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Paternal nicotine taking elicits heritable sex-specific phenotypes that are mediated by hippocampal Satb2

John J. Maurer[1,2], **Mathieu E. Wimmer**[3], **Christopher A. Turner**[1,2], **Rae J. Herman**[1,2], **Yafang Zhang**[1,2], **Kael Ragnini**[1,2], **Julia Ferrante**[1,2], **Blake A. Kimmey**[1,2], **Richard C. Crist**[2], **R. Christopher Pierce**[4], **Heath D. Schmidt**[1,2]

[1]Department of Biobehavioral Health Sciences, School of Nursing, University of Pennsylvania, Philadelphia, PA 19104, USA

[2]Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[3]Department of Psychology, College of Liberal Arts, Temple University, Philadelphia, PA, 19122, USA

[4]Brain Health Institute and Department of Psychiatry, Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ 08854, USA

Abstract

Nicotine intake, whether through tobacco smoking or e-cigarettes, remains a global health concern. An emerging preclinical literature indicates that parental nicotine exposure produces behavioral, physiological, and molecular changes in subsequent generations. However, the heritable effects of voluntary parental nicotine taking are unknown. Here, we show increased acquisition of nicotine taking in male and female offspring of sires that self-administered nicotine. In contrast, self-administration of sucrose and cocaine were unaltered in male and female offspring suggesting that the intergenerational effects of paternal nicotine taking may be reinforcer specific. Further characterization revealed memory deficits and increased anxiety-like behaviors in drug-naïve male, but not female, offspring of nicotine-experienced sires. Using an unbiased, genome-wide approach, we discovered that these phenotypes were associated with decreased expression of *Satb2*, a transcription factor known to play important roles in synaptic plasticity and memory formation, in the hippocampus of nicotine-sired male offspring. This effect was sex-specific as no changes in *Satb2* expression were found in nicotine-sired female offspring. Finally, increasing Satb2 levels in the hippocampus prevented the escalation of nicotine intake and rescued the memory deficits associated with paternal nicotine taking in male offspring. Collectively, these findings indicate that paternal nicotine taking produces heritable sex-specific molecular changes that promote addiction-like phenotypes and memory impairments in male offspring.

Corresponding Author: Heath D. Schmidt, Ph.D., Associate Professor & Killebrew-Censits Chair of Undergraduate Education, Department of Biobehavioral Health Sciences, School of Nursing, Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, 125 South 31st Street, TRL Room 2215, Philadelphia, PA, 19104, Phone: (215)-573-8291, hschmidt@nursing.upenn.edu.

Authors Contribution

J.J.M., M.E.W., R.C.P., and H.D.S. were responsible for the study concept and design. J.J.M., M.E.W., C.A.T., R.J.H., Y.Z., K.R., J.F., B.A.K., R.C.C. and H.D.S. performed experiments and analyzed the data. J.J.M., R.C.C. and H.D.S. prepared the figures. J.J.M., M.E.W., R.C.C., R.C.P. and H.D.S drafted the manuscript. All authors reviewed content and approved the final version for publication.

Keywords

self-administration; smoking; hippocampus; memory; intergenerational; epigenetics; e-cigarette; nicotine

Introduction

Tobacco smoking-related diseases including cancer cause 1 in 5 deaths annually and cost over $300 billion in healthcare costs and lost worker productivity[1]. In 2019, an estimated 672,000 adolescents (2.7%) smoked cigarettes in the past month[2]. Moreover, 1 of every 6 high school students reported smoking electronic nicotine delivery systems (i.e., e-cigarettes), a behavior that often leads to increased consumption of combustible tobacco products[3, 4]. Given the significance of cigarette smoking for public health, preventing nicotine use among adolescents is critical to ending tobacco use disorder and decreasing e-cigarette use[1].

Parental smoking is a significant risk factor for the development of smoking behavior in offspring[5–7]. Both maternal and paternal smoking have been consistently linked to nicotine dependence in male and female offspring[8–12]. These findings suggest that parental nicotine exposure may promote addiction-like behaviors in subsequent generations. In addition to genetic and environmental factors, epigenetic inheritance may provide a link between parental smoking and susceptibility to developing nicotine use disorder in offspring. Support for this hypothesis comes from recent preclinical studies showing that paternal nicotine exposure produces heritable phenotypes in offspring that are mediated, in part, by epigenetic mechanisms. For example, nicotine-sired offspring have cognitive deficits, altered depressive-like behaviors, enhanced fear memories, and increased locomotor activity[13–17]. Some of these phenotypes were associated with altered DNA methylation in the spermatozoa of nicotine-experienced sires suggesting that nicotine exposure results in epigenetic reprogramming of the germline[13, 14, 17]. With regard to nicotine taking, some studies showed that paternal nicotine exposure decreased nicotine self-administration in offspring while other studies found no change[16, 18]. While not clear, these discrepant findings could be due to different exposure methods in sires (i.e., consumption of nicotine in the drinking water versus chronic subcutaneous delivery via osmotic minipumps) and/or approaches to measuring acquisition of nicotine taking in the offspring (for review see,[19, 20]). However, no studies to date have investigated the heritable effects of paternal nicotine taking using an operant model in which sires voluntarily self-administer nicotine.

Here, we describe a novel rodent model of patrilineal transmission developed to delineate the intergenerational effects of voluntary nicotine taking. Using this model, we investigated the effects of paternal nicotine taking on drug self-administration, cognitive function, and anxiety-like behaviors in the first (F1) generation. We also characterized the F1 hippocampal transcriptome to identify molecular substrates associated with heritable sex-specific phenotypes resulting from paternal nicotine taking. We hypothesized that acquisition of nicotine taking, memory formation, anxiety-like behaviors and hippocampal gene expression would be altered in nicotine-sired offspring.

Materials and Methods

Details regarding all drugs used, surgeries, maternal behaviors and littermate measurements, locomotor tests, cocaine and sucrose self-administration, novel object recognition task, anxiety-like tests, tissue extraction, microarray analysis, RNA extraction, PCR and immunohistochemistry are available in the Supplement.

Animals and housing

For the F0 generation, male (sires) and female (dams) Sprague-Dawley rats (*Rattus norvegicus*) weighing 225–250 g were obtained from Taconic Laboratories (Germantown NY, USA). Sires were housed individually, except during the mating period. Food and water were available *ad libitum* and rats were housed on a 12h-12h reverse light-dark cycle with the lights off at 07:00 h. All behavioral tests were conducted during the dark phase. The experimental protocols were consistent with the guidelines issued by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

F0 nicotine self-administration

F0 male rats were mildly food restricted (25 g chow daily) to ~90% of their free-feeding body weight. Mild food restriction was used to facilitate acquisition and maintenance of nicotine self-administration per previously published reports[21, 22]. Water was available *ad libitum* in the home cage. Nicotine self-administration was performed as described previously[23–27]. Briefly, rats were placed in operant conditioning chambers (Med-Associates Inc., East Fairfield, VT, USA) and allowed to lever press for intravenous nicotine (0.03 mg/kg nicotine/59 μl saline, infused over 5 s) on a fixed-ratio 1 (FR1) schedule of reinforcement. Each nicotine infusion was paired with a contingent 10 s light cue that was illuminated directly above the active lever (i.e., drug-paired lever). Each infusion was followed by a 20 s timeout period. Responses made on the inactive lever, which had no scheduled consequences, were also recorded, and used as a measure of nonspecific behavioral activity. All self-administration sessions were two hours in duration. Rats self-administered nicotine for 60 consecutive days, the duration of spermatogenesis[28]. Each rat allowed to respond for contingent nicotine infusions was paired with a yoked rat that received infusions of saline. While lever pressing for the saline-yoked rats had no scheduled consequences, these rats received the same number and temporal pattern of infusions as self-administered by their paired nicotine-experimental rat.

