α2* Nicotinic acetylcholine receptors influence hippocampus-dependent learning and memory in adolescent mice

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The absence of α2* nicotinic acetylcholine receptors (nAChRs) in oriens lacunomuscular (OLM) GABAergic interneurons ablates the facilitation of nicotine-induced hippocampal CA1 long-term potentiation and impair memory. The current study delineated whether genetic mutations of α2* nAChRs (Chrna2L9′/S′ and Chrna2KO) influence hippocampus-dependent learning and memory and CA1 synaptic plasticity. We substituted a serine for a leucine (L9′) in the α2 subunit (encoded by the Chrna2 gene) to make a hypersensitive nAChR. Using a dorsal hippocampus-dependent task of preexposure-dependent contextual fear conditioning, adolescent hypersensitive Chrna2L9′/L9′ male mice exhibited impaired learning and memory. The deficit was rescued by low-dose nicotine exposure. Electrophysiological studies demonstrated that hypersensitive α2 nAChRs potentiate acetylcholine-induced ion channel flux in oocytes and acute nicotine-induced facilitation of dorsal/intermediate CA1 hippocampal long-term potentiation in Chrna2L9′/L9′ mice. Adolescent male mice null for the α2 nAChR subunit exhibited a baseline deficit in learning that was not reversed by an acute dose of nicotine. These effects were not influenced by locomotor, sensory or anxiety-related measures. Our results demonstrated that α2* nAChRs influenced hippocampus-dependent learning and memory, as well as nicotine-facilitated CA1 hippocampal synaptic plasticity.

Acetylcholine and nicotine activate nicotinic acetylcholine receptors (nAChRs) to influence learning and memory, via the release of neurotransmitters and neurotrophic factors. The specific contributions of selective nAChR subunits influencing learning and memory, particularly during development, are poorly understood. Genetic deletion of α2* nAChRs leads to subtle, yet discernible, potentiation of the first 2 d of nicotine self-administration, context specific withdrawal, emotional memory processing (Lotfipour et al. 2013), and baseline deficits in hippocampus-dependent memory (Kleeman et al. 2016). Developmental dysregulation of neural circuits in the absence of α2* nAChRs may be responsible for these modifications. α2 (and β2) mRNA are expressed within midbrain (interpeduncular nucleus) and limbic brain regions (hippocampus, amygdala, and neocortex) (Deneris et al. 1988; Wada et al. 1988, 1989; Ishii et al. 2005; Upton and Lotfipour 2013; Hilscher et al. 2017). During early postnatal rodent development, particularly during the first 2 wk of life, heightened expression patterns of α2* nAChRs are observed within the hippocampus and cortex (Son and Winzer-Serhan 2006). Due to the expression pattern of α2* nAChRs, studies have focused on dissecting the functional roles of these subunits within these brain regions (Nakauchi et al. 2007; Salas et al. 2009).

Within the dorsal through ventral CA1 of the hippocampus, α2* nAChRs are located on oriens lacunomuscular (OLM) GABAergic interneurons (Subweeks and Yakel 2000; Son and Winzer-Serhan 2006; Nakauchi et al. 2007, 2015; Leao et al. 2012; Chen et al. 2016; Kleeman et al. 2016). Studies suggest that α2* nAChRs play an important role in CA1 hippocampal OLM GABAergic interneurons (Ishii et al. 2005; Nakauchi et al. 2007, 2015; Chen et al. 2016; Kleeman et al. 2016). Deletion of Chrna2 induces an absence of nicotine facilitation and suppression of hippocampal long-term potentiation (LTP) (Nakauchi et al. 2007), likely via a dysregulation of OLM GABA neurotransmitter release (Leao et al. 2012). Furthermore, early developmental chronic nicotine exposure during peak mRNA expression periods of α2* nAChRs (i.e., the first 2 wk of rodent life) is known to influence hippocampal synaptic plasticity and learning and memory during adolescence, likely via α2* nAChRs (Nakauchi et al. 2015; Chen et al. 2016; Kleeman et al. 2016). Our current study assessed whether deletion or functional enhancement of α2* nAChRs are important for baseline and acute nicotine’s effects...
on learning and memory processing. We evaluated this using a dorsal CA1 hippocampus-dependent task of preexposure-dependent contextual fear conditioning in adolescent male mice (Fanselow 1990; Rudy et al. 2002; Cushman et al. 2011). Our findings provide evidence for a role of α2 nAChRs in hippocampus-dependent learning and memory and nicotine-facilitated synaptic plasticity in mice.

Results

Adolescent Chrna2L9S/19S generation, and developmental stereotypical behavioral characteristics

We genetically engineered the Chrna2L9S/WT mice in our laboratory (Fig. 1). Subsequently, Chrna2L9S/WT mice were backcrossed with C57Bl/6J mice for 3–4 generations. Heterozygous Chrna2L9S/WT mice were mated for experiments and offspring had expected mendelian genotype (1:2:1) and sex ratios (50% M, F). The adolescent male Chrna2L9S/19S mice exhibited good health, no gross anatomical morphological changes, no differences in stereotypical behaviors (Table 1a1; Fig. 2), and no differences in weight gain compared with wild-type littermate control mice (data not shown).

Oocyte expression

The use of oocyte expression studies (Fig. 3A) was applied as an initial screen to determine the functionality of a substitution of a serine for a leucine (L9S) in the α2 nAChR subunit. In the presence of acetylcholine (Ach), fitted Hill equations for αβ2 wild-type nicotinic receptors had an EC50 of 3.7 ± 1.0 μM, which is similar to that previously reported for αβ2 nAChRs, 5.07 ± 2.4 μM (Lipovsek et al. 2008). α2L9 mutations produced an increase in the apparent affinity for Ach when expressed in Xenopus oocytes together with the β2 subunit (Fig. 3A). Thus, the α2L9S mutation produced a 100-fold (EC50: 0.041 ± 0.015), the α2L9A a 10-fold (EC50: 0.37 ± 0.03) and the α2L9T a fivefold decrease in the Ach EC50 (EC50: 0.91 ± 0.04) when compared with α2L9WT-containing receptors (EC50: 3.7 ± 1.0) (n = 3 per group). Our results confirmed earlier findings in oocytes that had a serine for a leucine (L9S) substitution in a nAChR subunit induces a 100-fold leftward shift in the acetylcholine EC50 dose response curves (for review, please see Lester et al. 2003). Based on these data, α2L9S mutations were chosen for the design and development of the hypersensitive Chrna2L9S/19S mouse line (Fig. 1A), because they produced the greatest increase in Ach apparent affinity (Fig. 3A).

Dorsal/intermediate CA1 hippocampal nicotinic facilitation of LTP

We measured the impact of α2L9S nAChR expression on synaptic transmission at the Schaffer collateral (SC) pathway by monitoring stimulus–response relationships. We found no significant differences between slices from control and Chrna2L9S/19S mice in the stimulus–response relationships (Fig. 3B), suggesting that the expression of α2L9S nAChRs had no significant effect on basal synaptic transmission at the SC pathway. We then monitored LTP induction at the SC pathway using weak theta burst stimulation (TBS) protocol. Previous studies demonstrated that endogenous Ach-mediated activation of α2 nAChR-containing OLM cells did not occur during weak TBS at the SC pathway, but bath application of nicotine activated α2-nAChR-containing OLM cells to induce LTP (Nakauchi et al. 2007). Indeed, in both wild-type and Chrna2L9S/19S mice, LTP was not induced in the absence of nicotine (Fig. 3C); wild-type, 102.0 ± 2.8%, N = 6 and Chrna2L9S/19S, 100.7 ± 5.7%, N = 6). In wild-type mice, 1 μM nicotine was required for LTP induction (Nakauchi et al. 2007), whereas we found that in Chrna2L9S/19S mice, 0.1 μM nicotine (Fig. 3D), wild-type, 107.3 ± 3.4%, N = 9 versus Chrna2L9S/19S, 159.3 ± 21.4%, N = 6, P = 0.033, Bonferroni-corrected). 0.01 μM nicotine (Fig. 3D), wild type, 99.4 ± 7.8%, N = 7 versus Chrna2L9S/19S, 133.7 ± 9.3%, N = 8, P = 0.045, Bonferroni-corrected) was sufficient for LTP induction. These findings suggest that α2L9S nAChRs were activated by lower concentrations of nicotine than that used to induce LTP. Overall, the findings indicate that α2 nAChRs are capable of participating in nicotinic facilitation of LTP at the SC pathway.

