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Protease-activated receptor 1-dependent neuronal damage involves NMDA receptor function

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Abstract

Protease-activated receptor 1 (PAR1) is a G-protein coupled receptor that is expressed throughout the central nervous system. PAR1 activation by brain-derived as well as blood-derived proteases has been shown to have variable and complex effects in a variety of animal models of neuronal injury and inflammation. In this study, we have evaluated the effects of PAR1 on lesion volume in wild-type or PAR1^{-/-} C57Bl/6 mice subjected to transient occlusion of the middle cerebral artery or injected with NMDA in the striatum. We found that removal of PAR1 reduced infarct volume following transient focal ischemia to 57% of control. Removal of PAR1 or application of a PAR1 antagonist also reduced the neuronal injury associated with intrastriatal injection of NMDA to 60% of control. To explore whether NMDA receptor potentiation by PAR1 activation contributes to the harmful effects of PAR1, we investigated the effect of NMDA receptor antagonists on the neuroprotective phenotype of PAR1^{-/-} mice. We found that MK801 reduced penumbral but not core neuronal injury in mice subjected to transient middle cerebral artery occlusion or intrastriatal NMDA injection. Lesion volumes in both models were not significantly different between PAR1^{-/-} mice treated with and without MK801. Use of the NMDA receptor antagonist and dissociative anesthetic ketamine also renders NMDA-induced lesion volumes identical in PAR1^{-/-} mice and wild-type mice. These data suggest that the ability of PAR1 activation to potentiate NMDA receptor function may underlie its harmful actions during injury.

Keywords

N-methyl-D-aspartate; glutamate; thrombin; protease activated receptor; PAR; ischemia; stroke; neuronal death; neuroprotection

Introduction

Protease-activated receptors (PARs) are a family of four G-protein coupled receptors that are activated by proteolytic cleavage of the extracellular N-terminus by serine proteases such as thrombin. The new N-terminus revealed by this cleavage acts as a tethered ligand, activating a complex signaling cascade (Liu et al. 1991; Vu et al. 1991; Macfarlane et al. 2001; Trejo,

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2003; Traynelis and Trejo, 2007). PAR1 couples to at least three different G-proteins, $G_{\alpha_{i/o}}$, $G_{\alpha_{12/13}}$, and $G_{\alpha_{q/11}}$, to initiate intracellular signaling (Smirnova et al. 2001; Macfarlane et al. 2001; Klarenbach et al. 2003; Junge et al. 2003, 2004;). Although first described for its role in the coagulation cascade, PAR1 is also expressed throughout the central nervous system. *In situ* hybridization studies in rats show that PAR1 is expressed in select neuronal populations, including motoneurons, dopaminergic neurons in the substantia nigra pars compacta (SNc), as well as a subset of cortical neurons (Weinstein et al. 1995; Niclou et al. 1998). Similarly, in human brain tissue, certain neuronal populations show PAR1 protein expression (Junge et al. 2004; Ishida et al. 2006). Glial expression of PAR1, particularly in astrocytes, is consistently strong in all regions (Weinstein et al. 1995; Wang et al. 2002; Junge et al. 2004; Hamill et al. 2005). Microglia also express functional PAR1 (Suo et al. 2002). PAR1 expression changes in response to injury in a manner dependent on cell-type and the nature of injury (Striggow et al., 2001; Riek-Burchardt et al., 2002; Rohatgi et al., 2004; Henrich-Noack et al., 2006).

The effects of PAR1 activation on neuronal health are complex (Gingrich and Traynelis, 2000; Vivien and Buisson, 2000; Matsuoka and Hamada, 2002; Xi et al., 2003; Ruf, 2003; Sheehan and Tsirka, 2005). Several *in vitro* models show thrombin activation of PAR1 to be neuroprotective. For example, thrombin administration protected cultured hippocampal neurons from glucose deprivation (Vaughan et al. 1995). Similarly, thrombin at concentrations lower than 50 nM (or other PAR1-specific agonists) protected organotypic hippocampal slices from oxygen-glucose deprivation (Striggow et al. 2000). Several *in vivo* studies also have found that administration of thrombin or PAR1 specific agonists several days prior to an insult, such as focal ischemia or 6-hydroxydopamine, is neuroprotective (Masada et al. 2000; Jiang et al. 2002; Xi et al. 2003; Cannon et al. 2006). Activated protein C protects brain endothelial cells from hypoxia and cultured hippocampal neurons from N-methyl-D-aspartate (NMDA)-induced toxicity in a PAR1-dependent manner (Shibata et al. 2001; Cheng et al. 2003; Mosnier and Griffin 2003; Ruf 2003; Guo et al. 2004). It has also been proposed that PAR1 activation by activated protein C administration is protective against neuronal damage in both a focal ischemia model and following NMDA microinjection into the parenchyma (Shibata et al. 2001; Cheng et al. 2003; Guo et al. 2004).

In contrast to these neuroprotective effects, several lines of evidence suggest that known PAR1 activators as well as PAR1 activation can be detrimental to neuronal health. A number of studies show that thrombin inhibitors can reduce damage *in vitro* and *in vivo*, with apparent contributions from both neuroprotective mechanisms and effects on cerebral blood flow (e.g. Friedmann et al., 2001; Jin et al., 2002; Karabiyikoglu et al., 2004; Cuomo et al., 2007). In addition, global inhibition of protease activity led to neurite extension in murine neuroblastoma cells (Monard et al. 1983; Snider and Richelson 1983; Snider et al. 1984), and thrombin was subsequently identified as the first chemorepellant to neurite outgrowth (Hawkins and Seeds 1986; Jalink and Moolenaar 1992; Suidan et al. 1992). Neurons that showed neurite retraction in response to thrombin subsequently died, whereas inhibition of thrombin by hirudin supported neurite outgrowth and neuronal survival (Jalink and Moolenaar 1992; Suidan et al. 1992; Turgeon et al. 1998). Although these studies did not implicate PAR1 in the actions of thrombin, subsequent studies that utilized a selective peptide mimic of PAR1's new N-terminal following activation suggested that the changes in neuron shape and survival were dependent on PAR1 activation; neuronal death following PAR1 activation was at least in part apoptotic (Donovan et al. 1997; Turgeon et al. 1998). At high concentrations (>50 nM), thrombin is toxic to hippocampal cultures and organotypic slice cultures (Smith-Swintosky et al. 1995; Vaughan et al. 1995; Striggow et al. 2000; Xue et al., 2006; Fujimoto et al., 2008). Moreover, PAR1 activation appears to be proinflammatory (Suo et al. 2004). *In vivo*, thrombin pre-treatment exacerbates neuronal damage when administered close in time with an insult, such as transient ischemia (Henrich-Noack et al. 2006). Mice with PAR1 genetically removed (PAR1^{-/-}) are protected from global ischemia/hypoxia as well as from 30 min intracerebral hemorrhage and

