SYMPOSIUM REPORT

Compartmentalized NMDA receptor signalling to survival and death

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The ability of Ca^{2+} influx through the *N*-methyl D-aspartate subclass of glutamate receptor (NMDA receptor) to both kill neurons and to promote survival under different circumstances is well established. Here we discuss the signal pathways that mediate this dichotomous signalling, and the factors that influence whether an NMDA receptor-dependent Ca^{2+} signal results in a net pro-survival or pro-death effect. The magnitude of NMDA receptor activation, be it intensity or duration, is of course very important in determining the nature of the response to an episode of NMDA receptor activity, with excitotoxic death pathways requiring higher levels than survival pathways. However, the NMDA receptor is not merely a conduit for Ca^{2+} influx: the consequences of NMDA receptor activity can be influenced by signalling molecules that physically associate with the NMDA receptor or indeed the location (synaptic *versus* extrasynaptic) of the receptor. Furthermore, we discuss the possibility that the Ca^{2+} effectors of survival and death are in different subcellular locations, and thus depend on the spatial characteristics of the Ca^{2+} transient. A greater understanding of these issues may point to ways of selectively blocking pro-death signalling in neurological disorders such as stroke, where global NMDA receptor antagonists have proved ineffective.

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It is long-established that high levels of glutamate kill neurons (Lucas & Newhouse, 1957; Olney, 1969). During an ischaemic episode, extracellular glutamate builds up due to synaptic release and impaired/reversed uptake mechanisms (Rossi *et al.* 2000; Camacho & Massieu, 2006). This glutamate induces excessive activation of the *N*-methyl D-aspartate subclass of glutamate receptor (NMDAR) which results in Ca^{2+}-dependent cell death (Arundine & Tymianski, 2004). Zn^{2+} influx and release from internal sites also contribute to ischaemic injury (Sensi & Jeng, 2004). However, while influx has been reported to take place through NMDARs, elevation of Zn^{2+} levels takes place mainly via other routes (Sensi & Jeng, 2004); NMDAR signalling to cell death is predominantly Ca^{2+} dependent. Excessive NMDAR activity can lead to cell death in other acute events such as mechanical trauma and seizure, and may contribute to chronic neurodegeneration in Alzheimer's disease (Lipton & Rosenberg, 1994; Chohan & Iqbal, 2006). The destructive effects of excessive NMDAR activity are in no doubt, nor the protective effects of NMDAR antagonists in blocking several animal models of neuronal injury. Nevertheless, a number of recent studies have shown that in some circumstances, survival of several neuronal types is dependent on physiological synaptic NMDAR activity (Ikonomidou & Turski, 2002; Hardingham & Bading, 2003). While elimination of NMDA receptor activity *in vivo* has long been established as protective in many excitotoxic scenarios, it causes widespread apoptosis and enhances trauma-induced injury in developing neurons (Gould *et al.* 1994; Ikonomidou *et al.* 1999; Pohl *et al.* 1999; Adams *et al.* 2004). In the adult CNS, NMDAR blockade exacerbates neuronal loss when applied after traumatic brain injury and during ongoing neurodegeneration (Ikonomidou *et al.* 2000), and prevents the survival of newborn neurons in the adult dentate gyrus (Tashiro *et al.* 2006). In most of these *in vivo* studies, the pro-survival role of NMDAR activity is exposed by the harmful effects of pharmacological blockade of normal physiological NMDAR activity. It is unclear whether elevating activity above this level would have a greater protective

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effect, or begin to have an excitotoxic effect. Responses of neurons to glutamate or NMDA follow a bell-shaped curve: both too much and too little NMDAR activity can be potentially harmful (Lipton & Nakanishi, 1999). This dose–response is consistent with the observation that too much and too little intracellular Ca^{2+} is harmful to neurons (Lipton & Kater, 1989). However, the NMDAR is not merely a conduit for Ca^{2+} influx, the consequences of NMDAR activity can be influenced by signalling molecules that physically associate with the NMDAR, the location (synaptic *versus* extrasynaptic) of the receptor, or the nature of the stimulation (chronic/low level *versus* transient/saturating).

Clinical trials for stroke with NMDA receptor antagonists have been unsuccessful

Despite an overwhelming body of evidence from animal studies implicating NMDAR activity in neuronal loss following ischaemia, the many clinical trials of different NMDAR antagonists for stroke have failed due to poor tolerance and efficacy (Ikonomidou & Turski, 2002; Muir, 2006). The fact that the NMDAR plays a central role in synaptic plasticity and transmission, and learning and cognition accounts for the undesired psychomimetic and CNS-adverse effects of antagonists (Muir, 2006). However, trial design may have been erring too far on the side of caution in seeking to avoid psychosis and other CNS-adverse effects, when these side-effects are on-target and not off-target effects. Other issues cloud a clear assessment of NMDAR antagonists, such as numbers of patients within the trials and time taken to administrate the drug. With many large pharmaceutical companies shying away from NMDAR antagonists, these issues may not be resolved any time soon.

