Deletion of CREB1 from the Dorsal Telencephalon Reduces Motivational Properties of Cocaine

Transcriptional changes in neurons underpin the long-lived cellular response to environmental stimuli, and cAMP-responsive element-binding protein (CREB1) has been implicated in this process. Exposure to psychostimulants such as cocaine results in persistent neuronal plasticity in cortical circuitry that likely modulates the motivation to use the drug again. To examine whether CREB1 in cortical glutamatergic neurons was implicated in cocaine use, we developed conditional CREB1 mutants that exhibit ablation of functional CREB1 in the cortex and hippocampus. Here we report that CREB1 mutants show normal locomotor responses to acute and chronic cocaine and develop a place preference for cocaine. However, CREB1 mutants demonstrate a diminished drive to self-administer cocaine under operant conditions. We conclude that there is a specific role for CREB1 in telencephalic glutamatergic neurons regulating the motivational properties of cocaine.

Keywords: addiction, cocaine, CREB1, Cre-Lox transgenics, Emx1

Introduction

The Creb1 gene encodes the nuclear transcription factor cAMP-responsive element-binding protein (CREB1). CREB1 is an essential component of the molecular machinery that drives ongoing homeostatic regulation of cellular transcriptome. In turn, CREB1 acts as a key substrate mediating aspects of learning and memory (Dash et al. 1990, 1991), including long-lasting electrophysiological adaptations (Barco et al. 2002; Pittenger et al. 2002), memory consolidation, and retrieval (Pittenger et al. 2006; Isiegas et al. 2008). More recently, a role for CREB1 in the molecular actions of drugs of abuse has been established (Pliakas et al. 2001; Andersen et al. 2002; McClung and Nestler 2003).

Midbrain dopamine pathways are an established candidate for mediating the acute rewarding properties of drugs of abuse (Di Chiara and Imperato 1988; Cadoni and Di Chiara 1999). Furthermore, medium spiny neurons of the ventral striatum function as coincident detectors for the concomitant innervation by dopaminergic and glutamatergic afferents (Valjent et al. 2006), crucially integrating both the corticostriatal and mesostriatal pathways. These form an integral component of, and impact upon, reciprocal projections found within the basal ganglia circuit. Glutamatergic (N-methyl-D-aspartate receptor mediated) plasticity of D1 receptor-containing neurons in the striatal complex is required for the development of sensitization to cocaine (Heusner and Palmiter 2005). In addition, lasting plasticity at glutamatergic synapses onto ventral tegmental area dopamine cells is observed following a single injection of cocaine in mice (Unless et al. 2001), a mechanism thought to underpin the motivational and reinforcing properties of drugs.

Recent studies have demonstrated that cocaine sensitization may be driven by the persistent perturbation of corticostriatal synaptic transmission (Thomas et al. 2001). Kalivas and Volkow (2005) have noted that inactivation of the prefrontal cortex (PFC) can prevent all forms of reinstatement drug seeking, suggesting that this is a critical neurobiological substrate driving motivated behaviors and responses to external and internal stimuli. Given the role of CREB1 in the consolidation of synaptic plasticity (Nestler 2002), we hypothesized that corticaly derived CREB1 is involved in the affective drive to self-administer cocaine.

CREB1 deletion mouse models, thus far, have proved troublesome as total CREB ablation generates a perinatal lethality phenotype (Rudolph et al. 1998), whereas mice hypomorphic for CREB exhibit upregulation in the CREB1 β isoform. To overcome these problems, we employed the Cre-loxP targeted recombinase system (Tsien et al. 1996) to generate conditional CREB1 mutant mice, exhibiting the ablation of all CREB1 isoforms with a spatial resolution constrained to neurons of the developing dorsal telencephalon (Briata et al. 1996). To achieve this, we cross-bred 2 transgenic mouse lines, one which expressed the Cre recombinase transgene driven by the homeobox gene Emx1 (Iwasato et al. 2000) and the other having a critical exon of the Creb1 gene “floxed” (flanked with loxP sequences) (Mantamadiotis et al. 2002).

In the Creb1Emx1Cre model presented herein, subsequent to recombination, the truncated CREB1 protein is rendered unstable (Mantamadiotis et al. 2002) and, thus, will be absent in relevant cells. As the Creb1Emx1Cre model results in germline deletion of CREB1, certain of biological compensations are considered. Indeed, prior studies have observed that the expression of CREB1 during development contributes to neuronal survival and plasticity (Rudolph et al. 1998; Lonze et al. 2002; Chiamulera et al. 2008). Moreover, basic region/leucine zipper motif (bZIP) family member transcript levels may compensate in response to CREB1 mutations (Rudolph et al. 1998; Walters and Blendy 2001; Valverde et al. 2004). Therefore, we examined the brains of Creb1Emx1Cre mice to assess for any developmental plectropism induced by the germline deletion of CREB1. Furthermore, to determine possible ontogenetic compensatory effects of the mutation, we assayed transcript levels in related bZIP CREB/activating transcription factor-1 (ATF-1)/cAMP response element modulatory protein (CREM) family members, as well as the transcriptional coactivator CREB-binding protein (CBP) (Charvia et al. 1993).

