An Investigation into the Role of Proteinase-activated Receptor 2 on Neuronal Excitability and Synaptic Transmission in the Hippocampus

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Abstract

Proteinase-activated receptor 2 (PAR-2) belongs to a novel family of G-protein coupled receptors that are unique in their activation mechanism by which a proteolytic cleavage at N-terminus by a proteinase reveals a 'tethered ligand' to activate the receptor. Albeit at a low level, PAR-2 is extensively expressed in normal and pathological brains, including the hippocampus. Qualitative studies into the expression of PAR-2 in several disease conditions, including ischaemia, HIV-associated dementia, Parkinson's disease, Alzheimer's disease, as well as multiple sclerosis, have suggested that PAR-2 plays either degenerative or protective role depending on in which cell type an increase in PAR-2 expression is observed. However, its potential roles in modulating neuronal excitability, synaptic transmission as well as network activities remain to be determined.

Utilising the whole-cell patch clamp recording technique, I demonstrate, for the first time, that the activation of PAR-2 leads to a depolarisation of cultured hippocampal neurones following application of SLIGRL (100μM), a selective PAR-2 activating peptide (5.52 ± 1.48mV, n=16, $P<0.05$) and paradoxically a reduction of spontaneous action potential (AP) frequency (29.63 ± 5.03% of control, n=13, $P<0.05$). Pharmacological manipulation reveals that the PAR-2-mediated depolarisation is most likely dependent on astrocytic glutamate release, which takes effect on ionotropic glutamate receptors. In addition, an overt depression of synaptic transmission among the cultured neurones upon PAR-2 activation is more likely to cause the reduction of spontaneous APs.

In further experiments, I show, for the first time, that the activation of PAR-2 induces a long term depression (LTD) of glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices following SLIGRL (100μM) application (80.75 ± 2.54% of control at 30 minute, n=12, $P<0.05$). Additionally, this novel form of LTD is independent of metabotropic glutamate receptors but mediated by NR2B subunit-containing N-methyl-D-aspartic acid (NMDA) receptors. It is also suggested from these experiments that glial-neuronal signalling is contributing to this novel form of LTD.

In the final set of experiments, by monitoring field potentials in the *stratum pyramidale* of the CA3 area in acute hippocampal slices, I demonstrate that PAR-

2 activation depresses the frequency of epileptiform activities induced by the application of 4-AP/0 Mg^{2+}, an *in vitro* model of epilepsy (1.53 ± 0.21Hz to 1.18 ± 0.17Hz, n=13, P<0.05, 100μM SLIGRL). In contrast, PAR-2 activation has no effect on the frequency of epileptiform activities induced by bicuculline (0.14 ± 0.03Hz to 0.13 ± 0.03Hz, n=6, P>0.05, 100μM SLIGRL).

In summary, in this thesis, I demonstrate that PAR-2 modulates neuronal excitability and depresses excitatory synaptic transmission in the hippocampus. These data indicate that PAR-2 may play a regulatory role in neuronal signalling at single cell level by controlling neuronal intrinsic properties, as well as at synaptic level by tuning excitatory synaptic strength, which ultimately affects global excitability in the neural circuits as a whole. Therefore, this investigation suggests a novel physiological/pathophysiological role for PAR-2 in the brain. These data may reveal valuable clues for the development of drugs targeting a novel and potentially promising candidate.

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Acknowledgement

When I type the last letter into my thesis, it has jumped into the fifth year since I first landed onto the British Isles through Glasgow airport in a windy and raining evening. In contrast to this typical Glaswegian weather, my stay in Glasgow, Scotland, as well as the UK is always fulfilled by joy, surprise, passion and enlightenment. This is the land where I spend the longest time overseas. And eventually, when I have to leave, I have already found myself firmly clung to its gorgeous natural landscape, enthusiastic people, and more importantly elegant intellectual aura. When the time arrives, I can't help recalling a gentle poem written by Zhimo Xu, a Chinese student studying philosophy and economics at Cambridge 90 years ago. The poem comes as follows:

Very quietly I take my leave 轻轻地我走了，
As quietly as I came here; 正如我轻轻的来；
Quietly I wave good-bye 我轻轻的招手，
To the rosy clouds in the western sky. 作别西天的云彩。

The golden willows by the riverside 那河畔上的金柳，
Are young brides in the setting sun; 是夕阳中的新娘；
Their reflections on the shimmering waves 波光里的艳影，
Always linger in the depth of my heart. 在我的心头荡漾。

The floating heart growing in the sludge 软泥上的青荇，
Sways leisurely under the water; 油油的在水底招摇；
In the gentle waves of Cambridge 在康河的柔波里，
I would be a water plant! 我甘心做一条水草。

That pool under the shade of elm trees 那树荫下的一潭，
Holds not water but the rainbow from the sky; 不是清泉，是天上虹；
Shattered to pieces among the duckweeds 揉碎在浮藻间，
Is the sediment of a rainbow-like dream? 沉淀着彩虹似的梦。

To seek a dream? Just to pole a boat upstream 寻梦？撑一枝长篙，
To where the green grass is more verdant; 向青草更青处漫溯，

Or to have the boat fully loaded with starlight 满载一船星辉，

And sing aloud in the splendor of starlight. 在星辉斑斓里放歌。

But I cannot sing aloud 但我不能放歌，

Quietness is my farewell music; 悄悄是别离的笙箫；

Even summer insects heap silence for me 夏虫也为我沉默，

Silent is Cambridge tonight! 沉默是今晚的康桥！

Very quietly I take my leave 悄悄的我走了，

As quietly as I came here; 正如我悄悄的来；

Gently I flick my sleeves 我挥一挥衣袖，

Not even a wisp of cloud will I bring away. 不带走一片云彩。

Firstly, I would like to thank my two supervisors for guiding me through my PhD study, Dr. Stuart Cobb at University of Glasgow and Dr. Trevor Bushell at University of Strathclyde. Without their joint effort, this fascinating and challenging project would not be feasible from vision to reality at the very beginning. I learned so much from them in planning, designing, executing, and interpreting a research project. Their passion and rigorous attitude towards scientific research motivated me deeply in my PhD. I also personally own them a great favour in their attempts to transpose many of my Chinese idioms into concise and comprehensible academic English during the preparation of this thesis. Furthermore, without their extreme patience and encouragement, the completion of this thesis would be impossible.

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I also like to thank my Chinese colleague in the Glasgow lab, Dr. Shih-ming Weng (Jonas) from Taiwan. Not only is it a great feeling sometimes in a foreign country to communicate in our mother tongue, which makes the life much easier, but also with your help and advices I go through quite a few pitfalls in the past few years.

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Finally, I would like to thank all the people who love or loved me. I will give all my heart to my parents and grandparents in China. Without your physical as well as mental support and sacrifice, my stay overseas is empty. It is your determination that makes me determined. I own tremendous debt to Frau Dr. Med. Chengjing Zhu. I was so lucky to have you on my side when my heart was torn. It is your love that makes me beloved and fulfilled. Furthermore, if possible, I would like to say to my ex-girlfriend Ms. Le Cao in China: ‘With your companion on the other side of the world, I was not alone in the UK’.

Author's declaration

I declare that the work presented in this thesis is entirely my own with all exceptions being clearly indicated or/and properly cited in the context, and all citations have been provided in the reference list.

Signature:……Jian Gan……..

This work has not been presented in part or alone for any other degree programme. Some of the work contained here has been published in part: a list follows.

Gan, J., Cobb, S.R., and Bushell, T.J. 2008 Activation of Proteinase-activated Receptor 2 Inhibits Hippocampal Synaptic Transmission ***FENS Abstr.***, vol 4, A115.7

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Abbreviations

4-AP	4-aminopyridine
7TM	seven transmembrane
ACSF	artificial cerebrospinal fluid
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	action potential
ATP	adenosine triphosphate
BAPTA	1, 2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid
BLAST	basic localalignment search tool
CA	(1 or 3) cornus ammonis (1 or 3)
CaMKII	Ca^{2+}/calmodulin-dependent protein kinases II
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COS cell	CV-1 monkey cells infected with SV40 virus
CXCR2	CXC chemokine receptor 2
DAG	diacyl glycerol
DG	dentate gyrus
DHPG	(S)-3, 5-Dihydroxyphenylglycine
EAE	experimental autoimmune encephalomyelitis
EC	entorhinal cortex
EC_{50}	half maximal effective concentration
ED_{50}	half maximal effective dose
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
ERK	extracellular-regulated kinase
ETS	expressed sequence tag
fEPSP	field excitatory postsynaptic potential
G protein	guanine nucleotide-biding protein
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors

GIRK	G-protein coupled inwardly rectifying K^+ channels.
GPCR	G-protein coupled receptors
GRK	G protein-coupled kinase
GRO/CINC-1	growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1
GTP	guanosine triphosphate
HEL cell	human erythroleukemia cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	high frequency stimulation
HIV	human immunodeficiency virus
HPLC	high-pressure liquid chromatography
Hz	hertz
IL	interleukin
I/O	input/output
IP_3	inositol 1, 4, 5-triphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
JNK	c-Jun N-terminal kinase
LFS	low frequency stimulation
LTD	long term depression
LTP	long term potentiation
mA	milliampere
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MCPG	α-methyl-4-carboxyphenylglycine
mEPSC	miniature excitatory postsynaptic current
MF	mossy fibre
mGluR	metabotropic glutamate receptor
mIPSC	miniature inhibitory postsynaptic current
ml	millilitre
mm	millimetre
mM	millimolar
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
ms	millisecond

mV	milivolt
NF	nuclear factor
nM	nanomolar
NMDA	N-methyl-D-aspartic acid
OHSC	organotypic hippocampal slice cultures
O-LM	oriens-lacunosum moleculare
pA	picoampere
PAR-1(2 or 4)	proteinase-activated receptor 1 (2 or 4)
PAR-1(2 or 4)-AP	proteinase-activated receptor 1 (2 or 4) activating peptide
PAR-2-IP	proteinase-activated receptor 2 inactive peptide
PCR	polymerase chain reaction
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
pM	picomolar
PP	perforant path
PPF	paired-pulse facilitation
PTP	post-tetanus potentiation
PTX	pertussis toxin
Raf-1	proto-oncogene serine/threonine-protein kinase 1
RC	resistance and capacitance
SEM	standard error of mean
SIC	slow inward current
SNARE	soluble NSF attachment protein receptors
SNpc	substantia nigra pars compacta
Src	sarcoma
STD	short term depression
STF	short term facilitation
STP	short term plasticity
TNF	tumor necrosis factor
tPA	tissue-type plasminogen activator
TRP	transient receptor potential
TTX	tetrodotoxin

uPA	urokinase-type plasminogen activator
μA	microampere
μg	microgram
μm	micrometre
μM	micromolar

1 Introduction

1.1 General Introduction

It is widely estimated that 550 genes in the human genome encode proteolytic enzymes, termed proteinases or proteases (Southan, 2001; Puente *et al.*, 2003). Therefore, it is not surprising that proteinases take part in a variety of biological processes from digesting dietary proteins and peptides in the gastrointestinal tract to destroying antigen and aging cells in macrophages. Interestingly, proteolytic events occur on the cell membrane as well, initiating signal transduction through proteinase activated receptors (PARs).

PARs belong to a novel G-protein coupled receptor family which is unique in its activation mechanism. A proteolytic cleavage in the N-terminus unveils a sequence to act as "tethered ligand". This "tethered ligand" then binds to the second extracellular loop of the receptor, leading to the receptor activation (Macfarlane *et al.*, 2001; Ossovskaya & Bunnett, 2004; Bushell, 2007; Luo *et al.*, 2007; Hansen *et al.*, 2008; Ramachandran & Hollenberg, 2008). Whilst PARs are widely expressed in the mammalian body with extensive studies so far focusing on the cardiovascular, gastrointestinal, and immune systems, PARs are also expressed in the central nervous system (CNS) (Noorbakhsh *et al.*, 2003; Bushell *et al.*, 2006; Luo *et al.*, 2007), yet PARs involvement in the brain function under either physiological or pathophysiological conditions is poorly characterised.

This thesis therefore makes an initial attempt to elucidate if the activation of PARs modulates fundamental properties in the neuronal function such as neuroexcitability, synaptic transmission and synaptic plasticity in the hippocampus, a well organized brain structure with readily experimental access.

1.2 G-protein coupled receptors

As presented in figure 1.1, G-protein coupled receptors (GPCRs), also described as seven-transmembrane (7TM) receptors, represent the largest receptor superfamily to transfer extracellular stimuli into intracellular cascades of biochemical reactions through the signal transduction system of a cell in the animal kingdom (Findlay *et al.*, 1993; Baldwin, 1994; Pierce *et al.*, 2002). It is estimated that there are more than 1000 GPCRs encoded by the human genome (Premont & Gainetdinov, 2007). Ligands for these receptors are relatively diverse including biogenic amines, amino acids, ions, lipids, peptides to proteins, light, odorants, pheromones, nucleotides, opiates, cannabinoids, endorphins (Premont & Gainetdinov, 2007). Despite this diversity of ligands, the characteristic activation mechanism for GPCRs is relatively uniform (Strader *et al.*, 1994; McCudden *et al.*, 2005). The binding of an appropriate ligand to the second extracellular loop, in some cases, to the N-terminus or transmembrane domains, of a GPCR initiates conformational changes in the intracellular domain of the GPCR (Wess *et al.*, 2008), resulting in the substitution of GDP by GTP from the α-subunit of the inactive heterotrimeric (αβγ) guanine nucleotide-binding protein (G protein), which in turn dissociate the G protein to G_{α}-GTP and βγ dimer. This 'activated' G protein, through the dissociated G_{α}-GTP and βγ dimer, interacts with downstream effectors in the cytoplasm.

It is widely acknowledged that GPCRs coupled to at least 16 heterotrimeric G protein subtypes (Premont & Gainetdinov, 2007; Tuteja, 2009), leading to diverse second messenger cascades in the cytoplasm. They are functionally grouped into four major classes as G_s, G_i, $G_{q/11}$ and $G_{12/13}$. G_s primarily activates adenylate cyclase, which in turn, increases the cytoplasmic concentration of cAMP, which in turn upregulates the activity of cAMP-dependent kinase or protein kinase A (PKA)(Gilman, 1995). In contrast, G_i mainly inhibits adenylate cyclase activity, which therefore reduces the production of cytoplasmic cAMP from ATP, which in turn downregulates the activity of PKA (Gilman, 1995). $G_{q/11}$ subunit stimulates phospholipase C (PLC), and PLC cleaves a membrane phospholipid, the phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Then, IP_3 primarily binds to IP_3 receptors, which are Ca^{2+} channels in the endoplasmic reticulum (ER), leading

to Ca^{2+} release from the ER to increase cytoplasmic Ca^{2+} concentration (Majerus, 1992). Furthermore, Ca^{2+} and DAG together activate protein kinase C (PKC), which phosphorylates other downstream molecules (Tanaka & Nishizuka, 1994). Finally, $G_{12/13}$ regulates cellular responses through guanine nucleotide exchange factors (GEFs) (Dhanasekaran & Dermott, 1996), which are activators of small GTPases, notably the Ras GTPases superfamily, such as Ras, Rho, and Ran, which regulate cell growth, differentiation, and vesicle transport (Wennerberg *et al.*, 2005). For a single GPCR, it may interact primarily with only one class of G proteins, whereas it can interplay with all four G protein subtypes, which leads to a broad spectrum of cellular responses (see figure 1.1). It should also be noticed that GPCRs could activate signalling pathways independent of (or parallel to) all G proteins (Premont & Gainetdinov, 2007). Notably, the ß-arrestin pathway, where GPCR-ß-arrestin directly recruits several signalling proteins, such as Src family tyrosine kinases and components of mitogen-activated protein kinases (MAPKs), has been well documented (Luttrell & Lefkowitz, 2002; Lefkowitz & Shenoy, 2005; Shenoy & Lefkowitz, 2005).

A striking feature of all GPCRs is that their responsiveness to a given agonist is plastic (Freedman & Lefkowitz, 1996; Premont & Gainetdinov, 2007). When exposed to prolonged or repeated agonist stimulation, GPCRs desensitise, whereas it resensitises when not exposed to agonist for some time (Huganir & Greengard, 1990). It has been demonstrated that the G protein-coupled receptor kinase (GRK)-arrestin system is primarily responsible for this feature (Hausdorff *et al.*, 1990; Gainetdinov *et al.*, 2004). That is the GRK recognises the activated GPCR and phosphorylates the C-terminus of the GPCR. Once phosphorylated, the GPCR is recognised by arrestin, and the binding of GPCR with arrestin prevents the GPCR to activate more G proteins and therefore terminates its signalling (Premont & Gainetdinov, 2007). The arrestin binding also facilitates GPCR internalisation and trafficking. In addition, GPCRs have been documented to behave functionally as hetero-/homo- dimers or oligomers to transduce extracellular signals into cytoplasm, which attracts intensive investigations into its mechanism and functional consequences in recent years (Bai, 2004; Prinster *et al.*, 2005; Wess *et al.*, 2008).

In summary, GPCRs are widely found on the cell membrane with common seven transmembrane domains structure. Characteristic activation mechanisms and

signalling pathways of GPCRs have been described. GPCRs are major mediators of transmembrane information flow. They are key controllers of a variety of physiological processes such as neurotransmitter release, secretion, cellular metabolism, growth, and differentiation. Therefore, bearing these features, GPCRs are celebrated targets for the majority of medicine development.

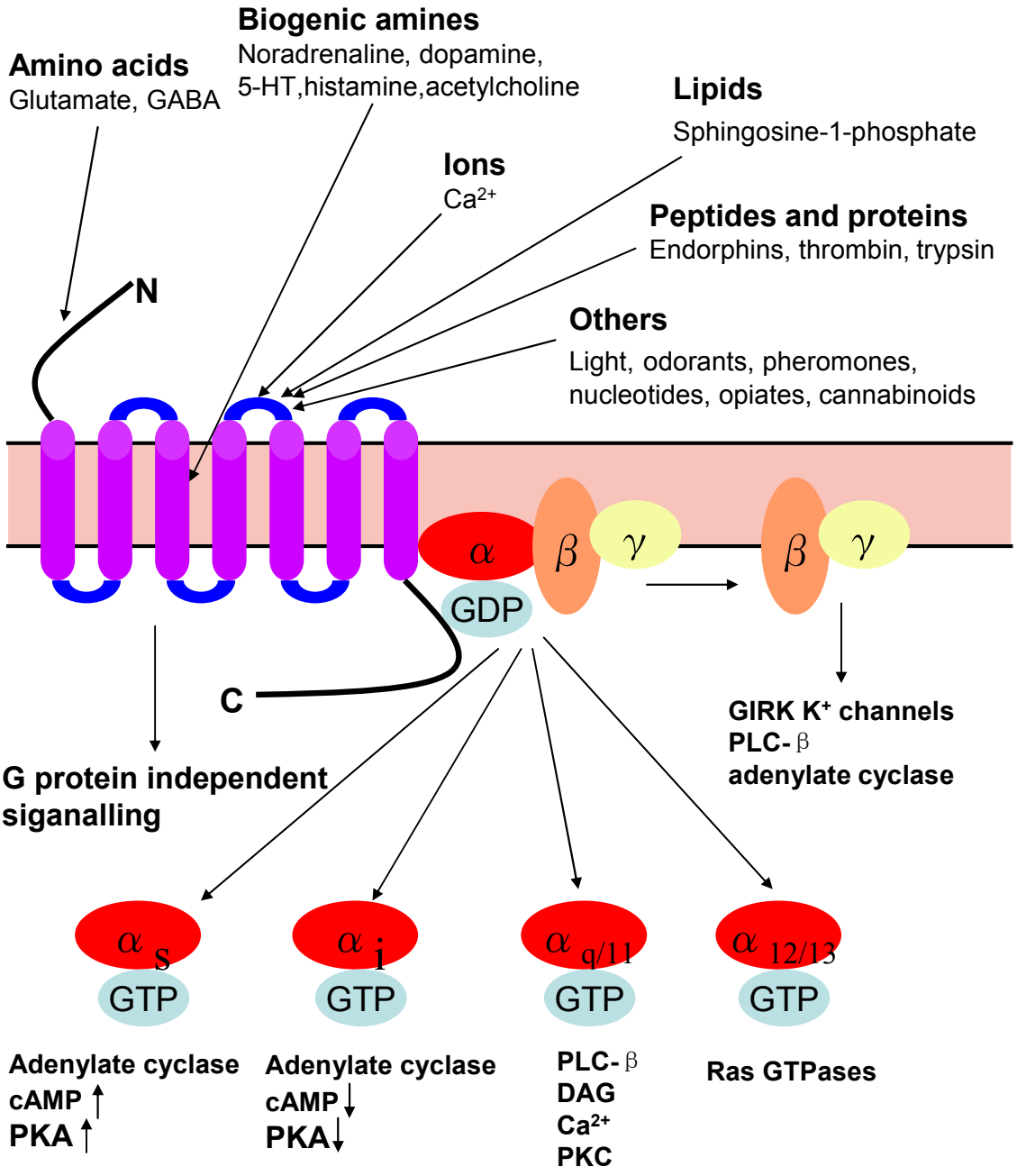


Figure 1-1 Summary of ligands, structure, activation mechanism, and signalling pathways of GPCR.

Adapted and modified from Marinissen et al.,2001 (Marinissen & Gutkind, 2001).

1.3 Proteinase activated receptors

PARs also described as protease activated receptors, are a recently characterised, novel family of GPCRs. Rather than being activated by the free ligand occupying the ligand-biding site, it is mobilised through a proteolytic cleavage in the N-terminus of the receptor by a serine proteinase, exposing a newly formed N-terminus to act as a "tethered ligand" that interacts with the receptor in the second extracellular loop. To date, four PARs have been discovered, with distinct cleavage sites as well as "tethered ligand", which give rise to differential molecular and cellular responses (Macfarlane *et al.*, 2001; Ossovskaya & Bunnett, 2004; Bushell, 2007; Luo *et al.*, 2007; Hansen *et al.*, 2008; Ramachandran & Hollenberg, 2008)

1.3.1 Cloning of PARs

The discovery of the first PAR was largely based on extensive research into the regulation of blood clot formation and platelet aggregation in the cardiovascular system by a serine proteinase, thrombin. Thrombin was the product of prothrombin by factor Xa and was regarded as a key player in the coagulation cascade linking tissue damage to wound healing (Davie *et al.*, 1991). In addition, several studies demonstrated that thrombin alone had a direct effect on different cell types other than blood cells, including endothelial cells, monocytes, lymphocytes and smooth muscle cells (Chen *et al.*, 1976; Bizios *et al.*, 1986; Daniel *et al.*, 1986; Shuman, 1986; Hattori *et al.*, 1989). Meanwhile, parallel studies suggested that the protelytic property of thrombin was required as proteinase inhibitors abolished thrombin's action (Shuman, 1986). Based on these experimental data, the existence of a thrombin receptor on the cell membrane was proposed, however initial screens failed to identify any such receptor (Okumura *et al.*, 1978; Gronke *et al.*, 1987).

1.3.1.1 Cloning of PAR-1

In 1991, two laboratories cloned the hamster and the human thrombin receptor respectively (Rasmussen *et al.*, 1991; Vu *et al.*, 1991a), later termed as proteinase activated receptor 1 (PAR-1), by expressing mRNA from thrombin-

responsive cells in oocytes of *Xenopus*. The putative protein contained 425 or 427 amino acids for human or hamster respectively. Hydropathy analysis revealed the receptor to be a member of the seven transmembrane domain receptor family. A remarkable feature of the receptor was its relatively long (75 amino acid) extracellular N-terminus, which includes a thrombin cleavage site. Furthermore, the PAR-1 response to thrombin was abolished by hirudin, a specific thrombin antagonist, confirming the selective nature of the receptor to thrombin (Vu *et al.*, 1991a). In the following years, PAR-1 was also cloned in rat (Zhong *et al.*, 1992) and mouse (Soifer *et al.*, 1994).

1.3.1.2 Cloning of PAR-2

As not all the actions of thrombin could solely be attributed to PAR-1 receptor activation (Vouret-Craviari *et al.*, 1992), it was widely believed that a second thrombin receptor must exist. By screening the mouse genome library using a mixture of two 60-oligomers corresponding to the second and sixth transmembrane domains of the bovine substance K receptor (Masu *et al.*, 1987), Nystedt and co-authors cloned a DNA sequence encoding a protein of 395 residues with seven transmembrane domain structure (Nystedt *et al.*, 1994; Nystedt *et al.*, 1995b). A putative proteinase cleavage site was deduced in its long N-terminus. This receptor shared 30% amino acids with human PAR-1 receptor, and was 28% identical with mouse PAR-1 receptor. Surprisingly, rather than been stimulated by thrombin, the receptor was highly sensitive to another proteinase, trypsin. Similar to PAR-1, cleavage of a trypsin cleavage site in the N terminus was shown to be required for the receptor activation (Nystedt *et al.*, 1994). This receptor was accordingly termed as proteinase activated receptor 2 (PAR-2). Subsequently, the human PAR-2 receptor was cloned in the following year (Nystedt *et al.*, 1995a).

1.3.1.3 Cloning of PAR-3

The cloning of PAR-2 opened doors for hunting for more members of the PARs family. Indeed, platelets in the PAR-1 deficient mice still responded strongly to thrombin (Connolly *et al.*, 1996). PAR-3 was subsequently cloned by polymerase chain reaction (PCR) using degenerate primers corresponding to conserved domains in PAR-1 and PAR-2 to screen platelet RNA from rat. Then, this rat

sequence was used to obtain mouse and human cDNA clones (Ishihara *et al.*, 1997). The human PAR-3 shares 27% sequence homology with human PAR-1, and 28% with human PAR-2.

1.3.1.4 Cloning of PAR-4

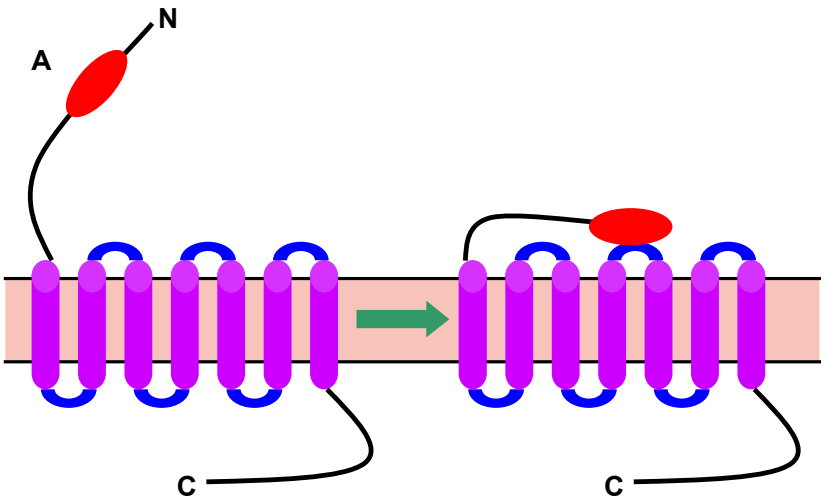
Coughlin's group found the thrombin responses in platelet from PAR-3 deficient mice was largely delayed, but not absent (Kahn *et al.*, 1998). As PAR-1 played no roles in mouse platelet aggregation (Connolly *et al.*, 1994; Connolly *et al.*, 1996), it indicated another receptor was activated in response to thrombin. Two laboratories using the strategy of GenBank BLAST (Basic Local Alignment Search Tool) based on the conserved Expressed Sequence Tag (ETS) among PARs, discovered the PAR-4 in mouse and human respectively (Kahn *et al.*, 1998; Xu *et al.*, 1998).

1.3.2 Structural features of PARs

Each PAR has a proteinase cleavage site within its long N-terminus, although distinctive sequence location and sensitivity to a variety of proteinase differ among PARs as illustrated in Figure 1.2. Taking human PARs for instance, proteinase cleavage site in PAR-1 is $LDPR^{41} \downarrow S^{42}FLLRN$ (↓ indicates specific cleavage position), $SKGR^{34} \downarrow S^{35}LIGKV$ for PAR-2, $LPIK^{38} \downarrow T^{39}FRGAP$ for PAR-3, and $PAPR^{47} \downarrow G^{48}YPGQV$ for PAR-4. The cleavage sites in PAR-1, PAR-3 and PAR-4 are preferentially sensitive to thrombin, whereas, PAR-2 is preferentially to trypsin. In addition, PAR-1 and PAR-3 have a hirudin-like domain in their N-terminals as demonstrated in Figure 1.2. This feature is distinct as this domain is not found in either PAR-2 or PAR-4. Experiments indicated the hirudin-like domain facilitated receptor-thrombin interaction by reducing kinetic barrier, leading to conformational change in the catalytic site of thrombin to perform proteolysis (Vu *et al.*, 1991b; Ishii *et al.*, 1993). The lack of a hirudin-like domain in PAR-2 also explains its irresponsiveness to thrombin.

Apart from the features in the N-terminus, the second extracellular loop in PARs is highly conserved in the four members across species. It has been confirmed the second extracellular loop alone has the ability to recognise "proteinase

identity" (Lerner *et al.*, 1996). Together with the N-terminus cleavage site, they jointly confer the specificity of proteinase binding (Bahou & Demetrick, 1997). Finally, the intracellular C-terminus of PARs has been shown to participate in PARs desensitisation and trafficking.



B Functional important domains on PARs

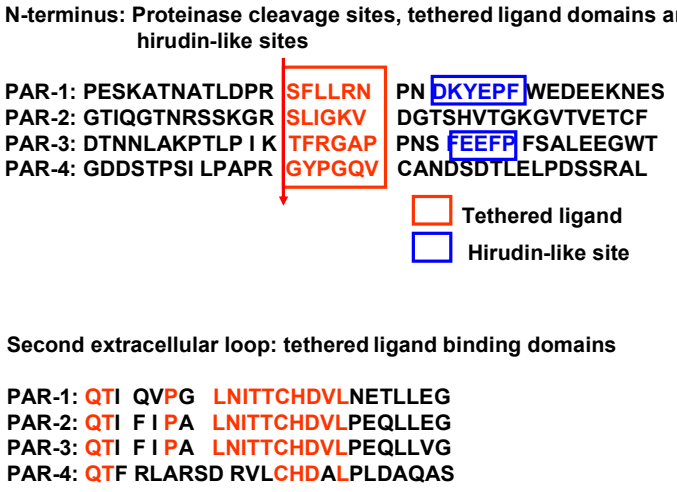


Figure 1-2 Structural features of PARs.

A. Schematic structure of PARs during rest (Left) and activation (Right) B. Functional important domains on PARs. Adapted and modified from Ossovskaya VS&Bunnett NW, 2004 (Ossovskaya & Bunnett, 2004)

1.3.3 Mode of activation of PARs

The default mechanism by which the proteinases cleave and activate PARs endogenously is identical. Proteinases interact with PAR, recognise the conserved domains in the N-terminal, and cleave at the proteinase cleavage site, unveiling a new N-terminal to serve as "tethered ligand", which binds to the second extracellular loop, initiating cellular signalling. In addition to their endogenous activation mode, all PARs with the exception of PAR-3, have been demonstrated to be activated by synthetic short peptides, which mimic the amino acid sequence of the "tethered ligand" (Vu *et al.*, 1991a; Nystedt *et al.*, 1994). These synthetic peptides with relatively high selectivity offer valuable tools to test function of specific PAR member in biological systems usually expressing more than one PAR. Furthermore, they provide an accessible experimental strategy to bypass the dilemma of destroying the integrity of PARs by using proteinases, such as thrombin and trypsin. (see figure 1.3) More importantly, synthetic "scrambled" peptides which are in reverse order of the amino acid sequence of activating peptide and not able to activate PARs, have been developed to use as "control peptides" in investigating PARs function. However, It has been noticed that in certain circumstances, the PAR activating peptides can stimulate a receptor other than any one of the four PARs (Vergnolle *et al.*, 1998; Hollenberg *et al.*, 2004). These data suggest it is necessary to use both PAR activating peptide and its corresponding PAR inactive peptide to study a given PAR rigorously (Ramachandran & Hollenberg, 2008).

Recently, two structurally different, potent, selective, and metabolically stable small-molecule PAR-2 agonists, AC-55541 and AC-264613, were discovered and characterised *in vitro* by in cellular proliferation assays, phosphatidylinositol hydrolysis assays, and Ca^{2+} mobilisation assays from cell culture preparations, with potencies ranging from 200 to 1000 nM for AC-55541 and 30 to 100 nM for AC-263613 (Gardell *et al.*, 2008; Seitzberg *et al.*, 2008). Although these PAR-2 agonist molecules were only tested in expression systems, they may be useful in probing the physiological functions of PAR-2 receptor as alternatives for short peptides in brain tissues.

As PARs are regularly co-expressed, it has been proposed that the interactions among PAR members within proximity would share a single proteinase agonist or react as activation facilitators. This appeared to be the case for PAR-3 and PAR-4 located in mouse platelets (Nakanishi-Matsui *et al.*, 2000). In mouse platelet, PAR-3 and PAR-4 are receptors for thrombin with differential affinity. PAR-3 responds to low concentration of thrombin, whereas PAR-4 activation needs high dose (Nakanishi-Matsui *et al.*, 2000). However, when PAR-3 alone was expressed, or even overexpressed in COS cells, it did not respond to thrombin at all (Nakanishi-Matsui *et al.*, 2000). Interestingly, when co-expressed PAR-3 and PAR-4, the signalling to thrombin through PAR-4 increased 6 to 15 fold, whereas PAR-4 response to its activating peptide was not changed (Nakanishi-Matsui *et al.*, 2000). This suggested a novel way that PAR-3 does not mediate signalling itself but plays as a co-factor for the cleavage and activation PAR-4 by thrombin.

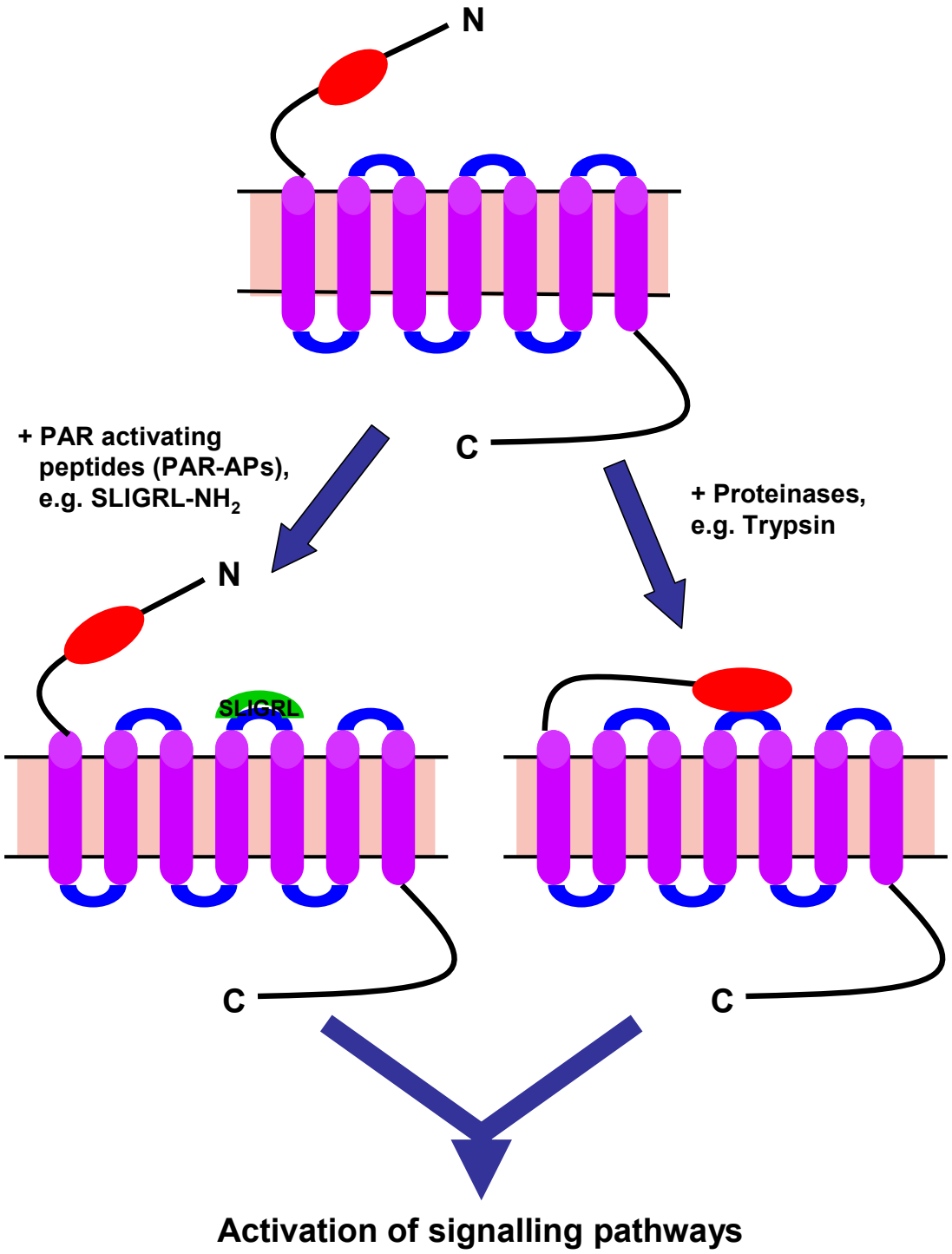


Figure 1-3 Schematic demonstration of activation mechanisms of PARs. Adapted and modified from Bushell,2007 (Bushell, 2007)

	PAR-1	PAR-2	PAR-3	PAR-4
Proteinase cleavage site	$R^{41}\downarrow S^{42}$FLLRN (Human) $R^{41}\downarrow S^{42}$FFLRN (Rat) $R^{41}\downarrow S^{42}$FFLRN (Mouse)	$R^{34}\downarrow S^{35}$LIGKV (Human) $R^{36}\downarrow S^{37}$LIGRL (Rat) $R^{34}\downarrow S^{35}$LIGRL (Mouse)	$K^{38}\downarrow T^{39}$FRGAP (Human) $K^{37}\downarrow S^{38}$FNGGP (Mouse)	$R^{47}\downarrow G^{48}$YPGQV (Human) $R^{58}\downarrow G^{59}$FPGKP (Rat) $R^{59}\downarrow G^{60}$ YPGKF (Mouse)
Proteinase agonists	Thrombin Plasmin Trypsin FXa FVⅡa APC Granzyme A Gingipians-R	Trypsin Tryptase Kallikrein-4, -5, -6,-14 FXa FVⅡa MT-SP1 Proteinase-3 Acrosien Gingipians-R	Thrombin	Thrombin Trypsin Kallikrein-14 Cathepsin G FXa FVⅡa Gingipians-R
Activating peptide	TFLLR-NH_2 SFLLR-NH_2	SLIGRL-NH_2 SLIGKV-NH_2 2-furoyle-LIGRLO-NH_2	None	AYPGKF-NH_2
Other agonists		PAR-2 molecule agonists AC-55541, AC-264613		
Antagonist	RWJ-56110 RWJ-58259 SCH-205831 SCH530348 BMS-200261 P1pal-12 pepducin	FSLLRY-NH_2 LSIGRL-NH_2 N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine (ENMD-1068)	None	trans-cinnamoyl-YPGKF-NH_2 P4pal-12 pepducin
PAR knockout mouse	YES	YES	YES	YES

Table 1-1 Summary of pharmacology of PARs.

1.3.4 Cellular signalling of PARs

1.3.4.1 PAR-1 signalling

PAR-1 has been confirmed to couple with three groups of G proteins as G_i, $G_{q/11}$, and $G_{12/13}$ as discussed in the following. The signalling through $G\alpha_i$ pathway, which inhibits adenylyl cyclase and suppresses formation of cAMP, was demonstrated in several cell types including HEL cells (Brass *et al.*, 1991), osteosarcoma cells (Babich *et al.*, 1990), vascular smooth muscle cells (Kanthou *et al.*, 1996), fibroblasts (Hung *et al.*, 1992), platelets (Kim *et al.*, 2002), astrocytes (Wang *et al.*, 2002), and endothelial cells (Vanhauwe *et al.*, 2002). Another major pathway for PAR-1 signalling is through $G\alpha_{q/11}$, which stimulates PLC-ß. PLC-ß generates IP_3, which mobilises intracellular Ca^{2+} and DAG that activates PKC. Coupling of PAR-1 to $G\alpha_{q/11}$ was evidenced by the blockade of PAR-1 mediated Ca^{2+} signalling by using antibody against $G\alpha_{q/11}$ subunit in fibroblast cells (Baffy *et al.*, 1994) and through co-immunoprecipitation of $G\alpha_{q/11}$ with PAR-1 (Ogino *et al.*, 1996). $G\alpha_{q/11}$ coupling to PAR-1 was found also in astrocytes (Wang *et al.*, 2002), and astrocytoma cells (LaMorte *et al.*, 1993). The interaction between PAR-1 and $G\alpha_{12/13}$ emerged in platelets and astrocytoma cells. $G\alpha_{12/13}$ interacts with Rho guanine-nucleotide exchange factors (GEFs), permitting Rho-mediated control of cell shape and migration by activating Rho-kinases (Offermanns *et al.*, 1994; Aragay *et al.*, 1995). The $G_{\beta\gamma}$ subunits released from the coupling of PAR-1 to heterogeneous G proteins also initiate signal transduction, notably, the activation of phosphatidylinositol (PI) 3-kinase which leads to changes in cytoskeletal structure, cell motility, survival, and mitogenesis (Wang *et al.*, 2002).

Apart from classical G protein signalling, PAR-1 activates other multiple intracellular kinase cascades in a G protein dependent or independent manner. One important substrate of PAR-1 is the MAPKs. The MAPK family has been characterised in mammalian cells with several MAPK signalling molecules, including extracellular-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAP kinase (Widmann *et al.*, 1999). They share a common activation mechanism by which a MAP kinase kinase Kinase (MAPKKK) phosphorylates and activates MAP kinase kinase (MAPKK), which in turn phosphorylates and activates MAP kinase. Then specific MAP kinase targets molecules in the cytoplasm and

nucleus, leading to a biological response such as mitogenesis, cytoskeleton change, and cell survival. PAR-1 has been confirmed to activate ERKs in astrocytes (Wang *et al.*, 2002), intestinal epithelial cells (Buresi *et al.*, 2002), and fibroblasts (Vouret-Craviari *et al.*, 1993). PAR-1 also activates p38 MAP kinase for example in fibroblasts (Sabri *et al.*, 2002; Wang *et al.*, 2006a), and human endothelial cells (Banfi *et al.*, 2009). JNK has also been recently shown to involved in PAR-1 signaling in astrocytes (Wang *et al.*, 2006b). PAR-1 signalling to the nuclear factor (NF)-κB has also been reported (Rahman *et al.*, 2002; Tantivejkul *et al.*, 2005), but the underlying mechanisms are still under debate.

1.3.4.2 PAR-2 signalling

Compared to extensive studies into PAR-1-mediated signalling, PAR-2 signalling was less investigated. However, it was widely reported that both trypsin and PAR-2 activating peptide (PAR-2-AP), elevated intracellular Ca^{2+} concentration subsequent to the release of IP_3 alongside with DAG in numerous cell types including transfected cell lines, ovary cells, tumor cells, neurons, and astrocytes (Nystedt *et al.*, 1995a; Bohm *et al.*, 1996; Ubl *et al.*, 1998; Bushell *et al.*, 2006). These data suggested that PAR-2 coupled to $G_{q/11}$. The PAR-2 coupling to G_i is disputed. One report showed the PAR-2 signalling was not affected when treated with pertussis toxin (PTX), a G_i blocker, in enterocytes, suggesting PAR-2 did not signal through G_i pathway (DeFea *et al.*, 2000). However, another report challenged this suggestion, and demonstrated the Ca^{2+} signalling in *Xenopus* oocytes in response to trypsin was PTX sensitive (Schultheiss *et al.*, 1997). So far, the coupling of PAR-2 with $G_{12/13}$ has not been reported.

A novel aspect of PAR-2 signalling is its ability to activate cellular kinase cascades in a ß-arrestin dependent, G protein independent mechanism. PAR-2 activators strongly activated ERK1/2 but p38 MAP kinase moderately (Belham *et al.*, 1996). Further studies demonstrated that trypsin and PAR-2-AP stimulated the formation of a multiprotein complex, containing internalised PAR-2, ß-arrestin, Raf-1, and activated ERK, which was required in ERK1/2 activation (DeFea *et al.*, 2000). A recent report showed PAR-2 promoted ß-arrestin dependent dephosphorylation and activation of the actin filament-severing protein (cofilin) independently of $G_{q/11}$ signalling (Zoudilova *et al.*, 2007). These findings indicate PAR-2 may signal through 'dual' mechanisms, and act via both

$G_{q/11}$ and ß-arrestin simultaneously to exert a large variety of cellular functions. In keratinocytes (Macfarlane *et al.*, 2005) and smooth muscle cells (Bretschneider *et al.*, 1999), the intracellular Ca^{2+} concentration induced by PAR-2 activation was an important determinant of NF-κB activation.

1.3.4.3 PAR-3 and PAR-4 signalling

PAR-3 does not appear to signal on its own. However, it indeed plays a role as a co-activator for PAR-4 activation. One intriguing aspect of this is the possible ability of PAR-3 to form heterodimers with other PARs, PAR-1 for instance, jointly exerting regulatory role for signal transduction (McLaughlin *et al.*, 2007). PAR-4 activation led to the aggregation of platelets in mouse and human platetes (Kahn *et al.*, 1998) and presumably via $G_{q/11}$ as PAR-4 resulted in the formation of intracellular IP_3 when transiently transfected into COS cells (Xu *et al.*, 1998). PAR-4 signalling through ERK1/2 was documented in smooth muscle cells (Bretschneider *et al.*, 2001). A recent study reported PAR-4 upregulated p38 MAP kinase in endothelial cells (Fujiwara *et al.*, 2005).

1.3.5 Expression and localisation of PARs in the brain under disease conditions

Various pathological processes have been shown to modulate the expression of PARs in the brain.

1.3.5.1 Ischaemia

Different experimental models of ischaemia, both *in vitro* and *in vivo*, have demonstrated a differential regulation of PARs in the brain. PAR-1 and PAR-3 are upregulated in rat hippocampal slices culture in an *in vitro* ischaemia model, oxygen-glucose deprivation (Striggow *et al.*, 2001). In one *in vivo* study, PAR-2 expression was strongly upregulated at 8-24 hours after transient middle cerebral artery occlusion in neurones in the cortex in mice (Jin *et al.*, 2005). In another report, the mRNA expression level of PAR1-4 was analysed in a transient focal ischaemia in rat brain, induced by microinjection of endothelin near the middle cerebral artery (Rohatgi *et al.*, 2004a). Interestingly, PAR-1 mRNA was strongly downregulated, whereas, PAR-2 mRNA was only decreased modestly

(Rohatgi *et al.*, 2004a). PAR-3 was upregulated transiently and then downregulated, and PAR-4 mRNA levels showed the most striking (2.5-fold) increase 12 hr after ischaemia (Rohatgi *et al.*, 2004a). This group further demonstrated a de novo expression of PAR-1 and PAR-3 in microglia in the penumbra zone of the infarct in rat 12 or 48 hours after endothelin-induced middlecerebral artery occlusion (Rohatgi *et al.*, 2004a).

1.3.5.2 Parkinson's disease

One Japanese group reported the upregulation of PAR-1 in astrocytes in Parkinson's brain (Ishida *et al.*, 2006). This group examined PAR-1 expression in substantia nigra pars compacta (SNpc) of brains from patients with Parkinson's disease. PAR-1 was exclusively found in astrocytes in these samples. Interestingly, the numbers of astrocytes expressing PAR-1 increased in the specimens of Parkinson's disease but not in healthy controls (Ishida *et al.*, 2006).

1.3.5.3 HIV infection

Human immunodeficiency virus (HIV) infection has several implications in the brain, in which PARs expression is altered. During HIV encephalitis, the expression of PAR-1 is upregulated at both mRNA and protein levels in astrocytes in humans *in vivo* (Boven *et al.*, 2003). Furthermore, PAR-2 is upregulated on neurones in conjunction with neuroinflammation in brain tissue from patients with HIV infection associated dementia (Noorbakhsh *et al.*, 2005).

1.3.5.4 Alzheimer's disease

Two studies demonstrated the altered levels of PARs expression during Alzheimer's disease (Pompili *et al.*, 2004; Afkhami-Goli *et al.*, 2007). Direct post-mortem study examining the brain tissue from patients with Alzheimer's disease revealed that PAR-2 expression was downregulated in neurones but upregulated in proximal astrocytes in Alzheimer's specimens compared to healthy controls (Afkhami-Goli *et al.*, 2007). Furthermore, one study utilising an in vitro model of Alzheimer's disease by trimethyltin administration demonstrated that all PARs particularly PAR-1 expression at both mRNA and

protein levels increased significantly in hippocampal astrocytes (Pompili *et al.*, 2004).

1.3.5.5 Multiple sclerosis and experimental autoimmune encephalomyelitis

PAR-2 expression is increased on astrocytes and infiltrating macrophages in brain white matter from human multiple sclerosis samples (Noorbakhsh *et al.*, 2006). Similar observation is reported in experimental autoimmune encephalomyelitis (EAE) in mouse (Noorbakhsh *et al.*, 2006).

1.3.5.6 Radiation damage

Expression of PAR-2 is investigated in a study into the consequences of single exposure to nuclear radiation. Significant PAR-2 immunoactivity in cell membrane is discovered in deeper cortical layers (Olejar *et al.*, 2002). At the same time, diffuse cytoplasmic PAR-2 immunoactivity is demonstrated in cortex and hippocampus (Olejar *et al.*, 2002). Increased cytoplasmic and polarised membrane positivity is also noticed on the neurones of hypothalamic nuclei (Olejar *et al.*, 2002).

In conclusion, a variety of pathological conditions differentially altered the expression of PAR1-4 in brain depending on diseases themselves or experimental disease models that were used and which cell type was studied. More precisely, the cell type specific, concentration/time course dependent dynamic expression profiles under pathological conditions indicates either harmful or protective roles for PARs in pathophysiology.

1.3.6 Dual functions of PARs in the brain under disease conditions

1.3.6.1 PARs in neurodegeneration

Numerous studies have demonstrated neurodegenerative role of PARs in a variety of conditions in the brain. Although most of current knowledge about PAR-mediated neurodegeneration is from PAR-1, PAR-2 and PAR-4 have been shown to participate in this process as well.

1.3.6.1.1 Ischaemia

Ischaemia has been shown to regulate PARs expression (Striggow *et al.*, 2001; Rohatgi *et al.*, 2004a; Jin *et al.*, 2005), and actually, PARs directly contribute to ischaemia-induced cell death. It is widely accepted that thrombin, the PAR-1 activator, is released into the brain tissue during the damage of the blood-brain barrier, which is a characteristic feature of brain ischaemia, such as stroke. Thrombin has been shown to be able to kill cultured hippocampal neurones and astrocytes mediated by PAR-1 activation (Donovan *et al.*, 1997). It has been shown that thrombin activity is increased in the ischaemic areas of the brain (Xi *et al.*, 2003). At high concentrations, thrombin deceases neuronal survival, and even causes neuronal cell death in a concentration dependent manner (50-500nM) in hippocampal slice culture following oxygen and glucose deprivation, an *in vitro* model of ischaemia (Striggow *et al.*, 2000). It is further demonstrated that blocking thrombin activity by selective inhibitor, hirudin, attenuated ischaemic damage before *in vitro* ischaemia was induced (Striggow *et al.*, 2000; Karabiyikoglu *et al.*, 2004). *In vivo* studies further reported that PAR-1 contributed to the infarct volume in the brain after transient focal cerebral ischaemia, which was significantly reduced in PAR-1 knockout mice (Junge *et al.*, 2003). Similar neuronal damage caused by PAR-1 activation was reported, and also prevented in PAR-1 knockout mice after unilateral cerebral hypoxia/ischaemia (Olson *et al.*, 2004).

