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The role of p21-activated kinase in hippocampal synaptic function

Miriam Durazo Barba

A dissertation submitted to the University of Bristol in accordance with the requirements for award of a degree of Doctor of Philosophy in the Faculty of Health Sciences.

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Abstract

Alzheimer's disease (AD) is the most common form of dementia with the characteristic symptom of progressive memory impairment. This is thought to be underpinned by the primary pathological features of neuronal and synaptic loss. The Group I p21-activated kinases (PAKs) are involved in regulating neuronal structure, which is crucial for the generation and maintenance of neuronal connections and functional signal transmission. Interestingly, evidence suggests that PAK levels are reduced in the brains of AD patients. The potential consequences of this for neuronal function will largely depend upon the roles PAKs play in neuronal physiology, the understanding of which is still being developed.

Synaptic plasticity is widely considered to underlie the cellular mechanisms of memory, and so has been the focus of extensive study in trying to understand the causes of cognitive impairment in AD. We studied the role of PAKs in the long-term potentiation (LTP) form of synaptic plasticity in acute hippocampal rat slices. We found that treatment of slices with IPA-3, a pharmacological PAK inhibitor, blocks LTP. However, another PAK inhibitor - FRAX486 - does not. As PAKs regulate cellular morphology, we stimulated synapse growth in cultured hippocampal neurons and examined the size of dendritic spines in the presence and absence of the PAK inhibitors. Consistent with our electrophysiology data, we found that IPA-3-treated neurons showed marked reductions in size, and FRAX486-treated neurons and controls did not. These observations suggest that IPA-3 and FRAX486 act differently on PAK function and that PAKs may have an influence on steps in the signalling cascade that triggers LTP. Further characterisation of the physiological roles PAK proteins play in neuronal function will be important to understand the possible consequence of their decline in AD.

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Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .... DATE: ....

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Abbreviations

Aβ	Amyloid-β
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPA	α-amino-3hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	α-amino-3hydroxy-5-methyl-4-isoxazole-propionic acid receptor
α-syn	α-synuclein
CA1-3	*Cornu Ammonis* 1-3
Ca^{2+}	Calcium
CaMK	Calcium calmodulin kinase
chemLTP	Chemical LTP
fEPSP	Field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
LTD	Long-term depression
LTP	Long-term potentiation
mGlu	Metabotropic glutamate
NMDA	N-methyl-D-aspartate

NMDAR	N-methyl-D-aspartate receptor
ANOVA	Analysis pf variance
PPR	Paired pulse ratio
GSK-3β	Glycogen synthase kinase 3β
HFS	High-frequency stimulation
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
K^+	Potassium
KAR	Kainate receptors
Mg^{2+}	Magnesium
MCI	Mild cognitive impairment
mAChR	Muscarinic acetylcholine receptor
PFA	Paraformaldehyde

CHAPTER 1

General Introduction

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Chapter 1 – Introduction

1.1 Dementia and aberrant protein aggregates

1.1.1 The epidemiology of dementia

Dementia is a syndrome characterised by a progressive deterioration in memory and higher cognitive functions such as reasoning, planning, language, and is usually accompanied by alterations in mood and behaviour (Johns & Johns, 2014). According to the World Alzheimer Report 2015, there were 46.8 million people living with dementia worldwide, but this number is expected to reach approximately 132 million in 2050 (Ali et al., 2015). As dementia is a condition which leads to a gradual loss of the patients' ability to execute everyday activities on their own, the worldwide cost of dementia is considerable and it is expected to increase from $818 billion US dollars in 2015 to $2 trillion US dollars in 2030 (Ali et al., 2015).

In addition to the economic cost, the main risk factor associated with the disease is age; the prevalence of this disease increases from approximately 6% in 65 to 69-year-old cohort, to 20% in 85 to 89-year-olds, and 40% in 90 to 94-year-olds (Corrada et al., 2010; Ebly et al., 1994; Fiest et al., 2016). It has been reported that the growing number of people aged 65 and older is a major cause of the predicted prevalence of dementia (Prince et al., 2016). As old age is a risk factor for dementia, and with an 'ageing population', it is clear that dementia is a growing public health concern globally. Therefore, developing a better understanding of neuropathology in the search of better therapeutics is of critical importance.

1.1.2 Unifying dementia: protein aggregates

There are various types of dementia and they are classified according to clinical and neuropathological features. The most common type of dementia is Alzheimer's disease, accounting for approximately two-thirds of all cases, followed by vascular dementia, dementia with Lewy bodies, and frontotemporal dementia.

The different aetiologies of dementia have been characterised by their neuropathology as well as their clinical features. Interestingly, the majority of these disorders share a central pathological phenotype which is defined by aberrant aggregation of different misfolded proteins. It is thought that the presentation of the different types of dementia depends on the amount and localisation of protein aggregates in the brain. However, the mechanisms underlying the pathological effects of protein aggregates – and whether they share similar molecular mechanisms – is currently unknown. Therefore, protein aggregation *per se* might be a causal factor that leads to degeneration.

1.2 Dementia with Lewy bodies and Alzheimer's disease – distinct diseases with shared aberrant protein aggregates

1.2.1 Pathophysiology of Dementia with Lewy bodies

Dementia with Lewy bodies (DLB) is one of the various types of dementia. This type of dementia accounts for approximately 4% of the dementia cases diagnosed in primary care, but its prevalence increases to 7% when diagnosed by specialists in secondary care (Vann Jones & O'Brien, 2014). The core clinical features of DLB include alterations in cognition and attention, recurrent visual hallucinations, motor symptoms which occur in later stages of the disease, and rapid eye movement sleep disorder, in addition to progressive memory loss (McKeith et al., 2017).

The hallmark pathological feature of this neurodegenerative disorder is the presence of Lewy bodies and Lewy neurites, which are intracellular inclusions composed of misfolded proteins (Spillantini et al., 1997). The key protein found in Lewy bodies is α-synuclein, a presynaptic 140-amino-acid protein abundantly expressed in the brain and encoded by the SNCA gene. The aggregated α-synuclein forms oligomers and insoluble unbranched filaments which have a cross-β pattern characteristic of amyloid proteins (Serpell et al., 2000); whereas under physiological conditions α-synuclein has been found to be a "natively unfolded" monomeric protein (Weinreb et al., 1996). This protein has a critical role in the neurodegenerative process as several studies found that mutations in the

SNCA gene or increases in SNCA gene dosage lead to Parkinson's disease (PD) and DLB (Goedert et al., 2013). Furthermore, even though PD is predominantly associated with motor symptoms, development of dementia is common in advanced cases of this disease due to the progressive deposition of abnormal protein aggregates reaching the neocortex (H. Braak et al., 2003). Therefore, cognitive problems observed in DLB and in advanced cases of PD are probably caused by neurodegeneration produced by α-synuclein aggregation in the neocortex.

1.2.2 Pathophysiology of Alzheimer's disease

In the case of Alzheimer's disease (AD), patients usually present with a history of memory loss, inability to generate or understand written and spoken language, decreased ability to perform daily tasks, and neuropsychiatric symptoms such as mood disorders. The majority of AD cases have a late onset form of the disease referred to as sporadic AD, whereas a small proportion of cases (~1%) develop an inherited form of the disease with early onset referred to as familial AD. Although most of the cases are sporadic, the pathological and clinical findings are similar in both types of AD. The defining pathological signs of AD are senile plaques, neurofibrillary tangles, neuronal death and synaptic loss (Serrano-Pozo et al., 2011). Senile plaques consist mainly of extracellular amyloid β (Aβ) deposits, while neurofibrillary tangles (NFTs) consist of intracellular deposits of hyperphosphorylated tau.

The Aβ peptide is produced from the cleavage of amyloid precursor protein (APP). The function of APP has not been elucidated, but it is thought to be involved in cell proliferation, differentiation, neurite outgrowth, synaptogenesis and synaptic plasticity (U. C. Müller et al., 2017). As shown in **Figure 1.1**, APP processing is initiated by various enzymes resulting in pathways yielding different fragments. In the amyloidogenic pathway, APP is cleaved by β-secretase into an intracellular C99 fragment and the extracellular soluble APPβ fragment (sAPPβ). Next, γ-secretase cleaves C99 into Aβ and the APP intracellular domain (AICD) fragment

(D. J. Selkoe et al., 1996). In the physiological non-amyloidogenic pathway, APP is cleaved into an intracellular fragment C83 and an extracellular soluble APPα (sAPPα) by α-secretase. Afterwards, C83 is cleaved into other fragments, P3 and AICD by γ-secretase (Dennis J. Selkoe, 1991).

The enzyme γ-secretase is part of both the amyloidogenic and non-amyloidogenic pathways, and it is an enzymatic complex composed of presenilin1 (PS1) or PS2, nicastrin, presenilin enhancer 2 (PEN-2) and anterior pharynx-defective 1 (Aph-1). The cleavage of APP is crucial for the pathological mechanisms of the disease as mutations in APP, PS1 and PS2 genes lead to early onset familial AD (Dennis J. Selkoe, 1991). These mutations alter APP processing resulting in increased production of different forms of Aβ that range from 39 - 42 amino acids and tend to aggregate to form dimers, oligomers and fibrils, each with different levels of toxicity (Borchelt et al., 1996).

In the case of neurofibrillary tangles, their main component tau is an axonal microtubule-associated protein which binds and stabilises microtubules thereby supporting axonal transport. Under physiologically normal conditions tau is natively unfolded, however, increased phosphorylation of tau diminishes its affinity to microtubules. It has been suggested that tau hyperphosphorylation promotes aggregation, as it precedes the formation of NFTs, and these pathological accumulations spread through the brain as the disease progresses (F. Braak et al., 1994); however, the mechanism by which tau mediates toxicity remains unclear. Tau deposits are not exclusive to AD, as they are also characteristic of neurodegenerative diseases called tauopathies, such as frontotemporal dementia with parkinsonism-17, progressive supranuclear palsy, sporadic corticobasal degeneration, agyrophilic grain disease, and Pick disease (V. M.-Y. Lee et al., 2001). Some of these diseases are caused by mutations in the tau encoding *MAPT* gene, and mutations within the microtubule-binding domain have been shown to enhance aggregation (Hong et al., 1998; S. Barghorn et al., 2000). Taken together, these findings support the hypothesis that protein aggregation is

central not only to AD pathogenesis but in other neurodegenerative disorders as well.

Aβ come in Aβ40 and Aβ42 isoforms. Aβ42 is more hydrophobic than Aβ40 and therefore more likely to bind to the cell membrane and form aggregates (Iljina et al., 2016). The ratio of Aβ40 to Aβ42 is normally around 9:1 but the ratio of Aβ42 is increased in some early onset forms of AD (Scheuner et al., 1996). An increased ratio of Aβ42 has been shown to correlate with toxicity in vitro and in vivo (Kuperstein et al., 2010; Pauwels et al., 2012).

The Aβ42 toxicity is linked with the disrution of cell membrane and bilayer disruption which are generated by secondary nucleations (aggregations of the primary oligomers) (Michaels et al., 2020). Flagmier *et al*. show that Aβ42 bilayer disrution correlates linearly with the amount of oligomers generated through secondary nucleations (Flagmeier et al., 2020).

De *et al*. found that small Aβ42 aggregates are more inhibited by antibodies at the C-terminal region but larger aggregates are more effective at causing an inflammatory response (De et al., 2019). The larger aggregates are inhibited by antibodies targetting the N-terminal region.

Aβ and α-syn have been found in vitro to form hetero-oligomers that promote the aggregation of each other (Chia et al., 2017). Bassil *et al*. found that mice injected with α-syn preformed fibrils accelerated Aβ deposits (Bassil et al., 2020). They also found that Aβ deposits promoted α-syn seeding in mice injected with α-syn preformed fibrils.

There is evidence that α-syn propagates from cell to cell through a process similar to prions (Jucker & Walker, 2018). Prions are misfolded proteins that induce other proteins to misfold in the same way causing the misfolded protein to further replicate. Lau *et al.* found that α-syn propagates similarly to prions by conformational templating (Lau et al., 2020). They demonstrated this through the innoculation of transgentic mice with brain derived and recombinant α-syn and

showed that the induced aggregates maintained their respective distinct biochemical and conformational properties. The preservation of these properties is a key feature of prion-like replication.

α-syn forms both nontoxic and toxic forms (type A, type B) oligomers (Cremades et al., 2012) which have similar sizes and morphologies. However the toxic form includes lipophilic elements that encourage a strong membrane interactions which disrupt cellular function and lipid bilayers (Fusco et al., 2017).

In summary, multiple lines of evidence suggest that different protein aggregates lead to loss of memory function in various diseases. One potential mechanism underlying the shared toxicity of different protein aggregates may be the similarities between their oligomeric species (Kayed et al., 2003). Indeed, mounting evidence suggests that small aggregates known as oligomers mediate neuronal dysfunction (Bucciantini et al., 2002; Hartley et al., 1999; Lambert et al., 1998; Lashuel et al., 2002). However, it is necessary to explain how memory functions under normal physiological circumstances before attempting to characterise how aggregated proteins might lead to memory impairment.

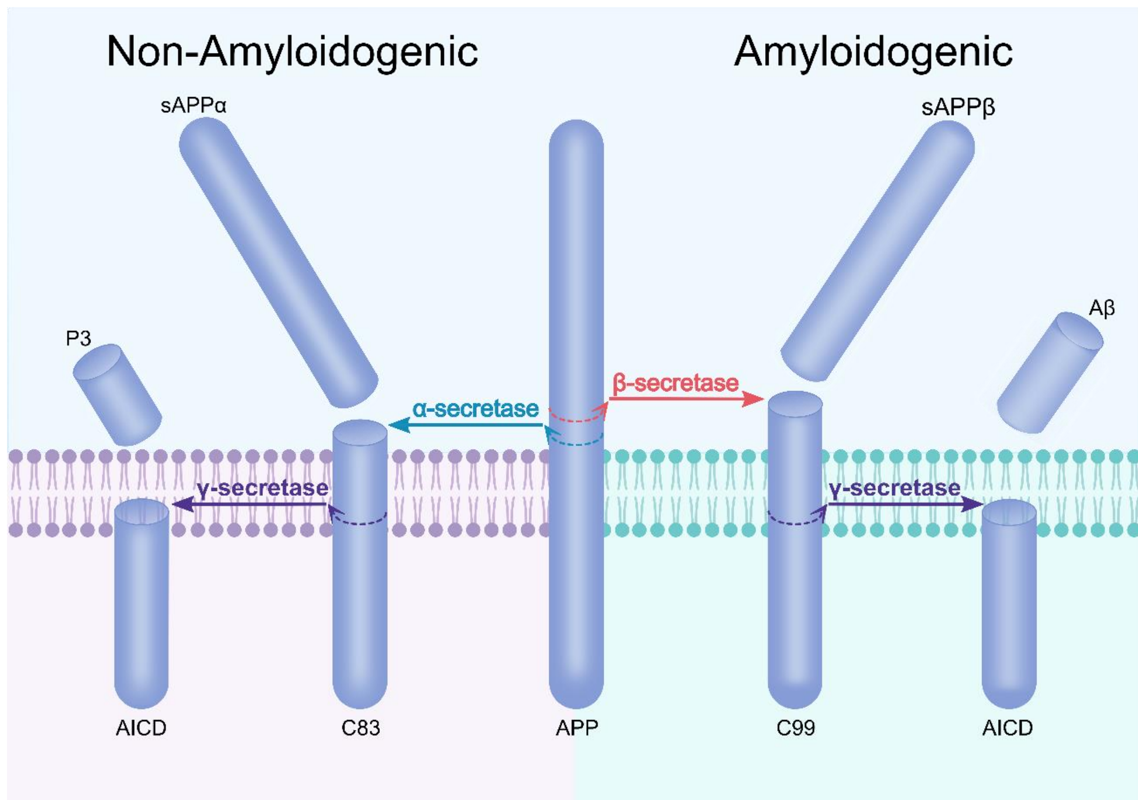


Figure 1.1 Schematic diagram of Amyloid Precursor Protein (APP) processing pathways. Left: the non-amyloidogenic pathway APP cleavage is mediated by α-secretase and γ-secretase. This pathway precludes the production of Aβ as the enzymes involved produce a smaller fragment (P3). **Right:** in the amyloidogenic pathway, the cleavage of APP by β-secretase and γ-secretase results in the production of Aβ. AICD, amyloid precursor protein intracellular domain; sAPPα, soluble amyloid precursor protein-α; sAPPβ, soluble amyloid precursor protein-β.

1.3 Memory as a cognitive function

1.3.1 Definitions of memory

There are different types of memory; the current subdivisions of memory depend on timing: short-term memory and long-term memory. Short-term memory is measured in seconds to minutes and it includes sensory memory and working memory (Nelson, 2008). Sensory memory is a mental representation of the sensory characteristics of a stimulus while working memory is the storage of information necessary to perform tasks. The second type of memory is long-term memory, which is also divided into explicit (declarative) and implicit (non-declarative) memory (Squire, 1987; Squire & Zola-Morgan, 1991). These further

divisions depend on the contents of memory; explicit memory includes memories that are recalled consciously, such as facts and events, while implicit memory includes procedural memory, which is the ability to remember sequences of motor movements such as riding a bicycle. The distinction between these types of memory allows for a focused analysis of the processes associated with each type.

Implicit memory is characterized by the nonconscious recall of motor skills. This type of memory includes procedural (skills and habits), priming, associative learning (classical and operant conditioning), and non-associative learning (habituation and desensitisation). The areas of the brain associated with these forms of memory are the cerebellum and basal ganglia (Squire, 1992). Explicit memory processes are supported by neural circuits and systems in the medial temporal lobe and structures such as the hippocampus, subiculum, and entorhinal cortex. Explicit memory, in contrast to implicit memory, involves conscious recall of personal experiences and knowledge about the world (Tulving, Endel Donaldson, 1972). Explicit memory is in turn subdivided into episodic and semantic memory; the first type consists of personal experiences involving places, time, and people while the latter represents the knowledge of concepts and facts (**Figure 1.2**).

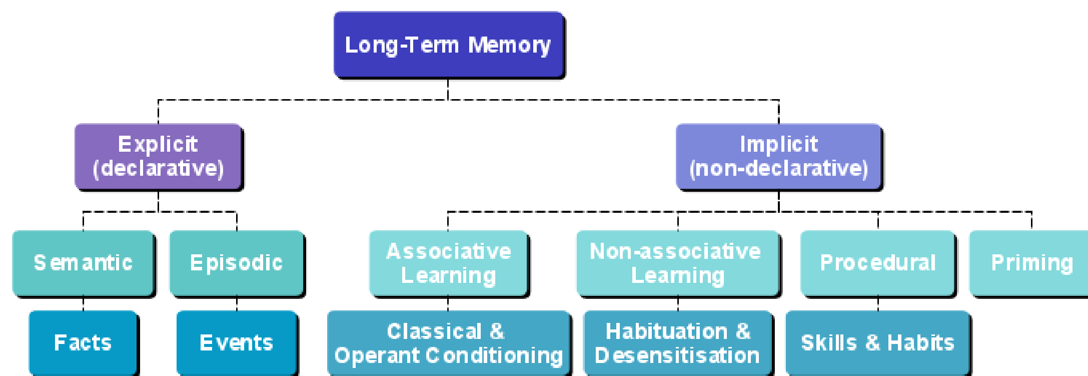


Figure 1.2 Classification of long-term memory. The types of long-term memory are based on the type of information that is stored. Explicit memory involves storage of information that can be recalled consciously whereas implicit memories are recalled unconsciously.

1.3.2 The role of the hippocampus in learning and memory

The distinction between explicit and implicit memory was suggested by studies of patients with damage to specific brain regions. An important case study was that of Henry Molaison (H.M.) who underwent a bilateral medial temporal lobectomy in 1953 to prevent his frequent epileptic seizures. The surgeons removed a large part of both his hippocampi and severed its connections to associated limbic structures, such as the amygdala. After his surgery H.M.'s seizures were significantly reduced, however, he also did not remember events in his life that occurred one year before his surgery and he lost the ability to form new episodic memories. Multiple tests were performed on H.M. to assess his memory (Scoville & Milner, 1957). The results of those tests suggested that H.M. could still remember details such as numbers or a few words at a time, at least for a short amount of time if he repeated them; and he could perform progressively better at motor tasks without remembering that he had tried to perform the task before. These tests suggested that his short-term and procedural memory were intact. Furthermore, H.M.'s older memories from one year before his surgery and his short-term memory were intact. Therefore, this clinical case led to the discovery that the hippocampus has a time-dependent role in information encoding and retrieval of memories (M. A. Lynch, 2004) and that it might be responsible for converting short-term memories into long-term memories.

Further evidence supporting the role of the hippocampus in explicit memory came from other clinical cases in which the severity of damage to the hippocampal formation corresponded to the level of memory impairment (Rempel-Clower et al., 1996). One of the cases was that of patient R.B. who exhibited anterograde amnesia after he suffered an ischaemic episode which specifically damaged the entire CA1 region of the hippocampus (Amaral et al., 1986). Therefore, findings from patients who exhibited bilateral damage specific to the hippocampus validate the function of the hippocampus in declarative memory. However, the underlying mechanisms of learning and memory have not been completely elucidated.

1.3.3 The hippocampal anatomy

The hippocampus is a structure located in the medial temporal lobe. It consists of circuits of neurons with specific inputs coming from surrounding cortical areas. The arrangement of these circuits is well known: the inputs to the hippocampus come mainly from the entorhinal cortex to form synapses with neurons in the dentate gyrus (DG) and area CA3 (*Cornus Amoni* 3); the DG neurons send their axons to form connections with CA3; then CA3 neurons send axons to form synapses with both the CA1 region, and the contralateral hippocampus in the adjacent hemisphere; CA1 neurons send axons to the subiculum; finally the latter sends output axons to the entorhinal cortex. As the neuroanatomical organisation of the hippocampus connects the DG with CA3 and CA3 to CA1, it is referred to as the trisynaptic pathway.

The arrangement of neurons in the hippocampus is layered. The first layer is formed by afferent and efferent fibres, interneurons and basal dendrites. This layer is called hilus in the DG, and stratum oriens in CA (van Strien et al., 2009). The adjacent layer is referred to as the cell layer because it is where neuronal bodies are situated. The cell layer is called the granule layer in the DG, and the pyramidal cell layer in CA, because the main types of neurons in these regions are granule neurons and pyramidal neurons respectively. Pyramidal neurons possess two different dendritic trees formed by basal and apical dendrites. Basal dendrites are located in the stratum oriens, and apical dendrites are located stratum radiatum, which extend into the stratum lacunosum-moleculare.

The well-defined arrangement of layers in this area of the brain is advantageous for the study of the physiological basis for learning and memory. The reason for this advantage is that we know that if we stimulate neurons in one area, in CA3 for example, we observe a response from the neurons in CA1, then the synaptic efficacy of the responses elicited can be compared under different experimental conditions. In this thesis, synaptic transmission was assessed using extracellular field recordings from the stratum radiatum in the CA1 region in rat hippocampal slices (**Figure 1.3A**).

1.4 Molecular mechanisms of memory

Memory function is underpinned by the ability of neurons to transmit information. This information transfer occurs at synapses which are anatomical regions that connect one neuron to the next. There are two main types of synaptic transmission: electrical and chemical. Electrical synapses are formed by gap junctions, which are clusters of intercellular channels, creating a bridge between the interior of adjacent neurons allowing the flow of electric current, small metabolites and signalling molecules (Bennett & Zukin, 2004). Conversely, in chemical synapses information is transferred from the presynaptic neuron to the postsynaptic neuron through the release of neurotransmitters. Upon the arrival of action potentials at the presynaptic terminal, voltage-gated calcium channels are activated, allowing calcium ions to flow into the presynaptic site. The increased concentration of calcium ions allows the fusion of vesicles containing neurotransmitters with the presynaptic plasma membrane, thereby releasing neurotransmitters into the space between the presynaptic and postsynaptic sites.

Neurotransmitters determine the opening or closing of ion channels which alter the synaptic potential of the postsynaptic neuron. The effect on the synaptic potential is usually excitatory or inhibitory, with the main excitatory neurotransmitter in the CNS being the amino acid L-glutamate and the main inhibitory neurotransmitter being γ-aminobutyric acid (GABA). These excitatory and inhibitory neurotransmitters bind to different types of receptors, glutamate receptors and GABA receptors.

1.4.1 Glutamate receptors

Depending on their mechanisms of action glutamate receptors are divided into two groups: ionotropic and metabotropic glutamate receptors. The first type, ionotropic receptors are ligand-gated ion channels; when glutamate binds to these receptors their ion channel opens and permits the movement of cations into the cell. The second type, metabotropic glutamate (mGlu) receptors are G-protein coupled

receptors which mediate the activation of proteins or other ion channels through secondary messengers.

Ionotropic glutamate receptors are classified into four classes according to the synthetic agonists that activate each receptor and structural homology: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), L-α-kainic acid (kainate), δ receptors, and N-Methyl-d-aspartic acid (NMDA) receptors (Traynelis et al., 2010).

1.4.1.1 AMPA receptors

AMPA receptors (AMPARs) are tetrameric receptors, that is, their structure consists of four protein subunits assembled together to form the receptor (Rosenmund et al., 1998). There are four types of AMPAR subunits called GluA1 to GluA4 which are encoded in different genes (Anggono & Huganir, 2012). AMPARs are composed of different combinations of subunits forming heteromeric assemblies (containing 2 or more different subunits). Each subunit possesses an extracellular N-terminal domain, a ligand-binding domain, transmembrane domains, and an intracellular C-terminal domain (Hollmann et al., 1994). Furthermore, AMPAR subunit composition confers different channel properties, ion selectivity and trafficking properties; therefore, the structural diversity of these receptors generates functional diversity (Greger et al., 2017).

In the adult hippocampus, two forms of AMPARs are thought to predominate: GluA1/GluA2 heteromers and GluA2/GluA3 heteromers (Lu et al., 2009; Wenthold et al., 1996). The majority of GluA2-containing AMPARs are impermeable to Ca^{2+}, due to the fact that these subunits contain a positively charged arginine (R) residue at the glutamine (Q)/R site located in the pore-lining region M2 (Sommer et al., 1991). The change of residue at the Q/R site results from a post-transcriptional modification of the genetically encoded adenosine into inosine by adenosine deaminases acting on RNA (ADAR) enzymes, producing the codon for arginine instead of the codon for glutamine (Pachernegg et al., 2015).

Alternatively, when GluA2 mRNAs are unedited so that a neutral glutamine residue is present at the Q/R site, the receptor becomes Ca^{2+} permeable which increases their channel conductance. This characteristic of the GluA2 subunit makes it a crucial determinant of AMPAR function (S. Cull-Candy et al., 2006). AMPARs are permeable to Na^{+} and K^{+}, and the excitatory postsynaptic currents (EPSCs) mediated by these receptors peak within approximately one hundred microseconds and decay within a few milliseconds (Colquhoun et al., 1992). Therefore, the activation and deactivation kinetics of AMPARs makes them key mediators of fast excitatory signal transmission.

1.4.1.2 NMDA receptors

Similar to AMPARs, NMDA receptors (NMDARs) are tetramers, with each subunit possessing an N-terminal domain, a ligand-binding domain, a transmembrane domain, and a C-terminal domain. NMDARs subunits belong to 3 subfamilies which include the GluN1 subfamily (GluN1 subunit), the GluN2 subfamily (GluN2A, GluN2B, GluN2C and GluN2D subunits), and the GluN3 subfamily (GluN3A and GluN3B subunits) (Traynelis et al., 2010). The NMDAR heterotetramers contain two obligatory GluN1 subunits together with two GluA2 or GluA3 subunits or GluA2 and GluA3 subunits combined (Ulbrich & Isacoff, 2008). This variability of the subunit composition of NMDAR subtypes contributes to the distinct physiological properties and functions (S. G. Cull-Candy & Leszkiewicz, 2004).

The properties conferred by the various NMDAR subunits make them distinguishable from the other types of ionotropic receptors. First, in addition to glutamate binding, two conditions are required for the channel ion to open: the binding of the cofactor glycine or D-serine (Kuryatov et al., 1994), and membrane depolarization need to occur. The reason why membrane depolarization is necessary is that at resting membrane potential the NMDAR channel is blocked by the presence of Mg^{2+} inside the pore of the channel (Mayer et al., 1984). When glutamate is released AMPARs activate producing depolarization which causes the Mg^{2+} block to be repelled allowing further ion flow through NMDARs into the

postsynaptic neuron. Second, they are highly permeable to Ca^{2+} as well as to Na^{+} and K^{+}. Third, NMDAR-gated currents have slow kinetics, of around a few hundred milliseconds (Lester et al., 1990). Finally, the function of these receptors is modulated by many small molecules which bind to specific subunits, allowing researchers to pharmacologically distinguish receptor subtypes (Paoletti et al., 2013).

Moving on now to consider the contribution of NMDARs and AMPARs to synaptic transmission, the release of glutamate activates both types of receptors producing EPSC consisting of an early component and a late component. The fast component is mediated by AMPARs while the slow component is mediated by NMDARs (Collingridge et al., 1988; Forsythe & Westbrook, 1988; Hestrin et al., 1990). In addition, both of these types of receptors are widely expressed at individual synapses in hippocampal neurons (Bekkers & Stevens, 1989). These findings support the idea that AMPARs and NMDARs are fundamental to neuronal circuit function.

1.4.1.3 Metabotropic glutamate receptors

mGlu receptors belong to the family of GTP-binding protein-coupled receptors which activate signalling cascades indirectly via second messengers. The structure of these receptors consists of a large extracellular amino-terminal domain which contains the binding site for glutamate, seven-transmembrane domains, and an intracellular carboxyl-terminal domain which activates G-proteins (Niswender & Conn, 2010). The classification of mGlu receptors subtypes is based on shared second messengers, sequence homology and sensitivity to pharmacological agents (Pin & Duvoisin, 1995). Group I consists of mGluR1 and mGluR5, Group II consists of mGluR2 and mGluR3, while Group III consists of mGluR4, mGluR6, mGluR7 and mGluR8. These receptors are widely expressed in neurons and their synaptic location is associated with their specific groups: Group I mGlu receptors are found in postsynaptic sites, Group II mGlu receptors are found in presynaptic and postsynaptic sites, while Group III mGlu receptors are found in presynaptic sites (Conn & Pin, 1997). Several studies have reported

that mGlu receptor activation leads to the modulation of a broad range of ion channels and signalling proteins (Anwyl, 1999). Thus, mGlu receptors have many physiologic roles making them key players in the modulation of neuronal excitability and synaptic transmission.

1.4.2 GABA receptors

GABARs are activated by the main inhibitory neurotransmitter in the adult brain. GABARs exist as ionotropic and metabotropic, whose activation mediates inhibitory postsynaptic potentials (IPSPs) composed of fast responses and slow responses (Connors et al., 1988). Ionotropic $GABA_AR$s consist of pentameric ligand-gated ion channels permeable to chloride ions (Cl^-) that mediate the fast component of IPSPs (Bormann, 1988). Activation of $GABA_AR$s leads to the influx of Cl^- and results in hyperpolarisation in mature neurons. It has been reported that another class of ionotropic GABA receptor exists, these are termed $GABA_CR$ and they are considered to be variants of $GABA_AR$s as they share sequence homology and they are also permeable to Cl^- (Bormann & Feigenspan, 1995). Conversely, metabotropic $GABA_BR$s are G-protein-coupled receptors that share sequence similarities with mGlu receptors (Kaupmann et al., 1997). $GABA_BR$s mediate the slow component of IPSPs via second messengers and their associated enzymes acting on Ca^{2+} and K^+ channels (Bormann, 1988). Therefore, GABA receptors are essential for the regulation of neuronal excitability.

In summary, a wide range of excitatory and inhibitory receptors are involved in processing and integrating neuronal signals. This information processing mediated by receptors contributes to the conversion of neuronal signals into long-term changes in synaptic strength (Voglis & Tavernarakis, 2006). Long-term changes in synaptic strength might underpin the ability of neurons for learning and memory. However, we will only focus experimentally on AMPARs. Thus, the next section describes the mechanisms that are thought to be involved in the regulation of synaptic strength.

1.4.3 Synaptic plasticity

Over 100 years ago, Santiago Ramón y Cajal suggested that learning relies on changes in the strength of synaptic networks (Mayford et al., 2012). This idea was developed further by Donald Hebb who proposed that if a neuron is able to activate a neighbouring neuron repeatedly, eventually it will become more efficient at activating that neuron by an unknown process (Hebb, 1949). Hebb's postulate is a potential mechanism by which information is stored. Accordingly, the repetition of a signalling pattern triggered after an experience would lead to the modification of neural circuits involved, producing long term changes in patterns of neural transmission which create a physical representation of the experience (Bliss & Collingridge, 1993). Synaptic plasticity shares characteristics with the mechanism described by Hebb, therefore it has been extensively studied in relation to learning and memory.

Synaptic plasticity is the ability of neuronal connections to adapt to different activity patterns to increase or decrease the intensity of their signal transmission. Different forms of synaptic plasticity have been observed, but the most studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP was first identified in rabbits' hippocampi by Bliss and Lomo by in 1973; they found that a brief train of high-frequency stimulation produced a long-lasting increase in the magnitude of neuronal responses (Bliss & Lømo, 1973). Their results demonstrated that activity-dependent changes in synaptic function resulted in increased synaptic efficacy specific to the neurons forming synapses with the stimulated neurons. Conversely, long-term depression (LTD) is the weakening of neuronal responses which results from low-frequency stimulation (G. S. Lynch et al., 1977). As both LTP and LTD result in long-lasting changes in synaptic efficacy produced by coincident pre- and post-synaptic signalling, they are useful experimental models to study memory encoding and storage.

1.4.3.1 Long-term potentiation

Synaptic strength is defined by the amplitude of the postsynaptic potentials produced in the postsynaptic neuron by neurotransmitter release from presynaptic

neurons. Synaptic activation during high-frequency stimulation triggers a sequence of biochemical reactions that result in a long-lasting increase in synaptic strength (**Figure 1.3B**). LTP has been observed in many different synapses and the molecular cascades that are initiated by it are also different depending on the synapses and circuits that are stimulated (Malenka & Bear, 2004). This thesis focuses on mechanisms triggered by the NMDAR-dependent form of LTP in synapses formed by Schaffer collaterals and pyramidal neurons in the CA1 area of the hippocampus.

LTP is a multi-step process that consists of at least two phases: early-LTP (E-LTP) and L-LTP (L-LTP). E-LTP consists of events leading to LTP induction. Usually triggered by high-frequency stimulation (HFS) or tetanus which activates biochemical processes. The activation of this process increases synaptic strength during the first 1 to 3 hours (Baltaci et al., 2019). Whereas, L-LTP or LTP maintenance is triggered by repeated stimuli and consists of the biochemical events produce a sustained increase in synaptic efficacy lasting from hours to weeks, or months (Baltaci et al., 2019).

Before LTP induction, a single stimulus releases glutamate which binds to AMPARs, metabotropic glutamate receptors and NMDARs present on the postsynaptic membrane (discussed in the previous section). However, NMDARs are only weakly activated because of the Mg^{2+} blocking the NMDAR channel. It is during and just after HFS when glutamate release from axon terminals is enhanced which activates AMPARs and therefore depolarises the postsynaptic neuron. This removes the Mg^{2+} block, allowing Ca^{2+} influx through NMDARs which depolarises the membrane further. Although the intracellular Ca^{2+} rise is short-lasting (a few seconds), it is a requirement for the induction of LTP as it triggers the activation of Ca^{2+}-dependent protein kinases such as Calcium-calmodulin (CaM), CaM kinase II (CaMKII), and protein kinase C (PKC) (Lisman et al., 2012; Malenka & Bear, 2004; Malinow et al., 1989). CaMKII is a key component of the LTP signalling cascade as its pharmacological or genetic deletion results in inhibition of LTP (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992;

Yamagata et al., 2009). In addition, PKC was found to contribute to LTP as PKC inhibitors block LTP induction if they are applied following the tetanus, whereas the application of PKC activators such as phorbol esters induces synaptic potentiation similar to LTP (Bliss & Collingridge, 1993a; Hu et al., 1988). It has been proposed that CaMKII and PKC are necessary for LTP because these kinases phosphorylate the C-tail of the GluA1 AMPAR subunit (Barria et al., 1997; Boehm et al., 2006; Mammen et al., 1997). The phosphorylation of this AMPAR subunit increases the conductance of the receptor, which is important for potentiation of synaptic strength (Lee et al., 2000). Then, LTP induction results from molecular cascades trigged by Ca^{2+}-dependent proteins leading to the modification of AMPARs. The phosphorylation of AMPARs is also a marker for their delivery and insertion into the synaptic membrane, which increases the number of receptors at the postsynaptic site (Hayashi et al., 2000; Shi et al., 1999). Finally, AMPAR phosphorylation and AMPAR trafficking to the postsynaptic cleft results in the sustained potentiation of the postsynaptic glutamatergic response. The changes in AMPAR trafficking is thought to produce an initial increase in synaptic strength lasting 30–60 min, sometimes referred to as LTP expression (R C Malenka & Bear, 2004).

