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DOCTORAL THESIS

Modulation of neuronal network activity in the primary motor cortex

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In the present study I investigated the mechanisms of modulation of neuronal network activity in rat primary motor cortex using pharmacological manipulations employing the *in vitro* brain slice technique. Preparation of the brain slice in sucrose-based aCSF produced slices with low viability. Introducing the neuroprotectants N-acetyl-cysteine, taurine and aminoguanidine to the preparatory method saw viability of slices increase significantly. Co-application of low dose kainic acid and carbachol consistently generated beta oscillatory activity in M1. Analyses indicated that network activity in M1 relied on the involvement of $GABA_A$ receptors.

Dose-response experiments performed in M1 showed that beta activity can be modulated by benzodiazepine site ligands. Low doses of positive allosteric modulators consistently desynchronised beta oscillatory activity, a mechanism that may be driven by α1-subunit containing $GABA_A$ receptors. Higher doses increased the power of beta oscillatory activity.

Whole-cell recordings in M1 uncovered three interneuronal subtypes regularly encountered in M1; Fast-spiking, regular-spiking non-Pyramidal and low threshold spiking. With the paradoxical effects of positive allosteric modulators in mind, subsequent voltage-clamp recordings in FS cells revealed a constitutively active tonic inhibitory current that could be modulated by zolpidem in two different ways. Low dose zolpidem increased the tonic inhibitory current in FS cells, consistent with the desynchronisation of network oscillatory activity seen at this concentration. High dose zolpidem decreased the inhibitory tonic current seen in FS cells, coinciding with an increase in oscillatory power.

These studies indicate a fundamental role for a tonic inhibitory current in the modulation of network activity. Furthermore, desynchronisation of beta activity in M1 decreased as viability of the *in vitro* brain slice increased, suggesting that the extent of desynchronisation is dependent upon the pathophysiological state of the network. This indicates that low dose zolpidem could be used as a therapeutic agent specifically for the desynchronisation of pathological oscillations in oscillopathies such as Parkinson's disease.

Key words: Oscillations, interneurones, zolpidem, tonic inhibitory current.

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Abbreviations

5-HT - 5-hydroxytryptamine

6-OHDA - 6-hydroxydopamine

Ach - Acetylcholine

Ach - Acetylcholine

aCSF – Artificial Cerebrospinal Fluid

AD - Alzheimer's disease

ADHD – Attention Deficit Hyperactivity Disorder

AHP – After Hyperpolarisation

AMPAR - 2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) propanoic acid Receptor

BG - Basal Ganglia

BL - Baseline

BZDs - Benzodiazepines

Ca^{2+} - Calcium Ion

CA3 - Cornu Ammonis 3

$CaCl_2$ – Calcium Chloride

CCh - Carbachol

CHPG - (RS)-2-Chloro-5-hydroxyphenylglycine

Cl^- - Chloride Ion

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

CNS – Central Nervous System

COX1- Cyclooxygenase-1

DAP - Depolarising After Potential

DHPG- (S)-3,5-Dihydroxyphenylglycine

EEG- Electroencephalogram

EPSPs – Excitatory Postsynaptic Potentials

FS - Fast Spiking

GABA - Gamma (γ)-Aminobutyric Acid

GAD - L-glutamic acid decarboxylase

$g_{GABA(A)}$ - Unitary Hyperpolarising $GABA_AR$ Mediated Conductance

GIRKs -Inwardly Rectifying K^+ Channels

GPCRs – G Protein Coupled Receptors

GPe - External Globus Pallidus

GPi - Internal Globus Pallidus

HCO_3^- - Bicarbonate Ion

IB - Intrinsically Bursting Cells

ICMS – Intracortical MicroStimulation

IEI –Interevent Interval

IEM 1460 - N, N, H,-Trimethyl-5-[(tricyclo [3.3.1.13, 7] dec-1-ylmethyl) amino]-1-entanaminiumbromide hydrobromide

ING - Interneurone Network Gamma

iNOS - Inducible Nitric Oxide Synthase

IPSCs – Inhibitory postsynaptic currents

IPSPs- Inhibitory postsynaptic potentials

IS - Irregular Spiking

I_{tonic} – Tonic Inhibitory Current

K^+ - Potassium Ion

KA - Kainate

KCC2 - Cl^-/K^+ co-transporter

KCl – Potassium Chloride

K_i - Affinity

KO – Knock Out

LFP – Local Field Potential

LS - Late Spiking Cells

LTS - Low Threshold Spiking

M1 - Primary Motor Cortex

mAchRs – Muscarinic Acetylcholine Receptors

MaCSF – Modified Artificial cerebrospinal fluid

MEG - Magnetoencephalography

Mg^{2+} - Magnesium Ion

mGluR – Muscarinic Glutamate Receptor

$MgSO_4$ – Magnesium Sulphate

mIPSC - Miniature Inhibitory Postsynaptic Current

ml - Millilitre

mM - Millimolar

MPTP - 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine

MΩ - Megaohm

NAC - N-Acetylcysteine

NaCl – Sodium Chloride

NaH_2PO_4 – Sodium Phosphate

$NaHCO_3$ – Sodium Hydrogen Carbonate

NF-kB - nuclear factor kappa-light-chain-enhancer of activated B cells

nM - Nanomolar

NMDAR - N-Methyl-D-aspartic acid receptor

NMDG - N-methyl d-glucamine chloride

NO - Nitric Oxide

NOS – Nitric Oxide Synthase

nRT - Thalamic Reticular Nucleus

NSAID – Non-steroidal anti-inflammatory Drug

P21 – Post-natal Day 21

Pyramidal cells – Principal/Pyramidal Cells

PD - Parkinson's disease

PING – Pyramidal Interneurone Network Gamma

PMA - Pre-Motor Area

PV - Parvalbumin

Rin - Input Resistance

RMP - Resting Membrane Potential

ROS - Reactive Oxygen Species

RSNP - Regular Spiking Non-Pyramidal

S1 - Somatosensory Cortex

sIPSC – Spontaneous Inhibitory Postsynaptic Current

SMA - Supplementary Motor Area

SNr - Substantia Nigra pars reticulata

STN - Subthalamic Nucleus

Tau - Decay Time Constant

$T_{GABA(A)}$ - $GABA_A$ Decay Time Constant

TNF–α - Tumor Necrosis Factor-Alpha

VGAT/VIAAT - Inhibitory Amino Acid/GABA Vesicular Transporter

VSD - Voltage Sensitive Dye Imaging

Chapter 1 Introduction

The role of neuronal network oscillations in brain function has become a focus of attention for neuroscientists worldwide and, more recently, the role of network oscillations in pathological states such as Parkinson's, Alzheimer's and epilepsy is increasingly being recognised. The work presented in this thesis makes use of an *in vitro* model of neuronal network oscillatory activity in primary motor cortex (M1). The oscillations described herein depend on gamma (γ)-aminobutyric acid (GABA) receptors and inhibitory interneurones in M1, and the focus of the project is the actions of benzodiazepine-site ligands on pathological beta-frequency activity related to Parkinson's disease. These key areas and concepts are discussed below.

1.1 *GABA and its Receptors*

1.1.1 GABA

GABA is synthesised in the cytosol by the decarboxylation of glutamate catalysed by L-glutamic acid decarboxylase (GAD) and accumulated into synaptic vesicles by an inhibitory amino acid/GABA vesicular transporter (VGAT/VIAAT) (McIntire et al., 1997; Sagne et al., 1997; Chaudhry et al., 1998; Wojcik et al., 2006). There are two isoforms of GAD; GAD65 and GAD67, with GAD67 distributed evenly throughout the neurone and GAD65 concentrated in the nerve terminals where it synthesises GABA for neurotransmission (Martin and Rimvall, 1993). Recent data have demonstrated that there is a functional coupling between GAD65 and VGAT (Jin et al., 2003), showing that VGAT preferentially transports newly synthesised GABA generated predominantly by GAD65 (Jin et al., 2003). This preference is abolished in GAD65 $^{-}$/ $^{-}$ mice (Wu et al., 2007). The vesicular concentration of GABA is thought to be in the range of several hundred millimolar (Axmacher et al., 2004). The fusion of a single vesicle to the presynaptic neurone terminal releases thousands of GABA molecules into the synaptic cleft, generating a GABA concentration that peaks in the millimolar range (Mody et al., 1994; Mozrzymas et al., 2003; Mozrzymas, 2004).

First conclusively identified in brain tissue in 1950 by two independent research groups (Roberts, 1950; Awapara, 1950), GABA was reported to be almost exclusively found in the CNS and not in peripheral tissue. However, it took a decade

of debate and further experimentation until GABA was accepted as a neurotransmitter. GABA was shown to have an inhibitory function on electrical activity in the brain (Florey et al., 1953; Hayashi and Nagai, 1956; Hayashi and Suhara, 1956; Bazemore, 1957), while Del Castillo et al., (1963, 1964), working with the nematode *Ascaris*, demonstrated that application of GABA (10 μM) abolished spontaneous action potentials in muscles. This inhibition was shown to be caused by a hyperpolarisation of the muscle from a resting potential of -30 mV to a lower value of -45 mV, and was mediated by increased chloride ion (Cl^-) conductance (see Bowery and Smart, 2006 for review). In the 1970s and 1980s, experiments were conducted to define the nature of the receptor through which GABA acts, culminating in elucidation of the structure of the ionotropic GABA receptor (Olsen & Tobin, 1990).

1.1.2 GABA Receptors

Two distinct classes of GABA receptors were later identified: the $GABA_A$ receptor ($GABA_AR$), a ligand-gated Cl^- channel; and the $GABA_B$ receptor ($GABA_BR$), a seven-transmembrane segment G protein-coupled receptor (Hill & Bowery, 1981; Bowery et al., 1983). Both types of receptor are present in postsynaptic membranes and also in presynaptic terminals (Axmacher and Draguhn, 2004), where they contribute to regulation of glutamate and GABA release. Hence, whilst ionotropic $GABA_ARs$ mediate fast, phasic synaptic inhibition and tonic extrasynaptic inhibition, $GABA_BRs$ are GPCRs that mediate the slow synaptic actions of GABA. Activation of $GABA_A$ and $GABA_BRs$ can be distinguished pharmacologically by addition of certain ligands. $GABA_ARs$ are activated by the agonist muscimol and blocked selectively by picrotoxin and bicuculline. $GABA_BRs$ are selectively activated by baclofen and selectively blocked by 2-OH-saclofen (see Terunuma et al., 2010 for review). This selective activation of either $GABA_A$ or $GABA_BRs$ provides many different functions, from feedback and feedforward inhibition, through to synchronising large networks of principal cells (Pyramidal cells) (see Farrant and Nusser, 2005, for review).

1.1.2.1 Postsynaptic GABA Receptors

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system and its principal action, which is mediated by ionotropic $GABA_A$Rs, is to increase membrane permeability to Cl^- and HCO_3^- ions. In the majority of mature neurones, this causes a net inward flow of anions and a hyperpolarising postsynaptic response - the inhibitory postsynaptic potential (IPSP). This event occurs when postsynaptic $GABA_A$Rs (from 10 to >100), clustered opposite the release site, are activated following exposure to a brief, high concentration of GABA released from presynaptic vesicles. GABA increases the permeability of membranes to specific ions in such a way as to cause the membranes to resist depolarisation. An increase in permeability to Cl^- ions measured as an increase in membrane conductance, hyperpolarises the membrane and decreases the excitability of the cell (Farrant and Nusser, 2005).

The action of the $GABA_A$R is dependent on the intracellular Cl^- concentration. As mentioned above, at low intracellular Cl^- concentrations, the equilibrium potential for Cl^- is negative to the resting membrane potential, and activation of the channel leads to Cl^- influx and, thus, hyperpolarisation. At high intracellular Cl^- concentrations, the equilibrium potential is positive with respect to the resting membrane potential. In this case, an activation of the Cl^- permeable channel will lead to an efflux of Cl^- ions and the membrane becomes depolarised (Rivera et al., 1999). In this latter instance, as the Cl^- equilibrium is more positive than the threshold for action potential triggering, activation of a Cl^- conductance will lead to the generation of action potentials (Staley, 1992; Owens et al., 1996).

GABA can also depress membrane excitability by increasing K^+ conductance by its action on $GABA_B$Rs that are not co-localised with $GABA_A$Rs. In general, GABA accelerates the rate of return of depolarised membrane segments to the resting potential and also stabilises membrane compartments by decreasing their sensitivity to stimulation (Roberts, 1950). Postsynaptically, $GABA_B$Rs activate inwardly rectifying K^+ channels (GIRKs), leading to hyperpolarisation of the postsynaptic membrane, whilst pre-synaptically they suppress voltage-gated N and P/Q type Ca^{2+} channels (that provide Ca^{2+} influx for release of vesicles (Dunlap, 1998), leading to reduced neurotransmitter release (Couve et al., 2004; Bettler et al., 2004, 2006).

1.1.2.2 Presynaptic GABA Receptors

$GABA_A$Rs can also be expressed presynaptically, where they act to control vesicle release into the synapse. Presynaptic inhibition was first suggested by Frank & Fuortes (1957), who studied the inhibition of the extensor spinal reflex on stimulation of antagonistic afferent inputs (from the flexor). This action has been shown to be either faciltatory or suppressive (Axmacher and Draguhn, 2004), dependent upon intracellular Cl^- concentration and thus the membrane Cl^- gradient and also the activation of voltage-dependent ion channels at the presynaptic terminals (see Schicker et al., 2008 for review). Dudel & Kuffler (1961) proposed that the activation of $GABA_A$Rs on the presynaptic terminals were likely to hyperpolarise the presynaptic terminal through an increase in the resting Cl^- conductance. Activation of $GABA_A$Rs drives the membrane potential towards the Cl^- reversal potential, which is set close to the resting membrane potential in adult neurones. This reversal potential is dependent on the concentrations of Cl^- in the extracellular space and in the cytoplasm. The extracellular concentration of Cl^- remains the same throughout development, whereas cytoplasmic Cl^- levels often decrease, especially during brain maturation (Staley et al., 1992). As a result, $GABA_A$Rs often elicit depolarisation early in development (Staley et al., 1992; Cherubini et al., 1991). During postnatal development, various growth factors lead to the expression of the Cl^-/K^+ co-transporter (KCC2). This leads to a switch of $GABA_A$ responses from depolarising to hyperpolarising within two weeks of postnatal development (Rivera et al., 1999, 2005). There are also examples of depolarising responses to GABA at presynaptic sites in the adult (Bracci et al., 2001) CNS. However, this appears to be due to the HCO_3^- ion and not Cl^- (Rivera et al., 2005).

$GABA_B$Rs can also cause an increase in opening of voltage-gated K^+ channels (Gage, 1992), which may contribute to presynaptic inhibition by hyperpolarising the membrane and/or shunting the presynaptic action potential.

This selective activation of either $GABA_A$ or $GABA_B$Rs provides many different functions; Feedback inhibition, whereby increased Pyramidal cell firing increases interneurone discharge frequency and may thus decrease the Pyramidal cell output; Feed-forward inhibition, where the increased discharge of the interneurone results in

the decreased activity of the Pyramidal cell and helping to increase the temporal precision of firing (Buzsáki, 1984); Synchronisation of large networks of Pyramidal cells (see Farrant and Nusser, 2005, for review).

1.1.3 $GABA_ARs$ - structure and diversity

$GABA_ARs$ are structurally comprised of 5 protein subunits arranged around a pore; an ion channel that is porous to chloride (Cl^-) and bicarbonate (HCO_3^-) under physiological conditions. Each subunit consists of several domains. A larger extracellular N-terminus provides the binding sites for the receptors agonists and antagonists. Three membrane spanning domains encompass an intracellular loop, followed by another membrane spanning domain. The C–terminus is also extracellular (Graham et al, 1996; Rudolph and Mohler, 2006; Mohler, 2006; Olsen and Sieghart, 2009). The second membrane spanning domain of each subunit creates the wall of the pore (Fig. 1.1).

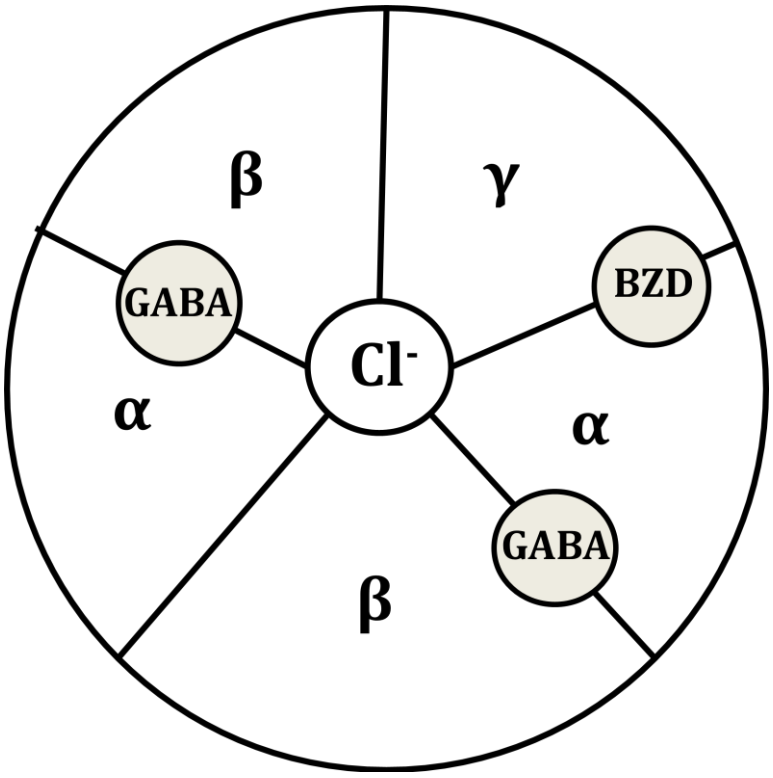


Figure 1-1 Structure and subunit composition of $GABA_ARs$. The composition of the $GABA_AR$; composed of 5 subunits, situated within the cell membrane. GABA binds at the interface of the α and β subunits. BZDs bind at the interface of the α and γ subunits.

Sixteen different subunits have been elucidated to date; α1-6, β1-3, γ1-3, δ, ε, π and θ. There are also a number of ρ subunits that make up a homomeric receptor and show distinct agonist and antagonist profiles. This ligand-gated ion channel is classified as the $GABA_C$ receptor. GABAc receptors can be found in the spinal cord, cerebellum and in the retina. However, in other CNS regions, the ρ subunits can be expressed and incorporated into heteropentameric GABA receptors (Milligan et al., 2004).

The diversity of the subunits and the number of receptors that can be formed (at least 11 are known to be found *in vivo*), creates a number of binding sites for different ligands including barbiturates, benzodiazepines (BZDs) and steroids, as well as the agonist site specific for GABA (Graham et al., 1996; Mohler, 1992). Combinations of α and β subunits are sufficient to form functional $GABA_A$Rs. However, the vast majority of native receptors contain a third subunit type, with data from various different techniques all indicating (Tretter et al., 1997; Baumann et al., 2002; Boileau et al., 2005; Baur et al., 2006) a stoichiometry of two α, two β and one of either γ, δ, ε, π or θ. The major receptor subtypes found naturally are α1β2/3γ2, α2β3γ2 and α3β3γ2 (McKernan and Whiting, 1996; Whiting, 2003; Benke et al., 2004). α1β2γ2 is the most abundant receptor subtype and is widely distributed throughout the CNS (Olsen and Sieghart, 2009; Davies et al., 2000; Barnard et al., 1998). Where α1β2γ2 is absent, the α2 and α3 subunits are found, although at much lower levels of expression (Wisden et al., 1992; Fritschy and Mohler, 1995; Pirker et al., 2000). With so much diversity, many physiological differences are seen between the subtypes. Differences in physiology include channel kinetics, affinity for GABA, rate of desensitisation and ability of the receptor to undergo chemical modification such as phosphorylation (Mohler, 2006). Diversity of subunit composition also creates cell type specific expression and domain specific location (Nusser et al., 1995; Fritschy and Mohler, 1995; Mohler, 2006). For example, δ subunits, usually expressed with α4 or α6, are not found synaptically (Nusser et al., 1998; Poltl et al, 2003) but are abundant at extrasynaptic dendritic and somatic membranes (Olsen and Sieghart, 2009). γ2 subunits are found synaptically and extrasynaptically (Semyanov et al., 2003). Synaptic and extrasynaptic subtypes also differ in their kinetic properties, which is particularly evident within the cortex. δ subunits show slow desensitisation properties extrasynaptically, whereas α2 subunits, found postsynaptically show fast

desensitisation properties (Mohler et al., 2002). The α4 subunit is expressed at very low abundance but is found most prominently in the thalamus and dentate gyrus (Pirker et al., 2000). The α6 subunit is confined to cerebellar granule cells and inferior colliculus granule cells (Luddens et al., 1990). Further to this, as $GABA_AR$s are compromised of two α and two β subunits, these themselves may contain two different isoforms (Benke et al., 2004; Minier and Sigel, 2004; Boulineau et al., 2005).

In the extracellular domain, five pockets are created at the five subunit interfaces (Graham et al., 1996, Rudolph and Mohler, 2006; Olsen and Sieghart, 2009). There are two GABA binding sites, created by two pockets at the α-β interface. The β subunit creates the primary bond, with the α subunit being complementary. Two GABA molecules are generally needed to open the Cl^- channel. $GABA_AR$s are important drug targets representing the sites of action of BZDs, barbiturates, and neurosteroids. At the interface of the α and γ subunits is a specific binding site for BZDs. The α-subunit creates the primary bonding site, with the γ subunit being complementary.

1.1.4 $GABA_A$ Receptor Distribution

α1, β1,2,3 and γ2 subunits of the $GABA_AR$ are found throughout the brain, with some slight variations in distribution, whilst α2, 3, 4, 5, 6, γ1 and δ subunits are confined to certain brain areas, such as α2 in the forebrain (Pirker et al., 2000; Sieghart and Sperk, 2002). The distribution of the $GABA_AR$ varies dependent upon the subtype. The α1 subunit is highly expressed in all layers of the cortex, whereas the α2 and α3 subunits are expressed to a lesser degree (Sieghart and Sperk, 2002). More specifically, the density of $GABA_AR$ distribution across laminae in the motor cortex and somatosensory cortex tends to vary, while distribution within laminae tends to be more evenly distributed (Huntley et al., 1990). Sensory areas appear to have a higher receptor density than motor areas (Zilles et al., 1995). In adult rats the highest receptor density was found in LII/III followed by V, coinciding with the majority reception of cortico-cortical connections (Jones and Wise, 1977; Jones et al., 1978; Jones, 1986).

Subunit	α1	α2	α3	α4	α5	δ	β1	β2	β3	γ2	γ3
Layer											
LI	xxx	xx	x	xx	x	xxx	xx	xxx	xxx	xxx	x
LII/III	xxx	xx	x	xx	x	xxx	xx	xxx	xxx	x	x
LIV	xxx	xx	x	xx	xx	xx	xxx	xxx	xx	xx	x
LV	xxx	x	xx	x	x	xx	xx	xxx	xxx	x	x
LVI	xxx	x	xx	x	xx	xx	xx	xxx		xx	x

Table 1. 1 **Relative density of receptor subunit staining in neocortex** (adapted from Pirker et al., 2000).

1.2 Types of Inhibition

Inhibition of neuronal excitability exists in more than one form. The actions of GABA can exist as synaptic transmission, involving the transient or 'phasic' activation of receptors, or they can exist as a persistent or 'tonic' activation of receptors, whereby the tonic conductance is temporally distinct from synaptic events (Mody, 2001; Semyanov et al., 2004; Farrant and Nusser, 2005).

1.2.1 Phasic Inhibition

Phasic inhibition involves the rapid and precise transmission of activity from the presynaptic neurone to the postsynaptic neurone. An action potential arriving at the nerve terminal of the presynaptic neurone will cause a calcium influx, which induces fusion of a GABA containing vesicle to the membrane. GABA is then released into the synaptic cleft at millimolar concentration. This release of GABA then activates the $GABA_ARs$ located on the postsynaptic membrane, causing the synchronous opening of Cl^- channels, which form the pore of the $GABA_ARs$ (Mody et al., 1994). Activation of these receptors induces a hyperpolarising inhibitory postsynaptic potential (IPSP) in the postsynaptic neurone. Even in the absence of hyperpolarisation (for example when the transmembrane Cl- gradient is symmetric and no net ion flow is observed), the actions of GABA can cause inhibition due to increased membrane conductance. So-called 'shunting' inhibition results in low membrane input resistance, acting as a brake on excitation and decreasing the likelihood of an action potential being fired (see Farrant and Nusser, 2005 for review; Mody et al., 1994). Of course, hyperpolarising and shunting inhibition are not exclusive and either or both effects may underlie the actions of GABA in given locations. Phasic inhibition is vital to the normal functioning of neuronal networks, preventing pathological states and also having a major role in more complex activities (Buzsaki and Chrobuk, 1995; Singer, 1996; Freund, 2003; Somogyi and Klausberger, 2005), such as rhythmic network activity (Traub et al., 1998, Galarreta and Hestrin, 2001).

1.2.2 Tonic Inhibition

GABA concentration decays rapidly from its post-release peak in the synaptic cleft (Overstreet et al., 2002) due to active uptake and diffusion away from the synaptic cleft. This diffusion, or spillover, may activate receptors away from the synapse, either those at adjacent postsynaptic densities, or those located peri- or extra-synaptically. Spillover can therefore be attributed to phasic activation as it is temporally related to a release event; however, spillover can also contribute to an ongoing or 'tonic' conductance. Persistent or 'tonic' activation of receptors occurs in a variety of neurones (Mody, 2001; Semyanov et al., 2004; Farrant and Nusser, 2005) and often appears to be independent of any identified release event. Tonic activity of $GABA_A$Rs seems to be a persistent activation of specific extrasynaptic or perisynaptic receptor populations by low concentrations (nM to mM) of ambient GABA (Cavelier et al., 2005; Farrant and Nusser, 2005; Santhakumar et al., 2006).

Tonic GABAergic signalling in mature neurones was first identified in cerebellar granule cells (Kaneda et al., 1995; Brickley et al., 1996; Tia et al., 1996; Wall and Usowicz, 1997). Further studies have identified a tonic conductance in dentate gyrus granule cells (Nusser and Mody, 2002; Stell and Mody, 2002; Stell et al., 2003; Mtchedlishvili and Kapur, 2006), Pyramidal cells and inhibitory interneurones in the CA1 region of the hippocampus (Semyanov et al., 2003; Scimemi et al., 2005), Pyramidal neurones and interneurones in the somatosensory cortex (Yamada et al., 2007; Keros and Hablitz, 2005) and thalamocortical relay neurones (Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005). Scimemi et al., (2005) noted that some of these studies used either additional GABA or GABA uptake blockers to record a tonic current, particularly in Pyramidal cells. However, interneuronal populations were likely to display a tonic current without the need for this enhancement of GABA concentration (Seymanov et al., 2003).

Many studies have reported that tonic inhibition requires $GABA_A$Rs to contain the δ-subunit, which confers high affinity for GABA and limited desensitisation (Mortensen et al., 2010), both of which are required for an extrasynaptically located, tonic current. However, a few of the studies have noted modulation of the tonic current by the BZD site ligand zolpidem, which by the nature of its action requires a γ subunit (Semyanov

et al., 2003; Yamada et al., 2007). In this context, it should be remembered that all $GABA_A$Rs contribute to the generation of a tonic conductance if the ambient concentration of GABA is high enough to activate them and although δ-subunit containing receptors are exclusively extrasynaptic, non-δ-subunit containing receptors are not exclusively synaptic (Nusser et al., 1995; Brunig et al., 2002). Hence, tonic conductance by non-δ subunit containing receptors could reflect either increased ambient levels of GABA or a selective increase in the affinity of extrasynaptic non-δ subunit containing receptors by, for example, positive allosteric modulators (Farrant and Kaila, 2007). BZDs have been shown to enhance tonic conductance in interneuronal populations in hippocampus (Semyanov et al., 2003) and cortex (Yamada et al., 2007), suggesting α1γ2 subunit containing $GABA_A$Rs can modulate tonic conductances. Increasing GABA concentrations uncovers a α5-subunit containing receptor mediated tonic conductance in hippocampal Pyramidal cells (Caraiscos et al., 2004), while under normal conditions the conductance is mediated by δ-subunit containing receptors. These studies imply that tonic activity is a dynamic process, with different $GABA_A$R populations playing a role under different conditions, with differences dependent upon neuronal populations, pharmacological intervention with positive allosteric modulators, such as BZDs or changes to GABA uptake, all which could have an effect on network activity (Scimemi et al., 2005; Glykys and Mody, 2006; Glykys et al., 2008; Farrant and Kaila, 2007; Mann and Mody, 2010).

The effect of tonic GABA conductance on cell excitability should not be disregarded. Recent work has shown that tonic inhibition has a biphasic effect on cell firing rates. Low tonic GABA conductances depolarise the cell and enhance voltage-dependent resting membrane potential (RMP) fluctuations that can lead to action potential generation. However, as tonic conductance increases, these RMP fluctuations are shunted and the cell cannot fire an action potential (Song et al., 2011).

1.3 Benzodiazepine Pharmacology

BZDs were first discovered in the 1950s with the synthesis of chlordiazepoxide (Sternbach, 1979), used as a tranquiliser for the relief of anxiety. Problems with the use of barbiturates as tranquilisers, due to their low therapeutic index and issues of tolerance and safety, then drove work to find a BZD compound with similar clinical utility. The synthesis of diazepam soon followed that of chlordiazepoxide and this new class of drugs was used clinically to replace barbiturates. The early group of drugs is known as the classical BZDs and all are allosteric modulators of the $GABA_AR$. Binding to the receptor causes a leftward shift in the dose response curve (Figure 1.2). $GABA_ARs$ containing α1, α2, α3 and α5 subunits in combination with any β subunit and the γ2 subunit are sensitive to BZDs. Rogers et al., (1994) proposed that diazepam altered $GABA_AR$ current by acting to increase the apparent agonist association rate, which in turn increases the conduction of Cl^- ions across the neuronal cell membrane. This increased conductance decreases the membrane potential of the postsynaptic neurone resulting in inhibition of neuronal firing (Choh et al., 1977; Macdonald and Barker, 1978). Once bound to the BZD binding site, the BZD keeps the $GABA_AR$ in a conformation in which it has a greater affinity for GABA.

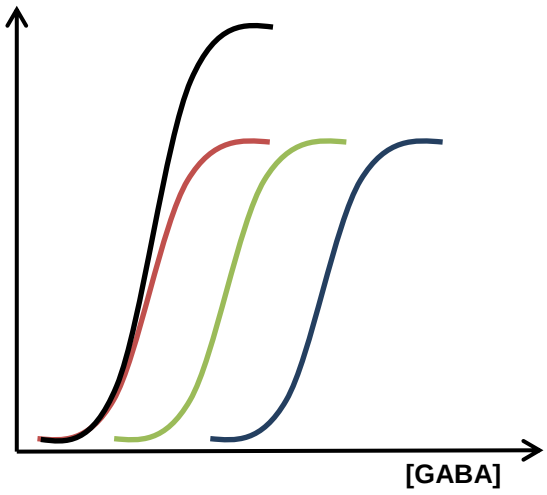


Figure 1-2 Effect of BZDs & Barbiturates on $GABA_ARs$. GABA alone causes a concentration dependent increase in Cl^- current into neurones upon $GABA_AR$ activation (green). BZD agonists or barbiturates in the presence of GABA shift the curve to left (higher GABA affinity, red). High dose barbiturates can also activate $GABA_ARs$ without the need for GABA and thus increase the current over GABA alone, leading to toxicity (black). BZD inverse agonists in the presence of GABA shift receptor function to the right (lower GABA affinity, blue).

GABA receptors containing α, β and γ subunits are sensitive to BZDs and within this group the ligand binding profile of the various forms of α subunit containing receptors varies dramatically. α1 subunit containing receptors have a much higher affinity for BZDs such as CL 218,872 and zolpidem than those containing the α2 and α3 subunits (Barnard et al., 1998; Olsen and Sieghart, 2009; Davies et al., 2000). α5 containing receptors are found in lower abundance and are differentiated from α1, α2 and α3 by extremely low affinity for CL 218,872 and zolpidem. α4 and α6 are thought to be insensitive to BZDs (Rudolph et al., 1999). Furthermore, the selective expression pattern of the subtypes has lead to selective modulation of distinct neuronal networks based on the efficacy of the different BZDs available (Mohler, 2006). For instance, those with high activity at α1 subunits are associated with hypnotic effects, whereas those with higher affinity for the α2 and/or α3 subunits have anxiolytic effects (Table 1.1 and associated references).

Subunit	Action	Reference
α1	Hypnotic, anti-convulsant, locomotor	McKernan et al., 2000, Rudolph et al., 1999, Low et al., 2000., Crestani et al., 2000
α2	Anxiolytic, muscle relaxant	Low et al., 2000
α3	Anxiolytic*, anti-absence effects, anti-pyschotic, muscle relaxant	Yee et al., 2005, * but see Low et al., 2000
α5	Anxiolytic, learning and memory, muscle relaxant	Crestani et al., 2002b
α4/6	BZD insensitive	Korpi et al., 1993, Yang et al., 1995

Table 1.2 BZD activity mediated by the different $GABA_AR$ alpha subunit containing receptors.

The generation of mice with a point mutation on specific subunits of the $GABA_AR$ revealed the pharmacological actions of the different subunit types and, therefore, their relevance in wild type mice. Hence, amino acid substitution (replacing histidine with arginine) within the binding pocket at the α–γ interface rendered target α subunits insensitive to diazepam. This is also why α4 and α6 subunits are BZD insensitive; they contain an arginine residue where the BZD binding site is located (Korpi et al., 1993; Yang et al., 1995). This research has helped to identify a group of novel drugs, specific for the different α subunits and therefore mediating specific effects, such as anxiolysis *or* sedation, which the classical BZDs could not do (Table 1.2 and associated references; Rudolph et al., 1999; Crestani et al., 2000; McKernan et al., 2000; Wingrove et al., 2002). The β subunit does not appear to significantly affect BZD binding to the $GABA_AR$ (Graham et al., 1995; Pritchett et al., 1989).

Drug	Activity	Interaction	Reference
Zolpidem	Hypnotic	α1	Damgen and Luddens, 1999
Zopiclone	Hypnotic	Little difference in affinity at α subunits	Davies et al., 2000
CL 218,872	Anxiolytic, anticonvulsant	α1	Sieghart, 1995
L-838,417	Anxiolytic	Comparable affinity at α1,2,3 and 5 but no efficacy at α1	McKernan et al,. 2000
Diazepam	Anxiolytic, anticonvulsant, hypnotic	Comparable affinity at all α subunits	Sieghart, 1994

Table 1.3 Interaction and activity of ligands that are active at the BZD site of the $GABA_AR$.

1.4 Local GABAergic Inhibitory Interneurones

Inhibitory GABAergic interneurones play an important part in cortical circuitry. Interneurones form distinct networks and play a part in oscillatory activity, with their axons commonly making short-range connections and releasing GABA onto their targets (McBain and Fisahn, 2001; Somogyi and Klausberger, 2005). Characteristics and types of interneurones vary between species (Zaitsev et al., 2009) with the number of interneurones much greater in primates than rodents (Gabbott et al., 1997). Firing properties also vary compared to those seen in rodents (Krimer et al., 2005). Their mutual inhibition is critical in cortical networks and oscillatory dynamics (McBain and Fisahn, 2001). Interneurone types are diverse and many characteristics have been used to define and categorise them, for example, characterisation by physiology, morphology, histochemistry, postsynaptic targets and the presence of electrical synapses are commonly used techniques (Kawaguchi, 1993; Jones 1993; McBain and Fisahn, 2001). GABAergic inputs from specific subtypes of inhibitory interneurone may also target spatially distinct zones on principal neurones (axonic, perisomatic, proximal or distal dendritic), and this is important in regulating neuronal actions (inhibition of spiking, synchronisation, integration of synaptic input) in the cortex (Miles et al., 1996; Pouille and Scanziani, 2001; Somogyi and Klausberger, 2005; Szabadics et al., 2006). It is often difficult to classify interneurones into one particular group due to significant overlap of characteristics. Dendritic morphology has the most variability, but axonal arborisation and connectivity with other cells can go some way to identify the interneurone (Markram et al., 2004). Electrophysiological characterisation classifies interneurone types into four broad groups; fast spiking cells (FS), low threshold spiking (LTS; or burst spiking cells), late spiking cells (LS) and regular spiking non-Pyramidal cells (RSNP) (Kawaguchi and Kubota, 1997). Of these four groups, FS and RSNP cells are more commonly found in the cortex, whereas LS and LTS cells only make up a small proportion of interneurones in the cortex.

What follows is an overview of characteristics of inhibitory interneurones found in the cortex and I will be using this description in this thesis, however, it should be noted that more recently, researchers have called for a change in how interneurones are

classified (Ascoli et al., 2008). Gupta et al., (2000) first noted that there are more electrophysiological characteristics than could be easily placed into four groups, and Steriade (2004), reported that characteristics defined *in vitro* are not consistent and that firing properties of individual neurones can change dependent on current injection intensity, but also, *in vivo*, firing characteristics show some dependence on the behavioural state of the animal. There is no agreement on the number and identity of interneurones within the cortex, partly due to a lack of agreement on what criteria to use to define a cell, therefore different researchers will use different criteria to define cells.

Fast spiking (FS) cells are identified by a non-adapting, high frequency pattern of firing in response to depolarising current injection. They are known to be immunopositive for the calcium binding protein, parvalbumin (PV), and are morphologically classed as basket or chandelier-like (Kawaguchi 1993a, 1993b, Kawaguchi, 1995; Kawaguchi and Kubota, 1997; Galarreta and Hestrin, 2002). FS cells display brief action potentials and a large afterhyperpolarisation (AHP) (Kawaguchi and Kubota, 1995). Their axons most commonly terminate on somata (Jones, 1993; Kawaguchi and Kubota, 1997; Mann et al., 2005) within motor cortical layer (L) II/III with dendrites extending to all layers of the motor cortex (McCormick et al., 1985; Connors and Gutnick, 1990).

Low threshold spiking (LTS) cells (Kawaguchi 1993a, 1993b; Kawaguchi and Kubota, 1997) show two or more spikes from hyperpolarising potentials after current injection. Neurochemically, they test positive for calbindin, also a marker for GABAergic cells (Kawaguchi and Kubota, 1997; McBain and Fisahn, 2001). Morphologically they are classed as double bouquet cells, where axon terminals are on dendritic shafts and spines of Pyramidal cells (Jones, 1993; Kawaguchi and Kubota, 1997). Spikes tend to be wider than those seen in FS cells and LTS cells have a much higher input resistance and a more positive resting membrane potential than most interneurones. LTS cells were also later referred to as Burst Spiking Non Pyramidal (BSNP) cells (Kawaguchi and Kubota, 1997). Their dendrites tend to be bitufted or multipolar. Their axons originate from the pial side of the soma, with the main axon ascending with collaterals into L1. Some axon collaterals are distributed throughout LII/III and

LV (Kawaguchi, 1993; Kawaguchi and Kubota 1993; Kawaguchi, 1995; Kawaguchi and Kubota, 1997).

Late spiking (LS) cells show a slow depolarising ramp to near threshold level before regular action potential firing after a considerable delay (Kawaguchi, 1995; Kawaguchi and Kubota, 1997; Chu et al., 2003) and can be induced to fire from both hyperpolarised and depolarised states. Morphologically, they are neurogliaform cells with multipolar symmetrical dendrites (Kawaguchi and Kubota, 1997) and their axons target dendrites and somata (Jones, 1993). Their input resistance and spike width are much greater than FS cells, despite having a non–adapting firing pattern and a tendency to rest at more positive potentials (Kawaguchi, 1995; Kawaguchi and Kubota, 1996).

Regular spiking non–Pyramidal (RSNP) cells are inhibitory cells (non-Pyramidal cells) that cannot be categorised into the above three groups. Regular spikes can be seen in response to current pulses and at depolarising potentials spiking is strongly adapting (Kawaguchi and Kubota, 1996, 1997 and 1998). They can display two types of behaviour depending on the depolarising current applied (Kawaguchi, 1995). One group consisted of RSNP cells that exhibited a fast depolarising “notch” after a spike when they were depolarised from hyperpolarised potentials and the second group did not exhibit these “notches”. Those with a depolarising notch are multipolar, bipolar or bitufted, with axons originating from the soma or dendrites. Cells that do not show a depolarising notch have axonal branches that extend vertically and are multipolar and bitufted cells (Kawaguchi and Kubota, 1996, 1997 and 1998).

Interneurones can also be divided according to their steady state response (Markram et al., 2004). Non-adapting, adapting, stuttering, irregular spiking and bursting types have been identified. These can then be subdivided dependent upon their onset response; bursting, delay and classic (with no bursts or delays and so their onset in indistinguishable from their steady state response (Markram et al., 2004)). It should be noted that a firing type does not automatically denote a morphological subtype. In fact, morphologically identified interneurones may have many firing behaviours (Kawaguchi and Kubota, 1997; Gupta et al., 2000; Wang et al., 2002; Kawaguchi, 1993; Kawaguchi, 1995).

Cell Type	RMP (mV)	Spike Width (ms)	Neurochemical substances	Morphological type	Synaptic targets
FS	-77.4 - -73.0	0.43 – 0.59	Parvalbumin, cholecystokinin	Basket, Chandelier	Soma, AIS, thick dendrites
RSNP	-66.0 - -60.6	0.75 – 1.06	somatostatin	Martinotti	Thin dendritic branches
LTS, BSNP	~ -57.5	0.94	VIP, calretinin, NPY	Double bouquet	Dendrites, soma
LS	~ -67.2	0.77	calbindin	Neurogliaform	Dendrtitic spines

Table 1.4 Properties of interneuronal subtypes that may exist in M1. Overview of the important properties that distinguish interneuronal subtypes from one another based on morphological and physiological and electrophysiological properties (Halasy et al., 1996; Kawaguchi, 1995; Kawaguchi and Kubotoa, 1997; Cauli et al., 1997).

1.5 Network Activity within the Cortex

Within the cortex, local networks of interneurones acting on the larger population of principal cells create synchronous oscillations from their electrical activity. First described in humans by Hans Berger (1929) from electroencephalographic (EEG) data, oscillatory activity could be split into certain bands based on the frequency of the oscillations. Each band was thought to relate to a certain type of behavioural state. Slower waves i.e. delta (less than 4 Hz) and theta waves (4-8 Hz) were associated with drowsy and sleeping states, delta waves, particularly, can be seen in deep sleep (Amzica and Steriade, 1998). Alpha waves (8-12 Hz) were associated with a resting state, and higher frequency activities such as beta (12-26 Hz) and gamma waves (26-100 Hz) were associated with active behavioural states (Traub et al., 1996). However, more recently it has become clear that particular behavioural states are not confined to specific frequency bands, such that bands can in fact be

associated with multiple behaviours and may also co-exist with one another during behaviour. Gamma rhythms have been of particular interest in the study of cortical oscillatory activity due to the fact that it is thought this particular rhythm is involved in higher cortical processes such as cognition and sensory input (Gray and Singer, 1989; Traub et al., 1999). Gamma oscillations were first reported in early 1930s through electroencephalogram (EEG) measurements. Wolf Singer was the first to hypothesise the importance for gamma oscillations by proposing the gamma binding hypothesis; that gamma oscillations could be the mechanism for perceptual binding (for review, see Singer, 2001). By synchronising assemblies of neurons, which process various features of an object within integrating time windows, gamma oscillations would allow neurons to synchronise and therefore multiply their output onto subsequently connected neurons. The gamma-binding hypothesis has been further extended to include binding across sensory modalities (e.g. audio-visual integration) (Tallon-Baudry and Bertrand, 1999).

Most work to date has been conducted in the hippocampus *in vitro,* while motor cortical oscillations have very rarely been studied *in vitro* although work carried out by Yamawaki et al., (2008) showed beta oscillations predominated in rat primary motor cortex (M1). These oscillations were driven by kainic acid (KA) and carbachol (CCh) induction of interneurone network activity and relied upon $GABA_A$Rs and gap junctions, but not AMPARs. The oscillation was likened to “interneurone network gamma” (ING), first shown in 1995 by Whittington et al., and proposed as a mechanism by Traub et al., (1996).

1.5.1 Interneurone Network Gamma (ING)

ING was considered as a mechanism for oscillation generation when gamma oscillations were observed without Pyramidal cell drive and by blockade of ionotropic glutamate receptors in the hippocampus *in vitro* (Whittington et al., 1995). Two main mechanisms for the generation of ING were shown; tetanic stimuli and mGluR activation accompanied by ionotropic glutatmate receptor block (Whittington et al., 1995; Traub et al., 1996; Traub et al., 1999). Experiments using 2 µM pentobarbital also showed that a network of interneurones was involved. At this concentration pentobarbital, a barbiturate that allosterically acts to enhance the effect of GABA on

$GABA_ARs$, has negligible effects on the intrinsic properties of the interneurones but can alter the frequency of the oscillation and can affect the conductance and/or time course of $GABA_A$ IPSCs. The frequency of oscillations within a mutually inhibiting interneurone network is dependent on three factors; the excitatory driving current to the network, the $GABA_A$ decay time constant ($T_{GABA(A)}$) and the amplitude of unitary hyperpolarising $GABA_AR$ mediated conductance ($g_{GABA(A)}$) (Traub et al., 1996).

1.5.1.1 Driving Current

Mathematical modelling has shown that if a network of mutually inhibiting interneurones are firing synchronously and if they become more depolarised (provided the amount of depolarisation is approximately the same to each cell in the network) they will begin to fire faster (Traub et al., 1996; Jeffreys et al., 1996; Traub et al., 1999). However, the linear relationship between driving current and frequency seen in the models was not entirely repeated in the experiments. In experiments, using puffs of glutamate to regulate the amount of drug to a specific area, frequency increased with driving force up until a point, where frequency would then begin to decrease as driving force continued to increase (Traub et al., 1996; 1999).

