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Excitotoxicity and stroke: Identifying novel targets for neuroprotection☆

Ted Weita Lai [a,b,*], Shu Zhang [b,c], Yu Tian Wang [c,**]

[a] Graduate Institute of Clinical Medical Science, China Medical University, 91 Hsueh-Shih Road, 40402 Taichung, Taiwan
[b] Translational Medicine Research Center, China Medical University Hospital, 2 Yu-De Road, 40447 Taichung, Taiwan
[c] Brain Research Center, University of British Columbia, 2211 Wesbrook Mall, V6T 2B5 Vancouver, Canada

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ABSTRACT

Excitotoxicity, the specific type of neurotoxicity mediated by glutamate, may be the missing link between ischemia and neuronal death, and intervening the mechanistic steps that lead to excitotoxicity can prevent stroke damage. Interest in excitotoxicity began fifty years ago when monosodium glutamate was found to be neurotoxic. Evidence soon demonstrated that glutamate is not only the primary excitatory neurotransmitter in the adult brain, but also a critical transmitter for signaling neurons to degenerate following stroke. The finding led to a number of clinical trials that tested inhibitors of excitotoxicity in stroke patients. Glutamate exerts its function in large by activating the calcium-permeable ionotropic NMDA receptor (NMDAR), and different subpopulations of the NMDAR may generate different functional outputs, depending on the signaling proteins directly bound or indirectly coupled to its large cytoplasmic tail. Synaptic activity activates the GluN2A subunit-containing NMDAR, leading to activation of the pro-survival signaling proteins Akt, ERK, and CREB. During a brief episode of ischemia, the extracellular glutamate concentration rises abruptly, and stimulation of the GluN2B-containing NMDAR in the extrasynaptic sites triggers excitotoxic neuronal death via PTEN, cdk5, and DAPK1, which are directly bound to the NMDAR, nNOS, which is indirectly coupled to the NMDAR via PSD95, and calpain, p25, STEP, p38, JNK, and SREBP1, which are further downstream. This review aims to provide a comprehensive summary of the literature on excitotoxicity and our perspectives on how the new generation of excitotoxicity inhibitors may succeed despite the failure of the previous generation of drugs.

Abbreviations: ADD1, adipocyte determination and differentiation-dependent factor 1; AIF, apoptosis-inducing factor; BDNF, brain-derived neurotrophic factor; CaM-KK, calcium–calmodulin dependent protein kinase kinase; CAPON, carboxyl-terminal PDZ ligand of nNOS; cdk5, cyclin-dependent kinase 5; CRE, cyclic adenosine monophosphate response element; CREB, CRE binding protein; CSAID, cytokine-suppressive anti-inflammatory drug; CSBP, CSAID-binding protein; DAPK1, death-associated protein kinase 1; DL-TBOA, DL-threo-benzyloxyaspartic acid; Drp1, dynamin-related protein 1; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluN2AR, GluN2A subunit-containing NMDA receptor; GluN2BR, GluN2B subunit-containing NMDA receptor; GSK3, glycogen-synthase kinase 3; INPP4A, inositol polyphosphate phosphatase 4A; insig1, protein encoded by insulin-induced gene 1; IRS-1, insulin receptor substrate-1; JBD, JNK-binding domain of JIP; JIP, JNK-interacting protein; JNK, c-Jun N-terminal kinase; JNK-1, JNK inhibitor 1; LTD, long-term depression; LTP, long-term potentiation; MAGUK, membrane-associated guanylate kinase; MAPK, mitogen-activated protein kinase; MCU, mitochondrial calcium uniporter; mGluR, metabotropic glutamate receptor; MMAC1, mutated in multiple advanced cancers 1; MMP-9, matrix metalloprotease-9; mNCX, mitochondrial sodium–calcium exchanger; NFI-A, nuclear factor I-A; NCX, sodium–calcium exchanger; NMDA, N-methyl-D-aspartate; NMDAR, NMDA-type of glutamate receptor; nNOS, neuronal nitric oxide synthase; NOS1AP, nitric oxide synthase 1 adaptor protein; p35, the 35-kDa regulatory activator of cdk5; PARP-1, poly(ADP-ribose) polymerase 1; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; PIKE-L, long form of phosphoinositide 3 kinase enhancer; PSD95, postsynaptic density protein 95; PtdIns(3,4)P2, phosphatidylinositol (3,4)-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; rac, protein kinase related to the A and C kinases; RK, MAPK-activated protein kinase-2 reactivating kinase; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; SCAP, SREBP cleavage-activating protein; SREBP1, sterol response element binding protein 1; STEP, striatal-enriched protein tyrosine phosphatase; TEP1, TGF-beta-regulated and epithelial cell-enriched phosphatase 1; TRPM7, transient receptor potential cation channel M7; TTX, tetrodotoxin; UCPs, uncoupling proteins.

* Corresponding author at: Graduate Institute of Clinical Medical Science, China Medical University, 91 Hsueh-Shih Road, 40402 Taichung, Taiwan. Tel.: +886 4 22052121x7638.
** Corresponding author. Tel.: +1 604 8220398.
E-mail addresses: ted.weita@me.com (T.W. Lai), ytwang@brain.ubc.ca (Y.T. Wang).

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Contents

1. Introduction

In the past several decades, excitotoxicity, a type of neurotoxicity mediated by glutamate, has been at the center stage of stroke research. Glutamate is the principle neurotransmitter in the adult central nervous system. In addition to being required for the rapid synaptic transmission that is critical for neuron-to-neuron communication, glutamate plays important roles in neuronal growth and axon guidance, brain development and maturation, and synaptic plasticity in health and disease. Among the ionotropic and metabotropic glutamate receptors in the adult central nervous system, the N-methyl-D-aspartate (NMDA) type of glutamate receptor (the NMDAR) acts as a hub, by detecting and processing extracellular glutamate signals into diverse intracellular signaling outputs. With the emergence of cellular and molecular biology, scientists are unraveling the mechanisms by which glutamate-mediated activation of the NMDAR in health and disease transmits so many different functional outputs, at both the microscopic neuron level and the macroscopic behavior level. These mechanisms have important implications for research concerning excitotoxicity and its role in ischemic neuronal death. The identification of distinct intracellular pathways linking NMDAR activation to neuronal death allows scientists to develop novel treatments that target specific death signaling pathways without affecting all the signaling pathways downstream of the receptor. This increased specificity not only translates into reduced side effects but also increases the therapeutic window in which the drug can be efficaciously administered.

2. Stroke and the NMDAR

Compared to other tissues and organs in the body, the brain is particularly prone to ischemic damage. Unlike the immediate ischemic damage that is observed in other tissues, a transient period of cerebral ischemia (approximately 10 min) can produce profound neuronal damage that only becomes evident 3d post-ictus and continues progressively for months. As relief of vascular occlusion is the primary method by which tissue ischemia is treated in the clinic, the propensity for ischemic damage to occur regardless of the recovery of blood flow highlights the need for an alternative method for treating cerebral ischemia. Importantly, the delayed and progressive nature of neuronal damage following cerebral ischemia points to a wide time window for therapeutic intervention and emphasizes the importance of understanding the nature of ischemic neuronal death. An improved understanding of the processes that translate cerebral ischemia into neuronal damage would highlight new therapeutic targets for stopping the seemingly inevitable progression from ischemia to neuronal death. One explanation for the peculiar susceptibility of the brain to ischemic damage is that brain tissue contains high levels of the neurotoxic excitatory neurotransmitter glutamate, and many neurons in the brain contain receptors that actively respond to

this neurotransmitter. Thus, because cerebral ischemia leads to a massive release of glutamate, which stimulates the NMDAR and induces calcium influx through these ionotropic receptors, the calcium-dependent activation of death-signaling proteins that are immediately downstream of the receptors triggers a plethora of signaling cascades that work synergistically to induce neuronal death.

2.1. Glutamate and excitotoxicity

Neuroscientists have made progress in understanding the role of the NMDAR in glutamate-mediated excitotoxic damage. Initially, interest in this topic was based on the observation that monosodium glutamate, a popular food additive in Chinese restaurants, is neurotoxic to the inner layer of the mouse retina (Lucas and Newhouse, 1957). This observation was soon followed by the finding that glutamate is excitatory: it can depolarize neurons, causing them to fire action potentials (Curtis et al., 1959). It has subsequently been shown that the neurotoxic effect of monosodium glutamate is not limited to the mouse retina but extends to the peripheral and central neurons in mice, rats, rabbits, and rhesus monkeys (Burde et al., 1971; Freedman and Potts, 1962, 1963; Olney, 1969a,b; Olney and Sharpe, 1969). In addition, this effect can be mimicked by other amino acids that are excitatory to neurons (Olney, 1971; Olney and Ho, 1970; Olney et al., 1971, 1974) but not by non-excitatory amino acids (Olney and Ho, 1970; Olney et al., 1971). Importantly, this neuronal damage can be induced following oral intake of relatively low doses of monosodium glutamate (Burde et al., 1971; Olney and Ho, 1970), which raises concerns about the common use of this additive in diets. Because glutamate is excitatory and other related excitatory amino acids are also neurotoxic, these amino acids are collectively called excitotoxins, and their associated neuronal damage is called excitotoxicity.

2.1.1. Ionic mechanism of excitotoxicity

In investigations concerning the mechanism of excitotoxicity, several hypotheses have been proposed that suggest an ionic basis for neurotoxicity. The concept of excitotoxicity originates from the idea that because neurotoxic glutamate analogs are structurally similar, these molecules are likely to elicit neurotoxicity by a common mechanism (Olney et al., 1971, 1974). The bold hypothesis that excitotoxicity is "in essence, an exaggeration of the excitatory effect" (Olney et al., 1974) follows from this theory. This hypothesis is supported by the findings that glutamate analogs that are excitatory are also neurotoxic and that the neurotoxic potency of these analogs correlates with their potency in inducing neuronal excitation (Olney et al., 1971). In addition, glutamate analogs that are not excitatory are not neurotoxic (Olney et al., 1971). Further, glutamate-mediated neuronal excitation is expected to occur in postsynaptic locations (soma and dendrites), and these locations are also the origin of rapid neuronal swelling, a pathological feature that is thought to underlie excitotoxicity (Olney et al., 1971, 1974). Finally, drugs that antagonize glutamate-mediated neuronal excitation also prevent the associated neurotoxicity. In contrast to the concept of excitotoxicity, which suggests that the sodium cation is a key molecule in neurotoxicity, many cytotoxic processes in neuronal and non-neuronal tissues, such as the heart, the muscles, and the liver, require the divalent calcium cation (Berdichevsky et al., 1983; Coyle, 1983). The extension of this calcium hypothesis to excitotoxicity is further corroborated by experimental evidence demonstrating that glutamate and other excitatory amino acids also increase calcium influx into neurons (Berdichevsky et al., 1983; Jancso et al., 1984) and that non-neurotoxic amino acids do not increase calcium influx (Berdichevsky et al., 1983). Importantly, among several glutamate analogs, N-methyl-DL-aspartate is the most potent in increasing calcium influx and is also the most potent in inducing neurotoxicity. This finding led to the initial hypothesis that the glutamate receptor subtype that is activated by N-methyl-DL-aspartate (now known as the NMDAR) underlies glutamate-mediated excitotoxicity (Berdichevsky et al., 1983).

Because it is virtually impossible to study the ionic mechanism of glutamate neurotoxicity in vivo, most studies are performed in primary neurons in vitro. Thus, controversies can arise from differences in experimental technique and differences in the endpoint used to define neuronal damage. The aforementioned rapid neuronal swelling, which occurs immediately upon treatment with glutamate, is completely abolished by the removal of sodium ions (Olney et al., 1986; Rothman, 1985), but it is not affected by the removal of calcium ions (Olney et al., 1986; Price et al., 1985; Rothman, 1985). In addition, in the absence of glutamate or other excitatory amino acids, excitation of neurons by increasing the concentration of potassium ions in the culture medium also induces neuronal swelling (Olney et al., 1986; Rothman, 1985). Together, these data support the excitotoxicity hypothesis that proposes that excitotoxicity is an exaggeration of neuronal excitation mediated by sodium ions and that any source of excitation (even those that are not mediated by excitatory amino acids) is potentially harmful. In contrast to the neuronal swelling that occurs immediately upon glutamate treatment, the delayed neuronal death that is observed 24 h after glutamate treatment is abolished by the removal of calcium and potentiated by increased calcium levels (Choi, 1985). In addition, the removal of sodium ions from the culture medium prior to glutamate treatment prevents neuronal swelling but does not affect the neuronal death that occurs 24 h later (Choi, 1987). More importantly, while the removal of calcium exacerbates acute glutamate-induced neuronal swelling, this change prevents the delayed neuronal death that typically occurs 24 h later (Choi, 1987). In addition, the rapid neuronal swelling and related morphological and pathological features that are caused by glutamate challenge eventually recover over time (Choi, 1987). This finding highlighted the critical role of calcium ions in glutamate-mediated neurotoxicity. At the same time, emerging experimental evidence began to suggest that NMDARs, the most calcium-permeable ionotropic glutamate receptors, are responsible for the ischemic neuronal damage that follows stroke.

2.1.2. Calcium homeostasis and distinct pathways toward death and survival

The calcium ion is one of the most important signaling molecules in cell biology, and tight regulation of intracellular calcium level by means of sequestration and extrusion is crucial for cellular function. Given the essential role of calcium in glutamate-mediated injury (see Section 2.1.1), dysfunction of calcium homeostatic machineries are expected to exacerbate calcium overload and contribute to excitotoxicity. Moreover, the initial calcium influx following excitotoxic glutamate stimulation is known to trigger a secondary intracellular calcium overload, and this secondary response strongly correlates with neuronal death (Randall and Thayer, 1992; Tymianski et al., 1993a). One important regulator of intracellular calcium level is the plasma membrane sodium-calcium exchanger (NCX), which extrudes calcium using driving force from sodium influx. Following glutamate stimulation, calcium extrusion by NCX partially recovers intracellular calcium concentration back to physiological level (White and Reynolds, 1995). More recently, it has been shown that NMDAR-mediated dysfunction of NCX explains the subsequent calcium overload that occurs following an excitotoxic stimulus, and that replacement of the defective NCX with a non-compromised isoform prevents excitotoxic neuronal death (Bano et al., 2005) (see Section 4.3). In

contrast to calcium extrusion, NCX operates in the reverse direction during the early phase of glutamate stimulation (Hoyt et al., 1998). While this contributes to early glutamate-mediated calcium loading, it does not contribute to the calcium overload and neuronal death upon prolonged glutamate exposure (Hoyt et al., 1998). Another major player in cellular calcium homeostasis is mitochondria. Mitochondria can recover intracellular calcium concentration by (1) itself taking up a huge amount of calcium (White and Reynolds, 1996, 1997), and by (2) facilitating ATP-dependent calcium extrusion (Budd and Nicholls, 1996a,b). Importantly, inhibition of both mitochondria and NCX completely prevents recovery of calcium level following glutamate stimulation (White and Reynolds, 1995). Upon excitotoxic glutamate stimulation, the mitochondrial uptake of calcium in turn results in the production of reactive oxygen species (ROS) (Castilho et al., 1999; Reynolds and Hastings, 1995), opening of the permeability transition pore that results in mitochondrial depolarization (Abramov and Duchen, 2008; Vergun et al., 1999; White and Reynolds, 1996), induction of calcium deregulation (Castilho et al., 1998, 1999; Keelan et al., 1999; Ward et al., 2000), and induction of neuronal death (Stout et al., 1998). In addition, the mitochondrial NCX (mNCX) mediates extrusion of calcium from mitochondria into the cytoplasm, and this also partially contributes to the delayed cytoplasmic calcium loading during excitotoxicity (White and Reynolds, 1996, 1997). Notably, the uncoupling proteins (UCPs) 2 and 3 have recently been identified to be the mitochondrial calcium uniporter (MCU) (Trenker et al., 2007) (but see also Brookes et al., 2008). Genetic knockdown of MCU prevents mitochondrial calcium uptake in response to glutamate and other stimuli (Brookes et al., 2008; Qiu et al., 2013), and prevents NMDAR-mediated mitochondrial depolarization and excitotoxic neuronal death (Qiu et al., 2013). In contrast, MCU overexpression promotes NMDAR-mediated mitochondrial calcium uptake, and thereby exacerbates mitochondrial depolarization and neuronal injury (Qiu et al., 2013).

The spatial and temporal regulation of intracellular calcium ion distribution entails that calcium input from different sources and under different conditions can have very different consequences. While the essential role of the calcium ion in excitotoxicity explains the prominent role of the NMDAR over less calcium-permeable types of glutamate receptors in this phenomenon, it does not explain why calcium input from the NMDAR is neurotoxic while calcium input from other sources can be neuroprotective. For example, stimulation of voltage-gated calcium channels promotes the survival of cultured neurons in vitro (Balazs et al., 1990; Gallo et al., 1987; Ghosh et al., 1994) and protects them from neuronal death mediated by the NMDAR (Balazs et al., 1990) or by nerve growth factor starvation (Koike et al., 1989). In vivo studies in the mouse brain demonstrated that reducing calcium loading by inhibiting voltage-gated calcium channels exacerbates, rather than attenuates, excitotoxicity induced by glutamate or N-methyl-D-aspartate (Price et al., 1985). The observed differential effects of different calcium sources could not be due to differences in calcium loading, because calcium loading induced by glutamate via NMDARs is more neurotoxic than equivalent calcium loading induced by depolarization via voltage-gated calcium channels (Tymianski et al., 1993b). The differential effects on neuronal death and survival that result from these different calcium inputs can be partially explained by their differential effects on gene expression (Bading et al., 1993; Ghosh et al., 1994), such as their differential effect on the expression of brain-derived neurotrophic factor (BDNF) (Ghosh et al., 1994). Thus, although calcium overload is almost always detrimental and has been shown to be tightly associated with neuronal death (Randall and Thayer, 1992; Tymianski et al., 1993a), transient calcium influx can have distinct functions, including neuroprotective and neurodegenerative effects, depending on the source of the calcium (Bading et al., 1993; Ghosh et al., 1994; Tymianski et al., 1993b) or the location of the calcium transient (Hardingham et al., 1997). The pivotal role of the NMDAR as a source of death-signaling calcium led to the prominent theory that some calcium-dependent death-signaling proteins must be closely associated with, if not physically bound to, the NMDAR (Tymianski et al., 1993b). Today, mounting evidence has indicated that the C-terminal domain of the NMDAR directly binds to many death-signaling proteins.