Using Emx1Cre, we have targeted the glutamatergic pyramidal neurons of the cerebral cortex and hippocampus.
(Iwasato et al. 2000). With the ablation of CREB1 from the hippocampus, we sought to determine the impact upon hippocampal-dependent learning and memory. Studies involving CA1 CREB1-deficient mice (Pittenger et al. 2002) have demonstrated a subtle deficiency in spatial learning and reference memory in the Morris water maze (MMW), although mutants lacking cortical or forebrain-restricted CREB1 did not appear to display such deficits (Balschun et al. 2003). Moreover, mice lacking CREB1 protein in the brain display an anxiety phenotype (Valverde et al. 2004). To assess the likelihood of any such phenotype in the Creb1<sup>Emx1Cre/Cre.Creblox/lox</sup> mutant, we conducted discrete experiments for anxiety, including the elevated plus maze and large open field.

As glutamatergic pyramidal neurons are implicated in the cortical modulation of striatal and tegmental neuronal change during acute and chronic drug abuse (Hyman 1996; White and Kalivas 1998; Nestler 2002), we investigated behavioral sensitization, an assay for drug-induced neural plasticity. Such plasticity underlies the enhancement of a behavioral response upon reexposure to cocaine and other drugs (Pierce and Kalivas 1997). Furthermore, as mice deficient in central CREB1 have been demonstrated to exhibit a place preference to the reinforcing effects from drugs of abuse (Valverde et al. 2004), we determined the impact of cortical and hippocampal CREB1 deletion in regards to place preference for cocaine. Ultimately, Creb1<sup>Emx1Cre/Cre.Creblox/lox</sup> mutants were assessed in the operant conditioning paradigm to address the motivational properties of natural and drug reinforcers.

Here we report the characterization of conditional CREB1 mutants. Our results demonstrate that excitatory forebrain systems expressing CREB1 are not necessary for the Pavlovian conditioned response to cocaine, however, appear to be critically involved in motivational properties of cocaine. Together, these data suggest a role for CREB1 in cortical glutamatergic neurons in behaviors that underpin continued cocaine use.

### Materials and Methods

**Animals**

Emx1<sup>Cre/Cre</sup> mice were obtained from Iwasato et al. (2000), whereas the floxed Creb<sup>lox/lox</sup> transgenics were procured from Mantamadiotis et al. (2002). Initially, all mice were backcrossed for 10 generations onto a C57/Bl6 background prior to use. Transgenes expressing Cre recombinase (Cre-Neo cassette insert into chromosome 6) driven by the Emx1 promoter were crossed with mice expressing floxed Creb1<sup>lox/lox</sup>. Control mice were taken as mice with the following genotypes: Emx1<sup>Cre/Cre</sup>, Creb1<sup>lox/lox</sup>, Emx1<sup>Cre/Cre.Creb1/llox</sup>, and Emx1<sup>Cre/Cre</sup>. Emx1<sup>Cre/Cre.Creb1/llox</sup> mice were regarded as those expressing one (Emx1<sup>Cre/Cre.Creb1/llox</sup>) or two (Emx1<sup>Cre/Cre.Creb1/llox</sup>/Emx1<sup>Cre/Cre.Creb1/llox</sup>) alleles of the Cre recombinase transgene, with both CREB alleles floxed (flanked by loxP).

All experiments described and performed with Creb1<sup>Emx1Cre/Cre.Creblox/lox</sup> mice were conducted in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Experiments were conducted within established experimental conditions that were separate from the housing environment. Where necessary, experimental subjects were acclimated to the experimental environment prior to experimentation. Only male mice were utilized for behavioral experiments. All mice were housed on a 12-h light/dark cycle (7 AM–7 PM), with free access (ad libitum) to water and rodent chow (Ridley AgriProducts, Pakenham, Victoria, Australia), upon cat litter (Breeders Choice, fibreCycle, Nerang, Queensland, Australia) with tissue supply for nesting. Those underlying intravenous self-administration of cocaine were singly housed under reverse-cycle lighting conditions.

**PCR Genotyping**

Mice were genotyped by polymerase chain reaction (PCR), using previously described Creb<sup>lox</sup> methods (Mantamadioti et al. 2002), as well as those provided by Takai Iwasato for Emx1<sup>Cre/Cre</sup> transgenic mice. The primers employed in PCR were (5’→3’): Creb1 (TATGTAAAG-CAGGGAGAATCTG, TAGAACATTTGAGCAGCATT), and Emx1 (TGAGTCACTGTCGACGCTG, TGGTATGCACGCTCTGGAA). Primers were ordered from Geneworks (Victoria, Australia).

**Quantitative PCR**

Quantification of bZIP CREB/ATF-1/CREM family and CBP transcripts were assayed by quantitative reverse transcriptase PCR (qRT-PCR). Cortical, striatal, and hippocampal nuclei were microdissected from brains of freshly sacrificed naive Creb1<sup>Emx1Cre/Cre.Creblox/lox</sup> mice. Total RNA was extracted using the Qiagen (Doncaster, Victoria, Australia) RNasy Mini Kit according to the manufacturer’s instructions and, subsequently, reverse transcribed with the Invitrogen SuperScript III Platinum Two-Step qRT-PCR Kit (random hexamers and oligo(dT)20), according to the manufacturer’s instructions. All quantitative polymerase chain reaction experiments were performed using the SYBR Green I intercalating dye and run on an Applied Biosystems ABI 7500 Fast thermal cycler in Normal Mode with 20 μl reaction volumes in 96-well microplates. Data were analyzed by ΔΔCt, described elsewhere (Livak and Schmittgen 2001).

