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Distinct regulation pattern of Egr-1, BDNF and Arc during morphine-withdrawal conditioned place aversion paradigm: role of glucocorticoids

Running title: Morphine withdrawal and corticoids

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Abstract

Negative affective aspects of opiate abstinence contribute to the persistence of substance abuse. Importantly, interconnected brain areas involved in aversive motivational processes, such as the ventral tegmental area (VTA) and medial prefrontal cortex (mPFC), become activated when animals are confined to withdrawal-paired environments. In the present study, place aversion was elicited in sham and adrenalectomized (ADX) animals by conditioned naloxone-precipitated drug withdrawal following exposure to chronic morphine. qPCR was employed to detect the expression of brain derived neurotrophic factor (*Bdnf*) and the immediate early genes (IEG) early growth response 1 (*Egr-1*) and activity-regulated cytoskeletal-associated protein (*Arc*) mRNAs in the VTA and mPFC at different time points of the conditioned place aversion (CPA) paradigm: after the conditioning phase and after the test phase. Sham+morphine rats exhibited robust CPA, which was impaired in ADX+morphine animals. *Egr-1* and *Arc* were induced in the VTA and mPFC after morphine-withdrawal conditioning phase. Furthermore, *Bdnf* expression was enhanced in the VTA during the test phase. *Bdnf* induction seemed to be glucocorticoid-dependent, given that was correlated with HPA axis function and was not observed in morphine-dependent ADX animals. In addition, BDNF regulation and function was opposite in the VTA and mPFC during aversive-withdrawal memory retrieval. Our results suggest that IEGs and BDNF in these brain regions may play key roles in mediating the negative motivational component of opiate withdrawal.

Keywords: Adrenalectomy; ventral tegmental area; medial prefrontal cortex; morphine withdrawal aversive memories

Introduction

Drug addiction is a chronic relapsing disorder, in part due to strong associations formed between drugs and stimuli associated with drug use [1]. One factor thought to be responsible for this vulnerability is conditioned withdrawal, which consists of somatic/affective withdrawal signs elicited in the presence of cues that were previously paired with withdrawal [2]. Reactivation of these withdrawal memories may trigger relapse of drug seeking behavior in abstinent opiate addicts [3, 4]. However, the neural circuitry that underlies conditioned-cued relapse is only now being clarified.

Several of the molecular and cellular adaptations involved in addiction are also implicated in models of learning and memory. The conditioned-place aversion (CPA) paradigm is a highly sensitive animal model used to measure the negative affective component of drug withdrawal as well as to investigate the neural substrates underlying the aversive memory associated with drug withdrawal [5, 6]. In the CPA, opiate withdrawal is paired with a particular environment, which results in the association between negative affective consequences of withdrawal with context. When animals are re-exposed to the paired environment in a drug-free state, they avoid the paired environment due to the association between the context and aversive memories of drug withdrawal.

The mesolimbic dopamine (DA) reward pathway represents a critical neural pathway that is likely to mediate different components of drug addiction, and consists of DA neurons originating in the ventral tegmental area (VTA) and extending to different brain areas [7]. DA neurons in the VTA are thought to play important roles in motivation and reward [7]. However, the mesolimbic DA system also plays a role in the negative affect.

The conditioning place preference (CPP) paradigm was used to examine the secondary reinforcing effects of D1 receptor antagonists and the neuroanatomical substrates mediating these effects [8]. Systemic administration, intra-ventricular injection and microinjection into the nucleus accumbens (NAc) produced dose-related conditioned aversion for the drug-associated place [8]. In contrast, microinjections of the same antagonist into the VTA, caudate putamen or medial prefrontal cortex (mPFC) were without effect [8]. The inhibition (by means of microinjections of the D2 receptor agonist quinpirole) of VTA neurons mediates a negative affective state, leading to negative affective encoding and place aversion, whereas blunting the activation of VTA neurons disrupts the positive affective encoding involving food reward [9]. The VTA presents differences in synaptic connectivity. On the one hand, laterodorsal tegmentum neurons preferentially synapse on dopamine neurons projecting to the NAc lateral shell, and elicit reward [10]. On the other hand, lateral habenula neurons synapse primarily on dopamine neurons projecting to the mPFC and elicit aversion [10]. Thus, these results establish that distinct VTA circuits generate reward and aversion although they interact anatomically and functionally with one another.

Experiences become long-lasting memories if experienced with a certain level of stress or arousal. Stress hormones such as glucocorticoids (GCs) mediate and modulate memory consolidation, the process that stabilizes newly formed memory [11]. Importantly, the VTA and mPFC are brain regions known to be sensitive to GCs and to express GC receptors (GR) [12-14]. On the other hand, CPA develops through associative learning and requires synaptic plasticity [15], which is implicated in adapted responses in brain, including memory formation and drug addiction and withdrawal [16]. Neuronal immediate early genes (IEGs) expression is regulated by neuronal activity and plays an important role in the neuroplastic mechanisms critical to memory

consolidation. IEGs can be divided into two functional classes: a) regulatory transcription factors, such as early growth response 1 (Egr-1), which can broadly influence cell function depending on the "downstream" genes they regulate, and b) "effector" proteins, such as activity-regulated cytoskeletal-associated protein (Arc), which may directly modulate specific cellular functions [17]. Accordingly, Arc and Egr-1 are required in late LTP and long-term memory [18, 19]. These genes are likely to have very distinct functions in late LTP: Arc mRNA is rapidly transported to dendrites and translated, whereas Egr-1 regulates the transcription of late response genes. Neurotrophic signaling has also been shown to modulate neural plasticity and behavior [20]. Of particular interest, brain derived neurotrophic factor (BDNF) mediates long-term synaptic plasticity and triggers LTP in vivo [21].

Therefore, we have used CPA to study the plasticity-related processes that occur within the VTA and mPFC during the conditioning of the aversive properties of opiate withdrawal (memory formation-consolidation), and after allowing their reactivation by re-exposure to the conditioned environment (memory retrieval). Specifically, we tested the expression levels of different genes that are known to accompany long-term plasticity and memory formation, including BDNF and the IEGs Egr-1 and Arc. Second, we hypothesized that GCs might play a crucial role in the aversive memories of drug withdrawal. To test this hypothesis we have used adrenalectomized (ADX) rats and examined the same neurobiological parameters.

