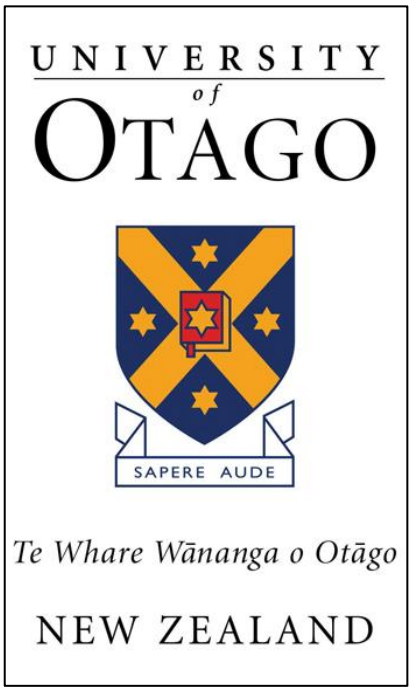
The Effects of Pharmacological Preconditioning with GYKI-52466 and Domoic Acid on LTP and LTD Induction in the Rat Hippocampus



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Abstract

Many neurodegenerative diseases are associated with severe memory loss and cognitive impairment, highlighting a critical demand for the development of neuroprotectants and nootropics. It has been shown that certain compounds can trigger lasting neuroprotective mechanisms. This phenomenon is called 'pharmacological preconditioning,' and it has recently been suggested that preconditioning may also enhance cognitive function. Indeed, preconditioning with GYKI-52466 and domoic acid (DOM) has prophylactic neuroprotective efficacy *in vivo* and *in vitro*, and preliminary *in vitro* results demonstrate their ability to enhance long term synaptic potentiation (LTP) and long term depression (LTD), thus denoting nootropic potential. The aim of the present study was develop an effective *in vitro* preconditioning strategy using GYKI-52466 or DOM, and clarify their effects on LTP and LTD induction in the rat hippocampus.

Hippocampal slices from male Sprague Dawley rats were subject to acute or chronic preconditioning with 6 µM GYKI-52466, or acute preconditioning with 50 nM DOM. Control slices were not preconditioned. Slices subsequently underwent LTP or LTD induction, and electrophysiological techniques were used to assess the response to this. Orthodromic Schaffer collateral-evoked CA1 population spikes and field excitatory postsynaptic potentials (fEPSP) were monitored before and after LTP or LTD induction. Data were expressed as mean percentage change from baseline (± SEM) and group differences compared to controls at a 30 minute time-point post LTP or LTD induction was determined by an unpaired student's *t*-test at a confidence level of $P<0.05$.

GYKI-52466 and DOM preconditioning failed to enhance LTP and LTD induction. Both control and preconditioned slices exhibited comparable magnitudes of

LTP and LTD for population spike amplitude, area and fEPSP slope, with no significant differences between control and preconditioned slices evident at a 30 minute time-point. These findings suggest that preconditioning with GYKI-52466 and DOM would not confer nootropic potential.

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List of Abbreviations

°C – Degrees Celcius

ACSF - Artificial Cerebrospinal Fluid

AIDA - 1-aminoindan-1,5-dicarboxylic acid

AMPA - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APV - D-2-amino-5-phosphonovaleric acid

CA1 - *cornu ammonis* 1

CA2 - *cornu ammonis* 2

CA3 - *cornu ammonis* 3

CA4 - *cornu ammonis* 4

$CaCl_2$ – Calcium Chloride

CaMKII - Calcium Dependent Protein Kinases

cAMP - Cyclic Adenosine Monophosphate

CNQX - 6-cyano-7-nitroquinoxaline-2,3-dione

CNS - Central Nervous System

CO_2 – Carbon Dioxide

CPPG - RS-alpha-cyclopropyl-4-phosphonophenylglycine

CSD - Cortical Spreading Depression

DG - Dentate Gyrus

DOM - Domoic Acid

EC - Entorhinal Cortex

EC_{50} – Half Maxima Effective Concentration

EGLU - 2S-alpha-ethylglutamic acid

EPSP - Excitatory Post Synaptic Potential

fEPSP - Field Excitatory Post Synaptic Potential

GABA - Gamma-aminobutyric acid

GPCR - G-protein Coupled Receptor

GTPase – Guanosine Triphosphatase

GYKI-52466 (GYKI) - 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine

IC_{50} – Half Maximal Inhibitory Concentration

KA - Kainic Acid

KCl – Potassium Chloride

LTD – Long term Depression

LTP – Long Term Potentiation

MF - Mossy Fibres

$MgCl_2$ – Magnesium Chloride

mGluR - Metabotropic Glutamate Receptor

$MgSO_4$ – Magnesium Sulfate

MK-801 - Dizocilpine

NaCl – Sodium Chloride

NaH_2PO_4 – Sodium dihydrogen Phosphate

$NaHCO_3$ – Sodium Hydrogen Carbonate

NMDA - N-methyl-D-aspartate

O_2 - Oxygen

OGD - Oxygen Glucose Deprivation

PKA - Protein Kinase A

PKC - Protein Kinase C

PP - Perforant Path

SC - Schaffer Collateral-Commissural Pathway

1 Introduction

1.1 The Need for 'Brain Medicine'

'Neurodegenerative disease' is an umbrella term used to describe the range of central nervous system (CNS) disorders encompassing neuronal damage or loss (1). Common examples of such diseases include Alzheimer's disease, Parkinson's disease and multiple sclerosis, however alternative medical conditions such as stroke and epilepsy can cause similar neuronal damage (2, 3). While many tissues throughout the human body are capable of replacing their dead or damaged cells, the human brain lacks this regenerative capacity, leaving it particularly vulnerable to injury or disease (4). Consequently, neurodegenerative diseases almost inevitably have detrimental effects on a patient's quality of life, frequently impairing cognition, memory, movement and coordination (1, 4). Although there are a number of treatment strategies available, the efficacy of these are limited and often temporary, tending to slow disease progression while having little or no impact on the final disease outcome (1, 5, 6).

The economic impact of neurodegenerative diseases is immense, and the emotional burden for patients and families is virtually incalculable (6). Treating Alzheimer's disease alone was estimated to have cost US $604 billion worldwide in 2010 (7) and with a rapidly aging population, the challenges that neurodegenerative diseases pose for our society will only increase (6). This highlights the critical demand for the development of neuroprotectants and nootropics (compounds that enhance mental processes such as memory, learning, attention and cognitive function), in a field almost completely devoid of effective pharmaceuticals thus far (8). However, due to the frequently irreversible nature of neuronal damage, prophylactic neuroprotection, as opposed to retrospective treatment, would be preferable (8-10). Prevention could

improve long term survival and functional outcomes for patients at a known risk of certain neurodegenerative disorders, and would be useful prior to any situations entailing probable neuronal damage, such as brain or cardiac surgery when blood flow to the brain may be disrupted (11).

Although the search for such agents is not easy, scientific interest in the field is growing rapidly, and a novel breakthrough may therefore be just around the corner. Indeed, the concept of preconditioning - or 'induced tolerance' - is gaining momentum as a prophylactic treatment strategy, and may well be the answer to the long awaited search for neuroprotectants.

1.2 Preconditioning

Traditional neuroprotective strategies have targeted the pathological processes behind neuronal damage (12, 13), such as excitotoxicity (14-16), apoptosis (17), inflammation (18) and oxidative damage (12). Such therapies include sodium and calcium channel blockers (13, 19, 20), gamma-aminobutyric acid (GABA) receptor agonists (21), and free radical scavengers (13, 22). Particular focus has also been on the use of glutamate receptor antagonists, given the pathological role of excess glutamate in neuronal damage and neurodegenerative disorders (15, 16). However, while these traditional approaches have demonstrated enormous capacity for neuroprotection in a range of preclinical studies, they have frequently failed in clinical trials, and have been associated with severe side effects (23-30). In fact, a review on stroke therapies published in 2002 stated that, "of more than 49 neuroprotective agents studied in over 114 stroke trials, none have proven successful clinically" (31).

However, one of the most promising approaches to prophylactic neuroprotection currently is the concept of induced tolerance, or 'preconditioning,' a term coined by

Janoff in 1964 (32). Since the initial suggestion by Noble over half a century ago (33), it has been repeatedly demonstrated that mild 'insults' to an organ confer tolerance to subsequent severe insults, signifying the presence of endogenous protective mechanisms throughout the body (34-37). In particular, the brain has an astounding capacity for self-protection - perhaps as a compensatory mechanism for lacking regenerative capacity – which makes it an ideal target for preconditioning (12). Indeed, noxious stimuli below the threshold for neuronal damage can activate endogenous neuroprotective mechanisms that ultimately lessen the impact of any further damage (34, 35, 38-49). Preconditioning can be triggered by a range of mild stimuli, such as hypoxia (35), cortical spreading depression (CSD;(44)), oxygen glucose deprivation (OGD;(45)), seizures (42), excitotoxins (47-49), hypothermia (15) and anaesthetics (46, 50), and has reduced damage associated with cerebral and cardiac ischemia (34, 38, 39), hypoxia (11, 35), seizures (40-43), OGD (45) and neurotoxins (42, 47-49). Endogenous protection is not a phenomenon restricted to the brain however, having also been demonstrated in the heart (34, 51, 52), lungs (36), liver (36, 53), and kidney (37).

The earliest demonstrations of preconditioning, which date back to 1986, focussed on rapidly inducible tolerance, whereby protection against tissue damage occurred within minutes of the preconditioning stimulus being administered (34, 35, 38). However, delayed preconditioning was later observed by Kitagawa *et al.*, who reported that mild ischemic insults in adult gerbils protected the brain from subsequent ischemia one day later (39). It has since been shown that repetitive preconditioning can induce protracted tolerance that lasts up to four weeks (54), sparking interest about the mechanisms involved, and the therapeutic implications of such prolonged protection.

1.2.1 Pharmacological Preconditioning

From a pharmacological and clinical viewpoint, the ability to precondition using noxious stimuli suggests certain drugs may have a similar capacity. Using pharmacological compounds rather than a physical stimulus to trigger the signalling cascades of preconditioning is a concept dubbed 'pharmacological preconditioning'. However, progress in this area is limited by our incomplete understanding of the mechanisms behind endogenous neuroprotection (9). Pre-clinical trials have assessed pharmacological preconditioning using drugs already tested in traditional, post-conditioning settings, namely glutamate receptor antagonists and GABA receptor agonists (55), and have also assessed novel drugs targeting molecules or pathways shown to be altered by preconditioning with noxious stimuli.

1.2.1.1 *GYKI-52466*

There has been particular focus in the Kerr lab at the University of Otago on the use of the benzodiazepine derivative GYKI-52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine; hereafter referred to as GYKI) as a pharmacological preconditioning agent. GYKI is conventionally considered a negative allosteric modulator of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA) type glutamate receptors, with IC_{50}'s around 11-22 μm (56, 57), and 7.5 μm (57), respectively. Consistent with its activity as an ionotropic AMPA receptor blocker, GYKI administration has been associated with an adverse side effect profile including sedation, confusion and ataxia, which limits its therapeutic utility at these doses (8, 58). However, GYKI has also recently demonstrated inverse agonist activity at G-protein coupled receptors (GPCRs), indicating a novel metabotropic mode of action (48). The exact mechanism remains somewhat elusive as the specific G-protein implicated is unknown, highlighting a focus for future studies.

GYKI is an effective anticonvulsant and anaesthetic in animal models (57, 59), and has also demonstrated prophylactic neuroprotective efficacy when administered acutely (8, 11, 48, 59). *In vivo* studies have tested GYKI preconditioning against a range of neurological insults (8, 11, 59). Block *et al.* reported that functional deficits and neuronal damage following global ischemia were reduced by GYKI preconditioning in rats (59), and likewise that KA induced seizure severity in rats was diminished by GYKI preconditioning (8). Furthermore, Nayak and Kerr have recently reported long term neuroprotection against hypoxic-ischemic brain injury, evidenced by an improvement in sensorimotor tests, and a reduction in seizure activity, infarct volume and ventricular enlargement in rats pre-treated with GYKI (11). *In vitro, l*ow dose GYKI preconditioning was shown to impart tolerance against KA-induced toxicity in the rat hippocampus (48). These results have not been reliably reproduced, however, and consequently GYKI has never been tested in clinical trials. Nevertheless, certain drawbacks of *in vitro* studies may mask a compound's true neuroprotective efficacy, such as the dissection potentially rendering the tissue too damaged to respond to preconditioning agents. The tolerance induced *in vivo* by GYKI has lasted for 12 weeks after preconditioning thus far (11), indicating that its lasting metabotropic action may underpin these protective actions, rather than its classical blockade of AMPA receptors.

1.2.1.2 *Domoic Acid*

Domoic acid (DOM) is a neurotoxin produced by marine dinoflagellates (49) and as a function of its structural similarity with glutamate, is also a potent agonist at AMPA and KA-type glutamate receptors. DOM administration has been shown to result in hyperexcitability and induced seizures in experimental animals (due to continuous activation of the AMPA and KA receptors), thus causing tonic depolarisation of neuronal cells (60). DOM has therefore been widely used to model neurodegenerative

diseases, and has provided a way of enhancing understanding of the pathology of many neurological disorders (61).

In 1987, DOM was identified as the causative agent in a case of human poisoning in Canada following ingestion of contaminated mussels, causing serious neurological deficits including amnesia, seizures, and in some cases, death, particularly in the elderly (47). Since this incident, studies have generally investigated the detrimental effects of high doses of DOM. However, recent work, particularly in the Kerr lab, has focussed on the potential therapeutic implications of low dose DOM.

Preconditioning hippocampal slices with excitotoxins such as KA and DOM at concentrations so low as to exert no observable effect itself, induces an acute tolerance to the electrophysiological effects of higher doses of these excitotoxins in the rat hippocampus (47-49). For example, *in vitro* preconditioning of hippocampal slices with 50 nM DOM for 30 minutes was sufficient to impart tolerance to the electrophysiological effects of 500 nM DOM (47). This tolerance remained detectable even upon washout of the low dose DOM, again pointing to the likelihood of a metabotropic mechanism underlying preconditioning. Furthermore, *in vivo* preconditioning of rats with low doses of DOM reduced the seizure intensity caused by subsequent high dose DOM 45-90 minutes later (47).

1.2.2 Metabotropic Involvement in Preconditioning

In addition to the lasting nature of tolerance, it is a phenomenon that persists after washout of the preconditioning agent, and can be induced at sub-therapeutic levels (11, 48, 50), all of which suggest metabotropic receptor involvement in preconditioning. This is owing to the fact that GPCRs and downstream second messenger cascades underlie long lasting cellular changes (11, 48), in contrast to the temporary and localised

cellular events mediated by ionotropic receptors. Metabotropic involvement was further evidenced by Hesp *et al.,* who showed that a range of established preconditioning agents uniformly reduced constitutive guanosine triphosphatase activity (GTPase; the intrinsic enzymatic activity of G-proteins) in hippocampal membranes (48). Furthermore, a recent study has identified that tolerance induced by mild ischemia is, in part, mediated by a GPCR coupled to phospholipase C (12).

