Brief Communications

Neuroprotection by the NR3A Subunit of the NMDA Receptor

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Hyperactivation of NMDA-type glutamate receptors (NMDARs) results in excitotoxicity, contributing to damage in stroke and neurodegenerative disorders. NMDARs are generally comprised of NR1/NR2 subunits but may contain modulatory NR3 subunits. Inclusion of NR3 subunits reduces the amplitude and dramatically decreases the Ca^{2+} permeability of NMDAR-associated channels in heterologous expression systems and in transgenic mice. Since excessive Ca^{2+} influx into neurons is a crucial step for excitotoxicity, we asked whether NR3A subunits are neuroprotective. To address this question, we subjected neurons genetically lacking NR3A to various forms of excitotoxic insult. We found that cultured neurons prepared from NR3A knock-out (KO) mice displayed greater sensitivity to damage by NMDA application than wild-type (WT) neurons. *In vivo*, neonatal, but not adult, WT mice contain NR3A in the cortex, and neonatal NR3A KO mice manifested more damage than WT after hypoxia–ischemia. In adult retina, one location where high levels of NR3A normally persist into adulthood, injection of NMDA into the eye killed more retinal ganglion cells in adult NR3A KO than WT mice. These data suggest that endogenous NR3A is neuroprotective. We next asked whether we could decrease excitotoxicity by overexpressing NR3A. We found that cultured neurons expressing transgenic (TG) NR3A displayed greater resistance to NMDA-mediated neurotoxicity than WT neurons. Similarly *in vivo*, adult NR3A TG mice subjected to focal cerebral ischemia manifested less damage than WT mice. These data suggest that endogenous NR3A protects neurons, and exogenously added NR3A increases neuroprotection and could be potentially exploited as a therapeutic.

Introduction

NMDA-type glutamate receptors (NMDARs) act as integrators of coincident synaptic signals (Malenka and Nicoll, 1999). Hyperactivation, however, causes neurodegeneration (Lipton and Rosenberg, 1994; Dingledine et al., 1999; Cull-Candy et al., 2001). Conventional NMDARs require two distinct subunits, NR1 plus NR2(A-D), to form functional channels (Hollmann and Heinemann, 1994). We and our colleagues identified a third family of NMDAR subunits, including NR3A (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998).

Expression of NR3A subunits in the brain is regionally and temporally restricted (Wong et al., 2002). The level of NR3A mRNA in the cerebrocortex peaks at postnatal day (P)7–10 and then rapidly decreases. In adult cortex and hippocampus, NR3A expression is very low. Interestingly, however, NR3A persists in the retina into adulthood (Wong et al., 2002).

In heterologous expression systems, the addition of NR3A decreases the unitary conductance and Ca^{2+} permeability of NR1/NR2 channels (Das et al., 1998; Pérez-Otaño et al., 2001; Sasaki et al., 2002). Consistent with these findings, the amplitude of NMDA whole-cell currents in NR3A KO neurons is larger than that of wild-type (WT) neurons and displays a larger Ca^{2+} conductance (Das et al., 1998; Sasaki et al., 2002). Conversely, transgenic (TG) mice expressing exogenous NR3A subunits in the adult brain manifest reduced Ca^{2+} permeability of NMDA-evoked currents (Tong et al., 2008). Since Ca^{2+} influx through NMDAR-operated channels mediates excitotoxic neurodegeneration, we postulated that NR3A might act as inhibitory molecule in this process.

