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Locomotor activity to nicotine and Fos immunoreactivity in the paraventricular nucleus of the hypothalamus in adolescent socially-stressed rats

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Abstract

We reported previously that social stressors in adolescence (SS: one-hour isolation and new cage partners daily for 16 days) increased locomotor activity to nicotine and to amphetamine in females, but not in males, when tested as adults. Here, we investigated whether effects of stressors in adolescence on locomotor responses to nicotine would be observed in both sexes if tested closer in time to the stressor exposure. We also tested whether social instability was necessary to alter nicotine′s effects on locomotor activity by including a group that underwent daily isolation but was housed with the same partner (ISO). The locomotor-activating effects of nicotine were lower in SS rats compared to ISO and non-stressed control rats. In males, but not in females, there were effects of nicotine treatment and of stress condition on Fos immunoreactive (Fos-ir) cell counts in the paraventricular nucleus (PVN) of the hypothalamus: SS males had higher Fos-ir counts than did ISO and non-stressed control males, and higher Fos-ir counts in the PVN were found in repeated-nicotine groups than in acute-nicotine and saline groups. These results add to evidence that adolescents are uniquely vulnerable to stressors due to ongoing brain development, and also indicate that effects are sex- and stressor-specific.

Keywords: Adolescence; Chronic stress; Sex differences; Locomotor activity; Nicotine; Locomotor sensitization; c-*fos*

1. Introduction

There is increasing evidence to suggest that vulnerability to drug abuse is more readily established in adolescence than in adulthood based on investigations in rodent models (Adriani et al., 2003; Elliott et al., 2005; Trauth et al., 2000). It is well-known that the behavioural and neurochemical effects of drugs differ markedly between adolescents and adults (e.g., Barron et al., 2005; Cruz et al., 2005; Faraday et al., 2001; Schochet et al., 2005; Shram et al., 2006; Vastola et al., 2002), and that exposure to drugs of abuse can lead to relatively permanent changes in the brain that may increase vulnerability in adulthood to psychopathology (Abreu-Villaca et al., 2003; Liu et al., 2005; McDonald et al., 2005; Slawecki et al., 2005). An important factor in the vulnerability of adolescents is that neural systems associated with the effects of drugs of abuse are continuing to develop over adolescence (reviewed in Spear, 2000b).

In people, stressful life events are important precursors to drug use in adolescence (Bruns and Geist, 1984; Hoffmann et al., 2000). Glucocorticoid hormones, which are released by activation of the hypothalamic–pituitary–adrenal axis when a stressor is perceived, have been implicated in the development of drug abuse (Marinelli and Piazza, 2002). For example, glucocorticoids increase the release of mesocorticolimbic dopamine (Barrott et al., 2000; Kalivas and Duffy, 1995), and the release of mesocorticolimbic dopamine is critical for the locomotor-stimulating and rewarding effects of psychostimulants (Vezina, 2004). In early life, glucocorticoid hormones are known to shape ongoing brain development and have "programming" effects on many brain functions (Meaney et al., 2002; Owen et al., 2005; Seckl, 2001). Glucocorticoids can thus impact brain function long after their stressor-induced

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release. However, there has been little research using animal models on the role of stressors in adolescence on risk for drug abuse, and yet adolescents typically have more prolonged release of glucocorticoids in response to a stressor than adults (reviewed in McCormick and Mathews, in press).

We recently reported that chronic exposure to social stressors over mid-adolescence (daily one-hour isolation and daily pairings with new cage partners for 16 days) increased locomotor responses to nicotine and to amphetamine in females, but not in males, when tested as adults several weeks after the stressor exposure (McCormick et al., 2004, 2005). Kabbaj et al. (2002) found that adolescent males had decreased behavioral sensitization to amphetamine when tested immediately after exposure to social stressors administered from age 28 to 56 (2 h daily: either isolation, novel environment, crowding, litter-shifting, subordination). Thus, it may be that adolescent social stressors affect behavioural responses to drugs of abuse, but that the effects dissipate with time in males. One of the aims of the present experiment was to investigate whether greater effects of our stressor procedure on locomotor sensitization to nicotine would be observed in both sexes if rats were tested closer in time to the stressor exposure in adolescence.

A second aim was to test the extent to which the social instability involved in our adolescent social stress procedure was necessary to alter behavioural responses to drugs in females and/or whether it served to protect males from the stress of daily isolation (i.e., change of cage mates as an "enriched environment" that possibly counters the effect of isolation). This was accomplished by including a second adolescent stressor group in which rats underwent daily one-hour isolation for 16 days but always were returned to the same cage and partner. We have found that repeated daily isolation leads to a different pattern of neuroendocrine activation when it also involves the social instability of daily change of cage partner (our social stress procedure: SS) than when it involves isolation only (ISO). ISO males and females showed more evidence of habituation to isolation than did SS males and females as indicated by plasma corticosterone and corticosteroid binding globulin levels after the 16th episode of isolation (McCormick et al., 2007). Therefore, we hypothesized that ISO and SS rats would also differ in locomotor sensitization to nicotine, with SS more likely to differ from controls than would ISO. If this hypothesis was confirmed, it would suggest that social instability is a critical factor in the effects of our stressor procedure on behavioural responses to nicotine.

