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Novel treatment of excitotoxicity: targeted disruption of intracellular signalling from glutamate receptors

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Abstract

Glutamate signalling plays key physiological roles in excitatory neurotransmission and CNS plasticity, but also mediates excitotoxicity, the process responsible for triggering neurodegeneration through glutamate receptor overactivation. Excitotoxicity is thought to be a key neurotoxic mechanism in neurological disorders, including brain ischemia, CNS trauma and epilepsy. However, treating excitotoxicity using glutamate receptor antagonists has not proven clinically viable, necessitating more sophisticated approaches. Increasing knowledge of the composition of the postsynaptic density at glutamatergic synapses has allowed us to extend our understanding of the molecular mechanisms of excitotoxicity and to dissect out the distinct signalling pathways responsible for excitotoxic damage. Key molecules in these pathways are physically linked to the cytoplasmic face of glutamate receptors by scaffolding proteins that exhibit binding specificity for some receptors over others. This imparts specificity to physiological and pathological glutamatergic signalling. Recently, we have capitalised on this knowledge and, using targeted peptides to selectively disrupt intracellular interactions linked to glutamate receptors, have blocked excitotoxic signalling in neurones. This therapeutic approach circumvents the negative consequences of blocking glutamate receptors, and may be a practical strategy for treating neurological disorders that involve excitotoxicity.

Keywords: Excitotoxicity; Glutamate receptors; Intracellular signalling; Neurological disorders

1. Introduction

Glutamate, the major excitatory neurotransmitter in the mammalian brain, is a key mediator of intercellular communication, plasticity, growth and differentiation. Through a family of membrane receptors glutamate transduces signals that govern postsynaptic depolarisation and its consequent functions. Although vital for brain function, glutamatergic signalling is also implicated in a variety of neurological disorders. Brain damage in stroke, CNS trauma and epilepsy occurs as a result of excessive stimulation of postsynaptic receptors by L-glutamate, resulting in dysfunction of downstream signalling systems (excitotoxicity). In theory, controlling excitotoxic glutamate signalling in these disease states should be as simple as blocking glutamate receptors. However, exhaustive studies using glutamate receptor antagonists for the treatment of CNS disorders have not resulted in finding clinically useful compounds, as these caused disruption of normal brain function and adverse side effects [1] (see www.stroketrials.org for clinical trials with glutamate antagonists). An alternative approach to blocking glutamate receptors focuses on the knowledge gained from studies of postsynaptic signalling pathways. This permits the dissection of those pathways that are neurotoxic from those signalling events that are vital for neuronal survival. Recent evidence suggests that clustering of glutamate receptors at the postsynaptic density (PSD) allows coupling of glutamate receptor activity to second messengers capable of mediating neurotoxicity. Understanding this arrangement has allowed us to separate neurotoxic signalling from signals essential to neuronal survival and to propose novel strategies for the treatment of CNS disorders. This review will summarise the organisation of the glutamatergic

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Abbreviations: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid; HIV-1 TAT, Tat-NR2B9C; NMDA, *N*-methyl-D-aspartate; nNOS, nitric oxide synthase; PSD, postsynaptic density; PDZ, PSD-95; Discs large, Zonula occludens-1.

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synapse, the events downstream of glutamate receptor activation, and attempt to outline potential therapeutic targets for controlling excitotoxic damage in neurological diseases, such as stroke.

2. Excitotoxicity

Since the late 1950s researchers have observed that glutamate can act as a neurotoxin. Lucas and Newhouse [2] first found that injection of L-glutamate into immature mice destroys the inner neural layers of the retina. Later work by Olney and colleagues confirmed this glutamate retinotoxicity and additionally showed that kainate (a glutamate receptor agonist) injections produce brain lesions. At the cellular level, Olney found that glutamate toxicity produced marked dendrosomal swelling. Thus, he coined the term 'excitotoxicity' whereby excitatory amino acids produce neurodegeneration [3,4]. Excitotoxicity is now considered to be a predominant mechanism of cell death in diseases, such as stroke, CNS trauma, epilepsy and chronic neurodegenerative disorders. Excitotoxicity results from an excessive release and inadequate uptake of synaptic glutamate. Its role in hypoxic neurodegeneration was established in the 1980s by studies that showed reduced neuronal sensitivity to hypoxia when postsynaptic glutamate receptors were blocked [5,6].

