AN IN VIVO NEUROPHYSIOLOGICAL MODEL OF CORTICAL ISCHEMIA IN THE RAT

by

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ABSTRACT

Spontaneous and evoked potentials (EPs) were recorded with cross-cortical microelectrode arrays following partial occlusion of the MCA and ACA in urethane-anaesthetised rats. The control group received no occlusion, while the treatment group was injected with anti-stroke peptide Tat-NR2B9c 5min before ischemia. Spontaneous EEG power significantly decreased in the stroke-only group when compared to controls (p<0.001). A greater loss of EEG power was observed on anterior electrodes closer to the occluded area versus posterior contacts in stroke-only rats (p<0.05). The Tat-NR2B9c+stroke group lost significantly less power when compared to stroke-only animals (p<0.05). EP amplitude in the stroke-only group was significantly reduced following ischemia when compared to control and Tat-NR2B9c+stroke animals (p<0.001). Epileptiform discharges were observed in 8/10 untreated stroke rats and 3/5 stroke rats treated with Tat-NR2B9c. The characteristic features of spontaneous and evoked potentials validate this rat focal stroke model for in vivo testing of pharmacological agents.
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LIST OF ABBREVIATIONS

ACA – anterior cerebral artery
AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AUC – area under curve
CSD – current-source density
DCR – direct cortical response
EAA – excitatory amino acid
EEG – electroencephalogram
EP – evoked potential
HFO – high frequency oscillation
HSD – hypoxic spreading depression
iGluR – ionotropic glutamate receptor
MCA – middle cerebral artery
MCAo – middle cerebral artery occlusion
mGluR – metabotropic glutamate receptor
ICA – internal carotid artery
IED – interictal epileptiform discharges
MAGUK – membrane associated guanylate kinase
NMDAR – N-methyl-D-aspartate receptor
NO – nitric oxide
nNOS – neuronal nitric oxide synthase
OGD – oxygen-glucose deprivation
PED – periodic epileptiform discharge
PSD – post synaptic density
PSD95 – post synaptic density 95 scaffolding protein
rCBF – regional cerebral blood flow
SD – spreading depression
SEP – somatosensory evoked potential
tPA – tissue plasminogen activator
VPL – ventral posterior lateral nucleus of thalamus
1 INTRODUCTION

1.1 Stroke: General Introduction

Stroke is defined as a "neurological deficit of a cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours" by the World Health Organisation. Stroke is the second most common cause of death worldwide after ischemic heart disease and a serious cause of disability in both developed and developing countries (Donnan et al., 2008). Because of the world’s ageing population, the burden on society due to stroke-related impairments is expected to greatly increase by 2030 (Murray & Lopez, 1997).

Development of treatment and prevention of stroke has advanced during the last decade. Primary treatment options currently include thrombolytic drugs that break down blood clots, such as the tissue plasminogen activator (tPA) and surgical intervention (Donnan et al., 2008). tPA is also currently the only stroke therapeutic approved by the Food and Drug Administration (FDA). Different forms of supportive care such as speech therapy, physiotherapy and occupational therapy are coupled with pharmacological interventions (Gilligan et al., 2005). Secondary prevention consists of antiplatelet drugs (eg. aspirin), regular blood pressure control and removal of arteriolar plaques and blockages in selected patients.

Although stroke awareness and prevention has increased clinical management of stroke, therapeutics which protect the brain from post-stroke deterioration are notably lacking. The translation from bench to bedside of potential ‘brain-protective’ drugs has largely failed due to combinations of insufficient preclinical validation, poor clinical trial
design, and deleterious side effects. Currently, the primary goal of stroke research is finding a treatment that stops the progression of stroke-related injury when given after initial attack, without having serious adverse effects on the patient.

1.2 Blood Supply to the Brain

Although the human brain is only 2% of the total body weight, it uses 15% of the total cardiac output via two vertebral and two internal carotid arteries (ICA) (Fix, 2008). The brain consumes 20% of the oxygen used by the body and is perfused at a rate of 50 ml/100 g of brain tissue per minute. Together with the anterior and posterior cerebral and communicating arteries, the ICA forms the Circle of Willis (Fig. 1). The ICA enters the cranium via the carotid canal of the temporal bone and lies within the cavernous sinus as the carotid siphon. It supplies direct branches to the optic nerve, optic chiasm, hypothalamus and genu of the internal capsule. ICA also divides into seven critical branches which supply most of the brain: ophthalmic artery, central artery of the retina, posterior communicating artery, anterior choroidal artery, anterior cerebral artery (ACA), anterior communicating artery and the middle cerebral artery (MCA). The MCA supplies most of the cerebral cortex including the lateral and insular cortices, trunk, arm, and face areas of the motor and sensory cortices and the speech areas. It also perfuses the caudate nucleus, putamen, globus pallidus and anterior and posterior limbs of the internal capsule. The ACA supplies the medial surface of the frontal and parietal lobes (surrounding the midline) and corpus callosum and is responsible for perfusing the leg and foot area of the
motor and sensory cortices. These arteries are easily accessible following craniotomies and their occlusion in the human often results in debilitating neuronal damage.

**Figure 1** Blood vessels of the brain viewed from the ventral surface. The Circle of Willis is made up of the proximal posterior cerebral arteries, the posterior communicating arteries, the internal carotid arteries just before their bifurcations, proximal anterior cerebral arteries, and anterior communicating artery. (Figure from Kandel *et al.*, 2000)

### 1.3 Neurophysiology

#### 1.3.1 Cerebral cortex

The cerebral cortex is composed of six distinct layers each containing a characteristic distribution of neuron types and connections to different cortical and
subcortical regions (Fig. 2) (Fix, 2008; Purves et al., 2007; Kandel et al., 2000). The molecular layer I is located just beneath the pial surface and is mostly comprised of dendritic extensions and horizontally-oriented axons. There are very few scattered neurons because of the small size of this layer. The external layer II contains small pyramidal cells and many stellate neurons. The external pyramidal layer III is made up of small and medium sized pyramidal neurons in addition to non-pyramidal neurons with vertically-oriented intracortical axons. Layer III is the main source of corticocortical commissural fibres whose primary targets are layers I through III within the same hemisphere. Internal granular layer IV contains a mixture of densely packed stellate cells and some pyramidal neurons. It is the primary target of horizontally running myelinated fibres from different thalamic centres. The internal pyramidal layer V is made up of large pyramidal cells and is the primary source of axons going to subcortical areas such as the basal ganglia. The deepest part of the cortex – multiform layer VI contains spindle-like pyramidal and multiform neurons that send fibres to the thalamus creating a precise corticothalamic connection.

The cerebral cortex sends information via efferent connections to various subcortical structures including the thalamus and the basal ganglia. It also receives signals from the same areas via afferent fibres. The thalamus acts as a relay for all sensory and motor connections going to and out of the cortex (Sherman, 2001).
Figure 2 Three different stains of neurons in the cerebral cortex arranged in distinctive layers. The Golgi stain reveals neuronal cell bodies and dendritic trees. The Nissl method shows cell bodies and proximal dendrites. A Weigert stain for myelinated fibers reveals the pattern of axonal distribution. (Figure from Kandel et al., 2000)

1.3.2 Electrophysiology

Extracellular field recordings are measured between two points in the extracellular space, as opposed to intracellular recordings which measure the voltage
difference across a neuronal membrane. As a result, the extracellular field potential is the summated activity of a population of neuronal elements (Johnston & Wu, 1995).

Since the active neuron is not uniformly polarised, the dendrites may be at a different potential from the cell body and cell body at a different potential from the axon. As the action potential propagates along the neuronal fibre, different regions of the axon are at different potentials with respect to each other. Since the extracellular fluid acts as a volume conductor, current flow in a volume conductor establishes fields that can exert force on electrical charge in the conductor. This force is measured in the form of a potential difference. The area of current flow into the axon or soma is termed current sink, while the area where current exits the axon/soma is called current source.

Extracellular recording at a current sink will yield a negative potential with respect to a distant ground. A positive extracellular potential is recorded at a current source. Given that an action potential is never static but is constantly propagating, the sink and source are always changing (Johnston & Wu, 1995).

Extracellular fields in the central nervous system can be classified into two different categories – open and closed. Open fields are characterised by a laminar organisation of neurons where the dendrites face in one direction and somata in another. The cortex and hippocampus are examples of open fields. If this population of neurons is activated by a summated synaptic input, a dipole is established between the dendrites and somata. Closed fields are made up of spherical arrays of neurons with the cell bodies localised either at the centre or periphery. The polarity of measurement depends on placement of electrode within the sphere, while recording outside yields zero potential.
Nuclei in the basal ganglia, such as GPi or SNr have neurons with radial dendrites and are often considered to have closed fields (Johnston & Wu, 1995).

Many studies have investigated *in vitro* and patch-clamp stroke models using oxygen-glucose deprivation (OGD), where a number of parameters can be closely controlled (Perez Velazquez *et al.*, 1997; Perez Velazquez *et al.*, 2000; Frantseva *et al.*, 2002a; Frantseva *et al.*, 2002b). Relatively few *in vivo* studies have looked at the neurophysiology of the ischemic events leading up to neuronal loss. What’s more, electrophysiology of acute focal ischemic injury has not been previously examined *in vivo*. Surface EEG recordings in humans (Trojaborg & Boysen, 1973) and rats (Hartings *et al.*, 2003; Kelly *et al.*, 2006) have shown loss of power following a focal ischemic attack. However, the topography of these findings was often too widespread for precise localisation of underlying neuronal activity (Kaiser & Lutzenberger, 2003).

### 1.3.2.1 Spontaneous cortical potentials

Spontaneous oscillations in both the low and high frequency ranges are common in many neural systems. The rhythmic nature of these potentials may be the result of pacemaker activity connected to a population with specific resonant properties (Steriade *et al.*, 1993). Conversely, it is also believed that individual elements do not necessarily need to be autorhythmic in order for larger neuronal systems to exhibit rhythmic oscillations (Contreras & Steriade, 1995). In other words, the rhythmicity is generated by the network properties not the individual nodes or cells.

Oscillations of a large neuronal population in the cortex and thalamus are reflected as waves in the electroencephalogram (EEG). Spontaneous EEG activity was
originally thought to represent the plastic and constantly changing condition of the brain (Bremer, 1958). It was later proposed that these oscillations directly correlate with the synaptic activity in cortical cells (Klee et al., 1965).

Delta waves (1-4 Hz) have been shown to appear during later stages of sleep while slow oscillations (<1 Hz) are present throughout resting sleep (Steriade et al., 1993). These two rhythms are also the predominant spontaneous oscillations recorded from the cortex of animals anesthetised with both urethane and ketamine / xylazine (Contreras & Steriade, 1995; Steriade et al., 1993). Delta rhythms are made up of two different components – cortical and thalamic. The thalamic inputs to the 4th layer of the cortex from thalamocortical cells generate an oscillation in the delta frequency range via the interplay between two of their voltage gated currents. Low frequency oscillations (<1 Hz) are also present in the sensory, motor and associated areas, however unlike their delta counterparts they are for the most part unaffected by thalamic lesions (Steriade et al., 1993). Steriade and colleagues suggested that the cortical slow rhythm organises the thalamically generated delta waves within slowly recurring oscillatory cycles. By analysing these deep cortical EEG patterns before, during and following focal ischemic injury we have the opportunity to look at neuronal impairment beyond the obvious cortical injury.

1.3.2.2 Evoked potentials

When inputs to a population of neurons are simultaneously activated the response of these neurons (assuming that input has the same effect on each) is synchronous and
there is an associated voltage fluctuation in the vicinity known as an evoked potential (Stevens 1966).

Cortical SEPs evoked from the trigeminal, sciatic and median nerves via bipolar stimulating electrodes were found to attenuate in rats following MCAo induction (Sakatani et al., 1990; Astrup et al., 1977). Also, Mun-Bryce et al. used bipolar stimulation of the contralateral snout to record from the SI cortex of the swine and reported similar findings following intracerebral haemorrhage (Mun-Bryce et al., 2001). Each SEP cortical recording site responds primarily to one somatosensory area of stimulation and as such simultaneous cross-cortical recordings using this method require multiple peripheral stimuli.