transient focal ischemia (Junge et al. 2003; Olson et al. 2004; Xue et al. 2006). Similar levels of neuroprotection in both hypoxia/ischemia and transient focal ischemia can be achieved in wild-type animals by pretreatment with a selective PAR1 antagonist (Junge et al. 2003; Olson et al. 2004).

N-methyl-D-aspartate receptors mediate a slow Ca^{2+} -permeable component of excitatory synaptic transmission, and are thought to be involved in learning and memory (Erreger et al., 2004). In addition, NMDA receptor overactivation is neurotoxic when studied *in vitro* (Koh and Choi 1987; Choi et al. 1988; Lei et al. 1992; Arundine and Tymianski 2003) as well as *in vivo* (Meldrum et al. 1987; Park et al. 1988; Swan and Meldrum 1990; Duncan et al. 1991; Uematsu et al. 1991; Miyabe et al. 1997; Dogan et al. 1999; Wang and Shuaib 2005). This toxicity is widely considered a leading cause of early neuronal death in situations in which extracellular glutamate is elevated, such as ischemia (Lipton 1993; Whetsell 1996; Dirnagl et al. 1999). PAR1 activation by a variety of agonists has been shown to potentiate neuronal responses to NMDA (Gingrich et al. 2000; Lee et al. 2007; Mannaioni et al. 2008) as well as increase spontaneous NMDA receptor mediated currents (Shigetomi et al. 2008). Given the prominent role of NMDA receptors in mediating excitotoxic injury during ischemia, potentiation of NMDA receptor responses by PAR1 activation, if it occurred during ischemia, should exacerbate neuronal damage.

One explanation for the apparent dual nature of PAR1 in neuronal injury is that PAR1 activation *in vivo* engages multiple signaling mechanisms in multiple cell types. We hypothesized that some of the harmful effects of PAR1 activation are due to its ability to potentiate NMDA receptor function in the ischemic penumbra (Gingrich et al. 2000; Henrich-Noack et al. 2006). If the harmful effects of PAR1 are primarily mediated through enhancement of NMDA receptor function, one would predict that the residual damage in the presence of NMDA receptor blockade will be insensitive to PAR1 antagonism or removal of the PAR1 gene. To test this idea we examined the lesion volume in PAR1^{-/-} and wild-type mice both in the presence and absence of NMDA receptor blockers (MK801 or ketamine) in models of ischemia in which the NMDA receptor contribution to cell death is important. Our data are consistent with the idea that PAR1 activation exacerbates neuronal damage in the absence of NMDA receptor blockers. However, blockade of NMDA receptors removes the harmful effects exerted by PAR1. Previous reports that PAR1 activation potentiates NMDA receptor function (e.g. Gingrich et al., 2000; Mannaioni et al., 2008) and enhances glutamate release (Lee et al. 2007; Ramos-Mandujano et al. 2007) are consistent with an extensive literature showing NMDA receptor activation is harmful in animal models of ischemia (Koh and Choi 1987; Meldrum et al. 1987; Choi et al. 1988; Swan and Meldrum 1990; Duncan et al. 1991; Lei et al. 1992; Lees 1997; Miyabe et al. 1997; Dirnagl et al. 1999; Uematsu et al. 1991).

Methods

Ca^{2+} imaging in striatal cultures

All procedures involving animals were performed in accordance with international, national, and local standards on animal welfare, and were reviewed and approved by the Emory University Institutional Animal Care and Use Committee. Rat striatal neuronal cultures were obtained from E17–E18 pups (Charles River Laboratories, Inc, Wilmington, MA). Briefly, pregnant rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation. The brains were removed and placed in ice cold HEPES buffered saline. The striatum was dissected, and placed in fetal bovine serum and triturated through a fire polished pipette. Cultures were plated onto poly-D-lysine (10 $\mu\text{g}/\text{ml}$) coated glass coverslips in MEM media (Invitrogen, Carlsbad, CA) supplemented with B27 defined nutrients (Invitrogen). Cultures were maintained at 37° C in a humidified 5% CO_2 incubator and the media replaced every 3 days. After 4–9 days, cells were placed (in mM) in 150 NaCl, 3 KCl, 10 HEPES, 2 CaCl_2 , 20 mannitol, 10 glucose,

pH 7.3. Cells were subsequently incubated for 30–45 minutes in this solution supplemented with 0.1% pluronic acid, 0.5 % DMSO and 3 μ M Fluo-3 acetoxymethyl ester (Molecular Probes, Eugene, OR), and transferred to the microscope stage for imaging. Images were recorded in response to 410 nm excitation and band-passed 500–550 nm emission at variable frequency (0.033 Hz for baseline and 0.5 Hz for drug application). After recording images for a three minute baseline period, cells were exposed to 30 μ M of the PAR1 activating peptide TFLLR-NH₂ (TFLLR, Emory Microchemical Facility) for three minutes in the presence of 1 mM Mg²⁺, 0.5 μ M tetrodotoxin (TTX), and 50 μ M D-2-amino-5-phosphonovalerate (D-APV), and the fluorescent responses were recorded. Following a three-minute wash, cultures were then exposed to 10 μ M NMDA in order to identify neurons.