1.5.1.2 $T_{GABA(A)}$

The decay time constant, or Tau, is defined as the time taken for a postsynaptic GABA current to decay to 1/e (1/2.718 or about 37 %) of its peak value and provides a means of cross-comparing PSC kinetics regardless of variations in amplitude. After three time constants have elapsed the value of any current is below 15% of the original peak current. Most IPSCs follow mono or biphasic decay profiles, and so it is sometimes necessary to calculate both slow and fast Tau values for a given synaptic IPSC. As the decay time constant of the $GABA_A$ IPSCs increase, a decrease in the frequency of the oscillations is seen (Whittington et al., 1995; Traub et al., 1996; Fishan et al., 1998), reflecting the direct relationship between the large conductance of synaptic $GABA_AR$ currents and observable changes in local field potentials. Experiments using the barbiturate thiopental, which increased tau, showed a steady decline in frequency with increasing concentrations, which, although non-linear, fitted well with modelling data (Traub et al., 1996).

1.5.1.3 $g_{GABA(A)}$

Using the $GABA_AR$ anatagonist bicuculline to decrease $GABA_A$ conductance within the network and the BZD diazepam to increase $GABA_A$ conductance, experimental data showed an increase in network frequency with decreasing conductance and a decrease in frequency with increasing conductance that fit well with modelling data (Traub et al., 1996). However, the slowing of the network frequency is probably also related to the affect of diazepam on $T_{GABA(A)}$ as well as $g_{GABA(A)}$ (Traub et al., 1996, 1999).

Network activity within the thalamic reticular nucleus (nRT), which consists entirely of inhibitory GABAergic neurones, has been suggested as a biologically relevant role for ING (Traub et al., 1996b, 1999b), however, in the motor cortex, and in other areas, ING would most likely constitute a local phenomenon only, as the axonal length of interneurones is short (Traub et al., 1996b, 1999b, 2001, 2004). Long-range synchronisation would therefore not be expected to occur via ING *in vitro* or indeed, *in vivo* (Whittington et al., 1997b).

For ING to be viable biologically, the interneuronal network would be required to be excited uniformly. Computer simulation of ING has suggested that this could be possible when interneuronal networks are linked via dendritic gap junctions providing such uniform excitation (Traub et al., 2001, 2003).

1.5.2 Pyramidal-Interneurone Network Gamma (PING)

During cortical oscillations, both Pyramidal neurones and interneurones are likely to participate (Jagadeesh et al., 1992). A recurrent synaptic feedback loop between Pyramidal cells and interneurones has been proposed to underlie network rhythms on a local scale (less than 400µm) (Freeman, 1968; Colling et al., 1998). Interneurones and Pyramidal cells fire in phase, to within a couple of milliseconds (Traub et al., 1996a; Whittington et al., 1997a). The frequency of the oscillation can be modified by driving currents to the Pyramidal cells as well as by IPSC parameters (Traub et al., 1997; Whittington et al., 1997b).

Network simulations indicate that long-distance synchronisation of gamma oscillations (Gray et al., 1989) could occur when both Pyramidal neurones and interneurones participate. Two-site tetanic stimulation in CA1 showed that both synaptic excitation and inhibition contribute. Using stimuli that are close to threshold for evoking a response, a transient oscillation is produced and provided the stimulus intensity is at least approximately balanced between sites, the oscillations at the two sites are synchronised to within <1 ms (Gray et al., 1989; Traub et al., 1996b; Whittington et al., 1997a, b). Intracellular recordings (Whittington et al., 1997a) show a slow (hundreds of ms) depolarisation, larger than 10 mV is evoked in both Pyramidal cells and interneurones and is blocked in Pyramidal cells when blockers of metabotropic glutamate receptors are applied (Congar et al., 1997). During PING induced by a tetanic stimulus, ING is produced in the interneurone population, under conditions when Pyramidal cell somatic firing is greatly reduced (Whittington et al., 2001). The resulting IPSPs are prominent in Pyramidal cells and can gate the timing of Pyramidal cell action potentials (Whittington et al., 1995). However, showing that it is distinct from ING, PING can synchronise over distances as long as 3.5 mm (Traub et al., 1996b), and is dependent upon AMPA receptors (Traub et al., 1999b; Whittington et al., 1997a). A combination of modelling, mathematical analysis (Ermentrout and Kopell, 1998) and experimental evidence (Traub et al., 1996b) indicate that long range synchronisation is dependent on two mechanisms; narrow action potentials generated by the interneurones that follow each other at short intervals and on the rapid time course and large amplitude of EPSCs in interneurones (Geiger et al., 1997; Miles, 1990). These properties are shown to drive action potentials that are precisely timed, and that give rise to interneurone doublets (double spikes) (Traub et al.,1996b, Whittington et al., 1997a; Bibbig et al., 2002). The within-doublet spike interval of which, provides to Pyramidal cells information about the firing time of Pyramidal cells at another site (Bibbig et al., 2002). Under certain circumstances, this information acts as a feedback signal that stabilises the firing pattern in which the two sites fire synchronously (Ermentrout and Kopell, 1998). The second spike of the doublet is thought to be produced by synchronisation of AMPAR mediated excitatory input from local and distant Pyramidal cells. The EPSP produced is powerful enough to overcome the strong AHP occurring immediately after the first spike of an inhibitory interneurone and generates the spike doublet. The resulting spike doublet summates IPSPs onto Pyramidal cells, which delays the

initiation of local Pyramidal cell spiking and, consequently, the phase lag of spikes between the local and distant Pyramidal cells is reduced. This long range synchronisation occurs at beta frequencies following a shift from gamma (Kopell et al., 2000). The inhibition of local Pyramidal cells following the spike doublets leads to a firing pattern by which interneurones only fire on every second gamma period (Bibbig et al., 2002).

1.5.3 Persistent Gamma

Bath application of pharmacological agents, such as KA and CCh, can induce oscillations lasting for hours (Buhl et al., 1998; Fisahn et al., 1998, 2002; Hormuzdi et al., 2001). Persistent gamma oscillations have been hypothesised to be an experimental model of hippocampal gamma that persists during the theta state (Sik et al., 1995; Ylinen et al., 1995) and may also represent an oscillation dependent on chemical synapses and also on electrical coupling of both axons (principal cell) and dendrites (interneurone), which PING does not rely upon. Persistent oscillations are a rhythmic field potential, < 1 mV in amplitude, reliant upon interneuronal firing, phasic excitation by AMPARs and gap junction coupling.

Traub et al., (2000, 2003a) developed a network model that took into account the above features, the key idea being that the oscillation is driven by the plexus of electrically coupled Pyramidal cell axons. Spontaneous action potentials in these axons pass from axon to axon, generating waves of firing, most of which do not propagate antidromically to somata because of synaptic inhibition and shunting. The firing of Pyramidal cell somata is rare (Traub et al., 2004), and so spikes spread throughout the axonal plexus. Such a mechanism requires AMPARs to propagate orthodromically and excite interneurones, and because the interneurone firing gates the gamma oscillation, $GABA_A$Rs are required. The interneurone gap junctions act to increase coherence of gamma oscillations by leading to higher interneurone firing rates, and so a reduction in gamma power is seen when interneurone gap junctions are removed transgenically (Traub et al., 2004; Hormuzdi et al., 2001). Functional evidence of electrical coupling has been shown in GABAergic interneurones of the hippocampus and neocortex (Beierlein et al., 2000; Galarreta and Hestrin, 1999; Gibson et al., 1999; Tamas et al., 2000; Venance et al., 2000).

This network model is consistent with data showing that KA excites interneurone axons (Semyanov & Kullmann, 2001), with GABA release perhaps exciting Pyramidal cell axons as well.

1.5.4 Electrical Coupling

Two types of electrical coupling have been shown to influence hippocampal population oscillations: those between dendrites of interneurones (Kosaka, 1983; Fukuda and Kosaka, 2000) and those between the axons of Pyramidal cells (Schmitz et al., 2001). The effects on oscillations of these two types of coupling appear to be quite different (Traub et al., 2003a).

Between interneurones, a slow hyperpolarisation or depolarisation in one cell produces a detectable slow hyperpolarisation or depolarisation, respectively, in the connected cell. In contrast, in the case of axonal electrical coupling, antidromic stimulation of hippocampal principal neurones (Schmitz et al., 2001) and somatosensory intrinsically bursting (IB) cells (Roopun et al., 2006) produces communication via spikelets. Pyramidal cell electrical coupling is low in comparison to interneurone electrical coupling (Traub et al., 2004); about 1% in hippocampus (Deuchars and Thomson, 1996), compared to more than 50% of nearby interneurones being electrically coupled (Gibson et al., 1999; Venance et al., 2000).

Connexin36 appears to be the major neuronal gap-junction protein required in interneurones of mature animals (Condorelli et al., 1998; Venance et al., 2000). Electrical coupling between principal neurones appears to be mediated by a protein other than connexin36 (Hormuzdi et al., 2001). Very fast oscillations involving hippocampal Pyramidal neurones, which are known to depend on electrical coupling (Draguhn et al., 1998), are indistinguishable in connexin36 knockout mice compared to wildtype mice (Hormuzdi et al., 2001). For oscillations to be generated in axonal networks, it is necessary that spikes be able to cross from axon to axon (LeBeau et al., 2003; Traub et al., 1999c).

1.5.5 Oscillations in the Primary Motor Cortex

The primary motor cortex (M1) plays an important role in the execution of voluntary movement and oscillations are characteristically seen at beta (15 - 30 Hz) frequencies, particularly before the initiation of movement in primates (Murthy and Fetz, 1992; Donoghue et al., 1998), using local field potential (LFP) recordings. It has long been known that movements elicit frequency specific changes in the EEG (Jasper and Penfield, 1949). *In vitro* electrophysiological studies have also shown beta oscillations in the motor cortex in control rats (Yamawaki et al., 2008), plus further distinguishable oscillations in the mu (8 - 14 Hz) and gamma (> 30 Hz) ranges using human MEG studies (Cheyne et al., 2008).

These beta oscillations reflect interplay between GABAergic and glutamatergic neurones producing synchronised network activity (Yamawaki et al., 2008). *In vivo*, beta oscillatory activity is associated with the resting state; pre-movement or anticipation (Cheyne et al., 2008) or sustained contraction (Baker et al., 2007). When movement is executed, a decrease in beta oscillatory power, coupled with an increase in gamma oscillations, is seen. It is therefore thought that beta represents the preparatory state for movement and/or maintenance of postural tone.

Sensorimotor gamma rhythms have been suggested to serve a similar role within the motor system as the gamma binding hypothesis serves in other cortical areas (Marsden et al., 2000; McAuley and Marsden, 2000). Using MEG studies, Cheyne et al., (2008) demonstrated that simple, self-paced movements of the upper and lower limbs in humans are accompanied by a burst of high-frequency (65 - 80 Hz) gamma activity that can be localised to somatotopically specific regions of M1.

However, paradoxical to the normal beta oscillations seen in M1, pathologically enhanced beta activity is associated with the loss of voluntary movement including akinesia/bradykinesia and rigidity associated with Parkinson's disease (PD) (Brown and Marsden, 1998) (See section 1.9 for further discussion). Beta in the normal brain, as discussed above is thought to be a preparatory state that switches to gamma when movement ensues. Enhanced beta oscillations would therefore be thought to disrupt movement.

1.6 Primary Motor Cortex

1.6.1 Overview

The motor cortex is comprised of three areas. Along with the pre-motor area (PMA) and the supplementary motor area (SMA), known as Brodmann Area (BA) 6, the primary motor cortex (M1) plays an important role in the execution of voluntary movement and is known as BA 4. In 1937, Penfield and Boldrey found that M1 was functionally organised in a somatotopic manner. They called this the "Motor Homunculus" and it depicts a map of M1, with each area innervating a particular body part. The complexity of movement is variable – the homunculus allocation is related to the motor innervation of an area, which underlies the ability for more complex movement. Stoney et al., (1968) followed up this work using intracortical electrical stimulation, where a microelectrode was inserted into M1 and then moved across the region using small steps. It has consistently been shown that there is indeed a topographic organisation to M1, but areas for particular body parts are multiple, overlapping and widely distributed (Nudo et al., 1992; Sanes and Donoghue, 2000). A medial to lateral organisation of leg, arm and head and face is crudely defined, but more recently, studies have suggested functional sub-regions within M1 (Sanes and Donoghue, 2000). Imaging techniques, such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have also confirmed these data by revealing functional overlapping within M1 (Colebatch et al., 1991). M1 is arranged in a columnar manner, each column consisting of five layers; layers I, II and III comprise the superficial M1 and layers V and VI comprise deep M1. There is no layer IV in M1, making it distinct from other cortical areas and therefore considered an agranular area (Donoghue and Wise, 1982). The motor cortex (M1) is well characterised in terms of motor output and long range cortical connections, however, its intrinsic connectivity is less well studied.

In primary somatosensory cortex (S1, BA 1, 2, 3), stellate cells provide dense innervation of the superficial layers (LIV to LII/III), however, M1 has no LIV and appears devoid of stellate cells. What, then, are the ascending circuits in M1? M1 receives and integrates inputs from motor and sensory systems and local circuits are involved in mediating motor-based behaviour. Weiler et al., (2008) found the strongest pathway in M1 is descending, from LII/III to LVa and the main ascending

pathway was from LVa to LII, which the authors thought to be similar to that of LIV - LII/III in S1. Top-down organisation appears to dominate in M1, particularly in excitatory circuits (Weiler et al., 2008) with LII descending pathways dominating. A strong LII/III to LV pathway, with recurrent excitation in upper layers, and which supplies feedforward excitation to lower layers that in turn project onto subcortical circuits may be a logical summary of network connectivity based on the extant literature. Using glutamate uncaging, Hook et al., (2011) have shown that the strongest excitatory connections within M1 are from LII/III to LVa and from LV to LII/III and also ascending within LV. The superficial layers (LII to Va) are spatially compressed within M1 as compared to LII-V within S1 and as compared to LVb and VI within M1.

Using anterograde tracing techniques, Capaday et al., (2004) reported horizontal connectivity within M1. In fact, boutons are located all along axonal projections, and not restricted to terminals (Capaday et al., 1998), allowing neuronal contact across large distances (Capaday et al., 2009). Collaterals extend throughout M1 and to S1, with the majority of boutons forming excitatory connections (Capaday et al., 2009). It is thought that this *en passant* structure, appearing along the axon as it extends, rather than a point-to-point structure, allows the binding of the representations of a variety of muscles, allowing for synergistic interaction between different cortical areas and allowing a wider range of possible movements (Capaday, 2004). Indeed, M1 has been shown to be a canonical circuit (Sanes and Donoghue, 1993) with excitatory and inhibitory neurones over a large region receiving inputs from a given site (Huntley and Jones, 1991; Keller, 1993; Capaday et al., 2004, 2009).

1.6.2 Layer I

Layer I (LI) of M1 lacks Pyramidal cells. It contains axons from LII-VI, from other cortical areas and from the thalamic nuclei, which reciprocally synapse on the dendrites of Pyramidal cells in LII-VI. Studies using GABAergic markers and looking at the morphological appearance of the cells in LI suggest that they are mainly inhibitory neurones. Work by Chu et al., (2003) showed that late spiking (LS) interneurones provide inhibitory input to Pyramidal cells in other layers and to other layer neurones.

1.6.3 Layer II/III

Layer II/III (LII/III) contains both Pyramidal cells and non-Pyramidal cells. Non-Pyramidal cells lack apical dendrites and their dendrites are aspiny. The non-Pyramidal cells in LII/III are mainly GABAergic and consist of four subtypes. Fast spiking (FS), low threshold spiking (LTS), late spiking (LS) and regular spiking non-Pyramidal (RSNP) cells. RSNP can be divided into two further subgroups depending on whether they are fast depolarising (Kawaguchi, 1995). Within LII/III, FS and RSNP cells are the most widely found interneurone types. The diversity of non–Pyramidal cells is also greater than in the deeper layers (Kawaguchi, 1995). Pyramidal cells in LII have undeveloped apical dendrites, while the main axons project to the white matter with many axon collaterals. Their axon varicosities tend to be closely associated with the dendrites of corticospinal cells (Cho et al., 2004). LIII Pyramidal cells have well developed apical dendrites, which ascend to the pial surface (Lev and White, 1997) and form clusters, with axon patterns similar to those in LII (Cho et al., 2004). The Pyramidal cells in these layers appear to have two output types; those which receive input from S1 and send axons to LV Pyramidal cells, including Betz cells (see section 1.6.6) and a more superficial Pyramidal neurone, which receives input from S1, but whose axon collaterals are restricted to LI-III. Pyramidal cells in these layers also project to ipsi/contra-lateral cortex (Kawaguchi and Kubota, 1997). Layer II/III receives multiple neurochemical projections including noradrenaline, 5-hydroxytryptamine (5-HT), and dopamine. Thalamic axons occur at their highest density in LII/III (Douglas and Martin, 2004).

1.6.4 Layer V

Layer V (LV) of M1 contains a large type of Pyramidal cell, known as the Betz cell. These are projection neurones of two types (see section 1.6.6 for a more detailed overview). Smaller Pyramidal cells, similar to those encountered in more superficial layers are also present. These smaller Pyramidal cells have prominent apical dendrites, which are thick, ascend towards the pial surface, and have a well-developed terminal tuft in the superficial layers (Cho et al., 2004). Axons from these cells appear to extend to the spinal cord, however, two types of axon collateral distribution are observed: the first is restricted to infragranular layers and tends to originate in bursting type cells. The second is widely distributed towards neighbouring

columns and supragranular layers and tends to originate in intrinsically bursting Pyramidal cells. Lev and White, (1997) showed that the apical dendrites of LV Pyramidal cells organised into clusters, whilst their cell bodies also formed groups. Activity within these clusters was synchronous and each cluster excited by different afferents, thus creating functional modules. Cells here project towards the brainstem and spinal cord (Kawaguchi and Kubota, 1997).

1.6.5 Layer VI

Pyramidal cells in layer VI (LVI) show reciprocal corticothalamic projections and widely project to other cortical areas and layers. Interneurones within this layer are mostly parvalbumin (PV) positive. Callosally projecting neurones synapse here and innervate interneurones in the contralateral hemisphere (Karayannis et al., 2007). Pyramidal cell apical dendrites terminate in LV with moderately developed apical tufts (Cho et al., 2004; Ledergerber and Larkum, 2010) and horizontally spread axon collaterals within infragranular layers that closely appose to corticospinal dendrites. Apical dendrites are thin and assemble into groups, terminating in LV (Lev and White, 1997). Pyramidal cells project towards thalamus (Jones, 1981; Kang and Kayano, 1994; Kawaguchi and Kubota, 1997). Two types have also been reported dependent upon the finding of a depolarising after potential (DAP). DAP positive cells have a shorter apical dendrite with extensive arborisation in LV and terminations in LIII, with axon collaterals that are less recurrent that DAP negative neurones. DAP negative neurones have long apical dendrites extending to LI/III and the main axon extends into the white matter with ascending recurrent axon collaterals. Dendritic branches were most prominent in LV/VI, and gradually become sparser throughout more superficial layers.

1.6.6 Betz Cells

In deep motor cortex, specifically LVa, a specialised giant Pyramidal cell exists that is not seen in any other cortical area (though Meynert cells of the visual cortex do share some features). First discovered by Betz in 1874, and thus termed the giant Pyramidal cells of Betz, they are approximately 20 times the volume of other Pyramidal cells in deep motor cortex and make up ~12% of the Pyramidal cell

population (Rivara et al., 2003). Although very similar to large Pyramidal cells, they can be distinguished by a number of features. Where Pyramidal cells have an apical dendrite and further dendrites leave the cell basally in a symmetrical fashion, Betz cell dendrites can leave the cells body at any site and have an asymmetrical nature (Wise, 1985). The cell bodies are also heterogeneous in nature, and therefore not necessarily Pyramidal, but can also exist in triangular and spindle like forms (Braak and Braak, 1976). They also appear to be distributed in a manner related to somatopy, with the densest distribution in large muscle representations (i.e. leg and foot) (Lassek, 1940). Betz cells can also be split into two types dependent upon their response to hyperpolarising current injection. Type one Betz cells show a decrease in firing rate after a hyperpolarising step, whilst type two Betz cells show an increase in firing rate (Spain, 1991a, 1991b, 1994). Further to this, Betz cells have been discussed in terms of their projections. One type of Betz cell sends cortical outputs to the striatum, superior colliculus, spinal cord, and basal pons. They have thick, tufted apical dendrites and exhibit intrinsic burst firing characteristics (see Molnár and Cheung, 2006 for review). A second type of Betz cell sends its axon to the contralateral hemisphere or ipsilateral striatum. These cells have slender, obliquely oriented apical dendrites and always show regular spiking characteristics (Molnár and Cheung, 2006). Betz cells that are found in LVb tend to possess smaller cell bodies than those in LVa. Those cells found in LVa are known to send axons to the basal ganglia (BG); in particular, the striatum is well innervated (Lei et al., 2004).

1.7 Primary Somatosensory Cortex

1.7.1 Overview

The primary somatsosensory cortex (S1), also referred to as BA 3b, consists of a mediolateral strip of cortex that extends from the medial wall of the cerebral hemisphere towards the lateral sulcus, where it curves anterior and ends on the ventral surface of the frontal cortex. The main function of rodent S1 lies in its vibrissal system, by which three parallel pathways run from the whiskers to the barrel area of S1. The lemniscal pathway is the classic route by which information reaches S1 after thalamic processing, although two other, parallel, pathways also exist; extralemniscal and paralemniscal. There are no striking differences in the appearance of S1 between species, apart from the absence of barrel cortex in man, most likely due to there being a similar evolutionary and functional importance for this cortical area across all mammals. Differences in long range and short-range connectivity are dependent on the cortical laminar separation. LIV spiny neurones are thought to have no long-range connections and are therefore termed “excitatory interneurones” (Staiger, 2008).

1.7.2 Layer I

LI has no Pyramidal cells (Ren et al., 1992) and is involved in direct regulation of feed forward information transfer from thalamus (Galazo et al., 2008) and feedback information from higher cortical areas, which could have reciprocal connections and could also innervate terminal tufts of Pyramidal cells arborising in LI (Zhu and Zhu, 2004).

1.7.3 Layer II/III

LII is densely packed with Pyramidal cells with no apical dendrite due to proximity to surface. Inputs to LII tend to be from LIV, but a prominent input from LVa also exists. Outputs preferentially target LII and LVa within home and neighbouring columns. LII of rodent S1 is responsible for long-range associational and some callosal

projections (Jones, 1984) and therefore sensory-motor integration as the major supragranular long range connections appear to be to M1 (Alloway, 2008). LIII has a high density of Pyramidal cells of which apical dendrites extend to LI where they tuft. Local intralaminar connections are outweighed by LIV translaminar input (Lefort, 2009). Outputs extend to LVb and VI.

1.7.4 Layer IV (The Barrel Cortex)

“The "canonical microcircuit", a closed loop of within-area connections consists of a series of excitatory feed forward projections, in which the thalamus innervates LIV via the lemniscal pathway then projects to LII/III. These superficial layers then innervate LV, which send projections to subcortical target sites, but also give rise to a collateral projection to LVI. LVI then closes the loop with the thalamus (Douglas et al., 1989).

The barrel cortex, is densely packed with small sized Pyramidal cells, spiny stellate cells (with no apical dendrite) and star pyramids. Two substantial input sources are known; the lemniscal thalamic projection (Gibson et al., 1999; Bureau et al., 2006) and local collaterals of neurones residing within the same column (Feldmeyer et al., 1999; Petersen and Sakmann, 2000). Also, intracolumnar feedback projections originating from all layers as well as transcolumnar projections directly originating in LIV of neighbouring columns can also be found (Martin and Whitteridge, 1984; Staiger et al., 2000; Schubert et al., 2003; Egger et al., 2008).

Each whisker is represented by a discrete and well-defined structure in LIV of S1 (Woolsey and van der Loos, 1970) and is arranged almost identically to the whisker layout. Functional operation of cortical circuits is under rapid and strong top down control (Gilbert and Sigman, 2007).

There exist two important parallel pathways for signalling whisker sensory information to the barrel cortex. The lemniscal pathway originates from neurones in the principal trigeminal nucleus that innervate VPM somatosensory thalamocortical neurones. VPM neurones then terminate in S1 LIV (and some LVI). Direction selective action potentials from mechanosensensitive neurones of the trigeminal ganglion are activated by deflections of the whiskers (Aronoff et al., 2010).

Trigeminal sensory neurones, innervating trigeminal brainstem nuclei, which project to spinal interpolaris nucleus and then posterior medial nucleus of thalamus, form the paralemniscal pathway (Furuta, 2009). Each whisker is represented by a termination field of somatotopically arranged thalamocoortical axons that define the cortical barrel map (Woolsey and van der Loos, 1970; Aranoff et al., 2010). Between the barrels of LIV S1 are areas known as septa, which have their own microcircuits separate from the barrels (Kim and Ebner, 1999).

Excitatory LIV neurones have dendrites and axon arbors confined to a single barrel (Feldmeyer et al., 1999; Petersen and Sakmann, 2000). LIV excitatory neurones primarily innervate LII/III in the immediate overlying area (Lefort et al., 2009). The spread from LI to VI and confinement to the width of one barrel is designated a column (Petersen and Sakmann, 2001). Arborisations of LII/III extend beyond the barrel boundary to other LII/III columns. LII/III to LV and LV to LII/III are other robust connections within a column (Reyes and sakmann, 1999; Bureau et al., 2006; Lefort et al., 2009). Single whisker deflections evoke only one action potential or less, with the contralateral hemisphere being where the response is found (Ferezou et al., 2007).

Neurones of S1 may also differ to those of M1. Egger et al., (2008), found that dendritic arbors were asymmetrical and were closely linked to cortical columns most frequently in LIV. In barrels, dendrites orientate away from barrel borders and are subject to intrabarrel confinement. The main neurones in barrel cortex are spiny stellate and star Pyramidal cells. Spiny stellate and star Pyramidal cells differed in their dendritic symmetry and integration into different cortical circuits. Star Pyramidal cells are radially symmetric and spiny stellate cells are asymmetric and most likely located near to barrel border.

1.7.5 Layer V

LVa consists of medium Pyramidal cells with terminal tufts in LI. Connections are mainly intra- and trans-columnar inputs from LVa and also trans-laminar from LIV. LVa Pyramidal cells have major projections to M1 and the striatum (Alloway, 2008), whereas the LVb projections are diverse, projecting downstream of the thalamus to

the trigeminal nucleus, spinal cord and other areas. IB cells in this layer are the major contributor to driving sub cortical circuits (de Kock and Sakmann, 2008). LVb consists of a mixture of different sized Pyramidal cells in much higher density than LVa. Also, very large Pyramidal cells are present (Schubert et al., 2001). Medium RS cells have home column connections while large IB cells connect trans-columnarly. Strong feedback is received from LVI (Mercer et al., 2005).

1.7.6 Layer VI

LVIa consists of spiny Pyramidal neurones. Intra-columnar, cortico-thalamic connections rarely target each other, but prefer interneurones. Neurones in this layer receive afferent input from local LV and LIV neurones (Mercer et al., 2005; Lefort et al., 2009). Efferent outputs project to the thalamus and also to LIV (Ledergerber and Larkum, 2010). LVI sub-cortical projections are restricted to the thalamic lemniscal ventrobasal nucleus and reticular nucleus, which themselves are reciprocally connected (Bourassa et al., 1995). LVIb is less densely packed than other layers and is tightly intermingled with horizontal pathways (Clancy and Cauller, 1999).

1.8 M1-S1 connections

Several pieces of evidence suggest that S1 modulates M1 neuronal activity. Electrophysiological studies have indicated that S1 modulates activity in M1 (Kosar et al., 1985; Zarzecki, 1989; Kaneko et al., 1994; Farkas et al., 1999; Kelly et al., 2001) and direct connections have been demonstrated repeatedly in cats and rodents (Asanuma et al., 1982; Donoghue and Parham, 1983; Porter and Sakamoto, 1988; Izraeli and Porter, 1995). Anatomical connectivity can be studied by various techniques, including directly injecting anterograde or retrograde tracers or viral vectors into the brain regions of interest, fluorescent proteins and by voltage-sensitive dye (VSD) imaging.

VSD imaging has shown that (whisker) motor cortex serves to integrate sensory input with motor commands (Ferezou et al., 2007). Whisker deflections evoked a response in M1. This begins in S1 and is relayed to M1 in a manner dependent on ongoing behaviour. Monosynaptic excitatory pathways from S1-M1 appear to dominate (Izraeli and Porter, 1995; Hoffer et al., 2003; Alloway et al., 2004; Ferezou et al., 2007; Chakrabarti et al., 2008). Reciprocal connections have, however, also been shown from M1 to S1 (Hoffer and Alloway, 2001).

S1 and M1 are often grouped together as the "sensorimotor cortex" and numerous connectivity studies show why. Peripheral inputs from particular body parts have also been shown to be relayed through somatosensory cortex to the area of motor cortex that produces the associated movement (Porter and Sakamoto, 1988). Further to this, destruction of S1 induces a slight loss of motor skills and also disrupts the ability of the animal to lean complex motor skill (Sakamoto et al., 1989).

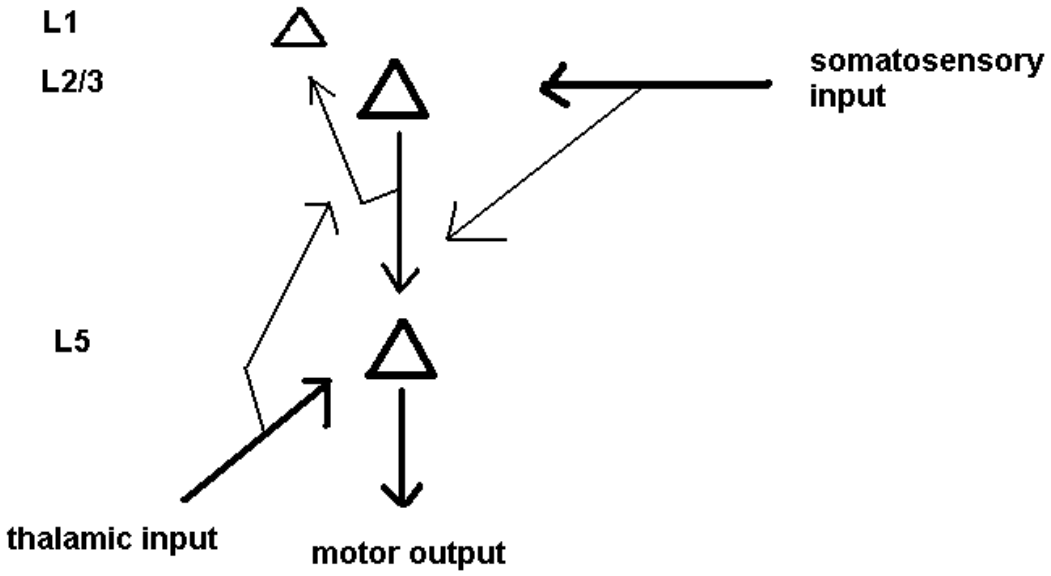


Figure 1-3 Connectivity of M1. Schematic diagram summarising the key connections revealed in connectivity studies (adapted from Kaneko et al., 1994). Major input to M1 is from the somatosensory cortex, mainly via a superficial connection. Thalamic input enters via the deeper layers, perhaps in relay with S1, with weaker inputs to the superficial layers. Descending pathways exit deep M1 to subcortical structures such as the basal ganglia and spinal cord.

Much of the connectivity of the sensorimotor cortices in rodents, as is the focus of the thesis, is conducted in barrel cortex. Of course, motor cortex and somatosensory cortex in rodents is not entirely a vibrissal representation and studies utilising similar tracing approaches in different motor and somatosensory representations have produced similar results. Kaneko et al., (1994a) showed two pathways projecting from S1 to M1. One direct route following superficial connections, later shown to be a definite cortico-cortical connection (Kaneko et al., 1994b) and one indirect route via the thalamus. There is a detailed somatotopic arrangement of the vibrissa in M1, consistent with the barrel cortex. Frostig et al., (2008), after postmortem LIV cytochrome oxidase staining of S1 and M1, showed the spread of activity away from a peak location evoked individually from stimulations of three large whiskers. Long-range horizontal connections to M1 were found and large, highly overlapping activity spread away from the peak in a decreasing gradient. This activity appeared to ignore functional and anatomical borders that have previously been set. Activity is therefore not necessarily restricted to vibrissal M1. Porter (1999) showed the synaptic relationships between neurons in a specific cortico-cortical pathway between

somatosensory cortex areas 3a and 2, and M1. Projections from area 3a to area 2, and from area 2 to MI have also been shown to be somatopically organised in cats (Waters et al., 1982a; Yumiya and Ghez, 1984; Avendano et al., 1992) and monkeys (Pons and Kaas, 1986). In area 2 of both species, layer II–III pyramidal cells give rise to the M1 projection (Ghosh et al., 1987; Porter, 1992), and area 3a axons terminate in layers II and III.

Connections from BA 2a, whereby projections from area 3 were thought to relay, appear to stay superficial (Kosar et al., 1985; Porter and Sakamoto, 1988; Porter et al., 1988) and connections from area 3, which have since been shown to be direct, appear to terminate in all layers of M1 (Herman et al., 1985; Porter, 1991). From barrel cortex to M1, the densest innervations come from cells in LII/III and project to LV/VI (Izraeli and Porter, 1995; Zarzeki, 1989; Kelly et al., 2001). Welker et al., (1988) showed projections from barrel columns via anterograde tracing. Prominent projections to motor cortex came via smooth medium-thick fibres that ran through deeper cortical layers, and branched in LI. Dual anterograde tracing paradigms were also used to characterise patterns of S1 projections to M1 (Hoffer et al., 2003). Many of these projections were shown to arise from septal regions, areas in-between the barrels (Alloway et al., 2004; Chakrabarti and Alloway, 2006). Some neurones in septal regions send the same information to M1 and S1. The barrel and septa represent two functionally distinct cortical circuits (Kim and Ebner, 1999), with S2 receiving information from both, whereas M1 receives information only from septal circuits (Chakrabarti and Alloway, 2006). Barrel and septal compartments have local intracortical connections that are largely segregated. Rostral barrels project to rostral parts of M1, and caudal to caudal. Labelling in M1 also appeared as a mirror image of that in S1, showing the topographic organisation of M1 and S1 to be a mirror image of each other (Hoffer et al., 2003). Sensory processing in motor cortex is most evident in LII/III and is differentially regulated as compared to action potential firing of LV/VI Pyramidal cells, which contribute directly to motor control.

1.9 Oscillations in Pathological states

1.9.1 Overview

Mechanisms that mediate the generation of activity in cortical circuits are prime candidates for understanding the pathophysiology of many neurological diseases. Neuronal network oscillations are a fundamental mechanism for enabling coordinated activity during normal brain functioning (Singer, 1999; Buzsaki and Draguhn, 2004). Parkinson's disease (PD), Alzheimer's disease (AD), schizophrenia, epilepsy and stroke have all been linked to a disruption of normal oscillatory activity (see below). Oscillations in the low (theta and alpha) and high (beta and gamma) frequency ranges establish precise temporal correlations between distributed neuronal responses. Oscillations in the high frequency range establish synchronisation in local cortical networks (Womelsdorf et al., 2007), and appear in all cortical structures. Lower frequency ranges tend to establish synchronisation over longer distances (von Stein et al., 2000); however long range communication may also be subserved at beta frequencies (Kopell et al., 2000). There is abundant evidence for a close relationship between the occurrence of oscillations and cognitive and behavioural responses, for example, gamma rhythms are associated with perception, attention and memory (Jefferys et al., 1995; Fries et al., 2001), whilst beta oscillations are associated with sensory gating, motor control and long range synchronisation (Murthy and Fetz, 1996; Kopell et al., 2000; Hong et al., 2008).

1.9.2 Parkinson's disease

PD is a progressive neurodegenerative disease affecting more than ten million people worldwide. It is characterised by symptoms of akinesia, bradykinesia, rigidity and tremor. The motor abnormalities associated with PD have been hypothesised to be related to changes in the firing activity of neurones in the basal ganglia (BG). In 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD in animals and also in PD patients taken off their medications certain changes have been identified. Disordered activity in the indirect pathway has been stipulated in the "rate model" of abnormal firing in PD (DeLong, 1990). Decreased firing in the external globus pallidus (GPe), along with increased firing in

internal globus pallidus (GPi) and subthalamic nucleus (STN) along with periodic bursts at tremor frequency has been noted (Wichmann et al., 1994; DeLong and Wichmann, 2007). This has lead to the conclusion that disinhibition in the STN leads to increased excitatory drive to GPi and therefore increased inhibition of thalamo-cortical circuits i.e. the motor circuitry. This causes the emergence of akinesia and bradykinesia. It has also been shown that alterations of GP and STN firing are affected by changes in cortical activity (Magill et al., 2000). As well as the rate model, changes in single cell and LFP firing rates have been of particular interest, particularly those in the beta range. Increased beta activity is seen in GPi, STN and SNr in both animal models and patients taken off their dopamine replacement therapy (Brown et al., 2001; Brown and Williams, 2005; Gatev et al., 2006; Hammond et al., 2007; Steigerwald et al., 2008). Excessive synchrony has been strongly linked with bradykinesia (Brown, 2007). Dopamine replacement therapy sees a decrease in beta power and the emergence of gamma oscillations associated with the return of movement (Brown et al., 2001; Magill et al., 2001; Steigerwald, 2008). It must be noted that beta synchrony within the motor system is not a cause of PD symptoms; rather, excessive, pathological synchrony is hypothesised to be the problem. Hence, beta synchrony is seen normally and effectively represents an idling state or the preparatory state before movement (Cheyne et al., 2008).

1.9.3 Schizophrenia

Schizophrenia is characterised by prominent psychotic symptoms that include hallucinations, delusions, reduction in affect and behaviour (negative symptoms) and disorganisation of thought and language/speech (thought disorder). In addition, patients with schizophrenia exhibit impairments in both basic sensory processing and higher cognitive functions, such as language, reasoning and planning and also display social dysfunction. The onset of symptoms usually occurs in young adulthood.

Patients with schizophrenia show a reduction in the amplitude and phase locking of evoked oscillations during the processing of visual information and also demonstrate reduced amplitude and synchronisation of self-generated, rhythmic activity in several cortical regions (Canive et al., 1996). This suggests an impaired ability to precisely

align oscillatory activity with incoming sensory information (Spencer et al., 2004; 2008). Deficits in the high gamma band have also recently come to light (Ford et al., 2007). An impaired performance in an organisation task also showed widespread reduction in gamma-band power in the right temporal lobe 50-300 ms after stimulus onset and further studies have also reported changes to low frequency oscillations (see Uhlhaas and Singer, 2010 for review).

1.9.4 Alzheimer's Disease

AD is associated with a wide range of cognitive dysfunctions that typically start with the characteristic memory impairment. This is followed by deficits in visuo-spatial and executive processes. EEG measurements during the resting state of AD patients show an increase in the theta and delta band (Poza et al., 2007) activity that coincides with a reduction in activity in the alpha and beta bands, associated with impaired synchrony. The reduction in alpha band activity has been shown to be well-correlated with the severity of the disease and the cognitive deficits observed (see Uhlhaas and Singer, 2006 for review).

1.9.5 Epilepsy

Epilepsy is characterised by recurrent seizures, representing pathological states of hypersynchrony that may spread to encompass the entire cortical mantle. Numerous pathological oscillations, including beta activity (Hirai et al., 1999), very fast oscillations (Fisher et al., 1992) and augmented theta activity (Clemens, 2004) have been associated with epilepsy of various forms.

1.9.6 Stroke

A stroke is characterised as the rapidly developing loss of brain function due to a disturbance in the supply of blood to the brain. Classified into two major forms based on aetiology, stroke can be due to ischemia, caused by an interruption of the blood supply caused by a blockage (thrombosis, arterial embolism), or a haemorrhage, resulting from the rupture of a blood vessel or abnormal vascular structure. As a

result, the affected area of the brain is unable to function resulting in certain symptoms such as the inability to move one or more limbs on one side of the body, the inability to understand or formulate speech, or an inability to see one side of the visual field (Donnan et al., 2008).

Recent work by Hall et al., (2010) has shown the emergence of abnormal oscillations in the peri-infarct area of an ischemic stroke patient, indicating that the neuronal tissue surrounding the lesion exhibits pathological oscillatory activity years after the initial injury. Increases in the power of the theta and beta frequency bands were most notable and could not been seen in the hemisphere contralateral to the infarct. Administration of zolpidem, but not zopiclone, at sub-sedative doses had a powerful desynchronising effect on the enhanced theta and beta oscillations that correlated with an improvement in cognitive and motor function. An increase of slow frequency band powers and a reduction of relative fast band powers were also found in stroke patients in the affected hemisphere (Tecchio et al., 2006).

1.10 Aims and Hypotheses

1.10.1 Hypothesis

Oscillations in M1 have shown to be pathologically enhanced in Parkinson's disease (PD). Modulation of these pathological oscillations would appear to be a prime target for treating PD. Zolpidem, an imadizopyridine, which acts at the benzodiazepine (BZD) site of α1 subunit containing $GABA_A$Rs, has recently been shown to modulate oscillatory activity *in vitro*. *In vivo* work with zolpidem at sub-sedative doses in PD and stroke patients has also shown modulation of beta oscillations, particularly the desynchronisation of such rhythms, corresponding to an improvement in motor function (Hall et al., 2010; 2011). If the action of zolpidem *in vivo* is indeed modulated via the α1 subunit, then we would expect low doses of zolpidem to be desynchronising *in vitro*. If this is true, then other non-α1 specific BZD site ligands would have a different profile of modulation compared to zolpidem. It is therefore hypothesised that zolpidem at low doses *in vitro* will modulate beta oscillations in M1 by its action at the α1 subunit of the $GABA_A$R and will act to desynchronise the oscillatory activity.

1.10.2 Aims

- To produce stable oscillations in M1 *in vitro*
- To determine the effects of different concentrations of zolpidem on beta oscillatory activity in M1
- To determine if the effects of zolpidem are mediated by the α1 subunit of the $GABA_A$R
- To determine the profile of modulation of beta oscillations by other BZD site ligands

Chapter 2 Methods

2.1 Cortical Slice Preparation

Coronal slices were prepared from male Wister rats (juvenile, ~postnatal day 20-21, 40-70g). Each rat was anaesthetised with isoflurane until no heartbeat was detected (following the Animals Scientific Procedures Act 1986, U.K.) and transcardially perfused with ice-cold sucrose-based artificial cerebrospinal fluid (aCSF) containing (in mM); 205 sucrose, 2 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 5 pyruvate and 2 $CaCl_2$ and saturated with 95% O_2 and 5% CO_2. Following perfusion with approximately 100 ml aCSF, the brain was extracted and incubated in the same sucrose-based aCSF. Indomethacin (45 µM), a cyclo-oxygenase inhibitor was added to the sucrose-based aCSF to improve cell viability (Pakhotin et al., 1997). The anti-oxidants ascorbic acid (300 µM) and uric acid (400 µM) were also added as neuroprotectants (Rice et al., 1994). Coronal slices were cut at 450 µm for extracellular recordings and 350 µm for whole–cell recordings and were cut in sucrose-based aCSF cooled to 3-4ºC, using a HM 650 V microslicer and CU 65 cooling unit (Microm, De.). Slices were subsequently stored in an interface chamber (for extracellular recordings) or a submersion chamber (for patch–clamp recordings) filled with oxygenated glucose-based aCSF containing (in mM); 126 NaCl, 3 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 2 $CaCl_2$ and 0.30 ascorbic acid at room temperature (20-25^{o}C). Storage aCSF also contained indomethacin (22.5 µM) and uric acid (400 µM). For extracellular recordings, slices were then transferred to a recording chamber (Scientific System Design Inc, Canada, figure 2.1) and continuously perfused with glucose-based aCSF for 60 minutes. The temperature of perfusing aCSF was maintained using a PTC03 proportional temperature controller (Scientific System Design Inc., Canada) at 33-34 ^{o}C. After 60 minutes, kainic acid (KA, 400 nM) and carbachol (CCh, 50 µM) were added to the perfusing aCSF (but see chapter 3). KA and CCh have been shown to elicit oscillations in cortical slice preparations. For intracellular recordings, slices were transferred to a submerged recording chamber where they were maintained at room temperature (see next section for more details).

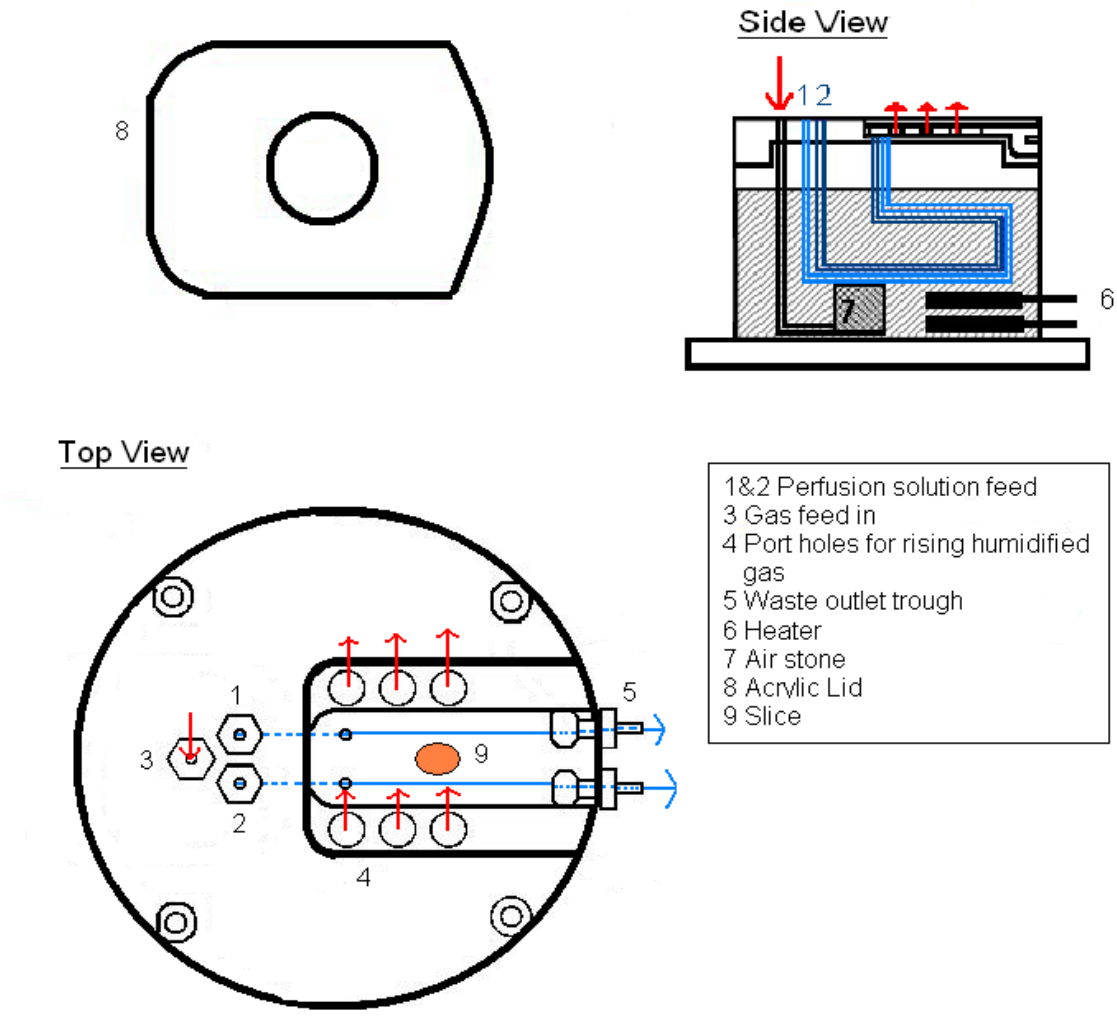


Figure 2-1 Recording Chamber. Diagram of the recording chamber consisting of two compartments. The upper compartment housed the slice at an interface between perfused aCSF and humidified air. The acrylic lid was placed over this to trap the humidified air. The lower chamber was filled with distilled water, the temperature of which was controlled by a thermoregulator, to which the heater was connected. The water was constantly bubbled with 95%O_2 - 5%CO_2. Red arrows indicate direction of gas flow. Blue arrows indicated direction of aCSF flow.

