

Elevation of BDNF Exon I-Specific Transcripts in the Frontal Cortex and Midbrain of Rat During Spontaneous Morphine Withdrawal is Accompanied by Enhanced pCreb1 Occupancy at the Corresponding Promoter

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Abstract Brain-derived neurotrophic factor (BDNF) is believed to play a crucial role in the mechanisms underlying opiate dependence; however, little is known about specific features and mechanisms regulating its expression in the brain under these conditions. The aim of this study was to investigate the effects of acute morphine intoxication and withdrawal from chronic intoxication on expression of BDNF exon I-, II-, IV-, VI- and IX-containing transcripts in the rat frontal cortex and midbrain. We also have studied whether alterations of BDNF exon-specific transcripts are accompanied by changes in association of well-known transcriptional regulators of BDNF gene—phosphorylated (active form) cAMP response element binding protein (pCreb1) and methyl-CpG binding protein 2 (MeCP2) with corresponding regulatory regions of the BDNF gene. Acute morphine intoxication did not affect levels of BDNF exons in brain regions, while spontaneous morphine withdrawal in dependent rats was accompanied by an elevation of the BDNF exon I-containing mRNAs both in the frontal cortex and midbrain. During spontaneous morphine withdrawal, increased associations of pCreb1 were found with promoter of exon I in the frontal cortex

and promoters of exon I, IV and VI in the midbrain. The association of MeCP2 with BDNF promoters during spontaneous morphine withdrawal did not change. Thus, BDNF exon-specific transcripts are differentially expressed in brain regions during spontaneous morphine withdrawal in dependent rats and pCreb1 may be at least partially responsible for these alterations.

Keywords BDNF · Exon-specific mRNA · Promoters · Rat brain · Morphine withdrawal

Introduction

According to recent findings, dependence on opiates induces a specific form of neuroplasticity [1]. Brain-derived neurotrophic factor (BDNF) may be involved in activity-dependent synaptic plasticity, linking synaptic activity with long-term functional and structural modification of synaptic connections [2]. BDNF-initiated signaling pathways can regulate structural and behavioral plasticity in the context of drug addiction [3, 4]. Chronic morphine exposure is associated with specific biochemical and morphological alterations in the mesolimbic dopamine system, and exogenous intracerebral BDNF infusions can alleviate these changes [5–7].

Opiates can alter expression of the BDNF gene in different regions of the brain [8–11] however, little is known about the transcriptional mechanisms of the control of BDNF expression under these conditions. The rodent BDNF gene is comprised of eight 5' exons (I–VIII) and the 3' coding exon (exon IX), each of the first seven exons having a putative promoter on its 5' flanking region and a splice donor site on its 3' end [12]. All BDNF transcripts containing the coding exon IX get translated into the pro-

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form of the protein, which, when cleaved, generates a functionally mature form of BDNF. Only a small number of published papers describe the expression pattern of BDNF exons in the brain after morphine administration. Particularly, morphine-conditioned place preference paradigm modulates expression of BDNF exons I, II, IV and VI in mouse brain regions [13]. In addition, chronic morphine treatment significantly augments the expression of BDNF exon I, IV and VI transcripts in the mouse periaqueductal gray matter [14]. According to Wang et al. [15], during conditioned place aversion extinction training BDNF exon I transcription can be diminished in the prefrontal cortex of acute morphine-dependent rats. In the rodent brain BDNF exons I, IV and VI are the most responsive exons to psychoactive drugs such as cocaine [16, 17] and benzodiazepines [18] as well as stimuli such as contextual fear learning [19] and social defeat stress [20]. Thus, BDNF transcription particularly expression of specific exons is altered by opiates; however the molecular mechanisms of dependence-related BDNF expression remain obscure.

