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Neonatal isolation alters stress hormone and mesolimbic dopamine release in juvenile rats

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Abstract

Rat pups were individually isolated from the mother and nest for 1 h/day from postnatal days (PND) 2 to 9 and tested as juveniles (PND 26–30) compared to nonhandled (NH) controls. In response to 1 h of restraint stress, NH rats increased locomotor activity and dopamine (DA) levels, but neonatally isolated (ISO) rats did not. Both groups had increased plasma corticosterone levels in response to restraint, but corticosterone levels were higher in ISO than in NH. Brain allopregnanolone (3α, 5α-THP) levels also increased in response to stress, but NH and ISO did not differ. Sex of the rats was not a factor for any of the measures except plasma corticosterone levels, where females had higher levels than males. These data indicate that the effects of neonatal isolation persist postweaning and that the effects are most evident in response to stress as opposed to under baseline conditions. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Neonatal isolation has been a useful model to study the long-term neurochemical and behavioural changes produced by moderately stressful experiences early in life. The paradigm that we have employed in a number of studies involves removing a rat pup to a temperature-controlled environment away from its nest, dam and siblings for 1 h/day from postnatal days (PND) 2 to 9. Neonatal isolation has immediate effects on various stress-responsive systems. Plasma corticosterone levels are significantly increased within 30 min of isolation (McCormick et al., 1998b). Although basal plasma corticosterone levels remain similar to those of control pups, by the eighth episode of neonatal isolation on PND 9, pups hypersecrete corticosterone compared to pups receiving their first episode of isolation on PND 9 (McCormick et al., 1998b). We have found that neonatal isolation increased brain levels of pregnane neurosteroids (Kehoe et al., 2000). Further, by PND 9, pups undergoing repeated daily neonatal isolation had higher levels of allopregnanolone (3α, 5α-THP) compared to pups undergoing their first episode of neonatal isolation (Kehoe et al., 2000). Increased neurosteroid release is also a consequence of exposure to stress in adult animals (Purdy et al., 1991, 1992). These data suggest that neonatal isolation is a stressor and that the magnitude of corticosterone release in response to such a stressor increases with repeated exposure.

Consistent with growing evidence that stress and the concomitant secretion of glucocorticoids facilitates dopaminergic activity (e.g., Piazza and Le Moal, 1998; Piazza et al., 1996), neonatal isolation also produces immediate changes in brain mesolimbic dopamine (DA) systems. Similar to the results for plasma corticosterone and pregnane neurosteroids, after several episodes of neonatal isolation, pups showed greater dopaminergic turnover in the hypothalamus, septum and striatum than pups undergoing their first neonatal isolation episode on PND 9 (Kehoe et al., 1997). No differences were found among groups in basal DA activity. These changes in brain mesolimbic DA systems persist at least until the pups are juveniles. Neonatally isolated (ISO) pups as juveniles continue to show enhanced locomotor activity and greater nucleus accumbens DA levels when challenged pharmacologically with amphetamine (Kehoe et al., 1996b).

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In the present experiments, we examined whether the potentiation of corticosterone release, pregnane neurosteroid metabolism and dopaminergic activity found on PND 9 in response to repeated episodes of isolation would be evident in response to a stressor when the animals were juveniles. Although differences on some of these measures were found in juveniles in response to a pharmacological challenge, these experiments address whether ISO and NH juveniles differ in their response to a restraint stress challenge.

2. Methods

2.1. Subjects

All procedures were conducted in accordance with National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and approved by the Trinity College IACUC. Female and male Sprague–Dawley rats were mated (Charles River Breeding Laboratories, Wilmington, MA) in the Trinity College Psychobiology Laboratory colony, and the offspring of these matings served as subjects. Pregnant females were housed individually in plastic cages with a stainless steel wire lid and allowed free access to Rat Chow (Ralston-Purina, St. Louis, MO) and tap water. Lights in the colony remained on from 7 a.m. to 7 p.m. to establish a 12-h light–dark cycle, and the colony temperature was maintained at 25 °C. Newborn litters born before 5 p.m. on a given day were considered born on that day (Day 0). Litters were culled to 12 on PND 1 with a goal of balancing the number of males and females equally. Each litter was randomly assigned to either the ISO or nonhandled (NH) condition. No more than one pup per sex per litter was used in any of the treatment conditions. All measures from the experimental animals were obtained when the rats were between PND 26 and 30.

