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Nicotine improves probabilistic reward learning in wildtype but not alpha7 nAChR null mutants, yet alpha7 nAChR agonists do not improve probabilistic learning.

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Abstract

Cognitive impairments, e.g., reward learning, are present in various psychiatric disorders and warrant treatment. Improving reward-related learning could synergistically enhance psychosocial treatments and cognition generally. A critical first step is to understand the mechanisms underlying reward learning. The dopamine system has been implicated in such learning, but less known is how indirect activation of this system may affect reward learning.We determined the role of alpha7 nicotinic acetylcholine receptors (nAChR) on a probabilistic reversal learning task (PRLT) in mice that includes reward and punishment. Male alpha7 knockout (KO), heterozygous (HT), and wildtype (WT) littermate mice (n=84) were treated with vehicle, 0.03, or 0.3 mg/kg nicotine. Two cohorts of C57BL/6NJ male mice were treated with various alpha7 nAChR ligands, including the full agonists PNU282877 and AR-R-17779, the positive allosteric modulator CCMI, the partial agonist SSR180711, and the antagonist methyllycaconitine. All mice were then tested in the PRLT. Nicotine (0.3 mg/kg) significantly improved initial reward learning in alpha7 WT and HT mice but did not improve learning in KO mice, suggesting an involvement of the alpha7 nAChR in the pro-learning effects of nicotine. Neither alpha7 nAChR treatments (PNU282987, AR-R-17779, CCMI, SSR180711, nor methyllycaconitine) affected mouse PRLT performance however. Nicotine improved reward learning via a mechanism that may include alpha7 nAChRs. This

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Contributors

JWY designed the study and wrote the protocol. KH, AG, DD, and MMP performed the studies and collected the data. JWY and MMP undertook the statistical analysis, and MMP wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of Interest

The remaining authors declare that there is no conflict of interest.

improvement unlikely relied solely on alpha7 nAChRs however, since no alpha7 nAChR ligand improved reward learning in normal mice. Future assessments of the effects of other nAChR subtypes on reward learning are needed.

Keywords

(3-6 from Index Medicus) probabilistic reversal learning; alpha7 nicotinic acetylcholine receptor; agonists; positive allosteric modulators; cognition; schizophrenia

1. INTRODUCTION

Individuals with serious mental disorders have poorer cognition than do healthy people, which negatively impacts their quality of life and also results in high societal costs due to lost earnings/productivity (Bellack et al., 1999). Schizophrenia is an example of such a disorder. Given that cognitive deficits predict psychosocial outcomes in schizophrenia as well as other disorders (Green, 1996, 2006), there has been a major drive to understand the mechanisms underlying these deficits in order to develop targeted treatments (Twamley et al., 2012). Deficient learning is a prime target for such investigations given its contribution to not only everyday life, but also in psychosocial treatments used to improve cognition in serious mental illness (Acheson et al., 2013; Brambilla et al., 2013; Eshel and Roiser, 2010). One important aspect of learning is the feedback-related learning that has been described as reward versus punishment learning. Psychosocial treatment typically involves aiding cognitive processing in psychiatric patients using positive and negative feedback during learning. Importantly, patients suffering from schizophrenia have been shown to have impaired learning from positive feedback (Waltz et al., 2007). Identifying treatments that could enhance reward-associative learning could therefore synergistically enhance neurocognitive treatment and cognitive behavioral therapy outcomes (Acheson et al., 2013; Tamminga, 2006). Understanding the mechanism(s) underlying such behavior is a crucial first step.

The dopamine system has been implicated in the feedback-related learning process (Frank and O'Reilly, 2006; Higa et al., 2017). The 'direct' dopamine D_1 receptor pathway in the basal ganglia has been hypothesized to mediate reward-associated learning, while the 'indirect' dopamine D_2 receptor pathway appears to mediate punishment-associated learning (Danjo et al., 2014; Hikida et al., 2010; Keeler et al., 2014; Kravitz et al., 2012; Nakanishi et al., 2014). Direct activation of the dopamine system could lead to unwanted side-effects (hypotension and dyskinesia), which limits the therapeutic use of dopamine agonists to enhance cognition in patients (Blanchet et al., 1998; Rosell et al., 2015). Identifying mechanisms to indirectly activate this system may circumvent such side effects, however.

