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# Tyrosine Phosphorylation of the NMDA Receptor Following Cerebral Ischaemia

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Additional information is available at the end of the chapter

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## 1. Introduction

Cerebral ischaemia (stroke) describes a condition wherein blood flow to the brain is reduced such that neurological function is disrupted, and neural cell death becomes possible. For several decades, stroke has remained a leading international cause of death and disability, which is the reason considerable effort has been applied to improve understanding of its pathogenesis; however, only a modest comprehension of the complex cellular processes underlying ischaemia-mediated cell death can currently be claimed. Our limited knowledge regarding how the brain is changed by an ischaemic event is part of the explanation for the absence of a successful clinical intervention, despite the examination of more than a thousand potential pharmacotherapies during the past fifty years [61, 69, 169].

Phosphorylation is the most broadly examined post-translational modification within the central nervous system [125, 222, 244]. Physiological shifts in neuronal activity, such as those that occur during memory formation, can lead to changes in protein phosphorylation; in a similar fashion, pathological changes in brain activity, such as those that occur during cerebral ischaemia, can also affect phosphorylation status. One principal means whereby the pattern of phosphorylation, especially at tyrosine residues [45, 80], can affect brain function is by regulating the activity of ionotropic receptors, which mediate the vast majority of rapid signal transmission. While the phosphoregulation of many ionotropic receptors has been examined, the NMDA sub-type of receptors that respond to the excitatory neurotransmitter glutamate has been the subject of a disproportionate level of attention due to its key role in neuronal communication.

To contribute to ongoing efforts directed at developing improved pharmacotherapies for stroke, the present review will provide a reflection on the manner in which ischaemic injury may alter neuronal physiology through changes in the tyrosine phosphorylation of the NMDA receptor; in particular, three goals will aim to be accomplished: (a) providing a

general review of the primary upstream changes initiated by cerebral ischaemia, and, in so doing, highlighting the importance of the NMDA receptor (b) offering a summary of the structure and function of the NMDA receptor, and the evidence that establishes how the receptor's function and cellular distribution are altered by tyrosine phosphorylation (c) outlining what is known about how ischaemia may set in motion cellular changes leading to the aberrant, potentially harmful, and possibly self-amplifying over-activation of the NMDA receptor.

## 2. Cerebral ischaemia

### 2.1. Definition, prevalence, and risk factors

Insufficient cerebral blood supply may result from either the collapse of systemic circulation (leading to global ischaemia), or from the occlusion of a vessel that supplies a discrete region of the brain (leading to focal ischaemia). Although there are several possible causes of focal occlusions, they are predominantly the result of a foreign substance travelling within the cerebral circulation until the lumen becomes too narrow to permit further movement (embolism) [240]; the principal source of emboli is believed to be atherosclerotic plaques [187]. While uncontrolled bleeding from a vessel (haemorrhage) can also cause ischaemia of a focal nature, occlusion is thought to account for approximately 80% of focal events [198, 230].

For several decades, stroke has consistently been recognised as one of the leading causes of death worldwide, and one of the major causes of severe disability. Globally, over 15 million people per year are diagnosed with stroke, and a third of those afflicted die from complications relating to the injury [255]. In addition to significant medical consequences for affected individuals, cerebral ischaemia also presents enormous socioeconomic costs; for example, recent estimates place the direct and indirect annual costs associated with stroke in the United States at approximately 65 billion USD [47], while similarly constructed European estimates place the annual costs at approximately 77 billion USD [172]. Given that those who survive an ischaemic attack must cope with a variety of significant cognitive deficits (including aphasia, hemiparesis, and memory problems) that often lack treatment, the social costs of stroke are as enduring as they are significant.

Understanding the underlying causes of cerebral ischaemia requires an appreciation for the numerous genetic and environmental factors that contribute to its development, and that its determinants may be divided into non-modifiable and modifiable categories. The primary, and most significant, non-modifiable risk factor is age. The incidence of stroke rises exponentially with age, and the majority of strokes are seen within individuals who are older than 65 years of age [83, 203]. Gender is also an important consideration, for stroke incidence among men has consistently been shown to be approximately one-third greater than among women [203]. In addition, numerous American studies have indicated that the occurrence of stroke among multiple non-white demographic groups is greater than among white individuals, even when socioeconomic factors are considered [95, 202]. The principal modifiable risk factor for cerebral ischaemia is hypertension, and a large body of work has

illustrated that the likelihood of stroke rises proportionately with increasing blood pressure [210, 256]. As well, cardiac disease, notably atrial fibrillation and coronary artery disease [203, 257], and metabolic disease, particularly type II diabetes and dyslipidaemia [189, 256], are also associated with elevated stroke risk. Finally, several lifestyle factors, including physical activity levels, cigarette smoking, alcohol consumption, and diet, have been shown to independently affect the potential for stroke development [13, 92, 93, 251].

## 2.2. Pathogenesis

Despite comprising only about 2% of total body weight, the brain receives 15% of cardiac output and consumes about 20% of the oxygen utilised by the body [28]. The brain's disproportionate circulatory demands are attributable to a high metabolic rate based almost exclusively upon cellular respiration; in addition, unlike most other organs, glucose stores in the brain are sufficient to cover energy requirements for only about one minute [83]. In a relatively quick manner, reduction of blood flow beyond a critical threshold results in the inability of neurones to fire action potentials, and, if sufficiently extensive, may lead to the failure of oxidative phosphorylation, which is the principal method of cellular energy production [5]. To avert the cellular energy crisis that rapidly follows reduced blood supply, cells in an affected area rely increasingly upon glycolysis; consequently, tissue concentrations of lactate and hydrogen ions increase dramatically, causing acidosis [214]. However, the comparatively meagre amount of energy provided by anaerobic metabolism provides limited compensation, and, in a short period of time, the lack of high-energy phosphate, combined with decreased pH, precipitates a multifactorial increase of membrane permeability.

A number of ionic gradients exist across the neuronal membrane (high intracellular  $[K^+]$  and low intracellular  $[Na^+]$ ,  $[Cl^-]$ , and  $[Ca^{2+}]$ ), and these are quickly disrupted by the collapse of various energy-dependent pumps and transporters. Of particular note is  $Na^+/K^+$ -ATPase pump failure, which allows  $Na^+$  to move into the cell causing neuronal depolarisation accompanied by the passive diffusion of  $Cl^-$  and water [126, 230]. In combination, the normalisation of ions across the cellular membrane and the concomitant movement of water lead to intracellular swelling that causes osmolysis (cytotoxic oedema), which significantly contributes to acute neuronal cell death [60].

Disrupted ionic homeostasis also leads to a dramatic and unregulated increase in the fusion of neurotransmitter storage vesicles with pre-synaptic membranes, which causes a massive release of vesicular content. Of the transmitters that flood the synapse following ischaemia, the most intensely studied has been the amino acid glutamate, which is the principal mediator of excitatory neurotransmission within the mammalian brain. The harm that might result from excessive glutamate was first observed in studies that found its systemic administration caused pronounced retinal degeneration [142, 173], a phenomenon described as "excitotoxicity". A substantial body of subsequent work has established that glutamate is a key element of neurodegeneration in general, and of ischaemic cell death in particular [119, 133, 199]. For example, glutamate efflux precedes widespread injury to cellular

membranes and enzyme systems [2], the extracellular concentration of glutamate rises dramatically during ischaemia [63, 84], glutamate release is correlated with insult severity [29, 224], and glutamate receptor antagonists provide significant protection against ischaemic brain damage [119, 155, 215].

