A Novel Protein Complex in Membrane Rafts Linking the NR2B Glutamate Receptor and Autophagy Is Disrupted following Traumatic Brain Injury

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Abstract

Hyperactivation of *N*-methyl-D-aspartate receptors (NRs) is associated with neuronal cell death induced by traumatic brain injury (TBI) and many neurodegenerative conditions. NR signaling efficiency is dependent on receptor localization in membrane raft microdomains. Recently, excitotoxicity has been linked to autophagy, but mechanisms governing signal transduction remain unclear. Here we have identified protein interactions between NR2B signaling intermediates and the autophagic protein Beclin-1 in membrane rafts of the normal rat cerebral cortex. Moderate TBI induced rapid recruitment and association of NR2B and pCaMKII to membrane rafts, and translocation of Beclin-1 out of membrane microdomains. Furthermore, TBI caused significant increases in expression of key autophagic proteins and morphological hallmarks of autophagy that were significantly attenuated by treatment with the NR2B antagonist Ro 25-6981. Thus, stimulation of autophagy by NR2B signaling may be regulated by redistribution of Beclin-1 in membrane rafts after TBI.

Key words: autophagy; excitotoxicity; membrane rafts; traumatic brain injury

Introduction

ACTIVATION OF *N*-METHYL-D-ASPARTATE (NMDA) receptors (NRs) play a critical role in glutamate excitotoxic damage in a variety of pathological conditions, including traumatic brain injury (TBI) (Arundine et al., 2003; Yurkewicz et al., 2005; Giza et al., 2006; Lei et al., 2006). Functional NRs contain heteromeric combinations of the NR1 subunit with one or more of NR2A-D (Nakanishi et al., 1992; Hollman et al., 1994). NR2A and NR2B are the major NR2 subtypes present in forebrain structures (Monyer et al., 1994). These receptors interact with 185 or more proteins, and form signaling complexes in the postsynaptic density (Grant, 2003). The NR complexes localize signaling molecules with Ca²⁺ influx and facilitate the activation of second messenger pathways that effect change in the channel (Lan et al., 2001), synapse (Lisman, 2001; Malinow and Malenka, 2002), and nucleus (Deisseroth et al., 1996; Hardingham et al., 2001). NR2B-containing receptors play a critical role in synaptic plasticity by activation and interaction with CaMKII at the synapse, a key component of the underlying signaling transduction pathway (Barria and Malinow, 2005). However, the mechanism regulating distribution and clustering of NR2B recep-

tors in the neuronal membrane, particularly following injury remains undefined.

Redistribution of NRs in membrane rafts is one possible mechanism for regulating the efficiency of NR signaling (Besshoh et al., 2005). Membrane rafts are heterogeneous, dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2006), and have been implicated in various neuronal processes, including maintenance of synapses and dendritic spines (Suzuki et al., 2001; Suzuki, 2002; Hering et al., 2003; Fortin et al., 2004), protein trafficking and channel localization (Nabi and Le, 2003; Wong et al., 2004; Besshoh et al., 2005), neurotrophic factor signaling, nerve growth cone guidance (Paratcha and Ibanez, 2002; Guirland et al., 2004), and the formation of death receptor signaling after TBI and spinal cord injury (SCI) (Lottocki et al., 2004; Keane et al., 2006; Davis et al., 2007). Thus, many important processes for neuronal function and viability could depend on localization of NRs in cholesterol-rich membrane rafts; however, this area has been little explored.

Intriguingly, one report provides evidence for a link between activation of the GluRδ2 receptor and stimulation of the autophagic pathway in dying *lurcher* Purkinje cells (Yue et al., 2002). In this excitotoxicity model, GluRδ2 interacts

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with a novel form of a post-synaptic density/Drosophila disc tumor suppressor/zonula occludens 1 (PDZ)-containing protein nPIST and the evolutionary conserved autophagy protein Beclin-1, thus activating the autophagy process. Autophagy is a highly regulated vesicular pathway for delivery of cellular proteins, membranes, and organelles to lysosomes for degradation and recycling (Shintanti and Klionsky, 2004). Here, we provide evidence that NR2B and signaling intermediates form protein interactions with Beclin-1 in membrane rafts in neurons in the normal rat cerebral cortex. TBI induced a rapid redistribution of Beclin-1 out of membrane rafts, altered association with NR2B signaling intermediates, and induced activation of the autophagy pathway. Thus, redistribution of Beclin-1 in membrane rafts and non-raft regions of the plasma membrane may regulate signaling responses initiated by these receptors in the normal brain and after TBI.

Methods

Parasagittal fluid percussion brain injury

All animal protocols were approved by the University of Miami Animal Care and Use Committee. Adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 250–300 g ($n = 64$, 8 per group) were surgically prepared under 3% halothane, 70% N₂O, and a balance of O₂, to achieve deep sedation. Animals were placed in a stereotaxic frame; then, a 4.8-mm craniotomy (3.8 mm posterior to bregma, 2.5 mm lateral to the midline) was performed, and an injury cap (18-gauge syringe hub; 8 mm length, PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ) was anchored over the exposed dura (Keane et al., 2001). Animals were allowed to recover for 24 h following craniotomy. The animals were then re-anesthetized and intubated endotracheally and mechanically ventilated using a Harvard rodent ventilator (Harvard Apparatus, Holliston, MA) at a mixture of 1.5% halothane, 70% N₂O, and a balance of O₂. Pancuronium bromide (0.5 mg/kg, intravenous [i.v.]) was administered every hour during the surgical procedure to facilitate mechanical ventilation. The femoral artery and vein were cannulated with a PE-50 cannula after sedation, for drug delivery and blood sampling for arterial blood pressure, arterial blood gases, and serum glucose. Arterial blood gases were measured 15 min before and after TBI. For NR antagonist experiments, NR2B antagonist Ro 25-6981 (Sigma, St. Louis, MO) or NR2A antagonist NVP-AAM077 (1 mg/kg, i.v.; Novartis Pharmaceuticals, Basel, Switzerland) was administered 30 min prior to injury (Liu et al., 2007). Rectal and temporalis muscle thermometers were used to maintain a core temperature of 37°C using self-adjusting feedback warming lamps.