Breeding

Twenty-four hours after the last self-administration session, nicotine-experimental and yoked saline F0 male rats (sires) were group housed with drug-naïve females. Male and female rats remained group-housed for 7–10 days, after which the sires were removed. The resulting F1 offspring were weaned at 21 days and remained group housed (2–3 per cage) by sex until the onset of experiments (beginning at ~postnatal day 60 (P60)). For each experiment, 1–2 rats per litter were selected to prevent over-representation of specific litters. The assignment of rats to each experiment was random. The sample size for each

experiment was based on a combination of power analyses and previous publications from our laboratories[28–31].

F1 nicotine self-administration

F1 offspring were randomly selected from litters, single housed and implanted with indwelling jugular catheters as described above. Following a 7-day recovery from catheter surgery, nicotine- and saline-sired rats were placed in operant conditioning chambers and allowed to lever press for intravenous nicotine infusions (0.03 mg/kg nicotine/59 μl, over 5 s) and contingent light cues. Rats were allowed to self-administer nicotine during daily 2 h operant sessions under a FR1 schedule of reinforcement for 10 consecutive days.

Object location memory task

Spatial object recognition tasks were conducted as described previously[30, 32]. Prior to the onset of training, drug-naïve F1 offspring were habituated to the training context (30” × 30” × 17”) during two 5 min sessions on two separate days. On the day of training, rats were placed in the training arena with two identical objects for a total of three 5 min sessions with an intersession interval of 1–2 min, during which rats were returned to their home cages. The objects used were two glass Erlenmeyer flasks fixed to the arena floor with double-sided tape. A visual cue (i.e., a white and black checkerboard figure) was affixed to one wall of the arena to help orient the rat to the training environment. The objects and the arenas were wiped with 70% ethanol before each session. Twenty-four hours after training, rats were placed back in the training context for 5 min with one object displaced to a new location (displaced object; DO), while the other object was not moved (non-displaced object; NDO). All sessions were videotaped, and time spent exploring each object was scored by investigators blind to experimental groups. Exploration of the objects was defined as the amount of time rats were oriented toward an object with their nose within ~1 cm of the object. Grooming near the object was not considered exploration. Percent preference was calculated as time spent exploring the displaced object relative to the total time spent exploring both objects (preference = DO/(NDO+DO) × 100).

Viral-mediated upregulation of hippocampal Satb2 expression

To study the functional significance of Satb2, F1 male offspring were infused with AAV8-hSyn-eGFP (control virus) or AAV8-hSyn-Satb2-V5 (Satb2-expressing virus) directly into the dorsal hippocampus (SignaGen Laboratories, Rockville, MD, USA). Briefly, rats were anesthetized as described above and mounted in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). 500 nl of viral stock (1.7×10^{12} vg/ml) was infused bilaterally into the dorsal hippocampus at a rate of 100 nl/min. The coordinates for the infusions, relative to bregma, were: A/P, −3.60 mm; M/L, ±2.00 mm; D/V, −3.50 mm[33]. Following infusion, microinjectors were left in place for an additional 5 min to allow for diffusion away from the injection site. Rats were allowed to recover for three weeks after infusions, a time point that coincides with maximal hippocampal Satb2 expression in rodents[34]. Three weeks post viral infusion, one cohort of saline-sired and nicotine-sired male offspring were allowed to self-administer nicotine. A separate cohort of saline-sired and nicotine-sired male offspring underwent object location memory tests. Acquisition of nicotine taking and object location memory tests were measured as described above to determine if increased Satb2 expression

in the dorsal hippocampus normalizes nicotine taking and rescues the cognitive deficits associated with paternal nicotine taking in male offspring.

Statistics

Statistical comparisons were performed using GraphPad Prism (La Jolla, CA, USA). Initial statistical analyses were performed using sex as a factor for all behavioral experiments. No significant interactions were found between the three factors (sex, sire, and day) in any experiment. Subsequent analyses were conducted using male and female offspring data separately. Data were analyzed using un-paired t-tests, one-way ANOVAs, two-way ANOVAs, or three-way ANOVAs as appropriate. Significant main or interaction effects were followed by Tukey's HSD *post hoc* comparisons. Repeated measures ANOVAs (RM-ANOVAs) were followed by Bonferroni *post hoc* tests with corrections for multiple comparisons. All data are expressed as mean± S.E.M.

Results

To investigate the heritable effects of paternal nicotine taking, we allowed male rats to self-administer nicotine daily for 60 consecutive days; control rats received yoked saline injections (Figures 1A & 1B). Nicotine-experienced rats initially self-administered 0.4–0.5 mg/kg of nicotine per day, with daily intake escalating to ~1.0–1.2 mg/kg per day after 60 days of self-administration (Figure 1C). This rate of consumption achieves plasma concentrations of nicotine comparable to those detected in human tobacco smokers[35, 36]. The day after the last self-administration session, sires were bred with drug-naïve females resulting in nicotine-sired and saline-sired first generation (F1) offspring (Suppl Figure 1A). A total of 35 nicotine-experienced sires were bred with 45 drug-naïve females and 35 saline-experienced sires were bred with 40 drug-naïve females resulting in 85 total litters. Paternal nicotine taking had no effect on litter size or sex ratio (Suppl Figure 1B). Sire experience also had no effect on maternal behaviors (Suppl Figure 1C **&** 1D). Baseline locomotor activity and growth curves were similar in nicotine-sired and saline-sired male (Suppl Figures 1E **&** 1F) and female (Suppl Figures 1G **&** 1H) offspring, respectively.

Enhanced vulnerability to nicotine taking in the offspring of nicotine-experienced sires

Acquisition of nicotine self-administration was assessed in 1–2 male and female offspring from each litter. Nicotine-sired male offspring (n=22) acquired nicotine self-administration faster than saline-sired controls (n=21) (Figure 2A). These data were analyzed with a repeated measures (RM) two-way ANOVA, which revealed significant main effects of sire [$F(1,41)=7.62$; $p<0.01$] and day [$F(9,369)=43.08$, $p<0.0001$]. Total infusions over the 10-day acquisition phase were significantly increased in nicotine-sired versus saline-sired male offspring [$t(41)=2.82$; $p<0.01$]. The rate of acquisition of nicotine self-administration in nicotine-sired female offspring (n=23) was also increased compared to saline-sired controls (n=16) (Figure 2B). These data were analyzed with a RM two-way ANOVA, which revealed significant main effects of sire [$F(1,37)=6.62$; $p<0.05$] and day [$F(9,333)=22.79$, $p<0.0001$]. Consistent with these effects, total infusions were significantly increased in nicotine-sired versus saline-sired female offspring [$t(37)=2.54$; $p<0.05$]. These results indicate that paternal

nicotine taking is associated with increased nicotine consumption in both male and female offspring.

Normal intake of sucrose and cocaine in the offspring of nicotine-experienced sires

Drug-naïve offspring were allowed to self-administer sucrose to determine if the heritable effects of paternal nicotine taking are reinforcer-specific. Nicotine-sired male offspring (n=11) had similar levels of sucrose self-administration compared to saline-sired controls (n=11) (Figure 2C). Rates of acquisition of sucrose self-administration were also similar in female offspring from nicotine-experienced (n=11) and saline-experienced (n=11) sires (Figure 2D). Reinforcer-specificity was also tested in F1 littermates self-administering cocaine, another stimulant drug of abuse. No effects of sire were observed in male (Suppl Figure 2A) and female (Suppl Figure 2B) offspring self-administering cocaine. Together, these findings indicate that enhanced vulnerability to drug taking in the offspring of nicotine-experienced sires is reinforcer-specific and not due to baseline differences in operant learning.

