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Research report

Perinatal protein deprivation facilitates morphine cross-sensitization to cocaine and enhances ΔFosB expression in adult rats

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ABSTRACT

Previous studies have indicated that neural changes induced by early nutritional insult cause an altered response to pharmacological treatments, including addictive drugs. This study evaluates the influence of perinatal protein malnutrition in developing cross-sensitization to cocaine-induced rewarding effects in animals pre-exposed to morphine.

Different groups of well-nourished (C-rats) and protein-deprived animals (D-rats) were treated twice a day for three days with increasing doses of morphine or with saline. After 3 days, the incentive motivational effects of cocaine were assessed in a Conditioned Place Preference paradigm in both groups. In saline pre-treated animals, dose-response curves to cocaine revealed a conditioning effect in D-rats at doses of 5, 7.5 and 10 mg/kg, while this effect was observed in C-rats only with 10 and 15 mg/kg. Furthermore, when animals of both groups were pre-treated with escalating doses of morphine, cross-sensitization to the conditioning effect of cocaine was elicited only in D-rats with low doses of cocaine (5 and 7.5 mg/kg). In contrast, under the same experimental conditions, C-rats show no cross-sensitization. To correlate this differential rewarding response with a molecular substrate linked to the behavioral changes observed after repeated drug exposure, ΔFosB expression was assessed in different brain regions. D-rats showed a significant increase in this transcription factor in the nucleus accumbens, amygdala and medial prefrontal cortex. These results demonstrated that perinatal protein deprivation facilitates rewarding effects and the development of cross-sensitization to cocaine, which correlates with an upregulation of ΔFosB in brain areas related to the reward circuitry.

1. Introduction

Drugs of abuse, despite their different mechanisms of action, converge on the brain's reward circuit, producing a series of common functional effects after both acute and repeated exposure [1–3]. Opiates and psychomotor stimulants produce their neural and behavioral effects through different mechanisms. Stimulant drugs directly increase dopaminergic transmission in the nucleus accumbens (NAc). Opiates do the same, but indirectly; they inhibit GABAergic interneurons in the ventral tegmental area (VTA), which disinhibits VTA dopamine neurons. Opiates also act directly, principally on mu and perhaps on delta receptors, on NAc neurons [4,5]. There is thus growing evidence that abuse-related effects of opiates and psychostimulants may be mediated by common neurobiological substrates, focused on the mesocorticolimbic brain areas.

Repeated intermittent exposure to addictive drugs has been consistently reported to induce a persistent increase in incentive motivational effects, a phenomenon known as behavioral sensitization [6–8]. Neural changes underlying this long-lasting phenomenon probably contribute to developing the compulsive patterns of drug-seeking and craving that characterize addiction [see reviews 9,10]. Moreover, cross-sensitization occurs, consistent with the involvement of common neurobiological substrates, between drugs with different pharmacological modes of action and could play a role in the escalation of drug use in populations characterized by polydrug abuse [11]. Several preclinical studies have indicated that cross-sensitization can develop between mu-opioid agonists and cocaine. For instance, repeated morphine administration increases the locomotor response [12,13] and conditioning rewarding effects of cocaine [14].

Common chronic functional adaptations at cellular and molecular levels in the VTA-NAc pathway are essential for acute drug reward and chronic changes related with addiction [1]. These adaptations appear to be complex, including other brain areas (amygdala, hypothalamus, hippocampus and regions from the frontal cortex, among others) that

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interact with the VTA-NAc and are also important for the rewarding effects of drugs associated with addiction [15–19].

The genesis of addiction and of long-lasting behavioral abnormalities is believed to be closely linked to the regulation of gene expression. In particular, the transcription factor ΔFosB is upregulated and plays an essential role in long-term adaptive changes in the brain associated with diverse conditions, such as repeated exposure to drugs of abuse [16]. Due to this protein's stability, ΔFosB remains elevated for long periods after its induction [20]. The induction and accumulation of ΔFosB in specific brain regions suggests that this transcription factor represents a mechanism by which drugs of abuse produce stable changes in the brain that contribute to the emergence of addiction [21,22][21,see review 22].

Altered reactivity to drugs of abuse has been reported in animal models of protein-malnutrition, with undernourished rats showing increased response to drug effects in their adult life. For psychostimulants, enhanced sensitization to the locomotor effect of amphetamine [23] and cocaine-induced stereotypy [24] has also been described. Furthermore, adult rats submitted to perinatal protein malnutrition (D-rats), after repeated administration of cocaine or morphine, showed facilitation for the development of behavioral sensitization [13,25–28]. In addition, the rewarding effects of cocaine and morphine were increased in D-rats, correlated with an overexpression of ΔFosB in brain areas related to the reward network [26,27]. All this information indicates a higher reactivity of the mesocorticolimbic dopaminergic pathway, resulting from early nutritional insult. Therefore, the aim of this study was to investigate the influence of perinatal protein malnutrition in developing cross-sensitization to cocaine-induced rewarding effects in animals intermittently pre-exposed to escalating doses of morphine, using a conditioned place preference (CPP) paradigm. In order to correlate the behavioral response with a neurobiological marker associated with repeated drug exposure, we also assessed ΔFosB expression in different brain areas of D-rats and control rats (C-rats).