A fluid-percussion injury device was connected to the injury cap. Animals were then subjected to moderate (1.7–2.1 atm) levels of TBI (Dietrich et al., 1994; Hicks et al., 1996;

Keane et al., 2001). After TBI, all animals were returned to their cages and allowed to recover from the surgical procedures. Sham-operated animals underwent all surgical procedures, but were not injured. If mortality or lung edema resulted from the injury, animals were excluded from analysis. For biochemical analysis, at the time of sacrifice, animals were deeply reanesthetized with halothane. Tissue samples were perfused and stored at 4°C, or snap-frozen in liquid nitrogen and stored at –80°C until the time of assay.

Perfusion fixation

Animals were anesthetized with an intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Complete anesthetization was determined by the lack of a stereotypical retraction of the hindpaw in response to a nociceptive stimulus. Animals then received an intracardial injection of heparin (0.1 cc) and were perfused transcardially with physiological saline, followed by 300 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed and placed in 4% paraformaldehyde at 4°C for 48 h, then transferred to 20% sucrose in 0.1 M PBS until they were ready to be sectioned.

Antibodies

Rabbit anti-ATG 5 polyclonal (1:1000) and rabbit anti-ATG 7 polyclonal (1:1000) were obtained from Abgent (San Diego, CA). Rabbit anti-LC3 polyclonal (1:1000) was obtained from Abnova (Tokyo, Japan). Mouse anti- β -tubulin monoclonal (1:3000), mouse anti-caveolin-1 monoclonal (1:250), mouse anti-flotillin-1 monoclonal (1:250), mouse anti-Homer monoclonal (1:500), mouse anti-NR2B monoclonal (1:250), mouse anti-PSD 95 monoclonal (1:250), mouse anti-shank monoclonal (1:500), and mouse anti-Thy-1 monoclonal (1:1000) were obtained from BD Biosciences (San Diego, CA). Mouse anti-pCaMKII monoclonal (1:1000), mouse anti-mTOR monoclonal (1:1000), mouse anti-pmTOR monoclonal (1:1000), mouse anti-p70 S6K monoclonal (1:1000), and mouse anti-p-p70 S6K monoclonal (1:1000) were obtained from Cell Signaling (Beverly, MA). Mouse anti-Na⁺/K⁺ ATPase monoclonal (1:1000) was obtained from Chemicon International (Temecula, CA). Rabbit anti-Beclin-1 polyclonal (1:1000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cholera toxin sub-unit B-coupled to horseradish peroxidase (HRP; 5 μ g/mL) was obtained from Sigma-Aldrich (St. Louis, MO).

Immunohistochemistry

Animals were perfused with 4% paraformaldehyde solution as described above, and brains were processed for cryostat sectioning (Leica, Bannockburr IL; SM 2000R sliding microtome). Sections (50 μ m) were stored in free-floating cryostat media (30% ethylene glycol, 30% sucrose, 0.1 M PBS,

FIG. 1. TBI induces changes in NR2B signaling intermediates and autophagy proteins in the injured cortex. **(A)** Immunoblot analysis of NR2B, pCaMKII, and PSD95. Rats were subjected to moderate TBI, and cerebral cortices were harvested at the indicated times after injury. Levels of NR2B and pCaMKII were increased at 1 and 4 h after injury. **(B)** Immunoblot analysis of Beclin-1, LC3, ATG5, ATG7, pmTOR, p-p70 S6K, mTOR, and p70 S6K. Levels of Beclin-1, LC3, ATG5, and ATG7 significantly increase after injury but with different time courses. In contrast, levels of the negative regulator of autophagy pmTOR and p-p70 S6K show significant decreases after trauma. * $p \leq 0.05$ versus sham at 15 min. mTOR and p70 S6K were used as internal standards. β -Tubulin was used as a loading control. $n = 8$ for each group.

pH 7.4) at 20°C and then rinsed with 0.1 M PBS (pH 7.4). Tissue sections were blocked by treatment with 5% goat serum (Vector Laboratories Inc., Burlingame, CA) and 0.4% Triton X-100 (Sigma). Sections were incubated for 48 h at 4°C with the primary antibodies. Primary antibody binding was detected with Alexa Fluor secondary antibody conjugates (1:200; Molecular Probes, Eugene, OR). Controls lacking the primary antibody were run in parallel. Sections were coverslipped with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) for confocal analysis (Zeiss, Thornwood, NY; LSM 510, scanning confocal microscope).

Preparation of lysate

For immunoblot analyses and membrane raft isolation, sections of cerebral cortex (2 mm²) were dounce homogenized in PTN 50 extraction buffer (50 mM sodium phosphate, pH 7.4, 1% Triton X-100, 50 mM NaCl) containing protease inhibitors (1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 µg/mL leupeptin, 1 µg/mL pepstatin A) at a concentration of 100 µg/mL as previously described (Lotocki et al., 2006). Homogenate was centrifuged at 12,000 rpm for 2 min to pellet cellular debris. Supernatants were saved and stored at -80°C.

Immunoblot analysis

Cortical lysates surrounding the injury epicenter were prepared from 15 min, and 1, 4, 8, 24, and 48 h TBI animals and from 15 min and 48 h sham-operated controls. Protein levels were resolved on 10–20% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Applied Biosciences, Foster City, CA). Membranes were blocked (0.1% Tween 20, 0.4% I-block in PBS; Applied Biosciences). Membranes were incubated with primary antibodies followed by the appropriate secondary antibody. Visualization of the signal was enhanced by chemiluminescence using a Phototope-HRP Detection Kit (Cell Signaling). To control for protein loading, the immunoblots were stripped with RestoreTM Western blot stripping buffer (Pierce, Rockford, IL), and probed for β -tubulin as a protein loading control. MTOR and p70-S6K were used as internal protein standards.