Sex-specific memory deficits in the offspring of nicotine-experienced sires

Memory formation was assessed in young adult drug-naïve offspring using a hippocampus-dependent object location memory task wherein rats were exposed to two identical objects in an open field with spatial cues. Twenty-four hours after training, offspring were returned to the arena in which one of the objects was in a novel location (Figure 3A). Saline-sired male offspring (n=14) spent equal time exploring both objects during training and showed a preference for the displaced object during the memory test. In contrast, nicotine-sired male offspring (n=16) spent equal time exploring both objects during training and during the memory test (Figure 3B). These data were analyzed with a RM two-way ANOVA, which revealed a significant sire × session interaction [$F(1,28)=4.34$, $p<0.05$]. Both saline-sired (n=12) and nicotine-sired (n=17) female offspring showed normal object location memory, with a preference for the displaced object (Figure 3C). These data were analyzed with a RM two-way ANOVA, which revealed a significant main effect of session [$F(1,27)=16.42$, $p<0.001$] and no sire × session interaction [$F(1,27)=0.05$, $p=0.82$]. These data indicate that the memory deficits associated with paternal nicotine taking were sex specific.

To determine the anatomical specificity of this cognitive impairment, separate cohorts of drug-naïve male and female F1 rats were subjected to a novel object recognition task (Figure 3D). Rats were trained in familiar arenas lacking spatial cues, two factors that have been shown to render object recognition independent of hippocampal function[37, 38]. Both saline-sired (n=18) and nicotine-sired male (n=19) offspring spent equal time exploring both objects during training and showed a preference for the novel object during the retrieval tests, indicating that novel object recognition is not influenced by paternal nicotine taking (Figure 3E). These data were analyzed with a RM two-way ANOVA, which revealed a significant main effect of session [$F(1,35)=31.6$, $p<0.001$] and no sire × session interaction [$F(1,35)=0.001$, $p=0.97$]. Saline-sired (n=18) and nicotine-sired (n=18) female offspring also showed normal object recognition memory (Figure 3F). Similar to F1 males, both groups of female offspring spent equal time exploring both objects during training and showed a

preference for the novel object during the object recognition tests [significant main effect of session, F(1,25)=16.18, $p<0.001$, and no sire × session interaction, F(1.25)=0.082, $p=0.78$].

Enhanced anxiety-like behaviors in male, but not female, nicotine-sired rats

The novelty-induced hypophagia (NIH) test was used to assess anxiety-like behavior in F1 male and female rats (Suppl Figure 3A). Latencies to feed in familiar (home cage) and novel environments were analyzed with RM two-way ANOVAs. Analyses of saline-sired ($n=18$) and nicotine-sired ($n=15$) male offspring revealed a significant sire × environment interaction [F(2,62)=3.45, $p<0.05$]. *Post hoc* tests indicated a significant increase in latency to feed in the novel environment in nicotine-sired males relative to saline-sired males (Bonferroni, $p<0.05$, Suppl Figure 3B). No differences in total food intake were observed between nicotine-sired and saline-sired male offspring in either environment (data not shown). Analyses of saline-sired ($n=15$) and nicotine-sired ($n=16$) female offspring demonstrated a significant main effect of environment [F(2,58)=93.80, $p<0.001$], but no significant effect of sire [$p=0.52$] and no significant sire × environment interaction [$p=0.50$] (Suppl Figure 3C). Total food intake in both environments was the same for both groups of female offspring (data not shown). Thus, siring had no influence on NIH in female offspring, but nicotine-sired males displayed a significantly enhanced latency to feed in a novel environment relative to saline-sired controls. Defensive burying (DB) was also measured to further assess anxiety-like behaviors in the F1 generation. Nicotine-sired male offspring ($n=12$) spent more time burying than saline-sired ($n=11$) control rats [t(21)=2.147, $p<0.05$] (Suppl Figure 4A). Female offspring spent equal time burying regardless of siring [t(17)=0.80, $p=0.44$] (Suppl Figure 4B). These results indicate that paternal nicotine taking is associated with increased baseline anxiety in male, but not female, offspring.

Paternal nicotine taking is associated with decreased *Satb2* expression in the hippocampus of male, but not female, offspring

To identify the molecular changes associated with the heritable cognitive effects of paternal nicotine taking, an unbiased, whole-genome microarray was used to characterize the hippocampal transcriptome of drug-naïve F1 males (Figure 4A). A total of 1,441 genes were differentially expressed (654 up-regulated genes and 787 down-regulated genes) in the dorsal hippocampus of nicotine-sired versus saline-sired male offspring (FDR, $p<0.05$; Suppl Table 1)(Microarray data were deposited to Gene Expression Omnibus; GSE199661) . Enrichment analysis of the DEGs revealed overrepresentation of genes associated with neural development, cell signaling, and cell motility (Figure 4B & Suppl Tables 2-5). The DEGs were also enriched for significant loci from genome-wide association studies (GWAS) of phenotypes frequently comorbid with nicotine use, including smoking status, body mass index, and schizophrenia (Figure 4C & Suppl Table 6). *Satb2*, a gene related to synaptic plasticity and hippocampus-dependent behaviors, appeared in many of the overrepresented gene sets (highlighted in red, Figure 4B–C). When ranked by fold change, *Satb2* was one of the most differentially expressed genes in the hippocampus (Figure 4D & Suppl Table 1). qPCR was used to validate *Satb2* changes in the same hippocampal RNA samples (i.e., technical replicates) screened in the microarray. Consistent with the array results, *Satb2* expression was significantly decreased in the hippocampus of nicotine-sired males ($n=4$) relative to saline-sired controls ($n=4$) [t(6)=2.70, $p<0.05$] (Figure 4E). To further confirm

these findings, qPCR was performed using hippocampal RNA extracts from drug-naïve, F1 male littermates that were not used in the array study (i.e., biological replicates). This study validated the previous two and confirmed that hippocampal *Satb2* expression is significantly decreased in nicotine-sired male offspring (n=5) relative to saline-sired controls (n=5) [t(8)=3.60, p<0.01] (Figure 4F). Consistent with these mRNA expression studies, we found decreased Satb2 protein expression in nicotine-sired male offspring (Suppl Figure 5). To determine if *Satb2* levels were altered in nicotine-sired female rats, qPCR was performed using hippocampal RNA extracts from drug-naïve F1 females. Hippocampal *Satb2* expression was not altered in nicotine-sired female offspring (n=11) relative to saline-sired controls (n=12) [t(21)=0.03, p=0.97] (Figure 4G). Together, these gene and protein expression studies identify a novel molecular substrate that may underlie the heritable sex-specific behavioral effects of paternal nicotine taking.