A single endogenous control gene was used for experiments, selected from four candidates using genNorm (Vandesompele et al. 2002). Primers for genes of interest were designed with Primer3 (Rozen and Skaletsky 2000) to generate an amplicon of between 130 and 150 bp, with a primer length of 20 nucleotides and mouse genome specificity confirmed with UCSC BLAT (basic local alignment search tool [BLAST]-like alignment tool) (Kent 2002) and BLAST (Altschul et al. 1990). The successful flanking of amplicon and intron was corroborated with the UCSC In Silico PCR (Hinrichs et al. 2006). Primers, ordered from Geneworks (Hindmarsh, SA, Australia) are listed by gene (forward, reverse, concentration):

- **Atf1**: (GGGTTGGCAAGTGGAGTAA, GAACAGGCTGAGATGAGCT, 300 μM).
- **Atf2**: (CAAGAGGGCTTCCGAAGATG, AGGTAAGGCGCTTCTGGT, 50 μM).
- **CBP** (GTCTTTGCGTTTTGTCGAC, CGGACATCTGCAGCTTC, 50 μM).
- **Crem** (AGTCCCGCAACTAGCAGA, GATTCTCAACACAGCACA, 300 μM).
- **Creb1** (CCAAACTAGCTTGGGACGAT, GAATGATAGACCCGGCTGA, 300 μM).
- **Creb1** (excised exons) (CTTCAGGCGGATGAAACAATGCA, CTCCTCTTCCG-TGCCGTCTTC, 50 μM).
- **β-Actin** (GATCTGGCACACACTCTC), GGAGGTGGAGTGGCTCACA, 50 μM).
- **Hprt1** (CTTTGCTAGTTCTGGTCTGATT, TAATGCCCCGTGAGCTGT, 300 μM).
- **Mthfd1** (AAGGAAAGTGGTGGGTAGTGT, GCTGGTGCTGCTTCAATGAT, 300 μM).
- **Tbp** (TTCGTCGCAAGAAGATTGAAA, TCTTGTCGACACCACTTTTTC, 300 μM).

**Immunohistochemistry**

Mice were transcardially perfused individually with 30-ml phosphate-buffered saline (PBS, 0.1 M, pH 7.4) followed by fixation with 30 ml of 4% paraformaldehyde (Sigma, Victoria, Australia) in PBS, followed by postfix in fixative containing 10% sucrose. Immunohistochemistry, previously described (McLean et al. 1999; McDougall et al. 2004; McPherson and Lawrence 2006; McPherson et al. 2007), was adapted for individual antibodies. Coronal 40-μm brain sections were...
preblocked with 10% normal serum (NS) and then incubated with rabbit anti-CREB1 (Cell Signalling Technology, Boston, MA; #9197, 1:1000) in PBS containing 1% NS and 0.3% TX-100 (PBS-NTx) for 48 or 24 h, respectively, at 4 °C. Subsequently, sections were washed, then incubated in PBS-NTx containing biotinylated secondary antibody (1:500 dilution; Vector, East Brisbane, Queensland, Australia) solution for 1 h at room temperature, again rinsed, then immersed in PBS-NTx containing streptavidin horse radish peroxidase (1:500; Vector) for 1 h at room temperature. After washing, sections were incubated with nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride (Sigma) solution (0.1 M PBS, 0.004% w/v ammonium chloride/ammonium nickel (II) sulfate hexahydrate) for 10–30 min, and immunoreactivity was then developed by addition of hydrogen peroxide (0.03%). The reaction was terminated by washing in 0.1 M PBS (3 × 10 min).

Sections processed for immunofluorescence were double stained for CREB1 and mouse anti-γ-aminobutyric acid (GABA) (Sigma Aldrich, A0310, 1:100) or mouse antiparvalbumin (Sigma Aldrich, Sydney, Australia; PS088; 1:1000) overnight at 4 °C, followed by incubation at room temperature for 4 h with relevant secondary antibodies, Alexa Fluor 594 goat α mouse IgG and Alexa Fluor 488 goat α rabbit IgG at 1:500 dilution (Invitrogen, Mount Waverley, Victoria, Australia) for parvalbumin/GABA and CREB1, respectively. Subsequently, sections were slide mounted and coverslipped with antifade fluorescent mounting medium (Dako, Kingsgrove, NSW, Australia). Analysis was performed on a Leica LB DM-2 microscope.

**Stereology**

To assess for any histological differences as well as cell number dysregulation in regions devoid of CREB1 in the Creb1flx/flxCre mice, stereology was performed on coronal brain sections (n(C) = 3, n(KO) = 3) (McPherson and Lawrence 2006; McPherson et al. 2007). Section analysis was performed on a Leica DM LB-2 microscope, using Stereo Investigator 7.0x (MicroBrightField, Inc., Williston, VT). Stereological analysis involved cellular quantification and volumetric analyses, with relative stereotaxic coordinates established according to a mouse atlas ( Paxinos and Franklin 2001). The cingulate cortex (1, 2) was subject to cellular quantification by fractional analysis, at 4 levels (Bregma 1.18, 0.98, −0.22, −0.82 mm) unilaterally per subject, generating an average cell count. Both the hippocampal CA1 region and striatum (caudate putamen, nucleus accumbens) were analyzed volumetrically with Cavalieri estimation, generating a predicted assessment of the regional area, and volume through the structure. Volumetric analyses were conducted at four separate levels in the CA1 (Bregma −2.06, −2.3, −2.54, −2.8 mm) and striatum (Bregma 1.42, 1.18 or 1.1, 0.86, 0.62 mm).