1.3.3 Spreading depression

Spreading depression (SD) is a fast depolarisation of a large population of neurons and glia, concomitant with a considerable ionic exchange between the intracellular and extracellular space (Sugaya et al., 1975; Somjen, 2004). It was first discovered by Leão and manifested as a flattening of the electrocorticogram (ECoG) from bipolar recordings in the exposed cortex of the anaesthetised rabbit (Leão, 1944). In subsequent studies Leão recorded a sustained extracellular potential shift during SD using direct current (DC)-coupled electrodes (Leão, 1947; Leão, 1951). Membrane potentials were measured before, during and after SD using intracortical microelectrodes (Brožek, 1966). Voltages were less negative during SD than prior to it, indicating depolarisation, and more negative after repolarisation, suggesting transient hyperpolarisation. Studies performed using ion-sensitive electrodes found that extracellular K⁺ levels increase
(Vyskocil et al., 1972), while extracellular Ca$^{2+}$, Na$^+$ and Cl$^-$ dramatically decrease during SD (Hansen & Olsen, 1980; Kraig & Nicholson, 1978). This seems to indicate that K$^+$ is leaving the neurons in exchange for Ca$^{2+}$ and Na$^+$ (Somjen, 2001).

Spreading depression in the presence of abnormally low oxygen levels is termed hypoxic spreading depression (HSD) and is thought to be the most recognisable predictor of rapid cell death following ischemia (Somjen, 2004). Normoxic spreading depression does not cause injury to the brain when experimentally induced on non-ischemic animals (Nedergaard & Hansen, 1988). Repolarisation and restoration of ion gradients following HSD requires energy, which requires a significant increase in blood flow to cover oxygen and glucose consumption (Mies et al., 1993; Hansen & Lauritzen, 1984). Thus, the membrane potential can recover from HSD only if oxygen levels recover shortly after depolarisation. The ischemic cortex, however, is not able to accomplish re-oxygenation due to the drastic and often prolonged decrease in blood flow.

1.4 Ischemia

Ischemia is defined as a lack of blood supply caused by obstruction of arterial inflow. Severe heart disorders, such as cardiac arrest, systemic hypotension and rhythm disorders cause a loss of blood flow to the entire brain, leading to global ischemia. On the other hand, acute disruption of brain circulation in one or several arteries is termed focal brain ischemia, which typically manifests as a syndrome of ischemic stroke or transient ischemic attack. Cerebral ischemia initiates a process called ischemic cascade, which in turn releases proteolytic enzymes, reactive oxygen species and other pathogens.
eventually causing brain damage (Martin, 1990). Physiological processes in the brain depend on the state of its energy metabolism, which is dependent on a near constant supply of oxygen and glucose via the bloodstream. Inadequate blood supply results in hypoxic or anoxic tissue as well localized hypoglycaemia. Glucose is the most important energy-providing substrate in the brain, however due to limited stores complete oxidation of brain glucose and glycogen takes only 5–7 min (Gusev & Skvortsova, 2003). At normal body temperature, the process of cell death as outlined later, takes 3 – 4 hours before becoming irreversible in aerobic tissues, such as the brain and heart. (Martin, 1990)

1.4.1 Ischemic core and penumbra

Upon focal ischemia, there are two regions of reduced blood flow in the cortex – the infarct core and bordering penumbra or ‘area at risk’ (Astrup et al., 1977). In animal models, specifically the middle cerebral artery occlusion (MCAo), the core is defined as the area of critical blood flow reduction (<15% of normal flow) (Tamura et al., 1981b;Duverger & MacKenzie, 1988). Cell death in the ischemic core usually occurs within minutes, as a result of rapid anoxic depolarization and a simultaneous rise in extracellular K⁺ (Nedergaard & Hansen, 1993). At 1 – 2 min of reduced blood flow, there is a significant decrease in extracellular Ca⁺ levels at the core, signifying entry into the tissue (Harris & Symon, 1984). Even if K⁺ levels return to normal after adequate reperfusion, the severity of the ischemic insult leads to considerable cell death (Gido et al., 1997).
The penumbra surrounds the core and is also supplied by the occluded artery but retains structural integrity due to residual perfusion from collateral blood vessels. Both silencing of the EEG (Strong et al., 1983) and reduced amplitude of somatosensory evoked potentials (Sakatani et al., 1990) have been recorded in the penumbral tissue minutes after infarct. Because blood flow to the penumbra is not completely blocked, this area experiences only moderate hypoxic spreading depression rather than complete anoxic depolarization (Hossmann, 1994; Nedergaard & Hansen, 1993; Somjen, 2001). Acute hypoxic periods in the ischemic penumbra have been shown to coincide with the passing wave of every peri-infarct depolarization (Back et al., 1994).

1.4.2 Ischemic cell death

Clearly defining cell death has proven to be difficult, but is typically thought to be the point at which the cell is unable to recover normal morphology and function even if all processes leading to damage have stopped (Lipton, 1999). However, we do not know enough about the development of neuronal death following ischemia to be able to identify this point with certainty. At present, the only clear definition of cell death includes morphological states thought to be precursors of disintegration and phagocytosis (Nedergaard, 1987). The process of cell death in ischemia is characterized by a delay between onset of injury and the first signs of major cellular damage. Depending on extent and duration of energy deprivation and the brain region affected, this delay can last days (Du et al., 1996) or only hours (McGee-Russell et al., 1970).
1.4.2.1 Apoptosis and necrosis

In general, there are two main types of cell death – apoptosis and necrosis (Majno & Joris, 1995). Apoptosis is also termed programmed or gene-directed cell death and in a healthy organism it serves as a normal method of eliminating nonessential tissue. Apoptosis involves a series of biochemical events leading to morphological changes and the formation of new structures that are integral to the cell death process (Kerr et al., 1972). After an insult to the plasma membrane, K\(^+\) and Cl\(^-\) leave the cell (Yu & Choi, 2000). Cell shrinkage occurs early in the process and the altered ion composition inside the cell leads to a swelling and malformation of the mitochondria (Lipton, 1999). This triggers a release of soluble mitochondrial proteins such as cytochrome C, into the cytosol. Cytochrome C in turn initiates a protein signal cascade which results in the activation various caspases (Siesjö et al., 1999). Caspases are a family of cysteine proteases that cleave a variety of cellular targets including other caspases, proteins, cytoskeletal elements and chromosomal DNA. The caspase proteins have been termed “executioners” because of their role in mediating the disintegration of the cell structure during programmed cell death.

Necrosis, on the other hand, is a premature or abnormal death of a cell or tissue that occurs following external trauma such as infection or bioenergetic failure as seen in ischemia. Unlike cell shrinkage seen in apoptosis, ischemic necrosis is marked by neuronal swelling and loss of membrane integrity (Duchen, 1992). In vitro studies of cortical neurons show that severe oxygen and glucose deprivation leads to purely necrotic death. When the same ischemic cultures were protected by glutamate receptor antagonists, cell death occurred via apoptosis (Gwag et al., 1995). In vivo studies are less
conclusive – some groups discovered signs of apoptosis following transient global cerebral ischemia in rats (Siesjö et al., 1999), while others found only necrotic neurons (Colbourne et al., 1999).

1.4.2.2 Excitotoxicity

Cell death in pathologies such as stroke, epilepsy and neurodegeneration is thought to be mediated primarily via excessive release and inadequate reuptake of excitatory amino acids (EAA), specifically glutamate (Aarts et al., 2003). Blocking postsynaptic glutamate receptors in vitro results in reduced sensitivity to hypoxia (Kass & Lipton, 1982; Rothman, 1983). It is also widely accepted that glutamate excitotoxicity depends largely on Ca$^{2+}$ (Choi, 1995). Cellular functions, such as excitability, growth and synaptic activity are governed by intracellular Ca$^{2+}$ ions. Calcium influx or release from intracellular stores elevates intracellular Ca$^{2+}$ to levels that exceed the capacity for homeostatic regulation (Tymianski & Tator, 1996). This activates maladaptive mechanisms, such as persistent accumulation of proteolytic and lipolytic enzymes that mediate cell death (Deshpande et al., 1987).

Although loss of Ca$^{2+}$ homeostasis is a significant factor in excitotoxic cell death, it may not be the exclusive mediator of calcium toxicity. The ‘source specificity hypothesis’ stipulates that calcium toxicity is not solely dependent on overall Ca$^{2+}$ concentration, but is also tied to the route of Ca$^{2+}$ entry into the cell and subsequent secondary messenger activation (Tymianski et al., 1993). To support this, studies have shown that blocking glutamate receptor-mediated Ca$^{2+}$ entry results in survival of neurons after many hours of hypoxia (Peng et al., 1991) while blocking voltage-gated
Ca\textsuperscript{2+}-channels does not prevent neurotoxicity under anoxia (Dubinsky & Rothman, 1991). Therefore, glutamate and calcium both appear to be crucial in mediating ischemic injury (Hossmann, 1994).

### 1.4.2.3 Glutamate Receptors

There are two functionally distinct families of glutamate receptors (GluR) –
ionotropic (iGluR) and metabotropic (mGluR).

Ionotropic GluRs are heteromeric ligand-activated ion channels that are selectively permeable to extracellular Na\textsuperscript{+}, K\textsuperscript{+} or Ca\textsuperscript{2+} when activated. Each receptor is thought to be comprised of four individual subunits. Each subunit of the channel has four transmembrane domains consisting of an extracellular N-terminus and an intracellular C-terminus (Hollmann et al., 1994). iGluRs are important in mediating the synaptic plasticity implicated in memory formation. Also, their activation has been associated with glutamate-mediated toxicity (Choi, 1988). The iGluR family is typically grouped into two pharmacologically different receptor subfamilies – \(\alpha\)-amino-3-hydroxy-L-5-methyl-4-isoxazolepropionic acid / kainate receptors (AMPARs / KARs) and \(N\)-methyl-\(d\)-aspartate receptors (NMDARs).

Metabotropic GluRs are monomeric proteins that activate intermediate intracellular molecules called G-proteins following neurotransmitter binding. They do not have ion channels as a part of their structure; instead G-protein subunits modulate ion channels directly or indirectly through enzymes and secondary messengers. mGluRs have been shown to play an important role in neuronal plasticity and nociception. Although they haven’t been implicated directly in neurodegeneration, agonists of certain mGluRs
increase and antagonists decrease NMDA-mediated excitotoxicity in stroke (Pellegrini-Giampietro et al., 1999; Nicoletti et al., 1999).

1.4.2.3.1 AMPARs / KARs

AMPAR / KAR activation by glutamate binding leads to changes in permeability of Na$^+$ and K$^+$ ions and consequently to a depolarisation of the post-synaptic membrane (Schousboe et al., 1994). This depolarisation results in increased Ca$^{2+}$ influx via both ligand-gated and voltage-gated channels (Li & Buchan, 1993; Sheardown et al., 1993). AMPARs are tetraheteromeric structures made up from combinations of GluR1-4 subunits. It is important to note that most AMPARs contain GluR2 subunits, which make them impermeable to extracellular Ca$^{2+}$ (Hume et al., 1991). Loss of the GluR2 subunit and a subsequent increase in Ca$^{2+}$ permeability has been implicated in delayed ischemic death of central neurons (Bennett et al., 1996).

A number of intracellular scaffolding proteins termed post-synaptic density (PSD), because of their cellular location, bind to GluR subunits and function by clustering receptors at the synapse, which is thought to be a modulator of synaptic plasticity (Srivastava et al., 1998; Rose & Konnerth, 2000). PSD proteins are also believed to mediate AMPAR-activated secondary-messenger cascades, but have not been directly associated with excitotoxic signalling. Some of these proteins, such as the Ras family of small GTPases have the potential for mediating toxic insults by interacting with other synaptic molecules. AMPAR activation may be coupled to Ras signalling via the binding of two neuronal scaffolding proteins, GRIP-1 and GRASP-1 (Ye et al., 2000). GRASP-1 (GRIP associated protein) is cleaved during ischemic apoptosis, thereby
disrupting its regulation of Ras signalling. It has also been shown that synaptic targeting of AMPARs is downregulated by GRASP-1 (Ye et al., 2002). It is possible that cleavage during ischemia results in increased synaptic AMPAR activity and thus increases vulnerability to glutamate overactivity (Aarts et al., 2003).

