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Activity-dependent *NR2B* expression is mediated by MeCP2-dependent epigenetic regulation

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ABSTRACT

Different NR2 subunits (NR2A-D) of NMDA receptors confer distinct properties on the receptors and the subunit composition of heteromeric NMDA receptor complex is tightly regulated. Here, we demonstrate that suppression of neuronal activity causes mRNA expression of the NR2B subunit to increase significantly, both *in vitro* and *in vivo*, and that this modulation of transcription is mediated by epigenetic mechanisms. Treating cortical neurons with TTX substantially increases the level of mRNAs for NMDA receptor subunits. Particularly, the *NR2B* expression increases over 2-fold, similar to the effects of dark-rearing. The increase of *NR2B* induced by TTX is occluded by inhibiting DNMTs. Furthermore, MeCP2 binds to *NR2B* and the association of MeCP2 with *NR2B* is reduced by TTX treatment. Together, these data indicate that DNA methylation as well as subsequent MeCP2 association mediates neuronal activity-dependent regulation of *NR2B* expressions.

Visual sensory experience during early postnatal development guides the refinement of visual pathways, and the properties of synaptic transmission mediated by *N*-methyl-D-aspartate receptors (NMDARs) change over this refinement period [1]. In the early days of postnatal development, NR2B is the major NR2 subunit found in heteromeric NMDARs in the visual cortex. In contrast, NR2A subunit expression is virtually absent at birth but appears at the time of eye opening and progressively increases over the course of postnatal development [2]. The reduction in NMDAR-mediated current duration during synaptic maturation depends on the changes in NR2 subunit composition because NR2A-containing NMDARs have faster desensitization kinetics [3]. When animals are deprived of normal visual experience during early postnatal development, the visual cortex retains the synaptic properties of the immature state [4], which is accompanied by a delay in the change in relative contribution of NR2A and NR2B subunits [5]. Therefore, activity-dependent regulation of NMDAR expression is crucial for optimal synaptic maturation and refinement.

Despite the importance of the regulation of NMDAR expression, how neuronal cells achieve the sophisticated control of subunit composition is poorly understood. It was recently shown that the epigenetic mechanisms play vital roles in brain functions such as memory formation, drug addiction, and stress responses [6,7]. Two interrelated events, changes in DNA methylation and the remodeling of local chromatin structure, largely mediate epigenetic regulation of gene expression [8]. These processes appear to be coordinated by methyl-DNA binding proteins. One such protein, MeCP2 is particularly abundant in brain. The fact that MeCP2 plays critical role(s) in postnatal brain development was clearly demonstrated when a mutation in *MeCP2* was found to be responsible for Rett syndrome, a neurodevelopmental disorder that is classified as a pervasive developmental disorder [9]. While some epigenetic modifications are quite stable, MeCP2 activity in neuronal cells is regulated dynamically in response to neuronal activity [10].

Here, we show that the expression of *NR2B* is regulated by neuronal activity both *in vitro* and *in vivo*. We found that the activity-dependent change in expression is dependent on proper DNA methylation and is mediated by the activity-dependent association of MeCP2 with *NR2B*. Our data suggest that epigenetic mechanisms play a crucial role in neuronal circuit maturation in the brain by controlling the activity-dependent regulation of NMDAR expression.

Materials and methods

Animals and housing. Juvenile (P25-28) Long Evans black hooded rats (Charles River Labs) were used for dark-rearing experiments. Dark-reared rats were raised in complete darkness from birth while light-reared rats were raised on a 12 h:12 h light:dark cycle.

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Pregnant Sprague–Dawley rats were purchased from Orient Bio and used for the preparation of primary cortical neuron cultures. All animals were raised and used in accordance with MIT and Korea University guidelines.

Dissociated cortical neuron cultures. Cortical neurons were isolated from the cortical tissue of embryonic day 18–20 (E18–E20) rats. Dissociated neurons were maintained in Neurobasal A-based medium with 2.5% serum. On the third day of culture *in vitro* (3DIV), cytosine arabinoside (5 μM) was added to prevent glial cell proliferation. For most experiments, cultures were treated with the following drugs at optimum concentrations for 3 days from 7 DIV to 10 DIV: TTX (1 μM, TOCRIS), 5azaC (5 μM, Sigma), and K252a (200 nM, Sigma).

