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Dynorphin up-regulation in the dentate granule cell mossy fiber pathway following chronic inhibition of GluN2B-containing NMDAR is associated with increased CREB (Ser 133) phosphorylation, but is independent of BDNF/TrkB signaling pathways

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ABSTRACT

Emerging evidence suggests that neuronal responses to *N*-methyl-D-aspartate (NMDAR) activation/inactivation are influenced by subunit composition. For example, activation of synaptic NMDAR (comprised of GluN2A > GluN2B) phosphorylates cAMP-response-element-binding protein (CREB) at Ser 133, induces BDNF expression and promotes neuronal survival. Activation of extrasynaptic NMDAR (comprised of GluN2B > GluN2) dephosphorylates CREB (Ser 133), reduces BDNF expression and triggers neuronal death. These results led us to hypothesize that chronic inhibition of GluN2B-containing NMDAR would increase CREB (Ser 133) phosphorylation, increase BDNF levels and subsequently alter downstream dynorphin (DYN) and neuropeptide Y (NPY) expression. We focused on DYN and NPY because these neuropeptides can decrease excitatory neurotransmission and seizure occurrence and we reported previously that seizure-like events are reduced following chronic treatment with GluN2B antagonists. Consistent with our hypothesis, chronic treatment (17–21 days) of hippocampal slice cultures with the GluN2B-selective antagonists ifenprodil or Ro25,6981 increased both CREB (Ser 133) phosphorylation and granule cell mossy fiber pathway DYN expression. Similar treatment with the non-subtype-selective NMDAR antagonists D-APV or memantine had no significant effect on either CREB (Ser 133) phosphorylation or DYN expression. In contrast to our hypothesis, BDNF levels were decreased following chronic treatment with Ro25,6981, but not ifenprodil, D-APV or memantine. Blockade of BDNF actions and TrkB activation did not significantly augment hilar DYN expression in vehicle-treated cultures and had no effect in Ro25,6981 treated cultures. These findings suggest that chronic exposure to GluN2B-selective NMDAR antagonists increased DYN expression through a putatively pCREB-dependent, but BDNF/TrkB-independent mechanism.

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Introduction

N-methyl-D-aspartate receptors (NMDAR) are heteromeric ionotropic glutamate receptors composed of obligatory GluN1 and variable, modulatory GluN2A-D subunits. GluN2A and GluN2B are the primary GluN2 subunits in hippocampus and cortex (Monyer et al., 1994; Yamakura and Shimoji, 1999) and differ in their subcellular localization, biophysical properties, signaling pathway coupling, and brain function contributions (Barria and Malinow, 2005; Flint et al., 1997; Lavezzari et al., 2004; Monyer et al., 1994; Vicini et al., 1998). Data emerging over the past decade suggest that neuronal responses to NMDAR activation/inactivation are influenced by receptor subunit composition and subcellular localization (Wyllie et al., 2013; Zhou and Sheng, 2013). For example, activation of synaptic NMDAR (comprised of GluN2A > GluN2B) phosphorylates cAMP-response-element-binding protein (CREB) at Ser 133, induces BDNF expression and promotes neuronal survival. Conversely, activation of extrasynaptic NMDAR (comprised of GluN2B > GluN2A)

Abbreviations: ABC, avidin–biotin–peroxidase complex; APV, D-(−)-2-amino-5-phosphonopentanoic acid; BCA, bicinchoninic acid; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CREB, cAMP response element-binding protein; DAB, 3,3′-diaminobenzidine; DMSO, dimethylsulfoxide; DYN, dynorphin; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GBSS, Gey's Balanced Salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horse-radish peroxidase; IBMX, 3-isobutyl-1-methylxanthine; IgG, Immunoglobulin G; -IR, immunoreactivity; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; NPY, neuropeptide Y; GluN2A, NMDAR 2A subunit; GluN2B, NMDAR 2B subunit; NSE, neuron specific enolase; PB, phosphate buffer; PBS, phosphate buffered saline; pCREB, phosphorylated CREB; PMSF, phenylmethanesulfonylfluoride; PVDF, polyvinylidene fluoride; RT, room temperature; SDS, sodium dodecyl sulfate; Ser, serine; TB, Tris buffer; TBS, Tris buffered saline; TrkB-Fc, tyrosine kinase, receptor/IgG Fc fragment chimera.

