Supporting Online Material

Materials and methods

Mice.

All experiments were conducted in accordance with the guidelines for care and use of animals provided by the National Institutes of Health, as well as, with an approved animal protocol from the California Institute of Technology animal use committee. Animals were kept on a standard 12 hr light/dark cycle and given food and water *ad libitum*. Mutant mice were back-crossed at least twice to the C57BL/6 strain.

Leu9'Ala Mouse Construction.

The strategy used to generate the Leu9'Ala line adapts the procedures used to generate the previous Leu9'Ser line. From previous work, we have in hand a 129/SvJ α4 genomic clone containing exon 5 with the L9'S mutation in the vector pKO Scrambler V907. A neomycin resistance cassette with a phosphoglycerate kinase promoter and polyadenylation signal flanked by loxP sites is present 163 bp downstream from exon 5 for positive selection. The diphtheria toxin A chain gene with an RNA polymerase II promoter has also been added to provide negative selection for random insertion.

A 4.1 Kb cassette containing exon 5 was removed from the pKO vector via digestion with Spe I and inserted into the Bluescript vector for modification. PCR mutagenesis using the QuikChange Site-directed Mutagenesis Kit was utilized to replace the Leu9'Ser mutation with the Leu9'Ala mutation (codon changed from

TCT to GCT). The presence of the mutation was confirmed by automated sequence analysis. A small 250 bp cassette containing the Leu9'Ala mutation was subsequently cut out from Bluescript with BsrGI and BglII, then unidirectionally ligated into Bluescript Leu9'Ser α4 exon 5. This was done to avoid sequencing the entire 4.1 Kb exon 5 cassette that went through PCR amplification. The 4.1 exon 5 cassette was then removed by digestion with Spe I and ligated back into the knock-in construct. Clones in the correct orientation yielded two fragments, 8 and 7 Kb in length, upon digestion with Nsi I (as opposed to 10 and 5 Kb fragments in the wrong orientation). The α4 Leu9'Ala knock-in construct was linearized with Not I and chloroform/phenol extracted in preparation for introduction into mouse embryonic stem cells (ES cells).

CJ7 mouse ES cells were electroporated with 25 μg of linearized knock-in construct. Six rounds of electroporation were performed using 9.9×10^6 ES cells/electroporation. After electroporation each group of cells was plated on 10 cm dishes containing single layers of mitotically inactivated mouse primary fibroblasts. To select for recombinant clones, G418 (180 μg/ml) was applied to ES cells 24 hours after plating. Cell death was apparent three days post treatment. After seven days of G418 treatment, 600 resistant colonies were picked and plated onto 96 well plates, grown for 48 hours, trypsinized, then split onto duplicate plates, one for freezing the other for screening. Correct recombinant colonies were screened for the presence of the Neo cassette and Leu9'Ala mutation by a combination of PCR and automated sequence analysis. DNA for PCR and sequencing was isolated by applying lysis buffer (10 mM Tris

pH 7.5, 10 mM EDTA, 10 mM NaCl, 1% SDS and 1 mg/ml preteinase K) to cells and incubating overnight at 60^{o} C followed by ethanol precipitation. To confirm the position of the Neo cassette, ES cells were screened via Southern blot. Positive clones were submitted for sequencing to verify the presence of the Leu9'Ala mutation. Karyotype analysis was utilized to assess chromosome number. Two positive clones with the highest percentage of proper chromosome number (90%) were selected for the subsequent round of electroporation.

Previous work with the Leu9'Ser mice has indicated that the presence of the Neo cassette reduces expression of the target gene by ~4-fold. To maximize expression of the mutated gene we have deleted the Neo cassette. This was done by electroporating two clones of the Neo-intact Leu9'Ala α4 ES cells with CMV CRE plasmid, leaving only a single 34-bp loxP. Electroporation was performed as described above. Cells were plated at low density (400 cells/plate) onto 10 cm dishes containing single layers of mitotically inactivated mouse primary fibroblasts. A total of 200 colonies were picked and plated onto 96 well plates, grown for 48 hours, trypsinized, and split onto triplicate 96 well plates, one for freezing, and two for screening. Clones were initially screened for Neo cassette deletion by G418 (180 μg/ml) treatment. G418 sensitive clones were then further screened using a combination of PCR and automated sequencing. Primers were designed to anneal to a DNA sequence upstream of the 5' loxP site and downstream of the 3' loxP site. All neo-deleted clones were sequenced to confirm the presence of the Leu9'Ala mutation. Karyotype analysis was used to assess chromosome number. Two Neo-deleted Leu9'Ala α4 clones were

injected into C57BL/6 blastocysts and chimeras were established. Chimeras were mated with wild-type animals and germline transmission was established as assessed by PCR analysis of tail DNA and sequence analysis.

