

● PERSPECTIVE

Molecular mechanisms of NMDA receptor-mediated excitotoxicity: implications for neuroprotective therapeutics for stroke

Excitotoxicity is a process observed in many disease states by which an excessive synaptic excitation causes neuronal death, and is thought to be triggered by the extracellular accumulation of the excitatory neurotransmitter glutamate, which binds and activates ionotropic N-methyl-D-aspartate glutamatergic receptors (NMDARs) in the brain. Normally, NMDARs mediate calcium entry into the cell to regulate physiological processes such as synaptic plasticity and memory, but excessive stimulation can cause a pathological rise in intracellular calcium, which activates cell death signaling to produce apoptosis. This phenomenon is implicated in numerous conditions such as Alzheimer's disease, traumatic brain injury, and alcohol withdrawal, and is widely studied to better understand disease processes and possible treatment strategies. Particularly in stroke, excitotoxicity has been demonstrated to be the primary mechanism by which neuronal damage occurs and is a popular target for many recent attempts at developing stroke therapeutics.

Stroke is an acute brain insult leading to neuronal damage that has virtually no effective neuroprotective treatments in clinical use. Immediately following stroke, brain tissue loses blood perfusion and the core of the infarct deteriorates rapidly. The surrounding penumbra experiences milder ischemia and many neurons within it will undergo delayed death that can take hours or even days. For these cells, studies show that the mechanism of death is primarily NMDA receptor-dependent excitotoxicity. In ischemic regions, extracellular glutamate levels acutely rise several fold *via* increased release and compromised uptake, while preventing glutamate release, synaptic activity, or NMDAR activation is able to rescue cell death in numerous stroke models (Lai et al., 2014). Therefore, blocking excitotoxicity should prove a viable strategy for mitigating brain damage and improving patient outcome following a stroke, and this has indeed promoted extensive academic and industrial efforts in developing NMDA receptor-based stroke treatments in the last two decades. Unfortunately, these have thus far largely met with rather disappointing results; several large scale clinical trials have failed to find the expected efficacy of NMDAR antagonists in reducing brain injuries (reviewed in Lai et al., 2014). The reasons underlying the apparent contradiction between basic research results and clinical trials remain uncertain, but several reasons have been proposed. These include, but are not limited to, the inability to use the antagonists at the doses required for neuroprotection due to side effects, the inability to administer the drugs within their neuroprotective windows, poor experimental designs, and heterogeneity in the patient population (Lai et al., 2014). However, as briefly summarized below, advancement in our understanding of the mechanisms of physiologic and pathologic NMDAR activation, and in particular, the distinct pathways linked to different NMDAR subtypes, has reignited hope and allowed scientists to develop novel treatments that improve therapeutic windows and increase specificity for death signaling pathways, achieving neuroprotection without indiscriminate disruption of other signaling pathways downstream of the receptor.

Developing novel and effective neuroprotectants by differentially targeting NMDAR subtypes

Different NMDAR subtypes serve opposing functions in normal physiology and excitotoxicity: The NMDAR is a heteromeric receptor consisting of four subunits. NMDARs generally contain two GluN1 (previously also known as NR1) subunits and two subunits from the GluN2 subfamily (GluN2A-2D, previously also known as NR2A-2D). In the cortex, the major subpopulations of NMDARs are GluN2A-, GluN2B-, or GluN2A and 2B-containing receptors. GluN2A-containing receptors are

preferentially localized at synapses, while GluN2B-containing receptors are predominantly found on extrasynaptic membranes. GluN2A- and GluN2B-containing receptors oppose each other as key mediators of plasticity, favoring either long-term potentiation (GluN2A) or depression (GluN2B) by virtue of their different electrophysiological and pharmacological properties and coupled downstream signaling proteins. Additionally, these receptors play an additional role in promoting cell survival (GluN2A) or death (GluN2B) following excitotoxic stimulation (Liu et al., 2007). Because GluN2A-containing receptors are primarily localized to synapses, while GluN2B-containing receptors are on both synaptic and extrasynaptic membranes, when excitotoxic conditions cause excess glutamate to spill beyond synapses, GluN2B-mediated death signaling becomes stronger relative to GluN2A-mediated survival signaling and results in increased death. Therefore, during stroke, indiscriminate targeting of NMDARs is unlikely to tip the balance to favor cell survival and could instead cause detrimental effects by inhibiting important normal physiological functions; Selfotel, a nonspecific NMDAR blocker, was neuroprotective against stroke *in vitro* and *in vivo*, but failed in clinical trials by causing intolerable side effects (Lai et al., 2014).

