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Differential expression of nicotine withdrawal as a function of developmental age in the rat

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ABSTRACT

Cigarette smoking and resultant nicotine dependence remain major public health problems. Most smokers begin before the age of 18, yet preclinical models have insufficiently characterized the development of nicotine dependence in adolescence. To categorize the short-term effects of chronic nicotine administration throughout adolescence and adulthood, we exposed male Sprague Dawley rats to 14 days of continuously delivered nicotine (0, 1.2 or 4.8 mg/kg/d) using a subcutaneous osmotic minipump, starting between postnatal day 33 (p33) and p96. Next, to explore the effects of extended exposure to chronic nicotine, we exposed male Sprague Dawley rats to 42 days of continuous nicotine starting in adolescence (p33) or early adulthood (p68). Somatic and affective signs of precipitated withdrawal (PW) were observed after a mecamylamine (1.5 mg/kg, i.p.) challenge as compared to a saline injection. Short term nicotine exposure starting at p96, well within the adult period, elicited a significant increase in somatic PW as measured by a composite behavioral score. In contrast, adolescent exposure to nicotine elicited a unique behavioral profile, dependent on the starting age of exposure. Late adolescence exposure was characterized by scratching while adult exposure was characterized by facial tremors and yawns. Extended exposure to nicotine resulted in age specific characteristic nicotine withdrawal behaviors, including scratches, ptosis and locomotion, distinct from the short-term exposure. Thus, nicotine dependence severity, based on the expression of total somatic PW behaviors, is not observed until the adult period, and differences between adolescents and adults are observed using a more nuanced behavioral scoring approach. We conclude that age of nicotine initiation affects somatic withdrawal signs and their magnitude. These data serve as a foundation for understanding the underlying brain mechanisms of nicotine dependence and their development over adolescence and early adulthood.

1. Introduction

Smoking remains a major public health concern worldwide. The yearly economic cost of smoking is estimated at 1.4 trillion USD (Goodchild et al., 2017), with past month use of tobacco products estimated at 24% (SAMHSA, 2015). Of regular smokers, ~90% initiated smoking before the age of 18, with earlier initiation associated with worse health outcomes later in life (Surgeon General, 2014). Despite age restrictions on cigarette purchases, 15% of adolescents aged 11 to 17 currently use at least one tobacco product, and by the age of 18, this proportion increases to 22% (SAMHSA, 2015). Furthermore, with the increasing prevalence of nicotine vaping among adolescents (Johnston et al., 2018) and the increased likelihood to smoke following vaping experience (Leventhal et al., 2015), understanding the consequences of adolescent nicotine exposure remains essential to minimize long term consequences. As the adolescent developmental period is characterized by profound changes in brain and behavior and is associated with an increased risk for substance abuse (Spear, 2016), including tobacco products (Silveri et al., 2016; Yuan et al., 2015), it is critical to characterize and understand the developmental trajectory of nicotine dependence inclusive of the adolescent period to reduce adult smoking addiction.

There are no objective brain-based biomarkers of addiction, including nicotine dependence, so behavioral characterization of withdrawal signs and symptoms are used to define and quantify dependence. In general, these symptoms increase with dose and duration of nicotine use in humans and preclinical models (Heatherton et al., 1991; Malin et al., 1992). Although both adult and adolescent smokers self-report withdrawal symptoms, adolescent withdrawal is generally less severe (Smith et al., 2008), varies as a function of the questionnaire and

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typically does not include changes in affect (O'Loughlin et al., 2002). Critically, avoidance of withdrawal symptoms may be a primary motivator to continue smoking for adults but seemingly not as much for adolescents (Smith et al., 2008; Surgeon General, 2014).

In rats, somatic withdrawal behavior is conventionally quantified by the summation of a suite of behaviors including, but not limited to, scratching, facial tremors and ptosis (Malin et al., 1992). Compared to adults, adolescent rats do not manifest significant precipitated or spontaneous somatic (O'Dell et al., 2004, 2006; Torres et al., 2013) or affective withdrawal (O'Dell et al., 2007) following high doses of nicotine exposure with the duration of exposure typically lasting 10–14 days, encompassing adolescence and early adulthood. Notably, the effects of nicotine in adolescents vary by method of assessment (Portugal et al., 2012; Smith et al., 2006), and as the adolescent period is dynamic, a thorough characterization of the developmental trajectory of nicotine dependence defined by the expression of withdrawal, including a range of starting ages, doses and extended periods of nicotine exposure encompassing adolescence and well into adulthood, is lacking in the extant literature.