Although a depressant effect on locomotor activity is often reported to a first injection of nicotine (e.g., Clarke and Kumar, 1983; Kanyt et al., 1999), progressive increases in locomotor activity are found to repeated injection of nicotine as are found to repeated injection of psychostimulants. The locomotor-activating and rewarding effects of nicotine are mediated primarily by increasing mesocorticolimbic dopamine function (Balfour et al., 2000; Clarke et al., 1988; Di Chiara, 2000; Omelchenko and Sesack, 2006). Nicotine also acts on other systems, notably the hypothalamic–pituitary–adrenal axis. Nicotine increases the expression of the immediate early gene c-*fos*, an indirect marker of neuronal activation, in many neural regions including the parvocellular paraventricular nucleus (pPVN) of the hypothalamus, the main neural regulator of hypothalamic–pituitary–adrenal function (e.g., Matta et al., 1993; Salminen et al., 2000; Schochet et al., 2005; Trauth et al., 2000). The pPVN integrates various neural inputs to determine the release of corticotropin releasing hormone (CRH) into hypophyseal portal veins, which leads to the release of ACTH from the anterior pituitary, and ultimately to the release of glucocorticoids from the adrenal cortex into general circulation. Nicotine, by increasing the release of CRH from the pPVN, increases circulating glucocorticoid levels (e.g., Cam and Bassett, 1984; Matta et al., 1998; Okada et al., 2003). We have evidence that our adolescent stressor procedure affects central levels of the HPA axis in addition to the peripheral effects described earlier. On the last day of stress procedures (45 days of age), both SS and ISO males and females had elevated baseline expression of CRH mRNA in the PVN of the hypothalamus compared to controls (McCormick et al., 2007). Further, compared to baseline (pre-isolation), the 16th episode of isolation increased expression of CRH mRNA in the central nucleus of the amygdala in SS males and not in ISO males. The central nucleus of the amygdala is involved in initiating HPA responses to stress via projections to the pPVN (Herman et al., 2005; Schulkin et al., 1998). Thus, it is possible that the effect of nicotine on the pPVN would differ in adolescent-stressed rats compared to control rats. Differential sensitivity of the HPA axis to nicotine may be related to group differences in the locomotor-stimulating effects of nicotine: Glucocorticoids augment locomotor sensitization to nicotine (e.g., Caggiula et al., 1998; Johnson et al., 1995; Kita et al., 1999). The third aim of the present experiment was to determine whether differences would be observed between SS, ISO, and controls in the pPVN in response to repeated or acute treatment with nicotine or saline by examining Fos immunoreactivity in the pPVN. Fos expression is an indirect measure of neuronal activation, and thus increased expression of c-*fos* in the pPVN in response to nicotine would suggest greater activation of the HPA axis.

2. Methods

2.1. Animals

Long-Evans rats were obtained at 22 days of age (Charles River, St. Constance QC) and were housed in same-sex pairs. Individuals within a cage were identified by tail markings made with a felt marker. Rats were kept on a twelve-hour light/dark cycle with lights on at 0800 h and with free access to food and water. Use of animals in this experiment was in adherence to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), and approved by the Brock University ACUC.

2.2. Stress conditions

Rats were randomly assigned to either the adolescent social stress (SS), isolation stress (ISO) or no stress control (CTL) conditions. Stress regimens began after one week of acclimation to the colony. The SS and ISO procedures both involved

Table 1
Experimental design and procedures

	Stress manipulation	Behavioural testing													
Age	30–45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
Procedures	Daily stress (ISO or SS) or no-stress controls. Half of each group began testing on day 46 and half on day 47	H	NT	LT 1	NT	LT2	NT	LT3	NT	LT4	NT	LT5	NT	C	
		NT	H	NT	LT1	NT	LT2	NT	LT3	NT	LT4	NT	LT5	NT	C

ISO: 1 h of isolation then returned to cage and original partner.
SS: 1 h of isolation then returned to new cage and new partner.
H: Habituation for 1 h to locomotor test chamber.
LT: Locomotor testing for 1 h after injection of either nicotine or saline (see Table 2).
C: Challenge day. Locomotor testing for 1 h after injection of either nicotine or saline (half of rats previously treated with saline receive a first dose of nicotine).
NT: No behavioural testing and no injection.

individual isolation of rats in ventilated, round plastic containers (approx. 10 cm in diameter, 10 cm in height) in a room separate from the colony for 1 h each of days 30 through 45 of age, which encompasses mid-adolescence for male and female rats (see reviews by McCormick and Mathews, in press; Spear, 2000b; Tirelli et al., 2003). Upon each return to the colony, SS rats were housed with an unfamiliar cage partner that also was undergoing the stress regimen, whereas ISO rats were returned to their original cage and partner. The one-hour isolation was carried out during the light phase of the diurnal cycle. Rats in the no stress (control) condition were not disturbed except for regular cage maintenance and to be weighed. All rats were weighed at day 30 and at day 45 of age to examine the effect of the stress procedure on weight gain.

2.3. Locomotor activity testing

Testing began for half the animals on day 46 of age (1 day after the last day of the stress procedures) or on day 47 of age. Rats were tested for locomotor activity in an open field made of black plastic acrylic and divided into four 58×58×58 cm chambers, thereby allowing four rats to be tested simultaneously. A colour video camera (Sony Instruments) mounted above the centre of the box was connected to a computer tracking system (SMART; San Diego Instruments, San Diego CA) which measured distance travelled (cm) in each chamber.

