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Female rats display greater nicotine withdrawal-induced cellular activation of a central portion of the interpeduncular nucleus versus males: A study of Fos immunoreactivity within provisionally assigned interpeduncular subnuclei

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Abstract

Background: The *interpeduncular nucleus (>1840)* (IPN) has been shown to modulate the behavioral effects of nicotine withdrawal in male rodents. To date, the contribution of this brain structure to sex differences in withdrawal is largely unexplored.

Methods: This study compared neuronal activation, as reported by observable Fos expression in the IPN of nicotine-dependent female and male rats experiencing withdrawal. We provisionally localized the Fos-expressing cells to certain IPN subnuclei within Swanson's standardized brain atlas (2018). Adult female and male rats were prepared with a pump that delivered nicotine (3.2 mg/kg/day; base) continuously. Controls received a sham surgery. Fourteen days later, the rats received administration of saline or the nicotinic receptor antagonist, mecamylamine (3.0 mg/kg; salt), and physical signs and anxiety-like behavior were assessed. The rats were then euthanized and brain sections containing the IPN were processed for Fos immunofluorescence to infer the possible IPN subnuclei displaying differential activation between sexes.

Results: Both female and male rats displayed withdrawal-induced Fos expression within the IPN. Compared to males, female rats displayed greater numbers of withdrawal-induced Fos-positive cells within a circumscribed portion of the IPN that may fall within the cytoarchitectural

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Contributors

Dr. Matos-Ocasio and Ms. Espinoza performed the experiments, conducted the statistical analyses, and wrote the initial drafts of this manuscript. They both contributed equally to this study. Ms. Correa-Alfonzo helped plan and conduct the experiments. Dr. Khan was critical in the implementation of the immunofluorescence work and provided critical feedback on the framing and interpretation of the anatomical aspects of this work. Dr. O'Dell is the PI of the grant that supported this work and was involved in all aspects of the project. All authors reviewed, edited, and approved the manuscript.

Conflict of Interest

No conflict declared.

boundaries of the *central subnucleus (>1840)* (IPNc). The withdrawal-induced activation of the IPN was correlated with negative affective states in females, but not males.

Conclusion: These data suggest that that a centrally located group of IPN cells, presumably situated partly or completely within the IPNc, play a role in modulating sex differences in negative affective states produced by withdrawal.

Keywords

Sex differences; Fos; immunofluorescence; dependence; standardized brain atlas

1. Introduction

Recent studies in male rodents have revealed that the *medial habenula (>1840)* (MH) and the *interpeduncular nucleus (>1840)* (IPN) play key roles in the expression of the behavioral effects of nicotine withdrawal (Antolin-Fontes et al., 2015; Fowler and Kenny, 2014; McLaughlin et al., 2017; Molas et al., 2017). The MH provides a major glutamatergic input to the IPN by way of neurons that co-release acetylcholine (ACh; Frahm et al., 2015). Blockade of nicotinic ACh receptors (nAChRs) in the IPN elicits withdrawal signs in nicotine-treated male mice, possibly by blocking presynaptic nAChRs on glutamate terminals (Dani and De Biasi, 2013; Salas et al., 2009).

Prior work has shown that the behavioral effects of nicotine withdrawal vary between female and male rodents (Gentile et al., 2011; Hamilton et al., 2010; Kota et al., 2008; Kota et al., 2007; Skwara et al., 2012; Tan et al., 2019). Work in our laboratory has found that female rats display greater anxiety-like behavior during nicotine withdrawal as compared to intact males or females lacking ovarian hormones (Flores et al., 2020; Torres et al., 2013, 2015). Recently, we reported that the magnitude of anxiety-like behavior produced by nicotine withdrawal was correlated with the expression of specific nAChR subunits (α4, α5, and β2) in the IPN, suggesting that this brain region contributes to sex differences in withdrawal (Correa et al., 2019).

