SUPPLEMENTARY METHODS AND MATERIALS

Shock sensitivity

To test if sensitivity to the footshock US increased, locomotor and vocal shock reactivity was tested in a separate cohort of NIC-Sired and SAL-Sired mice (n = 4 M and F per group). Each animal was tested individually in a conditioning chamber with no background noise to allow for scoring of vocal behaviors. Locomotor and vocal responses to shocks at 0.10, 0.20, 0.30, 0.50, and 0.70 mA were recorded across a 12-min session. Shocks were presented 3 times each in pseudo-random order at pseudo-random intervals of 30, 45, or 60 sec. Behaviors were scored by an experimenter blind to sire treatment and order of shock presentations. Locomotor behaviors were scored as follows: 0=no response, 1=run, 2=jump, 3=run and jump. Vocal behaviors were scored as follows, 0=no audible vocalization, 1=audible vocalization. Scores were averaged across the 3 shock presentations.

Open field

A drug-naive cohort of F1 mice was used to assess behavior in open field (OF), novel object recognition (NOR), and then elevated plus maze (EPM) (n=4M and 6F SAL-Sired, and 4M and 5F NIC-Sired). Baseline locomotor activity in F1 mice was assessed in a Plexiglas arena (49.5 cm × 59.7cm). Mice were placed in the center of the OF arena and locomotor activity was recorded for 5 min using a camera (Basler, Ahrensburg, Germany) attached to tracking software (Smart Tracking Software, Panlab, Barcelona, Spain). One subject (NIC-Sired) was removed because total distance traveled was 2 standard deviations above the mean.

Novel object recognition

NOR was used to examine non-emotional learning in F1 mice. NOR took place in the Plexiglas OF arena described above. Objects were an inverted 50 ml falcon tube (Fisher Scientific, Waltham, MA) filled with clean mouse bedding and a 10-cm high yellow and green plastic interlocking block tower affixed to gray Plexiglas (7.5 × 7.5 cm). Mice trained in NOR were allowed to explore the two identical objects (5 cm from the walls) for 10 min. Twenty-four hours after training, mice returned to the arena for 10 min with a novel object replacing one of the previously explored objects. Objects were counterbalanced across locations and conditions. Exploration was defined as a mouse directing its nose to the object within approximately 1 cm. Climbing and sitting on objects was not scored as exploration. One subject (SAL-Sired) was removed because time exploring the novel object was 2 standard deviations below the mean. Preference ratio was calculated as: (time spent exploring the novel object/ time spent exploring the novel object + time spent exploring the old object).

Elevated plus maze

Anxiety-like phenotype in F1 mice was assessed using the EPM. The EPM was a 62.6 cm tall opaque Plexiglas structure with two opposing open arms (7.6 × 30.6 cm), two opposing closed arms (7.6 × 30.6 × 15.5 cm) and a center area (7.6 × 7.6 cm). Mice were placed in the center of the EPM facing a closed arm and behavior was recorded for 5 min. One subject (SAL-Sired) was removed from analysis because open arm duration was 2 standard deviations above the mean. Percent open arm time was calculated as: ([total open arm time ÷ (total open arm time + total closed arm time)] * 100).

SUPPLEMENTARY RESULTS

Paternal nicotine has limited effects on baseline, pre-CS, and CS freezing.

