

NMDA receptor activation down-regulates expression of δ subunit-containing GABA_A
receptors in cultured hippocampal neurons

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List of non-standard abbreviations:

GABA_A receptor- γ amino butyric acid type A receptor, ERK1/2- extracellular signal-regulated kinase 1/2, MAP kinases- mitogen activated protein kinases, NMDA- N methyl D aspartic acid, BAPTA-AM- 1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl ester), UO126- 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene, APV- dl-2-amino-5-phosphonopentanoic acid, TTX- tetrodotoxin, CaMKII- Ca²⁺/calmodulin-dependent protein kinase II, 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), TrkB- tropomyocin receptor kinase B, BDNF- brain derived neurotrophic factor, PI3K- phosphoinositidekinase

Abstract:

Neurosteroids are endogenous allosteric modulators of γ amino-butyric acid type A receptors (GABARs), and 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. The molecular mechanisms triggering this GABAR plasticity were explored. In the cultured hippocampal neurons treatment with NMDA (10 μ M) for 48 hrs reduced surface expression of δ and α 4 subunits, but did not increase 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 effects on surface and total δ subunit expression were distinct; shorter (6 hrs) treatment decreased surface expression, whereas longer treatment reduced both surface and total expression. APV blocked NMDA effects on δ subunit expression. Chelation of calcium ions by BAPTA-AM or blockade of ERK1/2 activation by UO126 also prevented the NMDA effects. Thus prolonged activation of NMDA receptors in hippocampal neurons reduced GABAR δ subunit expression through Ca^{2+} 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; Kokate *et al.*, 1996). Neurosteroids convert GABA from a partial to a full agonist at δ subunit-containing GABARs (GABAR- δ) (Bianchi and MacDonald, 2003). GABAR- δ have 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 are localized to extrasynaptic membrane 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 2) are localized to synaptic and extrasynaptic membrane and have lower affinity for GABA as well as neurosteroids (Saxena and Macdonald, 1994; Belelli *et al.*, 2002).

In animal models of temporal lobe epilepsy (TLE) 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 subunit, 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). Up-regulation of γ 2 subunit-containing receptors maintained tonic current and enhanced synaptic inhibition of the DGCs of epileptic 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). Molecular mechanisms that trigger these changes are not known.

Prolonged seizures or status epilepticus (SE) often lead to the development of temporal lobe epilepsy (TLE) in the experimental animals (Turski *et al.*, 1987; Cronin and Dudek, 1988; Lothman *et al.*, 1990). One or more of the mechanisms activated during SE may down-regulate expression of GABAR- δ and up-regulate expression of GABAR- γ 2. 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 also terminate SE (Rice and DeLorenzo, 1999; Borris *et al.*, 2000; Martin and Kapur, 2008).

We tested whether NMDAR activation reduced GABAR- δ expression and up-regulated that of GABAR- γ 2. Our findings suggest that NMDAR activation reduced expression of GABAR- δ via ERK1/2 signaling. However an increase in the expression of GABAR- γ 2 did not occur in NMDA-treated neurons. NMDA treatment led to 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 Corporation (St. Louis, MO). Sulfosuccinimidyl-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). MEK inhibitor UO126 and BAPTA-AM were from calbiochem. Human TrkB-Fc chimera was from R & D Systems.

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 ~50 kDa (supplementary figure 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 (supplementary figure 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.

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 co-cultures were prepared from newborn rat pups of both sexes, as described previously (Ming *et al.*, 2006; Rannals and Kapur, 2011). Briefly, hippocampi from new-born (P0) pups were dissected free of meninges and treated with trypsin (0.125%) for 15 min at 37°C. Following denaturation of trypsin with fetal bovine serum, hippocampi were suspended in a medium containing Dulbecco's modified Eagle's medium (DMEM) and F-12 supplement (1 : 1), 10% fetal bovine serum (heat-inactivated), 2 mM L-glutamine (Sigma), penicillin (100 U/mL)-streptomycin (100 U/mL) and 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. 24 hrs after plating, the medium was changed to a serum free medium (DMEM medium containing 2% B27 and 2 mmol/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 3-5 day-old rat pups of both sexes, as described previously (Joshi and Kapur, 2009; Stoppini *et al.*, 1991). 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 μM NMDA or 2 μM TTX. The regular growth medium was exchanged with the growth medium containing NMDA or TTX and

cultures were incubated for 48 hrs. 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 hrs after removing them from the treatment medium. Biotinylation assay was performed immediately after the cultures were removed from the treatment medium. For co-treatment of NMDA (10 μ M) and different inhibitors such as APV (50 μ M), BAPTA-AM (10 μ M), UO126 (10 μ M), KN93 (10 μ M), KN62 (5 μ M), or TrkB-Fc chimera (2 μ g/ml), the inhibitors were added to the culture medium 30 min prior to addition of NMDA, and cultures were incubated for 48 hrs. To study whether the NMDA effect on δ subunit expression was reversible, cultures were incubated in the medium containing NMDA (10 μ M) for 48 hrs, washed twice with regular growth medium and incubated in it for additional 48 hrs.