Quantitative real-time PCR. RNA was harvested using the RNAqueous-4PCR kit (Ambion). RNA was reverse transcribed using the SuperScript III First Strand cDNA synthesis kit (Invitrogen) following the manufacturer's protocol (1 μg of total RNA was used). The same amount of cDNA (8 μl of the 200 μl sample) from each sample was used for amplification. Amplification of cDNA was performed on a Light cycler 480 system (Roche-applied science) in triplicate using the IQ-SYBR Green mixture (Bio-rad). All genes were standardized to *G6pdh*.

Primers. All primers were ordered from IDT (Coralville). For real-time PCR, the primers were: NR1 forward, ATC TGG CCA GGA GGA GAG AC; NR1 reverse, CTG GGT CAC CAT TGA CTG TG; NR2A forward, CCA GAG ACC TCA TGA CTA TTC TCC; NR2A reverse, ATA GAT GAA AGC GTC CAA CTT CC; NR2B forward, AGA GGT GGT TGA CTT CTC TGT ACC; NR2B reverse, TGA AGT ATT CAA AGA CAA AGA CAG C; BDNF forward, CCC CTT TTA ACT GAA GAG AAG G; BDNF reverse, TGC AAC CGA AGT ATG AAA TAA CC; G6PDH forward, CAG CGG CAA CTA AAC TCA GA; G6PDH reverse, GGA AGG CAT CAC CCT GGT A. The primers for chromatin immunoprecipitations were: 2BI1 forward, TCT TGG GTT TCT CCT CCT GA; 2BI1 reverse, CAA GAG AGC CCA GAT TCC GA; 2BI2 forward, CCC AAC CCA GGA GAC TGA TCC ATT TAT CC; 2BI2 reverse, TCC CCT TCC CCC TCC CCA AAA ATA TCC; 2BI3 forward, CCT CCA GTC CAA TCC TCT GG; 2BI3 reverse, CAC ACT GGG CAT TTC TTG G; 2BI4 forward, TGG AGG AGA TGA GGA GAG; 2BI4 reverse, CAC CAA TCT ATA AAC AAT TCC; 2BI5 forward, CTG GAT TCT GCA TTG TGA GC; 2BI5 reverse, AGA GTC CTC TTC TCG CTT GC; H19 forward, CCC GGT ATT GGA ATC CAC; H19 reverse, GAA ATG CAT GTG TCC TGC CCT CC.

Chromatin immunoprecipitation (ChIP). ChIP was performed as previously reported [11]. Briefly, cross-linked cell lysates were sheared by sonication to generate chromatin fragments of approximately 200–500 bp on average. Chromatin was then subjected to immunoprecipitation using antibodies specific to MeCP2 (Millipore) and control IgG (Sigma) at 4 °C overnight. Protein–DNA–antibody complexes were precipitated with Protein G-agarose beads and then subjected to a series of washes. The precipitated protein–DNA complexes were eluted from the antibody with elution buffer, and then incubated at 65 °C for 5–6 h in 300 mM NaCl to reverse the cross-links. After proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation, purified DNA was subjected to PCR amplification using quantitative real-time PCR, as described above. ChIP samples were standardized to their own input controls.

Results

Visual experience regulates the expression of NMDAR genes

The rodent visual cortex provides an excellent model system where the plastic nature of the neural system can be easily revealed by relatively simple experimental manipulations. Depriving visual experience by rearing animals in complete dark from birth is an effective way to shift the threshold for synaptic modification [4]. The history of integrated postsynaptic activity is hypothesized to adjust the modification threshold (θ_m) such that the value of θ_m falls as the average activity decreases (e.g., as in dark-rearing) [12]. Changes in NMDAR subunit composition alter the properties of NMDAR-mediated synaptic transmission and, therefore, may underlie the mechanism of metaplasticity (plasticity of plasticity) [13]. In order to determine whether the overall activity of neurons can regulate the transcription of NMDAR genes *in vivo*, we examined the levels of mRNAs encoding NMDA receptor subunits in the visual cortex of rats reared normally, or in the dark, from birth. While the level of NR2A mRNA in the visual cortex of dark-reared animals was almost identical to that of normally-reared animals, the mRNA level of the NR2B subunit was almost 2-fold higher in

