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ARTICLE

Pharmacological characterization of BDNF promoters I, II and IV reveals that serotonin and norepinephrine input is sufficient for transcription activation

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Abstract

Compelling evidence has shown that the effects of antidepressants, increasing extracellular serotonin and noradrenaline as a primary mechanism of action, involve neuroplastic and neurotrophic mechanisms. Brain-derived neurotrophic factor (BDNF) has been shown to play a key role in neuroplasticity and synaptic function, as well as in the pathophysiology of neuropsychiatric disorders and the mechanism of action of antidepressants. The expression of BDNF is mediated by the transcription of different mRNAs derived by the splicing of one of the eight 5′ non-coding exons with the 3′ coding exon (in rats). The transcription of each non-coding exon is driven by unique and different promoters. We generated a gene reporter system based on hippocampal and cortical neuronal cultures, in which the transcription of luciferase is regulated by BDNF promoters I, II, IV or by cAMP response element (CRE), to investigate the activation of selected promoters induced by monoaminergic antidepressants and by serotonin or noradrenaline agonists. We found that incubation with fluoxetine or reboxetine failed to induce any activation of BDNF promoters or CRE. On the other hand, the incubation of cultures with selective agonists of serotonin or noradrenaline receptors induced a specific and distinct profile of activation of BDNF promoters I, II, IV and CRE, suggesting that the monoaminergic input, absent in dissociated cultures, is essential for the modulation of BDNF expression. In summary, we applied a rapidly detectable and highly sensitive reporter gene assay to characterize the selective activation profile of BDNF and CRE promoters, through specific and different pharmacological stimuli.

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Introduction

Dysfunction in monoaminergic pathways has been implicated in the pathophysiology of depression and, consistently, selective serotonin (5-HT) and 5-HT/noradrenaline (NA) reuptake inhibitors (SSRIs and SNRIs, respectively) are the most frequently prescribed drugs as first line treatment for depression. However, the increase of extracellular levels of monoamines represents only the first step of the antidepressant effect. Indeed, mood disorders are associated with alterations in synaptogenesis, dendritic remodelling and neurogenesis in hippocampal, prefrontal cortical and limbic regions (McEwen, 2005; Pittenger and Duman, 2008; Gorman and Docherty, 2010; Sanacora et al., 2012). On the other hand, chronic antidepressants evoke adaptive changes in intracellular signalling pathways, ultimately inducing plastic modifications that could explain the need for long-term treatment to achieve clinical efficacy (Popoli et al., 2001; Tardito et al., 2006; Castrén et al., 2007; Racagni and Popoli, 2008; Ryan et al., 2009; Krishnan and Nestler, 2010).

In line with these observations, converging evidence has implicated changes in the neurotrophin brain-derived neurotrophic factor (BDNF)/TrkB signalling pathway both in the pathophysiology of depression, and in antidepressant action. Reduced BDNF expression has been observed in the hippocampus and prefrontal cortex of depressed patients (Chen et al., 2001; Dwivedi et al., 2003; Thompson et al., 2011; Birkenhäger et al., 2012). Moreover, changes in BDNF mRNA and protein levels were measured after chronic antidepressants, suggesting a role of BDNF in the action of the drugs (Dias et al., 2003; Russo-Neustadt et al., 2004; Dwivedi et al., 2006;

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Khundakar and Zetterström, 2006; Calabrese et al., 2007; Wang et al., 2008; Alboni et al., 2010; Baj et al., 2012).

The *bdnf* rat gene is composed of eight untranslated 5′ exons and one 3′ exon encoding the protein (Aid et al., 2007). Different transcript isoforms are expressed by splicing each of the non-coding exons to the coding exon (9). Although the function of the different BDNF transcripts is not yet completely understood, a differential role during plasticity-associated changes has been suggested (West et al., 2001; Chiaruttini et al., 2008). The different 5′ exons are transcribed from unique and alternative promoters, used as a versatile mechanism to create flexibility in the regulation of gene expression and subcellular mRNA localization (Aid et al., 2007; Chiaruttini et al., 2008; Pruunsild et al., 2011). To date, the promoters of BDNF exons 1, 2 and 4 (respectively, promoter I, II and IV) have been studied in more detail (Tabuchi et al., 2002; Takeuchi et al., 2002; Tao et al., 2002; Pruunsild et al., 2011). BDNF promoter I contains calcium response elements (CaREs), bound by CRE binding protein (CREB) and upstream stimulatory factors 1/2 (USF1/USF2), and a binding site for the nuclear factor kB (NF-kB) (Tabuchi et al., 2002; Kairisalo et al., 2009). BDNF promoter I and II are negatively regulated by repressor element-1 (RE-1)-silencing transcription factor (REST) on their neuron restrictive silencer elements (NRSE) (Timmusk et al., 1999; Hara et al., 2009). Promoter IV is strongly activity-responsive and is the most extensively studied at the molecular level (Shieh et al., 1998; West et al., 2001). A tight temporal, spatial and stimulus-specific regulation of BDNF promoter IV is achieved by a complex interplay between multiple activity-regulated transcriptional pathways. Three CaREs are selectively bound and cooperatively regulated by the transcription factor calcium-response factor (CaRF), USF1/USF2 and CREB (Shieh et al., 1998; Tao et al., 1998; Chen et al., 2003). Moreover, an alternative transcription start site is regulated by the association of NF-kB (Lipsky et al., 2001) and the basic helix–loop–helix (bHLH) protein bHLHB2 (Jiang et al., 2008), with elements localized between the two transcription start sites. Finally, neuronal activity-dependent activation of both BDNF promoters I and IV was found to be dependent on ARNT2/NPAS4 binding to an asymmetric E-box-like element (Pruunsild et al., 2011).

Although there is a general agreement that chronic antidepressants increase BDNF transcription *in vivo*, it is not known if this effect can be replicated *in vitro* in primary neuronal cultures. Therefore, in the present study, we used an *in vitro* gene reporter system based on primary neuronal cultures to characterize the transcriptional regulation of BDNF promoters I, II and IV, and CRE promoter after *in vitro* incubation with monoaminergic antidepressants (fluoxetine (FLX) and reboxetine (RBX)). Moreover, to verify if the monoaminergic input, absent in dissociated cultures, is necessary for the modulation of BDNF and CRE promoters activity, we also studied the effects of incubation of neuronal cultures with selective serotonergic or noradrenergic agonists.

We found that the incubation *in vitro* of neuronal cultures with FLX and RBX was not able to increase the transcription of BDNF promoters I, II, IV and CRE, likely due to the lack of serotonergic and noradrenergic subcortical afferents. However, the acute treatment of cultures with distinct serotonergic and noradrenergic agonists exerted a selective and distinct transcriptional activation of BDNF promoters I, II, IV and CRE.

