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Effects of age, but not sex, on elevated startle during withdrawal from acute morphine in adolescent and adult rats

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Abstract

Investigations into animal models of drug withdrawal have largely found that emotional signs of withdrawal (e.g., anxiety, anhedonia, and aversion) in adolescents are experienced earlier and less severely than in their adult counterparts. The majority of these reports have examined withdrawal from ethanol or nicotine. In order to expand our knowledge about the emotional withdrawal state in adolescent rats, we used potentiation of the acoustic startle reflex after an acute dose of morphine (10 mg/kg, s.c.) as a measure of opioid withdrawal. Startle was measured at four time points after morphine injection (2, 3, 4, and 5 h) in 28 and 90 day old male and female rats. The results of this experiment revealed that peak potentiation of the startle reflex occurred at 3 h in the adolescent rats and at 5 h in the adult rats, and that the magnitude of withdrawal was larger in the adults. No sex differences were observed. Overall, these results affirm that, similar to withdrawal from ethanol and nicotine, opiate withdrawal signs are less severe in adolescent than in adult rats.

Keywords

morphine; rat; startle; withdrawal; adolescent; adult

Introduction

Adolescents differ from adults along several dimensions that could make them more susceptible to addiction. Studies in animals have confirmed that adolescents are more sensitive than adults to the rewarding effects of drugs of abuse (Badanich et al. 2006; Brenhouse and Andersen 2008; Brenhouse et al. 2008; Brielmaier et al. 2007; Kota et al. 2007; Shram et al. 2006; Torres et al. 2008; Zakharova et al. 2009a; Zakharova et al. 2009b), and adolescents self-administer drugs in greater quantities than adults (Chen et al. 2007; Füllgrabe et al. 2007; Levin et al. 2003, 2007; Shahbazi et al. 2008; Vetter et al. 2007).

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The experience of drug withdrawal could contribute to future patterns of drug use, either by discouraging use or encouraging drug taking as a means of alleviating negative emotional signs and symptoms. Understanding how adolescents respond during withdrawal is therefore important in explaining their heightened vulnerability to drug abuse. Investigations into animal models of withdrawal have found that emotional signs of withdrawal (e.g., anxiety, anhedonia, and aversion) in adolescents are experienced earlier and are less severe than in their adult counterparts (Doremus-Fitzwater and Spear 2007; Doremus et al. 2003; Hodgson et al. 2009; O'Dell et al. 2006, 2007). The majority of these reports have examined withdrawal from ethanol or nicotine.

In order to expand the literature on the emotional withdrawal state in adolescents, we tested for potentiation of the acoustic startle reflex after an acute dose of morphine. Potentiated startle has been used to measure the anxiety-like component of withdrawal from morphine (Radke et al. 2011, 2013; Rothwell et al. 2009), nicotine (Engelmann et al. 2009), and ethanol (Rassnick et al. 1992). We hypothesized that morphine withdrawal would occur earlier and be lower in magnitude in adolescents vs. adults.

Methods

Subjects

Male and female Sprague-Dawley rats (Harlan) were bred and housed in plastic cages with free access to rat pellet chow (Purina Mills, Minneapolis, MN, USA) and water, at all times except during experimental sessions. The humidity, temperature (21–23 °C), and light–dark cycle (14 h–10 h; lights on at 06:00h) were all regulated. Litters were weaned at postnatal day 21 (P21). Adolescent and adult rats were gently handled for two consecutive days and began testing at approximately postnatal day 28 (P28) and day 90 (P90), respectively.