Figure 1. Genetic design of the hypersensitive Chrna2L9S/19S mouse line. (A) The generation of the hypersensitive Chrna2L9S/19S mouse line has been described in the Materials and Methods. Briefly, a leucine to serine substitution was engineered using gene synthesis. The targeting vector was electroporated into 129S5/SvEv embryonic stem (ES) cells and homologous recombinants were confirmed through DNA sequencing (B) and Southern blot analysis (using the probe denoted by the single red line) (C). Targeted ES cells were microinjected into C57Bl/6J blastocyst embryos and implanted in pseudopregnant female mice, with the DNA sequence of the germline transmitted offspring confirming the AGC (Serine)/CTC (Leucine) hypersensitive genomic modification (denoted by *). Genotyping of mice was through tail biopsy, MyTaq HS Red Mix (Bioline) with PCR primers (A2L9S_1.10, A2L9S_1.11, denoted by arrows flanking the 34 bp FRT Site) designed upstream of and downstream from the deleted PGK-NEO, as described (FRT site denoted by the single arrow).
Table 1. Statistics table

| Symbol | Result                                                                 | Data structure                  | Type of test                  | P-value, power, and LSN                              | N    |
|--------|------------------------------------------------------------------------|---------------------------------|-------------------------------|----------------------------------------------------|------|
| a1     | Figure 2, Stereotypic behaviors                                       | Normal distribution             | Two-way ANOVA                | n.s. for genotype, drug or Genotype × Drug         | 12–16|
| b1-3   | Figure 3F, Electrophysiology data, Chrna2^9.5/19.5 mice                | Normal distribution             | T-test (a1–3)                | (b1)p = n.s. (saline), (b2) P = 0.033, Bonferroni-corrected, power: 0.8 (0.1 µM nicotine), (b3)(P) = 0.045, Bonferroni-corrected, power: 0.7, (0.01 µM nicotine) | 6–9  |
| c      | Figure 4B D1 versus D3, Learning and memory data, Chrna2^9.5/19.5 mice  | Normal distribution             | Matched pairs t-test         | (***) P = 0.002, Bonferroni-corrected, power: 1.0 for nicotine-treated Chrna2^9.5/19.5 Mice, (****) P = 0.001, Bonferroni-corrected, power: 1.0 for saline-treated wild-type mice, (****) P = 0.0004, Bonferroni-corrected, power: 1.0 for nicotine-treated wild-type mice, LSN: 16 for saline-treated Chrna2^9.5/19.5 mice (current N = 12) | 12–16|
| d1-3   | Figure 4C D2, Learning and memory data, Chrna2^9.5/19.5 mice           | Normal distribution             | Two-way ANOVA                | (d1–3)p = n.s. n.s. for Genotype, Drug, or Genotype × Drug | 12–16|
| e1-2   | Figure 4B D3, Learning and memory data, Chrna2^9.5/19.5 Mice           | Normal distribution             | Two-way ANOVA (e1), least square means differences Student’s t-test (e2) | (e1)p = 0.02, Power: 0.7 for the interaction of Genotype × Drug, (e2) P = 0.03, Bonferroni-corrected, power: 0.8 for wild-type saline-treated mice versus saline-treated Chrna2^9.5/19.5 mice for day 3 (f1) = 0.02, Power = 0.7 for the main effect of genotype, (f2) = 0.03, Power = 0.6 for the Interaction of genotype × drug, Bonferroni-corrected, P = 0.02, power: 0.9 for saline-treated Chrna2^9.5/19.5 mice versus saline-treated wild-type mice, Bonferroni-corrected, P = 0.049, power: 0.8 for saline-treated Chrna2^9.5/19.5 mice versus nicotine-treated wild-type mice, Bonferroni-corrected, P = 0.06 for saline-treated Chrna2^9.5/19.5 mice versus nicotine-treated Chrna2^9.5/19.5 mice | 28(11) |
| f1-2   | Figure 5A, Rearing, Chrna2^9.5/19.5 mice                              | Normal distribution             | Two-way ANOVA, least square means differences Student’s t-test | (g1)(P) = 0.002, Power = 0.9 for main effect of genotype, (g2)P = n.s. for Genotype × Drug interaction | 28   |
| g1-2   | Figure 5B, Mid-line crosses, Chrna2^9.5/19.5 mice                      | Normal distribution             | Two-way ANOVA                | (g1)P = 0.004, power = 0.9 for the main effect of genotype | 28   |
| h      | Figure 5C,D, Anxiety: center/perimeter time, Chrna2^9.5/19.5 mice      | Normal distribution             | Two-way ANOVA                | (h)P = 0.02, power = 0.7 for the main effect of genotype | 28   |
| i      | Figure 5E, Locomotor activity, Chrna2^9.5/19.5 mice                    | Normal distribution             | ANOVA                        | (i)P = 0.004, power = 0.9 for the main effect of genotype | 28   |
| j      | Figure 5F, Shock reactivity, Chrna2^9.5/19.5 mice                      | Normal distribution             | Two-way ANOVA                | (j)P = 0.02, power = 0.7 for the main effect of genotype | 28   |
| k      | Figure 6, Stereotypic behaviors                                       | Normal distribution             | Two-way ANOVA                | n.s. for Genotype, Drug or Genotype × Drug         | 5–8  |
| l1-3   | Figure 7B D2, Learning and memory data, Chrna2^KO mice                 | Normal distribution             | Two-way ANOVA, least square means differences Student’s t-test | (l1)P = 0.03, power: 0.6 for main effect of genotype, (l2)P = 0.02, power: 0.7 for Genotype × Drug Interaction, (l3)Bonferroni-corrected, power:0.9 for nicotine-treated wild-type mice versus nicotine-treated Chrna2^KO mice | 11(1–2) |
| m      | Figure 7 D3, Learning and memory data, Chrna2^KO mice                  | Normal distribution             | Two-way ANOVA                | (m)P = 0.02, Power: 0.7 for main effect of genotype | 11–14|
| n      | Figure 8A, Rearing, Chrna2^KO mice                                    | Normal distribution             | Two-way ANOVA, Least Square means differences Student’s t-test | P = 0.052, LSN:49 for Genotype × Drug interaction (current N = 25 for total sample of N = 5–8 per group) | 5–8  |
| o1-4   | Figure 8B, Mid-line crosses, Chrna2^KO mice                            | Normal distribution             | Two-way ANOVA, least square means differences Student’s t-test | (o1) = 0.01 for main effect of genotype, (o2) = 0.04 for main effect of drug, (o3) = 0.04 for Genotype × Drug interaction, power = 0.6, (o4)P = 0.02, Bonferroni-corrected, power: 0.9 for nicotine-treated Chrna2^KO mice versus all other groups: nicotine-treated wild-type mice, saline-treated wild-type mice, and saline-treated Chrna2^KO mice | 5–8  |
| p      | Figure 8C,D, Anxiety: center/perimeter time, Chrna2^9.5/19.5 mice      | Normal distribution             | Two-way ANOVA                | (p)P = n.s. n.s. for Genotype, Drug, or Genotype × Drug | 5–8  |

Continued
Table 1. Continued

| Symbol | Result | Data structure | Type of test | P-value, power, and LSN | N |
|--------|--------|----------------|--------------|------------------------|---|
| q      | Figure 8E, Locomotor data, Chrna2<sup>L9S/L9S</sup> mice | Normal distribution | Two-way ANOVA | (***) P = 0.01, power = 0.8 for main effect of genotype | N = 28 |
| r      | Figure 8F, Shock reactivity, Chrna2<sup>L9S/L9S</sup> mice | Normal distribution | Two-way ANOVA, least square means Student’s t-test | P = 0.01, Power = 0.8 for Genotype × Drug interaction, Bonferroni-corrected post hoc comparison were not significant | N = 5–8 |

