1 Introduction

Evolving data implicate the synapse in the pathogenesis of ischemic brain injury and stroke. Ischemic conditions in the brain induce profound changes in synaptic function and synaptic morphology, which may account for early functional loss and deficits observed following stroke. A better understanding of the effects of ischemia on the synapse may help guide novel therapeutic approaches to reduce brain damage and aid in the recovery of function following a stroke.

2 Introduction to Stroke

2.1 The Challenge of Stroke

Stroke accounts for one in every 16 deaths in the United States (2004), making it the third most common cause of death after heart disease and cancer. Stroke is the leading cause of long-term disability, because 76% of people survive their stroke (14). The economic burden of stroke is huge, $63 billion dollars in 2007. Part of the challenge facing stroke research is the multifaceted etiology of the disorder in humans, with many cerebrovascular, cardiac and metabolite risk factors contributing to the condition.

2.2 Classification of Stroke

Stroke is the clinical syndrome resulting from impaired blood flow in the brain due to local disturbances in cerebral perfusion, produced by large vessel (thrombotic) or small vessel (lacunar) in situ vascular occlusion or occlusion by embolism from a
remote source (9, 103). Transient symptoms (TIA: transient ischemic attack) or permanent symptoms (stroke) result from these occlusions. The clinical manifestation of the stroke is defined by the region of brain infarcted: unilateral weakness with or without impairment of language function being the commonest impairment. A focal stroke syndrome may also result from rupture of a blood vessel within the parenchyma of the brain usually as the result of chronic hypertension. The basal ganglia is the most common location and unilateral paralysis is the most common presentation (46).

Global brain ischemia occurs when the blood supply to the entire brain is reduced, for example following cardiac arrest, or due to raised intracranial pressure resulting from the rupture of an intracranial aneurysm (46). Global ischemia produces damage in metabolically sensitive and selectively vulnerable brain regions, such as the hippocampus, basal ganglia and cortical lamina. Damage to the hippocampus has been associated with memory deficits in patients recovering from global ischemia (46).

Fig. 1. Different types of stroke result in different patterns of damage in the brain. Stroke can be caused by a global reduction in global brain perfusion, such as a heart attack or during heart bypass surgery. (Left) Global ischemia results in damage to selective metabolically brain regions such as the hippocampus (black circled region) and the cortical lamina. Focal ischemia can be induced by either a hemorrhage or by local blockade of perfusion, such as results from an embolism. Focal stroke results in local damage to the brain resulting in functional deficits based on that brain region’s function. (Middle) Following a hemorrhage in the brain parenchyma small ischemic regions occur, where as a sub-arachnoid bleed will result in the cortical lamina being subject to ischemic conditions. (Right) Ischemia induced by embolic stroke in a blood vessel result in wedge shaped pattern of damage to the cortical and subcortical regions which receive their greatest blood flow from the occluded vessel.
2.3 Therapeutic Management of Stroke

Each type of ischemic event requires different clinical management: symptomatic ischemia of thrombotic, embolic, or lacunar etiology may improve clinically by administration of thrombolytic therapy (tissue plasminogen activator: tPA) begun within three hours of the onset of symptoms (1). This is a so-called “clot-busting drug”, which will aid the dissolution of a clot. This treatment is counter indicated in hemorrhagic stroke. No neuroprotective drugs to preserve or protect brain parenchyma are currently available and approved by the FDA. Unfortunately with the recent failure of the SAINT II trial (Stroke Acute Ischemic NXY-059 Treatment), interest in spin trap reagents (free radical scavengers) has been reduced (101, 102). Given the increasing prevalence of stroke in the community, which may be related to the increasing population age, it seems imperative that additional therapeutic options be identified.

2.4 Experimental Models to Study Stroke and Ischemia – In vivo Models of Stroke

There are multiple experimental models used to study stroke, because of this caution is needed in interpreting sometimes conflicting results from different models. Different studies may employ different techniques of inducing ischemia, different species or strain of experimental animals, different brain regions for study and differing techniques to determine infarction area/volume and functional effects. Experimental ischemia can be permanent, or transient (temporary) whereby the cause of reduced perfusion is removed, i.e. withdrawal of an occluding filament.

The nature of damage following focal and global ischemia, as well as the development of damage (progressive vs. completed) is determined by the choice of model used to study ischemia. Global ischemia models are performed to reduce blood flow to the entire brain. Common models are the 2-vessel occlusion (whereby the bilateral carotid arteries are clamped and systemic blood pressure reduced) or 4-vessel occlusion (most commonly used in rat) whereby the vertebral arteries are permanently occluded by ligature or cauterizing and then the carotid arteries are transiently occluded by aneurysm clips (95, 113). Some studies have employed a cardiac arrest model whereby the heart is stopped for a varying duration (by KCl injection), and then resuscitated (chest compressions, intravenous epinephrine, and ventilation with 100% O₂).

Focal ischemic models attempt to recapitulate the situation of disrupted local blood flow to a specific area of the brain. Probably the most common model is the filament occlusion of the middle cerebral artery. While commonly used, due to the ease of controlling the reperfusion of the tissue, filament insertion may exacerbate injury following ischemia due to damage to the vascular endothelium (25). Other focal models include electro-coagulation and chemical-coagulation of blood vessels (112) on the surface of the brain. While similar to human stroke the duration of ischemia is more variable in these models, and distal thrombosis may induce secondary infarctions. As an alternative, endothelin-1 has been used to induce constriction in the cerebral blood vessels, which can be reversed by an antagonist,
thereby reducing filament damage to the vasculature endothelium (54). (For a review of ischemia models see (14, 113)).

