model of CNS disorders.**

Chapter II. MATERIAL AND METHODS.

2.1. Materials.

Materials	Company
bovine serum albumin (BSA), complete freunds adjuvant (CFA), D-glucose, dimethyl sulfoxide (DMSO), sodium chloride (NaCl), ethanol, ethylene-di-amine tetra-acetic acid (EDTA), Gey's balanced salt solution (GBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrochloride acid (HCl), kainic acid monohydrate, kynurenic acid, methanol, papain, poly-L-lysine hydrobromide, propidium iodide solution (1.0 mg/ml in water), sodium chloride (NaCl), sterile 3,2ml transfer pipette, trypsin, Tween 20 and Tween 80.	Sigma Aldrich, (UK).
calcium chloride ($CaCl_2$), boroscillate glass coverslips (round, 13 mm, thickness No. 1), magnesium sulphate ($MgSO_4$), microscope slides (superfrost, 76x26x1mm, white), optimal cutting temperature compound (OCT), pasteur pipettes (plain glass, 150mm), potassium	VWR International Ltd, (UK).

chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen carbonate ($NaHCO_3$), sodium hydroxide (NaOH), sucrose and sulphuric acid (H_2SO_4).	
B27, D-glucose, Dulbecco's Modified Eagle's Medium (DMEM), Fura-2 AM, foetal bovine serum (FBS), foetal calf serum (FCS), Fungizone, Hank's Balanced salt solution (HBSS), HEPES buffer solution, GlutaMAX supplement, L-glutamine, Lipofectamine 2000, Neurobasal-A minimum essential medium (MEM) and penicillin-streptomycin (5,000 U/mL)	Invitrogen, (UK).
D-glucose, 96 well ELISA plates, Roswell Park Memorial Institute -1640 (RPMI-1640) media and 50mm tissue culture dishes.	Fisher Scientific, (UK).
Millex syringe-driven filter unit (0.22µm) and millipore culture plate insert.	Merck Millipore, (Germany).
MOG^{35-55}.	China Peptides Co Ltd, (China).
Cell culture dishes (35 mm TC treated), Horseradish peroxidase (HRP), Lysis buffer, Mycobacterium tuberculosis H37Ra and Tetramethyl benzidine (TMB) reagents A and B.	BD Biosciences, (UK).

Nitex nylon mesh.	Cadisch precision meshes, (UK).
2-furoyl-LIGRL-NH_2 (2-f), SLIGRL-NH_2, TFLLR-NH_2 and AYPGKF-NH_2.	Peptides Synthetics, (UK).
Anti-green fluorescent protein (GFP).	Clontech, (Mountain View, USA).
AC-264613 and pertussis toxin (PTX).	Tocris Bioscience, (UK).
DM/8/36, GB88, GB110 and Jami1066a.	Dr C. Jamieson, University of Strathclyde (UK).
GFP and PAR2 YFP plasmid.	Dr Roth Tate, University of Strathclyde (UK).
AC-264613.	Eli Lilly, (UK).

2.2. Methods.

2.2.1. Primary hippocampal cultures.

2.2.1.1. Animals

Hippocampal primary cultures were prepared using Sprague Dawley rat pups (1 to 3 days old) that were obtained from the Biological Procedures Unit (University of Strathclyde, UK), and killed by cervical dislocation according to U.K. Home Office Schedule 1 guidelines under the authority of U.K. Animals (Scientific Procedures) Act, 1986.

2.2.1.2. Solutions

For cell culture, solutions were prepared as described below.

- Sterile enzyme solution consisting of 116mM NaCl, 5.4mM KCl, 26mM $NaHCO_3$, 1.3mM NaH_2PO_4, 2mM $MgSO_4$, 2mM $CaCl_2$, 25mM D-glucose (pH adjusted to 7.4).
- Serum free hippocampal culture medium consisting of Neurobasal A (97 %), B-27 supplement (2 %) and L-glutamine (1% from 200 mM stock concentration).

All solutions made for the cell culture preparations were filter sterilized and maintained at 37°C in a water bath until required.

2.2.1.3. Cell cultures

Autoclaved sterile coverslips (13mm diameter) were coated with filter sterilized Poly-L-lysine (0.1mg/ml) for one hour, then washed in autoclaved sterile water and dried before being plated in 33mm petri dishes (3 coverslips per dish). Under sterile conditions, rat brains were removed and hippocampi were dissected out, chopped into small pieces using a scalpel and placed into sterile enzyme solution containing papain (1.5%) and incubated at 37 °C for 20 min to allow enzymatical dissociation of cells. In order to stop the papain digesting activity, cell tissue was then transferred to a fresh enzyme buffer with containing BSA (1%) and triturated with a series of three flame-polished glass Pasteur pipettes of decreasing diameter. The cell suspension was then centrifuged at 2000 rotations per minute for 2 minutes producing a pellet, the supernatant was removed and the pellet re-suspended in 1 ml of serum free hippocampal culture media. Cells were counted using a haemocytometer, diluted in culture medium if required and plated at a density of 3 $X10^5$ cells/ml. Coverslips with cell suspension (100µl per coverslip) were maintained in a humidified atmosphere in an incubator at 95% O_2/ 5% CO_2 at 37 °C for at least 1 hour in order to allow cells

adhesion to coverslips. Each culture dish was then slowly flooded with an additional 2ml of culture media before being placed back in the incubator. Cultures were monitored regularly, with any culture showing signs of infection being discarded. Experiments were conducted on cells aged between 12-15 days *in vitro* (DIV).

2.2.2. Calcium Imaging.

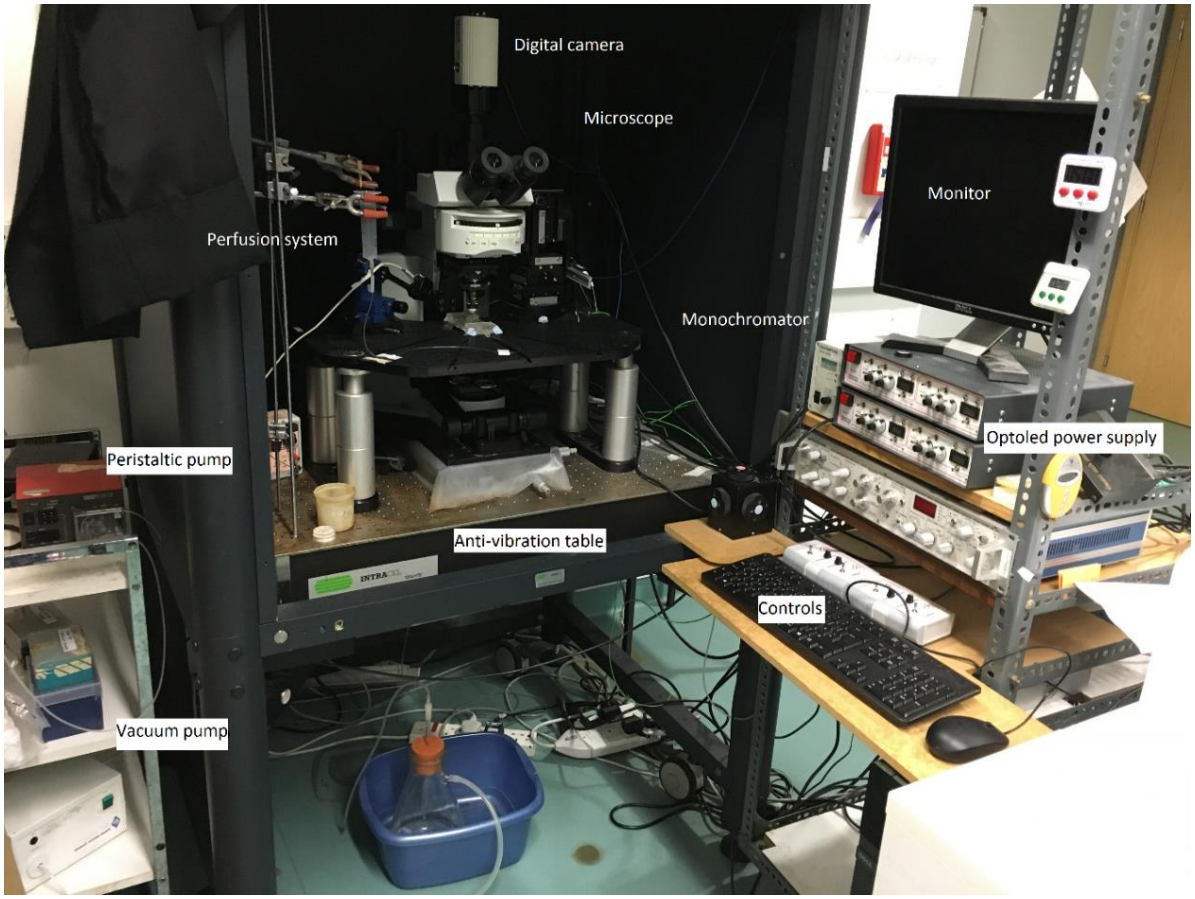


Figure 2.1 - Setup used in calcium imaging experiments.

2.2.2.1. Equipment

Calcium imaging experiments were performed using a Q-imaging digital camera mounted on an Olympus BX51W (Japan) microscope with a 40× water immersion objective (as seen in figure 2.1). An OptoLED was used to generate light of relevant excitation wavelengths, with analysis of emission intensity ratios at 350 nm/380 nm

excitation being performed off-line using WinFluor (J. Dempster, University of Strathclyde, UK).

2.2.2.2. Solution

For calcium imaging, a solution was prepared as described below.

- HEPES-buffered saline (HBS) containing: 140mM NaCl; 2.5mM KCl; 2mM $MgCl_2$; 10mM HEPES; 10mM D-glucose, 2mM $CaCl_2$. The pH was adjusted to 7.4 ± 0.02 and osmolarity was corrected with sucrose if required, to range between 310 ± 2 mOsm.

2.2.2.3. Procedure

Hippocampal cultures prepared from 1-2 days old rat pups were loaded with the ratiometric Ca^{2+} sensitive dye Fura-2 AM (0.33µM in HBS) and covered to avoid exposure to light for a 60 min incubation at room temperature (RT) prior to experiments. This incubation allowed Fura-2 AM to be taken up by the cells, cleaved and turned into the active dye Fura-2. Loaded cells were then washed twice in HBS and placed in a perfusion chamber designed to allow direct access of the microscope lens with the bottom of the coverslip, Fura-2 was excited using fluorescence light emitted from the OptoLED at 350 and 380 nm for periods of 500ms every 1s. Cells were continuously perfused with HBS at a flow rate of 1-2mL/min. Following a 5 min stable baseline period, drugs were added via the perfusate, subsequently cells were washed and further perfused with trypsin (100nM). Neurons and non-neuronal cells were identified at the end of the experiments based on their response to an application of a high potassium solution (20mM) as it results in increases in intracellular Ca^{2+} only for neurons. The same protocol was repeated for each of the drugs used in the calcium imaging experiments.

2.2.2.4. Analysis

Increases in intracellular Ca^{2+} levels was determined by analysis of the fluorescence emitted by the cells after a drug perfusion of activator (100 µM), for each cell the peak Ca^{2+} response to drug application was recorded. Images of a group of cells were captured every 1 second. Regions of interest (ROI) were drawn around individual cells, with the background fluorescence being subtracted from the ROI fluorescent intensity and relative fluorescence change (ΔF/F) versus time plots was generated for each ROI. Raw data in the form of emission intensities were recorded and stored using Winfluor, moreover, following background subtraction, emission ratios (350/380 nm) were calculated off-line. Data were calculated as changes in fluorescence ratio (350/380). All values shown are mean ± standard error of the mean (S.E.M.). Significance tests utilised were Student's paired t-tests with the differences being considered significant if $P<0.05$. For all experiments n = the number of cells with data obtained from at least three different cultures made from different animals.

2.2.3. Transfection.

2.2.3.1. Equipment

Confocal microscopy was used in order to determine localisation of the receptor within cells depending on the treatment used. Immunofluorescence microscopy was performed to detect the fluorophore-tagged receptors introduced into tsA-201 cells on a Leica SP5 DM600 (Leica Microsystems, UK) confocal microscope using a 20x water immersion objective. Fluorophores were excited in individual cells with an Argon laser (excitation wavelength 488 nm), the fluorescent emissions were recorded by 3 different photomultiplier tubes. Image stacks were collected reaching from top to bottom of the cells at 1µm intervals with every image being of size 1024 × 1024 pixels averaged over 8 lines of scan.

2.2.3.2. Solutions

For transfection, solutions were prepared as described below.

- Modified DMEM consisting of foetal calf serum (10%), penicillin/streptomycin (1%) and non-essential amino acids (1%) into sterilized filtered DMEM.
- HEPES-buffered saline (HBS) as previously described.

Modified DMEM was filtered, sterilized and maintained at 37°C in a water bath until required, while HBS was kept at room temperature, both solutions were prepared on the day of experiments.

2.2.3.3. Procedure

tsA-201 cells, a modified HEK cell line, were cultured and grown to confluence in a 75 cm^2 tissue culture flasks containing modified DMEM. When passaging cells, they were diluted at a 1:10 concentration and maintained in an incubator with 95% O_2 /5% CO_2 at 37°C. Once confluent, cells were harvested via a 5 minutes trypsinization (0.5%) in an incubator to allow dissociation from the flask surface and plated out onto 13mm glass coverslips coated with Poly-L-lysine (0.1mg/ml). To allow cell adhesion to coverslips, they were incubated for two hours following which, the cells were flooded with serum free DMEM and transfected. Plasmid DNA, here PAR2-YFP, was introduced into tsA-201cells using Lipofectamine 2000 transfection reagent, it was added into 50 µl of serum free DMEM in sterile 1.5 ml reaction tubes at a ratio of 1.5 µl of Lipofectamine 2000 for 1 µg of DNA. The solutions containing PAR2 plasmid DNA and Lipofectamine 2000 were then combined, pipetted several times and incubated for 25 minutes at room temperature. Subsequently the PAR2 plasmid-Lipofectamine mixture was added dropwise to the cells. Cells were incubated with the transfected expression vector for 5 hours in an incubator, the media was subsequently replaced by modified DMEM and cells were incubated at 95% O_2 / 5% CO_2 at 37 °C for 24 hours

prior to experiments. Before imaging, modified DMEM was replaced by 2ml of serum free DMEM and PAR2 activators were applied for 45 min (100µM) to examine whether they induce receptor internalisation. The media was then removed and replaced with 2ml of modified DMEM, coverslips were finally transferred into HBS just before imaging. We also transfected tsA-201 cells with GFP, PAR1-YFP, PAR4 -CFP and P2Y1-YFP DNAs, kindly provided by Dr. Charles Kennedy (University of Strathclyde, UK) or produced by Dr. Rothwell Tate (University of Strathclyde, UK) using Lipofectamine 2000.

2.2.3.4. Analysis

In order to investigate GPCR localisation and PAR2 receptor trafficking in the tsA-201 cells following activation caused by a range of PAR2 activators, ROIs were drawn in individual cells around the membrane prior to PAR2 activation and around a central area representing 25% of the cell surface toward the nucleus in the centre of the cell where the receptor would translocate after internalisation (figure 2.2). Data were calculated as a ratio, obtained by comparing the amount of fluorescence expressed in the region of interest compared to the total fluorescence expressed in the cell.

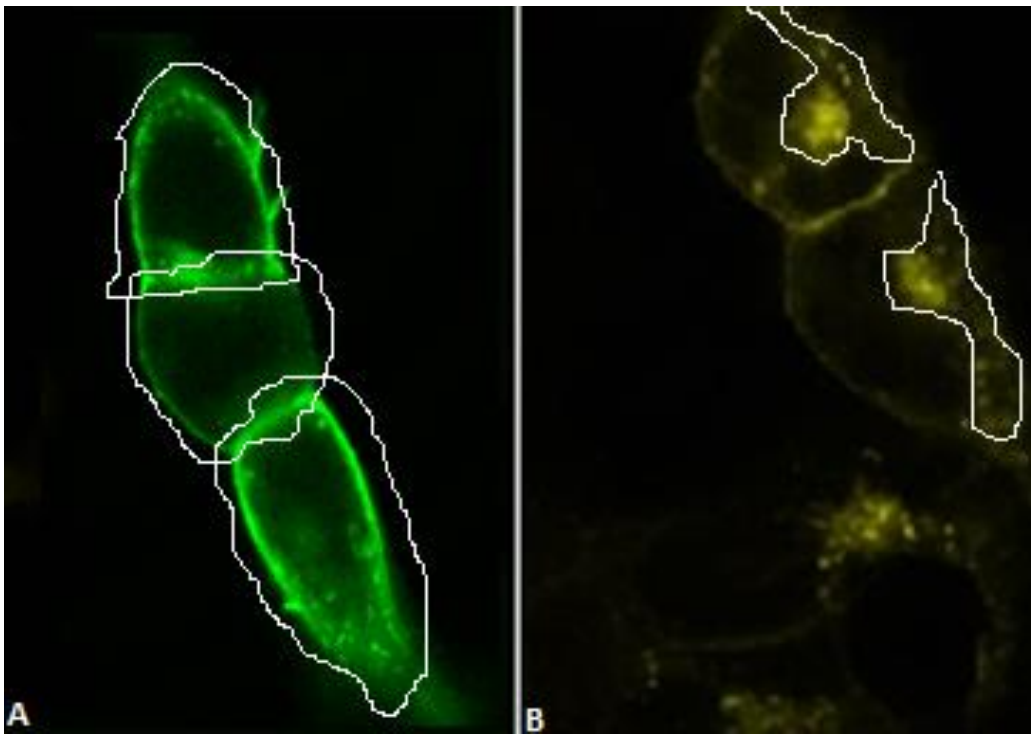


Figure 2.2 - **Description of the method used for study of receptor internalisation.** (A) Prior to activating the receptor, we monitor the level of fluorescence for the whole tsA-201 cell. (B) Following a 45 min application of PAR2 activator, once the receptor has been activated, we measure the level of fluorescence expressed in an area located around the nucleus, representing approximately 25% of the total cell surface. Finally, we compare the fluorescence expressed in the whole cells to that expressed in these central regions obtaining a ratio expressed in %. Image were visualised with confocal microscopy.

Data were extracted with the image processing software ImageJ (National Institutes of Health, USA) and were analysed using statistical software packages Microsoft Excel (Microsoft, USA), GraphPad Prism (GraphPad Software, La Jolla, USA) and Origin Pro 9 (OriginLab, Northampton, USA). All values shown are mean ± standard error of the mean (S.E.M.). Significance tests utilised was one-way ANOVA with Dunnett or Bonferroni post-hoc analysis where required, with the differences being considered significant if $p<0.05$, for all experiments n= the number of cells with data obtained from a minimum of 3 different cultures.

2.2.4. Organotypic slice cultures.

2.2.4.1. Animals

C57 mice pups aged 5 to 8 days old were obtained from Harlan UK Ltd, and killed by cervical dislocation according to U.K. Home Office Schedule 1 guidelines under the authority of U.K. Animals (Scientific Procedures) Act, 1986.

2.2.4.2. Solutions

For organotypic slice cultures, solutions were prepared as described below.

- Culture media (200 mls): MEM (50%), heat-inactivated horse serum (25%), Hank's Balanced salt solution (1%), HEPES buffer solution (1%), GlutaMAX supplement (0.5%), L-Glutamine (1%), Pen-Strep (1%), Fungizone (1%) and D-Glucose (1.5%) and filtered ddH2O (19%). It was then filter sterilised and pH was adjusted to be between 7.1 - 7.2 using NaOH (10M).
- Dissection buffer (200 mls): Gey's Balanced Salt Solution (98.5%), supplemented with D-glucose (1.5%) and filter sterilised with the pH adjusted to 7.1. Following the filter sterilisation kynurenic acid (1mM) was added to the buffer.

The culture media was maintained in an incubator at 95% O_2 / 5% CO_2 at 37 °C, until required, while the dissection buffer was then left to cool in a -20°C freezer for about an hour prior to the time of dissection, so that it would be ice cold but still not completely frozen (between 0 and 4°C).

2.2.4.3. Procedure

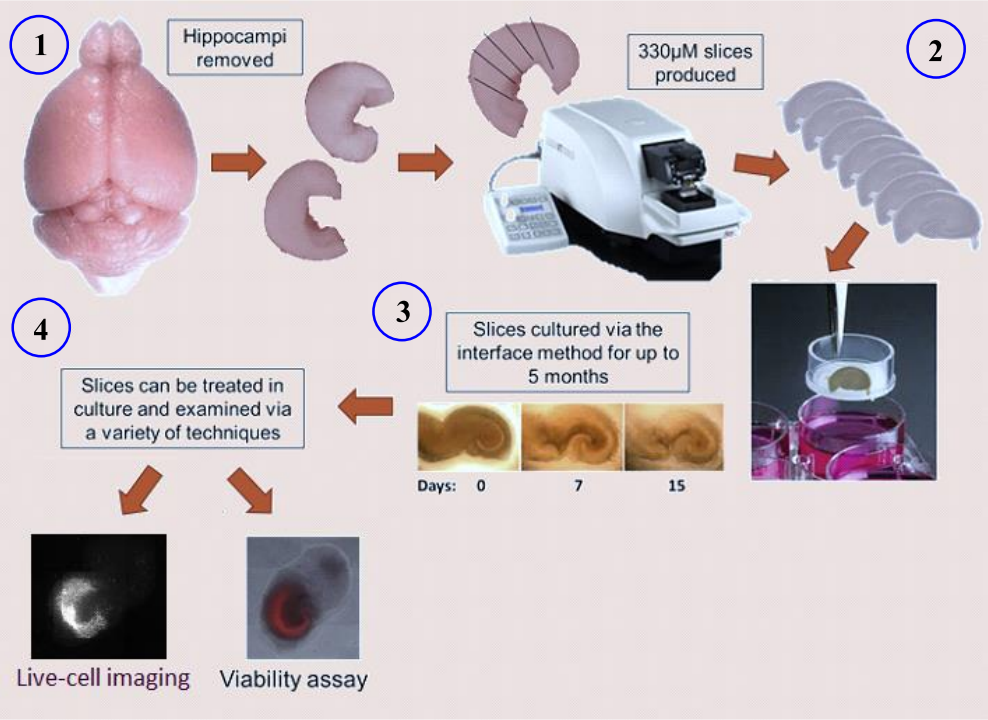


Figure 2.3 - **Organotypic slice culture protocol** (Images courtesy of Jim McCarthy, Eli Lilly). Hippocampi are dissected out in sterile conditions (1) 330µm slices are produced and placed in an insert (2) where they are cultured for 15 days in media, finally after 15 days in culture slices are used for experiments (4).

2.2.4.3.1. Preparation

Sterilization of dissection instruments was achieved by autoclaving for 30 minutes at 135°C or in cases where this was not possible, instruments were soaked in 70% ethanol for at least 15 minutes. Six well tissue culture plates were prepared for culturing of slices, with 1ml of organotypic slice culture media and a Millipore culture plate insert placed into each of the six wells. The plate and inserts were placed in a 37°C / 5% CO_2 incubator to enable the medium to equilibrate before the addition of slices. All work areas and Leica VT1200 vibratome were wiped down with 70% ethanol. Just prior to beginning the dissection, dissection buffer was oxygenated then added to the buffer tray attached to the vibratome with a constant input of 95% O_2 and 5% CO_2.

2.2.4.3.2. Dissection

Pups were killed by cervical dislocation according to UK Home Office Schedule 1 guidelines and decapitated using surgical scissors. The mouse pup head was sprayed with 70% ethanol and transferred to the laminar flow hood. All work from here on took place within the laminar flow hood. Working on sterile blotting paper, the skin over the skull was removed by cutting along the midline toward the nose with a dissection scissors. Excess skin was peeled back and cut off to prevent contamination of the brain. Using surgical scissors, the skull was cut bilaterally along the midline starting from the vertebral foramen towards the frontal lobes. With short, blunt forceps, the skull was peeled back, the brain was removed from the skull into a sterile 50mm tissue culture dish with dissection buffer. The brain was left cooling on ice for 1 minute and then dissected into two hemispheres. Hippocampi was removed and attached to single square blocks of agar glued onto the specimen disc of the vibratome. Hippocampal slices (330µm) were cut using a Leica VT1200S vibratome with the following settings: speed 0.14 mm/s, amplitude 2.00 mm and thickness 330µm. Using a sterile transfer pipette, slices were transferred onto millipore inserts in 6 well tissue culture plates with 1ml of pre-warmed organotypic slice culture media per well (figure 2.3).

2.2.4.3.3. Culture.

Slices were incubated in an environment of 95% O_2/ 5% CO_2 at 37 °C in culture media for 24 hours before it was first replaced, following this initial media change, the media was exchanged in totality every 48h.

2.2.4.3.4. Analysis.

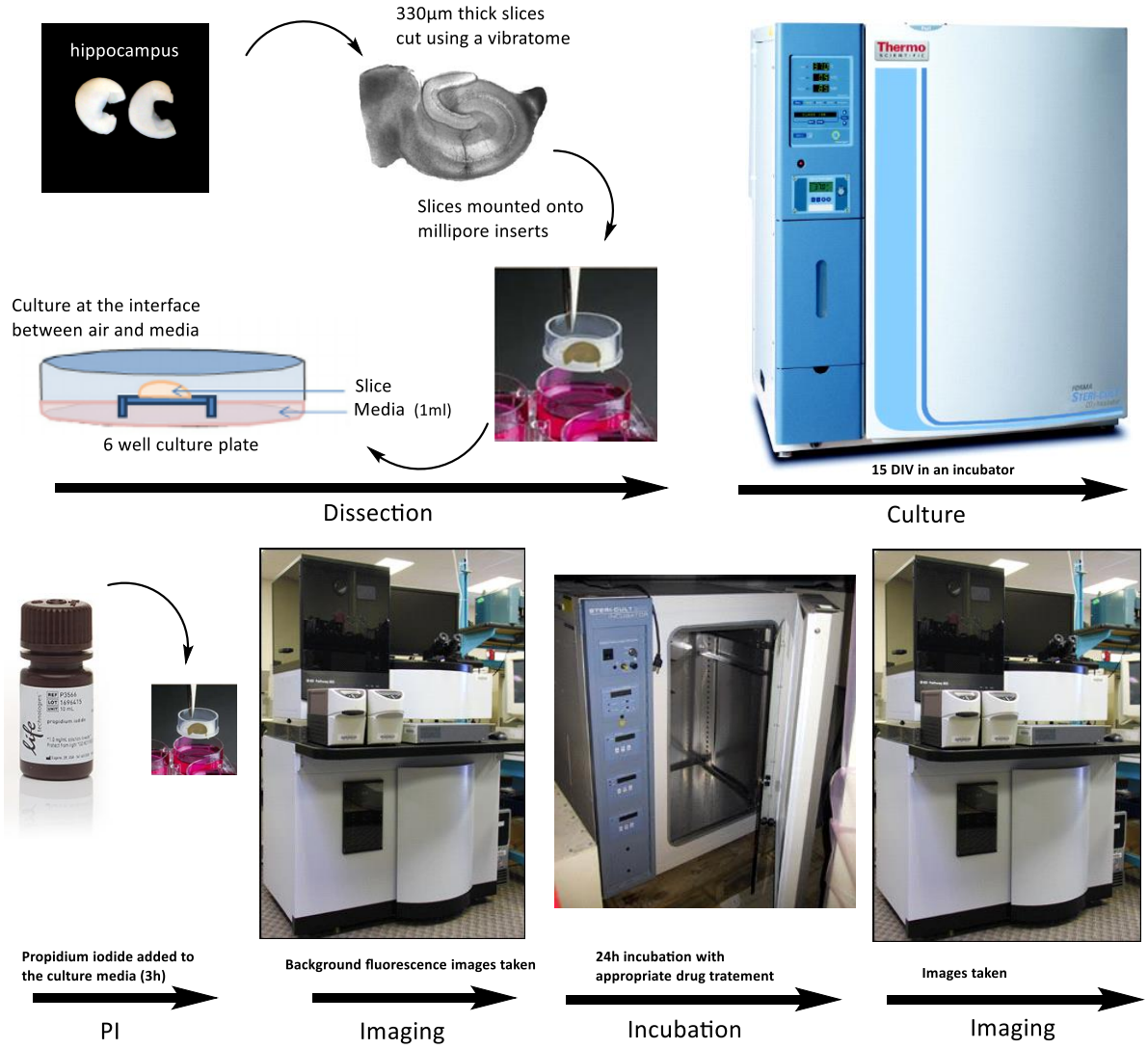


Figure 2.4 - **Time flow of organotypic slice culture imaging protocol**.

Prior to take the first images, propidium iodide (PI, 2.9µM) was added to the media. Following a 3-hour incubation, to allow PI uptake, slices were imaged using a BD pathway 855 High-Content Bioimager (BD Bioscience, UK) with a controlled atmosphere of 95% O2/ 5% CO2 at 37°C in order to asses PI related fluorescence baseline levels. Slices whose health was compromised during the first 14 days in culture, as assessed using bright field microscopy when feeding the slices, were

excluded (about 5% of the total slices). The remaining healthy slices were then taken back to a sterile hood with the media in each well appropriately replaced to fit the treatment plans (figure 2.4). Subsequently, slices were replaced into the incubator for 24h and imaged again in order to observe the effects of the treatment. We compared the level of PI fluorescence emitted in the CA3-CA1 and dentate gyrus area, and its related level of fluorescence in slices where neurotoxicity was induced using different concentrations of kainic acid (see figure 2.5). Data were extracted using ImageJ and analysed with Microsoft Excel, Prism 5 or Origin Pro9. All values shown are mean ± standard error of the mean (S.E.M.). Significance levels were assessed using one-way ANOVA with Dunnett's post-hoc analysis where required, with differences were considered significant if $p<0.05$. For all experiments n= the number of slices with data obtained from three different animals.

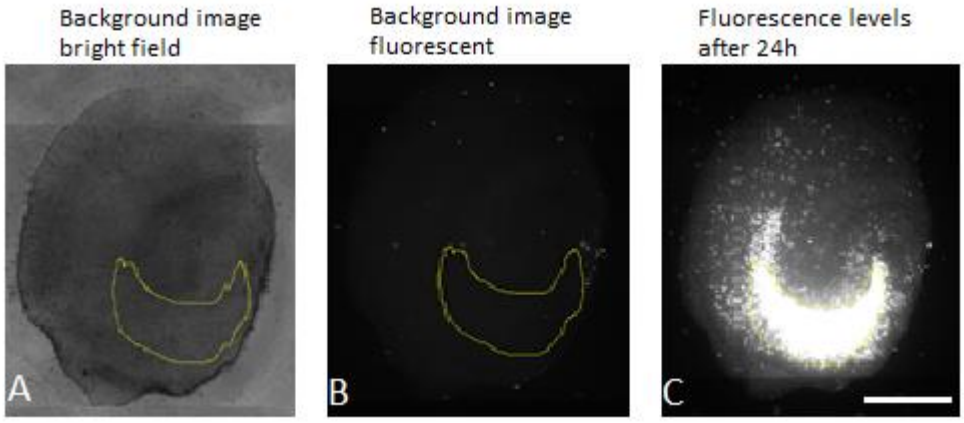


Figure 2.5 - Representative images of the area selection for organotypic slice culture analysis. Baseline image from a typical organotypic slice after 15 days in culture, in bright field (A) and using fluorescence imaging (B), following a 24h incubation in KA 20µM, the slice was imaged again (C). We selected the CA3-CA1 to dentate gyrus area and compare the fluorescence levels within this area over time depending on the treatment applied. Scale bar 450µm.

2.2.5. Open field experiments.

2.2.5.1. Animals

Female C57BL/6J mice aged between 6 and 8 weeks were obtained from an in-house colony and housed in cages of 6 animals. They were bred and maintained in a temperature-regulated environment with a 12-hour light/dark cycle with unrestricted access to food and water in the Biological Procedure Unit at University of Strathclyde. Experimental protocols were carried out under UK Home Office Project license no PP70/8520 according to U.K. Home Office guidelines under the authority of U.K. Animals (Scientific Procedures) Act, 1986. The basis of using animals to model human diseases relies on the idea that experiments carried out in animal can potentially predict the human outcome. However, a variety of bias exists which if not considered and avoided make it difficult to provide evidences that animal studies are relevant to translation in human health. In order to minimise these subjective bias in the work presented here, we have tried to designs experiments following as closely as possible the ARRIVE guidelines (Kilkenny et al., 2010). These guidelines where designed to promote ethical, high-quality reporting hence allowing a more accurate critical review of animal experiments and their results.

2.2.5.2. Equipment

The open field test apparatus consisted of four 40cm x 40cm x 40cm translucent Perspex arenas and positioned directly under a ceiling-mounted infra-red sensitive camera (figure 2.6), linked to a computer in an adjacent room. Anxiety and exploratory behaviour were recorded and analysed using EthoVision Pro, Version 3.0 video tracking software (Noldus Information Technology, Netherlands). The software allowed monitoring of the paths taken by the mice, measures the total distance travelled, time spent in the centre square and the number of entries into the centre of the arena during the sessions.

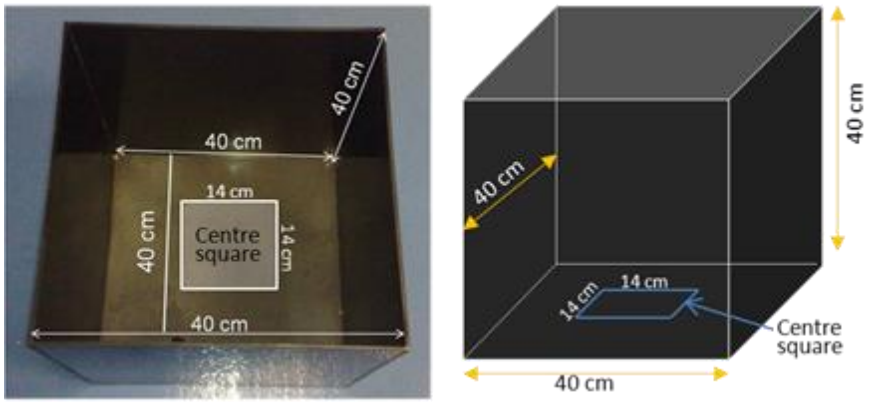


Figure 2.6 - Apparatus as used for open field experiments (from Abulkassim, 2014).

2.2.5.3. Procedure

Mice were carried to the test room in their home cages and were handled by the base of their tails at all times, with each mouse initially habituated to the open field arenas for 15 minutes prior to open field assessment following the appropriate drug treatment. Following habituation, mice received intraperitoneal (i.p.) injections of the PAR2 agonist AC-264613 (1, 10 and 100 mg/kg) dissolved in 0.9% saline solution with 1% Tween 80, and placed back into their cages. Two hours post-injection, mice were then placed back into the same open field arena in which they were habituated in, to assess locomotor activity over a 30-minute recording period. After the test, mice were returned in their home cages and the open field arena was cleaned with disinfectant between each session. The effect of PAR2 activation on locomotor activity was assessed by recording the distance travelled, the time spent in the inner square and the number of entries in the centre square. Finally, once *in vivo* data were acquired, animals were sacrificed by CO_2 asphyxiation and washed with 70% ethanol prior to a rapid spleen dissection. Spleens were placed in complete RPMI media consisting of RPMI-1640 supplemented with foetal calf serum (10%), penicillin/streptomycin (1%) and L-glutamine (1%) maintained at 37°C and filtered through nitex nylon mesh to form a cell suspension. Pellets were re-suspended in lysis buffer for 5 minutes before being washed twice with PBS, re-suspended in RPMI, counted and re-suspended again to an appropriate concentration. Spleen cells were

then cultured at 2.3 $X10^6$ cells/ml in 24-well tissue culture plates in an incubator for 48h before being analysed using ELISA.