LSN: Least significant number (i.e., total "n") needed to attain significance at an α = 0.05 and a power of 0.8 (see LSN definition in JMP, 12.0.1). Power values less than 0.8 are considered underpowered, if a significant difference is present (see parameter power definition in JMP, 12.0.1). While an interaction could provide a low power (e.g., e1), the post hoc test can illustrate sufficient power (e.g., e2). Please note that there are more cases that are underpowered in data collected from the Chrna2<sup>L9S/L9S</sup> mice versus the Chrna2<sup>L9S/L9S</sup> mice. This is mainly due to the reduced "n" in the Chrna2<sup>L9S/L9S</sup> mouse population (i.e., N = 5–8 per group for Chrna2<sup>L9S/L9S</sup> mice). For transparency, we have retained the data and power analyses in our study. We have not included LSN’s that require a high "n" to lead to significance. For anxiety behavior in Chrna2<sup>L9S/L9S</sup> mice, a normal distribution was found in two of four groups.

Preexposure-dependent contextual fear conditioning in adolescent mice

We assessed nicotine modulated hippocampus-dependent learning and memory in our Chrna2<sup>L9S/L9S</sup> mouse line compared with their wild-type littermate controls (heterozygous mice were not assessed in these studies) (Fig. 4A–C). The learning and memory task we used was a preexposure-dependent contextual fear conditioning procedure, which is dorsal hippocampus-dependent (Fanselow 1990; Wiltgen et al. 2001; Rudy et al. 2002; Kenney and Gould 2008; Cushman et al. 2011). In the current studies, we hypothesized that Chrna2<sup>L9S/L9S</sup> mice would exhibit modifications in nicotine-facilitated learning and memory. As a measure of conditioning, we examined changes in individual animals freezing from day 1 (preexposure) to day 3 (context test) using matched-pair t-tests (Bonferroni-corrected for multiple comparisons holding α = 0.05<sup>5</sup>) (**** P = 0.002, Bonferroni-corrected; (***) P = 0.001, Bonferroni-corrected; (** P = 0.004, Bonferroni-corrected, N = 12–16 per group) (Fig. 4B). A significant enhancement of freezing occurred for all genotypes and drug treatments on context test, except for the Chrna2<sup>L9S/L9S</sup> saline-treated mice (Fig. 4B). For day 2, using a two-way ANOVA, we observed no main effects for Genotype (F<sub>1,52</sub> = 0.07, P = n.s.51), Drug (Nicotine versus Saline) (F<sub>1,52</sub> = 1.68, P = n.s.52), or Genotype × Drug (F<sub>1,52</sub> = 1.51, P = n.s.53) interaction (Fig. 4C). For day 3, a two-way ANOVA revealed a Genotype × Drug Treatment (Nicotine versus Saline) interaction (F<sub>1,52</sub> = 6.12, P = 0.02, N = 12–16 per group), Fig. 4B). Bonferroni-corrected post hoc analysis demonstrated saline-treated Chrna2<sup>L9S/L9S</sup> mice had significantly reduced freezing behavior when compared with saline-treated wild-type mice (P = 0.03<sup>2</sup>, Fig. 4B). The reduced freezing behavior in Chrna2<sup>L9S/L9S</sup> mice was absent after nicotine treatment. The findings support the conclusion that saline-treated Chrna2<sup>L9S/L9S</sup> mice failed to exhibit enhanced freezing on day 3 when compared with day 1, likely due to a deficit to acquire and encode appropriate contextual representations during preexposure day 1. We refer to this loss of freezing behavior in saline-treated Chrna2<sup>L9S/L9S</sup> mice as a baseline deficit in hippocampus-dependent learning and memory.

Activity measures in Chrna2<sup>L9S/L9S</sup> mice

We tested whether or not changes in learning and memory (as assessed by freezing behavior) were confounded by modifications in other types of behaviors, including locomotor, sensory, and anxiety behavior (Fig. 5). After an acute treatment to saline or nicotine, we measured automated locomotor activity (a robust measure of locomotor activity, Cushman et al. 2011), rearing, mid-line crosses (exploratory behavior), center and perimeter time (anxiety behavior) as animals were preexposed to the conditioning chamber. An increase in center time was regarded as reduced anxiety. For center (and perimeter) time anxiety measures, a two-way ANOVA demonstrated no main effect for Genotype (F<sub>1,52</sub> = 2.92, P = n.s.), Drug (Nicotine versus Saline) (F<sub>1,52</sub> = 0.18, P = n.s.), or
Chrna2

Significant main effect analysis for Genotype demonstrated behavior only resolved hyperactivity in saline-treated Chrna2^{L9/S,195} mice. Hyperactivity may provide additional evidence of impaired hippocampal function in the Chrna2^{L9/S,195} mice, as one effect of hippocampal lesions is hyperactivity (Anagnostaras et al. 1999; Godsil et al. 2005). On the other hand, it is possible that the hyperactivity observed on day 1 in Chrna2^{L9/S,195} mice confounds learning and memory deficits on day 3. If this was the case, we predicted that there would be a significant correlation between activity on day 1 and freezing behavior on day 3, with greater activity associated with reduced freezing behavior. Given our learning and memory findings, we predicted that the relationships should be dependent on both genotype and drug treatment, since nicotine treatment rescued the baseline deficit selectively in Chrna2^{L9/S,195} mice. When we separated by genotype and drug treatment, significant correlations were not observed for any groups: saline-treated Chrna2^{WT} mice: \( R^2 = 0.004, \ P = n.s.; \) nicotine-treated Chrna2^{WT} mice: \( R^2 = 0.003, \ P = n.s.; \) saline-treated Chrna2^{L9/S,195} mice: \( R^2 = 0.07, \ P = n.s.; \) nicotine-treated Chrna2^{L9/S,195} mice: \( R^2 = 0.09, \ P = n.s., \ n = 12–16 \) per group. The results did not illustrate selective correlations in saline or nicotine-treated Chrna2^{L9/S,195} mice. Thus, we do not believe that hyperactivity on day 1 confounded interpretations of our learning and memory results on day 3.

For changes in sensory response, we assessed the impacts of shock reactivity for all groups of mice. For shock reactivity, a two-way ANOVA revealed a significant effect for Genotype (\( F_{(1,52)} = 5.81, \ P = 0.02, \ N = 28 \) per group) and no other main effect or interaction. Significant main effect analysis for Genotype demonstrated that Chrna2^{L9/S,195} mice have greater shock reactivity versus wild-type control mice, independent of drug exposure (Fig. 5E). Increased shock reactivity may indicate increased pain sensitivity (i.e., sensory response). In Chrna2^{L9/S,195} mice, however, this would be expected to produce increased freezing, rather than the decrease that was observed in saline-treated Chrna2^{L9/S,195} mice, arguing that altered sensory response cannot explain the results. Thus, our results suggest that our learning and memory observations are not confounded by modifications in other types of behaviors, including locomotor, sensory, or anxiety behaviors.

### Adolescent Chrna2^{KO} stereotypical behavioral characteristics

We have previously described the genetic design, absence of gross anatomical morphological changes and behavioral characterization of adult Chrna2^{KO} mice (Lotfipour et al. 2013).
Adolescent Chrna2KO mice had no differences in weight gain or stereotypical behaviors observed in a novel environment compared with wild-type littermate control mice (Fig. 6). Thus, no growth or development modifications were observed in adolescent Chrna2KO mice.