2. Materials and methods

2.1. Animals

The schedule of perinatal protein deprivation and diet composition employed has been described elsewhere [26]. Briefly, pregnant rats (Wistar strain from our own colony), were divided into two groups on the 14th day of pregnancy and fed isocaloric diets containing 24% and 8% casein, for well-nourished (C-rats) and deprived (D-rats), respectively. Litters from both groups were culled to eight pups. After weaning at 30 days, when the deprivation period was ended, both groups were given balanced standard chow (Gepsa feeds, Pilar Group, Argentina) for at least 40 days (nutritional recovery period) before the programmed experiments. Animals were maintained at 22 °C ± 2 under a 12-h light-dark cycle (lights on at 07:00 AM) with food and water ad-libitum. All experimental groups were composed of male animals from different litters, in order to avoid sibling replication. Animals were maintained and the studies conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, USA, 2010). Every effort was made to minimize animal suffering and to reduce their number.

2.2. Drugs

Morphine hydrochloride and cocaine hydrochloride (Verardo Laboratories, Buenos Aires, Argentina) were dissolved in 0.9% saline and injected in a volume of 1 ml/kg body weight. Drug dose is expressed in terms of the salt.

2.3. Conditioning place preference procedure (CPP)

The place conditioning procedure used in these experiments was similar to that described by Valdomero et al. [26] and included an unbiased design. The apparatus for CPP comprised two compartments distinguished by different patterns on the floors and walls, separated by a central neutral area. The two outer compartments were 32 × 25 × 35 cm. One compartment was black with a stainless steel rod floor, and the other was white with a polyethylene reticulate floor. The central compartment, with translucent walls and a smooth floor (11 × 22 × 25 cm), was connected to the outer compartments by guillotine doors.

The CPP procedure consisted of three phases and was as follows. In the preconditioning, animals were placed in the central compartment with free access to all three compartments and the time spent in each compartment was measured during 15 min. Conditioning was conducted during four days. Sessions were conducted twice per day with 5 h separating each. Rats were injected with saline (1 ml/kg) and confined to one of the outer compartments for 45 min. Five h later they received cocaine injections and were confined to the other compartment for 45 min. The treatment compartment was counterbalanced. The day after the last cocaine injection, the testing session was carried out by placing the animal in the central compartment as in the preconditioning test. Time spent in each compartment was recorded by two experimenters who were always unaware of the drug treatment and the nutritional conditions of the animals. Place preference was evaluated as time spent in the drug-paired compartment relative to the total time spent in outer compartments [preference score = time in paired/(time in paired + time in non-paired compartment)]. Under our experimental conditions, saline-treated rats from both groups did not show any preference for the outer compartments.

2.4. Cross-sensitization to cocaine in morphine pre-exposed C- and D-rats

To evaluate the influence of early undernutrition on the development of morphine-cocaine cross-sensitization, animals from both groups received pre-treatment with morphine. The pre-treatment protocol used was one previously shown to result in homologous sensitization only in deprived rats [27]. Thereafter, morphine was administered intermittently at escalating doses by twice-daily s.c. injections for three days: day 1, 5 mg/kg; day 2, 10 mg/kg and day 3, 20 mg/kg. Three days after the last injection, different groups of animals were conditioned in the CPP paradigm, as previously described, using increasing doses of cocaine (3, 5, 7.5, 10 or 15 mg/kg i.p.; mor-coc groups). This cross-sensitization schedule is context-independent, since morphine was administered in the home cage and sensitization expressed in the CPP apparatus. Another group of C- and D-rats was pretreated with morphine and paired with saline (mor-sal group). Finally, two additional groups of C- and D-animals received repeated saline injections in the same schedule as those which were treated intermittently with morphine, and then subjected to the CPP with saline or cocaine (sal–sal and sal-coc groups, respectively). Table 1 summarizes the different experimental groups employed.

Table 1
Experimental groups employed in this study.

C-rats	D-rats
sal-sal	sal-sal
sal-coc[a]	sal-coc[a]
mor-sal	mor-sal
mor-coc[a]	mor-coc[a]

[a] different groups of animals for each dose of cocaine (3, 5, 7.5, 10 and 15 mg/kg).

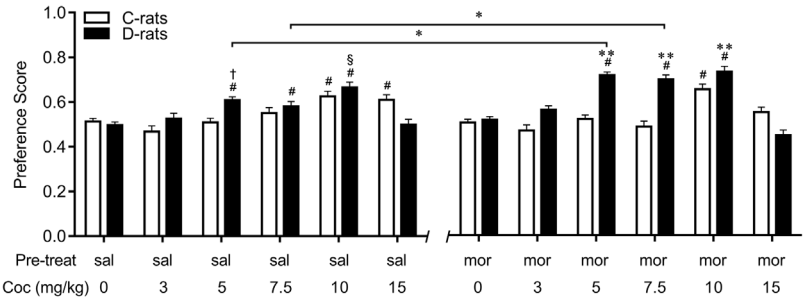

Fig. 1. Conditioning effect of cocaine in the CPP paradigm in different groups of C-and D-animals pre-treated with saline (1 ml/kg, s.c.) or escalating doses of morphine (twice-daily s.c. injections for 3 days: day 1: 5 mg/kg; day 2: 10 mg/kg; day 3: 20 mg/kg). Bars represent mean preference score ± S.E.M., N = 8–20 animals. # $P < 0.005$ vs. respective saline group; † $P < 0.0001$ vs. saline pre-treated C-rats conditioned with cocaine 5 mg/kg; § $P < 0.05$ vs. saline pre-exposed D-rats conditioned with 5 and 7.5 mg/kg of cocaine; ∗ $P < 0.0001$ vs. respective D-rats pre-treated with saline; ∗∗ $P < 0.05$ vs. respective C-rats pre-treated with morphine (LSD Fisher test).

