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Long-term Effects of Neonatal Stress on Adult Conditioned Place Preference (CPP) and Hippocampal Neurogenesis

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Abstract

Critically ill preterm infants are often exposed to stressors that may affect neurodevelopment and behavior. We reported that exposure of neonatal mice to stressors or morphine produced impairment of adult morphine-rewarded conditioned place preference (CPP) and altered hippocampal gene expression. We now further this line of inquiry by examining both short- and long-term effects of neonatal stress and morphine treatment. Neonatal C57BL/6 mice were treated twice daily from postnatal day (P) 5 to P9 using different combinations of factors. Subsets received saline or morphine injections (2 mg/kg s.c.) or were exposed to our neonatal stress protocol (maternal separation 8 h/d × 5d + gavage feedings ± hypoxia/hyperoxia). Short-term measures examined on P9 were neuronal fluorojade B and bromodeoxyuridine staining, along with urine corticosterone concentrations. Long-term measures examined in adult mice (>P60) included CPP learning to cocaine reward (± the kappa opioid receptor (KOR) agonist U50,488 injection), and adult hippocampal neurogenesis (PCNA immunolabeling). Neonatal stress (but not morphine) decreased the cocaine-CPP response and this effect was reversed by KOR stimulation. Both neonatal stress or morphine treatment increased hippocampal neurogenesis in adult mice. We conclude that reduced learning and increased hippocampal neurogenesis are both indicators that neonatal stress desensitized mice and reduced their arousal and stress responsiveness during adult CPP testing. Reconciled with other findings, these data collectively support the stress inoculation hypothesis whereby early life stressors prepare animals to tolerate future stress.

Keywords

kappa opioid receptor; morphine; dynorphin; neonatal stress; stress inoculation hypothesis

1. Introduction

Newborn infants hospitalized in neonatal intensive care units may experience significant stressors including maternal separation, repeated painful procedures, and transient hypoxia

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and hyperoxia. In order to reduce the pain and stress, clinicians commonly prescribe opiates despite uncertainty about analgesic efficacy in this population [1–2], and concern about both short- and long-term detrimental effects [3–4]. However, there is equal concern that neonatal stress may perturb brain development and produce a variety of both short and long-term effects on neuroendocrine and cognitive function [5–6]. Alternatively, the *stress inoculation hypothesis* asserts that brief intermittent neonatal stress prepares infants to resist future stress [7].

To investigate long-term effects of early exposure to opiates and stress, we developed an animal model that combined several common neonatal stressors, with and without morphine treatment. We observed that neonatal stress or morphine treatment altered hippocampal gene expression [8] and impaired adult morphine-rewarded CPP learning [9], and neonatal stress activated neonatal kappa opioid receptor (KOR) signaling [10]. In adult mice, KOR stimulation (via endogenous dynorphin or KOR agonist injection) produces dysphoria and distress and thereby shifts the dose-response curve for cocaine to the left and potentiates the behavioral effects of cocaine, and these effects are KOR dependent [11–18]. Because the hippocampus mediates spatial place learning and hippocampal processing and neurogenesis are suppressed by stress and glucocorticoids [19–21], hippocampal neurogenesis may be useful as an index of the stress state of animals undergoing CPP testing.

We previously combined daily injections of morphine with maternal separation and exposure to oxidative challenge to produce *neonatal stress* that simulates the combined stressors experienced by preterm infants during intensive care. We found that neonatal stress disrupted adult morphine-CPP learning [9]. It was undetermined from this early work whether individual stressors or the combined stress was responsible for this long-term change. It was also not known whether the response was specific to morphine, or generalizable to other reward stimuli. We now examine the effect of individual stressors on short term outcomes, and test whether the neonatal stress-induced effect on morphine-CPP was specific to opiate reward or not, by testing whether neonatal stress and/or morphine also decrease the adult CPP response to cocaine reward. We hypothesized that exposure to the neonatal stress protocol would disrupt adult mouse cocaine-CPP learning. We also tested whether the KOR agonist U50, 488 could potentiate the response to cocaine. Lastly, we examined both short- and long-term effects of combinations of neonatal stressors on hippocampal neurogenesis.