Methods

Plasmid construction

The BDNF promoter regions of exons 1, 2 and 4 (Timmusk et al., 1993) (from −528 bp upstream from the initiation site to +138 bp downstream, for promoter I; from −830 to +100 bp, for promoter II; from −629 to +281 bp, for promoter IV) were amplified from rat genomic DNA. Primers were designed in order that each promoter has two different digestion sites (XhoI and HindIII for promoters I and IV and BglII and MluI for promoter II), for a directional insertion in the *Firefly* luciferase expression vector (pGL3-Basic Vector, Promega Italy SRL, Italy) (Table 1). The amplified sequences were subcloned in pGEM-T Easy Vector (Promega). Promoter sequences were checked by sequencing, starting from both ends (Value Read DNA sequencing service, MWG-Biotech, Germany). Each promoter was excised from the cloning vector, purified by electrophoresis and ligated into pGL3-Basic Vector. The constructs obtained (pGL3-BDNF promoter I, pGL3-BDNF promoter II, pGL3-BDNF promoter IV) were sequenced, amplified and purified with Endo-Free Plasmid Maxi Kit (Qiagen, Italy).

As an internal control, we used *Renilla* luciferase vector (pRL Vector, Promega) carrying the rat elongation factor 1α promoter (EF1α), which is non-responsive to calcium signals and drug treatments (Tabuchi et al., 1998) (EF1α-pRL). This construct was generated in the same way as the experimental vectors (see Table 1 for primers used for cloning).

Primary neuronal cultures preparation

Primary cultures of rat hippocampal and cortical neurons were prepared from 17–18 d old rat embryos by trypsin +DNAse (Sigma-Aldrich Italy, Italy) treatment and mechanical dissociation in Hanks' Balanced Salt Solution (HBSS) (Invitrogen S.R.L., Italy). Cultures used for the measurement of endogenous expression of BDNF exons were not transfected; for other experiments, neurons were transfected before plating (see below). All cultures were grown in Neurobasal medium, containing B27 supplement, glutamine and penicillin/streptomycin (Invitrogen) and incubated at 37 °C in a humidified CO_2 (5%) incubator (Brewer et al., 1993). Experimental procedures on animals were performed in accordance with

Table 1. Sequences of primers used for the cloning of promoter regions from rat genomic DNA

Promoter	Primer forward	Primer review
BDNF I	ACACTCGAGGGATCCTCCCCTCCTAGCC	GAAAAGCTTCGCCTTGTCAGGCTAGGG
BDNF II	CCAACGCGTATTACCTTCCAGCATCTGTTGG	AATAGATCTAACTTCAGCGAGCTCAATGAGG
BDNF IV	CCGCTCGAGCAAGAGGCTGTGACACTATGC	CCGAAGCTTCAGTCACTACTTGTCAAAG
EF1α	GGCAGATCTAGAGTAATTCATACAAAAGGAGGG	GGCACGCGTGTTGCTTTGAATTAGCGGTGGCTTTC

Table 2. Sequences of primers used for RT-PCR

	Primer forward	Primer review
BDNF exon 1	ACTCAAAGGGAAACGTGTCTCT	GCCTTCATGCAACCGAAGTA
BDNF exon 2	CGGTGTAGGCTGGAATAGACT	GCCTTCATGCAACCGAAGTA
BDNF exon 4	CTCCGCCATGCAATTTCCACT	GCCTTCATGCAACCGAAGTA
BDNF exon 6	GTGACAACAATGTGACTCCACT	GCCTTCATGCAACCGAAGTA
GAPDH	CAATGACCCCTTCATTGACC	AGTTGTCATGGATGACCTTGG

the European Community Council Directive 86/609/EEC, and were approved by Italian legislation on animal experimentation (Decreto Legislativo 116/1992).

RNA isolation, cDNA synthesis and reverse transcription-PCR for BDNF isoforms

Neurons were seeded in Poly-D-Lisine (Invitrogen)/Laminin (Becton Dickinson Italy, Italy) coated six-well plates at 5×10^5/well. Depolarization was performed after 7 or 14 d *in vitro* (DIV) with 25 mM KCl for 6 h (Tabuchi et al., 2002). Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed with ImProm-II Reverse Transcription System (Promega) (Donnici et al., 2008). cDNA was amplified with selective primers (Table 2). BDNF exons were amplified together with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, used as housekeeping (Table 2). The number of cycles and primer concentrations were chosen experimentally to stay within the exponential phases of the amplification reaction. For semi-quantitative analysis, amplicons were detected by gel electrophoresis (0.8% agarose, ethidium bromide staining). Bands were acquired with Bio-Rad GelDoc System (Bio-Rad Laboratories, Italy), intensities were measured with Quantity One software (Bio-Rad Laboratories) and normalized on GAPDH.

Immunocytochemistry

Rat neurons were electroporated (3×10^6 per transfection), in Rat Neuron Nucleofector Solution, using Amaxa Nucleofector program G-13 (Amaxa Gmb, Germany), with 3 mg of a plasmid carrying the coding sequence of green fluorescent protein under SV40 promoter (pSV40-GFP) and plated at 1×10^5/well on coated 13 mm coverslips. After 7, 10 or 14 DIV, cells were fixed with 4% paraformaldehyde. Primary antibodies used were a monoclonal antibody against the neuron-specific nuclear protein NeuN (Chemicon, USA) and polyclonal antibodies against the glia-specific glial fibrillary acid protein (GFAP) (Dako Italy SpA, Italy) and microtubule-associated protein 2 (MAP-2) (Chemicon). Secondary antibodies used were anti-mouse Rhodamine-conjugated and/or anti-rabbit Cy5-conjugated antibodies. 40 fields were analysed with AxioVision 4.3 microscope (Carl Zeiss Vision GmbH, Germany); 40× magnification, 10 fields in four dishes.

Transfection of neurons with pGL3-BDNF promoter I, pGL3-BDNF promoter II, pGL3-BDNF promoter IV and pGL3-CRE

Rat neurons were electroporated ($2–5\times10^6$ per transfection) with 3 mg of total DNA in a 5:1 molar ratio between the experimental vector (pGL3-BDNF promoter I, pGL3-BDNF promoter II, pGL3-BDNF promoter IV or pGL3-CRE) and the internal control EF1α-pRL, in Rat Neuron Nucleofector Solution, using Amaxa Nucleofector, program G-13 (Amaxa GmbH). Neurons were then plated $7.5–10\times10^4$ on coated 96-well plates and kept in culture for 7, 10 or 14 DIV (for details, see below).

In vitro *drug treatment of neurons transfected with pGL3-BDNF promoter I, pGL3-BDNF promoter II, pGL3-BDNF promoter IV and pGL3-CRE*

In vitro depolarization was performed after 7, 10 or 14 DIV with 25 mM KCl for 6 h (Tabuchi et al., 2002).

Treatments with FLX (a selective 5-HT reuptake inhibitor) and RBX (a selective NA reuptake inhibitor) were performed by incubating cultures with 2, 5 or

10 μM FLX or RBX hydrochloride for 6, 24, 48 or 72 h before luciferase expression measurement; treatment with lithium was performed by incubating cultures with 1 mM lithium carbonate (Sigma-Aldrich) for 24 or 72 h before luciferase expression measurement (all cultures were maintained for 10 DIV).