**Ethology**

A prototypical battery of tests comprising relevant ethological assessments were conducted in order to establish any underlying phenotypic differences. A prototypical battery of tests comprising relevant ethological assessment were broken down by within-control group confounding phenotype, statistical analyses were conducted whereupon the submerged platform was removed. Mice were tracked for time spent and entries into individual quadrants (EthoVision 3.0).

To determine the impact of CREB1 deletion upon hippocampal-dependent learning tasks, we employed the MWM (Featherby et al. 2008). A large circular container (1.2 m diameter) was filled with opaque water such that the waterline was 1 cm above a submerged platform. Water temperature was maintained at 25 ± 1 °C. Mice were randomly assigned a quadrant as home for the platform placement. Spatial cues were housed in constant locations. Each mouse received four 2-min trials per day, with random seeding for the first trial placement, followed by sequential placement into remaining quadrants. Mice were allowed to remain on the platform for 30 s. Twenty four hours after the final training session, a single 2-min probe trial was conducted whereupon the submerged platform was removed. Mice were tracked for time spent and entries into individual quadrants (EthoVision 3.0).

**Behavioral Sensitization to Cocaine**

Spontaneous locomotor activity and sensitization to repeated cocaine was conducted in photo-optic locomotor chambers (Coulburn Instruments, Whitallham, PA) (Brown et al. 2009). Mice were initially habituated to the locomotor cells during 30-min sessions, across 3 sequential days. Control and CREb1flx/flxCre mice were randomized into different treatment groups to receive either cocaine 20 mg/kg body weight (intraperitoneal [i.p.]; Sigma) or saline 0.1 ml/10 g body weight (i.p.) once per day over 5 sequential days and tracked 30-min sessions. One week later, subjects were randomly assigned to challenge with either saline 0.1 ml/10 g or cocaine 10 mg/kg (i.p.) and tracked in the smaller open field. Final groups are denoted by pretreatment, followed by challenge: sensitized (Coc−Coc), n(C) = 11, n(KO) = 10); context (Coc-Sal, n(C) = 10, n(KO) = 5), and acute (Sal−Coc, n(C) = 8).

**Place Preference Conditioning**

Conditioned place preference (CPP; n(C) = 8, 12, n(KO) = 13) was undertaken to determine the response of CREb1flx/flxCre mice. The CPP apparatus (Hamilton Kinder) was of an enclosed, rectangular design, with two equally sized chambers made distinct by somatosenory (floor pad) and visual (patterned walls) cues, and a small central neutral zone for initial subject placement. Each chamber and the neutral zone were independently lit by its own 6 × 8 white light emitting diode grid. Retractable doors placed at the lateral sides of the neutral zone facilitated free access to both chambers or the confinement of subjects to a single chamber for the duration of a session (Brown et al. 2009). On a preliminary pretreatment day, mice had free access to both chambers to assess for any basal chamber preference. A treatment schedule involving alternating days of cocaine and salin control (1 week) and adaptation to saline (3 days saline). On every odd treatment day (1, 3, 5, 7), mice received cocaine 20 mg/kg (i.p.), whereas on every even treatment day (2, 4, 6), subjects received saline 0.1 ml/10 g body weight (i.p.). Treatments were matched with context in a counterbalanced manner to preclude a side preference. Mice were then immediately placed in the given chamber and monitored during a 30-min session. Twenty four hours following final cocaine treatment, mice were returned to the chambers and allowed free access. Time spent and entries into either chamber were monitored.

**Operant Conditioning**

Self-administration of a natural reinforcer, 10% sucrose (w/v) solution, was assessed as previously described (Brown et al. 2009). For cocaine self-administration, mice were surgically implanted with cannulae feeding into the jugular vein (Brown et al. 2009) and bi-daily flushed with 0.02 ml Heparin-antibiotic solution for 48 h following surgery. Mice were conditioned under a dual lever program, with the active lever programmed to deliver a cocaine infusion at a dose of 0.25 mg/kg/infusion (n(C) = 14, n(KO) = 15) or 0.5 mg/kg/infusion (n(C) = 20, n(KO) = 11). Each successful reinforcing response (active lever press) was conditioned to a light cue (conditioned stimulus), whereas an olfactory cue (vanilla essence) was presented beneath the active reward-associated lever (unconditioned stimulus). A 10-s time-out was imposed between reinforcers as well as a ceiling of 100 reinforcers per
2-h session. Mice were examined on a fixed ratio of 1 (FR1) lever press to one intravenous infusion of cocaine. Upon acquisition of stable operant responding for cocaine, mice were tested on a progressive ratio (PR) session (Brown et al. 2009). The criteria for stable responding was set at 10 infusions of cocaine per session, as well as an active lever discrimination of at least 75% versus inactive lever, consistently observed over 3 consecutive days. A break point was measured, taking the value of the step associated with the last completed ratio (number of reinforcers earned) after a 60-min hold (the period with no reinforcer earned) (Thomsen et al. 2005). In all sessions, active lever latency, lever presses, and active lever time-out responses were collected.

**Statistics**

All stereological analysis was performed using Prism 5.0, with specific between-genotype tests involving 2-tailed t-tests. qRT-PCR data were plotted in Prism 5.0, and linear regression was performed for data arising from the validation experiments. Data from all other qRT-PCR experiments were analyzed in SigmaStat 3.1 with 1-way analyses of variance (ANOVA), taking genotype as a factor and employing Student-Newman-Keuls (SNK) post hocs. All behavioral analysis was performed with SigmapStat 3.1 as 1-way or 2-way ANOVAs, using repeated measures where applicable. All post hocs were SNK, unless a Kruskal-Wallis test was applied on ranks, whereupon Dunn’s post hocs were used.

