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Brain-derived neurotrophic factor uses CREB and Egr3 to regulate NMDA receptor levels in cortical neurons

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Abstract

Regulation of gene expression via brain-derived neurotrophic factor (BDNF) is critical to the development of the nervous system and may well underlie cognitive performance throughout life. We now describe a mechanism by which BDNF can exert its effects on postsynaptic receptor populations that may have relevance to both the normal and diseased brain where BDNF levels either rise or fall in association with changes in excitatory neurotransmission. Increased levels of NMDA receptors (NMDARs) occur in rat cortical neurons via synthesis of new NMDA receptor 1 (NR1) subunits. The majority of synthesis is controlled by binding of cAMP response element binding protein (CREB) and early growth response factor 3 (Egr3) to the core NR1 promoter (*NR1-p*) region. BDNF-mediated NR1 transcription depends upon induction of the mitogen-activated protein kinase (MAPK) pathway through activation of the TrK-B receptor. Taken together with the fact that NMDAR activation stimulates BDNF synthesis, our results uncover a feed-forward gene regulatory network that may enhance excitatory neurotransmission to change neuronal behavior over time.

Keywords

BDNF; CREB; Egr3; NMDAR1; ZnEgr

The NMDAR is a ligand-gated ion channel that is composed of NR1 subunits and NR2 (NR2A-D) or NR3 subunits. Canonically, two subunits of NR1 form a heterotetramer with

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specific NR2 subunits and are required for functional NMDAR formation (Dingledine *et al.* 1999). The function of NMDARs is believed critical for memory processes that are modeled by long-term potentiation (Bliss and Collingridge 1993; Collingridge and Bliss 1995) and increased presence of membrane-bound NMDARs, rather than α -amino-3-hydroxy-5-methylisoxazole-4-propionate receptors, has been associated with the short-phase of long-term potentiation in adult animals (Grosshans *et al.* 2002). Increases in NR1 mRNA and protein levels are also described for the chemically kindled epileptic animal (Rafiki *et al.* 1998; Ekonomou and Angelatou 1999) and in human cases with temporal lobe epilepsy (Mathern *et al.* 1999; Doi *et al.* 2001). Increased NMDAR function has been implicated in pathological processes such as excitotoxic cell death (Choi *et al.* 1987; Choi 1992) and is hypothesized to play a role in the neurobiology of Rett syndrome (Johnston *et al.* 2001). Additionally, increases in both mRNA and protein levels of NR1 have been shown in cultured hippocampal neurons in response to BDNF treatment (Caldeira *et al.* 2007) suggestive of a general process in the brain that links BDNF levels to the availability of NMDARs at the cell surface.

The association of NMDARs with human disease and the importance of the NMDAR to normal brain physiology have prompted interest in the signal transduction and transcriptional mechanisms underlying the production of NMDAR subunit mRNAs. Genomic regulation of receptor subunits is especially of interest given the recent finding that there is an intracellular pool of NMDAR subunits that directly contributes to dynamic changes in NMDAR populations at the plasma membrane (Grosshans *et al.* 2002). It has been postulated that changes in such pools of subunits may occur in response to the activation of membrane-bound receptors and their coupled signaling pathways.

Data from our laboratory strongly suggest that NR1 synthesis is regulated by the cAMP signaling pathway after binding of CREB in response to signal-dependent phosphorylation (Lau *et al.* 2004). The core NR1 promoter region contains three CRE sites but little is known regarding the use of these sites by other signaling pathways. In an earlier study using PC12 cells that do not express functional NMDARs, it was shown that differentiation by nerve growth factor increases NR1 promoter activity by both the Ras/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase pathways (Liu *et al.* 2001), possibly mediated by Egr1 (Zif268 or NGFI-A; Bai and Kusiak 1997).

We have previously shown that BDNF causes phosphorylation of CREB at serine 133 (pCREB) and that heterodimers of pCREB and the inducible cAMP early repressor decrease transcription of *gabral*, the gene coding for the $\alpha 1$ subunit of the type A GABA receptor (GABAR). This down-regulation in subunit levels occurs in primary neurons as well as *in vivo* in models of epilepsy via activation of the Janus kinase/signal transducer and activator of transcription pathway (Lund *et al.* 2008). We have also shown that Egr3 (or Pilot) is regulated by BDNF via the MAPK pathway and that it controls high levels of *gabrb4* after status epilepticus (Roberts *et al.* 2005, 2006).