2.5 Experimental Models to Study Stroke and Ischemia – In vitro Models of Ischemia

Most studies of in vitro ischemia utilize primary cultures of cortical or hippocampal cells (18), although immortalized neuronal and neuronal like cells have been used (55, 56). These cultures however vary between study groups, regarding age of cultures, growth media, species (mouse vs. rat) and date of tissue preparation (embryonic vs. post-natal). Most studies focus on the effects of ischemia on neuronal cell populations, however in vitro systems can be used to study astrocyte and oligodendrocyte responses (4, 40). Of note while in general astrocytes tend to show higher resilience to ischemia than neurons, protoplasmic astrocytes may have a similar vulnerability to ischemia as neurons (40). Organotypic slices have also been used for the study of ischemia.

There are a number of methods to mimic ischemic conditions. Hypoxia/anoxia can be induced by placing cells in a solution where oxygen is removed or lowered by bubbling the solution with an anaerobic gas mixture or by placing cells into a hypobaric/anoxic chamber (80). Hypoglycemia can be modeled by the removal of glucose, or its replacement with a non-metabolized variant (2-deoxy glucose) (115). Mitochondrial inhibitors, for example potassium cyanide or 3-nitopropionic acid (115, 117), have been used to model the metabolic inhibition that occurs following ischemia. Ischemia also induces glutamate release and excitotoxicity, hence many studies have focused on the effects of high concentrations of glutamate or the selective agonist N-methyl-D Aspartate (NMDA).

2.6 Physiology and Biochemistry of Brain Ischemia

Ischemic conditions in the brain rapidly deplete neurons, and to a lesser extent astrocytes, of the metabolites oxygen and glucose that are necessary for energy production. Energy loss results in a rundown of the electrochemical gradients of ions across membranes, notably Ca$^{2+}$ and K$^+$, leading to depolarization of cells and neurotransmitter release (59). The rapid rise in intracellular calcium, via influx from voltage and ligand-gated calcium ion channels can activate calcium sensitive proteases such as calpain and cathepsin resulting in protein degradation.

The brain, when subject to focal ischemia, can be divided into two regions. The portion of the brain subject to the most extreme ischemic conditions exhibits rapid cell death and is termed the ischemic core. The region surrounding the ischemic core is termed the penumbra. The penumbra is also vulnerable to ischemia-induced cell damage but cell death evolves over a slower time course. The slower progression of cell death enables the penumbra to be salvaged by attenuating neurochemical cell death processes. In this regard, ischemia activates a complex series of biochemical and molecular processes in the core and penumbra, which have been covered extensively in a number of reviews (28, 34, 45, 69, 76); the following is a brief overview.
In the ischemic core, the combined loss of energy and ion gradients results in cell swelling, compromising the cell membrane and leading to the release of cell constituents and cell death. The mitochondria of cells in the ischemic core show swelling and rupture of contents. Associated with this rapid passive cell death is an accompanying inflammatory response. Released cell constituents (K$^+$ and glutamate) may then cause a cascade effect resulting in further damage to adjacent cells. Raised extracellular K$^+$ can also induce the release of glutamate from astrocytes via reversal of uptake transporters (24). Extracellular glutamate may evoke calcium mediated glutamate release from astrocytes (104). Hence once these conditions of high extracellular glutamate and potassium are obtained, they can overwhelm compensatory mechanisms leading to excitotoxic “necrotic” cell death.

**Fig. 2.** Ischemia induced excitotoxicity. Glutamate excitotoxicity is implicated in the cell damage following ischemic conditions. Excessive extracellular glutamate results in prolonged NMDA receptor activation leading to calcium overload in the cells. Calcium can activate Nitric oxide synthase leading to NO production and the generation of oxygen radicals and peroxynitrite. Calcium can also lead to the activation of proteases, protein kinases and phosphatases. Once a critical threshold is reached, bcl-2 proteins are activated and translocate to the mitochondria, resulting in a loss of mitochondrial membrane potential, and release of apoptogenic factors cytochrome C and AIF. Cytochrome C can then interact with APAF-1 to initiate the caspase cascade which ultimately results in cell death.

In contrast to the ischemic core, cells in the penumbra show transient damage and can die by programmed cell death mechanisms. Following reperfusion to these cells, oxidative stress and the release of free radicals can drive lipid peroxidation and membrane damage. Stress-activated protein kinases initiate the activation of intracellular signaling cascades associated with apoptosis/programmed cell death. The activation of the pro-cell death protein Bax compromises the mitochondria membrane resulting in the release of cytochrome c and other apoptosis-inducing factors, including AIF (apoptosis inducing factor) and SMAC/Diablo (second
mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) which neutralizes IAPs (inhibitor of apoptosis protein) (13, 41, 63, 68). Released mitochondrial cytochrome C interacts with APAF-1 (Apoptotic Protease Activating Factor-1) and ATP (adenosine 5’ triphosphate) to form the apoptosome, which catalyzes the cleavage and subsequent activation of the caspase family of proteolytic enzymes.

The initiator caspase, caspase 9 is directly activated by the apoptosome and can then cleave and activate caspase 3, which can further cleave and activate executioner caspases (caspases 6, 7 and 8). Caspase 3 also cleaves ICAD (inhibitor of CAD), resulting in the activation of CAD (caspase activated deoxynuclease), which cleaves DNA into regular sized packages (50–300-kb). These biochemical cascades and subsequent DNA damage render the cells committed to die. The complex nature of this cell death cascade reveals many potential therapeutic targets, although to date none have been used successfully in clinical trials (76).

3 Evidence Implicating the Synapse in Mediating Ischemia-Induced Brain Damage

3.1 Synaptically Released Glutamate Mediates Excitotoxicity Following Ischemia

Early work on the retina showed that excessive levels of the excitatory amino acid aspartate resulted in brain damage and neurodegeneration termed excitotoxicity (87). Excitotoxicity due to excessive brain glutamate levels was suggested as a potential mechanism of injury in stroke and other acute/chronic neurological disorders (77). Two pieces of evidence strongly support the role of the synapse in mediating the effect of ischemia. First, it was shown that cutting the pre-synaptic inputs to the CA1 region of the hippocampus blocks global ischemia induced neurodegeneration (11) and that ischemia induced cell death in vitro is attenuated by blocking synaptic release of glutamate with tetanus toxin (85) Second, glutamate receptor antagonists attenuate ischemia-induced brain damage in animal models of global ischemia and stroke (78). Indeed, this latter observation initiated the large interest in NMDA and glutamate receptor acting drugs as potential therapeutics for stroke.

