Inhibiting pro-death NMDA receptor signaling dependent on the NR2 PDZ ligand may not affect synaptic function or synaptic NMDA receptor signaling to gene expression

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NMDA receptors (NMDARs) mediate ischemic brain damage, in part through interactions of the PDZ ligand of NR2 subunits with the PDZ domain proteins PSD-95 and neuronal nitric oxide synthase located within the NMDAR signaling complex. We have recently shown that this PDZ ligand-dependent pathway promotes neuronal death via p38 activation. A peptide mimetic of the NR2B PDZ ligand (TAT-NR2B9c) reduces p38-mediated death in vitro and p38-dependent ischemic damage in vivo. In the absence of the PDZ ligand-p38 pathway, such as in TAT-NR2B9c-treated neurons, or in NMDAR-expressing non-neuronal cells, NMDAR-dependent excitotoxicity is mediated largely by JNK and requires greater Ca2+ influx. A major reason for blocking pro-death signaling events downstream of the NMDAR as an anti-excitotoxic strategy is that it may spare physiological synaptic function and signaling. We find that neuroprotective doses of TAT-NR2B9c do not alter the frequency of spontaneous synaptic events within networks of cultured cortical neurons nor is mini-EPSC frequency altered. Furthermore, TAT-NR2B9c does not inhibit the capacity of synaptic NMDAR activity to promote neuroprotective changes in gene expression, including the upregulation of PACAP via CREB, and suppression of the pro-oxidative FOXO target gene Txnip. Thus, while the NR2 PDZ ligand does not account for all the excitotoxic effects of excessive NMDAR activity, these findings underline the value of the specific targeting of death pathways downstream of the NMDAR.

It is long-established that high and prolonged levels of glutamate kill neurons.1 During an ischemic episode, extracellular glutamate builds up due to synaptic release and impaired/reversed uptake mechanisms.2 This glutamate induces excessive activation of the N-methyl D-aspartate subclass of glutamate receptor (NMDAR) which results in Ca2+-dependent cell death.3 The destructive effects of excessive NMDAR activity are in no doubt, and nor is the protective effects of NMDAR antagonists in blocking several animal models of neuronal injury. However, NMDARs also have an important role in normal physiology, so cannot be blocked with impunity. NMDARs are heavily involved in synaptic transmission and synaptic plasticity, so antagonists can have considerable CNS-adverse effects.4,5 Moreover, modest levels of NMDAR activity can exert a neuroprotective effect.6 In the adult CNS, NMDAR blockade exacerbates neuronal loss when applied after traumatic brain injury and during ongoing neurodegeneration,7 and prevents the survival of newborn neurons in the adult dentate gyrus.8 Also, ischemic tolerance is thought to be mediated, at least in part, by NMDAR activity.9

Neurons do not respond in a stereotypical way to Ca2+ influx: the channel through which Ca2+ enters can also affect the response.3,10 In the case of excitotoxicity, Ca2+ influx specifically through NMDARs promotes cell death more efficiently than through voltage-gated Ca2+ channels.3,11 An explanation for this: the ‘source-specificity hypothesis’ proposes that neuron-specific enzymes or substrates responsible for Ca2+ dependent excitotoxicity are co-localized with NMDARs.

The cytoplasmic tail of NMDAR subunits is linked to a network of neuronal proteins, the so-called NMDAR signaling complex (NSC). A role for the NSC in mediating NMDAR-dependent death was shown in the case of the PDZ proteins neuronal nitric oxide synthase (nNOS) and PSD-95.12 PSD-95 is linked to the C-terminal PDZ ligand of NR2, and also binds to nNOS. When the interaction of NR2 and PSD-95 is disrupted (using TAT-NR2B9c, a cell-permeable peptide mimetic of the NR2 PDZ ligand) the NMDAR becomes uncoupled from nNOS activation, reducing (but not eliminating) NMDAR-dependent excitotoxicity.12 The important role of nNOS and PSD-95 above any other PDZ proteins in mediating NMDAR-dependent excitotoxicity was recently demonstrated.13

Targeting interactions within the NSC in order to reduce excitotoxic
Uncoupling the NMDA receptor from death signals

signaling is therefore a potentially attractive proposition, since this may be more selective than simple NMDAR blockade.

We recently showed that reducing the activation of the pro-death p38 pathway is an important consequence of uncoupling NR2/PSD-95 interactions with TAT-NR2B9c. TAT-NR2B9c is able to achieve this without influencing NMDAR-dependent Ca\(^{2+}\) influx or interfering with a model of NMDAR-dependent synaptic plasticity, suggesting that targeting the NSC may spare some of the normal functions of the NMDAR. Further investigations revealed that TAT-NR2B9c does not affect either miniEPSC frequency, or the frequency of spontaneous EPSCs (Fig. 1A and B). Thus, by these metrics, synaptic properties are largely unaffected. Furthermore, TAT-NR2B9c’s effect on the p38 pathway was specific to its activation by the NMDAR: activation of p38 by peroxide was unaffected by TAT-NR2B9c (Fig. 2).