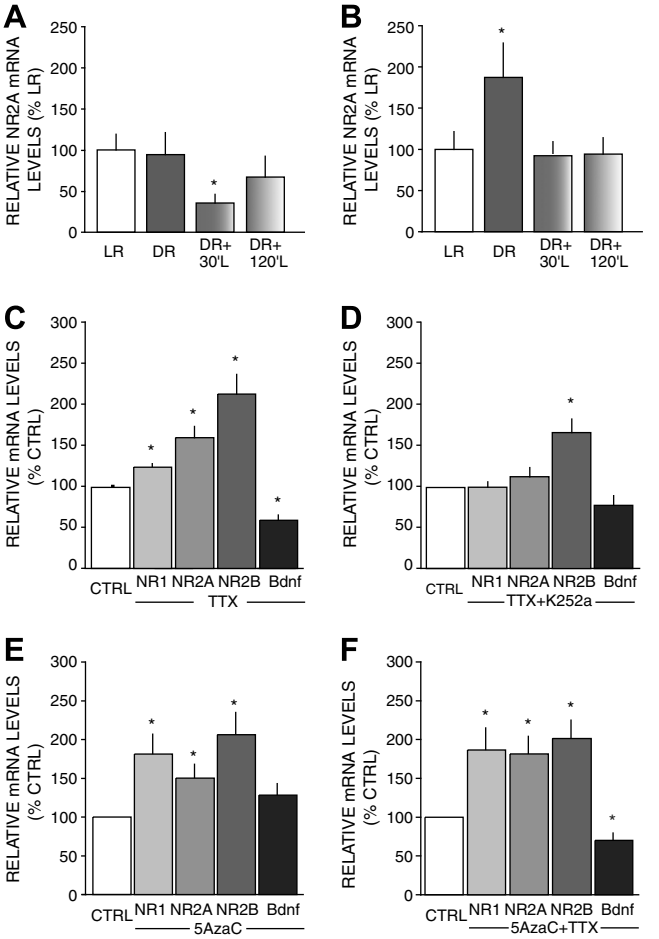

Fig. 1. *NR2B* expression is regulated by neuronal activity both *in vitro* and *in vivo* via epigenetic mechanisms. (A) Relative amounts of *NR2A* expression in the visual cortex from normally-reared (LR) and dark-reared rats (P25-28) are shown. Subsets of dark-reared animals were exposed to light 30 min (DR + 30′L) or 2 h (DR + 120′L) before sacrificing. The relative amount of *NR2A* (standardized to *G6pdh*) was measured by quantitative real-time PCR. Student's *t*-test indicates a significant reduction in *NR2A* mRNA from DR + 30′L ($p < 0.01$). (B) Relative amounts of *NR2B* expression are graphed. Dark-rearing significantly increased the amount of *NR2B* ($p < 0.05$). (C) Dissociated cortical neurons were maintained in the presence of TTX (1 μM) from 7 DIV to 10 DIV. The level of mRNA was then assessed by quantitative real-time PCR. *NR2B* expression was significantly increased compared to control (CTRL) cultures incubated in the absence of TTX. (D) Cortical neurons were incubated with TTX and K252a (200 nM), an antagonist for Trk receptor that binds to BDNF, for 3 days. The relative mRNA levels were indistinguishable from the TTX-only treated cells. (E) Cortical neurons were incubated in the presence of DNMT inhibitor, 5azaC, for 3 days (7–10 DIV). Expression of *NR1* and *NR2B* was significantly increased by 5azaC treatment. (F) Treatment with both TTX and 5azaC did not result in any further increase in *NR2B* mRNA, indicating that both drugs exert their effects through a common mechanism.

dark-reared animals (Fig. 1B) (LR vs. DR: 100 ± 21.4% vs. 187 ± 41.6%, $p < 0.05$, $n = 6$). Interestingly, when dark-reared animals were exposed to light, the level of NR2B mRNA was quickly reduced to the same level as that in light-reared animals (LR vs. DR + 30′L: 100 ± 21.4% vs. 92.2 ± 17%,), and the level of *NR2A* decreased significantly with 30 min light exposure (LR vs. DR + 30′L: 100 ± 18.9% vs. 35.4 ± 10.7%, $p < 0.01$, $n = 6$) (Fig. 1A).

The increase in *NR2B* expression in dark-reared animals significantly decreased the ratio of NR2A/NR2B mRNAs, which is consistent with the decrease in the ratio of the two proteins [14]. Slow desensitization, as well as its propensity for binding CamKII, has implicated NR2B as a candidate 'LTP molecule' [15]. Increasing the number of NMDARs that contain the NR2B subunit may lower the threshold for LTP induction, which could contribute to the maintenance of cellular homeostasis.