Methods

Animals

All surgical and experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012).

Male Wistar rats (Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments.

Adrenalectomy

Under deep intraperitoneal anaesthesia [100 mg/kg ketamine chlorhydrate (Imalgene 1000, Merial, Lyon, France) and 8 mg/kg xylazin (Xilagesic® 2%, Lab. Calier S.A., Barcelona, Spain)] rats were bilaterally adrenalectomized (ADX) via a dorsal approach and they were implanted subcutaneously (s.c.) with slow-release corticosterone pellets. The composition of steroid pellets (25 mg corticosterone plus 75 mg cholesterol) was chosen to provide stable corticosterone concentration corresponding to circadian nadir up to 20 days after implantation [22]. ADX rats with corticosterone replacement (ADX plus corticosterone) do not mount a challenge-induced increase of plasma corticosterone. After surgery, ADX plus corticosterone rats had free choice to drink isotonic saline (0.9% NaCl) to replace depleted sodium secondary to the loss of

aldosterone because of adrenalectomy. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation. Sham and ADX plus corticosterone rats were allowed to recover from surgery for 3 days before morphine or placebo pellet implantation. Successful bilateral adrenalectomy was confirmed by plasma concentration of corticosterone and ACTH and by post-mortem examination of the ADX animals.

Induction of Opiate Dependence

Morphine base was supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Morphine dependence was induced by subcutaneous implantation (lower back) under light ether anaesthesia of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Full dependence on morphine has been previously operationally defined in this model by using a complete naloxone dose effect on various behavioral parameters (spontaneous locomotor activity, operant responding for food, intracranial self-stimulation threshold, conditioned place aversion) [23], and the rating of abstinence signs showed that opiate dependence was achieved 24 h after implantation of the morphine pellets and remained constant for 15 d [24]. Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia. There were four experimental groups (sham + placebo pellets, sham + morphine pellets, ADX + placebo pellets and ADX + morphine pellets) and two experimental time-points (day 10 and day 11). 56 animals were employed in this study. Five animals were excluded due to unconditioned preference or aversion in the pre-test phase. 26 animal were subjected to

sham-surgery while 25 were subjected to adrenalectomy (one rat died the day following adrenalectomy). Conditioning memory (CM) rats were decapitated on day 10: sham + placebo pellets (n = 5), sham + morphine pellets (n = 6), ADX + placebo pellets (n = 5) and ADX + morphine pellets (n = 6). Conditioned place aversion animals (CPA groups) were killed on day 11: sham + placebo pellets (n = 7), sham + morphine pellets (n = 8), ADX + placebo pellets (n = 7) and ADX + morphine pellets (n = 6).

Drugs

Naloxone hydrochloride (N-7758, Sigma Chemical Co, St Louis, MO, USA) was dissolved in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain) and injected subcutaneously. Naloxone was administered at a dose of 0.1 mg/kg, 1 ml/kg body weight. Naloxone HCl doses are expressed as the weight of the salt. Sterile 0.9% saline was also injected subcutaneously at a dose of 1 ml/kg. Note that a slightly higher naloxone dose (0.120 mg/kg) has previously been shown to be without effect on place aversion conditioning in placebo control rats [25].

Conditioned Place Aversion Paradigm

Briefly, the conditioned place aversion apparatus (Panlab, Barcelona, Spain) used to induce a reliable aversion consists of a box with two equally sized chambers (40 *L* x 34 *W* x 45 *H* cm) interconnected by a rectangular corridor (25 *L* x 13 *W* x 45 *H* cm). Distinctive visual and tactile cues distinguish the compartments: the motifs painted on the walls (either black dots or grey stripes), the floor colouring (black or grey) and the floor texture (smooth or rough). The sensory cues that produce a balanced choice include walls and floor colouring and texture, respectively: (A) black dots, black smooth floor; (B) grey stripes, grey rough floor. The corridor walls were transparent. The weight transducer technology (and PPCWIN software) was used to detect and

analyze the position of the animal throughout the test and the number of entries in each compartment. The experimental protocol consists of three distinct phases [26, 27]: a preconditioning phase, a conditioning phase, and a testing phase. The weight gain of the rats was checked during the entire protocol to ensure that the morphine was released correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake [28, 29]. In addition, the animals were observed for body weight loss and opioid withdrawal behaviours for 15 min after the conditioning phase.

Pre-testing Phase

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. Animals showing strong unconditioned aversion (less than 40% of the session time) or preference (more than 60% of the session time) for any compartment were discarded. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Importantly, after the compartment assignments were completed, there were no significant differences between time spent in the naloxone-paired and the vehicle-paired compartments during the preconditioning phase. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted in Figure 1A.

Conditioning Phase

In the second phase (conditioning), rats received an injection of saline on days 7 and 9, prior to being confined to their randomly assigned saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opioid

withdrawal behaviours on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. CM rats were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

Testing Phase

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D0$) between the time spent in the naloxone-paired compartment after conditioning (D) minus the time spent in the same compartment before conditioning (preconditioning test $D0$) reflects the change of preference induced by opiate withdrawal. A negative score indicates a place aversion; a positive score indicates a place preference. CPA groups were killed 1 hour after starting the testing phase.

Measurement of the withdrawal syndrome

Sham + mor and ADX + mor rats were individually placed into transparent plastic cages after the conditioning phase and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 15 min on days 8 and 10 (naloxone injection). Subsequently, previously identified behavioural characteristics of the rat opiate abstinence syndrome [30] were evaluated. The number of jumping, wet-dog shakes, paw tremor, sniffing and writhing was counted as the number of events occurring during the total test time period. Tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble were scored 1 for appearance or 0 for non-appearance within each 5 min time. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight as previously reported [31]. Body weight loss was determined as the difference between

the weight determined immediately before naloxone injection and that determined 1 hour later, after the conditioning phase.