Restoring hippocampal Satb2 levels normalizes nicotine taking and rescues memory impairments in nicotine-sired male offspring

Viral-mediated gene delivery methods were used to increase Satb2 protein expression *in vivo* and test the hypothesis that the heritable behavioral deficits associated with paternal nicotine taking are due to decreased hippocampal Satb2 in F1 males. Drug-naïve, saline-sired and nicotine-sired male rats were injected with AAV8-hSyn-eGFP (control virus) or AAV8-hSyn-Satb2-V5 (Satb2-expressing virus) directly into the dorsal hippocampus. Three weeks post infusion, acquisition of nicotine self-administration was assessed in all four groups (i.e., saline-sired control (n=12), saline-sired Satb2 (n=15), nicotine-sired control (n=15), nicotine-sired Satb2 (n=17)) (Figure 5A). Increased expression of Satb2 was observed three weeks post infection in rats infused with AAV8-hSyn-Satb2-V5 (Figure 5B and Suppl Figure 5). In this experiment, no significant behavioral differences were produced by the control virus versus the Satb2 virus in saline-sired rats (Suppl Figure 6). Therefore, these data sets were combined to produce one control group (i.e., saline-sired controls). F1 males were allowed to self-administer nicotine for 10 consecutive days (Figure 5C). These data were analyzed with a RM two-way ANOVA, which revealed significant main effects of day [F(9,504)=29.88, p<0.001] and virus [F(2,56)=6.79, p<0.01]. *Post hoc* analyses revealed that nicotine-sired control rats self-administered significantly more nicotine than saline-sired rats on days 1, 5, 6, 7, 9 and 10 (Bonferroni, p<0.05). No differences were found between saline-sired controls and nicotine-sired rats infused with the Satb2 virus. Total nicotine infusions were analyzed with a one-way ANOVA, which revealed a significant main effect of treatment [F(2,56)=0.19, p<0.0001] (Figure 5D). Subsequent *post* hoc analyses showed that nicotine-sired control rats self-administered more nicotine than saline-sired rats and nicotine-sired rats infused with the Satb2 virus (Bonferroni, p<0.05). These findings indicate that increased Satb2 expression in the hippocampus prevents the escalation of nicotine intake in nicotine-sired male offspring. Since these studies only targeted the dorsal hippocampus, it is possible that Satb2 in other nuclei could also play an important role in the heritable effects of paternal nicotine taking.

Drug-naïve F1 male rats were used to determine if increased expression of Satb2 in the hippocampus would ameliorate the memory deficits associated with paternal nicotine taking. Saline-sired and nicotine-sired male offspring were injected with AAV8-hSyn-eGFP

(control virus) or AAV8-hSyn-Satb2-V5 (Satb2-expressing virus) directly into the dorsal hippocampus (saline-sired control (n=10), saline-sired Satb2 (n=8), nicotine-sired control (n=12), nicotine-sired Satb2 (n=13)). Consistent with our previous results, nicotine-sired offspring that received control virus showed object location memory deficits compared to saline-sired controls (Figure 5E). These results were analyzed with a RM three-way ANOVA, which revealed a significant main effect of session [$F(1,39)=47.02$, $p<0.0001$] as well as a significant session × virus interaction [$F(1,39)=5.56$, $p<0.05$]. Subsequent *post hoc* analyses showed that saline-sired controls and nicotine-sired rats infused with the Satb2 virus preferred the displaced object during testing sessions (Bonferroni, $p<0.05$). Taken together, these data demonstrate that decreased Satb2 in the hippocampus of nicotine-sired male offspring is functionally relevant since increased expression of Satb2 was sufficient to restore normal object location memory.

Discussion

To study the heritable behavioral effects of paternal nicotine taking and their underlying molecular mechanisms, we allowed male rats to self-administer nicotine prior to mating with drug-naïve females. Paternal nicotine taking did not affect average litter size, survival rates or pup development similar to intergenerational studies in which sires were exposed to nicotine via osmotic minipumps[15]. Both male and female offspring of nicotine-experienced sires acquired nicotine self-administration faster and consumed more nicotine than saline-sired control rats. Moreover, there were no effects of nicotine sire experience on the acquisition of cocaine or sucrose self-administration in the F1 generation suggesting that the heritable effects of paternal nicotine taking are reinforcer specific. These results are consistent with human epidemiological studies showing that paternal smoking status is a critical factor influencing intergenerational transmission of smoking behavior[7, 11, 12]. Interestingly, paternal nicotine taking was also associated with sex-specific behavioral responses in the F1 generation. Drug-naïve nicotine-sired male, but not female, offspring displayed increased anxiety-like behaviors as well as spatial memory impairments. These sex-specific phenotypes were associated with decreased expression of Satb2, a DNA-binding protein known to play important roles in synaptic plasticity and memory formation[34, 39], in the dorsal hippocampus of nicotine-sired male offspring. Restoring Satb2 expression in the dorsal hippocampus normalized nicotine taking and rescued memory deficits in the male offspring of nicotine-experienced sires. Taken together, these findings indicate that paternal nicotine taking promotes addiction-like phenotypes in male and female offspring and induces sex-specific molecular changes in the dorsal hippocampus that are associated with cognitive deficits in male offspring.

The present study discovered that the offspring of sires that self-administered nicotine were more susceptible to nicotine taking than control offspring. Both male and female nicotine-sired offspring acquired nicotine self-administration faster and consumed more nicotine than saline-sired controls. These results contrast previously published studies that used different models of paternal nicotine exposure. For example, Vallaster et al.[18] found no differences in nicotine-taking behavior between offspring of sires exposed to nicotine in the drinking water (200 μg/ml for 5 weeks) versus control sires. Another study found decreased nicotine-taking behavior in the offspring of sires exposed to chronic subcutaneous nicotine

(12.6 mg/day via osomotic minipump for 28 days) compared to saline-sired controls[16]. While not clear, these discordant findings could be due to differences in dose, route, and duration of nicotine exposure in the F0 generation (for review see[19]). In drinking paradigms, saccharin is added to the drinking water to facilitate consumption of nicotine, which has a bitter taste on its own. However, saccharin exposure alone produces significant behavioral changes in adult sires and their drug-naïve offspring[40]. Furthermore, oral nicotine exposure paradigms like the one used in Vallaster et al.[18] do not model the fluctuations in plasma nicotine concentrations seen in human smokers[20]. Nicotine delivery via subcutaneous osmotic minipumps results in continuous, non-volitional exposure throughout both the light and dark cycles. This exposure paradigm does not model volitional nicotine taking in human smokers[36]. Nicotine self-administration on the other hand has the highest degree of face validity of all animal models of nicotine use disorder, primarily because it mimics voluntary nicotine consumption in humans[41]. Another important consideration that distinguishes these studies is whether sires experienced nicotine withdrawal during or prior to breeding. In the present study mating began 24 hours after the last sire self-administration session. In contrast, sires began mating 4–7 days after their last nicotine exposure in studies by Vallaster et al.[18] and Goldberg et al.[16] to avoid the acute withdrawal effects of nicotine and nicotine in the seminal fluid. Thus, it is possible that intergenerational transmission produces distinct behavioral phenotypes depending on the duration of nicotine exposure, the route of nicotine administration and whether the germline was reprogrammed during acute versus prolonged withdrawal.

Another important consideration when comparing studies is how nicotine taking was modeled in the F1 generation. The present study investigated spontaneous acquisition of nicotine self-administration in F1 male and female rats. In contrast, Vallaster et al.[18] and Goldberg et al.[16] examined acquisition of nicotine self-administration in mice with prior operant experience. To facilitate subsequent nicotine taking, mice were food-restricted and trained to lever press for food pellets before and after catheterization surgeries. Nicotine taking was then assessed using the same operant conditioning chambers in which mice acquired food self-administration. This design is limited in that multiple behaviors are expressed during the nicotine self-administration phase[42]. Differences in nicotine taking between nicotine- and saline-sired offspring could be due to altered extinction learning (i.e., perseverance of responding for food reward), drug-seeking behavior on the first day of nicotine exposure, and/or cognitive flexibility when transitioning from responding for food to drug reward[16]. Thus, the complex interplay between different behaviors in these two studies make direct comparisons with the present study difficult at best.

