Review

Stroke intervention pathways: NMDA receptors and beyond

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Despite abundant evidence from basic/preclinical research that excessive NMDAR (*N*-methyl-D-aspartate receptor) stimulation is a crucial step required for brain damage following a stroke, clinical trials for NMDAR blockers have all ended with disappointments. The past decade of stroke research has revealed distinct NMDAR subpopulations and many specific effectors downstream of these receptors that are differentially responsible for neuronal survival and death. These new advancements provide promising targets for the development of novel NMDAR-based neuroprotective stroke therapies that could have greater therapeutic windows and reduced side effects. In this review, we discuss these advancements with a particular emphasis on the identification of novel signaling effectors downstream of pro-neuronal death NMDARs and the potential implications of these findings for the development of stroke therapeutics.

Stroke and *N*-methyl-D-aspartate receptors (NMDARs)

Stroke is a major cause of death and disability in developed countries, and represents a major economic burden in the world [1]. Because neuronal death in the brain following stroke is an active and prolonged process [2], understanding the underlying death-signaling mechanism can lead to therapeutics that minimize stroke damage even when administered several hours to days after a stroke. To date, unfortunately, no such neuroprotective therapeutics are in clinical use. The mechanisms mediating stroke damage are probably multifactorial [3], with NMDAR-mediated excitotoxicity being a primary factor [3–5]. However, selective compounds that block these receptors are not clinically feasible because of their side effects and short therapeutic windows (Box 1) [1,6]. Extensive research in the past decade has significantly advanced our understanding of the mechanisms mediating NMDAR functions, leading to the development of promising novel NMDAR-based therapeutics. This review highlights some of these key discoveries, with a particular emphasis on newly identified death-signaling proteins downstream of the NMDAR upon which treatments are being developed and tested in animal models of stroke.

Dual roles of NMDARs in neuronal survival and death: a stroke perspective

The NMDAR is a type of cation-permeable ionotropic receptor that mediates fast excitatory synaptic transmission by glutamate, the primary excitatory neurotransmitter in the mammalian central nervous system [7,8]. Physiological NMDAR activity is required for many important neurological functions including synaptic plasticity, memory formation, mood control, motivation for rewards, brain development and neuronal survival [3,7,9,10]. However, NMDAR overactivation under pathological conditions can lead to neuronal death in a process known as 'excitotoxicity' [3–5]. Not only has excessive NMDAR activation been considered a common pathological event leading to neuronal death in many neurological disorders [11], its role in ischemic neuronal death following stroke is also particularly strong. Indeed, NMDAR blockers protect neurons from ischemic neuronal injuries *in vitro* [4,11,12] and *in vivo* [4,5,11–13]. How the NMDAR mediates so many different functions, and in some cases apparently opposing functions such as neuronal survival and death, is a matter of great research interest (Box 1) and, as briefly discussed below, several hypotheses have been put forward in recent years.

Synaptic versus extrasynaptic NMDARs: the location hypothesis

Given that some signaling or scaffolding proteins such as PSD95 (postsynaptic density protein95) are distinctly located in the synapses, NMDARs at the synapse might be coupled to synapse-specific signaling proteins, thereby exerting functions distinct from extrasynaptic NMDARs. In support of this notion, Lu *et al.* provided the first proof-of-concept evidence that distinct NMDAR subpopulations in the synapses and extrasynaptic sites can mediate opposing functional outcomes [14]. They found that the preferential activation of synaptic NMDARs mediates postsynaptic AMPAR (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid type ionotropic glutamate receptor) insertion, resulting in long-term potentiation, a well-characterized form of synaptic plasticity; by contrast, the activation of extrasynaptic NMDARs mediates AMPAR endocytosis, leading to the opposing synaptic phenomenon, long-term depression (LTD) [14] (reviewed in [9]). Several subsequent studies have provided support for this 'NMDAR location' hypothesis in the field of neuronal survival and death [15–19]. Specifically, synaptic NMDAR

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 1471-4914/$ – see front matter doi:10.1016/j.molmed.2010.12.008 Trends in Molecular Medicine, May 2011, Vol. 17, No. 5

Box 1. NMDAR-based neuroprotective therapies: the past, present and future

Many explanations for the failure of NMDAR antagonists in clinical studies have been proposed by clinicians and scientists. These include poor experimental design, insufficient sample size because of heterogeneous patient populations, a lack of unified standards for outcome measures, side effects that limit the administration of effective doses and delay in patient admission and diagnosis, which prohibits early treatment (see reviews in [1,3,6,84]). Two of these factors, severe side effects because of blocking normal neuronal function of the NMDARs [1,6] and relatively short therapeutic time windows of these receptor blockers [1,6], are emphasized here because they can be minimized significantly by using novel therapeutics developed based on our new understanding of NMDAR signaling pathways. NMDAR functions largely depend on the activation of signaling complexes that are brought adjacent to the channel pore either through direct protein–protein interactions between one or more components of the signaling complex and the NMDAR [24–26,34,46] or by other subcellular compartmentalization mechanisms [14–20]. Normal NMDAR activity primarily activates the NSC and other functional signaling complexes involved in mediating neuronal functions including neuronal circuit maturation, learning and memory and other behavioral functions (Figure I). However, excessive NMDAR stimulation can also activate the NDC, especially when NDC molecules are recruited to the NMDAR under pathological conditions such as stroke [28,34]. The stimulation of the NDC under pathological conditions results in neuronal death (Figure Ia). Current treatments based on NMDAR antagonism block all signaling pathways downstream of NMDARs. The blockade of NMDAR neurological function can result in intolerable side effects such as psychomimetic effects and memory loss [6,28]. Moreover, the inhibition of the NSC under certain conditions can explain why the use of NMDAR blockers has exacerbated stroke outcome in some clinical trials [85,86] (Figure Ib). These undesirable actions have limited the clinical use of NMDAR blockers at the concentrations required to reduce ischemic damage. Newly developed therapeutics, however, selectively enhance NSC signaling [12,19,33] and/or block NDC signaling [24–26,34,46] without affecting other NMDAR signaling pathways, thereby resulting in therapeutic efficacy without the many side effects (Figure Ic). Moreover, because conventional NMDAR antagonists target the earliest step of NMDAR death signaling, they become ineffective shortly following stroke when the NDC death-signaling cascade has already been activated. In marked contrast to this, many of the new therapeutics targeting the NDC or signaling steps further downstream continue to be efficacious even when administered to experimental animals many hours after stroke [12,25,28,34,51].