3.2 NMDA Receptor Antagonists as a Therapy for Stroke: Potential and Pitfalls

Since these initial experiments, glutamate receptor blocking agents have been shown to be effective in reducing ischemia induced brain damage in a variety of species (rat, mouse, hamster, gerbil, cat, primate) and ischemia models (focal, global temporary permanent and in vitro models). However, it should be noted that some of the neuroprotective effects of MK801 may be due to its ability to reduce body temperature (61). Yet it is of note that even though it is over thirty years since the initial experiments proving that blockade of excitatory amino acid receptors blocks ischemia induced brain damage, not a single compound has to date been successful
enough in clinical trials to be approved for general clinical use (although the NMDA antagonist memantine was approved for treatment of Alzheimer’s disease by the FDA in 2003) (61).

The reasons behind this apparent failure of glutamate antagonists in clinical stroke trials may be due to multiple factors, including the time window of efficacy, pH modulation of the ligand gated ion channel (low pH in ischemia may reduce channel function) and acute toxicity of the compounds. Furthermore some NMDA blocking compounds show unacceptable side effects such a psychosis (ketamine) or vacuolation of the brain (MK801: dizocilpine) (51). One significant issue is the that time window for therapeutic NMDAR blockade, at least in rodents is slender, up to one hour following the ischemic event (97). Such a short therapeutic time window makes it unlikely that a patient presenting with stroke symptoms will be given an NMDA blocking compound within the effective time range. Indeed this may also be a reason for the failure of the SAINT II trial of free radical blocking agents in stroke (101). However, one potential solution is to identify therapeutic combinations that extend the therapeutic time window of NMDA receptor antagonists. It has recently been shown that combination of an ASIC1 receptor blocker PcTX (see previous chapter) with the NMDA antagonist memantine enhanced the therapeutic time window of the NMDA blocker following ischemia (92). This suggests that combinations of neuroprotective agents may provide more effective therapeutic options for treating stroke than a single compound alone. Indeed this approach has proved effective in cancer therapy, whereby multiple cytotoxic compounds are combined.

The failure in clinical trials of NMDA receptor antagonists and the SAINT trial also highlight some of the limitations of studies assessing neuroprotective potential of an agents using in vivo models of stroke. For example, in in vivo models the test agent is frequently administered at the time of ischemia induction, rather than at time points more equivalent to clinical response times (3–6 hours post ischemia). As such, numerous recommendations have been made from the review of failed clinical trials of neuroprotective agents with recommendations for future studies (27, 35, 101, 102).

### 3.3 Physiological and Pathological NMDA Receptor Signaling: Cell Fate in the Balance

Another compelling reason for the apparent failure of NMDA antagonists concerns the apparent Yin/Yang effects of NMDA receptor signaling. On the one hand, activation of NMDA receptors has been shown to mediate pro-survival physiological effects, such as CREB transcription factor activation and BDNF expression (50, 116). However, excessive activation of NMDA receptors promotes neurodegeneration and cell death. In a series of experiments, Biegnon showed that the prolonged blockade of NMDA receptors is detrimental if administered hours following traumatic brain injury. Indeed, administration of a low dose of NMDA agonist (NMDA) results in less brain damage than the administration of the NMDA antagonist MK801 in the TBI-injured mice (6).
The results of the Beignon study leads to two intriguing suggestions. First, that a weak partial agonist of NMDA receptors may actually be a more therapeutically effective therapy. A weak partial agonist would antagonize excessive endogenous activation of a given receptor, yet maintain a low level of tonic activation of the receptor if endogenous activation falters. This approach is currently in clinical trials; the NMDA antagonist memantine may be a weak partial agonist.

The second suggestion regards the nature of NMDA receptors and their contribution to physiological and pathological signaling. Hardingham showed that following blockade of synaptic receptors on neurons, further NMDA stimulation resulted in toxicity, which was attributed to the activation of extra-synaptic receptors (49, 50). Other groups have shown that NMDA toxicity is not blocked by loss of spines (100), e.g. following the removal of dendritic spines with latrunculin (an actin de-stabilizing agent). These data suggest that toxic NMDA signaling exists when either synaptic NMDA receptors are blocked or following removal of dendritic spines (and presumably the synaptic receptors). This implies that toxic NMDA signaling is mediated through extra-synaptic NMDA receptors.

However, ischemia induced excitotoxicity may exert its toxic signaling effects through synaptic NMDA receptors, and not extrasynaptic receptors. For example, removal of dendritic spine heads, following exposure of neurons to the actin depolymerizing agent latrunculin, blocks ischemia-induced cell death in vitro (100). This suggests that ischemia induced cell death may be reliant on synaptic signaling mechanisms and synaptic NMDA receptors that are activated by endogenous synaptic glutamate that is released during energy failure. This concept is supported by the observation that cutting pre-synaptic inputs to the CA1 neurons of the hippocampus blocks ischemia-induced neurodegeneration (11). Furthermore blocking synaptically released glutamate with tetrodotoxin blocks oxygen glucose modeled ischemia induced cell death in vitro (85). However what is not clear from the experiments of Sattler et al. (100) is what happens to synaptic NMDA receptor subunits following latrunculin treatment, and whether latrunculin induces changes to all NMDA receptors, or whether its effects are subtype specific (NR2A vs. NR2B).