In this study, we tested the hypothesis that the presence of the NR3A subunit affords neuroprotection. To this end, we applied various forms of excitotoxic insults to neurons lacking NR3A and compared the extent of neuronal damage with WT. In addition, we compared excitotoxic damage in WT mice at a developmental stage when they mostly lacked NR3A to age-matched TG mice overexpressing exogenous NR3A subunits. Specifically, we used the following experimental paradigms: (1) NMDA application on cultured cortical neurons prepared from NR3A KO and WT newborn pups, (2) hypoxic–ischemic insult induced by a modified Rice–Vannucci model in neonatal NR3A knock-out (KO)

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and WT mice, (3) NMDA injection into adult retinas of NR3A KO and WT mice, (4) NMDA application to cultured cortical neurons prepared from NR3A TG and WT mice, and (5) focal hypoxic–ischemic insult induced by the intraluminal suture method to produce transient middle cerebral artery occlusion/reperfusion (tMCAOr) in adult NR3A TG and WT mice. Remarkably, in each of these experiments, the presence of NR3A subunits led to a reduction in the extent of neuronal damage, whereas the absence of NR3A resulted in increased damage. Hence, NR3A subunits appear to be neuroprotective.

Materials and Methods

NR3A KO and TG mice. NR3A KO mice were generated as described previously (Das et al., 1998). We performed homologous recombination in an embryonic stem cell line (J1) that was derived from a 129/Sv mouse and then crossed founder chimera mice directly with 129/SvJ breeders. The line was then backcrossed into 129/SvJ for more than 10 generations.

The generation and electrophysiological characterization of NR3A overexpressing TG mice that were tetracycline (tet)-controlled have been described previously (Tong et al., 2008). Calmodulin-dependent kinase type II promoter (CaMKII)-tTA TG mice were purchased from The Jackson Laboratory. For the experiments described in this study, the double-transgenic NR3A and CaMKII-tTA TG mice have repeatedly been backcrossed into the C57BL/6 breeders. All experiments were performed in accordance with Institutional guidelines concerning care and treatment of vertebrate animals.

NMDA-induced cell death in cultured neurons. Cerebrocortical neurons were isolated from newborn pups and maintained in culture for up to 3 weeks as described previously (Okamoto et al., 2002). To induce apoptosis, cortical cultures were exposed for 20 min to 50–200 μM NMDA (plus 5 μM glycine, a coagonist of the NMDAR) in Earle's balanced salt solution (Budd et al., 2000; Okamoto et al., 2002). After NMDA exposure, cells were rinsed and replaced in their original conditioned medium. Eighteen to twenty-four hours after the NMDA treatment, apoptotic death was assessed on the basis of cellular morphology, nuclear condensation with Hoechst 33342 DNA dye (Invitrogen), and TUNEL staining. A DeadEnd Fluorometric TUNEL System (Promega) was used for TUNEL assays. To assess necrotic neurons, cells were incubated with propidium iodide (1 μg/ml) for 10 min before fixation. Unlike apoptotic cells, necrotic cells have compromised plasma cell membranes and thus take up and label with propidium iodide (Budd et al., 2000). Statistical analyses between two groups were performed using a one-way Student's *t* test in all experiments.

Neonatal model of hypoxia–ischemia. To produce cerebral ischemia in neonatal NR3A KO mice, we used a modified version of the Rice–Vannucci model (Rice et al., 1981). P7 mouse pups were lightly anesthetized with 1.5% isoflurane. Under anesthesia, a ligature was tied around the right common carotid artery (CCA). We used permanent ligation on these mouse pups for technical reasons, as they were smaller than rat pups of the same age. After recovery from anesthesia, pups were placed in jars submerged in a 37°C water bath for 2–2.5 h. Then the lids of the jars were tightened, and an 8% oxygen/92% nitrogen mixture was delivered to the pups for 2.5 h. After this period of hypoxia, the pups were returned to the mother. Seven days after the hypoxic–ischemic insult, the pups were perfusion-fixed and killed. The brains were removed and 50-μm-thick coronal slices were cut. The sections were then stained with cresyl violet and acid fuchsin. The area of infarction, corrected for possible edema (Wang et al., 1998), was measured using National Institutes of Health Image 1.62 software program. The infarct volume was calculated by summation of the areas of infarction, multiplied by the thickness of the slices. All histological analyses were performed blind to genotypes.