2.1. Glutamate receptors and excitotoxicity

Postsynaptic responses to glutamate are mediated *via* pharmacologically and functionally distinct metabotropic (mGluR) or ionotropic (iGluR) glutamate receptor families. mGluRs are G-protein-coupled membrane receptors that mediate their actions through GTP-binding protein-dependent mechanisms linked to phospholipase C (PLC) and phosphoinositide turnover that mobilise internal Ca^{2+} stores [7,8]. mGluR subtypes have been shown to downregulate K^+ channels and upregulate non-selective cation channels, inhibit GABA receptor activity and potentiate iGluR function, resulting in enhanced neuronal excitability [8–12]. Thus, these receptors play an important role in mediating neuronal plasticity, nociception, pain, and in some instances, neurodegeneration. iGluRs consist of ligand-gated ion channels exhibiting permeability to sodium, potassium or calcium ions. The ionotropic family of receptors can be divided into pharmacologically distinct subfamilies based on their affinity for *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid (AMPA) or kainate (for review of iGluRs see Refs. [13–15]). iGluRs play an important role in mediating the synaptic plasticity that is implicated in our ability to learn and form memories [16,17]. In addition, much of the toxicity associated with glutamate overactivity has been attributed to stimulation of the ionotropic receptors [18].

AMPA receptors (AMPARs) are heteromeric structures assembled from any of four subunits (GluR1–GluR4, or GluR-A–GluR-D), each having four transmembrane domains with an extracellular amino-terminus and an intracellular C-terminal tail [19]. AMPARs are permeable to Na^+ and K^+. They are also permeable to Ca^{2+} ions unless the receptor contains at least one GluR2 subunit [20]. The majority of neuronal GluR2 are impermeable to Ca^{2+} due to RNA editing within the second transmembrane domain so that a glutamine in the pore-lining region is replaced with a positively charged arginine [21,22]. Though the majority of AMPARs in the CNS contain GluR2, the loss of this subunit has been implicated in certain disease states, such as delayed death of CA1 hippocampal neurones in ischemic brain damage [23,24]. AMPARs are also considered to be key mediators of axonal and white matter damage during trauma and ischemia [25–27].

NMDA receptor (NMDAR) channels are made up of various subunits that impart the receptor with distinct kinetics and synaptic distribution, as well as pharmacological and signalling properties. NMDAR subunits share amino acid sequence and structural homology with AMPA/kainate receptors and similarly form penta- or tetrameric structures [28]. Three subfamilies of NMDAR subunits have been identified: the ubiquitously expressed NR1 subunit, four distinct NR2 subunits (A–D), and two NR3 members (A and B) [13]. Highly active, functional NMDARs are produced only when they contain both NR1 and NR2 subunits and in some cases NR3 subunits [29–31]. NMDARs are highly permeable to Ca^{2+} and Na^+ [32]. Permeability to Na^+ contributes to further membrane depolarisation, whereas the influx of Ca^{2+} generates the intracellular calcium transients responsible for the physiologic effects of NMDAR signalling. These calcium transients are also considered to trigger excitotoxic death, as blockade of NMDAR Ca^{2+} signalling is effective in preventing neuronal damage during anoxia or glutamate challenge [33,34].