Treatment with BDNF was performed in 10 DIV cultures with 60 ng/ml in HBSS (Sigma-Aldrich) for 10 min; luciferase expression was measured after 6 h. Incubations with selective receptor agonists and antagonists were performed in 10 DIV cultures, treating neurons for 10 min in HBSS with 3 or 10 μM 5-HT, (R)-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT) (a full 5-HT_{1A} serotonin receptor agonist; active enantiomer), (R)(−)-DOI hydrochloride (DOI) (a potent and selective 5-HT2 serotonin receptor agonist), NA, (−)-Isoproterenol hydrochloride (a β-adrenoceptor agonist) or (R)-(−)-Phenylephrine hydrochloride (a α_1-adrenoceptor agonist). Luciferase expression was measured after 60 min.

All drug concentrations were chosen to expose neuronal cultures to a drug concentration as close as possible to those reached in brain tissue after (systemic) administration *in vivo*. It was shown that the therapeutic concentrations of monoaminergic antidepressants in human plasma are around 1 μM (Kelly et al., 1989; DeVane, 1999), but the concentrations in brain are higher (Karson et al., 1993; Strauss et al., 1997). Clinically relevant extracellular concentrations of lithium are in the low mM range. Electrophysiological effects of 5-HT, NA and monoaminergic agonists and antidepressants have been studied in the micromolar concentration range (Mueller et al., 1981; Krause and Jia, 2005).

Measurement of luciferase expression

For the measurement of both *Firefly* and *Renilla* luciferases levels, Dual-Glo® Luciferase Assay System (Promega) was used, following manufacturer instructions. A plate reader beta counter (Beckman Coulter, Italy) was used for the detection of light emission.

Statistical analysis

To analyse the results of endogenous BDNF exons, two-way analysis of variance (ANOVA) for the variables days in culture and treatment (KCl), followed by Bonferroni's *post-hoc* multiple comparison test (BPHT) was used. To analyse luciferase expression induced by depolarization in neuronal cultures transfected with pGL3-BDNFprI, pGL3-BDNFprII and pGL3-BDNFprIV, two-tailed Student's *t*-test for unpaired samples was used. In time–course experiments of BDNF and CRE promoters activity after *in vitro* incubation with antidepressants, two-way ANOVA for the variables time and treatment, followed by BPHT, was performed. For the study of the effects of *in vitro* treatment with BDNF, serotonergic and noradrenergic agonists on transcription activity of BDNF and CRE promoters, one-way ANOVA, followed by BPHT, was applied.

In all tests used, significance was assumed at $p<0.05$. Statistical analyses were carried out using GraphPadPrism4 (GraphPadSoftwareInc., USA).

Results

Endogenous expression of BDNF exons 1, 2, 4 (but not 6) is induced by depolarization

Because it is well established that total BDNF mRNA transcription is activated by membrane depolarization (Tabuchi et al., 2000; Tao et al., 2002; Pruunsild et al., 2011), we first investigated the effects of KCl stimulation of hippocampal cultures on the endogenous expression of the most abundantly expressed BDNF exons (transcripts 1, 2, 4 and 6) by RT-PCR. We found that, both in 7 and 14 DIV cultured hippocampal neurons, the expression of exons 1, 2 and 4 containing transcripts was markedly up-regulated by membrane depolarization (two-way ANOVA, significant effect of KCl; exon 1: $F_{1,16}=88.04$ $p<0.001$, BPTH: $p<0.001$ KCl *vs.* respective CNT; exon 2: $F_{1,16}=16.53$ $p<0.001$, BPTH $p<0.01$ KCl 7 DIV *vs.* respective CNT, $p<0.05$ KCl 14 DIV *vs.* respective CNT; exon 3: $F_{1,16}=38.48$ $p<0.001$, BPTH $p<0.01$ KCl *vs.* respective CNT) (Fig. 1*a–c*), whereas the expression of the exon 6 was not changed by *in vitro* stimulation (two-way ANOVA; no effect of KCl: $F_{1,16}=0.07$ $p=0.79$) (Fig. 1*d*). Our results are in line with previous studies (Tabuchi et al., 2002; Pruunsild et al., 2011).

Evaluation of transfection efficiency and of in vitro *neuronal maturation*

Before transfecting neurons with BDNF promoter vectors, we used immunocytochemistry to assess the transfection efficiency and *in vitro* viability of rat neurons transfected with the Amaxa Nucleofector system. Moreover, to check for possible interference of glial cells, we also measured the percentage of astroglia and assessed whether astroglia could be transfected. Rat hippocampal cultures were transfected with a plasmid carrying the coding sequence of GFP under the constitutive promoter SV40 (see Material and Methods for details).

Interestingly, the percentage of GFP positive (transfected) cells was similar in 7, 10 and 14 DIV (66.17±3.47%, 63.44±3.063% and 65.03±3.29%, respectively, one-way ANOVA, $F_{2,58}=2.69$, $R^2=0.089$, $p=0.077$) (Supplementary Figure S1), suggesting high transfection efficiency (as previously reported, Gärtner et al., 2006) and good viability of transfected cells. However, GFAP positive (glia) cells were 32.37±3.09% in 7 DIV culture, 39.36±2,87% in 10 DIV and 52.79±4.08% in 14 DIV, suggesting *in vitro* growth of astroglia (one-way ANOVA, $F_{2,56}=9.65$, $R^2=0.28$, $p<0.001$, BPHT: $p<0.001$ 14 DIV *vs.* 7 DIV, $p<0.05$ 14 DIV *vs.* 10 DIV) (Supplementary Figure S1). Interestingly, we also found that the number