F1 generation: 3-way ANOVAs analyzing baseline or pre-CS freezing with sex, sire treatment, and acute drug treatment as factors found no significant effects or interactions. Subsequent 2-way ANOVAs collapsed across sex analyzing baseline or pre-CS freezing additionally found no significant sire or acute drug treatment effects. A 3-way ANOVA of CS freezing with sire treatment, acute drug treatment, and sex as factors revealed significant sex × sire treatment and sex × acute drug treatment interactions ($F_{(1,36)}$=11.82, p<.05 and $F_{(1,36)}$=11.46, p<.05, respectively). A subsequent 2-way ANOVA of CS freezing in only females revealed a significant main effect of sire treatment ($F_{(1,18)}$=5.71, p<.05) and a significant interaction between sire treatment and acute drug treatment ($F_{(1,18)}$=5.71, p<.05). Post-hoc comparisons indicated that nicotine-treated SAL-Sired females were lower than all other groups (mean±SEM; nicotine-treated SAL-Sired=15.0±0.69, nicotine-treated NIC-Sired=17.20±0.42, saline-treated SAL-Sired=17.0±0.40, saline-treated NIC-Sired=17.0±0.35, p<.05). A 2-way ANOVA of CS freezing in only F1 males revealed significant main effects of sire treatment ($F_{(1, 18)}$=6.11, p<.05), with NIC-Sired animals showing reduced cued freezing, and acute drug treatment ($F_{(1, 18)}$=8.07, p<.05), with acute nicotine treated animals showing enhanced cued freezing (mean±SEM; saline-treated SAL-Sired=16.83±0.34, nicotine-treated SAL-Sired=18.0±0.0, saline-treated NIC-Sired=15.6±0.91, nicotine-treated NIC-Sired=17.0±0.50).

F2 generation: 3-way ANOVAs analyzing baseline or CS freezing with sex, sire treatment, and acute drug treatment as factors found no significant effects or interactions. Subsequent 2-way ANOVAs collapsed across sex analyzing baseline or CS freezing additionally found no significant sire or acute drug treatment effects. A 3-way ANOVA of pre-CS freezing with sire treatment, acute drug treatment, and sex as factors revealed a significant main effect of acute drug treatment ($F_{(1,33)}$=4.59, p<.05) and a significant sex × sire treatment × acute drug treatment interaction ($F_{(1,33)}$=4.59, p<.05). A subsequent 2-way ANOVA of pre-CS freezing in only F2 females revealed a significant interaction between sire treatment and acute drug treatment ($F_{(1,17)}$=6.79, p<.05). Post-hoc comparisons indicated that saline-treated NIC-Grandsired females had enhanced pre-CS freezing compared to saline-treated SAL-Grandsired females (mean±SEM; saline-treated NIC-Grandsired=0.2±0.22, saline-treated SAL-Grandsired=1.4±0.45, p<.05), while there was no difference in pre-CS freezing between nicotine-treated NIC-Sired and SAL-Sired F1 females. Post-hoc comparisons found no significant differences in pre-CS freezing between nicotine versus saline treated SAL-Grandsired or NIC-Grandsired animals. A 2-way ANOVA of pre-CS freezing in only F2 males revealed a significant main effect of sire treatment ($F_{(1,16)}$=4.65, p<.05), with NIC-Grandsired males showing higher freezing (mean±SEM; SAL-Grandsired = 0.80±0.34, NIC-Grandsired mean = 1.80±0.34).

Paternal nicotine has limited effects on secondary behaviors:

Because changes in contextual fear conditioning could have been due to differences in shock sensitivity, locomotor activity, general learning mechanisms, or anxiety behaviors, we tested F1 mice for shock sensitivity, open field behavior, novel object recognition, and elevated plus maze behaviors.

<u>Shock Sensitivity</u>

A 3-way, mixed model ANOVA of locomotor reactivity to shock found no significant interaction between sex and sire treatment or shock level. A subsequent 2-way mixed model ANOVA collapsed across sex indicated a main effect of shock level ($F_{(4,56)}$=103.59, p <.001) but no main

effect of sire treatment ($F_{(1,14)}$=.10, p =.76). A 3-way, mixed model ANOVA of vocal reactivity to shock found no significant interaction between sex and sire treatment or shock level, a 2-way mixed model ANOVA collapsed across sex, with shock level as a within subjects factor and sire as a between subjects factor found a significant main effect of shock ($F_{(4,48)}$=49.01, p<.001) and sire ($F_{(1,12)}$=16.70, p<.01), as well as a significant interaction between sire and shock level ($F_{(4,48)}$=3.43, p<.05) (**Fig. S2**). Follow-up post-hoc comparisons indicated that SAL-Sired mice were more sensitive to shock in terms of vocal reactivity at shock intensities 0.30mA (p<.05) and 0.50mA (p<.05). Thus, it is unlikely that sensitivity to the shock US contributed to the effects of paternal nicotine exposure as no change in locomotion sensitivity to shock was seen, and the significant vocalization effects were opposite to the observed NIC-Sired fear conditioning phenotype.

