

## Full Length Article

## Developmental nicotine exposure and masculinization of the rat preoptic area



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## ABSTRACT

Nicotine is a neuroteratogenic component of tobacco smoke, e-cigarettes, and other products and can exert sex-specific effects in the developing brain, likely mediated through sex hormones. Estradiol modulates expression of nicotinic acetylcholine receptors in rats, and plays critical roles in neurodevelopmental processes, including sexual differentiation of the brain. Here, we examined the effects of developmental nicotine exposure on the sexual differentiation of the preoptic area (POA), a brain region that normally displays robust structural sexual dimorphisms and controls adult mating behavior in rodents. Using a rat model of gestational exposure, developing pups were exposed to nicotine (2 mg/kg/day) via maternal osmotic minipump (subcutaneously, sc) throughout the critical window for brain sexual differentiation. At postnatal day (PND) 4, a subset of offspring was analyzed for epigenetic effects in the POA. At PND40, all offspring were gonadectomized, implanted with a testosterone-releasing capsule (sc), and assessed for male sexual behavior at PND60. Following sexual behavior assessment, the area of the sexually dimorphic nucleus of the POA (SDN-POA) was measured using immunofluorescent staining techniques. In adults, normal sex differences in male sexual behavior and in the SDN-POA area were eliminated in nicotine-treated animals. Using novel analytical approaches to evaluate overall masculinization of the adult POA, we identified significant masculinization of the nicotine-treated female POA. In neonates (PND4), nicotine exposure induced trending alterations in methylation-dependent masculinizing gene expression and DNA methylation levels at sexually-dimorphic differentially methylated regions, suggesting that developmental nicotine exposure is capable of triggering masculinization of the rat POA via epigenetic mechanisms.

## 1. Introduction

Maternal tobacco smoke (TS) exposure has been extensively characterized as a major risk to fetal health. Epidemiological studies have found that smoking tobacco during pregnancy is not only related to increased infant mortality and decreased birth weight (Brooke et al., 1989; Picone et al., 1982b, a; Meyer and Comstock, 1972; Matsubara et al., 2000; Ernst et al., 2001; Hardy and Mellits, 1972) but also to persisting cognitive and behavioral impairments (Cornelius et al., 2011; Dunn and McBurney, 1977; Naeye and Peters, 1984; Rantakallio, 1983;

Eskenazi and Castorina, 1999; Ernst et al., 2001; Fergusson et al., 1993; Wakschlag et al., 1997; Dwyer et al., 2008). Children born to women who smoke tobacco are more likely to display attention deficit hyperactivity disorder (ADHD)-like behaviors (Dong et al., 2018; Streissguth et al., 1984; Ernst et al., 2001; Dunn et al., 1977; Kristjansson et al., 1989; Milberger et al., 1996; Naeye and Peters, 1984) and suffer learning impairment (Butler and Goldstein, 1973; Fried et al., 1992; Fried and Watkinson, 1990). Moreover, the behavioral effects of prenatal TS exposure are long-lasting and likely permanent (Cross et al., 2017). Despite the known detrimental effects of maternal TS exposure

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and ongoing public health efforts, nearly 9% of women in the United States smoke cigarettes while pregnant (Scherman et al., 2018).

Adult women are particularly vulnerable to the effects of tobacco smoking (Cross et al., 2017; Schnoll et al., 2007). Not only do they experience more difficulty with smoking cessation (Perkins, 2001; Piper et al., 2010), females experience higher rates of anxiety and depression associated with tobacco withdrawal than do males (Al'absi, 2006; Schnoll et al., 2007; Xu et al., 2008). Following acute nicotine exposure, females display a heightened hypothalamic pituitary adrenal (HPA) axis response and cortisol secretion as compared to males (Cao et al., 2010; Gentile et al., 2011). These effects are in part explained by sex hormones. Nicotine, the most addictive and neuroactive substance in tobacco smoke, exerts its effects via activation of the nicotinic acetylcholine receptor (nAChR). Estradiol and progesterone, the predominant circulating female sex hormones, are allosteric modulators of human nAChRs (Valera et al., 1992; Curtis et al., 2002), and have marked effects on nAChR expression in rats (Miller et al., 1984). In general, estrogens potentiate nAChR expression (Miller et al., 1984) and activity (Paradiso et al., 2001; Curtis et al., 2002; Jin and Steinbach, 2015), while progesterone inhibits activity (Ke and Lukas, 1996; Valera et al., 1992). Following the onset of estrous cycling during adolescence, fluctuations in estrogens and progesterone can have opposing effects on nAChR activity or expression (Cross et al., 2017).

Estrogen signaling is also critical to early developmental processes, including the sexual differentiation of the brain in rodents (Konkle and McCarthy, 2011; Rhoda et al., 1984; Amateau et al., 2004; Amateau and McCarthy, 2002, 2004; He et al., 2013; Lenz and McCarthy, 2010; Lenz et al., 2012; McCarthy et al., 2009, Schwarz and McCarthy, 2008; Wright et al., 2010; Wright and McCarthy, 2009; Lenz et al., 2011; McCarthy, 2008; McCarthy et al., 2002), a process initiated towards the end of gestation, well after sex determination, and continuing throughout early life to postnatal day (PND) 10 (Schwarz and McCarthy, 2008; McCarthy et al., 1997; McCarthy, 2008; McCarthy et al., 2002). The estrogens are derived locally in the brain following aromatization of androgens derived from the fetal testis. The differentially higher exposure of the developing male brain to estrogens results in brain regions that are sexually dimorphic in both structure and function (Lenz and McCarthy, 2010; Lenz et al., 2012; McCarthy, 2016; McCarthy et al., 2009; Schwarz and McCarthy, 2008; Wright and McCarthy, 2009; Wright et al., 2010; McCarthy et al., 1997; McCarthy, 2008). nAChR activity is detectable in the brain as early as gestational day (GD) 11 in rodents (Cross et al., 2017), well before the critical period for brain sexual differentiation (Dwyer et al., 2008, Schwarz and McCarthy, 2008; McCarthy et al., 1997; McCarthy, 2008; McCarthy et al., 2002). Exposure to nicotine during early development has been associated with sex-specific effects on neurotransmitter function (Slotkin et al., 2007), dendritic complexity (Mychasiuk et al., 2013), and even the inclusion of membrane potassium-chloride transport channels (Damborsky et al., 2012). While previous studies have examined the effects of nicotine exposure throughout the critical developmental period for brain sexual differentiation (GD18-PND10) (Klein et al., 2003; Lichtensteiger and Schlumpf, 1985; Chen et al., 2005; Dwyer et al., 2008), little is known about nicotine's effects on the differentiation of highly sexually-dimorphic brain regions, such as those that comprise the diencephalon (Konkle and McCarthy, 2011).

The preoptic area (POA) is one such region that displays robust structural and functional dimorphisms in rodents (Lenz et al., 2012; McCarthy et al., 2009, Schwarz and McCarthy, 2008; Wright et al., 2010). The rodent POA lies just anterior of the hypothalamus, and largely controls male sexual behavior (Schwarz and McCarthy, 2008; Dominguez et al., 2007; Wright and McCarthy, 2009; Hillarp et al., 1954; Larsson and Heimer, 1964; Markowski et al., 1994; McCarthy and Arnold, 2011). It is comprised of two neighboring subnuclei: the sexually dimorphic nucleus (SDN-POA), which is embedded with the larger medial preoptic nucleus, and the anteroventral periventricular nucleus (AVPV). Sexual differentiation of the POA is initiated around GD18,

when testosterone released by the developing testes is aromatized into estrogen in male POA neurons (McCarthy, 2008; McCarthy et al., 2002; Konkle and McCarthy, 2011). This local estradiol surge triggers a downstream cascade promoting neuronal survival in the SDN-POA (Schwarz and McCarthy, 2008; Wright and McCarthy, 2009; Wright et al., 2010). The absence of estradiol in the developing female POA leads to programmed cell death in the SDN-POA, resulting in dramatically smaller sub-nucleic size (Schwarz and McCarthy, 2008; McCarthy and Arnold, 2011). After sexual differentiation, the SDN-POA displays marked size differences between males and females: the male SDN-POA reaches nearly 5–7 times the volume of the female SDN-POA (Lenz et al., 2012; Gorski, 1978, Schwarz and McCarthy, 2008; McCarthy and Arnold, 2011; Wright and McCarthy, 2009; Wright et al., 2010).

Activation of the masculinized rodent POA via testosterone in adulthood results in male sexual behavior in the forms of mounting, intromissions, and ejaculations (McCarthy et al., 2009, Schwarz and McCarthy, 2008). Electro-stimulation of the medial POA increases male-like sexual behavior in both males (Malsbury, 1971) and females (Dominguez-Salazar et al., 2003). Correspondingly, adult males with lesioned POA lose their ability to perform copulatory behavior (Christensen et al., 1977; Larsson and Heimer, 1964; Malsbury, 1971). Females who were developmentally masculinized by estradiol treatment during the critical window for brain sexualization and gonadectomized as adolescents also display male-like sexual behavior if administered testosterone in adulthood (Lenz and McCarthy, 2010; Lenz et al., 2012; McCarthy, 2008; McCarthy and Arnold, 2011; McCarthy et al., 2002, 2009; Nugent et al., 2015). As such, adult male sexual behavior serves as a reliable readout for the masculinization status of the sexually-differentiated POA in both males and females.

Masculinization of the rodent POA was recently shown to require the inhibition of DNA methyltransferase enzymes (Dnmts), and an overall hypomethylated epigenetic state (Nugent et al., 2015). In contrast, active DNA methylation during the critical period was required for feminization of the POA. Estradiol mediates these effects; administration of estradiol during the critical window for POA sexual differentiation significantly reduces Dnmt activity in females, and masculinizes sexual behavior in adulthood (Nugent et al., 2015). Pharmacological inhibition of Dnmt activity in the female POA during this window similarly results in masculinized POA DNA methylation levels and adult sexual behavior (Nugent et al., 2015). Of note, Dnmt inhibition additionally upregulates expression of 24 of the 34 genes identified as sexually dimorphic in the POA, with higher expression in males (Nugent et al., 2015). This subset of 24 genes, termed methylation-dependent masculinizing genes (MDMGs), includes *Cyp19a1* encoding aromatase, the cytochrome p450 enzyme responsible for converting testosterone into estradiol in the masculinizing POA (Nugent et al., 2015; McCarthy, 2008, Schwarz and McCarthy, 2008; Wright et al., 2010). These findings suggest that hormone-induced alterations to DNA methylation in the developing POA are critical for POA sexual differentiation.

