Investigating The Role of Mitogen Activated Protein Kinase Phosphatase-2 on CNS function

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A thesis presented in fulfilment of the requirement for the degree of

Doctor of Philosophy

March 2015

Strathclyde Institute of Pharmacy and Biomedical Sciences

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AUTHOR'S DECLARATION

All the work in this thesis was carried out solely by the author. This thesis has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signature:

Date:

ACKNOWLEDGEMENT

First and foremost I would like to express my gratitude to Allah for blessing me to be able to complete this thesis.

I would like to thank my supervisor, Dr Trevor Bushell for guiding me through my PhD study. It is his guidance, patience and constant support that made this thesis completion possible. It has been a great experience for me to work under his supervision. I learned so much not only in designing the experiments to interpretations of results but also in work and time management. Also my thanks go to my second supervisor, Prof Robin Plevin for his advice, helpful suggestion and constructive comments through my Ph.D work.

My sincere gratitude goes to my sponsor, Ministry of Higher Education Malaysia and Universiti Sains Islam Malaysia for financing my study. I would like to extend my sincerest thanks to all the officers that I have been dealing with for making my study here smooth sailing.

It's been a great pleasure for me to have a chance to work with professionals in the lab. I'd like to convey my heartfelt thanks to Dr Sam Greenwood for helping me and getting me through for the few months when I first started with his warm welcome and very patience helping me with the dissection and extracellular electrophysiology, Dr Ka Ho Ho, Dr Emma Torance, Dr Katy, Dr Yuen for helping me and sharing their advice and expertise when I work with western blot in Prof Robin's lab. I would like also to extend my gratitude to BPU staff, especially Carol Whitehouse and Lee Wheeler in helping and ease my work in animal room.

Thanks to my labmate and officemate, Rajeev, Conor, Majed, Dima, Roua, Louise, Sajad, Sukrut, Serge and Molly for making my PhD life cheerful. I also like to thank my Malaysian community especially Kak Ros, Kak T, Wini and Zila for the ever being very generous in helping me and my small family here in Glasgow. It has been the best years in my life despite all the struggles.

My deepest gratitude goes to my parents, family and father and mother in-laws for always giving me physical and mental support, words of encouragement and prayers.

Saving the most important for the last, I am greatly indebted to my devoted husband, Afif for always being with me all this tough years for his unconditional love, patience, physical and mental support, and never ending sacrifices. Without him by my side this thesis would not be possible. For my son, Hasif, the one that could drain all my stress and lift me up just with his smile and kisses. I am sorry for all the time I gave to my research and thesis that I should spend with you. For this two important person in my life, I am forever in debt with you.

ABSTRACT

Mitogen-activated protein kinase phosphatases (MKPs) belong to the dual-specificity phosphatase (DUSP) family and are negative regulators of mitogen-activated protein kinases (MAPKs). Recently, MKP-2 has been shown to play a novel role in development, the immune system and cancer. However, our knowledge in relation to its function in the central nervous system (CNS) is limited. Hence, we have utilised novel MKP-2 knockout mice to investigate the role of this phosphatase in CNS function.

The effect of MKP-2 deletion on CNS was first investigated using primary hippocampal culture by examining astrocyte proliferation and neurite length using immunocytochemistry techniques performed on cultures 1-7 days *in vitro* (DIV). To investigate the functional consequence of MKP-2 deletion on CNS at 3, 7 and 11 DIV, intracellular calcium concentrations were determined by fluorescence imaging using Fluo-4 dye. Na^+, K^+ current, synaptic transmission was also investigated using patch clamp electrophysiology. To examine the effect MKP-2 deletion in whole tissue, standard western blotting techniques were utilised to examine the consequence of MKP-2 deletion on ERK activity in the hippocampus of 3 week old mice. The functional effect of MKP-2 deletion on basal synaptic transmission and paired pulse facilitation (PPF) on acute hippocampal slices, field excitatory postsynaptic potentials (fEPSPs) were recorded from acute hippocampal slices of 3 week old mice.

Astrocytes number and neurite length were reduced in 1–3 DIV in MKP-$2^{-/-}$ compared to MKP-$2^{+/+}$. However, there is no difference in astrocyte proliferation and neurite length in 4-7 DIV. Further investigation into the reduction of neurite length, it was due to impairment in astrocyte function and not account of reduction in astrocyte proliferation. Investigating further into the functional consequences, astrocytic intracellular calcium was reduced at 7 DIV however neuronal intracellular calcium was increased at 11 DIV in MKP-$2^{-/-}$ primary hippocampal culture. Furthermore, Na^+ and K^+ current were also reduced at 7 DIV. However, spontaneous excitatory postsynaptic current (sEPSC) and synapse number has been shown to increase at 7 and 11 DIV. The mechanism underlying this seems to be not related to ERK phosphorylation as no difference in ERK activation was evident when compared between MKP-$2^{+/+}$ and MKP-$2^{-/-}$.

In further experiments in acute hippocampal slices, MKP-2 deletion leads to a reduction in ERK activity within the hippocampus. In contrast, increased ERK activity was observed in the heart and liver. Basal synaptic transmission was enhanced in MKP-$2^{-/-}$ mice at high stimulus intensities compared to MKP-$2^{+/+}$ but PPF was unaltered at all in inter-stimulus times tested (10-500 ms).

In summary, in this thesis I demonstrate that even though MKP-2 deletion reduced astrocyte growth and neurite length at 3 DIV, it doesn't affect their functional properties at early development. However, alterations of functional activity at 7 and 11 DIV and acute hippocampal culture suggest that MKP-2 deletion might play a role in functional activity when the neuron is fully developed. This data suggest a novel physiological role for MKP-2 in the brain and might reveal valuable insight for the drug development.

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LIST OF ABBREVIATION

$[Ca^{2+}]i$	intracellular calcium
µg	microgram
µM	micromolar
µm	micrometre
ACSF	artificial cerebrospinal fluid
ADP	adenosine triphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AraC	cytosine arabinofuranoside
ATP	adenosine triphosphate
CaMKII	$Ca^{2+/}$calmodulin-dependent protein kinase II
CNS	central nervous system
CREB	cyclic AMP responsive element binding protein
DG	dentate gyrus
DHPG	(S)-3,5-Dihydroxyphenylglycine
DIV	days *in vitro*
DUSP	dual specificity phosphatases
EDTA	ethylenediaminetetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
ERK	extracellular-regulated kinase

fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
HFS	high frequency stimulation
IP3	inositol 1,4,5-triphosphate
ISI	Inter-stimulus interval
JNK	c-Jun N-terminal kinase
KAR	kainate receptor
LFS	low frequency stimulation
LTD	long term depression
LTP	long term potentiation
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
mGluR	metabotropic glutamate receptor
MKP	Mitogen Activated Protein Kinase Phosphatase
ml	millilitre
mm	millimetre
mM	millimolar
ms	millisecond
mV	milivolt
NMDAR	N-methyl-D-aspartic acid receptor
pA	picoampere
PAR	proteinase-activated receptor
pERK	Phospho-ERK

PPF	paired-pulse facilitation
SEM	standard error of mean
sEPSC	spontaneous excitatory postsynaptic current
tERK	total-ERK
TTX	tetrodoxin

1. INTRODUCTION

1.1 Brief overview of central nervous system

The central nervous system (CNS) can be regarded as a very complex and organized system, which is symmetrical in structure and is grouped into of three broad regions that consist of seven main parts: the spinal cord, the hindbrain (medulla oblongata, pons, cerebellum), midbrain, and forebrain (diencephalon and the cerebral hemispheres) (Kandel et al., 2000). The spinal cord functions to receive and process sensory information from the skin, joints and trunk and control the movement of limbs and the trunk (Bican et al., 2013). The midbrain and hindbrain are grouped as brain stem, which play a role in sensory and provide motor function sent from brain to the body. The midbrain specifically controls many sensory and motor functions (Breedlove and Watson, 2013). The medulla oblongata is responsible for autonomic function, such as digestion, breathing and heart rate while the pons connects and conveys information about movement from the cerebral hemisphere to the cerebellum. The diencephalon have two structures, the thalamus which process most of information reaching the cerebral cortex and the hypothalamus which regulates autonomic, endocrine and visceral function (Kandel et al., 2000).

1.2 The Neuron

The CNS consists of unique types of cells namely neurons and glia. Neurons are the building blocks of the CNS and the sole unit in the nervous system that transmit nerve impulses (Fodstad, 2002). The basic morphology of a neuron consists of four regions: the cell body, the dendrites, the axon and the presynaptic terminals (Kandel et al., 2000). The cell body, which gives rise to two regions, the dendrites and the

axon, contains the nucleus that stores genetic material. Dendrites play a key role in receiving incoming signals from other nerve cells, whereas the axon functions as the main conducting unit for carrying signals to other neurons (Kandel et al., 2000). The signals are called action potentials and together with glutamate, these are used to communicate signals from one neuron to another. At the end of the axon, there is a region that is used for communications with other neurons, which is called the synapse.

1.3 Synaptic transmission in the central nervous system

1.3.1 Overview of synaptic transmission

The special ability of neurons to communicate with each other is termed synaptic transmission, which can be either in electrical or chemical form. Electrical synaptic transmission is used primarily to send simple depolarizing signals between cells, which normally has limited function. It is primarily excitatory transmission, Ca^{2+} independent and has no synaptic delay (Dale et al., 2004). On the other hand, chemical transmission is a much complex process which requires presynaptic receptors to regulate neurotransmitter release upon depolarization of action potential. It has more variable signalling and can be either excitatory, with the main neurotransmitter being glutamate, or inhibitory, with the main neurotransmitter being gamma-aminobutyric acid, (GABA) (Kandel et al., 2000). The neurotransmitter is synthesized in the nerve terminal, stored in vesicles and released via exocytosis when an action potential invades the nerve terminal. The action potential depolarizes the nerve terminal and glutamate neurotransmitter release is initiated by influx of Ca^{2+} through Ca^{2+} channels which are voltage dependent and starts within 200μs of the

action potential arriving at the synaptic terminal (Barrett & Stevens, 1972). After being released, the neurotransmitter will diffuse across the synaptic cleft and interact with receptor proteins on the membrane of the postsynaptic cell, causing ionic channels on the membrane to either open or close (Figure 1.1). When the ionotropic channels open, depolarization occurs that will initiate another action potential in the postsynaptic region.

1.3.2 Glutamate receptors

The principal excitatory neurotransmitter in the CNS is glutamate, which exerts its action through two broad categories of receptors: ionotropic and metabotropic glutamate receptors (Goudet et al., 2009) (Figure 1.2). Ionotropic receptors are excitatory receptors and directly gated channels whilst metabotropic receptors (mGluR 1-8) are G-protein coupled receptors which activate secondary messengers (Traynelis et al., 2010). Unlike ionotropic receptors, activation of metabotropic receptor can be either excitatory or inhibitory. AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor), NMDAR (N-methyl-D-aspartate receptors) and KAR are the 3 major subtypes of ionotropic receptors, whose names are based on the type of synthetic agonist that activates them (Meldrum, 2000). NMDAR activation is blocked by D-AP5 (D-(-)-2-Amino-5-phosphonopentanoic acid) (Kantamneni et al., 2014) meanwhile AMPAR and kainate are also known as non-NMDAR as they are antagonised by CNQX and not affected by D-AP5 (Peng et al., 2011).

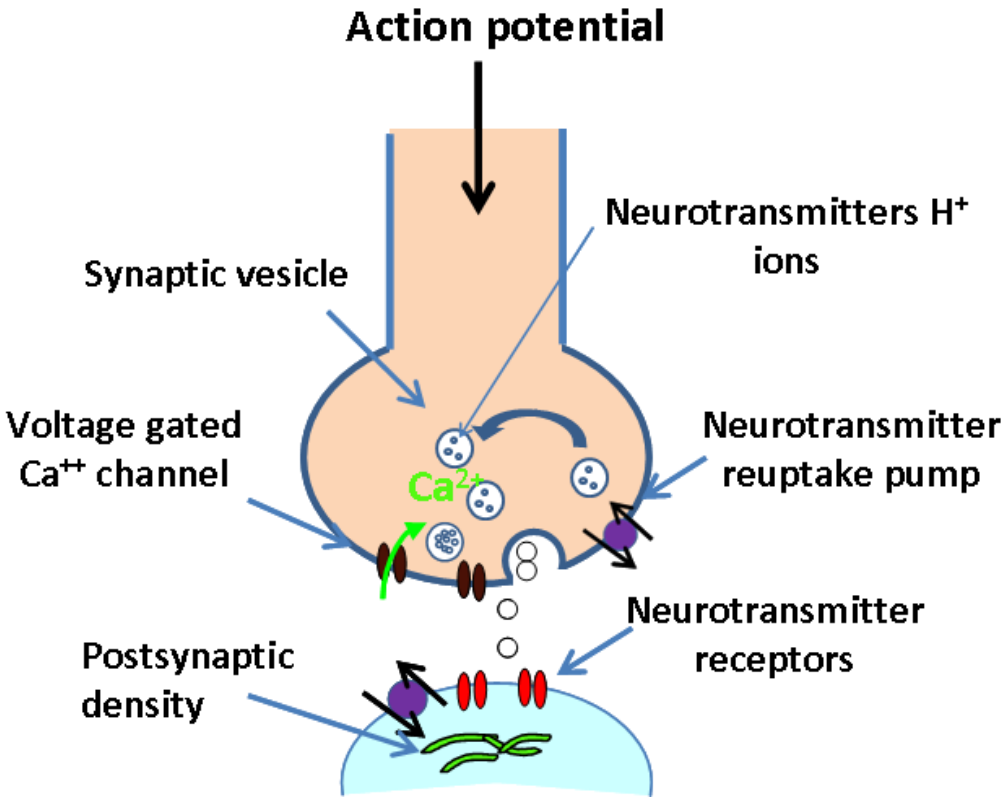


Figure 1.1 : A schematic diagram illustrating the process of synaptic transmission.

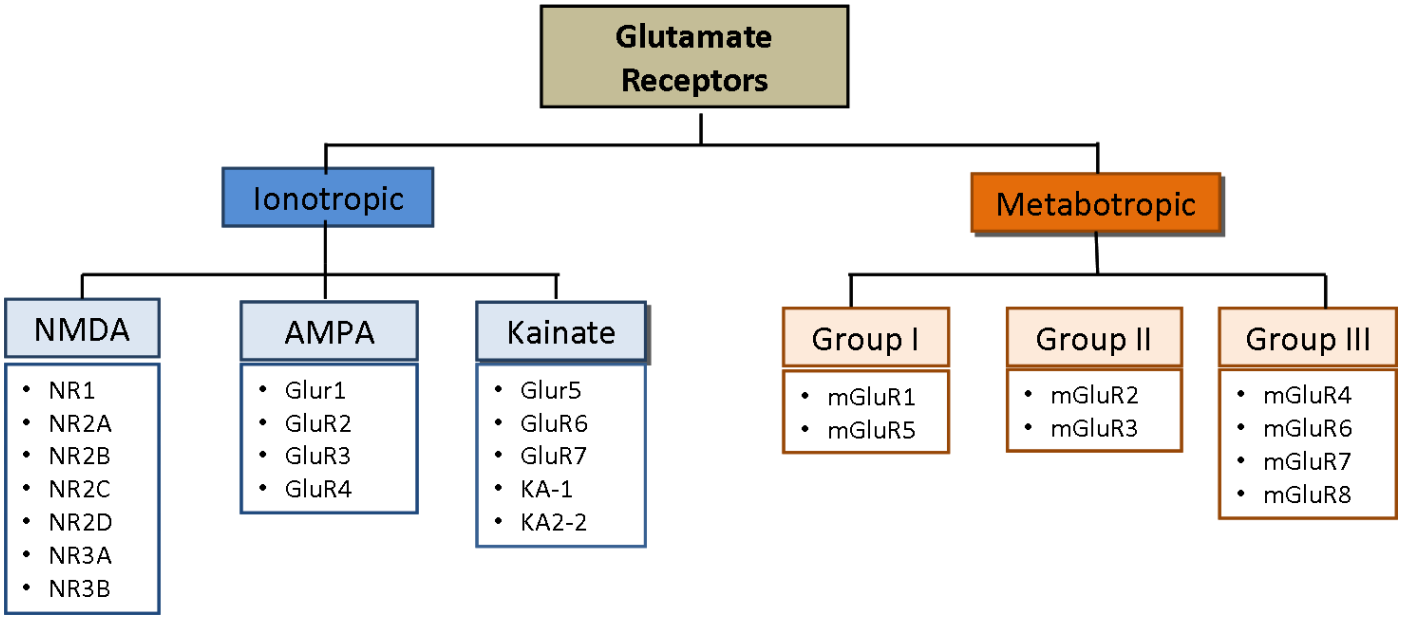


Figure 1.2: Classification of glutamate receptors

NMDARs are formed from hetero-tetrameric assemblies of GluN1 (previously NR1) subunits with GluN2A–D and GluN3A/B (previously NR2A–D and NR3A/B) subunits (Alexander et al., 2011). Classically, NMDARs were thought to only exist postsynaptically, however increasing evidence suggests the existence of presynaptic NMDARs (Tzingounis and Nicoll, 2004; Zorumski and Izumi, 2012). They are activated by the binding of glutamate and the co-agonist glycine and conducts Na^+, K^+ and Ca^{2+} ions. The voltage-dependent blockade of the NMDAR pore by Mg^{2+} produces an additional requirement for depolarization (Traynelis et al., 2010). The NMDAR NR3A subunit is important that it is able to modulate the Ca^{2+} permeability and Mg^{2+} sensitivity (Tong et al., 2008). AMPARs are glutamate gated cation channels composed of a homo or heteromeric complex of four subunits, GluA1–4 (previously GluR1–4) and are activated directly by glutamate binding. In contrast to NMDAR, AMPARs are largely Ca^{2+} impermeable (Jonas, 2000) and are the main contributors to excitatory neurotransmission, mediating the fast and rapid excitation of many synapses. Their activation opens the pore of the channel, permitting the inward flow of sodium, resulting in the depolarization of the neuronal membrane (Platt, 2007). This change in the intracellular charge releases the Mg^{2+}cation from the NMDARs channel, permitting passage of Ca^{2+} through the pore. AMPARs are responsible for the primary depolarization in glutamate-mediated neurotransmission and AMPAR trafficking has been reported as a major regulatory mechanism in controlling synaptic plasticity, where increased AMPAR insertion at synapses results in long-term potentiation (LTP) whilst removal of AMPAR leads to long-term depression (LTD) (Shepherd & Huganir, 2007).

Another non-NMDAR is the kainate receptors (KARs). KARs perform different roles from the other two ionotropic receptors (Copits & Swanson, 2013) and are able to bidirectionally regulate the release of glutamate at the mossy fiber to CA3 synapse (Schmitz et al., 2001). It is composed of two related subunit families, GluK5–7 and KA-1 and 2 (Kew and Kemp, 2005). GluK5 subunit is expressed predominantly in interneurons in the hippocampus, where it may play a role in receptors mediating the facilitatory effects of glutamate released by astrocytes (Liu et al., 2004). It shares many characteristics with AMPARs, but these two receptors do not cross-assemble (Pinheiro and Muller, 2006). Studies have shown that KARs have different roles in synaptic transmission including carrying part of current charge in the postsynaptic region, while in the presynaptic region, it has been proposed to regulate transmitter release at both excitatory and inhibitory synapse (Lerma, 2006).

The metabotropic glutamate receptors (mGlus) are coupled to GTP-binding proteins (Nicoletti et al., 1986) and classified into three groups based on sequence similarities, pharmacological properties and intracellular signal transduction mechanism and consist of eight different subtypes: mGlu1– 8 (for review see Gladding et al., 2009). The three groups of mGlus are Group I that includes mGlu1 and mGlu5 receptors, which couple to Gq and activate phospholipase C (PLC), Group II that consist of mGlu2, mGlu3 and Group III (mGlu4, mGlu6, mGlu7, and mGlu8), receptors couple to Gi/Go (Gladding et al., 2009). mGlus mediate long term depression as well as play a modulatory role in CNS function regulating both neuronal excitability and the release of neurotransmitters (Doherty and Dingledine, 2002).

1.3.3 Synaptic plasticity

The communication between neurons is mediated through synapses. Synaptic plasticity can be defined as the activity dependent changes in the efficacy of synaptic communication (Mayadevi et al., 2012). Among the well-studied mechanisms involved in synaptic plasticity are short term plasticity, long-term potentiation and long-term depression.

1.3.4 Short-term plasticity

Short-term plasticity is a type of plasticity that lasts from milliseconds to a few minutes (Zucker and Regehr, 2002) and is proposed to play a role in short-lasting forms of memory. It is triggered by short bursts of activity that causes transient accumulation of calcium in the presynaptic bouton and results in changes in the probability of neurotransmitter release by modifying biochemical mechanisms required for vesicle release. Short-term plasticity can be seen as either facilitation or depression based on a few factors. Paired-pulse depression (PPD) is caused by transient depletion of a readily releasable pool of vesicles docked at the presynaptic active zone or inactivation of voltage-dependent sodium or calcium channels. On the other hand, paired-pulse facilitation (PPF) caused at longer interstimulus interval (20 – 500 ms), is caused by residual calcium from the first action potential contributing to additional release during the second stimulation. Whether it will be PPF or PPD, it was all depends on the recent activity of transmitter release (p). Very high p tends to depress on the second response, meanwhile a very low initial p will subsequently increase p in second stimulation (Dobrunz & Stevens, 1997). However, a single synapse can change from facilitation to depression depending on its recent activity.

1.3.5 Long-term potentiation

Long-term potentiation (LTP) is a phenomenon first discovered by Bliss and Lomo based on experiments in anaesthetized rabbits, where population responses were recorded in the dentate gyrus when the perforant path was stimulated repetitively for the period of 30 min to 10 hr (Bliss & Lomo, 1973). LTP can be defined as repetitive activation of excitatory synapses in the hippocampus that will cause a potentiation of synaptic strength that could last for hours or days, which is proposed to be the cellular mechanism by which memories are formed (Whitlock et al., 2006). It can be either NMDAR-dependent or NMDAR-independent depending on the pathway examined. NMDAR-independent LTP has been found in mossy fiber pathway in hippocampus as well as very strong stimulation leading to activation of L-type Ca^{2+} channel has been proposed to induce this type of LTP (Johnston et al., 1992). Meanwhile, for the induction of NMDAR-dependent LTP, it is necessary that the membrane is sufficiently depolarized to expel Mg^{2+} from NMDAR channel, simultaneously with glutamate binding to NMDARs (Bliss and Collingridge, 1993; Malenka and Bear, 2004). The induction phase of potentiation also depends on protein phosphorylation by a persistently active Ca^{2+}/calmodulin-dependent protein kinase (Schulman and Hyman, 1999). Initial studies on LTP suggested that the locus of LTP was postsynaptic (Malenka et al., 1989) because NMDARs are assumed to be located on dendritic spines (Bliss and Collingridge, 1993). Inhibition of NMDARs inhibits LTP induction (Mulkey and Malenka, 1992), suggesting the induction of LTP in the CA1 area of the hippocampus requires influx of Ca^{2+} following activation of NMDA glutamate receptors. In contrast, a few reports later suggest that expression of LTP could involve presynaptic and postsynaptic loci, depending on the

brain region examined (Kullmann & Nicoll, 1993). Early-LTP lasts about 30 - 60 min but can be longer lasting from hours to days. In order for the plasticity to last hours or days, it requires new protein synthesis. It is well established that a number of protein kinase that involved in maintaining LTP including MAPK (English & Sweatt, 1997), CaMKII (Wu et al., 2006) and TrkB (Minichiello.,2009)

1.3.6 Long-term depression

In contrast to LTP, long-term depression (LTD) is an activity–dependent reduction in synaptic activity. There are a two established mechanisms to induce LTD which are applying low frequency stimulation (LFS, for example 1 Hz train of 900 paired pulse) in the hippocampal CA1 region (Kemp & Bashir, 2001; Mulkey and Malenka, 1992) or by chemical induction via the activation of group I mGluR by specific agonist, 3,5-dihydroxyphenylglycine (DHPG) (Fitzjohn et al., 1999). Induction of LTD is not solely dependent on mGlu, but also NMDAR activation and increases in intracellular Ca^{2+} that are smaller than those required for LTP (Collingridge et al., 2010). A recent study has shown LTD is not inhibited by MK-801, an NMDAR antagonist (Nabavi et al., 2013) suggesting LTD involves a metabotropic form of NMDAR with signalling similar to mGlu signalling (Babiec et al., 2014). It is well established that induction of LTD required p38 MAPK activation either in mGlu-induced LTD (Moult et al., 2008) or NMDAR-induced LTD (Collingridge et al., 2010). However, the direct downstream consequence of p38 MAPK activation and how it is contributes to LTD is still unclear.

1.4 Glial cells

All other neural cell that unable to transmit electrical signal are classified as glial cells, reported to constitute 50% of all the cells in the human brain (Azevedo et al., 2009). There are three principle types of glial cells in the CNS, which are astrocytes, oligodendrocytes (grouped as macroglia) and microglia (Kandel et al., 2000). They have a diverse range of functions in the CNS from forming cellular framework that aids the development of the nervous system, to immune system functions.

1.4.1 Function of macroglia in the CNS

Astrocytes can be characterized as star-shape glial cells that constitute in the brain. It is well accepted that the general function of astrocytes is to provide support and protection for neurons for instance, to maintain the right potassium ion concentration in the extracellular space between neurons (Holthoff and Witte, 2000). Astrocytes also function as the building blocks of the blood-brain barrier, together with capillary endothelial cells, vascular pericytes and the perivascular end feet of astrocytes. This creates a highly selective barrier that allows only oxygen and hormones to permeate into the brain while preventing the passage of other molecules in order to prevent possible harmful effects (Ota et al., 2013).

There is accumulating evidence showing that astrocytes contribute to synaptogenesis. In a study conducted using retinal ganglion cells (RCG), neurons grown in the presence of astrocytes had significantly more synapses compared to RCGs grown in serum free media (Pfrieger & Barres, 1997). This is hypothesized to be because astrocytes secrete a soluble factor called thrombospondins (TSP) (Ullian et al., 2004). Astrocytes are also reported to secrete cholesterol that binds to apolipoprotein which has been proposed to promote presynaptic function and transmitter release as

well as facilitate the development of dendrites (Goritz et al., 2005; Mauch et al., 2001). These findings suggest that soluble factors secreted by astrocytes contribute to not only synapse formation but also synapse function.

A main function of oligodendrocytes is the formation of myelin sheaths around axons that subsequently permit conduction of action potential (Jessen, 2004). Apart from that, they also maintain axonal integrity so as to mediate the rapid conductance of action potentials and support axonal survival (Fünfschilling et al., 2012; Watkins et al., 2005).

1.4.2 Function of microglia in the CNS

Microglia constitutes of 10% of glial cells in the CNS. Specifically described to function as the innate immune system in the brain, microglia are macrophages that act as the immune defence in the CNS (He and Sun, 2007). They act as sensors of the extracellular environment and respond to any changes, for example injury in the surrounding area (Barres, 2008). In the healthy normal brain, microglia are in a resting state and are activated and undergo morphological changes if injury occur (Nolte et al., 1996).

1.5 Neuron-astrocyte interaction

Classically, the function of glial cells, especially astrocytes was thought to be to support neurons and not be directly involved in information processing. Astrocytes have been neglected as signalling cells in neuronal network function mainly because an astrocyte is not an excitable cell. However, there is accumulating evidence that there is an important role of astrocytes in the physiology of the nervous system (Tasker et al., 2012) based on the excitability of astrocytes caused by mobilization of

Ca^{2+} stored in endoplasmic reticulum which then act as cellular signal (Perea & Araque, 2005; Araque & Navarrete, 2010) (Figure 1.3).

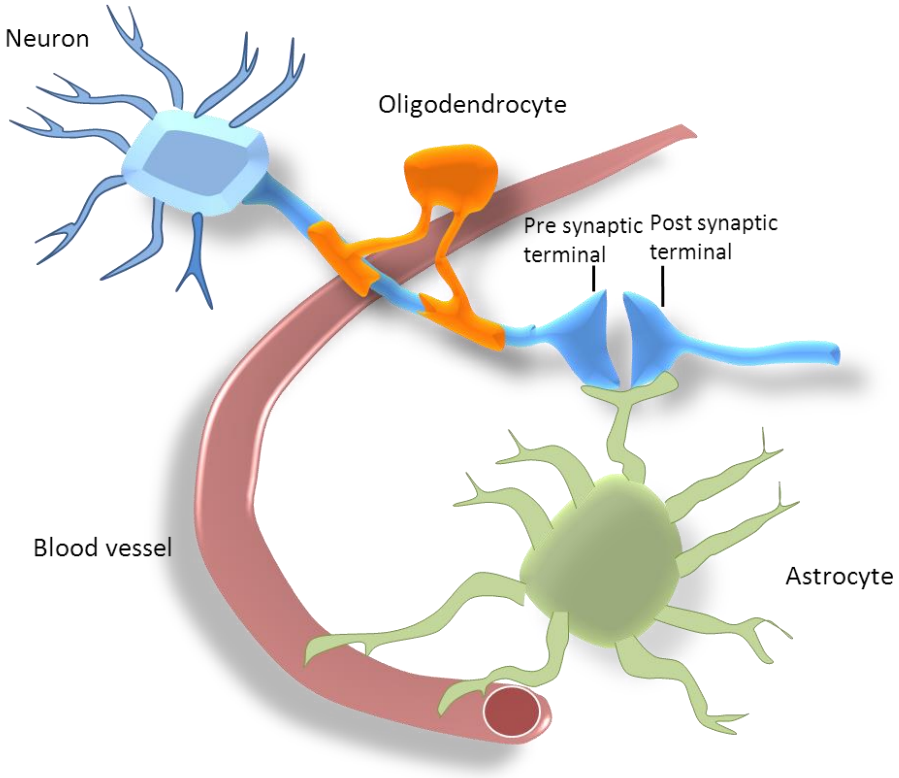


Figure 1.3: Neuron-astrocyte interaction

This Ca^{2+} elevation is activated either by neurotransmitter released during synaptic transmission and plasticity or spontaneously in the absence of neuronal activity and plays an important role in synaptic transmission.

This astrocyte-neuron relationship is termed the 'tripartite synapse', where astrocytes respond to neuronal activity as well as actively modulating synaptic activity. This will activate receptors at neighbouring astrocytes resulting in gliotransmitter release (termed as gliotransmission) including glutamate, ATP, ADP as well as D- serine, all of which have been shown to modulate synaptic function (Paixão & Klein, 2010; Perea & Araque, 2010). The evidence is not only *in vitro,* as a study conducted using human brain tissue has shown spontaneous Ca^{2+} elevation indicates that existence of bidirectional communication between astrocyte and neurons in human tissue (Navarrete et al., 2013) suggesting that neuron-astrocyte interactions also exist *in vivo*.

Moreover, astrocytes are also important for maintaining active neurotransmitter at excitatory terminals where the glutamate-glutamine cycle is required (Tani et al., 2014). Stimulation on transverse slices of the Schaffer Collateral region shows that glutamatergic synapses rely on astrocytes for recycling and generation of glutamate during prolonged periods of intermittent high frequency activity (Tani et al., 2014).

In terms of plasticity, there is growing evidence that astrocytes play a housekeeping role, which act to clear up neurotransmitter and may participate in synaptic plasticity in clearance and controlling the speed – it will increase post synaptic receptor activation and desensitization (Danbolt, 2001; Araque, 2001; Paixão and Klein, 2010).

1.6 Overview of protein kinase

Enzymes are naturally occurring substances that are responsible for a lot of metabolic reactions in the body. Each enzyme has a well-defined active site for a particular substrate to bind and consequently form a highly effective and selective catalyst but involved in a wide range of chemical reactions. They play a critical role in human health especially in regulating metabolic activities (Schomburg, 2001) including signal transduction and cell regulation via kinases and phosphatases. Protein phosphorylation regulates most aspects of cell life, whereas abnormal phosphorylation is a cause or consequence of disease. As a result of this, protein kinases have now become the second most prominent group of drug targets, after G-protein-coupled receptors (Cohen, 2002).

Protein kinase, a kinase enzyme that modifies other proteins by adding phosphate groups to it, is one important cell function regulator that constitutes one of the largest and most functionally diverse gene families. They mediate most of the signal transduction in eukaryotic cells as well as controlling many other cellular processes, including metabolism, transcription, cell-cycle progression, cytoskeletal rearrangement, cell movement, apoptosis and differentiation (Manning et al., 2002) with there being about 500 protein kinase genes in the human genome (Endicott et al., 2012). Abnormalities in protein kinases are implicated in a variety of human diseases such as cancer, rheumatoid arthritis, asthma, psoriasis, cardiovascular and neurological disorders (Melnikova & Golden, 2004). One important protein kinase signalling pathway is the mitogen-activated protein kinase (MAPK) cascade.

1.6.1 Mitogen Activated Protein Kinases

Mitogen Activated Protein Kinases (MAPK) are an evolutionary conserved cellular signalling pathway that is involved in cell functions and physiological process. It is a serine-threonine protein kinases that is stimulated in response to extracellular or intracellular stimulation. MAPK are regulated through tyrosine and threonine dephosphorylation (Caunt and Keyse, 2013) via a conserved cascade of upstream kinases, composed of MAPK, MAPK kinase (MAPKK, MKK or MEK and a MAPKK kinase or MEK kinase (MAPKKK or MEKK) (English, 1999). The phosphorylation is highly specific; for example, each MEK phosphorylates only one or a few of the MAP kinases (Raman et al., 2007). This will result in a conformational change and an increase in specific activity; so as a result, MAPKs are inactive unless phosphorylated by their respective upstream kinases (Figure 1.4). ERKs are normally activated by a mitogen and JNK and p38 MAPK are normally simulated by stress or inflammation. Once the upstream cascade is activated, it will lead to the sequential activation of MAPKs from MAPKKK to MAPK and eventually will activate the target protein (Cargnello and Roux, 2011). In mammals, three distinct groups of MAPK have been identified which are ERK 1/2, JNK and p38 MAPK based on their structure and functional reactivity (Zhang & Dong, 2007) (Figure 1.5).

1.6.2 ERK 1 and ERK 2 cascade

The best characterized MAPK are members of the ERK family that possess six isoforms (ERK1–5 and ERK7/8) (Bogoyevitch and Court, 2004). Within this family, the two most studied members are ERK1 and ERK2 with molecular weights of 42

kDa and 44kDa respectively (Sturgill & Wu, 1991). In the mammalian ERK1 and ERK2 signalling pathway, Ras associates with Raf-1 and B-Raf, which upon phosphorylation act as a MAPKKK and activates the dual function threonine and tyrosine kinases MEK 1/2 which in turn phosphorylates and activate ERK1 and ERK2 respectively (Cargnello and Roux, 2011). Upon stimulation, ERK1 and ERK2 can phosphorylate a variety of substrates that in turn are involved in proliferation, differentiation, neuronal plasticity, stress responses and apoptosis (Yoon and Seger, 2006). On the other hand, dysregulation of ERK1 and ERK2, is associated with various pathologies including cancer (Montagut & Settleman, 2009), Alzheimer's disease (Perry et al., 1999) and Parkinson disease (Kim and Choi, 2010).

Mounting evidence has shown that ERK is activated by neuronal activity and is involved in synaptic plasticity and learning and memory (Impey et al., 1999, Sweatt, 2011). ERKs are also activated in neurons by a variety of extracellular signals (Kurino et al., 2002; Widmann et al., 1999). Previous *in vivo* studies using MEK inhibitors further suggests a role for the ERK pathway in long-term memory formation (Cooke & Bliss, 2006; Thomas & Huganir, 2004). Further details of this part including the mechanisms proposed will be explained further in the next subtopic.

