N-Methyl-D-Aspartic Acid Receptor Activation Downregulates Expression of δ Subunit-Containing GABA_A Receptors in Cultured Hippocampal Neurons^[S]

Suchitra Joshi and Jaideep Kapur

Departments of Neurology (S.J., J.K.) and Neuroscience (J.K.), University of Virginia Health Sciences Center, Charlottesville, Virginia

Received December 27, 2012; accepted April 9, 2013

ABSTRACT

Neurosteroids are endogenous allosteric modulators of GABA_A receptors (GABARs), and they enhance GABAR-mediated inhibition. However, GABARs expressed on hippocampal dentate granule neurons of epileptic animals are modified such that their neurosteroid sensitivity is reduced and δ subunit expression is diminished. We explored the molecular mechanisms triggering this GABAR plasticity. In the cultured hippocampal neurons, treatment with *N*-methyl-D-aspartic acid (NMDA) (10 μ M) for 48 hours reduced the surface expression of δ and α 4 subunits but did not increase the expression of γ 2 subunits. The tonic current recorded from neurons in NMDA-treated cultures was reduced, and its neurosteroid modulation was also diminished. In contrast, synaptic inhibition and its modulation by neurosteroids were preserved in these neurons.

The time course of NMDA's effects on surface and total δ subunit expression was distinct; shorter (6 hours) treatment decreased surface expression, whereas longer treatment reduced both surface and total expression. DI-2-amino-5-phosphonopentanoic acid (APV) blocked NMDA's effects on δ subunit expression. Chelation of calcium ions by 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) or blockade of extracellular signal-regulated kinase (ERK) 1/2 activation by UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) also prevented the effects of NMDA. Thus, prolonged activation of NMDA receptors in hippocampal neurons reduced GABAR δ subunit expression through Ca²⁺ entry and at least in part by ERK1/2 activation.

Introduction

Neurosteroids are potent endogenous modulators of GABA_A receptors (GABARs) (Lambert et al., 2009). These compounds exert a strong anticonvulsant action, and the rank order potency of anticonvulsant action is similar to their potency at GABARs (Kokate et al., 1994, 1996). Neurosteroids convert GABA from a partial to a full agonist at δ subunit-containing GABARs (GABAR- δ) (Bianchi and Macdonald, 2003). GABAR- δ s have a high affinity for GABA and desensitize slowly and incompletely. Thus, once activated, these receptors remain open longer (Saxena and Macdonald, 1994; Haas and Macdonald, 1999; Bianchi et al., 2002; Bianchi and Macdonald, 2003).

GABAR- δ s are localized to extrasynaptic membranes and mediate a major fraction of tonic inhibition in hippocampal dentate granule neurons (DGCs) (Nusser et al., 1998; Wei et al., 2003; Glykys et al., 2007). In contrast γ 2 subunit-containing receptors (GABAR- γ 2s) are localized to synaptic and extrasynaptic membrane and have a lower affinity for GABA as well as neurosteroids (Saxena and Macdonald, 1994; Belelli et al., 2002).

In animal models of temporal lobe epilepsy, GABAR- δ expression is diminished in the hippocampal DGCs (Peng et al., 2004; Zhang et al., 2007; Rajasekaran et al., 2010). In contrast, the expression of α 4 subunits, which usually assemble with δ subunits, is increased (Peng et al., 2004; Rajasekaran et al., 2010). Reduction in GABAR- δ is associated with diminished neurosteroid modulation of tonic currents; however, total tonic current is preserved (Zhang et al., 2007; Rajasekaran et al., 2010). Upregulation of the γ 2 subunit-containing receptors maintained tonic current and enhanced synaptic inhibition of the DGCs of epileptic

This study was supported by National Institutes of Health National Institute of Neurological Disorders and Stroke [Grants RO1 NS 040337 and RO1 NS044370 (to J.K.)]. A part of the work was also supported by an American Epilepsy Society postdoctoral fellowship to S.J.
dx.doi.org/10.1124/mol.112.084715.

[S] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: APV, dl-2-amino-5-phosphonopentanoic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxymethyl ester); BDNF, brain-derived neurotrophic factor; CA, cornu ammonis; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; DGC, dentate granule neuron; DNQX, 6,7-dinitroquinoxaline-2,3-dione; ERK1/2, extracellular signal-regulated kinase 1/2; GABAR, GABA_A receptor; *I*_{hold}, current required to voltage clamp neuron to -60 mV; KN62, 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; KN93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; mIPSC, miniature inhibitory postsynaptic current; NMDA, *N*-methyl-D-aspartic acid; NMDAR, NMDA receptor; NO711, 1-[2-[[[diphenylmethylene]imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid; SE, status epilepticus; TrkB, tropomyocin receptor kinase B; TTX, tetrodotoxin; UO126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene.

animals, although neurosteroid modulation of synaptic currents is also diminished (Sun et al., 2007; Zhang et al., 2007; Zhan and Nadler, 2009; Rajasekaran et al., 2010). The molecular mechanisms that trigger these changes are not known.

Prolonged seizures or status epilepticus (SE) will often lead to the development of temporal lobe epilepsy in experimental animals (Turski et al., 1987; Cronin and Dudek, 1988; Lothman et al., 1990). One or more of the mechanisms activated during SE may downregulate the expression of GABAR- δ and upregulate the expression of GABAR- γ 2. *N*-methyl-D-aspartic acid (NMDA) receptors (NMDARs) are activated during prolonged SE. Their blockade protects animals from excitotoxic cell death and development of spontaneous seizures (Fujikawa, 1995; Rice and DeLorenzo, 1998; Prasad et al., 2002; Brandt et al., 2003). Furthermore, NMDAR antagonists in combination with benzodiazepines can terminate SE (Rice and DeLorenzo, 1999; Borris et al., 2000; Martin and Kapur, 2008).

We tested whether NMDAR activation reduced GABAR- δ expression and upregulated that of GABAR- γ 2. Our findings suggest that NMDAR activation reduced the expression of GABAR- δ via extracellular signal-regulated kinase (ERK) 1/2 signaling. However an increase in the expression of GABAR- γ 2 did not occur in NMDA-treated neurons. NMDA treatment led to a reduction in total GABAR-mediated tonic current and its augmentation by neurosteroids.