In a recent study it was suggested that blockade of NR2B subunits, irrespective of synaptic location mediates neurotoxic NMDA-receptor mediated signaling, but NR2A subunits were pre-survival. This suggests that selective enhancement of NR2A, but reduction of NR2B mediated signaling may offer a new therapeutic mechanism for stroke. Unfortunatly, the polyamine site NR2B glutamate receptor subunit selective antagonist Eliprodil was withdrawn from clinical trials in 1997, reducing the enthusiasm of NR2B subunit selective NMDA receptor antagonists as a therapeutic option for stroke (see (120)). Hence the challenge for one future direction of neuroprotective stroke research is to devise a mechanism whereby physiological signaling can be maintained, but toxic NMDA signaling can be selectively attenuated. Interestingly, endogenous protective mechanisms may indeed mimic this situation, and are considered later on in the chapter.

Taken together the role of the synapse in mediating ischemia induced damage to neurons is implicated by a number of studies, but ischemia can also result in profound changes to the synapse. These mechanisms may be part of a neuroprotective response to the ischemia, or a result of excitotoxic signaling.
Fig. 3. Different signaling modes of NMDA receptors. Synaptic NMDA receptors appear essential for physiological roles of NMDA in neurotransmission. Synaptic NMDA receptors may mediate ischemia induced cell death, in contrast to NMDA, which activates extrasynaptic NMDA receptors. Following ischemic preconditioning, NMDA receptors show reduced toxic signaling, but maintain physiological function.

4 Effect of Ischemia on Synaptic Morphology

The physical loss of neurons following an injurious ischemic episode will result in defective brain function. However, synaptic changes are evident in areas subject to non-harmful ischemic where the loss of function can be temporary or reversible. The form and function of the synapse are intertwined, for simplicity we consider the effect of ischemia on synaptic morphological and then the effect of ischemia on synaptic function.

4.1 Methods to Study Synaptic Structure Following Ischemia

Structural studies have been performed using both in vitro and in vivo stroke models to determine the morphological effects of ischemia. In vitro studies have allowed the “real time” assessment of dendritic structures through the application of fluorescence and confocal microscopy. Typically immunocytochemistry, fluorescent proteins, tagged actin or lipophilic fluorescent membrane dyes are used in these studies. In vivo studies have commonly used fixed sections to assess morphology either by confocal imaging, or electron microscopy. However, recently “real time” imaging of live neurons expressing yellow fluorescent protein (YFP) in layer V of the mouse cortex was reported (124).

4.2 Ischemia Induces Rapid Dendritic Spine Loss and Morphological Changes to Neurons

Long periods of ischemia induces neurons and other cells in the brain to die, whereas briefer ischemic periods may not be toxic. The initial response of neurons to both lethal and sub-lethal ischemia is similar regardless of the technique and model system used: ischemia induces a rapid loss of dendritic spines and an increase in varicosity formation along the dendrite. Spine loss can occur as quickly at 10
minutes following the onset of ischemia (124). The spine loss is recoverable if the ischemia is transient and non-harmful, but is persistent when toxic levels of ischemia occur (88, 124). It should be noted however that neurons in the penumbral region that recover spines may yet be lost at a later time point. The recovery of spines takes approximately 2–4 hours (88). Interestingly the recovery of the spines occurs at the same location on the dendrites that the spines were originally lost both in vitro (52) and in vivo (124). Spine loss is similar when toxic and non-toxic ischemia or excitotoxic stimuli are used, leading to the suggestion that spine loss may be a protective phenotype (see later).

Fig. 4. Overview of effect of ischemia on synapse structure. Following ischemia various modifications to synaptic structure have been noted, including, temporary swelling of presynaptic boutons and astrocytes. The post-synaptic regions show more profound changes, including loss of dendrites, disassembly of actin cytoskeleton and receptor scaffolds. Microtubules are cleaved and accumulate in varicosities along with swollen mitochondria.
The mechanisms regulating spine loss following ischemia have not been thoroughly studied, rather many studies have focused on the mechanisms of NMDA-induced morphological changes. NMDA activates protein phosphatase 2B, calcineurin, which causes actin depolymerization and may be responsible in part for spine loss. However, actin depolymerization alone is insufficient to remove spines from dendrites. Latrunculin treatment of hippocampal cells results in a loss of the spine head, but the shaft is still evident (100). In contrast, incubation of hippocampal cells with NMDA results in loss of the entire spine (44, 52, 53). This suggests additional mechanisms are responsible for the spine loss and rather than just actin remodeling. Interestingly, latrunculin treatment does prevent spine recovery following NMDA treatment (52).

NMDA may regulate spines via the activation of proteolytic enzymes. NMDA-mediated activation of the proteolytic enzyme cathepsin D degrades MARCKS: a protein, which is responsible for anchoring the actin cytoskeleton to the plasma membrane (44). Calpain however does not regulate spine loss, but calpain blockers slow the recovery of spines following NMDA treatment (32). The ubiquitin-proteasome system may also play a role in the loss of MARCKs following brief ischemia, and contribute to synaptic remodeling (Meller: unpublished results). This suggests that brief ischemia may remodel the synapse via the selective degradation of actin-associated elements.