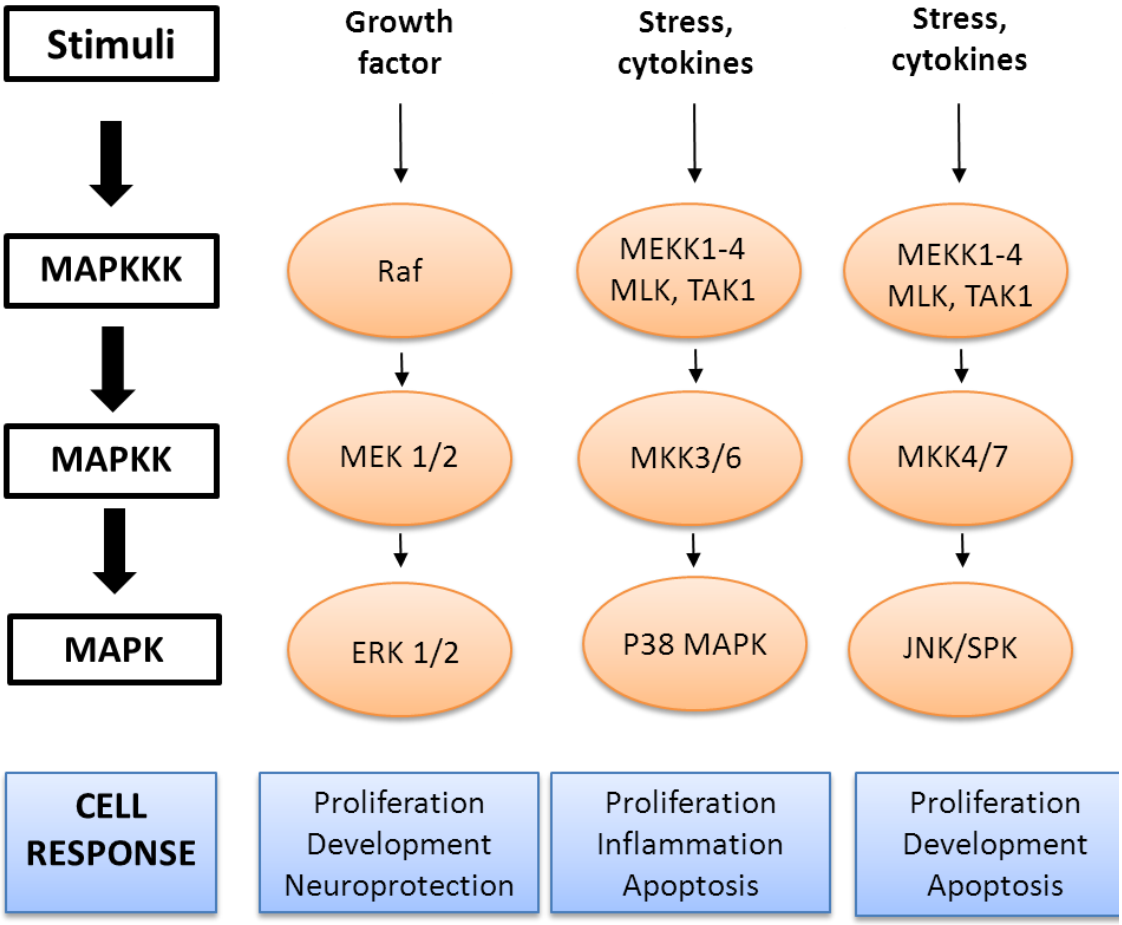


Figure 1.4: Schematic illustration of the MAP kinase cascades in mammalian cells

1.6.3 c-Jun N terminal kinases/stress activated protein kinases (JNK/SAPK)

There are three genes that encode for JNK which are JNK1, JNK2, and JNK3 with JNK3 being reported to be expressed primarily in the brain while JNK1 and JNK2 are more ubiquitous (Weston et al., 2003; Yamasaki et al., 2012). The weight of JNKs is around 46 to 54 kDa (Hibi et al., 1993; Kyriakis and Avruch, 2001) and they activate the transcription factor AP-1 (Weston and Davis, 2002) and c-Jun, which is a mediator of intra and extracellular stressors (Davis, 1994). JNKs are stimulated by a large number of external stressors including heat shock and UV irradiation as well as inflammatory cytokines (Johnson & Nakamura, 2007; Weston & Davis, 2002). Upon stimulation, JNKs are able to phosphorylate a large number of substrates in the nucleus and cytoplasm. This will further regulate many genes that mediate cellular processes including apoptosis (Dhanasekaran & Reddy, 2011), insulin signalling and neuronal activity (Haeusgen et al., 2009). JNK dysregulation is implicated in various diseases, including neurodegenerative diseases including Alzheimer disease (Giovannini et al., 2008), Parkinson's disease, amyotrophic lateral sclerosis (Kim and Choi, 2010) and Huntingtons Disease (Perrin et al., 2009). Moreover, it has been reported that mice lacking the JNK3 gene shown a reduction in excitotoxicity-induced apoptosis of hippocampal neurons, suggesting that the requirement of JNK3 activity for neuronal apoptosis (Yang et al., 1997).

1.6.4 p38 MAPK pathway

With regard to the p38 MAPK pathway, GTPases (Rac, Rho and Cdc42) are responsible for the transmission of stimuli to the MAPKKKs. The p38 MAPK family

has four isoforms, which are α, β, γ and δ (Han and Sun, 2007). Each isoform is subject to substrate specificity and differential localization with p38α and p38β MAPK having a wider expression pattern compared to p38γ and p38δ (Mayor et al, 2007) and they are responsible for the activation of heat shock proteins as well as many other substrates. The activation pathways for p38 MAPK includes stress, cytokines and growth factors (Roberts and Der, 2007). After stimulation, p38 MAPK activity is critical for normal immune and inflammatory responses (Roux & Blenis, 2004) and apoptosis (Xia et al., 1995). Furthermore, p38α MAPK has been established as a drug discovery target for inflammatory disease includes Crohn's disease and rheumatoid arthritis (Andreakos, 2003) as well as neurodegenerative diseases (Harper & Wilkie, 2003).

The function of p38 MAPK signalling in neurons is poorly investigated compared to ERKs and JNKs. Despite this, it is generally accepted that p38 MAPK has been associated with cell death. For example p38 MAPK activity has been shown to be increased after axotomy in retinal ganglion cell (Kikuchi et al., 2000). Furthermore, a previous research has shown that p38 MAPK pathway serves as a signal mediator in the induction of mGlu-dependent LTD at excitatory synapses between CA3 and CA1 pyramidal neurons in the mammalian hippocampus (Bolshakov et al., 2000). Similarly, a previous study has shown the involvement of p38 MAPK in translational control in the nervous system, but not ERKs, suggesting that p38 MAPK regulates synaptic plasticity in an opposing way compared to ERK (Dyer & Sossin, 2000).

1.6.5 The role of MAPK cascade in neuronal development

MAPK cascade has been shown to play important role in neuronal development. For instance, α-lipoic acid treatment has been shown to increase neurite outgrowth and its stimulation significantly increased ERK phosphorylation (Wang et al., 2011). A previous study conducted on embryonic stem cells revealed that after 5 days neural differentiation, there was dramatic increase in ERK phosphorylation which largely inhibited in the presence of MEK inhibitor (Li et al., 2006). In addition, knocking out the ERK gene in the early development resulted in the reduction of cortical size and reduced in brain size (Pucilowska et al., 2012).

As for JNK, its function on neuronal development has been associated with axon and neurite formation. For example, a studies on neuronal cultures shown that inhibition of JNK prevent axon degeneration but it does not alter neurite formation (Oliva et al., 2006). In another study, JNK activity was shown to be increase in PI-3 kinase that involve in neurite outgrowth in PC12 cells and blocking the pathway significantly inhibited the process formation suggesting JNK pathway is important in process formation (Kita et al., 1998). Based on the findings, it can be concluded that MAPKs play an important role in neuronal and axonal development.

1.6.6 The role of the MAPK cascade in synaptic plasticity

MAPK signalling components are highly enriched in the adult CNS and the expression of MAPKs signalling component is high in the hippocampal area (Impey et al., 1999). There is a number of previous studies that shown MAPK cascade plays a critical role of the in the regulation of synaptic plasticity, namely short-term plasticity or LTP and LTD. For example, NMDAR-dependent LTP in area CA1 is generally divided into two phases: early-LTP which last about 60-90 minute and late-

LTP which is longer lasting and is blocked by inhibitors of protein and RNA synthesis (Frey et al., 1988; Sweatt, 2001). Early LTP generally depends on CAMKII phosphorylation whereas late-LTP required new protein synthesis through activation of transcription factors for example cyclic AMP responsive element binding protein (CREB) (Adams et al., 2000; Alzoubi and Alkadhi, 2007). CREB is one transcription factor that is not only involved in hippocampal LTP (Alberini, 2009), but also critical in many important functions in the nervous system, including neurogenesis and neuronal survival, development and differentiation, as well as neuroprotection, axonal outgrowth and regeneration (Alberini, 2009). During high-frequency stimulation, Ca^{2+} influx activate adenylyl cycles type I which in turn will activate PKA through cAMP (Impey et al., 1999) or through Ras/RSK/ERK pathway. Activated ERK is translocated to nucleus and subsequently activates CREB to begin a new protein transcription (Choi et al., 2011). In addition, ERK2 has been shown to be activated in hippocampal area CA1 by NMDA receptor stimulation (English and Sweatt, 1996). Pharmacological studies have also shown that ERK signalling is involved in plasticity (English and Sweatt., 1997) through NMDAR stimulation that eventually leads to the activation of ERK2 in both cortical and hippocampal neurons in culture (Kurino et al., 1995).

JNK is suggested to be involved in cytokine-induced impairment of synaptic plasticity (Curran et al., 2003) as well playing an important role in long term memory and short term memory (Bevilaqua et al., 2003). Application of SP600125 (a JNK inhibitor to brain slices has significantly altered LTD but no changes in LTP compared to control (Curran, 2003). In contrast, electrophysiological studies on c-Jun mutant mice shows that LTP induction was impaired whereas there was no

change in LTD induction (Seo et al., 2012). Similarly, late-LTP examined in JNK2$^{-/-}$ mice was reduced whereas short-term plasticity was impaired in JNK1$^{-/-}$ mice (Li et al., 2007). Furthermore, the importance of JNK in synaptic plasticity shown as activating protein-1 (AP-1), an immediate early transcription factor for JNK (Curran and Morgan, 1995), positively regulates both synaptic strength and synapse number and in effect shows a greater effect compared to CREB (Sanyal et al., 2002).

In comparison with ERK and JNK, most of the studies regarding the role of p38 MAPK in synaptic plasticity are associated with LTD. For instance, whole cell recordings study on hippocampal slices has shown mGlu antagonist largely blocked the increase in p38 MAPK phosphorylation following the LTD-induction (Bolshakov et al., 2000). Moreover, perfusion of SB203580, a p38 MAPK inhibitor, on hippocampal slices completely blocked LTD induction whereas application of JNK inhibitor had no effect on LTD (Moult et al., 2008). Similar result obtained by another study showed that application of SB203580 prevented induction of LTD by DHPG (Rush et al., 2002).

Taken together, these findings suggest that the MAPK cascade plays an important role in synaptic plasticity, involved in the formation of both short-term and long-term memory.

1.7 Regulation of MAPK signalling pathways by protein phosphatase

MAPKs are deactivated following dephosphorylation by mitogen activated protein kinase phosphatase (MKPs). MKPs are a member of the threonine-tyrosine dual-specificity phosphatases (DUSP) family (Wu, 2007) which means it has the ability to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues

within one substrate (Boutrous et al., 2008; Petterson et al., 2009). The duration and magnitude of MAPK activity will determine the physiological outcome of its signalling; hence, it is extremely crucial to regulate its activity. Dysregulation of MAPKs has been implicated in the pathogenesis of many diseases including Alzheimer's disease (AD) and Parkinson's disease (Kim & Choi, 2010). Previously, it has been shown that the pathogenesis of AD is associated with the regulation of neuronal apoptosis, β- and γ-secretase activity, and phosphorylation of APP and tau (Kim and Choi, 2010) suggesting that the MAPK pathway may be targeted for the treatment of this neurodegenerative disease.

1.7.1 MAP Kinase Phosphatase Structure

MKPs belong to the large dual specificity family which have the ability to dephosphorylate serine/threonine and tyrosine residues in the activation loops of MAPKs (Lloyd & Wooten, 1992) (Figure 1.5).The inactivation of MAPK proceeds through a two-step dephosphorylation process: 1) binding of MAPK to MKP and the first residue to be dephosphorylated is phosphor-serine/tyrosine, 2) binding of MAPK to MKP, then phospho-threonine residue is dephosphorylated before both proteins are released (Alonso et al., 2004; Farooq and Zhou, 2004). Up until now, ten mammalian MKPs have been characterized based on four characteristics: gene structure, sequence similarity, substrate specificity and subcellular localization (Theodosiou and Ashworth, 2002; Caunt and Keyse, 2013).

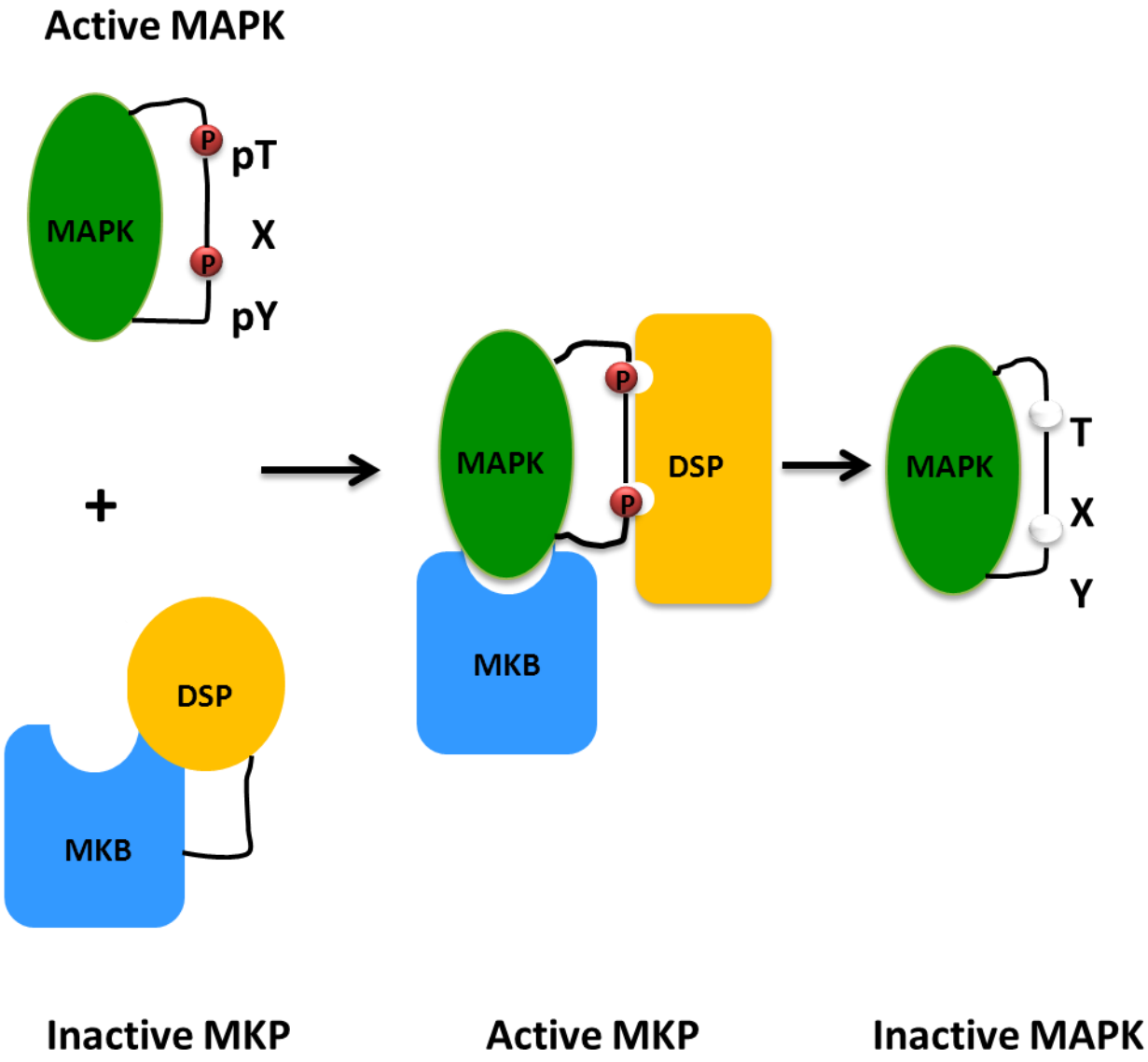


Figure 1.5: A model for the substrate-induced catalytic deactivation of MAPKs by MKPs

1.7.2 Classification of MAP Kinase Phosphatases

Based on these three characteristic; subcellular location, substrate preference and sequence homology, MKPs are grouped into three group. The first group comprises of DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2, and DUSP5/hVH-3, which all are mitogen and stress inducible nuclear MKPs. DUSP6/MKP-3, DUSP7/MKP-X and MKP-4/DUSP9 are in group II, which are cytoplasmic and ERK specific MKPs. The third group consists of MKP-5/DUSP10, MKP-7/DUSP16 and DUSP8 (MK3/6) which are JNK/p38 MAPK specific and located both in the nucleus and cytoplasm (Farooq and Zhou, 2004; Theodosiou and Ashworth, 2002) (Table 1.1).

1.7.3 Physiological function of MAP Kinase Phosphatase in CNS

Among the ten MKPs, MKP-1 is the most extensively studied, particularly in relation to the immune response and cancer. Even though the function of MKP-1 in the brain is less well understood, in previous studies on Multiple Sclerosis patients, MKP-1 has been found to be highly expressed in microglia cells as compared to control during CNS inflammation, suggesting MKP-1 plays an important role in regulation and control of inflammatory reactions and thus provides neuroprotection within the CNS during CNS inflammation (Eljaschewitsch et al., 2006). Moreover, an *in vitro* study using lentiviral MKP-1 expression vectors has shown that MKP-1 provides neuroprotection in primary striatal and cortical neuron models of Huntington's disease, *in vitro* as well as *in vivo* (Taylor et al., 2013). In term of neurodevelopment, MKP-1 is proposed to regulate neuron axonal development (Jeanneteau et al., 2010). A study on MKP-$1^{-/-}$ mice, in response to BDNF, showed a reduction in primary branch formation when compared to MKP-$1^{+/+}$.

Group	MKP	Substrate specificity	Gene
I	MKP-1	p38 ~ JNK>>ERK	DUSP1
	PAC-1	ERK>> p38~JNK	DUSP2
	MKP-2	ERK~JNK> p38	DUSP4
	hVH-3	ERK>> JNK ~ p38	DUSP5
II	MKP-3	ERK>> JNK ~ p38	DUSP6
	MKP-X	ERK>p38>>JNK	DUSP7
	MKP-4	ERK>>p38>JNK	DUSP9
III	MKP-5	P38>JNK>>ERK	DUSP10
	MKP-7	JNK~p38>>ERK	DUSP16
	MK3/6	JNK~p38>>ERK	DUSP8

Table 1.1: Summary of mammalian MAPK Phosphatase

This study suggests that regulation of MKP-1 protein level and BDNF may play an important role in axon branching as well as connectivity (Jeanneteau et al., 2010). Conversely, dysregulation of MKP-1 levels are also linked to pathophysiology in the brain. In a study on post-mortem tissue taken from hippocampus region of patients who suffered from major depressive disorder, MKP-1 expression is significantly increased (Duric et al., 2010). A subsequent study conducted in a rodent chronic unpredictable stress model suggests the dysregulation of MKP-1 will induce depressive-like behaviour.

In regard to MKP-3, in contrast to MKP-1, MKP-3 overexpression is not neuroprotective *in vitro* or *in vivo* (Taylor et al., 2013). Another study on MKP-3 however indicates that MKP-3 plays a role in regulating survival and programmed cell death of neurons under stress conditions (Mishra and Delivoria-Papadopoulos, 2004).

1.8 Mitogen-Activated Protein Kinase -2

MKP-2 is grouped in the type 1 DUSP family, a 42 kDa phosphatase with a substrate preference of ERK ~ JNK > p38 (Guan & Butch, 1995). It is induced by growth factors that have a distinct pattern of tissue distribution compared to DUSP1 (Guan and Butch, 1995) and has been reported to be expressed moderately in all tissues and cells including all areas of the brain (Kwak et al., 1994; Misra-Press et al., 1995). Furthermore, in contrast to MKP-1, which has been found in mitochondria, it is located exclusively in the nucleus (Rosini et al., 2004) and encodes a phosphatase

that inactivates MAP kinase *in vitro* and MAP kinase dependent gene transcription *in vivo* (Misra- Press et al., 1995). Despite the fact that MKP-2 binds strongly to p38 MAPK, it does not dephosphorylate it *in vitro* (Al-Mutairi et al., 2011). Indeed, MKP-2 specificity has been reported to be for ERK and JNK over p38 MAPK. However, selectivity in cells can be variable depending on the cell type investigated (Guan and Butch, 1995).

Among its physiological functions is the regulation of the immune system. Using a MKP-2 knockout mouse model, MKP-2 deletion compromises the immune system as evidenced by the increased the growth of parasite; *Leishmania mexicana* (Al-Mutairi et al., 2010). In addition, MKP-$2^{-/-}$ mice also exhibited a decreased inflammatory response subsequent to LPS stimulation; suggesting it functions to regulate the inflammatory response (Cornell et al., 2010). In cell proliferation studies, MKP-2 has a non-redundant function compared to MKP-1. Deletion of MKP-2 in mouse embryo fibroblast reduced the proliferation rate as shown by an increase in doubling time (Lawan et al., 2011). Furthermore, DUSP4 deletion is also found to be protective against TGFβ (transforming growth factor β)-induced apoptosis in B cells (Ramesh et al., 2008). These previous studies disclosed an improvement in our understanding of MKP-2 in physiological functions. In contrast, its function in the CNS is inadequately studied.

1.8.1 Function of MKP-2 in CNS

Production of MKP-2 knockout mice has enhanced understanding of its function in regulation of cellular proliferation and survival (Lawan et al., 2011), as well as genotoxic stress-induced apoptosis (Cadalbert et al., 2010). However, there is a lack

of understanding of the MKP-2 function in the CNS as no specific antagonist yet exists. Studies have shown that MKP-2 is expressed in the CNS suggest a potential role for this phosphatase in neuronal signalling pathways (Mishra-Press et al., 1995). Furthermore, based on a post-mortem study, it has been shown that a disturbance in MAPK signalling may contribute to major depression (Dwivedi et al., 2001). The level of ERK1 and ERK2 were also decreased in an animal model of depression (Feng et al., 2003; Gourley et al., 2008). A study on the post-mortem schizophrenic brain has shown that significant decrease in the levels of MKP-2 (Kyosseva et al., 1999). Based on these evidences, it is intriguing to speculate that removing the normal regulatory functions of MKP-2 will cause neuronal dysfunction.

1.9 Working hypothesis: aim and objectives

Extensive studies have also been done on the function of MKP-2 on proliferation and cell survival (Lawan et al., 2011), immunology (Gehart et al., 2010) and cancer (Haagenson and Wu, 2010; Haagenson et al., 2014). However, the regulation of MAPK cascade by MKP-2 in post mitotic cells of the CNS is just beginning to be investigated despite evidence that MAPKs play an important role in synaptic plasticity (Adams et al., 2000). So, this project will investigate the role MKP-2 in modulating neuronal function in the CNS and may provide key evidence for future behavioural investigations into the consequence of MKP-2 deletion.

Hence, the central hypothesis of my PhD is that MKP2 deletion will modulate hippocampal cell proliferation and neuronal function.

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

1. Is activation of MAPKs altered as a consequence of MKP-2 deletion?
2. Does MKP-2 affect astrocyte proliferation and have a knock-on effect on neuronal growth?
3. Does deletion of MKP2 affect hippocampal synaptic transmission?
4. Is short-term plasticity altered in the CA1 region of MKP-2 knock-out mice?

2 MATERIAL AND METHODS

2.1 Materials

Materials	Company
D-Glucose, sodium chloride (NaCl), ethylene-di-amine tetra-acetic acid (EDTA), EGTA, paraformaldehyde (PFA), sodium chloride (NaCl), bovine serum albumin (BSA), phosphate buffer tablet (PBS), methanol, ethanol, papain, triton X-100 solution, sodium dodecyl sulphate (SDS), Tween-20, glycine, Tris, TEMED, Kodak X-Omat Film, sodium hydrochloride (HCl), adenosine triphosphate (ADP), potassium methyl sulphate ($KMeSO_3$), Anti-β-III tubulin, HEPES, Acrylamide, bis-Acrylamide, cytosine β-D-arabinofuranoside (ArAc), poly-L-lysine hydrobromide.	Sigma Aldrich (UK)
Calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), potassium chloride (KCl), sodium dihydrogen phosphate	VWR Internatrional Ltd (UK)

(NaH_2PO_4), sodium hydrogen carbonate ($NaHCO_3$), sucrose, Coverslips, round, 13 mm, thickness No. 1 (Boroscillate Glass).	
Alexa Fluor anti-rabbit 488 IgG, Alexa Fluor 555 anti-mouse IgG, B-27, L-glutamine, Neurobasal-A, FLUO-4 AM, NP-40	Invitrogen (UK)
SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor), UO126 (ERK inhibitor) (S)-3,5-Dihydroxyphenylglycine (DHPG),	Ascent Scientific (UK)
Anti- Glial Fibrillary Acidic Protein (GFAP) antibody, Anti-Synaptophysin Antibody	Merck Millipore, Germany.
Anti-phospoERK antibody (for imuunocytochemistry)	Cell Signalling Technology, Inc (USA)
Anti-phospho ERK (for western blot), Anti-totalERK antibody	Santa Cruz Biotechnology Inc, (UK)
ECL detection reagents	Amersham International Plc, (UK)
Borosilicate glass micropipette (1.5 mm	Harvard Apparatus Ltd, (UK)

O.D x 0.86 mm I.D)	
Cell culture dishes, 35 mm (TC treated)	BD Biosciences (UK)
Serological pipette	Nunc / Nunclon, Thermoscientific Inc (UK)

2.2 Mice

MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were provided by Professor Robin Plevin. The mutation was generated by 129Sv mouse embryonic stem cell and male chimeras obtained were crossed with C57Bl/6 female to obtain the F1 generation, and these mice has been described in previous studies (Al-Mutairi et al., 2010). All animal care and experimental procedures were in accordance with UK Home Office guidelines.

2.3 Primary hippocampal culture

2.3.1 Primary hippocampal culture preparations

Autoclaved sterile coverslips were coated with filter sterilized 0.1 % poly-L-lysine for one hour before being washed rigorously and dried prior to being plated in 33 mm petri dishes (3 per dish). The hippocampal culture media, a serum-free media (97 % Neurobasal A, 2 % ml B-27 supplement and 1% L-glutamine (200 mM stock concentration) were prepared, filter sterilized and kept in a water bath (37°C) until required.

2.3.2 Primary hippocampal culture procedure

Mice pups (1-2 days old) were decapitated and killed in agreement with the UK Home Schedule 1 guidelines under the authority of the U.K Animals (Scientific Procedures) Act, 1986. The brain was removed, hippocampi were dissected out and

placed in a sterile buffered enzyme solution (116 mM NaCl; 5.4 mM KCl; 26 mM $NaHCO_3$; 1.3 mM NaH_2PO_4; 2 mM $MgSO_4$; 2 mM $CaCl_2$; 25mM D(+)-glucose) containing 1.5 % papain for 20 minutes. The cells were then dissociated by triturating, using a series of sterile flame-polished glass pasteur pipettes of decreasing tip diameter in 1 % bovine serum albumin (BSA), to prevent further papain catalysis. The cells were centrifuged for 2 minutes at 2000 rpm, producing a cell pellet that was then resuspended using 1 ml hippocampal culture media. The cells were counted using a haemocytometer and diluted to give a final density of 3.0 x 10^5 cells/ml. Once the appropriate final cell concentration was achieved, the cells (100 µl per coverslip) were seeded on glass coverslips and were incubated at 37 °C, 5% CO_2 incubator for 1 h. The excess cells were then discarded by pipetting off any remaining media and the petri dishes were then carefully flooded with 2 ml hippocampal culture media. The cultures were maintained in hippocampal media and incubated at 37 °C, 5% CO_2 incubator until required for experimentation.

2.4 Immunocytochemistry

2.4.1 Antibodies for immunohistochemistry

Anti-β-III tubulin (neuron specific cytoskeleton marker, 1:500 dilution) and glial fibrillary acidic protein (GFAP, astrocytic marker, 1:500 dilution) was used to identify neurons and astrocytes respectively with anti-synaptophysin (synaptic vesicle marker, 1:500 dilution) was used to identify synapses. Anti-phospho ERK and anti-total ERK were both used at a final dilution of 1:500.

2.4.2 Procedure for immunohistochemistry

Primary cultures of the appropriate days *in vitro* (DIV) were washed thrice with PBS before being fixed in ice-cold 4% paraformaldehyde for 10 minutes. The cells were washed again thrice with PBS prior to treatment with 100% ice-cold methanol for a further 10 minutes and again washed with PBS (3x washes) following which the cells were permeabilised with 0.01% Triton-X (in PBS) for 10 minutes. Blocking buffer (5% foetal bovine serum (FBS) v/v and 1% BSA w/v in PBS) was added to the cells and incubated for 1 hour to block non-specific binding. Primary antibodies were prepared at the appropriate dilution in blocking buffer to identify particular cells or cellular components. The block/antibody solution (100 µl) was applied directly to the coverslip and incubated overnight (16-20 hours) at 4 °C in a wet box. The cultures were washed three times with PBS to remove any unbound primary antibody and a fluorescent secondary antibody added. Alexaflour 488 goat anti-mouse and Alexaflour 555 goat anti-rabbit fluorescent secondary antibodies were used simultaneously. Secondary antibodies were prepared in blocking buffer at a 1:200 dilution and applied directly to each coverslip and kept in the dark for 1h. To remove the excess secondary antibodies, coverslips were washed three times with PBS. Coverslips were then kept in dishes (maximum 3 weeks) containing PBS until viewed using epifluorescent microscopy. Image acquisition was performed with an OLYMPUS BX51W1 microscope with a Q-imaging digital camera and using WinFlour v3.4.4 imaging software (J Dempster, University of Strathclyde). Cells were imaged using a 40x water immersion lens with excitation using excitation filters to visualize AlexaFlour 488 and 555 secondary antibody signals respectively.

2.5 Astrocyte number and neurite length

Preparation of hippocampal cultures was conducted as described in section 2.3.2 but with final density of cell is 1.5 x10^5 cells/ml. The cells were then fixed and underwent immunocytochemistry using identical procedures to that described in section 2.4.

2.5.1 Control experiments for astrocyte number and neurite length

2.5.1.1 MKP-$2^{-/-}$ cultured grown in MKP-$2^{+/+}$ conditioned media

MKP-$2^{+/+}$ hippocampal cultures were grown for 5 – 7 days and the conditioned media (CM) from these cultures was used to grow MKP-$2^{-/-}$ cultures from 0 DIV, instead of normal hippocampal culture media.

2.5.1.2 MKP-$2^{+/+}$ cultured grown in MKP-$2^{+/+}$ conditioned media

MKP-$2^{+/+}$ hippocampal cultures was grown for 5 – 7 DIV and the conditioned media (CM) was used to grow MKP-$2^{+/+}$ culture, instead of normal media.

2.5.1.3 Inhibition of astrocyte proliferation

MKP-$2^{+/+}$ and MKP-$2^{-/-}$ hippocampal cultures were supplemented with cytosine β-D-arabinofuranoside (AraC) (10 μM), an astrocyte inhibitor on day 1 to reduce astrocyte proliferation.

2.5.2 Analysis

Astrocytes: images were taken from 3 random microscopic fields per coverslip with the number of GFAP-positive astrocytes counted and reported as astrocytes per field within representing the number of fields taken. Neurons: images were taken of 10 randomly chosen neurons from each coverslip. Neurite length was then measured

using NeuronJ software (Meijering, 2010). For neurons, n represents the number of neurons from at least three separate cultures (n > 50). For controlled experiments, the astrocytes number and neurite length were measured for the first four days. All data are expressed as mean ± S.E.M. Data were compared by unpaired t-test or two-way analysis of variance with Tukey's comparison as appropriate with $P < 0.05$ considered significant.

2.6 ERK activity in primary hippocampal cultures

Primary hippocampal cultures were prepared as describe in section 2.3.2 and the cells were stained as described in immunocytochemistry procedure in section 2.4 using phosphoERK and ERK antibodies.

2.6.1 Analysis

Fluorescent intensity was analysed using ImageJ Software v1.46 (National Institute of Health) software. The images were normalised using Adobe Photoshop CS3 to a standard brightness and contrast. 10 neuronal cells per image were chosen that showed a growth independently from other cells and appeared healthy. A boundary around the cells was drawn with ellipse tool that include the nucleus and the soma. The multi measure plugin was used and integrated density was calculated. All data are expressed as mean fluorescent density ± S.E.M. with n representing the number of cells from at least three separate cultures. Data were compared by one-way analysis of variance with Tukey's comparison as appropriate with $P < 0.05$ considered significant.

2.7 Synaptophysin in primary hippocampal culture

Primary hippocampal cultures were prepared as describe in section 2.3.2 and the cells were stained as described in immunocytochemistry procedure in section 2.4 using anti-synaptophysin and anti-β-tubulin III antibody.

2.7.1 Analysis

To count the number of synapses, the field of view was divided into squares and the length of the neurites within the squares and the number of synapses along the neurites was determined. All data are expressed as mean of number of synapstic puncta per μm of dendritic length ± S.E.M. with n representing the number of cells from at least three separate cultures. Data were compared by one-way analysis of variance with Tukey's comparison as appropriate with $P < 0.05$ considered significant.

2.8 Electrophysiology in cultured primary neurones

2.8.1 Patch clamp electrophysiology recording procedure

Patch clamp recording setup; a submerged recording chamber, an inverted microscope (Nikon Eclipes TS100, Japan) and a manipulator (MP-365 Sutter instrument company, USA) were mounted on an anti-vibration table (Intacell Isolate System). The whole recording system was covered by a faraday cage to protect from external electrical noise (Figure 2.1). Signals in the experiments were captured by an Axopatch 1D amplifier (Molecular Devices, USA) digitized at 10 kHz and filtered at 2 kHz. Borosilicate glass micropipettes were pulled with an electrode puller (DMZ-Universal, Germany) and filled with an internal solution containing: 130 mM

$KMeSO_3$; 20 mM KCl; 10 mM HEPES; 0.5 mM EGTA; 4 mM MgATP; 0.3 mM GTP; pH was adjusted to 7.2 and osmolarity was corrected to 290 ± 2 mOsm with sucrose if required. HEPES based saline (HBS) solution (140 mM NaCl; 2.5 mM KCl; 2 mM $MgCl_2$; 10 mM HEPES; 10 mM D-glucose, 1 mM $CaCl_2$, pH 7.4, and osmolarity was corrected if required to range between 310 ± 2 mOsm with sucrose) was perfused (1-2 ml/min) to cultured neurons continuously. Voltage clamp recording were perfomed in 3, 7 and 11-14 DIV (days in vitro). Glass electrodes (resistance range between 4-8 Ω) were moved by using manipulator (MP-365 Sutter instrument company, USA) towards the cells, and once the tip of the glass micropipette touched the membrane (seen as an increase in pipette resistance), a slight negative pressure was applied to the cells to obtain a high resistance seal. Once a gigaseal was obtained, extremely gentle short periods of suction were applied to rupture the membrane patch. The patched cell was left to equilibrate with the internal solution for 5 minutes before any recordings were made. To analyze sodium and potassium currents, voltage clamp experiments was conducted using the WinWCP Software (J Dempster, University of Strathclyde) with 14 depolarization steps (-90mV to +40mV) every 10s with the initial holding potential being -70mV. Spontaneous synaptic activity was investigated using WinEDR Software (J Demspter, University of Strathclyde) with the holding potential and sampling frequency being -65mV and 10kHz respectively.