The current standard method for quantifying nicotine withdrawal (Malin et al., 1992), and by extension dependence, assumes that the frequencies of somatic behaviors are functionally equivalent, and a summed score best characterizes the complexity of withdrawal behavior. This method assumes equal potency and relevance of all behavioral signs. However, we don't know whether certain behaviors best characterize adolescent withdrawal. Examination of individual withdrawal-related behaviors may help determine whether across multiple ages, adolescents remain relatively insensitive to the expression of somatic withdrawal behaviors or simply display a characteristic behavioral profile, distinct from adults following chronic nicotine exposure. Here, we asked the following questions: 1) what is the developmental trajectory of nicotine dependence as defined by both somatic and affective withdrawal behaviors; 2) how does this differ with short *vs.* extended exposure; and 3) is this metric equally represented across all characteristic somatic withdrawal behaviors? To address these questions, we administered nicotine *via* osmotic minipumps for 14 days starting at six ages traversing the developmental spectrum from adolescence to adulthood (Spear, 2007, 2013; postnatal day (p) 33, p47, p61, p68, p82 and p96). Extended exposure to nicotine for 42 continuous days was also assessed, starting at two ages (p33 and p68) to better model the continuous duration of exposure from adolescence to adulthood experience by a human smoker. The frequencies of characteristic withdrawal behaviors were compared across groups. This study is the first to include such a broad range of starting ages as well as duration of exposure and the contribution of individual signs of somatic withdrawal in adolescent nicotine withdrawal behavior.

2. Materials and methods

An outline of the experimental timeline can be found in Fig. 1.

2.1. Subjects

Male Sprague-Dawley rats (Charles River Laboratories) arrived at the National Institute on Drug Abuse Intramural Research Program (NIDA-IRP) between the ages of postnatal day p25 and p28 (adolescents) and p59 and p62 (adults). Rats were given 4–7 days to acclimate to the housing facility, where they received *ad libitum* access to food and water in a temperature and humidity controlled facility and were housed in pairs with same age cage mates. Rats were weighed daily, and all procedures were conducted during the light phase of the cycle and in accordance to approved protocols by the Animal Care and Use Committee of the NIDA-IRP.

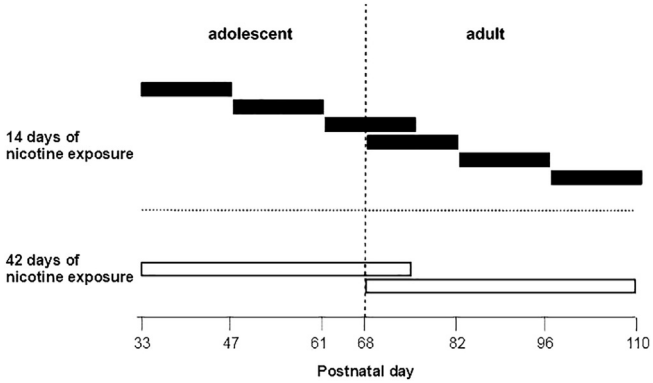

Fig. 1. Experimental timeline. Starting time points for behavioral assessment after 14 (top panel, black boxes) and 42 (bottom panel, write boxes) days of nicotine exposure. Starting age for treatment are represented with separate boxes, and for continuous exposure groups, pumps were replaced every 2 weeks. All groups were tested for differences in somatic and affective withdrawal behaviors at the end of their treatment period.

2.2. Surgery

Using aseptic techniques, rats were implanted with osmotic minipumps (Model #2002, Alzet, Cupertino, CA, USA) subcutaneously (s.c.), preloaded with either saline (SAL) or nicotine hydrogen bitartrate salt, with the latter calculated to deliver either 1.2 mg/kg/d (low nicotine; LN) or 4.8 mg/kg/d (high nicotine; HN) calculated as free base, dissolved in saline. These doses were chosen given that they produce reliable levels of blood nicotine and cotinine in adolescents and adults (O'Dell et al., 2006), decrease brain reward function in adults (Epping-Jordan et al., 1998) and produce reliable signs of precipitated nicotine withdrawal (Brynildsen et al., 2016) (Malin et al., 1992). Anesthesia was induced and maintained using 2.5% isoflurane in a 1:1 mixture of O_2 and air. An incision was made between the shoulder blades, the minipump was placed s.c., and surgical staples closed the wound, followed by application of a topical antibacterial and anesthetic cream. Directly after emergence from anesthesia, rats were given a single s.c. injection of buprenorphine (0.3 mg/kg) for pain management and were monitored for 3 days following implantation. LN and HN exposure *via* osmotic minipump began at 6 different ages: p33, p47, p61, p68, p82 and p96. SAL groups were included for both adolescents and adults. For all groups, $N = 8–9$ subjects.