### 4.3 Varicosity Formation in Neuronal Dendrites Following Ischemia

A second feature following ischemia is the appearance of beaded varicosities along the dendrites. These varicosities have been reported in response to excitotoxic stimuli and were originally considered cell death indicators resulting from excitotoxicity (86). The varicosities occur in the same time period as spine loss is observed (88). Interestingly, more varicosities are observed on proximal dendrites following ischemia, than those in close proximity to the cell soma (Meller; unpublished observations). Microtubule associated proteins (MAP2A/MAP2B etc.) have been shown to be cleaved following ischemia, and they accumulate into the varicosities (12). The formation of varicosities following ischemia is not inhibited by a calpain inhibitor, MDL 28, 170, however the recovery of varicosities following NMDA treatment was slowed in the presence of MDL28,170(32). More recent data show that varicosity formation and excitotoxic cell death occur through independent mechanisms; death is Ca\(^{2+}\) mediated while varicosities are mediated by Na\(^{+}\) flux and AMPA internalization (64). The functional consequence of varicosity formation is not clear, but may also serve as a protective function, as they may help compartmentalize calcium to reduce calcium overload (64). Indeed, varicosity formation by NMDA induces a transient, reversible neuroprotective state by attenuating excitatory neurotransmission (64). Thus the varicosity formation as well as spine loss following ischemia may be part of a protective response.
5 Ultrastructural Changes in the Brain Following Ischemia

A number of ultrastructural studies have helped to shed light on the effect of global ischemia on selectively vulnerable neurons, especially damage to the CA1 pyramidal neurons of the hippocampus. Most of these studies involve observations made on brain sections prepared for electron microscopy.

5.1 Presynaptic Membranes are Tolerant of Ischemia

Pre-synaptic membranes are not as affected by global ischemia as the post synaptic surface. The pre-synaptic membrane and glia show temporary swelling, which is reversible within one hour (31, 57). This is consistent with the lack of permanent cell damage to astrocytes and pre-synaptic neurons in global ischemia models (67, 82). One common observation is that astrocyte end feet are swollen, and appear to reduce the extracellular space (31), which may have implications for synaptic transmission. Following ischemia, vesicle aggregation is observed in pre-synaptic terminals, and some synaptic boutons show a reduction in vesicle number (31, 118). This may correlate to ischemia/reperfusion induced vesicular release of glutamate, and other transmitters. The morphological changes to the pre-synaptic terminal and astrocytes appear temporary and return to relatively normal levels within one to three hours following the ischemic insult.

Further support for the robustness of the pre-synaptic neurons in global ischemia comes from multiple experiments, and not just ultra-structural studies. Global ischemia induces cell death in the CA1 field of the hippocampus but not the innervating cells. Following global ischemia, potassium evoked glutamate release can still be measured using microdialysis a few days after the ischemic insult (82). Histological analysis of these brains show that the post-synaptic cells were lost, but the pre-synaptic axons appear to withstand the ischemic shock. In a study by Hasbani, the pre-synaptic labeled cells appear relatively stable, following exposure to NMDA (52). In comparison the post synaptic dendritic spine shrinks, but reappears following wash out of the NMDA. This apparent robustness of pre-synaptic boutons to ischemia may also serve to guide dendritic spines back to their original contacts. However, one week following global ischemia pre-synaptic bouton numbers are reduced, which may be due to a lack of target sites in fields where neurons have degenerated (58).

5.2 Postsynaptic Membranes are Vulnerable to Ischemia

Ischemia induces more morphological defects in the post-synaptic region, compared to the pre-synaptic terminal. Following ischemia, modifications of the post-synaptic density (PSD) have been reported, including projections of electron dense material (the consequence of this is as yet unclear). The PSD becomes irregular following the ischemic insult. The PSD is thicker and discontinuous 20–60 minutes following reperfusion (31). Apparent breaks in the PSD correspond to the breaks in the post-synaptic membrane at 1 hour. A disruption or compromise of the post-synaptic membrane is consistent with the observation that post-synaptic sites take up
exogenous HRP (horse radish peroxidase) following ischemia (although the HRP is associated with cell membranes and so may be due to endocytic mechanisms rather than cell lysis) (26). The potential of post fixation to induce the membrane breaks should also be considered. One unusual observation (albeit with unknown significance) is the formation of pockets in the post-synaptic membrane, which are filled by the pre-synaptic membrane, but no PSD is present in the “buckets” (31).

One hour following ischemia, many studies report the swelling of post-synaptic membranes and changes in the morphology of internal organelles: mitochondria appear swollen with a loss of the cristae, the endoplasmic reticulum appears swollen, vacuoles form in the cytoplasm and poly-ribosomes are disintegrated into single units (31, 57, 118). Some cells also start to show signs of chromatin clumping following ischemia. Microtubules in axons and dendrites also appear to show kinking and breaks following ischemia, which may correspond to the formation of varicosities (see above).

The thickening of the post synaptic density in ischemic-vulnerable CA1 hippocampal neurons can last 4–24 hours following global ischemia, where as in the dentate gyrus (which is less vulnerable to ischemia) changes in the PSD thickness reverse 24 hours following the ischemic insult (74). This thickening of the PSD may correspond to an enhanced NMDA receptor mediated facilitation (signaling) following ischemia in CA1 neurons or be associated with ischemic damage to the post-synaptic region. Hence the persistent modification of the PSD may be a marker or a cause of the delayed cell loss in the CA1 following global ischemia.

The thickening of the PSD following ischemia may also involve the denaturation of proteins. Ubiquitinated protein aggregates have been reported to occur following ischemia, especially in synaptic preparations (70). If proteins are present but denatured (62), this may lead to a decrease in signaling function of synaptic proteins. Since multiple proteins in the post-synaptic density are targets for ubiquitination (30), this may affect post synaptic receptor signaling following ischemia.