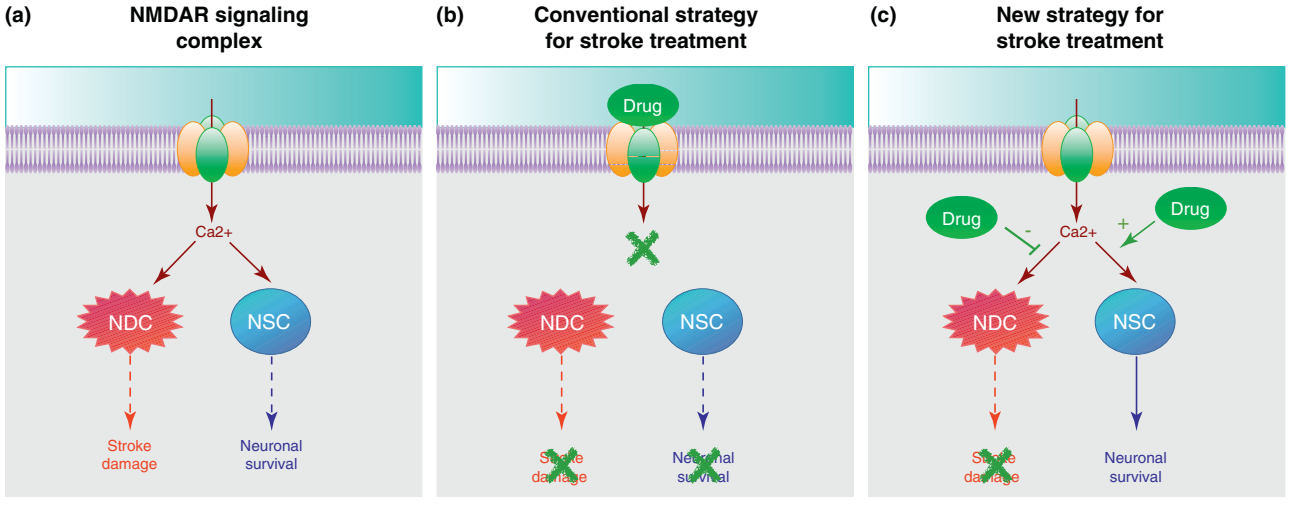

Figure I. NMDAR-based therapeutics. **(a)** The NMDAR can either maintain neuronal survival by stimulating the signaling components of the neuronal survival-signaling complex (NSC) (blue) associated with synaptic NR2ARs or mediate neuronal death by activating signaling components in the neuronal death-signaling complex (NDC) (red) bound to extrasynaptic NR2BRs. **(b)** Conventional NMDAR blockers inhibit both the NDC and NSC pathways by targeting the surface receptor. **(c)** New stroke therapeutics being developed will reduce neuronal damage by selectively stimulating the NSC or inhibiting the NDC, without affecting NMDAR activity.

stimulation has prosurvival effects owing to its activation of the downstream cyclic-AMP response element-binding protein (CREB) signaling pathway; by contrast, the activation of extrasynaptic NMDARs mediates prodeath effects by attenuating the CREB pathway [19] (see also [15–18] and the review in [20]). Whether the 'NMDAR location' hypothesis is instructive for explaining ischemic brain damage in *in vivo* animal models of stroke remains to be investigated. In this regard, it is interesting to note that memantine, a low-affinity uncompetitive NMDAR antagonist, can selectively block extrasynaptic NMDARs at specified low doses, thereby protecting against neuronal death in a mouse model of Huntington's disease [21]. Although memantine is also neuroprotective in stroke models, the concentrations used were much higher than the specified doses for a preferential blockade of extrasynaptic NMDARs [22].

The most important question concerning the 'NMDAR location' hypothesis is how survival- and death-signaling proteins are compartmentalized to different subcellular locations. Notably and contrary to this hypothesis, the best characterized synapse-specific protein PSD95 (with only scarce presence at extrasynaptic sites) [23] is required for NMDAR-mediated excitotoxic neuronal death [24–28] (Box 2). Moreover, the neurotoxic role of PSD95 is consistent with earlier experimental evidence suggesting that synaptic NMDAR activity can be important for ischemic neuronal death during stroke [4,29]. Although these controversies do not exclude the notion that synaptic NMDARs are primarily prosurvival, they do suggest that, at the very least, some synaptically localized NMDARs can exert prodeath effects, thereby having roles pathologically relevant to stroke damage.