Neuronal activity regulates NMDAR expression in cultured cortical neurons

In order to dissect the molecular mechanisms by which the expression of NMDAR subunits is regulated, we used cultures of dissociated cortical neurons which can easily be manipulated pharmacologically. Homeostatic upregulation of synaptic NMDARs has been observed in dissociated cortical cultures when neuronal activity was chronically suppressed [16], although the precise mechanism of the upregulation is not understood. We measured the mRNA levels of NMDAR genes upon changes in activity of dissociated cortical neurons in culture. Under chronic blockade of neuronal activity by tetrodotoxin (TTX), the NR2B mRNA level increased significantly (CTRL ($n = 10$) vs. TTX ($n = 9$): 100 ± 10.6% vs. 212.9 ± 24.1%, $p < 0.001$). The levels of NR1 and NR2A mRNAs also increased (*NR1*, 123.7 ± 5.4%, $p < 0.05$; *NR2A*, 159.6 ± 14%, $p < 0.01$) (Fig. 1C).

Brain-derived neurotrophic factor (BDNF) plays essential roles in neural development, as well as in neuronal survival. BDNF can induce or influence plastic changes at active synapses [17] and activity-dependent regulation of *Bdnf* expression has been demonstrated both *in vitro* and *in vivo* [10,18]. Therefore, we measured *Bdnf* expression as an indicator of changes in neuronal activity. In TTX-treated neurons, the level of *Bdnf* decreased significantly (58.9 ± 6.3, $p < 0.001$), confirming that the treatment suppressed neuronal activity (Fig. 1C). Exogenously added BDNF induces changes in NMDAR expression in hippocampal neurons [19], raising the possibility that the elevated expression of *NMDAR*s under TTX treatment may be due to changes in BDNF expression. In order to assess the influence of BDNF signaling on NMDAR expression, we measured the expression of NMDAR genes in response to TTX treatment in cultures in which BDNF signaling was blocked using a Trk receptor antagonist. We found that the increase of *NR1* and *NR2A* in the presence of TTX was blocked by inhibiting BDNF signaling (*NR1*, 100.3 ± 4.8%, $p > 0.9$; *NR2A*, 113 ± 9.5%, $p > 0.37$; $n = 6$), while *NR2B* was still increased regardless of BDNF signaling, suggesting that the increase of *NR2B* in response to TTX treatment is not mediated by changes in BDNF signaling (*NR2B*, 166.9 ± 14.8%, $p < 0.03$; *Bdnf*, 78.3 ± 10.1%, $p > 0.23$; $n = 6$) (Fig. 1D).

These results indicate that neuronal activity regulates the expression of NMDAR at the level of transcription in multiple mechanisms. Previous results showed that regulation of translation, as well as of protein degradation, plays important roles in activity-dependent control of NMDARs [14]. Our results add an additional layer of activity-dependent regulation. NMDAR expression may require dynamic range of regulations, employing fine controls at both transcriptional and translational steps.

Epigenetic regulation is a key mechanism subserving the activity-dependent expression of NMDAR

The regulation of gene expression through epigenetic mechanisms such as DNA methylation and chromatin structure modification seems to play important roles in many brain functions, including learning and memory [6] and visual cortical plasticity [20]. Recent studies indicate that DNA methylation and/or the association of methyl-DNA binding proteins with methylated DNA is reversible and dynamic in neurons, possibly reflecting integrated neuronal activity [6,21].