**Results**

**Emx1-Driven Ablation of CREB1**

To elucidate the specific contribution that CREB1 in cortically originating glutamatergic pathways makes to cocaine-related behaviors, we generated a mutant mouse by cross-breeding Emx1Cre mice (Iwasato et al. 2000) with (Creb1lox) mice such that the exon responsible for dimerization and response element association was excised (Mantamadiotis et al. 2002). The resulting mutant (Creb1Emx1Cre) was characterized against littermates without a recombination, designated as controls (Supplementary Fig. 1).

**CREB1 Ablation in the Cortex and Hippocampus**

Immunostaining of Creb1Emx1Cre mice brain showed that CREB1 protein was almost completely ablated through the cerebral cortex (Fig. 1A) and was similarly ablated from pyramidal cells of the hippocampus (Fig. 1C). Conversely, nuclear expression of CREB1 protein in control mice brain was clearly observed; both cingulate cortex (Fig. 1B) and hippocampus CA1–CA3 and dentate gyrus (Fig. 1D) replete with CREB1-immunopositive cells. Lateral amygdaloid CREB1 expression remained intact in Creb1Emx1Cre mice (Fig. 1E,F). Also, robust CREB1 immunoreactivity was apparent in the striatum of Creb1Emx1Cre and control mice (Fig. 1G,H).

A remaining, albeit diffuse, expression of CREB1 protein was observed in the cortex and hippocampus of knockout mice. In order to ensure that such remaining protein was restricted to GABAergic interneurons, double immunostaining for CREB1 and GABA or parvalbumin was performed. Immunofluorescent data confirmed that hippocampal and cortical neurons of Creb1Emx1Cre mutant mice which expressed CREB1 coexpressed parvalbumin (Fig. 2B) or GABA (Fig. 2C,D). In contrast, although control mice also demonstrated a subset of hippocampal neurons coexpressing CREB1 and parvalbumin (Fig. 2A), a predominance of strictly CREB1 single-labeled cells was revealed. Together, these data provide clear evidence for the fidelity of CREB1 ablation in mice and confirm the expected spatial resolution conveyed by the recombination event.

**Creb1Emx1Cre Mice Show No Evidence of Neurodegeneration**

Total estimated cell counts for the cingulate cortex of control mice were no different to those observed for knockout mice (F(1,4) = 1.441, P = 0.82) (Supplementary Fig. 2). Similarly, Cavallieri estimation of structural volume was not statistically different between control and knockout mutant mice in either the CA1 region of the hippocampus (Supplementary Fig. 2) or in the striatum (data not shown).

**Creb1Emx1Cre Mice Do Not Display an Anxiety Phenotype**

On the elevated plus maze, no significant difference between genotype or within-control subgroup was observed for either entries into, or time spent in, the open arms (Supplementary Fig. 3A,B). Further, mice tracked in the large open field for key parameters displayed no statistical difference between Creb1Emx1Cre and control genotypes nor within the control subgroups for these measures (Supplementary Fig. 3C,D).

**Creb1Emx1Cre Mice Demonstrate Intact Spatial Learning and Reference Memory**

To assess spatial learning, Creb1Emx1Cre mice were trained on the MWM to locate a submerged hidden platform. Control mice showed a typical pattern of task learning that was similar to Creb1Emx1Cre mice (Fig. 4A). Further, latency to platform was not different between genotypes on any individual day, F3,9,802 = 1.3, P = 0.233 (genotype × day). Indeed, these findings are broadly similar to mice with a dominant negative CREB isoform in the hippocampus (Pittenger et al. 2002). After learning, a probe trial was performed to test spatial reference memory. Creb1Emx1Cre and control mice displayed a similar latency to resolve the previous platform locus, and also similar time was spent in the previous platform locus and home quadrant (Fig. 4B), suggesting equivalent spatial reference memory under the conditions of the test.

**Creb1Emx1Cre Mice Exhibit a Hypoactive Phenotype, but Sensitization to Cocaine Is Normal**

**Spontaneous Locomotion—Habituation to Novel Environment**

Mice were introduced to a novel environment (photo-optic chambers) and their locomotor behavior monitored over 3 successive days. Both genotypes displayed a robust habituation to the context (Fig. 5A). Throughout the entire 3 days of habituation, Creb1Emx1Cre knockout mice displayed
a significantly diminished spontaneous locomotor activity profile relative to controls \( F_{3,232} = 2.98, P = 0.034 \) (genotype), and \( F_{2,232} = 116.4, P < 0.001 \) (day) (Fig. 5A).

**Behavioral Locomotor Sensitization to Cocaine**

Following habituation, both Creb1\(^{Emx1\text{Cre}}\) and control mice were randomly assigned to receive either saline or cocaine 20 mg/kg (i.p.) treatment daily for 5 days. The acute response to cocaine was similar between genotypes, and across the 5 days of treatment, mice of both genotypes similarly developed sensitization to the behavioral effects of repeated cocaine exposure (Fig. 5B). During the challenge day, mice challenged with cocaine (Sensitized) displayed a sensitized behavioral response to cocaine, which was observed in both the control (Fig. 5C) and knockout (Fig. 5D) genotypes, \( F_{5,57} = 1.99, P=0.093 \) (treatment group).