2.8.2 Analysis

Sodium, potassium current and spontaneous activity was analyzed off-line using WinWCP Software and MiniAnalysis Software (Synaptosoft, USA) respectively where n represents the number of cells recorded from at least three separate cultures.

All data are expressed as mean ± S.E.M. Data were compared by one-way analysis of variance with Tukey's comparison as appropriate with $P < 0.05$ considered significant.

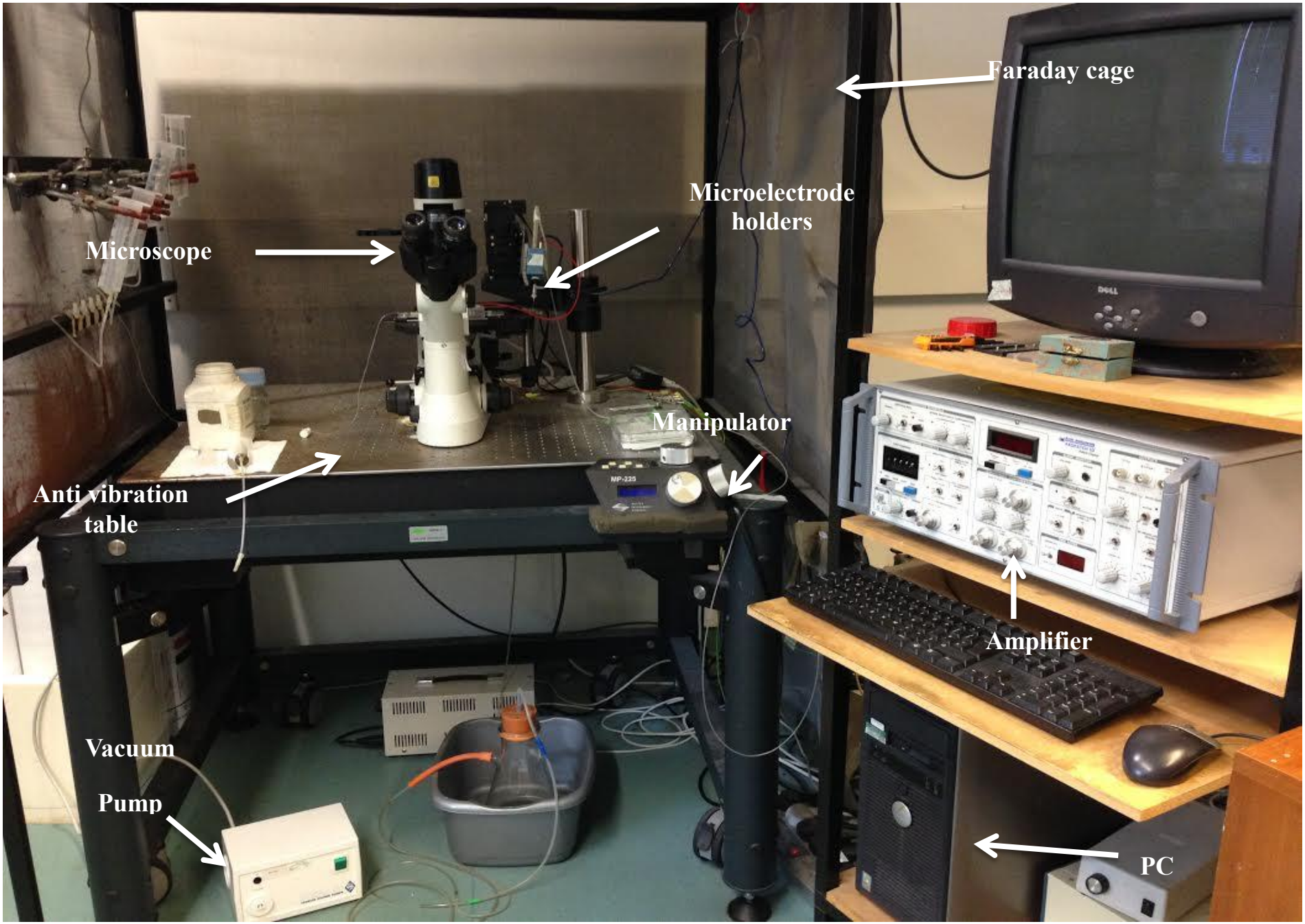


Figure 2.1: Image showing the setup used in patch clamp electrophysiology recordings

2.9 Calcium Imaging Experiments

2.9.1 Calcium imaging preparation

HBS solution was prepared as described earlier (see section 2.7.2). Fluo-4 AM (5µM) was prepared in HBS solution and hippocampal primary cultures (3, 7, 11-14 DIV) were washed with HBS solution before being incubated in FLUO-4 AM for 1 hour in the dark.

2.9.2 Calcium imaging procedure

Following incubation with Fluo-4 AM, the cultures were washed twice with HBS before being transferred to a perfusion system which allowed a constant flow of HBS solution (1-2 ml/min). All imaging experiments were performed using a 20X water immersion lens with excitation at 480nm using a fluorescence microscopy (OLYMPUS BX51W1) with a Q-imaging digital camera and WinFlour v3.4.3 software (J Dempster). Drugs were applied via the perfusate after 2 min stable baseline. (S)-3,5-Dihydroxyphenylglycine (DHPG) (100 µM, 5 min) and potassium chloride (25 mM, 30 s) were applied to activate neurons and astrocytes were activated by adenosine triphosphate (ADP) (1 µM, 2 min) and trypsin (100 nM, 2 min). Following drug applications, 5 min washout were applied to the cells.

2.9.3 Calcium Imaging Analysis

Image analysis was performed off-line using WinFluor v3.4.3. Regions of interest (ROI) were drawn around individual cells. The background fluorescence was subtracted from the ROI fluorescent intensity and then relative fluorescence change

(ΔF/F) versus time plots was generated for each ROI. n represents the number of cells from three separate cultures (n > 50 for each treatment). All data are expressed as mean ± S.E.M. Data were compared by unpaired t-test with $P < 0.05$ considered significant.

2.10 SDS PAGE and Western blotting

2.10.1 Sample collection

Male and female mice (19-25 days old) were decapitated and killed in agreement with UK Home Schedule 1 guideline under the authority of the U.K Animals (Scientific Procedures) Act, 1986. The brain, heart and liver were quickly removed and immersed in chilled artificial cerebrospinal fluid, (ACSF, 124 mM NaCl; 3 mM KCl; 26 mM $NaHCO_3$; 2.5 mM NaH_2PO_4; 1 mM $MgSO_4$; 2 mM $CaCl_2$; 10 mM D-glucose). Following the removal of the brain, the brain was hemisected and the cortex, hippocampus and cerebellum were carefully removed. The tissue was then transferred into a solubilisation buffer (0.3% NP-40; 150 mM NaCl; 25 mM Tris; 1mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM Na_3VO_4; 0.4 mM phenylmethylsulphonyl fluoride; 10 lg/mL leupeptin; 10 lg/mL pepstatin; 10 lg/mL aprotinin; pH 7.4) and incubated on ice for about 60 minutes following which the tissue was triturated using a 25g x 5/8" Becton Dickinson Microlance Needles needle.

2.10.2 Protein quantification

The total protein concentration was determined by Bradford reagent. Bovine serum albumin (BSA), used widely as a protein standard, was used to develop a standard curve with concentrations ranging from 0 - 20 μg/ml. Samples and the protein

standard (prepared in triplicate) were then transferred to cuvettes and absorbance were read at 595 nm. A Standard curve was plotted and used to calculate the amount of protein in 10 µl of each sample. 10 µg (cortex, hippocampus and cerebellum) and 5 µg (heart and liver) of total cellular protein was used for each experiment. The samples were then diluted by 25% with loading buffer (63 mM Tris; 50 mM dithiothreitol; 5 mM EDTA; 2 mM $Na_4P_2O_7$; 10% glycerol; 2% sodium dodecyl sulfate (SDS); 0.007% bromophenol blue; pH 6.8) and denatured by boiling for 5 min with the samples kept at -20ºC until used.

2.10.3 SDS-polyacrylamide gel electrophoresis

Glasss plates were cleaned and assembled in the appropriate casting frames. A 10 % resolving gel was prepared which contains 4.1 ml water, buffer 1 [1.5 M TRIZMA base, 0.4 % (w/v) SDS; pH 8.8], 4 ml acrylamide [30 % (w/v) acrylamide; 0.8 % (w/v) bis-acrylamide], 5 µl TEMED and 45 µl 10 % (w/v) Ammonium Persulfate (APS) in PBS. Resolving gels were poured between assembled plates and it was overlaid with 0.1 % SDS and allowed to set. While waiting for the resolving gel to set, stacking gels which contains 4.8 ml water, buffer 2 [0.5 M TRIZMA base; 0.4 % (w/v) SDS; pH 6.8], 0.75 ml acrylamide [30% (w/v) acrylamide; 0.8 % (w/v) bis-acrylamide], 10 µl TEMED 10 % (w/v) APS were prepared. In both resolving and stacking gel preparations, TEMED, a polymerization agent, was added last. Once the resolving gel was set, the SDS was discarded and stacking gels were poured on top of the set resolving gel. A comb was then inserted and everything was allowed to set for approximately 30 minutes. Once the gel was set, the comb was removed and the gel cassettes were then assembled in a Bio-Rad Mini-PROTEAN II™

electrophoresis tank. Both inner and outer tanks were filled with running buffer (24.8 mM Tris, 191.8 mM glycine, 3.46 mM SDS). A molecular weight marker (15 µl) was loaded into the first lane followed by samples (30 µl) into each lane using a Hamilton syringe. The gel electrophoresis was then run at 130 V for approximately 1 h 30 minutes or until the dye had run to the bottom of the gel.

2.10.4 Blotting to sample into nitrocellulose membrane

Once the gel had finished running, it was transferred to a nitrocellulose membrane. Nitrocellulose was soaked in transfer buffer (25 mM TRIZMA base, 19 mM glycine, 20% (v/v) methanol) and the gels were arranged in a transfer cassette in following order:

- Sponge
- Blotting paper
- Nitrocellulose
- Gel
- Blotting paper
- Sponge.

The assembled cassette was then placed in a Bio-Rad Mini Trans-Blot tank and filled with transfer buffer and run for approximately 1 hour 45 minutes at 300 mA.

2.10.5 Western blotting

Following the protein transfer to nitrocellulose, the membranes were then incubated in NaTT buffer (150 mM NaCl; 50 mM Tris; 0.2% Tween 20, pH 7.4), containing 4% bovine serum albumin (BSA) for 2 h to block non-specific binding. Blots were then incubated overnight on an orbital shaker at 4 °C in 0.4% BSA/NaTT containing

mouse anti- phosphoERK (1:3000 dilution). Blots were washed in NaTT for 2 h with a 15 min interval before incubation in 0.4% BSA with horseradish peroxidase-conjugated anti-mouse IgG antibody for 1.5 h. After further washing (NaTT; 2 h with 15 min interval), blots were developed using enhanced chemiluminescence (ECL) reagent for approximately 2 min. After the excess liquid was blotted with a paper towel, the membrane was then mounted into an exposure cassette and covered with cling film. Then, inside the dark room the membrane was then exposed to Kodak X-OMAT LS film for about 1-3 min before being developed using a Kodak M35-M X-OMAT processor.

2.10.6 Stripping for reblotting

Following detection of phospho-specific bands, blots were stripped by incubation in stripping buffer (100 mM 2-mercaptoethanol; 62.5 mM Tris; 2% SDS; pH 6.7) for 60 min at 75°C on a platform shaker at a speed of 55 rpm. Stripped blots were washed in NaTT thrice with 15 min interval, then re-blocked as in section 2.3.4 and reprobed with rabbit total ERK antibody (1:3000 dilution).

2.10.7 Western Blot Analysis

To determine the intensity of the bands, densitometry was used to quantify the bands using Scion Image Software (Scion, Frederick, MD) with the background intensity subtracted from total intensity. Values are expressed as percentage of phosphoERK over total ERK. All data are expressed as mean ± S.E.M. Data were compared by unpaired t-test with $P < 0.05$ considered significant.

2.11 Extracellular Electrophysiology

2.11.1. Preparation of acute hippocampal slices

Mice (male, 19 – 25 days old) were killed by cervical dislocation and decapitation in agreement with U.K Home Office Schedule 1 guideline under the authority of the U.K Animals (Scientific Procedures) Act, 1986. The brain was quickly removed and immersed in chilled (0-3°C), oxygenated (95% O_2/5% CO_2) ACSF. The brain was further hemisected, and each part was glued to the stage of a vibratome (Vibratome 1500 Intracel Ltd.) (Figure 2.2). Parasagittal brain slices (400 μm) were cut and placed in a holding chamber in continuously bubbled ACSF (95% O_2/5% CO_2), for at least 1 h at room temperature to equilibrate from slicing trauma.

2.11.2. Extracellular electrophysiology recording procedure

After at least 1 hour equilibrium time, hippocampal slices were transferred into the recording chamber (Scientific Systems Design Inc, UK) and continuously perfused with ACSF at a flow rate of 2 ml min^{-1} with the temperature maintained at 30 ± with ACSF at a flow rate of 2 ml min^{-1} with the temperature maintained at 30 ± 0.5°C by an automatic temperature control unit (Scientific Systems Design Inc, UK). Recordings were made with an Axoclamp 2A amplifier (Molecular Devices, USA) and signals were filtered at 2 kHz by using a 4-pole low-pass Bessel filter (Warner Instrument, Hamden, CT)(Figure 2.3).

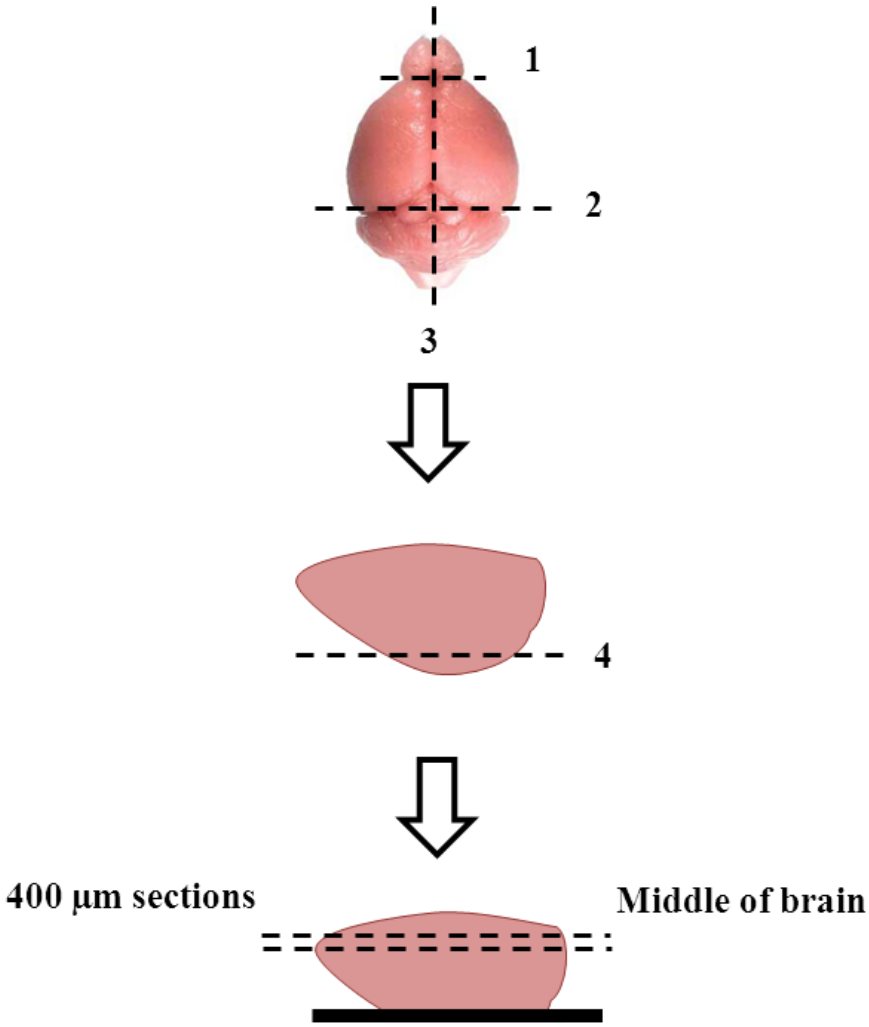


Figure 2.2: Process and orientation of parasagittal slice preparation.

The resulting signals were digitized at 10 kHz and transferred to the hard disc of a PC. Extracellular field excitatory postsynaptic potential (fEPSP) recordings were obtained in response to low frequency (0.033 Hz) stimulation of the Schaffer-collateral pathway. The pathway was stimulated with a bipolar tungsten stimulating electrode (Figure 2.4) and recordings made with a borosilicate glass recording electrode filled with ACSF positioned in stratum radiatum of area CA1. Once synaptic potentials were observed, the intensity of stimulating current was reduced to the lowest current that produced a response. An input output curve was generated by gradually increasing the intensity of the stimulating current until 10X the current required for the initial response was reached. For all other experiments, the stimulating current which gave the half maximum fEPSP response was then used for the remainder of the experiment. Paired pulse facilitation were conducted at interstimulus interval (10 - 500 ms).

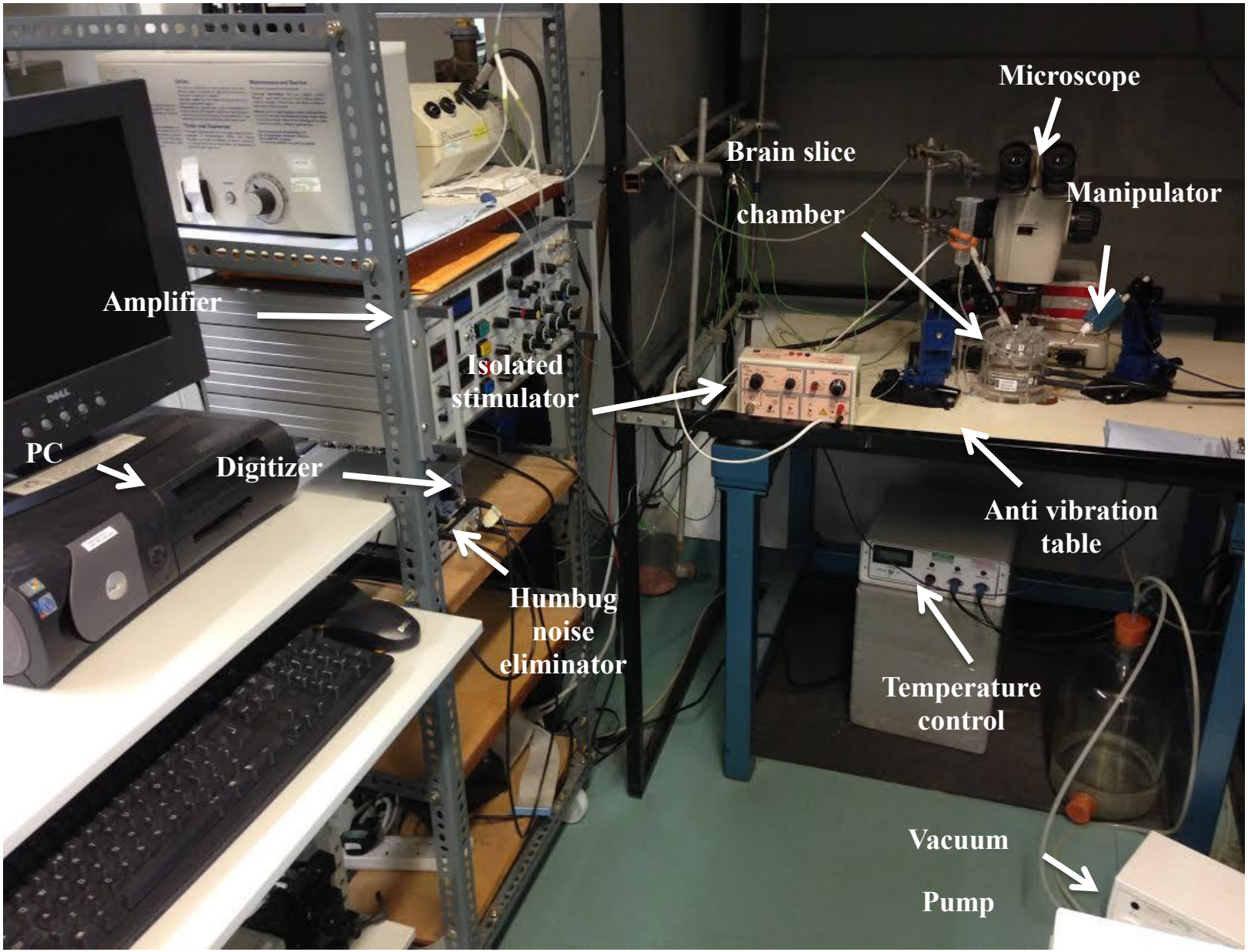


Figure 2.3: Setup used in extracellular electrophysiology recording

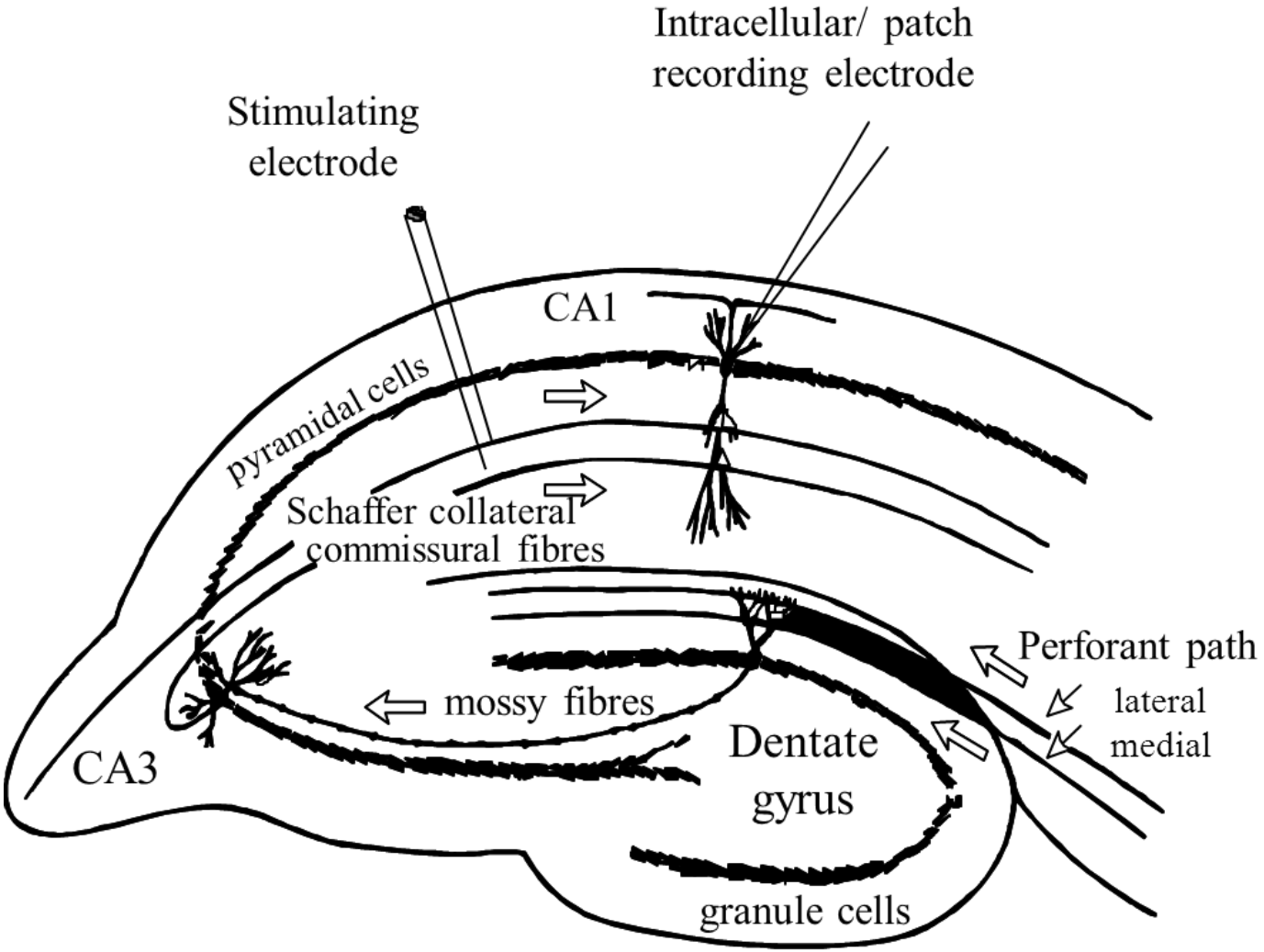


Figure 2.4: Schematic diagram illustrating the positions of electrodes for extracellular field recordings.

2.11.3. Pharmacological treatment

UO126 (20 μM, Favata et al., 1995), SP600205 (40 μM, Bennet et al., 2001; Costello and Herron, 2004)) and SB203580 (100 μM, Cuenda et al., 1995) were used as inhibitors of ERK, JNK and p38 MAPK respectively. Prior to application, a 20 minute stable baseline was obtained with all drugs administered for 20 min via addition to the perfusate.

2.11.4 Analysis

fEPSPs were acquired online and analysed offline using the WinFlour v3.4.4 software (J Dempster, University of Strathclyde). Synaptic efficacy was analysed by measuring the initial slope of the fEPSP and paired-pulse facilitation (PPF) was investigated at different inter-stimulus intervals (10 - 500 ms) as a ratio of the second fEPSP amplitude to the first fEPSP amplitude.

3 THE ROLE OF MKP-2 IN ASTROCYTE PROLIFERATION AND NEURITE DEVELOPMENT

3.1 Introduction and aims

Astrocytes, the most abundant cell in the CNS, are the most proliferative cell within the CNS, and they play a critical role in the development and normal physiological function of the brain. For example, astrocytes has been shown to play a role in synaptogenesis (He and Sun, 2007) by producing trophic factors (Christopherson et al., 2005; Diniz et al., 2012; Kucukdereli et al., 2011). In addition, the function of astrocytes to support neurite growth has been extensively described (Ferguson and Scherer, 2012; Pizzurro et al., 2014). Moreover, increasing evidence has shown that astrocytes modulate basal synaptic transmission (Bonansco et al., 2011; Navarrete & Araque, 2011; Panatier et al., 2011) as well as synaptic plasticity (Bonansco et al., 2011; Ota et al., 2013; Paixão and Klein, 2010).

The function of the MAPK pathway on the proliferation of astrocytes is well-described (Cheng et al., 2013). For example, ERK activation has been shown to induce glial fate specification and enhances differentiation of glia (Li et al., 2013). With regard to MAPK modulation, MKP modulation of MAPKs is dependent on the cellular response or cell type involved as outlined earlier (see Chapter 1). Indeed, recently a study of MAPK activity in astrocytes revealed that the preferred MKP-3 substrate is JNK > ERK (Ndong et al., 2014) even though MKP-3 has been identified to regulate mainly ERK > JNK (Owens and Keyse, 2007). Furthermore, a previous study has shown that the upregulation of MKP-1 mRNA expression in embryonic sympathetic neurons as a consequence nerve growth factor (NGF) (Peinado-ramon

and Hallbook, 1998), indicates that MKPs are involved in neuronal development. Similarly, a study revealed that MKP-$1^{-/-}$ neurons were unable to produce axon dendritic branches in response to the growth factor, BDNF (Jeanneteau et al., 2011). Even though MKP-2 has been shown to play a role in regulating embryonic fibroblast cell proliferation (Lawan et al., 2011), its function in astrocyte proliferation and neurite growth remains unknown.

The specific aim of the experiments described in this chapter was to investigate the function of MKP-2 in astrocytes proliferation and neurite development. The experiment was conducted using immunocytochemistry techniques performed on primary hippocampal cultures (see section 2.4).

3.2 Results

3.2.1 MKP-2 deletion affects astrocytes number

As the effects of MKP-2 deletion has been shown previously to regulate mouse embryonic fibroblast cell proliferation (Lawan et al., 2011), I investigated the effect of MKP-2 deletion on astrocyte proliferation. Astrocyte number was reduced significantly at 2 DIV (MKP-$2^{+/+}$: 4 ± 0.7, n =27) compared to MKP-$2^{-/-}$ (2 ± 0.4, n =27, $P < 0.05$) and 3 DIV (MKP-$2^{+/+}$: 5 ± 0.7, n = 27 vs MKP-$2^{-/-}$: 3 ± 0.5). However, at 4 DIV, there was a significant increase of astrocytes number in MKP-$2^{-/-}$: 8 ± 1.2 vs MKP-$2^{+/+}$: 4 ± 0.7, n = 27, $P < 0.05$) (Figure 3.1B). Starting from 5 to 7 DIV, astrocytes proliferation in MKP-$2^{-/-}$ was not significantly different from MKP-$2^{+/+}$.

3.2.2 MKP-2 deletion reduces neurite length

Having established a reduction in astrocytes number, I also examined the effect of MKP-2 deletion on neurite length. Neurite length was significantly longer in MKP-$2^{+/+}$ culture at 1 DIV (MKP-$2^{+/+}$: 27.9 ± 1.3 µm, n = 66, vs MKP-$2^{-/-}$: 20.5 ± 0.9 µm, $P < 0.001$, n = 78), 2 DIV (MKP-$2^{+/+}$: 42.7 ± 1.74 µm, n =75, vs MKP-$2^{-/-}$: 34.9 ± 1.78 µm, n =82) and 4 DIV (MKP-$2^{+/+}$: 51.7 ± 2.7 µm, n = 60 vs MKP-$2^{-/-}$: 40.2 ± 1.76 µm, n = 56, $P < 0.05$) (Figure 3.2B). However, from 5 DIV, the neurite length was similar in both genotypes (MKP-$2^{+/+}$: 54 ± 2.2 µm, n = 74, vs MKP-$2^{-/-}$: 49.8 ± 2.7, n = 51, $P > 0.05$), 6 DIV (MKP-$2^{+/+}$: 54.5 ± 2.5 µm, n = 55, vs MKP-$2^{-/-}$: 54.3 ± 2.7µm, n = 50, $P > 0.05$) and day 7 (MKP-$2^{+/+}$: 64.1 ± 2.1µm, n = 82 vs MKP-$2^{-/-}$: 59.1 ± 0.14 µm, n = 66, $P > 0.05$) (Figure 3.2B).

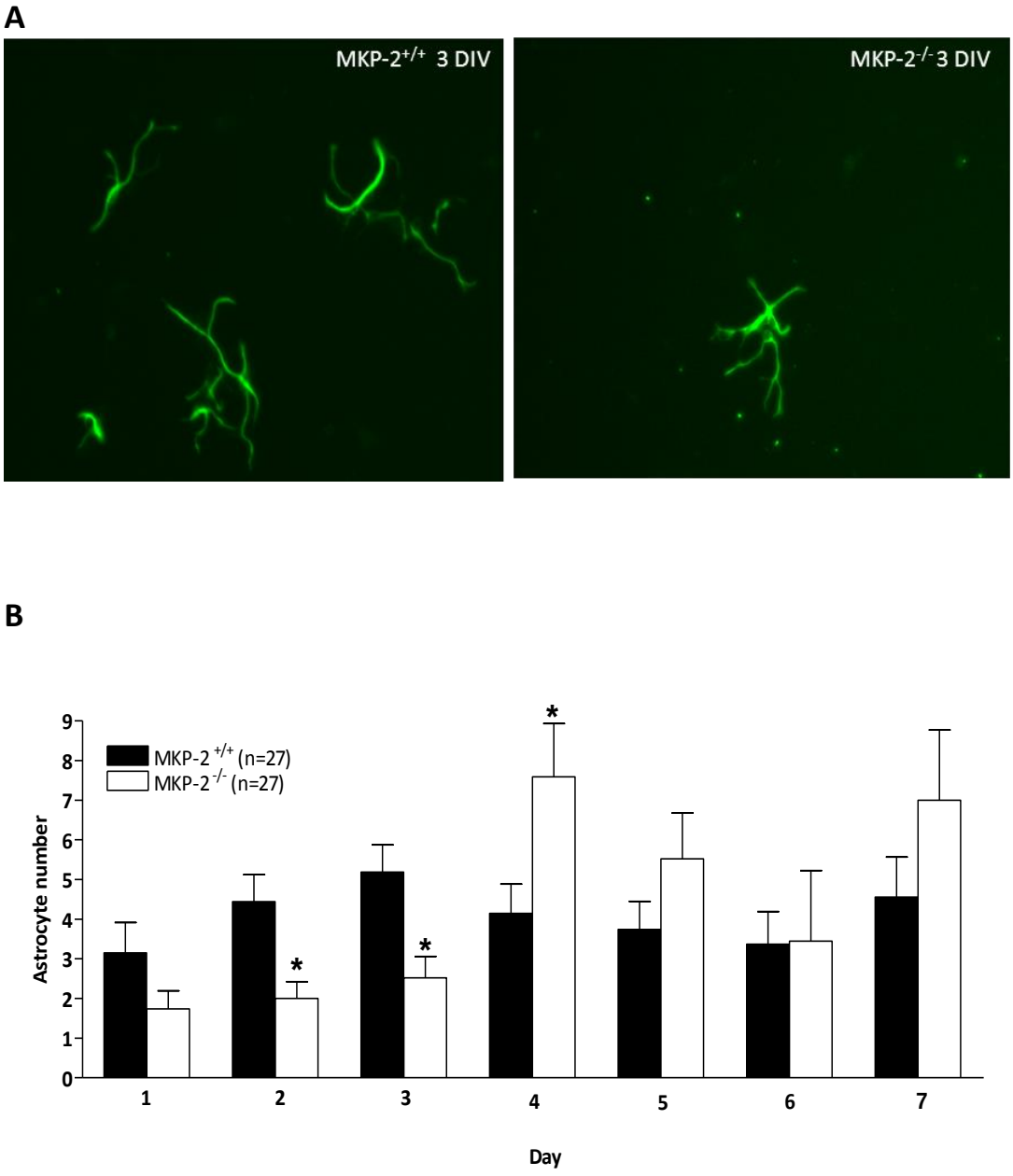


Figure 3.1: MKP-2 deletion affected astrocyte proliferation.

(A) Representative images show the differences of astrocytes number in MKP-$2^{+/+}$ compared to MKP-$2^{-/-}$ (B) Bar chart summarising significant difference in astrocytes number between MKP-$2^{-/-}$ and MKP-$2^{+/+}$ at 2 - 4 DIV. Data are mean ± S.E.M analysed with unpaired t-test between genotypes where appropriate with n being number of field per genotype, taken from 3 different cultures. (*$P < 0.05$).