Box 2. The deadly association of the NR2B–PSD95–nNOS signaling complex

The first set of death-signaling proteins to be identified was probably the NR2B–PSD95–nNOS signaling complex (Figure I) [24–28]. PSD95 is a synaptic scaffolding protein that binds NMDARs and brings several signaling molecules to the vicinity of the NMDAR channel pore [23,26,52,87]. It has three PDZ domains for binding to proteins containing specific PDZ ligands such as the carboxyl terminus of the NMDAR NR2B subunit (NR2B-CT) and the N terminus beta-finger of nNOS (Figure Ia) [26,27]. The simultaneous binding of PSD95 to NR2B-CT and nNOS is required for the NMDAR-mediated production of the neurotoxic molecule NO [24–28], and the inhibition of nNOS prevents NMDAR-dependent neuronal death following stroke [27]. Moreover, this NMDAR NDC formation is enhanced by the stimulation of the NR2BR during stroke, thereby forming a vicious cycle wherein NR2BR-mediated NO production can be progressively amplified [28]. Because both NMDAR and nNOS perform many important physiological functions in the brain, therapeutics that specifically disrupt this complex formation are expected to have fewer neurological side effects than do conventional drugs that directly block NMDAR or inhibit nNOS. The first compound of this kind is the 20 amino acid-long Tat-NR2B9c peptide (Figure Ib) [25], whose primary sequence includes the membrane transducing domain of the HIV1 Tat protein that renders the peptide membrane permeable, and the nine amino acid residues encoding the PSD95 binding region of the NR2B-CT. This peptide disrupts the NR2B–PSD95 interaction by competing with the native NR2B-CT for binding to PSD95; therefore, it has no effect on NMDAR channel activity or the NR2A–PSD95 interaction [25]. To increase bioavailability and facilitate drug administration, two recent studies developed nonpeptidic small molecules that mimic the interference peptides in disrupting this complex (Figure Ic) [28,44]. The first study [44] reported a small molecule IC87201, modified from a lead compound identified through the high-throughput screening of a chemical library of 150 000 small molecules. The second study [28] took a rationalized drug design approach, and designed and synthesized a *de novo* small molecule ZL006 based on the molecular properties of the interaction between nNOS and PSD95. As expected, these new therapeutics (Tat-NR2B9c and ZL006) can effectively reduce stroke damage and lack the neurological side effects commonly associated with NMDAR blockers or nNOS inhibitors [27,28].

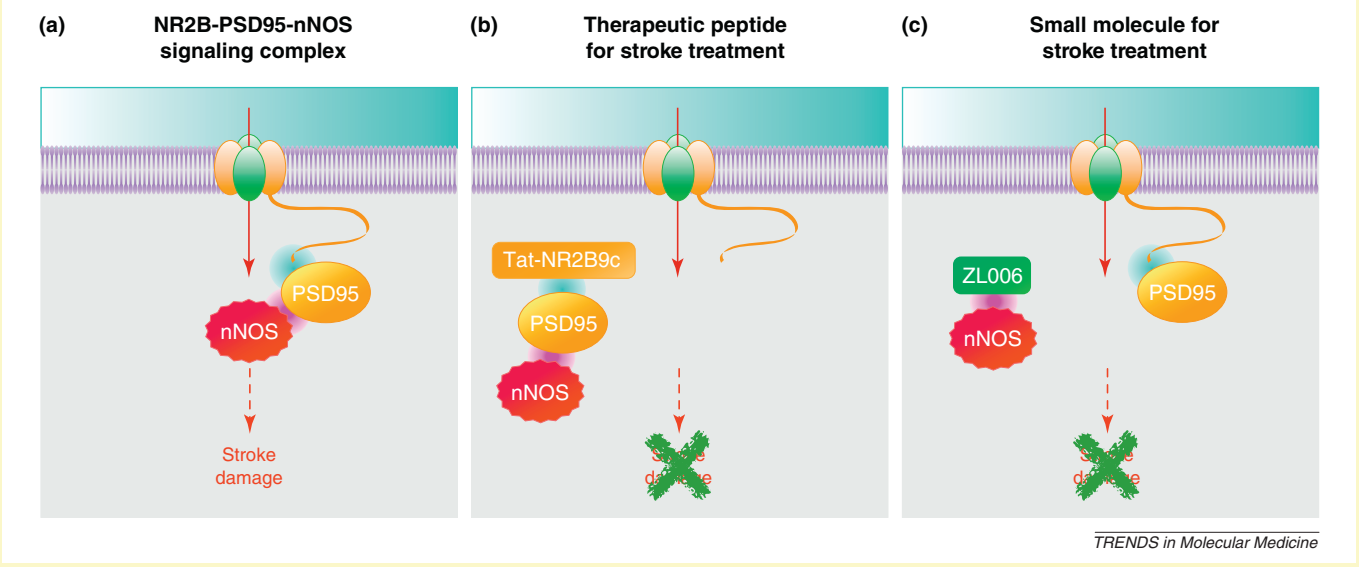

Figure I. The dissociation of the NR2B–PSD95–nNOS signaling complex prevents the NMDAR-mediated production of the neurotoxic molecule NO, reducing stroke damage. **(a)** The NR2B subunit (orange) of NMDAR forms a multimeric protein complex with PSD95 and nNOS. **(b)** Tat-NR2B9c dissociates PSD95–nNOS from NR2BRs by disrupting the NR2B–PSD95 interaction. **(c)** ZL006 dissociates nNOS from NR2B–PSD95 by disrupting the PSD95–nNOS interaction.

NR2A-containing versus NR2B-containing NMDARs: the subtype hypothesis

NMDAR subunits are classified into three subfamilies: NR1 (also called GluN1), NR2 (A–D; also called GluN2A–D) and NR3 (A and B; also called GluN3A,B). Native NMDARs are tetrameric complexes comprising two obligatory NR1 subunits with at least one type of NR2 subunit. Different NR2 subunits not only confer distinct electrophysiological and pharmacological properties on the receptors, but also couple the receptor to different signaling machineries because of the structural diversity of their carboxyl terminus (cytoplasmic tail) [7,8]. In addition, subunit compositions can influence the temporal and spatial distributions of the receptor [7,30]. The newborn forebrain is almost entirely populated by the NR2B-containing NMDARs (NR2BRs), but with developmental maturation the synapses become increasingly populated by NR2A-containing NMDARs (NR2ARs), whereas extrasynaptic sites remain populated by NR2BRs [7,30]. Together, subtype-specific location and subtype-specific downstream signaling can confer subtype-specific NMDAR functional outputs.