Nicotine exposure during development has been linked to alterations in DNA methylation patterning in the rodent brain in a limited number of studies. Developmental nicotine exposure is associated with a reduction in global 5-methylcytosine (CpG methylation) levels in both male and female mouse striatum and frontal cortex (FC) (Buck et al., 2019). A study examining DNA methylation patterns in mouse hippocampal and FC neurons noted that nicotine exposure downregulated Dnmt1 expression in both brain regions, and reduced promoter-specific CpG methylation in the FC. This downregulation was prevented in the FC following administration of mecamylamine, a noncompetitive nAChR channel blocker (Satta et al., 2008), suggesting that nicotine's ability to reduce Dnmt1 transcript levels is mediated via the nAChR, and providing important mechanistic links between nicotine, nAChRs, and DNA methylation.

Given the modulatory effects of hormones on nAChR expression and their critical role in the sexual differentiation of the rodent POA, we investigated the effects of developmental nicotine exposure on POA sexual differentiation by examining both structural and functional

outcomes in adults. Due to nicotine's ability to alter DNA methylation levels in the rodent brain (Buck et al., 2019; Satta et al., 2008), we further examined the epigenetic state of the neonatal rodent POA to elucidate potential developmental mechanisms underlying changes to the adult POA.

Here, we report masculinization of the nicotine-exposed female POA based on observed changes in 1) adult male-like sexual behavior and 2) adult SDN-POA area, supporting developmental impacts to POA structure and function. Nicotine-induced alterations in neonatal DNA methylation levels and MDMG expression levels further suggest that epigenetic mechanisms may at least partially underlie the phenotypes observed in the adult POA. Our findings are the first description of these associations and provide additional evidence corroborating females' enhanced vulnerability to nicotine's neuropsychological effects. This research not only contributes to our understanding of nicotine's ability to alter neurodevelopment, but also the sex-specific vulnerability of these alterations.

## 2. Methods

### 2.1. Animal husbandry, tissue collection, and behavioral testing

All experiments involving the handling of animals or processing of animal tissues were conducted with approval from the Duke Institutional Animal Care and Use Committee (IACUC) and in compliance with state and federal regulations. All animals were maintained on a reverse 12-h light/dark cycle, and provided with food and water *ad libitum*. Young adult male and female Sprague-Dawley rats between the weights of 225–250 g were obtained from Charles River Laboratories (Raleigh, NC, USA). Following two weeks of acclimation to our facilities, females were anesthetized (60 mg/kg ketamine +0.15 mg/kg dexmedetomidine administered intraperitoneally (ip), followed by 0.15 mg/kg atipamezole +5 mg/kg ketoprofen administered sc) as previously described (Hall et al., 2016), and surgically fitted sc with a Alzet 2ML4 mini-osmotic infusion pump (Durect Corp.; Cupertino, CA, USA) delivering either vehicle (dimethyl sulfoxide; DMSO) or nicotine (nicotine hydrogen tartrate salt, Sigma-Aldrich; Saint Louis, MO, USA) at a dose of 2 mg/kg/day via sc diffusion. A subset of females did not undergo surgery and were not fitted with pumps and served as our “no pump” controls.

The dose of nicotine we administered was chosen to model moderate maternal smoking based on previous reports that a sc dose of 2.0–3.0 mg/kg/day nicotine by osmotic minipump produces plasma nicotine levels in rats similar to those observed in pregnant moderate smokers (Trauth et al., 2000; Murrin et al., 1987; Benowitz and Jacob, 1984). A continuous infusion model of nicotine exposure was chosen to eliminate episodic hypoxia and ischemia that are associated with nicotine injections (Slotkin et al., 1987; Trauth et al., 2000), as well as the confound of maternal stress that accompanies forced smoke inhalation in rodents (Dwyer et al., 2008; Rees and Inder, 2005). Further, a gestational dose of 2 mg/kg/day of nicotine has been shown to cause neurochemical and behavioral changes in exposed offspring without impairing growth in either the exposed offspring (Slotkin et al., 1987; Slotkin, 1998) or the dam (Franke et al., 2007).

Nicotine dose was prepared based on the weight of the female on the day of surgery and adjusted for tartrate salt weight. Previous studies using an identical infusion model confirmed that despite pregnancy-related weight gain (at 2.0 mg/kg/day, the dose drops about one-third during pregnancy), the dose was maintained within a range comparable to moderate smoking (Hall et al., 2016). For early postnatal exposure, we relied on the lactational transfer of maternal nicotine to the pups (Oliveira et al., 2010) based on observations that the Alzet mini-osmotic pump continues to dose well past the 28-day marketed timeline (previous reports indicated up to a 39-day dose delivery period) (Hall et al., 2016). Following surgery, females were allowed to recover for three days.

Immediately following the three-day surgical recovery period, all females were mated with a drug-naïve adult male rat for five days. Females that developed an infection or displayed signs of injury or damage to the surgically implanted mini-osmotic pump following the five-day mating period were responsibly euthanized. Following mating, females were singly housed throughout pregnancy and allowed to undergo normal birthing processes. Cages were monitored for pups daily to detect timing of birth, and day of birth was considered PND0. Litter size and pup sex distributions were assessed on PND0. On PND0 and PND1, male and female pups from the vehicle (DMSO) treated group were injected sc with 100  $\mu$ L of either vehicle (sesame oil) or 100  $\mu$ g estradiol benzoate (Sigma-Aldrich) by litter, as previously described (Nugent et al., 2015). Pups treated with sesame oil were considered the “vehicle control” group, and pups treated with estradiol were considered the “estradiol-masculinized” group. PND0 and PND1 male and female pups from the nicotine-treated group were similarly injected sc with 100  $\mu$ L of vehicle (sesame oil) by litter, also as previously described (Nugent et al., 2015). Litters were culled to three males and three females on PND4, and the POA was harvested from culled animals for examination of epigenetic endpoints. To harvest the POA, a 1 mm section was taken at the optic chiasm on the ventral side of the whole PND4 brain using a cold brain block. The POA was then carefully dissected from this 1 mm section on a cold dissection platform, using the anterior commissure as a guide. Dissected POA tissue was flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processing for DNA and RNA extraction, as described below. Whole brain weight was also measured at this time. At PND21, pups were weaned and housed in same sex groups of three. At PND40, two males and two females were chosen from each litter from the vehicle control, estradiol-masculinized, and nicotine-treated groups to be surgically gonadectomized and implanted sc with a silastic capsule (30-mm length, 1.6-mm inner diameter, 3.17-mm outer diameter) containing crystalline testosterone (Sigma-Aldrich) to mimic male circulating levels of testosterone, as previously described (Nugent et al., 2015). Testosterone replacement is necessary to activate male sexual behavior in females masculinized during development (Nugent et al., 2015). All animals were single-housed following gonadectomy.

At PND50, animals from the vehicle control, estradiol-masculinized, and nicotine-treated groups were assessed for anxiety-like behavior and locomotion using the elevated plus maze (Med Associates, Inc.; Fairfax, VT, USA). The maze apparatus (142 cm x 104 cm x 76 cm) consists of two arms with high wall enclosures (15 cm) and two open arms with short railings (2 cm). Briefly, animals were placed in the center of the plus, and allowed to freely roam the apparatus for five minutes under dim light. Time spent in the open arms and the number of center crosses were recorded for each animal. At PND60, animals from the vehicle control, estradiol-masculinized, and nicotine-treated groups were assessed for male sexual behavior. Gonadectomized males and females were paired with a sexually receptive female (from the “no pump” control group) in a glass arena (50 cm x 25 cm x 30 cm) for 30 min under red light during the dark phase, and video recorded. Videos were later manually scored in a blinded fashion for male sexual behavior parameters, including the number of mounts and latency to first mount. Videos were also manually scored for female sexual behavior parameters (number of lordoses) in hormonally-primed females to ensure sexual receptivity. Lordosis quotients (LQ = number of receptive female lordoses per 10 mounts of paired animal) were calculated for all pairings to assess sexual receptivity, and those with LQ < 1 were discounted in subsequent analyses. Following sexual behavior assays, adult whole brain weight was measured after euthanasia between PND61 and PND72. Behavioral data was averaged within each litter by sex and is represented as a single data point for each sex and litter. Body weight was measured roughly twice per week between PND1-77, and anogenital distance (AGD) was measured using a digital caliper once per day between PND1-4. A full list of treatment groups is provided in Table S1.

## 2.2. Immunofluorescence microscopy

Male and female adults (PND75–77) were anesthetized and transcardially perfused with 200 mL of cold phosphate-buffered saline (PBS), followed by 250 mL of either 4% formalin or 10% neutral buffered formalin. Perfused brains were removed and placed in formalin overnight. Brains were then cryoprotected in 30% sucrose for four days, and subsequently stored in Tissue Tek O.C.T. freezing medium (Sakura Finetek USA; Dublin, OH, USA) at  $-80^{\circ}\text{C}$ . Brains were cryosectioned at  $40\ \mu\text{m}$ , and slices containing the SDN-POA were stored as floating sections in  $0.02\ \text{M}$  KPBS ( $1\times$ ). Following blocking with  $0.03\%$  Triton<sup>TM</sup> X-100 (Sigma-Aldrich) and  $2\%$  normal goat serum (NGS, Sigma-Aldrich) overnight at  $4^{\circ}\text{C}$ , sections were incubated with monoclonal mouse anti-calbindin D 28 K antibody (CB-955) (Sigma-Aldrich), a reliable and specific marker for the SDN-POA, diluted  $1:20,000$  in  $2\%$  NGS for  $72\ \text{h}$  at  $4^{\circ}\text{C}$ . Sections were then washed with  $1\times$  KPBS, and incubated for two hours at room temperature (RT) in goat anti-mouse IgG secondary antibody cross-adsorbed to Alexa Fluor 488 (Thermo Fisher Scientific; Waltham, MA, USA) diluted  $1:200$  in  $2\%$  NGS. Following incubation, sections were washed and mounted to glass microscope slides using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) and coverslipped. Mounted sections were dried overnight at RT, and subsequently imaged using the Keyence Fluorescence Microscope BZ-X800. Multiple sections were imaged per animal with both  $4\times$  and  $10\times$  objectives to capture the entirety of the SDN-POA, the area of which was measured in  $10\times$  images using the area measurement tool in Image J (Image Processing and Analysis in Java, NIH). The largest area measurement for each animal was used in final quantification.