Intrastriatal microinjection of NMDA or TFLLR

Male C57Bl/6 wild-type or PAR1^{-/-} mice (see below; 3–5 month) were anesthetized with chloral hydrate (400 mg/kg, Sigma, St. Louis, MO), isoflurane (5% in 100% oxygen for induction, 1–2% in 100% oxygen for maintenance) or ketamine (100 mg/kg, Allan Labs, Chicago, IL) and xylazine (10 mg/kg, Henry Schein Inc. Melville, NY). A subset of animals received 3 mg/kg of (–)MK801 (Tocris, Ellisville, MO). (–)MK801 is the less potent stereoisomer (Wong et al. 1986; Kant et al. 1991; Galbicka et al. 1994; Dravid et al. 2007) and is better tolerated by C57Bl/6 mice than (+)MK801. We have found that 1–3 mg/kg of either MK801 stereoisomer is equally effective at reducing infarct volume in C57Bl/6 mice following transient focal ischemia, with (–)MK801 showing a reduction in infarct volume to 55±10% of control (n=31) and (+)MK801 reducing infarct volume to 57±8% (n=26) of control. However administration of (+)MK801 causes higher mortality (41%) than (–)MK801 (10%). We subsequently utilized a well-tolerated dose (3 mg/kg) of the NMDA receptor antagonist (–)MK801 for experiments in PAR1^{-/-} and littermate controls.

Following induction of anesthesia, animals were placed on a homeothermic blanket and body temperature maintained between 36.5–37°C. The heads were immobilized in a small animal stereotaxic device (Model SAS 75, Cartesian Research Inc., Sandy, OR). A scalpel was used to expose the skull, a hole was drilled with a 0.0087 inch drill bit, and a 33 gauge needle was lowered into the striatum (rostral 1.000, lateral 2.150, ventral 3.450). The needle was left in place for two minutes prior to drug injection, then 1 μ l of 20 mM NMDA (20 nmol; Sigma) in phosphate-buffered saline was injected at a rate of 0.1 μ l per minute for 10 minutes or 0.5 μ l of 20 mM TFLLR in phosphate-buffered saline with 0.1% bovine serum albumin was injected at a rate of 0.1 μ l per minute for 5 minutes. The needle was left in place for five more minutes then slowly withdrawn and the wound closed with staples. For NMDA injections, the animals were allowed to recover for 24 hours, and then overdosed with sodium pentobarbital (390 mg/ml) and transcardially perfused with ice cold 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. The brain was removed and placed in 4% paraformaldehyde for 24 hours and cryoprotected in 20% sucrose in PBS at 4°C for 24 hours. The brains were then frozen and 20 μ m-thick coronal cryostat sections cut (Leica Model CM3050). Every tenth section was stained with cresyl violet, the lesion was identified by loss of staining, and the area was measured using Open Lab 3.08 (Improvision, Lexington, MA) and integrated to obtain the total lesion volume (Ayata et al. 1997; Guo et al. 2004). For TFLLR injections, mice were allowed to survive for five days and sacrificed in a similar manner. The sections from TFLLR-injected mice were blocked in 10% normal goat serum, and incubated overnight at 4°C in rabbit anti-GFAP antibody (Sigma, 1:5000). Sections were removed from primary antibody, washed in PBS, and incubated in secondary antibody for 1 hour at room temperature. Staining was visualized using a FITC-conjugated secondary antibody (goat-anti rabbit, Jackson ImmunoResearch, West Grove, PA, 1:200). All analysis was performed by a person blinded to genotype and condition (with or without MK801 or ketamine).

Intracerebroventricular injection of BMS200261

C57Bl/6 wild-type mice (3–5 month; Jackson Laboratory, Bar Harbor, ME) were anesthetized with isoflurane (5% in 100% oxygen for induction, 1–2% in 100% oxygen for maintenance). Animals were placed on a homeothermic blanket and body temperature was maintained between 36.5 and 37°C. The head was immobilized in a small animal stereotaxic device. A scalpel was used to expose the skull, a hole was drilled with a 0.04 inch carbide bit, and a 32-gauge needle was lowered into the brain (caudal, 2.000; lateral, 1.000; ventral, 3.000). One microliter of either the selective PAR1 antagonist BMS200261 (6 mM, Emory Microchemical Facility) or vehicle (10 mM HEPES-buffered saline) was delivered over 3 minutes. The needle was then slowly withdrawn, and the wound closed with staples.

Transient focal cerebral ischemia

Transient focal ischemia was performed and analyzed as previously described by persons blinded to the experimental conditions (Junge et al. 2003). Immediately prior to surgery, 3–5 month male wild-type or PAR1^{-/-} C57Bl/6 mice (see below) were injected intraperitoneally (IP) with either 0.9% NaCl or 3 mg/kg of (–)MK801 in saline and subsequently anesthetized with isoflurane (5% in 100% oxygen for induction, 1–2% in 100% oxygen for maintenance). Mice were placed on a homeothermic blanket and body temperature was maintained between 36.5–37°C. Relative changes in regional cerebral blood flow were monitored with a laser Doppler flow meter (Perimed Periflux System 5000, Jarfalla, Sweden). The probe was placed directly on the skull in the area of the middle cerebral artery (1 mm posterior and 5 mm lateral from bregma). The tip of an 11 mm 5-0 dermalon nylon suture was smoothed by heat, and the suture introduced into the left internal carotid artery through the external carotid artery stump until blood flow dropped. After 30 min of middle cerebral artery occlusion, blood flow was restored by withdrawing the suture. The incisions were surgically closed, and the mice immediately placed in warmed cages post-surgery. All mice had at least an 80% drop in blood flow during middle cerebral artery occlusion, as measured by laser Doppler flow. Mice that showed either no reperfusion following suture removal or a subdural hematoma were not studied further. After 24 hours survival, the brain was removed and cut into 2 mm sections. The lesion was identified by incubating the tissue in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS at 37°C for 20 min. The infarct area of each section was manually measured using NIH IMAGE (Scion Corporation, Beta 4.0.2 release), multiplied by the section thickness, and summed across sections to give the infarct volume. A ratio of the contralateral to ipsilateral hemisphere section volume was multiplied by the corresponding infarct section volume to correct for edema.

