Extrasynaptic NMDA receptors: mediators of excitotoxic cell death

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Abstract

The N-methyl-D-aspartate (NMDA) type of glutamate receptor is a calcium-permeable ion channel with important functions in the physiology and pathology of the mammalian brain. NMDA receptors are critical for long-lasting, activity-induced changes in synaptic transmission, a process thought to be involved in learning and memory. NMDA receptors also control neuronal survival and cell death. How can the biological consequences of NMDA receptor activation be so diametrically opposed? The outcome of NMDA receptor activation appears to be determined by its localization. Stimulation of synaptic NMDA receptors (by synaptically-released glutamate) activates gene expression mediated by the transcription factor, cAMP-response element-binding-protein (CREB) and induces pro-survival events. In contrast, calcium flux through extrasynaptic NMDA receptors overrides these functions, shutting off CREB activity, and causing mitochondrial dysfunction and cell death. These differences in the biological response are likely due to differences in the intracellular signaling complexes associated with synaptic vs. extrasynaptic NMDA receptors. As extrasynaptic NMDA receptors are thought to be activated following hypoxic/ischemic insults, specific blockade of extrasynaptic NMDA receptors or their signaling complex may efficiently reduce neuron loss following stroke.

The involvement of NMDA receptors in neuron death

NMDA receptor antagonists have long been known to reduce the early phase of post-ischemic neuron death in rats (Minematsu et al., 1993a, b; Simon et al., 1984). Brain ischemia causes elevated glutamate levels in the extracellular space (Benveniste et al., 1984; Stoffel et al., 2002) largely due to the reverse function of glutamate transporters (Rossi et al., 2000). Ischemia can also cause astrocyte dysfunction, necrosis and apoptosis, compromising the neuroprotective buffering of glutamate via the astrocyte specific glutamate transporter, GLT-1, and the conversion of glutamate to inactive glutamine in glial cells (Chen and Swanson, 2003; Schubert et al., 2000; Takuma et al., 2004; Tanaka et al., 1997). Excess extracellular glutamate and the resultant stimulation of ionotropic glutamate receptors is believed to be

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involved in subsequent excitotoxicity and active cell death (commonly termed apoptosis) leading to a penumbra of secondary neuron loss surrounding the focal lesion site (Bramlett and Dietrich, 2004; Lipton, 1999a).

Intervention with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or NMDA receptor antagonists is problematic because they also block normal and vital glutamate-mediated neurotransmission between non-injured neurons, inducing behavioral (psychotomimetic) side-effects, sedation and amnesia (Davis et al., 1997; Ikonomidou and Turski, 2002; Lees, 2000; Morris, 1989). More importantly, NMDA antagonists are known to induce or exacerbate apoptosis and neurotoxicity (Brenneman et al., 1990; Ciani et al., 1997; Ikonomidou et al., 1999, 2000; Low and Roland, 2004; Snider et al., 2002). This cell death caused by NMDA antagonists may be due to the inhibition of cell survival pathways (Hardingham et al., 2002; Yoon et al., 2003).

NMDA receptors have not been abandoned, however, in current clinical strategies against excitotoxicity as evidenced by the recent approval in the USA of memantine, an NMDA open channel blocker, for the treatment of advanced Alzheimer's disease (Farlow, 2004). More specific and efficacious pharmaceutical tools are needed, however, to prevent second-stage damage following stroke, to dampen glutamate-mediated excitation in epilepsy, and to interfere with the complex biochemical pathways that lead to cell death in certain neurodegenerative diseases including Alzheimer's disease, Huntington disease and AIDS (Kaul et al., 2001; Lancelot et al., 1998; Lipton and Rosenberg, 1994). Selective intervention in the role of NMDA receptors in these pathologies must distinguish between the aspects of NMDA receptors mediating neurotoxicity and those which protect against it.