Despite this strong body of evidence in favour of metabotropic involvement, intriguing results were obtained by Kerr *et al.* in their efforts to unravel the mechanism behind preconditioning (49). Agreeing with metabotropic involvement, they showed that DOM induced tolerance was not prevented by ionotropic glutamate receptor antagonists, namely the N-methyl-D-aspartate (NMDA) receptor blockers D-2-amino-5-phosphonovaleric acid (APV) and dizocilpine (MK-801), and the AMPA/KA blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (49). However, a range of metabotropic glutamate receptor (mGluR) antagonists, including 1-aminoindan-1,5-dicarboxylic acid (AIDA), RS-alpha-cyclopropyl-4-phosphonophenylglycine (CPPG) and 2S-alpha-ethylglutamic acid (EGLU), also failed to prevent tolerance induction. It therefore appears that a novel mGluR is likely involved, one that is not blocked by conventional antagonists (49). One such candidate that may fit this description is the involvement of G-Protein linked AMPA or KA receptors.

1.2.2.1 *G-protein Linked AMPA and KA-Rs*

Although AMPA and KA receptors are classically dubbed ionotropic, a number of studies to date have reported their ability to modulate CNS activity via G-proteins (62-66). In 1997, Wang *et al.* identified cortical AMPA receptors directly coupled to an inhibitory G-protein in rats (66), and this was shortly followed by reports of

metabotropic action of AMPA receptors both in mice cortical neurons (63) and rat retina (65). Furthermore, brief KA exposure was shown to mediate long lasting inhibition of post-spike potassium currents that was not only independent of ionotropic activity, but was also blocked by an inhibitor of pertussis toxin sensitive G-proteins (67). Two independent studies have since demonstrated the existence of G-protein coupled KA receptors (62, 64), but direct evidence for the involvement of metabotropic AMPA or KA receptors in the preconditioning phenomenon remains lacking. If AMPA and KA receptors do indeed display novel metabotropic mechanisms of action, they could represent very promising new therapeutic targets (48).

1.2.3 Alternative uses for preconditioning

Owing to the lasting effects that preconditioning can have on cellular functions and tissue health, this raises an interesting question about possible alternative implications of preconditioning. Indeed, the fact that preconditioning can protect tissues against damage may also mean that it can enhance tissue function, and consequently, natural physiological processes. In particular, preconditioning agents may have the capacity to improve neuronal functions, and therefore may represent a novel therapeutic strategy for enhancing mental processes such as learning and memory formation. While this concept is still in its infancy, preliminary results (especially from the Kerr lab) do indeed indicate nootropic potential of previously established preconditioning agents. These findings may pave the way for a host of ground-breaking discoveries for people suffering from the numerous detrimental effects of neurodegenerative disorders.

1.3 Synaptic Plasticity

The possibility that preconditioning may confer nootropic potential on top of its neuroprotective ability is an incredibly exciting, albeit uncertain, prospect. One of the

most significant challenges in neuroscience is to identify the cellular and molecular changes that underlie the processes of learning and memory formation (68-70). Learning involves the acquisition of knowledge or skills, while memory relates to the ability to retain and recall information (69). The earliest hypothesis, proposed a century ago by Cajal, suggested information storage is driven by changes in the synaptic efficacy between active neurons: a phenomenon called synaptic plasticity (68).

This suggestion was later refined in 1949 by psychologist Donald Hebb, who proposed that the efficiency of a synapse between two neurons will be strengthened if they are simultaneously active (71). Indeed, this suggestion was confirmed in 1966 and again in 1973, when it was repeatedly demonstrated that brief tetanic stimulation of the hippocampal perforant pathway elicited a lasting enhancement in the efficacy of the perforant path-granule cell synapse. This latter study is now widely considered to be the basic reference for the discovery and initial description of long term potentiation (LTP); the phenomenon by which tetanic stimuli of an afferent neural pathway results in a lasting enhancement of transmission at that synapse.

It was almost a decade later when the apparently opposing phenomenon of long term depression (LTD) was discovered in the cerebellum and hippocampus, whereby prolonged low frequency repetitive stimulation of an afferent neuron results in reduced transmission efficacy at the related synapse (72). LTP and LTD have since attracted immense interest in the search for the engram (73), and remain the leading experimental model for the synaptic basis of learning and memory to this day (68, 74, 75).

1.3.1 Hippocampus

The majority of plasticity related studies are based around the hippocampus, due to its proposed association with learning and memory formation. The hippocampus is a

scroll-like structure situated in the medial temporal lobe of the brain, and consitutes part of the limbic system (76). There are a number of distinct regions that comprise the hippocampus, namely the four *cornu ammonis* regions (CA1, CA2, CA3 and CA4), the dentate gyrus, and the entorhinal cortex. The relay of synaptic transmission within the hippocampus is commonly referred to as the 'trisynaptic loop', and is depicted in Figure 1.1. The first projection of this loop is the perforant path, which is the collective term for the fibres travelling from the entorhinal cortex to the granule cells of the dentate gyrus. The information is then transmitted to the dendrites of the pyramidal cells in the CA3 region via mossy fibres, and from CA3 it travels to CA1 pyramidal cells via the Schaffer collateral-commissural pathway. These connections are primarily excitatory glutamatergic synapses, and display a high propensity for both LTP and LTD. The trisynaptic circuit architecture connectivity patterns are similar between rodents and humans, making them an ideal model animal for hippocampal based research (77).

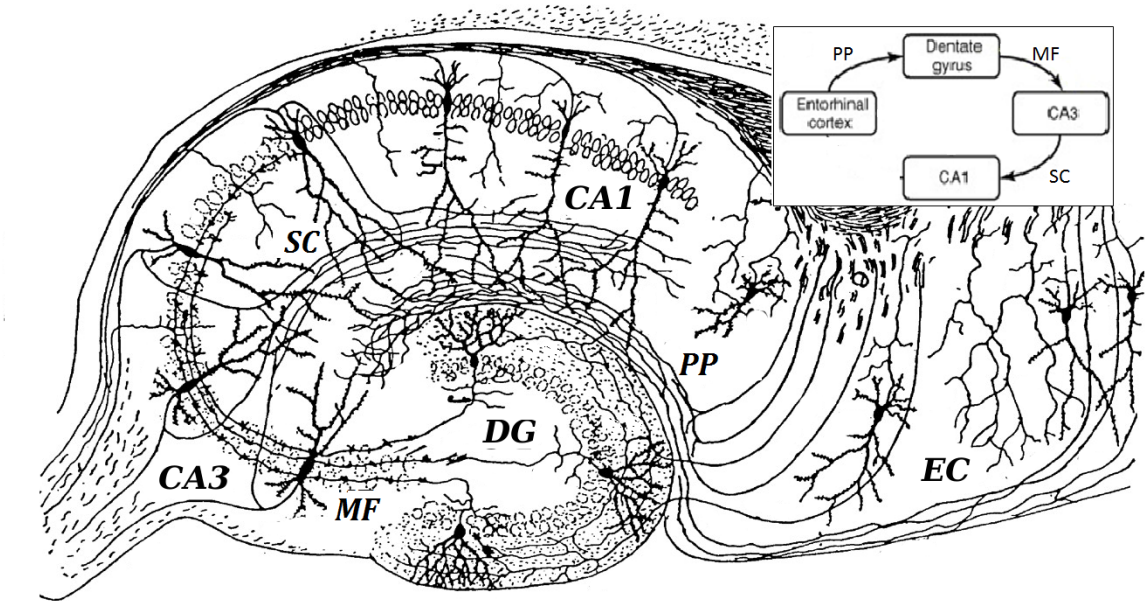

Figure 1.1: Diagram of Hippocampal Anatomy and the Trisynaptic Loop. The first projection of the trisynaptic loop is the perforant path (PP), which runs from the entorhinal cortex (EC) to the dentate gyrus (DG). The mossy fibres (MF) then carry information from the dentate gyrus to region CA3, and from CA3 it travels to CA1 via the Schaffer collateral-commissural pathway (SC). Image adapted from Nicoll, 2005 (78).

Nowadays it is widely accepted that the hippocampus plays a critical role in certain types of learning and memory formation, particularly declarative and spatial memory (69). The famous case of 'H.M', who suffered from severe anterograde amnesia after his hippocampus was removed in an effort to cure his epilepsy, was the first case highlighting the importance of the hippocampus in learning and memory processes. Since then, it has repeatedly been reported that damage to the hippocampus results in persistent memory impairments and learning difficulties, both in humans and other mammals, consolidating the findings of the initial study on H.M. (69, 79, 80). Due to this association, the hippocampus is frequently used as a model system for neurophysiological studies investigating processes related to learning and memory.

1.3.1.1 *Hippocampal Brain Slices*

When assessing characteristics of brain slices in order to garner information about synaptic transmission, *in vitro* hippocampal slices are valuable as they are easy to prepare, give stable responses over time, and have a well-defined, laminar architecture (81). From this structure it is possible to record excitatory post synaptic potentials (EPSPs) and population spikes (pyramidal cell firings), and assess the impact that different drugs or stimulatory paradigms may have on these recordings. These types of recordings can be used to assess a range of different neurophysiological processes, such as LTP and LTD, which can be measured at many of these synapses. The hippocampus is a particularly favourable site for studying LTP because of its densely packed and sharply defined layers of neurons. Hippocampal CA1 region is most commonly employed for electrophysiological procedures, as it readily yields the most robust and consistent recordings. Furthermore, *in vitro* measurements offer several advantages over *in vivo* recordings by enabling a greater degree of control over the external

conditions, reducing the number of animals that must be sacrificed, and enabling more simplistic recording techniques to be employed (81).

1.3.2 Long Term Potentiation

LTP may be operationally defined as a lasting enhancement in synaptic efficacy resulting from high frequency tetanic stimulation of afferent nerve fibres (73). LTP can be rapidly induced by applying one or more brief trains of tetanic stimuli to a presynaptic cell. However, the exact induction paradigm used has varied greatly between studies (82-87). Although LTP was initially discovered in the perforant pathway of the hippocampus, it has since been observed in all excitatory pathways in the hippocampus, as well as in a range of different brain regions such as the prefrontal cortex, cerebellum and amygdala (73, 88). Indeed, many scientists agree that memory formation utilises a number of these regions rather than just the hippocampus alone. Despite this, CA1 hippocampal region remains the prototypical site of mammalian LTP study, owing to its predictable organisation and readily inducible LTP.

While there are a number of different ‘types’ of LTP that can be induced, NMDA receptor dependent LTP remains the most extensively studied form of synaptic plasticity to this day. Research has indicated that the brain region involved and the age of the organism in question are key factors in the determination of what type of LTP is induced (89). Therefore, when discussing this phenomenon, it is critical to define the synapse in question, the time in development, and how the phenomenon is being triggered (89). For example, LTP in the Schaffer-collateral pathway of the hippocampus is NMDA receptor-dependent, whereas LTP in the mossy fibre pathway is NMDA receptor-independent (73, 90).

The underlying mechanism behind LTP in hippocampal CA1 region begins with tetanic stimulation of afferent fibres resulting in excessive glutamate release into the synaptic cleft. This glutamate is able to bind to postsynaptic glutamate receptors, in particular the AMPA receptor, resulting in sodium influx into the post-synaptic terminal. This depolarisation relieves the magnesium block in the NMDA receptor, enabling calcium influx into the post-synaptic terminal, which is the critical trigger for LTP induction (73). Calcium is able to activate a series of downstream signalling molecules such as calcium dependent protein kinases (CaMKII), protein kinase C (PKC) and protein kinase A (PKA), all of which contribute to phosphorylation of the AMPA receptor (73, 91, 92). This phosphorylation regulates AMPA receptor localisation by increasing its trafficking to the post-synaptic membrane, and also enhances the conductance and open probability of the receptor's ion channels (92). Taken together, these effects enhance the sensitivity of the synapse to any future presynaptic depolarisation, thereby accounting for the increased synaptic strength observed following LTP induction (91).

1.3.2.1 *Early and late LTP*

Memory is commonly categorised as either short term or long term. The former lasts for a matter of hours, while the latter persists for days, weeks or even years (69). The cellular changes underlying these two forms of memory differ with regard to whether protein synthesis is involved: long term memory is dependent on *de novo* protein synthesis, while short term memory is not (69). This highlights interesting parallels with LTP, as it has been shown that there are distinct phases of this phenomenon too, which also involve specific cellular processes. The early 'induction' phase of LTP is independent of protein synthesis, and only lasts a number of hours, while persistent LTP can last for weeks, and requires protein synthesis to occur (69).

1.3.3 Long Term Depression

While LTP is thought to underlie memory formation, LTD is required for the selective weakening of synapses, which ensures the constructive use of later synaptic strengthening. If LTP was allowed to continually strengthen synaptic connections, a plateau would eventually be reached, and encoding of new information would be impaired. By working in harmony, LTP and LTD therefore allow for the 'sculpting' of information in the most efficient manner possible.

Similarly to LTP, LTD occurs in various brain regions, with hippocampal LTD being the most well studied and best characterised type (88). LTD at Schaffer collateral synapses occurs in response to low frequency (approximately 1 Hz), repetitive stimulation for extended time periods (10–15 minutes), and results in a marked reduction in subsequent synaptic transmission. LTD can be induced by a number of different mechanisms. However, as was the case for LTP, intracellular calcium levels are crucial for triggering biochemical cascades required for the induction of LTD (73, 92). Changes in plasticity often occurs via the insertion or internalization of AMPA receptors into the postsynaptic membrane of the synapse undergoing plastic changes, and it is the magnitude of the calcium influx that modulates post-synaptic AMPA receptor density (91). While robust rises in intracellular calcium activates kinases that cause phosphorylation and insertion of AMPA receptors into the membrane during LTP, LTD is instead brought about by small, slow rises in calcium. This activates protein phosphatases, resulting in de-phosphorylation and internalisation of AMPA receptors. Internalization of post-synaptic AMPA receptors reduces sensitivity to pre-synaptic depolarisation, and thus reduced synaptic efficacy - or LTD - occurs (91).

1.3.4 Metaplasticity

In 1996, a novel form of persistent synaptic plasticity was described, and given the term ‘metaplasticity’ (93). This refers to the ability of synaptic plasticity to be modulated by prior synaptic activity; in other words, it is the ‘plasticity of plasticity’. This prior activity may not itself elicit any changes in normal synaptic transmission, but rather manifests as an alteration in the induction of subsequent plasticity (93).

Metaplasticity can be distinguished from conventional neuromodulation of plasticity, in which molecules present at the time of plasticity induction regulate the LTP or LTD elicited. Instead, metaplasticity refers to neuronal changes elicited at one point in time, that by virtue of their persistent nature, are able to regulate synaptic plasticity processes minutes, hours or days later (93). Functionally, metaplasticity therefore enables synapses to integrate plasticity-relevant signals across time.

The commonality between the lasting nature of pharmacological preconditioning and long term plasticity hints at a potential association between the two, and also at the involvement of metaplasticity. (9). This illustrates a potential role for neuroprotectants as nootropics, ultimately enhancing learning, memory and cognitive processes.