Materials and Methods

Materials. All common chemicals were obtained from the Sigma-Aldrich (St. Louis, MO). Sulfo-succinimidyl-6-(biotin-amido) hexanoate (sulfo-NHS-LC-biotin) and neutravidin agarose beads were obtained from Pierce Biotechnology (Rockford, IL). Allopregnanolone was procured from Steraloids Inc. (Newport, RI). Mitogen-activated protein kinase enzyme inhibitors UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) were from Calbiochem (Darmstadt, Germany). Human tropomyosin receptor kinase B (TrkB)-Fc chimera was from R&D Systems (Minneapolis, MN).

Antibodies. The mouse monoclonal anti- γ 2 subunit antibody 10F10-C1-B8 was prepared and characterized in our laboratory (Joshi et al., 2011; Rannals and Kapur, 2011). The anti- δ subunit antibody (clone N151/3.3) was prepared in our laboratory in cooperation with Neuromab (Davis, CA). The antibody was synthesized at the Lymphocyte Culture Center, University of Virginia. This antibody reacted with a protein of approximately 50 kDa (Supplemental Fig. 1), similar to that observed using previously characterized Millipore anti- δ subunit antibody (Joshi and Kapur, 2009; Rajasekaran et al., 2010). Antibody N151/3.3 also reacted with rat δ subunit expressed in HEK293 cells (Supplemental Fig. 1). In some experiments, rabbit polyclonal antibodies against GABAR γ 2 and δ subunits (Millipore, Billerica, MA) were also used (Joshi and Kapur, 2009; Rajasekaran et al., 2010). The results obtained using monoclonal antibodies were similar to those obtained with polyclonal antibodies. Anti- α 4 subunit antibody was also from Millipore. Anti pERK1/2 and ERK1/2 antibodies were from Cell Signaling Technology, Inc. (Danvers, MA).

Hippocampal Neuronal Cultures. All animals were handled according to a protocol approved by the University of Virginia Animal Care and Use Committee, and efforts were made to minimize animal stress and discomfort. Hippocampal neuron-glia cocultures were prepared from newborn rat pups of both sexes, as described previously (Ming et al., 2006; Rannals and Kapur, 2011). Briefly, hippocampi

from newborn pups were dissected free of meninges and treated with trypsin (0.125%) for 15 minutes at 37°C. After denaturation of trypsin with fetal bovine serum, hippocampi were suspended in a medium containing Dulbecco's modified Eagle's medium and F-12 supplement (1:1), 10% fetal bovine serum (heat-inactivated), 2 mM L-glutamine (Sigma-Aldrich), and penicillin (100 U/ml)-streptomycin (100 U/ml); cells were separated by trituration. Neurons were plated at a density of 1,00,000/35 mm on poly-L-lysine-coated culture plates at 37°C in a humidified incubator with 95% oxygen 5% CO₂. Twenty-four hours after plating, the medium was changed to a serum-free medium Dulbecco's modified Eagle's medium containing 2% B27 and 2 mM L-glutamine. Cultures were fed every 2 days. Cultures grown in vitro for 10–14 days were used for the experiments.

Organotypic Hippocampal Slice Cultures. Organotypic hippocampal slice cultures were prepared from 3- to 5-day-old rat pups of both sexes, as described previously (Stoppini et al., 1991; Joshi and Kapur, 2009). Cultures grown in vitro for 8 days were treated for 2 days and used in the experiments.

Treatment of Cultures. Cultured hippocampal pyramidal neurons or organotypic hippocampal slice cultures were treated with 10 μ M NMDA or 2 μ M tetrodotoxin (TTX). The regular growth medium was exchanged with the growth medium containing NMDA or TTX, and cultures were incubated for 48 hours. At the end of treatment, cultures were washed twice with regular growth medium to remove NMDA and used for experiments. To record tonic or synaptic currents, cultures were placed in an external recording solution and used for a maximum period of 3 hours after removing them from the treatment medium. Biotinylation assay was performed immediately after the cultures were removed from the treatment medium. For cotreatment of NMDA (10 μ M) and different inhibitors such as dl-2-amino-5-phosphonopentanoic acid (APV) (50 μ M), BAPTA-AM (10 μ M), UO126 (10 μ M), KN93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) (10 μ M), KN62 (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) (5 μ M), or TrkB-Fc chimera (2 μ g/ml), the inhibitors were added to the culture medium 30 minutes before the addition of NMDA, and cultures were incubated for 48 hours. To study whether NMDA's effect on δ subunit expression was reversible, cultures were incubated in the medium containing NMDA (10 μ M) for 48 hours, washed twice with regular growth medium, and incubated in it for additional 48 hours.

Biotinylation and Western Blotting. Surface proteins were biotinylated in organotypic hippocampal slice cultures as described previously (Joshi and Kapur, 2009). The experimental and control samples were processed simultaneously for all experiments at the end of treatment. Proteins from eight cultured slices were pooled for each treatment and constituted a single replicate. To determine the surface expression of GABAR subunits, tissue lysates corresponding to 100–200 μ g of protein were incubated with neutravidin agarose beads, and the pulled-down proteins were loaded onto the gels. The total expression of the subunits was determined using 10–20 μ g of protein. Standard electrophoresis, transfer, and Western blotting techniques were used to determine the expression of δ , α 4, and γ 2 subunits. Anti- δ (5 μ g/ml N151/3.3 antibody or 1:500 dilution of Millipore antibody), anti- γ 2 (2 μ g/ml) or anti- α 4 (1:250) antibodies were used. Western blots were imaged on a Kodak gel Logic 2200 imaging system (Carestream Health Molecular Imaging, New Haven, CT). The blots were reprobbed with a mouse monoclonal anti- β -actin antibody (1:1000 dilution; Sigma-Aldrich) to confirm the purity of surface samples. Signal intensity was determined by densitometric scanning of the Western blots. The total expression of the GABAR subunits was normalized to the expression of β -actin. The ratio of surface to total protein in the control and experimental treatment groups was compared.