2.2. Calcium and neurotoxicity

Calcium is a ubiquitous intracellular messenger, governing a wide range of cellular functions, including cell growth, membrane excitability, synaptic activity and cell death. Its intracellular concentrations are tightly regulated in order to efficiently control signalling. Localised increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are used to trigger physiological events, such as the activation of enzymes or ion channels. It is believed that excessive Ca^{2+} loading can exceed the capacity of Ca^{2+}-regulatory mechanisms and inappropriately activate processes (i.e. activation of proteases and endonucleases) that lead to cell death (for review see Refs. [35,36]). The majority of glutamate receptor subtypes have been implicated in mediating neurotoxicity and there is general agreement that the mechanism is largely calcium dependent [37,38]. It is also generally accepted that the NMDAR family plays a key role in

glutamate toxicity owing to their high Ca^{2+} permeability [18,34,39]. NMDAR activation causes influx of sodium ions that mediate osmotic swelling of the cell body and dendritic spines, and of calcium ions that are responsible for triggering neuronal degeneration. Choi *et al*. [37,40] demonstrated that removal of extracellular Ca^{2+} but not Na^{+} could reduce cell death in response to glutamate challenge. This and later works have established the critical role for Ca^{2+} influx in neurodegeneration [18,41–43]. However, there are differing opinions about the mechanism by which Ca^{2+} influx triggers neuronal cell death during excitotoxicity.

The 'calcium load hypothesis' suggests that neurodegeneration is simply a function of the quantity of Ca^{2+} entering the cell. This theory was based on the experiments showing that cultured neurones experience delayed Ca^{2+} accumulation and studies demonstrating that Ca^{2+} uptake and cell death were correlated with glutamate exposure [44–46]. However, several studies have shown that calcium channel blockers can prevent Ca^{2+} accumulation but not neurotoxicity during anoxia [47–50]. Thus, a general elevation in calcium does not necessarily predict neuronal death and additional factors may influence the outcome of Ca^{2+} influx. An alternative hypothesis is based on the knowledge that various calcium-dependent processes are regulated *via* distinct signal pathways linked to specific routes of Ca^{2+} influx [51,52]. The 'source specificity hypothesis' reasons that Ca^{2+} toxicity occurs not simply as a function of increased Ca^{2+} concentration, but is instead linked to the route of Ca^{2+} entry and the distinct second messenger pathways that are activated as a result. Source specificity was originally based on the experiments showing that Ca^{2+} loads produced by voltage-sensitive Ca^{2+} channels were not harmful whereas similar $[Ca^{2+}]_i$ increases *via* NMDARs were toxic [34]. Thus, distinct influx pathways, rather than calcium load, determine neuronal vulnerability to glutamate and calcium [53]. These findings directed research in our laboratory to further study and identification of the Ca^{2+}-activated processes that are associated with glutamate toxicity. The source specificity hypothesis proposes that molecular targets, such as rate-limiting enzymes, are physically linked or co-localised with glutamate receptors and can be manipulated to block Ca^{2+}-dependent neurotoxicity.

3. Postsynaptic organisation

Signal molecules with the potential to act as neurotoxic triggers most likely exist within the PSD. The PSD is a specialised structure located beneath the postsynaptic membrane aligned with active zones of presynaptic terminals within the CNS. It is a relatively insoluble, electron dense region comprised of multiple membrane-bound, scaffolding and cytoskeletal proteins [54,55]. Several functions, including cell-to-cell adhesion, regulation of receptor clustering and modulation of receptor function, have been attributed to the PSD. Excitatory CNS synapses are enriched in PSDs that contain dense populations of excitatory glutamate receptors [56]. The PSD at glutamatergic synapses is not a static entity and its morphology can be influenced by synaptic activity. It has been shown that events triggered by glutamate receptor activation affect not only signal transmission but also structural remodelling of the PSD [57]. There are four major types of molecules that constitute the PSD: membrane-bound, cytoskeletal and scaffolding proteins and modulatory enzymes. Knowledge about these is increasing as new PSD components continue to be identified.