Preparation of tissue extract

Rats were decapitated on days 10 and 11 (between 10:00-12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, and stored immediately at -80°C until use for quantitative real-time PCR (qPCR). Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane (Fig. 3A). For the VTA study, two consecutive 500-µm coronal slices were made corresponding to approximately -4.80 to -6.15 mm from bregma, according to the atlas of Paxinos and Watson [32] (Figure 2A). For the mPFC study, two consecutive 500-µm coronal slides were made corresponding to approximately +3.70 to +2.20 mm from bregma, according to the atlas of Paxinos and Watson [32] (Figure 3A). Tissues of interest were dissected using a punching device with a 1-mm internal diameter. Bilateral punches were collected into Eppendorf tubes, according to the method of [33].

RNA extraction and quantitative real-time PCR (qPCR)

One punch from the VTA and one punch from the mPFC were placed in an Eppendorf tube containing 30 µl of Trizol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and rapidly stored at -80°C. Frozen brain tissue samples were homogenized in Trizol® reagent (Invitrogen Corp., USA) and total RNA was isolated with QIAGEN miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To

eliminate genomic DNA contamination DNase I treatment were used and 100 µl Rnase-free DNase I (1 unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific, USA). Amplification was not detected in the RT-minus controls. The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed by Primer Express 3.0 Program. The primers (Microsynth, Balgach) were used in the Real-Time PCR reaction with Fast EvaGreen® qPCR Master Mix (Biotium, USA) on ABI StepOnePlus instrument was listed.

List of the genes: gapdh, arc, egr-1, bdnf

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to gapdh (glyceraldehyde-3-phosphate dehydrogenase) expression.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

bdnf for AAACGTCCACGGACAAGGCA

bdnf rev TTCTGGTCCTCATCCAGCAGC

Radioimmunoassay

Blood was collected on days 10 and 11 into ice-cooled tubes containing 5% EDTA and was then centrifuged (500 g; 4ºC; 15 min). Plasma was separated, divided into two aliquots and stored at -80ºC until assayed for corticosterone or ACTH. Plasma concentration of corticosterone and ACTH were quantified using specific corticosterone and ACTH antibodies for rats ([^{125}I]-CORT and [^{125}I]-ACTH RIA; MP Biomedicals, Orangeburg, NY, USA). The sensitivity of the assay was 7.7 ng/mL for corticosterone and 5.7 pg/mL for ACTH.

Reagents

Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany); HPLC reagents were purchased from Sigma Chemical Co.

Data Analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test to determine specific group differences. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of adrenalectomy on metabolic parameters, on HPA-axis activity and on the naloxone-induced conditioned place aversion in morphine dependent rats.

Data in the Figure 1B-E is represented as a before-and-after plot for each experimental group: it depicts the time that each rat spent in the naloxone-paired chamber during the pre-test and during the test phase. The difference between the time spent in the naloxone-paired compartment during the test phase minus the time spent in the same compartment during the pre-test reflects the CPA score, an index of the aversive withdrawal-memories. As illustrated in Figure 1F, during the test, morphine dependent rats spent less time in the naloxone-paired compartment than controls ($P < 0.001$). Furthermore, ADX diminished the naloxone-induced CPA in opiate-dependent animals ($P < 0.01$). Two-way ANOVA revealed a major effect of adrenalectomy ($F (1, 24 = 4.931$, $P = 0.0361$), treatment ($F (1, 24) = 20.03$, $P = 0.0002$) and interaction between factors ($F (1, 24) = 5.708$, $P = 0.0251$). We calculated the number of entries in the different compartments during the test phase for each experimental group (Figure 1G). Two-way ANOVA revealed a major effect of treatment ($F (1, 24 = 7.168$, $P = 0.0132$), but no effect of adrenalectomy ($F (1, 24) = 1.064$, $P = 0.3126$) nor an interaction between factors ($F (1, 24) = 0.4750$, $P = 0.4973$). Although sham+morphine rats tended to display higher number of entries during the test phase, Newman–Keuls post hoc test failed to detect any difference between groups.

Naloxone administration to sham- or ADX-operated rats dependent on morphine elicited the appearance of a number of behavioral symptoms characteristic of opiate withdrawal. It is noteworthy that, on day 10, the ADX + morphine group exhibited an enhancement of the withdrawal score when compared with both, sham + morphine day

10 ($P < 0.01$) and ADX + morphine day8 ($P < 0.05$) groups (Table 2). The body weight loss after saline or naloxone injection to sham or ADX placebo-pelleted and morphine-dependent rats was also recorded as a sign of opiate withdrawal (Table 1). Post hoc analysis showed that naloxone injection to sham or ADX morphine-dependent animals significantly increased ($P < 0.001$) the body weight loss when compared with their control placebo-pelleted group also receiving naloxone. We measured plasma corticosterone concentrations in blood samples obtained from morphine-dependent or control rats after injection of naloxone during the last conditioning session on day 10 (Table 1). Newman–Keuls post hoc test showed that naloxone-precipitated morphine withdrawal in sham animals evoked a marked increase ($P < 0.001$) in corticosterone secretion compared with sham placebo-treated rats receiving naloxone and morphine-dependent animals previously adrenalectomized.

To check the efficacy of the adrenalectomy, ACTH hormone concentrations were measured in plasma during the test phase (Table 1). As expected, Newman's post hoc test showed (Table 1) that in placebo- and morphine-ADX rats plasma concentration of ACTH was higher ($P < 0.001$) compared with sham-operated animals for the two treatment evaluated (placebo and morphine). On the other hand, we did not detect any significant change on corticosterone levels during the test day.

Effects of adrenalectomy on Egr-1, BDNF and Arc mRNA in the VTA during the naloxone-induced conditioned place aversion in morphine dependent rats.