2.5. FosB immunohistochemistry

Experiments were undertaken to evaluate the effects of cross-sensitization on FosB expression in brain areas related to the rewarding circuitry. Groups of C- and D-rats were submitted to a schedule of cocaine administration similar to that used in the CPP experiment with 5 mg/kg in morphine or saline pre-exposed rats. This cocaine dose was chosen since it was the lowest that elicited CPP in D-rats. Immediately after the testing session and twenty-four hours after the last cocaine injection, animals were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were then removed, left in fixative overnight and placed in phosphate-buffered 30% sucrose solution. Brains were sectioned on a cryostat into 40 μm slices and the sections immersed in 0.01 M PBS. Brain sections were first incubated for 1 h at room temperature in a solution of 3% hydrogen peroxide (H_2O_2) and 10% methanol in PBS 0.01 M. Afterwards, the sections were rinsed three times in 0.01 M PBS and placed in a blocking solution of 5% normal bovine serum (BSA) for 1 h. Then, brain sections were incubated for 48 h at 4 °C with a polyclonal FosB antibody (diluted 1:1500 in 0.01 M PBS containing 1% BSA). This antibody was raised against the residues 75–150 of the FosB molecule and it has been demonstrated by Western blotting analyses that proteins with molecular weight corresponding to ΔFosB can be recognized (SC-48; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following incubation in the primary antibody, sections were washed three times in 0.01 M PBS and incubated for 2 h in biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA.; diluted 1:200 in 0.01 M PBS containing 1% BSA), followed by an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Labs, Burlingame, CA, USA) for 1 h at room temperature. Finally, sections were incubated for 5 min with a solution containing 0.05% 3-3′-diamino-benzidine tetrahydrochloride (DAB, Sigma) and 0.01% H_2O_2. Sections were mounted onto gelatin-coated slides, dehydrated and coverslipped prior to viewing with a light microscope.

2.6. ΔFosB quantification

Positive ΔFosB cells were identified using light field microscopy at 200X and counted in selected brain areas using the SCION program from the NIH. The counting was carried out by a blinded investigator and performed using an identical area size (0.16 mm^2) of the same shape for each brain region. Automated counts of ΔFosB positive nuclei were obtained from each area of interest, maintaining constant background intensity across different areas. Counts from the left and right hemispheres were obtained from three sections within the NAc (core and shell), striatum, amygdala, hippocampus and the prefrontal cortex. The anteroposterior (AP) (coordinates from Bregma; [29]) of sections included for detailed analysis were AP 1.6, 1.2 and 0.7 (accumbens core and shell); AP −2.3, −2.56 and −2.8 (basolateral amygdala and hippocampus) and AP 2.2, 2.7 and 3.2 (anterior cingulate cortex, prelimbic cortex and infralimbic cortex). Schematic drawings of coronal sections of rat brain based upon the atlas of Paxinos and Watson are shown in Figs. 2B, 3B and 4B, indicating the different regions in which quantification of ΔFosB positive nuclei was performed.

2.7. Statistical analysis

Differences in behavioral scores were analyzed using three-way analysis of variance (ANOVA), with diet, pre-treatment and cocaine dose as independent variables. Post hoc comparisons were made using the LSD Fisher test, with values of $P < 0.05$ being considered as statistically significant. Since no significant differences were detected between the left and the right hemispheres in the ΔFosB positive nuclei, counts obtained from both hemispheres in different brain sections throughout an area of interest were averaged to generate one count of ΔFosB positive nuclei per region [26]. Data from each region were analyzed using two-way ANOVA (diet and pre-treatment). Results showing significant overall changes were subjected to a post hoc LSD Fischer test, with values of $P < 0.05$ being considered as statistically significant.

3. Results

3.1. Effect of morphine **pre-treatment** on the rewarding effects of cocaine assessed in a CPP paradigm

The rewarding effects of increasing doses of cocaine (3, 5, 7.5, 10 and 15 mg/kg, i.p.) were assessed in different groups of C- and D-rats pre-treated with saline (1 ml/kg, s.c. twice a day for 3 days) or escalated doses of morphine (day 1, 5 mg/kg; day 2, 10 mg/kg and day 3, 20 mg/kg, s.c.) in the CPP paradigm. Results are shown in Fig. 1. Three-way ANOVA conducted on these data revealed a significant interaction (pre-treatment × diet × cocaine dose, $F_{5237} = 2.33$, $P < 0.05$). Post hoc comparison revealed an effect in saline pre-treated C-rats, conditioned only with doses of 10 ($P < 0.0001$) and 15 mg/kg of cocaine ($P < 0.0005$). In contrast, saline pre-treated D-rats began to elicit conditioned place preference with lower doses of cocaine, 5 mg/kg ($P < 0.0001$) and 7.5 mg/kg ($P < 0.005$). This effect significantly increased with the 10 mg/kg dose ($P < 0.0001$), evidenced by an increase in the preference score compared with D-groups conditioned with 5 ($P < 0.05$) and 7.5 mg/kg ($P < 0.01$). In saline pre-treated D-rats, neither 3 nor 15 mg/kg of cocaine induced CPP.