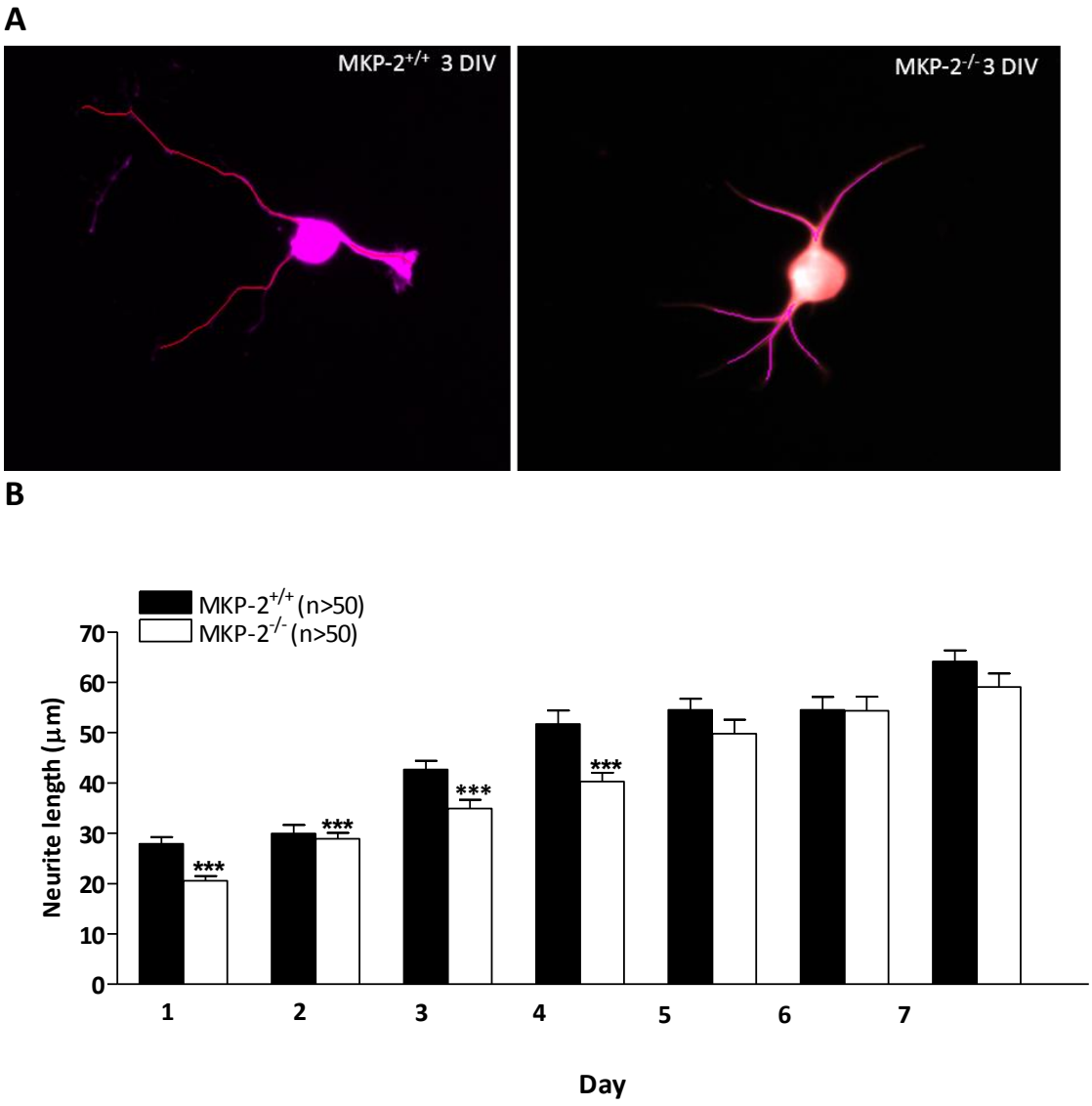


Figure 3.2: MKP-2 deletion reduced neurite length.

(A) Representative images show the differences of neurite length in MKP-$2^{+/+}$ compared to MKP-$2^{-/-}$ (B) Summary bar chart shows the significant reduction of neurite length at 1 - 4 DIV. Data are mean ± S.E.M analysed with unpaired t-test between genotypes where appropriate with n being number of neurons per genotype, taken from 3 different cultures. (***P < 0.001).

3.2.3 MKP-$2^{+/+}$ conditioned media rescues astrocyte number and neurite length in MKP-$2^{-/-}$ cultures

To investigate whether the reduced astrocyte proliferation is reversible, MKP-$2^{-/-}$ hippocampal cultures were grown in conditioned media from MKP-$2^{+/+}$ cultures (CM, 5 - 7 DIV) from 0 DIV. Astrocyte number was increased significantly in the first three days with at 1 DIV (MKP-$2^{+/+}$:1.7 ± 0.7, n = 27 vs MKP-$2^{-/-}$ + CM: 4 ± 0.7, n = 27, $P < 0.05$), 2 DIV (MKP-$2^{+/+}$: 2 ± 0.5, n = 27 vs MKP-$2^{-/-}$ + CM: 6.4 ± 1.5, n = 27, $P < 0.05$) and at 3 DIV (MKP-$2^{+/+}$: 2.5 ± 0.7, n = 27 vs MKP-$2^{-/-}$ + CM: 7.1 ± 1.4, n = 27, $P < 0.01$). In contrast, the number of astrocytes were similar at 4 DIV with astrocytes number in MKP-$2^{+/+}$ was 4.1 ± 1.0 and MKP-$2^{-/-}$ (7.5 ± 1.7) compared with MKP-$2^{-/-}$+ CM (5.5 ± 1.3, all $P > 0.05$, n = 27) (Figure 3.3A).

Having established that astrocyte proliferation was increased in CM, neurite length was also measured in MKP-$2^{-/-}$ cultures grown in CM to investigate the contribution of trophic factors released from astrocytes on neurite growth. As compared to MKP-$2^{-/-}$, neurite length in CM were increased significantly at 1 DIV (MKP-$2^{-/-}$: 20.5 ± 0.9 µm, n = 78 vs MKP-$2^{-/-}$ + CM: 27.1 ± 0.9, n = 80, $P < 0.01$). Similarly, the neurite length of MKP-$2^{-/-}$ + CM (44.3 ± 1.7 µm, n = 89) at 2 DIV is significantly increased between both MKP-$2^{+/+}$ (29.9 ± 1.7 µm, n = 75, $P < 0.01$) and MKP-$2^{-/-}$ (28.1 ± 1.1 µm, n = 82, $P < 0.01$). The differences in neurite length continued to be significant at 3 DIV between MKP-$2^{+/+}$ (42.6 ± 1.7 µm, n = 89) and MKP-$2^{-/-}$ (34.9 ± 1.7 µm, n =59) compared to MKP-$2^{-/-}$ + CM (61.6 ± 2.5 µm, n = 88, $P < 0.01$) as well as at 4 DIV (MKP-$2^{+/+}$: 51.7 ± 2.7 µm, n = 60) when compared to MKP-$2^{-/-}$ (40.3 ± 1.7 µm, n = 56) and MKP-$2^{-/-}$ + CM (69.1 ± 3.2 µm, n = 79, $P < 0.01$) (Figure 3.3B). This

result suggests that MKP-2 deletion regulates astrocytes proliferation and as a consequence, neurite growth is inhibited.

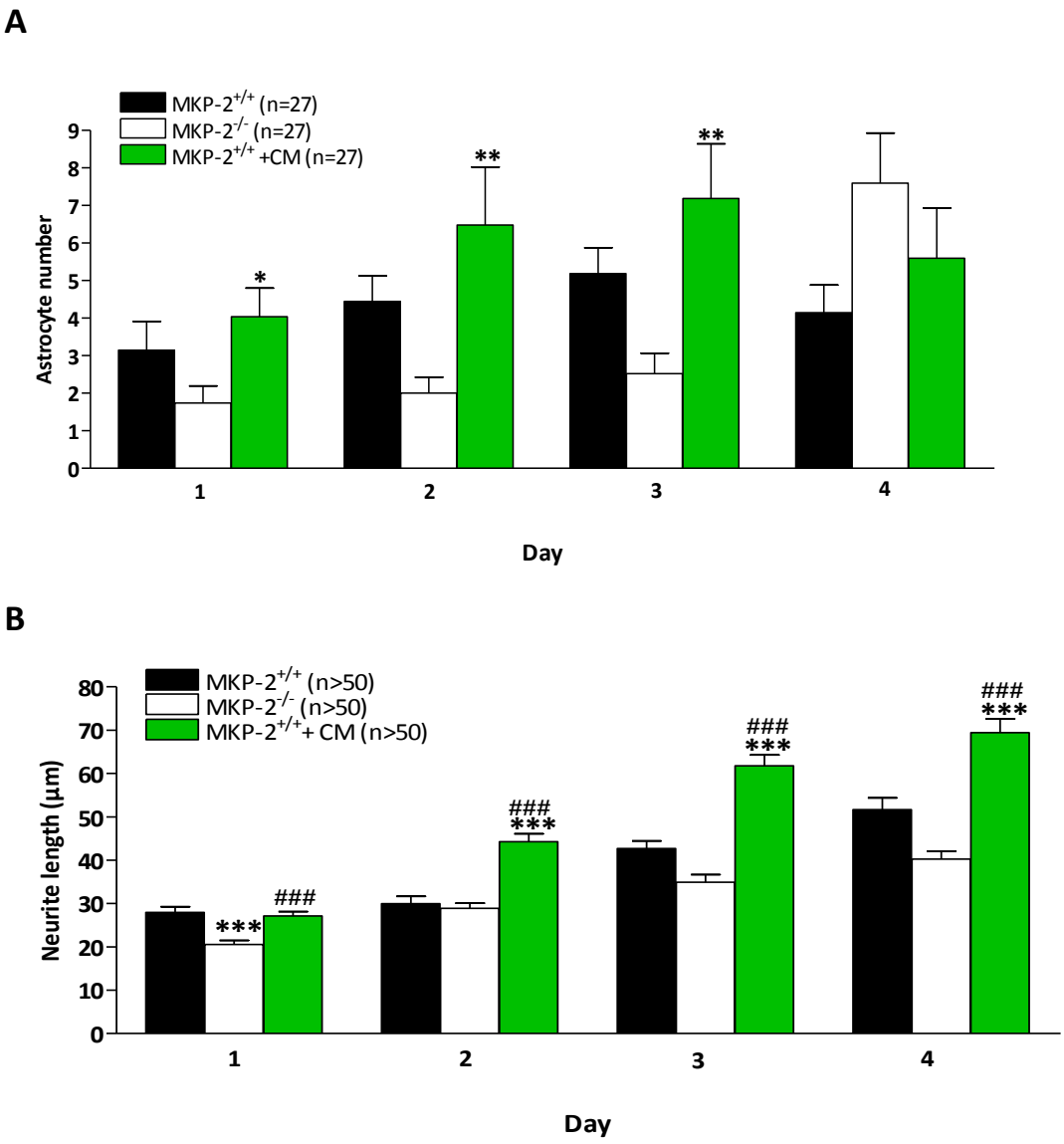


Figure 3.3: Conditioned media reverses reduced astrocytes proliferation and neurite physical properties.

A) Bar chart showing that astrocyte number was increased significantly at 1 to 3 DIV in the presence of CM when compared to MKP-$2^{-/-}$ alone. B) Bar chart summarising MKP-$2^{-/-}$ cultures grown in CM have a significant increase in neurite length. Data are mean ± S.E.M analysed with ANOVA with Tukey's post hoc test between genotypes where appropriate with n in A) being number of fields per genotype and in B) the number of neurons per genotype, taken from 3 different cultures. (*P < 0.05; **P < 0.01 compared to MKP-$2^{+/+}$, #P < 0.05; ##P < 0.01; ###P < 0.001 all compared with MKP-$2^{-/-}$).

3.2.4 Conditioned media increase astrocytes number and increase neurite length in MKP-$2^{+/+}$ cultures

Having established conditioned media are able to reverse reductions in astrocyte number and neurite length, conditioned media was also used withMKP-$2^{+/+}$ culture to act as a control. Generally, astrocytes number was increased significantly when MKP-$2^{+/+}$ + CM were compared either with either MKP-$2^{+/+}$ or MKP-$2^{-/-}$. Astrocyte number was increased significantly at 1 DIV (MKP-$2^{+/+}$: 3.1 ± 0.7, n = 27 and MKP-$2^{-/-}$: 1.7 ± 0.4, n = 27 vs MKP-$2^{+/+}$ + CM: 7.7 ± 0.8, n = 27, P < 0.01), 2 DIV (MKP-$2^{+/+}$: 4.4 ± 0.6, n = 27 and MKP-$2^{-/-}$: 2 ± 0.4, n = 27 vs MKP-$2^{+/+}$ + CM: 13.2 ± 2.2, n = 27, P < 0.01), 3 DIV (MKP-$2^{+/+}$: 5.1 ± 0.6, n = 27 and MKP-$2^{-/-}$: 2.5 ± 0.5, n = 27 vs MKP-$2^{+/+}$ + CM: 15.5 ± 1.4, n = 27, P < 0.01), and 4 DIV (MKP-$2^{+/+}$: 4.1 ± 0.7, n = 27 and MKP-$2^{-/-}$: 7.5 ± 1.3 vs MKP-$2^{+/+}$ + CM: 16.8 ± 2.4, n = 27, P < 0.01) (Figure 3.4A). Moreover, astrocytes number was also increased significantly when compared with MKP-$2^{+/+}$.

Similar to the increase in astrocytes number, neurite length was also increased significantly in MKP-$2^{+/+}$ + CM. There is a significant increase when compared to MKP-$2^{+/+}$ as at 2 DIV (MKP-$2^{+/+}$: 29.33 ± 1.6 μm, n = 359 vs MKP-$2^{+/+}$ + CM: 45.99 ± 2.4, n = 290, P < 0.001), 3 DIV (MKP-$2^{+/+}$: 42.68 ± 1.7 μm, n = 528 vs MKP-$2^{+/+}$ + CM: 54.10 ± 2.8 μm, n = 276, P < 0.001), and 4 DIV (MKP-$2^{+/+}$: 51.69 ± 2.7 μm, n = 341 vs MKP-$2^{+/+}$+ CM: 70.33 ± 3.3 μm, n = 275, P < 0.001) (Figure 3.4B). As compared to MKP-$2^{-/-}$, neurite length increase significantly at 1 DIV (MKP-$2^{-/-}$: 20.56 ± 0.9 μm, n = 345 vs MKP-$2^{+/+}$ + CM: 32.14 ± 2.131 μm, n = 225, P < 0.001), at 2 DIV (MKP-$2^{-/-}$: 28.91 ± 1.1 μm, n = 367 vs MKP-$2^{+/+}$ + CM: 45.99

± 2.4, n = 290, P < 0.001), at 3 DIV (MKP-2$^{-/-}$: 34.90 ± 1.7 μm, n = 345 vs MKP-2$^{+/+}$ + CM: 54.10 ± 2.8 μm, n = 276, P < 0.001), and at 4 DIV (MKP-2$^{-/-}$: 40.27.90 ± 1.7 μm, n = 447 vs MKP-2$^{+/+}$ + CM: 70.33 ± 3.3 μm, n = 275, P < 0.001) (Figure 3.4B).

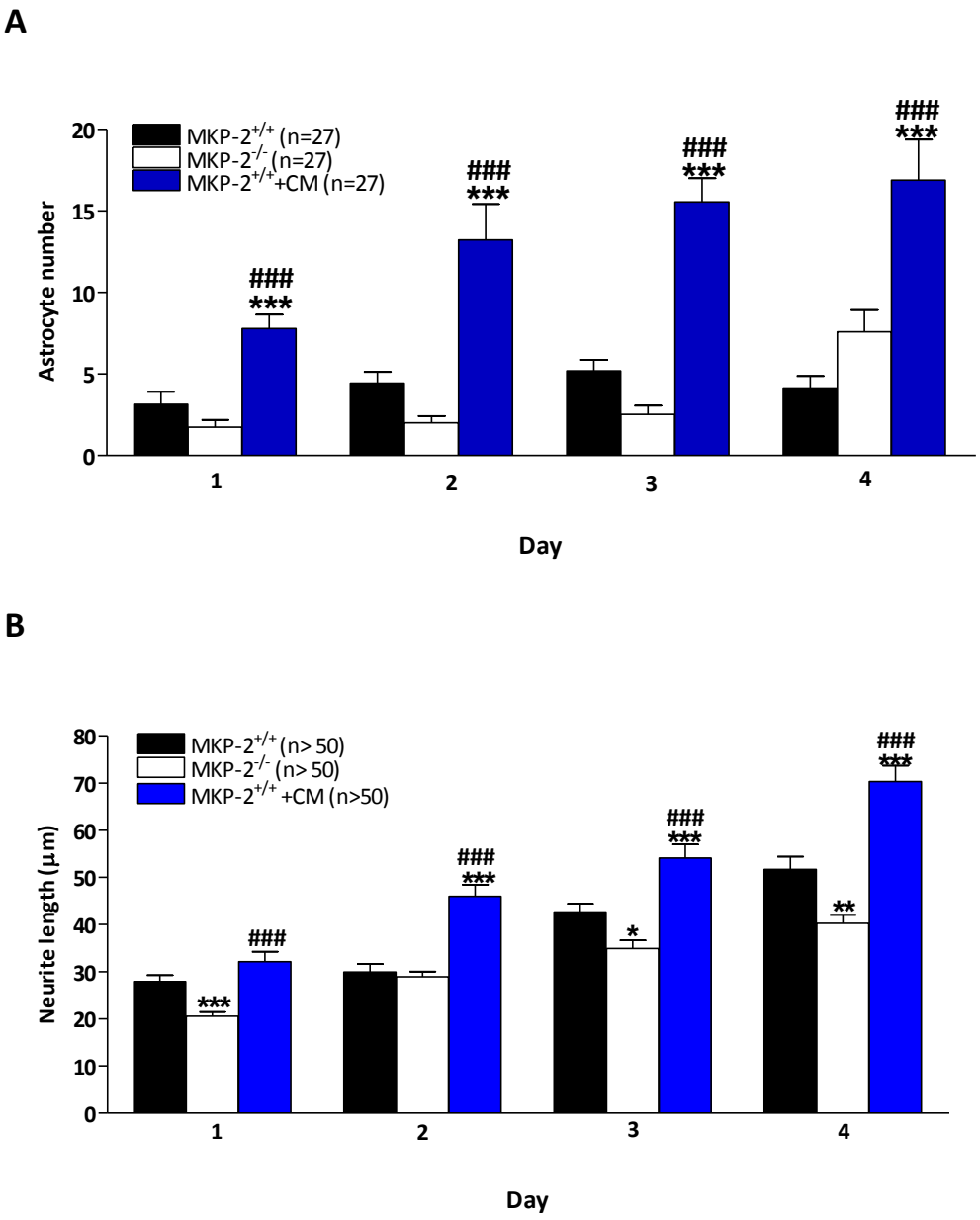


Figure 3.4: Conditioned media increases astrocytes number and neurite length in MKP-2$^{+/+}$ hippocampal cultures.

A) Bar chart summarising MKP-2$^{+/+}$ cultures grown in CM shows a significant increase in astrocytes number at 1-4 DIV. B) Bar chart shows neurite length was increased significantly at 1-4 DIV in MKP-2$^{+/+}$ cultures grown in MKP-2$^{+/+}$. Data are mean ± S.E.M analysed with ANOVA with Tukey's post hoc test between genotypes where appropriate with n in A) being number of fields per genotype and in B) the number of neurons per genotype, taken from 3 different cultures. (*P < 0.05; **P < 0.01; ***P < 0.001 compared to MKP-2$^{+/+}$; ###P < 0.001 compared with MKP-2$^{-/-}$).

3.2.5 AraC reduces astrocytes number but does not alter neurite length in MKP-$2^{+/+}$ cultures

As mentioned in 3.2.1, astrocytes number was reduced in MKP-$2^{-/-}$ primary hippocampal cultures. To further investigate whether neurite length reduction is due to reduction of astrocytes number alone or whether an impairment in astrocytes function contributes to this, cytosine arabinofuranoside (AraC, 10 μM), an inhibitor of glial proliferation, was applied to the MKP-$2^{+/+}$ cultures from 0 DIV. There is no significant difference in the number of astrocytes at 1 DIV between MKP-$2^{+/+}$ + AraC (3.2 ± 0.6, n = 27) vs MKP-$2^{+/+}$ (3.1 ± 0.7, n = 27) or MKP-$2^{-/-}$ (1.7 ± 0.4, n = 27 $P > 0.05$). At 2 DIV, there is no significant reduction in astrocytes number when comparing between MKP-$2^{+/+}$ + AraC (3.5 ± 0.6, n = 27) vs MKP-$2^{+/+}$ (4.4 ± 0.6, n = 27) or MKP-$2^{-/-}$ (2 ± 0.4, n = 27 $P > 0.05$), but significantly between MKP-$2^{+/+}$ and MKP-$2^{-/-}$. There is significant reduction of astrocytes number at 3 DIV (MKP-$2^{+/+}$ + AraC: 3.0 ± 0.6 vs MKP-$2^{+/+}$: 5.1 ± 0.6, $P < 0.05$), even though there is no significant difference with MKP-$2^{-/-}$ (2.5 ± 0.5, $P > 0.05$). At 4 DIV, significant difference on MKP-$2^{+/+}$ + AraC (3.1 ± 0.4) vs MKP-$2^{-/-}$ (7.5 ± 1.3, $P > 0.05$) but no difference on MKP-$2^{+/+}$ (4.1 ± 0.7, $P > 0.05$) (Figure 3.5A).

Even though there is reduction in astrocyte number at 4 DIV, neurite length was not affected as shown by significant increase in length despite a reduction in astrocyte number. As compared to MKP-$2^{-/-}$, there is significant increase at 1 DIV (MKP-$2^{-/-}$: 20.56 ± 0.9 μm, n = 345, $P < 0.001$ vs MKP-$2^{+/+}$ + AraC: 26.67 ± 0.9 μm, n = 276, $P < 0.001$), 2 DIV (MKP-$2^{-/-}$: 29.93 ± 1.6 μm, n = 359, $P < 0.001$ vs MKP-$2^{+/+}$ + AraC: 41.18 ± 2.1 μm, n = 261, $P < 0.001$), and 4 DIV (MKP-$2^{-/-}$: 51.69 ± 2.7 μm, n = 341, vs MKP-$2^{+/+}$ + AraC: 63.04 ± 4.4 μm, n = 182, $P < 0.01$). In contrast, compared with

MKP-2$^{+/+}$, a significant increase was seen at 2 DIV (MKP-2$^{+/+}$: 29.93 ± 1.6 µm, n = 359, vs MKP-2$^{+/+}$ + AraC: 41.18 ± 2.1 µm, n = 261, $P < 0.01$) and 4 DIV (MKP-2$^{+/+}$: 51.69 ± 2.7 µm, n = 341, vs MKP-2$^{+/+}$ + AraC: 63.04 ± 4.4 µm, n = 182, $P < 0.05$). No difference at 1 DIV (MKP-2$^{+/+}$: 27.93 ± 1.3 µm, n = 293, vs MKP-2$^{+/+}$ + AraC: 26.67 ± 0.9 µm, n = 276, $P > 0.05$ and at 3 DIV (MKP-2$^{+/+}$: 42.68 ± 1.7 µm, n = 528, vs MKP-2$^{+/+}$ + AraC: 39.06 ± 1.9 µm, n = 272, $P > 0.05$) (Figure 3.5B)

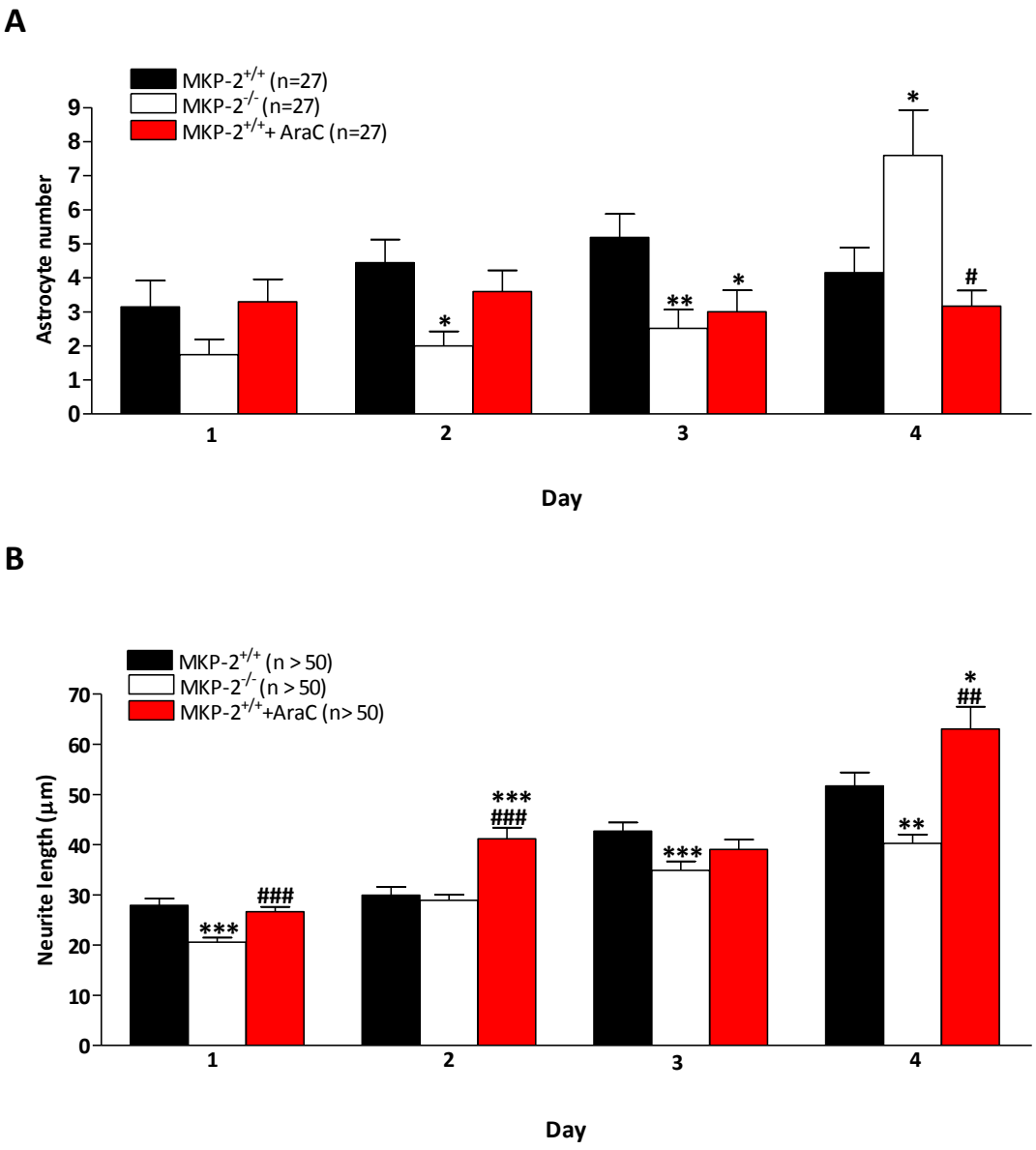


Figure 3.5: Astrocytes number is reduced but neurite length is increased in MKP-2$^{+/+}$ cultures supplemented with AraC. A) Bar chart showing astrocyte number in MKP-2$^{+/+}$ hippocampal cultures supplemented with AraC was decreased significantly at 3- 4 DIV B) Bar chart summarising neurite length was increased significantly at 1- 4 DIV. Data are mean ± S.E.M analysed with ANOVA with Tukey's post hoc test between genotypes where appropriate. n is A) being number of fields per genotype and B) the number of neurons per genotype, taken from 3 different cultures. (*P < 0.05; **P < 0.01; ***P < 0.001 compared to MKP-2$^{+/+}$, #P < 0.05; ##P < 0.01; ###P < 0.001 compared with MKP-2$^{-/-}$).

3.3 Discussion

In this chapter, I show for the first time that MKP-2 plays a role in regulating astrocytes proliferation and subsequently alter neurite length in primary hippocampal cultures. I also show that the condition is on account of impairment in astrocytes function and not solely due to the reduction in astrocytes number.

As the only proliferating cells in the CNS, astrocytes proliferation was investigated in this study. Within 7 DIV tested, astrocytes number was reduced in the first 4 DIV in MKP-$2^{-/-}$ culture compared to MKP-$2^{+/+}$. This data support our hypothesis that MKP-2 has a regulatory function in astrocyte proliferation. This result is consistent with a previous study that showed that MKP-2 deletion in mouse embryonic fibroblasts reduces cell proliferation compared to MKP-$2^{+/+}$ (Lawan et al., 2011). It is hypothesized that it is caused by a defect in proliferation at the G_2/M transition phase (Lawan et al., 2011). This result suggests that MKP-2 deletion reduces astrocyte proliferation by a defect in cell division or the cell cycle and implies that MKP-2 plays an important role in astrocytes proliferation. A study on astrocytic cultures has shown Endothelin-1 (ET-1) strongly promoted astrocytes proliferation and increase astrocytes number via activation of the JNK and p38 MAPK pathways (Gadea et al., 2008). Thus, it is reasonable to speculate that MKP-2 deletion modulates JNK and p38 MAPK activation that led to alteration of ET-1 and subsequently alter astrocytes proliferation. To confirm this, it would be interesting to investigate the selective activation of JNK and p38 MAPK as well as ET-1 expression in MKP-$2^{-/-}$ cultures. This could be done by using western blot analysis or manipulating immunocytochemistry technique. Alternatively, astrocytes proliferation could be further investigated by using flow cytometry to analyse the astrocytes cell cycle

progression through different phase of the cell. However, due to the time limit of my PhD, I did not investigate further the cellular mechanism underlying this; instead this thesis focusses on the functional consequence of MKP-2 deletion on neuronal and astrocytic function. This finding is in contrast with previous finding as a study has shown that ERK activation strongly regulates glial fate specification and enhances the differentiation of glia with MEK deletion leading to a failure in gliosis while hyperactivation of MEK increase production of astrocytes precursors (Li et al., 2013). Moreover, mitogens including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) stimulate DNA synthesis and proliferation in primary astrocytes (Kaufmann & Thiel, 2001) where EGF stimulation activates ERK and will then promote EGR biosynthesis and subsequently induce cell growth (Kaufmann and Thiel, 2001). In this study, it is suggested that the reduction of astrocytes proliferation may occur due to a reduction in EGF release and it is strongly related to ERK activation. This outcome lead us to investigate to determine the level of ERK activity in MKP-$2^{-/-}$ and MKP-$2^{+/+}$ cultures and it will be discussed further in the next chapter. Interestingly, at 4 DIV, astrocytes number was increased significantly in MKP-$2^{-/-}$, suggesting that after 3 DIV, astrocytes have produced enough trophic factor and enriched the media to support astrocyte proliferation. It is also reasonable to speculate that at different time points, different trophic factor are produced and would be able to promote astrocytic proliferation. This is in agreement with a previous study that has shown astrocyte-conditioned media (ACM) from 15 DIV promoted astrocyte proliferation at 15 and 30 DIV but the proliferation was blocked when treated with ACM derived from 30-60 DIV (Bramanti et al., 2007).

Subsequent to the reduction of astrocyte number, this study also shows that neurite length is reduced in MKP-$2^{-/-}$ mice compared to MKP-$2^{+/+}$. The reduction are significant for the first 3 DIV, however there is no differences starting from 5 DIV to 7 DIV. The reduction of astrocyte number in the first 3 DIV may explain this condition as suggested there is not enough trophic factor produced to support the neurite growth. It also seems reasonable to suggest that after 3 DIV, the media has enriched with enough substrate to allow neurite length to grow at 4 -7 DIV. This result is in agreement with a previous study that demonstrated neurons grown with astrocytes remain healthy for three weeks compared to neurons that grown without astrocytes (Jones et al., 2012). The concept that astrocytes contribute to the development of neurons by secreting trophic factors that induced neurite growth is well-established (Banker, 1980; Jean et al., 2009). Astrocytes has been shown to secrete numerous growth promoting molecules including fibronectin, N-cadherin, laminin-1, epidermal growth factor (EGF), basic fibroblast growth factor (bDNF) (Price, 1985; Mijller and Junghans, 1995; Bramanti et al., 2007) that contribute and support neurite development. Although the relationship of these trophic factors to the MKP and MAPK cascade is not clear, a lot of evidence suggests that activation of the MAPK cascade is involved in the production of these growth factors. For example, laminine and N-cadherine has been shown to induce neurite outgrowth by activation of the ERK pathway (Mruthyunjaya et al., 2010; Perron and Bixby, 1999) and nerve growth factor (NGF) was also identified to be among the strongest growth factors to stimulate ERK (Egea et al., 2000). To investigate further the role of astrocytes trophic factor in this study, MKP-$2^{-/-}$ hippocampal culture has been grown with conditioned media from MKP-$2^{+/+}$ media. As the reduction of astrocytes

number and neurite length were significant at the first 3 DIV, the reversal experiments were done for 4 DIV only. There is significant increase of astrocytes number in MKP-$2^{-/-}$ hippocampal culture grown in CM at 1 to 3 DIV compared to MKP-$2^{-/-}$ grown in fresh neurobasal media. The result suggests that the reduction of neurite length is due to impairment in astrocytes function and not because of any defect in the ability of the neurite to grow. This finding is consistent with a previous study that shown impairment of astrocytes by an oxidizing agent reduced fibronectin levels and subsequently reduced neurite length (Pizzurro et al., 2014). Similarly, astrocytic dysfunction caused by ethanol (Giordano, et al., 2011) and manganese (Giordano et al., 2009) led to a reduction of production of fibronectin and laminin-1 and eventually inhibit neurite outgrowth, even though astrocytic viability is not affected. In this study, MKP-$2^{+/+}$ hippocampal culture grown with MKP-$2^{+/+}$ conditioned media exhibited significant increase in astrocytes number and neurite length at 1 – 4 DIV as compared to MKP-$2^{+/+}$ and MKP-$2^{-/-}$. This result suggests that in conditioned media, there is a significant amount of growth factors that promote and stimulate the astrocyte proliferation and neurite growth. This finding is consistent with a recent study that shown significant increase of neurite outgrowth that incubated with astrocytes conditioned media (Wolf et al., 2014). This finding led us to investigate whether the reduction of neurite and astrocytes growth is due to the reduction in astrocytes number alone or impairment in trophic factor release by astrocytes. This has been done by supplementing the media with AraC, an astrocytic proliferation inhibitor, in MKP-$2^{+/+}$ primary hippocampal cultures to mimic the MKP-$2^{-/-}$ condition. There is significant reduction in astrocytes number, as expected. There is however, no differences in neurite length albeit the reduction in astrocyte

number. In fact, at the first 3 DIV, neurite length was increase significantly compared to MKP-2$^{-/-}$. This finding suggests that the reduction of neurite length in MKP-2$^{-/-}$ is probably due to the reduction in astrocytes number but further study needed to confirm whether this is solely because reduction in astrocyte number or on account of impairment of astrocyte function.

In summary, the data in this chapter shows that MKP-2 deletion reduced astrocytes proliferation and neurite length. To the best of my knowledge this is the first time that MKP-2 has been shown to implicate in regulation of astrocytes proliferation and neurite development.

4 DETERMINING THE FUNCTIONAL CONSEQUENCE OF MKP-2 DELETION IN THE CNS USING PRIMARY HIPPOCAMPAL CULTURES.

4.1 Introduction

The role of the MAPK pathway in CNS function has been well-characterized as it has been shown to regulate synaptic transmission (Liu et al., 2011; Sweatt, 2001), provide neuroprotection (Karmarkar et al., 2011) as well as involvement in development of functional synapses (Giachello et al., 2010a). On the other hand, the function of MKP-2 in the CNS is very limited despite the fact that it is widely expressed in the CNS (Misra-Press et al., 1995). Additionally, the involvement of MKP-2 in pathological conditions affecting the brain is also under studied. In fact the only previous study looking at this has shown that MKP-2 expression was increased in the cerebellum of depressed suicide victims (Dwivedi et al., 2001).