2. Material and Methods

2.1 Animals

All procedures were approved by the local Animal Care and Use Committee. Adult C57BL/6 wild-type mice were used. The day of birth was considered postnatal day (P) 1. P5 mice were weighed and distributed into weight-matched litters ($n = 5\text{--}7/\text{dam}$) and assigned to treatment groups. For short-term experiments examining acute effects of stress, both male and female mice were used. For long-term adult learning experiments, only male mice were used. Mice were housed under a 12 h light/dark cycle and fed ad libitum.

2.2 Neonatal Treatment Protocols

Neonatal treatments were administered every day from P5 to P9. Injections (10 μl s.c.) of either *saline* or *morphine* (2 mg/kg based on daily litter weights) were administered twice each day (08:00 and 16:00 h). To create an oxidative stressor, a subset of mice were exposed to *hypoxia/hyperoxia* shortly after drug treatment (100% N_2 1 min then 100% O_2 5 min). To create a prolonged physiological stressor, some mice were then exposed to 8 h of daily *maternal separation* by isolating individual mice in cups within a veterinary warmer and

gavage feeding those mice with 50 to 150 μ l of milk substitute three times per day. *Untreated* mice were dam-reared and only exposed to minimal handling on P5 for initial group assignment. No injections were given to these animals. Treatment groups were designed to evaluate possible interactions between specific combinations of different neonatal stressors. To track early cell division, all treated mice (but not untreated control mice) also received 10 μ l s.c. injections of BrdU (100 mg/kg) at 08:00 on P5 and P7.

2.3 Neonatal Treatment Groups for Short-term Experiments

For short-term (P9) experiments, 5 treatment groups were designed to combine morphine with individual neonatal stressors. The 5 groups were: untreated, morphine, morphine + maternal separation, morphine + hypoxia/hyperoxia, and morphine + maternal separation + hypoxia/hyperoxia. We hypothesized that each of these variables would incrementally exacerbate the deleterious acute effects of neonatal morphine on neurogenesis and neurodegeneration.

2.4 Neonatal Treatment Groups for Long-term Experiments

For long-term (adult) behavioral experiments, 5 treatment groups were combinations used previously [9]. The 5 groups were untreated, saline, morphine, saline + maternal separation + hypoxia/hyperoxia, and morphine + maternal separation + hypoxia/hyperoxia.

2.5 Conditioned Place Preference (CPP)

To measure learning, 142 adult male mice (> P65) underwent CPP training and testing [16]. The CPP apparatus had two 25-cm cubic chambers connected by a hallway with chambers differentiated by horizontal vs. vertical wall stripes. All behavior was video recorded and computer analyzed (Ethovision, Noldus, Wageningen, The Netherlands). On CPP training day 1, mice were allowed access to both chambers for 30 min of free exploration, and disqualified if aversion bias was present (< 300 s in exploration of each chamber). On CPP training days 2 and 3, specific associations were established by confining mice to each chamber for 30 min and injecting them with saline or cocaine (2 mg/kg). On CPP testing day 4, the mice were allowed 30 min of free exploration, and the net preference for the cocaine-paired chamber was calculated (cocaine minus saline time). Half of the adult mice were injected with the KOR agonist U50,488 (5 mg/kg) 1 hr before the final testing exploration.

2.6 Tissue Collection

For the short-term experiments, P9 pups were killed at 13:00 h on the last neonatal treatment day. For the long-term experiments, adult mice were killed after CPP testing. Mice were euthanized with a 2.2 mL/kg i.p. overdose of Euthasol (Virbac AH Inc., Fort Worth, TX) and then transcardially perfused with buffered 4% formaldehyde saline. Neonatal urine for corticosterone measurement was collected after Euthasol injection, prior to transcardial perfusion, by needle aspiration of the exposed bladder. Brains were removed after perfusion and immersion fixed overnight before being paraffin embedded, sectioned (7 μ M), and slide mounted.