Locomotor testing was conducted between 0900 h and 1500 h (during the light phase) under red light illumination. Rats were tested on 7 days over a 14 day period (tested on alternate days) in batches of four. Injections were administered only on test days. Order of testing was randomized except that males and females were never included in the same batch (i.e., run in separate groups of four on the same day), and cage partners were run within the same batch. Cage partners were in the same drug treatment group. Chambers were cleaned with 50% ethanol between batches. The first day consisted of 1 h of habituation to the apparatus after a SC injection of saline (1 ml/kg). For the next five test days, rats were given either SC injections of 0.5 mg/kg nicotine (nicotine bitartrate, doses calculated as a base, Sigma Aldrich Canada) or saline (1 ml/kg) immediately before being placed in the test apparatus for 1 h. The 7th time in the apparatus was a challenge test in which half of the rats treated with saline only over the test days were administered 0.5 mg/kg nicotine immediately before being placed in the test apparatus for 1 h. See Table 1 for the timeline of stress manipulations and of behavioural testing and Table 2 for experimental groups and sample sizes.

2.4. Immunohistochemistry

Two hours after the last injection (which included 1 h in the locomotor test chamber and 1 h in home cage), rats were deeply anesthetized and transcardially perfused with physiological saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were placed in a 30% sucrose and paraformaldehyde solution until they sank. Coronal sections (40 μm thick) were collected throughout the hypothalamus using a cryostat (Thermo-Shandon) and stored in cryoprotectant at −80 °C until the immunohistochemistry procedure could be performed according to adapted protocols on a subset of animals (n=5–7 per group). Every sixth section was collected for immunohistochemistry.

Sections were washed stringently in 0.01 M phosphate buffered saline (PBS; pH 7.4), then in PBS with 0.2% Triton-X (PBSx) with 0.3% hydrogen peroxide, and then again in PBSx. Next, the tissues were incubated for 48 h at 4 °C in 1% normal goat serum (Sigma) and Fos rabbit polyclonal primary antibody diluted at 1:2000 (Santa Cruz Biotechnology Inc.). After incubation, the sections were washed three times in PBSx before incubation for 2 h in biotinylated anti-rabbit immunoglobulin secondary antibody (Vector Laboratories) diluted at 1:500. The sections were again washed in PBSx and placed for 1 h in Avidin–Biotin Complex (Vector Laboratories). After another three washes in PBSx, tissues were placed in diaminobenzidine and nickel solutions according to the instructions on the substrate kit (DAB SK-4100, Vector Laboratories) for 5 min. Immunostained

Table 2
Sample size for each of the experimental groups over the locomotor test days

	Controls		Adolescent social stress		Isolation stress	
	CTL		SS		ISO	
	Males	Females	Males	Females	Males	Females
Saline[a]	n=12	n=12	n=16	n=16*	n=12	n=12*
Nicotine	n=8	n=8	n=10	n=10*	n=8	n=8

* n reduced by 1 in each of these 3 groups due to technical problems.
[a] Half of each saline group is administered nicotine on the challenge day, which occurs 48 h after the 5 sensitization test days.

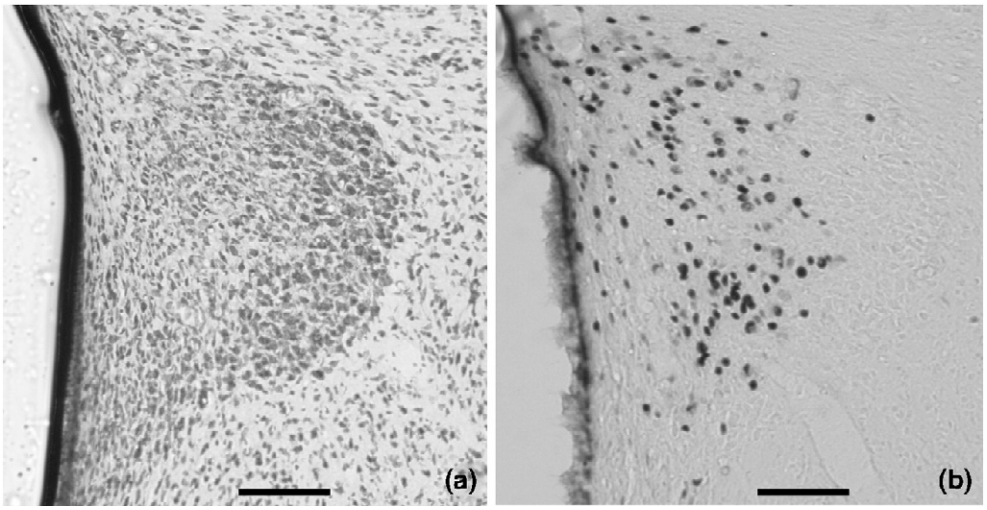

Fig. 1. Adjacent coronal sections through the paraventricular nucleus stained for (a) Nissl bodies or (b) Fos-immunoreactivity and photographed at 100× magnification. —=100 μm.

sections were mounted onto slides, dried, and coverslipped with Permount. Sections within approximately the coordinates from bregma −1.08 mm and −1.92 mm according to Paxinos and Watson (Paxinos and Watson, 2005) were photographed at 100× and 400× magnification with a Nikon Eclipse brightfield microscope (see Fig. 1 for examples of the immunostained sections). Fos-immunoreactive (ir) cell counts were made in a 250×250 μm area located adjacent to the third ventricle representing the medioparvocellular PVN in each hemisphere. No Fos-ir was found in the controls for immunostaining.