1.3.6.1.2 Alzheimer's disease

A high level of thrombin has been detected in brain tissues from Alzheimer's disease patients, especially in senile plaques, some diffuse amyloid deposits and neurofibrillary tangles (Akiyama *et al.*, 1992). The presence of thrombin in the Alzheimer's brain was also documented by other groups (Arai *et al.*, 2006; Grammas *et al.*, 2006). It was further demonstrated that thrombin in Alzheimer's brain induced rapid tau hyperphosphorylation and aggregation in hippocampal neurones via PAR-1 and PAR-4, resulting in delayed synaptophysin reduction and apoptotic neuronal death (Suo *et al.*, 2003). A recent report showed PAR-4 is involved in regulation of beta-secretase cleavage of the Alzheimer amyloid precursor protein (Xie & Guo, 2005). All these studies suggest the activation of PAR-1 and PAR-4 contribute to the pathogenesis of Alzheimer's disease.

1.3.6.1.3 Parkinson's disease

PAR-1 expression is up-regulated in astrocytes in substantia nigra pars compacta of Parkinson disease brains (Ishida *et al.*, 2006). It was further demonstrated that PAR-1 contributes to the pathogenesis of Parkinson's disease. Using a mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease, Hamill and colleagues found after MPTP injection, PAR-1 knockout mice showed significantly higher residual levels of dopamine, the dopamine transporter, tyrosine hydroxylase and diminished microgliosis compared with wild-type controls (Hamill *et al.*, 2007). Furthermore, PAR-1 antagonist BMS-200261 exerted comparable levels of dopaminergic neuroprotection from MPTP-induced toxicity (Hamill *et al.*, 2007). These data indicate that PAR-1 activation can contribute to dopaminergic terminal damage in this model of early Parkinson's disease.

1.3.6.2 Mechanisms underlying PAR-mediated neurodegeneration

Glutamate receptors, in particular NMDA receptors and Ca^{2+}-permeable AMPA receptors play a crucial role in maintaining homeostasis in brain morphology and activity. It is widely accepted that excessive activation of glutamate receptors is toxic to neurones via an excessive influx of Ca^{2+}. This mechanism has been

confirmed to associate with several neurodegenerative diseases, such as ischaemic insults, Alzheimer's disease, and HIV-associated dementia (Hardingham & Bading, 2003; Liu & Zukin, 2007). Interestingly, activation of PAR-1 in astrocytes induced the release of glutamate (Lee *et al.*, 2007; Shigetomi *et al.*, 2008). The elevated extracellular glutamate in turn activates NMDA receptors on the neighboring neurones (Lee *et al.*, 2007). Furthermore, it has been demonstrated that activation of PAR-1 on neurones potentiates NMDA receptor function (Gingrich *et al.*, 2000). These data suggest PAR-1 activation may further strengthen the NMDA receptor mediated neuronal toxicity in neurodegeneration during pathological conditions such as ischaemia and stroke.

Another possible mechanism underlying PAR mediated neurodegeneration may involve neuroinflammation. Up-regulation of inflammatory cytokines, such as TNF-α, TNF-ß, IL-1ß, and IL-6, was observed in ischaemia, Alzheimer's disease, Parkinson's disease and multiple sclerosis (Campbell, 2004; Lucas *et al.*, 2006). Interestingly, the up-regulation of cytokines is observed in a variety of neurodegenerative conditions involving neuroinflammation, such as HIV-associated encephalitis, EAE and multiple sclerosis, has been reported accompanied simultaneously with PAR's activation (Boven *et al.*, 2003; Noorbakhsh *et al.*, 2005; Noorbakhsh *et al.*, 2006). In one study, PAR-1 activating peptide significantly increased levels of IL-1ß in human astrocytes, and supernatants of these cells were neurotoxic (Boven *et al.*, 2003). Moreover, striatal implantation of the PAR-1-AP significantly induced brain inflammation and neurobehavioral deficits in mice (Boven *et al.*, 2003). In the other study, macrophages from PAR-2 knockout mice showed higher expression of IL-10, and released soluble oligodendrocyte cytotoxins after experimental autoimmune encephalomyelitis (Noorbakhsh *et al.*, 2006). Moreover, PAR-2 wildtype animals showed markedly greater microglial activation and T lymphocyte infiltration accompanied by worsened demyelination and axonal injury compared to controls (Noorbakhsh *et al.*, 2006). Taken together, these data demonstrate PAR-mediated neuroinflammation is an important contributor to neuronal death in neruodegeneration in the brain.

1.3.6.3 PARs in neuroprotection

The neurodegenerative effect of PARs activation is well documented and summarized above. However, it is also noticed that under certain conditions, PAR activation turns out to be neuroprotective.

Thrombin or PAR-1-AP at very low and moderate concentrations is suggested to be neuroprotective in the brain. Low concentration of thrombin at 50pM, or of PAR-1-AP at 10μM, induced significant neuroprotection against experimental ischaemia (Striggow *et al.*, 2000). Another study reported that pretreatment with a low dose of thrombin as preconditioning, attenuated brain edema induced by a subsequent intracerebral injection of a high dose of thrombin (Xi *et al.*, 1999). It was further demonstrated that the thrombin preconditioning would offer protection from ischaemic brain damage in vivo (Masada *et al.*, 2000). In addition, in vitro, low concentrations of thrombin protect neurones and astrocytes from cell death induced by hypoglycemia and oxidative stress (Vaughan *et al.*, 1995). Furthermore, an anticoagulant and cytoprotector blood serine, proteinase-activated protein C protected cultured hippocampal and cortical neurons against glutamate-induced excitotoxicity at low concentrations (0.01-10nM) via PAR-1 activation (Gorbacheva *et al.*, 2008).

Neuroprotective role of PARs has been demonstrated in several disease models as well. Thrombin preconditioning reduced dopaminergic terminal loss and ventricular enlargement as compared to saline-treated animals in 6-hydroxydopamine Parkinson's disease model (Cannon *et al.*, 2005). These authors further demonstrated the protective effect of thrombin preconditioning in this Parkinson's disease model was mediated PAR-1(Cannon *et al.*, 2006). In another study, neuronal PAR-2 activation protected human neurones against the toxic effects of fibrillar 42-aa form of beta-amyloid, a key component of neuropathogenesis in Alzheimer's disease (Afkhami-Goli *et al.*, 2007). During HIV infection induced dementia, PAR-2 activation has been confirmed to prevent neuronal cell death and induction of the tumor suppressor, p53, caused by the HIV-encoded protein, Tat, in brain tissue from human patients (Noorbakhsh *et al.*, 2005). In addition, PAR-2-AP also inhibited Tat-induced neurotoxicity in a mouse model of HIV neuropathogenesis (Noorbakhsh *et al.*, 2005). PAR-2

knockout mice displayed significantly increased infarct volume and the number of apoptosis cells at 24 hours of reperfusion in a model of acute focal ischemic brain injury (Jin *et al.*, 2005). Reiser's group has shown that activation of either PAR-1 or PAR-2 prevents ceramide-induced apoptosis in cultured rat hippocamapl astrocytes, providing a novel mechanism of PAR-dependant protection (Wang *et al.*, 2006a; Wang *et al.*, 2007). PAR-1 or PAR-2 activation induced release of a chemokine, growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) in primary rat astrocytes via c-Jun N-terminal kinase (JNK) activation (Wang *et al.*, 2006a; Wang *et al.*, 2007). Interestingly, GRO/CINC-1 inhibited cytochrome *c* release from mitochondria through chemokine receptor CXCR2 in a feedback manner to protect astryocytes from apoptosis (Wang *et al.*, 2006a; Wang *et al.*, 2007). One recent publication from our group further demonstrated that PAR-2 activation protects neurones in organotypic hippocampal slice cultures (OHSC) against kainate acid-induced neuronal death via activation of chemokine receptor CXCR2 and mGluRs in response to astrocytic release of gliotransmitter (Greenwood & Bushell, 2010). In addition, it is demonstrated that PAR-2-mediated neuroprotection in OHSC involves MAPK signalling pathways because PAR-2 activation significantly reduces both p38 MAPK and ERK activities in OHSC and co-application of antagonists for p38 MAPK and ERK can mimic PAR-2-mediated neuroprotection in OHSC (Greenwood & Bushell, 2010).

In summary, duality of function of PARs has been widely documented. However, the determining factors of this duality still remain to be elucidated. Initial suggestion, though not definite, is the final actions of PAR-1 in neurones, either degenerative or protective, depend on the magnitude and duration of activator exposure, such as thrombin, and PAR-1-AP. Low concentration or brief exposure leads to neuroprotection, whereas medium or high concentration results in degeneration. Similarly, the ultimate consequence of PAR-2 activation seems depending on the cell type in which the activation occurs. PAR-2 activation in astrycytes is neurodegenerative, and in contrast, its activation in neurones is protective (Bushell, 2007; Greenwood & Bushell, 2010).

1.3.7 Limitations in PAR research

As PARs activate a variety of second messenger cascades in different tissues and cell types, it is not surprising that the signal transduction mediated by PARs, ultimately gives rise to a wide range of physiological and pathophysiological responses. However, quantitative and specific analysis of individual PAR involvement in various processes was compromised by the following limitations:

Firstly, the system where PAR takes its function is complex. PARs' signalling needs a focal coherent interaction involving multiple proteinases, proteinase inhibitors, cofactors in a timing dependent manner. In many situations, the full components of this system remain unclear.

Secondly, selective, potent and readily accessible antagonists for all PARs are not available although much progress has been made. Indeed, compounds such as RWJ-56110 and RWJ-58259 were developed by the Johnson & Johnson team as potential PAR-1 antagonists to, at least, locally block thrombosis in arterial stent adducts (Andrade-Gordon *et al.*, 1999; Zhang *et al.*, 2001). Another compound SCH-205831 or PAR-1 antagonist 55, which competitively inhibits PAR-1-AP biding site on PAR-1, was reported to be antithrombotic in human (Chackalamannil *et al.*, 2005). Moreover, BMS-200261, another PAR-1 antagonist, which blocks the cleavage of Arg41 of PAR-1 by proteinases (Bernatowicz *et al.*, 1996), fully blocked PAR-1-induced Ca^{2+} signalling in astrocytes (Nicole *et al.*, 2005), and reduced dopaminergic terminal damage in a mouse model of Parkinson's disease (Hamill *et al.*, 2007). Very recently a new compound SCH530348 was described as a hope for orally available PAR-1 antagonist to target thrombosis (Camerer, 2007). Developing antagonists for PAR-2 has been much challenging. A few peptides, such as FSLLRY-NH_2 and LSIGRL-NH_2, were made but potency was too low (Al-Ani *et al.*, 2002). A recent progress was achieved by reporting a novel PAR-2 antagonist molecule N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine (ENMD-1068) attenuated PAR-2-mediated joint inflammation (Kelso *et al.*, 2006). Antagonist peptides for PAR-4, trans-cinnamoyl-YPGKF-NH_2 was also demonstrated by Hollenberg's group in 2001(Hollenberg & Saifeddine, 2001).

Novel strategies to antagonize PARs were attempted by 1) targeting the receptor intracellular loops with cell-penetrating membrane-tethered peptides termed pepducins (Covic *et al.*, 2002a; Covic *et al.*, 2002b; Kuliopulos & Covic, 2003). 2) disrupting proteolysis of PAR by antiserum or PAR specific monoclonal antibody (Kelso *et al.*, 2006).

These PAR antagonists have greatly facilitated PAR research, but limitations are still clear: 1) lack of selectivity 2) low potency 3) non-PAR actions 4) cost

Thirdly, to develop selective serine proteinase inhibitors has been proved difficult. Furthermore, even after a selective proteinase inhibitor was established, it would still be a challenge to interpretate PAR actions, because in most cases, PAR could be activated by more than one activator. Inversely, a given proteinase could activate more than one PAR. For example, upon the discovery of PAR-1 and PAR-2, it is evidenced clear that although trypsin at low concentration (<10U/ml, 20nM) activate selectively PAR-2, high concentration of trypsin (<=50U/ml, 100nM) also mobilises PAR-1 (Hollenberg & Compton, 2002).

Finally, the endogenous activators for PARs in the brain are largely based on speculation. Indeed, several studies have suggested possible endogenous activators for PARs in the brain, but whether they actually signal through PARs is far from certain. Thrombin expression *in situ* in the brain is wide spread (Yoshida & Shiosaka, 1999; Rohatgi *et al.*, 2004b). Tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and plasmin have functional roles in ischaemia, synaptic plasticity, neurodegeneration and neuroregeneration (Xi *et al.*, 2003; Sheehan & Tsirka, 2005). Signalling of thrombin or thrombin-like proteinase endogenously was hypothesized through PAR-1. Meanwhile, trypsin expression *in situ* in the brain is also demonstrated (Lohman *et al.*, 2008). Trypsin-like proteinase have also been detected in the brain including mast cell tryptase (Ibrahim *et al.*, 1996; Mitsui *et al.*, 1999; Kempuraj *et al.*, 2006), trypsin Ⅳ/ mesotrypsin (Toth *et al.*, 2007), kallikrein 6/neurosin (Oikonomopoulou *et al.*, 2006), Kallikrein 8/neuropsin (Mitsui *et al.*, 1999), neuropsin (Shimizu *et al.*, 1998; Matsumoto-Miyai *et al.*, 2003), and neurotrypsin (Gschwend *et al.*, 1997; Reif *et al.*, 2007). These trypsin-like proteinases modulate several physiological functions in the brain including

synaptogenesis, synaptic transmission, synaptic plasticity and learning and memory. However, the target of trypsin and trypsin-like proteinases endogenously in the brain, PAR-2 or not, is under investigation.

1.4 Electrical properties of a central neurone

Typically, normal brain function, from complex cognitive task to simple movement control, is achieved by coherent flow of information encoded by heterogeneous neuronal activities, which are, in physical nature, proved to be electrical activities. The fundamental unit of these electrical flows is the neurone, characterised by its ability to initiate, propagate, and terminate electrical signals in a timely order. Furthermore, this property of a neurone is determined by its capability to allow charged ions to cross its excitable membrane in a precisely regulated spatiotemporal manner. In the following section, the electrical properties of a central neurone are summarised.

1.4.1 Neuronal excitability

Neuronal excitability, in cellular neurophysiology, means the ability of neurone to generate an AP in response to stimulation (Hille, 2001b). It is attributed to the presence of a broad range of voltage-dependent ion channels across the neuronal membrane, in particular the voltage-dependent Na^+ channels and the voltage-dependent K^+ channels (Hodgkin & Huxley, 1952d). Neuronal excitability is the intrinsic property of a neurone. Several factors have been considered to influence neuronal excitability (Armstrong & Hille, 1998). Notable, three main factors determine the neuronal excitability, namely membrane potential, threshold level and voltage-dependent Na^+ channels. Membrane potential and threshold level are dependent on each other. Depolarising the membrane towards the threshold level is a prerequisite of generating an AP. So, the closer the two are, the higher the excitability is. The further apart the two are, the lower the excitability is. Furthermore, activation of the voltage-dependent Na^+ channels, in most cases, is the initiator of AP. The more functional voltage-gated Na^+ channels are expressed on a given neurone, the larger chance that the excitability of the neurone is higher.

1.4.2 Model equivalent circuit of a functional neurone

The plasma membrane of a neurone is a semipermeable lipid bilayer characterised in fluid mosaic model (Singer & Nicolson, 1972). It effectively separates the neuronal plasma from the extracellular environment both morphologically and electrically. In the majority of the time, the membrane of a neurone is of high electrical resistance because it is selectively impermeable to water soluble charged ions. As both extracellular and intracellular fluids are excellent conductors, this configuration confers a 'membrane capacitor' property. Meanwhile, the lipid bilayer of a neuronal membrane is anchored with ion channels, that are permeable to specific charged ions to convey electricity current under certain condition, leading to a 'membrane resistor' property. Thus, a functional neurone in fact could fit into a simplified model electrical circuit as illustrated in figure 1.1. This simplified circuit can be used to explain the passive electrical properties of a given neurone.

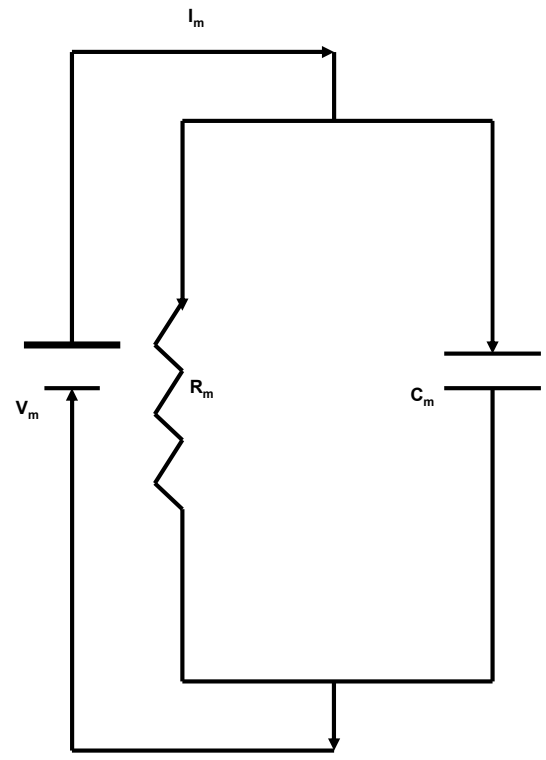


Figure 1-4 Schematic illustration of simplified model circuit of a neurone.

Modified from page 7, *Biophysics of computation: information processing in single neurons*, Christof Koch, 1999 (Koch, 1999) From an electrical aspect, lipid bilayers of a neuronal membrane confer a classical RC circuit, where membrane resistance (R_m) and membrane capacitance (C_m) are in parallel. Membrane ion flow (I_m) is driven by resting membrane potential (V_m) as a battery.

1.4.3 Resting membrane potential

The resting membrane potential is the difference of electrical potential across the membrane due to separation of charge by the lipid bilayer in the absence of stimulus. In most cases, neuronal membrane is negatively charged intracellularly, and positive charged extracellularly. If the extracellular potential is taken as reference as default, the membrane potential of a central neurone in the brain is typically about -65mV. Three factors contribute to the establishment of resting membrane potential in the central neurones. 1) Disparity of ions distribution across inside and outside of neuronal membrane. That is Na^+ and Cl^- are more concentrated outside the cell, whereas K^+ and in particular, organic anions are much more concentrated inside the cell. 2) Differential permeability for ions at resting situation. That is normally, without any stimulation, a neurone's membrane is almost only permeable to K^+, but not Na^+ due to the opening of K^+ channels and blockade of Na^+ channels. 3) Proper function of Na-K-ATPase. Na-K-ATPase pumps in two Na^+ ions to the plasma and pumps out three K^+ ions to extracellular fluid during each functional circle. Because Na-K-ATPase functions against concentration gradient and generating electrical field, it consumes energy which is supplied by ATP.

1.4.4 Action potential

An action potential (AP) is the 'signature character' of neuronal excitation and activity. It is also the coding unit of neural processing. An AP is characterised by transient alteration and re-establishment of the neuronal membrane potential in response to stimulus as illustrated in figure 1.5. The distinctive feature of an AP is its all-or-none property (Hodgkin, 1937a, b). That is, the shape and the amplitude of an AP do not change in response to the intensity of stimulus as long as the stimulus is suprathreshold. If a stimulus is subthreshold, a neurone can not generate an AP. In addition, an AP actively propagates across neurone's axon, without compromise of its shape and amplitude. AP's all-or-none phenomenon is in vast contrast with the electrotonic potential, which diminishes with distance. However, AP's all-or-none phenomenon has been complemented by the observation that APs can both actively and passively backpropagate into dendrites with diminishing amplitude across distance (Stuart *et al.*, 1997).

As illustrated in figure 1.5, the mechanism underlying the generation of an AP has been rigorously investigated and summarised by Hodgkin and Huxley in 1952 with the establishment of Hodgkin-Huxley model(Hodgkin & Huxley, 1952b, a, c, d). Briefly, the time course of an AP can be divided into five parts: the rising phase, the peak phase, the falling phase, and the hyperpolarisation phase. The rising phase is mediated by gradual activation of voltage-dependent Na^+ channels in a positive feedback manner. That is, a suprathreshold stimulus initiates the opening of voltage-dependent Na^+ channels, resulting in initial Na^+ influx. Therefore, this Na^+ influx depolarises the membrane further and drives more voltage-dependent Na^+ channels to open. The mass Na^+ influx, squeezed by both electrical potential and concentration gradient, gives rise to the faster kinetic character of the depolarising phase of AP compared to the repolarising phase. The opening of voltage-dependent Na^+ channels gradually reaches the maximum as positive feedback slows. Furthermore, because of the gradual inactivation of voltage-dependent Na^+ channels and accordingly the activation of voltage dependent K^+ channels, the AP reaches its peak and begins to decay swiftly. The falling phase is determined by the activation of voltage-dependent K^+ channels together with the inactivation of voltage-dependent Na^+ channels. The switch of neuronal membrane permeability towards K^+ drives efflux of K^+ to redepolarise membrane potential to restore its resting level. Because the redepolarisation reduces the opening of voltage dependent K^+ channels in a negative feedback manner, the falling phase is kinetically slower compared with the rising phase of an AP. The hyperpolarisation phase of an AP is caused by excessive opening of K^+ channels because the voltage dependent K^+ channels do not close immediately when membrane potential comes back to its resting level. Hyperpolarisation sustains until the normal permeability of K^+ is restored. In addition, the involvement of hyperpolarisation activated cation channels (Robinson & Siegelbaum, 2003), G-protein coupled inwardly rectifying K^+ (GIRK) channels and Ca^{2+} activated K^+ channels (Lujan *et al.*, 2009) in restoring membrane potential after hyperpolarization have been demonstrated.

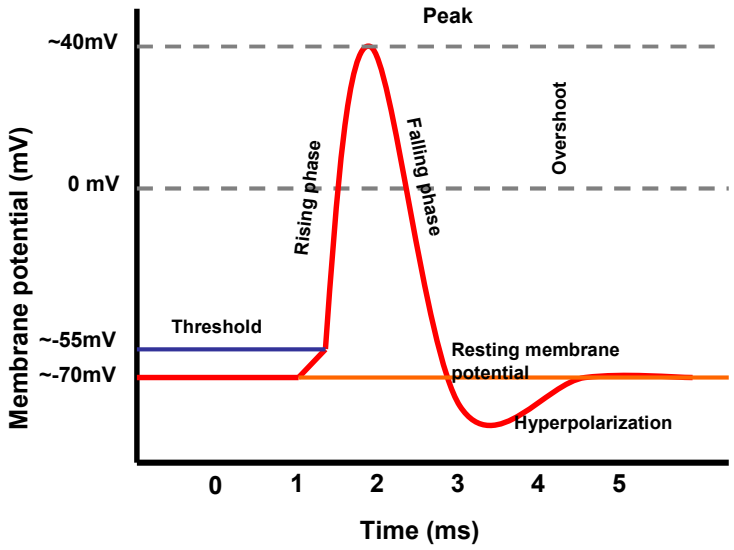


Figure 1-5 Schematic demonstration of an action potential.
Modified from page 28, *Ion channels of excitable membranes*, Bertil Hille, 2001 (Hille, 2001a)

1.5 Synaptic transmission

Neurones make contact with each other, and play the central role of structural and functional unit of neural activity within a highly interactive and coordinated circuitry. A contact between neurones is termed a ‘synapse’, which originates from ‘synaptein’, combing the Greek "syn-" ("together") and "haptein" ("to clasp"), first used by British neurophysiologist Charles Sherrington in the early 20th century. Interactions among neurones take place at synapses in the form of transmitting electrical signals from one neurone to the other. To date, two main forms of synaptic transmission are characterised as either electrical or chemical.

1.5.1 Chemical synaptic transmission

Chemical synaptic transmission is known as classical synaptic transmission. It has four phases:

1) AP initiated from presynaptic neurone activates voltage-dependent Ca^{2+} channels located within presynaptic terminal, permitting Ca^{2+} influx.
2) Ca^{2+} elevation triggers neurotransmitters released from presynaptic terminal.
3) Neurotransmitters cross over the synaptic cleft, acting on postsynaptic terminal.
4) The postsynaptic terminal responds with the change of local membrane potential.

In this process, the electrical signal coded in the pattern of AP is first "translated" into chemical signals carried by neurotransmitters, and "re-translated" back to electrical signal in the form of membrane potential alteration. Therefore, it is also termed as electrical-chemical-electrical transmission.

1.5.1.1 Transmitter release

The critical phase of chemical synaptic transmission is the release of neurotransmitters. Features of transmitter release were outlined as following: Firstly, transmitter release is characterised by a mechanism involving exocytosis and recycling of synaptic vesicles. Transmitter release could be divided into four steps: 1) Docking. Vesicles containing neurotransmitters approach and tether the active zone of presynaptic terminal. 2) Priming. This step includes all the protein and lipid modification and rearrangement in the active zone to make synaptic vesicles "readily releasable" and competent to fusion (Lloyd & Bellen, 2001). 3) Fusion. With the Ca^{2+} influx triggered by activation of Ca^{2+} channel, synaptic vesicles fuse with presynaptic membrane, leading to release of neurotransmitters into synaptic cleft. These three steps discussed above comprise the whole process of "excocytosis". 4) Recycling. Vesicles recycled back to presynaptic terminal, reloaded with neurotransmitters, and transported back to releasable pool.

Secondly, the process of docking, priming, fusion is tightly regulated by the interaction among SNARE complex and other proteins such as complexin, and synaptotagmin, which has been postulated as the "SNARE" hypothesis (Sollner *et al.*, 1993; Sudhof & Rothman, 2009).

Thirdly, the release of neurotransmitters is characterised by quantal release theory (Katz, 1971). As described above, neurotransmitters are stored and released from synaptic vesicles upon neural impulse. Thus functionally, each synaptic vesicle represents a release unit, which is termed as a “quantum”. The amount of synaptic vesicles released following one AP is regarded as “quantal content”. Likewise, the amount of neurotransmitter molecules loaded in single vesicle is described as “quantal size”. From analysis in nerve-muscle synapses, it is confirmed that the amount of vesicles released in response to a nerve impulse, the “quantal content” vary significantly. However, the amount of neurotransmitters in a single vesicle, the “quantal size” is more consistent. Therefore, the magnitude of a postsynaptic potential in response to a given impulse depends largely on the “quantal content” (Del Castillo & Katz, 1954).

1.5.1.2 Postsynaptic potential

Neurotransmitters released from presynaptic terminal diffuse through the synaptic cleft, and interact with specific receptors located on the postsynaptic terminal. This leads to the conformation change of receptor proteins, which subsequently opens ion channels (receptor and ion channel can coexist in the same protein complex or not) to change membrane conductivity to specific ions. Consequently, the ion influx/efflux results in membrane potential change in postsynaptic terminal, namely postsynaptic potential.

Depending on the time course, the postsynaptic potential can be divided into a fast or a slow response (Hille, 1994). A fast response, with a latency of 0.5-1.0ms after presynaptic AP, is mediated by ionotropic receptors, which are ligand-gated ion channels with the ability to change membrane conductance rapidly by swift conformation modification. In contrast, a slow response, with a latency of second to minute scale, is mediated by metabotropic receptors, which are not ion channels but indirectly coupled to ion channels on the membrane with signal transduction through G-protein-mediated second messenger systems.

Postsynaptic potentials can be excitatory. That is excitatory postsynaptic potential (EPSP), where the postsynaptic response depolarises the membrane

potential. Postsynaptic potential could also be inhibitory. That is inhibitory postsynaptic potential (IPSP), where a hyperpolarising response is observed. However, a given neurotransmitter could give rise to either excitatory or inhibitory postsynaptic response, depending on which type of receptor is activated as well as the resting membrane potential of the postsynaptic neurone (Ben-Ari, 2002). Thus, for simplicity, neurotransmitters leading to EPSPs in most cases are termed as excitatory neurotransmitters, whereas those resulting in IPSPs are classified as inhibitory neurotransmitters. In the brain, most prevalent excitatory neurotransmitter is glutamate. And the most important inhibitory neurotransmitter is γ–aminobutyric acid (GABA). In the brain, an EPSP is largely mediated by the Na^+ and/or Ca^{2+} influx through ligand-gated cation channels, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, N-methyl-D-aspartic acid (NMDA) receptors and kainate receptors. A fast IPSP is mainly from Cl^- influx through opening of $GABA_A$ receptors. Opening of K^+ channels by G_i-coupled $GABA_B$ receptors contribute to slow IPSP.

1.5.1.3 Integration of synaptic inputs

In the brain, where, when and how could a given postsynaptic neurone fire an AP depends on where, when and how the postsynaptic potentials it receives, either excitatory or inhibitory, are processed in a spatial and temporal manner. This process is synaptic integration (London & Hausser, 2005). Synaptic convergence, the number of presynaptic neurones innervating a given neurone, and divergence, the number of postsynaptic neurones innervated by a particular neurone, lay the structural foundation of this tuning (Bartos *et al.*, 2007).

EPSPs and IPSPs are local electrotonic potentials, without “all or none” nature, and can be summated. Single EPSP or IPSP generally is about 1mV. In order to make a neurone fire AP, the postsynaptic depolarisation must be strong enough to overcome threshold, normally around 10mV (depolarisation from -65mV to -55mV in a healthy neurone). Thus, spatial and temporal summation of EPSPs and/or IPSPs decides the magnitude and time course of gross synaptic inputs, which in turn dictates the output of postsynaptic neurone in the pattern of AP firing.

Synaptic summation can be divided into temporal summation and spatial summation (Bartos *et al.*, 2007). In case of temporal summation, a series of continuous post synaptic potentials come closely after each other before the last potential decays at a single synapse, thus the amplitude of a given potential will be "added", either more depolarised with an EPSP or less depolarised with an IPSP, into the previous one. On the contrary, spatial summation refers to the postsynaptic computation where synaptic potentials originated from multiple synapses, arrives simultaneously at a given neurone. Generally, spatial summation is linear, as the algebraic summation of post synaptic potentials from multiple synapses. Thus, in the spatial summation scenario, the membrane potential of postsynaptic neurone equals the algebraic sum of EPSPs and IPSPs. However, it must to be noticed that the discussion about spatial summation above is based on one assumption that an IPSP is hyperpolarising. Indeed, in most cases it is the case. However, IPSP can take its inhibitory function through an effect termed shunting inhibition, for instance in inhibitory interneurones in the brain (Alger & Nicoll, 1979; Gulledge & Stuart, 2003; Vida *et al.*, 2006). Because the reversal potential of Cl^- is about -65mV or less in interneurones, if the membrane potential is also -65mV, opening of Cl^- channels by neurotransmitters will not result in Cl^- influx with hyperpolarising potential. However, if an EPSP coincidently arrives to depolarise the membrane potential towards less negative, the opening of Cl^- channels will "offset" the magnitude of EPSP with Cl^- influx. Thus, the IPSP is still inhibitory although not hyperpolarising in the shunting inhibition scenario. This form of spatial summation is non-linear.

To sum up, synaptic integration includes temporal summation and spatial summation of synaptic inputs. They coexist and function simultaneously to steer a particular neurone to fire APs or not in response to EPSPs and IPSPs in a circuit.

1.5.2 Electrical synaptic transmission

Unlike electrical-chemical-electrical transduction in classical chemical synpases, the information flow among neurones could pass through direct electrical coupling established by the electrical synapses in the brain (Eccles, 1982; Jessell & Kandel, 1993). Electrical synapse, also know as gap junction, permit charged ions, and transmitters to flow freely through gap-junction channel, connexons,

between two tightly close neurones with only 2-3nm distance, much shorter compared to chemical synapse with 20-40nm (Bennett, 1997; Bennett & Zukin, 2004).

Compared to chemical synapse, transmission at electrical synapse does not delay, but without gain, and in most cases, biderectional. Electrical synapse and chemical synapse can coexist at one single synaptic formation (Shapovalov, 1980). Functionally, electrical synapses are particularly important in local inhibitory circuits comprised of GABA-releasing interneurones to generate gamma oscillation (Galarreta & Hestrin, 2001).

1.6 Synaptic circuits in the hippocampus

1.6.1 Overview of the hippocampus

The hippocampus belongs to the 'limbic system' in the mammalian brain. The necessity of the hippocampus in storing and retrieving memory as well as participating spatial navigation has been extensively documented (Bliss & Collingridge, 1993; Wilson & McNaughton, 1993; Malenka & Bear, 2004; McNaughton *et al.*, 2006). The hippocampus is one of the most widely investigated regions in the brain thanks to its relatively clear principle cell layers with their highly organized excitatory output as well as their local domain-specific inhibitory inputs from interneurones. Experimentally, the hippocampus is of readily access for neuroscientists, whose interests range from synaptic physiology, neural plasticity, spatial learning and memory and artificial remodeling to clinical conditions such as epilepsy, schizophrenia, Alzheimer' s disease, and autism (Bliss & Lomo, 1973; Best & White, 1999; Burke & Barnes, 2006; Boyer *et al.*, 2007; Squire & Kandel, 2009).

1.6.2 Excitatory circuits in the hippocampus

A 'banana shape' structure located deeply within the medial temporal lobe of the mammal's brain, the hippocampus has three main subdivisions: CA1, CA3 (CA: cornus ammonis) and the dentate gyrus (DG). Furthermore, the hippocampus is unique in conveying largely unidirectional flow of information through well characterised excitatory trisynaptic pathways (Bliss & Gardner-Medwin, 1973; Malenka & Nicoll, 1999; Andersen, 2007b). (see figure 1.3). Briefly, considering the most simplified scenario, pyramidal cells in the layer II of the entorihnal cortex (EC) send axon projections, among many destinations, into the DG. These projections are termed as perforant path (PP), which contributes to the major hippocampal input. Subsequently, information flows from the DG into the CA3 area of the hippocampus through mossy fibres (MF), which are axon projections from granule cells in the DG into the CA3. Likewise, the pyramidal cells in the CA3 innervate the CA1 area of the hippocampus by long axon projections called

Schaffer collaterals (SC) to allow information to flow from the CA3 into the CA1. To complete this loop, CA1 pyramidal cells project axons into subiculum, which directs projections back to the EC. Apart from this trisynaptic pathway, both through the PP, the pyramidal cells in layer II of the EC also send direct excitatory input into the CA3, and the pyramidal cells in layer III of the EC innervate the CA1 directly(Amaral *et al.*, 2007; Andersen, 2007b). Some axons of pyramidal cells in the CA1 also terminate directly into deep layers of EC (Amaral *et al.*, 2007; Andersen, 2007b). In summary, beginning from the superficial layers and finishing in the deep layers of the entorhinal cortex, information flow completes a cycle and is processed in the hippocampus through this main excitatory EC-DG-CA3-CA1-EC pathway.

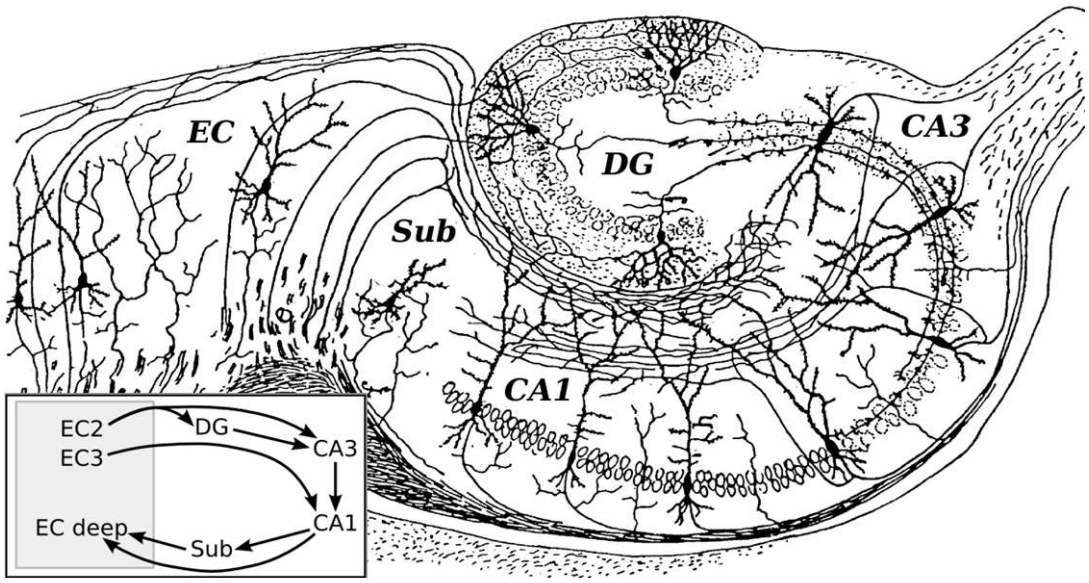


Figure 1-6 Classical illustration of the hippocampus showing the excitatory trisynaptic pathway.
Adapted and modified from classical illustration of hippocampal formation showing the excitatory trisynaptic pathway as drawn by Santiago Ramon y Cajal (Ramón y Cajal, 1952, 1955). Insert shows the schematic representation of main excitatory pathways and the direction of information flow. Source: http://en.wikipedia.org/wiki/Hippocampus

1.6.3 Inhibitory circuits in the hippocampus

Although pyramidal cells are the dominant cells in the hippocampus, there are indeed a large number of heterogeneous GABAergic interneurones, roughly 10% of the total hippocampal neuronal population, located across different hippocampal regions and layers, which provide general inhibition and modulate network activity (Freund & Buzsaki, 1996; Jonas *et al.*, 2004; Ascoli *et al.*, 2008;

Klausberger & Somogyi, 2008). GABAergic interneurones are recognised and classified by their firing signature, biochemical markers, morphology, innervating pattern, and location in the hippocampus, however overlapping among classifications is common (Ascoli *et al.*, 2008; Klausberger & Somogyi, 2008). Because my project, during its time course, is focused solely in the CA1 region of the hippocampus, only are interneurones in the CA1 discussed below.

One of the most studied subgroup of interneurones in the CA1 of hippocampus is fast-spiking, parvalbumin-expressing basket cells. These basket cells actually are pyramidal shape and reside in or close to the pyramidal cell layer. Their short range axons innervate mostly the soma and proximal dendrites of thousands of pyramidal cells to provide local inhibition. At the same time, a particular basket cell receives excitatory input from thousands of pyramidal cells, which indicates its high capacity of convergence and potentially critical role in controlling local pyramidal cells in feedforward manner and regulating the synchronisation of network activity (Cobb *et al.*, 1995; Andersen, 2007a; Bartos *et al.*, 2007; Klausberger & Somogyi, 2008). Indeed, it has been demonstrated that basket cells are the key factor to 'phase lock' AP firing in pyramidal cells to synchronise their activity in theta oscillation (Cobb *et al.*, 1995). Further studies have shown the fast inhibitory effect of basket cells promotes gamma oscillation (Bartos *et al.*, 2002) and shunting inhibition of basket cells consolidates gamma oscillation (Vida *et al.*, 2006).

The second group of fast-spiking, parvalbumin-expressing interneurones in the CA1 is axo-axonic cells, also termed as chandelier cells, which exclusively innervate the initial segment of axons of pyramidal cells. Like basket cells, they locate in or adjacent to the pyramidal cell layer. Because the initial segment of a axon is critical in generating AP in the pyramidal cells (Stuart *et al.*, 1997), the axo-axonic cells are thought to have ultimate control over the output of pyramidal cells and involved in the synchronisation of theta and ripple oscillation (Howard *et al.*, 2005).

Bistratified cells are also located in the CA1 pyramidal cell layer. In contrast to fast-spiking, parvalbumin-expressing basket cells and axo-axonic cells, bistratified cells innervate dendrites of pyramidal cells to provide dendritic

inhibition (Buhl *et al.*, 1996; Halasy *et al.*, 1996; Klausberger *et al.*, 2004). Bistratified cells have been found to fire at a phase when the dendrites of pyramidal cells are hyperpolarised (Klausberger *et al.*, 2004). Together with basket cells, bistratified cells provide precise 'phase locking' of theta oscillation in vivo (Klausberger *et al.*, 2004).

Apart from pyramidal cell layer, cell bodies of GABAergic interneurones distribute in the *stratum oriens*, *stratum radiatum*, and *stratum lacunosum-moleculare* of the hippocampus. One distinct group of these interneurones is somatostatin-containing O-LM cells, with cell bodies in the stratum oriens, dendrites parallel to pyramidal cell layers in the stratum oriens-alveus, axons in the stratum lacunosum-moleculare. O-LM cells innervate distal dendrites of CA1 pyramidal cells. O-LM cells fire preferentially in theta frequency, and are important in maintaining gamma oscillation (Tort *et al.*, 2007). Notably, O-LM cells has been found to express an "anti-hebbian" form of long term potentiation (see more in Chapter 1.7) (Lamsa *et al.*, 2007; Oren *et al.*, 2009).

In conclusion, GABAergic interneurones in the hippocampus provide feedforward and feedback inhibition in local circuits in both tonic and phasic means. The coherent activity of heterogeneous groups of interneurones in a timing dependent manner plays the key role in initiating, maintaining, and consolidating network oscillation.

1.7 Synaptic plasticity

It is widely observed that the efficacy of synaptic transmission between neurones could be strengthened or weakened depending on a variety of electrical or chemical stimuli in a time scale from a few milliseconds to a few days in different areas of the brain both *in vitro* and *in vivo* (Bliss & Collingridge, 1993; Bortolotto *et al.*, 1999; Kemp & Bashir, 2001; Zucker & Regehr, 2002; Whitlock *et al.*, 2006). This phenomenon has been described as synaptic plasticity comprising short-term plasticity (STP), long-term potentiation (LTP) and long-term depression (LTD). Synaptic plasticity has been a plausible candidate of molecular and cellular basis for learning and memory as information maybe stored and retrieved by transforming differential spatiotemporal patterns of neural activity into synaptic changes in different locations, circuits and neurones in the brain (Bliss & Collingridge, 1993; Martin *et al.*, 2000; Holtmaat & Svoboda, 2009; Wang & Morris, 2010). The term "synaptic plasticity" was first introduced by Dr. Jerzy Konorski in the late 1940s (Konorski, 1948). Subsequently refined by Dr. Donald Hebb in 1949 with proposed mechanism known widely as Hebb's postulate (Hebb, 1949):

'When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cell firing B, is increased'.

Since its first introduction, Hebb's rule has been verified in most types of activity-dependent synaptic plasticity in the brain. Hebbian LTP, which typically requires the depolarisation of postsynaptic NMDA receptors, has been characterised with three dogmas: input specificity, associativity, and cooperativity (Bliss & Collingridge, 1993). But exceptions do exist as the non-Hebbian LTP, where LTP induction is independent of the activation of NMDA receptors, and the anti-Hebbian form, where LTP is prevented by postsynaptic depolarisation during afferent activity, have been reported (Urban & Barrionuevo, 1996; Lamsa *et al.*, 2007; Kullmann & Lamsa, 2008). In the synapse made by mossy fibres onto pyramidal neurones in hippocampal CA3 area, non-

Hebbian LTP was observed as a result of sustained presynaptic activation (Urban & Barrionuevo, 1996). In the synapse made by pyramidal cells onto interneurones in the oriens of CA1 area, anti-Hebbian LTP was reported in response to activation of Ca^{2+} permeable AMPA receptors during hyperpolarization of postsynaptic membrane (Lamsa *et al.*, 2007).

1.7.1 Short-term plasticity

Short-term plasticity is short-term increase or decrease of synaptic strength that lasts for at most a few minutes. Such short lasting changes in synaptic efficacy may be expressed in two opposite directions as short-term facilitation or short-term depression.

1.7.1.1 Short-term facilitation

Experimentally, short-term facilitation (STF) is easily been observed upon two closely paired stimuli paradigm, termed paired-pulse, with an interval of, usually among 10ms to 300ms. The synaptic response of the second pulse can be several times stronger in relative to the first one at the Schaffer collateral-to-CA1 synapse. Short-term facilitation is, by its origin, presyanptic (Zucker & Regehr, 2002). It reflects an increase in the probability of release of readily vesicles and can be attributed to the transient accumulation of presynaptic Ca^{2+}, as Ca^{2+} influx triggered by the second AP is added into the residual Ca^{2+} which has not been depleted after the first pulse (Wu & Saggau, 1994). Another interesting observation about short-term facilitation is that the basal level of release probability will decide the magnitude of facilitation (McNaughton, 1982). The closer the basal release probability to 1, the less scope there is for subsequent facilitation.

1.7.1.2 Short-term depression

Short-term depression (STD) also occurs upon paired pulse paradigm in some synapses. The most widely accepted mechanism for STD is termed as the ‘depletion model’ (Zucker & Regehr, 2002). That is the STD is the result of a decrease of available vesicles in readily releasable pools in presynaptic terminals

in response to closely paired stimuli. The desensitization of responsible postsynaptic receptors is believed to contribute partially.

1.7.2 Long-term potentiation

Long term potentiation (LTP), as long lasting increase of synaptic transmission, was first described in the perforant path from entorhinal cortex into the dentate gyrus in an anaesthetised rabbit (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973). Subsequently, it is routinely observed in all excitatory pathways onto principal cells in the hippocampus and many other regions in the brain including neocortex, striatum, and cerebellum (Wilson & Racine, 1983; Calabresi *et al.*, 1992; Shibuki & Okada, 1992). Recently, LTP has also been discovered in excitatory synapses onto inhibitory interneurones in the hippocampus (Mahanty & Sah, 1998; Perez *et al.*, 2001; Kullmann & Lamsa, 2007; Lamsa *et al.*, 2007).

LTP has three distinct properties in input-specificity, associativity, and cooperativity (Bliss & Collingridge, 1993). Input-specificity refers to the fact that LTP does not spread. Only the pathway that has been activated expresses LTP even with other pathways in proximity. Associativity describes that a weak input may induce LTP as long as a separate strong input simultaneously stimulate the same target neurone. Cooperativity describes the existence of a threshold of input intensity for inducing LTP. LTP can be either induced by strong tetanus stimulation in a single pathway or by the summation of several small stimuli. Among these three LTP properties, input-specificity is relevant to the LTP expression, whereas, associativity and cooperativity are within the scope of LTP induction.

Based on whether or not NMDA receptor antagonist could block its induction, LTP can be classified as NMDA receptor dependent or NMDA receptor independent. In the hippocampus, the LTP in the Schaffer collateral-to-CA1 pyramidal cell synapses is the most studied type of NMDA receptor dependent LTP (Bliss & Collingridge, 1993). On the other hand, the LTP in the mossy fiber synapses onto CA3 pyramidal cells are typically NMDA receptor independent (Harris & Cotman, 1986; Nicoll & Schmitz, 2005). Schaffer collateral LTP is routinely induced by tetanus stimulation (100 pulses within 1 second for a tetanus, 1 to 3 tetani) or

theta burst stimulation (10 sets of burst of four stimuli in 100Hz, 200ms interburst interval). The induction of Schaffer collateral LTP classically follows the Hebb's postulate with the NMDA receptor functioning as a "coincidence detector" to pair presynaptic stimulation with postsynaptic depolarisation (Collingridge *et al.*, 1988; Kauer *et al.*, 1988; Huang *et al.*, 1992). Associativity, and cooperativity could be easily explained by the role of the NMDA receptor in Schaffer collateral LTP induction. The voltage dependent Mg^{2+} blockade of NMDA receptors in postsynaptic membrane installs the LTP threshold in cooperativity. Associativity is reflected by the fact that the requirement of removing Mg^{2+} blockade of NMDA receptors can be achieved by depolarising postsynaptic terminals experimentally by a voltage step or convergent excitatory inputs. Apart from NMDA receptor's voltage dependent Mg^{2+} blockade, its permeability to Ca^{2+} plays a key role in the Schaffer collateral LTP as Ca^{2+} influx has been confirmed to be necessary for its induction (Lynch *et al.*, 1983).

The time course of LTP can be divided into four phases as post-tetanus potentiation (PTP), short-term potentiation (STP), early-LTP, and late-LTP (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999). Post-tetanus potentiation (PTP), immediately following tetanus stimulation, is believed to be the response of enhanced transmitter release corresponding to residual Ca^{2+} accumulation presynaticly during tetanus stimulation. This phase is presynaptic, and NMDA receptor independent. Short-term potentiation (STP), usually lasting 30-60 minutes after tetanus, can be isolated by the presence of protein kinase inhibitors or routinely acquired by weak stimulation protocol. Early-LTP with the duration of less than 3-6 hours requires protein kinases such as CaMK II and PKC (Malinow *et al.*, 1988; Malenka *et al.*, 1989). Late-LTP, usually lasts more than 3 hours, and is protein synthesis dependent (Frey *et al.*, 1988).

1.7.3 Long-term depression

Long term depression (LTD) is the long lasting reduction of synaptic transmission opposite to LTP. This phenomenon was first recorded in a study that in the CA1 region of hippocampus, a LTP-inducing stimulation surprisingly resulted in a depressed synaptic efficacy in a separate, non-stimulated pathway, a phenomenon described later as "heterosynaptic LTD", by Lynch and co-workers

in 1977 (Lynch *et al.*, 1977). Another report in 1980 described that a low frequency stimulation effectively reversed established LTP in the hippocampus (Barrionuevo *et al.*, 1980) which was later termed as "depotentiation". Both phenomenon were largely neglected until the early 1990s, that a new form of "homosynaptic LTD" was discovered by Mark Bear's group and confirmed by Robert Malenka's group in 1992 that low frequency stimulation of 900 stimuli in 1 Hz produced a prolonged reduction of synaptic transmission in the CA1 region of hippocampus, without any initial LTP-inducing paradigm (Dudek & Bear, 1992; Mulkey & Malenka, 1992).

An intriguing aspect of homosynaptic LTD is its developmental dependency. One study showed the standard low frequency stimulation (LFS) protocol, 900 stimuli in 1 Hz, routinely produced LTD in the CA1 region of hippocampal slices from 2 weeks old animal, whereas LTD magnitude decreased by half in 5 weeks old animals (Dudek & Bear, 1993). This age-dependency was reproducible as another group independently showed LTD can be induced by LFS in animals younger than 40 days, however no LTD was acquired in animals between 40 days to 16 weeks (Kemp *et al.*, 2000). The loss of LTD induced by LFS is not likely due to the lack of ability to depress the Schaffer collateral-to-CA1 synapse as paired-pulse low frequency stimulation produced LTD in aged animals (Kemp *et al.*, 2000) as well as stimuli of 5-10Hz for 15 mins (Berretta & Cherubini, 1998). In summary, these data suggest LTD induction may require differential machineries responsible for LTD induction over development and upon stimulation protocols.

1.7.3.1 NMDA receptor dependent LTD

LTD can be classified as NMDA receptor-dependent or not depending on whether or not DL-AP5 or other NMDA receptor antagonists block its induction. The LTD induced by LFS (900 stimuli, 1Hz) in CA1 region of hippocampus has been confirmed as NMDA receptor dependent (Dudek & Bear, 1992; Mulkey & Malenka, 1992). NMDA receptor dependent LTD is also found in visual cortex (Kirkwood & Bear, 1994), perirhinal cortex (Ziakopoulos *et al.*, 1999), and barrel cortex (Feldman *et al.*, 1998).

It has been confirmed the induction of NMDA receptor dependent LTD requires a rise of postsynaptic Ca^{2+} concentration. Chelating Ca^{2+} postsynaptically by ethylene glycol tetraacetic acid (EGTA) or 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) blocked LTD induction (Brocher *et al.*, 1992; Mulkey & Malenka, 1992). In addition, Mulkey and Malenka also demonstrated that reducing extracellular Ca^{2+} resulted in the induction of LTD by a LTP-inducing paradigm (Mulkey & Malenka, 1992). Theses data suggest both LTD and LTP induction requires a rise of postsynaptic Ca^{2+} concentration. Lisman's study further proposed that because the postsynaptic enzymes such as phosphatases and protein kinases have differential affinity for Ca^{2+}, low level of Ca^{2+} may activate phosphatases to induce LTD, whereas, high level of Ca^{2+} may activate protein kinases to yield LTP (Lisman, 1989). To conclude these observations, it is further hypothesized that the spatiotemporal distribution and/or magnitude of Ca^{2+} transient postsynapticly may confer the polarity of long lasting changes of synaptic strength (Bliss & Collingridge, 1993; Bliss *et al.*, 2007).