We also studied the effect of TAT-NR2B9c on the activation of Akt by synaptic NMDAR activity, since this pathway is an important mediator of activity-dependent neuroprotection. As with previous studies, synaptic activity was enhanced by disinhibiting the neuronal cultures by treatment with a GABA\(_A\) receptor blocker, bicuculline, which induces action potential bursting and concomitant intracellular Ca\(^{2+}\) transients dependent on NMDAR activity and augmented by release of Ca\(^{2+}\) from internal stores. We found that Akt activation was not impeded by TAT-NR2B9c. One pro-survival consequence of NMDAR-dependent Akt activation is the nuclear export of FOXOs and the transcriptional suppression of the pro-oxidant gene Txinp, a thioredoxin inhibitor. We found that TAT-NR2B9c did not interfere with activity-dependent export of FOXO1, nor suppression of Txinp expression, in contrast to the NMDAR antagonist MK-801 (Fig. 3A and B). Activation of CREB-mediated gene expression is another contributor to activity-dependent neuroprotection. TAT-NR2B9c did not interfere with activity-dependent induction of the CREB target gene Adcyap1, which encodes the neuroprotective ligand, pituitary adenylate cyclase activating polypeptide (PACAP, Fig. 3C).

Whilst interfering with NMDAR-dependent p38 activation using TAT-NR2B9c was neuroprotective, the effect was not complete: death could still be achieved by using higher doses of NMDA. Other pro-death pathways remain unaffected by TAT-NR2B9c, including one mediated by JNK. Targeting JNK with the cell-permeable peptide inhibitor D-JNKI1, in addition to blocking p38 signaling by TAT-NR2B9c was additively protective in in vitro and in vivo models of excitotoxicity. These results indicate that activation of JNK did not require signaling via the NR2 PDZ ligand. Indeed, NMDAR-dependent JNK signaling appears not to require the NSC at all. NMDAR-dependent neuronal death can be recapitulated in non-neuronal cells simply by expression of functional NMDARs. When we studied the pathway responsible for this death in NMDAR-expressing AtT20 cells (NR-AtT20 cells), we found the death to be JNK-dependent. Therefore, it appears that the NMDAR can trigger pro-death pathways with differing reliance on neuron-specific signaling molecules. The protective effect of TAT-NR2B9c can be overcome in neurons by increasing the NMDAR-dependent Ca\(^{2+}\) load, by exposing neurons to higher concentrations of NMDA.

Thus, while NMDAR-dependent cell death can be reconstituted in non-neuronal cells, the p38 route to death is absent. One would predict therefore that the NMDAR would be less effective at promoting death when placed outside its neuronal context, away from neuron-specific signaling proteins. To obtain an indication of this, we performed a series of measurements to calculate the NMDAR-dependent Ca\(^{2+}\) load per unit cell volume required to kill a neuron, compared to a NR-AtT20 cell. We first determined the Ca\(^{2+}\) load associated with a half-maximally toxic dose of glutamate in neurons (Fig. 4A and B) and NR-AtT20 cells (see Fig. 1B and C from ref. 14). Our comparison took into account AtT20 transfection efficiency (based on NR1 immunoreactivity). To correct for...
Figure 3. TAT-NR2B9c does not interfere with synaptic NMDAR-dependent pro-survival signaling to CREB or FOXO1. (A) TAT-NR2B9c does not impair PI3K-dependent FOXO1 export. Neurons were transfected with vectors encoding eGFP and myc-tagged FOXO1. 24 h post-transfection the neurons were pre-treated with TAT-NR2B9c (2 μM) or LY294002 (a selective inhibitor of PI3-kinase 50 μM) for 1 h prior to bicuculline stimulation (50 μM, 1 h) to stimulate synaptic activity (reviewed in ref. 14). Cells were then fixed and processed for immunocytochemistry with an anti-myc antibody (9E10). FOXO1 subcellular distribution was scored for around 150 cells per treatment across 3 independent experiments. Graph shows quantification of the data; example pictures are also shown for each treatment. Immunofluorescent detection of proteins in neurons was performed as described previously.30 (B) TAT-NR2B9c does not impair synaptic NMDAR-dependent suppression of Tnpxp transcription. Neurons were pre-treated with TAT-NR2B9c (2 μM) or MK-801 (10 μM) for 1 h prior to bicuculline stimulation for 4 h. *p < 0.05 compared to unstimulated neurons (n = 5). For details of primers used see ref. 18 (C) TAT-NR2B9c does not impair synaptic NMDAR-dependent activation of Adcyap1 transcription. Neurons were pre-treated with TAT-NR2B9c (2 μM) or MK-801 (10 μM) for 1 h prior to bicuculline stimulation for 4 h. *p < 0.05 compared to unstimulated neurons (n = 5).

