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Targeted Deletion of the Mouse α2 Nicotinic Acetylcholine Receptor Subunit Gene (*Chrna2*) Potentiates Nicotine-Modulated Behaviors

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Abstract

Baseline and nicotine-modulated behaviors were assessed in mice harboring a null mutant allele of the nicotinic acetylcholine receptor (nAChR) subunit gene α2 (*Chrna2*). Homozygous *Chrna2*$^{-/-}$ mice are viable, show expected sex and Mendelian genotype ratios, and exhibit no gross neuroanatomical abnormalities. A broad range of behavioral tests designed to assess genotype-dependent effects on anxiety (elevated plus maze and light/dark box), motor coordination (narrow bean traverse and gait), and locomotor activity revealed no significant differences between mutant mice and age-matched wild-type littermates. Furthermore, a panel of tests measuring traits, such as body position, spontaneous activity, respiration, tremors, body tone, and startle response, revealed normal responses for *Chrna2*-null mutant mice. However, *Chrna2*$^{-/-}$ mice do exhibit a mild motor or coordination phenotype (a decreased latency to fall during the accelerating rotarod test) and possess an increased sensitivity to nicotine-induced analgesia in the hotplate assay. Relative to wild-type, *Chrna2*$^{-/-}$ mice show potentiated nicotine self-administration and withdrawal behaviors and exhibit a sex-dependent enhancement of nicotine-facilitated cued, but not trace or contextual, fear conditioning. Overall, our results suggest that loss of the mouse nAChR α2 subunit has very limited effects on baseline behavior but does lead to the potentiation of several nicotine-modulated behaviors.

Introduction

Transcription of the *Chrna2* gene is widespread within the central and peripheral nervous systems (Wada et al., 1988, 1989; Ishii et al., 2005; Whiteaker et al., 2009). In primates, α2 mRNA levels are highest in the thalamus, prefrontal cortex, and hippocampus (Han et al., 2000; Aridon et al., 2006), whereas in rodents, *Chrna2* transcripts are found primarily in the interpeduncular nucleus (IPN) (all subnuclei), with more restricted expression in scattered

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neurons of the amygdala (medial, central, and basomedial nuclei, anterior amygdaloid area), hippocampus (interneurons of the oriens layer and alveus of CA1 and CA3), olfactory bulb (internal plexiform layer), cortex (selected interneurons in layers V and VI), retina, optic lobe, ventral spinal cord (Renshaw cells), and cerebellum (a subset of Purkinje cells) (Wada et al., 1988; 1989; Han et al., 2000; Ishii et al., 2005; Aridon et al., 2006). Moreover, at least in rats, *Chrna2* transcription shows strong, transient upregulation during cortical and hippocampal development with ~10- to 20-fold higher levels of *Chrna2* mRNA at postnatal day 7 relative to postnatal day 1 and adult (Son and Winzer-Serhan, 2006).

Electrophysiological and pharmacological studies suggest that functional α2-containing nAChRs are expressed on GABAergic interneurons in the rodent IPN (Mulle et al., 1991) and hippocampus (McQuiston and Madison, 1999; Sudweeks and Yakel, 2000; Nakauchi et al., 2007). Moreover, α2-containing nAChRs are required for nicotine facilitation (Schaffer collateral pathway) and suppression (temporoammonic pathway) of long-term potentiation of synaptic transmission in a subset of hippocampal stratum oriens/alveus interneurons (Nakauchi et al., 2007). These findings, as well as more recent studies (Jia et al., 2009, 2010), suggest an important role for α2-containing nAChRs in hippocampal circuits and nicotine enhancement of hippocampal-dependent learning.

Behaviorally, lesions of rodent brain regions that express nAChR α2 mRNA (e.g., amygdala, hippocampus, IPN) result in altered motor, sensory, anxiety, reward, drug withdrawal, and memory functions (Meszaros et al., 1985; Kopchia et al., 1992; Phillips and LeDoux, 1992; Hentall and Gollapudi, 1995; Barros et al., 2005; Quinn et al., 2009; Glick et al., 2011). Moreover, compared with wild-type littermates, mice in which the *Chrna2* gene has been deleted show a marked decrease in aversive somatic behaviors after mecamylamine-precipitated withdrawal in mice treated with chronic nicotine (Salas et al., 2009). In humans, combined genetic and clinical studies suggest a role for α2-containing nAChRs in tobacco dependence, obesity, schizophrenia, bipolar disorder, epilepsy, and drug use during adolescence (Blouin et al., 1998; Gurling et al., 2001; Ophoff et al., 2002; Sullivan et al., 2004; Aridon et al., 2006; Corley et al., 2008; Heitjan et al., 2008; Kim, 2008).

To investigate the role of α2-containing nAChRs in normal and nicotine-modulated behaviors, we genetically engineered a *Chrna2*$^{-/-}$ mouse line. After an initial, wide ranging behavioral assessment of *Chrna2*$^{-/-}$ mice, we examined the effects of genotype and sex on locomotion and coordinated movement, drug reinforcement and withdrawal, sensory processing (nicotine-induced analgesia), and memory and cognitive function (cued, contextual, and trace fear conditioning).

Materials and Methods

Engineering and husbandry of Chrna2-null mutant mice

The *Chrna2* gene was isolated from a 129X1/SvJ mouse genomic library. Inactivation of the *Chrna2* gene was accomplished via insertion of a neomycin resistance cassette into exon 5. The targeting construct was electroporated into 129X1/SvJ embryonic stem cells and screened for homologous recombination using Southern blot analysis. Embryonic stem cell clones showing homologous recombination were injected into mouse blastocysts, implanted into pseudopregnant females, and chimeric pups scored for germline transmission of the targeted allele. Genotyping was performed using tail biopsy DNA, HotStar Taq DNA polymerase (QIAGEN) and PCR primers A213 (5′-GTA GAA CAC GAG CAC GGT GAG GCA-3′ and A216 (5′-CTA AAT GCA CAG AGT ACA GGG AGT-3′) to generate DNA fragments of 1042 and 1332 bp for the wild-type and targeted allele, respectively (Fig. 1). All mice used in this study were placed on a 12 h dark-light cycle with *ad libitum* access to

food and water. Behavioral experiments were performed during daylight hours using congenic animals (C57BL/6J, N9-N17) as per National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The UCLA, Scripps Research Institute, and Temple University Institutional Animal Care and Use Committees approved all animal experimentation. For contextual and cued fear conditioning, or intravenous nicotine self-administration, male and female *Chrna2*$^{-/-}$ mice and their wild-type littermates were bred at UCLA, transferred to Temple University or the Scripps Research Institute, at 4 weeks of age, and were tested ~4 weeks later. For trace fear conditioning experiments, mice arrived at 8–16 weeks of age and were conditioned a few weeks later. The *Chrna2*-null mutant mouse line (030508-UCD) is available from the Mutant Mouse Regional Resource Center, University of California, Davis.

RNA extraction and RT-PCR

Total RNA was extracted from adult mouse olfactory bulbs using an RNeasy Protect Maxi Kit and was reverse transcribed using random octamers and Omniscript reverse transcriptase as described by the manufacturer (QIAGEN). The resulting cDNAs were used as PCR template for transcripts encoding *Chrna2* (primers A222, 5′-GTA GAT CTC CGC GCA GCA GTC-3′, and A223, 5′-AGT GCA GAC GGG GAG TTT GCG) and *Omp* (olfactory marker protein) (primers Omp1, 5′-ATC CAG CAG CAG AAG CTG CAG-3′, and Omp2, 5′-TGC TGC GGC TCA CAG TGA TGT-3′).

Nicotine administration

Nicotine hydrogen tartrate salt (Sigma-Aldrich) was used to prepare stock solutions, pH 7.4, that were delivered via intraperitoneal or intravenous injections as well as via Alzet mini-osmotic pumps. Nicotine concentrations were calculated as the base. For acute injections, the nicotine tartrate salt was dissolved in sterile, physiological saline (0.9% NaCl) and injected in a volume of 10 ml/kg body weight.

SHIRPA behavioral phenotype assessment

We first compared wild-type and *Chrna2* mutant mice using SHIRPA (Table 1), a comprehensive screening and testing protocol designed to identify and characterize phenotype variation or behavioral disorders (Rogers et al., 1997). Behavioral measures included assessment of body position, spontaneous activity, respiration rate, tremor, transfer arousal, palpebral closure, piloerection, startle response, gait, pelvic elevation, tail elevation, touch escape, positional passivity, visual placing, grip strength, body tone, pinna reflex, corneal reflex, toe pinch, wire maneuver, and negative geotaxis. Observations were scored using standard methods.

Movement and coordination

To assess overall motor coordination, mice were tested on an accelerating rotarod. The apparatus consists of 1.25 inch diameter rod that is accelerated to a maximum speed of 18.3 revolutions per minute. Mice were observed for their latency to fall off the accelerating rotarod during a maximum period of 180 s. Each mouse underwent three consecutive trials per day for 5 d.

To evaluate gait, mice were trained (five trails per day for 3 d) to run through a narrow alley (5 cm wide, 43 cm long, 12.5 cm high). Subsequently, the paws of the mice were painted with nontoxic dye, a white sheet of paper was placed on the floor of the alley, and stride length and width were measured to assess aberrations in gait.

Mice were trained and tested for motor coordination using an elevated narrow beam traversal test. The square beam (25 cm long, 3.5 cm wide at the start) was reduced to 0.5 cm

in 1 cm stepwise increments. Mice were placed on the widest portion of the beam and allowed to walk to the opposite side. Training occurred across 2 d with five tests per day. On the third day, a 1 cm × 1 cm mesh grid was placed on the narrow beam to increase the difficulty of the traversal. Mice were assessed for a subsequent five trials, and the number of mean steps, step mistakes, and the total time taken to complete the walk were quantified from video recordings.

The pole test assessed the ability to coordinate movement across a 1-cm-diameter wooden pole, 50-cm-long situated above their home cage. Animals were placed at the top of the pole, with their head positioned away from their home cage. Subsequently, across five training sessions, animals had to learn to reorient their position and descend the pole toward their home cage. The time required to reorient their head position toward their home cage and descend to the bottom of the pole was measured. General locomotor activity was recorded in a novel environment for 30 min. The total number of beam breaks was digitally recorded across 16 photo beams in a polycarbonate plastic box (27 × 27 × 20.3 cm) (Med Associates). Mice were injected with saline (0.9%) or nicotine (1 or 3 mg/kg, i.p.) immediately before being placed into the locomotor activity chamber.

Anxiety

Behavioral measures of anxiety were assessed using an elevated plus maze and a light/dark box. The elevated plus maze consists of four polycarbonate arms extending 11.5 inches in length, two of which have walls 6.5 inches in height. The plus maze was elevated from the floor at a height of 24 inches. Mice were placed in an open square dividing the four arms of the chamber. An overhead camera recorded the time spent on the open arms of the plus maze during each 5 min test session. The light/dark box consisted of two polycarbonate chambers, one black covered compartment (10 × 12 × 7 inches) separated from the light chamber (10 × 12 × 12) through a trap door (2 × 2 inches). Mice were confined to the dark chamber for 5 min after which the trap door was raised and the latency to enter the light chamber was recorded, with a maximum cutoff time of 5 min. Time spent in, or latency in time to enter, the brightly lit side of the light-dark box are considered measures of anxiety-like behavior in the light/dark crossing test (Crawley, 1981).

Nicotine and morphine analgesia

The hotplate assay was performed using an AccuScan Instruments metal surface, with a perimeter Plexiglas cylinder (7.5 cm in diameter × 13 cm high). The temperature of the metal surface was set at 52.5°C. Animals were habituated to the testing room for 1 h and then assessed for baseline latency to flick or lick hindpaw or using a stopwatch. A 60 s cutoff time was implemented to avoid tissue damage from the test. After 30 min, mice were injected with nicotine (1.25 mg/kg, i.p.) and assessed for analgesia 5 min after injection. Maximum possible efficacy (MPE) was scored by the following formula: % MPE = [(test – baseline)/(cutoff – baseline)] × 100.

The tail withdrawal assay was performed using a constant temperature circulating water bath set to 48°C. Mice were habituated to the testing room for 1 h. Subsequently, mice were lightly restrained using a cotton sock and assessed for baseline latency to flick, withdraw, or flinch their tail, using a stopwatch. One hour later, mice were injected with nicotine (1.25 mg/kg, i.p.) and analgesia was tested 5 min after injection. The test was repeated for five consecutive days. A 15 s cutoff time was implemented to avoid tissue damage from the test. To assess sensitivity to persistent pain, mice were habituated to the testing chamber for 15 min and subsequently administered nicotine (1.25 mg/kg, i.p.) or 0.9% saline. After 5 min, 25μl of 5% formalin was administered (s.c.) in to the plantar surface of the right hindpaw.

The time spent licking or biting their hindpaw was recorded every other minute for a total of 60 min.