2.2.5.4. Enzyme linked immunosorbent assays (ELISA)

2.2.5.4.1. Solutions.

For ELISA, solutions were prepared as described below.

- ELISA wash buffer consisting of PBS, made up as previously described and supplemented with Tween 20 (0.05%).
- ELISA blocking solution consisting of PBS and complemented with FCS (10%).
- Horseradish peroxidase (HRP) solution was made up using HRP diluted to 1:500 in PBS.
- ELISA stop solution consisting of sulphuric acid (1M) diluted in PBS.

All solutions were kept at room temperature.

2.2.5.4.2. Protocol.

ELISAs were conducted using 96 well ELISA plates previously coated overnight with a primary 'capture' antibody diluted according to the manufacturer's instructions (see table 2.1) in PBS at 4 °C. The following day, capture liquid was removed and plates were blocked using 10% FCS in PBS for 2 hours. Plates were then washed in ELISA wash buffer and 50µl of standards and samples were added. Following 2 hours of incubation at room temperature, liquid from the samples/standards was removed and the plates were washed a minimum of 3 times with the wash buffer previously described. Secondary 'detection' antibody was added and incubated for a further hour at room temperature, following which the liquid was removed and the plate washed a further 3 times. HRP solution was then added to the plates and incubated for 30 min, the liquid was then removed and plates were washed a further 3 times in wash buffer. Tetramethyl benzidine (TMB) reagents A and B were mixed in equal volumes and 100µl was added to each well and incubated for 10 min. After a couple

of minute, when a definitive blue colour had sufficiently developed for recording by an ELISA reader (Epoch, BioTek Instruments Inc., USA.) the reaction was stopped by the addition of 100µl of ELISA stop solution, absorbance values were measured at 450nM. A standard curve was constructed from the absorbance values of the standards and cytokine concentrations were calculated.

Table 2.1 - Summary of antibodies and dilutions used for ELISA.

Antibody	Species	Manufacturer	Capture	Detection	Standard
IL-6	Mouse	eBioscience	1:500	1:500	1:2000
IL-17	Mouse/Rat	eBioscience	1:500	1:500	1:2500
TNF-α	Mouse	eBioscience	1:250	1:250	1:500

2.2.5.4.3. Statistical analysis.

For open field experiments, distance travelled was measured in centimetres, time spent in the centre area in seconds and the number of entries in the centre square was recorded, data was expressed using the mean ± S.E.M. for each condition. Data collected from the open field and from ELISA were analysed on Prism 5, using 1-way ANOVA with a Dunnett's post hoc analysis where appropriate, with differences considered significant if $P<0.05$.

2.2.6. Heart function experiments.

2.2.6.1. Animals

Female C57BL/6J mice aged between 6 and 8 weeks were obtained from an in-house colony and housed in cages of 5 animals. They were bred and maintained in a temperature-regulated environment with a 12-hour light/dark cycle with unrestricted access to food and water in the Biological Procedure Unit at University

of Strathclyde. Experimental protocols were carried out under UK Home Office Project license no PP60/4364 according to U.K. Home Office guidelines under the authority of U.K. Animals (Scientific Procedures) Act, 1986.

2.2.6.2. Equipment

Functional echocardiographic measurements of the heart, were carried out by Claire McCluskey and Margaret MacDonald at the University of Strathclyde. These were recorded on an HDI 3000CV echocardiography system (Advanced Technology Laboratories, Bothell, USA), a 13MHz linear array transducer and ultrasound transmission gel, in order to measure the cardiac output within the left ventricle. These metrics were obtained by non-invasively measuring the thickness of the posterior and anterior walls of the LV chamber and the LV chamber diameter during systole and diastole using two-dimensional short axis views and wall motion via M mode images, at the level of the papillary muscle. Fractional shortening (%FS) was assessed from M mode traces and is expressed as [LVEDD – LVESD/ LVEDD] x 100, where LVEDD is left ventricular end diastolic diameter and LVESD is left ventricular end systolic diameter. All parameters were measured over at least three consecutive cardiac cycles and an average for each variable was taken for each animal.

2.2.6.3. Procedure

Mice were anaesthetised in a Perspex chamber with 3% Isoflurane in the presence of 100% O_2 at a flow rate of 2L/min. After 2-3 minutes, anaesthetised mice were brought to a semiconscious state with 1.5-2% Isoflurane in the presence of 0.5-1L/min O_2 through a facemask and secured with masking tape in a supine position. Fur was removed by application of a topical depilatory cream in the pericardial region, from the neck and upper chest area. They were then maintained at these homeostatic conditions with the use of a heating lamp to help regulate the body temperature.

2.2.6.4. Analysis

For heart function assessments, experiments were carried out in triplicate, data was expressed using the mean ± S.E.M. for each experimental condition. Data collected were analysed on Prism 5, using 1-way ANOVA with a Dunnett's post hoc analysis where appropriate, with differences considered significant if $P<0.05$.

2.2.7. Experimental autoimmune encephalomyelitis model.

2.2.7.1. Animals

Female C57BL/6J mice aged between 6 and 8 weeks at the start of the experiments were used because they are known to develop more consistent EAE clinical signs than males (Rahn et al., 2014). They were housed, bred and maintained in a temperature-regulated environment with a 12-hour light/dark cycle with unrestricted access to food and water in the Biological Procedure Unit at University of Strathclyde. Experimental protocols were carried out under UK Home Office Project license no PP70/8520 according to UK Home Office guidelines under the authority of U.K. Animals (Scientific Procedures) Act, 1986.

2.2.7.2. EAE induction

2.2.7.2.1. Solutions

For EAE induction, solutions were prepared as below.

- Phosphate-buffered saline (PBS) was made up in distilled water to achieve the following final concentration; NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM,

KH_2PO_4 1.76 mM, the pH was then adjusted to 7.4 using HCL and it was autoclaved prior to use and kept at room temperature.

- 100 µl of a Myelin Oligodendrocyte Glycoprotein (MOG^{35-55}) emulsion was made up for each mouse consisting of a 1:1 ratio of MOG^{35-55} peptide solution and complete Freund's adjuvant (CFA) further complemented with Mycobacterium tuberculosis H37Ra (3.65mg). Final concentration achieved for both MOG^{35-55} and CFA was 2 mg/ml.

2.2.7.2.2. Procedure

The components were emulsified prior to injecting by repeatedly passing through a sterile needle until a thick homogenised emulsion was achieved. Mice were immunised with a 100 µl dose of an emulsion consisting of MOG^{35-55} and CFA via two 50 µl subcutaneous injections of MOG/CFA emulsion in each hind flank. Each mouse was also injected intraperitoneally with pertussis toxin (100µl, 2 µg/ml) diluted in PBS on days 0 and 2. Following injections, animals were randomly allocated to a group, however, due to limited resources, it was not possible to further randomise the injections or to conduct blind assessment of the clinical scores.

2.2.7.2.3. EAE disease rating scale

The mice were weighed and monitored for any signs of disease daily throughout the duration of the experiment. Mice were scored based on a 0-5 disease severity rating (Keating et al., 2009; Barbour et al., 2014; table 2.2). Animals were observed, scored and weighted daily for 35 days, with mice scoring a 4 rating and higher were further monitored regularly for a 24h period in search for any signs of improvement and euthanized if no improvement was observed.

Table 2.2 - Scoring system for clinical symptoms in mice.

SCORE	CLINICAL SYMPTOMS
0	NO CLINICAL SYMPTOMS
0.5	SLIGHT LOSS OF TAIL TONE/ALTERED GAIT MOVEMENT
1.0	TAIL PARALYSIS
1.5	TAIL PARALYSIS + ALTERED GAIT MOVEMENT
2.0	HIND LIMB WEAKNESS
2.5	SEVERE HIND LIMB WEAKNESS
3.0	COMPLETE HIND LIMBS PARALYSIS
3.5	PARALYSIS IN ONE FORELIMB
4.0	FORELIMB PARALYSIS
4.5	MORIBUND
5.0	DEATH

2.2.7.2.4. Injections

Mice were housed in cages (6 per cage). At the onset of EAE clinical signs (usually around day 9 to day 11) animals were injected intraperitoneally once per day for 7 days with 3 different doses of the PAR2 agonist AC-264613 (1, 10 and 100 mg/kg) dissolved in 0.9% saline solution with 1% Tween 80. For comparison purposes and to rule out any direct psychopharmacological effects of the injections, a 4th group of EAE control mice was injected with a vehicle solution (0.9% saline solution with 1% Tween 80) only for the same period.

2.2.7.2.5. Dissection

35 days after the first immunisation, mice were sacrificed by CO_2 asphyxiation and washed with 70% ethanol prior to dissection. Blood was harvested by cardiac puncture after cutting through the diaphragm and exposure of the heart. A 5ml

perfusion of sterile PBS was slowly injected into the left ventricle for fixation purposes, following which the spleen was removed. Blood and spleens were placed on crushed iced. Immediately after decapitation, brains were removed, submerged in OCT mounting medium and placed on dry ice. Following dissection, animal tissues were then placed in a -80°C freezer until needed.

2.2.7.2.6. Spleen cell culture

Following dissections, spleens were placed in complete RPMI media consisting of RPMI-1640 supplemented with FCS (10 %) Pen/Strep (1%) and L-glutamine (1%) maintained at 37°C and filtered through nitex nylon mesh to form a cell suspension. Pellets were re-suspended in lysis buffer for 5 minutes before being washed twice with PBS, re-suspended in RPMI, counted and re-suspended again to an appropriate concentration. Spleen cells were then cultured at 2.3 $X10^6$ cells/ml in 24-well tissue culture plates in an incubator and stimulated for 48 hours with RPMI media alone or with RPMI media supplemented with $MOG^{35–55}$ (40 µg/ml).

2.2.7.2.7. Enzyme linked immunosorbent assays (ELISA)

2.2.7.2.7.1. Solutions

For ELISA, solutions were prepared as described below.

- ELISA wash buffer consisting of PBS, made up as previously described and supplemented with Tween 20 (0.05%).
- ELISA blocking solution consisting of PBS and complemented with FCS (10%).
- Horseradish peroxidase (HRP) solution was made up using HRP diluted at a 1:500 in PBS.
- ELISA stop solution consisting of sulphuric acid (1M) diluted in PBS.

All solutions were kept at room temperature.

2.2.7.2.7.2. Protocol

ELISA were conducted using 96 well ELISA plates previously coated overnight with a primary 'capture' antibody in PBS at 4 °C. The following day, capture liquid was removed and plates were blocked using 10% FCS in PBS for 2 hours. Plates were then washed in ELISA wash buffer and 50µl of standards and samples were added. Following 2 hours of incubation at room temperature, liquid from the samples/standards was removed and the plates were washed a minimum of 3 times with the wash buffer previously described. Secondary 'detection' antibody was added and incubated for a further hour at room temperature, following which the liquid was removed and the plate washed a further 3 times. HRP solution was then added to the plates and incubated for 30 min, the liquid was then removed and plates were washed a further 3 times in wash buffer. Tetramethyl benzidine (TMB) reagents A and B were mixed in equal volumes and 100µl was added to each well and incubated for 10 min. After a couple of minutes, when a definitive blue colour had sufficiently developed for recording by an ELISA reader (Epoch, BioTek Instruments Inc., USA.) the reaction was stopped by the addition of 100µl of ELISA stop solution, absorbance values were measured at 450nM. A standard curve was constructed from the absorbance values of the standards and cytokine concentrations were calculated.

2.2.7.2.8. Statistical analysis

Behavioural and weight variables were calculated for each mouse as percent of baseline, by dividing each measured variable by the baseline measure taken on day 0, and multiplying it by 100. EAE clinical score data analysis was performed using one and 2-way ANOVAs with repeated measures and post hoc analysis performed using Bonferroni test, with differences considered significant if $p<0.05$.

Chapter III. CHARACTERISING PAR2 ACTIVATORS IN CENTRAL NERVOUS SYSTEM PREPARATIONS.

3.1. Rationale.

The recent development of novel PAR2 activators has allowed further investigation of PAR2 function in a range of different cell and tissue types (Suen et al., 2014; Zhao et al., 2016). When the current project was initiated, there had been no reported studies examining the properties of these new generation of PAR2 activators in central nervous system preparations. Regardless, it had been suggested based on studies in other cell types that activation of PAR2 stimulates multiple G protein-dependent pathways (Hollenberg et al., 1999) and leads to a cascade of events ending with receptor internalisation and degradation in a way common to most GPCRs (Bushell et al., 2015). More specifically, activation of these pathways following PAR2 activation, leads to the generation of second messengers, including IP_3 and DAG, which further trigger activation of protein kinase C (PKC), MAPK and Ca^{2+} dependent signalling pathways (Lappano, 2011). The main objective of the study described in this chapter was to characterise and compare the effect of PAR2 activation on CNS cells using a range of activators. We aimed to study any similarities and differences in order to contribute to a more detailed understanding of proteinase activated receptor 2 in the CNS. In order to achieve that objective, we mainly focused on two key processes induced following PAR2 activation, stimulation of Ca^{2+} pathways and receptor internalisation. These mechanisms have previously been thoroughly described for other GPCRs (Bunnett et al., 1996; Hollenberg et al., 1999; Bunnett et al., 2000; Scott et al., 2003; Stalheim et al., 2005; Krishnan et al., 2012), however, there is a very limited amount of available information specific to PAR2 particularly in relation to activation of these key processes in the CNS.

3.2. Characterisation of PAR2 activation in CNS preparations.

The hippocampus is a sea horse shaped structure of the cerebral cortex with a highly distinct morphology and is involved in a large range of normal processes including memory and learning but also and pathological processes including the progression of various degenerative diseases, for these reasons it is one of the most studied and characterised region in the mammalian brain (Duvernoy, 2005). Furthermore, the structure of the hippocampus is well conserved between the rat and human, making the rat a good model for studying human hippocampal functioning in health and disease (McClelland et al., 1995; Burgess et al., 2002; Clark & Squire, 2013). We have previously highlighted reports that in the CNS PAR2 is expressed on various types of cells (Steinhoff et al., 2000) and consistent with this broad expression, it has been linked with numerous physiological and pathophysiological processes in response to both endogenous and exogenous activation (Ubl et al., 2002; Bushell, 2006; Gieseler et al., 2013; Zhao, 2015; Jairaman, 2015). Knowing that it is expressed in the hippocampus (Smith-Swintosky et al., 1997; Noorbakhsh et al., 2003; Bushell, 2006) and considering the numerous suggestions that it might play functional roles in several pathological conditions of the CNS (Polymeropoulos et al., 1997; Jellinger, 2003; Andrade-Gordon et al., 2005; Stefanis, 2012; Liu et al., 2014), it is of interest to investigate PAR2 function in CNS preparation such as primary cultures and organotypic slice cultures. Culturing primary hippocampal neurons is a very established experimental model to study cellular mechanisms and neuronal functions *in vitro* (Ray, 1993). Despite a few shortcomings including the absence of normal patterns induced by brain connectivity, they still offer many technical benefits and are far less complex than normal neural tissue and therefore allow observation and manipulation of living neuronal cells at a lower cost, permitting for instance to record a single cell at the time (Kaech, 2006). Finally, PAR2 has been associated with the development of various diseases with an inflammatory component, including in the

central nervous system, therefore improving the understanding of the signalling pathways activated via PAR2 in the CNS might prove crucial for the design of new therapeutics.

3.2.1. Method.

Ca^{2+} is an important messenger mediating several physiological functions. At the cellular level, two of the main ways to control its cytosolic level involves DAG and IP_3 pathways. Briefly, DAG increases Ca^{2+} influx through a family of non-selective cation channels, whereas IP_3 promotes the release of Ca^{2+} from intracellular stores (McFarlane, 2005). Considering the fact that the Ca^{2+} signalling have been extensively described for GPCRs we decided to focus on pathways leading to Ca^{2+} mobilisation to measure and compare the effects of different PAR2 activators in CNS preparations. Ca^{2+} mobilisation experiments constitute an established method to measure Ca^{2+} signals and concentration in cultured cells. Ratio-metric dyes such as fura-2 change their excitation in response to the binding of Ca^{2+} ions, allowing the concentration of intracellular Ca^{2+} to be determined from the ratio of fluorescence emission or excitation at distinct wavelengths (Takahashi et al., 1999; Barreto-Chang & Dolmetsch, 2009), these experiments have therefore been extensively used to study Ca^{2+} signalling induced by the application of various agonist and antagonist compounds in slice or cell models. In this study, fluorescence ratio imaging of the Ca^{2+} indicator fura-2 was used to record and quantify Ca^{2+}, indicating the release of calcium ions into the cytosol of rat primary hippocampal cells following PAR2 activation. More specifically, we started by recording baseline levels of fluorescence for CNS cells studied in the experiments, subsequently we perfused PAR2 activators (100µM or 100nM for trypsin), and recorded peaks corresponding to the highest intracellular Ca^{2+} concentrations observed for a given cell. Following a washout period, we applied trypsin (100nM) to confirm the presence of PAR2 receptors and their excitability, finally following another washout period, we applied K^+ (20mM) and took note of its effect on Ca^{2+} increase, knowing that its impact would be mediated

by the Na^+/K^+ pump (Skou, 1957), which is exclusively found on neurons and not on non-neuronal cells, therefore allowing a simple option for cellular differentiation. Additionally, in order to rule out any effect of the vehicle used to dilute GB110, GB88 and AC, we recorded the effect of 0.1 % DMSO on changes of fura-2 ratio and observed no effect, in line with previous studies suggesting that it might have an effect only above 0.2% (Morley & Whitfield, 1993), trypsin, SLIGRL and 2-f were diluted in external solution with here again no effect on the variable studied. Fluorescence was measured every second with an exposure of 500ms, for periods of 15 to 20 minutes, and data representing the average increase recorded for a cell type following the treatment received was recorded. Finally, every measurement obtained was normalised to the values recorded for the baseline periods in order to allow better comparisons between different treatments studied.

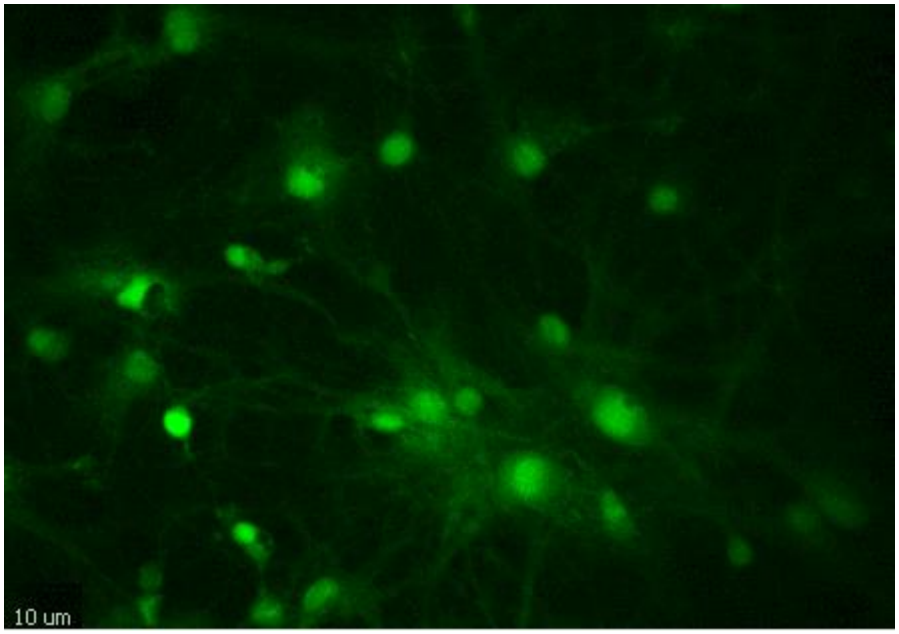


Figure 3.1 - Representative image of cells from a rat pup hippocampal primary culture. Cells after 10 DIV were incubated in fura-2 (0,33 µM) for 45min, images were visualised with fluorescence microscopy.

3.2.2. Results.

3.2.2.1. PAR2 activation via the endogenous activator trypsin increases Ca^{2+} concentration in hippocampal primary cultures.

Endogenously PAR2 is preferentially activated by the serine proteinase trypsin (Macfarlane et al., 2001) therefore, in order to get a broad picture of the impact of PAR2 in Ca^{2+} mobilisation experiments, we started by investigating it following activation via trypsin. Indeed, prior studies showed that PAR2 activation using trypsin induced a transient rise in Ca^{2+} concentration (Smith-Swintosky et al., 1997; Noorbakhsh et al., 2003; Bushell, 2006). Application of trypsin (100nM) resulted in a rapid increase in intracellular Ca^{2+} in both neurons (1.94 ± 0.15 increase; n=25, $p<0.001$ compared to baseline, figure 3.2) and non-neuronal cells (2.8 ± 0.23 increase; n=27, $p<0.001$ compared to baseline, figure 3.2), which was reversible upon washout.

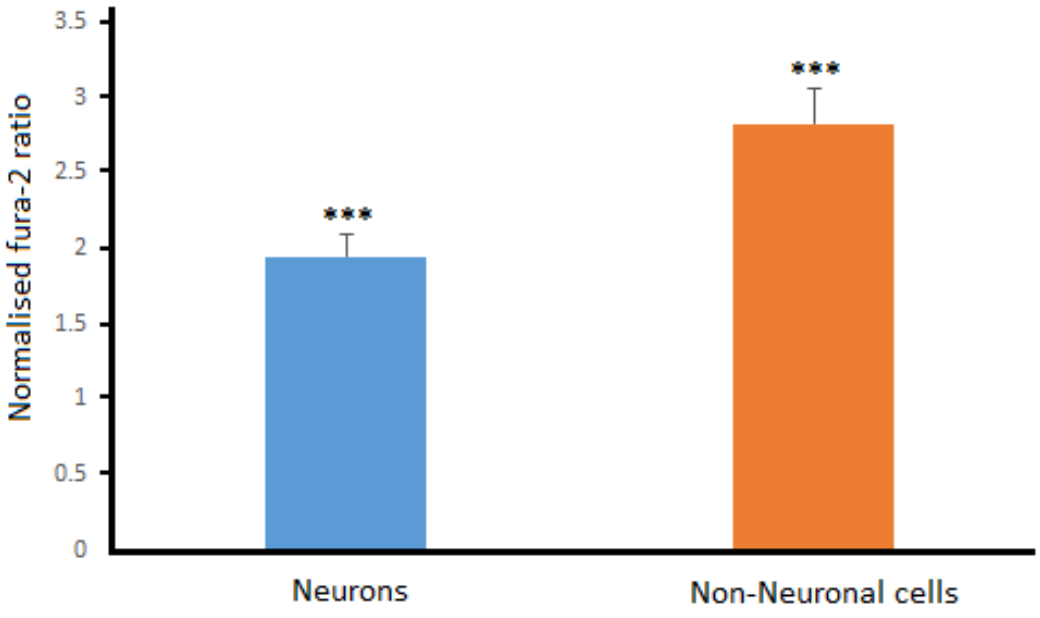


Figure 3.2 – Effect of trypsin application on intracellular Ca^{2+} in hippocampal cells from rat primary cultures. Bar chart summarising the effect of a trypsin (100nM) application on neurons and non-neuronal cells. All experiments were carried out on rat hippocampal cultures prepared from at least 3 separate animals with n≥25 for both cell types. Data were analysed using a Student's paired t-test and presented as mean ± S.E.M. ***= $p<0.0001$ compared to vehicle control baseline levels.

3.2.2.2. PAR2 activation via synthetic peptides increases Ca2+ concentration in hippocampal primary cultures.

Having confirmed a similar effect of trypsin in CNS preparation compared to other cell types with regards to Ca^{2+} mobilisation, we moved on to investigate the effect of PAR2 activation induced via non-endogenous activators. Keeping in mind the fact that trypsin selectivity for PAR2 have previously been challenged, SLIGRL-NH_2 (SLIGRL) was one of the first activators to be designed, it is an agonist peptide derived from the N-terminus tethered ligand of PAR2 with an increase selectivity to PAR2 (Santulli et al., 1995). Based on the concentrations used on previous work and in order to allow further comparisons we applied SLIGRL and all other PAR2 activators at 100µM (Bushell et al., 2006). Following SLIGRL application (100µM), we observed a significant increase in intracellular Ca^{2+} levels in neurons (1.83 ± 0.08 increase; n=38, $p<0.001$ compared to baseline, figure 3.3) and for non-neuronal cells (2.1 ± 0.12 increase; n=25, $p<0.001$ compared to baseline, figure 3.3 B). Having used SLIGRL, we then decided to study 2-furoyl-LIGRL-NH_2 (2-f), considered as the most potent non-enzyme agonist for PAR2 (Vergnolle et al., 1998). 2-f (100µM) application resulted in increases in intracellular Ca^{2+} in both neurons (3.1 ± 0.28 increase; n=28, $p<0.001$ compared to baseline) and non-neuronal cells (3.1 ± 0.31 increase; n=23, $p<0.001$, figure 3.3 B).

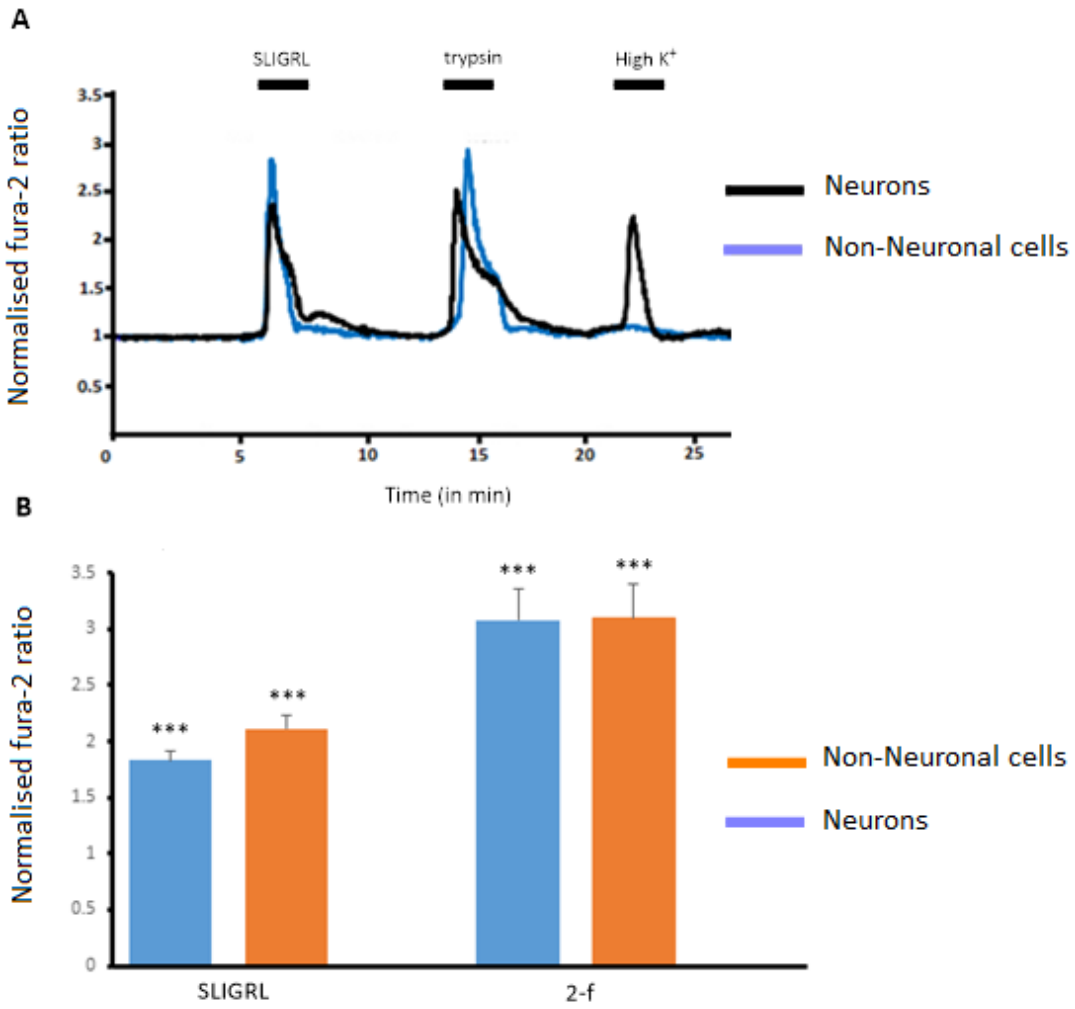


Figure 3.3 – Effect of PAR2 synthetic peptide application on intracellular Ca^{2+} in hippocampal cells from rat primary cultures. (A) Representative traces displaying the effect of PAR2 activation via SLIGRL (100µM) on intracellular Ca^{2+} in neurons and non-neuronal cells. (B) Bar chart summarising the effect of SLIGRL and 2-f applications (100µM) on neurons and non-neuronal cells. All experiments were carried out on rat hippocampal cultures prepared from at least 3 separate animals with n≥23 for both cell types. Data were analysed using a Student's paired t-test and presented as mean ± S.E.M. ***= p<0.0001 compared to vehicle control baseline levels.

3.2.2.3. PAR2 activation via non peptide and small molecule activators increases Ca2+ concentration in hippocampal primary cultures.

Having confirmed the effects of conventional PAR2 activators, we then investigated recently developed PAR2 activators. GB110 is a non-peptidic compound that has been shown to activate PAR2 *in vitro* and *in vivo* (Seitzberg, 2008). Application of GB110 (100µM) produced a rapid increase in intracellular Ca^{2+} in both neurons strong increase in Ca^{2+} concentration (2.4 ± 0.11 increase; n=35, p<0.001 compared to the baseline, figure 3.4) and non-neuronal cells (2.87 ± 0.16 increase; n=26, p<0.001). Furthermore, studies have demonstrated that AC-264613 is potent and specific PAR2 activator (Gardell et al., 2008) and also interestingly, it has been recently shown that it can be found in the brain after an Intraperitoneal injection (Abulkassim et al., 2016) suggesting that it can cross the blood brain barrier unlike other PAR2 activators described so far. In cells from hippocampal primary cultures, application of AC (100µM) resulted in a significant increase in intracellular Ca^{2+} in both neurons (1.84 ± 0.06 increase; n=28, p<0.001 compared to the baseline, figure 3.4) and non-neuronal cells (1.93 ± 0.06; n=31, p<0.001).

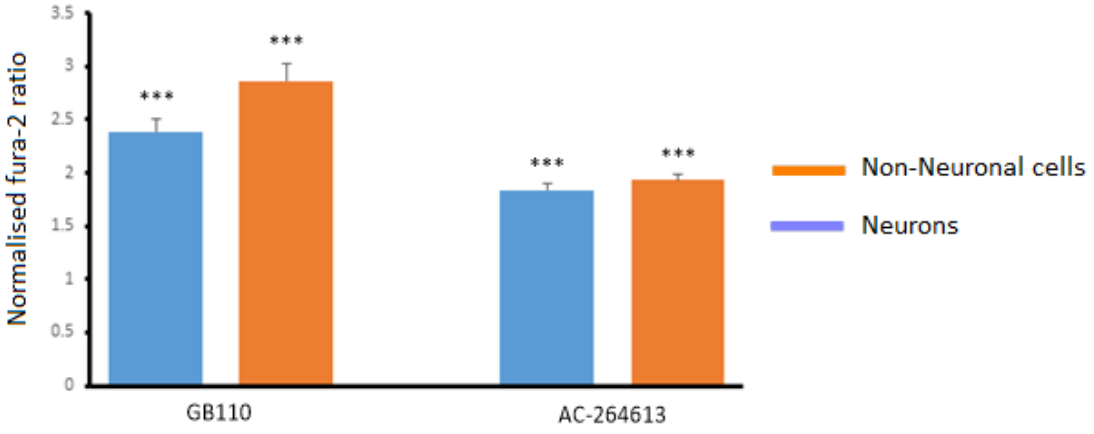
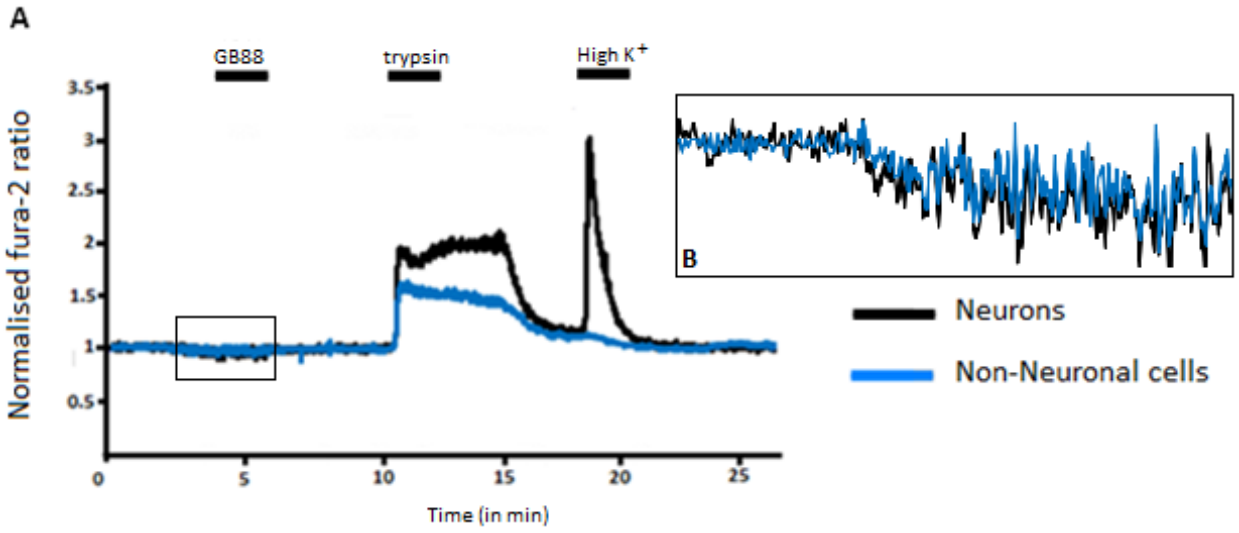


Figure 3.4 – Effect of PAR2 non-peptide and small molecules activators application on intracellular Ca^{2+} in hippocampal cells from rat primary cultures. Bar chart summarising the effect of GB110 and AC applications (100µM) on neurons and non-neuronal cells. All experiments were carried out on rat hippocampal cultures prepared from at least 3 separate animals with n≥26 for both cell types. Data were analysed using a Student's paired t-test and presented as mean ± S.E.M. ***= p<0.0001 compared to vehicle control baseline levels.

3.2.2.4. PAR2 activation via antagonist activators decreases Ca^{2+} concentration in hippocampal primary cultures.

Having initiated our study working with PAR2 agonists, we then moved on to studying the effects of PAR2 antagonists on intracellular Ca^{2+} levels. GB88 is the first non-peptidic antagonist developed and is based on the GB110 structure but is reported to confer PAR2 antagonism at low concentrations (Seitzberg et al., 2008). Application of GB88 (100µM) produced a significant decrease of intracellular Ca^{2+} levels in both neurons (0.90 ± 0.01 change; n=47, p<0.001 compared to the levels observed in the absence of drug) and non-neuronal cells (0.83 ± 0.02; n=33, p<0.001, figure 3.5 C). In addition, DM/8/36 and Jami 1066a were engineered to be non peptidic antagonists using some of the structural basis of GB88, therefore designed to work at low molecular concentrations with high selectivity for PAR2 (Jamieson, unpublished work). Application of DM/8/36 and Jami 1066a (both 100µM) also resulted in significant decreases in intracellular Ca^{2+} levels in both neurons (DM/8/36: 0.95 ± 0.01; n=17; Jami1066a: 0.94 ± 0.01; n=17, p<0.01 compared to baseline) and non-neuronal cells (respectively 0.9 ± 0.02; n=12, p<0.001 and 0.93 ± 0.02; n=14, p<0.01, figure 3.5 C).