As LFS-induced LTD in the hippocampal CA1 region has been shown to be NMDA receptor dependent, it is therefore, of interest to look into if the feature of developmental dependency of LFS-induced LTD in the CA1 is a result of the composition shift of NMDA subunits during development (Dudek & Bear, 1993; Monyer *et al.*, 1994; Kemp *et al.*, 2000). Evidence supporting this hypothesis came as one report discovered that overexpression of embryonic dominant NR2D subunit resulted in a loss of LTD in juvenile mice (Okabe *et al.*, 1998). Furthermore, an interdependency of NR2D and NR2B was noticed in this study with downregulation of NR2B accompanied with NR2D overexpression in 2 to 3 weeks mice (Okabe *et al.*, 1998), suggesting a potential role of NR2B in LFS-induced LTD in juvenile mice.

Subsequently, more studies investigated the possibility that specific NMDA receptor subunit is responsible for LTD. The critical role for NR2B subunit is demonstrated by a knockout-mice study that mice lacking the NR2B subunit-containing NMDA receptors did not express LTD (Kutsuwada *et al.*, 1996). However, pharmacological experiments provide conflicting data that one report using NR2B selective inhibitor, ifenprodil, enhances LTD (Hendricson *et al.*,

2002), whereas, another report, using the same drug, shows blockade of LTD (Liu *et al.*, 2004).

1.7.3.2 mGlu receptor dependent LTD

The involvement of mGlu receptors in LTD was first evidenced by the discovery that the group I/II mGluR antagonist, MCPG, blocked the induction of depotentiation by LFS protocol (900 stimuli, 1 Hz) following established LTP in the CA1 region of hippocampus (Bashir *et al.*, 1993; Bashir & Collingridge, 1994). Furthermore, several studies have demonstrated the requirement of activation of mGluRs in de novo LTD in the CA1 region of the hippocampus under certain conditions. In one study, MCPG also blocks the induction of LTD in hippocampal CA1 pyramidal neurones in neonatal rats induced by a stimulation of 5Hz for 3 minutes (Bolshakov & Siegelbaum, 1994). In another study, MCPG blocked de novo LTD following the same stimulation of 5Hz for 3 minutes in hippocampal CA1 region from both neonatal (6-7 days old) and juvenile (11-35 days old) rats (Oliet *et al.*, 1997). Subsequent studies demonstrate more evidence of mGluR involvement in LTD. A potent non-selective mGluR antagonist, LY341495 blocked depotentiation but not de novo LTD, suggesting they may not share a common induction mechanism (Fitzjohn *et al.*, 1998). LTD induced by low frequency stimulation of paired-pulse at 1Hz (PP-LFS) in adult (Kemp & Bashir, 1999) and juvenile anmimals (Huber *et al.*, 2000) were shown to require mGluRs.

Interestingly, NMDA receptor-dependent LTD induced by LFS is also present in the same synapses in the hippocampal CA1 but these two forms of LTD have been suggested to utilise distinct induction pathways: mGluR dependent LTD requires the Ca^{2+} influx from T-type Ca^{2+} channel, and protein kinase C, whereas, NMDA receptor dependent LTD requires Ca^{2+} influx through NMDA receptors and the activity of protein phosphatases (Oliet *et al.*, 1997). It has been further suggested that the requirement of NMDA receptors and mGlu receptors in induction of LTD may be mutually exclusive as where there is a role for NMDA receptors, there is no role for mGluRs and *vice versa* (Bortolotto *et al.*, 1999; Kemp & Bashir, 2001).

In addition to mGluR dependent LTD induced by synaptic activity, there is another form of long lasting depression of synaptic transmission caused by group Ⅰ mGluR agonist DHPG in areas such as CA1 (Palmer *et al.*, 1997; Fitzjohn *et al.*, 1999; Fitzjohn *et al.*, 2001) and DG (Camodeca *et al.*, 1999). This form of chemically-induced LTD is blocked by group I or broad-spectrum mGluR antagonists but not by NMDA receptor antagonists (Palmer *et al.*, 1997; Fitzjohn *et al.*, 1999; Fitzjohn *et al.*, 2001). The induction of DHPG-induced LTD is Ca^{2+}-independent with a postsynaptic locus. Inhibiting postsynaptic G proteins activity by loading a G protein inhibitor into CA1 pyramidal cells prevents the induction of DHPG-induced LTD (Watabe *et al.*, 2002), suggesting a postsynaptic induction. Furthermore, chelating Ca^{2+} postsynaptically with BAPTA does not block DHPG-induced LTD induction, suggesting a Ca^{2+}-independent mechanism (Fitzjohn *et al.*, 2001). A rapid protein synthesis in postsynaptic dendrites has also attributed to the induction of DHPG-induced LTD (Huber *et al.*, 2000). The locus of expression of DHPG-induced LTD is debatable. Several evidences have indicated a presynaptic locus. Fitzjohn and co-workers demonstrated that paired-pulse facilitation and the coefficient of variation of EPSCs were increased, with the reduction of frequency of miniature EPSCs but not amplitude (Fitzjohn *et al.*, 2001). Furthermore, DHPG-induced LTD is also not associated with changes in sensitivity to focally uncaged L-glutamate (Rammes *et al.*, 2003). These data support the argument that the locus of expression of DHPG-induced LTD is presynaptic. However, there is also evidence that removal of AMPA and NMDA receptors in postsynaptic terminal is responsible for the expression of DHPG-induced LTD, indicating a postsynaptic mechanism (Snyder *et al.*, 2001), indicating a postsynaptic expression locus.

1.8 Neurone-astrocyte interaction

Astrocytes are traditionally regarded as chemical and physiological insulators in the central nervous system, facilitating AP propagation, buffering glutamate induced neurotoxicity, and releasing neurotrophins to support neuronal growth (Galambos, 1961; Barres, 2008; Allen & Barres, 2009). This doctrine has been challenged by increasing evidence that astrocytes could not only respond to synaptic activity in their own right but also modulate synaptic communication actively both in culture and in intact brain slices (Agulhon *et al.*, 2008; Perea *et al.*, 2009; Hamilton & Attwell, 2010).

Astrocytes are passive cells without the ability to generate APs. However, temporal fluctuation of intracellular Ca^{2+} concentration has been widely accepted as a "signature" of excitability in astrocytes (Cornell-Bell *et al.*, 1990; Perea *et al.*, 2009). The elevation of Ca^{2+} could be evoked by at least three means (Agulhon *et al.*, 2010). Firstly, Ca^{2+} elevation can be induced as a result of the mobilisation of intracellular Ca^{2+} store by the binding of the second messenger IP_3 with IP_3 receptors located in the ER in response to stimulation or spontaneously (Pasti *et al.*, 1997; Bezzi *et al.*, 1998; Kang *et al.*, 1998; Kulik *et al.*, 1999; Araque *et al.*, 2002). Secondly, upon repetitive depolarisation of the astrocytic membrane, intracellular Ca^{2+} concentration elevates as a result of Ca^{2+} influx through voltage dependent Ca^{2+} channels or transient receptor potential (TRP) channels which are both confirmed to be expressed on the astrocytic membrane (Cahoy *et al.*, 2008). Thirdly, mechanical stimulation gives rise to Ca^{2+} elevation, possibly through mechanically sensitive receptors (Koizumi *et al.*, 2003). Among these mechanisms, nevertheless, Ca^{2+} elevation in response to the mobilisation of intracellular Ca^{2+} store is by far the most studied mechanism underlying the 'glial excitability' (Perea *et al.*, 2009). This mechanism is morphologically supported by the fact that astrocytes express most of the GPCRs expressed in neurones which are mostly receptors for neurotransmitters and G_q-coupled GPCRs(Porter & McCarthy, 1997).

The neurone-astrocyte communication is in fact bidirectional. On the one hand, several studies have demonstrated that astrocytes vividly respond to synaptic activities by transient intracellular Ca^{2+} fluctuation both in culture and in intact

brain slices. It has been confirmed that the firing of glutamatergic neuronal afferents triggers Ca^{2+} waves in acute and cultured organotypic slices of rat hippocampus (Dani *et al.*, 1992; Pasti *et al.*, 1997). Furthermore, intracellular Ca^{2+} was shown to increase upon stimulation at the Schaffer collaterals, and this effect was blocked by TTX as well as MCPG, suggesting a neuronal origin and mGluR dependency (Porter & McCarthy, 1996). More recently, Perea and Araque demonstrated that Ca^{2+} elevations were evoked by glutamate released from Schaffer collaterals and by acetylcholine, but not glutamate, released by alveus stimulation (Perea & Araque, 2005). In addition, neurotransmitters other than glutamate, such as acetylcholine and noradrenaline were also confirmed to evoke astrocytic Ca^{2+} signal (Kulik *et al.*, 1999; Araque *et al.*, 2002). These data confirm astrocytes could answer synaptic activity in a fashion of Ca^{2+} fluctuation in intact tissue. On the other hand, astrocytes could modulate the communication among neurones directly. Astrocytes release several neuroactive molecules such as glutamate, ATP, and D-serine, which can influence synaptic transmission (Agulhon *et al.*, 2008; Perea *et al.*, 2009).

Several studies showed glutamate release from astrocytes in a Ca^{2+}-dependent manner. An early study confirmed that astrocytes released glutamate in a Ca^{2+} -dependent manner in response to coactivation of the AMPA/kainate and mGluRs on astrocytic membrane both in culture and slices (Bezzi *et al.*, 1998). In another study direct stimulation of astrocytes potentiated miniature inhibitory postsynaptic currents (mIPSCs) in pyramidal neurons, and this effect was abolished by chelating astrocytic Ca^{2+} with BAPTA or by antagonists of the ionotropic glutamate receptors, suggesting a mechanism of Ca^{2+}-dependent glutamate release from astrocytes (Kang *et al.*, 1998). Furthermore, when astrocytes within the immediate vicinity of CA1 pyramidal cells were stimulated in hippocampal slices, the intracellular astrocytic Ca^{2+} wave coincided with a significant increase in the frequency of AMPA spontaneous EPSCs, and the increased frequency of AMPAR currents was sensitive to the group I mGluR antagonists, suggesting Ca^{2+}-dependent glutamate released from astrocytes activated presynaptic group I mGluRs to facilitate glutamatergic transmission (Fiacco *et al.*, 2007). Similarly, NMDA receptor-mediated slow inward currents (SICs) were recorded from CA1 pyramidal cells when DHPG was used, in the presence of TTX, to activate Gq-coupled group 1 metabotropic glutamate

receptors on astrocytes to elevate astrocytic Ca^{2+} concentration (Fellin *et al.*, 2004) Recently, Lee and co-workers reported activation of P2Y receptor, bradykinin receptors and PAR-1, all stimulated glutamate release from astrocytes, potentiating NMDA current in neurons in proximity (Lee *et al.*, 2007). Using PAR-1 as a model system, they further demonstrated that PAR-1 mediated glutamate release from astrocytes was Ca^{2+}-dependent in vitro, and astrocytic PAR-1 activation activated NMDA receptors on neurones in proximity in culture and induced APV-sensitive SICs in brain slices (Lee *et al.*, 2007). Collectively, these evidences strongly support the growing acceptance of Ca^{2+}-dependent release of glutamate from astrocytes.

However, conflicting results were also reported favouring the argument that the glutamate release from astrocytes was not Ca^{2+}-dependent. Different mouse models have been genetically engineered to allow astrocyte-specific manipulation of intracellular Ca^{2+} concentrations (Fiacco *et al.*, 2007; Petravicz *et al.*, 2008; Agulhon *et al.*, 2010). In one mouse model, the transgenic mice were introduced a Gq-coupled receptor MrgA1, which is normally not expressed in the brain, selectively into astrocytes (Fiacco *et al.*, 2007). Specifically elevating intracellular astrocytic Ca^{2+} concentration did not increase neuronal Ca^{2+}, produce neuronal SICs, or affect excitatory synaptic activity, which questioned the developing consensus that glutamate release by astrocytes in vivo is Ca^{2+}-dependent (Fiacco *et al.*, 2007). In addtion, in another mouse model where IP_3 mediated Ca^{2+} mobilisation was selectively prevented in astrocytes by deletion the gene PP3R2, which encodes the only astrocytic IP_3 receptor on the ER, spontaneous EPSCs and tonic NMDA-mediated currents recorded from CA1 pyramidal cells were unaffected, suggesting the removal of astrocytic Ca^{2+} increases did not affect excitatory neuronal activity and ambient glutamate levels (Petravicz *et al.*, 2008). Recently, utilising these two mouse lines, it was further demonstrated that neither increasing nor obliterating astrocytic Ca^{2+} fluxes affected spontaneous and evoked excitatory synaptic transmission together with short- or long-term synaptic plasticity at the Schaffer collateral-to-CA1 synapse (Agulhon *et al.*, 2010). Collectively, these data strongly challenge the developing general acceptance of the Ca^{2+} dependency of glutamate release from astrocytes.

How could this starkly contrasting conclusion among different labs be reconciled (Hamilton & Attwell, 2010; Kirchhoff, 2010)? Differences in experimental setups or method-induced artifacts may be responsible for the diverging results. For example, the introduction of an exogeneous G_q-coupled receptor MrgA1 in astrocytes (Fiacco *et al.*, 2007) would not necessarily overlap with the endogenous astrocytes signalling pathways. But rather focus on methodological reasons, it is perhaps more reasonable to consider the possibility of differential release mechanisms for glutamate may exist within potentially different groups of astrocytes. Moreover, it is also possible that within a given astrocyte, the requirement of Ca^{2+} in releasing glutamate or other gliotransmitters would vary in different subcellular regions of the cell (Kirchhoff, 2010). In other words, there could be more than one mechanism within a given astrocyte in governing the gliotransmitter release. Interestingly, one recent study has demonstrated the possible existence of two forms of astrocytic calcium excitability, whereby the activation of astrocytic PAR-1 receptors but not $P2Y_1$ receptors, although both elevated astrocytic Ca^{2+}, resulted in glutamate release, as APV-sensitive-NMDA-mediated SICs were recorded (Shigetomi *et al.*, 2008).

Apart from glutamate, D-serine, which is exclusively released from astrocytes has been demonstrated to modulate synaptic transmission and plasticity, and postulated as the endogenous ligand for the glycine site of NMDA receptors (Schell *et al.*, 1995; Mothet *et al.*, 2000; Panatier *et al.*, 2006). Recent literature further suggested that tonic D-serine release enhanced NMDA receptor activity in neurones, and the binding of D-serine to glycine site in NMDA receptor was required in LTP induction in cultured hippocampal neurones (Yang *et al.*, 2003). In addition, the degree of glycine site occupancy in NMDA receptors by D-serine affected the activation of NMDA receptor, thereby affecting long term synaptic plasticity (Panatier *et al.*, 2006). The concept that astrocytic release of D-serine regulates normal synaptic function was strongly boosted by a very recent paper reporting that the release of D-serine from astrocytes was required for establishing LTP at the Schaffer collateral-to-CA1 synapse (Henneberger *et al.*, 2010). Collectively, these data support the hypothesis that astrocytic release of D-serine is important in regulating synaptic transmission and plasticity.

ATP has also been demonstrated as a key player in astrocyte-to-neurone communication, inducing heterosynatpic depression of excitatory transmission (Koizumi *et al.*, 2003; Zhang *et al.*, 2003; Pascual *et al.*, 2005; Serrano *et al.*, 2006). It was first shown in hippocampal cultures that the activation of presynaptic P2 receptors depressed glutamatergic synaptic transmission in an ATP-dependent manner as a result of astrocytic Ca^{2+} wave in response to mechanical stimulation (Koizumi *et al.*, 2003). This discovery was independently confirmed by the following report that neuronal activity activated astrocytes to tonically release ATP, thus suppressing glutamatergic synapses via presynaptic P2Y receptors in culture and slices (Zhang *et al.*, 2003). The adenosine, the product of ATP degradation, was also found in modulating heterosynaptic depression in hippocampal slices by activating presynaptic A1 receptors (Serrano *et al.*, 2006). The involvement of ATP in mediating tonic heterosynaptic depression was further evidenced by using transgenic mice to selectively impair gliotransmitter release by inducing a dominant-negative SNARE domain (Pascual *et al.*, 2005). Collectively, these data demonstrated that astrocytic released ATP or its degradation product, adenosine, depresses glutamatergic synaptic transmission by activating presynaptic P2 or A1 receptors in the hippocampus.

In summary, as presented in figure 1.7, it appears that astrocytes not only listen to, and respond to synaptic activity but also bear the ability to release “gliotransmitters” to modulate synaptic activity. This bidirectional glia-neurone interaction has given rise to the concept of “tripartite synapses”, where in addition to pre and post synaptic neurones, there is a functional component of astrocytes. The functional cross talk among these three parties shed light on synaptic transmission and plasticity (Agulhon *et al.*, 2008; Perea *et al.*, 2009; Hamilton & Attwell, 2010).

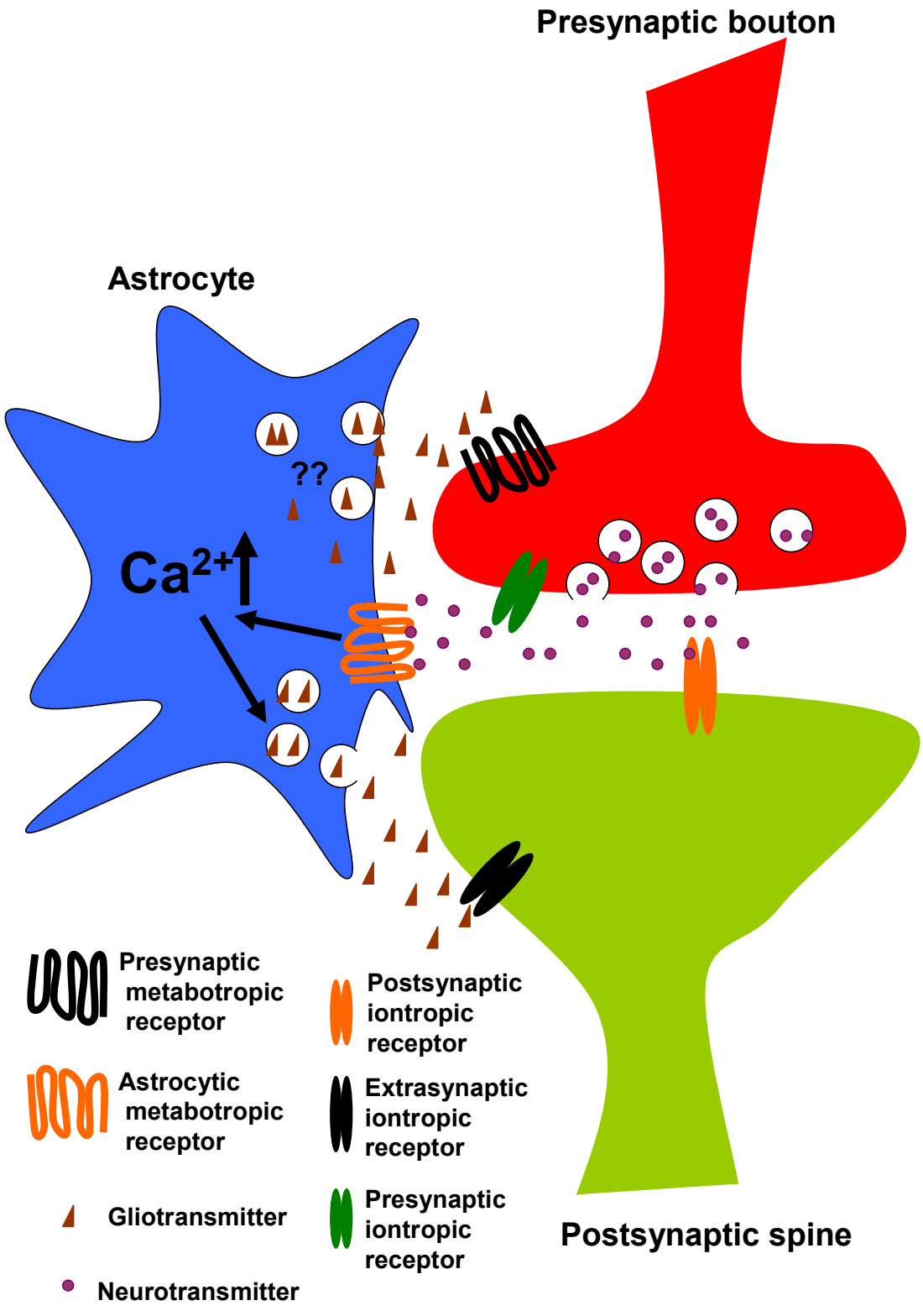


Figure 1-7 Schematic illustration of the concept of tripartite synapse.

(Adapted and modified from Agulhon C, et al., 2008 (Agulhon *et al.*, 2008))

1.9 Conclusion and aims

Albeit low level, PAR-2 is extensively expressed in normal and pathological brain, including the hippocampus. During pathological conditions, an increase of PAR-2 expression has been documented in diseases such as ischaemia, Parkinson's disease, HIV-associated dementia, Alzheimer's disease, and multiple sclerosis, suggesting that PAR-2 plays important roles in pathophysiological processes in the brain. Depending on the cell type in which increased PAR-2 expression is observed, PAR-2 has been proposed to play 'dual face' role, either degenerative (glial) or protective (neuronal). The hippocampus is the most studied region in the brain with detailed elucidation of its highly organized neural circuits. Acute slice preparation and primary hippocampal culture are well established experimental models in investigating neuronal and synaptic functions in the hippocampus *in vitro* with readily accessibility. Therefore, based on the fact that PAR-2 is indeed expressed in the hippocampus as verified independently by a number of groups, and given the functional roles that it plays in a variety of pathological conditions in the brain, it is reasonable to examine whether PAR-2 contributes to normal neuronal signalling under physiological conditions in the hippocampus, and if so, how this modulation behaves in terms of cellular and synaptic physiology. Furthermore, does PARs activation exert same or differential modulation of cellular neurophysiology in different neuronal circuits or neuronal types in the brain?

In conclusion, the central hypothesis of my PhD is that PAR-2 serves as 'molecular switches' in normal neural signalling in the hippocampus, modulating neuronal excitability, basal synaptic transmission and plasticity, as well as network activities.

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

1. HOW DOES PAR-2 ACTIVATION AFFECT EXCITABILITY OF HIPPOCAMPAL NEURONES?
2. HOW DOES PAR-2 ACTIVATION MODULATE SYNAPTIC TRANSMISSION AND SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS?

3. HOW DOSE PAR-2 ACTIVATION INFLUENCE ACTIVITIES AND GROSS EXCITABILITY OF NEURAL NETWORKS IN THE HIPPOCAMPUS?
4. HOW DOSE PAR-2 MEDIATE MODULATORY ACTIONS IN THE HIPPOCAMPUS AT THE CELLULAR AND SYNAPTIC LEVELS?

2 Materials and Methods

2.1 Primary hippocampal culture experiments

2.1.1 Preparation of primary culture of hippocampal neurones

Cultured hippocampal neurones were prepared as previously described (Greenwood et al., 2007). As presented in figure 2.1, 1-2 days old, male and female, Sprague Dawley rat pups were killed by cervical dislocation according to U.K. Home Office Schedule 1 guidelines under the authority of U.K. Animals (Scientific Procedures) Act, 1986. At least 3 rat pups were sacrificed in each culture preparation. The skull was opened through the midline by a sterilised No. 22 scalpel blade (Swann Morton Limited, UK). The whole brain was rapidly removed by a spatula, placed on a piece of sterilised filter paper and bisected by a sterilised No.11 blade (Swann Morton Limited, UK). Hippocampi were carefully dissected away from surrounding brain tissue from both hemispheres and submerged into a sterilised preparation buffer filtered by 0.2μm pore diameter syringe filter unit (Cronus, UK) of the following composition:

NaCl (116mM)

KCl (5.4mM)

$NaHCO_3$ (26mM)

NaH_2PO_4 (1.3mM)

$MgSO_4 \cdot 7H_2O$ (1mM)

$CaCl_2 \cdot 2H_2O$ (1mM)

Ethylenediaminetetraacetic acid (EDTA) (0.5mM)

Glucose (25mM)

PH was adjusted to 7.4

Hippocampi were then, transferred onto a sterilised lid of a 35mm diameter cell culture dish (BDbiosciences, UK), and finely chopped into pieces by a sterilised No.11 scalpel blade to increase surface area. Processed tissue was transferred into a 15ml centrifuge tube (Cronus, UK) filled with 5ml fresh preparation buffer supplemented with 1.5mg/ml papain (Sigma, UK) at 37 degree Centigrade for 20 minutes to allow enzymatical dissociation of cells. The tissue was then transferred to 6ml fresh preparation buffer supplemented with 10mg/ml bovine serum albumin (BSA) (Sigma, UK), to stop papain's digesting activity, and triturated with three flame-polished glass Pasteur pipettes of decreasing diameter from 3mm to 1mm. The tissue suspension was then centrifuged at 2000rpm for 3 minutes. A small pellet formed in the bottom of centrifuge tube following centrifuge, and was removed by a pipette (Gilson, UK) with minimal preparation buffer, and resuspended in a fresh 15ml centrifuge tube filled with Neurobasal-A media (Invitrogen, UK) supplemented by 2% (volume) B-27 (Invitrogen, UK) and 2mM L-glutamine (Invitrogen, UK) with a cell density of 5 X 10^5 cells/ml.

Following enzymatical and mechanical dissociation, cells were plated onto sterilised 13mm diameter glass coverslips coated with L-Lysine (200μg/ml, at least 1 hour to ensure good cell adhesion) at a density of $3.5X10^5$ cells/ml. Coverslips with cell suspension were maintained in 35mm diameter cell culture dishes (3 coverslips in 1 culture dish) in an incubator with an environment of 95%

O_2/ 5% CO_2 at 37 degree Centigrade for at least 1 hour to allow cells to settle down. After this period, each culture dish was supplied by 2ml Neurobasal-A media supplemented by B-27 and L-glutamine with same concentrations described above. No antibiotic agent was employed in all culture preparations. After 5 days in culture, cytosine-D-arabinofuranoside was added with 10μM final concentration to inhibit glial proliferation.

Cultures were monitored daily with any culture infected by bacteria or fungi as indicated by the change of media colour from pink to yellow being disregarded. Health conditions of neurones were examined under microscope in terms of gross morphology, processes branching pattern, and cell settlement status (clustered or scattered). Unhealthy cultures were excluded in the following experiments.

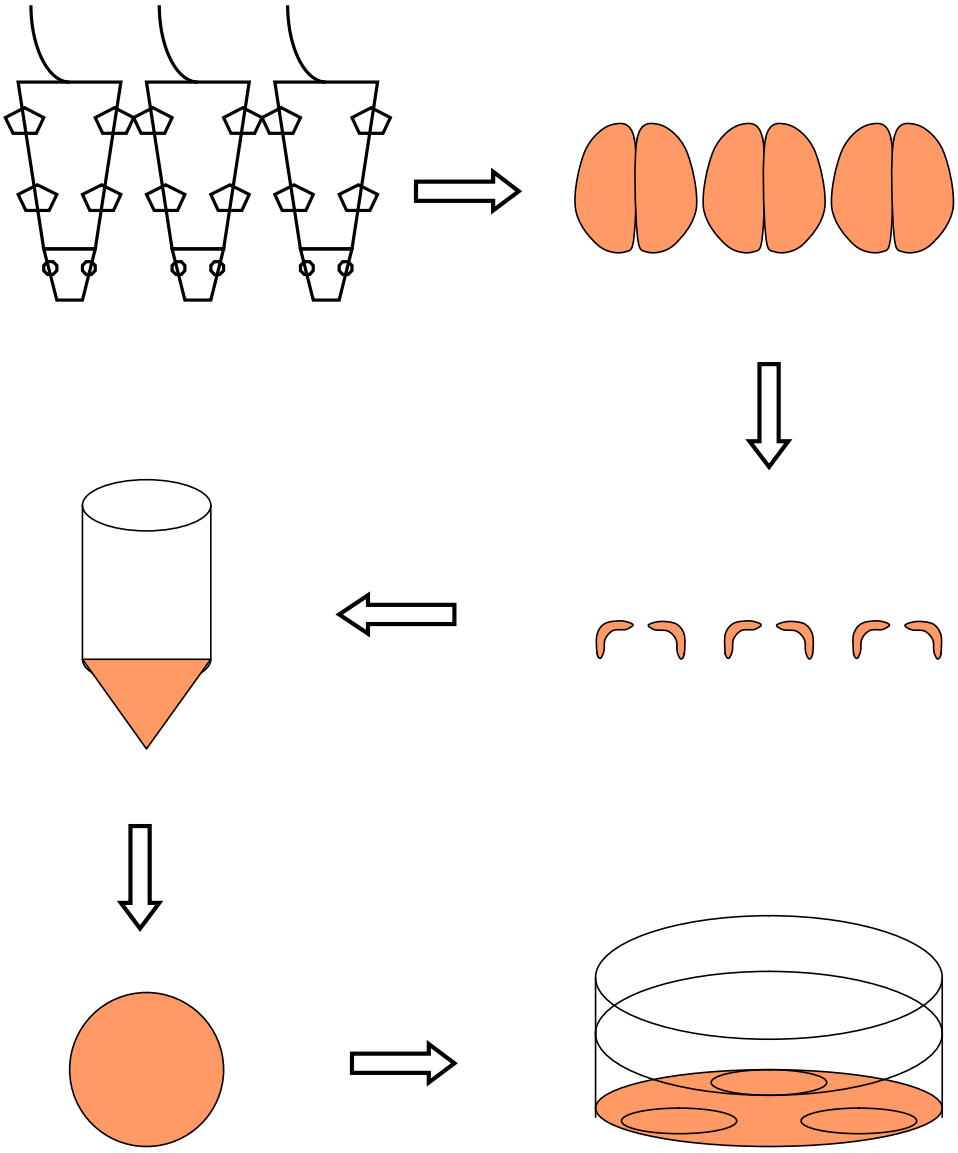


Figure 2-1 Schematic flow chart of primary hippocampal culture preparation.

2.1.2 Patch clamp recordings in cultured hippocampal neurones

2.1.2.1 Patch clamp recording setup

As shown in figure 2.2, a recording platform (Gibraltar Burleigh, Canada) was mounted with a submerged recording chamber (Custom design), a micromanipulator (EXFO Burleigh PCS-6000-150, Canada) and an upright microscope (Olympus, USA). This itself was fixed on an anti-vibration table (Intracel system 2000). A faraday cage covered the whole recording system to protect it from external electrical noises. All electrical equipment including the amplifier (Axoclamp 200B, Axon instrument, USA), the digitizer (Digidata 1322A, Axon instrument, USA), and the micromanipulator power supply (EXFO Burleigh PCS-6000-150, Canada) were secured in an equipment rig and commonly grounded from the anti-vibration table.

Filled glass pipettes were mounted into a head stage (Axon instrument, USA) with the intracellular solution making electrical contact with the silver chloride electrode. A silver chloride bath reference electrode was also connected to the head stage. Subsequently, the head stage was connected to the Axoclamp 200B amplifier. Neuronal activities in the experiments were captured by the amplifier and filtered by a built-in low pass Bessel filter at 2 KHz, digitised by a digitizer (Digidata 1322A, Axon instrument, USA) at 10 KHz and stored in a personal computer (Dell, USA).

2.1.2.2 Recording procedure

Both voltage clamp and current clamp recording were performed in DIV (days in vitro) 13-16 days cultured neurones because of their matured status with stable

intrinsic properties. Glass micropipettes (1.5mm O.D. x 0.86mm I.D. Harvard Apparatus, UK) were pulled by an electrode puller (DMZ-Universal, Germany), and filled with an intracellular solution with the following composition with a final resistance of 3-5MΩ:

KCl (150mM)

$MgCl_2$ (1mM)

$CaCl_2$ (1mM)

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10mM)

EGTA (0.5mM)

Mg-ATP (3mM)

GTP (0.3mM)

pH was adjusted to 7.2

Osmolarity was adjusted to 290mOsm

Cultured neurones were continuously perfused by a HEPES based saline solution with the following composition:

NaCl (140mM)

KCl (5mM)

$MgCl_2$ (2mM)

$CaCl_2$ (2mM)

HEPES (10mM)

D-Glucose (10mM)

pH was adjusted to 7.4

Osmolarity was adjusted to 310mOsm.

Whole cell voltage and current clamp recordings were obtained from cultured hippocampal neurones under microscopic visualization. Briefly, a healthy neurone was chosen by its shiny, smooth cell surface. No positive pressure was employed during advancing the electrode towards the targeted cell. A 10mV voltage step was applied through the pipette to generate a current pulse to calculate the electrode resistance. Once the electrode tip touched the cell membrane (seen by a robust reduction of the amplitude of the current pulse), a slight negative pressure was applied to the pipette to form a high resistance seal. A further application of -70mV voltage command facilitated the formation of 'gigoseal'. Once the 'gigoseal' was achieved, short suctions were given to rupture the membrane patch but leaving the 'gigoseal' intact to obtain the whole cell patch clamp configuration.

During pharmacological manipulation, chemical agents were supplied through the perfusate. Either whole cell voltage or current clamp mode was chosen according to experimental design. In voltage clamp mode, holding currents and spontaneous synaptic currents were recorded, whereas in current clamp mode, membrane potential and spontaneous synaptic potentials were monitored. During each experiment, fast capacitance was fully compensated. No slow

capacitance and series resistance compensation were applied. Input resistance was not monitored throughout the recording process.

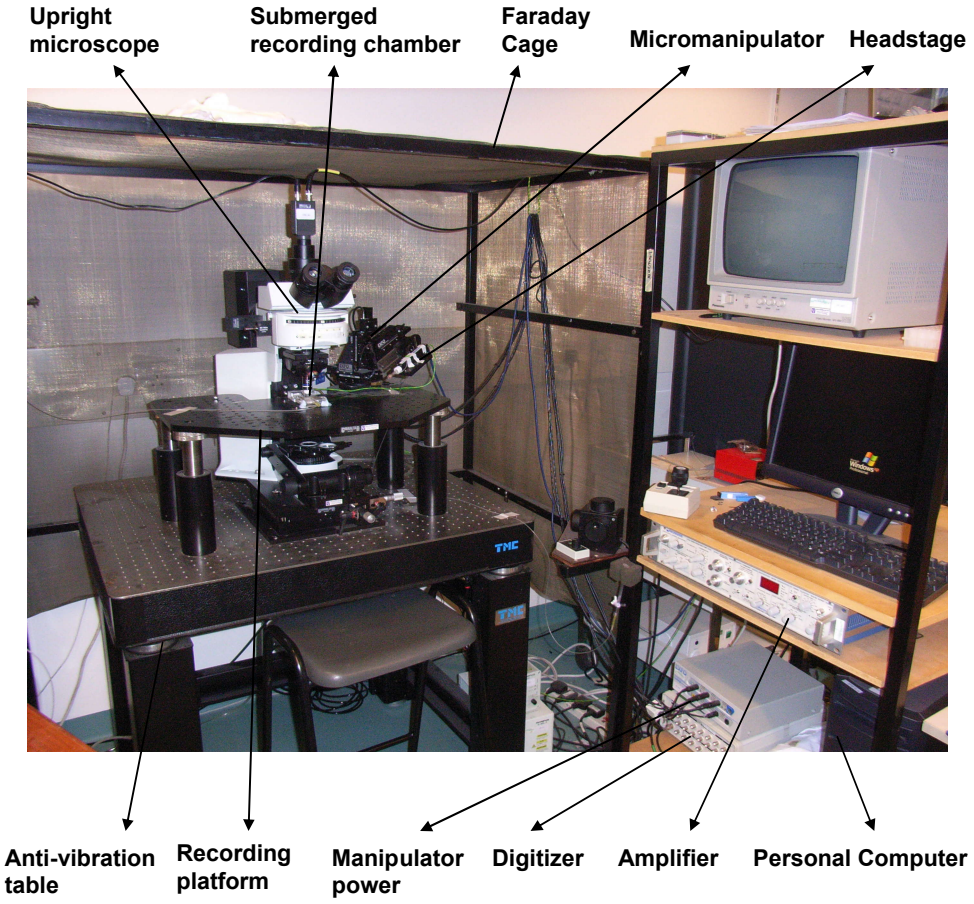


Figure 2-2 Photograph showing the actual setup used in patch-clamp experiments.

2.2 Acute hippocampal slice experiments

2.2.1 Preparation of acute hippocampal slices

Male and female Wistar rats (16-24 days old) were killed by cervical dislocation, followed by decapitation in accordance with U.K. Home Office Schedule 1 guidelines under the authority of the U.K. Animals (Scientific Procedures) Act, 1986.

The brain was removed quickly and immersed in ice-cold (0-4 degree Centigrade) oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) solution of the following composition (in mM): NaCl,124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $MgSO_4$ 1; D-glucose, 10; $CaCl_2$, 2 (McNair *et al.*, 2006). As shown in figure 2.3, the brain was further hemisected, and each part was glued to the stage of a vibrating microtome (Campden Instruments Ltd). Transverse whole brain slices (400μm) were cut in the parasagittal plane and hippocampal regions dissected free from surrounding brain tissue before being transferred to an interface holding chamber in a humidified and oxygen-enriched (95% O_2, 5% CO_2) atmosphere for at least 1 hour for equilibrium from slicing trauma. Subsequently, slices were transferred into a humidified and oxygen-enriched (95% O_2, 5% CO_2) atmosphere interface recording chamber maintanined at 28-32 degree Centigrade and perfused by ACSF oxygenated by 95% O_2 and 5% CO_2 at a flow rate of 6-8ml/minute powered by a peristaltic pump (Minipuls 3, Gilson, France) for subsequent recording.

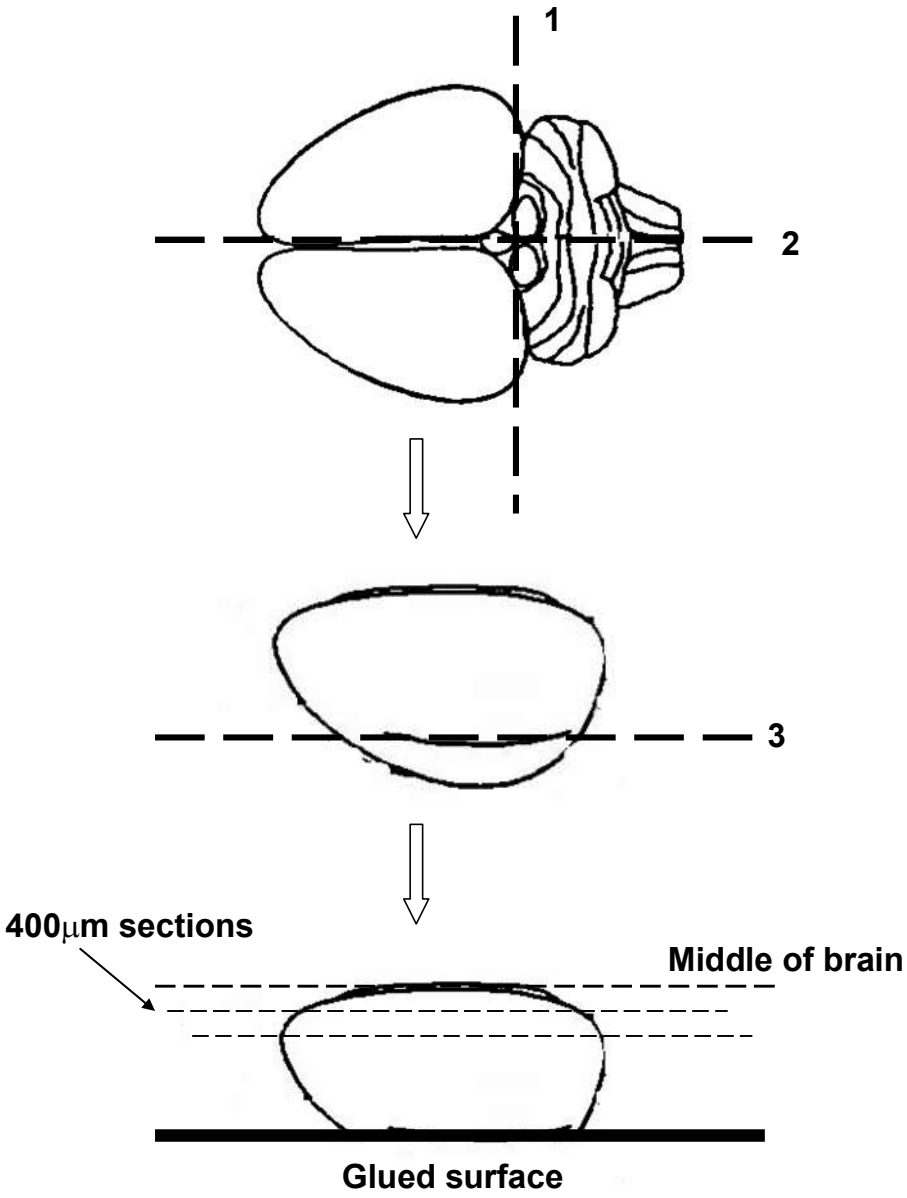


Figure 2-3 Process and orientation of parasagittal slice preparation.

2.2.2 Extracellular recording in acute hippocampal slice preparations

2.2.2.1 Extracellular recording setup

An interface recording chamber (HAAS-type, Berlin), a binocular (Zeiss, Germany), two micromanipulators (Narishige, Japan; Leica, Germany) and a lamp were secured on an anti-vibration table (Intracel, UK). Alongside, an amplifier (AxoClamp 2B, Axon instrument, USA), a digitizer (Digidata 1320A, Axon instrument), a 50Hz line noise eliminator (Hum Bug, Quest scientific), an instrumental amplifier (Model 440, Brownlee precision) and a PC were secured into a rig. A photo of this setup is presented in figure 2.4.

The experimental design is as described previously (McNair *et al.*, 2006). Stimulating current was delivered through a bipolar tungsten stimulating electrode of 3.5MΩ resistance (World Precision Instruments, USA) using an isolated constant current stimulator (Model D52A, Digitimer Ltd., UK). The current range is from 0-30mA. Pulse duration is 0.1ms. Recording electrodes were made from standard walled (1.2mm O.D. X 0.69 I.D.) borosilicate glass capillaries (Harvard Apparatus, UK) on a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Co., USA). The electrodes (3-5MΩ) were filled with ACSF solution and were connected via a silver chloride pellet containing electrode holder to a headstage (HS2A, Axon Instrumental).

Pre-amplification (x100) was carried out by AxoClamp 2B amplifier (Axon instruments, USA). Further amplification (x20) was carried out by a Brownlee instrumentation amplifier (Model 440, Brownlee Precision, USA) giving a total amplification of x2000. A Hum Bug 50/60Hz noise eliminator (Quest Scientific,

UK) was used to eliminate 50Hz line frequency noise where necessary. Data were captured by Digidata 1320A analogue-to-digital acquisition board (Axon Instrument) and transferred directly to the hard-disc of a PC (Viglen Pentium 4) at a rate of 10 KHz.

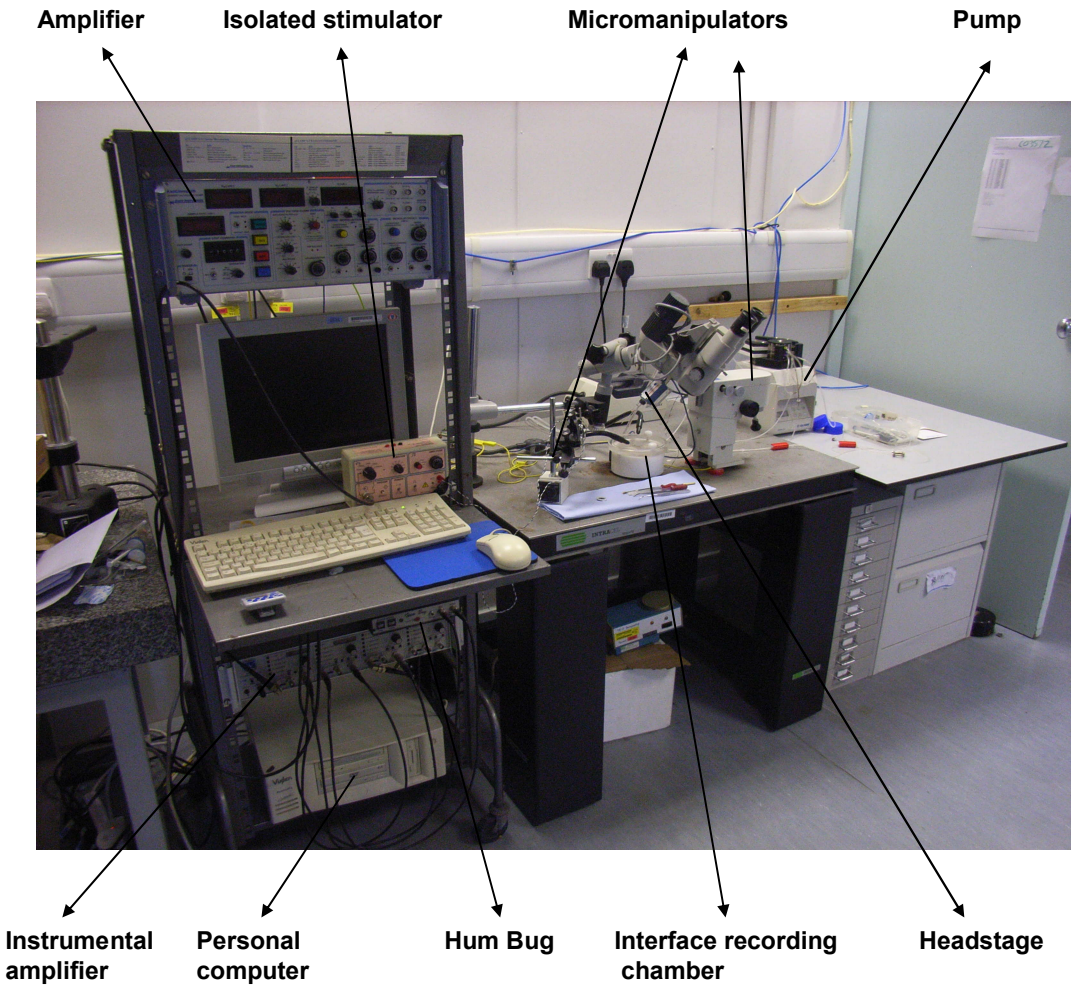


Figure 2-4 Photograph showing the actual setup used in extracellular recording experiments.

2.2.2.2 Extracellular recording procedure

After at least 1 hour equilibration time for hippocampal slices, a single recording electrode was placed on the *stratum radiatum* in the CA1 region of hippocampal slice. A single stimulating electrode was placed in *stratum radiatum* of either side of recording electrode to stimulate Schaffer collateral pathway. Small stimulating current pulses (0-30mA, 0.5ms) were administrated to test for the presence of field excitatory postsynaptic potentials (fEPSP) responses. Once synaptic potentials were observed, input-output (I/O) curve was obtained by gradually increasing stimulating current and monitoring fEPSP responses (see figure 2.5). The stimulating current which gave the half maximum fEPSP response was then used for the remainder of experiment. Measurements of synaptic response were typically monitored in terms of initial slope. The window for slope measurement was typically in the range of 2 to 6 ms following stimulation.

As shown in figure 2.6, fEPSPs were evoked at frequency of 0.05 Hz in the *stratum radiatum* of area CA1. Paired-pulse facilitation (PPF) was investigated at 50ms intersimulus interval. High-frequency stimulation paradigm of 100Hz high frequency stimulation (HFS) for 1 second was used to induce long term potentiation (LTP). Synaptic efficacy was monitored at least 20 minutes before and 60 minutes after induction by recording fEPSP responses every 20s (traces were averaged for every minute interval). Slices that did not exhibit stable fEPSP slopes during 20 minutes baseline recording were excluded from the study. For studies to investigate the action of PARs activation during *in vitro* epileptiform activity, field epileptiform activities were recorded from in *stratum pyramidale* of area CA3 in response to the application of the potassium channel

blocker 4-aminopyridine (4-AP, 100μM) & 0 Mg^{2+} or $GABA_A$ receptor antagonist bicuculline (20 μM) via the perfusate.

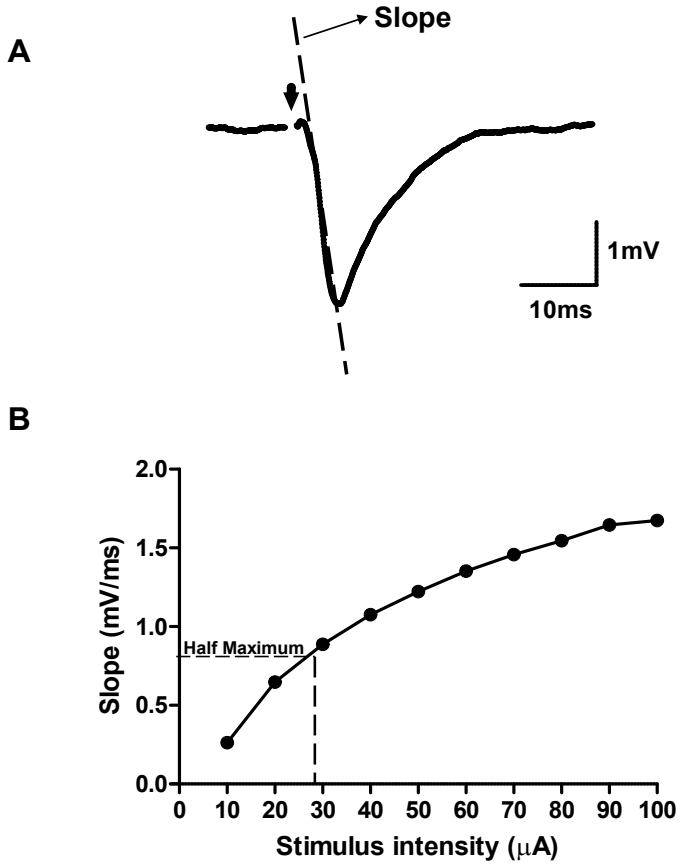


Figure 2-5 Glutamatergic synaptic transmission in the CA1 region of rat hippocampus.

A. Example of an excitatory post synaptic potential (EPSP) recorded in the *stratum radiatum* of area CA1 of rat hippocampus. Arrow represents time of stimulation. Note the dashed line indicates slope measurement.

B A representative Input-output curve recorded in the same experiment. Note the recorded pathway required approximately 28μA to reach a half maximum response (dotted line).

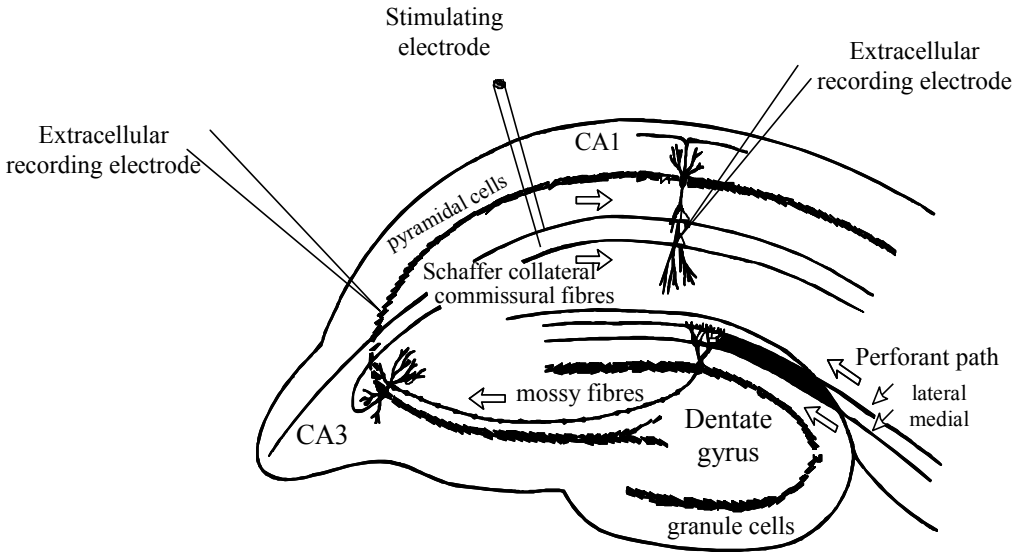


Figure 2-6 Schematic diagram illustrating the positions of electrodes for extracellular field recordings.