The recent availability of subtype preferential antagonists, mice lacking NR2A or NR2B NMDAR subunits, and proteomics techniques has allowed scientists to study the distinct functions mediated by the two major NMDAR subtypes in the adult forebrain where stroke most frequently occurs [12,13,31–34]. In particular, several groups have reported that NR2ARs and NR2BRs are required for glutamate-mediated neuronal survival and death in both *in vitro* and *in vivo* models of stroke and traumatic brain injuries, respectively [12,13,31–33]. Detailed pharmacological analyses further reveal that the selective stimulation of synaptically localized NR2BRs, however scarce, triggers excitotoxic neuronal death; moreover, the activation of extrasynaptic NR2ARs, despite their subcellular locations, promotes neuronal survival [12]. In addition, several survival-signaling pathways mediated by CREB [13,33], PI3K (phosphoinositide 3-kinase) [35] and Kidins220 (kinase-D-interacting substrate of 220 kDa) [36], have been

linked to NR2AR activation, whereas most death-signaling pathways are selectively activated by NR2BRs [27,37]. Because different NR2 subunits can bind distinct signaling proteins via direct protein–protein interactions [8], the 'NMDAR subunit' hypothesis might provide a better explanation for how survival- and death-signaling proteins are compartmentalized to distinct NMDAR subpopulations. Indeed, the many NMDAR death-signaling proteins identified thus far form complexes with NR2BRs through either direct or indirect protein–protein interactions [27,37].

Several remaining questions warrant future research and further modifications to the 'NMDAR subtype' hypothesis. First, most of the evidence supporting the 'NMDAR subtype' hypothesis came from experiments using subtype-specific antagonists. However, the selectivity of some of these antagonists has been questioned [7,38,39]. Second, in addition to the classic heterodimeric NR2ARs and NR2BRs, cortical neurons might also express heterotrimeric NMDARs containing both NR2A and NR2B subunits [7]. How these receptors fit into the 'NMDAR subunit' hypothesis remains to be determined. Third, under certain experimental conditions, NR2ARs can contribute to neuronal death and NR2BRs can promote neuronal survival [40,41]. Thus, the roles of specific NMDAR subtypes will undoubtedly vary depending on the nature of the signaling complexes that are bound to the receptor at that particular stage of development, the area of the brain and the model of disease.

A unified hypothesis

Given that in the adult forebrain, where stroke most frequently occurs, NR2ARs and NR2BRs are preferentially localized at the synaptic and extrasynaptic sites, respectively [7,12,30] (but, see also [42]), the 'NMDAR location' and 'NMDAR subtype' hypotheses are highly complementary.

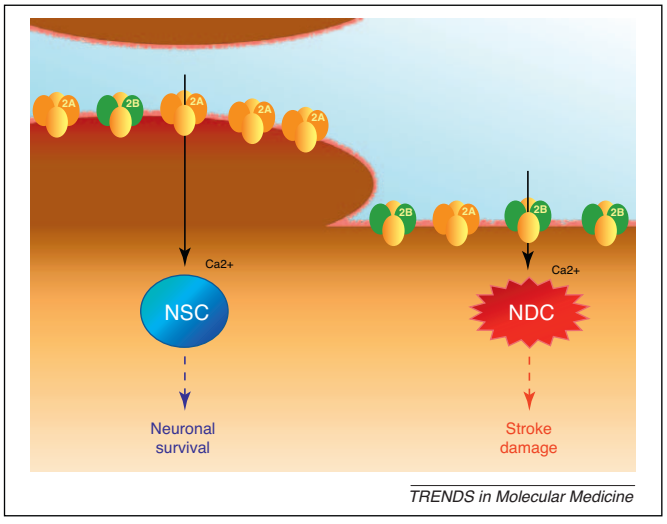

Figure 1. NMDARs mediate both neuronal survival and neuronal death. NMDARs are calcium-permeable ionotropic glutamate receptors that mediate many different neuronal functions in the brain, including opposing functions such as neuronal survival and death. In the adult forebrain, where stroke most frequently occurs, the two major subtypes of NMDARs are (i) those containing NR2A subunits (orange) primarily in the synapses and (ii) those containing NR2B subunits (green) primarily in extrasynaptic sites. Whereas the synaptic NR2A subpopulation activates the downstream NSC, leading to neuronal survival, the extrasynaptic NR2B subpopulation activates the downstream NDC, resulting in neuronal death under pathological conditions such as stroke.

Notably, stimulating synaptic and extrasynaptic NMDARs would predominantly activate NR2AR-dependent neuronal survival and NR2BR-mediated neuronal death pathways, respectively. From these points of view, a unified hypothesis can be synthesized (Figure 1). Normal synaptic transmission activates predominantly NR2ARs, resulting in the maintenance of neuronal survival via the activation of the neuronal survival-signaling complex (NSC) immediately downstream of these receptors. During stroke, glutamate surges primarily because of the reverse operation of the glutamate transporters [43], resulting in glutamate spillover to the extrasynaptic sites where the stimulation of NR2BRs mediates neuronal death by the activation of the neuronal death-signaling complex (NDC) associated with the receptors. Here, we loosely define the NSC and NDC to include all neuronal survival- and death-signaling proteins that closely associate with the NMDAR channel pore either through spatial compartmentalization to the synapses or extrasynaptic sites, or through direct or indirect protein–protein interactions with the NMDAR itself. Their close association with the NMDAR allows the efficient translation of calcium influx through the receptor channel into their respective functional outputs with high levels of spatial and subunit specificity.