PI Hydrolysis

Young mice (C57Bl/6, age P14–17) were deeply anesthetized with isoflurane and decapitated. The brain was rapidly removed and submerged in an ice-cold oxygenated artificial cerebrospinal fluid (ACSF) comprised of (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 1 CaCl₂, 3 MgCl₂ and 10 glucose saturated with 95% O₂/5% CO₂, at pH 7.4. The hemisected brain was glued onto the stage of a vibrating microtome (Leica VT1000 S) and sections of 300 µm thickness were cut and stored in an incubation chamber at room temperature for about 1 hour before use. Slices containing the striatum were removed, the striatum dissected, and the tissue labeled for 30 minutes with ³H-inositol (1 µCi/well) at 35°C. Slices were subsequently incubated for 15 minutes in LiCl, and the accumulation of radioactive inositol phosphates was measured after a 20 minute incubation with thrombin (30 nM; 3U/ml, Gingrich et al. 2000) or the group I mGluR agonist dihydroxyphenylglycine (30 µM). Tissue samples were extracted with chloroform/methanol and the ³H-inositol monophosphate fraction was separated by anion exchange chromatography (Dowex 1 X 4 400) using increasing amounts of ammonium formate. ³H-inositol monophosphate content was assessed by liquid scintillation

spectrometry. For each experiment, measurements were from three different reaction tubes (in triplicate), each of which had tissue samples from three different slices from an animal. Three separate experiments were performed using different animals.

Animals

Male PAR1^{+/-} mice (Connolly et al. 1996) were provided by Dr. Shaun Coughlin (UCSF), from which a colony of PAR1^{-/-} mice that were >99% C57Bl/6 (backcrossed >7 times) were derived. Wild-type male C57Bl/6 mice were obtained either from Jackson Laboratory (Bar Harbor, ME; BMS200261 experiments) or from a C57Bl/6 colony that was derived from littermates of PAR1^{-/+} animals used to establish the PAR1^{-/-} homozygous colony. All experiments involving PAR1^{-/-} animals were compared to mice from a littermate derived wild-type colony.

Statistics

All measurements are given as mean $\pm$ standard error of the mean (SEM) to 2 significant digits. Measured values were compared using t-test or ANOVA, as appropriate. Results were considered significant if $p < 0.05$.

Results

PAR1 is functionally expressed in the striatum of wild-type mice

Much of the damage induced by the injury models that we used in this study (middle cerebral artery occlusion, intrastriatal NMDA injection) is found in the striatum. Therefore, before examining the potential inter-dependence of PAR1 and NMDA receptors in these injury models, it was important to confirm that both receptors are expressed in striatal tissue. Although striatal neurons are well-known to express several subtypes of the NMDA receptors (Buller et al. 1994; Qin et al. 1996; Calabresi et al. 1998; Standaert et al. 1999; Kuppenbender et al. 2000; Cepeda et al. 2001; Dunah and Standaert, 2003), no data exists on PAR1 signalling in mouse striatum. We therefore initially examined whether PAR1 was also functionally expressed in the striatum of wild-type C57Bl/6 mice. PAR1 receptors are coupled to G α q/11 and mediate robust increases in intracellular calcium. We evaluated the fluorescent response of mixed striatal cultures containing neurons and glia that had been loaded with the Ca²⁺ sensitive dye Fluo-3. We found that exposing cultures to a maximally effective concentrations of the selective PAR1 activating peptide TFLLR (30 μ M) increased Fluo-3 fluorescence in both neurons and glia, which we interpret as an increase in intracellular Ca²⁺ (Fig. 1A, n=63 cells in three separate experiments). Several studies show that TFLLR induces Ca²⁺ signaling and ERK phosphorylation in astrocytes from wild type mice, however TFLLR has no effect on Ca²⁺ signaling and ERK phosphorylation in astrocytes prepared from PAR1^{-/-} mice (Lee et al., 2007; Mannaioni et al., 2008), consistent with previous suggestions that TFLLR is a selective PAR1 antagonist (Hollenberg et al., 1997). Experiments were performed in the presence of TTX and the NMDA receptor antagonist D-APV to eliminate signals from synaptic transmission or NMDA receptor activation secondary to glutamate release (Lee et al., 2007). Approximately 15 percent of the cells responded to both NMDA (10 μ M) and TFLLR, suggesting that PAR1 is functionally coupled to Ca²⁺ signaling in a subset of neurons. Astrocytes, which show strong PAR1 responses in cultures from other brain regions, likely comprise the remaining 85% of TFLLR-responsive cells in our preparation (Sorenson et al., 2003; Nicole et al., 2005; Lee et al., 2007).

Activation of PAR1 has previously been shown to stimulate inositol phosphate signaling in hippocampal cultures and slices (Macfarlane et al. 2001; Sorensen et al. 2003; Junge et al., 2003). Exposing intact striatal slices to 30 nM thrombin increased the accumulation of tritiated inositol phosphates in wild-type mice (n=3 experiments) but not in PAR1^{-/-} mice (n=3

experiments), suggesting that functional PAR1 is expressed in intact striatum (Fig. 1B). The group I mGluR1 agonist DHPG was used as a positive control to show that cells in slices from PAR1^{-/-} animals were capable of mediating signaling through the Gq/11 pathway. Previous work has shown that PAR1 activation will cause proliferation of cortical astrocytes *in vitro* (Wang et al. 2002; Sorensen et al. 2003) and increased expression of GFAP *in vivo* in wild-type but not PAR1^{-/-} mice (e.g. Nicole et al. 2005). Therefore, we tested whether injection of the selective PAR1 activating peptide TFLLR caused astrogliosis in the striatum, as has been previously shown in the cortex (Nicole et al. 2005). Figure 1C shows that TFLLR injection causes strong astrogliosis in wild-type mice in the ipsilateral striatum (n=4). In contrast, no gliosis was observed on the contralateral side (n=4) or in the striata of mice injected with vehicle (n=2; Fig. 1C). Together these data suggest that PAR1 is functionally expressed in the striatum of C57Bl/6 mice.