Expression of the BDNF gene can be modified by transcriptional regulators such as cAMP response element binding protein (Creb1) or methyl-CpG binding protein 2 (MeCP2) [21]. Expression of BDNF exon-specific transcripts is activity-driven and in part is mediated by the active (phosphorylated) form of Creb1–pCreb1. In many cell types, expression of BDNF is under control of the extracellular signal-regulated kinases (ERK) cascade, since ERK phosphorylates and activates Creb that directly binds to BDNF promoter(s) to induce gene transcription [22]. MeCP2 induced silencing of the BDNF gene may occur through chromatin remodeling by recruiting protein complexes containing co-repressors of transcription, histone deacetylases, histone methyltransferases, etc. [21]. Since Creb1 activity is strongly affected by opiate dependence [23], it is likely that Creb1 is involved in opiate-dependent BDNF gene expression. Indeed, a sole study performed by Wang et al. [15] reveals that conditioned place aversion extinction training induced an increase in recruiting pCreb1 to the promoter of BDNF exon I and increased the corresponding BDNF mRNA containing exon I in the prefrontal cortex of acute morphine-dependent rats. Furthermore, it is established that enrichment of pCreb1 and/or MeCP2 at BDNF promoters may be related to the altered expression of BDNF exons in specific brain regions during cocaine [16, 17] or benzodiazepines [18] exposure. Unlike Creb1, transcriptional activity of MeCP2 toward BDNF gene in the brain regions has not been studied during chronic morphine exposure.

Chronic morphine treatment or withdrawal after chronic drug administration is accompanied by neuroplastic processes throughout the brain; however, there are three main structural sites where morphological alterations are

regarded as most relevant and are well-described: the neocortex [24, 25], the midbrain [5, 26], and the corpus striatum [24, 27]. Although structural changes in a dependent brain are documented, little is known about the underlying molecular mechanisms, though BDNF gene expression is believed to be connected with altered brain morphology during morphine dependence. Although the BDNF mRNA level is rather high throughout the brain, the striatum demonstrates no detectable level of BDNF mRNA [28]. Lack of BDNF mRNA levels in this structure was noticed both under basal conditions and after cocaine treatment [29]. Taking into account that BDNF mRNA is virtually not transcribed in the striatum, we focused the study of BDNF transcriptional regulation by morphine in the frontal cortex and midbrain.

Thus, the aim of this study was to investigate whether morphine affects: (a) expression of BDNF exon I-, II-, IV-, VI-specific transcripts, as well as, BDNF exon IX levels (the sum of all isoforms transcribed) in the frontal cortex and midbrain, (b) association of transcriptional regulators pCreb1 and MeCP2 with corresponding regulatory regions of the BDNF gene.

Materials and Methods

Materials

All chemicals were purchased from Sigma (USA) unless otherwise stated. Morphine hydrochloride was obtained from Moscow Endocrine Plant (Russia).

Animals and Procedures

Male Wistar rats considered to be young adults (body weight 207 ± 5 g before the habituation period, 238 ± 5 g at the beginning of the experiments) from the “Stolbovaya” Breeding Center (Moscow Region, Russia) were used in this study. The experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals; the protocol was approved by the Institutional Ethical Committee.

Acute morphine intoxication was performed by an intraperitoneal injection of morphine hydrochloride (10 mg/kg); the animals were sacrificed 1 or 2 h later ($n = 5$ per group). Control animals ($n = 5$ per group) received equivalent volumes of saline instead of morphine.

For induction of a physical dependence, rats were subjected to a subchronic morphine administration ($n = 8$). Increasing doses of morphine hydrochloride (10–100 mg/kg) were injected intraperitoneally twice a day (at 8.00 and 20.00) for 6 days [30]. The animals of the control group received equivalent volumes of saline instead of morphine

($n = 8$). The specific somatic (jumping, wet dog shakes, writhing, chewing, teeth chattering and paw shakes) and autonomous signs (diarrhoea, ptosis and dyspnea) of the abstinence syndrome were recorded 38 h after the last injection of morphine for 5 min using the open field test performed in an arena of 120 cm in diameter with a wall of 40 cm in height. The control group was also subjected to the test. The “counted signs” and “checked signs” were multiplied by the respective “weighing factors” for evaluation of abstinence syndrome severity [30]. The animals were sacrificed 2 h after the evaluation of the morphine withdrawal syndrome (i.e. 40 h after the last morphine or saline injection), and the brain structures were isolated. Brains were removed from the skull, thoroughly washed in ice cold isotonic saline solution and dissected on ice. The crude samples of the frontal cortex and midbrain were taken for measurements. Frontal cortical area was defined as the most frontal part of the cortex located between +0.4 and +5.0 mm from bregma, excluding olfactory bulb tissue. The midbrain portion was dissected between –9.3 and –5.0 mm from bregma, which included tectum, tegmentum and cerebral aqueduct. Samples were frozen in liquid nitrogen and stored at –80 °C before use.