In the case of L-LTP, an increased number of tetanisations initialise molecular mechanisms that enable long-lasting increase in strength. This phase requires posttranslational protein modifications, *de novo* protein synthesis and gene transcription (Abraham & Williams, 2003; M. A. Lynch, 2004; R C Malenka & Bear, 2004). The signalling pathways involved in L-LTP are initiated by NMDAR stimulation, Ca2+ influx. Then CaM stimulates adenylate cyclase, increasing cyclic AMP (cAMP) levels (Nguyen & Woo, 2003). Increased cAMP in turn activates protein kinase A (PKA), which moves to the nucleus and activates the cAMP response elements-binding protein (CREB) following its activation (Nguyen & Woo, 2003). CREB and other proteins such as mitogen-activated protein kinase (MAPK) regulate the expression of transcription factors and gene expression. Those genes include targets such as the immediate early gene zif268, and Arc (Bramham et al., 2010; Veyrac et al., 2014). This produces the

synthesis of proteins necessary for the persistence of synaptic potentiation and changes in spine morphology (Baltaci et al., 2019; R C Malenka & Bear, 2004; Thomas & Huganir, 2004).

LTP has been observed in many different synapses and the molecular cascades that are initiated by it are also different depending on the synapses and circuits that are stimulated (Malenka & Bear, 2004). This thesis focuses on mechanisms triggered by the NMDAR-dependent form of LTP in synapses formed by Schaffer collaterals and pyramidal neurons in the CA1 area of the hippocampus.

1.4.3.2 Long-term depression

In this form of plasticity, postsynaptic potentials remain decreased in magnitude for hours following prolonged periods of repetitive stimulation (**Figure 1.3C**). Low-frequency stimulation induces a form of LTD in the area CA1 of the hippocampus that is dependent on NMDAR activation (Dudek & Bear, 1992). The activation of NMDARs causes small increases in intracellular postsynaptic Ca^{2+}, triggering the activation of phosphatases (Mulkey et al., 1994) which mediate dephosphorylation and removal of AMPARs from the postsynaptic membrane. This results in the weakening of postsynaptic responses.

Studies investigating synaptic plasticity mechanisms have provided evidence for the link between learning and LTP and LTD. For instance, experiments using rats showed that learning produces an enhancement of synaptic efficacy in the CA1 region of the hippocampus, and other biochemical changes associated with LTP induction (Whitlock et al., 2006), while inhibiting sustained potentiation leads to loss of long-term spatial memory without affecting short-term memory (Pastalkova et al., 2006). In terms of LTD, deficits in visual recognition memory have been observed following the blockade of AMPAR internalisation, a necessary step for the expression of LTD (Griffiths et al., 2008). Furthermore, it has been reported that hippocampus-dependent spatial learning is impaired when LTD is blocked, while learning is enhanced with LTD facilitation (Dong et al., 2013). These findings

indicate that understanding the regulation of synaptic strength might be crucial to elucidating the molecular mechanisms of learning and memory.

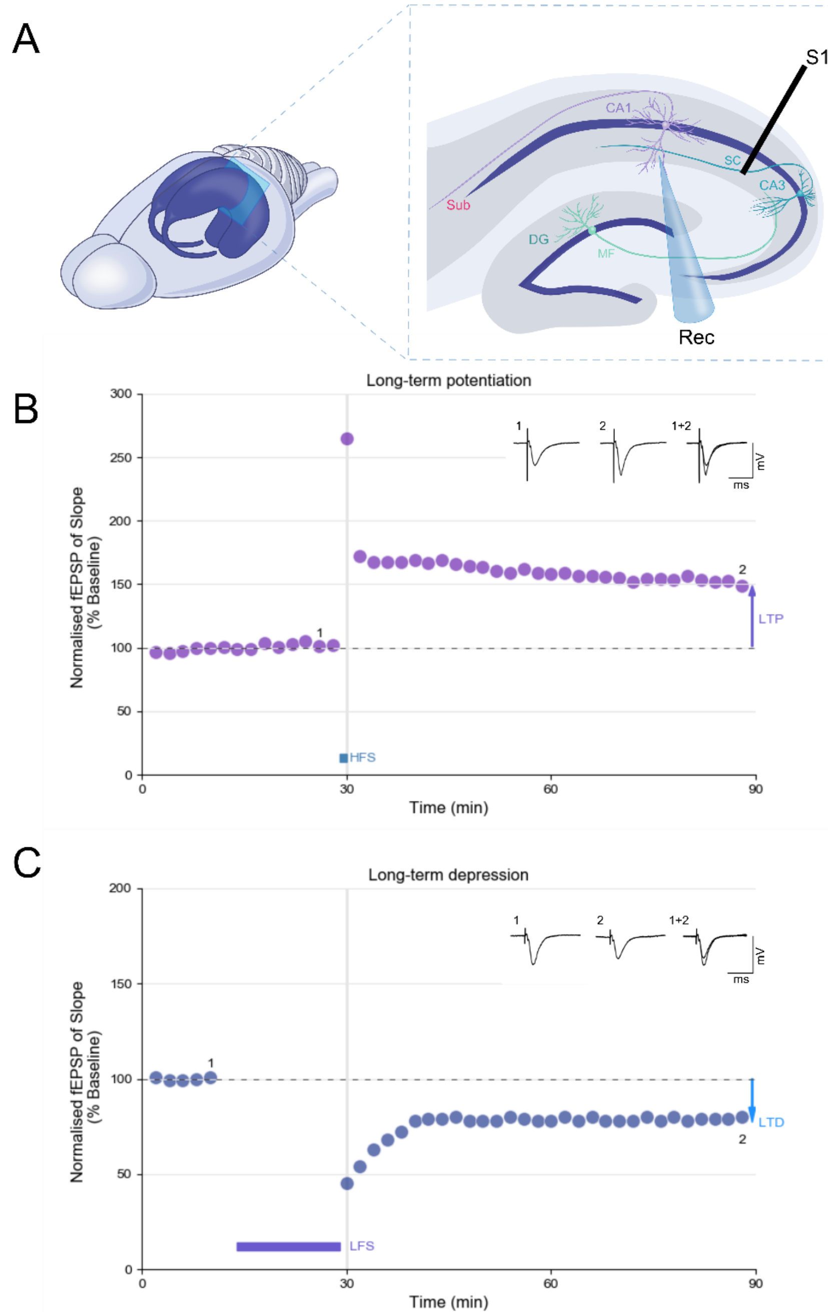


Figure 1.3 The hippocampus as an experimental model for studying synaptic plasticity. A. Left: A schematic diagram of the rodent brain showing both c-shaped hippocampi. **Right:** Schematic diagram of a transverse hippocampal slice showing the major excitatory pathways; the Perforant Path (PP) fibers terminate on granule cells (GCs) in the Dentate Gyrus (DG), then Mossy Fibers (MF) extend from GCs and terminate on CA3, and Schaffer Collateral (SC) fibers extend from CA3 to CA1. Responses elicited by electrically stimulating CA3 are measured in CA1 as field excitatory postsynaptic potentials (fEPSPs). **B.** Example of a typical LTP experiment induced by high-frequency stimulation (HFS; 100 Hz tetanus for 1 second). **C.** Example of a typical LTD experiment induced by low-frequency stimulation (LFS; 1 Hz stimulation for 15 minutes). The traces on the top-right side of B and C depict fEPSPs taken at the times indicated by the numbers on the graphs. Stimulating electrode (S1); recording electrode (Rec).

1.5 Regulation of neuronal structure in physiological and pathological conditions

1.5.1 Structural plasticity

In the central nervous system, the main sites for excitatory transmission are dendritic spines, tiny protrusions of various sizes and shapes (Bourne & Harris, 2008). They are classified in three groups according to their morphology; thin spines have a constricted neck and small heads, stubby spines have similar neck length and width, and mushroom spines have narrow necks and large heads (Tada & Sheng, 2006). Regarding the function of dendritic spines, it has been suggested that they may be involved in isolating and amplifying Ca^{2+} influx and Ca^{2+}-dependent biochemical cascades (Bloodgood et al., 2009; W. Müller & Connor, 1991). Consequently, dendritic spines are considered to be key structures for synaptic transmission.

Changes in neuronal morphology, such as the growth of new spines and remodelling of existing spines are referred to as structural plasticity. These structural changes have functional consequences as synapse formation, stabilisation, remodelling or elimination may result in altered connectivity (Caroni et al., 2012). Moreover, changes in synaptic connectivity are believed to be critical for learning. For instance, learning a motor skill task and exposure to an enriched environment where animals were exposed to novel sensory experiences promoted the formation of dendritic spines (Yang et al., 2009). In the same study, although only a small fraction of newly formed spines remained over weeks following training, the amount of newly formed spines correlated positively with the animal's performance on the motor task. Similarly, other studies have shown that motor and sensory learning paradigms promote rapid dendritic spine formation and stabilisation (Hofer et al., 2009; Roberts et al., 2010; Xu et al., 2009).

Interestingly, studies show that the induction of LTP is accompanied by structural changes in dendritic spines. Mounting evidence has shown that LTP induces dendritic spine growth, the formation of new spines, and PSD enlargement (Matsuzaki et al., 2004; Okamoto et al., 2004). LTP induces both the sprouting of

new protrusions and the growth of existing protrusions near the site of stimulation (Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999). Furthermore, LTP-inducing protocols promote the formation of new dendritic spines, some of which become stable and remain over periods of several days (De Roo et al., 2008). This indicates a relationship between synaptic plasticity and structural plasticity, however, any role either of these mechanisms plays in learning and long-term memory formation remains unclear.

1.5.2 Regulation of actin dynamics

Actin is a component of the cytoskeleton and it serves as an anchor that links scaffolding proteins and receptors to the cytoskeleton (Sheng & Pak, 2000). Structural plasticity is mediated by actin cytoskeleton rearrangements (Tada & Sheng, 2006). Actin exists in two forms: globular (G-actin) and filamentous (F-actin), the former polymerizes to form a double-stranded helical F-actin (Matus et al., 1982). Dendritic spine morphology changes are induced by different signalling cascades that involve actin regulatory proteins. These cascades activate the Rho family of guanosine triphosphatases (GTPases), which are small molecular switches that are active while they are bound to GTP and inactive while they are bound to GDP.

Regarding dendritic spine enlargement, the first steps of the mechanism correspond with events that trigger LTP. Glutamate release activates both AMPARs and NMDARs, which initiate calcium signals, that then activate CaMKII (Yamagata et al., 2009). Rho-GTPases, such as RhoA (Ras homolog family member A), and Cdc42 (Cell division cycle 42) have been found to become active following a protocol that induces spine enlargement, and this is inhibited after NMDAR inhibition (Hall, 2012). Additionally, RhoA and Cdc42 activation were partially suppressed (Hall, 2012), following CaMKII inhibition, which suggests that CaMKII relays activation signals through these Rho-GTPases. In contrast, RhoA inhibition resulted in decreased volume change on the initial phase of structural

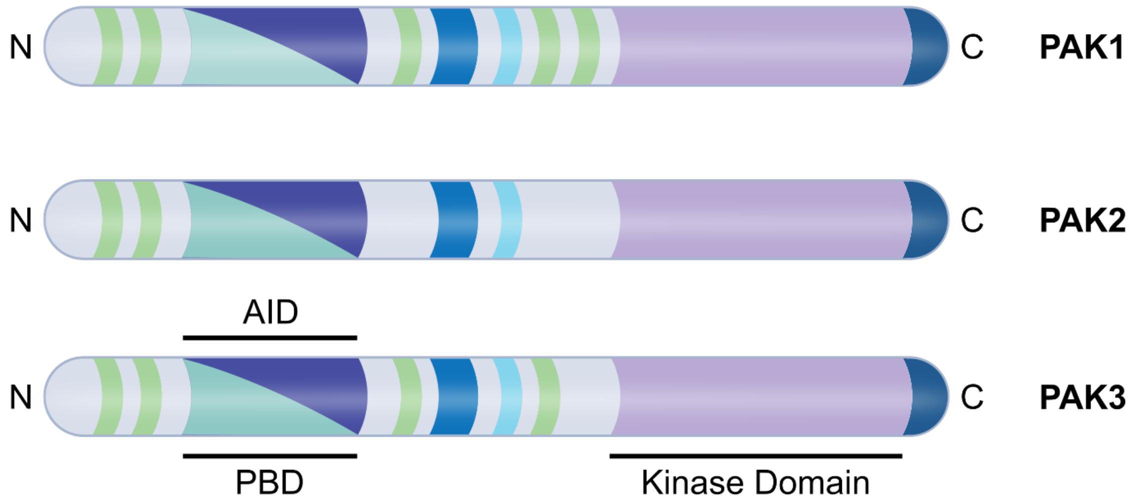
LTP (from 30 seconds up to 2 minutes after stimulation), whilst Cdc42 inhibition diminished the sustained phase of structural LTP (more than 30 minutes after stimulation) (Hedrick et al., 2016; Murakoshi et al., 2011). This indicates that RhoA and Cdc42 mediate different phases of dendritic spine enlargement.

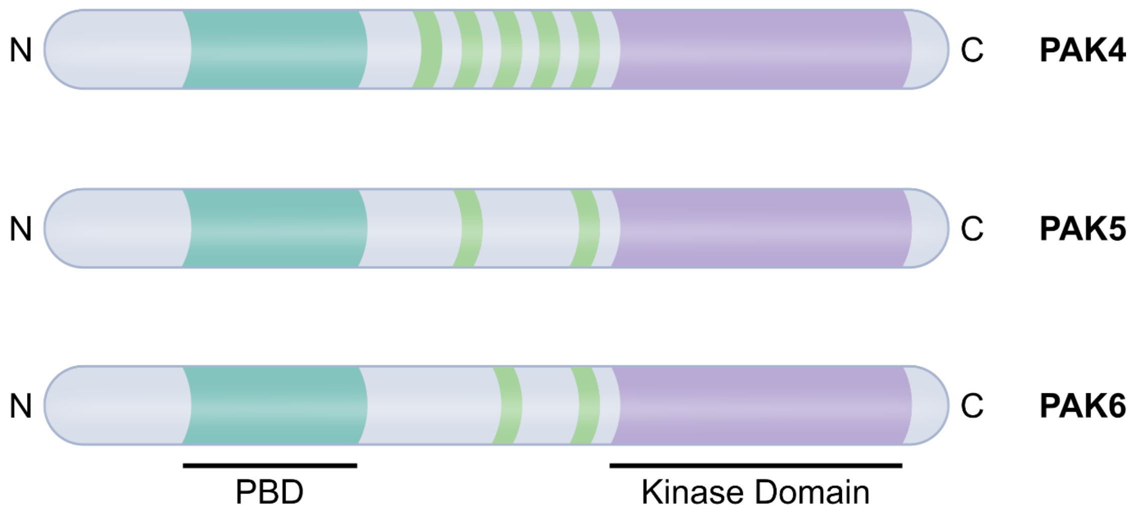
Previous studies evaluating downstream effectors of Cdc42 and Rac1 suggest that serine/threonine kinases known as p21-activated kinases (PAKs) are important mediators of structural plasticity in dendritic spines (Hall, 2012; Manser et al., 1994). In mammals PAKs are divided into two groups of proteins based on their structural domains and their regulatory mechanisms: PAK1, PAK2, and PAK3 are part of group I, while PAK4, PAK5 and PAK6 belong to group II. The six PAK isoforms have a regulatory domain on the N-terminal region and a kinase domain on the C-terminal region. In group I PAKs the regulatory domain contains a p21-binding domain (PBD) or Cdc42/Rac Interactive binding (CRIB) domain, polyproline motifs and a autoinhibitory domain (AID) (Bokoch, 2003) , see **Figure 1.4** Whereas group II PAKs do not contain a defined AID, which is consistent with their activation processes being different than group I PAKs activation (Civiero & Greggio, 2018; Eswaran et al., 2008). Group I PAKs are usually activated when they are bound with active GTPases, whereas group II PAKs kinase domains are constitutively active, independently of GTPases.

As can be seen in **Figure 1.5**, Group I PAKs exist as homodimers in their inactivated state, with the regulatory region (AID) of one PAK over the kinase domain of the other. This configuration blocks GTPase access to the PBD, preventing the activation of the two kinases of the homodimer (Bokoch, 2003). When GTP-bound GTPases, such as Cdc42 and Rac, bind to PAK homodimers the AID dissociates from the kinase domain, each of the two molecules undergoes conformational changes and autophosphorylation of the kinase domain, activating its activity towards downstream substrates (Bokoch, 2003; Manser et al., 1994). Once PAK is autophosphorylated, the active GTPase is released from the complex as the binding affinity of the activated kinase to the GTPase is reduced (Manser et al., 1994). PAK retains a high kinase activity until it is

dephosphorylated, which switches PAK to its closed and inactive conformation (Zenke et al., 1999).

Group I and II PAKs are expressed throughout the body, but some PAK isoforms have distinct expression patterns in humans and in mice. PAK1 is highly expressed in the brain, muscle and spleen; PAK2 is ubiquitously expressed; PAK3 is predominantly expressed in the brain (Koth et al., 2014); PAK4 is expressed in several tissues, and it is highly expressed in the prostate, colon and testis (Abo et al., 1998); PAK5 is highly expressed in the brain (Pandey et al., 2002); whilst PAK6 is highly expressed in the testis, prostate, brain, kidney and placenta (Arias-Romero & Chernoff, 2008; F. Yang et al., 2001). The expression of PAKs in the central nervous system makes them excellent candidates for the study of their role in neurons.





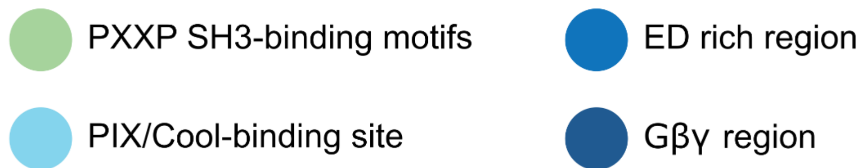


Figure 1.4 p21-activated kinase structure. Group I PAKs have a PBD that overlaps with an AID, and a kinase domain that is 93% similar (Arias-Romero & Chernoff, 2008). Group II PAKs also contain an N-terminal PBD and a C-terminal kinase domain, but they lack motifs found in group I and this group similarity among its members is lower than that of group I PAKs. Both groups contain many polyproline regions that serve as protein interaction sites.

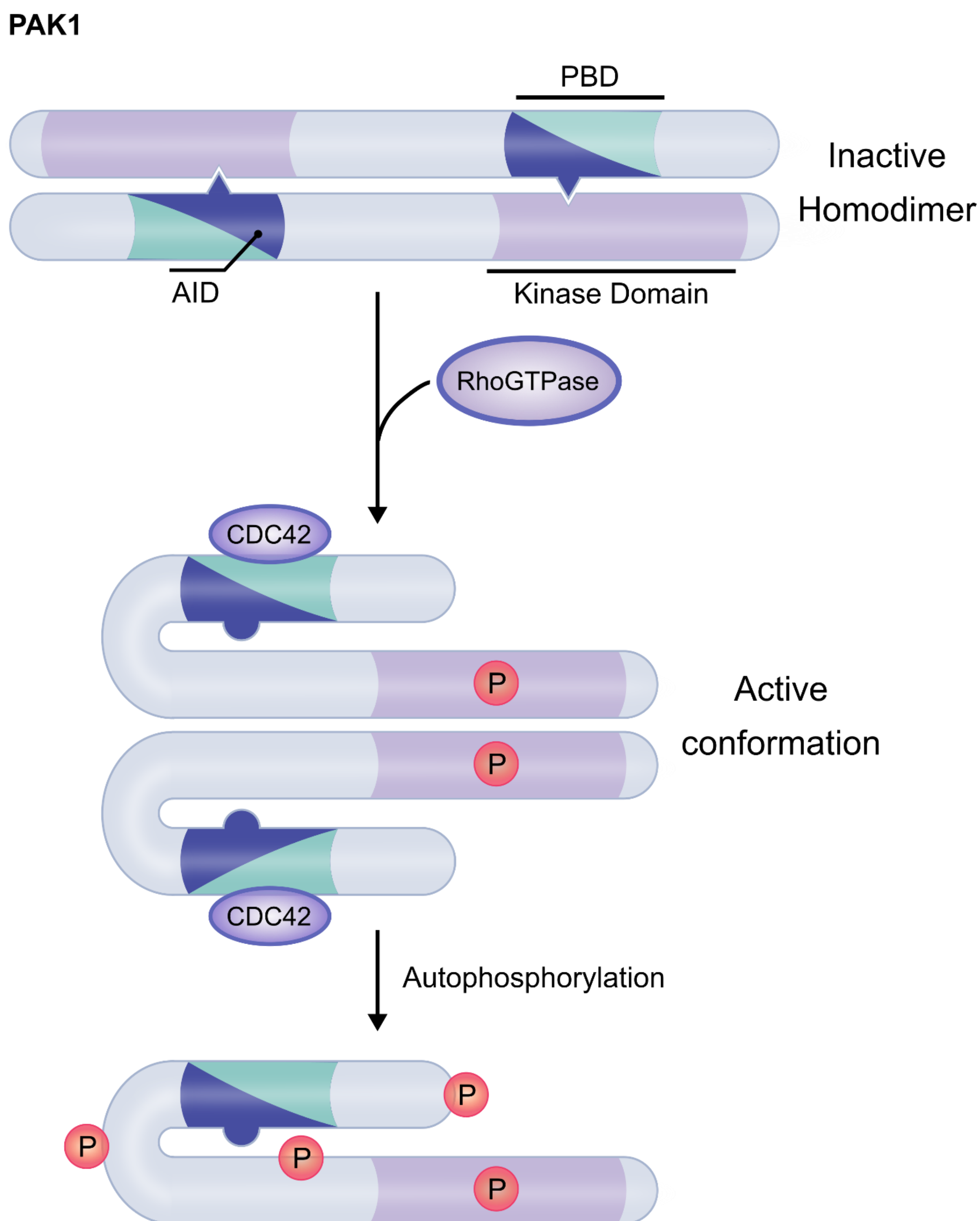


Figure 1.5 Mechanism for group I PAK GTPase-dependent activation. In their inactive form, PAKs exist as homodimers with the Autoinhibitory domain (AID) of one PAK protein overlapping the kinase domain of the second PAK protein, and vice versa. The homodimers dissociate following binding of an active RhoGTPase (Cdc42 or Rac1) to the p21-binding domain (PBD) of PAKs. The binding of the RhoGTPase leads to a conformational change that exposes the PAK activation loop allowing autophosphorylation. Then the RhoGTPase dissociates from its binding site, but kinase activity remains high as subsequent phosphorylation of various PAK residues allow PAK's structure to remain in an open and active state until PAK is dephosphorylated (Zenke et al., 1999).

In terms of the specific functions of PAK isoforms, studies targeting genetic deletions of specific PAK isoforms in mouse models have elucidated distinct roles of individual PAK members on biological processes. PAK1, PAK3, PAK5 and PAK6 knock-out mice are viable, whereas knocking-out PAK2 and PAK4 in mice leads to embryonic lethality (Arias-Romero & Chernoff, 2008; Li & Minden, 2003; Meng et al., 2005; Qu et al., 2003). PAK1 knockout mice show normal brain anatomy, basal synaptic strength, presynaptic function, but hippocampal CA1 LTP is dramatically impaired and F-actin content was reduced (Asrar et al., 2009). Mice lacking PAK3 showed normal neuronal structure and F-actin content, however, the late-phase of LTP was impaired and CREB phosphorylation was reduced (Meng et al., 2005). Furthermore, double PAK1/PAK3 knockout mice showed impairments in brain growth postnatally, less complex neuronal morphology, LTP impairment, reduced amount of F-actin, and memory deficits (Huang et al., 2011). These findings indicate that PAK1 and PAK3 functions are redundant as individual PAK1 and PAK3 knockout mice do not show aberrant neuronal morphology.

PAK4-null mouse embryos revealed that this isoform is necessary for axonal growth and neuronal development and that its absence resulted in embryonic lethality due to foetal heart defects (Qu et al., 2003). PAK5 knockout mice have no signs of gross abnormalities in tissues where PAK5 is usually expressed, which suggests that there might be functional redundancy between PAK5 and other PAK isoforms (Li & Minden, 2003). PAK6 knockout mice did not show an abnormal phenotype, whereas PAK5/PAK6 double knockout mice were found to have deficits in motor function and in learning and memory tests (Nekrasova et al., 2008).

In the central nervous system, PAK proteins have been found to be involved in the regulation of neuronal morphology, neuronal differentiation, brain development, dendritic spine maintenance, and synaptic activity. This is due to the broad range of substrates that are associated with PAKs. PAK phosphorylates the Myosin II

regulatory light chain (MLC) on Ser19, which promotes the formation of dendritic spines through the stabilisation of the actin network (Zhang et al., 2005). Other PAK substrates are also involved in cytoskeletal rearrangements, such as Filamin-A which is an actin-binding protein important for cross-linking actin filaments and for connecting them to the cell membrane (Vadlamudi et al., 2002); and, PAK phosphorylates the regulatory component of actin-related protein (Arp) 2/3 complex, which regulates actin nucleation and branching, inducing mammalian cell motility (Rane & Minden, 2014; Vadlamudi et al., 2004). Moreover, PAKs activates LIM kinase (LIMK), which in turn inactivates cofilin by phosphorylating it at Ser3. As cofilin is an actin-depolymerising protein, its inactivation facilitates actin polymerization (Arber et al., 1998; Koth et al., 2014; Yang et al., 1998).

Different approaches have helped identify the steps in the signalling cascades where PAKs contribute to actin dynamics. **Figure 1.6** shows that following activation of PAKs by Cdc42 or Rac1, PAK is able to phosphorylate LIM kinase (LIMK) which inactivates cofilin by phosphorylation (Cingolani & Goda, 2008a). Supporting evidence for this pathway comes from the finding that inhibiting PAKs resulted in decreased sustained volume change caused by glutamate uncaging (Hedrick et al., 2016; Murakoshi et al., 2011). These findings indicate that PAKs might be key mediators of dendritic structural plasticity.

Interestingly, PAKs have also been found to have effects that are independent of their role in the regulation of actin dynamics. Studies on PAK1 knockout mice have demonstrated that PAK dysfunction restricts GABAergic synaptic transmission by modulating the release of the inhibitory neurotransmitter GABA through an endocannabinoid receptor-mediated signalling mechanism (Xia et al., 2016, 2018).

PAKs are crucial components of many signalling cascades and their role in plasticity should be investigated further. Of particular concern is that Hayashi *et al.* found that dominant negative PAK (dnPAK)-expressing transgenic animals exhibited enhanced LTP (Hayashi et al., 2004), which contradicts the LTP

impairments found in PAK1 and PAK3 knockout animals. This indicates a need to understand the specific role of PAKs in synaptic plasticity because of its involvement in learning and memory as well as how PAK dysfunction could be associated with neurodegenerative disorders.

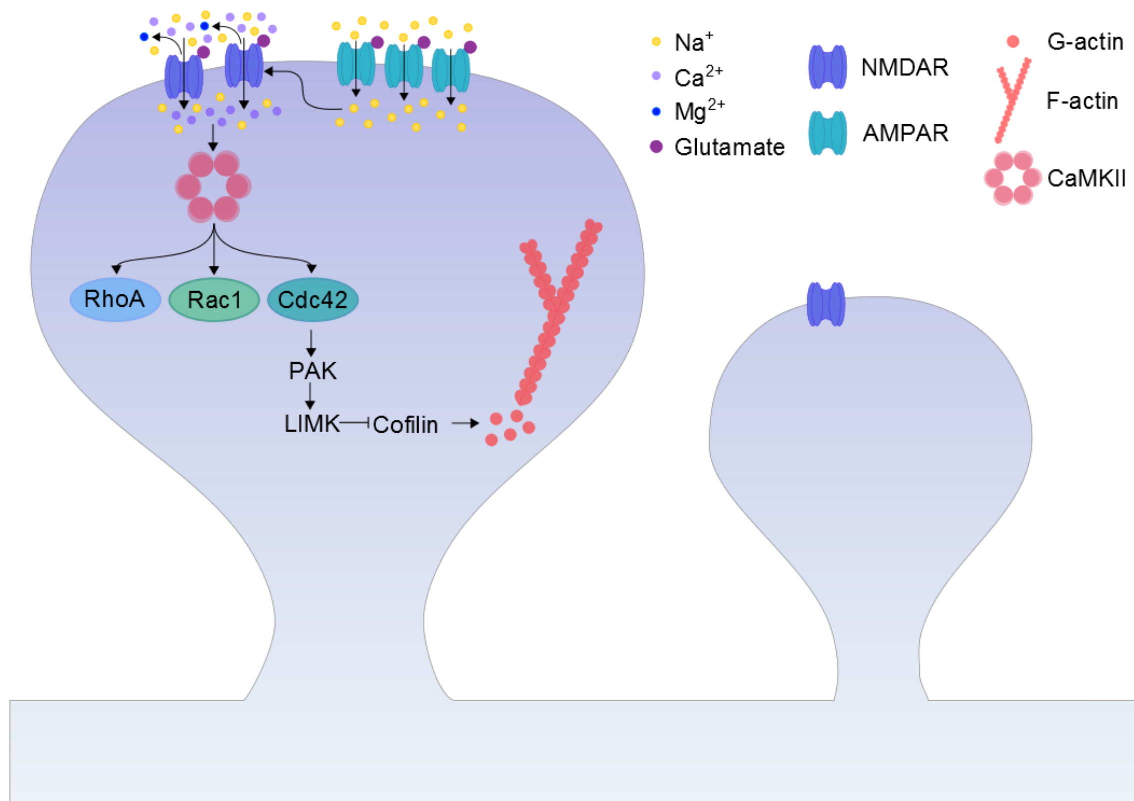


Figure 1.6 Molecular mechanism for spine morphology rearrangements. Following AMPAR and NMDAR activation, sodium permeates through AMPARs producing depolarization. This releases the magnesium block of the NMDAR ion channel. This receptor is permeable to sodium and calcium, which enter the spine. Calcium binds to calmodulin to activate CaMKII, which undergoes autophosphorylation and activates RhoA, Rac1 and Cdc42. The latter phosphorylates PAK, which activates LIMK to dephosphorylate cofilin, stopping it from disassembling actin filaments.

1.5.3 Pathophysiology

It has been proposed that synaptic function is affected early in the progression of Alzheimer's disease (Hardy & Selkoe, 2002). This hypothesis is supported by studies using animal models of AD and brain tissue samples from AD patients. In a mouse model of Alzheimer's disease, the number of spines has been found to be reduced in early stages (Spires et al., 2005). Further evidence comes from studies of tissue samples from AD and MCI patients, showing signs of damaged neurites, reduced dendritic complexity, and loss of dendritic spines in the hippocampus and cortex (Cochran et al., 2014; DeKosky & Scheff, 1990; Penzes et al., 2011). Moreover, there is positive correlation between synapse loss and decreased cognitive ability (Terry et al., 1991). Therefore, fewer synapses and connections may impair information processing because dendritic spines are main sites where excitatory synapses form.

Mounting evidence points to the role of actin cytoskeletal dynamics in dendritic spine loss in Alzheimer's disease. F-actin levels, number and size of spines, and inactive cofilin levels are all factors which are reduced both in mouse models of AD and in post-mortem samples of cortical tissue of Mild Cognitive Impairment and AD (Kommaddi et al., 2018). These results suggest that loss of F-actin may be caused by reduced inactive cofilin, leading to dendritic spine loss. One potential mediator of dendritic spine structural deficits could be PAK proteins, as deficits have been found in Alzheimer's disease pathology. PAK1 and PAK3 levels in the hippocampus, and phosphorylated PAK in the temporal cortex, were found to be reduced in post-mortem brain samples from patients with Alzheimer's disease compared to controls (Zhao et al., 2006). In the same study, PAK deficits were found in a transgenic mouse model of Alzheimer's disease; these deficits were found to be induced by amyloid-β and reversed by transfecting neurons with active wild-type PAK. Furthermore, PAK activity inhibition led to increased cofilin activity and memory deficits in adult mice (Zhao et al., 2006). These findings suggest that reduced PAK levels and activation might affect cognition.

In the case of DLB, it has been hypothesised that α-synuclein aggregates located near dendrites and spines may alter dendritic spine dynamics (Froula et al., 2018). Several studies provide evidence suggesting that α-synuclein aggregates decrease the levels of presynaptic and postsynaptic marker proteins (Kramer & Schulz-Schaeffer, 2007), and cause dendritic spine loss (Froula et al., 2018; Kramer & Schulz-Schaeffer, 2007). Furthermore, accumulation of α-synuclein aggregates in the somatosensory cortex not only triggers the progressive loss of dendritic spines, but it also alters spine dynamics such as spine turnover and stabilisation in transgenic mice overexpressing α-synuclein (Blumenstock et al., 2017). These structural consequences may be explained by the alteration of the actin cytoskeleton mediated by α-synuclein aggregates. Supporting evidence for this hypothesis comes from the findings that abnormal actin-rich inclusions which also contained cofilin were found in animal models of α-synucleinopathy and in samples from α-synucleinopathy patients (Ordonez et al., 2018). Therefore, DLB resembles AD given that synaptic dysfunction and synapse loss occur before neurodegeneration and cognitive decline. However, the detailed mechanisms underlying the pathophysiology of these neurodegenerative diseases remain undefined.

1.6 Aims and objectives

As previously mentioned, synaptic plasticity alterations are thought to be the first events in the progression of neurodegenerative disorders. The impairments also include the reduction in spine density.

There is evidence linking synaptic plasticity with synaptic remodelling. In addition, PAKs are proteins that mediate changes in the actin dynamics that regulate synapse remodelling and they may also have a role in long-term synaptic plasticity such as LTP.

The present study was designed to determine the consequences to neuronal function of exposure to protein aggregates and determine potential underlying molecular mechanisms that might be responsible.

The specific objectives are to:

1. Characterise the contribution of the oligomeric forms of Aβ and α-synuclein to LTP impairment.

2. Characterise the role of Group I PAKs in synaptic plasticity under non-pathological conditions.

3. Characterise the components on which Group I PAKs may act in order to mediate their effect on LTP.

CHAPTER 2

Materials and Methods

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Chapter 2 - Materials and Methods

2.1 Animals

All procedures involving animals were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. Acute hippocampal slices were obtained from postnatal day (P) 24 to 35 male Wistar rats (Sprague Dawley strain, Charles River, UK). These animals were housed in groups and exposed to 12 hours light / 12 hours dark cycle. Animals were housed in controlled environmental conditions with food and water available ad libitum. Hippocampal and cortical neurons for cell culture were obtained from P0-3 Wistar rats.

2.2 Slice preparation

Animals were sacrificed by cervical dislocation and then decapitated. The skull was cut along the longitudinal fissure, as well as on both sides from the midline to the orbit. Then both sides of the skull were separated so the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 1 and d-glucose 10 (bubbled with 95% O_2/5% CO_2). The brain was cut to separate the hemispheres, one of these was put back into ice-cold aCSF while the other was set on a filter paper medial side up. The cerebellum was folded towards the medial side using forceps and the cortex was gently pushed away from the transverse sinus using a spatula to reveal the hippocampus. The hippocampus was separated from the adjoining cortex, then lifted and turned 180° with a curved spatula; the remaining cortical tissue still attached to the hippocampus was removed. The hippocampus was placed on filter paper and set on a McIllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK) to cut transverse hippocampal slices (400 µm thick). These slices were placed in ice-cold aCSF to separate them. Finally, hippocampal slices were stored in a chamber containing aCSF (at 20–25°C) for 1–2 h before being used for experiments.

2.3 Electrophysiology

2.3.1 Recording equipment set-up

The recording chamber consisted of a polycarbonate recording chamber (RC-26G, Warner Instruments) with a glass coverslip base (22 x 40 mm, 64-0707, Warner Instruments, CT, USA). Leakage from the chamber was prevented by applying vacuum grease to the edges of the chamber before placing it on top of the glass coverslip. The chamber was then placed on an anodized aluminium platform (P-1, 64-0277, Warner Instruments, CT, USA), which in turn was mounted on the platform of an upright FN-S2N microscope (Nikon Instruments Inc., Japan). Prior to recording, a hippocampal slice was placed in the recording chamber. As the chamber was continuously perfused with aCSF and the flow (2-3 ml/min) caused movement, the slice was stabilised by placing on top of it a nylon mesh net held by a horseshoe-shaped wire made in-house. A peristaltic pump (Sci-Q 323; Watson-Marlow Ltd., Falmouth, UK) was used to deliver aCSF saturated with 95% O2/5% CO2 kept in a water bath at approximately 37°C (Clifton NE1-4, Nickel-Electro Ltd., Weston-Super-Mare, UK) to the chamber. The pump was connected to polyethylene tubing (2.42 mm outer diameter, 1.67 inner diameter; Smiths Medical, London, UK) and Tygon Norprene tubing (4.8 mm outer diameter, 1.6 mm internal diameter; Harvard Apparatus, Kent, UK). The aCSF was preheated using a TC-10 temperature control system (npi electronic GmbH, Tamm, Germany) connected to a heated perfusion tube system (HPT, ALA Scientific Instruments, Inc., USA) before flowing into the recording chamber to maintain a temperature of 29 ± 2°C. Excess aCSF in the chamber was removed by suction through a needle connected to a Dymax 30 vacuum pump (Charles Austen Pumps Ltd., Surrey, UK). Vibrations during recording were minimized by fixing the microscope to an air table (IsoStationTM, Newport, UK). An air compressor (JunAir 3-4, 11090, MI, USA) was used to fill the air table with compressed air. Interference caused by external electrical fields was prevented by covering the electrophysiology equipment with an in-house built Faraday cage.