Adult model of hypoxia–ischemia. Focal transient MCAOr was performed as described previously (Gu et al., 2002). Adult mice were anesthetized, and the right CCA was exposed and carefully dissected free from surrounding nerve and fascia through a midline neck incision under an operating microscope. After isolating the external carotid artery (ECA) and internal carotid artery (ICA), all of the extracranial branches of the right ICA were ligated. A microvascular clip (FE691; Aesculap) was temporarily placed on the right ICA and CCA. A 6-0 silk suture was tied loosely around the ECA stump, and a 10 mm length of 6-0 monofilament nylon suture, previously coated with silicon, was inserted via the proximal ECA into the ICA and thence into the circle of Willis, effectively occluding the MCA. After the insertion of the intraluminal nylon suture, the microvascular clip on the carotid artery was removed. Ischemia was induced for 2 h by leaving the tip of the nylon filament at the origin of the MCA. After the 2 h occlusion period, reperfusion was accomplished by withdrawing the intraluminal filament. Occlusion and reperfusion were verified by laser Doppler flowmetry to monitor relative cerebral blood flow. Twenty-four hours later, the animals were scored for neurological/behavioral impairments (see below) and then killed. The brains were removed and 1 mm coronal slices cut. The brain slices were immersed for 10–20 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C. Infarction was quantified and analyzed as described for the neonatal model of hypoxia–ischemia.

Mice were subjected to a neurologic evaluation (Mackensen et al., 2001) just before their killing. Each animal is assigned a score of 0–4 according to the following: 0, no observable neurological deficit; 1, failure to extend the left forepaw; 2, circling to the left; 3, falling to the left; 4, cannot walk spontaneously.

Excitotoxicity in the adult retina. Excitotoxicity in the retina was induced and quantified as described previously (Manabe and Lipton, 2003). Various doses of NMDA plus 5 nmol glycine in PBS were injected using a 33-gauge injection needle that was inserted into the vitreous just above the retina through the dorsal limbus of the eye. Animals with visible lens damage or vitreous hemorrhage because of the injection were excluded in the analysis.

Four days after intravitreal injection, retinal ganglion cells (RGCs) were retrogradely labeled by injection of 5% aminostilbamidine (FluoroGold; Invitrogen) into the superior colliculi. Three days later, mice were killed with an overdose of pentobarbital, and the eyes were removed. Eyecups were fixed in a 4% paraformaldehyde solution, and retinal flatmounts were prepared. The number of surviving RGCs was determined by counting FluoroGold-labeled neurons under epifluorescence microscopy, as described previously (Kikuchi et al., 2000).

Cell culture. High-density (4×10^5 cells per 35 mm dish) primary cerebrocortical cultures were prepared from newborn NR3A KO, TG, or WT control mice as described previously (Tu et al., 2007). In brief, cerebral cortices were enzymatically dissociated (papain; Collaborative Research) and mechanically dispersed into a single-cell suspension and plated onto glass coverslips coated with 0.1 mg/ml poly-L-lysine. The cells were maintained in neural basal medium supplied with B27, 0.5 mM glutamine, and 1× pen/strep (Invitrogen) for 2 weeks and used for biotin surface-labeling experiments.

Immunoblotting on biotin-labeled surface proteins. Cells were incubated in PBS containing 1.5 mg/ml EZ-link sulfo-NHS-SS-Biotin (Pierce) at 4°C for 20–30 min. After two rinses, cells were lysed in PBS with protease inhibitor mixture (Roche), 0.1% SDS, and 1% Triton X-100. Approximately10% of the lysates were saved to assess total protein. The rest of the lysates were incubated with NeutrAvidin agarose overnight at 4°C to isolate biotinylated proteins. The beads were then spun down and washed in PBS. Proteins were eluted in SDS-PAGE sample buffer and subjected to electrophoresis using NuPAGE 4–12% Bis-Tris gels (Invitrogen). Primary antibodies used were monoclonal anti-NR1 (1:4000–8000; Millipore Bioscience Research Reagents), polyclonal NR2A (1:2000; Millipore Bioscience Research Reagents), polyclonal anti-NR2B (1:1000; Millipore Bioscience Research Reagents), polyclonal NR3A (1:2000; Millipore Bioscience Research Reagents), and monoclonal anti-tubulin (1:5000–20,000; Sigma). Secondary antibodies were from mouse or rabbit and conjugated to HRP (The Jackson Laboratory). Proteins were visualized by ECL plus Western Detection Kit (GE Healthcare) on Kodak x-ray film Biomax MR-1 (VWR).