2.2. Dual role of the NMDAR in death and survival

The NMDAR is not always excitotoxic. This receptor is known to have dual-effects; it promotes neuronal death or survival in primary neuronal cultures in vitro and in the rat brain in vivo, depending on the level of receptor activity. For example, NMDAR stimulation with a low dose of N-methyl-D-aspartate promotes neuronal survival in granule cell cultures (Balazs et al., 1988a,b, 1989; Didier et al., 1989; Yan et al., 1994), and under the same culture conditions, NMDAR stimulation with a high dose of N-methyl-D-aspartate induces neuronal death (Yan et al., 1994). In comparison, NMDAR inhibition by a low dose of the antagonist 2-amino 5-phosphonovaleric acid promotes neuronal survival in spinal cord cultures, but a high dose of the same antagonist induces neuronal death (Brenneman et al., 1990a,b). Consistent with these in vitro data, pharmacological inhibition or genetic deletion of the NMDAR yields widespread neuropathy and neuronal death in the rat brain in vivo (Adams et al., 2004; Gould et al., 1994; Hansen et al., 2004; Ikonomidou et al., 1999) during a developmental period in which the rat brain is most prone to NMDAR-mediated excitotoxic neuronal death (Ikonomidou et al., 1989b). These findings suggested that the NMDAR could promote either neuronal death or survival under the same culture conditions in vitro or during the same stage of brain development in vivo, depending only on the level of NMDAR activity. This premature speculation, which is known as the set-point hypothesis, stipulates that there is an optimal level of intracellular calcium ions, such that too much or too little calcium input from the NMDAR or other sources can induce neuronal death (Choi, 1995; Franklin and Johnson, 1992; Koike et al., 1989). However, this hypothesis does not explain why the age at which the rat brain is most susceptible to NMDAR-inhibition is also the age at which the rat brain is most vulnerable to NMDAR-stimulation (Ikonomidou et al., 1989b, 1999). In light of the differential roles of distinct sources of calcium input on neuronal death and survival, emerging evidence points to distinct NMDAR subpopulations that differentially promote neuronal death and survival. Importantly, the disruptive effect of a neuropeptide on the excitotoxic but not the survival-promoting effect of the NMDAR suggests that these effects are mediated by distinct downstream mechanisms (Brenneman et al., 1990b).

2.2.1. Neuronal survival mediated by synaptic NMDAR

Neuronal survival is activity-dependent. The potentiation of neuronal activity by depolarization or NMDAR stimulation can enhance neuronal survival in cultures in vitro (Balazs et al., 1988a,b, 1989; Didier et al., 1989; Gallo et al., 1987), and the potentiation of neuronal activity by means of environmental enrichment induces a 45% decline in spontaneous neuronal death in vivo (Young et al., 1999). In addition, environmental enrichment protects the brain against excitotoxic injury (Young et al., 1999). Given the specific localization of many synaptic and extrasynaptic proteins, it is possible that synaptic and extrasynaptic NMDARs can be functionally coupled to different downstream signaling proteins, conferring differential effects on neurons. The propensity for synaptic activity to promote neuronal survival lends support to the hypothesis that synaptic NMDAR can promote neuronal

survival, while extrasynaptic NMDAR, which is activated when there is too much glutamate in the brain, such as during cerebral ischemia (Benveniste et al., 1984), can induce neuronal death. Although early attempts to differentiate the distinct capacities of synaptic and extrasynaptic NMDARs to induce excitotoxicity were unsuccessful (Sattler et al., 2000), subsequent work from several groups demonstrated that synaptic and extrasynaptic NMDARs have differential and sometimes opposing functions in neurons (Hardingham et al., 2002; Ivanov et al., 2006; Lu et al., 2001). These opposing functions include the induction of neuroprotective and neurodegenerative effects (Hardingham et al., 2002). In particular, synaptic NMDAR conveys the synaptic activity-driven activation of the survival-signaling protein extracellular signal-regulated kinase (ERK) (Chandler et al., 2001; Hardingham et al., 2001a; Leveille et al., 2008) and triggers an increase in nuclear calcium via release from intracellular stores, leading to the activation of the transcription factor CREB (Hardingham et al., 2001b) and the production of the survival-promoting protein BDNF (Hardingham et al., 2002). In contrast, global or extrasynaptic NMDAR stimulation decreases ERK activation (Chandler et al., 2001; Ivanov et al., 2006; Leveille et al., 2008), and extrasynaptic NMDAR stimulation decreases CREB activation and BDNF production (Hardingham et al., 2002). Most importantly, synaptic NMDAR stimulation protects neurons against staurosporine-induced and starvation-induced neuronal death (Hardingham et al., 2002; Papadia et al., 2005; Soriano et al., 2006; Zhang et al., 2007), while global stimulation of the NMDAR induces neuronal death (Gouix et al., 2009; Hardingham et al., 2002; Zhang et al., 2007). Consistent with the extrasynaptic localization of the GluN2B-containing NMDAR, selective inhibition of this NMDAR subtype protects neurons against global NMDAR stimulation (Hardingham et al., 2002). The finding that synaptic NMDAR promotes neuronal survival has important physiological implications (Fig. 1A and B): it provides a mechanistic explanation for the observation that synaptic activity mediates neuronal survival and provides neuroprotection against central distress (Young et al., 1999) and for the observation that NMDAR antagonism can be detrimental to the developing and adult brain (Gould et al., 1994; Ikonomidou et al., 1999) and hinder recovery of the brain from injury (Ikonomidou et al., 2000). In addition, the death-signaling property of extrasynaptic NMDAR provides an explanation for the finding that glutamate spillover under pathological conditions, such as cerebral ischemia, induces neuronal death.

Although synaptic NMDAR preferentially promotes neuronal survival (and extrasynaptic NMDAR preferentially promotes neuronal death), synaptic NMDAR is not exclusively pro-survival. First, the synaptic release of glutamate induces hypoxic neuronal death in primary hippocampal neurons (Rothman, 1983, 1984), and the inhibition of synaptic NMDAR protects neurons against hypoxic neuronal death (Wroge et al., 2012; Zhou et al., 2013). Likewise, a substrate inhibitor for the glutamate transporter induces prominent excitotoxicity in glial-lacking neuronal cultures at a dose that stimulates almost exclusively synaptic NMDAR (Gouix et al., 2009). Second, relieving synaptic NMDARs (allowing their relocation to extrasynaptic sites) failed to exacerbate NMDAR-mediated neuronal death (Sattler et al., 2000). In addition, postsynaptic density protein 95 (PSD95), the most well characterized NMDAR-conjugating death-signaling protein (see Section 4.2), is predominantly synaptic rather than extrasynaptic (Butko et al., 2012; Cho et al., 1992; Kornau et al., 1995), although exceptions have been observed (Petralia et al., 2010). In primary cortical neurons, knocking down PSD95 expression in the synapse prevents NMDAR-mediated excitotoxicity (Sattler et al., 1999). Third, in the presence of NVP-AAM007, an antagonist that preferentially inhibits the GluN2A-containing NMDAR, the direct stimulation of synaptic (presumably GluN2A-lacking) NMDAR in primary cortical neurons can induce neuronal death (Liu et al., 2007). Thus, some synaptic NMDARs can induce neuronal death. In addition, selective inhibition of synaptic NMDAR attenuates the excitotoxicity that is induced by global NMDAR stimulation in primary hippocampal neurons, further supporting the finding that synaptic NMDAR also contributes to excitotoxicity (Wroge et al., 2012). Finally, it is important to note that most of the studies on the distinct contributions made by synaptic and extrasynaptic NMDAR to neuronal death and survival took advantage of the pharmacological properties of MK-801, which is a high-affinity uncompetitive antagonist used to pre-block synaptic NMDAR for a prolonged period of time, and memantine (Milnerwood et al., 2010; Okamoto et al., 2009), which is a low-affinity uncompetitive antagonist thought to act specifically on extrasynaptic NMDARs (Leveille et al., 2008; Okamoto et al., 2009; Xia et al., 2010). However, recent work has questioned the duration of the synaptic block by MK-801 under different experimental paradigms (McKay et al., 2013) and the specificity of memantine at the doses used in previous studies (Wroge et al., 2012; Zhou et al., 2013). Several questions remain unanswered and warrant future research. How does the NMDAR exert location-specific output? Is the location of synaptic NMDAR mandatory for its promotion of neuronal survival? Does extrasynaptic NMDAR induce neuronal death as a result of its location? In light of the respective roles of synaptic and extrasynaptic NMDARs in neuronal survival and death, one important avenue of future research is to determine the mechanisms by which the location of these receptors promotes their coupling to specific downstream signaling proteins and biochemical cascades.

2.2.2. GluN2A versus GluN2B in neuronal death and survival

The molecular cloning of NMDAR subunits has provided an improved understanding of this receptor's pharmacology, electrophysiology, molecular biology, and cellular and physiological properties. The NMDAR is composed of an essential GluN1 subunit (also referred to as NR1) (Forrest et al., 1994; Moriyoshi et al., 1991; Yamazaki et al., 1992), and the ionotropic property of the receptor is fully established when it is coupled to at least one of the GluN2A–D subunits (otherwise known as NR2A–D) (Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992). The subunit composition of the NMDAR confers distinct channel kinetics, agonist affinity, and sensitivity to channel blockers (Ishii et al., 1993; Kutsuwada et al., 1992; Monyer et al., 1992), and NMDARs composed of different subunits are expressed in different brain regions (Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Watanabe et al., 1992). While the GluN1 subunit of the NMDAR is ubiquitously located in the adult brain, the forebrain is populated by NMDARs containing the GluN2A and the GluN2B subunits, and the cerebellum is populated by NMDARs containing the GluN2A and the GluN2C subunits (Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Watanabe et al., 1992). Importantly, different NMDAR subunits can play distinct physiological roles. Genetic deletion of GluN1 in mice completely abolishes NMDAR activity, and the animals die soon after birth (Forrest et al., 1994). Although GluN2A-knockout mice are viable, they display impaired synaptic plasticity, such as long-term potentiation (LTP) of synaptic efficacy, and poor spatial memory formation (Sakimura et al., 1995). In comparison, GluN2B-knockout mice are less viable (Kutsuwada et al., 1996), except when the knockout is specific to the pyramidal neurons of the cortex and the CA1 hippocampus (Brigman et al., 2010), and they display impaired long-term depression (LTD) and poor neuronal development (Brigman et al., 2010; Kutsuwada et al., 1996). In addition, many of the physiological roles of the NMDAR subunits depend on their large and distinct cytoplasmic tails, and genetic deletion of these C-terminal domains induces many of the features

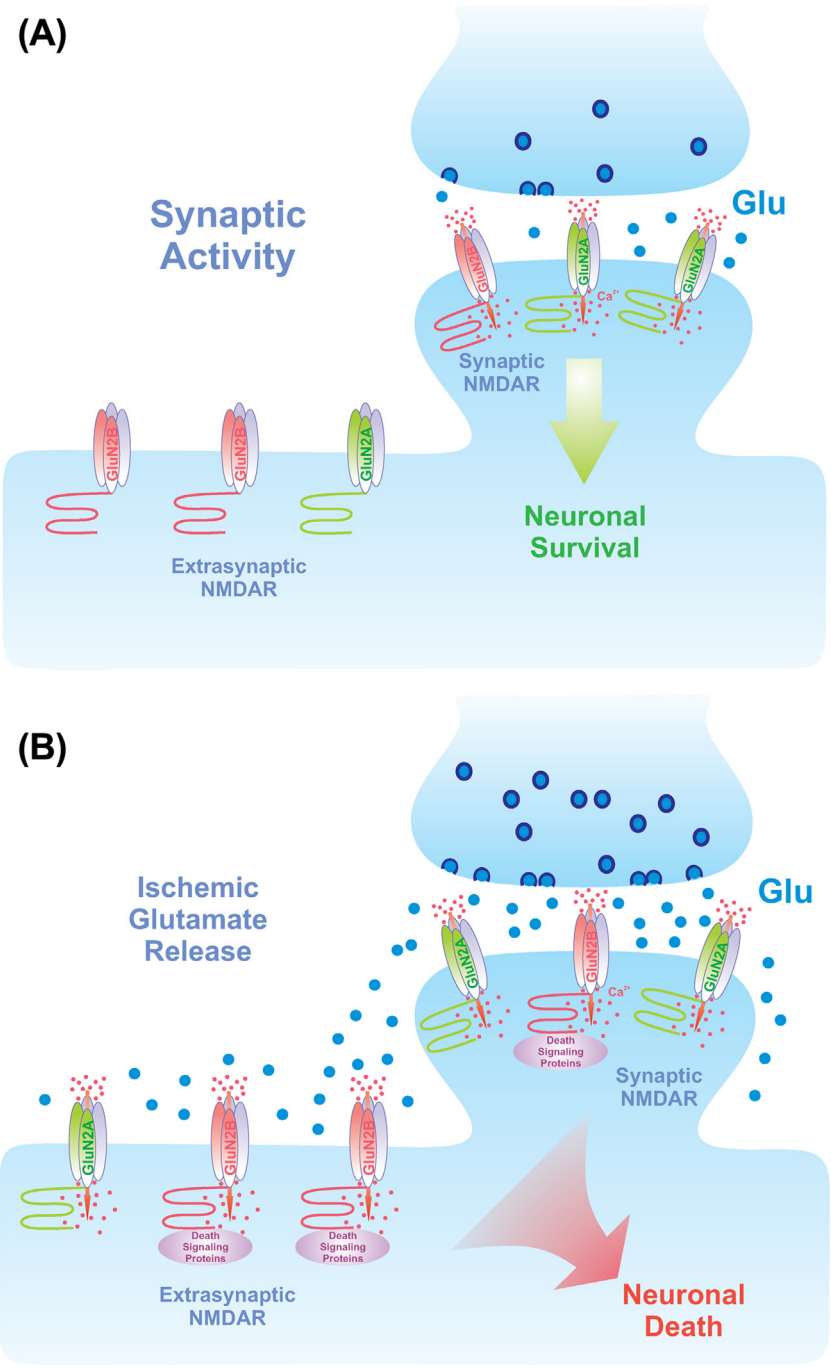

Fig. 1. Distinct subpopulations of the NMDA receptor (NMDAR) mediate neuronal death and survival. (a) Under normal conditions, synaptic activity maintains neuronal survival via activation of the synaptic NMDAR. This pro-survival effect is dependent on the calcium influx through the receptors. (b) During cerebral ischemia, excessive release of glutamate into the synapses and extrasynaptic sites causes global stimulation of NMDAR at both locations. The C-terminal domain of the GluN2B subunit acts as a major hub for recruiting death-signaling proteins, which in turn is activated by calcium influx through the receptors to induce neuronal death.

that are observed after genetic knockout of the entire receptor (Sprengel et al., 1998). The protein machinery required for synaptic plasticity, including proteins required for LTP and LTD, have been demonstrated to interact directly with the C-terminal domains of the GluN2A and GluN2B subunits of the NMDAR (Kim et al., 2005; Krapivinsky et al., 2003).

The subunit composition of NMDARs is specific not only to different regions of the brain but also to different subcellular locations (Harney et al., 2008; Liu et al., 2007; Stocca and Vicini, 1998; Thomas et al., 2006; Tovar and Westbrook, 1999) and changes dynamically throughout the process of brain development (Flint et al., 1997; Monyer et al., 1994; Sheng et al., 1994; Watanabe et al., 1992) and during responses to physiological stimuli (Bessho et al., 1994; Harney et al., 2008). In particular, the cortical and hippocampal neurons in the neonatal forebrain express GluN2B-containing NMDAR (GluN2BR) in both synapses and extrasynaptic sites, whereas the cortical neurons in the adult brain display an increased synaptic population of the GluN2A-containing NMDAR (GluN2AR) (Flint et al., 1997; Kirson and Yaari, 1996; Liu et al., 2007; Stocca and Vicini, 1998; Thomas et al., 2006; Tovar and Westbrook, 1999). By the adult age of P39–P92, the rat hippocampus displays a profound reduction in the proportion of functional GluN2BR, as measured by the degree of inhibition by selective antagonists (Harris and Pettit, 2007). Most importantly,

the specific localization of NMDAR subunits can be a mechanistic determinant of the differential effects of synaptic versus extrasynaptic receptors. For example, synaptic and extrasynaptic NMDARs have been demonstrated to govern different directions of synaptic plasticity, with synaptic NMDARs mediating the LTP of synaptic efficacy and extrasynaptic NMDARs mediating the opposing LTD (Lu et al., 2001). Notably, this finding can be partially explained by the different NMDAR subtypes that are present in these locations, with the GluN2AR subtype mediating the LTP and the GluN2BR subtype mediating the LTD (Dalton et al., 2012; Foster et al., 2010; Fox et al., 2006; Li et al., 2006; Liu et al., 2004; Massey et al., 2004) (but see also Berberich et al., 2005; Frizelle et al., 2006; Neyton and Paoletti, 2006; Weitlauf et al., 2005 for issues raised against the GluN2AR-specificity of NVP-AAM007 used in these and subsequent studies). Consistent with this finding, the differential roles of synaptic and extrasynaptic NMDARs in neuronal death and survival can also be partially explained by the presence of distinct NMDAR subunits in these sub-cellular locations (Fig. 1A and B). Selective GluN2BR antagonists are highly neuroprotective in models of cerebral ischemia and other neurodegenerative diseases (Chen et al., 2008; DeRidder et al., 2006; Gotti et al., 1988; Graham et al., 1992; Liu et al., 2007; O'Donnell et al., 2006; von Engelhardt et al., 2007; Zhou and Baudry, 2006), whereas selective GluN2AR antagonists are poorly neuroprotective and in several studies, exacerbated neuronal death (Chen et al., 2008; DeRidder et al., 2006; Liu et al., 2007). Importantly, in the adult rat forebrain, the GluN2AR and GluN2BR NMDAR subtypes can confer neuronal survival and neuronal death, respectively, independently of their subcellular location. Thus, synaptic GluN2BR is sufficient for the induction of neuronal death, and synaptic GluN2AR protects neurons against excitotoxic neuronal death mediated by synaptic GluN2BR (Liu et al., 2007). Similarly, extrasynaptic GluN2AR is pro-survival and protects neurons against extrasynaptic GluN2BR-induced and staurosporine-induced neuronal death (Liu et al., 2007). Consistent with the idea that NMDARs exert their subtype-specific outputs via their distinct C-terminal domains (Foster et al., 2010; Sprengel et al., 1998), replacing the C-terminus of GluN2B with that of GluN2A prevents GluN2BR-mediated excitotoxicity in vitro and in vivo (Martel et al., 2012). In contrast, replacing the C-terminus of GluN2A with that of GluN2B converts the pro-survival GluN2AR into an excitotoxic receptor (Martel et al., 2012). Taken together, these findings indicate that GluN2AR in the adult neuronal synapse confers activity-dependent neuronal survival signaling, while GluN2BR in extrasynaptic sites contributes to excitotoxicity following stroke and other neurodegenerative diseases (Fig. 1A and B).

