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Effects of the cell type-specific ablation of the cAMP-responsive transcription factor in noradrenergic neurons on locus coeruleus firing and withdrawal behavior after chronic exposure to morphine

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Abstract

Repeated exposure to opiates leads to cellular and molecular changes and behavioral alterations reflecting a state of dependence. In noradrenergic neurons, cyclic AMP (cAMP)-dependent pathways are activated during opiate withdrawal, but their contribution to the activity of locus coeruleus noradrenergic neurons and behavioral manifestations remains controversial. Here, we test whether the cAMP-dependent transcription factors cAMP responsive element binding protein (CREB) and cAMP-responsive element modulator (CREM) in noradrenergic neurons control the cellular markers and the physical signs of morphine withdrawal in mice. Using the Cre/loxP system we ablated the *Creb1* gene in noradrenergic neurons. To avoid adaptive effects because of compensatory up-regulation of CREM, we crossed the conditional *Creb1* mutant mice with a *Crem*–/– line. We found that the enhanced expression of tyrosine hydroxylase normally observed during withdrawal was attenuated in CREB/CREM mutants. Moreover, the withdrawal-associated cellular hyperactivity and *c-fos* expression was blunted. In contrast, naloxone-precipitated withdrawal signs, such as jumping, paw tremor, tremor and mastication were preserved. We conclude by a specific genetic approach that the withdrawal-associated hyperexcitability of noradrenergic neurons depends on CREB/CREM activity in these neurons, but does not mediate several behavioral signs of morphine withdrawal.

Keywords: cyclic AMP, Cre/LoxP, locus coeruleus, morphine dependence.

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Abbreviations used: ACSF, artificial CSF; ATF-1, activator transcription factor 1; cAMP, cyclic AMP; CREB, cAMP responsive element binding protein; CREM, cAMP-Responsive Element Modulator; DBH, dopamine β-hydroxylase; Forsk, forskolin; HPLC-ED, HPLC-electrochemical detection; LC, locus coeruleus; NA, noradrenaline; PAG, periaqueductal gray; PKA, protein kinase A; TH, tyrosine hydroxylase.

Several studies implicate noradrenergic systems in opiate withdrawal. In particular, adaptations in the locus coeruleus (LC), the major noradrenergic nucleus in the CNS, have been proposed to underlie the somatic manifestation of opiate dependence (Aghajanian 1978; Hayward *et al.* 1990; Koob *et al.* 1992; Maldonado and Koob 1993; Nestler 2004). At molecular level the up-regulation of the cyclic AMP-protein kinase A (cAMP-PKA) pathway contributes to the increased intrinsic excitability of LC neurons during opiate withdrawal (Nestler and Aghajanian 1997).

During opiate withdrawal cAMP activation leads to phosphorylation of the transcription factor cAMP response element binding protein (CREB) and binding to the cAMP response element in the promoters of target genes (Lonze and Ginty 2002). In fact, direct experimental evidence demonstrate that CREB is strongly activated in LC neurons during opiate withdrawal, as reflected by its enhanced phosphorylation (Guitart *et al.* 1992) and the induction of ß-galactosidase expression in LC neurons of transgenic mice expressing ß-galactosidase under the control of cAMP response elements (Shaw-Lutchman *et al.* 2002).

The approaches used to interfere with CREB function in noradrenergic neurons in rodents were in part based on the injection of CREB antisense oligonucleotides (Lane-Ladd *et al.* 1997) and more recently, on viral-mediated transfer of a dominant negative form of CREB into the LC (Han *et al.* 2006). Both studies showed the strong effect of CREB inhibition on neural activity and that some physical signs of morphine withdrawal were attenuated. However, these findings contrast with the observation that by neurotoxic lesions, thought to selectively damage the output of the LC, physical dependence was normal (Christie *et al.* 1997; Caille *et al.* 1999; Delfs *et al.* 2000; Cruz *et al.* 2008) raising the possibility that other brain regions surrounding the LC were involved. *Creb1* null mutants are lethal (Rudolph *et al.* 1998), thus mutants ubiquitously lacking the major transactivating $Creb1^{\alpha,\Delta}$ isoforms have been used to provide genetic evidence of CREB involvement in LC function and morphine withdrawal (Maldonado *et al.* 1996; Valverde *et al.* 2004). However, these approaches lacked the specificity of CREB genetic ablation specifically in noradrenergic neurons. In fact, in the conditional mutant characterized by *Creb1* loss in neural and glial progenitors CREB protein was still present in noradrenergic neurons (Maldonado *et al.* 1996; Valverde *et al.* 2004). Nevertheless, clear attenuation of physical dependence in combination with reduced firing activity of the LC neurons was observed in these mice (Maldonado *et al.* 1996; Valverde *et al.* 2004). Taken together, while there is general agreement that during withdrawal LC neurons are hyperactive and much evidence suggests a role of CREB in the cellular adaptation of LC neurons, its behavioral implications remain controversial.

To better understand the molecular and cellular basis underlying opiate effects we have analyzed the cell-autonomous role of CREB dependent gene expression in noradrenergic neuron activity and morphine withdrawal. Here, we have used conditional mutant mice in which *Creb1* gene is ablated exclusively in noradrenergic neurons (Parlato *et al.* 2007). However, the cAMP-responsive element modulator (CREM), another member of the same family of transcription factors, whose expression is otherwise undetectable in the brain, has been shown to be up-regulated upon *Creb1* ablation (Rudolph *et al.* 1998; Mantamadiotis *et al.* 2002; Parlato *et al.* 2006). Moreover, the presence of one CREM allele was sufficient to guarantee some of CREB functions (Mantamadiotis *et al.* 2002). One copy of CREM still induced most of the activity dependent CREB targets including *c-fos* (Lemberger *et al.* 2008). Because of this compensatory up-regulation, the functional, behavioral and molecular analyses were performed in mutants specifically lacking CREB in a CREM null context.

This is the first genetic model showing dissociation between the hyperexcitability of noradrenergic neurons abolished during opiate withdrawal and physical signs of opiate withdrawal that were mostly preserved.

Materials and methods

Mouse handling

Animals were housed in a temperature (21 ± 1°C) and humidity (55 ± 10%)-controlled environment, with a 12-h/12-h light-dark cycle (light on between 8:00 h and 20:00 h). Food and water were available *ad libitum*. Mice were allowed to become familiar to the experimental room and were handled during 1 week before initiating experiments. Behavioral tests and care of the animals were in accordance with standard ethical guidelines (NIH, 1985; European Communities Directives, 1986 86/609/EEC) and approved by a local ethical committee. All experiments were performed and analyzed by investigators blinded to treatment and genotype of the mice. The mice were single caged during the behavioral experiments.