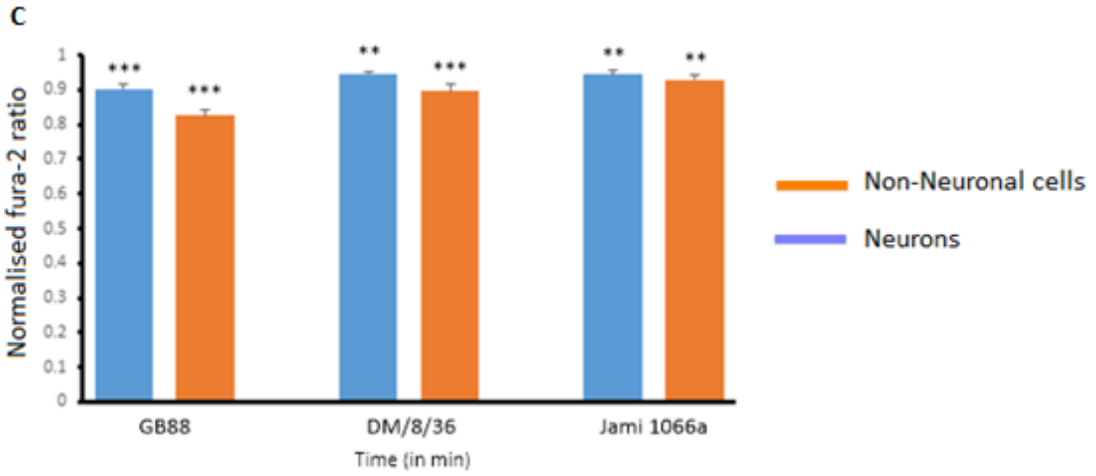


Figure 3.5 – Effect of PAR2 antagonists' application on intracellular Ca^{2+} in hippocampal cells from rat primary cultures. (A) Representative traces displaying the effect of PAR2 activation via GB88 (100µM) on intracellular Ca2+ in neurons and non-neuronal cells. (B) The boxed area in A was magnified (6x) to highlight the changes in Ca^{2+} concentrations. (C) Bar chart summarising the effect of GB88, DM/8/36 and of Jami 1066a applications (100µM) on neurons and non-neuronal cells. All experiments were carried out on rat hippocampal cultures prepared from at least 3 separate animals with n≥12 for both cell types. Data were analysed using a Student's paired t-test and presented as mean ± S.E.M. ***= p<0.0001 compared to vehicle control baseline levels.

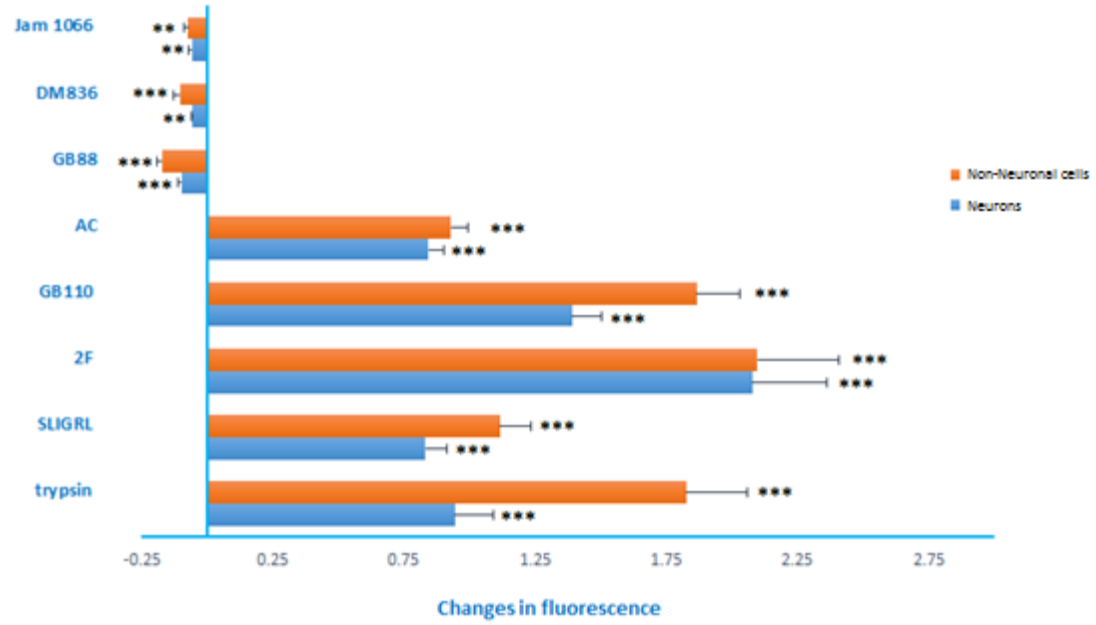


Figure 3.6 – Effect of PAR2 activation on intracellular Ca^{2+} in hippocampal cells from rat primary cultures. Bar chart summarising the effect of PAR2 activators on neurons and non-neuronal cells. All experiments were carried out on rat hippocampal cultures prepared from at least 3 separate animals with n≥12 for both cells. Data were analysed using a Student's paired t-test and presented as mean ± S.E.M. with **= p<0.001 and ***= p<0.0001 compared to vehicle control baseline levels.

3.3. Characterisation of PAR2 receptor internalisation.

3.3.1. Method.

Having previously established that the PAR2 agonists investigated elicited increases in intracellular Ca2+ while antagonists had no effect, we then decided to examine the effects of these compounds in another well documented GPCR mechanism, receptor internalisation. GPCR receptor internalisation into intracellular compartments of the cell is an important mechanism in regulating signal intensity, amplitude or duration (Lagerström & Schiöth, 2008). As such, it has been extensively detailed over recent years and the molecular mechanisms involved have now been well characterised (Conway et al., 1999; Drake et al., 2008; Kelly et al., 2009). More recently, studies investigating GPCRs receptor internalisation in a number of different cell types including HEK cell lines, have revealed a variety of patterns (Scarselli & Donaldson, 2009), suggesting that GPCR internalisation is mediated by multiple endocytic mechanisms. Experiments were carried out on tsA201 cells, a stable transformed human embryonic kidney (HEK293) modified cell line. These cells have been used in a variety of functional expression assays and have been reported to produce high levels of recombinant proteins (Baroudi & Chahine, 2000), expression of receptors in a cell line with a relatively simple spectrum of protein expression is a practical technique for the study of signal transduction pathways. Additionally, in order to investigate PAR2 receptor internalisation we had to ensure that the receptor would be expressed in tsA201 cells, that was achieved through transfection of PAR2 DNA, just like PAR1, PAR4 and P2Y1 receptors used for control experiments, were transfected using their human sequences. Transfection is the process of inserting exogenous genetic material, such as DNA, into mammalian cell lines to produce genetically modified cells, that insertion of DNA into cells enables the study of specific proteins expression produced using the cells own machinery (Kim, 2010; figure 3.7).

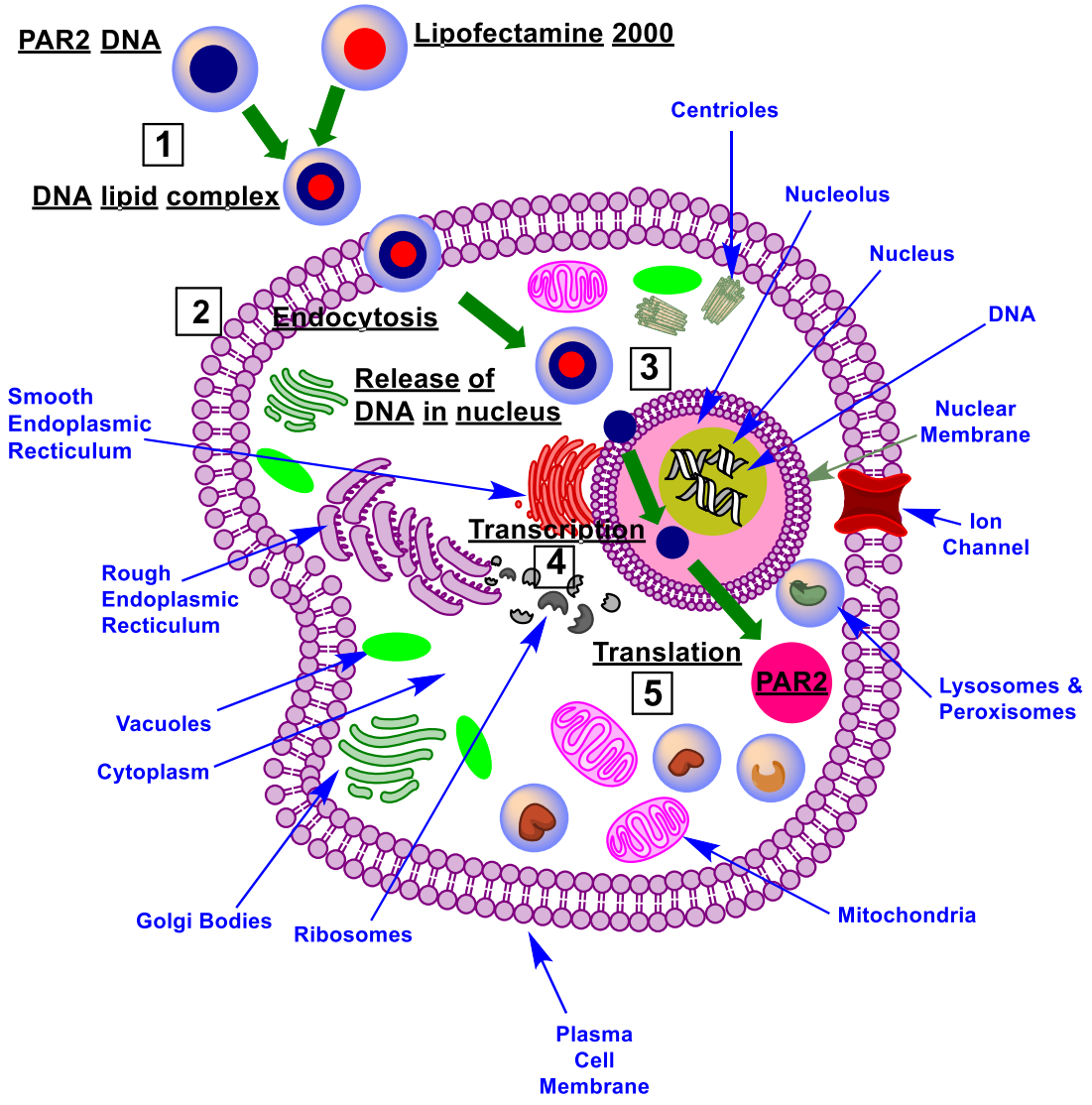


Figure 3.7 - **Schematic representation of the transfection of PAR2 process in tsA-201.** PAR2 DNA plasmid and Lipofectamine 2000 transfection reagent are combined to form a DNA lipid complex (1). This complex gets internalised into the cell via endocytosis (2), translocate to the nuclear membrane where it releases PAR2 DNA into the nucleus (3), where it is then transcripted (4) so that the cell starts producing PAR2 (5).

While splitting the cells, the number of passages was kept under 50 considering the fact that cells might not responding to transfection the same way after repeated passages (Geraghty et al., 2014). In addition to PAR2 DNA and in order to obtain satisfactory levels of gene transfer by transfection, we added the lipid reagent Lipofectamine 2000 to the plasmid DNA. Lipofectamine 2000 is used in transfection experiments to mediate efficient delivery into the cell's nucleus. We transiently expressed GFP, YFP and CFP-tagged receptors to assess their distributions in unstimulated cells, and in order to confirm transfection efficiency we examined the fluorescence probes binding before any agonist treatment. Once cells were transfected, PAR2 was activated using a range of activators (100µM) applied in the transfection media for 45min prior to imaging, receptor internalisation was finally assessed using confocal imaging and quantified using imaging software imageJ with data expressed as a ratio representing the percentage of total fluorescence expression expressed in the centre of the cell versus the fluorescence expressed in the whole cell.

3.3.2. Results.

3.3.2.1. PAR2 activation causes receptor internalisation.

We recorded the levels of fluorescence in the PAR2 transfected cells and compared the localisation of the receptors with or without PAR2 activation (figure 3.8). Under basal conditions, PAR2 receptors were located predominantly at the plasma membrane of the tsa-201 cells, however we observed a relatively small degree of constitutive internalisation of the receptor (figure 3.8 A), after ligand binding we observed a receptor translocation from the membrane to the nucleus of the cell thus confirming that it was being internalised (figure 3.8 B).

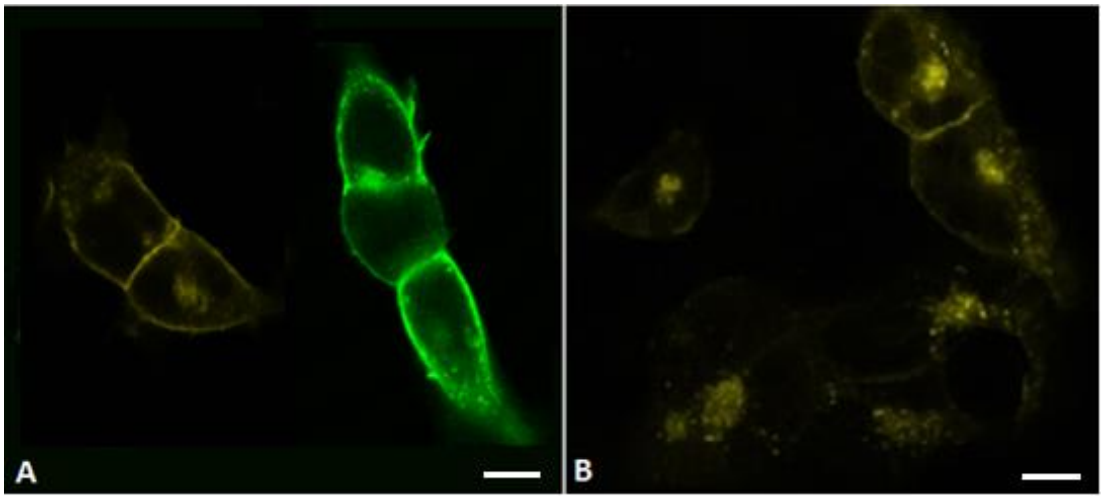


Figure 3.8 - **Typical representation PAR2 internalisation process in tsA-201.** Prior to stimulation PAR2 is expressed on the plasma membrane of the cell (A) once activated it gets internalised and translocate toward the nucleus (B). Image were visualised with confocal microscopy, scale bar; 25 µm (A) and 35 µm (B).

Using this method, we were able to monitor significant receptor translocation following trypsin (41.6 ± 3.87 internalisation ratio; n=6, p<0.001 compared to control conditions, figure 3.9), SLIGRL (39.3 ± 1.5 internalisation ratio; n=7, p<0.001, figure 3.9), and AC (46.7 ± 2.47 internalisation ratio; n=8, p<0.001, figure 3.9). Moreover, that observation was similar following an application of GB88 (38.7 ± 2.98 internalisation ratio; n=8, p<0.001), a proposed antagonist, suggesting that it too can induce receptor internalisation.

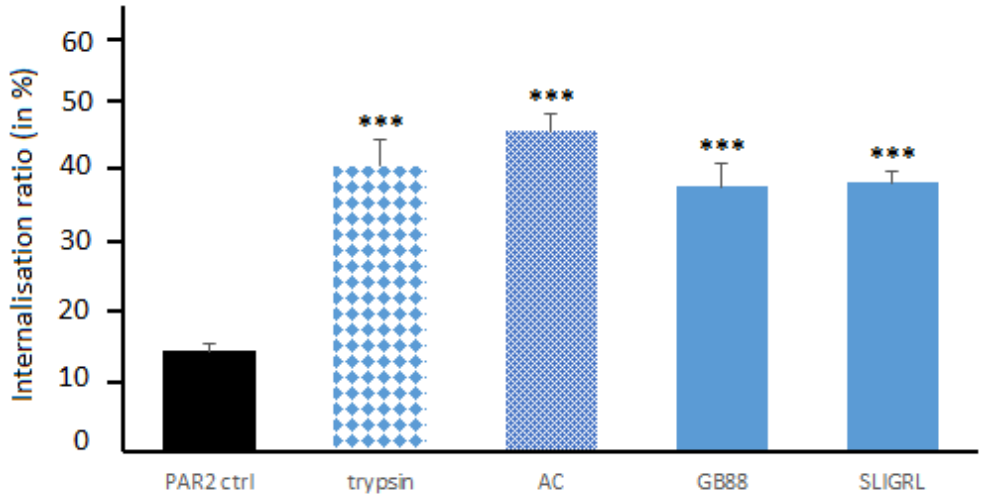


Figure 3.9 – Effect of PAR2 activation on receptor internalisation in PAR2 transfected tsA-201 cells. All 4 PAR2 activators tested (100µM and 100nM for trypsin) induced receptor internalisation in PAR2 transfected cells following a 45min incubation, when compared to cells where no activator was applied. All experiments were carried out on tsA-201 transfected cells prepared from different cultures with n≥6. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with ***= p<0.001, compared to negative control conditions.

3.3.2.2. Controls.

3.3.2.2.1. AC application doesn't induce PAR1 or PAR4 receptor internalisation.

In order to ensure the pertinence of our studies as well as the selectivity of the PAR2 activators used, we designed a few control experiments. We started by transfecting tsA201 cells with P2Y1 (another GPRC), PAR1 and PAR4 DNA, to which we subsequently applied PAR2 activators to see if they would induce internalisation, knowing that AC-264613 has been described as having the best selectivity and potency to activate PAR2 (Suen et al., 2014), we used only AC and trypsin for all control experiments. As expected using AC (at 100µM, for a 45 min incubation) the predominantly plasma membrane location of PAR1, PAR4 and P2Y1 transfected tsA-201 cells didn't change (14.1 ± 2 internalisation ratio; n=7, for PAR1, figure 3.10 A, 12.1 ± 1.28 internalisation ratio; n=6, for PAR4, figure 3.10 B and 13.5 ± 1.23 internalisation ratio; n=6, for P2Y1, data not shown) compared to negative control conditions where no activator was applied thereby suggesting a very specific effect of PAR2 activator AC. For trypsin however, which selectivity has previously been contested (Hollenberg et al., 2008), we observed a moderate degree of internalisation in PAR1 (21.4 ± 3.1 internalisation ratio; n=4, $p<0.05$, compared to vehicle figure 3.10 A), but not in PAR4 transfected cells (16.6 ± 3.4 internalisation ratio; n=5, figure 3.10 B). Additionally, specific PAR1 activator TFLLR (45.7 ± 3.08 internalisation ratio; n=6, $p<0.001$ compared to vehicle) and PAR4 activator AYPGKF (37.6 ± 5.53 internalisation ratio; n=4, $p<0.001$) did induce significant internalisation in PAR1 and PAR4 tsA-201 cells respectively (figures 3.10 A and B).

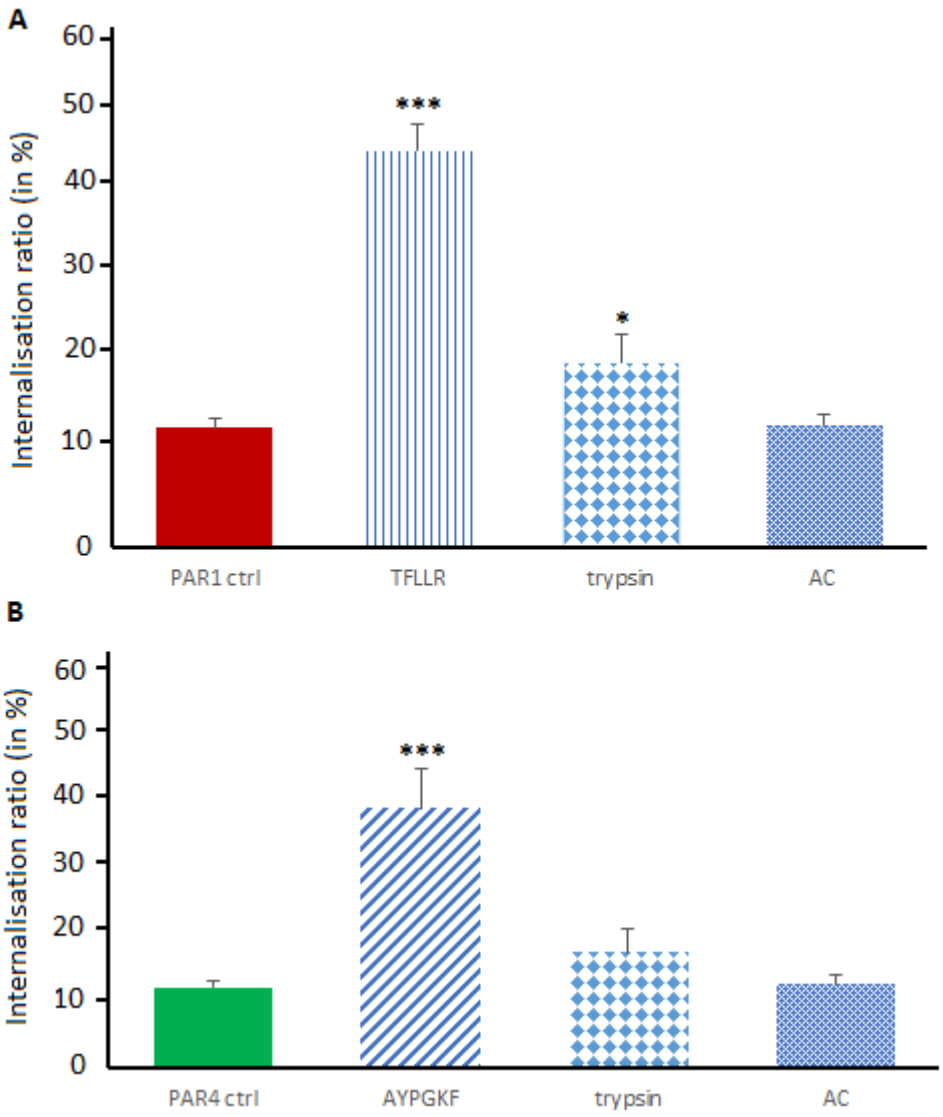


Figure 3.10 – Effect of PAR2 activator AC on receptor internalisation on PAR1 and PAR4 transfected tsA-201 cells. While trypsin induces some degree of receptor internalisation in PAR1 transfected cells, AC doesn't cause receptor internalisation in PAR1 (A) and PAR4 (B) when compared to cells where no activator has been applied. In comparison PAR1 activator TFLLR (100µM) induced significant receptor internalisation in PAR1 transfected cells while PAR4 activator AYPGKF (100µM) induced significant receptor internalisation in PAR4 transfected cells compared to negative controls. All experiments were carried out on tsA-201 transfected cells prepared from different cultures with n≥4 cells. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with *= $p<0.05$ and ***= $p<0.001$ compared to negative control conditions.

3.3.2.2.2. PAR1 and PAR4 activators don't induce PAR2 receptor internalisation.

Additionally, we used PAR1 (TFLLR-NH_2) and PAR4 (AYPGKF-NH_2) activators in PAR2 transfected tsA-201 cells to compare the receptor localisation and monitor any changes induced by these activators, similar experiments were conducted in our lab confirming that incubated P2Y12 transfected cells with their specific activator (ATP) resulted in receptor internalisation (data not shown). We observed no significant differences regarding receptor localisation after TFLLR (100µM) (11.6 ± 1.02 internalisation ratio; n=4 compared to vehicle, figure 3.11) and AYPGKF (100µM) (13.2 ± 0.9 internalisation ratio; n=4, figure 3.11) applications compared to negative conditions (14.6 ± 1.14 internalisation ratio; n=9, figure 3.11).

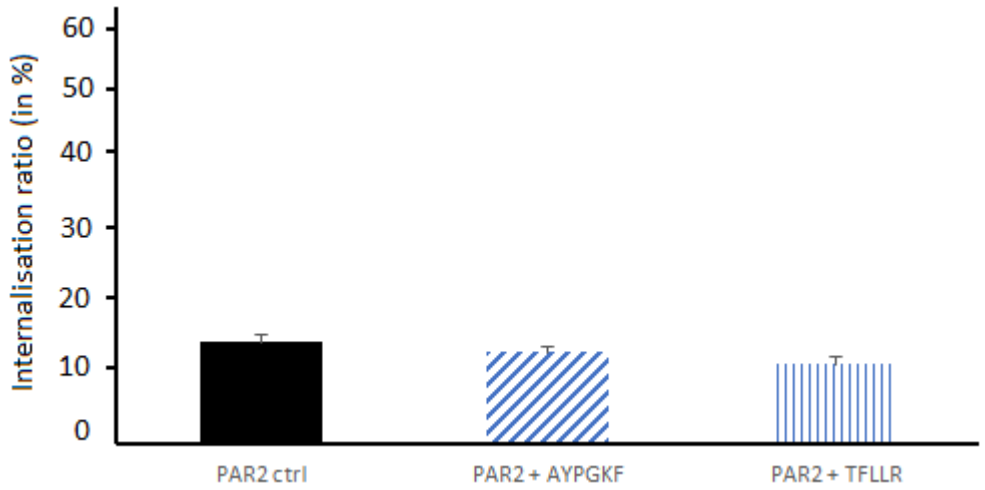


Figure 3.11 – Effect of PAR1 and PAR4 activators on receptor internalisation on PAR2 transfected tsA-201 cells. Bar chart showing that AYPGKF (100µM) and TFLLR (100µM) don't induce receptor internalisation in PAR2 transfected cells when compared to negative control where no activators was applied. All experiments were carried out on tsA-201 transfected cells prepared from different cultures with n≥4 cells. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. compared to negative control conditions.

3.3.2.2.3. *PAR1, PAR2, PAR4 and P2Y transfected cell show similar receptor localisation in the absence of activation.*

Finally, we compared plasma membrane expression basic levels of fluorescence for PAR2 transfected cells (14.6 ± 1.14 internalisation ratio; n=9) to PAR1 (11.8 ± 1.11 internalisation ratio; n=8), PAR4 (11.7 ± 0.74 internalisation ratio; n=5) and $P2Y_1$ (12.3 ± 0.79 internalisation ratio; n=5) transfected cells, observing no significant differences between them (figure 3.12).

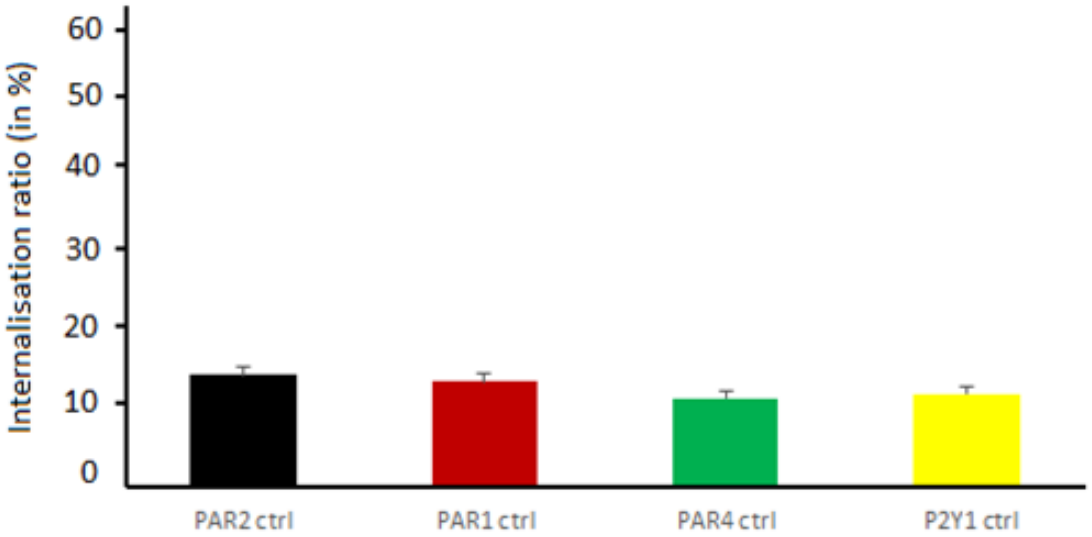


Figure 3.12 – Effect of PAR1, PAR2, PAR4 and P2Y1 transfected tsA-201 cells on basal fluorescence levels in the absence of activation. No differences were observed looking at PAR1, PAR2, PAR4 and P2Y1 transfected cells with regards to receptor localisation in basal conditions, before the application of agonists. All experiments were carried out on tsA-201 transfected cells prepared from different cultures with n≥4. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. compared to PAR2 control conditions.

3.4. Discussion.

Unlike most GPCR that are activated by direct binding of extracellular ligands, PAR2 is activated by N-terminal cleavage by proteolytic enzymes (Gainetdinov & Lefkowitz, 2004). That unusual activation mechanism (Nystedt et al., 1994) and its implication together with early indications of a suspected role in pathology explain why PAR2 have recently attracted more attention. Whilst studies aimed at investigating PAR2 have mostly focused outside of the CNS, PAR2 has been linked to several CNS diseases (Polymeropoulos et al., 1997; Jellinger, 2003; Andrade-Gordon et al., 2005). Hence it is important to examine PAR2 function in CNS cells and circuits to determine whether it is comparable in CNS preparation to other cell types. Moreover, having access to novel PAR2 activators, we were able to verify their properties in experiments designed to allow thorough comparisons in similar conditions. The results presented in this chapter describe the characterisation of PAR2 activators and this study demonstrate for the first time that PAR2 activation via AC-264613 and GB110 induces stimulation of Ca^{2+} pathways in rat primary hippocampal cultures while GB88, JAMI1066a and DM/8/36 seem to have no impact on this signalling pathway but still induce receptor internalisation. Furthermore, this work hints for the first time at the fact that GB88 might be a biased agonist rather than an antagonist as it was previously described.

3.4.1. PAR2 in neuronal cells: Ca^{2+} pathways as an insight into underlying mechanisms.

Previous studies have described that PAR2 activation initiates downstream pathways through coupling to G-proteins $G\alpha_q$, $G\alpha_i$, $G\alpha_s$, and $G\alpha_{12/13}$, as well as via β-arrestins 1 and 2 pathways (Soh et al., 2010; Yau et al., 2013; Kagota et al., 2016). Coupling of activated PAR2 with $G\alpha_q$ stimulates Ca^{2+} release through the PLC/IP_3 signalling pathway (Bushell, 2006) and causes the increase in intracellular Ca^{2+} we observed in CNS cells. Although it has been established that concomitantly to the activation of

this pathway, other signalling processes are initiated by PAR2 activation such as mitogen-activated protein kinase (MAPK), including phosphorylation of extracellular signal regulated kinases, cAMP, Rho kinase or nuclear factor κB pathways (Yau et al., 2013), we specifically intended to investigate $G\alpha_q$ /PAR2 coupling only at that stage. In this study, we demonstrate that PAR2 activation in hippocampal cells induced a rise in Ca^{2+} concentrations in an analogous manner to what was observed in previous studies on other cells types (Macfarlane et al., 2001; Kaufmann et al., 2011; Hennessey et al., 2015). In detail, we observed that trypsin application leads to an increase in intracellular Ca^{2+} concentration in neurons and non-neuronal cells from rat primary hippocampal cultures, furthermore, that increase was on average greater in non-neuronal cells compared to neurons as previously observed (Bushell, 2006). However, knowing that as we mentioned before, trypsin has been shown to also activate other PARs (Cottrell, 2003), it is reasonable to think that the results may be only partially due to PAR2 activation. Therefore, in order to overcome this issue and refine the experimental approach, activators displaying a greater PAR2 selectivity were also investigated. SLIGRL-NH_2 was engineered so that it would possess high levels of selectivity for PAR2, thus avoiding "off targets" effects seen with trypsin (Nystedt et al., 1994; al-Ani et al., 1995). Applied at 100µM a concentration that evokes a maximal PAR2 response in expression systems (Nystedt et al., 1995), but also in investigating PAR2 related Ca^{2+} release in CNS preparations (Bushell, 2006), SLIGRL-NH_2 elicited an immediate increase of intracellular Ca^{2+} concentration on both non-neuronal cells and neurons comparable with what we observed using trypsin. The response observed following SLIGRL application was comparable in shape to what was reported previously. For comparison purpose, the same concentration used for SLIGRL was applied for the other PAR2 activators used in Ca^{2+} mobilisation experiments. As previous works have shown, although SLIGRL might be more specific to PAR2 (Santulli et al., 1995; Suen et al., 2014), its potency had nonetheless been questioned over the last years, as it appears that it requires high concentrations to induce PAR2 activation, at which concentration it might become toxic (Vergnolle et al., 2009). Research has shown that substitution of the N-terminal serine residue by

a furoyl group in PAR2 resulted in a marked increase of agonistic properties (Ferrell et al., 2003; Kawabata et al., 2004). On this basis, 2-furoyl-LIGRL-NH_2 (2-f) was developed, addressing the concerns over the limited potency of SLIGRL; it is considered to be between 10 and 100 times more potent, possibly due to its resistance to amino-peptidase, the degrading enzyme (Kanke et al., 2005). Thus, the use of 2-f offered experimental advantages and constituted a valuable alternative to SLIGRL. Accordingly following 2-f application, we recorded a more robust increase in Ca^{2+} concentration in both neurons and non-neuronal cells in line with the findings previously reported (Hollenberg et al., 2008; Kanke et al., 2009). Considering the degree of specificity of that drug, we can consider that the 2-f related increase of Ca^{2+} concentration is an accurate image of PAR2 activation in primary hippocampal cells. Having confirmed the effect of previously documented PAR2 activators in CNS cells, we then moved on to study newly developed activating compounds. Recently developed GB110 is a non-peptidic organic compound with a low molecular weight designed so that it would be very specific to PAR2, therefore it was expected to induce PAR2 activation at low or limited concentrations (Suen et al., 2012). Previous studies have reported that it is as potent as 2-furoyl-LIGRLO-NH_2 in activating Ca^{2+} pathways *in vitro* (Barry et al., 2010). In this study, the significant increase in intracellular Ca^{2+} concentration induced by GB110 was lower than what we observed using 2-f, an observation that differs from that described previously albeit in different cell types (Barry et al., 2010; Suen et al., 2012). Also although a good understanding of PAR2 and its related Ca^{2+} mobilisation can be achieved using traditional activators *in vitro*, it is confirmed that these compounds are unable to cross the blood brain barrier therefore making it impossible to progress onto *in vivo* experiments and reducing their interest as a possible therapeutic tool (Barry et al., 2010). AC-264613 was developed to overcome this major downfall, it was designed to be very specific and is said to be active even at low concentrations (Gardell et al., 2008). Data presented in this chapter show for the first time in CNS preparations that AC-264613 (100µM) generates increases in intracellular Ca^{2+} concentration in both neurons and non-neuronal cells. The increase observed following AC application was however not

as substantial as what we recorded for GB110 and 2-f. At this stage it is difficult to interpret these results, however we think that it could be an evidence that AC mediated activation of PAR2 is more specific. In the experiments we ran it could mean that AC induces Ca^{2+} release only via PAR2 while other activators can have "off-targets" effects partially accounting for the results recorded. Investigating GB88, JAMI1066a and DM/8/36 we observed no increase of neuronal or non-neuronal Ca^{2+} concentration, contrariwise application of these compounds designed to produce antagonistic effects resulted in significant decreases compared to the baseline levels. However, following the small decrease in cellular Ca^{2+} concentration observed we demonstrated that the receptor wasn't being inactivated as it would subsequently still be excitable via trypsin or GB110.