1.4.2.3.2 NMDARs

Like its AMPAR counterpart, the NMDA receptor is a heteromeric integral membrane protein comprised of four subunits. Functional NMDARs are formed from the co-assembly of the obligatory NR1 subunit with the NR2 and/or NR3 subunits. The extracellular portion of the receptor is made up of two globular structures – the mandatory domain and the ligand-binding domain (Stephenson, 2006). NR1 subunits bind to the co-agonist glycine, while the NR2 subunits bind to glutamate. The NMDAR membrane domain contains a voltage-dependent Mg\(^+\) block obstructing a Ca\(^{2+}\) and Na\(^+\)-permeable pore. NMDA receptors are unique in that they require both membrane depolarization and ligand binding for full channel activity. Membrane depolarization is required to remove the magnesium block and allow the receptor to become highly conductive (Dale & Roberts, 1985). Na\(^+\) permeability contributes to further membrane depolarisation, while influx of extracellular Ca\(^{2+}\) generates intracellular Ca\(^{2+}\) transients mediating the primary physiological effects of NMDAR signaling (Waltereit & Weller, 2003). Each subunit also has a cytoplasmic domain with the C-terminal tails that contain a number of motifs for phosphorylation and protein-protein interactions.

There are four NR2 subunits (A-D), however, NMDARs in the mature forebrain consist of mostly NR2A and NR2B subtypes and are thought to have opposing roles in
synaptic plasticity (Massey et al., 2004). In vivo and in vitro studies implicate the activation of NR2B-containing NMDA receptors in excitotoxicity and subsequent apoptosis, while activation of NR2A subunits are thought to support neuronal survival (Liu et al., 2007).

1.4.2.3.3 The Post synaptic density (PSD)

The PSD is an electron dense, subcellular structure made up of multiple scaffolding and cytoskeletal proteins located beneath the postsynaptic membrane (Schikorski & Stevens, 1997). The PSD is also thought to contain signal molecules that can act as excitotoxic triggers downstream of glutamate receptors (Carlin et al., 1980). A number of functions have been ascribed to the PSD, such as cell to cell adhesion, regulation of receptor grouping and modulation of receptor coupling. In addition to modulating signal transmission, synaptic activity at glutamate receptors can also lead to structural remodelling of the PSD (Kennedy, 1993). Modulatory enzymes are associated with the PSD and have been implicated in iGluR signalling. For instance, neuronal nitric oxide synthase (nNOS) is activated by Ca\(^{2+}\) influx via NMDARs and can modulate NMDAR signalling. Cytoskeletal proteins in the PSD domain play an important role in receptor clustering, while scaffolding proteins function in clustering all the PSD components. PSDs are found at both excitatory and inhibitory synapses. The excitatory PSD is particularly abundant in scaffolding proteins containing protein-interaction PDZ domains (Fig. 3). The PDZ acronym stands for the first letters of three proteins which were first discovered to share the domain — post synaptic density protein (PSD-95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (ZO-1).
PDZ-containing scaffolds assemble specific proteins into large molecular complexes at defined locations in the cell (Kim & Sheng, 2004). In the postsynaptic density PDZ proteins organize glutamate receptors and their signalling proteins and regulate synaptic size and strength. PDZ scaffolds are also implicated in the dynamic trafficking of synaptic proteins by assembling cargo complexes for transport by molecular motors.

Synaptic PDZ-containing proteins fall into two families – GRIP and MAGUK. The GRIP (glutamate receptor signalling protein) family is involved in glutamate receptor synaptic targeting. GRIP1 and GRIP2/ABP are adaptor proteins for the AMPA receptor and contain seven PDZ domains, which are thought to link AMPARs to other GRIP-associated proteins (Kornau et al., 1995). The MAGUK (membrane associated guanylate kinase) family of synaptic proteins is composed of four members and each can undergo protein-protein interactions with multiple binding partners. MAGUK members include: Post-Synaptic Density 95 (PSD-95), Synapse Associated Protein 97 (SAP97), chapsyn-110/PSD-93 and SAP102. Each member is distributed differently across neuronal compartments. PSD-95 and PSD-93 are highly concentrated in the PSD, whereas SAP102 and SAP97 are found in dendrites and axons and are equally abundant in the cytosol and the synapse (Gardoni, 2008). SAP97 is thought to be particularly relevant for processing AMPA receptor subunits and NR2A subunits of NMDA receptors (Mauceri et al., 2004), whereas SAP102 is crucial for driving NR2B complexes to spines (Sans et al., 2003). PSD-95 preferentially binds NR2A in vivo, while SAP102 is mainly associated with NR2B. In mature synapses, the NR2B-SAP102 complex is replaced by the NR2A-PSD-95/PSD-93 complex. Also, overexpression of PSD-95 promotes synaptic insertion of NR2A and not NR2B, thus modifying the functional properties of synaptic NMDARs.
On the whole, PSD-95 and PSD-93 are thought to be specifically associated with synaptic functions, whereas SAP97 and SAP102 may be more important in trafficking. (Kim & Sheng, 2004)

Figure 3 A schematic diagram of the organization of PDZ proteins at a mammalian excitatory synapse. AKAP79, A-kinase anchor protein 79; AMPAR, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor; βPIX, PAAK-interactive exchange factor; CaMKIIα, α-subunit of Ca2+/calmodulin-dependent protein kinase II; GK, guanylate kinase-like domain; EphR, ephrin receptor; ErbB2, EGF-related peptide receptor; GKAP, guanylate kinase-associated protein; GRIP, glutamate-receptor-interacting protein; IP3R, IP3 receptor; IRSp53, insulin-receptor substrate p53; K ch, potassium channel; LIN7, lin7 homologue; LIN10, lin10 homologue; mGluR, metabotropic glutamate receptor; NMDAR, NMDA (N-methyl-D-aspartate) receptor; nNOS, neuronal nitric oxide synthase; PICK1, protein interacting with C kinase 1; PSD-95, postsynaptic density protein 95; SER, smooth endoplasmic reticulum; SH3, Src homology 3 domain; Shank, SH3 and ankyrin repeat-containing protein; SPAR, spine-associated RapGAP; SynGAP, synaptic Ras GTPase-activating protein. (Figure from Kim & Sheng, 2004)

It is widely believed that Ca$^{2+}$-dependent neuronal death is most efficiently mediated through NMDAR activation (Tymianski et al., 1993). NR2A and NR2B
subunits bind to first and second PDZ domains of PSD-95, indicating that the receptor’s interaction with PSD-95 plays an important role in synaptic NMDAR clustering. Still, others have shown that PSD-95 knockout or suppressing PSD-95 expression has no effect on normal NMDAR localisation and functioning (Migaud et al., 1998). Knockout mice also show a significant increase in long term potentiation (LTP), suggesting that interactions between NR2 subunits and PSD-95 play a key role in NMDAR signal transduction. It is hypothesised that PSD-95 forms a connection between the calcium ions entering the neuron and intracellular signal molecules (eg. nNOS – neuronal nitric oxide synthase) (Brenman et al., 1996). If the expression of PSD-95 is suppressed NMDAR excitotoxicity is selectively attenuated without affecting other GluR signalling and Ca\(^{2+}\) entry (Sattler et al., 2000). Selective suppression of PSD-95 also reduces Ca\(^{2+}\)-effected nitric oxide (NO) accumulation, but not nNOS expression. Recently it was shown that PSD-95 and nNOS above other PDZ proteins are keys in modulating NMDAR-dependent neurotoxicity (Cui et al., 2007). These data indicate that PSD-95 is necessary for coupling of NMDAR activation to NO signalling and excitotoxicity.

### 1.4.3 Uncoupling PSD-95 protein interactions as a therapeutic strategy

The aim of numerous previously published studies has been to save the ischemic penumbra and thus minimize total tissue damage (Mehta et al., 2007; Lo, 2008; Ginsberg, 2008). Excitotoxicity via glutamate receptor activation plays a key role in the necrotic and apoptotic death of neurons in the ischemic penumbra (Choi & Rothman, 1990). In light of this, many glutamate receptor blockers have been tested for stroke treatment. Of these, the most extensively studied was the non-competitive NMDA-type glutamate
antagonist, MK-801 (Ginsberg, 2008). To date none of the glutamate receptor antagonists have proven clinically useful for stroke due to poor pharmacology, poor neuroprotection in long-term recovery and severe side-effects of blocking excitatory communication including hallucination, catatonia and hypertension (Olney, 1994; Albers et al., 1995). Subsequently, it was discovered that a more sophisticated approach to preventing glutamate-mediated cell death would be to uncouple the NMDA receptors from their intracellular signal transduction cascades (Aarts et al., 2002; Sattler et al., 1999). To this end, Aarts and colleagues discovered that selective uncoupling of the scaffold protein PSD-95 from the NMDA receptor can prevent excitotoxic neuronal death without blocking normal NMDAR function. They accomplished this using a cell-permeable peptide that incorporates the last nine amino acids of the NMDA receptor 2B subunit and a membrane transduction domain from HIV-1 TAT protein (Tat-NR2B9c) (Fig. 4). The transport of proteins across the blood-brain barrier and consequent entry to neurons is limited. However, the HIV-1 TAT protein is able to enter the brain and cross cell membranes even when coupled with large peptide sequences TAT has been successfully used for therapeutic purposes following systemic application (Kilic et al., 2006). In short-term survival (24h) experiments Tat-NR2B9c significantly reduced focal ischemic brain damage in rats. It also improved neurological function when administered either one hour before or up to three hours after stroke onset (Aarts et al., 2002). In long-term survival (60 days) studies Tat-NR2B9c preserved motor, learning and behavioural abilities in ischemic rats with little or no brain lesion evident upon histological assessment. These results were significantly different from controls and the inactive Tat-NR2B-AA control peptide (Sun et al., 2008). Also, it has recently been shown that by uncoupling PSD-95
from NR2B subunit Tat-NR2B9c acts *in vivo* by impairing pro-death p38 MAP kinase signalling (Soriano *et al.*, 2008).

![Diagram](image)

**Figure 4** Illustration of Tat-NR2B9c selectively uncoupling the scaffolding protein PSD-95 from NMDAR (right). Glutamatergic excitotoxicity is inhibited without blocking normal NMDAR function. (Figure from Aarts *et al.*, 2003)

### 1.4.4 Rat models of stroke

There are many models of focal ischemic stroke in the rat cortex, but currently the most common techniques are modifications of the original middle cerebral artery occlusion (MCAo) model. Because it produces a survivable focal lesion, the MCAo is the most investigated ischemia model in animals and closely resembles focal strokes occurring in the human population. MCAo is a perfusion model that involves passing a removable suture through the internal carotid artery to lodge in the junction of the anterior and middle cerebral arteries (Koizumi *et al.*, 1986). Even though it is classified as a focal stroke, MCAo often results in extensive damage to the hemicortex, thalamus,
cervicomedullary junction, substantia nigra and hypothalamus (Kanemitsu et al., 2002; Garcia et al., 1995). As a result, additional models were introduced to create smaller infarcts. The so-called “Tamura MCAo” uses electrocoagulation to occlude the main trunk of the MCA at the lateral part of the cerebral hemisphere (Tamura et al., 1981a). An infarcted core is produced in the frontal and parietal cortices, while reperfusion via local collaterals from the anterior cerebral artery provides reflow (Lin et al., 2002; Guegan & Sola, 2000). The 3-vessel occlusion model (3VO) involves transient or permanent obstruction of the bilateral common carotid arteries and the MCA on the surface of the brain (Chen et al., 1986). Similar cortical infarcts as seen in the “Tamura MCAo” are induced; however prolonged common carotid occlusion often leads to striatal and pallidal damage (Carmichael, 2005). Both models create smaller infarcts, however extensive cortical and subcortical damage is still produced (Longa et al., 1989).