Neuroprotective phenotype of PAR1^{-/-} mice requires NMDA receptor activation in transient focal ischemia

Our working hypothesis is that the difference in the lesion volume previously observed between wild-type mice and PAR1^{-/-} mice (Junge et al., 2003; Olson et al., 2004) was due in part to PAR1-mediated potentiation of NMDA-receptor signaling (Gingrich et al. 2000; Lee et al., 2007; Mannaioni et al. 2008), which exacerbates NMDA receptor-mediated cell death in the penumbra. PAR1 potentiation of NMDA function has been observed using several enzymatic PAR1 activators (e.g. thrombin, plasmin) and selective PAR1 peptide agonists (Gingrich et al. 2000; Lee et al. 2007; Mannaioni et al. 2008). Furthermore, NMDA receptor potentiation was reduced in PAR1^{-/-} mice, and could be blocked using the PAR1 antagonist BMS200261 or direct inhibitors of the activating enzymes (Gingrich et al. 2000; Lee et al. 2007; Mannaioni et al. 2008). Our working hypothesis predicts that when NMDA receptor overactivation is blocked, there should be little difference in ischemia/hypoxia-induced lesion volume between PAR1^{-/-} and wild-type mice.

In order to test this hypothesis, we evaluated the effects of an NMDA receptor antagonist *in vivo* in a model of transient focal ischemia in wild-type and PAR1^{-/-} mice. Junge et al. (2003) observed that PAR1^{-/-} mice were protected against ischemic damage following 30 min transient focal ischemia. In our hands, this duration of ischemia produces lesion volumes of less than 1/4 hemispheric volume, which approximate relative infarct volumes in human stroke. Prolonged periods of focal ischemia in C57Bl/6 mice (e.g. 1 hour) produce large lesion volumes of over 50% of hemispheric volume and engage additional molecular mechanisms that occlude the effects of PAR1 removal (Junge et al., 2003). The neuroprotective effect of PAR1 deletion following 30 min ischemia did not involve changes in blood flow, blood gases, blood-brain-barrier breakdown, or cerebral blood vessel anatomy between wild-type or PAR1^{-/-} mice (Junge et al. 2003), which we interpreted as consistent with parenchymal effects of PAR1. If PAR1 activation during 30 min ischemia exacerbated NMDA receptor-mediated cell death, then we predict that administration of an NMDA antagonist would eliminate the harmful effects of PAR1 activation in wild-type mice, rendering lesion volumes in wild-type and PAR1^{-/-} mice similar.

We evaluated the effect of the NMDA receptor antagonist MK801 (3 mg/kg, see *Methods*) on the neuroprotective phenotype observed in 30 min transient focal ischemia (Junge et al., 2003). MK801 is an uncompetitive open channel NMDA receptor blocker that has been shown to be neuroprotective in animal models of neuronal damage (Park et al. 1988; McDonald et al. 1989; Swan and Meldrum 1990; Warner et al. 1991; Roussel et al. 1992). In the absence of NMDA receptor blockade, PAR1^{-/-} mice had significantly smaller lesions than their wild-type counterparts (Fig 2A, B; wild-type mice 57±9.4 mm³, PAR1^{-/-} mice 32±8.4 mm³; p<0.05; ANOVA; n=11–12), consistent with previously published results (Junge et al.,

2003). As expected, NMDA receptor blockade by MK801 administration produced significant neuroprotection in wild-type mice (Meldrum et al. 1987; Park et al. 1988; Swan and Meldrum 1990; Uematsu et al. 1991; Miyabe et al. 1997). However, lesion volumes in PAR1^{-/-} knockout mice were not further reduced by injection with MK801 (Fig 2B). Wild-type mice treated with MK801 had a mean lesion volume of $27 \pm 5.8 \text{ mm}^3$, which was virtually identical to the lesion volume in PAR1^{-/-} mice treated with MK801 ($27 \pm 7.8 \text{ mm}^3$; $p > 0.05$; ANOVA; $n = 10-12$). These data support our working hypothesis that the harmful effects of PAR1 during transient focal ischemia require functional NMDA receptors. These findings are consistent with the idea that PAR1 potentiation of NMDA receptor function exacerbates neuronal damage. However, the alternative hypothesis that a ceiling effect for neuroprotection obscured potential additive effects of PAR1^{-/-} mice and MK801 treatment cannot be ruled out.

Neuroprotection in PAR1^{-/-} mice requires NMDA receptor activation in a model of striatal toxicity

Because glutamate accumulation during brain ischemia can mediate neuronal damage through overactivation of NMDA receptors, a useful model for NMDA receptor-mediated damage has been intraparenchymal injection of the agonist NMDA, which selectively activates NMDA receptors. We applied the same experimental design described above to this model of intrastriatal NMDA injection *in vivo* to further examine whether NMDA receptor activity was involved in the actions of PAR1 (Portera-Cailliau et al. 1995; Ayata et al. 1997; Guo et al. 2004; Leavitt et al. 2006). PAR1^{-/-} and wild-type mice either with or without 3 mg/kg MK801 pretreatment received intrastriatal injections of 20 nmol of NMDA or vehicle under isoflurane anesthesia. After 24 hours survival, mice were sacrificed, 20 μm sections were cut spanning the striatum, sections were stained with cresyl violet (Fig. 3A), and lesion volumes were measured. Injection of saline into the striatum did not produce detectable lesions in any condition (data not shown).