1.4 Recent Findings in the Kerr Lab:

1.4.1 GYKI as a nootropic agent

In accordance with the purported association between neuroprotectants and nootropics, GYKI has recently emerged as a potential nootropic candidate (94). In a recent publication by Nayak and Kerr, GYKI preconditioning was reported to augment LTP induction *in vitro*, denoting nootropic potential (94). However, this contradicted a finding by Rammes *et al.* almost two decades earlier, that *in vitro* preconditioning with GYKI instead *inhibited* induction of LTP (95). Key differences in the preconditioning

paradigms between the studies may have accounted for this contradiction, however, as the former used a lower dose of GYKI and a shorter length of preconditioning time than the latter (6.25 μm for 20 minutes, and 10 μm for 65 minutes, respectively). Additionally, and perhaps most importantly, Nayak and Kerr performed LTP induction 30 minutes *after* GYKI had been washed out of the tissue preparation, while for Rammes *et al.* it was performed *during* preconditioning. This variation in experimental protocol appeared the root cause of the controversy, as Rammes *et al.* also reported successful induction of LTP upon washout of the GYKI. Unfortunately no controls were included as a comparison in this latter study, making it impossible to determine whether GYKI enhanced plasticity after washout or not (95).

Taken together, one can propose likely explanations for the above observations. Considering AMPA receptors are required for LTP induction, doses of GYKI at or near its effective concentration as a negative allosteric modulator at AMPA receptors would theoretically block this occurrence. Rammes *et al.* indeed reported that doses of GYKI near its effective concentration did impair LTP. However, the augmentation of LTP induction after washout seen by Nayak and Kerr supports GYKI's lasting metabotropic action independent of ionotropic AMPA-receptors. Importantly, the doses used in both studies fell below GYKI's IC_{50} at AMPA receptors, again implicating metabotropic receptors and the amplification of second messenger cascades.

1.4.2 Domoic Acid as a nootropic agent

Additional unpublished results from the Kerr lab also support the association between neuroprotectants and enhanced plasticity. Guy and Kerr demonstrated that low dose (50nM) DOM preconditioning augments subsequent induction of LTP and LTD in hippocampal slices 30 minutes later, and improves performance in the Morris water

maze – an experimental technique to assess spatial learning and memory (Guy and Kerr 2006, unpublished observations). Considering DOM had already demonstrated neuroprotective efficacy against severe excitotoxic insults (47, 49), these findings further corroborated the proposed association between neuroprotectants and nootropics.

1.4.3 Chronic GYKI Preconditioning

Recent unpublished observations (Macindoe and Kerr, 2012) investigated the efficacy of long term (chronic) pharmacological preconditioning with GYKI as a prophylactic neuroprotective strategy. Hippocampal slices were exposed to 6 µM GYKI for a prolonged period of time (2+ hours), and subsequently challenged with 3 µM KA for 30 minutes. However, preconditioned slices did not display signs of tolerance to KA, as they also exhibited considerable population spike and EPSP suppression within 10 minutes, comparable to that seen from control slices. This study therefore found no evidence to suggest that long term preconditioning of hippocampal slices with low dose GYKI confers neuroprotection to hippocampal slices.

1.5 Rationale and Aims

Taking everything into consideration, GYKI and DOM clearly possess novel mechanisms of action that are yet to be elucidated. Although preconditioning is a reasonably well-established pre-clinical phenomenon for neuroprotection, its ability to influence plasticity processes remains much less understood. Interestingly, the modulation of plasticity by preconditioning with these compounds may be explained by metaplasticity, by virtue of their ability to alter future induction of LTP/LTD. The emerging correlation between neuroprotection and nootropics suggests that therapeutic interventions harnessing these mechanisms may be a breakthrough for the treatment of neurodegenerative disorders, for which there are limited effective therapeutics thus far.

Nevertheless, extensive research investigating the safety and efficacy in human trials is required before preconditioning-based therapies will be included in our armamentarium for the battle against brain ageing and memory loss (12).

Although chronic preconditioning with GYKI did not confer neuroprotection against a KA challenge, it may still be capable of enhancing synaptic plasticity. Taking all factors into consideration, the aim of the current research was to develop an effective *in vitro* preconditioning strategy using GYKI or DOM, and to clarify their effects on LTP and LTD induction in the rat hippocampus. This was a further addition to a field so earnestly pursued, and may bring the final goal of combating neurodegenerative disorders one step closer.

1.6 Hypothesis

Based on the preliminary findings from the Kerr lab, the current hypothesis is that preconditioning hippocampal slices acutely or chronically with GYKI, or acutely with DOM, will enhance the magnitude of LTP and LTD *in vitro*.

2 Materials and Methodology

2.1 Animals

All experimental procedures were approved by the University of Otago Animal Ethics Committee (AEC# ET9/13), and conducted in accordance with the New Zealand Animal Welfare Act for the Care and Use of Laboratory Animals. Young (6-8 week old) male Sprague Dawley rats were obtained from the University of Otago Hercus-Taieri Resource Unit and acclimatized for at least 2 days in an animal room in the Department of Pharmacology and Toxicology, University of Otago. The animal room had a normal day/night light cycle (temperature 22°C), and rats had unrestricted access to both food and water.

2.2 Reagents

All carbogen (95% Oxygen, 5% Carbon dioxide) gas cylinders were obtained from BOC gases (Dunedin, New Zealand).

GYKI-52466 hydrochloride was purchased from Sigma-Aldrich, Australia.

DOM was purchased from Abcam Biochemicals, (distributed by Sapphire Bioscience) from Australia.

All other chemicals and reagents were supplied by Sigma or AnalaR.

2.3 Equipment

Drop-blade tissue chopper - Campus Mechanical, Dunedin, NZ

Grass SD9 stimulator - Grass Technologies, USA

Olympus stereo microscope (SZ40) - Olympus New Zealand Pty Ltd

Microinfusion pump (Beehive) - Bioanalytical systems Inc., USA

Water bath – Pharmacology and Toxicology, Sammut Lab

Brain Slice Recording Chamber – Kerr Scientific Instruments, Ltd., Christchurch

Powerlab – AD Instruments, Sydney

2.4 Hippocampal Slice Preparation

Animals were sacrificed via rapid decapitation under CO_2 anaesthesia, and brains were removed and cooled in ice cold, pre-gassed (95% O_2/5% CO_2) high sucrose solution containing 206 mM Sucrose, 26 mM $NaHCO_3$, 10 mM glucose, 2.8 mM KCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, and 0.4 mM ascorbic acid (pH 7.4). Hippocampi were dissected free, and 400 μm transverse sections were generated using a drop-blade tissue slicing apparatus. The fimbriae from hippocampi were trimmed, however region CA3 was left intact.

Slices were then maintained at room temperature in a holding chamber filled with a high calcium/low magnesium artificial cerebrospinal fluid (ACSF) containing 124.0 mM NaCl, 3.2 mM KCl, 1.25 mM NaH_2PO_4, 26.0 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 10.0 mM glucose (pH 7.4), unless otherwise stated for chronic GYKI preconditioning (Section 2.5.1.2). Slices were left undisturbed for 2 hours before commencement of electrophysiological recordings.

A minimum of eight rats from each group were sacrificed.

2.4.1 *In vitro* hippocampal electrophysiology

Slices were transferred into a prototype Kerr brain slice recording chamber (AD Instruments, Sydney), and placed between nylon mesh nets (Kerr Scientific Instruments Ltd., Christchurch). The slices were constantly superfused with oxygenated ACSF, and maintained at 31.5 ± 1°C using a water bath setup.

Orthodromic monosynaptic field potentials were evoked by positioning a twisted, bipolar 50 μm-diameter teflon-coated tungsten wire stimulating electrode (A-M Systems, Carlsborg, Washington, USA) in the Schaffer collateral-commissural pathway (*stratum radiatum)* of hippocampal area CA1. Biphasic 0.1 ms stimulus pulses were delivered to the afferent fibres at 0.18 Hz using a Grass SD9 stimulator. Low-impedance 50 μm tungsten recording microelectrodes were positioned just above the pyramidal cell layer (*stratum pyramidale*) and in the dendritic field (*stratum radiatum*) of area CA1, and inserted 200 and 100 μm deep, respectively. The former electrode was used to measure orthodromic population spikes, while the latter measured field excitatory postsynaptic potentials (fEPSPs). Cell layers in the hippocampal CA1 region were visualized by Olympus stereo microscope.

A typical recording set up is depicted below (Figure 2.1).

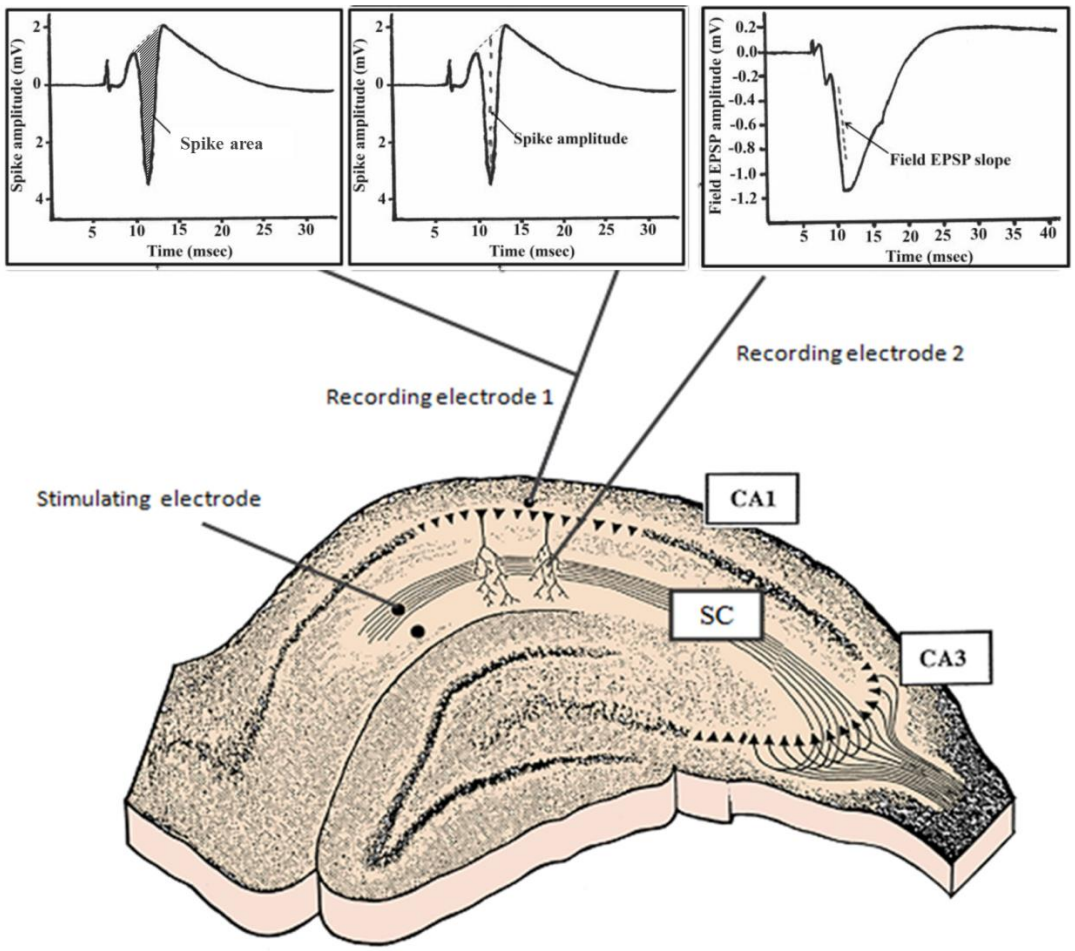

Figure 2.1: A simplified diagram of the extracellular recording setup. The stimulating electrode was placed in the Schaffer collateral-commissural pathway (*stratum radiatum*, CA3-CA1) of hippocampal region CA1, and the population spikes and fEPSPs were recorded from *stratum pyramidale* (cell body layer) and *stratum radiatum* (dendritic region), respectively.

Waveforms were digitally acquired at 10 kHz using a Powerlab analog-digital converter, and stored on a hard drive for off-line analysis using ChartTM software (Analog Digital Instruments Ltd, Dunedin, New Zealand). Population spike amplitude (mV) was calculated as the difference between the most negative point of the sharp negative-going spike and the two positive points of inflection on either side (Figure 2.2a), while population spike area (mV x ms) was defined as shown in Figure 2.2b. EPSP strength was assessed by measuring the initial negative going slope of the EPSP (corresponding to sodium influx through AMPA receptors; mV/ms; Figure 2.2c). Raw data were normalised and expressed a percentage change from the baseline.

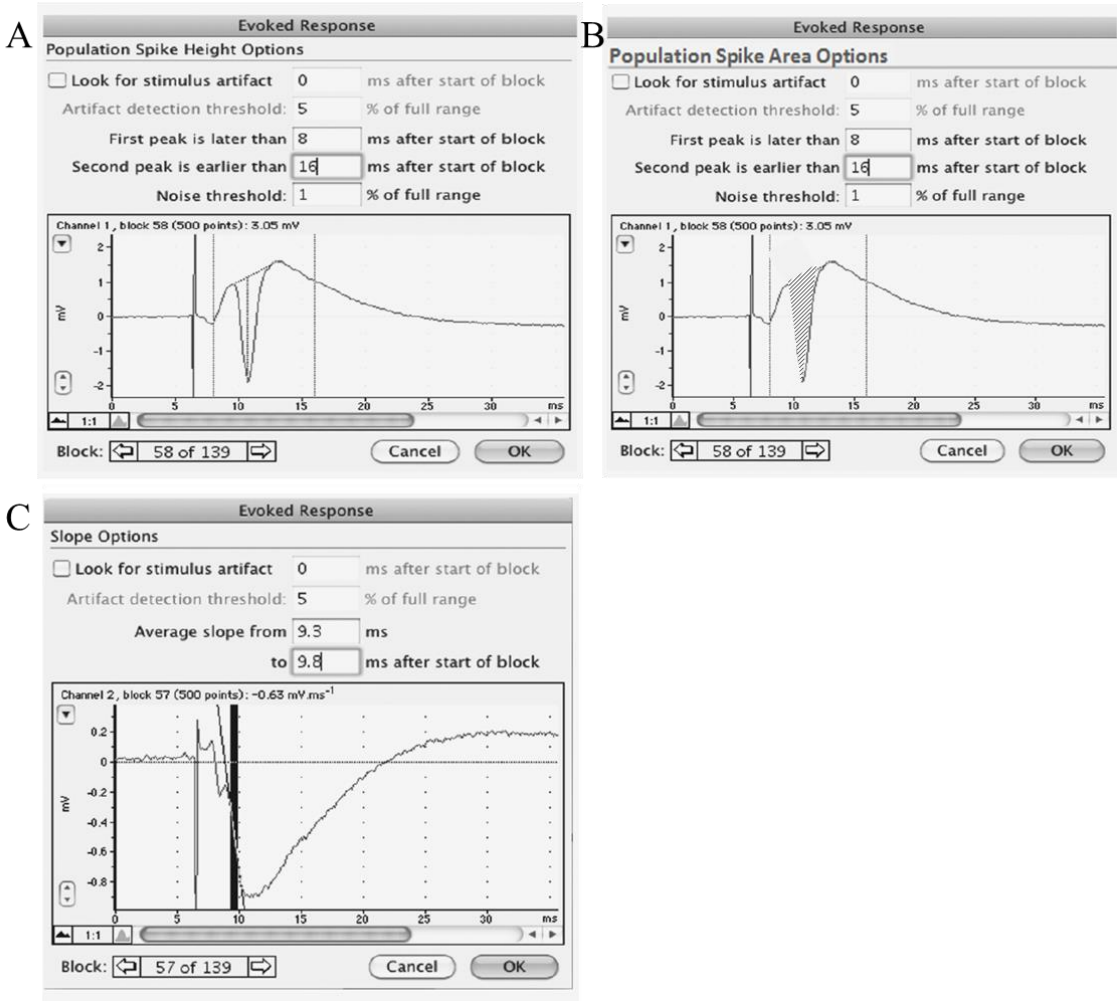

Figure 2.2: Waveform analysis through LabChart extension. A) Population spike amplitude measurement by placing the spike within the time window. B) Population spike area measurement by placing the spike within the time window. C) Measurement of fEPSP slope by selecting the steepest part of the negative going potential.