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Figure 1. Expression of CREB1 protein along the neuraxis of Creb1\(^{Emx1\text{Cre}}\) mice. (A-D) CREB1 immunoreactivity is replete through the cingulate cortex and pyramidal cell layers of the hippocampus in control mice (B, D), whereas almost devoid in that of knockout mice (A, C). (E-H) Subcortical structures including the lateral amygdala and basolateral amygdala (E–F) and dorsomedial caudate putamen (G–H) probed for CREB1 protein demonstrated an equivalent immunoreactivity. La, lateral amygdala; BLA, basolateral amygdala. Scale bar: 100 \( \mu \)m.
Creb1Emx1Cre mice show normal Pavlovian conditioning to cocaine

We analyzed Creb1Emx1Cre mice for expression of a CPP to cocaine. A robust preference toward the cocaine-paired chambers was observed for control and mutant mice when challenged 24 h after the cessation of conditioning. This result was similarly observed across all three control subgroups, \( F_{6,107} = 19.19, P < 0.001 \) (Fig. 6). Notably, however, the C2 and C3 groups appeared to be closer in their cocaine preference score to mutant mice compared with C1. This suggests that C2 and C3 are possibly the more robust control genotypes, with no apparent difference between them. Because previous studies have used exclusively what we term C2 as the control genotype for CREB deletion studies (Mantamadiotis et al. 2002; Valverde et al. 2004), we chose to focus upon this genotype as a valid control group for subsequent studies.

Cortical CREB1 Is Involved in the Motivational Properties of Cocaine

Self-Administration of a Natural Reinforcer (10% Sucrose)

Control and Creb1Emx1Cre mice were trained to self-administer a natural reinforcer, 10% sucrose (w/v) solution. Both genotypes displayed similar instrumental learning capabilities and reward discrimination, rapidly acquiring self-administration of the reinforcer via the active lever (Supplementary Fig. 4A).
showed a main effect of dose, \( F_{1,417} = 69.22, P < 0.001 \), a main effect of genotype, \( F_{1,417} = 45.48, P < 0.001 \), and a dose \( \times \) genotype interaction, \( F_{1,417} = 7.708, P < 0.05 \). Post hoc analysis indicated that control mice self-administered more cocaine than Creb\(^{F\text{Emx1Cre}}\) mice at both 0.5 mg/kg/infusion \((q = 10.225, P < 0.001)\) and at 0.25 mg/kg/infusion \((q = 3.73, P < 0.01)\) under FR1 conditions. Mice of both genotypes self-administered less cocaine when the dose was reduced to 0.25 mg/kg/infusion (Fig. 7C), suggesting that the lower dose was less reinforcing. The event records presented (0.5 mg/kg/infusion) demonstrate the "bunched" manner in which infusions are made in both genotypes (Fig. 7D).

To further examine the motivational properties of cocaine, Creb\(^{F\text{Emx1Cre}}\) and control mice were tested on a progressive ratio. The cumulative response record for cocaine at 0.25 mg/kg/infusion demonstrated no difference between genotypes, \( F_{1,119} = 0.23, P > 0.05 \) (Fig. 7G). Control mice demonstrated significantly higher cumulative responses on cocaine at 0.5 mg/kg/infusion versus Creb\(^{F\text{Emx1Cre}}\) mice, showing a main effect of genotype, \( F_{1,355} = 6.66, P < 0.05 \), a main effect of time, \( F_{1,355} = 19.34, P < 0.001 \), and a genotype \( \times \) time interaction, \( F_{1,355} = 4.4, P < 0.01 \) (Fig. 7H). Post hoc analysis demonstrated significantly greater cumulative responding for cocaine by control mice from 60 min into the progressive ratio session through session end (60 min: \( q = 2.96, P < 0.05 \); 70 min: \( q = 3.34, P < 0.05 \); 80 min: \( q = 3.81, P < 0.01 \); 90 min: \( q = 4.3, P < 0.01 \); 100 min: \( q = 4.53, P < 0.01 \); 110 min: \( q = 5.02, P < 0.01 \); 120 min: \( q = 5.57, P < 0.001 \)). A significant effect of genotype \((F_{1,37} = 4.33, P < 0.05)\) and dose \((F_{1,37} = 5.25, P < 0.05)\) was observed at 0.5 mg/kg/infusion for break point (Fig. 7E). Post hoc analysis demonstrated a significantly greater break point made by control mice at 0.5 mg/kg/infusion versus Creb\(^{F\text{Emx1Cre}}\) mice \((q = 4.32, P < 0.01)\). In addition, break point in control mice at 0.25 mg/kg/infusion was significantly lower than controls at the higher dose \((q = 3.6, P < 0.05)\), which was not the case in knockout mice. These data are consistent with a modulation in the motivational properties of cocaine in Creb\(^{F\text{Emx1Cre}}\) mice in a dose-specific manner.

**Discussion**

We have generated a conditional mutant mouse lacking CREB1 from Emx1-containing projection neurons of the dorsal telencephalon. Here we report that CREB1 mutants demonstrate diminished motivation to self-administer cocaine. In addition, although CREB1 mutants show a place preference for cocaine, the drug–environment association is not retained in the long term. We conclude that there is a specific role for CREB1 in telencephalic glutamatergic neurons regulating the motivational and associative properties of cocaine.