2.3.2 Electrodes

Recording electrodes were made using borosilicate glass capillaries (Standard wall, 1.5 mm outer diameter, 0.86 outer diameter, 100 mm long; Harvard Apparatus, Kent, UK). These capillaries were pulled with a P-100 Flaming/Brown micropipette puller (Sutter Instruments Co., CA, USA) to achieve a resistance of 5-7 MΩ, then they were filled with NaCl 3M before attaching them to an electrode holder (QSW-T15P, Warner Instruments, CT, USA) which was connected to a headstage (CV-203BU, Molecular Devices, CA, USA). The headstage was connected to a silver wire (99.9% purity, 0.20 mm diameter, Advent Research Materials Ltd., Oxford, UK) coated with silver chloride (AgCl) which allowed the transduction of ionic current from the internal solution to electron flow along the wire. The headstage was also connected to a second AgCl-coated silver wire; this second wire was submerged in the recording chamber to provide a ground reference. The position of the recording electrode was controlled by an electronic PatchStar micromanipulator (Scientifica, Uckfield, UK). The silver wires were re-chlorided approximately once every two weeks first by scraping the surface with a razor, then immersing them in a NaCl (1M) solution and applying an electrical current using a 9V battery for 5 minutes. The silver wires in the recording electrode and in the chamber were coated with AgCl only up to the point where they were in contact with the filling solution or aCSF, respectively.

Bipolar stimulating electrodes were made by tightly twisting nickel/chromium wires (80% Ni, 20% Cr, 0.050 mm diameter; Advent Research materials, Oxford, UK) and passed through a glass capillary (prepared as previously described). The stimulating electrodes were placed on a mechanical micromanipulator (Narashige International Ltd., Japan) for controlling their position on the hippocampus slice. These electrodes were connected to stimulating boxes (DS2A-Mk.II, Digitimer Ltd., Welwyn Garden City, UK), which delivered 5V monopolar 0.1 ms square-wave pulses triggered by a computer command.

2.3.3 Extracellular field recording

Acute hippocampal slices were used to record field extracellular postsynaptic potentials (fEPSPs). These potentials were recorded in the CA1 stratum radiatum (**Figure 2.1**) area of the hippocampus using a recording electrode back-filled with NaCl (3M) solution. Bipolar stimulation electrodes were used to evoke responses; the regions where the stimulating electrodes were placed were the Schaffer collateral-commissural pathway and the subiculum, where responses from the latter position were used as the non-tetanised control input.

A stimulus was delivered to each pathway every 30 seconds (0.033 Hz); the second pathway was stimulated 15 seconds after the first one, then the stimuli continued to be delivered alternately. The slope and peak amplitude (pkAmp) measurements of the evoked response were used to determine synaptic efficacy (**Figure 2.2**). The slope is the value of the rate of change of the voltage (mV/ms) and it was determined by setting start time and end time following stimulation. These time values were set so that the slope was between 20-80% of the peak fEPSP amplitude. The pkAmp is the difference between the peak of the response and the value of the DC baseline. The stimulation intensity was in the 5-15 V range; once a maximum response was reached, the intensity was lowered approximately 30% to prevent progressive rundown of responses by overstimulation. Each slope datapoint consisted of the average of 4 successive responses.

For LTP experiments, a baseline consisted of stable responses recorded for 30 minutes before any experimental manipulations. Following this, high-frequency stimulation (HFS) was used to induce LTP, which consisted of 2 trains of 100 pulses at 100 Hz delivered with a 30 s interval. After LTP induction, recording continued for 60 to 120 minutes. The slope values were normalised by expressing values as a percentage of the average of the responses obtained during the 30-minute baseline. Data from the same experimental condition were pooled together and data points were plotted as the mean ± standard error of the mean (SEM). Changes in fEPSP slopes of different groups were compared statistically using

unpaired t-tests based on the values of 5 data points before the end of the experiment, where a p-value of 0.05 was considered statistically significant. Representative traces are examples of the responses recorded at time points indicated by numbers above and below the data shown in graphs. These traces consisted of the average of 4 responses.

For paired-pulse ratio (PPR) experiments, slopes were measured for two stimuli using 50, 200, 500 and 1000 ms interpulse intervals. These pairs of stimuli were delivered every 30 s, and the responses for each pair were the average of 4 responses. The PPR results were obtained by dividing the slope of the second stimulus by the slope of the first (S2/S1 ratio) and the values from each experimental condition were plotted as the mean ± SEM. For analysis of input-output responses, the fibre volley amplitude and slope were measured at stimulus intensities of 2, 4, 8, 10, 15 and 20 mV. Data points were represented as the mean ± SEM of the data points from all the slices tested in each experimental condition. Experiments in which changes in the fibre volley occurred were discarded.

2.3.4 Data acquisition

The headstage was connected to an amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) with the low-pass filter set to 5 kHz. For storage and analysis of measurements, signals were converted from analogue to digital by connecting the amplifier to a BNC-2110 board (National Instruments, Austin, TX), which in turn was connected to a computer with an M-series data acquisition device board (National Instruments, Austin, TX). Each sweep consisted of 500 voltage measurements in 50 ms intervals. The LTP114J software was used to monitor recordings, set recording parameters and capture data online and to reanalyse offline. Data were saved on the computer’s hard drive before processing the results.

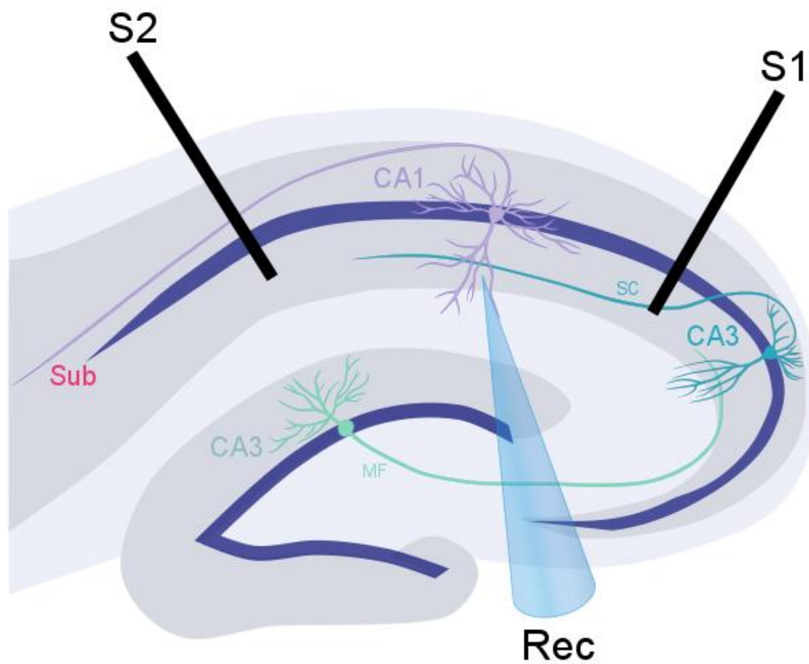

Figure 2.1 Acute hippocampal slice. Top: Photograph of a hippocampal slice; S1 and S2 indicate the positions of the stimulating electrodes and Rec indicates the position of the recording electrode. **Bottom:** schematic diagram showing the position of the stimulating and recording electrodes; S1 placed at the Schaffer collateral pathway (SC), S2 placed at the subiculum (Sub), and Rec placed at the CA1 region.

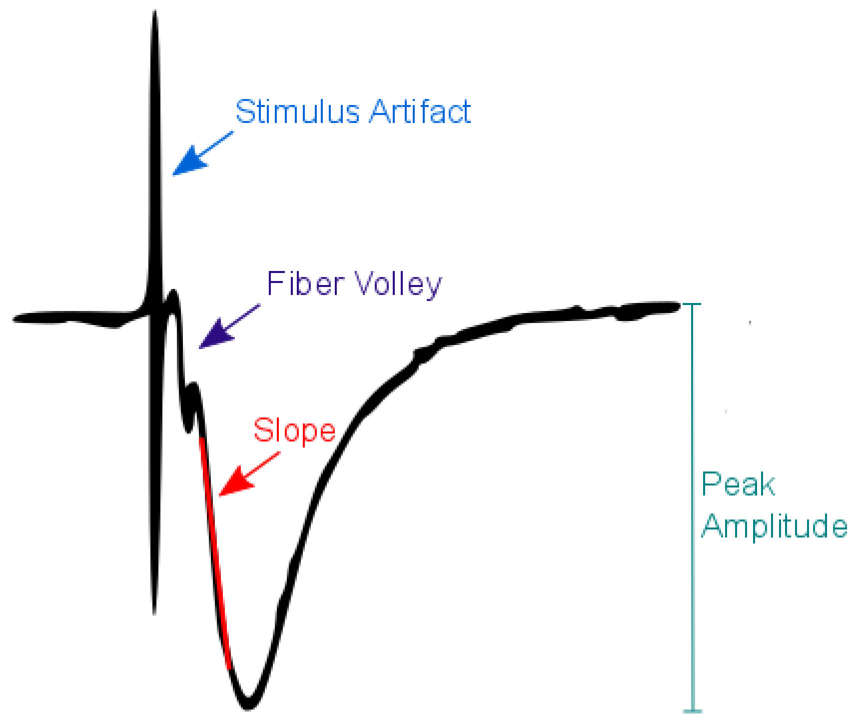

Figure 2.2 Schematic diagram of a typical fEPSP trace. The parameters of fEPSPs are indicated on the figure: the stimulus artifact, the fiber volley, the slope (determined by a start and end time after the stimulus), and the peak amplitude.

2.4 Amyloid-β preparation

HFIP (100% 1,1,1,3,3,3-hexafluoro-2-propanol; Sigma-Aldrich, 99.8% ACS reagent grade) was used to dissolve amyloid-β (Aβ) 42 peptide (Millipore, UK) at a concentration of 1mg/ml. This solution was incubated for 1 hour at room temperature with vortexing at moderate speed every 10 minutes. Then, the solution was sonicated for 10 minutes in a water bath sonicator; this was followed by drying under a gentle stream of nitrogen gas until the solution evaporated. The peptide was resuspended in DMSO (100%) and placed in a rotator for 12 minutes at room temperature with vortexing at moderate speed every 3 minutes. The resuspended solution was aliquoted into volumes of 10 µl and stored at -80°C. To induce Aβ peptide aggregation, D-PBS (Invitrogen, UK) was added to the aliquoted solution for a final concentration of 100 µM and this was incubated for 2 hours at room temperature in a rotator. The aggregated peptide was stored at -80°C after use.

2.5 α-synuclein preparation

The α-synuclein (α-syn) samples were obtained by collaboration with Cambridge University. These samples consisted of α-synuclein diluted in a Tris 25 mM and NaCl 100 mM buffer. The samples were diluted further to 70 µM using the same Tris/NaCl buffer, then the solution was aliquoted into smaller volumes and stored at -80°C. This solution was diluted in aCSF to a working concentration of 1 µM for experiments which involved incubation of acute hippocampal slices with α-syn monomers. To induce α-syn peptide aggregation, the solution was incubated in a rotating incubator at 37°C, 200 rpm for 12, 13 or 15 hours depending on the experimental condition.

2.6 Slice incubation and drug application

For slice incubation, drugs were added to aCFS in 35 mm plates. The aCSF was saturated with 95% O_2/5% CO_2 while the slices were left for different periods of time, depending on the experimental condition; incubation times are indicated in each figure description. In other cases, drugs were first diluted directly in aCSF which was delivered directly to the recording chamber during electrophysiological experiments; the specific drug used and time period of drug perfusion are indicated on each figure. Table 1 provides a summary of the pharmacological agents used, mode of action, suppliers, concentrations and solvents. Drug aliquots were stored at -20ºC.

Pharmacological Agent	Function	Supplier	Concentration used	Solvent
D-AP5	NMDAR antagonist	HelloBio HB0225	50 µM	ddH_2O
Bicuculline methiodide	$GABA_AR$ antagonist	HelloBio HB0893	20 µM	ddH_2O
CT-99021	GSK-3β inhibitor	Axon Medchem	1 µM	DMSO
MK-801 maleate	NMDAR antagonist	HelloBio HB0004	50 µM	ddH_2O
IPA-3	Group I PAK inhibitor	Abcam	25 µM	DMSO
FRAX486	Group I PAK inhibitor	Axon Medchem	500 nM	DMSO
Bradykinin	Cdc42 activator	HelloBio HB3101	85 nM 500 nM	Water

Picrotoxin	$GABA_AR$ antagonist	Abcam ab120315	1 μM	DMSO
Tetrodotoxin citrate	Voltage-gated Na^+ Channel blocker	HelloBio HB1035	5 μM	ddH_2O

Table 2.1 *Drugs used.*

2.7 Cell culture

Hippocampal and cortical neurons were cultured based on an existing method (Brewer and Torricelli, 2007). Animals were sacrificed by cervical dislocation. The brain was transferred to HABG medium (HibernateA, ThermoFisher #A1247501; B-27 Supplement, ThermoFisher #17504044; and Glutamax, ThermoFisher #35050038). The cerebellum and brainstem were removed, while the cortex and hippocampi were isolated from the midbrain and meninges were removed. The tissue was pulled apart into pieces of approximately 2 mm3, then digested with Trypsin-EDTA (ThermoFisher #25200056) in HibernateA and Glutamax for 15 minutes at 37°C. Trypsin was inactivated by addition of HABG, the tissue was further dissociated by pipetting tissue into and out of Pasteur pipettes with a flame polished tip. The neurons were isolated using OptiPrep Density Gradient Medium (Sigma-Aldrich #D1556) in HABG and quantified using Typhan-blue exclusion in a haemocytometer. The neurons were transferred to Neurobasal/B27 (Neurobasal-A medium, ThermoFisher #10888022, Gentamycin, ThermoFisher #15710049, B-27 Supplement and Glutamax). Neurons were plated onto 13-mm-diameter glass coverslips coated with poly-D-lysine (Sigma-Aldrich #P7280) at a density of 3 x 104 cells per square centimetre. Neurons were incubated at 20% O2, 5% CO2, 37 °C.

2.8 Calcium Imaging

The chemical calcium indicator Fluo4-AM (Life technologies F14201) was used to detect changes in intracellular calcium in primary cultured hippocampal neurons (DIV 14-29) cultured on 13 mm coverslips. Fluo4-AM stock solution was prepared by dissolving 50 μg of Fluo4-AM in 9.1 μl of DMSO, to a concentration of 5 mM. Neurons were washed 3 times with 0.1% BSA in HEPES Buffered Saline (HBS) containing (in mM): 119 NaCl, 5 KCl, 25 HEPES, 33 glucose, 2 $CaCl_2$, 2 $MgCl_2$, 0.001 glycine, 0.1 picrotoxin (pH 7.4) supplemented with BSA (1 mg/ml).

Neurons on coverslips were loaded with 5 μM Fluo4-AM diluted in HBS/BSA for 1 hour in an incubator at 20% O_2, 5% CO_2, 37°C in the dark. Subsequent to this, neurons were washed 3 times with HBS before placing them in the recording chamber, then they were perfused with HBS throughout the whole experiment at a flow rate of approximately 2ml/min. Fluo4 AM fluorescence was recorded for 25 minutes in total, an image was taken every 30 seconds. Cell imaging was performed using a Leica DM IRBE (Wetzlar, Germany) inverted microscope with a motorised stage (Optiscan II, Prior Scientific, Cambridge, UK) and a digital camera (ORCA 100 C4742-95, Hamamatsu Photonics, Hamamatsu, Japan) with a charge-couple device. All images were taken with a 20x objective. Images were captured using SimplePCI imaging software (Hamamatsu Photonics, K. K, 2017). A baseline of 10 minutes was recorded before adding DMSO alone or 500nM FRAX-486 diluted in DMSO for 10 minutes. KCl causes resting membrane potential depolarization which results in calcium influx into the neuron, therefore, it was added 5 minutes before the end of each experiment to identify neurons which showed increased fluorescence.

Icy software (Institut Pasteur, 2011) was used for image analysis. The total fluorescence intensity was obtained by selecting regions of interest (ROIs) on the somatic area of each neuron. Fluorescence intensity values were obtained for each ROI. Fluorescence was measured as the ratio of the fluorescence intensity as the experiment is running and the fluorescence intensity at the start of the experiment (F/F_0), which was calculated after subtraction of background

fluorescence. This measure relative to the initial signal is used as a way of normalising indicator concentration differences between neurons (Bootman et al., 2013). Pooled data from experiments were normalised to the average of values obtained during the baseline and were expressed as a percentage.

2.9 Chemical LTP

The coverslips used for these experiments were observed under the microscope to inspect neuronal morphology characteristics, such as the presence of viable cell bodies and neurites, before continuing with experiments. For glycine stimulation, hippocampal cultured neurons (DIV14-21) were incubated for 3 minutes in a Mg^{2+}-free bathing solution containing (mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 30 glucose, 0.001 strychnine, 0.02 bicuculline methiodide with (experimental group) or without (control group) 0.2 glycine (Sigma Aldrich) and 0.001 picrotoxin (Fortin et al., 2010; Groc et al., 2008). Following the 3 minutes incubation, neurons were kept in bathing solution without glycine for 15 minutes before fixing and staining them.

The application of glycine enhances NMDAR activation, picrotoxin and biccuculine are $GABA_A$ receptor antagonists which block inhibitory synapses, strychnine blocks glycine receptors and the absence of Mg^{2+} reduces NMDAR pore blocking (M. Patterson & Yasuda, 2011). The bath application of the solution with glycine strongly stimulates most synapses, triggering the synchronisation of nearby neurons and producing long-term strengthening of excitatory synapses (Molnár, 2011).

To test whether this chemical LTP stimulation depends on NMDAR, neurons were incubated with antagonists, such as MK-801 or AP5, diluted in culture medium for 30 minutes prior to stimulation. Other sets of neurons were incubated for ~1.5 - 2 hours with a group I PAK inhibitor, IPA-3 (25 µM) or FRAX486 (500 nM), diluted in the culture medium prior to chemical LTP stimulation. Neurons were left in an incubator at 20% O_2, 5% CO_2, 37°C while the incubations took place.

2.10 Phalloidin staining and quantification of spine density and morphology

Hippocampal neurons were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Neurons were washed three times in PBS and permeabilized in PBS containing 0.3% Triton for 15 minutes. After washing three times in PBS, neurons were stained with Alexa Fluor 568- phalloidin (Invitrogen) diluted with PBS at 1:40 to stain actin and visualize dendritic spines. Coverslips were washed and mounted onto slides using Hard-set Vectashield with DAPI. Fluorescent images were obtained using a Leica DFC7000T camera attached to a Leica DM2000 microscope with a 100x oil-immersion lens. The labels for all microscope slides were concealed from the experimenter until all images were taken and data was analysed. Spines were defined as visible protrusions extending from dendrites. From each neuron, up to 3 10 μm^2 sections of primary and secondary dendrites were analysed. Spine area, circularity, and spine number measurements were assessed using National Institutes of Health Image J software.

2.11 Immunodetection of surface GluA2-AMPARs

Following the chemical LTP protocol described above, surface GluA2-AMPARs were incubated for 15 minutes with an anti-GluA2 subunit mouse monoclonal antibody (1:100; Thermo Fisher Scientific) diluted in PBS containing 3% BSA. Hippocampal neurons were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. To add a postsynaptic location marker, neurons were washed 3 times in PBS and permeabilised in PBS containing 0.3% Triton X-100 for 15 minutes. Neurons were then washed and blocked with PBS containing 5% BSA for 60 minutes, followed by incubation with an anti-Shank3 rabbit monoclonal antibody (1:400; Cell Signalling Technology) diluted in PBS containing 3% BSA. After washing 3 times in PBS, neurons were stained with goat anti-mouse IgG secondary antibody Alexa

Fluor 555 (1:50; Thermo Fisher Scientific) and goat anti-rabbit IgG secondary antibody Alexa Fluor 488 (1:50; Thermo Fisher Scientific) diluted in PBS containing 3% BSA for 60 minutes. Coverslips were washed and mounted onto slides using Hard-set Vectashield with DAPI (Vector laboratories) and kept at 4°C until quantification.

Confocal images were obtained using a Leica SP5-AOBS confocal laser scanning microscope with a 63x 1.4 oil-immersion objective. Images of dendrites were digitally zoomed-in to reach a maximum resolution of approximately 70 nm. These images were taken as z projections with step intervals of 0.25 µm. The stacks contained 12-15 planes which encompassed a dendritic section from top to bottom. To measure changes in surface GluA2 in postsynaptic sites following chemical LTP, Shank staining was used as a mask filter to detect postsynaptic GluA2 staining. Then GluA2 integrated fluorescence levels within Shank clusters were measured. Two to 3 20 µm-long sections of primary and secondary dendrites from each neuron were analysed. The specific number of coverslips and neurons quantified for each experiment is stated within the corresponding figure description. Each experimental condition using the chemical LTP protocol was repeated at least 3 times with independent neuronal culture preparations. Fluorescence measurements were obtained using the National Institutes of Health Image J software (Abràmoff et al., 2004).

2.12 Statistical analyses

Data were analysed from hippocampal slices or neurons; numbers of slices, animals, neurons or coverslips are specified in each figure. Data pooled across slices or neurons are expressed as the mean ± SEM. For electrophysiology experiments, data are expressed relative to a normalised baseline (100 % = no change).

Statistical significance was considered at p-values lesser than 0.05. For LTP and calcium imaging experiments, statistical significance was tested using unpaired two-tailed t-tests. Two-way ANOVA was used for assessing statistical differences in input-output responses and PPR, while Kolmogorov-Smirnov was used for comparing differences on surface GluA2 staining between control and chemical LTP stimulation. Graphs were generated using a range of libraries in python, and SigmaPlot 13.0 (Systat Software, Inc., USA). Statistical analyses were conducted with SigmaPlot.

CHAPTER 3

The effects of oligomerised protein on synaptic function

3.1 Introduction

3.1.1 Aberrant protein aggregates and dysregulation of synaptic function

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3.1.3 Mechanisms of synaptic dysfunction induced by protein aggregates

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3.3 Results

3.3.1 Time-dependent effect of protein aggregation and synaptotoxicity

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3.4.1 Aggregation status and synaptotoxicity

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Conclusion

Chapter 3 - The effects of oligomerised protein on synaptic function

3.1 Introduction

3.1.1 Aberrant protein aggregates and dysregulation of synaptic function

The aberrant aggregation of proteins is a hallmark feature of many neuropathological diseases. Insoluble and diffusible oligomers composed of different proteins can be found in diseases that are clinically, genetically and pathologically distinct. For example, Aβ accumulates extracellularly and hyperphosphorylated tau accumulates intracellularly in Alzheimer's disease (AD); the accumulation of these aberrant proteins can be first seen in the hippocampus, and they spread to cortical areas with disease progression. In the case of Parkinson's disease (PD), PD dementia and dementia with Lewy Bodies (DLB), α-synuclein accumulates intracellularly in cell bodies and axons starting from the substantia nigra in the brainstem to then spread into the midbrain and cortical regions. Misfolded protease-resistant prion proteins accumulate intracellularly in Creutzfeldt-Jakob disease in various cortical areas. TDP-43 accumulates in amyotrophic lateral sclerosis which affects motor neurons and frontotemporal lobar dementia which affects the frontal and temporal lobes. Also, Huntingtin is a polyglutamine protein which accumulates intracellularly and primarily affects the striatum and cortex in Huntington's disease. However, though the specific mechanisms that underpin the development of pathology in these diseases differ, the ultimate consequences of the deposition of aggregated protein appear to be similar: neuronal impairment and synaptic dysfunction.

With the aim of elucidating how pathological protein aggregation leads to cognitive impairment, many studies have turned to *in vitro* models of disease and examined the consequences on synaptic plasticity. Many studies have shown that the application of Aβ (Klyubin et al., 2008; Shankar et al., 2007; Townsend et al., 2006; Walsh et al., 2002), α-synuclein (Diógenes et al., 2012), prion protein (Johnston et al., 1998), tau (Ondrejcak et al., 2018), or Huntingtin (Quirion & Parsons, 2019; Usdin et al., 1999) to hippocampal slices inhibits the induction of LTP. Whether

there is an ultimately shared mechanism across these diverse proteins which mediates this effect on the synapse, however, is unknown.

One potential consistent feature of these different proteins and their shared effect on synaptic plasticity is their aggregation status. Here, the extent of aggregation (or oligomerisation) is thought to determine the synaptotoxicity of the protein. Soluble Aβ oligomers inhibit LTP in hippocampal slices whereas insoluble amyloid plaques nor Aβ monomers do not (Shankar et al., 2008). Microinjection of medium containing Aβ oligomers and monomers inhibited LTP *in vivo*, but this effect was mediated by oligomers not monomers (Walsh et al., 2002). Another study found that Aβ monomers did not affect LTP, whereas trimers were more effective at inhibiting LTP than dimers and tetramers, which had an intermediate effect (Townsend et al., 2006). In turn, the anti-Aβ antibody reverses the effect of Aβ oligomers on LTP (Klyubin et al., 2008; Townsend et al., 2006).

The aggregation status of α-synuclein is thought to be the mechanism that leads to PD. A study by Winner *et al.* found that α-synuclein oligomerisation led to enhanced toxicity in the rat substantia nigra (Winner et al., 2011). Similar to Aβ, incubating rat hippocampal slices with α-synuclein oligomers but not monomers or fibrils resulted in inhibition of LTP induced by theta-burst stimulation (Diógenes et al., 2012). In another study, three types of α-synuclein oligomers co-localised with excitatory synapses and reduced LTP in mice hippocampal slice (van Diggelen et al., 2019). Moreover, LTP is impaired in striatal cholinergic interneurons of transgenic animals overexpressing wild type human α-synuclein and truncated α-synuclein (Tozzi et al., 2016). In the latter study, the findings were replicated by applying exogenous α-synuclein oligomers where LTP was adversely affected in a dose-dependent manner.

In terms of other types of aggregates, previous research has established that injection of abnormal prion proteins from brain homogenates of scrapie-infected mice leads to impairment in LTP stabilisation and maintenance in hippocampal slices of the injected mice 100 days post-incubation (Johnston et al., 1998).

Regarding huntingtin, studies have shown that LTP is impaired in transgenic mice expressing mutant huntingtin compared to wild-type animals (Quirion & Parsons, 2019; Usdin et al., 1999). The LTP impairment seen in the latter studies may be explained by the ability of mutant huntingtin to form oligomers (Stott et al., 1995). In addition, many recent studies suggest that application of exogenous soluble aggregates of wild type tau or human recombinant tau inhibited LTP while tau monomers and fibrils did not (Ondrejcak et al., 2018).

Together then, these studies indicate that a consistent factor across these divergent protein types, to determine the extent of their toxicity, is their level or type of aggregation. The consequences of specific protein aggregate isoforms for neuronal function, however, remain relatively poorly characterised.

3.1.2 Concentration threshold of aggregate-mediated pathogenesis

The link between the aggregation profile and synaptic impairment is currently unknown. One possible explanation could be the available concentration of toxic protein. In the case of Aβ, it has been reported that low concentrations (approximately 100 - 300 pM) of soluble Aβ low-n oligomers such as dimers, trimers and tetramers were found to robustly inhibit LTP (Townsend et al., 2006). However, studies have found that there is a concentration-dependent aspect of Aβ-induced synaptic depression as increasing the concentration from 1 μM Aβ40 and 50 nM Aβ42 to 10 μM Aβ40 and 500 nM Aβ42 led to LTP impairment (Kamenetz et al., 2003). In addition, exposing hippocampal slices to Aβ oligomers from cortical samples of AD patients adversely affected LTP in a dose-response manner (Shankar et al., 2008). It is important to note that the Aβ42 peptide is more prone to aggregate (Chen & Glabe, 2006; Marina et al., 2003) and genetic conditions in which mutations result in increased production of Aβ lead to early-onset familial AD (Citron et al., 1992; Rovelet-Lecrux et al., 2006).

With respect to α-synuclein, a study testing three types of α-synuclein, oligomers only reduced LTP at concentrations of 30 nM for a type of modified α-synuclein

and at 100 nM for unmodified α-synuclein (Diggelen et al., 2019). In contrast, incubating slices with α-synuclein oligomers at concentrations of 10-50 nM did not affect LTP, but incubating slices with α-synuclein oligomers at a concentration of 500 nM resulted in LTP impairment as the responses returned to baseline levels 60 minutes after induction (Diógenes et al., 2012).

3.1.3 Mechanisms of synaptic dysfunction induced by protein aggregates

Although the mechanisms by which protein aggregation contribute to neurodegeneration are still unknown, one potential mechanism may be that aggregates cause abnormal membrane permeabilization. Data from several studies suggest that Aβ and α-synuclein form "ring-like" oligomeric structures which form pores on the surface of membranes (Lashuel et al., 2003; Rochet et al., 2004; Volles & Peter T. Lansbury, 2002). The toxic effects of these "ring-like" structures led to dysregulated influx of ions, thereby affecting neuronal signal transmission (Furukawa et al., 2006). Furthermore, another study revealed that the interaction between Aβ and α-synuclein led to the formation of "ring-like" structures that form ion-channels, and that cells expressing α-synuclein and treated with Aβ showed altered synaptic dysfunction due to increased calcium influx (Tsigelny et al., 2008).

Alternatively, data from several studies suggest that protein aggregates disrupt the number and function of synaptic receptors. Several reports have shown that increased production of Aβ in cultured hippocampal neurons or hippocampal organotypic slices resulted in reduced synaptic surface AMPAR and NMDARs (Almeida et al., 2005; Hsieh et al., 2006a; Snyder et al., 2005). This effect might be mediated by oligomeric Aβ as it was found that exposure of neurons to Aβ oligomers resulted in decreased surface expression of NMDARs (Lacor et al., 2007). In terms of synaptic activity, studies have shown that Aβ production in neuronal slices overexpressing APP weakens glutamatergic synaptic transmission (Hsieh et al., 2006a; Kamenetz et al., 2003b).

Various studies have assessed the effect of oligomers on neuronal connectivity and plasticity. In mammalian brains, most excitatory connections are formed on small protuberances along dendrites termed dendritic spines. Dendritic spines are dynamic structures which undergo changes in number and morphology to establish or remodel neuronal circuit connectivity (Penzes, Cahill, Jones, Vanleeuwen, & Woolfrey, 2011). Indeed, increased levels of Aβ due to APP overexpression in hippocampal organotypic slices, as well as incubation of slices with Aβ for 7 days, reduce dendritic spine density (Hsieh et al., 2006a). It has also been reported that mature cultured hippocampal neurons exposed to Aβ oligomers (500 nM) for 24 hours result in disruption of dendritic spine morphology and reduced spine density (Lacor et al., 2007). In another study, neurons in organotypic hippocampal slices incubated with low-n number Aβ oligomers (approximately 100-300 pM) for 5 to 15 days showed significantly reduced spine density (Shankar et al., 2007a).

It has also been suggested that dysregulation of synaptic plasticity caused by small oligomers is an initial and contributing factor to progressive neurodegeneration (Selkoe, 2002). A considerable amount of research has shown that Aβ oligomers impair LTP in vivo and in vitro (Billings, Oddo, Green, McGaugh, & LaFerla, 2005; Chapman et al., 1999; Cleary et al., 2005; Cullen, Suh, Anwyl, & Rowan, 1997; Freir, Holscher, & Herron, 2001; Walsh et al., 2002b). Notwithstanding the extensive progress that has been made, the neurobiological basis of how protein aggregates disrupt synaptic plasticity needs to be elucidated.

3.2 Aims and Hypotheses

The aim of this chapter is to analyse the effects of α-synuclein aggregation on synaptic plasticity mechanisms in the hippocampus, specifically LTP induction.

3.3 Results

3.3.1 Time-dependent effect of protein aggregation and synaptotoxicity

As previously outlined, the aggregation status – and what synaptotoxicity this confers – is well established in the case of Aβ (Hsieh et al., 2006; Shankar et al., 2007, 2008; Townsend et al., 2006; Walsh et al., 2002). However, comparatively little is known about the relationship between aggregate and synapse dysfunction for other pathogenic proteins. In order to substantiate the hypothesis that it is protein aggregation *per se* that drives the synapse impairment (rather than a particular facet of the protein in question itself), it would need to be shown that, as with Aβ, there are aggregation status-dependent effects with other proteins. We decided to focus on the alpha-synuclein protein, the role of which in synaptic dysregulation is comparatively less well studied.

Synthetic α-synuclein was obtained from a collaborator (Klennerman Group, University of Cambridge). When initially derived, α-synuclein is in a monomeric form. However, following solubilisation in a Tris (25 mM)/NaCl (100 mM) solution, and incubation at 37 °C (as explained earlier in section 2.5), the protein forms aggregates. Interestingly, there is a time-dependence to this effect, where large n-aggregates form over time (Klennerman Group, personal communication).

We began by preparing 3 distinct α-synuclein preparations, which had been aggregated for 3 different time periods (12h, 13h and 15h). We then treated hippocampal slices with 1 μM of these preparations for 2-2.5h and examined the consequences on acute hippocampal slice synaptic plasticity. This was undertaken by means of measuring fEPSPs in area CA1, evoked by stimulation of Schaffer-collaterals (see Chapter 2, section 2.3.3).

When compared with control, untreated slices, we found that application of 12h-aggregated α-synuclein had no effect on the magnitude of LTP (α-syn 12h: 140 ± 9% of baseline, n = 3, closed circle; Control: 146 ± 12%, n = 3, open circles, p = 0.713, **Figure 3.1**). Similarly, when slices were treated with 13h-aggregated α-synuclein, there was also no effect on LTP (α-syn 13h: 153 ± 32% of baseline, n

= 3, closed circle; Control: 185 ± 17%, n = 3, open circles, $p = 0.430$, **Figure 3.2**). In contrast to these results, when slices were treated with 15h-aggregated α-synuclein, there was a robust inhibition of LTP (α-syn 15h: 122 ± 9% of baseline, n = 9, closed circle; Control: 152 ± 7%, n = 9, open circles, $p = 0.0218$, **Figure 3.3**). Taken together, these results indicate a time-dependent aggregation effect of α-synuclein on hippocampal LTP and could suggest that the synaptotoxicity of α -synuclein, as with Aβ, is determined by its aggregation status.

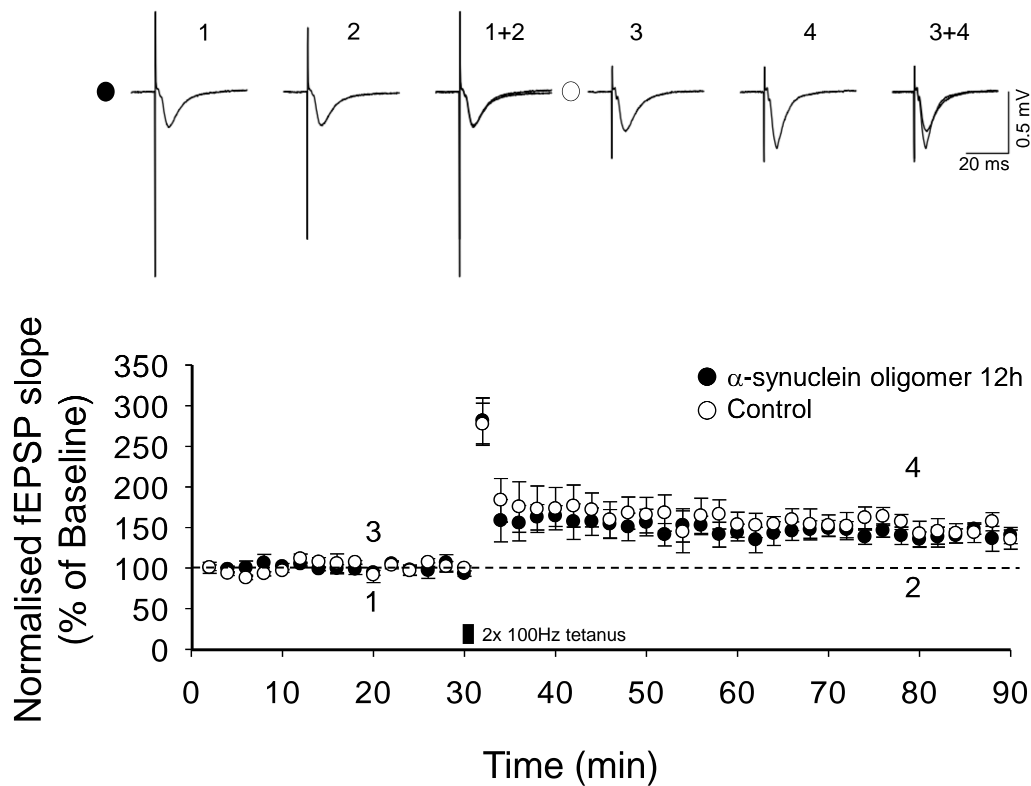

Figure 3.1 Application of α-synuclein oligomerised for 12h does not affect LTP. Slices incubated with α-synuclein oligomers (1 μM) for 2 - 2.5 hours (control n=3, α-syn n=3, *p*-value = 0.713) compared with untreated control.