## 2.3. Whole genome bisulfite sequencing analysis

Whole genome bisulfite sequencing (WGBS) data collected from male, female, and estradiol-masculinized PND4 POA (Nugent et al., 2015) was obtained from NCBI's Short Read Archive under BioProject ID 275796. Reads were mapped to the Rnor6 version of the rat genome using the Bismark alignment algorithm (Krueger and Andrews, 2011). Percent methylation was called via the *MethylKit* (Akalin et al., 2012) package from the R statistical programming environment (available at [www.r-project.org](http://www.r-project.org)). Due to the low coverage, percent methylation was called using  $250$  nucleotide-long bins tiling the whole genome every  $50$  nucleotides. Only windows that had at least four (un)methylated calls in each of the eight samples were considered for further analysis. Hierarchical clustering using a correlation distance with complete linkage was performed on the top  $25\%$  most variable sites to identify outlier samples and establish genome-wide similarity across samples. Sites were reported as differentially methylated if 1) all replicates of one condition were higher or lower than all replicates of the other condition, 2) the difference in percent methylation between all replicates was less than  $25\%$ , and 3) the difference in average percent methylation between the conditions was at least  $60\%$ . Using these thresholds,  $285$  differentially methylated regions (DMRs) were identified (Table S2), which mapped to  $134$  unique genes. Functional clusters from this gene list were derived using the DAVID Functional Annotation Tool (DAVID Bioinformatics Resources 6.7, NIAID/NIH) (Huang da et al., 2009; Huang et al., 2007).

## 2.4. Targeted DNA methylation quantification

Genomic DNA and total RNA were co-extracted from PND4 POA samples pooled in groups of two using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen; Valencia, CA, USA). DNA and RNA samples were eluted in nuclease-free water and assessed for concentration and purity using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). From each sample,  $800\ \text{ng}$  of genomic DNA was bisulfite-modified using the Zymo EZ DNA Methylation Kit (Zymo Research, Inc.; Irvine, CA, USA) for subsequent DNA methylation analyses. Bisulfite-treated samples were eluted in  $40\ \mu\text{L}$  nuclease-free water to achieve a final

concentration of  $20\ \text{ng}/\mu\text{L}$ , assuming  $100\%$  recovery. CpG methylation was examined at the *Dlgap1*, *Kcnn3*, *Nkain3*, and *Tab1* DMRs using bisulfite pyrosequencing as previously described (Murphy et al., 2012). Pyrosequencing assays for each DMR were validated for quantitative performance using methylation standards (EpigenDx Rat Methylation Standards, Cat No. 80-8060R-PreMix) of defined methylation levels:  $0\%$ ,  $25\%$ ,  $50\%$ ,  $75\%$ , and  $100\%$  methylated. Assay validation further informed the linearity and range of each assay. Validated pyrosequencing assay primers and PCR conditions are listed in Table S3. All pyrosequencing was performed using the Qiagen PyroMark Q96 MD Pyrosequencer, and percent CpG methylation was calculated using the PyroQ CpG Software (Qiagen) as an average across all CpGs within each sequence analyzed. Each pyrosequencing sample contained  $7\ \mu\text{L}$  PCR product generated using the following PCR conditions:  $95^{\circ}\text{C}$  for  $15\ \text{m}$ , then  $55$  cycles of  $94^{\circ}\text{C}$  for  $30\ \text{s}$ , [assay-specific] annealing temperature for  $30\ \text{s}$ ,  $72^{\circ}\text{C}$  for  $30\ \text{s}$ , followed by  $72^{\circ}\text{C}$  for  $10\ \text{m}$ . Assay-specific annealing temperatures were as follows: *Dlgap1*,  $58^{\circ}\text{C}$ ; *Kcnn3*,  $62^{\circ}\text{C}$ ; *Nkain3*,  $62^{\circ}\text{C}$ ; *Tab1*,  $62^{\circ}\text{C}$  (Table S3).

## 2.5. Quantitative real-time PCR

Gene expression levels for *Cyp19a1*, *Dnmt1*, *Dnmt3a*, *Ebf2*, *Ebf3*, and *Nr2f2* in PND4 POA were assessed using quantitative real-time PCR (RT-PCR) relative to the housekeeping gene *Gapdh*. Prior to quantification of experimental samples, RT-PCR assay efficiency and specificity were validated. Assay efficiency was calculated using a standard curve comprised of cDNA serial dilutions of  $16$ ,  $8$ ,  $4$ ,  $2$ ,  $1$ ,  $0.5$ , and  $0.25\ \text{ng}/\mu\text{L}$ . Standard curve amplicons were subsequently run on a  $2\%$  agarose gel to verify amplification specificity. Assays with targeted amplification of  $>90\%$  efficiency were used for subsequent analyses. Total RNA extracted from PND4 POA tissue was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences; Thermo Fisher Scientific). For each sample,  $500$ – $700\ \text{ng}$  of extracted RNA diluted in  $13.2\ \mu\text{L}$  nuclease-free water was combined with  $2\ \mu\text{L}$   $10\times$  RT buffer,  $0.8\ \mu\text{L}$   $25\times$  dNTP mix,  $2\ \mu\text{L}$   $10\times$  random primers,  $1\ \mu\text{L}$  reverse transcriptase ( $50\ \text{U}/\mu\text{L}$ ),  $1\ \mu\text{L}$  RNase inhibitor, and nuclease-free water to achieve a total reaction volume of  $20\ \mu\text{L}$ . Samples were reverse transcribed under the following conditions:  $25^{\circ}\text{C}$  hold for  $10\ \text{m}$ ,  $37^{\circ}\text{C}$  hold for  $120\ \text{m}$ , and  $85^{\circ}\text{C}$  hold for  $5\ \text{m}$ . Converted cDNA samples were diluted to a concentration of  $2\ \text{ng}/\mu\text{L}$  in nuclease free water prior to use in RT-PCR. RT-PCR assays were conducted using the SYBR Green reporting dye with ROX passive reference dye. cDNA ( $4\ \text{ng}$  in  $2\ \mu\text{L}$ ) was combined with  $12.5\ \mu\text{L}$  PowerSYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific),  $10.3\ \mu\text{L}$  nuclease-free water,  $0.1\ \mu\text{L}$  of  $10\ \mu\text{M}$  forward primer and  $0.1\ \mu\text{L}$  of  $10\ \mu\text{M}$  reverse primer to achieve a total reaction volume of  $25\ \mu\text{L}$ . RT-PCR was conducted using the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) and the following thermal cycler conditions:  $50^{\circ}\text{C}$  hold for  $2\ \text{m}$ ,  $95^{\circ}\text{C}$  hold for  $10\ \text{m}$ , then  $40$  cycles of  $95^{\circ}\text{C}$  for  $15\ \text{s}$  and  $62^{\circ}\text{C}$  (annealing temperature) for  $1\ \text{m}$ , followed by  $95^{\circ}\text{C}$  for  $15\ \text{s}$ ,  $60^{\circ}\text{C}$  for  $30\ \text{s}$ , and  $95^{\circ}\text{C}$  for  $15\ \text{s}$  to generate a melting curve. Cycle threshold (Ct) values were obtained for each sample, and difference in cycle threshold (Delta Ct) relative to *Gapdh* was used to determine fold change in expression. Fold change values for each gene were normalized to the vehicle control males. Primer sequences designed previously (Nugent et al., 2015) were as follows: *Cyp19a1* Fwd  $5'$  GCAAACCTACCACCTTCAAGAGT  $3'$ , Rev  $5'$  ATCTTGCTATTTTGCCTCAGAA  $3'$ ; *Dnmt1* Fwd  $5'$  GCTTTGACG GTGGCGAGAA  $3'$ , Rev  $5'$  TCTGCAAGAACTCGACCACAATC  $3'$ ; *Dnmt3a* Fwd  $5'$  TTTCTTGAGTCAACCCCGTGATG  $3'$ , Rev  $5'$  TGCAACTCAGCTTATCATTACA  $3'$ ; *Ebf2* Fwd  $5'$  ATGAGACGGTTT CAGGTCGTGTT  $3'$ , Rev  $5'$  TTTGATGCAGGGTGTAGCTTCTG  $3'$ ; *Ebf3* Fwd  $5'$  ACGCTTTGTCTACACTGCCCTAA  $3'$ , Rev  $5'$  TGCCGCCCTCTTCAGTAACA  $3'$ ; *Gapdh* Fwd  $5'$  TGGTGAAGTCGGTGTGAACGG  $3'$ , Rev  $5'$  TCACAAGAGAAGGCAGCCCTGGT  $3'$ ; *Nr2f2* Fwd  $5'$  CACGTCGACTCCGCCGAGTAC  $3'$ , Rev  $5'$  ACGAAGCAAAGCTTCCGAACCGT  $3'$ . All assays were optimized to an annealing temperature of  $62^{\circ}\text{C}$ .