In order to carefully study reparative processes in the post-ischemic cortex, Wei et al. selectively ligated small surface arterioles of the MCA with fine sutures (Wei et al., 1995). The outcome is a small cortical stroke in a functionally defined area of the rat barrel field. Whereas this method can be practical for chronic studies of ischemia, the necessary microsurgery cannot be performed while acutely recording with microelectrodes. Because of this, the Wei et al. ministroke was modified by electrocoagulating the same surface arteries. Forder et al. previously used this technique and showed a clearly reproducible infarct area in the rat whisker barrel cortex using triphenyltetrazolium chloride (TTC) staining of coronal brain sections (Forder et al., 2005). A similar microvessel coagulation technique has also been extensively studied in squirrel monkeys to evaluate reorganization of the motor cortex when it was necessary to
make lesions localized to the forelimb area without damaging subcortical structures (Nudo & Milliken, 1996).
2 OBJECTIVE AND HYPOTHESIS

2.1 Objective

Currently, there is a lack of viable therapeutics to protect neurons from ischemia and excitotoxicity and poor bench to bedside translation of stroke therapies. One of the reasons there is poor translation is because we don’t have a clear understanding of how neurons function following ischemic injury and whether anti-excitotoxic drugs actually preserve neuronal function. So, there is a need for an in vivo neurophysiologic model of ischemia that will allow us to accurately document what happens to neurons in stroke (not just changes in general tissue histology) and could be eventually used as a preclinical model to validate the effectiveness of neuroprotective compounds.

2.2 Hypotheses

1. Focal cortical ischemia in our 8-channel rostrocaudal rat model results in a loss of evoked and spontaneous potentials.

2. Systemic administration of Tat-NR2B9c leads to reduced ischemic damage as measured by both evoked and spontaneous potentials.
3 METHODS

3.1 Animals

Experiments were conducted using adult male Sprague Dawley rats (350-600 g) in compliance with the ethics protocol of the University Health Network Toronto Hospital Animal Care Committee. Rats were housed in the animal care facility at the Toronto Western Hospital with food and water ad libitum. Experiments were performed in the light phase of a 12-12hr light-dark cycle.

Pre-surgery, animals were exposed to 3 mL isoflurane (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) in a bell jar until loss of righting reflex and blink reflex was observed. The animals were then removed and anesthetised by an intraperitoneal (IP) injection of a urethane solution 0.2g/mL (Sigma Chemical Co., St. Louis, MO, USA) in saline; diluted and injected at a concentration of 1.2 g/kg. During surgery and the subsequent electrophysiology experiments anaesthesia was maintained by an additional dose (20% of the initial dose) of urethane IP in four rats. Spontaneous potentials measured in rats anaesthetised with urethane have similar characteristics to those generated in the conscious animal (Ebenezer, 1986).

Adequate levels of anaesthesia were confirmed every fifteen minutes during surgery and the experiment, by observation of a weak or absent withdrawal response to pinching the rat’s hind paws with blunt forceps. A Homeothermic Blanket Control Unit (Harvard Apparatus Ltd., Edenbridge, UK) was used to maintain the rat’s temperature at 36-37°C throughout the experiment via feedback of a rectal thermometer.
3.2 Pre-Experiment Surgery

After induction of anaesthesia, the hair on the dorsal surface of the head was shaved, to facilitate incision making. The rat’s head was then mounted in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA), with non-sharp earbars and a mouthpiece with an incisor bar. Upon fixing the rat’s head in position, local anaesthetic, 2% lidocaine (0.1-0.2ml) (Astra Pharma Inc., Mississauga, ON, Canada), was injected intradermally and after 5 minutes an incision was made along the anterior-posterior axis on the dorsal surface of the cranium. The skin was then retracted and clamped, exposing bregma and lambda. The anterior-posterior (AP) and medial-lateral (ML) coordinates for the area of stimulation and recording were located on the dorsal surface of the exposed skull. The Paxinos and Watson stereotactic atlas was used to target the ventral posterior lateral nucleus of thalamus (VPL) (coordinates AP -2.3, ML +1.8, DV 6.3mm). For experiments with the anteroposterior 8-channel array and the 3-channel probe, a right rostrocaudal cranial window (AP -5.0 to +4.0, ML midline to ridge) with the dura left intact was made for access to the somatosensory cortex using a high-speed drill (Dremel 10.8V Lithium-ion Cordless rotary tool, Dremel, Racine, USA). In two experiments with the mediolateral 6-channel array, another window was made on the left hemisphere (AP 0 to +4.0, ML midline to ridge), with the midline intact to prevent bleeding. In order to access the middle cerebral artery (MCA) for ischemia induction, a lateral extension of the craniotomy, approximately 5 mm in diameter, was made underneath the cranial ridge on the right hemisphere. Lastly, an incision was made in the dorsal neck of the animal for a common ground. The grounding consisted of a saline-soaked metal cup connected by a silver thread to a ground pin in the electrode array.
3.3 Microelectrodes

Microelectrodes were assembled from parlyene-C-insulated tungsten wires (Micro Probe, Inc., Potomac, MD, USA), with a 20 µm tip length. To decrease the impedance for recording and/or stimulation, the electrode tips (Fig. 5c,d) (tip size ~1 µm) were electroplated in 24 karat yellow gold electroplating solution (Krohn Technical Products, Carlstadt, NJ) and then platinizing solution (VWR Scientific Products, West Chester, USA) using a Stimulus Isolator (A360 World Precision Instruments, New Haven, CT, USA) and 1 µA of cathodal direct current applied to the electrode for approximately 10 s, giving final impedances of 200-400 kΩ. Finally, the microelectrodes were insulated by a sleeve of polyimide Kapton (Micro ML Tubing Sales Inc., College Point, NY, USA).

3.3.1 Triple recording electrode

Five experiments were performed using three microelectrodes glued together to form a triple electrode probe with tips equidistant from each other (~1 mm). The probe was inserted into the sensorimotor cortex (AP +2.0, ML +3.0, DV 2.1 mm). These coordinates have previously been used by Eckert and Racine to test long term potentiation in the cortex via thalamically evoked potentials (Eckert & Racine, 2006). We used these electrodes in preliminary experiments to verify current sources and sinks in the cortex by creating a depth profile of evoked field potentials. The electrodes were advanced from the cortical surface for 3000 µm in 200 µm increments using a hydraulic micropositioner (Scherr-Tumico Inc., St. James, MN, USA).
3.3.2 Anteroposterior 8-channel array

A total of 20 experiments were performed using an 8-channel microelectrode array, with 1 mm tip separation (Fig 5a). Electrodes were inserted into a 10-channel Omnetics connector (Omnetics Connector Corp, Minneapolis MN, USA) (Fig. 5 c,d). Upon insertion into the connector, the ends of each microelectrode were coated with a solvent based conductive compound (Permatex Inc., Hartford, CT, USA). Finally, the electrodes were secured with hot glue and the impedance was tested through the connector (200-400 kΩ) using NeuroAmp-1A (Axon Instruments, Union City, CA, USA). The array was arranged rostrocaudally across the fronto-parietal hemicortex with respect to Bregma (ML +3.0 to 3.5, DV 2.0 mm). Electrode 1 was the most anterior and electrode 8 the most posterior electrode, with inner electrodes 4 and 5 centred on bregma.

3.3.3 Medialateral 6-channel array

Additionally, to examine the neurophysiological effects of ischemia on the contralateral cortex, we performed two experiments using a 6-channel microelectrode array arranged mediolaterally, spanning both hemispheres and recording from 2 mm below cortical surface (Fig. 5b). The array was a modification of the 8-channel electrode (described in section 3.3.2) whereby electrodes 4 and 5 were removed in order to traverse the intact midline bone. Stimulation was given to the thalamic VPL nuclei ipsilateral to the ischemic cortex (see coordinates in section 3.2).
Figure 5 Illustration of electrode array and placement with respect to the rat cortex. Top two photographs are modified from Coyle et al (1982). Cerebral arteries have been injected with Vultex, a white latex based compound. Small circles indicate dorsal interarterial anastomoses. Numbers indicate electrodes or channels. MCA – middle cerebral artery, ACA – anterior cerebral artery, PCA – posterior cerebral artery. Blue X’s indicate points of occlusion corresponding to 3 branches of the MCA and one frontal branch of the ACA. A) Position of 8-channel frontoparietal electrode array in the rat cortex. Electrode spacing is 1 mm. B) Placement of the 6-channel mediolateral array. Ischemia is induced in only the left hemicortex. C) Photograph of the 8-channel array. The two middle electrodes are taken out in the 6-channel mediolateral array in order to avoid the cranial midline. D) Same array under a microscope.
3.4 Electrophysiological Recordings

Electrophysiological recordings of neuronal activity were made via a linear 8-channel microelectrode array oriented in the parasagittal plane 2 mm from the midline. In some cases a 6-microelectrode array was used, oriented in the coronal plane at the level of Bregma. Biphasic cathodal or anodal electrical stimulation in the thalamus using the A310 Accupulser and stimulus isolator (World Precision Instruments, New Haven, CT, USA) evoked field-potentials in the cortex. The signals detected by the triple electrode probe were amplified using the NeuroAmp-1A (Axon Instruments) (Gain 10,000, High Pass filter 3 Hz, Low Pass filter 5 kHz). Signals detected by the two microelectrode arrays were amplified using the Plexon Recorder/16 neural data acquisition systems (Plexon Inc., Dallas, TX, USA) (filter settings 3 Hz – 5 kHz, Gain 5000). Each output was digitized at a sampling rate of 12 kHz, displayed, and recorded using a MICRO 1401 mkII computer interface device, and Spike2 6.04 neurological capture software (Cambridge Electronic Design Ltd., Cambridge, England) for offline analysis. Three electrodes with the largest evoked potentials and most consistent components were selected for online display to be monitored throughout the experiment.

3.4.1 Input-Output Curve

Input-output curves were taken at the beginning of every experiment in order to determine the optimal stimulation intensity. Biphasic current pulses (200 µS per phase) of increasing intensity (20, 40, 60, 80, 100, 120 and 140 µA) were delivered at a rate of 0.1Hz. At each intensity level, the amplitude of the first positive peak evoked at 10 ms
was averaged (n = 10-20). The stimulation which yielded half of the maximal amplitude response was chosen for subsequent baselines and post ischemic tests pulses.

### 3.4.2 Stimulation parameters and ischemia induction

At the beginning of every experiment, a stable baseline of 5 - 10 min of both spontaneous and evoked potentials (EPs) was obtained. The EPs were grouped in clusters of 20 test pulses (0.1 Hz). The right middle cerebral artery (MCA) was localised under a high-power microscope (Carl Zeiss AG, Oberkochen, Germany). Ischemia was induced the stroke-only and Tat-NR2B9c + stroke groups at T = 0 by coagulating the visible arteriolar branches of the MCA using a microcautery pen (Fig. 6). This is a The control animals received no microcoagulation. This procedure is a modification of a similar focal coagulation from Forder et al. (2005).
Figure 6 Illustration of the surface vessel area pattern (modified from Forder et al. 2005). Distal arteries branching from the MCA are stained with Evans Blue dye in the picture (dark). Focal ischemia is induced by cauterising these critical branches with a microcautery pen as indicated by X.

After induction of stroke, stimulation pulses were administered every minute for 15 min, (n = 20 per cluster). Subsequently, the interval between clusters was increased to 5 minutes for the duration of 45 min (up to 1 h post stroke). From 1 h to 3 h after occlusion, each cluster was administered every 15 min (Fig. 7).
Figure 7 Schematic diagram of the thalamic stimulation parameters before, during and after induction of ischemia. Each horizontal line corresponds to a cluster of 20 test pulses (0.1Hz). Upon induction of ischemia (T = 0), a stimulation cluster was administered every minute for 15 min. Subsequently, the interval between clusters was increased to 5 minutes for the duration of 45 min (up to 1 h post stroke). From 1 h to 3 h post stroke, each cluster was administered every 15 min.