Elevated Plus Maze

Anxiety-like behavior was assessed in an EPM as a function of open arm time, closed arm time, and percent open arm time (**Fig. S3**). A 2-way ANOVA with sex and sire treatment as factors revealed a significant sex by sire treatment effect on open arm time ($F_{(1,14)}$=9.64, p<.01). As such, males and females were analyzed separately for all EPM-related phenotypes. NIC-Sired females displayed increased open-arm time compared to SAL-Sired females (p<.05), whereas NIC-Sired males did not differ from SAL-Sired males (p=.10). NIC-Sired females displayed increased percent open arm time compared to SAL-Sired females (p<.05), whereas NIC-Sired males did not differ from SAL-Sired males (p=.13). No significant effects of sire on closed arm time were found in females (p=.10) or males.

Locomotor Activity

To determine the effect of paternal exposure on locomotor activity, a 2-way ANOVA of baseline locomotor activity within the OF with sex and sire as independent factors was performed. Because there was no interaction of sex and sire treatment, a t-test collapsed across sex for main effect of sire treatment was performed and revealed no effect of sire treatment (t_{16}=.09, p=.93; **Fig. S4**).

Novel Object Recognition

To determine the effect of paternal nicotine exposure on non-emotional learning, a 2-way ANOVA with sex and sire treatment as independent factors was performed on NOR learning. Because there was no interaction of sex and sire treatment, a t-test collapsed across sex was performed and found no effect of sire treatment on NOR learning (t_{16}=1.81, p=.09; **Fig. S4**).

Paternal nicotine does not affect food self-administration.

Prior to behavioral testing, paternal exposure groups were examined for differences in baseline body weight; no differences were found (t_{20}=0.44, p=0.66; **Fig. S5a**). Thereafter, subjects were analyzed for their ability to learn an operant task to obtain food reward. Mice were first trained in the operant procedure across 8 sessions, and after the surgical and post-surgical recovery period, mice were then permitted to re-establish their responding to ensure behavioral recovery from the surgical procedure (sessions R1-R3). A 2-way mixed design ANOVA identified a main effect of session ($F_{(10,240)}$=43.90, p<0.0001). Post-hoc analyses indicated that the groups did not differ in the number of food pellets earned across all sessions (**Fig. S5b**). When the number of active and inactive lever presses were analyzed by 2-way mixed design ANOVA for food training, both of the paternal exposure groups exhibited similar acquisition with significance

preference found for the active lever over the inactive lever across sessions 2-8 and re-establishment sessions R1-R3 (Session: $F_{(10,480)}=55.93$, $p<0.0001$; Group: $F_{(3,54)}=391.8$, $p<0.0001$; Interaction: $F_{(30,480)}=19.24$, $p<0.0001$; Post-hoc ***$p<0.0001$ active lever vs. inactive lever sessions 2-8 & R1-R3 for both groups; **Fig. S5c**).