During the project, details of the slice cutting and storage procedures were altered in order to improve the viability of M1 slices, hence the final procedure used in slice preparation was as follows (but see chapter 3 for further details). Sucrose based aCSF was adjusted to contain extra neuroprotectants (in mM); Sucrose, 171; KCl, 2.5; $MgCl_2$, 10; NaH_2PO_4, 1.25; $NaHCO_3$, 25; $CaCl_2$, 0.5; Glucose, 10; Ascorbic acid, 1; NAC, 2; Taurine, 1; Ethyl Pyruvate, 20. The irreversible iNOS inhibitor

aminoguanidine (200 μM) was used for dissection and slice preparation, along with indomethacin and uric acid, to help circumvent cell death. This was shown to be the best method to improve cell viability, particularly when used for transcardial perfusion. Storage of slices was kept to the original method, with the concentration of indomethacin reduced to half the original concentration (now 22.5 μM). During the course of method development, it was discovered that cooling the sucrose based aCSF to 3-4 °C was unnecessary during the slice preparation (but was still needed during dissection and transcardial perfusion). Slices were therefore cut at room temperature.

2.2 Electrophysiological Recordings

2.2.1 Extracellular recording

Extracellular techniques were used to record local field potential activity (LFPs) of neuronal networks. LFPs are mainly thought to arise from dendro-somatic activity within 250 μm of the electrode tip. They therefore represent the inputs and local processing of a specific brain area. LFPs occur in low frequency ranges and are thought to represent the synchronous activity of neural populations (Logothetis, 2003; Mitzdorf, 1985; 1987; Rasch et al., 2007).

Borosilicate glass microelectrodes were pulled using a Flaming/Brown micropipette puller (P-97, Sutter instrument Co, U.S.A.) with an open tip resistance of 1-3 MΩ. The microelectrodes were filled with glucose-based aCSF and used to record LFPs. Silver wire coated with silver chloride was inserted into the microelectrodes, which were then mounted on to manually-operated micromanipulators (Kanetec, Japan).

Microelectrodes were placed in LV M1, which was located using a stereomicroscope (Leica Wild M3Z, U.K.) and the rat brain atlas of Paxinos and Watson as a reference (Figure 2.2). The recorded voltage was passed through an AI402 ultra-low noise amplifier headstage (Molecular Devices, U.S.A.), where it was amplified x50. The signals were further amplified x100 and low-pass filtered at 200 Hz through a programmable signal conditioner, CyberAmp 380 (Molecular Devices, U.S.A). Low-pass filtering was applied to expose the slower wave component of the voltage signal, which is thought to be the LFP. This low-pass filtration also separates

multiunit activity from the slower waveform. Neuronal network activity was visualised at 40 MHz on a HM507 oscilloscope (Hameg Instruments, U.K.) and signals were simultaneously digitised at 10 kHz sampling rate using an analogue to digital converter (Micro-1401 mk II; Cambridge Electronic Design, CED, U.K.). Spike2 software (CED, U.K.) was used for recording and for analysis both on- and off-line.

2.2.2 Whole-cell recordings

Whole-cell recordings were made using low resistance borosilicate electrodes applied to the surface of neurons identified visually using infra-red videomicroscopy with differential interference contrast (DIC 'Nomarski') optics (Olympus, BX51WI). Whole-cell patch-clamp access was achieved by rupturing the cell membrane so that the cell, whose interior then came into contact with the solution in the pipette, could be voltage or current-clamped. During patching, an area of membrane is electrically isolated from the external solution and the current flowing into the patch is recorded. This is achieved by pressing a glass pipette, which has been filled with a suitable electrolyte solution, against the surface of a cell and applying light suction. If the glass pipette and cell membrane are clean, a seal whose electrical resistance is >1 GΩ (Gigaseal) is formed. Under these conditions, the glass pipette and the cell membrane will be less than 1 nm apart. A high seal resistance will complete the electrical isolation of the membrane patch and reduce the current noise of the recording.

As the pipette was advanced through the surface of the bath solution, slight positive pressure was applied to the inside of the pipette to keep the tip free of contamination. Contact with the cell was identified by a slight rise in pipette resistance and indentation of the visualized cell surface. Gentle suction was applied to the pipette, and a seal formed, with the pipette holding current set to -70 mV, similar to the expected membrane potential of the cell. After forming a gigaseal, the fast capacity transients associated mainly with pipette capacitance to the bath were compensated. The membrane patch was ruptured by applying strong suction. A successful break-through was indicated by the sudden appearance of large capacitance transients at the leading and trailing edges of the pulse. Whole cell capacitance compensation was not applied, nor was series-resistance compensation.

Microelectrodes were filled with two different solutions (dependent on the experiment conducted) and attached to a microelectrode holder.

2.2.3 Voltage-Clamp

In order to characterise the contribution of phasic and tonic inhibitory currents during neuronal network oscillations, voltage-clamp recordings were conducted. The membrane potential is held constant in this technique, and the current flow due to ion channel opening can be measured, allowing the study of ion channel activity. Borosilicate glass microelectrodes with resistance of 3-5 MΩ were used and were fabricated using a P-2000 laser puller (Sutter Instruments, USA). The microelectrode holder was connected to a CV-7B headstage (Molecular Devices, U.S.A.). Voltage–clamp recordings were made in LV M1 in a submersion recording chamber, perfused at 5-7 ml/min with glucose–based aCSF, at 32°C. Slices were visualised at x400 magnification using infra-red differential interference contrast (DIC) 'Nomarski' optics on an upright microscope (Olympus BX51WI). Electrodes were filled with an internal solution containing (in mM): HEPES, 40; QX-314, 1; EGTA, 0.6; $MgCl_2$, 5; TEA-Cl, 10; CsCl, 100; ATP-Na, 80; GTP-Na, 6; and IEM 1460, 1 (titrated with CsOH to pH 7.25) at 295 mOsm. The chloride-loaded solution allowed the study of inhibitory postsynaptic currents recorded at -70 mV using an Axopatch 700A amplifier (Molecular Devices, U.S.A.). Series resistance (R_s) was measured regularly using the capacitance transient on a line-frequency voltage step (5 mV) during recording. Any recording showing >20 % change in R_s was discarded from analysis. Data were recorded and filtered above the Nyquist threshold for aliasing (sampled at 10 kHz, filtered (8-pole low-pass Bessel filter) at 2 kHz and digitised using a Digidata 1440A.

2.2.4 Current-Clamp

During current–clamp recording, membrane current is clamped at zero, allowing the study of changes to the membrane potential. To characterise the firing properties of an individual interneurone, electrodes were filled with an internal solution prepared such that no ion channels were blocked and containing (in mM): $KMeSO_4$, 130;

HEPES, 10; EGTA, 5; NaCl, 4; ATP-Mg, 4; GTP-Na, 0.4; at 295 mOsm. Borosilicate glass microelectrodes with resistance of 3-5 MΩ were pulled (P-2000, Sutter, USA), filled with the above solution and attached to a microelectrode holder. The microelectrode holder was connected to a headstage (CV-7B; Molecular Devices, U.S.A.) and current–clamp recordings were made in all layers of M1 as described above for voltage-clamp experiments. Active membrane properties were characterised using a current-step protocol. A current source can inject current into the cell in a series of DC current steps, to either hyperpolarise or depolarise the cell. A series of 250 ms current steps in increments of +0.1 nA from an initial holding current of -0.4 nA, until threshold for action potential firing was reached, were conducted. Different interneuron subtypes were identified dependent upon the firing response to the current injection.

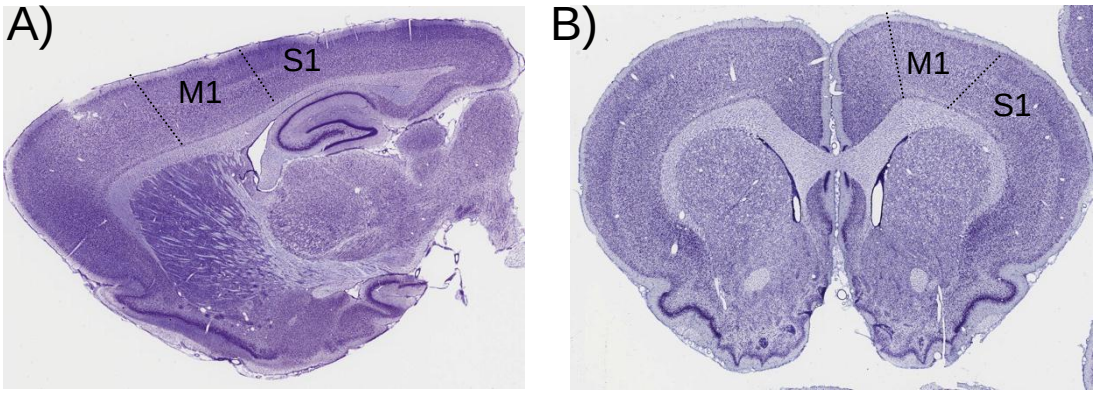


Figure 2-2 Location of M1 in the (A) sagittal and (B) coronal plane. Nissl stained slides showing placement of primary motor cortex (arbitrarily demarcated by black lines) and primary somatosensory cortex in both the A) sagittal and B) coronal plane of the rat brain. All recordings were conducted within this area unless otherwise stated and the majority of slices recorded from contained M1 and S1 and thus were "sensorimotor" slices. Adapted from The Rat Brain Atlas (Paxinos and Waston, 1998).

2.3 Noise concerns

Physical and electrical interference of the biological signals in electrophysiological recordings pose a problem. However, there are numerous ways in which most interference can be eliminated. Vibration, which could disrupt the recording, is caused mainly by three sources; ground vibration, acoustic noise and direct force disturbances. A vibration isolation table (TMC, USA), specifically designed to reduce these types of noise, was used. In addition, the use of well-designed micromanipulators ensured no destructive vibration was transmitted to the cell. Electrical noise is mainly derived from external sources; radiative electrical pickup (e.g. 50/60 Hz noise from lights/power sockets), magnetically-induced pick-up (e.g. electromagnets in power supplies), or ground-loop noise (e.g. when shielding is grounded in more than one place and differences in potential create currents and introduce noise into the shielding). Electrical noise was negated by shielding recording devices with a Faraday cage (TMC, USA) and connecting the shielding to a common ground (e.g. microelectrode amplifier). All signals from microelectrodes were additionally passed through a Humbug (Quest Scientific, Canada). Humbugs eliminate 50/60 Hz noise by cancelling out electrical interferences in real time. The hum bug is not a filter, so there were no phase delays in the signal processing, no amplitude errors and no waveform distortion.

2.4 Drug preparation and application

Stock solutions were prepared for each drug (purchased from Sigma or Tocris) at a known concentration and were stored at -20 °C before use. The required drug was directly applied to the perfusing aCSF after a stable control period of oscillatory activity (usually 20-40 minutes). Subsequent neuronal activity was then recorded for at least a further 30 minutes.

2.5 Data collection and analysis

2.5.1 Data analysis

All extracellular electrophysiological data were converted from analog to digital waveform and recorded in Spike 2. The sampling rate used for this conversion was chosen to be 10 KHz, which is sufficient for reconstructing the original signal of below 200 Hz. According to the Nyquist-Shannon sampling theorem, the sampling frequency must be more than twice the frequency (bandwidth) of the original input signal in order to perfectly reconstruct the signal digitally (Axon Guide, 1993). Time-series analysis was undertaken to examine the data, specifically the frequency–domain method, which uses a fast Fourier transform (FFT) algorithm to create a power spectrum, whereby the waveform is split into its frequency components and any distinct periodic component can be highlighted. The amplitude of the constituent sine/cosine waves into which the signal is decomposed is expressed as power at a particular frequency. All data were low-pass filtered at 80 Hz off-line in order to elucidate the low-frequency components of network activity. The FFT block size of 1.6384 seconds was used, as this gave a spectral resolution (frequency resolution of each bin) of 0.61 Hz, the highest available in Spike 2 software. Unless otherwise stated, 60 second epochs of sampled data were analysed. Pooled data are presented as mean peak power values ± SEM. All statistical analyses have been performed using these mean peak values, and, unless otherwise stated, it is these values that are reported in the text.

Spontaneous (s)IPSCs were recorded using Clampfit 10.2 (Molecular Devices, USA) and analysed using the Mini-Analysis programme (Synaptosoft, U.S.A.). At least 200 events were taken from each recording so that sIPSC amplitude and frequency (interevent interval) could be calculated for further analysis. Cumulative probability distributions (the probability that a real-value random variable *X* with a given probability distribution will be found at a value less than or equal to *x)* were plotted for each of the mentioned variables in control and drug conditions.

For cell characterisation analysis the following parameters were recorded; the input resistance (*R*in) was calculated by applying steps of a hyperpolarising current pulse (amplitude -0.4 nA, step change 0.1 nA, duration 250 msec). The analysis of the waveform of the first spike of a depolarising current pulse (+0.1 nA) was performed (250 msec, sampling rate 10 kHz). The amplitude of the first action potential was measured from the threshold to the peak of the spike. The duration of the action potential was measured at half amplitude (spike half width). The amplitude of the afterhyperpolarisations (AHPs) was measured between the spike threshold and the peak of the AHP.

2.5.2 Cross-correlation analysis

Cross-correlation analysis was used to reveal a temporal relationship between two different signals (X(t) and Y(t)). Cross-correlation estimates the degree to which two data series are correlated by assigning values between -1 and +1. The closer to 1, the more close the correlation. Information regarding common frequency components as well as phase differences between two signals can be obtained. Cross-correlation is calculated by multiplying two waveforms together and summing the products. The results are expressed as cross-correlation coefficient by Spike 2 (i.e. a normalised value, falling between -1 and +1). Positive values indicate series that are in phase, whilst negative values indicate series that are out of phase. Epochs of 60 seconds (the same timescale used for FFT analysis) were used from two waveforms recorded from the same slice.

2.5.3 Spectrogram analysis

The Mortlet - Wavelet time series analysis was used to calculate changes in power (amplitude) of the signal over time (Wallisch et al., 2009) in order to provide a better frequency/time representation of the signal than the FFT. The spectrogram created shows high power activity as “hot” colours (i.e. red/orange/yellow), while less powerful activity (or a less active state) are represented as “cold” colours (i.e. blue). Matlab was used for this analysis. A period of signals, recorded in Spike 2, was selected and stored initially as a notepad file. These data were subsequently converted by a Matlab script (written by Dr. S. D. Hall, Aston University), resampled to 1000 Hz and analysed in Matlab to create a spectrogram.

2.5.4 Statistical analysis

For extracellular data and changes in to the tonic inhibitory current, the statistical tests used were the paired Student’s t-test, unless normality failed where a Mann-Whitney test was used in its place. These tests were performed to determine statistical significance between two sets of data. For the Student’s t-test, one-tailed *P* values were selected when certain assumptions were made relating to the outcome, and two-tailed *P* values were used when no assumptions were made. All data are shown as mean ± standard error of mean (SEM) unless otherwise stated. Statistical significance ($P < 0.05$) is designated by *. A paired student t-test was conducted between the current and previous drug response for cumulative dose response experiments.

For changes to the phasic inhibitory currents, a cumulative probability distribution was plotted for sIPSC amplitude and inter event interval (IEI) during control and drug conditions and the two sample Kolmogorov–Smirnov test was used to see if the two distributions differed significantly.

2.5.5 Live/dead ratio

Cell counts were performed in superficial (LII) and deep (LV) motor cortex and the live/dead ratio determined as a representation of slice viability. LII recordings were taken at a depth from the pial surface no greater than 400 μm in an area 120 μm^2.

Readings from deep LV M1 were taken at a depth from the pial surface no greater than 1300 μm and, more specifically, where Betz cells began to appear in the visual field. Three readings were taken in each slice, one grid apart. Cell counts were taken within the confines of the grid, where cells were completely within the boundary lines, or touching the bottom or right-hand boundary line. Counts were taken 1 hour after incubation in the storage chamber to coincide with the timescale of recordings in experiments.

Live cells were considered viable when they had clear, smooth somata and, in the case of Pyramidal cells, when prominent apical dendrites were present. Cells with a shrunken appearance or cells that were swollen, with vacuolation and nuclear pyknosis were considered to be dead (unviable) cells.

Chapter 3 Increasing Viability of Slices Containing the Primary Motor Cortex

3.1 Introduction

The brain-slice technique (Yamamoto and McIlwain, 1966; Andersen et al., 1972; Alger et al., 1984) has greatly facilitated the investigation of the electrical properties of neurones and neuronal network activity. In acute brain slice preparations, the connections between neurones are preserved to a certain extent, however cell loss can be a large problem, either during the preparatory method or during storage. The slicing process causes some damage to the cells, a problem that may result in altered electrical properties or even cell death. The viability of acute brain slices is therefore essential for the success of *in vitro* experiments. Numerous studies have been undertaken with the aim of protecting neurones from damage, with varying success rates. Alterations to artificial cerebrospinal fluid (aCSF) have been the major target for neuronal protection. For example, replacing NaCl with sucrose was one of the first interventions to show prevention of cell swelling and lysis by reducing the acute neurotoxic effects of passive Cl^- entry and associated water transport and swelling (Aghajanian and Rasmussen, 1989). More recently, the replacement of sucrose with glycerol in the preparatory aCSF (Ye et al., 2006) had been shown to benefit viability, perhaps because sucrose is difficult for the cell to utilise and its entry into cells can lead to similar problems as seen with NaCl.

Work in M1 commenced by looking at P21, coronal, acute brain slices. With only 8.96 ± 0.98 % of slices used showing oscillatory activity, experiments started slowly, and colleagues from other laboratories noted that M1 was very difficult to work with and impossible to perform single-cell recordings in (M. Vreugdenhil, personal communication). I therefore commenced work trying to find a solution to the low viability of M1 coronal slices. Using a standard aCSF solution (Table 3.1) different neuroprotectants were added to the storage and cutting solutions in an effort to augment and prolong slice viability (Table 3.2). Nothing appeared to provide a long-term effect until a new aCSF solution was devised incorporating an array of neuroprotectants, a change in slice plane and a new dissection method (Table 3.1). This new method increased viability so that 20 % of slices now showed network oscillations on a regular basis. Subsequently, a change in the source of rats, along with the new slice and storage methods brought about an increase to almost 60 % of slices now showing oscillatory activity. This chapter describes work performed over 2

years in development of a reliable method for the production of slices of M1 in which the highly sensitive Betz cell population remains intact and functional.

3.2 Results

3.2.1 Changes to Storage aCSF

Work originally conducted in this laboratory showed that M1 exhibited oscillations at beta frequencies (27.8 ± 1.1 Hz) (Yamawaki et al., 2008). Using coronal slices from P21 rats, current experiments showed this to be the case (25.05 ± 0.39 Hz, Fig. 3.3). However, slice viability was low with a live/dead ratio (see methods) of cells of 2.25 in superficial layers and 0.79 in deep layers (photograph 1, Fig. 3.2), which meant that only 9 % of slices showed any oscillatory activity (figure 3.1). In order to improve the success rate, work from this low starting point began with alteration of the slice storage solutions and precise details of how the slices were stored. Various neuroprotectants were added to prolong the life of the slices prior to the use of excitants (KA and CCh) to try and induce oscillatory activity. Some neuroprotective agents were already used in the laboratory, such as uric acid and indomethacin, in storage and preparation of slices from entorhinal cortex.

3.2.2 Changes to cutting aCSF

After a period of time, with no long term-effects being seen through adjusting the storage solution only, steps were taken to create a new preparatory aCSF solution with enhanced neuroprotective properties and increased biological relevance. Numerous studies over the past few decades have attempted to protect neurones from damage and more recent reports (Hajos and Mody, 2009) have shown that adding certain compounds to the aCSF may create a more biologically relevant environment of neuromodulators that would normally be washed away in a standard *in vitro* experiment using a basic aCSF solution. Keeping these neuromodulators at levels found *in vivo* might be expected to maintain neuronal signalling patterns that would be seen in the intact brain. To this end, N-acetyl-cysteine (NAC), ascorbate, aminoguanidine and taurine were added as neuroprotectants to the cutting solution (see below for a more detailed description). This new cutting solution along with a

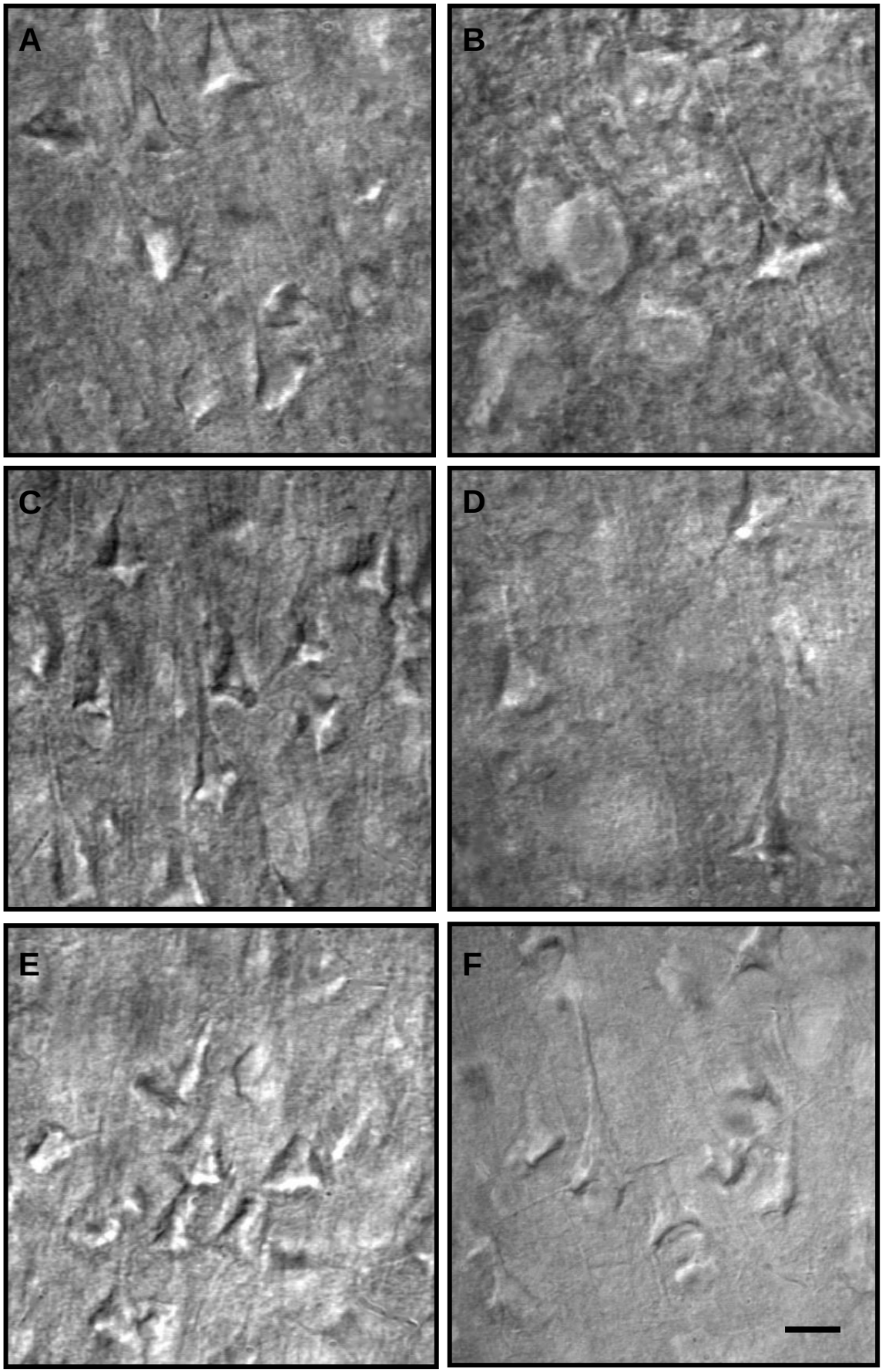
new method of dissection, transcardial perfusion, enhanced slice viability from only 8.96 ± 0.98 % of slices that oscillate to a much larger percentage (Fig. 3.1 13.16 ± 3.51 %), while the live/dead ratio did not alter in superficial layers (2.04) but increased to 1.16 in deep layers (Fig. 3.2).

Original aCSF		**Modified aCSF**	
Substance	**Concentration (mM)**	**Substance**	**Concentration (mM)**
Sucrose	206	Sucrose	171
KCl	2	KCl	2.5
$MgSO_4$	1.6	$MgSO_4$	10
$NaHCO_3$	26	$NaHCO_3$	25
NaH_2PO_4	1.25	NaH_2PO_4	1.25
Glucose	10	Glucose	10
$CaCl_2$	2	$CaCl_2$	0.5
Pyruvate	5	Pyruvate	20
		NAC	2
		Taurine	1
		Ascorbic Acid	1

Table 3.1 Original aCSF and the changes made to the modified aCSF. Note the main difference is the addition of Ascorbic acid, NAC and taurine and increased pyruvate.

Changes to aCSF composition are not uncommon and sucrose has been used in many laboratories as a replacement for NaCl in the cutting solution. The rationale underlying the use of sucrose was that a steady depolarisation of the cells when Cl^- was present in the aCSF led to an influx of Cl^- and associated water entry until swelling reached a point at which cell lysis would occur (Rothman, 1985). While this is also thought to be the case with sucrose (Ye et al., 2006), the process appears to take much longer than with NaCl based aCSF. Taurine, an abundant free amino acid (Jacobsen and Smith, 1968), has been implicated in mitochondrial function (Palmi et al., 1999), cell viability (Boldyrev et al., 1999) and intracellular Ca^{2+} homeostasis (Wu and Prentice, 2010). Therefore, addition of 1 mM to the aCSF has been suggested to prevent its depletion during acute brain slice preparation and thus helping to prevent excitotoxicity. Taurine is also recognised as an inhibitory neurotransmitter and has been shown to be a modulator of GABAergic function at glycine receptors at low concentrations (< 1 mM) and GABA receptors at higher concentrations (> 3 mM) (Wu and Xu, 2003). Taurine has also been shown to increase tonic inhibition in ventrobasal thalamus (Jia et al., 2008). Ascorbic acid, a naturally occurring antioxidant, synthesised from glucose in the liver of rats (Padayatty et al., 2003), is found at its highest concentration in the cerebral cortex of the brain, with neuronal concentrations reaching 10 mM and extracellular fluid concentrations around 500 µM (Rice, 2000). During brain slice preparation, up to 80 % of ascorbic acid is depleted. Addition of 1 mM ascorbate to the aCSF could mitigate this sudden decrease in extracellular levels. NAC, also an antioxidant, increases cellular pools of free radical scavengers. During traumatic brain injury, such as the acute brain slice preparation, NAC can help to restore mitochondrial function (Xiong, Peterson and Lee, 1999). Addition of 2 mM NAC to the modified cutting solution was therefore proposed. Finally, inducible nitric oxide synthase (iNOS) is known to be induced during ischemia (Rice, 2000). With a similar ischemic neuronal cell loss occurring during acute brain slice preparation, aminoguanidine, an irreversible iNOS inhibitor (Griffiths et al., 1993) known to improve neurological function, decrease cell swelling and attenuate cell death, was added to the solution at a concentration of 200 µM.

Opposite Page - **Photograph 3.1 Superficial and deep M1 neurones in different conditions**. A) Superficial layer cells in the original method. B) Deep layer cells in the original method. C) Superficial layer cells in the modified aCSF solution (MaCSF). D) Deep layer cells in the MaCSF. E) Superficial layer cells in MaCSF and after a change to the rat colony. F) Deep layer cells in MaCSF and after a change to the rat colony. Note particularly the difference in the deep layers. This is where the most noticeable difference occurred in cell viability. Scale bar 15 μm.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Substance	Action	Why and where used	Reference
Indomethacin	NSAID, inhibits COX1 and 2	used daily in preparatory and storage aCSF	Pakhotin et al 1997; Tutak et al., 2005
Uric Acid	Strong reducing agent and antioxidant	used daily in preparatory and storage aCSF	Proctor, 2008
Ketamine	NMDAR antagonist	can prevent excitotoxicty as an NMDAR antagonist but also implicated in apoptosis, used in storage and preparatory aCSF	Green and Cote, 2009
Kynurenic Acid	antiexcitotoxic and anticonvulsant	NMDAR antagonist, prevent excitotoxcity, used in storage	Fatokun et al.,2008
Ascorbic Acid	Strong reducing agent and antioxidant	Used in storage and circulating aCSF for expermients	Rice, 2000
NAC	Antioxidant	used in storage	Tian et al., 2003
CHPG	Selective mGlu5 agonist	used in circulating aCSF to induce oscillatory activity	Nistri et al., 2006
CNQX	AMPA/Kainate receptor antagonist	used in storage to prevent excitotoxicity	Margaryan et al., 2010
IEM	Voltage dependent open channel blocker of AMPAR	used in storage, blocks AMPAR, improves inhibitory functions, vital for oscillations	Buldakova et al., 1999
Glutathione	Antioxidant, free radical scavenger	used in storage for neuroprotection	Warner et al., 2004
α – lipoic acid	Antioxidant	used in storage for neuroprotection	Connell et al., 2011; Toklu et al., 2010

Table 3.2 Neuroprotective agents used for storage purposes. Over the course of altering the solutions used, various neuroprotective agents were added to the storage aCSF with varying results.

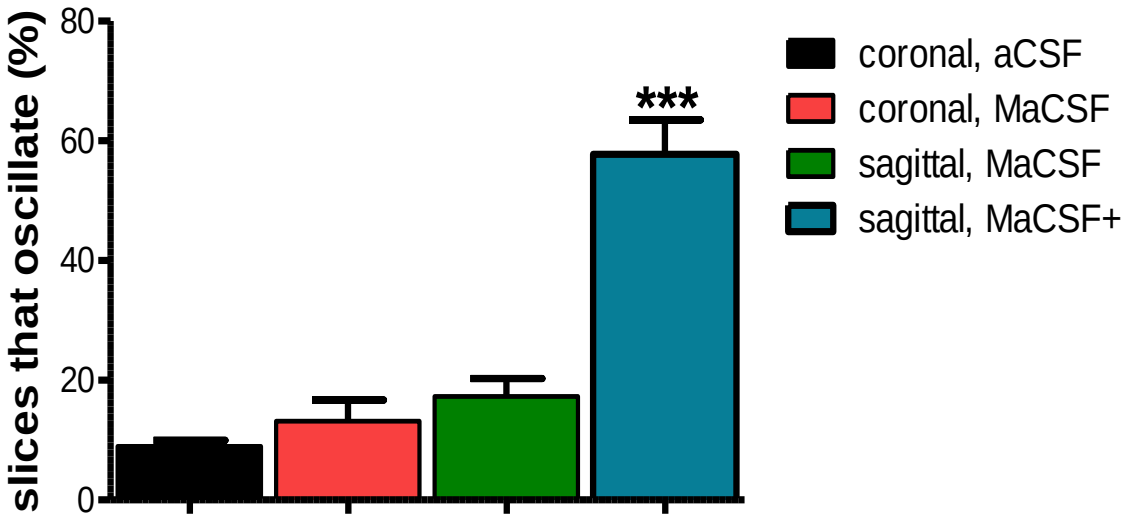


Figure 3-1 Changes in viability of slices Changes to the aCSF (modified (M) aCSF detailed above) increased the number of slices that produced oscillatory activity (8.96 ± 0.98 % to 13.16 ± 3.51 %). However, viability was still quite low and so further alterations were sought. A change to the slice plane (sagittal MaCSF) increased the viability further to 17.32 ± 2.97 %. Changes to the source of rats (sagittal, MaCSF +) more than tripled the number of viaibale slices again (57.73 ± 5.76 %, P < 0.0001).

3.2.3 Transcardial Perfusion

This method, as it stands, was first proposed by Palay et al., (1962) as a method of fixation, but more recently has been used for the improved viability of brain slices. Good tissue perfusion with cutting solution is critical to obtain healthy slices as many recent studies have shown (Michaloudi et al., 2005; Ye et al., 2006). An incision was made at the level of the xyphoid process, which was then opened and the diaphragm punctured. Ribs were sectioned bilaterally to open the thoracic cavity to expose the right atrium. A 20-gauge needle was used for the perfusion and was placed into the apex of the heart, piercing the left ventricle. 20-40 ml of cutting solution was injected until the outflow from the opened right atrium was clear. White colour of the brain indicated a good perfusion, while red or pinkish colour indicates poor perfusion.

3.2.4 Rat Colony

After changing the slice preparation procedure with a modified aCSF, the number of slices that showed oscillatory activity was still very low. Hence, although cell counts indicated that slices from the in house rats were indeed better in the modified aCSF, particularly in the deeper layers; eliciting oscillatory activity was still a struggle. At this point, a change in slice orientation was introduced, with activity now recorded in sagittal slices and the number of slices exbibiting beta activity increased to 17.32 ± 2.97 %. However, difficulty in eliciting oscillations was also found to be a problem in hippocampus and entorhinal cortex, suggesting a system wide issue, and it was at this point that we decided to introduce new breeding stock into the Wistar colony. Working with the new rats, along with the new cutting solution and a change in the slice orientation increased the number of slices showing oscillatory activity to nearly 60 %, and the live/dead ratio of cells increased dramatically to 10.00 in the superficial layers and 5.77 in the deep layers (Fig. 3.1 and 3.2). The most noticeable difference to the slices, which is most probably the determining factor in the increase in oscillatory activity, was the increased interneurone and Betz cell viability in the deep layers (Photograph 2. A-D). Photograph 2 shows interneurones adjacent to large Pyramidal cells (most likely Betz cells) in deep M1 along with a higher number of these preserved Pyramidal cells.

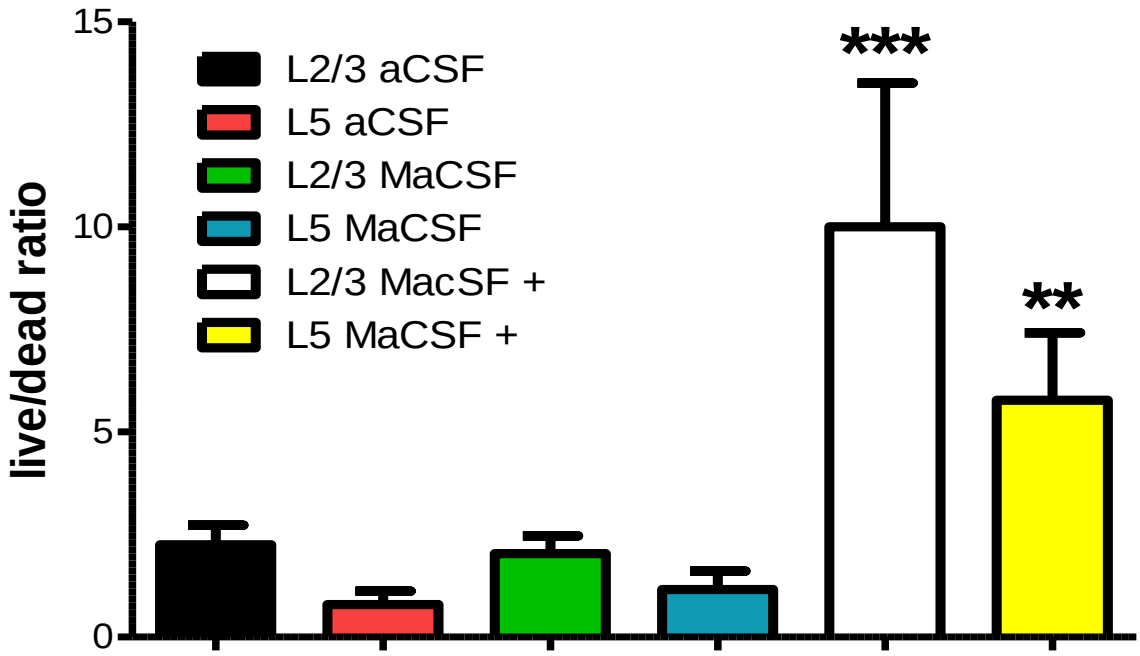
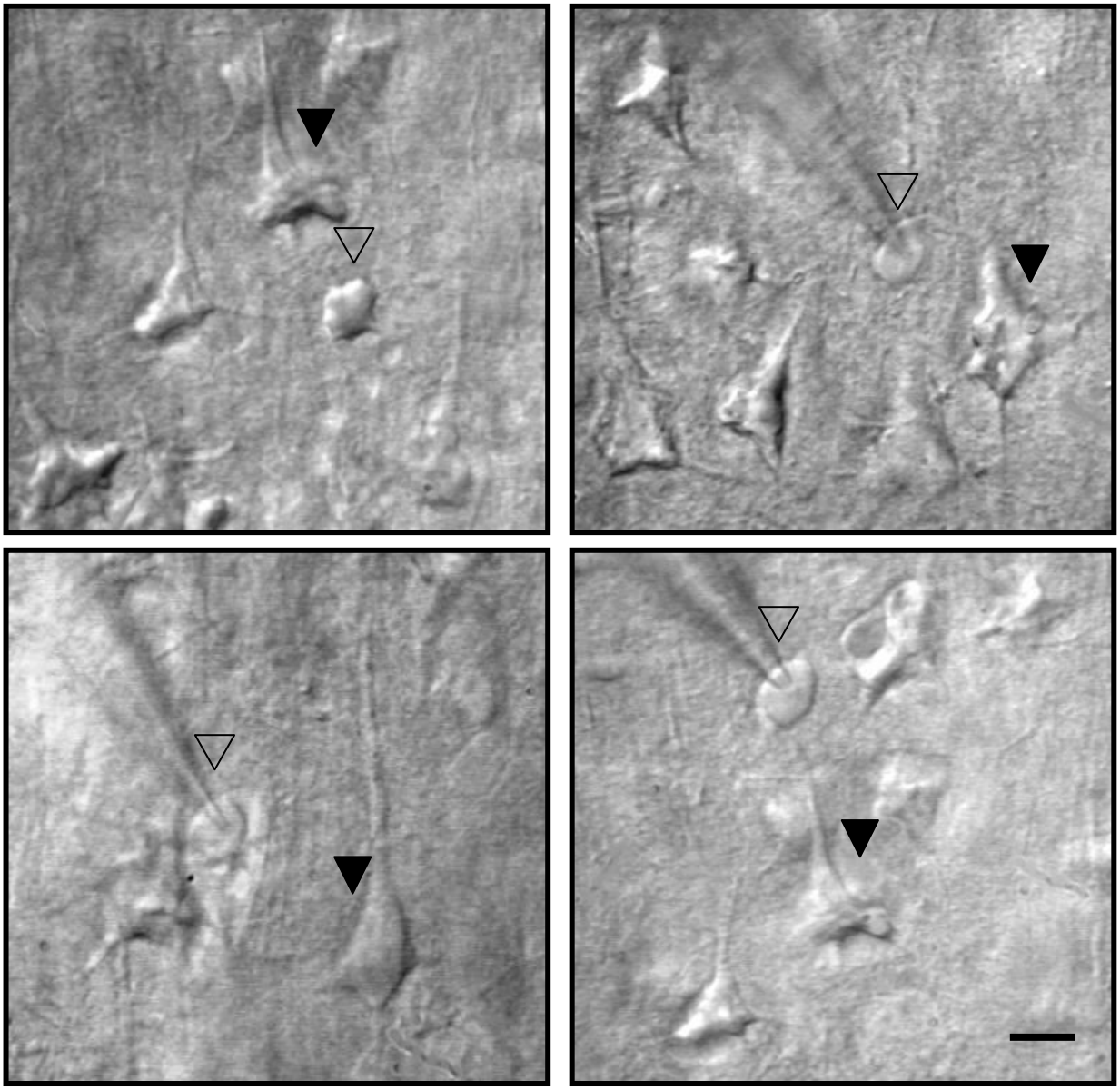


Figure 3-2 Changes to the live/dead ratio of cells in the three different conditions. Superficial layers generally had a greater number of cells than deep layers, as shown by the live/dead ratio. In the original method the live/dead ratio of cells viewed in the superficial layers (L2/3) in M1 was 2.25 and 0.79 in deep layers (L5) of M1. In the modified aCSF solution (MaCSF) there was no change to the live/dead ratio of cells seen in the superficial layers (2.04). An increase in the live/dead ratio of cells was observed in the deep layers (1.16). When a change to the source of rats was introduced (MaCSF +), the live/dead ratio increased dramatically to 10.00 ($P < 0.001$) and 5.77 respectively ($P < 0.01$).



Photograph 3.2 Increase in viability of interneurones– Betz cell pairs in the revised preparatory method.
The modified aCSF and, most importantly, a change to the rats resulted in the increased viability of interneurone – Betz cell pairs, as well as an increase in the number of deep cells leading an to an increase in the viability of slices. Photographs from four different preparations, all comprising of the modified aCSF and the new rat colony. Closed triangles indicate proposed Betz cells. Open triangles indicate proposed interneurones. Scale bar 15 µm.

3.2.5 Changes in Frequency and Power

Whilst the changes to the method were carried out, differences in frequency and power of the oscillation were also observed. Coronal slices cut in the original sucrose based aCSF solution had a mean peak frequency of 25.05 ± 0.39 Hz and a mean peak power of 129.5 ± 18.85 μV^2. Changes to the aCSF were carried out as detailed above, and mean peak frequency then increased to 29.9 ± 1.0 Hz and mean peak power was 50.64 ± 16.73 μV^2. The introduction of a change to the slice plane from coronal to sagittal saw increased viability and further alterations to the frequency and power of oscillations was observed. Mean peak frequency had increased to 32.66 ± 0.89 Hz and mean peak power was now 103.5 ± 23.19 μV^2. As mentioned in the previous section, problems were not only being encountered in the motor cortex and a change to the rat colony was introduced. Mean peak frequency was increased again to 34.15 ± 0.65 Hz and mean peak power was reduced to 46.81 ± 6.54 μV^2. The changes to the frequency do not appear to be totally dependent on the slice plane, but also due to the alteration of aCSF and the rat colony, indicating a correlation with viability.

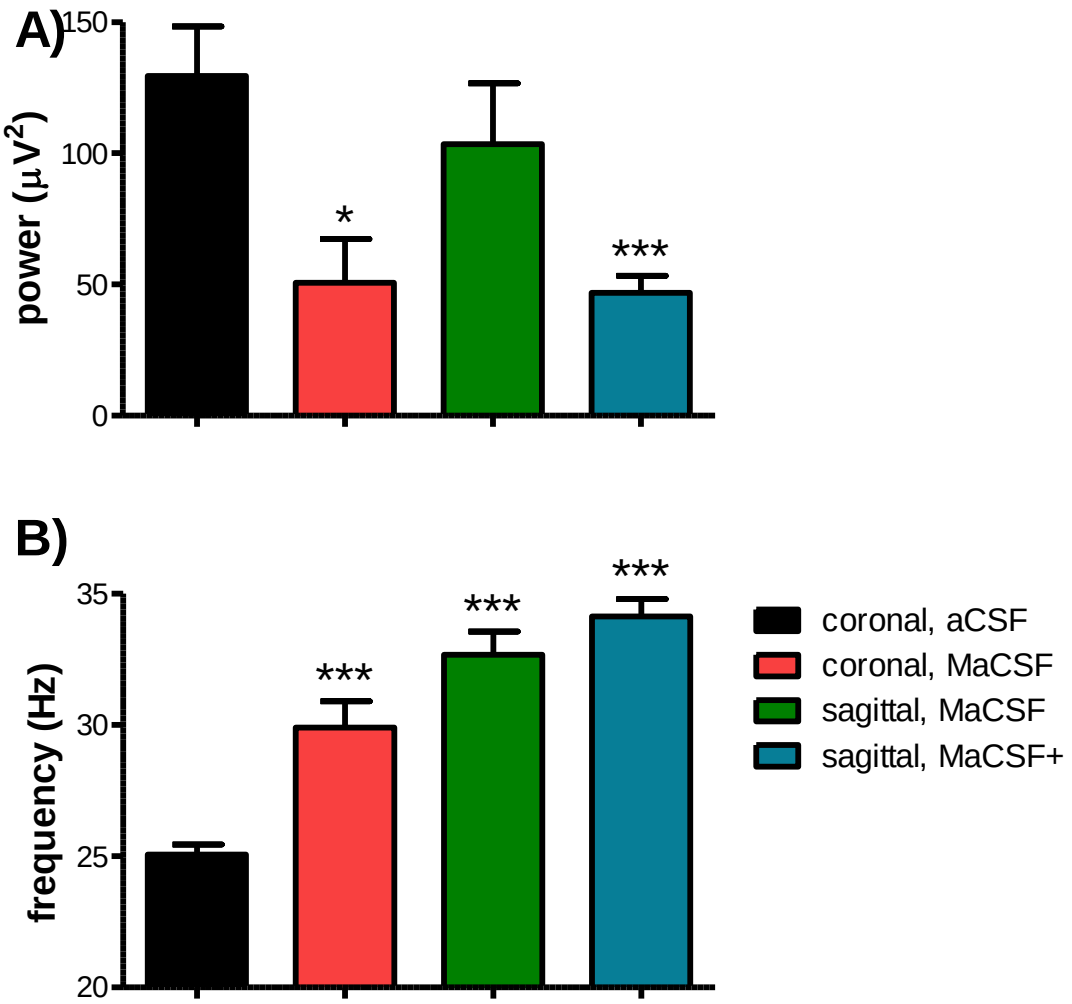


Figure 3-3 Differences in oscillation frequency and power before and after changes to the preparatory method. A) Mean peak power of the oscillations varied dependent upon the preparatory method. Coronal slices in the original aCSF solution had a mean peak power of 129.5 ± 18.85 μV^2, which decreased to 50.64 ± 16.73 μV^2 in the MaCSF ($P < 0.05$). When the slice plane was switched from coronal to sagittal, mean peak power rose to 103.5 ± 23.19 μV^2 ($P > 0.05$). When a change to the rat colony was introduced, mean peak power decreased to 46.81 ± 6.54 μV^2 ($P < 0.001$). B) Alterations to the aCSF resulted in a change in the frequency and power of the oscillations being observed. Frequency increased from 25.05 ± 0.39 Hz in coronal slices to 29.9 ± 1.0 Hz in MaCSF ($P < 0.001$). When the modified aCSF was used with an alteration to the slice plane, oscillation frequency increased further to 32.66 ± 0.89 Hz ($P < 0.001$). When a change to the rat colony was introduced frequency increased further to 34.15 ± 0.65 Hz ($P < 0.001$).

