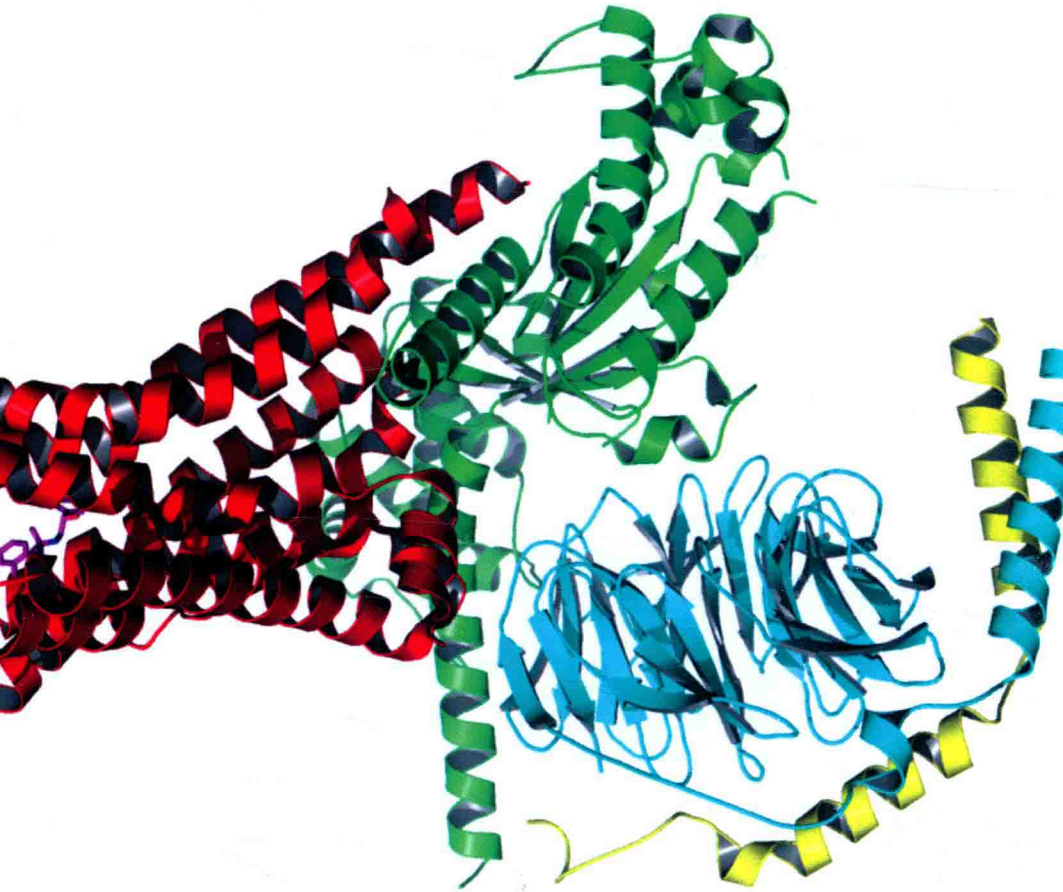
Protein Phosphorylation



Michelle McGuire

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Edited by **Michelle McGuire**

RC Callisto Reference

New York

Published by Callisto Reference,
106 Park Avenue, Suite 200,
New York, NY 10016, USA
www.callistoreference.com

Protein Phosphorylation
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International Standard Book Number: 978-1-63239-524-5 (Hardback)

Printed in China.

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Preface

This book compiles important information regarding protein phosphorylation and human health contributed by veteran scientists. The book elucidates the most significant research topics grouped under three sections: tyrosine kinases in receptor signaling and human diseases; protein kinases and phosphates in cell cycle regulation; and histidine kinases in two-component systems. It connects the basic protein phosphorylation channels with human health and diseases. This book also comprises of excellent figure illustrations and will be a valuable read for a broad spectrum of readers.

The information shared in this book is based on empirical researches made by veterans in this field of study. The elaborative information provided in this book will help the readers further their scope of knowledge leading to advancements in this field.

Finally, I would like to thank my fellow researchers who gave constructive feedback and my family members who supported me at every step of my research.

Editor

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Tyrosine Protein Kinases in Receptor Signaling and Diseases

Chapter 1

Tyrosine Phosphorylation of the NMDA Receptor Following Cerebral Ischaemia

Dmytro Pavlov and John G. Mielke

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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 rigourously 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 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 Ca^{2+} entry during and immediately after an ischaemic insult.

The dysregulation of intracellular 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 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 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 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.

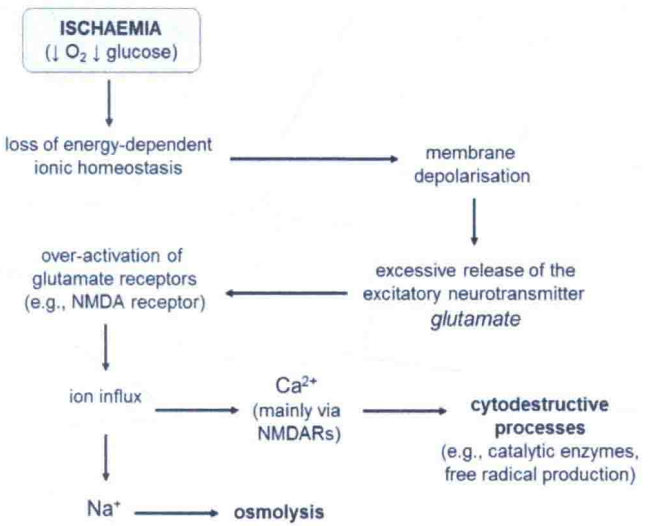

Figure 1. Summary of major elements in the early stages of ischaemic pathogenesis.

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), α-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].

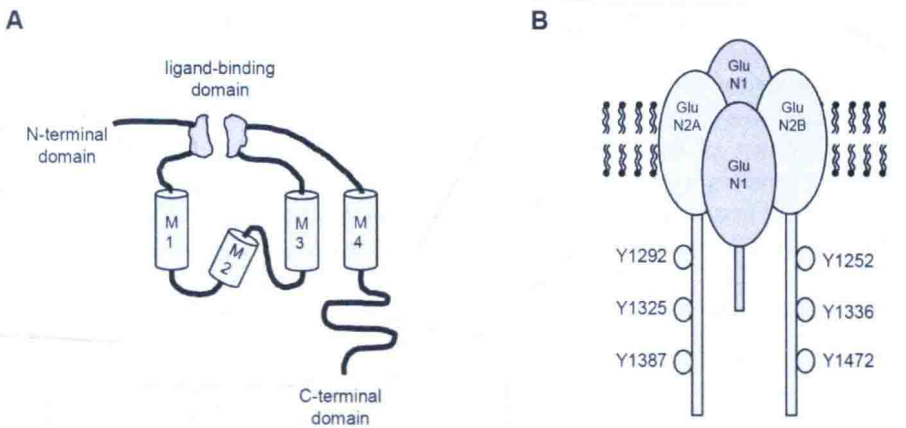

Figure 2. (A) Diagram of the structure for a typical NMDA receptor subunit. (B) Schematic illustrating one possible heterotetrameric combination of GluN subunits, along with identification of those GluN2 C-terminal tyrosine residues believed most relevant to phosphorylation-mediated changes in receptor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