SUPPLEMENTARY FIGURES

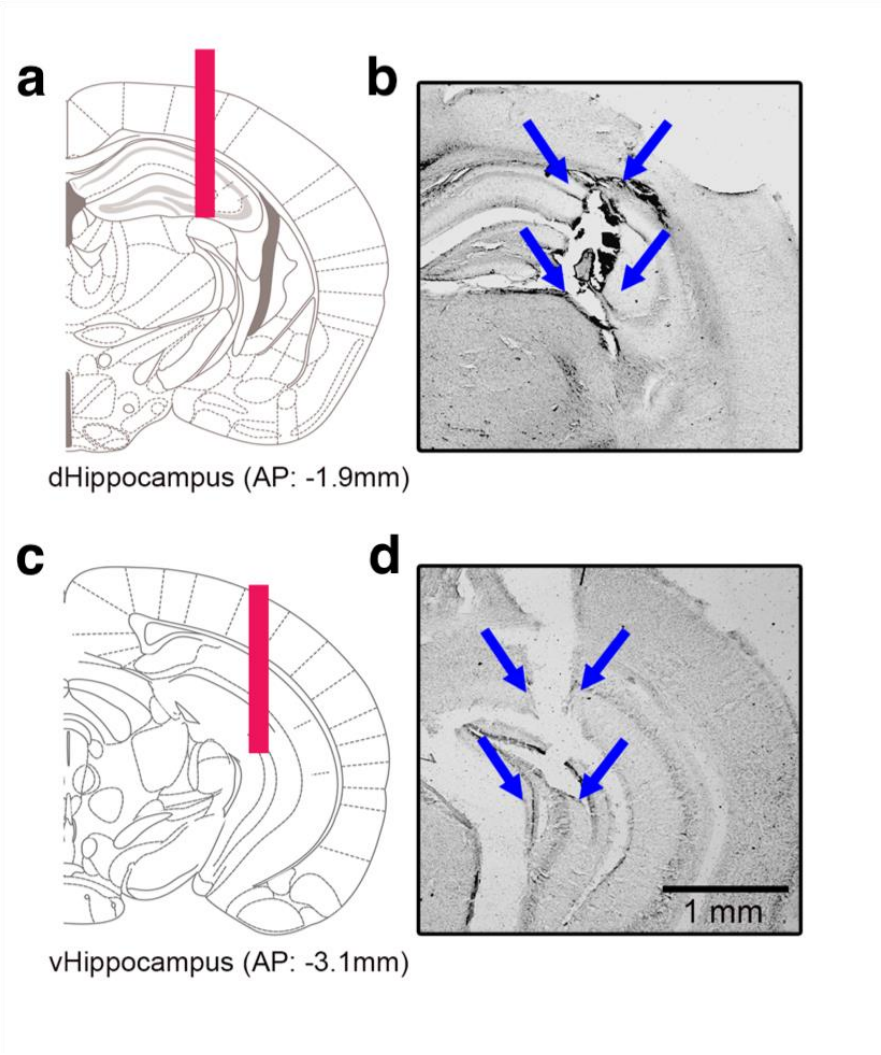

Figure S1: Placement of the electrochemical recording electrodes. Schematics for electrochemical recording electrode sites in the dorsal and ventral hippocampus **(A,C)**. Representative images of electrode placements **(B,D)**.