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 expression of δ , $\alpha 4$, and $\gamma 2$ subunits. Anti- δ (5 μ g/ml N151/3.3 antibody or

1:500 dilution of Millipore antibody), anti- $\gamma 2$ (2 $\mu\text{g}/\text{ml}$) or anti- $\alpha 4$ (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 re-probed with a mouse monoclonal anti- β -actin antibody (1:1000 dilution, Sigma, St. Louis, MO) 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 obtained at room temperature. Cultured hippocampal neurons were maintained in an external solution consisting of (in mM) 142 NaCl, 1 CaCl₂, 2.5 KCl, 6 MgCl₂, 10 glucose, and 10 HEPES, pH 7.4, and 310-320 mOsm. The neurons were visualized using an inverted Nikon Eclipse Ti microscope under a 40x objective lens (NA 0.8). Cultured hippocampal slices were incubated in oxygenated aCSF (containing in mM, 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 equipped with a 40x water-immersion objective (NA 0.8), IR-DIC 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-phosphonopentanoic acid (D, L-APV; 50 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM) to the external solution. Three micromolar

GABA and 10 μ M NO711 were also included in the external solution in some experiments to measure tonic current. To record miniature inhibitory post-synaptic 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 mM) 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 prior to 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, Sunnyvale, CA). Access resistance was monitored with a 10-ms –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). Thirty to fifty of these epochs were collected immediately prior to drug application and 5 min after drug application when a steady-state response was observed. 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 (supplementary figure 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 five times of 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 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 SEM (standard error of the mean). Data were compared using either a t-test or a one-way ANOVA followed by Dunnett's multiple comparison post-hoc test. In the voltage clamp recordings n

represents number of neurons, whereas it 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 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 hrs (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. 1A and 1B). 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, total and surface expression of the $\gamma 2$ subunit was unchanged in NMDA-treated cultures ($86 \pm 8\%$, $n=6$, $p>0.05$). Together these studies revealed that NMDA treatment reduced expression of $\alpha 4\delta$ subunit-containing GABARs but did not up-regulate GABAR- $\gamma 2$ expression.

The time course of NMDA effects was characterized by treating cultures for 3-24 hrs. NMDA had distinct effects on total and surface δ subunit expression (Fig. 1C and 1D). Reduced surface and total δ subunit expression was evident in cultures treated for 24 and 48 hrs ($n=5$, $p<0.05$). In contrast, after 12 hrs 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 hr 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 the cultures treated with 3 μM NMDA, δ subunit expression was $118 \pm 30\%$ ($n=4$, $p>0.05$), similar to that in untreated cultures, however it was lower in the cultures treated with 7.5 μM 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 down-regulation 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 μM) for 48 hrs followed by incubation in NMDA-free medium for additional 48 hrs, surface δ subunit expression showed a trend towards 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 are localized to the extrasynaptic membrane and mediate tonic current and its neurosteroid modulation (Nusser *et al*, 1998; Wei *et al*, 2003; Glykys and Mody, 2007). Hence the physiological significance of NMDA-induced down-regulation 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 non-competitive antagonist. In the dissociated cultured hippocampal pyramidal neurons, tonic current and its neurosteroid modulation were diminished following NMDA treatment (Fig. 2A and 2B). Tonic current recorded from

NMDA-treated neurons was only 18 ± 3 pA ($n=6$ neurons from 5 cultures, $p<0.05$), significantly smaller than that recorded from untreated neurons (28 ± 4 pA, $n=7$ neurons from 4 cultures). Furthermore, allopregnanolone (10 nM) enhanced tonic current recorded from NMDA-treated neurons only minimally compared to control neurons (8 ± 3 pA, $n=6$ neurons vs 23 ± 3 pA, $n=7$ neurons, $p<0.05$).

In some cells the effect of allopregnanolone was studied in the presence of GABAR blocker picrotoxin (50 μ M). Allopregnanolone did not enhance I_{hold} in these neurons ($I_{\text{hold}} -83 \pm 22$ pA vs. -82 ± 23 pA, $n=6$ neurons from 3 cultures, $p>0.05$), and confirmed that observed effect of allopregnanolone was through modulation of GABARs.

NMDA treatment could alter GABA release and/or uptake, which could influence allopregnanolone modulation of tonic current. Hence in some recordings external solution was supplemented with GABA (1 μ M) and GABA uptake blocker NO711 (10 μ M). Tonic current recorded from NMDA-treated neurons under condition of artificially increased GABA concentration was also less sensitive to modulation by allopregnanolone (7 ± 1 pA, $n=4$ vs 34 ± 7 pA, $n=5$ neurons from 4 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 ± 0.6 pA/pF, $n=6$ vs 6.4 ± 0.7 pA/pF, $n=7$, $p<0.05$). This indicated that observed reduction in neurosteroid modulation of tonic current was not

due to changes in capacitance.