We used for most of the analyses male and female mice and for the electrophysiology only male, thus our study does not address gender effects. Grouping by gender was provided for body weight or temperature.

Genotypic analysis

Generation of $Creb1^{fl/fl}$; *DBHCre* (abbreviated $Creb1^{DBHCre}$) mice was achieved by using $Creb1^{fl/fl}$ mice, where exon 10 is flanked by loxP sites (Mantamadiotis *et al.* 2002), crossed with DBHCre transgenic mice (Parlato *et al.* 2007). To generate $Creb1^{DBHCre}$; *Crem*−/− mice, we crossed $Creb1^{fl/fl}$ *Crem*+/− mice with $Creb1^{+/fl}$ *Crem*+/−; DBHCre mice. *Crem*−/− mice have been previously described (Blendy *et al.* 1996). All mice used were in a C57BL6 genetic background. For the analysis we used as controls $Creb1^{fl/fl}$ *Crem*+/− littermates. The analysis of the genotype for *Creb1* and *Crem* alleles was carried out as described (Mantamadiotis *et al.* 2002). iCre PCR primers were the following: iCre forward 5′-CTGCCAGGGACATGGCCAGG; iCre reverse 5′-GCACAGTCGAGGCTGATCAGC.

Histological analysis

Brains were perfused and post-fixed in 4% paraformaldehyde, pH7.2, and then sectioned at 50 μm on a vibratome. For the detection of β-galactosidase activity, sections were processed as described elsewhere (Hogan *et al.* 1994). For immunohistochemistry the following primary antibodies were used: anti-dopamine β-hydroxylase (DBH) (1 : 500) (DBH12-A, Alpha Diagnostics International, San Antonio, TX, USA), anti-CREB (1 : 3000), anti-CREM (1 : 500) (Mantamadiotis *et al.* 2002), anti-ATF-1 (1 : 1000) (Bleckmann *et al.* 2002), anti-tyrosine hydroxylase (TH) (1 : 1000) (AB1542, Chemicon International, Schwalbach, Germany). The primary antibodies diluted in 5% normal swine serum were incubated overnight at 4°C. Biotin-conjugated secondary antibody was diluted 1 : 400 in phosphate-buffered saline and detection was performed using the avidin-biotin system (Vector Laboratories, Peterborough, UK) with the VECTOR peroxidase kit. Staining was developed with 3,3′-diaminobenzidine and H_2O_2 (Sigma-Aldrich Chemie Gmbh, Munich, Germany). For double immunolabeling with anti-DBH and anti-CREB antibodies, the activity of the first antibody was blocked by Avidin/Biotin blocking kit (Vector Laboratories). Subsequently, the sections were stained for the second antigen using as a chromogenic reagent a 3,3′-diaminobenzidine yielding a red precipitate (Sigma-Aldrich Chemie Gmbh). In this way, we obtained brown–colored immunoreactivity for DBH and a red-colored nuclear immunoreactivity for CREB. For double immunohistochemistry with anti-TH (1 : 1000) we have used HistoGreen as a chromogenic substrate HISTOPRIME, E109 (Linaris, Wertheim-Bettingen, Germany). For double immunofluorescence, paraffin sections were incubated with anti-CREB (1 : 400) at 4°C overnight, followed by donkey anti-rabbit IgG coupled to Alexa Fluor 488 (A21206, Invitrogen, Carlsbad, CA, USA) for 1 h at 22°C. Afterwards, sections were incubated with anti-TH (1 : 500) (AB1542, Chemicon International) at 4°C overnight, followed by donkey anti-sheep IgG coupled to Alexa Fluor 594 (A11016, Invitrogen) for 1 h at 22°C. Fluorescent cells were viewed under a confocal laser-scanning microscope (LSM710, Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Non-radioactive *in situ* hybridization was performed on vibratome sections from mice killed 2 h after naloxone hydrochloride (same treatment as for the withdrawal syndrome assessment). After proteinase K digestion (20 μg/μL) for 10 min, sections were prehybridized in the hybridization solution without riboprobe at 70°C for 1 h. Sections were hybridized with specific digoxigenin–labeled riboprobes (DIG RNA labeling-kit, Roche, Basel, Switzerland) (c-*fos*, IMAGp966N2451Q; TH, gift of Prof. H. Rohrer, MPI, Frankfurt, Germany) at 70°C in hybridization solution overnight (50%formamide, 5xSSC pH4.5, 1% sodium dodecyl sulfate, 50 μg/mL yeast tRNA, 50 μg/mL heparin). After two washing steps (50% formamide, 5×SSC pH4.5, 1% sodium dodecyl sulfate) at 70°C and at 65°C the sections were incubated with the anti-digoxigenin antibody conjugated with alkaline phosphatase (1 : 10 000) (11093274910, Roche) in 20% normal swine serum and incubated overnight at 4°C. The development of the reaction was done with NBT/BCIP (Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt) according to the manufacturer instructions (Roche). Quantification of *Th* and *fos* expression was performed in a semiquantitative way by measuring the blue-stained area of the *Th* and *fos* mRNA signals in 6–8 vibratome stained sections containing the LC and located approximately between Bregma −5.38 mm and −5.85 mm. Each image was acquired at using a 20× objective and the stained area was measured with the ImageJ software (NIH, Bethesda, MD, USA) using manual adjustment of the threshold after background subtraction. The stained area was expressed in μm^2.

Relative densities of TH-positive neurons were quantified using an Axioplan 2 imaging microscope (Zeiss, Göttingen, Germany) as described previously (Zechel *et al.* 2006). In brief, the regions of interest in one focal plane were captured by a microscope-mounted Axiocam video camera (Zeiss) under the control of the software Axiovision (version 3.1; Zeiss). TH-positive neurons of the LC were photographed using a 10× objective. The areas of the LC and the number of cells within these areas were measured using the software ImageTool 3.0 (UTHSCSA, San Antonio, TX, USA). To avoid an overestimation of the counted objects, we corrected the counting using Abercrombie correction formula, as previously described (von Bohlen und Halbach and Unsicker 2003). The Linderstrom-Lang/Abercrombie (LLA) equation for estimating numerical neuronal densities is: $N = n[t/(t + H)]$ or $N/n = f = t/(t + H)$ N is an estimate of the number of objects; n is the counted number of objects; t is the mean thickness of the section; H is the mean height of the objects and f is the conversion factor for converting n to N. In a first step, n was determined. In a second step, H *and* t were estimated. These measurements were done using a computer-controlled motorized x-y-z stage under the control of the software NeuroLucida (MBF Biosciences, Williston, VT, USA). The surface areas of the LC as well as the size of the TH-immunopositive cells were measured using the combined hardware-software system NeuroLucida (MBF Biosciences). Both male and female mice were used in equal number.