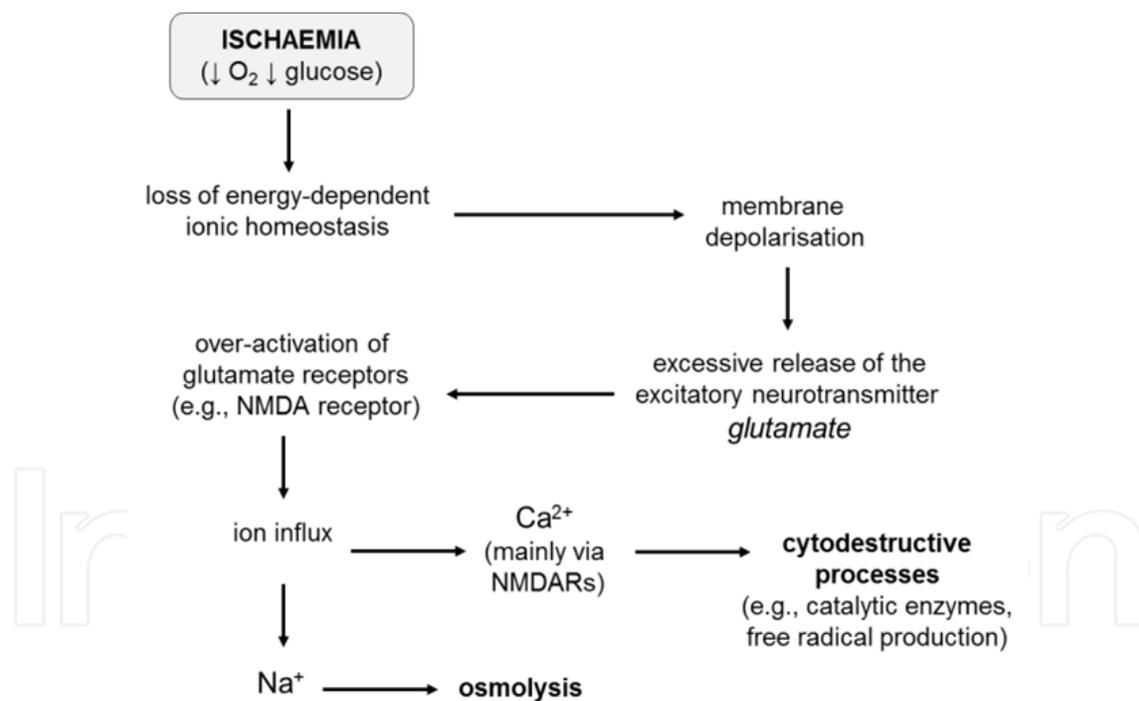
The widespread release of glutamate and the excessive stimulation of its high-affinity post-synaptic receptors are thought to act as critical elements that permit a profound rise of the intracellular calcium ion concentration. Calcium ions are involved in an array of neuronal functions, and their intracellular concentration is rigorously maintained at a level approximately  $10^4$  times lower than their extracellular concentration [143] by a combination of specialised binding proteins [8], sequestration within organelles [77], and extrusion [232]. One of the first studies to recognise the importance of calcium ions in cell death found that degeneration following axonal amputation occurred only when calcium ions were present in the bathing medium [208]. Subsequently, the essential role played by  $\text{Ca}^{2+}$  in glutamate-mediated cell death became established by studies that used mouse neocortical cultures [36, 88], rat hippocampal cultures [115, 190], and rat brain slices [57, 137]. Furthermore, work with culture [37, 67, 207], slice [276], and *in vivo* [15] models of ischaemia went on to reveal that a specific sub-type of glutamate receptor - the ionotropic NMDA receptor (section 3) - accounts for the majority of  $\text{Ca}^{2+}$  entry during and immediately after an ischaemic insult.

The dysregulation of intracellular  $\text{Ca}^{2+}$  has become recognised as a central branch point within the ischaemic cascade [11, 133, 213, 223], and serves as an important link between upstream activation of glutamate receptors and downstream stimulation of cell death mediators; for example, catalytic enzymes and free radicals. Several cytodestructive enzymes appear to be activated by cerebral ischaemia [119, 133, 185, 192], including proteases, phospholipases, and endonucleases. One set of enzymes that has received significant attention is the calpains, which are cytosolic cysteine proteases with variable  $\text{Ca}^{2+}$ -binding domains [217]. Calpains are ubiquitously expressed in the CNS, and a clear rise in their levels has been observed in models of both transient focal and global ischaemia [272, 280]. As well, activated calpains have been associated with damage to a variety of proteins [10, 241, 254], and calpain inhibitors have been found to provide a measure of protection in both culture [10] and *in vivo* models of ischaemia [12].

Free radicals have emerged as important players in the development of ischaemia-induced neuronal damage [3, 111, 133, 139]. The detection of free radical production following excitotoxicity caused by NMDA receptor stimulation has been clearly demonstrated in a variety of cultured rodent neurones [50, 76, 117, 194], and various groups have shown neuroprotection against excitotoxicity using antioxidant compounds [119]. As well, the mechanism of excitotoxicity-induced free radical production has been linked to  $\text{Ca}^{2+}$  by a report that demonstrated exposing isolated mitochondria to increasing calcium and sodium concentrations elevated free radical production [52], and another that showed removing extracellular calcium attenuated free radical production following NMDA application [50]. In addition to mitochondrial impairment, increased levels of reactive oxygen and nitrogen species are likely due to a combination of suppressed free radical scavengers and the

elevation of formative enzymes, such as xanthine oxidase, cyclooxygenase, and nitrogen oxide synthases. Collectively, the cellular changes caused by increased free radical activity are extensive, and include lipid peroxidation, protein denaturation, and nucleic acid modification.

Slight changes in cerebral blood supply can be effectively managed by autoregulatory mechanisms that govern blood flow and oxygen extraction; however, decreases beyond this primary threshold initiate numerous cellular changes that become more severe in direct relation to the extent of the disturbance. The critical stages of stroke pathogenesis (figure 1) develop following a rapid and sustained drop of neuronal energy supply, and are generally thought to include a loss of ionic homeostasis, the unregulated release of the excitatory transmitter glutamate, the profound over-activation of glutamate receptors (particularly, the NMDA receptor), the dysregulation of intracellular  $\text{Ca}^{2+}$  levels, and the activation of a number of calcium-mediated internal changes that broadly affect cellular structure and function. While the exact manner and time course of ischaemia-mediated changes can be varied, and is influenced by factors such as insult severity, neuronal maturation, phenotype, and connectivity, the one thing held in common is the ultimate development of extensive neuronal cell death.



**Figure 1.** Summary of major elements in the early stages of ischaemic pathogenesis.

### 3. The NMDA receptor

#### 3.1. Historical overview

Glutamate receptors (GluRs) mediate the majority of excitatory transmission in the vertebrate CNS, and participate in a number of physiological processes, including the

formation of neuronal networks during development [43, 110], the pattern of ongoing synaptic communication [236], and the cellular plasticity believed to underlie learning and memory [21, 146]. In addition to an intimate involvement with the brain's physiology, glutamate responsive receptors are also of central importance in several neuropsychiatric conditions. For example, the GluRs have been implicated in neurodevelopmental disorders (e.g., schizophrenia) [59], mood disorders (e.g., depression) [149], chronic neurodegeneration (e.g., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis) [1, 22, 100], and pain transmission [20], in addition to brain injury (e.g., head trauma and stroke).

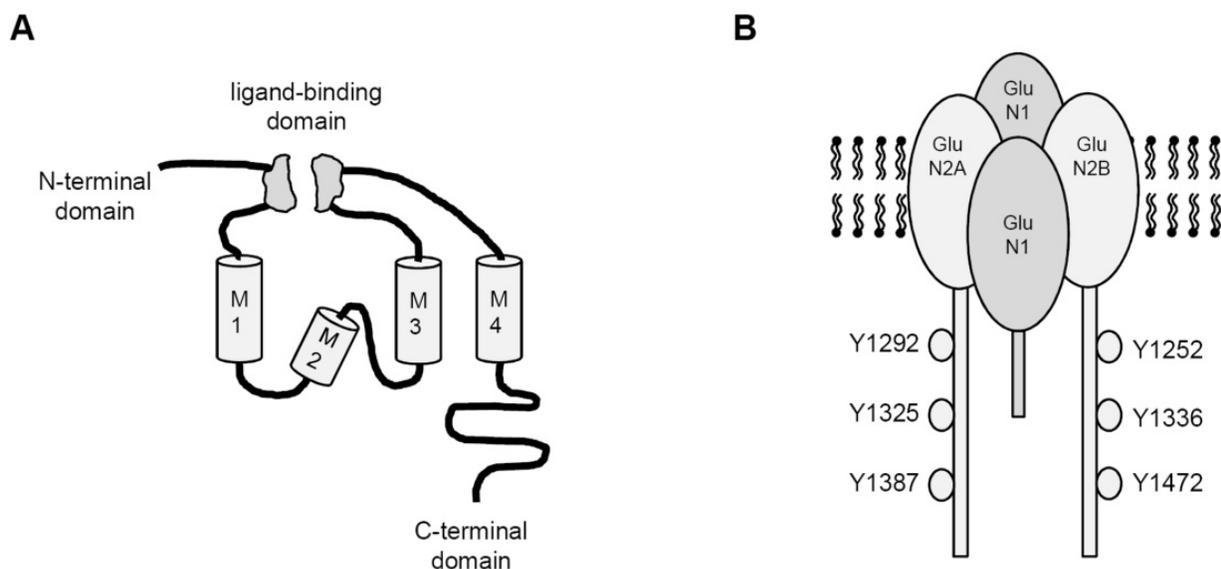
The broad influence of glutamate-mediated signalling upon synaptic function and dysfunction is attributable to the broad anatomical and cellular distribution of GluRs, and that they exist in two functionally and pharmacologically distinct varieties: metabotropic (mGluRs) and ionotropic (iGluRs). The metabotropic receptors are coupled to G-proteins, and, while structurally related to one another, do vary appreciably in their distribution and signal transduction mechanisms [175, 193]. The ionotropic receptors are non-specific cation channels that possess a common general structure, but vary considerably in both distribution and function [153, 175, 236]. As well, the iGluRs have been divided into three sub-types based upon relative selectivity to three exogenous agonists: N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate.