Membrane raft isolation

Detergent-resistant membranes from adult rat cortices were isolated on the basis of their insolubility in Triton-X 100 at 4°C and their ability to float in density gradients (Lotocki et al., 2006). Cortical lysates were placed in a centrifuge tube and mixed with an equal volume of 80% sucrose. The samples were overlaid with 30% and 5% sucrose, respectively. The discontinuous sucrose gradient was centrifuged at 130,000 $\times g_{avg}$ for 20 h and aliquoted into eight fractions, 0.60 ml each, with fraction 1 being the uppermost (lightest) fraction. The insoluble pellet (fraction 9) was re-suspended in 0.60 ml of PTN 50 extraction buffer plus protease inhibitors. Membrane raft containing fractions were identified by the raft marker proteins, caveolin 1, flotillin 1, Thy 1, GM 1, and Triton X-100 soluble fractions were tracked by the soluble marker protein Na⁺/K⁺ ATPase. Sucrose solutions were made using morpholino ethane sulfonic acid (MES; 25 mM, pH 6.5, 150 mM NaCl). All procedures for raft isolation were conducted at 4°C. Caveolin 1 was used as a protein loading control.

Immunoprecipitation in membrane raft and soluble fractions

Membrane raft-containing fractions were pooled and centrifuged at 130,000 $\times g_{avg}$ for 2 h to pellet the rafts. The membrane raft pellet was resuspended in PTN 50 extraction buffer plus protease inhibitor. Raft and soluble fractions were pre-cleared with 50 µL of mouse or rabbit immunoglobulin G (IgG) TrueblotTM beads (eBioscience, San Diego, CA) for 1 h. Beads were pelleted by centrifugation at 12,000 $\times g_{avg}$ for 30 sec. The resulting supernatants were mixed with 5 µg/mL of anti-NR2B (BD Pharmingen, San Diego, CA) or 5 µg/mL of anti-Beclin-1 (Santa Cruz Biotechnology) and rotated overnight at 4°C. Samples were incubated with 50 µL of mouse or rabbit IgG TrublottTM beads (eBioscience) and rotated for an hour. Beads were pelleted and washed six times in PTN 50 extraction buffer, resuspended in Laemmli loading buffer, and heated to 95°C for 3 min before analysis by immunoblotting using appropriate primary antibodies and HRP-conjugated mouse IgG TrueblotTM or rabbit IgG TrueblotTM (eBioscience) secondary antibodies (1:1000).

Transmission electron microscopy

Deeply anesthetized male Sprague-Dawley rats were perfused with saline followed by cold fixative, containing 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Regions of interest, including ipsilateral cortical layers IV–VI of injured and control animals, were grossly dissected under a microscope into ~1-mm tissue blocks and postfixed overnight, with 2% glutaraldehyde. Sections were then rinsed three times in 0.15 M phosphate buffer and fixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.4 for 2 h at room temperature followed by overnight fixation at 4°C. Sections were dehydrated using cold graded alcohols (25%, 50%, 70%, 95%, and 100% ethanol), and then rinsed in propylene oxide. Tissues were penetrated overnight with a 1:1 mixture of propylene oxide and Epon-Araldite (E/A) with DMP-30 (0.1 ml DMP-30 to 5 ml E/A). Sections were placed in fresh E/A with DMP-30 in embedding molds and placed in a 64°C oven overnight. Seventy nm thin sections were cut on a Reichert ultramicrotome, mounted on 200 mesh copper grids, contrasted with uranyl acetate and Sato's lead, then examined with a Phillips CM-10 electron microscope at 80 KV. Digital micrographs were acquired, and contrast, density, and sharpness of final images were adjusted using Adobe Photoshop CS.

Neuronal culture and glutamate treatment

Neuronal cultures were obtained by dissociation of 16–17-day Sprague-Dawley rat embryo brains. The tissue was disrupted into a cell suspension by gentle trituration, and the cells were grown on poly-L-lysine-coated tissue culture dishes in N5 medium that contained 5% serum fraction that supports the long-term survival of neurons as described elsewhere (Kaufmann and Barrett, 1983). Cultures were treated with 100 µM glutamate, or pretreated with 50–50,000 ng/ml Ro 25-6981 at 30 min prior to glutamate treatment. Cell viability was assayed by adding 50 µg (final concentration) of propidium iodide to neuronal culture for 20 min, and reading at 595 nm.

FIG. 2. Beclin-1 and LC3 autophagy proteins are present in cortical neurons, and TBI induces alterations in their expression patterns. Confocal images show cortical neurons near the injury epicenter of sham and injured brains at 24 h after TBI. Sections were stained for Beclin-1 and LC3 (red) and the neuronal markers NeuN and MAP2 (green). Beclin-1 immunoreactivity (red and merged, rows 1 and 3) was observed in a diffuse pattern in the perinuclear region of NeuN-positive cells and processes of MAP2-positive cells. At 24 h after TBI, there was increased immunostaining of Beclin-1 in cortical neurons that was distributed in a punctate pattern in the cell soma and in processes (rows 2 and 4). LC3 immunoreactivity was present as punctate inclusions in NeuN-positive cells in sham-operated animals (red and merged, row 5) and showed increased staining 24 h after injury (red and merged, row 6). Scale bar = 10 μ m.

Statistical analysis

Immunoblot analysis. Data are expressed as mean $\pm$ SEM. Quantification of bands corresponding to changes in protein levels was made using scanned densitometric analysis and NIH Image Program 1.62f. Between group differences in immunoblots were analyzed using one-way analysis of variance (ANOVA), followed by Tukey *post hoc* comparison. Cortical lysate and neuronal cultures were normalized to β -tubulin, and membrane rafts were normalized to caveolin 1. Significance level was as follows: $*p \leq 0.05$ versus sham at 15 min.

Autophagic vacuole quantification. For electron microscopy, a copper grid (200 mesh) was placed over mounted tissue and sections were randomly sampled such that every fifth grid was analyzed. Therefore, 40 grid sections per mounted tissue sample were analyzed. Five neurons per grid sections were randomly chosen for the determination of the number of autophagic vacuoles (AV) present in each neuron. AV were identified by morphological criteria as described previously (Liu et al., 2008). Briefly, initial AV (AVi) were identified by their hallmark double membrane morphology. Mitochondria were distinguished from AVi by the presence of cristae and relative size (AVi $\sim$ 300–900 nm, mitochondria $\sim$ 0.15–1 μ m). Degradative AV (AVd) were identified as having a single membrane structure containing electron dense material (Dunn, 1990; Eskelinen, 2005). These AVd are different from damaged mitochondria on the basis of electron density and relative size. Quantification of AV was by two-tailed student *t*-test. Significance level was as follows: $*p \leq 0.05$ versus sham at 15 min.