Epibatidine Binding.

Mice of wild-type, Leu9'Ala heterozygous and homozygous genotypes were sacrificed by cervical dislocation. Brains were removed, and the desired regions dissected on an ice-cold platform. Tissue was collected from the superior and inferior colliculi, midbrain, striatum, olfactory tubercles, and hypothalamus, then homgenized in ice-cold 0.1x binding buffer (mM: NaCl, 14.4; KCl, 0.2; $CaCl_2$, 0.2; $MgSO_4$, 1.0; HEPES, 2.0; pH = 7.5) using a glass-Teflon tissue grinder. Homogenates were washed three times by centrifugation (12,000 x *g*, 15 min, 4 °C) and resuspension into 0.1x binding buffer before a final centrifugation and storage at -70 °C.

[^{125}I]Epibatidine inhibition binding experiments were performed using 200 pM [^{125}I]epibatidine, equivalent to 25,000 cpm well^{-1}. The amount of membrane protein added was chosen to produce maximum binding of the ligand to the tissue of approximately 1000 Bq well-1, (< 5 % of total ligand added, minimizing the effects of ligand depletion), and a minimum of 300 cpm of specific binding. Membranes were incubated for 2 hours at 22 °C in 30 µl of 1x binding buffer. Non-specific binding was defined in the presence of 1 mM (-)-nicotine tartrate, and fell in the range of 20 – 50 cpm. Cytisine inhibition of [^{125}I]epibatidine was measured using drug concentrations in the range of 0.1 – 3000 nM, plus a no-

drug control. All determinations were performed in duplicate. Incubations were terminated by filtration onto Gelman GF/F fiber filters using an Inotech cell harvester, followed by six washes with ice-cold 1x binding buffer. Bound ligand was quantitated at 80 – 85 % efficiency using a Packard Cobra gamma counter. Specific binding was calculated for every region from every animal by subtraction of the relevant non-specific binding value. Protein concentrations were determined by the method of Lowry *et al*. (1953), using bovine serum albumin as the standard, and used to convert binding data to fmol [^{125}I]epibatidine bound mg^{-1} (protein).

Calculations.

Results for inhibition of [^{125}I]epibatidine binding were calculated using a two site fit: $B = B_1 / (1 + (I / IC_{50\text{-}1}) + B_2 / (1 + (I / IC_{50\text{-}2})$ where B is ligand bound at inhibitor concentration I, and B_1 and B_2 are binding sites sensitive to cytisine inhibition with $IC_{50\text{-}1}$ and $IC_{50\text{-}2}$, (cytisine-sensitive and cytisine-resistant sites, respectively).

Neuronal Cultures.

Brain tissue containing primary mesencephalic neurons of the ventral tegmental area from embryonic day 14 wild-type and Leu9'Ala heterozygous mouse embryos (Shimoda et al., 1992) were incubated in plating medium (Neurobasal, 2% B27, 0.5 mM glutamax, 5% horse serum) with 1 mg/ml papain at 37°C for 20 min. The digested tissue was mechanically dispersed, laid on 5% bovine serum albumin in PBS, spun for 6 min at 300 X g, and resuspended in plating medium. Cells (~150,000) were plated onto 35 mm petri dishes with glass coverslip

bottoms that were coated with poly-D-lysine 1mg/ml. Neurons were incubated at 37^0C, in 5% CO2 for 4 weeks before the start of each experiment. Cultures were treated with AraC (1-ß-D-Arabinofuranosylcytosine) overnight after 3-5 days in culture to inhibit glial growth.

Calcium Imaging.