RNA Isolation and RT-qPCR

The expression of mRNAs was measured by the quantitative real-time polymerase chain reaction (qPCR) combined with reverse transcription (RT). Total RNA was extracted using the method described elsewhere [31]. In brief, total RNA was extracted with acid guanidinium thiocyanate–phenol–chloroform mixture, RNA was precipitated from the aqueous phase using isopropanol. Then RNA was washed with 80 % ethanol and solubilized in sterile water. Isolated RNA was treated with DNase I (NEB, USA; cat. no. M0303). The purity and concentration of total RNA were determined by measurement of absorbance at 260 and 280 nm. We discarded all RNA samples that did not have a 260/280 ratio between 1.8 and 2.1. The integrity of RNA preparations was checked by performing agarose gel electrophoresis. RT was performed by using a standard kit for synthesis of cDNA containing reverse transcriptase from Moloney murine leukemia virus (MMLV) and random hexamer primers in accordance with the manufacturer protocol (Sileks, Russia; cat. no. K0202). cDNA quantities of BDNF exon I-, II-, IV- and VI-containing transcripts were detected by qPCR using exon-specific BDNF primers based on previously published sequences [17]. For the evaluation of total BDNF transcription we assessed BDNF exon IX mRNA levels (the sum of all isoforms transcribed) with primers described previously [32]. As a reference we used rpS18 mRNA quantities amplified with primers: forward 5'-TTTTGGGGCCTTCGTGTCCG-3' and reverse 5'-

CAGCAAAGGCCCAAAGACTCAT-3'. Threshold amplification cycle numbers (Ct) were used to calculate the content of cDNA followed by a comparison with the content of rpS18 mRNA as a reference gene, in according to the $2^{-\Delta\Delta Ct}$ method [33]. qPCR was done in two technical replicates in the presence of 5 pmol of synthetic oligonucleotides used as primers (supplied by Evrogen, Russia) and with the reagent kit containing hot start Taq DNA polymerase and an intercalating fluorescent dye SYBR according to the protocol provided by the manufacturer (Evrogen, Russia; cat. no. PK147). qPCR was performed using an ANK-32 thermocycler (Institute for Analytical Instrumentation RAS and Bauman Moscow State Technical University, Russia) using the following protocol: initial denaturation at 95 °C for 300 s; (1) 10 s at 62 °C; (2) 30 s at 72 °C; (3) 10 s at 95 °C for 50 cycles. The content of specific cDNA or DNA was estimated using the Ct recording method with ANK32 software for Windows, version 1.1 (Institute for Analytical Instrumentation, RAS, Russia).

Chromatin Immunoprecipitation (ChIP)

ChIP was done on additional cohorts of animals which have received morphine subchronically as described above: control group ($n = 7$) and morphine group ($n = 7$). The frozen brain regions (the frontal cortex or midbrain) were weighed minced on ice with a scalpel into small pieces ($\sim 1 \text{ mm}^3$) and deposited into tubes. To cross-link proteins to the genomic DNA, phosphate buffered saline (PBS) containing 1 % freshly prepared formaldehyde and 1 mM phenylmethanesulfonyl fluoride (PMSF) was added (1:9 w/v ratio). Brain pieces were incubated for 10 min at room temperature; 2.5 M glycine (125 mM final) was added to quench the formaldehyde and terminate the cross-linking reaction. Then the minced tissue was washed twice with ice cold PBS containing 1 mM PMSF. To isolate nuclei, tissue pieces were homogenized in 20 mM HEPES pH 7.4, 320 mM sucrose, 5 mM MgCl₂, 0.5 % NP-40, 0.25 % Triton X100, 140 mM NaCl, 1 mM Na₃VO₄ and 1 mM PMSF (1:9 w/v ratio) with a loose pestle in a glass homogenizer followed by centrifugation at 4 °C and 2,000 g for 10 min. The pellets were washed twice with the same buffer excluding detergents. The nuclear pellets were resuspended in SDS lysis buffer (50 mM Tris pH 8.1, 1 % SDS, 10 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄) and homogenized using the tight pestle of the homogenizer. The lysates were sonicated to shear DNA into 100–700 bp fragments (DNA length was verified on a 1 % agarose gel). The resulting homogenates were centrifuged for 20 min at 13,000 g at 4 °C to remove debris. Aliquots of total chromatin (1/10 part used for immunoprecipitation) were saved and designated as “Input DNA”. Chromatin immunoprecipitation was performed using ChromaFlash One-