Morphine-induced analgesia was also assessed in wild-type and *Chrna2* $^{-/-}$ mice using the tail withdrawal assay. On day 1, mice were tested three times for baseline tail withdrawal 20 min apart. The tail withdrawal assay was performed at 49°C using a constant temperature water bath. Mice were lightly restrained using a cotton sock and assessed for baseline latency to flick, withdraw, or flinch their tail, using a stopwatch. A 15 s cutoff time was implemented to avoid tissue damage. Subsequently, mice were injected with morphine (10 mg/kg, i.p.), analgesia was tested 30 min after injection, followed by a return to their home cage. On day 7, to assess tolerance to morphine, before opioid treatment, animals were reassessed for baseline latency to flick or flinch their tail, as described above. Subsequently, mice were injected with the last dose of morphine, tail withdrawal assessed 30 min after injection, followed 30 min later by the naloxone treatment to assess somatic withdrawal.

Fear conditioning

Fear conditioning experiments were performed in chambers made with Plexiglas front and back walls and aluminum side walls (18 × 19 × 38 cm Med Associates). Conditioning chambers contained stainless steel rod grid floors (2 mm diameter spaced 1 cm apart) inside sound-attenuating cubicles containing a house light (4 W) and ventilation fan that produced a constant white noise (69 dB). Grid floors were connected to a scrambled shock generator that delivered 0.57 mA foot shocks controlled by a computer running MedPC-IV software. All chambers were cleaned with 70% ethanol before and after each animal.

Cued fear conditioning testing took place in separate chambers (altered context, 20 × 23 × 19 cm) contained within sound attenuating cubicles (Med Associates). The altered context chambers differed in construction such that they had Plexiglas front and back walls, aluminum sidewalls, and a flat plastic floor. Additionally, vanilla extract was added to alter the olfactory stimuli present in the chambers. All chambers were cleaned with 70% ethanol before and after each use.

Mice were trained in contextual fear conditioning as previously described (Davis et al., 2005). Mice were placed into conditioning chambers and allowed to explore for 2 min, at which time a conditioned stimulus (CS, white noise, 85 dB) was presented continuously for 30 s and co-terminated with the unconditioned stimulus (US, shock) lasting 2 s. After the CS–US pairing, 2 min elapsed before a second CS–US pairing. Mice were returned to their home cages 30 s after the second CS–US pairing. At 24 h after conditioning, mice were returned to the training context and assessed for freezing to the context for 5 min.

Approximately 2 h after contextual testing, mice were placed into an altered context and assessed for freezing for 3 min. After 3 min, the CS was presented continuously for an additional 3 min. Freezing to the context was used as a measure of contextual memory. Freezing before the CS (altered context freezing) was used as a measure of generalized freezing, whereas freezing during the CS was used as a measure of fear memory (Gould and Lewis, 2005).

Trace fear conditioning took place in the same conditioning chambers (Davis and Gould, 2007). On conditioning day, mice were placed into the chambers and allowed to explore for 2 min before the first CS-trace-US pairing. The 30 s CS preceded a 30 s trace period, and the US was 2 s long (as in contextual fear conditioning experiment). Conditioning consisted of five CS-trace-US pairings separated by a pseudorandom interval that averaged 2 min. Freezing during conditioning was assessed using a 10 s time sampling procedure for the initial 2 min period (baseline), during each 30 s trace interval (traces 1–5), and between

pairings 4 and 5 (immediate). At 24 h after conditioning, mice were placed into altered context (described above) and were assessed for freezing for 3 min (pre-CS). After 3 min, the CS was presented for 3 min (CS).

For acute drug treatments, before training and testing procedures, mice were administered a single nicotine (0.09 mg/kg) or 0.9% saline injection 5 min before being placed into the behavioral testing apparatus. For measures of nicotine withdrawal, mice were subcutaneously implanted with an Alzet osmotic mini-pump delivering either saline or nicotine (12 mg/kg per day) for 12 d (Portugal et al., 2012). On day 12, the osmotic mini-pump was surgically removed. Cued and contextual training and testing occurred 24 and 48 h after mini-pump removal using the same paradigm described above.

Somatic nicotine withdrawal in novel and habituated environments

Male and female mice were group housed before evaluating nicotine withdrawal using an Alzet mini-osmotic pump (model 1002); saline or nicotine in PBS was delivered at a 24 mg/kg per day dose of nicotine for 14 d at a flow rate 0.25 μl/h, as described previously (Salas et al., 2009). After 13 d of chronic treatment, mice were placed in clear activity cages (a novel environment) and allowed 40 min to acclimate. After acclimation, mecamylamine (3 mg/kg, i.p.) was administered, and mice were evaluated for withdrawal signs over the next 20 min. All experiments were recorded using a video camera. Withdrawal signs were scored for 10 min before and 20 min after mecamylamine injection. The following somatic signs of withdrawal were monitored, with each event receiving a score of one point: paw tremors, head shakes, backing, curls, grooming, scratching, chewing, and shaking. Secondary parameters, scored as no more than one point per minute during behavioral observation, were as follows: cage scratching, head nodding, and jumping.

During the observation period, simultaneous measurements of locomotor behavior, center time, perimeter time, and rearing behavior were assessed, allowing for the quantification of physical and psychological components of withdrawal (Salas et al., 2007; Jackson et al., 2008). Rearing behavior was scored manually 10 min before and 20 min after mecamylamine injection, whereas locomotor behavior and center time were assessed during the entire 60 min using automated EthoVision XT software (version 7.0) and camera tracking system (data not presented). The 2 week length of nicotine exposure and dose of mecamylamine were chosen based on previously published work (Kenny and Markou, 2001; Damaj et al., 2003). Importantly, earlier work has reported that, in drug-naive C57BL/6J mice, the 3 mg/kg dose of mecamylamine does not increase somatic withdrawal signs under similar experimental conditions (Salas et al., 2009).

Experimental conditions for assessing nicotine withdrawal in the home cage (a habituated environment) were identical to those described above, except that male and female mice were single housed 2 weeks before the experiment. Given the presence of bedding in the home cage, we also included measures of burrowing behavior (i.e., using nose to dig in bedding, as well as pushing and playing with bedding).

Morphine withdrawal

Male and female mice were injected subcutaneously twice per day with escalating doses of morphine over 6 d (days 1 and 2, 10 mg/kg; days 3 and 4, 20 mg/kg; days 5 and 6, 40 mg/kg). On the seventh day, animals were injected with morphine (10 mg/kg), followed 1 h later with naloxone (2 mg/kg s.c.). Somatic withdrawal signs were measured for 20 min in a novel environment (Plexiglas cylinder, 17 cm in diameter × 34.5 cm high). Withdrawal symptoms included all measures identified under nicotine withdrawal as well as vigorous wet dog shakes.

Food and nicotine reinforcement behavior

Mice were first trained to respond on a lever to obtain food reward (25 mg pellets, TestDiet) under a fixed ratio time-out 20 s (FR5TO20 s) schedule of reinforcement (Fowler and Kenny, 2011) until stable levels of performance were achieved (≥25 pellets per 60 min session). Mice were then permitted to respond for nicotine (0.03 mg/kg per infusion) for 8 d until stable intake was established (<10% mean variation in intake across 3 consecutive days). Then, the unit dose of nicotine (0.01–0.4 mg/kg per infusion) available for self-administration was varied, and the effects on responding for the drug were assessed, thereby generating a nicotine dose–response curve. Mice were permitted ~5 d of access to each unit dose of nicotine, and between each dose mice reestablished stable responding for the 0.1 mg/kg per infusion dose of nicotine, as reported (Fowler and Kenny, 2011). After the experiment, catheters were tested for patency with brevital. If the catheter failed this test (or if it leaked during the experiment), data from these mice were excluded from analysis.

Neurotransmitter analysis

Two hours after withdrawal assessment, animals were killed by cervical dislocation, brains quickly removed, and frozen at −30°C with precooled isopentane for 90 s. Brains were stored at −70°C. Tissue punches from 300 μm cryostat sections of the IPN were acquired using a Harris Uni-Core micro-punch instrument (1 mm diameter). Tissue punches were transferred to precooled Eppendorf tubes, homogenized with 300 μl of 0.1 M perchloric acid with isoproterenol as an internal standard and centrifuged at 15,000 RPM for 10 min at 4°C. Supernatants were collected and purified through 0.22 μm Millipore centrifugal filter units (14,000 rpm for 5 min).

For quantification of neurotransmitter levels, homogenates were examined using high-performance liquid chromatography coupled with electrochemical (norepinephrine, dopamine, and serotonin levels) or fluorometric detection (GABA and glutamate). An isocratic mobile phase was used for electrochemical detection, and a gradient solution was used to resolve the o-phthaladehyde–amino acid adducts. The isocratic mobile phase was 0.1 M citrate-acetate buffer, 15% methanol, 110 mg/L sodium 1-octanesulfonate 5 mg/L EDTA pumped at a rate of 0.5 ml/min. Separation and detection of neurotransmitters were performed using an Eicompak SC-5ODS 3.0× 150 mm column and a working electrode (WE-3G) set at 0.75 V, against an Ag/AgCl reference, respectively.

Glutamate and GABA were derivatized with o-phthaladehyde immediately before being run on the high-performance liquid chromatography. A 3 × 150 mm Keystone Scientific reverse-phase column was used for amino acid separation. The gradient mobile phase consisted of an aqueous solvent mixture of sodium acetate (35 mM, pH 5.9 adjusted with glacial acetic acid) in ddH_2O. The organic mobile phase consisted of 70% acetonitrile, 15% methanol, and 15% sodium acetate (35 final concentration), pH 7.65 (adjusted with glacial acetic acid). The flow rate was 0.6 ml/min with the following gradient profile: 10.5–15% in 3 min, hold at 15% for 3 min, 15–33% in 9 min, and controlled through Gilson hardware and software. The concentrations of neurotransmitters and amino acids were calculated by comparison to standard curves (Lotfipour et al., 2011).

Statistical analyses

Data were analyzed using JMP statistical software (SAS Institute). ANOVA and repeated-measures ANOVA were performed to determine differences between groups for the behavioral and neurotransmitter measures assessed. For data reaching statistically significance main or interaction effects, subsequent *post hoc* analysis was performed as indicated using Dunnett's, Student's *t* test, or matched-pair *t* tests with Bonferroni correction for multiple comparisons, as appropriate.

Results

Heterozygous and homozygous *Chrna*2 mutant mice are viable, show expected sex and Mendelian genotype ratios, and exhibit no gross neuroanatomical abnormalities. The SHIRPA phenotype assessment panel was used to examine behavior in wild-type and *Chrna*2 mutant mice (Table 1). Across the 21 behavioral measures assessed, repeated-measures ANOVA revealed no main effects for genotype ($F_{(2,20)} = 1.62$, p = not significant), sex ($F_{(1,20)} = 0.54$, p = not significant), and no genotype × sex interaction ($F_{(2,20)} = 0.03$, p = not significant). Within-subject assessment revealed a significant main effect for SHIRPA tests ($F_{(20,400)} = 427.4$, $p = 0.0001$), but not for SHIRPA tests × genotype ($F_{(40,400)} = 1.14$, p = not significant), SHIRPA tests × sex ($F_{(20,400)} = 0.27$, p = not significant), or SHIRPA tests × genotype × sex three-way interaction ($F_{(40,400)} = 1.07$, not significant). Given these overall results, our findings suggest that, although the scores for the 21 listed SHIRPA measures significantly differ from one another, no major differences exist between wild-type and *Chrna*2 mutant mice across any of the behaviors.

We used the accelerating rotarod as a test for overall motor learning and coordination in wild-type and *Chrna*2 mutant mice (Fig. 2). A significant main effect for genotype ($F_{(2,41)} = 4.53$, $p = 0.02$) was observed for the latency to fall from the rotarod. Within-measures analysis reveals a significant main effect for time ($F_{(4,164)} = 71.3$, $p = 0.0001$) and a time × genotype inter action ($F_{(8,164)} = 2.45$, $p = 0.02$). *Post hoc* analysis revealed that, relative to wild-type, *Chrna*$2^{-/-}$ and *Chrna*$2^{-/-}$ mice show reduced coordination. We used the narrow beam traverse and pole tests as supplemental measures of coordination in wild-type and *Chrna*$2^{-/-}$ mice (Table 2). For the beam test, using a repeated-measure ANOVA, data across five training sessions revealed no overall significant effect for genotype on beam step mistakes ($F_{(1,44)} = 3.56$, p = not significant), the number of steps taken during the beam walk ($F_{(1,44)} = 2.02$, p = not significant), or the time taken to complete the walk ($F_{(1,44)} = 0.22$, p = not significant). Within-subject assessment for (1) beam step mistakes revealed a significant main effect for time ($F_{(19,836)} = 15.4$, $p = 0.0001$), but not for the interaction time × genotype ($F_{(19,836)} = 1.15$, p = not significant); (2) the number of steps taken during the beam walk revealed a significant effect for time ($F_{(4,176)} = 6.89$, $p = 0.0001$), but not for the interaction of time × genotype ($F_{(4,176)} = 0.19$, p = not significant); and (3) the time taken to complete the walk revealed no significant effect for time ($F_{(4,176)} = 1.26$, p = not significant) or a time × genotype interaction ($F_{(4,176)} = 0.65$, p = not significant). Similarly, for the pole test and gait analysis, which evaluated turns, total time to turn, stride length, and stride width, repeated-measures ANOVA revealed no significant main effect for genotype ($F_{(1,19)} = 2.96$, p = not significant). Within-subject assessment revealed a significant effect for pole test and gait analysis test ($F_{(4,76)} = 90.2$, $p = 0.0001$), but no significant interaction between pole test and gait analysis test × genotype ($F_{(4,76)} = 0.59$, p = not significant).

