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Activity-dependent regulation of NR2B translation contributes to metaplasticity in mouse visual cortex

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Abstract

Visual experience and deprivation bidirectionally modify the NR2A and NR2B subunit composition of NMDARs, and these changes in turn modify the properties of synaptic plasticity in the visual cortex. Deprivation-induced lowering of the NR2A/2B ratio can occur by altering either NR2A or NR2B protein levels, but how a reduction in synaptic activity regulates these changes in a subunit-specific manner is poorly understood. Here, we find that visual deprivation in juvenile mice by dark-rearing or monocular lid suture reduces the NR2A/2B ratio in the deprived cortex in temporally distinct phases—initially by increasing NR2B protein levels, and later by decreasing NR2A protein levels. Brief dark-exposure of juvenile rats likewise produces an increase in NR2B expression. Furthermore, we are able to model the early increase in NR2B by blocking NMDARs *in vitro,* and we find that translation of NR2B is likely a major point of regulation. Translation of NR2A is not regulated in this manner. Therefore, the differential translational regulation of NR2A and NR2B may contribute to experience-dependent modification of NMDAR subunit composition.

Keywords: Ocular dominance plasticity; Metaplasticity; Long-term potentiation; Long-term depression

1. Introduction

Primary visual cortex provides a useful model in which elementary mechanisms of synaptic plasticity, such as those revealed by NMDA receptor- (NMDAR-) dependent long-term potentiation (LTP) and long-term depression (LTD), can be related to experience-dependent modifications of brain function. The responsiveness of cortical neurons to visual stimulation can be depressed by sensory deprivation and potentiated by sensory experience. Theoretical analysis suggests that these changes can be accounted for by mechanisms of LTD and LTP if it is further assumed that the properties of synaptic plasticity are "metaplastic" to keep synaptic strengths within a useful dynamic range (reviewed by Bear et al., 1987; Bear, 2003). The notion that the LTD and LTP thresholds are lowered by reducing overall cortical activity and increased by elevating cortical activity has been confirmed experimentally (Kirkwood et al., 1996; Philpot et al., 2003). Furthermore, available evidence supports the hypothesis that these shifts in plasticity thresholds occur by activity-dependent changes in NMDAR function (Philpot et al., 2001, 2003). Key questions that remain concern the mechanisms responsible for altered NMDAR function and the contributions of these mechanisms to experience-dependent plasticity *in vivo.*

An appealing hypothesis is that metaplasticity results from activity-dependent regulation of NMDAR subunit

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composition. Biochemical analysis of protein expression in visual cortex of rats reared in complete darkness reveals a lower NR2A/2B ratio that can be increased rapidly by light exposure (Quinlan et al., 1999a,b; Tongiorgi et al., 2003; He et al., 2006). Since both the channel properties and the intracellular binding partners of NMDARs rely on the NR2 subunit present in the heteromer, experience-dependent changes in NR2A/2B ratio alter receptor function (Monyer et al., 1992; Flint et al., 1997; Vicini et al., 1998; Barria and Malinow, 2005; Kim et al., 2005). It has therefore been hypothesized that the LTP threshold is raised by increases, and lowered by decreases, in the NR2A/B ratio (Bear, 2003). However, this idea has been challenged by recent evidence from hippocampus suggesting that NR2A-containing receptors promote LTP and NR2B-containing receptors promote LTD (Liu et al., 2004; Kim et al., 2005).

Because it can be manipulated genetically, mouse visual cortex may offer a system in which the functional roles of NR2A/B regulation can be resolved *in vivo*. Indeed, recent studies have suggested a role for metaplasticity in the cortical response to monocular deprivation (MD) in mice (Frenkel and Bear, 2004). An essential first step in testing this hypothesis is to assess the degree to which NMDAR subunit expression is regulated by activity in the mouse visual cortex *in vivo*. Here we show there is a biphasic response of NMDAR subunits to light deprivation by both dark-rearing (DR) and MD. The deprivation-induced reduction in the NR2A/B ratio is mediated initially by an increase in total, whole-cell levels of NR2B and sustained by a later decrease in NR2A. The more rapid increase in NR2B is not peculiar to mice, as a reexamination of the first effects of visual deprivation in rats revealed a similar change.

To gain insight into the mechanisms responsible for the rapid deprivation-induced increase in NR2B, we studied the effects of activity blockade in dissociated cultures of cortical neurons. We found that selective blockade of synaptic NMDAR-mediated transmission produces a substantial increase in NR2B protein, likely due to de-repression of NR2B translation. Our data suggest that normal NMDAR activation initiates a negative feedback loop to limit surface and synaptic expression of NR2B-containing NMDARs, and this is relieved by sensory deprivation. In contrast, NR2A subunit expression is not subject to this mode of translational regulation. Therefore, we suggest that differential translational regulation of NR2 subunits underlie the changes in NMDAR subunit composition induced by manipulation of visual experience.

2. Methods

2.1. Animals and visual manipulation

For mouse dark-rearing experiments, pregnant C57/BL6 female mice (Charles River) were ordered in pairs, but were housed singly in cages maintained in a 12:12 h light:dark cycle. The pairs of pregnant mice typically gave birth within 24 h of one another. Two to three days after giving birth, one cage of mother and pups was left for light rearing (LR), and the other was transferred into a photon-free room for rearing in complete darkness (DR). For experiments extending past 4 weeks, LR and DR pups of similar birthdates were weaned on the same date into cages of 3–4. Both male and female mice were used, and preparation of homogenates from LR animals was interleaved with DR animals. For rat dark-exposure experiments, male and female Long Evans rats (Charles River) were weaned at P21 into cages of 2–3, and dark-exposed (DE) beginning at P23 for 3 days. Preparation of homogenates and synaptoneurosomes from LR animals was interleaved with LR + 3 day DE animals.

For monocular deprivation experiments, male C57/BL6 mice (Charles River) were group housed and maintained on a 12:12 h light:dark cycle. At P28, MD was performed by suturing either the left or right eyelid. Mice were anesthetized by inhalation of isoflurane (IsoFlo 2–3%) and placed under a surgical microscope. Lid margins were trimmed, and antibiotic ointment (Vetropolycin, Pharmaderm) was applied to the eye. Three mattress stitches were placed using 6-0 vicryl, opposing the full extent of the trimmed lids. Mice recovered by breathing room air and were monitored daily to ensure that the sutured eye remained closed and uninfected. Preparation of tissue from control, non-MD'ed animals was interleaved with MD'ed animals, and slice biotinylation of surface receptors was performed as described below.

2.2. Homogenate and synaptoneurosome preparation from frontal and visual cortex

Male and female C57/BL6 mice or Long Evans rats (Charles River) were given an overdose of barbiturate (Nembutal) and decapitated after disappearance of corneal reflexes in compliance with Brown University and MIT animal care guidelines. Synaptoneurosomes were prepared using a procedure adapted from Hollingsworth et al. (1985), described by Quinlan et al. (1999a). Primary visual and frontal cortices were rapidly dissected into ice cold dissection buffer (75 mM sucrose, 10 mM dextrose, 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.3 mM ascorbic acid, saturated with 95% O_2/5% CO_2), then homogenized in synaptoneurosome buffer (10 mM HEPES, 2.0 mM EDTA disodium salt, 2.0 mM EGTA, 0.5 mM DTT, 1:100 Calbiochem phosphatase inhibitor cocktail I, 1:100 Calbiochem phosphatase inhibitor cocktail II, 1:100 Calbiochem protease inhibitor cocktail III) using 20 even strokes in a glass-glass homogenizer. An aliquot of homogenate was mixed with 1/9 volume of 10% SDS (1% SDS final) and saved for later analysis. The remaining homogenate was passed through two 105 μm pore nylon meshes (Small Parts, Inc.) and one 5 μm filter (Millipore), then centrifuged at 1000 × *g* for 10 min to pellet synaptoneurosomes (P1). The P1 pellet was resuspended in 1% SDS. Homogenate and P1 samples were boiled, and an aliquot of each sample was saved at −80 °C for protein quantification. The remaining sample was diluted with 4× SDS-PAGE sample buffer (4× = 20% glycerol, 248 mM Tris–HCl pH 6.8, 12% SDS, 8% β-mercaptoethanol, bromophenol blue) to 1× final concentration and stored at −80 °C until SDS-PAGE analysis.

2.3. Cortical slice biotinylation of surface receptors

C57/BL6 mice (Charles River) MD'ed for 1, 3, 5, or 7 days, or age-matched, non-MD'ed control animals were given an overdose of barbiturate (Nembutal) and decapitated after disappearance of corneal reflexes in compliance with Brown University and MIT animal care guidelines. Both hemispheres of primary visual cortex from control or MD'ed mice were rapidly dissected into ice-cold dissection buffer (75 mM sucrose, 10 mM dextrose, 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.3 mM ascorbic acid, saturated with 95% O_2/5% CO_2). Left and right visual cortices were simultaneously sliced in ice-cold dissection buffer containing 2 μM jasplakinolide (Molecular Probes) into 300μm sections using a vibratome. Slices recovered in ice-cold artificial cerebrospinal fluid (ACSF, 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM dextrose, saturated with 95% O_2/5% CO_2) containing 2 μM jasplakinolide for 45–60 min. Slices were then transferred into ACSF containing 1 mg/ml Sulfo-NHS-SS-Biotin (Pierce) for 30 min on ice to allow conjugation of biotin to cell surface proteins. After the labeling period, slices were

rinsed 3× with ice-cold ACSF to remove unbound biotin. Cortical regions surrounding primary visual cortex were removed, the superficial layers (I–IV) were microdissected, then homogenized in 300 µl of modified 0.5% SDS-RIPA buffer (1% Triton X-100, 0.5% SDS, 0.5% deoxycholic acid, 10 mM NaH_2PO_4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium iodoacetamide, 1 mM sodium orthovanadate, 1:100 phosphatase inhibitor cocktails I and II, 1:100 protease inhibitor cocktail III (Calbiochem)) using 20 even strokes in a glass-glass homogenizer. The protein sample was incubated for 15 min on ice, then centrifuged at 14,000 × *g* for 15 min to pellet the unsolubilized fraction. Protein concentration of the soluble fraction was determined (DC Protein Assay Kit, BioRad), and 40 µg of protein sample was mixed with 80 µL Immobilized Neutravidin Protein (Pierce) in 0.5 mL of 0.1% SDS-RIPA buffer for precipitation of biotinylated proteins overnight (4 °C). The precipitation reaction was performed in duplicate for each sample. Biotin protein-avidin complexes were spun down (2500 × *g* for 1 min) and rinsed 3× with 0.1% SDS-RIPA to remove unbound protein. Bound protein was cleaved by boiling in 2× SDS-PAGE sample buffer (2× = 10% glycerol, 124 mM Tris–HCl pH 6.8, 6% SDS, 4% β-mercaptoethanol, bromophenol blue), yielding the surface protein fraction.

2.4. Quantitative immunoblotting and statistical analysis of in vivo data

Homogenate, synaptoneurosome, and biotinylated samples were assayed for protein concentration using the DC Protein Assay Kit (BioRad), a Bradford-based method. Homogenate (15 µg), synaptoneurosome (15 µg), or surface fractions (pulldown from 40 µg input) were loaded on 7.5% polyacrylamide gels, transferred to PVDF membranes (BioRad), and immunoblotted for NMDAR expression using subunit-specific antibodies against NR2A (1:250, Molecular Probes), NR2B (1:250, Santa Cruz), and NR1 (1:1000, Santa Cruz). Blots were then incubated with appropriate secondary antibodies coupled to HRP, immunoreactive bands detected by enhanced chemiluminescence (Amersham, ECL or ECL plus), and signal visualized using the Versadoc imaging system (BioRad). Optical densities of detected bands were quantified using Quantity One software (BioRad). A standard sample of wildtype mouse cortical tissue was run on each gel to gauge blot-to-blot variability, and a sample of NR2A K/O mouse cortical tissue was run on each gel to ensure specificity of NR2A antibody during each immunoblotting session.