Preexposure-dependent contextual fear conditioning in adolescent Chrna2KO mice

Using Chrna2KO mice, which exhibit an absence of nicotine-induced facilitation and depression of LTP during adolescence (Nakauchi et al. 2007), we predicted that we would observe an absence of nicotine-facilitated learning and memory in these mutant mice (Fig. 7). As a measure of conditioning, we examined changes in individual animals freezing to day 1 (preexposure) to day 3 (context test) using Bonferroni-corrected paired t-tests. Data demonstrated a significant enhancement of freezing behavior in all groups (\( P = 0.048 \), Bonferroni-corrected, \( \ast \) \( P = 0.01 \), Bonferroni-corrected, \( \ast\ast \) \( P = 0.049 \), Bonferroni-corrected, \( N = 5–8 \) per group, Fig. 7A). For day 2, using a two-way ANOVA, a main effect was observed for Genotype (\( F_{1,21} = 5.20, P = 0.035 \)) as well as a Genotype \( \times \) Drug Treatment (Nicotine versus Saline) interaction (\( F_{1,21} = 8.88, P = 0.02, N = 5–8 \) per group). Bonferroni-corrected t-test post hoc analysis revealed that wild-type nicotine-treated mice had significantly greater freezing behavior than nicotine-treated Chrna2KO mice (\( P = 0.025 \)), Fig. 7B). The findings suggest that nicotine-treated adolescent Chrna2KO mice had a deficit in learning and memory on day 2, when compared with nicotine-treated Chrna2NT mice. For day 3, a two-way ANOVA revealed a main effect for Genotype (\( F_{1,21} = 6.56, P = 0.021 \)) but no Genotype \( \times \) Drug Treatment (Nicotine versus Saline) interaction. Significant main effect analysis for Genotype revealed that wild-type mice had greater freezing behavior than Chrna2KO mice, independent of drug treatment (Fig. 7). Overall, the findings highlighted that Chrna2KO mice exhibited deficits in contextual learning during the preexposure that were not rescued by acute nicotine exposure.

Activity measures in Chrna2KO mice

We tested whether or not changes in learning and memory (as assessed by freezing behavior) were confounded by modifications in other types of behaviors, including locomotor, sensory, or anxiety-related behaviors (Fig. 8). After an acute treatment to saline or nicotine, we measured rearing, mid-line crosses, center time, and automated locomotor activity as animals were preexposed to the conditioning apparatus on day 1. We also assessed shock reactivity on day 2 (Fig. 8F). For rearing (exploratory vertical movement) behavior, two-way ANOVA failed to reach a significant main effect or interaction for Genotype \( \times \) Drug Treatment (\( F_{1,21} = 4.23, P = 0.052, N = 5–8 \) per group, Fig. 8A). For mid-line crossing behavior (exploratory horizontal movement), two-way ANOVA revealed a main effect for Genotype (\( F_{1,21} = 7.34, P = 0.01 \)), a main effect for Drug Treatment (\( F_{1,21} = 4.55, P = 0.049 \)) and an interactive effect for Genotype \( \times \) Drug Treatment (\( F_{1,21} = 4.97, P = 0.04, N = 5–8 \) per group, Fig. 8B). Bonferroni-corrected t-test post hoc analysis revealed nicotine-treated Chrna2KO had significantly greater mid-line crosses than all other groups (\( P = 0.024 \)). For anxiety behavior, we assessed center (Fig. 8C) and perimeter time (Fig. 8D) within the contextual fear conditioning chamber. For anxiety measures, a two-way ANOVA demonstrated no main effect for Genotype (\( F_{1,21} = 0.35, P = n.s. \)), Drug (Nicotine versus Saline) (\( F_{1,21} = 0.97, P = n.s. \)), or Genotype \( \times \) Drug (\( F_{1,21} = 0.74, P = n.s. \)) interaction (Fig. 8C,D). The findings highlighted that anxiety, as measured by an increase in center time, was not influenced by genotype or acute drug treatment. Independent of genotype or drug treatment, mice spent significantly greater time on the perimeter versus center of the open field chamber (\( 522.1 \) sec in perimeter versus \( 77.9 \) sec in center, \( P = 0.0001 \)).

For automated locomotor activity, two-way ANOVA demonstrated a main effect for Genotype (\( F_{1,21} = 9.09, P = 0.01 \)), but no other main effect or interaction. Greater activity was observed on day 1 in Chrna2KO versus wild-type mice, independent of drug treatment (Fig. 8E). To determine whether hyperactivity confounded our learning and memory results, we assessed simple linear correlations between activity on day 1 and freezing behavior on day 3 of context test. When we separated by genotype and drug treatment, significant correlations were not observed for...
Figure 5. Locomotor (rearing/vertical, mid-line crosses/vertical, automated vertical/horizontal), anxiety (center/perimeter time) and sensory response (shock reactivity) were assessed in wild-type littermate control and Chrna2<sup>L9S/19S</sup> mice. Behaviors were assessed in the contextual fear conditioning chamber during the preexposure period on day 1 (PE, Fig. 5A). Shock reactivity was assessed on day 2 (IS, Fig. 5A) using the Med Associated Automated video tracking system. (A) For rearing behavior, Bonferroni-corrected post hoc comparisons illustrate saline-treated Chrna2<sup>L9S/19S</sup> mice exhibited enhanced vertical movements versus saline and nicotine-treated wild-type mice littermate control mice. N = 12–16 per group, (*P* = 0.02, †*P* = 0.049). For mid-line crosses, Chrna2<sup>L9S/19S</sup> mice exhibited enhanced activity, independent of drug treatment. N = 28 per group, †*P* = 0.002 for mid-line crosses. (C,D) For anxiety, center or perimeter time did not differ in Chrna2<sup>L9S/19S</sup> of wild-type littermate control mice, independent of drug treatment. N = 12–16 per group. (E) For automated locomotor activity, Chrna2<sup>L9S/19S</sup> mice exhibited enhanced activity, independent of drug treatment. N = 28 per group, †*P* = 0.004 for automated locomotion. (F) For sensory response (shock reactivity), Chrna2<sup>L9S/19S</sup> versus wild-type littermate control mice exhibited enhanced shock reactivity on day 2, independent of drug treatment on preexposure day 1. N = 28 per group, †*P* = 0.02.

any groups: saline-treated Chrna2<sup>WT</sup> mice: *R*<sup>2</sup> = 0.11, *P* = n.s.; nicotine-treated Chrna2<sup>WT</sup> mice: *R*<sup>2</sup> = 0.36, *P* = n.s.; saline-treated Chrna2<sup>L9S</sup> mice: *R*<sup>2</sup> = 0.02, *P* = n.s.; saline-treated Chrna2<sup>L9S</sup> mice: *R*<sup>2</sup> = 0.002, *P* = n.s., *n* = 5–8 per group. Additionally, when we collapsed by drug treatment, again we observed no significant correlation between activity on day 1 and freezing behavior on day 3 of context test: Chrna2<sup>WT</sup> mice: *R*<sup>2</sup> = 0.12, *P* = n.s.; Chrna2<sup>KO</sup> mice: *R*<sup>2</sup> = 0.02, *P* = n.s.; *n* = 11–14. The results did not illustrate selective correlations in Chrna2<sup>KO</sup> mice. Thus, we do not believe that hyperactivity on day 1 confounded interpretations of our learning and memory results on day 3.

For shock reactivity across the 2 sec of the 0.75-mA shock and the 2 sec after, two-way ANOVA revealed a significant interaction for Genotype × Drug Treatment (*F*<sub>1,21</sub> = 9.05, *P* = 0.01, *N* = 5–8 per group) with no main effects observed. Bonferroni-corrected t-test post hoc analysis failed to reach statistical significance for any of the comparisons (Fig. 8F). The findings provide convergent evidence for impaired hippocampal function, similar to the pattern seen in Chrna2<sup>L9S/19S</sup> mice. The findings strongly suggest that our learning and memory observations are not confounded by modifications in other types of behaviors.