3.2.6 Changes in KA and CCh concentrations

A further difference that was found during the alterations to the preparatory method was the effect of changes to the concentrations of kainate (KA) and carbachol (CCh) required to elicit oscillatory activity. Previous studies in somatosensory (Buhl et al., 1998) and motor cortex (Yamawaki et al., 2008) had noted the need for 400 nM KA and 50 µM CCh to elicit oscillatory activity, where other areas needed either KA or CCh. In my experiments, a change from the original aCSF to the modified aCSF solution, a marked increase in epileptiform activity occurred at these high concentrations of KA and CCh and they therefore had to be reduced. In addition, changing the rat colony saw a significant reduction in the concentrations that were used in subsequent experiments, the optimal concentrations reduced to 100 nM KA and 5 µM CCh (for a further discussion, see Appendix 1).

3.2.7 Pharmacology

After changes to preparation to increase viability of slices, pharmacological experiments were conducted to ensure that responses of beta oscillatory activity to 10 nM zolpidem (discussed further in Chapter 4) were the same between experiments. All three experimental setups available in the laboratory showed a desynchronisation of beta oscillatory activity at 10 nM zolpidem (Fig. 3.4 – aCSF showed a reduction to 47.01 ± 16.09 % of BL, MaCSF showed a reduction to 54.26 ± 8.16 % of BL and MaCSF + showed a reduction to 75.27 ± 8.81 % of BL). Interestingly, the amount of desynchronisation of the beta oscillation decreased as viability of the slices increased.

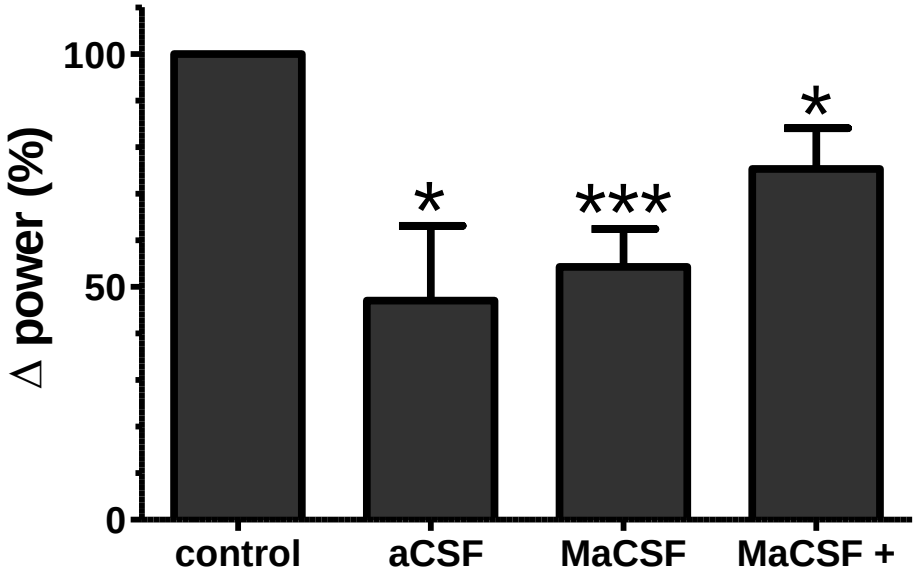


Figure 3-4 Pharmacological responses of beta oscillations in the three different conditions. Addition of zolpidem (10 nM) caused a desynchronisation of beta oscillations in M1 in all three conditions. Oscillations evoked in the original aCSF solution showed a reduction to 47.01 ± 16.09 % of BL ($P < 0.05$, n = 6). Oscillations evoked in the modified (M) aCSF showed a reduction to 54.26 ± 8.16% of BL ($P < 0.001$, n = 11) and oscillations evoked in the MaCSF when a change to the source of rats was also introduced showed a reduction to 75.27 ± 8.81% of BL ($P < 0.05$, n = 6).

3.3 Discussion

3.3.1 Changes to storage

The largest difference to slice viability was seen when the preparatory method was changed and alteration of storage solutions after this point made little or no difference to slice viability. This indicates that most of the damage is done either during dissection of the animal or during the preparation of the acute brain slice. Replacing anaesthesia and decapitation with transcardial perfusion helped to maintain neuroprotection and preserve the brain that would normally be exposed to greater anoxia and excitotoxic activity and thus appears vital to obtain healthy slices.

3.3.2 Changes to aCSF

Slice viability and neuronal network activity increased significantly when four core neuroprotectors were present in a modified aCSF solution used for preparation of acute brain slices. Hence, use of NAC, aminoguanidine, taurine and ascorbate induced a step-change in slice viability and delivered immediate benefits in terms of successful production of neuronal network oscillations in deep M1. NAC has been shown to have neuroprotectant actions, in that during a traumatic brain injury it helps to restore mitochondrial function and increase antioxidant availability (Xiong, Peterson and Lee, 1999). It acts to increase the pool of glutathione, a reactive oxygen species (ROS) scavenger, thus inhibiting the actions of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex involved in cellular responses to stimuli such as stress) and TNF–α (tumor necrosis factor-alpha, a cytokine involved in systemic inflammation) (Ferrari et al, 1995, Chen et al, 2008). This, along with reducing the activity of other inflammation related factors, helps to significantly decrease the likelihood of apoptosis (Ferrari et al., 1995; Chen et al., 2008). The possibility of this being achieved is due to the conversion of NAC to cysteine, a glutathione precursor. NAC has also been reported to prevent excitotoxic cell death by reducing N-Methyl-D-aspartate receptor (NMDAR) activation during anoxic and ischemic conditions (Monje et al., 2000). Along with decreased extracellular concentrations of Ca^{2+}, a loss of protein synthesis is avoided, thus preserving neuronal electrophysiology and morphology.

Another ROS scavenger associated with preventing apoptosis after ischemia is the biological compound aminoguanidine (Sun et al., 2010; Hunot et al; 1996, Dawson et al., 1993). Excessive production of Nitric Oxide (NO) is neurotoxic. NO is produced from L-arginine by nitric oxide synthase (NOS). NOS is found in three isoforms, and as well as being involved in neurotransmission also plays a role in plasticity, control of sleep, appetite, body temperature and neurosecretion (Calabrese et al., 2007). Inducible NOS (iNOS) is induced following inflammatory or traumatic events and not usually detectable in the normal brain (Sun et al., 2010), but may be produced in excess following brain damage. NO undergoes oxidative reduction to form reactive oxygen (nitrogen) species (Hunot et al., 1996; Dawson et al., 1993). Cytotoxicity after traumatic events is thought to be due to a process of excitotoxicity caused by NMDAR hyperfunction (Dawson et al., 1993), which in turn induces iNOS expression. Aminoguanidine is an inhibitor of iNOS (Griffiths et al., 1993; Sun et al., 2010). When given after a traumatic event, aminoguanidine improves neurological function, decreases brain swelling and attenuates necrotic and apoptotic cell death.

NMDAR mediated neurotoxicity has been linked with an influx of Ca^{2+} (Dawson et al., 1993; El Idrissi et al., 2008), which is a known cause of mitochondrial death. Taurine, a modulator of cytosolic and intra-mitochondrial calcium concentrations, can be employed to prevent mitochondrial death, and thus cell death, during traumatic events (Ellren and Lehmann, 1989). Increasing the taurine concentrations in the modified aCSF was of particular use, as taurine itself is depleted during ischemia (Torp et al., 1991). Cell volume regulation is a vital process (Inoue et al., 2005) and is regulated in part by taurine (Beetsch and Olson, 1998). Neuronal activity such as neurotransmission and neuronal discharge (Rothmann, 1985; Kimelberg and Kettenmann, 1990) contribute to changes in intracellular osmolarity by altering osmolyte transport. Cells readjust to this by regulatory volume decrease (RVD; Hoffman and Simonsen, 1989, Lang et al., 1998). Activation of the Cl^- current in response to cell swelling is one of the mechanisms that cells utilise to restore their volume. At normal resting potentials, this current causes the efflux of intracellular Cl^- (Hoffman and Simonsen, 1989, Kubo and Okada, 1992), thereby decreasing intracellular osmolarity. During brain slice preparation, along with storage in a solution that lacks the osmolytes (amino acids) required for maintenance of the cell

volume, taurine levels fall and the processes controlling cell volume are further affected (Kreisman and Olson, 2003).

Ascorbic acid, an important antioxidant and neuromodulator is found at its highest concentration in the brain (Rice, 2000). It is found in CSF and extracellular fluid and is taken up by neurones where it is found at its highest concentration at 10mM. High levels of ascorbic acid are required for protection against glutamate induced neuronal death. Loss of antioxidants from intracellular compartments during ischemia leaves cells vulnerable to oxidative damage. Providing ascorbic acid in the modified aCSF means it can directly scavenge ROS' and can be hetero-exchanged with glutamate, increasing levels in the extracelllar fluid to minimise excitotocity.

Taken together, these four neuroprotectants combined in a modified aCSF solution have increased the preservation of neurones in M1 and also helped to increase number of slices that display neuronal network activity and show a definitive rise in slice viability. The overwhelming theme of neuronal cell loss is that of an excitotoxic event by which prevention/correction cannot occur when the four neuroprotectors are not present in the aCSF and resulting from their concentrations being diminished during slice preparation. NMDAR mediated excitotoxicity is associated with an influx of Ca^{2+} and Cl^{-} ions; Ca^{2+} due to the activity of the receptor itself, which sets of a second messenger cascade resulting in necrotic or apoptotic cell death (Tymianski et al., 1993; Szydlowska and Tymianski, 2010) and Cl^{-} to compensate for the excessive depolarisation excitotoxicity causes (Rothman, 1985; Aghajanian and Rasmussen, 1989;, Olney et al., 1986). It is this process that resulted in sucrose replacing NaCl in preparatory methods for acute brain slices (Aghajanian and Rasmussen, 1989). Brain injury can cause ischemia, reduced blood flow to the injured areas followed by an accumulation of glutamate in the extracellular fluid. It is this build up of glutamate that induces excitotoxicity. More recently, further methods for maintaining viable slices have been proposed, including glycerol as a replacement for sucrose (Ye et al., 2006). Glycerol is a naturally occurring alcohol and in the Ye et al., study it prevented cell swelling and lysis, producing twice as many live cells as compared to sucrose based aCSF. Tanaka et al., (2008) have shown that replacing NaCl/sucrose with NMDG (*N*-methyl d-glucamine chloride) specifically helps to preserve GABAergic interneurones in cerebral cortex. They also showed that sucrose-,

NMDG-, choline- and glycerol-based cutting solutions all produced viable slices as opposed to a NaCl based solution.

As well as changes to the cutting solution, adding further biologically available compounds to the perfusing aCSF also makes a difference to neuronal activity. K^+, Ca^{2+} and Mg^{2+} are usually found in varying concentrations across laboratories, but even subtle differences can affect neuronal activity. Neurotransmitter molecules are normally not added to aCSF but are found in CSF and have been found sufficient to affect tonic conductances (Glykys and Mody, 2007) and oscillatory activity (Glykys et al., 2008). The amount of GABA and glutamate available in acute brain slice preparations can vary vastly this being dependent upon preparation and storage. Along with other neurotransmitters, such as taurine, ascorbate and serotonin, the aCSF could be used to increase slice viability as suggested by Hajos and Mody (2009).

3.3.3 Rat Colony

Changing the breeding pairs of the Wistar rats resulted in the greatest increase to the viability of the slices, increasing the occurrence of oscillatory activity to over 50%. Wistar rats are an outbred strain and are generally used where isogenicity is not required. Outbred animals should not be kept in a breeding program where breeding occurs between parent/offspring or between littermates (Harris, 1997). Due to heterogeneity, outbred strains are usually larger, more robust and more resistant to stress than inbred rat strains. However, over time a closed outbred colony, such as is kept at Aston University, will become inbred to a certain degree, the extent dependent upon the size of the starting population. Thus, a few points should be taken from this;

- The colony should be started with as many breeding pairs as possible
- The colony should be restarted every few years, either with new breeding pairs, or by replacing one sex of the breeding pairs from outside sources.

Inbreeding with parents/littermates produces a level of homozygosity that can result in inbreeding depression, a state where a population's ability to reproduce and survive is reduced. This arises due to an increase in harmful recessive alleles that

would normally be heterozygous and thus having a dominant normal gene. Inbreeding depression is most noticeable in its reduction of size, growth, fertility and resistance to stress. One of the most noticeable problems with the Wistar colony was the increased occurrence of enlarged ventricles. Enlarged ventricles are implicated in many neurological disorders, such as schizophrenia, Parkinson's disease, Alzheimer's disease and ADHD. Schizophrenia, Parkinson's disease and Alzheimer's disease are all known to affect normal oscillatory activity (Uhlhaas and Singer, 2006; 2010; Donnan et al., 2008; Hall et al., 2010).

3.3.4 Changes to KA and CCh concentrations

KA alone can generate oscillatory activity in hippocampus (Fisahn et al., 1998), entorhinal cortex (Cunningham et al., 2003) and somatosensory cortex (Roopun et al., 2006). However, KA and CCh are required for the generation of oscillatory activity in the primary motor cortex (Yamawaki et al., 2008). Kainate receptors (KAR) act principally as modulators of synaptic transmission and neuronal excitability (for review see Contractor et al., 2011). KARs perform this function through diverse mechanisms; Postsynaptic depolarisation at a subset of excitatory synapses (Campbell et al., 2007), presynaptic modulation of both excitatory and inhibitory transmission (Cossart et al., 1998; Frerking et al 1998.,) and balance of excitation and inhibition and thus control of oscillations (Huxter et al., 2007). KA is also a powerful neurotoxin implicated in seizures, as shown by Smolders et al., 2002, whereby KAR antagonists prevented pilocarpine induced epileptiform activity. Acetylcholine has many cognitive functions, including the cortical modulation of sensory information (Lucas-Meunier et al., 2003). Muscarinic acetylcholine receptors (mAChRs) are found as two subtypes; M1 like, consisting of the subunits M1, 3 and 5, and M2-like, consisting of the subunits M2 and M4 (Caulfield and Birdsall., 1998). Addition of CCh, a mAchR agonist, increases glutamate release, first in GABAergic interneurones, via a fast hyperpolarisation, then in Pyramidal cells, via a slow depolarisation (Lucas-Meunier et al., 2003).

The requirement of both KA and CCh to elicit oscillatory activity in M1 may be due to a consequence of reduced resilience of M1 during acute brain slice preparation, possibly due to a decline in the structural and functional properties of the neuronal

circuits. This led to the requirement of 400 nM KA and 50 μM CCh to elicit oscillatory activity in previous studies (Buhl et al., 1998; Yamawaki et al., 2008). During the present study, when the aCSF was modified, KA and CCh concentrations had to be reduced due to an increase in the frequency of epileptiform activity observed in the slices. The final concentrations required in the new preparation were 100nM KA and 5μM CCh. KA concentrations could be used up to 150 nM, as required, but the best network activity was acquired when CCh concentrations were retained at 5 μM. This adds credence to the hypothesis that damage caused by the acute brain slice preparation requires a higher concentration of excitatory agents to elicit oscillatory activity. *In vitro* preparations reduce synaptic background activity (Dexteshe and Pare, 1999), which is required for oscillation generation. Decreased synaptic background activity leads to decreased neuronal responsiveness (Ho and Dexteshe, 2000). Pyramidal cells in the neocortex receive 5,000-60,000 synapses, of which 70 % originate from other cortical neurones (Dexteshe and Pare, 1999). If the slice viability is dramatically reduced, this would account for the loss of interneurones and connectivity and thus the increased amount of excitation required to elicit oscillatory activity. Preservation of cortical neurones with the new preparatory method, and possibly increased synaptic activity in sagittal slices could account for the reduced requirement of KA and CCh required for network activity in M1.

3.3.5 Changes to Frequency

Traditionally, oscillations in the motor cortex have been characterised as beta frequency oscillations. However, the ranges of frequencies classified as beta (or gamma) are not consistent from one report to another. Murthy and Fetz (1992) define beta as 25-35 Hz and later (1996a, b) define beta as 20-40 Hz. Baker et al., (1999) define the range as 20-30 Hz, whilst it has been considered to be as low as 15 Hz (Traub et al., 1996). Beta has also been split into two frequency bands consisting of low beta 1 (15-20 Hz) and high beta 2 (20-30 Hz) (Roopun et al., 2006). The crossover with gamma has long been debated, with the range of gamma itself also varying, from 25 Hz and above (Whittington et al., 1995) to 30 Hz and above. Other studies have chosen not to define frequency bands, instead including all activity in the 15-50 Hz range (Sanes and Donoghue, 1993, 1998). The differences in oscillatory frequency seen here vary by up to 10 Hz, all of which could either be

considered either beta or gamma. Therefore, activity in the motor cortex may not be restricted to beta frequencies. Beta has been described as the resting frequency of the motor cortex (Murthy and Fetz, 1992) and is most often seen during gripping whilst awaiting a cue to move (Murthy and Fetz, 1992; 1996a; Baker et al., 1999), where a switch to gamma frequencies is then seen (Murthy and Fetz, 1996a; Donner and Siegel, 2011; Ball et al., 2008; Donner et al., 2009; Ricket et al., 2005). The only clear way to determine if an oscillation is the same between the acute brain slices in these experiments is via pharmacological manipulation. All oscillations were modulated in the same manner by 10 nM zolpidem, suggesting that although the frequency varied considerably, they are indeed the "same" oscillation. The differences seen between slices could mainly reflect differences between slice planes, as the major difference in oscillation frequency was the change in that seen between sagittal and coronal slices. Gamma and beta frequency oscillations have been proposed to reflect differences in circuitry. Gamma has been proposed as a local circuit phenomena (Kopell et al., 2000) encoding information within local neuronal populations (Donner and Siegel, 2011). Beta has been proposed to mediate long-range interactions that mediate integrative cognitive functions and therefore linking local neuronal populations (Kopell et al., 2000; Donner and Siegel, 2011). This would therefore indicate that coronal slices have more long range connections than are seen in sagittal slices. M1 is separated into distinct zones dependent upon muscle innervations. Aroniadou and Keller (1993) postulated that inhibitory networks limit postsynaptic responses of an organised excitatory network rather than to control spatio-temporal patterns of network activity in an unorganised network. Therefore, specific circuits of inhibitory interneurones could exist for this activity and control of the circuits would be dependent upon the power of the excitation.

Yamawaki et al., (2008) previously reported that the beta oscillation seen in M1 resembles the pharmacologically induced persistent gamma oscillation described by Fisahn et al., (1998) and subsequently Cunningham et al., (2003). Pyramidal cells were found to fire at a lower frequency than the field oscillation, while FS cells were found to fire on every gamma cycle (Fisahn et al., 1998). Furthermore, Pyramidal cells and FS cells were found to receive IPSPs at gamma frequency, which strongly correlated with field activity. Fisahn et al., (1998) attributed this to a mechanism

whereby Pyramidal cells fired producing monosynaptic EPSPs in neighbouring Pyramidal cell dendrites, which were curtailed by disynaptic perisomatic IPSPs from neighbouring interneurones. Yamawaki et al., (2008, thesis) found that EPSPs did indeed occur at a much lower frequency than IPSPs. It was also found that the beta oscillation appeared to be AMPA current independent, where the persistent gamma oscillation was AMPA current dependent, indicating a lesser role for recurrent phasic excitation in beta oscillation generation. The requirement of kainate-dependent and muscarinic-dependent tonic excitation, seen in coronal slices in the original aCSF solution (400 nM and 50 μM respectively), is much reduced in the modified aCSF, sagittal slices and new rat colony (100 nM and 5 μM respectively), perhaps indicating that the preservation of Pyramidal cells and interneurones is introducing an AMPA-dependent current to these networks that was not seen in previous studies and thus producing the higher frequency observed in these slices. Further experiments will be required to determine involvement of recurrent excitation and, thus, the involvement of an AMPA-dependent current in these newer slices.

3.3.6 Conclusion

In this chapter I have shown optimal neuronal network responses obtained from brain slices cut in a modified aCSF solution containing taurine, ascorbic acid, NAC and aminoguanidine. This was confirmed in extracellular studies of beta frequency network activity in the motor cortex and by cell counts. I could consistently obtain a stable oscillatory recordings in >50 % of slices, as compared to 9 % of slices cut in the original aCSF solution, with frequency and power responses mediated by the same properties to those of responses in the original aCSF as confirmed by pharmacological studies. What part of the altered process is most important remains unknown, but taken together healthy, viable slices have been obtained. In conclusion, I have here described a slice preparation method, which has allowed improved viability of primary motor cortical slices from rats. The slice preparation technique recommended in the present chapter is used throughout this thesis for electrophysiological studies on the neuronal circuits of the primary motor cortex.

Chapter 4 Pharmacological Modulation of Oscillatory Activity in the Primary Motor Cortex *in vitro*

4.1 Introduction

The primary motor cortex (M1) plays an important role in the execution of voluntary movement and is associated with characteristic neuronal oscillatory activity at beta (15-30 Hz) frequencies. These beta oscillations reflect interplay between GABAergic and glutamatergic elements producing synchronised network activity (Yamawaki et al., 2008). *In vivo*, beta oscillatory activity is associated with the resting state; pre-movement or anticipation (Cheyne et al., 2008) or sustained contraction (Baker et al., 2007). When movement is executed, a decrease in beta oscillatory power, coupled with an increase in gamma oscillations (>30 Hz), is seen. It is therefore thought that beta represents the preparatory state for movement and/or maintenance of postural tone. In PD, beta oscillations of an increased power are often observed, and are thought to be pathological, such that movement is inhibited and this results in the commonly seen symptoms of bradykinesia and rigidity (Brown, 2007). In recent years, transdural high frequency stimulation of M1 in animal models of PD (Drouot et al., 2004) and in groups of PD patients (e.g. Pagni et al., 2003) and transcranial magnetic stimulation (TMS) (Lefaucheur et al., 2004) of the same region have been shown to improve motor impairments.

Previous studies *in vivo* and *in vitro* have shown that beta oscillations can be modulated by zolpidem (Hall et al., 2010; Yamawaki et al., 2008), a non-BZD drug that acts at the α1 subunit of the $GABA_AR$. Zolpidem is a non-BZD imidazopyridine that at the BZD site of the $GABA_AR$ and acts to-augment the agonist action of GABA on the receptor (Crestani et al., 2000). GABA binding regulates the permeability of the $GABA_AR$ to Cl^- ions and application of a BZD can augment these effects. Binding of BZDs increases the frequency of the channel opening, but cannot directly open these channels without GABA having bound first. The α-subunit of the $GABA_AR$ determines the recognition of BZDs and the γ subunit must also be present (Cope et al., 2004; 2005) since the BZD binding site is shared between the α subunit and the γ subunit. BZD binding is therefore separate to GABA binding on the receptor.

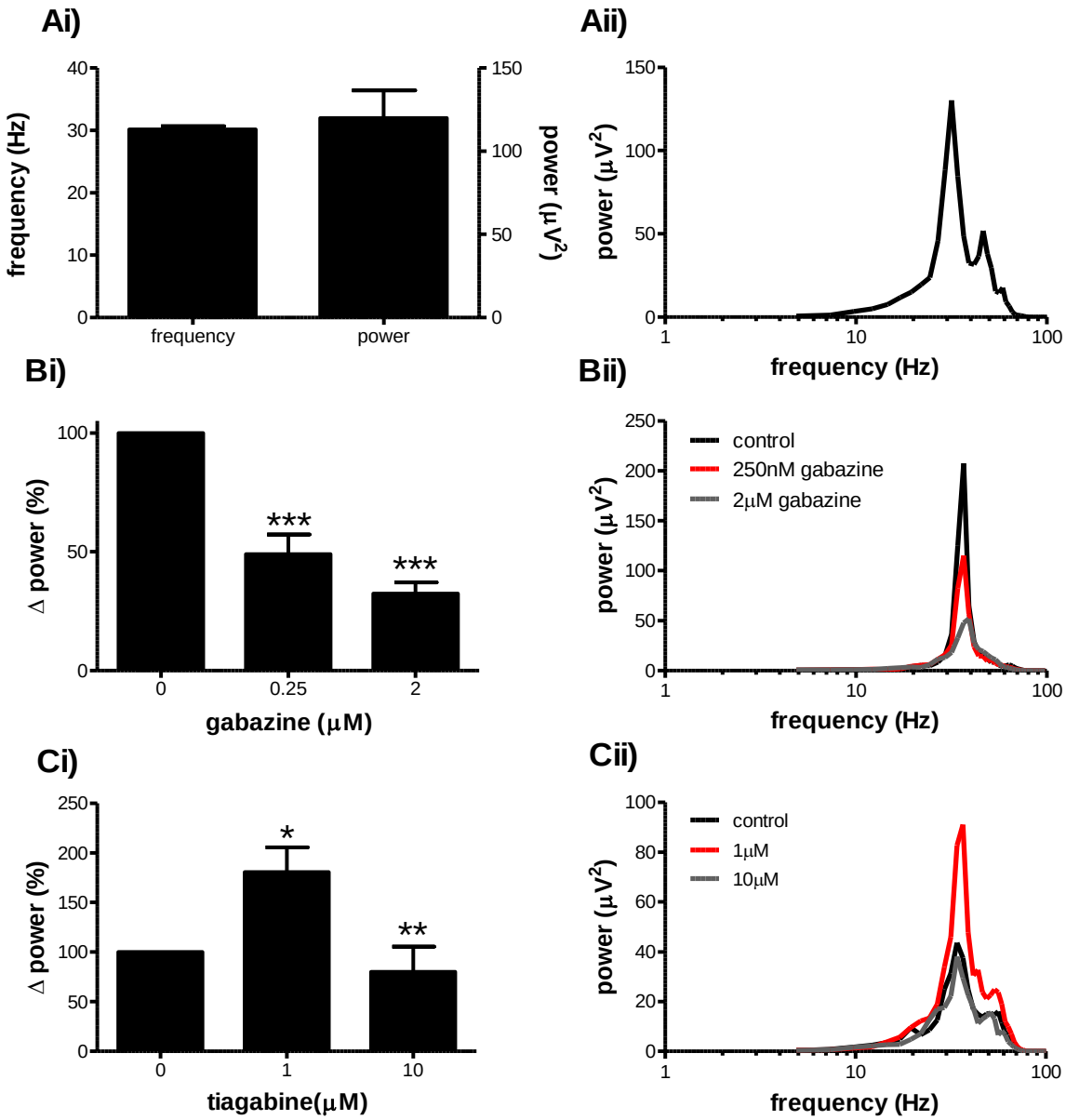


Figure 4-1 Oscillatory activity in M1 is dependent on GABAergic inhibitory mechanisms. Ai) Mean peak frequency (30.14 ± 0.50 Hz) and mean peak power (139.9 ± 6.53 μV^2) of a beta oscillation in M1 (n = 128). Aii) Representative power spectrum of a beta oscillation in deep (L5) M1. Bi) Bar chart showing the decline in beta power in response to increasing concentrations of gabazine, a $GABA_AR$ antagonist (n = 6). Gabazine (250 nM) caused a decline in oscillatory power to 49 ± 8.24% of baseline (BL) ($P < 0.001$) and further decline in power at 2 μM to 32.39 ± 4.64% of BL. ($P < 0.001$) Bii) Representative power spectrum showing the decline in beta power upon cumulative addition of gabazine at 250 nM and 2 μM. Ci) Bar chart showing the varying response of a beta oscillation to increasing concentrations of tiagabine, a GABA uptake inhibitor (n = 6). At low concentrations tiagabine (1 μM) increases beta power to 180.70 ± 24.72 % ($P < 0.05$) and at high concentrations tiagabine (10 μM) decreases power to 80.17 ± 25.4 % ($P < 0.01$). Cii) Representative power spectrum showing the varying responses of a beta oscillation to increasing concentrations of tiagabine.

Although zolpidem effects are thought to be specifically mediated through the α1 subunit containing $GABA_AR$ (Rudolph et al., 1999), at higher concentrations zolpidem will potentiate function at receptors containing α2/3 subunits (Pritchett and Seiburg, 1990). Zolpidem, used clinically as a sedative, was recently shown to awaken patients in a persistent vegetative state (Clauss et al., 2000), suggesting that it might have paradoxical effects under certain (pathological) conditions. In 2010, Hall et al. showed a reduction in beta power after the administration of sub-sedative doses of zolpidem in stroke patients, which correlated with an increase in cognitive ability and motor function. The role of zolpidem in treating these neuropatholgies may be to desynchronise aberrant oscillatory activity. This research has opened up the possibility of using non-invasive therapeutic approaches for diseases in which aberrant neuronal network oscillatory activity is a feature. Such “oscillopathies” include, stroke, PD, AD and some of the epilepsies. This paradoxical ability of zolpidem at sub-sedative doses to improve cognitive and motor function in patients suffering from neurological disease was the motive for the experimental work described below, which aimed to investigate possible mechanisms by which zolpidem might modulate network function in the *in vitro* M1 slice.

4.2 Results

4.2.1 Effect of zolpidem on beta oscillatory activity

Initial *in vitro* studies of the actions of zolpidem on beta oscillatory activity suggested that zolpidem (100 nM; Yamawaki et al., 2008) augmented beta power, and this was in apparent conflict with *in vivo* data suggestive of a decrease in beta power in human subjects (Hall et al., 2010). Hence, I began this programme of work with the question, why are there conflicting effects of zolpidem *in vivo* and *in vitro*?

Synchronous activity was primarily elicited by the co-application of 400 nM kainic acid (KA) and 50 μM carbachol (CCh) (or later, 100 nM and 5 μM respectively (see chapter 3)), as M1 does not show any robust spontaneous oscillatory activity (Yamawaki et al., 2008). Activation of KA receptors has repeatedly been shown to elicit oscillations in the cortex (Hormuzdi et al., 2001; Cunningham et al., 2003), however, KA receptor activation alone does not appear to be sufficient within the motor cortex. CCh was also co-applied after it was found that the addition of both receptor ligands would induce oscillations within the somatosensory cortex *(*Buhl et al., 1998) and was therefore thought to be sufficient to induce oscillations in M1. Oscillations were allowed to stabilise for 30-60 minutes before drug application, and measures of beta power were repeated during this time to ensure stability and consistency of the baseline activity so that any changes produced in the neuronal activity could confidently be attributed to drug application.

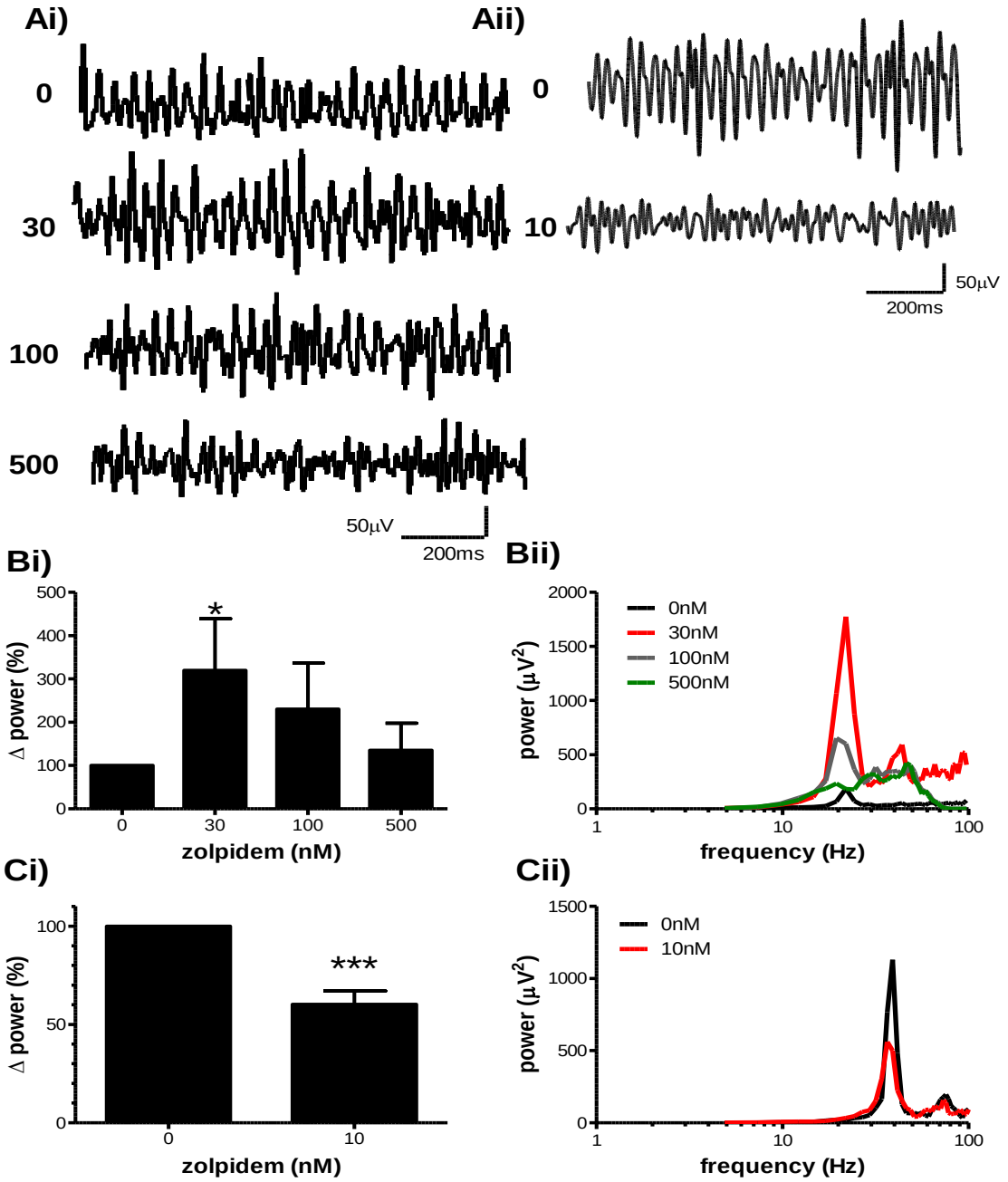


Figure 4-2 Concentration-dependent effects of zolpidem on beta oscillations in L5 M1. Ai) Raw data showing changes to oscillatory activity in 0-500 nM zolpidem and Aii) 10 nM zolpidem Bi) Bar chart showing the change in beta power in the presence of 30-500 nM zolpidem (n = 7). Mean oscillatory power would increase at 30 nM (319.17 ± 119.9 %, $P < 0.05$) and then showed a steady decline at higher concentrations (100 nM 230.2 ± 106.1 % and 500 nM 135.2 ± 62.67 %). Bii) Representative power spectrum showing changes to the beta oscillation in the presence of 30-500 nM zolpidem (n = 7). Ci) Bar chart showing the mean change in beta power in the presence of 10 nM zolpidem (60.15 ± 6.85%, $P < 0.001$, n = 21). Cii) Representative power spectrum showing the decline in beta power in the presence of 10 nM zolpidem (n=7).

All recordings were conducted in deep (L5) M1 as previous studies have shown this to be the source of oscillatory activity in M1 and also to have the most robust synchronous activity (Yamawaki et al., 2008). Mean oscillatory frequency was 30.14 ± 0.5 Hz and average power was 119.9 ± 16.53 μV^2 (Fig. 4.1A, n = 128). The oscillation in M1 is thought to be dependent on $GABA_AR$-mediated phasic inhibition (Yamawaki et al., 2008). I tested two concentrations of the selective $GABA_AR$ antagonist gabazine (Fig. 4.1B). At 250 nM, gabazine decreased the power (Fig. 4.1B 49.00 ± 8.24 % of baseline, $P < 0.001$, n = 6). At higher concentrations (2 µM, $n = 6$), gabazine decreased beta power further (Fig. 4.1B 32.39 ± 4.64 % of BL, $P < 0.001$). Tiagabine, the GABA uptake blocker, was also tested at two concentrations. At 1 µM, tiagabine increased the beta power (Fig. 4.2C, 180.7 ± 24.72 %, $P <0.05$, n = 6) and at 10 µM tiagabine decreased the beta power in M1 (Fig. 4.2C, 80.17 ± 25.38 %, $P < 0.01$, n = 6).

Zolpidem was applied in a cumulative manner at increasing concentrations. Each application of zolpidem was left for 30 minutes before application of the higher dose to allow any drugs effects to clearly appear and for activity to stabilise. A dose response curve was constructed and revealed a parabolic effect on the oscillations. A maximum response was reached at 30 nM (Fig. 4.2B & C 319.17 ± 119.9 %, $P < 0.05$, n = 7) and a decline was seen at further concentrations (Fig. 4.2B & C, 100 nM zolpidem, 319.17 ± 119.9 % to 230.2 ± 106.1 % was recorded and 500 nM zolpidem saw a further fall to 135.2 ± 62.67 %). To investigate this effect further, experiments including a lower dose of zolpidem were conducted. It was found that 10 nM zolpidem reduced the power of oscillations in M1 and therefore has a desynchronising effect (Fig. 4.2E & F, 10 nM zolpidem 60.15 ± 6.85%, $P < 0.001$, n = 21). It was also found that after application of 10 nM zolpidem, network activity could not be “resynchronised” with the addition of 30 nM zolpidem. 10 nM zolpidem experiments were therefore conducted separately to the dose response experiments.

To further investigate the role of the α1 subunit of the $GABA_AR$ in beta oscillatory activity in M1, a further dose response experiment was conducted with CL 218,872. CL 218,872 is a BZD agonist displaying selectivity for α1 subunit-containing $GABA_ARs$.

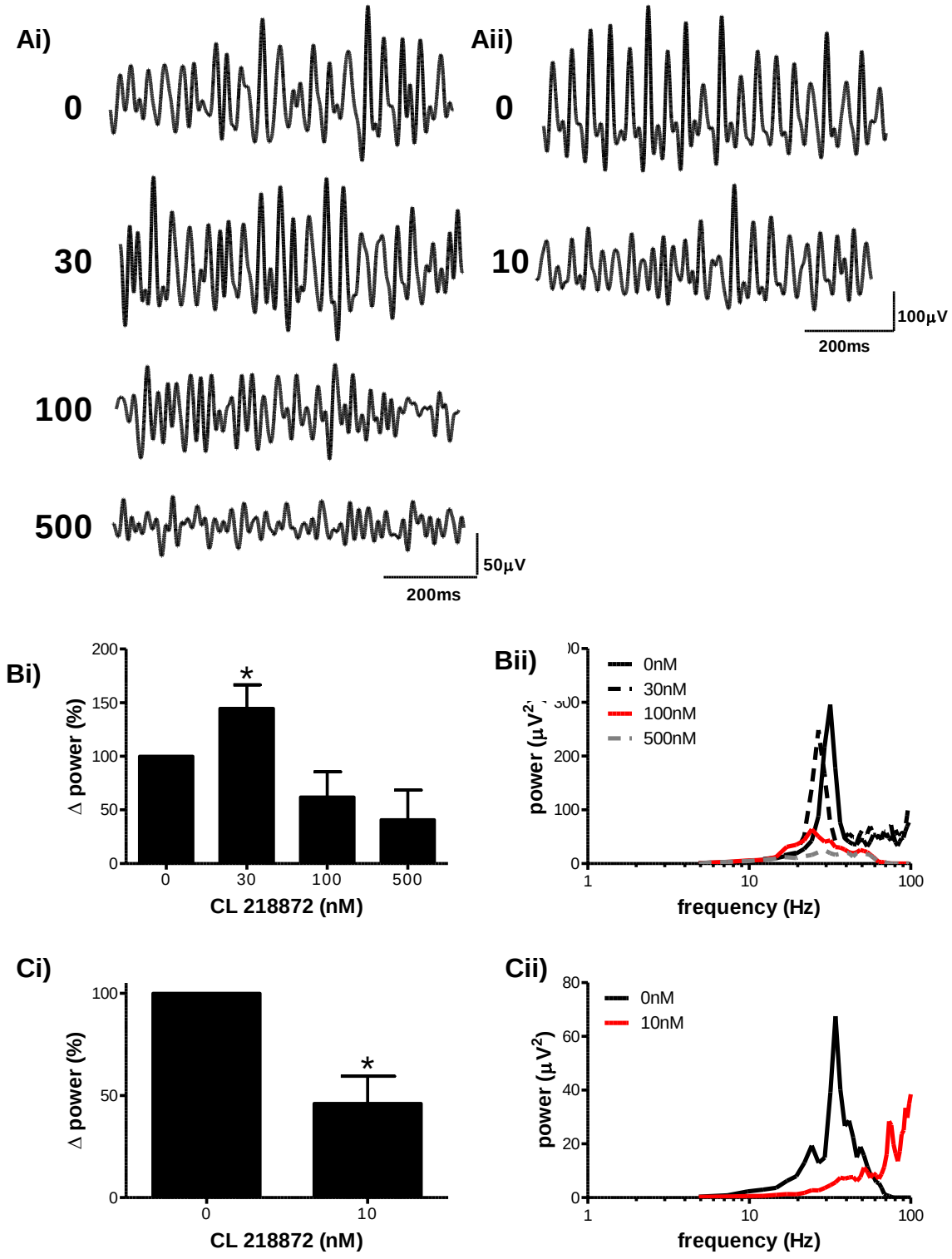


Figure 4-3 Concentration-dependent effects of CL 218,872 on beta oscillations in L5 M1. Ai) Raw data showing the changes in oscillatory activity in 0-500 nM CL 218,872. Aii) Raw data showing the change in oscillatory activity in the presence of 10 nM CL 218,872 Bi) Histogram showing the average changes in beta power in the presence of 30-500 nM CL 218,872. Bii) Representative power spectrum in the presence of 30-500nM CL 218,872 (30 nM 144.18 ± 21.79 % $P < 0.05$, 100 nM 62.15 ± 23.39 % and a further reduction to 40.99 ± 27.47 % at 500 nM, n = 7). Ci) Bar chart showing the mean change in beta power in the presence of 10 nM CL 218,872. Cii) Representative power spectrum showing a decline in beta power in the presence of 10 nM CL 218,872 (46.18 ± 13.24 %, $P < 0.05$, n = 7).

The amino acid determining the α1 subunit selectivity of CL 218,872 and zolpidem are the same, confirming a high affinity of the $GABA_A$ BZD site for these two ligands (Wingrove et al., 2002). Using CL 218,872 as a comparison with zolpidem, I sought to see if the desynchronising properties at lower doses were specific effects of the drugs at the BDZ site. With a similar outcome to zolpidem, 30 nM CL 218,872 significantly increased the power from control (Fig. 4.3B & C, 144.18 ± 21.79% of BL power, $P < 0.05$, n = 6). Higher concentrations then induced a desynchronisation of β synchrony in M1 (Figure 4.3B, C & F, 144.18 ± 21.79 % to 62.15 ± 23.39 % at 100 nM and a further reduction to 40.99 ± 27.47 % at 500 nM). 10 nM CL 218,872 also showed the desynchronisation of beta oscillations as with zolpidem (Figure 4.3E & F, oscillatory power fell to 46.18 ± 13.24 %, $P < 0.05$, n = 6). These data suggested that zolpidem's desynchronising effect was not due to a non-specific, non-BZD site action.

4.2.2 Effect of non-α1 subunit specific BZD agonists on beta oscillatory activity

To further characterise the role of $GABA_A$Rs on neuronal network activity in M1, dose response experiments were conducted for zopiclone and diazepam. Zopiclone is also used as a sedative, even though its effects are not specific to the α1-subunit. However, it does show higher affinity for α1-subunit containing $GABA_A$Rs over those containing α3, or α5 and its efficacy is similar at α1,2,3 and 5 (Brunello et al., 2009). Zopiclone is a non–α1 subunit selective BZD site agonist at the BZD site and was expected to show a steady increase of the power of the oscillations with increasing concentrations (Fig. 4.4B & C). Oscillatory power increased to 281.67 ± 142.6 % above BL and then again to 282.2 ± 142.6 % at 100nM and finally to 989.8 ± 812.5% at 500 nM ($P > 0.05$, n = 7). However, further experiments conducted with 10nM zopiclone showed the same effect as zolpidem; 10 nM zopiclone decreased the oscillatory power (Figure 4.4E & F - 27.64 ± 7.45% of BL, $P < 0.05$, n = 6). As the most commonly available BZD site agonist, I also repeated the experiments with diazepam. In knock-in point mutations, diazepam has been shown to mediate its effects through the α2 but not the α3 or α5 subunit containing $GABA_A$Rs (Löw et al., 2000; Crestani et al., 2002). The dose relationship of diazepam would then be expected to be similar to that of zopiclone. However, 30 nM diazepam showed a non-

significant tendency towards increased power, with similar effects at 100 nM and 500nM (Fig. 4.7B, 30 nM 115.5 ± 11.97 %, 100 nM 116.7 ± 48.07 % and 500nM 121.3 ± 48.55 of BL, $P > 0.05$, n = 6).