An additional cohort of rats was used to study the effects of extended nicotine administration. The adolescent and adult groups were implanted with minipumps starting at p33 and p68, respectively, containing SAL, LN or HN (N = 8–9 subjects/grp). Pumps were replaced every 14 days, requiring three separate surgeries, for a total of 42 days of continuous nicotine exposure in both adolescents and adult. To maintain a constant dosage, nicotine concentrations for each pump implantation were adjusted to account for weight gain.

2.3. Precipitated withdrawal (PW) test & conditioned place avoidance (CPA)

To determine the effect of age of onset and duration of nicotine dependence, PW testing occurred at baseline, prior to pump implantation and following maximum nicotine exposure on day 14. For the two extended exposure groups, withdrawal was assessed after 42 days of nicotine administration.

2.3.1. Testing apparatus

Two conditioning chambers that differed in three ways (shape, color and odor) were used as previously described for appetitive and aversive conditioning (Antoniadis and McDonald, 2000; Keeley et al., 2014). Chambers consisted of opaque Plexiglas boxes, custom made by Maze

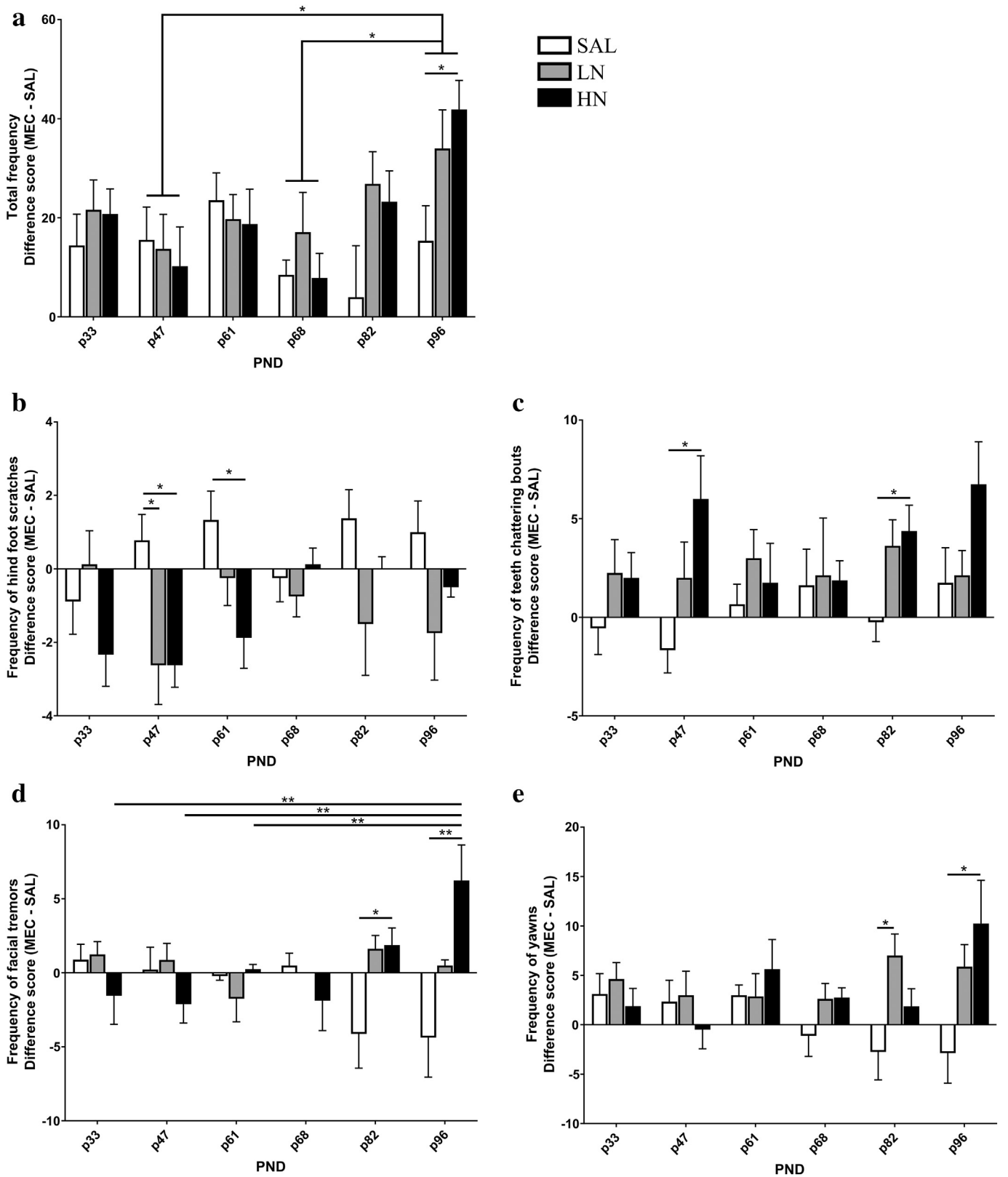

Fig. 2. Differences scores (MEC – SAL injection) after 14 days of nicotine exposure for all age groups for a) total composite score, b) hind foot scratches, c) teeth chattering, d) facial tremors and e) yawns. Bar graphs are expressed as the mean ± SEM. $^{*}p < 0.05$. $^{**}p < 0.001$. Note that negative values indicate a lower frequencies of a characteristic withdrawal behavior during a mecamylamine trial.