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dephosphorylates CREB (Ser 133), reduces BDNF expression and triggers neuronal death (Hardingham et al., 2002; Liu et al., 2004; Tovar and Westbrook, 1999).

Based upon these previous findings we hypothesized that chronic inhibition with GluN2B antagonists would increase CREB (Ser 133) phosphorylation and increase BDNF expression. We further hypothesized that chronic inhibition with GluN2B antagonists would alter dynorphin A (DYN) and neuropeptide Y (NPY) expression because CREB (Ser 133) phosphorylation can directly increase expression of DYN and NPY (Carlezon et al., 1998; Chance et al., 2000; Cole et al., 1995; Higuchi et al., 1988) and indirectly regulate their expression through altered BDNF levels (Croll et al., 1994; Nawa et al., 1993; Shieh et al., 1998; Tao et al., 1998). We focused on DYN and NPY because these neuropeptides can decrease excitatory neurotransmission and seizure occurrence (Baraban, 2004; Bausch et al., 1998; Chen et al., 1995; Chen and Huang, 1998; Simonato and Romualdi, 1996; Colmers et al., 1987, 1988; Klapstein and Colmers, 1997; Patrylo et al., 1999; Richichi et al., 2004; Wagner et al., 1993) and seizure-like events were decreased following chronic treatment with GluN2B antagonists (Wang and Bausch, 2004).

Results

Chronic treatment with GluN2B-selective, but not non-subunit-selective NMDAR antagonists, increased CREB phosphorylation

To test our hypothesis, we first performed Western blot analysis for CREB and CREB (Ser 133) phosphorylation on hippocampal slice culture homogenates. Chronic treatment with the GluN2B-selective NMDAR antagonist Ro25,6981, but not ifenprodil, as well as the non-subtype-selective NMDAR antagonist, APV increased CREB levels relative to NSE loading controls (Fig. 1A,B). However, chronic treatment with the GluN2B-selective NMDAR antagonists, Ro25,6981 and ifenprodil, but not the non-subunit-selective NMDAR antagonists, D-APV or memantine significantly increased CREB (Ser 133) phosphorylation relative to CREB levels (Fig. 1A,C). Homogenates prepared from vehicle-treated slice cultures displayed moderate levels of CREB (Ser 133) phosphorylation (Fig. 1A).

Chronic treatment with GluN2B-selective, but not non-subunit-selective NMDAR antagonists, increased DYN, but not NPY expression

To examine the potential down-stream consequences of CREB (Ser 133) phosphorylation on neuropeptide expression, we first examined effects of NMDAR antagonist treatment on DYN peptide levels using immunohistochemistry. Vehicle-treated hippocampal slice cultures displayed DYN immunoreactivity in puncta localized sparsely in the hilus and more prominently in CA3 stratum lucidum (Fig. 2A, vehicle), consistent with a previous report in neonatal, prepubescent rats (Shirayama et al., 2005). Given the documented expression of DYN in granule cells and their mossy fiber axons (McGinty et al., 1994; Pierce et al., 1999), these puncta most likely represent DYN immunoreactivity in dentate granule cell mossy fiber terminals. Chronic treatment of cultures with the non-subunit-selective NMDAR antagonists, APV or memantine, did not significantly affect DYN immunoreactivity (Fig. 2A–C). In contrast, chronic treatment with the GluN2B-selective NMDAR antagonists, Ro25,6981 or ifenprodil, significantly increased both the percentage of cultures exhibiting DYN immunoreactivity throughout the mossy fiber pathway (Fig. 2A,B) and the density of hilar DYN immunoreactivity (Fig. 2A,C).