While paternal nicotine taking increased nicotine consumption in male and female offspring, it did not alter cocaine- or sucrose-taking behaviors in the F1 generation. The mechanisms underlying the specificity of this heritable response are not clear but could be due to changes in dopamine and/or cholinergic signaling in the brains of nicotine-sired offspring. The reinforcing effects of nicotine are mediated, in large part, by activation of neuronal nicotinic acetylcholine receptors (nAChRs) in the ventral tegmental area (VTA) and increased dopamine release in the ventral striatum (for review see,[43, 44]). Emerging evidence indicates that paternal nicotine exposure alters dopamine signaling in the F1 generation. For example, one study found decreased dopamine content and dopamine receptor subtype mRNA

expression in the striatum of nicotine-sired male offspring[13]. Another study found increased extracellular levels of dopamine and decreased expression of the dopamine reuptake transporter (DAT) in the hippocampus of nicotine-sired female and male offspring[17]. While the exact relationship(s) between changes in central dopamine signaling and the heritable effects of paternal nicotine exposure is not clear, there is some evidence that enhanced striatal dopamine signaling in the F1 generation facilitates or augments the rewarding effects of nicotine[45]. These results are consistent with the hypothesis that increased vulnerability to nicotine taking in the offspring of sires that self-administered nicotine is due to increased dopamine receptor sensitivity in the striatum. Changes in cholinergic signaling are also likely to influence the intergenerational effects of paternal nicotine taking. Offspring of nicotine-exposed sires exhibit increased nAChR ligand binding and decreased nicotine-evoked acetylcholine release in the hippocampus[16]. To discover the neurochemical mechanisms underlying the heritable effects of paternal nicotine taking, future studies should investigate nAChR function in the VTA of nicotine-sired offspring and whether these changes are associated with altered excitability of VTA dopamine neurons, striatal dopamine signaling and/or nicotine-taking behaviors.

Previous studies investigating the heritable effects of paternal nicotine exposure on baseline anxiety-like behaviors have yielded conflicting results. While some studies have found no effects of paternal nicotine exposure on anxiety-like behaviors in the F1 generation as measured in the elevated plus maze (EPM)[14, 15, 18], a recent study showed increased anxiety-like behavior in nicotine-sired female, but not male, offspring using the same test[16]. In contrast, the present study found that voluntary paternal nicotine taking was associated with enhanced anxiety-like behaviors in nicotine-sired male, but not female, rats as measured in the novelty-induced hypophagia (NIH) and defensive burying tasks. Several differences in experimental design could account for these discordant findings including species studied, route and duration of paternal nicotine exposure, and phenotype measured (i.e., anxiety-like behaviors in EPM vs. NIH vs. defensive burying). These provocative findings suggest that paternal nicotine exposure may produce sex-dependent heritable changes in baseline anxiety-like behaviors and warrant further study.

Parental tobacco smoking is associated with decreased cognitive function in offspring including impairments in attention and memory[46–50]. Recent evidence indicates that paternal smoking is a significant risk factor for the development of cognitive impairments in offspring[51]. Consistent with these intergenerational effects in humans, preclinical studies show that paternal exposure to nicotine in the drinking water[13] or via minipumps[16] produces cognitive deficits in the F1 generation. We extend these findings and show that paternal nicotine taking produces memory impairments in drug-naïve male, but not female, offspring. Specifically, nicotine-sired male offspring displayed impaired hippocampus-dependent object location memory. In contrast, object location memory was normal in nicotine-sired female offspring. No effects of sire experience were found on novel object memory indicating that paternal nicotine taking impairs hippocampal-dependent memory formation while sparing traces that do not engage the hippocampus. In our novel object recognition experiments, rats were extensively familiarized with the training arena and spatial cues were removed from the training context, which diminishes the engagement and requirement of the hippocampus for novel object trace[37, 38, 52]. Similar sex-specific effects on object location

memory have been found in the offspring of sires that self-administered cocaine[30], which suggests that the hippocampus is vulnerable to the intergenerational effects of licit and illicit psychostimulants. The intergenerational effects of paternal nicotine taking on cognitive functions are likely to be complex as sex-specific deficits in object-based attention have also been observed in male, but not female, offspring of sires exposed to nicotine (albeit in the drinking water)[13]. Interestingly, the heritable effects of paternal nicotine exposure on hippocampal-dependent behaviors are not all sex-specific. Both male and female offspring of sires exposed to nicotine via osmotic minipumps displayed enhanced contextual and cued fear responses[16]. Since the amygdala plays an important role in these learned behaviors[53], it is possible that the amygdala is also altered by paternal nicotine exposure. Future studies should comprehensively determine how paternal nicotine taking affects learning and memory in both sexes and identify the neural circuits and neuroadaptations regulating these cognitive deficits.

To identify the molecular mechanisms underlying the heritable sex-specific effects of paternal nicotine taking, we used an unbiased, genome-wide approach to compare the hippocampal transcriptomes of drug-naïve nicotine-sired male offspring and saline-sired controls. Gene expression network analysis revealed a collection of genes related to synaptic plasticity and hippocampus-dependent behaviors including *Satb2*. Special AT-rich sequence-binding protein 2 (Satb2) is a transcription factor that regulates higher-order chromatin structure and gene expression[54–57]. Satb2 is highly conserved in different species including humans and rodents[39] and has been shown to play a critical role in the developing central nervous system[55, 58, 59]. The role of Satb2 in the adult brain, however, is less clear. Recent preclinical studies revealed an important role for Satb2 in hippocampus-dependent memory and synaptic plasticity. Specifically, decreased Satb2 expression in the adult hippocampus significantly impaired LTP along with spatial memory and working memory[34, 39, 60]. Given the emerging role of Satb2 in hippocampus-dependent memory and synaptic plasticity, it is likely that the intergenerational effects of paternal nicotine taking are due, in part, to decreased Satb2 expression in the hippocampus. Our microarray studies also identified altered expression of genes known to be targeted by Satb2 in the adult brain[61]. However, further studies are required to fully understand how Satb2 alters the hippocampal transcriptome to affect cognition in nicotine-sired offspring.

To test the functional significance of reduced *Satb2* expression in the sex-specific phenotypes associated with paternal nicotine taking, viral-mediated gene delivery methods were used to restore Satb2 protein expression in the dorsal hippocampus of nicotine-sired male offspring. We found that increased hippocampal expression of Satb2 normalized nicotine taking and rescued memory deficits in the male offspring of sires that self-administered nicotine. Thus, the heritable sex-specific behavioral effects of paternal nicotine taking can be reversed by increased Satb2 protein expression in the hippocampus. These results are consistent with studies of *Satb2* conditional knockout mice showing that re-established expression of Satb2 in the hippocampus rescues memory deficits[34]. The mechanisms by which increased Satb2 normalized nicotine taking to control levels are unclear but could be due to altered hippocampal-dependent learning and synaptic plasticity[62, 63]. In the adult brain, Satb2 regulates chromatin structure and expression of genes associated with cognitive function and neuropsychiatric disorders[64]. For example,

Satb2 binding is enriched at miRNA and immediate early gene promoters *in vivo*[34, 39]. Many of these targets play important roles in synaptic plasticity or learning and memory. Future studies should aim to identify Satb2 genomic binding sites as well as characterize the chromatin states of Satb2-bound promoters in the dorsal hippocampus of nicotine-sired male offspring to expand our understanding of the molecular mechanisms underlying the sex-specific effects of paternal nicotine taking on memory formation. These studies could focus initially on altered expression of downstream targets of Satb2 identified here, including *Htr5b*, *Penk*, *Kcnb2, Oxtr*, *Zfp423*, *Six4*, and *Lsm1*.