All procedures conformed to the eighth edition of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Academies Press 2011) and were approved by the University of Minnesota Institutional Animal Care and Use Committee under protocol numbers 1008A87754 and 0812A55002. All laboratory facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

Acoustic startle

Acoustic startle was tested in plastic cages resting on compression springs and located within individual ventilated sound-attenuating chambers. P28 and P90 rats were tested in separate cages adapted for their body size. Cage movement resulted in displacement of a piezoelectronic accelerometer (Model ACH-01, Measurement Specialties, Valley Forge, PA) attached to each cage. Voltage output from the accelerometer was filtered and amplified by a custom-built signal processor, digitized on a scale of arbitrary units ranging from 0–1000 (National Instruments SCB100 and PCI-6071E boards), and recorded using Matlab (The MathWorks, Natick, MA). Startle amplitude was defined as the peak accelerometer voltage during the first 200 ms after onset of the startle stimulus. High frequency speakers (Radio Shack Supertweeters, range = 5–40 kHz) located 10 cm beside each cage delivered the startle stimuli, which were 50 ms bursts of filtered white noise (low pass: 22 kHz, rise-

decay <5 ms) at intensities of 95 or 105 dB. Ventilating fans delivered background noise of approximately 60 dB.

Each startle test session consisted of a 5 min acclimation period followed by presentation of 40 startle stimuli (10 blocks of 4 stimuli, 2 each at 95 and 105 dB in random order) with a 30 s fixed inter-stimulus interval. Acoustic startle was first tested on 2 consecutive drug-free days and average startle amplitudes were used to match animals into groups with similar overall mean startle amplitude. Each test day began with a pre-drug exposure baseline startle session and concluded with a final post-drug exposure startle session (Figure 1A). A Latin square design was used so that each rat was tested once at each of the four post-injection time points (2, 3, 4, and 5 h) over a series of 4 days (Rothwell et al. 2009).

Drugs

Morphine sulfate was purchased from Mallinckrodt (Hazelwood, MO), dissolved in 0.9% saline, and injected s.c. All drug doses are expressed as the weight of the salt.

Data Analysis

Throughout the text and figures all data are expressed as mean $\pm$ SEM. Startle data were collapsed across both intensities (95/105 dB) before further statistical analyses were conducted, as the magnitude of potentiated startle does not depend on startle stimulus intensity (Walker and Davis 2002). In each experiment, analysis of variance (ANOVA) was conducted to verify similar baseline startle amplitude between experimental groups. Changes in startle after experimental treatment were calculated as percent change from baseline on the same day, that is percent change = [(test-baseline)/baseline] $\times$ 100 (Harris and Gewirtz 2004). To assess the magnitude of withdrawal, the area under the curve (AUC) was calculated by summing the potentiated startle values at each time point (Rothwell et al. 2009).

Data from all experiments were analyzed using factorial ANOVA, with repeated measures on within-subject factors. When appropriate, significant interactions were followed up with t-tests using the Holm-Sidak method to correct for multiple comparisons. All statistical analyses were conducted using SPSS (version 17.0) with a Type I error rate of $\alpha = 0.05$ (two-tailed).

Results

A three-way ANOVA of baseline startle values revealed a significant main effect of age ($F_{1,70} = 18.77$; $p < 0.001$) (Table 1). Differences in baseline startle can be explained by difference in weight of the P28 and P90 rats (Table 1). A three-way ANOVA of weights on the first day of testing revealed significant main effects of age ($F_{1,64} = 854.13$; $p < 0.001$) and sex ($F_{1,64} = 107.844$; $p < 0.001$) and a significant age $\times$ sex interaction ($F_{1,64} = 102.09$; $p < 0.001$).

Repeated-measures ANOVA of potentiated startle values 2, 3, 4, and 5 h after morphine exposure revealed a significant main effect of treatment ($F_{1,70} = 18.77$; $p < 0.001$). Significant interactions included treatment $\times$ age ($F_{1,70} = 4.83$; $p < 0.05$), treatment $\times$ hour