2.3. Excitotoxicity as the primary mechanism of ischemic damage

While many of the early studies concerning excitotoxicity focused on public concerns about food safety, the search for pharmacological inhibitors of the NMDARs becomes especially motivating when mountains of evidence begin to point to the major role of excitotoxicity in stroke and other neurodegenerative diseases. The first link between glutamate and stroke damage was reported in 1959, when Van Harreveld found that topical application of glutamate, which is abundant in brain extracts, induced spreading depression in the rabbit brain. Based on this observation, he proposed that glutamate might be involved in similar cortical changes following stroke (Van Harreveld, 1959). Subsequent studies have provided unequivocal evidence that glutamate-mediated excitotoxicity is a primary contributor to ischemic neuronal death. First, newly cultured hippocampal neurons lacking synapses are resistant to oxygen deprivation for up to 24 h, whereas mature cultured neurons with intact synaptic connections degenerate abruptly upon oxygen deprivation (Rothman, 1983). Importantly, attenuating synaptic activity, by either reducing presynaptic release with tetrodotoxin or magnesium chloride or inhibiting ionotropic glutamate receptors with the antagonist gamma-D-glutamylglycine, prevents anoxic neuronal death in mature cortical neurons (Rothman, 1983, 1984). These data provide strong evidence that the synaptic release of glutamate is required for anoxic damage. Second, intracerebral concentrations of glutamate and aspartate increase abruptly in rats subjected to cerebral ischemia (Benveniste et al., 1984; Drejer et al., 1985; Hagberg et al., 1985), partially due to facilitated release of the transmitters (Bosley et al., 1983) and to the dysfunction of their uptake transporters (Drejer et al., 1985; Silverstein et al., 1986). This 8-fold increase in extracellular neurotransmitter release provides an explanation for the observation that the abundant central neurotransmitter glutamate is normally benign in the brain but induces excitotoxicity during cerebral ischemia. Consistent with this finding, the removal of glutamatergic afferent neurons attenuates neuronal death in rats subjected to cerebral ischemia (Buchan and Pulsinelli, 1990; Johansen et al., 1986; Jorgensen et al., 1987; Onodera et al., 1986). Third, intracerebral injection of the NMDAR blocker 2-amino-7-phosphonoheptanoic acid into the rat brain prevents neuronal death following cerebral ischemia (Simon et al., 1984), providing the first clear evidence that glutamate, particularly its action on the NMDARs, is required for ischemic brain damage. This finding has been replicated extensively (Germano et al., 1987; Gill et al., 1987; Goldberg et al., 1987; Ikonomidou et al., 1989a; McDonald et al., 1987; Olney et al., 1989a; Rothman et al., 1987; Simon et al., 1986; Steinberg et al., 1988, 1989; Weiss et al., 1986), and one of the most important implications of this line of work is that drugs targeting the NMDAR or the downstream signaling cascade are effective against stroke damage. The enthusiasm is demonstrated by the large number of clinical trials since (Tables 1 and 2).

2.3.1. Ischemic glutamate release and its inhibition

The first step toward excitotoxicity during an acute episode of stroke is the rapid elevation of glutamate levels in the ischemic region of the brain (Benveniste et al., 1984; Dawson et al., 2000; Drejer et al., 1985; Globus et al., 1988; Hagberg et al., 1985; Mitani et al., 1990) (Fig. 1B). Thus, the inhibition of ischemic glutamate release could confer neuroprotection by terminating multiple downstream death signaling cascades at their converging upstream initiation point. The mechanism by which glutamate is released is likely multi-factorial and depends on the severity of stroke and the ischemic model used (Dawson et al., 2000; Drejer et al., 1985; Rossi et al., 2000). This phenomenon can involve synaptic release of glutamate due to excessive neuronal activity and action potentials (Dawson et al., 2000; Drejer et al., 1985) or potentiated release efficacy (Bosley et al., 1983). In vitro, hypoxic neuronal death can be inhibited by either tetrodotoxin (TTX), an inhibitor of voltage-gated sodium channels that inhibits action potentials, or magnesium, which inhibits synaptic glutamate release (Rothman, 1983). As a result, a number of sodium channel blockers, including BW1003C87 and sipatrigine (BW619C89), have been designed to prevent ischemic glutamate release, and these molecules effectively reduce the elevation of glutamate levels and the neuronal death that follow cerebral ischemia in vivo (Gaspary et al., 1994; Graham et al., 1993; Leach et al., 1993; Lekieffre and Meldrum, 1993; Meldrum et al., 1992; Okiyama et al., 1995). In addition, magnesium ions and the N-type calcium channel antagonist ziconotide (SNX-111; Prialt), which both prevent synaptic glutamate release, are efficacious against cerebral ischemic damage (Okiyama et al., 1995; Valentino et al., 1993). Ischemic glutamate release can also involve the impairment or reversal of the glutamate uptake system, resulting in spillover

Table 1
Inhibiting glutamate release in stroke patients.

	Clinical trial	Patient population	Outcome
Na^+ channel blocker			
Lifarizine (RS-87476)	Phase II	117	Treatment within 12 h of ischemic stroke results in favorable trend toward improvement in mortality and morbidity. The treatment was well tolerated, with no serious side effects. Squire et al. (1995)
Sipatrigine (BW619C89)	Phase II	48 + 27	Treatment within 12 h of stroke failed to improve outcome compared to placebo. Several side effects were observed: nausea and vomiting, irritation at the injection site, reduced consciousness, agitation, confusion, and hallucination. Muir et al. (1995, 2000)
N-type Ca^{2+} channel blocker			
Ziconotide (SNX-111; Prialt)			Approved for the treatment of chronic pain. Status of the stroke clinical trial is unclear.
Magnesium sulfate	Phase III	2589	Treatment within 12 h of acute stroke failed to improve mortality and morbidity. The treatment was well tolerated, with no serious side effects. Muir et al. (2004)

and/or the elevation of extrasynaptic glutamate levels from glial (Dawson et al., 2000; Drejer et al., 1985; Phillis et al., 2000) or neuronal origins (Drejer et al., 1985; Phillis et al., 2000; Rossi et al., 2000; Sanchez-Prieto and Gonzalez, 1988; Silverstein et al., 1986). In addition to their role in ischemic glutamate release, these glutamate uptake transporters are also essential for glutamate re-uptake and clearance during normal synaptic transmission and following ischemia–reperfusion (Colleoni et al., 2008). Thus,

Table 2
Antagonism of NMDA receptor in stroke patients.

	Clinical trial	Patient population	Outcome
Competitive: glutamate-binding site			
Selfotel (CGS 19755)	Phase II	389 + 87	Treatment within 6 h of acute ischemic stroke increased mortality due to brain insult and failed to improve morbidity. Several side effects were observed: agitation, hallucinations, confusion, paranoia, and delirium. Davis et al. (1997) and Grotta et al. (1995)
Midafotel (CPPene; SDZ EAA 494)	Preliminary trial with seizure patients		Intolerable side effects were observed: poor concentration, sedation, ataxia, dysarthria, depression, and amnesia. Sveinbjornsdottir et al. (1993)
Competitive: glycine-binding site			
Licostinel (ACEA 1021)	Phase I	64	Treatment within 48 h of ischemic stroke failed to improve outcome compared to placebo, despite reaching plasma concentration optimal for neuroprotection in animal studies. The treatment was well tolerated, with no serious side effects. Albers et al. (1999)
Gavestinel (GV150526)	Phase III	109 + 1804 + 1367	Treatment within 6 h of acute stroke failed to improve mortality and morbidity. The treatment was well tolerated, with no serious side effects. Anon. (2000), Lees et al. (2000) and Sacco et al. (2001)
Uncompetitive or non-competitive			
Aptiganel (CNS 1102)	Phase II/III	628	Treatment within 6 h of hemispheric ischemic stroke exacerbated mortality and morbidity. Several side effects were observed: hypertension, ventricular arrhythmia, cerebral edema, stupor, and confusion. Albers et al. (2001)
AR-R15896AR	Phase II	175 + 103	Treatment within 12 or 24 h of acute stroke had no effect on morbidity (preliminary finding). Several side effects were observed: dizziness, vomiting, nausea, fever, stupor, agitation, and hallucination. Diener et al. (2002) and Lees et al. (2001a)
Dextrorphan HCl (Ro 01-6794/706)	Phase I	67	Treatment within 48 h of ischemic stroke had no effect on morbidity (preliminary finding). Several side effects were observed: nystagmus, somnolence, nausea, vomiting, change in blood pressure, agitation, confusion, and hallucination. Albers et al. (1995)
Remacemide HCl	Phase II	61	The optimal neuroprotective dose cannot be achieved in early hours with the maximum well-tolerated dose. Several side effects were observed: nausea and vomiting, irritation at the injection site, headache, dizziness, tremor, agitation, confusion, somnolence, and hallucination. Dyker and Lees (1999)

chronic inhibition or knockdown of the neuronal and/or the glial uptake system induces excitotoxicity (Rothstein et al., 1993, 1996). In addition, gene-deletion of the glial glutamate transporters GLT-1 and GLAST exacerbates ischemic neuronal death (Rao et al., 2001) and other forms of excitotoxicity (Tanaka et al., 1997; Watase et al., 1998), while increased expression of GLT-1 protects neurons against ischemic injury (Rothstein et al., 2005). Importantly, the new transporter inhibitor hydroxy-tetrahydro-pyrrolo-isoxazole carboxylic acid (HIP), which selectively inhibits reverse glutamate uptake rather than glutamate uptake (Funicello et al., 2004), is protective against ischemic neuronal death (Colleoni et al., 2008). In comparison, the general glutamate transporter inhibitor DL-threo-benzyloxyaspartic acid (DL-TBOA) and nonselective high doses of HIP are not neuroprotective and can exacerbate ischemic neuronal death (Colleoni et al., 2008).

The inhibition of ischemic glutamate release allows for the termination of ischemic stroke-induced excitotoxicity at its most upstream initiation point. In addition, these agents have the benefit of inhibiting ischemic glutamate release by blocking reverse uptake while potentiating physiological glutamate transmission by blocking glutamate uptake. Unlike NMDAR antagonists, which produce neurotoxicity in some non-ischemic brain regions like the cingulate and the retrosplenial cortex, these agents are considerably safer (Graham et al., 1993). However, no feasible treatment candidates from this line of drugs have passed the rigorous clinical testing required for broad clinical use (Table 1). Lifarizine (RS-87476), which decreases glutamate release by modulating voltage-gated sodium and calcium currents, has shown promise in Phase II trials when administered to patients within 12 h of acute stroke (Squire et al., 1995). However, no follow-up studies have been reported. The previously mentioned sodium channel blocker sipatrigine caused nausea, vomiting, and severe neurological side effects in Phase II stroke trials, preventing further clinical investigations (Muir et al., 1995, 2000). Magnesium ions, which can both hinder presynaptic glutamate release and antagonize the NMDAR uncompetitively, failed to reduce mortality or morbidity when administered to 2589 patients within 12 h of stroke in a Phase III trial (Muir et al., 2004). The N-type calcium channel antagonist ziconotide, which protects the rat brain against cerebral ischemia by reducing ischemic glutamate release (Valentino et al., 1993), has been approved and marketed for the treatment of chronic pain, but its status in stroke clinical trials remains unclear.

2.3.2. NMDAR antagonists and calcium channel blockers

The critical role of NMDAR-mediated calcium influx in stroke pathogenesis suggests that NMDAR antagonists and post-synaptic calcium channel blockers may be effective against stroke (Choi, 1995). Notably, calcium accumulation occurs before and is a strong predictor of neuronal death following cerebral ischemia (Deshpande et al., 1987). Thus, protection against neuronal hypoxia in vitro and cerebral ischemia in vivo can be achieved by direct NMDAR antagonism (Goldberg et al., 1987; Rothman et al., 1987; Simon et al., 1984; Weiss et al., 1986), antagonism of the AMPA receptor, which is required for NMDAR activation (Sheardown et al., 1990), simultaneous antagonism of both the NMDAR and AMPA receptor (Germano et al., 1987; Goldberg et al., 1987; Rothman, 1984; Simon et al., 1986), antagonism of the L-type calcium channels, which can contribute to calcium overload (Uematsu et al., 1989), and intracellular calcium chelation (Tymianski et al., 1993c). In general, direct NMDAR antagonism appears to be sufficiently neuroprotective against hypoxic neuronal death compared to co-antagonism of different excitotoxic receptors (Goldberg and Choi, 1993; Goldberg et al., 1987) and calcium sources (see Section 2.2). It is worth mentioning that ketamine, phencyclidine, and dextromethorphan, three drugs that are commonly used in humans, are uncompetitive NMDAR antagonists that are neuroprotective in models of ischemic/anoxic neuronal death (Goldberg et al., 1987; Rothman et al., 1987; Steinberg et al., 1988, 1989; Weiss et al., 1986). The drug MK-801 is a potent and selective uncompetitive NMDAR antagonist that readily crosses the blood–brain barrier, allowing systemic administration (Foster and Wong, 1987; Wong et al., 1986). Notably, MK801 is one of the most widely studied NMDAR antagonists in neurodegeneration research, and has been widely demonstrated to be effective in in vitro and in vivo models of ischemic/anoxic neuronal death (Gill et al., 1987; Goldberg and Choi, 1993; Ikonomidou et al., 1989a; Lo et al., 1994; McDonald et al., 1987; Olney et al., 1989a; Park et al., 1988). Two other notable compounds are the NMDAR glycine-binding site antagonists gavestinel (GV150526) and licostinel (ACEA1021) (Bordi et al., 1997; Woodward et al., 1995), which were developed with the intent of reducing the side effects that are observed with conventional NMDAR antagonists that target the glutamate-binding site or the NMDAR channel pore (Chiamulera et al., 1990; Hargreaves et al., 1993). Like conventional NMDAR antagonists, gavestinel and licostinel are strongly protective against ischemic neuronal damage in vivo (Bordi et al., 1997; Pearlstein et al., 1998; Reggiani et al., 2001; Takaoka et al., 1997; Warner et al., 1995). Taken together, the experimental evidence demonstrates the potential for NMDAR antagonism, whether it is competitive or uncompetitive or occurs via inhibition of the glutamate-binding site, the glycine-binding site, or the channel pore, in the treatment of ischemic stroke damage.

Like the drugs designed to prevent ischemic glutamate release (see Section 2.3.1), none of the drugs designed to target the NMDAR directly have been approved for clinical use in stroke treatment, and several of these compounds have failed clinical studies in unexpected ways (Table 2). The reason for this lack of clinical success is likely multi-factorial. As previously reported (Lai et al., 2011), two possible explanations are that (1) NMDAR blockers produce many side effects due to the prevalence of neurological functions requiring these receptors and (2) NMDAR blockers have a short therapeutic window for drug administration, as they are effective only when administered before or immediately following stroke. A major drawback of NMDAR antagonists is their side effects, including the dysfunction of neurological processes that require the NMDAR and the reversible induction of pathology related to neuronal morphology. Indeed, a number of NMDAR antagonists that were under development have been discontinued from further human clinical studies, partially due to concerns about pathomorphological changes that were observed in neurons from animals treated with the earlier experimental compounds like MK-801 and phencyclidine (Olney et al., 1989b, 1991). Consistent with findings obtained in animal studies, NMDAR antagonists that bind to the glutamate-binding site and the channel pore cause sedation and psychotomimetic side effects in human subjects (Albers et al., 1995; Diener et al., 2002; Dyker and Lees, 1999; Grotta et al., 1995; Lees et al., 2001a; Sveinbjornsdottir et al., 1993). In some studies, the optimal neuroprotective plasma concentration of NMDAR antagonists cannot be achieved in a timely manner when they are administered to patients at the maximum well-tolerated dose (Dyker and Lees, 1999; Sveinbjornsdottir et al., 1993). In other studies, the optimal neuroprotective plasma concentration that was observed in animal models of stroke is achieved in human subjects, but clinical efficacy is lacking. This finding may be due to the delayed treatment that is typical in clinical settings (Albers et al., 1999; Diener et al., 2002). Antagonists that target the NMDAR glycine-binding site are well-tolerated and lack the above mentioned side effects, including pathomorphological changes and neurological dysfunction, in both animal studies (Chiamulera et al., 1990; Hargreaves et al., 1993)

Table 3
Inhibiting GluN2B-containing NMDA receptors and the death-signaling cascades downstream of the NMDA receptor in stroke patients.

	Clinical trial	Patient population	Outcome
GluN2B/NMDAR antagonist			
Eliprodil (SL-82.0715)	Phase III	114	Trial suspended due to lack of efficacy, and side effects make neuroprotective concentrations unlikely. Concerns with electrocardiographic effects. Reviewed by Doble (1999) and Lees (1997)
Traxoprodil (CP-101,606)	Phase II	53 + 30 + 404	Open-label: treatment within 12 h of traumatic brain injury or hemorrhagic stroke showed above-average outcome. Bullock et al. (1999) Double-blind: treatment within 12 h of traumatic brain injury or hemorrhagic stroke showed no difference between drug and placebo. Merchant et al. (1999) Double-blind: treatment within 8 h of traumatic brain injury improved mortality and morbidity at 6 months. Yurkewicz et al. (2005) In all studies, the treatment was well tolerated, with no serious side effects
GluN2B-PSD95–nNOS interaction			
NA-1	Phase II	185	Treatment initiated after the completion of endovascular aneurysm repair surgery resulted in significantly fewer ischemic infarction (iatrogenic stroke). The treatment was well tolerated, with no serious side effects. Hill et al. (2012)
Multiple mechanisms			
Lubeluzole	Phase III	721 + 725 + 1786	Treatment within 6 h of acute ischemic stroke had no effect on mortality and morbidity. The treatment was well tolerated. Diener (1998), Diener et al. (2000) and Grotta (1997)

and clinical trials (Anon., 2000; Albers et al., 1999; Lees et al., 2001b; Sacco et al., 2001). However, patients receiving these compounds demonstrated no improvement in stroke outcome (Anon., 2000; Albers et al., 1999; Lees et al., 2001b; Sacco et al., 2001). Thus, it appears that among experimental compounds that target the NMDAR, the side effects or lack of side effects that are observed in animal studies are a strong predictor of clinical side effects and drug tolerance, but the neuroprotective efficacy that is observed in animal models of stroke has failed to translate into clinical efficacy.