Table 1 Acute morphine administration does not affect the content of BDNF exon-specific transcripts in the frontal cortex and midbrain

Brain region	mRNA	Normalized mRNA amount			
		Control 1 h (n = 5)	Acute morphine 1 h (n = 5)	Control 2 h (n = 5)	Acute morphine 2 h (n = 5)
Frontal cortex	BDNF exon I	1.11 ± 0.24	1.39 ± 0.14	1.18 ± 0.33	1.46 ± 0.17
	BDNF exon IV	1.12 ± 0.28	1.22 ± 0.19	1.15 ± 0.27	1.58 ± 0.27
	BDNF exon VI	1.25 ± 0.35	1.86 ± 0.31	1.40 ± 0.58	1.90 ± 0.31
	BDNF exon IX	1.03 ± 0.12	1.04 ± 0.28	1.04 ± 0.15	1.27 ± 0.15
Midbrain	BDNF exon I	1.07 ± 0.19	1.46 ± 0.54	1.01 ± 0.08	1.56 ± 0.58
	BDNF exon IV	1.01 ± 0.07	0.83 ± 0.18	1.01 ± 0.06	1.69 ± 0.39
	BDNF exon VI	1.11 ± 0.24	1.54 ± 0.58	1.01 ± 0.07	1.52 ± 0.66
	BDNF exon IX	1.00 ± 0.04	0.95 ± 0.04	1.02 ± 0.10	1.04 ± 0.11

Step ChIP Kit (P-2025-96, Epigentek Group Inc., USA) according to the manufacturer instruction with 4 ug of antibodies (Santa Cruz Biotechnology, Inc., USA): pCreb1 (sc-7978X), MeCP2 (sc-137070X) or non-immune IgG (sc-2025) as a negative control for immunoprecipitation. DNA quantities in the immunoprecipitated samples were detected by qPCR using primers encompassing BDNF exon-specific regulatory regions based on previously published sequences [16, 19, 34–36]. Ct values were used to calculate immunoprecipitated DNA quantities as percentage of corresponding inputs. DNA quantities were then expressed as percentages of corresponding Input using the following equation:

$$\% \text{ of Input} = 100 \times 2^{\Delta\text{Ct}[\text{normalised ChIP}]},$$

where $\Delta\text{Ct} [\text{normalized ChIP}] = \text{Ct} [\text{ChIP}] - \text{Ct} [\text{Input}] - \log_2 (\text{Input dilution factor})$.

Statistics

The data are presented as $M \pm \text{SEM}$. Differences between groups were analyzed for statistical significance by using two-tailed Student's *t* test. When more than two groups of data were compared two-way ANOVA was used followed by Tukey's honestly significant difference (HSD) test post hoc test. Behavioral data (all-or-none) was analyzed using the Fisher's exact test. Statistical analysis of the data was performed using STATISTICA 7.0 software (StatSoft Inc., United States) and results were presented by means of GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA), results were considered to be significant at p value < 0.05 .

Results

The dynamics of changes in BDNF exon-specific transcripts induced by acute morphine administration was analyzed using two-way ANOVA; the test did reveal

significant main effects of morphine or time after treatment neither in the frontal cortex nor in the midbrain (Table 1).

Animals which received morphine chronically were considered dependent on morphine since they demonstrated typical somatic and autonomous signs of opiate abstinence 38 h after spontaneous morphine withdrawal (Table 2). Two-way ANOVA of body weight dynamics revealed significant main effect of morphine withdrawal ($F_{(1,56)} = 16.36$, $p = 0.00016$), but not of withdrawal duration. There was a significant loss of body weight 38 h after morphine withdrawal as compared with the control group (Table 3). Moreover, the animals showed a significant increase ($p = 0.000028$, Student's *t* test) of the intensity of morphine spontaneous withdrawal syndrome. Thirty-eight hours after the final morphine injection, the score reflecting intensity of specific signs of morphine withdrawal was 30.4 ± 4.3 while in the control group the score was 3.4 ± 1.2 . As compared with acute intoxication, the dependent animals demonstrated specific changes in levels of BDNF exon-specific transcripts in the brain regions. Analysis of the expression revealed significant main effect of morphine withdrawal in both the frontal cortex ($F_{(1,39)} = 19.90$, $p = 0.00007$) and midbrain ($F_{(1,38)} = 13.75$, $p = 0.00066$). In particular, the levels of BDNF exon I-containing transcripts, but not other exons studied, were elevated in both the frontal cortex (Fig. 1a) and the midbrain (Fig. 1b).