Locomotor behavior in a novel environment was assessed in *Chrna*$2^{-/-}$ and wild-type mice after an intraperitoneal injection of 0.9% saline or nicotine (1 or 3 mg/kg) (Table 2). Using a repeated-measure ANOVA, data reveal a main effect for nicotine treatment ($F_{(2,25)} = 20.5$, $p = 0.0001$) in reducing overall locomotor behavior, but no significant effect was observed for genotype ($F_{(1,25)} = 1.43$, p = not significant) or genotype × nicotine treatment interaction ($F_{(2,25)} = 0.17$, p = not significant). Within-measures analyses demonstrated a main effect for time (i.e., minutes in the locomotor chamber) ($F_{(5,125)} = 6.24$, $p = 0.0001$) and a time × nicotine treatment ($F_{(10,125)} = 3.11$, $p = 0.0014$) for reduced locomotor behavior, but not a time × genotype ($F_{(5,125)} = 1.60$, p = not significant) or a time × genotype × nicotine pretreatment interaction ($F_{(10,125)} = 0.84$, p = not significant). *Post hoc* analysis revealed that both doses of nicotine significantly reduced locomotor behavior compared with the saline injection control. At the doses tested, however, our findings also suggest that no major

differences exist between wild-type and *Chrna2*$^{-/-}$ mice in locomotor behavior after an acute injection of nicotine.

To determine whether injection stress influences locomotor behavior, wild-type and *Chrna2*$^{-/-}$ mice were observed after an intraperitoneal injection of saline, or no injection control (Table 2). Using a repeated-measure ANOVA, data reveal a significant effect of injection ($F_{(1,30)} = 11.67$, $p = 0.0018$) in reducing overall locomotor behavior, but no main effect for genotype ($F_{(1,30)} = 3.01$, p = not significant) or genotype × injection interaction ($F_{(1,30)} = 0.47$, p = not significant). Within-measures analysis reveals a main effect for time ($F_{(5, 150)} = 30.92$, $p = 0.0001$) in reducing locomotor behavior, but not for time × genotype ($F_{(5,150)} = 1.21$, p = not significant), time × injection ($F_{(5,150)} = 0.91$, p = not significant), or a time × genotype × injection interaction ($F_{(5,150)} = 1.12$, p = not significant). Our findings suggest that no major differences exist between wild-type and *Chrna2*$^{-/-}$ mice in stress sensitivity to the injection procedure.

Nicotine and morphine-induced analgesia

Nicotine-induced analgesia was assessed using tail withdrawal in wild-type and *Chrna2*$^{-/-}$ mice (Table 2). Using a repeated-measure ANOVA, data revealed no significant main effect for genotype ($F_{(1,18)} = 2.54$, p = not significant) for percentage MPE values. Within-measure analysis revealed no main effect for time ($F_{(4,72)} = 1.06$, p = not significant) or time × genotype ($F_{(4,72)} = 0.23$, p = not significant) for percentage MPE values. Our data suggest that *Chrna2*$^{-/-}$ mice do not differ from wild-type mice in nicotine-induced analgesia on the tail withdrawal.

Nicotine-induced analgesia was also assessed using the formalin test in wild-type and *Chrna2*$^{-/-}$ mice (Table 2). For the first 10 min, ANOVA revealed that nicotine increased percentage MPE ($F_{(1,32)} = 17.70$, $p = 0.0002$), but no main effect was observed for genotype ($F_{(1,32)} = 0.43$, p = not significant), or nicotine treatment × genotype interaction ($F_{(1,32)} = 1.06$, p = not significant). For the last 50 min of the formalin test, ANOVA revealed no statistically significant main or interaction effects for nicotine treatment ($F_{(1,32)} = 0.17$, p = not significant), genotype ($F_{(1,32)} = 0.002$, p = not significant), or treatment × genotype interaction ($F_{(1,32)} = 3.38$, p = not significant) in predicting percentage MPE values. Our results suggest that, although nicotine treatment induces analgesia during the first 10 min of assessment, *Chrna2*$^{-/-}$ mice do not differ from wild-type.

Next, nicotine-induced analgesia was assessed using a hotplate in wild-type and *Chrna2*$^{-/-}$ mice 5 min after nicotine injection, compared with baseline values with no nicotine injection (Fig. 3). Using ANOVA, analysis revealed a main effect for genotype ($F_{(1,21)} = 9.87$, $p = 0.0054$) for percentage MPE values. *Post hoc* analysis revealed that *Chrna2*$^{-/-}$ mice have enhanced nicotine-induced analgesia, an effect specific to the hotplate assay. A trend ($p < 0.06$) for reduced baseline values (before nicotine injection) was observed in *Chrna2*$^{-/-}$ versus wild-type control mice (data not shown).

To further assess a possible role for $\alpha 2$-containing nAChRs in pain processing, we used the tail withdrawal assay to assess morphine-induced analgesia in wild-type and *Chrna2*$^{-/-}$ mice across two time points (day 1 and day 7) (Table 2). Using a repeated-measures ANOVA, data revealed no significant main effects for genotype ($F_{(1,24)} = 0.17$, p = not significant), sex ($F_{(1,24)} = 0.89$, p = not significant), or a sex × genotype interaction ($F_{(1,24)} = 2.02$, p = not significant) in predicting percentage MPE values. Within-measure analysis revealed a main effect for time ($F_{(1,24)} = 44.03$, $p = 0.0001$) in reducing percentage MPE values (i.e., inducing tolerance), but no interactive effect for time × sex ($F_{(1,24)} = 0.88$, p = not significant) or time × genotype ($F_{(1,24)} = 0.16$, p = not significant) or a time × sex × genotype ($F_{(1,24)} = 1.99$, p = not significant). Our data suggest that *Chrna2*$^{-/-}$ mice do not

differ from wild-type mice in morphine-induced analgesia or tolerance as measured by the tail withdrawal assay.

Anxiety

We measured anxiety-like behavior in wild-type and *Chrna2* mutant mice using the elevated plus maze and the light/dark box (Table 2). For the elevated plus maze, ANOVA revealed that there was no main effect for the time spent on the open arms for sex ($F_{(1,21)} = 0.15$, p = not significant), genotype ($F_{(2,21)} = 0.76$, p = not significant), or genotype × sex ($F_{(2,21)} = 0.08$, p = not significant). For the light/dark box, using an ANOVA, the latency to enter the light chamber revealed no main effect for genotype ($F_{(2,22)} = 0.44$, p = not significant), sex ($F_{(1,22)} = 0.11$, p = not significant), or genotype × sex ($F_{(2,22)} = 2.30$, p = not significant). Our data suggest that *Chrna2* mutant mice do not differ from wild-type using these measures of anxiety.

Fear conditioning

We examined learning and memory in wild-type and *Chrna2*$^{-/-}$ mutant mice using cued and contextual fear conditioning (Fig. 4). Repeated-measures ANOVA revealed no differences in baseline, immediate, or precue stimulus values for main or any interaction effects in predicting freezing episodes (data not shown). For cued fear conditioning, ANOVA revealed a main effect for nicotine treatment ($F_{(1,70)} = 13.41$, $p = 0.0005$), a genotype × sex interaction ($F_{(1,70)} = 6.57$, $p = 0.01$), and a genotype × sex × nicotine treatment interaction ($F_{(1,70)} = 4.53$, $p = 0.04$) for freezing episodes. *Post hoc* analysis revealed significant enhancement of nicotine-facilitated cued fear conditioning in female *Chrna2*$^{-/-}$ mice. Statistically significant differences were not observed for genotype ($F_{(1,70)} = 0.21$, p = not significant), sex ($F_{(1,70)} = 1.12$, p = not significant), nicotine treatment × sex interaction ($F_{(1,70)} = 1.17$, p = not significant), or genotype × nicotine treatment interaction ($F_{(1,70)} = 0.08$, p = not significant). For contextual fear conditioning, analysis showed that there were no main effects for genotype ($F_{(1,70)} = 0.99$, p = not significant), nicotine treatment ($F_{(1,70)} = 0.93$, p = not significant), sex ($F_{(1,70)} = 0.03$, p = not significant), genotype × sex interaction ($F_{(1,70)} = 0.99$, p = not significant), nicotine treatment × sex interaction ($F_{(1,70)} = 0.08$, p = not significant), genotype × nicotine treatment interaction ($F_{(1,70)} = 0.15$, p = not significant), or nicotine treatment × sex × genotype three-way interaction ($F_{(1,70)} = 0.59$, p = not significant) in predicting freezing episodes. Although overall measures of contextual fear conditioning were not altered in *Chrna2*$^{-/-}$ mice, when the average of the last 2 (or 5) min of the test was evaluated, the nicotine-treated mutant mice had significantly higher freezing behavior than all other groups, independent of sex (data not shown). Our data suggest that $\alpha 2$-containing nAChRs play a role in nicotine-facilitated cued fear conditioning in female mice and may regulate extinction freezing behavior during contextual testing.

To further investigate the role of the *Chrna2* gene in learning and memory, we examined the effect of nicotine on trace fear conditioning in wild-type and mutant mice, a behavioral test that is highly dependent on hippocampal function (Table 3). Repeated-measures ANOVA revealed no differences in baseline, immediate, or precue stimulus values on main or interactive effects, with the exception of a significant within-measure main effect for test ($F_{(2,66)} = 143.59$, $p = 0.0001$) and an interaction between sex × testing condition ($F_{(2,66)} = 3.48$, $p = 0.04$) in predicting freezing episodes. *Post hoc* analysis did not reveal any specific test that was higher in males versus females. Using a repeated-measure ANOVA, across five training time-points during trace fear conditioning, data revealed no main effects for genotype ($F_{(1,33)} = 0.003$, p = not significant), nicotine pretreatment ($F_{(1,33)} = 0.02$, p = not significant), sex ($F_{(1,33)} = 0.59$, p = not significant), nicotine pretreatment × genotype ($F_{(1,33)} = 1.04$, p = not significant), sex × genotype ($F_{(1,33)} = 0.63$, p = not significant), nicotine pretreatment × sex ($F_{(1,33)} = 0.77$, p = not significant), or nicotine pretreatment ×

sex × genotype ($F_{(1,33)}$ = 1.29, p = not significant) for freezing episodes. Within-measures analysis demonstrated a main effect for time ($F_{(4,132)}$ = 89.52, p = 0.0001), time × drug ($F_{(4,132)}$ = 2.54, p = 0.04), and a time × drug × sex three-way interaction ($F_{(4,132)}$ = 2.74, p = 0.03) for freezing epi sodes. *Post hoc* analysis revealed that nicotine-treated female mice learned trace fear conditioning more quickly than males, particularly at time point 3 of the five training episodes (although the results do not pass Bonferroni correction for multiple comparisons). No significant effects were observed for time × genotype ($F_{(4,132)}$ = 0.45, p = not significant), time × sex ($F_{(4,132)}$ = 1.30, p = not significant), time × drug × genotype ($F_{(4,132)}$ = 0.41, p = not significant), time×sex×genotype ($F_{(4,132)}$=2.23, p=not significant), or a time×drug×sex×genotype interaction ($F_{(4,132)}$=0.47, p=not significant) for freezing episodes in trace fear conditioning training. During cued stimulus testing, ANOVA revealed no significant effects for sex ($F_{(1,40)}$ = 0.34, p = not significant), nicotine pretreatment ($F_{(1,40)}$=0.35, p=not significant), genotype ($F_{(1,40)}$=0.0032, p=not significant), sex×genotype interaction (F(1,40)=2.48, p=not significant), genotype × nicotine pretreatment interaction ($F_{(1,40)}$ = 0.29, p = not significant), genotype × sex interaction ($F_{(1,40)}$ = 2.48, p = not significant), or a sex × nicotine pretreatment × genotype three-way interaction ($F_{(1,40)}$ = 1.21, p = not significant) for freezing episodes. The data suggest that deletion of the *Chrna*2 gene does not significantly influence nicotine-facilitated trace fear conditioning, but that female mice treated with nicotine may learn trace fear conditioning more quickly than males.

To assess the role of α2-containing nAChRs in emotional memory deficits during nicotine withdrawal, cued and contextual fear conditioning was evaluated (Table 4). Using a repeated-measures ANOVA, baseline, immediate, or precue stimulus data revealed no significant effects for sex, genotype, nicotine treatment, or any interactive main effects for freezing episodes (data not shown). Within-measures analysis revealed a significant difference between test (baseline, immediate, or precue stimulus) ($F_{(2,64)}$ = 5.78, p = 0.0049), but not for any other group or interactions. *Post hoc* analysis revealed greater immediate and precue stimulus freezing episodes compared with baseline values. For cued fear conditioning, there was a trend for a nicotine treatment × sex interaction ($F_{(1,39)}$ = 3.0, p = 0.09), but not for any other group for freezing episodes. When splitting by sex, male mice demonstrated a significant nicotine treatment effect ($F_{(1,26)}$ = 8.0, p = 0.01) in reducing freezing episodes (i.e., inducing a greater memory deficit), driven primarily by the *Chrna*$2^{-/-}$ mice, although the results do not pass Bonferroni correction for multiple comparisons (Table 4). In female mice, no significant main or interactive effects were observed for predicting freezing episodes for cued fear conditioning during withdrawal. For contextual fear conditioning, a near-significant effect was observed for sex ($F_{(1,39)}$ = 3.34, p = 0.08), but not for genotype ($F_{(1,39)}$ = 0.56, p = not significant), nicotine treatment ($F_{(1,39)}$ = 0.14, p = not significant), genotype × sex ($F_{(1,39)}$ = 0.24, p = not significant), nicotine treatment × sex ($F_{(1,39)}$ = 0.37, p = not significant), genotype × nicotine treatment ($F_{(1,39)}$ = 0.02, p = not significant), or nicotine treatment × sex × genotype ($F_{(1,39)}$ = 0.01, p = not significant) in predicting freezing episodes. *Post hoc* analysis revealed no main or interactive effects for contextual fear conditioning during withdrawal. However, in male mice, the absence of α2-containing nAChRs produces nicotine withdrawal-induced deficits in cued fear conditioning.