Engineers (Glenview, IL, USA), connected *via* an alleyway. Both chambers were lit by overhead fluorescent lamps which provided illumination for behavioral observation without deterring emergence from the darker connecting alleyway.

2.3.2. Pre-exposure

Rats were placed in the middle alleyway and allowed to freely explore both chambers for 10 min the day before testing. Dwell time and number of entries were recorded in real time by an observer. Assignment to training pairings was counterbalanced across all rats and based on dwell time during pre-exposure.

2.3.3. PW test

PW was conducted as previously described (Brynildsen et al., 2016; Malin et al., 1992). Context pairings occurred in the morning and afternoon, with at least 6 h (equivalent to ~6 half-lives of mecamylamine (MEC)) between each injection. Rats were given a s.c. injection of saline or MEC (1.5 mg/ml, dissolved in sterile saline) at a dose of 1.5 mg/kg and placed into one distinct context chamber, with access to the alleyway and the other context chamber blocked. This dose of MEC is known to both precipitate withdrawal (Brynildsen et al., 2016) and induce avoidance behavior (Tzschentke, 2007). After 20 min of habituation, the frequency of PW behaviors was recorded by an observer blind to the injection. Observation following a 20 min habituation period captures a peak in precipitated withdrawal behavior (unpublished observations). Motor behaviors, including tail chasing, grooming, investigation and rearing were also recorded. Following observation, rats remained in the chambers for an additional 20 min, for a total of 60 min. In the afternoon session, rats received the alternative drug (either MEC or saline) and were placed in the other context chamber with behavior similarly recorded.

2.3.4. CPA

The day after the PW test, rats were once again placed in the middle alleyway of the chamber with both context chambers accessible and given free access to both chambers for 10 min. Dwell time and number of entries were recorded in real time by an observer blind to the MEC or saline paired contexts.

2.4. Statistical analysis

Scoring of somatic PW behaviors was conducted as previously described (Brynildsen et al., 2016; Malin et al., 1992), which included summing the frequency of all behaviors into a single score. To control for potential effects related to handling or to the injection, analyses were performed on differences in behavioral scores between that observed following MEC injection to that observed following saline injection (MEC – SAL). In addition to the summed behavioral score, we also conducted statistical tests for each behavior individually, as has been examined previously (Damaj, 2003; Epping-Jordan et al., 1998; Jackson et al., 2008; O'Dell et al., 2004; Watkins et al., 2000). To understand the relative contribution of each behavioral score to the summed score, a data reduction technique, partial least squares regression (PLSR), was conducted. However, PLSR failed to reduce the data to significant or meaningful components (see Supplementary information), supporting the independence of these behaviors.

Two methods of data analysis were conducted. First, *a priori* testing between treatment groups (SAL, LN and HN), using Bonferroni correction for multiple comparisons within each exposure age group were conducted to determine whether, within an age of exposure, there was a significant difference across doses of nicotine. Then, as an exploratory analysis, main effects of age and age × dose interactions were examined; *post hoc* Bonferroni corrections were applied when a main effect or interaction was observed. For the main effect of age, the starting age of nicotine administration (p33, 47, 61, 68, 82 or 96) was used as a continuous variable in all analyses.

3. Results

3.1. Withdrawal after 14 days of nicotine administration

First, we conducted an *a priori* analysis, testing whether within each age of exposure, there were differences between treatment groups (SAL, LN and HN). We observed that rats exposed to HN starting at p96 showed a significant elevation in the composite behavioral score ($p = 0.041$; Fig. 2a). When examining each behavior individually, earlier treatment with nicotine (p47 and p61) decreased hind foot scratching bouts in HN (p47: $p = 0.020$; p61: $p = 0.025$) or LN (p47: p = 0.020; Fig. 2b) treated rats as well as increased teeth chattering in HN treated rats (p47: $p = 0.014$; Fig. 2c). Later exposures to HN increased teeth chattering (p82: p = 0.041; Fig. 2c), bouts of facial tremors (p82: p = 0.041; p96: $p = 0.0050$; Fig. 2d) and yawns (p96: $p = 0.033$; Fig. 2e) in HN exposed rats. Increased number of yawns were observed in p82 LN exposed rats ($p = 0.0201$; Fig. 2e). There were no significant main effects of nicotine dose for any behavior examined for rats that began treatment starting at p33 or p68, and no significant difference in CPA was observed for any age group. A summary statistical table of all nicotine dose effects within each age group can be found in Table 1.