Immunoblotting postsynaptic density proteins. Forebrains were dissected out from P7 NR3A KO and WT control mice or adult NR3A TG and WT control mice. Tissues were homogenized in 0.32 M sucrose with 10 mM Tris-HCl, pH 7.4. The postsynaptic density (PSD) fraction was prepared as described previously (Tong et al., 2008) and immunoblotted as described above.

Results

No change in expression levels of NR1 and NR2 subunits in NR3A KO and TG mice

Before we subjected NR3A mutant mice to various paradigms of excitotoxicity, we tested the possibility that genetic manipulation of NR3A might lead to compensatory changes in other NMDAR subunits. To this end, we quantified protein levels of NR1, NR2A, and NR2B subunits in cerebrocortical neurons cultured from NR3A KO and WT mice (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Neither surface nor total expression of NMDAR subunits was altered in neurons deficient in NR3A. Furthermore, expression levels of NR1, NR2A, and NR2B proteins were unaltered in the PSD fraction of the forebrain of NR3A KO mice at P7 (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

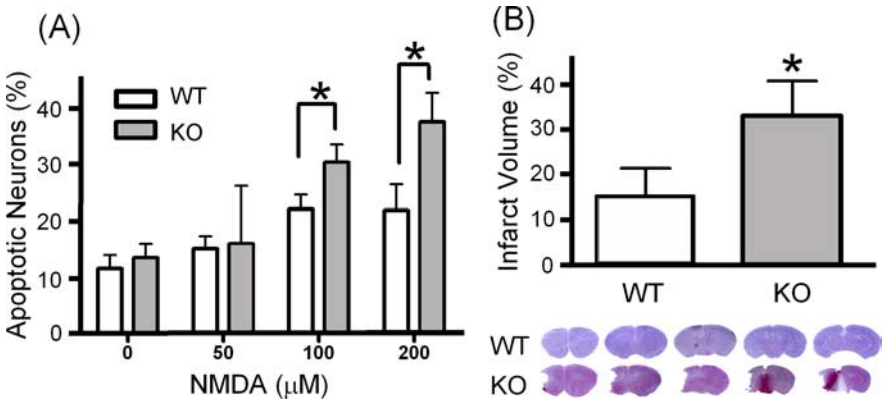

Figure 1. ***A***, NR3A-deficient neurons manifest increased cell death after exposure to NMDA. Cortical neuronal cultures ($n = 5$ for WT; $n = 6$ for NR3A KO) were prepared and exposed to various concentrations of NMDA. Apoptotic death was assessed by TUNEL staining. Neurons cultured from NR3A KO mice manifested significantly increased cell death compared with WT neurons when challenged with 100 or 200 μM NMDA. ***B***, Infarct size in the Rice–Vannucci model of cerebral ischemia is greater in NR3A KO mice ($n = 13$) than in WT ($n = 11$) mice. The images below the graph show representative brain sections with infarcts. P7 mice were used in this series of experiments because NR3A is maximal in the cerebrocortex at that developmental stage but decreases thereafter (*$p < 0.05$). Error bars indicate SEM.

Similarly, we quantified surface and total expression of NR1, NR2A, and NR2B in cerebrocortical neurons cultured from NR3A TG and WT mice (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Again, we did not detect any significant change in expression levels of NMDAR subunits in these mutant mice. Finally, expression levels of NR1, NR2A, or NR2B proteins were not altered in the PSD fraction of adult NR3A TG mice (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). These experiments show that the effects of NR3A genetic manipulation does not lead to compensatory changes in expression levels of other NMDAR subunits.