Primary hippocampal cultures are a standard and well-established method that is widely used for investigating the modulation of neuronal function because of the ease of recording and visualisation. Despite the obvious shortcomings in this technique in terms of network connectivity, the system has many technical benefits for example the ease in identifying the cell to patch from single neurons as well as being accessible to pharmacological manipulations. Taking these factors into consideration, I decide to utilise this technique in my experiments to provide an initial investigation of consequence of MKP-2 deletion in CNS function.

The specific aim of the experiments described in this chapter was to assess the functional consequence of MKP-2 deletion on neuronal excitability and synaptic transmission as well as determining whether ERK activity was altered. Having

established that astrocyte proliferation and neurite growth was reduced at 3 DIV, all the functional studies in the chapter were conducted and quantified at three time points: 3, 7 and 11 DIV. 11 DIV was chosen in addition to 3 and 7 DIV as the network circuit is well characterised in that full synaptic maturity (Wagenaar et al., 2006) is observed at this time point. All experiments were performed using whole cell patch clamp recording techniques as well as immunocytochemistry as explained in section 2.6 and 2.8.

4.2 Results

4.2.1 Neuronal intracellular calcium concentration is preserved in MKP-$2^{-/-}$ primary hippocampal cultures at 3 and 7 DIV, but increased at 11 DIV.

One of functional properties investigated in this study is whether MKP-2 deletion alters intracellular Ca^{2+} ($[Ca^{2+}]i$) release. Neuronal calcium was activated by using either an agonist of group I mGluRs, (S)-3, 5-Dihydroxyphenylglycine (DHPG, 20 µM) or by the application of high extracellular K^+ (25 mM). Activation by DHPG activates mGluR1 & 5 and induces elevation of $[Ca^{2+}]i$ through inositol receptor (IP3) or ryanodine receptor meanwhile application of high K^+ would depolarize the membrane potential that leads to the activation of voltage-gated calcium channels and subsequently elevate $[Ca^{2+}]i$ due to calcium flowing into the cell. Application of DHPG elevated activate neuronal $[Ca^{2+}]i$, however, no difference in neuronal $[Ca^{2+}]i$ at 3 DIV between $MKP^{+/+}$ (2.8 ± 0.3, n = 21) vs MKP-$2^{-/-}$ (2.5 ± 0.4, n= 12, P > 0.05) (Figure 4.1 A), at 7 DIV MKP-$2^{+/+}$ (3.1 ± 0.2946, n=39) vs MKP-$2^{-/-}$ (3.018 ± 0.2092, n=96, P > 0.05) (Figure 4.1 B) as well as at 11 DIV MKP-$2^{+/+}$ (3.1 ± 0.2, n=39) and MKP-$2^{-/-}$ (3.0 ± 0.2, n=96, P > 0.05) (Figure 4.1 C) was observed.

Application of high extracellular K^+ resulted in a significant increase in $[Ca^{2+}]i$ in the neurons at 3 DIV but no significant difference between genotype, as in MKP-$2^{+/+}$ (4.2 ± 0.18, n = 167) vs MKP-$2^{-/-}$ (4.56 ± 0.27, n = 80, P > 0.05) (Figure 4.1 A) and at 7 DIV with MKP-$2^{+/+}$ (6.0 ± 0.36, n=105) and MKP-$2^{-/-}$ (6.4 ± 0.29, n=157, P > 0.05) (Figure 4.1 B) cultures. However, at 11 DIV, application of high extracellular K^+ on primary hippocampal cultures produced robust elevations in MKP-$2^{-/-}$ $[Ca^{2+}]i$

(8.9 ± 0.4, n=265, P < 0.001) which was increased significantly from MKP-$2^{+/+}$ (5.8 ±0.3, n = 108) (Figure 4.1 C).

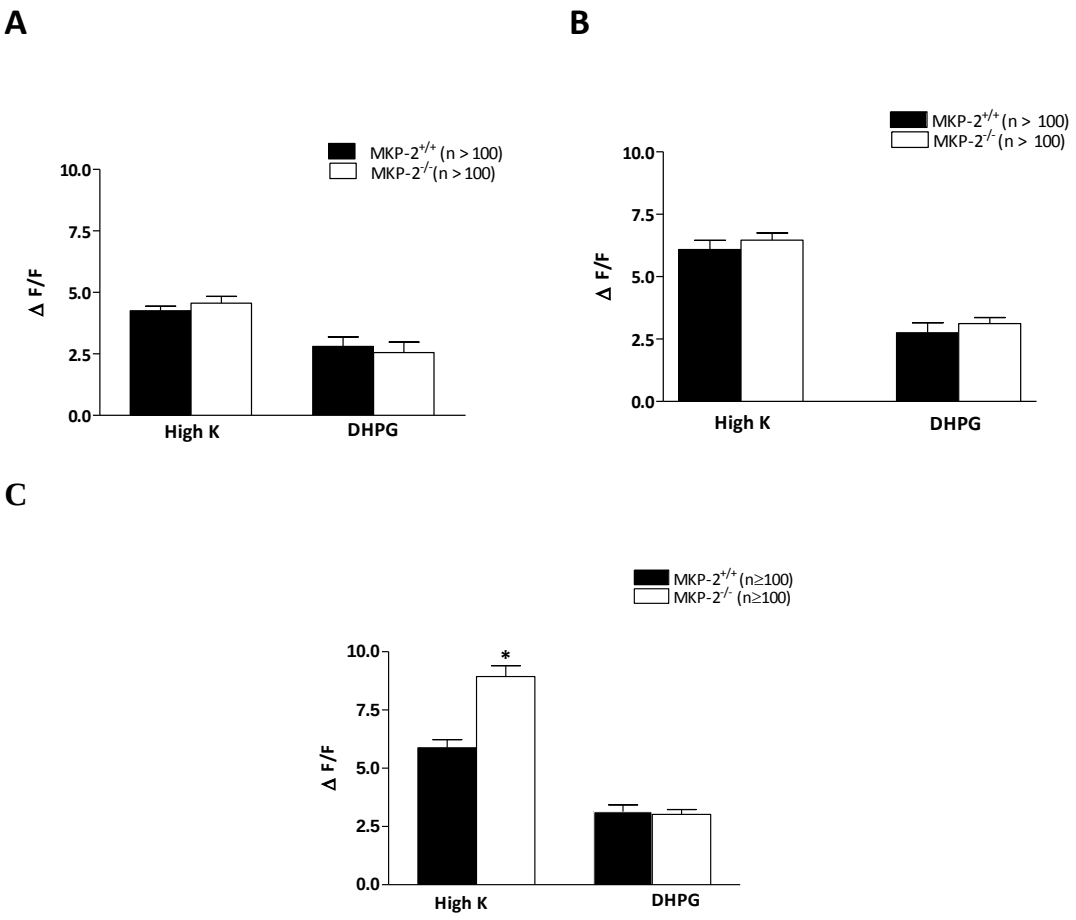


Figure 4.1: Neuronal [Ca^{2+}]i is increased in MKP-$2^{-/-}$ primary hippocampal cultures by high extracellular K^+ at 11 DIV, but is unaltered at 3 and 7 DIV.
Summary data showing that no difference in [Ca^{2+}]i changes at (A) 3 DIV and (B) 7 DIV in MKP-2 primary hippocampal cultures. (C) However, [Ca^{2+}]i is significantly increased in MKP-$2^{-/-}$ primary hippocampal cultures on 11 DIV activated by high potassium application. No differences in [Ca^{2+}]i activated by DHPG was observed. Data are mean ± S.E.M analysed with unpaired t-test between genotypes where appropriate (* = $P < 0.05$, n ≥ 100 ROI from 3 separate cultures).

4.2.2 Astrocytic [Ca^{2+}]i is preserved in MKP-$2^{-/-}$ primary hippocampal culture at 3, 7 and 11 DIV.

To investigate if there is any difference in astrocytic [Ca^{2+}]i, two different compounds were used: trypsin and adenosine triphosphate (ADP). Trypsin has been used widely to activate PAR-2 on astrocytes (Bushell et al., 2006; Fereidoni et al., 2013; Park et al., 2009), as PAR-2 receptors are found to be expressed on astrocytes (Bushell et al., 2006; Park et al., 2009). Meanwhile, ADP (1 μM) (Gallagher and Salter 2003) was used to evoke astrocytes through P2Y metabotropic receptors (Nicholas, 2001) as this receptor found to be expressed on astrocytes (Hashioka et al., 2014; Verkhratsky et al., 2009). Cells were identified as astrocyte during off-line analysis only if the cell did not respond to high extracellular potassium (as presented in 4.2.2). Application of trypsin resulted in an significant increase in astrocytic [Ca^{2+}]i at 7 DIV and there was significant decrease in MKP-$2^{-/-}$ (2.2 ± 0.2, n=27, $P < 0.05$) as compared to MKP-$2^{+/+}$ (3.1 ± 0.2, n=21) (Figure 4.2B). However, at 3 and 11 DIV, no significant different was observed between MKP-$2^{+/+}$ (3.7 ± 0.3, n=19) vs MKP-$2^{-/-}$ (2.8 ± 0.3, n=21, $P > 0.05$) (Figure 4.2A) and MKP-$2^{+/+}$ (3.01 ± 0.3, n=21) vs MKP-$2^{-/-}$ (2.4 ± 0.2, n=52, $P > 0.05$) (Figure 4.5C) respectively.

In contrast, following a 2 min application of ADP, a non-significant increase in astrocytic [Ca^{2+}]i was observed when comparing MKP-$2^{+/+}$ (3.6 ± 0.3, n = 14) vs MKP-$2^{-/-}$ (3.0 ± 0.38, n = 14) (Figure 4.2A) at 3 DIV. Similarly at 7 DIV, application of ADP resulted a non-significant increase in MKP-$2^{-/-}$ astrocytic [Ca^{2+}]i (3.5 ± 0.38, n=32, $P > 0.05$) when compared to MKP-$2^{+/+}$ (3.1 ± 0.49, n=12) (Figure 4.2B).

Similarly, at 11 DIV, application of ADP resulted in non-significant increase in MKP-$2^{-/-}$ (2.4 ± 0.2 n=52, P > 0.05) vs MKP-$2^{+/+}$ (3.0 ± 0.3, n=21) (Figure 4.2C).

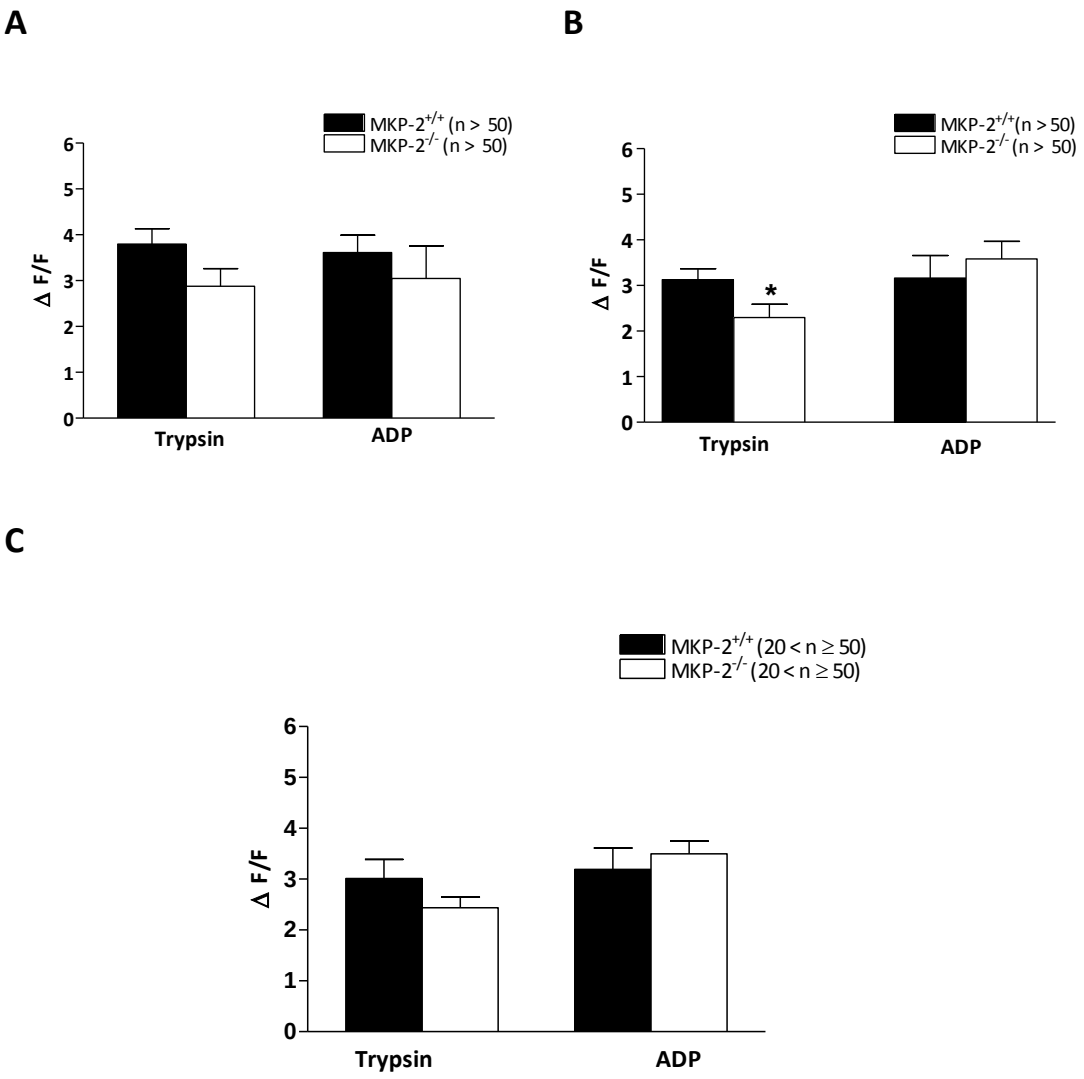


Figure 4.2: Significant change in astrocytic [Ca^{2+}]i activated by trypsin at 7 DIV MKP-$2^{-/-}$ primary hippocampal cultures.

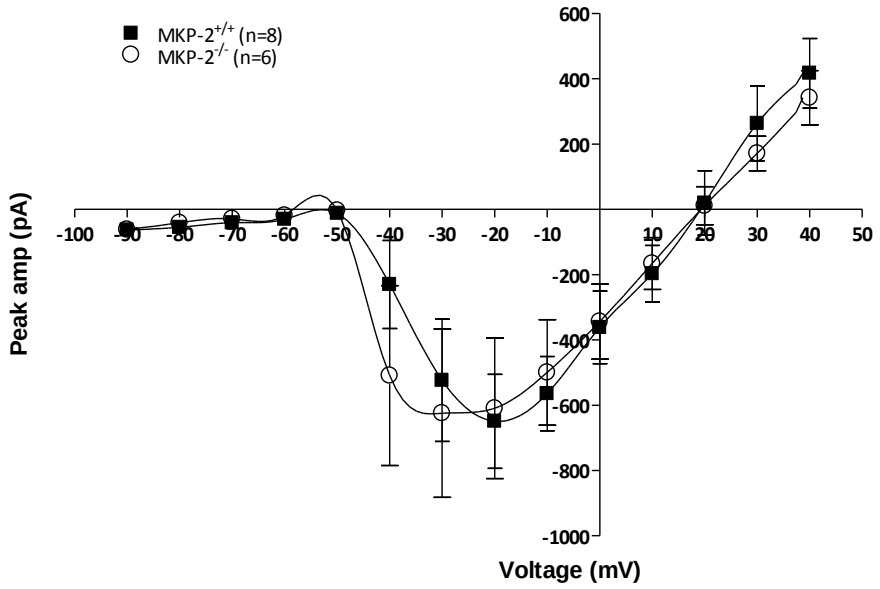
Bar chart summary revealing significant reduction at MKP-$2^{-/-}$ astrocytic [Ca^{2+}]i activated by trypsin at (B) 7 DIV but no difference in between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ cultures at (A) 3 and (C) 11 DIV. Data are mean ± S.E.M analysed with unpaired t-test between genotypes where appropriate (*P < 0.05, n ≥ 50 ROI from 3 separate cultures).

4.2.3 Na^+ and K^+ current is significantly reduced at 7 DIV, but no changes at 3 and 11 DIV in MKP-$2^{-/-}$ primary hippocampal culture.

Having established MKP-2 deletion leads to increased [Ca^{2+}]i following high K^+ application, I next examined the consequence of MKP-2 deletion on neuronal Na^+ and K^+ channel development using whole cell voltage clamp. At 3 DIV, there was no significant difference in Na^+ and K^+ current (Figure 4.3AB). However at 7 DIV, there was significant reduction in the maximum Na^+ current in MKP-$2^{-/-}$ (-1327 ± 157.27 pA, n=11) when compared to MKP-$2^{+/+}$ (-2034 ± 194.6 pA, n = 11, $P < 0.05$) (Figure 4.4A). Similarly, a significant reduction in K^+ current was also observed at maximum peak in MKP-$2^{-/-}$ (1404 ± 117.39 pA, n = 11) as compared to MKP-$2^{+/+}$ (1807 ± 174.8 pA, n = 11) (Figure 4.4B).

Na^+ and K^+ current were also investigated at 11 DIV. Even though there is significant reduction in Na^+ and K^+ current at 7 DIV in MKP-$2^{-/-}$, at 11 DIV, there was no significant difference at 11 DIV with Na^+ current at the peak in MKP-$2^{+/+}$ being -2148.05 ± 465.83 pA (n = 12) vs MKP-$2^{-/-}$ (-2342.13 ± 401.63 pA, n = 12)(Figure 4.5A). However, there was significant increase in MKP-$2^{-/-}$ as in K^+ currents MKP-$2^{+/+}$ being 2019.8 ± 276.14 pA (n = 12) as compared to MKP-$2^{-/-}$ (2704.8 pA ± 283.25 pA, n = 12, $P < 0.05$) (Figure 4.5B).

A



B

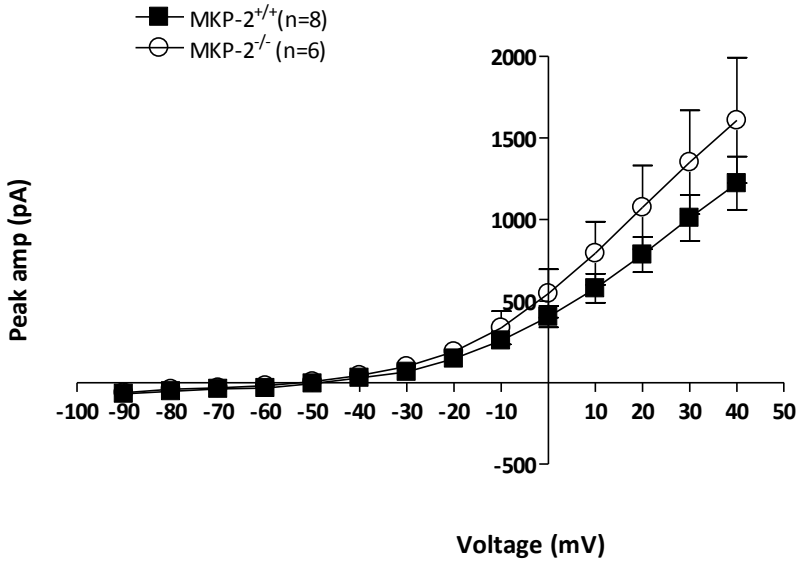


Figure 4.3: No significant difference in the (A) Na^+ and (B) K^+ current between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ neurons in primary hippocampal culture at 3 DIV.

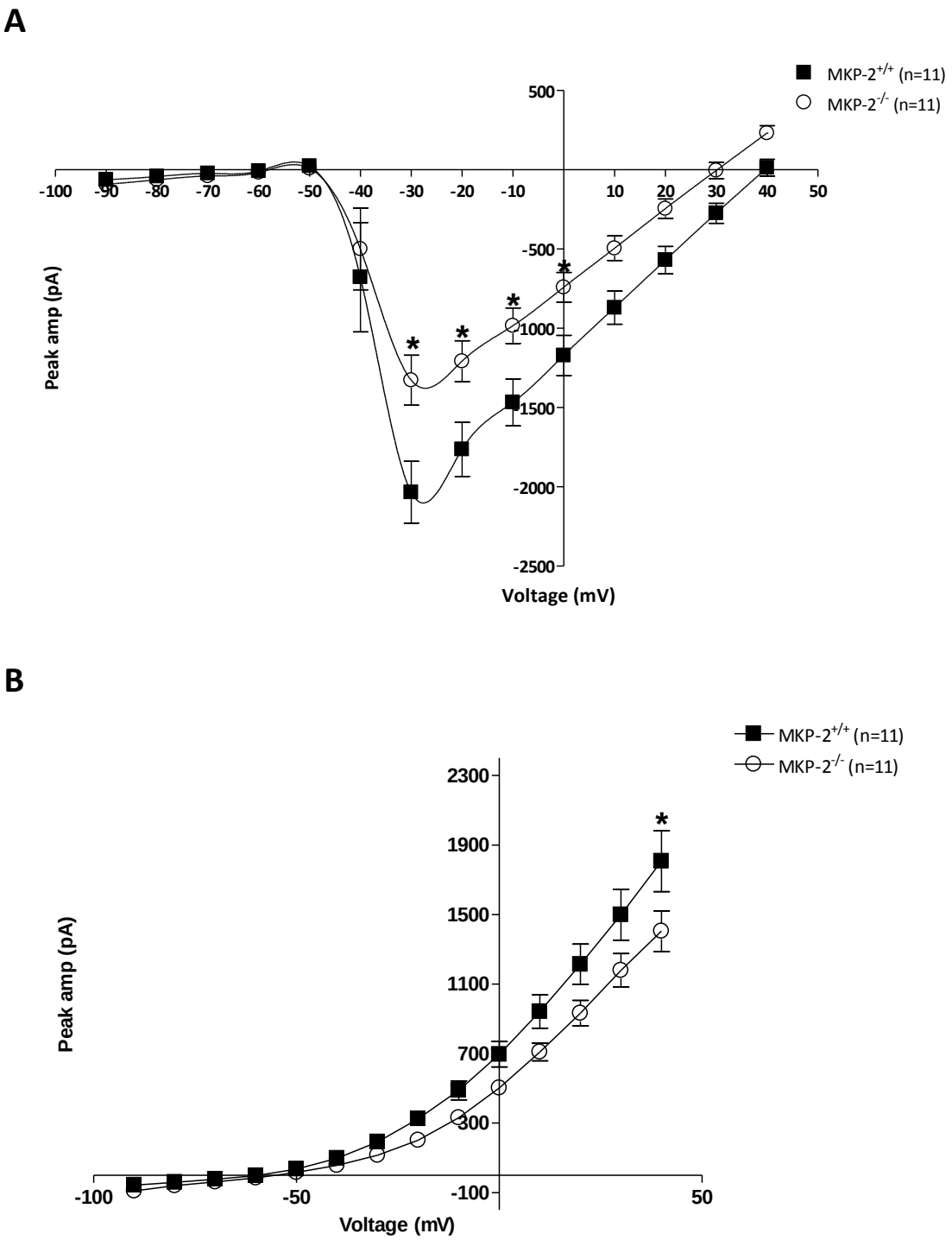


Figure 4.4: Na^+ and K^+ currents are significantly decreased at 7 DIV in MKP-$2^{-/-}$ neurons.

Pooled data showing a significant decrease of (A) Na^+ current and (B) K^+ current in MKP-$2^{-/-}$ neurons in primary hippocampal culture. Data are mean ± S.E.M analysed with two-way repeated measures ANOVA with Tukey's post hoc test between genotypes where appropriate with n being recordings, taken from 3 different cultures (*$P < 0.05$, n = 11 from 3 separate culture).

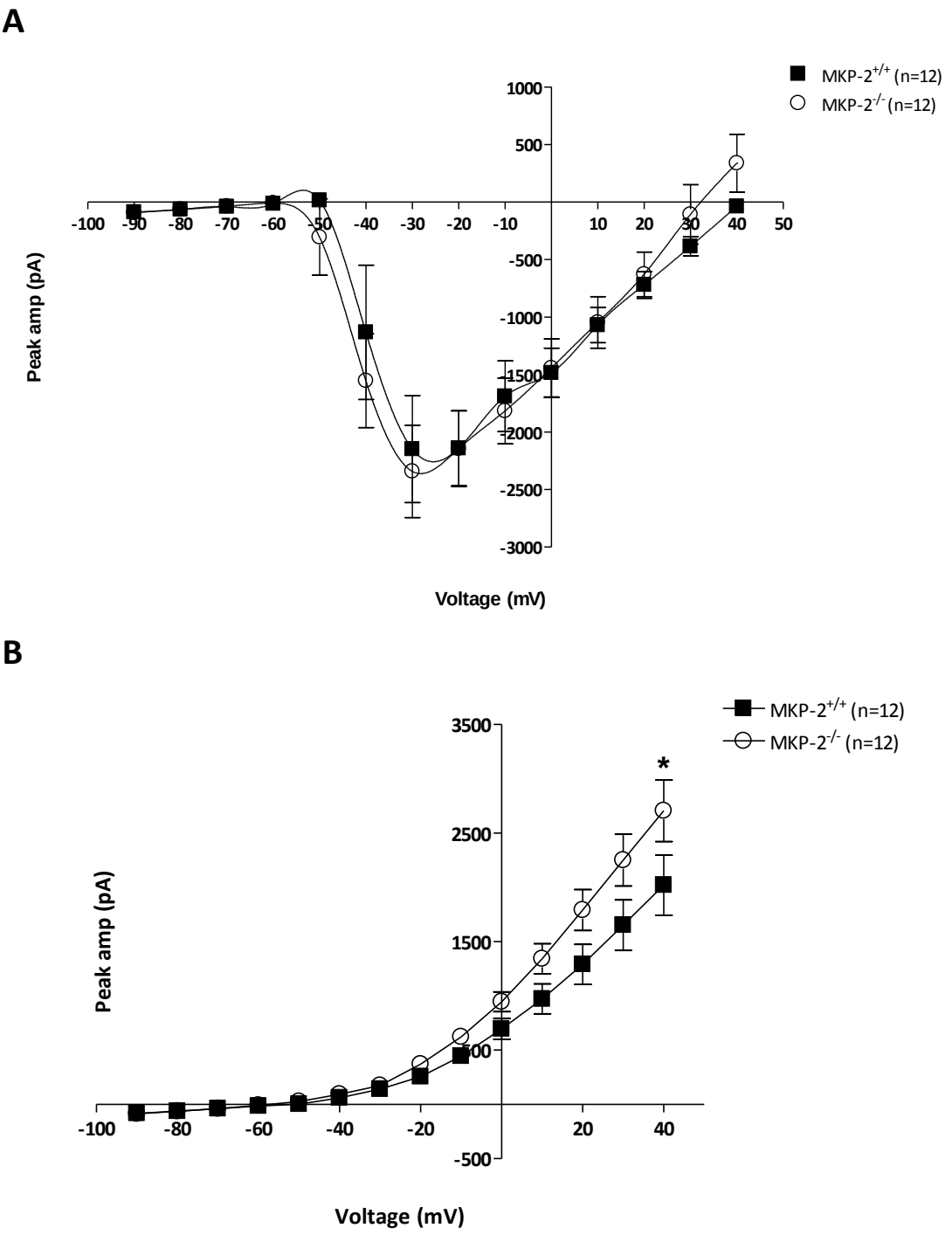


Figure 4.5: Significant increase in K^+ current but no significant difference in the Na^+ current at 11 DIV.

Pooled data showing significant increase in (B) K^+ in MKP-$2^{-/-}$ neurons in primary hippocampal culture at 11 DIV. Data are mean ± S.E.M analysed with two-way repeated measures ANOVA with Tukey's post hoc test with n being recordings, taken from 3 different cultures (*$P < 0.05$, n = 11 from 3 separate culture).

4.2.4 MKP-2 deletions significantly increases the frequency but not the amplitude of spontaneous excitatory postsynaptic currents at 7 and 11 DIV

Having established a reduction of sodium currents at 7 DIV, I next examined the consequence of MKP-2 deletion on spontaneous excitatory postsynaptic currents (sEPSC). There was a significant increase in sEPSC frequency in MKP-$2^{-/-}$ (21.9 ± 4.3 frequency/min, n=10) when compared to MKP-$2^{+/+}$ (7.3 ± 3.187 frequency/min, n=10, $P < 0.05$) (Figure 4.6A). However, there is no difference in sEPSC amplitude between MKP-$2^{+/+}$ (12.7 ± 1.8 pA, n=7) and MKP-$2^{-/-}$ (15.2 ± 3.2 pA, n=11, $P > 0.05$) cultures (Figure 4.6B).

To further investigate the effect of MKP-2 on sEPSC when the synapses are fully developed under normal culture conditions, sEPSC were investigated at 11 DIV. There was a significant increase in sEPSC frequency in MKP-$2^{-/-}$ neurons (249.8 ± 54.2 frequency/min, n=12) when compared to MKP-$2^{+/+}$ neurons (120.4 ± 15.49 frequency/min, n=12, $P < 0.005$) (Figure 4.7A). However, there was no difference in sEPSC amplitude detected between MKP-$2^{+/+}$ (17.0 ± 2.0 pA, n=13) vs MKP-$2^{-/-}$ (21.8 ± 3.9 pA, n=12, $P > 0.05$) (Figure 4.7B).

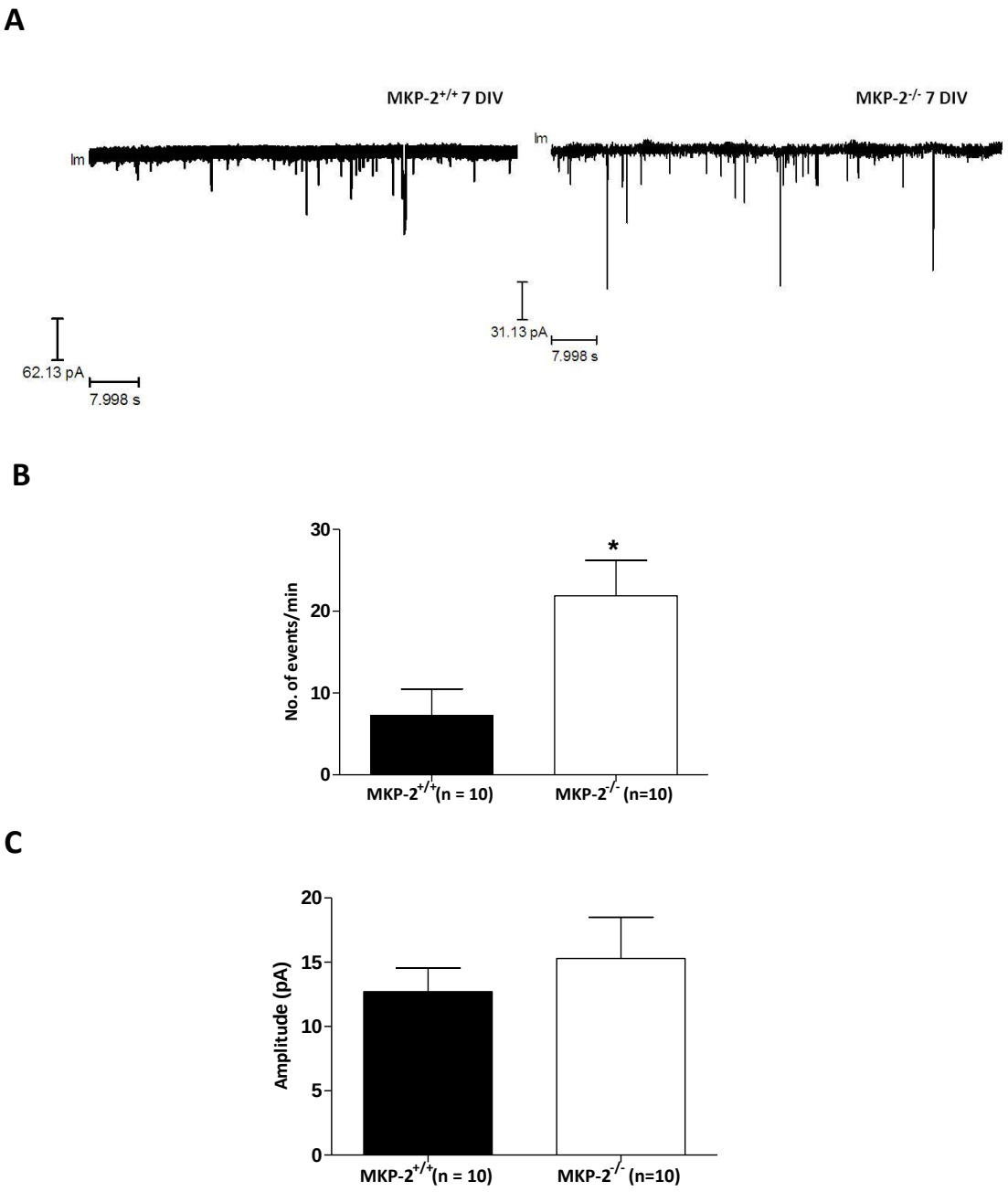


Figure 4.6: MKP-2 deletion increases sEPSC frequency but not amplitude in primary hippocampal cultures at 7 DIV

(A) Representative voltage clamp recordings showing increased sEPSC frequency in (ii) MKP-$2^{-/-}$ compared to (i) MKP-$2^{+/+}$. (B) Bar chart data showing a significant increase of sEPSC frequency but (C) not amplitude in MKP-$2^{-/-}$ neurons. sEPSC was determined by the shape of the amplitude and size (peak amplitude less than 150 pA). Data are mean ± S.E.M analysed with unpaired t-test with n being recordings, taken from 3 different cultures (*$P < 0.05$, n = 10 from 3 separate cultures).