HPLC–Electrochemical Detection (HPLC-ED)

For measurements of total noradrenaline and dopamine content, forebrain tissues from Bregma 2.54 mm to −0.48 mm from control and mutant mice were dissected and processed by HPLC-ED as previously described (Otto and Unsicker 1990). For this analysis $n = 5$ control (male and female) and $n = 6$ mutant (male and female) 9-month-old mice were used.

Morphine withdrawal syndrome

Opiate dependence was induced by repeated morphine injection. The morphine dose (i.p.) was progressively increased from 20 to 100 mg/kg over a period of 5 days. The first and second numbers inside the parenthesis represent the doses of morphine hydrochloride (mg/kg) injected at 9:00 h and 19:00 h, respectively: 1st day (20, 20); 2nd day (40, 40), 3rd day (60, 60), 4th day (80, 80), 5th day (100, 100), and 6th day (100 mg/kg, only in the morning). The morphine withdrawal syndrome was precipitated by naloxone hydrochloride injection (1 mg/kg, s.c.) 2 h after the last morphine administration. The drug doses were not corrected for salt and water contents. Mouse behavior was observed for 30 min beginning immediately after naloxone administration, as reported previously (Maldonado *et al.* 1996). A global withdrawal score was also calculated for each animal by giving to each individual sign a proportional weight as reported previously (Maldonado *et al.* 1992a,b). Mice from 4 to 8 months old were used for behavior analysis both male and female (control + saline: 11; control + morphine: 11; mutant + saline 9; mutant + morphine: 8).

Electrophysiology

Prior to the slice preparation, 3.5-month-old mice were either treated with saline or morphine for 5 days (same treatment as for the withdrawal syndrome assessment). On the morning of day 6, mice were injected with 100 mg/kg morphine and after 2 h, were killed to prepare acute brain slices. For morphine treated animals, the brain was cut and incubated in 5 μM morphine artificial CSF (ACSF) solution for 1–6 h before electrophysiological recording. The withdrawal was precipitated *in vitro* with 1 μM naloxone (Ivanov and Aston-Jones 2001). Current-clamp recordings ($I = 0$ pA) in loose-attached configuration were carried out in cooled ACSF containing (in mM): NaCl 119, KCl 2.5, $MgCl_2$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2 and glucose 11, bubbled with 95% O_2/5% CO_2. Slices were warmed to 34°C and transferred after 1 h to the recording chamber superfused (2.5 mL/min) with ACSF. Traces were filtered at 10 kHz and digitized at 20 kHz. To block synaptic transmission a cocktail consisting of kynurenic acid (2 mM), picrotoxin (250 μM) and strychnine (1 μM) was used. ANOVA were performed on the log transformed firing frequencies. Log transformation was used to reduce the variance heterogeneity and non-normality of the data and to better fulfill the underlying hypotheses of the ANOVA model. Only the interactions corresponding to a *p*-value lower than 0.05 for the Fisher test were kept in the selected final model. All male mice were used for the recordings (control: 11; mutant: 12).

Results

Generation of mutant mice lacking CREB in noradrenergic neurons

To achieve specific genetic ablation of CREB in noradrenergic and adrenergic cells, mice in which exon 10 of the *Creb1* gene is flanked by loxP sites ($Creb1^{flox/flox}$) (Mantamadiotis *et al.* 2002) were crossed with mice which express the Cre recombinase under the control of the *Dbh* gene contained in a P1 artificial chromosome clone (DBHCre) (Parlato *et al.* 2007). The DBHCre transgenic line fully reproduced the expression pattern of *Dbh* gene expression in the central and peripheral nervous system (Parlato *et al.* 2007). Specific loss of CREB immunoreactivity in $Creb1^{flox/flox}$; *DBHCre* mutants (abbreviated $Creb1^{DBHCre}$) was observed in DBH-expressing cells of the LC (Fig. 1a and b, insets). To further monitor the specificity of the transgenic recombinase activity, DBHCre animals were crossed with the ROSA26 reporter mouse line (Soriano 1999). As shown by the co-localization of β-galactosidase activity (blue) and DBH immunoreactivity (brown), reporter gene activity was restricted to DBH-positive neurons (Fig. 1c, inset). To further assess the specificity of this mutation, we have analyzed by double immunofluorescence CREB expression (green signals in Fig. 2) in noradrenergic neurons of the LC (Fig. 2a and b) and dopaminergic neurons of the ventral tegmental area (Fig. 2c and d) both characterized by the expression of the enzyme TH (red signals in Fig. 2a–d). In control LC CREB was clearly visible in the TH-positive cells