We next conducted an exploratory analysis and identified a significant main effect of age for the composite behavioral score ($F_{(5,130)} = 3.06$, $p = 0.012$; Fig. 2a), with rats exposed to treatment starting at p96 demonstrating a significantly higher composite behavioral score than those exposed starting on p47 ($p = 0.034$) and p68 ($p = 0.011$). There was also a significant age × dose interaction for the number of facial tremors ($F_{(10,130)} = 3.90$, $p = 0.00012$; Fig. 2d), and among HN exposed rats, rats exposed starting at p96 displayed significantly more tremors as compared to those starting at p33 ($p = 0.0087$), p47 ($p = 0.0047$) and p68 ($p = 0.0074$). There was a significant age × dose interaction for the number of yawns ($F_{(10,130)} = 2.00$, $p = 0.0382$; Fig. 2e); no additional comparison survived correction for multiple comparisons (see Table 2 for results). No differences in CPA behavior were observed in the secondary analysis.

3.2. Withdrawal after long term (42 days) of nicotine administration

Similar to the results observed after 2 weeks of nicotine exposure, we conducted an *a priori* analysis examining the effects of dose within each age of exposure (see Table 3). Adolescent (p33) rats exposed to HN for 6 weeks displayed increased hind foot scratching as compared to SAL exposed adolescents ($p = 0.036$; Fig. 3a). Rats exposed to nicotine for 6 weeks starting in early adulthood at p68 displayed significantly elevated ptosis in both LN ($p = 0.0094$) and HN ($p = 0.0065$; Fig. 3b) groups. Rats exposed at p68 also showed elevated frequency of rearing

Table 1
Summary of statistical results for *a priori* comparison of each starting age for effects of nicotine dose.

Age	Behavior difference score	Comparison			
		HN > SAL	HN < SAL	LN > SAL	LN < SAL
		p	p	p	p
p33	all n.s.				
p47	Teeth chattering	0.0138	n.s.	n.s.	n.s.
	Hind foot scratch	n.s.	0.0195	n.s.	0.0195
p61	Hind foot scratch	n.s.	0.0252	n.s.	n.s.
p68	all n.s.				
p82	Teeth chattering	0.0406	n.s.	n.s.	n.s.
	Tremors	0.0422	n.s.	n.s.	n.s.
	Yawns	n.s.	n.s.	0.0209	n.s.
p96	Total	0.0408	n.s.	n.s.	n.s.
	Tremors	0.00498	n.s.	n.s.	n.s.
	Yawns	0.0334	n.s.	n.s.	n.s.

Table 2
Summary of statistical results for significant main effect of age and age × nicotine dose interaction effects and all *post hoc* tests, where appropriate.

Behavior difference score	Main effects & interaction				*Post hoc* Bonferroni	
	Age		Age × dose		Age	Age × dose
	F	p	F	p	p	p
Total	3.096	0.0121	n.s.	n.s.	p47 < p96* p68 < p96*	–
Tremors	n.s.	n.s.	3.9	0.000117	–	HN: p33 < p96** p47 < p96** p68 < p96**
Yawns	n.s.	n.s.	2.00	0.0382	–	n.s.

* $p < 0.05$.
** $p < 0.01$.

Table 3
Summary of statistical results for *a priori* comparison of each starting age for effects of nicotine dose for extended (42 days) exposure to nicotine.

Age start	Behavior difference score	Comparison	
		HN > SAL	LN > SAL
		p	p
p33	Hind foot scratch	0.0355	–
p68	Ptosis	0.00654	0.00942
	Rearing	0.00877	–

when exposed to HN ($p = 0.0088$; Fig. 3c).

Exploratory analyses revealed a main effect of age only for the frequency of ptosis, wherein adults (p68) more frequently demonstrated ptosis than adolescents ($F_{(1,44)} = 4.35$, $p = 0.043$; Fig. 3b). There was also a significant age × dose interaction ($F_{(2,44)} = 3.37$, $p = 0.044$; see Table 4). *Post hoc* analysis revealed that rats exposed to HN starting at p68 displayed significantly more ptosis bouts than adolescent (p33) exposed rats (Fig. 3b). No differences in conditioned place aversion behavior were observed.