Neuronal damage after NMDA exposure of cerebrocortical cultures from NR3A KO and WT mice

We examined the sensitivity to NMDA of cultured neurons prepared from cerebral cortex of WT and NR3A KO mice. Cerebrocortical cultures were exposed to 0, 50, 100, and 200 μM NMDA plus 5 μM glycine for 20 min. Subsequent apoptotic and necrotic cell death was analyzed. As observed previously (Budd et al., 2000), exposure to these concentrations of NMDA for short periods induced apoptosis rather than necrosis. Neurons were identified by staining with antibodies to NeuN or microtubule associated protein 2. Apoptosis was scored by dividing the number of apoptotic neurons by the total number of neurons.

We found no difference in the number of apoptotic neurons in cultures prepared from NR3A KO and WT mice after exposure to 50 μM NMDA plus 5 μM glycine (Fig. 1*A*). However, neurons prepared from NR3A KO mice manifested significantly more apoptosis than WT neurons after exposure to 100 or 200 μM NMDA plus 5 μM glycine ($p < 0.05$, paired Student's t test). These results show that cultured NR3A KO neurons manifest a greater susceptibility to NMDA after exposure to moderate concentrations compared with WT control neurons.

Hypoxic–ischemic insult by a modified Rice–Vannucci model in neonatal NR3A KO and WT mice

We next asked if NR3A KO mice displayed increased vulnerability compared with WT mice in response to excitotoxic insults *in vivo*. To this end, we subjected neonatal NR3A KO and WT mice to a modified Rice–Vannucci model of hypoxia–ischemia (Rice et al., 1981). We chose neonatal stroke because expression of endogenous NR3A in the cerebrocortex peaks at ~P7–P10 and then rapidly decreases, and we wanted NR3A to be present in the control mouse brains. In this model, mice were subjected to unilateral cerebral ischemia by CCA ligation followed by systemic hypoxia (see Materials and Methods for details). Seven days after the hypoxic–ischemic insult, the pups were killed and the area of infarction measured.

We found that NR3A KO mice manifested significantly larger infarcts compared with control WT mice (15.1 ± 6.3 mm^3 for WT vs 32.9 ± 7.9 mm^3 for KO; $p < 0.05$) (Fig. 1*B*). Representative brain sections stained with cresyl violet and acid fuchsin show that NR3A KO mice are more susceptible to hypoxic–ischemic insult than WT mice. Thus, endogenous NR3A appears to protect cortical neurons from excitotoxic insults *in vivo* as well as *in vitro*.

NMDA injection into adult retina of NR3A KO and WT mice

The retina is one of only a few CNS regions that express high levels of NR3A into adulthood (Wong et al., 2002). This fact allowed us to ask if endogenous NR3A was neuroprotective in the adult CNS. To address this question, we injected a range of doses of NMDA into the vitreous of adult NR3A KO and WT mice. Seven days later, surviving RGCs were counted by visualization of the retrograde label, FluoroGold.

We found that the density of surviving RGCs was similar in both genotypes under control conditions (0 nmol NMDA/5 nmol glycine). As expected, a very large dose (4 nmol) of NMDA resulted in such a massive insult that KO and WT showed a similar degree of cell death. However, with more subtle insults (injection of 2 nmol NMDA), the mean value for the number of surviving neurons for NR3A KO retina was <75% of the control value ($p < 0.05$) (Fig. 2). This finding shows that the NR3A KO retina manifested an increase in cell death compared with WT and is consistent with the notion that endogenous NR3A is neuroprotective in the adult retina under conditions of moderate excitotoxic insult.