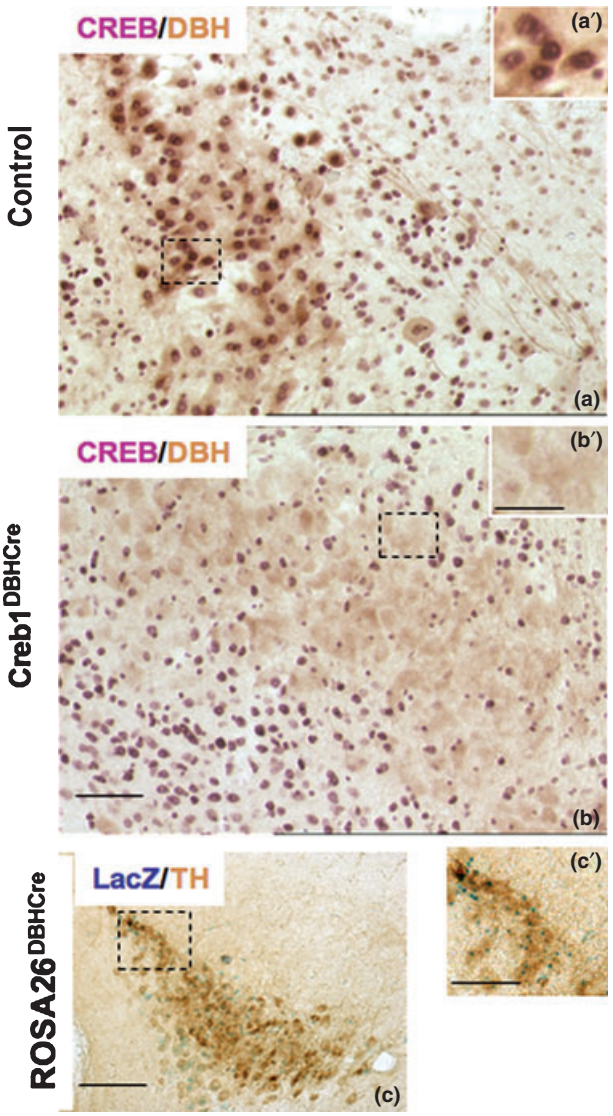


Fig. 1 Specific loss of cAMP response element binding protein (CREB) in noradrenergic neurons of $Creb1^{DBHCre}$ mice. (a, b) Immunohistochemistry of coronal vibratome sections with CREB (red) and dopamine-β-hydroxylase (DBH) (brown)-specific antibodies in control (a) and $Creb1^{DBHCre}$ mice (b) (2-month-old) in the locus coeruleus (LC). (c) Detection of Cre recombinase activity in the reporter mouse line ROSA26 crossed with the DBHCre transgenic line by β-galactosidase staining of coronal sections containing the LC identified because of tyrosine-hydroxylase (TH) positive immunolabeling. Inlays represent the high magnification of the respective boxed areas. Scale bar: 125 μm (a, b); 250 μm (c); 40 μm (a′, b′); 125 μm (c′).

(Fig. 2a), while in $Creb1^{DBHCre}$ mutant it was lost (Fig. 2b). No changes were observed in TH positive dopaminergic neurons of the ventral mesencephalon between control and mutant mice (Fig. 2c and d).

In line with other CREB mutant strains (Rudolph *et al.* 1998; Mantamadiotis *et al.* 2002; Parlato *et al.* 2006), we observed that CREM, another member of the same family of

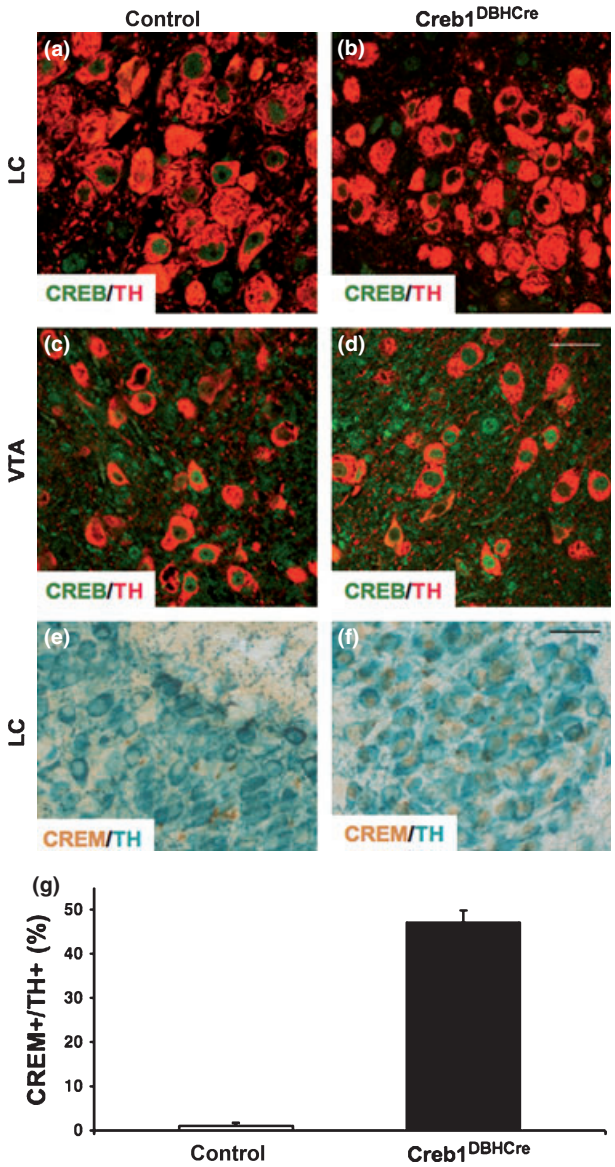


Fig. 2 Loss of cAMP response element binding protein (CREB) restricted to noradrenergic neurons results in cAMP-Responsive Element Modulator (CREM) up-regulation. (a–d) Double immunofluorescence analysis showing CREB (green) in tyrosine hydroxylase (TH) positive neurons (red) within the locus coeruleus (LC) (a, b) and the ventral tegmental area (VTA) (c, d) in control (a, c) and *Creb1*^{DBHCre} mutant (2-month-old) (b, d). (e, f) Immunohistochemistry with CREM (brown) and TH (blue)-specific antibodies in control (e) and *Creb1*^{DBHCre} mice (f) in the LC. (g) Quantification of the percent of TH positive neurons positive for CREM in the LC of control and *Creb1*^{DBHCre} mutant mice (Bregma −5.38 mm and −5.85 mm) (2-month-old) (n = 4). Scale bar: 40 μm (a–d); 60 μm (e, f).

transcription factors, was up-regulated in *Creb1*^{DBHCre} mutants (Figure S1a and b). To quantify the level of CREM up-regulation in noradrenergic neurons we analyzed the number of TH positive cells also CREM positive by double immunohistochemistry with CREM and TH specific antibodies (Fig. 2e and f). About 50% of TH positive neurons were also CREM positive, while in control mice CREM protein was mostly below detection threshold (Fig. 2g). To circumvent the effects of a possible adaptive up-regulation, we generated and further characterized *Creb1*^{DBHCre}/*Crem*−/− mutants by crossing *Creb1*^{DBHCre} mice with germ line *Crem*−/− mutants (Blendy *et al.* 1996). The *Creb1*^{DBHCre}/*Crem*−/− mutants were viable and did not differ from control littermates in size, body weight or temperature (male control: 35.9 g ± 4.8; male mutant: 36.5 g ± 6.2; female control: 28.6 g ± 4.5; female mutant: 27.7 g ± 3.1; control: 37.9°C ± 0.8; mutant: 37.8°C ± 0.7; n = 12 per group). Also in these double mutants, we have confirmed the loss of CREB in TH positive neurons (Figure S2c and d). In addition we have assessed in *Creb1*^{DBHCre}/*Crem*−/− mutants the expression levels of the activator transcription factor 1 (ATF-1) that also shares a high degree of homology with CREB, but found no changes between the genotypes (not shown).