There is an obvious limitation in our study: we could obtain reliable RT-qPCR results for BDNF exon II-containing transcripts neither in the frontal cortex nor in the midbrain (Ct values > 36 and ineffective PCR). Obviously, BDNF exon II-containing transcripts are expressed at very low level in the rodent brain and some groups reported lack of its detectable level both in the rat [16] and mouse brain [18].

Then we tried to find out whether changes of the expression of BDNF exon I-containing transcripts in the brain regions during spontaneous morphine withdrawal could be accompanied by changes to the association of transcriptional regulators with BDNF promoters in the frontal cortex and midbrain.

Table 2 The structure of abstinence syndrome 38 h after spontaneous morphine withdrawal

Withdrawal sign	Number of rats demonstrating a sign		<i>p</i> value (Fisher's exact test)
	Control (n = 8)	Morphine (n = 8)	
Jumping	0	5	0.026
Wet dog shakes	0	4	0.077
Writhing	1	5	0.119
Chewing	3	6	0.315
Teeth chattering	1	6	0.041
Paw shakes	1	8	0.001
Diarrhoea	1	3	0.569
Ptosis	2	5	0.315
Dyspnea	1	7	0.010

Table 3 Body weight dynamics after spontaneous morphine withdrawal

Hours after withdrawal (h)	Body weight (g)	
	Control (n = 8)	Morphine (n = 8)
0	245 ± 5	240 ± 6
12	248 ± 6	239 ± 5
24	248 ± 6	230 ± 5
38	250 ± 7	217 ± 5**

** $p < 0.005$, versus control group; two-way ANOVA followed by Tukey's HSD post hoc test

Analysis of ChIP-qPCR data revealed significant main effects of morphine withdrawal in both the frontal cortex ($F_{(1,51)} = 6.92$, $p = 0.01128$) and the midbrain ($F_{(1,46)} = 48.19$, $p < 0.00001$). Particularly, multiple comparisons showed that during spontaneous morphine withdrawal in the frontal cortex pCreb1 occupancy was enhanced at promoters of BDNF exon I (Fig. 2a) and in the midbrain at promoters of exon I, IV and VI (Fig. 2d, e, f respectively). Thus, ChIP-qPCR demonstrated that the enrichment of pCreb1 at BDNF promoters does not necessarily correlate with expression changes of corresponding BDNF exon-specific transcripts.

MeCP2 occupancy did not change during spontaneous morphine withdrawal at promoters of the BDNF gene in the frontal cortex (Fig. 2a–c) and the midbrain (Fig. 2d–f).

Discussion

The present study demonstrated that: (1) the expression of BDNF exons in brain regions depends on morphine exposure regimen (acute vs. chronic); (2) BDNF exons are