2.3 Data analysis

All data from patch-clamp recording in primary culture was analysed online and offline using the software package pClamp9 (Axon Instrument, USA). All data from extracellular recordings in acute hippocampal slices were analysed online and offline using the software packages WinLTP (Anderson & Collingridge, 2007), Excel 2003 (Microsoft, USA) and pClamp9 (Axon Instrument, USA). Epileptiform activities in chapter 5 were analysed using threshold based detection mode in pClamp9 (Axon Instrument, USA). All data were graphically illustrated using GraphPad Prism 5.0 (GraphPad Software, USA). Data are presented as means ± standard error of the mean (SEM). The n value refers to the number of times a particular experiment was performed.

Statistical significance was determined by Student t-test, paired Student t-test. Statistical test was performed using Graph Pad 5.0 (GraphPad Software, USA). If not indicated in the context, in chapter 3, either mean (for bar charts) or peak values (for time plots) of drug effect were compared with controls. In chapter 4 and 5, either mean values (for bar charts) or values from the last minute of drug application period (for time plots) were tested with controls. A probability value of less than 0.05 was taken as indicating statistical significance.

2.4 Drugs and Chemicals

PAR peptides, namely, PAR-2-APs, SLIGRL-NH_2 and 2-furoyl-LIGRL-NH_2, PAR-2 inactive peptide (PAR-2-IP), LRGILS-NH_2, PAR-1 activating peptide (PAR-1-AP), TFFLRL-NH_2, and PAR-4 activating peptide (PAR-4-AP), AYPGKF-NH_2 were purchased from either the Peptides Synthesis Facility at the University of Calgary (Canada) (http://wcm2.ucalgary.ca/peptides) or Peptides Synthetics (UK) (www.peptidesynthetics.co.uk). All the other chemicals employed in this thesis were obtained from Ascent Scientific (UK), Sigma-Aldrich (UK) or VWR (UK).

All drugs were dissolved in physiological saline employed according to experimental design (ACSF or HEPES based buffer). PAR peptides solutions were made fresh immediately before each experiment. All the other drugs were prepared in physiological saline by 1 in 1000 dilution from stock aliquots to the final working concentration.

3 The action of PAR activation on neuronal excitability and synaptic transmission in cultured hippocampal neurones

3.1 Introduction and aims

As described in Chapter 1, PARs are expressed widely throughout the central nervous system. In the hippocampus, PARs have been confirmed to be expressed in neurones, astrocytes, oligodendrocytes, and microglia (Striggow *et al.*, 2001; Noorbakhsh *et al.*, 2003; Bushell *et al.*, 2006; Luo *et al.*, 2007). In recent years, PAR involvement during pathological conditions in the brain have been extensively described (Noorbakhsh *et al.*, 2003; Luo *et al.*, 2007), however, their potential modulatory roles in regulating intrinsic neuronal and synaptic properties during physiological conditions are only partially understood. Indeed, cellular level study has shown PAR-1 involvement in normal neuronal signalling in the brain (Almonte *et al.*, 2007). However, much less is known about PAR-2 receptors in this context.

Primary hippocampal cultured neurones are extensively utilised as an *in vitro* system to investigate neuronal and synaptic properties of glutamatergic and GABAergic neurones. Despite obvious shortcomings of this system in terms of a lack of normal patterns of brain connectivity and the need to obtain samples from immature tissues, the system nevertheless has many technical benefits. For instance, it is easy to identify and patch or image single neurone or glia. Being a reduced system, it is also readily accessible to pharmacological and other manipulations. Due to these factors, I decide to utilise this culture system in my experiments to provide an initial characterisation of PAR-2 receptor actions in central neurones.

The specific aim of the experiments described in this chapter was to evaluate the possibility that PARs activation modulates neuronal excitability and synaptic transmission in cultured neurones. For this, I utilised whole cell patch clamp recording technique in dissociated hippocampal neurones.

3.2 Characterisation of spontaneous activities in cultured hippocampal neurones under whole-cell voltage clamp configuration

In order to evaluate whether PAR-2 activation has modulatory effect on synaptic transmission in the network formed by cultured hippocampal neurones, whole-cell voltage clamp recordings were made initially in 10 cultured hippocampal neurones from 5 separate culture preparations (~3-4 rats for each) to monitor spontaneous synaptic activities. Upon obtaining whole-cell recording configuration, membrane potentials of neurones was first monitored before the cell was voltage-clamped at -70mV. All neurones with a resting membrane potential more positive to -55mV were disregarded. Mean holding current in these experiments was -32.52 ± 11.52pA (n=10). As shown in one representative recording (Fig 3.1), the spontaneous whole cell synaptic currents consisted of two major activities. 1) Subthreshold excitatory post synaptic currents (EPSCs). 2) Superthreshold EPSCs compounded with AP discharges. This activity was characterised with the spatiotemporal summation of several EPSCs, which broke in the threshold, resulting in AP firing.

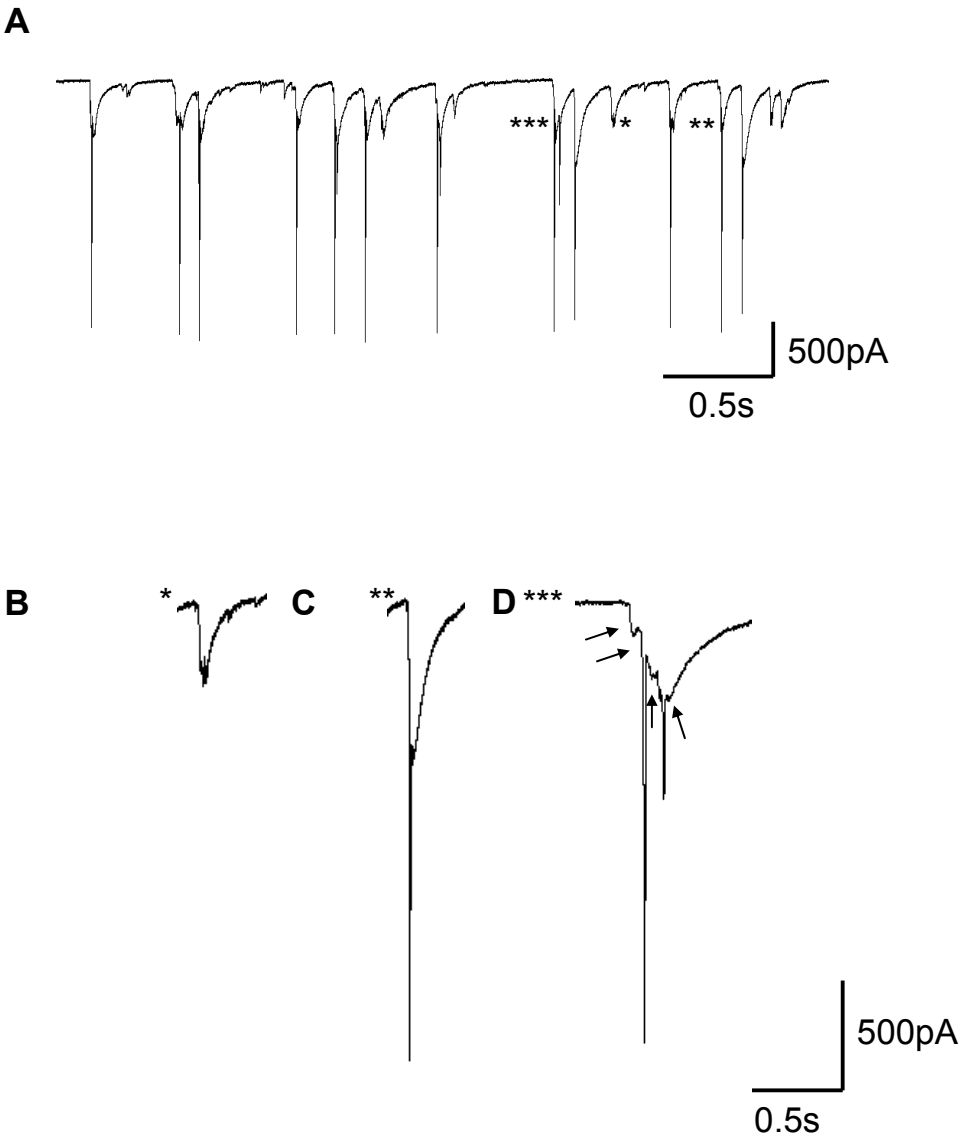


Figure 3-1 Characterisation of spontaneous activities in cultured hippocampal neurones under voltage clamp.

A. Representative current trace from a cultured hippocampal neurone (cell number 170507/1) clamped at -70mV. Note the presence of both spontaneous inward synaptic currents* (EPSCs) and large events representing unclamped action potentials. B. Excerpts of trace showing (B) a representative subthreshold EPSC and (C) a representative action potential event. D. Current trace showing summation of EPSCs (arrows) leading to an action potential event and loss of voltage clamp***.**

3.3 Trypsin increases holding current and spontaneous activity in cultured hippocampal neurone under voltage clamp

PAR-2 is preferentially activated by the proteinase, trypsin (Macfarlane *et al.*, 2001; Ossovskaya & Bunnett, 2004). Thus, trypsin was used as a PAR-2 activator to test the effect of PAR-2 activation on cultured hippocampal neurones in initial experiments. Whole-cell voltage clamp recordings were carried out on 10 cultured hippocampal neurones from 5 separate preparations. As presented in Fig 3.2B, application of 100nM trypsin of 5 minutes duration induced a significant yet reversible increase of holding current value from -27.84 ± 11.65pA to -548.62 ± 160.40pA (P=0.0055, n=10). Upon washout, the holding current recovered back to -58.13 ± 13.54pA. The sustained inward current upon trypsin application would be consistent with a membrane depolarisation where the cells were held under physiological current clamp conditions. The recordings also revealed a significant yet reversible increase of the frequency of spontaneous synaptic activities in these neurones from 5.51 ± 0.86Hz to 8.24 ± 1.05Hz (P=0.0092, n=10) in response to 100nM trypsin application as presented in Fig 3.2C. These data suggest that trypsin gives rise to an increase of frequency of synaptic inputs experienced by the recorded neurone.

One interpretation of this result is that the membrane depolarisation and increased EPSC frequency is due to activation of PAR-2 receptors in response to trypsin application. However, whilst trypin shows relative selectivity towards PAR-2, it has been shown to activate other PARs, albeit at higher concentrations (Noorbakhsh *et al.*, 2003; Ossovskaya & Bunnett, 2004). Moreover, the proteolytic action of trypsin on the cell membrane could lead to bioactive processes other than PARs activation. For instance, proteolytic action may release soluble ligands, activate enzyme precursors, and inactivate agonists (Ossovskaya & Bunnett, 2004). Thirdly, trypsin is potentially a stressor for the cultured neurones given its nature of digesting activity. Therefore, these data from the initial experiments suggest that the trypsin-induced membrane depolarisation and enhanced synaptic activities in cultured neurones may be partially mediated by PAR-2 activation, but the alternative explanations

described above can not be ruled out. In subsequent experiments, I therefore switched to the use of PAR-2-AP, SLIGRL.

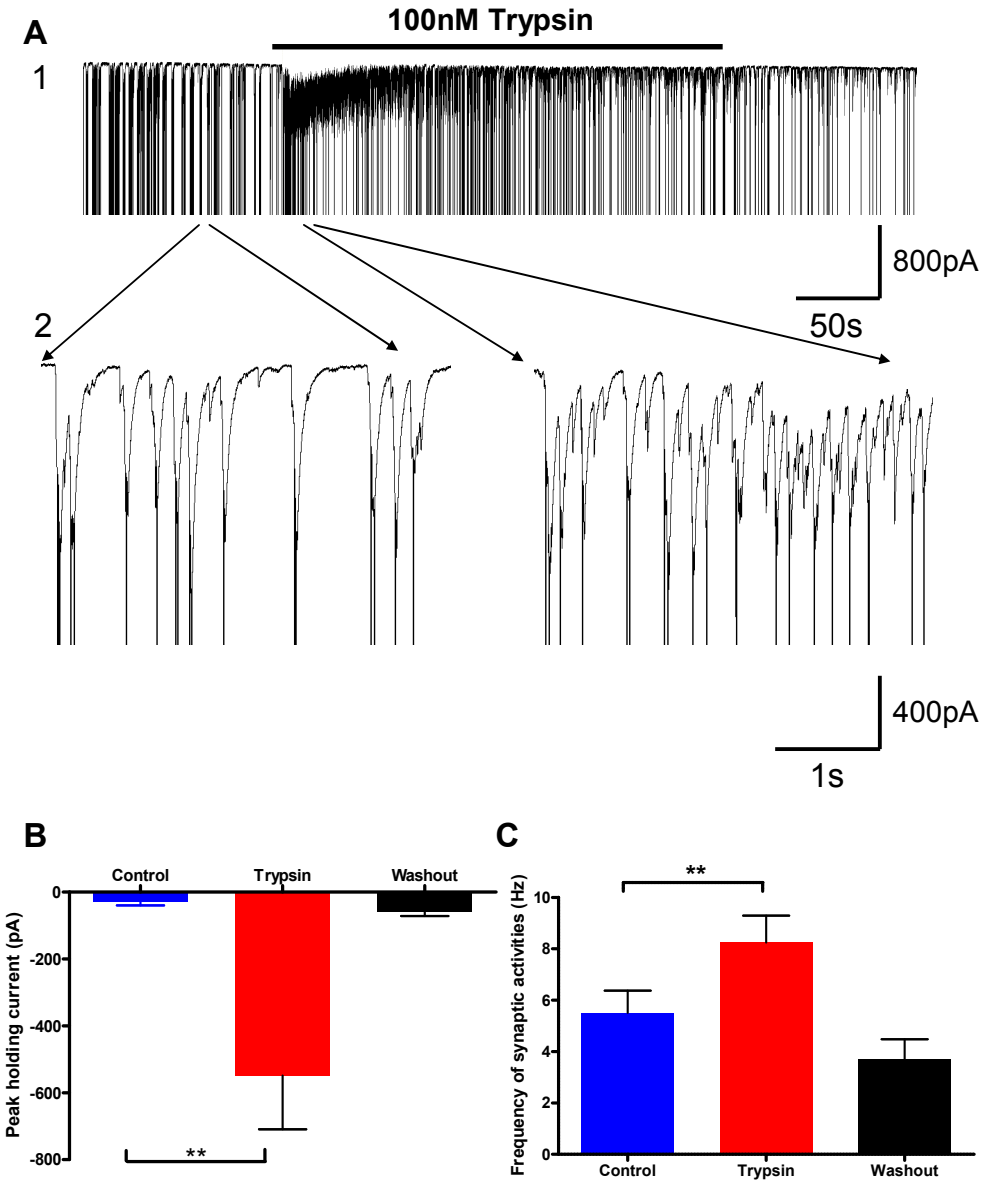


Figure 3-2 Action of trypsin on cultured hippocampal neurones.

A. Representative recording (cell number 200607/3) illustrating the effect of 100nM trypsin on holding current and spontaneous activities. Excerpts before and during trypsin application as shown on an expanded time scale below. Note an increase of the holding current immediately following trypsin (100nM) application and the substantial increase in the occurrence of subthreshold inward synaptic currents. B. Bar chart summarising quantitative analysis of holding current before and after trypsin application. Note that 100nM trypsin results in a significant but reversible increase in holding current (n=10, P=0.0055). C. Bar chart showing a significant increase in the frequency of spontaneous inward synaptic currents following 100nM trypsin application (n=10, P=0.0092).

3.4 Characterisation of spontaneous activities in cultured hippocampal neurones under whole-cell current clamp configuration

The high levels of spontaneous APs observed in the cultured neurones recorded previously under voltage clamp conditions demonstrated space clamp problems and specifically, an inability to adequately clamp neurones sufficient to avoid AP discharge. I therefore switched recording configuration in all subsequent cellular studies to current clamp mode. This had the added advantage of maintaining the neurones under more physiological conditions.

All neurones, with membrane potential more positive than -55mV, were excluded from the data set. Initially, 7 cultured hippocampal neurones from 4 separate preparations were recorded to characterise the electrical properties and the spontaneous activities in these cells. The mean resting membrane potentials of these neurones was -61.31 ± 0.56mV (n=7). As demonstrated by a representative voltage trace in Fig. 3.3, the cells recorded under baseline conditions displayed spontaneous activities consisted of two major events: 1) Subthreshold excitatory postsynaptic potentials (EPSPs), which had a mean amplitude of 8.46mV ± 0.58mV with a mean frequency of 0.72 ± 0.25 Hz (n=7) 2) Spontaneous overshooting APs with a mean amplitude of 88.12 ± 4.72mV with a mean frequency of 1.45 ± 0.25 Hz (n=7).

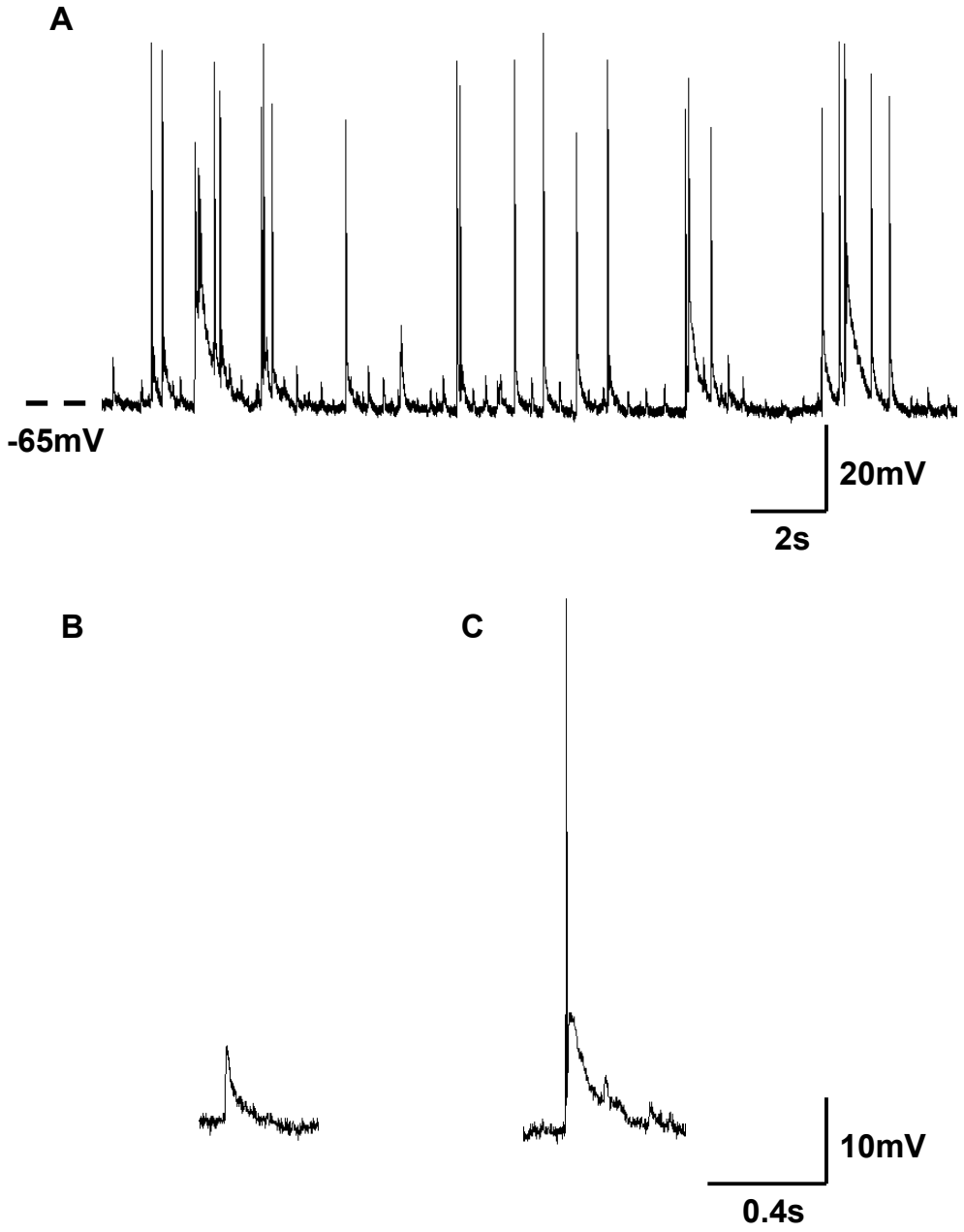


Figure 3-3 Characterisation of spontaneous activities in cultured hippocampal neurones under current clamp.

A. Representative voltage trace from a cultured hippocampal neurone (cell number 210607/2) at resting membrane potential (zero current injection). Note the presence of both spontaneous subthreshold excitatory postsynaptic potentials (EPSPs) and suprathreshold EPSPs giving rise to action potentials. B. Excerpts of trace above showing (B) a representative subthreshold EPSP and C. a representative suprathreshold EPSP / action potential.**

3.5 Trypsin depolarises cultured hippocampal neurones and increases the occurrence of spontaneous action potentials

Initially, trypsin was used to repeat the experiments conducted previously under voltage clamp conditions. Bath application of trypsin (100nM) significantly depolarised the membrane potential of the recorded neurones with the mean magnitude of 15.50 ± 5.29mV (n=7, P=0.026, figure 3.4C). An increase of AP frequency was also noticed following trypsin application. However, due to the high variance, this effect did not achieve statistical significance (251.11 ± 81.52% of control level, n=7, P=0.11, figure 3.4D). Given trypsin is reported to irreversiblely cleave and activate the functional PAR-2s on the cell membrane, a further application of trypsin was introduced to test whether the second application was able to evoke further membrane depolarisation and / or increase AP discharge. In contrast to the first application in which all cell respond with a depolarisation, overtly, the second application resulted in neither significant depolarisation (8.10 ± 4.90mV, n=7, P=0.1497, figure 3.4E) nor increase of AP frequency (174.14 ± 56.98% of control, n=7, P=0.2375, figure 3.4F). Dissecting into these cells more closely, only 3 of 7 cells responded to a second application with 4 out of all 7 neurones showing no detectable change in membrane potential (0.63 ± 0.99mV, n=4, figure 3.4B) or change in firing frequency (110.15 ± 26.77% of control, n=4, figure 3.4B). However, in 3 of 7 neurones examined, the second application of trypsin (100nM) produced a similar magnitude of depolarisation (mean magnitude = 19.72 ± 6.92mV, n=3) and increase in AP frequency (260.86 ± 121.66%, n=3). Again however, these effects did not achieve statistical significance, presumably due to low replicate numbers.

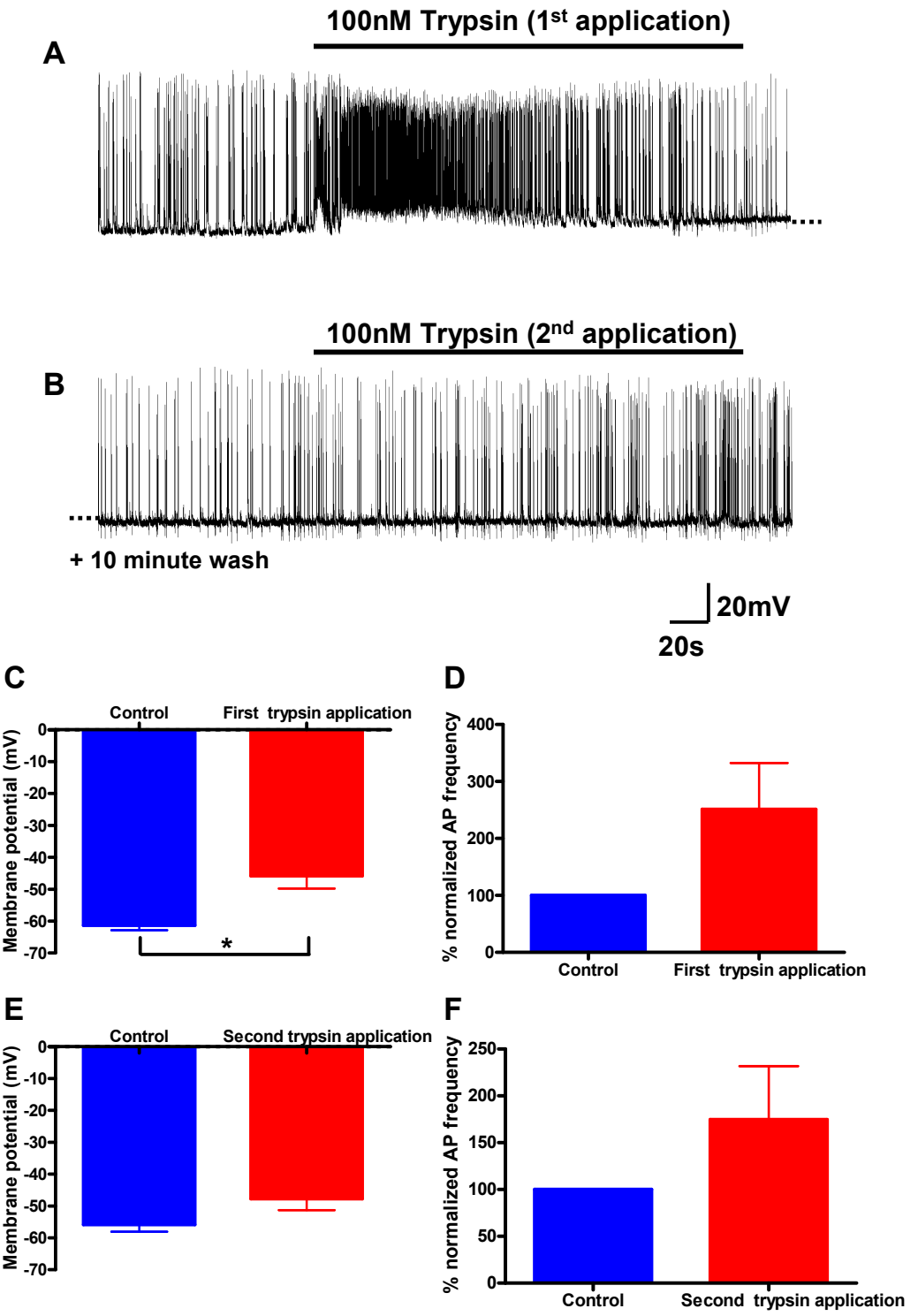


Figure 3-4 Effect of repeated trypsin application on membrane potential and action potential frequency.

A. Representative current clamp recording upon application of 10nM trypsin and showing the characteristic depolarisation and increase in EPSP and action potential frequency. B. Following a 10 minute washout period, a second application of 100nM trypsin did not produce any detectable response. C-E. Bar chart showing pooled data. Note that a significant membrane depolarisation was observed (C) yet increase of firing frequency (D) did not achieve significant upon 1st trypsin application. 2nd application of trypsin did not cause significant membrane depolarisation (E) and increase of AP frequency (F).

3.6 PAR-2 activating peptide, but not PAR-2 inactive peptide depolarises neuronal membrane and depresses spontaneous action potential firings

In order to refine the experimental approach and discriminate the involvement of PAR-2 receptors over other PAR subtypes, the synthetic PAR-2 activating peptide (PAR-2-AP), SLIGRL-NH_2, was used. This peptide agent has been confirmed previously to possess high selectivity for murine PAR-2 (EC_{50}=5μM) (Nystedt *et al.*, 1994; al-Ani *et al.*, 1995; Nystedt *et al.*, 1995b; Saifeddine *et al.*, 1996). It is utilised to overcome the disadvantage of lack of selectivity and the potential 'off-target' effect in previous experiments with trypsin. 100μM concentration, which has been used in expression system to evoke maximal response of PAR-2 (Nystedt *et al.*, 1995b), and was similar to previous study in investigating Ca^{2+} mobilisation by PAR-2 activation in cultured hippocampal neurones and astrocytes (Bushell *et al.*, 2006), was selected initially to test the effect of PAR-2 activation in cultured hippocampal neurones. Under current clamp recording, as presented in figure 3.5, bath application of PAR-2-AP significantly depolarised the neuronal membrane with the mean magnitude of 9.19 ± 1.22mV (n=5, P=0.0016). Furthermore, alongside the membrane depolarisation, a significant depression of spontaneous AP firings was observed (65.72 ± 4.62% compared to control level, n=5, P=0.013). These data suggested PAR-2 activation depolarised neuronal membrane in hippocampal culture under autonomous resting membrane potential conditions. However, the quantification of this depolarisation was hampered by the fact that the neurones being tested were of differential resting membrane potentials (range = -70mV to -60mV). To eliminate this variable, neurones were monitored under an equal baseline membrane potential of -65mV (close to the recognised physiological value), by tonic current injection (<200pA). Under these conditions, three concentrations of PAR-2-AP were tested. Interestingly, bath application of PAR-2-AP (1, 10, and 100μM) induced depolarisation of neuronal membrane in a concentration-dependent manner with the mean magnitude of 0.62 ± 0.86mV (1μM, n=12, P=0.4872), 1.39 ± 0.58mV (10μM, n=11, P=0.038), and 5.52 ± 1.48mV (100μM, n=16, P=0.026) respectively as shown in figure 3.6B. This was accompanied by a concentration-dependent depression of spontaneous APs to 85.36 ± 11.84% (1μM,

n=7, P=0.9314), 75.79 ± 6.11% (10μM, n=6, P=0.1403) and 29.63 ± 5.03% (100μM, n=13, P=0.0001) of control (drug free) conditions respectively as shown in figure 3.6C.

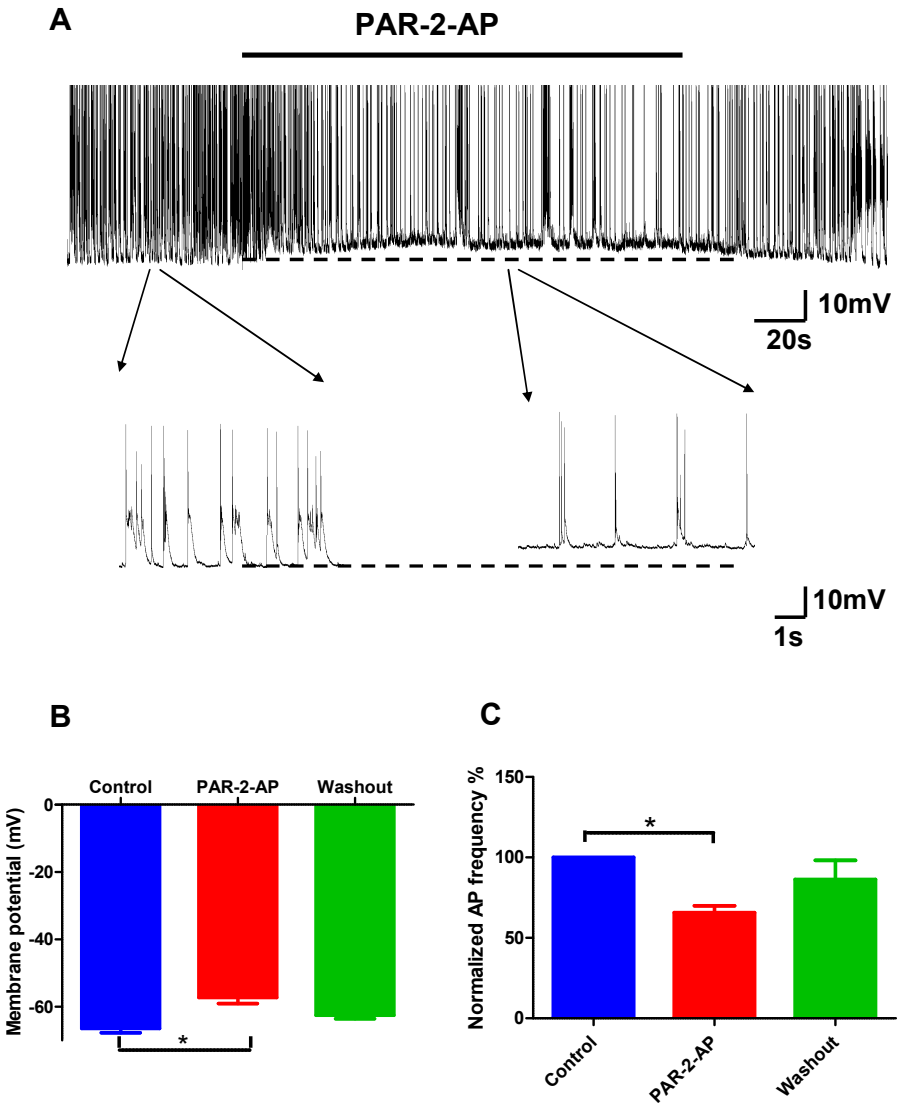


Figure 3-5 Action of the PAR-2-AP, SLIGRL, on cultured hippocampal neurones under autonomous membrane potential.

A. Representative recording (cell number 141107/1) illustrating the effect of 100μM PAR-2-AP on the holding current and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-2-AP application as shown on an expanded time scale below. Note the sustained depolarisation immediately following PAR-2-AP (100μM) application and the substantial decrease in the occurrence of spontaneous action potentials. B. Bar chart summarising quantitative analysis of membrane potential before and after PAR-2-AP application (n=5, P=0.0016). C. Bar chart showing a significant decrease in the frequency of spontaneous action potentials following 100μM PAR-2-AP application (n=5, P=0.013).

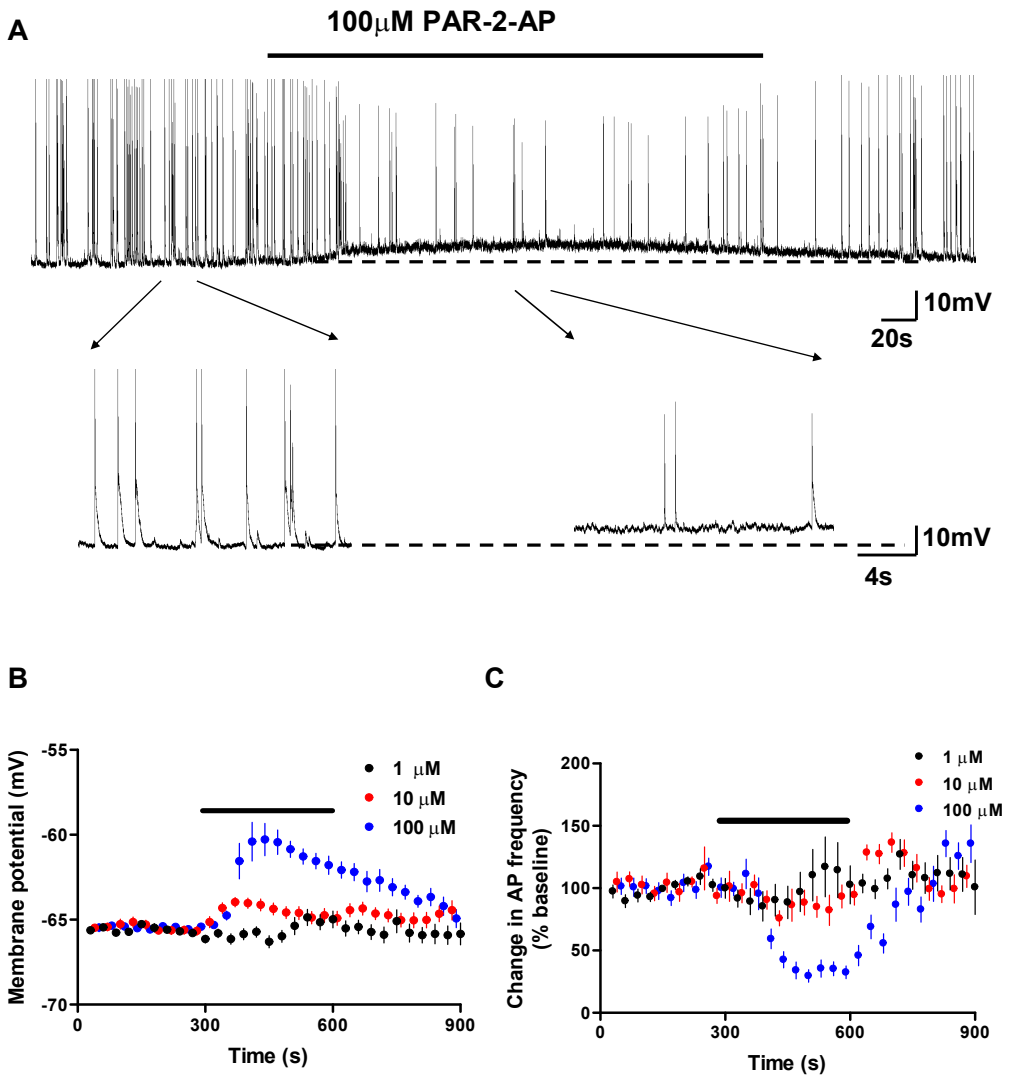


Figure 3-6 Action of the PAR-2-AP, SLIGRL on cultured hippocampal neurones clamped at -65mV.

A. Representative recording (cell number 121108/2) illustrating the effect of 100μM PAR-2-AP on holding current and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-2-AP application as shown on an expanded time scale below. Note that all cells received somatic current injection to set the membrane potential to -65mV prior to drug application. B. Time plot showing the action of 1, 10 and 100μM PAR-2-AP on mean membrane potential. Quantitative analysis revealed a concentration-dependent depolarisation (1μM, n=12, P=0.4872; 10μM, n=11, P=0.038; 100μM, n=16, P=0.026). C. Time plot showing a concentration-dependent decrease in the frequency of spontaneous action potentials following the application of 1, 10 and 100μM PAR-2-AP application (1μM, n=7, P=0.9314; 10μM, n=6, P=0.1403; 100μM, n=13, P=0.0001).

To further verify the membrane depolarisation and depression of spontaneous APs were indeed a result of selective PAR-2 activation rather an artefact introduced by the chemical nature of synthetic peptide, synthetic PAR-2 inactive peptide (PAR-2-IP), LRGILS-NH_2, which has a scrambled (reversed) amino acid sequence compared to PAR-2-AP was applied as a 'negative control'. Again, these experiments were conducted both under resting membrane conditions and with the cells maintained at -65mV through somatic current injections. As expected, bath application of 100μM PAR-2-IP did not depolarise neurones maintained at resting membrane potential (control: -63.04 ± 1.53mV, upon PAR-2-IP: -59.98 ± 2.64mV, n=10, P=0.16, figure 3.7B). Furthermore, no significant change in the frequency of spontaneous APs was observed (117.05 ± 10.62% compared to control level, n=9, P=0.15, figure 3.7C). Similarly, PAR-2-IP applied to neurones maintained at -65mV showed no change in membrane potential (0.61 ± 0.31mV, n=8, P=0.0915, figure 3.8B) or AP frequency (91.55 ± 7.63%, n=8, P=0.3049, figure 3.8C).

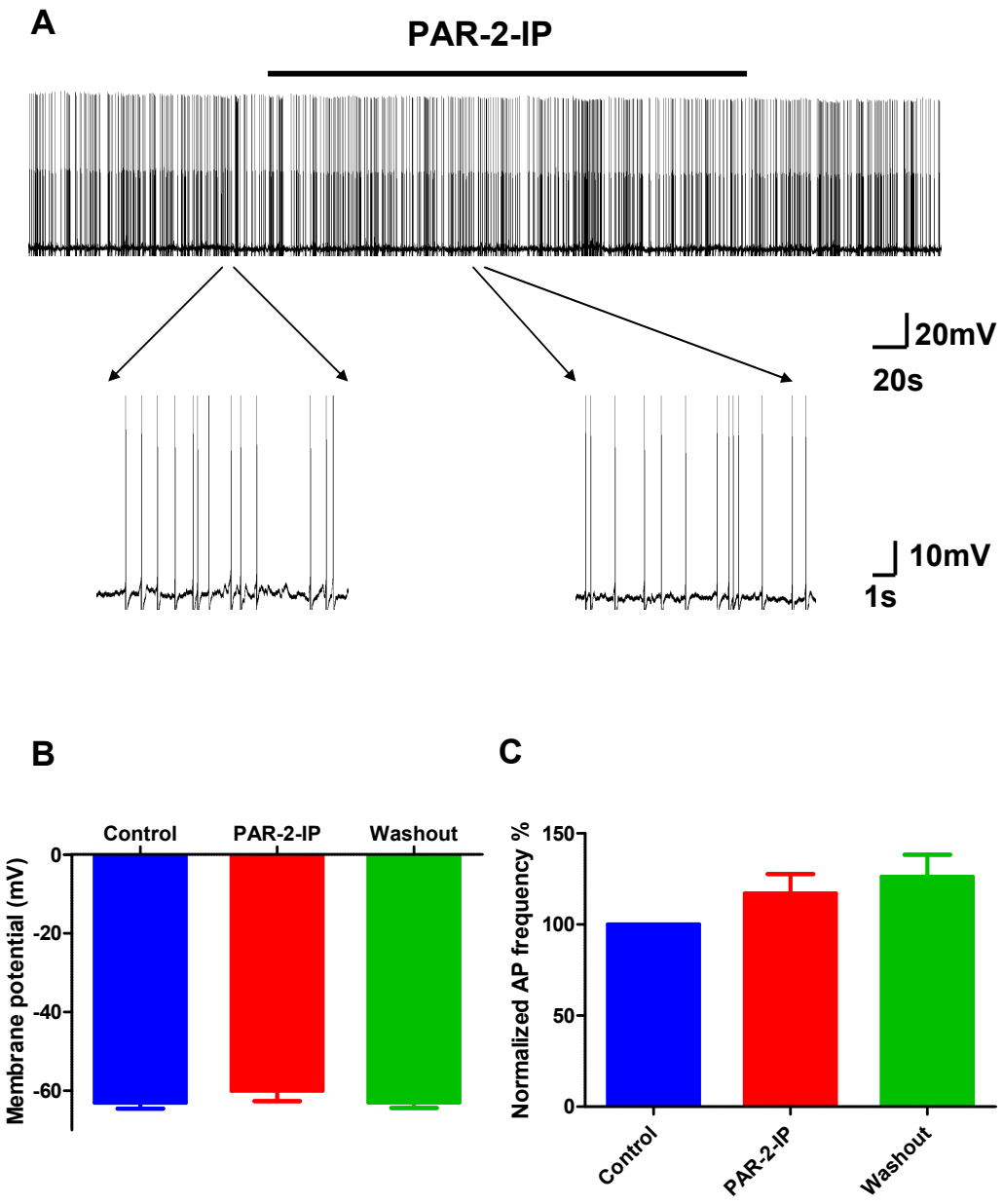


Figure 3-7 Action of the PAR-2-IP on cultured hippocampal neurones under autonomous resting membrane potential.

A. Representative recording (cell number 200308/2) illustrating the effect of 100μM PAR-2-IP on holding current and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-2-IP application as shown on an expanded time scale below. Note absence of any detectable change in membrane potential or spontaneous action potential frequency following PAR-2-IP application. B. Bar chart summarising quantitative analysis of membrane potential before and after PAR-2-IP application (n=10, P=0.16). C. Bar chart of pooled data showing no significant change in the frequency of spontaneous action potentials following 100μM PAR-2-IP application (n=9, P=0.15).

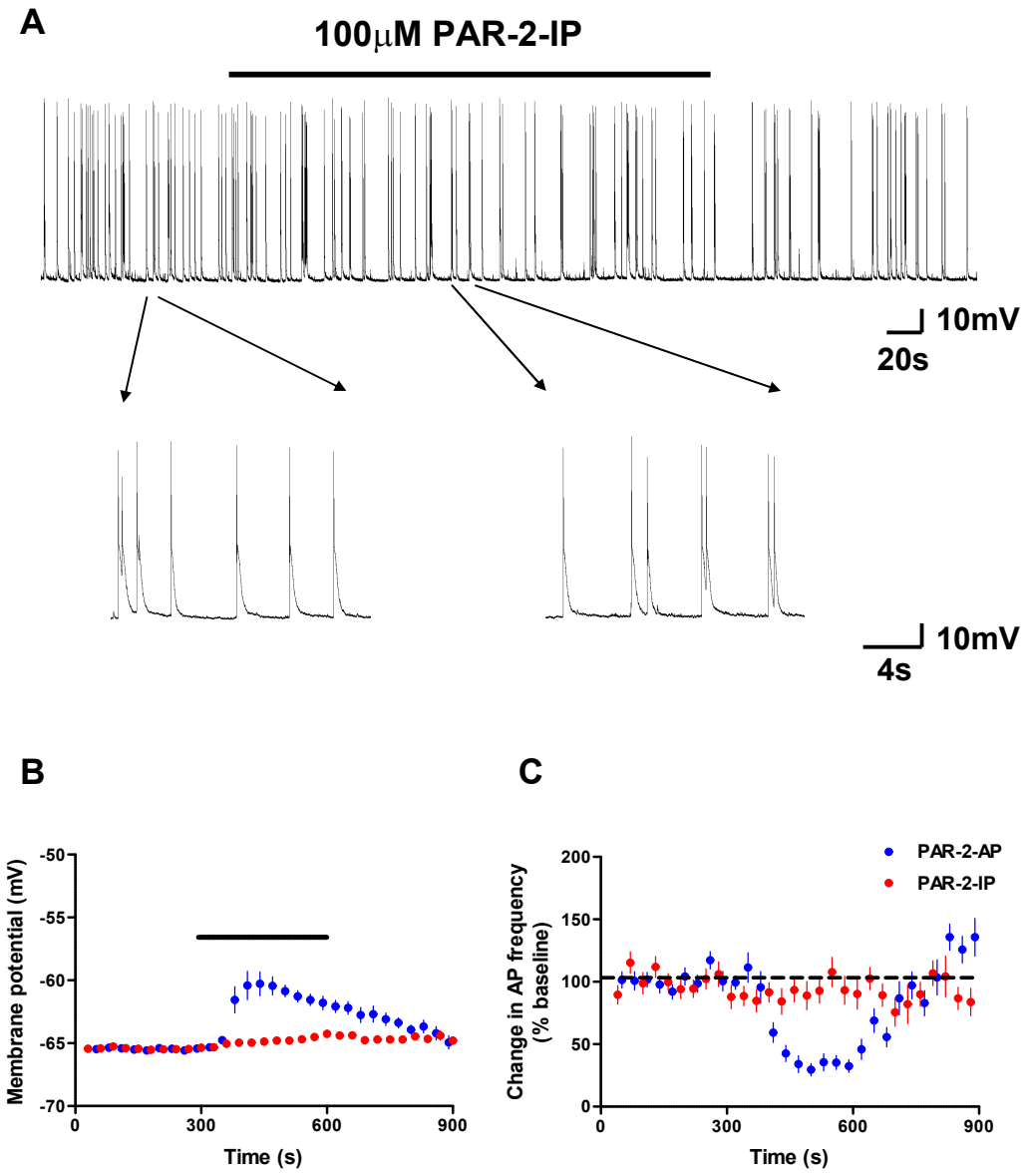


Figure 3-8 Action of the PAR-2-IP on cultured hippocampal neurones normalised to membrane potential of -65mV.

A. Representative recording (cell number 080409/1) illustrating the effect of 100μM PAR-2-IP on holding current and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-2-IP application as shown on an expanded time scale below. Note that all cells received somatic current injection to set the membrane potential to -65mV prior to drug application. B. Time plot showing the action of 100μM PAR-2-IP on mean membrane potential. Quantitative analysis revealed no significant change in membrane potential (n=8, P=0.09, cf. robust depolarisation following PAR-2-AP, blue symbols). C. Time plot showing no significant change in the frequency of spontaneous action potentials following the application of 100μM PAR-2-IP application (n=8, P=0.30, cf. robust decrease of action potential frequency following PAR-2-AP, blue symbols).

3.7 PAR-1 and PAR-4 activating peptides depolarise membrane of cultured hippocampal neurones.

After establishing the fact that PAR-2 activation induced membrane depolarisation and depressed spontaneous AP firings in cultured neurones, my investigation extended to assess whether the other two functional PAR family members, PAR-1 and PAR-4, have modulatory roles in cultured hippocampal neurones. To this end, 100μM PAR-1 activating peptide (PAR-1-AP), TFLLR-NH_2, was applied to cultured neurones under identical recording conditions. As presented in figure 3.9, a significant membrane depolarisation was observed (11.61±1.50mV, n=8, P=0.0078). However, unlike that seen with PAR-2-AP, the PAR-1-AP did not cause any significant change in the frequency of spontaneous APs (103.90±9.08%, n=8, P=0.68). This clear membrane depolarisation but lack of effect of AP frequency was apparent both when cells where maintained at resting membrane potential and at -65mV (membrane depolarisation: 3.26±0.27mV, n=12, P=0.0006, figure 3.10B; change of firing frequency: 105.89±14.06%, n=10, P=0.7301, figure 3.10C).

Finally, we also assessed the action of the PAR-4-AP, AYPGKF-NH_2. These studies were hampered by the very limited availability of the peptide but preliminary data showed a depolarisation (8.35±5.07mV, n=4, P=0.19, 100μM) but this did not achieve statistical significance. Similar to PAR-1 response, no change was detected in frequency of spontaneous APs upon PAR-4-AP application (110.68±13.80%, n=4, P=0.49).

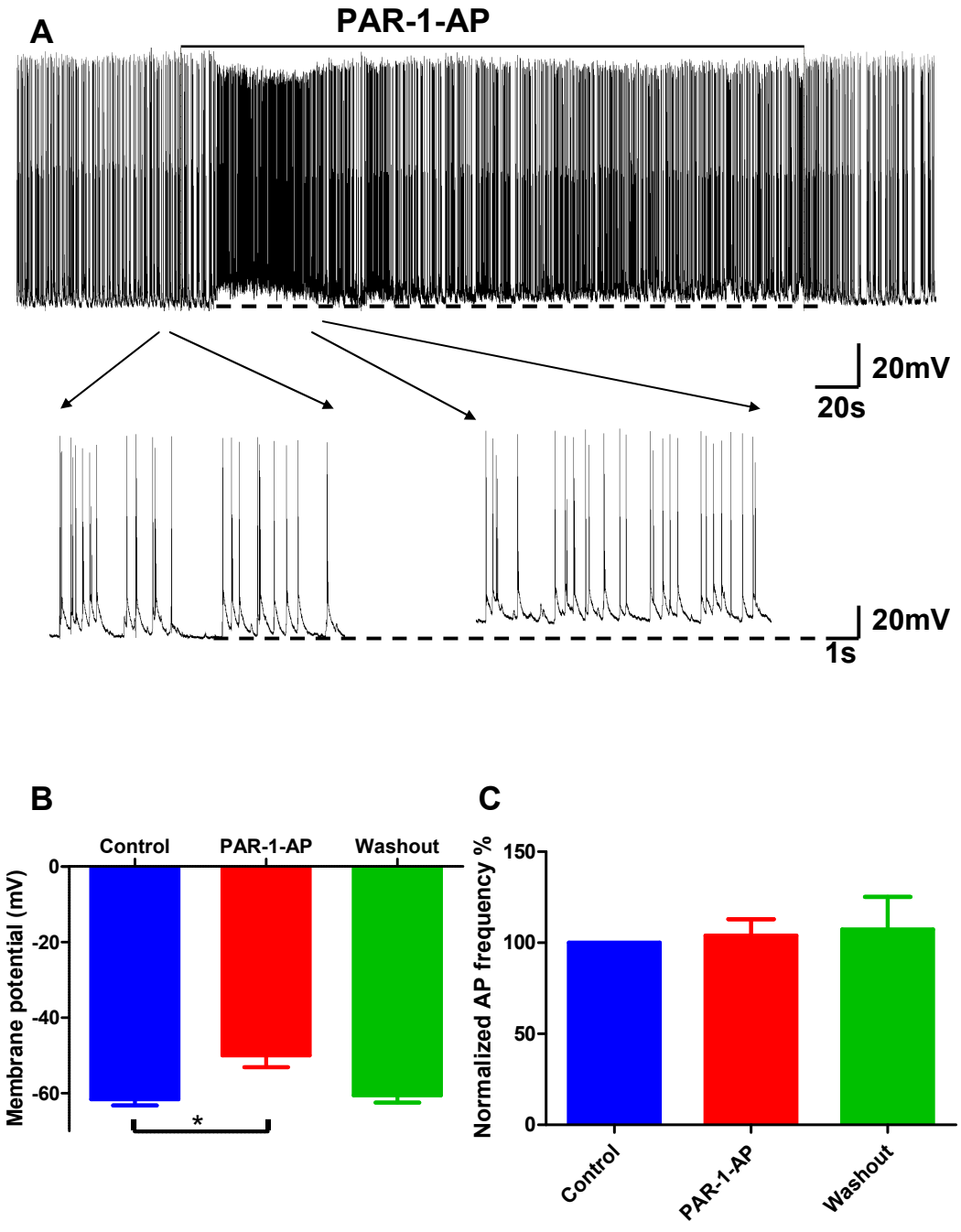


Figure 3-9 Action of the PAR-1-AP on cultured hippocampal neurones under autonomous resting membrane potential.