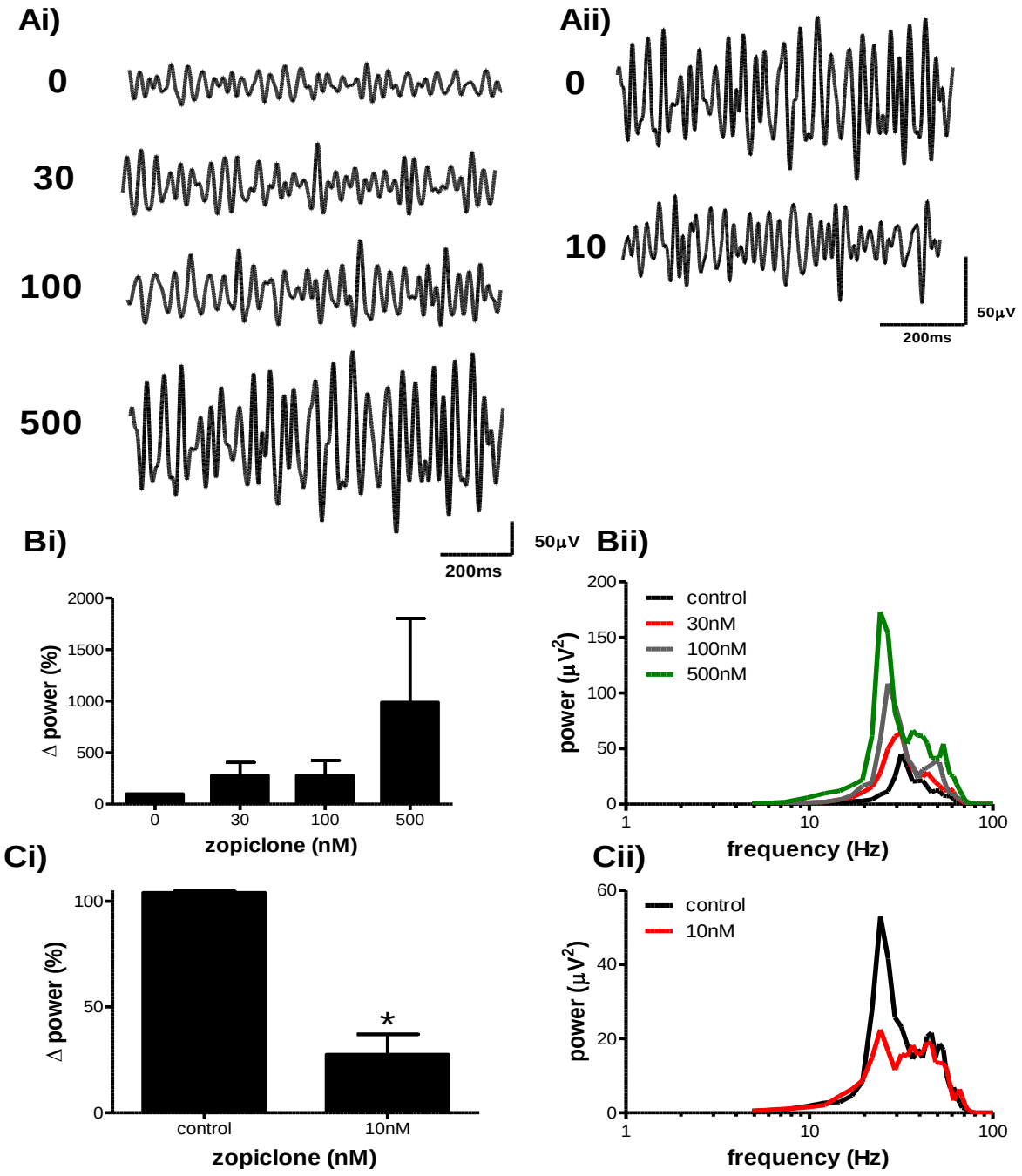


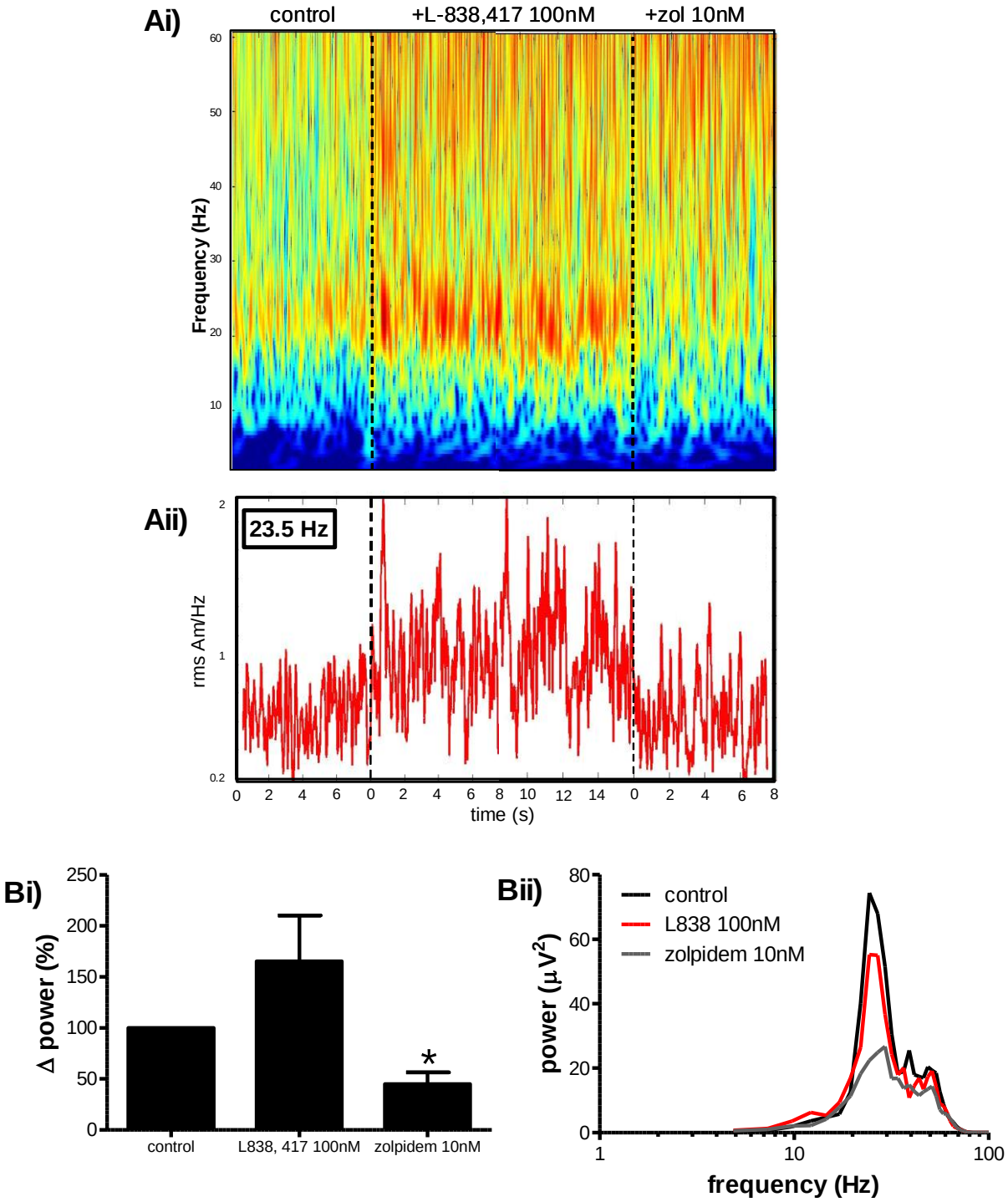
Figure 4-4 Concentration-dependent effects of zopiclone on beta oscillatory power in L5 M1. Ai) Raw data showing oscillatory activity in 0-500 nM zopiclone Aii) Raw data showing oscillatory activity in 0-10 nM zopiclone (Bi) Bar chart showing the mean change in beta power in the presence of 30-500 nM zopiclone. Oscillatory power increased to 281.67 ± 142.6 % above BL and then again to 282.2 ± 142.6 % at 100 nM and finally to 989.8 ± 812.5% at 500 nM (P > 0.05, n = 7). Bii) Representative power spectrum in the presence of 0-500 nM zopiclone. Ci) Bar chart showing the mean change in beta power in the presence of 0-10 nM zopiclone (27.64 ± 7.45% of BL, P < 0.05, n =6). Cii) Representative power spectrum showing a decrease in beta power in the presence of 0-10 nM zopiclone.

Given that the desynchronising effects of zolpidem could be achieved at very low doses, when it might be expected that only α1-subunit containing $GABA_A$Rs would be bound, I decided to test the effects of 100 nM L-838,417, a partial agonist which has the unique effect in that it shows equal affinity at α1, α2, α3, and has a threefold lower affinity at the α5 subunit containing $GABA_A$Rs, but has no efficacy at α1-subunit containing receptors. I hypothesised that L-838,417 would boost oscillatory power and also block the effects of zolpidem at the α1-subunit containing $GABA_A$R. On application of L-838, 417, the power of the oscillation increased (Fig. 4.5B, D & F, average increase from BL of 216.2 ± 58.24 % across the three conditions, n = 18). However, application of zolpidem (10 nM) in the presence of L-838,417 induced a marked desynchronisation of beta activity (Fig. 4.5A, B & C, 44.99 ± 11.59 % of BL, $P < 0.05$, n =6). The same was seen for zopiclone (Fig. 4.6A & B 42.67 ± 7.97 % of BL, $P < 0.05$, n = 6) and diazepam (Figure 4.6C & D 27.93 ± 9.13 % of BL, $P < 0.05$, n = 6).

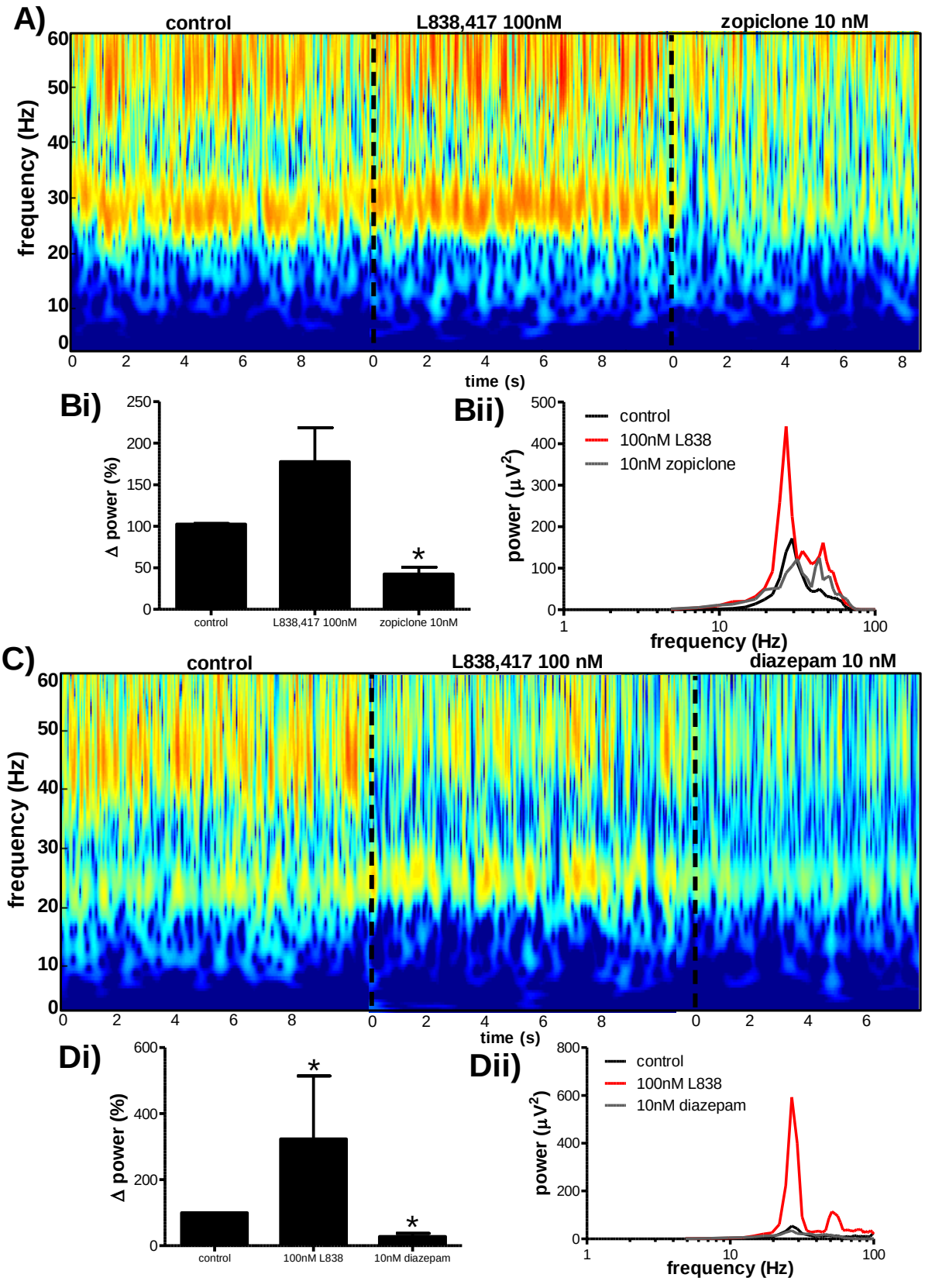
4.2.3 Effect of BZD site inverse agonist on beta oscillatory activity

Notwithstanding the effects of L-838,417, the data so far implicated the α1 subunit containing $GABA_A$R in desynchronisation of beta activity in M1. I hypothesised that an inverse agonist with selectivity for the α1 subunit might have opposite effects to zolpidem. β-CCM is an inverse agonist at the BZD site of the $GABA_A$R and cumulative application showed a steady decline in the power of oscillations (Fig. 4.7A). β-CCM was ineffective at doses selective for α1 subunit containing $GABA_A$Rs (Fig. 4.7A reduction to 77.64 ± 12.54 % of BL, $P > 0.05$, n = 6), while higher doses induced a steady decline in the power of the oscillations: 100 nM and 500 nM (Fig. 4.7A – reduction to 61.66 ± 15.71 %, $P < 0.01$ and 42.15 ± 14.20 % respectively, $P < 0.01$, n = 6).

Following Page - **Figure 4-5 The desychronising effects of zolpidem *in vitro* are mediated by the α1 subunit of the $GABA_AR$.** Ai) Morlet wavelet time – frequency plot (Matlab R2010a, Natick, USA) in control, L-838,417 (100 nM) and zolpidem (10 nM). Control conditions show peak amplitude within the beta (15-30 Hz) oscillatory band in M1. When 100 nM L-838,417 is added, a marked increase in amplitude is observed. When 10 nM zolpidem is added, this oscillation is abolished, as shown in the earlier experiments with zolpidem. Aii) Band – pass filtered envelope of RMS power in the 15 - 30 Hz range shows the increase in amplitude with 100 nM L-838,417 and the decline of the oscillation below that of control conditions with 10nM zolpidem. Bi) Bar chart showing the dose response of L-838,417 (100 nM, 165.20 ± 45.10 %, $P > 0.05$) followed by zolpidem (10 nM, 44.99 ± 11.59 % of BL, $P < 0.05$, $n = 6$). Bii) Representative power spectrum showing the response to L-838,417 (100 nM) followed by zolpidem (10 nM).


Ai)
control
+L-838,417 100nM
+zol 10nM
Frequency (Hz)
60
50
40
30
20
10
Aii)
23.5 Hz
rms Am/Hz
2
1
0.2
0 2 4 6 0 2 4 6 8 10 12 14 0 2 4 6 8
time (s)
Bi)
Δ power (%)
250
200
150
100
50
0
control
L838, 417 100nM
zolpidem 10nM
*
Bii)
power (μV²)
80
60
40
20
0
control
L838 100nM
zolpidem 10nM
1
10
100
frequency (Hz)

Following Page - **Figure 4-6 The desychronising effects of zopiclone and diazepam *in vitro* are mediated by the α1 subunit of the $GABA_AR$.** A) Morlet wavelet time – frequency plot (Matlab R2010a, Natick, USA) in control, L-838,417 (100 nM) and zopiclone (10 nM). Control conditions show peak amplitude within the beta (15-30Hz) oscillatory band in M1. When 100 nM L-838,417 is added, a marked increase in amplitude is observed. When 10 nM zopiclone is added, this oscillation is abolished, as shown in the earlier experiments with zolpidem. Bi) Bar chart showing the dose response of L-838,417 (100 nM, 177.9 ± 40.95 %, $P > 0.05$, n = 6) followed by zopiclone (10 nM, 42.67 ± 7.97 %, $P < 0.05$, n = 6). Bii) Representative power spectrum showing the response to L838, 417 (100 nM) followed by zopiclone (10 nM). C) Morlet wavelet time – frequency plot (Matlab R2010a, Natick, USA) in control, L-838,417 (100 nM) and diazepam (10 nM). Control conditions show peak amplitude within the β (15-30Hz) oscillatory band in M1. When 100nM L-838,417 is added, a marked increase in amplitude is observed. When 10nM diazepam is added, this oscillation is abolished, as shown in the earlier experiments with zolpidem. Di) Bar chart showing the dose response of L-838,417 (100 nM, 323.4± 191.0 %, $P < 0.05$, n = 6) followed by diazepam (10 nM, 27.93 ± 9.13 %, $P < 0.05$, n = 6). Dii) Representative power spectrum showing the response to L-838,417 (100 nM) followed by diazepam (10 nM).


A)
control
L838,417 100nM
zopiclone 10 nM
frequency (Hz)
time (s)
Bi)
Δ power (%)
control
L838,417 100nM
zopiclone 10nM
Bii)
power (μV²)
control
100nM L838
10nM zopiclone
frequency (Hz)
C)
control
L838,417 100 nM
diazepam 10 nM
frequency (Hz)
time (s)
Di)
Δ power (%)
control
100nM L838
10nM diazepam
Dii)
power (μV²)
control
100nM L838
10nM diazepam
frequency (Hz)

4.2.4 Effect of different ligands on oscillation frequency.

At 500 nM, zolpidem elicited a significant decline in the frequency of the oscillation (Fig. 4.8A, 20.75 ± 0.83 Hz to 19.94 ± 0.75 Hz, $P < 0.05$, n = 7). There was a trend for the frequency to decline in the dose response experiments for the other BZD site ligands also, but perhaps most surprisingly, this was not the case for CL 218,872. However, because of the size of the frequency bins used for analysis, this could be due to an averaging effect.

The work in this chapter shows that beta oscillations in M1 appear to be under GABAergic control, and can be modulated in one of two ways. BZD agonists at low concentrations desynchronise beta oscillations and this effect does not appear to be specific to one particular agonist as first thought. However, the desynchronising effect at agonist concentrations above 100nM does indeed appear to be specific to α1 subunit selective BZD site agonists.

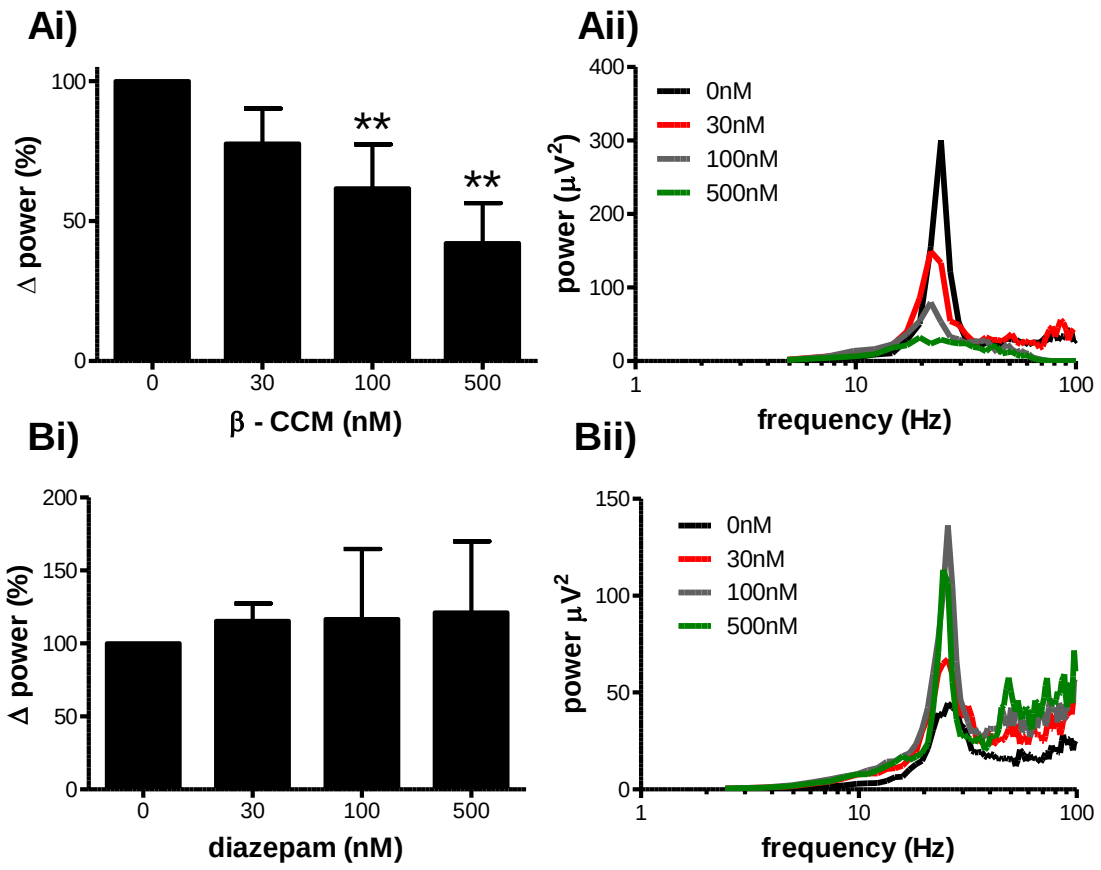


Figure 4-7 Concentration-dependent effects of β-CCM and diazepam on beta power. Ai) Bar chart showing the decline in beta power in the presence of 0-500 nM β-CCM (30 nM 77.64 ± 12.54 % of BL, $P > 0.05$, 100 nM 61.66 ± 15.71 %, $P < 0.01$ and 500 nM 42.15 ± 14.20 %, $P < 0.01$, n = 6). Aii) Representative power spectrum in the presence of 30-500 nM β-CCM (n=6), an inverse agonist at α1 containing $GABA_A$Rs. Bi) Bar chart showing the increase in beta oscillatory power in increasing concentrations of diazepam (30 mM 115.5 ± 11.97 %, 100 nM 116.7 ± 48.07 % and 500nM 121.3 ± 48.55 of BL, $P > 0.05$, n = 6). Bii) Representative power spectrum showing the changes to beta power in the presence of increasing concentrations of diazepam.

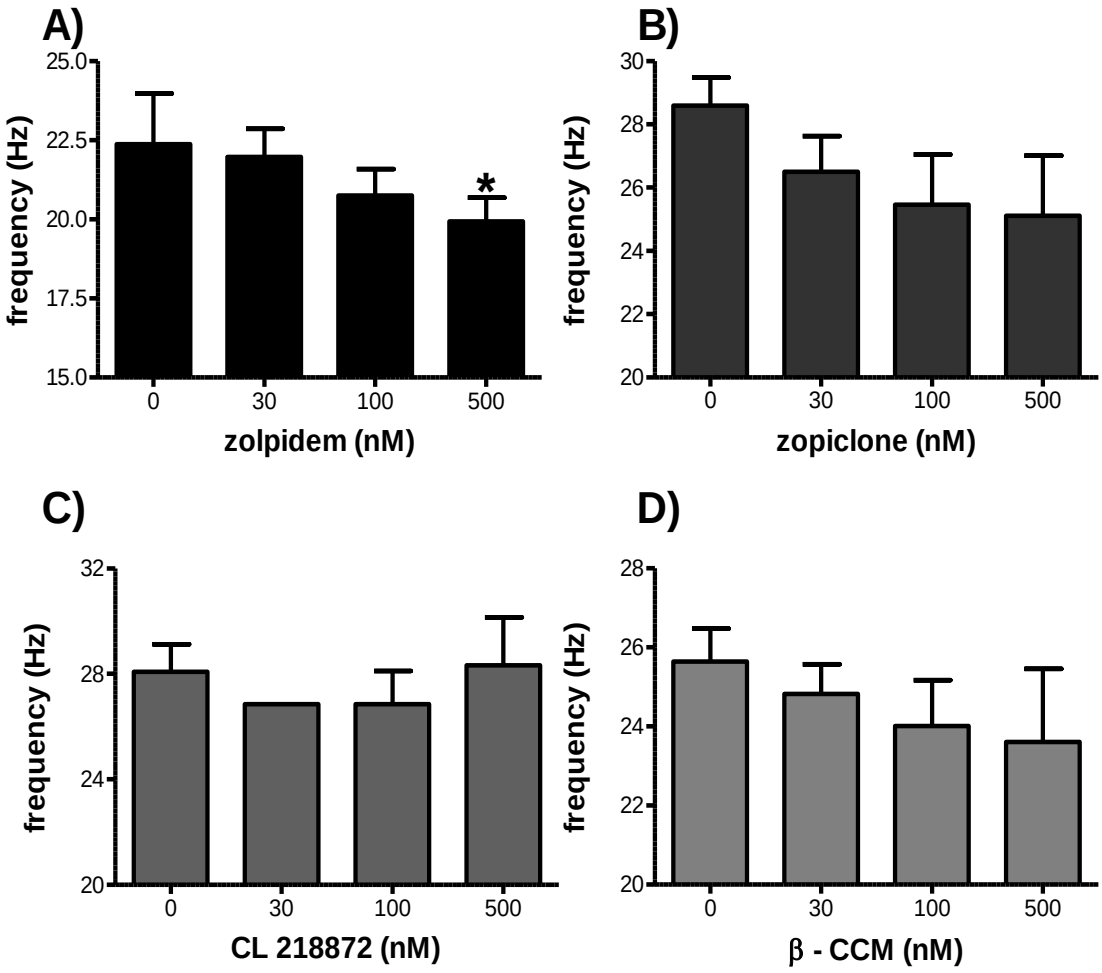


Figure 4-8 Dose-dependent effects of zolpidem, zopiclone, CL 218,872 and β -CCM on the frequency of β oscillations in M1. A) Bar chart showing the effect of increasing concentrations of zolpidem on frequency (n = 7). B) Bar chart showing the effect of increasing concentrations of zopiclone on frequency (n = 6). C) Bar chart showing the effect of increasing concentrations of CL 218,872 on frequency (n = 6). D) Bar chart showing the effect of increasing concentrations of β -CCM on frequency (n = 6).

4.2.5 M1-S1 interactions

If there is a sensory component to motor control, then we might expect the properties of zolpidem on beta oscillations to also be evident in the primary somatosensory cortex (S1). Sensory gating is known to be a problem in PD. For example, a PD patient who is walking will suddenly be faced with rigidity and akinesia when coming to a doorway. There is an obvious link between sensory and motor function and this is still to be explored in PD. M1 and S1 have already been shown to display long range synchronous oscillatory activity (Murthy and Fetz, 1992, 1996). Extracellular techniques were employed here to record oscillatory activity in S1 simultaneously with M1. In a combined slice, the mean frequency of beta oscillations in M1 was 29.3

± 1.2 Hz, whilst the mean frequency of beta oscillations in S1 was 31.3 ± 0.81 Hz (Fig. 4.9A, $P > 0.05$, n = 6). Cross-correlation analysis showed a link between M1 and S1 oscillatory activity with a positive correlation at beta frequency; the two signals co-varied at 31Hz (Fig. 4.9D). An auto-correlation in M1 (Fig. 4.9D, black line) showed a time-scale similar to that of the cross-correlation. Using 10 nM zolpidem, as seen in earlier experiments, oscillatory activity was reduced in M1, with the same occurring in S1 (Fig. 4.9C, 10 nM zolpidem decreased the power to 30.01 ± 7.2 % of BL and 13.37 ± 4.1 % respectively, $P < 0.001$, n = 4). However, a time course analysis revealed that S1 activity started to desynchronise before M1 (Fig. 4.9E). S1 began to desynchronise 25.0 ± 5.0 minutes after zolpidem application and M1 started to desynchronise 36.67 ± 3.33 minutes after zolpidem application (n = 4).

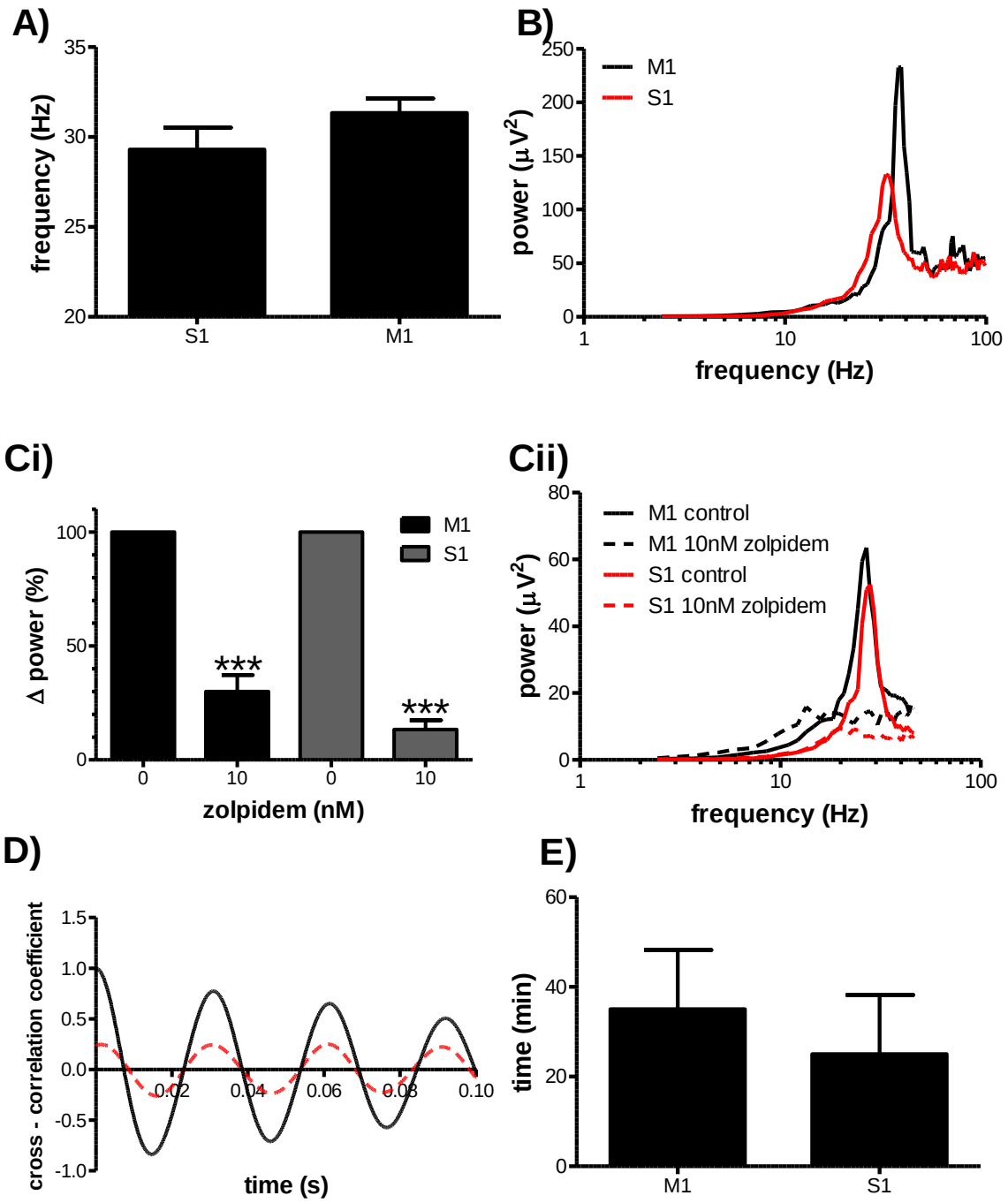


Figure 4-9 M1 – S1 Interactions. A) Histogram showing the frequency of beta oscillations in M1 and S1 (M1 29.3 ± 1.2 Hz, S1 31.3 ± 0.81 Hz, $P > 0.05$, n = 6). B) Representative power spectrum showing beta oscillatory power in M1 and S1 of a conjoined sensorimotor slice. Ci) Histogram showing changes to beta oscillatory power after application of zolpidem (10 nM M1 30.01 ± 7.2 %, S1 13.37 ± 4.1 %, $P < 0.001$, n = 4) to a sensorimotor slice. Cii) Representative power spectrum showing the effects of zolpidem (10 nM) on M1 and S1 in a sensorimotor slice. D) Cross–correlation showing the correlation between beta oscillations in a sensorimotor slice. M1/S1 (red) and M1/M1 (black). E) Bar chart showing that S1 activity would start to desynchronise before that in M1 (S1 25.0 ± 5.0 mins, M1 36.67 ± 3.33 mins, $P > 0.05$, n = 4).

4.3 Discussion

4.3.1 Changes in Power

In the present study, the concentration–effect relationship of zolpidem, a non-BZD that acts at the α1 subunit of the $GABA_AR$, on beta oscillations in M1 was studied *in vitro*. Beta oscillations are characteristic of M1 and reflect GABA network activity (Yamawaki et al., 2008). It has previously been shown that administration of zolpidem at sub–sedative doses (5 mg) reduces beta power and correlates with an improvement in motor function in PD and stroke patients (Brefel–Courbon et al., 2007; Hall et al., 2010). However, studies *in vitro* showed an increase in beta power at 100 nM (Yamawaki et al., 2008) in contrast to the *in vivo* data. This study therefore looked at the cumulative dose response of zolpidem on M1 oscillations *in vitro* and the effect that the different concentrations had on said oscillations. Here, it has been shown that sub-sedative doses of zolpidem do indeed dresynchronise beta oscillations. However, this is also the case for non-α1 subunit specific BZDs, suggesting a dose specificity that is not limited to a particular ligand.

It is widely acknowledged that BZD administration corresponds with an increase in beta power in the EEG (Baker and Baker, 2003; Van Lier et al., 2004). Power and frequency in the EEG represent the activity of underlying Pyramidal neurones within the cortex. In terms of motor activity, beta oscillations are associated with inactivity or a resting state; pre-movement, or anticipation (Cheyne et al., 2008), and sustained contraction (Baker et al., 1997). In Parkinson's disease (PD), increases in beta power are seen, and these are thought to inhibit movement resulting in the commonly seen symptoms; particularly bradykinesia and rigidity (Brown, 2007). These increases in EEG beta power after BZD administration would tie in with the effects of zolpidem as a sedative and with the cessation of movement associated with sedation. This is reflected in the 30 nM data that has been shown here. A corresponding increase in beta power is associated with sedation and the normal function of the drug. These sedative effects have been widely attributed to the α1 subunit of the $GABA_AR$ (Crestani et al., 2000), of which zolpidem shows its highest affinity. However, this does not explain the paradoxical effect seen at 10 nM zolpidem, where desynchronisation beta oscillatory power was observed.

Zolpidem shows selectivity for the α1 subunit, with its affinity in the nanomolar range (Crestani et al., 2000). However, the increase in beta power seen after zolpidem administration is not necessarily α1 subunit mediated. Lier et al., (2004) showed that diazepam and zolpidem increase beta activity in the EEG, as did Hall et al., (2009) using diazepam in MEG studies and this has previously been termed the "beta buzz" (Glaze et al., 1990). Diazepam shows no subunit selectivity, with equal affinity at all subunits (Sieghart, 1994). Work here with zopiclone, a non-subunit selective drug, showed the same increase in beta power at 30 nM as zolpidem, also showing that the increase in beta activity is not necessarily α1 subunit mediated. Work in this study with L-838,417 (100 nM) also showed an increase in beta oscillatory power. L-838, 417 has equal affinity at all subunits but no efficacy at the α1-subunit i.e. it is a mixed α2/3 agonist and neutral α1-subunit antagonist at the $GABA_AR$ (McKernan et al., 2000). As zolpidem also has efficacy at α2/3-subunits, albeit at higher concentrations, this could be where it is exerting its effects at 30nM and possibly at 100nM as seen in previous experiments (Yamawaki et al., 2008) and this may explain the increased beta power observed. Indeed, in studies in mice, where the α1 subunit has been made BZD-insensitive due to an amino acid substitution in the BZD binding site, hyperlocomotion is seen with diazepam administration, suggesting that movement is α2/3 subunit mediated and α1-subunit mediated sedation masks locomotion driven by the α2/3 subunits (Rudolph et al., 1999, Reynolds et al., 2001). However, McKernan et al., (2000), using α1 subunit knock-out mice noted that diazepam administration did not decrease locomotor activity to the same extent as wildtype mice, suggesting that locomotor activity is subject to modulation by α1-subunits. Further studies conducted using CL 218,872 were in agreement with the original zolpidem data. CL 218,872 is thought to have an affinity for the α1-subunit that matches that of zolpidem (Seighart, 1995). A decrease in beta synchronisation is noted with an increase in movement (Cheyne et al., 2008). If locomotor activity is tied to the α1 subunit, then this would explain the data seen in the McKernan study and the results seen in this study with 10 nM zolpidem.

How 10 nM zolpidem could out-compete 100 nM L-838,417 at the α1 subunit suggests an intriguing story as to how 10 nM zolpidem is exerting its effect. It could be as simple as that while L 838,417 is potentiating the effects of output neurones expressing the α2/3 subunits, zolpidem is affecting the output of a subset of

interneurones that preferentially express the α1 subunit. However, L-838,417 has been shown to be an antagonist at the α1 subunit (Mckernan et al., 2000) and it is unlikely that zolpidem could out-compete L 838,417 at the concentration ratio used above.

It seems likely that populations of receptors could show distinct pharmacological profiles at different zolpidem concentrations as they do at different GABA concentrations (Semyanov et al., 2003). The beta desynchronisation seen could in fact be due to different receptor localisations, i.e. synaptic or extrasynaptic, with distinct affinities for zolpidem. GABAergic signalling can be either phasic or tonic, with phasic signalling being mediated by inhibitory postsynaptic potentials (IPSPs) at the synapse. The second form of GABAergic signalling is mediated by receptor activity away from the synapse, and is known as tonic inhibition. $GABA_A$Rs involved in tonic signalling are thought to contain the δ subunit. However, Semyanov et al., (2003) found a tonic current in interneurones that used the α1γ2 subunits and was shown to be modulated by zolpidem. The work done here with 10 nM zolpidem may be increasing the tonic current, corresponding with a disruption to a class of interneurones and thus a decline in the overall power of the oscillations. Furthermore, distinct $GABA_A$Rs are found synaptically and extrasynaptically (Nusser et al., 1998; Fritschy and Brünig, 2003) and α1 subunit containing $GABA_A$Rs have been shown to be found extrasynaptically (Mohler, 2002, Semyanov et al., 2003).

FS, or fast spiking cells, are a specific type of interneurone found in M1. FS cells fire at the same frequency as the LFP (Yamawaki, 2008; thesis). Due to this strong phase relationship, any disruption to the FS cell would significantly affect the LFP. The tonic current is an important determinant of interneuronal excitability and firing and may determine firing rates of the neurones presynaptic to the interneurone. Strong mutual inhibition is required to produce a stable oscillation (Whittington et al., 1995; Pauluis et al., 1999). Inhibition is required to be strong enough to override any excitation within the network and to therefore set each cycle of oscillations by a train of synchronous IPSCs (Pauluis et al., 1999). It is possible that at 10 nM zolpidem disrupts mutual inhibition, rather than adding to it and therefore causes desynchronisation of the beta oscillations, and hence, it may be that the network in

the presence of 10 nM zolpidem is unable to sufficiently excite enough inhibitory interneurones to propagate synchronous IPSCs to the whole network.

The higher concentrations of zolpidem (i.e. 30 nM and 100 nM), could be affecting IPSC kinetics and/or having the opposite effect to that of the tonic current, creating the apparent paradox seen in these results. The GABA subunits are not only distinctly distributed by brain region, but also at the subcellular level (Fritschy and Mohler, 1995; Pirkir et al., 2000). Interneurone signalling properties are shaped by the type of $GABA_AR$ expressed either synaptically or extrasynaptically. For instance, the soma of a hippocampal Pyramidal cell is innervated by two types of interneurone. The FS cells form synapses containing α1 subunit containing $GABA_ARs$, which display fast kinetics of deactivation (Freund and Buzsaki 1996; Nyíri et al., 2001; Klausberger et al., 2002; Pawelzik et al., 2002), whereas the synapses of the RSNP cells contain α2 subunit containing $GABA_ARs$, which display slower kinetics than α1 subunit containing receptors (Brussaard and Herbison, 2000; Hutcheon et al., 2000; Nyíri et al., 2001; Jüttner et al., 2001; Vicini et al., 2001). Axon initial segments of Pyramidal cells also contain α2 subunit containing $GABA_ARs$, which appear to be responsible for simple on/off signalling (Rudolph and Mohler, 2004; Mohler, 2006). Activating α1 subunit containing $GABA_ARs$ could depress GABA release and therefore prohibit oscillatory activity generated by the output neurones, whereas activating α2/3 subunit containing $GABA_ARs$ will not depress GABA release, and therefore potentiate the oscillation. Although zolpidem has its highest affinity at the α1-subunit, its ability to increase GABA evoked currents (i.e. its efficacy) was shown to be higher at α2 (Sanna et al., 2002; Brunello et al., 2009; Petroski et al., 2006) and α3 (Petroski et al., 2006) subunits. Thus, different interneurones operate with the appropriate $GABA_AR$ subtypes to regulate network behaviour.

4.3.2 Changes in Frequency

Previous work by Yamawaki et al., (2008) has equated oscillations in M1 to an interneurone network gamma ("ING") like network. Theoretical and experimental work showed rhythmic network activity that could be generated by reciprocally connected and mutually inhibiting GABAergic interneurones. The generation of these oscillations is induced through excitation of metabotropic receptors (glutamate or

cholinergic/muscarinic acetylcholine) (Whittington et al., 1995; Traub et al., 1996 and 1999; Yamawaki et al., 2008). Within this “ING” network, three factors are known to affect frequency of the oscillations; excitatory driving current, the $GABA_A$ decay time constant ($T_{GABA(A)}$) and the amplitude of unitary hyperpolarising $GABA_AR$ mediated conductance ($g_{GABA(A)}$). Of these three, the latter two are attributed to decreases in frequency, showing an inverse, non–linear relationship and have been shown to be modulated by the BZD diazepam (Traub et al., 1996 and 1999; Fisahn et al., 1998). The work done in these experiments with various BZD site ligands have shown a trend to a decrease in the frequency of the oscillations with increasing concentrations of the drug, which appears to be independent of the changes in power observed. Zolpidem, in this situation, can be equated to the actions of diazepam in the Traub study, with particular emphasis on the changes in $T_{GABA(A)}$. It is also likely that zolpidem will affect the conductance.

Although “ING” was found to induce gamma oscillations in the Whittington and Traub studies, their definition of gamma was much broader, often including frequencies as low as 20 Hz, which encompasses the beta oscillations seen within M1. Beta oscillations are thought to be preferred when excitatory drive is low, decay time is slow and conductance is high. This fits with the beta model within M1 and further reduction in frequency when BZDs are added to the network.

4.3.3 *M1-S1 interactions*

Cross-correlation analysis performed on oscillatory activity in M1 and S1 in a sensorimotor slice revealed a cortical connection between the two areas, with no siginificant phase differences, indicating that M1 and S1 are in near synchrony with each other. Given that the delay seen between the two brain areas is minimal, a near-zero phase lag relation could arise from reciprocal communication between the two areas or from the two areas being primed for communication by a third set of areas (Rajagovindan and Ding, 2008). Connections between M1 and S1 are prominent, particularly between M1 and S1 vibrissal regions in rodent cortex (Izraeli and Porter, 1995; Welker et al., 1998; Hoffer et al., 2003; Chakrabarti and Alloway, 2006; Ferezou et al., 2007) and connections are also evident via spread of oscillatory activity (Crotchet and Peterson, 2006; Murthy and Fetz, 1996). Electrophysiological

studies have also indicated that S1 modulates M1 (Kosar et al., 1985; Zarzecki, 1989; Kaneko et al., 1994; Farkas et al., 1999; Kelly et al., 2001). Functional connectivity between the two areas has also been confirmed at the cellular level. Intracortical microstimulation of S1 caused short-latency, monosynaptic EPSPs in the neurones located in the superficial layers of M1 (Kosar et al., 1985) and antidromic stimulation studies have shown connectivity between M1 and S1 (Kelly et al., 1999; Zarzeki et al., 1989). Similar responses to pharmacological modulation by zolpidem represent two highly interconnected areas with a similar $GABA_AR$ subunit distribution, while the desynchronisation of S1 occurring first is most likely representative of activity in S1 collapsing and M1 following, suggesting that the S1 to M1 connections are either stronger than M1 to S1 or a reflection of the direction of connections that dominate. The response of M1 to desynchronise following S1 could therefore be due to disrupted signalling between the two areas and not just due to termination of synchronous oscillations occurring independently of one another. Learning of motor skills has been attributed to plasticity of synapses from projection neurones of S1 to neurones in the superficial layers of M1 (Kaneko et al., 1994) while inactivation of somatosensory cortex eliminates activity in M1 (Andersson, 1995).

S1 has a similar topographical organisation to M1 (Huffman and Krubitzer, 2001), and although connections are reciprocal between the two areas (Veinante and Deschenes, 2003) connections from S1 to M1 appear to dominate (Izraeli and Porter, 1995, Welker et al., 1998). From the way sensory information is interpreted and transmitted to M1 (Ferezou et al., 2007), it appears logical that abolition of S1 activity would precede that of M1, and thus be the cause of abolition of M1 activity.

4.4 Conclusion

Beta desynchronisation is seen when movement is initiated and beta activity is then kept at low levels during motor tasks, where gamma activity predominates (Baker et al., 1997; Cheyne et al., 2008), this is known as event related desynchronisation, (ERD) and a rebound in beta power is seen when movement has stopped, known as post movement beta rebound (PMBR) (Cheyne et al., 2008). In PD, the inability to desynchronise beta oscillations appears to be what causes the symptoms (Galvan and Wichman., 2008) and treatment with levodopa and deep brain stimulation

corresponds with a decrease in beta activity and a corresponding increase in gamma activity (Brown et al., 2001). Treatment with zolpidem may be useful as a non-invasive method of treating the pathological beta oscillations and helping to improve motor function.