Given that multiple lines of investigation implicate BDNF in epileptogenesis (McNamara *et al.* 2006; Brooks-Kayal *et al.* 2009; Kotloski and McNamara 2010; Heinrich *et al.* 2011), that spontaneous seizures occur after intrahippocampal BDNF infusion (Scharfman *et al.*

2002), and that BDNF-dependent long-term potentiation relies on post-synaptic mechanisms (Kovalchuk *et al.* 2002), we asked if BDNF regulates the expression of NR1 subunits via the same pathways that regulate GABARs. We now report that the MAPK pathway links these two receptor systems, GABAR and NMDAR, via coordinated gene regulation that is dependent upon CREB and Egr3.

Methods

Cell culture

Primary neocortical neurons were derived from E18 rat embryos and grown in defined media as described in Russek *et al.* (2000).

Transient transfections and reporter assays

Primary neurons at days *in vitro* (DIV) 7–8 were transfected with a modified calcium chloride mediated protocol (Xia *et al.* 1996). Promoter fragments for the human NR1 promoter were cloned upstream of the luciferase gene in the pGL2 vector (Promega, Madison, WI, USA). 24 h after transfection, cells were assayed for luciferase (Promega) using the Victor 1420 detection system (E. G. Wallace). Luciferase counts were normalized to protein within each dish. Co-expression of cmv- β gal was used to determine the variability in transfectional efficiency between sister dishes in establishing the best culturing conditions for the assay. Expression constructs for Egr3 and Egr zinc finger domain (ZnEgr) were generously provided by J. Milbrandt (Washington University, Saint Louis, MO, USA) and J. Baraban (Johns Hopkins University School of Medicine, Baltimore, MD, USA), respectively.

Western blot

Whole cell extracts from primary neocortical neurons at DIV 7–8 were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) under reducing conditions and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). Proteins were visualized using enhanced chemiluminescence (GE Healthcare Lifesciences, Piscataway, NJ, USA) following incubation with a mouse anti-NR1 Ab (BD Pharmingen, San Diego, CA, USA) and anti-mouse horseradish peroxidase-conjugated secondary Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti- β -actin Ab (Sigma) was used to normalize the NR1 protein levels in the whole cell extract preparation. Immunoblots were scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and the images were quantified using ImageQuantTM TL software (GE).

Chromatin immunoprecipitation (ChIP) using radioactive PCR

Chromatin immunoprecipitation was performed as previously described (Kuo and Allis 1999). Five to 10 million cells were used for each assay and were split into three aliquots for immunoprecipitation in the presence and absence of specific antibodies, such as those for Egr1 and Egr3 (Santa Cruz). Genomic DNA (gDNA) was sheared to produce fragments of 300–500 bps. Average size was verified by agarose gel electrophoresis. Immunoprecipitated gDNAs were isolated and dissolved in 100 μ L Tris-EDTA to be used as templates for PCR to amplify a fragment of NR1 surrounding the CREB and Egr3 binding sites. Primers for

CRE region: sense, 5'-CACCTGCACATGGTGACGAGAAAC-3'; antisense, 5'-GACTGCAGCAGC TGC GGAGTCATG-3'. PCR was also performed on gDNAs precipitated with rabbit IgG (Santa Cruz) as additional negative controls. ³⁵S or ³³P labeled PCR products were separated on 10% Tris/Borate/EDTA polyacrylamide gels and exposed to X-ray film. Data were normalized as percentage of antibody/input signal.

ChIP analysis using real-time PCR

PCR primers were designed using primer express software (PE Biosystems, Foster City, CA, USA) or SciTools (IDT, Coralville, IA, USA). *NR1-p* CRE region primers: sense (5'-GTTCTTCCGCT CAGGCTTTG-3'), antisense (5'-AGGGAAACGTTCTGCTTCCA 3'); probe: 5'-FAM-CGGCATGCGCAAGGACAGCC-TAMRA3'. *NR1-p* proximal region primers: sense (5'-CGGATGATTGCACC TAGTTCTCTG-3'), antisense (5'-ATGAGCGGGACCTA CGCG TTCTA-3'); probe: 5'-FAM-TATGCTAACGCGGTGCACACA CCCTCGTG-NFQMGB3'. *NR1-p* distal region primers: sense (5'-GGCTGTCTGGGATTTACTCTGAA-3'), antisense (5'-TCA AATGAAAGGCCCGGTGTAGTG-3'); probe: 5'-VIC-AGGCTG GCCT CAAACTCAG CGATCCACCT-NFQMGB3'. Thermocycling was done using the ABI7900HT in a final volume of 20 µL. PCR parameters were 50°C for 2 min, 95°C for 15 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. Standard curves were generated from rat gDNA (Clontech, Mountain View, CA, USA).