NMDA receptor overview

NMDA receptors are glutamate-gated cation channels whose activation contributes to depolarization by allowing sodium and calcium influx. The presence of both NR1 and NR2 subunits are required to form functional channels due to the presence of the glutamate binding domain at their junction. Four distinct subtypes (NR2A-D) of the NR2 subunit exist. A binding site for glycine is found on the NR1 subunit while the NR2B subunit possesses a polyamine binding site where regulatory molecules can modulate the activity of the NMDA receptor.

At resting membrane potentials, NMDA receptors are normally inactive due to a voltage-dependent block of the channel pore by magnesium ions. Activation of the NMDA channel occurs during simultaneous depolarization of the post-synaptic cell and the binding of glutamate and glycine. Bursting activity in a presynaptic glutamatergic cell can satisfy these conditions through co-activation of postsynaptic excitatory AMPA receptors. Alternatively, accumulation of extracellular glutamate following ischemia is expected to activate both synaptic and extrasynaptic NMDA receptors.

NMDA and other glutamate receptors cluster together in dendritic spines where they mediate synaptic transmission, with an adaptive nature evident in long-term potentiation (LTP) or long-term depression (LTD) involved in memory formation and learning (Bear and Malenka, 1994; Paulsen and Sejnowski, 2000). NMDA receptors are also found at extrasynaptic sites (Clark et al., 1997; Rao and Craig, 1997; Rao et al., 1998; Rosenmund et al., 1995; Tovar and Westbrook, 2002). NMDA receptor clusters have been detected colocalized (ie. synaptic) and non-colocalized (ie. extrasynaptic) with presynaptic markers using immunocytochemical methods in hippocampal and cortical neurons (Aoki et al., 1994; Liao et al., 1999; Pickard et al., 2000).

The distinguishing features responsible for the striking differences in the biological responses induced by extrasynaptic and synaptic NMDA receptors remain unclear. NMDA receptor activation in both cases leads to a calcium influx into post-synaptic cells, a signal crucial for the induction of NMDA-receptor dependent plasticity and learning on the one hand and excitotoxic cell death on the other (Bading, 2000; Hardingham and Bading, 2003).

Such contrasting actions of NMDA receptors may be due to differences in the downstream signaling complexes linked to synaptic and extrasynaptic NMDA receptors.

Signaling cascades regulating survival and death

Calcium influx through NMDA receptors can trigger LTP or LTD of synaptic connections, and can send signals to the nucleus to activate gene expression (Bading et al., 1993; Bito et al., 1996; Fink and Meyer, 2002; Hardingham et al., 1999, 1997; Malenka and Nicoll, 1999). These processes are thought to play a role in memory and learning as well as promoting cell survival (Fig. 1). Calcium acts as a second messenger to induce post-translational modifications including the activation of calcium calmodulin-dependent (CaM) kinases and the Ras–extracellular signal-regulated protein kinases (Ras-ERK1/2) pathway which phosphorylate and inactivate the pro-apoptotic protein BAD (Bonni et al., 1999; Yano et al., 1998). ERK1/2 activation is linked to both survival (Hetman and Gozdz 2004) and death pathway activation (Chu et al., 2004). Synaptic NMDA receptor activation *in vivo* also results in the transcription of several immediate early genes (Cammarota et al., 2000; Cole et al., 1989; Schulz et al., 1999; Wisden et al., 1990), many of which are controlled, at least in part, by the transcription factor cAMP-response element-binding-protein (CREB).