**Emx1-Driven CREB1 Ablation: Developmental Compensation**

The Creb\(^{F\text{Emx1Cre}}\) mouse has specific ablation of all CREB1 isoforms (\(\alpha, \beta, \delta\)) from cells expressing the Emx1 promoter, by removal of the exon responsible for DNA binding and dimerization, thus, making the protein unstable (Mantamadiotis et al. 2002). We confirmed the successful targeted ablation of CREB1 protein in Creb\(^{F\text{Emx1Cre}}\) mice in cortex and hippocampus. The contribution of CREB1 to normal cellular development is established (Rudolph et al. 1998). Previous studies of CREB1-deficient mice have demonstrated excessive apoptosis.
Lonze et al. (2002; Mantamadiotis et al. 2002), pre- or perinatal mortality (Bleckmann et al. 2002; Mantamadiotis et al. 2002), and developmental perturbations in neural structures (Valverde et al. 2004). In brain regions expressing the conditional ablation of CREB1, we found no differences in cell number or volume between genotypes.

Real-time PCR examined expression of endogenous bZIP CREB/ATF-1/CREM family members and CBP, in specific brain structures in the Creb1Emx1Cre mutants. Crem transcript was upregulated in both hippocampal and cortical brain regions, but not the striatum, of Creb1Emx1Cre mutants, consistent with the expression pattern of Emx1 (Iwasato et al. 2000). Previous studies investigating the neural knockdown or knockout of CREB1 isoforms have observed developmentally upregulated CREM (Blendy et al. 1996; Hummler et al. 1994; Rudolph et al. 1998; Valverde et al. 2004), which shares high sequence homology with CREB1 (Bleckmann et al. 2002; Shaywitz and Greenberg 1999). Presumably, this acts to circumvent untoward developmental deficits, given the similarity of function (Shaywitz and Greenberg 1999). Furthermore, we witnessed concurrent downregulation of excised Creb1 transcript in the cortex and hippocampus, but neither the amygdala nor the striatum, of mutants. These data confirm that the recombination event is constrained to the hippocampus and cortex and that in response to this developmental insult, Creb transcript is upregulated. It is important to note that the downregulation of excised Creb1 transcript here represents the downregulation of functional CREB1 as the proteome will lack the exon responsible for CRE binding and dimerization (Mantamadiotis et al. 2002). Within the striatum, Atf1 transcript was downregulated and CBP transcript upregulated. As described in Bleckmann (Bleckmann et al. 2002), ATF-1 plays a critical developmental role in mice, so the dysregulation of Atf1 transcript in the striatum of mutants may be due to alterations in the efferent innervation by glutamatergic projection neurons arising from the cortex. Within the cortex, however, Atf2 transcript was upregulated in the mutant, whilst CBP transcript was downregulated. CBP is a CREB co-activating factor (Chrivia et al. 1993), playing an important role in the functional effect of bZIP family member dimers, bridging at the TATA box the formation of stable RNA Pol-II transcription factor complexes (Shaywitz and Greenberg 1999). CBP also exhibits histone acetyltransferase activity (Fass et al. 2003), the subsequent perturbation of which may lend itself to explaining the altered sensitivity toward cocaine observed in the CREB1 mutants (Levine et al. 2005). Together, these data support spatial resolution of CREB1 ablation. Moreover, the data demonstrate the developmental plasticity of the nervous system, with compensatory regulation of other bZIP family members in the absence of functional CREB1.

**Spatial Learning Phenotype**

Given the apparent ablation of all CREB1 isoforms from the hippocampus, we assessed hippocampal-dependent learning.
The hippocampus is a component of limbic information processing, important for relating external cues and events to an internal affective state (Robbins and Everitt 1996; White 1996), providing a translational interface between motivation and subsequent action. We observed no spatial learning or reference memory deficit in the mutant. Although studies using a nestin (Valverde et al. 2004), CamKIIα-driven (Balschun et al. 2003), or hypomorphic (Gass et al. 1998) Creb1 knockout demonstrated no learning phenotype, other Creb1-αB (Bourtchuladze et al. 1994) and Crebc (Gass et al. 1998) research found impairments in learning and reference memory. Pittenger et al. (2002) generated a mutant with CREB/ATF-1/CREM bZIP family knockdown restricted to the dorsal CA1 region of the hippocampus. Their MWM findings were of essentially intact learning and short-term memory in the mutant, but impairments in longer term memory; moreover, LTD and LTP on hippocampal slices from mutant, but impairments in longer term memory; moreover, essentially intact learning and short-term memory in the region of the hippocampus. Their MWM findings were of Pittenger et al. (2002) generated a mutant with CREB/ATF-1/CREM bZIP family knockdown restricted to the dorsal CA1 region of the hippocampus. Their MWM findings were of essentially intact learning and short-term memory in the mutant, but impairments in longer term memory; moreover, LTD and LTP on hippocampal slices from mutant, but impairments in longer term memory; moreover, essentially intact learning and short-term memory in the region of the hippocampus. Their MWM findings were of Pittenger et al. (2002) generated a mutant with CREB/ATF-1/CREM bZIP family knockdown restricted to the dorsal CA1 region of the hippocampus. Their MWM findings were of essentially intact learning and short-term memory in the mutant, but impairments in longer term memory; moreover, LTD and LTP on hippocampal slices from C1 (black bars, n = 8), C2 (hatched bars, n = 12), and C3 (diagonally patterned bars, n = 21) all displayed a preference for the cocaine-paired side, \( *P < 0.05 \) (mean ± standard error of the mean).