## 2.6. Statistics

Prior to all statistical analyses, data sets were assessed for normality using the Shapiro Wilk's test. Normally-distributed data were analyzed using parametric statistics, including analysis of variance (ANOVA), analysis of covariance (ANCOVA), multivariate analysis of variance (MANOVA), and t-tests. Data that were not normally distributed were analyzed using non-parametric tests including the Kruskal-Wallis test. Student's *t*-test was used in *post hoc* analyses of multiple comparisons when appropriate. To account for testosterone-induced mounting behavior in females occurring as a result of testosterone replacement rather than developmental masculinization (Boehm et al., 1991), females with a mount count greater than – and an SDN-POA area less than – the average of the vehicle control males were excluded from all analyses. Four females met these criteria for exclusion. Prior to our comprehensive masculinization analyses, data were normalized to percent of the vehicle control males within each endpoint and log transformed. Variance within data sets was examined to ensure similarity across endpoints prior to combined repeated measures analysis. A mixed model approach was employed to examine repeated measures in our comprehensive masculinization analysis since we were only able to examine SDN-POA area in half of the animals evaluated for male sexual behavior. The mixed model approach is ideal for assessing repeated measures across incomplete data sets since individuals with missing values are not excluded from the analysis, unlike the MANOVA approach. In order to ensure that our masculinization parameters (male sex behavior and SDN-POA area) differed from non-masculinization parameters (anxiety-like behavior), our mixed model tested the interaction between the three main effects of treatment, sex, and measure type (positive or negative) with positive measure indicating masculinizing parameters and negative measure indicating non-masculinizing parameters. Due to the fact that our gene expression data sets were nearly complete, we employed a MANOVA approach for repeated measures analysis.

Statistical analyses were performed using either JMP Pro Version 13.0 (SAS Institute Inc.; Cary, NC, USA) or GraphPad Prism Version 7.00 for Mac OS X (GraphPad Software; LaJolla, CA, USA) software.

## 3. Results

### 3.1. Birth outcomes and weight characteristics

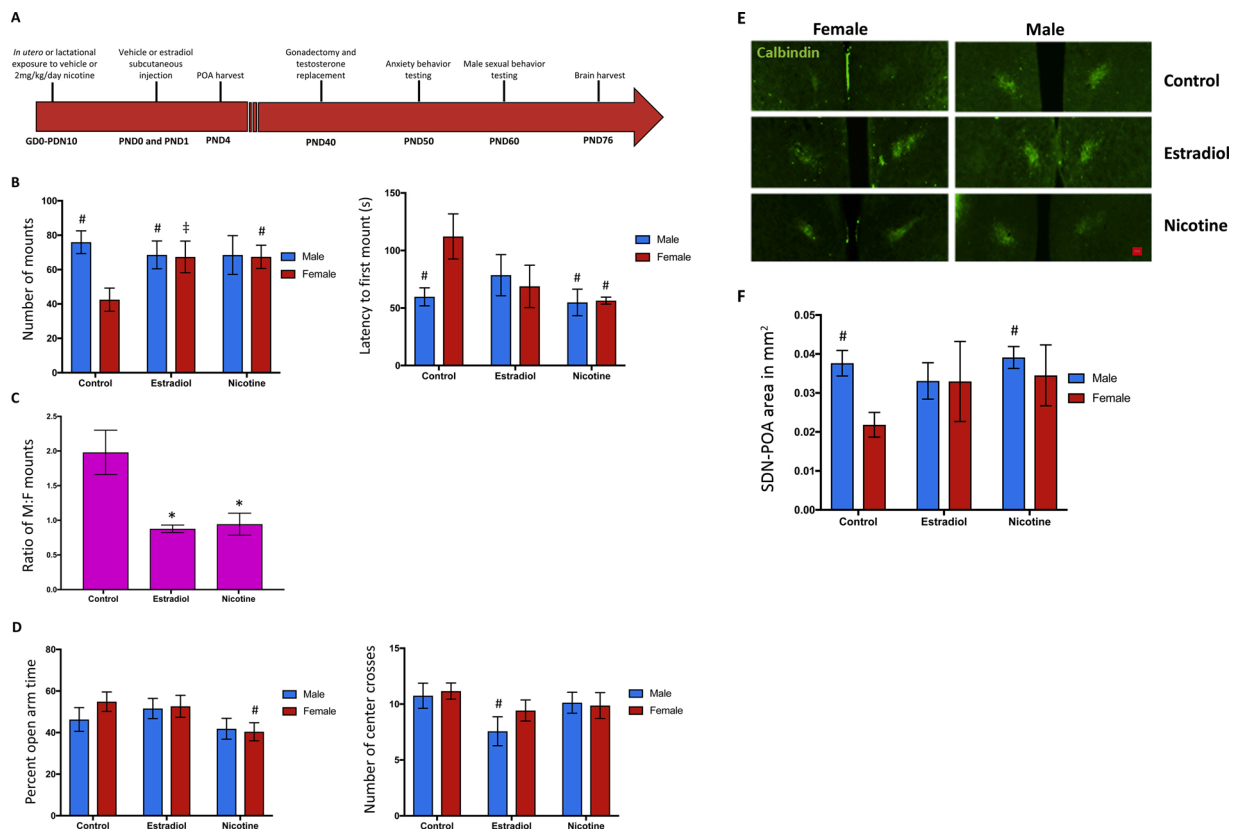
Birth outcomes and offspring weight were evaluated across all litters to examine potential confounding treatment effects. In alignment with previous findings (Bertolini et al., 1982; Slotkin et al., 1987) gestational treatment with 2 mg/kg/day nicotine had no effect on the number of pups born, the distribution of male and female pups within litters, (Table S4, Fig. S1A-B), sex-specific body weight (Fig. S2), or the emergence of sex differences in adult brain weight (Fig. S3A-B) relative to vehicle controls. In contrast, perinatal treatment with estradiol (PND0-PND1) affected body weight outcomes in both males and females (Fig. S2), and normal sex differences in adult brain weight (Fig. S3A-B), suggesting off-target developmental effects. Anogenital distance (AGD), an established indicator of perinatal androgen exposure, (van den Driesche et al., 2011) revealed sex-specific differences in PND1-4 neonates (Fig. S4). AGD was unaffected by gestational or perinatal treatment in male pups, indicating comparable androgen levels across all male exposure groups. Female pups injected with estradiol during PND0-1 displayed significantly higher AGD than all other treated females, indicating an expected androgen surge following neonatal estradiol administration (Fig. S4). Gestational nicotine exposure had no effect on AGD in either male or female pups (Fig. S4).

### 3.2. Developmental nicotine exposure eliminates structural and functional sex differences in the adult POA

Male sexual behavior and SDN-POA area were evaluated in adults developmentally exposed to either vehicle or nicotine in order to examine both the structural and functional masculinization of the POA. Animals perinatally exposed to estradiol (PND0-PND1) served as a positive control for POA masculinization. Prior to sexual behavior assessment, all animals were surgically gonadectomized and subcutaneously implanted with testosterone capsules, delivering adult male circulating levels of testosterone required to activate the masculinized POA in females (Fig. 1A). Three weeks following gonadectomy and testosterone replacement, animals were paired with a sexually receptive female, and scored for male sexual behavioral endpoints, including the number of mounts and latency to first mount. Female sexual behavior (number of lordoses) was simultaneously assessed among the sexually receptive females to examine potential confounding effects by treatment on their receptivity. We verified that receptive female lordosis quotient (LQ = # lordoses/10 mounts) was unaffected by the treatment of the paired animal (Fig. S5A), although sex of the paired animal did have an effect (Fig. S5B). Across both male sexual behavioral endpoints examined, we resolved expected sex differences among vehicle control animals that were lost in both estradiol-masculinized and nicotine-treated groups (Fig. 1B). Moreover, both the number of mounts and latency to first mount were masculinized in nicotine-treated females (Fig. 1B). The ratio of male to female mounts was significantly reduced in estradiol-masculinized and nicotine-treated animals compared to vehicle controls ( $p < 0.05$ ; Fig. 1C) due to an increase in mounting behavior in the females of both groups, supporting nicotine's ability to functionally masculinize the POA. In order to assess the selectivity of our experimental paradigm for testosterone-mediated behaviors, we additionally examined two non-testosterone mediated behaviors: anxiety-like behavior and locomotion (Fig. 1D). Sex differences in behavior were not observed among vehicle controls in either anxiety-like behavior or locomotion, validating the testosterone-mediated selectivity of our experimental paradigm (Fig. 1D). Shortly following the behavioral assays, the SDN-POA area was measured in all animals using immunofluorescent staining. Normal sex differences in adult SDN-POA area among vehicle control animals were not evident in both estradiol-masculinized and nicotine-treated groups (Fig. 1E), supporting nicotine's ability to eliminate structural sex differences in the POA.

### 3.3. Developmental nicotine exposure masculinizes the female POA

Masculinization of the POA was comprehensively assessed by combining metrics of POA structure (SDN-POA area) and function (male sexual behavior). We implemented two novel methods to comprehensively assess POA masculinization across multiple measurements (Fig. 2A). Using the first method, masculinization parameters (including male sex behavior and SDN-POA area) and non-masculinization parameters (including anxiety-like behavior) were normalized, log transformed, and combined in a mixed model repeated measures analysis. Within the repeated structure, the interaction between treatment, sex, and measure (masculinization or non-masculinization) was evaluated as a three-way ANOVA (Fig. 2A). This approach resolved POA masculinization in both nicotine-treated and estradiol-masculinized females, relative to vehicle control females (Fig. 2B). Further, vehicle control female masculinization parameters significantly differed from all other groups across masculinization and non-masculinization parameters ( $p < 0.05$ ), underscoring the ability of this model to differentiate between the two parameter types. Using the second approach, a masculinization index was calculated for each animal based on normalized and log transformed masculinization parameters (male sex behavior and SDN-POA area). This approach similarly resolved POA masculinization in nicotine-treated and estradiol-masculinized females, although to a lesser degree of statistical significance (Fig. 2B). Our findings support that both