2.2. Isolation procedure (PND 2–9)

Beginning on PND 2 through 9, pups designated for isolation were weighed and marked daily with marker pen for identification purposes. Each pup was placed in an individual round plastic container (9 cm diameter × 8 cm deep) with no bedding and remained isolated for 1 h in a humidity-controlled environmental chamber at 30 °C. The chamber produces white noise, which masks the hearing of other pups' calls even in the ultrasonic range as determined by an S200 Bat Detector (Ultrasound Advice, London, UK). Further, plastic containers were spatially separate (approximately 0.5 m apart) to minimize olfactory cues from siblings. At the end of the isolation period, pups were returned to the nest and the dam. Isolations were carried out between 9 a.m. and 12 noon each day. NH control litters were left undisturbed and not handled in any way throughout the treatment period except for weekly cage cleaning. All pups were weaned on PND 25; same-sex animals were housed three per cage.

2.3. Restraint procedure

Juvenile animals (between PND 26 and 30) designated for restraint were placed in a cylindrical Plexiglas tube that greatly inhibited movement. Each animal remained in the restrainer for 60 min and was then placed immediately into the activity-monitoring chamber.

2.4. Locomotor activity testing

In this experiment, juvenile male and female animals between 26 and 30 days of age that had been isolated as neonates were tested for the effects of immobilization stress on locomotor behaviour compared to NH controls. Behavioural testing was conducted over 2 consecutive days. The first day consisted of a 10-min habituation period. The animal was placed in an activity chamber and allowed to move freely while being monitored by an overhanging video recorder. On the following day, all animals were placed in the activity chamber for 30 min either following 60 min of restraint ($n = 29$) or without restraint ($n = 20$). Movement was recorded as distance traveled (in centimeters) by a Colbourn Instruments Video Path Analyzer Activity System and was used for analysis. The activity monitor was programmed to calculate numerical sums at 5-min intervals for the 30-min session.

2.5. In vivo microdialysis

In this experiment, we examined DA levels in the nucleus accumbens before, during and after restraint stress in juvenile male and female animals that had been exposed to neonatal isolation. Juvenile rats (PND 27–30) were anesthetized with a combination of sodium pentobarbitol (25 mg/kg body weight) and chloral hydrate (250 mg/kg body weight). The skin on the scalp was incised and a hole drilled for the insertion of a cannula into the nucleus accumbens. Coordinates for this positioning were determined to be +1.1 mm AP, +2.5 mm ML and −3.8 mm DV from bregma (Sherwood and Timiras, 1970). Once lowered, the cannula was kept in place with both Superglue and dental cement. Following implantation of the cannula, the animal was placed in a cage with clean bedding. The cage was placed on a heating pad, and the temperature was maintained at 30 °C until the animals recovered from anesthesia. The cage was then returned to the colony until in vivo microdialysis was carried out 24 h later. After the 24-h recovery, the rat was placed in the dialysis chamber consisting of a clear Plexiglas bowl (diameter = 40 cm, depth = 35.5 cm) containing bedding from its home cage. The CMA/12 microdialysis probe (CMA/Microdialysis, Acton, MA; cut-off 20,000 Da), which protrudes through the base of the cannula to the nucleus accumbens, was inserted into the cannula. Probes were used once only and had recoveries between 10% and 15%. The

outer diameter of the guide is 0.64 mm, and the outer diameter of the membrane is 0.5 mm. The probe apparatus was attached by PE-10 tubing to a syringe pump (Sage Model 341A), which distributed filtered artificial cerebral spinal fluid (composition: 150 mM Na, 3.0 mM K, 1.4 mM Ca, 0.8 mM Mg, 1.0 mM P, 155 mM Cl) through the probe at a rate of l.67 μl/min. After a 1-h habituation period, three 30-min baseline dialysate collections were taken prior to the restraint. The animal was restrained for 1 h in a specially designed cloth jacket immobilizing the animal's entire body except for the head. Dialysis continued with collections every 30 min during the restraint period and following release from restraint for an additional 1.5 h. Each dialysate sample was collected into a vial containing 10 μl of 0.1 M per-chloric acid.

2.6. Histology of brains for probe placement

To verify probe placement, animals were overdosed with sodium pentobarbitol and then perfused transcardially. After removal of the brain from the skull, a section of the brain around the cannula, approximately 2-mm thick, was removed and placed in a buffered 10% formalin solution. The tissue was viewed under a light microscope, and the location of the probe tip placement was determined. Rats that had microdialysis probes outside of the nucleus accumbens were excluded from the data set.