3.1. Membrane receptors and proteins

Cell junction proteins, including N-cadherin, densin-180, neuroligins and β-neurexin, play a key role in the co-localisation of the PSD with the appropriate presynaptic region [58–60]. mGluRs are considered to have a peripheral association with the PSD that may allow the receptors to modulate NMDAR and AMPAR signalling in response to glutamate [61–63]. Perhaps the most abundant and most important membrane proteins in the PSD are the iGluRs. NMDA and AMPA/kainate receptors are clustered within the PSD where they have access to the various components that modulate their function and transduce their signals [64,65].

AMPA and NMDA receptors possess intracellular C-terminal tails that contain consensus motifs that are critical for both the regulation of receptor activity and interaction with cytoskeletal and postsynaptic signal transduction cascades. Requirement for the intracellular motifs is particularly striking in the NMDAR NR2 subunits where targeted deletion of the NR2 cytoplasmic tails results in mice that are phenotypically indistinguishable from the corresponding NR2 subunit knockouts [66,67]. Phosphorylation of iGluRs by enzymes, such as protein kinase A (PKA), PKC, Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and Src-kinase, can influence receptor clustering and potentiation of receptor activity [68–72]. Interaction of NR1 and NR2B with α-actinin-2 and the actin cytoskeleton is important for the clustering of NMDARs at the synapse [73]. The GluR2 subunit associates with the ATPase, NSF (*N*-ethylmaleimide-sensitive fusion protein) [74]. Several studies indicate that NSF is critical to the rapid turnover and regulation of AMPAR function [75,76]. Most notably, the NMDAR NR2 subunits and AMPAR GluR2/3 end in short C-terminal motifs that enable the receptors to bind PDZ (PSD-95, Discs large, Zonula occludens-1) domain containing scaffolding proteins. NR2 subunits possess tSXV motifs that allow them to bind members of the PSD-95/synapse-associated protein 90 (SAP90) family of scaffolding proteins [77]. PSD-95 in turn binds multiple intracellular signalling and scaffold molecules and links NMDARs to signalling partners, regulatory enzymes and adaptor

molecules. AMPAR GluR2/3 contain a similar PDZ-binding motif (-SVKI) important for interaction with the PDZ domains of PICK1 (protein interacting with C kinase), GRIP1 (glutamate receptor interacting protein) and ABP (AMPAR-binding protein). These scaffolding proteins have been implicated in the clustering and trafficking of AMPARs at the synapse [78–80]. Recently, several studies have demonstrated links between the interactions of iGluR subunits with PDZ proteins and excitotoxic cell death. For example, our research has shown that NMDAR-mediated neurotoxicity in cortical cultures is regulated by the interaction of PSD-95 with NMDARs [81,82].

3.2. Enzymes and modulators

Several enzymatic systems exist within the PSD. Many of these regulate phosphorylation of PSD components, including iGluRs: members of the Src family of non-receptor protein tyrosine kinases, CaMKII, PKC and the phosphatase calcineurin are all implicated in regulating iGluR function [59,83,84]. Other enzymes found in the PSD are key effectors in the glutamate receptor signal pathway. Neuronal nitric oxide synthase (nNOS) has been shown to be activated by NMDAR Ca^{2+} influx and can modulate NMDAR signalling as well as contribute to neurotoxicity [85,86]. SPAR, SynGAP (synaptic GTPase activating protein) and the more recently identified citron are effectors of the Rap and Ras signalling pathways within the PSD [87–89].

3.3. Cytoskeletal and scaffolding proteins

The PSD is enriched in a number of cytoskeletal elements, including actin, fodrin, tubulin and neurofilaments, and scaffolding proteins, including spectrin, α-actinin-2, AKAP 79 (A kinase anchoring protein 79) and PDZ-containing proteins and their associated binding partners [90]. Cytoskeletal proteins are considered important to the localisation and clustering of PSD receptors and complexes whereas scaffolding proteins are the ‘glue’ that function in bringing the various PSD components into association. For instance, α-actinin-2 serves as the intermediary that links actin to NMDAR subunits, thereby influencing the clustering of the receptors at the synapse [73]. The PSD is particularly enriched in specialised scaffolding proteins that contain PDZ domains. The PDZ proteins of excitatory synapses fall into two main families related to either PSD-95 or GRIP1 [91,92].