For mouse DR experiments, frontal cortex samples were always run on the same gels as the visual cortex samples from the same animal as a within-animal control. For analysis of visual cortex or frontal cortex signal alone, signal intensities were normalized to the standard protein sample run on each blot to adjust for blot-to-blot variability. When appropriate, signal intensities of visual cortex samples were divided by the within-animal frontal cortex signal intensity to obtain the visual/frontal signal intensity ratio. Measurements greater than 2 standard deviations from the mean were excluded from the analysis. Data were tested for interaction between time (3–7 weeks for DR, or 3 days for DE) and group (LR and DR, or LR and LR + DE) using a 2-factor ANOVA. Unpaired *t*-tests were performed for each time point in data sets that resulted in a significant interaction of time and group. For graphical representation, the values measured from DR or DE samples were divided by the average values obtained from control, LR animals. Thus, data expressed is the NMDAR subunit protein level as a percentage of LR at each time point.

For MD experiments, surface fractions of left and right visual cortex were prepared in duplicate and always run on the same gels to allow within-animal comparison of deprived and non-deprived hemispheres. Measurements greater than 2 standard deviations from the mean were excluded from the analysis. Data were tested for interaction between time (1–7 days) and hemisphere (contralateral and ipsilateral for MD'ed animals, or left and right for non-MD'ed animals) using a repeated-measures ANOVA. Paired *t*-tests were performed for each time point in data sets that resulted in a significant interaction of time and hemisphere. For graphical representation, the values measured from contralateral and right hemispheres were divided by the average values obtained from ipsilateral and left hemispheres, respectively. Thus, data is expressed as the NMDAR subunit protein level as a percentage of IPSI (or LEFT) at each time point.

2.5. Dissociated cortical culture from occipital cortex

Occipital cortices of P0–P1 Long Evans rat pups were rapidly dissected in ice-cold Neurobasal A media (Gibco) containing 3 mM $MgCl_2$ and 1 mg/mL kynurenic acid to prevent excitotoxicity. Neurons were dissociated by incubating the tissue in 20 units/mL papain for 1 h at 37 °C, 5% CO_2, followed by trituration in feeding media (Neurobasal A, 2% B27 supplement, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.5 mM L-glutamine). Neurons were plated at high density (530 cells/mm^2) on poly-lysine coated 60 mm tissue-culture treated dishes and maintained in a 37 °C incubator with 5% CO_2. One-half of the feeding media was changed twice weekly to replenish nutrients. For pharmacologic experiments, treatments were performed at 22–30 div, with appropriate drugs diluted directly into a 1:1 mixture of new and conditioned feeding media.

2.6. Purification of postsynaptic density and surface fractions from cortical cultures

PSDs were prepared from dissociated cortical cultures using a procedure adapted from Ehlers (2003). Cultured neurons were pelleted and homogenized in HEPES-buffered sucrose (4 mM HEPES pH 7.4, 0.32 M sucrose, 1:100 phosphatase inhibitor cocktails I and II, 1:100 protease inhibitor cocktail III (Calbiochem)) in a glass-glass homogenizer using 40 even strokes, then centrifuged at 1000 × *g* for 10 min to remove nuclei and large debris (P1). The supernatant was spun at 10,000 × *g* for 15 min to pellet the synaptosome fraction (P2). P2 was washed in HEPES-buffered sucrose, then respun at 10,000 × *g* for 15 min to pellet the washed synaptosome fraction (P2′). Washed synaptosomes were lysed in 4 mM HEPES pH 7.4 by agitation for 30 min, then centrifuged at 25,000 × *g* for 20 min to pellet the synaptosomal membrane fraction (P3). P3 was agitated in 0.5% Triton X-100, 50 mM HEPES pH 7.4, 2 mM EDTA, followed by a 32,000 × *g* spin to isolate the Triton-extracted PSD (PSD-1T). PSD-1T was further purified by agitating in either a second round of 0.5% Triton X-100, 50 mM HEPES pH 7.4, 2 mM EDTA for 10 min and spun at 100,000 × *g* to isolate the 2× Triton-extracted PSD (PSD-2T), or PSD-1T was agitated in 3% sarcosyl, 50 mM HEPES pH 7.4, 2 mM EDTA for 10 min and spun at 200,000 × *g* to isolate the sarcosyl-extracted PSD (PSD-TS). All fractions were solubilized in 1% SDS, protein assayed, and processed by SDS-PAGE.

Surface fractions were prepared from cortical cultures following treatment with or without APV. Cultures were rinsed 3× with ice-cold ACSF, surface receptors were biotinylated, and 100 µg biotinylated protein was precipitated to isolate the surface fraction as described above.

2.7. Quantitative immunoblotting and statistical analysis of culture data

Protein concentration was determined for each fraction prepared from cortical cultures using the DC Protein Assay Kit (BioRad). Equal amounts of protein (20 µg homogenate, 2 µg PSD-TS fraction, or precipitation of 100 µg biotinylated protein) were loaded on 7.5% polyacrylamide gels, transferred to PVDF membranes, and immunoblotted for NMDAR expression using subunit-specific antibodies as described above. Samples were prepared from control, untreated cultures with every experiment, and the same control samples were run on every blot within the same experimental group to adjust for blot-to-blot variability. The average control value also served as a baseline reading, allowing comparison across different experimental sets. Signal intensities were normalized to the average CTRL value, and measurements greater than 2 standard deviations from the mean were excluded from the analysis. Data were tested for significant changes between multiple pharmacologic manipulations by 1-factor ANOVA. Fisher's PLSD post-hoc comparison was used to test for significant changes between individual pharmacologic treatment conditions.

3. Results

3.1. Dark-rearing modifies NMDAR subunit composition in mouse visual cortex

Visual deprivation by dark-rearing has been shown to alter synaptic NMDAR subunit composition in juvenile rats. The NR2A/2B ratio in synaptoneurosomes prepared from visual cortex of dark-reared (DR) rats is approximately 30% less than in light-reared (LR) rats. This change is thought to be due to decreases in synaptic NR2A levels, although there are also reports of elevated NR2B levels following DR in older rats (Quinlan et al., 1999a; He et al., 2006). Visual deprivation also produces a very modest (10%) decrease in the NR2A/2B ratio in homogenate and synaptic fractions prepared from visual cortices of juvenile mice, but it is not clear whether the lower NR2A/2B ratio is due to a reduction in NR2A or an elevation of NR2B levels (Yashiro et al., 2005).

To see if visual deprivation by dark-rearing has specific effects on the expression of NR2A versus NR2B in the mouse visual cortex, homogenates were prepared from primary visual cortices of C57/BL6 mice, raised either in a normal light:dark cycle (12:12 h/day, LR) or in complete darkness (DR) from birth. Since the changes in mouse visual cortex were expected to be small (~10%), several precautions were taken in the experimental design to minimize sources of variability. First, homogenate fractions were also prepared from frontal cortices of the LR and DR animals and run on gels alongside visual cortex samples. NMDAR subunit expression in the frontal cortex is not altered by visual experience (confirmed below) and serves as a within-animal comparison to control for animal-to-animal variability (cf. Jo et al., 2006). Since frontal and visual cortex samples from both LR and DR groups are prepared together, slight differences in immunoblotting that may skew comparisons from one preparation to another are minimized by normalizing to the frontal cortex value. Second, homogenate fractions from both frontal and visual cortex were assayed in triplicate for protein concentration, and equal amounts of protein (15 μg) were loaded in each lane and resolved on 7.5% polyacrylamide gels. Third, 15 μg of a standard protein sample (Std) was run on each gel and served as a standard to assess blot-to-blot variability, and a sample from NR2A−/− mice served to ensure NR2A antibody specificity. A representative immunoblot is shown in Fig. 1, illustrating visual and frontal samples from LR and DR animals run on the same blot as the Std and NR2A−/−. Finally, the experimenter was blind to the history of the animal's visual experience and origin of the tissue collected.

Because the aim of our experiment is to assess the effect of experience at any given age, the data are presented as a percentage of the average LR value; however, all statistical analyses were performed on the raw data. Analysis of the homogenate fraction from frontal cortices of LR and DR animals reveals no significant differences in NR2A, NR2B, or NR1 signal intensity between the LR and DR groups at time points examined (Fig. 2B–D, ANOVA for NR2A, NR2B, NR1, $p > 0.3$, $p > 0.7$, $p > 0.6$, respectively). Thus,

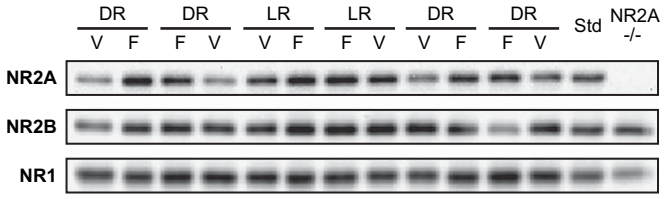

Fig. 1. Sample immunoblots of NR2A, NR2B, and NR1 of homogenate samples from visual (V) and frontal (F) cortices of 7 week old light-reared (LR) and dark-reared (DR) mice. For each animal, left and right hemispheres of visual or frontal cortex were pooled, homogenized, and protein assayed in triplicate. 15 μg of homogenate from visual cortex and frontal cortices of the same animal, a standard protein sample (Std), and a NR2A−/− sample were run on the same blot. Samples were transferred to PVDF membrane and immunoblotted for NMDAR proteins using subunit-specific antibodies. Sample signals from LR and DR animals were normalized to the Std signal to control for blot-to-blot variability. Analysis of frontal cortex samples alone shows no significant differences in NR2A, NR2B, or NR1 between LR and DR groups. Therefore, for some analyses, the visual cortex signal was normalized to the within animal frontal cortex signal to reduce animal-to-animal variability. The experimenter was blind to the rearing history and origin of tissue collected.

the frontal cortex serves as a within-animal control tissue in which NMDAR subunit composition is not altered by visual deprivation. Similar analysis of the homogenate fraction from visual cortices of LR and DR animals reveals a small but significant effect of visual deprivation on NR2A and NR2B, but not NR1 expression (Fig. 2F–H, ANOVA for NR2A and NR2B, $p < 0.008$ and $p < 0.04$; ANOVA for NR1, $p > 0.4$). Remarkably, we observe two temporally distinct changes in NR2A and NR2B protein levels. In the early period of visual deprivation, NR2B levels in the homogenate are significantly elevated at 4 and 5 weeks (Fig. 2A, G, $125.6 \pm 9.2\%$ and $114.7 \pm 2.2\%$ of LR, t-test, $p < 0.04$ and $p < 0.0001$). This elevation in NR2B is followed by a significant decrease in NR2A subunit expression at 6 and 7 wks (Fig. 2A, F, $77.0 \pm 2.3\%$ and $77.5 \pm 7.4\%$ of LR, t-test, $p < 0.002$ and $p < 0.02$).

Since (1) visual deprivation-induced changes in NMDAR subunit expression are detected only in visual cortex and not frontal cortex, and (2) signal intensities of visual and frontal cortex samples tend to co-vary from preparation to preparation, we reasoned that normalization of visual cortex values to frontal cortex would reduce variability introduced by the experimental preparation and allow for maximal detection of small changes (Jo et al., 2006). Indeed, when visual cortex values are normalized to their corresponding frontal cortex values, similar magnitude changes in NR2A and NR2B expression are detected, while the standard error of the mean of visual/frontal ratio values is reduced compared to the error of visual values or the frontal values alone (Fig. 2J–K, ANOVA for NR2A and NR2B, $p < 0.04$ and $p < 0.02$; NR2B: 3 weeks DR, $118.0 \pm 3.4\%$ and 4 weeks DR, $111.6 \pm 2.0\%$ of LR, t-test, $p < 0.002$ and $p < 0.0001$; NR2A: 6 weeks DR, $84.1 \pm 3.4\%$ and 7 weeks DR, $84.5 \pm 5.9\%$ of LR, t-test, $p < 0.006$ and $p < 0.03$). Again, no significant change is detected in NR1 protein levels (Fig. 2L, ANOVA, $p > 0.7$).