Results

Traumatic brain injury acutely increases cortical NR2B and pCaMKII

NR signaling at synapses is mediated by interaction with the scaffolding protein PSD 95 and other signaling intermediates (Kornau et al., 1995; Wu et al., 2005). Calcium influx through NR activates CaMKII (Ulrich-Bayer et al., 2006; Meng et al., 2003, 2004) that leads to downstream initiation of multiple signaling cascades. In order to define whether TBI altered proteins in NR signaling cascades, cortical lysates from control and injured animals at various times after trauma were analyzed by immunoblotting procedures (Fig. 1A). TBI induced an acute increase in the levels of NR2B and pCaMKII in cortical lysates from injured animals at 1, 4, and 8 h after TBI. However, levels of NR2B and pCaMKII had returned to baseline levels thereafter. TBI did not affect the levels of PSD 95 in lysates at any time-point measured. Thus, TBI induces significant and dynamic changes in NR2B and pCaMKII expression, but does not significantly alter the levels of PSD 95.

TBI induces an increase in Beclin-1 and key autophagic protein expression in the injured cortex

We next determined whether TBI induced changes in signaling intermediates in the autophagy pathway. Beclin-1 mediates the localization of autophagic proteins to nascent membranes and is required for the induction of autophagy (Liang et al., 1999; Petiot et al., 2000). Pro-autophagic proteins ATG 5 and ATG 7 are necessary for autophagic vacuolization

(Yu et al., 2004; Boya et al., 2005; Pyo et al., 2005; Komatsu et al., 2006). The processed form of LC3 (LC3II) localizes in autophagosome membranes (Kabeya et al., 2000) and can be used as a marker for AV formation (Kabeya et al., 2000; Mizushima et al., 2001; Boya et al., 2005). To determine the temporal profile of expression of autophagic proteins induced by TBI, cortical lysates were analyzed for Beclin-1, ATG 5, ATG 7, and LC3II from the ipsilateral hemispheres and sham-operated animals (Fig. 1B). Increased levels of Beclin-1 and ATG 7 were observed in traumatized cortices by 4 h following TBI, and elevated levels were maintained until 48 h post-trauma. Increases in the lipidated form of LC3 were observed as early as 1 h following TBI, and a significant increase in ATG 5 was observed by 24 h post-trauma. In contrast, the levels of pMTOR and p-p70 S6K, negative regulators of autophagy, decreased in lysates of injured animals at 24 and 48 h after TBI (Fig. 1B). No changes were observed in the levels of mTOR, p70 S6K, and β -tubulin that served as internal standards or loading controls. Thus, key proteins in the autophagic pathway are significantly up-regulated in the injured cerebral cortex, and negative regulators of the process are significantly down-regulated following TBI.

Beclin-1 is present in cortical neurons, and TBI induces alterations in protein expression pattern

Figure 2 shows confocal images of the cell type expression and regional distribution of Beclin-1 and LC3 in neurons in cerebral cortices of sham-operated animals and injured cortices at 24 h after injury. Sections were stained for Beclin-1, LC3 (red), and the neuronal markers NeuN and MAP2 (green). Beclin-1 immunoreactivity was seen in NeuN- and MAP2-positive cells, indicating that Beclin-1 is expressed in neurons in the cortex of sham animals. Beclin-1 immunoreactivity was observed in a diffuse pattern in the perinuclear region of NeuN-positive cells (red and merged, row 1), and less intense immunostaining was seen in MAP2-positive cells (red and merged, row 3). LC3 expression in cortical neurons of sham-operated animals was also detected using immunohistochemistry and confocal microscopy (red and merged, row 5).

Moderate TBI resulted in altered staining patterns of Beclin-1 and LC3 in cortical neurons (Fig. 2). At 24 h after injury, increased Beclin-1 immunoreactivity was present in neurons surrounding the injury epicenter and exhibited a punctate staining pattern in the cell soma (red and merged, row 2). Increased Beclin-1 expression was also present in dendritic processes of MAP2-positive cells and exhibited a punctate staining pattern (red and merged, row 4). Moreover, by 24 h after injury, increased LC3 immunoreactivity was seen in NeuN-positive cells located around the injury epicenter (red and merged, row 6). LC3 immunoreactivity exhibited a punctate staining pattern similar to that observed for Beclin-1. Thus, TBI induces alterations in Beclin-1 and LC3 immunoreactivity consistent with formation of AV.

Ultrastructural analysis of autophagic maturation after traumatic brain injury

Induction of autophagy is manifested by the formation of autophagosomes (APs) that enclose cytoplasmic constituents. APs are double membrane vacuoles and are readily identified by electron microscopy (EM) procedures, and are hallmarks

FIG. 3. Ultrastructural evidence for autophagy in normal cortical neurons and 3 days after TBI in adult male rats. Transmission electron micrographs of cortical neurons from injured animals near the injury epicenter at 3 days after trauma (**A,B**). Arrowhead points to double membrane vacuoles (**A**). Arrows point to initial autophagic vacuoles (AVi); degradative autophagic vacuoles (AVd). (**C**) Quantification of number of AVi and AVd per cortical neuron in sham and injured brains at 3 days after trauma. * $p \leq 0.05$ versus sham at 15 min. Scale bar = 500 nm. $n = 6$ for each group.