2.7 Histochemistry

Standard slide-mounted immunoperoxidase labeling was performed. Briefly, slides were dewaxed, rehydrated, boiled in citrate buffer, exposed to a primary then secondary antibody, then detected with an avidin or streptavidin-conjugated peroxidase and diaminobenzidine. Immunolabeled targets included glial fibrillary acidic protein (GFAP, ab68428, Abcam, Cambridge, MA, USA), proliferating cell nuclear antigen (PCNA, M0879, DAKO, Carpinteria, CA, USA), bromodeoxyuridine (BrdU kit 2760, Chemicon, Billerica, MA, USA). To detect degenerating neurons, fluorojade B (FJB) staining was performed by

bathing slides in 0.06% KMnO_4 followed by 0.0005% FJB (AG310, Chemicon, Billerica, MA, USA).

2.8 Image Analysis

Digital images were captured on an Olympus BX41 microscope (Olympus America Inc., Center Valley, PA, USA). Cell counts were performed by two blinded observers using AnalySIS software (Olympus, Münster, Germany). Multiple images were evaluated and replicates were averaged.

2.9 Statistical Analysis

Parametric or non-parametric analyses were conducted as appropriate using SPSS software (SPSS, Chicago, IL). ANOVA was followed with Dunnett's (multiple comparisons) or t-test (two groups) post hoc testing when warranted. Comparisons were two-tailed and used an alpha criterion of $P = 0.05$. Initially, two-way multivariate analysis using stress and drug treatment as factors was performed, and if significant effects were detected for only one variable, univariate analysis was subsequently performed prior to post hoc testing. Thus data from non-significant drug treatment groups (untreated, saline and morphine) may be combined together to permit meaningful comparison of significant stress effects (dam-reared vs. maternal separation + hypoxia/hyperoxia).

3. Results

3.1 Mortality

The neonatal mortality was 21% (23/111) for male and female mice in the short-term experiment with 0/23 deaths in controls (untreated plus saline) compared to 23/88 deaths in the combined morphine and stress-exposed groups (Fisher's exact test $P = 0.01$). The neonatal mortality was 12% for male mice assigned to the long-term experiment, with 0/62 deaths in controls compared to 22/115 in the morphine and stress groups (Fisher's exact test $P = 0.001$). Adult deaths were 10/62 in saline mice compared to only 3/93 in the morphine and stress groups (Fisher's exact test $P = 0.01$). Thus *neonatal stress* (the combination of maternal separation + hypoxia/hyperoxia) or morphine increased early mortality but decreased late mortality.

3.2 Short-term effects

Short-term data were collected from mice killed at P9. Urine corticosterone concentrations on P9 were difficult to collect because urine volumes were minimal (due to age and voiding) so the data are incomplete (only 19 samples obtained from 97 mice). Nevertheless, the data are novel and the mean ($\pm$ SEM) urine corticosterone concentration for 3 untreated mice was 12 ± 10 ng/mL compared to 515 ± 152 ng/mL (range 60 to 1763 ng/mL) for the 16 mice combined from all the morphine-treated groups ($P = 0.17$). These data are within the range reported for neonatal urine corticosterone values from stressed rats [22].

Neonatal treatments acutely increased neuronal degeneration in P9 mice. Table 1 lists the densities for FJB-positive cells in the hippocampal CA1 region, piriform cortex, and cingulate cortex. In CA1 there was a higher density of FJB-positive cells in the morphine + maternal separation + hypoxia/hyperoxia group compared to the untreated mice. In both piriform and cingulate cortex, both the morphine and the morphine + maternal separation + hypoxia/hyperoxia groups had increased density of FJB-positive cells compared to untreated mice.