2.7. Analysis of dialysates for amines

Dialysate samples were analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection (Kehoe et al., 1996a,b, 1998a). The samples were placed in the auto-injection unit of the HPLC system (Gilson). An MD-150 column (ESA; 3 μm, 15 cm × 3.0 mm ID) was used in conjunction with the electrochemical detector (Coulochem II; triple detector electrode system) to separate and oxidize the amines. The first guard detector was set at +350 mV, the second detector at −175 mV and the third at +175 mV. The mobile phase (75 mM $NaH_2PO_4 \cdot H_2O$, 1.4 mM OSA, 10 μM EDTA, 10% acetonitrile, pH 3.1 with H_3PO_4) was delivered via an ESA 580 HPLC pump at a flow rate of 0.6 ml/min. The electrochemical signal was acquired via an ESA 501 chromatography software package for data storage and analysis. Levels of DA were expressed as picograms per 20 μl of dialysate. The last baseline from each animal was used to calculate percent change of DA from baseline during restraint and following release from restraint for statistical analysis. The three baseline DA levels did not differ significantly ($P > .20$).

2.8. Plasma corticosterone and brain 3α,5α-THP in response to restraint

Changes in plasma corticosterone and brain 3α, 5α-THP levels in response to 1 h of restraint was examined in ISO

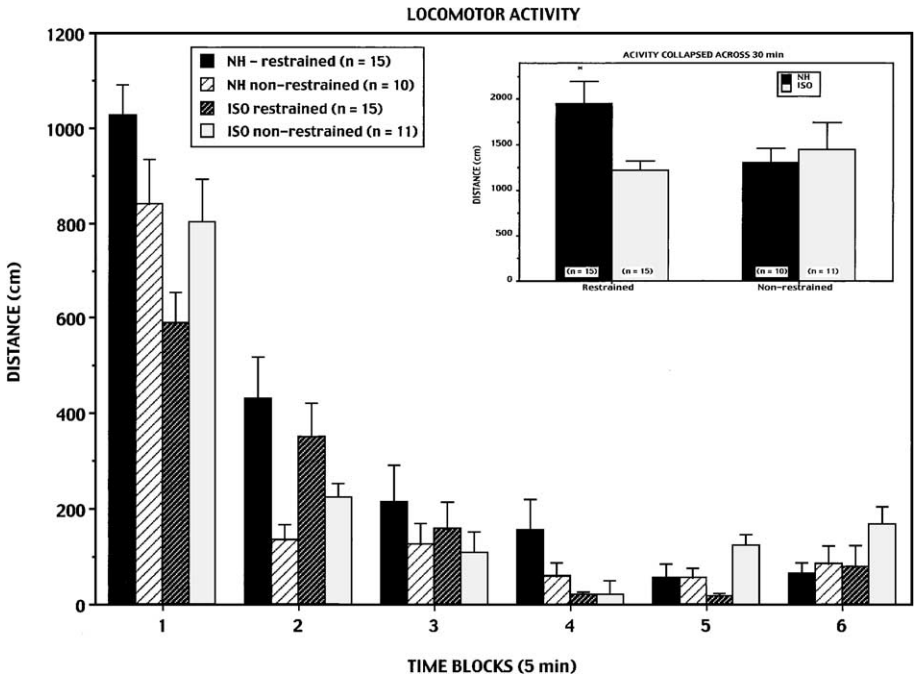

Fig. 1. Mean (±S.E.M.) distances (centimeters) traveled (movement in the horizontal plane as recorded by a video path analyzer) across 5-min blocks of time for nonhandled (NH) and neonatally isolated (ISO) rats. The interaction of condition (ISO vs. NH) and treatment (restrained vs. nonrestrained) was significant [$F(5,85) = 5.54$, $P < .001$]. The inset illustrates the latter interaction (distance traveled collapsed across time blocks). * Higher than all other groups, $P < .05$.

and NH rats between the ages of PND 26 and 29. The restraint experiment was conducted between 9 a.m. and 11 a.m. For determination of prerestraint (basal) corticosterone levels, rats were rapidly decapitated within seconds of removal from the home cage. The restraint procedure was as described above. After 60 min in Plexiglas restrainers, rats were decapitated. For both prerestraint and restrained rats, trunk blood was collected in ice-chilled microcentrifuge tubes containing 20 μl of EDTA. Whole brains were rapidly removed from the skull and stored at −70 °C until assayed for 3α, 5α-THP.