Important preliminary evidence for diversity within excitatory neurotransmission was found in the early 1960s when the synthetic GluR agonist NMDA was shown to potently excite neurones [44]. Subsequent work in the 1970s, using radioligand binding and specific antagonists, established the existence of a specific NMDA subtype of iGluR (NMDAR) [154]. Following the advent of molecular cloning technology in the 1980s, a receptor complex possessing the functional characteristics ascribed to the NMDAR was characterised [158], which confirmed the existence of this particular iGluR sub-type, and helped to foment investigation into its physiopathological roles.

### 3.2. Subunit structure and assembly

The NMDA receptor is thought to be a heteromeric complex formed from a combination of four subunits: GluN1, GluN2 (with four known sub-types, labelled A-D), and GluN3 (with two identified sub-types, labelled A and B); notably, the nomenclature for GluR subunits has recently changed [42]. The GluN1 subunit has been shown to be essential to the formation of functional receptors [55], while the GluN2 and GluN3 subunits are believed to impart distinct gating and ion conductance properties [236]. Although the stoichiometry of subunits remains to be definitively resolved, endogenous NMDA receptors are thought to require the assembly of two GluN1 subunits with either two GluN2 subunits, or a combination of GluN2 and GluN3 subunits [19, 236]. Regardless of their ultimate arrangement, similar to other iGluRs, NMDARs are thought to be held within the endoplasmic reticulum until they assemble in a manner sufficient to permit counteraction of a retention signal [183].

One important limitation to improved understanding of NMDAR composition is the significant degree of developmental and anatomical heterogeneity that exists within subunit expression. The GluN1 subunit displays a peak degree of expression late in embryonic life before slightly declining to a relatively stable level of post-natal expression, while the GluN2 subunits vary considerably in their expression across the lifespan [53, 252]. For example, the GluN2A and GluN2C subunits are found post-natally, the GluN2B is expressed both before and after birth, although expression levels decline considerably between the early post-natal period and adulthood, and the GluN2D subunit is overwhelmingly restricted to embryonic development. As well, the GluN1 subunit is found in all central neurones, but a significant degree of anatomical heterogeneity exists among GluN2 subunits; in particular, the GluN2A and GluN2B subunits are found throughout the forebrain, the GluN2C subunit is limited to the cerebellum, and the GluN2D subunit is found predominantly within the midbrain [53, 156, 252, 253].



**Figure 2.** (A) Diagram of the structure for a typical NMDA receptor subunit. (B) Schematic illustrating one possible heterotetrameric combination of GluN subunits, along with identification of those GluN2 C-terminal tyrosine residues believed most relevant to phosphorylation-mediated changes in receptor gating and surface expression.

Despite being variably expressed, each NMDA receptor subunit shares a similar general architecture: a large extracellular region that consists of the amino-terminal and ligand binding domains, a pore-forming transmembrane region, and an intracellular region containing the carboxy-terminal domain [19, 56, 236] (figure 2). The N-terminal domain, at least in certain GluN2 subunits, is believed to allow receptor activity to be non-competitively inhibited by ligands such as zinc [177], although this may be an artifact of heterologous expression [261]. The adjacent ligand-binding domain is elegantly formed by two, non-contiguous segments that are separated by a portion of the polypeptide sequence thought to weave its way through most of the transmembrane region; as a result, conformational changes within the ligand-binding domain are thought to influence opening

of the channel pore [56]. Four hydrophobic domains are believed to form the transmembrane region: the M1, M3, and M4 are predicted to cross the membrane as helices, while the M2, which lines the lumen of the pore, is expected to be a re-entrant loop that connects M1 and M3 [14, 19].

Among the NMDAR subunits, the C-terminal domain (CTD) is regarded as the most divergent region of the protein sequence [201], and can vary between 80-600 amino acids [56]. In addition to accounting for almost half the length of certain subunits (e.g., GluN2A and GluN2B), the CTD appears to be particularly important for intracellular signalling, trafficking, and localisation of the receptors due to the presence of multiple protein motifs that permit interaction with a variety of enzymes and scaffolding molecules. In particular, the intracellular region contains multiple locations for post-translational modifications, such as tyrosine phosphorylation [31, 125, 205, 236].

While the comparatively short CTD of the GluN1 does possess a tyrosine residue (Y837) [204], the subunit does not appear to experience tyrosine phosphorylation [121]; in contrast, each CTD of the GluN2 subunit contains 25 tyrosine residues, although not all of these residues will accept a phosphate group. On the GluN2A subunit, Y1292, Y1325, and Y1387 are thought to be the primary tyrosine residues subject to phosphoregulation [114]. On the GluN2B subunit, phosphorylation of Y1252, Y1336, and Y1472 has been reported [163]. Despite comprising a relatively small number of sites within the extensive CTD, tyrosine residues have become regarded as crucial points of convergence for signalling pathways that modulate NMDAR activity [170, 204, 205, 237].

### 3.3. Receptor function and cellular distribution

The basic pattern of excitatory signal transmission between the overwhelming majority of central neurones in the mammalian brain involves the pre-synaptic release of glutamate, its passage across the synaptic cleft, and its interaction with post-synaptically positioned GluRs. While basal synaptic transmission tends to be mediated by the AMPA sub-type of iGluR, periods of higher frequency synaptic activation (such as those that would tend to be present during an ischaemic event) recruit the NMDAR; the primary reason for the distinct activation profiles rests with a unique characteristic of the receptor. During basal transmission, the NMDAR's endogenous agonist (i.e., glutamate, which binds to an extracellular segment of GluN2 or GluN3 subunits) and its co-agonist (i.e., glycine, which binds to an extracellular segment of the GluN1 subunit) are present, yet the receptor remains functionally silent (i.e., ion conductance does not occur). The lack of basal NMDAR activity is attributable to a voltage-dependent blockade of the channel pore. At resting membrane potentials, external magnesium ions (which experience a significant inward driving force due to their high external concentration) enter the NMDAR pore, and bind in a manner that prevents further ion passage; however, membrane depolarisation of a sufficient magnitude and duration leads to the expulsion of  $Mg^{2+}$  from the pore, which permits the subsequent movement of cations [168].

Upon activation, glutamate receptors become permeable to both monovalent and, in some cases, divalent cations; however, the nature and degree of ion flux is not equivalent across iGluRs, and the NMDA sub-type has become acknowledged as the primary mediator of  $\text{Ca}^{2+}$  passage [57, 152, 236]. The ability to permit the entry of calcium ions is a primary reason that NMDARs make substantial contributions to both physiological and pathological phenomena [223, 236], although their pattern of cellular distribution also plays a role in their wide functional reach. Similar to many other ion channels, NMDARs are recognised as being widely dispersed across the cellular surface, however, information regarding the manner in which membrane bound receptors are distributed has only recently begun to be gathered. The available evidence indicates that NMDARs are not uniformly distributed at the plasma membrane, but can be divided into three spatially defined categories: synaptic, peri-synaptic and extra-synaptic [70].

The post-synaptic population exhibits the greatest density, relative to the other sub-regions, and responds directly to pre-synaptically released glutamate [70]. Peri-synaptic NMDARs have been operationally defined as those located within a 300 nm range of the post-synaptic terminal [70, 184, 275], and are believed to be activated by glutamate released from the pre-synaptic terminal following strong stimulation. Approximately half of all surface NMDARs, depending upon the developmental stage, are located extra-synaptically; however, relative to synaptic receptors, this compartment has a low density given the much broader area [72, 233]. Under physiological conditions, extra-synaptic NMDARs are unlikely to be activated by pre-synaptically released glutamate; however, they could be stimulated by glutamate released either from other sources [104, 128], or following the spillover of synaptic glutamate caused by ischaemia.

## **4. Regulation of NMDA receptors by tyrosine phosphorylation**

### **4.1. Phosphorylation as a key determinant of ligand-gated ion channel function**

Proteins exist as part of a complex system of elements that interact with one another to allow a cell to respond, directly or indirectly, to changes in its environment. Over several decades, the cellular distribution and molecular interactions of many proteins have been shown to be fundamentally regulated by post-translational modification in the form of phosphorylation [178, 238]. In essence, phosphorylation involves the protein kinase-mediated transfer of the  $\gamma$ -phosphate from adenosine triphosphate to a serine, threonine, or tyrosine residue of a substrate (although other amino acids may also be modified [40]), and is a process counteracted by protein phosphatases, which catalyse dephosphorylation.

Near the end of the 1980s, phosphorylation began to emerge as an important determinant in the function of a class of proteins essential for most neuronal communication – ligand-gated ion channels (LGICs). The first study to illustrate that LGICs could be regulated by phosphorylation revealed that NMDA currents recorded from cultured hippocampal neurones gradually declined during dialysis with an intracellular solution, but that the loss of receptor activity could be prevented by the addition of an ATP regenerating solution to

the dialysate [148]. In the mid-1990s, further work not only confirmed that the NMDA receptor was indeed regulated by phosphorylation, but also that a critical site for the modification was at tyrosine residues [249]. Subsequent reports established that a variety of other LGICs, such as the inhibitory GABA-A receptor [159] and the nicotinic acetylcholine receptor [246], were also regulated by the activities of tyrosine kinases and phosphatases, and helped to establish the importance of phosphorylation in the control of synaptic function.