3.4.2. PAR2 receptor internalisation studies introduce new elements toward a better characterisation of PAR2 functioning.

To further functionally characterise PAR2 using the set of activators we had available for this research, we next sought to determine how they will initiate receptor internalisation, moreover given that some of the activators we studied did not elicit increases in Ca^{2+}, but have been reported to activate other pathways, we investigated receptor activation via its internalisation. Therefore, we examined internalisation of PAR2 in transfected tsA-201 cells following a 45 min time incubation period, which has been described as corresponding to the time needed for this receptor to be activated in similar cell types (Dery et al., 1999; Soh et al., 2010). Within 45 min of PAR2 stimulation, we observed a clear receptor translocation from the membrane to the cytoplasm corresponding to receptor internalisation. A few key mechanisms have been described for GPCR internalisation and it has been established that there is a dynamic equilibrium between receptor synthesis, transport to the membrane, endocytosis, recycling and degradation (Drake, 2006). Endocytosis is the main process allowing cells to internalise their receptors following activation, it serves

different purposes including down-regulation of activated receptors and can involve a variety of pathways including phagocytosis, macropinocytosis, raft-mediated uptake caveolae-mediated uptake and clathrin mediated endocytosis which have been thoroughly characterised (Pearse, 1976). Previous studies have demonstrated that PAR2 is internalised via clathrin mediated endocytosis (Defea et al., 2000), which is the best understood endocytosis pathway and is mediated by the molecule clathrin. This protein induces the formation of a coated pit on the inner surface of the membrane, which then form a coated vesicle in the cytoplasm, by doing so it allows a small volume of fluid from outside the cell to be taken inside (Pearse, 1976). In the case of PAR2, β-arrestin1 and β-arrestin2 two proteins ubiquitously expressed and associated with most activated GPCRs (Gainetdinov & Lefkowitz, 2004; DeWire et al., 2007; Drake et al., 2008)), have been identified as essential to facilitate receptor uncoupling from G-proteins. Activated PAR2 is rapidly recruited to clathrin-coated pits, causing GRKs phosphorylation of the C-terminus which leads to β-arrestin recruitment and translocation to the plasma membrane where they bind to PAR2, thus resulting in desensitisation and internalisation through clathrin-coated pits (DeFea et al., 2000). Because PAR2 activation generates a tethered ligand that cannot be inactivated, the mechanisms that contribute to terminate PAR2 signalling are critical. Studies have described that, in contrast to most GPCRs, internalisation and lysosomal sorting of activated PAR2 is required for termination of signalling, this is achieved through a phosphorylation (Ricks & Trejo, 2009) and arrestin dependent mechanism resulting in PAR2 being marked for rapid lysosomal degradation (Stalheim et al., 2005) thereby promoting recovery only via recycling (Defea et al., 2000; Soh et al., 2010; Yau et al., 2013). Based on the results from our previous Ca^{2+} mobilisation experiments as well as from previous studies (Seitzberg, 2008), we have hypothesised that agonists trypsin, SLIGRL, 2-f, GB110 and AC would activate PAR2 and subsequently induce receptor internalisation while GB88 and other PAR2 antagonists wouldn't induce internalisation since they had been described to deactivate PAR2, therefore preventing the activation of any of its related pathways. Nevertheless, we observed similar receptor internalisation patterns using

proposed antagonist GB88 as we did with other established PAR2 agonists. These observations can be of crucial importance for future PAR2 studies as they suggest, first than PAR2 activation doesn't induce Ca^{2+} pathways, moreover they infer that GB88 and possibly JAMI1066a and DM/8/36 are not antagonists but biased agonists. The recent description of partial agonism, full agonism and inverse agonism (Simmons, 2005; Urban et al., 2006) exposed new angles for drug discovery, and contradicted preeminent models whereby a receptor could only adopt two states either fully activated or fully inactivated, thus it is now established that receptors can endorse distinct portions of their full signalling repertoire (Kenakin, 2011; Reiteret al., 2012; Violin et al., 2014). GB88 as we previously reported, doesn't activate Ca^{2+} pathways but still induce receptor internalisation and also been described as activating ERK1/2 (Suen et al., 2014), can therefore be described as biased towards specific PAR2 pathways compared to more classical PAR2 ligands who will activate all pathways.

3.4.3. Limitations, improvements and future experiments.

The idea behind the work presented in this chapter was to achieve a better understanding of PAR2 function in the CNS so that it might, in the future allow the design of more refined experiments toward the development of new pharmacological therapies. With that in mind, we considered essential to work with models mimicking as closely as possible what is known of human *in vivo* physiology. Nonetheless in order to obtain a better level of understanding we were required to start our investigations in cultured cells from primary cultures and cell lines, notwithstanding their disadvantages. It is not clear how the function of these cells relates to that of other cells, healthy or diseased *in vivo*, furthermore, in order to grow them efficiently, they also require the use of complex medium whose properties aren't identical to what is found in living organisms, additionally, we had to transfect and overexpress PAR2 in cells where it wouldn't normally be expressed at these levels. Therefore, even if we were trying to observe a specific mechanism, we can't

be sure that the cellular context had no influence on our results. Nevertheless, primary cells are morphologically similar to the parent tissue and thus considered by many researchers to be physiologically very close to *in vivo* cells, while cell lines have been used for decades, are very well understood and also allow easy comparisons between researches held in different labs at different times. From a purely experimental point of view, a few additional procedures could have been further explored to complement this study. For instance, differences in agonist potency were observed between the different compounds and even in neurons and non-neuronal cells for some drugs, which would possibly be something to investigate further considering that studies have suggested that cells types might play different roles in activating PAR2, with non-neuronal cells, including astrocytes being a central mediator (Greenwood & Bushell, 2010; Gan et al., 2011). From previous studies, the timing of PAR2 stimulation appears to be a crucial factor in cell activation which can also impact subsequent cellular responses, in our internalisation studies, other experimental settings would have allowed us to extend the levels of details we have looked at, for example studying potential differences with regards to the time needed for receptor internalisation, or time needed to recycle the receptor following activation. Studies have shown that some of the pathways activated by PAR2 are active for a limited period of time, in the case of ERK1/2 the activation phase has been estimated to be at its peak 30 to 60 min following its stimulation (Eishingdrelo & Kongsamut, 2013). Furthermore, we looked at a single aspect of PAR2 activation in the internalisation studies, it would have been interesting to also study other pathways and could have brought additional expertise for the next experiments we planned. Indeed, it would be interesting to study in more details the role of PAR2 in activating pathways linked to neuroprotection the main objective of this work, such as ERK1/2 (Macfarlane et al., 2005; Nikolakopoulou et al., 2016) and determine if the PAR2 activators we have identified as biased agonists activate these pathways in CNS preparations. Moreover, considering the fact that this chapter describes PAR2 biased properties for the first time in the CNS and knowing that various studies have highlighted potential protective and detrimental pathways initiated following PAR2

activation, it might be useful, once identified, to be able to specifically activate those pathways involved in neuroprotection.

3.4.4. Conclusions.

Proteinase activated receptor 2 is an interesting receptor due to its unique activation mechanism, moreover it has been shown to be targeted by multiple proteolytic enzymes involved in many biological functions (Yau, Liu, & Fairlie, 2013; Boitano et al., 2015; Kagota et al., 2016). Considering the numerous potential roles attributed to PAR2 in inflammatory, gastrointestinal, metabolic and neurological disorders (Kaufmann et al., 2011; Zhao et al., 2014; Mrozkova et al., 2016; Kagota et al., 2016), the recent development of new generations of PAR2 activators can possibly contribute to the development of new pharmacological therapies for the treatment of a large range of diseases. The aim of these first experiments was to confirm *in vitro* data previously obtained with classical PAR2 activators and ensure that the effects of activating compounds we will use for further studies are consistent with the data available for other PAR2 activators. Our initial findings using calcium imaging experiments involving a range of different PAR2 activators, peptide based agonist, small molecule agonist and biased agonist have highlighted PAR2 mediated Ca^{2+} responses in hippocampal neurons and non-neuronal cells. Results for trypsin, SLIGRL and 2-f are in line with data available in the literature (Bushell, 2006; Barry et al., 2010, Suen et al., 2012). Moreover, we investigated agonists GB110 and a new small molecule PAR2 activator AC-264613 for the first time in CNS preparations and this chapter demonstrates that PAR2 can be activated in CNS cells via these new activating compounds. Additionaly, we applied 3 proposed PAR2 antagonists GB88, JAMI1066a and DM/8/36 which induced no Ca^{2+} mobilisation. Furthermore, data presented in this chapter illustrate PAR2 internalisation in tsA-201 cells using different activators. We establish the specificity of PAR2 activators in line with data available in the literature (Yau et al., 2013). Interestingly and in opposition with previous studies (Barry et al., 2010), using GB88 we observed what can be understood

as PAR2 biased agonism for the first time, with the activators inducing receptor internalisation while, as we previously highlighted, it doesn't activate PAR2 related Ca^{2+} pathways.

Taken together, data presented in this chapter confirm that manipulation and investigation of PAR2 in rodent hippocampal cultures produces outcomes consistent with what was previously described for other cell types, establishing them as a good platform for further experimental designs, furthermore this chapter introduces important elements toward a better understanding of the underlying mechanisms of PAR2 activation and receptor internalisation. Finally, these studies provide evidences that it is possible to selectively target some of the PAR2 signalling pathways, therefore it will be interesting to use these biased agonists able to inhibit different pathways to identify and target protective and detrimental PAR2 signalling pathways more effectively.

Chapter IV. DETERMINING THE NEUROPROTECTIVE PROPERTIES OF PAR2 ACTIVATION AGAINST KAINATE-INDUCED NEUROTOXICITY IN HIPPOCAMPAL ORGANOTYPIC SLICE CULTURES.

4.1. Introduction.

4.1.1. Rationale.

Over the last decades, different approaches have been tested to maintain explants of nervous tissue in culture. The hippocampus is deeply embedded in the brain and is connected to several other brain regions in both hemispheres, it receives inhibitory fibres from the contralateral side via the associational/commissural fibres, as well as modulatory input from the hypothalamus, and the septal nucleus median and nucleus of the diagonal band of Broca by the fimbria/fornix (Noraberg et al., 2005). It is one of the best characterised region of the brain and has been linked to key processes including learning and memory, thereby justifying the interest in that structure. Hippocampal organotypic slice cultures (OSC) were first successfully implemented in the early 80's (Gähwiler, 1981) and slightly modified later for culture on inserts (Stoppini et al., 1991). They represent a widely accepted experimental platform to model the CNS and have been increasingly used in molecular biology, electrophysiology, immuno-histochemical studies and biological imaging (Simoni & Yu, 2006). Kainic acid (KA) is a natural marine acid originally isolated from seaweed in 1953 (Moloney, 1998). It is a potent neuro-excitatory amino acid that acts by agonising kainate receptors, a subtype glutamate receptor, the principal excitatory neurotransmitter in the central nervous system. It also non-specifically binds to GluR2 subunit on AMPA receptors allowing Ca^{2+} influx inside the cells (Zhu et al., 2011). KA administration has been shown to increase production of reactive oxygen

species, to promote mitochondrial dysfunction, and cause apoptosis in neurons in many regions of the brain, particularly in the CA1 and CA3 regions of the hippocampus (Wang, 2010), it has therefore been extensively used to study the neuroprotective effects of various agents in slices or cells models of neurodegeneration. For these reasons, having previously characterised the properties of novel PAR2 activators in intracellular Ca^{2+} and receptor internalisation assays and considering previous studies suggesting a neuroprotective role for PAR2 in OSC (Greenwood & Bushell, 2010), experiments were carried out investigating PAR2 potential neuroprotective properties. More specifically, the experimental aims described in this chapter were to study whether kainate-induced neuronal death in organotypic hippocampal slice cultures (see figure 4.1) could be prevented by PAR2 activation. All experiments were performed using fluorescence imaging as described in section 2.2.4.4.

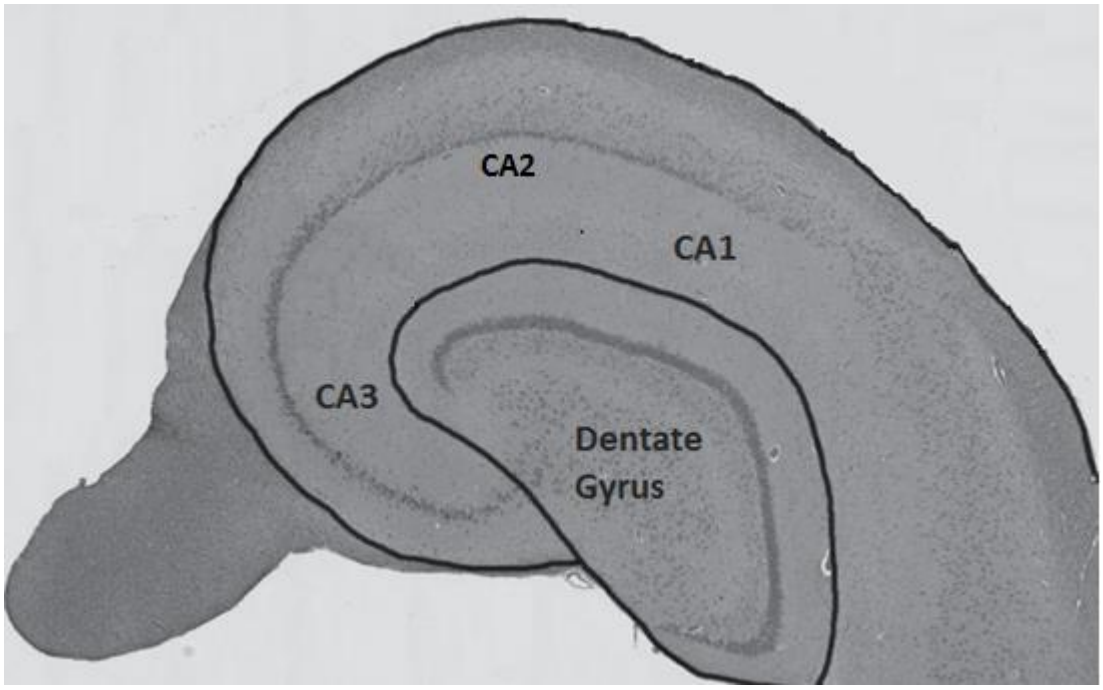


Figure 4.1 - **Representative image of an organotypic slice culture of mice hippocampus.** Slices after 15 DIV, image was visualised with bright field microscopy. CA1: cornu ammonis 1, CA2: cornu ammonis 2, CA3: cornu ammonis.

4.1.2. Method.

The dissection and slicing procedures lead to inevitable cell death and tissue debris, while also altering the metabolic state via the release of enzymes and ions. Previous work has looked in details into hippocampal slices and have revealed that following approximately one week *in vitro*, most cells on the slice surface are already healthy, receiving and sending inputs from intact axons (Stoppini et al., 1991), after 2 weeks in culture detrimental consequences of the dissection procedure were confirmed to be mostly completely reversed which is considered as an advantage over acute slices (Simoni et al., 2006). Therefore, in this study, we have worked with hippocampal slices cultured for at least 15 days, where cell death was assessed using staining via the nuclear marker propidium iodide (PI), a membrane impermeable fluorescent dye that only enters cells lacking an intact plasma membrane where it forms an irreversible bond predominantly with DNA thereby enhancing its fluorescence 20 to 30 fold (Riccardi & Nicoletti, 2006). It is known to be non-toxic to neurons, since interaction with DNA can only happen in dying cells, and is used frequently as an indicator of neuronal integrity and cell viability (Noraberg et al.,1999; Lee et al., 2012).

4.1.2.1. Kainic acid

For these experiments, we used two concentrations of KA, 20µM and 300µM for two different periods of 1h and 24h. These values were chosen as previous work from our lab (Greenwood & Bushell, 2010) and from the Lilly research group identified 300µM as the maximum concentration possible to carry out experiments on OSC over more than 24h, moreover using a lower concentration (20µM) has allowed to monitor cell death in a more progressive manner over longer periods of time (Noraberg et al., 2005). We considered that KA at 300µM for 24h would represent the maximum cell

death we can record, therefore, every measurement obtained was normalized to be compared to that maximum cell death.

4.1.2.2. Kynurenic acid

In order to assess the specificity of the results obtained in the study of PAR2 neuroprotective properties in OSC, we used a glutamate receptor blocker, kynurenic acid (KYN) as a positive control (Bertolino et al., 1989; Khan et al., 2000). Previous work has demonstrated that a large dose of KYN significantly reduce KA induced cell death in slices when added in co-application (Banerjee et al., 2012). The ability of glutamate receptor antagonists to inhibit KA-induced neuronal cell death also confirms that the mechanism of KA-induced excitotoxicity involves glutamate receptors.

4.1.3. PAR2 activators and their vehicles are not toxic to OSC.

4.1.3.1. Drug vehicles are not toxic to OSC.

In order to rule out any toxicity effect induced by the vehicle used to dilute chemicals in these experiments, vehicle control toxicity assays were carried out. Since PAR2 activators were diluted in distilled water (dH_2O) and DMSO while PI, KA and KYN were diluted in distilled water only, we monitored the effect of DMSO and dH_2O at the highest and lowest concentrations they were applied at. These experiments resulted in no changes in cell death (figure 4.2), indeed DMSO at 0.05% (5.1 ± 6.7 % of maximum cell death; n=6, compared to negative control) and distilled water at 0.001% (5.2 ± 7.6% of maximum cell death; n=6) and at 0.01% (4.8 ± 9.6% of maximum cell death; n=6) did not result in significant cell death when compared to negative control (4.4 ± 6.5%; n=18), where nothing was added to the slices but normal growing media (figure 4.2), suggesting that neither DMSO nor dH_2O caused any inherent toxicity.

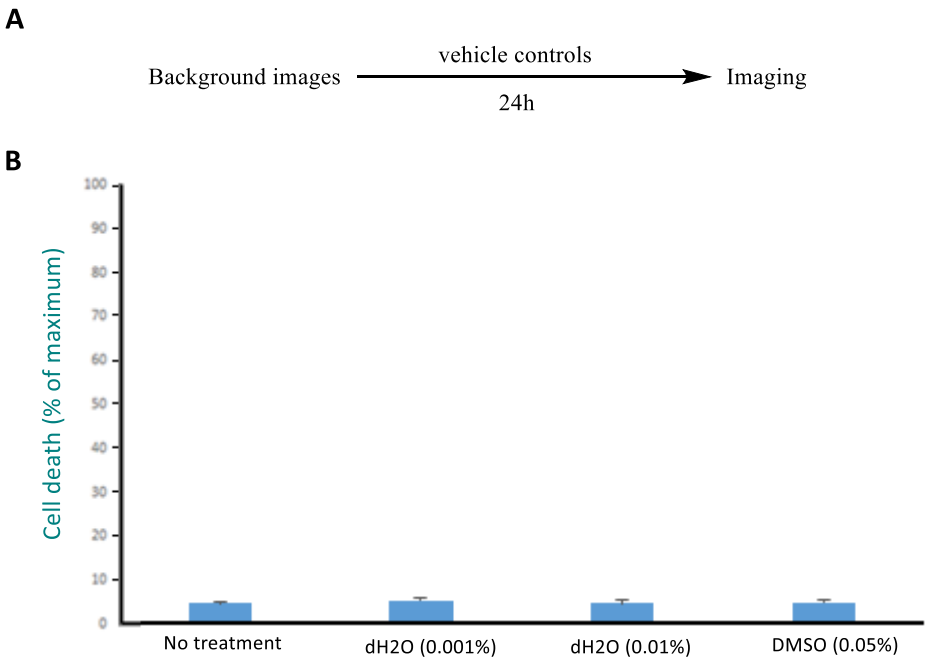


Figure 4.2 – Effect of vehicles on neuronal toxicity in mice hippocampal slice. (A) Time-flow representing experimental design for vehicle toxicity assays, in which slices (15 DIV) were incubated in media containing vehicles for 24 hours at the same concentrations used in the experiments. (B) Bar chart showing that vehicle didn't induce any significant cell death on OSC compared to negative control conditions where no treatment was added. All experiments were carried out on OSCs prepared from at least 3 separate animals with n≥4. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. compared to negative control conditions.

4.1.3.2. Vehicles are not neuroprotective against KA-induced toxicity.

Additionally, in order to exclude any protective effect induced by the vehicle used, experiments using a vehicle control against KA were carried out where we monitored the effect of DMSO and dH_2O, for 24h at the highest and lowest concentrations they were applied at. These control experiments resulted in no changes in the amount of cell death compared to a negative control where nothing was added to the slices but normal growing media containing KA (figures 4.3 A and 4.3 B). Hence KA (20µM for 24h) in the presence of DMSO (0.05%) resulted in 47.8 ± 4.1% of maximum cell death (n=4) and in the presence of dH_2O (0.01%) resulted in 47.4 ± 4.23% of maximum cell death (n=9) compared to KA alone (20µM) for 24h (52.5 ± 2.7% of maximum cell death; n=11). Furthermore, against KA (300µM for 24h), the presence of DMSO (0.05%) resulted in 94.1 ± 5.1% of maximum cell death (n=5) while the presence of dH_2O (0.001%), resulted in 98.6 ± 6.5% of maximum cell death (n=6) not significantly different when compared to the positive control, KA alone (300µM) for 24h (100 ± 3% of maximum cell death; n=19). Hence these data suggest that any results subsequently observed are not mediated by the drug vehicles.

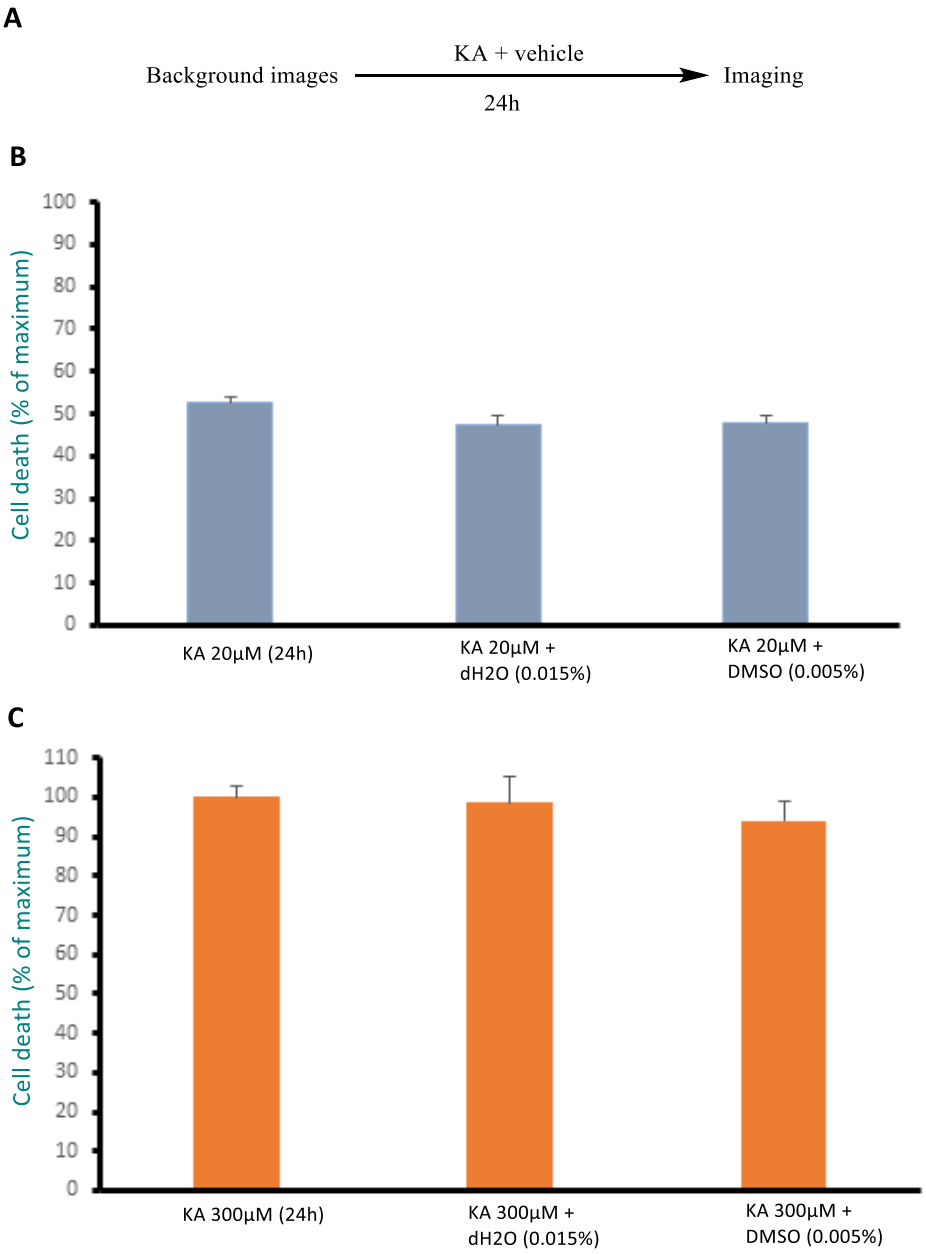


Figure 4.3 – Effect of drug vehicles on neuroprotection against KA-induced toxicity. (A) Time-flow representing experimental design for vehicle toxicity assays, in which slices (15 DIV) were incubated in media containing KA and vehicle for 24 hours. (B) Bar chart showing that vehicle was not neuroprotective against KA (20µM, 24h) on OSC compared to positive control conditions where KA alone was applied. (C) Bar chart showing that vehicle is not protective against KA (300µM, 24h) on OSC compared to positive control conditions where KA alone was applied. All experiments were carried out on OSCs prepared from separate animals with n≥3. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. compared to positive control conditions.

4.1.3.3. PAR2 activation is not toxic to OSCs.

As a final control, we conducted toxicity assays in order to determine any neurotoxic effect inherent to the drugs of interest, as has been previously suggested (Smith-Swintosky et al., 1997). Application of the PAR2 activator SLIGRL (100µM, 24h), a concentration previously reported as inducing a maximal response in CNS preparations (Bushell, 2006), did not induce cell death in OSCs (respectively 5.1 ± 9.1% of maximum cell death; n=5, and 4.1 ± 15.9% of maximum cell death; n=4, compared to negative control, figure 4.4 C). Similarly, other PAR2 activators of interest similarly exhibited no toxicity in OSCs when applied for 24h. As such, AC (5.3 ± 10.2 % of maximum cell death; n=5, figure 4.4 C), and GB88 (3.8 ± 12.6% of maximum cell death; n=5, compared to negative control, figure 4.4 C) resulted in no changes in the amount of cell death compared to a negative control. Hence these data suggest that the PAR2 activators are not neurotoxic to OSC in the experimental settings used here.

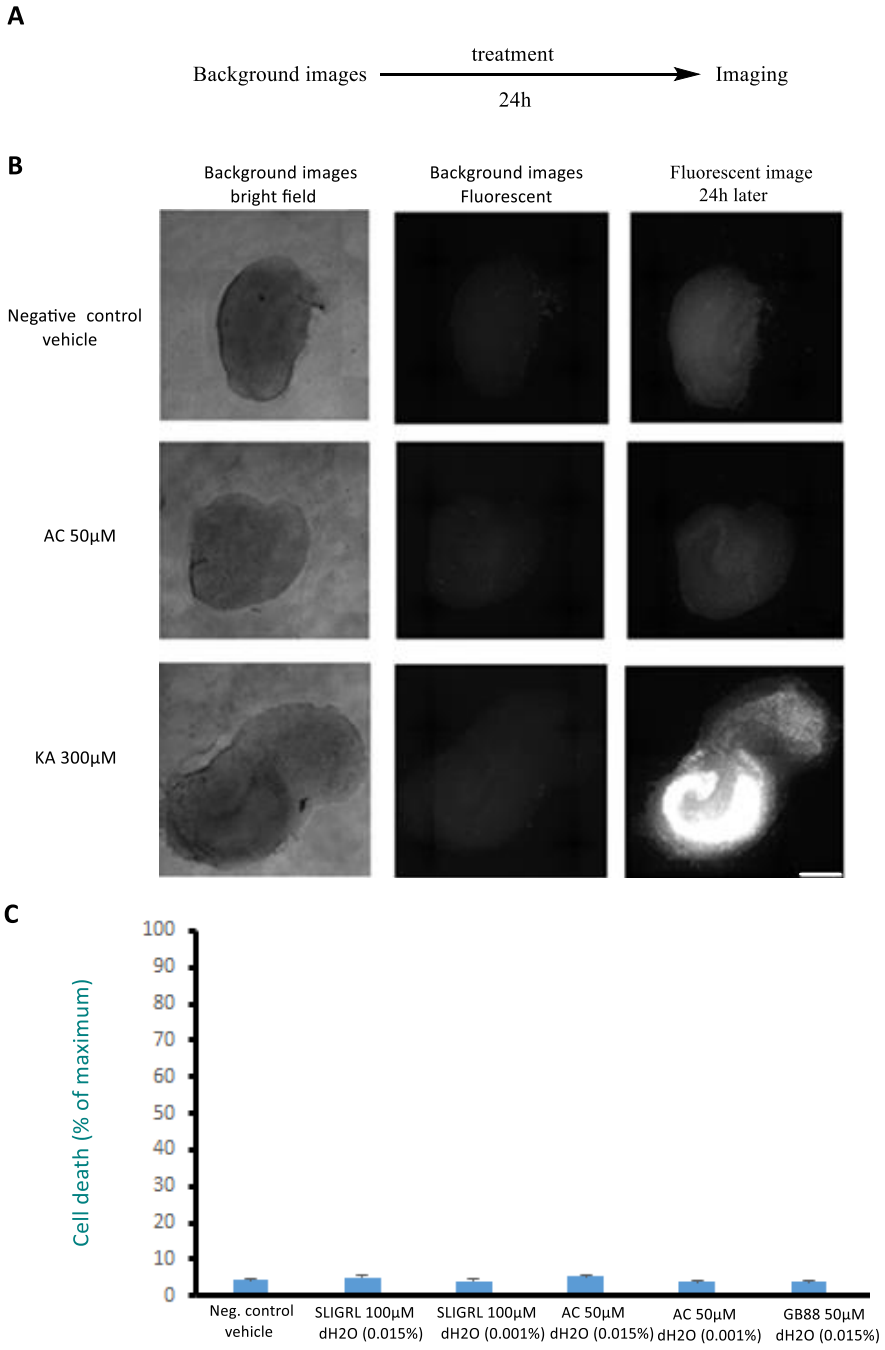


Figure 4.4 – Effect of PAR2 activators on toxicity on OSCs. (A) Time-flow representing experimental design for toxicity assays, in which slices (15 DIV) were incubated in fresh culture media containing vehicle only (DMSO 0.05%) or media containing AC 50µM or with KA 300µM for 24 hours, (B) representative images of slices showing propidium iodide induced fluorescence(C) Bar chart showing that PAR2 activators didn't induce any significant cell death on OSC compared to negative control conditions. All experiments were carried out on OSCs from at least 3 separate animals with n≥3. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. compared to negative control conditions. Scale bar: 250 µm.

4.2. Characterising the neuroprotective properties of PAR2 activators against KA-induced toxicity.

4.2.1. Co-application of PAR2 activators reduces KA-induced neurotoxicity.

Our experiments revealed that when co-applied with KA (20µM, 24h), PAR2 activation via SLIGRL 100µM (23.1 ± 5.6 % of maximum cell death; n=15, p<0.001), AC 50µM (21.9 ± 7.3 % of maximum cell death; n=11, p<0.001) and GB88 50µM (23.2 ± 6.4 % of maximum cell death; n=8, p<0.001) was neuroprotective compared to KA 20µM alone for 24h (52.6 ± 2.7 % of maximum cell death; n=11, figure 4.5 C). In a similar way, KYN (3M) also induced significant neuroprotection (12.9 ± 13.2 % of maximum cell death; n=16, p<0.001 in co-application with KA 20µM for 24h). Moreover, when co-applied against KA 300µM for 24h, PAR2 activation still resulted in significant neuroprotection (48.5 ± 4.6 % of maximum cell death; n=13, p<0.001 for SLIGRL; 42.5 ± 3.3 % of maximum cell death; n=15, p<0.001 for AC and 44.3 ± 4.5 % of maximum cell death; n=10, p<0.001 for GB88, figure 4.5 D) when compared to KA 300µM alone for 24h (100 ± 3 % of maximum cell death; n=19). As expected, where no treatment was applied, little cell death was observed (4.4 ± 6.5% of maximum cell death; n=11, p<0.001 compared to KA 300µM alone for 24h) but when KYN (3M) was applied in co-application with KA 300µM for 24h, this resulted in reduced cell death (32.7 ± 6.7 % of maximum cell death; n=16, p<0.001 compared to KA 300µM alone, figure 4.5 D).

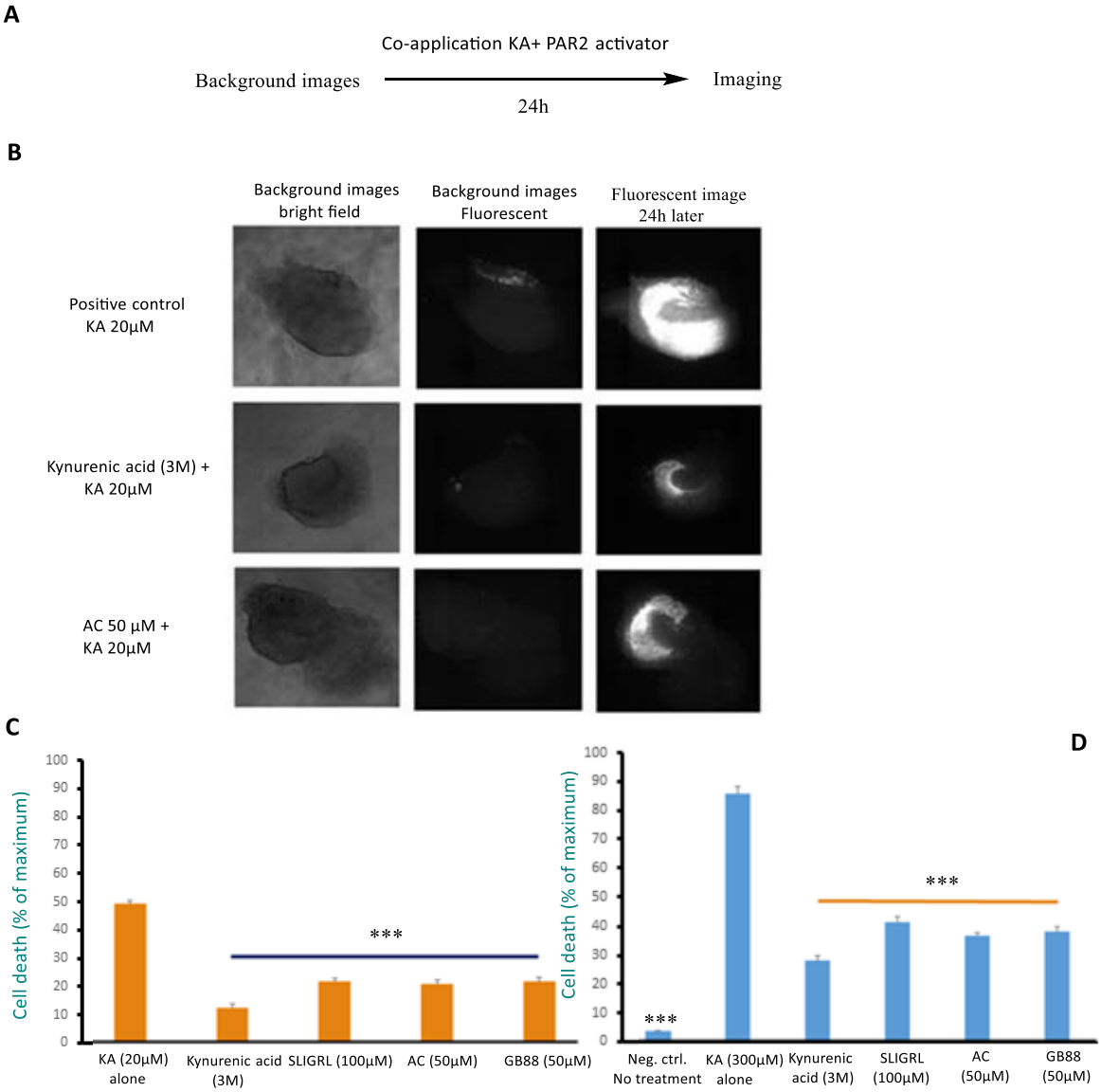


Figure 4.5 – Effect of co-application of PAR2 activators on neuroprotection against KA-induced cell death in OSCs. (A) Time-flow representing the experimental design for PAR2 co-application neuroprotective assays, in which slices (15 DIV) were incubated in media only, in media containing KA (20 and 300µM), in media containing KYN (3M) + KA (20 and 300µM) and in media containing PAR2 activators + KA (20 and 300µM) for 24 hours, as shown in representative images showing propidium iodide induced fluorescence in slices (B). (C) Bar chart demonstrating that PAR2 activation results in significant neuroprotection when applied in co-application with KA (20µM). (D) Bar chart showing that PAR2 activation results in significant neuroprotection when applied in co-application with KA (300µM). All experiments were carried out on OSCs prepared from at least 3 separate animals with n≥8. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with ***= $p<0.001$ compared to positive control conditions.