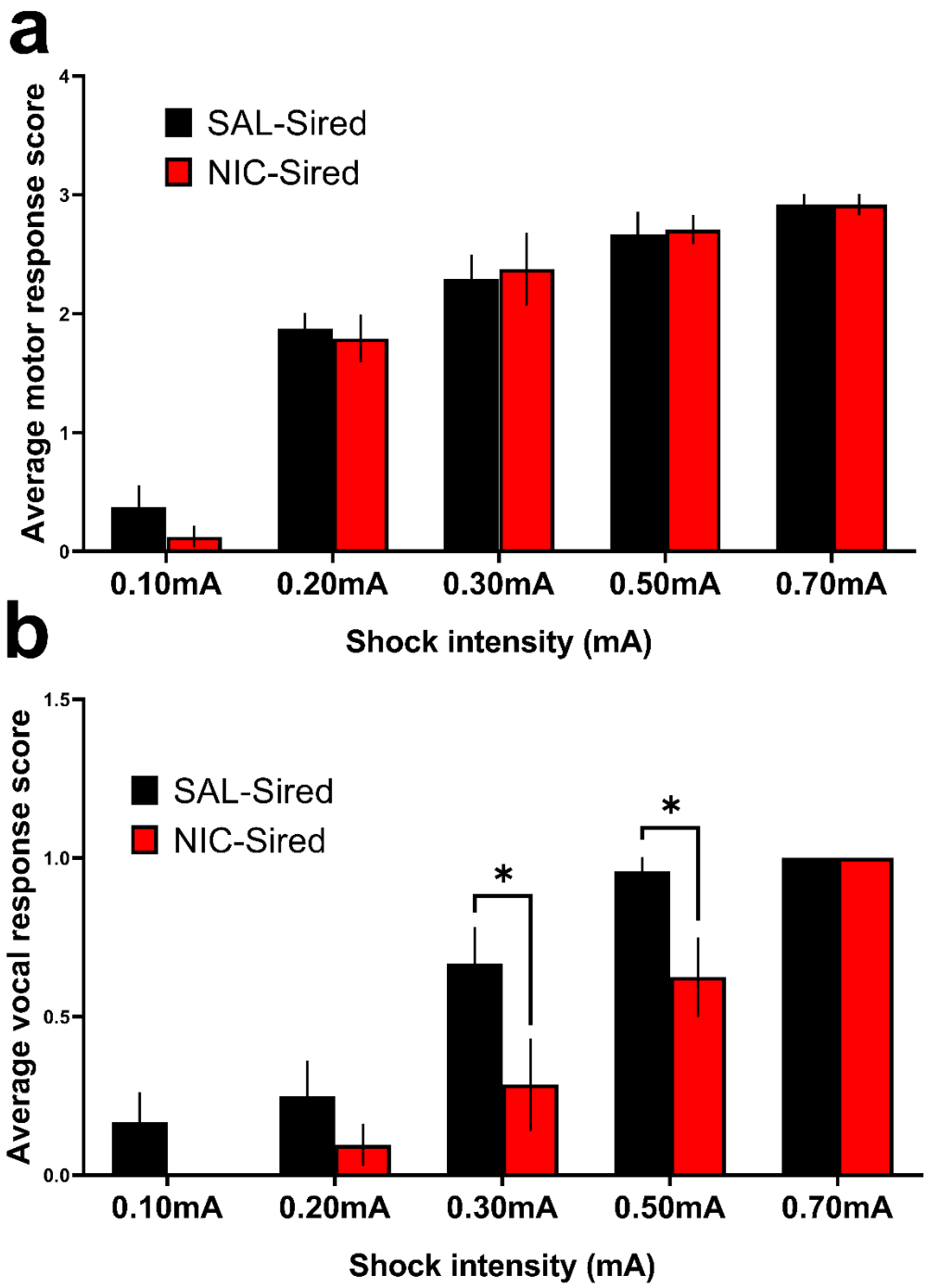

Figure S2: Effects of paternal nicotine on shock sensitivity. (A) No differences were observed in motor shock reactivity between NIC- and SAL-Sired mice. **(B)** NIC-Sired animals exhibited reduced vocal shock reactivity at shock intensities of 30mA and 50mA (n=8 per group). No effects of sex on shock sensitivity were observed. Error bars indicate standard error of the mean (SEM), *p<0.05.