CREB: A calcium regulated transcription factor

Synaptic NMDA receptor-mediated calcium signals activate DNA regulatory elements including the serum response element (SRE),

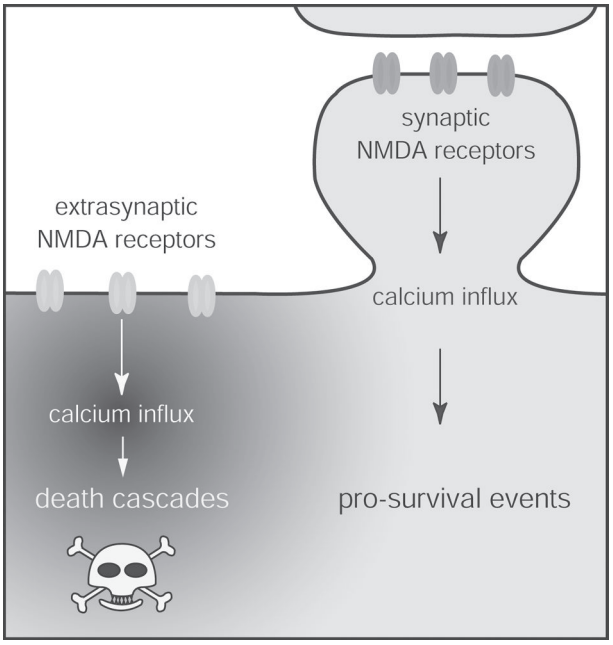

Fig. 1. Depending on their localization, NMDA receptors mediate contrasting effects: calcium influx through synaptic NMDA receptors triggers the activation of survival programs, while calcium influx via extrasynaptic NMDA receptors couples to cell death pathways (Hardingham et al., 2002).

which functions as a cytoplasmic calcium response element, and the cAMP response element (CRE) which responds to nuclear calcium signals (Hardingham et al., 1997). The CRE interacts with CREB to regulate the expression of several genes including brain-derived neurotrophic factor (BDNF) involved in cell survival (Bonni et al., 1999; Finkbeiner, 2000; Ghosh et al., 1994; Hardingham et al., 2002; Lonze and Ginty, 2002; Mantamadiotis et al., 2002). Mice lacking CREB and its relative, the cAMP response-element modulator (CREM), show extensive neuronal apoptosis and progressive neurodegeneration (Mantamadiotis et al., 2002). CREB may also be important for long-term synaptic plasticity, learning and memory (Barco et al., 2002; Cho et al., 1998).

CREB is also activated by hypoxia or transient ischemia *in vivo* (Lonze and Ginty, 2002; Mabuchi et al., 2001). Neurons that die following ischemia show only transient CREB phosphorylation, whereas surviving neurons have sustained CREB phosphorylation and express BDNF (Kokaia et al., 1995; Tanaka et al., 1999b; Walton et al., 1996; Walton and Dragunow 2000).

There are two principal calcium signaling pathways which can lead to CREB phosphorylation at its activator site, serine 133 (Fig. 2). One pathway involves the propagation of a calcium signal from the synapse to the nucleus. Nuclear calcium then activates calcium-calmodulin (CaM) dependent protein kinase IV, a potent CREB kinase (Finkbeiner and Greenberg, 1996; Hardingham et al., 2001b). The