Protection of NR3A TG neurons from NMDA exposure in culture

We next determined the sensitivity to NMDA insult of cultured neurons expressing exogenous NR3A. We had previously gener-

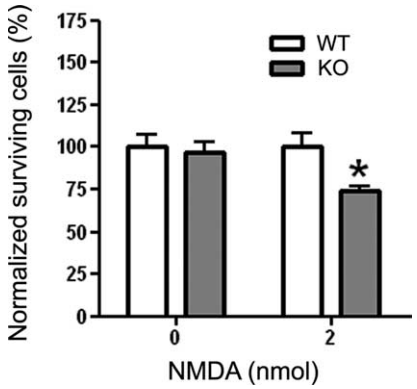

Figure 2. Adult NR3A KO mice manifest increased damage to retinal ganglion cells after intravitreal injection of NMDA. NMDA was injected into the vitreous, and retrogradely labeled RGCs were counted 7 d later. NR3A KO mice ($n = 4$ for untreated control; $n = 3$ for NMDA treated samples) displayed an increase in damage after the 2 nmol injection of NMDA compared with WT mice ($n = 4$ for untreated control; $n = 3$ for NMDA treated samples). Adult mice were used in this series of experiments because NR3A expression persists into adulthood in RGCs, unlike most other areas of the CNS ($*p < 0.05$). Error bars indicate SEM.

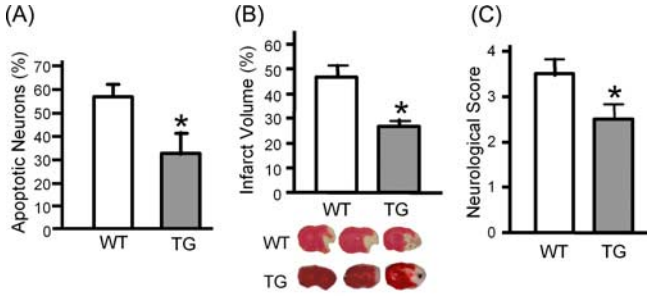

Figure 3. Protective effect of NR3A transgene overexpression. ***A***, NR3A TG neurons manifest decreased cell death after exposure to NMDA. Cortical neurons were cultured from mixtures of WT and NR3A TG newborn pups. Neurons expressing the NR3A transgene were identified by coexpression of EGFP. After mild NMDA exposure, apoptotic cell death was assessed by TUNEL staining. The degree of apoptosis for TG neurons was significantly less than that of WT neurons within the same culture ($n = 5$ for each genotype; $*p < 0.05$). ***B***, NR3A TG mice ($n = 7$) manifested a decrease in infarct size in the adult tMCAOr stroke model compared with WT mice ($n = 4$). The images below the graph show representative brain sections with infarcts. ***C***, NR3A TG mice ($n = 6$) exhibited an improvement in neurological behavior (lower disability score) after tMCAOr stroke compared with WT mice ($n = 6$; $*p < 0.05$). Error bars indicate SEM.

ated NR3A overexpressing TG mice (Tong et al., 2008). Briefly, NR3A cDNA is placed downstream of one of the two cytomegalovirus (CMV) promoters within the construct. The other CMV promoter drives enhanced green fluorescence protein (EGFP), allowing identification of cells expressing the transgenes by fluorescence microscopy. Furthermore, both CMV promoters are under the regulation of the tet responsive element, which is activated by the tet-controlled transactivator (tTA).

We crossed the NR3A TG mice with another TG mouse line carrying tTA under the CaMKII (Mansuy et al., 1998). The tet-off system was used in this line, and tTA was kept active by not feeding the mice with doxycycline. We crossed mice doubly transgenic for tTA and NR3A (heterozygous at both loci) with C57BL/6 mice. Using newborn pups, we generated cultures containing both TG and WT neurons. Neurons expressing EGFP (and thus also NR3A) were identified under fluorescence microscopy. Theoretically, the probability of producing a doubly transgenic pup in these litters was 25%. In practice, we routinely observed that 10–30% of the neurons in these cultures were EGFP positive after 8–10 d *in vitro*. These neurons were then stained with anti-NR3 antibody, and we observed that all EGFP-positive cells were strongly NR3 positive (Tong et al., 2008). In contrast, all EGFP-negative cells displayed either very low or background staining with anti-NR3A antibody. This very low-level of staining was likely attributable to the immunoreactivity of endogenous NR3A, which is expressed transiently early in development at a very low-level (Wong et al., 2002). Therefore, EGFP-positive neurons were considered transgenic NR3A expressors, whereas EGFP-negative neurons served as internal controls.