Death-signaling proteins in the NDC and beyond: a therapeutics perspective

Many signaling proteins that contribute to extrasynaptic/NR2B NMDAR-mediated neuronal death have been characterized [27,37]. These include the molecular components of the NDC immediately downstream of these receptors and those death-signaling proteins beyond the NDC. In translating these exciting findings to clinical use, several interference peptides and small molecules have been developed [27]. These novel NMDAR-based therapeutics inhibit protein–protein interactions or post-translational modifications specifically required for NMDAR-mediated death-signaling pathways, without affecting receptor activation. In doing so, they protect neurons against NMDAR-mediated excitotoxic damage with a wider therapeutic window, without many of the side effects commonly associated with NMDAR blockers.

The NR2B subunit is a major hub for NDC formation

Many death-signaling proteins downstream of the NMDAR specifically bind the NR2B subunit either directly or indirectly, thereby making the NR2BR a major hub for NDC formation.

NR2B–PSD95–nNOS signaling complex

The NR2B–PSD95–nNOS signaling complex is the first and best characterized example that neuronal death-signaling proteins can form a physical protein complex with NR2BRs [24–28] (Box 2). In this complex, PSD95 acts as a scaffolding protein to bring nNOS (neuronal nitric oxide synthase) into close vicinity of the NR2BR channel pore. This allows the efficient activation of nNOS by calcium influx running down the NR2BR channel pore, resulting in the NMDAR-mediated production of the highly neurotoxic molecule nitric oxide (NO) [24–28,44]. Several new experimental stroke treatments, including the peptide NR2B9c

Box 3. Recruitment of DAPK1 to the NR2B subunit following stroke

A recent study [34] identified DAPK1 as a new component of the NDC. DAPK1 is a member of a serine/threonine kinase family well known for its role in cell death [53]. In a search for the most prevalent molecules in the NR2B-associated NDC following stroke, the total mix of signaling machineries directly or indirectly bound to NR2BRs before and after a stroke insult to the rat brain were analyzed by means of coimmunoprecipitation with anti-NR2B antibodies followed by mass spectrometry [34]. The results demonstrated that following stroke insults, DAPK1 is activated and recruited to the NR2BR through its direct binding to the amino acid residues 1292–1304 of the NR2B carboxyl terminus (NR2B-$CT_{1292–1304}$). This direct DAPK1–NR2B interaction enables the DAPK1-mediated phosphorylation of the NR2B subunit at serine-1303, thereby potentiating NR2BR activity. To selectively and competitively disrupt the DAPK1–NR2B interaction, an interference peptide, Tat-NR2B-$CT_{1292–1304}$, was developed whose primary sequence includes the membrane transducing domain of the HIV1 Tat protein and the DAPK1-binding domain of NR2B (Figure Ic) [34]. This peptide not only prevents DAPK1-mediated NR2B subunit phosphorylation and thereby NR2BR activity potentiation, but also attenuates excitotoxic neuronal injuries. Importantly, when applied systemically to animals, Tat-NR2B-$CT_{1292–1304}$ prevents ischemic brain damage following stroke [34]. It is especially important to note that DAPK1 is not associated with NR2BRs under basal conditions, but is only recruited to the NDC of NR2BRs following stroke insults. This stroke-induced recruitment of NDC molecules can, in part, explain why NMDAR signaling is normally benign (Figure Ia), but under pathological conditions such as stroke, NMDAR stimulation contributes to neuronal death (Figure Ib). Additional research is also required to further characterize the detailed mechanism by which DAPK1 mediates neuronal death (Figure Ib). Because DAPK1 potentiates NR2BR activity, it would inevitably also promote neuronal death by potentiating functions of 'calcium-activated death-signaling proteins' in the NDC (Figure Ib; 'calcium current'); however, this is probably not the only mechanism. Given the fundamental role of DAPK1 as a pro-apoptotic protein and tumor suppressor [53], the recruitment of DAPK1 to the NDC following stroke is probably self-sufficient for inducing neuronal death by a direct activity on 'phosphorylation-dependent death-signaling proteins' in the NDC via its kinase activity (Figure Ib, '? arrow'). An example of such a death-signaling protein is protein kinase D, whose phosphorylative activation by DAPK1 is in turn required for its phosphorylation-mediated activation of the death-signaling protein JNK [88].