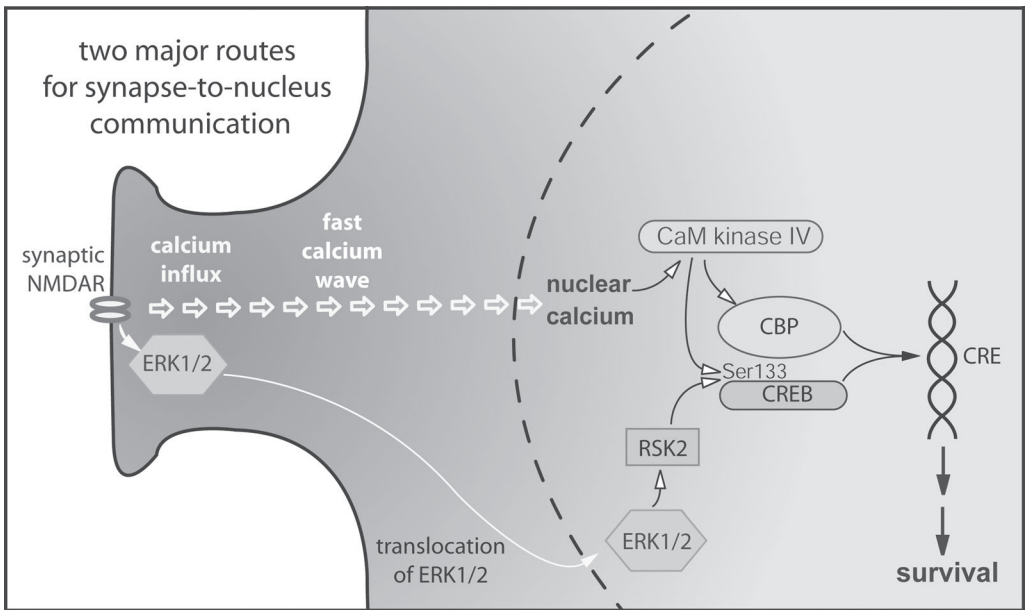

Fig. 2. Synaptic NMDA receptors signal to the nucleus to regulate neuronal survival via two major communication routes: a fast propagating calcium transient and a somewhat slower transduction mechanism involving ERK1/2 that translocate to the nucleus following their activation by calcium signals in the immediate vicinity of synaptic NMDA receptors (Hardingham et al., 2001a). Nuclear calcium activates CaM kinase IV, which leads to phosphorylation of CREB on Ser133, activation of CBP, and stimulation of CREB/CBP-mediated transcription (Hardingham et al., 1997; Chawla et al., 1998; Impey et al., 2002). ERK1/2 stimulate RSK2 (ribosomal S6 kinase 2) that can phosphorylate CREB on serine 133. The ERK1/2-RSK2 cascade is not sufficient for inducing CREB-dependent gene transcription, however, it can prolong CREB phosphorylation on serine 133 and thus serves as an auxiliary CREB activity-promoting pathway.

second signaling pathway is slower and involves ERK1/2 and RSK2 activation (Bading and Greenberg 1991; Bading et al., 1993; Hardingham et al., 2001a, 1999; Impey and Goodman 2001; Ginty et al., 1993; Wu et al., 2001).

BDNF is involved in CREB-activated survival

One of the target genes of CREB is BDNF which can promote neuron survival (Ghosh et al., 1994; Shieh et al., 1998; Tabuchi et al., 2002). In Huntington disease models, a decrease in BDNF production has been linked to the loss of striatal neurons while the expression of BDNF promotes survival (Kells et al., 2004; Zuccato et al., 2001). BDNF transcription is induced by KCl-induced membrane depolarization (activating L-type calcium channels) and upon stimulation of synaptic NMDA receptors (Ghosh et al., 1994; Shieh et al., 1998; Tao et al., 1998; Hardingham et al., 2002). Increased BDNF transcription leads to activation of TrkB, the BDNF receptor (Hardingham et al., 2002). The stimulation of TrkB receptors by BDNF can also increase CREB activity suggesting a cycle of positive feedback (Pizzorusso et al., 2000). Other neurotrophins such as nerve growth factor (NGF) may also exert their neuroprotective powers through the activation of CREB (Riccio et al., 1999).

Whereas the activation of synaptic NMDA receptors or L-type voltage-gated calcium channels can stimulate BDNF transcription, stimulation of extrasynaptic NMDA receptors with bath application of glutamate cannot (Hardingham et al., 2002). This failure to activate BDNF transcription most likely results from the dephosphorylation of CREB on its activator site serine 133 that is triggered by extrasynaptic NMDA receptors (Hardingham et al., 2002; Sala et al., 2000).