Electrophoretic mobility shift assay

Adult rat whole brain extracts were purchased from Active Motif. A ³²P radiolabeled probe corresponding to an Egr binding element of the rat NR1 promoter (5'-TTCACGCCAACGCAGGCG-3') was incubated with 10 µg of nuclear extract. Cold wildtype oligonucleotides were used to define specificity through competition. Cold oligonucleotides were added at 100-fold excess over probe. Antibodies to Egr3 and Egr1 were pre-incubated with the nuclear extracts. The protein-DNA complexes were then resolved with PAGE gel and exposed to X-ray film.

Fluorescent microscopy

Primary neocortical cultured neurons were transfected with pDsRed2-F vector (Clontech) at DIV 7–8 to identify both plasma membranes and transfected neurons. 18 h after transfection, neurons were treated for 6 h with BDNF (50 ng/mL) or vehicle. After treatment, cells were washed and fixed with 4% paraformaldehyde using a standard protocol. Immunocytochemistry was performed using the anti-NR1 antibody (BD Pharmingen) without permeabilizing cells at 1 : 200 and the anti-mouse secondary antibody conjugated to FITC was used at a dilution of 1 : 50 (Santa Cruz). After mounting, cells were viewed using an Olympus IX 71 fluorescence microscope equipped with an UPlanApo 60×/0.9 water objective lens (Olympus, Center Valley, PA, USA). Total cell fluorescence was quantified using IPLab software (Scanalytics, Inc., Rockville, MD, USA) after correcting for background with vector (backbone) transfected cells and normalized to the total area of a selected cell as calculated using DsRed fluorescence. Changes in subunit expression were monitored using a double blind cell selection procedure.

Results

Activation of signaling by BDNF or phorbol myristate acetate (PMA) up-regulates NR1 subunit levels in cortical neurons

To investigate whether increased NR1 gene expression following activation of MAPK leads to an increase in NR1 peptide levels, cortical cultures were treated with BDNF, the general protein kinase C/MAPK activator PMA, or vehicle for 6 h ($N=4$). Following drug treatment, cultures were harvested and extracts were subjected to western blot analysis. As shown in Fig. 1, NR1 peptide levels increase ($*p < 0.05$; Student's t -test) around 50% in response to either BDNF or PMA.

NR1 promoter activity is increased in response to BDNF and PMA through stimulation of a MAPK pathway

Both BDNF and PMA are capable of stimulating MAPK signaling pathways. To test the hypothesis that these pathways are important for *NR1* transcription in primary neurons, cultures were transfected with the *NR1-p*/luciferase reporter construct (−233/+276). After transfection, neurons were treated for 6 h with vehicle, PMA ($N=16$), or 4- α -PMA ($N=9$), its inactive analog (Fig. 2a). Results show that PMA increases NR1 promoter activity by almost twofold ($**p < 0.01$; Student's t -test) whereas 4- α -PMA has no effect. Additionally, the PMA-induced increase in promoter activity is inhibited by a 1 h pre-treatment with U0126, an inhibitor of the MAPK kinase (MEK) (Fig. 2b; vehicle, $N=7$; U0126, $N=4$; $**p < 0.01$; ANOVA with Tukey's *post hoc* analyses). In parallel to changes that occur in response to PMA, *NR1-p* activity is also increased by almost threefold by a 6-h treatment with BDNF and activation is fully reversed by a 1-h pre-treatment with a tyrosine kinase receptor B receptor signaling inhibitor (K252a; $N=4$) or by the MEK inhibitor U0126 ($N=7$). The antagonists K252a and U0126 are without effect on their own (Fig. 2c; $*p < 0.05$; $**p < 0.01$; ANOVA with Tukey's *post hoc* analyses). To further determine whether BDNF activation of the MAPK signaling pathway regulates *NR1-p* activity, primary neurons were transfected with an expression vector for the constitutively active MEK1 or an empty expression vector, and co-transfected with a luciferase reporter construct containing either (i) no promoter ($N=4$), (ii) the *SV40-p* ($N=4$), or (iii) the *NR1-p* ($N=8$). Constitutively active MEK1 significantly increased activity of *NR1-p* by over 2-fold, while having no effect on activity of the other two promoters ($*p < 0.05$; Student's t -test). Taken together these results demonstrate that *NR1-p* can be stimulated by BDNF through a MAPK-dependent pathway.