Overall, the present findings provide novel mechanistic insights into the heritable effects of paternal nicotine exposure. This study is the first to demonstrate that voluntary paternal nicotine taking increases vulnerability to nicotine dependence in male and female offspring and produces sex-specific cognitive deficits that are mediated by decreased Satb2 expression in the dorsal hippocampus. Future studies should expand these intergenerational studies and determine the stability and permanence of these heritable phenotypes by examining the behavioral and molecular effects of paternal nicotine taking in subsequent generations (i.e., the second (F2) generation). Indeed, some evidence supports transgenerational transmission of cognitive deficits in the F2 descendants of males exposed to nicotine in the drinking water or via osmotic minipumps[13, 16]. The heritability of these nicotine-induced phenotypes is likely transmitted via germ cells. Nicotine exposure is associated with epigenetic regulation of spermatozoa DNA and germline mutations in humans and rodents (for review see,[19]). Nicotine may also influence seminal fluid contents, which can affect embryo development and uterine environment[65–67]. Understanding how voluntary nicotine taking changes germ cells and/or seminal fluid and how these modifications translate into neuroadaptations and behavioral phenotypes in subsequent generations is necessary for understanding the heritability of parental drug taking. Further delineating the molecular and epigenetic mechanisms underlying the heritable effects of paternal nicotine taking is critical considering the prevalence of tobacco use and the dramatic rise in e-cigarette consumption. Findings from these studies highlight vulnerable populations at risk for developing nicotine dependence, cognitive impairments, and/or mental health disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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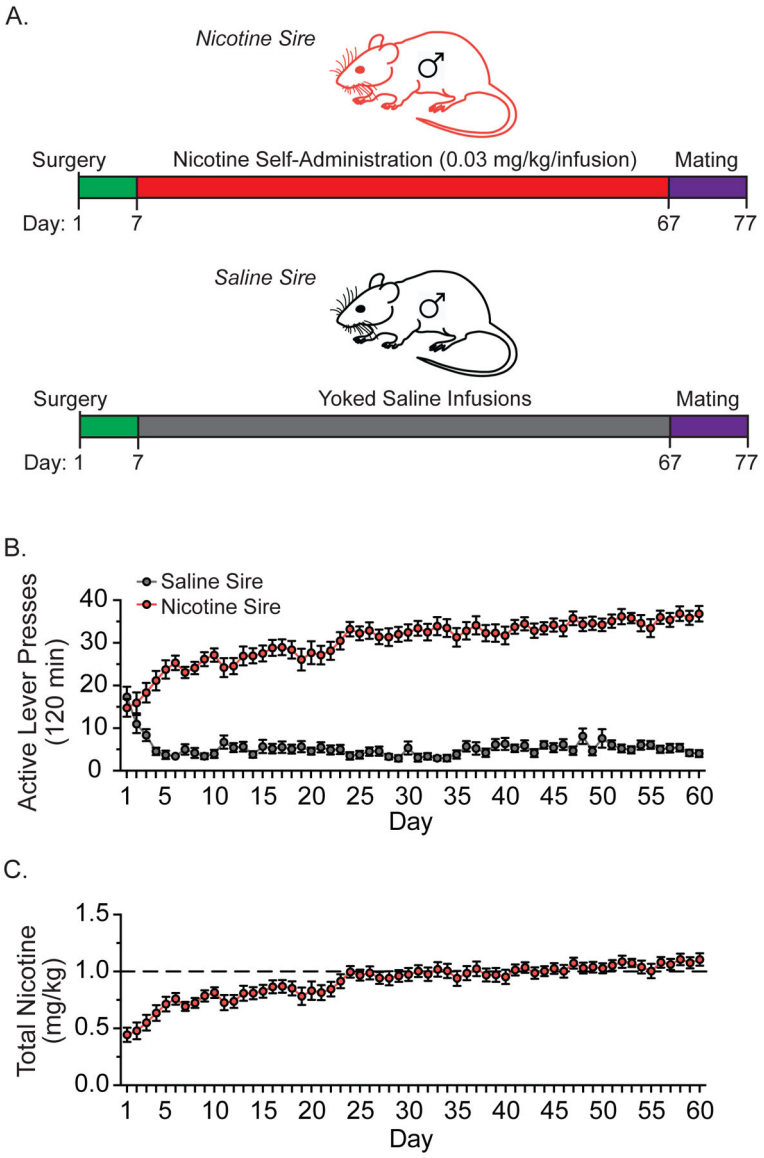

Figure 1: Nicotine self-administration by the F0 sires.

(A) Male rats were implanted with indwelling jugular catheters and allowed one week to recover. Rats were randomly assigned to a nicotine self-administration group and a yoked saline control group. Following 60 consecutive days of nicotine self-administration and a 24 h washout period, F0 sires were co-housed with drug-naïve female rats during the mating period. **(B)** Total number of active lever responses for F0 sires self-administering nicotine (n=35) or saline (n=35). **(C)** The amount of nicotine consumed daily by nicotine-experienced sires throughout the self-administration phase. Note the behavior escalates over the first four weeks until it stabilizes with nicotine sires self-administering ~1.0–1.2 mg/kg nicotine daily.

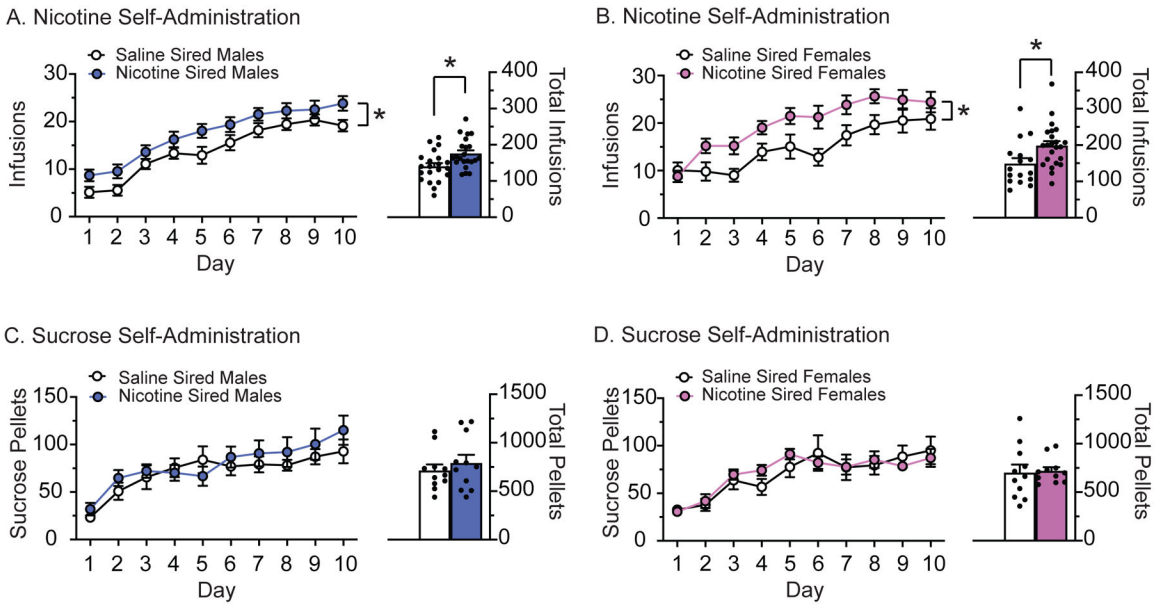

Figure 2: Increased vulnerability to nicotine taking in the offspring of nicotine-experienced sires.

Increased acquisition of nicotine self-administration in nicotine-sired male **(A)** and female **(B)** offspring compared to saline-sired controls (significant main effects of sire, Bonferroni's *post hoc* test, *$p<0.05$). Total nicotine consumed was significantly greater in nicotine-sired versus saline-sired progeny (un-paired t-tests, *$p<0.05$). In contrast, there were no significant differences in the acquisition of sucrose self-administration between nicotine-sired and saline-sired male **(C)** and female **(D)** rats. (n=11–23/treatment).