A. Representative recording (cell number 111107/3) illustrating the effect of 100μM PAR-1-AP on membrane potential and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-1-AP application as shown on an expanded time scale below. Note the depolarisation and transient decrease in the occurrence of spontaneous action potentials immediately following PAR-1-AP (100μM). B. Bar chart showing a significant membrane depolarisation following PAR-1-AP application (n=8, P=0.0078). C. In contrast, changes in the frequency of spontaneous action potentials following 100μM PAR-1-AP application did not achieve statistical significance (n=8, P=0.68).

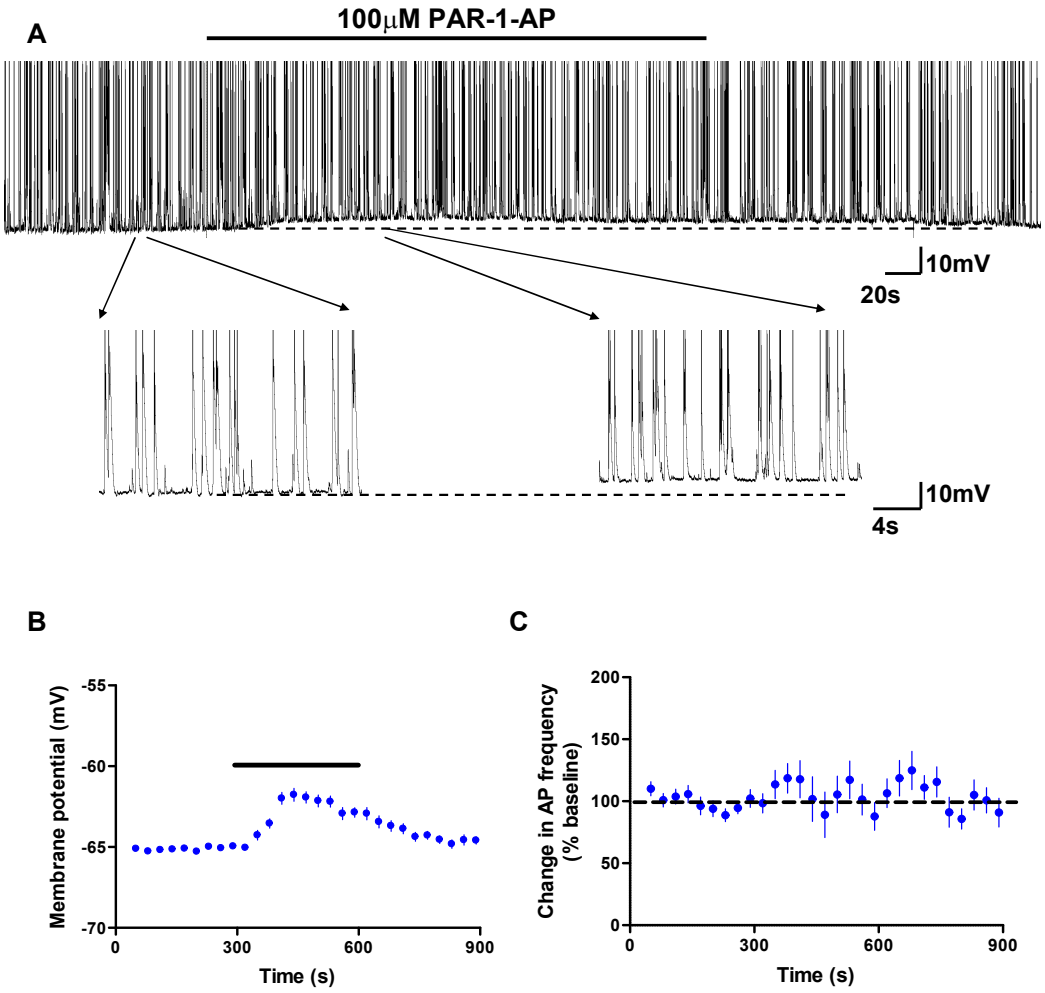


Figure 3-10 Action of the PAR-1-AP on cultured hippocampal neurones normalised to membrane potential of -65mV.

A. Representative recording (cell number 140609/1) illustrating the effect of 100μM PAR-1-AP on membrane potential and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-1-AP application as shown on an expanded time scale below. Note that all cells received somatic current injection to set the membrane potential to -65mV prior to drug application. B. Time plot showing the action of 100μM PAR-1-AP on mean membrane potential. Quantitative analysis revealed significant depolarisation upon drug application (n=12, P=0.0006). C. Time plot showing no significant change in the frequency of spontaneous action potentials following the application of 100μM PAR-1-AP application (n=10, P=0.73).

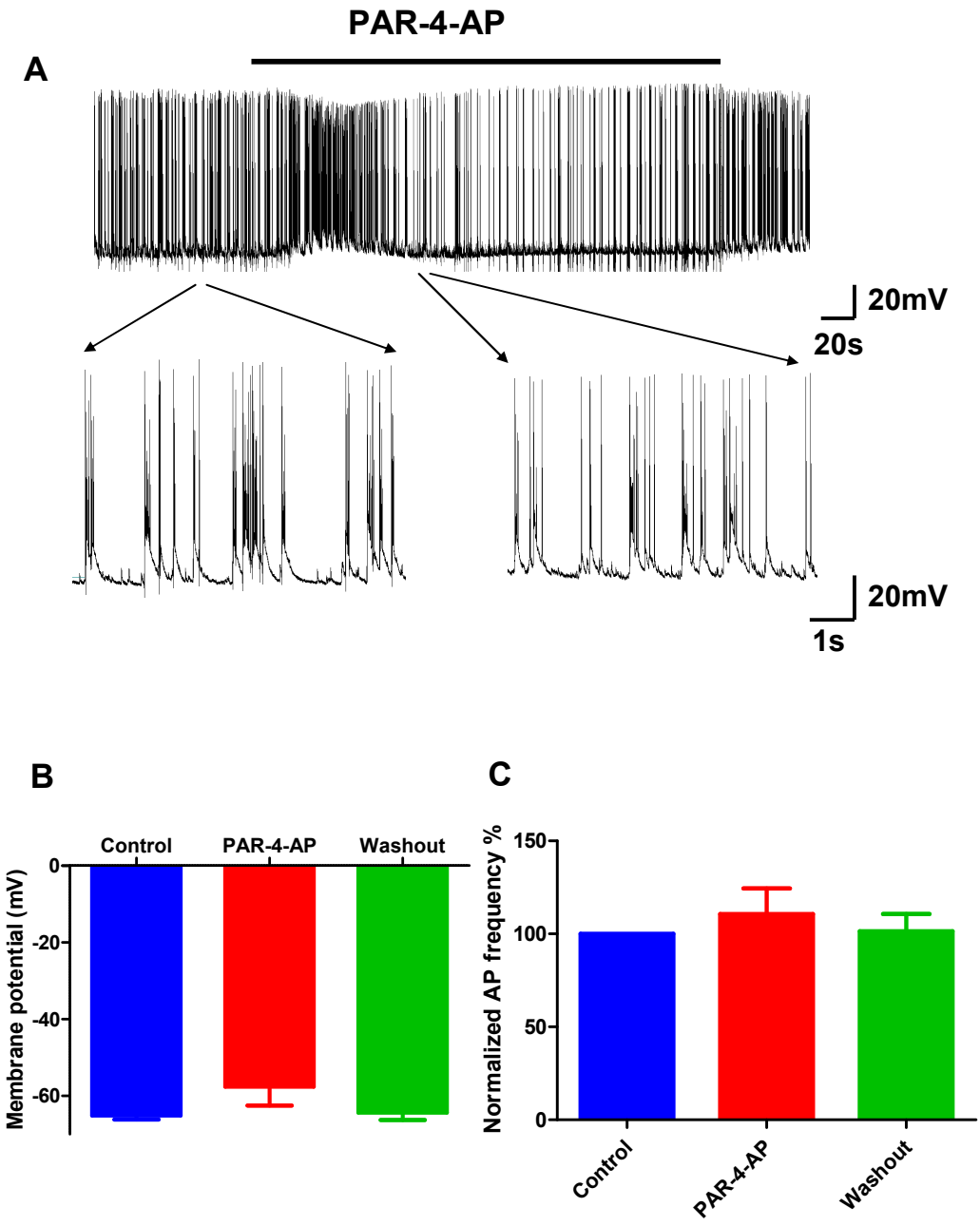


Figure 3-11 Action of the PAR-4-AP on cultured hippocampal neurones under autonomous resting membrane potential.

A. Representative recording (cell number 090807/1) illustrating the effect of 100μM PAR-4-AP on membrane potential and spontaneous activities in a cultured hippocampal neurone. Excerpts before and during PAR-4-AP application as shown on an expanded time scale below. Note the depolarisation and transient decrease in the occurrence of spontaneous action potentials immediately following PAR-4-AP (100μM). B. Bar chart showing no significant membrane depolarisation following PAR-4-AP application (n=4, P=0.19). C. In contrast, changes in the frequency of spontaneous action potentials following 100μM PAR-4-AP application did not achieve statistical significance (n=4, P=0.49).

3.8 Mechanism underlying PAR-2 activation-induced membrane depolarisation in cultured hippocampal neurones

3.8.1 PAR-2 activation-induced neuronal membrane depolarisation is dependent on glutamate

Previous report suggests that PAR-1 receptors in the brain may signal via astrocytes rather than direct neuronal mechanisms (Lee *et al.*, 2007). To test this hypothesis, the PAR-2-AP experiments were repeated in the presence of a cocktail of ionotropic and metabotropic glutamate receptor antagonists (20μM NBQX, 100μM DL-APV, and 500μM MCPG). Current clamp recordings from cultured hippocampal neurones held at -65mV and bathed in a cocktail of these glutamate antagonists revealed that application of 100μM PAR-2-AP, SLIGRL, was no longer able to induce any distinguishable membrane depolarisation in any neurones tested. (Control: -64.28±0.26mV, PAR-2-AP: -64.39±0.44, n=8, mean difference=-0.1±0.42mV, P=0.8157). These data are presented in figure 3.12 and suggest that glutamate is indeed required to achieve a PAR-2-AP-induced neuronal depolarisation.

After establishing this glutamate dependency, we dissected further into which class of glutamate receptors, either ionotropic or metabotropic, was contributing to the effect. Therefore, the nonselective group I/II metabotropic glutamate receptor antagonist, MCPG was first omitted from the glutamate antagonists cocktail. As shown in figure 3.13, application of 100μM PAR-2-AP, SLIGRL, did not cause significant depolarisation of neuronal membrane in present of ionotropic glutamate receptor blockade alone (Control: -65.26 ± 0.07mV, PAR-2-AP: -65.10 ± 0.18mV, n=8, mean difference=0.16 ± 0.06mV, P=0.4678). This data indicated that the PAR-2 modulation of neuroexcitability observed in current experimental model was dependent on iontropic glutamate receptors.

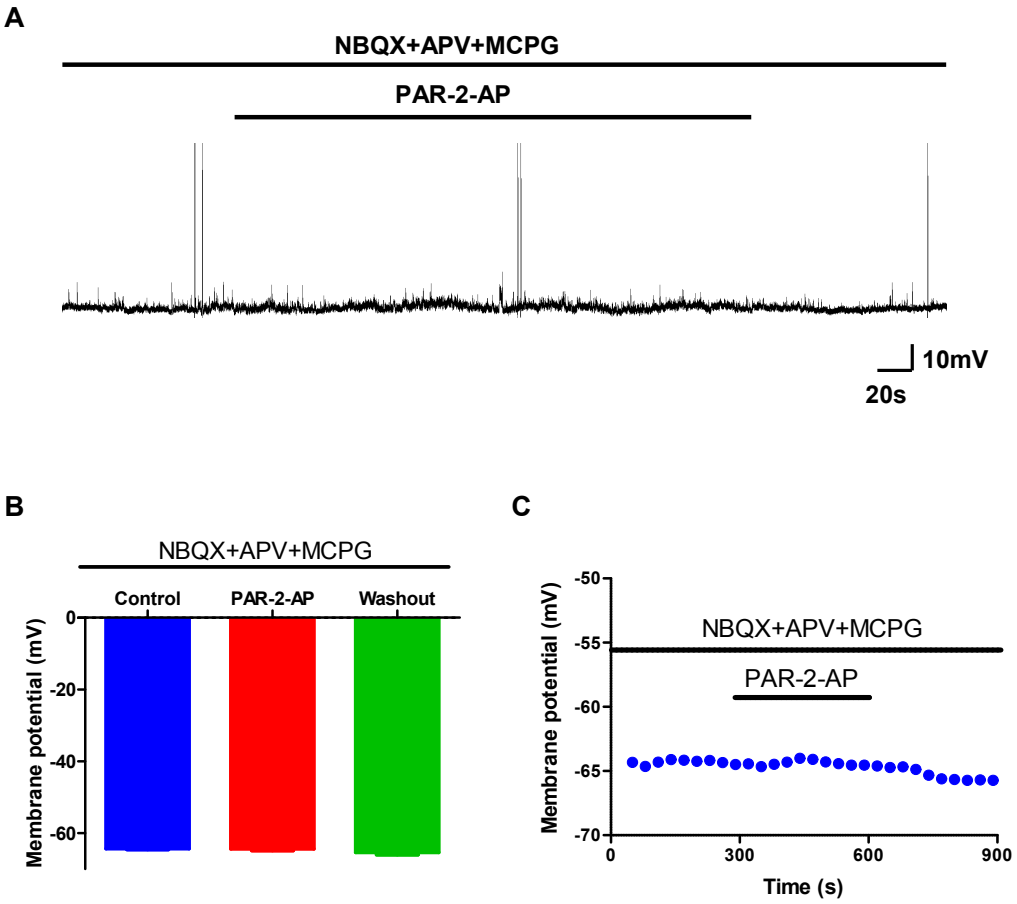


Figure 3-12 Glutamate receptor antagonism prevents PAR-2-induced depolarisation.

A. Representative current clamp recording showing a lack of membrane response to 100μM PAR-2-AP in a cell bathed in a cocktail of AMPA/Kainate, NMDA and metabotropic glutamate receptor antagonist. B. Pooled data showing a lack of any significant membrane depolarisation (n=8, P=0.82). C. Same data represented as a time plot. Horizontal bars in A and C represent time of drug administration.

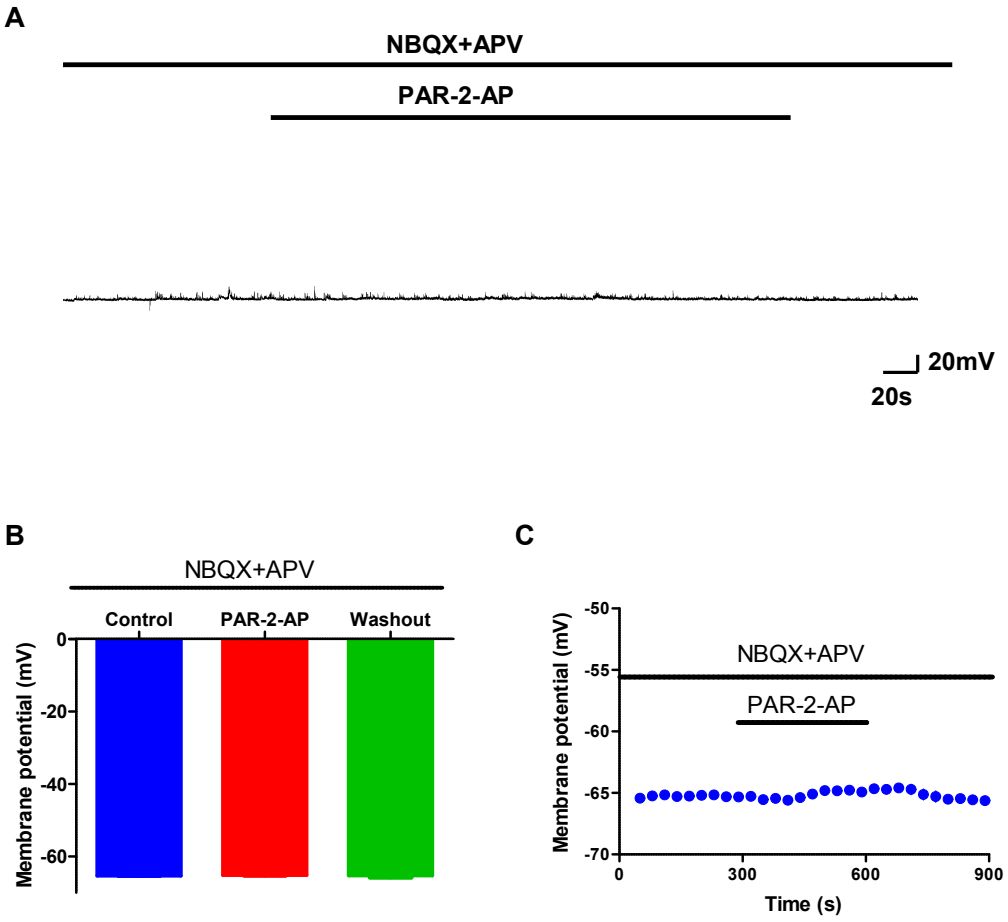


Figure 3-13 Iontropic glutamate receptor antagonism prevents PAR-2-induced depolarisation.

A. Representative current clamp recording showing a lack of membrane response to 100μM PAR-2-AP in a cell bathed in a cocktail of AMPA/Kainate, NMDA glutamate receptor antagonist. B. Pooled data showing lack of any significant membrane depolarisation (n=8, P=0.47). C. Same data represented as a time plot. Horizonal bars in A and C represent time of drug administration.

3.8.2 PAR-2 activation-induced neuronal membrane depolarisation requires normal astrocytic function

As glutamate was required for the modulation of neuroexcitability by PAR-2 activation in current experimental model, a natural question was to investigate further the possible source of glutamate. Given that glutamate could be released during normal synaptic transmission, experiments were carried out to test whether synaptically released glutamate was responsible for this dependency. Therefore, current clamp recordings were conducted in neurones normalised at -65mV in the presence of the sodium channel blocker tetrodotoxin (TTX), which was employed to prevent AP-dependent synaptic transmission. As shown in figure 3.14, 0.5μM TTX completely abolished the spontaneous AP activity as expected. However, surprisingly, application of 100μM PAR-2-AP, SLIGRL, was still able to produce a significant membrane depolarisation (3.3 ± 0.62mV, n=8, P=0.0011) in cultured neurones as presented in figure 3.14. Considering the fact that PAR-2-induced neuronal depolarisation was glutamate dependent, this data strongly suggested that synaptically released glutamate was not responsible for PAR-2-induced neuronal depolarisation.

After ruling out the involvement of AP-dependent release of glutamate, the possibility of astrocytically released glutamate was next investigated. Several studies have demonstrated that activation of GPCRs on astrocytes induce the release of gliotransmitters, including glutamate (Araque, 2008; Fiacco *et al.*, 2009; Santello & Volterra, 2009). Furthermore, recent publication has proposed that activation of PAR-1 in astrocytes led to astrocytic release of glutamate (Lee *et al.*, 2007). To test the possible involvement of astrocytically released glutamate in our experiments we suppressed the metabolic function of astrocytes using fluoroacetate (FAc) (Fonnum *et al.*, 1997). This strategy has been successfully utilised and verified to depress astrocytic functions in a range of culture and slice preparations (Swanson & Graham, 1994; Zhang *et al.*, 2003; Gordon *et al.*, 2005; Shigetomi *et al.*, 2008; Henneberger *et al.*, 2010).

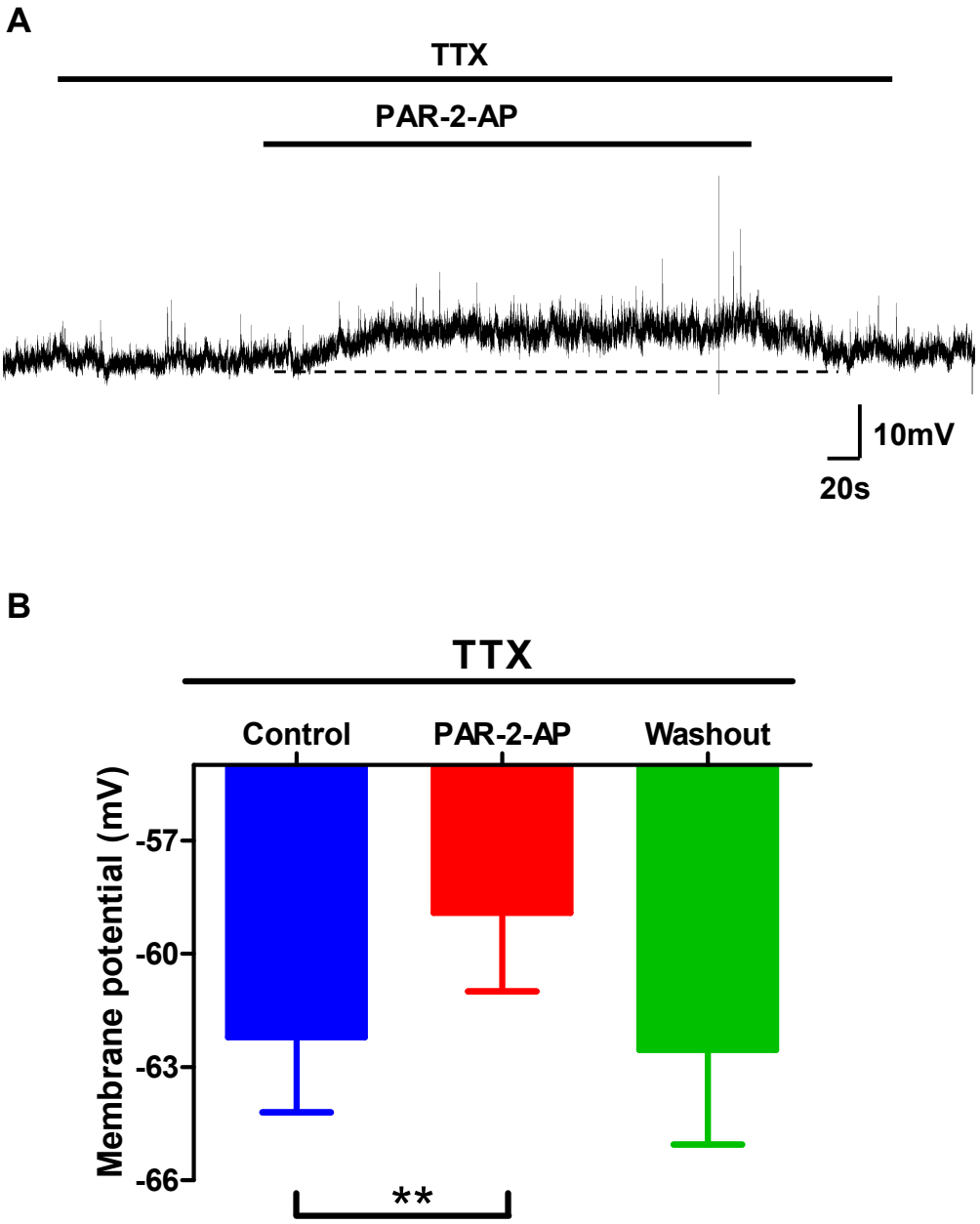


Figure 3-14 Blockage of action potential-dependent synaptic transmission failes to prevent PAR-2-induced depolarisation.

A. Representative current clamp recording showing a depolarising membrane response to 100μM PAR-2-AP in a cell bathed in the sodium channel blocker, Tetrodotoxin (500nM). B. Pooled data showing modest yet significant membrane depolarisation (n=8, P=0.0011).

Whole-cell current clamp recordings were carried out as described previously in neurones that had been preincubated with 10μM FAc for 3 hours. As shown in figure 3.15B, application of 100μM PAR-2-AP, SLIGRL, to such cells failed to induce any significant depolarisation of neuronal membrane (0.19 ± 0.32mV, n=10, P=0.5742) In contrast, neurones from the same culture preparation but which had not received FAc treatment continued to display a robust depolarising response upon 100μM SLIGRL application (mean depolarisation = 3.40 ± 0.43mV, n=6, P=0.0005). Furthermore, as shown in figure 3.16, no significant change of basal spontaneous AP frequency was observed between naïve neurones and FAc treated cells (drug free: 1.23 ± 0.08Hz, n=6; FAc treated: 1.30±0.10Hz, n=10, P=0.6030), which suggests that the 3 hours incubation with 10μM FAc did not cause overt physiological damage in cultured neurones.

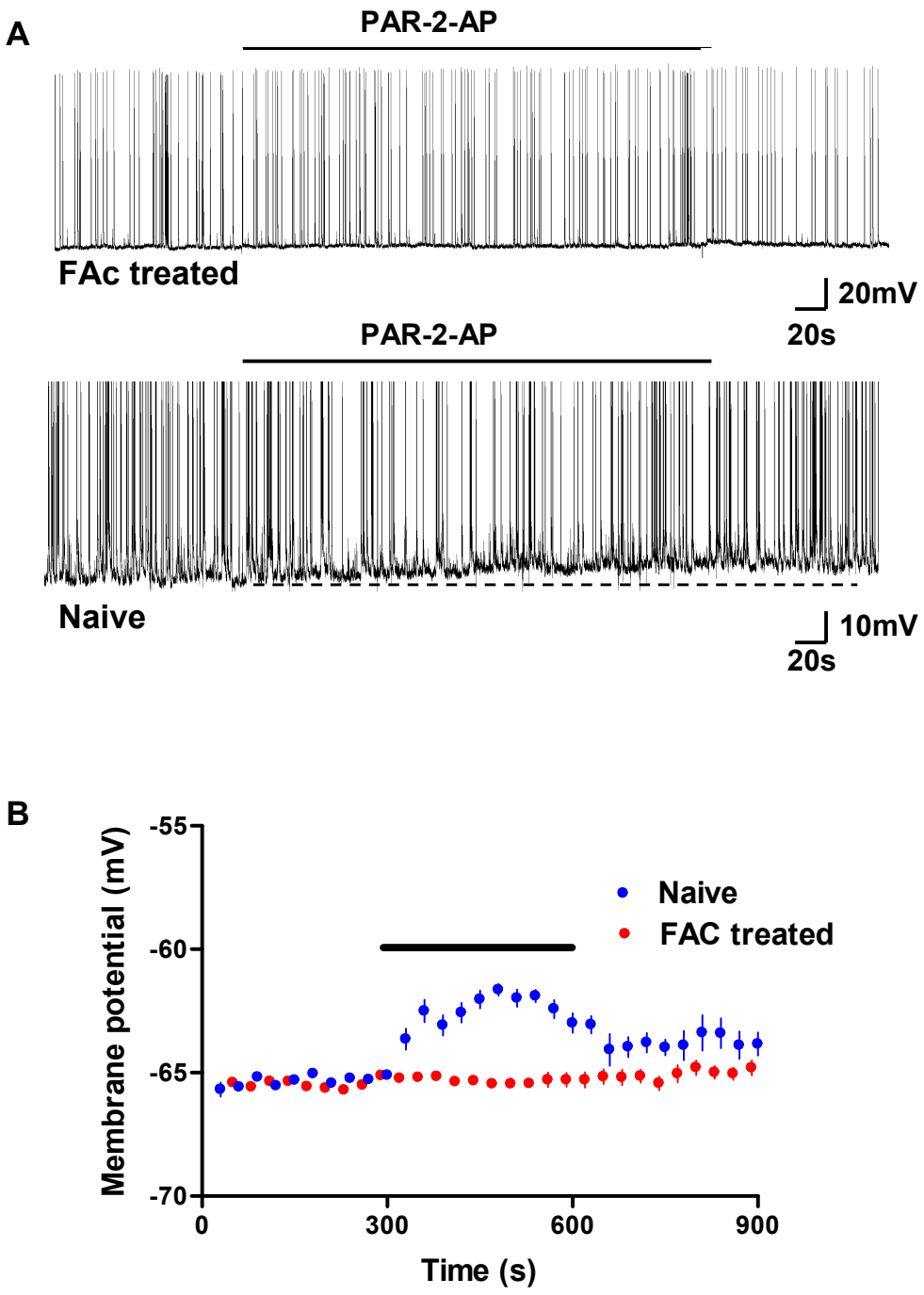


Figure 3-15 Inhibition of astrocytic function prevents PAR-2-AP-induced membrane depolarisation.

A. Representative current clamp recordings form FAc treated (upper trace) or naïve (lower trace) neurones obtained from the same culture preparation. Note the presence of a membrane depolarisation in the control but not in the FAc-treated cell. B. Time plot summarising pooled data confirms 100μM PAR-2-AP to produce a depolarisation in naïve neurones but not in those treated with FAc (FAc treated, n=10, P=0.57; Naïve, n=6, P=0.0005)

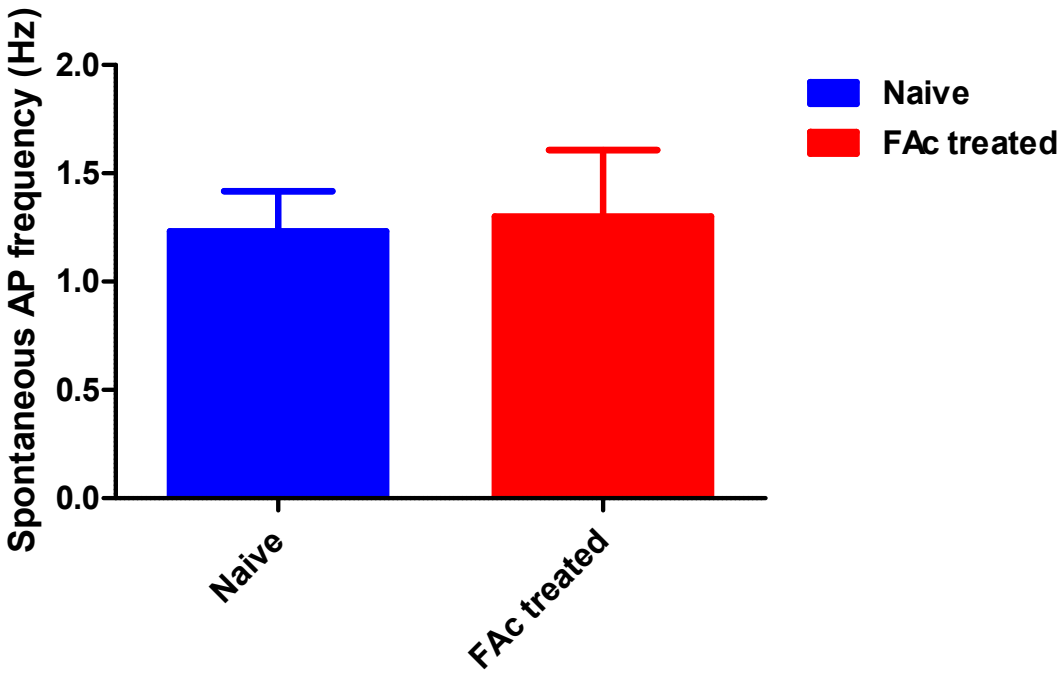


Figure 3-16 No significant difference in the frequency of basal spontaneous AP firings from naïve neurones and those treated with 10μM FAc.

3.9 Mechanism underling PAR-2 activation-induced depression of the frequency of spontaneous AP firings in cultured neurones

As shown in figure 3.5, PAR-2 activation-induced neuronal membrane depolarisation was associated with a profound but paradoxical depression of the frequency of spontaneous APs. To examine this phenomenon further, we investigated the mechanisms underlying the generation of APs in the dissociated culture preparation.

3.9.1 Spontaneous APs are synaptically driven in nature

It was first noticed that the spontaneous APs were significantly abolished (10.75 ± 2.97% of control, n=7) in the presence of the cocktail of glutamate receptor antagonists (20μM NBQX, 100μM DL-APV, and 500μM MCPG) as showed in figure 3.17. This observation suggested the occurrence of AP firings in these cultured neurones was largely dependent on glutamatergic synaptic transmission within the network of cultured neurones. Indeed, on closer inspection, recorded cultured neurones were found to receive a constant barrage of EPSPs, many of which were suprathreshold for the generation of APs (see figure 3.3). In order to further confirm that the majority of spontaneously occurring APs were synaptically driven, current-induced depolarisation was first applied by current injection through micropipette during current-clamp recording to the magnitude similar to that seen with PAR-2 activation (from -65mV to -60mV). In contrast to the decrease in firing rate caused by PAR-2 activation, this procedure resulted in a predicable and significant increase of the frequency of AP firings (220.85 ± 24.92% of control, n=15, P=0.0003, figure 3.18). This data supported the interpretation that the depression of AP firings seen associated with PAR-2 receptor activation was not due to a membrane depolarisation *per se*. We also calculated the frequency of sub-threshold EPSPs before and after the application of SLIGRL (100μM), as shown in figure 3.19. There was a significant reduction of sub-threshold EPSP (27.80 ± 5.57% of control, n=7, P=0.0006) occurrence in the presence of SLIGRL (100μM), suggesting there was indeed a reduction in the activity of neurones innervating the recorded neurone. Taken together, these

data suggest that the majority of spontaneous APs in the cultured neurones were synaptically driven, and furthermore the reduction of the frequency of spontaneous AP firings reflected that PAR-2 activation depressed synaptic transmission in the cultured neurones. This question is addressed further in the next chapter.

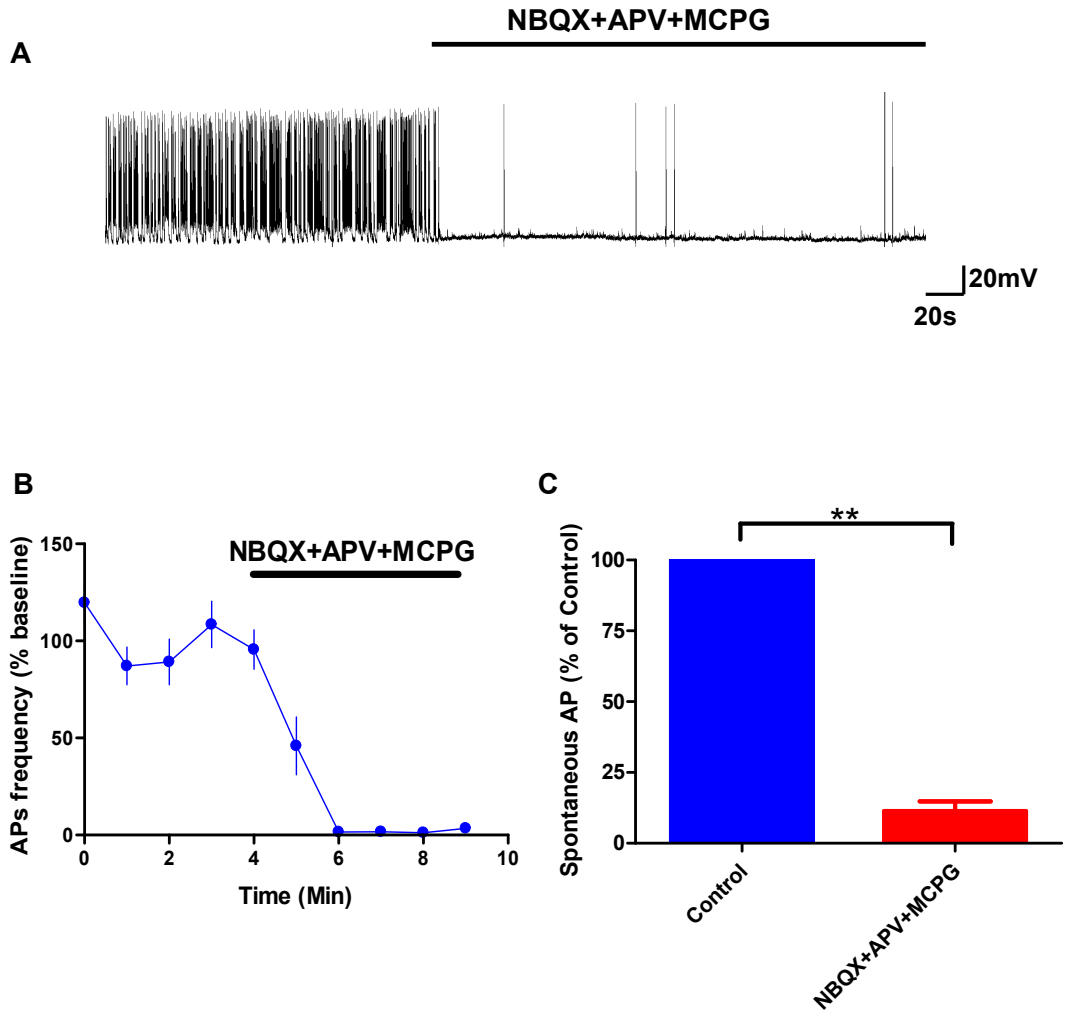


Figure 3-17 The occurrence of spontaneous APs is prevented in the presence of glutamate antagonism.

A. Representative recording of experiment B. Real time summary and C. pooled data show frequency of APs is depressed upon glutamate antagonism (NBQX+APV+MCPG)

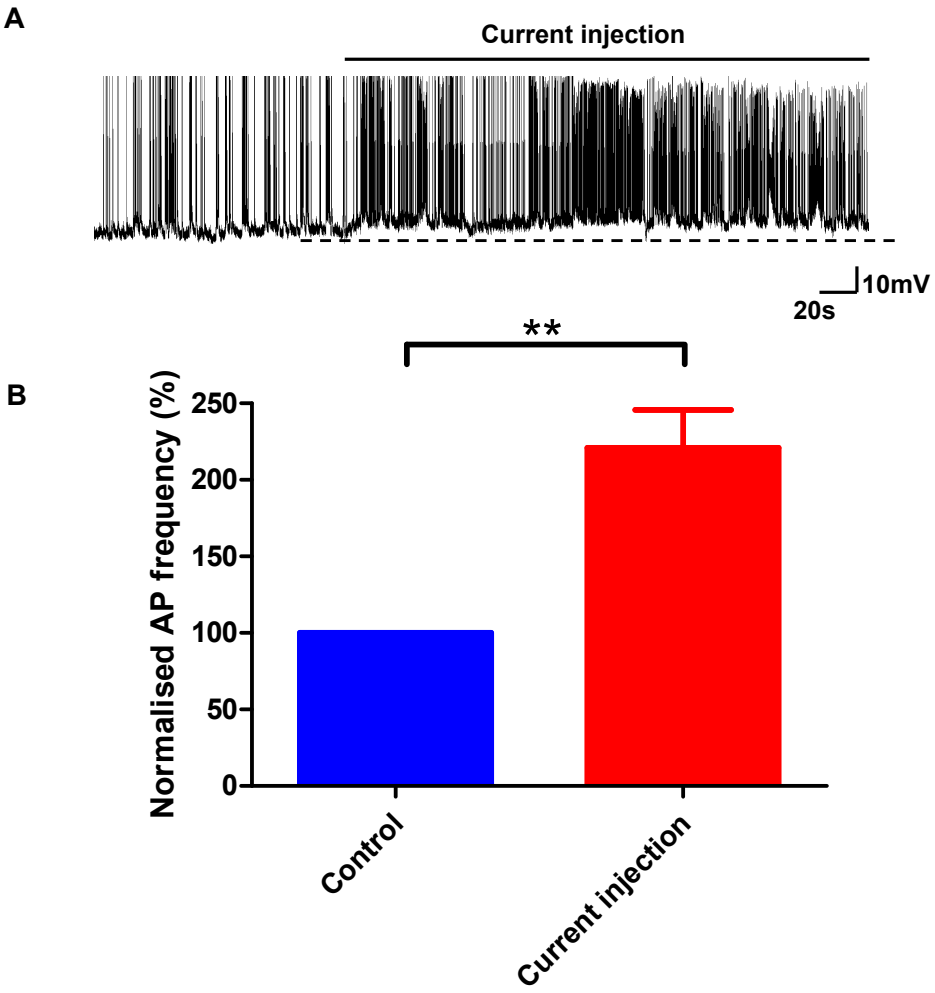


Figure 3-18 Control experiments showing membrane depolarisation in response to somatic current injection is associated with an increased frequency of action potentials.

A. Representative current clamp recording showing current injection to mimic PAR-2-AP-induced depolarisation results in increased action potential dischage (cf. the reduced frequency seen in figure 3.6). B. Bar plot of pooled data showing a significant increase of spontaneous AP frequency following current injection to shift membrane potential from -65mV to -60mV (n=15, P=0.0003).

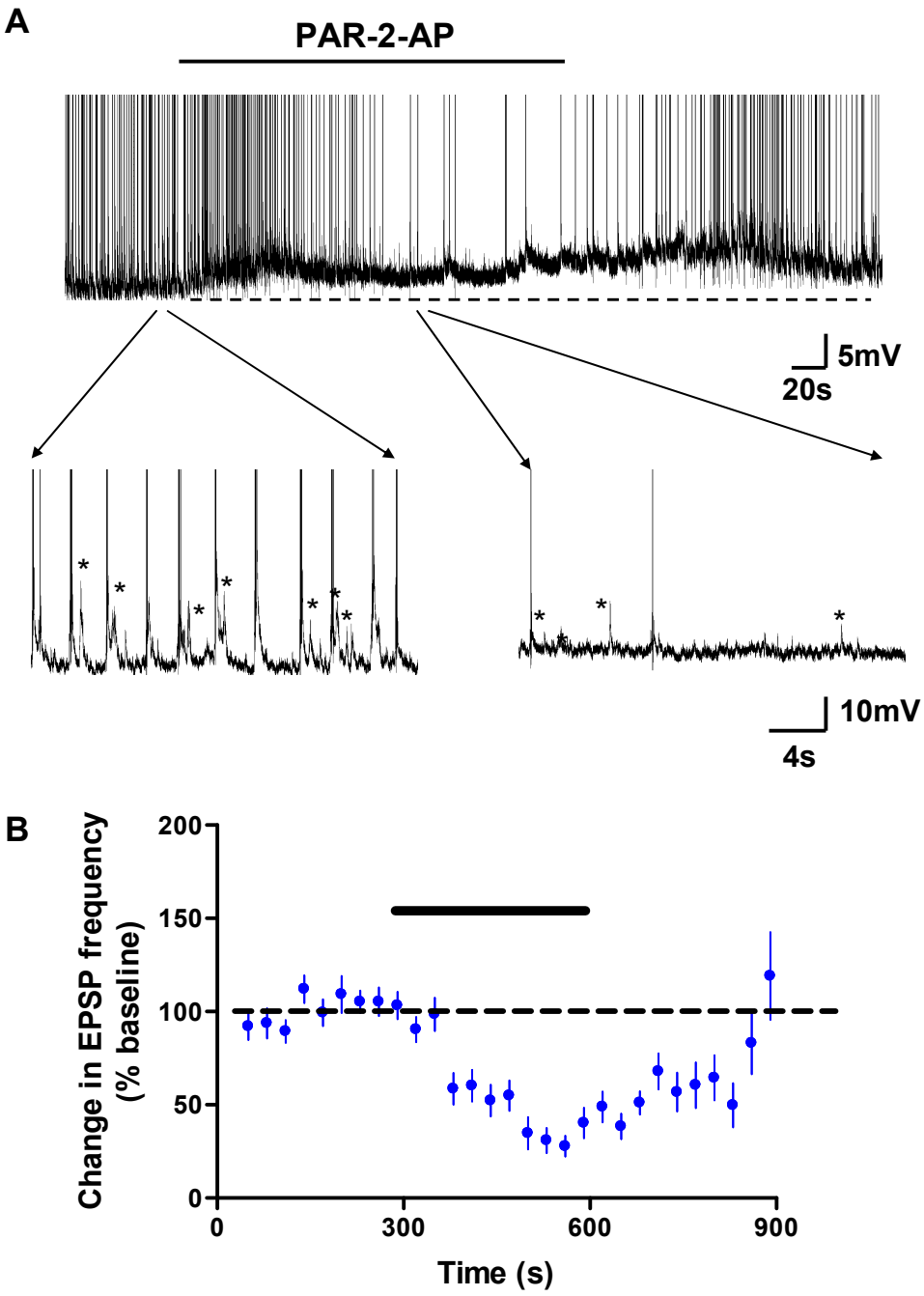


Figure 3-19 PAR-2-AP depresses frequency of EPSPs.

A. Representative traces of one experiment. Stars represent EPSPs and large positive deflections are APs. B. Real time summary of depression of EPSPs upon PAR-2-AP application (n=7, P=0.0006)

3.9.2 Glial-neuronal signalling contributes to the PAR-2 activation induced depression of the frequency of spontaneous AP firing in cultured neurones

As discussed in 3.8.2, FAc, the selective astrocytic function inhibitor, was employed to test if glial-neuoronal signalling was involved in the PAR-2 activation induced depolarisation. Apart from the fact that 3 hours incubation of 10μM FAc completely abolished the PAR-2 activation-induced depolarisation in neurones, it was also noticed that the suppression of frequency of spontaneous APs was reduced. As shown in figure 3.20, the maximal reduction of AP suppression observed at the time point of 480 second (67.23 ± 9.12% of control, n=8), compared to neurones from naïve cultures (41.63 ± 11.5% of control, n=6), was statistically significant (P=0.0308). However, the gross pattern of the reduction of AP suppression following FAc treatment was nevertheless modest. These data suggest that PAR-2 activation, in addtion to its roles on glial cells, may have direct influence on neurones over their intrinsic properties, which are responsible for generating APs.

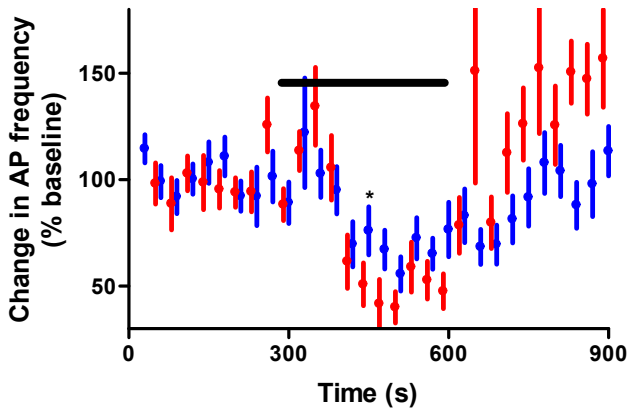


Figure 3-20 Depression of the frequency of spontaneous AP firings is modestly reduced in neurones treated with FAc.

Star point shows the time point of statistical significance.

3.10 Discussion

In this chapter, I show, for the first time, that the PAR-2 receptor activation modulates neuroexcitability and depresses spontaneous APs in cultured hippocampal neurones. Furthermore, this PAR-2-mediated regulation is dependent on iontropic glutamate receptor activation and normal astrocytic function. This latter result suggests that glial-neuronal signalling may be an important element in PAR-2-mediated actions in the nervous system.

In this chapter, I first demonstrate that trypsin, which at the similar concentration (80nM) used is reported to preferentially activate PAR-2 (Bushell *et al.*, 2006), induced significant depolarisation of neuronal membrane and increased spontaneous activities (APs and spontaneous synaptic events under both voltage clamp and current clamp configurations (See figure 3.2 and 3.4)). These data support our hypothesis that PAR-2 has a modulatory role in central neurones under non-disease conditions. These results are distinct from previous studies which have tended to focus upon PAR-2 actions being solely related to disease conditions (Striggow *et al.*, 2001; Jin *et al.*, 2005; Noorbakhsh *et al.*, 2005; Afkhami-Goli *et al.*, 2007; Lohman *et al.*, 2008). However, this interpretation could be compromised by serious concerns over the selectivity of trypsin for activating PARs and its 'off target' effects in terms of digesting other surface proteins. It has been reported, at high concentrations (mM range), trypsin could activate PAR-1 and PAR-4, although it is more selective to PAR-2 at low concentration (EC_{50}=1nM in *Xenopus* oocytes (Macfarlane *et al.*, 2001)). Therefore, it is difficult to rule out the possible activation of PAR-1 and PAR-4, albeit at low levels, in the experiments using 100nM trypsin. In addition to its action on PARs, trypsin is a potent digesting enzyme for very many proteins with 80% of proteins possessing at least one trypsin-like proteinase cleavage site (Southan, 2001; Puente *et al.*, 2003). Taking this into account, the suspicion that observed depolarisation upon trypsin application could be due to cleavage of other membrane proteins important in regulating membrane potential and cellular excitability is credible. In the current clamp recording, overtly, second application of 100nM did not cause significant depolarisation in neurones tested as shown in figure 3.4E, F. This is consistent with a cleavage of PAR2 receptors

(a 'one hit' stimuli) and corresponding membrane depolarisation. However, it is possible that cleavage of off-target proteins could produce a similar one-hit response. Interesting, 3 out of 7 neurones did indeed responded to the 2nd trypsin application but this was typically smaller and overall did not achieve statistical significance. However, this can be explained in terms of the initial application causing a partial cleavage of the PAR2 population. Given that the second application was 10 minutes following the initial response, this might be explained by a number of mechanisms including 1) receptor desensitisation, 2) trafficking of new PAR2 receptors to the membrane surface or to downregulation of the signalling processes. These possibilities have been considered previously for PAR2-induced calcium signalling responses (Bushell *et al.*, 2006). Overall, it is concluded from the trypsin experiments that the membrane depolarisation and increased synaptic activities observed upon trypsin treatment are partially mediated by PAR-2 activation. However, to confirm this suspicion, all further experiments were conducted using more selective PAR-2-AP, SLIGRL and its inactive negative controls, PAR-2-IP, LRGILS.

The neuronal depolarisation observed upon SLIGRL application is consistent with previous trypsin experiments. Taken together, these experiments support the interpretation that PAR-2 activation leads to membrane depolarisation in cultured hippocampal neurones. Whilst the finding that PAR-2 induces neuronal depolarisation in central neurones is completely novel, it nevertheless mirrors previous reports that PAR-2 receptor activation excites dorsal root ganglia (DRG) neurones (Kayssi *et al.*, 2007). It also mirrors reports that PAR-1 receptor activation excite hippocampal neurones (Kayssi *et al.*, 2007; Lee *et al.*, 2007). Kayssi and co-workers demonstrated that membrane depolarisation in colonic DRG neurone was attributed to PAR-2-induced suppression of delayed rectifier K^+ currents (Kayssi *et al.*, 2007). In the current investigation, our primary focus was to characterise the overt actions of PAR2 activation on membrane excitability (membrane potential) and synaptic transmission and I did not investigate possible intracellular or ion channel signalling processes. Nevertheless, a reduction in potassium conductance as seen in the Kayssi paper would be consistent with the depolarising responses observed in the current studies.

In the study by Lee and co-authors, the authors proposed that the membrane depolarisation in CA1 hippocampal neurone was in response to glutamate release

from astrocytes upon astrocytic activation by PAR-1 (Lee *et al.*, 2007). In this chapter, I demonstrated glutamate is also required during PAR-2-mediated depolarisation in cultured hippocampal neurones. Specifically, as ionotropic glutamate receptors blockade alone sufficiently prevented this depolarisation, it is highly likely that mGluRs are not required under current experimental condition and in contrast, this modulation is dependent on signalling through iontropic glutamate receptors. Furthermore, when it comes to the origin of glutamate, the hypothesis that astrocytically released glutamate is underlying this glutamate dependency is suggested by the fact that TTX (to block neuronal AP- dependent glutamate release) does not block PAR-2-mediated depolarisation. This hypothesis is strengthened by the result that PAR-2-induced neuronal depolarisation is abolished when normal astrocytic function is inhibited in hippocampal cultures. In summary, these results are consistent with a dependency for astrocytic (but not neuronal) release of glutamate.