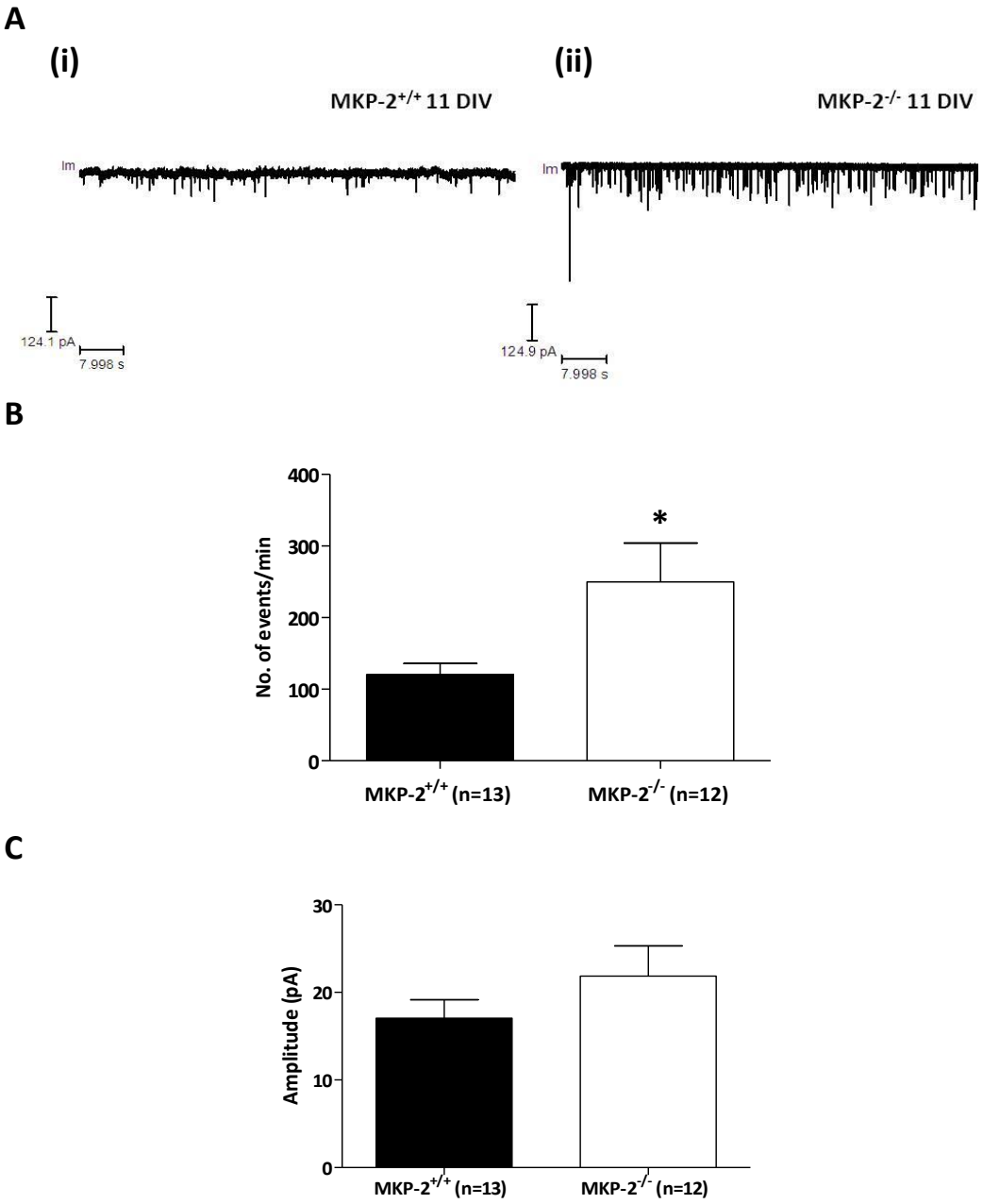


Figure 4.7: A significant increase in sEPSC frequency but not amplitude in 11 DIV in MKP-2$^{-/-}$ cultures.

(A) Representative voltage clamp recordings showing an increase in sEPSC frequency in (ii) MKP-2$^{-/-}$ compared to (i) MKP-2$^{+/+}$ neurons. (B) Bar chart data showing a significant increase of sEPSC frequency but (C) not amplitude in MKP-2$^{-/-}$ neurons. sEPSC was determined by the shape of the amplitude and size (peak amplitude less than 150 pA). Data are mean ± S.E.M analysed with unpaired t-test with n being recordings, taken from 3 different cultures (*P < 0.05, n = 10 recordings from 3 separate cultures).

4.2.1 Synapse number is significantly increased in MKP-2$^{-/-}$ primary hippocampal culture at 7 and 11 DIV.

Having established an increase in sEPSC frequency at 7 and 11 DIV, I next investigated the possibility that an increased synapse number accounted for this. At 7 DIV, there was a significant increase in synapse number in MKP-2$^{-/-}$ neurons (9.8 ± 0.23 synapse number/30 µm, n=188, $P < 0.0001$) when compared to MKP-2$^{+/+}$ (8.0 ± 0.1 synapse number /30 µm, n=229) (Figure 4.8B). Similarly, the synapse number was also increased at 11 DIV in MKP-2$^{-/-}$ neurons (15.9 ± 0.3 synapse number /30 µm, n=178, $P < 0.0001$) (Figure 4.8B) when compared to MKP-2$^{+/+}$ neurons (13.1 ± 0.2 synapse number/30 µm, n=172).

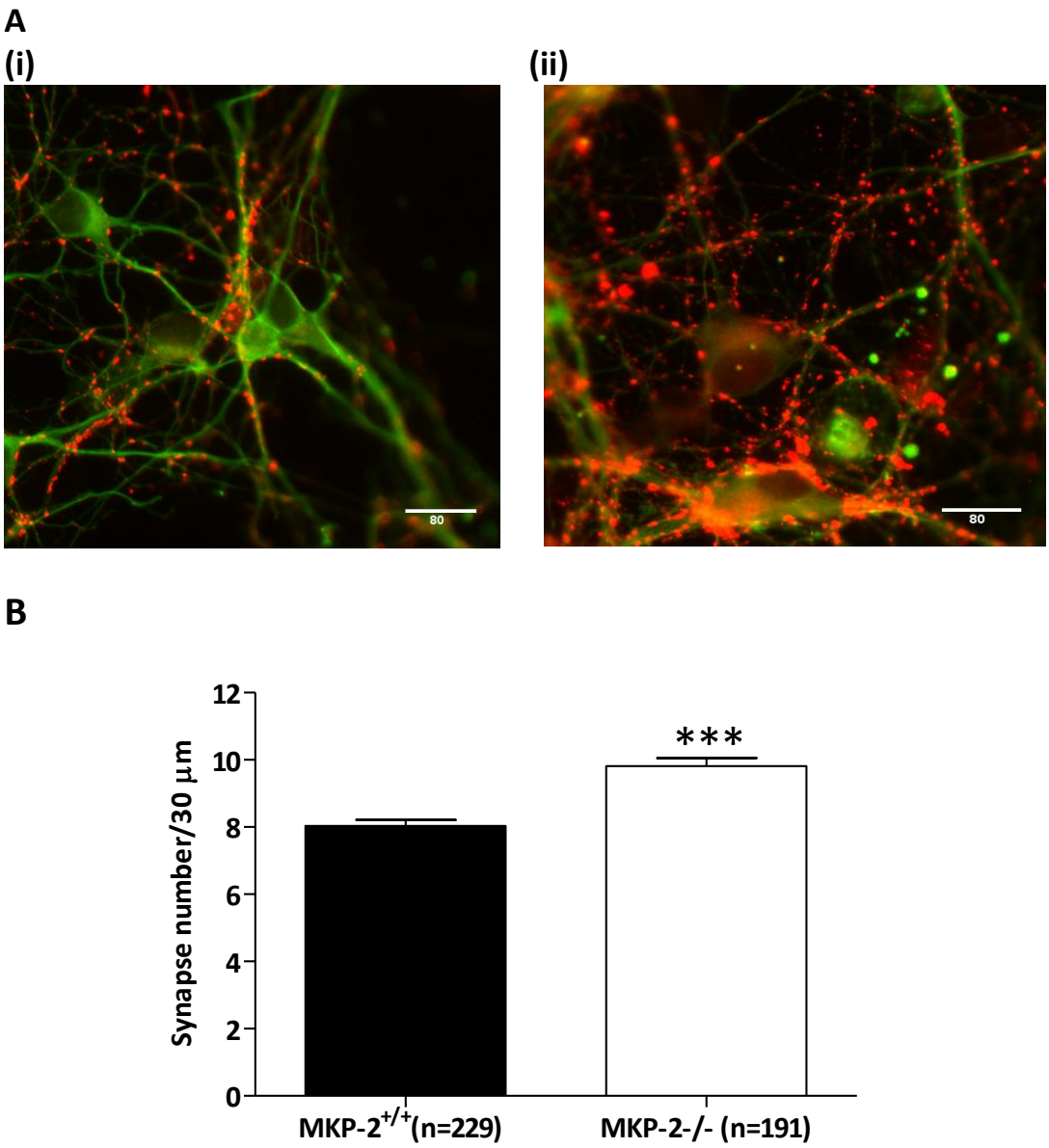


Figure 4.8: Synapse numbers are significantly increased in MKP-2$^{-/-}$ primary hippocampal cultures at 7 DIV.

(A) Representative images illustrating the number of synaptophysin (red) on neurite (green) in (i) MKP-2$^{+/+}$ and (ii) MKP-2$^{-/-}$ at 7 DIV. (B) Bar chart summarising the significant increase in synapse number in MKP-2$^{-/-}$ neurons as compared to MKP-2$^{+/+}$. Data are mean ± S.E.M analysed with unpaired t-test with n number of field, taken from 3 different cultures (***P < 0.001, n > 190 from 3 separate cultures).

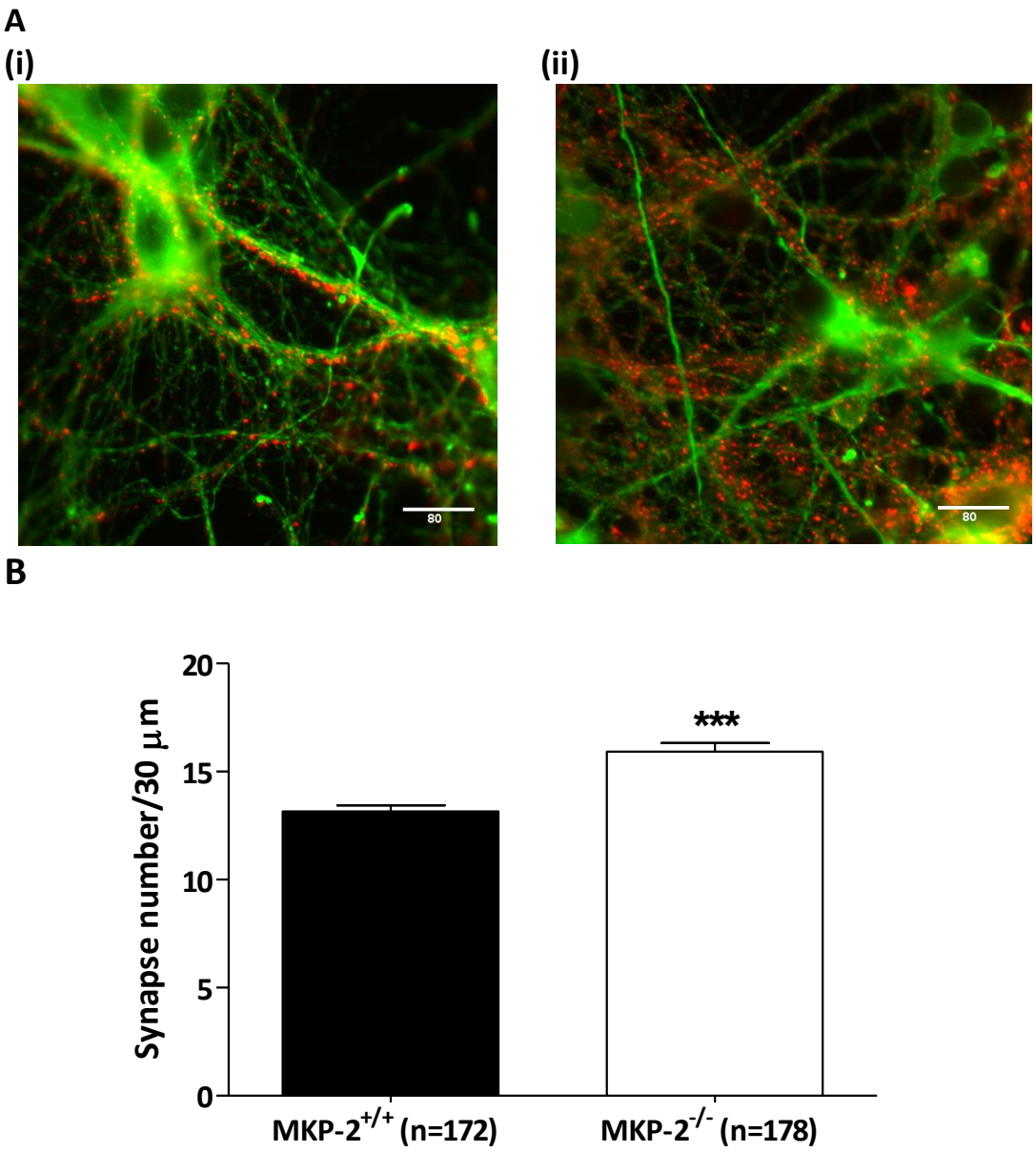


Figure 4.9: Synapse numbers are significantly increased in MKP-2$^{-/-}$ primary hippocampal cultures at 11 DIV.

(A) Representative images illustrating the number of synaptophysin (red) on neurite (green) in (i) MKP-2$^{+/+}$ and (ii) MKP-2$^{-/-}$ at 11 DIV. (B) Bar chart summarising significant increase in synapse number in MKP-2$^{-/-}$ as compared to MKP-2$^{+/+}$ neurons. Data are mean ± S.E.M analysed with unpaired t-test with n number of field, taken from 3 different cultures (***P < 0.0001, n > 170 from 3 separate cultures).

4.2.5 MKP deletion has no effect on ERK activation on 3, 7 and 11 DIV in primary hippocampal cultures.

As a negative modulator to the MAPK pathway, the consequence of MKP-2 deletion on ERK activation was investigated at 3, 7 and 11 DIV cultures. ERK has been chosen over JNK and p38 as it has been well established that the preferred substrate for MKP-2 is ERK > JNK > p38. As compared to MKP-$2^{+/+}$ (63090 ± 4328 arbitrary unit (a.u.), n=126), at 3 DIV, there is no significant difference in phospho-ERK (pERK) in MKP-$2^{-/-}$ (58880 ± 2934 a.u, n=195) (Figure 4.10A), even though there is a significant increase in total ERK (tERK) in MKP-$2^{-/-}$ (60590 ± 1948 a.u., n=195, $P < 0.05$) vs MKP-$2^{+/+}$ (53890 ± 2403 a.u., n=126) (Figure 4.10B). Nonetheless, there is no significant different of ERK activation when normalised to total ERK in MKP-$2^{-/-}$ (99.4 ± 4.6 a.u, n=199, $P > 0.05$) as compared to MKP-$2^{+/+}$ (99.2 ± 5.7 a.u, n=127) (Figure 4.10C).

Similarly, at 7 DIV, there is significant increase in pERK in MKP-$2^{-/-}$ (51750 ± 3878 arbitrary unit, n=85, $P < 0.05$) vs MKP-$2^{-/-}$ (37560 ± 3936 a.u, n=129) (Figure 4.11A). This is probably due to significant increase in tERK in MKP-$2^{-/-}$ (71830 ± 3033 arbitrary unit, n=85, $P < 0.001$) as compared to MKP-$2^{+/+}$ (48900 ± 2044 arbitrary unit, n=129) (Figure 4.11B), because even though there is significant increase in both pERK and tERK, no significant difference was detected in the ERK activation ratio in MKP-$2^{-/-}$ (74.5 ± 5.549 arbitrary unit, n=86, $P > 0.05$) vs MKP-$2^{+/+}$ (69.1 ± 4.90 arbitrary unit, n=130) (Figure 4.11C).

ERK activation was further investigated at 11 DIV. There is no significant difference in both pERK (MKP-$2^{+/+}$: 57440 ± 3945 a.u., n=128, vs MKP-$2^{-/-}$:51300 ± 3565 a.u., n=101, $P > 0.05$)(Figure 4.12A) and tERK (MKP-$2^{+/+}$: 58400 ± 2558 a.u., n=101 vs

MKP-2$^{-/-}$: 59930 ± 2839 a.u., n=128) (Figure 4.12B). Similar to 3 and 7 DIV, there is a non-significant increase in the ERK activation ratio in MKP-2$^{-/-}$ (91.4 ± 3.9, n=132, $P > 0.05$) as compared to MKP-2$^{+/+}$ (86.0 ± 6.721, n=109) (Figure 4.12C).

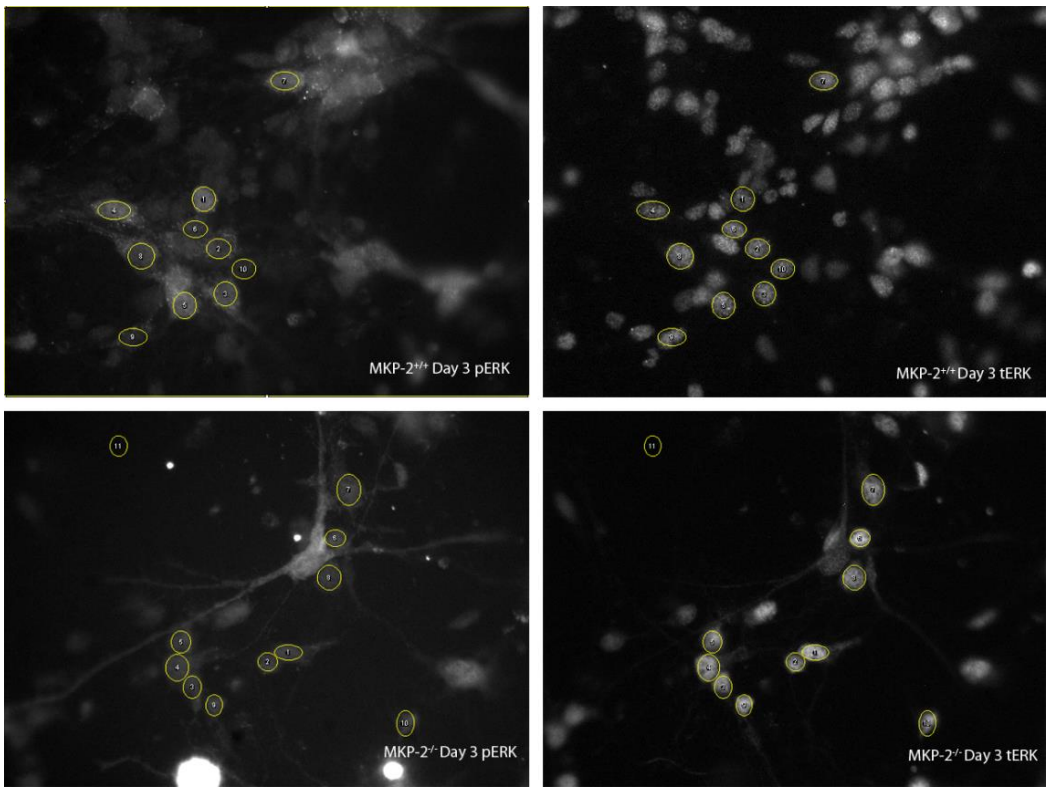


Figure 4.10: No differences in ERK phosphorylation ratio between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ at 3 DIV

Representative images showing no differences in ERK phosphorylation ratio at 3 DIV.

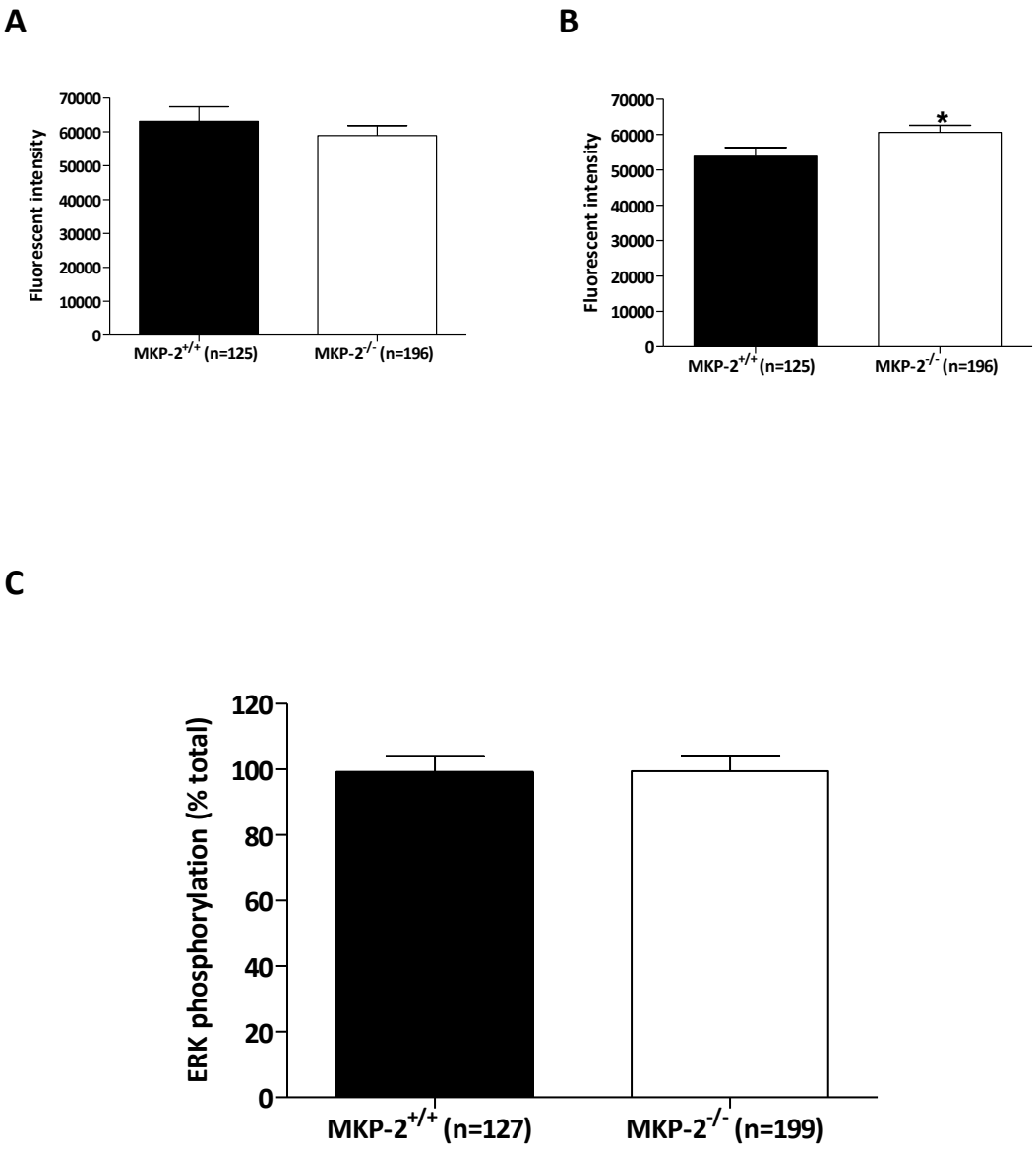


Figure 4.11: No differences in ERK phosphorylation ratio between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ at 3 DIV

Bar chart summaries reveal no significant difference in (A) pERK and (D) ERK activation, even though (C) tERK is significantly increased in MKP-2$^{-/-}$. Data are mean ± S.E.M analysed with unpaired t-test with n is number of cells, taken from 3 different cultures (* = $P < 0.05$, n > 50 cells from 3 separate cultures).

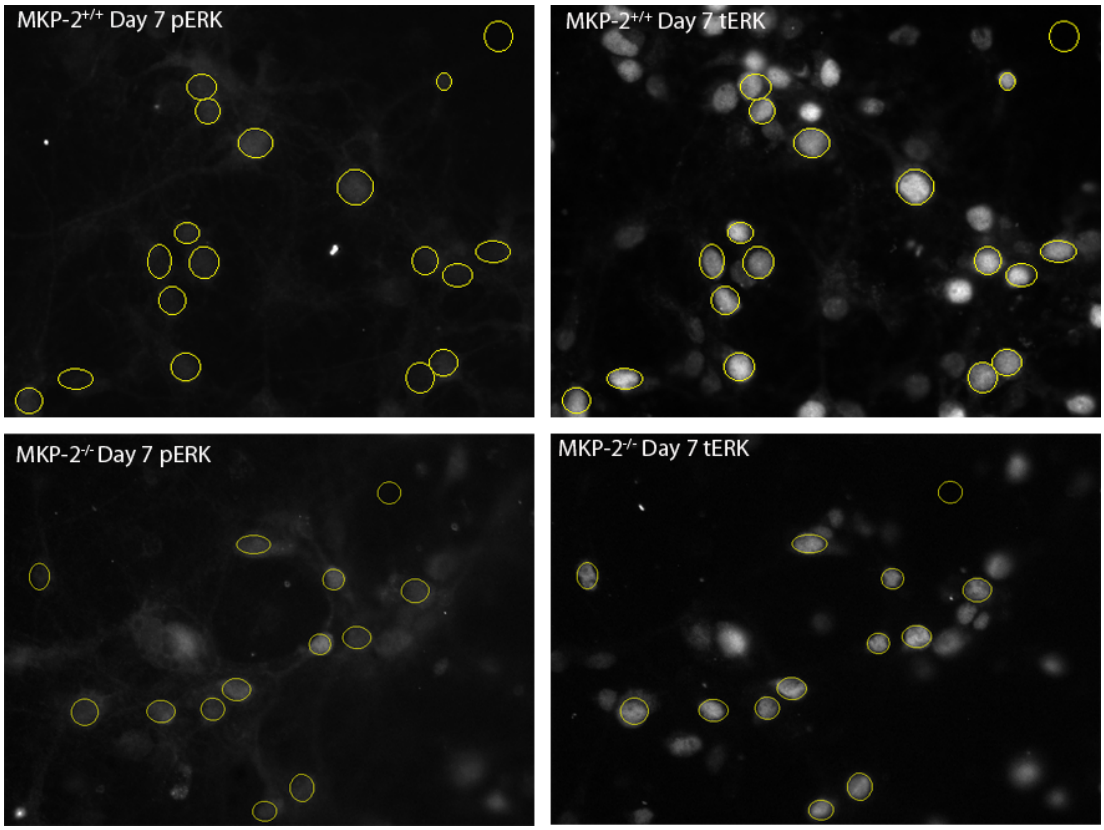


Figure 4.12: No differences in ERK phosphorylation ratio between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ at 7 DIV

Representative images showing no differences in ERK phosphorylation ratio at 7 DIV.

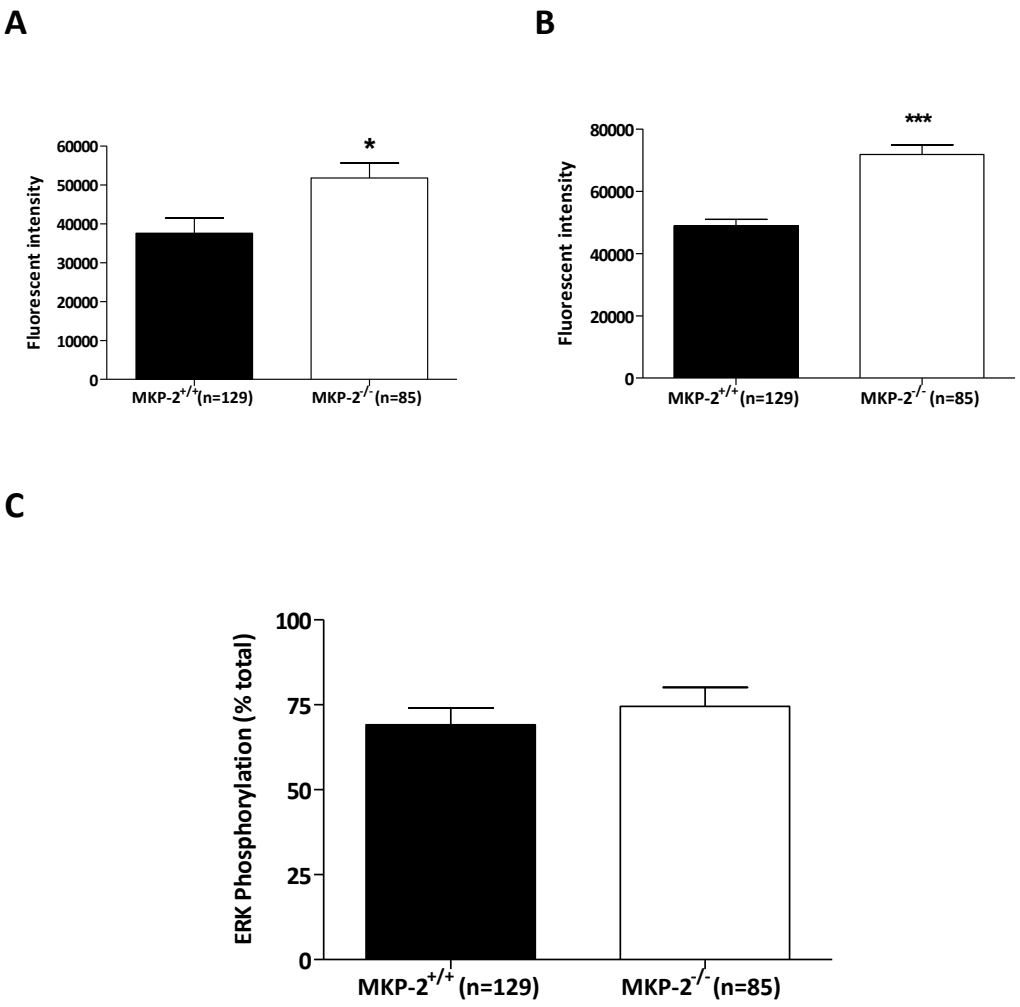


Figure 4.13: No differences in ERK phosphorylation ratio between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ at 7 DIV

Bar chart summaries reveal no significant difference in (C) ERK activation, even though (A) pERK and (B) tERK is significantly increased in MKP-2$^{-/-}$. Data are mean ± S.E.M analysed with unpaired t-test with n is number of cells, taken from 3 different cultures (* = $P < 0.05$, $n > 50$ cells from 3 separate cultures).

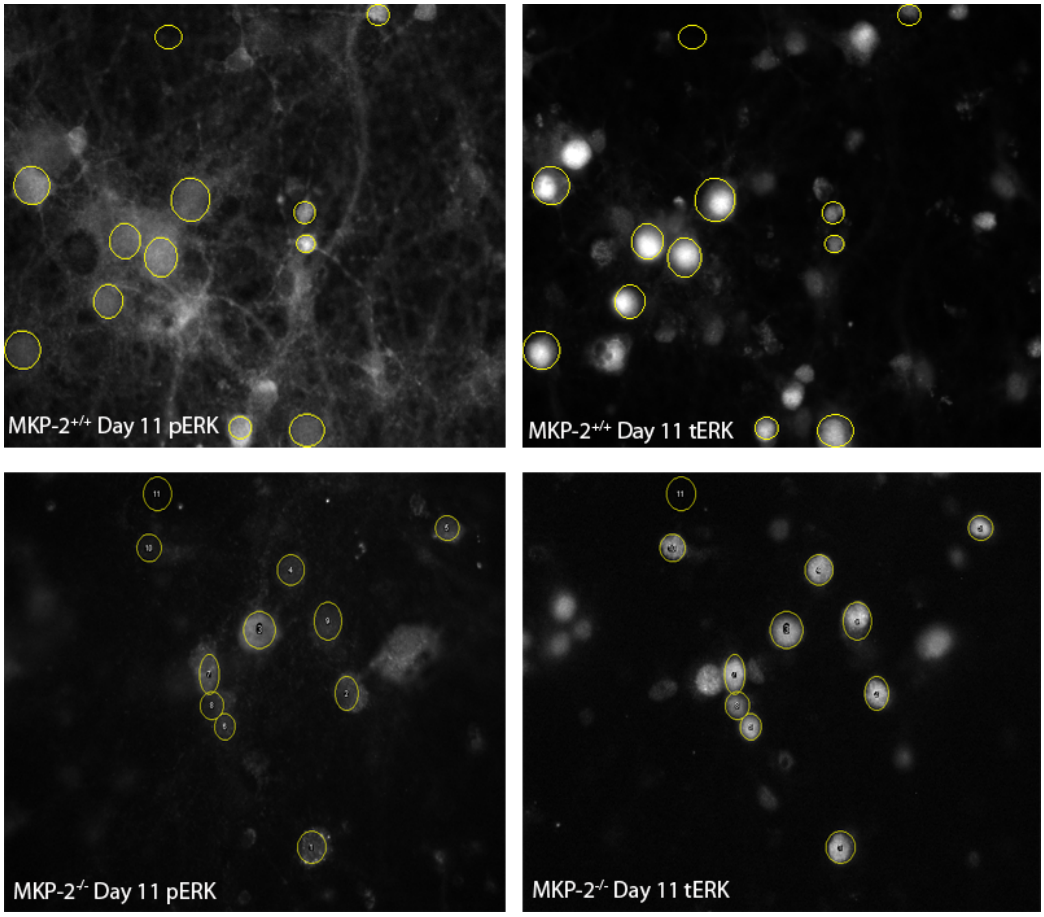


Figure 4.14: No differences in ERK phosphorylation ratio between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ at 11 DIV

Representative images showing no differences in ERK phosphorylation ratio at 11 DIV.

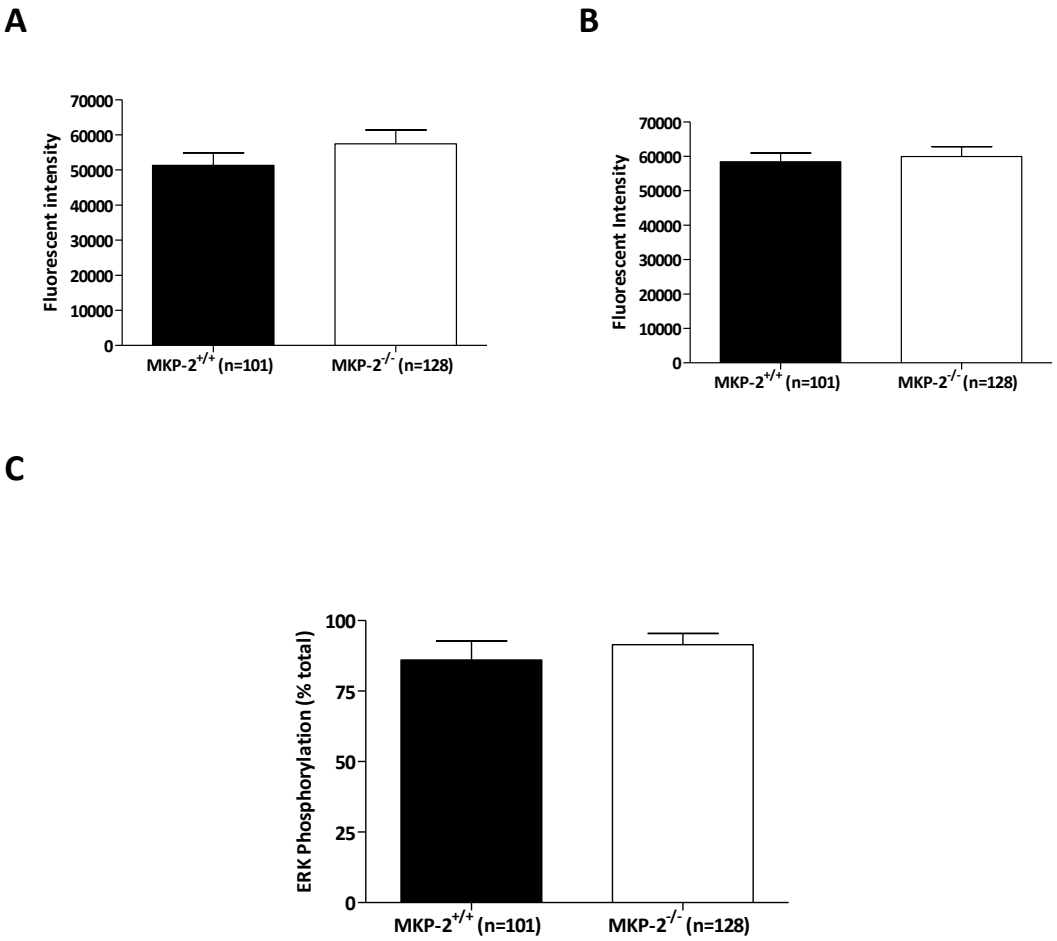


Figure 4.15: No differences in ERK phosphorylation in 11 DIV cultures.

Bar chart summaries showing no significant increase in (A) pERK, (B) tERK and (C) ERK activation ratio at 11 DIV.