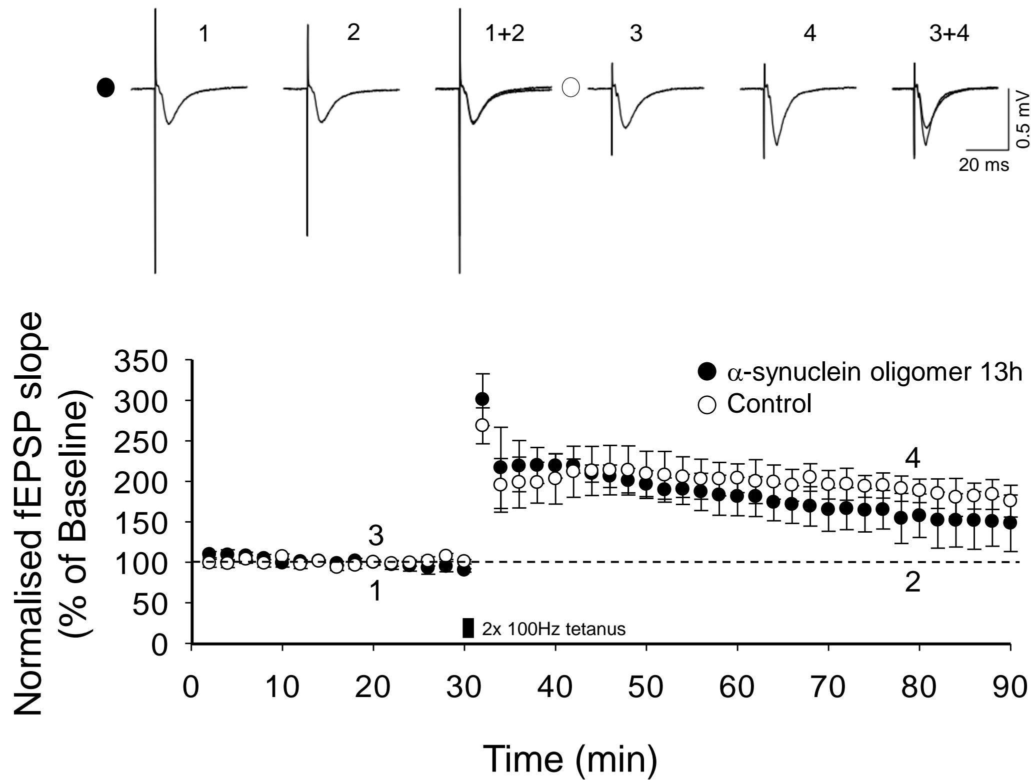

Figure 3.2 LTP can be induced following application of α-synuclein oligomerised for 13h. Slices were incubated with α-synuclein oligomers (1 μM) for 2 - 2.5 hours (control n=3, α-syn n=3, *p*-value = 0.430) compared with untreated control.

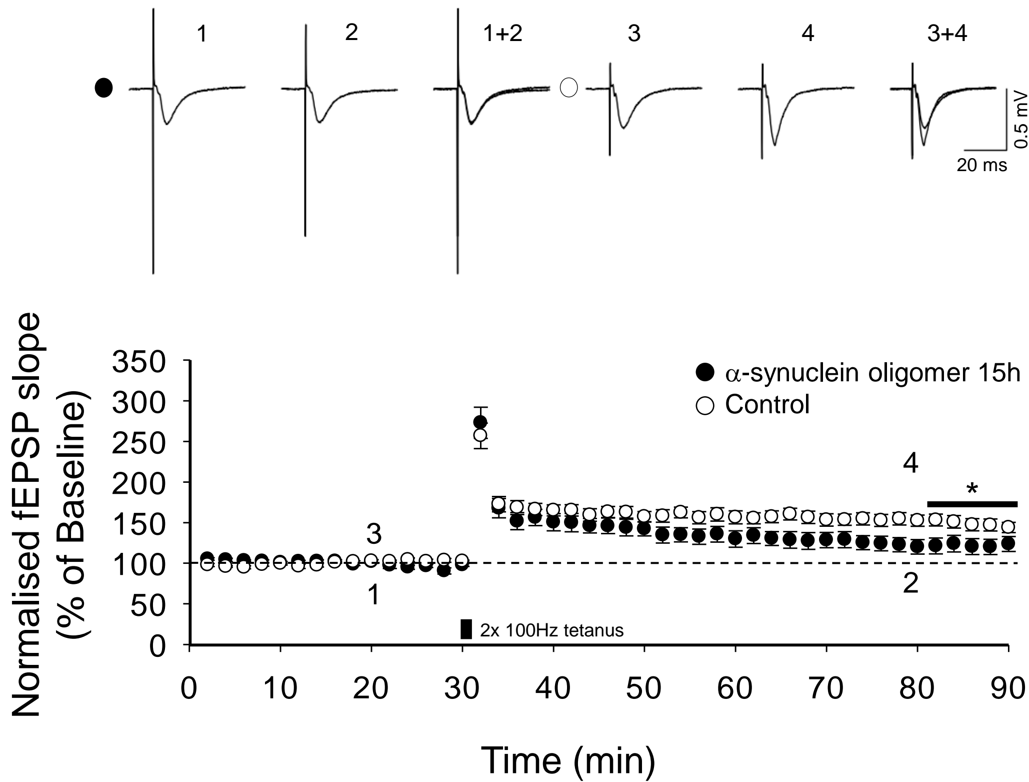

Figure 3.3 Application of α-synuclein oligomerised for 15h inhibits LTP. Slices were incubated with α-synuclein oligomers (1 μM) for 2 - 2.5 hours (n=8) compared with untreated slices as control (n=9, *p*-value = 0.0218) .

3.3.2 No effect of α-synuclein on tetanus-evoked synaptic transmission

Our previous experiments have shown that LTP inhibition by α-synuclein oligomers was dependent on its aggregation status. Strong depolarisation is necessary to activate enough NMDARs to reach a critical level of intracellular Ca^{2+} that ensures LTP stabilisation (Bliss & Collingridge, 1993; Robert C Malenka & Nicoll, 1993). Accordingly, our next aim was to determine whether α-synuclein oligomers affected the induction of LTP by decreasing the level of depolarisation induced by tetanic stimulation. To test this hypothesis, we assessed synaptic efficacy as the cumulative amplitude of fEPSPs evoked by each of the 2 trains of 100 pulses delivered at 100 Hz in slices preincubated with α-synuclein oligomers and in control slices using the data from the experiments described in section 3.3.1.

When cumulative fEPSP amplitude profiles were compared to control, there were no significant differences in slices incubated with α-synuclein oligomerised for 12 hours in response to tetanus 1 (α-syn 12h: 3349 ± 621% of the first fEPSP, n = 3, red; Control: 3856 ± 618%, n = 3, black, $p = 0.594$, **Figure 3.4, left**) or tetanus 2 (α-syn 12h: 1963 ± 227% of the first fEPSP, n = 3, red; Control: 2345 ± 386%, n = 3, black, $p = 0.441$, **Figure 3.4, right**). No significant differences in slices incubated with α-synuclein oligomerised for 13 hours in response to tetanus 1 (α-syn 13h: 2100 ± 114% of the first fEPSP, n = 3, red; Control: 1528 ± 186%, n = 3, black, $p = 0.059$, **Figure 3.5, left**) or tetanus 2 (α-syn 13h: 1453 ± 176% of the first fEPSP, n = 3, red; Control: 1425 ± 136%, n = 3, black, $p = 0.905$, **Figure 3.5, right**) were observed either. Finally, there was no significant difference in the cumulative fEPSP amplitude profile in α-synuclein oligomerised for 15 hours compared to controls in response to tetanus 1 (α-syn 15h: 3619 ± 265% of the first fEPSP, n = 9, red; Control: 3561 ± 352%, n = 9, black, $p = 0.897$, **Figure 3.6, left**) or tetanus 2 (α-syn 15h: 2105 ± 244% of the first fEPSP, n = 8, red; Control: 2440 ± 373%, n = 9, black, $p = 0.464$, **Figure 3.6, right**). This combination of findings suggests that the inhibitory aggregation effect of α-synuclein on hippocampal LTP is not produced by decreased fEPSPs during tetanic stimulation.

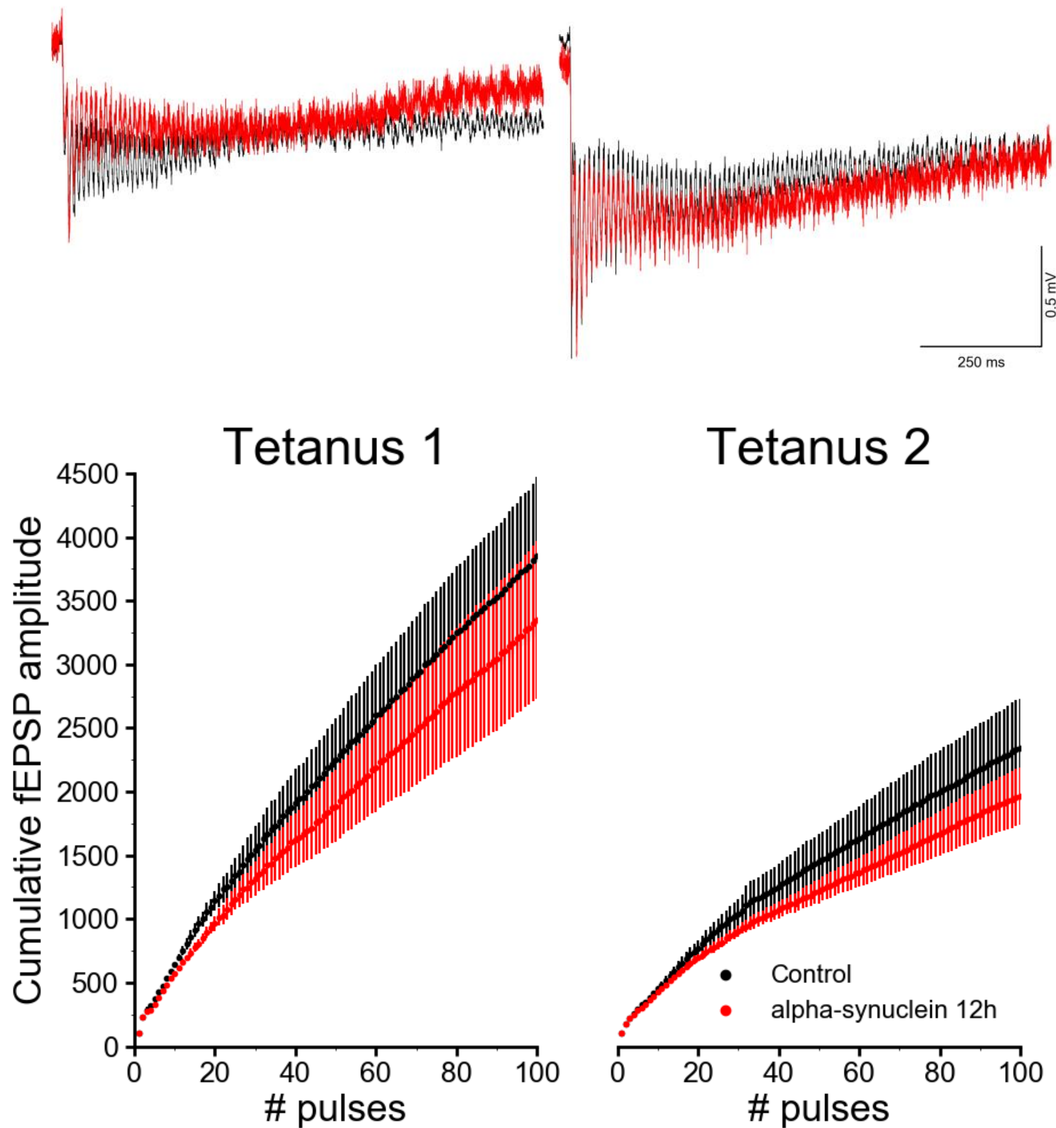

Figure 3.4 α–syn does not affect the cumulative depolarisation evoked by tetanic stimulation. The cumulative pulse amplitude expressed as the fold increase from the first pulse amplitude in each train of high-frequency stimulation (two trains of 100 pulses, 100 Hz) in control slices (black symbols) and in slices incubated for 2-2.5 hours with α–syn oligomerised for 12 hours (red symbols). Data from Fig 3.1; all values are expressed as means ± SEM.

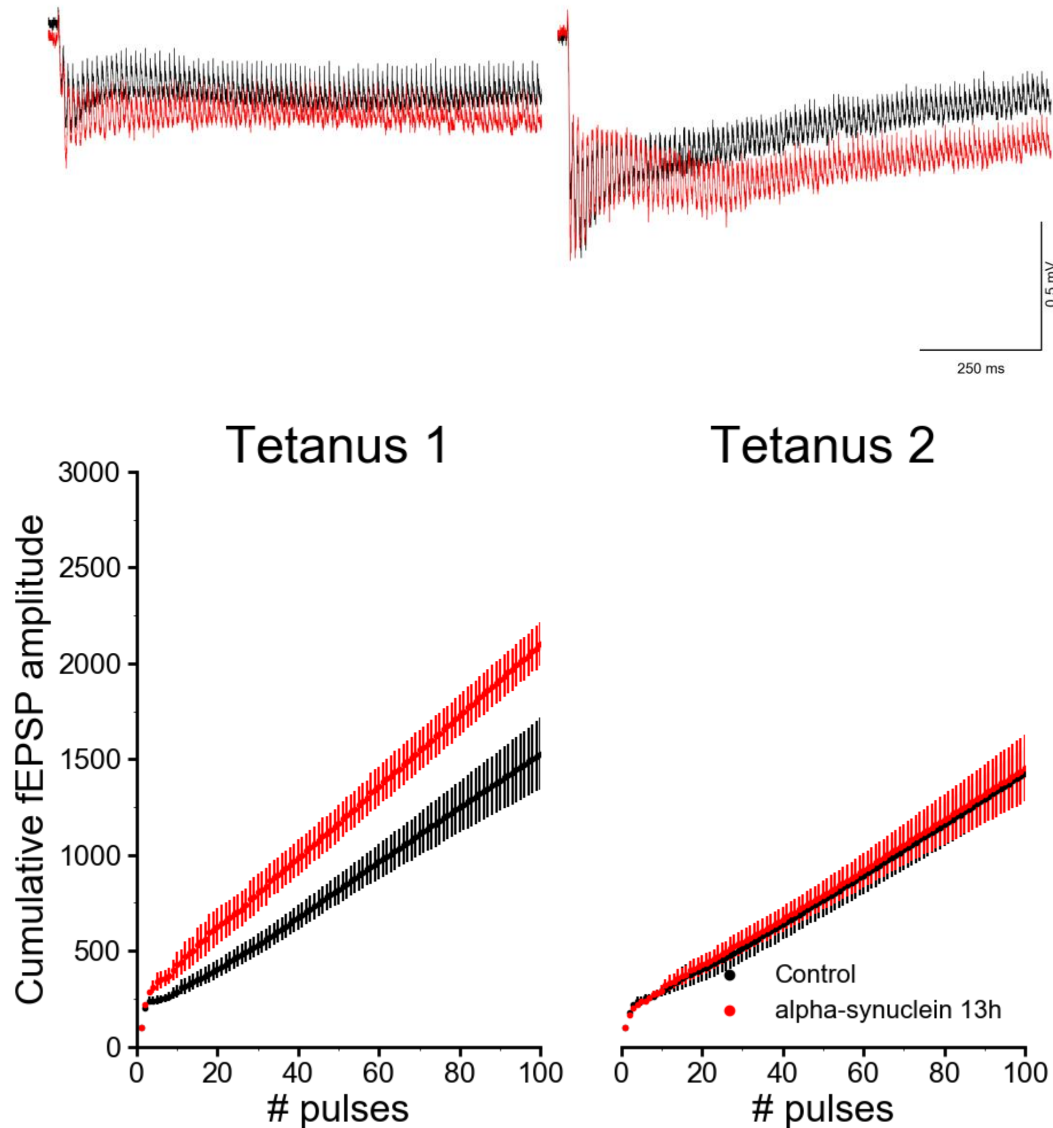

Figure 3.5 Oligomerising α–syn for 13h does not affect the cumulative depolarisation evoked by tetanic stimulation. The cumulative pulse amplitude expressed as the fold increase from the first pulse amplitude in each train of high-frequency stimulation (two trains of 100 pulses, 100 Hz) in control slices (black symbols) and in slices incubated for 2-2.5 hours with α–syn oligomerised for 13 hours (red symbols). Data from Fig 3.2; all values are expressed as means ± SEM.

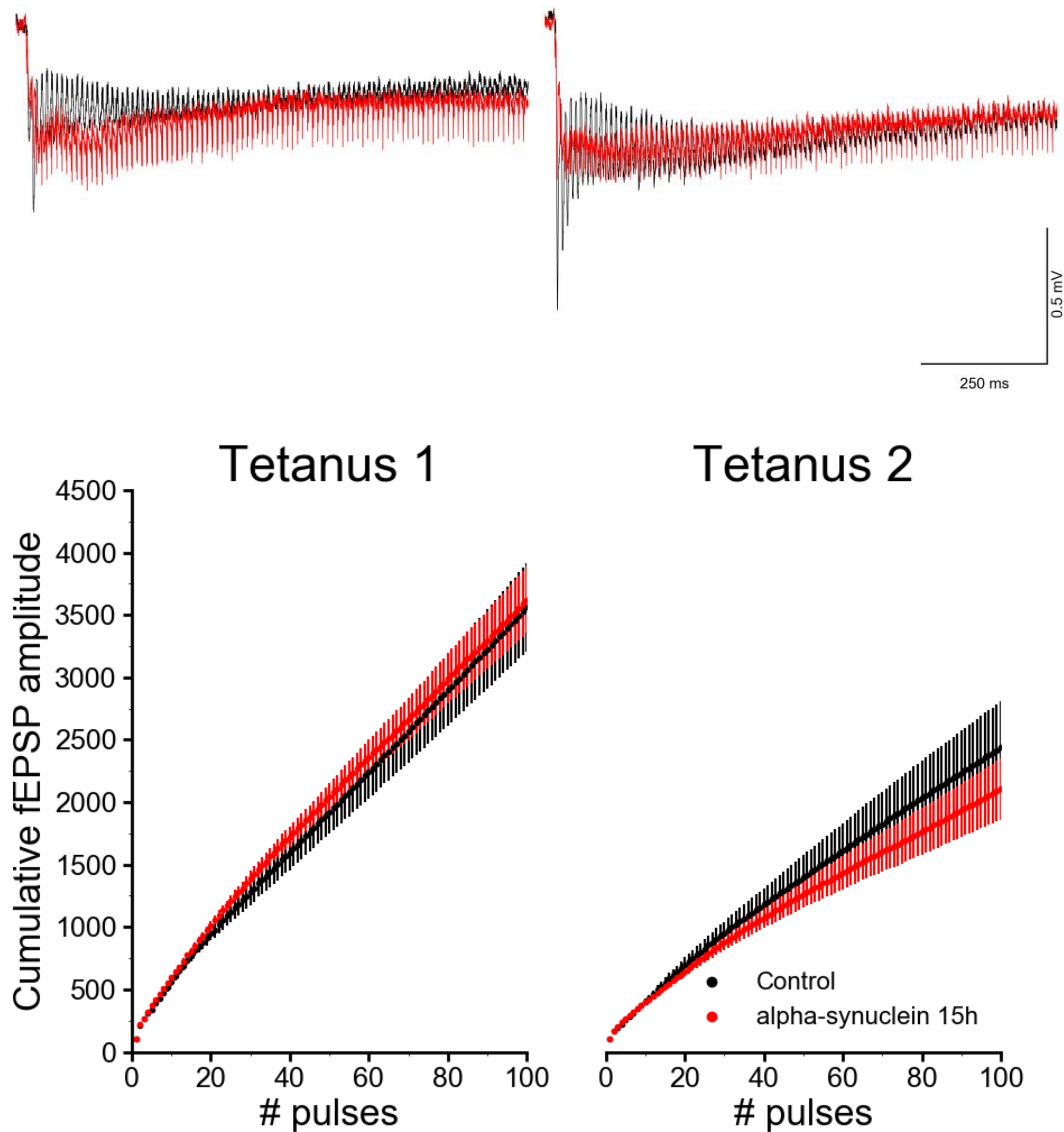

Figure 3.6 α–syn oligomerised for 15h does not affect the cumulative depolarisation evoked by tetanic stimulation. The cumulative pulse amplitude expressed as the fold increase from the first pulse amplitude in each train of high-frequency stimulation (two trains of 100 pulses, 100 Hz) in control slices (black symbols) and in slices incubated for 2-2.5 hours with α–syn oligomerised for 15 hours (red symbols). Data from Fig 3.3; all values are expressed as means ± SEM.

3.3.3 No effect of α-synuclein monomers on hippocampal LTP induction

Several lines of evidence suggest that oligomeric species of proteinaceous aggregates are the drivers of synaptic dysfunction (Hsieh et al., 2006; Shankar et al., 2007, 2008; Townsend et al., 2006; Walsh et al., 2002). However, it was necessary to confirm whether the synthetic α-synuclein in monomeric form that we prepared affected LTP, to control for presence of the protein alone. The samples we used for these experiments were solubilised and diluted in Tris 25 mM and NaCl 100 mM buffer. Afterwards, hippocampal slices were incubated with 1 μM of monomeric α-synuclein for 2-2.5h before measuring extracellular fEPSPs in area CA1, evoked by stimulation of Schaffer-collaterals to assess the effect on synaptic plasticity.

As shown in **Figure 3.7**, LTP was unaffected by application of monomeric α-synuclein when compared with untreated slices (α-syn monomer: 140 ± 8% of baseline, n = 5, closed circle; Control: 166 ± 13%, n = 5, open circles, $p = 0.124$). This data suggests that application of α-synuclein monomers does not affect LTP.

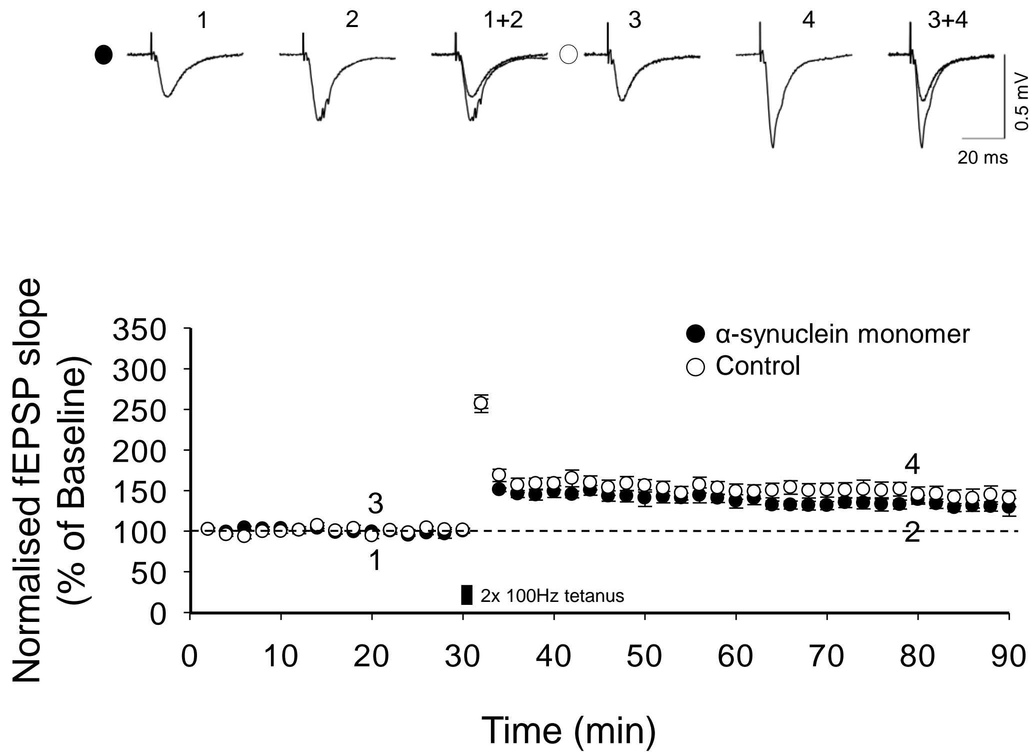

Figure 3.7 α-synuclein monomers do not affect LTP. Slices incubated with α-synuclein monomers at a concentration of 1 μM for 2 hours (n = 5) exhibited LTP similar to control slices (n = 5).

3.3.4 No role for GSK-3β in the a-synuclein mediated inhibition of LTP

Aβ inhibits LTP by a pathway that activates caspase-3, which cleaves Akt1 and leads to activation of glycogen synthase kinase-3β (GSK-3β) (Jo et al., 2011). We hypothesised that this could underpin a shared mechanism of synaptic impairment, and that this pathway would be activated by protein oligomers per se, regardless of their specific molecular composition. To test this, we pre-incubated hippocampal slices with a GSK-3β inhibitor, CT-99021 (1 μM) for 30 minutes before incubating them for 2 - 2.5 hours with α-synuclein oligomers (1 μM) oligomerised for 15 hours. As shown in **Figure 3.8**, CT-99021 did not prevent the inhibition of LTP caused by α-synuclein (α-syn: 122 ± 9% of baseline, n = 9, closed circle; α-syn + CT-99021: 132 ± 12%, n = 6, open circles, p = 0.522). Therefore, this result suggests that α-synuclein may exert its inhibitory effect through a mechanism independent of GSK-3β activation.

To reduce GSK-3β activity slices were exposed to 1 μM CT-99021 30 minutes prior to incubation with α-synuclein. However, we did not assess whether the CT-99021-treated slices exhibited reduced activation of GSK-3β compared to control slices. LTP was blocked in both conditions, in slices incubated with α-synuclein and in slices incubated with CT-99021 and α-synuclein, therefore there was no effect of CT-99021 pre-incubation on LTP inhibition. However, if the degree to which CT-99021 inactivated GSK-3β activity was not sufficient, then it remains unclear whether LTP is not rescued by GSK-3β inhibition because of GSK-3β was still active or because the effect of α-synuclein does not trigger the same pathogenic pathway as Aβ.

GSK-3β is inactivated by phosphorylation at the serine 9 residue (Stambolic & Woodgett, 1994). Therefore, to confirm our results further experiments should assess GSK-3β activity by determining the protein levels of phosphorylated and unphosphorylated GSK-3β via Western Blot comparing slices incubated with CT-99021 (1 μM) and control slices.

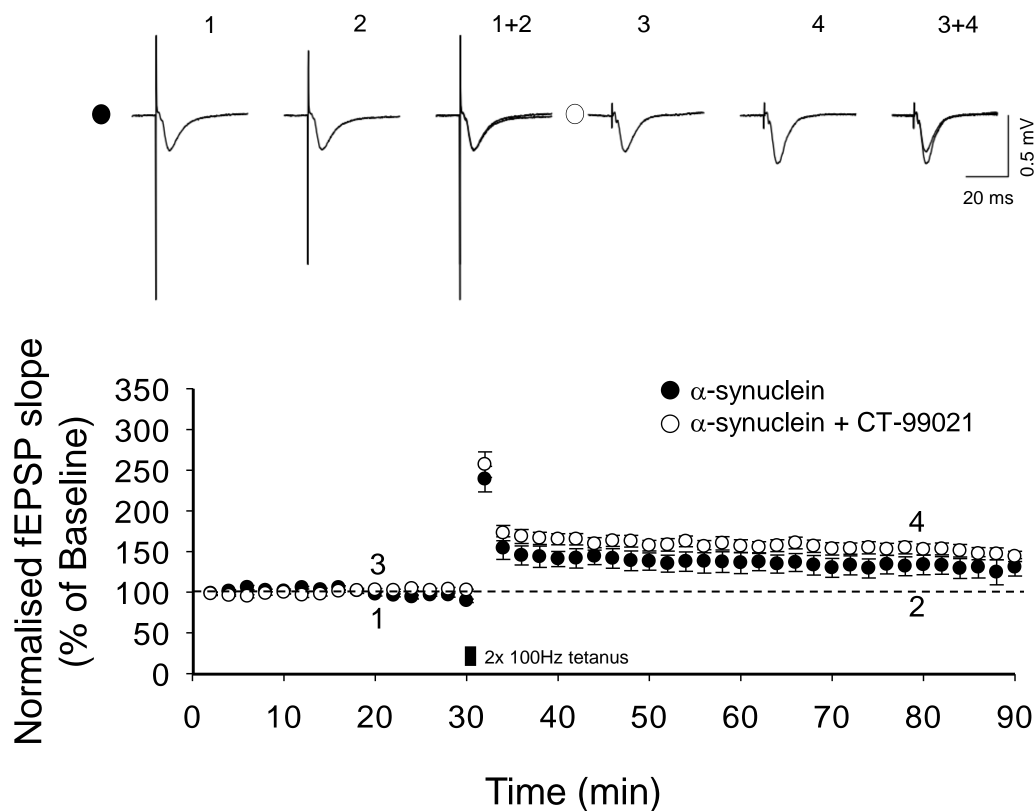

Figure 3.8 LTP inhibition mediated by α-synuclein oligomers is not prevented by CT-99021. Slices were incubated with a GSK-3β inhibitor, CT-99021, for 30 minutes prior to α–synuclein oligomers (15h) incubation, (n = 6) compared to slices incubated with α–synuclein oligomerised for 15h (n = 9) , $p = 0.552$.

3.3.5 Subthreshold concentrations of protein aggregates are additive to inhibit LTP

It is widely accepted that Aβ soluble oligomers block LTP (Jo et al., 2011; Klyubin et al., 2008; Shankar et al., 2008; Townsend et al., 2006; Walsh et al., 2002). Interestingly, in our previous experiments detailed above, we found that α-synuclein oligomers also significantly reduce LTP. Some evidence suggests that Aβ and α-synuclein can act synergistically (Bate et al., 2010; Clinton et al., 2010; Lashley et al., 2008; Masliah et al., 2001). We therefore wanted to test our hypothesis that reaching a threshold of aggregated protein *per se* was the key determinant for synaptic impairment. It has been suggested that synthetic Aβ reliably inhibits LTP at a minimum concentration of 500 nM (H.-W. Wang et al., 2002; Q. Wang et al., 2004). Acute hippocampal slices were therefore pre-incubated for 2 hours with Aβ and α-synuclein oligomers at lower concentrations (250 nM and 50 nM, respectively) than those needed to significantly reduce LTP independently.

LTP was not affected in co-incubated slices when compared with that observed in untreated (control) slices (Aβ (250 nM) α-syn (50 nM): 139 ± 26% of baseline, n = 3, closed circle; Control: 141 ± 8%, n = 4, open circles, $p = 0.948$, **Figure 3.9**). However, increasing the concentration of α-synuclein up to 250 nM produced LTP impairment in slices pre-incubated with Aβ (250 nM) and α-synuclein (250 nM) for 2 hours (Aβ (250 nM) α-syn (250 nM): 117 ± 9%, n = 6, closed circle; Control: 165 ± 9% of baseline, n = 7, open circles, $p = 0.004$, **Figure 3.10**). Taken together, these results suggest that when a threshold is reached for the presence of oligomerised protein, α-synuclein and Aβ oligomers may act in a cooperative fashion to augment toxicity.

This work suffers from a number of limitations, as the level of aggregation and biological activity of Aβ and α-synuclein were not tested. As previously mentioned in section 3.3.1, confirming that the Aβ and α-synuclein were aggregated could be confirmed in future experiments by western blots (Shankar et al., 2008; Walsh et al., 2002); while the biological activity of the protein aggregates could be tested by quantifying the integrity of the membrane using an LDH assay.

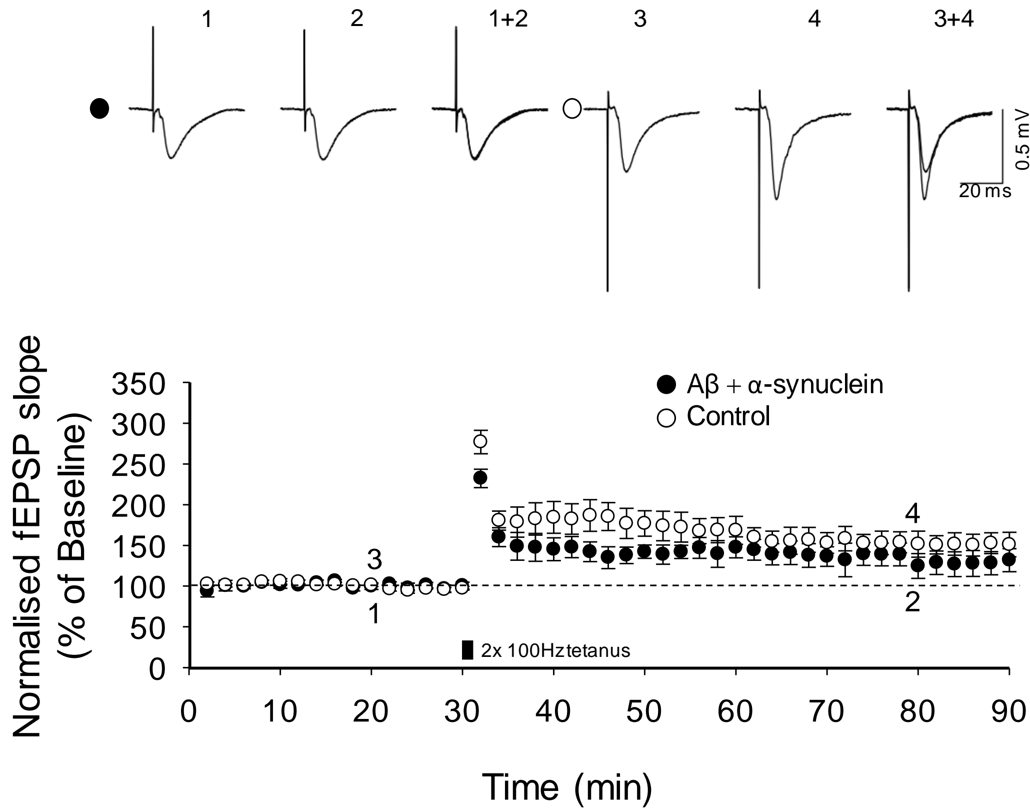

Figure 3.9 Aβ oligomers (250 nM) and α-synuclein oligomers (50 nM) combined not affect LPT. Slices were incubated with 250 nM Aβ oligomers + 50 nM α-synuclein oligomers (n = 3) control (n = 4), $p = 0.948$.

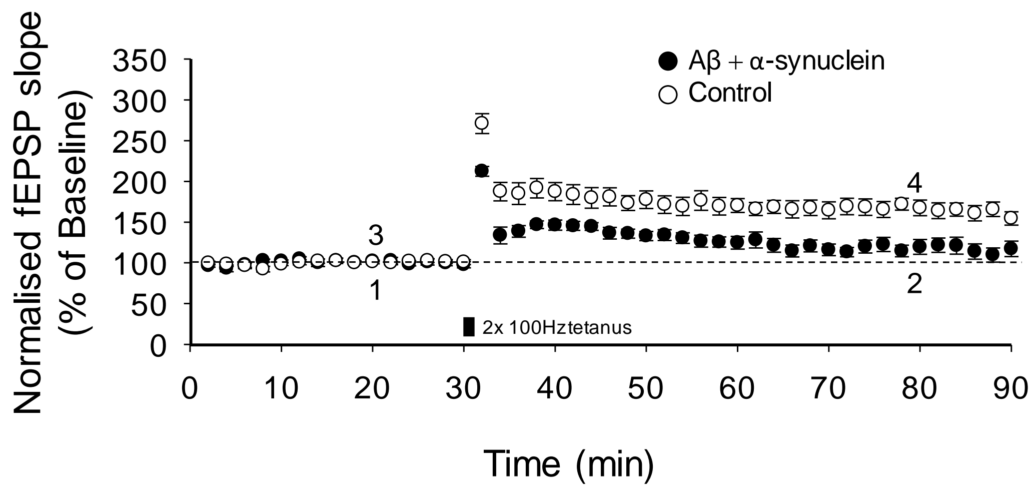

Figure 3.10 Aβ oligomers (250 nM) combined with α-synuclein oligomers (250 nM) impaired LTP. Slices were incubated with 250 nM Aβ (oligomer) + 250 nM α-synuclein (oligomer) (n = 6) control (n = 7) $p = 0.004$.