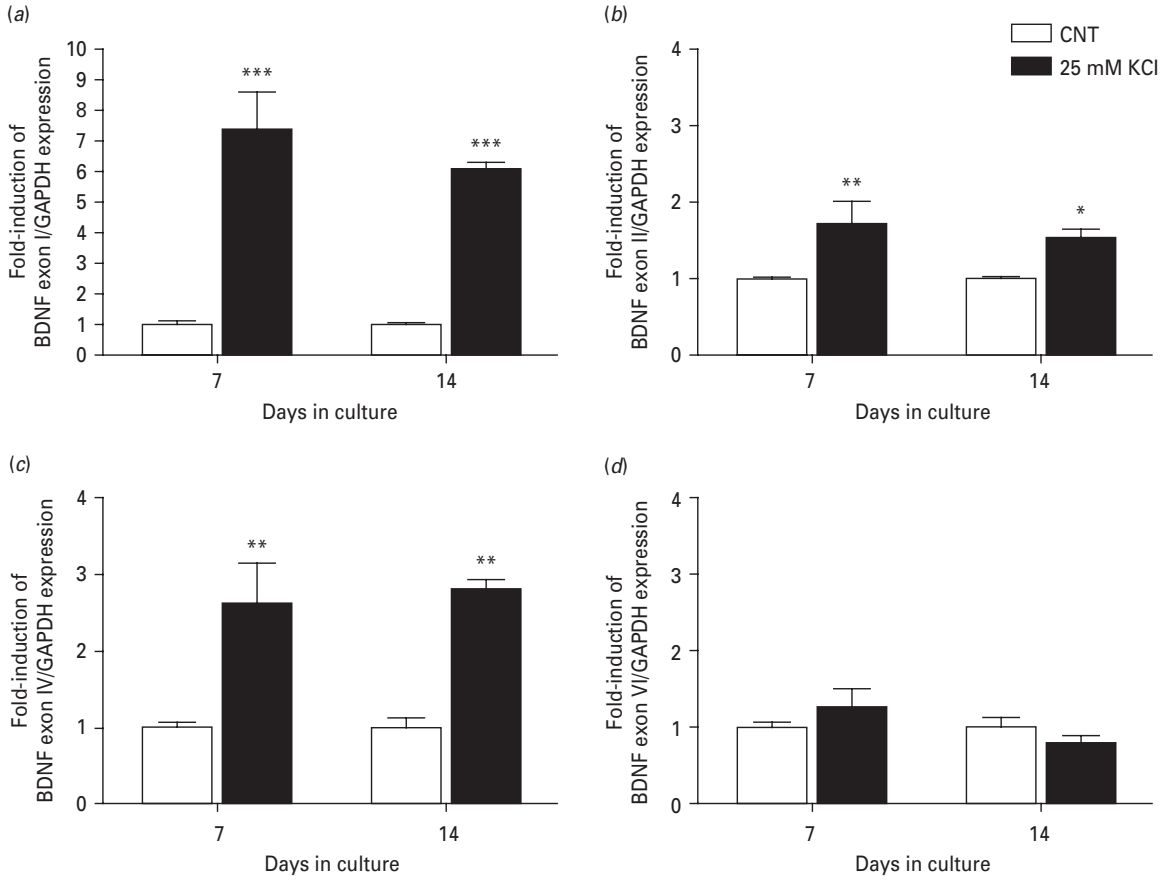

Fig. 1. Endogenous expression levels of BDNF exon I, II, IV and VI in 7 and 14 d *in vitro* (DIV) hippocampal cultures. Expression level of BDNF exon I (*a*), II (*b*), IV (*c*), VI (*d*) in 7 and 14 DIV hippocampal cultures in basal conditions (CNT, white bars) and after 6 h *in vitro* depolarization with KCl 25 mM (black bars), measured with RT-PCR. Data are normalized on GAPDH levels and expressed as fold change *vs.* CNT±S.E.M. Statistics: two-way ANOVA followed by BPHT. *$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs.* CNT, $n=6$, two independent experiments.

of NeuN positive (neurons) transfected cells was significantly reduced in 14 DIV compared with 7 DIV (7 DIV 84.28±3.74%, 10 DIV 75.87±4.83%, 14 DIV 69.61±2.89%; one-way ANOVA, $F_{2,59}=3.36$, $R^2=0.10$, $p<0.05$; BPHT: $p<0.05$ 14 DIV *vs.* 7 DIV) (Supplementary Figure S1).

We also used immunocytochemistry to assess the level of neuronal maturation, immunostaining cultures with MAP-2, allowing visualization of dendritic arborization. We found that all cultures were MAP-2 positive, but 7 DIV cultures showed low dendritic ramification, compared to 10 and 14 DIV (compare Supplementary Figure S2*b* with Supplementary Figure S2*e–h*).

Together, these results showed that, on the one hand, in 7 DIV cultures, astroglia contamination is low and the high majority of transfected cells were neurons, but dendritic arborization is poor. On the other hand, in 14 DIV cultures, despite neurons reaching a good state of maturation, the astroglia percentage significantly increased, and neurons percentage in the transfected population decreased. Therefore, in all further experiments, we used 10 DIV cultures, which showed an adequate neuronal maturation with lower interference of transfected glial cells compared with 14 DIV cultures.

In vitro *depolarization of neurons transfected with pGL3-BDNF promoter I, II and IV constructs and luciferase assay*

In hippocampal cultures transfected with pGL3-BDNF promoter I, we observed a 10-fold increase of the expression of luciferase after depolarization (Student's *t*-test, $p<0.001$) (Fig. 2*a*) while, after transfection with pGL3-BDNF promoter II or IV vectors, KCl evoked a two- to three-fold increase of luciferase transcription (Student's *t*-test, $p<0.001$) (Fig. 2*b*, *c*). Interestingly, in hippocampal cultures, the induction of luciferase expression, resulting from depolarization-dependent activation of BDNF promoters I, II and IV, was in line with the results obtained with endogenous transcription of exons I, II and IV (compare Fig. 1 and Fig. 2*a–c*). Also in cortical cultures we measured a significant increase of luciferase expression induced by *in vitro* depolarization, similar to the endogenous transcription of exons