Strategies to reduce unwanted side effects: glycine site antagonists and NMDAR subtype-specific developments: In light of the importance of sparing the physiological functions of NMDARs, one alternative approach was to reduce side effects by targeting the allosteric glycine binding sites on the GluN1 subunits with licostinel and gavestinel rather than by directly blocking the receptor. These drug candidates performed well in preclinical tests, but also failed clinical trials due to low efficacy despite minimal side effect profiles (Lai et al., 2014). The negative results are likely due to a missed short window of time following stroke that receptor blockers are effective in blocking the initiation of death signaling.

A better method for reducing side effects of targeting the NMDAR is to exploit the differences between its variants. For example, the GluN2B-specific inhibitor traxoprodil is neuroprotective in stroke studies and has minimal side effects, but has also failed clinical trials (Gogas, 2006). Similar to the glycine site antagonists, it likely required earlier administration to be effective. GluN2A agonists should also promote cell survival signaling that could act to counteract GluN2B death signaling and allow greater cell survival and recovery following stroke. Indeed, activation of GluN2A-containing receptors with high dose glycine was neuroprotective in an animal model of stroke (Liu et al., 2007), but more work is required to evaluate GluN2A activation as a therapeutic target in humans.

While NMDAR antagonists and modulators are effective at attenuating excitotoxicity in experimental models, their shortcoming is the difficulty in applying treatments early enough to coincide with the peak of excitotoxic glutamate release. Most stroke patients present to hospital after many hours and have no chance of receiving these treatments in time. However, the issue can be avoided if receptor blockers can be used prophylactically in at-risk populations. One study has shown that low doses of prophylactic memantine, an NMDAR noncompetitive antagonist with few side effects, can substantially reduce brain damage and functional deficits following a stroke (Trotman et al., 2015). Whether any other drugs are tolerable and effective when taken this way remains to be seen, but other creative solutions may yet address how to deliver these drugs at the time they are most potent.

One consideration in light of these failed clinical trials is that the interplay of NMDARs in cell survival may be incompletely understood. In recent years, there has been accumulating evidence that synaptic NMDARs may also cause cell death, and that GluN2A and GluN2B do not always have dichotomous functions in excitotoxicity (Zhou et al., 2015). Further studies may be needed to resolve this controversy and identify more nuanced receptor inhibitor strategies.

Developing novel and effective neuroprotectants by specifically targeting cell death signaling molecules downstream of the NMDAR

An alternative approach to NMDAR inhibitors is to target the downstream signaling events specific to cell death that occur over a much longer period of time following receptor activation. A number of cell death pathways following NMDAR activation have been discovered, and several groups have recently provided proof-of-principle evidence

that many of these pathways can be successfully targeted with peptides to protect against excitotoxicity following stroke, without any substantial off-target effects.

The earliest reported and most explored peptide approach in stroke targets nitrous oxide synthase (nNOS)-mediated cell death. nNOS binds to postsynaptic protein 95 (PSD95), which in turn binds to the C-terminal tail of the GluN2B subunit specifically. NOS is a calcium-activated enzyme that catalyzes production of nitrous oxide (NO), and its position in the receptor complex places it in proximity to the concentrated stream of calcium entering from activated GluN2B. In stroke, the excessive calcium influx activates GluN2B-coupled nNOS to produce large amounts of NO that can induce cell death. To prevent NO production, an interference peptide is used to dissociate the complex. The peptide, Tat-NR2B9c, consists of an HIV-1 Tat-derived cell penetration sequence, which allows passage through the blood brain barrier and cell membranes, linked to a copy of the binding site on the GluN2B for PSD95. Once in the cell, the peptide dissociates PSD95 from GluN2B, thereby decoupling nNOS from the locally high levels of calcium without significantly disrupting the normal function of the enzyme in other pathways. Application results in significant protection against tissue and functional damage with no side effects *in vitro* and *in vivo* after a single dose given before or up to several hours after ischemia *in vivo* (Aarts et al., 2002). The peptide has recently succeeded in a Phase II clinical trial wherein it reduced iatrogenic infarcts during intracranial aneurism repair (Hill et al., 2012). This is the first time an NMDAR-based strategy has shown efficacy in humans, and lends a great deal of credibility that targeting NMDAR downstream cell death signaling with an interference peptide strategy can be effective against excitotoxic/ischemic neuronal injuries.