We examined whether activity-dependent regulation of NMDAR gene expression is mediated by epigenetic mechanisms. We measured the expression of NMDAR in the presence of a DNMT inhibitor 5azaC, in order to examine whether a change in DNA methylation influences NMDAR expression. After 3 days in the presence of 5azaC, the NR2B mRNA level increased approximately 2-fold, and *NR1*, *NR2A* also showed statistically significant increases (Fig. 1E) (*NR1*, 182 ± 25.1%, $p < 0.01$; *NR2A*, 150.5 ± 17.6%, $p < 0.05$; *NR2B*, 206.8 ± 28.2%, $p < 0.01$; BDNF, 128.7 ± 14.2%, $p > 0.06$; $n = 7$). This result indicates that *NMDARs* expression is sensitive to the state of DNA methylation. To further explore the possibility that the enhanced expression of *NMDARs* in response to TTX treatment is mediated by DNA methylation-dependent mechanism, we treated cortical neurons with both TTX and 5azaC together for 3 days. Treatment with both TTX and 5azaC did not increase *NR2B* expression further, indicating that 5azaC and TTX both modulate *NR2B* expression by the same mechanism, i.e. changes in DNA methylation (*NR1*, 186.6 ± 27.8%, $p < 0.01$; *NR2A*, 181.6 ± 21.9%, $p < 0.01$; *NR2B*, 201.6 ± 22.9%, $p < 0.002$; BDNF, 69.9 ± 9%, $p < 0.02$; $n = 7$) (Fig. 1F). Although both *NR1* and *NR2A* expression all increased in the presence of TTX, 5azaC, and TTX + 5azaC, their regulation mechanism seems to be different from that of *NR2B* on the following grounds. First, the magnitude of *NR1* and *NR2A* increases in the presence of TTX + 5azaC is greater than that in the presence of TTX alone, which suggests that neuronal activity regulates the expression of *NR1* and *NR2A* through additional mechanisms independent of DNA methylation. Second, inhibiting BDNF signaling blocked the TTX-mediated increases in *NR1* and *NR2A* but not the increase in *NR2B*.

Changes in DNA methylation could either directly influence the efficiency of transcription initiation or exert an effect by recruiting chromatin remodeling complexes through methyl-binding proteins such as MeCP2. To determine whether the regulation of *NR2B* expression is mediated by MeCP2, we analyzed the promoter area of *NR2B* and identified five putative MeCP2-binding sites (designated 2BI1 to 2BI5, Fig. 2A). Chromatin immunoprecipitation (ChIP) assays demonstrated that two (2BI3 and 2BI5) of the five candidate sites bind MeCP2 in cortical neurons (Fig. 2B). Next, we examined whether these interactions are altered by changes in neuronal activity to assess the role of the interactions in the activity-dependent regulation of NR2B gene expression. The interaction of MeCP2 with the 2BI3 and 2BI5 sites was strikingly reduced in neurons maintained in the presence of TTX (Fig. 2C). This result is consistent with the classical view of MeCP2 as a general transcriptional repressor, in that the reduced association leads to increased expression of *NR2B*. The association of MeCP2 with DNA can be regulated either by direct changes in DNA methylation or by changes in phosphorylation status of MeCP2, possibly without changes in DNA methylation. In order to determine if the change in MeCP2 binding to *NR2B* is accompanied by changes in DNA methylation, we examined the extent of DNA methylation in the vicinity of 2BI5. We found a significant demethylation in the promoter of *NR2B* upon TTX treatment (Fig. 3).

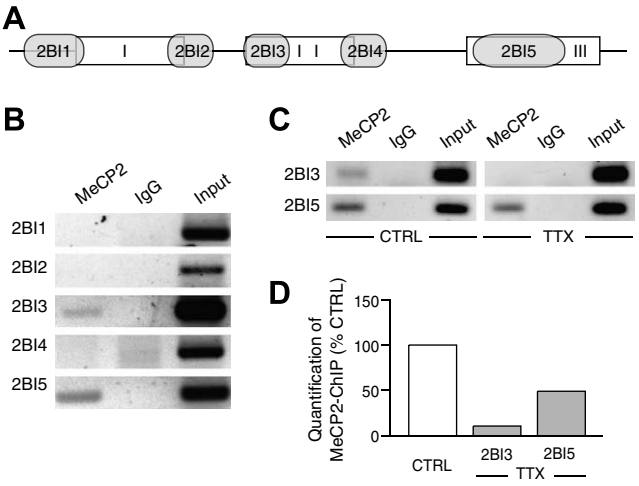

Fig. 2. MeCP2 binds to *NR2B* and its association is regulated by neuronal activity. (A) Schematic diagram showing the relative positions of *NR2B* exons (open boxes, I–III) and putative MeCP2 binding sites that are CpG-rich (shaded boxes, 2BI1–2BI5). (B) ChIP assays demonstrated the interaction of MeCP2 with 2BI3 and 2BI5. 10 DIV cortical neurons were subjected to ChIP analysis using an anti-MeCP2 antibody (Millipore). (C) Cortical neurons were treated with TTX for 3 days before they were subjected to ChIP assay. The interaction between MeCP2 and 2BI3 and 2BI5 is clearly reduced when neuronal activity was suppressed. (D) ChIP products were quantified using real-time PCR.