Electrophysiology. Whole-cell patch-clamp technique was used to record GABAR-mediated currents from cultured hippocampal pyramidal neurons (Rajasekaran et al., 2010; Rannals and Kapur, 2011). All recordings were performed at room temperature. Cultured hippocampal neurons were maintained in an external solution

consisting of (in millimolars) 142 NaCl, 1 CaCl₂, 2.5 KCl, 6 MgCl₂, 10 glucose, 10 HEPES (pH 7.4), and 310–320 mOsm. The neurons were visualized using an inverted Nikon Eclipse Ti microscope (Nikon Instruments, Inc., Melville, NY) under a 40 $\times$ objective lens (NA 0.8). Cultured hippocampal slices were incubated in oxygenated artificial cerebrospinal fluid containing (in millimolars), 127 NaCl, 2 KCl, 1.5 MgSO₄, 1.1 KH₂PO₄, 25.7 NaHCO₃, 10 dextrose, and 1.5 CaCl₂, 300 mOsm, in a recording chamber mounted on the stage of an Olympus BX51 microscope (Olympus Corporation, Center Valley, PA) equipped with a 40 $\times$ water-immersion objective (NA 0.8), infrared differential interference contrast (Olympus) optics, and video. External solutions were perfused using a peristaltic pump at a flow rate of 2–3 ml/min. Glutamatergic currents were blocked by adding dl-2-amino-5-phosphopentanoic acid (D, L-APV; 50 μ M) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μ M) to the external solution. Three micromolar GABA and 10 μ M 1-[2-[[[diphenylmethylene]imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO711) were also included in the external solution in some experiments to measure tonic current. To record the miniature inhibitory postsynaptic currents (mIPSCs), TTX (1 μ M) was added to the external medium.

Patch pipettes (resistance 5–8 M Ω) were pulled on a P-97 Flaming/Brown puller (Sutter Instrument Company, Novato, CA) using a three-stage pull protocol and filled with a recording solution containing (in millimolars) 153 CsCl, 1 MgCl₂, 10 HEPES, and 5 EGTA (pH 7.3). Mg²⁺-ATP (5 mM), and 5 lidocaine-N-methyl bromide (10 μ M) were added to the internal solution immediately before use (280–290 mOsm).

The neurons were voltage-clamped to –60 mV with an Axopatch 1D (cultured hippocampal neurons) or 200B (organotypic hippocampal slice cultures) amplifier (Molecular Devices, Sunnyvale, CA). The currents were low-pass filtered at 5 kHz and digitized at 10 kHz using a 1322A Digidata A/D converter and acquired using Axoscope 10.2 software (Molecular Devices). Access resistance was maintained with a 10-millisecond 5-mV test pulse. It was 10.4 ± 0.6 m Ω ($n = 38$) in the dissociated cultured hippocampal neurons and 22.7 ± 2.2 m Ω ($n = 28$) in the DGCs of organotypic hippocampal slice cultures. The recordings were terminated if access resistance changed more than 25% at any time.

Analysis of Currents. The current required to clamp neurons to –60 mV (I_{hold}) was measured. I_{hold} was determined by averaging the mean current in a 250-ms epoch sampled every 2500 ms using MiniAnalysis (Synaptosoft, Fort Lee, NJ). Immediately before drug application and 5 minutes after drug application, when a steady-state response was observed, 30 to 50 of these epochs were collected. The contribution of synaptic currents to I_{hold} measures was eliminated by removing epochs containing synaptic events. The drug effects on individual neurons were assessed by comparing the mean holding current before and after drug application using a paired t test. The shift in the mean I_{hold} (ΔI_{hold}) after drug application relative to the baseline was calculated (Supplemental Fig. 2). At the end of recording, total GABAR-mediated tonic current was determined by applying picrotoxin to block all GABARs. The I_{hold} recorded from control (226 ± 20 pA, $n = 10$) and NMDA-treated cultured hippocampal neurons (224 ± 19 , $n = 12$) was similar ($P > 0.05$, t test). The baseline current recorded from DGCs of untreated (97 ± 13 pA, $n = 19$) or NMDA-treated (74 ± 8 pA, $n = 18$) organotypic hippocampal slice cultures was also similar ($P > 0.05$, t test).

The mIPSCs were analyzed using MiniAnalysis software as before (Sun et al., 2007). The detection threshold was set at 5 times the root mean square noise. After detection, decay time constants and peak amplitude were analyzed in individual mIPSCs. The decay was analyzed in 10–90% of the peak amplitude, and 100 iterations were used for each event. The mIPSCs could be fitted with double-exponential decay time constants. The weighted decay time constant (τ_w) was then calculated with the following equation: $\tau_w = \tau_1 A_1 + \tau_2 A_2 / (A_1 + A_2)$, where τ_1 and τ_2 are fast and slow decay time constants (first and second exponential functions), and A_1 and A_2 represent the magnitude of fast and slow components, respectively.

Statistical Analysis. The results are presented as the mean $\pm$ S.E.M. Data were compared using either a t test or a one-way analysis of variance followed by Dunnett's multiple comparison post hoc test. In the voltage-clamp recordings, n represents number of neurons, whereas i represents number of replicates in the biochemical experiments.

Results

NMDA Treatment Reduced GABAR- δ Expression in Cultured Hippocampal Neurons. The effect of prolonged NMDA treatment on the surface and total expression of δ , $\alpha 4$, and $\gamma 2$ subunits was studied in organotypic hippocampal slice cultures. The cultures were maintained in a medium supplemented with 10 μ M NMDA for 48 hours (NMDA-treated neurons) or in the regular culture medium (control neurons), and GABAR surface expression was studied by a biotinylation assay.

Surface expression of δ and $\alpha 4$ subunits was reduced in NMDA-treated cultures, and that of $\gamma 2$ subunits was unaltered (Fig. 1, A and B). In NMDA-treated cultures, surface δ subunit expression was only $40\% \pm 7\%$ of that in controls ($n = 7$, $P < 0.05$). Total δ subunit expression was also diminished in NMDA-treated cultures ($70\% \pm 7\%$, $n = 7$, $P < 0.05$). Surface expression of $\alpha 4$ subunits was also less in NMDA-treated cultures ($52\% \pm 9\%$, $n = 4$, $P < 0.05$). In contrast, the total expression and surface expression of the $\gamma 2$ subunit were unchanged in NMDA-treated cultures ($86\% \pm 8\%$, $n = 6$, $P > 0.05$). Together, these studies revealed that NMDA treatment reduced the expression of $\alpha 4\delta$ subunit-containing GABARs, but it did not upregulate GABAR- $\gamma 2$ expression.