3.4.3 Tat-NR2B9c peptide synthesis and administration

Tat-NR2B9c was custom synthesized at the Advanced Protein Technology Centre at The Hospital for Sick Children in Toronto. It was generated by solid phase peptide synthesis and cleaved from the solid substrate using TFA (trifluoroacetate) then lyophilised. Tat-NR2B9c was reconstituted in Dr. Michelle Aarts’ lab at a concentration of 1M in water and dialysed overnight at 4°C against 10mM ammonium bicarbonate buffer pH 7.0 to remove TFA. The dialysed peptide was aliquoted and stored at -80°C. Tat-NR2B9c concentration was determined by the O.D. at 280nm, which is feasible for small peptides because it contains a tyrosine residue. In the present study, Tat-NR2B9c peptide (3 nmol/g of body weight) was hand injected via tail vein in 5 animals 5 minutes before ischemia induction.
3.4.4 Regional cerebral blood flow (rCBF)

Cerebral blood flow was measured in one stroke rat and three control animals for the duration of the experiment (3 h) with a Moore Instruments Doppler Flowmeter (Devon, UK). The laser probe was placed 2 – 3 mm above the surface of the cortex near in the vicinity of the 3rd and 4th electrode. Large veins and arteries were avoided in order to obtain regional blood flow of multiple vessels.

3.5 Data Analysis

Following experiments, extracellular evoked potentials were smoothed, averaged (Spike2, CED, Cambridge, UK) and plotted (Sigma Plot, SPSS, Chicago, USA). Fast Fourier transforms (FFT) were used to analyze acute loss of power spectra following stroke. Spontaneous EEG power was obtained from 1 min time windows before and after ischemia without any overlap. The frequency spectrum was largely restricted to the 2-8 Hz range across all electrodes and animals. The delta slow wave activity (2-4 Hz) was the most prevalent frequency band and was readily observed in ischemic and sham rats. In order to characterize loss of FFT power across animals we calculated the area under curve (AUC) by taking 0-20 Hz range on the x-axis (Fig. 8).
Figure 8 Power histograms of spontaneous cortical potentials taken over periods of 1 min before and after stroke. Delta waves 2-4 Hz are the most prevalent oscillatory frequencies. The gray shading indicates the area under curve (AUC) which was subsequently averaged across channels and animals in order to calculate loss of EEG power as a result of stroke.

Two-way ANOVAs were used to test main effects and a p value of 0.05 was taken as significant (Sigma Stat, Systat Software, Inc., San Jose, USA). Suppression of evoked potentials was evaluated using the biphasic W_FP script in Spike2. Amplitudes of the first positive (P1) and first negative (N1) components were calculated and summated to obtain a peak-to-trough (P1+N1) measurement. These measurements were then normalised to baseline for channel 3 in each animal and baselines averaged across experiments.

Filtering for different frequency bands was done by processing the raw waveform data through a fourth order bandpass filter. A type of rectification function called root mean squared (RMS) was then applied to the filtered data to render a rectified waveform (positive only) of the specific oscillation.
CSD analysis was used to determine the net extracellular current flow into and out of the active neuronal tissue as a function of distance (Fig. 9). The current flow along an axon is known to be equal to the second spatial derivative of the membrane potential.

\[ i_m = \frac{\partial^2 V_m}{\partial X^2} \quad i_m = \text{units of current/area or current density} \]

\[ K = \text{proportionality constant} \]

The current source density function was obtained by calculating the second derivative of the voltages from the depth profile:

\[ i_m(t) = \frac{\partial^2 V(t)}{\partial X^2} = \frac{V_b + V_a - 2V_0}{\Delta X^2} \]

\[ V_0 = V(x, t) \]

\[ V_a = V(x - \Delta x, t) \]

\[ V_a = V(x + \Delta x, t) \]

Figure 9 Diagram for current source-density analysis. Representations of two neurons is shown on the left. The dots represent point for measuring extracellular fields and \( \Delta x \) represents the distance between them (Johnston & Wu, 1995).
4 RESULTS

4.1 Early and late components

Evoked potentials in a rostrocaudal axis of the somatosensory cortex were mapped out across time. Baseline potentials at a 2 mm depth had comparable components across rats. The evoked potential had 4 discernable synaptic components and one early presynaptic fibre volley across animals (Fig. 10). The fibre volley (FV) was the most consistent component in all animals. It always had a negative deflection and a poststimulus peak latency of 3.1±0.2ms (mean±S.E., n = 21) at this depth. The subsequent components were named according to their orientation and assigned a number based on order of latency. The first synaptic component (P1) had a positive peak at 12.9±1.7ms (mean±S.E., n = 20). The second component (N1) was negative and had a maximum deflection at 34.2±2.2ms (mean±S.E., n = 19). Both P1 and N1 were readily noticeable and consistent across rats and have both been previously documented (Eckert & Racine, 2006;Di & Barth, 1991). The third component (P2) was positive and displayed a late peak at 103.0±8.8ms (mean±S.E., n = 19). The fourth and final component (N2) had a very late negative deflection at 208.5±9.8ms (mean±S.E., n = 19). P2 and N2 were not as consistent in amplitude and latency as the earlier P1 and N1, which has been shown before (Di & Barth, 1991). Lastly, a few very late components that had an onset at 300ms were found to be particularly susceptible to change and have been previously recorded in SEP studies (Henninger et al., 2007).
Figure 10 Illustration of a cortical potential evoked via ventrolateral thalamic stimulation. Typically, four distinct (P – positive, N – negative) components and a fibre volley (FV in inset) were present. The early P1/N1 biphasic component was followed by positive (P2) and negative (N2) slow waves.

4.2 Depth Profile

Figure 11 shows the primary responses to stimulation of the sensorimotor cortex to VPL stimulation. In order to determine the evoked responses of neurons in the sensorimotor cortex, potentials were evoked in increments of 200 um from the cortical surface until the triple recording electrodes reached a depth of 3 mm which corresponds to the deeper layer of the cortex (Paxinos & Watson, 1986). This yielded a depth profile of individual components – the fibre volley (FV), the first positive component (P1) and
the first negative component (N1). The peak latency of all three components was shorter in the deeper layers of the cortex.

Classic experiments have shown that small components with early post stimulus latencies (<5ms) tend to represent afferent fibre volleys, while later larger components represent synaptic activity in the neuron (Malis & Kruger, 1956; Shaw & Teyler, 1982; Mitzdorf & Singer, 1978). In the present study, FV had a negative deflection and was present most clearly at 1.6 – 2.0 mm below the cortical surface (see also Fig. 10). The amplitude of the volley reached approximately 100 uV at a depth of 1.8 mm after which it decreased until 2.4 mm below surface, where it reached a stable inflection point.

The first positive component (P1) was found to be present throughout the cortex. Like the FV, P1 also increased in amplitude until it reached a maximum of 140 uV at 1.8 mm below surface. It did not, however, decrease in amplitude at greater depth. The latency of P1 also became shorter as the recording depth increased from cortical surface. At 0 mm the component peaked at 10 ms, while at its 1.8 mm maximum it had a shorter latency of 6.8 ms. P1 retained this latency until 3.0 mm.

The first negative component (N1) increased in amplitude with depth being smallest at the surface and largest in the deep layers. N1 reached a maximal magnitude of 100uV at 2.0 mm, which was observed in all deeper recordings. The peak latency of this component also changed with depth. At 0.4 mm N1 peaked at 30 ms, while at its maximal amplitude the component had a latency of 19 ms. N1 did not further change its latency with depth.

Figure 12 shows one example of activity of spikes to single pulse stimulation in the thalamus. In this example, the post-stimulus time histogram (PSTH, bottom trace)
shows greatest spiking during the negative part of the EP (at 10-15 ms), and less average spiking during the earlier (P1) and later (P2) components of the EP. Spontaneous spiking neurons and spikes driven by thalamic stimulation most often occurred at depths of 2 mm.
Figure 11 Illustration of the depth profile of the thalamically evoked field potential in the rat sensorimotor cortex. The negative fibre volley (FV) is best seen at 1.6-1.8 mm (see arrows). P1 increased in amplitude with depth but did not attenuate. N1 was not present at the surface but was evoked at 0.4 mm and subsequently increased with depth until reaching a maximum at 2.0 mm. All three components had earlier latencies in the deeper layers of the cortex.
Figure 12 The top illustration is a raw trace of one evoked potential showing neuronal activity superimposed on N1. Recording was taken at depth of 2 mm below cortical surface. Middle trace is an averaged EP. Bottom trace is a post-stimulus time histogram (PSTH) corresponding to the average neuronal activity during the field evoked potential.

4.3 Current-source Density (CSD) Analysis

Figure 13 shows an example of a colour contour plot of the CSD associated with the evoked potentials shown in figure 11. There are two apparent current sinks (blue) and two current sources (red). The first sink appeared at a depth of 0.7 mm and was attenuated at 1.2 mm. It peaked at a depth of 1 mm, which corresponds to the largest amplitude of the fibre volley. The sink became active at a post-stimulus latency of 2 ms and terminated at 7 ms. The first current source had a small area of activation from 1.2
mm to 1.5 mm below cortical surface while the highest power was at 1.4 mm. The source started at 2 ms and terminated at 8 ms post stimulus. The second current sink covered a large area from 1.5 mm to 2.0 mm below surface, with a power maximum at 1.8 mm. This power maximum coincided with the maximum of P1. The current sink was observed starting just before 2 ms and had two points of termination. At 1.6 mm it terminated at 9 ms, but at deeper levels (1.7-2.0 mm) the same sink terminated early at 4 ms. The second current source was spatially localised at 2.2 mm below the surface of cortex, initiating at 2 ms and terminating at 4 ms.

Figure 13 Spectral contour diagram of the current source density calculations from one depth profile of the sensorimotor cortex in the anaesthetised rat. The large dark blue areas at depths of 1.0 mm and 1.8 mm below the cortical surface correspond to localised current sinks. The smaller bright red areas at subcortical depths of 1.4 and 2.2 mm are current sources.
4.4 Regional Cerebral Blood Flow

Regional cerebral blood flow (rCBF) as measured by a Doppler blood flux meter in one rat dropped in a gradual manner over a period of 3 h to 27% of baseline following induction of ischemia (Fig. 14). Three control experiments showed that rCBF steadily increased over the duration of the experiment to 140% of baseline.

Figure 14 Cerebral blood flow versus time following a stroke (n = 1) and in control rat (n = 3) as measured with a Doppler laser probe placed in the middle of the array just above the cortical surface. The stroke rat experienced a slow drop in blood flow reaching 27% of pre-ischemic levels. The control group displayed a gradual increase in flow reaching 140% of baseline at 3 h.
4.5 Spontaneous Potentials

4.5.1 Total loss of EEG power across channels after stroke

Figure 15 illustrates the loss of spontaneous potentials across all 8 electrodes in one animal after induction of ischemia. The stroke-only group displayed significantly less power of spontaneous potentials when compared to control at both 20 min (two way ANOVA F = 16.286, d.f. = 23 p<0.001) and 1 h after ischemia (p<0.001) (Fig. 16). Twenty minutes after occlusion, the average area under the curve (AUC) of the power histogram on all channels in the stroke group (n = 8) was 35.2±4.3% of baseline (Fig. 16). At the same time point the control animals (n = 5) displayed an AUC average of 103.5±4.8% of baseline. One hour after infarct, there was a partial recovery of power in the stroke animals - average stroke AUC was 50.4±6.4% of pre ischemic levels. The control group displayed an average of 100.9±6.6% of baseline.

4.5.2 Greater recovery of EEG power in Tat-NR2B9c + stroke group

Ischemic animals treated with Tat-NR2B9c also showed a drop in EEG power after 20 min to an average across channels of 51.5±4.8% baseline (Fig. 16). Partial recovery was observed at 1 h with an average of 69.4±5.7% of pre-stroke levels. However, the spontaneous potentials in the Tat-NR2B9c + stroke treatment displayed significantly more power than the stroke-only group at both 20 min (p<0.05) and 1 h (p<0.05) after infarct.
Figure 15 FFT waterfall illustration of EEG power loss after stroke (T = 0 min). Each trace is a power histogram of a 1 min section of spontaneous cortical rhythms. Suppression of spontaneous potentials directly follows stroke on every channel. Epileptiform discharges were present in this particular experiment at 10 min and 30 min after stroke. Frequency scale on x-axis is from 1 – 25 Hz.
Figure 16 Total EEG power in control (n = 6), stroke (n = 8) and TAT-NR2B9c + stroke (n = 5) animals. Areas under curve (AUC, 0-20Hz, predominantly 3 – 4 Hz delta oscillations) were averaged across experiments and channels and normalized to % of baseline. Loss of power was not seen in control animals at either 20 min (104% baseline) or 1 h (101% baseline) post baseline. The stroke only group displayed a significant loss of power across channels compared to controls at 20 min (35% baseline) and 1 h (52.4% baseline) post stroke (p<0.001). The Tat-NR2B9c+stroke group lost significantly less power at both 20 min (51.5% baseline) and 1 h (69.4% baseline) when compared to stroke-only rats (p<0.05, *).