Examination of the NR2A/2B ratio in the homogenate fraction from visual cortex reveals that DR mice exhibit a lower

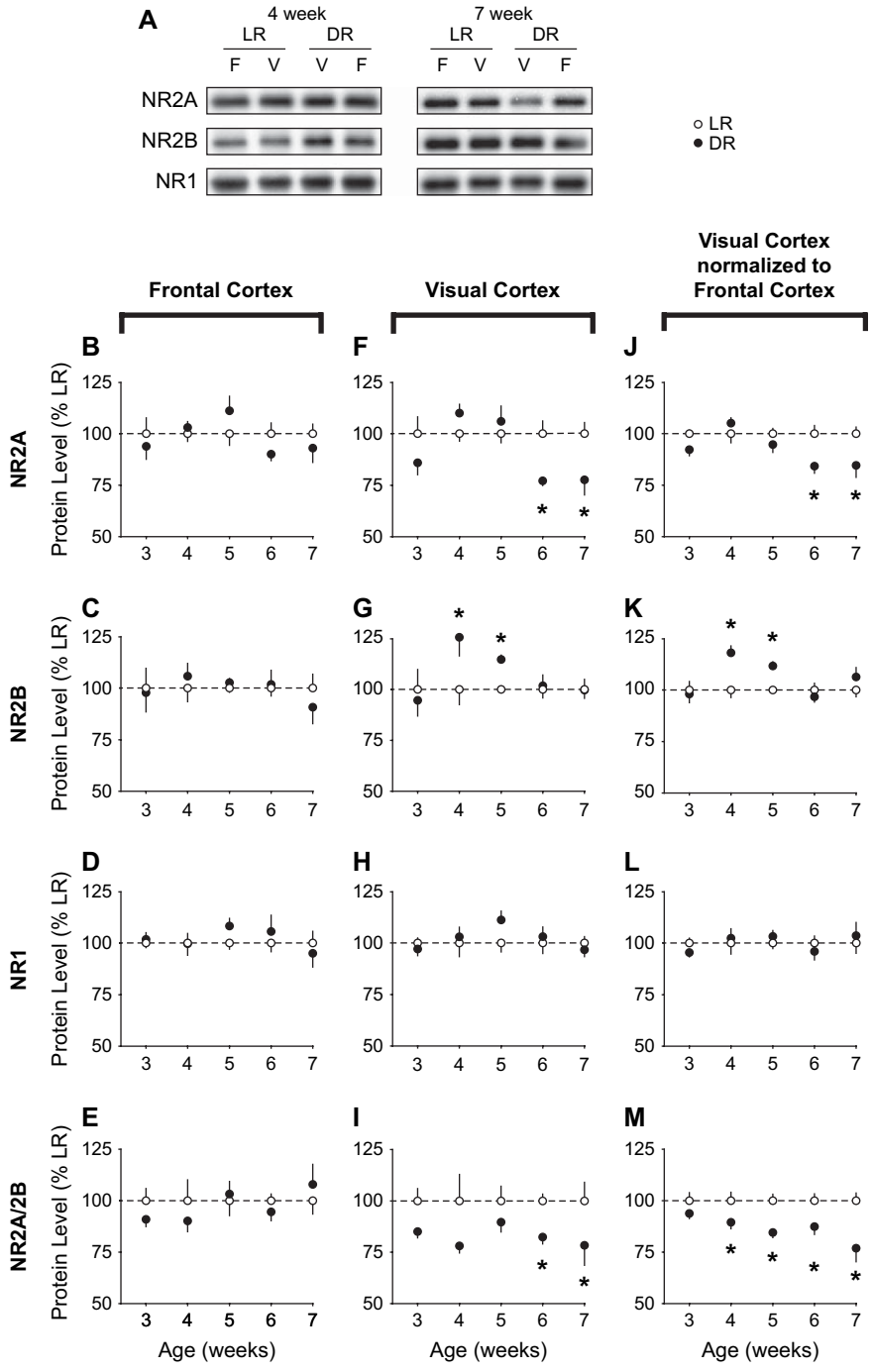

Fig. 2. Dark-rearing modifies NMDAR subunit expression in the homogenate fraction of mouse visual cortex. (A) Representative immunoblots of homogenate samples prepared from frontal (F) and visual (V) cortices of light-reared (LR) and dark-reared (DR) mice at 4 and 7 weeks of age. Blots were probed with subunit-specific antibodies for NR2A, NR2B, and NR1. (B–M) Summary of immunoblot analysis of homogenate samples prepared from frontal (B–E) or visual cortices (F–I) of LR and DR mice (white and black symbols, respectively, $n = 10–14$ for each data point, with mean values ± SEM normalized to the average LR protein level). (B–E) NR2A, NR2B, NR1 protein levels, and the calculated NR2A/2B ratio from homogenates of frontal cortex do not differ between LR and DR groups (ANOVA, $p > 0.3$, $p > 0.7$, $p > 0.6$, $p > 0.6$, respectively). (F) NR2A protein in the visual cortex is significantly reduced after 6–7 weeks of DR (ANOVA, $p < 0.008$, and G) NR2B protein is significantly elevated after 4–5 weeks of DR (ANOVA, $p < 0.04$), while (H) NR1 protein is not altered (ANOVA, $p > 0.4$). (I) The NR2A/2B ratio is significantly reduced after 6–7 weeks of DR in the visual cortex (ANOVA, $p < 0.0001$). (J–M) Data expressed as visual cortex protein level normalized to frontal cortex of the same animal reduces SEM, but still shows the same NR2A, NR2B, NR1, and NR2A/2B trends as visual cortex values (ANOVA, $p < 0.04$, $p < 0.02$, $p > 0.7$, $p < 0.0001$, respectively). The * indicates $p < 0.05$ for unpaired t-test performed between LR and DR groups.

NR2A/2B ratio than LR mice, consistent with published changes, while no significant change is observed in the frontal cortex (Fig. 2E, I, ANOVA for frontal cortex, $p > 0.8$; ANOVA for visual cortex, $p < 0.0001$). Analysis of the visual cortex data alone indicates that the decrease in the NR2A/2B ratio is significant at 6–7 weeks of dark-rearing (78.5 ± 5.5% and 75.9 ± 5.1% of LR, t-test, $p < 0.008$ and $p < 0.005$). When the visual cortex data are normalized to the frontal cortex

values, the NR2A/2B ratio is significantly reduced by 4 weeks of dark-rearing, and the change is maintained through 7 weeks (Fig. 2M, ANOVA, $p < 0.0001$; $89.3 \pm 3.1\%$, $84.3 \pm 2.5\%$, $87.2 \pm 3.8\%$, $76.7 \pm 6.6\%$ of LR, *t*-test, $p < 0.05$, $p < 0.0005$, $p < 0.02$, $p < 0.009$ for 4, 5, 6, and 7 weeks, respectively). These results confirm that the NR2A/2B ratio is lowered by dark-rearing in mice and extend previous findings by defining temporally distinct changes in the protein expression of NR2A and NR2B.

3.2. Monocular deprivation modifies surface NMDAR subunit composition in juvenile mouse visual cortex

Visual deprivation by dark-rearing alters NMDAR subunit composition in juvenile mice, with temporally distinct effects on the expression of NR2A and NR2B protein. We wondered if a reduction of visual cortical activity by monocular deprivation (MD) also alters NMDAR subunit composition. MD in juvenile mice induces two distinct electrophysiological responses in visual cortex contralateral to the deprived eye. MD induces a rapid depression of deprived eye responses after 3 days, followed by a delayed potentiation of non-deprived eye responses after 7 days (Frenkel and Bear, 2004). It has been hypothesized that a decrease in the NR2A/B ratio, caused by the reduction in cortical activity over the first 3–5 days of MD, is permissive for the subsequent potentiation of open-eye responses.

To detect small changes in NR2 expression, we compared visual cortex in the hemisphere contralateral to the deprived eye with the same-animal visual cortex from the other hemisphere. This procedure is justified by the finding that overall visual responsiveness is significantly depressed in the hemisphere contralateral to the deprived eye relative to the other hemisphere after brief periods of deprivation (Heynen et al., 2003). Therefore, visual cortex ipsilateral to the deprived eye serves as within-animal control tissue, and paired comparisons can be made between contralateral and ipsilateral hemispheres (Fig. 3A). We chose to examine protein levels of surface-expressed NMDARs since those are the biochemical changes most relevant to the functional changes after MD. C57/BL6 mice were MD'ed at P28, and 300 μm slices from the two hemispheres of visual cortex were prepared 1, 3, 5, and 7 days post-MD. Slices were allowed to recover briefly in ice-cold ACSF, surface receptors were labeled with biotin, and superficial layers (I–IV) of visual cortex were microdissected. The microdissected tissue was homogenized, biotinylated proteins were precipitated using avidin-agarose beads, and surface fractions were analyzed by quantitative immunoblotting for NR2A, NR2B, and NR1 (Fig. 3A).

Analysis of the precipitated surface fractions using NMDAR subunit-specific antibodies indicates that NR2B levels are significantly increased in visual cortex contralateral to the deprived eye at 3 days MD (Fig. 3C, ANOVA, $p < 0.008$; $126.5 \pm 11.3\%$ of IPSI, *t*-test, $p < 0.01$). In addition, NR2A levels are significantly decreased in the contralateral visual cortex at 7 days MD (Fig. 3B, ANOVA, $p < 0.004$; $79.7 \pm 11.8\%$ of IPSI, *t*-test, $p < 0.004$). Surface NR1 levels do not differ significantly between ipsilateral and contralateral visual cortices (Fig. 3D, ANOVA, $p > 0.9$). The resultant NR2A/2B ratio in the contralateral visual cortex is significantly reduced at 5 days and 7 days following MD (Fig. 3E, ANOVA, $p < 0.005$; $90.2 \pm 5.2\%$ and $86.7 \pm 8.0\%$ of IPSI, *t*-test, $p < 0.03$ and $p < 0.05$). These changes in NR2A and NR2B expression are remarkably reminiscent of changes seen following extended DR. Of particular interest is the compressed time scale over which we observe the MD-induced changes (on the order of days) compared to DR-induced changes (on the order of weeks).

To verify that the changes in NMDAR subunits were specific to monocular deprivation, left and right visual cortices were prepared from control, non-MD'ed mice. Analysis of surface fractions reveals no left-right differences in expression of NR2A, NR2B, or NR1 proteins, and correspondingly, there is no change in the NR2A/2B ratio (data not shown, ANOVA, $p > 0.6$, $p > 0.8$, $p > 0.6$, and $p > 0.1$ for NR2A, NR2B, NR1, and NR2A/2B ratio, respectively). These results suggest that lowering cortical activity by monocular deprivation has similar effects on NR2A and NR2B subunit expression as dark-rearing, and these changes are specific to the deprived visual cortex. However, the timecourse over which these changes occur is substantially shorter. The significant reduction of the NR2A/B ratio occurs at a time post-MD when non-deprived eye response potentiation is initiated (5–7 days post-MD).