Figure 2 summarizes the changes in Egr-1, BDNF and Arc mRNA during both, the conditioning (day 10) and the test phase (day 11) of morphine withdrawal CPA in the VTA. The overall ANOVA on Egr-1 mRNA content in the VTA during the

conditioning session revealed significant effects of morphine treatment ($F(1, 18) = 25.21$, $P < 0.0001$), but not adrenalectomy ($F(1, 18) = 0.8635$, $P = 0.3651$) or significant interaction between adrenalectomy and treatment ($F(1, 18) = 2.799$, $P = 0.1116$) (Figure 2B). Post hoc analysis showed that the Egr-1 levels were significantly higher ($P < 0.001$) in sham+morphine group as compared with the sham+placebo group, and also were enhanced in ADX+morphine ($P < 0.05$) compared to ADX+placebo animals. The ANOVA for BDNF mRNA expression after the conditioning session showed no significant effect of adrenalectomy ($F(1, 18) = 1.245$, $P = 0.2791$), morphine treatment ($F(1, 18) = 0.1886$, $P = 0.6693$) or a significant interaction between adrenalectomy and treatment ($F(1, 18) = 0.5584$, $P = 0.4645$) (Figure 2C). Results for the two-way ANOVA for Arc mRNA in the VTA revealed no significant effect of adrenalectomy ($F(1, 18) = 0.5428$, $P = 0.4708$), chronic morphine treatment ($F(1, 18) = 0.6840$, $P = 0.4191$) and significant interaction between adrenalectomy and treatment ($F(1, 18) = 2.877$, $P = 0.1071$) (Figure 2D).

When we performed the analysis of Egr-1 mRNA levels in the VTA during the expression of opiate-withdrawal CPA, two-way ANOVA revealed no effects of adrenalectomy ($F(1, 20) = 1.761$, $P = 0.1995$), chronic treatment ($F(1, 20) = 0.05192$, $P = 0.8221$) or significant interaction between adrenalectomy and treatment ($F(1, 20) = 2.990$, $P = 0.0992$) (Figure 2E). The ANOVA for BDNF on the test phase failed to detect significant effect of adrenalectomy ($F(1, 21) = 1.368$, $P = 0.2552$), morphine treatment ($F(1, 21) = 4.073$, $P = 0.0565$) or significant interaction between adrenalectomy and treatment ($F(1, 21) = 3.311$, $P = 0.0831$) (Figure 2F). However, post hoc analysis showed that the BDNF mRNA levels were significantly higher in sham+morphine group when compared with the sham+placebo ($P < 0.05$) group. Results for the two-way ANOVA for Arc mRNA in the VTA revealed no significant

effect of adrenalectomy (F (1, 20) = 0.06290, P = 0.8045), morphine (F (1, 20) = 1.524, P = 0.2313) or interaction (F (1, 20) = 2.831, P = 0.1080) (Figure 2G).

Effects of adrenalectomy on Egr-1, BDNF and Arc mRNA in the mPFC during the naloxone-induced conditioned place aversion in morphine dependent rats.

Figure 3 summarizes the changes in Egr-1, BDNF and Arc mRNA during both, the conditioning (day 10) and test phase (day 11) of morphine withdrawal CPA in the mPFC. The overall ANOVA on Egr-1 mRNA content in the mPFC during the conditioning session resulted in significant effect of morphine (F (1, 18) = 17.01, P = 0.0006), but no effect of adrenalectomy (F (1, 18) = 1.128, P = 0.3023) or interaction (F (1, 18) = 0.002110, P = 0.9639) (Figure 3B). Post hoc *analysis* showed that naloxone injection to sham or ADX morphine-dependent animals significantly increased (P < 0.05) Egr-1 mRNA when compared with their control placebo-pelleted group also receiving naloxone. The ANOVA for BDNF mRNA on the conditioning phase showed no effect of adrenalectomy (F (1, 17) = 1.490, P = 0.2388), morphine treatment (F (1, 17) = 3.194, P = 0.0917) or interaction between adrenalectomy and treatment (F (1, 17) = 3.341, P = 0.0852) (Figure 3C). Moreover, results for the two-way ANOVA for Arc mRNA in the mPFC detected a significant effect of chronic morphine treatment (F (1, 17) = 34.04, P < 0.0001), but was not significant for adrenalectomy (F (1, 17) = 0.02380, P = 0.8792) or interaction between adrenalectomy and treatment (F (1, 17) = 0.3840, P = 0.5437) (Figure 3D). Thus, post hoc analysis showed that naloxone injection to sham or ADX morphine-dependent animals significantly increased (P < 0.01) Arc mRNA when compared with their control placebo-pelleted group also receiving naloxone.

When we performed the analysis of Egr-1 levels in the mPFC during the expression of opiate-withdrawal CPA, two-way ANOVA unveiled no effect of adrenalectomy ($F(1, 22) = 1.960$, $P = 0.1755$), morphine ($F(1, 22) = 0.2170$, $P = 0.6459$) or significant interaction between adrenalectomy and treatment ($F(1, 22) = 0.4924$, $P = 0.4902$) (Figure 3E). The ANOVA for BDNF mRNA expression on the test day also exposed no effect of adrenalectomy ($F(1, 22) = 0.1644$, $P = 0.6891$), morphine ($F(1, 22) = 0.006146$, $P = 0.9382$) or significant interaction between adrenalectomy and treatment ($F(1, 22) = 0.7553$, $P = 0.3942$) (Figure 3F). In addition, results for the two-way ANOVA for Arc mRNA (Figure 3G) in the mPFC on the test day revealed no significant effect of adrenalectomy ($F(1, 22) = 3.282$, $P = 0.0837$), morphine ($F(1, 22) = 0.4911$, $P = 0.4908$) or interaction ($F(1, 22) = 2.824$, $P = 0.1070$).

Relationship between Egr-1, BDNF or Arc mRNA and behavioral or HPA-axis activity during the CPA test

Once we assessed that sham rats that were rendered dependent on morphine avoided the compartment where they had previously experienced opioid withdrawal, we analyzed if the expression of CPA was correlated with the induction or repression of different mRNAs. Importantly, rats that displayed the higher aversion score exhibited enhanced BDNF mRNA levels in the VTA (Figure 4A) but reduced BDNF mRNA levels in the mPFC (Figure 4B). As shown in Figure 4C, HPA axis activation during the test phase (measured as ACTH levels) was positively correlated with BDNF mRNA in the VTA. When we compared BDNF expression in the different brain areas, we detected that BDNF mRNA in the VTA and BDNF mRNA in the mPFC were inversely regulated (Figure 4D).