## 4.2. Regulation of NMDA receptors by tyrosine phosphorylation

Electrophysiological studies have established that NMDAR function is regulated by a balance between phosphorylation and dephosphorylation of tyrosine residues [31, 204, 205]. Within mammalian neurones, introducing an exogenous protein tyrosine kinase (PTK), or inhibiting endogenous protein tyrosine phosphatase (PTP) activity enhances NMDAR currents [249]; in contrast, inhibiting PTK activity [249, 250], or introducing an exogenous PTP [250] suppresses NMDAR currents. In addition, exogenous PTKs have been shown to potentiate currents mediated by recombinant NMDARs [32, 108]. During the past twenty years, members of the Src family of PTKs have emerged as the predominant tyrosine kinases responsible for mediating activity of the NMDA receptor.

### 4.2.1. Src Family Kinases (SFKs) and the brain: An introduction

The Src family of non-receptor, protein tyrosine kinases (SFKs) consists of several lower molecular weight proteins (52 to 62 kDa) that share a common domain organisation, which includes a catalytic region (the Src homology 1 or SH1 domain) and two regions that guide protein-protein interactions (the SH2 and SH3 domains) [170, 204]. Given that Src, the prototypical SFK, was initially identified as a proto-oncogene [218], SFKs in the brain were originally believed to influence only those processes related to the regulation of neuronal proliferation and differentiation [102, 116]. However, SFKs were subsequently shown to be expressed in differentiated, post-mitotic neurones, which suggested that these kinases might participate in neural activity past the point of early development [86, 220]. Notably, significant changes in neuronal plasticity and behaviour have been observed in adult mice lacking certain SFKs [68, 239], while the expression and activity of Src was shown to increase during spatial learning [278].

Within the mammalian nervous system, the expression of five members of the SFKs has been established; of these, Src [101], Fyn [221], and Yes [105] have been found within the post-synaptic density (PSD), which is a specialised region of the post-synaptic terminal thought to provide a molecular scaffold that helps regulate proper protein placement. In addition, Src [270], Fyn [267], and Yes [105] have been shown to be components of a large complex in the PSD that includes the NMDA receptor. Considered together, the biochemical analyses reveal that several SFKs have a spatial distribution appropriate to permit regulation of NMDAR function.

#### 4.2.2. Evidence to support SFKs as modulators of NMDAR channel gating

When activated, ion channels, such as the NMDAR, passively conduct charged particles (i.e., they permit current flow) across the plasma membrane in a direction influenced by electrostatic forces and the ionic concentration gradient. The transition of a channel between closed and open states (i.e., from non-conducting to conducting) is referred to as gating, and requires a dramatic, albeit temporary, change in the channel's structure. The first evidence that SFKs might affect NMDAR function through alterations in channel gating was provided by a study that recorded channel currents from dissociated neurones [270]. A specific, high-affinity activator of SFKs [134] increased synaptic NMDAR-mediated currents, while an antibody that inhibited SFKs (anti-cst1; [195]), but not other protein tyrosine kinases, depressed NMDAR channel gating. A subsequent report, which recorded activity within neurones from acutely prepared brain slices, confirmed that the phosphopeptide SFK activator was able to significantly enhance NMDAR gating [141].

Additional support for the ability of SFKs to mediate NMDAR function was provided by a study that found protein tyrosine phosphatase alpha (PTP $\alpha$ ) enhanced NMDAR-mediated synaptic currents in both cultured neurones and brain slices, while reducing PTP $\alpha$  activity with an inhibitory peptide reduced NMDAR currents [127]. Although the findings appear superficially paradoxical, PTP $\alpha$  is thought to activate SFKs by selectively dephosphorylating a residue in their regulatory domain, and thereby interfering with an intramolecular interaction that maintains the kinases in an inactive state [176]. In support of the proposed mechanism of action, PTP $\alpha$  has been shown to activate several different SFK members within cell lines [17, 87, 279], and SFK activity is substantially reduced in PTP $\alpha$  deficient mice [186]. As well, in cells lacking SFKs, or in which SFK activity is inhibited, PTP $\alpha$  has no effect on NMDAR currents [127].

While little doubt can exist that SFKs are involved in regulating NMDAR activity, the identities of the family members that might contribute to the process are not definitively known; however, considerable evidence has drawn attention to at least two kinases. The first candidate to be examined was Src, which was implicated by experiments illustrating that application of Src-specific inhibitors (the antibody anti-src1, and the peptide Src(40-58), which was the immunogen used to create the antibody) significantly decreased synaptic NMDAR-mediated currents and NMDAR channel gating [270]. As well, the Src-specific inhibitors were found to prevent the increased channel activity produced by application of a high-affinity SFK-activating peptide, which suggested that endogenous Src is required for SFK-mediated upregulation of NMDAR activity. To provide a structural complement to the functional studies, recent work with GluN2A subunits expressed in a heterologous expression system has found that Src directly interacts with segments in the C-terminus [75].

The second SFK member considered to have a role in NMDAR activity is Fyn, which was initially implicated by experiments wherein exogenous Fyn was shown to modulate glutamate-evoked currents mediated by recombinant NMDARs expressed in HEK293 cells [108]. Subsequently, co-expression of a constitutively active form of Fyn with GluN1 and GluN2B subunits in cerebellar granule cells was found to cause a significant increase in the

amplitude of NMDA miniature excitatory post-synaptic currents [188]. In addition, several groups have reported that Fyn is able to phosphorylate both GluN2A and GluN2B within post-synaptic densities of the rodent forebrain [237].

Along with a direct effect upon NMDARs, SFKs may influence channel activity by phosphorylating proteins that associate with the receptor. For example, the post-synaptic density contains several proteins that are both tyrosine phosphorylated and potentially connected with NMDAR function, such as the scaffold protein PSD-93 [161, 206]. As well, phosphorylation of GluN2B may recruit signalling proteins, such as phosphatidylinositol 3-kinase, which has been shown to associate with the subunit [94, 182]. In addition, tyrosine phosphorylation of GluN2 subunits may prevent the loss of signalling molecules from the NMDA receptor by limiting degradation caused by calpain [18, 197], one of the principal proteases activated following ischaemia (section 2.2). Taken together, the variety of evidence suggests that SFKs may indirectly influence NMDAR function by altering the manner in which scaffolding and signalling proteins interact with GluN subunits.

#### *4.2.3. Evidence to support SFKs as modulators of NMDAR trafficking*

Rapid synaptic communication was traditionally believed to be altered by structural changes in ligand-gated ion channels that affected gating properties, such as mean open time and open probability. However, during the past fifteen years, cellular trafficking events, which modify the surface density of ion channels, have attracted considerable attention as an additional means whereby synaptic transmission can be regulated [41]. Although the inhibitory GABA-A receptor was the first LGIC found to undergo changes in cell surface expression in response to extracellular signals [245], the trafficking of other receptors, notably the NMDAR, has been the subject of growing interest over the past decade [70, 120, 183]. In particular, a number of studies have revealed that NMDARs are quite mobile, and undergo regulated trafficking between intracellular organelles and the plasma membrane [51, 64, 74, 124, 188, 191] and between extra-synaptic and synaptic sites [65, 71, 234].

The earliest evidence that NMDAR trafficking could be influenced through tyrosine phosphorylation was provided by a study wherein the repeated application of glutamate to heterologously expressed GluN1 and GluN2A subunits lead to an increase in receptor internalisation that could be prevented by application of Src [242]; using site-directed mutagenesis, the authors found that agonist-mediated dephosphorylation of a single tyrosine residue in the C-terminus of the GluN2A subunit was responsible for the reduced surface expression of functional channels. Subsequent studies wherein tyrosine phosphatase activity was reduced have demonstrated that phosphorylation of the NMDAR is positively associated with its surface expression. For example, brief treatment of cultured striatal neurones with a general inhibitor of tyrosine phosphatases increased the level of GluN2 subunit tyrosine phosphorylation and their surface expression [85]. As well, treatment of cultured cortical cells with a short interfering RNA to reduce the expression of a tyrosine

phosphatase known to interact with the NMDAR lead to both an increase in its surface expression and the level of  $\text{Ca}^{2+}$  influx after agonist stimulation [24].

While enhanced surface expression of a receptor would reasonably be expected to increase its activity, the possibility that such a change will have a functional outcome is greater if the insertion occurs at synaptic membranes. In acutely prepared striatal slices from adult animals, a tyrosine phosphatase inhibitor was observed to increase the phosphorylation state of GluN2 subunits and increase their association with synaptosomal membranes; notably, treatment with a tyrosine kinase inhibitor had effects in the opposite direction [51]. An ensuing study that used tissue slices from the adult hippocampus and a separate set of tyrosine kinase and phosphatase inhibitors found the same general pattern tying together changes in GluN2 subunit phosphorylation status with synaptosomal membrane density [64]. Intriguingly, a further report revealed that the surface location to which GluN2 subunits are trafficked may be differentially affected by changes in tyrosine phosphorylation [65]. In particular, increasing the phosphorylation level of GluN2B subunits through the application of a tyrosine phosphatase inhibitor tended to increase the extra-synaptic expression of the NMDAR more greatly than its synaptic expression.