Cultured ventral midbrain neurons were loaded in the dark for 35 min with fura-2 AM (1.25 μM) in pluronic acid (0.01%). During imaging, cells were perfused continuously with extracellular solution. Images were obtained with an inverted fluorescence microscope. Ratio images were obtained by acquiring pairs of images at alternate excitation wavelengths (340/380 nm, Lambda 10–2 filter wheel, Sutter Instrument Co) with an 80 nm bandpass emission centered at 510 nm. Images were collected with a 40X oil immersion objective (UApo/340 1.35 NA) and captured (one pair per 2 sec) on a Photometrics Cascade CCD camera (16 bit resolution) under the control of Slidebook 4.0 imaging software.

Extracellular solution (ECS) composition was identical to that reported in Nashmi et. al. (*28*) and contained 2 mM ascorbic acid. ECS [methyllycaconitine (MLA)] and without nicotinic receptor blockers were delivered via bath perfusion using a peristaltic pump. For imaging in midbrain neurons, sodium channels, NMDA, AMPA, and GABAA receptors were blocked by including 0.5 μM TTX, 50 μM AP5, 10 μM CNQX, and 20 μM bicuculline in the bath perfusate. To block muscarinic responses, 0.5 μM atropine was included in the extracellular solution (ECS). The agonist nicotine or ACh was delivered for either 2 or 5 sec using a

U-tube. A silica tube (150 μm inner diameter and 360 μm outer diameter) projected from the pore of the U-tube and directed agonist outflow. Negative pressure was constantly applied to the U-tube to prevent agonist leakage and desensitization of receptors. For this reason, agonists were applied for at least 2 sec to overcome the delay (~500 msec) in delivery of agonist produced by the negative pressure. The agonist delivery via the U-tube was tested each day before and intermittently during the experiment by visual inspection of the output of phenol red added to the ECS.

Electrophysiology.

Anaesthetized (halothane) mice (postnatal day 12-25) were decapitated and brains were placed in an ice-cold artificial cerebrospinal fluid (ACSF containing, in mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, pH 7.3, bubbled with 95% O_2 and 5% CO_2). 300 μm-thick coronal slices containing midbrain were cut with a vibratome, and stored in bubbled ACSF with kyurenic acid (1 mM) for 30 min. and without kyurenic acid for at least another 30 min. In midbrain, horizontal slices include the pedunculopontine tegmental nucleus, which along with the laterodorsal tegmental nucleus is a major source of cholinergic projections to the VTA (*29*).

During recordings, slices were perfused with ACSF at 32-33°C. Somatic whole-cell recordings were made from visually identified VTA neurons (medial in respect to fibres of the oculomotor nerve). Patch electrodes were pulled from borosilicate glass on a Narishige PP-38 pipette puller and filled with a solution

containing (in mM): 144 K-gluconate, 2 $MgCl_2$, 10 HEPES, 0.2 EGTA and titrated to pH 7.2. The resistance of the electrodes was 3 - 6 MΩ. Series resistance was compensated 70-85% using lag values of 7-8 µs. Recordings were low-pass filtered at 2 kHz and digitized on-line at 20 kHz. Dopaminergic neurons were identified by anatomy, the presence of I_h, baseline AP frequency (1-4 hz), AP length, and pronounced after-hyperpolarization. Nicotine was applied using a glass pipette connected to a picospritzer. The glass pipette was moved within 20 µm of the recorded cell using a piezoelectric device over a period of 250 ms starting 300 ms prior to drug application. Nicotine (1 µM) was then applied for 250 ms at 10-20 psi . 50 ms after the end of this puff, the glass pipette was retracted over a period of 250 ms.

Telemetry Probes.

Vital View PDT-4000 temperature and activity telemetric probes were used. For implantation, mice were anesthetized with halothane, and a 1 cm incision was made at the back of the neck. Probes were inserted subcutaneously into the back of the animal. The incision was sealed with surgical glue and the mice were allowed to recover for 48 hours. Temperature and activity data were acquired using vital view software and analyzed in Origin.

Nicotine Administration

Nicotine base was used in all behavioral experiments and administered through i.p. injection with 300 µL insulin syringes. Animals were injected daily with saline

for at least seven days prior to the start of each temperature and activity experiment. Saline and nicotine were warmed to 37^{o}C prior to injection.

Conditioned Place Preference Methods.