Discussion

In the present study we demonstrated that naloxone-precipitated withdrawal from chronic morphine administration produced a conditioned place aversion following two conditioning trials. This effect was prevented by adrenalectomy. *Egr-1* and *Arc* were induced in the VTA and mPFC after morphine-withdrawal conditioning in both sham and ADX rats. *Bdnf* expression was enhanced in the VTA during the test phase of the sham rats, but not the ADX rats and was correlated with HPA axis function. Rats that displayed a higher aversion score exhibited enhanced *Bdnf* mRNA levels in the VTA, but reduced *Bdnf* mRNA levels in the mPFC during retrieval.

According to the CPA paradigm, reduced CPA expression in ADX+morphine animals could be a consequence of decreased aversive valence for morphine-withdrawal. However, we observed that ADX+morphine animals did not differ on body weight loss and even exhibited a greater global withdrawal score compared to the sham+morphine group during the naloxone-conditioning sessions. Thus, the present data do not support a role for the HPA axis in the somatic expression of morphine withdrawal. On the other hand GCs are known to modulate memory consolidation and memory retrieval [11]. All major drugs of abuse stimulate the HPA axis during acute withdrawal from the drug, with a common response of elevated ACTH and corticosterone [34, 35]. Our results reflected that naloxone administration to morphine dependent rats induced an increase in GC plasma levels, which was not observed in ADX animals due to the adrenalectomy. Thus, given that increased GC levels enhance memory consolidation [11], these differences in corticosterone plasma levels during the consolidation phase are likely to mediate the differences observed between sham- and ADX-dependent rats during CPA performance.

An important observation from our data is that all the genes we analyzed tended to show increased expression in sham+morphine animals when compared to their sham+placebo controls both, during memory consolidation and during memory retrieval in the VTA. So, our results are in agreement with previous observations describing that the VTA is activated during both processes [36]. Specifically, VTA activation during the conditioning phase resulted in increased *Egr-1* levels in sham+morphine animals. Previous data showed unaltered/decreased activity of VTA DA neurons following opiate withdrawal [37, 38]. Moreover, withdrawal from cocaine administration results in unaletered/reduced Egr-1 VTA levels [39]. According to this, the increased *Egr-1* expression that we detected in the VTA during the conditioned-withdrawal must be due to memory-consolidation related mechanisms. Contrary to us, re-exposure to cocaine–conditioned stimulus (CS) increased *Egr-1* expression in neurons of the VTA [40]. A plausible explanation might be that *Egr-1* expression in the VTA is increased by appetitively (drug) CS after Pavlovian learning, but not by aversive (withdrawal) CS.

We observed that aversive-memory consolidation in the VTA resulted in unaltered *Bdnf* levels. By contrast, memory retrieval elicited marked increase in *Bdnf* expression in sham morphine-dependent rats. Importantly, it has been demonstrated that chronic morphine suppresses *Bdnf* gene expression in the VTA [41-43]. On the other hand, the level of BDNF in the VTA progressively increases during abstinence [44]. However, it cannot be argued that the augmented *Bdnf* levels we observed on day 11 were caused by opiate-abstinence, given that pelleted-morphine was still released during the test phase. Thus, we postulate that *Bdnf* induction in the VTA following the test phase is the result of memory retrieval and not due to morphine pharmacologic effects. Moreover, while *Bdnf* has been shown to reduce CPP [42], we detected that *Bdnf* promotes CPA, as a significant positive correlation exists between *Bdnf* levels and CPA score. In addition

our results suggest that *Bdnf* induction during memory retrieval is influenced by the HPA axis, as previously described [45]. Therefore, ADX+morphine rats do not exhibit enhanced *Bdnf* levels and do not express CPA.

When we studied gene expression regulation in the mPFC, we detected that during conditioned morphine-withdrawal, *Egr-1* and *Arc* mRNA levels were increased in the experimental groups that received chronic morphine treatment. In accordance with our results, enhanced expression of the IEG *c-fos* in the mPFC has been observed during the withdrawal consolidation phase [36]. However, our results do not support a role for GCs on conditioning-mediated increased *Egr-1* expression in the mPFC, since *Egr-1* levels do not differ between sham- and ADX-dependent rats. Furthermore, a previous study demonstrated that Arc was induced in the mPFC by conditioned naloxone-precipitated drug withdrawal following exposure to a single dose of morphine [46]. Collectively, these data indicate that the mPFC contributes to the formation of the place aversion regardless of morphine is administered acutely or chronically. Arc is a marker for excitatory neurotransmission, which is important for synaptic plasticity, and is required for memory consolidation in the hippocampus [18, 47]. While the function of Arc in the PFC has not been studied to a similar degree, it is conceivable that Arc has a similar role in the mPFC, where it is almost exclusively expressed in pyramidal cells [48]). *Arc* mRNA expression therefore provides an indirect measure of pyramidal cell excitability, which is coupled to cognitive function. PFC plays an important role in a variety of cognitive and executive processes, including working memory, planning and decision making, and behavior inhibition [49]. It is also important for addiction-related behaviors such as impulsive [50] and reward-related learning [51]. So, we propose that *Arc* induction in the mPFC plays a role in the process whereby the conditioned-withdrawal

rats could easily identify the naloxone-paired environment during the conditioning phase, because this cognitive ability requires the PFC function.

However, we did not detect significant differences between groups during memory retrieval, adding further evidence to the notion that the mPFC is not involved in the retrieval process of withdrawal memories [36]. In contrast, re-exposure to a CS previously associated with cocaine increased *Egr-1* mRNA levels in the mPFC [40]. As stated above, Egr-1 may participate in the molecular mechanisms underlying the retrieval-reconsolidation process of Pavlovian stimulus–reward drug associations but not Pavlovian stimulus-withdrawal associations.