2.3.3. Selective GluN2BR antagonists and targeting NMDAR signaling proteins

A hallmark of conventional NMDAR antagonism is the generation of psychomimetic side effects, which are a major cause of clinical failure. In addition, as in animal studies (Ikonomidou et al., 2000; Liu et al., 2007), NMDAR antagonism can worsen clinical stroke outcome in some trials (Albers et al., 2001; Davis et al., 1997; Lees et al., 2001b). Given the pivotal role of the GluN2AR in neuronal function, including the LTP of synaptic efficacy (Liu et al., 2004; Massey et al., 2004; Sakimura et al., 1995) and NMDAR-mediated neuronal survival signaling (see Section 2.2.2), there may be several advantages of selectively inhibiting the GluN2BR and its downstream death signaling pathways. In particular, ifenprodil and related compounds are a distinct class of NMDAR antagonists that act non-competitively at the GluN2BR with high subunit-specificity (Fischer et al., 1997; Gallagher et al., 1996; Kew et al., 1996; Malherbe et al., 2003; Priestley et al., 1994; Williams, 1993; Williams et al., 1993). These GluN2B-selective compounds have been shown to be effective against ischemic neuronal death in vitro and in vivo (Chen et al., 2008; DeRidder et al., 2006; Gotti et al., 1988; Graham et al., 1992; Liu et al., 2007; O'Donnell et al., 2006; von Engelhardt et al., 2007; Zhou and Baudry, 2006), and they are not associated with the neurological side effects that are commonly seen with conventional NMDAR antagonists (Blanchet et al., 1999; Jackson and Sanger, 1988). In addition, like glycine-binding site antagonists, these compounds do not induce the pathomorphological changes in the rat brain that are characteristic of conventional NMDAR antagonists (Duval et al., 1992). Two ifenprodil derivatives, eliprodil (SL-82.0715) and traxoprodil (CP-101,606), have been demonstrated to cause no neurological side effects, and traxoprodil has been demonstrated to have therapeutic efficacy in small double-blinded placebo-controlled Phase II studies (Bullock et al., et al., 1999; Merchant et al., 1999; Yurkewicz et al., 2005). A Phase III trial for this drug is currently underway (Table 3). While the clinical use of eliprodil in stroke management can be of cardiovascular concern due to its nonspecific effects on other ion channels, traxoprodil has been found to be safe and tolerable (Bullock et al., 1999; Merchant et al., 1999; Yurkewicz et al., 2005).

Although selective inhibitors of the pro-death GluN2BR circumvent nonspecific blockade of the pro-survival GluN2AR and appear to lack the clinical side effects seen with conventional NMDAR antagonists, these treatments could have clinical limitations for similar reasons. First, in addition to their role in excitotoxicity, these pro-death receptors have important physiological functions, such as their roles in the extinction of fear memory, the manifestation of stress responses, and the learning of new memory. Thus, even with the most selective GluN2BR antagonists, some adverse neurological effects may be overlooked when low doses are used in small clinical trials that would manifest when larger (and more effective) doses are used to treat a larger patient population. Second, like nonspecific NMDAR blockers, ifenprodil-related drugs are only effective when administered immediately following stroke, and they quickly lose their efficacy when treatment is delayed. In an animal model of focal ischemic stroke, the highly selective GluN2BR antagonist Ro 25-6981 is neuroprotective when administered prior to, but not 4.5 h after, the onset of ischemia (Liu et al., 2007). This limited time window for effective drug administration makes NMDAR antagonists, whether or not they are selective for the GluN2B-containing NMDAR subtype, therapeutically impractical. In an effort to circumvent these limitations, recent studies have attempted to target death signals downstream of the NMDAR, rather than the receptor itself (see Section 4 and Table 3). Because there are many cellular signaling proteins downstream of the NMDAR, it is

possible to differentiate between the signaling proteins that are responsible for NMDAR-mediated excitotoxicity and those that are responsible for important physiological functions. By specifically targeting downstream death signaling proteins, and avoiding signaling proteins with other functions, it may be possible to develop therapeutics that are as effective as NMDAR blockers with fewer side effects. In addition, because of the sequential nature of signaling cascades, blocking downstream death signals may allow for a wider or delayed window for effective treatment compared to receptor blockers.

3. The NMDAR and neuronal survival signaling pathways

In the non-excitotoxic brain, the NMDAR is critical for neuronal development and the maintenance of neuronal survival post-development, and in the ischemic or traumatized brain, certain subpopulation of the NMDAR is neuroprotective (see Section 2.2) and plays an important role in the recovery of neurological functions. As discussed in Section 2.2, the NMDAR subpopulation in the synapse that contains the GluN2A subunit tends to promote neuronal survival, while the receptor subpopulation in extra-synaptic sites that contains the GluN2B subunit tends to promote neuronal death. This finding partially explains how this notorious death-associated receptor can be a major player in neuronal survival and neuroprotection under both physiological and pathological conditions. Thus, in some neurons cultured in vitro, NMDAR stimulation promotes neuronal survival (Balazs et al., 1988a,b, 1989; Didier et al., 1989), and NMDAR inhibition induces neuronal death (Brenneman et al., 1990a,b). Similarly, in the rodent brain in vivo, GluN2AR stimulation in particular and synaptic activity in general decreases spontaneous (physiological) neuronal death and protects the brain against excitotoxic injury (Chen et al., 2008; Liu et al., 2007; Yao et al., 2012; Young et al., 1999), while NMDAR inhibition induces neuronal death in the developing brain (Adams et al., 2004; Gould et al., 1994; Ikonomidou et al., 1999) and hinders recovery of the injured brain (Ikonomidou et al., 2000). Importantly, stimulation of the GluN2AR survival signaling pathway appears to be the primary mechanism by which ischemic preconditioning of the brain, a highly effective regimen for increasing the tolerance of the brain to subsequent ischemic injury, protects the brain against stroke damage (Chen et al., 2008; Terasaki et al., 2010), and direct stimulation or expression of the synaptic NMDAR in general or the GluN2AR in particular protects the brain from excitotoxic injury in rodent models of ischemic stroke (Liu et al., 2007; Yao et al., 2012).

3.1. Survival signaling by Akt

The NMDAR promotes neuronal survival via the activation of the protein kinase Akt (Bhave et al., 1999; Lafon-Cazal et al., 2002; Yano et al., 1998; Zhang et al., 1998). The cellular Akt (c-Akt), also known as protein kinase B (PKB) and protein kinase related to the A and C kinases (rac), is a serine threonine protein kinase that is homologous to the viral oncogene product v-Akt (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). One mechanism by which the NMDAR induces Akt activation is via calcium-dependent tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), which subsequently binds to and activates the well characterized pro-survival protein phosphatidylinositol 3-kinase (PI3K) (Zhang et al., 1998), which is critical for the phosphorylative activation of Akt (Alessi et al., 1996; Burgering and Coffer, 1995; Cross et al., 1995; Franke et al., 1995; Kohn et al., 1996) (Fig. 2). Active PI3K phosphorylates phospholipids in the cell membrane to generate phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P3), which bind to the pleckstrin homology (PH) domain of Akt, facilitating its dimerization and phosphorylative activation (Datta et al., 1995, 1996; Franke et al., 1995, 1997; Frech et al., 1997; Klippel et al., 1997; Stephens et al., 1998; Stokoe et al., 1997), which result in PI3K/Akt mediated neuronal survival signaling (Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kulik et al., 1997). Alternatively, the NMDAR can directly activate Akt via a non-canonical mechanism that involves calcium-dependent activation of the calcium–calmodulin dependent protein kinase

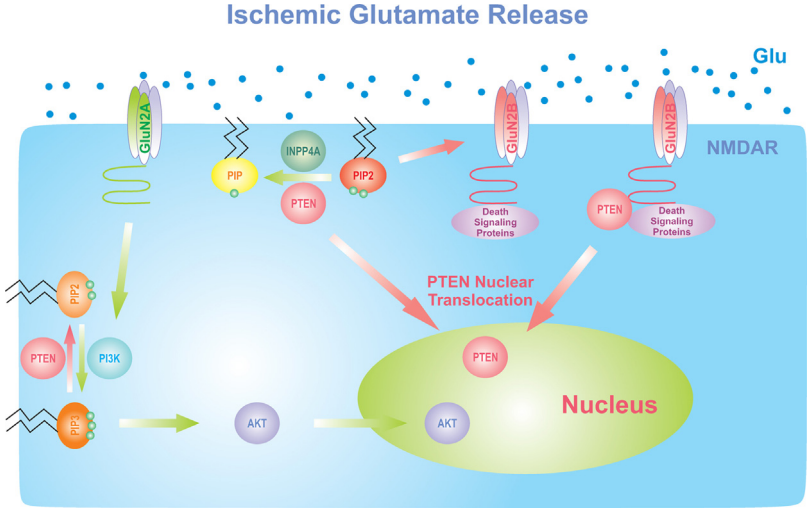

Fig. 2. The phospholipid pathways leading to neuronal death and survival. Synaptic activity induces neuronal survival in part by NMDA receptor (NMDAR)-mediated activation of the survival-promoting protein kinase Akt. Phosphorylative activation of Akt can occur via several pathways, and the best characterized one involves Akt binding to the phosphatidylinositol (3,4,5)-triphosphate (PIP3), a phospholipid product of phosphatidylinositol 3-kinase (PI3K) generated from the plasma membrane phosphatidylinositol (4,5)-bisphosphate (PIP2, *orange colored*). The activated Akt translocates into the nucleus, a process known to promote cell survival. During cerebral ischemia, the death-promoting protein lipid and protein phosphatase known as phosphatase and tensin homolog deleted on chromosome ten (PTEN) de-phosphorylates PIP3 into PIP2 to terminate Akt activity. PTEN also directly bind to the C-terminal domain of the GluN1 subunit in GluN2B-containing NMDARs to augment excitotoxicity, and translocates into nucleus to induce neuronal death in a GluN2B subunit-dependent manner. Recently, it is demonstrated that a different form of PIP2 (*red colored*), phosphatidylinositol (3,4)-bisphosphate, is excitotoxic by recruiting the death-promoting GluN2B subunit of the NMDAR into the synapse. Dephosphorylation of this bad PIP2 into phosphatidylinositol monophosphate (PIP) by inositol polyphosphate phosphatase 4A (INPP4A) is neuroprotective. Since this survival-promoting dephosphorylation can also be catalyzed by PTEN, this finding can in part explain the mechanism by which nuclear translocation of PTEN is excitotoxic.

kinase (CaM-KK) in a PI3K-independent manner (Yano et al., 1998). Consistent with the survival-promoting role of synaptic NMDAR, basal PI3K/Akt survival signaling depends on ongoing synaptic activity and activation of the synaptic NMDAR (Sutton and Chandler, 2002), and augmented PI3K/Akt activity can be triggered by increased synaptic NMDAR stimulation (Papadia et al., 2005; Soriano et al., 2006). Importantly, stimulation of the PI3K/Akt pathway via the NMDAR or other mechanisms is neuroprotective against hypoxic and excitotoxic neuronal death in vitro (Jo et al., 2012; Soriano et al., 2006; Yamaguchi et al., 2001) and ischemic neuronal death in vivo (Endo et al., 2006; Jo et al., 2012; Kawano et al., 2001; Noshita et al., 2003), and inhibition of the PI3K/Akt pathway exacerbates ischemic neuronal death (Endo et al., 2006; Noshita et al., 2001). In addition, cerebral ischemia induces neuronal death in part by inhibiting Akt activity (Kawano et al., 2001; Yano et al., 2001), and ischemic preconditioning confers resistance to further ischemic challenge by stimulating Akt (Miao et al., 2005; Yano et al., 2001).

Although the essential role of Akt in NMDAR-mediated neuronal survival signaling has been well established, the exact mechanism by which Akt promotes survival remains unclear. Several mechanisms by which PI3K/Akt promotes neuronal survival have been reported. For instance, Akt phosphorylates and thereby inhibits the death-signaling protein kinase glycogen synthase kinase-3 (GSK3) (Cross et al., 1995; van Weeren et al., 1998), and this is an important mechanism by which the NMDAR and PI3K/Akt promote survival of neurons and other cell types (Cross et al., 2001; Crowder and Freeman, 2000; Endo et al., 2006; Hetman et al., 2000; Pap and Cooper, 1998; Soriano et al., 2006). Consistent with the role of PTEN in terminating PI3K/Akt activity during excitotoxicity (Ning et al., 2004) (see also Section 3.1.1), GSK3 contributes to excitotoxic neuronal injury and inhibition of GSK3 protects neurons against excitotoxicity (Facci et al., 2003; French and Heberlein, 2009; Kelly et al., 2004). Other mechanisms by which PI3K/Akt promotes neuronal survival include Akt-mediated phosphorylation and inhibition of the death-signaling BCL-2 family member BAD (Datta et al., 1997; Noshita et al., 2002; Yano et al., 1998), the forkhead transcription factor FKHR (Abid et al., 2004; Kawano et al., 2002; Nakae et al., 1999, 2000; Soriano et al., 2006; Trotman et al., 2006), and the proline-rich Akt substrate (PRAS) (Kovacina et al., 2003; Saito et al., 2004). In addition, the finding that Akt translocates to the nucleus soon after its activation at the plasma membrane suggests that many of its targets are nuclear rather than cytosolic (Andjelkovic et al., 1997; Borgatti et al., 2003; Meier et al., 1997; Pekarsky et al., 2000). Nuclear Akt is required for its tumorigenic activity, and specific inhibition of nuclear Akt by dephosphorylation prevents tumorigenesis (Trotman et al., 2006). Finally, PI3K/Akt survival signaling may also be induced by BDNF (Bhave et al., 1999; Zheng and Quirion, 2004; Zhu et al., 2002). Thus, the NMDAR can induce the release of BDNF via the CREB-signaling pathway (see Section 3.2), indirectly inducing neuronal Akt activation. At least one study has demonstrated that appreciable Akt activation is observed only after prolonged NMDAR stimulation that is sufficient to induce BDNF expression and that NMDAR-mediated Akt activation is blocked by an antagonist to the BDNF receptor Trk (Bhave et al., 1999).

3.1.1. Inhibition of neuronal survival by nuclear and cytosolic PTEN

While the PI3K–Akt pathway mediates NMDAR survival signaling, the opposing phosphatase and tensin homolog deleted on chromosome ten (PTEN) pathway mediates NMDAR-induced death signaling. PTEN, also known as mutated in multiple advanced cancers 1 (MMAC1) and TGF-beta-regulated and epithelial cell-enriched phosphatase 1 (TEP1), is a tumor suppressor that is mutated in multiple types of cancer cells (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997) and mediates its apoptotic effects primarily via dephosphorylation of PtdIns(3,4,5)P3 at its 3-position, which terminates the PI3K–Akt signaling pathway (Maehama and Dixon, 1998; Myers et al., 1998; Stambolic et al., 1998). Consistent with its role opposing PI3K/Akt survival signaling, the introduction of PTEN inhibits cell growth and survival, and the mutation of PTEN confers cell resistance to a variety to apoptotic stimuli (Furnari et al., 1997; Groszer et al., 2001; Li et al., 2002; Stambolic et al., 1998). In addition, the expression of PTEN has been correlated with certain forms of neuronal death (Kyrylenko et al., 1999) and sensitizes neurons to excitotoxic neuronal death in vitro and in vivo (Gary and Mattson, 2002). Interestingly, PTEN forms a death-signaling complex with the NMDAR (Fig. 2). Consistent with the death-signaling role of GluN2BR, PTEN directly interacts with the cytoplasmic tail of the GluN1 subunit of GluN2BR but not that of GluN2AR (Ning et al., 2004). In contrast to the lipid phosphatase activity of PTEN that is known to terminate PI3K-to-Akt signaling, the protein phosphatase activity of PTEN is required for the potentiation of extrasynaptic NMDAR current (Ning et al., 2004). Knockdown of PTEN expression restores PI3K/Akt signaling and dampens extrasynaptic NMDAR current. These effects contribute to neuroprotection via independent mechanisms, leading to substantial protection against ischemic neuronal death in vitro and in vivo (Ning et al., 2004). Interestingly, while PTEN directly potentiates the GluN2BR-dependent neuronal death-signaling pathway that leads to neuronal injury, it can also indirectly potentiate the GluN2AR-mediated neuronal survival-signaling pathway via a PTEN-induced kinase 1 (PINK1)-dependent mechanism (Chang et al., 2010). In contrast to modulation of the NMDAR by PTEN, the NMDAR can also exert its effect directly by modulating PTEN nuclear translocation (Zhang et al., 2013; Zheng et al., 2012). Consistent with the recently reported critical role of nuclear PTEN in the induction of apoptosis and growth inhibition (Liu et al., 2005; Song et al., 2011; Trotman et al., 2007), excitotoxic stimulation of the NMDAR triggers nuclear translocation of PTEN in a GluN2B-dependent manner, resulting in a marked decrease in nuclear PtdIns(3,4,5)P3 and nuclear Akt phosphorylation in injured neurons (Zhang et al., 2013). In contrast, GluN2AR not only fails to induce PTEN nuclear translocation (Zhang et al., 2013) but also impairs the nuclear translocation of PTEN (Zheng et al., 2012). GluN2AR-mediated impairment of PTEN translocation can confer neuroprotection, in part via the up-regulation of nuclear TAR DNA-binding protein-43 (TDP-43) (Zheng et al., 2012). Likewise, the inhibition of PTEN nuclear translocation via the interference peptide Tat-K13 (K13: KEIVSRNKRRYQED), whose sequence flanks the critical lysine-13 mono-ubiquitination site that is required for PTEN nuclear translocation (Trotman et al., 2007; Walker et al., 2004), protected neurons from GluN2BR-mediated excitotoxic neuronal death (Zhang et al., 2013). Importantly, the Tat-K13 peptide, which is rendered membrane-permeable by the Tat sequence from the membrane transduction domain of the HIV1 Tat protein (Tat: GRKKRRQRRR), protected the rat brain from ischemic stroke damage even when administered up to 6 h post-ictus.