The IPN is a *midbrain* (Baer, 1837) structure that is situated in the ventral portion of the *tegmentum* (Swanson, 2000). Across different taxa, the IPN contains neuronal cell types that display heterogeneous morphology (see Figs. 8 and 9 in Cajal, 1895), cytoarchitecture (Groenewegen et al., 1986), and chemoarchitecture (Contestabile et al., 1987; Groenewegen et al., 1986; Hemmendinger and Moore, 1984). The IPN features a narrow rostral, dorsal portion and a wider ventral portion that is further subdivided by Swanson (2018) into a *central subnucleus (>1840)* (IPNc), which is flanked bilaterally by both *intermediate subnuclei (>1840)* (IPNi) and *lateral subnuclei (>1840)* (IPNl) (Hemmendinger and Moore, 1984; Quina et al., 2017). The ventral portion of the IPN may be functionally distinct, given that it receives the largest input from the MH (Yamaguchi et al., 2013). Indeed, a prior study suggested (on the basis of male rodent data) that the ventral portion of the IPN modulates negative affective states, whereas the rostral portion modulates physical signs produced by nicotine withdrawal (Molas et al., 2017). Another report using nicotine-dependent male mice, showed that administration of the nicotine receptor antagonist, mecamylamine elicited neuronal activation in the intermediate portion of the IPN (Zhao-Shea et al., 2015). The

present study builds upon and extends existing literature by documenting region-specific activation of the IPN in nicotine-dependent female and male rats during withdrawal. Immunofluorescence methods were used to visualize Fos expression in the IPN as a cellular marker of neuronal activation, and high-resolution imaging to quantify and contextualize sub-regional activation patterns within the IPN. This work constitutes an important step towards understanding sex differences for a brain region believed to promote nicotine withdrawal in females.

2. Methods

2.1. Nomenclature

The present study sought to interrelate our Fos activation patterns with those produced by other behaviors such as hunger/satiety and context fear learning, which have used Swanson's spatial framework (*e.g.*, see Zséli et al., 2016; Santarelli et al., 2018). To this end, we used *standard terms* for brain structures as defined by Swanson (2015; 2018). These terms are listed in italics together with the associated citation that first uses the term as defined. If a definitive assignment of priority for the term was not possible, it was assigned by Swanson the citation "(>1840)"; that is, "defined sometime after the year 1840". Refer to Swanson (2015, 2018) for further details regarding this standard nomenclature system.

2.2. Subjects

Fully out-bred adult Wistar rats (mean body weight at time of sacrifice = 260 g (females) 400 g (males); n = 4–8 per group) were housed on a 12-hr reverse light cycle (lights off at 6 a.m.) with *ad libitum* access to food and water. All procedures adhered to the NIH guide for the care and use of laboratory animals and approved by our Institutional Animal Care and Use Committee.

2.3. Procedures

The rats were anesthetized with isoflurane (1–3 %) and prepared with an osmotic pump (model 2ML2; 5.0 μL/hr; Durect Corporation, Inc.) that delivered nicotine continuously (3.2 mg/kg/day; base). Control rats received a sham surgery. This nicotine dose produces similar levels of nicotine in female and male rats (Torres et al., 2013). Fourteen days after surgery, rats received saline or mecamylamine (3.0 mg/kg, sc; salt) to precipitate withdrawal. Physical signs of withdrawal (eyeblinks, writhes, body shakes, teeth chatters, and gasps) were assessed for 10 min. Following physical signs testing, the rats were transported to another room and acclimated for 5 min. Negative affective states were then assessed for 5 min using the light dark transfer (LDT) test. Anxiety-like behavior was defined as a decrease in time spent in the lit versus dark side of the LDT apparatus. Ninety min after withdrawal induction, the rats were anesthetized (200 mg/kg, ip; Fatal–Plus) and perfused transcardially with 200 mL of ice-cold phosphate-buffered saline (PBS; 0.1 M; pH 7.4 at room temperature) followed by 200 mL of ice-cold paraformaldehyde (PFA; 4%). The brains were post–fixed in 4% PFA overnight and transferred to a 30% sucrose solution in PBS at 4°C for 48–72 hrs. The brains were sectioned at 30 μm (−5.80 mm to −6.12 mm from Bregma) using a cryostat (Leica CM1850). Sections were then washed with PBS and then incubated in a blocking solution (2% normal goat serum made with 2% BSA and 0.2% Triton–X–100) for

2 hrs. Sections were then incubated at 4°C in rabbit anti–cFos (diluted 1:5,000 in 2% BSA; ab190289, Abcam) for 48 hrs. The sections were then washed and incubated in a dark room in a donkey anti–rabbit IgG (diluted 1:1,000 in 2% BSA; Alexa 488, Abcam) for 3 hrs. Following incubation with the secondary antibody, the sections were mounted onto slides. Images were acquired using an Axio Imager M.2 upright epifluorescence microscope (Carl Zeiss, Inc.) and analyzed using Fiji software. The number of Fos-positive cells was quantified using a manual cell counter within ImageJ software (NIH). Fos-positive cells were included if their fluorescence values exceeded background levels. The average of 3 sections were analyzed for each animal.