The schedule of increasing morphine administration induced subsequent cross-sensitization to rewarding effects for lower doses of cocaine in D-rats, an effect that was absent in controls. Post hoc comparison showed that only D-animals conditioned with 5 ($P < 0.00001$) and 7.5 mg/kg ($P < 0.0001$) of cocaine developed cross-sensitization, spending significantly more time in the drug-paired compartment compared with the respective saline pre-treated D-group. Under our experimental conditions, cross-sensitization was not induced in C-rats with any of the cocaine doses used.

As perinatally undernourished rats have significantly lower body weights than control animals, the drug doses were recalculated in relation to body surface area (mg drug/100 cm^2), calculated by the formula: body surface area = 9.1 × body weight$^{0.66}$ [30]. All recalculated

Table 2
ΔFosB expression in different brain areas from C- and D-rats pre-treated with saline or morphine.

Brain region	Group			
	C- sal-coc	D- sal-coc	C- mor-coc	D- mor-coc
Accumbens core	131 ± 11	201 ± 10†	128 ± 9	270 ± 29$^{*}_{**}$
Accumbens shell	121 ± 18	187 ± 13†	152 ± 11	248 ± 21$^{*}_{**}$
Basolateral amygdala	56 ± 10	107 ± 6†	66 ± 12	152 ± 20$^{*}_{**}$
Cingulate cortex	29 ± 3	52 ± 4	44 ± 10	83 ± 11$^{*}_{**}$
Prelimbic cortex	49 ± 11	93 ± 9†	54 ± 5	130 ± 13$^{*}_{**}$
Infralimbic cortex	61 ± 9	117 ± 5†	60 ± 5	158 ± 16$^{*}_{**}$
Hippocampus	72 ± 7	89 ± 6	79 ± 4	80 ± 8

Values represent number of positive nuclei in 0.16 mm^2 (mean ± SEM, n = 4).
$^{*}P < 0.05$ vs. D-rats pre-treated with saline.
$^{**}P < 0.05$ vs. C-rats pre-treated with morphine.
$^{\dagger}P < 0.05$ vs. C-rats pre-treated with saline.

morphine doses showed a pattern similar to the dose of 10 mg/kg: 0.406 ± 0.002 mg of morphine/100 cm^2 for C-rats and 0.382 ± 0.002 mg of morphine/100 cm^2 for D-rats ($F_{1159} = 96.86$, $P < 0.0001$). Similarly, all recalculated cocaine doses showed a pattern to the dose of 5 mg/kg: 0.406 ± 0.002 and 0.380 ± 0.002 mg/100 cm^2/conditioning session for C- and D-rats, respectively ($F_{1170} = 108.04$, $P < 0.0001$). These results indicate that, expressed as mg/100 cm^2 body surface area, D-rats were administered significantly lower amounts of drug than C-rats.

3.2. Impact of early undernutrition on ΔFosB levels in different brain areas of the reward circuit following morphine pre-treatment and cocaine exposure

ΔFosB expression was assessed in several brain areas of the reward circuit in different groups of C- and D-rats pre-exposed to either saline or morphine and conditioned with 5 mg/kg of cocaine in the CPP paradigm. Consistent with previous studies [26,27], no significant differences were found in ΔFosB levels between C- and D-animals both pre-treated and conditioned with saline (data not shown).

Table 2 shows brain regions in which immunohistochemical ΔFosB expression was analyzed. No significant differences were detected between left and right hemispheres in the ΔFosB positive nuclei. The following results were obtained from two-way ANOVA (diet x pre-treatment) conducted on these data. In morphine pre-treated D-rats only, cocaine administration induced a significant increase in ΔFosB positive nuclei in both core and shell subareas of NAc [$F_{1,12} = 39.16$, $P < 0.001$ (core); $F_{1,12} = 24.52$, $P < 0.001$ (shell)], in the basolateral amygdala ($F_{1,12} = 28.55$, $P < 0.001$) and in the anterior cingulate, prelimbic and infralimbic subareas of the medial prefrontal cortex ($F_{1,12} = 15.82$, $P < 0.005$; $F_{1,12} = 38.457$, $P < 0.001$; $F_{1,12} = 58.25$, $P < 0.0001$, respectively). Thus, D-rats pre-treated with morphine showed a higher levels of ΔFosB than saline pre-exposed D-animals ($P < 0.05$) and than morphine pre-treated C-rats ($P < 0.05$). In contrast, C-rats did not increase ΔFosB expression in any of the regions assessed. Figs. 2 A, 3 A and 4 A show representative photomicrographs from brain areas where ΔFosB was induced. Figs. 2C, 3C and 4C represent the ΔFosB levels of C- and D-rats pre-exposed to saline or escalating doses of morphine and conditioned with 5 mg/kg of cocaine. No significant differences were found in the hippocampus when ΔFosB levels were analyzed, in either group of rats (C- or D-rats), pre-treated with either saline or morphine and conditioned with cocaine ($F_{1,12} = 3.25$, N.S.).

4. Discussion

Modifications in perinatal brain development linked to early protein malnutrition could lead to permanent alterations in neuronal pathways, resulting in altered reactivity upon exposure to different drugs of abuse [see reviews 31,32]. The present study evaluated the influence of perinatal protein malnutrition on developing cross-sensitization to cocaine-induced rewarding effects in animals pre-exposed to morphine. In agreement with our earlier findings, we observed enhanced rewarding effects of cocaine, since cocaine dose-response curves in saline pre-treated animals revealed a conditioning effect in D-rats, with doses of 5, 7.5 and 10 mg/kg, while this effect in C-rats was observed only with higher doses (10 and 15 mg/kg). In D-rats, place conditioning was evident at a dose of 5 mg/kg, increasing significantly for the 10 mg/kg dose. In full agreement, adult malnourished rats showed a greater responsiveness than controls to the rewarding effects of cocaine, as evidenced by a shift to the left of the D-R curves obtained with increasing doses of cocaine.