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, $n=18$ vs 84 ± 12 pA, $n=18$ neurons from 5-7 cultures, $p<0.05$) and its allopregnanolone (10 nM) modulation (25 ± 4 pA, $n=8$ vs 47 ± 6 pA, $n=8$ neurons from 4-5 cultures, $p<0.005$) were diminished in DGCs of NMDA-treated slices (Fig. 2C and 2D). Together these studies revealed that NMDA-induced down-regulation of GABA 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 due to 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. 3A and 3B). Synaptic currents recorded from NMDA-treated neurons tended to be more frequent (0.51 ± 0.2 Hz vs 0.3 ± 0.12 Hz, $n=7$ neurons from 4 cultures), and with larger amplitudes (73 ± 7 pA vs 63 ± 3 pA, $n=7$ neurons from 4 cultures) compared to those recorded from untreated neurons, however these differences were not statistically significant ($p>0.05$). The 10-90% rise time (2.5 ± 0.4 ms vs 2.6 ± 0.4 ms, $n=7$), and decay (39 ± 7 ms vs 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 ms, $n=7$ vs 16 ± 2 ms, $n=5$ neurons, $p>0.05$), without altering other parameters of mIPSCs (data not shown). Thus NMDA treatment neither altered GABAR- $\gamma 2$ expression nor changed characteristics of mIPSCs.

Activity blockade by TTX increased GABAR- δ subunit expression.

In the subsequent experiments the effect of activity blockade on GABAR- δ expression was determined. TTX treatment of slice cultures ($2 \mu\text{M}$, 48 hrs) increased GABAR- δ surface expression (Fig. 4A and 4B) ($152 \pm 13\%$ $n=4$, $p<0.05$), 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, $n=5$ vs 25 ± 4 pA, $n=7$ neurons from 4 cultures), but its modulation by allopregnanolone was enhanced (38 ± 5 pA, $n=5$ vs 24 ± 3 pA, $n=7$, $p < 0.05$) (Fig. 4C and 4D).

NMDAR activation and calcium influx was necessary for NMDA-induced down-regulation of δ subunit expression.

To confirm that NMDA-induced NMDAR activation led to down-regulation of δ subunit expression, cultures were co-treated with NMDA and APV. APV blocked NMDA-induced reduction in surface GABAR- δ expression without altering its expression under basal conditions (Fig. 5A and 5B). Surface δ subunit expression in the neurons co-treated 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 co-treated 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 down-regulation of δ subunit expression was determined by chelating intracellular calcium by BAPTA-AM. Calcium ion chelation blocked NMDA-induced down-regulation of δ subunit expression without affecting basal δ subunit expression (Fig. 5C and 5D). Surface δ subunit expression in cultures co-treated 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 co-treated cultures was comparable to that in controls; however its neurosteroid modulation remained diminished (table 1).

ERK1/2 activation was associated with NMDA-induced down-regulation of δ subunit expression.

Calcium flux following NMDAR activation could activate CaMKII (Fukunaga and Soderling, 1990; Fukunaga *et al.*, 1992). We determined whether activation of CaMKII was necessary for NMDA-induced down-regulation of GABAR- δ expression, by co-treating 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 co-treatment of cultures with KN62 or KN93 did not prevent NMDA-induced down-regulation of GABAR- δ expression (Fig. 6A and 6B). Surface δ subunit expression in slices co-treated 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 (data not shown). 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 following NMDA treatment, and it appeared to increase as early as 1 hr after NMDA treatment and was sustained even after 6 hrs of NMDA treatment (Fig. 6C and 6D). Levels of pERK1/2 were $140 \pm 22\%$ ($n=5$, $p<0.05$) of that in controls after 1hr of NMDA treatment, increased to $214 \pm 33\%$ and $243 \pm 35\%$ after 3 and 6 hrs of NMDA treatment respectively ($n=4$, $p<0.05$). We then determined whether blockade of ERK1/2 activation prevented NMDA-induced down-regulation δ subunit expression. Protein kinases MEK1/2 phosphorylate and activate ERK1/2, and their inhibition by UO126 prevents activation of ERK1/2 (Zheng and Guan, 1993; Favata *et al.*, 1998; Roskoski, 2012). Co-treatment of NMDA and UO126 (10 μM) prevented δ subunit down-regulation (Fig. 6E and 6F). Surface expression of δ subunit in NMDA and UO126-treated cultures was $120 \pm 23\%$ of that in 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 UO126 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 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 down-regulation of GABAR- δ expression (Binder *et al.*, 2001; He *et al.*, 2004; Kotloski and McNamara, 2009). The role of TrkB receptors in mediating the NMDA effect was determined in cultures co-treated with NMDA and TrkB-Fc (2 μ g/ml, 48 hrs), which is known to block biological activity of BDNF. Surface expression of GABAR- δ was lower in cultures treated with NMDA in the presence or absence of TrkB-Fc (Fig. 7A and 7B). 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 NMDA and TrkB-Fc treated cultures (table 1). Hence the NMDA effects did not require activation of TrkB receptors.