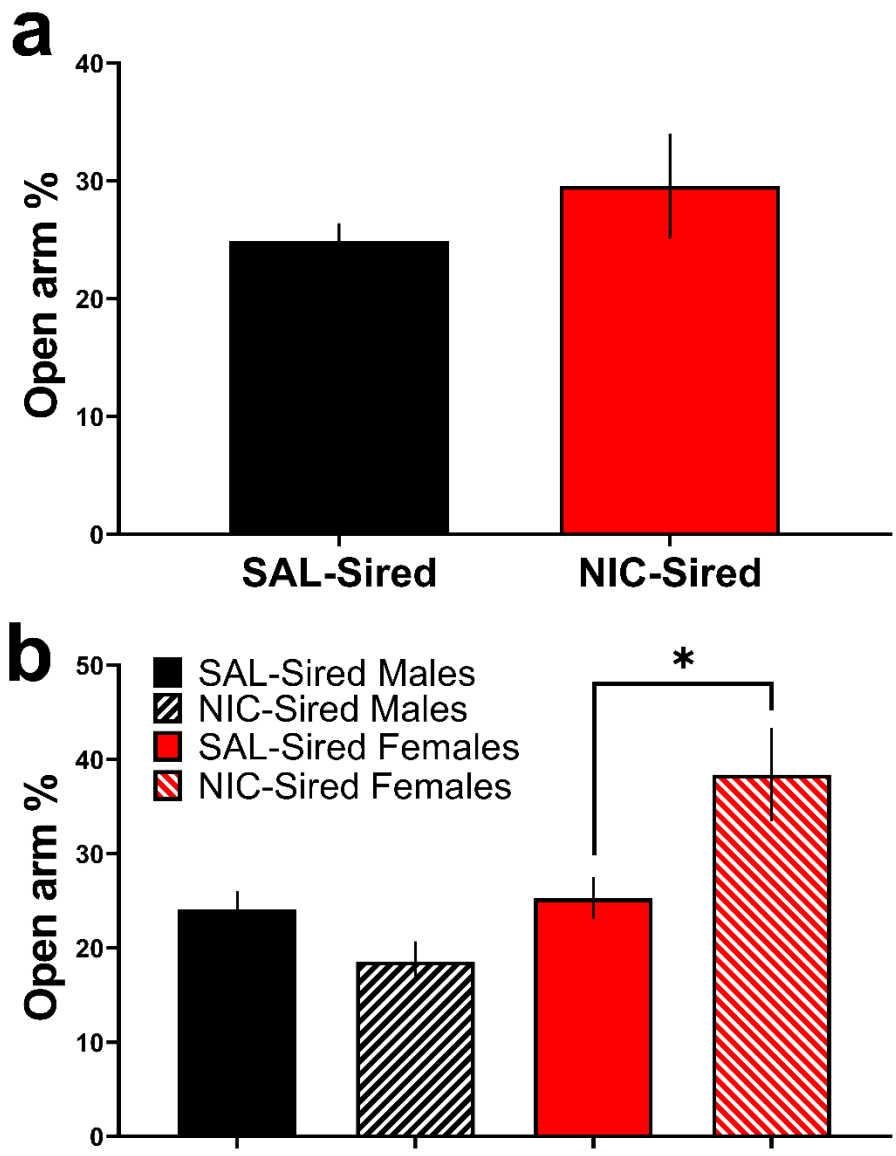

Figure S3: Effects of paternal nicotine on anxiety-like behaviors. (A) In analyses collapsed across sex, no differences in open arm percent between NIC- and SAL-Sired mice were found (n=9-10 per group). **(B)** Percent time in open arm was increased only in NIC-Sired females. Error bars indicate standard error of the mean (SEM), *p<0.05.