Extrasynaptic NMDA receptor activation leads to death

Several conditions including the exposure of neurons to hypoxic/low glucose media or the stimulation of extrasynaptic NMDA receptors with bath-applied glutamate causes rapid CREB dephosphorylation of its activator site serine 133 (Hardingham et al., 2002). A similar CREB dephosphorylation has also been observed following stroke *in vivo* (Tanaka et al., 1999a; Walton and Dragunow, 2000). One possible mechanism through which extrasynaptic NMDA receptors lead to CREB-shut off involves direct interaction with HDAC1 (histone deacetylase 1, a class I HDAC), and protein phosphatase 1 (PP1) (Canettieri et al., 2003). PP1 is also part of a signaling complex consisting of Yotiao, a scaffolding protein beneath the NMDA receptor, and PKA (protein kinase A), that is involved in regulating NMDA receptor activity (Westphal et al., 1999). Although direct evidence for PP1-induced cell death exists, blockade of PP1 has also been shown to promote cell death *in vitro* (Jiang et al., 2000). This points to a complex role of PP1, the precise action of which may depend on cofactors and the association with particular signaling complexes.

Histone deacetylases can be divided into class I and class II HDACs. A key to transcriptional regulation by class II HDACs lies in the control of their subcellular localization (de Ruijter et al., 2003). Death-promoting stimuli cause the caspase-dependent cleavage of class II HDAC4 and translocation of the amino-terminal fragment into the nucleus, which then induces cell death (Paroni et al., 2004). In contrast, synaptic activity in hippocampal neurons promotes nuclear export of class II HDACs (Chawla et al., 2003). One protein controlled by class II HDACs is the transcription factor MEF-2, which links the localization of HDACs to a possible survival event (Mao et al., 1999). The emerging view is that death-promoting stimuli cause the import of class II HDACs into the nucleus; this silences certain transcription factors and leads to the activation of death cascades. Synaptic NMDA receptors can counter-

act these mechanisms by inducing nuclear export of class II HDACs. This underscores the opposing roles of synaptic and extrasynaptic NMDA receptors in transcriptional regulation and the promotion of cell survival/cell death pathways.

ERK, JNK, and p38 MAP kinases

Many of the effects of NMDA receptor activation on gene transcription, survival and death are mediated by protein kinases including CaM kinases, ERK1/2 and the p38 MAP kinase (Fig. 3). The ERK1/2-pathway as well as the JNK (c-Jun N-terminal kinase) pathway have been shown to mediate pro-survival events (Dougherty et al., 2002; Li et al., 2003; Xia et al., 1995), while the induction of apoptosis correlates with the activation of the p38 MAP kinase (Cheng et al., 2001; Kawasaki et al., 1997; Xia et al., 1995). ERK1/2 may achieve this by phosphorylating and inactivating the pro-apoptotic factor BAD (Jin et al., 2002), while JNK phosphorylates Bcl-2 (Deng et al., 2001), which is able to inhibit efflux of cytochrome C from mitochondria, thereby preventing apoptosis (Yang et al., 1997). The actions of