While use of peptides in a clinical setting is effective and achievable, a similar efficacy has been achieved with small molecule drugs which act on the same target and function like the peptides in a laboratory setting. For mimicking Tat-NR2B9c, two small molecules, IC87201 (Florio et al., 2009) and ZL006 (Zhou et al., 2010) have been independently found that compete at the same GluN2B-specific binding site without affecting the binding of PSD95 to other proteins. Furthermore, ZL006 mimics the peptide's neuroprotection without introducing any significant adverse effects (Zhou et al., 2010). By identifying the effective targets and the specific binding sites, research using peptides can help prototype small molecule drugs and accelerate their discovery and fine-tuning in their application towards excitotoxicity and stroke.

Other GluN2B-specific pathways have been targeted in a similar fashion and are showing promise in their various stages of development. One such pathway that is activated following GluN2B activation is the potentiation and recruitment of GluN2B at the cell membrane by death-associated protein kinase 1 (DAPK1). DAPK1 is a protein that binds to calmodulin to initiate apoptosis, but is normally phosphorylated in an inactive form incapable of binding calmodulin and causing cell death. Following excitotoxicity, calcineurin activation dephosphorylates and activates DAPK1, contributing to cell death. Additionally, active DAPK1 is able to bind to and phosphorylate the C-terminal tail of GluN2B receptors, but not GluN2A receptors, to potentiate their function, exacerbating calcium influx and excitotoxicity. A Tat-linked interference peptide containing the GluN2B C-tail phosphorylation site was able to block the interaction of active DAPK1 with GluN2B and mitigate excitotoxicity. Once administered in mice, the peptide, dubbed Tat-NR2B-CT, was able to improve outcome following ischemia (Tu et al., 2010). However, Tat-NR2B-CT was only capable of preventing runaway GluN2B insertion and activity, and not DAPK1's downstream apoptotic signaling. By adding a lysosome-targeting sequence at the end of the interference peptide to create a degradation peptide, we were additionally able to bind and direct active DAPK1 towards lysosomes for degradation and clearance. The effect was an acute and temporary drop in active DAPK1 levels with a corresponding decrease in infarction when administering the peptide hours after ischemia (Fan et al., 2014).

The c-Jun N-terminal kinase 3 (JNK) acts upon many pathways and is a significant mediator for cell death in excitotoxicity. JNK interacting protein (JIP) binds and inhibits JNK activity through a JNK binding domain (JBD) that spans 20 residues. When these residues are attached to Tat as in the Tat-JBD20 interference peptide, they are capable of

inhibiting JNK activity and preventing cell death in stroke models when administered before or a few hours after ischemia (Borsello et al., 2003). Interestingly, the Tat-JBD20 peptide has also been constructed using D-amino acids instead of L-amino acids to resist degradation by endogenous proteases. Doing so greatly extends the peptide's half-life and does not negatively affect its binding affinity and selectivity (Borsello et al., 2003), suggesting that this modification may be applied to any interference peptide to increase efficacy and bioavailability.

New targets are continually being discovered and explored. While presently no new stroke therapeutics have been implemented for widespread use, a great deal of progress has been made towards developing new therapeutics by targeting the excitotoxic processes that occur during stroke. With the advent of the success of numerous interference and degradation peptides targeting GluN2B-specific death signaling events, there is hope that new therapies are on the horizon for stroke and potentially many other neurological diseases that have excitotoxicity at the core of their pathogenesis.

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