NMDA Receptors in Stroke: Pathways and Potential Treatments

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Abstract

Ischemic strokes are the second-most common cause of death worldwide, with cerebral excitotoxicity following the cessation of oxygen and glucose delivery to the brain inducing confusion, changes in vision, sensation, and language capabilities. Poststroke brain injuries can persist for years, making post-ischemic recovery a crucial target for therapeutic treatments. NMDAR, a glutamate receptor, has been implicated in stroke pathology, with subunit GluN2B having been shown to promote cell death signal cascades. Stroke treatments must prevent apoptotic signals without impeding normal glutamatergic cellular function, propelling the search for treatments beyond mere GluN2B antagonists. This review examines pathways for treatment of ischemic stroke, beginning with low affinity extrasynaptic NMDAR antagonist memantine, then moving to the use of GlyT1 antagonist NFPS to increase glycine-induced NMDAR internalization and decrease excitotoxicity, and finally examining recent debates on CaMKII versus DAPK1 binding to GluN2B in post-ischemic cell death signaling.

Keywords: ischemia, excitotoxicity, NMDA receptors.

I. Introduction

Strokes represent the second-most common cause of death worldwide according to Wu & Tymianski (2018), with ischemic strokes comprising 87% of all strokes (Donnan et al., 2008). The pathology of a stroke lies within the brain and involves cessation of blood flow to the brain resulting in the disruption of a variety of cognitively mediated functions such as speech, physical sensation, vision, and understanding. Beyond stroke mortality, cognitive damage induced by ischemic stroke can take years to heal, making stroke the third most common cause of neural disabilities (Wu & Tymianski, 2018). Treatments for ischemic stroke are extremely limited, as only a single FDA-approved treatment currently exists: recombinant tissue plasminogen activator (rtPA). Furthermore, most stroke patients are disqualified from rtPA due to the associated cerebral hemorrhage risks (Wahlgren et al. 2007), or delays in seeking medical care, as the drug must be administered intravenously within 4.5 hours of a stroke.

In addition to cognitive impairment resulting from immediate post stroke brain injury, recent developments have indicated that ischemic stroke might increase risk for vascular dementia and Alzheimer's disease (Vijayan et al., 2016), although the exact molecular mechanisms linking these pathologies are not yet understood. There is thus an urgent need for better stroke treatments in order to combat stroke mortality as well as to improve recovery rates and limit cognitive damage caused by ischemic stroke. This damage can persist for years, severely impact patient quality of life, and potentially increase risk for the development of other neurological diseases.

One potential target in stroke pathology is N-methyl-D-aspartate receptors (NMDAR). Following the onset of stroke, when blood delivery to the brain has ceased, energy resources in the brain become depleted, leading to ionic dysregulation—as ATP-dependent ion exchange is no longer possible—and to subsequent uncontrolled glutamate release (Gasull et al., 2022). Excitotoxicity ensues, with incessant glutamate activation of NMDA and AMPA receptors prompting increased calcium release within neuronal membranes (Mao et al., 2022), triggering Ca2+ dependent cell death pathways and resulting in neuronal death. Research into the mechanisms of NMDAR excitotoxicity and the discovery of molecular pathways specific to ischemic stroke can inform crucial targets for treatments that decrease excitotoxicity following stroke onset, as well as potentially decrease the spread of post-ischemic necrosis and improve the rate of repair for stroke-induced brain injury.

II. NMDAR implication in stroke pathology

NMDAR plays a dual role in cell survival and death. A receptor localization hypothesis put forth by Sattler et al. (2000) theorizes that synaptic NMDAR contribute to cell survival through the activation of CREB, which suppresses pro–cell death genes (Hardingham, 2009) and promotes the expression of growth factor signal BTG2 found in BDNF and anti-apoptotic. In addition, synaptic NMDAR activation promotes calcium signaling which leads to the phosphorylation of Akt. Akt suppresses the apoptotic signaling protein BAD (Kim et al., 2001). In contrast, extrasynaptic NMDARs are believed to play the opposite role and promote cell death signals, including CREB dephosphorylation and inactivation. They also suppress environmental signals to the nu-

cleus via interference with the ERK pathway, which results in decreased gene expression and the promotion of cellular death (Hardingham, 2009). The role of extrasynaptic NMDARs in pro–cell death pathways may be rationalized by considering how tightly glutamate signaling is regulated in the brain and considering that extrasynaptic NMDAR activation would be indicative of excessive glutamate release and excitotoxicity.