3.4 Discussion

3.4.1 Aggregation status and synaptotoxicity

Increasing evidence supports the notion that there is overlap between pathological characteristics of different neurodegenerative diseases (Baker & Götz, 2015). Various studies suggest that the accumulation of protein aggregates is damaging to synaptic plasticity. Therefore, our aim was to test whether the presence of protein aggregates, other than the typical Aβ oligomers, affected LTP. To test this hypothesis, we incubated slices with α-synuclein oligomerised for different periods of time then tested whether this affected long-term synaptic plasticity. Our results show that only the samples which had been left to oligomerise for 15 hours impaired LTP. Consequently, our findings suggest that there might be a concentration needed to be surpassed of α-synuclein to produce LTP impairment. However, one limitation with this explanation is that we did not assess the sizes of α-synuclein oligomer assemblies contained in our samples. In future investigations, it will be necessary to identify the specific fragments that inhibit LTP and which components of the LTP signalling cascade are involved in this process.

In this study, incubation of slices with low concentrations of Aβ (250 nM) + α - synuclein (50 nM) did not impair LTP. However, increasing the concentration to Aβ (250 nM) + α -synuclein (250 nM) impaired LTP. These concentrations of both α-synuclein or Aβ do not inhibit LTP on their own, but they impair LTP when applied together as long as the concentration of α -synuclein was increased to at least 250 nM. Our results are consistent with previous studies in which concentrations of α-synuclein oligomers ranging from 10 to 50 nM do not produce significant changes in LTP, whereas incubation of slices with α-synuclein oligomers at a concentration of 500 nM resulted in LTP impairment (Diógenes et al., 2012). In another study, a concentration-dependent effect was also shown for different species of Aβ oligomers as increasing the dose from 1 μM Aβ40 + 50 nM Aβ42 to 10 μM Aβ40 + 500 nM Aβ42 lead to a significant difference on depression of synaptic transmission in acute hippocampal slices (Kamenetz et al., 2003).

As previously mentioned, Aβ inhibits LTP through the caspase-3-GSK-3β cascade and α-synuclein promotes GSK-3β activation. Although LTP impairment mediated by α-synuclein oligomers was not prevented by CT-99021, one possible explanation for the mechanism for LTP inhibition in our experiments involving both Aβ (250 nM) and α-synuclein (250 nM) might be that activation of GSK-3β by α-synuclein enhances the effect of the caspase-3-GSK-3β cascade triggered by a lower concentration of Aβ. Alternatively, various studies have shown that Aβ and α-synuclein adversely affect glutamatergic synaptic transmission (Diógenes et al., 2012; Kamenetz et al., 2003; Shankar et al., 2007), a natural progression of this work is to analyse the effects of these oligomers on postsynaptic receptors that mediate LTP.

It was observed by the Klenerman research group that the aggregation protocol produced small fibrils after 24 hours, therefore we chose three different incubation periods that were shorter to avoid the production of fibrils. However, one limitation of these experiments is that we did not quantify the specific amount of protein aggregation for the different incubation periods used. In future work, it may be useful to measure the level of aggregation at 12h, 13h and 15h using gel electrophoresis for separating the aggregates by molecular weights and Western blot for detecting α-synuclein with an anti-α-synuclein primary antibody (Newman et al., 2013). We expect that the resulting banding pattern would show higher molecular bands for samples incubated for longer periods. However, the absence of a band would suggest that α-synuclein is highly aggregated so the protein would not be able to run through the gel. Then, the time course of aggregation could be monitored using a real-time Thioflavin T (ThT) fluorescence aggregation assay as the ThT dye gives a fluorescence signal upon binding to protein aggregates (Sulatskaya et al., 2017).

To confirm whether the α-synuclein samples we used were biologically active, we could use methods that assess the effects of aggregates on the integrity of the cell membrane by quantifying the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the bathing medium. Cultures of primary hippocampal

neurons would be incubated with media-alone, α-synuclein oligomerised for 12h, 13h, and 15h before LDH quantification, as previously demonstrated by (Wogulis et al., 2005). We would expect to observe greater LDH concentrations with higher levels of aggregation suggesting further damage in cultured neurons incubated with α-synuclein oligomerised for longer periods of time.

Finally, another limitation is that the low number of repetitions of experiments using α-synuclein oligomerised for 12h and 13h provided us with insufficient information to infer that the amount of oligomers present in these samples did not reach the concentration necessary to block LTP. Therefore, further work will be necessary to confirm that the effect of α-synuclein on LTP is caused by a time-dependent effect on the formation of toxic aggregates.

3.4.2 Plasticity dysfunction in the absence of transmission dysfunction

Our experiments show that sufficiently oligomerised α-synuclein inhibits LTP, therefore, we tested whether this inhibition was caused by an effect of α-synuclein oligomers inducing suboptimal depolarisation following HFS. The cumulative fEPSP amplitude following each train of 100 pulses at 100 Hz was not significantly different in the treated than control slices, thus it is unlikely that the α-synuclein oligomers-mediated inhibition of LTP was due to a reduced response to HFS. This is consistent with other results which indicate that Aβ does not have an effect on HFS induced depolarisation (Townsend et al., 2006). Therefore, the next step will be to identify which components of the LTP signalling cascade are involved in the inhibition of LTP mediated by α-synuclein.

3.4.3 Distinct signalling pathways mediate protein aggregate synaptotoxicity

Previously, it was shown that Aβ oligomers result in caspase-3-mediated cleavage of Akt, which prevents Akt from supressing the activity of GSK-3β, resulting in LTP impairment (Jo et al., 2011). In the same study, LTP was rescued by pre-incubating slices with the GSK-3β inhibitor CT-99021. Therefore, to test whether α-synuclein operates to inhibit LTP through the canonical caspase-GSK3β

pathway, we pre-incubated hippocampal slices with CT-99021 before incubating with α-synuclein oligomerised for 15 hours. Our data suggest that α-synuclein does not exert its inhibitory effect through GSK-3β activation. This is an interesting finding as it has been suggested that α-synuclein activates GSK-3β through phosphorylation at Tyr-216 following a parkinsonism-inducing neurotoxin MPP/MPTP, and that phosphorylated GSK-3 β is increased in samples from PD patients (Duka et al., 2009), and that α-synuclein stimulates autophosphorylation of GSK-3β (Kawakami et al., 2011). However, our results suggest that α-synuclein oligomers do not inhibit LTP via the caspase-3-GSK-3β pathway, as opposed to Aβ oligomers.

Another significant aspect of protein aggregate pathogenesis is their effect on neuronal structure. As indicated previously, exposure to small Aβ oligomers resulted in altered spine morphology and reduced spine density in hippocampal neurons, and these effects were caused by oligomers, as exposure to monomers did not result in significant differences compared to controls (Lacor et al., 2007; Shankar et al., 2007). Furthermore, Aβ oligomers-mediated effects on spine density were prevented by the expression of a constitutively active isoform of cofilin, a filamentous actin severing protein (Shankar et al., 2007). As structural dendritic spine changes depend on actin dynamics, and they are tightly related to synaptic plasticity (Kasai et al., 2010), these findings indicate that dendritic spine pathology could underpin the loss of LTP.

3.5 Conclusion

These data tentatively indicate that the extent of protein aggregates *per se* – rather than the actual identity of the aggregated protein – might confer the impairing effects. Wider evidence suggests that pathological protein aggregates could fundamentally affect neuronal structure and the regulation of proteins that govern neuronal structure. Whether and how that links with the effects of aggregated proteins and plasticity, is unknown.

Critically, how the regulation of neuronal structure itself contributes to the normal physiological expression of plasticity is still not fully understood. Therefore, in order to determine whether structural dysregulation links aberrant proteins and plasticity loss, we must first understand how the regulation of structure underlies plasticity expression.

CHAPTER 4

The role of Group I PAKs in hippocampal synaptic function

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Chapter 4 - The role of Group I PAKs in hippocampal synaptic function

4.1 Introduction

4.1.1 The regulation of neuronal structure

Synapses are critical for signal transmission, and the majority of excitatory synapses form on dendritic spines which are small membrane protrusions on dendrites (Fiala et al., 2002). Although the shape and size of dendritic spines is variable, three main types of dendritic spines have been described: mushroom, thin, and stubby spines. Mushroom spines have narrow necks and large spherical heads, thin spines have a constricted neck and small heads, while stubby spines have similar neck length and width (Tada & Sheng, 2006).

The major cytoskeletal component of dendritic spines is branched filamentous actin (F-actin) (Korobova & Svitkina, 2010). The arrangement of F-actin in dendritic spines is constantly subject to activity-dependent reorganisation. Changes in synaptic structure such as growth and shrinkage, synapse formation and pruning are regulated by signalling pathways acting on the actin polymerisation and depolymerisation. The main regulators of actin dynamics are the family of Ras homologous guanosine triphosphatases (Rho GTPases). These small GTPases are monomeric G-proteins which alternate between their active state (GTP-bound) and their inactive state (GDP-bound). Once active, they act as molecular switches activating subsequent elements of various signalling cascades.

These changes are triggered during development, but also after learning and experience. Extracellular signals activate receptors which in turn activate key regulators of the actin cytoskeleton. It was shown that strong synaptic inputs activate three Rho GTPases: Rho (Ras homolog family member A), Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (Cell division cycle 42) (Murakoshi et al., 2011). These GTPases have many effectors which participate in signalling pathways involved in cytoskeletal dynamics. The downstream

effectors of Rho GTPases include several protein kinases and actin binding proteins (ABPs).

ABPs have distinct roles in actin assembly, disassembly and stabilisation (Borovac et al., 2018). For instance, the Arp2/3 protein complex mediates actin polymerisation and branching (Mullins et al., 1998); cofilin induces actin filament depolymerisation, but it is inhibited by phosphorylation (Theriot, 1997); α-actinin facilitates the formation of cross-links between actin filaments and is involved in spine maturation (Hodges et al., 2014); drebin mediates the clustering of actin fibres by regulating the activity of other ABPs and is involved in spine morphogenesis (Takahashi et al., 2003). Thus, various ABPs with different roles regulate the organisation of the actin cytoskeleton.

Another class of Rho GTPase effectors are a family of proteins called p21-activated kinases (PAKs), serine/threonine kinases which are activated by binding to Rac1 or Cdc42 (Manser et al., 1994). PAKs phosphorylate and thereby activate LIM-domain-containing kinase (LIMK), which in turn phosphorylates cofilin, preventing it from severing actin filaments (Edwards et al., 1999). This pathway is important for cytoskeleton regulation as inactivating cofilin facilitates actin polymerisation. Furthermore, PAKs have been reported to mediate dendritic spine formation as expression of wild type PAK1 resulted in an increase in the number of dendritic spines, whereas inhibiting PAK1 activity produced the opposite effect (Zhang et al., 2005). These results suggest that PAKs might mediate the formation of spines by regulating cytoskeleton dynamics.

Mounting evidence suggests that synaptic plasticity is accompanied by dynamic actin cytoskeleton changes, however, the precise links between structure-regulators and synaptic plasticity remain poorly characterised.

4.1.2 Molecular mechanisms of LTP

Glutamate receptors are crucial for the expression of LTP. First, there should be a sufficient amount of glutamate to bind and activate AMPARs. This in turn depolarises the postsynaptic neuron which repels the NMDARs Mg^{2+} ions block the ion channel pore of these receptors. Then Na^{+}, K^{+} and Ca^{2+} flow into the cell. Calcium is an important part of many signalling cascades; after NMDARs are activated there is a fast influx of calcium in the postsynaptic neuron. Calcium ions bind to the messenger protein Calmodulin or CaM (calcium-modulated protein), which senses intracellular calcium concentration and then transduces signals to various downstream proteins. One of the most important of those proteins for synaptic plasticity is calcium-calmodulin-dependent kinases (CaMK). These kinases are serine/threonine kinases; as their name implies, their activation depends on binding of Ca^{2+}-calmodulin, but CaMKs can remain activated after they have been activated and autophosphorylated. These kinases are crucial to LTP expression because once they have been activated, CaMKIIs phosphorylate GluA1 subunits of AMPARs which increases their conductance and increases their trafficking to the postsynaptic membrane where they can be activated by glutamate release, leading to increased postsynaptic potentials (Lisman et al., 2012).

Receptor changes at the synapse are ultimately underpinned by changes to the physical structure of the synapse and the neuron. It has been shown that a positive correlation between spine volume and the amount of AMPARs (Matsuzaki et al., 2001). Indeed, such physical changes in size and shape are governed by a distinct set of mechanisms that respond to the need for structural changes during synaptic plasticity.

4.1.3 Structural changes in synaptic plasticity

Synaptic plasticity has been regarded as the cellular basis for learning and memory, however, mounting evidence suggests that structural changes to

synaptic networks may also have functional implications for learning and memory (Caroni et al., 2012). Recent studies have reported a strong relationship between synaptic plasticity and morphology changes of dendritic spines (Bosch & Hayashi, 2012; Kasai et al., 2010). Glutamate release triggered by glutamate uncaging or HFS induces a rapid input-specific enlargement of dendritic spines (Matsuzaki et al., 2004; Okamoto et al., 2004). Furthermore, the enlargement in dendritic spine, referred to as structural LTP (sLTP), shares similarities with the induction of LTP. For instance, dendritic spine enlargement is also dependent on the activation of NMDARs as it is prevented by the NMDAR antagonist AP5, it is associated with increased AMPAR current increase, and it is long-lasting (Matsuzaki et al., 2004). There is also experimental evidence suggesting that both LTP and dendritic spine enlargement share components of signalling cascades, as blocking CaM, CaMKII, and actin filament polymerisation blocks LTP induction and spine enlargement (Fukazawa et al., 2003; Krucker et al., 2000; R C Malenka et al., 1989; R Malinow et al., 1989; Matsuzaki et al., 2004).

The aforementioned studies and additional experiments suggest that there is substantial overlap between the signalling pathways implicated in LTP and sLTP. One potential link between these two aspects of synaptic plasticity are PAKs. In terms of LTP, it has been shown that LTP induction using TBS results in increased phosphorylation of PAK (L. Y. Chen et al., 2007). Furthermore, this phosphorylation of PAKs might be necessary for LTP induction, as knocking out or supressing expression of Group I PAKs results in LTP impairment (Asrar et al., 2009; Boda et al., 2004; Meng et al., 2005). However, other experiments observed that suppressing PAK1 activity leads to increased potentiation compared to controls in the forebrain, but has no effect on hippocampal LTP (M. L. Hayashi et al., 2004). Thus, the precise reasons for why and how PAKs are important for LTP induction need to be investigated further.

4.2 Aims and Hypotheses

Characterise the role of Group I PAKs in synaptic plasticity under non-pathological conditions. Synaptic dysfunction is one of the first steps in the progression of neurodegenerative diseases (Hardy & Selkoe, 2002), and many studies have found abnormalities in dendritic spines associated with cognitive disorders, such as mental retardation, schizophrenia, in addition to Alzheimer’s disease (Holtmaat & Svoboda, 2009). Considering that synaptic plasticity is closely related to structural plasticity and cytoskeletal changes, the aim of this study is to characterise the role of group I PAKs in LTP, by pharmacological inhibition.

4.3 Results

4.3.1 Group I PAKs are required for hippocampal LTP

Various studies have reported altered late-phase LTP in the hippocampus and deficits in learning and memory in animals or brain slices deficient in PAK1 and PAK3 expression (Asrar et al., 2009; Boda et al., 2004; M. L. Hayashi et al., 2004; Meng et al., 2005). Since our aim is to identify the role of PAKs in synaptic plasticity, we used the Group I PAKs pharmacological inhibitor IPA-3 to examine whether pharmacological PAK inhibition would also produce deficits in LTP. Therefore, we examined LTP in hippocampal slices following incubation in aCSF with IPA-3 at 25 µM for an hour prior to recording and perfused during recording. Whilst LTP could be readily induced in control slices (control, 150 ± 9% of baseline, n = 8, **Figure 4.1A**), LTP was significantly reduced in incubated and perfused slices compared to controls assessed at 55 minutes after HFS (IPA-3: 125 ± 8% of baseline, n = 7; control, 161 ± 7%, n = 8, $p = 0.004$, **Figure 4.1B**) and at 115 minutes after HFS (IPA-3: 115 ± 8% of baseline, n = 7, closed circle; control, 150 ± 9%, n = 8, open circles, $p = 0.012$, **Figure 4.1B**). Thus, these results suggest that Group I PAKs are required for LTP.

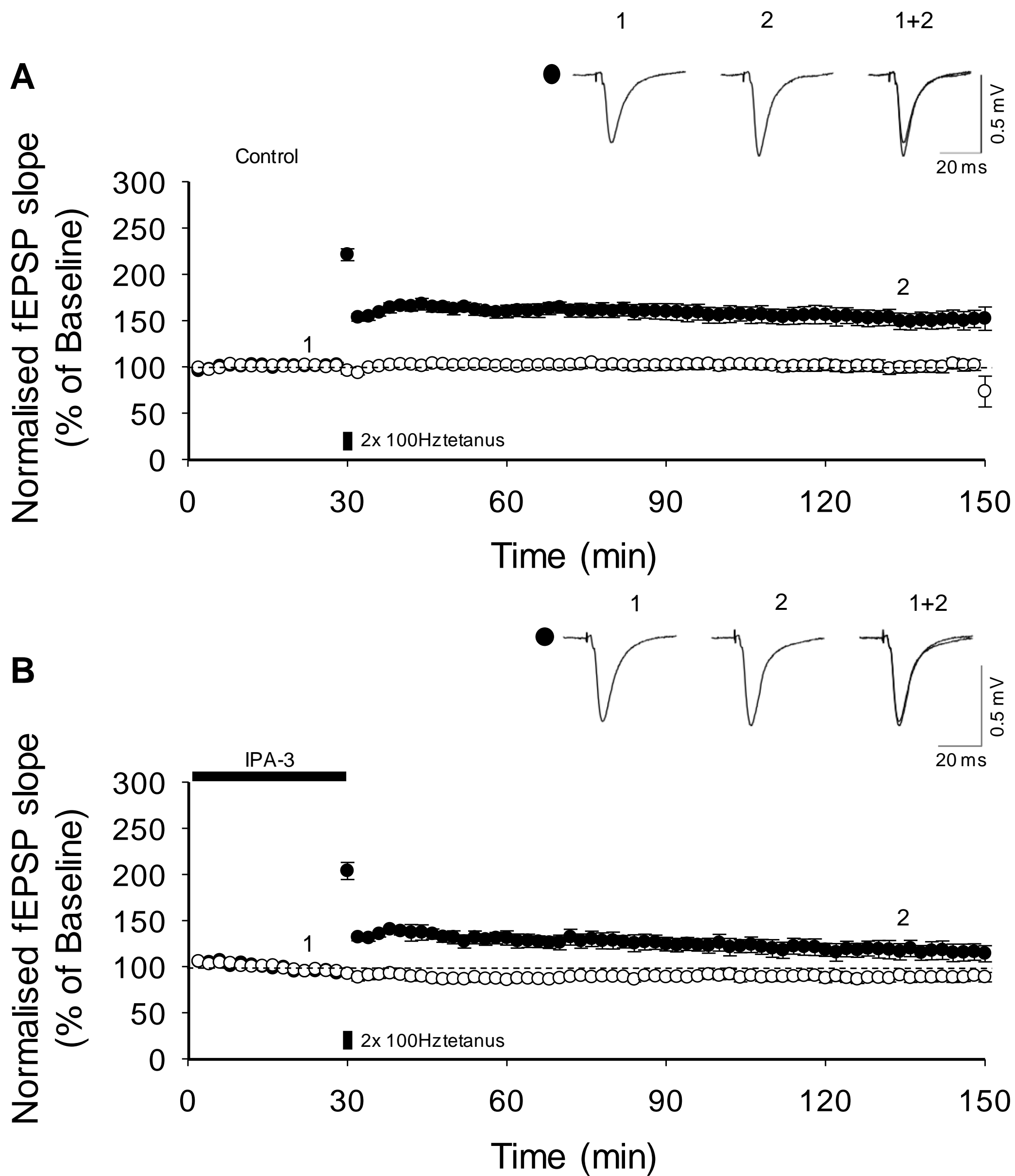

Figure 4.1 LTP is impaired in IPA-3 treated hippocampal slices. A. LTP can be induced in control slices (n = 8). **B.** Incubation of slices with IPA-3 (a group I PAK inhibitor, at 25 μM) for 1 hour before recording and perfusion of the drug in aCSF during baseline (n = 7. Tme after HFS: 55m minutes p = 0.004, 115 minutes *p* = 0.012). All symbols represent the mean ± SEM. Inset represents traces of fEPSPs recorded and averaged at the times specified by the numbers on the graphs.

4.3.2 Group I PAK inhibitor does not affect basic synaptic function

We were next interested in understanding why inhibition of Group I PAKs impaired LTP. One possible explanation is that treatment of slices with the PAK inhibitor IPA-3 impairs fundamental synaptic function, which could prevent normal physiological signalling and therefore LTP expression. Paired pulse facilitation is a form of short-term plasticity which depends on presynaptic function and it is measured by the ratio of the slope of the second stimulus by the slope of the first (Byrne & Roberts, 2009). Changes to this form of plasticity as well as basal synaptic strength are likely to alter LTP (Roberto Malinow & Malenka, 2002). Therefore, we examined whether pharmacological inhibition of group I PAKs affected pre-synaptic function by comparing paired-pulse ratios (PPR). We found that there was no significant difference in PPRs between slices incubated in IPA-3 for 1 hour and perfused slices versus controls (IPA-3: PPF at 50 ms intervals: 1.76 ± 0.08, n = 7; controls: PPF at 50 ms intervals: 1.76 ± 0.06, n = 8; two-way ANOVA p = 0.198, **Figure 4.2A**). These results indicate that presynaptic neurotransmitter release was not affected by group I PAK inhibition, and therefore impairment to LTP induction is unlikely to be explained by changes to presynaptic function.

Basal synaptic strength is an important property of synaptic physiology, therefore, we sought to assess whether inhibiting group I PAKs with IPA-3 affected synaptic transmission. For this purpose, we measured basal synaptic strength by recording fEPSPs and presynaptic volley evoked by various stimulation intensities. As the presynaptic volley represents the presynaptic action potentials occurring near the recording electrode, its magnitude conveys information about the number of axons firing action potentials, which allows us to compare fEPSP slope between different slices (Byrne & Roberts, 2009). Therefore, if the presynaptic volley/fEPSP relationship increases, it would mean that synaptic transmission is increased. However, as shown in **Figures 4.2B** and **4.2C** we found no differences between controls and slices incubated with IPA-3 for an hour and perfused with the drug during recording; the maximal fEPSP slope was 0.49 ± 0.05 mV/ms (n = 8) in

control slices and 0.55 ± 0.05 mV/ms (n = 7) in IPA-3 incubated and perfused slices (two-way ANOVA $p = 0.726$). Thus, these results suggest that IPA-3 does not alter basal synaptic strength.

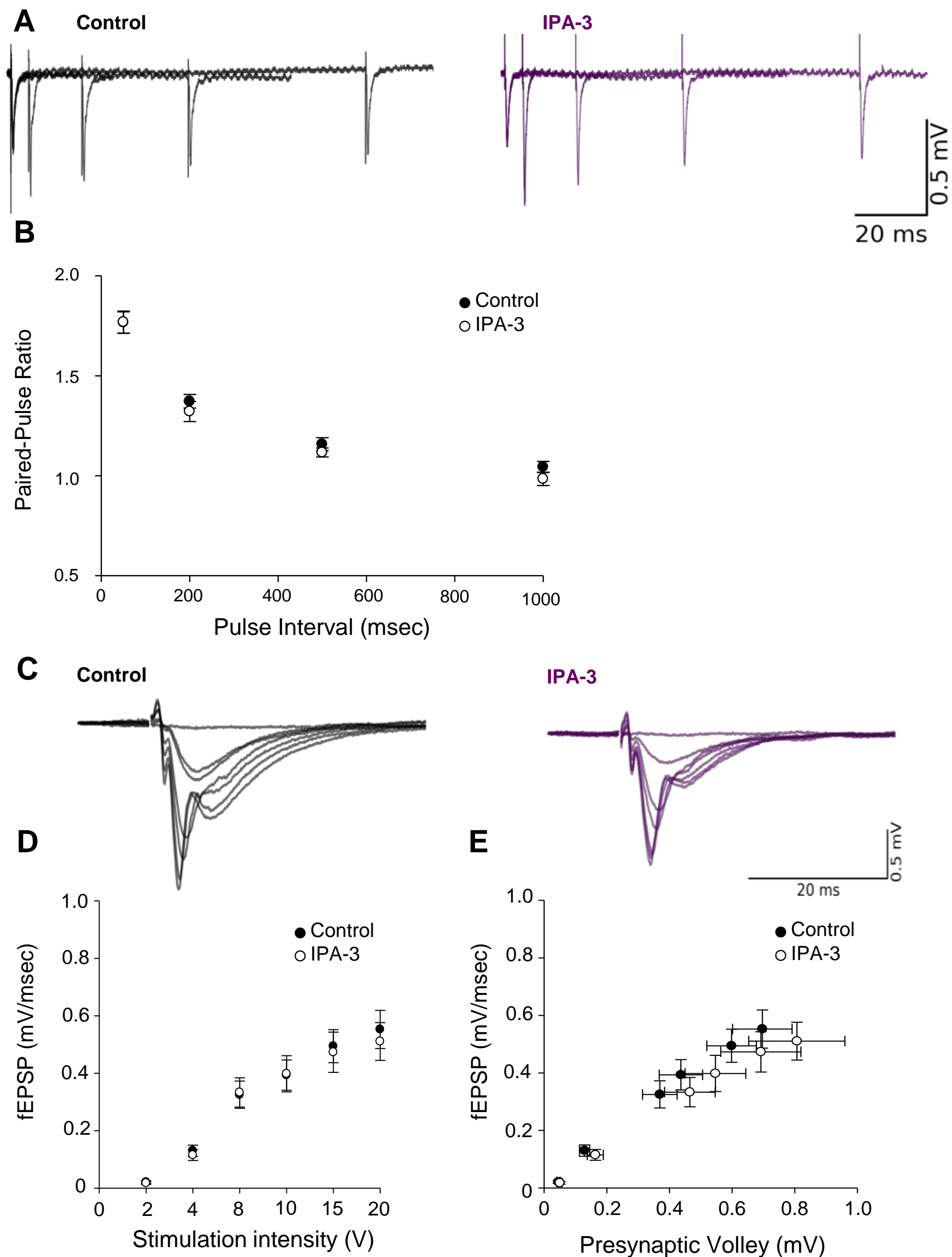

Figure 4.2 No observed effect of IPA-3 incubation and perfusion on short term plasticity or synaptic efficacy. Slices were incubated with IPA-3 (25 μM) for 1 hour before and perfused during recording (IPA-3, n = 7; control, n = 8). **A.** Representative fEPSPs traces evoked by paired-pulse-stimulation. **B.** Paired-pulse ratios were not significantly different in incubated slices compared to controls (two-way ANOVA p = 0.198). **C.** Examples of fEPSPs of CA1 synapses at increasing stimulation strengths. **D.** No significant difference was found in fEPSP slopes at various stimulation intensities **E.** Input-output curves were not significantly different in incubated slices compared to controls (Control Slope: 0.49 ± 0.05 mV/msec; IPA-3 Slope: 0.55 ± 0.05 mV/msec; two-way ANOVA p = 0.726).

4.3.3 Group I PAK inhibition does not affect cumulative depolarisation evoked by tetanic stimulation

As indicated in the previous chapter (section 3.3.2), LTP induction stimulation patterns are required to produce a sufficiently strong depolarisation to produce stable long-term potentiation. Hence, a possible explanation for Group I PAK inhibition effect on LTP may be that it lessened the amplitude of fEPSPs evoked by HFS, thereby impairing LTP. To test this hypothesis, we calculated the cumulative amplitude of responses elicited by HFS in control slices and in slices incubated and perfused with IPA-3 analysing data from the results in section 4.3.1.

Overall, Group I PAK inhibition did not produce significant differences in cumulative fEPSP amplitude profiles when compared to untreated slices in response to tetanus 1 (IPA-3: 3202 ± 149% of the first fEPSP, n = 7, purple; Control: 3082 ± 196%, n = 8, black, $p = 0.641$, **Figure 4.3, left**) or tetanus 2 (IPA-3: 2166 ± 131% of the first fEPSP, n = 7, purple; Control: 2244 ± 150%, n = 8, black, $p = 0.704$, **Figure 4.3, right**). It can therefore be assumed that the inhibition of LTP resulting from the application of a Group I PAK inhibitor was not caused by decreased depolarisation following HFS.

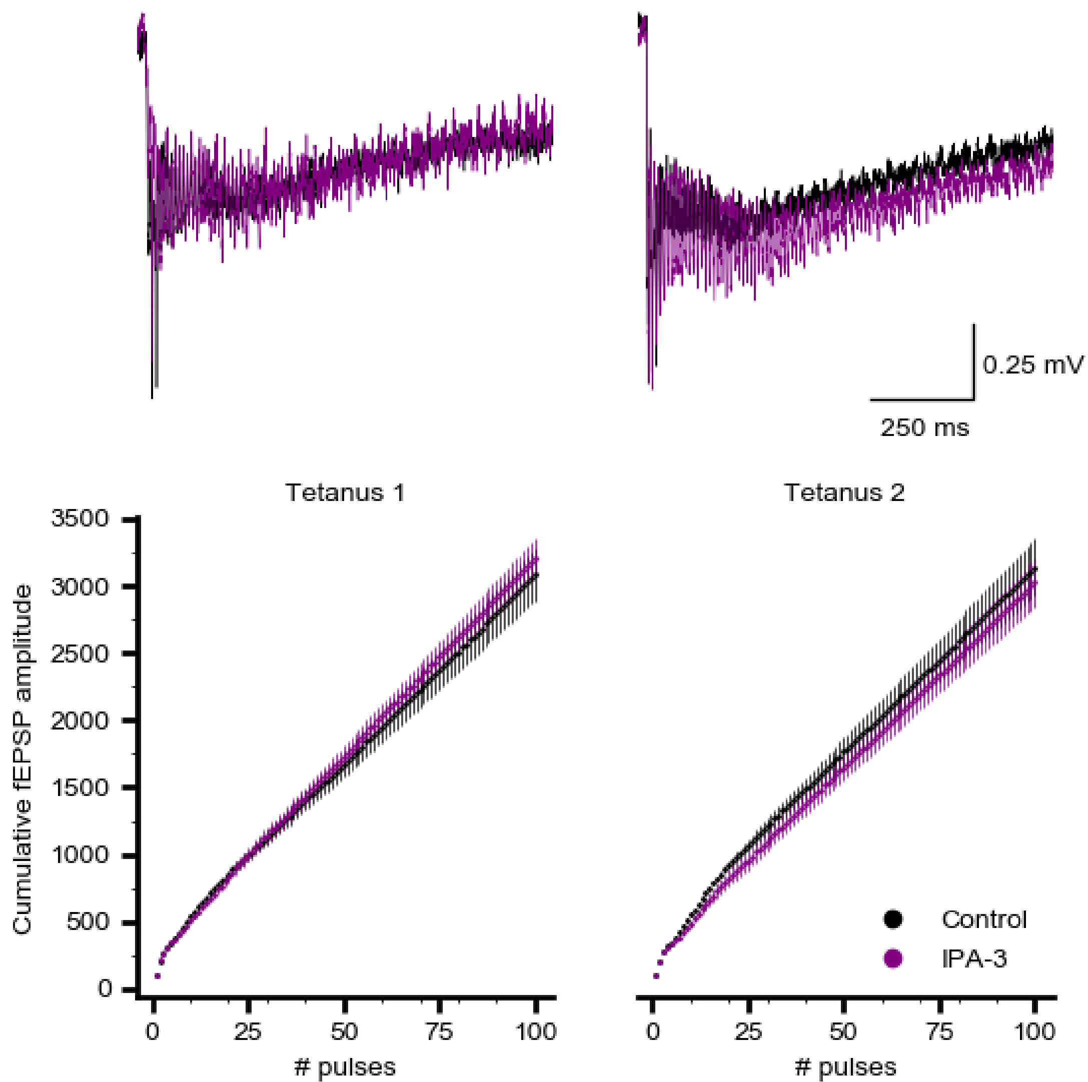

Figure 4.3 PAK inhibition does not affect the cumulative depolarisation evoked by tetanic stimulation. The cumulative pulse amplitude expressed as the fold increase from the first pulse amplitude in each train of high-frequency stimulation (two trains of 100 pulses, 100 Hz) in control slices (black symbols) and in slices incubated for 1 hour and perfused during baseline with IPA-3 (25 μM). Data from Fig 4.1; all values are expressed as means ± SEM.

4.3.4 Group I PAKs are not required for hippocampal LTP maintenance

It is thought that components of LTP, such as induction, expression and maintenance involve different molecular events (R C Malenka & Bear, 2004). Therefore, as it is apparent that inhibiting PAKs leads to LTP impairment, the next step was to examine whether IPA-3 inhibits LTP at a particular phase. To test this, we applied IPA-3 10 minutes after delivering high frequency stimulation to the Schaffer collateral pathway but found no significant difference between perfused slices and controls (IPA-3: 141 ± 4% of baseline, closed circles, n = 6; control: 149 ± 10%, n = 5, open circles, p = 0.449, **Figure 4.4A**). We also applied IPA-3 10 minutes before tetanus but the results match the previous experiment (IPA-3: 133 ± 8% of baseline, closed circles, n = 5; control 126 ± 10%, n = 6, open circle, p = 0.577, **Figure 4.4B**). Taken together, these results indicate that PAK inhibition impairs LTP induction, not maintenance.

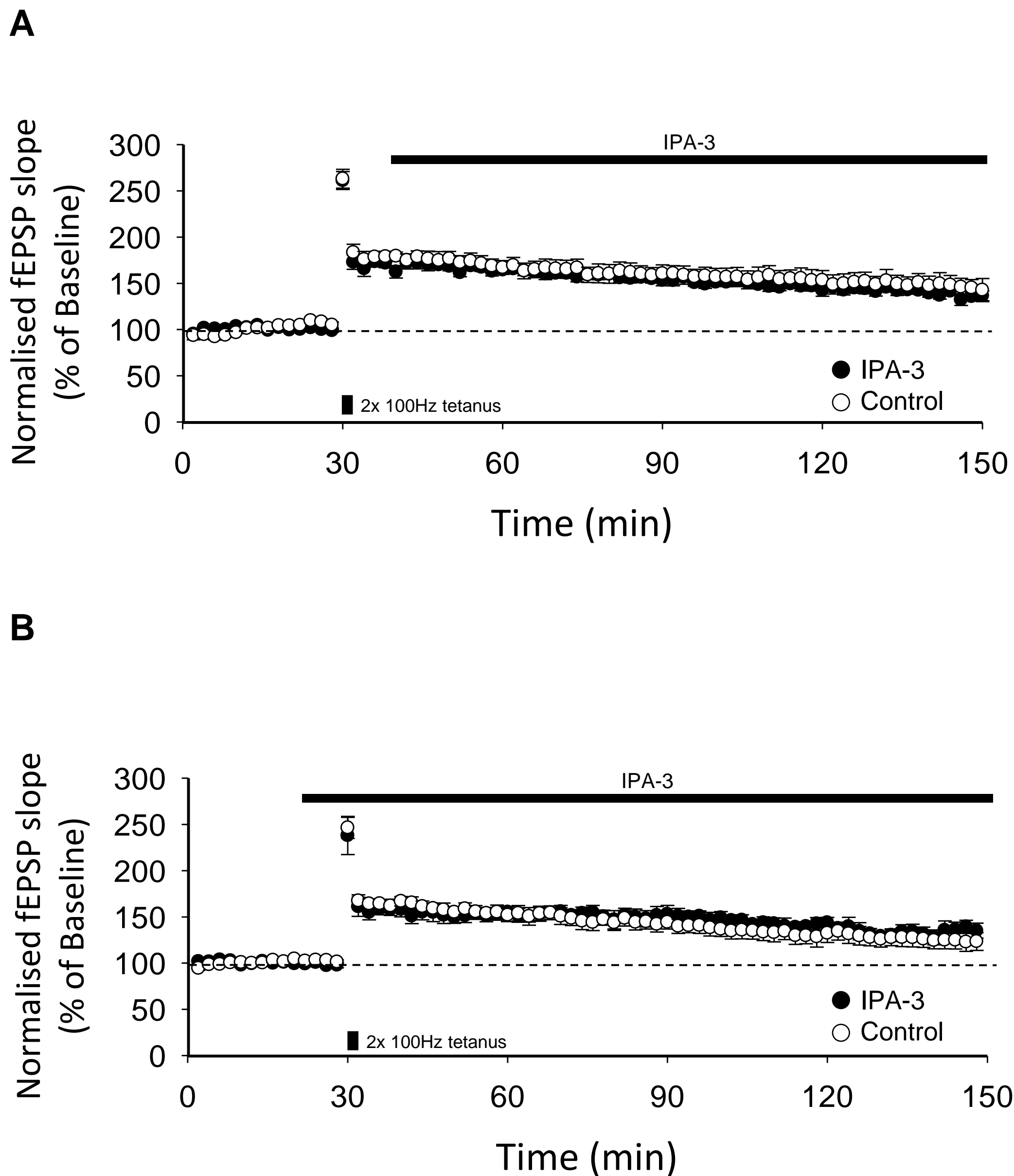

Figure 4.4 LTP maintenance was not impaired by PAK inhibition. LTP is similar in control slices and slices perfused with IPA-3 (a group I PAK inhibitor, at 25 μM). **A.** Perfusion of IPA-3 10 minutes after tetanus (IPA-3: 141 ± 4% of baseline, closed circles, n = 6; control: 149 ± 10%, n = 5, open circles, *p* = 0.449). **B.** Perfusion of IPA-3 10 minutes before tetanus (IPA-3: 133 ± 8% of baseline, closed circles, n = 5; control 126 ± 10%, n = 6, open circle, p = 0.577).