2.5. Statistical analyses

Statistical analyses consisted of repeated measure and between-group factor analyses of variance (ANOVA). *F* tests for simple effects and Fisher's Protected Least Square Differences (for between group differences) and Bonferonni-corrected paired *t*-tests (for repeated measures) were used for *post hoc* analysis where appropriate. Alpha level to determine statistical significance was $p<0.05$. However, because of the limits on statistical power due to the number of factors in the design and the corresponding loss of degrees of freedom, post hoc analyses were conducted for statistical interactions that failed to meet this criterion of statistical significance to test a priori hypotheses regarding the main factor of interest, that of Stress Condition.

3. Results

3.1. Weight

A Stress Condition×Sex×Age ANOVA of weight on first and last day of the stress procedures found a significant three-way interaction [$F(1, 126)=4.84$, $p=0.01$ (greenhouse-geisser corrected epsilon=1.0)] (see Fig. 2). Post hoc analysis indicated that there was no effect of Stress Condition at 30 days of age or at 45 days of age for females (which weighed less than males at both ages, $p<0.001$), whereas for males, the three Stress Conditions weighed the same at 30 days of age, but 45 days of age, control males weighed more than ISO males ($p=0.002$) and than SS males ($p=0.03$).

3.2. Habituation

For locomotor activity during habituation to the chamber, Stress Condition×Drug Treatment×Sex ANOVA found that

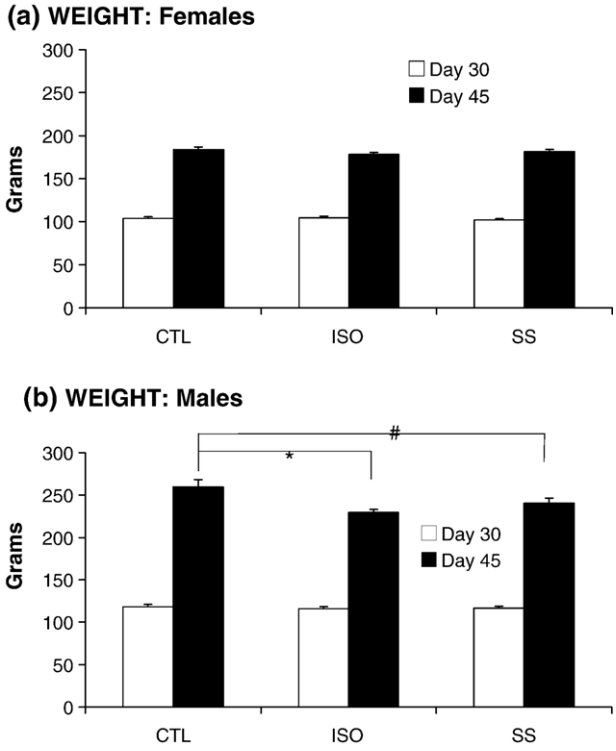

Fig. 2. Mean (S.E.M.) weight for females (a) and males (b) on the first and last day of the stress procedure. CTL: controls; ISO: rats returned to their original partner and cage after daily isolation; SS: rats returned to a new partner and cage after daily isolation. * $p=0.002$; # $p=0.03$.

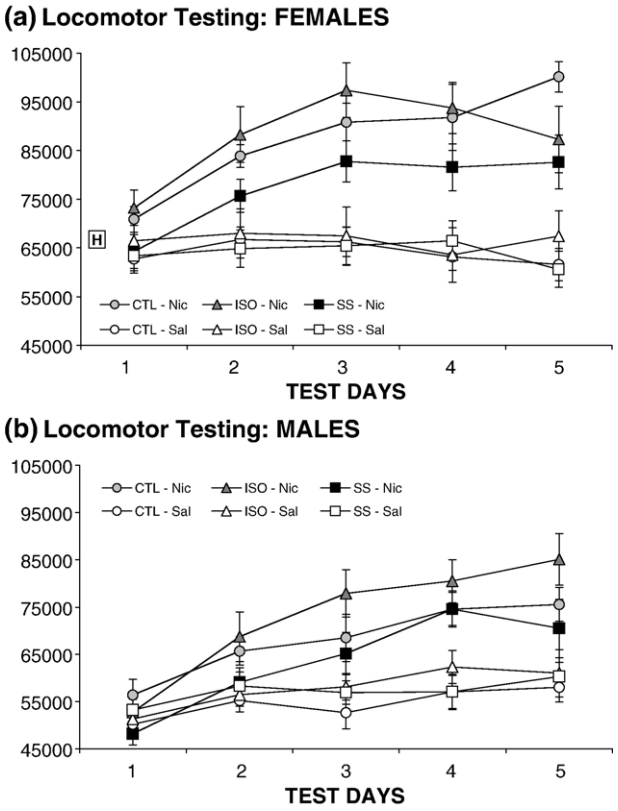

Fig. 3. Mean (S.E.M.) locomotor activity in females (a) and males (b) over the 5 days of treatment with either nicotine or saline. CTL: controls; ISO: rats returned to their original partner and cage after daily isolation; SS: rats returned to a new partner and cage after daily isolation. H indicates the mean locomotor activity of the group on the day of habituation. For Nicotine groups: Locomotor activity increased across test days ($p<0.0001$); locomotor activity was higher in females than in males ($p<0.001$); and SS rats had lower levels of locomotor activity than ISO ($p=0.003$) and than CTL ($p=0.02$). The interaction of Sex and of Stress Condition was not significant. For Saline groups: Test Day and Sex interaction ($p<0.0001$) based on an effect of Test Days in males only: Locomotor activity was higher on days 2 and day 5 of testing than on day 1 in males ($p<0.01$).

females had higher levels of activity than males, but no other factor or interaction among factors was significant (mean activity for males and females is in Fig. 3a–d).