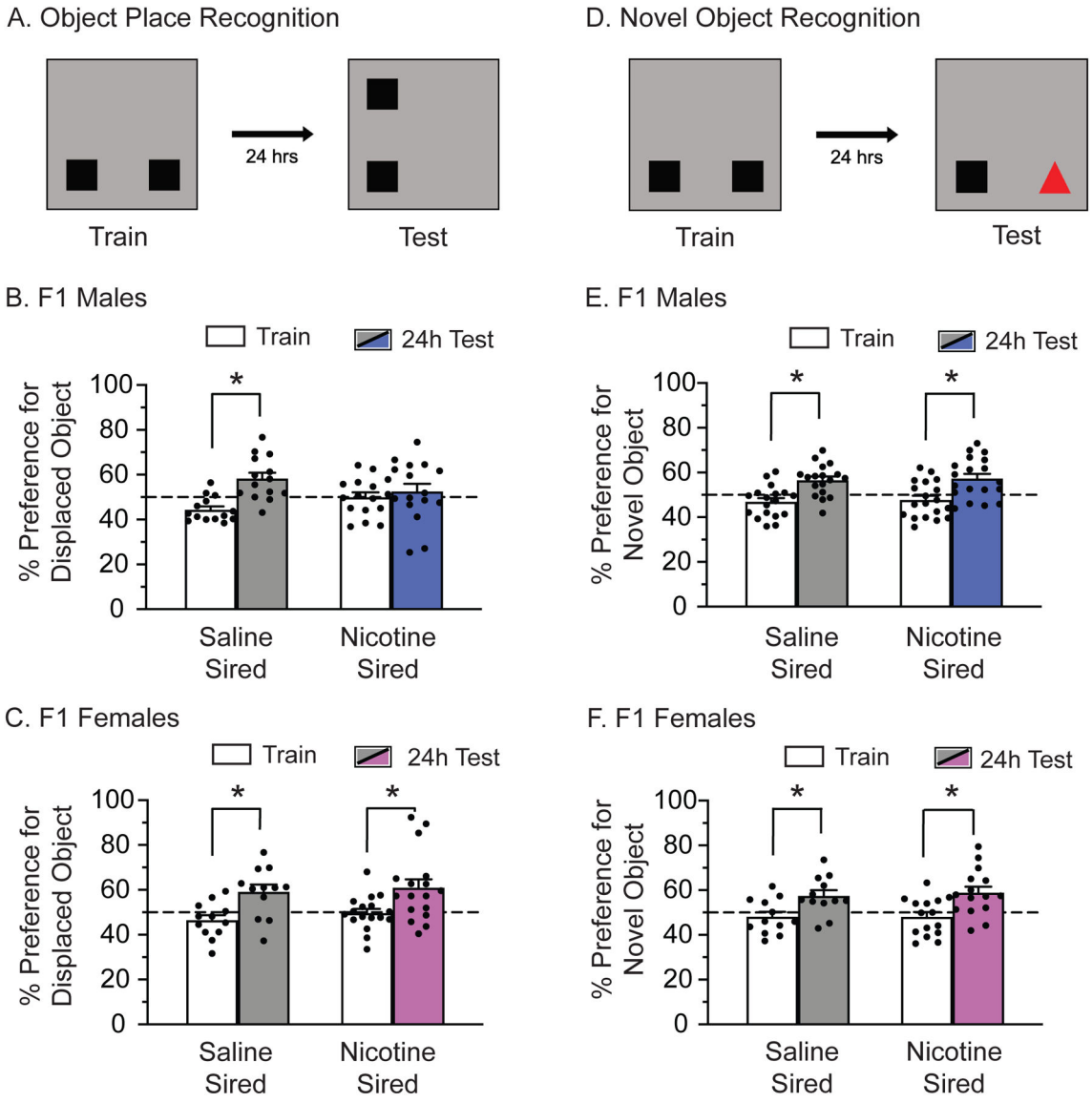

Figure 3: Paternal nicotine taking is associated with sex-specific spatial memory impairments in the F1 generation.

(A) Male and female offspring were exposed to two identical objects in an arena with spatial cues and time exploring each item was recorded. After a 24 h delay, rats were placed back into the same arena with one of the objects displaced to a new location. Saline-sired male **(B)** and female **(C)** offspring spent equal time exploring both objects during training and showed a preference for the displaced object during the spatial memory test. In contrast, nicotine-sired male offspring **(B)** spent equal time exploring both objects during training and during the memory test indicating a spatial memory deficit. Similar to controls, nicotine-sired female offspring **(C)** also displayed a significant preference for the displaced object during the memory test. **(D)** To determine anatomical specificity, separate cohorts of F1 rats were pre-exposed to the training context lacking spatial cues for 5 days prior to training with two identical objects. A novel object was introduced during the object recognition memory test 24 h after the training session. Nicotine-sired and saline-sired male **(E)** and female **(F)**

rats spent equal time exploring both objects during training and showed a preference for the novel object during the memory tests. (*p<0.05 comparing train vs test, Bonferroni's *post hoc* test, n=12–19/treatment).