Loss of CREB/CREM does not influence the survival of noradrenergic neurons but modulates the induction of TH expression

Previous studies have shown severe neurodegeneration in hippocampus and striatum of conditional forebrain-specific *Creb1* mutants in a *Crem*−/− background (Mantamadiotis *et al.* 2002) and to a lesser extent also in mutants with targeted *Creb1* deletion in dopaminergic neurons (Parlato *et al.* 2006). Thus, we estimated the effects of CREB/CREM on survival of LC noradrenergic neurons by the analyzing the density, the surface area and the mean size of TH positive neurons in the LC of animals between the age of 2–6 months. No significant changes were observed, indicating that CREB and CREM are not essential for survival of noradrenergic neurons in the LC (Figure S2).

Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of noradrenaline (NA) and its expression in the LC is regulated by opiates (Lane-Ladd *et al.* 1997; McClung *et al.* 2005). While precipitation of opiate withdrawal in control mice led to an increase in the *Th* mRNA (Fig. 3a, b and e), in *Creb1*^{DBHCre}/*Crem*−/− mice the induction of *Th* expression was abolished in LC neurons (Fig. 3c–e). Quantification of *Th* mRNA levels confirmed that *Th* expression was in part regulated by CREB, although, because of the small sample size, our conclusions could rather indicate a trend (Fig. 3e) (*genotype* $F(1,8) < 1$, NS; *treatment* $F(1,8) = 36.60$, $p = 0.0003$; *genotype* × *treatment* $F(1,8) = 10.24$ $p = 0.01262$).

Using HPLC-ED we have assessed the functional status of the noradrenergic neurons by measuring NA levels in the forebrain, known to receive substantial noradrenergic innervations. We found that NA levels in the *Creb1*^{DBHCre}/*Crem*−/− mutants were lower than in control mice, whereas the

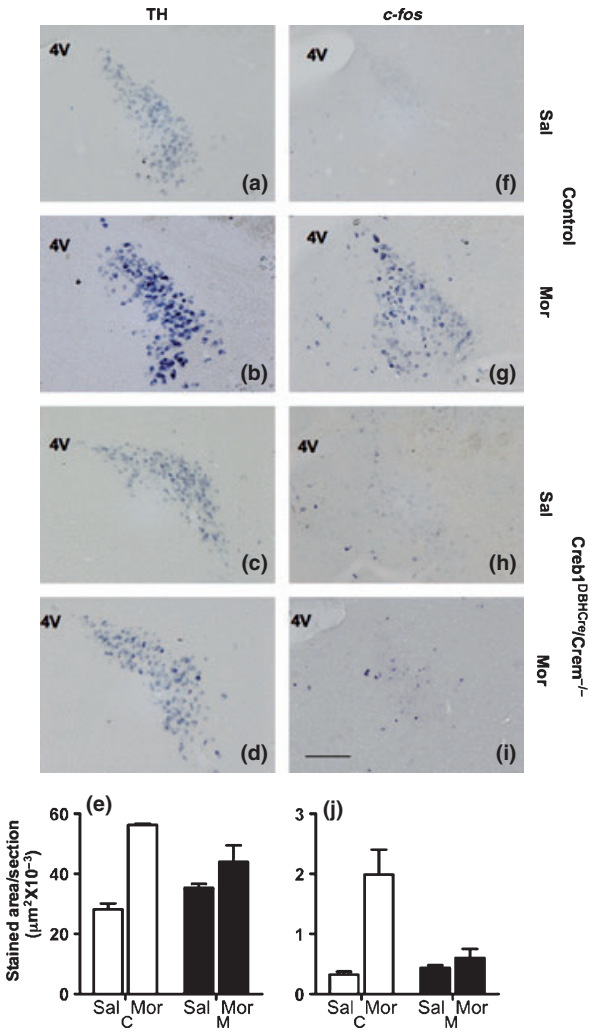


Fig. 3 Expression of cAMP response element binding protein (CREB)-dependent genes in the locus coeruleus (LC) after naloxone-induced morphine withdrawal**.** (a–d, f–i) Representative images of non-radioactive *in situ* hybridization experiments showing that *Th* (a–d) and *c-fos* mRNA (f–i) are stimulated in the LC by naloxone-induced morphine withdrawal in control mice (a, b, f, g) but not in *Creb1*^{DBHCre}/*Crem*−/− mutants (5-month-old) (c, d, h, i). All the analyses were performed in the same conditions. (e, j) Semiquantitative analysis of the stained area per section (expressed in μm^2) in vibratome sections containing the LC between Bregma −5.38 mm and −5.85 mm ($n = 3$ in each group). Scale bar: 125 μm.

dopamine content in the same tissues was similar (Figure S3).

Cell autonomous firing activity of LC neurons in *Creb1*^{DBHCre}*/Crem*−/− mutants

There is general agreement that LC neurons are hyperactive during withdrawal. Increased neuronal firing can also be observed *ex vivo* in the acute slice preparation of the brainstem (Aghajanian 1978). This hyperactivity is reflected by the up-regulation of immediate early genes like *c-fos* (Curran *et al.* 1996). We examined whether *c-fos* is induced after morphine withdrawal in mutant animals by non-radioactive *in situ* hybridization. By measuring the stained area in each experimental condition we found that precipitation of opiate withdrawal in control mice led to an increase in *c-fos* mRNA in the LC (Fig. 3f, g and j), but not in *Creb1*^{DBHCre}*/Crem*−/− mutants (*treatment* $F(1,8) = 16.43$, $p < 0.01$; *genotype* × *treatment* $F(1,8) = 11.08$ $p < 0.05$; Tukey's HSD (for Honest Significant Difference) post-tests for morphine vs saline $p < 0.01$ for controls and NS for double-mutants). However, as for the TH mRNA analysis, given the small sample size, these conclusions can be considered as a clear trend (Fig. 3h–j).