2.5 Experimental design and drug treatments

The effects of acute or chronic preconditioning with low-dose GYKI (6 μM), and of acute preconditioning with DOM (50 nm), on LTP and LTD induction in naïve hippocampal slices were examined.

An input/output response curve was completed in order to determine the maximal response and the response threshold of the population spike and fEPSP for each slice. Slices with 5 mV maximal population spike and 0.5 mV fEPSP amplitude or greater

were considered acceptable for LTP and LTD studies. A baseline recording at a stimulus intensity that evoked approximately 50% of the maximal population spike amplitude was performed for 20 minutes at 0.18 Hz to ensure slice health. Slices not exhibiting stable baselines (more than 10% change in baseline over the 20 min period) were excluded from the final analysis.

2.5.1 Drug preconditioning Paradigms

2.5.1.1 *Acute GYKI Preconditioning*

Following a 20 minute baseline recording, GYKI wash in was performed. A 3 mM stock solution of GYKI was microinjected into the ACSF perfusion stream via micro-infusion pump for 20 minutes, at a rate that resulted in a final concentration of 6 µM in the recording chamber. This was followed by a washout period of 30 min, whereby slices were exposed to ACSF alone. Following washout, LTP was induced by high frequency stimulation, or LTD was induced by low frequency stimulation (Section 2.5.2). Recording was continued for 30 minutes at baseline frequency and intensity following LTP or LTD induction.

2.5.1.2 *Chronic GYKI Preconditioning*

Hippocampal slices were placed into a holding chamber containing 6 µM GYKI in ACSF at room temperature for at least 2 hours, prior to being transferred into the brain slice recording chamber. After a 20 minute baseline recording, either LTP or LTD was induced (Section 2.5.2), and this was followed by a further 30 minutes of recording at baseline frequency and intensity.

2.5.1.3 *Acute DOM Preconditioning*

Following a 20 minute baseline recording, DOM wash in was performed. A 100 µM stock solution of DOM was microinjected into the ACSF perfusion stream via microinfusion pump for 15 min, at a rate that resulted in a final concentration of 50 nM in the recording chamber. This was followed by a washout period of 30 minutes. Following washout, LTP was induced by high frequency stimulation, or LTD was induced by low frequency stimulation (Section 2.5.2). Recording was continued for 30 minutes at baseline frequency and intensity following LTP or LTD induction.

2.5.1.4 *Control Slices*

Control slices were not preconditioned with GYKI or DOM. Following a 20 minute baseline recording, LTP was induced by high frequency stimulation, or LTD was induced by low frequency stimulation (Section 2.5.2). Recording was continued for 30 minutes at baseline frequency and intensity following LTP or LTD induction.

2.5.2 LTP and LTD Induction Paradigms

LTP was induced by tetanic stimulation of Schaffer-collateral fibres. This was delivered as two bursts of high frequency stimulation at 85 Hz for 1 second each, with an inter-burst interval of 30 seconds. LTD was induced by low frequency stimulation of Schaffer-collateral fibres at 1 Hz for 15 minutes.

2.6 Statistics

Data were analysed using Graphpad Prism software and Microsoft Excel. Data from slices in each treatment group on a particular day were averaged to yield a single set of values. The final number of data sets in each treatment group is therefore representative of the number of rats from which slices were obtained, rather than the

total number of slices that were recorded from. Data were normalised and expressed as a mean percentage of baseline values ± SEM. An unpaired, two-tailed student's t-test was used to compare the last five minutes of data from control and preconditioned slices, and statistical significance was determined at confidence level of $P<0.05$.

2.6.1 Exploratory analysis

To assess whether LTP and LTD had successfully been induced, a one-sample t-test was used to determine whether the field potentials from the last five minutes of each recording had exhibited a significant change following LTP or LTD induction.

3 Results

The primary aim of this research was to determine whether preconditioning hippocampal slices with GYKI or DOM enhanced the subsequent induction of long term potentiation (LTP) and long term depression (LTD).

3.1 General slice health

On a daily basis, the majority of slices exhibited field potentials that met the criteria to be used for LTP and LTD studies (minimum of 5 mV population spike and 0.5 mV fEPSP). However, due to natural biological variability, each slice was unique in terms of the voltage required to achieve a half maximal response, and in the size of the maximal response. Obtaining stable baseline responses for 20 minutes occasionally proved difficult, with some slices exhibiting drifting population spike amplitudes, areas and fEPSP slopes. On a small number of occasions, no slices from a particular rat showed sufficiently stable baselines, and no recordings were able to be made.

3.2 Slice response to preconditioning

Although the concentration of GYKI used for acute preconditioning was well below its reported IC_{50} at AMPA and KA receptors, slices generally exhibited a noticeable reduction in population spike amplitude, area, and fEPSP slope during GYKI wash in. For the majority of slices, the evoked potentials returned to baseline values upon drug wash out (Figure 3.1), however in a number of cases they remained below baseline values even after the wash out was complete. In these cases, stimulus voltage was adjusted in order to return the responses to baseline levels.

Figure 3.1 shows a dot plot of a single slice's response to acute GYKI preconditioning.

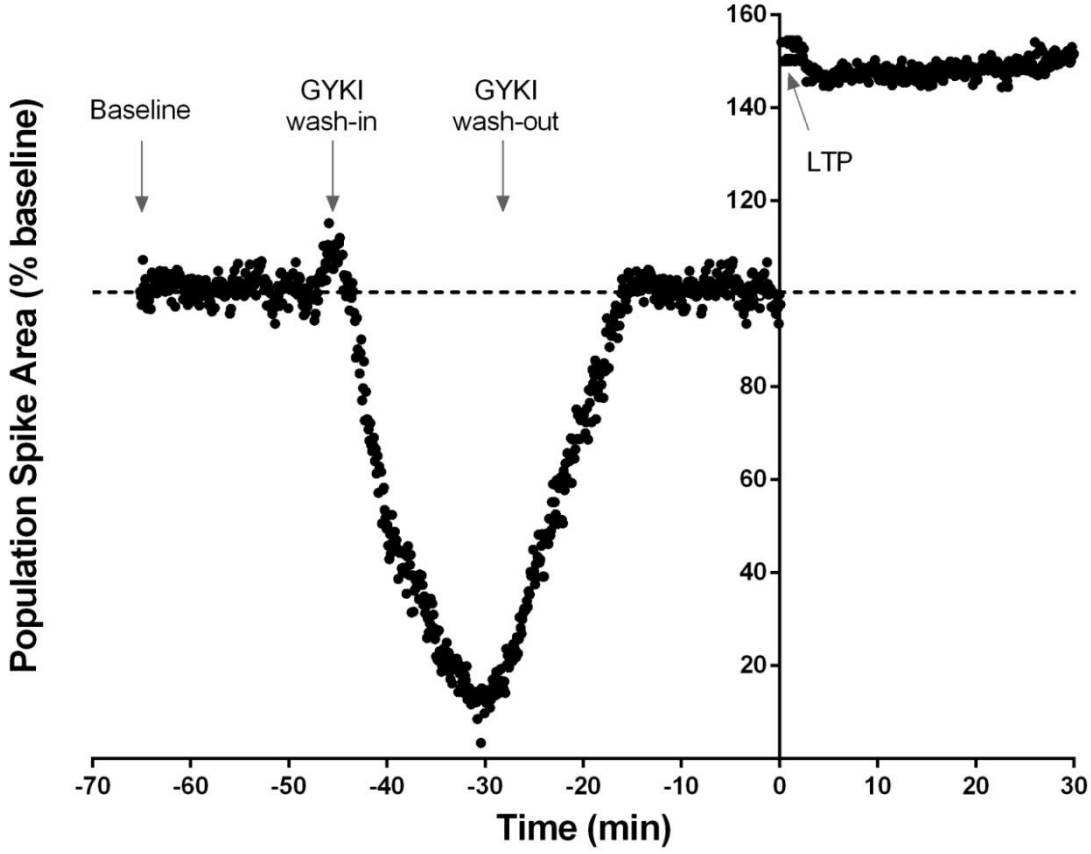

Figure 3.1: Dot plot showing the effect of acute GYKI preconditioning on population spike area in the CA1 region of a single hippocampal slice. After a 20 minute baseline (first arrow) 6 µM GYKI was washed into the slice for 20 minutes (second arrow), and this was followed by a 30 minute washout period (third arrow). LTP was induced at time point zero, using two 1 second bursts at 85 Hz with an inter-stimulus interval of 30 seconds, and recording was continued for a further 30 minutes.

Chronic preconditioning with GYKI caused no observable change in slice health compared to control slices, and likewise, slices exhibited no detectable response to 50 nM acute DOM wash in or wash out.

3.3 LTP Induction

Initially LTP induction in both control and preconditioned slices was unsuccessful, therefore a high calcium/low magnesium ACSF solution was employed to help promote LTP. Natural biological variability meant that each slice exhibited slightly

different responses to LTP induction, and this was seen both in control and preconditioned slices.

LTP was readily induced in the majority of control slices and in all preconditioned hippocampal slices, evidenced by a statistically significant increase in the population spike amplitude, area and fEPSP slope following high frequency stimulation (Table 3.1). Immediately following LTP induction, some slices exhibited transient post-tetanic facilitation, while others exhibited transient post-tetanic depression. However, following this, the response from all slices stabilised.

Table 3.1: Success of LTP induction in control and preconditioned slices. Ticks indicate successful LTP induction according to a one sample t-test. NS = not significant.

	AMPLITUDE	**AREA**	**EPSP SLOPE**
Control	✓	✓	✓
Acute GYKI	✓	✓	✓
Control	✓	✓	NS
Chronic GYKI	✓	✓	✓
Control	✓	✓	✓
DOM	✓	✓	✓

Data from slices in each preconditioning or control group were normalised to the last five minutes of baseline values, and averaged to form representative dot plots (Figure 3.2). This was done for all the preconditioned and control groups, however a single example is included below**.**

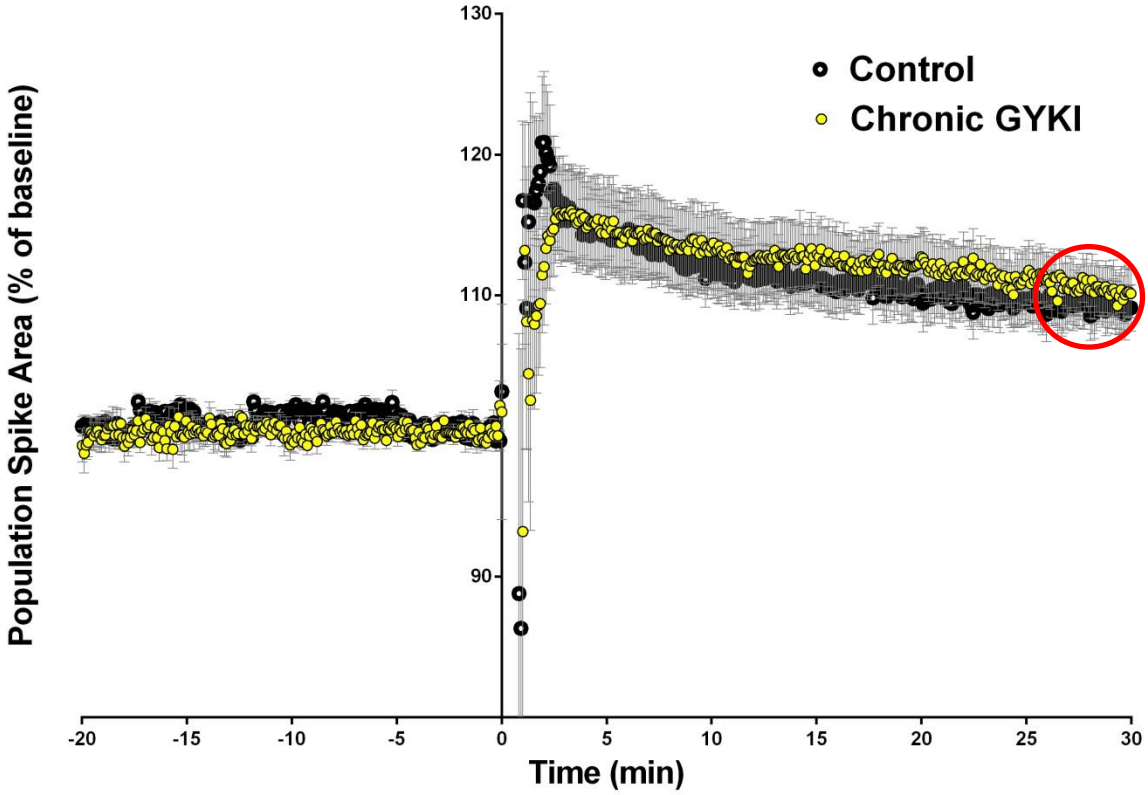

Figure 3.2: Dot plot showing LTP in the CA1 region of the rat hippocampus for control and chronic GYKI preconditioned slices. Data shows population spike area (mean % of baseline ± SEM) before and after tetanic stimulation (time point zero). Data represents control slices (n = 8; black dots) and GYKI preconditioned slices (n=9; yellow dots). Statistical analysis was performed using an unpaired student's t-test, comparing the last 5 minutes of data from each group (red circle).

3.3.1 Acute GYKI Preconditioning

Acute GYKI preconditioning did not enhance subsequent induction of LTP (Figure 3.3). Control slices (n=12) exhibited on average a 36.4 ±7.8% increase in population spike amplitude, a 24.1 ±6.1% increase in population spike area, and a 25.8 ±4.2% increase in EPSP slope. In comparison, acute GYKI preconditioned slices (n=8) exhibited on average a 30.2 ±6.7% increase in population spike amplitude, a 17.9 ±4.8 % increase in population spike area, and a 25.3 ±7.2% increase in EPSP slope. The

magnitude of LTP from acute GYKI preconditioned slices was not significantly different from the control slices for any of the field potentials measured (P>0.05).