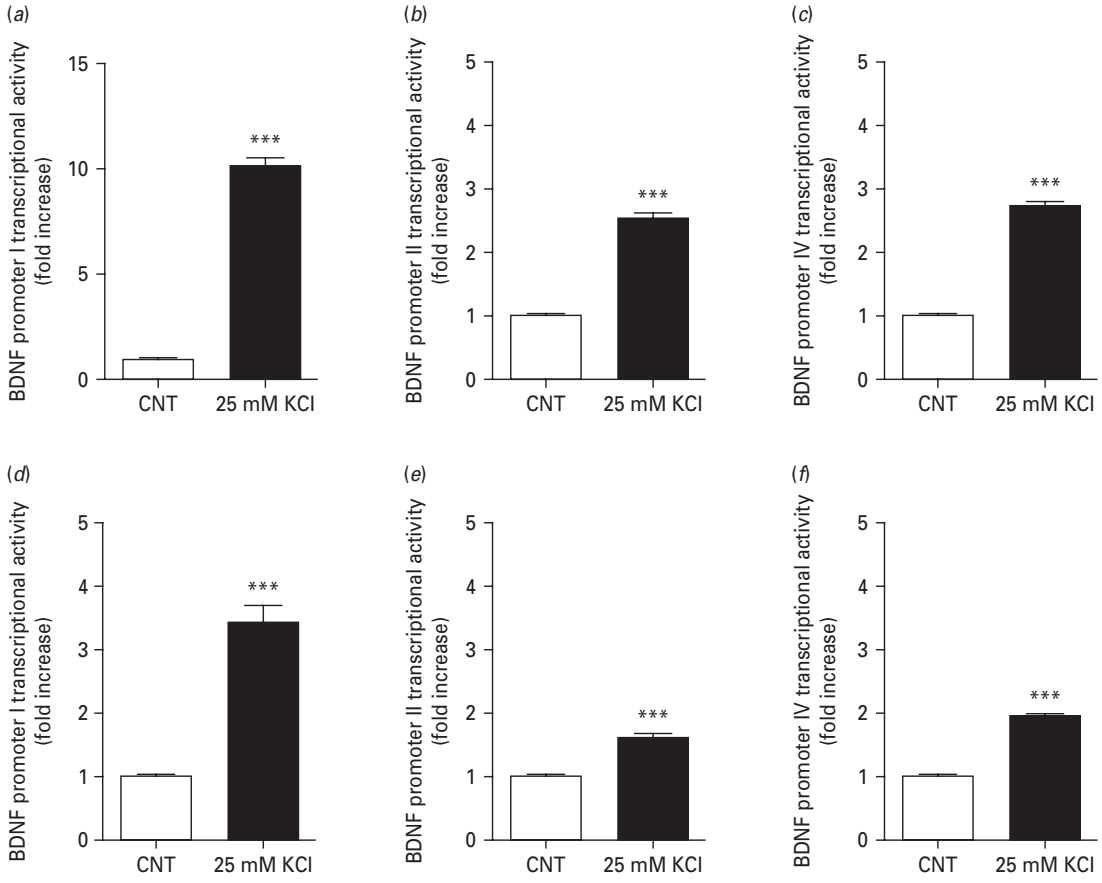

Fig. 2. Luciferase expression levels induced by *in vitro* depolarization of neuronal cultures transfected with pGL3-BDNFprI, pGL3-BDNFprII and pGL3-BDNFprIV. Firefly luciferase expression levels induced by 6 h depolarization with KCl 25 mM of 10 d *in vitro* hippocampal (*a–c*) or cortical (*d–f*) cultures alternatively transfected with pGL3-BDNFprI/EF1α-pRL (*a, d*), pGL3-BDNFprII/EF1α-pRL (*b,e*) or pGL3-BDNFprIV/EF1α-pRL (*c,f*). Data are normalized on *Renilla* Luciferase expression levels and expressed as fold change *vs.* CNT±S.E.M. Statistics: two-tailed Student's *t*-test for unpaired samples. ***$p<0.001$ *vs.* CNT, $n=6$–10, two independent experiments.

1, 2 and 4 previously reported (Tabuchi et al., 2002). In particular, we found that depolarization induced a more than three-fold increase in the expression under BDNF promoter I and about a two-fold increase of luciferase in neurons transfected with pGL3-BDNF promoter II or IV (Student's *t*-test, $p<0.001$) (Fig. 2*d–f*).

Effect of in vitro *treatment with fluoxetine and reboxetine on transcriptional activity of BDNF promoter I, II and IV*

We used the described gene reporter system to assess the effect of treatment with clinically used antidepressants on BDNF promoters activity. Since we have obtained similar results with both hippocampal and cortical cultures (see Fig. 2 above), we performed all subsequent experiments on cortical neurons to limit the number of animals used. Rat cortical neurons were alternatively transfected with pGL3-BDNF promoter I, II or IV and cultures were treated with 2, 5 and 10 μM FLX or RBX, for 6, 24 and 48 h. 25 mM KCl for 6 h was used as a positive control and luciferase expression was measured at 10 DIV. As previously found, KCl depolarization activated BDNF promoter activity (BDNF promoter I: CNT 100±4.60, KCl 392.4±10.98, $n=6$; BDNF promoter II: CNT 100±3.88, KCl 177.00±6.08, $n=6$; BDNF promoter IV: CNT 100±4.80, KCl 340.7±9.19, $n=6$). However, we found no significant increase of luciferase expression after incubation with FLX and RBX at any concentration and time point, with the single exception of BDNF promoter IV after 6 h of RBX (two-way ANOVA; BDNF promoter I FLX: no effect of time $F_{3,36}=0.57$ $p=0.67$, concentration $F_{2,36}=2.94$ $p=0.07$, interaction $F_{6,36}=1.80$ $p=0.13$; BDNF promoter I RBX: no effect of time $F_{3,36}=2.95$ $p=0.06$, concentration $F_{2,36}=0.71$ $p=0.50$, interaction $F_{6,36}=1.33$ $p=0.27$; BDNF promoter II FLX: no effect of time $F_{3,36}=0.55$ $p=0.58$, concentration $F_{2,36}=2.98$ $p=0.06$, interaction $F_{6,36}=1.36$ $p=0.26$; BDNF promoter II RBX: no effect of time $F_{3,35}=2.97$ $p=0.06$, concentration $F_{2,35}=0.09$ $p=0.91$, interaction $F_{6,35}=1.11$ $p=0.37$; BDNF promoter IV FLX: no effect of time $F_{3,36}=2.93$ $p=0.06$, concentration $F_{2,36}=0.75$ $p=0.46$, interaction $F_{6,36}=1.26$ $p=0.31$;