To further analyze this hyperactive state, we monitored the firing frequency of LC neurons (i.e. the number of action potential per second) in acute slices of the brainstem *ex vivo* after 1 week of morphine treatment and during the induction of naloxone-precipitated withdrawal. When control mice were challenged with naloxone, the firing frequency in LC neurons was significantly higher in slices from morphine-treated animals compared with saline-injected mice (Fig. 4a and b). In slices from *Creb1*^{DBHCre}*/Crem*−/− mutants that were saline-treated, baseline firing was very sparse, while after morphine withdrawal the firing frequency was similar to naïve control mice (Fig. 4a and b). There was a significant effect of the genotype on the basal firing of LC neurons and the magnitude of the increase of the firing frequency was larger in the controls compared with the mutant mice (2ANOVA: genotype $F(1,17) = 35.15$, $p < 0.001$; morphine exposure $F(1,17) = 38.01$, $p < 0.001$; interaction $F(1,17) = 5.95$, $p = 0.026$). We therefore repeated these experiments in the presence of a pharmacological cocktail blocking synaptic transmission by inhibiting GABAA, glycine, α-amino-3-hydroxy-5-methylisoxazole-4-propionate and NMDA receptors. This approach also allowed us to determine the contribution of synaptic input to this increase of the firing rate. There was again a major difference between control and *Creb1*^{DBHCre}*/Crem*−/− mutant mice. In these conditions, the increased firing frequency normally associated with morphine withdrawal was abolished in the mutant mice. When in the end of each recording, we applied forskolin (Forsk) to maximally activate the cAMP-PKA pathway, we observed a significant increase of the basal firing frequency in saline injected control mice, an effect that was occluded in slices from morphine-withdrawn control mice (3ANOVA: interaction of genotype with morphine exposure $F(1,51) = 12.67$, $p = 0.001$; interaction of genotype and Forsk treatment $F(1,51) = 4.89$, $p = 0.032$). In stark contrast, in slices from *Creb1*^{DBHCre}*/Crem*−/− mutants baseline firing was virtually abolished and could not be increased by Forsk, both in saline and morphine-treated mice (Fig. 4c and d).

Taken together, we conclude that CREB and CREM in the LC are necessary for the maintenance of normal firing

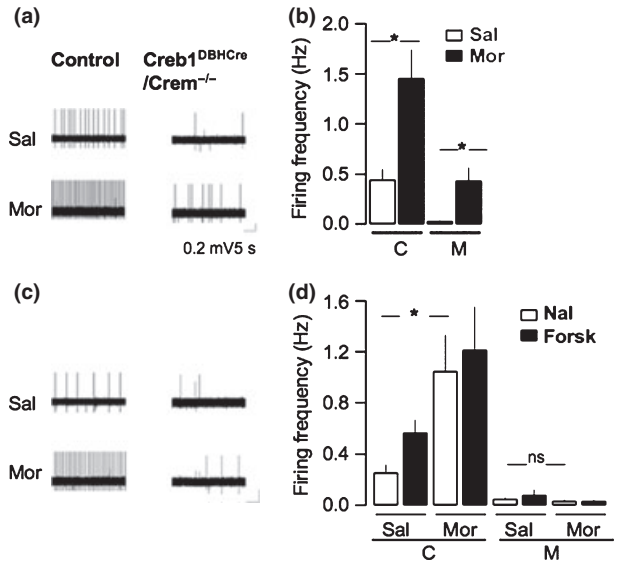


Fig. 4 Reduced spontaneous firing and after withdrawal in the locus coeruleus (LC) neurons of *Creb1*^{DBHCre}/*Crem*–/– mice in comparison to respective controls. (a) Representative traces of loose-cell-attached recordings in brainstem slices of control (C) and *Creb1*^{DBHCre}/*Crem*–/– (M) mice after bath application of naloxone 1 μM, in saline (sal) and morphine (Mor)-treated animals. (b) Bar graph showing mean firing rates at baseline and during withdrawal. During withdrawal in both control and mutant mice, there is a significant increase in LC neuron firing rate, which is lower in *Creb1*^{DBHCre}/*Crem*–/– mice. (c) Representative traces as above, but in the presence of a cocktail blocking synaptic transmission (see methods). (d) Bar graph showing mean firing rates after bath application of naloxone (nal) 1 μM and after application of forskolin 10 μM (Forsk). Notice that in mutant mice basal firing is virtually abolished and that withdrawal and Forsk are without effect. Six to twelve neurons were recorded per experimental condition. Control and *Creb1*^{DBHCre}/*Crem*–/– mice (n = 6 in each group) were between 2 and 5 month-old.