Dopamine neurons can be activated indirectly via nicotinic acetylcholine receptor (nAChR) activation, e.g., the prototypical ligand nicotine (Wonnacott et al., 2005). The predominant nAChRs in the brain are the α4β2- and α7-nAChRs (Mansvelder et al., 2006), widely expressed in brain areas important for cognition [e.g. hippocampus, thalamus, etc. (Mamede et al., 2004)]. Nicotine enhances the burst firing of dopamine neurons likely through activation of α7 nAChR located presynaptically on glutamatergic afferent neurons; this

activation results in increased glutamate release onto the dopamine cell body region where it acts on NMDA receptors and stimulates dopamine release (Schilstrom et al., 2003). This released dopamine acts preferentially on dopamine D1 receptors (Wonnacott et al., 2005). Consistent with this observation, mice lacking α7 nAChR expression (knockouts; KO), exhibit impaired reward learning (Young et al, 2011; Young et al, 2004; Keller et al, 2005), but normal aversive/punishment associative learning (Young et al, 2011; Paylor et al, 1998). In addition, activation of α7 nAChRs via the full agonist AR-R-17779 improved within-session learning in the radial-arm maze (Levin et al., 1999). Conversely, blockade of the ventral tegmental area (VTA) nAChR with methyllycaconitine (MLA; a selective α7 nAChR antagonist) selectively blocked the rewarding effects of nicotine and switched the motivational valence from rewarding to aversive (Laviolette and van der Kooy, 2003). The α7 nAChR may therefore be a viable target for improving reward- associative learning.

Impaired reward-associative learning in schizophrenia patients has been identified using probabilistic learning-based tasks (Strauss et al., 2011; Waltz et al., 2007). One example is the probabilistic reversal learning task (PRLT) which measures both reward and punishment learning within the same task and can be conducted in animals (Bari et al., 2010; Young et al., 2015). Testing whether α7 nAChR agonists could improve such learning could be directly applicable to the clinical population. The availability of various α7 nAChR agonists and modulators, including full or partial agonists and positive allosteric modulators (PAMs), offers numerous opportunities to improve reward-associated learning (Lightfoot et al., 2008). Further, the availability of mutant knockouts (KOs) of the α7 nAChR - whom exhibit deficits in reward- but not punishment-related learning (Young et al., 2011) - provides the opportunity to test the selectivity of any α7 nAChR-mediated effects, given that no drug is purely selective to the α7 nAChR. This complementary approach provides greater selectivity than either alone.

The present studies sought to determine whether the non-specific nAChR agonist nicotine could improve PRLT and whether any observed effect would be absent α7 nAChR KO mice. In addition, we tested whether various α7 nAChR agonists would improve learning in C57BL/6 mice.

2. EXPERIMENTAL PROCEDURES

Animals

Male alpha7 KO mice, heterozygous mice (HT), and their wildtype (WT) littermates (n=27, 27, and 30 respectively) were generated via HT breeding pairs and aged 4 months for experiment 1. The strain originated from a mixed 129/SvEv and C57BL/6 background and was backcrossed to C57BL/6. Two groups of male C57BL/6NJ mice (n=60 each) were used for experiments 2 - 5. Mice were purchased from Jackson Laboratories at 3 months old. At start of training, all groups of mice were 3 months old and weighed between 23 - 30 g.

Mice were group housed (max. 4/cage) and maintained in a temperature-controlled vivarium (21±1 °C) on a reversed day-night cycle (lights on at 19:00 h, off at 07:00 h). All mice were food deprived to approximately 85% of their free-feeding body weights during periods of training or testing and tested during the dark phase of the day-night cycle between 08:00 h

and 18:00h. Mice had *ad libitum* access to water. All of the behavioral testing procedures were approved by the UCSD Institutional Animal Care and Use Committee. The UCSD animal facility meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care.