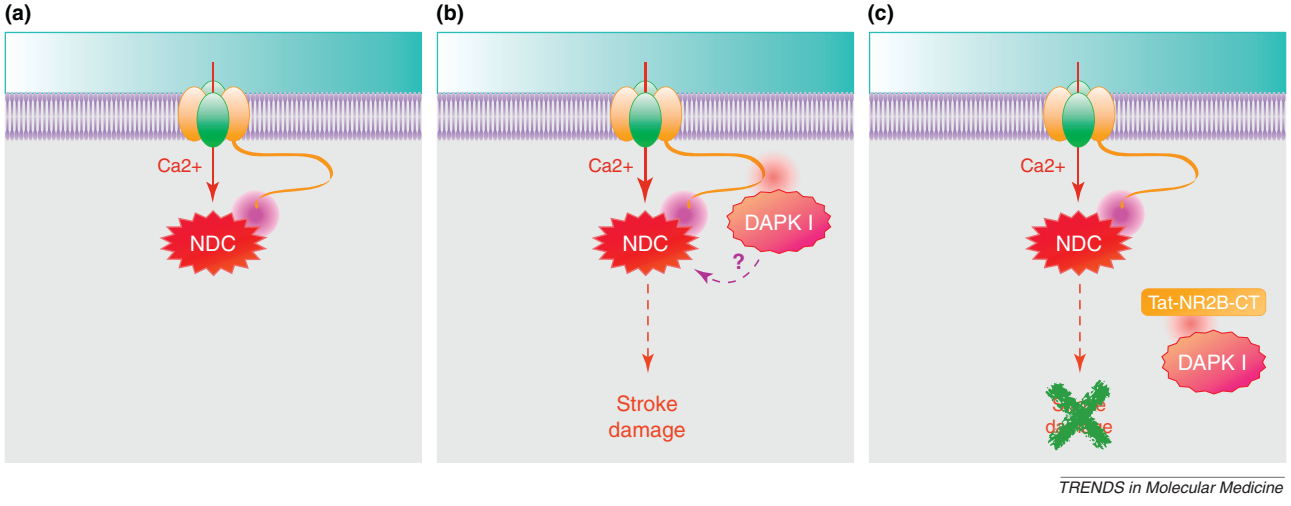

Figure I. DAPK1 as a component of the NDC. **(a)** The NR2B-associated NDC is not active under normal conditions. **(b)** DAPK1 is recruited to the NDC through its direct binding to the carboxyl terminus of NR2B during stroke, resulting in NDC activation either by potentiating NR2B activity or stimulating other NDC components. **(c)** Tat-NR2B-CT prevents the recruitment of DAPK1 to the NDC and the subsequent activation of the NDC by disrupting the NR2B–DAPK1 interaction.

[25] and a new drug ZL006 [28], disrupt the protein–protein interactions required for the formation of this signaling complex, thereby preventing NMDAR-mediated NO release and neuronal death. Because these new therapeutics specifically disrupt NR2B–PSD95–nNOS with no effect on other NMDAR signaling complexes, they retain the therapeutic efficacy of conventional NMDAR blockers but lack the neurological side effects associated with blocking NMDARs [24,25,27,28] (Box 2). These studies provide the proof-of-concept evidence that the specific disruption of the NDC can confer the therapeutic efficacy seen with conventional NMDAR blockers while minimizing side effects.

PTEN

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [45] is a well-characterized cell death-promoting molecule that was recently identified as a crucial component of the NDC [46–51]. PTEN is recruited to the NR2BRs (but not to the NR2ARs) via interactions with the NR1 subunit [46] and with PSD95 in a manner dependent on NMDAR calcium influx [52]. Moreover, the NR2BR–PTEN association potentiates NR2BR channel activity [46], and this can further enhance their association. This self-propagating mechanism, along with the well-known inhibition of the PI3K survival-signaling pathway by PTEN contributes to NR2BR-mediated neuronal death following stroke [46]. Interestingly, ischemia in animals subjected to stroke also triggers a time-dependent translocation of PTEN into the nucleus [51]. Consistent with the selective binding of PTEN to NR2BRs, this nuclear translocation of PTEN requires NR2BR, but not NR2AR, stimulation. Unlike in other cell types where the monoubiquitination of PTEN at residues K13 and K289 is thought to be required for nuclear translocation and tumor suppressive effects [45], only the K13 site is required for PTEN nuclear translocation and consequent excitotoxic neuronal injuries in cortical neurons [51]. In an effort to translate this exciting finding into clinical use, an

interference peptide Tat-K13 was developed. It contains the amino acid residues flanking the K13 ubiquitination site on PTEN and the membrane transduction domain of the HIV1 Tat protein, which renders the peptide membrane permeable [25]. When given to rats subjected to focal ischemia, the interference peptide Tat-K13, but not the control Tat-K289, strongly protects against cerebral infarction even when administered 6 h after stroke onset [51]. These studies add to the growing evidence supporting that the specific inhibition of the NDC can confer much wider therapeutic time windows than can blocking NMDARs with conventional blockers.

DAPK1

Death-associated protein kinase 1 (DAPK1) is a death-signaling protein [53] that is recruited to the NDC via its interaction with NR2B following stroke challenge [34] (Box 3). This NR2B–DAPK1 interaction allows DAPK1 to specifically potentiate NR2BR function and thereby the receptor-mediated death signaling. The disruption of the NR2B–DAPK1 interaction by Tat-NR2B$_{CT1292–1304}$ prevents the potentiating effect on NR2BR channel activity and attenuates neuronal death in rats subjected to focal ischemia [34] (Box 3). By identifying the recruitment of DAPK1 to the NDC via its direct binding to NR2B, but not NR2A, this study lends strong evidence to the hypothesis that NR2BRs mediate prodeath signaling, thereby supporting the 'NMDAR subunit' hypothesis.

Beyond the NDC: death processes further downstream

The new targets for NMDAR-mediated excitotoxic neuronal death are not only limited to the NDC but also include the death-signaling proteins further downstream of the NDC. Because neurons can die of slow processes over hours to days following a stroke insult [2], these newly identified death-signaling proteins and pathways further downstream of the NMDAR are promising targets for novel NMDAR-based therapeutics with wider therapeutic time windows.

Calpains

This family of calcium-activated cysteine proteases plays a major role in translating the calcium influx of NMDAR into neuronal injuries [31,32,36,54,55]. In line with the 'NMDAR subtype' and 'NMDAR location' hypotheses, calpains are only activated by NR2BRs, but not NR2ARs [31,32,36], and only by extrasynaptic, but not synaptic, NMDARs [17]. The inhibition of calpains [31,54,55] or their downstream death-signaling pathways [17,56] is strongly protective against NMDAR-mediated neuronal damage *in vitro* [17,31,54,56] and *in vivo* [55,56]. In support of the notion that blocking these downstream death-signaling proteins can confer a wider therapeutic time window than can blocking NMDARs, the calpain inhibitor SNJ-1945 protects mice against permanent focal ischemia even when treatment is delayed for up to 6 h post-stroke [55].