4.3 Discussion

In this chapter, I show for the first time that, in MKP-$2^{-/-}$ primary hippocampal culture, astrocytic [Ca^{2+}]i was decreased and neuronal [Ca^{2+}]i was increased at 7 and 11 DIV respectively. Furthermore, Na^+ and K^+ was decreased at 7 DIV and sEPSCs was increased at 7 and 11 DIV. Eventhough there is reduction is Na^+ and K^+ current, however, spontaneous EPSC and synapse number was increased significantly at 7 and 11 DIV. It seems the differences in functional activities were not related with ERK activation as no differences was evident in ERK activation at 3, 7 and 11 DIV.

Intracellular Ca^{2+} signalling within the CNS plays a vital role in a diverse range of functionality, from regulation of gene transcription to neuronal firing. The results in this study shown that neuronal [Ca^{2+}]i that depolarized with application high extracellular K^+ was increased at 11 DIV, but not at 3 and 7 DIV. The elevation of extracellular K^+ leads to depolarization of the neuron thus activates voltage-gated calcium channels meanwhile application of DHPG; activates mGluR1 & 5 which resutls in increases in [Ca^{2+}]i via the Gq and IP_3 pathway. As the changes in [Ca^{2+}]i in the presence of DHPG has no difference between genotype, there is probability that the calcium increase could be due to activation of voltage gated calcium channels that induced the increasing of [Ca^{2+}]i. Thus, it is reasonable to speculate that MKP-2 deletion modulate the expression of voltage gated calcium channel. Previous study has shown that the inhibition of ERK by UO126 reduced calcium current by 45 % (Fitzgerald, 2000). However, as there was no significant difference in ERK activation in this study, there mechanism underlying this still unknown and worth to be investigated further. In contrast to neuronal [Ca^{2+}]i, application of trypsin on MKP-$2^{-/-}$ primary cultures activates astrocytes leading to a significant decrease in

MKP-2$^{-/-}$ astrocytic [Ca^{2+}]i at 7 DIV but no significant difference in the presence of ADP. As both P2Y and PAR-2 receptor activate astrocytic [Ca^{2+}]i through phospholipase C (PLC)/Inositol triphosphate (IP_3) pathway that subsequently stimulate calcium release from the endoplasmic reticulum (Bushell et al., 2006; Neary et al., 1999), it is difficult to assume that the impairment was in regulation of astrocytic receptor expression. However, this interpretation could be compromised by a concern that purinergic agonist used in this study was ADP, which only activates certain type of P2Y receptor (Scemes and Nichia., 2011) compared to majority of studies that used ATP to activate P2Y receptor (Hashioka et al., 2014; Neary et al., 1999). Furthermore, previous study has shown that activation of PAR-2 involved p38 MAPK and ERK modulation (Greenwood and Bushell, 2010). As in this study no differences in ERK activation and p38 MAPK activation was not be able to investigate due to time constraint, the mechanism underlying this is still unknown.

Given the changes observed following neuronal depolarisation, I next examined the possibility that MKP-2 regulates Na^+ and K^+ channel expression and function. In this study, at 7 DIV, the maximum Na^+ and K^+ current was decreased in MKP-2$^{-/-}$ but K^+ was increase at 11 DIV. These results suggest that MKP-2 play a role in Na^+ and K^+ development, which at 7 DIV, Na^+ and K^+ channel function is not fully developed. However, as at 11 DIV no significant difference in Na^+ current between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ was seen, this suggests that as network connectivity is well-established (Wagenaar et al., 2006), the Na^+ and K^+ channels becomes fully functional. Previous studies have shown the direct involvement of p38 MAPK in Na^+ channel development as co-expression exists and the activation of p38 MAPK significantly

decreased peak current density (Wittmack et al., 2005) . In this study, there is probability that MKP-2 deletion increases p38 MAPK expression during development and this affects Na^+ channel development and subsequently reductions in Na^+ current and worth to investigate further. Apart from p38 MAPK, pERK was also shown to be involved in modulating the gating properties of Na_v 1.7 as Na_v 1.7 and ERK are co-localised in dorsal root ganglion neurons and the ERK inhibitor, UO126, shifts the activation and fast inactivation kinetics of Na_v1.7 (Stamboulian et al., 2010). Similarly with K^+ channel, previous study has shown the basal phosphorylation of ERK with Kv4.2 suggesting ERK plays a role in expression K^+ channel (Yuan, et al.,, 2002). It is suggested to investigate the expression of p38 MAPK at two different time point, during development, as in 7 DIV and late development, as in 11-14 DIV, as well as immunolabelling to detect co-localization of MAPK and Na^+ and K^+ channel. Investigating sodium channel development as well as sodium current is important as an impairment in sodium and potassium channels is implicated in action potential firing and disorders such as epilepsy (George, 2005).

Having established MKP-2 deletion impacted in Na^+ channel development as well as $[Ca^{2+}]i$, I next investigate the effect of MKP-2 deletion on spontaneous neurotransmitter release (sEPSCs) as to know its potential role in modulating synaptic function and regulating neurotransmitter release. Results in this study showed that the frequency of sEPSC was increased at 7 and 11 DIV. The increase in frequency of sEPSC but not the amplitude imply that the modulation is pre-synaptic and not post-synaptic (Pinheiro and Mulle, 2008). Furthermore; sEPSCs is a mix of synaptic vesicle release and signal derived from action-potential dependent release.

In this study, as Na^+ current has been shown to be decreased at 7 DIV, and the effect was presynaptic, it is possible reasonable to speculate that the MKP-2 deletion affect spontaneous vesicle release at 7 DIV. To confirm this, it would be interesting to investigate the quantal vesicle release by conducting mEPSC experiment by adding TTX (tetrodotoxin) to block Na^+ channel function. Taking this into account, it would be reasonable to speculate that in MKP-$2^{-/-}$ culture, there is probability that there is increasing synapse formation that contribute to the increasing of the frequency spontaneous EPSC. Previous study has shown that ERK dependent synapsin I phosphorylation increases paired pulse facilitation and docked vesicles (Giachello et al., 2010). Thus, it is reasonable to speculate that there is increasing of pool of vesicles, known as readily released pool (RRP) in MKP-$2^{-/-}$ culture. This could be investigate further by using patch clamp electrophysiology and applying rapid train of electrical stimuli and counting the number of vesicle per synapse that released.

To determine whether synapse number accounts for the changes in sEPSC frequency, the number of synaptophysin puncta per 30 μm neurite length was taken as a measure (Dityatev et al., 2000). Synapse number was increased significantly at both 7 and 11 DIV, implying that MKP-2 regulates the formation of hippocampal synapses. Thus it is reasonable to speculate that this might account for the increasing sEPSC frequency at 7 and 11 DIV. This finding is consistent with a previous study that shown increasing in sEPSC is contributed by increasing of synapse number (Groc et al., 2002). As at 7 DIV astrocyte proliferation is increased (chapter 3), this result suggests the possibility that the enrichment in trophic factor might contribute to the development of synapse. This hypothesis is consistent with previous finding

that shown thrombospondins has induced production of synapse (Christopherson et al., 2005). Similarly, application of the astrocyte tropic factor hevin, to the primary cultures shows increasing synapse number (Kucukdereli et al., 2011). Similarly, BDNF also has been shown to be involve in promoting synapse formation (Bamji et al., 2006; Bramham and Messaoudi, 2005) To confirm this, it is worth to investigate the trophic factor that produced by MKP-$2^{-/-}$ in younger and older culture. In term of synapse development, it would be also worth to investigate the formation of synapse in acute hippocampal slice by using immunohistochemistry method.

Even though most of the results in this study shows a link with ERK activation, however, contrary to expectation, no significant difference in ERK activation at 3, 7 and 11 DIV. It is important to highlight that the ERK quantification in this study is in the soma area meanwhile the main change observed in this study is in the functional in the synapse area. Thus, it would be interesting to investigate whether there is changes in activation of localized ERK in presynaptic and postsynaptic terminals. This could be done by conducting an experiment by using immunogold labelling with immune-electron microscopy. In this study, there were increasing in ERK phosphorylation at 3 and 7 DIV, but this probably due to the increasing in total ERK. It is also important to note that the cells quantified in this study were not differentiating between neuron and astrocyte. This result is distinct from previous studies that show increasing of PDGF-mediated ERK phosphorylation in embryonic fibroblast cell (Lawan et al., 2011) and decreasing of ERK activation upon increasing MKP-1 expression (Duric et al., 2010). However, this result is consistent with a previous study on macrophage derived from MKP-$2^{-/-}$ that showed no significant

enhancement in ERK activation (Al-Mutairi et al., 2010). Taken together, this results support the interpretation that the regulation of MKP-2 is could be cell-specific. It is also reasonable to speculate that no alteration in activation of ERK suggests that apart from MKP-2, there might be crosstalk between pathways that compensate for the MKP-2 activity. This result supports the finding of previous studies which shown that JNK pathway also has an antagonistic effect on ERK activity (Dong & Bode, 2003; Friedman and Perrimon, 2006) as well as activation of p38 MAPK signalling causing inactivation of the ERK pathway (Junttila et al., 2008). It is important in the future studies to investigate the effect of MKP-2 deletion on p38 MAPK and JNK activity, both in primary hippocampal culture or acute hippocampal slices to confirm this.

In summary, in this chapter, I demonstrated for the first time that MKP-2 deletion increases neuronal $[Ca^{2+}]i$ elicited by high K^+ as well as the frequency of sEPSC. However, Na^+ and K^+ currents and astrocytic $[Ca^{2+}]i$ was reduced at 7 DIV. My findings indicate that the increased sEPSC frequency is due to the increase in number of synapses and the changes in functional activities was not due to ERK phosphorylation as no significant difference in ERK activation. However further experiments are required to identify the exact mechanisms underlying this.

Days in vitro	Changes in CNS function
3 DIV	Reduction in astrocyte number Reduction in neurite length No changes in neuronal and astrocytic intracellular calcium No changes in ERK activation
7 DIV	No changes in astrocyte number No changes in neurite length Astrocytic intracellular calcium decreased Neuronal intracellular calcium decreased Na^+ current reduced K^+ current reduced sEPSC increased Synapse number increased No changes in ERK activation
11 DIV	Neuronal intracellular calcium increased K^+ current increased sEPSC increased Synapse number increased No changes in ERK activation

Table 4.1: Summary of changes in neuronal and astrocytic function consequence MKP-2 deletion in primary hippocampal culture.

5 DETERMINING THE CONSEQUENCE OF MKP-2 DELETION ON SYNAPTIC TRANSMISSION IN ACUTE HIPPOCAMPAL SLICE PREPARATIONS

5.1 Introduction

As mentioned in chapter 1, the role of MAPK cascade in synaptic transmission and plasticity is well-established. Mounting evidence has shown that ERK is largely involved in synaptic transmission and plasticity (Impey et al., 1999; Peng et al., 2010; Sweatt, 2004; Thomas and Huganir, 2004). Furthermore, another member of MAPK family, p38 MAPK, found to be highly expressed in mature neurons has also been implicated to play a key role in in neuronal function (Corrêa & Eales, 2012). Another MAPK subfamily, JNK has also been shown to be involved in synaptic transmission and plasticity (Yang et al., 2011). Despite the fact that MKP-2, a negative regulator of the MAPK cascade, is widely expressed in the brain, its role in the CNS, especially in basal synaptic transmission and plasticity, has not been investigated.

Having conducted experiments in primary neuronal culture, I further investigate the consequence of MKP-2 deletion using acute hippocampal slices. Acute hippocampal slices are a well-established method used extensively to study the effect of role of numerous receptors and signalling pathways in synaptic transmission and plasticity. Among distinct advantages of acute hippocampal slices includes it is able to mimic many *in vivo* aspects as the tissue architecture of brain region and synaptic circuits is

still intact (Lein et al., 2011). The experiments were conducting using well-established extracellular electrophysiology method.

Therefore, the specific aim of this chapter is to investigate the consequence of MKP-2 deletion on ERK activity in a number of brain regions including the hippocampus. As the two previous chapters were done in primary hippocampal culture, I aimed in this chapter to further examine whether MKP-2 deletion leads to alterations in synaptic transmission and short term synaptic plasticity in the CA1 region by using acute hippocampal slices. This synapse has been selected because the pathway is well-studied and its anatomy is well characterized.

5.2 Results

5.2.1 MKP-2 deletion enhances basal synaptic transmission at high stimulus intensities.

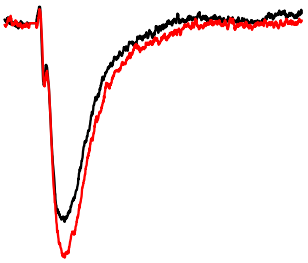
The first functional consequences of MKP-2 deletion in acute hippocampal slices was investigated was basal synaptic transmission. Input output curves were generated to compare the basal synaptic transmission between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices. The fEPSP slope was significantly enhanced in MKP-$2^{-/-}$ slices compared to MKP-$2^{+/+}$ at high intensities stimulations (Figure 5.3). The fEPSP slope increased from -0.11 ± 0.02 mV ms^{-1} to -0.79 ± 0.12 mV ms^{-1} in MKP-$2^{+/+}$ whereas in MKP-$2^{-/-}$, the slope increased from -0.06 ± 0.01 mV ms^{-1} to -1.26 ± 0.35mV ms^{-1}. There were no significant differences between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ in the basal synaptic transmission at lower stimulus intensities. However, there was significant increase in the transmission observed at 5x (MKP-$2^{+/+}$: -0.4 ±0.05 mV ms^{-1} vs MKP-$2^{-/-}$: 0.96 ± 0.25 mV ms^{-1}, $P < 0.05$, n = 12) and 10x (MKP-$2^{+/+}$: -072 ±0.75 mV ms^{-1} vs MKP-$2^{-/-}$: -1.67 ± 0.09 mV ms^{-1}, $P < 0.05$, n = 12).

5.2.2 Short-term plasticity is unaltered in MKP-$2^{-/-}$ mice

To determine whether MKP-2 plays a role in short-term plasticity, paired pulse facilitation (PPF) with increasing inter-stimulus intervals was investigated. In MKP-$2^{-/-}$ hippocampal slices, PPF at inter-stimulus intervals (ISI) of 10 ms and up to 500 ms consistently yielded facilitation as determined by the ratio of the evoked response to the second stimulus compared to the first stimulus (10 ms: 1.9 ± 0.07; 25 ms: 1.9 ± 0.04; 50 ms: 1.98 ± 0.38; 100 ms: 1.24 ± 0.02, n = 8, $P < 0.05$). However, there are

no significant differences between PPF in MKP-$2^{+/+}$ compared to MKP-$2^{-/-}$ observed at all ISIs investigated (10 ms: 2.2 ± 0.21; 25 ms: 2.0 ± 0.2; 50 ms: 2.1 ± 0.09; 100 ms: 1.9 ± 0.11, n = 8, $P < 0.05$) (Figure 5.4).

A



B

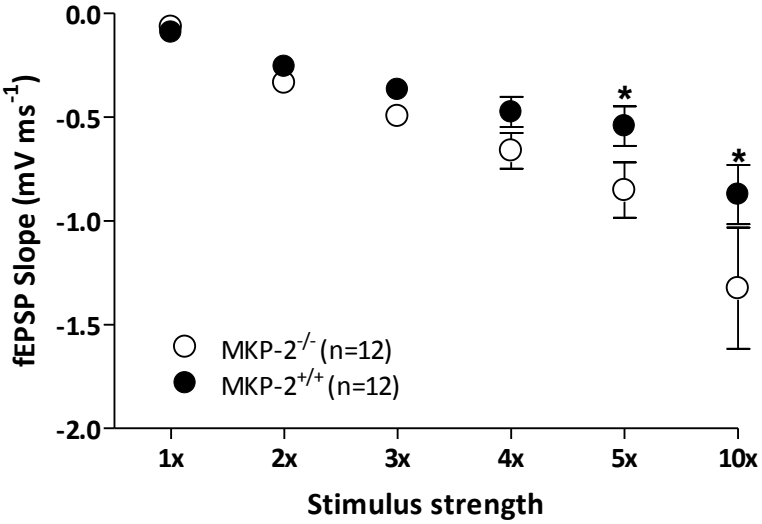


Figure 5.1: Basal synaptic transmission was increased in MKP-2 slice at high stimulus strength. (A) Representative traces illustrating fEPSPs obtained from MKP-$2^{+/+}$ (black) and MKP-$2^{-/-}$ (red) at high stimulus strength (B) Summary illustrating MKP-2 deletion increased basal synaptic transmission at higher stimulus strengths. Data are mean ± S.E.M analysed with two-way repeated measures with n is number of recordings, taken from 3 different cultures (*P < 0.05).

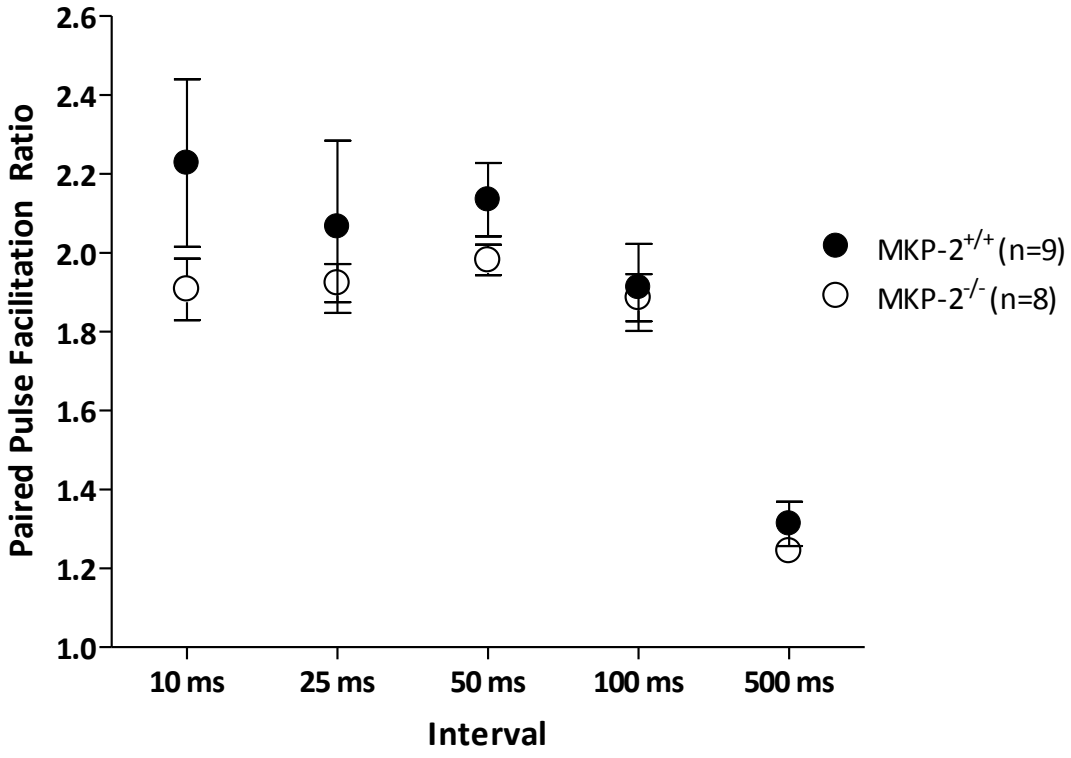


Figure 5.2: Short-term plasticity is unaltered in MKP-2$^{-/-}$ mice.

There is no significant difference in paired-pulse ratio in MKP-2$^{+/+}$ slices compared to MKP-2$^{-/-}$ slices across all ISIs investigated.

5.2.3 Does MAPK inhibition affect synaptic transmission and PPF?

5.2.3.1 ERK inhibition has no effect on basal synaptic transmission and short-term potentiation in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices.

The input-output curve revealed significant enhancement of baseline synaptic transmission at high stimulation in MKP-$2^{-/-}$ slices. In order to examine whether MAPKs have an effect on synaptic transmission, pharmacological inhibition of MAPKs was studied. To investigate the effect of ERK inhibition on synaptic transmission, UO126, an upstream inhibitor of ERK activation which specifically inhibits MEK1 and MEK2, was used (Favata et al., 1995). Application of UO126 (40 μM) led to a small but non-significant increase in synaptic transmission in MKP-$2^{+/+}$ (8 ± 12%, n = 7, P > 0.05) (Figure 5.5 A (i), B). Similar findings were seen in MKP-2 knock-out slices, in which a small but non-significant increase in synaptic transmission was observed after perfusion with UO126 (10 ± 12%, n = 7, *P*> 0.05, Figure 5.5 A (ii), B). In addition, the presence of UO126 did not alter short term plasticity, with only a small reduction in PPF being observed in both MKP-$2^{+/+}$ (2.0 ± 1, n = 7, *P*> 0.05) and MKP-$2^{-/-}$slices (2.0 ± 0.7, n = 7, P> 0.05).

5.2.3.2 p38 MAPK inhibition increases basal synaptic transmission but does not alter PPF in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices.

SB203580, a specific inhibitor of p38 MAPK (Cuenda et al., 1995), has been used to investigate the effect of p38 MAPK inhibition on synaptic transmission. Application of SB203580 (100 μM) resulted in a significant increase in synaptic transmission in both MKP-$2^{+/+}$ (31 ± 7%, n = 8, P < 0.05) (Figure 5.6 A (i), B) and MKP-$2^{-/-}$ slices (31 ± 5%, n = 5, P < 0.05, Figure 5.6 A (ii), B). However there is no significant

difference between the increase in synaptic transmission between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices. The effect of SB203580 on short-term plasticity in MKP-$2^{-/-}$ was also measured. A small but non-significant decrease in PPF was observed in MKP-$2^{+/+}$ slices (2.14 ± 0.6, n = 8, P > 0.05) as well as in MKP-$2^{-/-}$ slices (2.0 ± 0.1, n = 5, P > 0.05).

5.2.3.3 JNK inhibition has no effect on basal synaptic transmission and PPF in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices.

The effect of JNK activity on MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were investigated using SP600125 (20 μM), a selective JNK inhibitor (Bennet et al., 2001; Costello and Herron, 2004). Perfusion of SP600125 led to a small non-significant increase in basal synaptic transmission in MKP-$2^{+/+}$ slices (31 ± 9%, n = 6, P > 0.05). On the other hand, there was no significant different increase in basal synaptic transmission in MKP-$2^{-/-}$ (10 ± 11%, n = 6, P > 0.05) (Figure 5.7 A (i), B) subsequent to SP600125 application. The PPF ratio was not significantly different in MKP-$2^{+/+}$ and MKP-$2^{-/-}$ following SP600125 application with the PPF ratio in MKP-$2^{+/+}$ slices being 2.0 ± 0.12, n = 6 and 1.9 ± 0.12 in the absence and presence of SP600125 respectively (Figure 5.7 C). Similarly, the PPF ratio was unaltered in MKP-$2^{-/-}$ slices following SP600125 application with a value of 2.3 ± 0.19 and 2.5 ± 0.38 respectively.

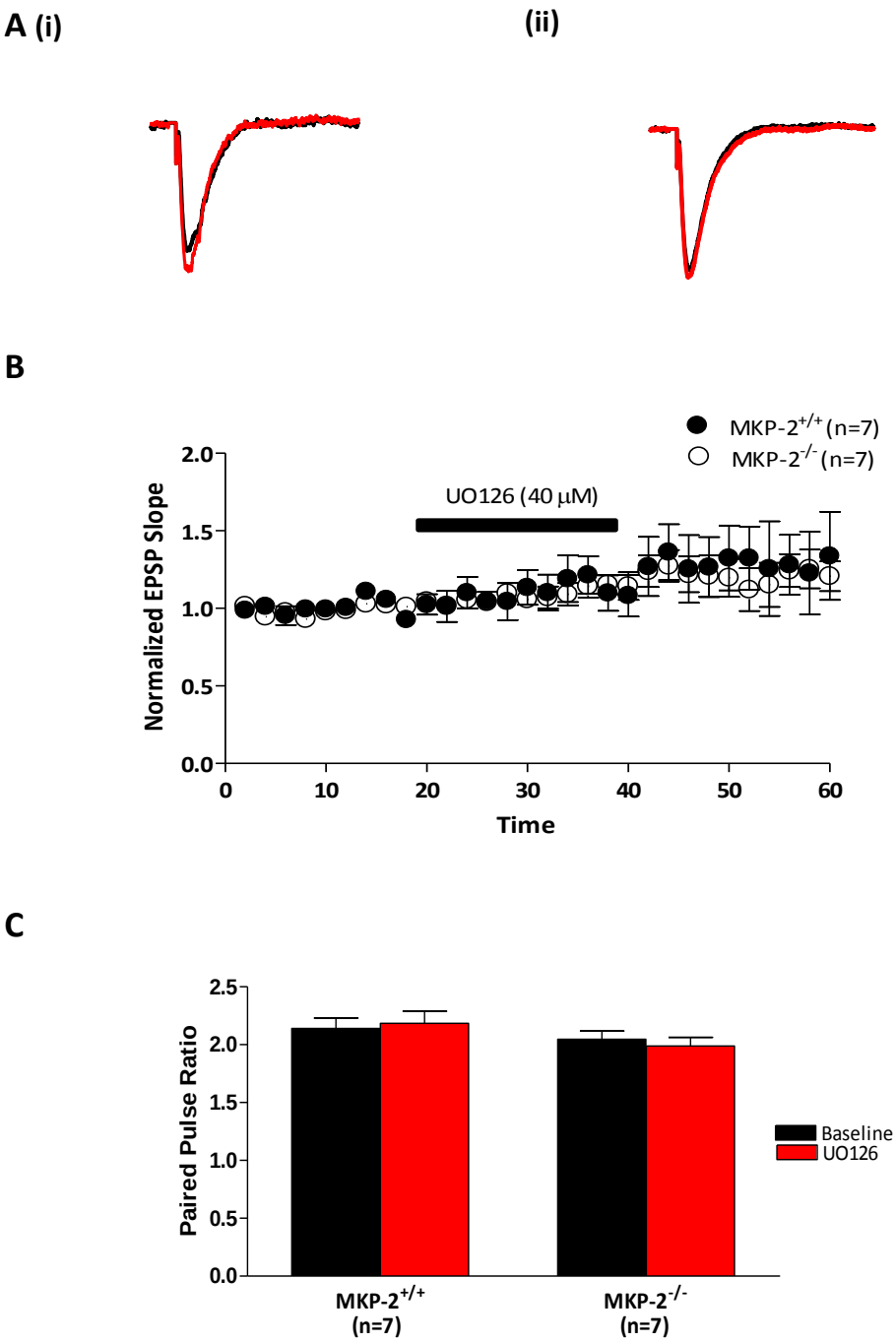


Figure 5.3: ERK inhibition does not alter the synaptic transmission.

(A) Representative traces illustrating the effect of ERK inhibition by UO126 (40 µM, red traces) in (i) MKP-$2^{+/+}$ slices and (ii) MKP-$2^{-/-}$ slices. B) Application of UO126 did not affect synaptic transmission in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices (C) Bar chart illustrates that UO126 application does not affect paired-pulse facilitation ratio.

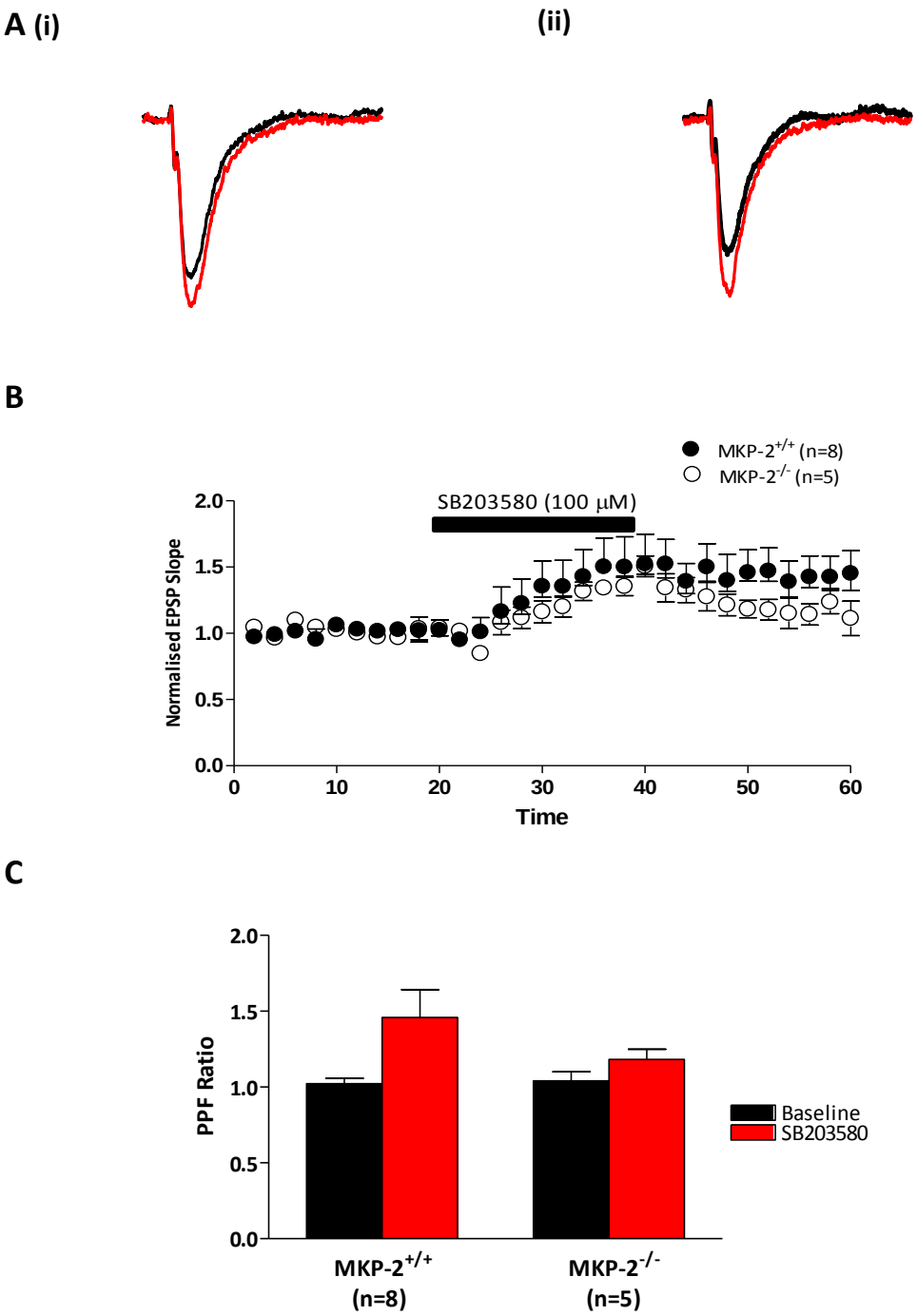


Figure 5.4: Application of SB203580 increase synaptic transmission but does not affect short term plasticity. (A) Representative traces illustrating the effect of p38 MAPK inhibition by SB203580 (100 µM, red traces) in (i) MKP-$2^{+/+}$ mice and (ii) MKP-$2^{-/-}$ mice. (B) Application of SB203580 increases synaptic transmission in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices. (C) Summary reveals no significant difference in PPF ratio was evident subsequent to SB203580 application.

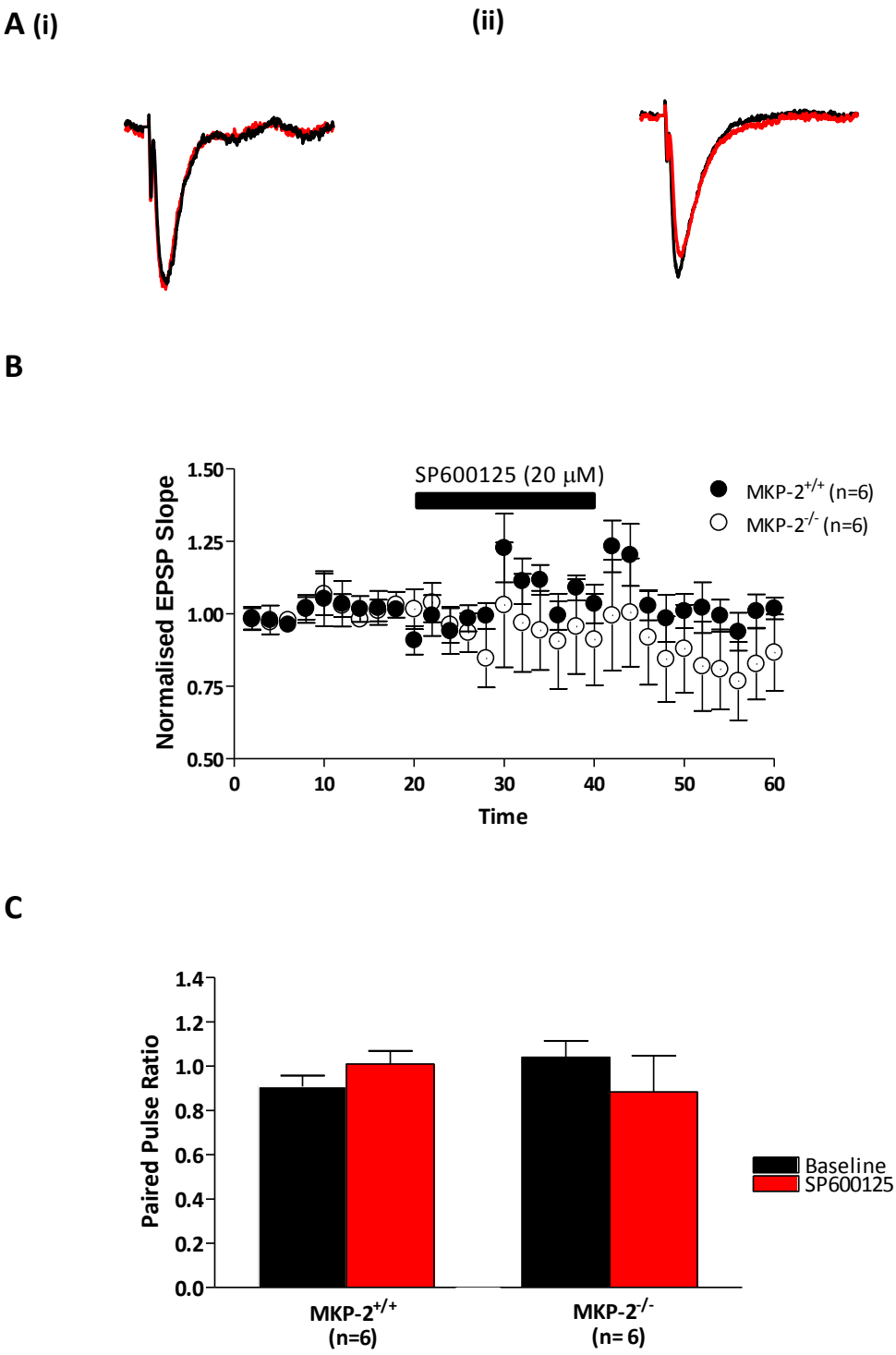


Figure 5.5: JNK inhibition does not alter the synaptic transmission or PPF ratio

(A) Representative traces illustrating the effect of JNK inhibition by SP600125 (20 µM) in (i) MKP-$2^{+/+}$ slices and (ii) MKP-$2^{-/-}$ slices B) Application of SP600125 on MKP-$2^{+/+}$and MKP-$2^{-/-}$ slices did not affect synaptic transmission. (C) The summary shows that SP600125 application does not affect PPF ratio.