Another theory for the role of NMDAR in ischemic stroke is the "NMDAR subtype" hypothesis, which claims that differences in the NMDA-binding subunits that make up a receptor determine the receptor's role in promoting either survival or cell death (Liu et al., 2007). NMDAR is usually comprised of four subunits: two GluN1 and two GluN2. The relevant differentiation for excitotoxicity is between GluN2A and GluN2, as it has been demonstrated that activation of NMDAR with the GluN2A subunit promotes cell survival, whereas activation of receptors containing GluN2B promotes cell death (Liu et al., 2007). To test this hypothesis, selective antagonists for each subunit were applied to cultures of cortical neurons, which were then bathed in glutamate. Inhibition of GluN2B activity resulted in reduced infarct volume when the antagonist was applied before the simulated stroke, demonstrating the implication of GluN2B in excitotoxicity. Moreover, activation of GluN2A via glycine agonist, as well as normal GluN2A activity in the presence of the GluN2B antagonist, resulted in decreased brain damage after stroke onset, evidencing the role of GluN2A in promoting cell survival and post-ischemic repair.

Subtype distribution shows trends of GluN2B subunits belonging primarily to extrasynaptic NMDAR, whereas GluN2A is more prevalent in synaptic NMDAR (Tymianski, 2011). This distribution is not absolute; therefore hypothetical treatments using GluN2B antagonists to target ischemic overexcitation might end up harming synaptic NMDA activity towards promoting cell survival. However, GluN2B has been shown to induce excitotoxicity at both synaptic and extrasynaptic NMDAR sites (Liu et al., 2007), a result that prompted research efforts to understand the role of GluN2B in mediating and prolonging excitotoxicity, and to develop treatments to suppress its interaction with downstream death-signaling pathways.

III. Mediating consequences of GluN2B excitotoxicity

One possible research avenue for inhibiting the spread of GluN2B excitotoxicity is finding antagonists that possess enough strength to block the death signals without impeding NMDAR function at the synapse. Difficulties in examining the specificity of antagonists for differentiating between GluN2 subtypes have resulted in few viable treatment options, none of which have been FDA-approved. However, Wang et al. (2017) recently proposed the use of GluN2B antagonist memantine as a potential therapeutic for increasing post-ischemic recovery. Memantine has been used to treat Alzheimer's disease and exhibits a lower affinity for NMDAR compared to other antagonists. It was shown to preferentially bind extrasynaptic NMDARs, as well as to reduce brain injury in mice in the acute stroke phase (Lipton, 2004). Memantine binds to excessively open NMDARs, which explains its greater affinity for high-activity extrasynaptic NMDARs during stroke. Memantine also dissociates rapidly, meaning it will not interfere significantly with normal signaling (Lipton, 2004).

Wang et al. (2017) used memantine to treat mice for 4 weeks, beginning 3 days poststroke. Memantine was administered via pump directly to the cortex, in doses of either 4 mg/kg of mouse body weight or 20 mg/kg daily, with a control population receiving saline. Motor coordination tests (tight rope test and rotarod) were administered and evidenced significant improvements for the 20 mg/kg/day memantine group from the first data points onwards, while the 4 mg/kg/day group remained at the same performance level as the control group of mice (Wang et al., 2017, figure 2). This result suggested that direct administration of memantine can improve motor coordination recovery time following ischemic stroke. Because the progression of the control mice's recovery over time was not followed, it cannot be concluded whether memantine increases the overall post-ischemic recovery from brain injury or if it solely increases the rate of recovery. The researchers also tested the different treatment groups for spatial memory using the Barnes maze test, through which they observed that the 20 mg/kg/day group showed decreases in total errors and latency during both the learning and retention phases. This result displays evidence related to the impact of memantine on cognitive repair (Wang et al., 2017, figure 3). Again, results were comparable for the 4 mg/kg/day group and the control group, and the lack of longitudinal study raises the question of the potential effects of memantine on the overall volume of recovery.