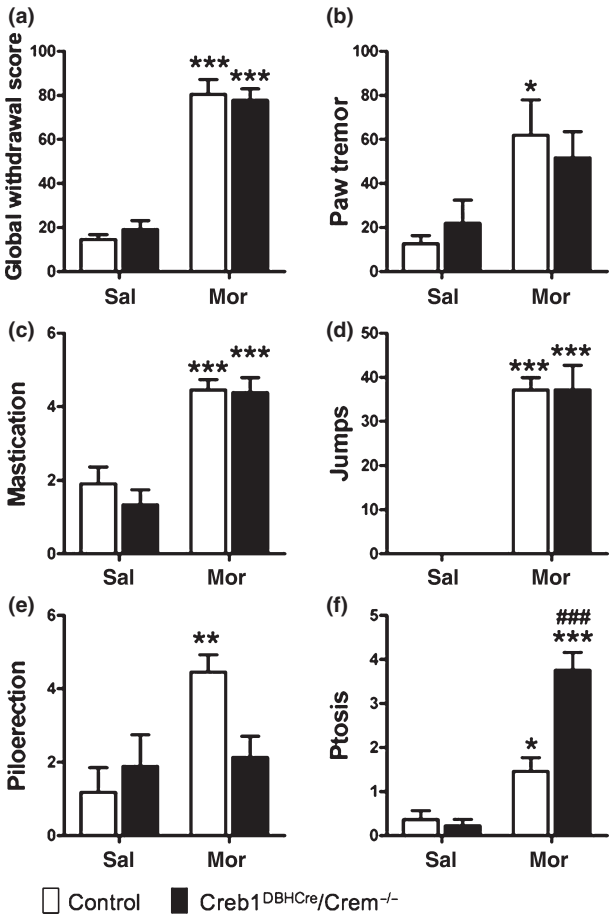


Fig. 5 Analysis of morphine withdrawal in *Creb1*^{DBHCre}/*Crem*–/– mutants. (a–f) Morphine withdrawal was precipitated by naloxone injection (1 mg/kg, s.c.) in mice intermittently treated with morphine or saline as indicated below the graphs. Open bars represent control (*Creb1*$^{fl/fl}$/*Crem*+/–) animals and black bars correspond to *Creb1*^{DBHCre}/*Crem*–/– mutant mice ± SEM (n = 8–11). Behaviors were scored for 30 min starting from naloxone treatment. A global withdrawal score was calculated by giving a range of possible scores from 0 to 100 for each animal taking into account all the individual behavioral signs. Significant difference between mean scores in saline vs morphine treated mice of same genotype are indicated by '*' for $p < 0.05$, '**' for $p < 0.01$ and '***' for $p < 0.001$ (Tukey's HSD post-test). Difference in mean scores of withdrawal symptoms in morphine treated controls vs mutants is labeled with '###' for $p < 0.001$. Comprehensive results of statistical analyses are provided in Table S1.

rates under baseline conditions and for the cell-autonomous increase in cellular excitability during morphine withdrawal.

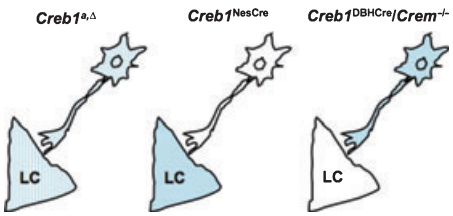
Loss of CREB and CREM in noradrenergic neurons does not affect opiate withdrawal

Next, we tested opiate withdrawal syndrome in the *Creb1*^{DBHCre}/*Crem*–/– mutants (n = 8–11 per group) (Table S1 and Fig. 5 and Fig. S4). Naloxone challenge in mice treated for 5 days with increasing doses of morphine (20–100 mg/kg) resulted in a severe withdrawal syndrome, manifested by the precipitation of several stereotypical behavioral responses, including paw tremor, mastication, jumping, piloerection and ptosis. As shown in Fig. 5a, the global withdrawal score for the total number of checked signs did not change between control and mutants. For all the withdrawal physical signs, there were no significant differences between *Creb1*^{DBHCre}/*Crem*–/– mutants and control mice (Fig. 5b–d; for complete overview of statistical analysis see Table S1). Withdrawal-induced piloerection was attenuated in *Creb1*^{DBHCre}/*Crem*–/– mutants (*genotype* $F(1,35) = 1.54$, NS, *treatment* $F(1,35) = 8.88$, $p = 0.005$; *genotype* × *treatment* $F(1,35) = 5.28$, $p = 0.028$), while the occurrence of ptosis was enhanced (*genotype* $F(1,35) = 12.08$, $p = 0.001$; *treatment* $F(1,35) = 59.88$, $p < 0.001$; *genotype* × *treatment* $F(1,35) = 18.89$, $p < 0.001$) (Fig. 5e

and f). Severity of opiate withdrawal symptoms in *Crem*−/− mutants was similar to controls (data not shown).

Discussion

The purpose of the present study was to determine the role of cAMP-dependent transcription in noradrenergic neurons for firing activity and opiate physical dependence using cell type-specific genetic manipulation. Our cellular analysis focused on the LC asking whether cAMP-dependent transcription is necessary for the hyperactivity of these noradrenergic neurons during morphine withdrawal, a highly debated matter. In fact, only few studies have tried to address the direct role of the effects of cAMP-dependent transcription by using specific genetic approaches. Figure 6 summarizes the phenotypic alterations reported for the two previously generated mouse lines with mutated CREB (*Creb1*$^{\alpha\Delta}$ and *Creb1*^{NestinCre}) (Valverde *et al.* 2004) and for the *Creb1*^{DBHCre}/*Crem*$^{-/-}$ mutants. Here, we show that a loss of cAMP cascade by conditional ablation of *Creb1* in noradrenergic neurons in a *Crem*$^{-/-}$ background strongly decreases the intrinsic activity of noradrenergic neurons, prevents opiate-induced expression of TH and *c-fos* but has only minor consequences on the behavioral opiate withdrawal syndrome.



	Creb1$^{a,\Delta}$	Creb1^{NesCre}	Creb1^{DBHCre}/Crem$^{-/-}$
Firing rate	-	-	-
TH/c-fos	nd	nd	-
Piloerection	-	-	-
Paw tremor	-	-	=
Ptosis	nd	=	+
Jumping	=	=	=
Body tremor	-	=	=
Mastication	nd	nd	=
Global Withdrawal score	-	-	=

–, decreased; +, increased; =, not changed; nd, not determined

Fig. 6 Schematic representation of the pattern of cAMP response element binding protein (CREB) expression in the locus coeruleus (LC) and surrounding neurons in different CREB genetic mutants. Loss of CREB in the LC and in its afferents is indicated in white. The loss of *Creb1*$^{\alpha\Delta}$ isoforms is indicated as a pattern of white and color in the LC and its afferents. The possible afferent types are discussed in the text. The table summarizes the analysis of firing rate, tyrosine hydroxylase/c-*fos* induction and physical signs of morphine withdrawal in the *Creb1*^{DBHCre}/*Crem*−/− mutants in comparison with other genetic CREB mutants.

Our findings contrast with the previous observation in germ line mutants of CREB signaling (*Creb1*$^{\alpha\Delta}$), where the global withdrawal score was significantly reduced. Our findings are also surprising in light of reports that in rats where CREB antisense oligonucleotides and dominant negative form of *Creb1* were injected into the LC, some signs of morphine withdrawal were attenuated (Lane-Ladd *et al.* 1997; Han *et al.* 2006). On the other hand we reported that CREB activity in forebrain-specific mutants was independent of the development of drug dependent behaviors (Bilbao *et al.* 2008). These findings are not necessarily contradictory. For example, dominant-negative proteins may affect protein-protein interactions of CREB, which is not the case in the knock-out mice. Moreover, compensatory changes in *Creb1*^{DBHCre}/*Crem*−/− mice must be considered. We have excluded effects as a result of CREM up-regulation and ATF-1 expression is undetectable in the mutant mice. Moreover, the *Creb1*^{DBHCre}/*Crem*−/− mice show no obvious impairments and we find no difference in the survival of LC noradrenergic neurons compared with controls. On the other hand, intrinsic activity of LC neurons was strongly attenuated (and noradrenaline content slightly decreased) in basal conditions in *Creb1*^{DBHCre}/*Crem*−/− mice. Such reduction of basal firing rates reveals that CREB and CREM contribute to the regulation of the basal firing rate of LC neurons. Similar decrease in activity was reported after expression of dominant-negative variant of the CREB protein in medium spiny neurons of the nucleus accumbens (Dong *et al.* 2006), which was attributed to changes in expression of ion channels controlling resting membrane potential. A similar mechanism could also explain the decrease in basal activity of LC neurons observed in *Creb1*^{DBHCre}/*Crem*−/− animals.