Removal of PAR1 or administration of MK801 significantly reduced lesion volume in wild-type mice receiving intrastriatal NMDA injection (Fig 3AB). Wild-type mice without MK801 treatment had a lesion volume of $11 \pm 1.0 \text{ mm}^3$ ($n = 12$; Fig 3C), which was significantly higher than that of PAR1^{-/-} mice ($5.6 \pm 0.4 \text{ mm}^3$; $p < 0.05$; ANOVA; $n = 6$) or that of wild-type mice receiving 3 mg/kg MK801 ($5.3 \pm 1.8 \text{ mm}^3$; $p < 0.05$; ANOVA; $n = 3$). Similar to results obtained with transient focal ischemia, PAR1^{-/-} mice did not show any further protection following MK801 injection, and had a lesion of $5.5 \pm 1.1 \text{ mm}^3$ ($n = 4$), similar to the lesion volume for PAR1^{-/-} mice without MK801 injection. There was no significant difference between lesion volumes in PAR1^{-/-} mice, MK801-injected wild-type mice, and MK801-injected PAR1^{-/-} mice ($p > 0.05$; ANOVA; $n = 3-6$; Fig 3C). These data are consistent with our findings in transient focal ischemia, and suggest that the harmful actions of PAR1 require functional NMDA receptors.

An important caveat to the use of knockout animals is the potential for compensatory mechanisms to alter gene regulation and animal response to various perturbations. One important control for this is to test whether a selective antagonist can produce the same effects as a receptor knockout. We have previously confirmed that pharmacological blockade of PAR1 is neuroprotective in transient focal ischemia (Junge et al., 2003). In order to insure that the results found in PAR1^{-/-} mice receiving intrastriatal NMDA injection were not due to an epigenetic phenomena, we treated wild-type C57Bl/6 mice (Jackson Labs) with the selective PAR1 antagonist BMS200261, (Bernatowicz et al. 1996; Kawabata et al. 1999; Mannaioni et al. 2008) injected intracerebroventricularly (ICV) 30 minutes prior to the intrastriatal NMDA injection. The mice pretreated with the PAR1 antagonist had significantly smaller lesions than those pretreated with vehicle control (Fig 4). In these studies, mice receiving vehicle ICV injections showed a mean NMDA-induced lesion volume of $8.8 \pm 1.1 \text{ mm}^3$ ($n = 6$), compared to

those receiving antagonist, which had a lesion volume of $4.8 \pm 0.3 \text{ mm}^3$ (Fig. 4B; ANOVA; $p < 0.01$; $n = 6$). Injection of PBS into striatum alone produced no detectable lesion (data not shown). This result strengthens our interpretation that effects seen in PAR1 $^{-/-}$ mice reflect the absence of the PAR1 receptor. The results further suggest that PAR1 activation, rather than just expression alone, is necessary to exacerbate NMDA-induced intrastriatal damage.

The NMDA antagonist ketamine eliminates neuroprotection in PAR1 $^{-/-}$ mice in a striatal model of toxicity

The dissociative anesthetic ketamine is a well known use-dependent uncompetitive NMDA receptor antagonist at all NMDA receptor subtypes (Harrison and Simmonds 1985; Thomson et al. 1985; Beal et al. 1988; Brady and Swann 1986; Dravid et al. 2007). Moreover, the anesthetic effect of ketamine is related to its ability to block NMDA receptor function, suggesting that NMDA receptor block will persist as long as animals show evidence of anesthesia. To assess whether ketamine can also reduce lesion volume in wild-type animals, we made intrastriatal injections of NMDA or vehicle in wild-type and PAR1 $^{-/-}$ mice anesthetized with ketamine/xylazine. We found that the lesion volume in wild-type mice receiving ketamine ($5.9 \pm 1.2 \text{ mm}^3$; $n = 9$) was significantly reduced compared to wild-type mice anesthetized with isoflurane ($11 \pm 1.0 \text{ mm}^3$; $p < 0.05$; ANOVA; $n = 12$). The lesion volume we found in ketamine-anesthetized wild-type mice (5.9 mm^3 ; Fig 5) was identical to previously reported values (6 mm^3 ; Guo et al. 2004), and similar to the volume observed in mice treated with the well-known NMDA receptor antagonist MK801 (see Fig 3). Consistent with data described above for MK801, ketamine had no effect on lesion volume in PAR1 $^{-/-}$ mice ($7.3 \pm 0.6 \text{ mm}^3$; $n = 5$; Fig 5), which in our hands was indistinguishable to previously reported findings (7 mm^3 ; Guo et al. 2004) and not significantly different from wild-type mice receiving ketamine (Fig 5; $p > 0.05$; ANOVA). These data are consistent with our working hypothesis that the harmful effects of PAR1 activation require functional NMDA receptors.

Isoflurane, like most anesthetics, also shows a modest level of NMDA receptor blockade itself and some level of neuroprotection (Criswell et al. 2004; Kawaguchi et al. 2005; Ogata et al. 2006). The IC₅₀ value for isoflurane inhibition of neuronal NMDA receptor function was 1 mM, or about 3 times the minimum alveolar concentration (MAC) of 0.32 mM (Yang and Zorumski, 1991; Yamakura et al., 2005; Ogata et al. 2006). Ketamine inhibits recombinant NMDA receptor function with an IC₅₀ in the 1–3 μM range (Dravid et al. 2007), which is roughly equal to 1 MAC. These IC₅₀ and MAC values suggest that anesthetic concentrations of isoflurane will have significantly less affect on NMDA signaling than those of ketamine. Consistent with this idea, we found no significant difference on lesion volume between mice treated with isoflurane and those treated with chloral hydrate, one of the few anesthetics not thought to be neuroprotective. Wild-type mice treated with isoflurane had an NMDA-induced lesion volume of $11 \pm 1.4 \text{ mm}^3$ ($n = 8$), which was identical to the NMDA-induced lesion volume of wild-type mice treated with chloral hydrate $11 \pm 0.7 \text{ mm}^3$ ($p = 0.85$; $n = 3$; unpaired t-test). We used isoflurane for these studies because chloral hydrate may not fully prevent pain transmission (Ozden and Isenmann 2004; Silverman and Muir 1993).

Discussion

Our working hypothesis at the outset of these studies was that PAR1 activation, through its complex and multi-faceted signaling properties (Coughlin 1999; Macfarlane et al. 2001; Trejo 2003; Traynelis and Trejo, 2007), has both positive and negative effects on neuronal survival in various models of ischemia and excitotoxicity (Shibata et al., 2001; Ruf 2003; Cheng et al., 2003; Guo et al. 2004; Junge et al. 2003; Suo et al. 2004). Here we have shown that the pro-neurodegenerative effects of PAR1 activation are dependent on the activation of the NMDA receptor in two separate *in vivo* models of neuronal injury. The results of these studies clarify

the relationship between PAR1 and the NMDA receptors during ischemia, and suggest that the ability of PAR1 to potentiate NMDA receptor function may be one important factor exacerbating neuronal death during ischemia.