FIG. 4. Partitioning of NR2B, signaling intermediates, and Beclin-1 in membrane rafts in the normal and injured cortex. (**A**) Density gradient profile of detergent-resistant membranes from cortices of sham-operated animals. Cortical membranes were extracted with 1.0% Triton X-100 at 4°C and separated on density gradients formed from 40%, 30%, and 5% sucrose. Eight fractions (from top to bottom of the gradient) were immunoblotted for the indicated proteins. Fraction 2 of the gradient was enriched for caveolin 1, flotillin 1, Thy 1, and GM1, indicating the membrane raft containing fraction (R), while fraction 8 contained the Na⁺/K⁺-pump, indicating the non-membrane raft or soluble fraction (TS). (**B**) TBI induces recruitment of NR2B and pCaMKII into membrane rafts and translocation of Beclin-1 out of membrane rafts. Equal aliquots of the fractions were subjected to SDS-PAGE, and the protein distribution was assessed by immunoblotting using specific antibodies against NR2B, pCaMKII, PSD 95, Beclin-1, and caveolin 1. * $p \leq 0.05$ versus sham at 15 min. $n = 8$ for each group.

FIG. 5. Membrane raft associated NR2B signaling complex is altered following TBI. (A) Co-immunoprecipitation with anti-NR2B of membrane raft fractions of cortical lysates obtained from sham animals and at 15 min to 48 h after TBI. NR2B immunoprecipitates were blotted for NR2B, PSD 95, Shank, Homer, Beclin-1, pCaMKII, and Flotillin 1. In sham animals, anti-NR2B immunoprecipitated NR2B, PSD 95, Shank, Homer, and Beclin-1, thus indicating association of these proteins in a multiprotein complex. TBI-induced disassociation of this multiprotein signaling complex within 15 min. (B) Co-immunoprecipitation using anti-NR2B demonstrated similar protein associations of NR2B signaling intermediates in cortical membrane rafts in the contralateral hemisphere of sham animals. However, the NR2B multiprotein signaling complex was not present in Triton X-100 insoluble fractions (TS) isolated from cortical lysates. (C) Reciprocal co-immunoprecipitation with Beclin-1 of membrane rafts from sham and injured cortices, and rafts obtained from injured animals. Anti-Beclin-1 immunoprecipitated Beclin-1 and NR2B in sham animals only, further indicating association of these proteins in a multiprotein complex in membrane rafts.

of autophagy. To establish whether TBI-induced increased expression of autophagic proteins resulted in autophagic maturation, cortical sections of sham and injured animals at 3 days after trauma were examined by transmission EM.

AVi were identified as double membrane structures (Fig. 3A, arrowhead) and were seen in cell processes and the axon trunk, as well as in perinuclear areas in both sham and injured animals, thus indicating that autophagy is a constitutive physiological process in cortical neurons. Degradative AV (AVd) were identified as single-membrane bound degradative vacuoles containing electron dense material (Fig. 3B). Loss of the inner membrane is presumably due to hydrolysis or fusion with the outer membrane (Dunn, 1990). Cortical neurons in sham-operated animals contained a small number of initial AVi and AVd (Fig. 3C). Cortical neurons of rats at

3 days after TBI contained significantly more AVi and AVd (Fig. 3C).

TBI alters Beclin-1, NR2B, and pCaMKII distribution in membrane rafts in the injured cortex

To investigate whether membrane rafts are involved in NR2B signal transduction pathways after TBI, membrane rafts were isolated from sham-operated and traumatized cortices by discontinuous sucrose gradient centrifugation. The purity of membrane raft preparations was confirmed by immunoblotting gradient fractions with the membrane raft markers: flotillin 1, cholesterol-binding protein caveolin 1, GPI-anchored cell surface protein Thy 1, and sphingolipid ganglioside G_{M1} , and Triton X-100 soluble (TS) fractions were identified by the

FIG. 6. NR2B antagonist but not NR2A antagonist inhibits TBI-induced increases in expression of NR2B signaling intermediates in the injured cortex. Representative immunoblots of cortical lysates from sham animals and injured animals injected intravenously with 1 mg/ml/kg of Ro 25-6981 or NVP0AAM077 at 30 min prior to TBI. (A) Ro 25-6981 (NR2B antagonist) blocked TBI induced increases in NR2B signaling intermediates. (B) NVP-AAM077 (NR2A antagonist) had no effect on expression of NR2B signaling intermediates, where protein profiles were similar to those in untreated animals subjected to TBI (Fig. 1). * $p \leq 0.05$ versus sham at 15 min. $n = 6$ for each group.

non-membrane raft marker Na^+/K^+ -ATPase (Fig. 4A). Low-density membrane raft fractions (fraction 2, labeled R) of gradients from both sham-operated and injured animals were enriched in flotillin 1, caveolin 1, Thy 1, and G_{ML} , and the high-density Triton X-100 soluble fraction (fraction 8, labeled TS) was enriched in Na^+/K^+ -ATPase. Although small amounts of NR2B, pCaMKII, PSD 95, and Beclin-1 were associated with membrane rafts in cortices of sham animals, most of these proteins were excluded from these microdomains (Fig. 4B). Association of NR2B and pCaMKII with membrane rafts was significantly increased by 1–8 h after TBI, but levels declined thereafter. In contrast, there was a significant translocation of Beclin-1 out of membrane rafts, rapidly at 15 min and 1 h after TBI. Significant increases in NR2B, pCaMKII, and Beclin-1 were also observed in TS fractions of the gradient, exhibiting a similar temporal profile (Fig. 1A,B) as that observed in cortical

lysates (data not shown). Thus, a portion of NR2B, pCaMKII, PSD 95, and Beclin-1 are present in membrane rafts, and TBI signals recruitment of NR2B and pCaMKII to rafts, but rapid translocation of Beclin-1 out of rafts.