These cysteine proteases exert their neurotoxic actions mainly by the proteolytic destruction/inhibition of survival-signaling proteins [17,36,57] and by the proteolytic activation of death-signaling proteins [56,57]. First, calpains directly cleave survival-signaling proteins STEP61 (striatal-enriched tyrosine phosphatase-61) [17] and Kidins220 [36], thereby contributing to extrasynaptic NMDAR- and NR2BR-mediated neuronal death, respectively. Therefore, the inhibition of STEP61 cleavage by the Tat-STEP peptide rescues the STEP61-mediated inhibition of p38 death signaling and protects neurons against NMDAR-mediated excitotoxic damage [17]. Second, the calpain-mediated cleavage of p35 into p25 and the subsequent activation of cyclin-dependent kinase 5 contribute to glutamate-mediated excitotoxic neuronal death following stroke [58]. Likewise, the NR2BR-mediated proteolytic activation of the prodeath protein SREBP1 (sterol response element binding protein1) requires calpain activity; however, it remains unclear whether calpains directly cleave SREBP1 [56]. Third, calpains cleave mGluR1α (metabotropic glutamate receptor 1α), thereby converting this natively pro-survival glutamate receptor into a prodeath receptor [57]. Indeed, the Tat-mGluR1α peptide protects neurons against excitotoxic death *in vitro* and *in vivo*.

Clathrin-mediated endocytosis of AMPARs

One of the best characterized functional outputs of NR2BR is regulated AMPAR endocytosis, a common mechanism responsible for the expression of various forms of LTD [9,59–61]; nevertheless, it was not until recently that this was recognized as an essential step downstream of NMDAR-mediated excitotoxic neuronal death [62]. The inhibition of AMPAR endocytosis by two structurally different inhibitors of clathrin-mediated endocytosis prevents NMDAR-mediated neuronal death without affecting NMDAR activity [62]. More importantly, the specific inhibition of AMPAR endocytosis with an interference peptide GluR2$_{3Y}$, whose sequence is derived from the GluR2 tyrosine phosphorylation sites required for regulated AMPAR endocytosis and LTD [63], prevents neurons against excitotoxic damage *in vitro* [62]. Whether this process is involved and whether the GluR2$_{3Y}$ peptide is effective in animal models of stroke *in vivo* remain to be investigated.

How AMPAR endocytosis mediates excitotoxic neuronal death remains unclear. First, whereas an earlier study reported that AMPAR endocytosis mediates neuronal death both by enhancing the caspase-3 death-signaling pathway and inhibiting the PI3K survival-signaling pathway [62], a recent study showed that increased caspase-3 activity and decreased PI3K signaling is actually required for AMPAR endocytosis [61]. Together, these two studies suggest that an amplification of death signaling via positive feedback interplay between AMPAR endocytosis and caspase-3 following NR2BR activation is required for excitotoxic neuronal death. Second, clathrin-mediated rhodopsin endocytosis promotes its interaction with the clathrin adaptor protein arrestin, and this rhodopsin–arrestin interaction induces retinal neuronal death in *Drosophila* [64]. Given that β-arrestin also interacts with activated c-Jun amino-terminal kinase 3 (JNK3) [65] (see below), it is possible that a clathrin adaptor protein such as β-arrestin also activates and/or recruits death-signaling molecules such as JNK3 to endocytosed AMPARs, thereby mediating AMPAR endocytosis-dependent neuronal apoptosis. Third, because GluR2-containing AMPARs are specifically endocytosed following NR2BR stimulation, the endocytosis could lead to a compensatory increase in the expression

of surface GluR2-lacking AMPARs following stroke [66,67]. These GluR2-lacking receptors are characterized by high calcium permeability and, as such, they might contribute to neuronal death following ischemic stroke [66,67].

MAPKs

Mitogen-activated protein kinases (MAPKs) are traditionally known for transducing extracellular signals from neurotransmitters and hormones to the nucleus, resulting in gene expression changes [68]. There are three main types of MAPKs: (i) the extracellular signal-regulated kinases (ERKs) that are generally prosurvival and are activated by growth factors and other survival factors, (ii) the p38 protein kinase and (iii) JNK, which mediate cell death in response to inflammatory cytokines and cellular stress. Consistent with their roles in neuronal survival and death, synaptic/NR2A NMDAR stimulation induces ERK activation [59], whereas extrasynaptic/NR2B NMDAR stimulation inhibits ERK activation (indirectly via synGAP [59] or DAPK1 [69]) and induces p38 activation [17]. Moreover, the upregulation of the NR2B subunit in the spinal cord has been implicated in JNK activation [70].

Notably, recent studies have demonstrated that p38 activation is probably a downstream effector of the NDC given that NMDAR-mediated p38 activation requires intact NR2B–PSD95–nNOS formation [71,72] and the calpain-mediated proteolysis of STEP61, yielding STEP33 [17]. In agreement with the prodeath role of p38, the inhibition of p38 with selective inhibitors SB203580 and SB239063 prevents NMDAR-mediated neuronal death in *in vitro* [71,73,74] and *in vivo* [75] models of stroke.

Unlike many transient signaling molecules downstream of NMDARs, JNK remains strongly activated for as long as 24 h post-stroke [76]. A peptide inhibitor D-JNKI-1, which mimics the JNK-binding domain (JBD20, residues 143–163) of the JNK-interacting protein-1 (JNK1), protects cultured cortical neurons against NMDA-induced neuronal death *in vitro* and reduces ischemic brain damage in rat models of both transient and permanent focal ischemia [76–78]. Notably, and consistent with the long-lasting activity of JNK following stroke, inhibition by D-JNKI-1 has a relatively long post-stroke therapeutic time window of up to 12 h following transient focal ischemia [76,78], and 3 h following permanent focal ischemia [77].