The conditioning place preference test apparatus consists of a rectangular cage with overall inside dimensions of 46.5(L) x 12.7(W) x 12.7(H) cm. This includes a center neutral gray compartment that is 7.2 cm (L), and a hinged clear polycarbonate lid for loading the test animal. The adjacent conditioning compartments measure 16.8 cm (L). One compartment is black with a stainless steel grid rod floor and the other is white with a 6.35 mm square stainless steel mesh floor. Guillotine doors separate the chambers and can be fixed in the closed or opened position.

The test consists of three stages over a 10 day period. In the first stage a mouse is placed in the central compartment and allowed free access to all chambers. The time spent in each chamber is recorded over a 20 min period. During the second stage, i.p. injections of nicotine are paired with one of the conditioning chambers while injections of saline are paired with the other. The mouse receives nicotine injections and is placed in the isolated nicotine conditioning chamber on days 2, 4, 6 and 8. The mouse receives saline injections and is placed in the saline chamber on days 3, 5, 7, and 9. Each training trial lasts 20 min. During stage 3, the testing phase, the mouse is once again given free access to all chambers for 20 min. The time spent in each chamber during stage 1 (baseline) is subtracted from the time spent in each

chamber during stage 3. A preference toward the nicotine associated chamber compared to baseline is a measure of the reward behavior associated with nicotine. Typical wild-type mice display a maximal preference for the nicotine associated chamber when challenged with 0.5 mg/kg nicotine during stage 2. Control mice receive saline in both chambers. Drug naïve mice that exhibit a severe bias towards one of the two conditioning chambers during stage 1 are excluded from the study. We define bias as > 65 % of total time spent in one conditioning chamber. On average, 13 % of wild-type and 10 % of mutant animals show severe bias towards one chamber before training.

Statistics.

Statistical significance in the place preference assay was determined using two-way ANOVA between subjects with genotype and treatment acting as variables. One-way ANOVA was used between same-subject groups to assess significance between nicotine preference and saline. For epibatidine binding, within-region effects of genotype on B1 and B2 (the size of the cytisine-sensitive and –resistant [^{125}I]epibatidine binding populations, respectively) were tested using one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test, where $p < 0.05$ was considered significant. Significance in all additional experiments was determined by one-way ANOVA or two sample t-test as indicated. In addition, post-hoc analysis (Tukey test) was performed after analysis of variance in all cases unless otherwise noted.