Both Egr1 and Egr3 bind to the endogenous NR1 promoter

In a previous study, *in vitro* synthesized Egr1 protein was reported to bind to a GCG₅CG element in *NR1-p* (Bai and Kusiak 1997; Fig. 3a, purple box). In our studies bioinformatics was used to confirm the presence of this element (GCG₅CG), as well as an additional Egr site (Fig. 3a, red box). Results of ChIP using genomic DNA isolated from primary neocortical neurons indeed demonstrate that both Egr1 and Egr3 are present at the endogenous *NR1-p* of cortical neurons, with little or no association of Egr2 and Egr4 (Fig. 3b).

To determine whether these endogenous binding activities might be specific to the additional site in *NR1-p* (Fig. 3a, red box), as detected by ChIP, electrophoretic mobility shift assay was performed with a radiolabeled probe containing the sequence displayed in Fig. 3(a) (red box). As shown in Fig. 3(c), addition of either Egr3 or Egr1 antibodies to the reaction mixture containing adult rat whole brain nuclear extracts causes loss of a specific DNA-protein complex. These results strongly suggest that both Egr1 and Egr3 can bind to the newly identified Egr regulatory site in *NR1-p*.

NR1 promoter activity is increased by over-expression of Egr3

Previously, co-transfection of Egr1 was shown to increase *NR1-p* reporter activity in PC12 cells (Bai and Kusiak 1997). To determine whether there is a role for Egr3 in regulation of *NR1-p*, the expression construct for Egr3 or Egr1 and the *NR1-p*/luciferase reporter construct (−233/+276) were co-transfected (Fig. 4a). We now show that in primary neurons, over-expression of Egr3, but not Egr1, increases *NR1-p*/luciferase activity by two fold ($N=5$; $**p<0.01$; Student's *t*-test). Neurons transfected with *NR1-p* reporter constructs containing a 2 bp mutation in the Egr binding site, and Egr3 expression vectors, show that Egr3-dependent increases in promoter activity rely on the newly identified Egr element in the core promoter region (red box, Figs 3a and 4b; $N_{NR1}=6$, $N_{mutNR1}=3$; *, $p<0.05$; Student's *t*-test).

BDNF increases association of Egr3 with the proximal NR1 promoter

To investigate whether increases in the levels of NR1 subunits in response to BDNF signaling could be because of increased binding of Egr3 to endogenous *NR1-p*, ChIP was performed, as described above, using genomic DNA from primary neuronal cultures that were treated with either BDNF or vehicle for 2 h. After sonication that produced a majority of DNA fragments of 300–500 bps, protein-DNA complexes were immunoprecipitated with an Egr3 Ab, reverse-cross-linked and purified for quantitative PCR analysis using amplicons that were either proximal (within 200 bases from the predicted *NR1-p* start site, including the sequence within the red box in Fig. 3a) or distal (selected ~6 kb upstream of the start site; Fig. 5a). Two specific products were precipitated with the Egr3 Ab (data not shown); we refer to them here as 'proximal' and 'distal'. While there was no change in the intensity of the signal at the 'distal' site upon BDNF treatment, there was a significant increase (twofold; $N=5$; $*p<0.05$; Student's *t*-test) at the 'proximal' site that contains the newly identified Egr binding sequence (red box, Figs 3a and 5b). Taken together with the finding that Egr3 functions as an activator for the transfected *NR1-p*/luciferase reporter (Fig. 4a), our current results suggest a novel mechanism whereby BDNF alters the presence of NR1 subunits in neurons via the activation of transcription factors that bind close to the core *NR1-p* region.

Phosphorylation of CREB by BDNF and PMA at Ser133 is paralleled by presence of activated CREB at endogenous NR1

Our laboratory has previously shown that activation of a cAMP signaling pathway, with the subsequent phosphorylation of CREB, increases *NR1-p* activity (Lau *et al.* 2004). In order to determine whether BDNF signaling also leads to phosphorylation of CREB, western blot analysis was conducted using whole cell neocortical extracts with and without BDNF or

PMA treatment. Antibodies to either the N-terminus of CREB or to the pCREB were used. CREB becomes phosphorylated at Ser133 as early as 30 min after stimulation, and this effect is maintained for 4 h. Total CREB levels remain elevated after 6 h (Fig. 6a).

To determine if there is a change in the presence of phosphorylated CREB at *NR1-p* in response to BDNF (or PMA) treatment, ChIP was performed with antibodies to either the N-terminus of CREB or to pCREB, using NR1 primers as previously described (Lau *et al.* 2004). *NR1-p* specific genomic DNA was quantified by quantitative PCR. While a 1 h BDNF treatment does not change the amount of DNA precipitated with the antibody that recognizes both the phosphorylated and non-phosphorylated forms of CREB (data not shown), increased precipitation is clearly seen as detected by the pCREB antibody (Fig. 6b; $N=4$; $*p<0.05$; Student's *t*-test). This finding strongly suggests that BDNF regulates not only the activation of CREB through its serine 133 site but also its specific presence at *NR1-p*.