This study extends previous research related to psychostimulants and opioid drugs in animal models of protein-malnutrition, with undernourished rats showing increased response to drug effects in their adult life. For psychostimulants, enhanced sensitization to locomotor effects of amphetamine [23] and to cocaine-induced stereotypy [24] has also been described. Our D-rats, after repeated administration of cocaine or morphine, showed a facilitated development of sensitization [13,25–27]. These data indicate a greater reactivity of the mesocorticolimbic dopaminergic pathway resulting from this early nutritional insult. Thus, morphine pre-treated D-rats showed cross-sensitization to the rewarding effects of cocaine. Only D-rats showed a significant increase in the preference score with 5 and 7.5 mg/kg of cocaine compared to the respective saline pre-treated D-rats, while no cross-sensitization was observed under our experimental conditions for any doses of cocaine in C-rats. The lack of development of this phenomenon in C-rats can be explained by the use of a sensitization protocol similar to that described by Cadoni and Di Chaira [33], but employing doses that were half the concentrations used by these authors.

In agreement with the above findings, cross-sensitization has been consistently reported between morphine and cocaine. For instance, pre-treatment with opioid (morphine or heroin) intensifies the subsequent behavioral response to different direct and indirect DA agonists [4,11–13] and the conditioned rewarding effects of cocaine [14,34]. The mu-opioid agonists inhibit GABAergic interneurons in VTA, thus increasing the activity of mesolimbic and mesocortical dopamine systems [35]. Related to these neuronal systems, our previous reports demonstrated that early malnutrition results in permanent alterations in neuronal pathways, which may account for the altered reactivity to different pharmacological treatments, such as facilitated development and expression of behavioral and rewarding sensitization to cocaine or morphine, correlated with an increased response of the mesocorticolimbic system [13,25], and ΔFosB overexpression in areas associated with the brain reward network [26,27].

Repeated pre-treatment with opioids can increase the response to a subsequent challenge with psychostimulants, but this effect depends on several parameters including dose, route, time between withdrawal and challenge treatment, as well as the genetic background of the animals and species differences [6,12,36–38]. Our findings also indicate that both the development and the expression of cross-sensitization depend on the prior nutritional status during the perinatal period, since this phenomenon was evident in D-rats for lower doses of these drugs.

It is well documented that stabilized isoforms of ΔFosB accumulate with repeated, but not acute, exposure to many drugs of abuse and remain elevated in the brain's reward regions for weeks, suggesting that this transcription factor could be associated with the mechanisms underlying the long-lasting neuroadaptation process that contributes to a state of addiction. The expression of the transcription factor ΔFosB varies dependent on the administered substance, showing region-specificity for different drug stimuli [22,39][22,see review 39]. In contrast, in acute treatments, induced proteins (due to molecular instability) return to basal levels within hours of the initial drug administration [40].