Fewer studies have investigated long term changes to the structure of the brain following harmful ischemia. There is evidence to support reinnervation of some areas lost following an ischemic insult. One week following ischemia there is a persistent decrease in synaptic number in the hippocampus. In particular there is a reduced number of asymmetrical synapses, which correlate with excitatory synapses (58). However there is an increase in glutamate in the remaining synaptic boutons of the hippocampus, which may be a compensatory mechanism for the initial loss of cells following ischemia (58). In the short-term, presynaptic markers levels (GAP43) do not change following ischemia, but at 2 weeks changes were apparent (increased protein expression) (47, 71). This may coincide with reinnervation of damaged areas or a recovery in function. Recently, realtime in vivo two-photon imaging has demonstrated up to 5-fold higher rates of spine formation in neurons near the infarct borders (10). It is notable that the brain is capable of recovering function following the loss of discrete brain regions due to ischemia.
6 Functional Effects of Ischemia on Synaptic Transmission

Many studies have been performed using global ischemia to determine the effects of harmful ischemia on synaptic transmission (123). Such studies are performed using the global model of ischemia, where the maturation of injury in the vulnerable CA1 region of the can be compared to dentate gyrus or CA3 cells, which are resistant to the effects of ischemia. While it is assumed that regions of the brain exposed to hypoxic conditions will show greatest functional deficits following ischemia, it is of note that in a receptor study Zhang and Murphy showed that functional deficits extended on average up to 400 µm from areas with damaged dendrites indicating synaptic function may even be compromised in the structurally intact penumbra (125).

6.1 Rapid Onset of Synaptic Depression During Ischemia

When cells are subject to hypoxia there is an initial depression or failure of synaptic transmission (108). This may be due to the rapid energy deletion in the cells following the onset of ischemic conditions. The synaptic depression is reversible if the duration of ischemia is short. The CA1 cells of the hippocampus show a rapid decrease in population spike response to stimulation following ischemia, compared to dentate gyrus cells.

6.2 Anoxia-Induced Spreading Depression

If ischemia is prolonged the cells suffer a spreading depression type event, which has some similarities to normoxic spreading depression (108). Interestingly while some drugs are able to delay the time of onset of spreading depression in brain slices, no drugs have been shown to produce blockade (108). The consequence of this period of depolarization is not fully understood but may play a role in the post ischemic enhancement of synaptic transmission. The ability of the brain to recover from this depolarization is inversely proportional to the time exposed to spreading depression (108). The timing of the spreading depression may contribute to two features of post ischemic responses. First the spreading depression may result in excessive release of the excitatory neurotransmitter glutamate, which may activate NMDA receptors leading to enhanced intracellular calcium levels. Second, the spreading depression may also account for some of the post-ischemic abnormalities in synaptic transmission.

Following recovery from ischemia the neuronal responses may appear normal or enhanced, but drop off between 8 and 24 hours following ischemia, a time interval that corresponds to that of CA1 neuronal loss. It must be noted that this mechanism of cells dieing off after a period of re-perfusion in global ischemic models is distinct from the cell death following irreversible immediate hypoxic damage usually observed in the core of a focal ischemic infarct (commonly referred to apoptosis vs. necrosis, but see (106)). Following global ischemia, there is a temporary decrease in inhibitory synaptic transmission, but this is not as prolonged as the enhancement of excitatory transmission (15).
6.3 Enhanced Postsynaptic Responses Following Ischemia

Prolonged post-synaptic glutamate receptor activation may result in an apparent increase in EPSP amplitude up to 12 hours following an ischemic episode (21, 22, 37, 38, 83). Enhanced NMDA responses have been termed post ischemic long term potentiation, in that it shows some similarities to long term potentiation induced by a tetanic stimulation under normoxic conditions. The potentiation is due to the increased synaptic efficiency in post ischemic hippocampal CA1 neurons following ischemia. Post ischemic LTP can be blocked by NMDA receptor antagonists, which may account for the ability of some glutamate antagonist studies to show a reduction in cell damage when administered following global ischemia (84). Calcium chelating agents can also block post ischemic LTP, but unlike tetanic LTP AMPA antagonists are not effective (21).

A number of mechanisms may account for the enhanced response of NMDA receptors following ischemia. Ischemia decreases ATP, changes pH, increases glycine release, activates protein kinases (PKC) and results in reducing conditions, all of which have been shown to enhance NMDA receptor currents (21). Some studies suggest that the increased expression of the NMDA receptor 2C subunit (NR2C) following ischemia may account for enhanced NMDA responses (66, 107). NR2C subunits have a reduced magnesium blockade of the channel resulting in enhanced calcium influx and prolonged opening of the channel. Hypoxic LTP requires a strong depolarizing event to be induced, and one potential candidate could be anoxic spreading depression, which is observed following ischemia.

The enhanced NMDA response in CA1 regions may also lead to additional complications in the brain following stroke. Following ischemia there is an increased occurrence of epileptic seizures. NMDA receptors regulate burst firing in the CA1 post ischemic, but in some recent studies the CA3 was shown to have a lowered seizure threshold following global ischemia (19). Enhanced seizure susceptibility occurs 2–3 weeks following the primary ischemic insult.

6.4 Changes in NMDA Receptors Following Ischemia

Following global ischemia there is a reduced expression of NMDA receptor subunits (123) which probably correlates with the loss of cells from the CA1 following injury. These decreases are blocked by administration of NMDA receptor antagonists at the time of ischemia (again supporting the view that ischemia induced cell death involves excitotoxicity). Following ischemia there is also a calpain sensitive cleavage of the NR2A and NR2B receptor subunit C termini (39, 121), which may also enhance receptor signaling. Interestingly the post ischemic induction of LTP, cell death and reduction of NR2B subunits is blocked by the NMDA receptor 2B subunit selective antagonist ifenprodil (91). Other studies have reported that NR2B mediated signaling is toxic (50).

The interaction of the NMDA receptor with the postsynaptic scaffold may also result in enhanced NMDA function. Following ischemia there is an increased phosphorylation of NMDA receptor subunits by the tyrosine kinases Src Fyn, and Pyk (17). The phosphorylation of NR2A and NR2B receptor subunits has been
linked to hyper-excitability of the cell and cell death. Disruption of PSD-95 interactions with NMDA receptors, or reduction in its expression by antisense oligonucleotides reduced NMDA receptor subunit phosphorylation and ischemia induced cell death (2, 60). Hence the downstream intracellular signaling components of the NMDA receptor scaffold may offer new targets for anti-stroke therapies.