3.3. Locomotor activity over test days

For locomotor activity over the 5 days of repeated nicotine or saline treatment, a Stress Conditio × Drug Treatment × Sex × Test Day ANOVA determined a near significant four-way interaction [$F(8, 468)=1.94$, $p=0.052$ (greenhouse-geisser corrected epsilon=0.82)]. To explore the primarily variable of interest, Stress Condition, and because Stress Condition differences were predicted for nicotine-treated rats and not for the saline-treated rats, subsequent analyses were conducted separately for Nicotine and Saline treatments after verifying that there was the expected difference between locomotor activity after nicotine treatment compared to after saline treatment: There was a significant interaction between Drug Treatment and Test Day [$F(4, 124)=31.2$, $p<0.0001$], with the higher locomotor activity to nicotine than to saline significant on every day ($p<0.0001$) except the first test day.

For Nicotine-treated rats, a Stress Condition × Sex × Test Day ANOVA found significant main effects of all three factors, and no significant interactions (see Fig. 3a–b). Locomotor activity increased across Test Days [$F(4, 180)=53.21$, $p<0.0001$ (greenhouse-geisser corrected epsilon=0.75)] and females had higher levels of locomotor activity than males [$F(1, 45)=32.07$, $p<0.001$]. Post hoc analysis of the effect of Stress Condition [$F(2, 45)=5.01$, $p=0.01$] found that SS rats had lower levels of locomotor activity than ISO ($p=0.003$) and than CTL ($p=0.02$), and that ISO and CTL did not differ significantly. Effects of Stress Condition persisted when separate analyses were conducted for females, but not when conducted for males.

For Saline-treated rats, a Stress Condition × Sex × Test Day ANOVA found a significant interaction between Test Day and Sex [$F(4, 288)=6.09$, $p<0.0001$ (greenhouse-geisser corrected epsilon=0.85)] (see Fig. 3c–d). F tests for simple effects found a significant effect of Test Days in males only [$F(4, 156)=8.71$,

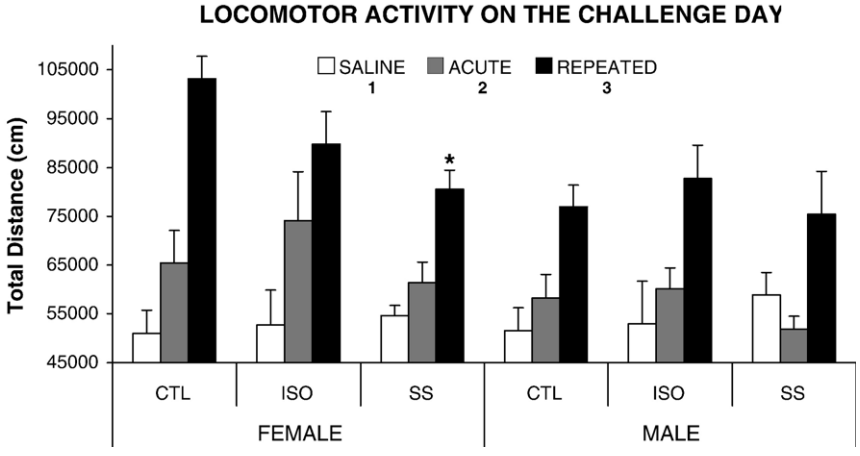

Fig. 4. Mean (S.E.M.) locomotor activity on the challenge day of testing in females and males receiving either saline[1], acute-nicotine[2], or repeated-nicotine[3] treatment. CTL: controls; ISO: rats returned to their original partner and cage after daily isolation; SS: rats returned to a new partner and cage after daily isolation. Females > Males ($p<0.0001$). 1<2 ($p=.058$; 1<3 ($p<0.0001$); 2<3 ($p<0.0001$). * For repeated-nicotine treatment, SS females < CTL females ($p=0.01$).