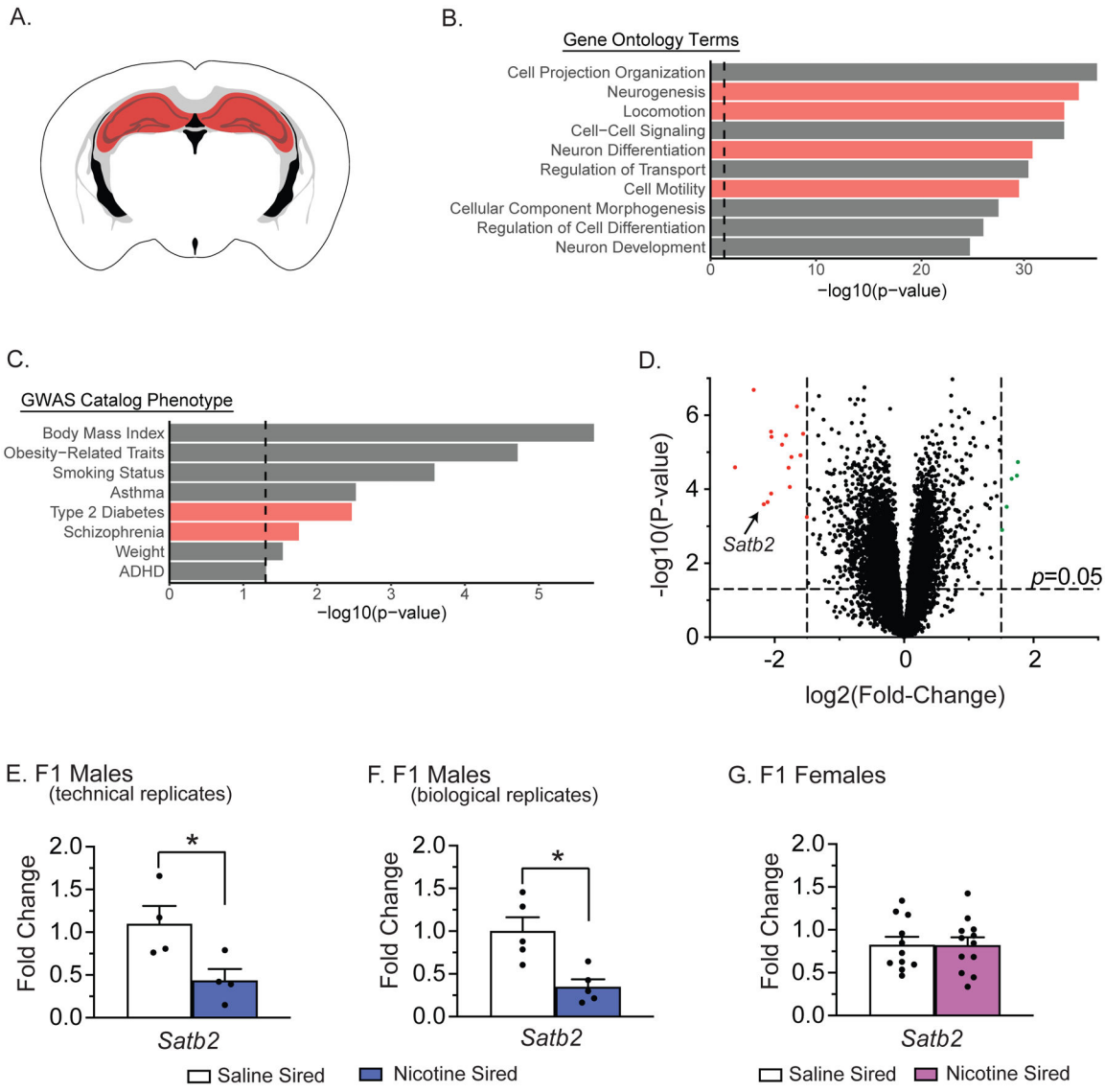

Figure 4: Satb2 is down-regulated in the hippocampus of nicotine-sired male offspring.

Differentially expressed genes in the dorsal hippocampus **(A)** of nicotine-sired versus saline-sired male offspring (n=4/treatment) are enriched for genes associated with Gene Ontology (GO) biological processes **(B)** and genome-wide association study (GWAS) loci **(C)**. Gene sets containing *Satb2* are highlighted in red. Dashed lines are p=0.05. **(D)** Volcano plot of microarray data showing global transcriptome changes in the dorsal hippocampus of nicotine-sired versus saline-sired male offspring. Genes above the dashed horizontal line indicate DEGs based on raw p values less than 0.05. The dashed vertical lines indicate a +/− 1.5 fold-change cutoff (log2 transformed), where upregulated and downregulated genes are on the far right and far left, respectively. **(E)** RT-PCR was used to verify that *Satb2* expression was decreased in the dorsal hippocampus of nicotine-sired males relative to saline-sired controls in the same RNA extracts used in the microarray. **(F)** *Satb2* expression was also decreased in the hippocampus of drug-naïve male littermates further validating the array findings (n=5/treatment). **(G)** In contrast to the F1 males, there were

no differences in hippocampal *Satb2* expression between nicotine-sired and saline-sired females (n=11–12/treatment). (un-paired t-tests, *p<0.05); smoking status=ever versus never smokers; ADHD=attention deficit hyperactivity disorder.

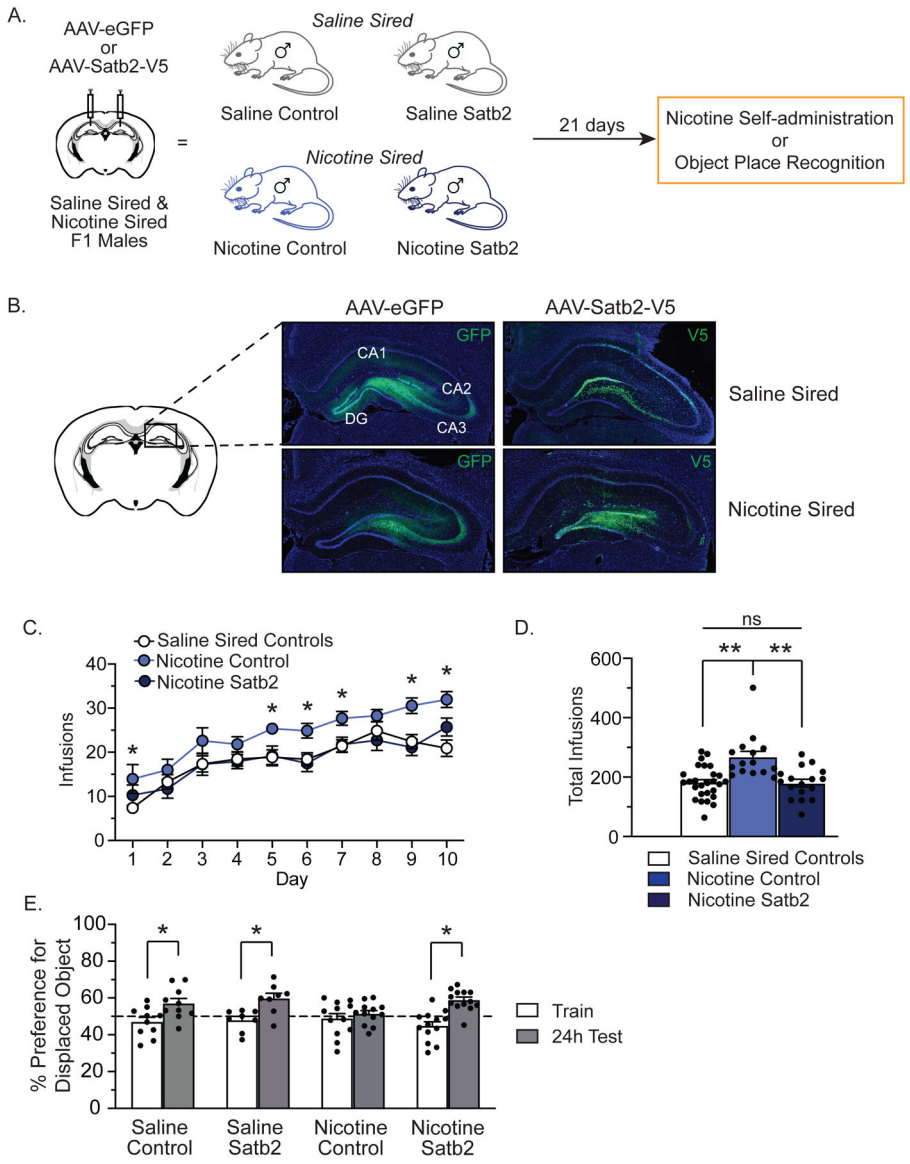

Figure 5: Increased Satb2 expression in the hippocampus of nicotine-sired male offspring rescues the behavioral deficits associated with paternal nicotine taking.

(A) Saline-sired and nicotine-sired male offspring were infused with a control virus (AAV-eGFP) or a virus expressing Satb2 (AAV-Satb2-V5) directly into the dorsal hippocampus. **(B)** Immunofluorescence showed increased eGFP expression in control rats and increased Satb2 expression via the V5 tag in saline-sired and nicotine-sired rats three weeks post infusion. Separate cohorts of rats were used to study the effects of increased Satb2 protein expression on the acquisition of nicotine taking and memory formation. **(C)** Satb2 overexpression normalized nicotine taking in the male offspring of nicotine-experienced sires (n=15–27/treatment). Nicotine-sired controls (i.e., Nicotine Control) self-administered significantly more nicotine on days 1, 5, 6, 7, 9, and 10 when compared to saline-sired controls and nicotine-sired rats with increased Satb2 expression (i.e., Nicotine Satb2). **(D)** Over the 10-day acquisition phase, nicotine-sired control rats self-administered more nicotine than saline-sired controls and nicotine-sired rats with increased Satb2. **(E)** In

memory formation tests, nicotine-sired male offspring infused with the control virus did not show a preference for the displaced object during the memory test compared to saline-sired controls. This memory impairment was rescued by increased Satb2 protein expression in the dorsal hippocampus of nicotine-sired male offspring (n=8–13/treatment). (Bonferroni's *post hoc* test, *p<0.05)