Nevertheless, the growing body of evidence that physiological synaptic NMDAR activity exerts a neuroprotective effect has led to suggestions that it may play a role in promoting recovery and preventing delayed neuronal loss in the penumbra (Albers *et al.* 2001; Ikonomidou & Turski, 2002). Thus, global NMDAR antagonists may block NMDAR-activated pro-survival signals triggered in response to an ischaemic challenge, but interfere with some recovery or preconditioning processes in the penumbra. The anti-excitotoxic effects of NMDAR antagonists have never been in question, but until relatively recently the pro-survival role of the NMDAR was not known and so antagonists were not tested in contexts that would expose their harmful effects. In treating disorders associated with pro-death NMDAR signalling, it may be desirable to block pro-death signalling, without affecting pro-survival signalling or synaptic plasticity. This will require a thorough understanding of the nature of both survival and death pathways triggered by the NMDAR, and the factors that make an episode of NMDAR activity promote survival or death. Although the signals that mediate NMDAR signalling to death and survival are discussed in more detail elsewhere (Hardingham & Bading, 2003; Arundine & Tymianski, 2004; Hardingham, 2006; Hetman & Kharebava, 2006), there follows a very brief overview.

Death and survival signalling from the NMDAR

There are several fundamental mechanisms implicated in NMDAR-dependent cell death. In instances of extremely high NMDAR activity, simple Ca^{2+} overload may mediate fast necrotic cell death. However, in many cases, active mechanisms are implicated, even in what would be classically described as necrotic cell death. Mitochondrial dysfunction caused by excessive Ca^{2+} uptake by the mitochondria through the potential-driven uniporter (Stout *et al.* 1998) is one mechanism. The mitochondrial membrane becomes depolarized due to this uptake, which inhibits ATP production, and can cause depletion of cytosolic ATP due to reversal of the mitochondrial ATPase, resulting in loss of ion homeostasis and rapid cell death. In cases where mitochondrial depolarization and dysfunction are only partial, neuronal apoptosis may be triggered (Ankarcrona *et al.* 1995), for example due to cytochrome *c* release. In addition to causing mitochondrial dysfunction, toxic levels of NMDAR activation cause Ca^{2+} efflux to be impaired (Bano *et al.* 2005; Pottorf *et al.* 2006). Calpains, Ca^{2+}-dependent proteases, are activated by the excessive NMDAR-mediated Ca^{2+} influx and cleave a major isoform of the plasma membrane Na^{+}–Ca^{2+} exchanger (NCX3) impairing its function (Bano *et al.* 2005). The plasma membrane Ca^{2+}-ATPase pump (PMCA), which would utilize the energy from ATP hydrolysis to transport Ca^{2+} across the plasma membrane, is inactivated by excitotoxic insults via mechanisms attributed to both caspases (Schwab *et al.* 2002) and calpains (Pottorf *et al.* 2006). NMDAR activity-regulated overactivation of the Ca^{2+}-dependent nNOS (neuronal nitric oxide synthase) also has toxic downstream responses, including mitochondrial dysfunction, p38 mitogen-activated protein kinase signalling and TRPM (transient receptor potential melastatin) channel activation (Aarts *et al.* 2003; Arundine & Tymianski, 2004).

The PI3K (phosphoinositide-3-kinase)–Akt kinase cascade is a key signalling pathway responsible for the pro-survival effects of NMDAR activity (Lafon-Cazal *et al.* 2002; Papadia *et al.* 2005; Soriano *et al.* 2006). Akt phosphorylates and inactivates GSK3 (glycogen synthase kinase-3β; Cross *et al.* 1995), and also triggers phosphorylation and nuclear export of the FOXO (forkhead box O) subfamily of forkhead transcription

factors, promoting down-regulation of pro-death genes such as Fas ligand (Brunet *et al.* 1999). NMDAR signalling to Akt-dependent FOXO export has also been observed in the context of NMDAR signalling (Soriano *et al.* 2006). Akt also inactivates the pro-apoptotic Bcl-2 family member BAD (Bcl-2/Bcl-X_L-antagonist causing cell death), the JNK/p38 activator ASK1 (Kim *et al.* 2001), and also the pro-death transcription factor p53 (Yamaguchi *et al.* 2001).