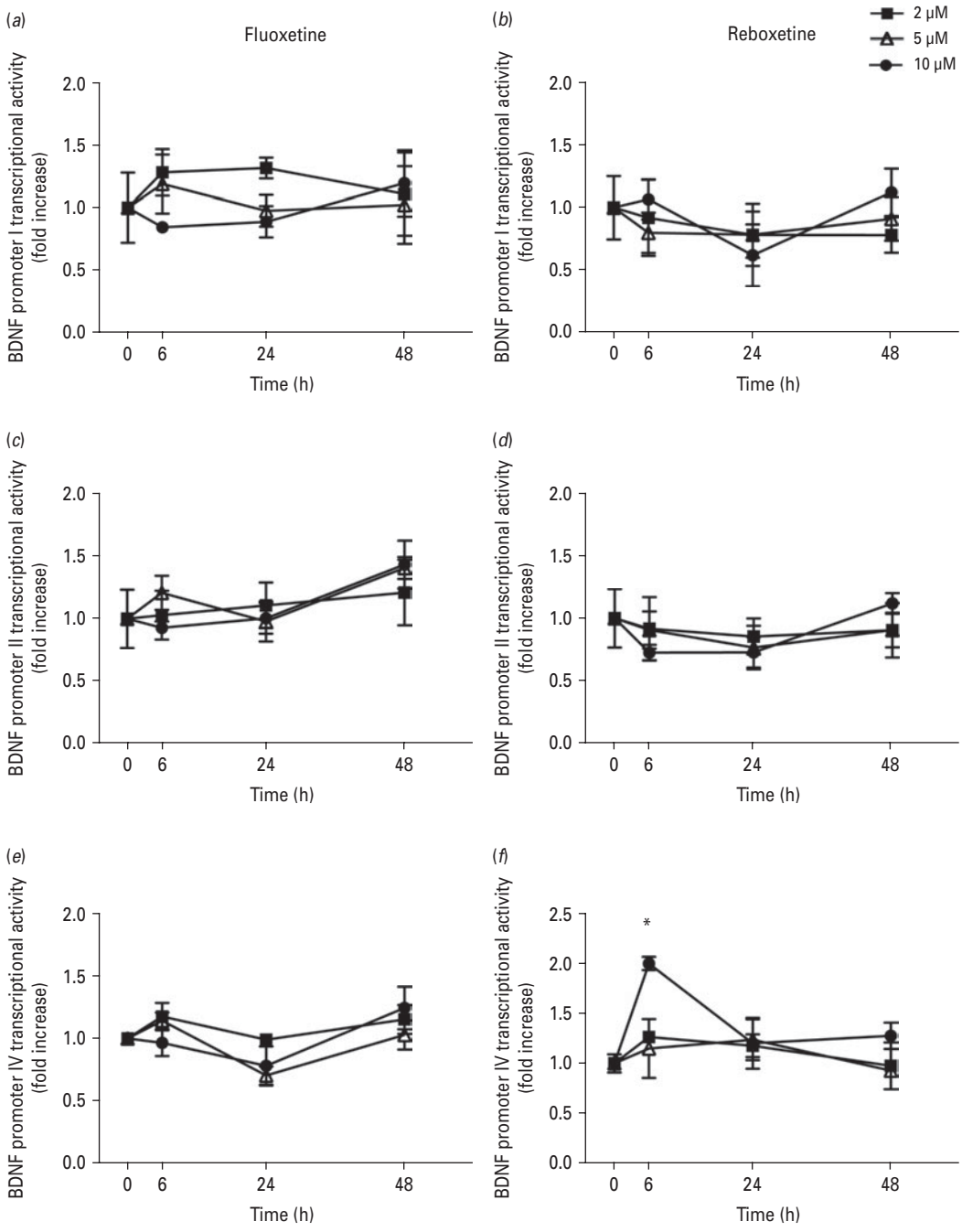

Fig. 3. Time–course of BDNF promoters activity after incubation *in vitro* of transfected cortical cultures with increasing concentrations of fluoxetine and reboxetine. Firefly luciferase expression levels in cortical cultures transfected with pGL3-BDNFprI/EF1α-pRL (*a,b*), pGL3-BDNFprII/EF1α-pRL (*c,d*) or pGL3-BDNFprIV/EF1α-pRL (*e,f*) and incubated for 6, 24 and 48 h with 2, 5 10 μM fluoxetine (*a,c,e*) or reboxetine (*b,d,f*). Data are normalized and expressed as in Fig. 2. Statistics: two-way ANOVA, followed by BPHT; *$p<0.001$ *vs.* untreated cultures, $n=5$.

BDNF promoter IV RBX: significant effect of time $F_{3,33}=18.49$ $p<0.001$, concentration $F_{2,33}=14.75$ $p<0.001$, interaction $F_{6,33}=6.59$ $p<0.0001$, BPHT $p<0.001$ 10 μM 6 h *vs.* $t=0$) (Fig. 3).

These results suggested that *in vitro* treatment of neuronal cultures with FLX or RBX does not cause induction of BDNF promoters I, II, IV.

Effects of fluoxetine, reboxetine and lithium on CRE promoter activity in neurons transfected with pGL3-CRE

Since monoamine reuptake inhibitor antidepressants increase CRE activity (for a review, see Tardito et al., 2006), we analysed the *in vitro* effect of FLX and RBX on

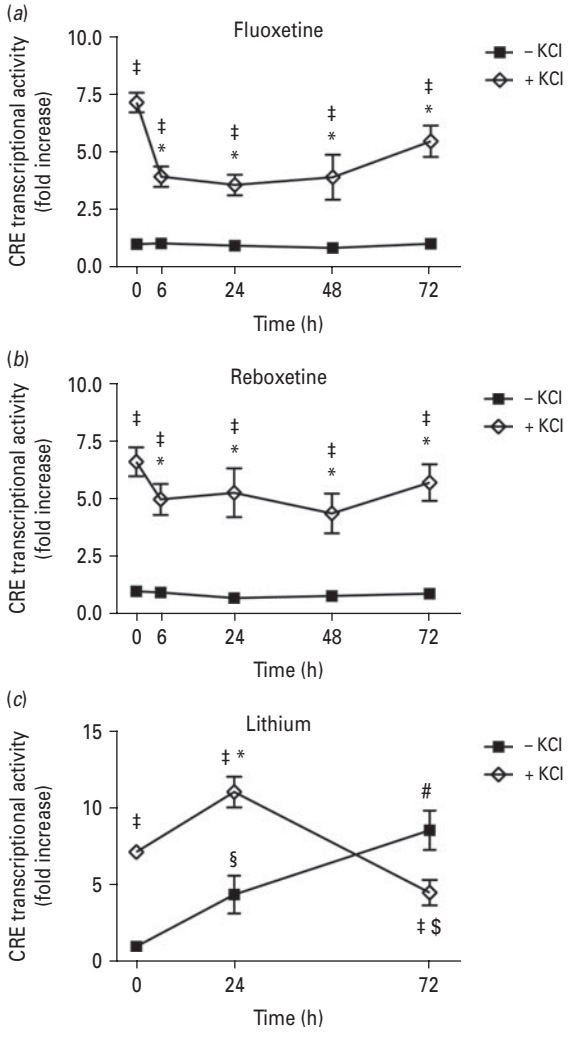

Fig. 4. Time–course of CRE promoter activity after incubation *in vitro* of transfected cortical cultures with fluoxetine, reboxetine and lithium carbonate, under depolarization conditions. Firefly luciferase expression levels in cortical cultures transfected with pGL3-CRE/EF1α-pRL, incubated for 6, 24, 48 and 72 h with 2 μM fluoxetine (*a*) or reboxetine (*b*) and depolarized *in vitro* with 25 mM KCl. (*c*) Firefly luciferase expression levels in cortical cultures transfected with pGL3-CRE/EF1α-pRL, incubated for 24 and 72 h with 1 mM lithium carbonate and depolarized *in vitro* with 25 mM KCl. Data are normalized and expressed as in Fig. 2. Statistics: two-way ANOVA followed by BPHT; ‡$p<0.001$ *vs.* basal condition, untreated, for each experimental time ($t=0$, 6, 24, 48 or 72 h); *$p<0.001$ *vs.* depolarized untreated cultures ($t=0$); §$p<0.001$ *vs.* basal untreated ($t=0$); #$p<0.001$ *vs.* basal untreated $t=24$ h; \$$p<0.001$ *vs.* depolarized treated $t=24$ h; $n=6$.

the transcriptional activation of CRE (present in the sequences of BDNF promoters I and IV (Tabuchi et al., 2002; Tao et al., 2002)). We also tested lithium (a mood stabilizer), a drug with a mechanism different from monoamine reuptake inhibitors, which is known to increase phosphorylation of CREB and CRE activity (Einat et al., 2003). With these experiments we also assessed if the effects on CRE activity of FLX, RBX and lithium were modified by *in vitro* depolarization.

We transfected cortical neurons with pGL3-CRE and treated neurons with 2 μM FLX or RBX for 6, 24, 48 and 72 h or with 1 mM lithium carbonate for 24 and 72 h. At 10 DIV, cultures were depolarized (with 25 mM KCl, as above) and luciferase expression was measured. As expected, depolarization caused a high induction of CRE transcriptional activity with about a seven-fold increase of luciferase expression in untreated cultures (two-way ANOVA followed by BPHT; FLX: significant effect of KCl $F_{1,49}=1073$ $p<0.001$, time $F_{4,49}=33.26$ $p<0.001$, interaction $F_{4,49}=30.37$ $p<0.001$, BPHT $p<0.001$; RBX: significant effect of KCl $F_{1,50}=905.61$ $p<0.001$, time $F_{4,50}=7.39$ $p<0.001$, interaction $F_{4,50}=5.31$ $p=0.001$, BPHT $p<0.001$ *vs.* respective CNT) (Fig. 4*a*, *b*). Instead, both FLX and RBX were not able to induce activation of CRE and rather decreased its activation after depolarization (BPHT $p<0.001$ *vs.* KCl $t=0$), suggesting a reduction of neuronal responsiveness (Fig. 4*a*, *b*).