Discussion:

Key findings of this study were 1) 48 hrs NMDA treatment of hippocampal neuronal cultures reduced δ and $\alpha 4$ subunit expression but did not change expression of $\gamma 2$ subunits. 2) NMDA treatment also reduced tonic current and its neurosteroid modulation but synaptic currents and their neurosteroid modulation was preserved. 3) Activity blockade by TTX induced changes opposite to those triggered by NMDA. 4) The NMDA effects were mediated via NMDA receptor (NMDAR) activation and required calcium flux. 5) The NMDA effects were mediated at least in part via ERK1/2 phosphorylation.

In the DGCs of epileptic animals, expression of δ subunit is reduced whereas that of $\alpha 4$ and $\gamma 2$ subunits is up-regulated (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 expression of $\alpha 4$ and $\gamma 2$ subunits. BDNF activated TrkB signaling increases the $\alpha 4$ subunit expression (Roberts *et al.*, 2006). In contrast, down-regulation of δ subunit expression in NMDA-treated hippocampal slice cultures was independent of BDNF signaling. Thus the signaling pathways underlying reduction in δ subunit expression and up-regulation of $\alpha 4$ and $\gamma 2$ subunits expression appear to be distinct.

Hippocampal slice cultures were used to determine expression of δ and $\alpha 4$ subunits because they yield sufficient protein for biochemical analysis. In the hippocampus, δ subunit is primarily expressed in the DGCs as well as 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 δ subunit originating from interneurons should be minor compared to 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 and Kapur, 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 tonic current recorded from DGCs of NMDA-treated slice cultures was also reduced. GABARs containing a δ subunit have higher neurosteroid sensitivity than 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 which prevented NMDA-induced down-regulation of δ subunit expression also blocked diminution in neurosteroid modulation of tonic current. However in the cultures co-treated 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 expression of δ subunit mRNA but increased expression of $\gamma 2$ subunit mRNA (Memo *et al.*, 1991). These differences could be due to 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 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 hr of NMDA treatment. Expression of δ subunits in the ER fraction is greater in epileptic animals, suggestive of their potential ER 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 down-regulation of δ subunit expression (Choi *et al.*, 1988; Rothman, 1985; Rothman and Olney, 1987). However, previous studies have indicated that CA3 and CA1 neurons are predominately susceptible to cell death, whereas DGCs are comparatively resistant (Engel Jr 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 non-specific action of NMDA on δ subunit expression. In the cultures in which NMDA was

washed out, δ subunit expression showed a trend towards recovery, suggesting that the NMDA effects could be partially reversed. Importantly, cell death can not 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, MAP 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 down-regulation of δ subunit expression. Increased ERK1/2 phosphorylation levels could originate from neurons and/or glia. However, previous immunohistochemical studies in brain slices have revealed increased pERK1/2 immunoreactivity in the neurons during SE or following 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 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). Interestingly, 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, PI3K-Akt, adenylyl cyclase, and heterotrimeric G proteins, which may play a role in calcium-induced activation of ERK1/2, could provide more

insights on NMDA-induced ERK1/2 activation (Agell *et al*, 2002; Holstein *et al*, 2004; Lopez-Illasaca 1998; Xia and Storm, 2012). NMDA-induced oxidative stress could also lead to ERK1/2 activation (Lafon-Cazal *et al*, 1993; Rybakova *et al*, 2012).

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 can not 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 knockout mice threshold to pentelenetetrazole-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 entorhinal cortex (EC) (Stringer and Lothman, 1989; Stringer *et al*, 1989). However if the dentate gate is compromised, activity from EC can spread into hippocampal CA subfields forming a re-entrant loop, which could amplify seizure activity. Previous studies have revealed a rapid reduction in the δ subunit expression following 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 following SE (Pathak *et al*, 2007). Hence, future studies in the experimental animals will be important to determine

whether blocking ERK1/2 activation following SE can prevent down-regulation of δ subunit expression, maintain the dentate gating function, and alter the course of epileptogenesis.

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Authorship contribution:

Participated in research design: Suchitra Joshi and Jaideep Kapur.

Conducted experiments: Suchitra Joshi.

Performed data analysis: Suchitra Joshi.

Wrote or contributed to the writing of the manuscript: Suchitra Joshi and Jaideep Kapur.