3.3. Brief dark exposure modifies NMDAR subunit composition in synaptoneurosomes from rat visual cortex by increasing NR2B

Previous reports of dark-rearing (DR) rats from birth, or dark exposing (DE) juvenile rats beginning at P21–28 for ~5 days, describe reductions in the NR2A/2B ratio that are attributable entirely to decreases in the NR2A subunit (Quinlan et al., 1999a,b; Philpot et al., 2001). Our results in *mice* suggest that in addition to reductions in NR2A protein expression, there is a substantial increase in NR2B protein expression in homogenate and surface fractions following visual deprivation. To see if NR2B protein levels are similarly modified in juvenile *rats* by very brief dark exposure, animals aged P23 were transferred to a photon-free room for dark exposure for 3 days, and synaptoneurosome fractions were prepared from visual cortices for analysis of NMDAR subunit expression by quantitative immunoblotting (Fig. 4A). Brief, 3 days dark exposure of P23 rats (DE) results in a significant increase in NR2B and NR1 expression in synaptoneurosomes (Fig. 4C, D, $125.7 \pm 4.5\%$ and $117.9 \pm 5.7\%$ of LR, *t*-test, $p < 0.005$ and $p < 0.03$ for NR2B and NR1, respectively). NR2A protein levels do not differ significantly between LR and DE groups (Fig. 4B, $104.4 \pm 3.7\%$ of LR, *t*-test, $p > 0.4$), but the NR2A/2B ratio is significantly reduced by DE (Fig. 4E, $80.9 \pm 3.2\%$ of LR, *t*-test, $p < 0.002$). These data indicate that a deprivation-induced reduction of the NR2A/2B ratio can be achieved by increasing NR2B expression in juvenile rats, as we have shown in juvenile mice and

as was recently reported in adult rats and mice (Yashiro et al., 2005; He et al., 2006).

3.4. Reducing glutamate receptor activation of the NMDAR subtype in dissociated cortical cultures mimics the effects of visual deprivation

The studies of visual cortex *in vivo* indicate that the earliest response to visual deprivation, in both rats and mice, is an increase in NR2B protein expression that is manifest as a decreased NR2A/B ratio of surface expressed NMDARs. The notion that this change is caused by a reduction in overall cortical activity is supported by findings that chronic activity blockade by infusion of TTX into the hippocampus *in vivo* also induces a post-synaptic change in NMDA receptors to an NR2B-predominant subunit composition (Galvan et al., 2003). These experiments imply, importantly, that the effects of decreasing visual cortical activity by visual deprivation can be mimicked by reducing activity at the synaptic receptor level. Remarkably, activity-dependent changes in NMDA receptor expression and subunit composition can be modeled in an even more reduced system: dissociated neuron cultures. Chronic blockade of activity by TTX or APV upregulates synaptic NMDA receptors in cortical and hippocampal dissociated cultures (Rao and Craig, 1997; Rutherford et al., 1998; Turrigiano et al., 1998; Watt et al., 2000), and in some cases, with differential effects on NR2A and NR2B expression (Follesa and Ticku, 1996; Ehlers, 2003). Activity-dependent changes in NR2A and NR2B expression can therefore be studied *in vitro*, and the nature of this reduced preparation facilitates detailed study of mechanisms regulating these changes.

To see if we could model deprivation-induced NMDA receptor subunit changes in a dissociated neuron culture system, high density, mature cultures from occipital cortex (22–30

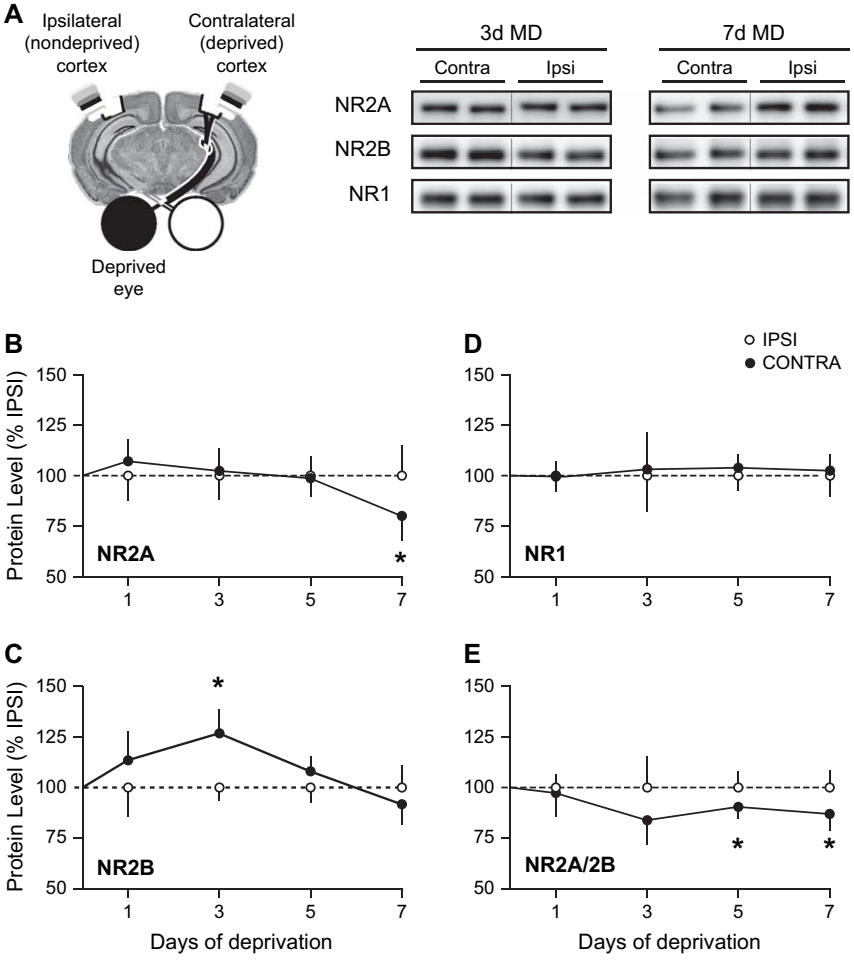

Fig. 3. Monocular deprivation (MD) modifies surface expressed NMDAR subunits in mouse visual cortex. (A) Schematic of mouse visual cortex hemispheres ipsilateral and contralateral to the deprived eye and representative immunoblots of surface fractions prepared from visual cortex of MD'ed mice. (B–E) NMDAR subunit expression was measured by quantitative immnoblotting of surface fractions prepared by biotinylating visual cortical slices ipsilateral and contralateral to the deprived eye (white and black symbols, respectively, $n = 11–12$ for each data point, with mean values ± SEM normalized to the average IPSI protein level). Blots were probed with subunit-specific antibodies for NR2A, NR2B, and NR1. (B) NR2A protein in visual cortex contralateral to the deprived eye is significantly reduced after 7 days of MD (ANOVA, $p < 0.008$). (B) NR2B protein in visual cortex contralateral to the deprived eye is significantly elevated after 3 days of MD (ANOVA, $p < 0.004$). (C) Surface NR1 levels do not differ significantly between IPSI and CONTRA hemispheres (ANOVA, $p > 0.9$). (D) The NR2A/2B ratio is reduced in the visual cortex contralateral to the deprived eye at 5 and 7 days after MD (ANOVA, $p < 0.005$). The * indicates $p < 0.05$ for paired *t*-test performed between IPSI and CONTRA hemispheres.

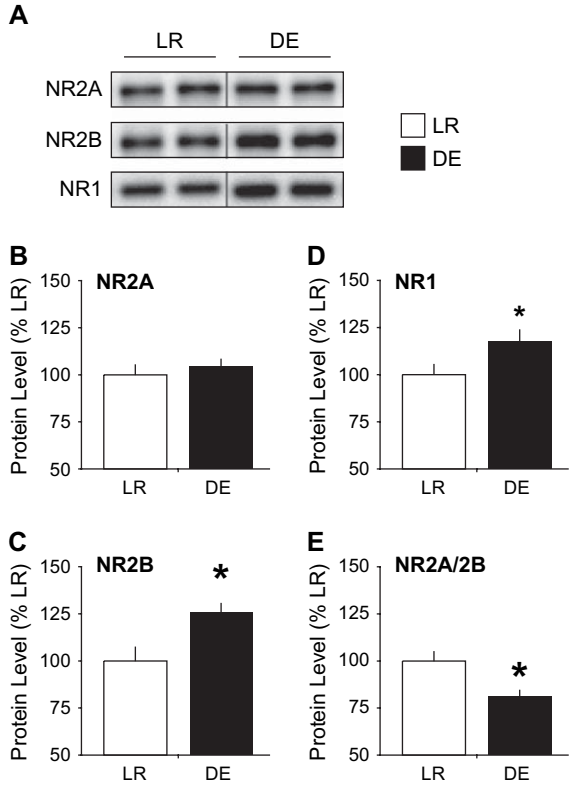

Fig. 4. Brief dark exposure modifies NMDAR subunit composition in synaptoneurosomes from rat visual cortex. (A) Representative immunoblots of synaptoneurosome samples prepared from rats that were light-reared (LR) until P26 or dark-exposed (DE) from P23–26. Blots were probed with subunit-specific antibodies for NR2A, NR2B, and NR1. (B–E) Summary of immunoblot analysis of synaptoneurosome samples from LR and DE rats (white and black bars, respectively, $n = 22$ measurements from 11 animals for each group, with mean values ± SEM normalized to the average LR protein level). (A) NR2A protein levels are not modified by brief dark exposure (t-test, $p > 0.4$). (B) NR2B protein levels are significantly elevated by 3 days DE (t-test, $p < 0.005$). (C) NR1 protein levels are also elevated (t-test, $p < 0.03$). (D) The NR2A/2B ratio is reduced by brief dark exposure (t-test, $p < 0.002$).

div) were treated for 1 day with TTX (1 μM) to reduce activity. Consistent with previous reports, we observe an increase in NR2B expression in the homogenates prepared from TTX-treated cultures compared to control, untreated cultures (Fig. 5A, $164.0 \pm 8.5\%$ of CTRL, $p < 0.003$). However, NR2A and NR1 levels are not significantly altered by TTX treatment ($95.4 \pm 6.9\%$ and $79.3 \pm 7.8\%$ of CTRL, $p > 0.5$ and $p > 0.06$). These changes in NMDAR subunit expression are similar to those seen following brief visual deprivation periods.

Since TTX eliminates synaptically evoked glutamate release, we wondered if its effects were mediated by decreased activation of a specific subtype of glutamate receptor. To see if AMPAR or NMDARs, specifically, were involved, we treated cultures with various antagonists and measured NMDAR subunit expression in homogenates. We find that inhibition of NMDA receptors, but not AMPA receptors, induces a change in NMDAR subunit composition similar to TTX treatment (Fig. 5B). Total NR2B protein levels increase dramatically following treatment with the competitive NMDAR antagonist d-APV (50 μM, $240.8 \pm 19.3\%$ of CTRL, $p < 0.0001$), but not the AMPAR antagonist CNQX (20 μM, $105.2 \pm 7.7\%$ of CTRL, $p > 0.7$). In addition, blockade of NR2B-containing receptors with ifenprodil is sufficient to induce NR2B upregulation (3 μM, $235.0 \pm 3.6\%$ of CTRL, $p < 0.0001$). Blockade of synaptically activated NMDARs with the open-channel antagonist MK801 is likewise sufficient to increase NR2B levels (15 μM, $270.7 \pm 23.0\%$ of CTRL, $p < 0.0001$). NR2A and NR1 expression are not significantly altered by any of the above treatments (ANOVA, $p > 0.7$ and $p > 0.1$). Together, these data suggest that tonic activation of ifenprodil-sensitive (NR2B-containing) NMDARs normally represses expression of NR2B protein, and this repression is relieved by selective NMDAR blockade.