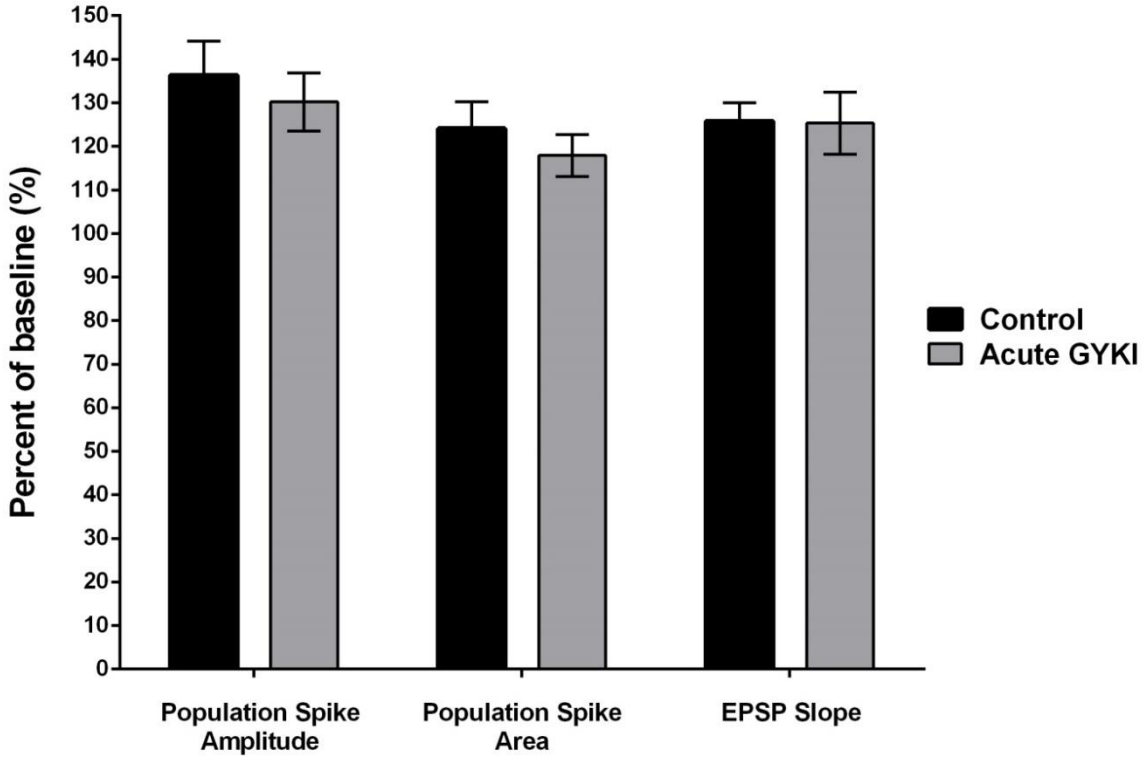

Figure 3.3: Effects of acute GYKI preconditioning on LTP in the CA1 region of the hippocampus. Acute GYKI preconditioning was performed as described Section 2, however this preconditioning did not enhance LTP of the population spike amplitude, area or EPSP slope compared to control slices. Data represents mean % of baseline ±SEM for control slices (n=12; black bars) and preconditioned slices (n=8; grey bars). Statistical analysis was performed using an unpaired student's t-test, comparing the last five minutes of data from each group. * indicates P<0.05 versus control slices.

3.3.2 Chronic GYKI Preconditioning

Chronic GYKI preconditioning did not enhance subsequent induction of LTP (Figure 3.4). Control slices (n=8) exhibited on average a 12.9 ±3.0% increase in population spike amplitude, a 9.3 ±1.8% increase in population spike area, and a 5.6 ±4.0% increase in EPSP slope. In comparison, acute GYKI preconditioned slices (n=9) exhibited on average a 18.8 ±3.9% increase in population spike amplitude, a 10.6 ±1.9

% increase in population spike area, and a 9.4 ±3.4% increase in EPSP slope. The magnitude of LTP from chronic GYKI preconditioned slices was not significantly different from the control slices for any of the field potentials measured (P>0.05).

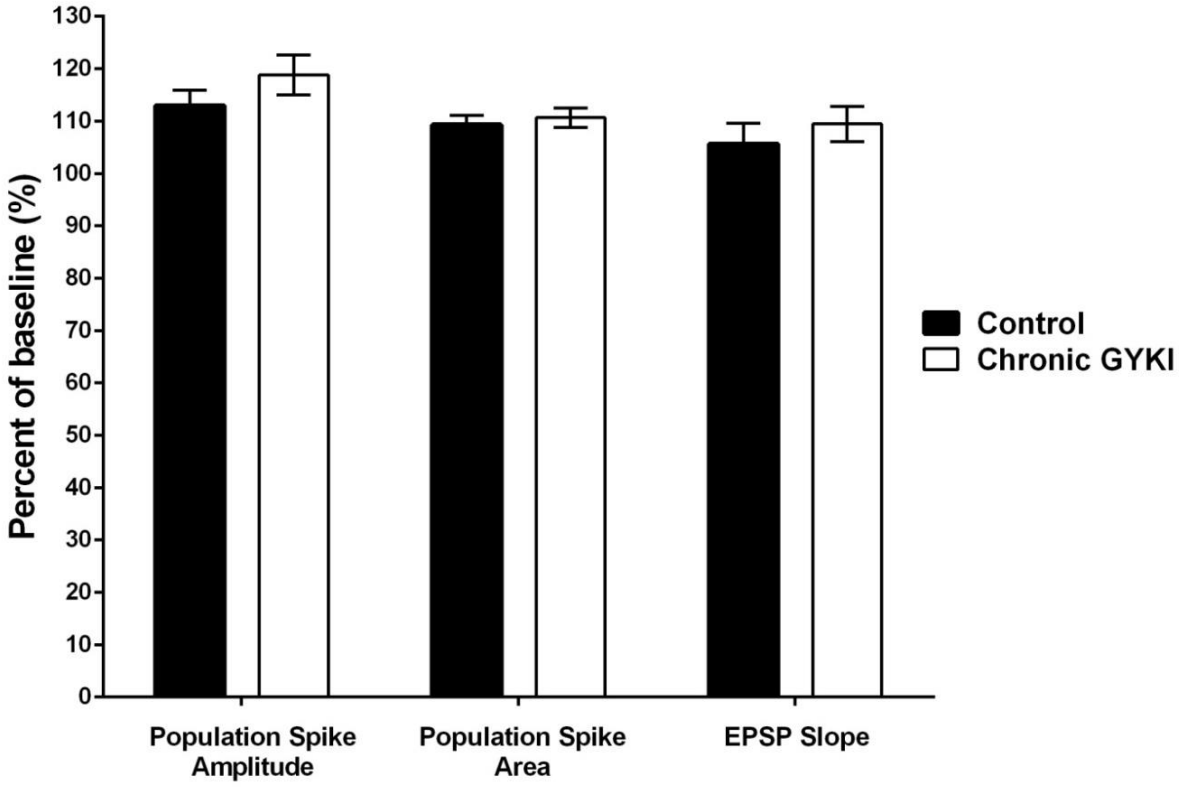

Figure 3.4: Effects of chronic GYKI preconditioning on LTP in the CA1 region of the hippocampus. Chronic GYKI preconditioning was performed as described in Section 2, however this preconditioning did not enhance LTP of the population spike amplitude, area or EPSP slope compared to control slices. Data represents mean % of baseline ±SEM for control slices (n=8; black bars) and preconditioned slices (n=9; white bars). Statistical analysis was performed using an unpaired student's t-test, comparing the last five minutes of data from each group. * indicates P<0.05 versus control slices.

3.3.3 Acute DOM Preconditioning

Acute DOM preconditioning did not enhance subsequent induction of LTP (Figure 3.5). Control slices (n=8) exhibited on average a 26.4 ±4.0% increase in population spike amplitude, a 14.7 ±2.3% increase in population spike area, and a 17.8

±4.0% increase in EPSP slope. In comparison, acute GYKI preconditioned slices (n=10) exhibited on average a 35.4 ±7.2% increase in population spike amplitude, a 23.4 ±3.8% increase in population spike area, and a 24.7 ±7.2% increase in EPSP slope. The magnitude of LTP from acute DOM preconditioned slices was not significantly different from the control slices for any of the field potentials measured (P>0.05).

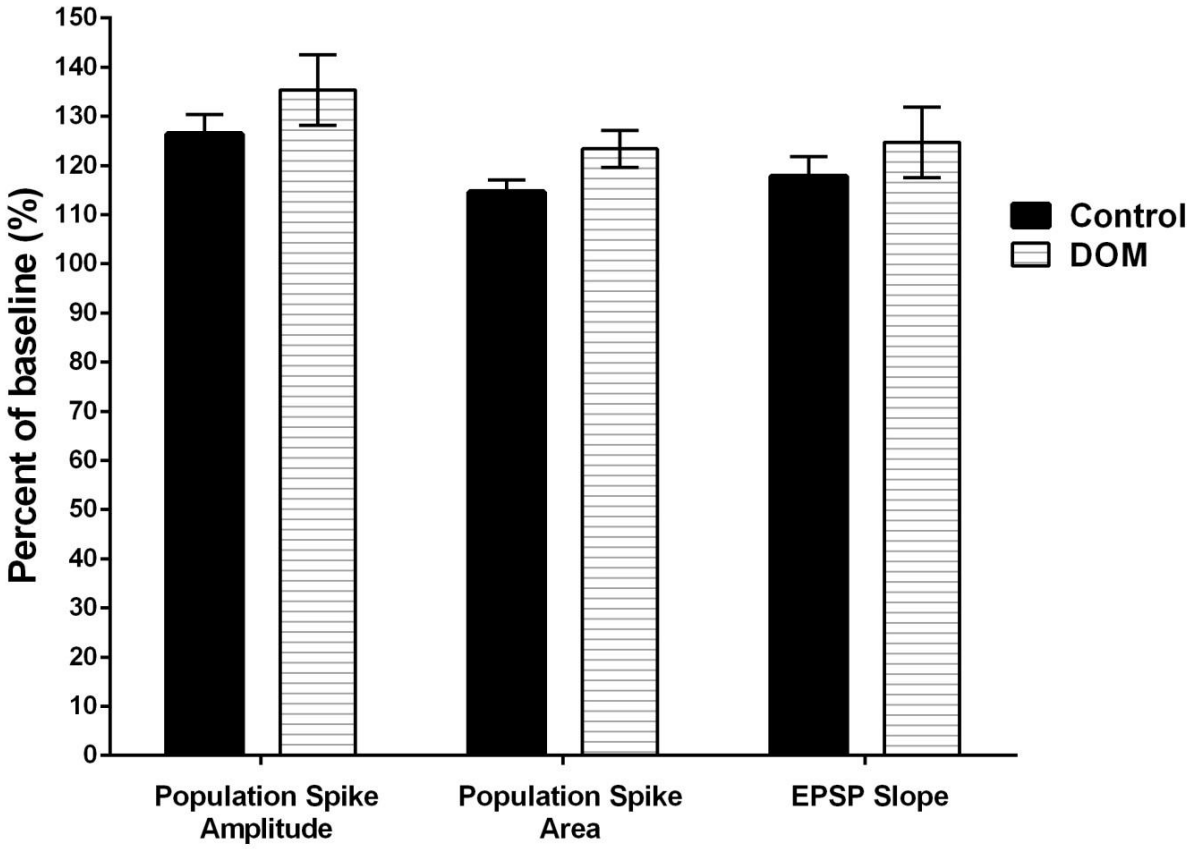

Figure 3.5: Effects of acute DOM preconditioning on LTP in the CA1 region of the hippocampus. DOM preconditioning was performed as described in Section 2, however this preconditioning did not enhance LTP of the population spike amplitude, area or EPSP slope compared to control slices. Data represents mean % of baseline ±SEM for control slices (n=8; black bars) and preconditioned slices (n=10; striped bars). Statistical analysis was performed using an unpaired student's t-test, comparing the last five minutes of data from each group. * indicates P<0.05 versus control slices.

3.4 LTD Induction

Natural biological variability meant that each slice exhibited slightly different responses to LTD induction, and this was seen in both control and preconditioned slices.

LTD was readily induced in the majority of control slices and all preconditioned hippocampal slices, evidenced by a statistically significant decrease in the population spike amplitude, area and fEPSP slope following prolonged, low frequency stimulation (Table 3.2). The magnitude of the evoked potentials was often facilitated immediately after the onset of LTD induction, however this was almost always followed by a progressive decline in the response magnitude during the following 15 minutes to values below baseline levels (data not shown). Immediately after LTD induction, there was usually some recovery in response magnitude over the next 30 minutes, however the evoked potentials on average reached a plateau at a value that was significantly lower than baseline (Figure 3.6 and Table 3.2). This occurred for both control and preconditioned slices.

Table 3.2: Success of LTD induction in control and preconditioned slices. Ticks indicate successful LTP induction according to a one sample t-test. NS = not significant.

	AMPLITUDE	**AREA**	**EPSP SLOPE**
Control	✓	NS	✓
Acute GYKI	✓	✓	✓
Control	✓	✓	✓
Chronic GYKI	✓	✓	✓
Control	✓	✓	✓
DOM	✓	✓	✓

Data from slices in each preconditioning or control group were normalised to the last five minutes of baseline values, and averaged to form representative dot plots (Figure 3.6). This was done for all the preconditioned and control groups, however a single example is included below.

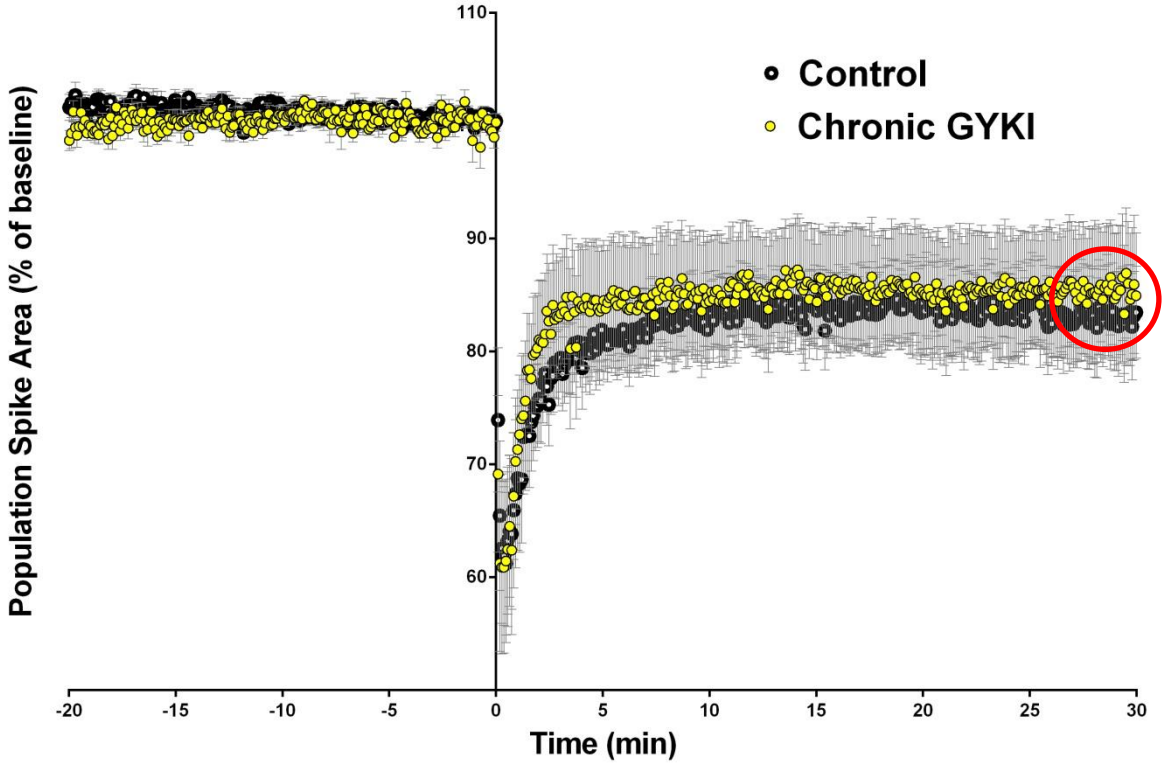

Figure 3.6: Dot plot showing LTD in the CA1 region of the rat hippocampus for control and chronic GYKI preconditioned slices. Data shows population spike area (mean % of baseline ± SEM) before and after low frequency stimulation (time point zero). Data obtained during the 15 minute LSF has been omitted. Data represents control slices (n=9; black dots) and GYKI preconditioned slices (n=8; yellow dots). Statistical analysis was performed by unpaired student's t-test, comparing the last 5 minutes of data from each group (red circle).