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**Telencephalic CREB1 Regulates Motivational Properties of Cocaine and Creb1Emx1Cre Mutants Demonstrate No Deficits in Pavlovian Conditioning to Cocaine**

Behavioral sensitization is well established (Downs and Eddy 1952; Lett 1989; Kalivas and Duffy 1993). As CREB1 is involved in sensitization to psychostimulants (Turech et al. 1997; Brehenhouse et al. 2007; McPherson et al. 2007), we assayed Creb1Emx1Cre mice for sensitization to cocaine. Creb1Emx1Cre and control mice showed similar hyperactivity to acute cocaine and equivalent development and expression of locomotor sensitization to repeated cocaine. Data from the Creb1Emx1Cre model corroborate our observation, with equivalent locomotor activity observed to cocaine acutely and also in the development of cocaine sensitization (Walters and Blendy 2001). These data collectively suggest that the molecular events underpinning the development of sensitization, and the stability of these changes which accompany expression, are not perturbed significantly in the Creb1Emx1Cre mutants. Despite the relevance of the medial PFC to the attentional/motive circuits driving relapse behavior (Grusser et al. 2004), it is possible that compensatory upregulation of CREM ameliorated any deficits which may have arisen due to ablation of CREB1, in this specific context.

Previous studies have been equivocal regarding the place preference properties of cocaine in mice hypomorphic for CREB1 (Walters and Blendy 2001; Kreibich and Blendy 2004). Thus, to further explore this domain of cocaine/context-conditioned behavior, we assayed for a place preference to cocaine. Creb1Emx1Cre mutants demonstrated a similar place preference for cocaine as controls when challenged at 24 h following the final conditioning session. These data suggest that Pavlovian conditioning within the mutant remains intact and that the mutant finds cocaine reinforcing at the given conditioning dose (20 mg/kg).

Creb1Emx1Cre mice were trained on an instrumental operant task to self-administer a natural sucrose reinforcer. Mice of both genotypes successfully acquired this task and demonstrated high discrimination between the active and inactive levers. Thus, the impact of the recombination appears to not affect this form of cue-conditioned instrumental learning. In contrast, testing Creb1Emx1Cre mutants for intravenous cocaine self-administration showed a profound phenotype. A dose-dependent selectivity of responding in Creb1Emx1Cre mice was apparent, such that responding for cocaine at 0.25 and 0.5 mg/kg/infusion was diminished in mutants. This finding was corroborated by latency data, such that mutants took longer to obtain their first cocaine reinforcement. Furthermore, on an FR1 schedule, mutants increased self-administration to a lesser extent than did control mice when the cocaine dose was elevated. In concert, on a progressive ratio, a reduced break point at 0.5 mg/kg/infusion, but not at 0.25 mg/kg/infusion, was observed in Creb1Emx1Cre mice. Notably, cumulative responding under PR was similar for mutants at both cocaine doses, but PR responding in controls was significantly greater for the higher dose of cocaine. These data suggest that Creb1Emx1Cre mice have a diminution in the motivation to self-administer cocaine intravenously, consistent with a reduced reward valence of intravenous cocaine in Creb1Emx1Cre mutants. An alternative interpretation for these data is that mutant mice are more sensitive to the aversive properties of cocaine, a finding previously documented with

![Place Preference for Cocaine](image-url)

**Figure 6.** Creb1Emx1Cre knockout mice express a place preference to cocaine. Mice conditioned to cocaine (20 mg/kg) were tracked for time spent in the saline-paired (SP) or cocaine-paired (CP) chambers. Creb1Emx1Cre mice (clear bars, n = 13), control mice subgroups C1 (black bars, n = 8), C2 (hatched bars, n = 12), and C3 (diagonally patterned bars, n = 21) all displayed a preference for the cocaine-paired side, \( *P < 0.05 \) (mean ± standard error of the mean).
accumbal CREB overexpression (Carlezon et al. 1998; Pliakas et al. 2001; Sakai et al. 2002; Dong et al. 2006). It should, however, be noted that Creb1Emx1Cre mice still develop a place preference to cocaine in a manner similar to controls and

while they obtain more cocaine under FR1 conditions at the higher dose offered, it is this higher dose where the reduced break point occurs under PR. It would therefore seem likely that a motivational component exists in this phenotype, but

Figure 7. Self-administration of cocaine in Creb1Emx1Cre knockout mice is reduced in a dose-dependent manner. (A–D) Mice trained to self-administer cocaine intravenously (IVSA) on an FR-1 schedule demonstrated (A) significantly greater active to inactive lever presses (*P < 0.05), with control mice making more active lever presses than knockout mice (#P < 0.05) and (B–C) self-administering more cocaine during the 8 days of treatment than knockout mice (*P < 0.05). Control and knockout mice alike (C) increased cocaine responding at the higher dose of cocaine (#P < 0.05). (D) A representative event record during cocaine IVSA at 0.5 mg/kg/infusion during a single FR-1 session. Individual vertical lines represent discrete intravenous infusions of cocaine. (E–H) Upon acquisition of stable responding, knockout mutant mice run on a single 2-h PR schedule session demonstrated a significantly lower break point (E) and received fewer infusions (#) than control mice at 0.5 mg/kg/infusion (*P < 0.05). Control mice displayed an increased break point and propensity for cocaine at the higher cocaine dose (#P < 0.05). Cumulative active lever responses during FR were overlapping at a cocaine dose of 0.25 mg/kg/infusion (G); however, a significantly greater cumulative response ratio was observed in control mice at 0.5 mg/kg/infusion (H) (*P < 0.05); error bars: standard error of the mean.
we cannot at this stage rule out a contribution of altered aversion in addition.

In summary, our findings provide a robust characterization of the importance of CREB1 in cortically derived glutamatergic neurons in the motivational properties of cocaine, highlighting the critical role that CREB1 contributes to downstream signaling pathways in these networks following substance abuse.

Supplementary Material

Supplementary Figs 1–4 can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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