Dominant negative early growth response factor or dominant negative CREB reduce basal levels of *NR1-p*/luciferase reporter activity

To investigate whether CREB and Egrs might regulate basal levels of NR1 transcription in primary neocortical neurons, *NR1-p*/luciferase reporter assays were performed in the presence and absence of co-transfected constructs that express the dominant negatives K-CREB or ZnEgr (Fig. 7a). K-CREB contains an Arg to Leu mutation of the CREB DNA binding domain that prevents it from binding to DNA (Walton *et al.* 1992). K-CREB will continue to form dimers with CREB family members, thereby inhibiting binding of complexes. ZnEgr contains the highly conserved zinc finger domain of Egr1–4 and blocks the ability of Egr proteins to modulate gene expression by preventing the binding of Egrs to promoters (Levkovitz and Baraban 2001). Over-expression of K-CREB causes a reduction of basal *NR1-p*/luciferase activity to 35% of control while ZnEgr reduces activity to 25% of control, suggesting that both CREB and Egrs mediate activity-dependent NR1 expression in developing neocortical neurons ($N=3$; $*p<0.05$; $**p<0.01$; ANOVA with Tukey's *post hoc* analyses).

Co-expression of ZnEgr and K-CREB abolishes BDNF gene regulation. To test whether CREB or Egrs are downstream targets for MAPK stimulation that results in an increase in *NR1-p* activity seen after BDNF or PMA treatment, ZnEgr and K-CREB were co-transfected with the *NR1-p*/luciferase construct, either independently or together in drug treated cultures. Independently, both ZnEgr and K-CREB fail to decrease the response to BDNF (Fig. 7b; $*p<0.05$; ANOVA with Tukey's *post hoc* analyses). However, with combined expression of ZnEgr and K-CREB, the response to BDNF is lost. There is also a strong indication that a similar loss occurs in response to PMA.

ZnEgr and K-CREB attenuate BDNF-induced increases in endogenous NMDARs at neuronal membranes

To determine whether BDNF alters the levels of cell surface NMDARs in a transfection-dependent manner, neocortical neurons co-transfected with the dominant negatives ZnEgr and K-CREB were treated with BDNF or vehicle control ($N=4$; 8–12 cells from each N

were selected for analysis). In the presence of ZnEgr and K-CREB, BDNF-mediated increases in cell surface expression are blocked ($***p < 0.001$; ANOVA with Tukey's *post hoc* analyses), suggesting that the pool of surface NMDARs can indeed be regulated by changes in the activity of the endogenous *NR1-p* as it responds to activation by these two major transcription factor families (Fig. 8).

Discussion

BDNF is a member of the neurotrophin superfamily and recent studies suggest that while it is one of the most critical signaling molecules for learning and memory, it is also a major contributor to disease. The majority of BDNF signaling takes place via its recognition of the tyrosine kinase receptor B receptor that is coupled to several signaling pathways that include MAPK/protein kinase C, phosphatidylinositol 3-kinase, and phospholipase C γ (Kaplan and Miller 2000; Patapoutian and Reichardt 2001). Based on the observation that BDNF may play a major role in epilepsy and that NR1 subunit levels are found to change markedly in human cases of temporal lobe epilepsy (Mathern *et al.* 1999; Doi *et al.* 2001), as well as in primary hippocampal cultures in response to BDNF, we focused our studies on how BDNF signaling may regulate NR1 gene expression. Our data strongly suggest that BDNF activation of the MAPK pathway regulates the amount of NR1 subunits in single neocortical neurons and that a major portion of this regulation is dependent upon transcriptional mechanisms. Our data also show that coordinated activation of the endogenous *NR1-p* by CREB and Egrs is critical for the BDNF response.

Previous investigation of the transcriptional mechanisms for Ras/ERK activation of *NR1-p* has focused on the importance of a GSG/Sp1 element in the minimal promoter as assayed in PC12 cells. The following observations were made: the GSG/Sp1 element binds recombinant Egr1 in a gel mobility shift experiment; *NR1-p*/reporter is potentiated by co-transfection with Egr1 (Bai and Kusiak 1997) in PC12 cells; and the GSG/Sp1 element may mediate enhanced promoter activity during development (Okamoto *et al.* 2002). Our results from functional promoter analysis also demonstrate that Egrs may be bound, however, to a consensus Egr element (Fig. 3). This Egr binding element confers sensitivity to activation by Egr3 in close association with a functional CRE site in the proximal promoter region (Fig. 3).