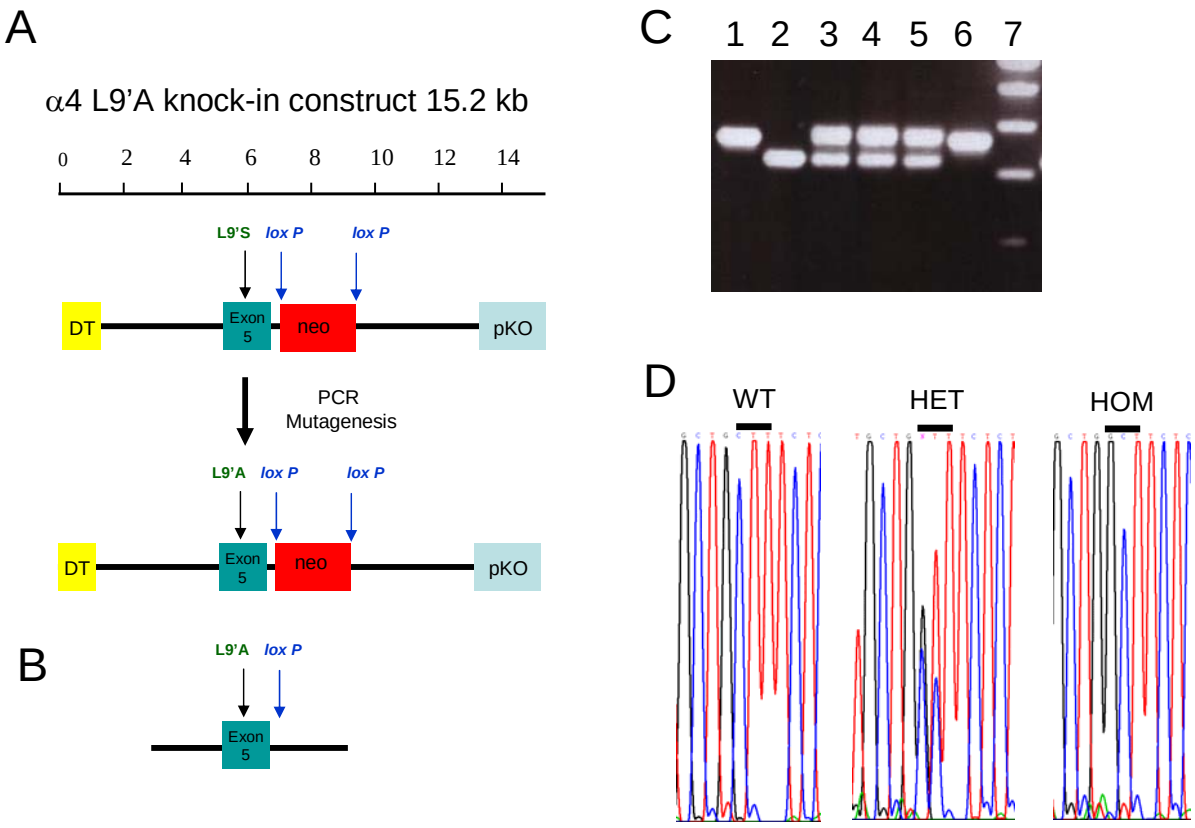

Fig. S1: Leu9'Ala mouse construction. A) Targeting construct containing exon 5 with the Leu9'Ser mutation was used as the template for the Leu9'Ala construct. Quickchange PCR mutagenesis was utilized to replace the Leu9'Ser mutation with the Leu9'Ala mutation. In addition to exon 5 and the targeted mutation the knock-in construct contains a neomycin resistance gene (neo) flanked by loxP sites, a diphtheria toxin A chain gene (DT), and the pKO V907 vector (pKO). B) The Neo cassette was deleted by transfecting recombinant stem cells with a cytomegalovirus-Cre plasmid. The resulting clones contain the targeted mutation

and a single 34 bp loxP site. C) Agarose gel illustrating amplified genomic DNA fragments stained with Ethidium Bromide from Leu9'Ala homozygous (lanes 1 and 6), heterozygous (lanes 3, 4, and 5) and wild-type (lane 2) mice. Bands migrating at 218 bps represent wild-type alleles while bands at 252 bps represent mutant alleles. The 34 bp difference between mutant and wild-type alleles is due to the lox P site present in the mutants. Lane 7 contains 100 bp ladder molecular weight marker. D) Sequence analysis of DNA extracted from wild-type (left graph), heterozygous (middle graph) and homozygous (right graph) animals. The WT sequence corresponding to the 9' position in M2 is CTT which is mutated to GCT in the Leu9'Ala mutants.