4.2.2. PAR2 activation is neuroprotective when applied after induction of KA-induced neurotoxicity.

Having established the neuroprotective properties of PAR2 activators when co-applied with KA thereby confirming previous work from our lab (Greenwood & Bushell, 2010), we decided to set up experiments to investigate whether PAR2 activation was neuroprotective after KA-induced neurotoxicity had been initiated. Therefore, we assessed the effect of PAR2 activation following the application of KA (20µM and 300µM, both for 1h) with a media change (figure 4.6). Our experiments revealed that when applied following KA (20µM for 1h), AC (50µM for 24h) remains neuroprotective (14.4 ± 4.2 % of maximum cell death; n=6, $p<0.01$, figure 4.6 B) compared to KA 20µM for 1h followed by a media replacement (20.6 ± 8.4 % of maximum cell death; n=8). However, none of the other PAR2 activators were neuroprotective under these experimental conditions (16.1 ± 4.2 % of maximum cell death; n=5 for SLIGRL and 15.7 ± 3.5 % of maximum cell death; n=4 for GB88). On the other hand, when applied following KA (300µM for 1h), PAR2 activation via SLIGRL (56.3 ± 2.1 % of maximum cell death; n=10, $p<0.001$), AC (52.6 ± 2 % of maximum cell death; n=12, $p<0.001$) and GB88 (55.8 ± 3.1 % of maximum cell death; n=9, $p<0.001$) were neuroprotective compared to KA 300µM alone for 1h (71.6 ± 3.9 % of maximum cell death; n=11, figure 4.6 C). In addition, KYN (3M) was not neuroprotective against both KA concentrations (19.8 ± 6.9 % of maximum cell death, n=5 against KA 20µM for 1h followed by media change and 68.1 ± 2.6 % of maximum cell death, n=8 against KA 300µM).

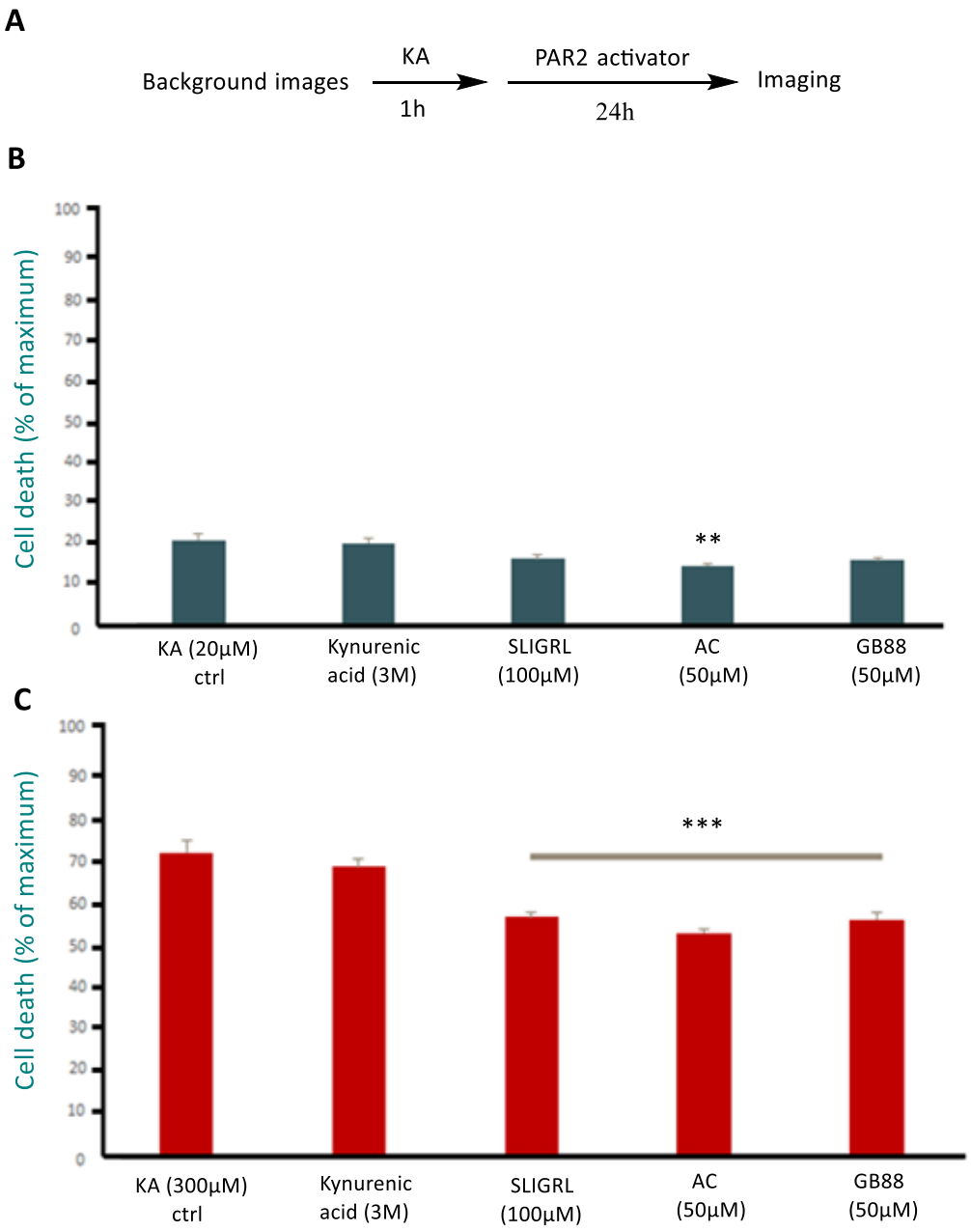


Figure 4.6 – Effect of PAR2 activators on neuroprotection when applied 1h after KA-induced neurotoxic insult. (A) Time-flow representing the experimental design for PAR2 post-treatment neuroprotective assays, in which slices (15 DIV) were incubated in media containing KA 20µM or 300µM for 1h, media was then replaced for a fresh media containing PAR2 activator for 24h. (B) Bar chart validating that PAR2 activation via AC for 24 hours post KA (20µM for 1h) insult, induces significant neuroprotection compared to positive control conditions. (C) Bar chart confirming that PAR2 activation for 24 hours post KA (300µM for 1h) insult induces significant neuroprotection compared to positive control conditions. All experiments were carried out on OSCs prepared from at least 3 separate animals with n≥5. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with **= $p<0.01$ and ***= $p<0.001$ compared to positive control conditions.

4.2.3. Pre-treatment with PAR2 activators does not protect against KA-induced neurotoxicity.

Having demonstrated that PAR2 activation has a positive effect on cell survival even when applied following the KA insult, we decided to investigate the effect of a pre-treatment with PAR2 activators on KA-induced neurotoxicity. Experiments were performed where following a 24h pre-treatment with PAR2 activators, slices were incubated for 1h with KA and cell death examined 24h later. We demonstrate that when applied before KA (20µM for 1h), PAR2 activation via SLIGRL (17.8 ± 13.5 % of maximum cell death, n=4), AC (16.9 ± 6.1 % of maximum cell death; n=5) and GB88 (17.6 ± 7.4 % of maximum cell death; n=4) doesn't induce neuroprotection against KA-induced toxicity (20.6 ± 8.2 % of maximum cell death, n=8, figure 4.7 B). In contrast, when applied before KA (300µM for 1h), AC was indeed neuroprotective (65.2 ± 3.7 % of maximum cell death, n=5, $p<0.05$ compared to KA alone, figure 4.7 C), however none of the other PAR2 activators induced neuroprotection compared to KA (300µM for 1h) alone (71.6. ± 3.9 % of maximum cell death, n=11, figure 4.7 C).

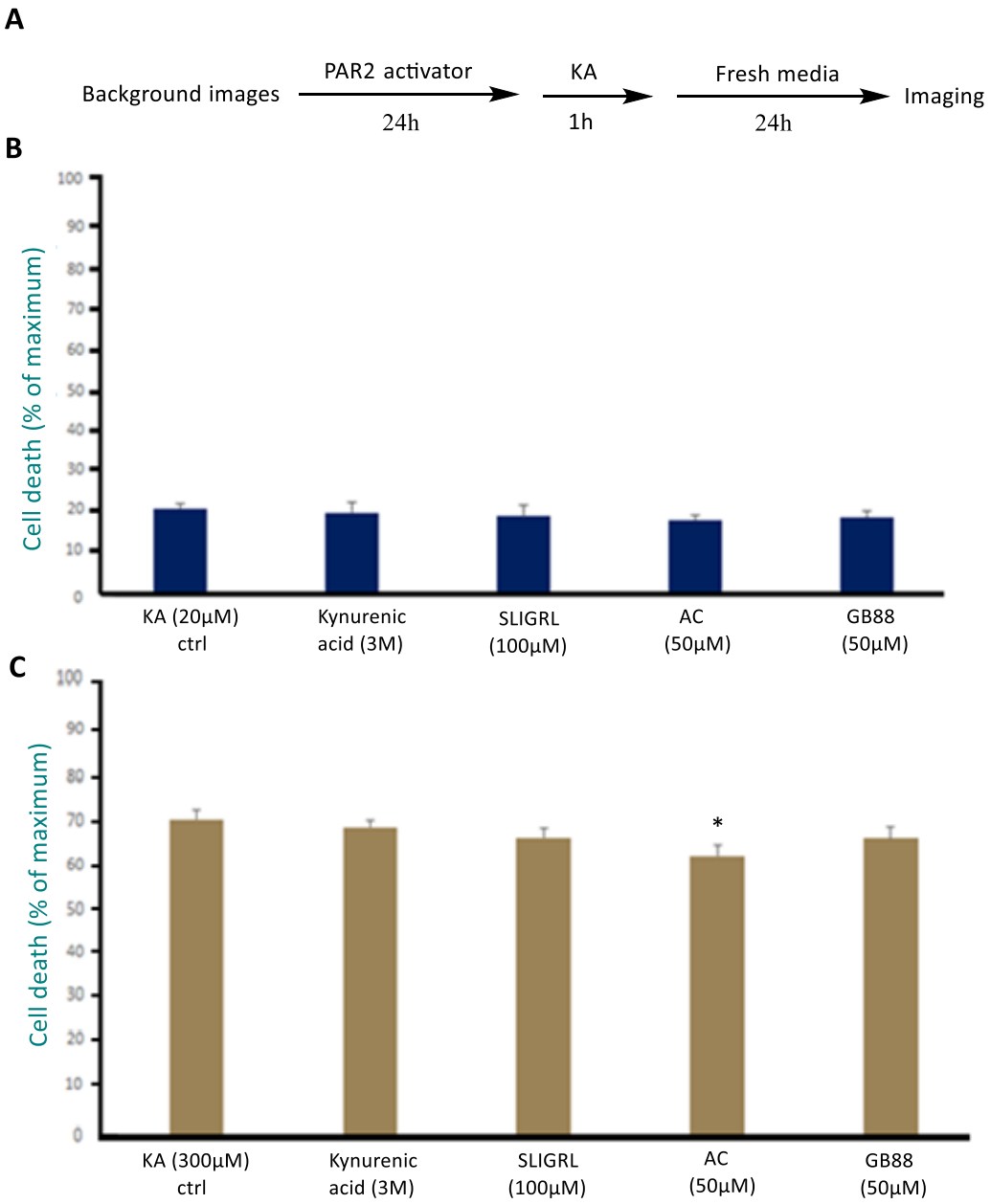


Figure 4.7 – Effect of PAR2 activation on neuroprotection when induced in a pre-treatment before KA-induced neurotoxic insult. (A) Time-flow representing the experimental design for PAR2 pre-treatment neuroprotective assays, in which slices (15 DIV) were incubated in media containing PAR2 activators for 24h, replaced for a media containing KA 20µM or 300µM for 1h, followed by 24h in fresh media. (B) Bar chart demonstrating that PAR2 activation before an application of KA (20µM) doesn't induce significant neuroprotection compared to positive control conditions. (C). Bar chart confirming that PAR2 activation via AC, before an application of KA (300µM) for 1 hour, induce significant neuroprotection compared to positive control conditions. All experiments were carried out on OSCs prepared from at least 3 separate animals with n≥3. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with *= $p<0.05$ compared to positive control conditions.

4.2.4. PAR2 activation is neuroprotective against KA-induced neuronal cell death when applied as a pre-treatment followed by a post treatment.

Having previously established that PAR2 activation is neuroprotective when used following an hour of neurotoxic KA application, but is not following pre-treatment, we then combined both approaches. In this set of experiments, we assessed the effects of pre and post treatment with PAR2 activators against KA application (20µM and 300µM, 1h). Additionally, KYN (3M) was again used as a comparison experiment as it was previously shown to have no effect in pre or post treatment. Our results revealed that a conjunction of pre and post treatment doesn't prevent neuronal cell death induced by KA (20µM for 1h). Indeed, SLIGRL (15.2 ± 10.4 % of maximum cell death, n=4), AC (14.2 ± 10.9 % of maximum cell death, n=5) and GB88 (15.8 ± 6.6 % of maximum cell death, n=4) were not neuroprotective when compared to KA (20µM for 1h) alone (19.4 ± 7.1 % of maximum cell death; n=4, figure 4.8 B). However, against KA (300µM for 1h), PAR2 activation resulted in a significant reduction in cell death via SLIGRL (65.3 ± 5% of maximum cell death; n=4, $p<0.05$), AC (57.4 ± 7.3 % of maximum cell death; n=4, $p<0.001$) and GB88 (58.0 ± 4.8 % of maximum cell death, n=4, $p<0.01$) compared to KA (300µM for 1h) alone, (76.9 ± 1.8 % of maximum cell death; n=5, figure 8 C). Furthermore, no neuroprotective effects were induced by KYN (72.6 ± 4.4% of maximum cell death; n=3, figures 4.8 B and C).

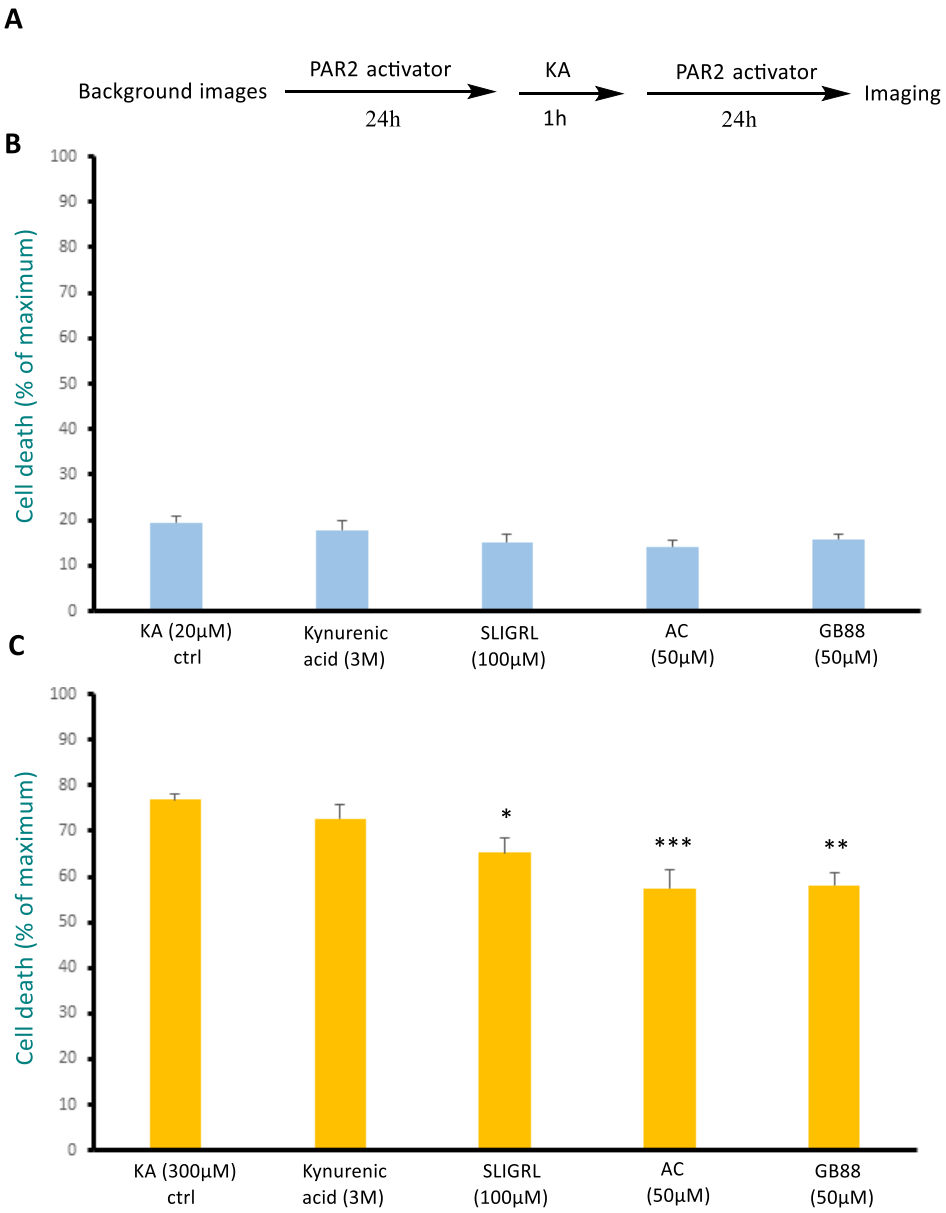


Figure 4.8 – Effect of PAR2 activation on neuroprotection when given as a pre-treatment before a 1h application of KA (300µM) **followed by a post-treatment in OSCs**. (A) Time-flow representing the experimental design for PAR2 combined pre/post treatment neuroprotective assays, in which slices (15 DIV) were incubated in media containing PAR2 activators for 24h, replaced for a media containing KA 20µM or 300µM for 1h, followed by 24h in media containing PAR2 activators. (B) Bar chart demonstrating that PAR2 activation combined before and after an application of KA (20µM) doesn't induce significant neuroprotection compared to positive control conditions. (C). Bar chart confirming that PAR2 activation combined before and after an application of KA (300µM) for 1 hour, induce significant neuroprotection compared to positive control conditions. All experiments were carried out on OSCs prepared from at least 3 separate animals with n≥3. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ compared to positive control conditions.

4.2.5. Combined pre and post-treatment with PAR2 activators is neuroprotective against KA-induced neurotoxicity.

Finally, having established that a combination of PAR2 pre and post treatment only induced neuroprotection against KA (300µM for 1h) alone but not against KA (20µM for 1h), we investigated the effects of combining pre/post treatment with co-application of PAR2 activators and KA. Our experiments revealed a neuroprotective effect of PAR2 activation in co-application for both KA concentrations. Hence SLIGRL (13.6 ± 2.3 % of maximum cell death; n=3, $p<0.001$), AC (14.2 ± 6 % of maximum cell death; n=4, $p<0.001$) and GB88 (13.8 ± 5 % of maximum cell death; n=3, $p<0.01$) were neuroprotective when compared to KA (20µM for 1h) alone (20.6 ± 5 % of maximum cell death; n=4, figure 4.9 B). Similarly, KYN was also neuroprotective (8.4 ± 13.9 % of maximum cell death; n=4, $p<0.001$) under these experimental conditions. Moreover, when the concentration of KA was increased to 300µM (for 1h), PAR2 activation was still protective. Specifically, SLIGRL (61.3 ± 6.2% of maximum cell death; n=3, $p<0.05$) and AC (56.9 ± 9.1 % of maximum cell death; n=4, $p<0.01$) significantly reduced cell death compared to KA (300µM for 1h) alone (81.2 ± 2.4% of maximum cell death; n=3, figure 4.9 C). Finally, KYN (3M) also induced significant neuroprotection (47.1 ± 5.7% of maximum cell death; n=3, $p<0.001$, figures 4.9 B and C).

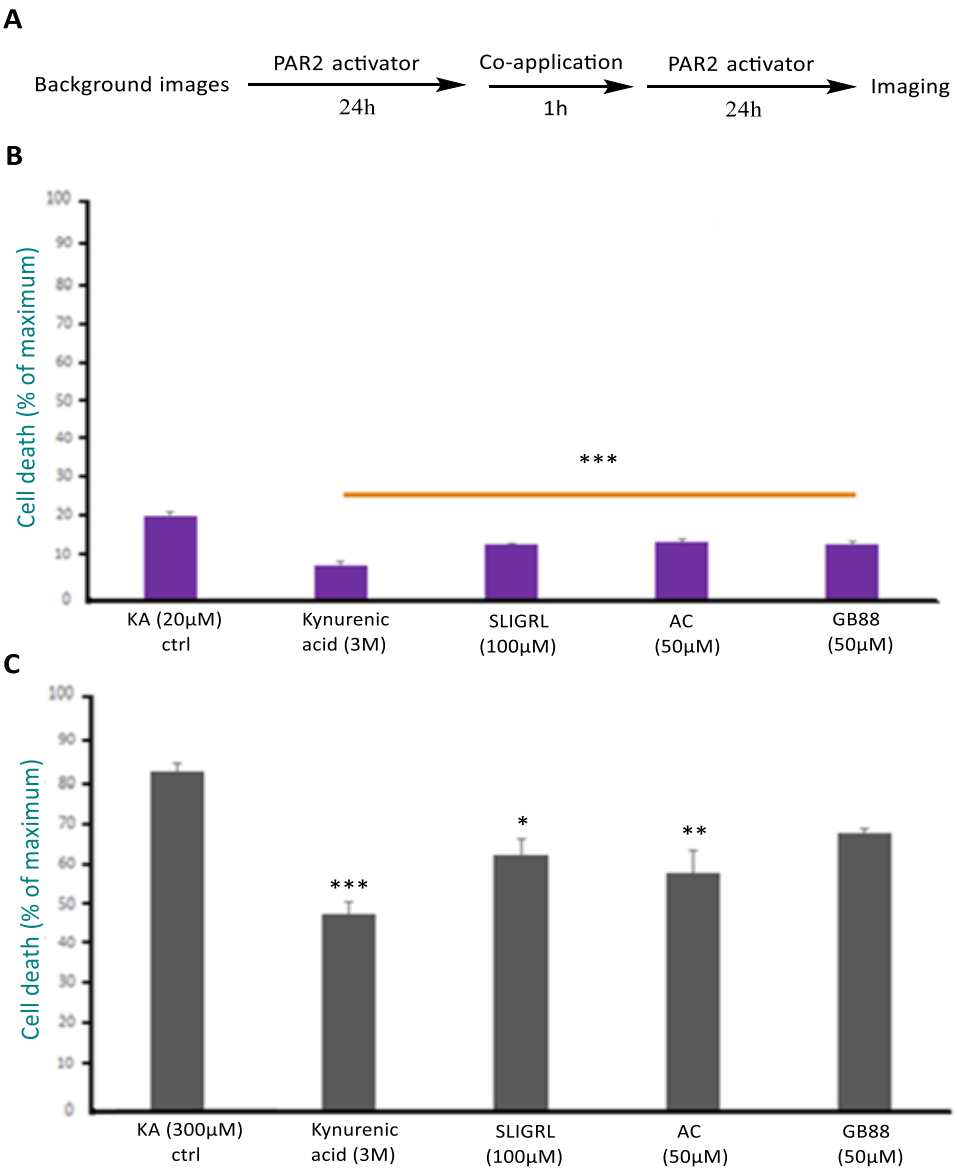


Figure 4.9 – Effect of PAR2 activation on neuroprotection when given as a combined pre + post treatment before and after 1h in co-application with KA in OSCs. (A) Time-flow representing the experimental design for PAR2 combined pre + post treatment neuroprotective assays, in which slices (15 DIV) were incubated in media containing PAR2 activators for 24h, replaced for a media containing KA 20µM or 300µM for 1h combined with PAR2 activators, followed by 24h in media containing PAR2 activators. (B) Bar chart demonstrating that PAR2 activation before and after an application of KA (20µM) combined to PAR2 activators induce significant neuroprotection compared to positive control conditions. (C) Bar chart demonstrating that PAR2 activation before and after an application of KA (300µM) combined to PAR2 activators induce significant neuroprotection compared to positive control conditions. All experiments were carried out on OSCs prepared from at least 3 separate animals with n≥3. Data were analysed using a one-way ANOVA and Dunnett's post-test and presented as mean ± S.E.M. with *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ compared to positive control conditions.

4.3. Discussion.

4.3.1. Organotypic slice cultures; an *ex vivo* representation of the *in vivo* brain.

Accurately modelling the underlying mechanisms of complex neurodegenerative diseases *in vitro* isn't an easy task. No experimental protocol allows a perfect observation and manipulation of neuronal cells and all their characteristics so that what happens in a real disease can be scrupulously reproduced and observed. However, a few options are available allowing the study of some of the key aspects of cell degeneration and death in the CNS, including primary cultures, acute slices or organotypic slice cultures, with the option selected seriously impacting the relevance of any experimental results and the possibility to translate them *in vivo*. Both acute slices and organotypic slices preserve the anatomical structure, neuronal circuitry and heterogeneous cell populations found in an *in vivo* brain whereas dissociated cell cultures typically contain a single cell type (Zhu et al., 2011). However, there is a major limitation to the use of acute slice preparations, unlike organotypic slices, which can be cultured for months before experimentation and therefore have the opportunity to recover and return to a stable state, acute slices require experiments to be performed on the day of dissection and slicing and don't have time to recover from that initial trauma (Stoppini et al., 1997). For that reason, in order to study cellular death over a large period of time, it seemed more relevant to work with cultured organotypic slices. During the transversal slicing of the hippocampus, the commissural fibres connections are lost, but the tri-synaptic loop, which connects the hippocampus to the cortex, is well preserved (Simoni & Yu, 2006). Moreover, it was found that organotypic slices cultured for 14 days and more were the developmental equivalent of slices taken from aged-matched pups *in vivo*, with regards to spine density, growth of dendrites, axonal length (Simoni & Yu, 2006). Furthermore, no differences were observed in the frequency of action potentials, immunological responses as well as in signalling pathways, thus allowing functional analysis of CNS

tissue (Huuskonen et al., 2005; Morin-Brureau et al., 2013). Hippocampal slice cultures can be maintained for weeks to months and are readily accessible for optical imaging or electrophysiological studies. Moreover, the method generates several repetitions from each animal and thus greatly reduces the number of animals required. Finally, the extracellular environment can be precisely controlled and manipulated including when investigating the mechanism of action of drugs in order to correlate molecular changes with pathological outcomes, which was particularly useful in the current study as it allowed direct control over KA and PAR2 concentrations across the slices.

4.3.2. The kainic acid model of excitotoxicity: an amenable representation of neurodegeneration.

Neurotoxicity is a pathological process by which neuronal cells are damaged following excessive stimulation. It is a major detrimental mechanism involved in the physiopathology of various CNS diseases including neurodegenerative disorders (Mazzone et al., 2013; Bevinahal et al., 2014). Kainic acid is a non-degradable and extremely potent glutamate analogue, it induces excitotoxicity by overstimulating glutamate receptors, and causing membrane depolarisation thereby inducing a large influx of $Ca^{2+,}$ which in turn triggers a large production of free radicals and subsequent neuronal death (Mazzone et al., 2013). The mechanisms involved in KA-induced neurodegeneration are also thought to increase oxidative stress responsible for damaging cell physiology causing structural and functional changes at molecular, protein and cellular levels (Moloney, 2002). It was demonstrated that due to a high density of kainate and AMPA receptors, the hippocampus is particularly vulnerable to KA-induced excitotoxicity (Zhang & Zhu, 2011). Furthermore, in mice hippocampal slices cultures, KA application initially causes significant neuronal damage in the CA3 region where kainate receptors are more concentrated, and from there the excitotoxicity spreads to the CA1 region through connecting fibres and axons before

dissipating to other hippocampal regions (Noraberg et al., 1999; Jarvela et al., 2011). Our data is in line with these previous observations, specifically using KA (20µM) we could observe a primary damage in CA3 followed by CA1 before spreading to the other hippocampal areas. Moreover, previous work has described how the application of KYN would reduce KA induced neuronal cell death (Bertolino et al., 1989). Findings in our lab have shown that effect to be due to high affinity competitive binding at the receptor site (Eli Lilly, unpublished data), therefore, we have used kynurenic acid as a control for neuroprotection. Studies investigating the role of different potential neuroprotective compounds in the KA model of neuronal death have played a fundamental role in providing preliminary data for the development of a variety of therapeutics currently in use for the treatment of neurodegenerative diseases (Lipton, 2007). In the present study, mouse hippocampal organotypic slices were exposed to toxic doses of KA to model neurodegeneration and to determine the potential neuroprotective role of PAR2 activation.

4.3.3. PAR2 activation is neuroprotective against KA-induced neuronal cell death in organotypic slice cultures.

Previous studies have investigated PAR2 expression within the CNS in the context of cellular degeneration (Andrade-Gordon et al., 2005; Noorbakhsh et al., 2006; Bushell, 2006; Luo et al., 2007; Peng et al., 2013; Gieseler et al., 2013; Hurley et al., 2015), however, very few studies have examined its functional effects. Contradictorily to what was originally reported in primary cultures (Smith-Swintosky et al., 1997), this research shows that PAR2 activation via SLIGRL is not toxic to neurons from OSC and even leads to neuroprotection against KA induced neuronal cell death, in agreement with previous studies from our laboratory (Greenwood & Bushell, 2010). In addition, we also demonstrated for the first time that PAR2 activation via AC-264613 and GB88 also produce significant levels of neuroprotection. That observation is made for different KA concentrations (20µM and 300µM) and for different periods of time (1h

and 24h). Knowing that KA excitotoxicity affects a variety of cellular pathways (Mazzone et al., 2013), it is interesting as it suggests than PAR2 might promote neuroprotection in a variety of situations involving neuronal toxicity. The neuroprotective properties observed following PAR2 activation via GB88 are interesting, having previously shown that GB88 doesn't signal via the Ca^{2+} signalling pathway, these data tend to infer that PAR2 neuroprotective activity isn't mediated by the Ca^{2+} pathway but by another of the pathways proposed to be activated by PAR2 in the CNS. Previous studies have proposed that these neuroprotective properties might be mediated by inhibition of p38 MAP kinase and reduced ERK activity (Greenwood & Bushell, 2010) or by ERK1/2 activation (Eishingdrelo & Kongsamut, 2013; Boitano et al., 2015; Mrozkova et al., 2016; Nikolakopoulou et al., 2016). Previous investigations have also examined the role of PAR2 in CNS diseases and have suggested that its activation can be neuroprotective. Indeed, it has been demonstrated that PAR2 activation prevents neuronal cell death during HIV infection (Andrade-Gordon et al., 2005), protects neuronal cells against Aβ-induced toxicity (Afkhami-Goli et al., 2007), while PAR2 deletion led to an increased infarct volume in a model of acute focal ischemic brain injury (Jin et al., 2005). These observations from diseases samples and models could be related to our observation of a neuroprotective effect of PAR2 activation as a post-treatment, when the insult is already present. Furthermore, this study establishes the fact that the mechanisms underlying PAR2-induced neuroprotection are not based on competitive antagonism of KA receptors, as illustrated by the effect of kynurenic acid. KYN is known to cause neuroprotection by competing with KA for the KA receptors (Bertolino et al., 1989), accordingly its neuroprotective effect was observed only in co-application and not in a set-up where KYN was applied following a 1h KA (300µM) administration and media change thereby preventing competitive binding. However, when applied in the same conditions PAR2 activation still resulted in a significant decrease in cell death. When activated following a 1h KA (20µM) application, PAR2 activation via AC only resulted in significant neuroprotection, which might be explained by a better selectivity and potency of AC over the other PAR2 activators used for these experiments (table 1.4).

4.3.4. Prolonged PAR2 activation is neuroprotective.

Overall these observations lead to two conclusions, firstly, these experiments have shown that the neuroprotective pathways activated by PAR2 are able to protect cells from degenerating when PAR2 is activated at the same time as KA application, but also when the damage has already been initiated, which might have more relevance from a therapeutic point of view as we previously highlighted it. Second and contrastingly, it was also found that consecutively to a 24h PAR2 activation preceding OSC exposure to KA for 1h, the PAR2 neuroprotective effect was abolished. It has previously been established that PAR2 activation is irreversible and lead to receptor degradation and recycling (Stalheim et al., 2005; Soh et al., 2010; Boitano et al., 2015), therefore knowing that the receptor is recycled suggests that there might be a specific time-window in which a PAR2 receptor can be activated before it gets destroyed, thus a 24h activation could lead to receptor depletion (Jackson et al., 2014; Suen et al., 2014) and explain the absence of PAR2-induced neuroprotection when prompted for a 24h pre-treatment. Additionally, we conducted experiments where pre and post treatments were combined hoping to gather more information about the temporality of the effects induced by PAR2 activation. On account of the lack of significant protection following a 24h pre-treatment, it is intriguing to notice that when we combined it with a 24h treatment following a 1h KA application (300µM) we still managed to monitor a significant neuroprotection, suggesting that it is possible to recycle PAR2 receptors so that they will be able to prevent cell death *de novo* in a 24h time-window. That longer term effect was even more significant when PAR2 was applied in conjunction with the 1h KA application (for both 20 and 300µM). Previous researches have shown that PAR2 requires a full cellular re-sensitisation which is not immediate as it involves a complete new receptor synthesis and demands a reserve receptor pool (Böhm et al., 1998; Soh et al., 2010), still the exact necessary time-frame for replenishment of these reserves hasn't yet been elucidated. Nevertheless, recent studies have suggested that PAR2 activation via

SLIGRL and peptide based activators is achieved from 5 min to 2h *in vitro* depending on the pathways observed (Suen et al., 2014) while *in vivo* it has been determined that AC-264613, at least in the CNS, is at its peak after about 2h (Eli Lilly, unpublished data). It might be interesting in further studies, to determine the duration for which proteinase activated receptors 2 are active in time-window specific studies, thereby establishing the peak for effects mediated by PAR2 activation but also determining the time needed for the receptor to be recycled and re-sensitised. It would also be of interest to investigate the links between PAR2 activation and time-windows of established neuroprotective pathways such as ERK (Eishingdrelo & Kongsamut, 2013).