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Figure legends

Figure 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 μ M) for 48 hrs. 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 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) following NMDA treatment relative to that at time 0. Data represent average and standard error from 5 experiments. * $p < 0.05$.

Figure 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 hrs. 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 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 hrs) organotypic hippocampal slice cultures. Tonic current was determined by bath application of picrotoxin (50 μ M, PT, arrow) following 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.

Figure 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 hrs). The external solution contained TTX. **B:** Averaged currents from representative control and NMDA-treated neurons before (black) and after application of allopregnanolone (gray).

Figure 4: Activity blockade by TTX increased GABAR- δ expression. **A:** A representative western blot demonstrating surface and total expression of δ and γ 2 subunits of GABARs in organotypic hippocampal slice cultures treated with TTX (2 μ M, 48 hrs). 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 γ 2 subunits of GABARs in TTX-treated cultures. n=4 experiments, *p<0.05. **C:** Representative current traces demonstrating effect of allopregnanolone (10 nM, arrow) on tonic current in

cultured hippocampal pyramidal neurons treated with TTX (2 μ M, 48 hrs). Picrotoxin (50 μ M, PT) was applied after recording allopregnanolone effect to determine tonic current. Gray line running through the trace represents position of basal holding current. **D:** Magnified portions of traces from A showing the effect of allopregnanolone and picrotoxin.

Figure 5: NMDA effects on δ subunit expression were mediated by activation of NMDARs and calcium flux. **A:** A representative Western blot showing surface and total expression of δ subunit in NMDA-treated (48 hrs) 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 hrs. Expression of β -actin demonstrates purity of surface samples. Anti δ subunit antibody (N151/3.3) was used in these studies. **D:** Bar graph showing relative surface δ subunit expression. $n = 5$ experiments, * $p < 0.05$.

Figure 6: Blockade of ERK1/2 activation prevented NMDA-induced down-regulation of δ subunit expression. **A:** A representative Western blot showing 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 relative surface expression of δ subunit. $n = 4$, * $p < 0.05$. **C:** A representative Western blot demonstrating the time course of ERK1/2 phosphorylation following 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 min prior to NMDA and surface δ subunit expression was determined after 48 hrs. 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.

Figure 7: Activation of TrkB receptors was not necessary for NMDA effects. **A:** A representative Western blot demonstrating surface and total δ subunit expression in slice cultures treated with NMDA without or with TrkB-Fc chimera (2 μ g/ml) for 48 hrs. Expression of β -actin demonstrates purity of surface samples. Millipore anti- δ subunit antibody was used in these experiments. **B:** Relative surface δ subunit expression from 4 experiments. *p<0.05.

Table 1

Treatment	Tonic current	Neurosteroid modulation of tonic current
Control	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+UO126	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

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), UO126 (10 μ M) or TrkB-Fc (2 μ g/ml) for 48 hrs. Tonic current and its neurosteroid modulation was recorded from DGCs. Cultures without any treatment grown in parallel were used as controls. Data represents mean \pm SEM.* $p < 0.05$.