There were no acute effects of neonatal treatments on P9 astrocyte activity or neuronal proliferation in hippocampus. The mean ($\pm$ SEM) GFAP-positive cell densities (count/mm²)

were untreated = 702 ± 181 , morphine = 733 ± 318 , morphine + hypoxia/hyperoxia = 743 ± 273 , morphine + maternal separation = 687 ± 367 and morphine + maternal separation + hypoxia/hyperoxia = 775 ± 234 . In the granule cell layer of the dentate gyrus, the mean ($\pm$ SEM) BrdU-positive cell densities (count/mm²) were morphine = 4008 ± 480 , morphine + hypoxia/hyperoxia = 4007 ± 654 , morphine + maternal separation = 3623 ± 352 and morphine + maternal separation + hypoxia/hyperoxia = 3161 ± 270 . Untreated mice were not injected with BrdU because that would have confounded their untreated status.

3.3 Long-term effects

Long-term data were collected from adult mice. Neonatal stress decreased adult cocaine-CPP response time and this effect was reversed by injection of the KOR agonist U50,488 prior to testing. The top panel in Figure 1 shows that early exposure to maternal separation + hypoxia/hyperoxia (with or without morphine) significantly decreased the CPP time in adult mice. In both panels, statistical comparison found that responses from dam-reared saline-treated mice and untreated mice did not differ, so those two control groups are combined into a single control group. The bottom panel in Fig 1 shows a larger analysis comparing CPP times between adult mice treated with or without U50,488. KOR activation increased the CPP times of neonatally-stressed mice to levels matching control.

Adult neurogenesis was affected by neonatal treatments as indicated by differences in adult PCNA immunolabeling. Table 2 lists the densities for PCNA-immunoreactive neurons in the granule cell layer of the hippocampal dentate gyrus. As indicated, early exposure to saline + maternal separation + hypoxia/hyperoxia or early morphine produced an increase in the number of hippocampal neurons undergoing neurogenesis in adult mice. In contrast, there were no group differences detected for adult BrdU-immunolabeling (data not shown).

4. Discussion

4.1 Overview

We examined both short- and long-term effects of neonatal stress combined with neonatal morphine exposure to further knowledge of the developmental consequences of these factors. Acute experiments were conducted both to corroborate prior studies, and to test new combinations of neonatal treatments. Both short- and long-term effects of treatments were evident. The principal findings of this report are that neonatal stress reduces CPP learning and increases adult hippocampal neurogenesis. These effects are consistent with the explanation that neonatal stress reduces adult arousal.

4.2 Short-term effects

Neonatal morphine (with and without maternal separation) increased early neuronal degeneration in hippocampus, piriform and cingulate cortex, and these data agree with similar experiments measuring neonatal neuronal apoptosis [23–24]. In fact, a number of reports have established that early morphine exposure disrupts DNA synthesis and triggers neuronal death and apoptosis [25–28]. It is possible that early hippocampal neuronal degeneration contributed to the adult CPP response of morphine-treated mice.

We have previously collected mouse plasma and rat urine to confirm that neonatal rodents mount an acute adrenal response to neonatal stress [9,22]. In this experiment, neonatal mouse urine volumes were too small to be reliably collected by needle aspiration. Although we did not detect significant group effects, we did identify that neonatal mouse corticosterone can be measured in urine. In future experiments, this may provide a simple non-invasive measure of the neonatal stress response, especially if voided urine can be more reliably collected.

In our prior report, neonatal stress and morphine treatment greatly increased GFAP immunofluorescence in the molecular layer of the hippocampus [10], and others found that either high-dose morphine or maternal separation increased hippocampal GFAP [23,29]. In this report, GFAP-positive cell counts were not increased. These data may reflect methodological differences since GFAP-positive cell counts assess the size of the reactive astrocyte population, whereas total GFAP immunofluorescence reveals the degree of GFAP expression within the astrocyte population.