4.3.5 Strong tetanus stimulation is not sufficient to induce LTP following Group I PAK inhibition

The BCM (Bienenstock, Cooper and Munro) theory of modification states that when a pattern of activity is stronger than the “modification threshold” potentiation occurs at the active synapses, but when the pattern of activity is weaker than the threshold active synapses undergo depression (Bear, 1996). Accordingly, inhibition of LTP by Group I PAKs may be caused by a shift in the modification threshold of synapses treated with the drug, then increasing tetanic stimulation may rescue LTP. Studies have shown that an LTP induction protocol using 4 trains of HFS produces a form of strong L-LTP which lasts for more than 3 hours (Alarcon et al., 2006; Y.-Y. Huang et al., 2005; Y. Y. Huang & Kandel, 1994; Pavlowsky & Alarcon, 2012; Villers et al., 2012). Therefore, to test whether Group I PAK inhibition on LTP is prevented by stronger tetanic stimulation, we induced LTP by 4 trains of 100 Hz (separated by 30 sec intervals) in slices incubated with IPA-3 (25 μM) for 1 hour prior to recording and perfused during recording and in untreated, control slices. As can be seen in **Figure 4.5**, inhibition of Group I PAKs resulted in decreased LTP compared to controls (IPA-3: 90 ± 12% of baseline, n = 3, closed circle; control: 130 ± 6%, n = 3, open circles, $p = 0.044$). These results suggest that Group I PAKs may have a role in LTP induction rather than increasing the threshold for LTP induction.

As in section 4.3.3, it may be the case that depolarisation following the trains of HFS was not sufficient to induce LTP. Therefore, we compared the cumulative fEPSP amplitude produced by each of the 4 trains of HFS, but found no differences between the slices incubated with the Group I PAK inhibitor compared to controls in response to tetanus 1 (IPA-3: 3061 ± 592% of the first fEPSP, n = 3, red; Control: 3226 ± 331%, n = 3, black, $p = 0.821$, **Figure 4.6, top left**), tetanus 2 (IPA-3: 2127 ± 147%, n = 3, red; Control: 2202 ± 201%, n = 3, black, $p = 0.779$, **Figure 4.6, top right**), tetanus 3 (IPA-3: 1501 ± 188%, n = 3, red; Control: 2244 ± 55%, n = 3, black, $p = 0.019$, **Figure 4.6, bottom left**) or tetanus 4 (IPA-3: 2131 ±

269%, n = 3, red; Control: 2587 ± 657%, n = 3, black, p = 0.556, **Figure 4.6, bottom right**). It can thus be suggested that HFS-induced depolarisation was not the factor responsible for the inhibition of L-LTP in slices which were incubated and perfused with IPA-3.

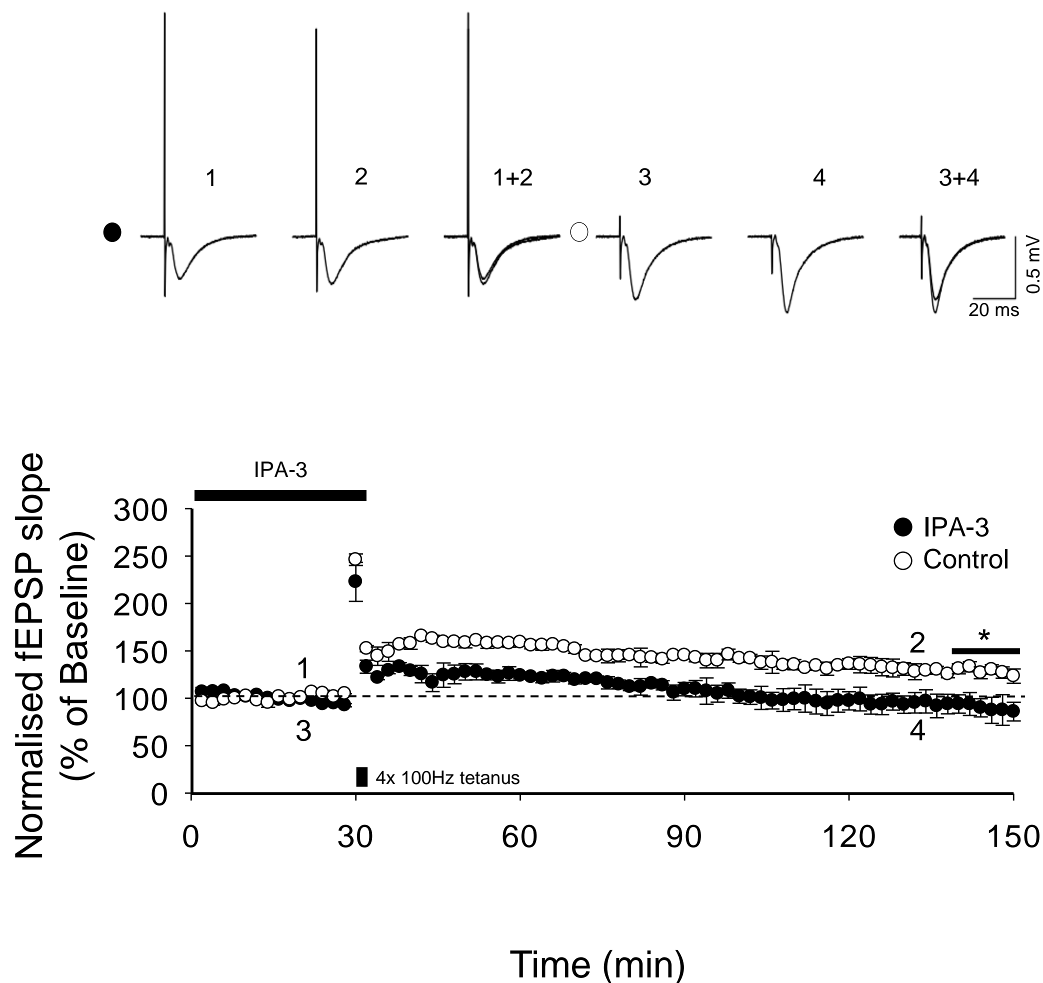

Figure 4.5 Strong LTP is blocked by PAK inhibition . LTP is inhibited in slices pre-incubated for 1 hour and perfused during baseline with IPA-3 (25 μM) following 4 trains of 100Hz tetanus (n = 3, p = 0.044).

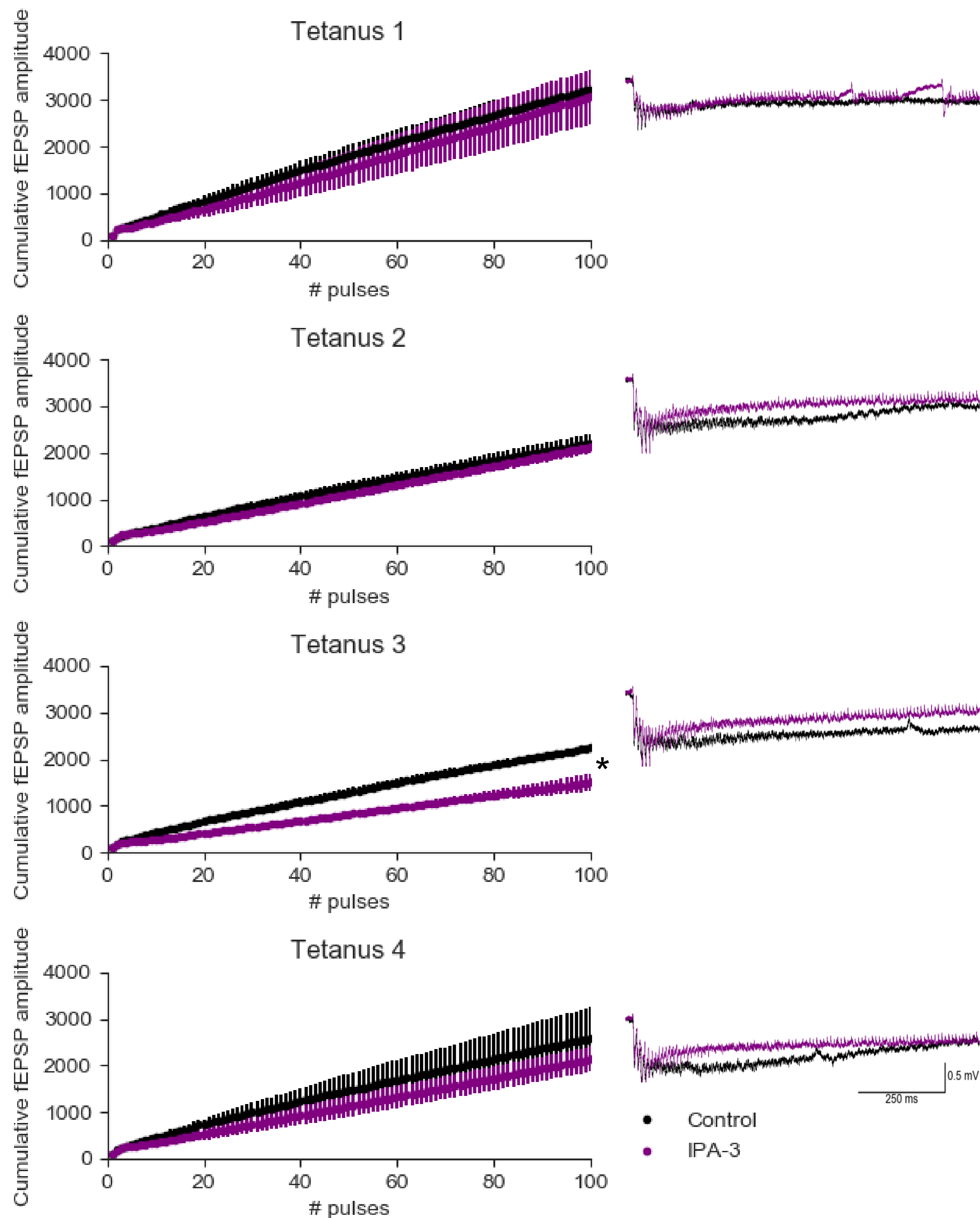

Figure 4.6 PAK inhibition does not affect the cumulative depolarisation evoked by 4 trains of HFS. The cumulative pulse amplitude expressed as the fold increase from the first pulse amplitude in each train of high-frequency stimulation (four trains of 100 pulses, 100 Hz) in control slices (black symbols) and in slices incubated for 1 hour and perfused during baseline with IPA-3 (25 μM, purple symbols). Data from Fig 4.5; all values are expressed as means ± SEM (T1: p = 0.821; T2: p = 0.779; T3: p = 0.019; T4: p = 0.556).

4.3.6 Group I PAK inhibition does not change LTP threshold

As inhibition of Group I PAKs impaired LTP in slices stimulated with both 2 and 4 trains of HFS, it was hypothesised that these effects might be explained by a change in the modification threshold (described in the previous section) in favour of synaptic depression. To test this hypothesis, we applied a subthreshold LTP induction stimulus (20 pulses at 100Hz) to untreated slices and to slices incubated for 1 hour and perfused during baseline with IPA-3. However, as can be seen in **Figure 4.7,** no significant differences were found between the two groups (IPA-3: 96 ± 9% of baseline, n = 5, closed circle; control: 89 ± 2%, n = 6, open circles, p = 0.126). This finding suggests that the modification threshold was not affected, therefore inhibition of Group I PAKs effect on LTP might be caused by other molecular mechanisms.

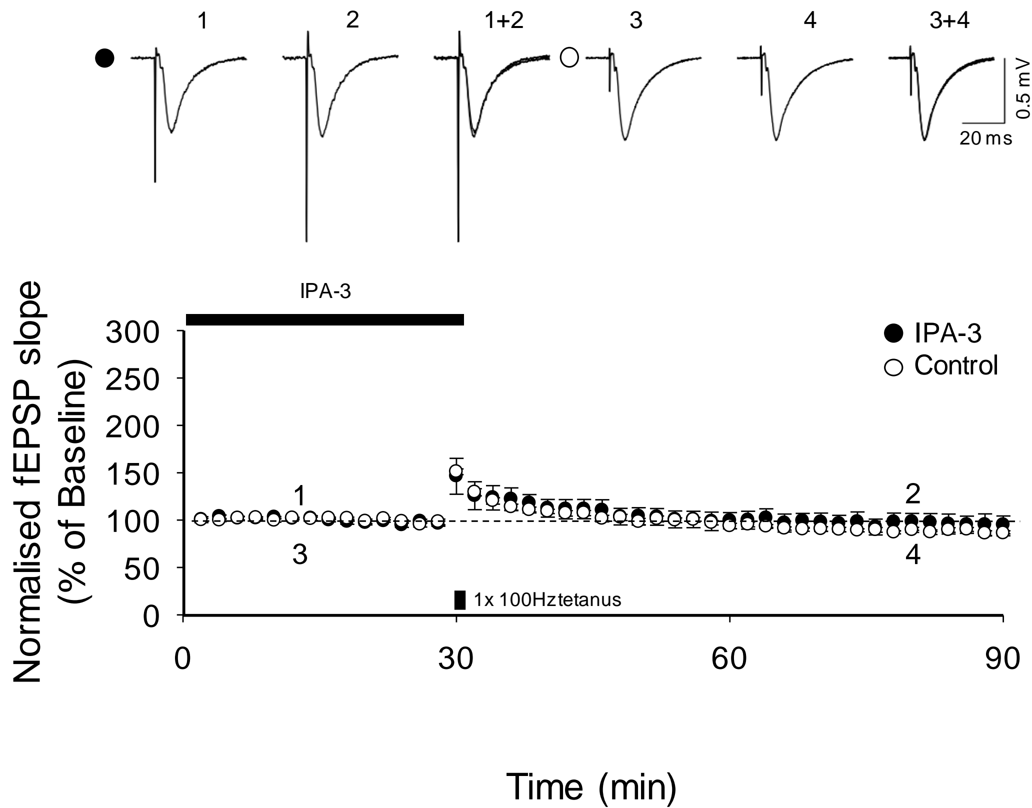

Figure 4.7 PAK inhibition has no effect on LTP induction threshold. Responses in control and slices pre-incubated and perfused with IPA-3 were similar after 1 train of 20 pulses of 100Hz (n = 3 slices from 3 animals, $p = 0.126$).

4.3.7 Group I PAK inhibitor FRAX does not affect LTP

To validate the effects of group I PAK inhibition on LTP induction a different inhibitor was used. FRAX486, a selective group I PAK inhibitor, was discovered by performing a high-throughput screen of a 12,000 kinase-focused small library and was found to alleviate abnormalities in dendritic spines in a mouse model of fragile X syndrome (Dolan et al., 2013). Surprisingly, LTP in slices incubated and perfused with FRAX486 at a concentration of 500 nM impaired LTP but the result was not statistically significant (FRAX: 141 ± 8% of baseline, n = 5, closed circles; control 161 ± 5%, n = 6, open circles, p = 0.056, **Figure 4.8A**). To test whether the concentration used was sufficient to produce the necessary group I PAK inhibition to affect LTP, we incubated slices in aCSF with FRAX486 at a concentration of 1 μM, however, the results were similar in control and incubated slices (FRAX: 132 ± 2% of baseline, n = 8, closed circles; control 139 ± 5%, n = 7 open circles, p = 0.189, **Figure 4.8B**), however, this could be explained by low LTP levels in controls. This suggests that pharmacological inhibition of group I PAKs with FRAX486 may have no effect on LTP.

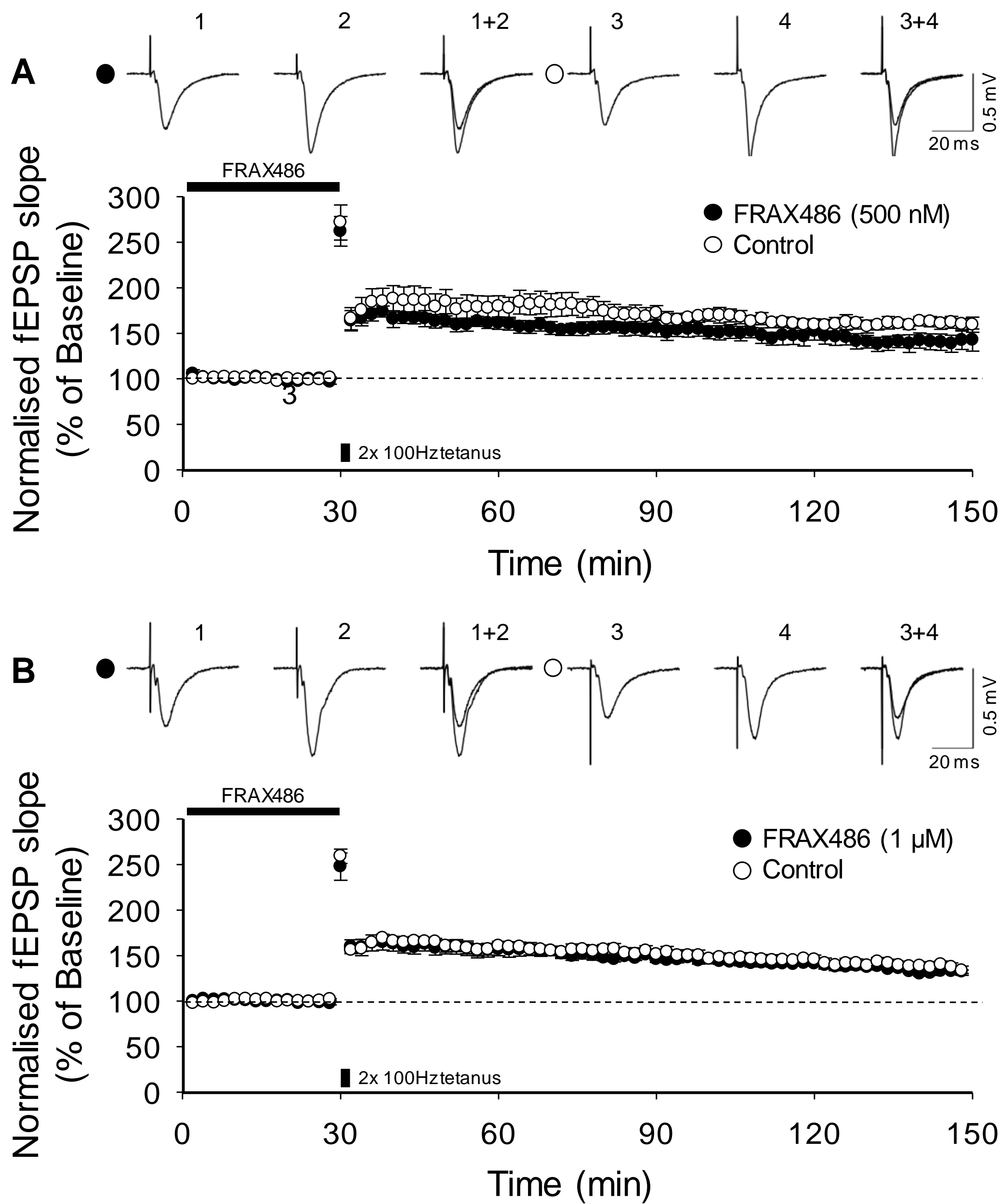

Figure 4.8 Group I PAK inhibitor FRAX486 has no effect on LTP. Slices were incubated with FRAX486 at 500 nM and 1 µM, for 1 hour before recording and perfusion of the drug in aCSF during baseline did not significantly change potentiated responses after tetanus **A** (p = 0.056) and **B** (p = 0.189), respectively.

4.3.8 Cdc42 activator does not affect LTP induction

It is known from the literature that bradykinin activates the GTPase Cdc42 (Kozma et al., 1995), which is an activator of PAKs. In previous research bradykinin was used at a concentration of 85 nM to investigate the effect of Cdc42 activation on synaptic maturity in hippocampal neuronal cultures (Shen et al., 2006). Therefore, we used bradykinin to test whether PAK activation by Cdc42, would increase LTP. To do this, we incubated slices in aCSF with bradykinin 85 nM for 30 min prior to recording but there was no difference between incubated slices and controls (Bradykinin: 142 ± 4% of baseline, n = 3, closed circles; control 144 ± 6%, n = 3 open circles, *p* = 0.756, **Figure 4.9A**) or in slices incubated for 30 minutes and perfused with bradykinin during baseline (Bradykinin: 141 ± 10% of baseline, n = 4, closed circles; control 146 ± 4%, n = 4 open circle, *p* = 0.549, **Figure 4.9B**). In addition, to test whether the effects of Cdc42 activation occurred during late-phase LTP, we incubated the slices with bradykinin at a higher concentration (500 nM) for 30 min and perfused during baseline and recorded for 2 hours after delivering high frequency stimulation. As can be seen from Figure 4.10, there was no significant difference between incubated slices and controls (Bradykinin: 114 ± 4% of baseline, n = 8, closed circles; control 119 ± 6%, n = 8 open circles, *p* = 0.574, **Figure 4.10**). These results suggest that Cdc42 activation does not have an effect on LTP.

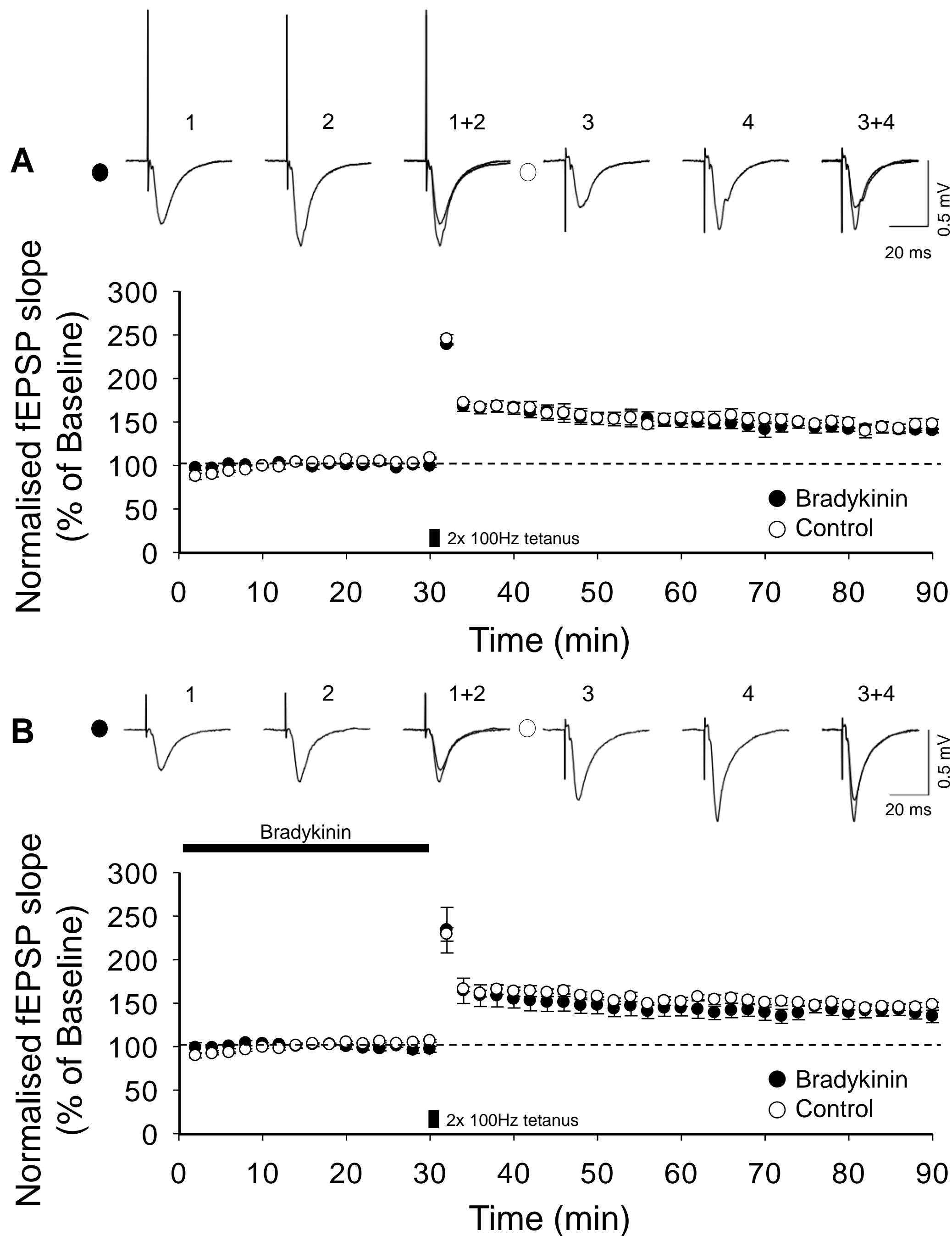

Figure 4.9 Bradykinin has no effect on LTP. A. Incubation of slices in Cdc42 activator Bradykinin (85 nM) does not affect LTP. Slices were incubated for 30 minutes before recording (n = 3, p = 0.756). **B.** Slices were incubated for 30 minutes before recording and bradykinin (85 nM) was perfused during baseline (n = 4, p = 0.549).

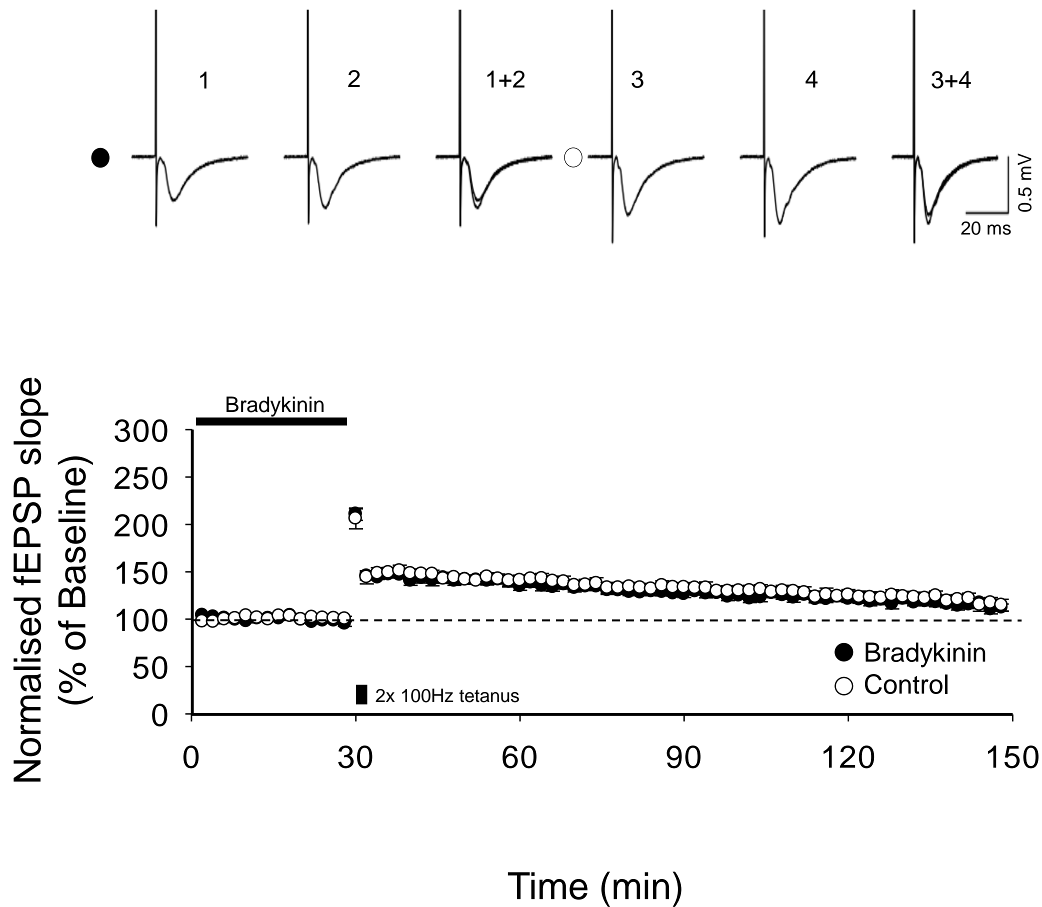

Figure 4.10 Cdc42 activator has no effect on LTP at high concentration. Slices incubated with Bradykinin for 30 minutes before recording and bradykinin was perfused during baseline were potentiated at a level similar to control slices (n = 8, $p = 0.574$).

4.4 Discussion

4.4.1 Group I PAKs and LTP induction

As was mentioned earlier, there might be interplay between Group I PAKs and the mechanisms of LTP induction. Previous experiments have indicated that Group I PAKs have a role in spine morphology changes, as well as in synaptic transmission and plasticity (Asrar et al., 2009; Boda et al., 2004; Chen et al., 2007b; Hayashi et al., 2004; Meng et al., 2005). The latter studies used genetic techniques, such as gene knockdown, interference RNA, or expression of dominant-negative proteins to suppress PAKs, while we inhibited PAKs using pharmacological inhibitors in our experiments. The use of pharmacological inhibitors allowed us to analyse the effect of PAK function on LTP maintenance. Our results suggest that inhibiting Group I PAKs 10 minutes before or after LTP induction does not affect LTP maintenance.

However, one key issue with our experiments is that we did not test activation of Group I PAKs following HFS. Other studies have shown that PAK phosphorylation peaks 7 minutes after the induction of LTP using TBS (Chen et al., 2007b; Rex et al., 2009). These findings suggest that if the pattern of activation of Group I PAKs is similar in LTP induced by HFS, infusing IPA-3 10 minutes after HFS would be rather late to observe an effect on maintenance. Another possibility is that perfusing IPA-3 10 minutes before HFS does not provide sufficient time for the drug to effectively suppress Group I PAKs.

Although our results indicate that PAKs are not necessary for maintenance, another alternative is that PAK signalling may be required for LTP stabilisation. It has been reported that Latrunculin A, which prevents F-actin polymerisation, inhibits LTP maintenance when infused within 10 minutes after TBS (Rex et al., 2009). In the same study, infusion of a low concentration of the Group I PAK inhibitor IPA-3 (2 μM) for 50 minutes before TBS and until the end of the experiment did not affect LTP. However, infusing latrunculin A 30 minutes after TBS in slices already perfused with IPA-3 diminished potentiation. As the effect of

latrunculin A alone has a time window of 10 minutes, these results suggest that LTP induction requires actin filament assembly, and that inhibition of PAKs perturbs F-actin stabilisation. Therefore, it would be interesting to test whether infusion of latrunculin A produces similar effects on LTP induced by HFS.

4.4.2 No effect of FRAX486 on LTP induction

As discussed above, PAKs have been found to have a role in LTP, and our results were consistent with the literature as using IPA-3 to inhibit Group I PAKs resulted in LTP impairment. However, when using the Group I PAK inhibitor FRAX486, incubating and perfusing slices in the same manner as with IPA-3, we observed no effect on LTP. Indeed, both IPA-3 and FRAX486 have been reported to inhibit all three Group I PAK isoforms (Deacon et al., 2008; Dolan et al., 2013). Therefore, it is somewhat surprising that the pharmacological inhibitor FRAX486 had no effect on LTP.

Our results may be explained by FRAX486 producing insufficient PAK inhibition necessary to result in LTP impairment. Although dose-response curves of PAK activity *in vitro* suggest that the concentrations of FRAX486 used in our experiments would inhibit all three Group I PAKs (Dolan et al., 2013), this assay tests kinase activity in isolation; whereas a more complex environment such as hippocampal slices could require a higher concentration or longer incubation time to produce alterations in LTP. This hypothesis is supported by a study in which suppression of PAK1 activity using transgenic mice expressing a dominant negative isoform of PAK1 resulted in increased potentiation in the cortex and normal LTP in the hippocampus (Hayashi et al., 2004). In the same study, it was found that PAK activity levels were reduced in the cortex to a greater extent than in the hippocampus. Furthermore, the observed levels of activated PAK were similar in the cortex of wild type mice and in the hippocampus of transgenic mice. The contradictory LTP results by Hayashi *et al* were attributed to PAK inhibition not reaching a threshold required to produce changes on hippocampal LTP.

Despite these contradictory results, further research could be done testing activity levels of Group I PAKs in hippocampus slices incubated with different concentrations of FRAX486 and IPA-3 to confirm their efficacy.

4.5 Conclusion

The results of this investigation support the idea that PAKs may have a specific role in LTP induction, as inhibiting Group I PAKs with IPA-3 had an adverse effect on LTP induction, but no effect on LTP maintenance. However, the precise mechanism used by PAKs in LTP induction remains to be elucidated. Therefore, it is necessary to assess if and how PAKs interact with crucial components of signalling pathways that are required for LTP induction.

CHAPTER 5

The effect of PAK inhibition on cellular functions underlying LTP induction

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Chapter 5 - The effect of PAK inhibition on cellular functions underlying LTP induction

5.1 Introduction

5.1.1 The form and function of field excitatory postsynaptic potentials

The synchronous responses evoked by activation of a population of neurons in the hippocampus can be measured as field potentials, and if these potentials are measured extracellularly, they are known as field excitatory postsynaptic potentials (fEPSPs) (Andersen et al., 1978). These fEPSPs consist of an early component, a rapid decline in voltage mediated by the influx of ions through the AMPAR channel, and a late component elicited by the influx of ions through NMDARs (Spruston et al., 1995). The preferred measurement to assess recorded fEPSPs is the initial slope, as it is less prone to be altered by other sources of current in brain slices compared to peak amplitude (Sweatt, 2010). However, there are other quantitative indices of fEPSP shape that can be used to compare neuronal responses (these waveform shape indices of fEPSPs are depicted in **Figure 5.1)**. In fact, a study by Petersen *et al.* analysed fEPSP waveform kinetics as method to distinguish whether responses were from the medial or the lateral perforant path inputs to the dentate gyrus (Petersen et al., 2013).

The fEPSP waveform parameters included in the study by Petersen *et al.* were onset latency, which is the time measured from when the stimulus is delivered to "foot" of the fEPSP (the point at which the fEPSP initial deflection can be detected); the peak latency which is the time measured from the time values of the foot to the peak of the fEPSP; and the half-width, which is defined as the duration of the fEPSP at half its peak amplitude. Changes in fEPSP parameters may indicate changes in receptor conductances (Fuenzalida et al., 2007). Measuring fEPSP kinetics may give insights into the kinetic properties of synaptic conductance which can affect how synapses integrate signals to produce enduring changes in synaptic plasticity.

5.1.2 The role of calcium in LTP

NMDARs are highly permeable to Ca^{2+} (Jahr & Stevens, 1987), and they are critical for the induction of LTP (Bliss & Collingridge, 1993). The requirement of NMDAR activation was explained by experiments in which preventing a rise in Ca^{2+} by loading the Ca^{2+} chelator, EGTA, into neurons resulted in the LTP inhibition (Lynch et al., 1983). Further evidence came from a study by Malenka *et al,* where synaptic potentiation occurred in hippocampal neurons using a photolabile chelator, nitr-5 preloaded with Ca^{2+}, which releases Ca^{2+} in response to ultraviolet light; whereas non-photolysed nitr-5 blocked LTP induction; in addition, the same researchers found that preventing Ca^{2+} influx by depolarising the postsynaptic neuron also prevented LTP (R C Malenka et al., 1988).

Prior studies have noted that a brief high rise in intracellular Ca^{2+} in the postsynaptic site initiates biochemical processes necessary for LTP induction, whereas prolonged lower concentrations of Ca^{2+} influx activate processes needed for LTD (Artola & Singer, 1993; S.-N. Yang et al., 1999). The calcium-dependent processes are amplified by many proteins that are activated or inactivated by Ca^{2+}, such as calmodulin (CaM), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), calcineurin and protein kinase C (PKC). The change in synaptic transmission is determined by which Ca^{2+}-binding proteins are activated following Ca^{2+} influx. For instance, a molecular cascade for LTP is initiated by Ca^{2+} influx through activated NMDARs, then Ca^{2+} binds to CaM complex, which in turn leads to the phosphorylation and thereby activation of CaMKII. Then, active CaMKII phosphorylates and potentiates AMPAR-mediated responses at the synapse (Barria et al., 1997). Furthermore, loss of CaMKII decreases AMPAR-mediated EPSCs and inhibits LTP (Incontro et al., 2018). Therefore, Ca^{2+} influx and the subsequent biochemical processes triggered by Ca^{2+} are necessary for the induction of LTP.