To see if the effects of NMDAR antagonists on NMDAR subunit composition are reflected at the synapse, we prepared purified postsynaptic density (PSD) fractions from CTRL and treated cultures. First, to verify that our preparation was enriching for postsynaptic proteins, we examined protein levels of NR2A, NR2B, NR1, PSD95, and Transferrin receptor (TfnR) in fractions of homogenate versus progressively purified PSD fractions (Fig. 5C). The preparation is effective in enriching for postsynaptic proteins NR2A, NR2B, NR1, and PSD95, while excluding the non-synaptic protein TfnR. Examination of the most highly enriched PSD-TS fraction reveals that NR2B upregulation induced by NMDAR blockade with d-APV, ifenprodil, or MK801 is reflected at the synaptic level (Fig. 5D, $264.9 \pm 55.9\%$, $286.4 \pm 51.6\%$, and $312.1 \pm 28.4\%$ of CTRL, $p < 0.0001$ for all). In contrast, CNQX has no effect on NR2B subunit expression ($113.9 \pm 21.3\%$ of CTRL, $p > 0.7$). Again, no significant changes are detected for NR2A expression with NMDAR or AMPAR blockade (ANOVA, $p > 0.06$), although NR1 is slightly elevated following d-APV treatment ($157.2 \pm 17.4\%$ of CTRL, $p < 0.0005$). These results suggest that activity blockade *in vitro* mimics the effect of visual deprivation on the expression of NR2B protein at the homogenate and synaptic levels. In addition, the results expand on previous reports of inactivity-driven increase in NR2B by specifically implicating the inhibition of NMDARs, but not AMPARs in regulation of NR2B. Moreover, these results suggest that reduced ligand binding, reduced ion influx through NMDARs, or reduced activation of NR2B-containing receptors can all serve as the initial sensor for signaling NR2B upregulation.

3.5. NR2B expression is bidirectionally modified by NMDAR deactivation and activation in cortical cultures

An important feature of NMDARs *in vivo* is that their subunit composition can be bidirectionally modified by visual deprivation and experience (Quinlan et al., 1999a). To see if NR2B expression can be bidirectionally modified by changing NMDAR activity *in vitro*, we blocked NMDAR activation with APV for 1 day, then washed the drug out and 2 h later, measured NMDAR subunit expression in homogenates by quantitative immunoblotting with NR2A, NR2B, and NR1 subunit-specific antibodies (Fig. 6A). The analysis shows that APV induces a robust

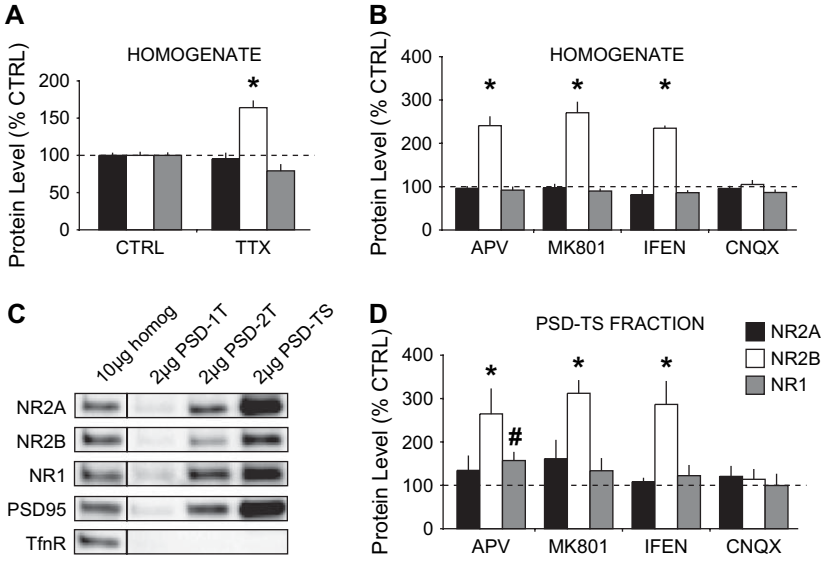

Fig. 5. Reducing glutamate receptor activation of the NMDAR subtype in dissociated cortical cultures mimics the effects of visual deprivation. NMDAR subunit expression was analyzed by quantitative immunoblotting for NR2A, NR2B, and NR1 (black, white, and gray symbols, respectively). Data expressed is the mean value ± SEM normalized to the average CTRL protein level. (A) TTX treatment (1 μM) of cultures for 1 day significantly increases NR2B protein levels in the homogenate ($n = 3$; $^*p < 0.003$ versus CTRL). No significant change in NR2A or NR1 expression is detected following TTX treatment ($p > 0.5$ and $p > 0.06$). (B) One day blockade of NMDARs with APV (50 μM), MK801 (15 μM), or ifenprodil (3 μM) induces a robust upregulation of NR2B protein in the homogenate fraction ($n = 3$–6, $^*p < 0.0001$ versus CTRL and CNQX). AMPAR blockade for 1 day with CNQX (20 μM) does not change NR2B expression compared to CTRL conditions ($p > 0.7$). No significant changes in NR2A or NR1 are detected following treatment with either NMDAR or AMPAR antagonists. (C) Representative immunoblots showing progressive enrichment of postsynaptic proteins NR2A, NR2B, NR1, and PSD95 in 2 μg of PSD fractions versus 10 μg of homogenate. Transferrin receptor (TfnR) is excluded entirely from PSD fractions. (D) Increases in NR2B protein induced by NMDAR blockade are reflected in the PSD-TS fraction ($n = 3$–6, $^*p < 0.0001$ versus CTRL and CNQX). NR2A levels are not significantly different with any drug treatment, but NR1 is slightly increased following APV treatment ($n = 6$, $\#p < 0.01$ versus CTRL). CNQX does not alter PSD levels of NR2A, NR2B, or NR1.

upregulation of NR2B protein (Fig. 6B, 193.4 ± 15.7% of CTRL, $p < 0.0003$) that is quickly reversed by washout of the drug (66.4 ± 4.0% of CTRL, $p < 0.03$ versus CTRL and APV). NR2A protein levels also decrease following APV washout (69.0 ± 4.6% of CTRL, $p < 0.002$ versus CTRL and APV), as do NR1 protein levels (89.8 ± 3.7% of CTRL, $p < 0.01$ versus APV). However, note that the initial APV treatment does not significantly alter NR2A and NR1 protein levels (110.2 ± 2.1% and 107.6 ± 1.8% of CTRL, $p > 0.1$ versus CTRL), and that the normal NR2A/B ratio is quickly restored after washout despite the decrease in NR2A. These data show that NR2B expression can be bidirectionally modified by NMDAR blockade and activation.

3.6. Increases in NR2B protein induced by NMDAR blockade are reflected in the surface fraction of cultured cortical neurons

Activity has been shown to differentially affect NR2A versus NR2B trafficking in hippocampal slice cultures. Blockade of glutamate or glycine ligand binding to NMDARs with APV or diclorokynurenic acid prevents the insertion of overexpressed recombinant NR2A, but not NR2B subunits at the synapse (Barria and Malinow, 2002). One interpretation of these data is that if NR2B subunits are available, they are automatically delivered to synapses, regardless of activity levels. However, it is not known whether surface trafficking (e.g., reduced NMDAR internalization) as well as overall expression is also affected by inactivity. If trafficking was affected, we would expect to see a disproportionally larger increase of cell surface versus total NR2B levels in APV versus CTRL cultures. To test this idea, we isolated surface expressed proteins by biotinylation. Control, untreated cultures and cultures treated with 50 μM d-APV for 1 day were incubated for 30 min with Sulfo-NHS-SS-Biotin in ice-cold artificial cerebrospinal fluid (ACSF) to label surface proteins. After rinsing off excess unconjugated biotin, cells were harvested, biotinylated proteins were precipitated using an avidin-agarose conjugate, separated by electrophoresis, and analyzed by quantitative immunoblotting (Fig. 7A). Quantification of immunoblots shows a modest increase in the surface:total ratio of the NR2B subunit in APV-treated cultures that nears significance (Fig. 7B, 130.4 ± 4.8% of CTRL, $p = 0.09$), and no change in NR2A or NR1 (96.8 ± 8.5% and 90.8 ± 9.5% of CTRL, $p > 0.2$ and $p > 0.4$). Examination of the surface fraction alone, however, shows a great increase in NR2B levels (Fig. 7C, 306.9 ± 46.9% of CTRL, $p < 0.003$), as does the total pool (Fig. 7D, 234.0 ± 43.8% of CTRL, $p < 0.0002$). Again, no significant changes in NR2A or NR1 expression are seen in the surface or total fractions (Fig. 7C, D, surface NR2A: 82.7 ± 15.1% of CTRL, $p > 0.2$; total NR2A: 86.1 ± 6.9% of CTRL, $p > 0.1$; surface NR1: 95.2 ± 4.1% of CTRL, $p > 0.5$; total NR1: 104.3 ± 9.2% of CTRL, $p > 0.7$). These results suggest that APV-induced increases in NR2B expression are reflected at the cell surface, in agreement with previous findings that NR2B trafficking to synaptic membranes

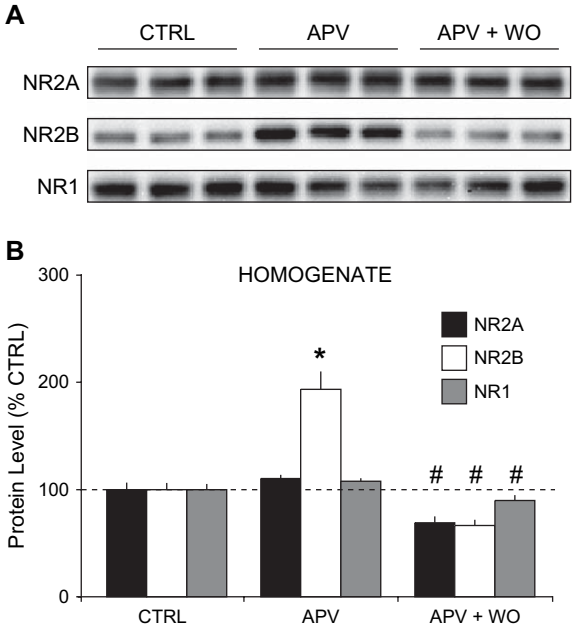

Fig. 6. NR2B expression is bidirectionally modified by NMDAR deactivation and activation in cortical cultures. NMDAR subunit expression in the homogenate fraction was analyzed by quantitative immunoblotting for NR2A, NR2B, and NR1 (black, white, and gray symbols, respectively). Data expressed is the mean value ± SEM normalized to the average CTRL protein level ($n = 3$–4). (A) Representative immunoblots of homogenate samples collected from control, untreated cortical cultures (CTRL), cultures treated with APV for 1 day (APV, 50 μM), or cultures after 2 h of washout of APV (APV + WO). (B) Summary of immunoblot analysis, indicating that APV induces a robust upregulation of NR2B protein (*$p < 0.001$ versus CTRL) that is reversed by washout of the drug (#$p < 0.0001$ versus APV). NR2A protein levels also decrease following washout of APV (#$p < 0.001$ versus APV), as do NR1 protein levels (#$p < 0.01$ versus APV), but they are not significantly altered by the initial APV treatment.

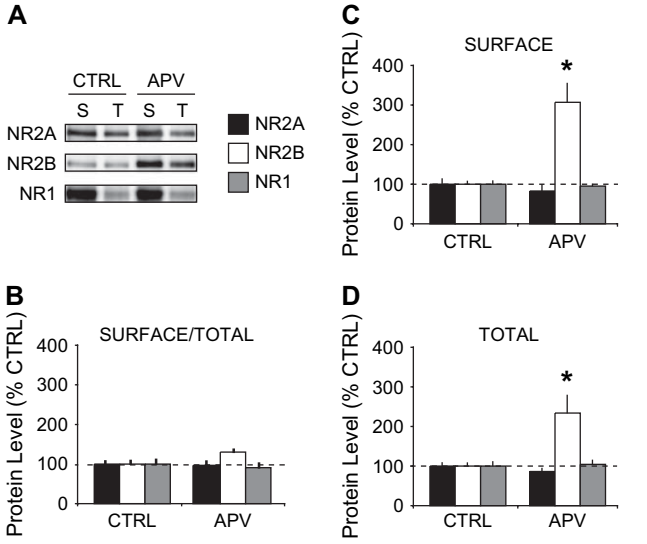

Fig. 7. Increases in NR2B protein induced by NMDAR blockade are reflected in the surface fraction of cultured cortical neurons. Surface fractions from cortical cultures were prepared by avidin precipitation of biotinylated surface-expressed proteins, and NMDAR subunit expression was subsequently measured by quantitative immnoblotting using subunit-specific antibodies for NR2A, NR2B, and NR1 (black, white, and gray symbols, respectively). Data expressed is the mean value ± SEM normalized to the average CTRL protein level ($n = 3$). (A) Representative immunoblots of homogenate samples collected from control, untreated cortical cultures (CTRL) and cultures treated with APV for 1 day (APV, 50 μM). (B) Analysis of the ratio of surface to total expressed protein level in APV-treated cultures shows a slight elevation of NR2B that nears significance ($p = 0.09$) and no change in NR2A or NR1 ($p > 0.2$ and $p > 0.4$). (C) Analysis of the surface fraction, alone, reveals a robust increase in NR2B in APV-treated cultures (*$p < 0.003$), but not NR2A or NR1 ($p > 0.2$ and $p > 0.5$). (D) Analysis of the total fraction, alone, likewise shows an increase in NR2B in APV-treated cultures, but of slightly smaller magnitude than the surface fraction (*$p < 0.0001$). No difference in total NR2A or NR1 expression is detected between CTRL and APV conditions ($p > 0.1$ and $p > 0.7$).

is independent of activity. However, the proportion of the expressed NR2B that is retained at the cell surface compared to total levels following APV treatment is only marginally larger. Therefore, retention of NR2B subunits likely plays only a minor role in increasing surface NR2B under conditions of NMDAR inactivity.