3.1.2. Excitotoxicity mediated by PtdIns(3,4)P2

In marked contrast to the neuronal survival signaling that is mediated by the PtdIns(3,4,5)P3-Akt pathway, the other PI3K product, PtdIns(3,4)P2, exerts excitotoxic effects by potentiating NMDAR-induced death signaling (Sasaki et al., 2010) (Fig. 2). Direct application of PtdIns(3,4)P2 or gene-deletion of the synapse-enriched PtdIns(3,4)P2 phosphatase inositol polyphosphate phosphatase 4A (INPP4A) renders neurons susceptible to NMDAR-mediated excitotoxicity, in part via recruitment of the death-promoting GluN2B subunit from the extrasynaptic site into the

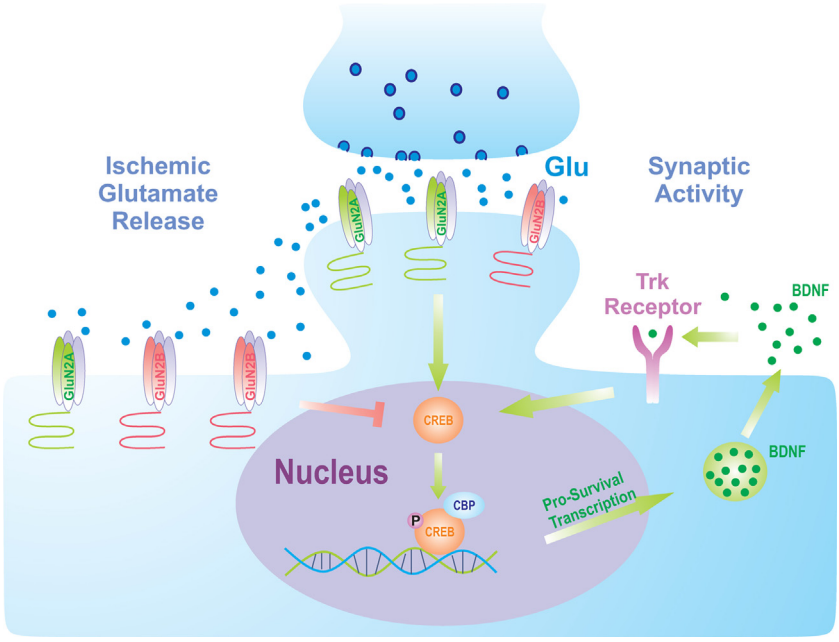

Fig. 3. Synaptic activity mediates prolonged neuronal survival via a positive feedback loop between CREB and BDNF. Synaptic activity promotes neuronal survival in part by NMDA receptor (NMDAR)-mediated phosphorylative activation of cyclic adenosine monophosphate response element (CRE) binding protein (CREB). The phosphorylated CREB binds to the CREB-binding protein (CBP) to regulate the transcription of CRE-responsive genes. One survival gene that is unregulated is brain-derived neurotrophic factor (BDNF), and BDNF release mediated by NMDAR stimulation and BDNF activation of the Trk receptor can further promote CREB activation. During cerebral ischemia, stimulation of the extrasynaptic NMDARs induces a CREB shut-off pathway, leading to neuronal death.

synapse. Potentially as a result of synaptic GluN2B recruitment, mice that lack INPP4A or express a mutant INPP4A that lacks phosphatase activity exhibit substantial neurodegeneration in the striatum and related behavioral deficits, even in the absence of any exogenous excitotoxic input (Sasaki et al., 2010). Interestingly, PTEN is also a phosphatase for PtdIns(3,4)P2, and this activity is comparable to its phosphatase activity for PtdIns(3,4,5)P3 (Myers et al., 1998). Given that PtdIns(3,4)P2 confers excitotoxicity via its effect on the synapse, this finding could partially explain why translocation of PTEN into the nucleus, and away from the synapse and the cytoplasm, is excitotoxic (see Section 3.1.1). Consistent with the death-signaling role of this non-canonical PI3K product, a recent study reported that PI3K is required for NMDAR-mediated and GluN2B-dependent stimulation of the NADPH oxidase and the production of superoxide (Brennan-Minnella et al., 2013). In this study, direct application of the supposedly pro-survival PtdIns(3,4,5)P3, which has no direct effect on superoxide production, facilitated NMDAR-mediated superoxide production independent of PI3K (Brennan-Minnella et al., 2013). Because PtdIns(3,4,5)P3 is also a substrate for the production of PtdIns(3,4)P2 (Lioubin et al., 1996), the apparently contradictory death-signaling effect of PtdIns(3,4,5)P3 could be due to its conversion to the excitotoxic PtdIns(3,4)P2.

3.2. CREB-mediated regulation of neuronal survival

NMDAR-mediated survival signaling is transcription-dependent, and the primary transcription factor involved in this process is likely the cyclic adenosine monophosphate response element (CRE) binding protein (CREB) (but see also NFI-A in Section 4.2.1). The activation of the NMDAR, particularly via synaptic activity, induces prominent activation of both CREB and its co-activator CREB-binding protein (CBP), resulting in the transcription of many CREB-responsive genes (Deisseroth et al., 1996; Hardingham et al., 2001a,b; Hardingham et al., 2002; Hu et al., 1999; Impey et al., 2002; Mabuchi et al., 2001; Monti et al., 2002) (Fig. 3). While the phosphorylative activation of CREB at serine-133 can occur via multiple mechanisms (Gonzalez and Montminy, 1989; Mayr and Montminy, 2001), its activation by the NMDAR involves (Hardingham et al., 2001a,b): (1) the faster-acting calmodulin kinase IV (CaMKIV) of the calmodulin-dependent protein kinase family (Sheng et al., 1991) and (2) the slower-acting ERK-mediated activation of the p90 ribosomal S6 kinase Rsk-2 (De Cesare et al., 1998; Xing et al., 1996). Thus, neuronal activity-dependent CREB activation depends on the CaMKIV pathway in the first hour, with the ERK pathway prolonging CREB activation by more than an hour (Hardingham et al., 2001a; Wu et al., 2001). The requirement for ERK in NMDAR-mediated CREB signaling raises the possibility that long-lasting NMDAR-mediated ERK survival signaling involves CREB as a major player. In addition, consistent with its long-lasting activity, neuroprotection after a brief episode of synaptic NMDAR activity is long-lasting, and the delayed-phase, but not the acute-phase, of neuroprotection is dependent on CREB signaling (Papadia et al., 2005). Consistent with the essential role of CBP in CREB-mediated transcription (Arias et al., 1994; Chrivia et al., 1993; Kwok et al., 1994), synaptic NMDAR activation induces a prominent increase in nuclear calcium, leading to the CaMKIV-mediated phosphorylative activation of CBP at serine-301, which promotes CREB/CBP dependent transcription (Chawla et al., 1998; Hardingham et al., 1997, 2001b; Hu et al., 1999; Impey et al., 2002). Most importantly, the activation of CREB and CREB-mediated transcription are required for NMDAR-dependent and other neurotrophic factor-dependent neuronal survival during development and in adulthood (Hansen et al., 2004; Lonze et al., 2002; Mantamadiotis et al., 2002; Monti et al., 2002) and can render neurons resistant to excitotoxicity and other apoptotic stimuli (Bonni et al., 1999; Hardingham et al., 2002; Mabuchi et al., 2001; Papadia et al., 2005; Riccio et al., 1999; Soriano et al., 2006; Walton et al., 1996, 1999). Finally, in addition to the set of genes that are directly regulated by CREB, CREB can also confer synaptic NMDAR-mediated neuronal survival signaling by regulating the expression

of other transcription factors. In particular, CREB induces the expression of activating transcription factor 3 (ATF3), which acts as a transcription repressor for death-signaling genes and thereby protects neurons against excitotoxic and ischemic neuronal death in vitro and in vivo (Zhang et al., 2011).

3.2.1. BDNF and other CREB-promoted gene products

Synaptic and extrasynaptic NMDARs affect the transcription of independent sets of largely non-overlapping genes and as such, mediate transcription-dependent neuronal survival and death, respectively (Lau and Bading, 2009; Leveille et al., 2010; Papadia et al., 2008; Qiu et al., 2013; Zhang et al., 2007). CREB appears to be the primary transcription factor involved in the transcriptional regulation of genes that are required for neuronal survival, and the inhibition of CREB signaling by the extrasynaptic NMDAR likely contributes to excitotoxicity (Hardingham et al., 2002; Tan et al., 2012). Among the pro-survival genes that are upregulated by CREB, BDNF has been extensively characterized, and it is critical to NMDAR/CREB-mediated neuronal survival signaling (Favaron et al., 1993; Hansen et al., 2004; Shieh et al., 1998; Tao et al., 1998). The transcription-dependent production of BDNF in the brain is neuronal activity-dependent and requires calcium influx through the NMDAR (Favaron et al., 1993; Ghosh et al., 1994; Zafra et al., 1991) or other calcium channels (Ghosh et al., 1994). The potentiation of synaptic activity by dis-inhibition of neurons and environmental enrichment promotes BDNF transcription (Hardingham et al., 2002; Young et al., 1999; Zafra et al., 1991), and inhibition of the NMDAR but not inhibition of the other ionotropic glutamate receptors prevents BDNF transcription (Favaron et al., 1993; Hardingham et al., 2002; Zafra et al., 1991). Consistent with the finding that distinct sub-populations of the NMDAR mediate neuronal death and neuronal survival (see Section 2.2), NMDARs in the synapse that contain the GluN2A subunit are primarily responsible for neuronal activity-dependent BDNF gene expression (Chen et al., 2007). In contrast, synaptic GluN2BR has no effect on BDNF gene expression (Chen et al., 2007), and extrasynaptic NMDAR has been shown to inhibit CREB-mediated BDNF gene expression (Hardingham et al., 2002). Importantly, the release of BDNF and subsequent activation of the Trk receptor can induce further BDNF production via an ERK-dependent signaling cascade (Bonni et al., 1999; Hu et al., 1999; Riccio et al., 1999), resulting in a positive-feedback loop that promotes long-lasting neuroprotection following a brief episode of NMDAR stimulation (Jiang et al., 2005). Consistent with the requirement for BDNF in NMDAR-mediated survival signaling, this neurotrophic factor exerts neuroprotective effects in vitro and in vivo (Alderson et al., 1990; Hofer and Barde, 1988; Hyman et al., 1991; Knusel et al., 1992; Morse et al., 1993; Widmer et al., 1993), and the absence of this factor leads to neurodegeneration in response to the depletion of NMDAR activity (Hansen et al., 2004). During focal ischemic stroke, BDNF is released into the brain in response to cerebral ischemia as a self-protective mechanism (Comelli et al., 1992), and this release requires activation of the NMDAR (Comelli et al., 1992), specifically the NMDAR subtype that contains the GluN2A subunit (Chen et al., 2008). Thus, exogenous administration of BDNF into the rat brain is strongly neuroprotective against focal ischemic stroke damage (Zhang and Pardridge, 2001).

4. The NMDAR and neuronal death signaling pathways

In view of the primary role of the NMDAR in excitotoxic neuronal injury following ischemic stroke and other neurodegenerative diseases, several pharmacological treatments have been developed to prevent excitotoxic glutamate release during cerebral ischemia (Table 1), to antagonize the NMDAR competitively or uncompetitively (Table 2), or to inhibit receptors that contain the GluN2B subunit (Table 3). These compounds target the NMDAR at the membrane surface receptor level (or at points further upstream), leading to a short time window for effective drug administration. Thus, drugs that target excitotoxic glutamate release are no longer effective once glutamate is released into the ischemic brain, and drugs that antagonize the NMDAR or other excitotoxic receptors are no longer effective once these receptors and their downstream death signaling proteins are activated. In addition to excitotoxic death signaling proteins, there are a variety of other NMDAR signaling proteins that are responsible for normal neuronal functions. These include signaling proteins with important roles in learning and memory, cognitive processing, motivation and rewards, and neuronal survival. As a result, NMDAR antagonists typically cause intolerable side effects, such as psychotomimetic effects, memory deficits, sedative effects, and exacerbation of stroke outcome, in both animal models and stroke patients (Tables 1–3). In the past decade, a number of studies have attempted to identify the precise death-signaling mechanisms or proteins that are downstream of the NMDAR and elucidate the mechanisms by which these downstream events contribute to excitotoxic neuronal death following ischemic stroke. By developing novel treatments that inhibit these downstream targets, it may be possible to overcome many of the clinical limitations that are observed with conventional treatments that target the NMDAR death-signaling cascade at the receptor level. Drugs that target delayed death-signaling events or proteins, specifically those that are activated several hours or days following stroke, can remain efficacious in the stroke clinic even when treatment is delayed. In addition, drugs that target death-signaling events or proteins that have no acute physiological functions should lack the severe side effects that are observed with conventional NMDAR blockers.

4.1. The cytoplasmic tail of the NMDAR

Because NMDAR death signaling requires an influx of calcium ions (see Section 2.1.1) and the NMDAR is more neurotoxic than other sources of calcium (see Section 2.1.2), it has been postulated that the excitotoxic neuronal death-signaling protein must be closely associated with, if not physically bound to, the NMDAR (Tymianski et al., 1993b). Consistent with this theory, the cytoplasmic tail of the NMDAR is a major hub for signaling proteins, and direct protein–protein interactions between the cytoplasmic tail of the NMDAR and its associated signaling proteins confer many of the known NMDAR functions (Kim et al., 2005; Krapivinsky et al., 2003; Li et al., 2006). In addition, genetic deletion of the NR2 cytoplasmic tail results in the subtype-specific loss of NMDAR function (Sprengel et al., 1998), and swapping the cytoplasmic tail of one NMDAR subunit with that of another NMDAR subunit modifies the functional output of the receptor accordingly (Foster et al., 2010). Importantly, swapping the cytoplasmic tail of GluN2A with that of GluN2B converts the pro-survival receptor into an excitotoxic receptor, while swapping the cytoplasmic tail of GluN2B with that of GluN2A prevents excitotoxicity in vitro and in vivo (Martel et al., 2012). Many neuronal death-signaling proteins are known to bind directly to the cytoplasmic tail of the NMDAR (Ning et al., 2004; Tu et al., 2010; Wang et al., 2003), and other death-signaling proteins associate with the NMDAR indirectly by binding the PSD95 (see Section 4.2) (Aarts et al., 2002; Sattler et al., 1999). These proteins include PTEN (see Section 3.1.2), which binds to the GluN1 subunit (Ning et al., 2004), cyclin-dependent kinase 5 (cdk5) (see Section 4.3.1), which binds to the GluN2A subunit (Wang et al., 2003), and death-associated protein kinase 1 (DAPK1) (see Section 4.1.1), which binds to the GluN2B subunit (Tu et al., 2010). Notably, the

binding of these neuronal death-signaling proteins to the NMDAR can be especially pronounced following cerebral ischemia (Tu et al., 2010; Zhou et al., 2010). This increased binding may partially explain the observation that the NMDAR is benign and promotes neuronal survival under normal conditions but becomes abruptly excitotoxic under pathological conditions, such as stroke (Fig. 1A and B). Importantly, disruption of the binding of NMDAR to death-signaling proteins using an interference peptide that mimics the amino-acid sequence of the NMDAR cytoplasmic tail prevents excitotoxic neuronal death following ischemia in vitro and in vivo, without affecting NMDAR channel activity or other downstream signaling proteins (Aarts et al., 2002; Sattler et al., 1999; Tu et al., 2010; Wang et al., 2003). By disrupting the excitotoxic output but not the functional output downstream of the NMDAR, these interference peptides have the potential to confer therapeutic efficacy in the stroke clinic without the intolerable neurological side effects that are observed with conventional NMDAR blockers that target surface receptors.

4.1.1. DAPK1 is the predominant protein recruited to the NMDAR

DAPK1 is of particular interest because a recent quantitative proteomic analysis of the death-signaling proteins that are recruited to the cytoplasmic tail of the NMDAR during cerebral ischemia revealed DAPK1 as the most prevalent protein (Tu et al., 2010) (Fig. 4). DAPK1 is a 160-kDa calmodulin-dependent serine/threonine protein kinase whose phosphorylation activity is known to be responsible for certain forms of apoptotic cell death (Cohen et al., 1997, 1999; Deiss et al., 1995; Kissil et al., 1997). Under basal conditions, inactive DAPK1 is subject to a negative-feedback loop that results in autophosphorylation at serine-308 of its calmodulin-binding domain, rendering DAPK1 insensitive to calmodulin-binding and preventing its death-promoting function (Shohat et al., 2001). In response to apoptotic stimuli, such as cerebral ischemia or NMDAR-mediated excitotoxicity (Shamloo et al., 2005), DAPK1 is dephosphorylated and its death-promoting activity resumes (Shamloo et al., 2005; Shohat et al., 2001). One mechanism by which cerebral ischemia induces activation of DAPK1 is via NMDAR-mediated calcineurin activation, which subsequently induces the dephosphorylation and activation of DAPK1, leading to ischemic neuronal death (Shamloo et al., 2005). Consistent with this finding, inhibition of the NMDAR or calcineurin prevents dephosphorylation of DAPK1 in cultured neurons in an in vitro model of ischemia, and inhibition of DAPK1 prevents ischemic neuronal death in vitro and in vivo (Shamloo et al., 2005). Interestingly, the pro-survival nuclear signaling factor ERK, which is downstream of the NMDAR, can directly interact with DAPK1, leading to ERK-mediated phosphorylation of DAPK1 at serine-735 and enhancing DAPK1 death-signaling activity (Chen et al., 2005). Thus, the NMDAR can also stimulate DAPK1 via the NMDAR–ERK signaling pathway (see Section 3.1.1) (Kim et al., 2005; Krapivinsky et al., 2003). Importantly, this DAPK1–ERK interaction causes ERK to be retained in the cytoplasm and prevents its pro-survival nuclear signaling, further enhancing the propensity for neuronal death (Chen et al., 2005). Once activated following cerebral ischemia, DAPK1 is recruited to the NMDAR, where it binds to the cytoplasmic tail of GluN2B at residues 1292–1304 (NR2B-CT: KKNRNKLRRQHSY) (Tu et al., 2010). This GluN2B–DAPK1 interaction leads to phosphorylation of GluN2B at serine-1303, which potentiates the NMDAR channel current. Disruption of this GluN2B–DAPK1 interaction using the interference peptide Tat-NR2B-CT, whose Tat sequence represents the membrane transduction domain of the HIV1 Tat protein to allow membrane permeability, prevents DAPK1-mediated GluN2B phosphorylation and potentiation of the NMDAR current in vitro and excitotoxic neuronal death in a mouse model of ischemic stroke in vivo (Tu et al., 2010). In addition, reversal of the ischemia-induced phosphorylation of serine-1303 by protein phosphatase 1 (PP1) also reverses the ischemic potentiation of NMDAR activity and results in neuroprotection in primary hippocampal neurons subjected to oxygen–glucose deprivation (Farinelli et al., 2012).