When PAR-1 is investigated, the result that a significant depolarisation was observed upon PAR-1-AP, TFLLR, is consistent with that previously reported (Lee *et al.*, 2007) and also consistent with the PAR-2-AP induced responses reported for the first time here. Taken together, these data suggest that the membrane depolarisation induced by activation of PAR-1 and PAR-2 receptors may share a common glial-dependent mechanism. In terms of cellular signalling as a result of PAR-1/PAR-2 activation, although PAR-2 and PAR-1 are activated differentially by distinct proteinases (both experimentally and presumably under endogenous conditions) there could be a common downstream mechanism which underlies the way they modulate neuroexcitability in the central neurones. However, it was also noticed the depression of spontaneous APs firings associated with PAR-2 activation, was not seen with PAR-1 activation, suggesting although PAR-1 and PAR-2 are in the same receptor family and have potentially common downstream modulatory target and mechanism in neuroexcitability, there may also be important differences in the way they control network excitability (see also chapter 5).

In addition to membrane depolarisation brought about by PAR-2 activation, it is of special interest to note the paradoxical decrease in the frequency of spontaneous APs. This was not observed upon trypsin treatment or upon selective activation of PAR-1. The reduction in AP frequency is unexpected

because a depolarisation usually gives rise to increased APs. Indeed, simulation of PAR2-induced depolarisation through intracellular current injection did cause an increase in AP frequency. However, it seems that in the cultured neuronal preparation, the vast majority of spontaneously generated APs are in fact driven by ongoing synaptic activity. This is confirmed by the finding that most spontaneous APs are prevented upon application of a cocktail of glutamate receptor antagonists shown in figure 3.17. Taken together, data from these two series of experiments suggest that the depression of APs seen associated with PAR-2 receptor activation was not due to a membrane depolarisation but instead the action of PAR-2 receptor activation on synaptic transmission. This is addressed experimentally in the following chapter. The finding that inhibiting astrocytic function modestly reduced the magnitude of SLIGRL-induced depression of spontaneous APs, as shown in figure 3.20, suggests that astrocytic-neuronal signalling partially contribute to the reduction of APs upon PAR-2 activation. Considering the fact that glutamate-dependent PAR-2-induced depolarisation is abolished during astrocytic blockade, this may suggest astrocytically released glutamate at least partially contribute to PAR-2-mediated depression of APs in cultured hippocampal neurones. Alternatively, other gliaotransmitters, such as ATP, may be involved (Koizumi *et al.*, 2003; Zhang *et al.*, 2003). However, the dependency of glutamate receptor activation as shown in figure 3.12 in PAR-2-induced neuronal depolarisation suggests that glutamate from astrocytes is the most likely the primary source of the modulation. However, it is also acknowledged that the astrocytic blockade did not abolish the PAR-2-mediated depression of APs in cultured neurones (figure 3.20). This indicates PAR-2 activation may have direct influence over neuronal intrinsic properties by modulating, for instance, ion channels. It has been well established that the activation of GPCRs has direct modulatory effect on ion channel conductances such as two-pore-domain K^+ channels (Mathie, 2007) and GIRK channels (Luscher & Slesinger, 2010), which modulate neuronal intrinsic properties and ultimately finetune the generation of APs. Moreover, it has been demonstrated that PAR-2 activation modulates neuronal K^+ channel conductances (Kayssi *et al.*, 2007). Therefore, in order to test this possibility, the input resistance of the recorded neurone has to be monitored before and after the PAR-2 activator is applied. This input resistance measurement could give a clue that whether channel conductances on the neuronal membrane are altered upon

PAR-2 activation. If so, a series of screening experiments could be performed to identify which ion channels are likely to be modulated directly by PAR-2 activation.

In summary, in this chapter, I demonstrated for the first time that the activation of PAR-2 receptor depolarises membrane and depresses spontaneous APs in cultured hippocampal neurones. Furthermore, this PAR-2-mediated regulation of neuroexcitability is dependent on iontropic glutamate receptor activation and normal astrocytic function. It is also suggested that PAR-2-mediated depression of spontaneous APs is the consequence of reduced synaptic transmission within the network which is modulated by astrocytes.

4 Actions of PAR-2 activation on synaptic transmission in acute hippocampal slice preparations

4.1 Introduction

In addition to its expression in neurones as well as astrocytes in primary hippocampal culture, PAR-2 has also been demonstrated to be present in neurones of the mature nervous system (Bushell *et al.*, 2006). Specifically, immunocytochemistry has revealed PAR-2-immunoreactivity at both excitatory (glutamatergic) and interneuronal (GABAergic) cell populations in the hippocampus (Bushell *et al.*, 2006) as well as GFAP-positive astrocytes. Neuronal immunostaining revealed strong dendritic labelling suggesting PAR-2 receptors may modulate aspects of synaptic signalling in addition to the intracellular Ca^{2+} signalling in the cell body reported previously (Bushell *et al.*, 2006). However, direct investigation of the actions of PAR-2 receptor activation on synaptic transmission in intact brain slices has not previously been investigated.

Having conducted initial experiments (chapter 3) in a cultured system, subsequent experiments to assess the action of PAR-2 on synaptic transmission was conducted in the acute hippocampal slice preparation. Acute hippocampal slices have been extensively employed as an *in vitro* model in studying neuronal and synaptic properties in the CNS thanks to many advantages such as the ability for direct visualization of neuronal layers to allow precise manipulation of stimulating or/and recording electrode at defined synapses. Moreover, synapses in the acute slice have developed normally and are likely to reflect more accurately the properties of synapses in the intact brain.

Therefore, the specific aim of this chapter was to investigate the actions of PAR-2 activation on synaptic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices using established extracellular recording technique. This synapse was chosen as it represents a well studied and well characterised connection in the brain. It is also a relatively straightforward synapse to isolate and stimulate. Finally, our earlier immunocytochemistry data suggested that

PAR-2 receptors are expressed both within the dendritic zone in area CA1 as well as in CA3 pyramidal cells providing the efferent output.

4.2 PAR-2 activation depresses synaptic transmission at the Schaffer collateral-to-CA1 synapse

To examine the consequences of PAR-2 activation on synaptic transmission, standard extracellular recordings were carried out in parasagittal slices of 400μm thickness prepared from 16-24 days old Wistar rats. fEPSPs were evoked by single stimulus at a frequency of 0.05Hz through a stimulating electrode positioned 2-3mm adjacent to a recording electrode positioned within *stratum radiatum* (see methods chapter). Electrical stimulation of the Schaffer collateral afferent evoked a characteristic fEPSP which, when recorded in mid *stratum radiatum*, comprised of a 1) stimulus artefact, 2) presynaptic volley, 3) negative deflecting EPSP (Figure 4.1A). Slices were deemed acceptable if they produced a clear fEPSP that was stable (in amplitude or slope measurement) over a 20 minutes period. Following 20 minutes of stable baseline measurements, drugs would then be applied to the bath via the perfusion media. Our initial studies attempted to use low concentrations of trypsin as a selective PAR-2 activator. However, pilot studies revealed unacceptable toxicity associated with the use of trypsin (100nM-10μM) in which synaptic responses were completely and irreversibly abolished (data not shown). This may have been due to digestion of glutamate receptors (Grosshans *et al.*, 2002) or other essential membrane proteins. However, in light of the pilot experiments it was decided to focus all further experiments on the use of non-proteolytic activators of PAR-2.

As presented in figure 4.1B, 100μM SLIGRL applied via the perfusate for 30 minutes resulted in a slow but profound depression in the slope of fEPSPs to 80.75 ± 2.54% of control (n=12, P=0.0008). These data suggest that PAR-2 activation depresses basal glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse. However, it was also apparent that the SLIGRL-induced synaptic depression did not recover upon washout of drug, suggesting PAR-2 activation provoked a long lasting plasticity (depression) of synaptic transmission. An alternative possibility was that SLIGRL was neurotoxic to the acute slices. To discriminate between these possibilities, a reanalysis of the data

was conducted. Specifically, analysis of the fibre volley, a direct indicator of presynaptic input integrity, was conducted in SLIGRL-treated slices to establish whether the integrity of axonal / synaptic terminal function was impaired by SLIGRL treatment. Overt and unambiguous presynaptic volleys were observed 7 of 12 slices and were used for subsequent analysis. As presented in figure 4.1B, no significant change of fibre volley was noticed (to 101.38 ± 4.82% of control at 30 minute, n=7, P=0.5334), suggesting SLIGRL-induced long lasting synaptic depression was not a consequence of slice deterioration.

To strengthen this argument, one high frequency stimulation (HFS, 100 stimuli at 1s) was delivered through the stimulating electrode to produce LTP after SLIGRL-induced long lasting depression was established. As shown in figure 4.2, the time course and the amplitude of responses following HFS were indistinguishable to those obtained upon HFS from naïve slices (naïve slices: 148.82 ± 9.39% of baseline, n=9; PAR-2-AP treatment: 142.64 ± 7.43% of baseline, n=10, at 60 minute following HFS, P=0.6090), suggesting the synaptic structure and mechanism underlying LTP in the Schaffer collateral-to-CA1 synapse were intact in acute hippocampal slices after SLIGRL treatment.

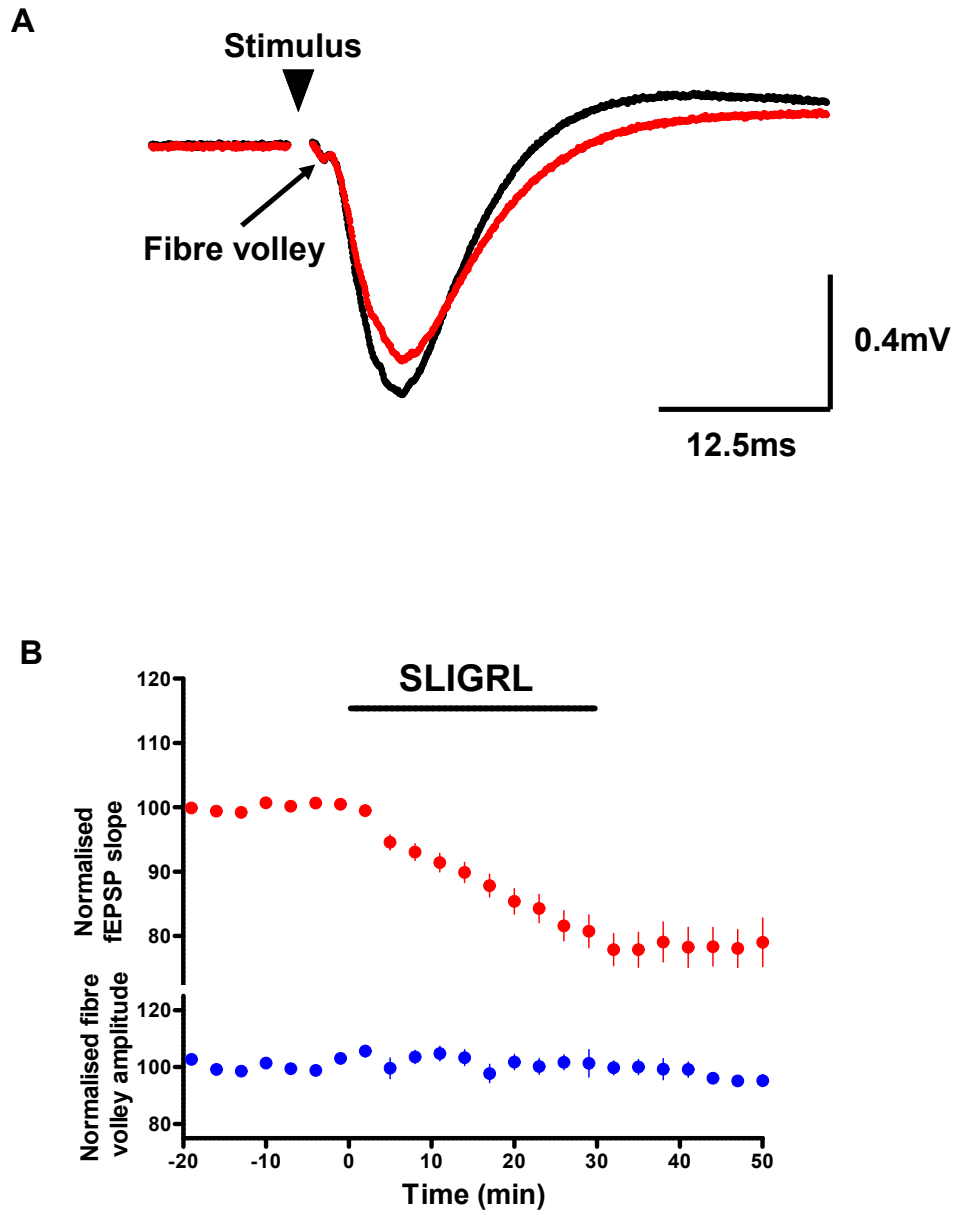


Figure 4-1 SLIGRL depresses synaptic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices.

A. Representative raw traces of extracellular field EPSPs recorded from *stratum radiatum* in are CA1. Note the presence of a presynaptic volley following the stimulus (artefact removed for clarity purposes) which is preceded by a negatively deflecting field EPSP. In all experiments, the magnitude of the response was quantified by measuring the initial slope of the fEPSPB using the LTP software. B. Time plot of pooled data showing the effect of PAP2-AP (SLIGRL, 100μM, n=12, period indicated by horizontal bar) on fEPSP slope (red symbols). Note the gradual reduction in fEPSP slope following SLIGRL application. Corresponding measurement of presynaptic fibre volleys (blue symbols, n=7) confirm no significant change in the integrity of presynaptic stimulation/input.

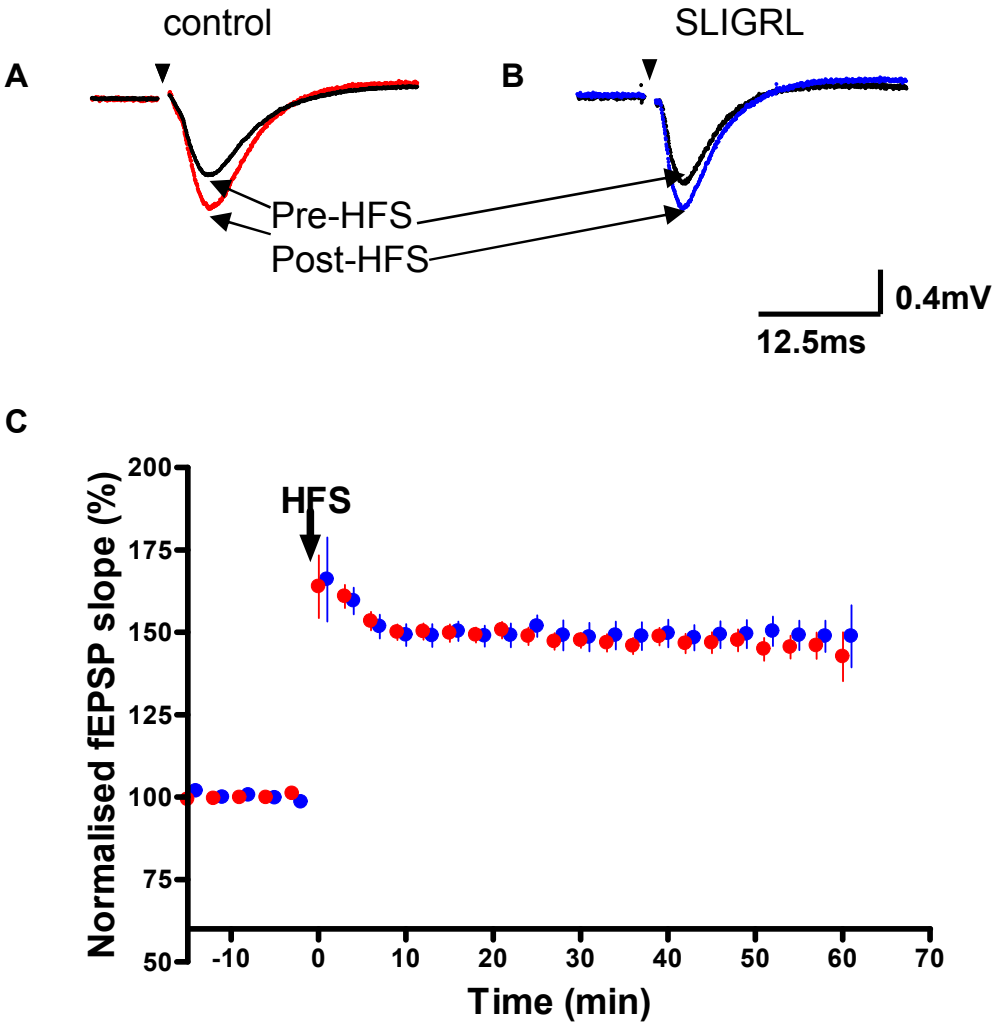


Figure 4-2 Preincubation of slices with PAR-2-AP does not affect the induction of LTP.

A. Representative control (drug-free) fEPSPs before and 60 minutes following high frequency stimulation (HFS). B. Similar traces in a slice preincubated with the PAR2-AP SLIGRL (100μM) produces a similar robust LTP following HFS. C. Pooled time series data showing LTP in control (blue symbols, n=9) and SLIGRL treated (red symbols, n=12) slices and revealing no significant difference in the propensity for LTP induction between groups.

In light of the relatively high concentration (100μM) of SLIGRL used in the study, control experiments were conducted to exclude the possibly of 'off-target' effect of this peptide. Thus, the biologically inactive reverse peptide, PAR-2-IP, LRGILS, which had been successfully used in previous study in primary culture (Bushell *et al.*, 2006), was employed as a negative control. As presented in figure 4.3, application of 100μM LRGILS for 30 minutes did not a cause significant change in the slope of fEPSPs (100.87 ± 0.88% of baseline at 30 minute, n=9, P=0.8751), suggesting LRGILS has no effect on synaptic transmission at the Schaffer collateral-to-CA1 synapse. This data is consistent with SLIGRL having a discrete and specific action on synaptic transmission in the acute slice.

To further address the concern over selectivity of SLIGRL, we utilised a recently reported potent peptide agonist, 2-furoylated-LIRL-NH_2 (2f-LIGRL) (Kawabata *et al.*, 2004; Kawabata *et al.*, 2005; Kanke *et al.*, 2009). 2f-LIGRL has been demonstrated to be 10 times more potent relative to the native SLIGRL peptide by Ca^{2+} signalling assays in cultured cell lines (2f-LIGRL EC_{50} =1μM) (Kawabata *et al.*, 2004). In the salivation bioassay in anesthetized mice, 2f-LIGRL was found to be approximately 100 times more potent than SLIGRL (2f-LIGRL ED_{50}=0.15μmol/kg) (Kawabata *et al.*, 2004). The higher potency of 2f-LIGRL might be explained by its resistance to the degrading enzyme, aminopeptidase by virtue of its furoylated C-terminus (Kawabata *et al.*, 2004). As presented in figure 4.4, 100μM 2f-LIGRL was applied in the perfusate after 20 minutes stable baseline was obtained. Interestingly, the application of 2f-LIGRL for 30 minutes produced a slow and profound depression of the slope of fEPSPs (82.07 ± 2.42% of control at 30 minute, n=9, P=0.0026), suggesting glutamatergic synaptic transmission was depressed. This data was in agreement with the experiments employing the native PAR-2-AP, SLIGRL, and further demonstrated PAR-2 activation induced a long lasting depression of the Schaffer collateral-to-CA1 synapse. However, it was also noticed that the prolonged synaptic depression produced by 2f-LIGRL had similar time course and magnitude of response compared with that of SLIGRL-induced depression. In contrast to previous studies that 2f-LIGRL was shown 10-100 times more potent than SLIGRL (Kawabata *et al.*, 2004; Kawabata *et al.*, 2005; Kanke *et al.*, 2009), our data suggest that at the concentration tested (100μM), 2f-LIGRL and SLIGRL were equally efficacious in suppressing synaptic transmission.

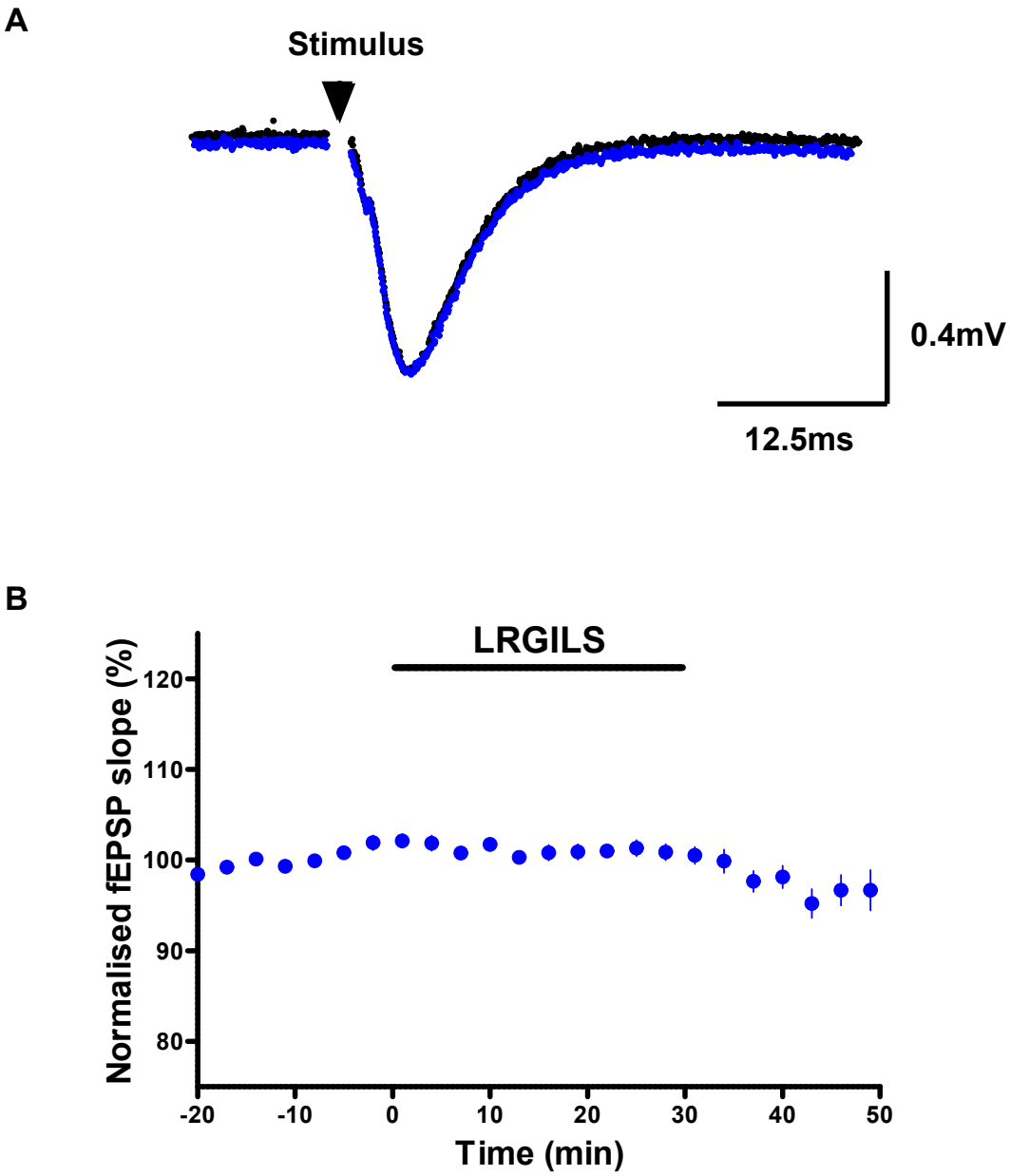


Figure 4-3 The PAR-2-IP LRGILS does not depresses synaptic transmission at the Schaffer collateral-to-CA1 synapse.

A. Representative raw traces of extracellular field EPSPs before (black) and following (blue) LRGILS (100μM) application. B. Time plot of pooled data showing the effect of PAP2-IP (period indicated by horizontal bar, n=9) on fEPSP slope (blue symbols). Note the absence of any significant change in fEPSP slope following LRGILS application.

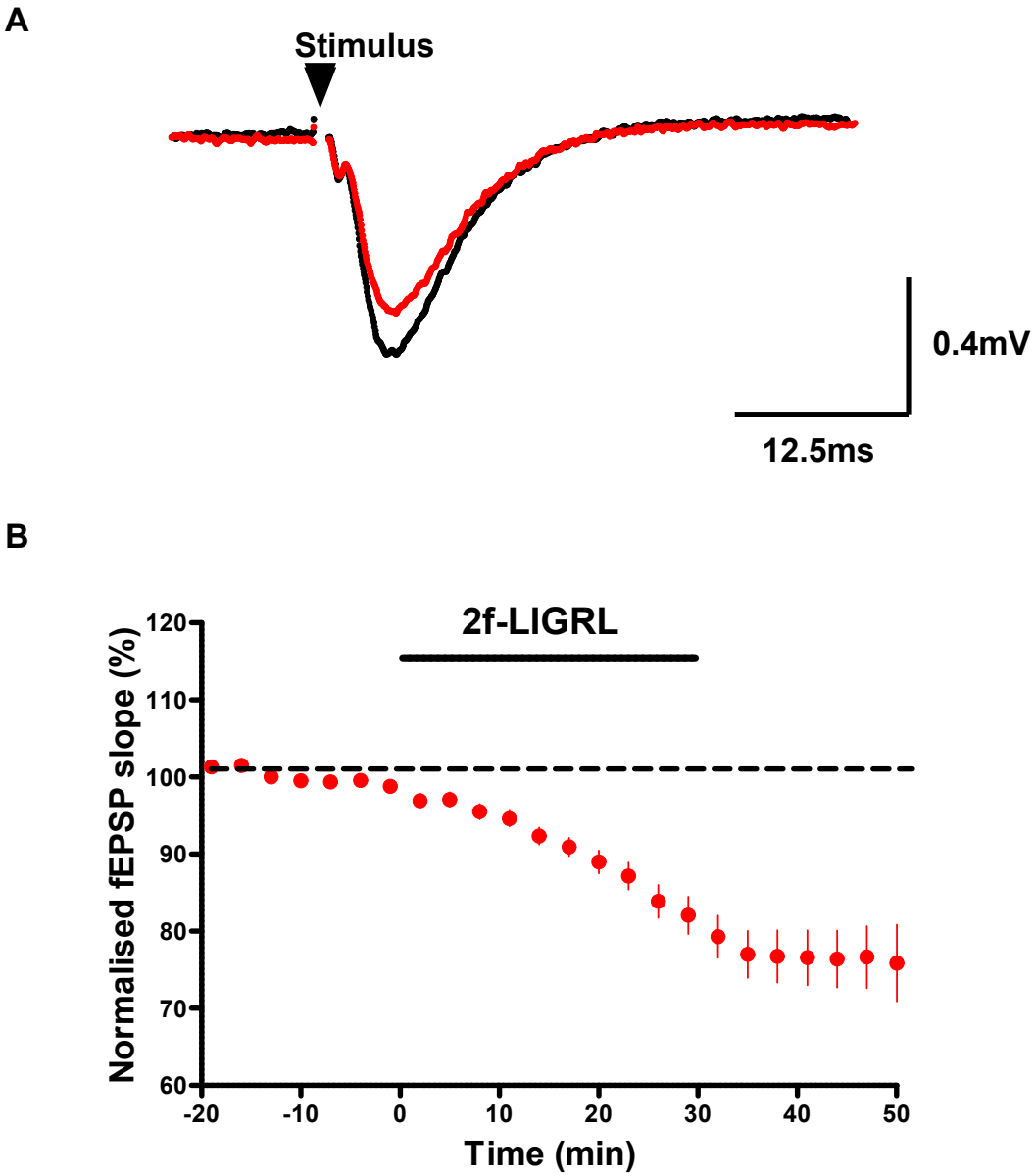


Figure 4-4 2f-LIGRL depresses synaptic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices.

A. Representative raw traces of extracellular field EPSPs recorded from *stratum radiatum* in are CA1 before (black) and following (red) 2f-LIGRL application. B. Time plot of pooled data showing the effect of PAP2-AP (2f-LIGRL, 100μM, n= 9, period indicated by horizontal bar) on fEPSP slope (red symbols). Note the gradual reduction in fEPSP slope following peptide application.

To sum up, in the current study, two independent PAR-2-APs, SLIGRL and 2f-LIGRL, induced a similar long lasting depression of glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices. This effect was unlikely due to slice deterioration. Furthermore, PAR-2-IP, the negative control, did not produce any measurable effect, consistent with the PAR-2-APs producing a discrete and specific suppressant action.

4.3 PAR-1 activation does not modulate synaptic transmission at the Schaffer collateral-to-CA1 synapse

After establishing that PAR-2 activation induced synaptic depression in the acute slices, my investigation extended further to assess whether the activation of another widely studied member of PAR family, namely, PAR-1, would display a role in modulating synaptic transmission in acute hippocampal slices. Therefore, the same extracellular recording configuration was used to investigate the actions of PAR-1 activation. Application of the selective PAR-1-AP, TFLLR (100μM), for 30 minutes did not cause any significant change of the slope of fEPSPs recorded at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices (103.10 ± 0.98% of baseline at 30 minute, n=11, P=0.0578, Figure 4.5), suggesting PAR-1 did not modulate glutamatergic synaptic transmission at this synapse under our experimental conditions.

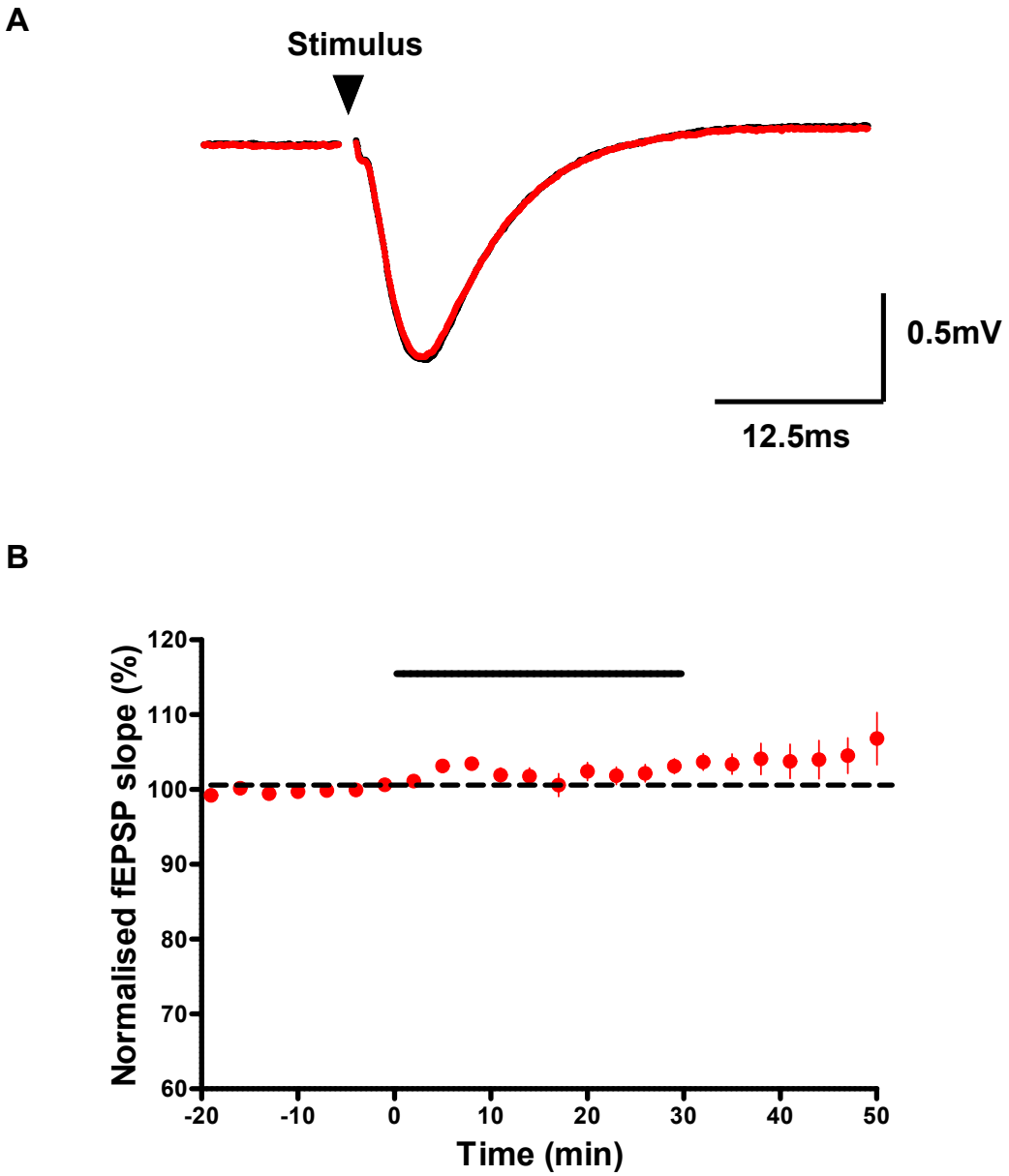


Figure 4-5 The PAR-1-AP, TFLLR, does not depresses synaptic transmission at the Schaffer collateral-to-CA1 synapse.

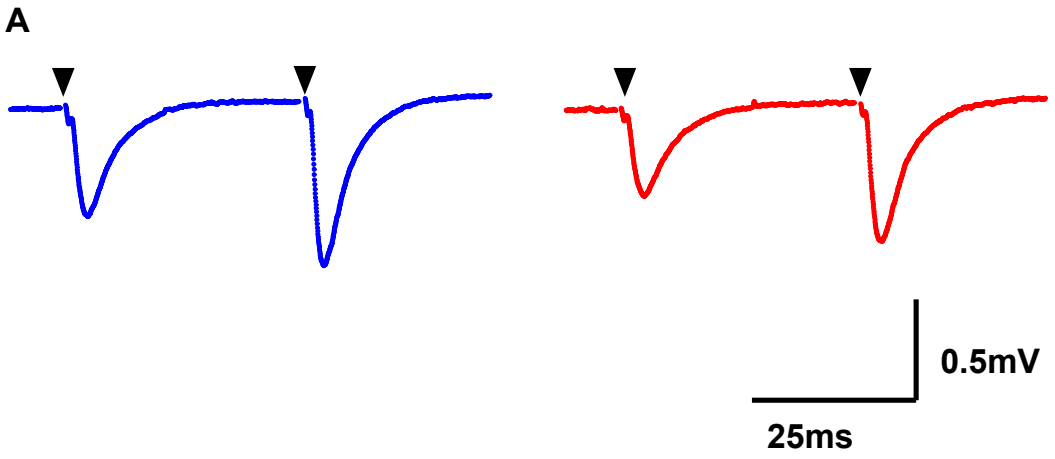
A. Representative raw traces of extracellular field EPSPs before (black) and following (red) TFLLR (100μM) application. B. Time plot of pooled data showing the effect of PAP1-AP (n=11, period indicated by horizontal bar) on fEPSP slope (red symbols). Note the absence of any significant change in fEPSP slope following TFLLR application.

4.4 Mechanisms underlying the PAR-2-induced long term depression of synaptic transmission.

In the following experiments, I investigated further the potential mechanisms underlying PAR-2 activation-induced long lasting synaptic depression at the Schaffer collateral-to-CA1 synapse.

4.4.1 Paired-pulse facilitation is enhanced during PAR-2 activation-induced long lasting synaptic depression

To dissect whether the induction of this novel form of long lasting synaptic depression triggered by PAR-2 activation was of presynaptic or postsynaptic locus, I examined paired pulse facilitation (PPF) at the Schaffer collateral-to-CA1 synapse under first naïve (drug-free) conditions and subsequently in the presence of 100μM SLIGRL. PPF was tested by measuring fEPSP responses following two stimuli delivered at short intervals (50 ms). PPF is a commonly used paradigm to test presynaptic function, where the response to the second stimulus is enhanced due to the residual calcium in the presynaptic terminal following the first stimulus (Malinow & Malenka, 2002). I examined PPF in response to a 50ms interval as described previously (Asaka *et al.*, 2006; Moretti *et al.*, 2006). Whilst the response to the 1st stimulus was consistently smaller in the drug-treated group, the degree of potentiation seen following the 2nd stimulus was in fact enhanced in the presence of SLIGRL. As presented in figure 4.6, the ratios of PPF was significantly increased in the presence of 100μM SLIGRL, compared to naïve control baseline (Baseline: 1.41 ± 0.02, n=7; SLIGRL-LTD: 1.48 ± 0.03, n=7, P=0.0044). However, the absolute value of this change is modest. These data may suggest a presynaptic locus of SLIGRL-induced changes in synaptic signalling.



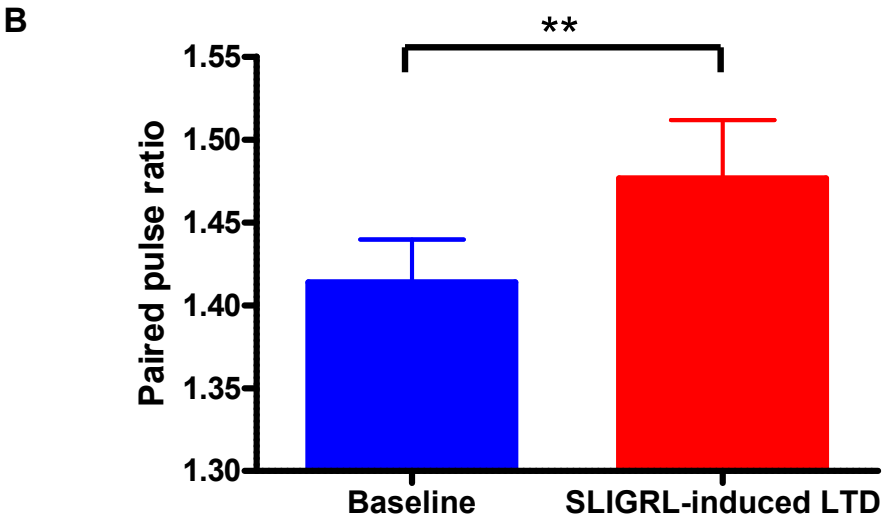


Figure 4-6 SLIGRL-induced long lasting synaptic depression is associated with an increased short term plasticity as revealed by paired-pulse facilitation.

A. Representative extracellular recordings showing paired synaptic responses at 50 ms inter-stimulus interval. Note the presence of paired-pulse facilitation as shown by the increased amplitude of the second pulse in both naïve and SLIGRL treated slices.

B. Bar chart of pooled data reveals a modest but significant enhancement in paired-pulse facilitation following SLIGRL treatment (Control: n=7, SLIGRL treatment: n=7).

4.4.2 mGluRs are not involved in the PAR-2-mediated synaptic depression

mGluRs have been suggested to mediate a variety of chemically-induced forms of long term depression at the Schaffer collateral-to-CA1 synapse (Palmer *et al.*, 1997; Fitzjohn *et al.*, 1999; Fitzjohn *et al.*, 2001). Thus, in the following experiments, I examined whether mGluR activation was implicated in the PAR-2 activation-induced synaptic depression. As presented in figure 4.7, the non-selective group I/II mGluR antagonist, MCPG (500μM), did not prevent LTD induced by SLIGRL (100μM) at these synapses (81.62 ± 2.18% of baseline at 30 minute, n=8, P=0.0043). And this reduction is not significantly different from that observed in SLIGRL-induced LTD in the absence of MCPG (80.75 ± 2.54% of control at 30 minutes, n=12, see figure 4.1, P>0.05). Furthermore, as shown in figure 4.8, following the establishment of well-characterised form of LTD induced by group I mGluRs agonist, DHPG at 50μM (50.99 ± 6.12% of baseline at 20 minute, n=4, P=0.0041), the application of 100μM SLIGRL was still able to elicit further synaptic depression with time course and magnitude similar to that observed in naïve slices. (80.64 ± 3.35% of baseline at 30 minute, n=4, P=0.0383, figure 4.8 and 4.9). Taken these two sets of experiments together, these results strongly suggest that mGluRs are unlikely to be directly involved or required in PAR-2 activation-induced LTD.

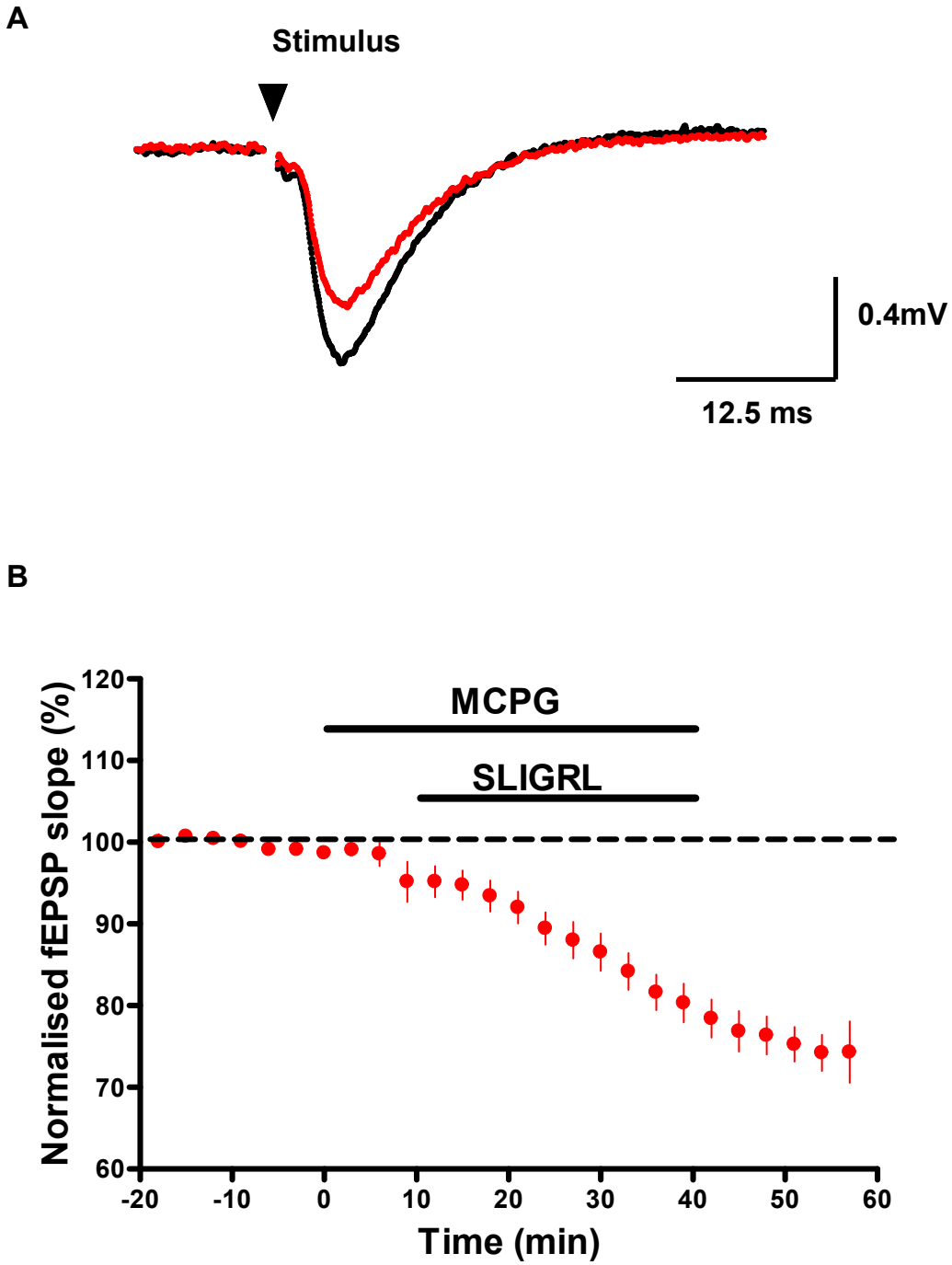


Figure 4-7 MCPG does not block SLIGRL-induced synaptic depression at the Schaffer collateral-to-CA1 synapse.

A. Representative fEPSPs before and following SLIGRL treatment in slices preincubated with the non-selective group I/II mGluR antagonist MCPG (500μM). Colour code: Black-drug free, Red: SLIGRL+MCPG B. Time plot of pooled data showing that MCPG does not prevent SLIGRL-induced depression (n=8).

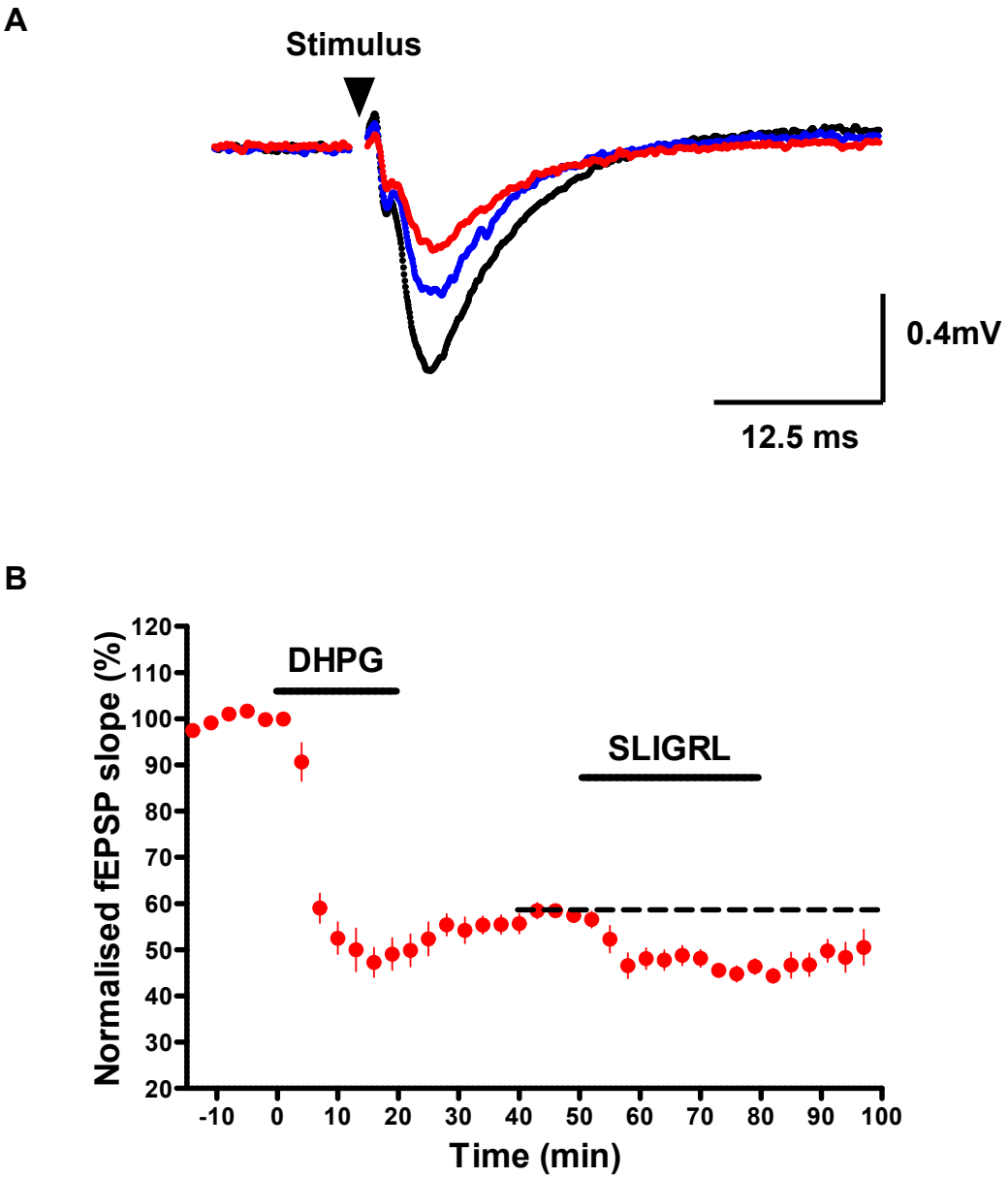


Figure 4-8 DHPG-induced LTD does not occlude SLIGRL-induced synaptic depression at the Schaffer collateral-to-CA1 synapse.

A. Representative extracellular recordings from a single experiment in which DHPG produces a robust synaptic depression which can increased by subsequent SLIGRL application. Colour code: Black-drug free, Blue-DHPG, Red-SLIGRL

B. Time plot of pooled data showing summation of synaptic depression by sequential DHPG (n=4) and SLIGRL (n=4) application. Note there is no evidence for occlusion of synaptic depression by DHPG and indeed the relative depressant action of SLIGRL following DHPG treatment is equal to that seen in naïve slices (see text).

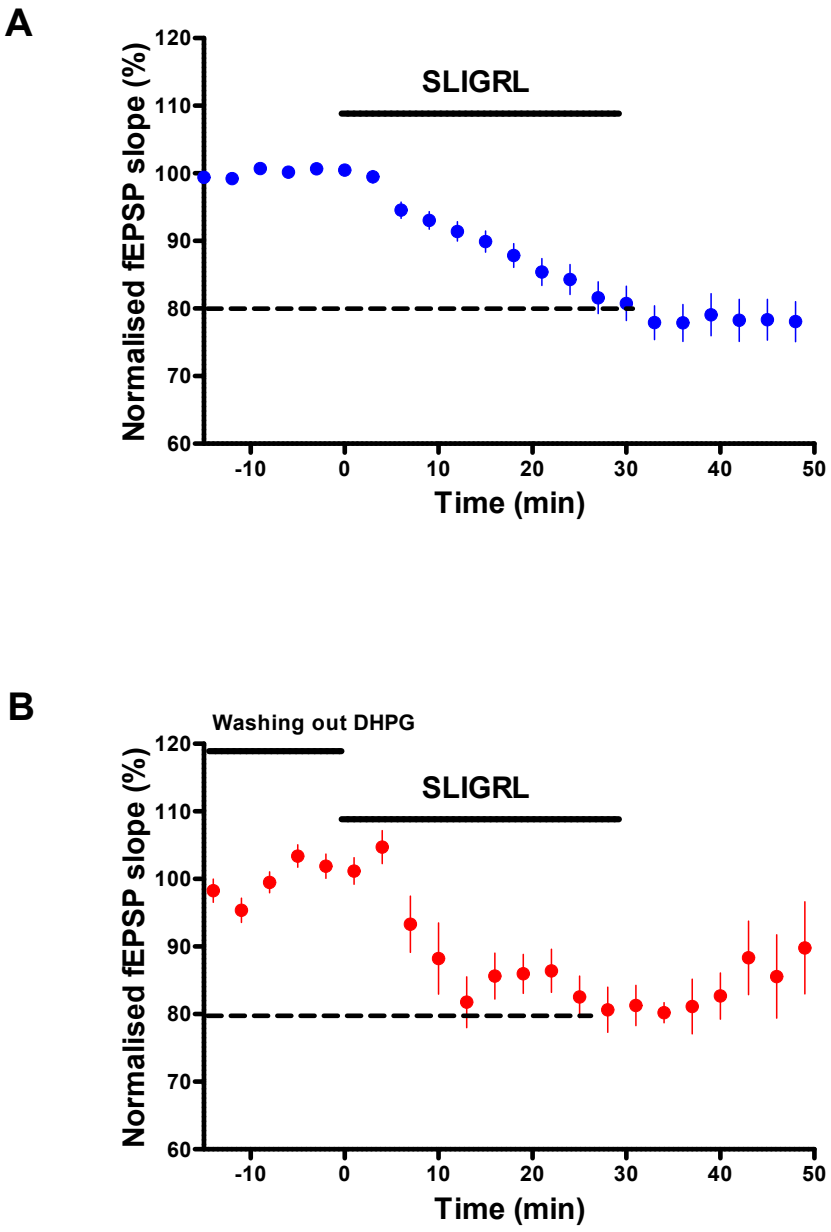


Figure 4-9 Time course and magnitude of SLIGRL-induced LTD following DHPG-LTD (n=4) (B) is similar to that observed in naïve tissue (n=12) (A).