The time course of NMDA effects was characterized by treating cultures for 3–24 hours. NMDA had distinct effects on both total and surface δ subunit expression (Fig. 1, C and D). Reduced surface and total δ subunit expression was evident in cultures treated for 24 and 48 hours ($n = 5$, $P < 0.05$). In contrast, after 12 hours of NMDA treatment, although total δ subunit expression was decreased ($n = 5$, $P < 0.05$), its surface expression was unaltered ($n = 5$, $P > 0.05$). Furthermore, 6 hours of NMDA treatment reduced surface δ subunit expression without altering its total expression ($n = 5$, $P < 0.05$).

The effect of various NMDA concentrations on δ subunit expression was also determined. In cultures treated with 3 μ M NMDA, δ subunit expression was $118\% \pm 30\%$ ($n = 4$, $P > 0.05$), similar to that in untreated cultures; however, expression was lower in the cultures treated with 7.5 μ M NMDA ($64\% \pm 17\%$, $n = 3$, $P < 0.05$).

NMDA induces excitotoxic damage in hippocampal slice cultures (Sakaguchi et al., 1997), which may play a role in the downregulation of δ subunit expression; however, such effects should not be reversible. Hence, the effect of washout of NMDA was determined. In the slice cultures exposed to NMDA (10 μ M) for 48 hours, followed by incubation in NMDA-free medium for an additional 48 hours, surface δ subunit expression showed a trend toward recovery. In four replicates, surface δ subunit expression was $74\% \pm 17\%$, which was similar to that in controls ($P > 0.05$) and slightly higher than δ subunit expression in NMDA-treated cultures without washout ($38\% \pm 18\%$).

NMDA Treatment Also Reduced Tonic Current and its Neurosteroid Modulation. GABAR- δ s are localized to the extrasynaptic membrane and mediate tonic current and

Fig. 1. NMDA treatment reduced surface expression of GABAR- δ . (A) Representative Western blots showing the surface and total expression of the δ , $\alpha 4$, and $\gamma 2$ subunits in organotypic hippocampal slice cultures incubated in regular growth medium or medium supplemented with NMDA ($10 \mu\text{M}$) for 48 hours. The expression of β -actin is shown to demonstrate the purity of the surface samples. Rabbit polyclonal antibodies against the δ , $\gamma 2$, and $\alpha 4$ subunits (Millipore) were used in these experiments. (B) Relative surface expression of the δ , $\alpha 4$, and $\gamma 2$ subunits of GABARs in NMDA-treated cultures. $n = 4$ –6 experiments. * $P < 0.05$; ** $P < 0.005$. (C) A representative Western blot demonstrating the time course of changes in δ subunit surface expression in NMDA-treated slice cultures. Mouse monoclonal anti- δ subunit antibody (N151/3.3) was used in these experiments. (D) Time course of change in GABAR- δ surface (black solid line) and total expression (red dotted line) after NMDA treatment relative to that at time 0. Data represent average and standard error from five experiments. * $P < 0.05$.

its neurosteroid modulation (Nusser et al., 1998; Wei et al., 2003; Glykys and Mody, 2007). Hence, the physiologic significance of NMDA-induced downregulation of GABAR- δ expression was determined by measuring tonic current and its modulation by neurosteroids. Tonic current was the change in holding current induced by blocking GABARs with picrotoxin, a noncompetitive antagonist. In the dissociated cultured hippocampal pyramidal neurons, tonic current and its neurosteroid modulation were diminished after NMDA treatment (Fig. 2, A and B). Tonic current recorded from NMDA-treated neurons was only $18 \pm 3 \text{ pA}$ ($n = 6$ neurons from five cultures, $P < 0.05$), significantly smaller than that recorded from untreated neurons ($28 \pm 4 \text{ pA}$, $n = 7$ neurons from four cultures). Furthermore, allopregnanolone (10 nM) enhanced tonic current recorded from NMDA-treated neurons only minimally compared with control neurons ($8 \pm 3 \text{ pA}$, $n = 6$ neurons compared with $23 \pm 3 \text{ pA}$, $n = 7$ neurons, $P < 0.05$).

In some cells, the effect of allopregnanolone was studied in the presence of GABAR blocker picrotoxin ($50 \mu\text{M}$). Allopregnanolone did not enhance I_{hold} in these neurons ($I_{\text{hold}} -83 \pm 22 \text{ pA}$ versus $-82 \pm 23 \text{ pA}$, $n = 6$ neurons from

three cultures, $P > 0.05$) and confirmed that the observed effect of allopregnanolone was through the modulation of GABARs.

NMDA treatment could alter GABA release or uptake, which could influence allopregnanolone's modulation of tonic current. Hence, in some recordings, external solution was supplemented with GABA ($1 \mu\text{M}$) and GABA uptake blocker NO711 ($10 \mu\text{M}$). Tonic current recorded from NMDA-treated neurons under the condition of artificially increased GABA concentration was also less sensitive to modulation by allopregnanolone ($7 \pm 1 \text{ pA}$, $n = 4$ versus $34 \pm 7 \text{ pA}$, $n = 5$ neurons from four cultures, $P < 0.05$).

Changes in cell volume and expression of ion channels in NMDA-treated neurons may alter whole-cell capacitance, which could influence neurosteroid modulation of tonic current. Therefore, the change in tonic current was normalized to whole-cell capacitance. Allopregnanolone modulation of tonic current was significantly smaller in NMDA-treated neurons ($1.6 \pm 0.6 \text{ pA/pF}$, $n = 6$ versus $6.4 \pm 0.7 \text{ pA/pF}$, $n = 7$, $P < 0.05$). This finding indicated that the observed reduction in neurosteroid modulation of tonic current was not due to changes in capacitance.