2.9. Corticosterone radioimmunoassay

Trunk blood was spun at 500 × *g* for 10 min. Plasma was collected and stored at −70 °C until time of assay. Plasma corticosterone was measured as previously reported (McCormick and Mahoney, 1999; McCormick et al., 1998a) with a radioimmunoassay using a highly specific corticosterone antiserum (B3-163, Endocrine Sciences, Tarzana, CA) and [^{3}H]corticosterone (88.0 Ci/mmol; New England Nuclear, Boston, MA) as tracer. Measurements were made in duplicate from plasma aliquots of 10 μl. The minimum level of detection with the assay is 15 pg/tube. The antiserum cross-reacts slightly with deoxycorticosterone (approximately 4%), but not with cortisol, aldosterone or progesterone (< 1%). Intra- and interassay variability are consistently < 5% and 10%, respectively.

2.10. 3α, 5α-THP assay

3α, 5α-THP was measured according to previously reported methods (Frye and Bayon, 1999). Briefly, steroids were extracted from homogenized whole brain samples in 50% MeOH, 1% acetic acid through a series of centrifugation and filtrations. A total of 300 μl of 0.1 M phosphate assay buffer (pH = 7.4) was added to test tubes containing steroid extracts and equilibrated. The antibody, purchased from Dr. Robert Purdy (Veterans Medical Affairs, La Jolla, CA), is very specific to 3α, 5α-THP (Finn and Gee, 1994). The 1:5000 dilution of this antibody bound between 40% and 60% of [^{3}H]3α, 5α-THP (NET-1047, 51.3 Ci/mmol; New England Nuclear). The minimum detection limit of the assay was 100 pg. The intra- and interassay coefficients of variance were 12.0% and 15.6%, respectively.

2.11. Statistical analyses

Statistical analyses consisted of between-group analysis of variance (ANOVA) and mixed-factor ANOVA, with sex, condition (NH or ISO) and treatment (restraint or nonrestraint) as independent measures, and time as a repeated measure. Post-hoc tests consisted of *F* tests for simple effects and pairwise comparisons using Fisher's procedure of least-squared differences when appropriate. An alpha level of $P < .05$ was used to determine statistical significance.

3. Results

3.1. Locomotor activity

There was no effect of the isolation procedure on body weight in juveniles. There were no sex differences in any activity scores across conditions, thus, male and female data were collapsed. All groups travelled similar distances during the 10-min habituation session (means ± S.E.M.: NH males = 1008 cm ± 117.0; NH females = 1197 cm ± 155.7; ISO males = 1275 cm ± 144.7; ISO females = 1219 cm ± 134.9). The next day, rats were tested after 60 min of restraint or immediately upon removal from the home cage (nonrestrained). Mixed-factor ANOVA conducted on locomotor activity over six 5-min blocks of time indicated a significant interaction between condition (ISO vs. NH) and treatment (restrained vs. nonrestrained) [$F(5,85) = 5.54$, $P < .001$]. Post-hoc analysis indicated that NH rats increased their activity levels in response to restraint ($P < .05$), whereas