2.4. Statistics

The data for physical signs and Fos-positive cells were analyzed using analysis of variance (ANOVA) with sex and treatment as between–subject factors. Separate analyses were conducted for the total IPN, presumptive IPNi, and presumptive IPNc. *A priori* comparisons assessed sex differences in neuronal activation using the Fisher's LSD test ($p \leq .05$). The relationship between neuronal activation and behavioral outcomes were assessed using a Pearson correlation coefficient analysis. Data were analyzed using IBM SPSS Statistics for Windows, version 27 (IBM Corp.).

3. Results

Figure 1 displays representative images of Fos-positive cells in female and male rats. No activation was observed in any portion of the IPN in nicotine-treated or sham controls that received saline administration (data not shown). Thus, our statistical analysis included sham controls and nicotine–treated rats that received mecamylamine to precipitate withdrawal.

Figure 2 displays activation of the total IPN (A), IPNi (B), and IPNc (C) as well as our correlational analysis of activation of the total IPN with physical signs (D), dark side of the LDT (E) and lit side of the LDT (F). The analysis of neuronal activation in the total IPN revealed that there was no interaction between sex and treatment [$F_{(1, 15)} = 0.75$, $p = .39$]. However, there was a main effect of treatment [$F_{(1, 15)} = 5.51$, $p = .03$], with nicotine-treated rats displaying more Fos-positive cells during withdrawal relative to mecamylamine controls. There was a trend towards a main effect of sex [$F_{(1, 15)} = 3.147$, $p = .09$]. The analysis of the presumptive IPNi revealed that there was no interaction between sex and treatment [$F_{(1, 15)} = 0.23$, $p = .63$]. There was also no main effect of sex [$F_{(1, 15)} = 2.05$, $p = .17$] or treatment [$F_{(1, 15)} = 1.54$, $p = .23$]. The analysis of the presumptive IPNc revealed that there was no interaction between sex and treatment [$F_{(1, 15)} = 2.32$, $p = .14$]. However, there was a main effect of treatment [$F_{(1, 15)} = 20.50$, $p < .001$], with all nicotine-treated rats displaying more Fos-positive cells during withdrawal relative to mecamylamine controls. There was also a significant main effect of sex [$F_{(1, 15)} = 4.97$, $p = .04$], with females displaying more Fos-positive cells in the presumptive IPNc as compared to males. An *a priori* comparison revealed that nicotine-treated females displayed greater withdrawal-induced activation of the presumptive IPNc as compared to males ($p < .05$). The correlational analysis revealed that there was no relationship between IPN activation and physical signs in female ($r = -.12$, $p = .85$) and male ($r = -.01$, $p = .98$) rats. In contrast,

there was a significant positive relationship between IPN activation and time spent in the dark side of the LDT in female (r = .70, p = .05), but not male rats (r = −.47, p = .29). Accordingly, there was a negative correlation between activation of the IPN and time spent in the light side of the LDT in female (r = −.70, p = .05), but not male rats. (r = .47, p = .29).

4. Discussion

This study extends prior work by illustrating sex- and region-specific differences in withdrawal-induced activation patterns in the IPN, placing these patterns within an open–access spatial model of the rat brain (Swanson, 2018). First, nicotine withdrawal did not elicit neuronal activation of the rostral or most lateral portions of the ventral IPN, consistent with previous reports (Zhao-Shea et al., 2015). The latter report also established that the intermediate sub-region of the IPN modulates withdrawal-induced increases in anxiety-like behavior in male mice. The present study expands prior work by assessing *sex differences* in withdrawal-induced activation within sub-regions of the IPN. A major finding of this report is that nicotine-treated females displayed greater withdrawal-induced activation of the presumptive IPNc as compared to males. These data suggest that the *central subnucleus (>1840)* of the IPN contributes to sex differences produced by nicotine withdrawal. To our knowledge, our report is the first demonstration of sex differences produced by withdrawal in the presumptive IPNc. Future studies are needed to determine the type of neurons that were activated in the IPNc. Based on prior reports, we suggest that sex differences in the IPNc are due to withdrawal-induced activation of GABAergic interneurons. This is based on prior work in male mice showing that 80% of neurons expressing Fos within the IPN during nicotine withdrawal are GABAergic (Zhao-Shea et al., 2013). There is also a high distribution of corticotropin-releasing factor receptor 1 (CRFr1) in the IPN that modulates local GABA release (Zhao-Shea et al., 2015). Thus, the possibility exists that sex differences produced by nicotine withdrawal are modulated via CRF peptidergic systems that are anatomically positioned to facilitate GABAergic inhibitory tone in the IPNc. Another finding of this report is that withdrawal-induced activation of the IPN was correlated with negative affective states in female, but not male rats. Future studies are needed to carefully distinguish the cytoarchitectural boundaries within the IPN in order to ascribe how each subnucleus modulates physical signs versus negative affective states produced by withdrawal in females versus males. Importantly, future work is also needed to determine the influence of ovarian hormones on the mechanisms that subserve withdrawal severity in females. This avenue of research is important given clinical reports showing that fluctuations in estrogen and progesterone influence nicotine use patterns, withdrawal severity, and the efficacy of cessation medications (Carpenter et al., 2006; O'Hara et al., 1989; Pang et al., 2018). This work will be important for informing optimal quit dates during the menstrual cycle and the possibility that hormones may be used to alleviate withdrawal in women (Weinberger et al., 2015).