4.2. PSD95/nNOS: the nitric oxide pathway

In neurons, the NMDAR is particularly effective at inducing calcium/calmodulin-dependent nNOS-mediated production of nitric oxide (Garthwaite et al., 1988), and this effect is calcium source-specific (Kiedrowski et al., 1992). Several lines of biochemical analysis have elucidated the mechanism underlying this calcium source-specificity. The GluN2A–D subunit of the NMDAR binds directly to PSD95 and several other members of the membrane-associated guanylate kinase (MAGUK) family (Brenman et al., 1996b; Kornau et al., 1995; Muller et al., 1996; Niethammer et al., 1996) (Fig. 5A). PSD95 contains three PDZ domains for binding to PDZ ligands. The PDZ1 and the PDZ2

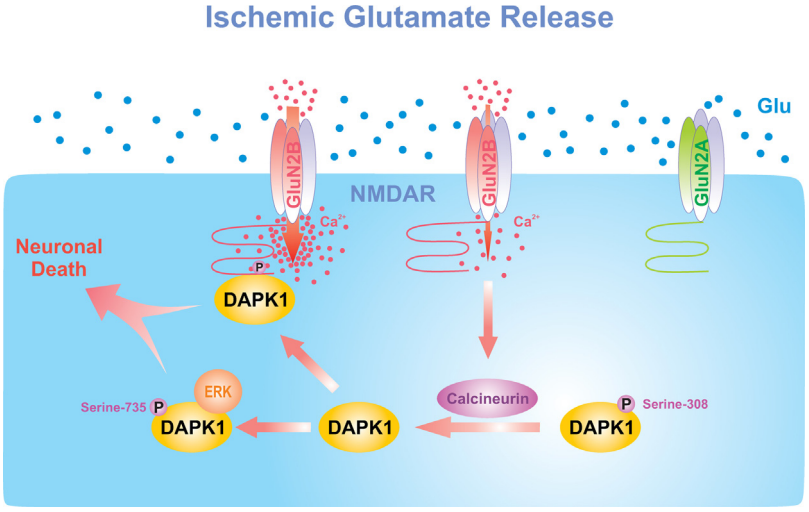

Fig. 4. Excitotoxicity recruits death-associated protein kinase 1 (DAPK1) to the cytoplasmic tail of the GluN2B subunit. During cerebral ischemia, influx of calcium ion through the NMDA receptor (NMDAR) induces calcineurin-mediated dephosphorylation and activation of DAPK1 at serine-308. The activated DAPK1 binds to the C-terminal domain of the GluN2B subunit of NMDAR, and augments the activity of the receptor to promote excitotoxicity. In addition, DAPK1 can be phosphorylated by extracellular signal-regulated kinase (ERK) at serine-735, and this phosphorylation induces neuronal death directly by augmenting DAPK1 activity and by hindering the survival-promoting activity of ERK.

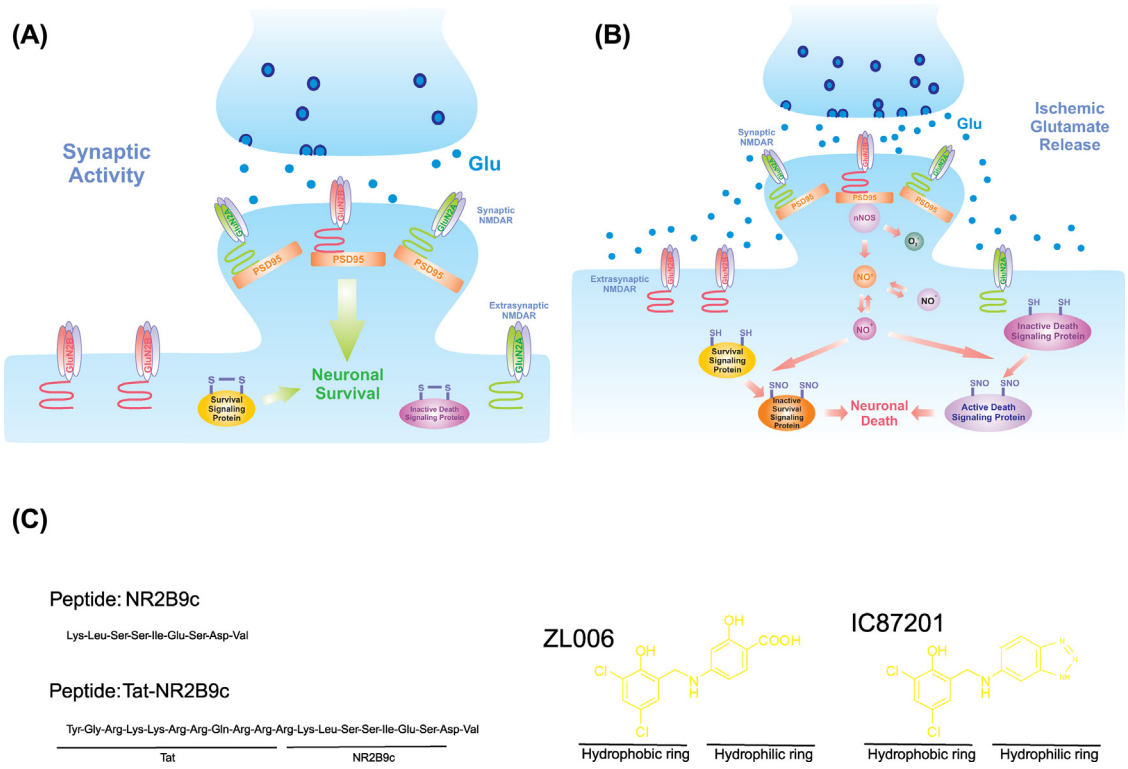

Fig. 5. The postsynaptic density protein 95 (PSD95) brings neuronal nitric oxide synthase (nNOS) into close proximity with the NMDA receptor (NMDAR) channel pore. (a) Under normal conditions, synaptic activity promotes neuronal survival in large by activation of the NMDAR. In addition, protein residues are protected from S-nitrosylation by forming disulfide bonds. (b) During cerebral ischemia, GluN2B subunit of the NMDAR forms a death-signaling complex with PSD95 and nNOS, leading to calcium-dependent production of the superoxide anion (O2−) and nitric oxide (NO*). The formation of peroxynitrite (ONOO−) from O2− and NO* can induce neuronal injury via a number of mechanisms. In addition, the NO* congener nitrosonium cation (NO+) can induce S-nitrosylation of free cysteine residues in proteins when the disulfide bonds are broken under oxygen deprivation. S-nitrosylation has been shown to induce neuronal death both by activating death-signaling proteins and inhibiting survival-promoting proteins. (c) The GluN2B–PSD95–nNOS death signaling complex can be disrupted using the peptides NR2B9c and Tat-NR2B9c, which is rendered membrane permeable by inclusion of the membrane transduction domain sequence from the HIV1 Tat protein. In addition, this complex can be disrupted using recently developed small molecules ZL006 and IC87201.

domains bind strongly to the genetically conserved threonine/serine-X-valine-COOH (T/SxV) motif of PDZ ligands, including the C-termini of all four GluN2 subunits and some GluN1 splice variants (Kornau et al., 1995). The PDZ2 domain of PSD95 also binds to the N-terminal linker of neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996a; Hillier et al., 1999; Tochio et al., 2000). Simultaneous binding of PSD95 to the NMDAR and nNOS brings nNOS into close proximity with the NMDAR channel pore and ensures efficient activation of nNOS by the calcium that fluxes through the NMDAR (Christopherson et al., 1999; Sattler et al., 1999). This finding has important implications for excitotoxicity and ischemic brain damage, because inhibiting nNOS activity by gene-deletion or by pharmacological means prevents NMDAR-mediated excitotoxicity in cultured neurons (Brorson et al., 1995; Dawson et al., 1991, 1993, 1996) and attenuates cerebral infarct and behavioral deficit in animals subjected to cerebral ischemia (Huang et al., 1994). Likewise, the direct application of nitric oxide donors to cultured neurons is potently neurotoxic (Dawson et al., 1991; Lipton et al., 1993). Mice in which exon2 of nNOS is disrupted express a catalytically active nNOS that lacks the PDZ domain and thus cannot interact with PSD95 (Brenman et al., 1996a). These mice are resistant to ischemic stroke damage (Huang et al., 1994). Consistent with the finding that PSD95 is required for NMDAR-mediated production of neurotoxic nitric oxide, knock-down of PSD95 using anti-sense mRNA attenuates both NMDAR-mediated nitric oxide production and NMDAR-mediated excitotoxicity (Sattler et al., 1999). The indirect interaction of nNOS with the NMDAR provides an explanation for the observation that calcium influx from NMDARs is especially neurotoxic and an underlying mechanism for the distinct calcium pathway hypothesis for excitotoxicity (Sattler et al., 1998; Tymianski et al., 1993b). In addition, a recent study demonstrated that the NMDAR–PSD95–nNOS interaction is augmented following cerebral ischemia (Zhou et al., 2010) (Fig. 5A and B). This specific priming of the NMDAR for excitotoxicity during ischemia may explain why the NMDAR is especially neurotoxic during ischemia.

4.2.1. Neuronal death mediated by nitric oxide

Nitric oxide can exist in its native free radical form (NO*), as a nitrosonium cation (NO+), and as a nitroxyl anion (NO−). There are a number of mechanisms by which this gaseous molecule induces neuronal death, but they can be largely differentiated by the congener in question (Fig. 5B). For example, the free radical form of nitric oxide can react with the superoxide anion to form the highly reactive molecule peroxynitrite (ONOO−), which triggers lipid peroxidation and damage. Importantly, early work comparing different nitric oxide congeners suggested that peroxynitrite is the primary nitric oxide derivative responsible for nitric oxide-mediated neurotoxicity (Lipton et al., 1993). Interestingly, nNOS can be a major source of superoxide anion (Xia et al., 1996), especially when the level of L-arginine decreases, such as during excitotoxicity (Parathath et al., 2007). Consistent with the primary role of peroxynitrite in nNOS death signaling, preventing superoxide formation prevents nNOS-dependent cell death in vitro and excitotoxic neuronal damage in vivo (Dawson et al., 1993, 1996; Lipton et al., 1993; Parathath et al., 2007; Xia et al., 1996). In

particular, nitric oxide-mediated DNA damage and the resulting activation of poly(ADP-ribose) polymerase 1 (PARP-1) is a critical mechanism leading to excitotoxic and ischemic neuronal death (Eliasson et al., 1997; Goto et al., 2002; Mandir et al., 2000; Zhang et al., 1994). In addition to causing energy deprivation with depletion of ATP and NAD, poly(ADP-ribose) polymer production by PARP-1 induces neuronal death by triggering the mitochondrial release and nuclear translocation of the apoptosis-inducing factor (AIF) (Wang et al., 2004; Yu et al., 2002, 2006). Consistent with the cytotoxic actions of the poly(ADP-ribose) polymer, its sequestration by the NMDAR survival-signaling protein Iduna leads to neuroprotection against excitotoxic and ischemic neuronal injury in vitro and in vivo (Andrabi et al., 2011) (see also Kang et al., 2011 for other cytoprotective mechanisms of Iduna). In addition to damages to DNA and other biomolecules, peroxynitrite activates a deadly current from transient receptor potential cation channel M7 (TRPM7) in neurons subjected to oxygen–glucose deprivation (Aarts et al., 2003). Knockdown of TRPM7 using siRNA prevents anoxic neuronal death in cortical neurons (Aarts et al., 2003), and knockdown of TRPM7 using a lentivirus carrying a shRNA that targets TRPM7 mRNA prevents hippocampal neuronal death in rats subjected to global cerebral ischemia (Sun et al., 2009). In contrast to the nitric oxide free radical, the nitrosonium cation preferentially promotes thiol group-nitrosylation (S-nitrosylation) of free cysteine residues on target proteins, leading to their activation, deactivation, or inactivation. This phenomenon is especially important during oxygen deprivation, as disulfide bonds (in their oxidized form) are reduced into individual free thiol-groups (in their reduced form), making them targets for nitric oxide-mediated S-nitrosylation (Takahashi et al., 2007). Recent evidence demonstrated that protein S-nitrosylation stimulates activation of death-signaling pathways and inhibition of survival-signaling pathways in cortical neurons subjected to excitotoxicity by direct NMDAR stimulation.

A comprehensive review of the evidence supporting the role of S-nitrosylation in neuronal death is outside the scope of this review, so we present here a brief summary of the findings that are directly relevant to NMDAR-mediated excitotoxicity. S-nitrosylation of enzymes can either stimulate or inhibit catalytic activity. For example, S-nitrosylation of matrix metalloprotease-9 (MMP-9) leads to the activation of downstream cell death signaling cascades and contributes to neuronal death in neurons subjected to NMDAR stimulation (Manabe et al., 2005) and cerebral infarction in rats subjected to 2 h of focal ischemia (Gu et al., 2002). In both cases, the excitotoxic damage can be prevented by nNOS gene-knockout or MMP inhibition (Gu et al., 2002; Manabe et al., 2005). In addition, S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is required for its interaction with Siah1, which promotes the nuclear translocation of GAPDH that is essential for excitotoxic neuronal injury (Hara et al., 2005). Moreover, NMDAR and nNOS-mediated S-nitrosylation of the GluK2 (GluR6) subunit of the kainate receptor facilitates its interaction with PSD95 and mixed-lineage kinase-3 (Yu et al., 2008), which subsequently leads to neuronal death following focal ischemic stroke (Yu et al., 2009), global cerebral ischemia (Pei et al., 2006), and other forms of excitotoxic neuronal death (Liu et al., 2006). More recently, S-nitrosylation of dynamin-related protein 1 (Drp1) was shown to contribute to mitochondrial fission, synaptic loss, and neuronal death in neurodegenerative diseases (Cho et al., 2009). In contrast to its role in the stimulation of neuronal death signaling, S-nitrosylation of protein–disulphide isomerase inhibits its neuroprotective activity in neurons subjected to NMDAR stimulation (Uehara et al., 2006), and S-nitrosylation of src homology-2 domain-containing phosphatase (SHP-2) inhibits its ERK-dependent neuronal survival signaling and contributes to NMDAR-mediated excitotoxic neuronal death in vitro and ischemic neuronal death in vivo (Shi et al., 2013).

It is worth noting that NMDAR-to-nNOS signaling may involve a mix of neuroprotective effects in addition to neurotoxic effects. For instance, NMDAR-mediated release of nitric oxide is required for neuronal survival signaling by the transcription factor nuclear factor I-A (NFI-A) (Gonzalez-Zulueta et al., 2000; Zheng et al., 2010), and this is one mechanism by which ischemic preconditioning confers neuroprotection (Gonzalez-Zulueta et al., 2000). When selectively removing this beneficial effect of nNOS by gene deletion of NFI-A, mice became especially susceptible to excitotoxicity (Zheng et al., 2010). Thus, selective targeting of nNOS death-signaling pathways may be more neuroprotective than directly targeting nNOS, which would also block the survival-signaling pathways. Finally, while the NMDAR promotes protein S-nitrosylation by releasing nitric oxide via the PSD95–nNOS pathway, the activity of the NMDAR is subject to feedback-regulation by this process (Lipton et al., 1993; Takahashi et al., 2007). In particular, the GluN2A subunit of the NMDAR is inhibited by S-nitrosylation under conditions of low oxygen supply (Takahashi et al., 2007), and this inhibition likely accounts for the depression of synaptic NMDAR activity following oxygen–glucose deprivation (Aarts et al., 2003). Although this anoxic suppression of NMDAR activity was once widely believed to be an evolutionarily favored mechanism by which the brain protects itself from excessive NMDAR stimulation during stroke (Lipton et al., 1993), recent evidence indicating that the GluN2A-containing NMDAR subpopulation promotes survival highlights the need for further research on this subject.

4.2.2. The Tat-NR2B9c peptide and clinical success

Disrupting the interaction between the NMDAR and nNOS has several therapeutic advantages over pharmacological inhibition of either or both of these molecules. As discussed earlier in this review, the NMDAR has several important functions in the brain, and as a result, NMDAR blockers failed to be clinically useful due to excessive side effects. Like the NMDAR, nNOS has several important physiological functions in the brain (Irikura et al., 1995; Nelson et al., 1995). Thus, it would be ideal to disrupt NMDAR-mediated nNOS activation without affecting normal NMDAR function or normal nNOS activity. This goal can be achieved by targeting PSD95. Knockdown of PSD95 using antisense mRNA attenuates NMDAR-mediated nitric oxide production and excitotoxicity (Sattler et al., 1999), but this strategy has no effect on NMDAR channel conductance and/or calcium loading or non-NMDA-dependent activation of nNOS (e.g., via voltage-gated calcium channels) (Sattler et al., 1999). In addition, disrupting the NMDAR–PSD95 interaction by loading neurons with NR2B9c (Fig. 5C), a small interference peptide that resembles the last 9 amino acid residues of the GluN2B C-terminal domain (Kornau et al., 1995), has no effect on NMDAR current (Sattler et al., 1999) but protects neurons against NMDAR-mediated excitotoxicity (Aarts et al., 2002). Both of these strategies allow for the inhibition of NMDAR/nNOS-mediated neuronal damage while leaving native NMDAR function and nNOS activity intact.