The researchers then euthanized the mice to observe the effects of memantine on synaptic plasticity and determine the molecular changes that resulted in greater behavioral improvements. Cresyl violet staining showed that the 20 mg/kg/day group displayed greater striatal volume 7 weeks post-stroke than the other two groups, as well as lower astrogliogenesis as determined through GFAP immunohistochemistry (Wang et al., 2017, figure 4, a—b). Researchers also found that memantine increases growth factor concentrations and regulates GluN2B, GluN2A, and PSD95 abundance in the contralesional motor cortex. Following a stroke, these proteins decreased in abundance, but memantine 20 mg/kg/day administration decreased GluN2B concentration after 14 days and partially restored GluN2A and PSD95 abundance after 28 days (Wang et al., 2017, figure 7, a—c). Decreased GluN2B reduced

NMDAR death signaling, while increased GluN2A increased cell survival. Furthermore, these results indicate that recovery improvements are still possible with later treatment administration. Memantine is thus a promising potential treatment for ischemic stroke patients. Future research should examine the effect of memantine on other brain areas and potentially other model organisms before attempting to administer it to human patients and should determine more precise dosages.

IV. Receptor endocytosis: a novel indirect mediator of excitotoxicity

NMDAR activation requires not only glutamate binding to the GluN2 subunit but also glycine as a co-agonist binding to the GluN1 at the glycine binding site (Rosenmund et al., 1998). Furthermore, Nong et al. (2004) demonstrated that glycine "priming" on NMDAR induces endocytosis through a clathrin- and dynamin-dependent pathway, with increased glycine signaling resulting in greater NMDAR internalization in hippocampal neurons, evidenced through weaker signals in response to NMDA application. This phenomenon, which requires the presence of both glutamate and glycine in the synaptic cleft, is called glycine-induced NMDAR internalization (GINI) and shows promise as a target for indirect mediation of excitotoxicity.

Cappelli et al. (2021) investigated GINI as a means of reducing NMDAR toxicity in ischemic stroke. First, they used cultures of CA1 pyramidal hippocampal neurons exposed to high concentrations of glycine to confirm that NMDAR-EPSC amplitude reduction was due to glycine-induced internalization. This hypothesis was verified when the effect of GINI was blocked by clathrin and dynamin inhibitors, preventing the endocytosis mechanism from operating. (Cappelli et al., 2021, figure 1). Cappelli et al. (2021) then demonstrated that Ca2+ influx is required for GINI, as NMDAR-EPSC increased with low extracellular calcium compared to normal amounts, indicating that a certain concentration of calcium is required. Application of nimodipine—a Ca2+ channel antagonist—to these cell cultures also decreased GINI amplitude, further confirming the need for calcium influx (Cappelli et al., 2021, figure 1). Further investigations into the mechanisms of glycine release into the synaptic cleft found that glycine is released during oxygen-glucose deprivation (Muller et al., 2013), conditions that correspond to the ischemic stroke environment.

GINI is countered by glycine uptake performed by glycine transporters like GlyT, which remove GINI from the synapse and therefore from glycine binding sites on NMDAR. Cappelli et al. (2021) hypothesized that GlyT +/- mice would experience greater GINI, as lower glycine concentrations would provoke the same amount of NMDAR signaling. Furthermore, glycine is neuroprotective (Chen et al., 2015), and it increases CREB phosphorylation and gene expression. Cappelli et al. (2021) additionally hypothesized that the GlyT +/- mice would experience reduced stroke volume, owing to the neuroprotective effects of increased GINI, which endocytoses NMDAR and prevents GluN2B cell death signaling pathways. TTC staining of cortex slices poststroke resulted in smaller infarct volume for the GlyT +/- mice, demonstrating the role of GINI in stroke mediation (Cappelli et al., 2021, figure 2).

Following the confirmation of GINI as a potential therapeutic target, Cappelli et al. (2021) tested the use of NFPS, a selective glycine transporter antagonist, as a means of increasing GINI. They found that mice pretreated with NFPS had decreased stroke volume, evaluating this decrease through TTC cortex staining for the infarct volume. Pretreated mice also experienced improved motor behavioral deficits compared to controls. Motor behavior was evaluated through a horizontal ladder task requiring dexterity. Although recovery was not extensively studied, these results were extremely promising for GINI as a stroke therapy. The mechanism of protection was further tested by transfecting HEK293 cells with a mutated GluN1 subunit which could not bind glycine. The mutant GluN1 cells treated with NFPS showed no difference in stroke volume compared to the control mice transfected with WT GluN1, proving that glycine binding to NMDAR was crucial to the neuroprotection mechanism (Cappelli et al., 2021, figure 3). This experiment served to show the possibility of GlyT antagonists as promoters of ischemic recovery, and provided possible directions for future experiments to examine cerebral glycine concentration determination in excitotoxicity in human patients to ascertain how GlyT antagonism might be targeted to reduce stroke pathology.