BDNF signaling mediates different morphological and behavioral effects depending on the brain region examined. For instance, it has been described that stimulating BDNF receptors in the VTA promotes [52] while BDNF microinjection into the PFC inhibits [53] drug-seeking. In concordance, we observed a negative correlation between VTA and mPFC *Bdnf* mRNA levels. In addition, while *Bdnf* induction in the VTA seemed to promote CPA, *Bdnf* expression in the mPFC exhibited an opposite relationship with CPA.

In summary, these experiments represent the first demonstration that *Egr-1* and *Arc* are induced in the VTA and mPFC during the consolidation of morphine-withdrawal aversive memories, thus suggesting that these genes contribute to the neural plasticity that accompanies the consolidation process. Besides, *BDNF* in the VTA seems to play an essential role during the retrieval of long-term withdrawal-aversive memories. GCs play a prominent role in *BDNF* regulation during memory retrieval. Given that subgroups of VTA DA neurons play different functions [10], future research needs to address which selective subgroups of DA neurons are responsible for CPA expression.

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Authors Contribution

DG-P designed and performed the research, analyzed the data and wrote the paper. SF assisted with PCR data analysis and interpretation. KJK guided the PCR project and provided critical revision of the manuscript. MVM guided the project. All authors critically reviewed content and approved final version for publication.

Conflict of Interest

Authors declare no conflicts of interest.

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Figure Legends

Figure 1. Effects of adrenalectomy on the naloxone-induced conditioned place aversion in morphine dependent rats. (A) Schematic overview of the animals' treatment and behavioral experiments time course. Four and six days after the morphine or placebo pellet implantation, sham and ADX rats were challenged with naloxone (0,1 mg·kg^{-1} s.c.) immediately prior to confinement in the naloxone-paired compartment for 1 hour. Then, immediately were tested for the occurrence of somatic signs of opiate withdrawal for 15 min. (B-E) Each graph depicts a different experimental group. For each animal it is represented the time spent in the naloxone-paired chamber during pretest phase and during the test phase. (F) Scatter dot plot graph indicates the difference between the time spent in the naloxone-paired compartment after conditioning sessions minus the time spent in the same compartment before conditioning sessions. (G) Column graph reflects the number of entries on both chambers during pretest phase for each experimental group. ***P < 0.001 versus sham+pla; $^{++}$P < 0.01 versus sham+mor.

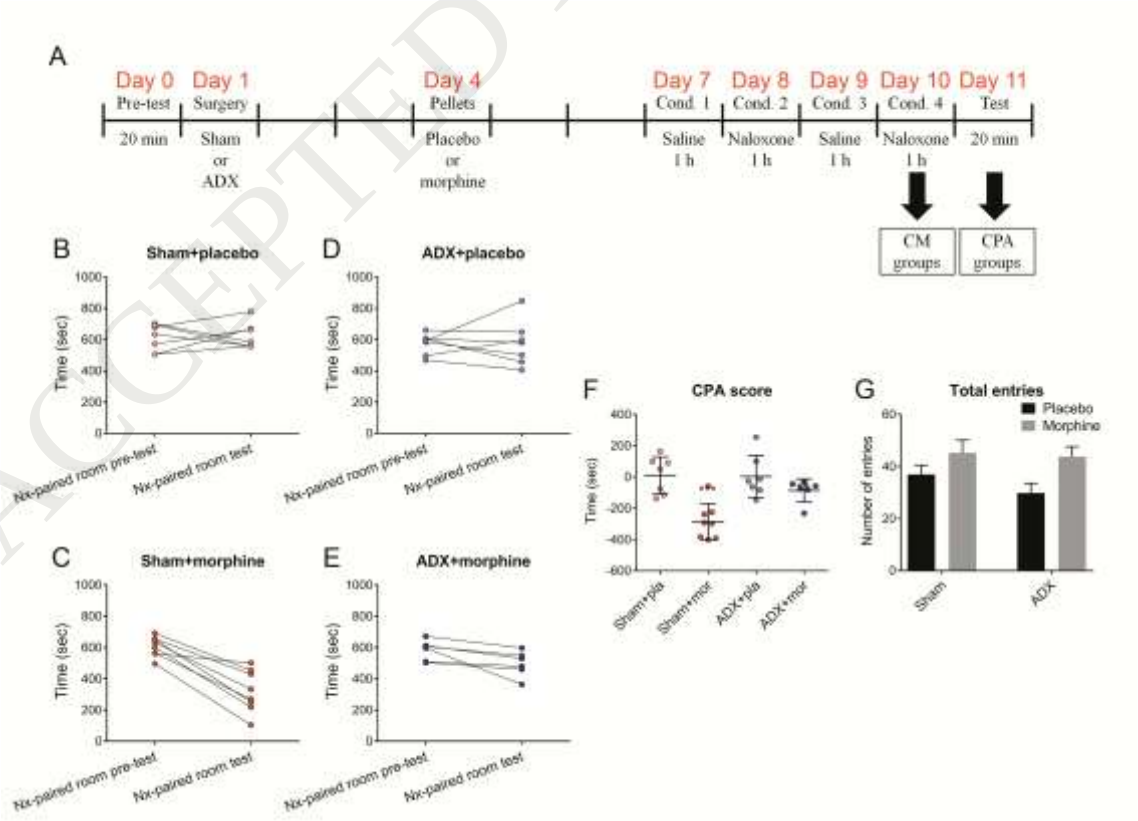

Figure 2. (A) Regions of the postmortem tissue preparation. Schematic illustration showing the punches placement in a coronal section (red circle). Forebrain regions of interest were dissected by pushing micropunch needles of 1-mm diameter into the posterior face of the coronal sections. Microphotograph showing representative VTA punches placement. (B-D) Egr-1, BDNF and Arc mRNA analysis in the VTA during the conditioning phase. (E-G) Egr-1, BDNF and Arc mRNA analysis in the VTA during the test phase of morphine withdrawal CPA. *P < 0.05, ***P < 0.001 versus sham+pla; $^{\#}$P < 0.05 versus ADX+pla.