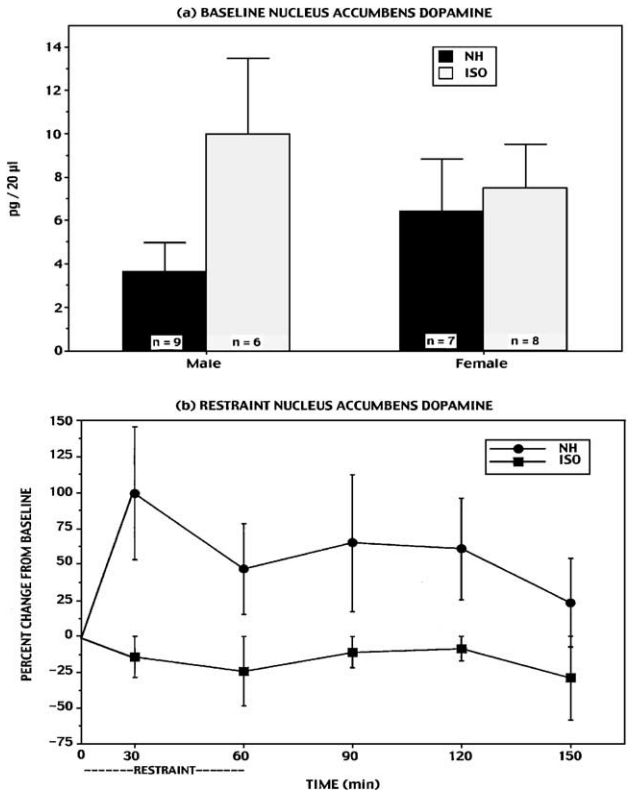

Fig. 2. (a) Mean (± S.E.M.) DA in the nucleus accumbens under baseline conditions for NH and ISO. (b) Mean (± S.E.M.) percent change from baseline levels taken at two intervals (30 and 60 min) during 1 h of restraint and at three intervals after restraint (90, 120 and 150 min). NH rats had a greater percent change in DA levels from baseline than ISO rats ($P = .03$).

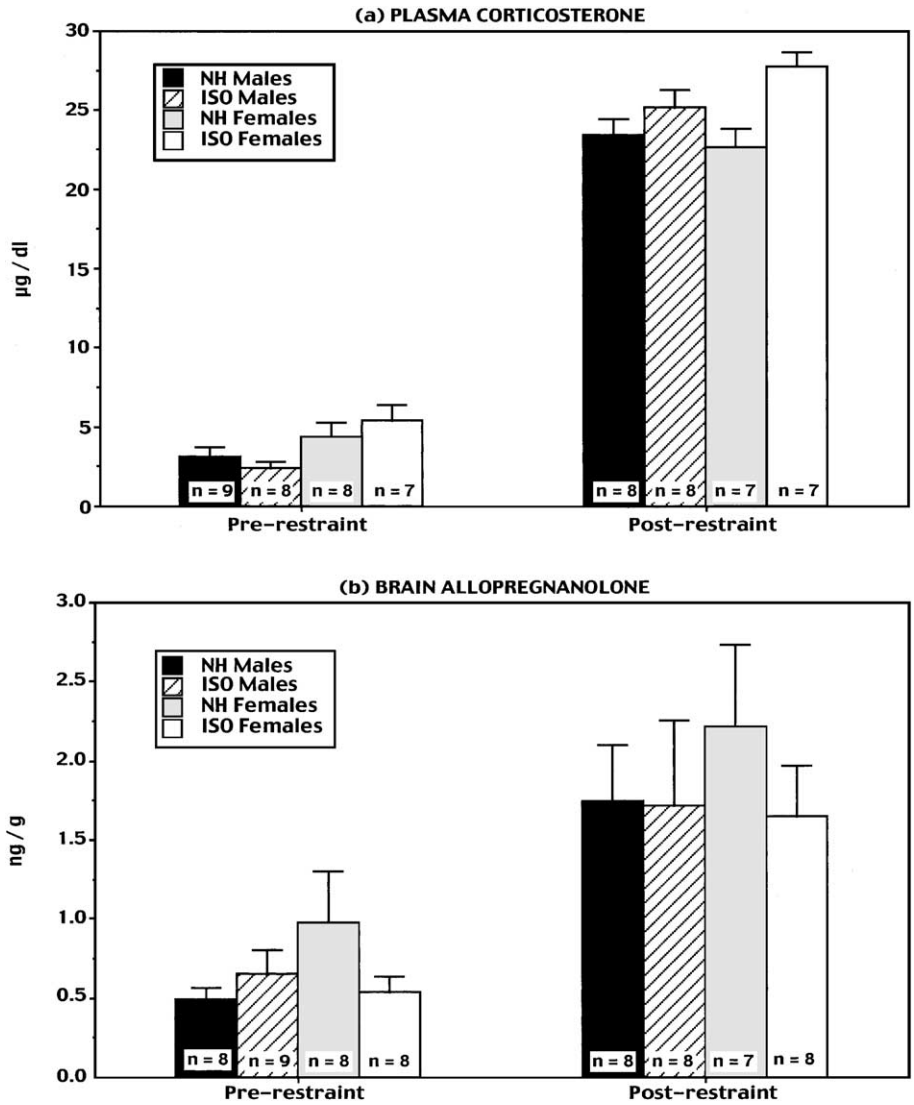

Fig. 3. (a) Mean (±S.E.M.) plasma corticosterone levels immediately upon removal from the home cage (prerestraint) or after 60 min of restraint (postrestraint) for NH and ISO males and females. Females had higher corticosterone levels than males ($P=.02$). ISO rats had higher corticosterone levels than NH rats in postrestraint samples ($P<.05$). (b) Mean (±S.E.M.) prerestraint and postrestraint brain 3α, 5α-THP levels.