Another key mediator of activity-dependent gene expression is the transcription factor CREB, which binds to the cAMP response element (CRE) and promotes expression of a number of pro-survival genes. CREB-dependent gene expression is causally linked to the long-lasting phase of synaptic NMDAR-dependent neuroprotection against apoptotic insults (Papadia *et al.* 2005). A recent study identified two genes that contribute to synaptic NMDAR-dependent neuroprotection (Zhang *et al.* 2007): *Btg2*, a potentially anti-apoptotic CREB target gene, and *Bcl6*, a transcriptional repressor implicated in suppression of p53, also potentially a CREB target. Another CRE-regulated candidate is the pro-survival neurotrophin BDNF (brain-derived neurotrophic factor), which is up-regulated by NDMAR activity (Hardingham *et al.* 2002) and is known to promote neuronal survival (Shieh *et al.* 1998; Hetman & Kharebava, 2006). NMDAR blockade *in vivo* reduces BDNF mRNA expression and *in vitro* supplementation of neurons with BDNF can rescue them from NMDAR antagonist-caused neuronal death (Hansen *et al.* 2004).

Stimulus intensity is important in determining the outcome of an episode of NMDAR activity

The magnitude of activation, be it intensity or duration, is very important in determining the nature of the response to an episode of NMDAR activity. The classical bell-shaped curve model of the neuronal response to NMDA or glutamate implies that the Ca^{2+} effectors of pro-survival signalling have a higher affinity (i.e. considerably lower requirements for Ca^{2+}) than the Ca^{2+} effectors of death. Therefore the Ca^{2+} concentration threshold for activating key pro-survival signalling cascades such as PI3K, ERK1/2 (extracellular signal-regulated kinase 1/2) and CREB's activator CaMKIV (Ca^{2+}–calmodulin-dependent protein kinase IV) must be lower than that necessary to trigger toxic levels of calpain activation, mitochondrial Ca^{2+} uptake or NO production. The key Ca^{2+}-dependent components of the survival pathways are in the main activated not by Ca^{2+} directly, but by Ca^{2+}–calmodulin. Calmodulin is a ubiquitous Ca^{2+}-binding protein which changes conformation when it binds to Ca^{2+}. Ca^{2+}–calmodulin then activates a number of downstream signals, including CaMKIV, PI3K and upstream activators of ERK1/2. As a physiological sensor of elevated Ca^{2+} levels, calmodulin is designed to be significantly activated by relatively modest increases in Ca^{2+} (around 1 μM), particularly *in vivo*, or in the presence of its downstream effectors (Bayley *et al.* 1996; Torok *et al.* 1998).

In contrast, central mediators of NMDAR-dependent cell death, calpains, are not Ca^{2+}–calmodulin dependent but are activated by Ca^{2+} directly, and require higher levels to be fully induced: half-maximal activity is around 4 μM for μ-calpain, and far higher for m-calpain, even in the presence of μ-calpain (Baki *et al.* 1996; Tompa *et al.* 1996). The same can be said of the mitochondrial uniporter, which senses Ca^{2+} directly and is only weakly active at < 2–3 μM Ca^{2+} (Nicholls, 2004). Furthermore, it is likely that toxic levels of matrix Ca^{2+} are only achieved when cytoplasmic levels are far higher than this. Another Ca^{2+}-dependent promoter of neuronal death, nNOS, *is* Ca^{2+}–calmodulin dependent and indeed plays important roles in non-pathological signalling processes such as synaptic plasticity. However, nNOS is also regulated by phosphorylation, and a recent study showed that, in contrast to non-toxic stimuli, high levels of glutamate fail to trigger an inhibitory phosphorylation event that ensures that nNOS activation is transient (Rameau *et al.* 2007). Thus, nNOS becomes excessively active and contributes to excitotoxic cell death (Rameau *et al.* 2007).

Where in the neuron are the Ca^{2+} effectors of survival and death?

As stated earlier, in treating disorders associated with pro-death NMDAR signalling, it would be desirable to be able to interfere with pro-death signalling from the NMDAR, while sparing pro-survival signals. While death and survival signalling pathways are both activated by Ca^{2+} influx, a greater knowledge as to the spatial Ca^{2+} requirements to activate these pathways may aid in their selective inhibition, and enhance our understanding of the dichotomous nature of NMDAR signalling. Recent studies suggest that the Ca^{2+} effectors of survival and death exist in different parts of the cell, potentially requiring different protein–protein interactions thus pointing to more specific ways of targeting of pro-death events.