Training and testing

Training and testing took place, during weekdays, in 15 operant chambers that consisted of one wall with five square holepokes arranged horizontally and on the opposite side a wall with a food magazine (25 × 25 × 25 cm; Med Associates, St. Albans, USA). The operant chamber was located in a sound-attenuating box that was ventilated by a fan also providing a low level of background noise. Liquid reinforcement in the form of strawberry milkshake (Nesquik® plus non-fat milk, 30μl) was used and was delivered by peristaltic pump (Lafayette Instruments, Lafayette, IN) to a well located in the food magazine. Holepokes and magazine entries were monitored using infrared beams and control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates Inc., St. Albans, VT) using custom programming.

Training consisted of two phases [consistent with previous reports (Milienne-Petiot et al., 2016)], beginning with an initial training phase (Hab1) during which mice were required to respond to magazine illumination and delivery of liquid reinforcement (~30 (μL strawberry milkshake; Nesquik) as a reward. Reward was delivered every 15 sec for 20 min with collection recorded once per each 15 sec timeframe for a possible count of 80 total responses. Once at 60 responses per session for two consecutive sessions, mice were moved on to the second training phase (Hab2) during which two lit apertures were illuminated after nosepoking in the magazine and mice had to nosepoke in one aperture to obtain the reward. Criterion was set at 70 correct nosepokes per session for two consecutive sessions, with stability of responding over 4 consecutive days in Hab2, prior to testing in the PRLT.

Probabilistic reversal learning task (PRLT)

During the 60 min PRLT, the same two stimulus locations as during Hab2 training were presented, but with altered contingencies [Fig. 1A; (Milienne-Petiot et al., 2016)]. The target hole provided a high probability of reward (80%) and low probability (20%) of ‘punishment’ (house light illumination for 4 sec). Although not strictly a punishment, the illuminated house- light is used to indicate the lack of reward for the selection made - similar to human PRLTs wherein the subject is simply informed they were ‘wrong’ [via lack of correct feedback or a red frowny face; (Cools et al., 2002; Reddy et al., 2016)]. The non-target hole provided a low probability of reward (20%) and high probability of punishment (80%). After 8 consecutive responses at the target hole, the hole-contingencies were reversed. The primary outcome measures of the task were total trials to criterion and number of reversals. Secondary outcome measures included are Dprime (optimal performance, wherein target win-stay is subtracted from target lose-shift), target win-stay behavior (reward sensitivity, specifically the tendency of the mice to reselect the target side following a reward after selecting that side), latency measures (mean response latencies to select target and non-target

stimuli), and % premature responses (% of times responding in a hole prior to target/non-target stimuli presentation).

Drug treatment

All treatments were created using the highest dose as stock solution and diluted to create each lower dose using 0.9% saline (vehicle). Descriptions of each treatment and their mechanisms of action are provided in Table 1. (−)Nicotine hydrogen tartrate (Sigma-Aldrich, St Louis, USA) was administered at doses of 0.03 and 0.3 mg/kg (of free base after pH neutralization using sodium hydroxide) via subcutaneous (s.c.) injections 5 min prior to testing based on previous studies (Hoyle et al., 2006; Young et al., 2004; Young et al., 2013). Methyllycaconitine citrate (MLA) was administered at doses 1.0, 3.0, and 10.0 mg/kg (free base) via intraperitoneal (i.p.) injections at a volume of 10 ml/kg 25 min prior to testing (Chilton et al., 2004; Quarta et al., 2009). PNU282987 hydrate (PNU; Sigma-Aldrich, St Louis, USA) was administered at doses of 1.0,1.7, 3.0, 5.6, 10.0, and 17.0 mg/kg (free base), via i.p. injection 10 min prior to testing in a volume of 10 ml/kg (Redrobe et al., 2009). CCMI (Tocris Bioscience) was administered at doses of 0.1, 0.3, and 1.0 mg/kg. CCMI was administered by i.p. injections 60 min prior to testing in a volume of 10 ml/kg (Nikiforuk et al., 2016; Nikiforuk et al., 2015). SSR180711 hydrochloride (SSR; Tocris Bioscience) was administered at doses of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 mg/kg (free base), via s.c. in a volume of 5 ml/kg 5 min prior to testing (Barak et al., 2009). Finally, AR-R-17779 hydrochloride (AR-R; (Tocris Bioscience) was administered at doses of 0.6, 2.0, and 6.0 mg/kg (free base) and injected via i.p. route 30 min prior to testing and in a volume of 5.0 ml/kg (Levin et al., 1999). When AR-R-17779 was tested in mice, amphetamine (Sigma-Aldrich, St Louis, USA) was used as a positive control and administered at 1.0 mg/kg in a volume of 5.0 ml/kg and a pre-injection time of 5 min based on previous studies (Young et al., 2015). A detailed overview of sample sizes per treatment is provided in Table 2.