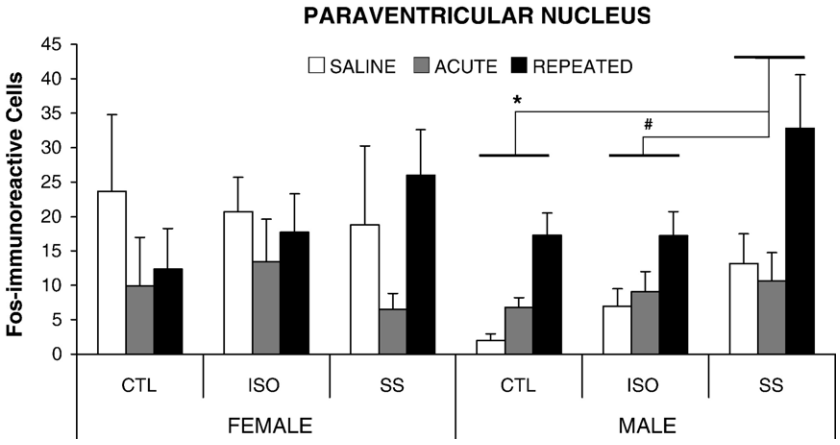

Fig. 5. Mean (S.E.M.) number of Fos-labelled immunoreactive cells in the paraventricular nucleus (pPVN) of the hypothalamus in females and males receiving either repeated-nicotine, acute-nicotine, or saline treatment. CTL: controls; ISO: rats returned to their original partner and cage after daily isolation; SS: rats returned to a new partner and cage after daily isolation (n=5–7 per group). For females, none of the main effects or interactions was significant. For males, SS higher than CTL (p=0.003) and than ISO (p=0.02), and repeated-nicotine treatment higher than did acute-nicotine and saline treatments (both p<0.0001).

p<0.0001 (greenhouse-geisser corrected epsilon=0.78)]: Locomotor activity was higher on days 2 and day 5 of testing than on day 1 (p<0.01).

3.4. Challenge day

For locomotor activity on the challenge day, a Stress Condition×Drug Treatment×Sex ANOVA indicated that males had lower activity than females [F(1, 111)=6.34, p=0.01] and that there was sensitization to nicotine [F(2, 111)=47.63, p<0.0001] (see Fig. 4): Post hoc analysis indicated that repeated-nicotine-treated rats had higher activity than acute-nicotine-treated rats (p<0.0001) and than saline-treated rats (p<0.0001), whereas the higher activity of acute-treated rats compared to saline-treated rats only approached statistical significance (p<0.058). Although the interactions of Sex×Drug Treatment [F(2, 111)=2.60, p=0.08] and Stress Condition×Drug Treatment [F(4, 111)=1.66, p=0.16] failed to meet statistical significance, to explore our a priori hypothesis of an effect of Stress Condition, post hoc one-way ANOVAs were conducted for each Sex and Drug Treatment condition separately to test for differences that may not be detectable in an analysis involving a large number of comparisons. SS females differed from CTL females only in the repeated nicotine condition (SS<CTL, p=0.01). For males, there were no differences among the Stress Conditions for any of the Drug Treatment conditions.

3.5. Fos immunoreactivity in the parvocellular paraventricular nucleus (pPVN)

Stress Condition×Drug Treatment ANOVAs of number of Fos-labelled immunoreactive cells in the pPVN were conducted for each sex separately. For females, none of the main effects or interactions was significant. For males, both the effects of Stress Condition [F(2, 43)=4.54, p=0.02] and of Drug Treatment [F(2, 43)=10.96, p<0.0001] were significant: SS had higher numbers of Fos-labelled cells in the pPVN than did CTL (p=0.003) and than did ISO (p=0.02). The repeated nicotine condition had higher numbers of Fos-labelled cells in the pPVN than did the acute nicotine and saline conditions (both p<0.0001) (Fig. 5).

4. Discussion

4.1. Overview

In brief, the effects of stressors in adolescence that did or did not include social instability were investigated for two neural systems; the mesolimbic dopamine system as indicated by the locomotor-activating effects of nicotine, and the hypothalamic–pituitary–adrenal axis, as indicated by nicotine-induced Fos-immunoreactive cell counts in the parvocellular paraventricular nucleus (pPVN) of the hypothalamus. Both stress procedures resulted in reduced weight gain in males, but there was no effect of the stressors on weight in females. The locomotor-activating effects of nicotine were reduced by social instability, particularly in females. The expression of Fos protein in the pPVN was increased by social instability irrespective of nicotine treatment, but only in males. These results are discussed in greater detail below.

4.2. Locomotor activity to nicotine after social stressors in adolescence

Adolescent social stress rats (SS: stressed for 16 days over mid-adolescence by daily one-hour isolation and change of cage partner) had reduced locomotor activity to repeated doses of nicotine than did control rats. These groups (SS and control) did not differ in locomotor activity during habituation or over the test days when treated with saline. The effect of SS was primarily due to effects in females than in males (as evident in Figs. 3 and 4).

We previously reported that social stress in adolescence increased the locomotor-activating effects of nicotine in females and not in males when tested several weeks after the stress exposure when the animals were adults (McCormick et al.,

2004). We hypothesized that perhaps the effects of adolescent stress dissipate in males over time, and that effects of the stressor procedure would be evident in males and perhaps greater in females if tested closer in time to the stress exposure. The present data are contrary to this hypothesis. Nonetheless, consistent with our previous reports (McCormick et al., 2004, 2005), SS had greater effects on nicotine-induced locomotor behaviour in females than in males. However, the direction of the effect of SS in females differed when tested soon after the SS exposure in late-adolescence than in our previous reports which involved testing in adulthood several weeks after the SS exposure.