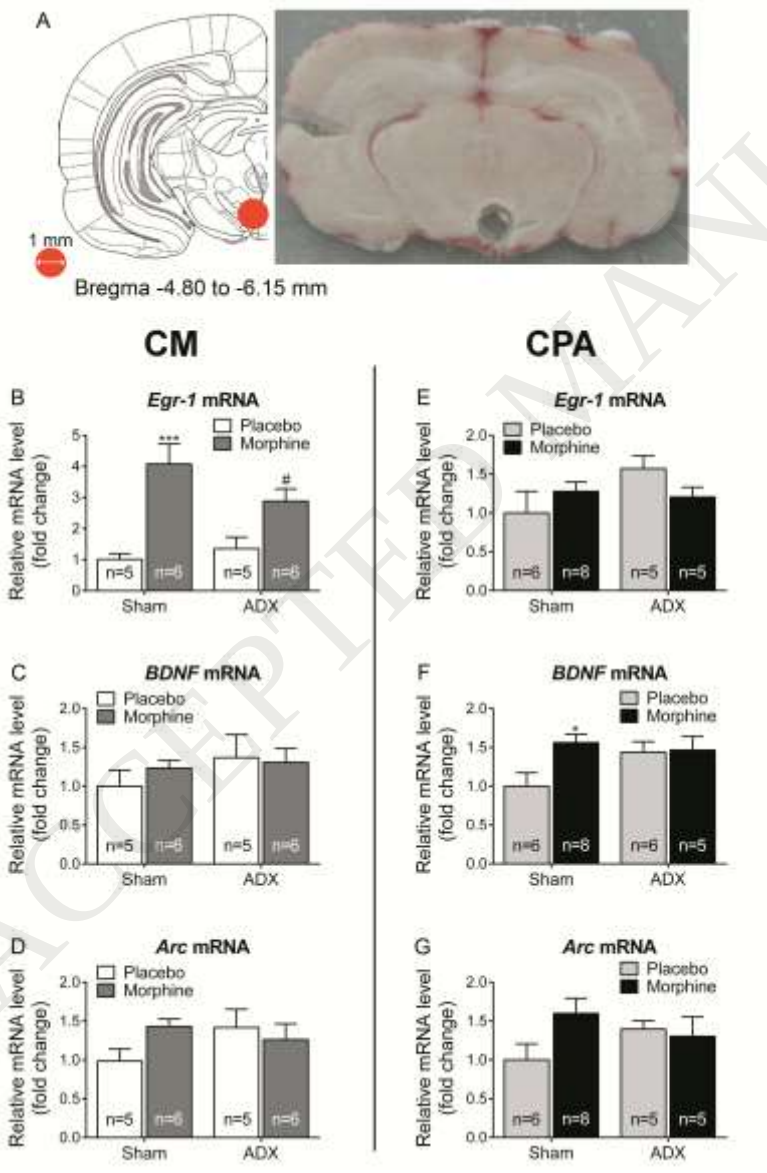

Figure 3. (A) Regions of the postmortem tissue preparation. Schematic illustration showing the punches placement in a coronal section (red circle). Forebrain regions of interest were dissected by pushing micropunch needles of 1-mm diameter into the posterior face of the coronal sections. Microphotograph showing representative mPFC punches placement. (B-D) Egr-1, BDNF and Arc mRNA analysis in the mPFC during the conditioning phase. (E-G) Egr-1, BDNF and Arc mRNA analysis in the mPFC during the test phase of morphine withdrawal CPA. *P < 0.05, **P < 0.01 versus sham+pla; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 versus ADX+pla.

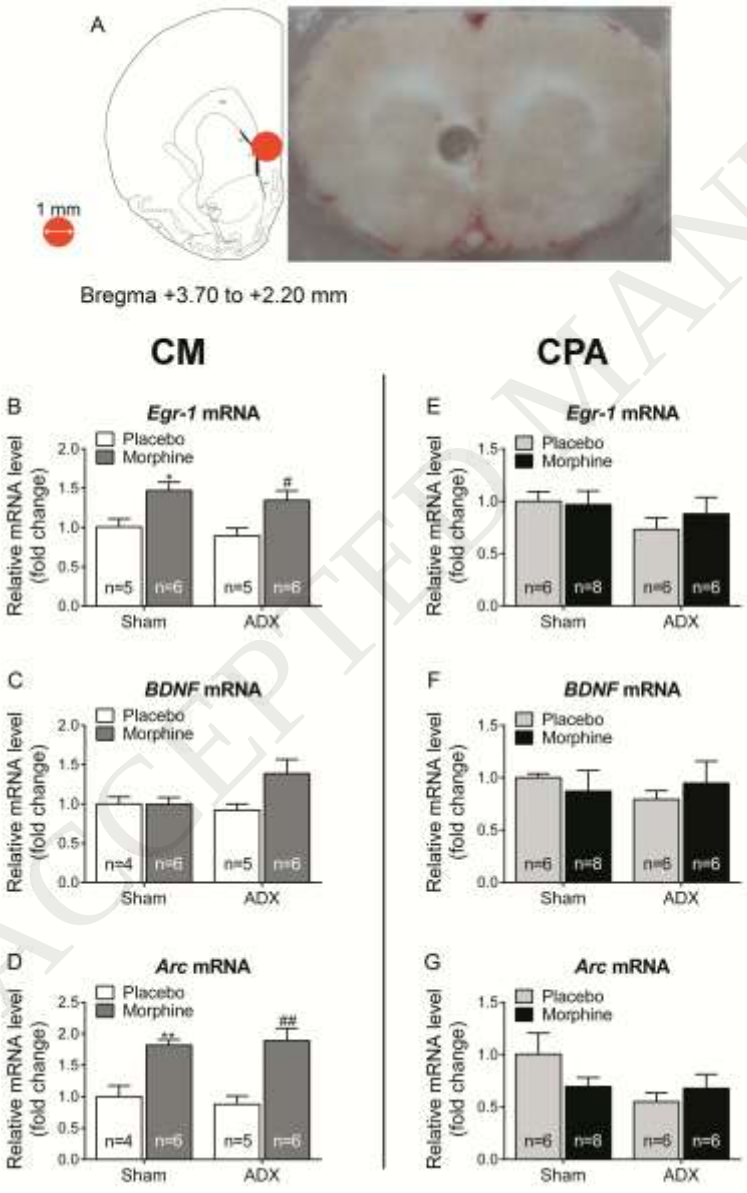

Figure 4. Correlation analysis between BDNF mRNA and behavioral or HPA-axis activity during the expression of CPA test in sham-operated rats rendered dependent on morphine.