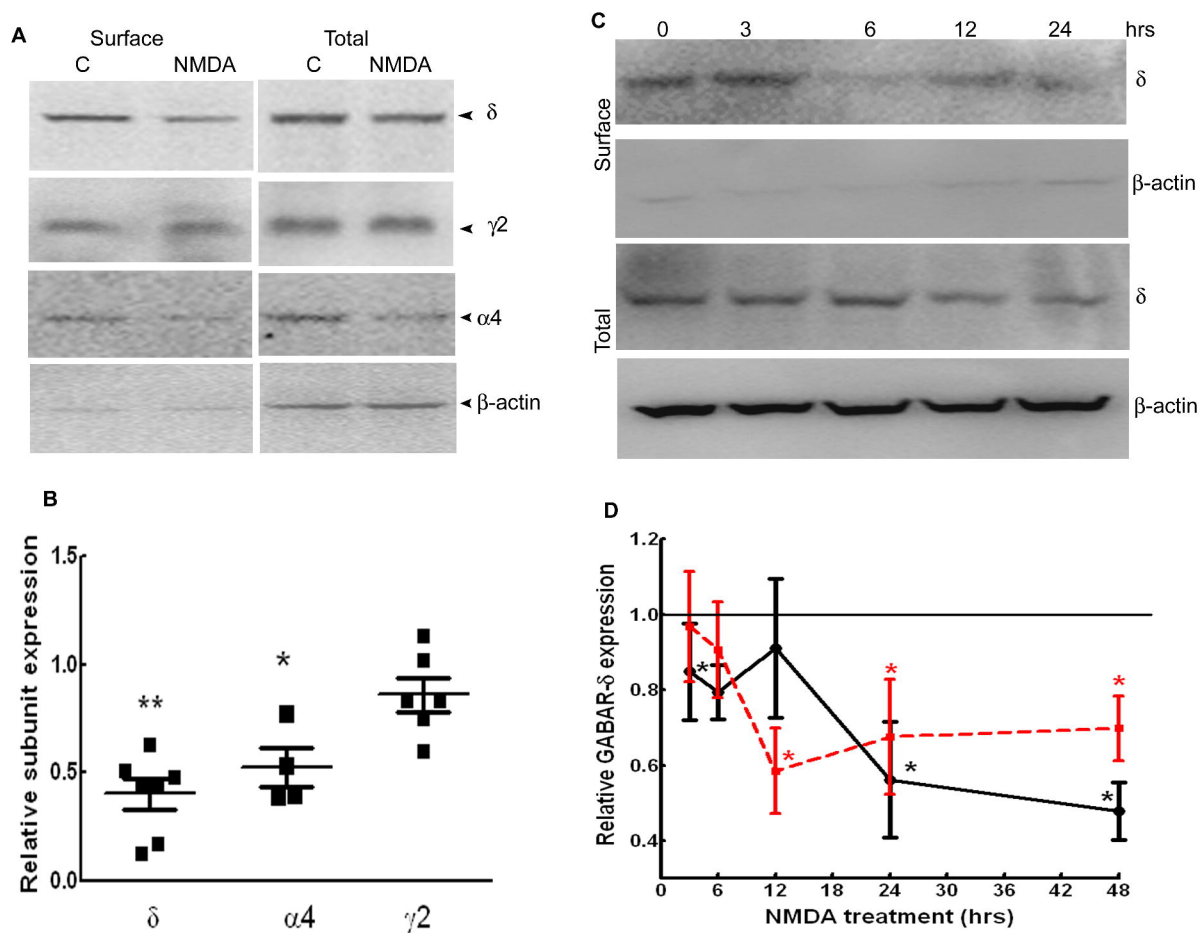


Figure 1

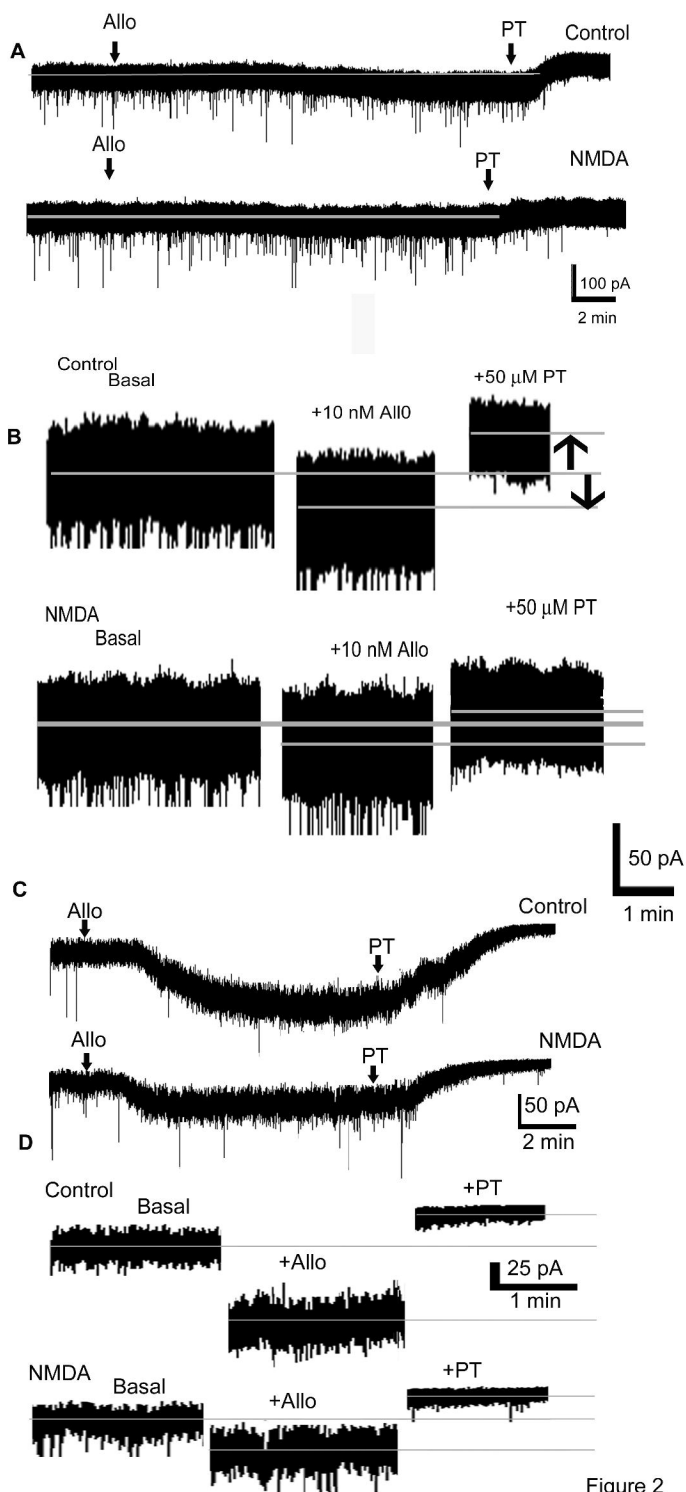


Figure 2

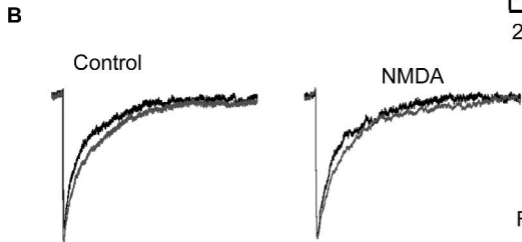
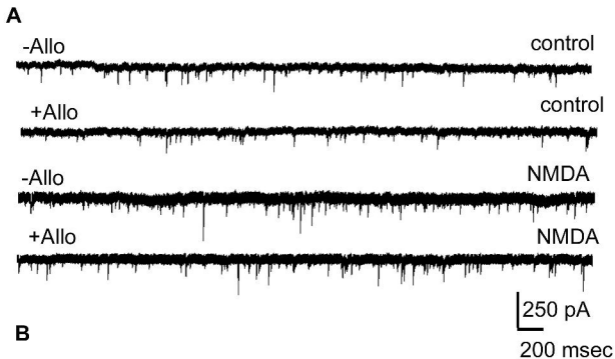