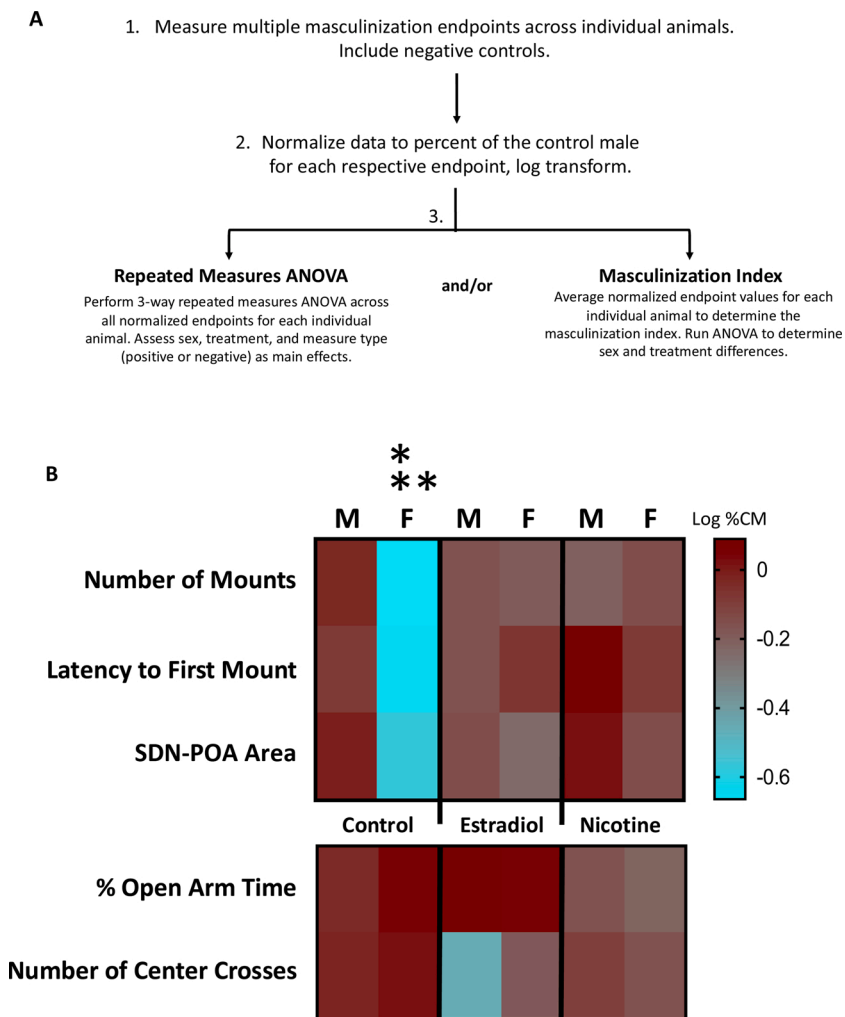
**Fig. 1.** Developmental nicotine exposure eliminates sexual dimorphisms in male sexual behavior and SDN-POA area. **A)** Animals were developmentally exposed to either vehicle (DMSO) or nicotine (2 mg/kg/day) throughout the window for POA sexualization (GD18-PND10). At PND0 and PND1, male and female pups were subcutaneously injected with either vehicle (sesame oil) or estradiol to induce masculinization. Animals were gonadectomized at PND40 and fitted with a testosterone replacement capsule. In all animals, anxiety-like behavior and locomotion were assessed at PND50, and male sexual behavior at PND60. Brains were harvested at PND76 to examine SDN-POA area. **B)** Male sexual behavior parameters measured in PND60 animals in the presence of a sexually receptive female. Vehicle control females displayed fewer mounts than vehicle control males (*a-priori t-test*,  $p = 0.0036$ ), estradiol-masculinized males (*a-priori t-test*,  $p = 0.0325$ ), and nicotine-treated females (*a-priori t-test*,  $p = 0.0227$ ). Number of mounts: (Vehicle) Control  $n = 9$  male,  $n = 7$  female; Estradiol  $n = 8$  male,  $n = 8$  female; Nicotine  $n = 8$  male,  $n = 7$  female. Vehicle control females took longer to mount a paired receptive female than vehicle control males (*a-priori t-test*,  $p = 0.0214$ ), nicotine-treated males (*a-priori t-test*,  $p = 0.022$ ), and nicotine-treated females (*a-priori t-test*,  $p = 0.0249$ ). Mount latency: (Vehicle) Control  $n = 8$  male,  $n = 7$  female; Estradiol  $n = 8$  male,  $n = 8$  female; Nicotine  $n = 8$  male,  $n = 6$  female. **C)** The ratio of male to female mounts was significantly reduced in nicotine-treated and estradiol-masculinized animals (one-way ANOVA  $p = 0.0022$ ,  $F = 8.751$ ). Mount ratio: (Vehicle) Control  $n = 7$  litters; Estradiol  $n = 7$  litters; Nicotine  $n = 7$  litters. **D)** Parameters of anxiety-like behavior and locomotion measured at PND50 using the elevated plus maze apparatus. Vehicle control females spent more time in the open arm of the elevated plus maze apparatus than nicotine-treated females (*a-priori t-test*,  $p = 0.0442$ ). Percent time in open arm: (Vehicle) Control  $n = 6$  male,  $n = 6$  female; Estradiol  $n = 7$  male,  $n = 7$  female; Nicotine  $n = 8$  male,  $n = 8$  female. Vehicle control females performed more center crosses than estradiol-masculinized males (*a-priori t-test*,  $p = 0.0418$ ). Center Crosses: (Vehicle) Control  $n = 6$  male,  $n = 6$  female; Estradiol  $n = 7$  male,  $n = 7$  female; Nicotine  $n = 8$  male,  $n = 8$  female. **E)** SDN-POA area visualized in 40 μm sections using immunofluorescent staining for calbindin. Scale bar represents 100 μm. Vehicle control female SDN-POA area was less than vehicle control males (*a-priori t-test*,  $p = 0.0133$ ) and nicotine-treated males (*a-priori t-test*,  $p = 0.0047$ ). SDN-POA Area (mm<sup>2</sup>): (Vehicle) Control  $n = 4$  male,  $n = 4$  female; Estradiol  $n = 3$  male,  $n = 3$  female; Nicotine  $n = 5$  male,  $n = 4$  female. **B-E) ‡**  $P = 0.05$  *a-priori t-test* compared to vehicle-treated females. #  $P < 0.05$  *a-priori t-test* compared to vehicle-treated females. \*  $P < 0.005$  Student's *t-test* post hoc analysis compared to vehicle-treated females following significant one-way ANOVA. N indicates the number of litters. Error bars represent standard error of the mean.

analysis methods are capable of statistically resolving POA masculinization in a comprehensive manner.

### 3.4. Nicotine exposure evokes changes in epigenetic mechanisms required for POA masculinization

To identify candidate mechanisms underlying nicotine-induced POA masculinization, we investigated DNA methylation levels in the neonatal POA based on evidence suggesting that developmental alterations to DNA methylation are critical to POA masculinization (Nugent et al., 2015). Using publicly-available whole genome bisulfite sequencing (WGBS) data obtained from neonatal male, female, and estradiol-masculinized female POA (see Fig. S6 for full methylation distributions and hierarchical clustering), we first identified differentially methylated regions (DMRs; 250 nucleotides in length) between males and females unexposed to nicotine. Specifically, we analyzed

those that were hypomethylated in males, based on previous work outlining POA masculinization to be, in part, due to the suppression of global DNA methylation (Nugent et al., 2015). To select for regions that were associated with masculinization, regions in which methylation levels were similar between males and estradiol-masculinized females were further prioritized. Finally, a stringent differential methylation threshold of 60% between unexposed males and females was employed to examine the most highly differentially-methylated, masculinizing regions. 285 DMRs were identified as statistically significant using these criteria (listed in Table S2). Using distance to nearest gene as a proxy for genomic location, we mapped 46% of these 285 DMRs to functional regions of the genome, including the proximal promoter region (up to 1000 bp upstream of gene), 5'UTR, 3' UTR, intron, and exon (Fig. 3A). Based on nearest gene name, our top 285 DMRs mapped to 134 unique genes (Table S2). Using the DAVID Functional Annotation Tool (DAVID Bioinformatics Resources 6.7, NIAID/NIH) (Huang da et al., 2009;



**Fig. 2.** Novel methods for assessing masculinization of the rat POA. **A)** Schematic outlining statistical approaches to comprehensively assess POA masculinization, including repeated measures analysis and masculinization index. **B)** Heat map representing normalized and log-transformed values for masculinization parameters, including male sex behavior (number of mounts, latency to first mount), and SDN-POA area, as well as negative control parameters, including anxiety-like behavior (% open arm time, number of center crosses) across individual animals. The normalized and log transformed values for masculinization parameters in vehicle control females significantly differed from all other groups and behaviors when assessed as a repeated measures three-way ANOVA (treatment\*sex\*measure  $p = 0.0234$ ,  $F = 3.853$ ) and masculinization index two-way ANOVA (treatment\*sex  $p = 0.0357$ ,  $F = 3.586$ ). (Vehicle) Control  $n = 9$  male,  $n = 9$  female; Estradiol  $n = 9$  male,  $n = 9$  female; Nicotine  $n = 8$  male,  $n = 8$  female. \* $P < 0.02$ , \*\* $P < 0.05$  Student's *t*-test post hoc analysis compared to vehicle-treated females following significant three-way repeated measures ANOVA or two-way masculinization index ANOVA, respectively. N indicates the number of litters.