3.7. A greater contribution of translation than transcription to regulation of NR2B expression

Our results and others (Barria and Malinow, 2002) suggest that trafficking of NR2B is not highly regulated by activity. Rather, the surface and synaptic levels of NR2B protein are governed largely by the total amounts available. Total NR2B protein levels are conceivably regulated by two opposing processes: synthesis and degradation of the protein. To see if the APV-induced increase in NR2B is protein synthesis-dependent, cultures were treated simultaneously with 50 μM d-APV and 5 μM cycloheximide (CHX) or 1 μM anisomycin (ANISO), two mechanistically different translation inhibitors.

Interestingly, we found that inhibition of mRNA translation, by itself, causes a slight but significant increase in NR2B relative to untreated controls (163.0 ± 8.6% and 140.4 ± 15.3% of CTRL, $p < 0.01$ versus CTRL). However, no further increase was observed in the cultures treated with APV and the protein synthesis inhibitors (Fig. 8A, B from 278.1 ± 21.1% in APV alone to 153.6 ± 9.3% for APV + CHX, to 135.7 ± 4.6% for APV + ANISO, $p < 0.0001$ for APV + CHX and APV + ANISO versus CTRL and APV). Thus, translation inhibitors completely abrogate the effect of APV on NR2B levels.

Homogenate NR2A protein levels are significantly reduced in all treatment conditions compared to CTRL, except for APV + ANISO (84.0 ± 7.1%, 68.4 ± 4.4%, 90.2 ± 7.1% 57.9 ± 5.5%, 65.7 ± 8.9% of CTRL for APV, APV +vCHX, APV + ANISO, CHX, and ANISO, respectively, $p > 0.1$ for APV + ANISO versus CTRL, $p < 0.03$ for all others versus CTRL). Homogenate NR1 protein levels are also significantly reduced in all treatment conditions compared to CTRL, except for APV (90.3 ± 8.3%, 79.4 ± 8.5%, 70.5 ± 4.1%, 77.6 ± 2.9%, and 82.3 ± 7.0% of CTRL for APV, APV + CHX, APV + ANISO, CHX, and ANISO, respectively, $p > 0.1$ for APV versus CTRL, $p < 0.03$ for all others versus CTRL).

If synaptic levels of NR2B simply depend on the amount of available NR2B, then reducing levels of NR2B by blocking

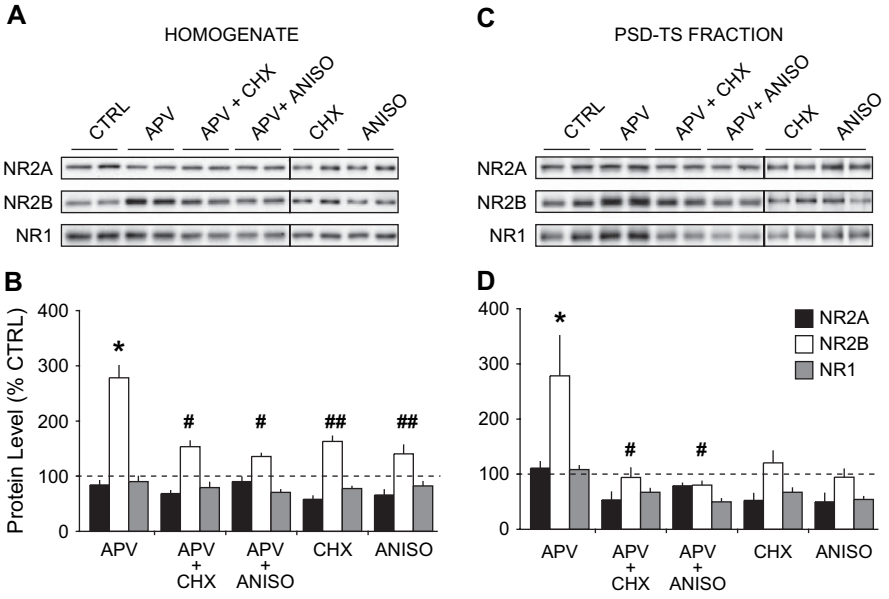

Fig. 8. Protein synthesis inhibitors block APV-induced increases in NR2B. NMDAR subunit expression in the homogenate or PSD-TS fraction was analyzed by quantitative immunoblotting for NR2A, NR2B, and NR1 (black, white, and gray symbols, respectively). Data expressed is the mean value ± SEM normalized to the average CTRL protein level ($n = 5$–7). p values reflect post-hoc comparisons by Fisher's PLSD after significance was found by ANOVA analysis across all treatment groups in both fractions ($p < 0.003$ for NR2A, NR2B, and NR1). Symbols denoting significant changes in NR2A and NR1 have been omitted for clarity. (A) Representative immunoblots of homogenate samples collected from control, untreated cortical cultures (CTRL), cultures treated with APV for 1 day (APV, 50 μM), cultures co-treated with APV and cycloheximide (CHX, 5 μM) or anisomycin (ANISO, 1 μM), or cycloheximide or anisomycin alone. (B) Summary of immunoblot analysis of homogenates, indicating that APV induces a robust upregulation of NR2B protein (*$p < 0.0001$ versus CTRL) that is partially blocked by CHX and ANISO (#$p < 0.0001$ for APV + CHX and APV + ANISO versus CTRL and APV). CHX and ANISO alone also slightly elevate NR2B levels (##$p < 0.01$ for CHX and ANISO versus CTRL and APV). NR2A is reduced in all groups except APV + ANISO compared to CTRL ($p > 0.1$ for APV + ANISO, $p < 0.03$ for all others). NR1 is reduced in all groups except APV compared to CTRL ($p > 0.1$ for APV, $p < 0.03$ for all others). (C) Representative immunoblots of the PSD-TS fraction collected from cultures exposed to various treatment conditions. (D) Summary of immunoblot analysis of PSD-TS fractions, indicating that APV induces a robust upregulation of NR2B protein (*$p < 0.0001$ versus CTRL) that is entirely blocked by CHX and ANISO (#$p < 0.001$ for APV + CHX and APV + ANISO versus APV). CHX and ANISO alone do not significantly alter NR2B levels ($p > 0.6$ versus CTRL). NR2A is significantly reduced in all groups except APV and APV + ANISO compared to CTRL ($p > 0.1$ for APV, APV + ANISO, $p < 0.003$ for all others). NR1 is significantly reduced in all groups except APV compared to CTRL ($p > 0.3$ for APV, $p < 0.002$ for all others).

protein synthesis should likewise reduce synaptic levels of NR2B. To see if this is true, we examined NR2A, NR2B, and NR1 subunit expression in the PSD-TS fraction after treatments with protein synthesis inhibitors. Quantitative analysis shows that APV induces a robust upregulation of NR2B at the synapse, and CHX or ANISO completely block the increase (Fig. 8C, D, from 278.3 ± 72.0% to 94.2 ± 16.1% for APV + CHX, to 80.3 ± 5.9% for APV + ANISO, $p < 0.001$ for APV + CHX and APV + ANISO versus APV). CHX and ANISO alone do not significantly change NR2B levels compared to CTRL in this biochemical fraction (CHX: 120.3 ± 20.9% of CTRL, $p > 0.6$; ANISO: 94.5 ± 13.9% of CTRL, $p > 0.8$). NR2A is significantly reduced in all groups except APV and APV + ANISO compared to CTRL (111.1 ± 10.7%, 53.3 ± 13.3%, 79.0 ± 3.9%, 52.4 ± 11.6%, 49.7 ± 14.8% of CTRL for APV, APV + CHX, APV + ANISO, CHX, and ANISO, respectively, $p > 0.4$ for APV versus CTRL, $p > 0.1$ for APV + ANISO versus CTRL, $p < 0.003$ for all other comparisons versus CTRL). NR1 is also significantly reduced in all groups except APV compared to CTRL (108.2 ± 6.4%, 67.3 ± 5.8%, 50.0 ± 4.7%, 67.3 ± 6.8%, 54.1 ± 4.4% of CTRL for APV, APV + CHX, APV + ANISO, CHX, and ANISO, respectively, $p > 0.3$ for NR1 in APV versus CTRL, $p < 0.002$ for all other comparisons versus CTRL).

The observation that blockade of protein synthesis for 24 h depletes protein levels of NR2A and NR1 in both homogenate and PSD fractions suggests that these proteins are constitutively synthesized at a higher rate than NR2B. This difference could account for the seemingly paradoxical increase in NR2B following treatment with CHX and ANISO. If baseline levels of other proteins (such as NR2A and NR1), but not NR2B are dependent on protein synthesis, the molar ratio of other proteins will decrease, and the molar ratio of NR2B will increase in CHX or ANISO conditions relative to CTRL conditions. Another possibility is that low concentrations of protein synthesis inhibitors may paradoxically increase synthesis of some proteins (Scheetz et al., 2000). Or, it is also possible that the effects of the protein synthesis inhibitors indirectly affected other cellular processes, such as degradation pathways, thereby preventing the breakdown of NR2B. This seems unlikely, however, since there was no additive effect of APV and CHX or ANISO treatment on NR2B levels. In any case, changes in NMDA receptor subunit expression are relayed to the PSD fraction, suggesting that the availability of NMDAR subunits, in general, determines their expression at synaptic sites.

In addition to translation, another point of regulation in the biosynthesis of NR2B may occur at the transcriptional level. To see if the APV-induced increase in NR2B was dependent on transcription, cultures were co-incubated with d-APV and 1 μg/mL actinomycin D (ActD), a transcription inhibitor. Quantification of NMDAR subunit expression by immunoblotting shows that APV induces a robust increase in homogenate NR2B levels (Fig. 9A, B, 313.8 ± 32.5% of CTRL, $p < 0.0001$) that is modestly, but significantly blocked by ActD (to 226.7 ± 20.8% of CTRL, $p < 0.02$ versus CTRL and APV). Blockade of transcription also attenuates NR2A and NR1 levels (77.2 ± 5.5% and 83.3 ± 4.1% of CTRL, $p < 0.02$ and $p < 0.001$ versus CTRL), suggesting that transcription may contribute to steady state levels of these proteins. Unlike the robust, near complete blockade of APV-induced NR2B expression by translation inhibitors, the transcription inhibitor ActD has only a modest effect. These results suggest that new transcripts are not essential for the APV-induced upregulation of NR2B, but transcription must occur at a baseline rate to maintain expression of all NMDAR subunits.