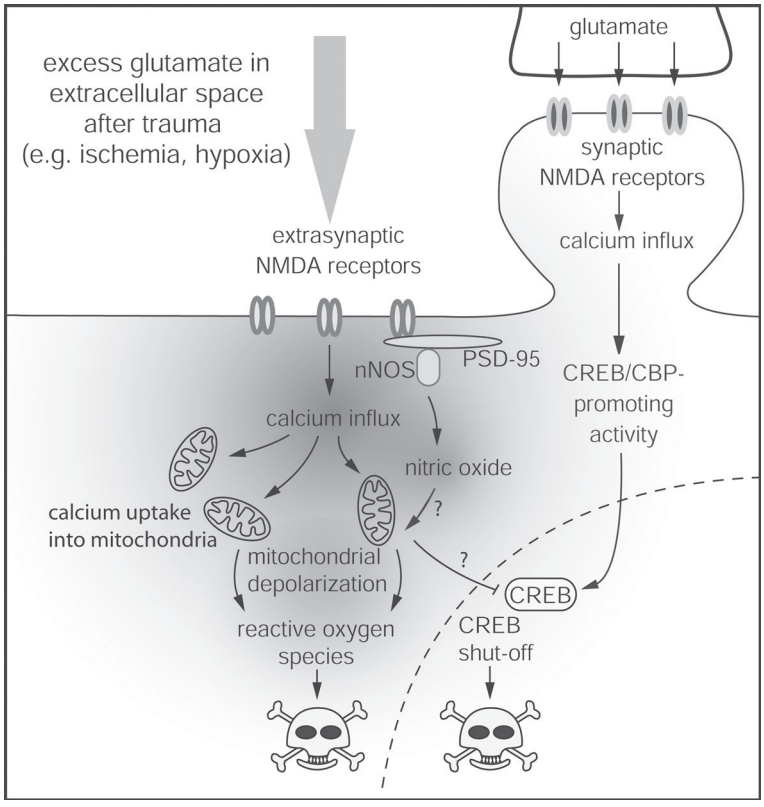

Fig. 3. Extrasynaptic NMDA receptors are thought to be activated by increases in glutamate concentrations in the extracellular (non-synaptic) space, which occur following hypoxic/ischemic insults. Calcium entry though extrasynaptic NMDA receptors leads to calcium uptake into mitochondria and to their depolarization; it also activates nNOS, and through an unknown mechanism, leads to the shut-off of CREB function. Mitochondrial dysfunction and NO synthesis lead to the production of reactive oxygen species that promote cell death.

ERK1/2 on survival/death, however, remain controversial. Evidence from animal models indicates that the ERK1/2 pathway is activated during focal cerebral ischemia and that its pharmacological blockade could significantly reduce the focal infarct volume following a transient middle cerebral artery occlusion (Alessandrini et al., 1999; Mori et al., 2002).

There is evidence for the involvement p38 MAP kinase in apoptosis (Bossy-Wetzel et al., 2004; Cao et al., 2004; Xia et al., 1995). Apoptosis is attenuated in a dose-dependent manner in cerebellar granule neurons by the p38 MAP kinase inhibitors SB203580 and PD169316 (Nath et al., 2001). P38 MAP kinase is a downstream target of Fas-mediated apoptosis in cerebellar granule neurons (Hou et al., 2002) and is capable of activating nuclear factors, including the pro-apoptotic factor Rb (Wang et al., 1999b). Hyper-phosphorylation of Rb leads to its dissociation from E2F1, a potent activator of apoptosis (Hou et al., 2000, 2001; O'Hare et al., 2000). P38 MAP kinase is also activated in response to neuronal stresses like glutamate toxicity (Kawasaki et al., 1997) and cerebral ischemia (Barone et al., 2001; Sugino et al., 2000).

The "source specificity" vs "calcium load" hypotheses

Although calcium influx clearly is an initiator of neurotoxicity, conflict exists as to the dependence of toxicity on a particular route of entry (the "source specificity" model) or whether the calcium source is irrelevant and toxicity relates simply to the intracellular calcium concentration (the "calcium load" hypothesis) (Eimerl and Schramm, 1994; Lu et al., 1996). The degree of cell death evoked by persistent glutamate or NMDA application is clearly related to the duration and concentration of intracellular calcium increases and the overload of mitochondria and their release of pro-apoptotic proteins such as cytochrome C (Hartley et al., 1993; Lu et al., 1996; Luetjens et al., 2000; Pivovarova et al., 2004). However equivalent calcium loads through L-type calcium channels are not (or much less) toxic (Hardingham and Bading, 2003; Sattler et al., 1998; Tymianski et al., 1993). Furthermore, calcium influx evoked by intense activation of synaptic NMDA receptors *in vitro* is not toxic whereas similar calcium loads following extrasynaptic NMDA receptor stimulation promote breakdown of the mitochondrial membrane potential and cell death (Hardingham et al., 2002).