V. GluN2B cell death signaling

Precise mechanisms of GluN2B apoptotic signaling remain unclear. The prevailing hypothesis within the field of NMDAR ischemic pathology has been that GluN2B interacts directly with death-associated protein kinase 1 (DAPK1) at extrasynaptic sites, prompting downstream signaling cascades that lead to apoptosis (Tang et al., 2018). However, a recent paper from Buonarati et al. (2020) rejects this long-standing claim and instead proposes a GluN2B-CaMKII interaction as the mediator of the death signal, challenging previously held findings.

CaMKII and DAPK1 bind GluN2B at the same location and their interactions are mutually exclusive (Goodell et al., 2017). CaMKII-GluN2B binding is a feature of long-term potentiation, and displacement of this interaction by DAPK1 competition is a feature of long-term depression. However, there is confusion surrounding the pathways of both enzymes: species designed to displace one must also displace the other, owing to overlap in binding sites, yet the resulting effects of

DAPK1 displacement (promotion of cellular survival) do not appear to correlate with the effects of CaMKII binding inhibition (promotion of cellular death). Further research is needed to elucidate the exact interactions of both species with GluN2B.

Buonarati et al. (2020) blocked the respective effects of CaMKII and DAPK1 to examine subsequent effects on GluN2B excitotoxicity. They created a GluN2B variant with two point mutations which prevented binding of either enzyme to the subunit. They then induced cardiac arrest in mice and stopped the arrest using epinephrine injections and increased oxygen supply. They then used H&E staining to examine the extent of cortical damage, which was found to be extremely limited compared to the damage inflicted on controls with wild-type GluN2B. This supported the involvement of either CaMKII or DAPK1 in GluN2B cell death signaling, and binding assays were subsequently performed to determine which one was implicated.

In vitro, pull-down assays were performed for both enzymes. CaMKII binding to GluN2B was significantly impaired for the mutant subunits compared to the wild-type, while DAPK1 binding was not impaired. These results surprisingly point towards CaMKII as the signal mediator. Moreover, immunofluorescence colocalization tests showed that DAPK1 colocalized to the cell membrane near NMDAR that contains mutant GluN2B. CaMKII must be activated by Ca2+ pathways to bind GluN2B, so no colocalization was observed initially, but CaMKII remained separate from mutant GluN2B following stimulation to induce Ca2+ influx, even though it was shown to colocalize with the wild-type subunit (Buonarati et al., 2020, figure 1). This shows that mutation of the GluN2B binding region and disruption of cell death signaling prevents CaMKII mediation of NMDAR apoptotic signaling, and not DAPK1 as previously thought. If these findings are correct, this may signify incredibly important progress towards our understanding of NMDAR-mediated ischemic stroke, and open up new avenues for treatment exploration.

However, it is difficult to discredit decades of research proclaiming DAPK1 signaling as the pathway associated with NMDAR cell death. As recently as 2018, publications have evidenced this pathway, with Tang et al. (2018) showing that different mutations of GluN2B blocked DAPK1 pathways. The actual cell death cascade is likely more complex than previously thought, with new research perhaps implicating DAPK1 signaling downstream of the CaMKII-GluN2B interaction. Further research is needed to determine the veracity of either of the two proposed pathways, with possible directions including knockout or attenuation studies of CaMKII to determine whether DAPK1 might take over as a secondary signaler, or perhaps knocking out DAPK1 to see whether it is required for NMDAR cell death signals to progress.

VI. Conclusions

While the full extent of the molecular mechanisms mediating ischemic stroke pathology in NMDAR are not yet entirely understood, many recent advances have suggested new avenues for research and treatment development. Memantine and other low-affinity NMDAR antagonists show great promise for improving post-ischemic recovery for both motor and cognitive function, while pre-stroke treatment with GlyT antagonists reduces stroke volume, pointing towards GINI as a target pathway to weaponize for therapeutic treatment. Finally, the implication of CaMKII as the secondary messenger in NMDAR cell death signaling contradicts many previous findings which attribute this role to DAPK1, but this evidences greater complexity than previously understood in the GluN2B excitotoxic pathway. Future research will hopefully elucidate further details of this pathway and the interactions between these two opposing enzymes, and allow for more targeted treatments for NMDAR-induced ischemic stroke.

VII. Bibliography

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I am a junior majoring in Biochemistry and Philosophy with a minor in chemistry. My research interests include protein-folding, biosynthesis, drug delivery vectors and metabolism. After graduating, I hope to pursue a PhD. in Pharmacology and contribute to improving existing therapeutics treatments and developing novel pathways for drug delivery, particularly in the field of neuropharmacology.