Four members of the PSD-95 family are found in mammalian synapses: PSD-95/SAP90, SAP97, PSD-93/chapsyn-110 and SAP102. These are membrane-associated guanylate kinases (MAGUKs), each characterised by three N-terminal PDZ domains followed by a Src homology 3 (SH3) and guanylate kinase-like (GK) domain. Each domain is capable of mediating protein–protein interactions with multiple binding partners. The PDZ domains bind to small consensus motifs (tSXV) at the carboxyl-terminus of associated proteins, such as NMDAR NR2 subunits. In addition, PDZ domains can self-associate, both within PSD-95 family members and with the PDZ domains of other proteins. This feature furthers their ability to cluster PSD proteins into functional complexes. For instance, PSD-95 forms PDZ–PDZ interactions with nNOS, CRIPT (cysteine-rich interactor of PDZ3—a microtubule-binding protein) and citron [89,93–95]. The SH3 domains of PSD-95 and SAP102 bind Pyk2, which in turn localises and activates Src-kinase within the PSD [96]. The GK domain of PSD-95 binds to SPAR and GKAP (guanylate kinase-associated protein). SPAR links PSD-95 to both the actin cytoskeleton and the Rap signalling pathway [88]. GKAP may functionally link PSD-95 to mGluRs *via* its interaction with Shank and Homer [62,97,98].

A second family of PDZ proteins includes GRIP and ABP, with seven and six PDZ domains, respectively [79,80]. These proteins do not contain SH3 or GK domains but are capable of forming homo- and heteromultimers through PDZ–PDZ interactions [80]. PICK1 (protein interacting with C kinase) is yet another PSD scaffolding molecule initially identified by its ability to bind *via* its single PDZ domain to the catalytic domain fragment of PKCα [99]. PICK1 binds *in vivo* to a variety of transmembrane proteins, including the AMPAR GluR2 subunit, the eph receptor tyrosine kinases, and to mGluR7 [78,100].

4. Neurotoxic signalling by glutamate receptors within the PSD

Yeast two-hybrid screening has been instrumental in identifying binding partners for both AMPAR and NMDAR channels, including the variety of PDZ-containing proteins found associated with the receptors [101]. It is now established that the wide array of proteins found in the PSD interact to form the glutamatergic signal transduction machinery. Such interactions have been proposed to govern the activity-dependent and -independent receptor targeting and trafficking that is important for long-term potentiation (LTP) and long-term depression (LTD) [17,102,103]. Indeed, AMPAR stability and movement at synapses are important factors controlling synaptic strength. Preventing the interaction of GluR2 with GRIP1/ABP proteins impairs LTD in both the hippocampus and cerebellum [104].

Synaptic localisation of Ca^{2+}-impermeable GluR2 subunits is thought to be important in modulating the neurotoxic effects of AMPAR signalling. Several studies suggest that CA1 hippocampal neurones, the cells that exhibit the greatest vulnerability to delayed death following global ischemia, also downregulate AMPAR GluR2 subunits [24,105,106]. This may induce a delayed increase in Ca^{2+} permeability in these cells that correlates with their degeneration. However, although increased Ca^{2+} permeability may play a role in the vulnerability of CA1 neurones

to degeneration, there are also studies that indicate that additional processes could be involved, as the GluR2 subunit mediates many of the interactions of AMPARs with intracellular proteins, and regulates the affinity of AMPARs for extracellular ligands [107]. To date, none of the AMPAR-associated proteins have been ascribed a role in excitotoxic signalling. However, these proteins have the potential for mediating excitotoxic insults *via* interactions with other synaptic molecules. For example, GRIP1 binding to GRASP-1 may couple AMPAR activation to the Ras signalling pathway [108]. GRASP-1 is cleaved in apoptotic neurones during ischemia disrupting its regulation of Ras signalling. In addition, GRASP-1 has been shown to down-regulate synaptic targeting of AMPARs [109]. Thus, its cleavage during ischemia may result in increased synaptic AMPAR activity and vulnerability to glutamate overactivity.