Mitochondria

The close relationship between NMDA receptors and mitochondria has been proposed to explain the source specificity model (Peng and Greenamyre, 1998). Calcium entry through NMDA receptors is more rapidly absorbed by mitochondria than calcium entry from kainate activated or voltage dependent channels (Peng and Greenamyre, 1998) and has a lower threshold than that of L-type calcium channels for inducing mitochondrial depolarization (Keelan et al., 1999).

Mitochondria are closely linked to neurotoxicity (Nicholls and Budd, 2000). Focal ischemic lesions *in vivo* are associated with calcium dysregulation and mitochondrial collapse (Dirnagl et al., 1999) and the inhibition of mitochondrial calcium uptake greatly attenuates glutamate-induced cell death (Stout et al., 1998). Calcium entering the cell through NMDA receptors is absorbed by mitochondria through a uniporter whose function depends on the mitochondrial membrane potential. Collapse of this potential results in calcium and cytochrome C release, production of superoxides and finally cell death (Luetjens et al., 2000). Cell viability is also critically dependent on mitochondrial respiration and maintenance of glucose levels, achieved by glucokinase, which is regulated by BAD, and dephosphorylation of BAD during glucose-deprivation induces BAD-dependent cell death (Danial et al., 2003). BAD is also dephosphorylated by calcineurin (protein phosphatase 2B) after glutamate-induced calcium influx (Wang et al., 1999a).

PSD-95 and the coupling of NMDA receptors to mitochondria and nNOS production

NMDA receptors couple directly via their intracellular carboxyl terminus of either the NR1 or NR2 subunits to large complexes of cytoplasmic proteins including scaffolding, adaptor, cell adhesion and cytoskeletal proteins, as well as components of signal transduction pathways, some of which are calcium regulated (Husi et al., 2000; Pawson and Scott, 1997; Sheng and Pak, 2000). A structure in the postsynaptic membrane called the postsynaptic density (PSD) binds several scaffolding proteins including PSD-95, thereby linking NMDA receptors to signaling molecules important for synaptic plasticity (Migaud et al., 1998; Sheng and Kim, 2002). PSD-95 also links NMDA receptors to nitric oxide (NO) production that plays a role in NMDA-induced excitotoxicity (Sattler et al., 1999). The toxic effects of NMDA receptor activation may be mediated by a specific coupling between PSD-95 and neuronal NO synthase (nNOS) (Brenman et al., 1996) which catalyzes NO production (Dawson et al., 1991) leading to neurotoxicity (Lipton, 1999b). In addition, the coupling of NMDA receptors to the molecular machinery of the PSD may facilitate uptake of calcium into the mitochondria (Peng and Greenamyre, 1998) which can also lead to cell death (see above).

The deletion of the cytoplasmic carboxyl terminus of either the NR1 or NR2A subunits has been shown to reduce NMDA induced toxicity *in vitro* (Anegawa et al., 2000; Rameau et al., 2000). The disruption of the NR2B-PSD-95 interaction with short peptides has also been shown to partially protect from excitotoxicity both *in vitro* and *in vivo* (Aarts et al., 2002). While such treatments do not affect gating of the NMDA channel (Aarts et al., 2002), they may compromise or abolish NMDA receptor-mediated intracellular signalling or alter the localization or even surface expression of NMDA receptors (Sans et al., 2003; Sprengel et al., 1998; Steigerwald et al., 2000). Thus, either changes in the localization or surface expression of the NMDA receptor or its dissociation from NO production or mitochondrial calcium uptake may underlie the neuroprotective effect of disrupting the coupling between NMDA receptors and PSD-95.