Rats in the untreated stroke group exhibited stronger reduction of EEG power in the anterior channels (Ch1-4) when compared to the posterior ones (Ch5-8). Anterior channels recorded an average reduction in EEG power to 24.2±2.7% baseline 20 min after stroke (Fig. 17c). The posterior channels showed significantly less reduction in EEG
power to 35.5±4.2% of baseline compared to anterior channels (one way ANOVA $F = 15.885$, d.f. = 7, $p<0.05$, n = 8). At 1 hour after ischemia, anterior electrodes displayed significantly less recovery of spontaneous potentials when compared to posterior channels ($p<0.01$) (Fig. 17d). Ch1-4 averaged 45.8±7.3% of baseline, whereas Ch5-8 averaged 64.9±10.9% of baseline. Differences between anterior and posterior electrodes in both the controls and ischemic rats treated with the Tat-NR2B9c peptide were not found to be significantly different (Fig. 17e,f).
Figure 17 Bar graphs illustrating change in EEG power. One bar in each graph corresponds to one channel on the electrode. Ch1 is the most anterior, Ch8 the most posterior and Bregma is located between channels 4 and 5 (refer to fig. 5a) A-B) Control animals (n = 6) did not lose power of spontaneous potentials at 20 and 1h after baseline; C) Stroke-only (n = 8) rats showed a decrease in EEG power 20 min after infarct with greater suppression in the anterior of the array (p<0.05, *); D) Partial recovery was observed in the stroke-only group at 1 h after ischemia. Anterior array still showed increased loss of EEG power (p<0.01, **); E) Ischemic rats treated with Tat-NR2B9c (n = 5) showed no difference in power loss between anterior and posterior contacts; F) At 1 h the Tat-NR2B9c recovered more than the stroke-only group.
4.5.3 Hemicortical localisation of post stroke EEG power loss

Experiments in two animals were performed with a mediolateral array spanning both sides of the cerebrum, with the ischemic infarct localised to the right hemicortex. Figure 18 illustrates the averages of the EEG power recorded by three electrodes on either side of the brain. At 20 min post stroke, the ischemic hemicortex had a drop in EEG power to 38.4±15.9% of baseline, while the control side averaged 79.9±16.7% of pre-stroke levels. One hour after stroke, the ischemic hemicortex recovered only minimally to 40.3±11.9% of baseline. At the same time the contralateral control cortex recovered fully to 100.6±8.0% of baseline.

**Figure 18** EEG power measured on both the L (stroke) and R (control) cortices. 20 min after infarct the stroke side lost more power (38.4% baseline) than control (79.9% baseline). No EEG recovery was observed on the stroke cortex (40.3% baseline), while the control half returned to pre-ischemic levels (100.7%).
4.6 Effects of stroke on evoked potentials

Suppression of evoked potentials (EP) after occlusion was ubiquitous across both the stroke-only group and the ischemic rats treated with Tat-NR2B9c. This initial flattening always occurred within 3 min of stroke (Fig. 19).

After partial recovery, further spontaneous attenuations of EPs occurred in both stroke-only rats and ischemic animals treated with the TAT-NR2B9c peptide. This decrease in amplitude occurred on all channels at the same time. In the stroke-only group EPs were spontaneously suppressed in 4 out of 5 animals, ranging from 2 – 4 times during the 3 h experiments. Figure 19 illustrates one such example where attenuation of EP amplitude occurred at 5, 8 and 56 min after occlusion. We found a similar phenomenon in the TAT-NR2B9c + stroke group. All 5 animals treated with the peptide showed spontaneous EP suppression, ranging from 1 – 3 times during the experiment. Lastly, the control animals (n = 5) showed no decrease in evoked potentials at any point. Regional cerebral blood flow (rCBF) was measured in one rat after arteriolar occlusion. Acute spontaneous decreases in EP amplitude coincided with short term increases in rCBF (Fig. 20).
Figure 19 Illustration of the evoked potential across time in one rat. Note the complete EP suppression at 1 min post-infarct. Also, in this particular animal spontaneous attenuation is seen at 7, 20 and 50 min. The evoked components P1 and N1 are increased in amplitude between 2 - 4 min.
Figure 20 Illustration of the increased amplitude of the P1/N1 biphasic component correlated with an acute decrease of regional cerebral blood flow (rCBF, middle trace). The top four traces are averaged EPs at four different time points corresponding to events in the trigger channel.
4.6.1 Biphasic component P1/N1

The absolute value of the amplitudes of the first positive (P1) and the first negative components (N1) were added together (P1/N1) in order to determine the total change in evoked potential amplitude over time. In all cases following stroke (including Tat-NR2B9c treated animals) the P1/N1 amplitude decreased to zero by 3 min. However, in the minutes after infarct there was considerable variation in the peak-to-peak amplitude across rats in both the stroke-only and the Tat-NR2B9c+stroke groups. In 3 out of 6 stroke-only experiments the P1/N1 amplitude recovered and exceeded baseline levels at some point during the first 15 min after ischemia induction. An example of this is shown in Fig. 21. The remaining 3 cases showed no post-stroke P1/N1 potentiation and either remained partially attenuated or transiently returned to pre-ischemic levels. In the Tat-NR2B9c treated animals 2 out of a total of 5 cases exhibited P1/N1 potentiation in the minutes after infarct, while the remaining were suppressed below baseline, much like the stroke cases. This early short-lasting potentiation was not observed in the controls (n = 4), where the biphasic levels remained at baseline.
Figure 21 Overlay of the baseline and post-stroke evoked potentials. Note the increase in the P1/N1 biphasic component after stroke.

Once the evoked potential recovered, either partially or completely, P1/N1 amplitudes in all three groups (stroke, Tat-NR2B9c+stroke and control) were stable to the 3 h end point. All treatments were averaged across time from 15 min to 3 h and normalised to their baselines (Fig. 22). In stroke-only group (n = 4) the average P1/N1 amplitude was 61.9±4.3 % of baseline. This was significantly less than the controls (n = 4), where the P1/N1 biphasic amplitude average was 97.8±3.7% of baseline (two way ANOVA, F = 49, d.f. = 35, p<0.001). There was only slight suppression of the evoked potential components in the Tat-NR2B9c + stroke treatment (n = 4), P1+N1 averaging...
91.7±5.2% of baseline. This was significantly greater than the stroke-only group (p<0.001), but not different from the controls (p=0.642).

![Graph showing P1/N1 biphasic amplitudes](image_url)

**Figure 22** P1/N1 biphasic amplitudes averaged across all time points (15 min to 3 h) and normalised to baseline for each treatment. The evoked potential was significantly less in the stroke-only group (61.9% baseline, n = 4, p<0.001) when compared to controls (97.8% baseline, n = 4). Treatment with Tat-NR2B9c in stroke rats rescued the evoked potential (91.7%, n = 4) to the levels seen in control animals (p=0.642).

### 4.6.2 Bilateral suppression of the evoked potentials following stroke

The evoked response was also measured using the mediolateral array. A phase shift was observed, whereby the primary components of the EP have changed orientation (Fig. 23). Following ischemia on the right side of the cortex, attenuation of EP was seen
on both the ischemic and control hemispheres (n = 2). EP amplitudes recovered on both sides within minutes as seen in the anteroposterior recordings.

**Figure 23** Illustration of evoked potentials from both the stroke (R) and control (L) rat cortices. Loss of the evoked response is present on both sides to unilateral R side stimulation. Also, note the phase shift manifesting in inversion of the post-stimulus components of the EP.
4.7 Periodic Epileptiform Discharges (PEDs)

Upon induction of focal ischemia, 5Hz rhythmic, periodic epileptiform discharges (PEDs) were observed in 8 out of 10 stroke-only rats. The duration of these events varied widely across animals, ranging anywhere from 5 s to 5 min. In all 8 cases the discharges began within an hour of infarct, which has been previously reported in an EEG-monitored rat medial carotid artery occlusion (MCAo) model (Hartings et al. 2003). However, in 5 out of the 8 animals the discharges were recorded as early as 15 min after stroke. The PEDs were localized on a single recording electrode in 5 out of 8 rats, whereas in 3 animals they propagated across contacts, often with a single point of origin. Three out of five ischemic animals treated with Tat-NR2B9c also displayed PEDs. Discharges presented within 1 h of ischemia in all three rats. In one instance the PEDs began 15 min after stroke. In all Tat-NR2B9c treatments, the discharges propagated across electrodes.

High frequency oscillations (HFO) in the low and high gamma range (75 – 500 Hz) range were found to precede almost every post-ischemic epileptiform wave (Fig. 24a). HFOs started 5 – 15 s before the onset of the discharge and were terminated at the onset. The actual PED was composed of mainly 5-6 Hz rhythmic discharges manifested in sharp positive spikes and longer negative waves. Band pass analysis uncovered high beta (11 – 30 Hz) activity during the negative slow waves in all cases of epileptiform discharge (Fig. 24b).

Isolated interictal epileptiform discharges (IED) or single spike-wave discharges were observed in four out of ten rats following ischemia (Fig. 24c). The IED consisted of a short negative spike followed by a longer-lasting positive slow wave. Band pass analysis uncovered low gamma oscillations superimposed on the spike and beta activity.
was observed on the subsequent slow wave. In one experiment, a drop in blood flow was shown to coincide with onset of IEDs (Fig. 24d).
Figure 24 Illustrations of epileptiform activity in the rat following cortical ischemia. Frequency analyses in A, B and C were performed using band pass filters. (hms – hour minute second from the start of the experiment); A) Illustration of intracortical EEG showing the gamma (31-175Hz) and ripple (200-500Hz) oscillations preceding the wave-like periodic epileptiform discharge (PED) (bottom trace). Beta oscillations (11-30Hz) are only present during epileptiform wave; B) Interictal beta waves were present between individual spikes of the discharge; C) Isolated interictal epileptiform discharges (IED) were also observed after ischemia (bottom). High frequency oscillations (middle) were present during the negative spike, while beta waves (top) were superimposed on the postspike slow wave; D) Regional cortical blood flow (rCBF) dropped during one instance of particularly strong IED activity.
5 DISCUSSION

5.1 Current-source Density Analysis and Unit Discharges

Current-source density analysis of the rat somatosensory cortex revealed a strong current sink within the middle of the cortex, approximately corresponding to layer IV, which contains afferent input from the thalamus. From there, the sink displayed a strong spread to the upper cortical layers. Previous studies reported evoked responses in the rat cortex following VPL stimulation and described a similar profile and localisation of current sinks upon CSD analysis (Castro-Alamancos & Connors, 1996). The authors show that relatively large negative field potentials were evoked, corresponding to a large current sink that started at short latency (3 ms) within layer IV of the cortex. Within the next 10 ms, this sink shifted higher into layer II-III, whereas a current source shifted in the opposing direction.

Spike-like unit discharges were observed in the middle and late cortical layers of the rat somatosensory cortex. Similar neuronal excitation was previously reported in awake and ketamine anaesthetised rats (Kandel & Buzsaki, 1997) and typically indicates a nearby excitatory field. The spiking occurred 15 ms after the stimulus and often rode upon the first negative component (N1), which indicated post-synaptic activation (Fig. 11). As noted in the introduction, this corresponds to the region containing largest pyramidal cell bodies (cortical layer V). Microelectrodes are more likely to record extracellular spikes from large versus small cell bodies (so-called electrode sampling bias).
5.2 Regional Cerebral Blood Flow (rCBF) Following Ischemia

The present study found a gradual drop in blood flow over a 3 h time course following cauterisation of the distal branches of the MCA. The total occlusion of a cerebral artery leads to initial cessation of blood flow in that vessel, so one would expect this to be reflected in the recording as a sharp drop in the flux reading. However, the Doppler blood flow monitor was raised 3 mm above the cortex so that it measured rCBF over a large area, presumably deriving most of the signal from brain tissue with multiple anastomoses and not the flow through a single blood vessel. Taking this into consideration it is possible that when the primary arteries have been occluded, adjoining vessels partially compensate for the lack of perfusion. Figure 20 illustrates these short-lasting increases in blood flow, the largest deflections occurring at 45 min, 118 min and 135 min after stroke. It has been previously shown that rCBF increases after ischemia and replenishes the affected area (Shi & Liu, 2007). Also because of artery-to-artery anastomoses between the MCA and ACA, the affected area receives additional perfusion even after a permanent occlusion (Coyle & Jokelainen, 1982). Depending on the type of stroke, additional reflow may or may not be adequate for complete recovery to baseline levels. In the present study, a decrease in rCBF to 20% of baseline occurred 3 h after occlusion. Furthermore, it should be noted that only one stroke experiment was performed while measuring blood flow. A larger sample size could yield different degrees of flow changes over the course of the experiment.