Chapter 5 Uncovering a Tonic Inhibitory Current in Interneurones in Primary Motor Cortex

5.1 Introduction

As discussed above, primary motor cortex (M1) *in vitro* exhibits ongoing neuronal network oscillatory activity in the beta (15-30 Hz) frequency range, as has also been shown *in vivo* at rest (Baker et al., 1997; Murthy and Fetz, 1996). The power of this oscillatory activity is dynamic, changes being coupled with the anticipation, initiation and termination of movement (Baker et al., 2007; Cheyne et al., 2008). Thus, excessive synchronisation and raised oscillatory power may underlie motor and cognitive deficits associated with the 'oscillopathies' which include stroke (Tecchio et al., 2006; Hall et al., 2010), Alzheimer's disease (Poza et al., 2007), Parkinson's disease (Brown, 2003; Kuhn et al., 2006) and schizophrenia (Canive et al., 1996; Ford et al., 2007).

Using magneto-encephalographic techniques it has recently been shown that cognitive and motor deficits observed in stroke (Hall et al., 2010) and Parkinson's disease (Hall et al., 2011) are associated with an increase in slow wave (4-12 Hz) and beta (~20 Hz) activity. Sub-sedative doses of the hypnotic drug zolpidem, which acts at the BZD binding site of $GABA_A$Rs (Crestani et al., 2000; Cope et al., 2004) reduced oscillatory power, an action which correlated with improved clinical scores (Hall et al., 2010). However, *in vitro* studies in this report and previously (Yamawaki et al., 2008) have shown that zolpidem, at a concentration of 30 nM and 100 nM, increases beta power and at lower (10 nM) concentrations, reduces beta power. Here, data is presented to reconcile these contradictory observations and provide a mechanistic understanding of neuronal network desynchronisation in M1.

As with gamma (30-80 Hz) frequency activity observed in hippocampus and other cortical regions (Whittington et al., 1995; Traub et al., 1996), beta oscillatory activity in M1 is generated as consequence of mutual inhibitory connectivity, where simultaneously activated fast spiking (FS) inhibitory interneurones are able to entrain each other to fire in a synchronous manner. In this way Pyramidal cell activity is sculpted by repetitive (phasic) inhibitory discharges, the frequency of which is directly dependent upon the kinetics of the inhibitory postsynaptic potentials (Whittington et al., 1995; Fisahn et al., 1998; Traub et al., 2000). Recently, evidence has emerged

that a sustained inhibitory membrane conductance (I_{tonic}), arising from spillover of GABA and mediated by high affinity extrasynaptic $GABA_AR$s (Farrant and Nusser 2005; Bright et al., 2007), plays a fundamental role in shaping network excitability (Semyanov et al., 2004; Mann and Mody 2010; Kochubey et al., 2011). I_{tonic} has been extensively studied in the thalamus (Belelli et al., 2005; Cope et al., 2005, 2009; Bright et al., 2007), the granule cells of dentate gyrus (Nusser and Mody 2002) and cerebellum (Brickley et al., 1996; 2003). In these regions I_{tonic} is maintained by the activity of GABA receptors containing the α4 or α6 subunit and the ∂ subunit. However, I_{tonic} may also be mediated by receptors containing the $\alpha 5$ and ∂ or γ subunits (Clarkson et al., 2010, Glykys and Mody, 2006, Glykys et al., 2008) and, under some circumstances by α1 and γ2 subunits which renders I_{tonic} BZD-sensitive (Semyanov et al., 2003; 2004, Yamada et al., 2007). As I_{tonic} is present in interneurones but not Pyramidal cells under normal conditions (Semyanov et al., 2003), any effect of BZD on I_{tonic} in a single FS cell could have a profound effect on the synchronous activity in a large number of Pyramidal cells. Thus, the paradoxical actions of zolpidem observed may be explained through receptor subunit expression, subcellular location and differences in receptor affinity for GABA yielding concentration-dependent effects.

5.2 Results

5.2.1 Inhibitory interneurone types in M1 as determined by electrophysiological characterisation.

I set out to specifically determine the different types of inhibitory interneurone located in deep layers of M1. Using whole–cell recordings in current clamp mode, I identified interneurones based on their non–Pyramidal shape and smaller size as compared to Pyramidal cells in deep (LV) M1. Specific targets were small, round cells located next to or near to the soma of large putative Betz cells. Current injections at hyperpolarising and depolarising levels were used to determine the spiking characteristics of putative inhibitory interneurones (Figure 5.1). The most commonly encountered cell type was the low-threshold spiking cell (LTS) (n = 10), characterised by a rebound spike on hyperpolarising current injections (Figure 1c). Regular spiking non-Pyramidal cells (RSNP) were also encountered (n = 7) (Figure 5.1b) and fast spiking (FS) cells were encountered less frequently (n = 6) (Figure 5.1a). All neurons sampled by whole-cell recording from the motor cortex had resting potentials more negative than -40 mV and overshooting spikes.

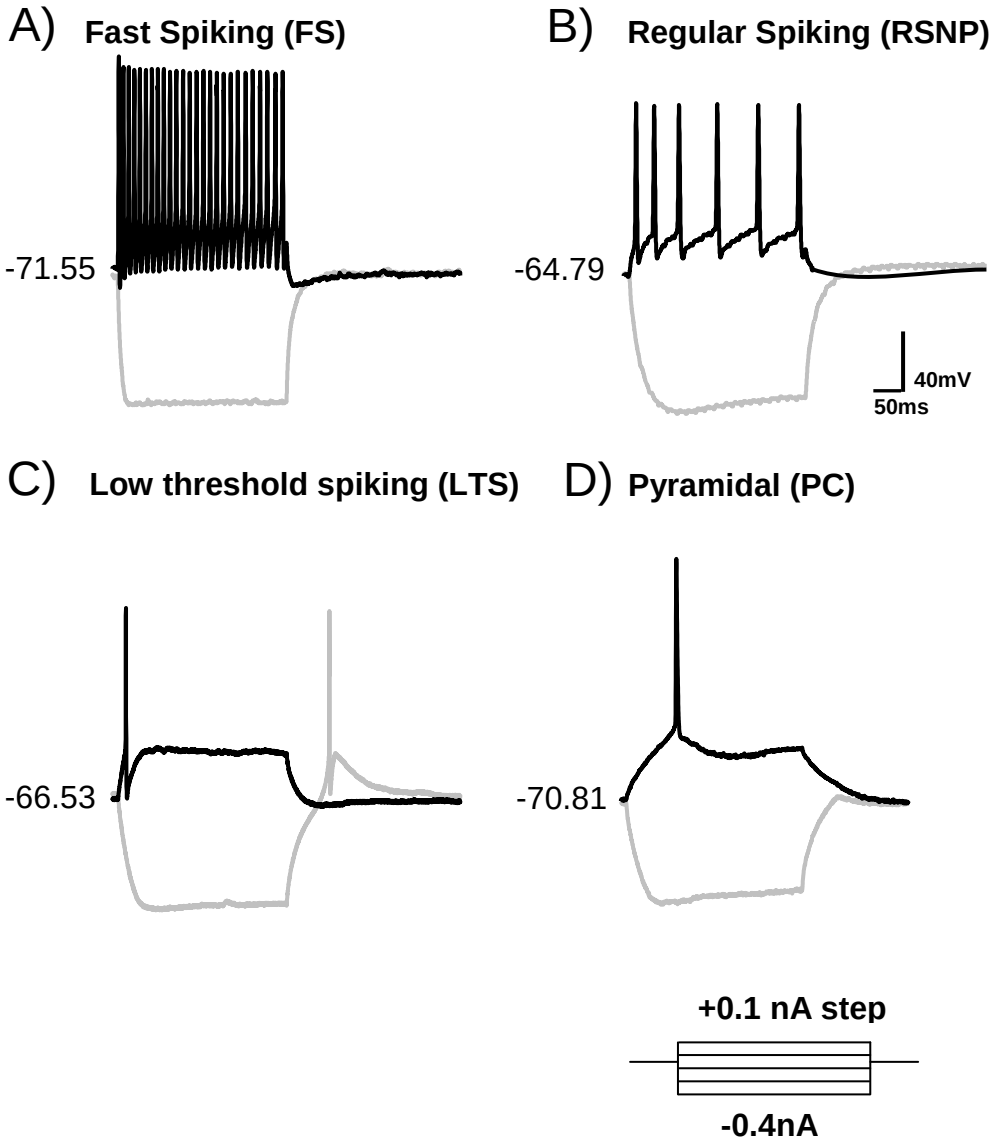


Figure 5-1 Neuronal subtypes located in deep (LV) M1. A) A fast spiking interneurone characterised by its short spike width, large AHP and high (>100Hz) firing frequency. B) A regular spiking non–Pyramidal cell, characterised by an adapting firing pattern at depolarising currents. Spike width is greater than that of FS cells and the AHP is much less pronounced. C) A low threshold spiking interneurone, most commonly identified by the rebound spike seen after hyperpolarising current injection. Adapting action potentials can also be seen at larger depolarising steps (not shown). D) A Pyramidal cell charaterised by larger spike width and low amplitude AHP. Current injection protocol used to study the firing properties of inhibitory interneurones in deep (LV) M1 consisted of steps ranging from -0.4 nA to + 0.1 nA.

The most distinguishable feature of FS cells was their short-duration action potentials with AHPs that were much larger than the other classes of cells (see Table 5.1, further properties shown in appendices). Another feature that was frequently encountered was their lower input resistances as compared to the other cell types. FS cells had spike widths at half amplitude shorter than 0.5 msec and input resistances that were relatively small, usually lower than 120 MΩ, and the resting membrane potentials of FS cells were more negative than those of LTS and RSNP cells. The discharge frequency of FS cells increased with stimulation intensity, but adaptation of firing was minimal or not seen at all. The maximum frequency measured for high intensities of depolarising currents ranged from 80 to 198 Hz.

	PC (n = 6)	**FS (n = 6)**	**LTS (n = 10)**	**RSNP (n = 7)**
Resting Potential (mV)	-84.9 ± 1.95	-71.55 ± 2.85	-66.53 ± 2.97	-64.79 ± 2.58
Input resistance (MΩ)	107.3 ± 19.93	98.95 ± 5.97	145.7 ± 18.22	119.8 ± 5.93
Spike width at half amplitude (msec)	1.75 ± 0.19	0.76 ± 0.03	1.31 ± 0.16	1.13 ± 0.19
Amplitude of AHP (mV)	-8.59 ± 1.38	-17.35 ± 1.56	-11.62 ± 1.88	-11.07 ± 1.41
Spike amplitude (mV)	78.48 ± 2.79	91.96 ± 5.73	75.84 ± 2.75	92.50 ± 5.74

Table 5.1 Properties of four types of motor cortical cells.

The other two classes of interneurones, LTS and RSNP, were distinguished from FS cells by longer duration action potentials, larger input resistances, less negative resting potentials and lower amplitude AHPs. Further, LTS interneurones were discriminated by the presence of one or more rebound spikes on the hyperpolarising current injection steps, although it has been reported that LTS cells are a type of RSNP cell (Cauli et al., 1997). In motor cortex, the largest proportion of interneurones encountered, which could not sustain high frequency repetitive discharges, were classified as RSNP cells. Spike width at half amplitude evoked by current injection in RSNP cells had a longer duration and a small AHP. RSNP and Pyramidal cells could be differentiated by the amplitude and duration of their action potentials. Action potentials emitted by Pyramidal cells were slower and had a smaller AHP than those of RSNP cells (Table 5.1). The most common defining feature that was used to distinguish interneurones from Pyramidal cells, was the size and shape of the soma, however, this was not used to categorise the cells.

In LTS cells, application of a hyperpolarising current injection produced a depolarising rebound that triggered action potentials. This rebound or low-threshold spike (LTS) is thought to be mediated by low voltage-activated Ca^{2+} channels (Cauli et al., 1997). Trains of frequency-adapting action potentials can also be observed at depolarising current steps, making them a type of RSNP cell (Connors and Gutnick, 1990; Kawaguchi, 1993, Cauli et al., 1997).

5.2.2 Oscillatory activity in LV M1 is dependent on GABA inhibitory mechanisms

Several reports have indicated that $GABA_A$Rs containing α1 and Y2 subunits are present on inhibitory interneurones, including FS cells involved in the generation of network oscillations in hippocampus (Thompson, 2000; Bartos et al., 2002; Klausberger et al., 2005) and neocortex (Bacci et al., 2003). Since the BZD binding site lies at the interface between these subunits (Crestani et al., 2000; Cope et al., 2004), and GABAergic interneurones have been shown to be subject to cell-type specific GABA tone (Semyanov et al., 2003), I hypothesised that BZD site activation would act, via GABAergic interneurones, to modulate network oscillatory activity. I generated persistent beta oscillations (119.9 ± 16.53 μV^2; modal peak frequency 27.8 Hz; range 22-31 Hz; n = 128) in slices of rat primary motor cortex (M1) using KA (100 nM) and CCh (5 μM). Since beta oscillations may (Roopun et al., 2006) or may not (Roopun et al., 2010) involve activation of local circuit GABA interneurones, I tested for involvement of GABAergic mechanisms using the $GABA_A$R antagonist, gabazine. The beta oscillations were highly sensitive to gabazine, being reduced to 49.0 ± 8.24 % of baseline in the presence of 250 nM gabazine and to 32.39 ± 4.64 % of baseline in the presence of 2 μM gabazine (n=10). Fig. 5.2Bi shows the effects of gabazine on beta activity in M1.

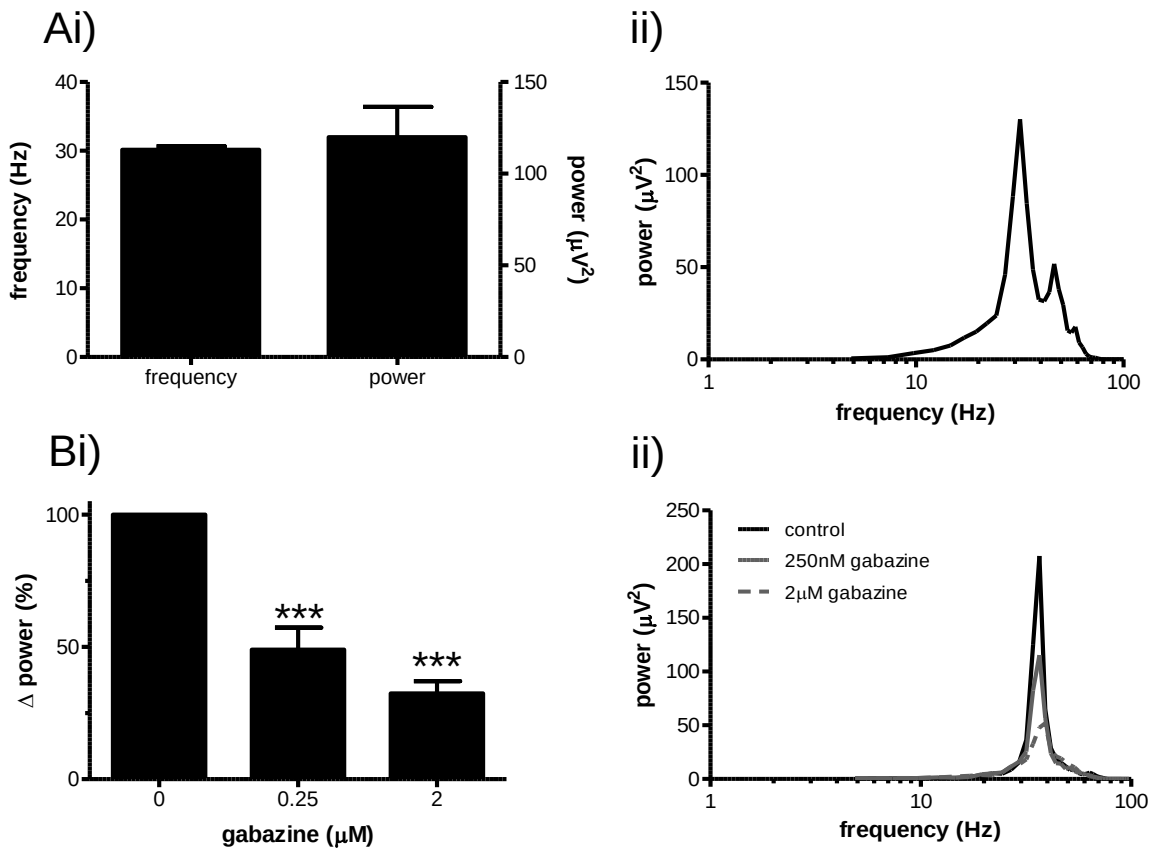


Figure 5-2 Beta oscillations in M1 are dependent on GABA inhibitory mechanisms. Ai) Bar chart detailing Mean frequency (30.14 ± 0.5 Hz) and mean power (119.9 16.53 μV2) of beta/gamma oscillations recorded in deep (LV) M1 (n = 128). Aii) Power spectrum showing a representative oscillation seen in M1. Bi) Bar chart showing the decline in beta power in response to increasing concentrations of gabazine, a $GABA_AR$ antagonist. Gabazine (250nM) caused a decline in oscillatory power to 49 ± 8.24% of baseline (BL) ($p < 0.001$, n = 10) and further decline in power at 2μM to 32.39 ± 4.64% of BL. ($p < 0.001$) Bii) Representative power spectrum showing the decline in betapower upon cumulaticve addition of gabazine at 250 nM and 2 μM.

5.2.3 Oscillatory activity in LV M1 can be modulated by benzodiazepine site ligands

In order to investigate the role of the BZD site in modulation of beta oscillatory activity, I again generated beta activity and applied the imidazopyridine, zolpidem. In initial dose-ranging experiments, zolpidem application generated a bell-shaped dose-response curve, with reduction of beta power at very low (10 nM) or high (>500 nM) concentrations (see chapter 4) and a clear augmentation of beta power at 30-100 nM, consistent with our previous observations (Yamawaki et al., 2008). As Fig.5.3Ai shows, application of 30 nM zolpidem potentiated network oscillations (319.7 ± 119.9%, n = 7). However, at 10 nM, zolpidem reliably decreased oscillatory power (60.15 ± 6.83%, n = 21). In order to rule out non-BZD site effects, I repeated these experiments in the presence of the BZD site antagonist, flumazenil. At 1 μM, flumazenil prevented the potentiation of oscillatory power previously observed with zolpidem at 30 nM (Fig. 5.3Cii), and also blocked the desynchronising effects of zolpidem at 10 nM, suggesting that zolpidem exerted both facilitatory and suppressive effects on beta power through the BZD binding site. Interestingly, application of flumazenil alone augmented beta power (276.7 ± 111.4% at 100 nM, 292.1 ± 90.8% at 200 nM and 273.2 ± 90.02% at 500 nM from BL, n = 6, Fig.5.3Bi), suggesting a tonic suppressive effect of endogenous BZD activity on neuronal network oscillations.

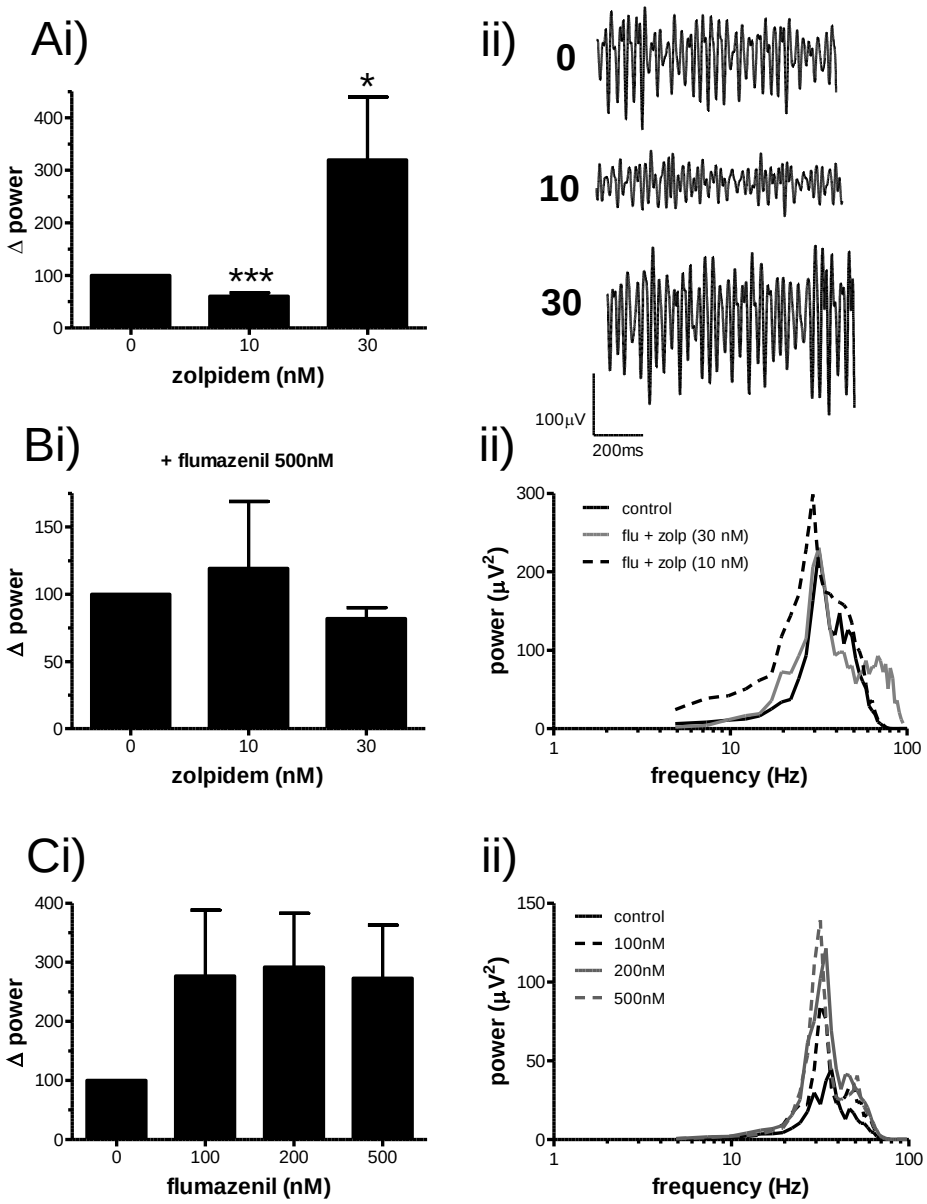


Figure 5-3 Concentration-dependent modulation of beta oscillations in LV of deep M1 by zolpidem. Ai) Bar chart showing the decline in beta oscillatory power to 60.15 ± 6.83% ($p < 0.001$, n = 21), induced by zolpidem (10 nM) and the increase in oscillatory power to 319.7 ± 119.9% of BL ($P < 0.001$, n = 7) induced by zolpidem (30 nM). Aii) Representative oscillatory traces from a single experiment showing the concentration-dependent effect of zolpidem on beta oscillations in M1. Bi) Bar chart showing flumazenil (500 nM) block of both the suppressive and facilitatory effects of zolpdem (n = 6). Bii) Representative power spectrum showing the lack of effect of zolpidem (10 and 30 nM) in the presence of 500 nM flumazenil. Ci) Dose response effects of flumazenil, showing an increase in beta power ($P > 0.05$, n = 6). Cii) Representative power spectrum showing the dose-dependent increase in beta power.

5.2.4 Low doses of zolpidem uncover a constitutively active tonic current in FS interneurones in LV M1

The action of zolpidem in causing desynchronisation of beta activity at low dose (10 nM) was of particular interest, since we have recently described a stroke patient in whom sub-sedative doses of zolpidem desynchronise a pathological theta/beta oscillation, restoring cognitive function (Hall et al., 2010) and similar actions may also be seen in Parkinson's disease (Hall et al., 2011). Hence, I explored this effect further in the *in vitro* brain slice. It is clear that zolpidem might affect phasic and/or tonic inhibition (Nusser and Mody, 2002; Semyanov et al., 2003; Gao and Smith, 2010) and so I investigated these aspects of GABAergic inhibition by recording directly from FS interneurones in deep layers of the motor cortex using whole-cell recording in current and voltage clamp. Betz cells in layer V of M1 were first identified by their characteristic size, orientation and morphology, and FS cells initially located through their own characteristic morphology and location. Fig. 5.4Ai shows a Betz cell with adjacent non-Pyramidal, presumably GABAergic interneurones. The stereotypical arrangement between Betz and GABAergic cells was readily observable in numerous experiments, and provided the basis for selection of cells from which to record. In current-clamp, FS cells showed relatively low input resistance (98.95 ± 5.97 MΩ) and a pronounced mAHP (-17.35 ± 1.56 mV) with no sAHP and upon depolarising current injection elicited non-adapting spikes at high frequency (Fig. 5.4Aii, n = 6). Following characterisation of FS cells, in a second series of experiments I applied zolpidem at 10 nM during recordings of spontaneous inhibitory postsynaptic currents made in voltage-clamp and using internal chloride loading to maximise currents through $GABA_A$Rs. As zolpidem is active at α1 subunit containing $GABA_A$Rs, which are located synaptically and extrasynaptically, I examined phasic responses as well as tonic responses to the drug. As Fig.5.4.Bi & Bii show, application of 10 nM zolpidem had no effect on the distribution of inter-event intervals (IEI) between sIPSCs (control IEI 221.9 ± 3.4 ms in control versus 246.0 ± 2.9 ms in zolpidem; $P > 0.05$ Kolmogorov-Smirnov test, n = 6 recordings). Similarly, no significant effect on amplitude was found (control amplitude 58.00 ± 1.05 pA in control versus 53.08 ± 1.1 pA in zolpidem; $P > 0.05$ Kolmogorov-Smirnov test, n=6 recordings).

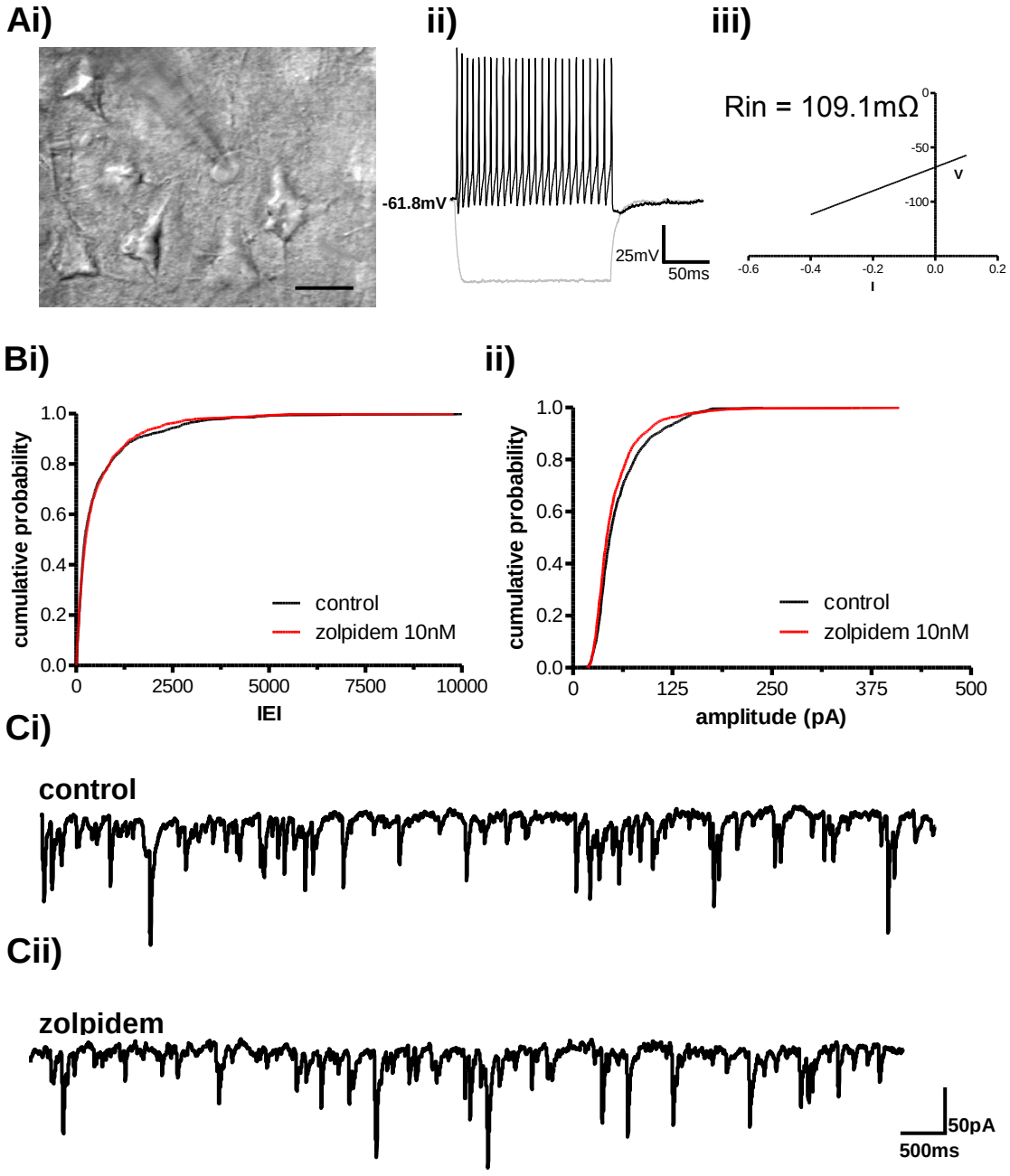


Figure 5-4 Zolpidem (10nM) has no effect on phasic inhibitory activity in FS interneurones. Ai) Photo-micrograph showing recording electrode and putative FS cell close proximity to Betz cells in LV of M1. Aii) Voltage traces of the FS cell identified in Ai), in response to -0.4 nA and + 0.1 nA current steps. Note the high frequency (>100 Hz) of short duration action potentials and large AHP. Aiii) Current (I) versus voltage (V) plot and calculated input resistance. Bi) Cumulative probability plot showing change in the interevent interval (IEI) of sIPSCs with the addition of zolpidem (10 nM, mean IEI 221.9 ± 3.4 ms vs. 246.2 ± 2.9 ms, P > 0.05). Bii) Cumulative probability plot showing there was no siginificant difference to sIPSC amplitude with the addition of zolpidem (10 nM, mean amplitude 58.00 ± 1.05 pA vs. 53.08 ± 1.1 pA, P > 0.05). Ci) Representative traces from a single experiment showing sIPSCs in control and Cii) zolpidem (10 nM).

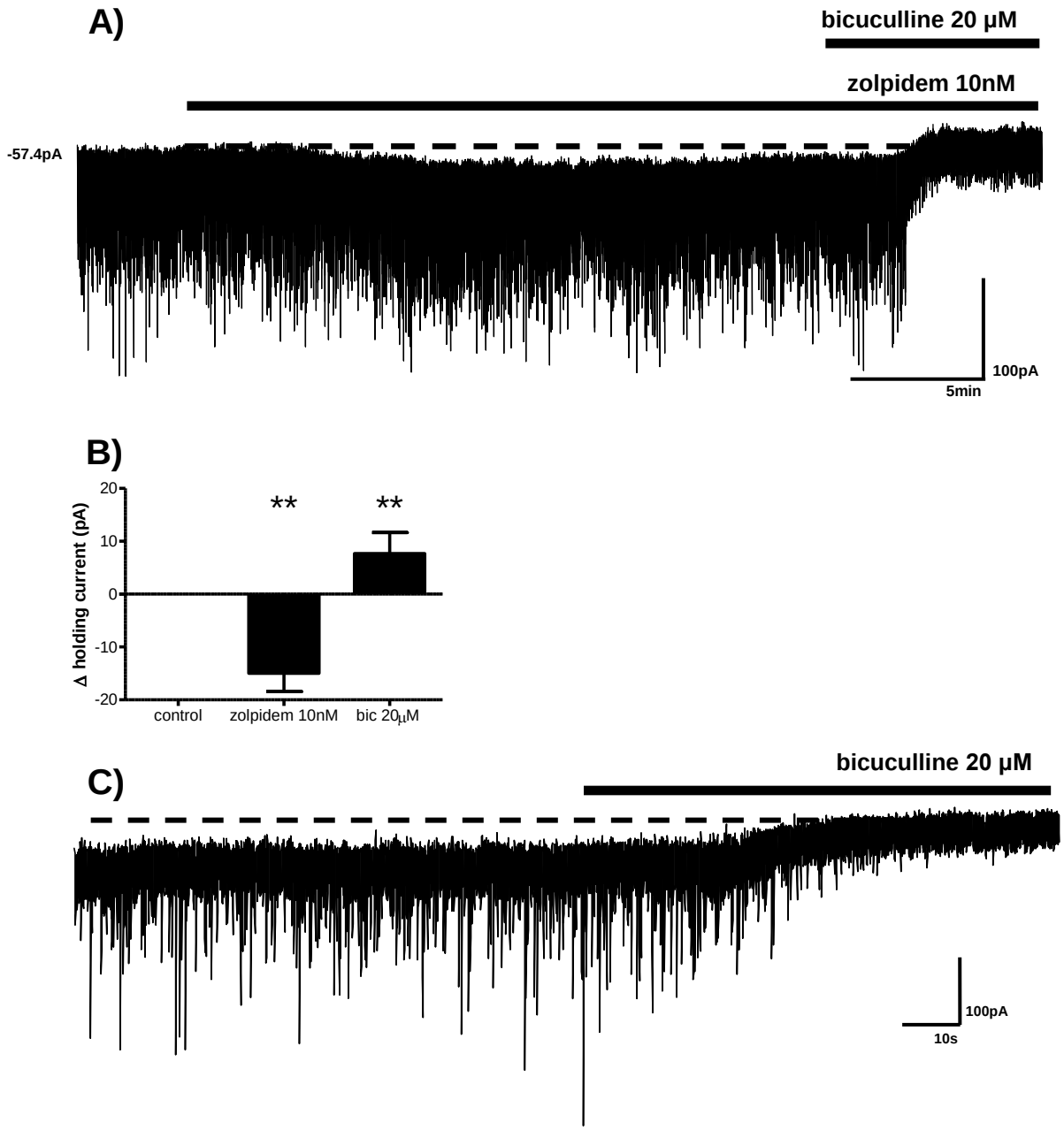


Figure 5-5 Zolpidem (10nM) causes an increase in tonic inhibitory current in FS interneurones. A) Representative voltage clamp recording from a FS interneurone at a holding potential of -70mV (I = -57.4 pA). Inward deflections show numerous phasic sIPSCs. Addition of zolpidem (10 nM, at time point marked by black bar) induced an inward current (14.98 ± 3.4 pA), which stabilised after ~10 minutes. Subsequent addition of bicuculline (20µM) abolished both phasic IPSCs and zolpidem-induced tonic current. However, the bicuculline-sensitive current is larger than the zolpidem-induced current, evidence for a constitutively active tonic current in FS interneurones. B) Bar chart showing change in holding current induced by zolpidem (mean amplitude of inward current 14.98 ± 3.4 pA, n = 6, P < 0.001) and bicuculline (22.66 ± 3.55 pA, P < 0.001, n = 6). C) Addition of bicuculline alone (20 µM) uncovered a constitutively active tonic current in FS interneurones.

Given that I could find no effect of 10 nM zolpidem on phasic inhibition impinging on GABAergic neurones in M1, I analysed the effects of zolpidem application on tonic inhibition. As Fig.5.5A shows application of 10 nM zolpidem generated a slow, inward current (mean amplitude 14.98 ± 3.4 pA, n = 6 recordings), suggesting augmentation of a tonic $GABA_AR$ mediated conductance. This was confirmed when I applied bicuculline (20 μM), which abolished both phasic and tonic components of inhibitory activity in FS cells (Fig. 5.5A & C).

The effects of bicuculline on I_{tonic} suggested that this form of inhibition was constitutively active in the sagittal M1 slice (Fig.5.5C). I investigated the effects of the BZD site antagonist flumazenil on both phasic and tonic inhibition in FS cells in deep M1. As Fig.5.6Bi & Bii show, flumazenil (500 nM) had no effect on phasic inhibition onto FS cells. In 6 recordings, control IEI was 416.5 ± 14.7 ms in control versus 488.5 ± 19.6 ms in flumazenil (P > 0.05 Kolmogorov-Smirnov test). Similarly, sIPSC amplitude was 81.8 ± 1.4 pA under control conditions versus 80.01 ± 1.4 pA in flumazenil (P > 0.05 Kolmogorov-Smirnov test, n=6 recordings). When I investigated the effects of flumazenil alone (Fig. 5.7A), I_{tonic} was decreased (mean change in holding current 31.3 ± 13.9 pA), suggesting that tonic inhibition is constitutively active in FS cells in deep M1.

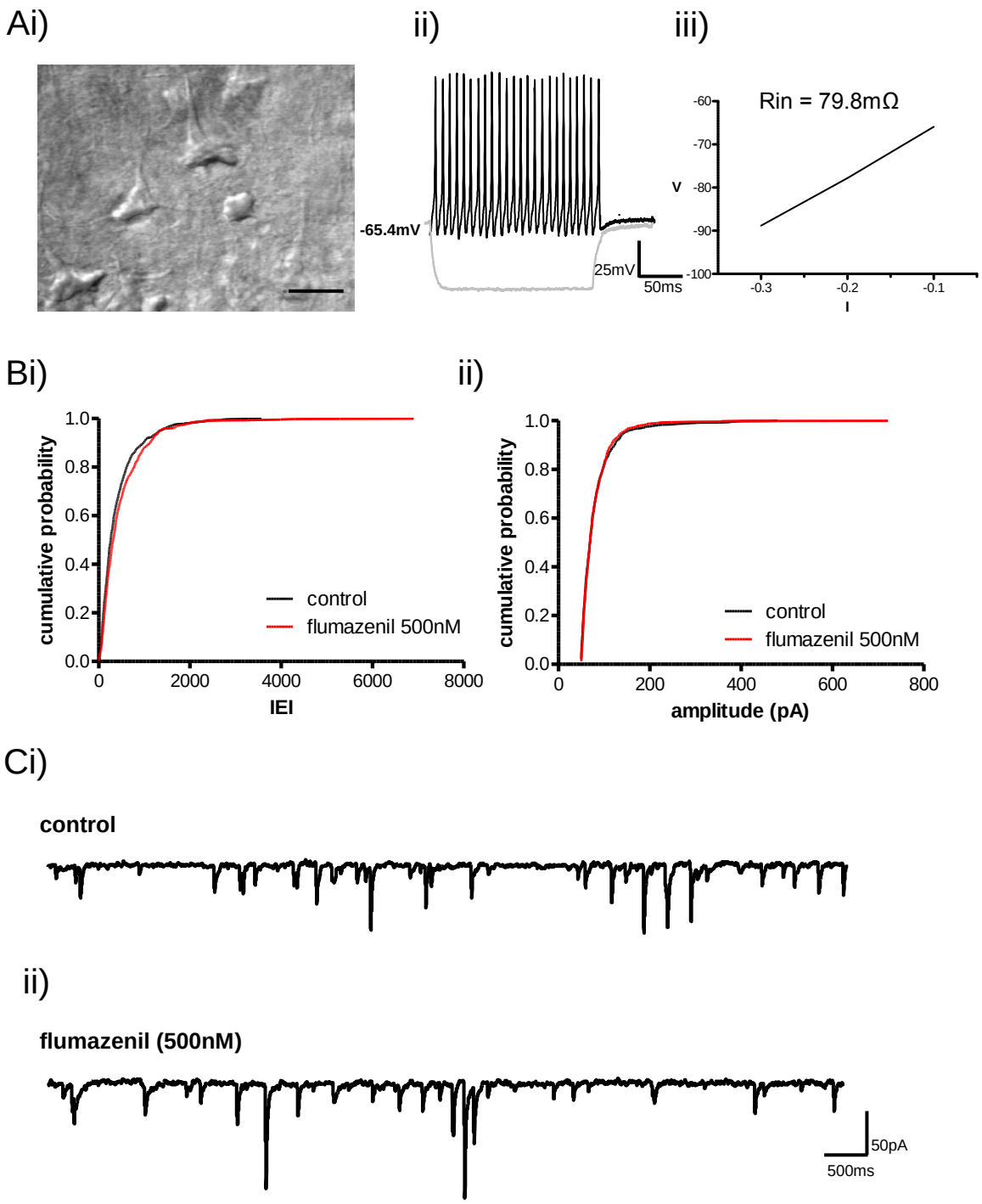


Figure 5-6 Flumazenil (500nM) has no effect on phasic inhibitory activity in FS interneurones. Ai) Photo-micrograph showing recording electrode and putative FS interneurone in close proximity to Betz cells in LV of M1. Aii) Voltage traces of the FS cell identified in Ai), in response to -0.4 nA and + 0.1 nA current steps. Aiii) Current (I) versus voltage (V) plot and calculated input resistance. Bi) Cumulative probability plot showing there was no significant change to the frequency sIPSCs with the addition of flumazenil (500 nM, mean IEI 416.5 ± 14.7 vs. 488.5 ± 19.6ms, P > 0.05). Bii) Cumulative probability plot showing there was no significant difference in sIPSC amplitude with the addition of flumazenil (500 nM, mean amplitude 81.8 ± 1.4 vs. 80.01 ± 1.4 pA, P > 0.05). Ci) Representative traces from a single experiment showing sIPSCs in control and Cii) flumazenil (500 nM).

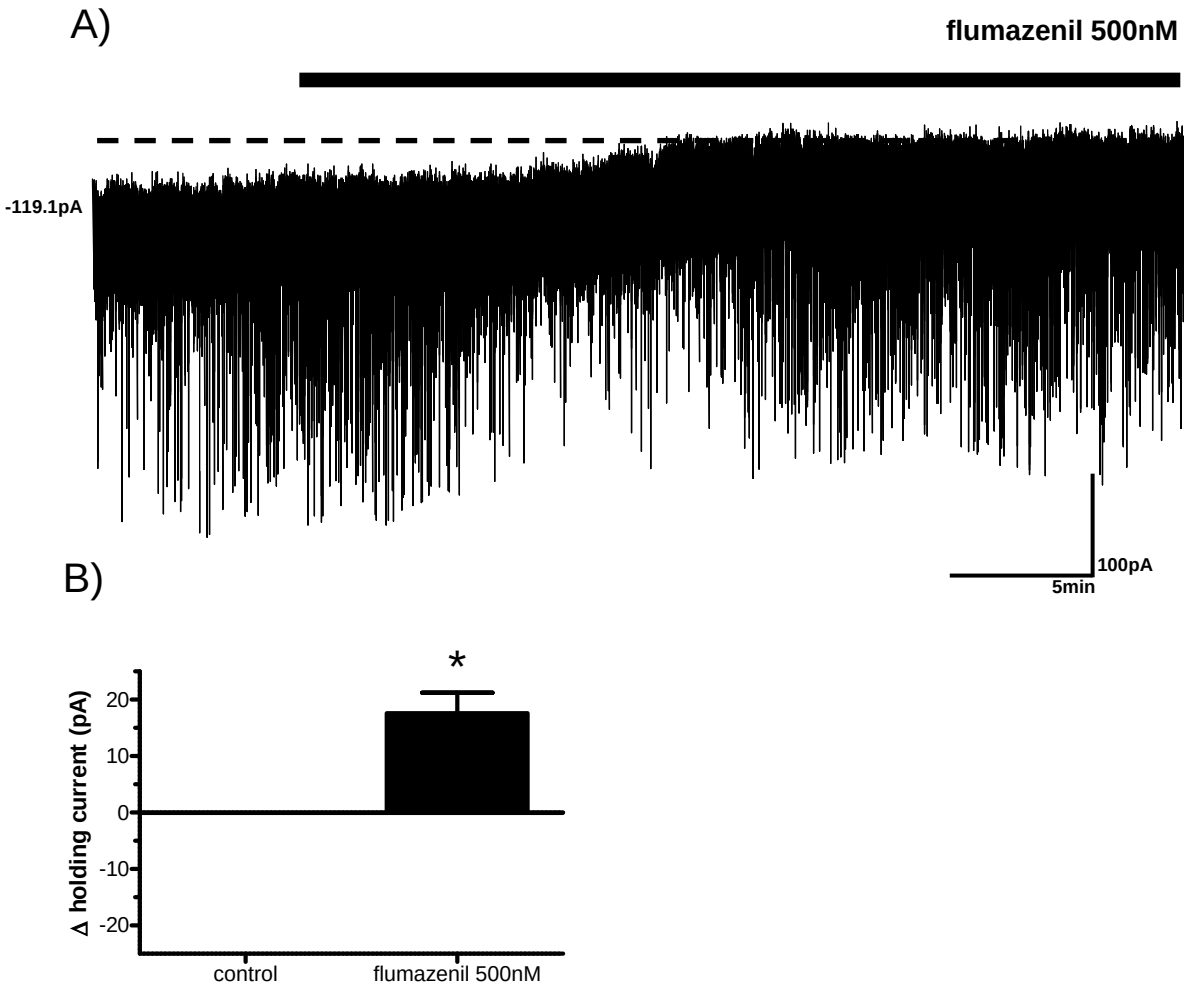


Figure 5-7 Flumazenil (500nM) causes a decrease in tonic inhibitory activity in FS interneurones. A) Representative voltage-clamp recording from an FS interneurone at a holding potential of – 70 mV (I = -119.11 pA). Addition of flumazenil (500 nM) induced a slow outward current. B) Bar chart showing the change in holding current upon addition of flumazenil (500 nM, 22.3 ± 13.9 pA, $P < 0.05$).

If the desynchronising effect of zolpidem on beta oscillations was mediated via the tonic current in FS cells, then I may expect zolpidem induced augmentation of I_{tonic} to be blocked by flumazenil. When I applied zolpidem (10 nM) in the presence of flumazenil, no significant change in I_{tonic} was seen (Fig. 5.8A), indicating that alterations in I_{tonic} were indeed due to activation of the BZD site (I_{tonic} amplitude 101.3% of the value in flumazenil (n = 3, $P > 0.05$ Student's t-test) and suggesting strongly that I_{tonic} modulation underlies the effects of zolpidem on network oscillations. These data indicate that at 10 nM, zolpidem acts to desynchronise beta-gamma rhythms in M1, via the BZD binding site of $GABA_A$Rs containing the α1 subunit.