4. Discussion

We investigated the trajectory of nicotine dependence across development through the quantification of somatic withdrawal signs following 14 or 42 days of continuous nicotine administration. Since human smokers' report differences in withdrawal as a function of age, we were particularly interested in the intensity of nicotine withdrawal observed in adolescent- *vs.* adult-exposed rats. When using the 'gold standard' behavioral assay of the summed frequency of PW behaviors (Malin et al., 1992), only rats starting nicotine administration well beyond adolescence and into the adult period (p96) showed elevated somatic withdrawal behaviors, consistent with the extant literature (Malin, 2001; Malin et al., 1992). However, 14 days of nicotine exposure produced unique behavioral profiles dependent on the age of exposure, with late adolescent nicotine exposure characterized by hind foot scratches and adult nicotine exposure characterized by facial tremors and yawns. In contrast, extended exposure (42 days) to nicotine decreased the number of hind foot scratches in adolescents (p33), while increased ptosis bouts and decreased locomotor behavior was seen in adult-exposed rats (p68). No differences were observed with nicotine in the composite behavioral score following extended exposure to nicotine. When considering the complexities of age, nicotine dose and length of exposure, rodent nicotine dependence may be better assessed using specific somatic PW behaviors, while summing these behaviors into a single metric results in an apparent loss of detection power. Finally, we observed no significant affective signs of withdrawal, as assessed through CPA behavior, suggesting that affective and somatic PW signs reflect different mechanisms.

4.1. Withdrawal is expressed only among older adults using a composite behavioral score

The developmental trajectory of nicotine dependence was protracted and expressed only when HN was administered during the adult period (p96), which is consistent with previous work reporting the absence of PW in adolescents administered chronic nicotine (Kota et al., 2007; O'Dell et al., 2004, 2006; Torres et al., 2013). Even after extended exposure (42 days), starting at p33 or p68, PW was not observed. Decreased withdrawal in combination with enhanced sensitivity to reward in human adolescents is thought to increase the risk of developing nicotine dependence, such that adolescents experience few to none of the aversive effects until they are adults, at which point they have already progressed to nicotine addiction (O'Dell, 2009). Indeed, early (< p35), middle (p35-p48) and late (p50-60) adolescent exposure to nicotine changes susceptibility to the effects of nicotine (Adriani et al., 2002). Adolescent exposure to nicotine is associated with a suite of behavioral changes, including decreased cognitive performance (Counotte et al., 2009; Portugal et al., 2012; Spaeth et al., 2010) and increased anxiety- and depression-like behaviors (Iñiguez et al., 2009; Smith et al., 2006) in adulthood. In combination with previous work, the present results highlight that the increased period of risk to develop nicotine dependence in adolescents and may extend well beyond adolescence and in to early adulthood.

4.2. Individual withdrawal behaviors vary with age, nicotine dose and exposure duration

In contrast to the composite score, discrete withdrawal behaviors, when quantified individually, distinguished age- and duration-dependent nicotine withdrawal severity, demonstrating that somatic withdrawal signs (and presumably dependence severity) vary with age of initiation of nicotine exposure. Thus, withdrawal is not entirely absent in the adolescent but is differentially and minimally expressed. Depending on the behavior examined, PW was observed as a function of age and dose of nicotine exposure. These results may partially explain why research to date has not reported heightened somatic withdrawal, as the expression of these behaviors is so highly dependent on the age of the individual.

In rodents, adolescent-typical behaviors, with and without pharmacological manipulation, are observed for many metrics (Spear, 2007). In humans, the Hooked on Nicotine Checklist (HONC) was developed specifically to detect self-report withdrawal in adolescents (Jacobsen et al., 2005; O'Loughlin et al., 2002; Smith et al., 2008). Our results provide a behavioral framework that parallels human research indicating that different metrics, reflecting perhaps age appropriate behavioral/motoric capability, may be required to characterize the adolescent-specific withdrawal syndrome. We propose the inclusion of