In the current study, the schedule of morphine pre-treatment used

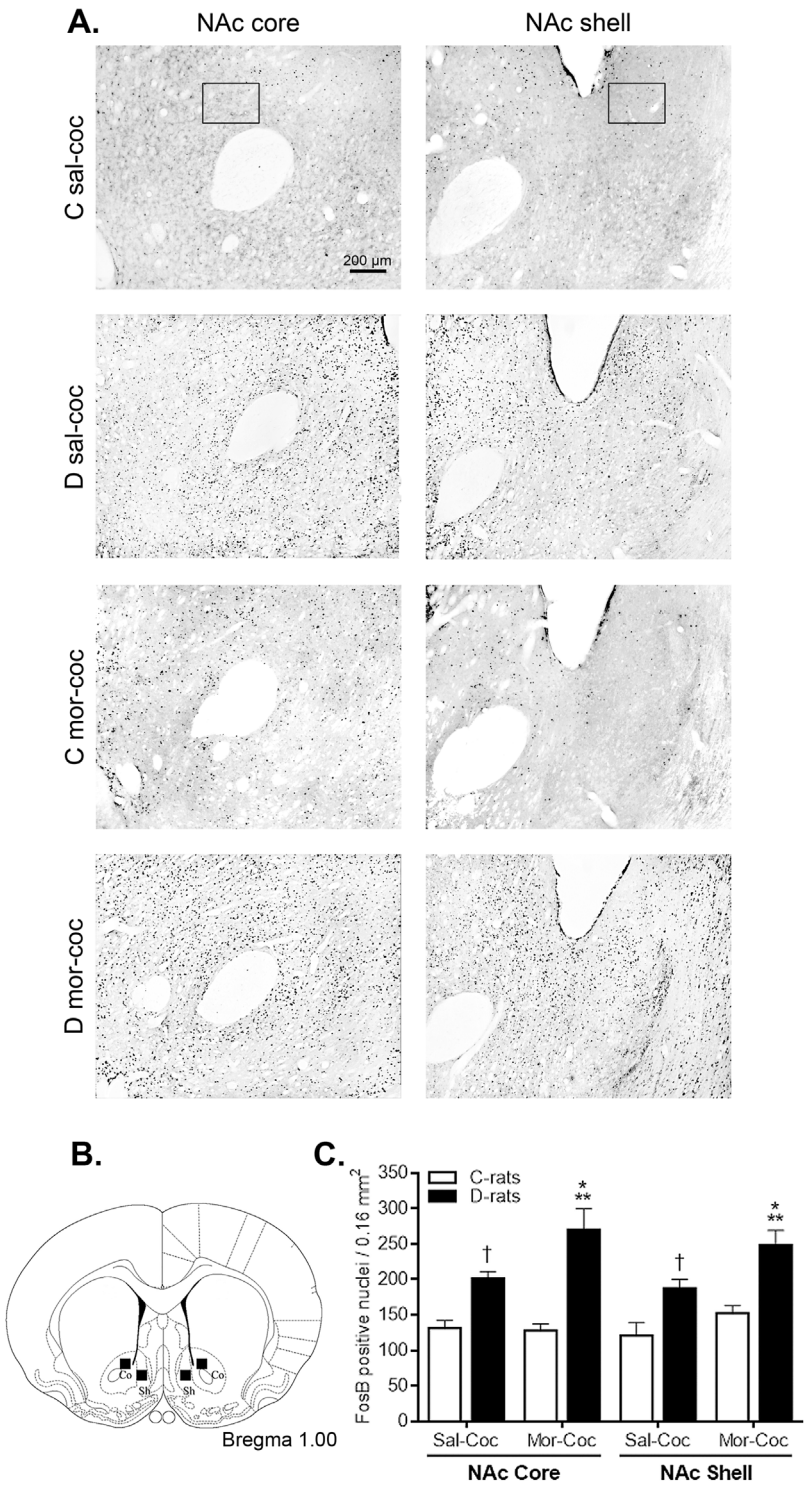

Fig. 2. A. Photomicrographs (50 X) of ΔFosB immunoreactivity in the NAc (core and shell) from C- and D-rats pre-treated with saline or escalating doses of morphine, and conditioned with 5 mg/kg of cocaine. Squares represent the areas from which the cell count was made. **B**. Schematic drawing of a coronal section of rat brain at Bregma 1.0. Squares represent the sites at which photomicrographs were taken. **C.** Quantification of ΔFosB positive nuclei. Bars represent mean ± S.E.M., N = 4. $*P < 0.05$ vs. D-rats pre-treated with saline; † $P < 0.05$ vs. C-rats pre-treated with saline; $**P < 0.05$ vs. C-rats pre-treated with morphine (LSD Fisher test).

induced a selective cross-sensitization to the rewarding effects of cocaine in the D-rats, as well as a significant induction of ΔFosB in the NAc (core and shell), the basolateral amygdala and medial prefrontal cortex (cingulate, prelimbic and infralimbic). It has been shown that the induction of ΔFosB in the NAc and dorsal striatum increase locomotor and reward responses to both cocaine and morphine [21,41,42]. Furthermore, the importance of FosB transcription factors and neuroadaptive response in amygdala and prefrontal cortical brain regions in opiate sensitization, highlighting that diverse pathways contribute to opiate sensitization including cognitive, motivational, motor and associative neural circuits has been demonstrated [43].

Our results are consistent with studies that highlight the importance of accumbal ΔFosB induction as a plasticity marker in opiate addiction [39,40]. Interestingly, cross-sensitized D-rats also showed a selective, significant increase in ΔFosB expression in other brain areas beyond the reward circuit of the nucleus accumbens and VTA, such as the amygdala and prefrontal cortex. In agreement with Kaplan et al. [43], the present study shows that opiate induces cross-sensitization to the reinforcing effects of cocaine, and also produces neuroadaptive effects in motivational (NAc), associative learning (BLA) and executive (PFC) pathways. The lack of such an effect in C-rats may be explained by the mild dosage of morphine used, which selectively produces cross-