3.4.1 Acute GYKI Preconditioning

Acute GYKI preconditioning did not enhance subsequent induction of LTD (Figure 3.7). Control slices (n=9) exhibited on average a 22.3 ±5.6% decrease in

population spike amplitude, a 10.2 ±5.2% decrease in population spike area, and a 10.7 ±4.6% decrease in EPSP slope. In comparison, acute GYKI preconditioned slices (n=8) exhibited on average a 22.3 ±2.5% decrease in population spike amplitude, a 10.7 ±2.9 % decrease in population spike area, and a 21.5 ±3.7% decrease in EPSP slope. The magnitude of LTD from acute GYKI preconditioned slices was not significantly different compared to control slices for any of the field potentials measured (P>0.05).

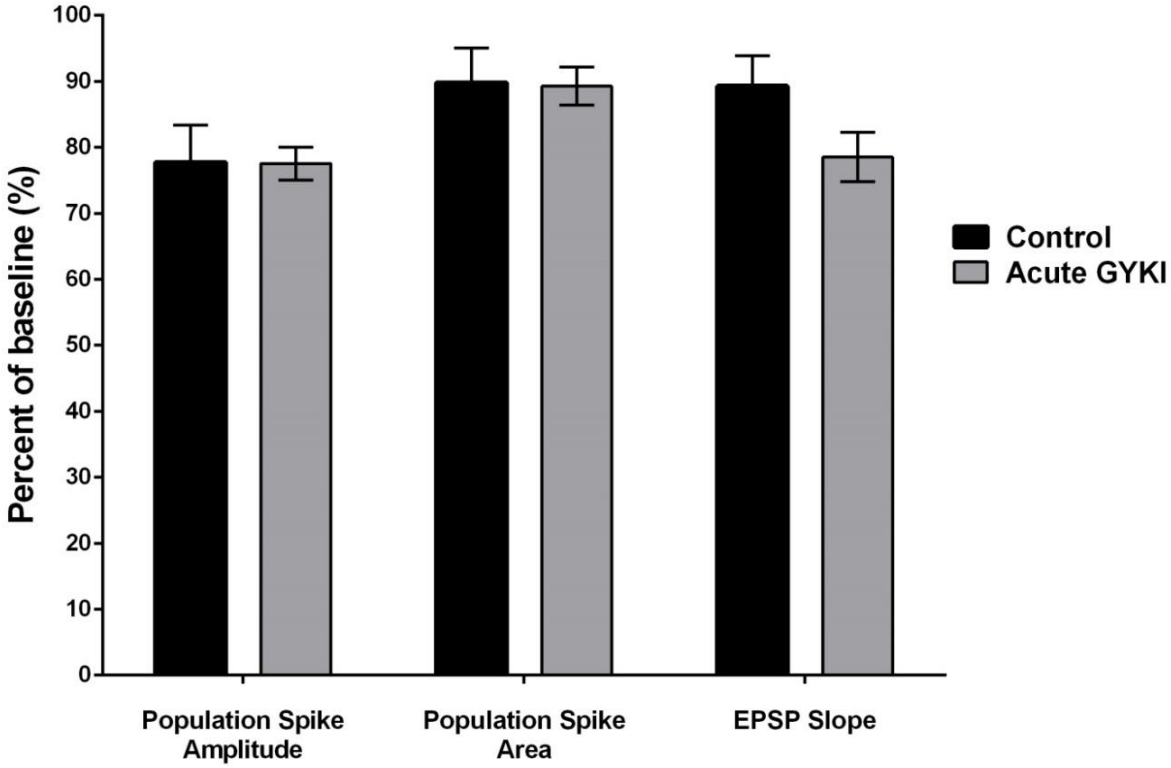

Figure 3.7: Effects of acute GYKI preconditioning on LTD in the CA1 region of the hippocampus. Acute GYKI preconditioning was performed as described in Section 2, however this preconditioning did not enhance LTD of the population spike amplitude, area or EPSP slope compared to control slices. Data represents mean % of baseline ±SEM for control slices (n=9; black bars) and preconditioned slices (n=8; grey bars). Statistical analysis was performed using an unpaired student's t-test, comparing the last five minutes of data from each group. * indicates P<0.05 versus control slices.

3.4.2 Chronic GYKI Preconditioning

Chronic GYKI preconditioning did not enhance subsequent induction of LTD (Figure 3.8). Control slices (n=9) exhibited on average a 30.8 ±5.4% decrease in population spike amplitude, a 16.7 ±4.3% decrease in population spike area, and a 14.7 ±3.4% decrease in EPSP slope. In comparison, acute GYKI preconditioned slices (n=8) exhibited on average a 24.0 ±6.4% decrease in population spike amplitude, a 14.6 ±5.9 % decrease in population spike area, and a 10.2 ±2.7% decrease in EPSP slope. The magnitude of LTD from chronic GYKI preconditioned slices was not significantly different compared to control slices for any of the field potentials measured (P>0.05).

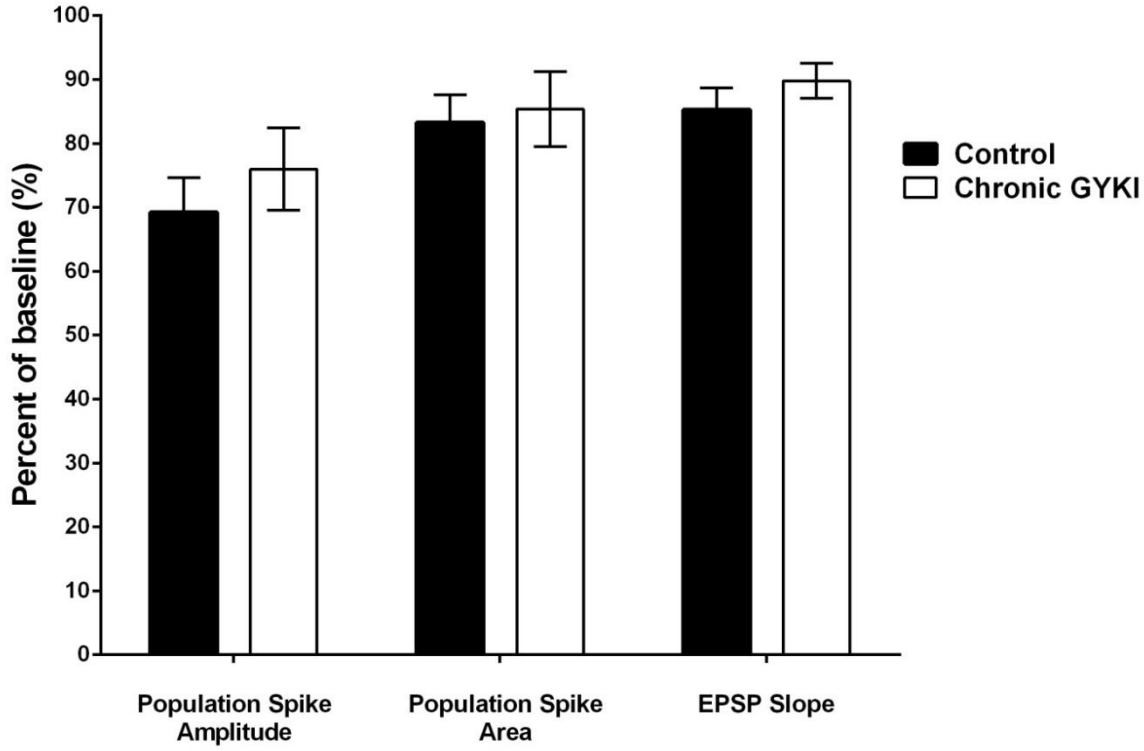

Figure 3.8: Effects of chronic GYKI preconditioning on LTD in the CA1 region of the hippocampus. Chronic GYKI preconditioning was performed as described in Section 2, however this preconditioning did not enhance LTD of the population spike amplitude, area or EPSP slope compared to control slices. Data represents mean % of baseline ±SEM for control slices (n=9; black bars) and preconditioned slices (n=8; white bars). Statistical analysis was performed using an unpaired student's t-test, comparing the last five minutes of data from each group. * indicates P<0.05 versus control slices.

3.4.3 Acute DOM Preconditioning

Acute DOM preconditioning did not enhance subsequent induction of LTD (Figure 3.9). Control slices (n=11) exhibited on average a 16.1 ±3.5% decrease in population spike amplitude, a 8.5 ±2.9% decrease in population spike area, and a 17.4 ±3.7% decrease in EPSP slope. In comparison, acute GYKI preconditioned slices (n=8) exhibited on average a 18.8 ±4.7% decrease in population spike amplitude, a 10.4 ±3.9 % decrease in population spike area, and a 20.2 ±4.4% decrease in EPSP slope. The magnitude of LTD from acute DOM preconditioned slices was not significantly different compared to control slices for any of the field potentials measured (P>0.05).

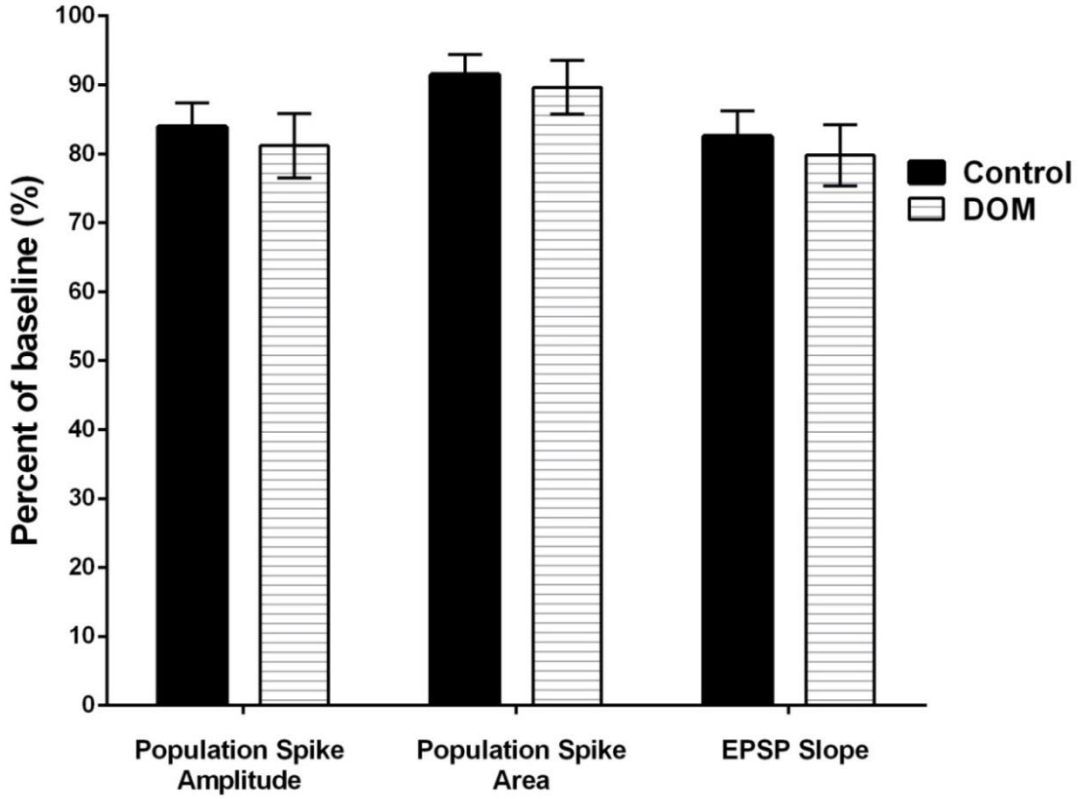

Figure 3.9: Effects of acute DOM preconditioning on LTD in the CA1 region of the hippocampus. Acute DOM preconditioning was performed as described in Section 2, however this preconditioning did not enhance LTD of the population spike amplitude, area or EPSP slope compared to control slices. Data represents mean % of baseline ±SEM for control slices (n=11; black bars) and preconditioned slices (n=8; striped bars). Statistical analysis was performed using an unpaired student's t-test, comparing the last five minutes of data from each group. * indicates P<0.05 versus control slices.

4 Discussion

The results of this study indicate that pharmacological preconditioning with GYKI or DOM does not enhance long term potentiation (LTP) or long term depression (LTD) in the rat hippocampus.

Synaptic plasticity is the leading experimental model for the neurophysiological changes underlying learning and memory formation. Consequently, compounds with the capacity to alter synaptic strength offer a novel approach for the discovery and development of nootropic agents. In the present study, an *in vitro* approach was used to assess the effects of pharmacological preconditioning with GYKI or DOM on the subsequent induction of LTP or LTD in the rat hippocampus. Electrophysiological techniques were employed to measure three field potentials (population spike amplitude, area and fEPSP slope), before and after LTP or LTD induction, from both control and preconditioned slices. This approach varied slightly from previous studies, whereby only one field potential was recorded. However, all three were measured in the current experiment to maximise thoroughness and accuracy of the results.

4.1 Interpretation of Results

Neither acute or chronic preconditioning with GYKI, nor acute preconditioning with DOM enhanced subsequent induction of LTP or LTD in hippocampal slices (Figures 3.3 - 3.5, and 3.7 - 3.9). This was the case for all three field potentials measured, suggesting that neither drug is an effective nootropic candidate. However, these findings were not in accordance with previous studies, which have shown that acute preconditioning with either drug does enhance subsequent LTP and LTD induction (Guy and Kerr 2006, unpublished observations; (94)

In a brain slice recording, LTP manifests as a sustained increase in the magnitude of extracellular field potentials. Therefore, if a drug was said to enhance LTP, this would be evident by a significantly larger increase in the magnitude of these field potentials following tetanic stimulation compared to that seen in control slices. However, in the current study, preconditioned slices exhibited a similar increase in population spike amplitude, area and EPSP slope compared to the control slices, indicating that pharmacological preconditioning with GYKI or DOM does not enhance LTP induction in the rat hippocampus (Figures 3.3 - 3.5). In contrast, LTD involves a sustained reduction in the magnitude of field potentials, and therefore a drug capable of enhancing LTD would be expected to cause a significantly larger decrease in the magnitude of these field potentials compared to that seen in control slices. However, in the current study, preconditioning with GYKI or DOM did not significantly enhance LTD, evident by a comparable change in field potentials in control and preconditioned slices (Figures 3.7 - 3.9).

For both LTP and LTD, a 30 minute time-point post plasticity induction was chosen for statistical comparison between preconditioned and control groups. This is a widely accepted time point for long-term plasticity, which must be distinguished from non-persistent forms of short-term plasticity. Consequently, comparing the groups at an earlier time-point would be misleading, as short term plasticity may have confounded results. Performing statistical analysis at an even later time point (for example, 1 hour after LTP or LTD induction) would arguably have been best. However, due to time constraints the 30 minute time-point was deemed acceptable.

4.1.1 GYKI Preconditioning

Despite an overall lack of significance between control and GYKI preconditioned slices, some trends in the results were observed. Acute preconditioning with GYKI noticeably augmented LTD of the fEPSP, evidenced by greater reduction in fEPSP slope from the preconditioned slices compared to the control slices (Figure 3.7). While this difference was close to reaching significance, the fact that this trend was not mirrored by the population spike amplitude or area suggests it was simply an anomaly.