4.3.5. Limitations, improvements and future experiments.

One of the major limitation to the use of OSC is the degree to which they are faithfully reproducing what happens in a living being. Knowing that one objective of this study is to investigate neurodegeneration and protection as it would occur in a mature CNS, it is important to note that due to poor viability, tissue is explanted and prepared from neonatal pups rather than from adults (Stoppini et al., 1997). Therefore, that needs to be taking into account when considering translating these results into *in vivo* experiments on adult mice and even more importantly when considering a translation of these results into human therapeutics. Another issue lies in the fact that it was reported that there is in organotypic slice cultures an increase in glutamatergic miniature synaptic currents compared to what is observed *in vivo* (Simoni & Yu, 2006), seeing that these currents are those targeted by KA, it suggests that the excitotoxicity observed could possibly be over-accentuated. It is intriguing to note here that earlier studies have described that PAR2 reduces synaptically driven spontaneous action potential frequency (Gan et al., 2011) potentially hinting that PAR2 neuroprotection might be mediated by a reduction of synaptic action potentials. Nonetheless as shown in this chapter, and in spite of these drawbacks, OSC still constitutes a good, although not perfect, platform to study neuroprotection

therefore justifying their use to investigate the neuroprotective potential of PAR2. The work presented in this chapter open up some attractive directions for the understanding of PAR2 related mechanisms of neuroprotection. The data presented here demonstrates a robust neuroprotection by PAR2 against KA induced excitotoxicity when used at high concentrations (100µM for SLIGRL and 50µM for AC and GB88). Knowing that these values are much higher than the EC_{50} for these compounds (table 1.4), it would be interesting to lower the concentration of PAR2 activators to see if a similar level of neuroprotection is still achieved, also from a therapeutic point of view it would also be more relevant. A further attractive avenue of study would be to use OSC cultured from other brain regions, known to exhibit a lower sensitivity to KA induced excitotoxicity because of a lesser receptor density, which would allow an investigation of PAR2 neuroprotective properties against a less aggressive insult. Finally, these data showing the neuroprotective role of PAR2 in OSC against KA excitotoxicity could be linked with studies where PAR2 was investigated in relation to CNS diseases from an expression rather than from a functional point of view. Interestingly it was found that PAR2 immuno-reactivity is increased in the white matter of patients with multiple sclerosis (Noorbakhsh et al., 2006), while PAR2 is decreased in neurons from post-mortem brains from patients with Alzheimer's disease (Afkhami-Goli et al., 2007), additionally an increase of microglia expressing PAR2 was observed in patients with Parkinson's disease (Hurley et al., 2015). For these reasons it might be interesting in the future to further investigate of the functional role of PAR2 in animal models of CNS disorders.

4.3.6. Conclusions.

In summary, the data in this chapter shows that PAR2 activation is neuroprotective in mice organotypic hippocampal slices cultures, reducing KA-induced neuronal cell death. This study has confirmed the neuroprotective properties of PAR2 activation in co-application, moreover it has also identified for the first time different settings where PAR2 neuroprotection can be observed against KA excitotoxicity in *ex vivo* CNS explants. Indeed this chapter demonstrate that PAR2 generate neuroprotection when activated after KA induced degenerative pathways have been initiated. Another important aspect of this study is the fact that it involved a range of different PAR2 activators, peptide based agonist, small molecule agonist and biased agonist, the ability to show PAR2 neuroprotective effects using different PAR2 activators confer a greater consistency to the results obtained. In this chapter, we have also been able to show that although GB88 doesn't activate PAR2-mediated Ca^{2+} pathways, as seen in the previous chapter, it still induces PAR2-mediated neuroprotection in OSC, therefore suggesting that Ca^{2+} are not involved in PAR2 neuroprotective properties. In order to further characterise PAR2 mechanisms of action, important follow-up studies will have to include a throughout investigation of the different pathways activated by PAR2, including of those beneficial to cell survival, as observed in these OSC studies. Elucidation of the pathways involved in neuroprotection, neurodegeneration or regulation of CNS inflammation would prove crucial as it will open-up the possibility to target those specifically, throught several CNS modulators, including PAR2.

Chapter V. INVESTIGATING THE *IN VIVO* EFFECTS OF PAR2 ACTIVATION.

5.1. Introduction.

The modern paradigm for drug discovery, as currently widely used in academic groups and pharmaceutical companies, consist in a series of steps designed to efficiently achieve establishment of a "proof of principle" (Leestemaker & Ovaa, 2015), so that an interesting concept can be taken from bench studies into clinical trials. These steps generally include, validation of a target, *in vitro* and *ex vivo* studies, toxicity assays and analysis of the structural and functional effect of the potential drug (Hughes et al., 2011). Once a compound has been assessed at the cellular level, it is usually tried on animal models of relevant diseases in order to determine any therapeutic effects. Animals have been used repeatedly through the history of biomedical research, with references reporting animal testing go as far back as the 4th century BC (Henrique Franco, 2013). As such, animal experimentation has become a cornerstone of biomedical research (Baumans, 2004), contributing significantly to the increase in life expectancy observed since the beginning of the 20th century (figure 1.2). This is clearly illustrated by the fact that 94 of the 106 Nobel Prizes awarded for physiology or medicine since 1901 directly or indirectly relied on experiments carried out on animals (Henrique Franco, 2013). These allowed extending and improving the quality of life for millions, many of these crucial discoveries would not have been possible without experiments on animals (Dey et al., 2010). It is clear that all animals, particularly mammals have large differences but also key similarities and animal research is based on both of these to gain insight into the many complex human biological systems (Baker, 1998). Nowadays mice and rats play a central role in animal experimentation and have allowed the development of new pharmacological therapies for a variety of diseases, thus it is estimated that in the UK approximately 75% of all experimental procedures are conducted on rats and

mice (Home Office report, 2016). Rats and mice are one of the best option available to biomedical research for a combination of reasons, the first one being convenience, rodents are relatively easy and cheap to breed, maintain and house comparatively to other mammals (Henrique Franco, 2013). Additionally, they have a quick reproduction cycle, allowing observation of large populations of subjects over a short period of time (Mural, 2002). Furthermore, a number of genetic and phenotypic resemblances have been observed between rodents and humans (Baker, 1998), for example, rodent genome is almost as long as the human genome and we share more than 97.5% of our working DNA and more than 80% of identical genes with them (Mural, 2002). Moreover, some specific strains of rats and mice have been found to be naturally susceptible to diseases also affecting humans such as cancers, infections or high blood pressure (Baker, 1998). However, rodents develop these diseases over a span weeks or months when it takes years in human (Semple et al., 2013), therefore offering a simpler platform to study disease formation despite a few limitations. Over the last decades, advances in the understanding of genetics have also allowed the design of animals whose genome has been modified so that it would express specific genes or genetic mutations responsible for certain diseases (Dey et al., 2010). This allows translational research relevant to humans and making it possible to model a variety of human physiological mechanisms in simplified systems easily controllable and specifically designed so that they would mimic at least partially human physiology with a high degree of similarity (Semple et al., 2013). These models have been essential to elucidate a wide variety of scientific questions in fields such as physiology, nutrition, cardiovascular science, immunology, drug discovery to behaviour and learning (Baumans, 2004; Leestemaker & Ovaa, 2015). Indeed, studies conducted in the past decades on experimental models involving rodents have provided a wealth of information constituting the basis for most of our current understanding of the pathogenic and pathophysiological mechanisms of human physiology and diseases, including neurodegeneration and CNS diseases (Dey et al., 2010). As such they have been implicated in major advances and prompted new insights into the understanding of human physiology. For the CNS specifically, it

would have been difficult to apprehend the mechanisms underlying neuronal damage and loss, but also neuro-inflammatory processes, blood brain barrier disruptions or pathological protein aggregation if not for animal experimentation (Chesselet & Carmichael, 2012). Indeed, despite a disappointing translational value from animal experiments to efficient therapeutics (Hughes et al., 2011; Pratt et al., 2012) there is still potential to apply further principles and results from animal research to human medicine. In addition to facilitating the understanding of physiological processes, animal testing allows the observation and analysis of behaviour, the study of cognition and motor activity (Nestler & Hyman, 2010) and in drug discovery it provides fine and precise indications with regards to toxicity and dosage, which are critical when trying to bring a new drug on the market (Hughes et al., 2011). It is important to stress that over the years, there have been a rising concern in the general public as well as within the scientific community surrounding animal research with regards to animal welfare and the potential experiments to cause pain and distress (Henrique Franco, 2013). Reducing animal suffering induced by biomedical research has become a central priority which have prompted strict regulatory laws and guidelines ensuring that no experiments are carried out if causing unnecessary harm to animals, as a results research within the EU is based on the framework for humane research introduced in 1959 (Russell & Burch, 1959) which is exemplified by the 3Rs (Replacement, Reduction and Refinement) in the UK. Experimental animal research has assumed a crucial role in scientific and biomedical advances and is likely to keep on doing so in the future. Still, it is vital to continue to aim for constant improvement of the wellbeing of laboratory animals, as well as for alternatives able to replace or greatly reduce the number of animal experiments. Due to its promising characteristics as described earlier, interest in the discovery of new pharmaceutical therapeutics based on PAR2 is increasing and *in vivo* investigation will constitute a crucial step to help determine its mechanisms and role in human physiology (Ramelli et al., 2010; Wang et al., 2010; Hughes et al., 2013; Boitano et al., 2015; Mrozkova et al., 2016). However, the objective of most of these *in vivo* studies has been to look at short term effects of PAR2 activation (Ramachandran et al., 2012)

so there are is a significant need for additional information about activation of PAR2 *in vivo* regarding longer-term effects, but also regarding time course and dose dependent effects on behaviour and in animal models of disease.

5.2. Investigating the effect of PAR2 activation on behaviour.

5.2.1. Introduction.

Findings from the previous chapter describing neuroprotective properties of PAR2 activation *ex vivo* are important as they open the possibility to explore new therapeutic routes for the treatment of CNS diseases. Therefore, as part of the drug discovery process it is crucial to also study the effect of PAR2 activation *in vivo*. Behavioural testing has been used extensively as an initial assay to predict potential therapeutic efficacy of a new drug in humans (Arthur & Hancock, 1999). Moreover, it allows measurements and comparison of behavioural parameters including motor coordination, locomotor activity, anxiety and memory skills (Wahlsten, 2011). Because of the characteristics of PAR2 activators previously available, including the fact that they can't cross the blood brain barrier, it was difficult to investigate PAR2 in the CNS except using PAR2 genetically modified mice (Ramachandran et al., 2012; Abulkassim 2014, unpublished work; Abulkassim et al., 2016), however the recently developed small molecule AC-264613 having been confirmed to be able to access the brain following I.P. injections (Eli Lilly, unpublished data), it therefore open new possibilities for research. Because of these previous restrictions there isn't many data currently available regarding the role of PAR2 activation in behaviour, therefore, building on previous work in our lab (Abulkassim et al., 2016), we decided to investigate the role of PAR2 activation via AC-264613 *in vivo*. With that objective, we started by utilising the open-field (OF) test to examine potential effects of PAR2 activation on mice, particularly on locomotor activity and on anxiety-like behaviour. The OF test was originally developed by Calvin Hall in order to study the natural or

spontaneous direction of rodent behaviour (Hall & Ballachey, 1932; Hall, 1934), it is one of the better validated and the most widely used test to study rodent behaviour, allowing measurements of activity such as motor function and emotions such as anxiety levels with thigmotactic behaviour (or wall-seeking behaviour) being one of the most validated parameter assessed (Prut & Belzung, 2003). The OF test was specifically designed for rats, much like most of the experimental models used for preclinical research on anxiety-like behaviour at that time (Buccafusco, 2001). However, recent technical advances in molecular genetics have established mice models as a central element of modern CNS research (Gould, 2009), accordingly this has resulted in the adaptation of most of the well-validated behavioural tests from rats to mice, including for the OF test (Buccafusco, 2001; Gould, 2009; Wahlsten, 2011). Indeed, OF apparatus have been adapted to mice and also allow testing of locomotor activity and anxiety levels by exploiting a natural internal conflict between their natural tendency for exploration particularly of new environments and the necessity to be cautious when exposed to something unknown (Prut & Belzung, 2003).

5.2.2. Rationale.

Although other elements such as genetics, molecular biology or electrophysiology are critically important tools to elucidate and understand molecular mechanisms in the CNS, behaviour and cognition eventually represents the main visible outputs of the CNS functioning. However, despite interesting results regarding the role of PAR2 *in vitro* prompting suggestions that it might be a viable target for the treatment of CNS diseases, the translation of this role on cognition and behaviour haven't been extensively examined so far, therefore, the aim of this study was to provide insights into the effects of PAR2 activation in behaviour.

5.2.3. Method.

We focused on investigating two specific traits: locomotor activity, and anxiety in mice. Initially, we controlled the heterogeneity of our 4 groups by comparing the data obtained during the habituation session in order to ensure that no group effect would impact the results. Then 2 hours after an intraperitoneal injection (IP) of PAR2 activator AC-264613 at different concentrations (100mg/kg, 10mg/kg and 1mg/kg) or of vehicle only (1% Tween 80 in 0.9% saline solution), we measured locomotor activity via measures of the total distance (in cm) travelled (TDT) and of the average velocity (cm/s) of the mice movements during the test session. Finally, we measured anxiety expressed in the mice through thigmotaxic behaviour, by recording the time spent (in seconds) in the centre square of the platform (TIC) but also the number of entries in the centre square (EIN). Following behavioural experiments, we decided to investigate whether PAR2 activation via AC-264613 had a measurable effect on cytokine production in spleen, therefore we subsequently harvested spleens from the experimental animals, prepared single cell suspensions and measured the level of 3 cytokines, Tumour Necrosis factor alpha (TNF-α), Interleukin 6 (IL-6) and Interleukin 17 (IL-17).

5.2.4. Results.

5.2.4.1. No differences were observed during the habituation stage.

Mice were randomly split into 4 groups, and recordings made during the habituation stage confirmed that no significant group effect was observed for all of the 4 parameters investigated in this study. Moreover, no overall interaction was observed (figures 5.1), therefore suggesting that any difference noted subsequently can only be explained by the effect of PAR2 activation. Additionally, the measures recorded to investigate anxiety-like behaviour, time spent in centre square and number of entries, and the measures used to assess locomotor activity, total distance travelled and average velocity displayed similar overall patterns.

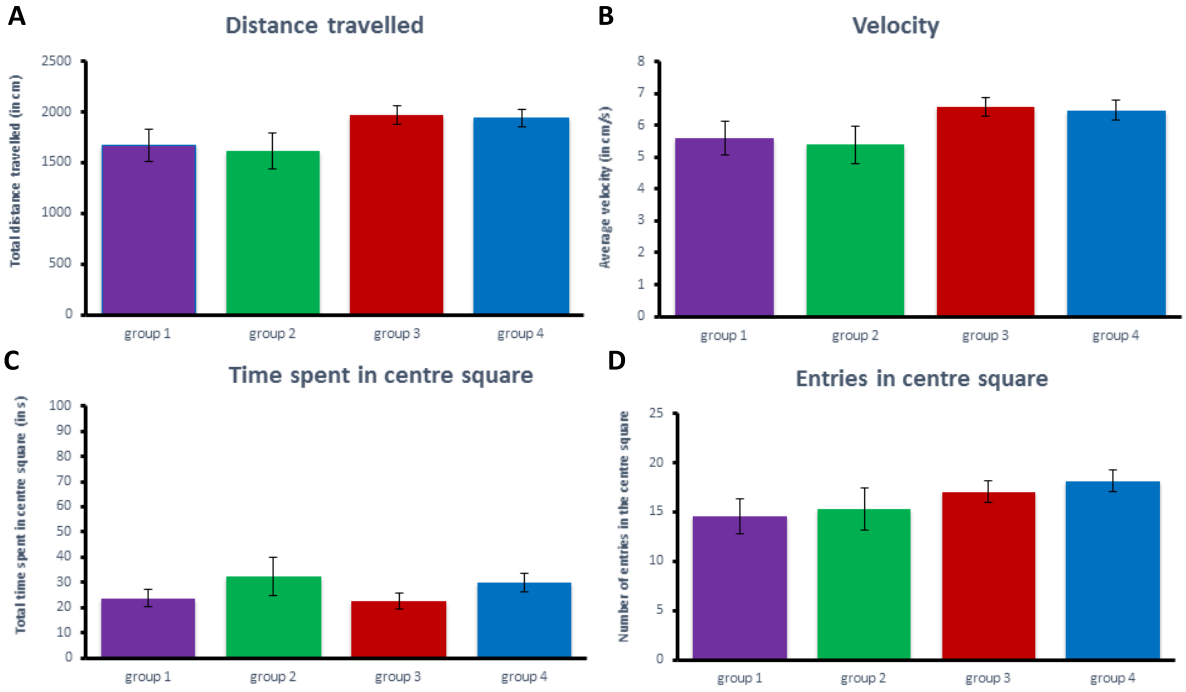


Figure 5.1 – Identification of potential group effects in the open-field test during the habituation session in C57BJ/6 mice. (A) Bar chart summarising the total distance (in cm) travelled by mice during the habituation session of the open-field test. (B) Bar chart summarising the recording of average velocity (in cm/s) displayed by mice in the open-field arena during the habituation session. (C) Bar chart summarising the time (in seconds) spent in centre square of the open-field arena during the habituation session. (D) Bar chart summarising the number of entries in the centre square of the open-field arena during the habituation period. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=6 for each group.

5.2.4.2. AC-264613 reduces locomotor activity and increases anxiety-like behaviour in the OF test.

In order to determine the effects of PAR2 activation on behaviour, we decided to record 4 parameters and compare the results following injections of different doses of the PAR2 activator AC-264613 100mg/kg i.p. resulted in a significant reduction in locomotor activity as demonstrated by measures of the total distance travelled (figure 5.3) and average velocity during the open field recording session (278 ± 38.91 cm; n=6, $p<0.001$, figure 5.2 A and figure 5.2 B and 0.93 ± 0.13 cm/s; n=6, $p<0.001$, respectively compared to the control condition where only vehicle was injected). However, injections of AC-264613 at lower concentrations (10mg/kg and 1mg/kg i.p.) resulted in no significant changes in measures of distance travelled (1116 ± 113 cm; n=6, $p<0.001$, figure 5.2 A for 10mg/kg and 1166 ± 81.76 cm; n=6, $p<0.001$, figure 5.2 A for 1mg/kg, compared to the control condition where only vehicle was injected; 1357 ± 50.34 cm; n=6). Looking at measures of anxiety, injections of AC-264613 (100mg/kg and 10mg/kg) resulted in increased anxiety-like behaviour as illustrated by measures of the time spent in the central square (2.49 ± 1.23 seconds; n=6, $p<0.001$, for 100mg/kg and 13.45 ± 2.1 seconds decrease; n=6, $p<0.001$, figure 5.2 C for 10mg/kg, compared to the control condition where only vehicle was injected) and the total number of entries in the centre square (0.97 ± 0.38 entries in centre square; n=6, $p<0.001$, figure 5.2 D for 100mg/kg and 9.03 ± 1.38 entries in centre square; n=6, $p<0.001$, figure 5.2 D for 10mg/kg, compared to the control condition where only vehicle was injected). However, injections of PAR2 activator at 1mg/kg didn't induce any significant changes in mice anxiety-like behaviour compared to the control group injected with vehicle only (13.11 ± 1.25 entries in centre square; n=6 and 23.88 ± 2.25 seconds; n=6 for time spent in centre square compared to the mice having received only vehicle injections 14.86 ± 0.97 entries in centre square; n=6 and 27.15 ± 2.21 seconds; n=6 respectively, figure 5.2).

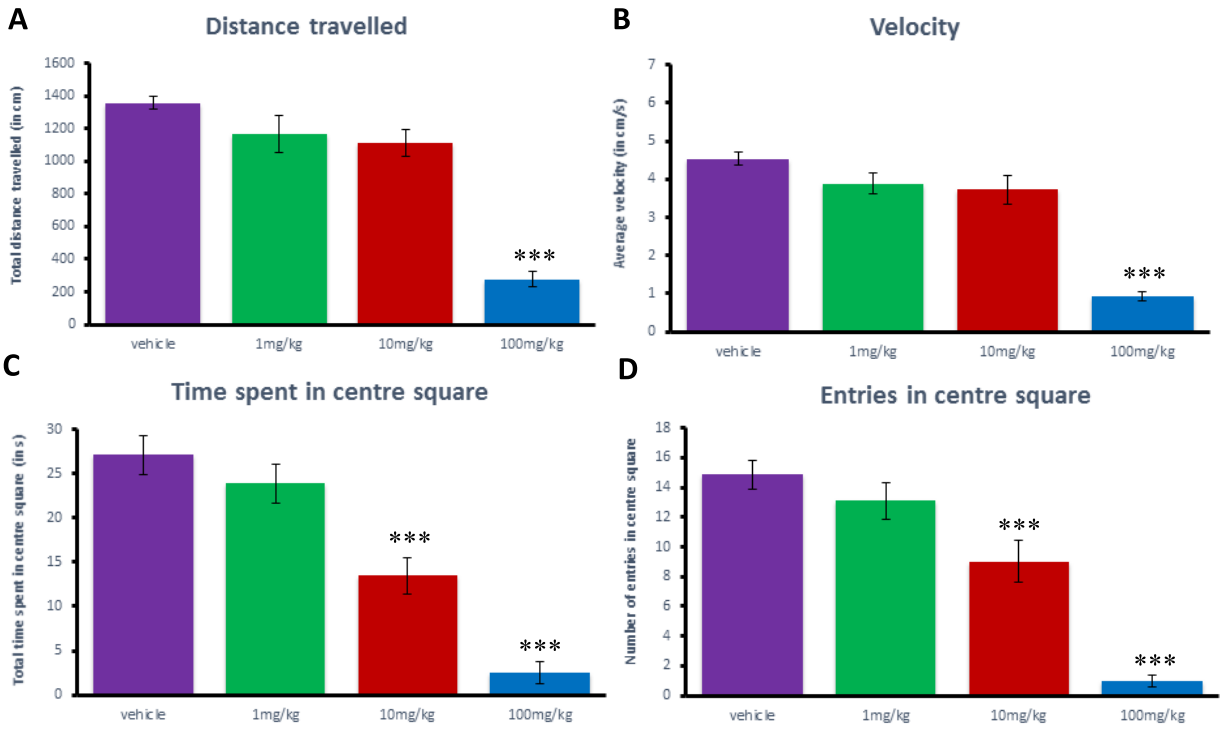


Figure 5.2 – Effect of PAR2 activation on locomotor activity and anxiety-like behaviour in C57BJ/6 mice. (A) Bar chart summarising the total distance travelled (in cm) by mice during the open-field test. (B) Bar chart summarising the recording of average velocity (in cm/s) displayed by mice in the open-field arena. (C) Bar chart summarising the time spent (in seconds) in centre square of the open-field arena. (D) Bar chart summarising the number of entries in the centre square of the open-field arena. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=6 for each group: ***= p< 0.001 compared to vehicle control group.

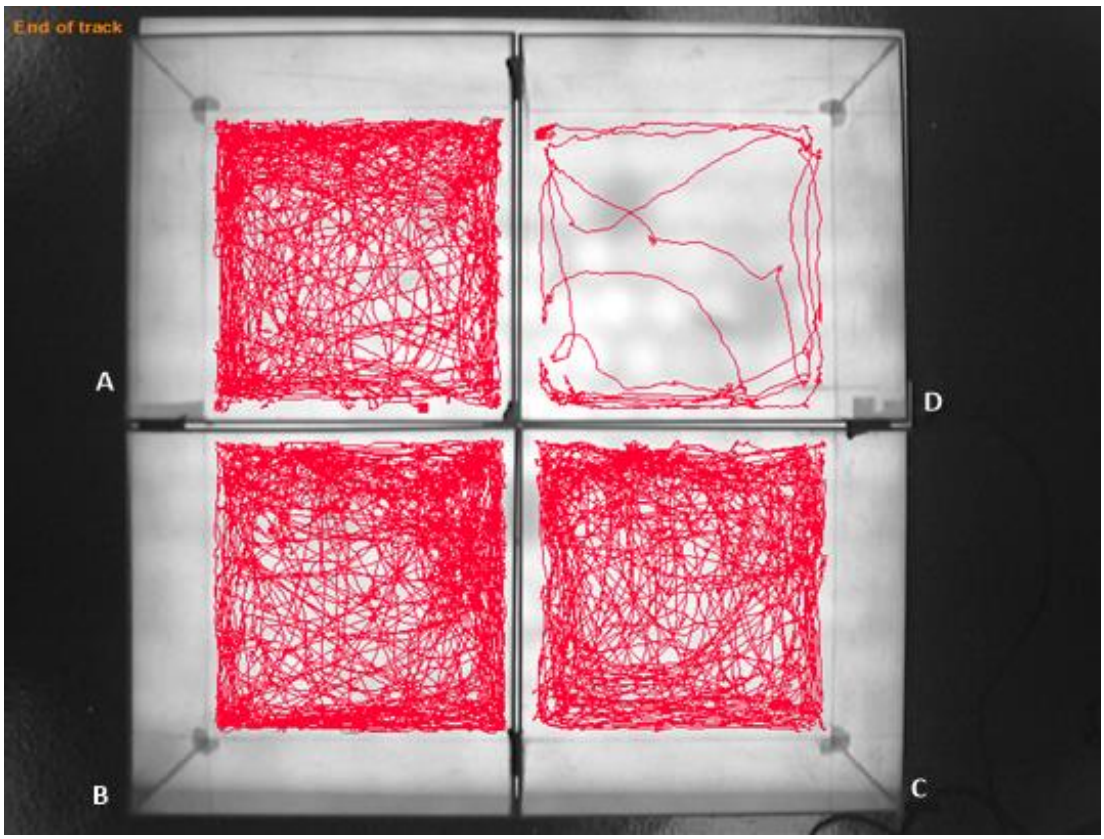


Figure 5.3 - Trajectories track plots showing C57BJ/6 mice exploration during the open-field test exploration. (A) Animal injected with vehicle, (B) animal injected with 1mg/kg, (C) animal injected with 10mg/kg and (D) animal injected with 100mg/kg. Track plot recorded over a 30 min period.

5.2.4.3. PAR2 activation induces increases in splenic IL-6 production.

Given that PAR2 is closely linked to inflammatory process in CNS disorders and it has previously been shown that PAR2 contributes to sickness-like behaviour (Abulkassim et al., 2016), we decided to determine whether AC-264613 injection elicited an inflammatory response. Hence we used spleen cultures to analyse TNF-α, IL-6 and IL-17 levels in all 4 test groups. Using an ELISA, we observed that AC-264613 injection increased splenic IL-6 levels in a dose-dependent manner with both 100mg/kg (131.3 ± 13.5 pg/ml; $p<0.001$,) and 10mg/kg (100.5 ± 11.5 pg/ml; $p<0.001$, figure 5.4 A) being significantly elevated compared to vehicle injected controls (58.56 ± 6.9 pg/ml; $p<0.001$). In contrast, AC-264613 injections did not affect splenic IL-17 (133.6 ± 7.7

pg/ml; for 100mg/kg, 144 ± 5.9 pg/ml; for 10mg/kg and 130.5 ± 7.78 pg/ml; figure 5.4 B for 1mg/kg) or TNF-α levels (62.7 ± 4.5 pg/ml; for 100mg/kg, 60.4 ± 2.5 pg/ml; for 10mg/kg and 69.6 ± 4.9 pg/ml; figure 5.4 C for 1mg/kg) when compared to vehicle injected controls (112 ± 10.26 pg/ml; and 61.6 ± 5 pg/ml; figure 5.4).

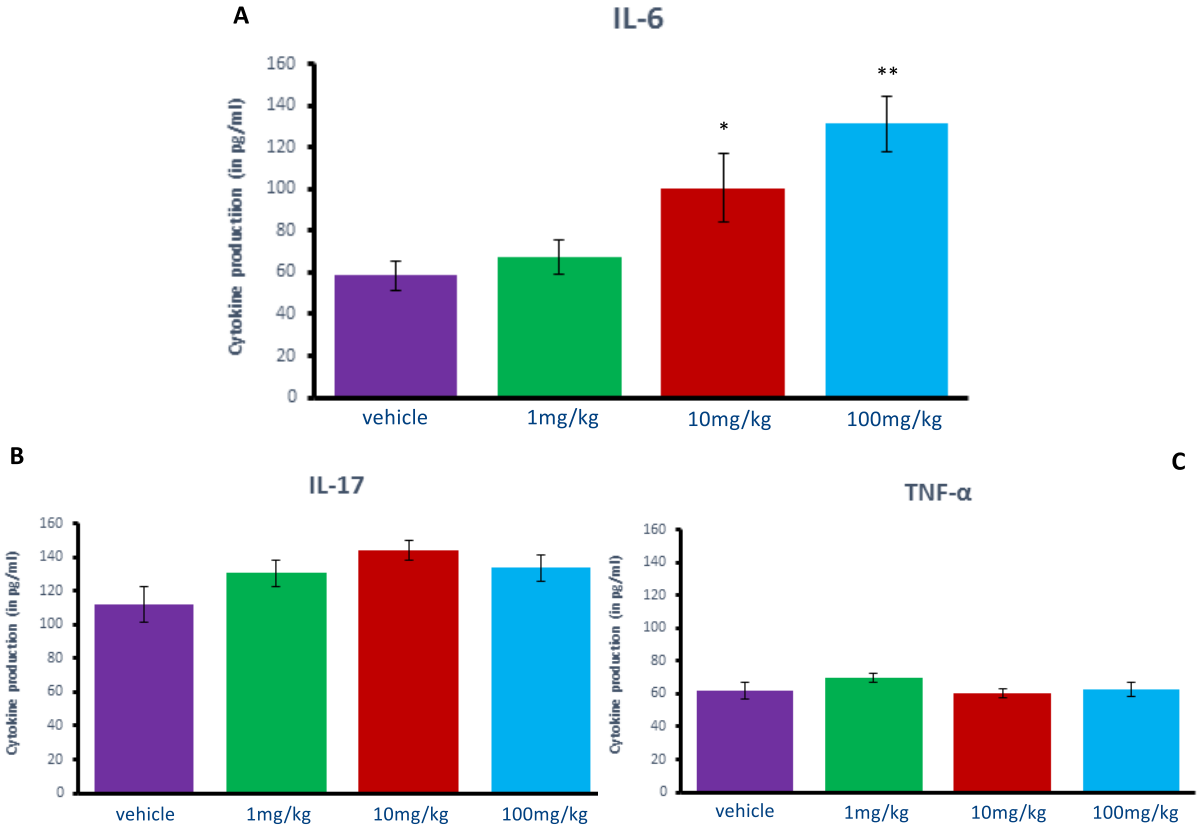


Figure 5.4 – Effect of PAR2 activation on IL-6 production by splenic cells in C57BJ/6 mice. (A) Bar chart summarising spleen IL-6 production in mice following injection of PAR2 activator AC-264613. (B) Bar chart summarising spleen IL-17 production in mice following injection of PAR2 activator AC-264613. (C) Bar chart summarising spleen TNF-α production in mice following injection of PAR2 activator AC-264613. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=6 for each group: *= $p<0.05$ and ***= $p<0.001$ compared to vehicle control.

5.3. Investigation of the effect of PAR2 activation on cardiovascular function.

5.3.1. Introduction.

Preliminary *in vitro* studies are an important part of the drug discovery process as they provide an early indication of potential toxic effects (Hughes et al., 2011), but there is still a subsequent need for *in vivo* testing in order to determine and analyse other unwanted physiological effects. Safety pharmacology has obvious clinical importance as a pharmaceutical company will not commit large amount of resources for the development of a drug which they will be unable to market. Thus being able to detect such side effects as early as possible regardless of the drug potential is critical. Testing the effects of potential new drugs on cardiovascular functions *in vivo* is a particularly critical stage, as such, determining the effect of a new drug on heart rate, arterial blood pressure, electrocardiogram (ECG) and contractile function, mostly in rodents is now routinely conducted as a proactive way to support drug development (Baumans, 2004; Guth, 2007). For reasons of practicality and to ease animal handling, it was necessary to use anaesthetised mice, however the effects of anaesthesia on cardiac function have been well established (Roth et al., 2002; Constantidines et al., 2010). We resolved to the use of 1.5-2% of isoflurane, previously identified as an optimum concentration allowing sufficient anaesthetic depth while ensuring stability of the murine cardiovascular functions while having low toxicity and allowing rapid recovery (Constantidines et al., 2010), we also used results obtained from the vehicle controls groups and baseline measurements to minimise the effect of the anaesthesia on the results.

5.3.2. Rationale.

Recent advances in the understanding of PAR2 and its role as potential target for a variety of diseases, will undoubtedly drive more and more interest in PAR2 based therapeutics interventions (Yau et al., 2013) which explain why understanding the effects of PAR2 activation on vital peripheral functions need to be comprehensively scrutinised. Previous studies had reported potential detrimental effect of PAR2 activated peptides on endothelium function when administered intravenously in rodents (Damiano et al., 1999). However, because AC-264613 is a different type of PAR2 activator, suitable for CNS studies since it can cross the blood brain barrier, but also more specific and requiring lower concentration than most PAR2 activating peptides, we considered it necessary to examine its specific impact on cardiac function. Furthermore, considering previous results from the open-field test, where we observed significant decrease in locomotor activity and increased anxiety-like behaviours for the 100mg/kg and 10mg/kg doses, it was important to be able to determine whether the differences observed could be explained by peripheral effects. Additionally, to measures of cardiac viability, measurements of heart rate (HR) per minute, central to understanding cardiovascular control under normal conditions (Ho et al., 2012) were taken from echocardiographic recordings and used to quantify any direct effect of PAR2 activation on this variable. Mice provide a useful platform as miniaturised equipment allow simple measurements with either conscious or anaesthetised animals, therefore allowing us to use 6/8 weeks old females C57BJ/6, similar to those used for behavioural experiments. Murine electrocardiogram and heart rate analysis allowed non-invasive *in vivo* assessment of cardiac function under normal physiological conditions and also 2 hours after the animals were injected with PAR2 activators.

5.3.3. Method.

The animals were lying on their back and breathing trough a mask with 1.5-2% isoflurane in O^2. During the cardiac cycle, ejection phase (systole) and during the relaxation phase (diastole), we monitored the left ventricle to measure both diameter and cavity dimensions, measurements of wall thickness were carried out at the thickest part of the wall (figure 5.5 A), additionnally, we measured the number of heart beats over 1000ms periods (figure 5.5 B). On the first day we recorded baseline levels for each mice, then a week later we took the same measurements again 2-hours following injections of PAR2 activator AC-264613 at different concentrations (100mg/kg, 10mg/kg and 1mg/kg with a control group receiving only a vehicle injection), finally, to obtain the more accurate possible results three typical cycles were analysed and averaged.