5.1.3 Dynamic changes in spine structure

As explained earlier, the morphology changes in dendritic spines is dependent on actin cytoskeleton rearrangements. Indeed, *in vivo* visualisation of dendritic spines revealed that changes in shape are driven by actin dynamics (Fischer et al., 1998). The molecular events that are thought to underlie experience-dependent morphology changes in dendritic spines have been previously described in sections *1.5.1*, *1.5.2* and *4.1.1*.

In addition to experience-dependent changes in morphology, LTP is associated with the persistent enlargement of dendritic spines, termed structural LTP (sLTP) (Nakahata & Yasuda, 2018). Previous studies have shown that LTP induction using high-frequency stimulation, glutamate uncaging or chemically-induced LTP (chemLTP), produces dynamic changes in dendritic spine morphology (Engert & Bonhoeffer, 1999; Kopec et al., 2006; Maletic-Savatic et al., 1999; Okamoto et al., 2004). For example, theta burst stimulation (TBS) paired with postsynaptic depolarisation induced an increase of spine volume which persisted for 45 minutes after TBS as well as LTP (Yang et al., 2008); tetanic stimulation induced enlargements in dendritic spine head size that persisted for 30 minutes after stimulation (Matsuzaki et al., 2004; Okamoto et al., 2004); glutamate uncaging caused a long-lasting increase in spine head diameter (Matsuzaki et al., 2004) and it promoted the persistence of newly formed spines following the LTP-inducing stimuli (Hill & Zito, 2013); Chemically-induced LTP using bath application of glycine also produces long-term spine enlargement (Fortin et al., 2010; Park et al., 2006). These results suggest that both functional and structural forms of plasticity occur in stimulated synapses, however, whether and how spine remodelling supports LTP is still not fully understood.

Interestingly, PAKs may be involved in structural changes in dendritic spines that lead to increased content of AMPARs on the synaptic surface. It has been shown that the expression of GluA1 and GluA2 AMPAR subunits at the synaptic surface increases following chemLTP stimulation protocols (Groc et al., 2008; Kopec et al., 2006; Lu et al., 2001). The precise mechanisms underlying the trafficking of

AMPARs are not completely understood. However, researchers have identified a novel signal transduction pathway in which phosphorylation of GluA1 AMPAR subunits by PAK3 increases expression of these subunits at the synaptic surface in neurons (Hussain et al., 2015). This finding suggests that PAKs might have a role in different mechanisms necessary for LTP induction, regulation of AMPAR expression at the synapse as well as the control of dendritic spine structural changes. Therefore, this chapter will examine the impact of PAK inhibition on spine morphology changes and AMPAR trafficking to the synaptic surface.

The chemLTP protocol is increasingly being used to assess synaptic plasticity related changes as it shares several features with LTP induced by high-frequency stimulation. ChemLTP is dependent on NMDAR activation, it requires Ca^{2+} influx and CaMKII activation, it is accompanied by the incorporation of AMPARs at the synaptic surface mediated by exocytosis, actin cytoskeleton rearrangements, and it is associated with potentiated excitatory signals (Kopec et al., 2006; Lu et al., 2001). Thus, the glycine-induced form of chemical LTP was selected in order to identify the effects of PAK inhibition on LTP.

5.2 Aims and Hypotheses

In the previous chapter, our results indicated that pharmacologically inhibiting Group I PAKs using IPA-3 resulted in LTP impairment. However, the second inhibitor, FRAX486, did not produce any change in LTP magnitude. Therefore, the aim of this chapter is to analyse the effects of these PAK inhibitors on components of the signalling cascades leading to LTP induction, such as intracellular calcium levels, dendritic spine morphology, and receptor trafficking to the synaptic surface.

5.3 Results

5.3.1 Group I PAK inhibition does not affect fEPSP waveform

The fEPSP waveform features can be measured to assess whether there are changes in postsynaptic receptor kinetics that can affect synaptic integration (Petersen et al., 2013). Therefore, we performed an analysis of the kinetics fEPSP recorded from control (untreated) and IPA-3 treated slices. The fEPSP waveform measurements were taken from data from **Figure 4.1A** and **Figure 4.1B** 5 minutes before HFS, and 5 minutes before the end of the experiments. The parameters measured included onset latency, peak latency, half-width, and decay time (see **Figure 5.1**). The onset latency, width at half-amplitude, decay time and decay slope of responses from both groups were not significantly different (**Figure 5.2** and **Table 5.1**). However, peak latencies were significantly longer in slices incubated and perfused with IPA-3 (**Figure 5.2** and **Table 5.1**). This is consistent with our previous experiments in which LTP was inhibited following Group I PAK inhibition suggest that inhibition of Group I PAKs by IPA-3 incubation attenuates synaptic conductance.

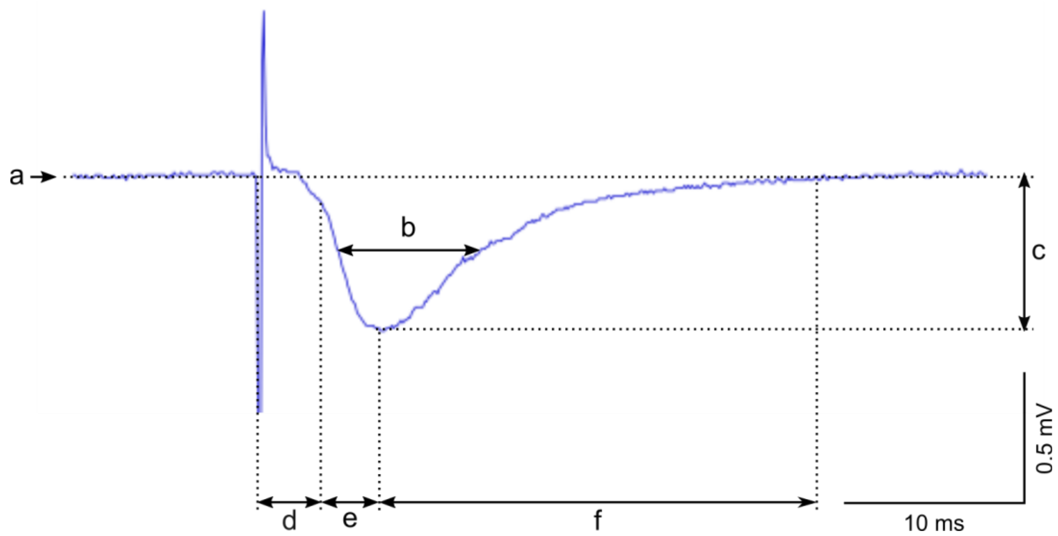


Figure 5.1 Field EPSP parameters. Representative fEPSP waveform. Measurements of field excitatory postsynaptic potentials: Baseline (a), half-width (b), amplitude (c), onset latency (d), peak latency (e), and decay time (f). Peak amplitude (mV) was calculated from the DC baseline and the slope was measured by defining the start and stop time after the stimulus.

Table 5.1 Summary of fEPSP kinetics parameters before and after HFS for control and IPA-3 treated slices. The data were taken from experiments from **Figure 4.1A** (n = 8) and **Figure 4.1B** (n = 7). Values are means ± SEM (msec). Statistical significance between control and IPA-3 set at $p < 0.05$, tested using two-way ANOVA (denoted by an asterisk).

	Onset latency	Peak latency	Half-width	Decay time
Before HFS				
Control	1.72 ± 0.06	1.59 ± 0.09	3.80 ± 0.19	13.96 ± 0.95
IPA-3	1.68 ± 0.07	1.80 ± 0.11	3.80 ± 0.13	13.07 ± 0.80
After HFS				
Control	1.68 ± 0.07	1.42 ± 0.06	3.38 ± 0.30	12.70 ± 1.21
IPA-3	1.71 ± 0.06	1.77 ± 0.13	3.09 ± 0.12	13.45 ± 1.27
p-value	0.431	0.008*	0.481	0.948

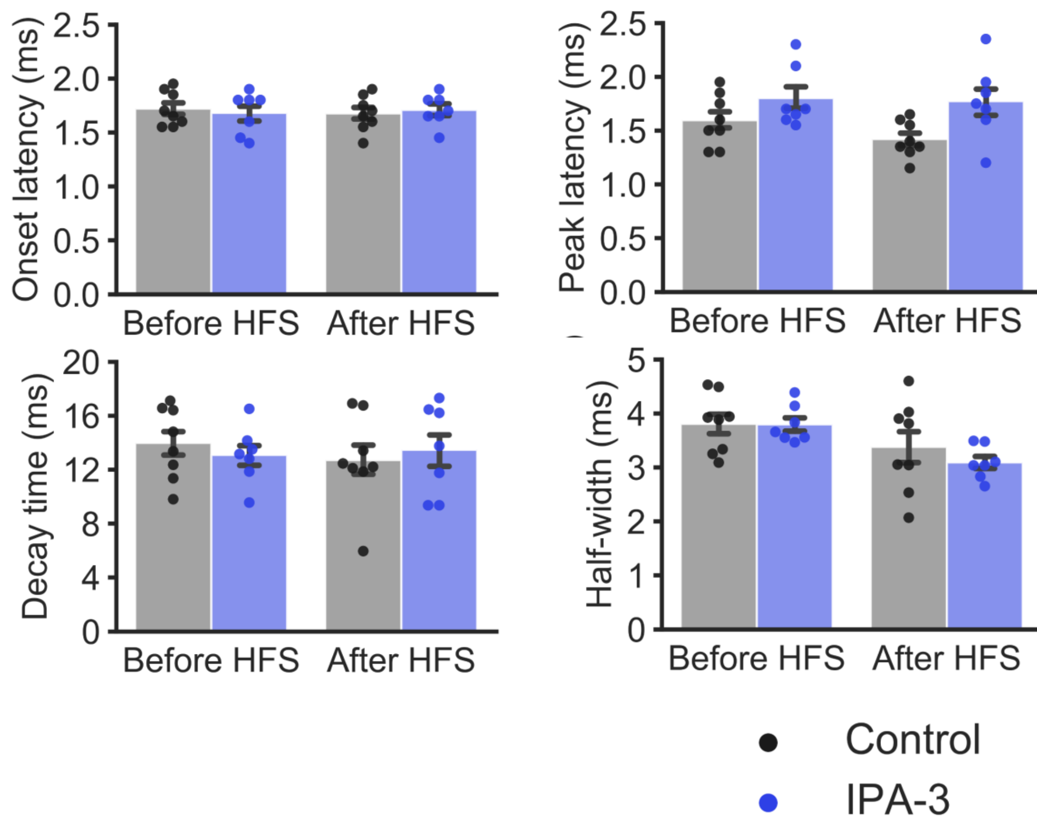
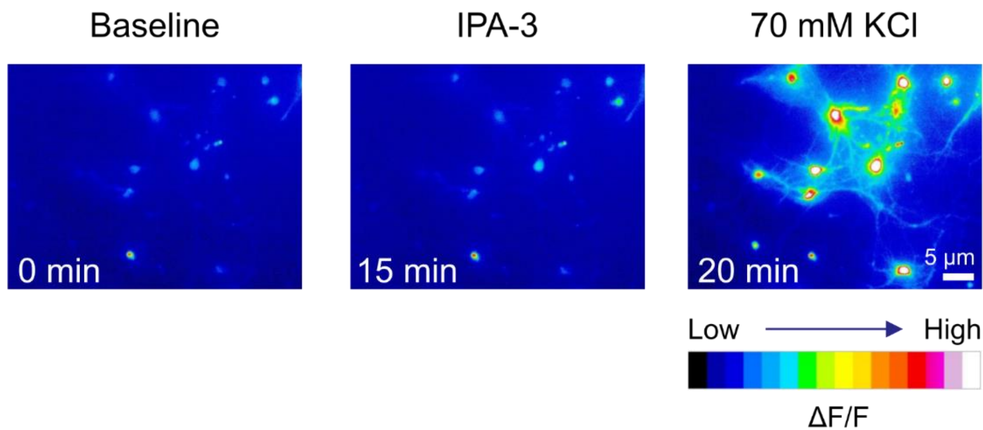


Figure 5.2 Field EPSP parameters before and after HFS. The onset latency, width at half-amplitude, and decay time of responses from both groups were not significantly different

5.3.2 Transient Group I PAK inhibition does not induce calcium flux

Changes in intracellular calcium concentration occur in many cellular signalling cascades. In neurons, calcium entry through NMDARs is necessary for LTP induction (Bliss & Collingridge, 1993). Moreover, blocking calcium release from intracellular stores lead to a inhibition of LTP induction but not maintenance (Harvey & Collingridge, 1992). In our previous electrophysiology results, LTP was inhibited by IPA-3 but not by FRAX486; this effect might be explained by IPA-3 altering Ca^{2+} entry to neurons. Therefore, we performed calcium imaging to evaluate whether inhibition of group I PAKs affected intracellular Ca^{2+}. Fluo4-AM, a chemical calcium indicator was used to observe calcium concentration changes in primary cultured hippocampal neurons (DIV 14-29). Following Fluo4-AM loading, neurons were placed in the recording chamber, then perfused with HBS buffer to measure baseline fluorescence of 10 minutes before bath application of IPA-3, FRAX486 or vehicle (DMSO) for 10 minutes. Neither, IPA-3 FRAX486 nor DMSO application produced changes in fluorescence intensity, see **Figure 5.3, Figure 5.4** and **Figure 5.5** (IPA-3: 99 ± 6% of baseline, n = 6 coverslips, 42 neurons, FRAX486: 107 ± 5%, n= 3 coverslips, 49 neurons, DMSO: 95 ± 2%, n = 9 coverslips, 38 neurons, $p > 0.05$); whereas, KCl application to neurons in all experimental groups led to a similar increase in fluorescence intensity which is consistent with previous studies (Cameron et al., 2016). These results suggest that application of group I PAK inhibitors IPA-3 or FRAX486 do not alter intracellular calcium signals, therefore, the effect of IPA-3 inhibition of LTP cannot be explained by altered calcium influx.



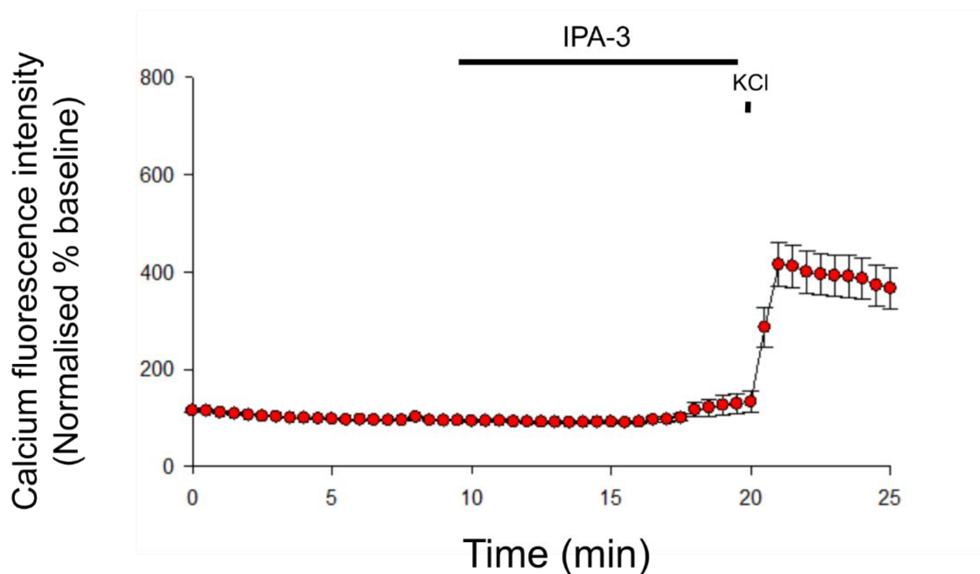


Figure 5.3 Intracellular calcium concentration is not affected by Group I PAK inhibitor IPA-3. Primary cultured hippocampal neurons (DIV 14-29) were incubated in 5mM Fluo4 AM for 60 min. After a baseline of 10 min, IPA-3 (25 µM) was perfused (n = 6 coverslips, 42 neurons). The background intensity was subtracted from fluorescence intensity of ROIs at all time points (each time point refers to 1 frame at 30 sec intervals).

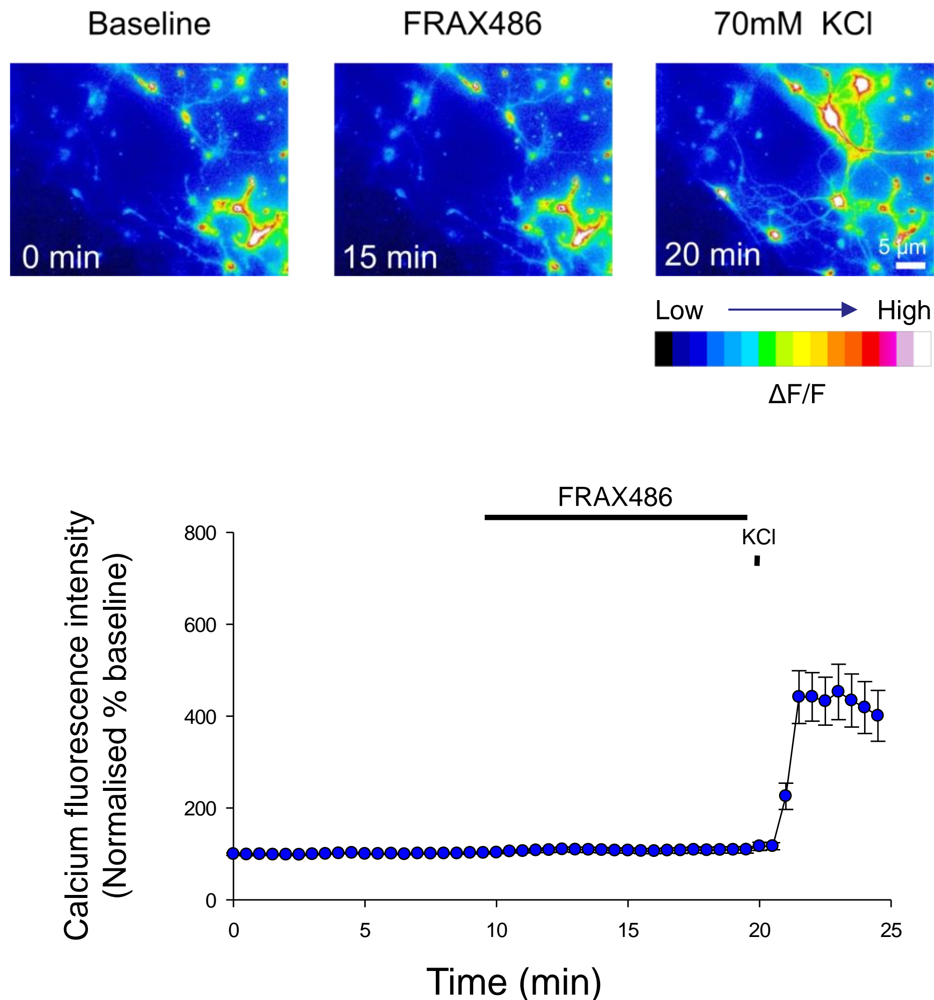
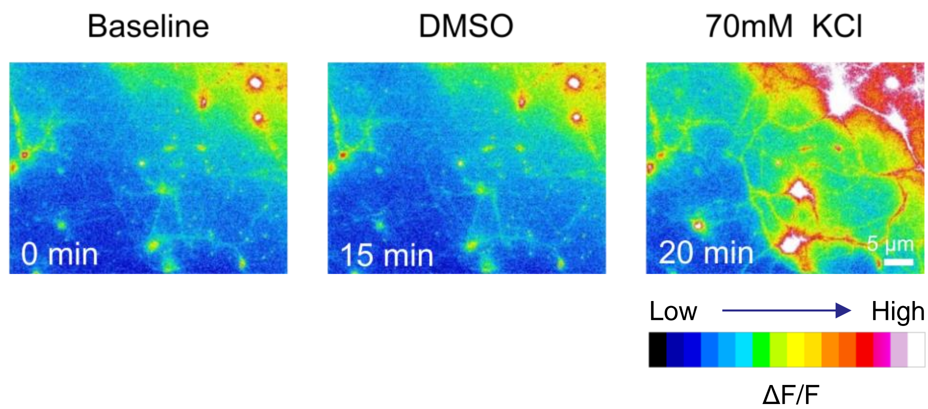


Figure 5.4 Intracellular calcium concentration is not affected by Group I PAK inhibitor FRAX486. Primary cultured hippocampal neurons (DIV 14-29) were incubated in 5mM Fluo4 AM for 60 min. After a baseline of 10 min, FRAX486 (500 nM) was perfused (n = 11 coverslips, 49 neurons). The background intensity was subtracted from fluorescence intensity of ROIs at all time points (each time point refers to 1 frame at 30 sec intervals).



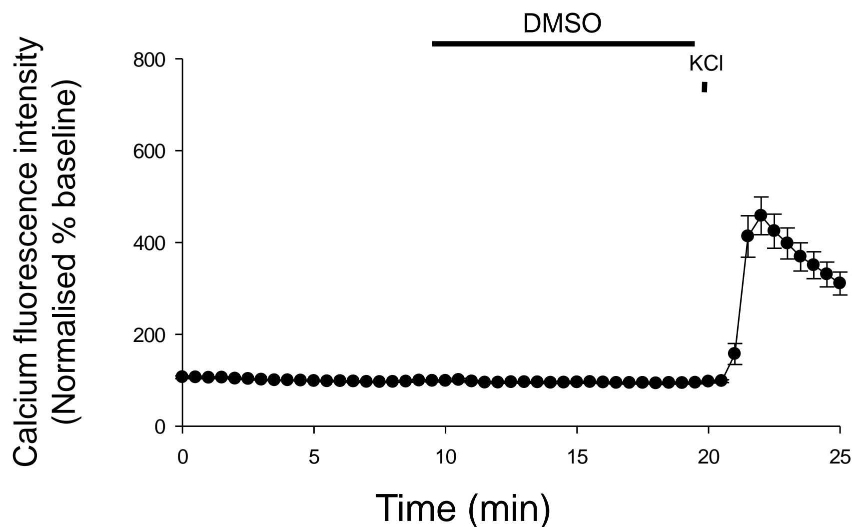


Figure 5.5 Intracellular calcium concentration is not affected by vehicle, DMSO. Primary cultured hippocampal neurons (DIV 14-29) were incubated in 5mM Fluo4 AM for 60 min. After a baseline of 10 min, DMSO was applied as a control (n = 9 coverslips, 38 neurons). The background intensity was subtracted from fluorescence intensity of ROIs at all time points (each time point refers to 1 frame at 30 sec intervals).

5.3.3 Group I PAK inhibition does not cause structural modifications

Prior studies that have noted the importance of Group I PAKs in dendritic spine morphology. For instance, mutations that inhibit PAK3 kinase activity alter spine morphology and decrease spine density (Kreis et al., 2007). Moreover, inhibition of PAK3 in rat cultured hippocampal slices resulted in activity-dependent increased numbers of unstable spines and reduced spine stabilisation stabilization (Dubos et al., 2012). Thus, to determine whether disturbing Group I PAKs function alters spine morphology thereby resulting in LTP impairment, we incubated primary cultured hippocampal neurons (DIV 21) with DMSO (control), IPA-3 (25 µM) or FRAX486 (500 nM) before fixing and staining these samples with phalloidin to analyse the area of protrusions on 10 μm^2 dendritic sections. However, no significant difference was observed between the groups (Control: 0.53 ± 0.05 Area(μm^2) n = 3 coverslips, 8 neurons; FRAX486: 0.66 ± 0.06, n = 3 coverslips, 7 neurons, IPA-3: 0.65 ± 0.06, n = 3 coverslips, 10 neurons; One-way ANOVA on Ranks p = 0.397, **Figure 5.6** and **Figure 5.7**). These results suggest that incubation of hippocampal neurons with Group I PAK inhibitors do not produce striking morphological changes on dendritic protrusions. Therefore, LTP inhibition produced by IPA-3 may be explained by an activity-dependent effect on morphology.

In a previous study, the overexpression of PAK1 or PAK3 in cultured hippocampal neurons resulted in the increase of the number of dendritic spines and in the number of clusters of PSD-95 indicative of an increase in excitatory synapse formation (Zhang et al., 2005). On the other hand, the expression of the dominant-negative form of PAK1 or a kinase dead form of PAK3 had the opposite effect: a dramatic decrease in dendritic spines and in the number of PSD-95 clusters (Zhang et al., 2005). These PAK constructs were expressed in cultured neurons for 7 days before analysing the results on neuronal morphology. In our experiment cultured neurons were incubated with PAK inhibitors for only 2 – 2.5 hours prior to fixing and staining with phalloidin. This suggests that the lack of changes on

dendritic spine morphology observed in our experiments might be due to insufficient exposure of hippocampal neuronal cultures to PAK inhibitors.

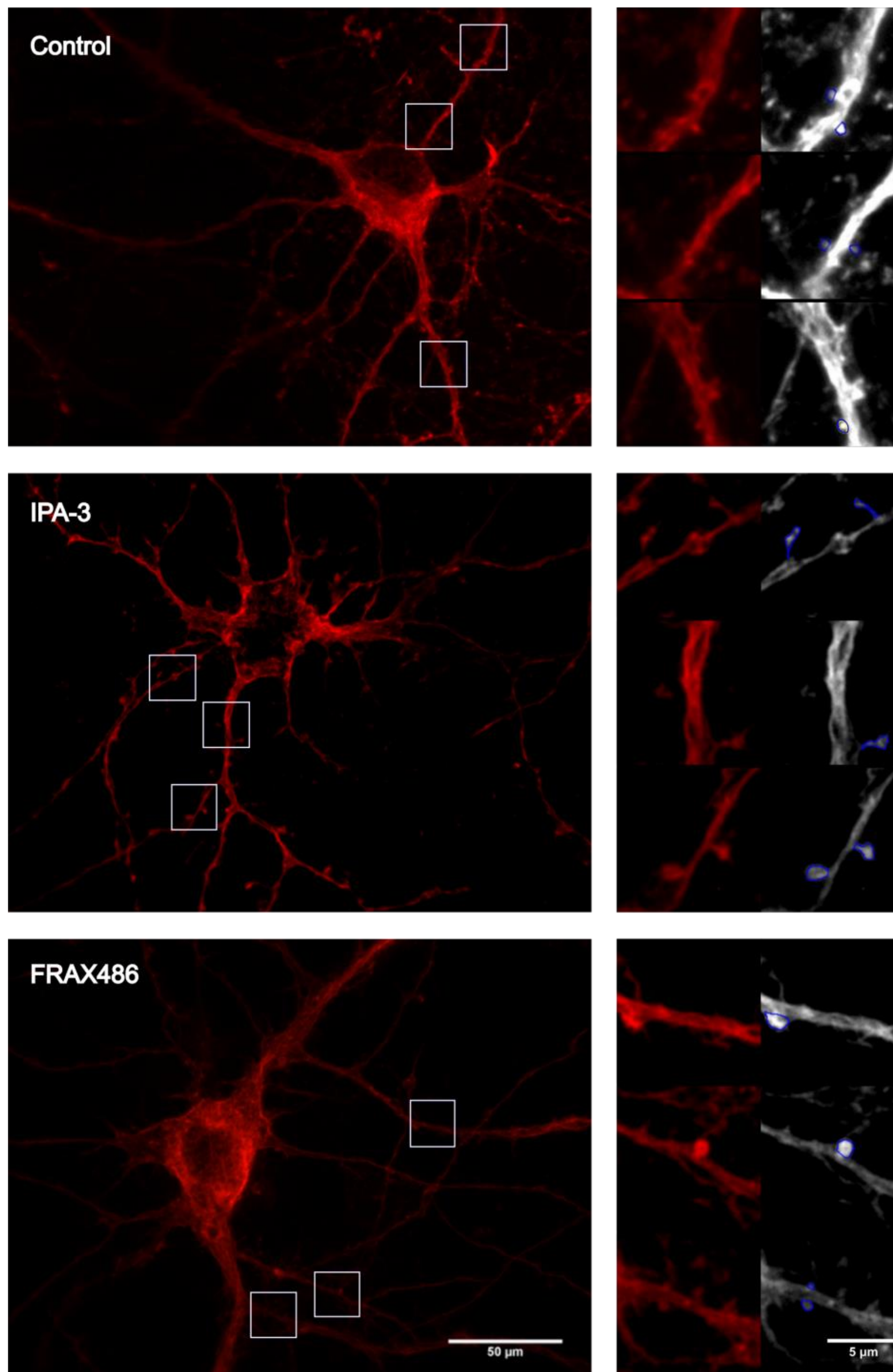


Figure 5.6 PAK inhibition does not affect basal dendritic spine morphology. Primary cultured hippocampal neurons (DIV 14-29) were incubated with vehicle (DMSO), IPA-3 (25 µM), or FRAX486 (500 nM) before fixing and staining with phalloidin (Control: 0.53 ± 0.05 Area(µm2) n = 3 coverslips, 8 neurons; FRAX486: 0.66 ± 0.06, n = 3 coverslips, 7 neurons, (IPA-3: 0.65 ± 0.06, n = 3 coverslips, 10 neurons; One-way ANOVA on Ranks p = 0.397).

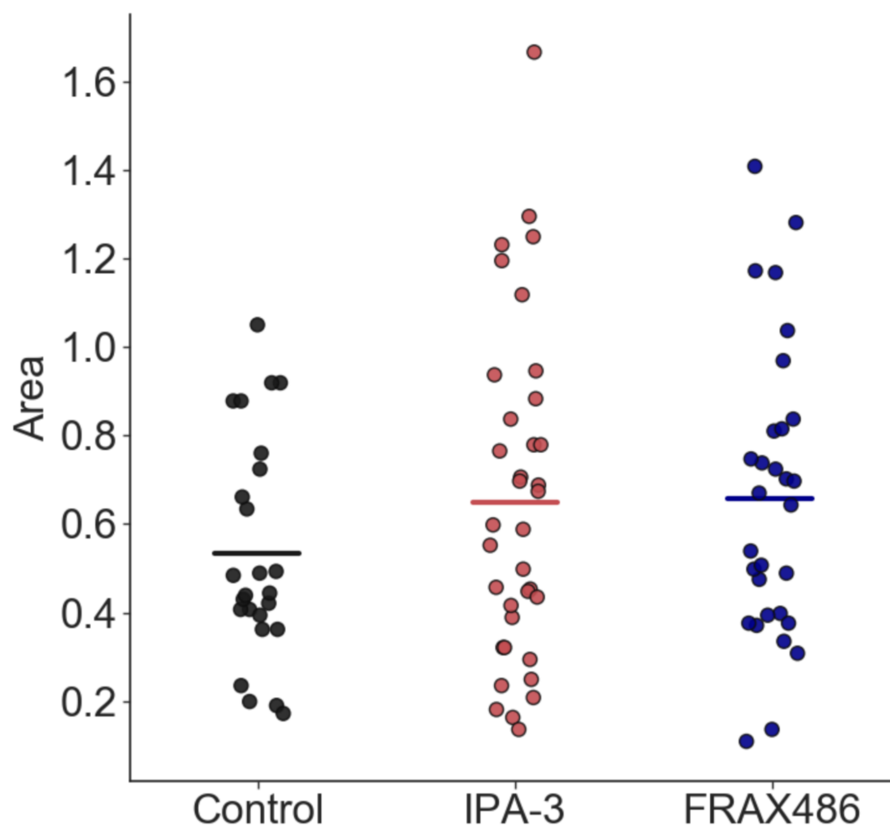


Figure 5.7 Quantification of dendritic spine area reveals no changes produced by Group I PAK inhibitors. Primary cultured hippocampal neurons (DIV 14-29) were incubated with vehicle (DMSO), IPA-3 (25 μM), or FRAX486 (500 nM) before fixing and staining with phalloidin (data from experiments shown in **Figure 5.6**).

5.3.4 A chemical-LTP stimulus in conjunction with Group I PAK inhibition reduces dendritic spine size

There are structural changes following glutamate uncaging (Matsuzaki et al., 2004), high-frequency electrical stimulation or theta-burst stimulation. LTP increases F-actin content which suggests that spine enlargement requires actin polymerization (L. Y. Chen et al., 2007; Fukazawa et al., 2003). LTP is impaired when cytoskeletal actin assembly is blocked (Fukazawa et al., 2003; Kim & Lisman, 1999). PAK proteins are involved in molecular cascades that mediate spine morphology via cofilin, an actin depolymerizing protein (L. Y. Chen et al., 2007). PAK phosphorylation activates its kinase activity, and this has been observed shortly after LTP induction (L. Y. Chen et al., 2007). As in previous experiments we found that inhibition of PAK blocked LTP induction but not maintenance we hypothesized that inhibition of PAK may block actin polymerization necessary for LTP.

Previously, bath application of glycine was found to promote dendritic spine growth (Groc et al., 2008; W. Y. Lu et al., 2001; Shahi & Baudry, 1993). Therefore, we analysed whether F-actin stained protrusions on dendrites of cultured hippocampal neurons were larger in neurons treated with glycine and whether group I PAK inhibitors would inhibit glycine-induced growth. Primary cultured hippocampal neurons (DIV 21) were treated with aCSF without glycine (control), aCSF with glycine (200 µM). In addition, two sets of neurons were pre-incubated with group I PAK inhibitors IPA-3 or FRAX486 for 1.5 – 2 hours before incubating them with aCSF + glycine (200 µM) + IPA-3 (25 µM), and aCSF + glycine (200 µM) + FRAX486 (500 nM), respectively.

Figure 5.8 shows representative images of F-actin stained neurons. As the data failed the Equal Variance Test, we analysed whether there were statistical differences between the groups using Kruskal-Wallis statistical test. Quantitative analysis of the area of individual protrusions indicate that there is no significant difference between groups (Control: 0.53 ± 0.05 Area(μm^2), n = 3 coverslips, 8 neurons, Gly: 0.65 ± 0.05, n = 9 coverslips, 15 neurons, FRAX486+Gly: 0.79 ±

0.09, n = 2 coverslips, 4 neurons, Gly+IPA-3: 0.38 ± 0.04, n = 3 coverslips, 4 neurons, **Figure 5.8** and **Figure 5.9**). The results from glycine treated neurons were highly variable. However, there was a significant difference between groups that were pre-incubated with IPA-3 and FRAX486, while data from other measurements, such as protrusion shape and number of protrusions analysed per 10 μm^2 were homogenous. These results suggest that IPA-3 and FRAX486 have distinct pharmacological actions on neurons, IPA-3 seems to reduce dendritic spine growth while FRAX486 does not. These results are consistent with our previous results in which IPA-3 incubation and perfusion hampers LTP whilst FRAX486 incubation and perfusion does not.

One of the limitations of this experiment is that the protrusions are close to the resolving limit of wide-field microscopy fluorescence imaging. The contrast and spatial resolution are affected by light scattering of approximately 250 nm (laterally) (Chazeau & Giannone, 2016) while the diameter of the largest spine heads, mushroom spine heads, measures up to 1 µm and spine neck diameter ranges from 100 to 200 nm (Fiala et al., 2002). Accordingly, these limitations might prevent accurate observation and measurements of structural changes, indicating an alternative approach may be required.