4.3 Long-term effects

Similar to our findings that neonatal stress decreased morphine-CPP [9], we now show that neonatal stress (but not morphine) also decreased cocaine-CPP performance. Changes in CPP performance may be interpreted as reflecting either modulation of responsiveness to cues (learning), or responsiveness to pharmacologic reward. In adult mice, stress can provoke drug-seeking behavior, possibly by activation of KOR, because both stress or U50,488 injection increase cocaine-CPP [16]. We found that while mice that had been stressed in the neonatal period showed less drug seeking behavior (cocaine and morphine-CPP), stimulation of KOR with U50,488 increased the cocaine-CPP response, effectively reversing the decrease due to neonatal stress. Thus it appears that neither neonatal stress nor neonatal morphine exposure prevent the KOR-mediated modulation of reward-mediated learning in adult mice. Moreover, because neonatal stress disrupts CPP using either morphine or cocaine reward, it is more likely an effect on arousal or learning rather than responsiveness to a specific pharmacologic reward. However, these data do not disqualify the more remote explanation that the decreases in both morphine-CPP and cocaine-CPP reflect reduced hedonic response after exposure to neonatal stress.

To understand how our neonatal stress-induced decrease in cocaine-CPP and corresponding increased adult hippocampal neurogenesis (PCNA data) may both be indicators of reduced arousal and decreased psychological response to environmental stress, some additional background is warranted. A substantial literature has established that the amygdala and hypothalamic-pituitary-adrenal axis (HPA) regulate hippocampal processing, and the affective aspects of learning, via noradrenergic and glucocorticoid receptor-mediated pathways. Emotional balance modulates learning such that moderate arousal improves learning, while reduced or excessive arousal inhibits learning [30]. As mentioned briefly before, adult-born hippocampal neurons are essential features of the endocrine and behavioral response to stress [19], brain-derived neurotrophic factor (BDNF) mediates adult neurogenesis [31–32] and stress and glucocorticoids potently inhibit adult neurogenesis [20]. One more key fact is that neonatal stress decreases hippocampal glucocorticoid receptor expression [21]. Collectively, these observations provide a mechanism whereby neonatal stress may decrease hippocampal glucocorticoid receptors, prevent the HPA-induced glucocorticoid suppression of hippocampal BDNF (thereby preserving neurogenesis) and reduce the emotional salience of cues during cocaine-CPP testing. In addition, our separation protocol, with return to the home cage each night, may result in recuperative maternal care which is also thought to reduce hippocampal glucocorticoid receptor responsiveness and thereby desensitize mice to stressors [33]. These explanations are not mutually exclusive.

The hypothesis that stressful neonatal experiences may influence adult susceptibility to drug seeking is controversial because the data are mixed. For example, neonatal Wistar rats exposed to daily handling stress develop decreases in adult dopamine metabolism, dopamine agonist-induced sugar consumption, and sugar-rewarded CPP, but an overall increase in consumption of sweet foods [34]. In contrast, prolonged maternal separation lowered the threshold doses for morphine-rewarded CPP, and KOR agonist-mediated place aversion [35], but decreased adult self-administration of cocaine in a manner that was sensitive to

both the degree of neonatal separation, and the dose of cocaine reward in adult Long-Evans rats [36]. Moreover, clinical outcomes for adult subjects born prematurely consistently show that prematurity predisposes to lower IQ and poorer academic achievement, but also lower risk of alcohol and drug abuse [37–38]. We conclude that our mouse data best support these clinical observations.

5. Conclusions

We conclude that neonatal stress impairs adult cocaine-CPP and increases hippocampal neurogenesis. We speculate that neonatal stress may permanently habituate animals to subsequent handling, thereby reducing their overall arousal and stress responsiveness during testing, and that this reduces the salience of learning cues and prevents the stress-induced decreases in adult neurogenesis. Further study of the interaction between stress and pharmacologic agents, particularly those used to treat neonates, is needed in order to improve neonatal analgesic therapy and alleviate neonatal stress, while simultaneously optimizing neurodevelopmental outcomes for these high-risk patients.