We calculated the ratio of apoptotic neurons in EGFP-negative and EGFP-positive cells in these cultures after exposure to 50 μM NMDA and 5 μM glycine. EGFP-positive (NR3A-containing) neurons manifested a significantly reduced apoptotic ratio ($p < 0.05$) (Fig. 3*A*). These results show that exogenously expressed NR3A protects cultured neurons from apoptosis after NMDA exposure *in vitro*.

Ischemic insult after tMCAOr in adult NR3A TG and WT mice

We next tested whether the exogenously expressed NR3A transgene could be neuroprotective *in vivo*. To this end, we subjected adult (6–8 week old) NR3A TG mice and control littermates to focal transient ischemia/reperfusion (see Materials and Methods). Since NR3A is normally downregulated in the adult cerebrocortex, we could use WT mice as a control without significant expression of NR3A. We chose this stroke model because the CaMKII promoter that drives tTA expression is highly active in the forebrain cortex at this age. Additionally, this model mimics stroke in human patients because of the presence of ischemia followed by reperfusion injury. Cerebral blood flow was monitored during the occlusion to assure that ≥80% of the blood flow was blocked by this procedure. Similarly, reperfusion led to recovery of blood flow to >50%. Twenty-four hours later, the animals were scored for neurological impairments and then killed for histological analysis. The brains were removed and the area of infarcts measured.

We found that NR3A TG mice had significantly decreased infarct size compared with WT control littermates (47.0 ± 4.7 mm^3 for WT compared with 27.0 ± 2.6 mm^3 for NR3A; $p < 0.05$) (Fig. 3*B*). In accord with these histological findings, the neurological scores of the NR3A TG mice manifested significant improvement (indicated by a lower deficit score) compared with WT control littermates 24 h after tMCAOr (2.5 ± 0.3 for NR3A compared with 3.5 ± 0.3 for WT; $p < 0.05$) (Fig. 3*C*). These data show that the tMCAOr results in less histological and neurological damage in NR3A TG mice compared with WT mice. Together with our data in culture, exogenously expressed NR3A transgene appears to be neuroprotective both *in vitro* and *in vivo*.

Discussion

In the current study, we present several lines of evidence that NR3A subunits are neuroprotective. Cultured neurons prepared from NR3A KO mice exhibited a greater susceptibility to excitotoxicity after exposure to NMDA. *In vivo*, in a neonatal model of hypoxia ischemia where NR3A is present in the cortex, we found greater neuronal damage in NR3A KO mice than in WT mice. In the adult retina where NR3A normally persists into adulthood, NMDA injection into the vitreous induced greater RGC loss in NR3A KO mice than in WT mice. These results are consistent with the notion that endogenous NR3A is neuroprotective against various excitotoxic insults.

We next examined NR3A TG mice generated in our labora-

tory to determine whether overexpression of NR3A could lead to enhanced neuroprotection. *In vitro*, we found that neurons expressing NR3A transgenes were protected from NMDA exposure to a greater extent than WT control neurons. Since the EGFP/NR3A-expressing and control EGFP-negative/nontransgene-expressing neurons were grown in the same culture dish, the difference in susceptibility to excitotoxicity cannot be attributed to a paracrine or toxic effect exerted from one cell to another. Rather, NR3A overexpression likely endows neurons with increased resistance to excitotoxicity by directly interfering with hyperactivation of the NMDAR.

Additionally, on adult mice, which normally no longer express the NR3A subunit in the cerebrocortex, focal cerebral ischemia resulted in less neuronal damage and improved neurological behavior in NR3A TG mice compared with WT control mice. Hence, reintroduction of the NR3A subunit into the adult CNS can offer protection during excitotoxic insults.