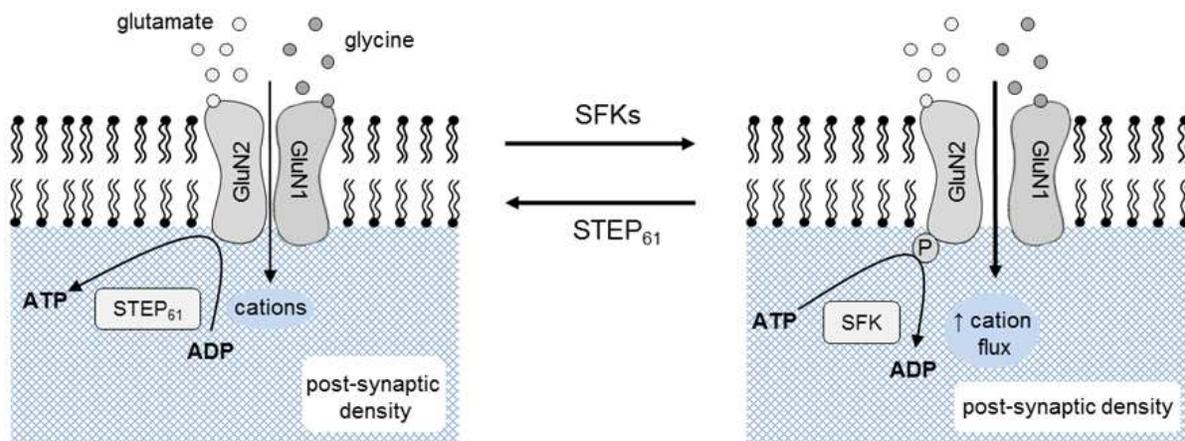
#### *4.2.4. Balancing of SFK activity by tyrosine dephosphorylation*

The level of NMDAR tyrosine phosphorylation is currently understood to be regulated by a balance between the activity of SFKs and protein tyrosine phosphatases [170, 204, 205] (figure 3). The PTPs are a large, structurally diverse family of enzymes [247] that play a number of important roles in the CNS, including contributing to the regulation of neural development [162, 219]. The striatal enriched phosphatase (STEP) is a brain-specific, non-receptor PTP that was originally found to be highly expressed within the adult rodent striatum [66, 138], and has subsequently been identified in other regions, such as the hippocampus [23]. While STEP immunoreactivity can be observed throughout the soma and processes of neurons [23], its presence at the post-synaptic density [174], a prominent structure at excitatory synapses, and its direct interaction with NMDARs [181], strongly suggest that the phosphatase contributes to signal transduction.

The ability of broad PTP inhibition to increase, and broad PTP activation to decrease, NMDAR gating in spinal cord neurones provided the initial evidence that the receptor was modulated by a phosphatase [250]. However, the identification of STEP as the putative candidate was not proposed until a report using the same experimental preparation showed that recombinant STEP reduced NMDAR activity, while the intracellular application of a function-blocking STEP antibody increased synaptic NMDAR-mediated currents [181]. Furthermore, within hippocampal slices, inhibition of STEP was shown to increase NMDAR activity, while recombinant STEP was able to occlude the development of a form of NMDAR-dependent synaptic plasticity.

The modulation of NMDA receptor function by STEP has become associated with its ability to affect the level of phosphorylation at a single tyrosine residue (Y1472) located in the distal

portion of the GluN2B C-terminus [123, 196]. When dephosphorylated, the unique residue, which is part of a short motif (YXX $\phi$ , where X is any amino acid and  $\phi$  = a bulky hydrophobic amino acid), promotes endocytosis by allowing the development of hydrophobic interactions between cargo molecules and clathrin adaptor proteins, such as AP-2 [235]. Notably, the phosphorylation of Y1472 and the association of GluN2B with adaptor proteins are inversely associated [164], and the overexpression of a Y1472 mutant unable to interact with AP-2 leads to a significant increase in the number of NMDA receptors at the synapse [188]. As well, STEP co-immunoprecipitates with the GluN1 subunit [181], directly dephosphorylates Y1472 [216], and, when deleted from the mouse genome, causes hyperphosphorylation of Y1472 in synaptosomal membranes prepared from the hippocampus [277]. Along with a direct effect upon Y1472, STEP may also act indirectly by dephosphorylating regulatory residues in the catalytic domains of Fyn, which is the principal SFK acting at Y1472 [165], and proline-rich tyrosine kinase 2 (Pyk2), which is an upstream activator of SFKs [262].



**Figure 3.** A simplified illustration of the key elements that regulate tyrosine phosphorylation of the NMDA receptor at the post-synaptic terminal.

## 5. Ischaemia-related changes in tyrosine phosphorylation of the NMDA receptor

### 5.1. Effects of ischaemia upon NMDA receptor subunit expression

Ischaemia causes a dramatic change in gene expression, which is largely attributable to a broad reduction in gene transcription and/or the inhibition of protein translation [109, 112]. While the general pattern of change suggests the level of most genes (and their protein products) will be reduced after ischaemia, several genes do show an increased level of expression; for example, certain ones associated with neuronal survival, such as members of the heat shock family of proteins. The first study to examine the effect of transient global ischaemia upon GluN2 subunits found that mRNA of both the GluN2A and GluN2B subunit declined in a progressive manner over 24 h of reperfusion, and that the transcriptional change was reflected in a pronounced loss of the proteins over the same time

period [274]; notably, the study employed an antibody that detected an epitope shared between the subunits, so the relative nature of the protein loss was not apparent. A subsequent study that used subunit specific antibodies revealed that the pattern of reduced protein expression may not be equal, for the loss of the GluN2A subunit was greater [228]. In agreement with the possibility that GluN2 subunits may be differentially affected by ischaemic insult, hypoxia-ischaemia applied to rats at post-natal day seven caused a reduction of GluN2A protein levels within one hour of reperfusion, while a reduction in the GluN2B subunit was not observed; in contrast, similar experiments performed with animals at post-natal day 21 showed a significant reduction of only the GluN2B subunit [82].

While evidence does exist that the expression of GluN2 subunit proteins may be decreased by ischaemia, a comparatively greater amount of work suggests that the level of the subunits is not altered. For example, no change in GluN2B immunolabelling was detected in hippocampal synaptosomes prepared six hours after a period of transient global ischaemia in gerbils [179]. As well, the level of GluN2A and GluN2B subunit proteins in either hippocampal homogenates [227], or forebrain post-synaptic densities [226] prepared six hours following global ischaemia in rats did not appear to differ from the levels seen in control samples [227]; in agreement, a large group of studies that employed the same experimental approach failed to find an effect of the insult upon hippocampal protein expression of the GluN2A subunit (the GluN2B subunit was not examined) [33, 98, 136, 144, 248]. When the period of reperfusion was extended to 24 h, one study continued to find no change [97], while another found a slight, albeit significant, decline in GluN2A protein levels in whole hippocampal homogenates (again, the GluN2B subunit was not assessed) [135]. Collectively, the data strongly suggest that GluN2 subunit protein expression, particularly of the GluN2A variant, is not likely to be altered within the first 24 h after a brief period of global ischaemia in adult animals.

## 5.2. Ischaemia and the general cellular pattern of tyrosine phosphorylation

In the early 1990s, a brief stimulation of cultured hippocampal neurones with either glutamate, or NMDA was shown to cause a significant increase in tyrosine phosphorylation of mitogen activated protein kinase (MAPK, a class of kinases that respond to extracellular signals and mediate proliferation, differentiation, and cell survival) in a manner sensitive to blockade of the NMDA receptor [7]. Given the establishment of a connection between NMDA receptor activation and downstream tyrosine phosphorylation, subsequent reports sought to determine whether the excessive stimulation of the receptor that occurs during ischaemia would cause a similar pattern of change. Indeed, a brief period of bilateral carotid artery occlusion in gerbils (which would cause global ischaemia) was found to very quickly evoke a significant and transient increase in hippocampal MAPK phosphorylation that could be prevented through NMDAR antagonism [30, 107].

Once the association between ischaemia-mediated activation of the NMDA receptor and MAPK phosphorylation had been confirmed, a search for other proteins that might experience an induced change in tyrosine phosphorylation began in earnest. Within gerbils,

global ischaemia was clearly shown to cause a rapid and significant increase in the level of tyrosine phosphorylation of a number of hippocampal proteins, particularly those with a higher molecular weight [171, 179, 269]. Within rats, both transient forebrain [99, 209] and global [34, 225, 228] ischaemia were found to induce a sustained increase in tyrosine phosphorylation of higher molecular weight proteins in the hippocampus. In addition, acutely prepared hippocampal slices subjected to *in vitro* models of ischaemia (i.e., oxygen-glucose deprivation) also displayed changes in the general level of tyrosine phosphorylation; however, while the magnitude of the effect was similar to that observed with the *in vivo* models, the direction of the effect was the opposite [6, 26].