Fig. 2. NMDA treatment reduced tonic current and its neurosteroid modulation. (A) Representative voltage-clamp recordings from control and NMDA-treated cultured hippocampal pyramidal neurons show the effect of the bath application of allopregnanolone (10 nM, allo) and picrotoxin (50 μ M, PT). Neurons were incubated in normal growth medium (control) or in a medium containing 10 μ M NMDA for 48 hours. Gray line running through the trace indicates basal holding current. (B) Magnified portion of traces from (A) showing tonic current before and after drug application, when a steady response was obtained. Gray lines through the trace mark the position of mean holding current. Upward arrow indicates tonic current, and downward arrow indicates neurosteroid modulation of tonic current. (C) Representative voltage-clamp recordings demonstrating the effect of bath application of 10 nM allopregnanolone (arrow, allo) on tonic current recorded from DGCs of untreated (control) and NMDA-treated (10 μ M, 48 hours) organotypic hippocampal slice cultures. Tonic current

NMDA-induced reduction in tonic current and its neurosteroid modulation was also confirmed in DGCs of organotypic hippocampal slice cultures. Similar to cultured hippocampal pyramidal neurons, tonic current (54 ± 7 pA, $n = 18$ compared with 84 ± 12 pA, $n = 18$ neurons from five to seven cultures, $P < 0.05$) and its allopregnanolone (10 nM) modulation (25 ± 4 pA, $n = 8$ compared with 47 ± 6 pA, $n = 8$ neurons from four or five, $P < 0.005$) were diminished in DGCs of NMDA-treated slices (Fig. 2, C and D). Together, these studies revealed that NMDA-induced downregulation of GABAR- δ was associated with reduced tonic current and its neurosteroid modulation.

Allopregnanolone Modulation of mIPSCs Was Preserved in NMDA-Treated Neurons. Synaptic inhibition is augmented in epileptic animals; however, its neurosteroid modulation is reduced likely as a result of the presence of $\alpha 4$ subunit containing receptors at the synapses (Sun et al., 2007). We measured synaptic currents and their neurosteroid modulation in NMDA-treated dissociated hippocampal neuronal cultures (Fig. 3, A and B). Synaptic currents recorded from NMDA-treated neurons tended to be more frequent (0.51 ± 0.2 Hz compared with 0.3 ± 0.12 Hz, $n = 7$ neurons from four cultures) and with larger amplitudes (73 ± 7 pA compared with 63 ± 3 pA, $n = 7$ neurons from four cultures) compared with those recorded from untreated neurons; however, these differences were not statistically significant ($P > 0.05$). The 10–90% rise time (2.5 ± 0.4 milliseconds compared with 2.6 ± 0.4 milliseconds, $n = 7$) and decay (39 ± 7 ms compared with 31 ± 4 ms, $n = 5$ –7) of mIPSCs recorded from NMDA-treated and -untreated neurons were comparable ($P > 0.05$). Furthermore, allopregnanolone (10 nM) prolonged the decay of mIPSCs recorded from NMDA-treated and -untreated neurons to a similar extent (change in the decay: 18 ± 4 milliseconds, $n = 7$ compared with 16 ± 2 milliseconds, $n = 5$ neurons, $P > 0.05$) without altering other parameters of mIPSCs (unpublished data). Thus, NMDA treatment neither altered GABAR- $\gamma 2$ expression nor changed the characteristics of mIPSCs.

Activity Blockade by TTX Increased GABAR- δ subunit Expression. In subsequent experiments, the effect of activity blockade on GABAR- δ expression was determined. TTX treatment of slice cultures (2 μ M, 48 hours) increased GABAR- δ surface expression (Fig. 4, A and B) ($152\% \pm 13\%$, $n = 4$, $P < 0.05$), whereas surface $\gamma 2$ subunit expression was decreased ($78\% \pm 8\%$, $n = 4$, $P < 0.05$). Consistent with the biochemical findings, tonic current recorded from TTX-treated neurons was unaltered (36 ± 6 pA, $n = 5$ compared with 25 ± 4 pA, $n = 7$ neurons from four cultures), but its modulation by allopregnanolone was enhanced (38 ± 5 pA, $n = 5$, compared with 24 ± 3 pA, $n = 7$, $P < 0.05$) (Fig. 4, C and D).

NMDAR Activation and Calcium Influx Were Necessary for NMDA-Induced Downregulation of δ Subunit Expression. To confirm that NMDA-induced NMDAR activation led to downregulation of δ subunit expression, cultures were cotreated with NMDA and APV. APV blocked NMDA-induced

was determined by bath application of picrotoxin (50 μ M, PT, arrow) after allopregnanolone application. The external solution contained GABA (1 μ M) and NO711 (10 μ M). (D) Magnified portions of traces from (A) to demonstrate the effect of allopregnanolone and picrotoxin on holding current.

Fig. 3. NMDA treatment did not affect synaptic currents or their neurosteroid modulation. (A) Representative voltage clamp recordings showing effect of allopregnanolone (10 nM) on cultured dissociated hippocampal neurons either untreated or treated with NMDA (10 μ M, 48 hours). The external solution contained TTX. (B) Averaged currents from representative control and NMDA-treated neurons before (black) and after application of allopregnanolone (gray).

reduction in surface GABAR- δ expression without altering its expression under basal conditions (Fig. 5, A and B). Surface δ subunit expression in the neurons cotreated with NMDA and APV was ($113\% \pm 14\%$, $n = 5$) greater than that in slices treated with NMDA alone ($30\% \pm 11\%$, $n = 5$, $P < 0.05$) but similar to that in cultures treated with only APV ($100\% \pm 31\%$, $n = 5$). Tonic current and its neurosteroid modulation were also preserved in the DGCs of organotypic slice cultures cotreated with APV and NMDA (Table 1). Thus, the NMDA effects were mediated via NMDAR activation.

NMDAR activation leads to calcium flux, and the role of calcium in NMDA-induced downregulation of δ subunit expression was determined by chelating intracellular calcium by BAPTA-AM. Calcium ion chelation blocked NMDA-induced downregulation of δ subunit expression without affecting basal δ subunit expression (Fig. 5, C and D). Surface δ subunit expression in cultures cotreated with NMDA and BAPTA-AM was $109\% \pm 24\%$ ($n = 5$), higher than that in cultures treated with NMDA alone ($33\% \pm 6\%$, $n = 5$, $P < 0.05$), but similar to that in cultures treated with BAPTA-AM alone ($107\% \pm 14\%$, $n = 4$). The tonic current recorded from DGCs of NMDA and BAPTA-AM cotreated cultures was comparable to that in controls; however, its neurosteroid modulation remained diminished (Table 1).