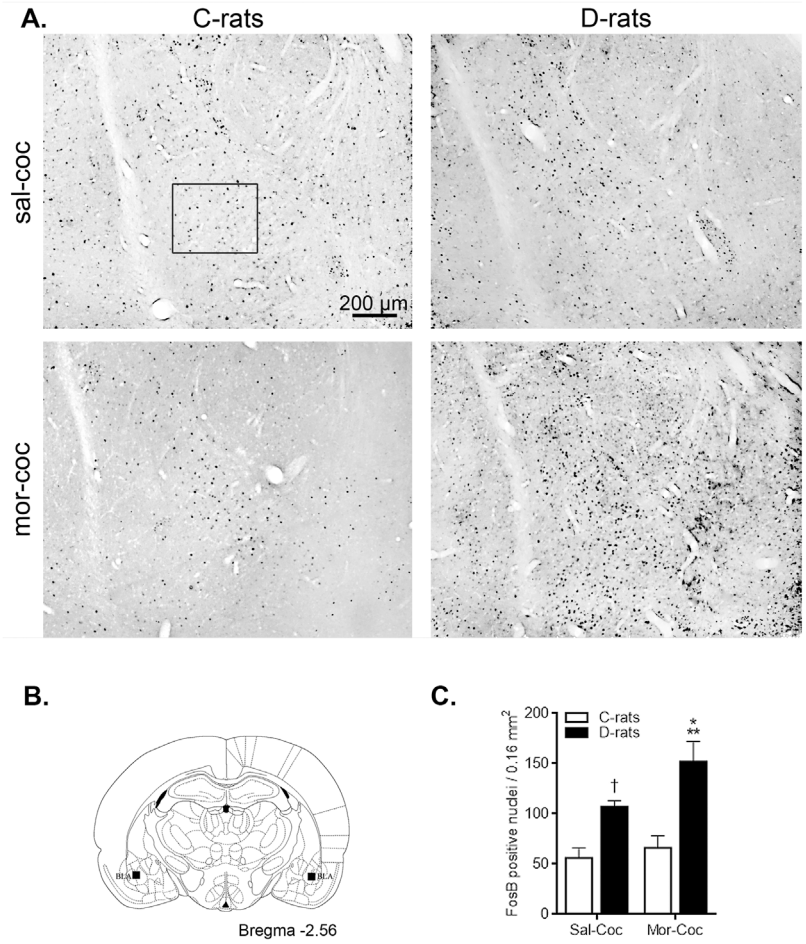

Fig. 3. A. Photomicrographs (50 X) of ΔFosB immunoreactivity in the basolateral amygdala from C- and D-rats pre-treated with saline or escalating doses of morphine, and conditioned with 5 mg/kg of cocaine. The square represents the area from which the cell count was made. **B**. Schematic drawing of the coronal section from which the photomicrographs were taken. **C**. Levels of ΔFosB in the basolateral amygdala of C- and D-rats pre-exposed to saline or escalating doses of morphine and conditioned with 5 mg/kg of cocaine. Bars represent mean ± S.E.M., N = 4. $*P < 0.05$ vs. D-rats pre-treated with saline; † $P < 0.05$ vs. C-rats pre-treated with saline; $**P < 0.05$ vs. C-rats pre-treated with morphine (LSD Fisher test).

sensitization in D-rats. Experimental protocols with higher doses or prolonged pre-treatment with morphine are generally used to induce sensitization and/or ΔFosB expression in normal rats [33,44].

The present results are also consistent with, and extend our previous studies related to higher ΔFosB expression in specific brain areas of the reward circuit only in D-Rats, after repeated administration of either cocaine or morphine independently (homologous sensitization) [26,27]. In relation to the altered responses to pharmacological treatments induced by early malnutrition, as well as showing facilitation to develop cross-sensitization, the D-rats showed a lower threshold for the reinforcing effects of cocaine reflected in a shift to the left of the D-R curve. This increased rewarding effect of cocaine observed in the saline pre-treated group correlated with a slight but significant increase in ΔFosB positive nuclei in the NAc (core and shell), the basolateral amygdala and the medial prefrontal cortex (prelimbic and infralimbic).

As regards the functional significance of prolonged ΔFosB expression in the NAc, it has been reported that ΔFosB expression increases the rewarding effects of cocaine, and mice over-expressing ΔFosB in the NAc showed increased place conditioning and incentive motivation for cocaine [16]. It is well known that, in normal animals, the transcription factor ΔFosB is induced only slightly after acute exposure to drugs. However, these isoforms begin to accumulate with repeated administration of the drug [16]. The slight increase in ΔFosB levels, observed in D-rats pre-treated with saline after only four administrations with low doses of cocaine (5 mg/kg) as in the conditioning protocol, could be one of the markers or molecular changes underlying facilitation or increased behavioral responses that malnourished animals present when they are exposed to different drugs of abuse.

These results indicate that neural alterations induced by nutritional insult in early life facilitate the rewarding effects and the development of cross-sensitization to cocaine, which correlates with an upregulation of ΔFosB in brain areas related to the neuronal reward circuits. They thus emphasize the vulnerability of the CNS during early life to harmful factors, such as early undernutrition, that may predispose subjects to consume drugs of abuse in adulthood, in agreement with several studies that have shown the high vulnerability of developing or immature brains to different drugs of abuse, characterized by changes in reactivity to challenges in adulthood [45–48]. Moreover, this study demonstrates the selective plasticity of a critical molecule in the malnourished animal's brain that regulates, at least in part, the mechanisms underlying addiction and suggests that these neuroadaptive changes may be involved in the mediation of enhanced addictive tendencies in subjects suffering severe malnutrition in early life.

It could be argued that changes in reactivity to different pharmacological treatments described in D-rats may be a consequence of pharmacokinetic alterations induced by nutritional insult. However, in our previous studies no significant differences were detected between C- and D-animals in plasma or brain levels of morphine or cocaine, after either acute or repeated administration of each drug [13,25–27]. These data are in agreement with other reports from our laboratory that demonstrate similar plasma and brain levels of diazepam, pentobarbital or ethanol from C- or D-rats following acute or chronic treatments [49,50]. Furthermore, since D-rats showed differences in body weight compared with controls, the morphine or cocaine doses used (mg/kg) were recalculated in both groups, considering body surface area (mg/100/cm^2) and the values obtained from D-rats were always lower than those from C-rats. Altogether, these data indicate that changes observed in the rewarding effects of cocaine and ΔFosB immunohistochemical expression may be associated with pharmacodynamic rather than pharmacokinetic factors.