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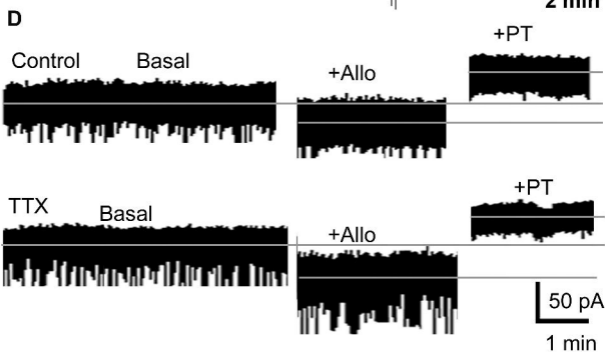
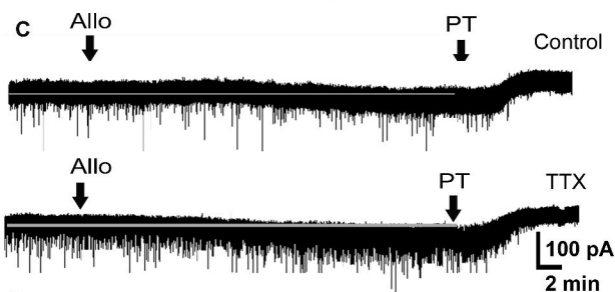
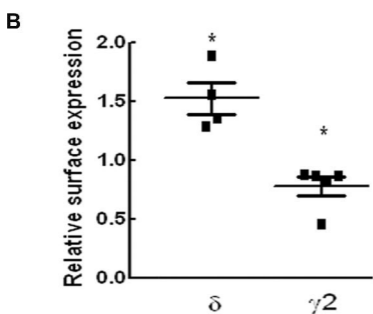
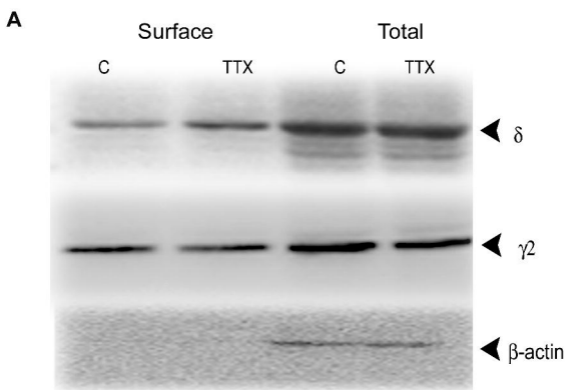