SREBP1

Although SREBP1 usually controls lipid biosynthesis genes [79], it was recently identified as a downstream death-signaling protein for NR2BR-mediated excitotoxic neuronal death following stroke [56]. Because transcriptional activities can contribute to 'slow' neuronal death signaling, prodeath transcription factors are promising targets for developing novel stroke therapeutics that reduce neuronal death with a prolonged therapeutic window. A recent non-biased screen [56] identified SREBP1 as a transcription factor whose activation by NR2BRs is required for neuronal death following stroke. The stimulation of NR2BRs, but not NR2ARs, triggers the calcium-dependent ubiquitination and proteasomal degradation of INSIG1 (protein encoded by insulin induced gene1), an important inhibitory binding partner that normally retains inactive SREBP1 in the endoplasmic reticulum [79], thereby allowing SREBP1 to travel to the Golgi apparatus where it is cleaved to generate the active N terminus of SREBP1 (nSREBP1). nSREBP1 then translocates into the nucleus where it carries out the transcriptional activities required for delayed neuronal death [56]. As expected, the suppression of SREBP1 signaling by either oversupplying cholesterol or siRNA-mediated knockdown protects cultured neurons against excitotoxic/ischemic neuronal death *in vitro* [56]. Inhibiting INSIG1 degradation with an interference peptide Indip (Insig1 degradation inhibiting peptide), a Tat-linked peptide with sequence flanking the two ubiquitination sites K156 and K158, prevents SREBP1 activation and subsequent neuronal death in *in vitro* and *in vivo* stroke models [56]. Indip remains effective when administered 2 h post-stroke onset, and the improved morphological and behavioral outcomes remain evident up to 7 days. Moreover, because SREBP1 activation can take up to 6 h following excitotoxic stimulation, targeting SREBP1 with therapeutics such as Indip could have significantly wider therapeutic windows than the 2 h tested in this study [56].

Detailed mechanisms underlying SREBP1-mediated neuronal damage remain to be established. SREBPs are the major transcription factors that regulate the expression of a large number of gene products involved in cellular cholesterol and lipid biosynthesis [79], and metabolic alterations in some of these lipid products were recently implicated in mediating neuronal damage following stroke insults [80,81]. In addition, SREBP1 also regulates the expression of proteins not directly involved in lipid metabolism, including G proteins [82] and voltage-gated ion channels [83]. Thus, SREBP1 can contribute to neuronal damage via a mechanism independent of, or in addition to, alterations in lipid metabolism.

Concluding remarks

NMDARs are important for many neuronal functions, including opposing functions such as neuronal survival and death. Based on the current 'NMDAR location and subtype' hypotheses, physiological synaptic transmission maintains neuronal survival by activating the downstream NSC of synaptic/NR2A NMDAR, whereas glutamate spillover during stroke stimulates the downstream NDC of extrasynaptic/NR2B NMDAR. Given that the neuronal death function of NMDAR is a primary mechanism of stroke damage, NMDAR blockers have been developed and tested in human clinical studies. However, because NMDARs mediate so many different neuronal functions, blocking these receptors results in undesirable side effects. Moreover, because these receptors have a neuronal survival function, blocking these receptors can exacerbate stroke outcomes. Finally, once the downstream death-signaling proteins of NMDARs are activated, including those immediately downstream of the NDC and those beyond the NDC, blocking these receptors is no longer useful.

The successful identification of these downstream death-signaling pathways has made it possible to develop several novel Tat-linked interference peptides that can selectively inhibit protein–protein interactions or the post-translational modifications required for NMDAR-me-

diated excitotoxic neuronal death following stroke. These novel NMDAR-based therapeutics specifically inhibit downstream death signaling without affecting other functional signaling or survival signaling of the receptor. Thus, they do not have the side effects seen with traditional NMDAR blockers. Moreover, because some of these downstream pathways are activated long after NMDAR activation, these new therapeutics provide a much wider therapeutic time window, thereby remaining effective long after NMDAR activation following stroke.

Although the medical use of 'peptidic biologics' is generally limited by routes of drug administration because of their susceptibility to proteolytic enzymes, expensive cost of production and risk for immunorejection [27], these limitations should not discourage the use of these interference peptides for stroke treatment. First, low bioavailability because of susceptibility to proteolytic enzymes can be minimized by intravenous injections in a hospital setting, where stroke patients are often treated. Secondly, the high cost and risk of immunorejection might not be a major issue because a single post-stroke dose might be sufficient to achieve substantial neuroprotection [25,56]. Moreover, small molecules that mimic these interference peptides are already being developed through either high-throughput screening [44] or rationalized drug design [28]. These small molecules retain most of the advantages of the interference peptides but are more clinically applicable [27].

Finally, because NMDAR-mediated excitotoxicity is thought to be a common neuropathology associated with a large number of neurological disorders ranging from acute brain insults such as brain trauma to chronic neurodegenerative disorders such as Huntington's disease and Parkinson's disease [11], these interference peptides and their small molecule mimetics designed to specifically target the neuronal death cascade downstream of NMDARs can have broad implications beyond stroke, raising the exciting potential for designing new therapeutics for the clinical treatment of these neurological disorders.

Acknowledgments

Y.T.W. is a Howard Hughes Medical Institute International Scholar, and Heart and Stroke Foundation of British Columbia and Yukon Chair in Stroke Research. T.W.L. receives financial support from Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship (CGS) Doctoral Award, National Science and Engineering Research Council CGS Master's award, Michael Smith Foundation for Health Research (MSFHR) Junior Graduate Scholarship and CIHR/MSFHR Strategic Training Scholarship in Neurobiology and Behaviour.

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