NR2B and signaling intermediates form a novel protein complex with Beclin-1 in membrane rafts that is disrupted after traumatic brain injury

When proteins are present in membrane rafts, association of those proteins is favored compared with that of proteins excluded from membrane rafts (Friedrichson and Kurzchalia, 1998). To assess the protein composition and association of signaling proteins in the NR2B signaling complexes, membrane rafts were isolated and immunoprecipitated with anti-NR2B antibody (Fig. 5A). In membrane rafts of cortices from sham-operated animals, NR2B was associated with PSD 95,

Homer, Shank, and surprisingly with the autophagy protein Beclin-1. Within 15 min after TBI, the composition of the complex changed. There was a substantial loss of NR2B-associated adaptor proteins, PSD 95, Homer, and Shank, and the autophagic signaling protein Beclin-1. A transient association of pCaMKII was observed in the NR2B signaling complex at 15 min, and 1 and 4 h after TBI. Anti-NR2B did not immunoprecipitate the raft-associated protein flotillin 1, demonstrating specificity in this membrane raft associated protein complex. In soluble fractions of cortical lysates of sham animals, anti-NR2B antiserum immunoprecipitated NR2B, but did not immunoprecipitate NR2B signaling intermediates PSD 95, Shank, Homer and the autophagic protein Beclin-1 (Fig. 5B), indicating that these proteins are not associated with each other in non-membrane raft fractions. In a reciprocal immunoprecipitation, from membrane rafts, using anti-Beclin-1 (Fig. 5C), NR2B was immunoprecipitated in the sham brain, thus serving as a positive control. Taken together, these findings show that NR2B, PSD 95, Homer, Shank, and Beclin-1 form a novel complex in membrane rafts of cortical neurons in the normal brain. TBI induces translocation of NR2B into membrane rafts and Beclin-1 out of membrane rafts, altering formation of receptor-associated signaling complexes that may regulate different biological outcomes dictated by these complexes.

NR2B but not NR2A antagonist inhibits NR2B signaling and delays TBI-induced increases in Beclin-1 and key autophagic protein expression in the injured cortex

We next tested whether NR2B and NR2A-containing receptors exert differential roles in mediating NMDA-induced signaling and autophagy in traumatized brains using subunit-specific NMDA receptor antagonists: Ro 25-6981, which specifically blocks NR2B containing receptors (Fischer et al., 1997), and NVP-AAM077, which preferentially inhibits NR2A-containing receptors (Liu et al., 2004). As proof of concept, animals were injected intravenously with 1 mg/mL/kg of antagonist 30 min prior to TBI, whereas sham animals served as controls. Cortical lysates were prepared at various times after injury and immunoblotted for NR2B signaling intermediates (Fig. 6) and autophagic proteins (Fig. 7). Pretreatment with Ro 25-6981 inhibited the TBI-induced increase in NR2B and pCaMKII (Fig. 6A), whereas NVP-AAM077 had little or no effect (Fig. 6B), indicating a critical involvement of NR2B but not NR2A-containing NR subtypes in mediating TBI-induced excitotoxic effects.

To examine antagonist effects on the autophagic signaling pathway, Ro 25-6981- and NVP-AAM077-treated animals were subjected to TBI, and cortical lysates were analyzed by immunoblotting procedures (Fig. 7). Animals pretreated with the NR2B antagonist showed increases in Beclin-1, LC3, and ATG 7, but the rise in these protein levels were delayed when compared to the acute increase observed in untreated animals (Fig. 7A). Levels of ATG 5 and pMTOR did not change after

TBI, whereas levels of p-p70 S6K showed a significant decrease at 24 and 48 h after TBI (Fig. 7A). Animals pretreated with the NR2A antagonists (Fig. 7B) demonstrated increases and time-courses of expression of autophagic proteins similar to those observed in untreated animals (Fig. 1B), except a significantly earlier decreases in pMTOR and p-p70 S6K were observed. These results support the idea that NR2B-containing NRs specifically mediate TBI-induced up-regulation of key proteins in the autophagic pathway.

NR2B but not NR2A antagonist alters the protein composition and association of NR2B signaling complexes with Beclin-1 in membrane rafts

As shown in Figure 5, a portion of NR2B in cortical tissue is associated with membrane rafts, where it forms a signaling complex that includes NR2B, PSD95, Shank, Homer, and Beclin-1. TBI disrupts protein associations in this complex and induces translocation of Beclin-1 out of membrane raft fractions. To determine whether NMDA antagonists altered protein associations of NR2B and Beclin-1, membrane rafts were isolated from cortices of injured rats pretreated with Ro 25-6981 and NVP-AAM077 and immunoprecipitated with anti-NR2B (Fig. 8). NR2B, PSD 95, Homer, Shank, and Beclin-1 were immunoprecipitated with NR2B in sham animals. However, pretreatment of injured animals with the NR2B, but not the NR2A antagonist, significantly delayed the disassociation of Beclin from NR2B signaling intermediates and blocked the translocation of Beclin-1 out of membrane rafts. Thus, it appears that the dissociation of NR2B signaling intermediates and Beclin-1 after TBI in membrane rafts is dependent on NR2B activity.

NR2B antagonist blocks glutamate excitotoxic death and induction of autophagy in primary neuronal cultures

Glutamate excitotoxicity contributes to neuronal cell death in primary neuronal cultures (Regan and Choi, 1994) and following TBI (Faden et al., 1989). To determine whether NR2B antagonists blocked glutamate excitotoxicity, we pretreated primary neuronal cultures with Ro 25-6981 followed by glutamate treatment (100 μ M). Figure 9A,B shows a dose response of neuroprotective effects of Ro 25-6981. At concentrations of 50–10 $\times 10^3$ ng/ml, the Ro 25-6981 significantly blocked neuronal death. At doses higher than 50 $\times 10^3$ ng/ml, Ro 25-6981 was toxic. Thus, NR2B antagonist significantly blocks glutamate excitotoxicity of primary neurons. To investigate whether glutamate exposure induces autophagy protein expression in primary neuronal cultures, we treated cortical neurons with 100 μ M glutamate and analyzed autophagy protein expression by immunoblot analysis. Significant increases in Beclin-1 and LC3 and lipidated LC3II were observed at 2–6 h after glutamate treatment (Fig. 9C). Pre-treatment with the NR2B antagonist Ro 25-6981 blocked

FIG. 7. NR2B antagonist but not NR2A antagonist inhibits TBI-induced increases in expression of autophagic proteins in the injured cortex. Representative immunoblots of cortical lysates from sham animals and injured animals injected intravenously with 1 mg/ml/kg of Ro 25-6981 or NVP-AAM077 at 30 min prior to TBI. (A) Pretreatment with the Ro 25-6981 (NR2B antagonist) blocked the TBI-induced increases in protein expression of autophagic proteins after TBI. (B) NVP-AAM077 (NR2A antagonist) did not alter expression of autophagic proteins after TBI, where protein profiles in both signaling pathways were similar to untreated animals subjected to TBI (Fig. 1). * $p \leq 0.05$ versus sham at 15 min. $n = 6$ for each group.