7 Role of the Synapse in Ischemic Tolerance

Tolerance is the biological phenomenon whereby a brief sub toxic exposure to a stimuli (preconditioning) renders the cell, organ or organism protected against a normal toxic exposure to the same stimuli. In ischemic tolerance, brief exposure to subtoxic ischemia (albeit sufficient to elicit a response in the tissue) renders the brain (or heart) tolerant to subsequent normally injurious levels of ischemia (29). The protection induced by preconditioning can render the system protected from other harmful stimuli as well, for example endotoxin (lipo-poly saccharide¨LPS) can induce tolerance to ischemia, and seizures or spreading depression can also induce ischemic tolerance (29). Tolerance to ischemia can occur over two time frames, rapid tolerance occurs within minutes and lasts approximately one hour, delayed tolerance require 24–72 hours to be maximal and is lost after 7 days (29).

7.1 Multiple Intracellular Mechanisms Regulate Ischemic Tolerance

Studies of neuroprotection in ischemic tolerance usually revolve around the study of an induced protective gene following preconditioning. Central to this is the observation that long term ischemic tolerance is blocked by protein synthesis inhibitors, which suggests that it requires de-novo protein synthesis (5, 79, 80). Long-term ischemic tolerance is also blocked by RNA synthesis inhibitor actinomycin D (Meller, unpublished observation). Ischemic tolerance following preconditioning with ischemia is inhibited by NMDA receptor antagonists (7, 111), but this may be specific to the nature of ischemic preconditioning (i.e. requiring a synaptic event).

Ischemic tolerance induced by ischemic preconditioning is blocked by pharmacological and genomic inhibition of Protein Kinase A (PKA), p42/44 mitogen-activated protein kinase (MAPK), calcium/calmodulin activated protein kinase (CAMK) and AKT (42, 80). Each of these protein kinases have been shown to be activated following synaptic activation of NMDA receptors, although the exact mechanism linking these protein kinases remains to be determined. Activation of these protein kinases has been shown to drive multiple transcription factors and CREB activation has been shown by a number of groups to regulate ischemic tolerance. Blocking CREB phosphorylation, interaction with CBP or CRE promoters has been shown to block ischemic tolerance (48, 80). Interestingly, the prosurvival protein bcl-2 contains a CRE promoter (36, 94, 119) and blocking CREB function blocks bcl-2 expression and ischemic tolerance (48, 72, 80).

However, apart from bcl-2, numerous genes and proteins have been touted as mediating protection in tolerance paradigms, including heat shock proteins, other
anti-apoptotic proteins e.g. Bcl-w, changes in expression of glutamate transport proteins EAAT2/3 and erythropoietin (23, 75, 81, 98, 99). When one considers a mechanism for ischemic tolerance, one must take into account the nature of the preconditioning agent as well the nature of the harmful event, as this may have lead to misleading generalizations regarding common mechanisms of ischemic tolerance. For example, induction of heat shock proteins have been shown as a potential mechanism of ischemic tolerance (16, 122), but ischemic tolerance is induced at a lower insult threshold than required to induce HSP expression (3). This does not rule out heat shock proteins as a relevant mechanism of inducing tolerance to ischemia, but suggests other mechanisms may be more relevant for spreading depression induced protection.

Many agents may induce long term (protein synthesis dependent) tolerance, however the mechanisms by which this tolerance is acquired may be quite different. Comparison of the genomic profile of ischemic preconditioning is quite different from other preconditioning agents, and no one common mechanism appears to result in protection. One potential explanation for this is not that preconditioning agents cause the same induction of protective genes, but rather they cause a reprogramming in the genomic response to normally harmful ischemia, thereby changing the cells fate. This has indeed been shown in ischemic tolerance using microarray profiling. Stenzel Poore et al. showed that the genomic overlap of infarcted cortex and tolerant cortex subject to harmful ischemia show no common regulated genes (110). Thus the genomic response to ischemia was “reprogrammed” by preconditioning the brain by brief ischemia and that this reprogramming produced protection. What remains to be determined is whether the different preconditioning agents also induce the same reprogramming effect or whether each stimuli not only induces its own pattern of protective gene expression but also its own re-programming effect, based of the genes expressed by the preconditioning agent. Stenzel-Poore has suggested that the tolerizing agent (ischemia, LPS) dictates the mechanism of protection to subsequent ischemia (109). Ischemic preconditioning induces a hibernation-like phenotype, the down regulation of high energy utilizers such as transporters and channels (110). In contrast LPS down regulated inflammatory mediators and upregulates anti-inflammatory mediators (109).

7.2 Is Synaptic Function Changed in Delayed Ischemic Tolerance?

Since ischemia tolerance induced by ischemic preconditioning can be blocked by NMDA receptor antagonists (43, 111), this would suggest that tolerance pathways are activated following ischemia-induced activation of NMDA receptors. However, no clear pattern of expression of synaptic proteins is induced by preconditioning or the tolerant state in microarray studies (110).

However, preconditioning ischemia may induce changes to synaptic transmission via indirect mechanisms. Microinjection of Bcl-XL into pre-synaptic terminals enhances post-synaptic responses and the rate of recovery from synaptic depression (65). In contrast an N-terminal cleaved Bcl-XL, which is pro-apoptotic, reduced post-synaptic responses. The effect of bcl-2 family proteins on the cell may depend on the developmental stage of the neuron. Loss of Bak results in reduced
GABA-ergic inhibition and enhanced seizure-like activity in young mice exposed to the excitatory amino acid analogue, kainite (33). However, in mature mice, loss of Bak renders cells protected against ischemia (33). Therefore in the case if ischemic tolerance, where Bcl-2 expression is increased (8, 80, 105), a change in the balance of pro-survival vs. pro cell death proteins may result in changes in mitochondrial function and therefore synaptic function.

