Behavioural Brain Research 227 (2012) 7–11

Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Long-term effects of neonatal stress on adult conditioned place preference (CPP) and hippocampal neurogenesis

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ARTICLE INFO

Article history:
Received 3 September 2011
Received in revised form 14 October 2011
Accepted 21 October 2011
Available online 28 October 2011

Keywords:
Kappa opioid receptor
Morphine
Dynorphin
Neonatal stress
Stress inoculation hypothesis

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 × 5 d + 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.

1. Introduction

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

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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doi:10.1016/j.bbr.2011.10.033

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 = 5–7/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 was 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 50–150 μL 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 μL 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 h 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 μM), 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 $P \leq 0.05$. 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 $P \leq 0.01$). 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 $P \leq 0.001$). Adult deaths were 10/62 in saline mice compared to only 3/93 in the morphine and stress groups (Fisher's exact test $P \leq 0.01$). 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 (±SEM) urine corticosterone concentration for 3 untreated mice was 12 ± 10 ng/mL compared to 515 ± 152 ng/mL (range 60–1763 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].