The lower basal activity of noradrenergic LC neurons was increased by naloxone in *Creb1*^{DBHCre}/*Crem*−/− mice, but only if synaptic transmission was intact indicating that a combination of extrinsic and intrinsic mechanisms controls the hyperactivity during withdrawal. Indeed, the application of a cocktail blocking pre-synaptic input completely abolished this residual activity, confirming that the PKA/CREB cascade is completely inactive in LC neurons of *Creb1*^{DBHCre}/*Crem*−/− mutants. Moreover, this analysis shows that normal firing rates of noradrenergic LC neurons are supported by the input from other regions and are not exclusively mediated via the post-synaptic PKA pathway. The observation that the increase in LC firing frequency during withdrawal in *Creb1*^{DBHCre}/*Crem*−/− mutants is present when synaptic transmission is intact suggests a role for afferents from tegmental and medullar neurons (Rasmussen 1995) or the inhibitory neurons projecting from the periaqueductal gray (PAG) (Bajic *et al.* 2000). It is possible that these afferents undergo adaptive changes during chronic exposure to morphine in wild-type mice and thus

they contribute to the opiate withdrawal syndrome. In the mutant such adaptive changes may occur to a lesser extent, because noradrenergic neurons that now lack CREB, indirectly influence the function of the neurons upstream, thus blunting the increase in firing frequency. The reduction of the withdrawal symptoms observed after acute, localized but not cell type-specific interference with CREB function (Lane-Ladd *et al.* 1997; Han *et al.* 2006), could result from disrupted CREB-dependent transcription in nearby non-noradrenergic neurons.

However, based on observations that rely on pharmacological interventions and chemical or mechanical lesions, the noradrenergic system has been implicated in opiate withdrawal. Which parts of the noradrenergic system are responsible for the opiate withdrawal however is hotly debated and several reports argue that the LC is not essential (Christie *et al.* 1997). Our data supports this view, as in the absence of CREB activity in the LC withdrawal symptoms, such as jumping, paw tremor, tremor and mastication remain unchanged. However, our findings do not preclude a modulatory role for CREB for instance by enhancing withdrawal symptoms, which may explain some of the observation in response to the acute over-expression of CREB (Carlezon *et al.* 1998).

Our data do not identify the actual locus that controls opiate withdrawal, which may actually be distributed among several brain regions. For example, projectiosn from the amygdala to the PAG may be involved in the dysphoria and hyperalgesia during withdrawal (Chieng and Christie 2009), while increased noradrenergic release in the bed nucleus of the stria terminalis may underlie anxiety (Aston-Jones and Harris 2004). The underlying biochemical adaptation may involve several pathways, including mitogen activated protein kinase cascades and cAMP mediated cascades (Christie 2008). Within the PAG, the opiate- sensitive GABAergic neurons have been strongly implicated in opiate withdrawal (Chieng and Christie 2009). It is possible that such cellular changes eventually lead to changes in synaptic connectivity and feedback circuit adaptations.

Our findings indicate that loss of CREB/CREM in *Creb1*^{DBHCre}/*Crem*−/− mice is not essential for most of withdrawal behaviors. However, looking at individual withdrawal signs, piloerection was attenuated and ptosis enhanced in *Creb1*^{DBHCre}/*Crem*−/− mice. Ptosis is caused by an inhibition of sympathetic activity and piloerection reflects an increase in sympathetic tone. The increased ptosis but reduced piloerection observed during withdrawal in *Creb1*^{DBHCre}/*Crem*−/− mutants cannot be explained by a reduced number of noradrenergic sympathetic neurons. We have previously reported that in *Creb1*^{DBHCre}/*Crem*−/− mutants the survival of sympathetic neurons was not impaired and rather an increase in their number was observed because developmental cell death was inhibited by down-regulation of proapoptotic CREB-dependent mechanisms (Parlato *et al.* 2007). However, we cannot exclude that the changes in ptosis and piloerection may reflect a mild sympathetic dysfunction caused by loss of CREB and CREM. Taken together these observations suggest that sympathetic dysfunction might support some signs of morphine withdrawal and that withdrawal syndrome is not homogeneous but it is a combination of symptoms related to the dysfunction of several systems.

In conclusion, while CREB/CREM activity is necessary for opiate-induced increase in expression of TH and increase of intrinsic activity of noradrenergic neurons, neither of these molecular changes appear essential for the expression of most behaviors associated with opiate withdrawal. Nevertheless, we do not suggest that this argues against a role of the noradrenergic system as such in withdrawal. Plasticity within the noradrenergic system is necessary for development of opiate dependence and withdrawal (Delfs *et al.* 2000; Cruz *et al.* 2008). Moreover, the CREB/CREM role in noradrenergic neurons may be still essential in the development of dependence as it is blocked in mice with broad genetic ablation of CREB.

Our results illustrate the complexity underlying the development of drug-induced behaviors. We however also demonstrate that molecular effects caused by drugs of abuse can in some cases be dissociated from behaviors that normally occur in parallel. This does not completely exclude an effect of CREB-dependent transcription for tuning drug-induced behaviors, but argues against the notion that CREB activity has specific role in the process. A possible conclusion is that dependence involves the reorganization of neuronal circuits or systems, and cannot be fully understood by studying adaptive changes of specific neurons. Future experiments, taking advantage of cell type-specific and circuit-specific expression of optogenetic tools may help to address these questions.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. cAMP-responsive element modulator up-regulation in absence of CREB and loss of CREB in *CrebDBHCre/Crem−/−* mutants.

Figure S2. Survival program of noradrenergic neurons in the LC is unaffected in the absence of CREB/CREM.

Figure S3. Catecholamine content in CREBDBHCre/CREM−/− mutants.

Figure S4. Weight loss after morphine withdrawal.

Table S1. Statistical analysis of withdrawal symptom scores.

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