The prevalence of NR2B subunits in extrasynaptic NMDA receptors

The subunit composition of NMDA receptors varies with their location. While NR2A containing receptors are predominantly confined to synapses, NR2B containing receptors are preferentially distributed extrasynaptically in rats (Charton et al., 1999; Lopez de Armentia and Sah, 2003; Tovar and Westbrook, 1999). Current evidence indicates that native NR2C subunit containing receptors are only present in cerebellum and NR2D containing receptors are not present within synapses in the brain (Brickley et al., 2003; Cull-Candy et al., 2001, 1998; Momiyama et al., 1996).

Electrophysiological evidence using NR2B selective antagonists and the kinetic characteristics of NMDA receptor currents has indicated that NR2B and not NR2A-containing receptors dominate NMDA receptor mediated synaptic transmission in young rats. However, as NR2A mRNA expression begins from around postnatal day 7, they begin contributing to, and by postnatal day 30, dominating synaptic NMDA currents. This developmental regulation of NR2 subunit distribution is qualitatively common to most brain regions examined to date including the hippocampus, cortex, cerebellum and lateral (but not central) amygdala (Flint et al., 1997; Lopez de Armentia and Sah, 2003; Monyer et al., 1994; Stocca and Vicini 1998; Zhong et al., 1995). Immunohistochemical and electrophysiological evidence has shown a similar redistribution of NR2 subtypes also occurs during the second and third weeks in cultured cortical neurons (Li et al., 1998; Tovar and Westbrook, 1999). This developmental regulation of NR2A and NR2B subunit distribution parallels the contribution of each receptor subtype to LTP induction (Kohr et al., 2003) and to the emergence of synchronous neuronal activity in cortical cultures (Opitz et al., 2002).

NR2B-containing receptors and neuronal death

Although NMDA receptor antagonists are known to induce neuronal apoptosis, antagonists selective for NR2B subunit containing receptors provide a degree of neuroprotection against cell death in ischemic and glutamate excitotoxicity models (Kundrotiene et al., 2004; Reyes et al., 1998; Williams et al., 2003). NMDA-induced apoptotic cell death appears to increase in cells transfected with mutant huntington and the NR1/NR2B but not the NR1/NR2A subunits (Zeron et al., 2001). In line with this evidence, NR2B subunits are highly expressed in medium spiny neurons of the striatum, the neuronal population selectively lost in Huntington disease. Not surprisingly, a potential therapeutic role of NR2B antagonists is currently emerging (Chazot, 2004).

It remains unclear whether the involvement of NR2B-containing NMDA receptors in neuron death relates to their localization, conductance characteristics or intracellular signaling mechanisms. NR2B-containing receptors have higher calcium permeability (Dingledine et al., 1999), show less desensitization (Krupp et al., 1996) and produce slower post-synaptic potentials (Carmignoto and Vicini, 1992; Flint et al., 1997; Vicini et al., 1998) than NR2A-containing receptors. The deactivation time constant for currents mediated by NR1/NR2A assemblies comprises tens of milliseconds, compared to hundreds of milliseconds for NR1/NR2B and several seconds for NR1/NR2D receptors (Cull-Candy et al., 2001; Monyer et al., 1994; Vicini et al., 1998; Wyllie et al., 1998). Thus the activation of NR2B-containing receptors will carry substantially more calcium into the neuron than would the activation of NR2A-containing receptors. Increased calcium entry via predominantly extrasynaptic NR2B-containing receptors may generate high calcium concentrations in specific micro-domains that may initiate death processes.

Conclusions

Synaptic and extrasynaptic NMDA receptors have fundamentally different effects on neuronal fate. Synaptic NMDA receptors promote survival, whereas extrasynaptic NMDA receptors trigger mitochondrial dysfunction and transcription shut-off pathways, and lead to neuronal degeneration and cell death. These findings have wide-ranging clinical implications, in particular for acute brain injury, hypoxia/ischemia and stroke during which extrasynaptic NMDA receptors are being activated. The development of drugs that specifically interfere with extrasynaptic NMDA receptors or their associated signaling complexes could be a novel avenue for therapeutic intervention in these pathological conditions.

Acknowledgments

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