4.4.3 PAR-2-mediated synaptic depression is dependent on NR2B subunit containing-NMDA receptors

As NMDA receptor dependency has been found to be a common mechanism in the induction of several forms of LTD (Dudek & Bear, 1992; Mulkey & Malenka, 1992), after ruling out the involvement of mGluRs in PAR-2 activation-induced LTD, I further tested whether NMDA receptors were involved in PAR-2 activation induced LTD. Surprisingly, as presented in figure 4.10, application of 100μM SLIGRL failed to produce any significant depression at the Schaffer collateral-to-CA1 synapse when applied in the presence of the selective NMDA receptor antagonist DL-APV (100μM, 99.20±1.97% of baseline at 30 minute, n=9, P=0.7852). These initial experiments suggest NMDA receptor activation to be an essential requirement for PAR-2-induced synaptic depression. To investigate this further, 100μM SLIGRL was co-applied in the presence of the selective NR2B-containing NMDA receptor antagonist, Ro 25-6981(3μM)(Fischer *et al.*, 1997). Again, under these conditions, I observed no detectable or significant change in the slope of fEPSPs (100.24±2.35 of baseline at 30 minute, n=5, P=0.9257, figure 4.11). These data are consistent with NMDA, and specifically NR2B-subunit containing NMDA receptors being an absolute requirement for PAR-2 activation-induced LTD.

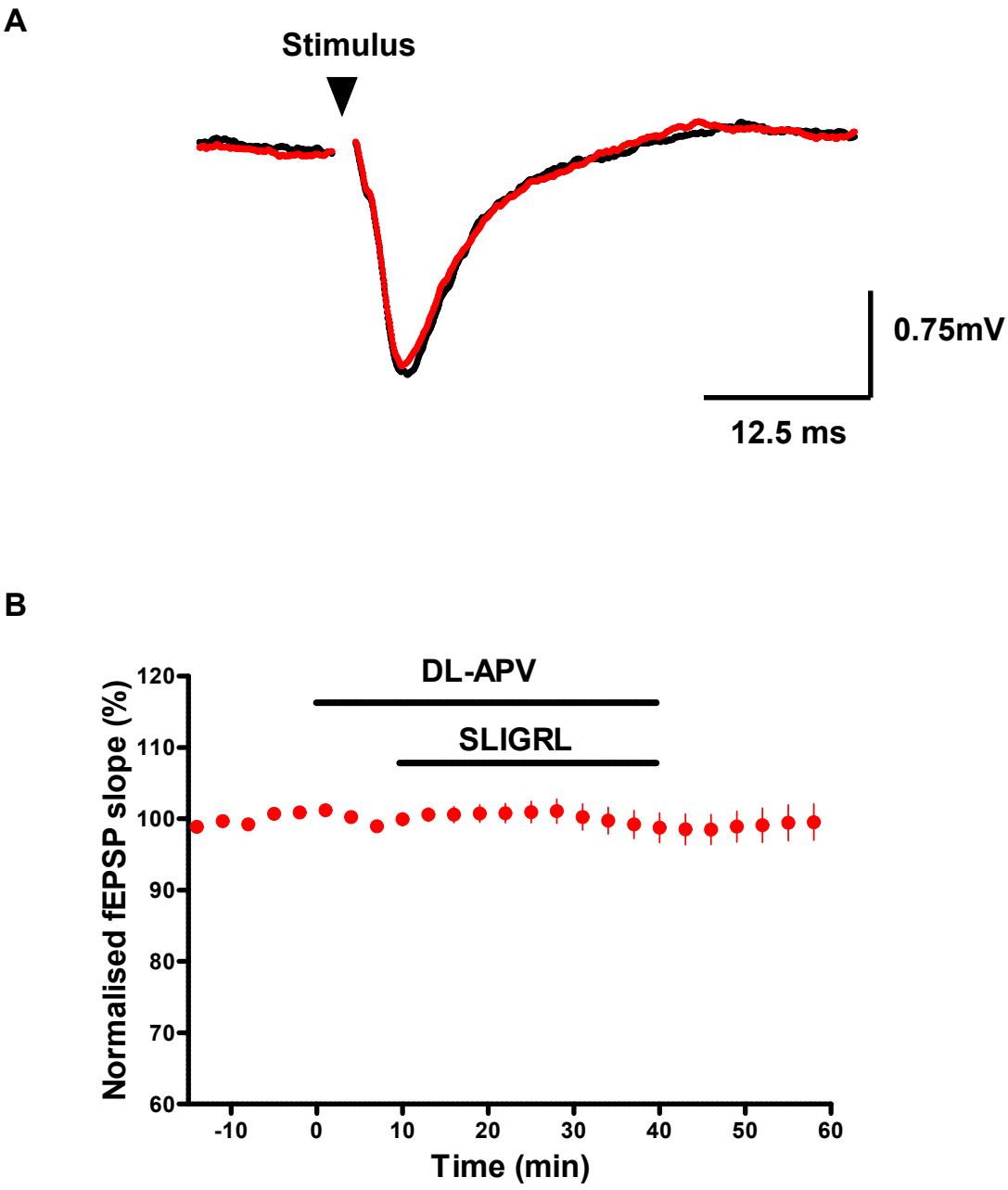


Figure 4-10 DL-APV blocks SLIGRL-induced LTD at the Schaffer collateral-to-CA1 synapse.

A. Representative fEPSPs before (black) and following SLIGRL application (100μM, red trace) in the presence of the NMDA receptor antagonist D-AP5. B. Time plot of pooled data showing PAR2 activation fails to elicit synaptic depression when co-applied in the presence of DL-APV (100μM, n=9) and suggesting the requirement for NMDA receptor activation in PAR-2-induced LTD

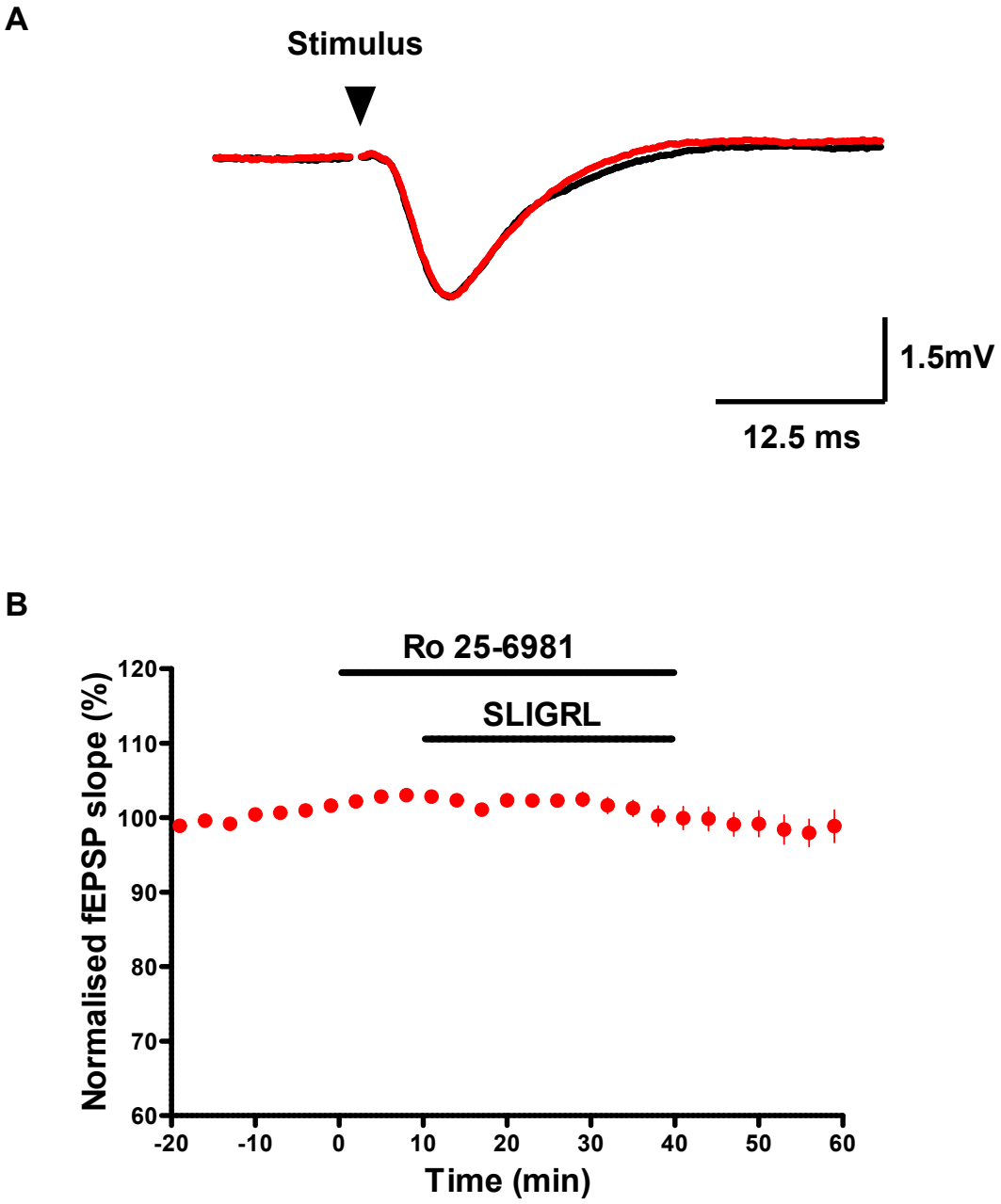


Figure 4-11 Ro 25-6981 blocks SLIGRL-induced LTD at the Schaffer collateral-to-CA1 synapse.

A. Representative fEPSPs before (black) and following SLIGRL application (100mM, red trace) in the presence of the NR2B subunit containing NMDA receptor antagonist Ro 25-6981 (3μM) B. Time plot of pooled data showing PAR2 activation fails to elicit synaptic depression when co-applied in the presence of Ro 25-6981 (3μM, n=5) and suggesting the requirement for NR2B-contaning NMDA receptor activation in PAR2-induced LTD.

4.4.4 Glial-neuronal signalling contributes to the PAR-2-mediated synaptic depression

It has been demonstrated in previous chapter that glial-neuronal signalling was contributing to the PAR-2 activation-induced depression of synaptic transmission in the cultured hippocampal neurones. Here, I examined, under more physiological conditions, whether glial mechanisms also contribute to the PAR-2-induced synaptic depression in acute hippocampal systems. To this end, hippocampal slices were preincubated with the selective glial function inhibitor FAc (10μM) for 3 hours. Initially, in order to test whether this procedure would be detrimental to the health of hippocampal slices, I examined the general properties of neurotransmission in naïve and FAc treated slices. Thus, experiments were performed to record synaptic responses at the Schaffer collateral-to-CA1 synapse to varying intensities of single electrical stimulation so that an input-output relationship could be established. Increasing stimulating intensity resulted in the expected accompanying increase in fEPSP slope (Figure 2.5). However, as also shown in figure 4.12, no significant difference in input-output curve was observed between naïve (drug-free) slices and those treated with FAc (naïve: n=12, FAc treated: n=9. stimulus intensity: 30μA, P=0.7095; 60μA, P=0.6659; 90μA, P=0.9440), suggesting basal integrity of synaptic transmission was not altered by 3 hours treatment of 10μM FAc.

After confirming the synaptic integrity in slices treated by FAc, extracellular recording was repeated as previous described. As shown in figure 4.13, the SLIGRL-induced LTD was significantly reduced in FAc treated slices compared to those recorded from naïve slices (naïve: 78.48 ± 3.14% of baseline at 30 minute, n=4; FAc treated: 94.11 ± 1.48% of baseline at 30 minute, n=6, P=0.0287), suggesting glial-neuronal signalling was indeed contributing to PAR-2 activation-induced LTD in hippocampal slices.

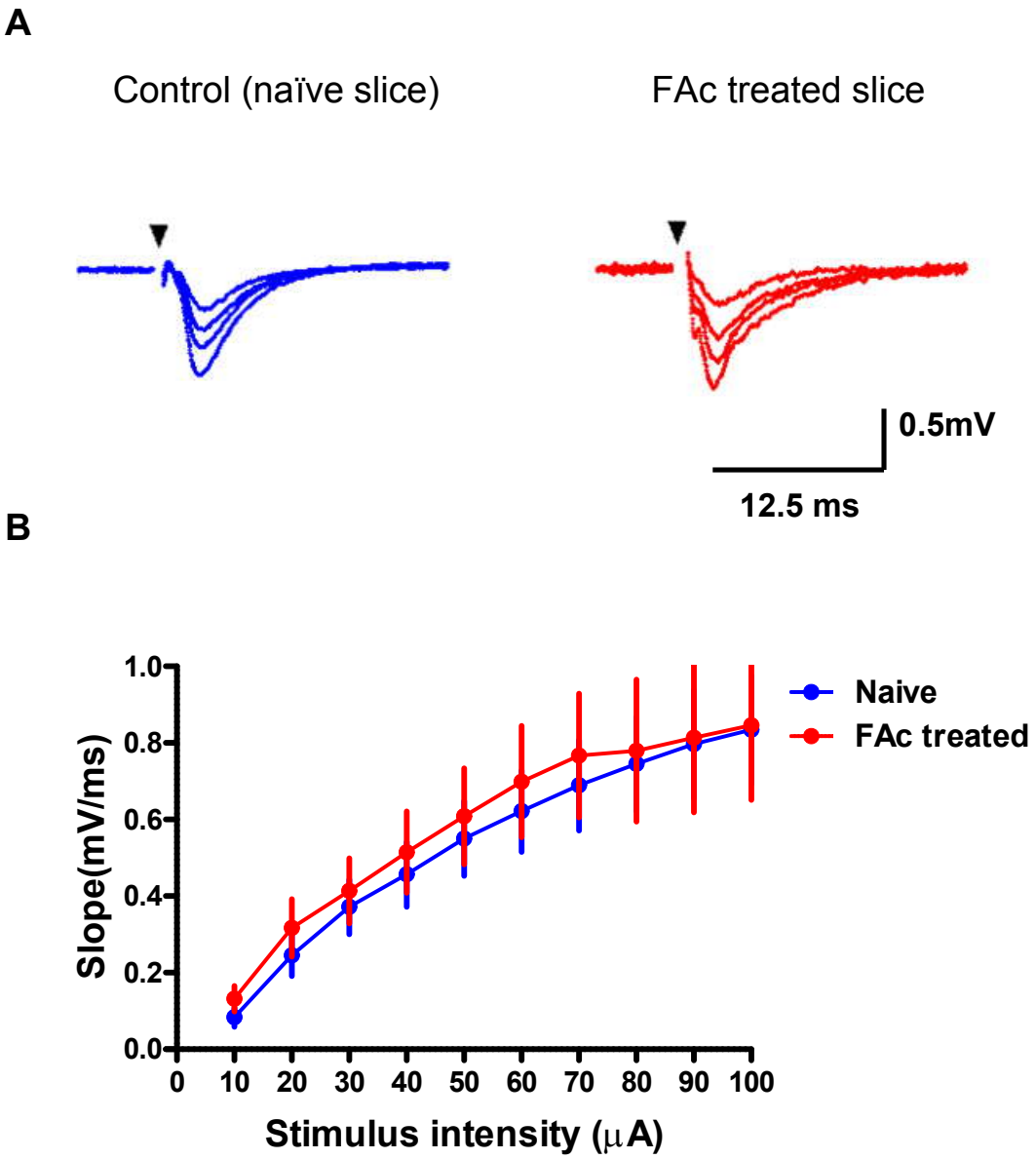


Figure 4-12 Input-output curve in naïve and FAc treated slices.

A. Representative fEPSPs in response to increasing stimulation (arrowhead) intensity. B. Similar experiment in performed in slice preincubated with the astrocyte inhibitor FAc.

B. Pooled input-output plot showing the characteristic relationship between stimulation intensity and fEPSP magnitude. Note there is no significant difference between control (blue symbols, n=12) and FAc treated slices (red, n=9).

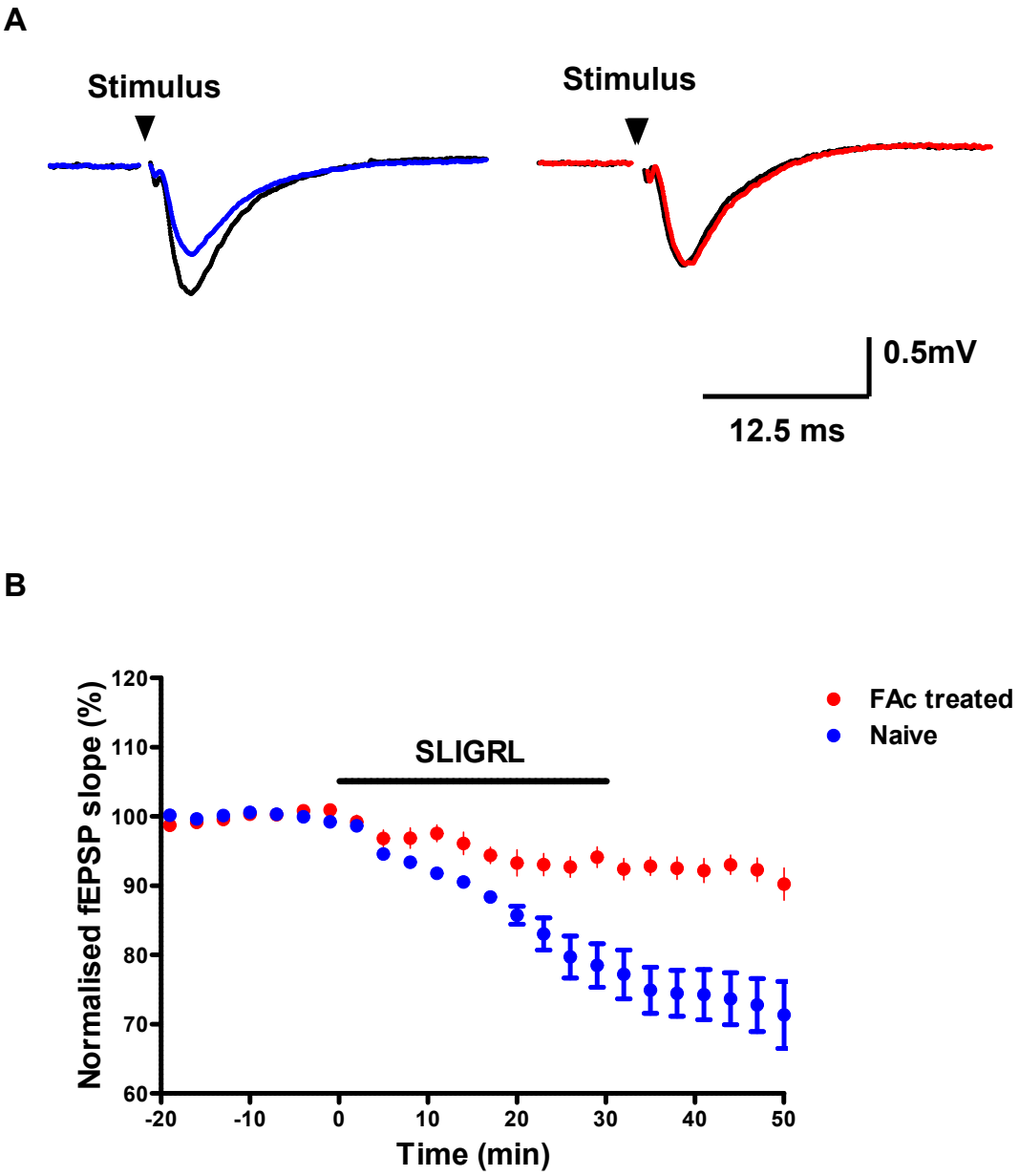


Figure 4-13 SLIGRL-induced LTD is significantly reduced in FAc treated slices.

A. Representative fEPSP recordings before and after SLIGRL treatment in naïve slices (blue symbols) and in slices preincubtated in FAc (Red symbols). B. Time plot of pooled data showing actions of SLIGRL on naïve slices (blue, n=4) and FAc treated slices (n=6). Note a significant reduction of the magnitude of SLIGRL-induced LTD in FAc treated slices (P=0.0287)

4.5 Discussion

In this chapter, I demonstrated, for the first time, that activation of PAR-2 induced a novel form of long lasting depression of glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices. Furthermore, I provided evidences that this PAR-2 activation-induced LTD is dependent on NR2B subunit-containing NMDA receptors and suppressed by disrupting glial cell function.

In this chapter, I first demonstrate that 100μM PAR-2-AP, SLIGRL, which at the concentration used is reported to elicit the maximal Ca^{2+} mobilisation in expression system (Nystedt *et al.*, 1995b), and was similar to previous study in investigating Ca^{2+} mobilisation by PAR-2 activation in cultured hippocampal neurones and astrocytes (Bushell *et al.*, 2006), induced a novel form of long lasting depression of glutamatergic transmission at the Schaffer collateral-to-CA1 synapse (see figure 4.1). The specific nature of the PAR-2-mediated response is confirmed by the lack of effect using the control peptide, 100μM LRGILS (see figure 4.3). This selectivity is further strengthened by the fact that another independent PAR-2-AP, 2f-LIGRL, induces similar LTD at the same synapses under equivalent conditions (see figure 4.4). Thus, these data support our hypothesis that PAR-2 activation modulates basal synaptic transmission and agree with the indication discussed in chapter 3 that PAR-2 activation induces a reduction of synaptic transmission in the cultured hippocampal neurone network (see figure 3.18 and 3.19).

However, this interpretation could be hampered by the concern that the irreversibility of PAR-2-induced LTD may be the consequence of the deterioration of the integrity of presynaptic terminals perhaps due to the neurotoxic effect of the chemical nature of these peptides. Therefore, this concern is addressed by the reanalysis of presynaptic fibre volley amplitude, which is an indicator of integrity of presynaptic terminals. My data demonstrated no significant change of presynaptic fibre volley amplitude during PAR-2-mediated LTD (see figure 4.1B), which confirms the integrity of presynaptic input. Furthermore, intact LTP induced by HFS in slices conditioned with 100μM SLIGRL incubation (see figure 4.2C) provides further evidence to support this

interpretation. Thus, the PAR-2-mediated LTD is not due to the deterioration of slice quality.

It was recently reported that PAR-2 activation caused a temporary deficit in the formation and/or recollection of experience-dependent learning and memory in a strain of rat (Lohman *et al.*, 2009). Considering the long term plasticity in the brain is well recognised cellular basis for learning and memory (Bliss & Collingridge, 1993; Martin *et al.*, 2000), it is of special interest to investigate into if PAR-2 activation in acute hippocampal slices would modulate long term plasticity. Interestingly, my data show that in the slices preconditioned with 100μM PAR-2-AP, SLIGRL, the LTP is indistinguishable from that elicited from naïve slices (see figure 4.2). Furthermore, this LTP is induced upon previously established synaptic depression by PAR-2-AP application. Thus, on one hand, this data suggest that the LTD induced by 100μM PAR-2-AP is reversible, confirming the integrity of the synapses after the concerns over potential neurotoxic effect of relatively high concentration of PAR-2-AP. On the other hand, this result suggests that the activation of PAR-2 before the input-postsynaptic association, as given by the HFS, has no disruption on the mechanisms underlying the induction and expression of NMDA-receptor dependent LTP at the Schaffer collateral-to-CA1 synapse. However, it is acknowledged that in order to address the question whether PAR-2 activation modulates hippocampal long term plasticity more convincingly, a better solution is to investigate LTP induction and expression in the presence of PAR-2-AP. But this was rather unfeasible in my hand, because PAR-2-AP depresses basal synaptic transmission (see figure 4.1). Alternatively, LTP could be thoroughly investigated utilising the PAR-2 knockout animals (Ferrell *et al.*, 2003) and their littermate controls. However, due to the time limit of my PhD, this is the subject of further experimentation.

An unexpected finding in this chapter is, in my hand, the PAR-1 activation has no modulatory role in the basal transmission of glutamatergic transmission at the Schaffer collateral-to-CA1 synapse in acute hippocampal slices (see figure 4.5). This result is in contrast to a previous report which showed the PAR-1-AP, SFLLRN (15μM), induced a gradual robust increase (50% increase of baseline) of fEPSPs recorded at the Schaffer collateral-to-CA1 synapse, which reach a plateau after 40 minutes, in rat hippocampal slices (Maggio *et al.*, 2008). The

disagreement between these two investigations could be explained by firstly, different PAR-1-APs employed in these studies. In my study, I used the TFLLR rather than SFLLRN as the PAR-1-AP because the substitution of threonine for serine prevents the cross-activation or desensitisation of PAR-2 (Hollenberg *et al.*, 1997). Thus, because of the lack of selectivity for PAR-1, theoretically, the robust increase of fEPSPs observed by Maggio and co-workers using SFLLRN could be a synergy effect of activation of both PAR-1 and PAR-2. If this is the case, combining my results, these data suggest the synergy consequence on synaptic transmission by dual activation of both PAR-1 and PAR-2 is distinct from that upon activation of individual PARs. Secondly, the concentration of PAR-1-AP utilised is different. Maggio and co-workers used 15μM as working concentration, which is close to the concentration used (10μM) in investigating PAR-1 action in acute hippocampal slices (Gingrich *et al.*, 2000) and slightly higher than the EC_{50} of SFLLRN (0.5-6μM) (Macfarlane *et al.*, 2001). In contrast, I used a relatively high concentration of TFLLR of 100μM although the peptide itself is more selective than SFLLRN. To reconcile the disagreement, it is possible that PAR-1 potentiates synaptic transmission under relatively modest activation upon low concentration of activator, whereas, this modulation of basal transmission could be masked upon strong activation of the receptor itself through an unknown mechanism involving perhaps feedback inhibition. Considering that in disease conditions, modest activation of PAR-1 tends to be protective and strong activation of PAR-1 is degenerative (see Chapter 1.3.7.4), it is reasonable to speculate a scenario that 'multi-face' modulation of synaptic transmission by PAR-1 is upon the magnitude of its activation under normal brain physiology.

In this chapter, I investigated further into whether the presynaptic mechanism is involved in the expression of the PAR-2-mediated LTD at the Schaffer collateral-to-CA1 synapse. I demonstrate that the paired-pulse ratio, which is an indicator of presynaptic involvement, is increased during the PAR-2 activation-induced LTD, suggesting a presynaptic locus. However, the change of absolute value of paired pulse ratio from 1.41 to 1.48 (see figure 4.6), though statistically significant, is relatively very modest to support a strong presynaptic contribution to the PAR-2-mediated LTD. On the contrary, another interpretation of this data is that because the synaptic transmission is reduced during the PAR-2-mediated LTD at the first place, the small increase in the paired pulse ratio could be

attributed to a reduced transmitter release probability at presynaptic terminals in agreement with the depressed synaptic transmission, which in turn results in an increase of fEPSP amplitude upon second stimulus as a result of increased residual Ca^{2+} in the presynaptic terminals in response to the first stimulus corresponding to the paired-pulse paradigm. Nevertheless, in order to clarify the presynaptic contribution for PAR-2-mediated LTD, a positive control experiment employing, for instance baclofen (1μM), the agonist for $GABA_B$ receptors which locate presynaptically, could be introduced to test the magnitude of paired pulse ratio under identical recording conditions. This experiment will give some clues that whether this established presynaptic inhibition (Mott & Lewis, 1994; Misgeld *et al.*, 1995) elicits comparable increase of paired pulse ratio as what was observed in PAR-2-induced synaptic inhibition.

Finally, in this chapter, I investigated further into the cellular mechanism underlying PAR-2 activation-induced LTD. LTD at the Shaffer collateral-to-CA1 synapse has been demonstrated to be NMDA-receptor dependent or NMDA-receptor independent (Dudek & Bear, 1992; Mulkey & Malenka, 1992; Bashir *et al.*, 1993; Bashir & Collingridge, 1994; Fitzjohn *et al.*, 1998; Citri & Malenka, 2008; Rebola *et al.*, 2010). Especially to our interest, a widely reported form of chemically-induced LTD expresses at this synapse in response to group I mGluRs agonist, DHPG (Palmer *et al.*, 1997; Fitzjohn *et al.*, 1999; Fitzjohn *et al.*, 2001; Gladding *et al.*, 2009; Luscher & Huber, 2010). To differentiate whether PAR-2 activation-induced LTD is dependent on mGluRs, I used two strategies. Firstly, I demonstrated 500μM MCPG, a non-selective antagonist of group I/II mGluRs, has no effect on PAR-2 activation-induced LTD (see figure 4.7). Secondly, 100μM SLIGRL still produced a further long lasting depression following LTD induced by the group I mGluR agonist, DHPG (50μM) (see figure 4.8) with the amplitude of this further synaptic depression being similar to that induced in naïve slices (see figure 4.9). Taken together, these data strongly suggest at least group I/II mGluRs are not involved in the PAR-2 activation-induced LTD at the Schaffer collateral-to-CA1 synapse. Therefore, I tested whether this novel form of LTD is NMDA receptor dependent. Surprisingly, the NMDA receptor antagonist DL-APV (100μM) completely abolished the SLIGRL-induced LTD (see figure 4.10). In addition, a specific NR2B subunit-containing NMDA receptor antagonist, Ro 25-6981(3μM) (Fischer *et al.*, 1997) also prevented the SLIGRL-induced LTD (see

figure 4.11). These data suggest the NR2B subunit-containing NMDA receptor is required during the PAR-2 activation-induced LTD. Previous studies showed conflicting data regarding the role of NR2B subunit-containing NMDA receptors in the induction of LTD (Kutsuwada *et al.*, 1996; Hendricson *et al.*, 2002; Liu *et al.*, 2004). However, my results here suggest that the NR2B subunit-containing NMDA receptors may be important in governing at least PAR-2 activation-induced LTD in the hippocampus. As PAR-2-induced LTD is suggested to be NMDA receptor dependent, it is possible that PAR-2-mediated LTD may utilise a common induction mechanism that has been observed in other forms of NMDA receptor dependent LTD. Clearly dinstinct from mGlu receptor dependent LTD, NMDA receptor dependent LTD requires intracellular Ca^{2+} singal (Gladding *et al.*, 2009; Collingridge *et al.*, 2010). The Ca^{2+} sensor protein, calmodulin, detects the entry of extracellular Ca^{2+} through NMDA receptors. The binding of Ca^{2+} with calmodulin activates protein phosphatase 2B, which in turn dephosphorylates inhibitor-1 to activate protein phosphatase 1 (Mulkey *et al.*, 1993). Protein phosphatase 1 is the key enzyme in NMDA receptor dependent LTD, as it dephosphorylates Ser845 on the AMPA receptor subunit GluR1, to initiate clathrin-mediated endocytosis of AMPA receptors (Collingridge *et al.*, 2004), which leads to LTD. Therefore, it is of special interest to further test whether employing protein phosphatase 1 inhibitors, for instance, okadaic acid (1μM) or calyculin A (1μM) (Mulkey *et al.*, 1993), could prevent the synaptic depression induced by PAR-2 activation in identical preparations. These experiments are likely to give futher evidences for the NMDA receptor dependency of PAR-2-mediated LTD and to shed light on its induction mechanisms.

Based on the suggestion from Chapter 3 that neurone-glial signalling may be important in the modulation of neuronal excitability and synaptic transmission upon PAR-2 activation in hippocampal cultures, I further investigated into the whether this signalling is also contributing the PAR-2 activation-induced LTD in acute hippocampal slices. Selective blockade of glial metabolism in acute slices by FAc (10μM) incubation significantly attenuated the magnitude of SLIGRL-induced LTD (see figure 4.13). In addition, the indistinguishable input-output curves obtained from FAc-treated and naïve slices confirmed no significant neuronal damage was brought in during the FAc incubation (see figure 4.12). These data are in agreement with my observation that neurone-glial signalling

was important in hippocampal cultures as discussed in chapter 3. They also demonstrated that neurone-glial signalling we observed in culture system was not a culture artefact that due to *in vitro* manipulations.

In conclusion, in chapter 4, I demonstrated for the first time that the activation of PAR-2 in acute hippocampal slices induces a novel form of LTD at the Schaffer collateral-to-CA1 synapse. Furthermore, the PAR-2-mediated LTD is dependent on NR2B subunit-containing NMDA receptor activation as well as normal glial-neuronal signalling.

5 Modulation of epileptiform activity in the hippocampus by PARs activation

5.1 Introduction

PARs are confirmed to be expressed in a variety of cell types, including neurones and astrocytes, in the CNS (Bushell *et al.*, 2006; Bushell, 2007; Luo *et al.*, 2007), and their involvement in the pathology of the brain has been widely reported (Noorbakhsh *et al.*, 2003; Luo *et al.*, 2007). In particular, recent evidences suggest PARs are crucial players in modulating epileptogenesis both *in vivo* and *in vitro*. One *in vivo* study demonstrated that intravenous application of the selective PAR-2-AP, SLIGRL protected rats against kindling-induced seizures (Lohman *et al.*, 2008). This led the authors to conclude that PAR-2 may have a protective role during epileptogenesis. This is in contrast with a recent *in vitro* study showing PAR-1 activation to facilitate epilileptogenesis in rat hippocampal slices (Maggio *et al.*, 2008), advocating a pro-epilepicactivity role for this PAR subtype. These studies suggest, despite being within the same receptor family and activation by common mechanisms, though possibly they are activated under different conditions, PARs may have differential modulatory effect on cortical network activity such as epilepsy. Moreover, the potential underlying mechanisms for this differential regulation are unclear. In this regard, this chapter makes an initial attempt to assess the influence of both PAR-2 and PAR-1 on epileptiform activity *in vitro* in the hope of gaining a better understanding of how PAR-2 and PAR-1 modulate cortical networks.

Acute hippocampal slice preparations have been extensively utilised to develop a range of different *in vitro* pharmacology-based models of experimental epileptogenesis (Jefferys, 1994, 2003). These have been invaluable for investigating some of the mechanisms underlying the genesis and control of epilepsy-like behaviours in hippocampal circuits (Rutecki *et al.*, 1985; Voskuyl & Albus, 1985; Tancredi *et al.*, 1990). Epileptiform activity can be induced in acute hippocampal slices by changing the ion composition of the perfusate, or applying convulsant agents such as ion channel blockers or modulators of excitatory or inhibitory synaptic transmission. Two models of epileptiform activity used in the

current investigations were 1. application of 4-aminopyridine (4-AP)/ low magnesium perfusate and 2. application of bicuculline. Both models produce robust and stereotyped patterns of epileptiform activity (rhythmical synchronised field potentials) over long periods. 4-AP is a non-selective K^+ channel blocker, which is used clinically in treating demyelinating disorders (Hayes, 2004; Judge & Bever, 2006) and used widely as an experimental tool in studying K^+ channels (Storm, 1987; Lesage, 2003). It has also be used widely to produce epilepsy *in vivo* (Morales-Villagran *et al.*, 1996; Pena *et al.*, 2002) and epilepsy-like activities in acute hippocampal slices (Voskuyl & Albus, 1985; Roshan-Milani *et al.*, 2003). At least two main factors contribute to 4-AP-induced epileptiform activity: firstly, by blocking K^+ channels in soma-dendritic membrane in postsynaptic neurone to depolarise the cells and drive it closer to AP threshold (Roshan-Milani, 2004). Secondly, by blocking K^+ channels in presynaptic axon terminals, 4-AP enhances neurotransmitter release by prolonging AP duration (Molgo *et al.*, 1977). Omitting Mg^{2+} from the perfusate removes the voltage-dependent Mg^{2+} blockade of NMDA receptors, which in turn allows free Na^+, Ca^{2+} and K^+ to move through the channels in response to glutamate and subsequent hyperexcitability. A unique feature of 4-AP-induced epileptiform activity is that synchronisation of firings occurs despite normal even enhanced inhibitory inputs (Rutecki *et al.*, 1987). In contrast, the $GABA_A$ receptor antagonist, bicuculline, produces eplileptiform activity in hippocampal slices by simple disinhibition of hippocampal microcircuits which then results in synchronised and unchecked firing of cells in area CA3 through recurrent excitatory collaterals. This enhancement is maintained by strengthening recurrent excitatory synapses among CA3 pyramidal neurones (plasticity) following disinhibition (Miles & Wong, 1983, 1987). As these two models induce *in vitro* epilepsy-like activities by either enhancing gross excitation (4-AP/low Mg^{2+}) or depressing overt inhibition (bicuculline), their combination provides a unique approach to assess whether PAR-2 activation modulates epilepsy-like activities differentially through these two opposite mechanisms.

To sum up, the specific aims of this chapter were to assess whether PAR-2 and PAR-1 exert any modulatory influence over pathological network activities under conditions where network excitation is enhanced (4-AP/0 Mg^{2+} model) or when synaptic inhibition is compromised (bicuculline model).

5.2 Characterisation of epileptiform activity in 4-AP/0 Mg^{2+} model

Extracellular recordings were obtained from the CA3 *stratum pyramidale* in hippocampal slices prepared from 16-24 days old Wistar rats. No detectable spontaneous field events were recorded during control period (drug free, normal ACSF) in all slices tested (n=33). Subsequent perfusion with 0 Mg^{2+} ACSF supplemented with 4-AP (100μM) resulted in the gradual appearance of intermittent, large amplitude, rhythmical field events (see figure 5.1A, B). These intermittent events of short burst duration mirrored the inter-ictal-like activity described in previous reports (Avoli et al., 1993; Mattia et al., 1994; Roshan-Milani et al., 2003).

Individual inter-ictal-like events (see figure 5.1C) comprised of (1) a large, fast negative deflection followed by (2) an initial fast and later slower positive potential, which overshot baseline to reach peak amplitude and then (3) a slow negative potential, some of which were nested by a high frequency oscillatory burst component (see figure 5.1). The application of 4-AP with 0 Mg^{2+}ACSF resulted in the occurrence of inter-ictal-like events within 10-20 minutes. The instantaneous frequency of these events increased to eventually plateau at a stable and stereotyped discharge with a mean instantaneous frequency of 1.51 ± 0.16Hz (n=33). The mean amplitude of these events was 2.03 ± 0.27mV (measured baseline to positive peak; n=33). However, the amplitude was rather variable and exquisitely sensitive to the precise placement of the recording electrode. Therefore, all subsequent analyses focused on the temporal domain rather and response amplitude.

The occasional appearance of prolonged seizure-like discharges or 'ictal-like' events was observed only in a small minority of experiments (7 in 33 slices) (see figure 5.2). Because the occurrence of ictal-like activity was rare, inconsistent and irregular across this set of experiments, the following analysis of epileptiform activity focused on the inter-ictal-like activity.

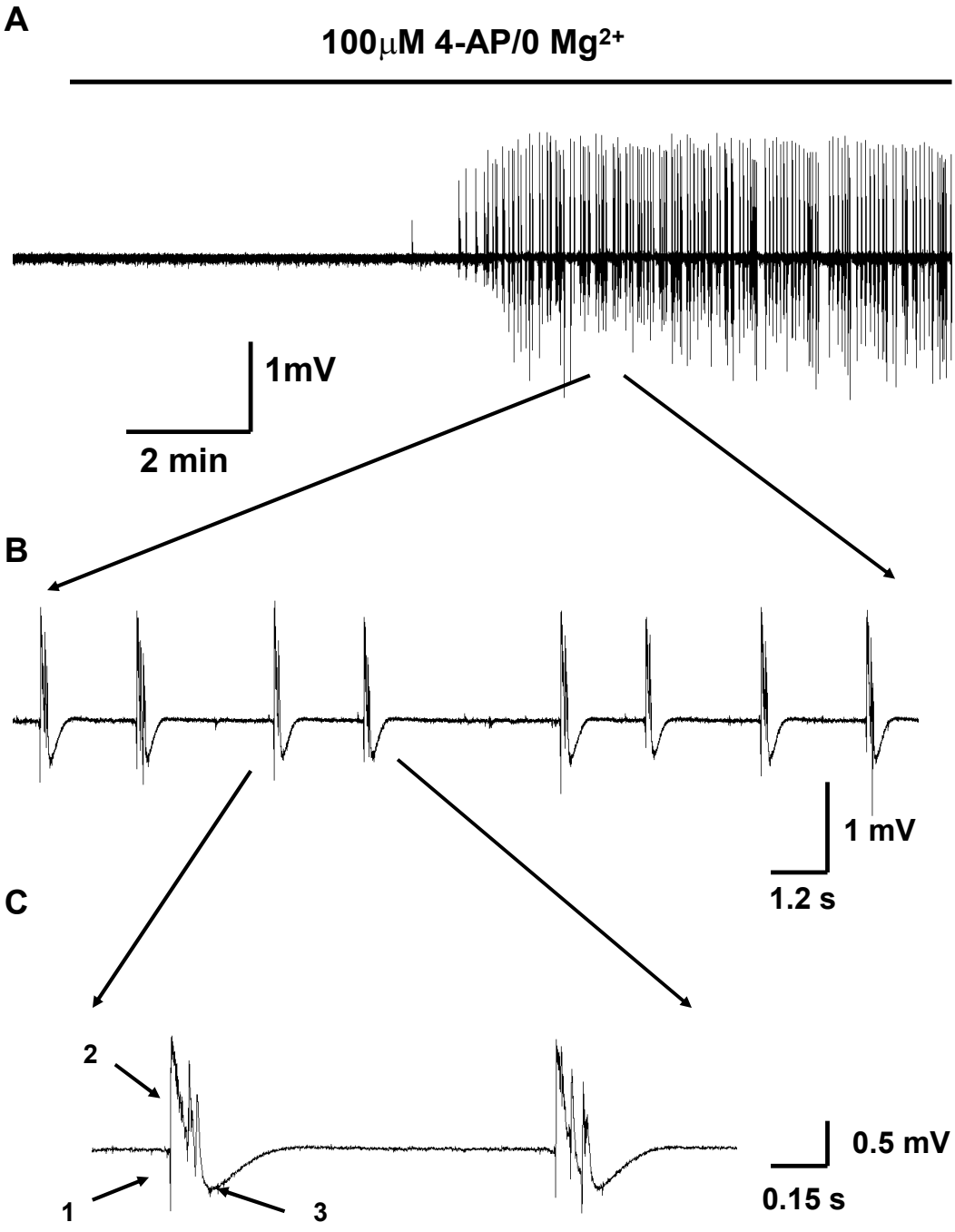


Figure 5-1 4-AP/ 0 Mg^{2+}-induced epileptiform activity in the CA3 area of acute hippocampal slices.

A. Representative extracellular recording upon bath application of the 0 Mg^{2+} ACSF supplemented with convulsant compound, 4-AP (100μM) as indicated by the solid bar. Note the appearance of rhythmical field events within 10 minutes of 4-AP/0 Mg^{2+} application. B. Expanded trace of the same recording shows intermittent and coherent inter-ictal-like events. C. Further expansion of trace to resolve the waveform of individual spontaneous field events show then to comprise of: (1) a fast negative potential followed by a (2) a series of fast positive potentials (extracellular action potential burst) followed by a (3)slow negative potential.

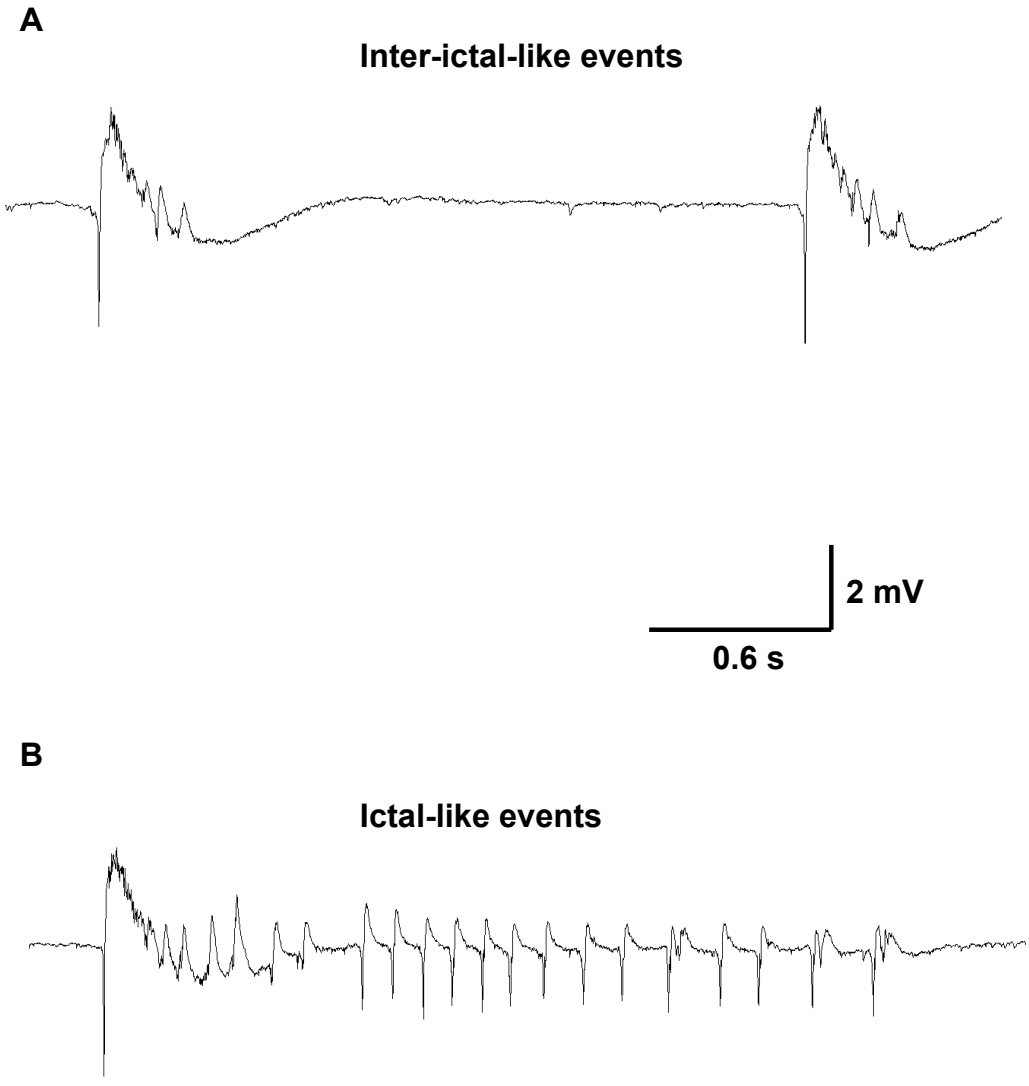


Figure 5-2 Examples of two types of 4-AP/0 Mg^{2+}-induced epileptiform activity in the CA3 area of acute hippocampal slices.

A. Representative trace of intermittent regular inter-ictal-like activity (spontaneous field bursts) which was the most dominant form of epileptiform activity induced by 4-AP/0 Mg^{2+} application (seen in all 33 slices). B. Example of ictal-like discharge pattern which was observed (in addition to inter-ictal bursts) in 7 of 33 slices recorded. Note the prolonged burst discharge separated by periods of relative network quiescence. Note that the initial response resembles a burst-like response but activity is thereafter on terminated but shows a prolonged discharge pattern consistent with a breakdown of inhibition.

5.3 PAR-2 activation depresses the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activities in acute hippocampal slices

Sustained and regular inter-ictal-like activities were recorded in the CA3 cell body layer following the application of 0 Mg^{2+} ACSF supplemented with 4-AP (100μM). 10 minutes baseline of inter-ictal-like activities with stable bursting frequency was required before 100μM PAR-2-AP, SLIGRL, was applied through the perfusate. As presented in figure 5.3B, application of SLIGRL for 30 minutes significantly depressed the bursting frequency of inter-ictal-like activities from 1.53 ± 0.21Hz to 1.18 ± 0.17Hz (n=13, P=0.0006). Individual experiment time plots revealed a gradual yet profound SLIGRL-induced depression of the bursting frequency with slow time course resembling that seen for the depression of evoked synaptic responses reported in chapter 4 (see figure 4.1), (normalised burst frequency, control: 100.98 ± 0.90%, drug: 80.46 ± 4.40%, n=13, P=0.0004, figure 5.3C). Furthermore, this depression of epileptiform burst frequency persisted following the washout of SLIGRL as was also seen with the synaptic depression experiments (chapter 4, see figure 4.1). In contrast, the application of negative control, 100μM PAR-2-IP, LRGILS, for 30 minutes, did not lead to any significant change in 4-AP/0 Mg^{2+}-induced bursting activities (control: 1.60 ± 0.40Hz, drug:1.71 ± 0.40Hz, n=6, P=0.1721,figure 5.4B). This trend is also seen in the normalised pooled data sample as shown in (figure 5.4C, normalised burst frequency, control: 98.40 ± 2.46%, drug: 106.73 ± 6.14%, p=0.2043, n=6). Together, these data suggesting the depression of bursting frequency was indeed a specific action of PAR-2 activation.

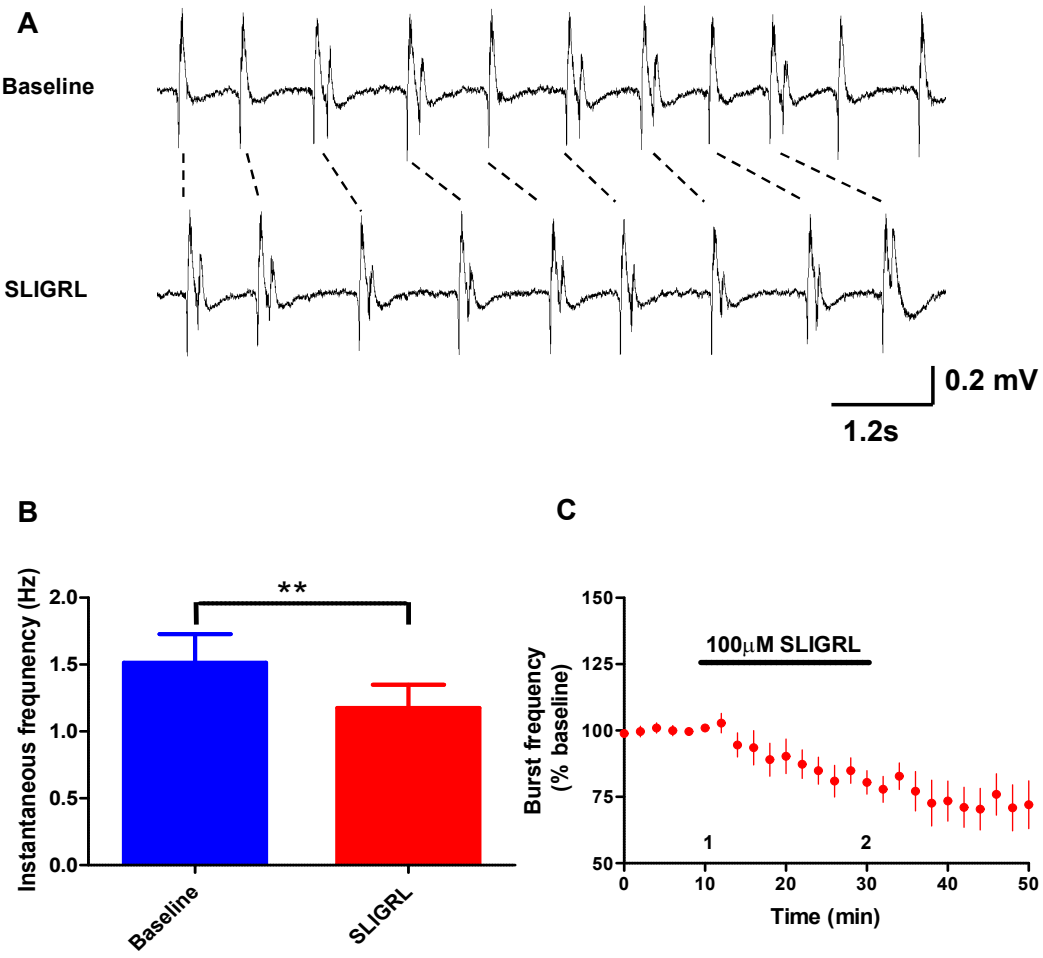


Figure 5-3 PAR-2 activation reduces the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activity in the CA3 area of acute hippocampal slices.

A. Representative traces (20080805/2) show the occurrence of inter-ictal-like activities during baseline period (upper trace) and after the PAR-2-AP, SLIGRL (100μM) is applied (lower trace). Note the frequency of inter-ictal-like events is reduced upon SLIGRL application. B. Bar chart summarising quantitative analysis of instantaneous frequency before and during SLIGRL application (n=13, P=0.0006). C. Time plot of the same pooled experiments normalised to baseline frequency show the gradual but robust reduction of epileptiform burst frequency upon SLIGRL application (n=13, P=0.0004, measured at last minute of baseline and SLIGRL application).