Exp. 1 - Effects of nicotine in α7 nAChR KO, HT, & WT mice

Mice (α7 nAChR KO, HT, & WT mice) were matched counterbalanced into three groups based on average total trials during the last two days of Hab2 training after achieving stable performance on Hab2. The mice received saline or nicotine at 0.03 mg/kg or 0.3 mg/kg in a within subjects design. An overview of the study outline is provided in Fig. 1B.

Exp. 2 - Effect of an α7 nAChR antagonist Methyllycaconitine on PRLT in C57BL/6 mice performance

Once responding consistently, C57BL/6 mice were baseline-tested in PRLT. Subsequently, groups were baseline-matched based on total number of reversals (switches), total number of trials, and the number of training days needed to reach criterion in Hab2. Mice received either saline, methyllycaconitine at 1 mg/kg, 3 mg/kg, or 10 mg/kg in a between-subject study design. An overview of the study outline is provided in Fig. 1C.

Exp. 3 - Effects of α7 nAChR full agonists on PRLT performance

Expt. 3A - PNU282987 in C57BL/6 mice—A wash-out period of three weeks was used between testing of methyllycaconitine and drug testing of PNU282987 in the PRLT.

C57BL/6NJ mice were used for this experiment. During the wash-out period, mice were trained twice a week on Hab2 maintenance after which mice were divided into equal groups receiving either vehicle or PNU282987 at 1.0, 1.7, 3.0, 5.6, 10.0, and 17.0 mg/kg in a between-subject study design based on Hab2 trials to criterion.

Exp. 3B - AR-R-17779 or amphetamine in C57BL/6 mice—After a wash-out period including maintenance training on Hab2 (2 training days per week), mice from group 2 (n=60) were divided randomly into equal groups receiving either saline vehicle, various doses of AR-R-17779, or 1 mg/kg of amphetamine in a between-subject design. Groups were baseline-matched based on Hab2 trials to criterion and previous treatment.

Exp. 4 - Effects of a positive allosteric modulator for α7 nAChR CCMI on PRLT performance in C57BL/6 mice

After being tested in the PRLT with PNU282987 treatment and a long wash-out period (6 weeks) with intermittent training on Hab2, group 1 (n=60) of the C57BL/6J mice were counter-balanced into equal groups based on Hab2 performance. Each group received either saline vehicle or various doses of CCMI in a between-subject design.

Exp. 5 - Effects of α7 nAChR partial agonist SSR180711 on PRLT performance in C57BL/6 mice

Finally, following a wash-out period (6 weeks) and Hab2 maintenance training, group 1 was tested again twice in the PRLT while treated with SSR180711. Mice were divided into equal groups and administered three different doses of SSR180711 (3.0, 10, or 30 mg/kg) or vehicle. 14 days after the initial testing with SSR180711, mice were divided once more into groups and treated with lower doses of S SR180711 (0.1, 0.3, and 1.0 mg/kg) and tested in the PRLT as done previously, counter-balanced based on previous treatment. During the second testing session, lower doses of the drug (0.1, 0.3, and 1.0 mg/kg) were used due to the reduction of overall activity seen in mice treated with the higher doses of SSR180711.