Although acute preconditioning has previously been investigated, the present study is the first of its kind to assess the effects of chronic preconditioning with GYKI. It was postulated that prolonged exposure to GYKI may exacerbate a preconditioning effect, but this was not apparent for either LTP or LTD (Figures 3.4 and 3.8). Although no difference in slice health was observed following chronic exposure to GYKI, the results obtained indicate that prolonged exposure to GYKI may actually *adversely* affect synaptic plasticity. Namely, chronic GYKI preconditioning demonstrated a subtle tendency to impair LTD induction, whereby preconditioned slices on average showed a smaller percentage reduction in population spike amplitude, area and fEPSP slope compared to controls (albeit not significant; Figure 3.8). Furthermore, not only did chronic GYKI preconditioning subtly impair LTD, but it also resulted in the smallest percentage increase in population spike amplitude, area and fEPSP slope out of all the preconditioned groups.

This highlights an interesting relationship between acute and chronic GYKI preconditioning. Although neither preconditioning paradigm elicited any significant effect compared to their respective control groups, acute GYKI preconditioning caused trends in the *expected* direction, while chronic GYKI caused trends in the *opposite*

direction to that anticipated. Although drawing comparisons between different preconditioning groups was not the focus of this study, this observation was nevertheless an intriguing finding, and may lend itself to further investigation.

GYKI's role as a negative allosteric modulator of AMPA receptors renders its potential nootropic capacity paradoxical. AMPAkines (drugs that modulate AMPA receptors) are one of the most abundant groups of nootropic agents available on the market (96), however these drugs commonly act as positive allosteric modulators of AMPA receptors. The enhanced activity of AMPA receptors has been shown to promote synaptic plasticity (96), which is accordance with the purported involvement of AMPA receptors in LTP. Phosphorylation of AMPA receptors during LTP up-regulates their trafficking to the membrane, their conductance and their open probability, all of which act to enhance the function of these receptors (92) (73).

Considering enhanced AMPA receptor function is associated with LTP, it seems contradictory that GYKI could demonstrate nootropic potential. However, in the present study both acute and chronic GYKI preconditioning were performed using a concentration of 6 μM, which falls well below GYKI's reported IC_{50} at AMPA receptors. Considering preconditioning with GYKI has previously proven effective at this low dose, it has been postulated that a novel action at metabotropic receptors may underlie GYKI's beneficial effects (8, 48). This supports the amplifying nature of metabotropic signalling, whereby the cellular response to a weak stimulus (i.e., low dose GYKI) is amplified by second messenger cascades, hereby eliciting a more significant and lasting effect than would otherwise be expected.

However, in the present study it was surprising to see a dramatic reduction in field potentials during GYKI wash in, indicating that GYKI is still acting at AMPA

receptors despite the low dose (Figure 3.1). Considering a 30 minute washout was performed following acute preconditioning, it was expected that all the GYKI would have been flushed out of the slices by the time LTP or LTD induction occurred. Nevertheless, it is possible that a small amount of the drug may still have been present. If this was the case, and if 6 µM GYKI was indeed sufficient to reduce AMPA receptor activity, this may have inhibited LTP induction and contributed to the negative results obtained. Although previous studies reporting GYKI's augmentation of plasticity induction also utilised a 30 minute washout, it is crucial to consider the impact of flow rate. The ACSF used for wash out was flowing from an elevated beaker through a plastic tube that relied upon gravity to function. The diameter of the tube and the elevation of the beaker are therefore hugely influential on the final flow rate of ACSF, which in turn will dictate the efficiency of the wash out. To this end, if the flow rate was slower in this experiment than in previous studies, the GYKI wash out may have been incomplete, thus providing an explanation for the negative results obtained.

The claim that AMPAkines 'enhance synaptic plasticity' suggests they would promote both LTP and LTD formation. However, considering the contrasting role that the AMPA receptor plays in each process, it seems logical that a particular ampakine would have opposing effects on LTP and LTD. While AMPA receptor up-regulation is central to LTP, the opposite is true for LTD. With this in mind, GYKI could theoretically promote LTD induction at concentrations near its IC_{50}, by virtue of its negative allosteric modulation of AMPA receptors. If the GYKI washout was not entirely successful, it could be argued that LTD should have been enhanced following acute GYKI preconditioning. However, this was not the case (Figure 3.7), which may have been a result of the low concentration of GYKI, or a consequence of the high calcium ACSF solution used (see Section 4.6.3).

4.1.2 DOM Preconditioning

Considering DOM is a potent neurotoxin at high doses, its purported ability to enhance mental functions seems paradoxical. However this highlights the importance of dose; many drugs are effectively poisons at high concentrations, despite exerting therapeutic effects at lower doses. This dose-dependent response is generally a function of which receptors the drug is acting at, which may explain DOM's ability to elicit seemingly opposing effects. The fact that such low doses (50nM) of DOM have proven effective for preconditioning when its EC_{50} at ionotropic glutamate receptors is approximately 200 nM (60), suggests an alternate metabotropic receptor target for DOM, just as has been proposed for GYKI. Indeed, a 2004 study demonstrated that 100 nM DOM caused a significant reduction in GTPase activity in hippocampal membranes, adding credence to this suggestion (48).

In line with the aims of the present experiment, a 2009 study examined the effects of neonatal exposure to serial low dose DOM injections on learning and memory tasks in the rat (97). DOM treated rats displayed significant improvements in choice accuracy in the 8-arm bait version of the radial arm maze, and also manifested improvements in several performance measures in the Morris water maze task. Although this study provides further evidence in favour of a positive correlation between low dose DOM and enhanced cognitive function, it can be difficult to draw dose comparisons between *in vivo* and *in vitro* studies. While the daily dose administered to the rats was 20 μg/kg s.c, a number of pharmacokinetic factors, such as volume of distribution and blood brain barrier permeability, must be considered when estimating what *in vitro* concentration this would equate to. Nevertheless, this study is yet another in the growing body of evidence that underscores the importance of glutamatergic signalling in optimal cognitive function.

In the present study, wash in of DOM caused no detectable change in the field potentials of hippocampal slices, which suggests that 50 nM was low enough to avoid DOM's actions at ionotropic glutamate receptors. However, preconditioning with DOM also failed to impart a detectable enhancement in plasticity induction (Figures 3.5 and 3.9). Although this dose has previously proven effective both for neuroprotection and enhancing plasticity, it is likely that the weak induction paradigms used (discussed in Section 4.3), natural biological variability and other limitations discussed in Section 4.6 may help to explain the non-concordant results.

An interesting consideration is that while DOM's neurotoxicity occurs at high doses, and neuroprotection and cognitive enhancement can occur at low doses, a therapeutic window that may lie between these extremes that could provide an even stronger beneficial effect. Considering the NMDA and AMPA receptors have repeatedly been demonstrated to be vital for LTP, it seems logical that slightly higher doses of DOM could enhance LTP, due to its agonistic effects at these receptors. This is especially likely considering that LTP is readily blocked by NMDA receptor antagonists (91). Furthermore, DOM has been shown to induce the enzyme cyclooxygenase 2, which is known to promote formation of LTP via an NMDA receptor dependent mechanism (98).

However, delving back into the fundamental concept of preconditioning, a potential problem becomes clear. Considering preconditioning is based upon the premise that exposure to an 'insult' triggers endogenous protective mechanisms that enable a tissue to tolerate a subsequent insult, DOM preconditioning would theoretically trigger processes rendering the tissue less responsive to subsequent DOM. It is reasonable to believe that this may involve changes to ionotropic glutamate receptors

which, considering the critical role these receptors play in LTP/LTD induction, could theoretically have a dramatic inhibitory effect on both these phenomena.

4.2 Variation in controls

An interesting observation upon graphing the final results was the variation in the magnitude of LTP and LTD induced in each control group. Considering the slice preparation and stimulation protocol remained unaltered between control groups, one would expect similar results from each, and yet this was not always the case. A likely reason for the variable results was the quality of the hippocampal dissection. A fast yet careful dissection minimises damage to the hippocampal tissue, which would likely result in more robust field potentials and improved plasticity induction. In accordance with this theory, the smallest magnitude of LTP overall was seen in the chronic GYKI preconditioning group and their respective controls. This happened to be the first set of experiments carried out, and therefore likelihood of tissue damage and impaired LTP induction was heightened due to the lack of dissection experience. However, it was the *relative* difference in the magnitude of plasticity between control and preconditioned groups that was of interest in the current study. Consequently, this factor should not affect the conclusions regarding preconditioning, as similar damage would have been imparted to both control and preconditioned slices.

Another factor contributing to the irregular results is natural biological variability. Despite this being an *in vitro* experiment, the hippocampal slices were still living tissue, and were therefore liable to exhibit similar biological variability to that expected from humans. Indeed, this variability was apparent in the large size of a number of the error bars, both for control and preconditioned groups.

4.3 The effect of Plasticity Induction Paradigms

When looking at previous related studies, it becomes clear that there are countless different induction paradigms for LTP and LTD. Considering the delicate nature of *in vitro* work, it is entirely plausible that small variations in experimental protocol could have a dramatic effect on the outcome of plasticity induction.

In the present study, control slices from all groups on average exhibited 25%, 16% and 16% LTP for population spike amplitude, area and fEPSP slope, respectively (combined from Figures 3.3, 3.4 and 3.5). While this is lower than previously reported in the hippocampus (82-85, 99), a likely explanation is that the present LTP induction paradigm was relatively weak compared to that used in past studies. More specifically, common LTP induction paradigms involve either tetanic bursts of at least 100 Hz (86, 87), or instead deliver tetanic stimulation for at least 5 seconds (83-85, 99). Furthermore, previous studies have reported delivering multiple tetanic trains, as opposed to the two delivered presently (87, 100). It has been repeatedly demonstrated that the strength of the tetanus is proportional to the degree of LTP induced (101, 102). Therefore, it stands to reason that the weak tetanus used presently would elicit weak LTP induction. However, LTP is a saturable phenomenon, in that it reaches a plateau upon which there can be no further increase in LTP regardless of an increase in tetanic stimulation (103). The reasoning behind the use of a weak induction paradigm here was to deliberately elicit a sub-maximal LTP response, thereby enabling room for preconditioning to detectably augment this phenomenon. Arguably, a strong induction paradigm may have elicited a maximum, or 'saturated' LTP response, leaving no room for the phenomenon to be enhanced by preconditioning. However, this decision may have been flawed. Considering that evidence for preconditioning as a pro-cognitive strategy is still in its infancy, and that the current study aimed to replicate preliminary

findings, employing experimental methods in line with those used previously may have lessened speculation regarding non-concordant results.

A similar trend was seen with the LTD results. On average, control hippocampal slices exhibited 23% LTD of the population spike amplitude, 12% LTD of the population spike area, and 14% LTD of the fEPSP slope (combined from Figures 3.6, 3.7 and 3.8), which is lower than previously described (103, 104). However, as discussed above for LTP, it is likely that the weak LTD induction paradigm chosen may provide an explanation for this. Many previous research groups have delivered multiple 'bouts' of low frequency stimulation (i.e., 2 x 1 Hz for 15 min), or alternatively have used slightly higher stimulation frequencies during (i.e., 10 Hz rather than 1 Hz) (87) Guy and Kerr 2006, unpublished observations; (11). However, LTD is also a saturable phenomenon, so for the reasons described for LTP, a weak LTD induction paradigm was once again chosen (103).

Following on from this, Mulkey *et al.* investigated the effect that the length of low frequency stimulation had on the subsequent magnitude of LTD induced (103). In accordance with previous data, they showed that continuous 1 Hz stimulation for 10-15 min reliably produced robust LTD (36% reduction in EPSP slope). However, they also investigated whether shorter bursts of activity, which more likely mimic endogenous firing patterns, also elicited LTD. Interestingly, they found that multiple short bursts (30 s) of 1 Hz stimulation were capable of inducing LTD to a similar extent as prolonged, low frequency stimulation. While the initial 30 second stimulus train only evoked a modest (13%) depression of synaptic strength, by the sixth stimulus burst, synaptic strength was reduced by 35%, similar to that seen as a result of 10-15 min of low frequency stimulation (103).

With this in mind, it could be reasoned that similar principles may apply to LTP induction. If shorter bursts of electrical activity are more representative of physiological brain activity during learning and memory, employing LTP induction paradigms that mimic this closely would theoretically provide the best platform for assessing nootropic potential of drugs. For this reason, it is plausible that alternative induction paradigms may have been the key to unveiling a preconditioning effect in the present study.

4.3.1 LTP and LTD Induction

A critical question that arises as a consequence of the weak induction paradigms used is whether or not LTP and LTD were actually induced. There is no defined threshold for what is considered 'significant' LTP or LTD, and consequently one could question whether the slices had experienced these phenomena at all. Interestingly, a study many decades ago set the threshold for LTP and LTD in their experiment at a 20% and 15% change for population spike amplitude and fEPSP slope, respectively, and as a consequence of these criteria, 35% of the slices exhibited no long-term plasticity (72). Arguably, a statistically significant increase or decrease in the field potentials compared to baseline could be deemed LTP and LTD, respectively. Indeed, this form of comparison was chosen in the present study, whereby a one sample t-test was used to determine whether the field potentials had exhibited a significant change following LTP or LTD induction. Although the majority of slices did exhibit significant changes, on a small number of occasions the LTP or LTD of a particular field potential did not reach significance (Table 3.1 and 3.2). When bearing in mind the previously reported effects of preconditioning with GYKI and DOM, it was particularly interesting to note that all the instances of unsuccessful LTP or LTD came from control, as opposed to preconditioned slices. Although preconditioning did not result in any significant differences compared to control slices, the fact that LTP and LTD induction

were more successful overall in preconditioned slices was intriguing. This indicates that perhaps preconditioning is having a subtle beneficial effect on plasticity, by lowering the threshold required to induce LTP and LTD as opposed to increasing the magnitude of the LTP/LTD induced. If this were true, this would suggest that preconditioning may be related to the realm of phenomena often referred to as metaplasticity, whereby neuronal changes elicited at one point in time are able to regulate subsequent synaptic plasticity (93). Although this suggestion is intriguing and may warrant further investigation, within the realms of the present study it was a *relative* increase or decrease in LTP/LTD compared to control slices that was of interest, and therefore this observation does not impact the final conclusions of the study.

4.3.2 Temperature effects

The effect of temperature on enzyme activity is well documented. It is therefore reasonable to expect the magnitude of LTP and LTD to vary as a function of temperature, due to the involvement of kinase and phosphatase enzymes in these processes. The normal body temperature of rats is approximately 37.5-37.8°C (105, 106). Interestingly, however, it has been documented that LTP in hippocampal slices occurs best at temperatures around 31-32°C, whereas LTD is strongest at room temperature (Avshalomov and Kerr 2007, unpublished observations). One could argue that all experiments should be carried out at physiological temperatures in order to mimic the true environment the drugs would be used in. However, in order to maintain consistency with previous studies, both LTP and LTD were carried out at 31 ±1°C in the current experiment, which may have contributed to the lower than normal levels of LTD seen.