Figure 4

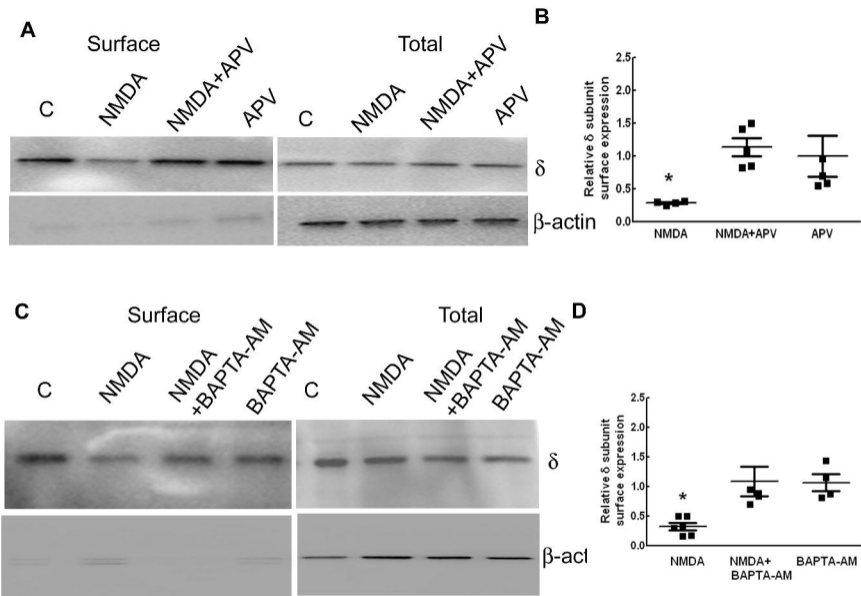


Figure 5

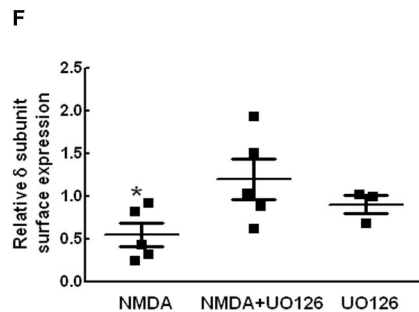
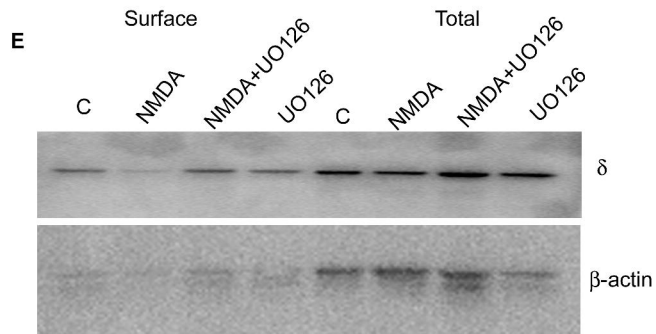
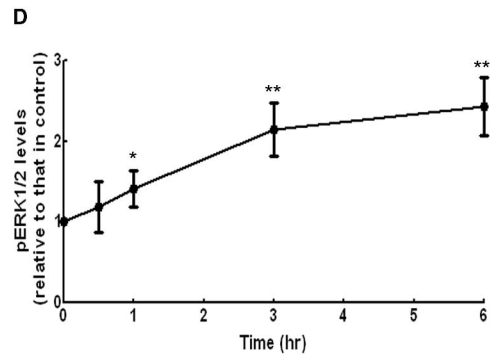
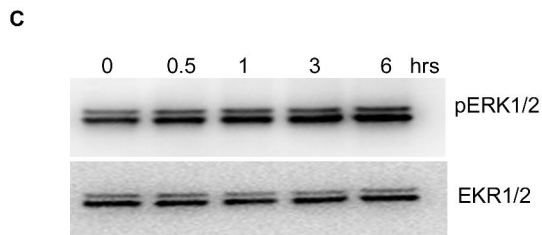
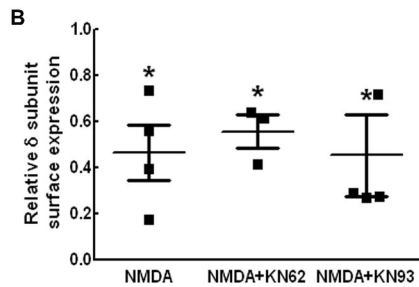
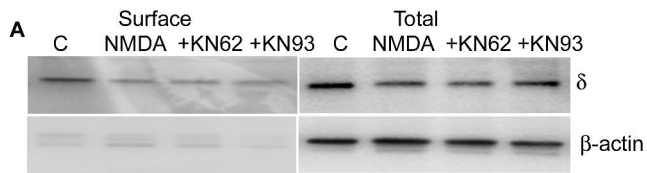


Figure 6

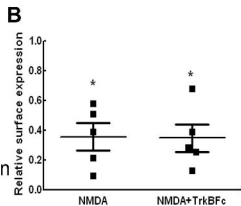
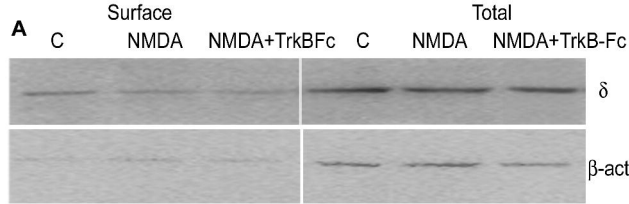


Figure 7