A **B**

the expressional changes observed in Beclin-1 and LC3 treated with glutamate (Fig. 9D). Taken together, these results indicate that glutamate treatment of cortical neurons results in significant cell death and increases expression of autophagy proteins. NR2B antagonist Ro 25-6981 blocks cell death and the induction of autophagy proteins by glutamate treatment.

Discussion

Our data show that, in the normal cortex, a portion of the NR2B receptor, the adaptor proteins PSD95, Shank and Homer, and the autophagic protein Beclin-1 localize in membrane rafts forming a novel multi-protein complex. TBI induced a dissociation of the protein complex and caused a rapid increase in the levels of NR2B, a translocation of pCaMKII to membrane rafts, and a translocation of Beclin-1 out of membrane raft microdomains. These findings suggest that membrane rafts mediate trafficking and signaling of NR2B and Beclin-1 following TBI.

The role of membrane rafts in the signal transduction of NRs has been established *in vitro* (Frank et al., 2004) and in rat models of ischemia (Hu et al., 1998; Hering et al., 2003; Besshoh et al., 2005), and NRs located in membrane rafts mediate neurotoxicity (Frank et al., 2004; Abulrob et al., 2005). Recent reports demonstrate that NR activation can produce either neuronal survival or death-promoting actions (Liu et al., 2007; Zhang et al., 2007) and that this dual action can be modulated by receptor subunit composition (Liu et al., 2007). NR2B-containing receptors have been shown to mediate pro-survival effects in several neuronal populations (Takadera et al., 2004; Hetman and Kharebava, 2006) and to contribute to cytotoxic cell death and apoptosis (Liu et al., 2007). Our findings extend these observations and indicate that following TBI, the composition of NR2B receptor-associated signaling complex that is found in membrane raft microdomains, undergo changes that may contribute to the activation of signal transduction pathways involving autophagy.

Post-translational modifications of several complex-associated proteins occur in membrane rafts in traumatized brains. For example, influx of extracellular calcium through activated NRs following TBI increases CaMKII phosphorylation, and pCaMKII has been shown to interact transiently with NR2B (Ulrich-Bayer et al., 2001; Meng et al., 2002). However, prolonged NR2B stimulation results in a transition from reversible to persistent binding of pCaMKII to NR2B (Ulrich-Bayer et al., 2006), thus causing phosphorylation of the receptor. NR2B phosphorylation (Meng et al., 2002) and subsequent internalization (Vissel et al., 2001; Aarts and Tymanski, 2004) occurs via membrane raft microdomains (Besshoh et al., 2005). Moreover, pCaMKII binding to NR2B

decreases NR2B association with PSD95 (Gardoni et al., 2001), thus exposing the internalization motif on the receptor (Roche et al., 2001). Disruption of NR2B/PSD95 interaction contributes to receptor destabilization at the membrane. Therefore, the phosphorylated state of NR2B may contribute to its internalization via membrane raft microdomains following TBI.

The data reported here identify novel protein interactions among the evolutionarily conserved autophagic protein Beclin-1, the NR2B receptor, and the synaptic scaffolding proteins PSD95, Shank, and Homer within membrane raft microdomains in the normal cortex. These interactions provide a physical linkage between the NR2B receptor and Beclin-1. At the synapse, NR2B and PSD95 have a known interaction through their common PDZ ligand domain (Roche et al., 2001; Dong et al., 2004; Kim and Sheng, 2004; Sheng, 2007). PSD95 interaction with Shank via PDZ/SH3 domains, and Shank interaction with Homer via proline rich motifs, contributes to synaptic organization, the regulation of protein interaction and cytoplasmic signaling pathways (Migaud et al., 1998; Sprengel et al., 1998), acting as a key modular subdomain of the post-synaptic specialization (Sheng, 2001). It is likely that Beclin-1 is associated with this NR2B protein complex via interaction with Homer through their common coil-coil domains, and that this interaction is facilitated within membrane raft microdomains.

TBI induced a rapid recruitment of NR2B into membrane rafts, but caused a translocation of Beclin-1 out of these microdomains. Co-immunoprecipitation of the NR2B signaling complex revealed that NR2B/PSD95/Shank/Homer/Beclin-1 interactions in membrane rafts were lost after TBI, suggesting that release of Beclin-1 or PSD95/Shank/Homer/Beclin-1 from the complex in response to excessive stimulation of NR2B following TBI may be a critical event required for activation of autophagy in neurons. In support of this idea is our observation that inhibition of NR2B receptor signaling by the NR2B antagonist Ro 25-6981 delays the triggering of intracellular cascades that lead to autophagy and NMDA- or ischemia-induced neuronal apoptosis. The inability of NVP-AAM077 to block TBI-induced changes in NR signaling and autophagy is an additional indication that NR2B receptor pathways have specific biological effects. Moreover, in the Lurcher mouse, GluR δ 2 is linked to the autophagy process through similar protein-protein interactions involving an isoform of PIST and Beclin-1 (Yue et al., 2002). Delineation of the precise mechanism by which NR2B leads to induction of autophagy will require additional studies aimed at determining the properties of NR2B/PSD95/Shank/Homer/Beclin-1 complex. For example, it is not clear whether Beclin-1 is covalently modified in response to signaling through the receptor and whether this modification leads to release of one or

FIG. 8. NR2B but not NR2A antagonist inhibits TBI-induced alterations of protein associations of NR2B signaling proteins in membrane rafts. Co-immunoprecipitation with anti-NR2B of membrane raft fractions of cortical lysates obtained from sham animals and animals pretreated with injected intravenously with 1 mg/ml/kg of Ro 25-6981 (A) or 1 mg/ml/kg of NVP-AAM077 (B) at 30 min prior to injury. Membrane rafts were isolated from animals at 15 min to 48 h after TBI and immunoprecipitated with anti-NR2B. NR2B immunoprecipitates were blotted for the indicated proteins. In sham animals, anti-NR2B immunoprecipitated NR2B, PSD 95, Shank, Homer, and Beclin-1, thus indicating association of these proteins in a multiprotein complex. Animals pretreated with Ro 25-6981 (NR2B antagonist) did not show TBI-induced disassociation of this multiprotein signaling complex at 15 min after trauma as observed in untreated animals subject to trauma (Fig. 5A). However, animals pretreated with the NVPAAM077 (NR2A antagonist) demonstrated similar pattern of association of NR2B signaling proteins as those observed in untreated sham and injured animals (Fig. 5).