NMDAR signalling to neuronal death may involve mitochondrial, and membrane proximal events

Neurons do not respond in a stereotypical way to Ca^{2+} influx: the channel through which Ca^{2+} enters can affect the response (Bading *et al.* 1993; Hardingham *et al.* 1999; Dolmetsch *et al.* 2001). In the case of excitotoxicity, Ca^{2+} influx specifically through NMDARs promotes cell death more efficiently than through voltage-gated Ca^{2+} channels (Tymianski *et al.* 1993; Arundine & Tymianski, 2004). An explanation for this, the 'source-specificity

hypothesis', proposes that neuron-specific enzymes or substrates responsible for Ca^{2+}-dependent excitotoxicity are co-localized with NMDARs. The cytoplasmic tail of NMDAR subunits are linked to a network of neuronal proteins, the so-called NMDAR signalling complex. A role for this complex in mediating NMDAR-dependent death was shown in the case of nNOS, which is linked to NR2 subunits via PSD-95. When the interaction of NR2B and PSD-95 was disrupted, the NMDAR was uncoupled from NO production, reducing excitotoxic signalling (Aarts *et al.* 2002). An important target of NMDAR signalling to NO production in excitotoxicity is the cation channel TRPM7 (Aarts *et al.* 2003). NMDAR-dependent Ca^{2+} influx triggers both NO production via nNOS activation and superoxide production via mitochondrial Ca^{2+} uptake, which combine to form $ONOO^{-}$, an activator of TRPM7. Since TRPM7 itself passes Ca^{2+}, this results in a positive feedback loop.

Other membrane-proximal events that contribute to excitotoxicity involve impairment of Ca^{2+} efflux (Schwab *et al.* 2002; Bano *et al.* 2005; Pottorf *et al.* 2006). As stated earlier, both NCX and PMCA are targeted for cleavage and inactivation by calpains and caspases. While by definition these events take place at the plasma membrane, the locus at which Ca^{2+} acts to initiate these events is unclear, although caspase activation may require mitochondrial depolarization to initiate cytochrome *c* release. Indeed, as discussed earlier, mitochondrial dysfunction caused by excessive Ca^{2+} uptake by the mitochondria through the potential-driven uniporter is a key mechanism by which NMDAR activity promotes cell death (Stout *et al.* 1998).

NMDAR-activated survival pathways involve nuclear Ca^{2+} signalling

Activation of CREB-dependent gene expression is strongly induced by synaptic NMDAR activity (Hardingham *et al.* 2002). Ca^{2+} influx through the synaptic NMDAR activates the Ras/ERK pathway in the cytoplasm and the nuclear Ca^{2+}–calmodulin-dependent protein kinases, principally CaMKIV. CaMKIV mediates fast CREB phosphorylation at Ser-133, whereas the ERK1/2 pathway promotes CREB phosphorylation in a slower, more long-lasting manner (Hardingham *et al.* 2001*a*; Wu *et al.* 2001). Ser-133 phosphorylation of CREB is necessary to recruit CBP (CREB-binding protein), a transcriptional cofactor, to CREB. The transactivation potential of CBP is itself positively regulated by NMDAR activity (Hardingham *et al.* 1999) by a mechanism involving its phosphorylation on Ser-301 by CaMKIV (Impey *et al.* 2002). Gene expression mediated by CREB can also be mediated by another family of CREB coactivators, the TORCs (transducers of regulated CREB activity; Screaton *et al.* 2004).

Elevation of Ca^{2+} within the nucleus is important for full activation of CREB-dependent gene expression (Hardingham *et al.* 1997, 2001*b*; Papadia *et al.* 2005), probably due to the nuclear localization of CREB and CBP's activator CaMKIV. CREB-dependent gene expression is causally linked to the long-lasting phase of synaptic NMDAR-dependent neuroprotection against apoptotic insults (Papadia *et al.* 2005). This long-lasting phase is also dependent on nuclear Ca^{2+}–calmodulin signalling (Papadia *et al.* 2005), consistent with the requirement for nuclear Ca^{2+} in CREB activation. Furthermore, activity-dependent induction of pro-survival genes *Bcl6* and *Btg2* also rely on nuclear Ca^{2+} signalling. Interestingly, nuclear Ca^{2+} is also implicated in regulating memory consolidation (Limback-Stokin *et al.* 2004), though whether this is via CREB activation is not known. Thus the nucleus is a major site for physiological effects of NMDAR signalling. However, it appears not to play a role in excitotoxic cell death since inhibition of Ca^{2+}–calmodulin signalling does not interfere with NMDAR-dependent excitotoxic cell death (G. E. Hardingham, unpublished observations).