5.2.4 Hippocampal ERK activity is reduced in MKP-2$^{-/-}$ mice in a gender specific manner

To determine the effect of MKP-2 deletion on the MAPK signalling cascade, the level of ERK activity in brain (cortex, hippocampus and cerebellum), heart and liver tissue was investigated using western blotting. When ERK activity in MKP-2$^{-/-}$ mice from both genders combined was examined, its activity was not significantly altered in both the cortex, (MKP-2$^{-/-}$: 44.5 ± 8.7 %, n=10 vs MKP-2$^{+/+}$: 51.3 ± 9.9 %, n = 11, P > 0.05, Figure 5.1AB) and the cerebellum (MKP-2$^{+/+}$: 60.2 ± 8.91%, n=11 vs MKP-2$^{-/-}$: 54.2 ± 8.8 %, n=10, Figure 5.1AB) as well as in hippocampus (MKP-2$^{+/+}$: 60.44 ± 8.298 %, n=11 vs MKP-2$^{-/-}$: 40.13 ± 9.2 %, n=11, P > 0.05, Figure 5.1AB) when compared to MKP-2$^{+/+}$ but there is a general trend for its activity to be reduced. Similarly, there is non-significant reduction of ERK activity in the in the heart (MKP-2$^{+/+}$: 49.33 ± 9.2 %, n=10 vs MKP-2$^{-/-}$: 43.02 ± 9.6 %, n=9, P > 0.05, Figure 5.1AB) or the liver (MKP-2$^{+/+}$: 46.39 ± 9.3 %, n =10 vs MKP-2$^{-/-}$: 42.13 ± 10.41 %, n=9, P > 0.05, Figure 5.1AB) when compared to the brain regions. Total ERK protein in the protein samples did not significantly differ between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ (Figure 5.1A) indicating that the amount of activated protein, but not total protein, was reduced. Despite a non-significant reduction when tissue from both genders was combined tested, closer analysis of the data when broken down into genders revealed a possibility that the modulation of ERK activity in MKP-2$^{-/-}$ mice may be gender specific. In a breakdown of data according to gender, there is a significant difference in ERK activation in the female hippocampus, as in MKP-2$^{+/+}$ tissue the activation was 52.62 ± 11.29 %, (n = 6) compared to MKP-2$^{-/-}$ was 23.73 ±

8.5% ($n = 5$, $P < 0.05$, Figure 5.2 A). In contrast, there is no significant difference of ERK activation in male hippocampus and in other tissues observed.

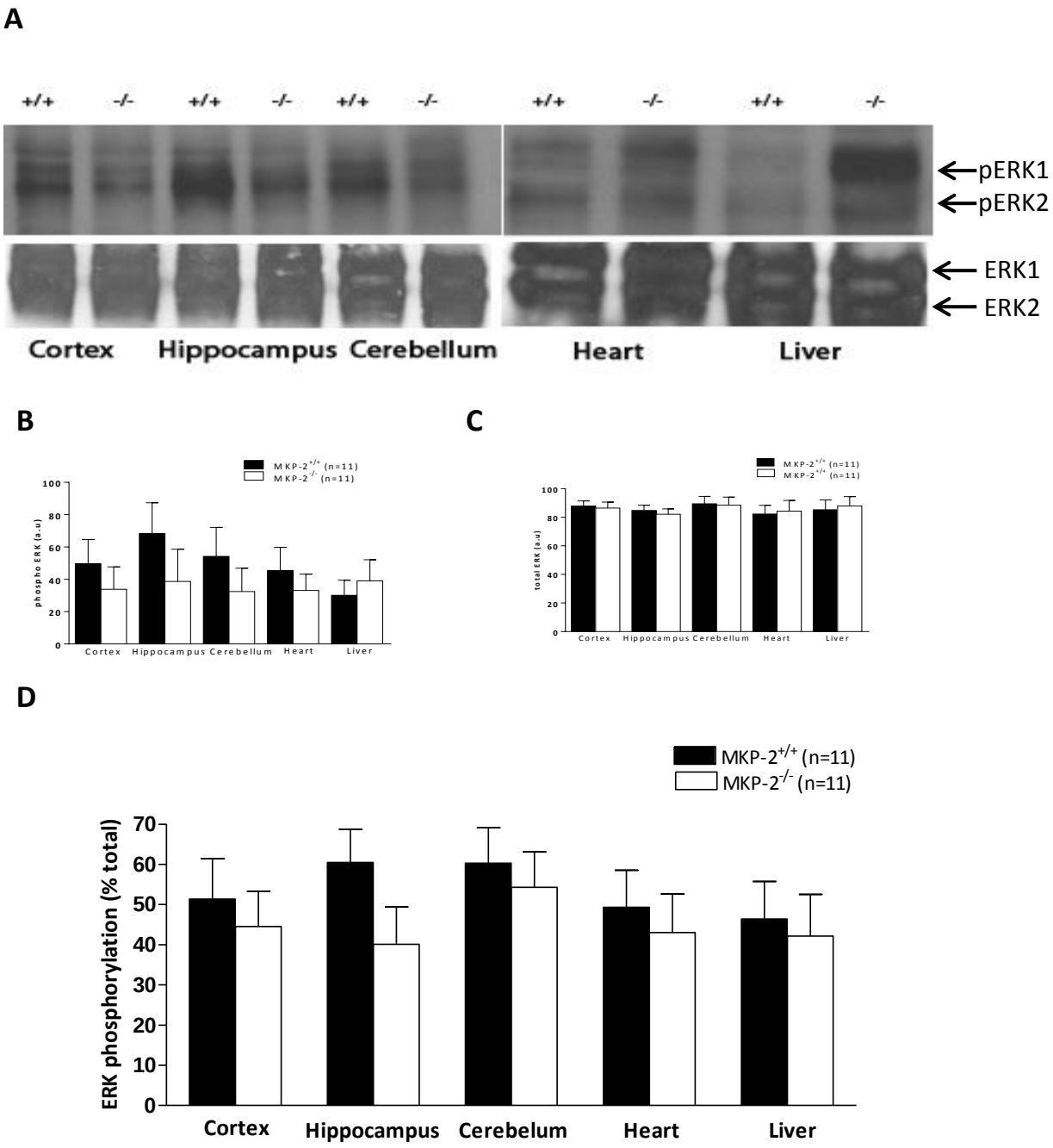


Figure 5.6: No alteration of ERK activity consequence of MKP-2 deletion

A) Representative blot shows no differences in pERK and tERK in the MKP-$2^{-/-}$ mouse brain, heart and liver tissues. B) Summary illustrating the percentage of ERK activation as a percentage of total ERK in the cortex, hippocampus, cerebellum, heart and liver. Data are mean ± S.E.M with n being number of mice used.

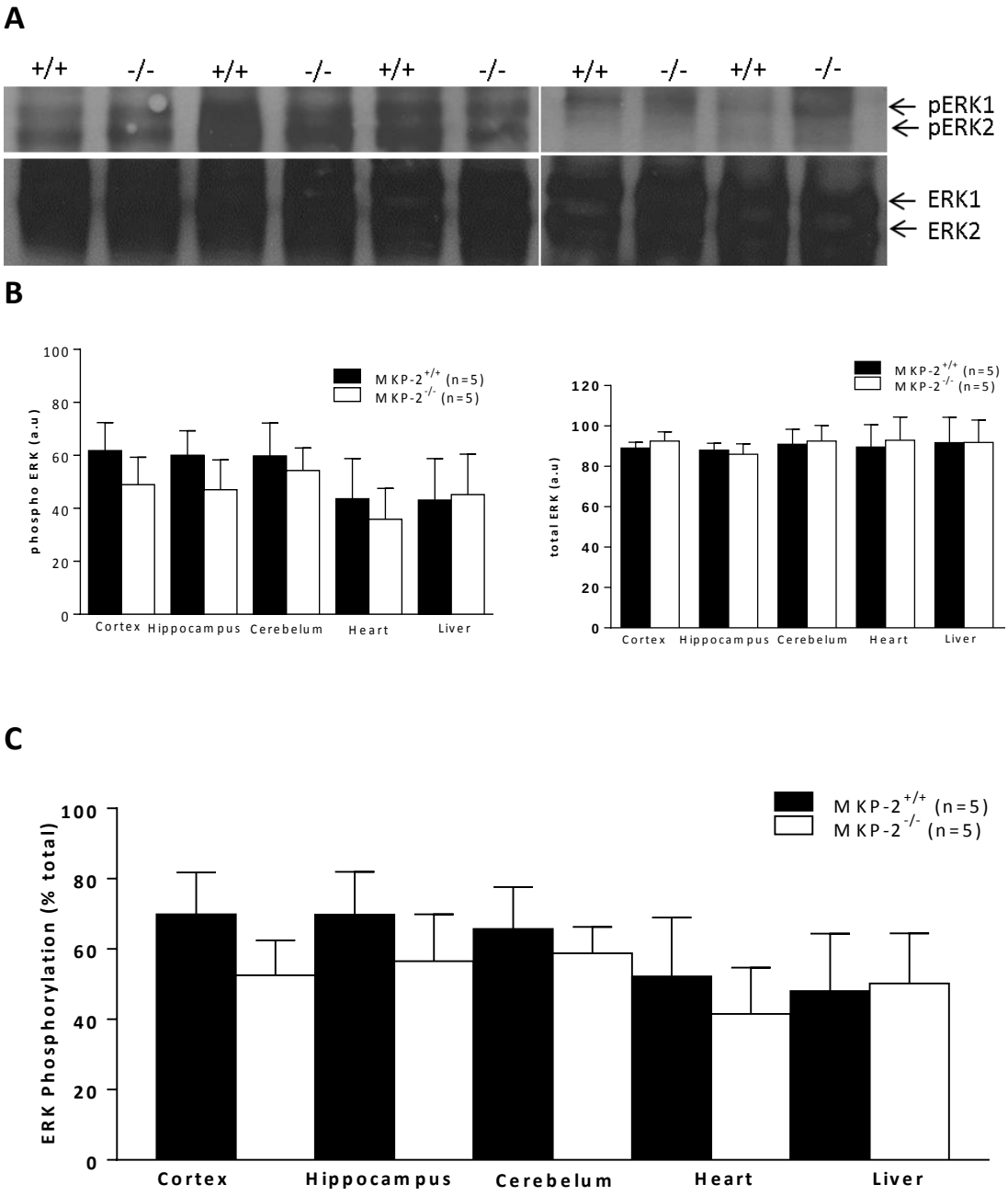


Figure 5.7: No differences in ERK activity consequence of MKP-2 deletion in tissues taken from male mice

Representative blot and summary graphs show no differences in (B) pERK (C) tERK and (D) ERK activation in MKP-$2^{-/-}$ when compared with MKP-$2^{+/+}$

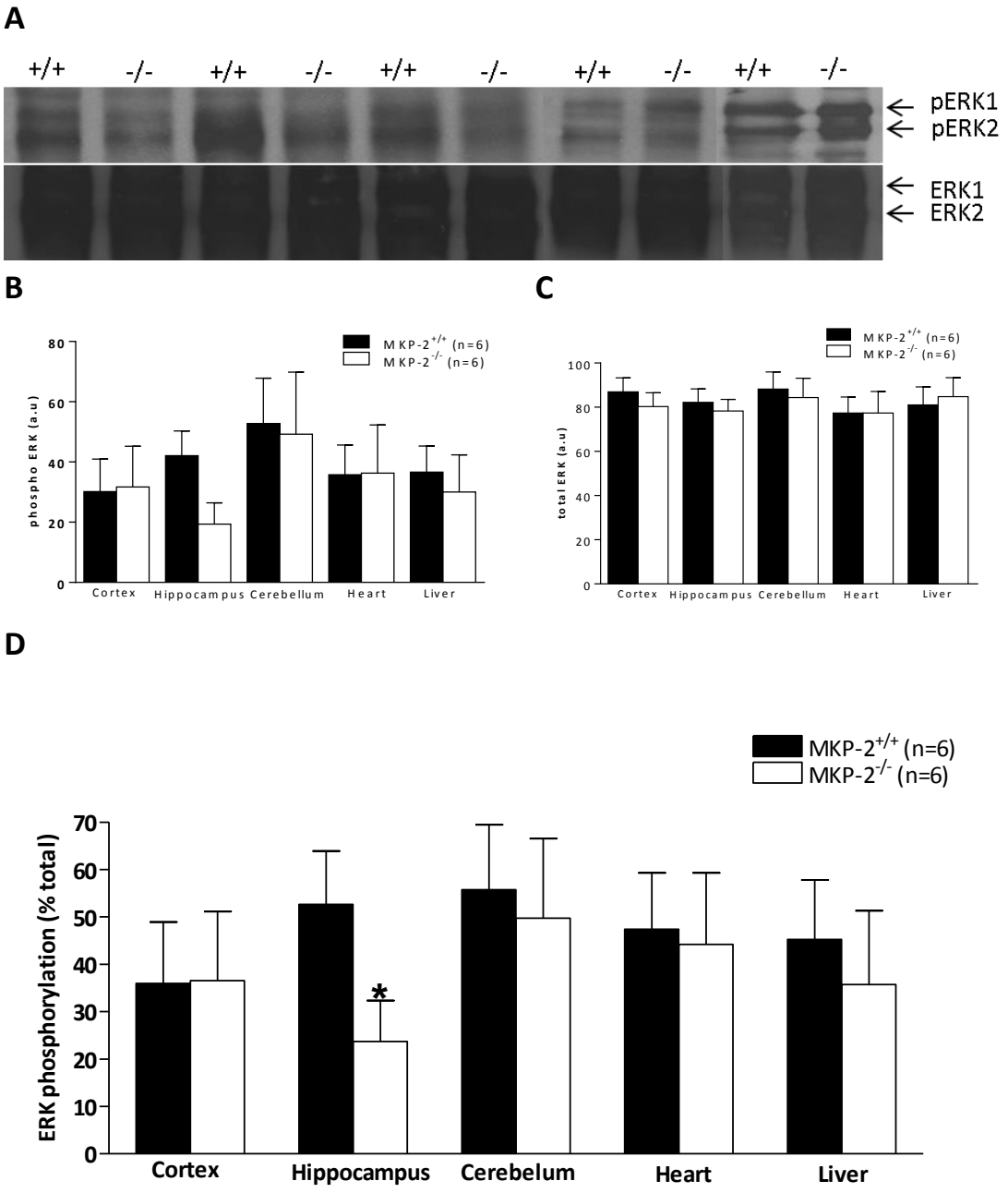


Figure 5.8: ERK activity is reduced hippocampal tissue taken from female MKP-2$^{-/-}$ mice A) Bar chart summary showing there is no significant difference between ERK phosphorylation in male MKP-2$^{+/+}$ and MKP-2$^{-/-}$ B) Summary illustrating significant differences in the percentage of ERK activation in female hippocampus tissue. Data are mean ± S.E.M analysed with unpaired t-test with n being number of mice used. (*P < 0.05).

5.3 Discussion

In this chapter, I demonstrated, for the first time that MKP-2 deletion increased basal synaptic transmission at higher intensities in MKP-$2^{-/-}$ slices when compared to MKP-$2^{+/+}$ implies that the deletion of MKP-2 regulates synaptic transmitter release. Previous study has shown that post tetanic potentiation at synapse depends on MAPK/ERK activation (Giachello et al., 2010). However, as no differences in ERK activation between genotype in this study suggested that there probably other mechanisms that involved underlying this. In the last few years there has been increasing evidence that shown astrocyte plays an important role in basal synaptic transmission. It would be very interesting to investigate the astrocyte production or expression in MKP-$2^{-/-}$ by using immunohistrochemistry method. In this study, short-term plasticity is preserved as measured by PPF. This suggests that even the deletion of MKP-2 enhances neurotransmitter release at higher stimulation intensities, but as there is no effect on PPF, it indicates that the effect does not affect residual calcium levels (Zucker and Regehr, 2002) which are responsible for this phenomenon. Previous study has shown that inhibition of ERK reduced the expression of post-tetanic potentiation, as type of short term homosynaptic plasticity (Giachello et al., 2010). In relation to LTP previous study has shown that ERK plays a role in early phase of LTP elicited by 5 Hz stimulation but not 100 Hz stimulation (Winder et al., 1999). Furthermore, the involvement of MAPK in late phase LTP has been widely accepted through the production of protein synthesis via CREB pathway (English & Sweatt, 1997; Impey et al., 1999). The function of MKP-2 in synaptic plasticity is really worth to investigate, however due to time constraint in this study, this part was not able to accomplish.

Application of SB203580 resulted in an increase in synaptic transmission both in MKP-$2^{+/+}$ and MKP-$2^{-/-}$ slices. However, there were no significant difference between basal synaptic transmission between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ upon SB203580 application. This result suggests that p38 MAPK plays a role in inhibiting synaptic transmission but that this role is not altered by the deletion of MKP-2. This result is consistent with previous study that shown application of SB203580 leads to small increase in basal synaptic transmission (Coogan, et al., 1999) and suggesting that p38 MAPK expression is high in basal state condition (Coogan et al., 1999). Thus, it is reasonable to speculate in this study that there is possibility that no alteration in p38 MAPK expression consequence of MKP-2 deletion. However, due to time constraint this was not possible to be investigated and it would be interesting to investigate the activation of p38 MAPK in consequence MKP-2 deletion by using western blot technique.

The role of JNK in CNS is highly investigated for example a previous study has shown that JNK$3^{-/-}$ mice exhibited a reduction in excitotoxicity-induced apoptosis of hippocampal neurones (Yang et al., 1997). It also has been a target for preventing neurodegenerative diseases (Recipi and Borsello, 2006; Bendotti et al., 2006; Resnick and Fennel., 2004). In this study, SP600125, a selective JNK inhibitor (Bennet et al., 2001) was used to investigate the role of JNK in synaptic transmission. Application of SP600125 non-significantly increased the basal synaptic transmission in MKP-$2^{+/+}$. However, inhibition of JNK did not alter the baseline in MKP-$2^{-/-}$ slices or affect PPF when compared to MKP-$2^{+/+}$ acute hippocampal slices. My data suggest that deletion of MKP-2 does not alter JNK activity and that JNK

plays no significant role of controlling synaptic transmission. This finding is inconsistent with a previous report in which SP600125 induced an increase in synaptic transmission and shown a reduction in PPF (Costello & Herron, 2004). With regard to JNK and synaptic plasticity, previous studies have shown that JNK1 does not significantly affect LTP in the dentate gyrus or CA1 (Curran, 2003; Bennett et al., 2001). However, it has been reported that there is high basal expression of certain JNK isoforms in the hippocampus (Carletti et al., 1995; Kumagae et al., 2011). Therefore, similar with p38 MAPK, it is worth to investigate the JNK activation if there is any difference in expression of certain type of JNK consequence MKP-2 deletion.

ERK is important for the induction and regulation of synaptic protein synthesis (Gallagher et al., 2004) as well as for rapid change in AMPAR transmission that occurs during synaptic plasticity. However, in this study, the application of UO126 on both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ did not significantly alter synaptic transmission. This study suggests reduction of ERK activity in MKP-$2^{-/-}$ mice is not directly linked to basal synaptic transmission. The finding is consistent with previous studies that shown application of U0126 did not affecting basal synaptic transmission even though post tetanic potentiation was reduced (Giachello et al., 2010b). Similarly, a study investigating the role of ERK in synaptic plasticity shown ERK plays no role in basal synaptic transmission but may have a role in LTP (English and Sweatt, 1997; Toyoda et al., 2007). However it should also be noted that another study indicates that ERK does not play a significant role in early LTP (Winder et al., 1999).

In relation to MAPK pathway, contrary to expectation that the ERK would increase subsequent MKP-2 deletion, however, the data in this study show no significant difference of ERK activation in MKP-$2^{-/-}$ and MKP-$2^{+/+}$, except in the female hippocampus. As mentioned in chapter 3, the result is in contrast with previous study on mouse embryonic fibroblast that showed an increase in ERK induction (Lawan et al., 2011). On initial interpretation, it would be very tempting to speculate that in this particular experiment, whole tissue were used, despite quantitative analysis is a bit difficult, it shows a global physiological effect compared than samples that derived from primary culture. However, as the result is consistent with the result in primary hippocampal culture (section 3.2.8), it is reasonable to speculate and further confirm that the activation of MKP-2 is cell type specific (Al-Mutairi et al., 2010). Moreover, previous studies has shown that JNK pathway also has an antagonistic effect on ERK activity (Dong & Bode, 2003; Friedman & Perrimon, 2006) as well as activation of p38 MAPK signalling causes inactivation of ERK pathway (Junttila et al., 2008). Based on these previous findings, the result in this study suggests that apart from MKP-2, there might be crosstalk between pathways that compensate for the MKP-2 activity. To confirm this, it would be very interesting and important to investigate the activation level of JNK and p38 MAPK using western blot analysis.

In summary, in this chapter I showed for the first time that MKP-2 deletion increase basal synaptic transmission at high stimulation but no differences was evident in short-term plasticity. Similarly, no significant differences was observed in basal synaptic transmission and PPF when specific MAPK cascade (ERK, JNK and p38 MAPK) was inhibited. Furthermore, there was no differences in ERK activation

when compared between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ even though there was a significant reduction was observed in ERK activation in female hippocampus.

6 GENERAL DISCUSSIONS

The main purpose of this thesis was to investigate the consequence of MKP-2 deletion on astrocytic and neuronal development as well as the functional consequences in primary hippocampal cultures and acute hippocampal slices. The central hypothesis of this thesis was that MKP-2, as a negative regulator of the MAP kinase pathway modulates functional activity within the CNS. It was hoped that this investigation would provide, for the first time, information on the role of MKP-2 function in the CNS as well as giving experimental evidence as to the potential of MKP-2 as therapeutic target for neurodegenerative diseases. Thus, in this chapter, I am going to summarize the major findings in this study, followed by the general discussion. Finally, I am going to make suggestions for future studies based on my data.

6.1 Major findings

The major findings in this thesis are listed below:

Firstly, from immunocytochemistry of primary hippocampal culture experiments.

1. Astrocyte number was affected in the first three days but no difference was observed at 5-7 DIV.
2. Neurite length was reduced in the first 3 days with no differences at 4-7 DIV.
3. Conditioned media was able to reverse the astrocyte number in MKP-$2^{-/-}$ culture and enhance the growth of astrocyte and neurite in MKP-$2^{+/+}$ culture.

Secondly, from functional experiments in primary hippocampal culture.

1. Na^+ and K^+ currents were reduced in MKP-$2^{-/-}$ culture at 7 DIV.
2. Neuronal [Ca^{2+}]i was increased in MKP-$2^{-/-}$ culture at 11 DIV.
3. Astrocytic [Ca^{2+}]i was increased in MKP-$2^{-/-}$ at 7 DIV.
4. sEPSCs was increased in MKP-$2^{-/-}$ culture at 7 and 11 DIV.
5. Synapse number was increased at 7 and 11 DIV.
6. No changes in ERK activation detected.

Finally, from acute hippocampal slice experiments

1. High stimulation increased basal synaptic transmission at the Schaffer collateral-to-CA1 synapse.
2. No differences in paired-pulse facilitation (PPF) between MKP-$2^{+/+}$ and MKP-$2^{-/-}$
3. UO126 has no effect on basal synaptic transmission and PPF.
4. SP125600 has no effect on basal synaptic transmission and PPF.
5. SB203580 increased basal synaptic transmission but no significant different between MKP-$2^{+/+}$ and MKP-$2^{-/-}$.
6. Reduction in hippocampal ERK activation in female MKP-$2^{-/-}$ but no general differences in between MKP-$2^{+/+}$ and MKP-$2^{-/-}$.

6.2 MKP-2 modulates astrocyte proliferation and neurite development

As MKP-2 has been shown to be involved in the cell proliferation (Lawan et al., 2011) and astrocytes is the only proliferative cell in the CNS, I investigated the role of MKP-2 in astrocyte proliferation and neurite growth. Here I showed that MKP-2 deletion reduced the astrocyte number in young cultures (1-3DIV). This result suggests MKP-2 plays a significant role in the astrocyte proliferation. This is, to the best of my knowledge, a novel finding as no other study shows the role of MKP-2 in astrocyte proliferation. This result is consistent with Lawan et al (2011) that show MKP-2 plays an important role in cell cycle progression as MKP-2 deletion reduced cell proliferation in embryonic fibroblast cell and hyphothesized that this was due to the cell being stuck in the G2/M phase. However, in my study, I did not further investigate the cellular mechanisms by which astrocytic proliferation was reduced and focussed on neuronal function. Neurite length was also reduced at 1-3 DIV in MKP-$2^{-/-}$ but starting from 4-7 DIV, neurite length was no different to MKP-$2^{+/+}$. A possible explanation for this result is as astrocyte proliferation is reduced in the early stages, there is not enough trophic factors or support proteins required for normal neurite growth. Having established that astrocyte proliferation and neurite length was reduced in the early development, I investigated further whether this is due to the reduction in astrocyte number or due to an impairment of astrocyte function and thus the production of support/growth factors. The results shown that the conditions is reversible when MKP-$2^{-/-}$ primary hippocampal culture was grown in MKP-$2^{+/+}$ conditioned media as well as growing of MKP-$2^{+/+}$ primary hippocampal culture in MKP-$2^{+/+}$ conditioned media lead to increased neurite outgrowth and astrocyte

proliferation at 1-4 DIV suggesting that MKP-$2^{+/+}$ provide significant amount of trophic factors and further support the growth of neurite length. In addition, astrocyte proliferation was reduced but no changes in neurite length in MKP-$2^{+/+}$ primary culture supplemented with AraC suggesting that the reduction of neurite length was on account of impairment in astrocyte function and not due to reduction of astrocyte number. Therefore, taken all the results together it is reasonable to make a general suggestion that MKP-2 plays a role in astrocyte proliferation and function and impairment in astrocyte function subsequently alter neurite length. Even though the fact that neurite length development is supported by trophic factor produced by astrocyte is well-established, however in a recent study, NCAD loss from astrocyte has been shown led to decerases to neurite growth and do not increases other cadherins (Ferguson and Scherer, 2012). This finding suggests that there are specific types of trophic factor who's role are not able to be compensated for by other factors. It is interesting to investigate whether trophic factor release is altered in MKP-$2^{-/-}$ astrocytes. This could be invetigate by using microarray that detecting different typr of trophic factor gene. Apart from producing trophic factors that support the growth of neurites, astrocytes also function to synthesis neurotransmitter and for maintenance of glutamate level (Hertz, 2006) that could potentially involved in increasing neurotransmitter release.

6.3 MKP-2 deletion modulates neuronal and astrocyte function in primary hippocampal cultures

Having established that MKP-2 plays a role in astrocyte and neurite development, I further investigated the role of MKP-2 in neuronal function in primary hippocampal culture in three different time point as at 3, 7 and 11 DIV as described at chapter 4.

MKP-2 deletion led to several functional changes including reduced astrocytic $[Ca^{2+}]i$ at 7 DIV and increased neuronal $[Ca^{2+}]i$ at 11 DIV. Moreover, the frequency of sEPSCs was increased at 7 and 11 DIV, but not the amplitude suggesting that presynaptic changes in neurotransmitter release. Similar findings from our lab in a study using acute hippocampal slices from 3 week old mice have previously demonstrated an increase in the frequency of sEPSC in the hippocampal CA1 region (Dr. Sam Greenwood, unpublished data), which show that the effects of MKP-2 deletion on sEPSC frequency is maintained through development. As in this study, at 4 DIV, astrocytes number in MKP-2 started to increase probably due to trophic factor there was also probability that the trophic factor produced by astrocyte plays a role in formation of excitatory synapses. This finding is supported by a previous study that shown significant increase of excitatory synapse in hippocampal primary culture applied with transforming growth factor β1 (Diniz et al., 2012). Similarly, postsynaptic silent excitatory synapse was increase when hevin was added to astrocyte free cultures (Kucukdereli et al., 2011). Another possible explanation in relation with trophic factor is there is probability of increasing in trophic factor produced in MKP-$2^{-/-}$ older cultures that subsequently promote spontaneous release. This is supported by a recent study shows that application of BDNF on primary hippocampal culture enhances spontaneous and activity-dependent neurotransmitter release (Shinoda et al., 2014). It would be worth to investigate the relationship of trophic factor produced by MKP-2 and astrocyte proliferation. Furthermore, a previous study shown ERK phosphorylated synapsin I increased the size of docked vesicles at presynaptic area (Kushner et al., 2005) suggesting that ERK plays a role in neurotransmitter release in presynaptic area. As the ERK activation investigated in

this study is in soma area and not in presynaptic area, this requires further investigation.

Most of the functional results seems to be related with modification of MAPK pathway, particularly ERK. However, in this study, no significant difference was seen in ERK activation at 3, 7 and 11 DIV. Even though there was significant increase of pERK at 3 and 7 DIV, it was probably because tERK was increasing significantly. This results is consistent with previous study that shown ERK activation was not altered (Al-Mutairi et al., 2010). It has been suggsted that ERK was not able to access to phosphatase because the inability of ERK to translocate to nucleus (Al-Mutairi et al., 2010). It is important to note that the level of ERK activation quantified in this study is not differentiated between neuron and astrocyte and it was taken on the soma. It would be interesting to investigate the activation of localized ERK in presynaptic and postsynaptic terminals. This could be done by conducting an experiment by using immunogold labelling with immune-electron microscopy

6.4 MKP-2 modulates synaptic transmission in acute hippocampal slices

Having established MKP-2 regulates the synaptic transmission and CNS functions in primary hippocampal culture, I next investigate the consequence or the translation of this condition in acute hippocampal slices preparation. In the acute hippocampal slice, I demonstrate for the first time that MKP-2 plays a role in synaptic transmission. Basal synaptic transmission was increased at Schaffer-Collateral-CA1 region at high stimulation (50x and 100x) in MKP-$2^{-/-}$. This MKP-2 regulation of synaptic transmission seen in acute slices is consistent with my data in chapter 4 as it

has shown MKP-2 deletion affecting neurotransmitter release in primary hippocampal culture. Even though there was a difference in the experimental preparation tested, the significant was seen at high stimulation and in hippocampal culture spontaneous release was investigated, however, the similarity that can be interpreted from this result as it MKP-2 may regulates neurotransmitter release. In initial interpretation, as ERK role in synaptic transmission is widely established (J D Sweatt, 2001; Sweatt, 2004; Thomas and Huganir, 2004), it was tempted to speculate that consequence of MKP-2 deletion, increasing ERK phosphorylation and eventually affecting synaptic transmission. However, as there was no difference in ERK activation in my study, the mechanism underlying this is still unknown. Furthermore, a recent publication also demonstrated that astrocytes plays a role in modulating basal synaptic transmission (Bonansco et al., 2011; Panatier et al., 2011). Considering there was alteration in astrocyte proliferation in primary hippocampal culture, it would be interesting to investigate the astrocyte proliferation in acute slices as well as to record its gliotransmission activity. In this study, short-term plasticity did not change as indicated by no difference in paired-pulse facilitation (PPF) measured. This result is consistent with the finding demonstrated in our lab that shown PPF was not changes in a patch-clamp experiment in acute hippocampal slices (Dr. Sam Greenwood, unpublished data). Even though there was modification in presynaptic activity in MKP-2 primary hippocampal culture, however, in acute hippocampal slices, as no changes in the PPF indicates that there was no differences in probability release (Zucker and Regehr, 2002) and no modification in residual calcium (Fioravante and Regehr, 2011). Moreover, the involvement of MAPK cascade in synaptic transmission and plasticity is well-established. The activation of

ERK is important in induction of LTP (Impey et al., 1999; Winder et al., 1999; Sweatt, 2004; Welsby et al., 2009) meanwhile JNK has been shown to contribute to impairment of LTP (Costello & Herron, 2004) and inducing LTD (Li et al., 2007) and p38 MAPK activation has been shown to be involved in the induction of LTD (Bolshakov et al., 2000). In this study, inhibition of ERK and JNK do not change basal synaptic transmission as well as PPF. However, application of p38 MAPK inhibitor, increased the basal synaptic transmission but no difference was observed between MKP-$2^{+/+}$ and MKP-$2^{-/-}$. As there was a lot of studies shown the significance of MAPK cascade in synaptic plasticity, it is also very tempting to know the role of MKP-2 in synaptic plasticity. However, time limitation did not allow this investigation. Some of the results in this study interpreted assuming there was alteration in ERK activation. However, contrary to expectation, taking into consideration of MKP-2 has been knock out and ERK is its preferential substrates (Chen et al., 2001), there is no difference in ERK activation when compared between brain tissue (cortex, hippocampal, and cerebellum) with other organ tissue (heart and liver). This result is consistent with my data on ERK activation in primary hippocampal culture. Previous studies has shown the possibility of cross-link in signalling pathway that induces compensatory effect (Repici et al., 2009). It is also interesting to know the activation of p38 MAPK and JNK consequence of MKP-2 deletion.

Taken together, the data from my study suggesting that MKP-2 modulate astrocyte proliferation and subsequently affecting neurite growth and a few neuronal functions such as Na^+ and K^+ channel development, $[Ca^{2+}]i$ as well as neurotransmitter release.

These findings clearly provide a significant insight into the function of MKP-2 in the CNS.

6.5 Future studies

The results detailed in this thesis have opened up the possibilities of investigation of MKP-2 in CNS development and function. Based on the studies and pioneering investigations discussed in this thesis, there are other interesting questions that needed to be answered and several further experiments are obviously needed. The following are some of the unresolved questions that may be worth investigating:

1. Astrocyte proliferation was reduced at 1-3 DIV but was increased significantly at 4 DIV. How MKP-2 modulates astrocyte proliferation. This could be investigated by quantify DNA incorporation of radioactive nucleotide [3H] thymidine into DNA or by using flow cytometry analysis of cell cycle progression at different phase of cell.
2. Is growth/neurotrophic factor production reduced in the MKP-$2^{-/-}$ astrocytes? Investigate this in primary cultures using microarrays to detect different neurotrophin levels.
3. Does MKP-2 modulate synaptic plasticity? Extracellular or whole cell electrophysiology could be used to examine LTP and LTD at CA1 region of hippocampal slices.
4. Is JNK and p38 MAPK activity altered as a consequence of MKP-2 deletion? Immunocytochemistry in primary hippocampal culture and western blotting using brain tissue could be used to examine the level of expression of p38 MAPK and JNK.

5. As sEPSC and synapse number was increase at 7 and 11 DIV in this study, it is interesting to investigate is MKP-2 involved in quantal vesicular release? Whole-cell patch clamp electrophysiology supplemented culture with TTX could be used to investigate miniature EPSC.
6. How MKP-2 regulates the calcium channel development? Investigate this using patch clamp electrophysiology by examining neuronal Ca^{2+} channel function.
7. Does MKP-2 regulate synaptic ERK activation differently from that in the soma? Analyzing spatio-distribution of ERK activation by using immunogold labelling with immune-electron microscopy.
8. How MKP-2 regulates the astrocyte and neural circuit in CNS? Immunohistochemistry of brain slices would be able to use to investigate the differences of astrocyte development in primary culture and in brain tissue.

PUBLICATIONS

Paper in preparation

Nor Zaihana Abdul Rahman, Sam Greenwood, Robin Plevin, Trevor J. Bushell (2015). MKP-2 deletion reduces hippocampal astrocyte proliferation but increases spontaneous EPSCs frequency. *The Journal of Neuroscience*.

Submitted

Neil Dawson, Ros Brett, Sam Greenwood, Nor Zaihana Abdul Rahman, Robin Plevin, Trevor J. Bushell. Mitogen-activated protein kinase phosphatase-2 modulates reward processing and hippocampal-dependent memory processes via impaired brain connectivity and synaptic plasticity. *The Journal of Neuroscience.*

Abstracts for poster presentations

Abdul Rahman, N. Z., Plevin R., and Bushell, T.J. (2012). Determining the consequence of MAP kinase phosphatase 2 (MKP-2) deletion on CNS function.*9th Scottish Neuroscience Meeting* (pp.65). Dundee, Scotland. University of Dundee

Abdul Rahman, N. Z., Plevin R., and Bushell, T.J. (2013). Determining the role of MKP-2 in the CNS. Paper presented at the *Glasgow Neuroscience Day*. Glasgow, Scotland. University of Glasgow.

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