We also provide evidence that Egr3, in addition to CREB, is a likely mediator of increased NR1 levels in response to BDNF. Evidence includes the observations that Egr3 binds to the endogenous *NR1-p* as assayed by ChIP; that such binding (specifically at the 'proximal' promoter region) increases upon BDNF treatment; that luciferase activity driven by *NR1-p* increases in the presence of co-transfected Egr3; and finally, that dominant negative Egr (ZnEgr), in concert with dominant negative CREB (K-CREB), blocks *NR1-p*/luciferase activity that is regulated by BDNF.

Binding of the activated form of CREB (phosphorylated at Ser133) is also increased at the endogenous *NR1-p* after BDNF stimulation (Fig. 6). Phosphorylation of CREB was previously described by our group to occur at *NR1-p* after protein kinase A stimulation by forskolin (Lau *et al.* 2004). Given that protein kinase A does not increase Egr3 binding to

NR1-p (data not shown), regulation by BDNF provides a novel mechanism of combinatorial gene regulation that enhances expression of the subunit by bringing transcriptional activation under the control of two distinct activity-dependent regulators. Activation of CREB by MAPK/ERK through the CREB kinase ribosomal protein S6 kinase (for review, see Lonze and Ginty 2002) is well documented in the literature.

Additional evidence for the concerted role of CREB and Egrs in the maintenance of basal levels of NMDARs in developing neocortical neurons comes from the use of two dominant negatives, ZnEgr and K-CREB, that block regulation via Egr and CREB, respectively. Co-transfection of expression constructs for either dominant negative decreases *NR1-p*/luciferase activity to about a third of control. Most importantly, co-expression of ZnEgr and K-CREB also inhibits BDNF-mediated increases in endogenous membrane-associated NMDARs (Fig. 8), suggesting that the BDNF-induced transcriptional mechanism under study may be biologically relevant.

The fact that K-CREB, only in combination with ZnEgr, blocks BDNF-induced increases in *NR1-p* activity was surprising given the results of these dominant negatives on levels of basal activity in cortical neurons where both CREB and Egr proteins may be limiting. This result may reflect, however, that the core promoter contains three distinct CRE sites and as the levels of pCREB rise in response to BDNF, activation driven by more than one of these sites may now be sufficient for a full transcriptional response. We previously have shown that mutation in all three CRE sites is necessary to knockout *NR1-p* transcription, as single and double mutations are without effect (Lau *et al.* 2004). Likewise, BDNF causes a striking increase in the levels of Egr3 that on their own may be sufficient for full activity of the promoter region.

CREB and Egr proteins may account for altered NR1 subunit levels observed in the epileptic brain. Levels of Egr3 increase after electroconvulsive shock (O'Donovan *et al.* 1998) and CREB phosphorylation rapidly occurs after seizure activity in the hippocampus and the cortex (Moore *et al.* 1996). In an animal model of epilepsy, NR1 immunoreactivity increases in dentate granule cells and these changes are correlated with the time scale for recurrent mossy fiber reinnervation of the dentate (Mikuni *et al.* 2000), a process that is believed to be critical in epileptogenesis (Sutula *et al.* 1988, 1989). Based on our observations that CREB and Egrs mediate the majority of *NR1-p* activity in primary neocortical neurons, and that their transcriptional function is regulated by the Ras/Erk MAPK pathway, current studies in our laboratories are underway to determine whether they may be critical factors that mediate excitatory synaptic connections both in the developing brain and in neurological diseases, including epilepsy and its comorbidities of cognitive disorders and depression.

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Abbreviations used

BDNF	brain-derived neurotrophic factor
ChIP	chromatin Immunoprecipitation
CREB	cAMP response element binding protein
DIV	days <i>in vitro</i>
Egr	early growth response factor
ERK	extracellular signal-regulated kinase
GABAR	GABA receptor
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
NMDAR1	NMDA receptor 1 subunit
NMDARs	NMDA receptors
PAGE	polyacrylamide gel electrophoresis
pCREB	phosphorylation of CREB at serine 133
PMA	phorbol myristate acetate
ZnEgr	Egr zinc finger domain

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Fig. 1. BDNF and PMA increase NR1 proteins levels. Rat neocortical cultures, 7–9 days *in vitro* (DIV), were treated with vehicle (H_2O), BDNF (50 ng/mL), or phorbol 12-myristate 13-acetate (PMA, 1 μ M). After 6 h, whole cell extracts were prepared as described in the methods section for detection of NR1 protein via western blot. A representative western blot is shown. NR1 peptide levels normalized to β -actin levels in the vehicle-treated cultures are defined as 100%. $N = 4$; * $p < 0.05$; Student's t -test.