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Abbreviations

CPP	conditioned place preference
KOR	kappa opioid receptor
P	postnatal day
GFAP	glial fibrillary acidic protein
PCNA	proliferating cell nuclear antigen
BrdU	bromodeoxyuridine

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- Neonatal mice underwent repeated maternal separation and hypoxia/hyperoxia exposure.
- Subsets of neonatal mice were also given morphine injection (2 mg/kg bid).
- Adults showed decreased cocaine place-preference conditioning after neonatal stress.
- Adults showed increased hippocampal neurogenesis after neonatal stress.

Figure 1.

Repeated neonatal exposure to maternal separation stress + hypoxia/hyperoxia (stressed) decreases adult conditioned place-preference (CPP) learning (top panel), and the kappa opioid receptor (KOR) agonist U50,488 reverses the learning impairment due to neonatal stress (bottom panel). CPP data are mean ($\pm$ SEM) net preference times for adult mice after cocaine-reward. As neonates, mice were either dam-reared or exposed to a neonatal stress protocol (stressed). The top panel compares control (white bars) to morphine-treated mice (gray bars) and ANOVA found an effect of stress ($F_{1,37} = 4.3$, $P = 0.047$) and the post hoc t-test comparing all stressed mice to all dam-reared mice is indicated as * = $P < 0.05$, with $N = 6$ to 19 per group. In the bottom panel, control and morphine treated mice are combined to compare those that did not receive any post-training injection (diagonal lines) with those that were given the KOR agonist U50,488 prior to testing (black bars), and ANOVA found a KOR agonist $\times$ stress interaction ($F_{1,125} = 5.4$, $P = 0.024$) and post hoc tests are indicated as † = $P < 0.01$ compared to dam-reared control, * = $P < 0.05$ compared to stressed KOR agonist treated. Data from control and morphine-treated animals are combined because there were no significant effects of morphine and $N = 40$ to 70 per group.

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Density of FJB-positive cells per mm² in hippocampus CA1 layer, piriform cortex, and cingulate cortex in groups of neonatal mice (mean ± SEM, N).

Table 1

Morphine	-	+	+	+	+	+
Hypoxia/hyperoxia	-	-	+	+	-	+
Maternal Separation	-	-	-	-	+	+
Hippocampus CA1	0 ± 0, 11	8 ± 6, 11	1 ± 1, 11	8 ± 4, 9	20 ± 9, 11 [*]	
Piriform Cortex	54 ± 20, 11	368 ± 116, 11 [†]	123 ± 28, 11	66 ± 31, 9	342 ± 61, 11 [*]	
Cingulate Cortex	42 ± 23, 11	425 ± 148, 11 [†]	112 ± 23, 11	159 ± 49, 9	458 ± 96, 11 [†]	

Plus signs indicate the treatments given in combination to groups of mice in each column. Multivariate ANOVA (drug × stress × hypoxia) for hippocampus (main F_{4,52} = 2.8, P = 0.05 and interaction F_{1,53} = 4.1 P = 0.05), piriform cortex (main F_{4,52} = 6.2, P = 0.001 and interaction F_{1,53} = 17.7 P = 0.001), and cingulate cortex (main F_{4,52} = 5.1, P = 0.01 and interaction F_{1,53} = 12.6 P = 0.001). Dunnett's t-test compared to untreated mice (first column).

^{*} P = 0.05
[†] P = 0.01

Table 2

Adult neurogenesis in the dentate gyrus of hippocampus as indicated by density (cells/mm²) of PCNA-immunopositive neurons (mean $\pm$ SD, N) from groups of adult mice exposed as neonates to a routine of repeated maternal separation + hypoxia/hyperoxia (stressed) vs. dam rearing, with (+) or without (–) neonatal morphine treatment.

Morphine	–	+
Dam-reared	0.95 $\pm$ 0.46, 20	2.32 $\pm$ 2.81, 10 [*]
Stressed	2.12 $\pm$ 1.06, 4 [*]	1.6 $\pm$ 0.98, 10

Significant Mann-Whitney U comparisons to dam-reared control are indicated as

^{*}
P < 0.05.