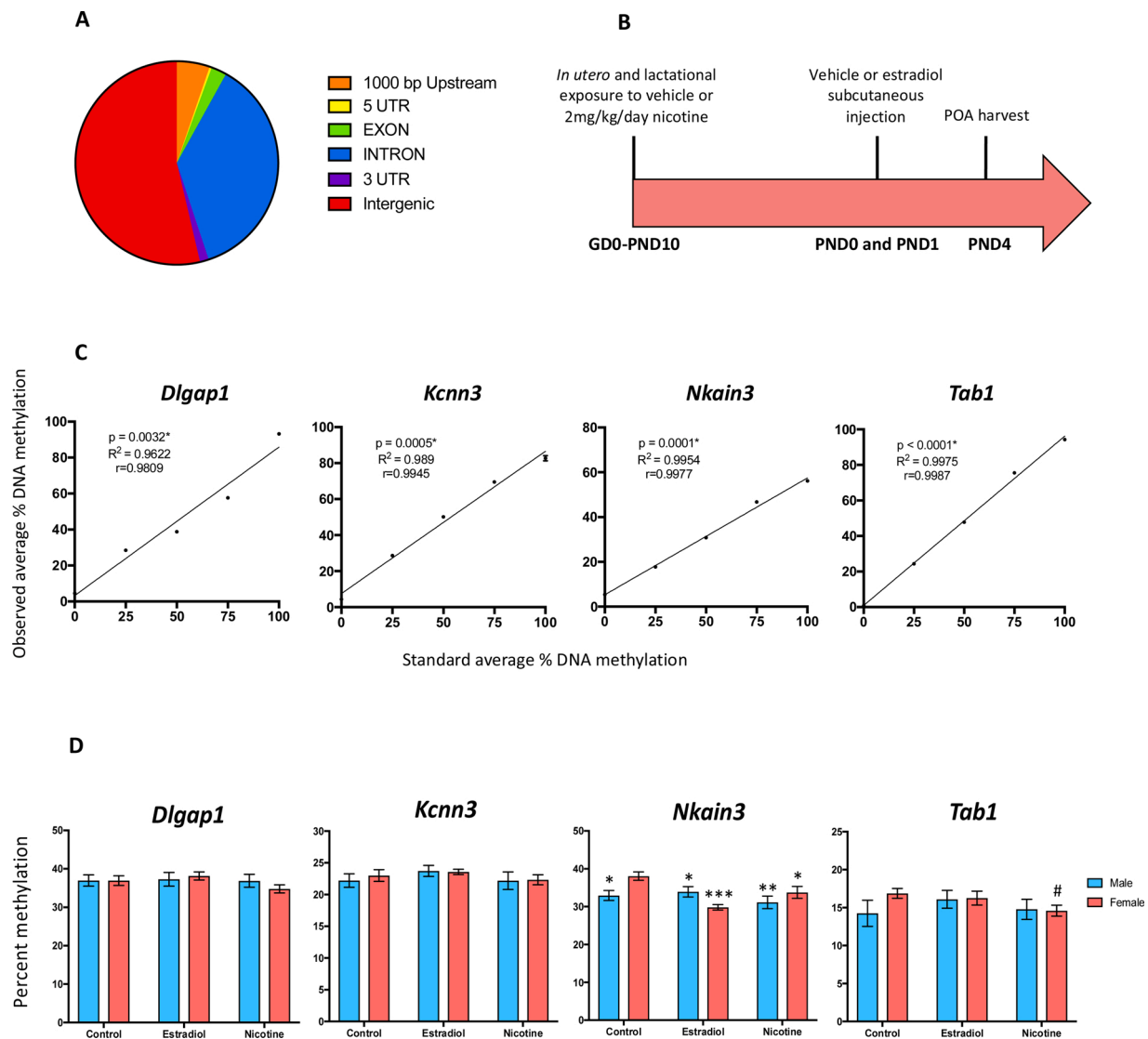
Huang et al., 2007), we identified the following enriched functional clusters among these genes: phosphatidylinositol binding, the phox homologous domain, and synapse/cell junction formation (Table S5). However, only the phox homologous domain functional cluster maintained statistical significance following Benjamini correction for multiple comparisons.

DNA methylation levels were examined at four of the top identified DMRs in neonatal (PND4) POA (Fig. 3B), based on their genomic locations, levels of differential methylation, and cellular function (Table S6). Bisulfite pyrosequencing assays were optimized within these DMRs (#CpGs): *Dlgap1* (2 CpGs), *Kcnn3* (2 CpGs), *Nkain3* (2 CpGs), and *Tab1* (1 CpG) (Table S3), and subsequently validated for their ability to detect methylation across the dynamic range (Fig. 3C). In examination of DNA methylation levels, we were not able to validate the expected magnitude of sex difference among vehicle control animals (Table S6, Fig. 3D). However, exposure and sex effects, albeit of smaller magnitude, were detected at both *Nkain3* and *Tab1* (Fig. 3D). DNA methylation levels at the *Tab1* DMR in nicotine-treated females were significantly lower than vehicle control females (*a-priori t*-test,  $p = 0.0410$ ; Fig. 3D). At *Nkain3*, DNA methylation was significantly increased in the vehicle control females relative to all other groups ( $p < 0.05$ , Student's *t* post hoc analysis following two-way ANOVA:  $p = 0.0061$ , sex\*treatment  $p = 0.0097$ ; Fig. 3D), suggesting masculinization of the *Nkain3* DMR DNA methylation levels in both estradiol-masculinized and nicotine-treated neonatal females.

Expression of MDMGs, or genes upregulated by *Dnmt* inhibition in the masculinized POA (Nugent et al., 2015), were additionally evaluated

given their association with alterations to neonatal global DNA methylation in the POA. Normalized gene expression levels were measured across four previously identified MDMGs: *Cyp19a1*, *Ebf2*, *Ebf3*, and *Nr2f2*. Expression levels of *Dnmt1* and *Dnmt3a* were concurrently quantified based on our expectation that their expression should not be altered by treatment or sex (Nugent et al., 2015). While expected sex differences in MDMG expression were not statistically resolved among vehicle control animals (Fig. 3E) trending sex differences were observed when relative expression values were normalized as a ratio of male to female expression (Fig. 3F). This ratio was diminished in both estradiol-masculinized and nicotine-treated animals compared to vehicle controls in all four genes examined, supporting a treatment-induced loss of normal sex difference in MDMG expression. This trend was significant in only one case, *Nr2f2* (*a-priori t*-test,  $p = 0.0499$ ) (Fig. 3F). As expected, *Dnmt1* and *Dnmt3a* expression levels were similar across sexes and treatments (Fig. 3E–G).

Repeated measures analysis indicated that MDMG expression levels across *Cyp19a1*, *Ebf2*, *Ebf3*, and *Nr2f2* were higher in nicotine-exposed animals compared to vehicle controls (two-way MANOVA  $p = 0.0716$ , treatment main effect  $p = 0.0113$ , sex main effect  $p = 0.9752$ , sex\*treatment  $p = 0.8749$ ) (Fig. 3G). This trend was significant at *Ebf2* (two-way ANOVA between nicotine and vehicle control groups,  $p = 0.1146$ , treatment main effect  $p = 0.0259$ ) (Fig. 3D and G), underscoring nicotine's ability to collectively alter MDMG expression in the developing POA.



**Fig. 3.** Nicotine exposure evokes upregulation of epigenetic mechanisms required for POA masculinization in neonates. **A)** Pie chart outlining genomic location distribution of the top 285 masculinizing differentially methylated regions (DMRs) between male and female POA. Differential methylation was calculated using publicly available WGBS data obtained from neonatal male, female, and estradiol-masculinized female POA (Nugent et al., 2015). **B)** Prior to targeted validation of DMRs, animals were developmentally exposed to either vehicle (DMSO) or nicotine (2 mg/kg/day) throughout the window for POA sexualization (GD18-PND10). At PND0 and PND1, male and female pups were subcutaneously injected with either vehicle (sesame oil) or estradiol to induce masculinization. At PND4, POA was isolated in male and female pups, and co-extracted for DNA and RNA. **C)** Validation of range and linearity of bisulfite pyrosequencing assays designed for four of the top 285 DMRs. Bisulfite-treated rat DNA methylation standards serially prepared at 0%, 25%, 50%, 75%, and 100% methylation were used to correlate the input versus measured methylation values for the following DMRs: *Dlgap1*, *Kcnn3*, *Nkain3*, and *Tab1*. Data generated was analyzed using Pearson’s correlation (*r*). *Dlgap1*: *r* = 0.9808, *p* = 0.0032; *Kcnn3*: *r* = 0.9945, *p* = 0.0005; *Nkain3*: *r* = 0.9977, *p* = 0.0001; *Tab1*: *r* = 0.9987, *p* < 0.0001. *N* = 3 replicates for each serial DNA standard. **D)** Average percent DNA methylation across each DMR measured in control and treated PND4 POA. DNA methylation at the *Nkain3* DMR was significantly hypermethylated in vehicle control females compared to all other groups (two-way ANOVA *p* = 0.0061, *F* = 4.111, sex\*treatment *p* = 0.0097). DNA methylation at the *Tab1* DMR was hypermethylated in vehicle control females compared to nicotine-treated females (*a-priori t-test*, *p* = 0.0410). *Dlgap1*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 6 male, *n* = 7 female. *Kcnn3*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 6 male, *n* = 7 female. *Nkain3*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 6 male, *n* = 7 female. *Tab1*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 6 male, *n* = 7 female. **E)** Expression of methylation-dependent masculinizing genes (MDMGs=*Cyp19a1*, *Ebf2*, *Ebf3*, and *Nr2f2*) and Dnmts (*Dnmt1* and *Dnmt3a*) in control and treated PND4 POA. For each gene, expression was measured across multiple cohorts as fold change relative to the vehicle control male within each cohort, and subsequently normalized as percent of the control males across combined cohorts. Although there was no significant main effect of sex, *Ebf2* expression was significantly higher in nicotine treated animals compared to vehicle controls (two-way ANOVA between nicotine and vehicle control groups *p* = 0.1146, *F* = 2.340, sex main effect *p* = 0.8612, treatment main effect *p* = 0.0259, sex\*treatment *p* = 0.6165). *Cyp19a1*: (Vehicle) Control *n* = 6 male, *n* = 5 female; Estradiol *n* = 5 male, *n* = 4 female; Nicotine *n* = 7 male, *n* = 6 female. *Ebf2*: (Vehicle) Control *n* = 4 male, *n* = 6 female; Estradiol *n* = 4 male, *n* = 3 female; Nicotine *n* = 4 male, *n* = 5 female. *Ebf3*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 6 male, *n* = 6 female. *Nr2f2*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 7 male, *n* = 7 female. *Dnmt1*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 7 male, *n* = 7 female. *Dnmt3a*: (Vehicle) Control *n* = 6 male, *n* = 6 female; Estradiol *n* = 5 male, *n* = 5 female; Nicotine *n* = 7 male, *n* = 6 female. **F)** Heat map representing sex differences in normalized fold change values across MDMGs and Dnmts. Expression represented as the ratio of male to female normalized fold change values. M:F expression of *Nr2f2* was higher in vehicle controls compared to nicotine treated animals (*a-priori t-test*, *p* = 0.0499). *Cyp19a1*: (Vehicle) Control *N* = 5 litters; Estradiol *N* = 4 litters; Nicotine *N* = 6 litters. *Ebf2*: (Vehicle) Control *N* = 4 litters; Estradiol *N* = 3 litters; Nicotine *N* = 4 litters. *Ebf3*: (Vehicle) Control *N* = 5 litters;