Fig. 2.

BDNF and PMA up-regulates *NR1-p* activity in a MAPK dependent fashion. (a) Rat neocortical cultures were transfected with the *NR1-p*/luciferase reporter construct, *NR1-p* (-233/+276). Cultures were treated for 6 h with vehicle (0.5% DMSO), PMA (1 μ M; $N=16$), or 4- α -PMA (1 μ M; $N=9$), an inactive analog of PMA. Data represent mean $\pm$ SEM and are expressed as a percentage of activity from control dishes (% Control). ** $p < 0.01$; Student's *t*-test. (b) Cultures transfected with the *NR1-p* (-233/+276) were pre-treated with vehicle (0.5% DMSO; $N=7$) or U0126 (20 μ M; $N=4$) for 1 h followed by a 6-h treatment with vehicle or PMA (1 μ M). Note that control cultures received vehicle during both treatment phases. Data are presented as mean $\pm$ SEM and are expressed as a percentage of activity from control dishes (% control). $N_{\text{vehicle}} = 7$, $N_{\text{U0126}} = 4$; ** $p < 0.01$; ANOVA with Tukey's *post hoc* analyses. (c) Cultures transfected with the *NR1-p* (-233/+276) were pre-treated with vehicle (0.5% DMSO), K252a (200 nM; $N=4$), or U0126 (20 μ M; $N=7$) for 1 h followed by a 6-h treatment with vehicle or BDNF (50 ng/mL). Data are presented as mean $\pm$ SEM and are expressed as a percentage of activity from control dishes (% control). * $p < 0.05$; ** $p < 0.01$; ANOVA with Tukey's *post hoc* analyses. (d) Cortical cultures were cotransfected with an expression vector coding for constitutively active MEK1 (S218/222E) and either one of the following luciferase reporter constructs: (i) a promoterless reporter construct ($N=4$), (ii) a reporter construct containing the SV40 promoter ($N=4$), or (iii) the NR1 promoter (-233/+276; $N=8$). Control dishes were co-transfected with the corresponding luciferase reporter and an empty expression vector. After 20–24 h, cultures were harvested and luciferase assays were performed. Results are expressed as a percentage of activity from control dishes defined as 100%. Error bars denote SEM. * $p < 0.05$; Student's *t*-test.

Fig. 3. Binding of Egr3 and Egr1 to the NR1 promoter. (a) A schematic of the NR1 promoter region. Transcriptional start sites are indicated by arrows. Sequence corresponding to transcription factor binding sites that are validated in the literature are indicated. (b) Chromatin immunoprecipitation assays were performed with E18 rat neocortical cultures. Cells were fixed, sonicated to produce 300–500 bp DNA fragments, and protein-DNA complexes were precipitated with antibodies to Egr1–4 (Santa Cruz, CA, USA). Genomic DNA specific to the NR1 subunit gene promoter was amplified using a PCR primer pair containing the Egr binding element and visualized by autoradiography. Three independent experiments showed similar results. (c) Electrophoretic mobility shift assay was performed with a nuclear fraction of the adult rat whole brain extracts, incubated with a ^{32}P radiolabeled probe corresponding to an Egr binding element of the rat NR1 promoter. Cold wildtype oligonucleotides, added at 100-fold excess, were used to define specificity through competition. Antibodies to Egr3 and Egr1 were pre-incubated with the nuclear extracts. The conditions for each lane are as indicated. Specific binding complexes are shown with arrowheads. Open arrowhead shows an Egr1 specific complex and the asterisk shows a complex specific to Egr3. The supershift assay was performed three times with similar results.

Fig. 4.

Egr3 increases NR1 promoter activity. (a) Vectors containing the minimal *NR1-p* were transfected into primary neocortical cultures in the presence and absence of an expression construct containing either the Egr3 or Egr1 cDNA under the control of the CMV promoter ($N=5$). Sister dishes were co-transfected with a CMV construct that did not contain an Egr transgene to control for promoter competition indicated as 'vector'. Data are presented as mean $\pm$ SEM. $**p < 0.01$; Student's t -test. (b) Vectors containing the minimal *NR1-p* with (mutNR1; $N=3$) and without (NR1; $N=6$) a 2-bp mutation in the Egr binding site (red box, Fig. 3a) were transfected into primary neocortical cultures in the presence and absence of an expression construct containing the Egr3 cDNA under the control of the CMV promoter. Sister dishes were co-transfected with a CMV construct that did not contain an Egr3 transgene to control for promoter competition indicated as 'vector'. Data are normalized to the NR1 promoter co-transfected with the 'vector' control set at 100%. Data are presented as mean $\pm$ SEM. $*p < 0.05$; Student's t -test.