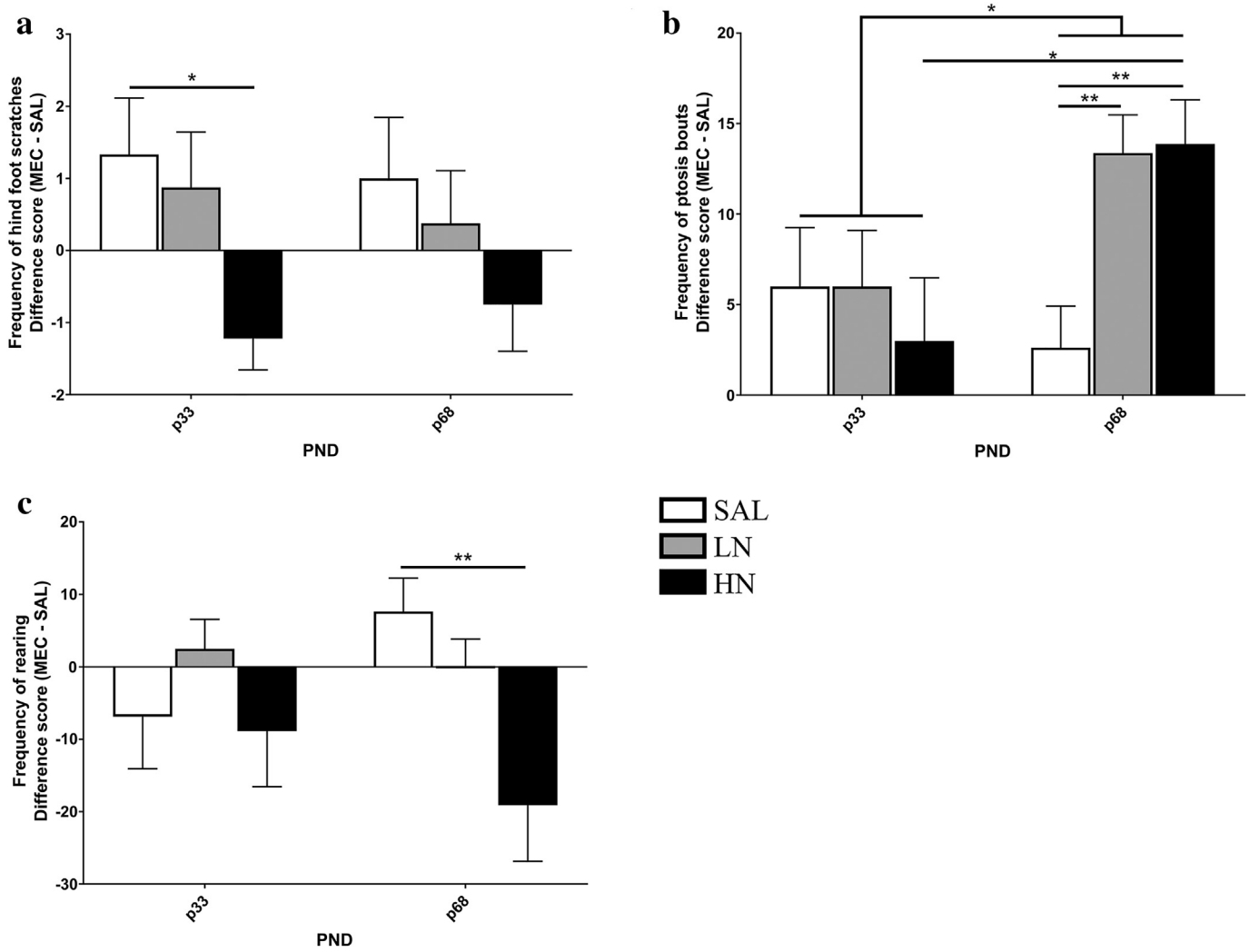

Fig. 3. Difference scores (MEC – SAL injection) after 42 days of nicotine exposure starting at p33 or p68 for a) hind foot scratches, b) ptosis bouts and c) rearing. Bar graphs expressed as the mean ± SEM, N's inset. *p < 0.05. **p < 0.01. Note that negative values indicate a lower frequencies of a characteristic withdrawal behavior during a mecamylamine trial.

individual signs in addition to a summed behavioral score to characterize adolescent nicotine withdrawal.

Extended chronic nicotine exposure, which is the case for most human smokers, leads to different behavioral outcomes depending on when the exposure began and total cigarette intake (sometimes quantified as pack years). Much of the rodent literature to date has focused on exposure in the range of a few weeks, which does not parallel the human condition. The majority of smokers start before the age of 18 (Surgeon General, 2014), and the average age of a successful quitter (*i.e.* remaining abstinent for 6–12 months) is ~40 years old (Schauer et al., 2015). So, animal models starting in adolescence, with nicotine treatment constrained to only the adolescent and young adult period may not adequately model the trajectory and duration of nicotine dependence in humans. This is best exemplified by our finding of differences in withdrawal signs following extended exposure between adolescent and adult rats unless we separately examined individual behaviors. In addition, of all the behaviors expressed during PW, only some were affected depending on the length of exposure, and no behavioral score of a single type could distinguish between short and prolonged exposure. Thus, somatic signs of nicotine withdrawal over development and with longer durations is not a simple construct, and the duration of exposure should be carefully considered when designing

Table 4
Summary of statistical results for significant main effect of age and age × nicotine dose interaction effects and all *post hoc* tests, where appropriate, for rats exposed to extended (42 days) nicotine.

Behavior difference score	Main effects & interaction				*Post hoc* Bonferroni	
	Age		Age × dose		Age	Age × dose
	F	p	F	p	p	p
Ptosis	4.35	0.0428	3.37	0.0436	Adol < adult*	HN: p33 < p68*
Yawn	–	n.s.	3.51	0.0387	–	n.s.