Estradiol N = 5 litters; Nicotine N = 6 litters. *Nr2f2*: (Vehicle) Control N = 6 litters; Estradiol N = 5 litters; Nicotine N = 7 litters. *Dnmt1*: (Vehicle) Control N = 6 litters; Estradiol N = 5 litters; Nicotine N = 7 litters. *Dnmt3a*: (Vehicle) Control N = 6 litters; Estradiol N = 5 litters; Nicotine N = 5 litters. **G**) Heat map representing treatment differences in normalized fold change values across MDMGs and Dnmts. MDMG expression was significantly increased in the POA of nicotine-treated neonates as compared to vehicle controls (two-way MANOVA  $p = 0.0716$ ,  $F = 2.909$ , treatment main effect  $p = 0.0113$ , sex main effect  $p = 0.9752$ , sex\*treatment  $p = 0.8749$ ). *Cyp19a1*: (Vehicle) Control n = 6 male, n = 5 female; Estradiol n = 5 male, n = 4 female; Nicotine n = 7 male, n = 6 female. *Ebf2*: (Vehicle) Control n = 4 male, n = 6 female; Estradiol n = 4 male, n = 3 female; Nicotine n = 4 male, n = 5 female. *Ebf3*: (Vehicle) Control n = 6 male, n = 6 female; Estradiol n = 5 male, n = 5 female; Nicotine n = 6 male, n = 6 female. *Nr2f2*: (Vehicle) Control n = 6 male, n = 6 female; Estradiol n = 5 male, n = 5 female; Nicotine n = 7 male, n = 7 female. *Dnmt1*: (Vehicle) Control n = 6 male, n = 6 female; Estradiol n = 5 male, n = 5 female; Nicotine n = 7 male, n = 7 female. *Dnmt3a*: (Vehicle) Control n = 6 male, n = 6 female; Estradiol n = 5 male, n = 5 female; Nicotine n = 7 male, n = 6 female. **C-G**) \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  Student's *t*-test post hoc analysis compared to vehicle-treated females following significant two-way ANOVA. #  $P < 0.05$  a-priori *t*-test compared to vehicle-treated females. ##  $P < 0.05$  treatment as main effect between vehicle control and nicotine-treated groups following two-way ANOVA. ###  $P < 0.05$  treatment as main effect between vehicle control and nicotine-treated groups in a repeated MANOVA. N indicates the number of litters. Error bars represent standard error of the mean.

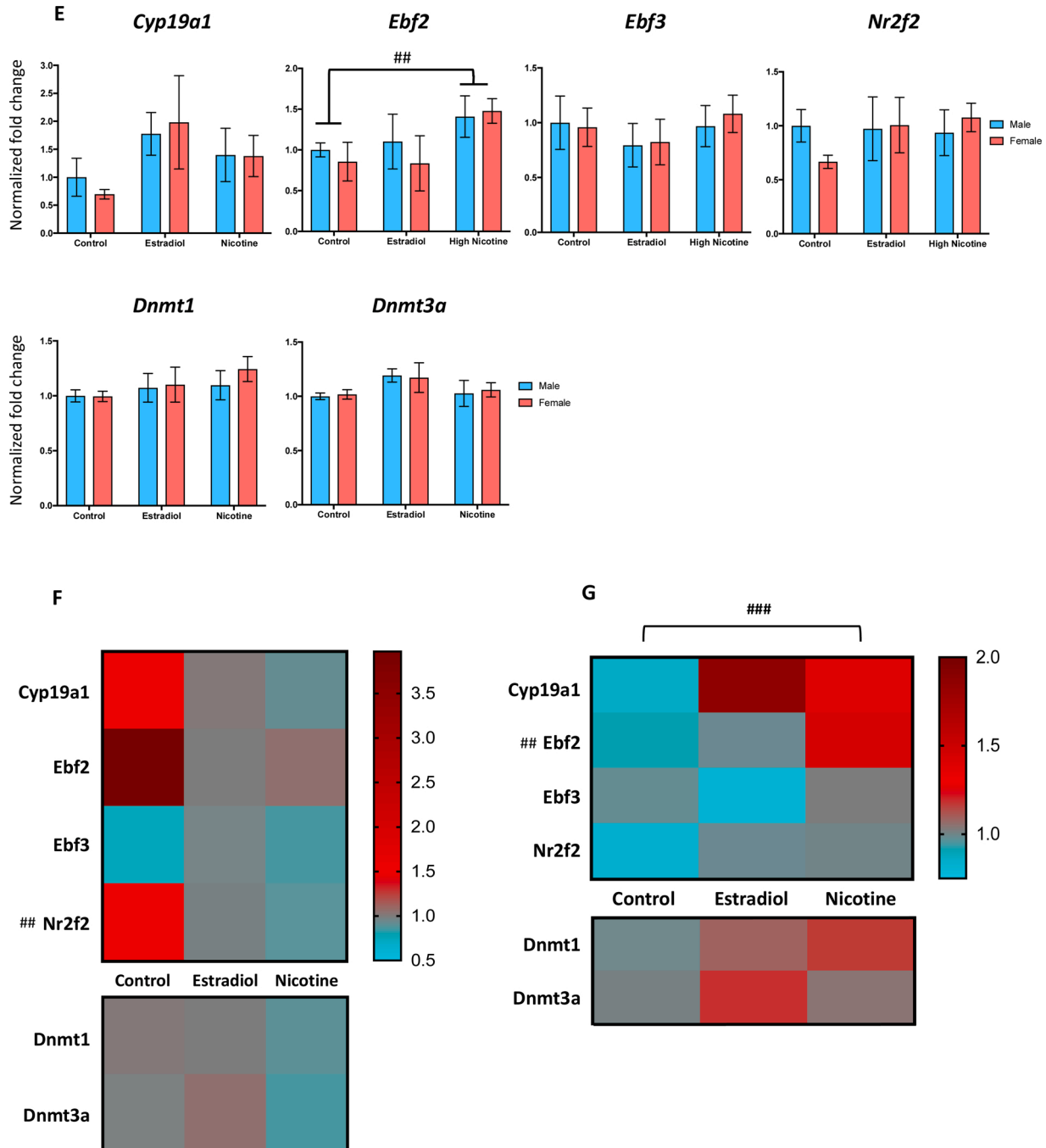


Fig. 3. (continued).

#### 4. Discussion

Nicotine induces sexually-dimorphic changes during neurodevelopment, likely through interactions between sex hormones and the nicotinic acetylcholine receptor (nAChR), as both estradiol and progesterone directly modulate nAChR expression and activity (Cross et al., 2017). These interactions support the notion that hormonally regulated developmental windows like the sexual differentiation of the brain might be particularly vulnerable to nicotine exposure. In this study, the effects of developmental nicotine exposure on the sexual differentiation of the POA were investigated using a rat model. We report that exposure to nicotine during the critical window for brain sexual differentiation masculinized the female POA by 1) eliminating normal sex differences in POA structure and function, and 2) altering the epigenetic mechanisms that induce POA masculinization during perinatal development. In order to comprehensively evaluate masculinization, we combined behavioral and structural endpoints into both a repeated measures analysis and masculinization index. Both approaches revealed significant masculinization of the nicotine-exposed female POA. This study is the first to demonstrate that exposure to nicotine during the perinatal period for brain sexual differentiation is capable of comprehensively masculinizing the female rodent POA, both in structure and function.

There were several advantages to evaluating POA masculinization across neonatal and adult timepoints. In addition to demonstrating the persistence of developmental alterations to SDN-POA structure, we were able to examine the extent to which testosterone replacement following surgical gonadectomy induced mounting behavior in females, independent of developmental POA masculinization. This additional measure is imperative, as testosterone-induced mounting behavior in females has been reported (Boehm et al., 1991) and yet is seldom controlled-for in masculinization studies. Both the repeated measures analysis and masculinization index enabled the assessment of multiple measurements across individual animals, all of which contributed to the same phenotype of POA masculinization. The masculinization index analysis approach is best applied to data sets with missing values, whereas the repeated structure is ideal for complete data sets. Despite having data sets for behavior and SDN-POA area that were not equal in size, we resolved slightly higher statistical significance among the nicotine-treated female group using the repeated measures approach. To account for missing values in our repeated measures analysis, we employed a mixed model approach, as opposed to the traditional MANOVA. We encourage future studies examining POA masculinization to adopt a comprehensive analytical approach as described above, and to use these measurements to control for testosterone-induced mounting behavior in female subjects.

Another strength of our experimental model was our ability to examine exposure effects during both the organizational and activational periods of POA sexual differentiation. While structural differences within the POA appear by the end of the organizational period in early development, functional differences, such as the ability to perform male sexual behavior, can only be resolved following hormonal activation in adolescence and adulthood, or the activational period (Schwarz and McCarthy, 2008; Wright et al., 2010; McCarthy, 2016, 2008; McCarthy and Arnold, 2011; Nugent et al., 2015). Indeed, our findings underscore the idea that deleterious exposure during critical, organizational periods of development can exhibit persistent, functional outcomes later in life. This phenomenon, known as the Developmental Origins of Health and Disease, or DOHaD (Suzuki, 2018), has not been previously examined within the context of nicotine and the sexual differentiation of the POA.