Intriguingly, the post-ischaemic increase in tyrosine phosphorylation observed with *in vivo* injury did not appear uniformly throughout the hippocampus, which is composed of three major sub-fields: *cornu ammonis* (or CA) sector 1, CA3, and the dentate gyrus [54, 131]. Immunohistochemical labelling of hippocampal slices prepared at several time points following global ischaemia indicated that the CA3 and dentate gyrus appear to have the most intense initial increases in phosphorylation, but that, over several days, the CA1 region becomes more strongly labelled [269]. Immunoblotting revealed that the CA3 and dentate gyrus regions displayed greater tyrosine phosphorylation, particularly of higher molecular weight proteins, during the first two days after insult [228]. A disproportionate increase of tyrosine phosphorylation in the CA3-dentate gyrus was also observed in synaptosomes (sub-cellular fractions of pre-synaptic terminals that also include remnants of many post-synaptic sites) during the first day after ischaemia [99].

The elevated level of tyrosine phosphorylation routinely observed in various models of ischaemic brain injury would intuitively be attributable to increases in the activity of tyrosine kinases, decreases in the activity of tyrosine phosphatases, or a combination of the two. Within whole hippocampal homogenates, the level of Src was not found to change during a six hour period of reperfusion following either global ischaemia in adult animals [273], or hypoxia-ischaemia in pre-weanling animals [103]. In contrast, during the same length of time after global ischaemia the level of both Src and Fyn proteins were observed to experience about a twofold increase within the post-synaptic density [16, 34, 226].

Autophosphorylation of Y416 in the catalytic domain of SFKs is necessary to permit enzyme activity [204, 211, 266]. Within several hours of reperfusion following global ischaemia, a general increase in the level of tyrosine phosphorylation at hippocampal Src was observed [135, 136]; as well, a specific increase in Y416 phosphorylation was found within the whole hippocampus [38, 79, 248, 258, 273], and the CA1 [130, 264, 273] and CA3-dentate gyrus [79] regions. The level of phosphorylated Y416 was also found to be increased in both synaptosomes [160] and post-synaptic densities [16, 35, 160] prepared from the rodent forebrain region after the return of blood supply to the insulted area. In agreement with the immunoblotting data, *in vitro* enzyme activity assays confirmed that Src function in either whole hippocampal homogenates [78], or hippocampal synaptosomes [180] was clearly greater several hours after a brief period of global ischaemia.

While understanding how ischaemia may alter tyrosine kinase activity has been the principal focus of many studies, a developing body of work has chosen to examine changes at the level of tyrosine phosphatases; in particular, attention has been drawn to STEP. Glutamate-mediated excitotoxicity within cultured cortical neurones lead to the cleavage of STEP (i.e., STEP<sub>61</sub>, a membrane-associated isoform localised to the endoplasmic reticulum and post-synaptic density) into a lower molecular weight isoform [66, 166, 263]. In agreement with the cell culture work, transient hypoxia-ischaemia in younger rats [81], brief focal ischaemia in adult rats [25], and global ischaemia in gerbils [89] caused the loss of STEP<sub>61</sub>, and a concomitant rise of lower molecular weight STEP in the affected brain areas. Quite likely, STEP degradation is mediated by Ca<sup>2+</sup>-activated proteases, such as calpain; for example, the *in vitro* incubation of isolated PSDs with calpain causes STEP<sub>61</sub> breakdown [81, 166], while treatment with a calpain inhibitor (calpeptin) prevents glutamate-activated STEP<sub>61</sub> cleavage [263]. Ischaemia also appears to reduce STEP levels at the transcriptional level by causing a rapid and significant reduction in its mRNA expression [25]. In addition, the exposure of cultured neurones to oxidative stress, using a free radical that undergoes increased production after ischaemia [4], results in greater oligomerisation of STEP<sub>61</sub>, which causes a reduction in its activity level [46]. Considered together, the data indicate that ischaemia initiates a series of different processes that reduce STEP activity, and thereby contributes to enhanced levels of tyrosine phosphorylation.

### 5.3. The NMDAR as a specific substrate of ischaemia-mediated tyrosine phosphorylation

#### 5.3.1. Evidence to illustrate ischaemic alteration of GluN2 phosphotyrosine status

The knowledge that ischaemia can bring about changes in the broad pattern of tyrosine phosphorylation, particularly of a glycoprotein with a molecular weight of 180 kDa, began to acquire additional significance after the identification of the GluN2B subunit as the major tyrosine phosphorylated, higher molecular weight glycoprotein associated with the post-synaptic density [157]. Shortly thereafter, came the first direct demonstration that ischaemia could specifically alter the tyrosine phosphorylation of NMDA receptor subunits; in particular, for several hours after a brief period of global ischaemia in adult rats, Takagi et al. [225] observed a rapid and dramatic rise in tyrosine phosphorylation of hippocampal GluN2A and GluN2B subunits. Although the modified phosphorylation pattern of the subunits was still clear 24 h after the insult, the GluN2A subunit consistently displayed a degree of change several times greater than that observed for the GluN2B subunit. As well, the magnitude of the effect was substantially greater in the hippocampus than either the cerebral cortex, or the striatum. The seminal report showed that ischaemia can cause a sharp and sustained rise in NMDAR tyrosine phosphorylation that differentially affects the receptor's constituent subunits and develops in an anatomically heterogeneous manner; in addition, the findings helped to influence a number of studies that confirmed ischaemia initiates cellular changes that ultimately modify the degree of tyrosine phosphorylation at GluN2 subunits (Table 1).

As noted, one of the first details to emerge regarding the pathological modification of NMDAR phosphorylation was the swift manner in which the change developed. Subsequent work revealed that within twenty minutes of reperfusion following a brief period of global ischaemia, a substantial rise in tyrosine phosphorylation of the GluN2A subunit could be detected [33, 34, 135]. As well, the magnitude of the GluN2A subunit modification was shown to increase during at least the first six hours of reperfusion, and was seen to slowly return to basal levels over 2-3 days [135, 228]. Using a similar *in vivo* model of ischaemia coupled with immunoprecipitation-based experiments, several groups confirmed a significant rise in the tyrosine phosphorylation of GluN2A subunits in either whole hippocampal homogenates [98, 136, 144, 226, 248], or CA1 homogenates [264] prepared within six hours of reperfusion.

While an increase in tyrosine phosphorylation of the GluN2B subunit was also detected after ischaemia, those studies that examined both subunits tended to find that the effect upon the GluN2A subunit was more pronounced [16, 160, 225, 226, 228]. One exception, was a study that applied global ischaemia to gerbils; however, the variation may have been, in part, attributable to a species difference [271]. In contrast to adult animals, ischaemic injury in weanling rats (post-natal day 21) was found to affect tyrosine levels of the GluN2A and GluN2B subunits in a similar fashion, and was shown to have a significantly greater effect upon the GluN2B subunit in pre-weanling rats (post-natal day 7); as well, the change in phosphorylation appeared to be more transient, for the levels had returned to baseline within a day of reperfusion [82, 103]. While the effect of oxygen-glucose deprivation (OGD) upon cultured neurones tended to match well with the effect of *in vivo* ischaemia upon the GluN2A subunit [248], the application of OGD to acutely prepared hippocampal slices caused an apparent decrease in the level of tyrosine phosphorylation at both subunits [6, 26]; notably, Src protein levels have been shown to rise in hippocampal slices after OGD [9], which suggests that the unexpectedly reduced level of phosphorylation might be attributable to a comparatively greater elevation in the expression and/or activity of tyrosine phosphatases.

In addition to an obvious effect upon GluN2 subunit phosphorylation at the cellular level (i.e., within whole homogenates of a brain region), several reports observed that ischaemia also clearly modified subunits located within synaptic compartments. For example, the immunoprecipitation of GluN2A and GluN2B subunits from post-synaptic densities after global ischaemia in the rat revealed a clear rise in tyrosine phosphorylation during the first six hours of reperfusion [34], while immunoblotting with antibodies directed against phosphotyrosine residues in the GluN2A (Y1387) and GluN2B (Y1472) subunits displayed a similar effect [16]. In agreement, several hours after transient restriction in blood supply to the rat cerebral cortex, tyrosine phosphorylation of both the GluN2A and GluN2B subunits was found to be increased in both post-synaptic densities and synaptosomes [160]. As well, the phosphotyrosine level of the GluN2B subunit in synaptosomes enriched from gerbil hippocampus was markedly increased within six hours of reperfusion after global ischaemia [179].