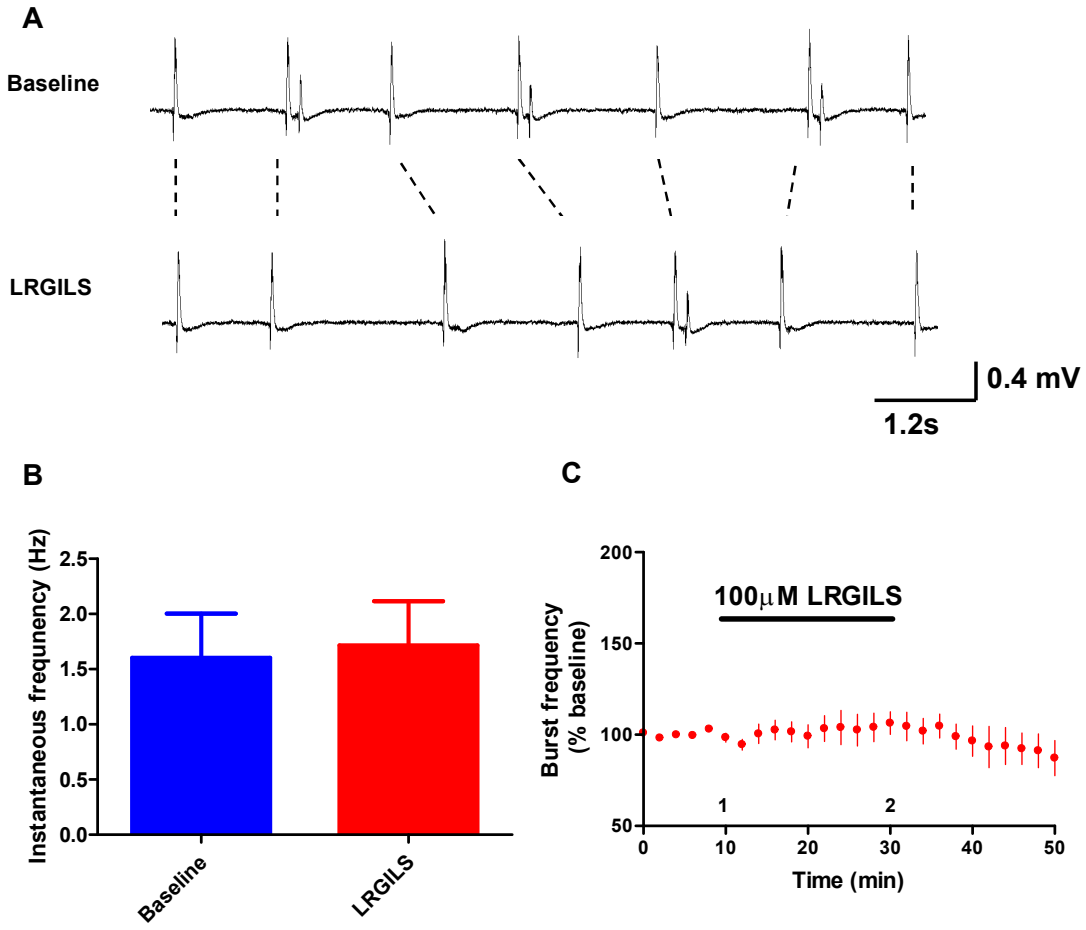


Figure 5-4 The PAR-2-IP, LRGILS has no effect on the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activity in hippocampal area CA3.

A. Representative traces (20080729/1) show the occurrence of inter-ictal-like activities during baseline period (upper trace) and after the PAR-2-IP, LRGILS (100μM) is applied (lower trace). Note the absence of any detectable change in burst frequency upon LRGILS application. B. Bar chart summarising quantitative analysis of instantaneous frequency before and after LRGILS application (n=6, P=0.1721) C. Time plot showing no significant change in mean burst frequency over time as the LRGILS is applied (n=6, P=0.2043).

5.4 PAR-1 activation does not affect the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activities in acute hippocampal slices

In order to assess whether PAR-1 activation may have a modulatory role on 4-AP/0 Mg^{2+}-induced epileptiform activities, extracellular recording was repeated in the CA3 *stratum pyramidale* in acute hippocampal slices. 10 minutes stable inter-ictal-like bursting activities was monitored before 100μM PAR-1-AP, TFLLR, was applied. As shown in figure 5.5B, no detectable change in bursting frequency was observed following TFLLR application (control: 1.10 ± 0.13Hz, drug: 1.14 ± 0.11Hz, n=13, P=0.3730). Time plot analysis confirmed the same observation (normalised burst frequency: control: 101.65 ± 1.19%, drug: 104.30 ± 3.52%, n=13, P=0.3781, figure 5.5C). Overall, this experiment using the selective activator of PAR-1 did not modulate the frequency of 4-AP/0 Mg^{2+}-induced epileptiform bursting.

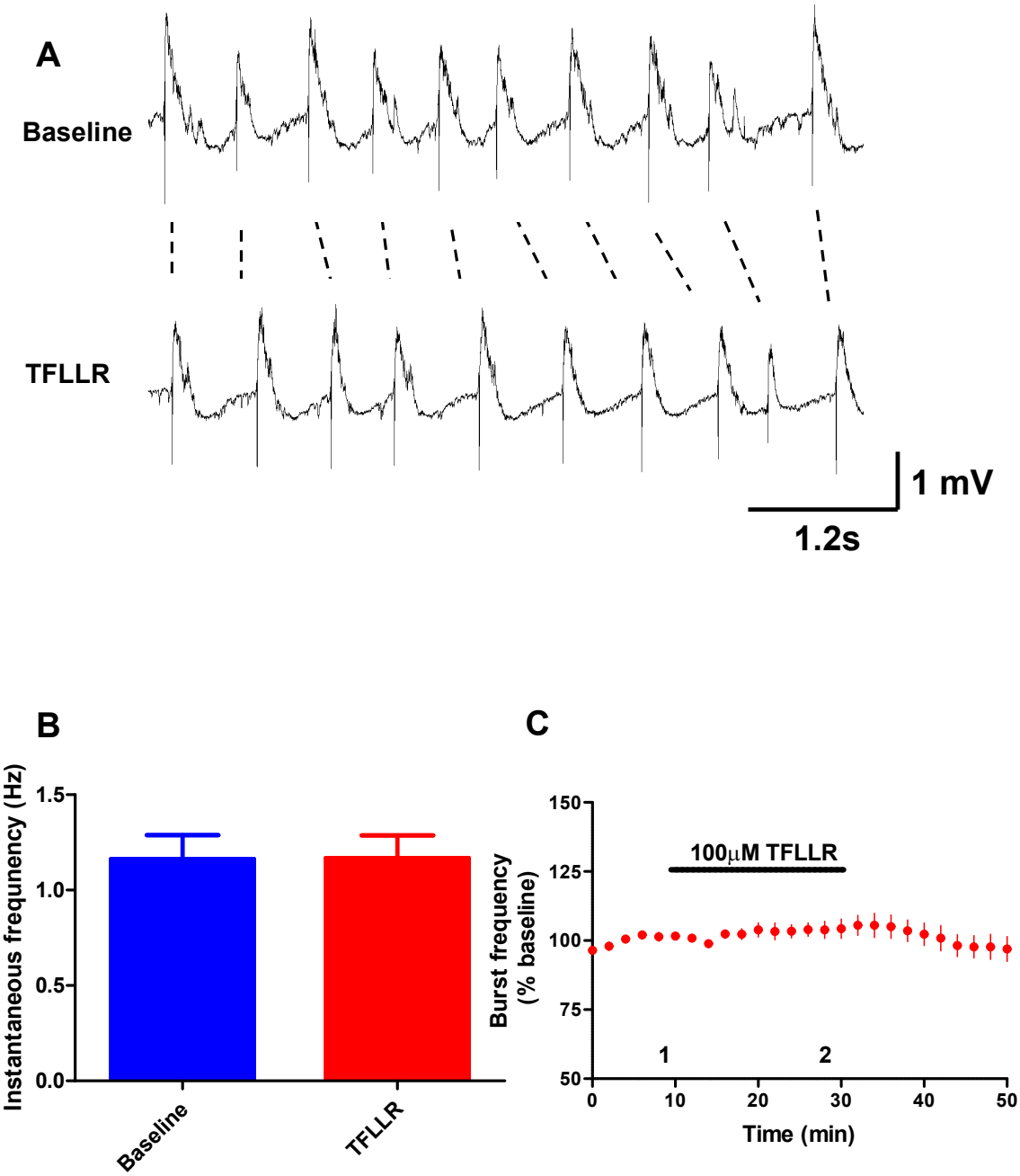


Figure 5-5 The PAR-1-IP, TFLLR, has no effect on the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activity in hippocampal area CA3.

A. Representative traces (20080819/1) show the occurrence of inter-ictal-like activities during baseline period (upper trace) and after the PAR-1-IP, TFLLR (100μM) is applied (lower trace). Note the absence of any detectable change in burst frequency upon TFLLR application. B. Bar chart summarising quantitative analysis of instantaneous frequency before and after TFLLR application (n=13, P=0.373) C. Time plot showing no significant change in mean burst frequency over time as TFLLR is applied (n=13, P=0.378).

5.5 Characterisation of epileptiform activity in bicuculline model

Extracellular recordings were obtained from the CA3 *stratum pyramidale* in hippocampal slices prepared from 16-24 days old Wistar rats. No detectable spontaneous field events were recorded during control period (drug free, normal ACSF) in all slices tested (n=10). Subsequent disinhibition by the bath application of $GABA_A$ receptor antagonist, bicuculline (20μM), resulted in a gradual appearance of intermittent rhythmical field events. These inter-ictal-like events occurred every 9.7±1.1s (n=10) and were 100 times less frequent compared to those observed in 4-AP/0 Mg^{2+} model (interevent interval: 0.10 ± 0.01s for 4-AP/0 Mg^{2+} model, n=33, 9.7 ± 1.1s for bicuculline model, n=10).

Bicuculline-induced inter-ictal-like events observed (shown in figure 5.6) were different in waveform from those seen in 4-AP/0 Mg^{2+} model. They were typically characterised by a primary large inter-ictal-like event with triphasic waveform: (1) a fast negative deflection followed by (2) a fast positive deflection which overshot baseline to reach peak amplitude (3) slow negative decay phase. This was followed by another burst or a train of repetitive smaller and shorter bursts. Bicuculline-induced epileptiform events occurred with a mean instantaneous frequency of 0.40 ± 0.27Hz (n=10) and the mean peak amplitude (measured from baseline to positive peak) of these events was 2.56 ± 0.43mV. (n=10).

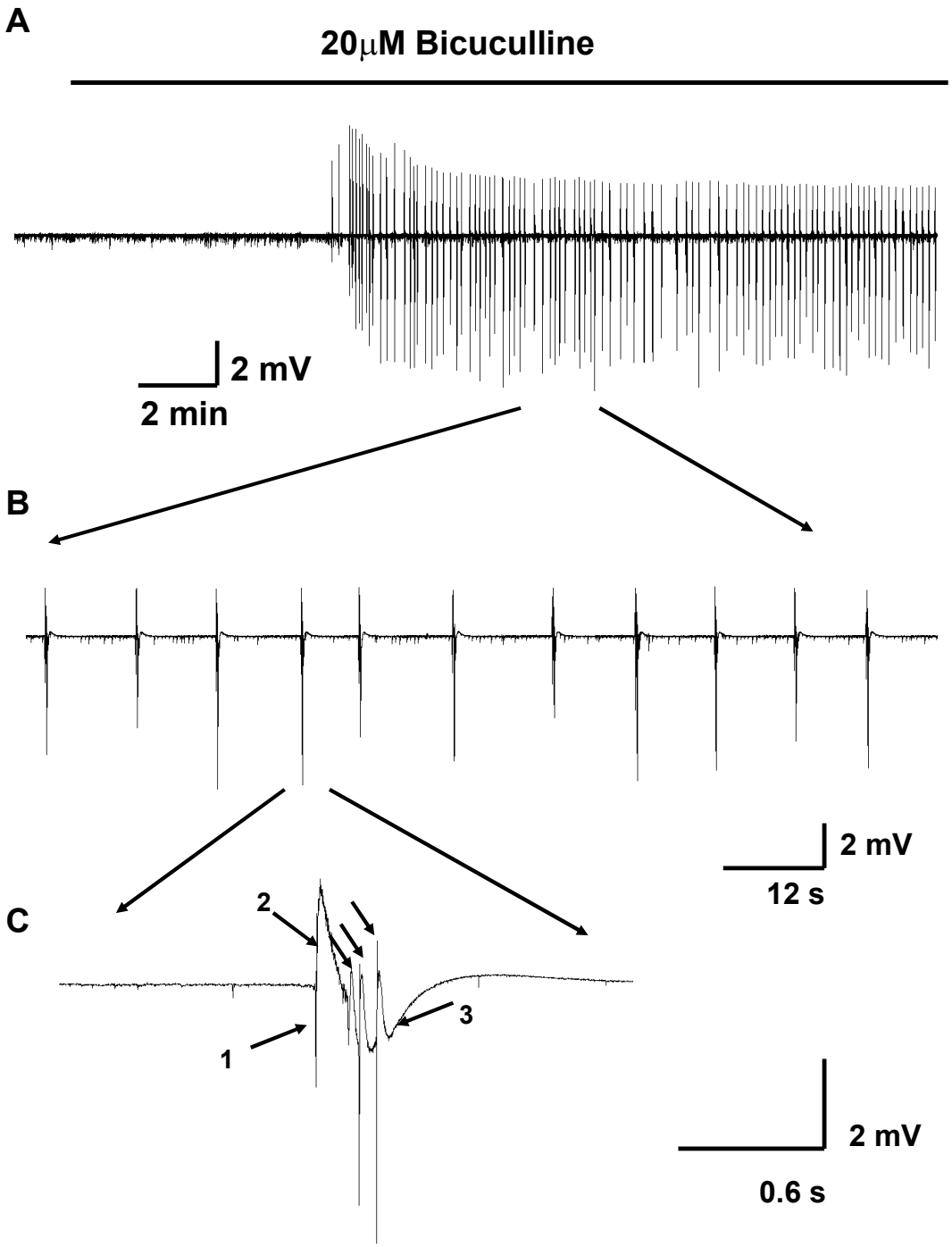


Figure 5-6 Bicuculline-induced epileptiform activity in the CA3 area of acute hippocampal slices.

A. Representative extracellular recording upon bath application of the $GABA_A$ receptor antagonist bicuculline (20μM) as indicated by the solid bar. Note the appearance of rhythmical field events within 10 minutes of bicuculline application. B. Expanded trace of the same recording shows intermittent and coherent inter-ictal-like events. C. Further expansion of trace to resolve the waveform of individual spontaneous field events show then to comprise of: (1) a fast negative potential followed by a (2) a series of fast positive potentials (extracellular action potential burst) on top on a (3)slow negative potential.

5.6 Both PAR-2 and PAR-1 activation have no effect on the frequency or occurrence of bicuculline-induced epileptiform activities in hippocampal slices

Once sustained and regular inter-ictal-like activities were recorded from the CA3 cell body layer following the application of 20μM bicuculline, 10 minutes baseline of inter-ictal-like activities with stable bursting frequency was recorded as control period before either the PAR-2-AP (SLIGRL, 100μM) or the PAR-1-AP (TFLLR, 100μM) was applied through the perfusate. Application of SLIGRL or TFLLR for 30 minutes induced no significant or detectable change in the frequency of inter-ictal-like activities (0.14 ± 0.03Hz to 0.13 ± 0.03Hz, n=6, P=0.5282, figure 5.7B for PAR2-AP) (0.13 ± 0.02Hz to 0.13 ± 0.02Hz, n=4, P=0.9893, figure 5.8B for PAR1-AP) respectively. Time plot summary confirmed the absence of any detectable drug effect over the period of drug application (normalised burst frequency, PAR-2-AP: from 98.01 ± 3.21% to 99.85 ± 4.60%, n=6, P=0.6043, figure 5.7C; PAR-1-AP: from 96.59 ± 0.35% to 89.28 ± 8.73 n=4, P=0.5385, figure 5.8C). These data suggest PAR-2 and PAR-1 activation had no modulatory influences on bicuculline-induced epileptiform activities, at lease in terms of overt changes in burst frequency.

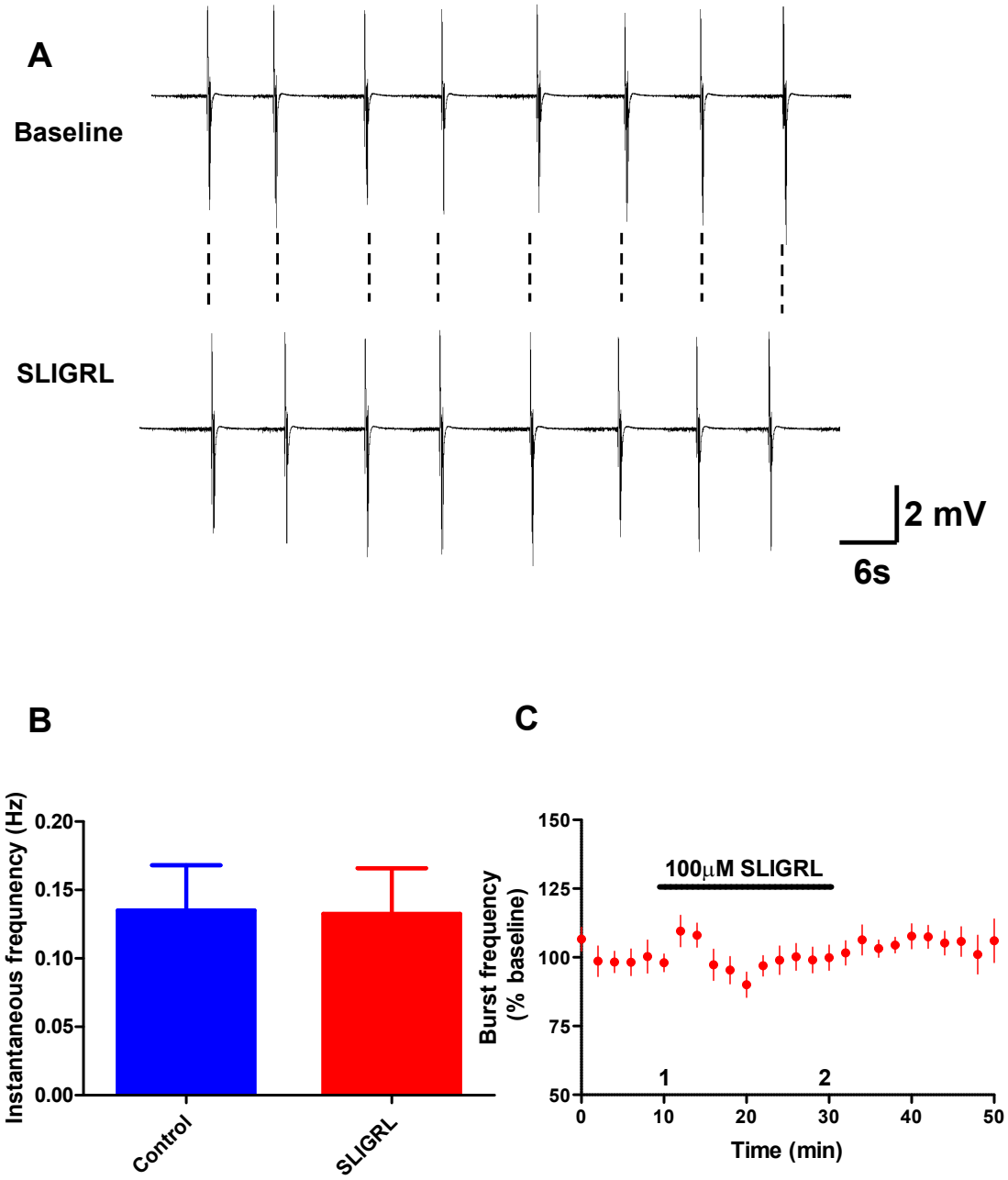


Figure 5-7 The PAR-2-AP, SLIGRL, has no effect on the frequency of bicuculline-induced epileptiform activity in hippocampal area CA3.

A. Representative traces (20080812/1) show the occurrence of inter-ictal-like activities during baseline period (upper trace) and after the PAR-2-AP, SLIGRL (100μM) is applied (lower trace). Note the absence of any detectable change in burst frequency upon SLIGRL application. Stippled indicated matching of traces indicative of no change in burst frequency. B. Bar chart summarising quantitative analysis of instantaneous frequency before and after SLIGRL application (n=6, P=0.5281) C. Time plot showing no significant change in mean burst frequency over time as the SLIGRL is applied (n=6, P=0.604).

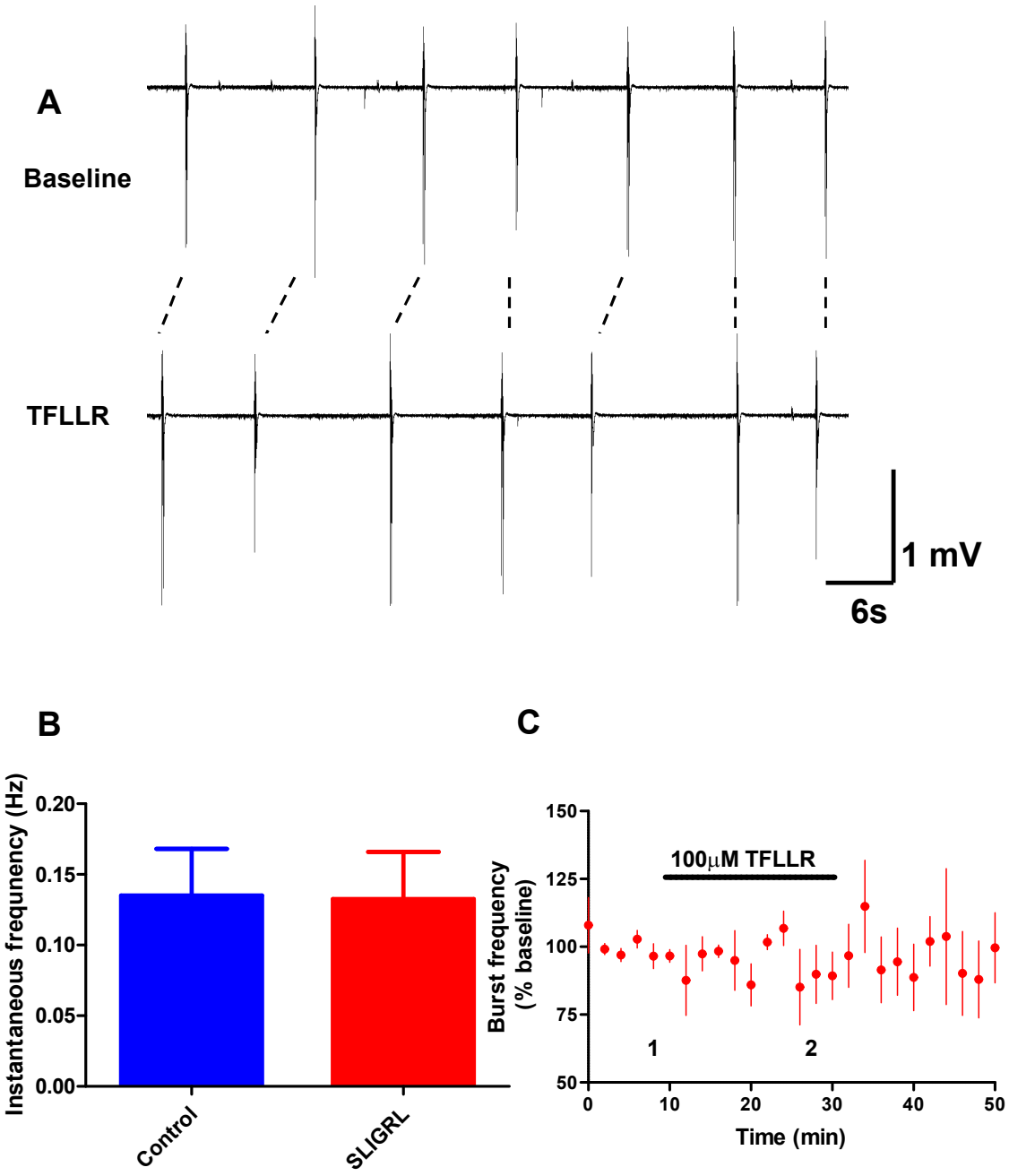


Figure 5-8 The PAR-1-AP, TFLLR has no effect on the frequency of bicuculline-induced epileptiform activity in hippocampal area CA3.

A. Representative traces (20081017/1) show the occurrence of inter-ictal-like activities during baseline period (upper trace) and after the PAR-1-AP, TFLLR (100μM) is applied (lower trace). Note the absence of any detectable change in burst frequency upon TFLLR application. Stippled indicated matching of traces indicative of no change in burst frequency. B. Bar chart summarising quantitative analysis of instantaneous frequency before and after TFLLR application (n=4, P=0.989) C. Time plot showing no significant change in mean burst frequency over time as the TFLLR is applied (n=4, P=0.539).

5.7 Discussion

In this chapter, I demonstrated, for the first time, that activation of PAR-2 significantly depresses the frequency of epileptiform activities induced by 4-AP/0 Mg^{2+} in acute hippocampal slices, suggesting that PAR-2 activation exerts an anti-epileptic effect in the 4-AP/0 Mg^{2+}-induced epilepsy model. In contrast, PAR-1 activation is ineffective. Furthermore, neither PAR-2 nor PAR-1 activation changes the frequency of epileptiform activities induced by bicuculline.

In this chapter, I first demonstrate that PAR-2 activation depresses the frequency of epileptiform activities in 4-AP/0 Mg^{2+} induced epilepsy (see figure 5.3). This result is in agreement with previous report that PAR-2 activation is protective against seizure in vivo (Lohman *et al.*, 2008). Considering the fact that enhanced excitatory input in the network is the main driving force to the epileptogenesis in 4-AP/0 Mg^{2+}-induced epileptiform activities in slices, the observed depression of epileptiform activities may reflect the nature that PAR-2 activation reduces excitatory synaptic transmission, which has been demonstrated in chapter 3, and 4 (see figure 3.17,3.19 and 4.1). Therefore, one rational interpretation based on this result is PAR-2 activation weakens excitatory synaptic transmission, which in turn reduces excitatory driving force, and thus depresses the frequency of epileptiform activity in 4-AP/0 Mg^{2+} model. In stark contrast, PAR-2 activation has no detectable effect on the frequency of bursting in bicuculline-induced epileptiform activities. This inconsistency may be explained by considering the mechanism underlying bicuculline-induced epileptiform activity. In this model, epileptiform activity is induced as a result of the collapsed balance of excitation and inhibition due to silenced inhibition by $GABA_A$ receptor blockade. Thus, as the disinhibition dominates the driving force for epileptogenesis in bicuculline model, a modest reduction of excitatory input as expected from PAR-2 activation (about 20%, see figure 5.3 and figure 4.1) may not be able to compensate the gross loss of inhibition. Therefore, no observable effect by PAR-2 activation could be rationalised.

It is surprising to notice in this chapter that PAR-1 activation has no detectable effect on the frequency of epileptiform activity in either 4-AP/Mg^{2+} or bicuculline model. Although this observation is consistent with the results reported in Chapter 4 (see figure 4.5) that PAR-1-AP, TFFLR (100μM) has no detectable effect on synaptic transmission at the Schaffer collateral-to-CA1 synapse, it is, at first glance, inconsistent with the previous report that PAR-1 activation facilitates epileptogenesis in hippocampal slices (Maggio *et al.*, 2008). This disagreement could be reconciled by the following consideration. Maggio and co-workers investigated the frequency of spontaneous spikes under normal saline solution and the threshold of seizures by elevating extracellular K^+ concentration (Maggio *et al.*, 2008). Therefore, the interpretation from their results is PAR-1 may facilitate the occurrence of epileptiform activity. However, in their report, it remains void that whether PAR-1 modulates the epileptiform activity once it is established. My result, on the other hand, suggests PAR-1 has no regulatory role after the establishment of epileptiform activity. To conclude, PAR-1 activation may facilitate epileptogenesis, but it may not have modulatory role in established epilepsy.

Taken together, in chapter 5, I demonstrate that PAR-2 may suppress epileptiform activities in the area of CA3 in acute hippocampal slices due to heightened excitation but does not regulate epileptiform activities as a result of disinhibition of hippocampal circuits.

6 General discussion

The main purpose of this thesis was to investigate whether activation of PAR-2 in the hippocampus under non-diseased conditions modulates neuronal intrinsic properties, synaptic transmission and plasticity as well as network activities. The central hypothesis of this investigation was that PAR-2, by its unique activation mechanism, may serve as 'molecular switch' in tuning neuronal signalling in the hippocampus, a brain structure important in aspects of learning and memory. It was hoped that by examining neuronal excitability and synaptic transmission in primary hippocampal culture and acute hippocampal slice preparations, such *in vitro* investigations would provide valuable insights about the action of PAR-2 activation in the cells of the CNS. It was hoped that these experiments might ultimately provide experimental evidence to whether PAR-2 is a plausible candidate for therapeutic interventions. Thus, in the final chapter, I will firstly summarise the major findings of this investigation with highlighted novel discoveries. Then, I will discuss the significance of this investigation, in particular in relation to established knowledge of PARs in the CNS as well as modulators of neural transmission and plasticity. Furthermore, I will reflect the limitations of this project in terms of methodology, data interpretation, and completeness. Finally, I will make suggestions to directions that future studies could take based on my current data.

6.1 Major findings

The major findings reported in the thesis are the following:

Firstly, from primary hippocampal culture experiments

1. Trypsin depolarises cultured hippocampal neurones and increases the occurrence of spontaneous APs.
2. Spontaneous APs are synaptically driven in nature.
3. The PAR-2-AP (SLIGRL) but not PAR-2-IP (LRGILS) depolarises cultured hippocampal neurones and depresses spontaneous occurring APs.
4. The PAR-1-AP (TFLLR) and PAR-4-AP (AYPGKF) depolarises cultured hippocampal neurones. However, neither PAR-1-AP nor PAR-4-AP

significantly changes the frequency of spontaneous AP firings.

5. SLIGRL-induced neuronal depolarisation is dependent on glutamate.
6. SLIGRL-induced neuronal depolarisation requires normal astrocytic function.
7. Astrocytic-neuronal signalling contributes to SLIGRL-induced depression of spontaneous AP firing.

Secondly, from acute hippocampal slice experiments

1. Two independent PAR-2-APs, SLIGRL and 2f-LIGRL, but not the PAR-2-IP (LRGILS) induce LTD of glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse.
2. mGluRs are not required in the induction of PAR-2 activation-induced LTD
3. PAR-2 activation-induced LTD is dependent on NR2B subunit-containing NMDA receptors
4. Glial-neuronal signalling contributes to PAR-2-mediated LTD.
5. The PAR-1-AP (TFLLR) has no effect on basal glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse.

Finally, from pilot study investigating PAR-1 and PAR-2 actions in *in vitro* models of epilepsy

1. The PAR-2-AP (SLIGRL) but not the PAR-2-IP (LRGILS) depresses the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activity in the CA3 of acute rat hippocampal slices.
2. The PAR-1-AP (TFLLR) has no effect on the frequency of 4-AP/0 Mg^{2+}-induced epileptiform activity in the CA3 of acute hippocampal slices.
3. Neither PAR-2-AP (SLIGRL) nor PAR-1-AP (TFLLR) has any effect on the frequency of bicuculline-induced epileptiform events in the area CA3 of acute rat hippocampal slices.

6.1.1 PAR-2-mediated neuronal depolarisation in hippocampal culture

In my thesis, firstly, I demonstrate that activation of PAR-2 leads to membrane depolarisation of cultured hippocampal neurones. To my knowledge, this is the first report that a member of PAR family modulates the excitability (membrane potential) of a central neurone. However, the data is nevertheless consistant with previous studies showing PAR-2 activation to depolarise membrane of submucosal neurones (Reed *et al.*, 2003), colonic DRG neurones (Kayssi *et al.*, 2007) in the digestive tract as well as nociceptive DRG neurones (Amadesi *et al.*, 2004) in the spinal cord, in response to 50 to 100μM PAR-2-AP, SLIGRL, application. In addition, the average magnitude of depolarisation reported in these studies is all in the range between 5 to 10mV (from normal resting conditions), which is comparable to that I observe in my preparation. Thus, my result reported here is in agreement with previous observations in other neuronal types. Furthermore, considering PAR-2-mediated neuronal depolarisation is independently verified in four neuronal types in three systems (submucosal neurones, colonic DRG neurones in the digesting tract, nociceptive DRG neurones in the spinal cord, and hippocampal neurones in the brain), it is suggesting that the modulation of neuronal excitability is perhaps a common regulatory role that PAR-2 plays in tuning neuronal function. However, it is also noticed that the proposed mechanism underlying neuronal depolarisation mediated by PAR-2 activation in all four types of neurones may not be the same. Reed and co-workers showed the depolarisation observed in submucosal neurones were associated with an increase of input resistant of the recorded neurone (Reed *et al.*, 2003), and this association was also apparent when colonic DRG neurones were tested (Kayssi *et al.*, 2007). In the later study, it was demonstrated that PAR-2-mediated depolarisation together with the increase of input resistance was due the blockade of delayed rectifier K^+ channels (Kayssi *et al.*, 2007), arguing a direct channel-modulating effect by the activation of neuronal PAR-2. In contrast, my results suggest PAR-2-mediated neuronal depolarisation in the cultured hippocampal neurones is dependent on glutamate release from nearby astrocytes, favouring an indirect effect from the activation of astrocytic PAR-2. Indeed the phenomena that gliotransmitters release, including glutamate, in response to GPCR activation on glial cells is very well

established (see Chapter 1.8). Notably, several recent publications reported that PAR-1, another member of PAR family, produced neuronal activity (enhancing neuronal NMDA receptor mediated component of mEPSCs (Lee *et al.*, 2007), eliciting NMDA receptor mediated SIC (Shigetomi *et al.*, 2008), and evoking Ca^{2+} signal in neurones from the nucleus of the solitary tract (Hermann *et al.*, 2009) respectively) through its activation on astrocytes in diverse experimental preparations, including hippocampal culture as well as hippocampal and medullary slice preparations. Thus, in terms of the mechanism underlying PAR-2-mediated neuronal depolarisation, my result is in agreement with the previously established astrocytic PAR-1-to neurone pathway. However, regarding neuroexcitability, it has to be acknowledged that the possibility that a direct influence on neuronal ion channels following neuronal or/and astrocytic PAR-2 activation, which is not examined in the current study, has to be considered. Ca^{2+} imaging experiments have demonstrated a small yet significant rise of intracellular Ca^{2+} upon SLIGRL application in the cultured hippocampal neurones (Dr. Sam Greenwood, unpublished data), suggesting a possible modulation of channels whose activation may need the Ca^{2+} signal, for instance the Ca^{2+}-activated K^+ channels.

Therefore, taking all these considerations into account, it is reasonable to make a general suggestion that neuronal depolarisation is maybe a common feature of PAR-2-mediated modulation of neuronal excitability. But at lease two independent or overlapping mechanisms could underlie this, either a direct effect of neuronal PAR-2 activation (via ion channels) or/and an indirect effect of astrocytic PAR-2 activation (via glutamate receptors).

6.1.2 PAR-2-mediated depression of the frequency of spontaneous firings

Another novel finding from my thesis is that in the cultured hippocampal neurones, a transient modest depolarisation is associated with paradoxically reduction of AP firing frequency upon PAR-2 activation (see figure 3.6). This observation is unusual because a membrane depolarisation normally results in increased excitability, therefore facilitating the occurrence of APs. Thus, in order to address the mechanism underlying this paradox. I first demonstrate that

the AP firing is largely dependent on glutamatergic synaptic transmission because a cocktail of glutamate receptor antagonists totally prevents the generation of APs (see figure 3.17). Secondly, I confirm the recorded neurone receives a constant barrage of EPSPs, many of which are suprathreshold for the generation of APs (see figure 3.3). Moreover, within closer inspection, I show the frequency of sub-threshold EPSPs are significantly reduced following PAR-2 activation (see figure 3.19). Thirdly, when somatic current injection is applied to depolarise neuronal membrane from -65mV to -60mV, the magnitude similar to that seen upon PAR-2 activation, a predictable and significant increase of APs is observed (see figure 3.18). Taken together, these data from my thesis, although indirect, suggest the occurrence of APs in the cultured hippocampal neurone is largely not intrinsic, but synaptically driven. Therefore, the reduction of spontaneous AP firings may be as a result of significant reduction of glutamatergic synaptic transmission onto the recorded cell. To combine the observed depolarisation data, this paradox that depolarisation associated with a reduction of AP firings could be rationalised by dissecting this simultaneously occurring event by separate mechanisms. That is PAR-2-mediated neuronal depolarisation is due to astrocytic release of glutamate, whereby, at the same time, PAR-2 activation depresses glutamatergic synaptic transmission in the network formed within the cultured hippocampal neurones.

The precise mechanisms underlying the PAR-2-mediated depression of glutamatergic synaptic transmission in the cultures was not established in unequivocal terms during the course of this thesis. Nevertheless, my data in Chapter 3 again favours an indirect action of PAR-2 activation whereby glutamate of astrocytic origin results in the suppression of fast glutamatergic synaptic transmission. Consistent with this, glutamate released from astrocytes have been demonstrated to depress excitatory synaptic transmission in hippocampal culture following activation by a number of other G-protein coupled receptors including presynaptic MAP-4 sensitive group III mGluRs (Araque *et al.*, 1998). Group I (Gereau & Conn, 1995) and group II mGluRs (Bushell *et al.*, 1996) can also be potential candidates as their involvement have been confirmed depending on types of synapses, animal age and species. Thus, it is of special interest to examine if presynaptic mGluRs are contributing to this PAR-2-mediated synaptic depression in culture using selective mGluR antagonists.

Moreover, as the rise of intracellular Ca^{2+} is confirmed in astrocytes in our primary hippocampal culture in response to PAR-2 activation (unpublished data by Dr. Sam Greenwood), it is reasonable to expect that gliotransmitters, including glutamate, could be released upon astrocytic PAR-2 activation. Therefore, the depression of synaptic transmission in the culture system could be as a result of other molecules. One potentially plausible candidate would be astrocytically released ATP. Recent studies demonstrated that ATP released from astrocytes induces synaptic depression in cultured hippocampal neurones via presynaptic purinergic receptors (Koizumi *et al.*, 2003; Zhang *et al.*, 2003). Thus, it would be interesting to employ purinergic antagonists to examine the consequence of the frequency of spontaneous APs and EPSPs in the culture system upon application of PAR-2-AP.

Although all my results support the explanation that a reduction of glutamatergic synaptic transmission leads to the depression of the frequency of synaptcally driven APs, alternative mechanisms for the reduction of spontaneous AP firings upon PAR-2 activation may still exist in parallel because the proposed mechanism with reduced glutamatergic synaptic transmission may not be the sole contributing factor. One possibility is perhaps the direct modulation of ion channel conductances, most likely enhanced potassium conductances via activation of Ca^{2+}-activated potassium channels, and perhaps decreased Ca^{2+} conductances. It has been demonstrated that PAR-2 activation could modulate membrane channels (Kayssi *et al.*, 2007). In order to test this hypothesis, one may have to first inspect the input resistance of the recorded neurone before and after the application of PAR-2-AP, SLIGRL.

6.1.3 PAR-2-mediated long term depression

In the acute slice preparation, I demonstrate for the first time that the application of PAR-2-AP induces a long lasting depression of glutamatergic synaptic transmission at the Schaffer collateral-to-CA1 synapse. This PAR-2-mediated depression of glutamatergic synaptic transmission seen in acute slices is consistent with my interpretation in chaper 3 that PAR-2 activation depresses glutamatergic synaptic transmission in the cultured hippocampal neurones as

demonstrated by the reduction of synaptically-driven APs upon PAR-2-AP application (see figure 3.6,17,18 and 19.). However, it is also noticed that the timecourse of PAR-2-induced synaptic depression is different under these two experimental conditions. In the cultured system, the PAR-2-mediated depression of synaptic transmission is rather transient as the frequency of synaptically-driven APs return to control level after 5 minutes PAR-2-AP perfusion (see figure 3.6), whereas, in acute slices, PAP-2-AP produced a relatively long term depression (up to 30mins) and this depression is not reversible upon washout PAR-2-AP. This timecourse difference may be explained by the fact that the cultured neurones have much less exposure time to PAR-2-AP (5mins) compared to neurones in slices (30mins). If this was the case, this might suggest that a short time exposure to PAR-2 activation leads to transient synaptic depression, whereas long term exposure gives rise to long lasting effect. However, this timecourse difference in two systems may also suggest there could be two independent mechanisms underlying short term modulation and long term modulation of basal synaptic transmission by PAR-2 activation, which would require perhaps differential signalling pathways and molecules. But nevertheless, a depression of glutamatergic synaptic transmission is observed in both culture and slice preparations. A recent publication demonstrated that certain serine proteinases are capable of inducing LTD at the Schafer collateral-to-CA1 synapse (MacGregor *et al.*, 2007). Considering PAR-2 is very likely endogenously activated by proteinases, it is of interest to speculate that PAR-2 might be activated tonically by the endogenous ligand and that this enduring action might indeed be able to modulate basal excitatory synaptic transmission *in vivo*, which would provide a novel target for pharmacological and therapeutic intervention. In addition, it is also acknowledged that in this study, only the Shaffer collateral-to-CA1 synapse is investigated. However, whether this PAR-2-induced long lasting synaptic depression is universal to all excitatory synapses in the hippocampus is unkonwn. Furthermore, it would be also interesting to know whether PAR-2 activation modulate inhibitory synapses.

It is noticed in the slice preparation that the PAR-2-mediated long lasting synaptic depression is irreversible. After ruling out that the irreversibility is due to the deterioration of slice quality in the process of these experiments (see figure 4.1, and figure 4.2), this feature may indicate that PAR-2-mediated LTD

could persist even without the ligand binding. In terms of mechanisms underlying this phenomenon, it might be plausible to firstly consider possible postsynaptic modifications associated with this form of LTD once established via PAR-2 activation, because the magnitude of paired-pulse ratio alteration upon PAR-2-AP application, though statistically significant, is relatively very modest (see figure 4.6). Thus, immediate speculations would include, for instance, a reduced number of functioning postsynaptic iontropic glutamate receptors. Because AMPA receptor internalisation has been widely proposed as the cellular response upon the expression of different forms of LTD (Malinow & Malenka, 2002; Kessels & Malinow, 2009), it may be of interest to compare the membrane expression level of AMPA receptors during normal synaptic transmission and the PAR-2-mediated LTD. Another aspect consider would be a possible modulation of shapes and numbers of postsynaptic spines following PAR-2 activation because structural plasticity, which associates with different forms of synaptic plasticity including LTD, has been manifested by the dynamics of spines (Bourne & Harris, 2008; De Roo *et al.*, 2008; Bhatt *et al.*, 2009; Yang & Zhou, 2009) and this has not yet been investigated in the PAR-2 knockout mouse.

Finally, a question emerged from the PAR-2-mediated LTD is whether it is functionally physiological or pathophysiological. It has been recently demonstrated in our group that PAR-2 activation is neuroprotective in slice culture preparations (Greenwood & Bushell, 2010). My data in Chapter 4 may support this hypothesis from a synaptic aspect that a reduced excitatory synaptic transmission upon PAR-2 activation might be beneficial in nature for certain pathological conditions, for instance, in ischaemia and epilepsy. It has been widely documented that the penumbra surrounding stroke regions show aberrant plasticity (Calabresi *et al.*, 2003; Di Filippo *et al.*, 2008) and it is possible that this is accounted for by region experiencing PAR-2 activation as an upregulation of PAR-2 expression in neurones has been recorded in a mouse model of ischaemia (Jin *et al.*, 2005). Furthermore, during these insults, the gross excitability in the neural network increases due to a pathologically increased glutamate level, and it has been confirmed excessive excitability is harmful to neurones and synapses (Arundine & Tymianski, 2004; Greenwood & Connolly, 2007). Therefore, it seems logical that PAR-2 activation reduces global excitability by depressing excitatory synaptic transmission in the synapses as

well as network (see Chapter 5), which in turn protects the functionality of the synaptic signalling within the network. Furthermore, as PAR-2 might be activated endogenously, this mechanism could be utilised to provide tonic inhibiting force for the synapses against trauma-induced hyperexcitability insults.

6.1.4 Glial involvement in the PAR-2-mediated modulation of neuronal signalling

In my thesis, the involvement of glial function has been strongly indicated by experimental data from both primary culture and acute slice preparations. This consistency may suggest glial cells are indeed underlying the PAR-2-mediated modulation of neuroexcitability and synaptic transmission. In the culture experiments, my data strongly suggest that astrocytically released glutamate is causing membrane depolarisation and at least is contributing to the depression of spontaneous APs in neurones (see Chapter 3). In the acute slice experiments, normal glial function is at least partially contributing to the PAR-2-mediated LTD (see Chapter 4). However, it has to be acknowledged that this hypothesis suffers significantly due to the lack of direct evidence of astrocytically released gliotransmitters, most notably, glutamate. Without a direct measurement of glutamate, or perhaps other gliotransmitters, released from astrocytes, the basic conclusion that astrocytically released glutamate is contributing to the PAR-2-mediated modulation is only suggestive. Initial screen of astrocytic release of glutamate has been conducted in primary culture but the sensitivity of this assay was not optimal (unpublished observations, Dr. Sam Greenwood). However, several approaches are available to address this question, for instance the high-pressure liquid chromatography (HPLC) analysis, radioactive labelled glutamate assay, and perhaps 'sniffer-patch' technique (Allen, 1997). Nevertheless, several indirect but independent evidences from both culture and acute slice preparations (see figure 3.14, 3.15, 3.20, 4.13) point to a role of glial cells actively contributing to the PAR-2-mediated modulation.

6.2 Significance of this study

The importance of this investigation is outlined as following key points.

1. My thesis provides first evidence to address what is the physiological function of PAR-2 in the CNS at single neurone and synaptic levels, which is urgently needed as biochemical studies of PAR-2 in the CNS are already abundant.
2. My thesis reveals a novel form of chemically-induced LTD which is NR2B subunit-containing NMDA receptor dependent.
3. My thesis provides evidence for a potentially unique new form of synaptic modulation which might be endogenously activated upon enzymatic activation of PAR-2 to act as tonic suppressor for excitatory synaptic transmission.
4. By studying the mechanisms underlying the modulatory role of PAR-2 in neuronal signalling, my thesis strongly suggests the functional importance of glial cells which provide key molecules to regulate neuronal excitability and synaptic transmission.

6.3 Technical considerations

From the aspect of methodology, the major disadvantage of this study is the concern over the selectivity of pharmacological manipulations. As discussed in the introduction, the selectivity of the PAR-2-AP, SLIGRL, when applied at a relatively high concentration at 100μM, is the first consideration. Because at this concentration (100μM), SLIGRL has been demonstrated to elicit the maximal responses in different cell types and tissue preparations (al-Ani *et al.*, 1995; Saifeddine *et al.*, 1996; Kawabata *et al.*, 2004) as its EC_{50} is approximately 5μM in rat aortic ring preparation and in rat gastric longitudinal muscle preparations respectively (al-Ani *et al.*, 1995; Saifeddine *et al.*, 1996). In order to minimise the potential 'off-target' effect at this high concentration, I carefully employed the 'control peptide', PAR-2-IP, LRGILS at same concentration of 100μM, as vehicle control. Furthermore, I utilised PAR-2-AP from different sources to avoid potential 'false positive' responses due to purity or different solvents employed in the production process. Finally, in the slice preparation, I utilised a new PAR-2-AP, 2f-LIGRL, whose potency relative to SLIGRL is approximately 10,20 and 100 as determined by the Ca^{2+} singnalling assay in cell line, the vasorelaxation assay in rat superior mesenteric artery and the *in vivo* salivation assay in mice (Kawabata *et al.*, 2004; Kawabata *et al.*, 2005), to independently confirm the

observed phenomenon. However, it is acknowledged that all these considerations are in the aspect of pharmacological manipulation. In order to make firm conclusions, one might need to repeat the whole experiments employing the PAR-2 knockout mouse (Ferrell *et al.*, 2003) (discussed further in 6.4).

Additional consideration is the employment of the glial cell metabolism inhibitor, FAc. Although it has been confirmed to selectively block the Krebs circle enzyme , aconitase (Fonnum *et al.*, 1997), and it has been extensively used to depress glial functions in both culture and acute slice preparations in a variety of studies (Swanson & Graham, 1994; Zhang *et al.*, 2003; Gordon *et al.*, 2005; Shigetomi *et al.*, 2008; Henneberger *et al.*, 2010), the selectivity towards glial cells are still debated (unpublished communications during peer-review). In order to overcome this disadvantage, one may have to repeat culture experiments using a pure neuronal culture preparation (Bushell *et al.*, 1999; Zhang *et al.*, 2003). However, preliminary experiments suggest that this approach may be problematic as the procedure still results in a residual glial component in the culture. In the acute slice preparations, one may have to perform experiments using transgenic mouse whose glial cells are genetically silenced (Fiacco *et al.*, 2007; Petravicz *et al.*, 2008; Agulhon *et al.*, 2010). Alternatively, one might be able to locally inhibit astrocytes by loading Ca^{2+} chelator BAPTA into visually identified astrocytes (Shigetomi *et al.*, 2008).

6.4 Future studies

The studies detailed in this thesis has opened the window for a full investigation of PARs involvement in modulating neuronal as well as synaptic properties with a detailed characterisation of underlying mechanisms. These pioneering experiments have laid the groundwork for future more detailed studies. Several further experiments needed urgently emerged from this thesis have been indicated in previous discussion. Here are a few unresolved questions may be worth further efforts:

1. Is it possible to provide unequivocal evidence for the modulation of neurones/synaptic transmission by PAR-2 activation? Strategies to address

this would be the development of new more selective peptide and non-peptide ligands. Especially, the development of potent PAR-2 antagonist is urgently required, which is currently lacking. Additionally, several experiments could be repeated in the PAR-2 knockout mouse preparations. These would require repeating whole cell recordings from hippocampal culture prepared from PAR-2 knockout mouse and its wildtype littermate. It is expected to observe similar membrane depolarisation/reduction of spontaneous APs in the cultured neurones upon SLIGRL application from the wildtype littermate but not from the PAR-2 knockout mouse. Subsequently, it would be a strong evidence to demonstrate that there is comparable LTD at the Schafer collateral-to-CA1 synapses upon SLIGRL application in the acute slices prepared from wildtype littermate but not from PAR-2 knockout mouse.

2. Does PAR-2 receptor modulate all excitatory synapses in the hippocampus? Is there synapse-specific differences? Further field recording experiments will perhaps look at other major synapses such as mossy fibre-to-CA3 synapses and perforant path-to-granule cell synapses.

3. Does PAR-2 activation modulate inhibitory synapses? Whole cell recording could be used to examine evoked pharmacologically isolated IPSCs at CA1 pyramidal neuones to start with.

4. In order to have single cell resolution of PAR-2-mediated modulation of neuronal intrinsic properties and synaptic transmission *in situ*, the following experiments would be interesting to consider: Whole-cell patch clamp recordings using intracellular solution supplemented with biocytin in acute slices prepared from PAR-2 knockout mouse and its littermate control to characterise:

    a. The cell type of recorded neurones following morphological reconstruction.
    b. Basic intrinsic properties of each neurone including current-voltage relationship, input resistance, maximal firing frequency, ‘Sag’, etc., before and following the application of PAR-2-AP.
    c. Evoked EPSCs upon long term application of PAR-2-AP to have better resolution of the PAR-2-mediated LTD with possible pre and post synaptic

manipulation to distinguish pre or post synaptic mechanism.

In summary, it is clear that the current investigation has opened up a new area of research that PAR-2 has an overt action on central neurones and synapses. Clearly, this research has laid a strong foundation for a number of other important questions which will need to be resolved in the coming years if we are to understand fully the role and significance of PAR-2 actions in the physiology and diseases in the brain and indeed where there are potential therapeutic applications.

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