PAR1 signaling and NMDA receptor function

Gingrich et al. (2000) were the first to predict that extravasation of blood-derived serine proteases during ischemia could trigger PAR1-mediated NMDA receptor potentiation, which could exacerbate neuronal damage (see also Gingrich and Traynelis 2000; Mannaioni et al., 2008). The data described here are consistent with this prediction, and suggest that NMDA receptor potentiation by PAR1 has detrimental effects during ischemia *in vivo*. In particular, the lack of further neuroprotection by the NMDA receptor antagonist MK801 in PAR1^{-/-} mice subjected to transient focal ischemia or intrastriatal NMDA injection suggests that PAR1 activation facilitates penumbral damage in a manner that involves NMDA receptor activation. Although the mechanism by which PAR1 activation potentiates NMDA receptor function requires additional study, the voltage- and Mg²⁺-dependence described in the initial report (Gingrich et al., 2000) suggest that PAR1 may trigger a depolarization-induced relief of Mg²⁺ blockade. This idea is supported by a recent study showing that PAR1 stimulates release of glutamate which can subsequently potentiate postsynaptic NMDA receptor function through depolarization-mediated relief of voltage-dependent Mg²⁺ blockade (Lee et al., 2007; Ramos-Mandujano et al. 2007). This would facilitate the NMDA receptor response of penumbral neurons to rising levels of extracellular glutamate that occur during ischemia.

One alternative hypothesis to this interpretation is the idea that PAR1 activation sensitizes neurons to NMDA receptor-mediated toxicity, without directly potentiating the NMDA receptor responses. This could occur by a number of mechanisms, including altered divalent cation homeostasis, activation of pro-apoptotic pathways, stimulation of glutamate release during ischemia, or inhibition of glutamate uptake. Some of these possibilities, although not directly involving potentiation or NMDA receptor function, would still have the effect of enhancing NMDA receptor signaling. Although the *in vivo* models we employed do not allow us to rule out these alternative hypotheses, we should note that PAR1 potentiation of synaptically-activated NMDA receptors has recently been described (Lee et al., 2007). These electrophysiological studies suggest that PAR1 activation is likely to potentiate NMDA receptor function *in vivo*. Thus, the most parsimonious interpretation at present is that PAR1 activation, perhaps secondary to blood-brain-barrier breakdown and entry into brain parenchyma of PAR1 activators, potentiates synaptic and non-synaptic NMDA receptor function in penumbral tissue, which exacerbates excitotoxicity. The effect of this would be the enhancement of neuronal death in the earliest phases following ischemia; the 24 hours survival time in this study does not allow evaluation of the mechanisms of cell death that are active at later times.

It is important to point out that PAR1^{-/-} mice and PAR1-antagonist-treated mice are not fully protected from either NMDA toxicity or from transient focal ischemia. Rather, a portion of the lesion is independent of PAR1 activation. For direct intrastriatal injections of NMDA, we hypothesize that this reflects a threshold effect of NMDA receptor overactivation on individual neuronal health, as shown in Figure 6. Following intrastriatal NMDA injection, a concentration gradient of NMDA is established centered at the injection site. This concentration gradient subsequently sets up a gradient of NMDA receptor activation. In regions close to the injection site, the degree of NMDA receptor activation is supramaximal with respect to neurotoxicity, and leads to rapid and perhaps immediate cytotoxic accumulation of divalent ions, cell swelling, and death. In regions distributed from this core, the concentration of NMDA falls as does the intensity of NMDA receptor activation. We predict that neurons outside this core area undergo modest NMDA receptor activation that is partially blunted by voltage-dependent

channel block of NMDA receptors by extracellular Mg^{2+} . Furthermore, their health and survival likely hang in the balance, as the level of NMDA receptor activation hovers near the threshold required to induce cell death. If PAR1 is present and becomes activated during injury, for example by brain-derived or blood-derived proteases (Gingrich and Traynelis 2000; Junge et al. 2003), the neurons in this peripheral region would show enhanced responses to extracellular glutamate. In this distal portion of the developing lesion, enhancement of NMDA receptor function would tip the balance towards neuronal death, expanding the lesion volume. By contrast, in the core of the lesion, the degree of NMDA receptor activation is supramaximal in terms of its cytotoxic processes. Thus, any effect of PAR1 activation on NMDA receptor function would have little effect on the survival of neurons in the core, since they are destined to die regardless of PAR1 activation.

PAR1 signaling and neuroprotection with NMDA receptor antagonists

How can application of an NMDA receptor antagonist block the hypothesized role of PAR1 without blocking the full effect of intraatrial NMDA? If an NMDA receptor antagonist was administered at a high enough concentration, the lesion volume including the core region of damage immediately adjacent to the injection site should be virtually eliminated. However, if a submaximally effective level of the uncompetitive antagonist is present, one would expect differential effects within the lesion core and distal regions. This is because the level of NMDA receptor activation is supramaximal near the injection site with respect to its ability to cause neurotoxicity. Removal of a portion of the active NMDA receptors by submaximal concentration of an uncompetitive channel blocker like ketamine would not prevent neuronal death in this core region; there is already more than enough NMDA receptor activation to cause neuronal death. However, in the penumbral regions, a submaximal dose of ketamine (or any NMDA receptor antagonist) would partially block NMDA receptor signaling in all neurons. We predict that NMDA receptor blockade in these penumbral regions could protect the neurons whose survival hangs in the balance from reaching a threshold level of NMDA receptor-induced intracellular divalent load, which is thought to drive neurotoxicity (Fig. 6). Thus, partial NMDA receptor block would effectively reduce the degree of penumbral cell death, which is a threshold event. Our data suggest that partial blockade of NMDA receptor activation in the penumbral region is sufficient to reduce lesion volume to a level observed in mice lacking PAR1. The similarity in the lesion volume between ketamine/MK801-treated mice and PAR1 $-/-$ mice further suggest that PAR1 potentiation of NMDA receptor function is a critical factor in tipping the balance towards cell death in penumbral regions.