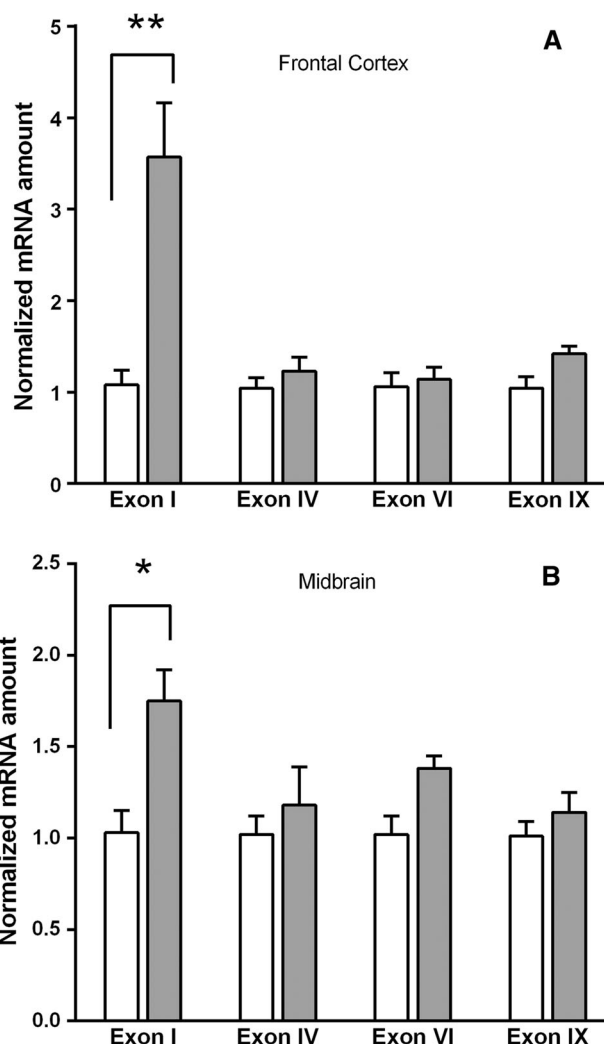
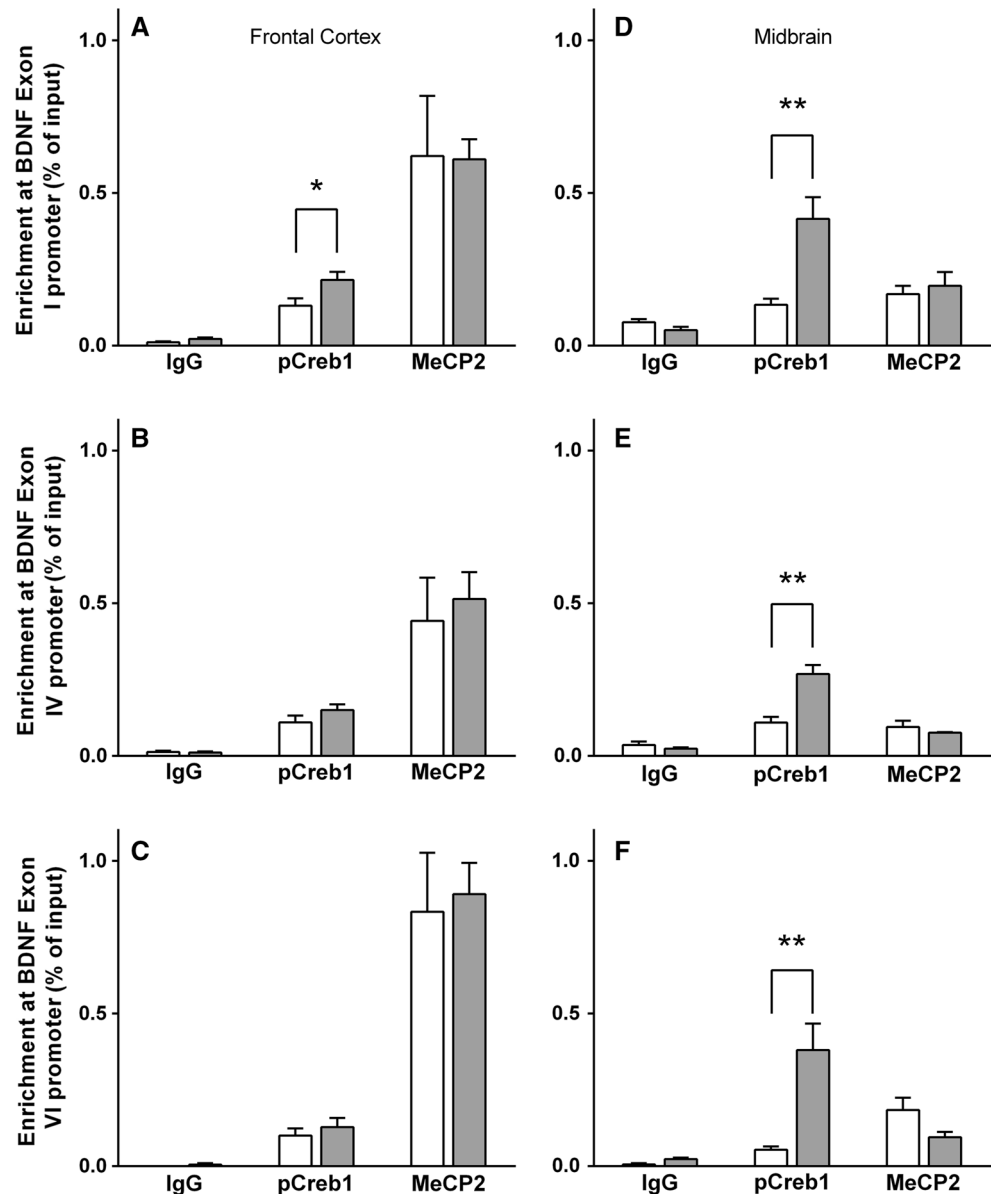