Close coupling of receptor channels to downstream signal machinery allows for efficient, local Ca^{2+}-dependent activation of signalling cascades. Unlike the AMPARs, compelling evidence exists demonstrating that Ca^{2+}-dependent neuronal death is triggered most efficiently through NMDARs [34,53,110]. These studies suggest that the synaptic organisation of NMDARs brings them into close association with intracellular pathways capable of causing neurotoxicity. NR2A and NR2B subunits bind to PSD-95 and this interaction may mediate NMDAR clustering. However, PSD-95 knockout mice demonstrate normal synaptic localisation of NMDARs suggesting interaction with PSD-95 is not essential for clustering [111]. Instead, synaptic localisation of NMDARs is governed by the interaction of the NR1 subunit α-actinin-2 and the actin cytoskeleton [112,113]. Studies in our laboratory however indicate that synaptic and extrasynaptic NMDARs are equally capable of mediating neurotoxicity [113] and that synaptic localisation alone does not govern signalling from iGluRs. It is notable that the hippocampal neurones of PSD-95 mutant mice exhibit a dramatic increase in LTP, indicating an important role for NR2/PSD-95 interactions in NMDAR signal transduction. Indeed, through its various protein-binding domains PSD-95 brings an array of signal molecules into close approximation with Ca^{2+} influx through the NMDAR channel.

The discovery of distinctive signal molecules as binding partners of PSD-95 lead our laboratory to test the hypothesis that PSD-95 acts as the scaffolding link between incoming calcium ions and intracellular signal molecules, such as nNOS [93,114–116]. By suppressing the expression of PSD-95, we selectively attenuated excitotoxicity triggered by NMDARs but not other glutamate receptor or Ca^{2+} channels [113]. Further study of NMDAR signalling mechanisms revealed that suppressing PSD-95 selectively reduced Ca^{2+}-activated production of nitric oxide (NO), a second messenger implicated in excitotoxic mechanisms, without affecting NOS expression [81]. Thus, PSD-95 was required for efficient coupling of NMDAR activation to NO signalling and toxicity.

5. Targeting intracellular signal pathways

In theory, a CNS disorder in which neuronal loss is caused by glutamate overactivity has the potential to be treated by blocking the receptors that transduce the neurotransmitter signal. To this end, numerous specific NMDAR and AMPAR channel blockers have been developed and studied in the treatment of brain damage during stroke. However, antagonism of glutamate receptor activity has not proven clinically useful in part due to the pharmacokinetic difficulties and adverse side effects. The high levels of NMDAR antagonists needed to treat excitotoxic damage produce undesirable effects in the healthy brain and can induce hallucinations, centrally mediated hypo- or hypertension, catatonia and sometimes anaesthesia. AMPAR blockers have shown more potential for therapeutic use, especially in the protection of CA1 hippocampal neurones during global ischemia, yet their clinical applications have been limited by poor solubility, poor CNS penetration and nephrotoxicity [117,118].

Excitatory synaptic activity through glutamatergic signalling, and the resulting synaptic Ca^{2+} influx, is vital to neuronal function and survival. Blocking NMDARs has been shown to cause extensive apoptosis in perinatal rats [119,120], suggesting that glutamate receptor antagonists have direct neurotoxicity. To correct this, alternative strategies for treating excitotoxic damage have been proposed to prevent the complete blockade of the receptor. These strategies include the use of antagonists selective for particular receptor subunits (ifenprodil for NMDAR NR2B), partial blockade of receptor activity (such as glycine antagonists) and the use of low affinity blockers whose binding is more easily displaced allowing for some glutamatergic signalling. However, another approach in the search for a therapeutic agent in excitotoxicity is to target the specific intracellular signal pathways that propagate excitotoxic signals downstream from the glutamate receptors. Given the current knowledge of the molecular organisation of the PSD, it is possible to derive a strategy to target specific protein–protein interactions in order to uncouple glutamate receptors from their potentially neurotoxic downstream effectors.