**Discussion**

The current study tested the hypothesis that genetic mutations of α<sup>2</sup> nAChRs in Chrna2<sup>L9S/19S</sup> mice, but not Chrna2<sup>KO</sup> mice, would produce modifications in nicotine-induced hippocampus-dependent learning and memory. When we substituted a serine for a leucine (L9S) in the α2 subunit (encoded by the Chrna2 gene) to make a hypersensitive nAChR, we confirmed the mutation potentiates acetylcholine-induced ion channel flux in oocytes. Subsequently, in adolescent Chrna2<sup>L9S/19S</sup> mice, we observed a potentiation of nicotine-induced facilitation of synaptic plasticity in hippocampal electrophysiological studies at subthreshold doses of nicotine (0.01 and 0.1 μM). Using a preexposure-dependent contextual fear paradigm (Willigen et al. 2001; Rudy et al. 2002; Stote and Fanselow 2004; Cushman et al. 2011), we observed an impairment in freezing behavior (i.e., baseline deficit) in both adolescent Chrna2<sup>L9S/19S</sup> and Chrna2<sup>KO</sup> mice. A subthreshold dose of nicotine (0.09 mg/kg per injection), was able to rescue the baseline deficit in Chrna2<sup>L9S/19S</sup>, but not Chrna2<sup>KO</sup> mice, which have potentiated and impaired nicotine-facilitated CA1 hippocampal synaptic plasticity, respectively. The findings support the hypothesis that α<sup>2</sup> nAChRs influence hippocampus-dependent learning and memory via alterations in nicotine-facilitated CA1 hippocampal synaptic plasticity in adolescent mice (Fig. 9).

**Figure 6.** Stereotypic behaviors were assessed in wild-type and Chrna2<sup>KO</sup> mice on day 1. Results illustrated that stereotypic behaviors do not differ based on drug treatment or genotype. *N* = 5–8 per group.
changes in hippocampus-dependent learning and memory during adolescence. Based on the in vivo electrophysiology studies in the Chnma2 KO mutant mouse line, we did not predict that baseline behavior would be different, as we found no significant differences between slices from control and Chnma2 KO mice in the stimulus–response relationships (Fig. 3B). When we performed the behavioral tasks, however, we observed a hippocampal deficit in saline-treated Chnma2 KO versus wild-type mice (i.e., reduced freezing behavior), which was rescued by an acute exposure to nicotine. We propose the mechanisms mediating the nicotine-induced rescue of learning and memory are through enhanced potentiation of CA1 hippocampus-synaptic plasticity, via direct actions on GABAergic OLM\(^2\) neurons. Alternatively, the increase in freezing may be due to excessive plasticity in response to nicotine. Future studies are needed to assess the mechanisms mediating the baseline deficit in hippocampus-dependent learning in Chnma2 KO mice. We speculate this may be mediated via alterations in cholinergic tone, induced by enhanced or absent activity within \(\alpha^2\)-containing GABAergic interneurons.

The alteration in activity may influence medial septum and/or CA1 hippocampal OLM\(^2\) neurons that regulate fimbria fornix septal-cholinergic input, thereby disrupting learning and memory (Ishii et al. 2005; Lovett-Barron et al. 2014). The hypothesis is derived from studies illustrating that dendritic inhibition of hippocampal neurons influence fear memory (Lovett-Barron et al. 2014).

The interpretation of our learning and memory results is based on assessing freezing behavior on days 2 and 3 using the preexposure-dependent contextual fear conditioning paradigm. A failure to exhibit enhanced freezing on days 2 and 3 is likely due to the inability to acquire appropriate contextual representations during preexposure day 1. In our results, greater variability in freezing behavior was observed on day 2 after immediate-shock versus day 3 of contextual testing in Chnma2 KO and Chnma2 KO mice. Previous studies using alcohol demonstrated that day 2 freezing behavior does not always predict day 3 freezing data (Cushman et al. 2011). While alcohol was able to reduce freezing on day 2 after an immediate-shock, it had no effect on day 3. The findings highlight that alcohol may impair the mechanisms mediating pattern completion in the presence of an immediate-shock; this deficit is not present during pattern completion of the shock–context pairing on day 3. Pattern completion refers to the ability of the hippocampus to use a subset of features experienced previously to recall or activate a conjunctive representation (Rudy et al. 2002, 2004; Matus-Amat et al. 2004; Nakashiba et al. 2012). In our current analysis, we did not observe a matched-pair correlation between days 2 and 3 freezing behavior in Chnma2 KO mice. The data confirmed that freezing behavior on day 2 did not predict freezing on day 3. The prior results with alcohol are in contrast with our current study, which illustrate that behavioral deficits are more prevalent on day 3 of contextual testing. This is likely due to a difference in methods, i.e., alcohol versus nicotine treatment.
Figure 8. Locomotor (rearing/vertical, mid-line crosses/vertical, automated vertical/horizontal), anxiety (center/perimeter time), and sensory response (shock reactivity) were assessed in wild-type littermate control and Chrna2KO mice. Behaviors were assessed in the contextual fear conditioning chamber during the preexposure period on day 1 (PE, Fig. 8A). Shock reactivity was assessed on day 2 (IS, Fig. 8A) using the Med Associated Automated video tracking system. (A) For rearing behavior, no main effects or interactions were observed, although there was a trend for an interaction, \( N = 5–8 \) per group. (B) For mid-line crosses, Chrna2KO versus wild-type littermate control mice exhibit enhanced activity on day 1, independent of drug treatment, \( N = 11–14 \) per group, (** \( P = 0.01 \)). (C–E) For anxiety, center (C) or perimeter time (D) did not differ in Chrna2KO versus wild-type littermate control mice, independent of drug treatment. \( N = 11–14 \) per group. (E) For automated locomotor activity, Chrna2KO versus wild-type littermate control mice exhibit enhanced automated activity on day 1, independent of drug treatment, \( N = 11–14 \) per group, (** \( P = 0.01 \). (F) For sensory response (shock reactivity), Bonferroni-corrected t-test post hoc analysis demonstrated that Chrna2KO versus wild-type mice did not exhibit any differences in shock reactivity on day 2 with or without drug treatment, \( N = 5–8 \) per group.

For studies performed in the absence of a preexposure day 1, zero to minimal freezing behavior (i.e., immediate-shock deficit) is regularly observed post-shock, illustrating a deficit in contextual encoding. In the presence of preexposure day 1, a rescue of the immediate-shock deficit is observed, associated with a significant enhancement of freezing behavior post-shock. The findings highlight that preexposure to a context develops a conjunctive representation of that environment which can be recalled on light that preexposure to a context develops a conjunctive representation, i.e., the environment into a stable contextual representation, i.e., the common-deficit hypothesis (Godsil et al. 2005). Further exploration is needed on whether similar mechanisms as proposed by Godsil and colleagues may be influencing our results. Prior results have demonstrated that Chrna2KO mice, our results demonstrated that the mutant mice have heightened activity. If hyperactivity on day 1 was confounding learning and memory deficits on day 3, we predicted that nicotine-treated Chrna2/19.5/19.5 mice independent of nicotine treatment, would illustrate significant association between hyperactivity and freezing behavior. When Chrna2/19.5/19.5, Chrna2KO or their wild-type littermate control mice were separated by treatments and/or genotype, activity on day 1 did not predict freezing behavior on day 3. Given that the results did not illustrate selective correlations in nicotine-treated Chrna2/19.5/19.5 or Chrna2KO mice independent of nicotine treatment, we conclude that hyperactivity on day 1 does not confound interpretations in our learning and memory results on day 3. Indeed, the hyperactivity that is induced by dorsal hippocampal lesions has been argued to result from the same learning impairment that disrupts contextual learning: impaired ability to encode the multisensory features of the environment into a stable contextual representation, i.e., impaired ability to encode the multisensory features of the environment into a stable contextual representation, i.e., the common-deficit hypothesis (Godsil et al. 2005). Further exploration is needed on whether similar mechanisms as proposed by Godsil and colleagues may be influencing our results. Prior results have demonstrated that Chrna2/19.5 mice exhibit deficits in hippocampus-dependent memory (Kleeman et al. 2016). Thus, our findings fit well with recent data in the Chrna2KO mouse as assessed via an alternative behavioral task, i.e., the object location task but not the hippocampus-independent novel object recognition task (Kleeman et al. 2016). If locomotor modifications confound learning and memory deficits, we would predict that both behaviors would be altered in Chrna2KO mice, which was not observed.
Thus, we conclude that the hippocampus-dependent learning and memory observations in the current studies were not confounded by modifications in locomotor hyperactivity.