This study was additionally strengthened by our targeted approach to identifying differentially methylated regions across the POA epigenome. Using publicly-available WGBS data and stringent thresholds for differential methylation, we mapped highly differentially methylated regions to functional areas of the genome. A previous examination of these data using lower thresholds for differential methylation (>10%) mapped less than 25% of differentially-methylated CpGs to functional

genomic locations (Nugent et al., 2015). Almost half of the masculinizing DMRs identified in this study using more stringent thresholds for differential methylation (>60%) mapped to functional genomic regions in the POA. Our results suggest that masculinizing DMRs characterized by high levels of differential methylation more strongly localize to functional genomic regions than those with low differential methylation, potentially underscoring their increased functional relevance in the POA. These results not only guided our targeted studies, in which we validated sex differences and treatment-associated masculinization at one of the four DMRs examined, but also revealed potential mechanistic underpinnings of altered DNA methylation in the masculinizing POA. Functional annotation clustering of the top masculinizing DMRs in the POA revealed the phox homologous domain as the most highly enriched functional cluster. The phox homology (PX) domains interact with the cell membrane and bind phosphatidylinositol phospholipids, often used as organelle identity markers (Chandra et al., 2019). Genes associated with top identified masculinizing DMRs that were enriched in this cluster included those from the sorting nexin (Snx) protein family, all of which contain PX domains (Shin et al., 2007; Chandra et al., 2019) and are implicated in membrane signaling and trafficking (Chandra et al., 2019).

Snx proteins have also been associated with neurological function (Shin et al., 2007; Binda et al., 2019; Mizutani et al., 2012). *Snx27*, a gene that was among our top masculinizing DMRs, was found to localize to the post-synaptic density and facilitate excitatory glutamergic neurotransmission via AMPA-type glutamate receptors (Binda et al., 2019), which are also critical to the early organization of the masculinizing POA in which dendritic spine density is increased (Lenz et al., 2011, 2012). Although not among our list of top DMRs, *Snx9* and *Snx12* have been associated with presynaptic vesicle endocytosis (Shin et al., 2007) and neurite outgrowth (Mizutani et al., 2012), respectively. *Snx12* was further implicated in cortical neurodevelopment, with expression levels increasing during embryogenesis, and decreasing shortly after birth, closely following the developmental period for brain sexual differentiation (Mizutani et al., 2012). Although we did not perform targeted DNA methylation analyses on *Snx* genes, results from our functional annotation clustering can inform future studies examining epigenome-wide changes in the neonatal POA.

In the neonatal POA, we determined that nicotine-induced masculinization might be the result of developmental alterations to DNA methylation patterning and MDMG expression that are characteristic of the masculinized POA. These findings corroborate the limited evidence supporting nicotine's ability to alter DNA methylation patterns in the rodent brain, specifically via downregulation of *Dnmt1* expression (Satta et al., 2008) and CpG methylation levels (Satta et al., 2008; Buck et al., 2019). Although we did not observe exposure-induced changes to *Dnmt1* transcript levels in the POA, it would be of interest to examine nAChR expression and activity in the developing rodent POA given the reported associations between nAChR activity and CpG methylation, albeit tissue-specific (Satta et al., 2008). This is especially important in light of 1) nAChR's mechanistic links to estradiol, the sex hormone responsible for developmentally masculinizing the POA (Konkle and McCarthy, 2011; Rhoda et al., 1984; Amateau et al., 2004; Amateau and McCarthy, 2002, 2004; He et al., 2013; Lenz and McCarthy, 2010; Lenz et al., 2012; McCarthy et al., 2009; Schwarz and McCarthy, 2008; Wright et al., 2010; Wright and McCarthy, 2009; Lenz et al., 2011; McCarthy, 2008; McCarthy et al., 2002), 2) nAChR's mechanistic links to *Bcl2* (Grando, 2014), an anti-apoptotic protein involved in POA masculinization (Wright et al., 2010), and 3) nAChR activity detected in the developing rodent brain as early as GD11 (Cross et al., 2017), nearly a week before the sexual differentiation of the brain is initiated. Nicotine exposure inhibits aromatase, the enzyme critical to the conversion of testosterone into estradiol, in hypothalamic-related brain regions including the POA (Barbieri et al., 1986). As aromatization is necessary for POA masculinization (Nugent et al., 2015; Wright and McCarthy, 2009; Wright et al., 2010), this interaction would potentiate a

phenotype opposite to that described in this study. However, as reported aromatase inhibition was not POA-specific, it is possible that nicotine does not inhibit aromatization in the POA, or at least to an extent that does not impede brain masculinization. Further, our observations that sex-specific androgen levels were not disrupted during the perinatal period by developmental nicotine exposure support the notion that nicotine-induced masculinization of the POA is occurring independently of hormone-mediated pathways.

In our model, nicotine exposure was administered during the early, organizational window for POA sexual differentiation, spanning from late gestation through the early postnatal period. With osmotic diffusion of nicotine during gestation and lactational transfer during early postnatal development, our exposure model was ideal for covering the full developmental window for POA sexual differentiation (GD18-PND10) (Schwarz and McCarthy, 2008; McCarthy et al., 1997; McCarthy, 2008; McCarthy et al., 2002). However, recent evidence suggests this window may extend beyond the perinatal period (Nugent et al., 2015). While adulthood has been traditionally considered the “activational” period for brain sexual differentiation, organizational events can occur up until, and even during, adolescence (Lenroot et al., 2007; Yates and Juraska, 2008; Herting et al., 2014, 2012; Nunez et al., 2001, 2002; Willing and Juraska, 2015; Pinos et al., 2001). This idea is supported by the fact that exposure to nicotine during adolescence alone can have sex-specific effects in the brain (Cross et al., 2017), simultaneously challenging the permanence of developmentally-established sex differences in the brain, as well as understanding of the DOHaD phenomenon (Suzuki, 2018). Given our findings, it will be of utmost importance to examine the effects of exposure during adolescence both as a separate exposure window, and in combination with developmental exposure, especially given the recent uptick of nicotine use during adolescence via electronic nicotine delivery systems (ENDS) (England et al., 2015).

Limitations to this study include the fact that DNA methylation and gene expression were analyzed in POA tissue samples containing both neurons and glia, which display considerable differences in DNA methylation patterning (Lister et al., 2013), potentially reducing our ability to detect changes in the reported differential methylation (Nugent et al., 2015). Additionally, because examination of adult male-like sexual behavior required testosterone administration during adolescence, we were limited in our ability to detect potential exposure-related disruptions to endogenous hormone levels during adolescence and adulthood. This is of particular relevance as prenatal nicotine exposure has been correlated with reduced testosterone levels in adult male rats (Segarra and Strand, 1989). However, by measuring AGD during the perinatal period (PND1-4), we were able to verify that developmental nicotine exposure did not alter normal androgen levels in neonates (van den Driesche et al., 2011).

As mentioned previously, adult women are particularly vulnerable to nicotine’s psychopharmacological effects, experiencing more difficulty with smoking cessation and abstinence than men. Although the underlying mechanisms remain unclear, this sex-specific vulnerability is suspected to have developmental origins, particularly during the perinatal and adolescent hormone-mediated sexual differentiation of the brain (Cross et al., 2017). Our findings support this idea by demonstrating that an additional sex-specific outcome in adult females is linked to developmental exposure: nicotine-associated reorganization during the early perinatal period for brain sexual differentiation resulted in pronounced behavioral impacts later in life. While drawing parallels in humans is difficult, especially given the fact that eliciting male sexual behavior in our model required activation via exogenous hormones in females, there are human studies that corroborate the phenotypes observed in this study. First, the higher incidence of same-sex sexual orientation among

women suffering from congenital adrenal hyperplasia (CAH), a genetic abnormality that leads to hyper-elevated androgen production throughout life (Gondim et al., 2018), suggests that constant, elevated androgen levels can both developmentally-masculinize and adolescently-activate sexual behavior.

While sexual preference is not entirely indicative of POA masculinization, same-sex sexual orientation in young adult females is additionally associated with gestational exposure to tobacco smoke (Ellis and Cole-Harding, 2001). This association is derived using robust surveying methods across over 7,000 mother-offspring pairs to assess the incidence of tobacco use, and other stressors, during pregnancy (Ellis and Cole-Harding, 2001). Complementing this finding, recent genome-wide association studies (GWAS) and phenotypic-wide association studies (PheWAS) across large human cohorts reveal that maternal smoking at the time of birth is associated with the same single nucleotide polymorphisms (SNPs) implicated in adult same-sex sexual behavior (Ganna et al., 2019). Although sex-specific effects were not elucidated, the observed associations were directionally similar, suggesting that maternal smoking at the time of birth could be associated with same-sex preference in human adults (Ganna et al., 2019). As neurobehavioral mechanisms underlying same-sex attraction are manifold, certainly further research is needed before drawing any firm conclusions.

## 5. Conclusions

Our results indicate that exposure to nicotine during the critical window for brain sexual differentiation is capable of masculinizing the female POA, both in structure and function. These phenotypes were further associated with disruption of epigenetic mechanisms required for POA masculinization during perinatal development, providing insights into organizational mechanisms and the persistence of developmental effects. Using novel statistical approaches, we combine multiple measurements of masculinization to demonstrate comprehensive exposure-associated effects. Results from this study underscore our previous understanding of female’s enhanced vulnerability to nicotine’s effects, and importantly provide developmental links to these events. Examining estradiol’s interaction with the nAChR in the developing POA might provide causative mechanistic insights into the phenotypes observed here. This would be of particular interest given estradiol’s critical role in POA masculinization (Konkle and McCarthy, 2011; Rhoda et al., 1984; Amateau et al., 2004; Amateau and McCarthy, 2002, 2004; He et al., 2013; Lenz and McCarthy, 2010; Lenz et al., 2012; McCarthy et al., 2009; Schwarz and McCarthy, 2008; Wright et al., 2010; Wright and McCarthy, 2009; Lenz et al., 2011; McCarthy, 2008; McCarthy et al., 2002) and established modulatory effect on nAChR expression in the rodent brain (Cross et al., 2017; Miller et al., 1984). Finally, further examination of the effects of other common environmental compounds or mixtures on POA sexual differentiation is needed to widen our understanding of DOHaD. Such studies are likely to improve our understanding of the processes associated with the sexual differentiation of the brain, and how the environment, long suspected of being a contributing factor (McCarthy, 2016), can impinge on these pathways.

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### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.neuro.2022.01.005>.

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