When the NR2B9c peptide is rendered membrane permeable by fusion with the membrane transduction domain of the HIV1 Tat protein, the new fusion peptide Tat-NR2B9c (Fig. 5C), which also disrupts NMDAR-mediated nNOS activation and excitotoxicity without affecting NMDAR current and calcium loading (Aarts et al., 2002), has the potential to be administered intravenously in rats subjected to focal ischemia, in other in vivo stroke models, and in human patients to treat stroke in the clinic. Consistent with the finding that non-excitotoxic NMDAR function is unaltered by this peptide, Tat-NR2B9c has no effect on NMDAR-dependent synaptic plasticity or neuronal survival signaling (Martel et al., 2009;

Soriano et al., 2008). In rats subjected to permanent focal ischemia or 90 min of transient focal ischemia, a single dose of Tat-NR2B9c (3 nmol/g, i.v., 1 or 3 h post-ischemia onset), but not the control peptide Tat-NR2B9c-AA, reduced infarct volume and improved neurological outcome for up to 62 d (Aarts et al., 2002; Sun et al., 2008). In gyrencephalic non-human primates subjected to 90 min, 3.5 h, or 4.5 h of transient focal ischemia, Tat-NR2B9c (2.6 mg/kg, i.v., 1 or 3 h post-ischemia onset) reduced MRI-measured infarct volume and non-human primate stroke scale (NHPSS)-based neurological outcome for up to 30 d (Cook et al., 2012b). In addition, in a preclinical study using non-human primates that modeled the design of the parallel and concurrent human clinical study ENACT (Evaluating Neuroprotection in Aneurysm Coiling Therapy), Tat-NR2B9c (2.6 mg/kg, i.v.) reduced the number and the volume of embolic stroke infarcts, as measured by T2-weighted MRI (Cook et al., 2012a). Most importantly, the recently reported outcome of the ENACT clinical trial (ClinicalTrial.gov NCT00728182) demonstrated that Tat-NR2B9c (NA-1, 2.6 mg/kg, i.v.) reduced the number of iatrogenic ischemic infarcts in patients receiving endovascular repair for intracranial aneurysm (Hill et al., 2012) (Table 3). After a multitude of clinical failures (Tables 1–3), this finding lends strong support to the theory that NMDAR-based treatment is indeed clinically feasible for the management of stroke and for the first time, demonstrates clinically that successful stroke intervention can be accomplished by targeting death-signaling events that occur downstream of the NMDAR.

4.2.3. Noncanonical mechanism of Tat-NR2B9c: inhibition of the PSD95–nNOS interaction

While the neuroprotective efficacy of the Tat-NR2B9c peptide has been consistently demonstrated in both pre-clinical tests using a variety of animal models and clinical trials in patients, the detailed mechanisms by which the peptide exerts its activity remain unclear. Given that the T/SxV motif of the NMDAR binds to several other members of the MAGUK family (Brenman et al., 1996b; Kornau et al., 1995; Muller et al., 1996; Niethammer et al., 1996), it is not surprising that the Tat-NR2B9c peptide can disrupt more than just the GluN2B–PSD95 interaction (Cui et al., 2007). However, among a group of PDZ-domain containing proteins that includes many of the MAGUKs, PSD95 and nNOS appear to be the most neurotoxic (Cui et al., 2007). Thus, although PSD95 binds to many other signaling proteins downstream of the NMDAR, the neuroprotective effect observed upon knockdown of PSD95 and administration of Tat-NR2B9c is likely due to the disruption of NMDAR-mediated nNOS activation, rather than the disruption of other downstream signaling cascades. In a recent screen of protein–protein interactions specifically disrupted by Tat-NR2B9c, it was demonstrated that Tat-NR2B9c preferentially disrupts the PSD95(PDZ2)–nNOS interaction with a potency (IC50 at 0.2 μM) an order of magnitude higher than that required for disruption of other PDZ–domain interactions, including that of GluN2A–PSD95(PDZ2) (IC50 at ~0.5 μM), GluN2B–PSD95(PDZ2) (IC50 at ~8 μM), and GluN2C–PSD95(PDZ2) (IC50 at 0.75 μM) (Cui et al., 2007). This finding suggests that the Tat-NR2B9c peptide is more effective at disrupting the PSD95–nNOS interaction than the intended GluN2B–PSD95 interaction. However, it should be cautioned that this study used the PDZ2 domain as a source of GluN2B interaction, whereas in the NMDAR–PSD95–nNOS signaling complex, the interaction presumably occurs between NMDAR–PSD95(PDZ1), as the PDZ2 domain would be occupied by nNOS. Inconsistent with the higher potency observed for Tat-NR2B9c against the GluN2A–PSD95(PDZ2) interaction than the GluN2B–PSD95(PDZ2) interaction that was observed in this study (Cui et al., 2007), Tat-NR2B9c disrupts the GluN2B–PSD95–nNOS interaction but not the GluN2A–PSD95–nNOS interaction in the rat forebrain (Aarts et al., 2002). Regardless, the finding that the PSD95(PDZ2)–nNOS interaction can be disrupted at an IC50 of 0.2 μM (Cui et al., 2007) suggests that Tat-NR2B9c, at a reasonable dose (3 nmol/g), acts in part by disrupting the PSD95–nNOS interaction. This high potency may also provide an explanation for the observation that Tat-NR2B9c is neuroprotective in rats subjected to ischemic stroke even when administered at very low doses (0.03, 0.3, and 1 nmol/g; 3 h post-stroke) (Sun et al., 2008).

4.2.4. Small molecule mimetics for interference peptides

Interference peptides such as Tat-NR2B9c have several clinical disadvantages compared to conventional small drug compounds, including poor pharmacokinetics, limited administration routes, expensive production cost, and immunological concerns (Lai and Wang, 2010). To overcome these pitfalls, several studies have attempted to identify small drug compounds that can mimic the effects of the interference peptides. A high throughput screen of a 150,000-compound library identified several small molecules that effectively disrupted the PSD95–nNOS interaction, and one of these compounds was further modified to yield IC87201 (Fig. 5C), which has an IC50 of 31 μM (Florio et al., 2009). Notably, IC87201 disrupts the PSD95–nNOS interaction without affecting the interaction of PSD95 with other proteins. This finding raises the intriguing possibility that interference peptides like Tat-NR2B9c can be mimicked using small molecular compounds. In addition, it may be possible to design these small molecules to achieve a high specificity for the disruption of a particular protein–protein interaction without affecting related protein–protein interactions. More recently, an independent study designed and synthesized the de novo small molecule ZL006 based on the presumable structural requirements of the PSD95–nNOS interaction (Tochio et al., 2000) (Fig. 5C), and this molecule has high specificity and potency for disrupting the PSD95–nNOS interaction without noticeable effects on the interaction of PSD95 with other proteins (Zhou et al., 2010). Consistent with the effect of Tat-NR2B9c on excitotoxic and ischemic neuronal injury, ZL006 is neuroprotective against NMDAR-mediated excitotoxicity in vitro and ischemic neuronal injury in vivo (Zhou et al., 2010). ZL006 readily crossed the blood–brain barrier, a potential limitation for interference peptides and neuroprotective proteins (Chen et al., 2013; Yen et al., 2013), when administered intravenously and had no effect on NMDAR channel function, nNOS activity, or related neurological functions, such as learning and memory (Zhou et al., 2010). Interestingly, the structures of IC87201 and ZL006 are highly similar, suggesting that the two compounds may act via the same mechanism and reconfirming the possibility that molecules with this type of two-ring structure can disrupt the PSD95–nNOS interaction. Future structure–activity relationship studies using these two compounds and other related compounds may allow for the development of compounds that are even more potent than IC87201 and ZL006.

4.3. The calpain pathway

The diverse proteolytic functions performed by calpains, a group of enzymes collectively named due to their calcium-dependent (cal-) cysteine endopeptidase activity (-pain) (Murachi et al., 1980), are involved in NMDAR/calcium-mediated neuronal damage (Fig. 6). These signaling proteases, which were initially isolated in the rat brain (Guroff, 1964), include a number of distinct isoforms that are either tissue-specific or ubiquitously expressed in animal cells (Murachi, 1983). There are at least two forms of calpain, which differ according to their sensitivity to activation by calcium (Mellgren, 1980), and each form contains a catalytic subunit of approximately 80 kDa (Ohno et al., 1984) and a regulatory subunit of approximately 30 kDa (Sakihama et al., 1985). NMDAR stimulation specifically activates the neuron-specific calpain I, but not the more ubiquitous calpain II, in the rat

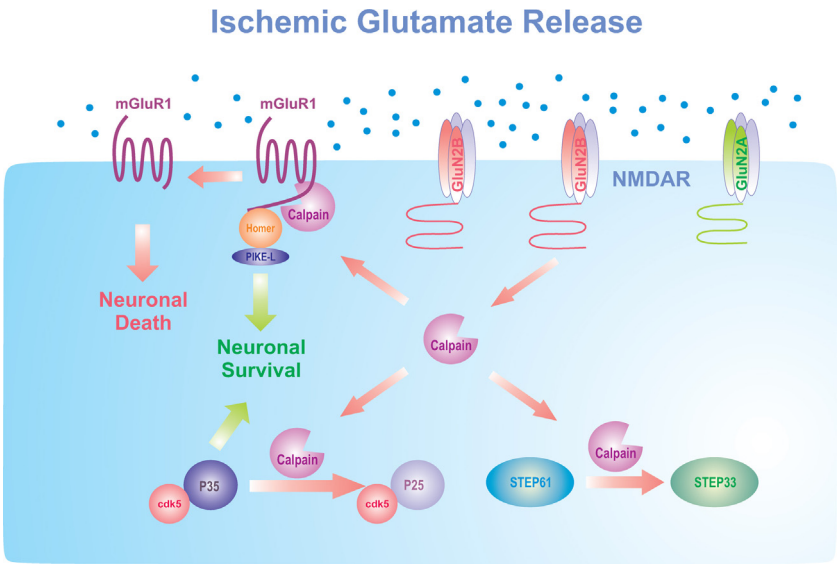

Fig. 6. Calpain mediates excitotoxicity via protein cleavage. GluN2B-containing NMDA receptors can induce neuronal injury by calcium-dependent activation of the death-promoting protease calpain. Calpain-mediated truncation of the C-terminal domain of the metabotropic glutamate receptor 1 (mGluR1), which promotes neuronal survival via activation of the long form of phosphoinositide 3 kinase enhancer (PIKE-L), converts the receptor into a death-signaling receptor. In addition, calpain-mediated truncation of p35, which promotes neuronal survival by cdk5-dependent activity, into p25 induces aberrant target redirection of cdk5 to induce neuronal death. Finally, calpain-mediated truncation of striatal-enriched protein tyrosine phosphatase 61 (STEP61), which inhibits death-signaling by p38 of the mitogen-activated protein kinase family, into the inactive STEP33 contributes to excitotoxic neuronal death.

brain (Seubert et al., 1988; Siman and Noszek, 1988), and this activation has been demonstrated to be the primary mechanism by which cerebral hypoxia and/or ischemia triggers proteolysis in the brain (Arai et al., 1991; Lee et al., 1991; Seubert et al., 1989), depression of synaptic efficacy (Arlinghaus et al., 1991; Hiramatsu et al., 1993; Lee et al., 1991), and cerebral damage (Lee et al., 1991; Rami and Krieglstein, 1993). In addition, excitotoxic calpain activation can induce calcium overload via inhibitory cleavage of plasma membrane NCX3 (see Section 2.1.2 on NCX), and inhibition of calpain or replacing endogenous NCX3 with the non-degradable NCX2 prevents NMDAR-mediated calcium overload and neuronal death (Bano et al., 2005). This positive-feedback mechanism may partially explain how a small calcium transient through the NMDAR can trigger a subsequent calcium overload (Randall and Thayer, 1992; Tymianski et al., 1993a). Consistent with the role of the GluN2B subunit in excitotoxicity (see Section 2.2.2), NMDAR-mediated calpain activation is blocked by a selective antagonist of the GluN2BR, but not by an antagonist that preferentially inhibits the GluN2AR (DeRidder et al., 2006; Gascon et al., 2008; Zhou and Baudry, 2006). Consistent with the theories that calpain acts downstream of the NMDAR and that the targeting of downstream mechanisms may offer a wider window of therapeutic opportunity, the calpain inhibitor MDL-28170 protected neurons against NMDAR-mediated excitotoxicity when administered 1 h after receptor activation (Brorson et al., 1995). In comparison, the NMDAR antagonist AP5, while equally neuroprotective when applied prior to NMDAR stimulation, had no effect when applied 1 h after NMDAR stimulation (Brorson et al., 1995). Like other treatments designed to target death-signaling cascades downstream of the NMDAR, calpain inhibition has no effect on NMDAR channel function (Brorson et al., 1995) and should have no effect on non-calpain-dependent NMDAR signaling cascades and functions. However, a number of calpain functions are required for normal cellular function, and nonspecific inhibition of all calpain activity can have adverse effects. This is an especially important consideration, as calpain is ubiquitously expressed in almost all animal cells. The identification of the specific downstream neuronal substrates of calpain that contribute to excitotoxicity could lead to the development of stroke treatments with an improved safety profile compared to that of direct calpain inhibition. Because many proteins are cellular targets for calpain cleavage, it is expected that the diverse and widespread cleavage of essential cellular proteins could result in multifactorial damage. However, recent studies suggest that the calpain-mediated cleavage of several distinct targets and their downstream signaling pathways are especially neurotoxic (see Sections 4.3.1 and 4.3.2).

4.3.1. p25–cdk5 death-signaling

The 35-kDa regulatory activator (p35) of cdk5 is a brain-specific calpain-substrate (Lew et al., 1994; Tsai et al., 1994), and both p35 and cdk5 are important signaling proteins required for maintaining brain development and survival (Chae et al., 1997; Nikolic et al., 1996; Ohshima et al., 1996; Paglini et al., 1998). Stimulation of the NMDAR in primary cortical neurons by glutamate triggers calcium-dependent calpain-mediated proteolytic cleavage of p35 into a smaller 25-kDa form (p25) (Lee et al., 2000; O'Hare et al., 2005), which induces neuronal death, in part by aberrant activation and target-redirection of cdk5 (Patrick et al., 1999) (Fig. 6). As a result, in these neurons, the inhibition of calpain or cdk5 prevents p25-mediated neuronal damage (Alvarez et al., 2001; Lee et al., 2000; O'Hare et al., 2005). It should also be noted that neurons undergoing certain forms of non-excitotoxic damage also require calpain-mediated production of p25 (Kusakawa et al., 2000; Nath et al., 2000), suggesting that calpain inhibitors possess therapeutic potential beyond excitotoxic neuronal damage. Consistent with its essential role in excitotoxicity, increased levels of p25 are found in the brains of mice subjected to focal ischemic stroke (Lee et al., 2000; Nath et al., 2000) and global cerebral ischemia (Garcia-Bonilla et al., 2006; Wang et al., 2003; Wen et al., 2007) and in brain specimens from patients with Alzheimer's disease (Patrick et al., 1999) and ischemic stroke (Mitsios et al., 2007). Importantly, conditional overexpression of p25 in the forebrain of transgenic mice triggers neurodegeneration (Cruz et al., 2003), and inhibition of cdk5 or its downstream targets prevents neuronal damage induced by p25 and cerebral ischemia (Kim et al., 2008; Rashidian et al., 2009; Wang et al., 2003). Although many proteins are

phosphorylative substrates of cdk5, neuronal damage induced by p25 has been shown to be induced by cdk5-mediated phosphorylation of the GluN2A subunit of the NMDAR (Wang et al., 2003), phosphorylation and inhibition of the transcription factor myocyte enhancer factor 2 (Rashidian et al., 2009; Smith et al., 2006), phosphorylation and inhibition of the antioxidant enzyme Prx2 (Qu et al., 2007; Rashidian et al., 2009), inhibition of histone deacetylase 1 (Kim et al., 2008), and phosphorylation and inhibition of apurinic/apyrimidinic endonuclease (Huang et al., 2010).

4.3.2. Calpain-mediated cleavage of the NMDAR and mGluR

Among the substrates that are cleaved by calpain following NMDAR stimulation, it is interesting to note that the GluN1, GluN2A, and GluN2B subunits of the NMDAR are also substrates for calpain cleavage (Bi et al., 1998). Like the S-nitrosylation-dependent inhibition of GluN2A by nitric oxide that was described earlier in this review (Takahashi et al., 2007), NMDAR stimulation induces calpain-mediated cleavage of GluN2A-B, which results in the depression of the NMDAR channel expression and function (Guttmann et al., 2002; Wu et al., 2005). Interestingly, the GluN1/GluN2A-containing NMDAR appears to be resistant to calpain cleavage in hippocampal neurons (Bi et al., 1998; Simpkins et al., 2003), despite being an active substrate when it is expressed in a cell line (Guttmann et al., 2002). This resistance to calpain cleavage in neurons appears to be due to the GluN2A–PSD95 interaction, as calpain vulnerability can be alleviated by PSD95 overexpression in a cell line and reinstated by disrupting the GluN2A–PSD95 interaction genetically or pharmacologically (Dong et al., 2004). In contrast, calpain-mediated cleavage of GluN2B occurs in neurons following only a brief exposure to NMDAR-mediated excitotoxicity in vitro. More recently, a study demonstrated that after prolonged in vitro NMDAR stimulation for 4 h as opposed to the 30 min stimulation used in previous experiments, or after focal ischemic stroke in vivo, both GluN2A and GluN2B are truncated by calpain in neurons (Gascon et al., 2008). The observed lack of PSD95-mediated resistance to calpain cleavage can be explained by the finding that PSD95 itself was also lost to calpain cleavage (Gascon et al., 2008) under these conditions. Interestingly, the truncated NMDAR subunits can remain stable for some time post-excitotoxicity (up to 48 h post ischemia) (Gascon et al., 2008; Simpkins et al., 2003), but as their cytoplasmic tails, which are critical for functional output, have been truncated (see Sections 2.2.2 and 4.1), these subunits are expected to have limited or altered signaling output.