Food and nicotine reinforcement

We used male *Chrna*$2^{-/-}$ mice to determine whether α2-containing nAChRs played a role in food or nicotine reinforcement (Fig. 5; Table 5). Using a repeated-measures ANOVA for time/nicotine dose demonstrated main effects for time during food training sessions ($F_{(6,84)}$ = 41.0, p < 0.0001) and nicotine dose ($F_{(4,16)}$ = 35.1, p < 0.0001) during nicotine dose–response testing. *Post hoc* analysis revealed that mice increased food intake over training

days and nicotine intake at higher drug doses. No significant main effects were observed for genotype during 7 d food training ($F_{(1,14)} = 0.63$, p = not significant), 8 d acquisition of nicotine self-administration ($F_{(1,8)} = 0.50$, p = not significant), or the nicotine dose–response for the four doses tested ($F_{(1,4)} = 0.33$, p = not significant). In addition, there were no significant genotype × time (or dose) effects for any of the measures. Using a repeated-measure ANOVA on the sum of the first 2 d of acquisition of nicotine self-administration, we observed a main effect of genotype ($F_{(1,8)} = 11.8$, $p < 0.0089$), a within-measure effect on reinforced versus nonreinforced responding ($F_{(1,8)} = 224.2$, $p < 0.0001$), and a genotype × reinforced/nonreinforced responding interaction ($F_{(1,8)} = 6.71$, $p < 0.032$). *Post hoc* analysis revealed that *Chrna2*$^{-/-}$ mice show enhanced nicotine self-administration behavior during the first 2 d of the acquisition of nicotine self-administration (Fig. 5*B*,*D*; Table 5) and during the highest dose of nicotine tested (0.4 mg/kg/infusion) on the dose–response (Fig. 5*C*,*E*,*F*; Table 5).

Somatic aspects of nicotine or morphine withdrawal

Mice were assessed for somatic nicotine withdrawal behavior in a novel environment (Fig. 6*A*). For paw tremors, head shakes, backing, and curls, our results showed that both wild-type and *Chrna2*$^{-/-}$ mice exhibit significant enhancement of mecamylamine (vs saline) precipitated withdrawal scores. Using a three-way ANOVA, significant effects were observed for drug pretreatment (nicotine vs saline) ($F_{(1,85)} = 25.5$, $p < 0.0001$), sex ($F_{(1,85)} = 5.23$, $p < 0.03$), and a drug pretreatment × genotype (wild-type, *Chrna2*$^{-/-}$, and *Chrna2*$^{-/-}$) interaction ($F_{(2,85)} = 3.76$, $p < 0.03$) but not for genotype ($F_{(2,85)} = 2.00$, p = not significant), sex × genotype ($F_{(2,85)} = 0.67$, p = not significant), sex × drug pretreatment ($F_{(1,85)} = 2.13$, p = not significant), or sex × genotype × condition ($F_{(2,85)} = 0.48$, p = not significant) for the above somatic signs. *Post hoc* analysis revealed that *Chrna2*$^{-/-}$ mice have enhanced somatic withdrawal behaviors, versus wild-type control mice. When other somatic behaviors were quantified (grooming, scratching, chewing, cage scratching, head nodding, and jumping; Fig. 6*B*), there was a main effect for drug pretreatment (nicotine vs saline) ($F_{(1,85)} = 14.7$, $p < 0.0003$), genotype ($F_{(2,85)} = 7.40$, $p < 0.0012$), but no significant effect for sex ($F_{(1,85)} = 1.04$, p = not significant) or any of the interactions. *Post hoc* analysis demonstrated suppression of these behaviors in wild-type mice, which failed to reach statistical significance in the *Chrna2*$^{-/-}$ mice. When comparing behaviors in nicotine-treated animals, *Chrna2*$^{-/-}$ mice again had potentiated somatic withdrawal signs. The combined findings suggest that the absence of $\alpha 2$-containing nAChRs potentiates mecamylamine-precipitated somatic nicotine withdrawal signs, and for certain somatic signs, in apposing directions.

We also assessed somatic nicotine withdrawal (paw tremors, head shakes, backing, curls, grooming, scratching, chewing, cage scratching, head nodding, and jumping) in a habituated environment (the home cage) for *Chrna2*$^{-/-}$ and wild-type mice (data not shown). Using ANOVA, our data reveal a main effect of sex in predicting somatic withdrawal signs ($F_{(1,43)} = 8.18$, $p = 0.007$). When the data were split by sex, we observed an interactive effect for genotype by drug pretreatment (nicotine vs saline treatment) in predicting somatic withdrawal signs in males only ($F_{(1,19)} = 4.75$, $p = 0.04$). *Post hoc* analysis demonstrated that wild-type male mice exhibited a significant enhancement of somatic withdrawal symptoms in their home cage, which was absent in *Chrna2*$^{-/-}$ mice. The overall findings suggest that the environment (i.e., novel vs habituated) in which somatic signs are assessed influences whether *Chrna2*$^{-/-}$ mice exhibit potentiated withdrawal behaviors.

To further investigate the role for $\alpha 2$-containing nAChRs in withdrawal, morphine withdrawal was assessed in a novel environment. ANOVA demonstrated a main effect of drug pretreatment (morphine vs saline; $F_{(1,31)} = 15.2$, $p = 0.0007$), but not for genotype or drug treatment × genotype interaction on expression of somatic withdrawal signs (paw tremors, head shakes, backing, curls, grooming, scratching, chewing, cage scratching, head

nodding, and jumping). When we assessed individual somatic signs, we observe a main effect for drug pretreatment ($F_{(1,31)} = 12.0$, $p = 0.002$) as well as a drug pretreatment × genotype interaction ($F_{(1,31)} = 4.36$, $p < 0.05$) in predicting head shakes/vigorous wet dog shakes (Fig. 7). *Post hoc* analysis revealed a significant enhancement of head shakes/vigorous wet dog shakes in *Chrna2*$^{-/-}$ versus wild-type mice, suggesting that similar circuitry may mediate naloxone and mecamylamine-precipitated morphine and nicotine withdrawal, respectively.

Neurotransmitter levels in the IPN after mecamylamine precipitated nicotine withdrawal

Given the high level of *Chrna2* mRNA in the IPN, we asked whether neurotransmitter levels (dopamine, norepinephrine, serotonin, glutamate, GABA) were altered within the IPN during mecamylamine-precipitated nicotine withdrawal in wild-type and *Chrna2*$^{-/-}$ mice (Table 6). Repeated-measure ANOVA on neurotransmitter levels within the IPN revealed a main effect for genotype ($F_{(1,28)} = 5.98$, $p = 0.02$), drug pretreatment (saline vs nicotine) × genotype interaction ($F_{(1,28)} = 8.51$, $p = 0.007$), and drug pretreatment × sex interaction ($F_{(1,28)} = 4.57$, $p = 0.04$). Within-measures analysis demonstrated a significant effect for neurotransmitter ($F_{(4,112)} = 683.3$, $p = 0.0001$), neurotransmitter × genotype interaction ($F_{(4,112)} = 5.87$, $p = 0.0003$), neurotransmitter × sex interaction ($F_{(4,112)} = 3.52$, $p = 0.01$), neurotransmitter × drug pretreatment × genotype interaction ($F_{(4,112)} = 8.58$, $p < 0.0001$), and neurotransmitter × drug pretreatment × sex interaction ($F_{(4,112)} = 4.47$, $p < 0.002$) for neurotransmitter levels within the IPN. When neurotransmitters were evaluated separately, for glutamate, ANOVA revealed a significant effect for genotype ($F_{(1,38)} = 4.61$, $p < 0.04$), genotype × drug pretreatment ($F_{(1,38)} = 7.08$, $p < 0.02$), and sex × drug pretreatment ($F_{(1,38)} = 4.02$, $p < 0.054$) but no other main or interactive effects. For IPN GABA levels, ANOVA revealed a significant effect for genotype ($F_{(1,35)} = 4.36$, $p < 0.05$), sex ($F_{(1,35)} = 6.02$, $p < 0.02$), and sex × condition interaction ($F_{(1,35)} = 4.21$, $p < 0.05$) but no other main or interactive effects. *Post hoc* analysis demonstrated that glutamate and GABA levels were significantly elevated in *Chrna2*$^{-/-}$ mice, whereas no genotype effects are observed for serotonin, dopamine, or norepinephrine. Furthermore, nicotine treatment significantly reduced IPN-glutamate levels in *Chrna2*$^{-/-}$ mice while potentiating sex differences for GABA levels (i.e., female nicotine withdrawal group had significantly greater levels than the male nicotine withdrawal group), independent of genotype (data not shown). Sex differences by drug treatment independent of genotype were also observed for glutamate levels, where saline-treated male (vs female) mice administered mecamylamine had significantly greater glutamate levels ($p = 0.053$, Bonferroni corrected for multiple comparisons; data not shown). Our data suggest that genotype-specific effects during withdrawal may be mediated, at least in part, through increased glutamate and/or GABA neurotransmission in the IPN, whereas nicotine withdrawal can induce sex-dependent effects on GABA and glutamate neurotransmitter levels, independent of genotype.

Discussion

Chrna2$^{-/-}$ mice show deficits in coordinated movement and enhanced nicotine-induced analgesia, nicotine reinforcement, as well as nicotine and morphine withdrawal behaviors. Female *Chrna2*$^{-/-}$ mice exhibit a sex-dependent potentiation of nicotine-facilitated cued fear conditioning, suggesting an amygdala-mediated mechanism. On the other hand, deletion of the *Chrna2* gene did not influence anxiety, locomotion, drug-induced analgesia on the tail withdrawal or formalin test, or nicotine-facilitated trace or contextual fear conditioning. Together, the data suggest that the absence of $\alpha 2$-containing nAChRs reduces motor coordination and potentiates nicotine's effects on selected behavioral tasks.

The role of *Chrna2* in locomotion and coordinated movement

Our results demonstrate that, although general locomotor behavior (with and without nicotine pretreatment) appears normal in *Chrna2*$^{-/-}$ mice, significant reductions are evident in coordination on the accelerating rotarod. To determine whether coordination is compromised across other motor tasks, we also examined the behavior of *Chrna2*$^{-/-}$ mice on the pole test and the narrow beam traversal. Our findings revealed no genotype-specific effects on these tasks, suggesting a selective role for $\alpha 2$-containing nAChRs during performance on the rotarod.

In the rat spinal cord, *in situ* hybridization experiments show that *Chrna2* transcripts are only detected in scattered neurons within lamina VII, which have tentatively been identified as Renshaw cells (Wada et al., 1988; Ishii et al., 2005). Renshaw cells are inhibitory interneurons that receive excitatory cholinergic input from motor neuron axon collaterals and send glycinergic inhibitory signals to α-motor, Ia inhibitory, and γ-motor neurons as well as other Renshaw cells. The role of Renshaw cells in recurrent inhibition of selected spinal cord circuits and control of posture and muscle coordination during movement is well documented (Alvarez and Fyffe, 2007; Lamy et al., 2008). Hence, loss of $\alpha 2$-containing nAChRs at motor neuron–Renshaw cell synapses might be expected to alter recurrent inhibition, impair muscle interaction or synergy, and disrupt voluntary movement. What remains unanswered, however, is why the observed deficit in *Chrna2*$^{-/-}$ mice is task-specific (i.e., seen in rotarod, but not in narrow beam traverse or gait). One possible explanation is that recurrent inhibition salience is controlled by the level of muscle activity and, therefore, varies with distinct motor tasks, as seen in humans during standing, walking, or bicycling (Iles et al., 2000).

Role of *Chrna2* in drug reinforcement and withdrawal

Our findings show that *Chrna2*$^{-/-}$ mice exhibit enhanced nicotine self-administration behavior on the first 2 d of acquisition with a preference for the higher dose of nicotine (0.4 mg/kg/infusion; Fig. 5*B*). Our findings are similar to recent data showing that deletion of *Chrna5* or *Chrnb4*, both of which are actively transcribed in the IPN, results in greater levels of nicotine intake in null and overexpressing mutant mice, respectively (Fowler et al., 2011; Frahm et al., 2011). Interestingly, in these studies, enhanced nicotine intake was observed when high unit doses of nicotine were available for intake, suggesting diminished sensitivity to the aversive properties of higher nicotine concentrations or a shift in the dose–response properties to nicotine. In the *Chrna2*$^{-/-}$ mice, increased nicotine intake was only observed during the first 2 d of access to the drug, suggesting that $\alpha 2$-containing nAChRs may underlie aversive responses to nicotine exposure, but that tolerance develops soon thereafter. It should be noted that mice were initially responding for food reward. Hence, the nicotine acquisition data might not represent responding for the drug alone, but rather food-seeking behavior coincident with nicotine reinforcement. Thus, an alternate possibility is that $\alpha 2$-containing nAChRs mediate behavioral plasticity that permits mice to respond appropriately when a reinforcer is switched or no longer present. In translating these findings to a clinical population, we speculate that individuals with altered functionality of the *Chrna2* gene may be more susceptible to reinforcing properties of nicotine and may be more likely to become addicted to nicotine, which if true may explain the association of human *Chrna2* gene polymorphisms with tobacco dependence (Corley et al., 2008).