Since expression levels of NR1 and NR2A/B subunits are unaltered in NR3A KO and TG mice, it is unlikely that the neuroprotection effects of NR3A are solely attributable to drastically changing the number of functional NMDA receptors in these mutant mice. Rather, we know that knockout of NR3A increases, whereas exogenous expression of NR3A decreases the amplitude of NMDA-evoked currents and the entry of Ca^{2+} through NMDAR-associated channels (Das et al., 1998; Sasaki et al., 2002; Tong et al., 2008). Since it is known that Ca^{2+} entry through NMDARs contributes to neuronal cell death after exposure to glutamate (Rothman and Olney, 1986; Choi, 1988), the decrease in Ca^{2+} influx engendered by NR3A in NMDARs composed of NR1/NR2/NR3 subunits likely underlies its neuroprotective effect.

In seeming contrast to our findings, Ulbrich and Isacoff (2008) recently performed subunit-tagging experiments and reported that NR3A did not form heterotrimeric complexes with NR1 and NR2 at 12–24 h after cRNA injection into *Xenopus* oocytes. However, the findings of Ulbrich and Isacoff (2008) contradict published observations by three groups, including our own (Pérez-Otaño et al., 2001; Matsuda et al., 2002; Sasaki et al., 2002). All of these groups have found that inclusion of NR3A with NR1/NR2 subunits results in the formation of glutamate- and glycine-activated (i.e., NMDAR type) channels that are distinct in Ca^{2+} permeability, Mg^{2+} sensitivity, and pharmacology from NR1/NR2 NMDAR-operated channels. These changes in ion selectivity and pharmacology cannot be explained by the rules of engagement proposed by Ulbrich and Isacoff (2008). Importantly, the evidence for formation of NR1/NR2/NR3 channels from the three other laboratories has been obtained not only in heterologous expression systems but also in primary neurons, arguing that these "triplet" receptors form endogenously.

To potentially explain this discrepancy, we also note that Ulbrich and Isacoff (2008) performed their subunit-tagging experiments 12–24 h after cRNA injection. From our published work, we know that this is too short a period of time for oocyte expression of NR1/NR2/NR3 channels. In our hands using the same vector that Ulbrich and Isacoff used, functionally active NR3-containing channels are not detectable within the first 12–24 h but instead require several days to appear. Hence, taking the work of the various laboratories together, we conclude that NR3 may initially interact only with NR1 but later may associate with NR1 and NR2 subunits to form functional channels.

The neuroprotective action of NR3A subunits described here is in some ways reminiscent of that of the NMDAR inhibitor memantine, which has been approved by the Food and Drug Administration for the treatment of moderate-to-severe Alzheimer's disease in the United States of America and is also used for Vascular dementia in Europe. Along these lines, our group has previously shown that the inhibitory mechanism of action of memantine involves open-channel block of the NMDAR channels, and in a somewhat similar manner the inhibitory action of NR3A involves blocking of the outer vestibule of the channel (Chen et al., 1992; Chen and Lipton, 2005; Lipton, 2006, 2007). We speculate, therefore, that a more detailed understanding of how NR3A interacts with NR1/NR2 channels may eventually lead to the development of new therapeutic tools that could combat excitotoxicity.

Intriguingly, a study examining postmortem human brains reported that the number of mRNA transcripts encoding NR3A was significantly altered in the dorsolateral prefrontal cortex but not in other contiguous brain regions in the major psychotic conditions bipolar disorder and schizophrenia. In the case of bipolar disorder, NR3A transcripts were decreased, in contrast to schizophrenia in which they were increased (Mueller and Meador-Woodruff, 2004). In the context of our findings, it is possible that decreased NR3A expression might lead to increased vulnerability of the neurons in this brain area in bipolar patients. Conversely, in schizophrenia, the increased expression of NR3A might lead to decreased NMDAR-mediated signaling because of the known modulatory role of NR3A in downregulating current influx, similar to the hallucinogen phencyclidine. Thus, the animal models that we have developed for knocking out or overexpressing NR3A may potentially serve as models of these neuropsychiatric disorders to facilitate development of future therapeutic agents.

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