Conclusions

Multiple reports have previously shown that PAR1 activation can potentiate NMDA receptor function in acutely prepared hippocampal slices (Gingrich et al., 2000; Lee et al., 2007; Mannaioni et al., 2008). Given the well known contribution of NMDA receptor overactivation to cell death in ischemia, we have focused in this study on the interaction of PAR1 and NMDA in two *in vivo* brain injury models. The data presented in this report are consistent with the idea that PAR1 activation during transient focal ischemia can exacerbate neuronal damage through enhancement of NMDA receptor signaling.

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Figure 1. PAR1 is functionally expressed in the striatum

A) Striatal cultures containing both neurons and glia were loaded with the Ca^{2+} -sensitive fluorescent dye Fluo-3 and subsequently exposed to 30 μM TFLLR, which induced an increase in fluorescence, which we interpret to reflect an increase in intracellular calcium. Both neurons and glia responded to TFLLR. B) Striatal slices were metabolically labeled with ^3H -myo-inositol, stimulated with 30 nM thrombin for 20 minutes, and accumulation of ^3H -inositol phosphates was measured. The group I metabotropic glutamate receptor agonist dihydroxyphenylglycine (DHPG, 30 μM) was used as a positive control, and evoked robust response in both experiments, demonstrating that the slices were viable and signaling through the phosphoinositide pathway was functional. All measurements were performed in triplicate;

data is the mean of 3 experiments; error bars are SEM. C) GFAP immunohistochemistry was performed on 20 μ m mouse brain sections 5 days after an intrastriatal injection of TFLLR (*right panels*) or vehicle (PBS–BSA 0.1%; *left panels*) in a volume of 0.5 μ l over 5 min using a Hamilton syringe (n=2 animals for PBS control, 4 animals for TFLLR). TFLLR injection caused increased GFAP reactivity in the ipsilateral striatum of all wild-type mice compared to the contralateral striatum or mice injected with vehicle.

Figure 2. PAR1^{-/-} mouse neuroprotection is dependent on NMDA receptor activation in transient focal ischemia

A) Representative TTC-stained brain sections from wild-type and PAR1^{-/-} mice at 24 hours recovery following 30 min occlusion of the middle cerebral artery under isoflurane anesthesia. B) Quantification of lesion volumes is shown in both genotypes in the presence (first two bars) and absence (last two bars) of 3 mg/kg MK801. PAR1^{-/-} mice are significantly protected from ischemia-induced damage in the absence of the NMDA receptor blocker. There is no difference between lesion volume of PAR1^{-/-} mice and wild-type mice in the presence of MK801; * p<0.05 indicates measurements different than wild-type control (ANOVA, n=10–12 per condition). Error bars indicate SEM.

Figure 3. The neuroprotective phenotype of PAR1 $-/-$ mice is dependent on NMDA receptor activation

A,B) Cresyl violet staining is shown for striatal lesions 24 hours after intrastratial NMDA injection (1 μ l of 20 mM NMDA administered over 10 minutes) in wild-type and PAR1 $-/-$ mice under isoflurane anesthesia in the presence or absence of MK801. The lesion is indicated by pallor in staining and surrounded by a black line. C) Mean lesion volumes are shown from NMDA-injected animals. Error bars indicate SEM; * $p < 0.05$ indicates measurements different than wild-type control (ANOVA, $n = 3-12$ per condition). There was no significant difference between PAR1 $-/-$ animals treated with MK801, wild-type animals treated with MK801, or

vehicle treated PAR1^{-/-} animals. D) Intrastriatal injection of PBS did not produce measurable lesions (n=2–4 per condition).

Figure 4. Wild-type mice treated with PAR1 antagonist are protected from NMDA-induced damage
A) Cresyl violet staining is shown for wild-type mouse striatal sections 24 hours after NMDA injection. B) Mean lesion volume induced by intrastratial NMDA injection into wild-type mice pretreated with either vehicle or PAR1 antagonist delivered ICV (1 μ l of PBS or 6 mM antagonist in PBS given over 3 minutes); * $p < 0.01$ (ANOVA $n = 6$ animals per condition). Lesion volumes from mice injected with phosphate-buffered saline (PBS) were undetectable (not shown). Error bars indicate SEM.

Figure 5. The NMDA antagonist ketamine eliminates the neuroprotective phenotype of PAR1^{-/-} mice

A) Typical lesion that develops 24 hours after intrastratial NMDA injection (see Methods) in mice anesthetized with ketamine/xylazine is shown. The lesion, indicated by pallor in staining, is surrounded by a black line showing how the volume was measured. B) Mean lesion volumes 24 hours following intrastratial NMDA or PBS injection are shown; error bars indicate SEM. *ns* indicates that there is no significant difference between wild-type and PAR1^{-/-} mice treated with ketamine/xylazine ($p > 0.05$; ANOVA $n = 3-12$).

Injury in control mice

Injury in mice with NMDA-R antagonist

Figure 6. Working model of exacerbation of excitotoxin damage by PAR1 potentiation of NMDA receptor function

A) Injection of NMDA creates a concentration gradient focused at the injection site. This concentration gradient creates two regions of toxicity—a region of assured cell death, where NMDA receptor activation is sufficient to be nearly uniformly toxic, and a region in which NMDA receptor activation may or may not cause cell death. In the presence of PAR1 activation, the cell death in the core of the lesion is unchanged. However, PAR1 increases NMDA signaling in the more peripheral regions thereby increasing toxicity. B) In the presence of submaximal levels of NMDA receptor blockers, neurotoxicity in the core of the lesion is unchanged. However, in surrounding areas, blockade of the NMDA receptor protects neurons from toxicity. By blocking NMDA receptor signaling, the channel blockers favor cell survival and prevent PAR1 activation from exacerbating the toxic effects of injected NMDA.