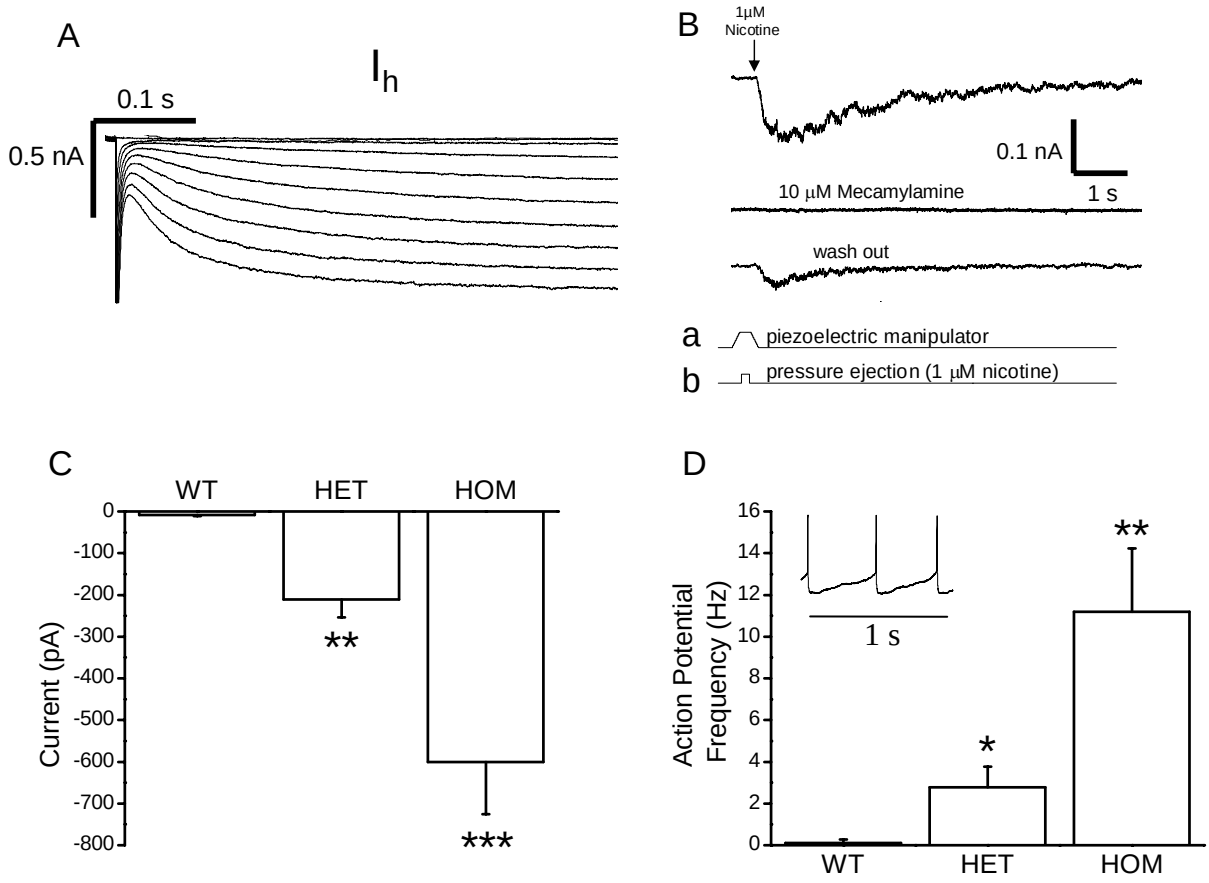

Fig. S2: Hypersensitive nicotinic responses in Leu9'Ala midbrain slice dopaminergic neurons. Whole-cell patch-clamp recordings from dopaminergic neurons in Leu9'Ala midbrain slice. A) Dopaminergic neurons were identified by activation of the hyperpolarizing activated potassium current, I_h, Voltage was held at -70 mV and jumped to test potentials between –60 mV and -150 mV at 10 mV increments. B) Representative recordings from a dopaminergic neuron in response to nicotine. The top traces show a response, followed by a response during perfusion in 10 μM mecamylamine, followed by a wash at least 5 min. The latter shows partial recovery, confirming that the response arises from nicotinic receptors. The bottom traces show the command (a) to the piezolectric manipulator, which places the pipette tip within 20 μm of the cell, and (b) to the pressure apparatus, which ejects a 250 ms puff of 1 μM nicotine. C) Average whole-cell responses from VTA dopaminergic neurons in midbrain slices from wild-type (n = 6), Leu9'Ala heterozygous (n = 8) or homozygous (n = 5) mice in response to 1 μM nicotine. Responses were also observed in I_h-negative (GABAergic) cells from mutant mice, but not from WT mice (data not shown). Current traces are filtered at 100 Hz. D) Average increased action potential frequency in response to 1 μM nicotine in wild-type (n = 6), Leu9'Ala heterozygous (n = 8), and homozygous (n = 5) mice. Inset: Representative baseline action potential frequency in a dopaminergic VTA neuron. Significance was determined via two sample independent t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