Conversely, blood flow in the three control rats increased steadily over the recording period to an average of 140% of baseline across animals. This rise may be due to the initiation of a compensatory cardiac mechanism. Since a large part of the animal’s
cortex was exposed during the experiment it is possible that this brain region required an increasing amount of blood in order to compensate for the stress. However, the observed increase in blood flow was most likely due to brain swelling after prolonged exposure following craniotomy.

5.3 Suppression of Spontaneous Potentials After Stroke

The EEG power of spontaneous potentials was significantly decreased at 20 min and one hour after occlusion. While there was partial recovery at one hour, the power was still considerably lower when compared to controls. In a seminal study, changes in human EEG waveforms were monitored following a critical drop in blood pressure (Trojaborg & Boysen, 1973). The authors performed a test occlusion of the internal carotid arteries in patients undergoing reconstructive surgery to fix lesions in the ICA. They observed a flattening of the EEG as the cerebral blood flow significantly dropped. Subsequently, others have reported a long lasting (2 h) decrease of 60% in surface EEG recordings following permanent rat MCAo (Hartings et al., 2003). Our study has reported comparable power loss to 35% of baseline 20 min and 52% of baseline one hour after stroke. These results show that loss of intracortical spontaneous oscillations occur after a localised stroke to the distal MCA region of the anterior sensorimotor cortex in the urethane anaesthetised rat, and underlie the EEG flattening observed in clinical studies.

The stroke-only group showed significantly more attenuation of spontaneous potentials in the anterior cortex compared to the posterior cortex. This is a novel finding made possible via the use of the 8-channel anteroposterior electrode array. One
explanation for this spatial localisation of EEG flattening can be found by looking at the arteriolar regions of perfusion and middle-anterior cerebral artery anastomoses. Our coagulation surgeries targeted the frontal and parietal branches of the MCA as well as the fronto-dorsal protrusions of the ACA (Fig. 5). In this manner, the localisation of the infarct was frontal (sensorimotor) and not parietal. Despite the fact that our model induced a permanent occlusion of cerebral arteries, increases in rCBF are known to occur after stroke and serve to replenish the affected area (Shi & Liu, 2007). However, only regions where the arterial inflow is not completely compromised will get the benefit from this reperfusion. The posterior parietal area, approximately corresponding to posterior contacts Ch5 – Ch8, is farthest away from the stroke and additionally perfused by the posterior cerebral artery (PCA) (Coyle & Jokelainen, 1982). The parietal region is thus much less not affected by the stroke. Also, since the ischemia primarily affects the frontal branches of the ACA, blood flow to the parietal cortex from the PCA is expected to remain fairly stable.

5.4 Evoked Potentials

The present study reported an evoked response in the sensorimotor rat cortex upon stimulation of the ventrolateral thalamus. The EP profile consisted of four post-stimulus components – a positive-negative biphasic component (P1/N1) followed by two late slow waves (P2 and N2).

A very similar pattern of EP components has previously been described using somatosensory evoked potentials (SEP) recorded from the rat barrel cortex upon vibrissal
stimulation (Di & Barth, 1991). The authors reported four distinct response peaks - P1, N1, P2 and N2. P1/N1 was a fast positive-negative biphasic wave sequence which was followed by positive (P2) and negative (N2) slow waves. They also presented topographical findings of evoked responses, which give us some idea as to the nature of these individual components. The authors showed that while P1 is much more focal than both N2 and P2, it is distributed over twice the epicortical area of the later N1. These findings are in agreement with a previous study of the topography of auditory evoked potentials (AEP) in the rat auditory cortex. The early positive P1 wave also covered approximately twice the epicortical area of N1 (Barth & Di, 1990). The authors thus concluded that the early positive P1 component of the EP was produced by distributed neural processing through polysynaptic activation of pyramidal axon collaterals. Conversely, the later negative N1 peak is thought to lead to focal monosynaptic and disynaptic activation and is probably not exclusively the result of specific thalamocortical afferents (Di & Barth, 1991).

In contrast to the early components of the evoked potential, the slow P2 and N2 waveforms have been shown to be widely distributed on the surface of barrel cortex (Di & Barth, 1991). We have also observed that amplitude and latency of these peaks are much more variable than that of the early sharp waves. Di and Barth (1991) hypothesised that given their long latency and wide spatial distribution the later slow waves may reflect integrative processing involving much of the cortex. They are thought to be the result of both excitatory and inhibitory postsynaptic potentials. Studies of epicortical evoked responses in the cat have categorised these longer latency peaks as associative responses, produced outside of primary somatosensory cortex (Dong et al., 1982; Thomson et al.,
1963). However, data from Di and Barth (1991) study does not support this conclusion. They discovered that all components of the evoked potential were approximately localised to the primary somatosensory cortex and additional recordings posterior and inferior to the barrel field yielded no response. Early studies of evoked potentials from the human scalp also suggest that both late and early components of the SEP are generated only in the primary sensory cortex (Stohr & Goldring, 1969). In the present study the ventral posterior lateral nucleus of the thalamus in the rat sensorimotor cortex was consistently stimulated according to previously verified coordinates (Eckert & Racine, 2006). In agreement with Stohr and Goldring 1969, potentials evoked from the most posterior electrodes (Ch7, 8) show weaker evoked responses when compared to the stronger anterior potentials (Ch1, 2).

5.4.1 EP attenuation following ischemia

Results from our study show an immediate and complete attenuation of the evoked response following ischemia. The P1/N1 biphasic component of the EP was significantly attenuated in stroke animals when compared to controls. A similar experiment was performed by Sakatani et al investigating SEPs at the hindlimb, forelimb and trigeminal cortical regions after a standard MCAo in the rat (Sakatani et al., 1990). They report a lasting suppression of the cortical potentials evoked by sciatic, median and trigeminal nerves within 3 minutes of MCAo. The authors also show a spatial localisation of the most profound SEP suppression to occur around the trigeminal region. They show that once perfusion from the MCA is abolished, the trigeminal sensorimotor tissue has no additional arteriolar inflow of blood. Poor arteriolar perfusion combined with inadequate
collateral circulation at the periphery of a circulatory field creates what is known as a ‘watershed effect’. The authors posit that a widespread loss of SEPs reflects a transient ischemia in the entire MCA territory before compensating collateral blood flow takes place. Artery-to-artery anastomoses connect the MCA to the anterior and posterior cerebral arteries (Coyle & Jokelainen, 1982). Retrograde blood flow has been observed in the distal ends of the ACA, but this process takes minutes to start. We have evidence of a comparable time course of EP attenuation and partial recovery and hypothesise that a similar loss and recovery of blood flow occurs in our model. Focal ischemia was induced by cauterising the main distal branches of the MCA and one frontal branch of the ACA instead of performing a more global version of the MCAo. Because of artery-to-artery anastomoses between the MCA and ACA the affected area received additional perfusion even after a permanent occlusion. It is possible that the acute recovery of evoked and spontaneous potentials occurred as a result. However, the inadequate blood flow from these anastomoses was probably the reason why full recovery of the evoked response and EEG power was not observed 20 min and 1 h after stroke.

What’s more, we observed a short period of spontaneous suppression of evoked potentials after the initial cautery-induced attenuation. If we assume that the loss of EP’s is primarily due to the loss of blood perfusion to the affected area (i.e. stroke), then we may suggest that any subsequent attenuation is also the result of additional drop in perfusion. This was, however, not the case in the present study, since a correlation between EP suppression and blood flow increase was found (Fig. 20). This unexpected result may be a phenomenon called post-ischemic reperfusion injury. Histological evidence has shown that excessive acute reperfusion and increased rCBF correlate with
more severe brain damage (Tamura et al., 1980). It is possible that the increased rCBF associated with a decrease in absolute EP amplitude seen in the present study presents early neurophysiological evidence of reperfusion injury.

There are however other explanations for the post-ischemic EP attenuation. Specifically, K$^+$ is known to be released from cells following an ischemic attack. Hence, diffusion of K$^+$ out of neurons may also be responsible for the widespread EP suppression. Additionally, K$^+$ is also thought to diffuse into white matter which underlies the cortex and block axons from conducting the evoked potentials from the thalamus (Sakatani et al., 1990). This idea is also supported by evidence of thalamic damage following cortical stroke (Garcia et al., 1995; Kanemitsu et al., 2002). Sakatani and colleagues suggest that spreading depression may be intimately linked with loss of SEP. They hypothesised that neurons in the ischemic cortex may become excited as they depolarise, releasing neurotransmitters into other areas of the cortex, thus transiently inhibiting neuronal excitability.

5.4.2 Post-ischemic P1/N1 acute, transient potentiation

We have observed acute potentiation of the P1/N1 component following ischemia in some rats from both the stroke-only rats and ischemic animals treated with Tat-NR2B9c. Plasticity has been extensively described in stroke literature as both aberrant and beneficial to the recovery of synaptic function (Calabresi et al., 2003). Early anoxic plasticity has been classified as aberrant if it occurs minutes after energy or oxygen deprivation ensues (Hammond et al., 1994). Conversely, long-term potentiation in the
days and weeks after stroke has been shown to lead to beneficial reorganisation of the motor cortex in non-human primates (Nudo et al., 2001)

Specifically, the acute increase of the evoked response following ischemia observed in the present study has been previously described with SEPs in the rat barrel cortex (Sakatani et al., 1990). The authors hypothesised that cellular swelling may have reduced the extracellular volume in the cortical area and increased the electrical impedance of extracellular diffusion paths. Given that field potentials result from extracellular currents (Johnston & Wu, 1995), an increase in tissue impedance would lead to an increase in evoked potential amplitude (Sakatani et al., 1990). However, the amplitude change of biphasic components P1/N1 in our study was not consistent. That is, in some animals potentiation of either P1 or N1 was observed but in many cases there was no change of the evoked response with respect to baseline. It is therefore possible that variation in P1/N1 potentiation was the result of a change in extracellular currents due to post-ischemic cell swelling. Another possibility is that the increase in components of the EP represents synaptic alterations as a result of excess glutamate release. However, since a consistent change was not observed in N1 or P1, the change in amplitude is most likely not due to the release of one specific neurotransmitter. In order to confirm this, subsequent experiments selectively targeting individual neurotransmitters would have to be performed.
5.5 Transhemispheric Changes in Cortical Potentials

The mediolateral array was used to test evoked and spontaneous potentials on both hemispheres after ischemia. Our results show that both the ischemic cortex and the contralateral control side displayed an acute attenuation of EPs followed by a lasting recovery. So why were both sides of the brain affected by an occlusion that was completely localised to one half of the brain? The answer may have something to do with the diaschisis phenomenon.

Diaschisis is defined as a series of remote changes in brain function following stroke (Reinecke et al., 1999; von Monakow, 1969). Cerebral ischemia is known to affect the function of widespread ipsilateral and contralateral brain regions in addition to the area immediately affected by the reduced blood flow. The brain hemispheres are connected through fibre tracts in the corpus callosum. Von Monakow posited in a seminal study of diaschisis that loss of excitatory input from the injured area will render other regions less responsive to stimuli (von Monakow, 1969). Currently there are competing thoughts on this topic. A number of researchers found no significant change in contralateral electrical activity following cortical stroke (Andrews, 1991). However, others reported a contralateral decrease in EP amplitude, which is in agreement with the results presented here and von Monakow’s original hypothesis (Molnar et al., 1988; Kempinsky, 1958).