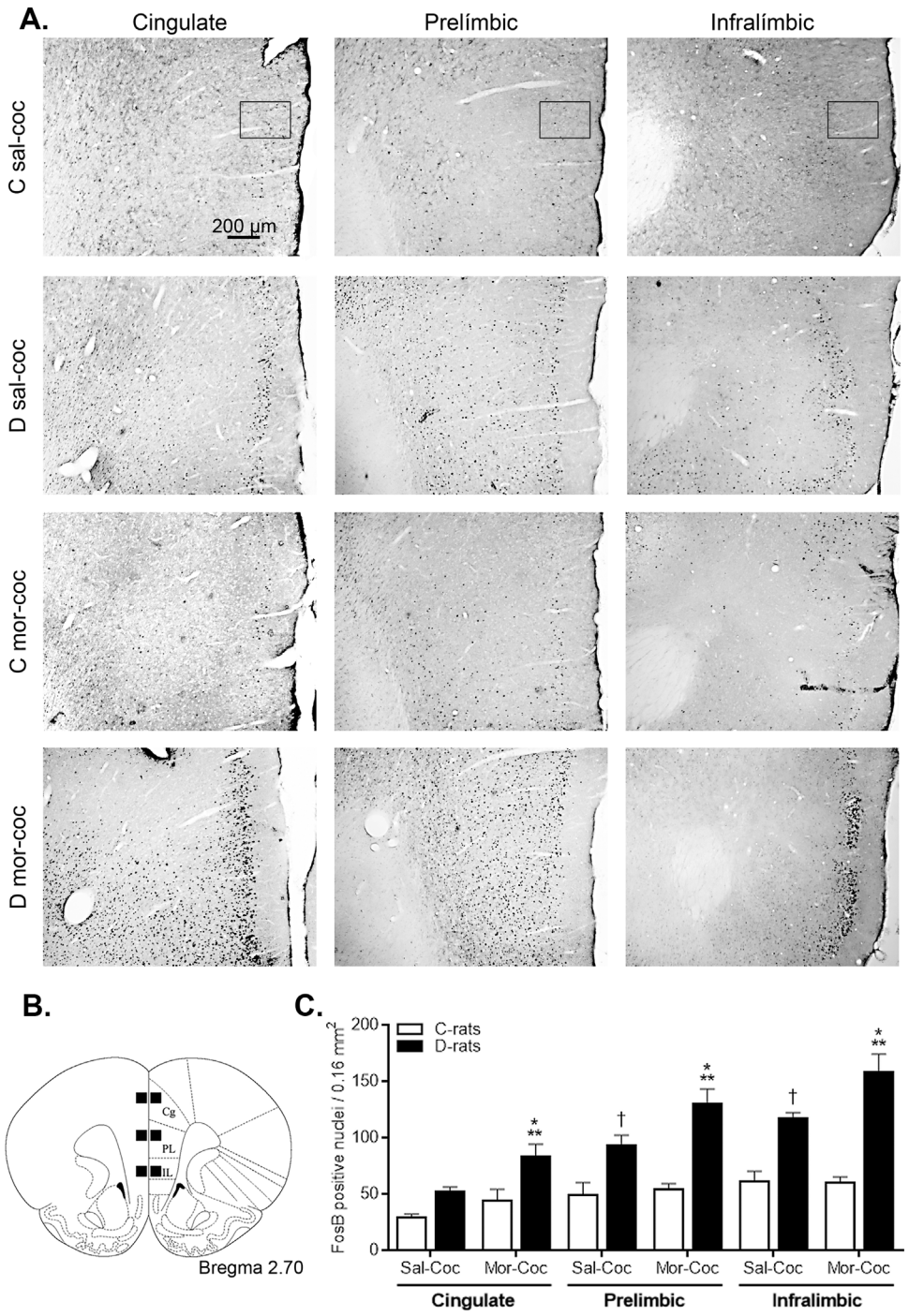

Fig. 4. A. Photomicrographs (50 X) of ΔFosB immunoreactivity in the cingulated, prelimbic and infralimbic cortex from C- and D-rats pre-treated with saline or escalating doses of morphine, and conditioned with 5 mg/kg of cocaine. Squares represent the areas from which the cell count was made. **B**. Photomicrographs were obtained from coronal sections 2.7 mm anterior to Bregma. **C.** Quantification of ΔFosB positive nuclei in the medial prefrontal cortex of C- and D-animals conditioned with cocaine (5 mg/kg, i.p.) and pre-treated with saline or escalating doses of morphine. Bars represent mean ± S.E.M., N = 4. $*P < 0.05$ vs. D-rats pre-treated with saline; † $P < 0.05$ vs. C-rats pre-treated with saline; $**P < 0.05$ vs. C-rats pre-treated with morphine (LSD Fisher test).

5. Conclusion

The present findings extend and complement knowledge of the incidence of early nutritional injury and its potential involvement in vulnerability to drug abuse in adult animals. Like previous reports, they demonstrate that early malnutrition may be considered as an additional risk factor to be taken into consideration in the etiology of addictive processes. Malnourished individuals not only have a greater propensity for neurobiological changes underlying addiction, but also this plasticity can be induced with lower doses and after shorter periods of consumption. Moreover, early nutritional insult can facilitate the use of a new drug of abuse (cocaine) in subjects with a history of opioid consumption. Such polydrug abuse is associated with more serious health consequences and additional problems for the therapy of addiction.

Conflicts of interest

All authors declare that they have no conflicts of interest.

Authors contribution

MCP, AV and GRC were responsible for the study concept and design. MCP and AV acquired data and performed the statistical analysis. MCP performed behavioral and immunohistochemical analyses. MCG contributed to behavioral experiments. MCP, AV and GRC interpreted the findings. GRC drafted the manuscript. MCP, AV and GRC provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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