4. Discussion

Changes in NMDAR subunit composition alter the properties of visual cortical plasticity and are a putative molecular mechanism for metaplasticity *in vivo* (Kirkwood et al., 1996; Philpot et al., 2003). Previous reports of the effect of dark-rearing rats from birth until P21-28, or of dark-exposing juvenile P21–28 rats for 3–5 days, suggested that the deprivation-induced reduction in the NR2A/2B ratio and the corresponding lengthening of NMDAR-mediated currents are due to a loss of synaptic NR2A-containing receptors (Quinlan et al., 1999a,b; Philpot et al., 2001). However, our re-examination of the effects of brief dark-exposure in rats (3 days, Fig. 4), as well as our examination of the effects of deprivation in mice, revealed a rapid increase in NR2B protein expression without a concurrent change in NR2A. The discrepancy with previous studies is likely due to slight differences in the length of deprivation since we found in mice that longer periods of deprivation invariably also cause a reduction in NR2A expression. In any case, the current data, taken together, clearly show that an early consequence of visual deprivation—by dark rearing, dark exposure, or monocular deprivation—is an increase in expression of the NR2B subunit in the visual cortex of both rats and mice.

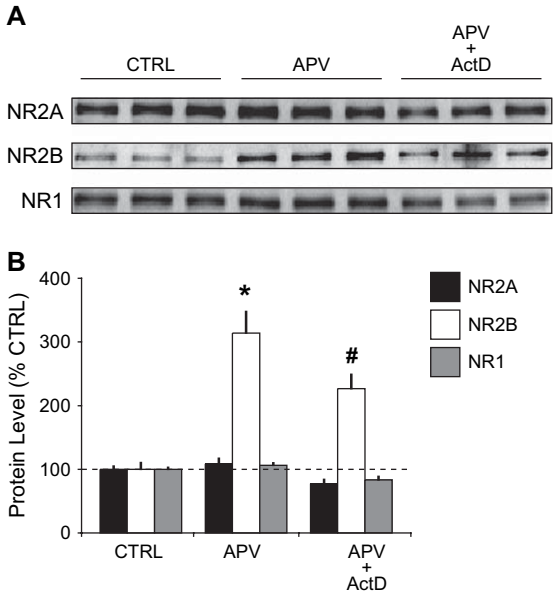

Fig. 9. The transcription inhibitor actinomycin D partially blocks APV-induced increases in NR2B. NMDAR subunit expression in the homogenate fraction was analyzed by quantitative immunoblotting for NR2A, NR2B, and NR1 (black, white, and gray symbols, respectively). Data expressed is the mean value ± SEM normalized to the average CTRL protein level ($n = 6$). (A) Representative immunoblots of homogenate samples collected from control, untreated cortical cultures (CTRL), cultures treated with APV for 1 day (APV, 50 μM), or cultures co-treated with APV and actinomycin D (ActD, 1 μg/mL). (B) Summary of immunoblot analysis, indicating that APV induces a robust upregulation of NR2B protein (*$p < 0.0001$ versus CTRL) that is partially blocked by ActD (#$p < 0.02$ versus CTRL and APV). ActD treatment also reduces NR2A and NR1 protein levels ($p < 0.02$ versus CTRL and APV, symbols indicating statistical significance are omitted for clarity).

Both dark exposure and monocular deprivation of juvenile animals caused a rapid increase in NR2B after 3 days of deprivation, followed at later time points by a decrease in NR2A. These substantially different types of sensory deprivation have in common the fact that they reduce activity in visual cortex. We therefore suggest that decreased cortical activation is the trigger for the changes in NMDAR subunit composition. It is interesting that the same bidirectional changes in NR2B and NR2A occurred after dark rearing mice from birth, but on a much slower time scale. This difference is likely explained by the fact that dark rearing, unlike the other forms of deprivation, greatly slows visual cortical development.

MD-induced changes in NR2B and NR2A expression map closely onto timepoints when physiological changes are known to occur. Although these changes are purely correlative, we speculate that they contribute to the features of OD plasticity. We suggest that modification of NMDAR function via alteration of subunit composition promotes experience-dependent increases in visual responsiveness to the non-deprived eye by altering the properties of NMDAR-dependent LTP and LTD (Philpot et al., 2003). However, the relative contribution of NR2A versus NR2B to LTP or LTD is still a hotly debated issue (see Morishita et al., 2007). Our finding that NR2B and NR2A changes are temporally separated, but correlated with naturally occurring depression and potentiation *in vivo* gives us an opportunity to probe the relative importance of NR2 subunits to the direction of plasticity. Overexpression or knockdown of NR2A or NR2B subunits during the MD period may help establish a causal relationship between NMDAR subunit changes, LTP and LTD, and metaplasticity.

Our work here also begins to address the question of how changes in synaptic activity regulate NMDAR subunit composition. Reduction of glutamate receptor activation by TTX treatment in cortical cultures successfully models the visual deprivation-induced changes in NMDAR subunit composition. Specifically, we find that inhibition of glutamate receptors of the NMDAR, but not AMPAR subtype, is

responsible for NR2B upregulation. Like experience-dependent changes in NMDAR subunit composition, deactivation and reactivation of NMDARs bidirectionally modifies NMDAR subunit composition *in vitro*. Interestingly, the effects of NMDAR reactivation on reversing NR2B expression are rapid and complete by 2 h following washout of APV. The half-life of NR2B is reportedly ~20 h, but shortens with increasing levels of activity in culture which is attributable to enhanced turnover of the protein (Ehlers, 2003). In addition, endocytosis of NMDARs is regulated by activity via membrane-proximal internalization motifs on NMDAR subunits. NR2A and NR2B internalization mediated by membrane-proximal signals in the C′-terminal domain near the fourth transmembrane segment are sufficient to target NR2A and NR2B to late endosomes (Scott et al., 2004) and for NR2A, involves binding to μ2 of the AP2 complex (Vissel et al., 2001). It has been proposed that together with a similar motif on the NR1 subunit, these membrane proximal signals form an ''endocytic ring'' around the intracellular channel opening, which may sense a conformational change in the receptor upon ligand binding and initiate agonist-induced endocytosis (Scott et al., 2004). Endocytosis of NR2A and NR2B can also be mediated by distal C′-terminal motifs, and differentially traffic NR2A to late endosomes and NR2B to recycling endosomes, but the influence of activity on endocytosis via these distal internalization motifs has not yet been tested (Roche et al., 2001; Lavezzari et al., 2003; Lavezzari et al., 2004; Prybylowski et al., 2005). Taken together, it is possible that both endocytosis and subsequent degradation of NR2B is accelerated by NMDAR reactivation, contributing to the rapid downregulation of NR2B following APV washout. These processes may also account for the reduction of NR2A and NR1 seen in the washout condition.

Changes in total levels of NR2B expression following APV treatment are directly reflected at the cell surface and at synaptic sites. Since NR2B levels are elevated, but not disproportionally, in the surface fraction compared to total, it appears that activity-regulated trafficking of NR2B plays only a minor role. In addition, pharmacologic blockade of transcription or translation in this system implicates translation as a major regulator of inactivity-driven NR2B upregulation. Since the effects of translation inhibitors on suppression of APV-induced NR2B expression could be indirect, metabolic labeling will be needed to confirm that NMDAR blockade does, in fact, stimulate synthesis of new NR2B. Although we do not observe a large effect of the transcriptional inhibitor ActD on APV-induced upregulation of NR2B, NR2B message levels may still be regulated by activity. For example, reduction of NR2B mRNA degradation may facilitate enhanced production of the protein, but concomitant APV and ActD treatment would not distinguish this possibility. Direct measurement of RNA levels under conditions of NMDAR blockade may help to answer this question.

Translation may also be important for visual experience-dependent and NMDAR activation-dependent expression of synaptic NR2A (Quinlan et al., 1999a,b). It is somewhat surprising that translation seems to play a role either when NMDARs are active or inactive. However, there is some evidence that NMDARs can mediate both the activation and suppression of translation. For example, brief NMDAR activation has been shown to generally inhibit translation via phosphorylation of eukaryotic elongation factor 2 (eEF2), while paradoxically triggering the translation of a subset of plasticity-related proteins, including CamKII (Marin et al., 1997; Wu et al., 1998; Scheetz et al., 2000; Wells et al., 2001; Sutton et al., 2004). To date, very little is known about translational regulation of the NR2A and NR2B subunits themselves, although the highly conserved nature and complexity of their 5′-UTRs suggest that regulation at the protein synthesis level may occur through secondary structure of the mRNA (Wood et al., 1996; VanDongen and VanDongen, 2004). There is precedent for this type of regulation with AMPAR subunits, where the 5′-UTR of GluR2 contains a 34–42 nucleotide imperfect GU repeat that forms secondary structure *in vivo* that suppresses translation (Myers et al., 2004). Another interesting question is whether translation of NMDAR subunits, like AMPAR subunits, can occur locally in dendrites in response to changes in activity (Kacharmina et al., 2000; Ju et al., 2004). The rapid, protein synthesis-dependent appearance of NR2A following light exposure, in particular, makes this an attractive hypothesis. In addition, our current studies suggest that synaptically expressed NR2B subunits are also sensitive to translation inhibition. It is plausible that NMDARs act as their own local sensors for decreased activity, and in response, stimulate local translation of NR2B subunits that can be inserted directly into nearby synaptic membranes. NR2A, NR2B, and NR1 mRNAs have been identified in dendritic fractions from cultured hippocampal neurons, but further study is required to see if cortical dendrites can support local translation of these transcripts in a NMDAR- and activity-dependent manner (Miyashiro et al., 1994; Gazzaley et al., 1997; Eberwine et al., 2002).

Although we have only begun to examine the role of biosynthetic processes in regulation of NR2B expression in this study, it should be noted that a substantial amount of work has already been done on post-translational regulation of the different NR2 subunits. Using electrophysiologically and optically tagged recombinant NR1/2A and NR1/2B receptors, Barria and Malinow (2002) demonstrate that activity promotes removal of existing NR2B and insertion of new NR2A, while inactivity promotes stabilization of NR2B and inhibition of NR2A insertion at synapses. In addition, NR2A and NR2B endocytosis is mediated by clathrin-dependent mechanisms, but different motifs in the membrane-proximal and distal C′-terminal regions of NR2A and NR2B specify whether the receptors are trafficked into late or recycling endosomes (Roche et al., 2001; Vissel et al., 2001; Lavezzari et al., 2003, 2004; Scott et al., 2004; Prybylowski et al., 2005). Association with the postsynaptic scaffolding molecule PSD95 stabilizes NR2B at synapses by preventing its endocytosis (Roche et al., 2001; Chung et al., 2004; Prybylowski et al., 2005). One could imagine that shifting preference from trafficking NR2B from late to recycling endosomes, or promoting PSD95 binding to NR2B could facilitate expression of

NR2B at synaptic sites during NMDAR blockade. Finally, activity-dependent expression of many classes of postsynaptic proteins, including NR2A and NR2B, is regulated by the ubiquitin-proteasome system. Although NR2A and NR2B are not directly ubiquitinated following activity modulation, their abundance and stability at the synapse are likely controlled via ubiquitination and proteasome-regulated degradation of their associated scaffolding molecules (Ehlers, 2003).

Our current data support a model for differential regulation of NR2A and NR2B translation by synaptic NMDAR activation and deactivation (Fig. 10). Under baseline conditions of moderate synaptic NMDAR activation, translation of NR2B is suppressed, thereby limiting the synaptic expression of NR2B-containing NMDARs. However, in conditions of reduced NMDAR activation (such as with visual deprivation), inhibition of NR2B translation is relieved, allowing the production of NR2B subunits that are shuttled to the cell surface and synaptic sites independent of activity levels. In contrast to NR2B, NR2A translation is not inhibited by baseline NMDAR activation, and NMDAR inactivation has no added effect. Therefore, an early effect of visual deprivation is disinhibition of NR2B translation, thereby increasing NR2B protein levels and decreasing the overall NR2A/2B ratio of synaptic NMDARs. In summary, we propose that differential translational regulation is one mechanism for controlling the relative abundance of NR2A and NR2B at synaptic sites and may underlie experience-dependent changes in the NMDAR subunit composition in the visual cortex.