The difference in the direction of the effects of SS in females on the locomotor-stimulating effects of nicotine as a function of time since stress exposure may be due to either of the following possibilities, which are not mutually-exclusive. First, there may be an interaction of SS with developmental stage of the animal on drug responses. Drugs have different effects in adolescents than in adults: For example, adolescents exhibited greater locomotor-stimulating effects to a first treatment with nicotine (Collins and Izenwasser, 2004; Cruz et al., 2005; Faraday et al., 2001) and lower locomotor-stimulating effects to a first treatment with amphetamine (Adriani and Laviola, 2000; Spear and Brake, 1983) than did adults. The neural structures that mediate the effects of nicotine are developing over adolescence; thus drugs are acting on neural substrates that differ in adolescents compared to in adults (reviewed in (Andersen, 2005; Spear, 2000a). Because there is overlap in the substrates for drugs with those for stressors (de Jong and de Kloet, 2004; Lu et al., 2003; Saal et al., 2003), interactions between developmental stage and stress history on drug responses are likely to occur. A second possibility is that stressors in adolescence initiate a cascade of effects that require an extended period of time to complete. Thus, the stage in the cascade at time of testing may be a basis for differences in the immediate and enduring effects of stressors on drug responses. Others have reported different immediate and enduring effects of stressors in adolescence on brain morphology and behaviour. For example, repeated exposure to physical stressors in adolescence led to decreased hippocampal volume and deficits in spatial learning in a water maze in males that were evident several weeks, but not 24 h, after the last stressor (Isgor et al., 2004). These findings contrast with the effects of repeated stressors in adulthood on hippocampal morphology and spatial learning, which are evident soon after the stressor exposure and tend to dissipate with time (e.g., McEwen, 2000; Sousa et al., 2000). Together with the findings described above, the present results indicate the importance of developmental stage, time since stress exposure, type of stressor, and sex on the consequences of stressors on behavioural responses to drugs and other aspects of brain function.

4.3. Role of social instability in adolescence on locomotor activity to repeated nicotine

We tested the role of social instability as a factor in the effects of SS on locomotor responses to nicotine by including a group that underwent the same 16 days of daily isolation but that was always with the same partner in the home cage (isolation only; ISO). Whereas SS had significantly lower locomotor activity to nicotine over the test days compared to controls and to ISO, ISO and controls did not differ significantly. The difference between the two stress groups may be because the effects of ISO on neuroendocrine function are milder than those of SS: SS rats are exposed to higher levels of bioactive corticosterone over the course of the stress period than are ISO, in that corticosterone levels remained high longer after isolation for SS rats and resulted in lower levels of corticosteroid binding globulin in SS rats (McCormick et al., 2007). The present results indicate that SS in adolescence has a greater impact on the locomotor-activating effects of nicotine in rats than does ISO, which may be related to the greater, more prolonged activation of the limbic-HPA axis due to the social instability that occurs after daily isolation in SS and that does not occur in ISO.

4.4. Age-related effects of nicotine on locomotor sensitization

The challenge day of testing compared groups of rats at 58 or 59 days of age exposed to nicotine repeatedly over late adolescence (over days 48 to 57 of age) to groups receiving a first injection of nicotine. There was clear evidence of locomotor sensitization at this age as indicated by the higher locomotor-activating effects of nicotine to repeated exposure compared to acute exposure. Locomotor sensitization was evident even in SS females despite their reduced locomotor activity to repeated nicotine compared to control females. Differences between adolescents and adults in locomotor sensitization to nicotine have been reported, with some studies finding less sensitization to nicotine in adolescent males compared to adolescent females and adult males and females (Collins and Izenwasser, 2004; Collins et al., 2004; Cruz et al., 2005; Schochet et al., 2004), and others finding greater sensitization in adolescent males compared to adult males (Elliott et al., 2004; Faraday et al., 2003). In the experiments reporting greater sensitization in adolescent males than in adults, testing continued into late adolescence, whereas those that found less sensitization in adolescent males than in adults involved younger rats. However, stage of adolescence may not be the critical factor in these discrepant results. Our rats were tested in late adolescence, and yet responded to nicotine similarly to younger adolescents (Collins and Izenwasser, 2004; Cruz et al., 2005; Faraday et al., 2001; Schochet et al., 2004), with a greater response to a first injection and less marked increases in activity to repeated injection than we have previously observed in adults (McCormick et al., 2004). The discrepancies in the literature may be related to other factors (e.g., number of dosage days, lag between test days and challenge day, mode of administration). We have also found that rats differed in their response to amphetamine in later adolescence (day 46 of age) compared to adults (Mathews and McCormick, 2006, and unpublished results). These results indicate that differences between adolescents and adults in behavioural drug responses likely remain post-pubertal into late adolescence.

4.5. Sex differences in locomotor activity

There are reports of greater locomotor-activating effects of nicotine in females than in males in the literature in adult (e.g., Booze et al., 1999; Kanyt et al., 1999; McCormick et al., 2005) and adolescent rats (e.g.,Collins et al., 2004), and there are reports of qualitative and quantitative sex differences in the effects of nicotine for a variety of measures in studies of rodents (e.g., Chaudhri et al., 2005; Cheeta et al., 2001; Donny et al., 2000; Elliott et al., 2004; Faraday, 2002; Faraday et al., 2005; File et al., 2001; Rhodes et al., 2001) and of people (e.g., Field and Duka, 2004; File et al., 2002; Perkins et al., 2002). Gonadal hormones and estrous/menstrual cycle phase have been implicated in sex differences in the effects of nicotine in adults (Booze et al., 1999; Epperson et al., 2005; Franklin et al., 2004; Sluzen and Anderson, 1997), although the effects are modest at best (Donny et al., 2000; Kanyt et al., 1999; Kuo et al., 1999; Perkins et al., 1999; Terner and de Wit, 2006). There are numerous sex differences in the mesolimbic dopamine system that may underlie differences between females and males in the behavioural effects of drugs (Hu et al., 2004). In the present study, the magnitude of the sex difference in locomotor activity tended to be greater in nicotine-treated rats than in saline-treated rats, which is consistent with the sex differences reported in the literature.