We also evaluated somatic and affective measures of withdrawal behavior after 2 weeks of chronic nicotine treatment. When withdrawal behavior was measured in a novel environment, *Chrna2*$^{-/-}$ mice exhibited higher overall withdrawal scores than their wild-type littermates. These results are surprising because *Chrna2*$^{-/-}$ mice exhibit a complete absence of withdrawal symptoms when assayed in a habituated environment (i.e., their home

cage) (Salas et al., 2009). Thus, it appears that expression of aversive somatic signs in *Chrna2*$^{-/-}$ mice depends on assay conditions (novel vs habituated environment). For example, relative to wild-type, *Chrna2*$^{-/-}$ mice show more paw tremors, head shakes, backing, and curls when withdrawal is precipitated in a novel environment, whereas in their home cage they show no aversive withdrawal behaviors. In addition, our data show that male *Chrna2*$^{-/-}$ mice have potentiated deficits in cued fear conditioning during nicotine withdrawal (Table 4). Although the results of such emotional memory measures of withdrawal are subtle, they provide a rationale to assess other affective (i.e., psychological) measures of withdrawal in *Chrna2*$^{-/-}$ mice. Together, our findings suggest that the absence of the *Chrna2* gene potentiates nicotine reinforcement and components of nicotine-induced somatic and affective withdrawal. When we assessed naloxone-precipitated somatic morphine withdrawal, our findings demonstrate that, whereas *Chrna2* genotype did not influence over-all scores, *Chrna2*$^{-/-}$ mice showed a significant increase in the number of head shakes/wet dog shakes relative to their wild-type littermates (Fig. 7). The mechanism mediating this effect is unknown; however, given the interaction of nicotinic and opioid systems in modulating addiction (Panchal et al., 2005; Taraschenko et al., 2007), it is possible that similar/overlapping neural circuits could influence withdrawal behavior via $\alpha 2$-containing nAChRs.

$\alpha 2$-containing nAChRs modulate neurotransmitter systems within the rat IPN (Lena et al., 1993). Indeed, our neurochemical analyses showed that glutamate and GABA are elevated within the IPN of *Chrna2*$^{-/-}$ mice, whereas serotonin, dopamine, and norepinephrine levels remain unchanged. The findings suggest that $\alpha 2$-containing nAChRs modulate expression of nicotine's withdrawal effects via selective regulation of GABA and glutamate levels. Whether similar mechanisms exist for morphine withdrawal requires further analysis. Indeed, IPN acetylcholine release can modulate GABA and glutamate release through both muscarinic and nicotinic receptor-mediated mechanisms (Lena et al., 1993; Girod et al., 2000; Girod and Role, 2001; Covernton and Lester, 2002). Such mechanisms may influence the functional control of the IPN efferent projections, including the serotonergic neurons that act as an important excitatory IPN output neurotransmitter system (Maciewicz et al., 1981; Morley, 1986). These circuits could manipulate direct and indirect negative feedback control over dopaminergic systems in the nucleus accumbens (Sutherland and Nakajima, 1981; Nishikawa et al., 1986), thereby influencing motivated behavior (Ellison, 2002).

Role of $\alpha 2$-containing nAChRs in memory

Nicotine enhances hippocampus-dependent learning (Davis et al., 2007). Moreover, $\alpha 2$-containing nAChRs are required for nicotine-mediated facilitation and depression of long-term-potentiation at two excitatory hippocampal synaptic inputs, the Schaffer collateral and temporoammonic pathways, respectively (Nakauchi et al., 2007). Based on these observations, we hypothesized that nicotine-facilitated contextual fear conditioning might be modified in *Chrna2*$^{-/-}$ mice. Figure 4 presents data showing that, although nicotine-facilitated contextual fear conditioning is the same in wild-type and *Chrna2*$^{-/-}$ mice, nicotine-facilitated cued fear conditioning is modified in females but not in males. Whereas contextual fear conditioning is amygdale- and hippocampal-dependent, cued fear conditioning is considered to be hippocampal-independent and, at least in part, amygdala-dependent (Phillips and LeDoux, 1992; Weinberger, 2011). These findings suggest a role for amygdala $\alpha 2$-containing nAChRs in sex-dependent modulation of cued fear conditioning. Finally, using trace fear conditioning, which is a prefrontal cortex-hippocampal-dependent and amygdale-independent task (Davis and Gould, 2007; Raybuck and Gould, 2010; Raybuck and Lattal, 2011), we showed that acquisition and expression of memory are unaltered in *Chrna2*$^{-/-}$ mice. The data suggest that $\alpha 2$-containing nAChRs modulate

nicotine enhanced emotional memories via a sex-dependent mechanism, possibly through actions within the amygdala.

Role of α2-containing nAChRs in sensory processing

Relative to wild-type, *Chrna2*$^{-/-}$ mice showed enhanced nicotine-induced analgesia in the hotplate test, but not the tail withdrawal or formalin test. The mechanisms influencing *Chrna2* gene-dependent effects on the hotplate test are currently unknown. Caggiula et al. (1995) have argued that central mechanisms versus a spinal reflex may mediate analgesic effects of nicotine on the hotplate versus the tail withdrawal test, respectively. Whether such mechanisms are at play needs further analysis. Also, the absence of saline injection controls in our current studies limits conclusions based on these findings. Future studies are needed to determine how these mechanisms may interact to influence nicotine-modulated behaviors, as well as identifying central neural circuits influencing nicotine-induced sensory processing in the *Chrna2*$^{-/-}$ mice.

In conclusion, the results of our studies demonstrate that α2-containing nAChRs play subtle yet discernible roles in regulating task-specific motor coordination, nicotine-induced analgesia, memory, reinforcement, and drug withdrawal behavior. Additional molecular and genetic studies are needed to understand how the *Chrna2* gene influences these basal and nicotine-facilitated behaviors.

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References

Alvarez FJ, Fyffe RE. The continuing case for the Renshaw cell. J Physiol. 2007; 584:31–45. CrossRef Medline. [PubMed: 17640932]

Aridon P, Marini C, Di Resta C, Brilli E, De Fusco M, Politi F, Parrini E, Manfredi I, Pisano T, Pruna D, Curia G, Cianchetti C, Pasqualetti M, Becchetti A, Guerrini R, Casari G. Increased sensitivity of the neuronal nicotinic receptor α 2 subunit causes familial epilepsy with nocturnal wandering and ictal fear. Am J Hum Genet. 2006; 79:342–350. CrossRef Medline. [PubMed: 16826524]

Barros DM, Ramirez MR, Izquierdo I. Modulation of working, short-and long-term memory by nicotinic receptors in the basolateral amygdala in rats. Neurobiol Learn Mem. 2005; 83:113–118. CrossRef Medline. [PubMed: 15721794]

Blouin JL, Dombroski BA, Nath SK, Lasseter VK, Wolyniec PS, Nestadt G, Thornquist M, Ullrich G, McGrath J, Kasch L, Lamacz M, Thomas MG, Gehrig C, Radhakrishna U, Snyder SE, Balk KG, Neufeld K, Swartz KL, DeMarchi N, Papadimitriou GN, et al. Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. Nat Genet. 1998; 20:70–73. CrossRef Medline. [PubMed: 9731535]

Caggiula AR, Epstein LH, Perkins KA, Saylor S. Different methods of assessing nicotine-induced antinociception may engage different neural mechanisms. Psychopharmacology (Berl). 1995; 122:301–306. CrossRef Medline. [PubMed: 8748400]

Corley RP, Zeiger JS, Crowley T, Ehringer MA, Hewitt JK, Hopfer CJ, Lessem J, McQueen MB, Rhee SH, Smolen A, Stallings MC, Young SE, Krauter K. Association of candidate genes with antisocial drug dependence in adolescents. Drug Alcohol Depend. 2008; 96:90–98. CrossRef Medline. [PubMed: 18384978]

Covernton PO, Lester RA. Prolonged stimulation of presynaptic nicotinic acetylcholine receptors in the rat interpeduncular nucleus has differential effects on transmitter release. Int J Dev Neurosci. 2002; 20:247–258. CrossRef Medline. [PubMed: 12175860]

Crawley JN. Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacol Biochem Behav. 1981; 15:695–699. CrossRef Medline. [PubMed: 6118883]

Damaj MI, Kao W, Martin BR. Characterization of spontaneous and precipitated nicotine withdrawal in the mouse. J Pharmacol Exp Ther. 2003; 307:526–534. CrossRef Medline. [PubMed: 12970387]

Davis JA, Gould TJ. β2 subunit-containing nicotinic receptors mediate the enhancing effect of nicotine on trace cued fear conditioning in C57BL/6 mice. Psychopharmacology (Berl). 2007; 190:343–352. CrossRef Medline. [PubMed: 17136517]

Davis JA, James JR, Siegel SJ, Gould TJ. Withdrawal from chronic nicotine administration impairs contextual fear conditioning in C57BL/6 mice. J Neurosci. 2005; 25:8708–8713. CrossRef Medline. [PubMed: 16177040]

Davis JA, Kenney JW, Gould TJ. Hippocampal α4β2 nicotinic acetylcholine receptor involvement in the enhancing effect of acute nicotine on contextual fear conditioning. J Neurosci. 2007; 27:10870–10877. CrossRef Medline. [PubMed: 17913920]

Ellison G. Neural degeneration following chronic stimulant abuse reveals a weak link in brain, fasciculus retroflexus, implying the loss of forebrain control circuitry. Eur Neuropsychopharmacol. 2002; 12:287–297. CrossRef Medline. [PubMed: 12126867]

Fowler CD, Kenny PJ. Intravenous nicotine self-administration and cue-induced reinstatement in mice: effects of nicotine dose, rate of drug infusion and prior instrumental training. Neuropharmacology. 2011; 61:687–698. CrossRef Medline. [PubMed: 21640128]

Fowler CD, Lu Q, Johnson PM, Marks MJ, Kenny PJ. Habenular α5 nicotinic receptor subunit signalling controls nicotine intake. Nature. 2011; 471:597–601. CrossRef Medline. [PubMed: 21278726]

Frahm S, Slimak MA, Ferrarese L, Santos-Torres J, Antolin-Fontes B, Auer S, Filkin S, Pons S, Fontaine JF, Tsetlin V, Maskos U, Ibañez-Tallon I. Aversion to nicotine is regulated by the balanced activity of β4 and α5 nicotinic receptor subunits in the medial habenula. Neuron. 2011; 70:522–535. CrossRef Medline. [PubMed: 21555077]

Girod R, Role LW. Long-lasting enhancement of glutamatergic synaptic transmission by acetylcholine contrasts with response adaptation after exposure to low-level nicotine. J Neurosci. 2001; 21:5182–5190. Medline. [PubMed: 11438593]

Girod R, Barazangi N, McGehee D, Role LW. Facilitation of glutamatergic neurotransmission by presynaptic nicotinic acetylcholine receptors. Neuropharmacology. 2000; 39:2715–2725. CrossRef Medline. [PubMed: 11044742]

Glick SD, Sell EM, McCallum SE, Maisonneuve IM. Brain regions mediating α3β4 nicotinic antagonist effects of 18-MC on nicotine self-administration. Eur J Pharmacol. 2011; 669:71–75. CrossRef Medline. [PubMed: 21871879]

Gould TJ, Lewis MC. Coantagonism of glutamate receptors and nicotinic acetylcholinergic receptors disrupts fear conditioning and latent inhibition of fear conditioning. Learn Mem. 2005; 12:389–398. CrossRef Medline. [PubMed: 16077017]

Gurling HM, Kalsi G, Brynjolfson J, Sigmundsson T, Sherrington R, Mankoo BS, Read T, Murphy P, Blaveri E, McQuillin A, Petursson H, Curtis D. Genomewide genetic linkage analysis confirms the presence of susceptibility loci for schizophrenia, on chromosomes 1q32.2, 5q33.2, and 8p21–22 and provides support for linkage to schizophrenia, on chromosomes 11q23.3–24 and 20q12.1–11.23. Am J Hum Genet. 2001; 68:661–673. CrossRef Medline. [PubMed: 11179014]

Han ZY, Le Novère N, Zoli M, Hill JA Jr, Champtiaux N, Changeux JP. Localization of nAChR subunit mRNAs in the brain of Macaca mulatta. Eur J Neurosci. 2000; 12:3664–3674. CrossRef Medline. [PubMed: 11029636]

Heitjan DF, Guo M, Ray R, Wileyto EP, Epstein LH, Lerman C. Identification of pharmacogenetic markers in smoking cessation therapy. Am J Med Genet B Neuropsychiatr Genet. 2008; 147B:712–719. CrossRef Medline. [PubMed: 18165968]