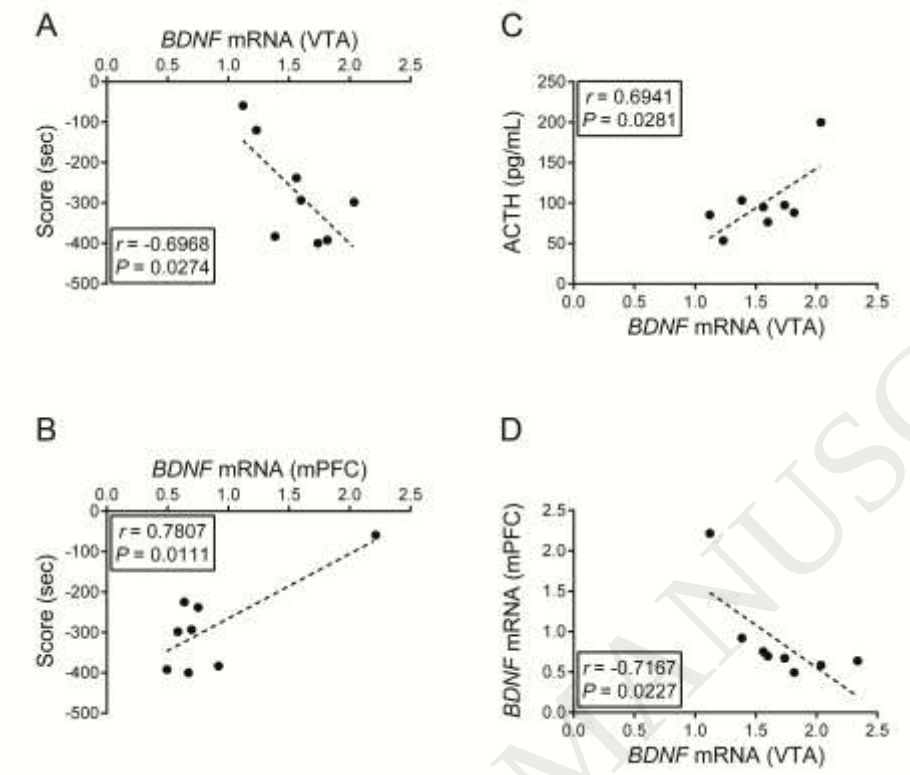

Table 1. Effects of adrenalectomy on metabolic parameters and on HPA-axis activity in morphine dependent rats. Two-way ANOVA with surgery (sham vs. ADX) and chronic-pelleted treatment (placebo vs. morphine) as between-subjects factors was employed. When significant interactions in surgery, treatment or between these two factors were observed, a subsequent *post hoc* test was applied. CM: Conditioning memory. CPA: Conditioned place aversion. Score entries test: number of entries during the test phase minus number of entries during the pre-test phase for the same animal. ***P < 0.001 vs sham+placebo; $^{+++}$P < 0.001 vs sham+morphine; $^{\#\#\#}$P < 0.001 vs ADX+placebo.

Parameters	Experimental groups				Two-way ANOVA					
					Surgery (sham vs. ADX)		Treatment (placebo vs. morphine)		Interaction	
	Sham+placebo	Sham+morphine	ADX+placebo	ADX+morphine	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
Corticosterone (CM)	54.10 ± 11.53	935.1 ± 44.76***	43.80 ± 11.64	43.67 ± 8.66^{+++}	F (1, 18) = 294.7	P < 0.0001	F (1, 18) = 281.2	P < 0.0001	F (1, 18) = 281.4	P < 0.0001
Body weight loss (g)	-3.73 ± 0.59	-12.66 ± 0.92***	-3.30 ± 0.41	-14.24 ± 0.92$^{\#\#\#}$	F (1, 46) = 0.5597	P = 0.4582	F (1, 46) = 167.9	P < 0.0001	F (1, 46) = 1.724	P = 0.1957
Corticosterone (CPA)	24.82 ± 4.61	30.72 ± 3.50	16.60 ± 3.38	26.77 ± 10.87	F (1, 24) = 1.099	P = 0.3050	F (1, 24) = 1.916	P = 0.1791	F (1, 24) = 0.1353	P = 0.7163
ACTH (CPA)	44.45 ± 5.81	99.90 ± 15.27	417.8 ± 47.94***	511.3 ± 40.80^{+++}	F (1, 24) = 159.1	P < 0.0001	F (1, 24) = 5.730	P = 0.0248	F (1, 24) = 0.3739	P = 0.5466
Score entries test	2.42 ± 2.03	8.87 ± 5.63	-7.26 ± 4.14	3.33 ± 3.72	F (1, 24) = 3.134	P = 0.0894	F (1, 24) = 3.921	P = 0.0593	F (1, 24) = 0.2345	P = 0.6326

Table 2. Global withdrawal syndrome. Two-way ANOVA with surgery (sham vs. ADX) and time (day 8 vs day 10) as between-subjects factors was employed. Since significant interactions were observed, a subsequent *post hoc* test was applied. **P < 0.01 vs sham+morphine day 10; $^{+}$P < 0.05 vs ADX+morphine day 8.

Parameters	Experimental groups				Two-way ANOVA					
					Surgery (sham vs. ADX)		Time (day 8 vs. day 10)		Interaction	
	Sham+morphine Day 8	ADX+morphine Day 8	Sham+morphine Day 10	ADX+morphine Day 10	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
Global withdrawal score	12.56 ± 1.192	11.25 ± 1.270	11.83 ± 1.783	20.14 ± 2.575$^{** +}$	F (1, 26) = 3.968	P = 0.0570	F (1, 26) = 5.4	P = 0.0282	F (1, 26) = 7.478	P = 0.0111