We next examined the effects of chronic NMDAR antagonist treatment on NPY expression using immunohistochemistry. Vehicle-treated hippocampal slice cultures showed NPY immunoreactivity throughout the slice culture (Fig. 3A, vehicle) in scattered, non-principal neuronal somata and dendrites (Fig. 3B1-3). NMDAR antagonists did not significantly affect the density of NPY immunoreactivity (Fig. 3D,E) or the number of hilar/granule cell layer NPY-immunoreactive neurons (Fig. 3C). The exception was D-APV, which significantly reduced the number of hilar/granule cell layer NPY-immunoreactive neurons (Fig. 3C). Reduced neuronal survival following chronic D-APV treatment (Wang and Bausch, 2006) is likely to account for decreased numbers of hilar NPY-immunoreactive neurons.

Dynorphin up-regulation by chronic treatment with GluN2B-selective NMDAR antagonists was independent of BDNF/TrkB signaling pathways

We next investigated the effects of chronic NMDAR antagonist treatment on BDNF and its effects on DYN expression. We first examined BDNF levels using ELISA. Chronic treatment of cultures with the non-subunit-selective NMDAR antagonists, D-APV or memantine, did not significantly alter BDNF levels (Fig. 4), consistent with their lack of effect on CREB (Ser 133) phosphorylation. Treatment of cultures with GluN2B-selective NMDAR antagonists showed differential effects on BDNF. Compared to vehicle, chronic Ro25,6981 decreased, while ifenprodil had no significant effect on BDNF levels (Fig. 4). The lack of association between BDNF levels and DYN expression in both Ro25,6981- and ifenprodil-treated cultures suggests that altered BDNF expression did not underlie GluN2B antagonist-induced increases in DYN expression. However, BDNF was measured in whole slice cultures, while increased DYN occurred only in the dentate gyrus/hilar mossy fiber pathway. Therefore, we directly examined the influence of BDNF and activation of its receptor, TrkB on DYN immunoreactivity. Vehicle- and Ro25,6981-treated cultures were treated concomitantly with the BDNF scavenger, TrkB-Fc or the broad spectrum Trk tyrosine kinase inhibitor, K252a. If decreased BDNF contributed to increased DYN expression, then TrkB-Fc and/or K252a should increase DYN expression in vehicle-treated cultures. Conversely, if increased BDNF expression and subsequent TrkB activation/phosphorylation lead to increased DYN expression, then TrkB-Fc and/or K252a co-incubation should decrease DYN expression in Ro25,6981-treated cultures. In vehicle-treated cultures neither TrkB-Fc nor its respective control, IgG-Fc significantly affected hilar DYN immunoreactivity (Fig. 5A,B). Opposite to what we expected, K252a caused a strong trend toward reduced hilar DYN immunoreactivity compared to both chronic vehicle treatment alone and its respective DMSO control (Fig. 5A,B). However, in Ro25,6981-treated cultures neither TrkB-Fc nor K252a significantly affected hilar DYN immunoreactivity (Fig. 5A,C). Ro25,6981 did not increase hilar DYN levels by the same magnitude (Fig. 5C) as seen in Fig. 2C due to the higher basal levels of hilar DYN expression in vehicle-treated cultures for this subset of experiments (compare Fig. 2A top to Fig. 5A left). However, the effect of Ro25,6981 on hilar DYN expression was still significant ($p = 0.012$, Mann–Whitney Rank Sum test). Taken together these data suggest that BDNF/TrkB receptor activation may contribute partially to basal DYN expression levels, but that alterations in BDNF and TrkB activation do not underlie chronic GluN2B antagonist-mediated increases in DYN expression.

Discussion

In this study we tested the hypothesis that chronic inhibition of GluN2B-containing NMDAR would increase CREB (Ser 133) phosphorylation, increase BDNF levels and alter downstream DYN and NPY expression. Consistent with our hypothesis, chronic treatment with the GluN2B-selective antagonists ifenprodil or Ro25,6981 increased both CREB (Ser 133) phosphorylation relative to CREB levels and granule cell mossy fiber pathway DYN expression. Chronic treatment with the non-subtype-selective NMDAR antagonists D-APV or memantine had no effect on either CREB (Ser 133) phosphorylation or DYN expression. In contrast to our hypothesis, BDNF levels were decreased following chronic treatment with Ro25,6981, but not ifenprodil. Hilar DYN expression was not significantly increased in vehicle-treated cultures or decreased in Ro25,6981-treated cultures following blockade of BDNF