Our data also suggest that our learning and memory results are not influenced by changes in sensory response (i.e., pain sensitivity as determined by shock reactivity) or anxiety-related behavior (center time). The preexposure-dependent contextual fear procedure was specifically designed to minimize any potential analgesic effects of memory modulating drugs by separating drug administration from shock presentation. For sensory response, we observed increased shock reactivity in Chrna2<sup>L9<sup>−/−</sup></sup>,<sup>L9<sup>′</sup>/L9<sup>−/−</sup></sup> mice, independent of drug exposure. If increased shock reactivity is a measure of enhanced sensory response, we would predict increased freezing behavior during contextual testing in saline-treated Chrna2<sup>L9<sup>−/−</sup></sup>,<sup>L9<sup>′</sup>/L9<sup>−/−</sup></sup> mice; rather, we observed a decrease in freezing behavior. No significant effects were observed for shock reactivity in Chrna2<sup>L9<sup>−/−</sup></sup> mutant mice. Thus, our findings argue that altered sensory response cannot explain changes in freezing behavior. Previous studies in adult Chrna2<sup>L9<sup>−/−</sup></sup> mice have indicated differences in nicotine-induced hyperalgesia in the hotplate test, but not when assessing baseline or nicotine-induced analgesia to the tail withdrawal or the formalin test (Lottipour et al. 2013). The findings suggest that nicotine-induced pain response to selective behaviors may be influenced in Chrna2<sup>L9<sup>−/−</sup></sup> and Chrna2<sup>L9<sup>−/−</sup></sup>,<sup>L9<sup>′</sup>/L9<sup>−/−</sup></sup> mice. However, given that (i) our nicotine exposure did not occur during day 2 immediate-shock presentation, (ii) adolescent Chrna2<sup>L9<sup>−/−</sup></sup> mice did not exhibit significant differences in shock reactivity, and (iii) Chrna2<sup>L9<sup>−/−</sup></sup>,<sup>L9<sup>′</sup>/L9<sup>−/−</sup></sup> mice had increased shock reactivity with reduced (instead of increased) freezing behavior in saline-treated mice, we do not believe that pain sensitivity is a plausible explanation of our results. In regards to anxiety-related behaviors, our current studies did not observe differences in center time in Chrna2<sup>L9<sup>−/−</sup></sup>,<sup>L9<sup>′</sup>/L9<sup>−/−</sup></sup> or Chrna2<sup>L9<sup>−/−</sup></sup> mice. Given that ventral (versus dorsal) hippocampal circuits are more involved in mediating anxiety-related behaviors and we did not observe differences in center time, we are confident that our results are not confounded by changes in anxiety-related circuits. Therefore, indirect effects, including sensory response to shock or anxiety, do not appear to account for our findings. Rather they implicate learning and memory processes. Our findings are consistent with previous studies, which found that hippocampal nAChR antagonists disrupt nicotine-facilitated learning and memory processing, as tested by contextual fear conditioning (Davis et al. 2007). Furthermore, genetic studies have demonstrated an absence of nicotine-facilitated contextual fear conditioning in β<sub>2</sub>−/− (but not a7<sup>−/−</sup>, β3<sup>−/−</sup>, or β4<sup>−/−</sup>) mutant mice (Wehner et al. 2004; Davis and Gould 2007; Semenova et al. 2012). Such findings suggest that nAChRs assembled from select subunits regulate nicotine’s effects on learning and memory processing.

Our results are not without limitations. For example, authors have argued that nAChR expression and/or function in oocytes may not be identical to native tissues (Buller and White 1990; Sivilotti et al. 1997). In particular, differences in nAChR subunit composition and/or molar concentrations of individual subunits are known to influence “channel open times, ion conductance, desensitization rates” as well as the “sensitivity to agonists and antagonists” (Boulter et al. 1987; Deneris et al. 1988; Wada et al. 1988; Papke et al. 1989; Luetje and Patrick 1991; Paradiso et al. 2001; Grady et al. 2007; Lipovsek et al. 2008; McIntosh et al. 2009; Jin and Steinbach 2011, 2015). Neurons in animal and human studies contain a diversity of neuronal α/β nAChR subunits at different levels, which would differentially alter the subunit composition and stoichiometry. For example, interneurons of the stratum oriens (where OLM neurons reside) contain more than α2 and β2 nAChR subtypes (Sudweeks and Yakel 2000; Jia et al. 2009). Thus, applicability of our studies in oocytes to function in neurons in vivo needs to be interpreted cautiously. Our studies in oocytes were applied only as an initial screen to confirm the functionality of the serine for a leucine (L9<sup>′</sup>) substitution in the α2 nAChR subunit. Future studies could use, for example, single-cell electrophysiology and neurotransmitter release assays or in vivo imaging coupled with behavioral techniques to better confirm the biophysical and pharmacological properties of the hyper-sensitive nAChR in Chrna2<sup>L9<sup>−/−</sup></sup>,<sup>L9<sup>′</sup>/L9<sup>−/−</sup></sup> mice (Sudweeks and Yakel 2000; Grady et al. 2007; Jia et al. 2009; Lovett-Barron et al. 2014). Another potential limitation authors have reported regarding oocyte studies is the possibility of low level endogenous expression of peripheral α1 nAChR subunits (Buller and White 1990). Such a finding will not have an impact in our studies, given that neuronal α2 or β2 nAChR subunits when expressed, alone, in oocytes do not form functional nAChRs (Boulter et al. 1987; Deneris et al. 1988; Wada et al. 1988). Prior work has used similar oocyte expression methodologies assessing knock-in mutations for different nAChRαs to validate behavioral outcomes in mice and humans (Lester et al. 2003; Klaassen et al. 2006; Lipovsek et al. 2008). Thus, the oocyte expression studies have been helpful in identifying function in vivo, with our current studies providing supportive evidence for their validation.

In addition to limitations on oocyte studies, other concerns should be highlighted. In particular, heightened expression of α2<sup>−</sup> nAChRs appear during the first 2 wk of rodent life, while our behavioral and electrophysiological studies took place during adolescence (i.e., 4 wk of life). Therefore, learning and memory modifications induced by developmental nicotine exposure could
be potentiated if nicotine was delivered during this heightened period of expression during the first weeks of early postnatal development. Furthermore, it is possible that Chrna2<sup>2L9S/1L9S</sup> mice have modified α2<sup>+</sup> nAChRs expression patterns within the brain, particularly since prior results demonstrate that knock-in mutations within the TM2 may decrease receptor expression, but not mRNA levels (O’Neill et al. 2013). Therefore, future studies should aim to determine whether the Chrna2<sup>2L9S/1L9S</sup> mice exhibit differences in nAChR binding within the brain. Given the selectivity of α2<sup>+</sup> nAChRs within the stratum oriens GABAergic OLM interneurons, it is possible that such effects may be negligible in the dorsal hippocampal CA1. Prior studies have demonstrated that maternal nicotine exposure does reduce the number of nAChR binding within the brain. Given the selectivity of α2<sup>+</sup> nAChRs within the stratum oriens GABAergic OLM interneurons, it is possible that such effects may be negligible in the dorsal hippocampal CA1, i.e., one brain region where the current electrophysiological slice recording took place in the current study (Chen et al. 2016). Therefore, it is possible that similar effects would be observed within the Chrna2<sup>2L9S/1L9S</sup> mice, with likely more significant consequences observed within the ventral (versus dorsal) hippocampus (Chen et al. 2016). Given our anxiety results, we do not believe that ventral hippocampus nAChRs would confound interpretation of our current findings. Indeed, we found no significant differences between slices from control and Chrna2<sup>2L9S/1L9S</sup> mice in the stimulus–response relationships in our electrophysiological studies within the dorsal/intermediate CA1 hippocampus (Fig. 3B), suggesting that the modified expression of α2<sup>+</sup> nAChRs had no significant effect on basal synaptic transmission at the SC pathway.