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Highlights

- Nicotine withdrawal induces sex-dependent neuronal activation of the IPN
- Female rats display greater withdrawal-induced activation of the IPNc versus males
- IPN activation correlates with affective states of withdrawal only in female rats

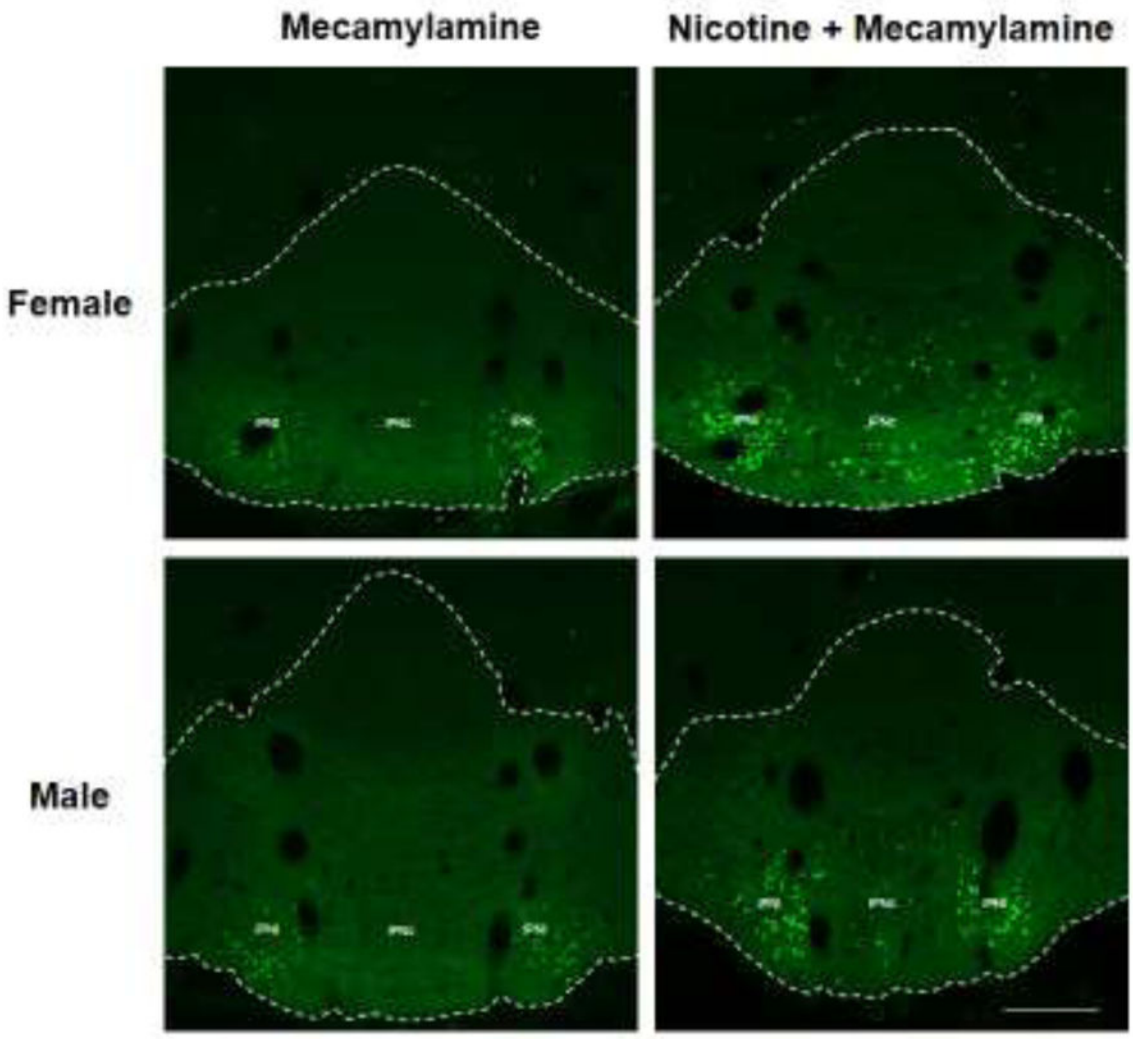

Figure. 1.