4.4 The link between nootropics and LTP/LTD

When considering GYKI and DOM's effect on cognitive function, it is interesting to look back at previous studies investigating the effect that proven nootropic agents have on LTP and LTD. These studies were conducted in a retrospective manner compared to the present experiment; rather than test LTP/LTD and extrapolate those findings to make claims about cognition, they instead tested established cognitive enhancers and looked back to see if they actually influenced LTP/LTD. Interestingly, the results have shown distinct irregularity.

Notably, there has been significant discrepancy regarding the effects of these drugs on baseline field potentials. For instance, piracetam (a positive allosteric modulator of AMPA receptors), has been found to increase the amplitude of the population spike in the CA1 region, without any effect on the fEPSP slope or LTP (107). Likewise, it has been demonstrated that oxiracetam is capable of inducing an LTP-like increase in neurotransmission efficacy in the CA1 region, while having no effect on the magnitude of subsequent LTP induction (86). In contrast, other studies report that certain nootropics exert no noticeable effects on the baseline field potentials, despite influencing the magnitude of subsequent LTP (82-84, 99). For example, bath application of cognitive enhances such as bifemelane, idebenone, vinpocetine, indeloxazine, aniracetam and piracetam, enhanced the magnitude of LTP produced in the CA3 region of the guinea-pig hippocampus, while having no effect on basal synaptic responses (83-85, 87, 99). However, one notable point of interest was that aniracetam significantly augmented LTP at lower concentrations (10^{-7} and 10^{-8} M), but not at higher concentrations (10^{-6} M). This provides further evidence in favour of using low drug doses to obtain a nootropic effect; a finding that was also supported by results from a clinical trial (108). Interestingly, in our hands, acute GYKI preconditioning

reduced the size of the baseline potentials while having no effect on subsequent LTP, which adds further variation to the pool of findings on this topic (Figures 3.1 and 3.3).

In vivo studies have also investigated the effects of nootropics, such as piracetam, hydergine, vinpocetine and physostigmine, on the magnitude of LTP. Intravenous administration of these four drugs did not influence the amplitude of the population spikes. However, while the two former did not have an effect on LTP, the two latter induced a significant potentiation of subsequent LTP (100). Interestingly, this potentiation was only observed if drug administration occurred 5 minutes before tetanic stimulation. Paradoxically, a significant inhibition of LTP was obtained when the drugs were administered 30 minutes prior to tetanic stimulation.

Studies investigating the effects of nootropics on LTD are far less numerous. Although LTD is also implicated in learning and memory, it plays a more sculpting role, and therefore more weight is often given to LTP related research. However, one group sought to investigate the effects of a novel glycine site partial agonist, GLYX-13, on both LTP and LTD induction in the CA1 region of *in vitro* hippocampal slices. GLYX-13 is both nootropic and neuroprotective *in vivo*. Interestingly, the investigators found that this drug simultaneously enhanced the magnitude of LTP, while reducing the magnitude of LTD. This brings the role of LTD in cognition into the spotlight. Considering the virtually contrasting physiological mechanisms of LTP and LTD, it may not be possible for a drug to enhance both processes. Perhaps it is inevitable that if a drug enhances LTP, then it must also inhibit LTD, and *vice versa*.

However, when considering the results of these previous studies, it is imperative not to overlook the fact that they are not preconditioning based. Rather, they look at the effect of drugs during the time that the slice is exposed to it. Furthermore, the majority

of studies also employed concentrations close to the therapeutic dose, whereas the present study utilised sub-therapeutic doses. These factors will undoubtedly have a heavy influence on a drug's effect, and thus it would be interesting to test these established nootropic agents in a preconditioning setting.

4.5 Pros and cons of the *in vitro* hippocampal slice

As previously described, the hippocampus is an ideal model system for experiments pertaining to synaptic plasticity, due to the integral role this structure plays in memory formation and learning. The majority of hippocampal based research uses rodents as a model animal, as the trisynaptic circuit architecture and patterns of connectivity are similar between rats and humans (77). Furthermore, the easily recorded field potentials provide a valuable tool for detecting changes in synaptic strength, and are often used for assessment of basic neurophysiological characteristics of the neurons in the hippocampus (109). The population spike is a compound action potential of a large number neurons measured at the cell layer; the amplitude gives an indication of the synchronicity of the neuronal firing, whereas the area reflects the total number of active neurons. The fEPSP, measured at the level of the dendrites, is representative of the degree of inward ion flow through AMPA receptors in response to excitatory stimulation of the Schaffer-collateral fibres. By strengthening the connection between neurons, this increases both the strength of the fEPSP, and also increases the likelihood of neurons firing in response to stimulation. This is the reason why an increase in pop spike amplitude and area, and in the slope of the fEPSP occurs upon LTP induction, while a decrease occurs following LTD induction. However, considering the complex nature of learning and memory formation, it goes without saying that numerous other brain regions are also implicated. Consequently, it would be unwise to rely solely upon

results from experiments based in the hippocampus to claim conclusively whether or not a drug can enhance cognition.

An *in vitro* approach can be an invaluable experimental tool in science. It is frequently able to minimise any ethical concerns associated with a particular experiment by reducing the number of animals sacrificed (such as was the case in the current study, in which it was possible to record from multiple slices from one animal). It also offers the immense advantage of enabling a greater degree of control over the experimental conditions, and provides the ability to deliver drugs directly to the site of interest. Furthermore, *in vitro* studies also are generally more cost effective, enable simple recording techniques to be employed, and may also require less extensive training for experimenters, all of which makes this approach particularly favourable.

However, despite these numerous advantages, it is also important to reflect on the drawbacks of *in vitro* work. Naturally, a solid argument in favour of *in vivo* models is that they more closely reflect a true physiological setting in which a drug may act. Furthermore, *in vitro* work is often associated with significant damage to the tissue of interest, which is particularly true for the brain due to its naturally delicate nature. For example, in the hippocampal slice all extralamellar connections are severed and the surface contains dead and damaged cells, which may render the hippocampal tissue unable to respond effectively and consistently to preconditioning agents (100, 110). Indeed, unreliable results have plagued efforts to assess the efficacy of preconditioning for neuroprotection *in vitro*. Despite certain drugs consistently demonstrating significant neuroprotection using *in vivo* models, these results have not been reliably mirrored *in vitro.* It is therefore entirely possible that a similar problem presents itself in the current study; perhaps an *in vitro* setting is not capable of unveiling a compound's true

nootropic potential. For this reason, it would be imprudent to claim outright that preconditioning with GYKI or DOM is not pro-cognitive.

With this in mind, an interesting extension of this study would be to administer the preconditioning agents to the rat 1-2 hours prior to sacrifice, rather than directly preconditioning the hippocampal slices. This would give the preconditioning agents an opportunity to act upon a healthy, intact brain, while still retaining the advantages of an *in vitro* hippocampal recording system.

4.6 Critical evaluation of past and present studies

4.6.1 Animal Models

When selecting an animal model to use in a study, it is critical to consider how closely that model represents both the human physiology and the disease of interest. This highlights a significant drawback of the model used presently; although the rat hippocampus shares close similarities with that of the human, the rats themselves were young and healthy. The majority of diseases encompassing memory loss and cognitive impairment are those that affect the elderly, and therefore young rats do not accurately reproduce the true situation in which these drugs would be employed.

However, preconditioning embodies a preventative approach in which currently healthy individuals at high risk of neurodegenerative diseases would receive treatment to prevent or minimise potential future damage. As such, a healthy animal model is appropriate. Unfortunately, good animal models for neurodegenerative diseases are rare, and thus developing an ‘at risk’ model to assess the ability of preconditioning to alter disease outcome would be incredibly challenging.

4.6.2 Duration of drug exposure and recording time

Without doubt, every study encounters limitations, and finding ways to reduce the impact of these represents a significant aspect of study design. One unavoidable limitation of this study was the variability in the length of time that slices were chronically preconditioned with GYKI. As explained in Section 2.5.1.2, slices were placed into a holding chamber containing 6 µM GYKI in ACSF, and left there for at least 2 hours prior to a recording being made. However, on any given day, the order in which the slices were recorded from was varied to reduce any 'time of day' effects. Consequently, if another recording was made first on a particular day, the chronically preconditioned slice may have been in the holding chamber for well over two hours before being used. Considering there was only one tissue recording system available this limitation could not be avoided. Despite this, it was reassuring to note that there were no obvious differences between slices that had been preconditioned for two hours, and those that had been preconditioned for up to seven hours.

A further limitation that could have been more easily amended was the variation in the total length of recording time for control or chronic preconditioned slices compared to slices that underwent acute preconditioning. The time invested in performing both wash-in and wash-out with GYKI or DOM added almost an hour to the recording time prior to LTP or LTD induction. It has previously been demonstrated that prolonged stimulation at baseline frequencies and intensities does not negatively impact slice health (48). However, one cannot be certain that it would not negatively impact subsequent plasticity induction. This is particularly important when factoring in the effects of temperature; not only are acutely preconditioned slices subject to prolonged stimulation, but also are exposed to non-physiological temperatures for a longer period of time. A simple solution to this problem would have been to either perform much

longer baseline recordings for control and chronically preconditioned slices, or to actually perform a wash-in and wash-out using saline. However, this was not done due to time constraints.

4.6.3 The effect of a high calcium/low magnesium ACSF solution

Considering the critical role that calcium plays in both LTP and LTD, it goes without saying that the high calcium/low magnesium ACSF solution used would have significantly impacted results. During LTP, calcium activates protein kinases that cause phosphorylation and insertion of AMPA receptors into the postsynaptic membrane. In contrast, calcium instead activates protein phosphatases in LTD, resulting in de-phosphorylation and internalisation of AMPA receptors (73)(91)(92). Although both processes are calcium dependent, it is the magnitude of the post-synaptic calcium that largely determines whether LTD or LTP occurs (91). Robust rises in intracellular calcium activates the signalling molecules required to induce LTP, whereas LTD is brought about by small, slow rises in post-synaptic calcium levels, which result in activation of LTD associated phosphatases (91)(93). Simply put, when calcium entry is above a certain threshold, LTP will occur, and when calcium entry is below a certain threshold, LTD will take place. Consequently, the high calcium/low magnesium solution used would tend to favour LTP induction, while simultaneously blocking LTD induction, and this may contribute to the lower than normal levels of LTD.

Interestingly, a much stronger preconditioning effect was seen for LTD than LTP by Guy and Kerr (Guy and Kerr 2006, unpublished observations). Aside from the different induction paradigm, the ACSF solution used was the only difference between that study and the present one. This indicates that the high calcium/low magnesium ACSF may indeed have been an adverse factor for LTD induction.

4.6.4 Positive Control

Considering that a preconditioning effect was not seen following acute GYKI, chronic GYKI or DOM exposure, one cannot firmly conclude that the results obtained were not simply due to the experimental design failing to detect a preconditioning effect. Considering that GYKI and DOM are only putative nootropic candidates, including a positive control in the experiment may have been worthwhile. By administering a compound that has been repeatedly shown to enhance synaptic plasticity, it would have been possible to determine whether the induction paradigm and experimental setup was capable of detecting this effect, giving more credence to any negative results obtained.

4.6.5 Do LTP/LTD equal memory?

Despite the extensive evidence that the two are related, measuring synaptic plasticity is nonetheless a relatively indirect measure of memory and learning. Therefore, a critical question is whether LTP and LTD actually equal memory. Although strong evidence has been published suggesting the two do equate (111, 112) (69, 70, 113), several recent studies have questioned whether these phenomena are actually used for memory at all (114-117). Ideally it would be better to do behavioural testing instead, such as the Morris water maze or radial arm maze, to enable measurement of a functional outcome, as this is more relevant to the intended use of the drugs. Furthermore, the unpredictable nature of the effects of nootropics on LTP and LTD also indicate that relying upon a presumed direct correlation between plasticity and memory may be inadvisable.

4.7 Future directions for study

4.7.1 Other Hippocampal regions

The next logical step would be to record from other areas of the hippocampus, such as region CA3 and the dentate gyrus. While CA1 is often used as it gives the most robust and consistent recordings, other areas play equally important roles in hippocampal function. Not only would this provide subtle anatomical and physiological differences, but it has also been documented that the 'type' of LTP and LTD can vary between hippocampal regions. For example, LTP induction at Schaffer-collateral CA1 synapses is NMDA receptor dependent, in contrast to the NMDA receptor independent LTP that occurs at mossy fiber-CA3 synapses (84). Due to the different mechanisms of action for each nootropic compound, it therefore it stands to reason that the efficacy of each nootrpoic is liable to change as a function of hippocampal location.

Indeed, one particular point of interest that arises from reading past studies is that it appears nootropic compounds tend to have a stronger effect in region CA3 compared to CA1. For example, Satoh *et al.* reported that piracetam and bifemelane enhanced LTP in CA3 but not in CA1 of the guinea pig hippocampus. Similarly, somatostatin was shown to augment LTP in CA3 but not CA1 (118). Kaneko *et al.* also demonstrated that bifemelane acts at CA3 not CA1, all of which suggest that testing GYKI and DOM in these regions may unveil a nootropic effect (84).

4.7.2 Investigate proposed metabotropic mechanism

If GYKI and DOM do indeed have a novel metabotropic action, elucidating the particular receptor involved and developing drugs to specifically target that receptor could be a significant breakthrough in the quest for effective preconditioning agents. This could be done by performing two-dimensional gel electrophoresis, and detecting

which proteins are significantly altered in the hippocampus as a result of preconditioning. One potential downstream target is cyclic adenosine monophosphate (cAMP), a ubiquitous second messenger implicated in a range of cellular responses mediated by metabotropic receptors. It has been shown that elevated cAMP levels enhance memory (119, 120). As such, this may be the mechanism behind the proposed metabotropic involvement in enhancing synaptic plasticity, and may represent a promising target for therapies enhancing cognitive function.

4.7.3 Test other drugs, doses and temperatures

Considering the dose-dependent effects that a number of nootropics have, it would be interesting to test preconditioning with other doses of GYKI and DOM. Additionally, it would be good to examine whether established nootropics that have been shown to enhance LTP while exposed to the slice would also do so in a preconditioning setting. Furthermore, as highlighted earlier, performing experiments at normal rat physiological temperatures would help to provide an environment that most closely resembles that in which these drugs would be employed.

4.7.4 Test GYKI and DOM *in vivo*

In view of the limitations of *in vitro* work, testing these drugs *in vivo* would be a logical next step. Preliminary work has already done this using DOM, and has indicated that preconditioning with DOM improves performance in the Morris water maze (Guy and Kerr 2006, unpublished observations). However, no *in vivo* studies investigating the effect of GYKI preconditioning on synaptic plasticity have been performed to date.

4.8 Conclusion

Due to the rapidly aging population, the challenges that neurodegenerative diseases pose for society are bound to increase, which highlights a critical demand for

the discovery and development of cognitive enhancers (6). Although the present study provides no evidence to suggest that preconditioning with GYKI or DOM can enhance synaptic plasticity, it cannot be unequivocally stated that pro-cognitive agents will enhance LTP and LTD. It is clear that some drugs can have a clinical benefit while seemingly having no impact on plasticity. To this end, it would be presumptive to claim GYKI and DOM have no use as cognitive enhancers.

Although the present results are not in accordance with preliminary findings on the topic, it is highly possible that protocol variations may account for this discrepancy. Nevertheless, these results are yet more to be added to the vast array of literature addressing the critical demand for pro-cognitive agents, and may still bring us one step closer to combatting neurodegenerative diseases once and for all.

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