In addition to ionotropic glutamate receptors like the NMDAR, calpain also directly cleaves the metabotropic glutamate receptor-1 (mGluR1) following excitotoxic injury (Xu et al., 2007) (Fig. 6). Importantly, this post-translational modification of mGluR1 transforms it from a pro-survival receptor into a pro-death receptor. Native mGluR1 forms a pro-survival complex with Homer and long form of phosphoinositide 3 kinase enhancer (PIKE-L) upon agonist-stimulation, and this multimeric mGluR1–Homer–PIKE-L interaction is required for mGluR1-dependent activation of the well-known pro-survival protein PI3K (see Section 3.1) and neuroprotection against apoptotic cell death induced by staurosporine (Rong et al., 2003). Upon activation of the NMDAR, however, calpain cleaves the cytoplasmic tail of mGluR1 at serine-936, and this post-translational modification reduces mGluR1-mediated calcium release from internal stores, cationic current from GIRK1, GIRK2, and TRPC1, and induction of PIK3 activity, as measured by the level of Akt phosphorylation (Xu et al., 2007). In addition, this modification transforms the pro-survival effect of mGluR1 stimulation into a pro-death effect (Xu et al., 2007). Based on the amino acid sequence of the calpain cleavage site of mGluR1, the interference peptide Tat-mGluR1 was developed to selectively block this post-translational modification. Like Tat-NR2B9c (see Section 4.2.2), the Tat sequence rendered the peptide membrane-permeable. Tat-mGluR1 prevents calpain-mediated mGluR1 truncation in a dose-dependent manner and rescues mGluR1-mediated pro-survival PI3K signaling. Notably, Tat-mGluR1 selectively inhibits calpain-mediated cleavage of mGluR1 at low concentrations (1–2 μM), but at marginally higher concentrations (4–8 μM), Tat-mGluR1 becomes non-selective and inhibits calpain-mediated cleavage of spectrin and possibly other proteins. Consistent with the role of mGluR1 in excitotoxic neuronal death, Tat-mGluR1 prevents excitotoxic neuronal death in vitro and in vivo (Xu et al., 2007). Importantly, the partial selectivity of Tat-mGluR1 for inhibition of the calpain-mediated cleavage of mGluR1 but not other calpain-mediated cleavages suggests that interference peptides can be designed to be more specific than conventional pharmacological calpain inhibitors.

4.3.3. STEP on death and survival

Another calpain target that mediates excitotoxicity is the brain-specific death-signaling protein striatal-enriched protein tyrosine phosphatase (STEP) (Fig. 6). Excitotoxicity induced by glutamate triggers the activation of the two alternatively spliced variants of STEP, STEP61 and STEP46, resulting in the dephosphorylation of the key neuronal survival signaling proteins ERK and CREB (Paul et al., 2003). Excitotoxic activation of the STEP splice variants, via dephosphorylation of their kinase-interacting domains, which bind to ERK and other substrates, requires activation of the NMDAR, but not other glutamate receptors, and requires calcium influx from the NMDAR, but not other sources of calcium (Paul et al., 2003). Importantly, synaptic NMDAR stimulation leads to STEP61 ubiquitination and degradation, prolonging the ERK activation that is needed for neuronal survival (Xu et al., 2009). In contrast, extrasynaptic/GluN2B NMDAR stimulation triggers calpain-mediated cleavage of STEP61 into STEP33, resulting in a lack of p38 dephosphorylation (Mesfin et al., 2012; Xu et al., 2009). Activated p38 (see Section 4.4.1) is thought to contribute to extrasynaptic NMDAR-mediated excitotoxicity. Future studies are needed to determine the mechanisms by which decreased levels of STEP61, produced as a result of ubiquitination/degradation or calpain-mediated cleavage, selectively lead to ERK stimulation versus p38 activation, respectively. Importantly, the interference peptide Tat-STEP, whose sequence resembles the calpain-cleavage site of STEP61 and is rendered membrane permeable by the Tat sequence, is protective against excitotoxic and ischemic neuronal injury (Xu et al., 2009).

4.4. Transcription-dependent NMDAR death signaling

One of the hallmarks of ischemic neuronal death is the delayed progression from the initial ischemic event to the resulting neuronal death. As the long-term memory of many cellular events involves transcriptional consequences, transcription is expected to be required for neurons to "remember" that they were previously ischemic and that they were programmed to undergo death at a later time point. Several lines of evidence have demonstrated that ischemic neuronal death is a transcription-dependent process that requires the expression of death-signaling genes, as well as the repression of survival-promoting genes (Dick and Bading, 2010; Wahl et al., 2009; Zhang et al., 2007). Thus, targeting the transcription factors that are responsible for neuronal death may be a therapeutically favorable strategy, as it prevents the up-regulation of many death-signaling genes and the down-regulation of many survival-promoting genes at the latest converging points. This strategy would allow for the widest therapeutic window for effective drug administration without the need to target individual genes for up-regulation or down-regulation. Among the signaling

proteins that regulate death-related transcription is the mitogen-activated protein kinase (MAPK) family. Excluding ERK (also known as classical MAPK), which is largely pro-survival and participates in CREB activation (see Section 3.2), MAPK family members, including p38 and c-Jun N-terminal kinase (JNK), are stress-signaling proteins that translate cellular stress into transcription-dependent responses (Derijard et al., 1994; Galcheva-Gargova et al., 1994; Gupta et al., 1996; Han et al., 1994; Kyriakis et al., 1994; Lee et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994). Thus, cellular stress is often accompanied by a decrease in the phosphorylative activation of ERK, and apoptosis can be prevented by ERK stimulation (Xia et al., 1995). In contrast, apoptosis is often dependent on p38 and JNK activation and can be ameliorated by inhibition of either of these kinases (Graves et al., 1996; Verheij et al., 1996; Xia et al., 1995). A recent non-biased screening assay identified sterol response element binding protein 1 (SREBP1), which is best known for its fundamental role in lipid metabolism (Brown and Goldstein, 1997; Goldstein et al., 2006), as a critical transcription factor for excitotoxicity and ischemic stroke injury (Taghibiglou et al., 2009). Given the pivotal role of lipids in neuronal death and survival (see Section 3.1), the novel excitotoxic role of this transcription factor warrants future research.

4.4.1. From GluN2B-PSD95–nNOS to p38

The death-signaling MAPK p38 is a well-characterized contributor to NMDAR-mediated excitotoxicity, and recent studies report that it is involved in death signaling downstream of the GluN2B–PSD95–nNOS pathway (see Section 4.2). p38, also known as MAPK-activated protein kinase-2 reactivating kinase (RK) or cytokine-suppressive anti-inflammatory drug (CSAID)-binding protein (CSBP), is activated by phosphorylation in response to endotoxin and cellular stress (Graves et al., 1996; Han et al., 1994; Lee et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994). Excitotoxic stimulation of the NMDAR triggers p38 activation (Cao et al., 2004, 2005; Kawasaki et al., 1997), and this activation requires influx of calcium through the NMDAR, but not concomitant calcium influx through voltage-gated calcium channels (Kawasaki et al., 1997). Selective inhibition of p38 with the inhibitors SB203580 and SB239063 prevents excitotoxic and anoxic neuronal death in vitro (Barone et al., 2001b; Cao et al., 2004, 2005; Kawasaki et al., 1997; Legos et al., 2002), and oral and intravenous prophylactic administration of SB239063 and SB202190 protects the rat brain against focal ischemic stroke damage in vivo (Barone et al., 2001a,b; Soriano et al., 2008). Interestingly, recent studies suggest that NMDAR-mediated p38 activation requires an intact GluN2B–PSD95–nNOS interaction. Selective disruption of either the GluN2B–PSD95 interaction or the PSD95–nNOS interaction via a variety of interference peptides or protein fragments prevents NMDAR-mediated p38 activation and attenuates neuronal death (Cao et al., 2005; Soriano et al., 2008), whereas disruption of other PSD95 binding proteins has no effect (Cao et al., 2005). Likewise, glutamate-mediated p38 activation and neuronal death can be attenuated by direct nNOS inhibition and exacerbated by nNOS stimulation (Cao et al., 2005; Soriano et al., 2008). In addition, the direct application of nitric oxide donors activates p38 and induces neuronal death, and because it bypasses the GluN2B–PSD95–nNOS pathway, this exogenous nitric oxide-mediated effect is not affected by interference peptides that target PSD95–protein interactions (Cao et al., 2005). Notably, two recent studies have identified Rho and NOS1AP (nitric oxide synthase 1 adaptor protein or CAPON (carboxyl-terminal PDZ ligand of nNOS)) signaling pathways in NMDAR/nitric oxide-mediated p38 activation (Li et al., 2013; Semenova et al., 2007). The relative contribution by each pathway to p38 activation and whether these two pathways interact or act independently of each other remains unresolved. Thus, the evidence indicates that p38 acts downstream of the GluN2B–PSD95–nNOS pathway in excitotoxicity. However, p38 is likely not the only death signaling molecule downstream of this pathway. Glutamate-mediated p38 activation peaks around 5 min post-treatment in cultured neurons, and it is greatly reduced by 30 min and completely abolished by 60 min post-treatment (Cao et al., 2004, 2005; Semenova et al., 2007). As a result, SB203580 protects neurons against excitotoxicity when applied 30 min before excitotoxic glutamate treatment, but not when applied 30 min afterwards (Cao et al., 2005). In contrast, although pretreatment with Tat-NR2B9c prevents glutamate-mediated p38 activation and neuronal death, its therapeutic efficacy against neuronal death remains evident when it is applied up to 60 min post-excitotoxic treatment (Aarts et al., 2002), a time point when p38 is no longer active (Cao et al., 2004, 2005; Semenova et al., 2007). This short therapeutic window would render p38 inhibitors impractical as clinical stroke treatments. The wider time window for effective administration of Tat-NR2B9c suggests that p38 is not likely to be the only death signal downstream of the GluN2B–PSD95–nNOS pathway.

4.4.2. JNK and the development of Tat-JBD20

The stress-associated MAPK JNK, also known as stress-activated protein kinase (SAPK), is a primary target of excitotoxic neuronal death. These kinases are notorious for their role in the death of different cell types and can be activated via phosphorylation by a variety of factors, including many non-excitotoxic apoptotic factors, such as cellular stress and tumor necrosis factor (Derijard et al., 1994; Galcheva-Gargova et al., 1994; Graves et al., 1996; Kyriakis et al., 1994; Matsuda et al., 1995; Verheij et al., 1996). There are three major subtypes of JNK, each with several sub-isoforms that are generated by alternative mRNA splicing (Gupta et al., 1996; Kuan et al., 1999; Martin et al., 1996). JNK1 and JNK2 are ubiquitously expressed and have been shown to be critical for functional apoptosis during development (Kuan et al., 1999; Martin et al., 1996). In contrast, JNK3 is largely expressed in the nervous system (Kuan et al., 1999; Martin et al., 1996) and has been directly implicated in excitotoxic neuronal death in vivo, as JNK3-knockout mice are resistant to excitotoxic neuronal injury (Yang et al., 1997). Because JNK activation is inhibited by binding of the JNK-binding domain (JBD) of the JNK-interacting protein (JIP) at the critical amino-acid-residues R156, R157, L160, and L162 (Barr et al., 2002; Bonny et al., 2001), overexpression of either full-length JIP or the JBD fragment prevents JNK activity and phosphorylation of its downstream substrates c-Jun and Elk-1 (Borsello et al., 2003). As such, JNK activity can be effectively inhibited by Tat-JBD20 (also known as JNK inhibitor 1 (JNKI-1)), whose sequence represents the JBD (amino acid residues 143–163 of JIP-1) fused to the membrane-permeability domain of the HIV1 Tat protein (Barr et al., 2002; Bonny et al., 2001; Borsello et al., 2003). Although Tat-JBD20 inhibits JNK activity, this interference peptide does not inhibit the phosphorylative activation of JNK itself (Borsello et al., 2003). Due to sequence similarity with the JBD of JIP-1, inhibitory effects on JNK activity can also be achieved with peptides resembling the JBDs of JIP-2 and JIP-3 (Barr et al., 2002). In addition, when this interference peptide is synthesized with D amino acids, resulting in the peptide D-JNKI-1, it is resistant to intracellular peptide degradation and therefore more potent than the original L-JNKI-1 peptide that was synthesized with L amino acids (Bonny et al., 2001; Borsello et al., 2003). This finding raises the possibility that the bioavailability of other interference peptides designed to inhibit NMDAR excitotoxicity or other biological pathways may also be enhanced using D amino acids. It is worth noting that the selectivity of JBD20 has been extensively tested against a wide range of protein kinases, and the peptide fragment potently inhibits JNK activity at 2.5–25 μM, but it has no effect on the activity of ERK-2, p38, PKC, P34, CaMK, and PKA at

concentrations up to 500 μM (Borsello et al., 2003). For JNK and other protein kinases that lack a small molecule-based drug inhibitor, the large margin of selectivity offered by this peptide suggests that the use of interference peptides is an important area of research for future drug design and development.

Like Tat-NR2B9c, D-JNKI-1 is one of the earliest NMDAR-based Tat-linked peptides to target death-signaling proteins downstream of the surface receptor, and it has been shown in multiple studies to be effective against stroke, even when administered several hours after the ischemic event. JNK is activated as early as 10 min post-excitotoxic NMDAR stimulation, and continues to be activated for at least 1 h (Borsello et al., 2003; Kawasaki et al., 1997). In mice subjected to 30 min of transient focal ischemic stroke, JNK is activated as early as 1 h post-ictus, and it remains activated up to 24 h post-ischemia (Borsello et al., 2003). Pretreatment with D-JNKI-1 protected cortical neuronal cultures against excitotoxic neuronal death in vitro and reduced ischemic neuronal death in rats/mice subjected to focal ischemic stroke in vivo (Borsello et al., 2003; Centeno et al., 2007; Esneault et al., 2008; Hirt et al., 2004). Impressively, D-JNKI-1 has been shown to be effective against stroke damage whether the drug is administered before stroke or 3–12 h after the initial ischemic event (Borsello et al., 2003). In addition, the observed improvements in neurological outcome in animals treated with D-JNKI-1 parallel a reduction of cerebral infarction and are evident from 6 h up to 2 weeks post-ischemia (Borsello et al., 2003). This wide time window for effective drug administration supports the theory that inhibition of death-signaling proteins downstream of the NMDAR can confer a wider window of therapeutic opportunity than conventional treatments that terminate NMDAR excitotoxicity at the receptor level. It should also be noted that this window of opportunity is dependent on the stroke model studied. In young P14 rats subjected to permanent focal ischemia, D-JNKI-1 is neuroprotective when infused before ischemia and at 3, 6, and 12 h post-ischemia (Borsello et al., 2003). In contrast, in adult mice subjected to transient 30 min focal ischemia, D-JNKI-1 is only effective when administered before ischemia and at 3–6 h post-ischemia, but not when administered 12 h post-ischemia (Borsello et al., 2003). In addition, in adult rats subjected to permanent focal ischemia, D-JNKI-1 is neuroprotective when administered 3 h but not 6 h post-ischemia (Hirt et al., 2004).

4.4.3. New role of SREBP1 in neuronal death

SREBP1 (also known as adipocyte determination and differentiation-dependent factor 1 (ADD1)), the principal transcription factor for genes related to lipid metabolism (see review (Brown and Goldstein, 1997; Goldstein et al., 2006)), was recently identified as a death-signaling protein involved in NMDAR-mediated excitotoxicity (Taghibiglou et al., 2009). NMDAR stimulation triggers calcium-dependent and calpain-dependent proteolysis of protein encoded by insulin-induced gene 1 (insig1), the inhibitory protein that retains the inactive SREBP1 and SREBP cleavage-activating protein (SCAP) complex in the endoplasmic reticulum (Gong et al., 2006; Yang et al., 2000), allowing for the proteolytic activation of SREBP1 and its subsequent translocation into the nucleus (Taghibiglou et al., 2009). Consistent with the subunit specificity of NMDAR in death signaling (see Section 2.2.2), excitotoxic stimulation of SREBP1 is blocked by the GluN2BR antagonist but not by the GluN2AR antagonist. Consistent with the role of nuclear SREBP1 in excitotoxicity, the degree of nuclear translocation of SREBP1 parallels the induction of neuronal death. Importantly, genetic knockdown of SREBP1 or inhibition of SREBP1 activation using the interference peptide Tat-INDIP (insig1-derived interference peptide: GEPHKFKREW), whose sequence flanks the lysine-156 and 158 ubiquitination sites of insig1 that are required for proteolysis (Gong et al., 2006) and is rendered membrane permeable with the membrane permeability sequence of the HIV1 Tat protein, attenuates NMDAR-mediated excitotoxic neuronal injury in vitro and ischemic stroke damage in vivo (Taghibiglou et al., 2009).

5. Perspectives

Research into excitotoxicity was initially based upon food safety concerns related to the use of monosodium glutamate, but this process was quickly revealed as the principle mechanism underlying neurodegeneration following cerebral ischemia and many other neurodegenerative diseases, including brain trauma, Huntington's disease, Alzheimer's disease, and ALS. Many questions about the molecular mechanisms that underlie excitotoxicity remain unanswered. As scientists begin to understand the critical role of the NMDAR and its calcium input in excitotoxicity, it becomes important to determine the mechanisms by which different subpopulations of the NMDAR and different sources of calcium input can lead to vastly different cellular responses, including opposing responses like neuronal death and survival. Although evidence points to the cytoplasmic tail of the NMDAR as the primary determinant of the receptor's functional output, the mechanisms by which death signaling proteins are specifically recruited following cerebral ischemia and the mechanisms by which death signaling proteins that do not bind to the NMDAR cytoplasmic tail, such as calpain and JNK, are activated with receptor subtype specificity and calcium source specificity remain unclear. The emergence of molecular and cell biology has allowed scientists to dissect the individual death-signaling proteins involved in the progression from NMDAR stimulation to excitotoxic neuronal death. Despite decades of research, new death-signaling proteins downstream of the NMDAR are still emerging. Studies have demonstrated that inhibiting these downstream proteins is neuroprotective, but few studies have addressed how these different death-signaling proteins work together, including whether they work synergistically or in parallel to trigger excitotoxicity. The downstream transcription factors and related proteins, including p38, JNK, and SREBP1, are particularly attractive therapeutic targets, because inhibition of these factors is likely to affect the expression of large groups of excitotoxicity-related genes without requiring treatment cocktails that individually target multiple genes.

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