If zolpidem (10 nM) increases I_{tonic}, then perhaps higher doses of zolpidem (30 nM) decrease I_{tonic} and thus reconciling the paradoxical effect seen in the dose response experiments. Addition of zolpidem (30 nM) induced a slow outward current in FS interneurones. No significant change to the IEI (440.9 ± 19.98 ms vs. 437.7 ± 21.27 ms, n = 6, $P > 0.05$) and amplitude (62.76 ± 2.26 pA vs. 58.88 ± 1.65 pA, n = 6, $P > 0.05$) of sIPSCs was observed. I_{tonic} was significantly decreased (-47.7 pA, n = 6, $P < 0.05$).

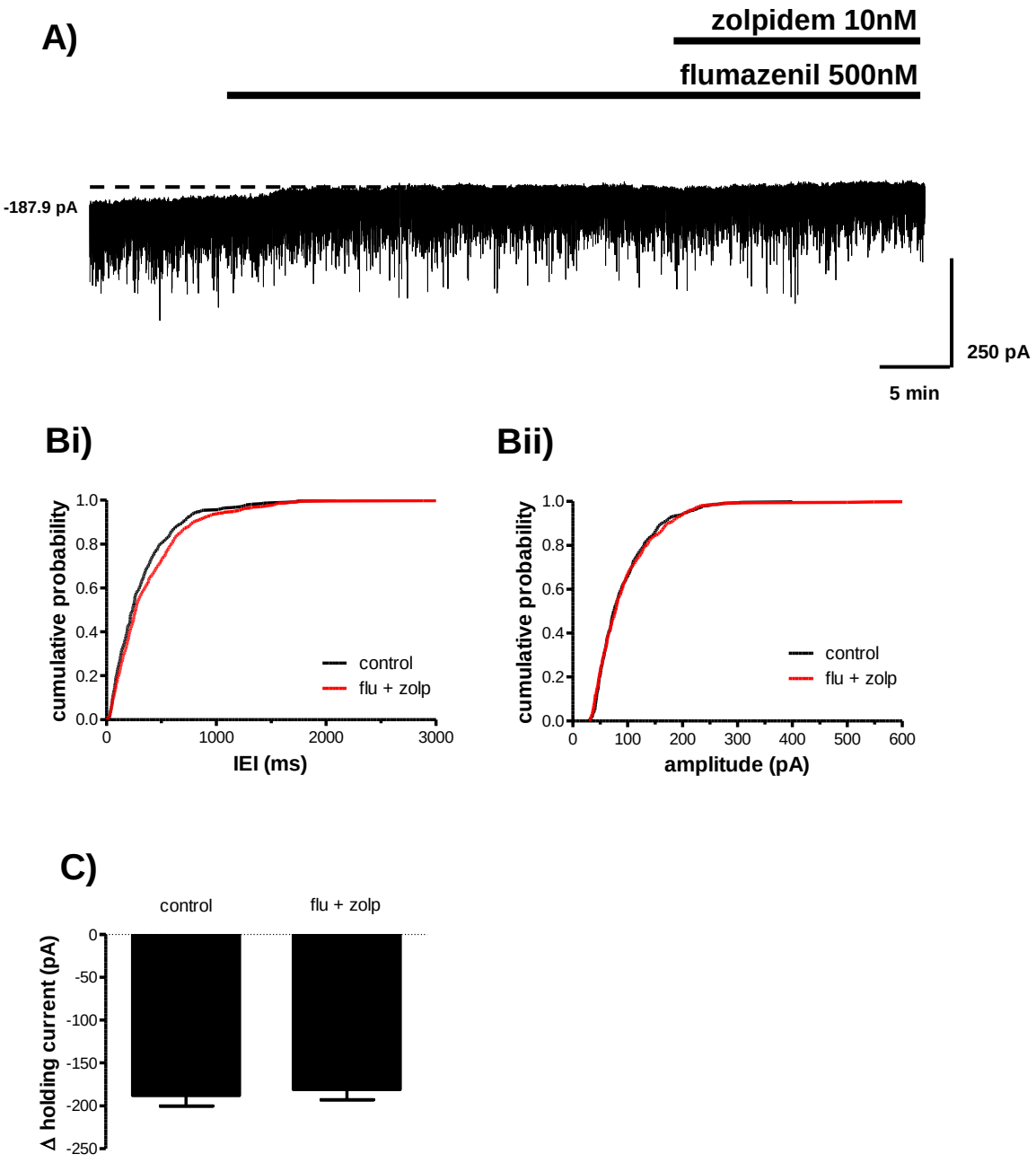


Figure 5-8 Flumazenil blocks the zolpidem-induced tonic current in FS interneurones. A) Representative voltage-clamp recording from an FS interneurone at a holding potential of –70 mV (I= -187.9 pA). Addition of flumazenil (500 nM) induced a slow outward current as previously described. Addition of zolpidem (10 nM) 20 minutes later showed no change to the tonic current. Cumulative probability plots showing no significant change to the IEI Bi) and amplitude Bii) sIPSCs (IEI 326.4 ± 13.79 ms vs. 386.8 ± 17.06 ms, amplitude 93.01 ± 2.25 pA vs. 95.47 ± 2.81 pA, *P* > 0.05). Ci) Bar chart showing no significant effect of zolpidem in the presence of flumazenil.

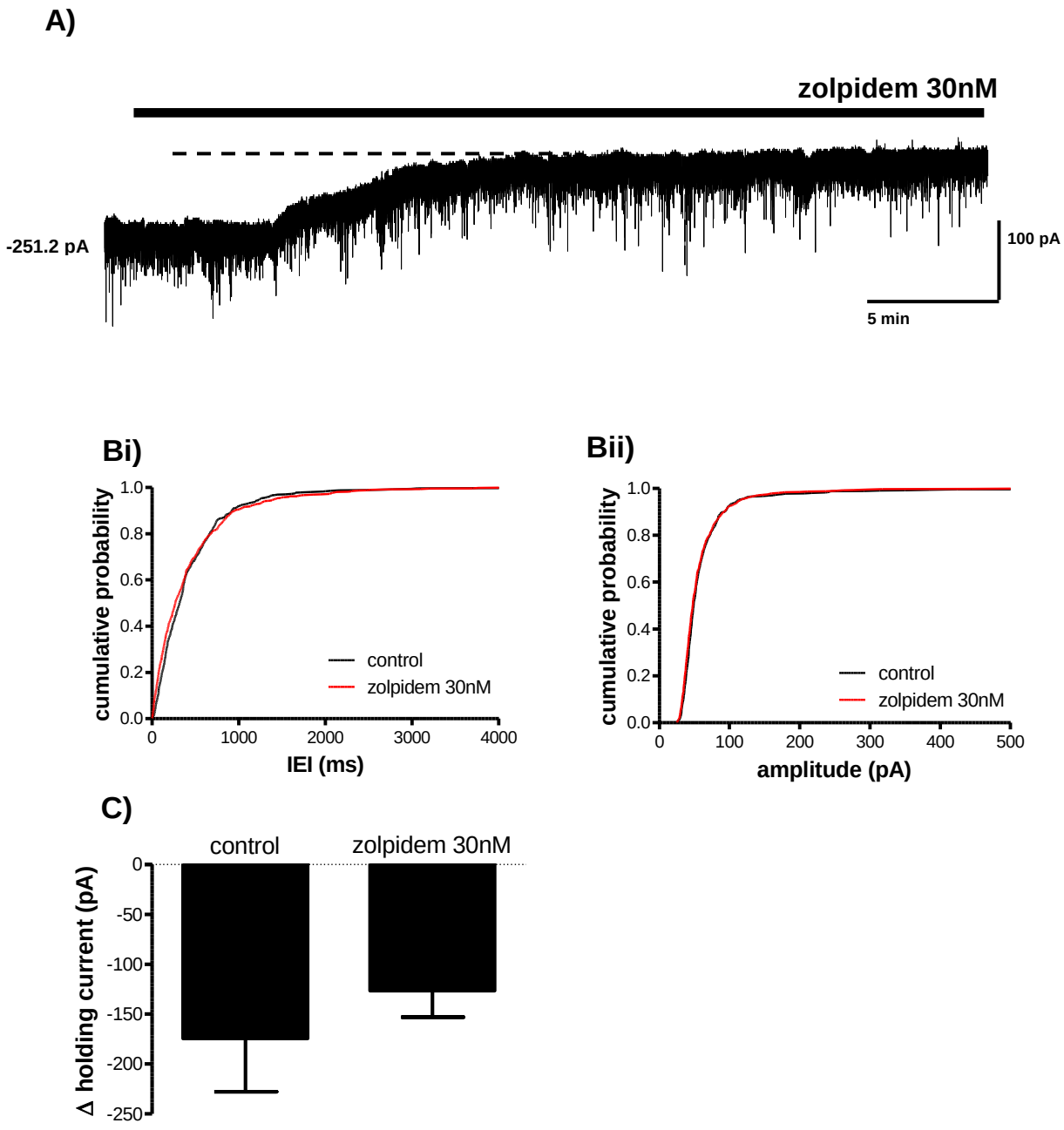


Figure 5-9 Zolpidem (30 nM) decreases tonic current in FS interneurones. A) Representative voltage-clamp recording from an FS interneurone at a holding potential of –70 mV (I= -251.2 pA). Addition of zolpidem (30 nM) induced a slow outward current. Cumulative probability plots showing no significant change to the IEI Bi) and amplitude Bii) sIPSCs (IEI 440.9 ± 19.98 ms vs. 437.7 ± 21.27 ms, amplitude 62.76 ± 2.26 pA vs. 58.88 ± 1.65 pA, $P > 0.05$). Ci) Bar chart showing decrease in I_{tonic} with addition of zolpidem (30 nM).

5.3 Discussion

5.3.1 Overview

I have shown in this chapter that a tonic inhibitory current (I_{tonic}), is increased by activation of α1-subunit containing $GABA_ARs$ by low doses of zolpidem, on putative FS interneurones, consistent with a decrease in oscillatory network activity in the primary motor cortex (M1) *in vitro*. The primary motor cortex (M1) plays an important role in the execution of voluntary movement and is associated with neuronal oscillatory activity at beta (15-30 Hz) frequencies. Here I have shown that these beta oscillations reflect GABA network activity and can be modulated by BZD site ligands. Low doses of zolpidem have been shown to decrease oscillatory power, and through further investigation using whole-cell recordings, I have shown that this desynchronisation may occur via an increase in I_{tonic}. Application of bicuculline and the BZD site partial agonist flumazenil blocks this increase in I_{tonic} and also reveals a constitutively active tonic current in FS interneurones.

5.3.2 Role of Inhibitory Interneurones

In the neocortex, inhibitory GABAergic interneurones make up a small percentage (~25 %) of the entire neuronal population (Benardo and Wong, 1995), yet their activity is crucial for cortical function. Interneurones provide the cortical feedforward and feedback inhibition that is necessary to shape the several types of cortical oscillation that underlie various brain functions (McBain and Fishan, 2001). The interneurone cell types in neocortex have been described many times (Kawaguchi and Kubota, 1997; Cauli et al., 1997), as well as those of the hippocampus (Klausberger et al., 2003, 2004, Freund and Buzsaki, 1996, Somogyi and Klausberger, 2005) and can be differentiated on the basis of their electrophysiological characteristics, expression of calcium-binding proteins, and neuropeptides, as well as axonal and dendritic arborisation morphologies (Kawaguchi and Kubota, 1993, 1997, 1998; Cauli et al., 1997; Gupta et al., 2000, Karagiannis et al., 2009). Different classes of interneurones have been shown to form synaptic contacts on different domains of Pyramidal cells in both the neocortex (Deuchars and Thomson, 1995; Thomson et al., 1996; Tamas et al., 1997) and

hippocampus (Halasy et al., 1996; Miles et al., 1996; Klausberger et al., 2003, 2005), suggesting that each interneurone type has a distinct inhibitory role.

Work here has identified three cell types that appear to be in agreement with these studies. RSNP cells were identified most regularly; indeed, RSNP cells appear to be the most widespread interneurone within the frontal cortex (Kawaguchi, 1995; Cauli et al., 1997). Low threshold spiking (LTS) cells were also regularly encountered, and it has been postulated that this cell type could in fact be a subgroup of RSNP cells (Cauli et al., 1997). Other cells types, such as the late spiking (LS) cell (Kawaguchi, 1995; Brecht et al., 2004) and the irregular spiking (IS) cell (Cauli et al., 1997) were not encountered, but this may be due to the low sample group compared to other studies, which focused on the identification of cells. Morphological identification and synaptic targeting were not studied in these experiments.

In sensorimotor cortex, FS cell terminals have been shown to preferentially target the perisomatic region of postsynaptic Pyramidal cells (Kawaguchi and Kubota, 1997; Cauli et al., 1997). Perisomatic targeting interneurones are thought to be responsible for regulating the overall output of the Pyramidal cells (Freund and Katona, 2007), as they induce IPSPs with faster kinetics and greater amplitude than those induced by dendrite-targeting interneurones (Miles et al., 1996). Furthermore, perisomatic-targeting interneurones were reported to have larger synaptic terminals more closely spaced around the soma and have the ability to generate simultaneous IPSPs at different postsynaptic cells in the absence of excitatory synaptic inputs (Miles, 1990; Freund and Katona, 2007), whilst dendritic targeting terminals were distant from the soma and often contacted different postsynaptic dendrites (Miles et al., 1996). It has been suggested that FS, perisomatic-targeting cells are likely to be responsible for recruiting and pacing the activity of networks of neurones in deep M1 (Yamawaki et al., submitted). Indeed, rapidly decaying IPSCs, as are characteristic of FS cells, are thought to be crucial for network oscillation coherence and frequency control (Bartos et al., 2001, 2002). FS cells are characterised by a repetitive discharge of action potentials whose duration is significantly shorter compared to that of Pyramidal cells. The maximum “steady state” firing rate of a FS cell, as determined from the number of spikes at the highest current strength before depolarisation block, can reach 100 Hz (Erisir et al., 1999; Tateno et al., 2004). Fast spiking behaviour can be achieved

because of the characteristic monophasic, short duration AHP. These cells preferentially form synaptic connections at perisomatic and proximal dendritic regions of neighbouring Pyramidal cells and are thought to exert powerful control over large assemblies of target cells (Cobb et al., 1995; Tamas et al., 1997) by precisely timed phasic synaptic inhibition. FS cells also form electrically coupled pairs (Galarreta and Hestrin, 1999, Gibson et al., 1999). A network of coupled FS interneurones, whose firing is synchronised by this electrical coupling, could co-ordinate activity across the cortex (Thomson, 2000, Traub et al., 2001) and over distance (Traub et al., 2001). FS cells are also known to preferentially express $GABA_AR$ α1 subunits over other interneurone types, such as the LTS cell (Bacci et al., 2003).

The contribution of other interneurone types to network activity is relatively understudied compared to that of FS cells, however, it has been suggested that the large number of interneuronal subtypes in the cortex is due to their distinct roles in shaping network activity (Klausberger et al., 2003). Klausberger et al., (2003, 2004) have shown that different types of interneurones in CA1 fired at different phases of the theta oscillation (4-8 Hz) and sharp wave ripples (120-200 Hz) in rats under urethane anaesthesia. Indeed, recent evidence suggests that a balance of perisomatic and dendritic inhibition is essential in maintaining normal cortical rhythmogenesis (Cossart et al., 2001; Szabadics et al., 2001).

5.3.3 Role of the $GABA_AR$ α - 1 subunit

Sedative-hypnotic effects of BZDs in the brain are mediated through $GABA_AR$, and all of the Z-drugs (i.e. zolpidem and zopiclone) are sedative-hypnotic in man through this mechanism. Zolpidem was the first subtype-selective ligand to be used clinically, and when tested on recombinant receptors, zolpidem displayed a high potency at α1-containing $GABA_ARs$ (α1β2γ2, α1β3γ2: K_i=20 nM). Zolpidem had medium potency at α2 and α3 containing $GABA_ARs$ (e.g. α121γ2, α3β1γ2: K_i=400 nM) and was ineffective at α5 subunit containing receptors (α5β3γ2, α5β2γ2: K_i 5000 nM) (Langer et al., 1992; Pritchett & Seeburg, 1990). In 2000, Crestani and colleagues showed unequivocally, using mutated α1 subunits, that zolpidem acted as a sedative exclusively through α1-subunit containing $GABA_ARs$. Direct functional effects of the α1-specific action of zolpidem have been described, for example Thomson *et al*.,

(2000) have shown that IPSPs mediated by FS allocortical interneurones are greatly enhanced in comparison to those mediated by regular spiking (RS) basket cells. These data are confirmed and extended by recent observations that only specific subclasses of inhibitory interneurones make synapses that exhibit α2 subunits within their $GABA_A$Rs (Nyiri et al., 2001). In addition to zolpidem action at α1 subunit containing $GABA_A$Rs, it is now known that γ2 subunits are also critical for zolpidem activity (Cope et al., 2004) and this has been resolved down to the level of a single amino-acid point mutation (Cope et al., 2005). The effects of 10 nM zolpidem appear to be specific to FS cells. Spiking characteristics indicated that the interneurones targeted were indeed FS cells, whilst the α1 subunit has been shown to specifically mediate the effects of zolpidem on sIPSCs in FS cells (Bacci et al., 2003), but not LTS cells. However, the expression of the α1 subunit has been shown to be heterogenous in LTS cells, so an effect of zolpidem on a subgroup of LTS cells cannot be completely ruled out (Bacci et al., 2003, Thomson et al., 2000).

FS cells were most commonly encountered in deep layer M1, in LVa where Betz cells are located, seemingly creating an FS cell – Betz cell pairing. However, this is not an exclusive arrangement and other interneurone subtypes were found in this layer and FS cells found in other layers. FS cells have been shown to specifically modulate oscillation at high frequencies (beta, gamma) (Gulyas et al., 2010), whereas the role of RSNP and LTS interneurones is less well understood, though they may play a role in lower frequency (alpha) oscillations (Gloveli et al., 2005; Vierling-Claassen et al., 2010). This suggests that FS cells are modulating the output frequency of excitatory (Betz) cells in M1.

5.3.4 Role of a tonic inhibitory current in putative fast spiking interneurones

As early as 1997, Eghbali et al., postulated that the major effect of BZDs may not be on synaptic transmission but on tonic inhibition. The extracellular concentration of GABA in the central nervous system is much lower than at the synapse (Lerma et al., 1986), but crucially it is also more stable and it has been proposed that this would produce a tonic background of extrasynaptic $GABA_A$R activity that would oppose excitation.

Tonic inhibition is mediated by $GABA_A$Rs localised outside the synapse with kinetics and pharmacological properties that make them distinct from synaptic receptors (Farrant and Nusser, 2005, Belelli et al., 2009). Extrasynaptic $GABA_A$Rs are thought to most commonly consist of the ∂ and α5 subunits and, indeed, ∂ subunits are exclusively extrasynaptic, however, no $GABA_A$R subunit has been found to have an exclusively synaptic location. One explanation for the presence of a tonic conductance is that GABA or another endogenous $GABA_A$R agonist must be present in the extracellular space at a sufficiently high concentration to cause persistent $GABA_A$R activation.

Recently, CA1 and CA3 Pyramidal cells have shown a residual tonic current in the mice lacking the α5 subunit (Glykys and Mody, 2006; Glykys et al., 2008), suggesting that other subunits could mediate tonic inhibition in these neurones. This residual current may be mediated by $GABA_A$Rs containing α and β subunits, as has been shown in cultured rat hippocampal neurones (Mortensen and Smart, 2006). It has also been shown in *in vitro* cultures and *in vivo*, that α1 subunits are located extrasynaptically in substantial quantities (Nusser et al., 1995; Brunig et al., 2002). Indeed, even the α1β2/3γ2 $GABA_A$R, where zolpidem is known to exert its major effects, is highly enriched in synapses but more receptors overall are found outside than inside synaptic junctions (Nusser et al., 1995). This is notable as the γ2 subunit is known to be required for synaptic clustering (Farrant and Nusser, 2005). Diazepam can also modulate extrasynaptic currents, indicating the need for α and γ subunits (Eghbali et al., 1997; Linquist et al, 2003); however, this has always been attributed to α5 subunit containing $GABA_A$Rs, which are known to be abundantly located extrasynaptically.

Where previous experimental studies have shown an enhancement of oscillations by decreasing tonic inhibition, I have shown an increase in tonic inhibition produced by 10 nM zolpidem on putative FS cells, which was not associated with changes in phasic inhibition. This increase in I_{tonic} correlated well with the reduction in oscillatory activity seen in extracellular experiments, and thus providing evidence for the dampening role of tonic inhibition on network excitability. Tonic $GABA_A$R activity is not generally seen in Pyramidal cells in acute brain slices from adult animals unless

GABA uptake or degradation are blocked (Stell et al., 2003; Stell and Mody, 2002), or GABA receptor affinity is increased (Wisden et al., 2002; Bieda and MacIver, 2004), but not in all cases (Bai et al., 2001; Yamada et al., 2007).

Small but persistent increases in Cl^- conductance can modulate synaptic efficacy and synaptic integration (Bai et al., 2001). Although the amplitude of the inhibitory tonic current is significantly less than synaptic currents, the persistence of the tonic current results in a substantial integrated charge transfer (Bai et al., 2001, Semyanov et al., 2003). The desynchronising effect of zolpidem may also reflect the differential distribution of α1 subunit containing $GABA_AR$ between specific interneurone subtypes sub-serving oscillatory activity (Thomson et al., 2000). Partially blocking tonic GABAergic signaling results in increased excitability of interneurones and an enhancement of GABAergic drive to principal cells (Semyanov et al., 2003). Flumazenil blocked the changes to the tonic current induced by zolpidem, in agreement with Thomson et al., (2000); however, in contrast to this study flumazenil alone had no effect on phasic currents, which could be attributed to differences in concentration. Flumazenil alone did, however, decrease the tonic current constitutively active in these interneurones and this could explain the increases in beta oscillatory power observed in extracellular field experiments. Zolpidem has also been shown previously to disrupt septo-hippocampal theta oscillations (Ujfalussy et al., 2007) and can affect a tonic inhibitory current in interneurones, but not Pyramidal cells in hippocampus (Semyanov et al., 2003). Flumazenil blocked the changes to the tonic current induced by zolpidem, in agreement with Thomson et al., (2000) and this could explain the block of desynchronisation observed in extracellular field experiments when applied with zolpidem. However, in contrast to this study, flumazenil alone had no effect on phasic currents, which could be attributed to differences in concentration. Flumazenil did, however, increase beta oscillatory activity, which could be due to its agonist action at α4 subunit containing $GABA_ARs$ (Whittemore et al, 1996) or its ability to decrease tonic currents, which as mentioned previously, can induce gamma oscillatory activity in hippocampus (Glykys et al., 2008).

The greatest increase in $GABA_AR$ activity by BZDs is shown to occur when low concentrations of GABA activate the receptors (Harris et al., 1995) and it has been

predicted that receptors mediating the tonic current would respond to BZDs differently from receptors activated during synaptic transmission (Bai et al., 2001), which could explain the differences of the dose response seen at varying concentrations of zolpidem. Indeed, it has been shown that pharmacological modification of $GABA_A$Rs is dependent upon the occupancy of the receptor by GABA (Bai et al., 2001) and the state of receptor activation, dissociation and desensitization (Mortensen et al., 2010).

5.3.5 The effects of increasing tonic inhibition on beta oscillatory activity in LV M1

Tonic inhibition has been postulated to have large effects on the firing and excitability of individual neurons (Farrant and Nusser, 2005) and it has been shown that in the CA3 region of the hippocampus from *Gabra5/Gabrd* $^{-/-}$ KO mice displays spontaneous gamma oscillations *in vitro*, and that gamma oscillations can also be induced in slices from WT mice by low concentrations of L-605,788, an α5 benzodiazepine site antagonist (Glykys et al., 2008). However, a small residual tonic inhibition was detected in these KO mice, suggesting non - ∂ containing $GABA_A$Rs do contribute to tonic inhibition. It is known that excitation of the hippocampal CA3, (by CCh and KA) leads to the generation of persistent gamma oscillations in mice and rats (Traub et al., 2004) and it has been shown here in M1, and previously in hippocampus (Glykys et al., 2008; Mann and Mody, 2010) and in modelling studies (Kochubey et al., 2011), that tonic inhibition can have an effect on oscillatory activity. Mann and Mody (2010) demonstrated that reducing tonic inhibition uncovers an NMDAR mediated excitation, whilst Kochubey et al., (2011) demonstrated that increasing tonic inhibition, in the presence of tonic excitation, causes network oscillations to become desynchronised and with further inhibition, network activity can cease altogether.

In most instances, under normal conditions, pharmacological blockade of tonic inhibition selectively enhances the excitability of interneurones, leading to an increase in the frequency of IPSCs in CA1 Pyramidal cells (Semyanov et al., 2003) and also increases power of gamma oscillations and spontaneously occurring oscillations (Glykys et al., 2008). It would thus appear logical that an increase in tonic

inhibition would disrupt oscillatory activity, as seen here. A baseline level of tonic inhibition is observed in FS cells, as uncovered by bicuculline administration, suggesting that some tonic inhibition is required for normal physiological functioning, along with phasic inhibition, but alteration to this has effects on network activity. At the other end of dose response experiments (see chapter 4), at doses of zolpidem above 30nM, a decrease in oscillatory power was seen.

5.3.6 Conclusion

Increasing concentrations of zolpidem have opposing effects on a tonic inhibitory current in FS interneurones in M1. Low doses of zolpidem (10 nM) increase I_{tonic} and appearing to coincide with the desynchronisation of network activity at this concentration. This concentration of zolpidem does not appear to have an affect on phasic sIPSCs, which are the most commonly considered variable when discussing oscillatory network activity. Work here has shown the possible importance of tonic inhibition on network activity in M1. However, due to the limitations of recording oscillatory activity in a submerged chamber, this has not been shown conclusively. However, it is good to speculate that increasing I_{tonic} may disrupt oscillations via a shunting inhibition i.e. by affecting feedback from interneurones and Pyramidal cells within the network and thus the main output of the LFP. In addition, higher doses of zolpidem (30 nM) decrease tonic inhibition and at this concentration oscillatory activity is augmented. Perhaps this is due to an increased ability to recruit more neurones to the network without the dampening effects of a tonic inhibitory current.

Chapter 6 General Discussion and Further Work

Work for this thesis commenced with alterations to the dissection and preparation of acute brain slices. Viability of neuronal networks in primary motor cortex (M1) was enhanced by the addition of neuroprotectants to the sucrose based aCSF (chapter 3), providing acute brain slices that lasted longer and also of which a greater proportion showed oscillatory activity. Previous work in M1 has been hindered by low viability figures. Not only did this new aCSF enhance viability, it is also more biologically relevant (Hajos and Mody, 2009), incorporating endogenous neuromodulators that would normally be washed away during the preparatory method. Keeping these neuromodulators at levels found *in vivo* might be expected to maintain neuronal signalling patterns that would be seen in the intact brain. Indeed, with this new aCSF solution, the concentrations of exitants (Kainic acid, KA and carbachol, CCh) required to evoke oscillatory activity was significantly reduced. Whether this solution would enhance viability in other brain regions would require further testing, however, experiments here suggest it is also relevant for work in primary somatosensory cortex (S1), as oscillatory activity was also more readily observed when dual recordings were conducted.

What mechanisms underlie modulation of beta oscillatory activity by benzodiazepine site ligands in M1? To address this question I first investigated the effects of numerous ligands on extracellular field potentials. Application of KA and CCh generated persistent synchronous beta frequency network activity in M1, consistent with *in vivo* observations (Murthy and Fetz, 1992). $GABA_AR$ modulation was also shown to be essential for oscillatory activity in M1 by application of gabazine and tiagabine. The significance of subtype specific $GABA_AR$ modulation soon became apparent (chapter 4). Recording of LFPs uncovered a paradoxical effect of zolpidem on beta oscillatory activity. Low doses of zolpidem (10 nM) desynchronised oscillatory activity, whereas higher doses (30 nM and 100 nM) augmented oscillatory activity. These effects were not specific to zolpidem either. Although different dose response profiles were obtained for zolpidem and zopiclone, and indeed, the desynchronising effect of zolpidem at very high doses (500 nM) does appear to be α1 specific, 10 nM of both drugs caused a desynchronisation of beta oscillatory activity. A dose response obtained for CL 218,872, an α1-subunit specific drug, confirmed these observations. If the desynchronising effects at low doses are not specific to

zolpidem, what is causing these effects? Uncovering a tonic current that increased in response to 10 nM zolpidem appears to provide the answer (chapter 5).

Tonic inhibition has recently been shown to affect oscillatory activity *in vitro* (Glykys et al., 2008; Mann and Mody, 2010). However, historically, tonic inhibition has been associated with the δ subunit of the $GABA_AR$ (Farrant and Nusser, 2005). The effect of zolpidem on tonic currents here and in previous studies (Semyanov et al., 2003; Song et al., 2011) suggests that the α1 and γ subunits must also have a role in controlling tonic inhibition. Indeed, for zolpidem to exert its effects the γ subunit is required (Cope et al., 2004), and this cannot be co-expressed with the δ subunit. As early as 1997, Eghbali et al., postulated that the major effect of benzodiazepines may not be on synaptic transmission but on tonic inhibition and this does appear to be the mechanism, at least in M1, by which zolpidem desynchronises oscillatory activity. However, the role of phasic inhibition cannot be ruled out. It is well known that the frequency of oscillations within a mutually inhibiting interneurone network is dependent on three factors; the excitatory driving current to the network, the $GABA_A$ decay time constant ($T_{GABA(A)}$) and the amplitude of unitary hyperpolarising $GABA_AR$ mediated conductance ($g_{GABA(A)}$) (Traub et al., 1996; Jeffreys et al., 1996; Traub et al., 1999) and beta oscillations in M1 have been likened to an ING type mechanism (Yamawaki et al., 2008). The persistence of these oscillations, sometimes seen for hours, also makes them mechanistically similar the persistant gamma oscillations described by Fisahn et al., (1998). Here it appears likely that the increase in amplitude of oscillatory activity with high concentrations of zolpidem (30 nM and 100 nM) is due to its action on phasic inhibition.

The results presented in chapters 4 and 5 indicate how modulation of oscillatory activity in M1 by zolpidem may alleviate the symptoms of PD and other "oscillopathies". Desynchronisation of pathologically enhanced beta oscillations, as seen in stroke (Hall et al., 2010) has recently been shown *in vivo* to be correlated with an improvement in cognitive and motor functions. However, in contrast to the *in vitro* experiments here, low doses of zopiclone did not result in desynchronisation of these oscillations. This could perhaps be due to discrepancies in drug concentrations or there could be further network mechanisms that are yet to be uncovered. Further

studies with zopiclone should be considered, particularly to see if its effect at low concentrations is also mediated by tonic inhibition.

To mimic the pathologically enhanced beta oscillations PD more closely, recordings obtained from dopamine-depleted animals *in vitro* and *in vivo* should be considered. The beta oscillations observed here in control animals may be mechanistically different from pathological beta oscillations seen in PD.

The tonic inhibition constitutively active in FS interneurones could also indicate how network activity is controlled. FS cells appear to fire in time with the network oscillation, while IPSCs are also occurring in these cells at a similar frequency. Their control is vital for a functioning network (McBain and Fisahn, 2001). Disruption of FS intereurones specifically via an enhancement of tonic inhibition could override, or shunt any excitatory input to the cell from Pyramidal cells during the oscillation thus disrupting the output of the FS cell. In a wider context this could indicate how oscillatory frequencies shift in M1 from beta to gamma and allow motor function (Brown et al., 2001). Indeed, Mann and Mody (2010), have shown that altering the tonic current in hippocampus, alters the frequency of oscillations. In δ subunit knockout mice, the firing frequency of interneurones was increased. The abolition of a tonic current enhanced the interneuronal sensitivity to NMDAR mediated excitation, and thus resulting in the increased frequency observed. Altering the tonic inhibition does not necessarily cause any changes to phasic activity (Glykys and Mody, 2006) that is also seen in the results shown here. FS cells are also known to be potently self-inhibitory and can use autaptic transmission to desynchronise networks (Manseau et al., 2010). FS interneurones have been shown to filter network activity by switching between two modes of GABA release at their autaptic and synaptic nerve terminals, allowing them to break synchrony when necessary (ie to prevent epileptic discharges). This cannot be ruled out as a mechanism and should be considered with the results of tonic inhibitory activity in FS interneurones seen here.

Anatomical studies indicate that sensory input to motor cortex originates from somatosensory cortex and thalamus (Miyachi et al., 2005). Results here, in a sensorimotor slice (Rocco et al., 2007), indicate that zolpidem can also desynchronise activity in S1, and also, that this desynchronisation precedes that of

M1. Does this mean that reciprocal activity been M1 and S1 results in coupled desynchronising abilities? Is this desynchronisation always coupled? Further work using disconnected areas may be appropriate, but studies using a stimulation paradigm may provide results as to whether S1 is causing the desynchronisation seen in M1. It seems unlikely, however, that the two cortices are always coupled.

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Appendix 1

A1.1 Introduction

Prior to the discovery of kainate receptor (KAR), fast neurotransmission was thought to be mediated via NMDA, AMPA and GABAARs, however, KARs are found throughout the brain and KAR-mediated synaptic responses have been shown in interneurones (Cossart et al., 1998, Frerking et al., 1998, Semyanov and Kullman, 2001, Fisahn et al,. 2004, Fisahn, 2005). Network pathways terminate on glutamatergic pyramdidal cells and local inhibitory GABAergic interneurones. Excitatory and inhibitory responses are elicited in Pyramidal cells by activation of these pathways. The output of these Pyramidal cells is controlled by the interaction between excitatory and inhibitory synaptic inputs, both to the Pyramidal cells and to the local interneurones. KAR mediated responses have been found to be vital for oscillation generation in various cortical areas (Fisahn et al., 1998; Buhl et al., 1998; Cunningham et al., 2003; Yamawaki et al., 2008) but their powerful effects on network activity mean they are also implicated in epileptiform activity (Ben-Ari, 1985; Ben - Ari and Cossart, 2000; Fisahn et al., 2004; Fisahn, 2005).

The kainate receptor (KAR) family consists of five glutamate receptor subunits; GluR5, GluR6, GluR7 (also known as GluK1-3), which can form homomeric functional channels (Hollmann and Heinemann, 1994; Pinheiro and Mulle 2006), and KA1 and KA2 (also known as GluK4 and 5), which can combine with GluR5-7 to create a heteromeric very high affinity receptor (Chittajallu et al., 1999). KAR are tetrameric structures, which span the lipid bilayer, forming three transmembrane domains, an extracellular N-terminal, a P–loop that forms part of the pore and an intracellular C–terminal. GluR5 and GluR6 form the ligand-binding domain. KARs have a variety of expression levels dependent upon brain region, which consist of different subunits with distinct pharmacological and electrophysiological properties. KARs are permeable to cations and show rapid activation and densensitisation properties.

Activation of KARs with submicromolar concentrations of KA increases excitability of neurons and enhances release of glutamate or GABA. It has been shown that at inhibitory synapses KA depolarises interneurones leading to continuous neuronal firing, and thus increasing the frequency of spontaneous (s)IPSCs on Pyramidal

neurons (Cossart et al., 1998; Frerking et al., 1998) and frequency and amplitude on interneurones (Semyanov and Kullman, 2001). Cossart et al., (2001) have also shown a significant increase in AP-independent miniature (m)IPSC frequency by KA, indicating a direct pre-synaptic effect.

At excitatory synapses, nanomolar concentrations of KA increased both sEPSC and mEPSC frequency in Pyramidal cells (Campbell et al., 2007). Campbell et al., (2007) attributed a role for presynaptic KARs in layer 2/3 Pyramidal cells as facilitators of glutamate release and modulators of synaptic transmission. These effects appear to involve GluR5 subunit-containing receptors. Postsynaptic KAR mediated EPSCs also appear to contribute to synaptic activation of Pyramidal cells and thus providing multiple roles for KARs in synaptic transmission in neocortex. However, the contribution of KAR-mediated currents to evoked (e) EPSC amplitude was found to be negligible in layer 5 Pyramidal cells of M1 (Ali, 2003). The pharmacological blockade of glutamate uptake required to observe a KA-mediated current in M1 suggested an extrasynaptic or perisynaptic location of KARs on Pyramidal cells but not FS interneurones.

Acetylcholine (ACh) has many cognitive functions, including the cortical modulation of sensory information (Lucas-Meunier et al., 2003). Muscarinic acetylcholine receptors (mAChRs) are found as two subtypes; M1 like, consisting of the subunits M1, 3 and 5, and M2 like, consisting of the subunits M2 and M4 (Caulfield and Birdsall., 1998). Muscarinic agonists have been shown to facilitate EPSPs and enhance the post-synaptic response to NMDA (Markram and Segal, 1990). Carbachol (CCh) acts primarily via M1 like mAChRs, which cause a membrane depolarization in the postsynaptic cell by inhibiting K^+ conductances; namely the rectifying outward current I_m (McCormick and Prince, 1986), the slow post–hyperpolarisation inducing I_{AHP} (McCormick and Prince, 1986), the voltage dependent K^+ current I_K and the leakage current I_{leak} (Zhang et al., 1992), and an increase in non-specific cation currents; an increase in I_{cat} (Ca^{2+} - dependent non-specific cation current) and I_h (the hyperpolarisation activated current) in Pyramidal cells (Fisahn et al., 2002), both, of which, have been shown vital for oscillation generation (Traub et al., 2000; Fisahn et al., 2002). The mAchR M1 has been found to be important for oscillation generation in the hippocampus (Fisahn et al., 2002). Addition of CCh, a

mAChR agonist, increases glutatmate release, first in GABAergic interneurones, via a fast hyperpolarisation, then in Pyramidal cells, via a slow depolarisation (Lucas–Meunier et al., 2003).

In order to induce network oscillations in M1, CCh was co-applied with KA. The need for both KA and CCh to induce robust oscillatory activity could reflect a reduction in neuronal network excitability in the *in vitro* slice preparation as compared to *in vivo*. In contrast to a number of other brain regions, including the hippocampus (Fisahn et al., 1998), entorhinal cortex (Cunningham et al., 2003), and somatosensory cortex (Roopun et al., 2006), the application of KA or CCh alone does not generate oscillatory activity in M1 (Yamawaki et al., 2008). It is possible that the structural and functional resilience, or indeed the survival, of Pyramidal cells and interneurones in M1 during the acute brain slice preparation may be less compared to other cortical areas. However, Buhl et al., (1998) noted that somatosensory cortex required a similar need for KA and CCh in generating persistent gamma oscillations. In a previous chapter (Chapter 3 – viability), it was noted that after changes to the solutions and rats, the amount of KA and CCh required to induce oscillatory activity had to be reduced significantly, due to the increased occurrence of epileptiform like events in M1 (and sometimes S1). The final concentrations required in the new preparation were 100 nM KA and 5μM CCh. KA concentrations could be used up to 150 nM, as required, but the best network activity was acquired when CCh concentrations were retained at 5 μM. These are just a quarter and a tenth, respectively, of the original concentrations required to elicit oscillatory activity in these and previous studies (Buhl et al., 1998; Yamawaki et al., 2008). Here, I present data and analysis of the epileptiform activity induced by the higher concentrations of KA and CCh in M1.

A1.2 Results

Epileptiform events began to occur in M1 after the aCSF was modified (for further details see chapter 3). In short, slice viability was low in older preparations and in order to increase the number of slices that produced oscillatory activity, neuroprotectants were added to sucrose based aCSF. After these changes were made, a period of experiments was conducted using the original concentrations of KA and CCh: 400 nM and 50 µM respectively. Epileptiform events occurred regularly until changes were made. The concentrations of KA and CCh, originally required to evoke oscillatory activity, now appeared to be causing these epileptiform events. In order to use produce viable slices with no epileptiform events, concentrations of KA and CCh were reduced to 100 nM and 5 µM. Epileptiform events were not evident in older preparations, possibly due to the fact that the interneuronal population was not surviving.

A1.2.1 Characteristics of Epileptiform events

The most common characteristic of the events observed was a large burst followed by an inward deflection of the baseline potential (Fig. 1A&B). However, these bursts were regularly preceded by a train of single spikes and then a quiescent period before the burst itself. Events were relatively short (Fig. 3A, mean 6.5 s, median 6.0 s, range 2–16s, n = 41) and rarely occurred more than once in a recording. This is likely due to the fact that after such an event, any preceding activity in the acute brain slice, whether it was oscillatory or not, was abolished. The bursts themselves were approximately ten times the amplitude of the existing baseline activity. In 2 out of 43 events, activity was not one single burst event but reoccurring bursts throughout the whole recording (Fig. 2A & B). One recording consisted of evenly spaced bursts for over two hours (Fig. 2A) whilst another consisted of a larger burst followed by a series of shorter bursts (Fig. 2B). During these situations, inter-burst activity was particularly silent and the epileptiform events were of similar amplitude to baseline activity.

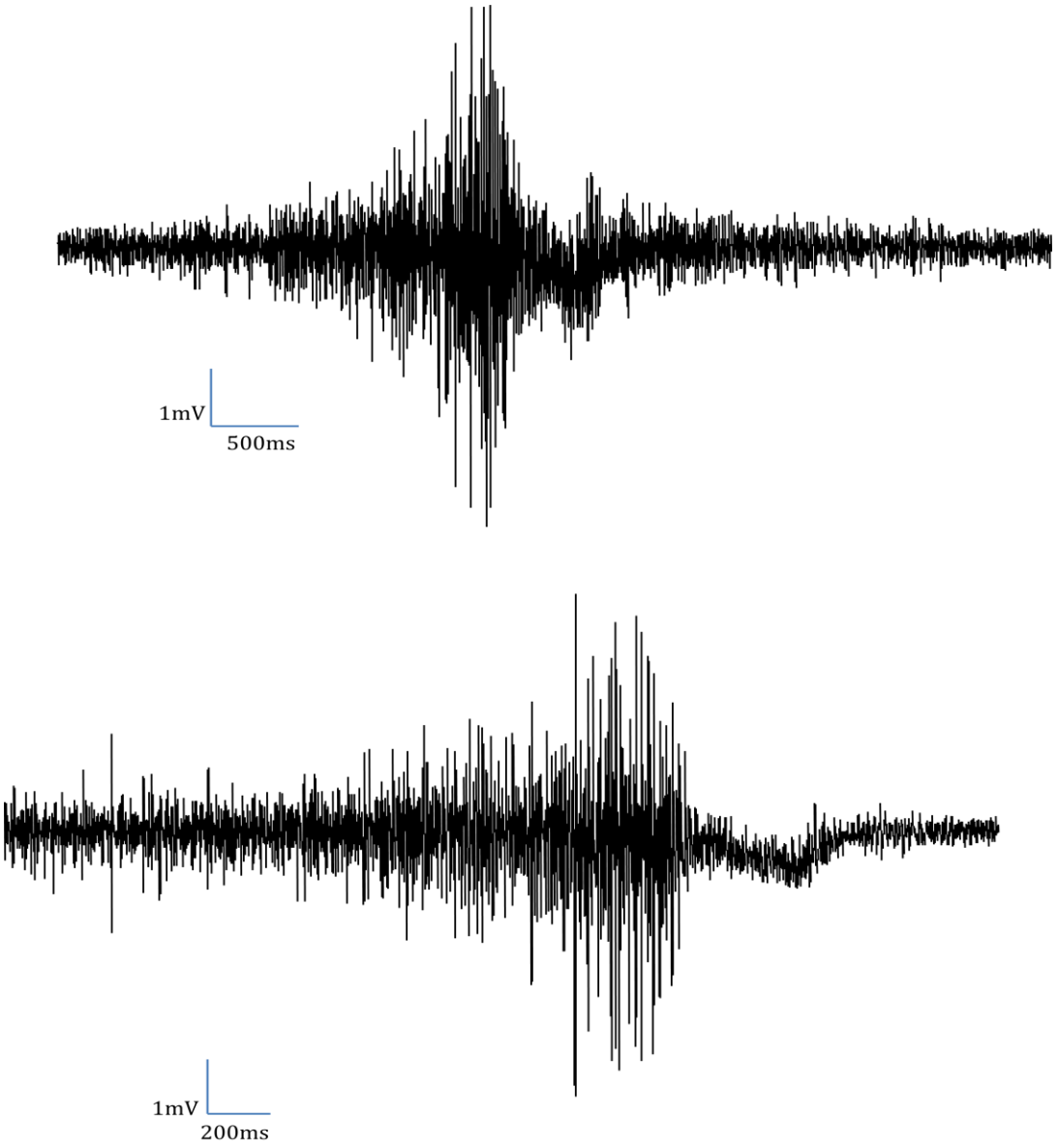


Figure A1.0-1. **Epileptiform events in M1.** Typical epileptiform events encountered in M1 after changing solutions, but not adjusting KA and CCh concentrations. Note the burst followed by an inward deflection of the baseline.

The frequency of the epileptiform events occurring in M1 generally consisted of high gamma (Fig. 3C; 70-100 Hz) with smaller peaks seen at very high frequency ranges. Perhaps due to online low pass filtering, no peaks were detected over 400 Hz. Peaks were extremely powerful in comparison to normal oscillatory events, reaching nearly 15 mV^2 (Fig. 3C), and no activity was detected in the beta/low gamma frequency ranges. No two events were the same, even though similar in appearance. Within the same event a variety of peaks was encountered.