ERK1/2 Activation Was Associated with NMDA-Induced Downregulation of δ Subunit Expression. Calcium flux after NMDAR activation could activate Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) (Fukunaga and Soderling, 1990; Fukunaga et al., 1992). We determined whether activation of CaMKII was necessary for NMDA-induced downregulation of GABAR- δ expression by cotreating cultures with NMDA and KN62 (5 μ M) or KN93 (10 μ M), which are known to block CaMKII activity (Tokumitsu et al., 1990; Sumi et al., 1991). However cotreatment of cultures with KN62 or KN93 did not prevent NMDA-induced downregulation

Fig. 4. Activity blockade by TTX increased GABAR- δ expression. (A) A representative Western blot demonstrating surface and total expression of δ and $\gamma 2$ subunits of GABARs in organotypic hippocampal slice cultures treated with TTX (2 μ M, 48 hours). Expression of β -actin demonstrates purity of the surface samples. Millipore anti- δ subunit antibody was used in these experiments. (B) Relative surface expression of δ and $\gamma 2$ subunits of GABARs in TTX-treated cultures, $n = 4$ experiments, * $P < 0.05$. (C) Representative current traces demonstrating the effect of allopregnanolone (10 nM, allo, arrow) on tonic current in cultured hippocampal pyramidal neurons treated with TTX (2 μ M, 48 hours). Picrotoxin (50 μ M, PT) was applied after recording the effect of allopregnanolone to determine tonic current. Gray line running through the trace represents the position of the basal holding current. (D) Magnified portions of traces from (A) showing the effect of allopregnanolone and picrotoxin.

Fig. 5. NMDA effects on the δ subunit expression were mediated by activation of NMDARs and calcium flux. (A) A representative Western blot showing surface and total expression of the δ subunit in NMDA-treated (for 48 hours) slices in the presence or absence of APV (50 μ M). (B) Relative surface δ subunit expression. $n = 5$, $*P < 0.05$. (C) A representative Western blot demonstrating δ subunit surface and total expression in slices treated with NMDA in the presence or absence of BAPTA-AM (10 μ M) for 48 hours. Expression of β -actin demonstrates the purity of the surface samples. Anti- δ subunit antibody (N151/3.3) was used in these studies. (D) Bar graph showing the relative surface δ subunit expression. $n = 5$ experiments, $*P < 0.05$.

of GABAR- δ expression (Fig. 6, A and B). Surface δ subunit expression in slices cotreated with NMDA and KN62 or KN93 was $55\% \pm 7\%$ and $45\% \pm 17\%$, respectively ($n = 4$, $P < 0.05$), which was similar to surface δ subunit expression in slices treated with NMDA alone ($54\% \pm 16\%$, $n = 4$). CaMKII blockade did not alter basal GABAR- δ subunit expression (unpublished data). Thus, activation of CaMKII did not appear to mediate NMDA effects. In accordance with the biochemical studies, allopregnanolone did not enhance tonic current recorded from the DGCs of slice cultures treated with NMDA and KN62 (Table 1).

Activity of ERK1/2 is increased in the neurons during SE, which could involve NMDAR activation (Bading and Greenberg, 1991; Kim et al., 1994; Garrido et al., 1998; Berkeley et al.,

2002). ERK1/2 phosphorylation was studied after NMDA treatment, and it appeared to increase as early as 1 hour after NMDA treatment and was sustained even after 6 hours of NMDA treatment (Fig. 6, C and D). Levels of pERK1/2 were $140\% \pm 22\%$ ($n = 5$, $P < 0.05$) of that in controls after 1 hour of NMDA treatment, increased to $214\% \pm 33\%$ and $243\% \pm 35\%$ after 3 and 6 hours of NMDA treatment, respectively ($n = 4$, $P < 0.05$). We then determined whether blockade of ERK1/2 activation prevented NMDA-induced downregulation of δ subunit expression. Protein kinases mitogen-activated protein kinase enzyme 1/2 phosphorylate activate ERK1/2, and their inhibition by U0126 prevents activation of ERK1/2 (Zheng and Guan, 1993; Favata et al., 1998; Roskoski, 2012). Cotreatment of NMDA and U0126 (10 μ M) prevented δ subunit downregulation (Fig. 6, E and F). Surface expression of the δ subunit in NMDA- and U0126-treated cultures was $120\% \pm 23\%$ that of control cultures ($n = 5$), greater than that in cultures treated with NMDA alone ($55\% \pm 13\%$, $n = 5$, $P < 0.05$). Blockade of ERK1/2 under basal conditions did not affect δ subunit expression ($85\% \pm 16\%$ of that in controls, $n = 3$, $P > 0.05$). Tonic current recorded from the DGCs of NMDA- and U0126-treated slice cultures was also comparable to that recorded from control neurons and was enhanced by allopregnanolone (Table 1). Thus, the NMDA effects were at least in part mediated by the activation of ERK1/2.

Activation of TrkB Receptors Did Not Mediate NMDA Effects on δ Subunit Expression. Activation of TrkB receptors is an important epileptogenic stimulus and could play a role in NMDA-induced downregulation of GABAR- δ expression (Binder et al., 2001; He et al., 2004; Kotloski and McNamara, 2010). The role of TrkB receptors in mediating the NMDA effect was determined in cultures cotreated with NMDA and TrkB-Fc (2 μ g/ml, 48 hours),

TABLE 1

Effect of NMDA receptor activation on tonic currents and their neurosteroid modulation is mediated at least in part by ERK1/2 activation

Organotypic hippocampal slice cultures were treated with NMDA (10 μ M) without or with APV (50 μ M), BAPTA-AM (10 μ M), KN62 (5 μ M), U0126 (10 μ M), or TrkB-Fc (2 μ g/ml) for 48 hours. Tonic current and its neurosteroid modulation were recorded from dentate granule neurons. Cultures without any treatment grown in parallel were used as controls. Data represent mean $\pm$ S.E.M.