($F_{3,210} = 5.01$; $p < 0.01$), hour $\times$ age ($F_{3,210} = 6.46$; $p < 0.001$), and treatment $\times$ hour $\times$ age ($F_{3,210} = 5.30$; $p < 0.01$). Potentiated startle peaked at 3 h in the P28 rats ($42.99 \pm 7.96\%$) (Figure 1C) and at 5 h in the P90 rats ($59.25 \pm 14.01\%$) (Figure 1E). A two-way ANOVA of AUC data revealed a significant main effect of treatment ($F_{1,74} = 19.31$; $p < 0.001$) and a significant age $\times$ treatment interaction ($F_{1,74} = 5.07$; $p < 0.05$). Follow-up t-tests on AUC values (data from males and females combined) demonstrated significant differences between morphine and saline groups in P90 ($t_{37} = 4.12$; $p < 0.01$), but not P28 rats (Figure 1F).

4. Discussion

The results of this study agree with previous findings with other drugs of abuse, such as ethanol and nicotine. Withdrawal, expressed as potentiation of the startle reflex, was both lower in magnitude and occurred earlier in the adolescent rats, regardless of sex. These results are the first to demonstrate a relationship between age and anxiety-like behavior during withdrawal from opiate exposure.

Differences in the time course of changes in startle potentiation may be attributable to differences in pharmacokinetics between the groups. While specific reports of morphine clearance are difficult to find, studies of morphine-induced analgesia in adolescent vs. adult rats suggest the drug is cleared quicker in younger animals (Jøhannesson and Becker 1973; White et al. 2008). Our initial studies on the time course of morphine (10 mg/kg) withdrawal-induced startle potentiation (Harris and Gewirtz 2004; Rothwell et al. 2009) observed a peak at 4 h in P60 rats, suggesting that peak startle potentiation is progressively delayed as the rats age and/or gain weight. In this study, by summing the area under the curve for each group we were able to demonstrate that, despite differences in time course, the magnitude of total withdrawal is greater in the adults.

We found no differences between male and female rats in the time course or magnitude of withdrawal-induced startle. This finding is consistent with our previous studies of morphine withdrawal in Sprague Dawley rats selectively bred for high or low saccharin preference (Radke et al. 2013) but conflicts with other investigations of drug withdrawal in male rodents and monkeys (Brasser and Spear 2002; Carroll et al. 2009; Cicero et al. 2002; Craft et al. 1999; Gatch and Lal 2001; Kest et al. 2001; Perry et al. 2006; Radke et al. 2013). Given the evidence, the lack of a relationship between sex and anxiety-like behavior during withdrawal may be specific to opiates, but further experiments using additional measures of withdrawal will be necessary to confirm this conclusion.

Overall, these results are consistent with the literature on adolescent drug withdrawal by confirming that anxiety-like behavior during opiate withdrawal follows a similar pattern as that observed with other drugs of abuse. Given the widespread availability and use of opiate-based painkillers, these results also have potential clinical relevance.

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Figure 1. Startle potentiation 2, 3, 4, and 5 hours after morphine exposure

a) Rats were tested for startle before and 2, 3, 4, or 5 h after a morphine (0 or 10 mg/kg) injection. b-c) Male (circles) and female (squares) P28 rats. d-e) Male and female P90 rats. f) Potentiated startle at the four time points was summed to produce a general measure of the magnitude of withdrawal in P28 (white bars) and P90 (black bars) rats. ***p < 0.001 compared to saline group.

Table 1

Experimental group information.

Group	Sex	N	Age (days) $\pm$ SEM	Weight (g) $\pm$ SEM	Baseline startle amplitude (arbitrary units)
P28	Male	20	27.5 $\pm$ 0.6	94.0 $\pm$ 4.8	1.93 $\pm$ 0.12
	Female	19	29.2 $\pm$ 0.8	91.8 $\pm$ 5.2	1.93 $\pm$ 0.11
P90	Male	19	93.9 $\pm$ 0.8	413.5 $\pm$ 14.5	4.35 $\pm$ 0.54
	Female	20	94.8 $\pm$ 2.4	247.5 $\pm$ 4.4	2.82 $\pm$ 0.26