On the contrary, the incubation of neuronal cultures with 1 mM lithium (Fig. 4*c*) induced a marked and time-dependent activation of CRE in basal conditions (two-way ANOVA followed by BPHT; FLX: significant effect of KCl $F_{1,27}=87.11$ $p<0.001$, time $F_{2,27}=46.95$ $p<0.001$, interaction $F_{2,27}=124.32$ $p<0.001$): we found a four-fold increase of luciferase expression after 24 h and a further increase, up to more than eight-fold, after 72 h (BPHT $p<0.001$ 24 h *vs.* $t=0$, 72 h *vs.* 24 h and $t=0$). In depolarized cultures, after incubation with lithium for 24 h, we registered an increase of luciferase expression (significantly higher than the levels measured in both lithium-untreated depolarized, BPHT $p<0.001$, and 24 h lithium-treated non-depolarized neurons, BPHT $p<0.001$). Instead, in cultures treated with lithium for 72 h, depolarization did not induce an increase of CRE-mediated transcription and, on the contrary, luciferase activity was significantly lower than that found in both lithium-untreated depolarized (BPHT $p<0.001$) and 72 h lithium-treated non-depolarized neuron (BPHT $p<0.001$).

These results suggested that: (1) neuronal cultures do not respond to FLX and RBX; (2) in neuronal cultures CRE activity is increased by chronic lithium; (3) lithium biphasically modulates CRE transcriptional activity depending on the length of treatment and on neuronal activation.

Effect of in vitro *treatment with BDNF, serotonergic and noradrenergic agonists on transcriptional activity of BDNF promoter I, II and IV*

Finally, we characterized the role of monoaminergic input in the activation of selected BDNF promoters. To this aim, we measured luciferase expression in cortical neurons transfected with pGL3-BDNF promoter I, II and IV or pGL3-CRE, after treatments of cultures with 5-HT, 8-OH-DPAT (5-HT1A agonist), DOI (5-HT2 agonist),

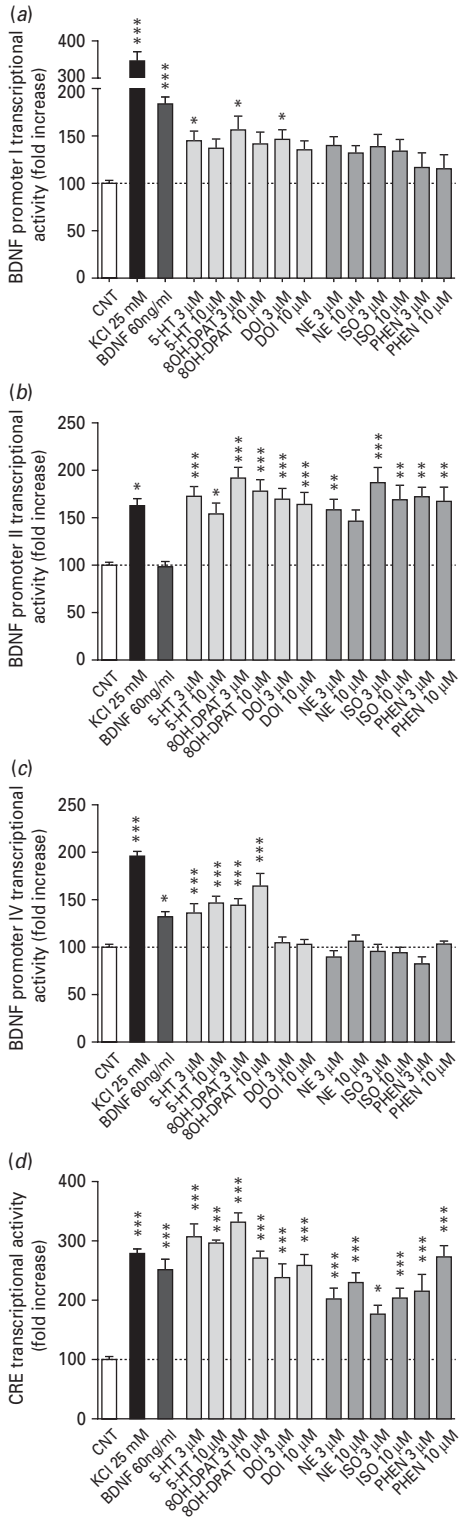

Fig. 5. Effect of *in vitro* treatment with BDNF, serotonergic and noradrenergic agonists on transcription activity of BDNF promoter I, promoter II and promoter IV. *Firefly* Luciferase expression levels in cortical neurons transfected with pGL3-BDNFprI/EF1α-pRL (*a*), pGL3-BDNFprII/EF1α-pRL (*b*) or pGL3-BDNFprIV/EF1α-pRL (*c*) treated 10′ with BDNF 60 ng/ml or serotonin (5-HT), 8-hydroxy-DPAT hydrobromide (8-OH-DPAT), (R)(−)-DOI hydrochloride (DOI), norepinephrine (NE), isoproterenol (ISO) and phenylephrine (PHEN) 3 and 10 μM. Luciferase expression was assayed after 5 h for BDNF and after 1 h for the other drugs. 6 h of depolarization with 25 mM KCl was used as internal control. Data are normalized and expressed as in Fig. 2. Statistics: one-way ANOVA, BPHT; *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* untreated neurons, $n=6$–10, two independent experiments.

NA, isoproterenol (β-adrenoceptor agonist) and phenylephrine (α1-adrenoceptor agonist). Moreover, since it is well established that BDNF activates a positive feedback mechanism (Acheson et al., 1995; Yoshii and Constantine-Paton, 2010), we also evaluated the effect of treatment with BDNF. Luciferase expression was assayed after 6 h for BDNF and after 1 h for the other substances (see Materials and Methods for details). 6 h of depolarization with 25 mM KCl was used as a positive control.

Our results showed different responses, suggesting a specific regulation of each BDNF promoter. BDNF promoter I activity was significantly increased by treatment with BDNF and showed a general trend to increase after both serotonergic and noradrenergic stimuli, that reaches significance only for serotonergic agents at the lower concentration (one-way ANOVA, $F_{14,250}=18.53$, $R^2=0.52$, $p<0.001$; BPHT: see Supplementary Table S1) (Fig. 5*a*). BDNF promoter II-dependent transcription was induced by both serotonergic and noradrenergic stimuli, but not by BDNF (one-way ANOVA, $F_{14,211}=5.93$, $R^2=0.30$, $p<0.001$; BPHT: see Supplementary Table S2) (Fig. 5*b*). We measured a selective induction of BDNF promoter IV transcription after treatment with BDNF and 5-HT1A agonists (5-HT and 8OH-DPAT), but no effect of DOI and noradrenergic inputs (one-way ANOVA, $F_{14,194}=14.74$, $R^2=0.54$, $p<0.001$; BPHT: see Supplementary Table S3) (Fig. 5*c*). Finally, all compounds, although to different degrees, markedly increased CRE transcriptional activity (one-way ANOVA, $F_{14,191}=14.83$, $R^2=0.73$, $p<0.001$; BPHT: see Supplementary Table S4) (Fig. 5*d*).