Another limitation of our current studies is that we have not assessed sex-dependent effects. Given prior findings that emotional memory processing is modified in a sex-dependent manner in adult Chrna2<sup>KO</sup> mice (Lotfrpoul et al. 2013), future studies should assess sex-dependent effects in hippocampal-dependent learning and memory in adolescent Chrna2<sup>KO</sup> and Chrna2<sup>2L9S/1L9S</sup> mice. In addition, future studies should assess nicotine dose–response relationships and age-dependent effects using the Chrna2<sup>KO</sup> and Chrna2<sup>2L9S/1L9S</sup> mice. Our current results used the 0.09 mg/kg/injection nicotine dose based on adult data illustrating enhancement in wild-type mice (Kenney and Gould 2008; Kutlu et al. 2016). Therefore, we were initially surprised that 0.09 mg/kg/injection had no effects in wild-type mice. Subsequent studies, however, confirmed our findings in adolescent C57BL/6 mice using the 0.09 mg/kg/injection nicotine dose (Kutlu et al. 2016). Kutlu et al. (2016), have performed a nicotine dose response during adolescence and adulthood using the preexposure-dependent contextual fear conditioning paradigm. They have demonstrated that adolescent C57BL/6 wild-type mice are less sensitive to low-dose nicotine exposure, at the 0.09 mg/kg/dose, which we have replicated with our findings in adolescent wild-type mice.

Our findings illustrated that while low-dose nicotine exposure had no effect on adolescent wild-type mice, as reported (Kutlu et al. 2016), nicotine exposure was able to rescue the baseline deficit in Chrna2<sup>2L9S/1L9S</sup>, but not Chrna2<sup>KO</sup> mice. The findings support the rationale that the hypersensitive mutation potentiated synaptic plasticity in addition to hippocampal-dependent learning and memory at nicotine doses that did not influence wild-type mice. Given that nicotine pretreatment was not able to rescue the baseline deficit in hippocampal-dependent learning and memory in Chrna2<sup>KO</sup> mice, we propose that this was mediated by the known absence of nicotine-facilitated synaptic plasticity (Nakauchii et al. 2007). Thus, nicotine-facilitated synaptic plasticity plays an important role in how nicotine can rescue baseline deficits in learning and memory. Whether these results remain at higher doses of nicotine needs to be tested in future studies. Because the Chrna2<sup>KO</sup> mice were backcrossed for >12 generations (>99.98% of the percentage of the C57BL/6j host strain) while the Chrna2<sup>2L9S/1L9S</sup> mice were backcrossed for significantly fewer generations (N = 3–4, 75%–87.5% of C57BL/6j host strain), the reader is cautioned to the interpretations made when comparing and contrasting the effects of L9S with those of the null mutation (% host strain attained from Fox and Witham 1997).

**Materials and Methods**

All animal procedures were conducted in accordance with the National Institute of Health Guide for the care and use of laboratory animals and with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Los Angeles and Irvine. Mice used in this study were placed on a 12-h dark–light cycle with ad lib access to food and water. Mice were group housed and tested during the light phase of the light cycle.

**Animals**

Construction and characterization of the Chrna2 null mutant mouse line (backcrossed to C57BL/6j, N17–18 generations, RRID: MGI:3790875) were described earlier (Lotfrpoul et al. 2013). Chrna2<sup>2L9S/1L9S</sup> mice (backcrossed to C57BL/6j, N3–4 generations) were engineered similar to (Taranda et al. 2009), with the exception that (i) an FRT flanked positive selection marker phosphoglycerate kinase gene promoter-neomycin resistance (PGK-NEO) cassette was inserted within an Agel restriction enzyme genomic site, upstream of the fifth exon of the Chrna2 gene (Fig. 1A) and (ii) a gene synthesized (GENWIZ) AatII/Ndel segment of DNA with a modified AGC codon for Serine corresponding to the 9<sup>+</sup> amino acid within the transmembrane region 2 (TM2) (Revah et al. 1991; Labarca et al. 1995; Lester et al. 2003; Tapper et al. 2004; Plazas et al. 2005; Drenan et al. 2008) was inserted within the 5th exon of the Chrna2 gene (Fig. 1; Klaasen et al. 2006). The targeting vector was electroporated into Sv/J129 embryonic stem (ES) cells and homologous recombinants were confirmed through DNA sequencing (Fig. 1B) and Southern blot (Fig. 1C) using a XmnI/Ndel 1007 base pair fragment upstream of exon 1. Genomic DNA from electroporated ES cells were cut with BstZ171/Swal restriction enzymes with a targeted Southern blot fragment of 9500 bp (Fig. 1C). Targeting efficiency of homologous recombination within ES cells was calculated at 43% (31 of 72 ES cells had the targeted fragment). Targeted ES cells were microinjected into C57BL/6j blastocyst embryos at the UCLA Transgenic Core and implanted into pseudopregnant female mice. Germline transmitted agouti colored embryos at the UCLA Transgenic Core and implanted into pseudopregnant female mice. Germline transmitted agouti-colored offspring were DNA sequenced at the UCLA genetic core to confirm that they were carriers of the AGC/CTC heterozygous genomic modification within exon 5 (Fig. 1B). The introduction of the AGC site at the correct location within the fifth exon introduced a SacI restriction enzyme site, which acted as a quick screening strategy for confirmation of the genetic mutation (not shown). After confirming the presence of the mutation and the correct orientation of the FRT flanked PGK-NEO cassette, the mice were mated with congenic FLPer mice (#009086, The Jackson Laboratory) to delete the positive selection marker, leaving a 34-bp FRT finger print in the intronic genomic region. PCR primers (A2L9S_1.10) GGCATGCTAGAAGCTGACACA and (A2L9S_1.11) GAAACAGGAGAATTCCGCAGG (Eurofins MWG Operon LLC) were designed upstream of and downstream from the deleted PGK-NEO cassette (Fig. 1A) to provide PCR-amplified fragments for wild-type and mutant carriers (not shown).

**Oocyte expression studies**

The use of oocyte expression studies (Fig. 3A) was applied as an initial screen to determine the functionality of a substitution of a serine for a leucine (I.9S) in the α2 nAChR subunit. Nicotinic receptor subunits were subcloned into a pSGEM vector, which contains X.lespedeza S-U TR and 3′-U TR segments that markedly increase expression of cloned cDNAs (Liman et al. 1992; Lipovsek et al. 1992).
Hippocampal synaptic plasticity studies

Slice preparation and extracellular field recordings within the dorsal/intermediate CA1 hippocampal slice were performed using the methods described by Nakauchi et al. (2007), Figure 3B–F. Briefly, slices of 375–400 μm were attained from 15 wild-type and Chrna2<sup>L9</sup>/S<sup>L9</sup> adolescent mice (4 wk of age). Animals included in the study were: (i) C57BL/6J wild-type male mice (n = 5), (ii) Chrna2<sup>L9</sup>/S<sup>L9</sup> homozygous S9<sup>S</sup> mice, (iii) Chrna2<sup>L9</sup>/S<sup>L9</sup> wild-type male (n = 3) and female (n = 2) mice, with the reported “n” representing the numbers of experiments in our study. Slices from these animals were maintained in artificial cerebral spinal fluid at 30°C, containing (mM): NaCl, 124; KCl, 2.5; NaH2PO4, 25; MgSO4, 2; CaCl2, 2; NaHCO3, 22; and glucose, 10, and oxygenated with 95% O2 and 5% CO2. A stimulating electrode activated the glutamatergic stratum radiatum schaffer collateral projections arriving from the CA3 hippocampus. Field excitatory postsynaptic potentials (fEPSPs) were recorded within the stratum radiatum using a glass electrode filled with 2 M NaCl (3–8 MΩ). We monitored LTP induction at the SC pathway using a subthreshold stimulation protocol (weak 0.5 burst stimulation (TBS); two 0.5 bursts of four pulses at 100 Hz), which induces LTP in the presence, but not absence, of the activation of α2*-nAChRs-expressing O/A interneurons (Nakauchi et al. 2007). To evaluate the magnitudes of LTP, the mean value for the slopes of fEPSPs recorded 50–60 min after weak TBS was calculated and concentration–response curves to ACh were performed under two-electrode voltage clamp, as described in Lipovsek et al. (2008).