Statistical Analyses

We first confirmed that all data were distributed normally and displayed equal variances. When analyzing the data for total trials to criterion, mice were only included if they met criterion. Stable performance during training was assessed using a repeated measure analysis of variance (ANOVA) with days as a within-subject factor. The primary measures for each experiment were analyzed using a one- or two-way ANOVA, with treatment and/or genotype as between-subject variables. Tukey *post hoc* analyses of statistically significant or relevant main and interaction effects were performed where applicable, with Bonferroni corrections conducted for multiple comparisons. All data are reported as mean and standard error of the mean (S.E.M.). The level of probability for statistical significance was set at 0.05. All statistics were performed using SPSS (22.0, Chicago, USA).

3. RESULTS

Exp. 1 - Nicotine improved reward-learning in WT and HT mice but not in mice lacking α7 nAChRs (KO mice)

Nicotine exerted significantly positive effects on learning in the PRLT. Specifically, nicotine improved initial learning as measured by total trials to criterion ($F_{(2,75)}$=4.9, p<0.01; Fig. 2A). Importantly, nicotine and genotype tended to interact ($F_{(4,75)}$=3.0, p=0.076). When analyzed separately, as per our *a priori* hypothesis, nicotine significantly reduced trials to criterion in WT ($F_{(3,36)}$=3.1, p<0.05) and HT ($F_{(3,36)}$=5.3, p<0.005) mice, but no improvement was seen in KO mice ($F_{(3,36)}$=2.1, p=0.13). *Post hoc* analyses revealed that 0.3 mg/kg nicotine reduced total trials to criterion in WT and HT mice, compared with vehicle treatment (p<0.05). When compared across genotype, KO mice treated with vehicle took longer to meet criterion than vehicle-treated WT mice (p<0.05). Nicotine significantly increased target win-stay behavior ($F_{(2,75)}$=6.5, p<0.05; Fig. 2B), but no interactions with genotype, with increased target win-stay in mice treated with 0.3 mg/kg nicotine compared with vehicle (p<0.05). Nicotine slowed reward latency ($F_{(2,75)}$=21.4, p<0.001; Fig. 2C), at 0.3 mg/kg compared with vehicle (p<0.05) with no effect on any other measure including Dprime ($F_{(2,75)}$=2.1, p=0.13; Fig. 2D). Nicotine also improved reversal learning by increasing the number of reversals made by the mice ($F_{(2,75)}$=6.0, p<0.01; Fig. 2E), however there was no interaction with genotype (F<1, n.s.) and genotype also had no effect on reversal learning by itself (F<2, n.s.).

No interaction between nicotine and genotype was observed in any other measure.

Exp. 2 - Effects of α7 nAChR antagonist (methyllycaconitine).

C57BL/6 mice were tested in the PRLT and treated with three different doses of methyllycaconitine or vehicle. There was no main effect of treatment on total trials to criterion (F<1, ns; Fig. 3B) or on number of reversals (F<1, ns; Fig. 3A). There was no effect of methyllycaconitine treatment on strategy measure Dprime (F<1, ns; Table 3) or any of the other measures.

Exp. 3 - Effects of full selective α7 nAChR agonists (PNU282987 & AR-R-17779).

Several doses of PNU282987 were administered to C57BL/6 mice when performing the PRLT. There was no main effect of PNU treatment on total trials to criterion (F<1, ns; Fig. 4A), or reversals performed by the mice (F<1, ns; Fig. 5A). There was no main effect of treatment on the strategy measures combined in Dprime (F<1, ns; Table 3) or any other measure.One group of mice was treated with AR-R-17779 at different doses and there was no main effect of treatment on total trials to criterion (F<1, ns; Fig. 4B), or number of reversals (F<1, ns; Fig. 5B). The positive control treatment given to the same group of mice revealed no main effect on total trials to criterion (F<1, ns; Fig. 4B), but amphetamine did significantly increased number of reversals ($F_{(1,18)}$=4.6, p<0.05; Fig. 5B). Finally, there was no main effect of AR-R-17779 or amphetamine treatment on strategy measures (Dprime $F_{(4,49)}$=1.05, p=0.39; Table 3) or any other measure.