Fig. 1 Content of BDNF exon I-containing transcripts is elevated in the frontal cortex and the midbrain during spontaneous morphine withdrawal. Relative levels of BDNF exon-specific transcripts were determined by RT-qPCR in the frontal cortex (a) or midbrain (b) of rats 40 h after morphine withdrawal (grey boxes, $n = 5-6$) or control group (white boxes, $n = 5-6$). * $p < 0.01$, ** $p < 0.005$, versus control group; two-way ANOVA followed by Tukey's HSD post hoc test

differentially expressed in brain regions after morphine withdrawal in dependent rats; and (3) pCreb1 occupancy at BDNF promoters is partially associated with changes in the content of respective BDNF exons.

So far, the expression of BDNF exon-specific transcripts in brain regions after acute morphine intoxication has not been documented, though some data are reported concerning total BDNF transcripts. Vargas-Peres et al. [8] showed that after withdrawal from repeated exposure to heroin, BDNF mRNA levels increased in the ventral tegmental area, however, there were no increases in BDNF if rats received a single injection of heroin. Likewise, Lunden and Kirby reported that total mRNA level of BDNF in rat

Fig. 2 pCreb1 occupancy at BDNF promoters is enhanced in the frontal cortex and the midbrain during spontaneous morphine withdrawal. ChIP was performed in the frontal cortex (a–c) or midbrain (d–f) of rats with IgG, pCreb1 or MeCP2 antibody 40 h after morphine withdrawal (grey boxes, $n = 4–7$) or control group (white boxes, $n = 4–7$). Occupancy level of pCreb1 or MeCP2 at BDNF promoters was detected with qPCR analysis using primers amplifying promoters of BDNF Exon I (a, d), IV (b, e) or VI (c, f). $*p < 0.05$, $**p < 0.005$, versus control group; two-way ANOVA followed by Tukey's HSD post hoc test



serotonergic dorsal raphe nucleus did not change after a single morphine administration, but was elevated after precipitated withdrawal from morphine [11]. Thus, the results of the present study are in agreement with previous data and demonstrate that the level of BDNF mRNA is affected by chronic but not acute opiate treatment. We suggest that transcriptional machinery responsible for the expression of BDNF gene is not activated after acute morphine administration as compared with chronic exposure.

We have demonstrated that BDNF exon I-specific transcripts, but not other exons IV, VI or common exon IX, are specifically elevated both in the frontal cortex and the midbrain after morphine withdrawal, and, most important, the level of pCreb1 associated with promoter of exon I also

increased. Thus, the expression of BDNF exon I-specific transcripts in the brain after morphine withdrawal could at least partly depend on pCreb1 transcriptional activation.

In general, our results are in agreement with previous data showing expression specificity of BDNF exon-specific transcripts in the frontal cortex and the midbrain after psychoactive drug treatment. During conditioned place aversion extinction training BDNF exon I transcription increases in the prefrontal cortex of acute morphine-dependent rats [15]. BDNF exon I-containing transcripts in the midbrain structure ventral tegmental area are selectively increased in cocaine-experienced animals following 7 days of forced drug abstinence [17].

The study of Matsushita and Ueda demonstrated that pretreatment with curcumin, an inhibitor the histone

acetyltransferase activity of CBP (CREB binding protein; co-activator for Creb1), prevented from upregulation of the expression of BDNF exon I and IV transcripts induced by chronic morphine exposure in the mouse midbrain structure periaqueductal gray matter [14]. Post-transcriptional modifications of histones at a particular promoter region define a specific epigenetic state encoding gene activation versus gene silencing [37]. Thus, upregulated expression of BDNF exons upon chronic morphine exposure can be associated with active state of chromatin at corresponding regions. Indeed, Mashayekhi et al. [38] reported that histone methylation and/or acetylation at BDNF promoters II and III may be involved in the regulation of the BDNF gene expression in the ventral tegmental area and locus coeruleus of rats during forced abstinence of morphine.