Hentall ID, Gollapudi L. The interpeduncular nucleus regulates nicotine's effects on free-field activity. Neuroreport. 1995; 6:2469–2472. CrossRef Medline. [PubMed: 8741744]

Iles JF, Ali A, Pardoe J. Task-related changes of transmission in the pathway of heteronymous spinal recurrent inhibition from soleus to quadriceps motor neurones in man. Brain. 2000; 123:2264–2272. CrossRef Medline. [PubMed: 11050026]

Ishii K, Wong JK, Sumikawa K. Comparison of $\alpha 2$ nicotinic acetylcholine receptor subunit mRNA expression in the central nervous system of rats and mice. J Comp Neurol. 2005; 493:241–260. CrossRef Medline. [PubMed: 16255031]

Jackson KJ, Martin BR, Changeux JP, Damaj MI. Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. J Pharmacol Exp Ther. 2008; 325:302–312. CrossRef Medline. [PubMed: 18184829]

Jia Y, Yamazaki Y, Nakauchi S, Sumikawa K. $\alpha 2$ nicotine receptors function as a molecular switch to continuously excite a subset of interneurons in rat hippocampal circuits. Eur J Neurosci. 2009; 29:1588–1603. CrossRef Medline. [PubMed: 19385992]

Jia Y, Yamazaki Y, Nakauchi S, Ito K, Sumikawa K. Nicotine facilitates long-term potentiation induction in oriens-lacunosum moleculare cells via Ca^{2+} entry through non-$\alpha 7$ nicotinic acetylcholine receptors. Eur J Neurosci. 2010; 31:463–476. CrossRef Medline. [PubMed: 20113344]

Kenny PJ, Markou A. Neurobiology of the nicotine withdrawal syndrome. Pharmacol Biochem Behav. 2001; 70:531–549. CrossRef Medline. [PubMed: 11796152]

Kim J. Association of CHRNA2 polymorphisms with overweight/obesity and clinical characteristics in a Korean population. Clin Chem Lab Med. 2008; 46:1085–1089. CrossRef Medline. [PubMed: 18588430]

Kopchia KL, Altman HJ, Commissaris RL. Effects of lesions of the central nucleus of the amygdala on anxiety-like behaviors in the rat. Pharmacol Biochem Behav. 1992; 43:453–461. CrossRef Medline. [PubMed: 1438482]

Lamy JC, Iglesias C, Lackmy A, Nielsen JB, Katz R, Marchand-Pauvert V. Modulation of recurrent inhibition from knee extensors to ankle motoneurones during human walking. J Physiol. 2008; 586:5931–5946. CrossRef Medline. [PubMed: 18936080]

Léna C, Changeux JP, Mulle C. Evidence for "preterminal" nicotinic receptors on GABAergic axons in the rat interpeduncular nucleus. J Neurosci. 1993; 13:2680–2688. Medline. [PubMed: 8501532]

Lotfipour S, Arnold MM, Hogenkamp DJ, Gee KW, Belluzzi JD, Leslie FM. The monoamine oxidase (MAO) inhibitor tranylcypromine enhances nicotine self-administration in rats through a mechanism independent of MAO inhibition. Neuropharmacology. 2011; 61:95–104. CrossRef Medline. [PubMed: 21419142]

Maciewicz R, Foote WE, Bry J. Excitatory projection from the interpeduncular nucleus to central superior raphe neurons. Brain Res. 1981; 225:179–183. CrossRef Medline. [PubMed: 7296274]

McQuiston AR, Madison DV. Nicotinic receptor activation excites distinct subtypes of interneurons in the rat hippocampus. J Neurosci. 1999; 19:2887–2896. Medline. [PubMed: 10191306]

Mészáros J, Gajewska S, Tarchalska-Kryñska B. Habenulointerpeduncular lesions: the effects on pain sensitivity, morphine analgesia and open-field behavior in rats. Pol J Pharmacol Pharm. 1985; 37:469–477. Medline. [PubMed: 3909126]

Morley BJ. The interpeduncular nucleus. Int Rev Neurobiol. 1986; 28:157–182. CrossRef Medline. [PubMed: 2433243]

Mulle C, Vidal C, Benoit P, Changeux JP. Existence of different subtypes of nicotinic acetylcholine receptors in the rat habenulointerpeduncular system. J Neurosci. 1991; 11:2588–2597. Medline. [PubMed: 1869929]

Nakauchi S, Brennan RJ, Boulter J, Sumikawa K. Nicotine gates long-term potentiation in the hippocampal CA1 region via the activation of $\alpha 2$* nicotinic ACh receptors. Eur J Neurosci. 2007; 25:2666–2681. CrossRef Medline. [PubMed: 17466021]

Nishikawa T, Fage D, Scatton B. Evidence for, and nature of, the tonic inhibitory influence of habenulointerpeduncular pathways upon cerebral dopaminergic transmission in the rat. Brain Res. 1986; 373:324–336. CrossRef Medline. [PubMed: 2424555]

Ophoff RA, Escamilla MA, Service SK, Spesny M, Meshi DB, Poon W, Molina J, Fournier E, Gallegos A, Mathews C, Neylan T, Batki SL, Roche E, Ramirez M, Silva S, De Mille MC, Dong P, Leon PE, Reus VI, Sandkuijl LA, et al. Genomewide linkage disequilibrium mapping of severe bipolar disorder in a population isolate. Am J Hum Genet. 2002; 71:565–574. CrossRef Medline. [PubMed: 12119601]

Panchal V, Taraschenko OD, Maisonneuve IM, Glick SD. Attenuation of morphine withdrawal signs by intracerebral administration of 18-methoxycoronaridine. Eur J Pharmacol. 2005; 525:98–104. CrossRef Medline. [PubMed: 16289028]

Phillips RG, LeDoux JE. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci. 1992; 106:274–285. CrossRef Medline. [PubMed: 1590953]

Portugal GS, Wilkinson DS, Kenney JW, Sullivan C, Gould TJ. Strain-dependent effects of acute, chronic, and withdrawal from chronic nicotine on fear conditioning. Behav Genet. 2012; 42:133–150. CrossRef Medline. [PubMed: 21822688]

Quinn JJ, Wied HM, Liu D, Fanselow MS. Post-training excitotoxic lesions of the dorsal hippocampus attenuate generalization in auditory delay fear conditioning. Eur J Neurosci. 2009; 29:1692–1700. CrossRef Medline. [PubMed: 19419430]

Raybuck JD, Gould TJ. The role of nicotinic acetylcholine receptors in the medial prefrontal cortex and hippocampus in trace fear conditioning. Neurobiol Learn Mem. 2010; 94:353–363. CrossRef Medline. [PubMed: 20727979]

Raybuck JD, Lattal KM. Double dissociation of amygdala and hippocampal contributions to trace and delay fear conditioning. PLoS One. 2011; 6:e15982. CrossRef Medline. [PubMed: 21283812]

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome. 1997; 8:711–713. CrossRef Medline. [PubMed: 9321461]

Salas R, Main A, Gangitano D, De Biasi M. Decreased withdrawal symptoms but normal tolerance to nicotine in mice null for the $\alpha 7$ nicotinic acetylcholine receptor subunit. Neuropharmacology. 2007; 53:863–869. CrossRef Medline. [PubMed: 17920082]

Salas R, Sturm R, Boulter J, De Biasi M. Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. J Neurosci. 2009; 29:3014–3018. CrossRef Medline. [PubMed: 19279237]

Son JH, Winzer-Serhan UH. Postnatal expression of $\alpha 2$ nicotinic acetylcholine receptor subunit mRNA in developing cortex and hippocampus. J Chem Neuroanat. 2006; 32:179–190. CrossRef Medline. [PubMed: 17046198]

Sudweeks SN, Yakel JL. Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. J Physiol. 2000; 527:515–528. CrossRef Medline. [PubMed: 10990538]

Sullivan PF, Neale BM, van den Oord E, Miles MF, Neale MC, Bulik CM, Joyce PR, Straub RE, Kendler KS. Candidate genes for nicotine dependence via linkage, epistasis, and bioinformatics. Am J Med Genet B Neuropsychiatr Genet. 2004; 126B:23–36. CrossRef Medline. [PubMed: 15048644]

Sutherland RJ, Nakajima S. Self-stimulation of the habenular complex in the rat. J Comp Physiol Psychol. 1981; 95:781–791. CrossRef Medline. [PubMed: 6975784]

Taraschenko OD, Shulan JM, Maisonneuve IM, Glick SD. 18-MC acts in the medial habenula and interpeduncular nucleus to attenuate dopamine sensitization to morphine in the nucleus accumbens. Synapse. 2007; 61:547–560. CrossRef Medline. [PubMed: 17447255]

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. Distribution of $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\beta 2$ neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J Comp Neurol. 1989; 284:314–335. CrossRef Medline. [PubMed: 2754038]

Wada K, Ballivet M, Boulter J, Connolly J, Wada E, Deneris ES, Swanson LW, Heinemann S, Patrick J. Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. Science. 1988; 240:330–334. CrossRef Medline. [PubMed: 2832952]

Weinberger NM. The medial geniculate, not the amygdala, as the root of auditory fear conditioning. Hear Res. 2011; 274:61–74. CrossRef Medline. [PubMed: 20466051]

Whiteaker P, Wilking JA, Brown RW, Brennan RJ, Collins AC, Lindstrom JM, Boulter J. Pharmacological and immunochemical characterization of $\alpha 2^*$ nicotinic acetylcholine receptors (nAChRs) in mouse brain. Acta Pharmacol Sin. 2009; 30:795–804. CrossRef Medline. [PubMed: 19498420]

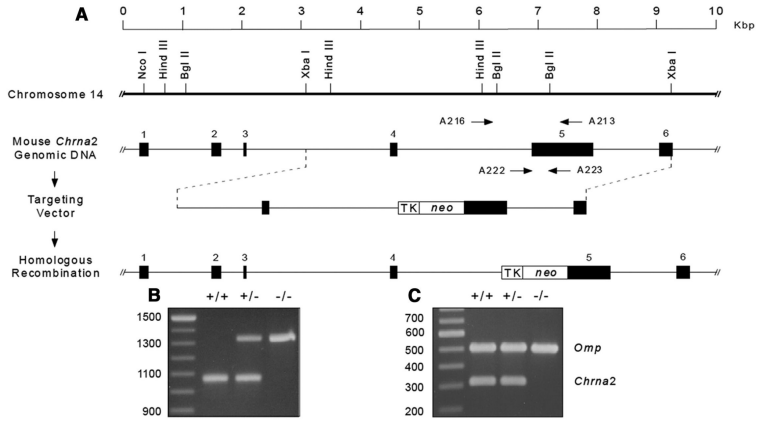

Figure 1.

Genetic engineering of *Chrna2*-null mutant mice. ***A***, The targeting construct and generation of *Chrna2*$^{-/-}$ mice are described in Materials and Methods. ***B***, Genotyping was performed using tail biopsy DNA samples and PCR primers A216 and A213 as described. Ethidium bromide-stained PCR products were analyzed on 1% agarose gels and photographed using a BioDoc-It Imaging System (UVP). ***C***, Total RNA was extracted from the olfactory bulbs of adult mice of the indicated *Chrna2* genotype and used as template for cDNA synthesis as described in Materials and Methods. Aliquots of the resulting cDNA reactions were used as PCR templates to detect transcripts encoding *Chrna2* (primers A222 and A223, 308 bp) or olfactory marker protein (*Omp*, 500 bp).

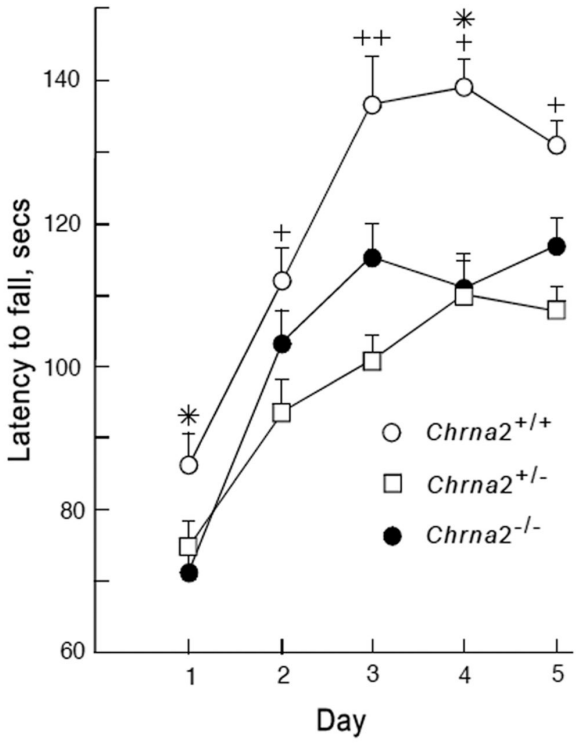

Figure 2.

Chrna2 mutant mice exhibit reduced motor coordination on the accelerating rotarod. Mice were observed for their latency to fall during a 3 min test period on each of five consecutive days. Using a Dunnett's test, *Chrna2*$^{-/-}$ and *Chrna2*$^{+/-}$ mice demonstrated significant reductions versus *Chrna2*$^{+/+}$ mice in the latency to fall over the 5 d of testing. *p<0.05 versus *Chrna2*$^{-/-}$ mice. $^{++}p < 0.01$ versus *Chrna2*$^{+/-}$ mice (n = 10–17/group). $^{+}p < 0.05$ versus *Chrna2*$^{+/-}$ mice (n = 10–17/group).