Photomicrographs illustrating Fos immunolabeling in coronal–plane sections containing the putatively assigned IPNi and IPNc subnuclei of the IPN. The scale bar denotes 200 μm and applies to all panels.

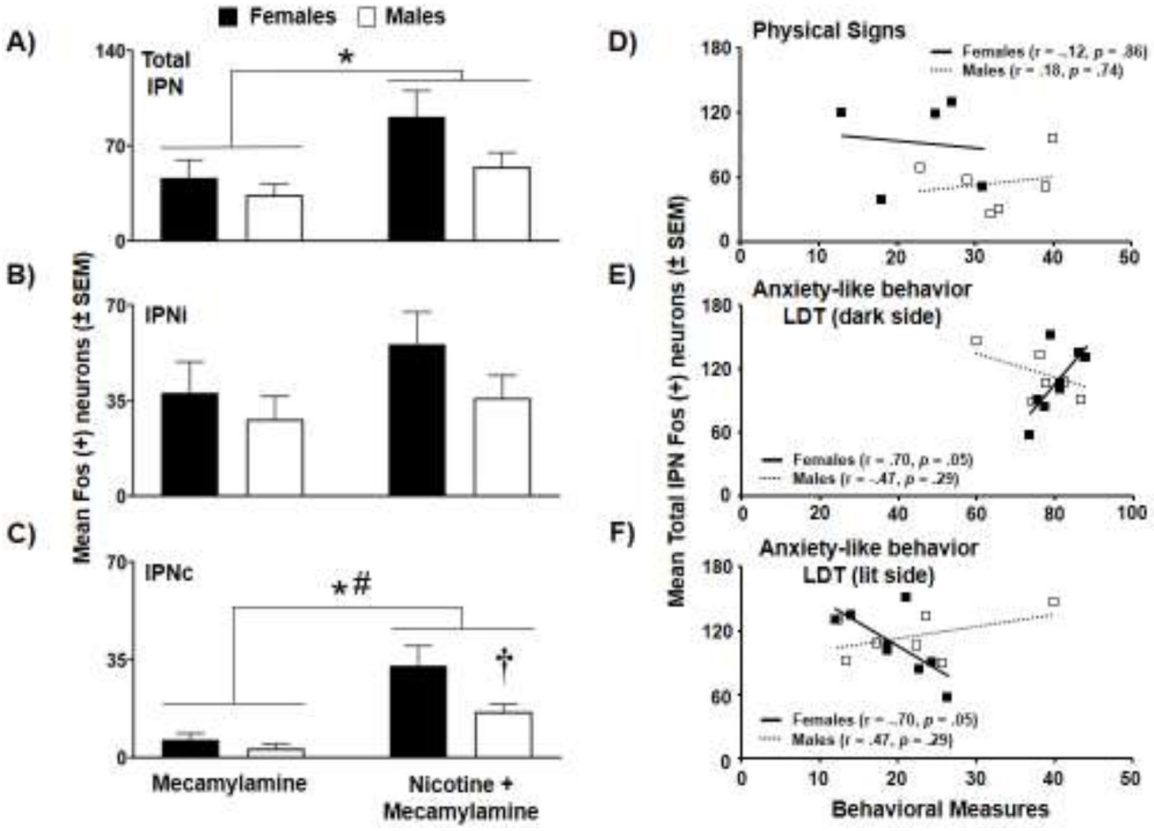

Figure. 2.

The data on the left side reflect Fos (+) cells (mean ± SEM) expressed in the total IPN (A) and putatively assigned IPNi (B) and IPNc (C) in mecamylamine controls and nicotine + mecamylamine-treated female and male rats. The data on the right side of this figure reflect the correlation between total IPN activation with physical signs (D) or anxiety-like behavior as measured by % time spent in the dark (E) or lit (F) side of the LDT apparatus. Asterisks (*) denote a main effect of treatment, the pound sign (#) denotes a main effect of sex, and the dagger (†) denotes a difference between nicotine + mecamylamine-treated female and male rats ($p < .05$).