Upon transhemispheric investigation of spontaneous potentials, we report a predominantly localised attenuation of EEG power loss to the ischemic cortex. However, at 20 min after occlusion there was partial suppression of the EEG on the control hemisphere (80% baseline). A previous study reported a comparable decrease in EEG
power less than an hour after infarct (79% of baseline) (Hossmann et al., 1985). Contralateral power loss may be the result of the primary flow response following ischemia. In order for the blood flow to be restored to the affected area rCBF is partially decreased to the healthy brain early after infarct. This has been previously reported in the Tamura rat model of MCA occlusion (Tamura et al., 1981b). However, diaschisis is the more probable cause for this short-term partial suppression of EEG power as also evidenced by the aforementioned EP attenuation. In our study, intracortical oscillatory EEG power returned to baseline in the control hemisphere within 1 h of stroke. Conversely, the spontaneous potentials on the control side remained suppressed.

5.6 Tat-NR2B9c Application Following Cortical Ischemia

It was recently discovered that selectively uncoupling the PSD-95 scaffolding protein from the NR2B subunit of the NMDA receptor inhibits excitotoxic signalling via oxidative compounds such as nNOS (Sattler et al., 1999; Sattler et al., 1999; Aarts et al., 2002). Tat-NR2B9c is a membrane-permeable peptide that selectively uncouples the PSD-95 protein from the NR2B subunit, without disrupting NMDA activity. It was demonstrated with morphological analysis that Tat-NR2B9c improves stroke outcome and limits infarct size when administered up to 3 h after stroke in vitro. Also, post-stroke application of Tat-NR2B9c in vivo significantly improves behavioural performance following transient MCAo. Rats displayed improved cognition and faster escape times in the Morris water maze (Sun et al., 2008). The present study aims to show the in vivo effect of Tat-NR2B9c on spontaneous and evoked potentials in the ischemic rat cortex.
In our preliminary studies we have provided new evidence of slowed stroke-related neurophysiological dysfunction in anaesthetised rats following Tat-NR2B9c injection in vivo. Neurophysiological parameters such as EEG suppression and EP attenuation are known correlates of brain damage following stroke (Astrup et al., 1977; Trojaborg & Boysen, 1973). We report less suppression of spontaneous intracortical potentials and less attenuation of the evoked response in five ischemic rats treated with Tat-NR2B9c when compared to stroke-only animals. However, epileptiform activity was also observed in ischemic rats following Tat-NR2B9c administration, indicating that the peptide did not have anti-epileptic effects. To our knowledge, these are novel findings not previously reported in stroke literature.

Previously studied anti-stroke compounds such as MK-801 reduce glutamatergic signalling through strong, non-competitive blockade of NMDA receptors (Ginsberg, 2008). This treatment was found to improve synaptic performance by decreasing post-stroke-excitotoxicity but had a poor neuroprotective mechanism and led to serious side effects upon human administration (Olney, 1994; Albers et al., 1995). In contrast we know that Tat-NR2B9c does not alter glutamatergic calcium signalling via NMDARs (Aarts et al., 2002). Disrupting PSD-95 with antisense oligonucleotides or PSD-95 inhibitors did not affect function or normal excitatory transmission (Sattler et al., 1999; Aarts et al., 2002; Migaud et al., 1998). In addition, mutant mice lacking PSD-95 showed facilitation of long-term potentiation indicating that this scaffolding protein acts as an inhibitor of LTP. At the same time, NMDAR currents, normal subunit expression and synaptic morphology have all been unaffected by the selective uncoupling of PSD-95 from the NMDA receptor.
In regards to our studies, we cannot comment with certainty on the precise effect the Tat-NR2B9c peptide that led to recovery of aforementioned neurophysiological parameters. It is possible that the same mechanism which leads to LTP inhibition in PSD-95 knock-outs is responsible for enhanced EP recovery post stroke. However, if this is the case, then we would expect to see increased potentiation of the early P1/N1 wave in Tat-NR2B9c treated animals, which was not observed in our study. It is more probable that the uncoupling of PSD-95 from the NMDA receptor disrupts neuronal excitotoxicity, which not only has as effect on the size of ischemic infarct (Aarts et al., 2002; Sattler et al., 1999), but also on neuronal excitability as evidenced by improved recovery of cortical potentials in the present study.

5.7 Post-ischemic Epileptiform Discharges

We have shown evidence of periodic wave-like and isolated interictal spike-wave discharges in ischemic rats occurring within an hour of infarct. These epileptiform phenomena were observed on the intracortical EEG in 8 out of 10 stroke animals and 3 out of 5 stroke animals treated with Tat-NR2B9c.

Post-stroke epilepsy has been extensively documented in ischemic patients (Myint et al., 2006; Afsar et al., 2003). The Oxford Community Stroke Project reported that 11.5% of patients with stroke were at risk of developing seizures by five years (Burn et al., 1997). Epileptiform activity was also reported and studied in animal studies of stroke (Hartings et al., 2003). Hartings and colleagues offer a detailed description of the phenomena using EEG recordings in vivo following middle cerebral artery occlusion.
(MCAo) in the rat. The authors report 80% incidence of non-convulsive seizures during the first hour of cortical ischemia, which we have replicated in our model of focal cortical stroke. Previous studies have shown that neuronal depolarisations in the ischemic core are accompanied by a high rise in extracellular potassium, manifesting as waves of spreading depression throughout the ischemic hemisphere (Nedergaard & Hansen, 1993). It is hypothesised that these potassium-induced depolarisations are responsible for mediating epileptiform discharges in the MCAo model (Hartings et al., 2003).

Periodic oscillating wave-like discharges seen in our animal experiments are comparable to those reported by Hartings et al., which they classified as intermittent rhythmic delta activity (IRDA). Like our periodic epileptiform waves, IRDA was shown to occur against a background of flattened or desynchronised EEG activity. We report 5 – 6 Hz primary oscillations, whereas the Hartings study observed a slightly faster oscillatory activity in the 6 – 8 Hz range. In the epileptic patient population IRDA is primarily made up of delta (0 – 4 Hz) and lower theta bursts (4 – 6 Hz) and reported incidences are largely confined to the frontal cortex (Neufeld et al., 1999; Fariello et al., 1982). We have also noticed a more frontal concentration of epileptiform activity in our model. However, it is probable that the actual physical insult to the anterior cortex as a result of the focal cautery is responsible for this localisation.

Isolated epileptiform spike-wave discharges (IEDs) not occurring in a wave-like or oscillatory pattern were also observed in our model. Hartings and colleagues have reported similar seizures in their MCAo model and have termed this activity periodic lateralised epileptiform discharges (PLEDs). PLEDs were characterised as biphasic with
an initial negative phase followed by a positive wave-like component, which is comparable to IEDs seen in our rat model of focal ischemia.

5.7.1 High-frequency oscillations coinciding with post-stroke epileptiform activity

In the present study, high frequency oscillations (HFOs) in both gamma (80 – 200 Hz) and ripple (200 – 500 Hz) ranges occurred immediately before all epileptiform wave seizures in the ischemic rat. During each seizure-like wave, oscillations predominantly in the beta (10 – 30 Hz) but also in the gamma frequencies were observed. Lastly, we have observed HFOs to occur during isolated interictal epileptiform discharges (IED) in ischemic animals.

Oscillations in the 100 – 600 Hz frequency range indicate synchronisation of neuronal ensembles (Jones et al., 2000). However, it is not known whether HFOs and epileptic discharges are produced by the same neuronal generators. Previous studies in humans with temporal lobe epilepsy and rodents with kainic acid (KA)-induced seizures have reported ripples to be closely linked to zones of seizure onset (Bragin et al., 1999; Jacobs et al., 2008). During ictal recordings, HFOs have been shown to occur mostly in regions of primary epileptogenesis and less frequently in areas of secondary spread. Bragin and colleagues have shown ripples to precede interictal spikes in the entorhinal cortex of KA kindled rats (Bragin et al., 1999). HFO spiking was also reported to occur on localised EEG contacts in human recordings immediately preceding seizures that is comparable to our findings in the rat (Fisher et al., 1992). HFOs in the human were largely localised to the seizure focus, which was not observed in our experiments. When the epileptiform discharges propagated to different electrode contacts each recording also
displayed a local pre-epileptic gamma discharge. The authors suggest that 80Hz+ activity at seizure onset may be due to several different mechanisms including synchronous action potentials at seizure focus or harmonics of synaptic potentials of high frequency transients associated with sharp EEG activity. Fast ripples (250-600 Hz) have also been reported to occur in the hippocampus between the initial epileptic insult and the onset of recurrent spontaneous discharges (Bragin et al., 2000). The authors hypothesise the fast ripples reflect activity of the so-called pathologically interconnected clusters of neurons (PIN). These clusters are proposed to play a role in initiating epilepsy by acting as a trigger for pathologic synaptic modification in target brain areas. Subsequently, other pathological ripple networks have been identified in epileptic mesial temporal areas of humans (Staba et al., 2004). A higher density of local synaptic connections in the epileptic brain has been attributed to neuronal death and subsequent synaptic reorganisation. It has been suggested that these new regional collaterals may be a substrate for synchronisation of activity resulting in spontaneous bursts of action potentials or population spikes (Bragin et al., 2002). Other experts propose that fast ripples reflect inhibitory post synaptic potentials of principal neurons as a result of synchronised discharges of the interneuronal networks (Ylinen et al., 1995). To our knowledge, this high frequency activity has not been previously reported in the post-ischemic brain preceding epileptic discharges.

We have also observed individual large spike-wave discharges in the rat sensorimotor cortex following stroke. IEDs are thought to reflect summated membrane potentials from abnormally synchronised neurons within epileptic tissue. Population spikes are believed to represent paroxysmal depolarisation shifts, while slow waves
following spikes are the result of prolonged hyperpolarisation (Matsumoto & Ajmone-Marsan, 1964). Upon filtering for high frequency activity, we report gamma activity to coincide with the negative spike of the IED. Beta activity in the 10-30 Hz range was superimposed on the longer positive wave. A recent study of patients with mesial temporal lobe epilepsy has also reported HFOs within the spike and a decrease in the power of high-frequency bands and an increase in beta activity during the postspike slow wave (Urrestarazu et al., 2006). Previous studies have shown that a decrease in excitability following a depolarisation associated with an epileptiform EEG spike may result from both intrinsic and synaptic currents. Seminal studies of penicillin-induced epilepsy have demonstrated that postspike inhibition is widely spread around the epileptic focus (Prince, 1968). This decrease in high frequency activity following the IED spike may represent the suppression of post-synaptic potentials and neuronal firing as a result of the widespread inhibition.
6 CONCLUSION

Presently, a viable treatment for brain ischemia is still being researched. Presented here is an 8-channel model of focal cortical stroke that illustrates the spatial distribution of EEG power loss and attenuation of evoked potentials following infarct. Cortical ischemia results in loss of EEG power and initial suppression of evoked potentials, which confirms the first hypothesis of this study. The Tat-NR2B9c peptide was used to validate this model as a neurophysiological method of assessing anti-stroke therapy. Application of Tat-NR2B9c via tail-vein injection 3 min before ischemia resulted in less EEG flattening and partial recovery of the evoked response. Therefore, systemic application of Tat-NR2B9c slows the progression of stroke-related neurophysiological dysfunction, which confirms our second hypothesis.

7 FUTURE STUDIES

This study validates the focal ischemic model for investigation of novel stroke therapies. In the future the 8-channel neurophysiological model will be used to evaluate the control peptide (Tat-NR2B-AA), which does not uncouple the PSD-95 scaffolding protein from the NMDA receptor. This will confirm whether the improved recovery of EEG power and EP amplitude is mediated by anti-excitotoxic properties of NMDAR-PSD-95 perturbation. It would also be of interest to test the effectiveness of other drugs that haven’t been typically used to treat stroke. For instance, memantine is part of a new class of Alzheimer’s disease medications, which targets neuronal excitotoxicity. As such, memantine could serve as an effective treatment of excitotoxic sequelae associated with ischemia.
Reference List