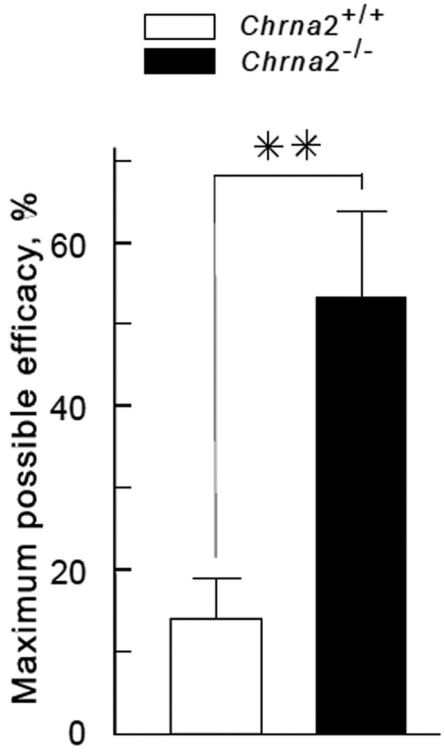

Figure 3.

Chrna2$^{-/-}$ mice exhibit enhanced nicotine-induced analgesia on the hotplate assay. Mice were assessed for nicotine (1.25 mg/kg, i.p.) induced analgesia 5 min after injection. Relative to *Chrna2*$^{+/+}$ mice, *post hoc t* test analysis revealed a significant enhancement of nicotine-induced analgesia on the hotplate assay in *Chrna2*$^{-/-}$ mice: $^{**}p < 0.01$. A trend ($p<0.06$) for reduced baseline values (before nicotine injection) was observed in *Chrna2*$^{-/-}$ versus wild-type control mice (data not shown); $n = 10–11$/group.

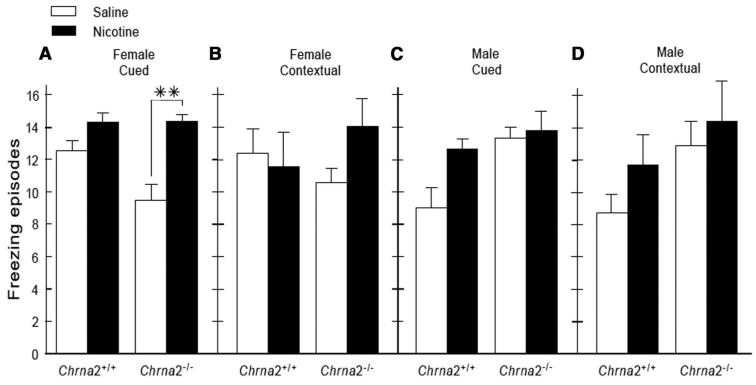

Figure 4.

An enhancement of nicotine-facilitated cued fear conditioning is seen in female *Chrna*2$^{-/-}$ mice. Female (***A***, ***B***) and male (***C***, ***D***) mice were trained on both cued and contextual fear conditioning after pretraining and testing administrations of saline or nicotine (0.09 mg/kg, i.p.). *Post hoc t* test comparisons demonstrate a significant enhancement of nicotine-facilitated cued fear conditioning specifically in female mice (***A***). $^{**}p < 0.01$, Bonferroni corrected for multiple comparisons (n = 5–12/group).

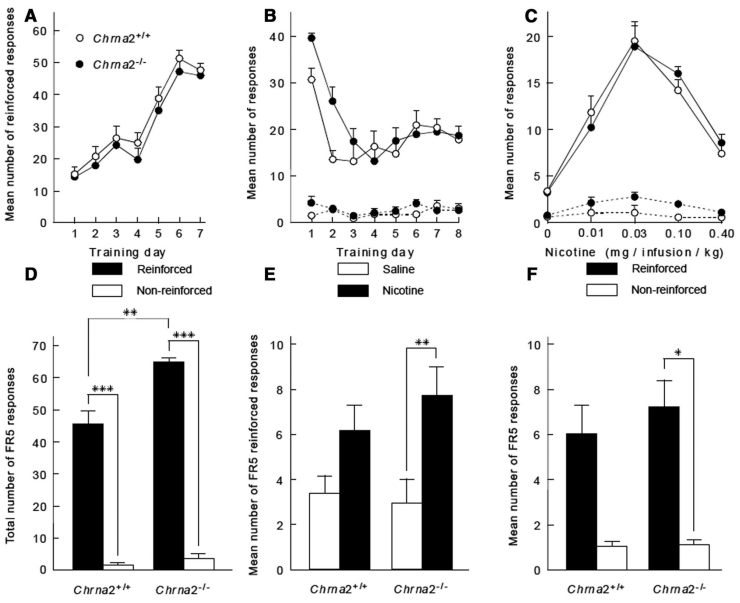

Figure 5.

Enhanced nicotine self-administration by *Chrna2*$^{-/-}$ mice is observed during the first 2 d of acquisition and at the highest dose of nicotine examined compared with saline responding. ***A***, Male mice were food trained up to a fixed ratio 5 (FR5), time out 20 s (TO20 s) schedule of reinforcement. Data are presented as the mean number of food rewards earned (± SEM). ***B***, Mice were then transitioned to 0.03 mg/infusion/kg of intravenous nicotine (acquisition period at an FR5, TO20 s schedule of reinforcement). Data are presented as the mean number of nicotine infusions earned (± SEM) with the active lever (solid line). Values for the inactive lever represent the mean number of lever presses divided by 5 for comparison purposes with the FR5 schedule of the active lever (dotted line). ***C***, Both *Chrna2*$^{+/+}$ and *Chrna2*$^{-/-}$ mice were then given access to a range of nicotine doses, which resulted in an inverted U-shaped dose–response curve. Data for the active lever are presented as the mean number of nicotine infusions earned (± SEM, solid line), and for the inactive lever as mean number of lever presses/5 (± SEM, dotted line). ***D***, *Post hoc* comparisons demonstrate significantly enhanced nicotine self-administration during the first 2 d of nicotine self-administration. $^{**}p < 0.01$, Bonferroni corrected for multiple comparisons. $^{***}p < 0.001$, Bonferroni corrected for multiple comparisons. ***E***, Matched pair *t* test comparisons between saline- and nicotine-reinforced responses demonstrated that *Chrna2*$^{-/-}$ mice had significantly enhanced responses at the highest dose of nicotine tested (0.4 mg/kg), which was not observed in *Chrna2*$^{+/+}$ mice.$^{**}p<0.001$, Bonferroni corrected for multiple comparisons. ***F***, At the highest dose of nicotine tested, *Chrna2*$^{-/-}$ mice demonstrated significant preference for the reinforced lever versus the nonreinforced lever, which was not observed in *Chrna2*$^{+/+}$ mice. $^{*}p < 0.05$, Bonferroni corrected for multiple comparisons (n = 4–10/group).

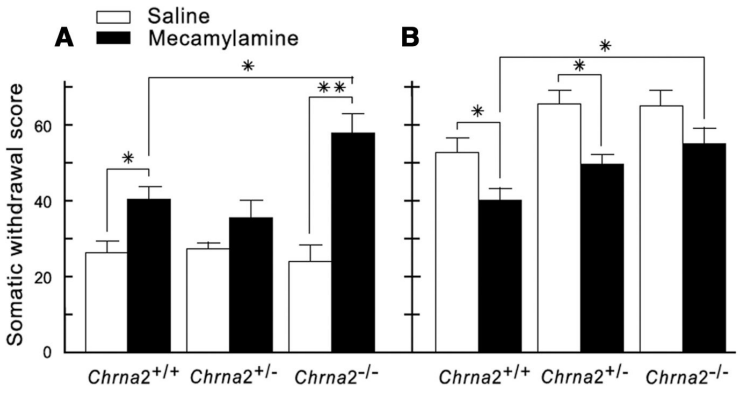

Figure 6.

When assayed in a novel environment, a significant increase in mecamylamine-precipitated withdrawal scores was observed in nicotine-treated *Chrna*$2^{+/+}$ and *Chrna*$2^{-/-}$ mice. Mice were administered nicotine (24 mg/kg per day) using an Alzet osmotic minipump. On the 13th day of nicotine exposure, mice were placed into a novel environment, habituated for 40 min, administered mecamylamine (3 mg/kg, i.p.), and evaluated for somatic symptoms of withdrawal for an additional 20 min. ***A***, Quantifying paw tremors, head shakes, backing, and curls, *t* test *post hoc* analysis revealed a significant enhancement of behavioral signs during nicotine withdrawal. $^{**}p < 0.01$ (Bonferroni corrected for multiple comparisons). $^{*}p < 0.05$ (Bonferroni corrected for multiple comparisons). Using a Dunnett's test (*post hoc* comparisons), *Chrna*$2^{-/-}$ versus *Chrna*$2^{+/+}$ wild-type mice demonstrated significant enhancement of withdrawal signs after nicotine, but not saline, treatment. $^{*}p < 0.0575$. ***B***, Quantifying grooming, scratching, chewing, cage scratching, head nodding, and jumping, *t* test *post hoc* analysis revealed a significant reduction of behavioral signs during nicotine withdrawal. Using a Dunnett's test (*post hoc* comparisons), *Chrna*$2^{-/-}$ versus wild-type mice demonstrated significant enhancement of withdrawal signs in nicotine-, but not saline-treated, mice. $^{*}p<0.05$, Bonferroni corrected for multiple comparisons (n = 11–20/group).

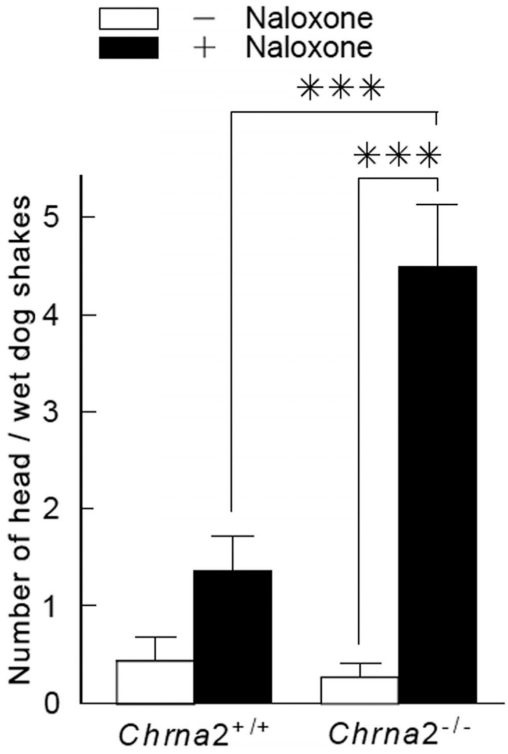

Figure 7.

An increased number of head shakes/wet dog shakes is observed in *Chrna2*$^{-/-}$ during naloxone-precipitated morphine withdrawal. Male and female mice were injected twice per day with morphine on an escalating dose over 6 d (day 1–2, 10 mg/kg; day 3–4, 20 mg/kg; day 5–6, 40 mg/kg, s.c.). On the seventh day, mice were injected with a challenge dose of morphine (10 mg/kg, s.c.) followed 1 h later with naloxone (2 mg/kg, s.c.). Somatic withdrawal signs were measured for 20 min in a novel environment. *t* test comparisons revealed that *Chrna2*$^{-/-}$ mice had significant enhancement of naloxone-precipitated head shakes/wet dog shakes, which was not significant in *Chrna2*$^{+/+}$ mice. *t* test comparisons revealed that *Chrna2*$^{-/-}$mice had significantly greater head shakes/wet dog shakes than wild-type control mice. ***$p < 0.001$, Bonferroni corrected for multiple comparisons (n = 3–13/group).

Table 1

SHIRPA behavioral phenotype assessment of wild-type and *Chrna2* mutant mice[a]

Behavioral phenotype	*Chrna2*$^{+/+}$	*Chrna2*$^{+/-}$	*Chrna2*$^{-/-}$
Body position	3.9 ± 0.1 (10)	4.0 ± 0.0 (10)	4.0 ± 0.0 (6)
Spontaneous activity	2.1 ± 0.1 (10)	1.8 ± 0.1 (10)	1.8 ± 0.2 (6)
Respiration rate	2.0 ± 0.0 (10)	2.0 ± 0.0 (10)	2.0 ± 0.0 (6)
Tremor	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (6)
Transfer arousal	3.5 ± 0.3 (10)	2.8 ± 0.2 (10)	3.2 ± 0.4 (6)
Palpebral closure	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (6)
Piloerection	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (6)
Startle response	1.0 ± 0.0 (10)	1.0 ± 0.0 (10)	1.0 ± 0.0 (6)
Gait	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (6)
Pelvic elevation	2.0 ± 0.0 (10)	2.0 ± 0.0 (10)	2.0 ± 0.0 (6)
Tail elevation	1.0 ± 0.0 (10)	1.0 ± 0.0 (10)	1.0 ± 0.0 (6)
Touch escape	2.0 ± 0.0 (10)	2.1 ± 0.1 (10)	2.0 ± 0.0 (6)
Positional passivity	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.0 ± 0.0 (6)
Visual placing	2.0 ± 0.0 (10)	2.0 ± 0.0 (10)	2.0 ± 0.0 (6)
Grip strength	2.0 ± 0.1 (10)	2.0 ± 0.0 (10)	2.0 ± 0.0 (6)
Body tone	1.0 ± 0.0 (10)	1.0 ± 0.0 (10)	1.0 ± 0.0 (6)
Pinna reflex	1.0 ± 0.0 (10)	1.0 ± 0.0 (10)	1.0 ± 0.0 (6)
Corneal reflex	1.0 ± 0.0 (10)	1.0 ± 0.0 (10)	1.0 ± 0.0 (6)
Toe pinch	3.0 ± 0.0 (10)	3.0 ± 0.0 (10)	3.0 ± 0.0 (6)
Wire maneuver	0.5 ± 0.2 (10)	0.4 ± 0.2 (10)	0.2 ± 0.2 (6)
Negative geotaxis	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.2 ± 0.2 (6)
Total score	28.0 ± 0.4(10)	27.1 ± 0.3 (10)	27.3 ± 0.5 (6)

[a]Behavioral scores were measured as described in Materialsand Methods. Values are presented as mean ± SEM (*n*).