Ischemic preconditioning may induce long-term changes to the synapse. A recent study reported that 3–30 days following preconditioning ischemia there is a higher number of dendritic spines compared to control (20). Increased spine number is usually associated with an increase in vulnerability to ischemia and other excitotoxic stimuli. This suggests that long-term changes occur in the brain following preconditioning, out lasting the neuroprotective period.

### 7.3 Rapid Ischemic Tolerance

Neurons also become tolerant to harmful ischemia one hour following preconditioning ischemia: a process termed rapid ischemic tolerance (79, 89, 90, 96). This form of tolerance is mediated by biochemical mechanisms in the brain. Blocking protein synthesis does not block rapid ischemic tolerance (79), suggesting it is not dependent on new protein synthesis. A complement of protein kinases appear to mediate rapid ischemic tolerance including p42/44 mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and AKT (79, 96), but not protein kinase A (PKA) or calcium calmodulin dependent protein kinase (CAMK) (Meller unpublished results).

### 7.4 Proteasomal Degradation of Structural Proteins in Rapid Ischemic Tolerance Leads to Dendritic Spine Remodeling

Rapid ischemic tolerance is blocked by inhibition of the ubiquitin-proteasome system (79), which is implicated in the regulation of synaptic signaling (30). Our recent studies have identified a number of proteins implicated in post-synaptic density regulation and signaling as being ubiquitinated, and potentially degraded one hour following preconditioning ischemia. Of particular focus, we identified the actin binding proteins MARCKS and fascin as being ubiquitinated and degraded. The loss of these proteins appears to result in re-organization of the actin cytoskeleton, although rapid acting calcium mediated processes following NMDA receptor activation, such as calpain, may also account for some of the rapid re-organization of actin.

A direct consequence of actin reorganization is the loss of dendritic spines on the neurons, which are therefore unable to make synaptic contacts. The loss of dendritic spines following ischemia has been reported in neurons in vitro following ischemia (53), but the role of the ubiquitin proteasome system has not yet been investigated. We showed that the proteasome inhibitor reduces the number of spines lost at one hour. Whether the proteasome directly mediates spine loss or speeds up the spine recovery is currently under investigation.
Fig. 5. Rapid ischemic tolerance induced protection is mediated by changes to the synapse. (a) Ischemic tolerance is induced by pairing a preconditioning brief ischemic stimulus (30 min OGD: PC) with a prolonged harmful ischemic challenge (120 min OGD: Isch). (b) The neuroprotection in ischemia tolerance is reversed by the proteasome inhibitor MG132. Compared to untreated cells, cells treated with the proteasome inhibitor MG132 do not acquire tolerance to harmful ischemia. (c–f). Compared to control (c), following preconditioning
The loss of dendritic spines may be a feature of endogenous protective mechanisms resulting in tolerance to ischemia. A reduction in dendritic spine number has been associated with the ischemia tolerant effect of hibernation in artic ground squirrels and European hamsters (73, 93, 114). Hence the remodeling of dendritic spines following transient sub-toxic ischemia appears to be a protective mechanism.

7.5 Reduced Excitotoxicity of NMDA in Rapid Ischemic Tolerance

The remodeling of the post-synaptic membrane may not just be mediated by changes to the actin cytoskeleton. The loss of dendritic spines one hour following preconditioning ischemia, would no doubt have an effect on synaptic transmission. We have also discovered that at the time of spine loss in preconditioned cells, NMDA-mediated cell death is reduced. The tolerance to NMDA excitotoxicity is blocked by a proteasome inhibitor, which also blocks the reduction in spine number at one hour following preconditioning ischemia (See Fig. 5). This suggest that preconditioning ischemia results in a selective inhibition of post-synaptic toxic glutamate signaling in cells, via the ubiquitin-proteasome pathway, yet NMDA function (CREB phosphorylation) and receptor subunit expression levels were not effected by the preconditioning ischemia (not shown).

The loss of NMDA toxicity following preconditioning ischemia, suggest a selective loss in pathological NMDA signaling that may account for the tolerance to ischemia. These studies agree with that of Aarts, who showed that disruption of the interaction of PSD-95 with NMDA2A/2B receptors blocks ischemia induced cell death, but not NMDA electrophysiological responses (2). Further this result agrees with Sattler whereby remodeling of dendritic spines results in protection to ischemia (100). However in that study, dendritic remodeling using latrunculin (an actin depolymerizing agent) only protected against ischemia and not NMDA toxicity. In contrast ischemic preconditioning protects against toxicity induced by both ischemia and NMDA. Our data suggest that preconditioning induced protection, involves more than just actin re-organization, rather the ubiquitination of post-synaptic density associated proteins results in a protective phenotype. Hence preconditioning cells with ischemia results in protection to toxic NMDA signaling from extra-synaptic sites as well as synaptically derived ischemia – induced neurotoxicity.

Summary and Concluding Remarks

Ischemia induces profound changes to the synapse, both in terms of mediating the death of cells following toxic ischemia, and the remodeling of synapses in response
to sub-toxic ischemia. By further investigating the mechanisms that separate physiological from pathological NMDA signaling, potential therapeutic options may be identified with significant therapeutic protection from stroke potential and a more favorable therapeutic index than currently available investigational tools. Such an intervention may be of use post-stroke as well as in situations whereby ischemic brain conditions can be predicted, for example heart bypass surgery.

Acknowledgements

Thanks to Simon Thompson Ph.D for images of MAP2 staining in cells. We apologize to those whose work we could not cite due to space limitations. Work in the Robert Stone Dow Neurobiology Laboratories is supported by the National Institute for Health (NINDS), the American Heart Association, the Medical Research Foundation of Oregon and The Good Samaritan Foundation.

References


78. Mitani A, Namba S, Ikemune K, Yanase H, Arai T, and Kataoka K. Postischemic enhancements of N-methyl-D-aspartic acid (NMDA) and non-NMDA receptor-mediated