We suggest that increased binding of pCreb1 to defined promoters of BDNF exons can precede recruitment of co-activators such as CBP and consequent activating histone modifications, finally resulting in a facilitation of the expression of BDNF exons. Indeed, Wang et al. [15] revealed that conditioned place aversion extinction training induced an increase in recruiting pCreb1 to and acetylation of histone H3 at the promoters of BDNF exon I and increased corresponding BDNF mRNA containing exon I in the ventromedial prefrontal cortex of acute morphine-dependent rats. In addition, experiments with cocaine exposure demonstrated similar pattern of epigenetic and transcriptional changes. BDNF exon I-containing transcripts were significantly increased in the ventral tegmental area of cocaine-experienced rats, and these changes were associated with increased acetylation of histone H3 and binding of CBP to exon I-containing promoters [17].

Surprisingly, in the midbrain we detected an elevation of pCreb1 occupancy at promoters of exon IV and VI after morphine withdrawal, however, the levels of corresponding BDNF exon IV- and VI- containing transcripts did not change. Thus, binding of pCreb1 to the regulatory region of the BDNF gene is not necessarily related to transcriptional activation. One possible scenario could be the following: during morphine withdrawal Creb1 could be phosphorylated and recruited to promoters of the BDNF gene non-specifically, while additional factors such as association with co-regulators, could determine specific transcription of BDNF exon I-containing transcripts only.

We did not reveal any changes in MeCP2 binding to BDNF promoters during morphine withdrawal. Thus MeCP2 may not be involved in the mechanisms of BDNF expression upon morphine exposure. However, there is a limitation of our study demanding further investigations of this issue. We studied binding of MeCP2 using antibody recognizing total MeCP2 protein, while the activity of MeCP2 toward the BDNF gene is tightly regulated by post-transcriptional modifications [35]. It should be also noted

that MeCP2 has not been studied during morphine treatment yet. We found only a study showing morphine withdrawal-induced phosphorylation of MeCP2 both in the lateral septum and the nucleus accumbens [39].

The biological importance of a diversity of BDNF exon-specific transcripts remains obscure. The distinct BDNF mRNAs can be localized in different subcellular compartments. Following neuronal activation, exons II and VI were localized in distal dendrites, whereas exons I and IV were restricted to the soma of neurons [40]. Moreover, silencing of individual endogenous transcripts in cultured neurons demonstrated that, whereas some transcripts (exons I and IV) selectively affected proximal dendrites, others (II and VI) affected distal dendrites [41]. The targeting of certain mRNAs to specific subcellular compartments, particularly to dendrites, for local protein synthesis is linked to synaptic plasticity. Although the functional significance of dendritic mRNA localization is not completely understood, the general underlying assumption is that mRNA targeting is restricted to local synthesis of proteins involved in synaptic plasticity. Repeated morphine treatment is able to remodel synaptic connectivity in a regionally specific manner. Morphine-treated animals showed considerable alterations in morphology of pyramidal cells of the neocortex (prefrontal and parietal cortex); in particular chronic morphine or withdrawal decreased the total dendritic length, the dendritic spine density, the complexity of dendritic branching [24, 25]. Degenerative processes affect midbrain structures: chronic morphine treatment and withdrawal from a chronic morphine treatment resulted in a reduction in the area and perimeter of ventral tegmental dopamine neurons [5, 26]. Bearing in mind our results that morphine withdrawal is associated with upregulation of BDNF exon I both in the frontal cortex and midbrain, we can speculate that enhancement of these exons can reflect mechanism of homeostatic compensations directed to region-specific morphological alterations during chronic morphine exposure.

Weight loss at withdrawal of chronic morphine administration is a well-defined phenomenon that may be connected with a decline in food and water intake at abstinence [42]. BDNF is involved in physiological mechanisms of feeding behavior and body weight control in the brain; particularly, elevated BDNF level in the brain is associated with a decrease in nutrient consumption and weight loss [43]. Thus, we speculate that there is a potential connection between elevated level of BDNF exon I-containing transcripts in the frontal cortex and/or midbrain during morphine abstinence and weight loss detected in the present study.

In conclusion, the present study has demonstrated that the content of BDNF exon I-containing transcripts both in the frontal cortex and midbrain during spontaneous morphine withdrawal in dependent rats but not after acute

morphine intoxication. Increased expression of BDNF exon I is accompanied by an increased occupancy of pCreb1, but not MeCP2 at corresponding promoter.

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