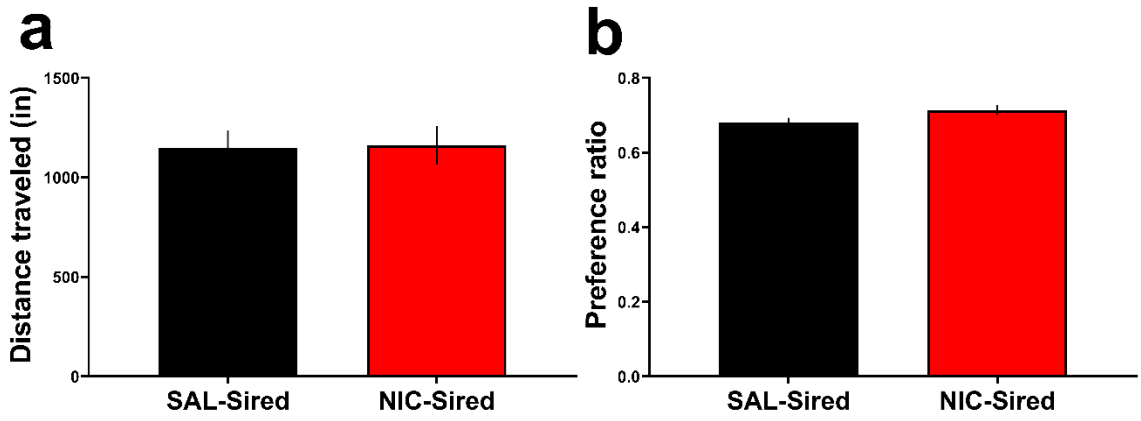

Figure S4: Paternal nicotine does not affect locomotor behavior or object location memory. (A) There was no effect of paternal nicotine exposure on locomotor activity in the Open Field paradigm (n=9-10 per group). (B) The NIC-Sired group did not differ in the Novel Object Recognition paradigm compared to SAL-Sired controls (n=9-10 per group). Error bars indicate Standard Error of the Mean (SEM).

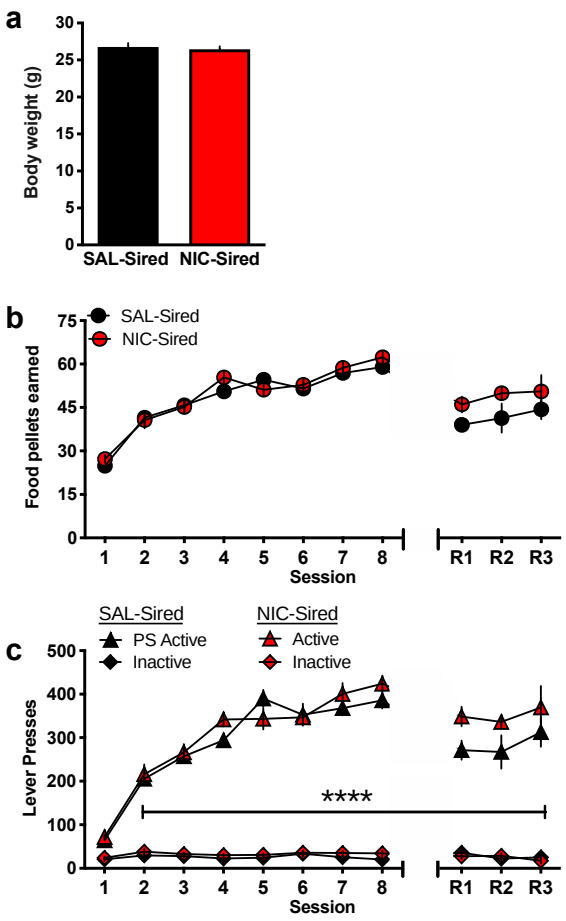

Figure S5: Paternal nicotine does not affect ability to learn an operant task. (A) NIC- and SAL-Sired mice did not differ in baseline free feeding body weight. **(B)** NIC- and SAL-Sired mice demonstrated a similar learning curve to obtain food reward up to a fixed ratio 5, time out 20 second schedule of reinforcement. Following the intravenous surgery and post-surgical recovery period, the groups equally resumed a high level of responding for food in the operant chambers (Re-established responding sessions, R1-R3). **(C)** During food training, both groups displayed a preference for the food-reinforced active lever from session 2 to 8 and during the re-establishment of food responding post-surgery on sessions R1-R3. The groups did not differ in their number of active lever presses, or in their number of inactive lever presses. Error bars indicate Standard Error of the Mean (SEM), ****p<0.0001, Active lever vs. inactive lever.

Table S1: Targeted bisulfite sequencing list
Table S2: vHPC and dHPC differential gene expression
Table S3: vHPC and dHPC IPA networks
Table S4: vHPC, dHPC, overlap dHPC and vHPA IPA diseases and disorders/molecular and cellular functions
Table S5: vHPC and dHPC EnrichR list
Table S6: Unique to vHPC or dHPC IPA canonical pathways