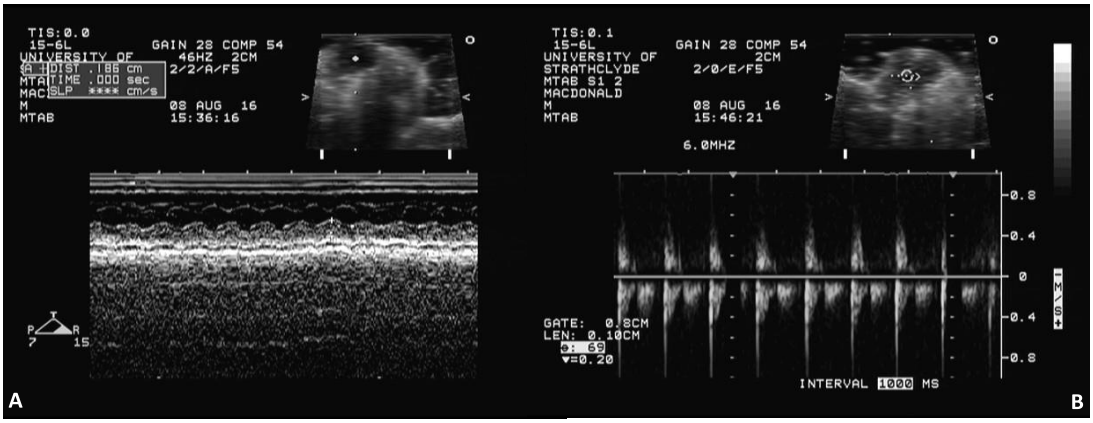


Figure 5.5 - Representative M-mode echocardiographic images as used to calculate fractional shortening and heart rate in C57BJ/6 mice. (A) Measurement of left ventricular end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD) was obtained from M-mode. (B). HR was obtained by monitoring the number of R waves during 1000ms intervals in the ECG tracing.

5.4. Results.

5.4.1. Baseline measurements revealed no difference in HR and fractional shortening between test groups.

Baseline recordings were taken for each mouse, using M-mode ECG images. Initially, cardiac dimensions were measured, which subsequently allowed calculation of fractional shortening, an estimate of myocardial contractility often used as a guide to cardiac function (Baicu et al., 2005) and heart rate, whose variation can reflect changes in physiological condition (Ho et al., 2012). These values were used as baseline controls. For both of these parameters, no significant differences between test groups was observed (figures 5.6 A and 5.6 B), therefore suggesting that any difference noted following injections can only be explained by the effect of PAR2 activation.

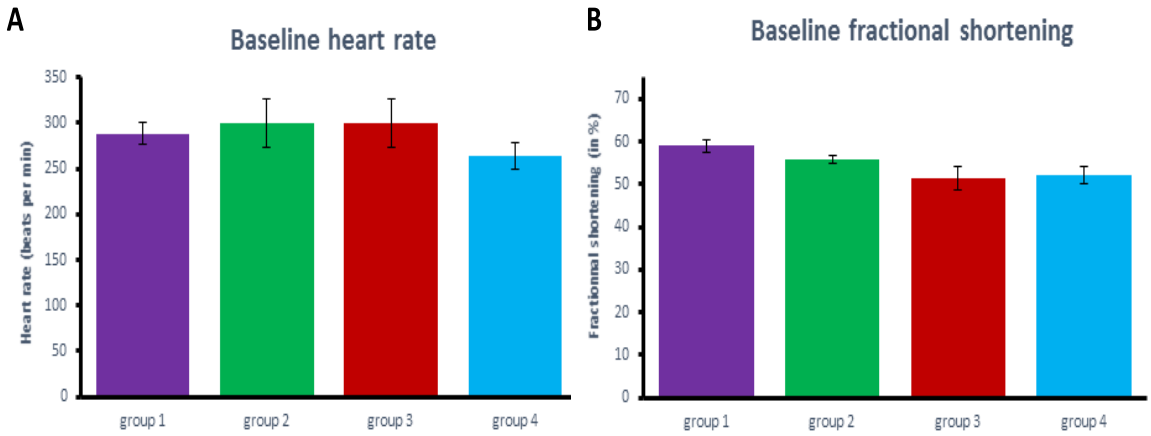


Figure 5.6 – Identification of potential group effects on heart rate and fractional shortening during the baseline measures in C57BJ/6 mice. (A) Bar chart summarising the heart rate measured during the baseline experiments across the 4 test groups. (B) Bar chart summarising measures of left ventricular fractional shortening as calculated during the baseline experiments for all 4 test groups. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=5 per group.

5.4.2. PAR2 activation has no significant effect on heart rate.

In order to observe potential peripheral effects of PAR2 activation, we started by monitoring heart rate (HR). We observed no effect of PAR2 activation on HR compared to the control group where mice were injected with vehicle only. In details the group where mice had been injected with 100mg/kg (360 ± 26.8 beats per min; n=5, figure 5.7), the group where mice had been injected with 10mg/kg (336 ± 24 beats per min; n=5), and the group where mice had been injected with 1mg/kg (336 ± 14.7 beats per min; n=5), were not significantly different from the control group where mice had been injected with vehicle only (312 ± 12 beats per min; n=5, figure 5.7).

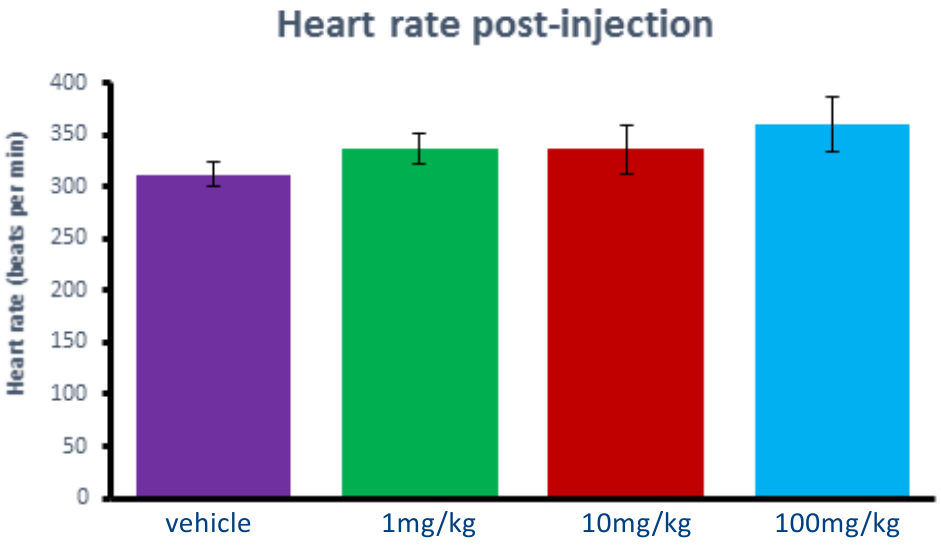


Figure 5.7 – Effect of AC-264613 injections on heart rate measures in C57BJ/6 mice. Bar chart summarising the measures of heart rate taken two hours following injections of PAR2 activator AC-264613. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=5 for each group.

5.4.3. PAR2 activation improves cardiac function in C57BJ/6 mice.

Having established that AC injections had no effect on HR, we then decided to look at fractional shortening, which is considered a good measure for observing changes in cardiac function (Ho et al., 2012). We observed a significant improvement of fractional shortening following injections of PAR2 activator AC-264613 at 10mg/kg (50.25 ± 0.67% fractional shortening; n=5, $p<0.05$, figure 5.8) and at 1mg/kg (55.45 ± 0.78% fractional shortening; n=5, $p<0.001$). It is important however to note that we were only able to correctly calculate fractional shortening from 1 mouse out of 6 from the 100mg/kg group due to incoherent data, suggesting that the sample might not be representative, more information will be needed to confirm these results.

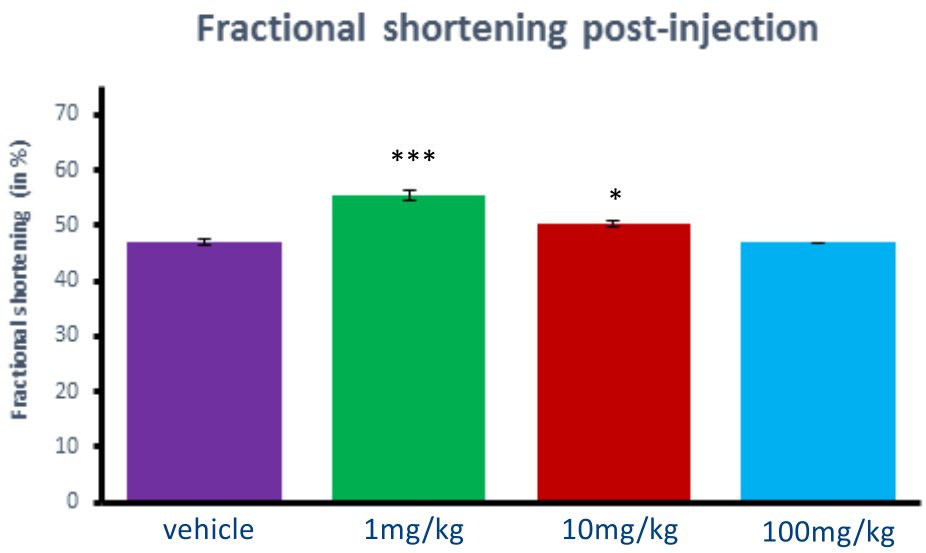


Figure 5.8 – Dose dependent effect of AC injections on fractional shortening in C57BJ/6 mice. Bar chart summarising measures of left ventricular fractional shortening taken two hours following injections of PAR2 activator AC-264613. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=6 per group: *= $p<0.05$ and ***= $p<0.001$ compared to vehicle control.

5.5. Investigating the effect of PAR2 activation in the EAE mice model of autoimmune disease.

5.5.1. Introduction.

Animal modelling allowed the emergence of insights into basics of human physiology and disease, it has become a keystone for scientific studies of disease mechanisms and for drug discovery through pre-clinical studies. Over the past several decades, a number of animal models have been specifically developed in order to understand the complexity of the CNS and the variety of its related diseases (Chesselet & Carmichael, 2012). Of those, because of the good level of understanding of the mechanisms involved in its physiopathology, multiple sclerosis (MS) has now been modelled on reliable experimental animal models who have allowed majors advances in drug development (Denic et al., 2011). A number of different animal models have been used to explore MS physiopathology including drug induced demyelination models, genetically modified animal models and viral or antigen-induced encephalomyelitis (Mix et al., 2010). Of the latter type, experimental autoimmune encephalomyelitis (EAE) is the most extensively studied model of autoimmune disease and it has been used to develop many of the current drugs used in the treatments of this diseases (Constantinescu et al., 2011). EAE is a model of MS in which a combination of pathological immunological responses leads to inflammation of the CNS (Rivers et al., 1933), thus approximating some of the key pathological features of MS (Lebar et al., 1986). In mice models of EAE, MOG_{35-55} emulsified in complete Freund's adjuvant (CFA) is the most common approach to generate active immunisation (Stromnes & Goverman, 2006). Myelin oligodendrocyte glycoprotein (MOG), a member of the immunoglobulin family is expressed exclusively in the central nervous system and is involved in myelin production (Slavin et al., 1998), therefore models using MOG_{35-55} allow to reproduce demyelination as seen in the EAE model (Ichikawa et al., 1999). By inducing auto-antibody production MOG_{35-55} peptide induce a monophasic EAE with first symptoms after 9-14 days, disease peak 3 to 5 days after the onset, followed by a slow symptom recovery over the next 7

days (Bittner et al., 2014). However, as the immunogenic potential of MOG_{35-55} peptide alone is not sufficient to induce EAE disease, it needs to be complemented with adjuvants such as CFA, able to activate mononuclear phagocytes thus inducing the phagocytosis of these molecules and the secretion of cytokines (Constantinescu et al., 2005), EAE induction is further facilitated by application of pertussis toxin which accelerate the immunological response (Hofstetter et al., 2002). This combination results in microglial, astrocytic and macrophages activation and thus blood brain barrier disruption, thereby leading to T-cells and macrophages recruitment from the periphery into the CNS, where they initiate autoimmune inflammation, thus leading to further demyelination (Stromnes & Goverman, 2006). Axonal and neuronal losses and in some cases relapses, which are all important features of MS physiopathology and have been described in EAE (Constantinescu et al., 2011), hence making it an excellent model to study MS aetiology as well as to explore potential drugs for CNS diseases. It is also important to note that following a few days of clinical signs, partial or complete remission of EAE symptoms is normally observed (Stromnes & Goverman, 2006), but not always, therefore it is important to be able to observe the effects of potentially disease modifying drugs at different time point to match the observations made in MS.

5.5.2. Rationale.

Multiple Sclerosis is the most common autoimmune disease affecting the CNS and usually causes significant disability (Constantinescu et al., 2011), although the exact causes of MS are still unknown, it is considered to be mainly mediated by T-cells and also B-cells and plasma cells (Stromnes & Goverman, 2006). Considering PAR2 links with inflammation in the periphery and its suggested role in CNS inflammation (Suen et al., 2014), we decided, in order to probe the effects of PAR2 activation, to use the EAE model, a well characterised model of CNS inflammation, as a platform to investigate its role in CNS diseases, in a similar manner to what was previously done, however relying mostly on PAR2 KO animals rather than PAR2 activators (Noorbakhsh

et al., 2006). Moreover, recent studies have suggested a neuroprotective role for alpha-crystallin in both EAE and MS (Ousman et al., 2007) and it has been proposed that these properties might be mediated by PAR2 (Li et al., 2009). Additionally, it has been proposed that PAR2 might be involved in the transduction of inflammatory signalling particularly of IL-6 and IL-17 (Liu et al., 2014), while the important role of these inflammatory cytokines has previously been highlighted as key event in EAE development (Penkowa & Hidalgo, 2001). Finally, the EAE model offers a good platform to study and to observe the effects of PAR2 activation over different period of time, including the onset, the peak and the first resolution of the disease, which is particularly useful to study relapses.

5.5.3. Method.

In order to generate EAE, age-matched female C57BJ/6 mice were immunised with a combination of synthetic MOG and CFA, followed by pertussis toxin. Using this model, it is estimated that more than 80% of the animal will develop a severe, chronic disease expression, from 7 to 14 days following immunisation (Stromnes & Goverman, 2006). For the duration of the experiments, animals were handled daily, weighted and assessed for EAE severity using a 0–5 rating scale (Keating et al., 2009; table 2.2). Additionally, from the onset of clinical signs and for 7 days, mice were injected with PAR2 agonist AC-264613 at different concentrations (1, 10 and 100 mg/kg) with a control group injected with vehicle only (1% Tween 80 in 0.9% saline solution). Finally, at the end of EAE experiments after 32 days we harvested spleens from the animals and analysed cytokine expression using ELISA in MOG-stimulated spleen cells consecutively to 48 hours in culture.

5.5.4. PAR2 activation decreases EAE clinical signs in mice.

We sought to evaluate the effect of PAR2 activation on EAE induced clinical signs, which we achieved by averaging the clinical scores recorded daily for every group of mice. We observed that the group injected with AC 10 mg/kg i.p. displayed significantly reduced clinical signs (0.30 ± 0.8 average clinical score; n=10, $p<0.001$ compared to the vehicle injection group, figure 5.9 A). On the other hand, we observed no overall effect of the 100mg/kg and 1mg/kg injections on clinical scores compared to the group injected with vehicle only (0.57 ± 0.14 average clinical score; n=9 and 0.53 ± 0.08 average clinical score; n=11 respectively compared to the control group 0.68 ± 0.08 average clinical score; n=12, figure 5.9 A). However, it is important to underline the fact that 3 animals from the group of mice injected with 100mg/kg died or had to be sacrificed for ethical reasons.

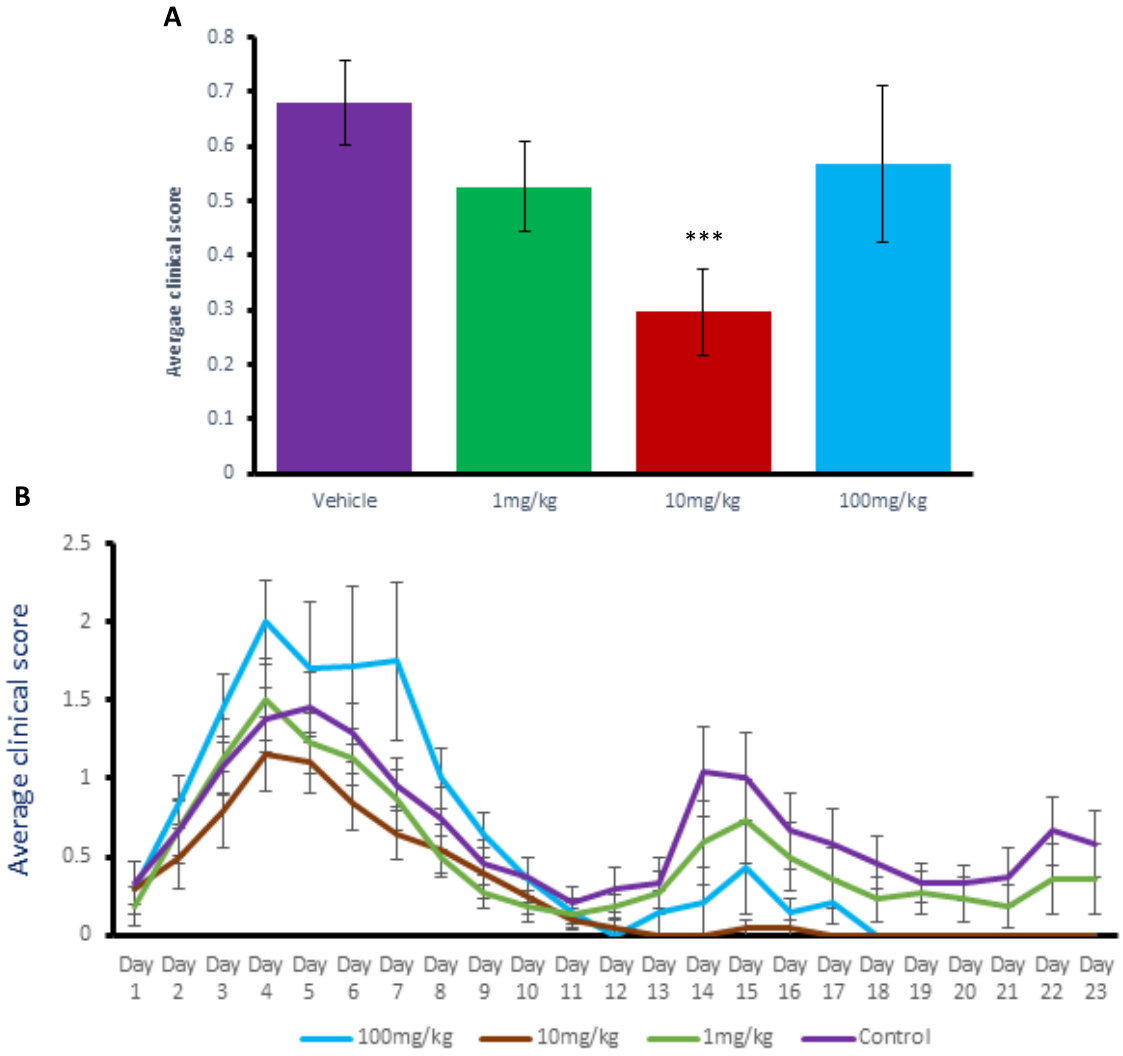


Figure 5.9 – Effect of AC-264613 on EAE clinical score in C57BJ/6 mice. (A) Bar chart summarising the effects of PAR2 activation on clinical scores observed over the whole experiment following injection of PAR2 activator AC-264613. (B) Line chart summarising the average clinical scores observed in the EAE mice model following injection of PAR2 activator AC-264613. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons (A) and a 2-way ANOVA with Dunnett's post hoc analysis (B), presented as mean ± S.E.M. with n≥10 for each group: **= $p<0.01$ and ***= $p<0.001$ compared to vehicle control.

5.5.5. EAE clinical signs are reduced at the peak of disease following PAR2 activation in mice.

We then tried to determine if we could identify the moment where injections of AC-264613 at 10mg/kg were inducing a change in clinical expression. Therefore, we studied averages daily clinical scores from the onset of clinical signs considered as day 1 (figure 5.10) but also from the peak of the disease severity which was reached at day 5 post disease onset and finally we looked at the clinical signs expressed after the first resolution after day 12 to study what can be considered as relapses. When looking at the clinical expression from the peak of disease severity (day5), we observed that AC-264613 injections at 1mg/kg, at 10mg/kg and at 100mg/kg induced reductions in clinical score (0.45 ± 0.07 average clinical score; n=11, $p<0.001$, 0.21 ± 0.08 average clinical score; n=10, $p<0.001$ and 0.44 ± 0.14 average clinical score; n=10, $p<0.05$ respectively compared to the control group, 0.64 ± 0.08 average clinical score; n=12 figure 5.10).

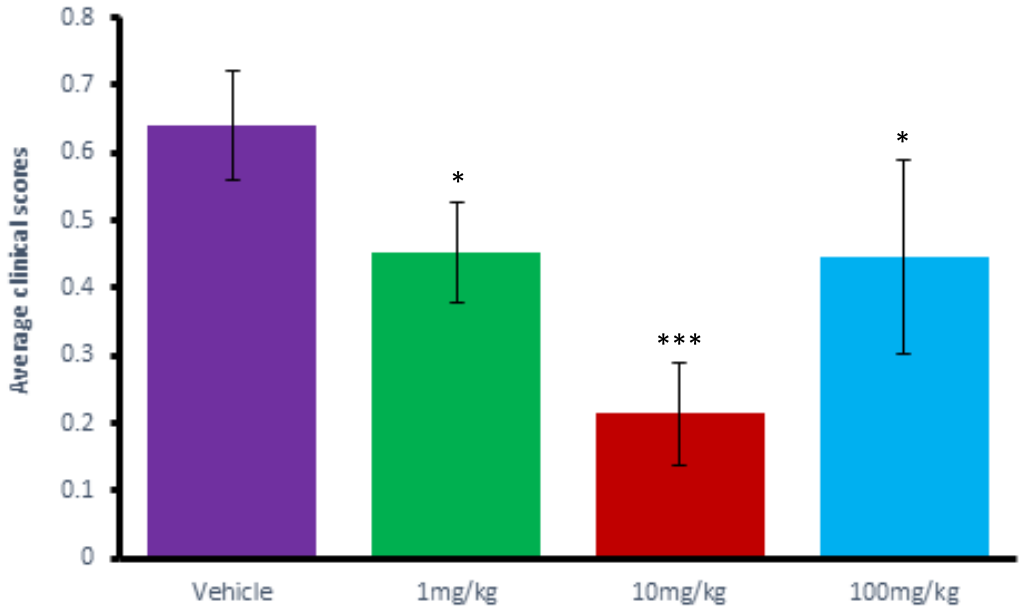


Figure 5.10 – Effect of AC-264613 on EAE clinical scores from the peak of the diseases in C57BJ/6 mice. (A) Bar chart summarising the effects of AC-264613 on clinical scores observed from the peak of clinical signs (day 5). Data were analysed using a one-way ANOVA and Dunnett's post-test for multiple comparisons and presented as mean ± S.E.M. with n≥10 for each group: *= $p<0.05$ and ***= $p<0.001$ compared to vehicle control.

5.5.6. EAE clinical signs are reduced from the resolution of disease following PAR2 activation in mice.

We then studied the clinical signs from the resolution (day12) and observed that PAR2 activation at 100mg/kg, at 10mg/kg and at 1mg/kg induced a reduction in average clinical scores (0.06 ± 0.03 average clinical score; n=10, p<0.001 for the group injected with 100mg/kg doses, 0.01 ± 0.006 average clinical score; n=10, p<0.001 for the group injected with 10mg/kg doses, and 0.36 ± 0.05 average clinical score; n=11, p<0.001 for the group injected with 1mg/kg doses, compared to the vehicle control group, 0.56 ± 0.08 average clinical score; n=11 respectively, figure 5.11).

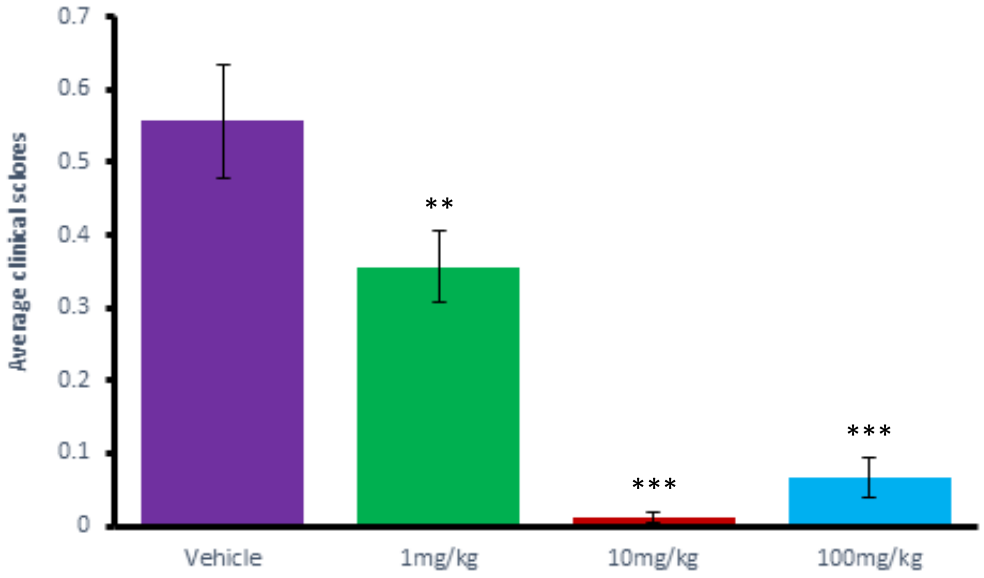


Figure 5.11 – Effect of AC-264613 on the incidence of relapse of EAE scores in C57BJ/6 mice. Bar chart summarising the relapsing clinical scores following injection of PAR2 activator AC-264613. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons and presented as mean ± S.E.M. with n ≥10 per group: *= p<0.05, **= p<0.01 and ***= p<0.001 compared to vehicle control.

5.5.7. PAR2 activation increases splenic cytokine production in the EAE mice model.

As we previously established that AC-264613 selectively increased IL-6 production in naïve mice, we next studied whether it modulated their cytokine production in the EAE mouse model. We therefore, analysed IL-6, TNF-α and IL-17 concentrations in spleen cultures from EAE mice using ELISA. We observed that PAR2 activation resulted in an upregulation of all 3 cytokines examined. IL-6 levels were upregulated in splenic cultures from the groups injected with AC-264613 for 7 days at all 3 concentrations used in this study (1479.54 ± 68.3 pg/ml; $p<0.001$, for the mice injected with 100mg/kg, 1428.65 ± 142.2 pg/ml; $p<0.001$ for mice injected with 10mg/kg and 658.21 ± 48.65 pg/ml; $p<0.001$ for mice injected with 1mg/kg compared to the vehicle control group, 468.07 ± 152.33 pg/ml, figure 5.12 A). IL-17 levels were upregulated in splenic cultures from the groups injected with AC-264613 for 7 days with 100mg/kg and for the group receiving 10mg/kg (1875.16 ± 60.5 pg/ml; $p<0.001$, 1833.89 ± 70.88 pg/ml; $p<0.001$ respectively compared to the vehicle control group) while there were no significant effect of the 1mg/kg injections on splenic IL-17 production (1070.5 pg ± 95.36 pg/ml; compared to the vehicle control group, 635.9 ± 112.15 pg/ml, figure 5.12 B).TNF-α levels were increased in splenic cultures from the groups injected for 7 days with 100mg/kg and 10mg/kg respectively (100 mg/kg: 1211.14 ± 204.5 pg/ml; $p<0.001$ compared to vehicle control; 10 mg/kg: 901.34± 113.94 pg/ml; $p<0.001$, compared to the vehicle control, figure 5.12 C) while there were no significant effect of the 1mg/kg injections on splenic TNF-α production (541.43 ± 57.47 pg/ml; compared to the control group 690.86 ± 101.68 pg/ml).

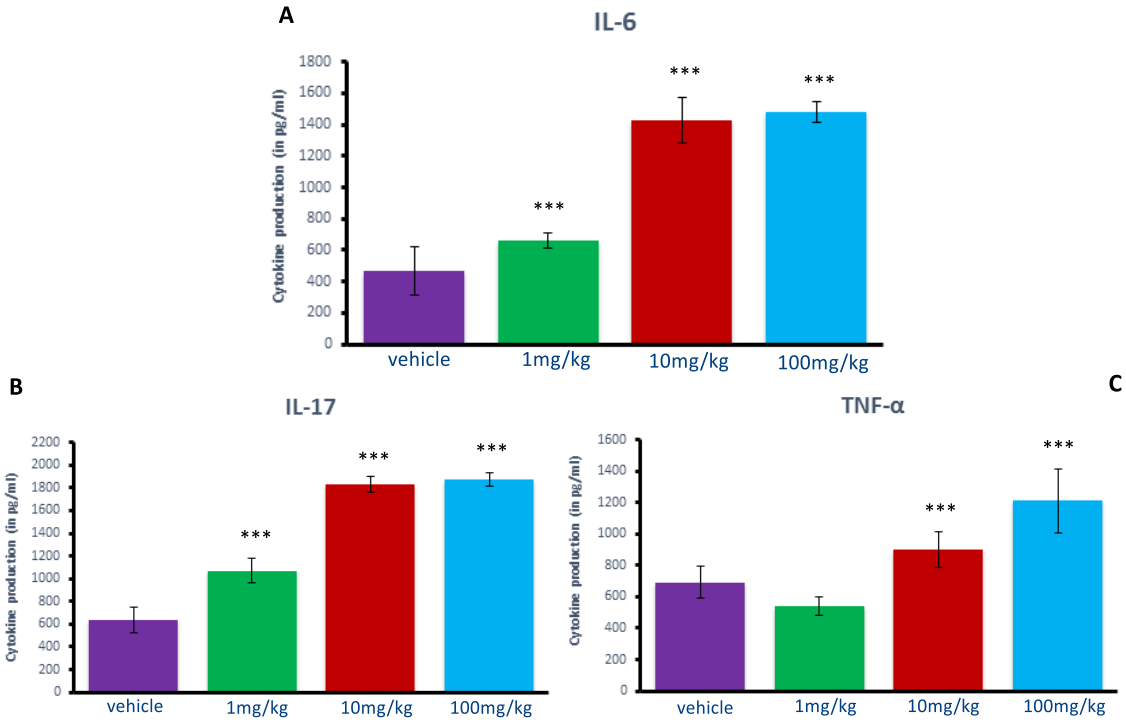


Figure 5.12 – Effect of AC-264613 on splenic cytokine expression in EAE mice. (A) Bar chart summarising spleen IL-6 production in mice following injection of PAR2 activator AC-264613. (B) Bar chart summarising spleen IL-17 production in mice following injection of PAR2 activator AC-264613. (C) Bar chart summarising spleen TNF-α production in mice following injection of PAR2 activator AC-264613. Data were analysed using a one-way ANOVA and Bonferroni post-test for multiple comparisons, presented as mean ± S.E.M. with n=6: *= $p<0.05$ and ***= $p<0.001$ compared to vehicle control.

5.6. Discussion.

5.6.1. PAR2 activation increases anxiety-like behaviours and might affect locomotor activity in mice.

The open-field test is a very established and simple sensorimotor assessment used to measure general activity levels and exploration habits in mice through recording of a variety of parameters including distance moved and velocity which can be correlated with locomotive function (Prut & Belzung, 2003). It is also used to model human pathological anxiety in animals by exploiting internal conflicts between natural behaviours such as exploration of new places and spontaneous aversion for open environments normally inducing a fearful response, this is normally assessed via analysis of the time spent in pre-defined zones and can be correlated with anxiety levels in mice (Buccafusco, 2001). Hence, because it allows to record a variety of behaviours in flexible experimental designs the open-field test is now one of the most validated and popular *in vivo* tools available to initially evaluate the effect of novel drugs on general activity as well as on CNS function (Hånell & Marklund, 2014). In this study we aimed to study the effects of PAR2 activation on locomotor function and on anxiety levels, considering pharmacokinetics data obtained from Eli Lilly suggesting that AC-264613 reaches its peak concentration in the CNS about 2 hours post injections (Eli Lilly, unpublished data) we analysed the effects of PAR2 activation after 2 hours. We first assessed locomotor function by recording distance travelled and velocity, since in mice very significant decreases in locomotor activity are considered as symptoms of sickness behaviour (Huang et al., 2009; Maes et al., 2012). Then, in order to study anxiety, we recorded thigmotaxis, which refers in the open-field test to mice natural tendency to stay in the closest contact possible with the wall. It is one of the most employed and best validated parameters to measure anxiety-like and depressive-like behaviours (Gould, 2009), indeed, pharmacological studies, have demonstrated that anxiogenic drugs induce increased thigmotaxis (Simon et al., 1994). We observed that AC-264613 injections at 100 mg/kg induced very significant changes in all the parameters measured, in details, we recorded large decreases in

locomotor activity and velocity combined with increased anxiety-like behaviour in these mice, which might also be correlated. Because previous studies have identified a correlation between sickness-like behaviour and elevated levels of anxiety (Biesmans et al., 2013), we can assume that these high doses of AC-264613 might have induced sickness-like behaviour which would be the cause for both decreased locomotor activity and increased anxiety-like behaviours we measured. These might also be impacted by peripheral effects induced by the high concentration of PAR2 activator, therefore, more specific experiments are required in order to precise the causes of these results.

Additionally, the 10mg/kg doses had no significant effect on distance travelled or on velocity, suggesting that only higher concentrations might impact mice locomotor abilities. However, when looking at both parameters used to assess anxiety-like behaviour, we observed significant reductions, in the amount of time spent in centre square as in the number of individual entries made into the centre square. Accordingly, we can assume that in this study, the increased thigmotaxis observed in both groups of mice injected with 100mg/kg and with 10mg/kg doses is a reflexion of increased anxiety-like or depression-like behaviour induced by PAR2 activation in these mice. Finally, we observed small but not significant reductions in both these parameters for the group of mice injected with 1mg/kg of AC-264613. The rare studies investigating the effect of PAR2 activation in behaviour have reported limited general effects on locomotor activity and anxiety except for increased anhedonia and sickness-like behaviour (Abulkassim, 2014, unpublished work). Although these observations were made on male animals and only 30 min post-injections, they still can be linked with the changes we monitored for the mice injected with 10mg/kg as both anhedonia and sickness-like behaviour have been correlated and associated with increased levels of anxiety and depression (Strekalova et al., 2004). Additionally, no difference was recorded for mice injected with the lower 1mg/kg doses, therefore suggesting that only elevated concentration of PAR2 activators might induce anxiogenic effects. This suggestion is further supported by the fact that previous experiments in our lab looking at the effect of PAR2 KO on behaviour, haven't

observed any increase in anxiety-like behaviours or locomotor activity (Abulkassim et al., 2016).