Treatment	Tonic Current	Neurosteroid Modulation of Tonic Current
Controls	84 ± 12 pA ($n = 18$)	47 ± 6 pA ($n = 8$)
NMDA	54 ± 7 pA* ($n = 18$)	25 ± 4 pA* ($n = 8$)
NMDA + APV	98 ± 17 pA ($n = 7$)	37 ± 8 pA ($n = 5$)
NMDA + BAPTA-AM	93 ± 14 pA ($n = 5$)	24 ± 4 pA* ($n = 5$)
NMDA + U0126	72 ± 14 pA ($n = 11$)	39 ± 6 pA ($n = 8$)
NMDA + KN62	63 ± 9 pA ($n = 7$)	16 ± 7 pA* ($n = 5$)
NMDA + TrkB-Fc	90 ± 22 pA ($n = 4$)	15 ± 5 pA* ($n = 4$)

* $P < 0.05$.

Fig. 6. Blockade of ERK1/2 activation prevented NMDA-induced downregulation of the δ subunit expression. (A) A representative Western blot showing the surface and total δ subunit expression in slice cultures treated with NMDA in the presence or absence of CaMKII inhibitor KN62 (5 μ M) or KN93 (10 μ M) (+KN62, +KN93). (B) Graph shows the relative surface expression of the δ subunit. $n = 4$, $*P < 0.05$. (C) A representative Western blot demonstrating the time course of ERK1/2 phosphorylation after NMDA treatment. (D) The ratio of pERK1/2 to total ERK1/2 relative to that in controls was plotted as a function of time, $n = 4$, $*P < 0.05$; $**P < 0.005$. (E) Blocking ERK1/2 activation prevented NMDA effects on δ subunit surface expression. UO126 (10 μ M), an inhibitor of MEK1/2, was added 30 minutes before NMDA, and the surface δ subunit expression was determined after 48 hours. Mouse monoclonal anti- δ subunit antibody (N151/3.3) was used in the experiments in (A) and (D). (F) Bar graph showing relative surface expression of δ subunit. $n = 5$, $*P < 0.05$.

which is known to block biologic activity of brain-derived neurotrophic factor (BDNF). Surface expression of GABAR- δ was lower in cultures treated with NMDA in the presence or absence of TrkB-Fc (Fig. 7, A and B). The δ subunit surface expression in cultures treated with NMDA without or with TrkB-Fc was $47\% \pm 6\%$ and $38\% \pm 8\%$ of that in controls, respectively ($n = 4$, $P < 0.05$). Neurosteroid modulation of tonic current was also diminished in DGCs of cultures treated

with NMDA or TrkB-Fc (Table 1). Hence, NMDA's effects did not require activation of TrkB receptors.

Discussion

Key findings of this study were 1) 48-hour NMDA treatment of hippocampal neuronal cultures reduced δ and $\alpha 4$ subunit expression but did not change the expression of $\gamma 2$ subunits;

Fig. 7. Activation of TrkB receptors was not necessary for NMDA effects. (A) A representative Western blot demonstrating the surface and total δ subunit expression in slice cultures treated with NMDA without or with TrkB-Fc chimera (2 μ g/ml) for 48 hours. The expression of β -actin demonstrates the purity of the surface samples. Millipore anti- δ subunit antibody was used in these experiments. (B) Relative surface δ subunit expression from four experiments. $*P < 0.05$.

2) NMDA treatment also reduced tonic current and its neurosteroid modulation, but synaptic currents and their neurosteroid modulation were preserved; 3) activity blockade by TTX induced changes opposite those triggered by NMDA; 4) NMDA's effects were mediated via NMDAR activation and required calcium flux; and 5) the NMDA effects were mediated at least in part by ERK1/2 phosphorylation.

In the DGCs of epileptic animals, expression of the δ subunit is reduced, whereas that of $\alpha 4$ and $\gamma 2$ subunits is upregulated (Peng et al., 2004; Sun et al., 2007; Zhang et al., 2007; Rajasekaran et al., 2010). Current study demonstrated that NMDAR activation diminished δ subunit expression but did not increase the expression of $\alpha 4$ and $\gamma 2$ subunits. BDNF-activated TrkB signaling increases the $\alpha 4$ subunit expression (Roberts et al., 2006). In contrast, the downregulation of δ subunit expression in NMDA-treated hippocampal slice cultures was independent of BDNF signaling. Thus, the signaling pathways underlying a reduction in δ subunit expression and upregulation of $\alpha 4$ and $\gamma 2$ subunits expression appear to be distinct.

Hippocampal slice cultures were used to determine the expression of δ and $\alpha 4$ subunits because they yield sufficient protein for biochemical analysis. In the hippocampus, the δ subunit is expressed primarily in the DGCs, as well as in hilar interneurons (Sperk et al., 1997; Wei et al., 2003; Glykys et al., 2007). However, in the proteins isolated from hippocampal slice cultures, the fraction of the δ subunit originating from interneurons should be minor compared with that originating from the DGCs. Consistent with this interpretation, reduced δ subunit expression was associated with diminished tonic current recorded from DGCs of NMDA-treated slice cultures. High-affinity, slowly desensitizing receptors mediate tonic inhibition of DGCs (Mtchedlishvili et al. 2006). The properties of δ subunit-containing receptors are uniquely suited for this function (Saxena and Macdonald, 1994; Nusser et al., 1998; Haas and Macdonald, 1999; Bianchi et al., 2002; Bianchi and Macdonald, 2003; Wei et al., 2003).

Neurosteroid modulation of the tonic current recorded from DGCs of NMDA-treated slice cultures was also reduced. GABARs containing a δ subunit have greater neurosteroid sensitivity than do other GABAR subtypes, and neurosteroids convert GABA from a partial to a full agonist at these receptors (Belelli et al., 2002; Wohlfarth et al., 2002; Bianchi and Macdonald, 2003). Animals lacking the δ subunit also have attenuated neurosteroid sensitivity (Mihalek et al., 1999). Increased phosphorylation of the $\beta 2/3$ subunits of GABARs is also associated with reduced neurosteroid enhancement of synaptic and tonic GABAR currents (Fáncsik et al., 2000; Kia et al., 2011). The role of GABAR phosphorylation was not determined in this study. Pharmacological perturbations that prevented NMDA-induced downregulation of δ subunit expression also blocked diminution in neurosteroid modulation of tonic current. However, in the cultures cotreated with BAPTA-AM and NMDA, neurosteroid modulation of tonic current did not recover. The reason for this discrepancy is unclear. It is possible that calcium-regulated mechanisms play a role in neurosteroid modulation of tonic current under basal conditions.