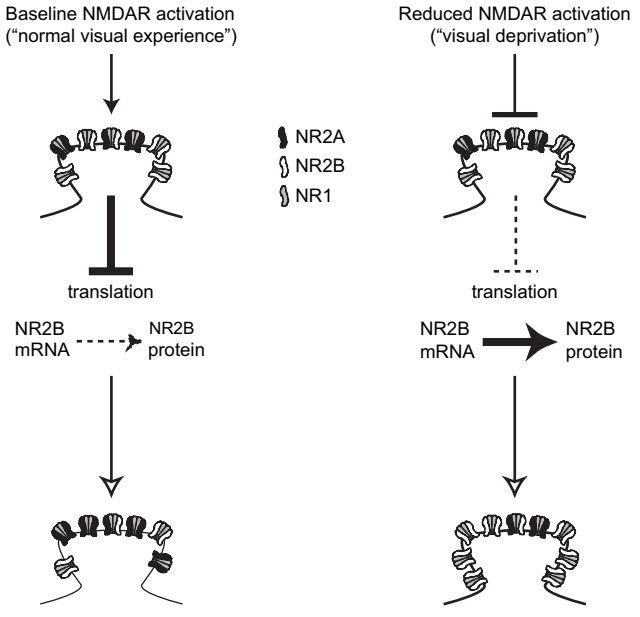

Fig. 10. Proposed model for translation-dependent regulation of NMDAR subunit composition by visual experience. Under baseline conditions of moderate synaptic NMDAR activation (such as with normal visual experience), translation of NR2B is suppressed, thereby limiting the synaptic expression of NR2B-containing NMDARs. However, in conditions of reduced NMDAR activation (such as with visual deprivation), inhibition of NR2B translation is relieved, allowing the production of NR2B subunits that are shuttled to the cell surface and synaptic sites independent of activity levels. In contrast to NR2B, NR2A translation is not inhibited by baseline NMDAR activation, and NMDAR inactivation has no added effect. Therefore, normal visual experience promotes a progressive increase in the NR2A/2B ratio by favoring NR2A translation, and visual deprivation promotes a decrease in the NR2A/2B ratio by disinhibition of NR2B translation.

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References

Barria, A., Malinow, R., 2002. Subunit-specific NMDA receptor trafficking to synapses. Neuron 35, 345–353.

Barria, A., Malinow, R., 2005. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48, 289–301.

Bear, M.F., 2003. Bidirectional synaptic plasticity: from theory to reality. Philos. Trans. R. Soc. Lond. B Biol. Sci. 358, 649–655.

Bear, M.F., Cooper, L.N., Ebner, F.F., 1987. A physiological basis for a theory of synapse modification. Science 237, 42–48.

Chung, H.J., Huang, Y.H., Lau, L.F., Huganir, R.L., 2004. Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. J. Neurosci. 24, 10248–10259.

Eberwine, J., Belt, B., Kacharmina, J.E., Miyashiro, K., 2002. Analysis of subcellularly localized mRNAs using in situ hybridization, mRNA amplification, and expression profiling. Neurochem. Res. 27, 1065–1077.

Ehlers, M.D., 2003. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. Nat. Neurosci. 6, 231–242.

Flint, A.C., Maisch, U.S., Weishaupt, J.H., Kriegstein, A.R., Monyer, H., 1997. NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J. Neurosci. 17, 2469–2476.

Follesa, P., Ticku, M.K., 1996. NMDA receptor upregulation: molecular studies in cultured mouse cortical neurons after chronic antagonist exposure. J. Neurosci. 16, 2172–2178.

Frenkel, M.Y., Bear, M.F., 2004. How monocular deprivation shifts ocular dominance in visual cortex of young mice. Neuron 44, 917–923.

Galvan, C.D., Wenzel, J.H., Dineley, K.T., Lam, T.T., Schwartzkroin, P.A., Sweatt, J.D., Swann, J.W., 2003. Postsynaptic contributions to hippocampal network hyperexcitability induced by chronic activity blockade in vivo. Eur. J. Neurosci. 18, 1861–1872.

Gazzaley, A.H., Benson, D.L., Huntley, G.W., Morrison, J.H., 1997. Differential subcellular regulation of NMDAR1 protein and mRNA in dendrites of dentate gyrus granule cells after perforant path transection. J. Neurosci. 17, 2006–2017.

He, H.Y., Hodos, W., Quinlan, E.M., 2006. Visual deprivation reactivates rapid ocular dominance plasticity in adult visual cortex. J. Neurosci. 26, 2951–2955.

Heynen, A.J., Yoon, B.J., Liu, C.H., Chung, H.J., Huganir, R.L., Bear, M.F., 2003. Molecular mechanism for loss of visual cortical responsiveness following brief monocular deprivation. Nat. Neurosci. 6, 854–862.

Hollingsworth, E.B., McNeal, E.T., Burton, J.L., Williams, R.J., Daly, J.W., Creveling, C.R., 1985. Biochemical characterization of a filtered synaptoneurosome preparation from guinea pig cerebral cortex: cyclic adenosine 3′:5′-monophosphate-generating systems, receptors, and enzymes. J. Neurosci. 5, 2240–2253.

Jo, J., Ball, S.M., Seok, H., Oh, S.B., Massey, P.V., Molnar, E., Bashir, Z.I., Cho, K., 2006. Experience-dependent modification of mechanisms of long-term depression. Nat. Neurosci. 9, 170–172.

Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T.J., Adams, S.R., Garner, C.C., Tsien, R.Y., Ellisman, M.H., Malenka, R.C., 2004.

Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. Nat. Neurosci. 7, 244–253.

Kacharmina, J.E., Job, C., Crino, P., Eberwine, J., 2000. Stimulation of glutamate receptor protein synthesis and membrane insertion within isolated neuronal dendrites. Proc. Natl. Acad. Sci. U.S.A 97, 11545–11550.

Kim, M.J., Dunah, A.W., Wang, Y.T., Sheng, M., 2005. Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46, 745–760.

Kirkwood, A., Rioult, M.C., Bear, M.F., 1996. Experience-dependent modification of synaptic plasticity in visual cortex. Nature 381, 526–528.

Lavezzari, G., McCallum, J., Dewey, C.M., Roche, K.W., 2004. Subunit-specific regulation of NMDA receptor endocytosis. J. Neurosci. 24, 6383–6391.

Lavezzari, G., McCallum, J., Lee, R., Roche, K.W., 2003. Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. Neuropharmacology 45, 729–737.

Liu, L., Wong, T.P., Pozza, M.F., Lingenhoehl, K., Wang, Y., Sheng, M., Auberson, Y.P., Wang, Y.T., 2004. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science 304, 1021–1024.

Marin, P., Nastiuk, K.L., Daniel, N., Girault, J.A., Czernik, A.J., Glowinski, J., Nairn, A.C., Premont, J., 1997. Glutamate-dependent phosphorylation of elongation factor-2 and inhibition of protein synthesis in neurons. J. Neurosci. 17, 3445–3454.

Miyashiro, K., Dichter, M., Eberwine, J., 1994. On the nature and differential distribution of mRNAs in hippocampal neurites: implications for neuronal functioning. Proc. Natl. Acad. Sci. U.S.A 91, 10800–10804.

Morishita, W., Lu, W., Smith, G.B., Nicoll, R.A., Bear, M.F., Malenka, R.C., 2007. Activation of NR2B-containing NMDA receptors is not required for NMDA receptor-dependent long-term depression. Neuropharmacology 52, 71–76.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., Seeburg, P.H., 1992. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256, 1217–1221.

Myers, S.J., Huang, Y., Genetta, T., Dingledine, R., 2004. Inhibition of glutamate receptor 2 translation by a polymorphic repeat sequence in the 5′-untranslated leaders. J. Neurosci. 24, 3489–3499.

Philpot, B.D., Espinosa, J.S., Bear, M.F., 2003. Evidence for altered NMDA receptor function as a basis for metaplasticity in visual cortex. J. Neurosci. 23, 5583–5588.

Philpot, B.D., Sekhar, A.K., Shouval, H.Z., Bear, M.F., 2001. Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. Neuron 29, 157–169.

Prybylowski, K., Chang, K., Sans, N., Kan, L., Vicini, S., Wenthold, R.J., 2005. The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron 47, 845–857.

Quinlan, E.M., Olstein, D.H., Bear, M.F., 1999a. Bidirectional, experience-dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. Proc. Natl. Acad. Sci. U.S.A 96, 12876–12880.

Quinlan, E.M., Philpot, B.D., Huganir, R.L., Bear, M.F., 1999b. Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat. Neurosci. 2, 352–357.

Rao, A., Craig, A.M., 1997. Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. Neuron 19, 801–812.

Roche, K.W., Standley, S., McCallum, J., Dune Ly, C., Ehlers, M.D., Wenthold, R.J., 2001. Molecular determinants of NMDA receptor internalization. Nat. Neurosci. 4, 794–802.

Rutherford, L.C., Nelson, S.B., Turrigiano, G.G., 1998. BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. Neuron 21, 521–530.

Scheetz, A.J., Nairn, A.C., Constantine-Paton, M., 2000. NMDA receptor-mediated control of protein synthesis at developing synapses. Nat. Neurosci. 3, 211–216.

Scott, D.B., Michailidis, I., Mu, Y., Logothetis, D., Ehlers, M.D., 2004. Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals. J. Neurosci. 24, 7096–7109.

Sutton, M.A., Wall, N.R., Aakalu, G.N., Schuman, E.M., 2004. Regulation of dendritic protein synthesis by miniature synaptic events. Science 304, 1979–1983.

Tongiorgi, E., Ferrero, F., Cattaneo, A., Domenici, L., 2003. Dark-rearing decreases NR2A N-methyl-D-aspartate receptor subunit in all visual cortical layers. Neuroscience 119, 1013–1022.

Turrigiano, G.G., Leslie, K.R., Desai, N.S., Rutherford, L.C., Nelson, S.B., 1998. Activity-dependent scaling of quantal amplitude in neocortical neurons. Nature 391, 892–896.

VanDongen, A.M., VanDongen, H.M., 2004. Effects of mRNA untranslated regions on translational efficiency of NMDA receptor subunits. Neurosignals 13, 194–206.

Vicini, S., Wang, J.F., Li, J.H., Zhu, W.J., Wang, Y.H., Luo, J.H., Wolfe, B.B., Grayson, D.R., 1998. Functional and pharmacological differences between recombinant *N*-methyl-D-aspartate receptors. J. Neurophysiol. 79, 555–566.

Vissel, B., Krupp, J.J., Heinemann, S.F., Westbrook, G.L., 2001. A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. Nat. Neurosci. 4, 587–596.

Watt, A.J., van Rossum, M.C., MacLeod, K.M., Nelson, S.B., Turrigiano, G.G., 2000. Activity coregulates quantal AMPA and NMDA currents at neocortical synapses. Neuron 26, 659–670.

Wells, D.G., Dong, X., Quinlan, E.M., Huang, Y.S., Bear, M.F., Richter, J.D., Fallon, J.R., 2001. A role for the cytoplasmic polyadenylation element in NMDA receptor-regulated mRNA translation in neurons. J. Neurosci. 21, 9541–9548.

Wood, M.W., VanDongen, H.M., VanDongen, A.M., 1996. The 5′-untranslated region of the *N*-methyl-D-aspartate receptor NR2A subunit controls efficiency of translation. J. Biol. Chem. 271, 8115–8120.

Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M.A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J.R., Richter, J.D., 1998. CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses [see comments]. Neuron 21, 1129–1139.

Yashiro, K., Corlew, R., Philpot, B.D., 2005. Visual deprivation modifies both presynaptic glutamate release and the composition of perisynaptic/extrasynaptic NMDA receptors in adult visual cortex. J. Neurosci. 25, 11684–11692.