Based on our previous findings that linked NMDAR activation to downstream NO toxicity through the scaffolding protein PSD-95 [81], we investigated the idea that the NMDAR NR2/PSD-95 interaction might constitute a therapeutic target for diseases involving excitotoxicity. Targeting the PDZ interaction with the C-terminal tSXV motif of NR2B represents a therapeutic strategy that may circumvent the negative consequences of blocking NMDAR function. We questioned whether interfering with NR2B/PSD-95 interactions could suppress excitotoxicity in a manner similar to the knockdown of PSD-95 expression. We designed a targeted peptide comprised of the nine C-terminal residues of NR2B (NR2B9c), including the tSXV motif which is anticipated to bind the second

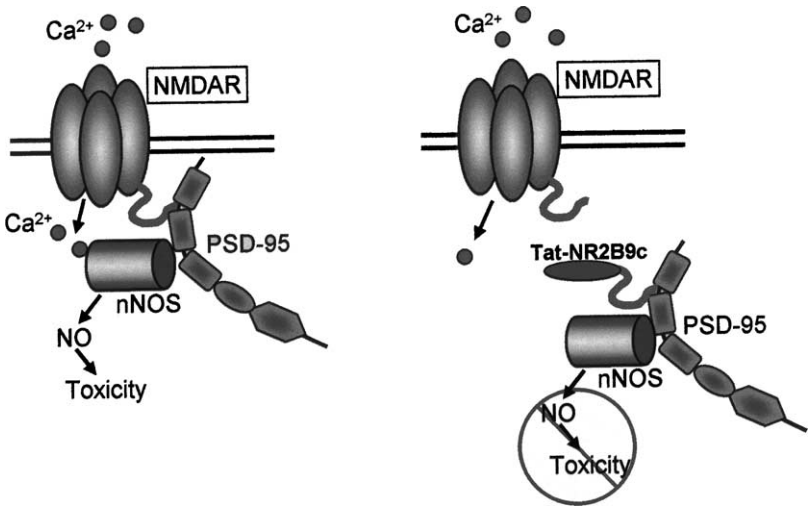

Fig. 1. Therapeutic model: the NMDAR/PSD-95 complex (left) may be dissociated using a peptide corresponding to the C-terminus of NR2B, thus reducing the efficiency of excitotoxic signalling *via* Ca^{2+}-dependent signalling molecules. The NR2B peptide is fused to the protein transduction domain of HIV-1 TAT for efficient delivery into cells (Tat-NR2B9c; right). NMDAR, NMDA receptor; PSD-95, postsynaptic density protein 95; nNOS, neuronal nitric oxide synthase; NO, nitric oxide.

PDZ domain of PSD-95. In order to allow for efficient delivery of the peptide into cells *in vitro* and across the blood–brain barrier *in vivo*, we conjugated NR2B9c to the cell membrane transduction domain of HIV-1 TAT protein (Tat-NR2B9c). The TAT-transduction domain is able to transport proteins of variable sizes across membranes in a rapid, dose-dependent manner independent of receptors or transporters [121–123]. We predicted that our Tat-NR2B9c peptide would disrupt the interaction between NMDAR NR2B and PSD-95, protecting treated neurones from NMDAR-activated NO production and excitotoxic death (Fig. 1).