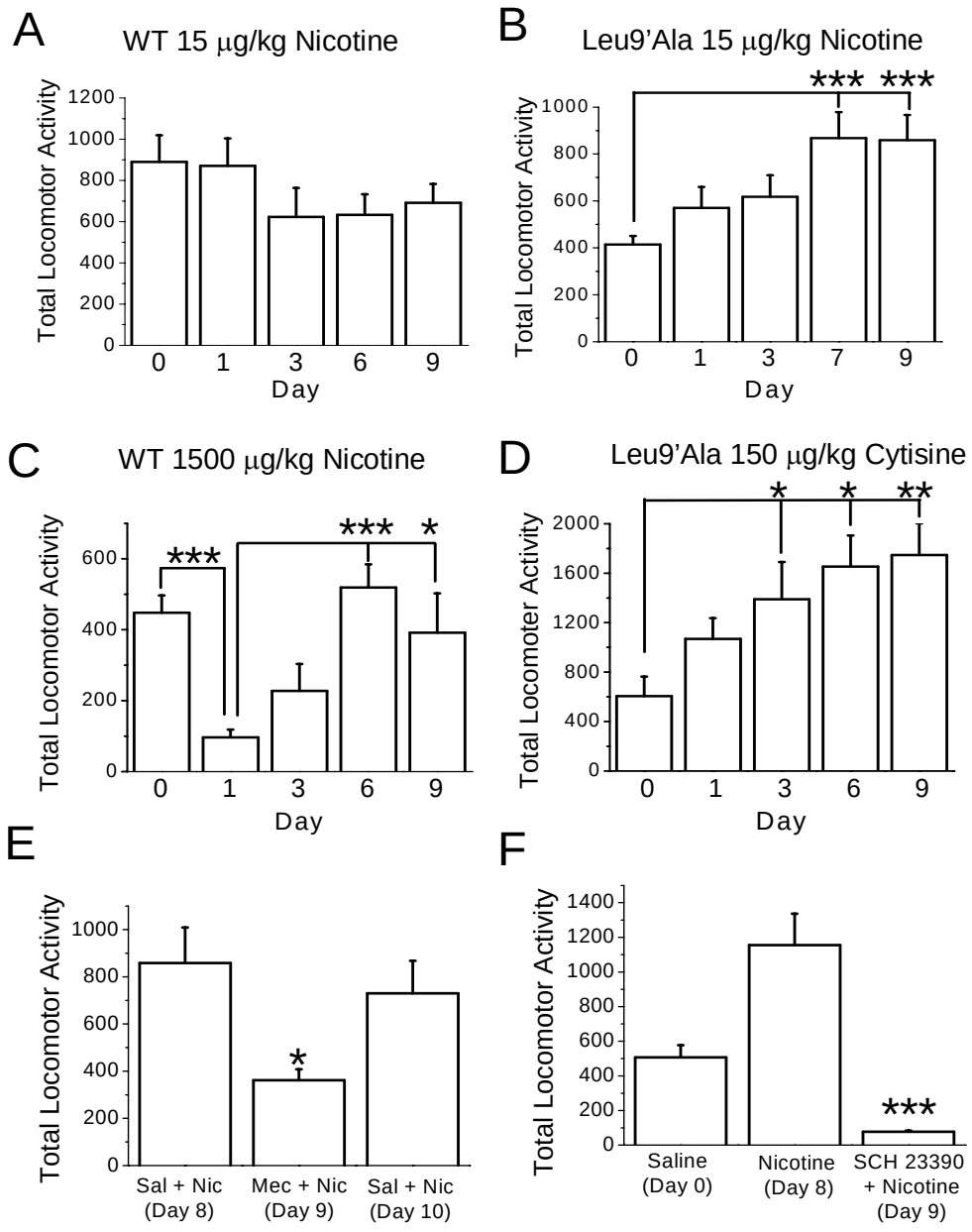

Fig. S3: Nicotinic agonist-induced locomotor activity in wild-type and Leu9'Ala male homozygotes: Locomotor activity was elicited by single daily i.p. injections of 15 μg/kg nicotine in A) wild-type (n = 7) or B) Leu9'Ala homozygous mice (n = 12). C) Locomotor effects in wild-type elicited by 1500 μg/kg nicotine. D)

Sensitization in Leu9'Ala homozgotes induced by 150 μg/kg cytisine. Each bar graph represents the averaged sum of 1 hour home cage activity immediately following injection. Baseline activity after saline injection is indicated as Day 0. E) Nicotine-induced locomotion is blocked by mecamylamine. Animals were sensitized via daily injections of 15 μg/kg nicotine as in A. On days 8, 9, and 10, animals were given saline or 1 mg/kg mecamylamine 15 minutes prior to nicotine as indicated (n = 7). F) Sensitization was induced as in A. Animals were administered 1 mg/kg SCH 23390, a D1 antagonist, 15 minutes prior to nicotine injection (day 9, n = 5). Data are expressed as mean +/- SEM. All animals were injected with saline once daily for at least 7 days prior to the start of the experiment. Significance was determined by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).