ISO rats did not. Fig. 1a shows locomotor activity for ISO and NH for each of the six 5-min blocks, and Fig. 1b shows total locomotor activity for ISO and NH (blocks collapsed).

3.2. In vivo microdialysis

No statistically significant differences were found in baseline DA levels, although ISO had higher values than NH ($P=.11$) (see Fig. 2a). For DA values during and after restraint, data were collapsed for males and females as there were no sex differences ($P=.87$). Mixed-factor ANOVA (Timepoint × Condition) indicated a significant main effect of condition [$F(1,27)=5.25$, $P<.03$], whereas ISO rats showed only a slight drop in DA levels from baseline during 60-min restraint that persisted for 90 min after restraint, DA levels of NH rats increased during 1 h of restraint and remained high for 90 min after restraint (Fig. 2b).

3.3. Plasma corticosterone and brain 3α,5α-THP

ANOVA for plasma corticosterone levels indicated that females had higher levels than males [$F(1,54)=5.58$, $P=.02$] that ISO had higher levels than NH [$F(1,54)=7.60$, $P=.008$], and that prestress levels were lower than poststress levels [$F(1,54)=1036.4$, $P<.0001$]. The interaction of condition and restraint was significant [$F(1,54)=6.22$, $P=.016$]: ISO rats had higher corticosterone levels than NH rats in response to restraint, but did not differ in prerestraint (basal) levels (see Fig. 3a). Brain levels of 3α, 5α-THP increased in response to restraint [$F(1,56)=22.28$, $P<.0001$], but there was no effect of sex or of neonatal condition (see Fig. 3b).

4. Discussion

The present experiments indicate that neonatal isolation alters how juvenile rats respond to a stressor. ISO and NH control rats did not differ in locomotor activity in either the 10-min habituation period or in the 30-min "nonrestraint" condition the following day. Whereas NH rats increased locomotor activity after restraint, ISO rats after restraint were no different in locomotor activity from nonrestrained rats. Apparently, a greater challenge is needed than simply a new environmental surrounding (i.e., habituation in the chamber) to find locomotor activity differences in ISO rats. Similarly, the higher DA levels of ISO juveniles (males in particular) were not statistically significant under baseline conditions. In addition, whereas DA levels in the nucleus accumbens were only slightly lower compared to baseline during and after restraint stress in ISO rats, levels were markedly higher in NH rats. Restraint caused no change in either locomotor activity or DA levels in ISO rats, whereas restraint elevated locomotor activity and DA levels in NH controls. As found in the present experiment with juveniles, adult rats that had been isolated as neonates do not increase locomotor activity following restraint as do control rats (Kehoe et al., 1998b). Further, pilot work from our laboratory (Shoemaker et al., 1998) suggests that, as adults, ISO rats decrease DA release in response to restraint as we found in juveniles. Previous research has indicated increased DA release in the striatum and nucleus accumbens in response to stress in adult rats as we have found for NH juvenile rats (e.g., Abercrombie et al., 1989).

In juvenile rats, we previously found no difference between ISO and NH in locomotor activity or in DA levels in the nucleus accumbens in response a saline injection (Kehoe et al., 1996b). However, ISO juveniles had increased locomotor activity and increased DA release in the nucleus accumbens compared to NH juveniles in response to an amphetamine injection (Kehoe et al., 1996b). In combination with the results of the present study, these data suggest that changes to mesolimbic DA systems are a consequence of neonatal isolation. However, the direction of differences between ISO and NH juveniles depends on the specific challenge; that is, amphetamine that has direct effects on mesolimbic DA or restraint stress that involves glucocorticoid-mediated effects on mesolimbic DA. It also likely depends on developmental stage. When challenged with amphetamine a day after the last episode of neonatal isolation (Day 10 of life), pups showed sensitization of the mesolimbic DA system (Kehoe et al., 1998a). Developmental stage may also account for the lack of sex differences in our DA measures in juvenile rats. DA receptors are in flux over the peripubertal period (Andersen et al., 2000; Teicher et al., 1995), with males and females exhibiting a different pattern of development (Andersen and Teicher, 2000; Andersen et al., 1997). Sex differences are thus more readily apparent postpuberty. We have found that the pattern of sex-specific effects of neonatal isolation on hippocampal electrophysiology is different in juvenile rats than in adult rats (Bronzino et al., 1996; Kehoe and Bronzino, 1999), which also highlights the importance of developmental stage.