Reference	Species	Age or Weight	Ischaemia Model	GluN2A pY Change Relative to Control	GluN2B pY Change Relative to Control	Sample Size
16	rat	200-250 g	4VO & 6 h REP	pY1387: ~3.5 fold increase	pY1472: ~2.8 fold increase	5
26	rat	150-200 g	OGD	↓ in sample blot, no measurement	↓ in sample blot, no measurement	-
33	rat	250-300 g	4VO & 15 min REP	~4 fold increase	not examined	3
34	rat	200-250 g	4VO & 6 h REP	↑ in sample blot, no measurement	~4.0 fold increase	3
35	rat	200-250 g	4VO & 45 min REP	not examined	↑ pY1472 in sample blot, no measurement	1
82	rat	PND 7, PND 21	permanent right CCAO + hypoxia & 1 h REP	PND 7: similar PND 21: ~3.2 fold increase	PND 7: ~3.5 fold increase pY1472 (PND 7): ~2 fold increase PND 21: ~3.2 fold increase pY1472 (PND 21): ~8 fold increase	3-4
98	rat	250-300 g	4VO & 6 h REP	~6.2 fold increase	not examined	3
103	mouse	PND 7	permanent right CCAO + hypoxia	~2 fold increase (15 min REP)	~3 fold increase (15 min REP)	7
135	rat	200-250 g	4VO & 0-72 h REP	~6.8 fold increase (6 h REP)	not examined	3
136	rat	220-270 g	4VO & 6 h REP	~2.8 fold increase	not examined	4
144	rat	250-300 g	4VO & 6 h REP	~4.8 fold increase	not examined	3
160	rat	7-8 wks; 240-360 g	right MCAO & 4 h REP	~4.7 fold increase	pY1336: ~3.9 fold increase pY1472: ~3.3 fold increase	5
179	gerbil	60-75 g	BCAO & 6 h REP	not examined	~1.9 fold increase	3
225	rat	200-250 g	4VO & 6 h REP	~30 fold increase	~2.2 fold increase	5
226	rat	200-250 g	4VO & 1 h REP	~6.6 fold increase	~3.1 fold increase	4
228	rat	200-250 g	4VO & 1 h REP	CA1: ~13.5 fold increase CA3/DG: ~14.0 fold increase	CA1: ~3.0 fold increase CA3/DG: ~5.4 fold increase	5 5
248	rat	220-250 g	4VO & 6 h REP	~2.6 fold increase	not examined	4
264	rat	250-300 g	4VO & 6 h REP	~2 fold increase	pY1472: ~1.5 fold increase	5
268	rat	16 DIV	OGD	~5 fold increase	not examined	5
271	gerbil	50-70 g	BCAO & 3 h REP	~1.6 fold increase	~2.2 fold increase	6
273	rat	250-300 g	4VO & 6 h REP	~1.9 fold increase	not examined	4

**Table 1.** Studies examining GluN2 tyrosine phosphorylation following experimental ischaemia. 4VO, four vessel occlusion; BCAO, bilateral carotid artery occlusion; CCAO, common carotid artery occlusion; DIV, days *in vitro*; OGD, oxygen-glucose deprivation; PND, post-natal day; pY, phosphotyrosine; REP, reperfusion

### *5.3.2. Interaction of GluN2 with synaptic elements may influence its phosphorylation*

Along with changes in the synaptic concentration and/or activity of those tyrosine kinases and phosphatases that act upon the NMDA receptor, the increased degree of tyrosine phosphorylation observed at GluN2 subunits after an ischaemic insult may also be attributable to changes in how the receptor directly interacts with SFKs. In particular, there may be greater association between phosphotyrosine sequences within GluN2 subunits and the segment of SFKs containing SH2 domains, which are relatively short amino acid modules believed to be necessary to permit the interactions that underlie many tyrosine based signalling cascades [150, 243]. Notably, ischaemia-mediated tyrosine phosphorylation was found to increase, by approximately twofold, the association of Src and Fyn with both GluN2A and GluN2B during reperfusion [226]. In agreement, a number of subsequent studies also observed greater association of SFKs with GluN2A during the first six hours following global ischaemia in adult rats [97, 98, 135, 248, 273]. As well, hypoxia-ischaemia applied to neonatal rats [103] and OGD of cultured neurones prepared from late-stage embryonic animals [268] also lead to greater co-immunoprecipitation of SFKs and the GluN2A subunit. Despite being examined to a lesser degree, the interaction between GluN2B and SFKs has also been clearly demonstrated after neonatal hypoxia-ischaemia [103], following global ischaemia in adult rats [179], and in response to global ischaemia within adult gerbils [271].

An additional component that may facilitate ischaemia-mediated changes in GluN2 phosphorylation is its enhanced interaction with a prominent post-synaptic scaffolding protein. The post-synaptic density 95 kDa (PSD-95) protein is a member of the membrane-associated guanylate kinase (MAGUK) family, and functions as an integral scaffolding protein in excitatory post-synaptic terminals [106, 265]. Like other MAGUK relatives, PSD-95 displays a modular structure that consists of three N-terminal PDZ domains (PDZ 1-3), an SH3 domain, and C-terminal guanylate-kinase domain. Through the PDZ1/2 domains, in particular, PSD-95 has been shown to bind with a conserved ES(E/D)V amino acid sequence located in the distal portion of GluN2 C-termini [113, 167], which permits PSD-95 to influence gating and surface expression of the receptor [39, 132]. A series of studies that employed multiple co-immunoprecipitation experiments helped to establish that an enhanced association of PSD-95 with the GluN2A subunit develops during the first few hours of post-insult reperfusion [33, 97, 144, 248, 273]. As well, an increased degree of binding with PSD-95 has been displayed by GluN2A subunits after OGD with cultured neurones [268] and by hippocampal GluN2B subunits within a few hours after global ischaemia in the gerbil [271].

Ischaemia clearly enhanced the interaction of GluN2 subunits with both SFKs and PSD-95, and increased the level of interaction between SFKs and PSD-95 [33, 49, 97, 144], presumably at its SH3 domain. As well, SFK-mediated tyrosine phosphorylation of GluN2 fusion proteins augmented their binding to PSD-95 [197], and co-expressing PSD-95 with the GluN2A subunit improved its ability to be phosphorylated by SFKs [231]. Considered together, these data suggest that the post-ischaemic increase of GluN2 tyrosine

phosphorylation (section 5.3.1) is attributable to a pathologically heightened and, potentially, self-amplifying degree of interaction among GluN2 subunits, PSD-95, and SFKs. Our understanding of how the tripartite complex operates following ischaemia has been greatly aided by a set of elegant studies that used molecular level approaches to alter its assembly. Reducing by approximately one-third the hippocampal expression of PSD-95 protein, through the repeated intracerebroventricular injection of antisense oligonucleotides, sharply attenuated both the post-ischaemic rise in GluN2A tyrosine phosphorylation and the elevated binding of SFKs with the GluN2A subunit [98]. In addition, adenoviral-mediated overexpression of the PSD-95 PDZ1 domain was able to nearly eliminate ischaemia-mediated changes in GluN2A phosphorylation, and prevented the expected increased interactions between the GluN2A subunit, PSD-95, and Src [248].

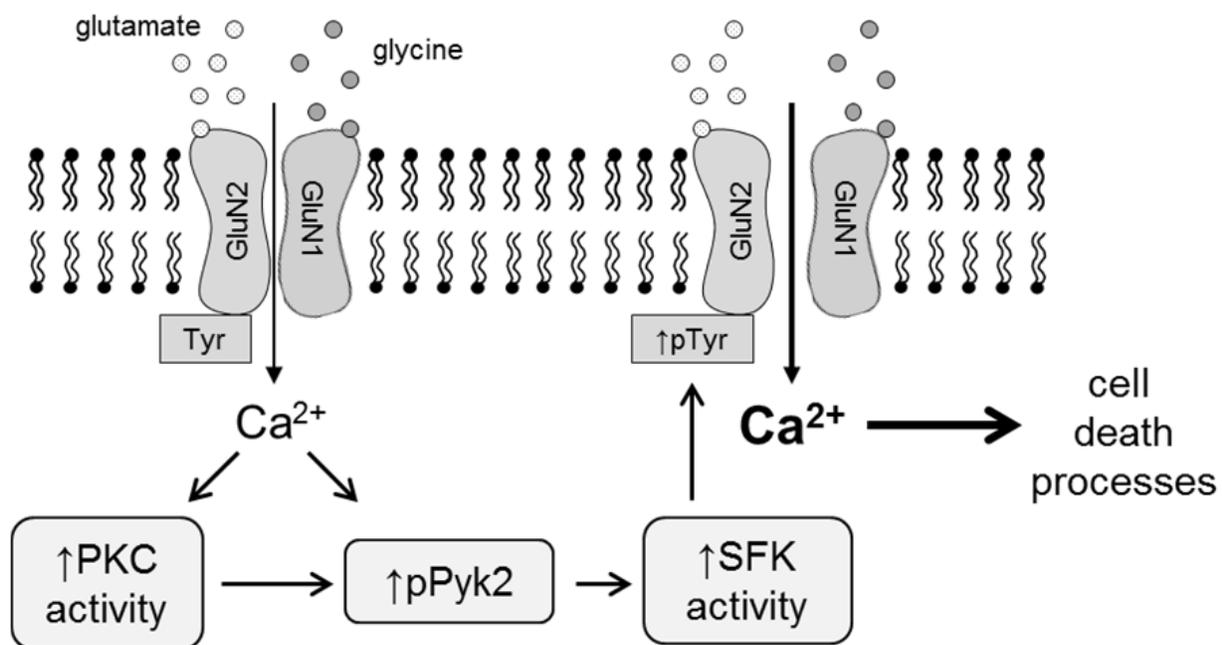
#### **5.4. Ischaemic changes that may contribute to increased SFK activation**