Table 2

Chrna2 mutant mice are indistinguishable from wild-type mice in measures of anxiety, coordination, locomotion, and sensory processing[a]

Analysis columns	*Chrna2*$^{+/+}$	*Chrna2*$^{+/-}$	*Chrna2*$^{-/-}$	Sex assessed
Light dark box, % time in light	31.2 ± 16.3 (7)	48.1 ± 15.8(9)	26.0 ± 12.7 (7)	Yes
Elevated plus maze, % time in open arms	29.8 ± 1.8 (7)	30.1 ± 1.3(9)	26.4 ± 3.7 (6)	Yes
Beam mistakes (total)	32.0 ± 3.1 (24)	NA	40.9 ± 4.5 (23)	No
Beam steps (average)	19.6 ± 0.5 (24)	NA	20.4 ± 0.3 (23)	No
Beam time (s)	21.9 ± 1.5 (24)	NA	22.7 ± 1.8(23)	No
Average beam mistakes/average steps taken	0.3 ± 0.0 (24)	NA	0.4 ± 0.0 (23)	No
Pole test, time tot-turn (s)	2.2 ± 0.6 (11)	NA	1.4 ± 0.2 (11)	No
Pole test, time to descend (s)	6.9 ± 0.7 (11)	NA	6.0 ± 0.3 (11)	No
Gait test, left stride length (cm)	7.6 ± 0.2 (11)	NA	6.9 ± 0.2 (10)	No
Gait test, right stride length (cm)	7.5 ± 0.3 (11)	NA	7.3 ± 0.3 (10)	No
Gait test, stride width (cm)	4.5 ± 0.1 (11)	NA	4.6 ± 0.2 (10)	No
Locomotion (no injection control), mean distance traveled per min for 30 min (cm)	160.8 ± 10.4(9)	NA	196.1 ± 8.8(9)	No
Locomotion (saline injection), mean distance traveled per min for 30 min (cm)	120.9 ± 22.2 (6)	NA	136.3 ± 16.4(10)	No
Locomotion (nicotine, 1 mg/kg), mean distance traveled per min for 30 min (cm)	66.11 ± 31.8(3)	NA	74.8 ± 6.04 (3)	No
Locomotion (nicotine, 3 mg/kg), mean distance traveled per min for 30 min (cm)	10.9 ± 2.3 (6)	NA	33.2 ± 26.8 (4)	No
Nicotine tail withdrawal (% MPE)	39.3 ± 6.5 (20)	NA	32.4 ± 4.4 (22)	No
Formalin nicotine, time licking paw every other min for 10 min (s)	3.5 ± 1.4(10)	NA	4.5 ± 2.8 (8)	No
Formalin saline, time licking paw every other min for 10 min (s)	17.6 ± 3.8 (8)	NA	13.0 ± 2.6 (7)	No
Formalin nicotine, time licking paw every other min for last 50 min (s)	206.3 ± 17.3 (10)	NA	249.1 ± 22.8 (8)	No
Formalin saline, time licking paw every other min for last 50 min (s)	259.9 ± 31.9 (8)	NA	214.9 ± 22.5 (7)	No
Morphine-induced analgesia, tail withdrawal (% MPE)	100 ± 0.0 (14)	NA	100 ± 0.0 (16)	Yes
Morphine-induced tolerance, tail withdrawal (% MPE)	72.1 ± 7.2 (13)	NA	66.0 ± 6.2 (15)	Yes

[a]Behavioral scores were measured as described in Materials and Methods. Values are presented as mean ± SEM (*n*).

NA, Not applicable.

Table 3

α2-containing nAChRs do not influence nicotine-facilitated trace fear conditioning[a]

	Chrna2$^{-/-}$ nicotine		*Chrna2*$^{-/-}$ saline		*Chrna2*$^{+/+}$ nicotine		*Chrna2*$^{+/+}$ saline	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Trace fear conditioning training								
Trace 1	0.00 ± 0.00 (5)	0.50 ± 0.50 (4)	0.00 ± 0.00 (5)	0.00 ± 0.00 (4)	0.00 ± 0.00 (4)	0.13 ± 0.13(8)	0.00 ± 0.00 (4)	0.29 ± 0.29 (7)
Trace 2	0.80 ± 0.37 (5)	1.00 ± 0.71 (4)	1.00 ± 0.45 (5)	0.00 ± 0.00 (4)	1.00 ± 0.41 (4)	0.5 ± 0.27 (8)	1.25 ± 0.48(4)	0.71 ± 0.42 (7)
Trace 3	2.20 ± 0.37 (5)	2.25 ± 0.48 (4)	1.20 ± 0.37 (5)	2.00 ± 0.00 (4)	3.00 ± 0.00 (4)	1.4 ± 0.38 (8)	1.75 ± 0.63(4)	1.71 ± 0.36(7)
Trace 4	2.40 ± 0.24 (5)	2.00 ± 0.71 (4)	2.60 ± 0.24 (5)	2.25 ± 0.48 (4)	2.50 ± 0.29 (4)	1.9 ± 0.35 (8)	2.00 ± 0.41 (4)	2.86 ± 0.14(7)
Trace 5	2.60 ± 0.24 (5)	2.50 ± 0.29 (4)	2.40 ± 0.24 (5)	2.75 ± 0.25 (4)	2.25 ± 0.75 (4)	1.75 ± 0.41 (8)	3.0 ± 0.0 (4)	2.57 ± 0.20 (7)
Baseline, immediate, pre-CS, and CS values								
Baseline	0 ± 0 (6)	0 ± 0 (3)	0 ± 0 (5)	0 ± 0 (4)	0 ± 0 (5)	0.0 ± 0.0 (7)	0 ± 0 (6)	0.4 ± 0.4 (5)
Immediate	8.17 ± 0.98 (6)	7.67 ± 2.33 (3)	8.2 ± 1.07 (5)	6.25 ± 1.7(4)	8 ± 0.71 (5)	6.71 ± 1.01 (7)	9.5 ± 0.85 (6)	8.8 ± 1.02 (5)
Pre-CS	3 ± 0.73 (6)	9 ± 1.53(3)	4.2 ± 1.32 (5)	4 ± 2.04 (4)	4.4 ± 1.44(5)	3.57 ± 0.81 (7)	4.17 ± 1.19(6)	4.6 ± 1.86(5)
CS	7.83 ± 1.6(6)	7 ± 2.65 (3)	8.2 ± 1.77 (5)	6.75 ± 2.29 (4)	6.6 ± 1.36 (5)	7 ± 0.88 (7)	5.67 ± 1.5 (6)	10.4 ± 1.47 (5)

[a]Behavioral scores were measured as described in Materials and Methods. Values represent number of freezing episodes and are presented as mean ± SEM (*n*).

Table 4

Sex-dependent influence on spontaneous nicotine withdrawal-induced deficits in cued fear conditioning[a]

	Chrna2$^{-/-}$ nicotine		*Chrna2*$^{-/-}$ saline		*Chrna2*$^{+/+}$ nicotine		*Chrna2*$^{+/+}$ saline	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Baseline	0.00 (2)	0.00 ± 0.00 (9)	0.00 ± 0.00 (3)	0.00 ± 0.00 (5)	0.00 ± 0.00 (6)	0.00 ± 0.00 (5)	0.00 (2)	0.00 ± 0.00 (8)
Immediate	0.00 (2)	0.22 ± 0.15(9)	0.33 ± 0.33 (3)	0.60 ± 0.24 (5)	0.17 ± 0.17(6)	0.60 ± 0.40 (5)	1.00(2)	0.63 ± 0.32 (8)
Context	8.00 (2)	11.00 ± 1.34 (9)	9.67 ± 4.10(3)	11.00 ± 1.70 (5)	8.50 ± 1.12(6)	13.40 ± 2.64 (5)	10.00 (2)	12.63 ± 1.63(8)
Pre-CS	0.50 (2)	0.22 ± 0.15(9)	0.00 ± 0.00 (3)	0.00 ± 0.00 (5)	0.00 ± 0.00 (6)	0.00 ± 0.00 (5)	0.00 (2)	1.25 ± 0.49 (8)
CS	12.00 (2)	9.67 ± 0.67 *(9)	10.67 ± 1.67(3)	12.40 ± 0.75 (5)	12.00 ± 2.00 (6)	9.80 ± 0.80 (5)	12.67 (2)	11.25 ± 0.65 (8)

[a] Values represent number of freezing episodes and are presented as mean ± SEM (*n*).

* $p < 0.05$ versus *Chrna2*$^{-/-}$ male saline-treated mice (uncorrected).

Table 5

Nicotine self-administration dose–response comparisons[a]

Comparisons	*Chrna2*$^{+/+}$	*Chrna2*$^{-/-}$
Matched pair *t* test comparisons for reinforced responses across nicotine dose versus saline self-administering animals		
Saline reinforced responses versus nicotine dose (0.01 mg/kg/injection) reinforced responses	$p = 0.0146$ (NS); $n = 4$	$p = 0.0368$ (NS); $n = 4$
Saline reinforced responses versus nicotine dose (0.03 mg/kg/injection) reinforced responses	$p = 0.0035$ (NS); $n = 5$	$p = 0.0106$ (NS); $n = 5$
Saline reinforced responses versus nicotine dose (0.1 mg/kg/injection) reinforced responses	$p = 0.0013$ *; $n = 5$	$p = 0.0011$ **; $n = 5$
Saline reinforced responses versus nicotine dose (0.4 mg/kg/injection) reinforced responses	$p = 0.0264$ (NS); $n = 5$	$p = 0.0009$ **; $n = 5$
Reinforced versus nonreinforced responding across dose		
Saline reinforced versus nonreinforced responses	$p = 0.0143$ (NS); $n = 5$	$p = 0.0409$ (NS); $n = 5$
Nicotine dose (0.01 mg/kg/injection) reinforced versus nonreinforced responses	$p = 0.0233$ (NS); $n = 4$	$p = 0.0119$ (NS); $n = 4$
Nicotine dose (0.03 mg/kg/injection) reinforced versus nonreinforced responses	$p = 0.0099$ (NS); $n = 5$	$p = 0.0073$ (NS); $n = 5$
Nicotine dose (0.1 mg/kg/injection) reinforced versus nonreinforced responses	$p = 0.0006$ **; $n = 5$	$p = 0.0002$ ***; $n = 5$
Nicotine dose (0.4 mg/kg/injection) reinforced versus nonreinforced responses	$p = 0.0191$ (NS); $n = 5$	$p = 0.0045$ *; $n = 5$

[a] Listed *p* values are not corrected for multiple comparisons.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$ (Bonferroni-corrected for multiple comparisons).

NS, Not significant.

Table 6

Interpeduncular nucleus neurotransmitter levels 2 h after nicotine withdrawal[a]

Neurotransmitter	*Chrna2*$^{+/+}$	*Chrna2*$^{-/-}$	*Chrna2*$^{-/-}$ nicotine	*Chrna2*$^{-/-}$ saline	*Chrna2*$^{+/+}$ nicotine	*Chrna2*$^{+/+}$ saline
Serotonin	0.028 ± 0.002 (15)	0.032 ± 0.002 (24)	—	—	—	—
Norepinephrine	0.020 ± 0.001 (15)	0.021 ± 0.001 (24)	—	—	—	—
Dopamine	0.008 ± 0.002 (15)	0.009 ± 0.001 (24)	—	—	—	—
Glutamate	12.79 ± 0.57 (15)	14.94 ± 0.87* (24)	12.89 ± 0.97 (10)	17.24 ± 0.86**,*** (14)	13.41 ± 1.17(7)	12.29 ± 1.09(8)
γ-Aminobutyric acid	0.0814 ± 0.006 (14)	0.0986 ± 0.006* (22)	—	—	—	—

[a] Values are represented as ng/μg protein/25 μl injection, mean ± SEM (*n*).

* $p < 0.05$ versus wild-type mice (uncorrected).

** $p < 0.05$ versus *Chrna2*$^{-/-}$ nicotine-treated mice (Bonferroni-corrected).

*** $p < 0.01$ versus wild-type saline-treated mice (Bonferroni-corrected).