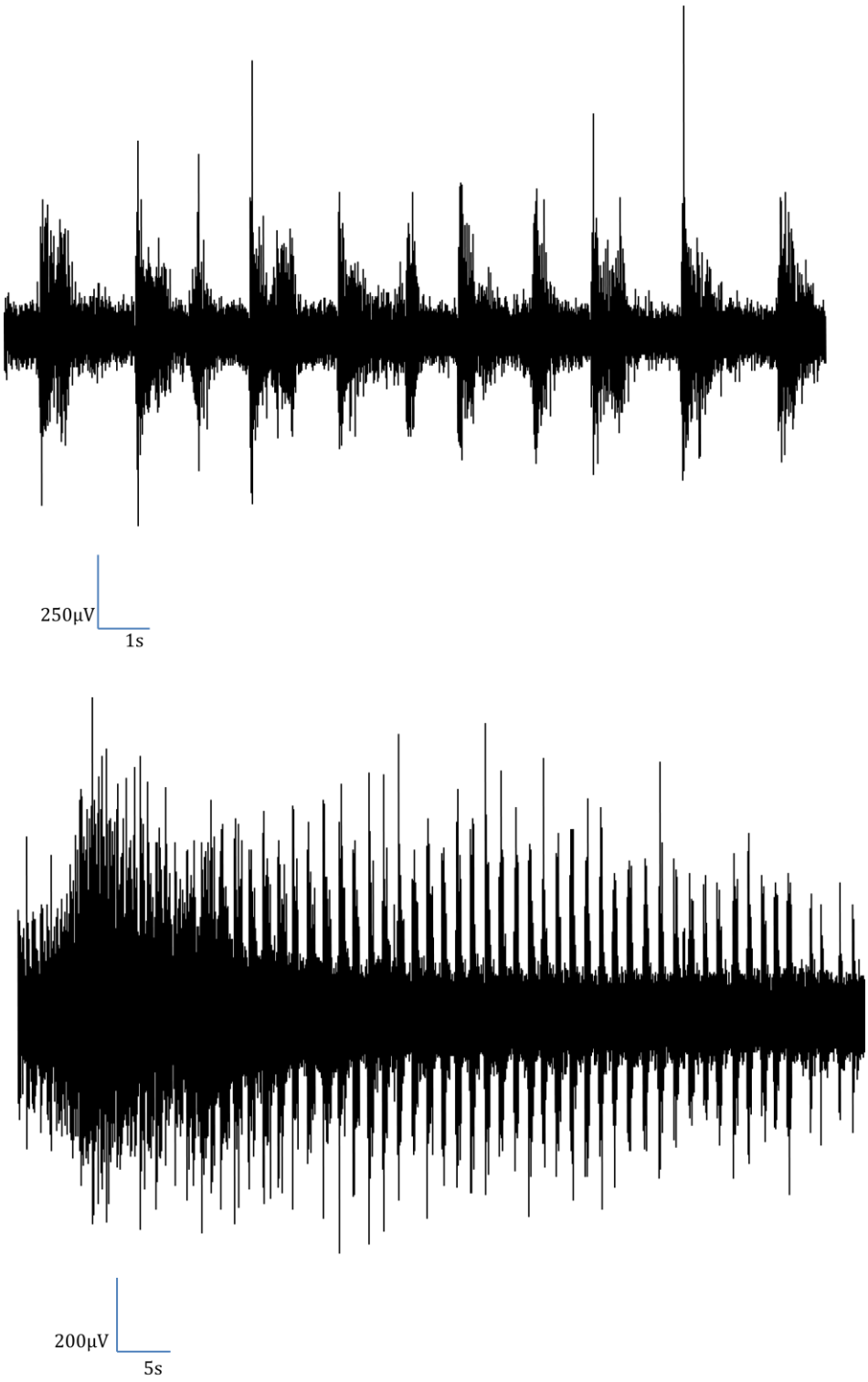


Figure A1.0-2. Other **forms of epileptiform activity encountered in M1.** Whilst most epileptiform events resembled a burst, followed by an inward deflection of the baseline, bursting events were encountered twice. A) Shows one type of bursting event that was encountered throughout the whole recording, while B) shows bursting events after one large preceding burst.

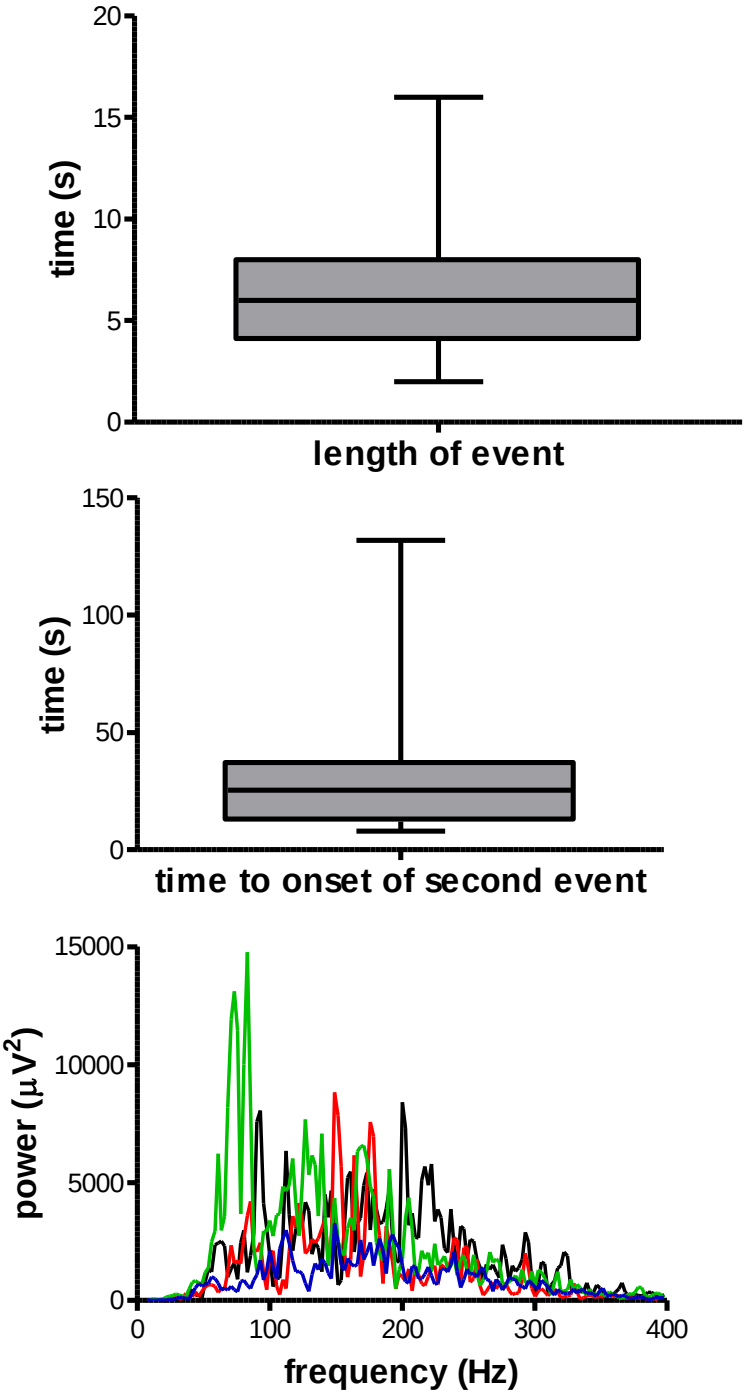


Figure A1.0-3. Epileptiform **events in sensorimotor slices**. (A) Length of the epileptiform events observed in M1. (B) Time to onset of second event when conducting dual recordings in M1 and S1. (C) Power spectrum showing the variety and high frequency of the epileptiform events in four different slices. Notice the variety of peaks and the differences from slice to slice.

A1.2.2 M1 – S1 Dual Recordings

When activity in one acute brain slice was recorded from both M1 and S1, epileptiform events were found to occur in both areas, with S1 events consistently leading that of M1 (Fig. 4). In 7 out of 10 recordings S1 would show an epileptiform event 34.9 seconds on average before M1 (Fig. 3B; mean 34.9 s, median 25.5 s, range 8–132 s). As stated previously, events were relatively short and rarely occurred more than once in a recording. There were no significant differences between the events seen in M1 and S1 (Fig. 4). This is likely due to the fact that after such an event, any preceding activity in the acute brain slice, whether it was oscillatory or not, was abolished.

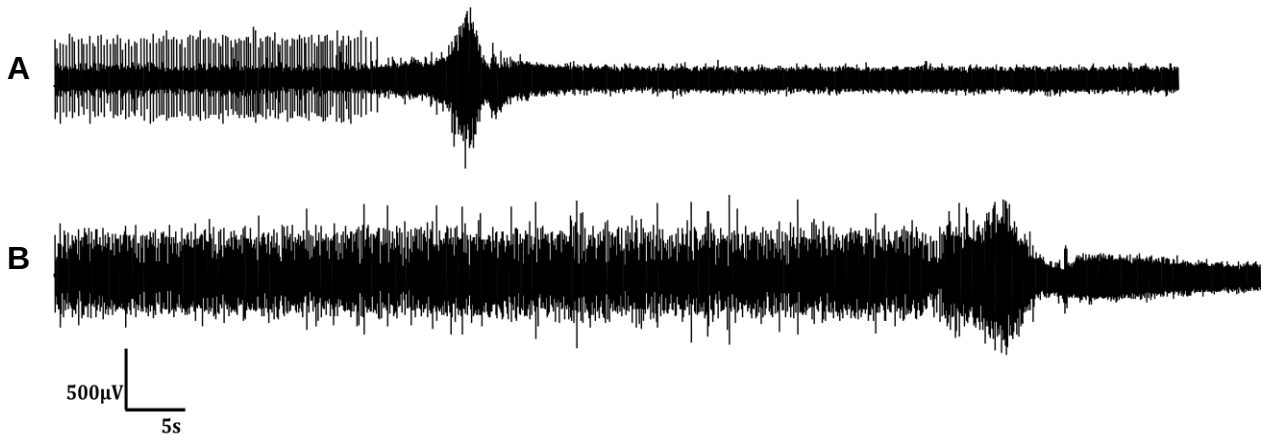


Figure A1.0-4. **Epileptiform events in S1 followed by M1.** (A) S1 and (B) M1 dual recordings show an epileptiform event in S1 followed by M1 ~20s later.

A1.3 Discussion

Here, it has been shown that in sensorimotor slices with increased interneurone and Pyramidal cell viability, concentrations of KA and CCh required in the past to elicit oscillatory activity now caused epileptiform discharges. It is well known that sub-micromolar concentrations of KA generate epileptiform activity in slice preparations in CA3. Pyramidal cells in CA3 have many high affinity binding sites for KA (Monaghan and Cotman, 1982; Bureau et al., 1999) and it has been shown that 100-500 nM KA causes a powerful postsynaptic depolarisation and readily generates spontaneous action potential discharges and bursts (Vincent and Mulle, 2008). A dense network of recurrent glutamatergic collaterals means that Pyramidal cell firing is able to generate synchronous activity that can propagate (Wong et al., 1984; Miles and Wong, 1987). Regulation of inhibition appears to play a strong part in KA induced epileptiform activity, whilst balance of excitation and inhibition has been attributed to KARs (Ali et al., 2001). Also, oscillation generation relies on intact inhibitory neurotransmission (Fisahn et al., 2005; Traub et al., 2000).

KA decreases evoked IPSCs and in parallel markedly increases sIPSCS through somatodendritic KARs in interneurones (Ali et al., 2001). The GluR6 subunit appears to be required for KA induced epileptiform activity and gamma oscillation generation (Fisahn, 2005). Receptor knock out (KO) studies in mice have revealed the differential function of GluR5 and GluR6. GluR6 appears to be vital for normal oscillation generation (Fisahn et al., 2004); while a GluR6 deficient mouse will have less susceptibility to seizures (Ben–Ari and Cossart 2000; Khalilov et al., 2002; Fisahn et al., 2004). A GluR5 KO mouse requires less KA for a comparable oscillation to that seen in a wild type mouse and a further increase in concentration induces epileptiform discharges (Fisahn et al., 2004). GluR6 has also been postulated to preferentially be found on Pyramidal cells, whilst GluR5 is thought to be specific to interneurones (Cossart et al., 1998; Ali et al., 2001). It therefore appears likely that GluR6 is involved in mediating KA induced excitation, whilst GluR5 is involved in setting the level of inhibition within a network. Indeed, Cossart et al., (1998) have shown that GluR6 containing synapses on Pyramidal cells mediate epileptogenic effects, whilst activation of GluR5 containing KAR on interneurones

enhances tonic inhibition, thus providing a regulatory mechanism to prevent epileptogensis. GluR5 has also been shown to promote spontaneous GABA release from interneurons (Semyanov and Kullman, 2001; Fisahn et al., 2004).

Frerking et al., (1998) have associated the generation of epilepsy to an excitotoxic loss of interneurones due to their high affinity expression of KARs. The balance of inhibition and excitation would be disrupted causing disinhibition of Pyramidal cells. This would be caused by the loss of tonic inhibition produced by GluR5 containing KAR on interneurons that are thought to prevent epileptogenesis (Cossart et al., 1998). Buhl et al., (1998) have also shown that a decrease in oscillatory power caused by bicucculline, resulted in epileptiform activity, further showing the need for an inhibitory input to provide stable network activity. In this study, an increase in the viability of slices, along with, relatively, high concentrations of KA and CCh, induce epileptiform activity in M1 and S1. This could be attributed to over-excitation, or indeed, excitotoxicity, due to an imbalance of excitation and inhibition. When the concentrations were reduced significantly, normal network activity resumed. The increase in susceptibility to seizures in the new preparatory method could indicate a greater involvement of KARs located on Pyramidal cells, and thus increased excitation (Fisahn et al., 2004), but without further studies into the mechanisms of this epileptiform activity, for example, the contribution of interneurone and Pyramidal cell generated I/EPSCs, it is impossible to know the full extent of the mechanism.

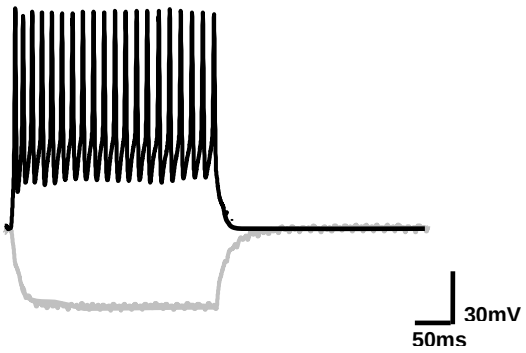
The activity of CCh cannot be disregarded in terms of epileptiform activity generation. Pilocarpine, a non-selective muscarinic receptor agonist, injection is widely used as a model for temporal lobe epilepsy (Cavalheiro, 1995) and its effects are thought to be mediated through M1 mAChRs. CCh application to hippocampal and other neocortical acute brain slices also induces epileptiform events (Dickson and Alonso, 1997; Williams and Kauer, 1997; Gloveli et al., 1999). Higher concentrations of CCh seem to preferentially produce epileptiform events (Gloveli et al., 1999, Alonso and Dickson, 1997). In previous experiments, 50 µM CCh was required to induce oscillatory activity (Buhl et al., 1998; Fisahn et al., 1998; Yamawaki et al., 2008) along with the dual application of 400nM KA (Buhl et al., 1999, Yamawaki et al., 2008) and this was also the case here. However, when viability of the slices was increased the requirement of CCh dropped to a tenth of what was previously required

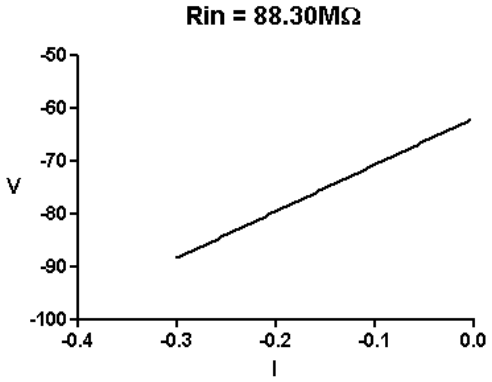
(from 50 to 5 µM). Indeed, it appeared that the induction of epileptiform activity was more sensitive to increased CCh concentrations than increased KA concentrations. KA could be increased to 150 nM (from the new concentration of 100 nM) as and when required, but any alteration to CCh concentrations would swiftly cause an epileptiorm event. During $GABA_AR$ blockade, epileptiform discharges increase with exogenous (25 µM CCh) and endogenous (eserine) acetylcholine agonists (Psarropoulou et al., 1998; Gruslin et al., 1999). Perhaps disinhibition, caused by KA, followed by an increase in excitability, caused by CCh, elicits the epileptiform discharges seen in M1.

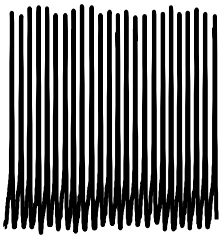
A1.3.2 Conclusion

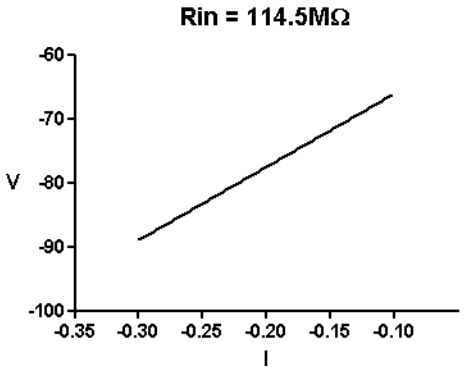
Changes to the preparation of sensorimotor slices have improved slice viability, as evidenced by the increase in occurrence of oscillatory activity and the survival of the interneuronal network and Pyramidal cell-interneurone pairing (chapter 3). This increase in viability has improved network activity within the motor cortex, but this has also increased its susceptibility when high doses of KA and CCh are used to evoke this oscillatory activity. The fact that low doses of these excitatory agents, even lower than those used to evoke network activity in hippocampus (Fisahn et al., 1999) and entorhinal cortex; (Morgan et al., 2008) is evidence of this improved viability, although neither one alone can generate network activity suggesting a functionally distinct network to that seen in hippocampus.

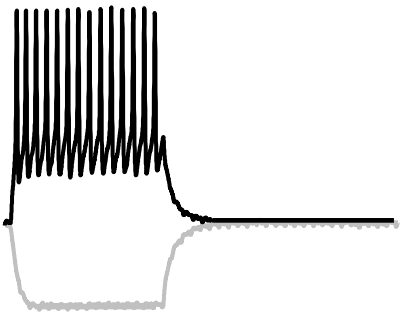
Appendix 2

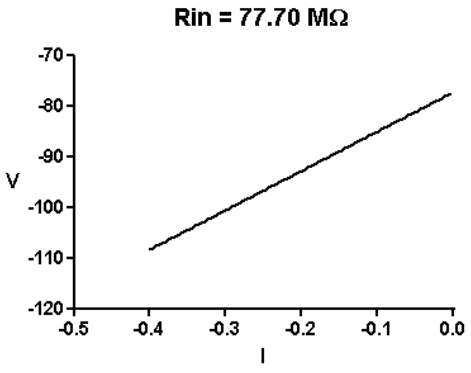




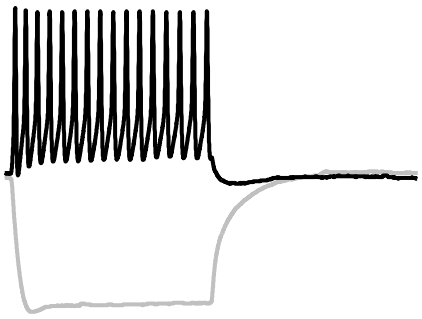


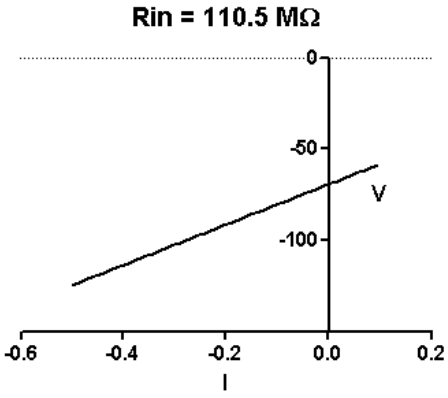






Appendix 2. Fast spiking (FS) interneurons as found in deep (L5) M1. Putative FS cells as recorded in L5 M1 (n = 6). Traces show current injection at -0.4 nA and +0.1 nA. Input resistance of putative FS cells as calculated from hyperpolarising current steps (-0.4 nA to 0 nA).

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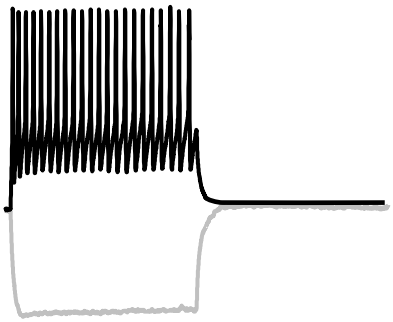
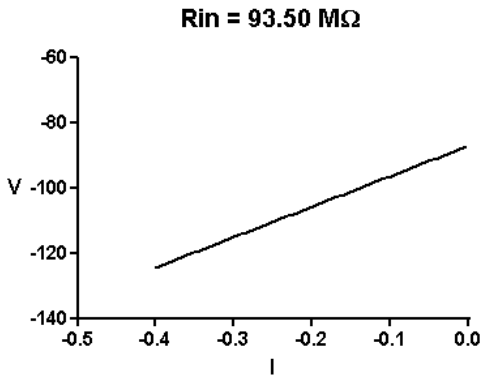
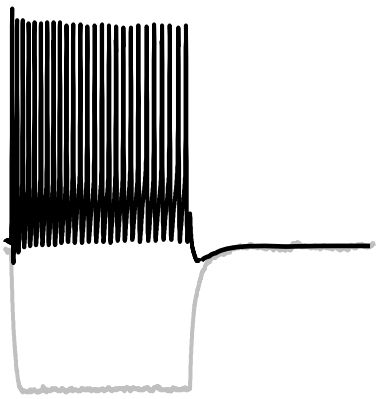
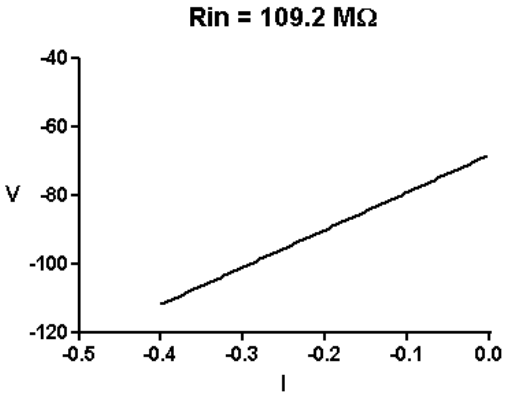
0
-50
-100
V
-0.6
-0.4
-0.2
0.0
0.2
I




Rin = 93.50 MΩ
-60
-80
V -100
-120
-140
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
I

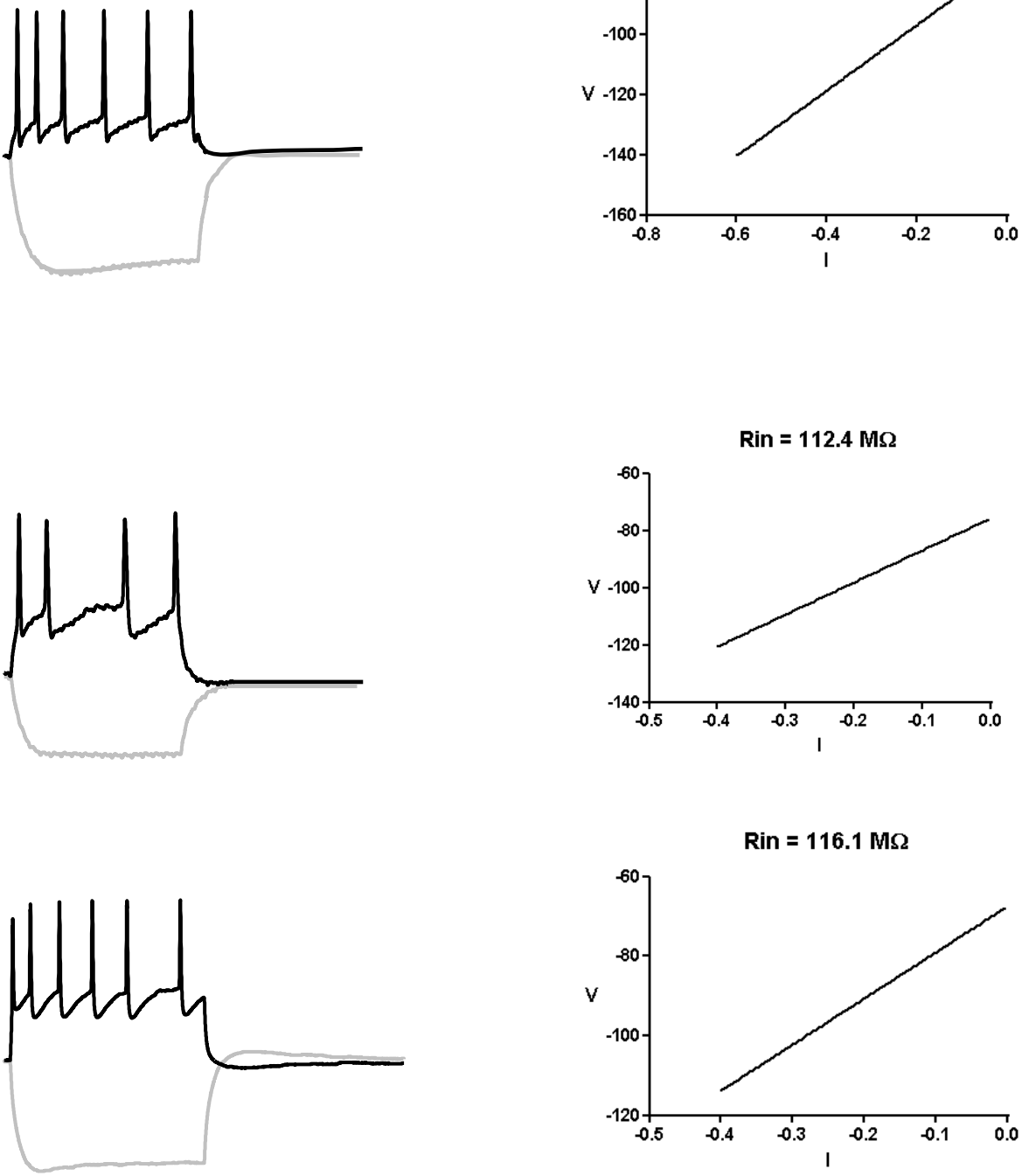



Rin = 109.2 MΩ
-40
-60
V -80
-100
-120
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
I

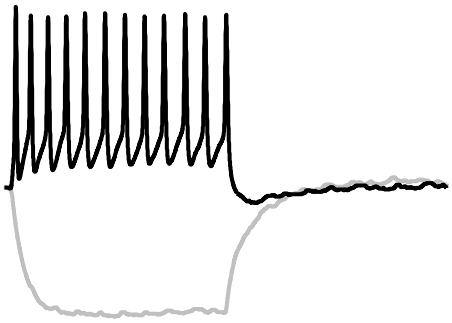
Amp (mV)	Rise (ms)	Decay (ms)	Area (mVms)	Spike Thresh-old	Rise 10-90	Half-width	AHP (mV)	BL (mV)	Rin
102.3	4.1	0.6	116.9	-57.1	2.1	0.7	-17.7	-82.3	93.5
90.9	2.3	0.6	79.4	-53.3	0.8	0.7	-22.2	-69.2	109.2
86.5	2.2	0.6	84.0	-48.4	1.2	0.8	-21.4	-71.0	110.5
97.5	3.7	0.6	113.9	-36.4	2.5	0.7	-15.0	-62.1	88.3
67.6	2.5	0.7	68.9	-56.2	0.8	0.9	-15.2	-68.2	114.5
106.9	5.8	0.6	173.5	-41.8	4.4	0.7	-12.6	-76.3	77.7
91.95	**3.42**	**0.59**	**106.11**	**-48.86**	**1.97**	**0.75**	**-17.4**	**-71.55**	**98.95**

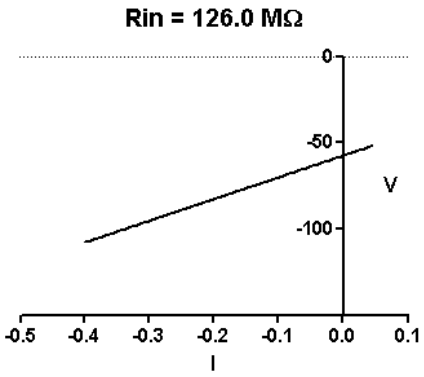
Table A2.1 Properties of fast spiking interneurones located in the deep (LV) M1 *in vitro*.
AHP = After hyperpolarisation. Rin = Input Resistance.

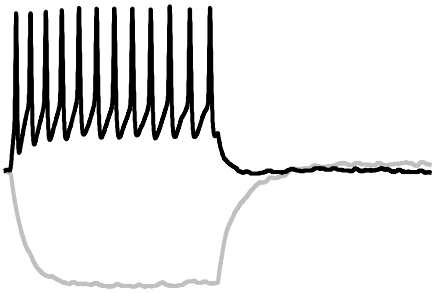
Appendix 3

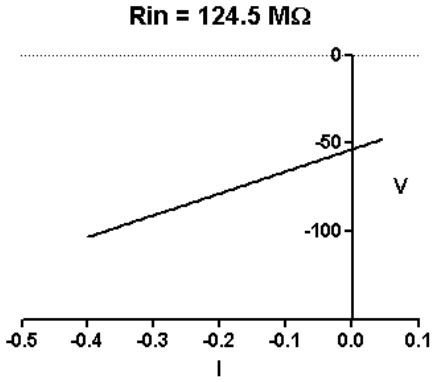


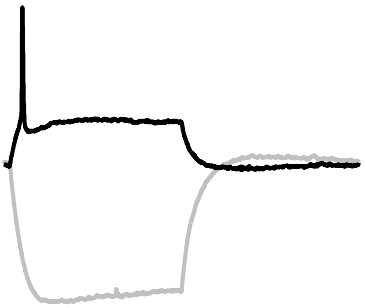
Appendix 3. Regular spiking non-Pyramidal (RSNP) interneurons as found in deep (L5) M1. Putative RSNP cells as recorded in L5 M1 (n = 7). Traces show current injection at -0.4 nA and +0.1 nA. Input resistance of putative RSNP cells as calculated from hyperpolarising current steps (-0.4 nA to 0 nA).

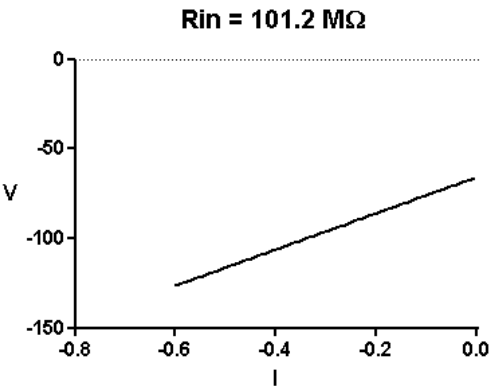


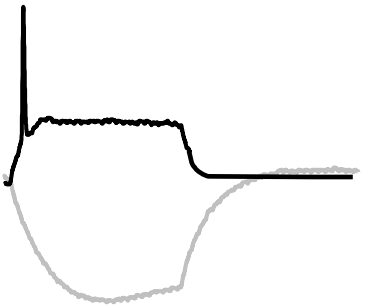

Rin = 126.0 MΩ
0
-50
-100
V
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
0.1
I

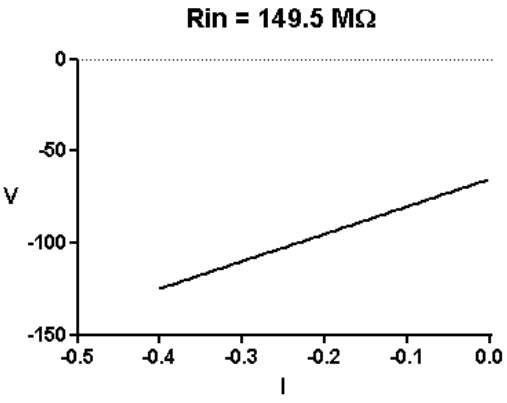



Rin = 124.5 MΩ
0
-50
-100
V
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
0.1
I




Rin = 101.2 MΩ
0
-50
V
-100
-150
-0.8
-0.6
-0.4
-0.2
0.0
I

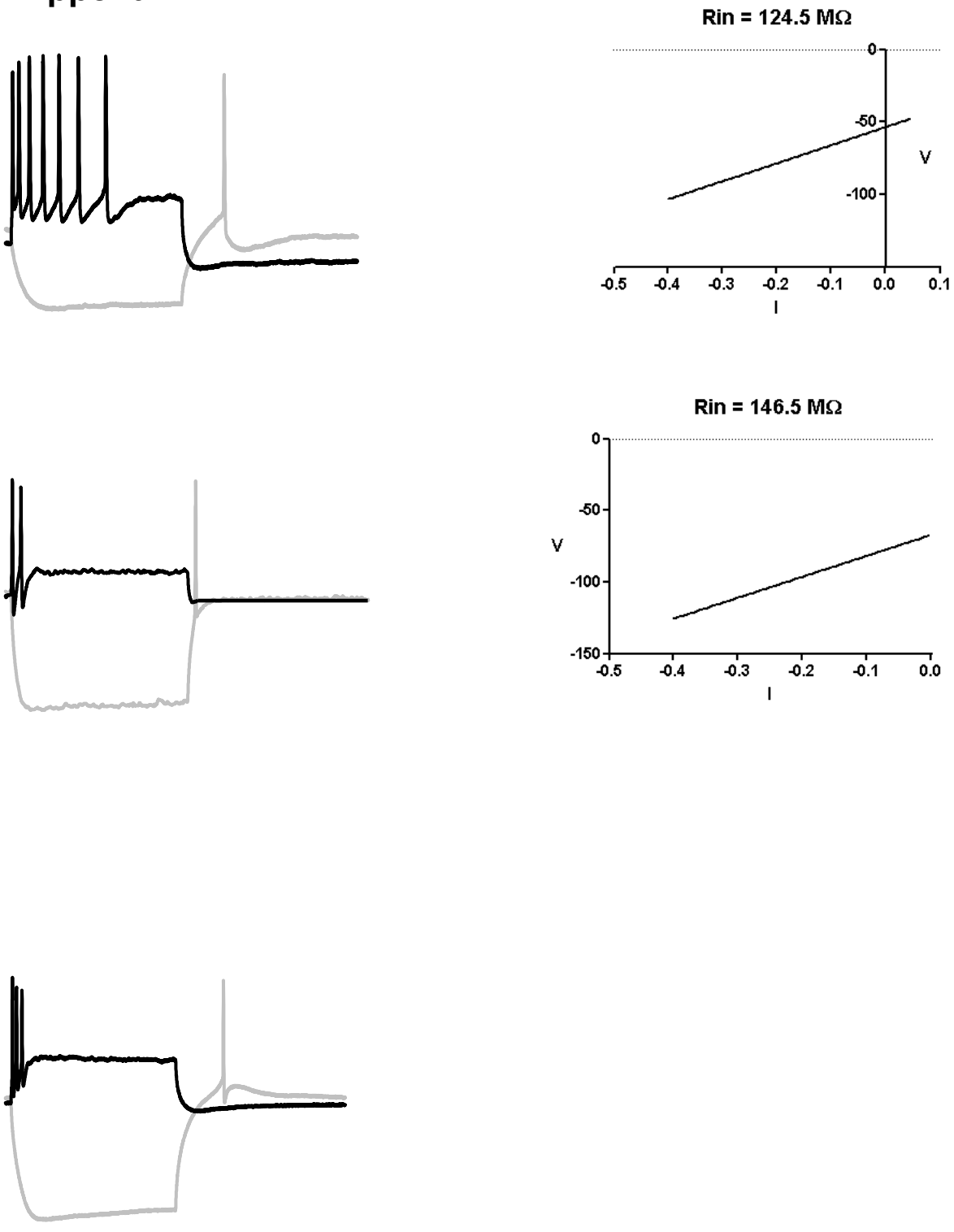



Rin = 149.5 MΩ
0
-50
V
-100
-150
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
I

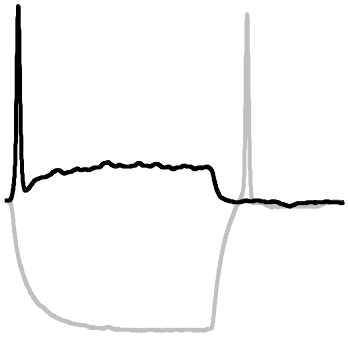
Amp (mV)	Rise (ms)	Decay (ms)	Area (mVms)	Spike Threshold	Rise 10-90	Half-width	AHP (mV)	BL (mV)	Rin
71.9	7.2	1.8	198.2	-41.2	3.5	2.0	-8.5	-63.8	101.2
109.9	6.4	1.6	255.3	-35.7	3.7	1.6	-4.5	-64.6	149.5
97.3	3.3	0.6	116.6	-30.5	2.2	0.8	-12.8	-52.6	124.5
92.6	2.3	0.6	87.1	-39.7	1.0	0.8	-14.1	-56.9	126
71.4	4.1	1.8	164.3	-42.7	3.1	1.6	-15.2	-66.6	116.1
104.2	4.2	0.7	128.9	-59.8	1.1	0.9	-12.5	-74.6	108.8
99.9	5.2	1.0	193.9	-44.9	3.7	1.2	-9.8	-72.6	112.4
92.50	**4.69**	**1.22**	**163.50**	**-42.07**	**2.66**	**1.30**	**-11.07**	**-64.55**	**119.79**

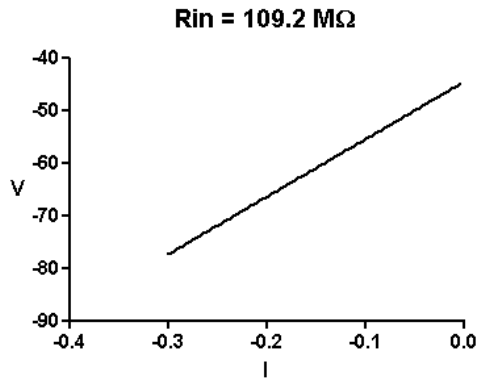
Table A3.1 Properties of Regular Spiking Non-Pyramidal Interneurones found in deep (LV) M1 *in vitro*.

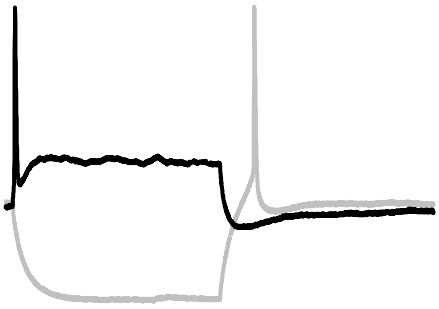
Appendix 4

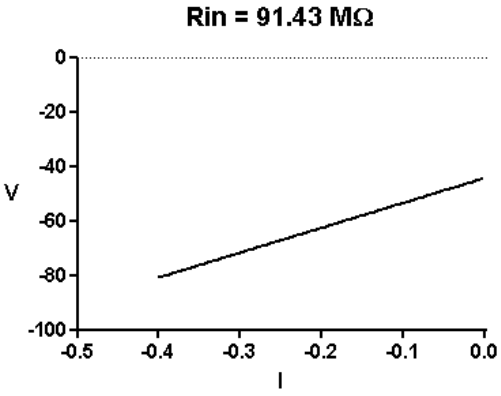


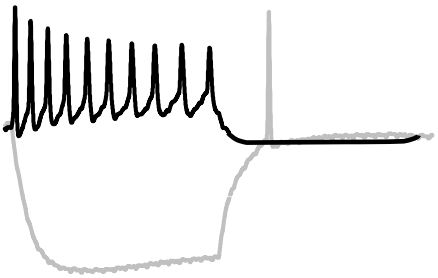
Appendix 4. Low-threshold spiking (LTS) interneurons as found in deep (L5) M1. Putative LTS cells as recorded in L5 M1 (n = 10). Traces show current injection at -0.4 nA and +0.1 nA. Input resistance of putative LTS cells as calculated from hyperpolarising current steps (-0.4 nA to 0 nA).

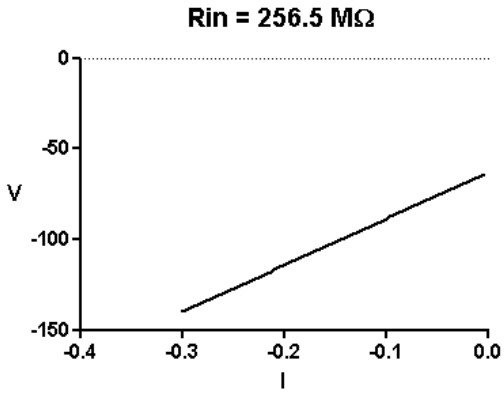



Rin = 109.2 MΩ
-40
-50
-60
V
-70
-80
-90
-0.4
-0.3
-0.2
-0.1
0.0
I




Rin = 91.43 MΩ
0
-20
-40
V
-60
-80
-100
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
I

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V
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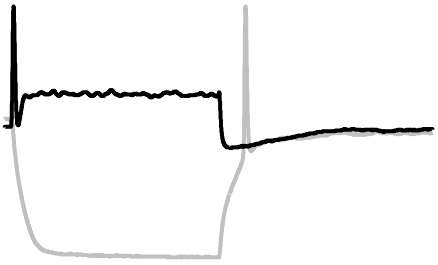
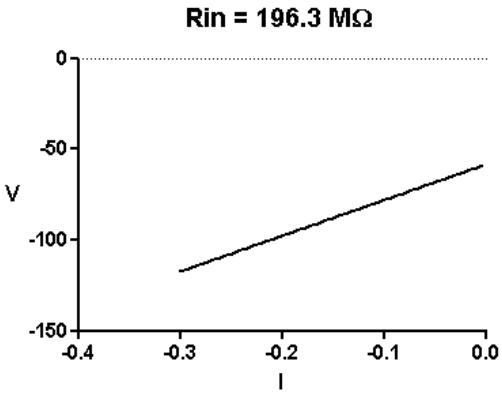
-0.4
-0.3
-0.2
-0.1
0.0
I

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0
-50
V
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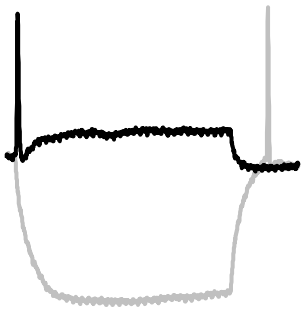
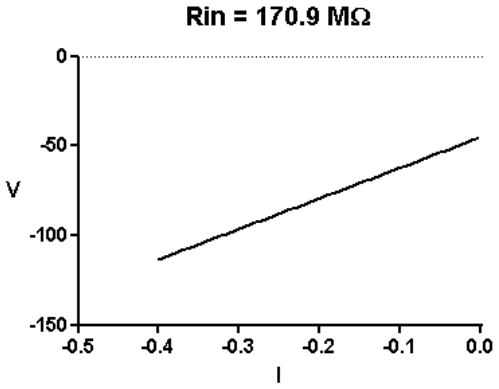
-0.1
0.0
I




Rin = 170.9 MΩ
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V
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-0.3
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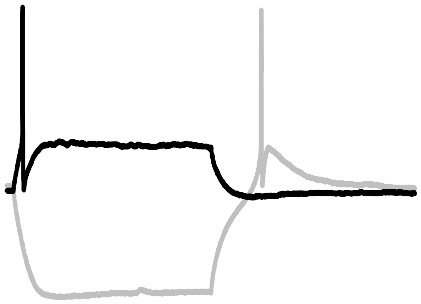
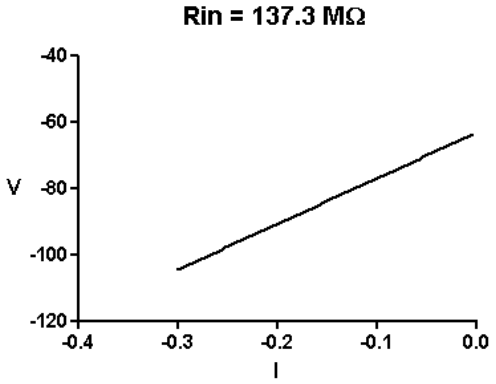
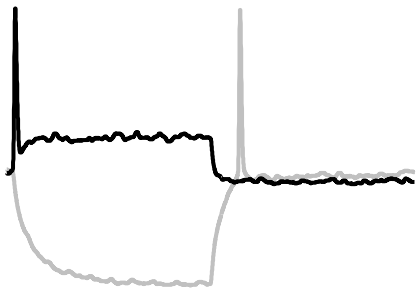
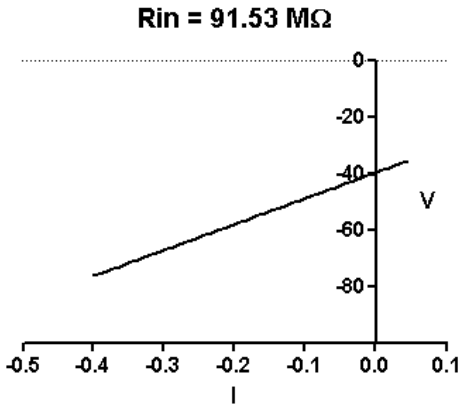
-0.1
0.0
I




Rin = 137.3 MΩ
-40
-60
-80
-100
-120
V
-0.4
-0.3
-0.2
-0.1
0.0
I




Rin = 91.53 MΩ
0
-20
-40
-60
-80
V
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
0.1
I

Amp (mV)	Rise (ms)	Decay (ms)	Area (mVms)	Spike Thres-hold	Rise 10-90	Half-width	AHP (mV)	BL (mV)	Rin
89.59	2.414	0.6	85.70	-54.60	1.05	0.77	-14.3	-66.8	146.5
74.06	1.73	1.4	111.50	-34.50	0.562	1.54	-3.7	-68.2	91.53
64.66	1.044	1.18	91.86	-47.60	0.6	1.51	-7.4	-53.9	196.3
73.42	4.537	0.57	83.54	-45.93	2.372	0.74	-20.1	-60.1	137.3
71.01	5.067	0.73	85.32	-45.84	1.408	0.90	-14.5	-57.5	111.7
73.94	3.55	1.23	120.88	-33.87	1.594	1.43	-12.3	-47.1	109.2
76.00	4.067	2.15	172.96	-45.42	1.58	2.16	-5.1	-46.8	91.43
88.84	1.817	0.77	96.94	-41.50	0.731	1.05	-17.7	-57.3	256.5
70.97	1.329	1.58	114.57	-45.20	0.654	1.68	-9.5	-50.9	170.9
75.84	**2.84**	**1.15**	**107.04**	**-43.83**	**1.17**	**1.31**	**-11.6**	**-56.5**	**145.7**

Table A4.1 Properties of Low Threshold Spiking Cells found in deep (LV) M1 *in vitro*.