* $p < 0.05$.

animal models of human nicotine dependence and when treating withdrawal symptoms in smokers attempting to quit.

An alternative explanation to these findings is that adolescents simply lack the motoric capacity to express somatic withdrawal. Like an infant learning to walk, although all the required 'equipment' is present, time and experience are required to shape this behavior. It is possible that results observed using the individual behavioral scores are, in fact, false positives, although the likelihood is diminished given our experimental design and statistical analyses. Future experiments should consider the possibility that the physical capacity of adolescents to express somatic withdrawal is diminished, which does not necessarily denote that they are immune to the dependence-inducing effects of nicotine. Instead, we should consider alternative methods for quantifying dependence that go beyond precipitated withdrawal in adolescent rats. For example, physical signs of withdrawal are not the sole determinant of smoking relapse following a quit attempt, as self-reported cravings and affective withdrawal severity are a known determinant of quitting success (Nakajima and al'Absi, 2012). Here, we observed no differences for any age group for CPA behavior. Reported age differences in affective signs of nicotine PW in rats are mixed, with decreased aversion (O'Dell et al., 2007), enhanced anxiety (de la Peña et al., 2016) and cognitive ability (Portugal et al., 2012; Wilmouth and Spear, 2006) during withdrawal in adolescents as compared to adults. In humans, adolescents self-report fewer depression-like withdrawal symptoms (O'Loughlin et al., 2002), indicating less of a change in affect in adolescents compared to adults. Thus, age differences in affective measures of withdrawal are mixed and require further research. However, characterizing a suite of behaviors from a single individual may help fully characterize adolescent withdrawal, and research in this field is currently lacking

4.3. Limitations

We observed changes in somatic withdrawal signs after 14 and 42 days of continuous nicotine exposure. Somatic signs of withdrawal encapsulate only one facet of the withdrawal experience and thus only accounts for one aspect of nicotine dependence. For example, testing cognitive abilities throughout the adolescent and adult period, sated or during withdrawal, may have further elucidated age-related differences. In addition, our use of precipitated withdrawal may not have revealed all differences between groups, and quantification of spontaneous somatic withdrawal may have further elucidated age differences. It is unclear whether the absence of aversion behavior was a function of the short training period employed, implemented to avoid lengthy behavioral training in adolescents, or indicative of blunted aversive properties of withdrawal.

Nicotine metabolism and pharmacokinetics change as a function of age (Craig et al., 2014), and we administered the same doses of nicotine, normalized for body weight, possibly resulting in different concentrations of nicotine between age groups. Although others have reported no effect of nicotine on withdrawal behavior in adolescents with the inclusion of blood nicotine and cotinine concentrations (O'Dell et al., 2006), the inclusion of these metric could have been used as a correlate with severity of withdrawal and would have served to further elucidate whether individual differences in nicotine metabolism were driving our observed effects.

Finally, continuous delivery of nicotine, as conducted here using osmotic minipumps, does not model the human smoking experience, and pulsatile drug exposure may more effectively mimic nicotine intake and subsequent dependence in humans (Kawa et al., 2016). Previous work from our group observed that using a modified version of the osmotic minipump, which delivered a discrete nicotine dose each hour, resulted in enhanced and prolonged withdrawal symptoms (Brynildsen et al., 2016); note that in that study, rats continuously exposed to 4.8 mg/kg/d of nicotine displayed increased withdrawal signs relative to the saline control group. For the current study, the size of the modified pump was too large relative to adolescent body size and weight, preventing its use. Furthermore, the inclusion of exclusively male rats in this study precludes the ability to extend these findings to females, shown to differentially form nicotine dependence (Cross et al., 2017).

4.4. Conclusions

We observed that the expression of nicotine withdrawal behavior is not present until the adult period when using standard behavioral metrics, regardless of nicotine dose or duration. Partially, this can be explained by the differential sensitivity of each behavior as a function of developmental age, nicotine dose and duration of exposure, although the possibility of differential motoric capacity to manifest PW in adolescents remains. Thus, age of initiation affects withdrawal signs, which may account for the increased difficulty to quit smoking in long-term smokers, the majority of whom start in adolescence (Surgeon General, 2014). Since no objective brain-based biomarkers of nicotine dependence have been developed, only behavior is currently available as an index of dependence severity. Refinement of behavioral measures should help improve the ability to quantify nicotine dependence, which, in turn, will help to identify brain-based biomarkers of dependence, increasing understanding of brain mechanisms of nicotine dependence and how early life experience can shape the brain to be susceptible to nicotine dependence later in life.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbb.2019.172802.

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