Figure 4. Increased NMDAR-mediated Ca2+ influx is required to kill NMDAR-expressing AtT20 cells than is required to kill neurons. (A) Dose-dependent NMDAR-dependent cell death in cortical neurons. Cortical neurons were treated with glutamate for 1 h and then returned to glutamate-free medium. Cells were fixed at 24 h, DAPI stained, and cell death counted (n = 3). Line indicates the concentration of glutamate required to achieve 50% death. (B) Dose-dependent NMDAR-dependent Ca2+ influx. Cortical neurons were incubated in 45Ca2+-containing medium and treated with glutamate (10 min). Line indicates the Ca2+ load associated with 50% death. (C) Example 3-D representations of eGFP-expressing AtT20 cells and neuron (cell body), obtained by confocal microscopy and image deconvolution [Scale bar 5 μm]. Also shown is a maximum intensity projection (MIP) of the confocal stack of a region of neuronal processes (90 μm x 90 μm). D) Ca2+ entry through NMDARs is more efficient at killing cortical neurons than NR-AtT20 cells. Relative Ca2+ load required to half-maximally kill NR-AtT20 cells vs. cortical neurons, taking into account relative cell volume. *p < 0.05, 2-tailed Student’s t-test.
the differences in cell volume, which was measured by acquiring 3-D deconvoluted models of eGFP-expressing cells (examples in Fig. 4C, see Methods). AtT20 cell volume was found to be 1.84 ± 0.16 pl (n = 6) and neuronal volume found to be 10.4 ± 0.9 pl (neuron, n = 6). Based on this, NR-AtT20 cells need significantly more NMDAR-dependent Ca2+ load to kill them than neurons (Fig. 4D). This is consistent with the absence of NMDAR induction of p38-dependent pro-death signaling in NR-AtT20 cells.14

To conclude, pro-death signaling by excessive NMDAR activity is mediated by multiple pathways, including p38 and JNK, which have differing requirements for the NSC. Using combined approaches to block specifically individual pathways may provide optimal protection against excitotoxic insults, while sparing physiological NMDAR signaling. At present it is not clear whether specific interactions involving NMDAR-associated proteins are important for activation of other mediators of excitotoxicity, such as calpains23 and TRPM7 activation.24 Moreover, it remains to be seen whether differential coupling of the NMDAR to Ca2+ effectors of cell death underlies the reported differences in pro-death signaling by synaptic versus extrasynaptic NMDARs18,25-27 or NR2A- vs. NR2B-containing NMDARs.28

Materials and Methods

All methods except those listed below were described previously. 14

Cell volume measurements. Cell volume measurements were performed using cortical neurons or AtT20 cells transfected with eGFP-N1 and were imaged using a Zeiss LSM 510 Axiovert confocal laser-scanning microscope. Data were acquired at Nyquist sampling rates with voxel dimensions of 59.509 mm (X) x 59.509 mm (Y) x 170 mm (Z), using a Zeiss C-Apochromat 1.2 NA 63x water corrected immersion objective lens. Z-stacks were acquired optimally with intensity values falling within the dynamic range of the detectors and extending above and below the cell to obtain all available fluorescence. Imaging was performed on living cells, maintained at 37°C in 5% (v/v) CO2, 95 % (v/v) air. Image data were deconvolved using Huygens software (Scientific Volume Imaging) before volume calculation using Volocity (Improvison). In the case of neurons, this technique was used to calculate the volume of the cell body. To estimate the mean diameter of a process, the total area contained within a network of processes, from a most intense pixel projection of a z-stack, was divided by the total process length calculated using ImageJ (http://rsb.info.nih.gov/ij/) and the NeuronJ plugin.29 Using this technique, the mean process diameter was found to be 0.74 μm, comfortably above the resolution of the technique which is 223 nm (0.61*wavelength (512 nm)/NA(1.4)). Moreover, the average diameter of the top 3% of widest processes was only 0.81 μm, demonstrating that the processes had a fairly narrow distribution of diameters, and that the average was not skewed by outliers. The average process volume per neuron was calculated by determining the average length of process per neuron. To calculate this, a number of images were taken and the total process length traced (as above). This length was then divided by the number of cell bodies in the fields (a total of 25, within six fields) to give the average process length per neuron. For display, representative images are shown surface rendered using Imaris (Bitplane).

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