Consistent with our previous research showing potentiation of corticosterone release in neonatal rats following repeated episodes of isolation (McCormick et al., 1998b), juvenile rats that had undergone neonatal isolation showed increased corticosterone release in response to 1 h of restraint stress compared to control (NH) rats. Pups that were exposed to higher corticosterone levels as neonates due to the addition of corticosterone to dams' drinking water during the first postnatal week also showed moderate elevations in corticosterone release in response to stress as juveniles (McCormick et al., 2001). Prenatal stress also causes long-term changes in hypothalamic–pituitary–adrenal (HPA) function (McCormick et al., 1995), and produces changes in DA receptors in the nucleus accumbens and sensitization to amphetamine (Henry et al., 1995). These data suggest the possibility that changes in the HPA axis may underlie the changes in mesolimbic DA systems in ISO rats. There is increasing evidence that dopaminergic activity in the nucleus accumbens is dependent upon stress and glucocorticoids (Barrot et al., 2000; Nakahara et al., 2000; Rouge-Pont et al., 1998). For example, in adult animals, stressors have been found to cause sensitization to the effects of amphetamine (Diaz-Otanez et al., 1997). In turn, dopaminergic drugs can activate the HPA axis (Swerdlow et al., 1993). Neonatal isolation also enhances acquisition of cocaine self-administration in adult rats (Kosten et al., 2000), which further suggests alteration of the mesolimbic DA system and possibly altered glucocorticoid control of the mesolimbic system. This possibility does not preclude that other changes in neurochemistry may be involved, such as in noradrenergic or serotonergic systems (e.g., Liu et al., 2000; Matthews et al., 2001).

Increased neurosteroid release is a consequence of exposure to stress in adult animals (Purdy et al., 1991, 1992). In addition, neurosteroids can modulate HPA function (Patchev et al., 1994) and can regulate mesolimbic DA (Grobin et al., 1992). 3α, 5α-THP is metabolized from progesterone by 5α-reductase (Karavolas and Herf, 1971; Karavolas and Hodges, 1990), and is synthesized de novo by glial cells (Jung-Testas et al., 1989). After repeated episodes of isolation, neonates showed much higher brain levels of 3α, 5α-THP in response to isolation than did control pups undergoing their first episode of isolation (Kehoe et al., 2000). The present experiment found that 3α, 5α-THP levels in the brain increased in response to restraint stress in juveniles, but levels were not different between ISO and NH rats. It may be that differences would emerge between the groups in discrete brain areas or with other stress paradigms. Alternatively, it may be that the effects of neonatal isolation on 3α, 5α-THP are short-lived, or that isolation-induced elevations in neonatal 3α, 5α-THP produces long-term changes in substrates for neurosteroid

actions. Maternal separation and neonatal handling have been shown to alter the development of the $GABA_A$ receptor systems, (Caldji et al., 2000), and 3α, 5α-THP is an effective modulator of $GABA_A$ receptor complexes (Majewska et al., 1986).