We found that fluorescent-labelled Tat-NR2B9c rapidly crossed the plasma membrane of cultured mouse neurones and accumulated in the brains of mice after intraperitoneal injection. Tat-NR2B9c but not control peptides protected cultured neurones from NMDA-mediated excitotoxicity without affecting NMDAR Ca^{2+} signalling or electrophysiology [82]. Tat-NR2B9c disrupted the interaction of NR2B and PSD-95 in co-immunoprecipitation and significantly depressed NMDAR-evoked stimulation of NO-cGMP signalling. Since Tat-peptides that target the NMDAR/PSD-95 interaction protect against NMDA toxicity without blocking NMDARs, we reasoned that treatment with Tat-NR2B9c *in vivo* could serve as an improvement on NMDA blockers in the treatment of ischemic brain damage. We found that Tat-NR2B9c but not control peptide dramatically reduced cerebral infarction and improved neurological function in rats subjected to transient focal cerebral ischemia. The peptide treatment was effective 1 hr before and most importantly, 1 hr after the onset of excitotoxicity and cerebral ischemia [82]. These results indicate that the strategy of treating neurones with Tat-fusion peptides is effective in reducing vulnerability to excitotoxicity *in vitro* and stroke damage *in vivo*. As this occurs without affecting NMDAR activity, then adverse consequences of blocking NMDARs are not expected. Efficacy after the insult onset suggests that targeting the NMDAR/PSD-95 interaction is a practical future strategy for treating stroke. It is also likely that targeting other intracellular proteins using the same approach could be used to modulate additional signalling mechanisms mediated by protein–protein interactions that lead to other human diseases.

6. Future directions

The use of peptides and small proteins in *in vivo* therapies has met the limitations of poor permeability of the cell membrane and in the instance of neurological disorders, poor delivery across the blood–brain barrier. However, research over the last decade has revealed that a series of small protein domains, termed protein transduction domains (PTDs), can cross cell membranes independent of specific receptors or transporters. Peptide PTDs may simply be synthetic polycation sequences (arginine or lysine rich) or they may be derived from proteins, such as *Drosophila* Antennapeadia protein Herpes virus VP22 and HIV-1 TAT [121,124–129]. The use of PTDs can ensure efficient delivery of attached proteins into cells and across the blood–brain barrier [130]. PTD conjugation to other macromolecules, such as DNA, also represents an effective, non-viral approach to gene therapy. Such a mechanism could be used in the delivery of PSD-95 antisense DNA for the treatment of neurodegenerative conditions, such as epilepsy. However, the use of peptides to target protein–protein interactions rather than a genetic intervention allows for the rapid treatment and short-term modulation of signalling cascades, a strategy particularly suited to the narrow therapeutic window offered during stroke.

The strategy outlined above, targeted disruption of protein–protein interactions based on a molecular understanding of excitotoxic mechanisms, may amount to practical future treatments for human neurological disorders. As our understanding of protein-binding domains has grown, so has the potential for therapeutic intervention. SH2, SH3, and ligand-binding domains, enzyme active sites and protein dimerisation sites have all been investigated as targets for therapeutic intervention [131,132]. The coupling of Ca^{2+}-dependent nNOS signalling to NMDAR activation is but one possible pathway in glutamate-mediated excitotoxicity. As we expand our knowledge of the signalling machinery attached to glutamate receptors, new potential therapeutic targets may also arise. For instance, little is yet known about the role of GRIP1/ABP multimers in clustering synaptic proteins with AMPARs at the synapse. It may be that specific enzymes or signal molecules, clustered with AMPARs, are deregulated upon glutamate overactivity or cell stress and mediate a toxic second messenger cascade. In addition, nNOS may not be the only signal pathway influenced by our peptide that disrupts the NR2B/PSD-95 interaction. As discussed above, PSD-95 binds and clusters a wide variety of enzymes and modulators that may be important in NMDAR signalling and/or neurotoxicity. Further investigation into the organisation of the excitatory PSD and of the molecular mechanisms of glutamate-mediated excitotoxicity should reveal additional targets for pharmacological intervention in the treatment of stroke and other neurodegenerative conditions.

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