The other key pro-survival signalling cassette induced by synaptic NMDAR activity is the PI3K–Akt pathway (Hetman & Kharebava, 2006). This pathway exerts multiple effects in the cytoplasm (see above) and is thought to be activated by cytoplasmic Ca^{2+} elevation, probably due to the Ca^{2+}–calmodulin dependence of PI3K (Joyal *et al.* 1997). While synaptic activity is ongoing, the PI3K–Akt pathway is sufficient to mediate NMDAR-dependent neuroprotection (i.e. CREB activation is not required; Papadia *et al.* 2005). Activation of the PI3K–Akt pathway does not rely on nuclear Ca^{2+} signalling (G. E. Hardingham, unpublished observations) and consistent with this, the PI3K-dependent, CREB-independent phase of neuroprotection also does not require nuclear Ca^{2+} signalling (Papadia *et al.* 2005). Furthermore, PI3K activation requires elevation of bulk cytoplasmic Ca^{2+} and is not activated by submembranous Ca^{2+} (G. E. Hardingham, unpublished observations). Thus, it appears that in cortical neurons, the NMDAR-activated mediators of survival and death have different spatial requirements for Ca^{2+}.

Influence of the location of the receptor on the nature of NMDAR signalling

Aside from stimulus intensity, the location of the NMDAR may also profoundly affect the signals that emanate from the NMDAR. Developing neurons have sizeable pools of NMDARs at extrasynaptic, as well as synaptic locations, which signal very differently. Ca^{2+} influx dependent on intense synaptic NMDAR activation is well tolerated by cells whereas activation of extrasynaptic NMDARs, either on their own or in the presence of synaptic NMDAR

activation, causes a loss of mitochondrial membrane potential and cell death (Hardingham *et al.* 2002).

Differential synaptic *versus* extrasynaptic NMDAR effects also extend to other signal pathways. While synaptic NMDAR activity strongly induces CREB-dependent gene expression, extrasynaptic NMDARs are coupled to a CREB shut-off pathway (Hardingham *et al.* 2002) in a developmentally regulated manner (Hardingham & Bading, 2002). It has also been shown that there is opposing regulation of the ERK1/2 pathway by synaptic and extrasynaptic NMDARs in hippocampal neurons: synaptic NMDARs activate the ERK pathway whereas extrasynaptic NMDARs evoke ERK inactivation (Ivanov *et al.* 2006). A recent study involving genome-wide expression analysis has extended our understanding of synaptic *versus* extrasynaptic signalling (Zhang *et al.* 2007). While synaptic NMDARs activated a number of pro-survival genes (including the aforementioned *Btg2* and *Bcl6*), extrasynaptic NMDARs failed to do this, and in fact activated expression of a gene, *Clca1*, which kills neurons. It will be of interest to know whether *Clca1* is activated *in vivo* during excitotoxic trauma, and whether it represents a potential therapeutic target.

The molecular basis for the apparent differences in synaptic/extrasynaptic NMDAR signalling could be due to differences in the way these two populations of receptors become stimulated, since the former are stimulated trans-synaptically with transiently saturating levels of glutamate, while the latter can only be activated chronically by extracellular glutamate. Thus, generating similar Ca^{2+} loads via these different stimulation protocols could conceivably activate different signalling cascades. Also, the composition of the synaptic *versus* extrasynaptic NMDAR signalling complexes as opposed to the location of the receptors *per se* may be critical, though these potential differences await study. Tied in with this is the possibility that differences in receptor subunit composition, rather than location, are important, although there is little evidence that differences in subunit composition are dramatic enough to explain the effects observed (Thomas *et al.* 2006). However, a recent study has contended that NR2B-containing NMDARs tend to promote neuronal death, irrespective of location (synaptic or extrasynaptic) while NR2A-containing NMDARs promote survival (Liu *et al.* 2007). The investigation of subunit-specific differences in NMDAR signalling is hampered by the lack of a NR2A-specific antagonist that is sufficiently selective to discriminate in the physiological scenario of trans-synaptic stimulation (Frizelle *et al.* 2006; Neyton & Paoletti, 2006).

Conclusions

We have an increasing understanding as to the molecular mechanisms of NMDAR signalling to survival and death. This has led to an appreciation of how different signal cascades require different levels of Ca^{2+}, and indeed how the spatial properties of the Ca^{2+} signal may also be important. Recent studies suggest a central role of nuclear Ca^{2+} signalling in regulating pro-survival gene expression. In contrast, mitochondria, and certain membrane-proximal interactions appear to be important for pro-death signalling. A knowledge of where in the neuron Ca^{2+} is acting to exert pro-death or pro-survival effects may aid in selectively uncoupling the NMDAR from harmful consequences.

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