However, we must be cautious in comparing results with our neonatal isolation paradigm to other paradigms involving separation of pups from dams. Maternal separation procedures vary in the literature as to the length of, and number of, episodes of separation and ages at which the separation occurs. The paradigms have been loosely grouped as either "early handling" involving short (<30 min), but typically repeated, episodes or, "maternal separation" involving longer (1–24 h) episodes that occur once or several times (Lehmann and Feldon, 2000). Duration of episode has been shown to be a critical factor in long-term effects. For example, short bouts (15 min) dampened HPA stress responsiveness and longer bouts (3 h) increased HPA stress responsiveness (Plotsky and Meaney, 1993). As mentioned above, short bouts as in the present experiment (1 h) increased DA release to injections of amphetamine later in life (Kehoe et al., 1996b) and longer bouts (6 h) decreased DA release in response to amphetamine (Hall et al., 1999). Further, the procedures also vary in ways that are secondary to the dam/pup separation but that are also important variables such as temperature of the environment in which separated pups are kept, whether the pups are isolated or in proximity of cues from siblings, and the familiarity of the environment during separation (reviewed in McCormick et al., 1998b). Maternal separation is often without observable effect unless pups are separated from their dams for extended periods — as much as 24 h (Levine et al., 1992; Rosenfeld et al., 1992). When pups are isolated from siblings and/or other familiar stimuli in a maternal separation procedure, effects are observed over much shorter time periods; from 5 min to an hour, depending on the neurochemical or behavioural variable concerned (Carden et al., 1996; Hennessy, 1997; Kehoe et al., 1996a; Shoemaker and Kehoe, 1995). For example, in a variety of maternal separation/maternal deprivation paradigms, pups separated from dams, but in contact with cues from siblings, do not show elevated plasma corticosterone levels unless the separation persists beyond 2 (Kuhn et al., 1990; Pihoker et al., 1993) to 8 h (Levine et al., 1992; Stanton et al., 1988). In contrast, we found significant elevations in corticosterone levels within 30 min in our neonatal isolation paradigm in which pups are also deprived of familiar cues from siblings (McCormick et al., 1998b). Thus, inconsistencies in the long-term biobehavioural effects in the literature may be due to such procedural differences across paradigms (Lehmann and Feldon, 2000). And, when somewhat different paradigms produce similar effects, it may be due to a common mechanism; for example, alterations in subsequent maternal–pups interactions (Liu et al., 1997; Smotherman, 1983; Wilkins et al., 1997). Alternatively, different mechanisms across paradigms may be affecting the same underlying developing substrates (McCormick et al., 2001).

We have previously argued that our isolation paradigm may be viewed as a "psychological" stressor (McCormick et al., 1998b); a stressor that is dependent upon lack of familiar cues, and thus different from both "early handling" and "maternal separation" paradigms. Maternal separation is different than both neonatal isolation and early handling in that it may be more of a "physiological" stressor due to the lengthy disruptions of maternal regulatory influences (Hofer, 1994). We used the following evidence for our designations of "psychological" versus "physiological." Firstly, brief episodes of isolation do not affect weight and growth even when the episodes are repeated over several days (Kehoe et al., 1996b), whereas long episodes of maternal separation do (Zimmerberg and Shartrand, 1992). Secondly, the effects of brief periods of isolation (5 min) on analgesia were attenuated by the presence of familiar olfactory cues in the isolation chamber (Kehoe and Blass, 1986), whereas the effects of long bouts (4–24 h) of maternal separation occur *despite* the presence of familiar cues including the home cage and siblings (Matthews et al., 1996; Pihoker et al., 1993; Suchecki et al., 1995; Walker et al., 1991). Thirdly, some of the effects of long bouts of maternal separation on the HPA axis were prevented by mimicking aspects of maternal regulation of physiology (i.e., feeding or anogenital stimulation) (Suchecki et al., 1993). Fourthly, the elevation in corticosterone levels induced by a short period (30 min) of isolation of a pup in a novel cage was attenuated by including the mother, either conscious or anesthetized (thereby limiting her regulatory influence but keeping her sensory stimuli), in the cage (Hennessy and Weinberg, 1990).

Overall, the present data indicate that the changes produced by repeated, brief episodes of neonatal isolation are enduring. We previously suggested that both alterations in subsequent maternal interactions and altered HPA function as neonates may be underlying mechanisms of the long-term effects of neonatal isolation (McCormick et al., 1998b, 2001). In juveniles, as we previously found for neonates, changes in locomotor activity, mesolimbic DA and plasma corticosterone levels are most evident when the animal is confronted with a stressor. Differences between ISO and control animals are less evident under baseline conditions. In other research, we have found differences between ISO and NH animals to emerge in response to pharmacological stimulation in neonates, juveniles and adults (Kehoe et al., 1996a,b, 1998b). Whether or not the pattern of differences observed are specific to duration, intensity or type of stressor is unknown, but these parameters are likely important (e.g., Antelman et al., 1991; Carlson et al., 1991; D'Aquila et al., 2000), as is developmental stage. Even in adult animals, a single exposure to a stressor or to a pharmacological challenge can have long-lasting consequences (e.g., Vanderschuren et al., 1999). Thus, neonatal isolation likely involves a cascade of effects, the precise

course of which depends on the environmental events the animal encounters over the life span.

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