5.6.2. PAR2 activation increases splenic IL-6 production in mice.

We have investigated cytokine production in these mice in order to observe potential changes correlated with the behavioural changes monitored in the open-field test and with the doses of PAR2 activators injected. Given that PAR2 has previously been described as pro-inflammatory (Cattaruzza et al., 2014), we have looked at the effect of PAR2 activation on splenic production of 3 cytokine markers of inflammation, TNF-α, IL-6 and IL-17 and we only observed increased IL-6 production on these naïve mice. IL-6, it is secreted by macrophages and T-cells to stimulate the immune response (Hama et al., 1989), with altered levels associated with the physiopathology of diseases such as diabetes, MS, atherosclerosis and depression (Erta et al., 2012). Indeed, both diabetes and depression, have also recently been linked with PAR2 and inflammation (Berk et al., 2013; Kagota et al., 2016) with IL-6 antibody based therapies being investigated for treatment of major depressive disorders (Hodes et al., 2016). If we consider the potential role of IL-6 in depression, we can assume that PAR2 related increases of IL-6 production might have a causative role in the elevated anxiety levels we observed in these mice, manifested via increased thigmotaxis measured at the peak of AC-264613 concentration in the brain. Additionally, because previous studies have suggested that upregulation of IL-6 might not only cause anxiety but also anhedonia (Miller & Raison, 2016), we can speculate that, had it been measured, we would have observed increased anhedonia following PAR2 activation, similarly to what was in previous studies (Abulkassim, 2014, unpublished work). Moreover, previous researches have described that prolonged IL-6 like exposure in the CNS is associated with inducing and maintaining sickness behaviour (Huang et al., 2009), which might be an explanation to the observations made for the higher doses of PAR2 activator AC-264613. Interestingly, this pro-inflammatory cytokine is not

only involved in inflammation responses but also in the induction of anti-inflammatory response in the CNS through regulation of metabolic, regenerative, and neural processes (Scheller et al., 2011) and therefore might play a role in neuroprotection.

5.6.3. PAR2 activation has no detrimental effect on heart function in mice.

Early in the 90's, terfenadine (Hoechst AG) an antihistamine developed to treat allergic conditions including hay fever was found to have potentially lethal cardiac consequences even in patients showing no pre-existing problems and therefore withdrawn from the market (Monahan et al., 1990). Since that episode, safety pharmacology aimed at predicting the toxicity of a drug in order to establish if when taken within the recommended doses or in excess by human or animal subjects is likely to cause adverse effects (Pugsley, 2008), has become a central element of drug discovery (Hughes et al., 2011). Thus, considering the severe detrimental effects observed in locomotor activity for the group of mice injected with 100mg/kg and on anxiety-like behaviour for the groups injected with 100mg and 10mg/kg in the open-field test, it was necessary to evaluate whether these detrimental effects could be accounted for by peripheral activity. Therefore, consecutively to behavioural assessments, we have in this study, investigated the effect of PAR2 activation on 2 measures of cardiac function, fractional shortening and heart rate measured using echocardiographic measurements, by applying the same concentrations we previously used in behavioural testing. Echocardiography is an examination of the heart using ultrasound to detect movements of the left ventricle (Gardin et al., 1995), such as movement and velocity of its walls and valves (Lang et al., 2006), but it is also useful to evaluate the morphology of cardiac structures (Gao et al., 2012). It is traditionally achieved through M-mode which provides a mono-dimensional view of the heart (Gardin et al., 1995). Multiple electrophysiological parameters can be measured, however we mainly focused on analysing measures of heart rate (HR) and

of fractional shortening (FS) considered as the gold standard to assess cardiac viability (Lang et al., 2006) and representing the fraction of any diastolic dimension that is lost in systole (Antoniak et al., 2013). Our results demonstrate that PAR2 activation in naïve animals doesn't negatively impact heart rate and cardiac function, yet more data are needed regarding the effect of 100mg/kg doses on FS that we couldn't measure here. Overall, these results are in line with previous observations suggesting that PAR2 activation albeit chronically, had no negative impact on cardiac function (Hughes et al., 2013). Furthermore, we observed a significant beneficial effect of lower concentrations of AC-264613 on fractional shortening, interestingly and in link with our observation that PAR2 activation induces increase in splenic IL-6 production, previous studies have highlighted a potential beneficial role for IL-6 in the pathophysiology of cardiovascular diseases where it is involved not only in inflammation (Tamariz & Hare, 2010) but also in the regulation of cardiac metabolism (Kanda & Takahashi, 2004).

5.6.4. PAR2 activation reduces clinical signs and relapse occurrence in the EAE mice model.

Multiple sclerosis is an inherently human disease, it has not been observed occurring naturally in any other animal (Denic et al., 2011). Therefore in order to understand and to study specific aspects of the disease, particularly neuro-inflammation, it is at the moment necessary to work on experimental animal models. In the present study, the clinical signs expressed by EAE mice were studied, mice injected from the onset of disease with different doses of PAR2 activator AC-264613 were investigated and compared to mice only injected with vehicle. Furthermore, and also taken into account previous results on naïve mice, we sought to investigate any potential changes caused by PAR2 activation on inflammatory markers well described in the EAE physiopathology such as TNF-α, IL-6 and IL-17 (Penkowa & Hidalgo, 2001; Erta et al., 2012; Jin & Dong, 2013) which were here investigated through splenic cytokine production. The present study is the first to investigate the role of PAR2 activation in

disease symptoms over the whole course of the EAE model from a clinical point of view. We observed an overall beneficial effect, since PAR2 activation significantly reduced the average clinical signs scored in C57BJ/6 mice, this is particularly true for the mice injected with 10mg/kg. Hence on all the parameters we measured, these mice exhibited lower clinical signs than mice from all of other groups. Overall, on the whole course of the EAE disease, no differences were observed between the vehicle control group and both the 100mg/kg and the 1mg/kg groups, however when looking at it in more details we were able to observe interesting changes. Indeed, other than looking at the effects of PAR2 activation over the whole course of the EAE disease, we have also chosen to focus on different time points to study the stage specific impact of PAR2 activation (Bittner et al., 2014), with a particular focus on relapses (Pachner, 2011). Data from this study showed that 7 days of PAR2 activators injections induced very significant reductions in EAE clinical severity when measured from the onset, from the peak and from the resolution of the disease. Looking at the disease from the peak of clinical signs, we observed a very significant decrease induced by 10mg/kg but also by 100mg/kg and 1mg/kg injections. More interesting again, when looking at the clinical signs recorded from the resolution stage when clinical signs have decreased to a minimum and which are often referred to as relapses, PAR2 activation at 100mg/kg and at 10mg/kg induced very significant reductions in the number of relapses, mice having received 1mg/kg also shown significantly reduced relapse severity and incidence compared to the vehicle group. Additionally, we observed that although injections of AC-264613 at 100mg/kg proved to be potentially unsafe for already weakened animals, as highlighted by the death of 3 animals ensuing a few days of injections was still inducing beneficial effects, since surprisingly, other mice in that group still displayed significantly decreased clinical symptoms of EAE. Furthermore, it is interesting to note that PAR2 treatment effects persisted until the end of the experiment, about 2 weeks after the latest injections, this long lasting effect on reducing EAE induced relapse incidence has already been described, including for drugs currently on the market for treatment of MS (Makar et al., 2008; Steinman, 2014). However, intriguingly this finding can be seen as a

contradiction of the only other report specifically focused on PAR2 in EAE, which has suggested a neuroprotective effect of PAR2 deficiency in EAE mice (Noorbakhsh et al., 2006), although the experimental design was significantly different and involved PAR2 KO mice rather than PAR2 activators.

5.6.5. PAR2 activation increases inflammatory cytokine production in the EAE mice model.

Once, we had been able to establish beneficial effects of PAR2 activation on the clinical outcomes on the EAE mice model, our next aim was to determine the impact of PAR2 activation on other key aspects of this disease model such as markers of inflammation. It has already been established that activation of the peripheral immune system can in some cases, exacerbates the physiopathology of neurodegenerative diseases such as Alzheimer's and multiple sclerosis (Dantzer et al., 2008; Lull & Block, 2010). Increased CNS cytokine production might happen as peripheral immune challenge can be communicated to the brain and reciprocally (Huang et al., 2009), potentially causing persistent microglial activation and hypersensitivity to further peripheral or central immune challenge (Lull & Block, 2010). EAE being a model of autoimmune inflammation it was expected that it would comprise an increased cytokine production, indeed this has been largely reported (Penkowa & Hidalgo, 2001; Makar et al., 2008; Mix et al., 2010; Constantinescu et al., 2011; Bittner et al., 2014). Furthermore, studies aimed at investigating PAR2 in EAE, have also described how PAR2 activation could be linked with increased peripheral cytokine production (Noorbakhsh et al., 2006). In EAE, it has been suggested that the enhanced production of a variety of cytokines often pro-inflammatory, which occurs from the early stages of the disease, could potentially be one of the causes of detrimental effects observed in the CNS (Pachner, 2011). In this study we have then decided to focus on the same 3 pro-inflammatory cytokines previously investigated for behavioural experiments IL-6, IL-17 and TNF-α, and previously described (see 5.6.2. for a more complete description), these cytokines have been identified as

critical regulatory actors of inflammatory diseases via T and B cell regulation (Lim, 2010; Erta et al., 2012; Jin & Dong, 2013) but also for the potential links they might have with both EAE physiopathology and PAR2 activation (Noorbakhsh et al., 2006; Liu et al., 2014; Suen et al., 2014). We were able to observe very significant increases in IL-6, IL-17 and TNF-α splenic production which correlated with improved clinical signs in EAE mice, in contrast to what was previously described (Penkowa & Hidalgo, 2001). Compared to the levels monitored in the vehicle group, PAR2 activation induced very significant increases in IL-6 and IL-17 splenic production at all 3 concentrations (100mg/kg, 10mg/kg and 1mg/kg), additionnaly TNF-α production was also significantly increased for the groups injected with 100mg/kg and 10mg/kg but not for the lower dose compared to what we detected in EAE mice having only received vehicle injections. IL-6 is a cytokine identified as an important modulator involved B-cell maturation (Erta et al., 2012) and CNS upregulation of IL-6 was previously described in the context of neuroinflammation in a variety of CNS diseases including but not limited to MS and EAE (Serada et al., 2008) which is thought to be mediated throught astrocytic activation (Erta et al., 2016). Intriguely it has also previously been suggested that PAR2 induced neuroprotection is also mediated throught astrocytic activation (Greenwood & Bushell, 2010). Additionnaly, studies have proposed that activation of PAR2 is central to the secretion of IL-6 from CNS stromal cells in a dose-dependent manner (Hirota et al., 2005; Radulovic et al., 2015). IL-6 which can act as a neurotrophic factors in the CNS, and has as such been linked to the promotion of a variety of CNS cells survival including of cholinergic, catecholaminergic neurons, retinal ganglion cells and dorsal root ganglia (Hama et al., 1989), these neuroprotective effects might involve the inhibition of glutamate release (Hirota et al., 2005). Therefore, we can hypothetise that increased IL-6 production induced by PAR2 activation might be one of the explanation of the reduced clinical scores observed in EAE mice. IL-17 on the other hand, has been closely linked with the development of autoimmune disorders including of MS and of its EAE animal model, as one the major driving force of inflammation in the CNS (W. Jin & Dong, 2013). Moreover, previous studies have observed that PAR2 activation is

correlated with increased levels of IL- 17 within the CNS (Takei-Taniguchi et al., 2012). It has been suggested that IL-17 driven apoptotic effects might occur through increases of glutamate levels thus increasing glutamate-induced neurotoxicity (Kostic et al., 2014). Strikingly, we have previously described that recent studies have identified IL-6 as involved in reducing glutamate-induced neurotoxicity (Erta et al., 2012), thus it is possible to infer that, in the context of a CNS disease, increased IL-6 production partly compensate for the detrimental effects induced by increased IL-17 production. Moreover we can relate this interpretation with observations made in the previous chapter while investigating the effects of PAR2 activation in OSC where it reduced KA-mediated glutamate neurotoxicity, hence possibly throught increased IL-6 production. Finally TNF-α is a cytokine critically involved in systemic inflammation and acute phase reaction by regulating immune cells. As such dysregulation of its production has been linked with the physiopathology of a variety of diseases with an inflammatory component, including autoimmune disorders, cancers, inflammatory bowel disease, MS, Alzheimer's disease, but also depression with TNF-α antibody therapies are being investigated (Idriss & Naismith, 2000). Intriguingly, some of these diseases have also been linked with changes in PAR2 expression (Ramachandran et al., 2012). Looking at the role of TNF-α in MS and EAE, previous studies have reported that its production by increasing synaptic instability in the CNS induce apoptosis and therefore is mostly detrimental (Lim, 2010). However, although being able to reduce TNF-α for the treatment of EAE via anti-TNF therapy is mostly beneficial, in MS patients these type of therapies have been described as prejudicial by increasing disease severity and progression (Lim, 2010). Early studies have suggested that PAR2 activation leads to the production of TNF-α, thereby resulting in increased inflammatory responses (Nystedt et al., 1996), this interpretation has however been contradicted in more recent studies showing that PAR2 activation reduces TNF-α induced apoptosis, potentially throught regulation of JNK activity (McIntosh et al., 2010; Iablokov et al., 2014). In our experiments, although we haven't specifically looked at the level of CNS cell death, we have nonetheless observed reduced clinical signs and relapses following PAR2

induced increase of TNF-α production, which might also be correlated with reduced apoptosis.

5.6.6. Limitations, improvements and future experiments.

Even though the experimental evaluations conducted in these studies provided attractive insights into the role of PAR2 in the CNS it is important to stress that they have limitations and should be improved and complemented in the future. In the open-field test studies, and knowing that there seem to be a dose-dependent effect of PAR2 activation in the factors we measured, it could have been interesting to test additional doses such as 3, 5 and maybe 25mg/kg, to elucidate under which conditions PAR2 activation induces detrimental effects on anxiety levels. Likewise, because they have been well validated, we have decided to only examine a limited number of measures in the open-field test, but other behaviours could have been considered such as rearing, freezing, grooming or irritation (Wahlsten, 2011). To explore the effect of PAR2 activation on behaviours, it would also be interesting in future experiments to use alternative tests including the Morris water maze, the forced swim test, balance beam, novel object recognition tests to study behaviours such as memory, spatial memory, learned fear, implicit learning or social behaviour for example. Future work is also necessary to characterise the long-term behavioural effects as well as the peripheral impact of PAR2 activation if given as a regular CNS treatment for example. Additionally, since we observed a PAR2 mediated change of cytokine profile, more studies can be done to precise the amplitude of these changes in different parts of the body and also in different brain regions or cell types including microglia, previously linked with the regulation of CNS inflammation. Here, we investigated splenic cytokine production only, considering our interest for the role of PAR2 in the CNS, it would be interesting to study cytokine expression in the brain following PAR2 activation using brain homogenates for these animals and microarray analysis of brain mRNA for example. Also, it would be important to study brain region specificity as the cytokine profile are different depending on the region considered,

indeed some regions such as the hippocampus express IL-6 cytokine receptors while other don't (Erta et al., 2012), accordingly excessive exposure to inflammatory cytokines is detrimental in some regions and functions while beneficial in others (Huang et al., 2009). Also, considering the importance of cardiovascular function, it is necessary to investigate the effect of any new pharmacological target on cardiac viability. Here we have observed that PAR2 activation had no direct detrimental impact on cardiovascular function, however, we studied a relatively small sample size in each group to study the short-term effect of PAR2 stimulation, it will be important in future settings to review bigger samples, using different genders and at different ages but also different species over longer periods of time. Furthermore, we have been able to study only 2 parameters of cardiovascular function due to limited availability of equipment specifically designed for these studies and further investigations are required to measures any PAR2-mediated changes in cardiovascular function, including measures of blood pressure, particularly considering that previous studies have suggested that chronic treatment with PAR2 activators could be a strategy to treat elevated blood pressures (McGuire et al., 2004). Additionally, we have previously studied the effect of PAR2 activation on splenic cytokine production, it would be interesting to further investigate cardiac cytokine production, as previous work has suggested a correlation between cytokine concentration and disease severity in the cardiovascular system with IL-6 being a strong predictor of subsequent outcomes (Kanda & Takahashi, 2004). Finally, in the EAE mice model, this study also suggests intriguing modulatory effect of PAR2 activation on EAE clinical signs as well as on cytokine expression. It would be important in future experiments to investigate different doses of PAR2 activators to examine for example whether doses between 10mg/kg and 100mg/kg could further reduce clinical signs and relapses occurence while still being safe for animals, also of interest would be to study if PAR2 induced effect on reducing relapses can be maintained with doses lower than 1mg/kg, additionally since the half-life of the PAR2 activators used in this study hasn't been established in the CNS, the daily dosing of AC might have resulted in over or under treatment. Furthermore, it is likely that the

way PAR2 regulate these cytokine's expression is dynamic and vary depending on the cytokine in question but also depending on the physiological context. Here we have only looked at the long term effect of PAR2 activation on splenic cytokine production on EAE mice and it is possible that the effects induced by PAR2 activation might have been different if observed at the onset, peak or resolution stages of the disease, that would be something to investigate in future studies. Also of interest would be to study the effects of PAR2 activation on other markers of inflammation, including in the CNS as microglial activation previously mentionned but also BBB permeability, or neuronal cell death. Finally we have observed a slightly increased variability in EAE clinical symptoms compared to what was reported from other laboratories (Constantinescu et al., 2011; Barbour et al., 2014) particularly for the groups injected with PAR2 activators, a variety of factors might provide an explanation such as variation in immunisation or injections efficiency and technique, and previous studies have reported similar effects affecting disease intensity (Steinman & Zamvil, 2005; Constantinescu et al., 2011; Bittner et al., 2014). It could also be due to the fact that AC-264613 solubility being poor (Gardell et al., 2008; Adams et al., 2011) it was therefore difficult to control with absolute precision slight variations in the amount of drug injected, thus potentially inducing variability.

Because animal models, although useful to dissect pathogenic mechanisms, recapitulate the complexity of the clinical features found in CNS diseases only partially the understanding we can gather from these models is likely to be only partial too, which can be correlated with unsatisfying levels of translation into new treatments. Nevertheless, disease modelling through animals have been critical in identifying basic physiological mechanisms involved in a variety of pathologies, but also in allowing the screening of potential therapeutic targets, as such they remain a major tool in current drug discovery toward the identification of new and effective therapies.

5.7. Conclusions.

It has been demonstrated in the previous chapter that PAR2 activation is neuroprotective in an *in vitro* model of neuronal toxicity, therefore potentially providing an interesting new target for CNS diseases. Due to these intriguing abilities, we considered as necessary to further investigate the role of PAR2 activation *in vivo*. Therefore, in this chapter, we have tried to get a good overview of the effects of PAR2 activation in behaviour, on cardiac function and in an animal model of CNS disease. Previous work has described that mice and humans share similar behavioural reactions including during anxiety and depressive states (V. Krishnan & Nestler, 2011), additionally neurotransmitters, neuromodulators and neuro-hormonal systems are highly conserved in mammalian biology with a strong functional homology across different species (Gould, 2009). Here we have started by observing PAR2 induced changes in behaviour, by looking at locomotor activity and anxiety. Some obvious difficulties and limitations exist in modelling human anxiety since it is not possible to precisely reproduce most aspects of such complex disorders and it wouldn't be reasonable to consider that responses deemed appropriate and adaptive for a given situation in rodents can be compared to anxiety mechanisms as observed in human where they constitute maladaptive or pathological responses to the existing situation, however because of the highly conserved mechanisms between both species (Buccafusco, 2001), we can expect some degree of similarity allowing for basic preliminary research in mice. Since the conditions under which PAR2 activation contributes to CNS function and behaviour under normal conditions was previously only partially documented and building on previous work within our lab (Abulkassim et al., 2016), we have in this study taken advantages of these similarities between humans and rodents to study the effects of PAR2 activation on locomotor activity and anxiety-like behaviours in C57BJ/6 mice. We have observed increased anxiety-like behaviours in the mice injected with high doses of AC-264613 (100mg/kg and 10mg/kg) in the open-field test, furthermore, these effects on behaviour were

correlated with increased levels of splenic IL-6 production. Additionally, we observed reduced locomotor activity using 100mg/kg doses, however this might be influenced by other factors, in addition to motor output or even by the anxiety-like behaviours we monitored. It is therefore difficult to conclude that PAR2 activation causes motor dysfunction and additional experiments, more specific to the study of motor function such as the rotarod test would be needed in order to further understand the results we observed. We have also observed that PAR2 activation had no effect on HR and a beneficial effect on fractional shortening, therefore suggesting that anxiety-like or locomotor deficits monitored aren't due to cardiac dysfunction. Finally, we observed no significant differences in the mice injected with 1mg/kg compared to the control group, injected with vehicle only, hence suggesting that PAR2 activation at lower concentrations wouldn't induce behavioural changes at least for the parameters we have investigated here. Furthermore, this is the first study to examine the long term effect of PAR2 activation via injections of AC-264163 in an *in vivo* model of CNS disease. We show that, in line with previous results establishing neuroprotective properties of PAR2 activation *ex vitro*, PAR2 activation *in vivo* reduces clinical symptoms induced in the EAE mice model of CNS inflammation. In details, we observed very significant beneficial effects of the 10mg/kg doses on the average clinical scores recorded, but also in reducing the occurrence and intensity of relapses, these effects could also be observed for the other doses investigated in this study (100mg and 1mg/kg), albeit with less consistency. Moreover, knowing that we observed lethal effects of high PAR2 activator concentration, it would be interesting in the future to run appropriate dosage adjustments studies to be able to identify the optimum dose for such experimental designs. Our results also propose for the first time that in the EAE model, PAR2 is involved in regulating the production of different pro-inflammatory cytokines IL-6, IL-17 and TNF-α. PAR2 treatment was applied only for 7 days and we were only able to look at cytokine expression at the end of the EAE experiment, more than 2 weeks after the last injections, interestingly we observed long lasting effects of PAR2 activation as it still impacted splenic cytokine profiles. Since we previously contemplated PAR2 related increases in IL-6 production in naïve

mice but not of IL-17 and of TNF-α, we can deduct that any beneficial effects induced by PAR2 activation via cytokine production is mediated by IL-6.

Chapter VI. GENERAL DISCUSSION.

Central Nervous System (CNS) diseases, afflict more than two billion people worldwide, in addition to causing great individual distress the worldwide economic cost of brain related conditions is estimated at over $1 trillion a year. Some of the most devastating CNS diseases are neurodegenerative and these specifically are getting more and more prevalent with age, since neuronal cells are not efficiently replaced endogenously, affected patients must cope with the loss of cognitive and physical abilities for the rest of their lives. Unlike for the other main types of human diseases including cancers, heart and infectious diseases, advances in pharmacological therapies to efficiently cure neurodegenerative diseases have not matched the increasing impact of these diseases in a globally ageing population, mostly because of the unique complexity of the CNS. Drug discovery in this area remains profoundly challenging, while the need for CNS disease modifying therapies remains extremely high. Neuroprotective strategies are one of the areas currently investigated to address this need and proteinase activated receptor 2 (PAR2) might be an interesting target in this regards. This discussion will start by summarising the main findings of this thesis, we will then examine the consistency of these findings in relation to previously established knowledge of PAR2 and neuroprotection in the CNS, we will then examine limitations before finally discussing the significance of this study with regard to future work in this area.

6.1. Summary of Main Findings.

The overall aim of this thesis was to test the hypothesis that PAR2 activation in the CNS is neuroprotective. To assess this hypothesis and based on the knowledge accumulated in the literature at the start of this project, we initially set out different aims for this study. (1) Advance the characterisation of PAR2 activators, including of GB88 described as the first specific antagonist designed for PAR2 and of the agonist

AC-264613 the first small molecule PAR2 activator. (2) Investigate the potential neuroprotective properties of PAR2 activation in an established model of glutamate induced excitotoxicity. (3) Determine the behavioural and cardiovascular effects of PAR2 activation *in vivo*. (4) Study the therapeutic effects of PAR2 activation in an *in vivo* animal model of CNS disease. We had hoped that investigations of PAR2 activation and pathways, *in vitro* including in CNS preparation, *ex vivo* brain cultures and finally *in vivo* on behaviour and in an animal model of CNS disease, like we have in the preceding chapters would provide valuable insights into the role of PAR2 in the CNS, thereby contributing to the current understanding of both PAR2 and neuroprotection as well as the potential of targeting PAR2 for CNS pharmacological therapies in the future.

In the first place, we sought to compare and investigate novel PAR2 activators in calcium imaging experiments and in internalisation studies. We observed that similarly to what was previously reported for other classical PAR2 activators, AC-264613 induces increases in Ca^{2+} concentrations in rat neuronal primary cultures, while GB88 previously described as an antagonist doesn't seem to affect PAR2 activated Ca^{2+} pathways. Moreover, in transfected tsA-201 cells we observed that all PAR2 activators used in this study induced receptor internalisation including GB88, therefore suggesting a mechanism of biased agonism for PAR2 rather than of antagonism. Data presented in this first chapter are the first demonstrations of GB88 biased agonism and also of AC-264613 mediated PAR2 activation in CNS preparation. Using that knowledge and building on previous work within or lab, we then moved on to investigate the neuroprotective properties of PAR2 against kainic acid induced neuronal cell death. We were able to show in mice organotypic slice cultures that PAR2 activation promote neuroprotection, in co-application with 300µM and 20µM of KA as previously suggested, but also when used as a post treatment, following the KA-induced insult to neuronal cells. Interestingly, these neuroprotective properties were maintained when PAR2 activation was induced using GB88, thereby suggesting that this bias agonist can activate pathways responsible for PAR2 neuroprotective features, but also that PAR2-mediated neuroprotection doesn't require activation of

the Ca^{2+} pathways in the CNS. Finally, we investigated the effect of AC-264613 mediated PAR2 activation *in vivo*, by recording potential behavioural differences in the open-field test, where we monitored increased levels of anxiety-like behaviours following injections of both 100mg/kg and 10mg/kg doses as well as decreased locomotor activity following 100mg/kg injections. Additionally, these behavioural changes were correlated with increased splenic productions of IL-6 but had no detrimental effect on heart function. Furthermore, in the EAE mice model of auto-immune CNS inflammation, PAR2 activation induced a significant reduction of clinical scores but also of relapse occurrence and intensity.

6.2. AC-264613 is an improved tool to investigate PAR2 in the CNS.

Given the vast range of roles that PAR2 have been linked with in normal and pathological tissue function, these receptors are emerging as potential therapeutic targets for several diseases including neurodegenerative conditions. Thus, considerable efforts have been made to understand and take advantage of PAR2 signalling and unusual mechanisms of activation, at the time this project was initiated, most of the pharmacological and physiological studies of PAR2 had been ran using synthetic peptides such as SLIGRL-NH_2 and 2-furoyl-LIGRLO-NH_2. The selectivity of these activators for PAR2 over other PARs is now disputed (Stenton et al., 2002), thus inferring that some of the results observed in these studies might be only partially PAR2 mediated, therefore, further characterisation was necessary to complement the knowledge established from studies based on these activators. In this thesis, we have measured lower increases in Ca^{2+} concentration following application of AC-264613 compared to what we observed following applications of 2-f, which could be understood as an evidence for AC greater selectivity, thereby inducing increases by activating PAR2 only while 2-f would have off-target effects on other PARs, thus explaining larger Ca^{2+} releases. Furthermore, because previous investigations have established that these peptides-based activators are unable to

access the brain (Ramachandran et al., 2012), AC-264613, because of its pharmacokinetics including its ability to by-pass the blood brain barrier (Eli Lilly, unpublished data), appeared as a critical tool when considering the effect of PAR2 in CNS related conditions.

In this report we have been able to use AC-264613 to specifically study the effects of PAR2 activation in the CNS for the first time. We have shown in an *ex vivo* model of excitotoxicity, consistent and long lasting beneficial effects of PAR2 activation thereby confirming that PAR2 activation has neuroprotective properties (Greenwood & Bushell, 2010). These results confirming the potential of using AC-264613 to achieve neuroprotection allowed us to move on to a comprehensive investigation of the effect of AC-264613 mediated PAR2 activation in living animals. We have recorded increased anxiety-like behaviours following high doses of PAR2 activator AC-264613, however these were only correlated with locomotor dysfunctions at very high drug concentrations. Furthermore, looking at the cytokine profiles, we were able to identify links between PAR2 activation and IL-6 production, knowing that IL-6 has been recently linked with neuroprotection (Scheller et al., 2011; Erta et al., 2012), this finding might be relevant for subsequent studies in animal models of CNS disease.

Interestingly, and in contrast with previous studies describing a detrimental role for PAR2 over-expression in heart failure (Antoniak et al., 2013) we have not monitored any detrimental effect on cardiac function as shown by comparable measures of HR regardless of the doses of AC-264613 received and even by slight improvements in fractional shortening. However, in this study, we have looked at naïve mice whose heart function was supposed normal at the time of the experiment, while preceding researches have investigated the role of PAR2 activation in a context of heart dysfunction. It might be suggested that while PAR2 activation can aggravate cardiac function once it is already challenged, it would have no detrimental effect in a normally functioning cardio-vascular system. This interpretation would be in line with studies highlighting the role of PAR2 in disease state (Antoniak et al., 2013; Kagota et al., 2016) while still being compatible with other investigations describing no negative

impact (Hughes et al., 2013) or even a positive impact of PAR2 activation on cardiac function (Steinberg, 2004). It will therefore be important to pursue investigations toward the understanding of PAR2 activation in heart functioning, as adverse effects might happen for patients suffering of cardiac dysfunctions and affected by CNS diseases in conjunction, especially those very vulnerable following a severe trauma as it is believed that PAR2 might then induce critical exacerbation.

Knowing the suggested links between PAR2 and inflammation (McIntosh et al., 2010; Takei-Taniguchi et al., 2012; Suen et al., 2014), we have decided to further investigate the effect of PAR2 activation in an animal model we considered relevant to studies of inflammation in the CNS (Steinman & Zamvil, 2005; Constantinescu et al., 2011), the experimental autoimmune encephalomyelitis (EAE) mice model. In this disease model, we have detected beneficial effects induced by PAR2 activation including decreases in intensity of the clinical signs exhibited by the animals as well as in the relapses occurrence. Additionally, we observed a correlation between PAR2 activation and increased cytokine expression as these PAR2 activator injections were correlated with increases in splenic production of IL-6, IL-17 and of TNF-α, which tend to confirm the pro-inflammatory properties of PAR2 previously suggested in other studies (Ramachandran et al., 2012; Cattaruzza et al., 2014), therefore also implying that PAR2 in disease state can to an extend contribute to increase the inflammation. A few studies have observed that initial increased inflammation is not always detrimental and is even neuroprotective (Correale & Villa, 2004). Thus, it is possible to suppose that in our experiments, while contributing to the EAE-induced inflammation, PAR2 activation is nevertheless neuroprotective, hence still reducing the clinical scores measured in mice.

6.3. Limitations and future work.

Although the data presented here advance the understanding of the role of PAR2 activation in the CNS and the implication of the potential use of PAR2 activation in order to achieve neuroprotection, a number of limitations and technical considerations of the current study exist and need to be mentioned as well as potential ways to complete this study in future work. Initially, in order to be consistent and to approximate as much as possible the experimental design of previous studies (Greenwood & Bushell, 2010), we have worked in this experiments with relatively high concentrations of PAR2 activators particularly *in vitro*, considering the modulatory roles of PAR2 in a variety of pathways, it is likely that a better awareness of doses requirement would prove beneficial and allow more specific observations. Furthermore, when looking at pharmacological agents for instance at AC-264613, factors such as pharmacokinetics, therapeutic time windows, receptor subunit selectivity, toxicity or blood brain barrier penetration also need to be investigated in greater details (Hughes et al., 2011). The study presented in this thesis was principally aimed at investigating the neuroprotective properties of PAR2, and thus we haven't designed experimental setups allowing a throughout exploration of the mechanisms involved, although a superficial investigation of mechanisms underlying PAR2 internalisation was achieved including during transfection studies, further details are lacking. Insights into these mechanisms will be critical for further understanding of PAR2 and it is required that future studies can examine the pathways involved in the neuroprotective properties we observed as this will permit to understand how to target PAR2 in future therapeutics aimed at slowing CNS diseases. Although intriguing, these results can only be understood as preliminary indications, additional investigations with refined experimental protocols are required to fully elucidate the exact mechanisms of action of PAR2, these would also greatly enhance the knowledge in this area by opening up novel avenues to stimulate neuroprotective pathways. Mechanistic studies would allow addressing some of the key limitations we have previously underlined including optimum dosing parameters,

therapeutic time windows, peripheral effects, but would also allow to take advantage of some of the findings we described such as the bias agonism properties of GB88, additionally, the development of efficient small molecule antagonists for PAR2 will help define new ways to modulate PAR2 pathways. Unfortunately, over the years a wide variety of agents have been identified as displaying interesting neuroprotective properties, still to fail in human clinical trials (Leestemaker & Ovaa, 2015), this problem could be explained by numerous suggested reasons, of which inconsistencies or inadequacies of pre-clinical models used to model the CNS *in vitro* and *in vivo* have been identified as a critical factor. In the present study we have looked at a specific *ex vivo* model of cell death involving glutamate-induced neuronal toxicity and subsequent apoptosis (Zhu et al., 2011), although the detrimental impact of excessive glutamate has been extensively described has a major factor of neuronal degeneration (Wang et al., 2005; Sheldon & Robinson, 2008), other neurotransmitters have been identified for their role in neurodegeneration (Burke et al., 2004; Stocchetti et al., 2015) and thus PAR2 neuroprotective properties could be investigated in other cellular pathways. Moreover, knowing that necrosis and autophagy have also been described in CNS disease physiopathology (Majno & Joris, 1995; Thompson, 1995; Jellinger, 2001; Ghavami et al., 2014), it might be relevant to investigate the effect of PAR2 in *in vitro* models reproducing other mechanisms of cell death. In animal model of disease, we have decided to study a model of CNS inflammation, considering the links previously established between PAR2 and inflammation it appeared like an ideal platform for an initial evaluation in the CNS (Noorbakhsh et al., 2006; Gieseler et al., 2013; Suen et al., 2014), nonetheless because more neurodegenerative diseases have been linked with CNS inflammation (Amor et al., 2010), it would have been interesting to examine the effect of PAR2 activation in other animal models of diseases, involving different physiopathological pathways. Finally, although the proximity of rodent and human DNA has been established and detailed (Clark & Squire, 2013), it might be worth considering, for successful translation of these preliminary studies into the development of potential disease modifying therapeutics, to use animal models approximating human

characteristics in a more accurate manner, in order to address the growing burden of CNS related disorders. Thus the work presented in this thesis open up some interesting avenues for further investigation in order to characterise PAR2 mechanisms of action further but also to address some of the issues highlighted in this discussion.

6.4. Conclusion.

Over the last several decades, a variety of useful and relevant *in vitro* and *in vivo* models have been designed to reproduce specific aspects of human CNS disease. Depending on the specific research question and on the relevance of the model chosen, it is possible to extract valuable insights in order to understand a variety of physio-pathological mechanisms, hence allowing the identification of new therapeutic pathways able to improve the current CNS pharmacology, despite clear limitations. The results presented in this thesis have addressed the relevance of proteinase activated receptor 2 as a viable target for CNS diseases by describing its neuroprotective properties in an *in vitro* model of excitotoxicity and in an *in vivo* model of CNS disease, this study proposes the first characterisation of novel PAR2 activators GB88 and AC-264613 in CNS preparations, additionally these results also hint at mechanisms and modulators underlying PAR2 mediated neuroprotection. Finally, this work provides insights into the effect of PAR2 activation on behaviour and on cardiac function. Taken together, the results presented in this thesis form a solid foundation to establish PAR2 as an intriguing potential target for CNS drug discovery and suggest that modulation of its expression or function may be a viable strategy in the treatment of CNS diseases, still, further studies are needed to attempt to elucidate the mechanisms by which PAR2 mediated neuroprotection is achieved.

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