The elevation of tyrosine phosphorylation within neurones, in general, and at GluN2 subunits, in particular, would seem to be a consistent feature in the array of changes that follows cerebral ischaemia. Proximally, the ischaemia-mediated interactions of a complex formed between GluN2 subunits, synaptic scaffolding proteins, and members of the SFKs provide at least one working explanation for the increased pattern of phosphorylation; however, the fashion in which these components begin to assemble is still unclear. A starting point in understanding, from a slightly more distal perspective, the reason for heightened GluN2 phosphotyrosine levels may be to focus on SFKs; specifically, to ask why might SFK activity rise after ischaemia? Fortunately, the answer to the question is beginning to take shape, and appears to involve the activity of two other, putatively interconnected, kinases. The first one is protein kinase C (PKC), which acts upon serine-threonine residues and exists in at least ten different isoforms, most of which are heterogeneously distributed within the brain [229]. While the various members of the PKC family have different mechanisms underlying their activation, many of them are stimulated by changes in  $Ca^{2+}$  dynamics and the generation of free radicals [27], which are key consequences following from the over-activation of NMDARs that characterises ischaemic injury (section 1). Also, the translocation of PKC to the post-synaptic density after ischaemia has been well established [35, 151].

The signalling cascade initiated by the stimulation of PKC is quite diverse, however, the ability of the activated enzyme to enhance NMDA receptor function [147, 260] and surface expression [118], is, to a large extent, attributable to its engagement of SFK activity. For example, the ability of PKC activators to potentiate NMDA-evoked currents in dissociated neurones was blocked by both tyrosine kinase inhibitors and Src-specific blocking peptides; as well, the PKC-dependent upregulation of the receptor was absent in neurones isolated from mice with a targeted deletion of the Src gene [140]. In addition, the stimulation of PKC activity in hippocampal slices was able to dramatically increase the level of phosphotyrosine detected within immunoprecipitated GluN2A and GluN2B subunits [73]. Direct support for the possibility that a PKC-dependent tyrosine kinase signalling cascade contributes to

ischaemic changes in GluN2 tyrosine phosphorylation was provided by a study wherein a PKC inhibitor administered immediately after an ischaemic challenge was able to reduce, by approximately half, Src Y416 phosphorylation, general GluN2A tyrosine phosphorylation, and GluN2B Y1472 phosphorylation [35].

The second kinase that may serve to regulate post-ischaemic SFK activity is the proline-rich tyrosine kinase 2, which is a member of the focal adhesion kinase family and highly expressed within the brain [62, 259]. Through mechanisms that are still being uncovered, Pyk2 stimulation (permitted by phosphorylation of its Y402 residue) can be initiated through either depolarisation-induced  $\text{Ca}^{2+}$  influx, or PKC activation [101, 129, 212]; notably, the level of total and phosphorylated Pyk2 in the post-synaptic density increases sharply less than an hour after post-ischaemic reperfusion has begun [34, 35]. Upon phosphorylation, Pyk2 has been observed to interact with the Src SH2 domain to form a complex that enhances Src function [48, 101, 122]. In addition, the degree of interaction between Pyk2 and GluN2A is quickly amplified in the hippocampus during reperfusion after global ischaemia in either rat [135, 145], or gerbil [271]. Together, the data strongly suggest that ischaemia actuates Pyk2 and enhances its interaction with GluN2 subunits, which, in turn, likely allows Pyk2 to bind and activate the SFKs that would also have been drawn into a complex with NMDA receptors (figure 4).



**Figure 4.** Model summarising the proximal steps leading to the ischemia-mediated increase in tyrosine phosphorylation of the NMDA receptor, and the possible association of its post-translational modification with upstream processes leading to cell death.

Direct support for Pyk2 as a critical component in the pathologic increase of GluN2 phosphorylation has been provided by a set of elegant studies that used either pharmacologic, or genetic approaches to downregulate the kinase's activity. The first study used lithium chloride (LiCl), which has been shown to protect neurones from ischaemic

injury [200]. The application of LiCl for several days to cultured cortical neurones was able to dramatically reduce the basal level of both activated Src and phosphorylation at Y1472 of GluN2B [90, 91]; more importantly, administration of LiCl to animals for several days prior to global ischaemia was able to reduce insult-mediated increases in tyrosine phosphorylation of GluN2A [144]. As well, pre-ischaemic LiCl was observed to reduce both the phosphorylation of Y402 of Pyk2 and Y416 of Src, and to diminish the association of both Pyk2 and Src with GluN2A that generally accompanies ischaemic reperfusion [145]. The second study involved the intracerebroventricular injection of Pyk2 antisense oligonucleotides, which lead to a reduction of nearly one-quarter in the enzyme's protein expression within the hippocampus [136]. Animals in which Pyk2 levels had been downregulated displayed not only very little post-ischaemic change in the level of GluN2A tyrosine phosphorylation, but also very little change in the activity of Pyk2 and Src and their ability to be co-immunoprecipitated with GluN2A.

## 6. Conclusions

A short period of time after the circulation of blood to a region of the brain is substantially reduced, a series of escalating changes is initiated that can very quickly place neurones on a path leading to cell death. One of the principal elements in the pathologic cascade is the excessive stimulation of the excitatory NMDA receptor, which initiates a broad array of potentially cytotoxic changes; most germanely, a disruption in the usually strict regulation of the intracellular calcium ion concentration. One consequence of the elevated level of internal  $Ca^{2+}$  is the initiation of an enzyme cascade that involves either the direct, or indirect (through the serine-threonine PKC) stimulation of the tyrosine kinase Pyk2, followed by the subsequent activation of SFK members that go on to increase the tyrosine phosphorylation of GluN2 subunits. Notably, the post-translational modification of the GluN2 subunits is likely both a consequence, and, potentially, a cause of the post-ischaemic enhancement of the degree of interaction between the subunits and Pyk2, SFKs, and PSD-95. One important outcome of the ischaemia-mediated increase of GluN2 tyrosine phosphorylation that is quite likely concerns the enhancement of NMDA receptor gating properties and surface expression, which would serve as a form of signal amplification with the undesirable effect of contributing to increased cell death.

Preliminary support for the possibility that elevated NMDA receptor phosphorylation may contribute to post-ischaemic neuronal loss is found in a collection of studies that have sought to manipulate the cellular level of tyrosine phosphorylation for a neuroprotective effect. The first report in the area revealed that the intracerebral injection of specific inhibitors of protein tyrosine kinases minutes prior to a brief period of global ischaemia was able to prevent the significant degree of neuronal loss that would normally have been seen within the CA1 sub-field of the gerbil hippocampus a week after the insult [107]. Subsequently, the intraperitoneal injection of a selective inhibitor of Src shortly after forebrain ischaemia was found to significantly reduce not only the usual increases in Src activity and general tyrosine phosphorylation, but also the level of cell death observed in the

hippocampus four days after the insult [171]. A group of successive studies observed that pretreatment of rats with PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), a selective SFK inhibitor, 0.5 h prior to global ischaemia was able to reduce by greater than half the usual increase in phosphorylated Src [130, 273] and GluN2A tyrosine phosphorylation [96, 273] seen after six hours of reperfusion; as well, cell density of the hippocampal CA1 sub-field five days after the insult was approximately 7-8 fold greater in the treated animals [96, 130, 273]. In addition, the injection of PP2 into neonatal mice shortly after hypoxia-ischaemia was able to reduce by about a third the degree of hippocampal cell loss observed several days after the insult [103].

By using tyrosine kinase inhibitors to control the level of GluN2 tyrosine phosphorylation after an ischaemic challenge, the excessive NMDA receptor activation that plays a critical upstream role in the resulting cell death may be, at least partially, addressed. As a result, a foundation may begin to be constructed that will serve as inspiration for the careful development of new approaches to address the discouraging absence of treatments for brain injury. Regardless of the outcome displayed by future studies that explore how understanding the causes and consequences of NMDA receptor tyrosine phosphorylation may be applied therapeutically, the activity will undoubtedly continue to add important details to our understanding of how the phosphorylation of brain proteins influences neuronal communication and synaptic dysfunction

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## 7. References

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