Fig. 5.

Binding of Egr3 to the NR1 promoter is increased with BDNF treatment. (a) A schematic of the NR1 promoter region. Egr3 is shown to bind to sites that are 'distal' (~6 kb upstream) and 'proximal' (within 200 bps of the start site) to the promoter in cultured primary neurons. (b) Presence of Egr3 at the proximal region of the NR1 promoter significantly increases upon BDNF treatment (2.02 ± 0.46 -fold) but not at the distal region (1.04 ± 0.44 -fold) where there is also specific binding activity. Data are presented as mean $\pm$ SEM. $N=5$; * $p < 0.05$; Student's t -test.

Fig. 6.

Activated pCREB binding is increased at endogenous NR1 after BDNF stimulation. (a) Rat neocortical cultures, 7–9 days *in vitro*, were treated with vehicle, BDNF (50 ng/mL), or PMA (1 μ M) for 30 min, 1, 2, 4, or 6 h. Whole cell extracts were prepared and resolved by sodium dodecyl sulfate–PAGE under reducing conditions. Proteins were visualized using enhanced chemiluminescence following incubation with an anti-CREB Ab or an Ab to its activated form pCREB (Ser133 phosphorylated). A representative western blot is shown. (b) Chromatin immunoprecipitation (ChIP) was used to determine whether pCREB is bound to the endogenous NR1 gene in primary cortical neurons after BDNF treatment. Real-time PCR was used to monitor the ability of pCREB antibodies to precipitate sonicated genomic fragments of the endogenous NR1 promoter after 1 hr treatment with BDNF (or PMA; see insert). $N = 4$; * $p < 0.05$; Student's t -test.

Fig. 7.

CREB and Egr are responsible for *NR1-p*/reporter activity in cortical neurons. (a) Vectors containing the minimal *NR1-p* driving expression of the luciferase reporter were transfected into primary neocortical cultures in the presence of an expression construct containing one of the following dominant negatives: ZnEgr and K-CREB. Sister dishes were co-transfected with only the vector backbone, a CMV construct as indicated by 'vector'. Neocortical cultures were assayed 24 h post-transfection. Data are normalized to the *NR1-p*/luciferase reporter construct co-transfected with the 'vector' control set at 100%. Data are presented as mean $\pm$ SEM. $N = 3$; * $p < 0.05$; ** $p < 0.01$; ANOVA with Tukey's *post hoc* analyses. (b) Vectors containing the minimal *NR1-p*/reporter were transfected into primary neocortical cultures in the presence and absence of an expression construct containing the dominant negatives ZnEgr and K-CREB under the control of the CMV promoter. 18 h after transfection, neocortical cultures were treated with BDNF (50 ng/mL; $N = 5$) or PMA (1 μ M; $N_{\text{K-CREB}} = 4$; $N_{\text{K-CREB+ZnEgr}} = 4$; $N_{\text{ZnEgr}} = 10$) for 6 h and assayed for luciferase activity. Data are presented as mean $\pm$ SEM and are expressed as a percentage of activity from control sister dishes (% control; 100% is represented as a dashed horizontal line). * $p < 0.05$; ANOVA with Tukey's *post hoc* analyses.

Fig. 8. K-CREB and ZnEgr attenuate BDNF-induced NR1 surface expression. Cultured neocortical neurons were co-transfected with a vector coding for a dsRED-farnesylated fusion protein targeted to plasma membranes and those containing dominant negatives K-CREB and ZnEgr or vector backbone. Neurons were treated with vehicle or BDNF (50 ng/mL) for 6 h. Presence of endogenous NR1 subunits was detected in non-permeabilized neurons using an NR1-specific antibody and a FITC-labeled secondary antibody. Fluorescence microscopy was performed to image single neurons. Red labeling: membrane-targeted dsRED-farnesylated fusion protein. Green labeling: endogenous surface NR1 subunits. Yellow: merge indicating relative presence of dsRED-farnesylated fusion proteins and NR1 subunits in the same cell. The histogram shows data from a total of four experiments (8–12 transfected neurons (per dish/experiment) were chosen without knowledge of condition). *** $p < 0.001$; ANOVA with Tukey's *post hoc* analyses.