4.6. Nicotine and Fos-immunoreactive cells in the parvocellular paraventricular nucleus (pPVN)

c-*fos* is an immediate early gene that is expressed in low levels in the brain under baseline, unstimulated conditions. The expression of c-*fos* is increased rapidly and transiently by changes in signal transduction pathways, most typically those occurring by neuronal depolarization (for reviews, see Hoffman and Lyo, 2002; Kovacs, 1998). It is widely used to map functional neuroanatomy to various stimuli, with maximal levels of the c-*fos* protein occurring within one to 3 h. Nicotine increases the expression of Fos in the pPVN, the compartment of the PVN containing CRH neurons, by increasing the release of norepinephrine from brainstem nuclei (e.g., Matta et al., 1993; Salminen et al., 2000; Sharp et al., 1993; Valentine et al., 1996), which in turn may act on CRH neurons indirectly through glutamatergic interneurons (for a review, see Herman et al., 2003). In the present experiment, we did not label sections for CRH immunoreactivity, therefore we cannot determine whether Fos immunoreactive (Fos-ir) labeling was occurring primarily in CRH neurons. However, other studies have shown increased c-*fos* expression in CRH-labelled neurons in the pPVN after treatment with nicotine (e.g., Loughlin et al., 2006).

We found a significant effect of nicotine treatment and of stress condition on Fos-ir cell counts in the pPVN, but in males only. Repeated nicotine treatment increased Fos-ir in the pPVN compared to acute treatment and to saline treatment, consistent with previous research. SS males had higher Fos-ir cell counts than did ISO and control males, which did not differ significantly. The interaction of the nicotine treatment and stress condition in males was not significant, and inspection of the means indicates that a difference between SS and controls is as apparent in saline-treated groups as it is in repeated-nicotine groups. We previously found increased expression of CRH mRNA in the PVN under baseline conditions in both SS and ISO compared to controls, and increased expression of CRH mRNA in the central nucleus of the amygdala in SS males compared to ISO males, which suggested that exposure to repeated stressors in adolescence increased the central drive of the hypothalamic–pituitary–adrenal axis (McCormick et al., 2007). Thus, the Fos-ir cell counts are likely related to functional differences in the PVN in socially-stressed adolescent males that are then exploited by nicotine.

Although Fos-ir cell counts are known to be influenced also by behavioural responses to nicotine (e.g., Sharp et al., 1993), there was no relationship between locomotor activity measures and Fos-ir cell counts in the pPVN. For example, SS had the highest Fos-ir cell counts in the pPVN and the lowest amount of locomotor activity in response to nicotine. Thus, we cannot determine the extent to which differences in HPA function may underlie differences in locomotor responses to nicotine. The lack of an effect of nicotine treatment or an effect of stress condition on Fos-ir cell counts in females may in part be a reflection of sex differences in response to the test situation and sex differences in neural structures. A study of chronic stress in adult rats found a stress-induced increase in Fos-ir cell counts in males only, because both control females and stressed females had high Fos-ir cell counts (Westenbroek et al., 2003). The increased variability evident among the female groups is not likely the result of estrous cycle variation, as c-*fos* expression in the PVN in response to stress does not appear to vary across the cycle (Figueiredo et al., 2002), despite estrous cycle variation in c-*fos* expression in other neural structures (Figueiredo et al., 2002) and in hypothalamic–pituitary–adrenal hormonal responses to stressors (Carey et al., 1995; Rhodes et al., 2002; Viau and Meaney, 1991). Thus, the effects of adolescent social stressors on Fos-ir in the PVN and on behavioural responses to nicotine are independent and sex-specific, which is consistent with out previous findings of more robust effects of SS on limbic–hypothalamic–pituitary adrenal function in males more robust effects of SS on locomotor-activating effects of nicotine and amphetamine in females.

5. Conclusion

The present results are consistent with our previous findings of a greater impact of social stressors in adolescence on locomotor responses to nicotine in females than in males. The results contradict our hypothesis that greater effects of social stressors on locomotor responses to drugs may be found in males if time between stress exposure and testing were reduced. The present results for Fos expression in the pPVN add to our evidence of altered limbic–hypothalamic–pituitary–adrenal (HPA) function in socially-stressed adolescent males as determined by neuroendocrine measures. These results suggest that social stressors impact both males and females, but in sex-specific ways. Greater effects of adolescent social stress might be found in males using behavioural measures other than responses to drugs; For example, behavioural measures of

anxiety and fear are more likely to reflect the differences in SS males in the limbic-HPA axis. Lastly, the present results also indicate that social instability is a critical aspect to our adolescent social stressor procedure, in that rats that underwent daily isolation stress only did not differ from controls in either the locomotor effects of nicotine or effects of nicotine on Fos expression in the pPVN, whereas those that were faced with new cage mates after isolation differed from controls. This may be because the neuroendocrine consequences of isolation are greater when accompanied by the social instability of the SS procedure (McCormick et al., 2007). In conclusion, these results add to the growing evidence that adolescents may be uniquely vulnerable to stressors due to ongoing brain development, and that stressors in adolescence may contribute to risk for psychopathology.

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