Nicotine studies in adolescent mice using the preexposure-dependent contextual fear conditioning procedure

Male adolescent (4 wk of age) Chrna<sup>KO</sup>, Chrna2<sup>L9</sup>/S<sup>L9</sup> or wild-type littermate control mice of each respective line were used in the current studies. Importantly, Chrna2<sup>L9</sup>/S<sup>L9</sup> mice used for the Chrna2<sup>L9</sup>/S<sup>L9</sup> studies were littersates of the Chrna2<sup>L9</sup>/L9<sup>L9</sup> mice. Similarly, Chrna<sup>KO</sup> mice used for the Chrna<sup>KO</sup> studies were littersates of the Chrna<sup>KO</sup> mice. Three days before day 1 of preexposure (Fig. 4A, PE), animals were handled and habituated to their transport cage for 5 min for 2 d, similar to Kenney and Gould (2008) and Kutlu et al. (2016). On the third day, animals were briefly weighed, exposed to their transport cage, and experiments began on the next day. On day 1 of preexposure, mice were transferred to the behavioral testing suite and allowed to acclimate to the new environment for at least 1 h prior to experimentation. Subsequently, on day 1 of preexposure, using random assignment, mice were placed into transport cages, administered either a pretraining injection of nicotine or vehicle (0.09 mg/kg, i.p., base) 3–5 min prior to being preexposed to a novel conditioning chamber for 10 min. Chamber dimensions were four identical 30 cm × 24 cm × 21 cm (Med Associates, Inc.) boxes fitted with grid floors and a digital video camera (Fig. 4A, PE). On day 2 (Immediate-Shock, IS, Fig. 4A), mice were placed in the same chamber and within 10 sec of entry given a single electric shock (0.75 mA, 2 sec) and assessed for shock reactivity and freezing behavior post-shock (30 sec). On day 3 (Context Test, CT, Fig. 4A), mice were administered a pretesting injection of vehicle or nicotine (0.09 mg/kg, i.p.), and 3–5 min later assessed for freezing behavior in the previously shocked environment for 8 min. The nicotine dose and dual injection approach was based on the study of Kenney and Gould (2008) and the time period for PE and CT was based on the study of Cushman et al. (2011). The subthreshold dose of nicotine was previously shown to enhance learning and memory in adult wild-type mice (Kenney and Gould 2008), therefore this dose was chosen for the current studies. The time periods for these two phases are based on the fact that different processes are engaged. During the preexposure period the animals are forming a new hippocampus-dependent contextual representation of the conditioning chamber whereas on day 3 they are retrieving this representation and expressing fear based on the strength of the context–shock association. Ten minutes for the preexposure was based on prior piloting to determine the necessary length of time for the contextual representation to be formed. 8 min for the context test is based on the most commonly used length duration during test day (Kim and Fanselow 1992; Bissiere et al. 2011). For consistency in our analyses between day 1 and 3, a 2 min period is reported in our results, even though the findings remained consistent if the 10-min period was reported instead. Freezing and shock reactivity were monitored through near infrared lighting and a digital video camera recording at 30 frames per second placed in front of the chambers, and scored through a Med Associates fear conditioning software package (Med Associates Video Freeze system). This system calculates the average motion in the video as activity units, which are determined based on the amount of gray scale pixel change in the image for each frame normalized by background noise when no mouse is present. Freezing was defined as activity below 19 activity units for 1 sec (30 frames). Shock reactivity on day 2 was used as a measure of sensory pain response in the presence of a shock, as determined by the activity units measured during the 2 sec of the 0.75-mA shock and the following 2 sec. Animals which have greater shock reactivity, likely have enhanced pain sensitivity to the shock (King et al. 1996). Freezing varies positively with shock intensity and pain sensitivity, so increased pain sensitivity should increase freezing (Fanselow and LeDoux 1979).

To illustrate conditioning (i.e., a significant increase in freezing on context test after shock training), freezing data were graphed together as PE (day 1) and CT (dy 3). The data are visualized using the same time scale on PE (day 1) and CT (day 3) of 8 min. We separated PE/CT and IS (day 2) data results primarily because of the different time scales used in the analyses. The gain for PE and CT versus 30 sec for IS (Figs. 4, 7) in order to reduce confusion about the different time scales used. The results remained consistent if data were illustrated by individual day of experimentation instead.

Growth, exploratory, stereotypical, and anxiety behaviors

Animal weight was measured on the day before preexposure day 1 and on day 2 of immediate-shock. Manually scored exploratory (vertical-rearing and horizontal-midline crosses), anxiety (center and perimeter time), and stereotypical mouse behavior were quantified in the conditioning chamber, by a blind observer during the 10-min preexposure period on day 1. Manually scored exploratory behavior was scored as a dichotomous score of either 0 for the absence of automated locomotor activity, as previous work had demonstrated that rearing and crossings did not individually resolve automated locomotor activity (Cushman et al. 2011). Stereotypical behaviors included natural mouse behavior observed in a novel environment, including grooming, scratching, chewing, head nodding, paw tremors, head shakes, jumping and backing behavior (Lotfipour et al. 2013). Center time was quantified by manually recording the seconds spent in the center versus parameter of the
apparatus for individual animals. An increase in center time is regarded as reduced anxiety. A mouse was defined as “in center” when its entire body was a tail length away from the nearest wall. Weight gain was used, in part, to calculate the injection volume. Weight gain and stereotypical behaviors were used to determine any growth or development modifications in adolescent 

\[ \text{Chrna2}^{S/L} \] mice. We assessed automated locomotor activity, rearing, mid-line crosses, center time, and shock reactivity, to determine genotype and drug-induced changes in exploratory activity, anxiety and pain sensitivity (as a measure of sensory response).

**Statistics**

Descriptive data were analyzed using the JMP Pro statistical software (SAS Institute Inc., version 12.0.1) and OriginPro 8.1 (OriginLab). Power analyses were determined using JMP Pro and G*Power 3.1. Electrophysiology data were assessed with t-test analysis and Bonferroni-corrected for multiple comparisons for each of the three doses tested. As our primary interests were within dose assessments for the electrophysiology data, across dose comparisons and multiple comparisons correction were not made. Data from wild-type mice from the C57Bl/6 and 

\[ \text{Chrna2}^{S/L} \] line or the male and female data did not differ, thus results were pooled for analysis. Each data point collected was assessed as a separate experiment and not as a within-subject analysis during t-test analysis. For behavioral data, day 1 versus day 3 freezing data as well as days 2 and 3 freezing data were assessed using a matched-pair within subject analysis, followed by Bonferroni-correction for all four comparisons. The association between day 1 activity and day 3 freezing data were assessed as a simple linear correlation using bivariate analysis. For day 1 stereotypical and activity data as well as days 2 and 3 freezing data, significant effects were assessed using analysis of variance (ANOVA). When there were more than two groups compared, Bonferroni-correction for multiple comparisons was applied. Outliers were determined and excluded based on Box-and-Whisker plot outlier analysis, identifying data outside the interquartile range (Tukey 1977). For the 

\[ \text{Chrna2}^{S/L} \] mouse line, a total of four mice were identified as outliers for freezing data (the primary outcome variable) and excluded from all analyses. For the 

\[ \text{Chrna2}^{2KO} \] mouse line one mouse was identified as an outlier for freezing data and excluded from all analyses.

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