Discussion

Generation of a gene-reporter system for the study of BDNF promoters activity

In the present study we first assessed basal and depolarization-evoked endogenous transcription of the four most highly expressed non-coding exons of the rat BDNF gene (1, 2, 4, 6). In line with previous studies, we found that the expression of exons 1, 2 and 4 was markedly up-regulated by membrane depolarization, while expression of the exon 6 was unchanged (Tabuchi et al., 2002; Pruunsild et al., 2011). Then, we generated a reporter gene assay based on transfection of rat primary neuronal cultures with a high-efficiency transfection system. At 10 DIV, we found around 60% of transfected cells (Gresch et al., 2004; Leclere et al., 2005; Gärtner et al., 2006), neurons showed good dendrite arborization

and the astroglial population showed low transfection efficiency and moderate growth, suggesting that the majority of transfected cells in culture were neurons and ensuring lack of interference from astroglia. In neurons transfected with BDNF promoters I, II and IV, we found that *in vitro* depolarization caused a marked induction of luciferase expression, similar to the observed depolarization-evoked transcription of endogenous BDNF exons. Thus, we generated a highly sensitive and rapidly detectable gene reporter system, able to reproduce the activation of endogenous BDNF promoters in response to stimuli.

Characterization of the effects of treatments with fluoxetine and reboxetine on the transcriptional activity of BDNF promoters and CRE. Bimodal action of lithium on CRE-driven transcription

We then evaluated the direct effects of monoaminergic antidepressants on BDNF promoters, measuring luciferase expression in neuronal cultures alternatively transfected with BDNF promoter I, II and IV and incubated with 2, 5 or 10 μM FLX or RBX for 6, 24 and 48 h. We found that neither FLX nor RBX were able to induce BDNF promoters transcriptional activities, at any time point considered. Interestingly, the same results were obtained in cultures transfected with pGL3-CRE. Since transcriptional activation of CRE is a common target of antidepressants (Carlezon et al., 2005; Malberg and Blendy, 2005; Duman and Monteggia, 2006; Tardito et al., 2006, 2009; Pittenger and Duman, 2008), the lack of changes in luciferase expression under CRE promoter after *in vitro* incubation with FLX and RBX suggested that their effects are mediated by changes in the monoaminergic input. Indeed, cortical neurons are mainly glutamatergic or GABAergic and, contrary to cerebral cortex *in vivo*, primary cortical cultures completely lack a serotonergic/noradrenergic input from subcortical areas. Although this hypothesis may appear somewhat obvious there is little or no direct evidence for this thus far.

We also showed lithium, a mood stabilizer with a mechanism independent of extracellular monoamine levels, induced a marked and time-dependent increase of CRE-driven luciferase transcription in pGL3-CRE transfected cultures. These data were consistent with previous evidence showing that lithium activates endogenous CRE transcriptional activity (Ozaki and Chuang, 1997). Intriguingly, it was shown that treatments of cortical neurons with lithium or valproic acid increase both the levels of BDNF exon IV-containing transcripts, and the activity of BDNF promoter IV (Yasuda et al., 2009). Moreover, our data showed that chronic lithium basally increases CRE activity, suggesting the activation of neurotrophic mechanisms, but at the same time, reduces the response to depolarization, suggesting a bimodal action of the drug, as previously argued by others (Jope, 1999). According to this hypothesis, lithium could stabilize intracellular signalling (including CRE-related pathways) in an optimal range, which may contribute to avoiding mood swings.

Characterization of the pharmacological modulation of BDNF and CRE promoters by serotonergic and noradrenergic stimuli

We found that FLX and RBX did not induce BDNF promoter I, II, IV and CRE transcriptional activity in neuronal cultures, most likely due to the absence of monoaminergic inputs. To understand if an increase of extracellular monoamine concentration is necessary to activate BDNF- and CRE-promoted transcription, we incubated with 5-HT or NA cortical neurons alternatively transfected with pGL3-BDNF promoter I, II, IV or pGL3-CRE. Moreover, to analyse what receptor subtypes mediate the effects of 5-HT and NA, we also applied a full 5-HT_{1A} agonist (8OH-DPAT), a selective 5-HT2 agonist (DOI), a β-adrenoceptor agonist (isoproterenol) and an α_1-adrenoceptor agonist (phenylephrine). The results obtained suggest a selective and distinct modulation of BDNF transcription mediated by each specific promoter in response to different receptor ligands.

We demonstrated that BDNF exerts a positive feedback on its transcription levels by selectively activating promoters I and IV. We also observed that BDNF activates CRE-mediated transcription. Since CRE is a responsive element present in BDNF promoters I and IV (Shieh et al., 1998; Tabuchi et al., 2002), we speculate that BDNF-dependent activation of BDNF promoters I and IV was partly dependent by the induction of a CRE element.

5-HT induced a marked activation of both CRE and BDNF promoters. However, while for CRE and BDNF promoters I and II the induction of transcription caused by 5-HT seems to be mediated by both 5-HT_{1A} and 5-HT_2 serotonin receptors, BDNF promoter IV-mediated transcription was increased selectively by the activation of 5-HT_{1A} receptors.

We also observed a specific regulation of selected BDNF promoters after noradrenergic inputs. Indeed, while CRE and BDNF promoter II were markedly activated by all noradrenergic stimuli, BDNF promoter IV transcription was totally independent of noradrenergic stimuli, while BDNF promoter I activation by noradrenergic agents did not reach statistical significance. Therefore, although the sequences of BDNF promoters I and IV contain a CRE element and CRE activity was markedly up-regulated by noradrenergic inputs, BDNF promoters showed a differential pattern of activation. It is known that in BDNF promoters I and IV there are additional responsive elements other than CRE (Tabuchi et al., 2002; Tao et al., 2002; Pruunsild et al., 2011), that could selectively modulate and partly reduce or eliminate the response of BDNF promoters I and IV to noradrenergic stimuli.

In the present study we investigated only BDNF promoters I, II and IV because they have brain-enriched expression patterns and are activated by depolarization (Aid et al., 2007). However, we cannot exclude that other promoters could contribute to the regulation of BDNF gene transcription induced by monoaminergic agonists. Moreover, extracellular levels of 5-HT or NA could affect not only total BDNF mRNA level, but also the intracellular trafficking of the transcripts (Baj et al., 2012).

In summary, our results suggest that cortical neuronal cultures, due to the lack of serotonergic and noradrenergic/monoeminergic afferents, are not a suitable system to study the effects induced by antidepressants that act primarily by increasing extracellular levels of monoamines, such as FLX and RBX. On the other hand, neuronal cultures transfected with BDNF-promoter reporters may constitute a simple and reliable system to characterize the effect of selective monoaminergic receptor agonists on neurotrophin signalling.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713001685.

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Statement of Interest

JR and ED were full-time employees of GlaxoSmithKline when the present study was performed. GR has scientific collaborations with and is a member of the scientific board for Eli Lilly, Innova Pharma, and Servier. ED is full-time employee of F. Hoffmann La-Roche. MP received research support and/or has been consultant for Abiogen, GlaxoSmithKline, Merck Sharp & Dohme, Abbott, Servier, Fidia. LM, and AI report no biomedical financial interests or potential conflicts of interest.

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