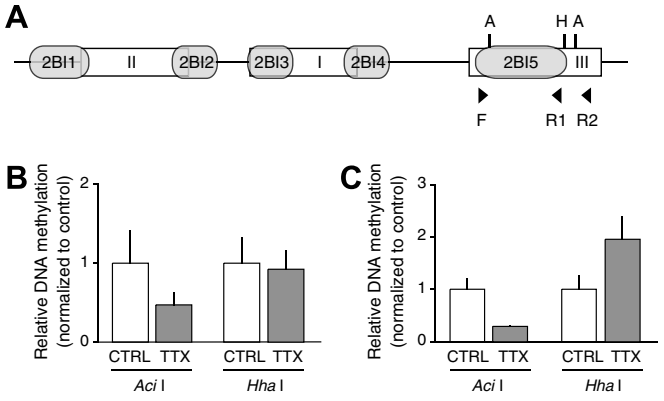

Fig. 3. Changes in local DNA methylation accompany changes in MeCP2 association with *NR2B*. (A) Schematic diagram showing the relative positions of *NR2B* exons (open boxes, I–III) and putative MeCP2 binding sites (2BI1–2BI5) along with methylation sensitive AciI (A) and HhaI (H) recognition sites around the 2BI5. The relative location of primers used are shown as triangles. Primer F and R1 are identical to the primers used for ChIP analysis (2BI5 forward and 2BI5 reverse). Primer R2 (5′-AGAGTCCTCTTCTCGCTTGC-3′) was used for assessing methylation status at HhaI site and another AciI site. (B) Quantitative analysis of relative methylation at the first AciI site. Genomic DNA from control neurons and TTX-treated neurons was fully digested with AciI or HhaI and was subjected to real-time quantitative PCR using a F-R1 primer pair. TTX-treated sample showed significantly reduced amplification when digested with AciI, indicating the loss of methylation at the AciI site. HhaI digestion did not affect the F-R1 amplification because the amplified region does not include a HhaI site, therefore it served as a negative control. PCR quantification with digested samples was carried out by standardizing them to PCR with undigested samples. (C) The same samples as in (B) were subjected to PCR amplification using a F-R2 primer pair. AciI digestion caused a further decrease in amplification with TTX-treated samples, suggesting demethylation at the second AciI site as well. In contrast, HhaI digestion caused increase in amplification with TTX-treated samples, which might indicate an increase in methylation at the HhaI site when neuronal activity is suppressed.

Discussion

NMDARs play critical roles in diverse brain functions such as neuronal circuit development, plasticity, and excitotoxicity. The different channel properties of NR2A-containing NMDARs and NR2B-containing NMDARs can critically influence the expression of bidirectional synaptic plasticity, for example through the modulation of the modification threshold θ_m [12]. Activity-dependent regulation of NMDAR subunit switching, therefore, may be a critical component of the mechanism that controls metaplasticity in the visual cortex.

Here, we have studied the regulation of NMDAR subunit gene expression and found that NMDAR expression is regulated at the transcriptional level. Reduced neuronal activity increases the expression of *NR2B* both *in vitro* and *in vivo*. Responses to dynamic changes in neuronal activity often are rapid but transient at the synaptic level. However, when overall activity changes at the cellular level persist, these changes are accompanied by slower but lasting cellular responses to maintain neuronal homeostasis. Increased *NMDAR* expression, in conjunction with the increase in the relative contribution of NR2B, in response to suppression of neuronal activity may be a homeostatic response. NR2B has been implicated in the induction of long-term potentiation because of its slower decay kinetics as well as its association with CaMKII, which is an essential mediator of synaptic potentiation. Therefore, the increased contribution of NR2B-containing NMDARs may help to boost neuronal output.

We have shown here that the expression of *NR2B* increases in response to chronic TTX treatment, concurrently with decrease in association of MeCP2 with *NR2B*. It was also accompanied by local DNA demethylation. Neuronal activity signals to MeCP2 through Ca^{2+}-dependent cascades [22]; however, it is to be determined if the same signaling pathway functions at *NR2B* locus. Different loci may engage different chromatin remodeling complexes, while including MeCP2 as a common factor. However the mechanisms differ from one locus to others, epigenetic regulation appears to be an important mechanism in neurons for fine-tuning the neural system in response to perturbations in neuronal activity.

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