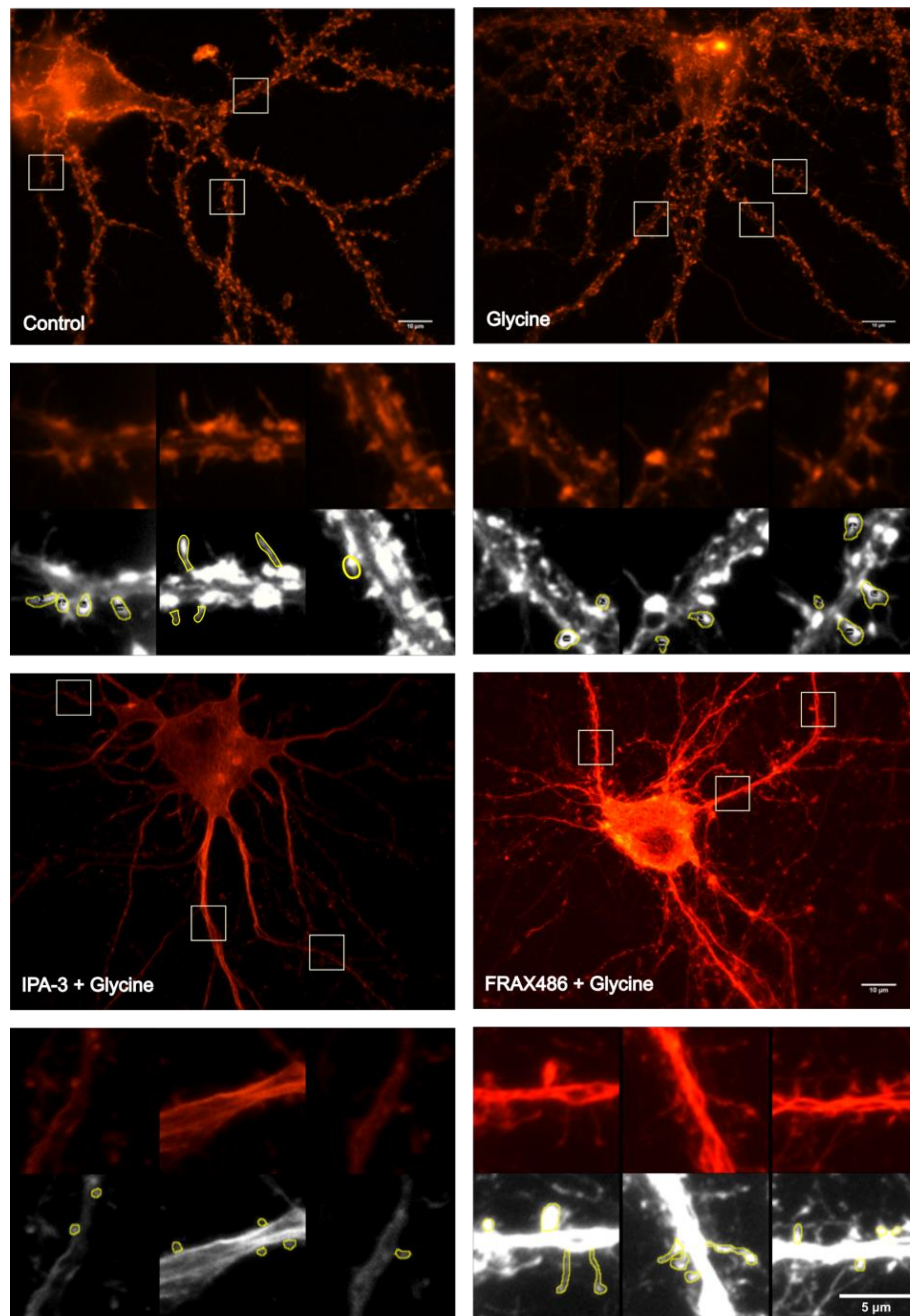


Figure 5.8 Group I PAK inhibition causes dendritic spine morphology alterations in primary cultured hippocampal neurons. Top left: control neuron incubated with aCSF for 10 minutes. **Top right:** neuron treated with Glycine (200 µM) tor 10 minutes. **Bottom Left:** neuron incubated with aCSF with Glycine (200 µM) for 10 minutes following preincubation with IPA-3 (25 µM). **Bottom right:** neuron pre-incubated with FRAX486 and treated with aCSF + Glycine (200 µM) tor 10 minutes. Below each condition examples of analysed ROIs (10 µm^2) are shown.

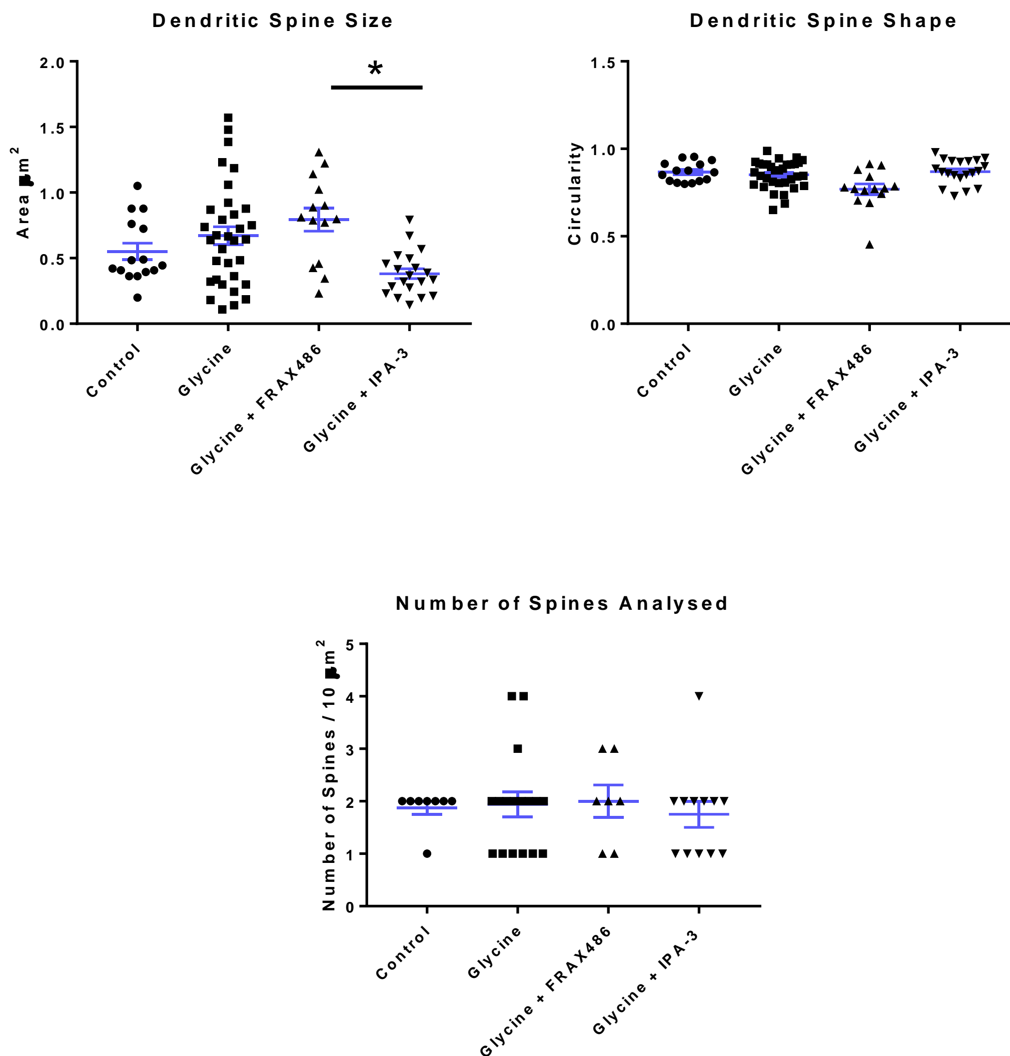


Figure 5.9 Different Group I PAK inhibitors produce distinct effects on structural plasticity. Area (µm²): Control: 0.53 ± 0.05, n = 3 coverslips, 8 neurons, Gly: 0.65 ± 0.05, n = 9 coverslips, 15 neurons, FRAX486+Gly: 0.79 ± 0.09, n = 2 coverslips, 4 neurons, Gly+IPA-3: 0.38 ± 0.04, n = 3 coverslips, 4 neurons. Data shown as mean ± SEM. *$p < 0.05$ by Kruskal-Wallis.

5.3.5 Chemical LTP induces GluA2-AMPAR synaptic expression

The heteromeric AMPARs containing GluA1/GluA2 and GluA2/GluA3 are the most commonly expressed in adult neurons in the CA1 region of the hippocampus, whereas only 8% of total AMPARs are homomeric GluA1 receptors (Lu et al., 2009; Wenthold et al., 1996). A substantial body of evidence suggests that the recruitment of additional AMPARs to the synaptic surface is necessary for LTP (Granger & Nicoll, 2014; Penn et al., 2017). The reason behind the impairment of LTP observed following inhibition of Group I PAK using IPA-3 might be explained by a reduction in activity-dependent AMPAR recruitment to the synapse. Therefore, in order to identify a method to test the mechanism by which Group I PAK inhibition impairs LTP, we tested a different stimulation protocol found to produce an increased amount of synaptic surface AMPARs (Groc et al., 2008; Lu et al., 2001).

First, we aimed to test whether the chemical LTP (chemLTP) protocol consisting of a 3-min application of glycine (200 μM) and picrotoxin (1 μM) elicited significant changes in GluA2-AMPAR expression at the synapse. We observed that Gly/Pic increased GluA2-AMPAR density at the synapse (Control: 967 ± 396 Intensity/Area(pixels), n = 2 coverslips, 3 neurons; Gly: 1867 ± 342, n = 2 coverslips 4 neurons, KS statistic = 0.1563, $p = 0.0003$, **Figure 5.10** and **Figure 5.11**). This result indicates that cLTP could be used to test whether preincubation with Group I PAK inhibitors prevent the activity-dependent increase of surface GluA2-AMPARs at the synapse, thereby providing a method to test the mechanism involved in IPA-3-mediated LTP impairment.

One limitation is that we did not test the specificity of the GluA2 antibody. If the specificity of the GluA2 antibody is low, then it means that the antibody might not have recognised the target protein correctly or that there was non-specific binding with other proteins and therefore our GluA2-containing AMPAR quantification would be inaccurate. For future experiments, it would be useful to test the GluA2-antibody specificity by omitting this anti-GluA2 primary antibody, and by using neurons from GluA2-knockout mice or cell line as negative controls. However, the

anti-GluA2 antibody we used for this experiment has been previously used by other researchers in immunocytochemistry experiments to stain receptors in dissociated neuronal cultures from mice and rats (Ho et al., 2014; Vazquez-Sanroman et al., 2015).

Future experiments should examine whether the GluA1-containing AMPARs recruitment to the synaptic surface are dysregulated by PAK inhibition as LTP induction triggers the trafficking of GluA1 homomers to the synapse, but these receptors remain there transiently before they are replaced by GluA2-containing AMPARs (Plant et al., 2006).

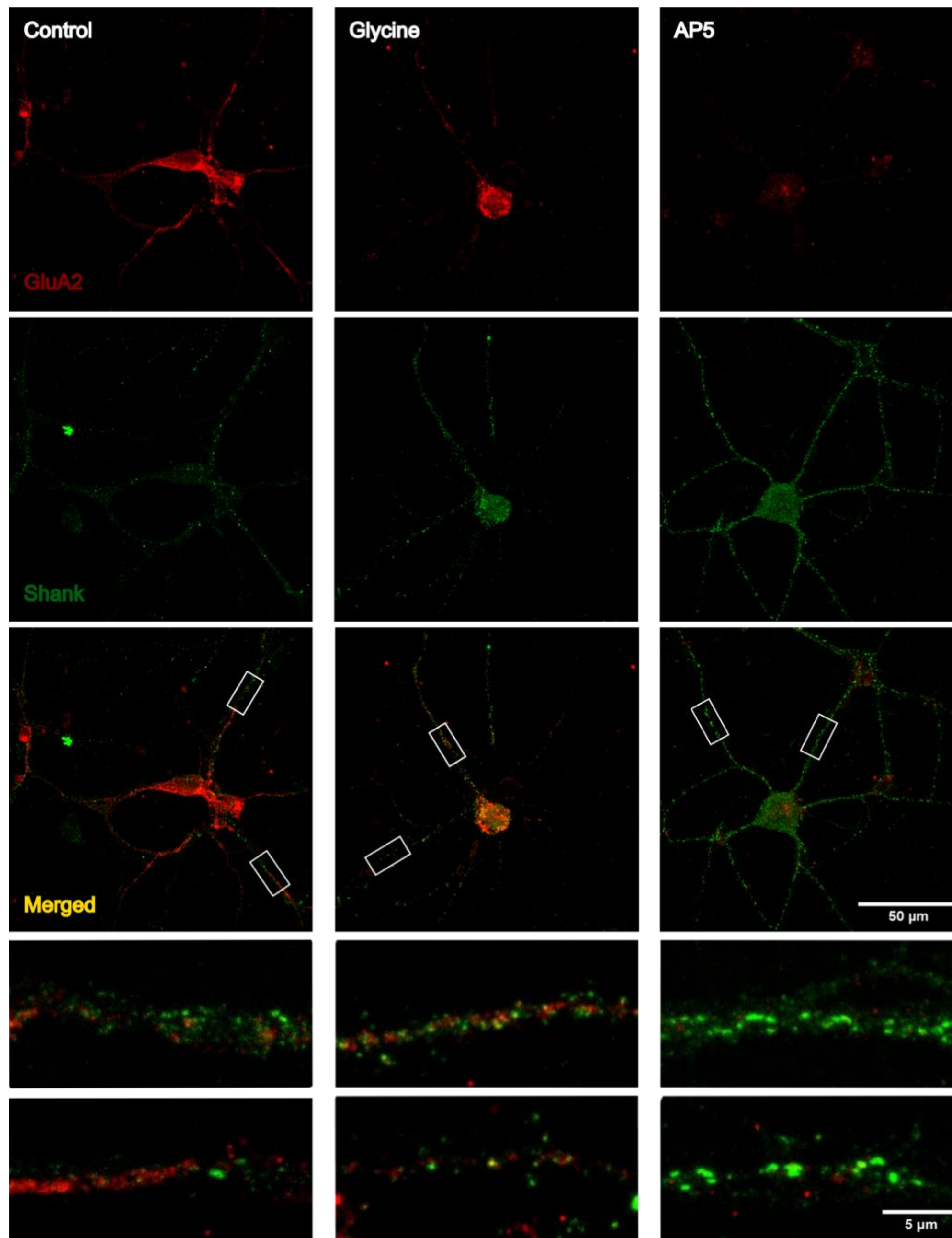


Figure 5.10 ChemLTP increases GluA2-AMPAR subunits on the dendritic spine surface of primary cultured hippocampal neurons. Top left: example of control neuron incubated in aCSF for 10 minutes. **Middle:** example of neuron incubated in aCSF with Glycine (200 µM) for 10 minutes. **Right:** example of neuron preincubated with AP5 before treating with aCSF and Glycine (200 uM) for 10 minutes. **Bottom:** examples of analysed ROIs of dendritic branches of 20 pm in length.

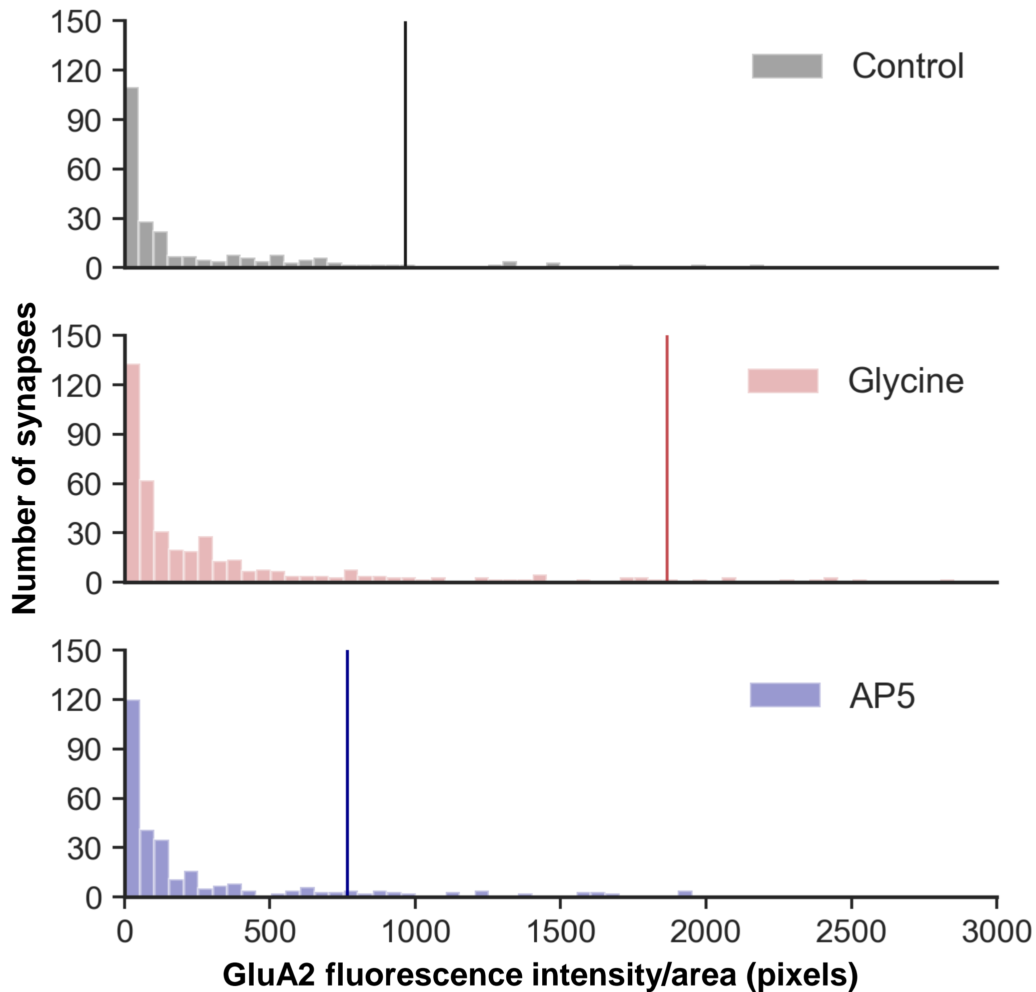


Figure 5.11 Effect of glycine on the content of synaptic GluA2-AMPARs. Distributions of the synaptic density of GluA2-AMPARs. **Top:** control (average Fluorescence Intensity/Area(pixels): 967 ± 396, n = 2 coverslips, 3 neurons). **Middle:** 10 minutes after glycine application (average: 1867 ± 342, n = 2 coverslips, 4 neurons; Kolmogorov-Smirnov statistic = 0.1563, p = 0.0003 compared to control). **Bottom:** 10 minutes after glycine application in neurons preincubated with AP5 (average: 767 ± 118, n = 2 coverslips, 4 neurons).

5.3.6 Chemical LTP is dependent on NMDAR function

Importantly, induction of LTP in the area CA1 of the hippocampus is dependent on NMDAR activation (Bliss & Collingridge, 1993). Our results thus far indicate that we could test whether Group I PAK inhibition by IPA-3 impairment of LTP is mediated by reduced synaptic AMPAR expression. However, we first aimed to assess whether chemLTP stimulation-mediated increase in synaptic surface GluA2-AMPARs is dependent on NMDAR activation. In order to test this, we incubated cultured hippocampal neurons with the NMDAR inhibitor AP5 (50 µM) before applying the chemLTP stimulus. Incubation of AP5 prior to chemLTP stimulus prevented the significant increase of synaptic surface GluA2-AMPARs observed with the chemLTP stimulus alone (Control: 967 ± 396 Intensity/Area(pixels), n = 2 coverslips, 3 neurons, Gly: 1867 ± 342, n = 2 coverslips, 4 neurons, AP5: 767 ± 118, n = 2 coverslips 4 neurons, ANOVA on Ranks $p = 0.001$, **Figure 5.10** and **Figure 5.11**). These findings provide support for the use of chemLTP as a method to assess whether Group I PAK inhibition of LTP might be mediated by reduced AMPAR recruitment to the synapse.

5.4 Discussion

5.4.1 PAK inhibition does not change the fEPSP waveform

Detailed analysis of fEPSP waveform kinetics have been used to determine if there are differences in synaptic integration between groups of neurons (Petersen et al., 2013). Therefore, we analysed fEPSP waveform parameters to assess whether there were changes in synaptic integration in untreated (control) slices and slices treated with the Group I PAK inhibitor IPA-3. However, the only significant changes observed were in the values of peak latency of control slices after LTP induction. This was expected as the responses measured in control slices underwent potentiation, measured by increased fEPSP slope values which in turn would reduce the peak latency.

One limitation of this analysis is that we did not properly assess the kinetics of the decay phase of the fEPSP responses. This could be done by using a curve fitting function to determine if there were differences in the rate of decay between fEPSPs from controls and IPA-3. It was found that NMDAR-mediated currents show a biexponential decay phase in dentate granule cells in rat hippocampal slices (Keller et al., 1991). Therefore, further work should include this analysis as it may provide an estimate for testing whether postsynaptic receptor NMDAR open-channel properties in the area CA1 of the hippocampus also decay biexponentially, and if their properties are modified by PAK inhibition.

5.4.2 Calcium signalling and PAK inhibition

Calcium influx through NMDARs is essential for the initiation of biochemical cascades that induce LTP and dendritic spine structural changes (Kennedy et al., 2005). If inhibition of Group I PAKs resulted in dysregulation of Ca^{2+} influx, then the signalling cascades necessary to induce LTP would be altered, consequently this result would explain LTP impairment caused by the Group I PAK inhibitor IPA-3. To test whether Group I PAK inhibition resulted in Ca^{2+} dysregulation, we analysed intracellular Ca^{2+} changes using the Ca^{2+} indicator Flou4-AM. However, our results suggest that perfusion of neither IPA-3 nor FRAX486, Group I PAK inhibitors produced significant changes in intracellular Ca^{2+}, while perfusion of KCl which produces neuronal depolarisation led to an immediate increase in intracellular Ca^{2+}. Thus, the inhibition of LTP mediated by IPA-3 cannot be explained by dysregulated Ca^{2+} signals.

One limitation of these experimental protocol is that PAK inhibition-mediated LTP impairment may specifically affect dendritic spine Ca^{2+} signals, as these structures have been hypothesised to be isolated signalling compartments (Bloodgood et al., 2009; Müller & Connor, 1991). However, our results were taken as measurements of intracellular Ca^{2+} concentration from the soma of cultured hippocampal neurons. It is now possible to image Ca^{2+} signalling in dendritic spine heads using two-photon laser scanning microscopy, glutamate uncaging and Ca^{2+}-indicators in acute hippocampal brain slices (Sobczyk et al., 2005). As glutamate uncaging

mimics neurotransmitter release from presynaptic inputs, using this paradigm in future studies may provide a better estimate of Ca^{2+} concentration changes in order to assess whether inhibition of Group I PAKs dysregulates Ca^{2+} signalling.

5.4.3 Chemical LTP: a model for rapid pharmacological assays

Although the labelling and imaging methods used in this study to asses spine morphology changes were not sufficiently sensitive, our results suggest that Group I PAKs are involved in chemLTP-induced morphology changes. However, whether PAKs are also involved in the accumulation of AMPARs at the synaptic surface is still unclear. Our results using chemLTP to assess the expression of GluA2 at the synaptic surface suggest that the protocol used might be a useful protocol to assess whether PAKs are involved in AMPAR trafficking to the synapse. Therefore, future work should focus on determining whether Group I PAKs mediate the expression of AMPAR GluA2 or GluA1 subunits using the chemLTP and GluA subunit labelling protocol. As AMPAR incorporation at postsynaptic sites is crucial for LTP induction (Granger et al., 2013), the results of the aforementioned future experiments may explain how Group I PAK inhibition results in LTP impairment.

These data must be interpreted with caution because we did not confirm experimentally if chemLTP was induced in cultured neurons. Several reports have shown that glycine stimulation leads to activation of NMDARs, increase in intracellular calcium, activation of CaMKII, insertion of AMPARs at the synaptic surface, in addition to enhanced amplitude and frequency of mEPSCs (Lu et al., 2001; Molnár, 2011). We confirmed that glycine stimulation was followed by an increased in synaptic GluA2-containing AMPARs, and that this effect was dependent on NMDAR activation.

However, future investigations could confirm whether chemLTP was induced in cultured neurons by measuring whether intracellular calcium is increased following glycine stimulation using the calcium indicator Fluo-4AM; the activation of CaMKII could be detected by assessing whether CaMKII inhibitors KN-62 or KN-93 block

the increase in surface AMPAR at the synapse (Matsuzaki et al., 2004; Steiner et al., 2008), or by identifying whether glycine stimulation increases CaMKII autophosphorylation at Thr286 (Oh & Derkach, 2005); The insertion of GluA1-containing AMPARs at the synaptic surface by staining cultured hippocampal neurons with an anti-GluA1 antibody against the amino-terminal extracellular epitope under non-permeant conditions and measuring colocalization with a synaptic marker; Finally, we could also measure whether the frequency and amplitude of mEPSCs are increased following glycine perfusion (Lu et al., 2001).

Another limitation is that the cultured hippocampal neurons used in these experiments were not preconditioned with the NMDAR antagonist APV prior to glycine stimulation. The presence of APV in the growth medium promotes the upregulation of NMDARs and when the antagonist is removed, glycine application enhances the activation of NMDARs (Molnár, 2011). Further research should be undertaken to investigate the effects of PAK inhibition on dendritic spine morphology and receptor trafficking by preconditioning cultured neurons before glycine-induced LTP.

CHAPTER 6

General Discussion

5.1 Summary of the results

5.2 The role of PAKs in synaptic plasticity: regulation of receptors or structure?

5.3 PAKs in synaptic regulation and possible therapeutic translation for dementia

5.4 Conclusion

Chapter 6 – General discussion

6.1 Summary of the results

In Chapter 3, we analysed the effects of Aβ and α-synuclein oligomerisation on LTP impairment. The results of this chapter suggest that a sufficient amount of oligomerisation is required to inhibit LTP. Additionally, the pathophysiology of Aβ and α-synuclein may be different as the canonical GSK-3β pathway triggered by Aβ was not involved in α-synuclein mediated pathogenesis on LTP. Finally, when oligomers from different sources are found together their adverse effects are magnified.

In Chapter 4, Group I PAKs were found to have a role in LTP induction, although different inhibitors produced different results. While analysing the mechanisms underlying the effect of PAKs on LTP, we found that inhibition of Group I PAKs do not interfere with the level of depolarisation resulting from tetanic stimulation. Furthermore, artificially stimulating Group I PAKs activator Cdc42 using bradykinin did not affect LTP.

In Chapter 5, we aimed to test whether PAK inhibition affects the molecular signals required for LTP induction. It was found that PAK inhibitors do not alter intracellular calcium concentration. Our results show that Group I PAKs are involved in activity-induced morphology changes in dendritic spines. Furthermore, the chemLTP protocol used in this study results in increased GluA2-AMPARs at the synaptic surface, making it a good protocol to test whether PAKs are involved in AMPAR trafficking to the synapse.

6.2 The role of PAKs in synaptic plasticity: regulation of receptors or structure?

In this study, it was found that applying the PAK inhibitor IPA-3 only blocked LTP at certain time-points which suggests that the target of PAKs is required for certain parts of the LTP process. This is consistent with the findings of Murakoshi *et al* 2011. In this paper, they found that sLTP has two phases: a transient phase and a sustained phase where RhoGTPases were activated Rho, Rac1 and Cdc42. Additionally, when PAK was inhibited only the sustained phase was affected. These findings suggest that PAK function allows LTP to progress into subsequent steps. However, the specific targets or function of PAKs on components of the signalling cascades leading to LTP expression is still unclear.

To explore the possible specific functions of PAKs cultured hippocampal neurons were used instead of hippocampal slices to analyse a larger proportion of synapses in a simpler system. First, we measured intracellular calcium concentration as the influx of calcium is essential for CaMKII activation and LTP induction. We found that PAK inhibitors do not alter calcium influx. However, these results were obtained by studying neurons under basal conditions and future work should test the effects of PAK inhibition on calcium uptake following activation. The next step was to assess the effects of PAKs on activity-induced morphology changes in dendritic spines, as PAKs are known regulators of the actin cytoskeleton. To do this, we used a chemLTP protocol consisting of bath application of glycine and picrotoxin to cultured hippocampal neurons known to produce spine enlargement. Our results suggest that PAK inhibition reduce spine growth triggered by chemLTP. This result is consistent with other studies demonstrating a role of PAKs on activity-dependent dendritic spine morphology changes. Indeed, structural modification is thought to be required for the expression of plasticity as blocking actin polymerisation impairs LTP (Fukazawa et al., 2003) and patterned stimulation results in LTP and spine enlargement

(Matsuzaki et al., 2004; Okamoto et al., 2004). Therefore, this role of PAKs in the regulation of the actin cytoskeleton could link them with plasticity regulation.

In addition, we explored the involvement of PAKs in AMPAR trafficking. We observed that the induction of chemLTP resulted in increased GluA2-AMPARs at the synaptic surface, which was dependent on NMDAR activation. Our results are consistent with experiments that observed an increase in GluA2 labelling at the PSD in response to a similar glycine-based chemLTP stimulation protocol (Tao-Cheng et al., 2011). However, we did not test the effect of PAK inhibition on AMPAR trafficking. Therefore, the problem we cannot conclude from our results that PAK inhibition interferes with AMPAR exocytosis. Nevertheless, there is evidence that glycine stimulation enhances GluA1 and GluA2 trafficking into spines, via a Rac1-PAK-LIMK-dependent pathway (Fortin et al., 2010). Therefore, these findings suggest that PAK might have a role in AMPAR trafficking necessary for NMDAR-dependent LTP.

One possible explanation for the involvement of PAKs in receptor trafficking is that PAK may be required for the proper function of other proteins involved in exocytosis. Direct evidence suggesting that the source of AMPARs mobilised during LTP are transported from endosomes to the synaptic membrane came from studies by Park *et al.* (2004, 2006). The small GTPase Rab11a and Rme1 are proteins required for recycling endosome transport. The expression of dominant negative forms of Rab11a and Rme1 prevented transport from recycling endosomes resulting in the inhibition of glycine-dependent increase in AMPARs at the synaptic surface (Park et al., 2004, 2006). Moreover, blocking SNARE-complex dependent recycling endosome fusion to the plasma membrane by the expression of a transmembrane domain lacking syntaxin13 (syn13ΔTM) abolishes glycine-induced AMPAR exocytosis, spine growth and LTP (Park et al., 2004, 2006). And, exocytosis in spines not only provides a method to transport proteins to the plasma membrane, it could also provide membrane components that would

expand the spine surface area (Patterson & Yasuda, 2011). These results suggest that LTP stimuli increase recycling endosome trafficking to the plasma membrane, which is necessary for spine growth and increased AMPAR-mediated synaptic transmission.

Turning now to more specific mediators of AMPAR exocytosis during LTP, the insertion of GluA-1 containing AMPARs into the plasma membrane in response to glutamate uncaging was found to be mediated by the small GTPase Ras-Raf-MEK-ERK pathway (Patterson et al., 2010). In addition, the Ras signalling pathway drives AMPAR delivery to the plasma membrane during LTP. A study by Zhu *et al.*, showed that the expression of constitutively active Ras mimics LTP by enhancing AMPAR-mediated synaptic transmission, whereas neurons expressing the dominant negative form of Ras did not exhibit pairing-induced LTP. Furthermore, it was reported that pairing-induced LTP was blocked by a MEK inhibitor (Zhu et al., 2002). Returning to the issue of the role of PAKs in exocytosis, PAKs could have an influence in Ras-mediated trafficking of receptors as it has been reported that PAK activates Raf1 (King et al., 1998) and MEK1 (Frost et al., 1997). Therefore, these findings suggest the existence of biochemical pathways linking PAK activity with AMPAR trafficking to the synapse which is associated with both functional and structural LTP.

Another interesting point to note that one member of the Group I PAKs was found to be involved in regulating glutamate receptor trafficking. In a study by Hussein *et al.* a previously unknown GluA1-AMPAR subunit phosphorylation site serine 863 (S863) was found to regulate GluA1 trafficking. The specific signalling pathway is triggered by EphB2 receptor activation, where EphB2 interacts with a guanine nucleotide exchange factor (Zizimin1) that activates Cdc42. This, in turn activates PAK3, and ultimately resulting in phosphorylation of S863 by PAK3 (Hussain et al., 2015). Recruitment of glutamate receptors to the postsynaptic membrane is required for the manifestation of synaptic plasticity, as hindering

AMPAR trafficking to the synapse inhibits synaptic potentiation (Granger & Nicoll, 2014; Penn et al., 2017). Therefore, these findings provide supporting evidence for the existence of a link between PAKs with synaptic plasticity through the regulation of AMPAR trafficking.

Furthermore, a possible role for PAK is the maintenance of RhoGTPase activity to mediate local protein synthesis which sustains LTP. RhoGTPases are activated by guanine nucleotide exchange factors (GEFs). It was found that PAK forms binds to α and β-PAK-interacting GEFs (αPIX and βPIX) which drives activation of Rac1 or Cdc42 (Manser et al., 1998). In turn, the scaffolding protein Shank was reported to form a complex with βPIX and PAK (Park et al., 2003). And, in a study by Saneyoshi *et al.* showed that the overexpression of Shank led to an increase of phosphorylated βPIX and PAK in dendritic spines. In the same study, the inhibition of NMDAR activity suppressed the phosphorylation of βPIX and the activation of Rac1, which resulted in decreased spine density. The effects of inhibiting this pathway were prevented by the expression of a constitutively active PAK1 (Saneyoshi et al., 2008). Furthermore, activation of Rac1 activates the PI3K-Akt-mTOR pathway which regulates ribosomal translation. This pathway may drive local dendritic protein synthesis necessary to stabilise LTP (Kennedy et al., 2005; Klann & Dever, 2004). Therefore, it might be the case that once PAKs form complexes with PIX and binds to Rac1 the complex retains its activation, and that produces local protein synthesis necessary for LTP.

An important limitation of this study was that we did not assess the effects of chemLTP on the delivery of GluA2-lacking AMPARs to the synaptic surface and whether PAK inhibition affected it. This is an important issue as trafficking of GluA2-lacking AMPARs to the synapse is crucial for LTP induction and as previously discussed, PAKs could be involved in AMPAR exocytosis (Y. Hayashi et al., 2000; Zamanillo et al., 1999). The omission of this experiment means that we cannot definitively link PAK function with AMPAR trafficking. However, given that we do show that blocking PAK during LTP induction in hippocampal slices

prevents LTP expression a likely explanation still remains a mechanistic relationship between PAK and glutamate receptor regulation.

The next limitation is that we only assessed hippocampal samples. Synaptic plasticity occurs in other areas, e.g., the motor cortex, the visual cortex, and the amygdala (De Pasquale et al., 2014; Iriki et al., 1989; Maren, 1999). Indeed, PAK is located in different regions not only the hippocampus (Koth et al., 2014). Therefore, whilst we can only argue with some certainty that PAK is required for LTP in the hippocampus whether its role extends to plasticity in other regions remains to be shown.

Finally, another limitation is that we only used pharmacological compounds to inhibit PAKs, therefore cannot account for possible off target effects of the inhibitors used. For instance, it was assumed that IPA-3 blocked LTP but another unknown factor affected by the inhibitor may have mediated the LTP inhibition effect. Alternatively, studies genetically manipulation by shRNA and transgenic animals to target a specific PAK family member have also reported that both PAK1 and PAK3 impair LTP (Asrar et al., 2009; Meng et al., 2005). Research using shRNA and transgenic tools are more specific, but they also have the disadvantage of the possibility of compensation by other PAK isoforms. Whilst our approach has limitations, its advantage is that it allowed us to block PAKs at different stages of LTP induction through extracellular perfusion. Therefore, our approach targeted PAKs directly and to test effects at different timepoints which could not have been achieved by genetic manipulation.

In conclusion, LTP is a complex process that is triggered by a combination of a plethora of postsynaptic events that lead to actin cytoskeleton rearrangements and AMPAR trafficking to synaptic surfaces, among others (Cingolani & Goda, 2008; Derkach et al., 2007; Murakoshi & Yasuda, 2012). A likely scenario is that

PAKs are involved in a dual-pathway, where they, in plasticity, are required for both the trafficking of receptors and synapse remodelling.

6.3 PAKs in synaptic regulation and possible therapeutic translation for dementia

In this study Group I PAKs were found to be necessary for synaptic plasticity and involved in dendritic spine structural changes, given that inhibition of Group I PAKs impaired LTP and chemLTP-associated spine enlargement. However, we did not assess the effects of PAKs in under pathological conditions.

PAK has previously been shown to be critical in postnatal growth and attainment of normal brain size and function in mice (Huang et al., 2011). It has also been reported to participate in spine stabilisation and spine growth associated with learning (Dubos et al., 2012). Arsenault *et al.* postulate that PAK pathways are crucial to the health of synapses and therefore are a likely therapeutic target for AD (Arsenault et al., 2013). Accumulating evidence from neuropathological studies suggests that synapse loss is a major component of many neurodegenerative diseases associated with dementia. Structural changes in dendritic spines have been observed in samples from AD patients (Cochran et al., 2014; DeKosky & Scheff, 1990; Penzes et al., 2011). Although neuronal loss in dementia with Lewy bodies is less prevalent than in Parkinson's disease, animal models of α-synucleinopathies suggest that α-synuclein alters dendritic spine morphology (Froula et al., 2018; Kramer & Schulz-Schaeffer, 2007). Interestingly, a study found that PAK4 has a neuroprotective role for signalling pathways and suggested that therefore may be a useful therapeutic target for PD (Won et al., 2016).

Collectively, these findings indicate that more research needs to be done to understand the role of PAKs in the context of neurodegenerative disorders. This is a compelling issue for future research as spine density reductions correlate

negatively with cognitive ability (Terry et al., 1991). Therefore, the understanding of the roles of PAKs could offer a unique way to find therapies address both synapse loss and synapse dysfunction.

6.4 Conclusion

In general, these experiments have confirmed that neurodegeneration is likely produced by aggregated protein oligomers which may trigger various adverse signalling cascades resulting in alterations in synaptic function and structure. In addition, we found that Group I PAK proteins are involved in both synaptic plasticity and synaptic morphology changes. This makes Group I PAKs key targets for study in the search for developing new treatments to ameliorate the symptoms of dementia.

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