FIG. 9. Glutamate treatment of primary neuronal cultures results in activation of autophagy and cell death that is blocked by the NR2B antagonist Ro 25-6981. **(A)** Primary cortical neurons were treated with 100 μ M glutamate for 8 h resulted in cell death as assayed by propidium iodide. Ro 25-6981 (10 ng/ml to 25 μ g/ml) administered 30 min prior to glutamate treatment significantly decreased glutamate induced cell death. **(B)** Immunoblots of primary cortical neurons treated with 100 μ M glutamate for 8 h resulted in significant increases in Beclin-1 and LC3II at 2–6 h after glutamate treatment. **(C)** Ro 25-6981 (100 ng/ml) administered 30 min prior to glutamate treatment prevented increases in Beclin-1 and LC3II. * $p \leq 0.05$ versus control. $n = 6$ wells per each group.

several of these proteins from the complex. As well, Beclin-1 is a Bcl-2 interacting protein (Liang et al., 1998a,b) and is found as a component of the class II PI3 kinase complex, which is involved in signal transduction pathways involved in both apoptosis and autophagy. The anti-apoptotic protein Bcl-2 directly binds to Beclin-1, attenuating autophagy-dependent cell death (Kihara et al., 2001; Levine and Yuan, 2005; Pattingre et al., 2005), as well as regulating autophagy under non-toxic conditions (Shimizu et al., 2004; Luo and Rubenstein, 2007), suggesting Bcl-2 may function as an anti-apoptotic and anti-autophagic protein.

Autophagy is a pathway for lysosome-mediated bulk degradation of subcellular constituents, and contributes to routine turnover of cytoplasmic components (Shintanti and Klionsky, 2004). This process has been implicated in a number of neurodegenerative pathologies (Larsen and Sulzer, 2002), and significant increases in Beclin-1 have been observed in TBI (Diskin et al., 2005; Clark et al., 2008; Liu et al., 2008). Whether increased Beclin-1 expression leads to the induction of autophagy or whether it contributes to apoptotic signaling is currently under debate. Several key autophagic proteins have a direct interaction with the apoptotic pathway, and the interplay between these processes remains unclear. Inhibition of autophagy can trigger apoptosis (Boya et al., 2005; Takacs-Vellai et al., 2005) and apoptotic signals can activate autophagy (Xue et al., 1999). Recent studies report that ATG5 interacts with the Fas-associated protein with death domain (FADD), a component of the extrinsic apoptotic pathway, which leads to interferon-induced cell death (Pyo et al., 2005), and thus provide another point of convergence of apoptotic and autophagic mechanisms. ATG5 is cleaved following Fas receptor activation, which can lead to cell death through interaction with Bcl-xl promoting Bax-induced mitochondrial dysfunction and apoptosis (Yousefi et al., 2006). Alternatively, autophagy may be a process that promotes survival. Autophagy is constitutively active at low basal levels to perform homeostatic functions, contributing to nutrient and energy conservation, subcellular remodeling and protein turnover (Baehrecke, 2005; Levine and Yuan, 2005). Increased death has been demonstrated in rodent models lacking ATG5, ATG7, and Beclin-1 gene products (Liang et al., 1998a,b; Lum et al., 2005). Specifically in the nervous system, loss of ATG7 has been observed to lead to neurodegeneration, confirming autophagy as a critical function for survival in neurons (Komatsu et al., 2006), and in this regard, may provide a mechanism of re-establishing homeostasis following injury. Moreover, pro-autophagic proteins ATG 5 and ATG 7 are necessary for autophagic vacuolization (Yu et al., 2004; Boya et al., 2005; Pyo et al., 2005; Komatsu et al., 2006). The processed form of LC3 (LC3II) localizes in autophagosome membranes (Kabeya et al., 2000) and can be used as a marker for AV formation (Kabeya et al., 2000; Mizushima et al., 2001; Boya et al., 2005). Here we observed an increase in these and other key autophagic proteins early after TBI, and a significant decrease in the negative regulator of autophagy mTOR that may contribute to increases in the number of autophagosomes in neurons observed after trauma.

Glutamate excitotoxicity contributes to secondary injury following TBI (Faden et al., 1989). Our data demonstrates that glutamate induced cell death of primary neuronal cultures, activates the autophagy pathway. This activation is blocked by the NR2B antagonist Ro 25-6981, thus demonstrating a link

between NR2B signaling, induction of autophagy and cell death. In addition, we have shown that Ro 25-6981 blocked the dissociation of the NR2B-Beclin-1 containing signaling complex, and significantly delayed the induction of autophagy *in vivo*, further supporting the link between NR2B signaling and autophagy. As proof of concept, the NR2B antagonist was administered intravenously 30 min prior to the traumatic insult. Since Ro 25-6981 has a half-life of dissociation of approximately 5 hrs (Mutel et al., 1998), it is likely that further studies are needed to determine whether repeated administration of the antagonist will completely block autophagy induction following TBI.

Our findings demonstrate that moderate TBI induces a rapid redistribution of a novel NR2B multi-protein signaling complex, where the key autophagic protein Beclin-1, is translocated out of membrane raft microdomains. Thus, the release of Beclin-1 or PSD95/Shank/Homer/Beclin-1 from the complex in response to excessive stimulation of NR2B following TBI may be a critical event required for activation of autophagy in neurons. Autophagy has been implicated as an active process in other models of TBI (Diskin et al., 2005; Clark et al., 2008; Liu et al., 2008); however, its role as a protective or detrimental process is still unclear. Understanding of glutamatergic signaling through NR2B, and its relationship to autophagic activation, may provide insight into the effects of excitotoxicity on cellular processes after injury and lead to development of pharmacological management of pathological cell loss.

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Author Disclosure Statement

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