In a previous study, prolonged NMDA treatment of cultured cerebellar granule neurons did not affect the expression of δ subunit mRNA but increased expression of $\gamma 2$ subunit mRNA (Memo et al., 1991). These differences could be due to the

different neuronal types studied and because mRNA and protein expression may be distinctly regulated. Further studies are needed to determine the effect of NMDA treatment on the expression of mRNA encoding the δ and $\alpha 4$ subunits in hippocampal neurons.

The time course of reduction in surface and total expression of δ subunits in NMDA-treated cultures was distinct; shorter treatment reduced only surface expression of δ subunits, whereas prolonged treatment also decreased total expression. Altered kinetics of receptor trafficking could reduce surface expression of δ subunits after 6 hours of NMDA treatment. Expression of δ subunits in the endoplasmic reticulum fraction is greater in epileptic animals, suggestive of their potential endoplasmic reticulum retention and a likely impairment in surface membrane insertion (Rajasekaran et al., 2010). In contrast, prolonged NMDA treatment likely involved trafficking and genomic changes.

NMDAR activation could induce excitotoxic cell death, which may play a role in the downregulation of δ subunit expression (Rothman, 1985; Rothman and Olney, 1987; Choi et al., 1988). However, previous studies indicated that cornu ammonis (CA)3 and CA1 neurons are predominately susceptible to cell death, whereas DGCs are comparatively resistant (Engel, 1996; Sakaguchi et al., 1997). Furthermore, the time course of NMDA effects on surface and total expression of δ subunits was distinct, which argues against a nonspecific action of NMDA on δ subunit expression. In the cultures in which NMDA was washed out, δ subunit expression showed a trend toward recovery, suggesting that the NMDA effects could be partially reversed. It is important to note that cell death cannot account for diminution in tonic current and its neurosteroid modulation recorded from the DGCs of NMDA-treated slice cultures.

NMDAR activation leads to calcium influx, which could activate CaMKII, PKC, mitogen activated protein kinases, and protein phosphatases (MacDermott et al., 1986; Berridge, 1998; DeLorenzo et al., 1998; Hardingham and Bading, 2003). Phosphorylation of ERK1/2 was increased in the NMDA-treated slice cultures and appeared to play a role in the downregulation of δ subunit expression. Increased ERK1/2 phosphorylation levels could originate from neurons or glia. However, previous immunohistochemical studies in brain slices revealed increased pERK1/2 immunoreactivity in the neurons during SE or after spontaneous seizures (Kim et al., 1994; Garrido et al., 1998; Berkeley et al., 2002; Houser et al., 2008).

This study revealed a connection between NMDAR-induced calcium flux, ERK1/2 activation, and δ subunit expression. An interaction between the NR2B subunit of NMDARs and CaMKII or RasGRF1 may lead to ERK1/2 activation (Kurino et al., 1995; Krapivinsky et al., 2003; El Gaamouch et al., 2012). It is noteworthy that activation of CaMKII was not necessary for the NMDA effects on δ subunit expression. In a previous study, bath application of glutamate reduced CaMKII activity in cultured hippocampal neurons (Churn et al., 1995), which also supports the findings presented here. Future studies addressing the role of Ras, phosphoinositide 3-kinase-Akt, adenylyl cyclase, and heterotrimeric G proteins, which may play a role in calcium-induced activation of ERK1/2, could provide additional insight on NMDA-induced ERK1/2 activation (Lopez-Illasaca 1998; Agell et al., 2002; Holstein et al., 2004; Xia and Storm, 2012). NMDA-induced oxidative

stress could also lead to ERK1/2 activation (Lafon-Cazal et al., 1993; Rybakova et al., 2012).

The expression of $\gamma 2$ subunits was not altered in NMDA-treated hippocampal slice cultures, and synaptic inhibition recorded from NMDA-treated cultured hippocampal pyramidal neurons was unchanged. However, these electrophysiological and biochemical studies cannot be directly correlated at this point because of the use of different culture preparations. Future studies are necessary to address whether synaptic inhibition of DGCs in NMDA-treated slice cultures was also preserved.

The δ subunit-containing GABARs play an important role in regulating neuronal excitability (Stell et al., 2003). In the δ subunit, the knockout mice threshold to pentetazetate-induced seizures is diminished (Spigelman et al., 2002). The dentate gating hypothesis suggests that an impaired inhibitory function of DGCs could allow spread of seizures into CA subfields of the hippocampus (Lothman et al., 1992). DGCs shield excitable pyramidal neurons of CA3 and CA1 regions from spread of the activity from the entorhinal cortex (Stringer and Lothman, 1989; Stringer et al., 1989). However, if the dentate gate is compromised, activity from the entorhinal cortex can spread into the hippocampal CA subfields, forming a re-entrant loop, which could amplify seizure activity. Previous studies revealed a rapid reduction in δ subunit expression after SE, which is not accompanied by increased expression of $\alpha 4$ and $\gamma 2$ subunits (Peng et al., 2004). The dentate gating function appears to be impaired after SE (Pathak et al., 2007). Hence, future studies in experimental animals will be important to determine whether blocking ERK1/2 activation after SE can prevent downregulation of δ subunit expression, maintain the dentate gating function, and alter the course of epileptogenesis.

Acknowledgments

The authors thank Kendra J. Keith for the hippocampal neuronal cultures and Suji Cha for technical assistance.

Authorship Contributions

Participated in research design: Joshi, Kapur.

Conducted experiments: Joshi.

Performed data analysis: Joshi.

Wrote or contributed to the writing of the manuscript: Joshi, Kapur.

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Address correspondence to: Dr. Jaideep Kapur, Department of Neurology, Box 800394, University of Virginia-HSC, Charlottesville, VA 22908. E-mail: jk8t@virginia.edu