Exp. 4 - Effects of positive allosteric modulator of α7 nAChR (CCMI).

One group of C57BL/6 mice was treated with increasing doses of CCMI and tested in the PRLT. There was no main effect of treatment on total trials to criterion ($F<1$, ns; Fig. 4C), or number of reversals ($F<1$, ns; Fig. 5C). There was also no main effect of treatment on strategy measures (Dprime $F<1$, ns; Table 3) or any other measure.

Exp. 5 - Effects of partial agonist of α7 nAChR (SSR180711).

One group of C57BL/6 mice was treated twice with SSR180711 at different doses (3.0, 10.0, or 30.0 mg/kg) and tested in the PRLT. When treated with SSR180711 at there was no main effect of treatment on total trials to criterion ($F<1$, ns; Fig. 4D), and no main effect of treatment on number of reversals ($F=1.0$, ns; Fig. 5D). There was no main effect of treatment on strategy measures (Dprime ($F_{(3,33)}=1.6$, $p=0.22$; Table 3). When mice were treated with SSR180711 at lower doses (0.1, 0.3, or 1.0 mg/kg), there was still no main effect of treatment on total trials to criterion ($F<1$, ns; Fig. 4D), number of reversals ($F<1$, ns; Fig. 5D), or strategy measures (Dprime ($F_{(3\ 52)}=1.06$, $p=0.37$; Table 3).

4. DISCUSSION

Nicotine significantly improved learning in α7 WT (and HT) mice on a C57BL/6J background.

In contrast, nicotine did not improve learning in mice lacking α7 nAChRs, suggesting that the nicotine-induced improvement in reinforcement learning was, at least partially, mediated by α7 nAChRs. Surprisingly, acute antagonism of the α7 nAChR with methyllycaconitine did not deleteriously affect performance of C57BL/6 mice on the PRLT. Similarly, none of the α7 nAChR agonists improved initial or reversal learning in C57BL/6 mice, while importantly, our positive control, amphetamine, significantly improved reversal learning. These data confirm a positive role of nicotine on reward learning in mice, an effect not replicated by any α7 nAChR agonist tested. α7 nAChRs may contribute to nicotine-induced improvement in reward learning, but additional mechanisms likely play a primary role in such improvement.

4.1. Nicotine, an α4β2 and α7 agonist, improved probabilistic reversal learning in mice.

Nicotine improved initial and reversal reinforcement learning in mice, consistent with previous reports (D'Souza and Markou, 2012; Heishman et al., 2010; Levin et al., 1998; Poltavski and Petros, 2006). Given that schizophrenia patients exhibit impairments in such learning, this finding may explain why so many people with schizophrenia smoke (Kumari and Postma, 2005; Mackowick et al., 2014). Nicotine (0.3 mg/kg) significantly enhanced reward learning in both α7 WT and HT mice in the PRLT as measured by reduced trials to reach criterion. This improvement was likely a result of enhanced reward-associated learning since nicotine increased target win-stay. Nicotine at high doses can bind to α7 nAChRs and likely activates dopamine D_1 receptors (Hamada et al., 2004; Livingstone and Wonnacott, 2009), driving this increased reward-associated learning as reducing dopamine D1 receptor expression impairs reward learning (Higa et al., 2017). Hence, the mechanism of nicotine-

induced improvement in reward-related learning may be driven by α7 nAChR mediated activation of these receptors.

In support of this premise, we observed that mice lacking α7 nAChRs (KO mice) did not exhibit improved learning following nicotine treatment. Although nicotine reduced the trials to reach criterion in these mice, this effect was not statistically different from vehicle treatment at any dose of nicotine tested. Why nicotine remained as efficacious in HT mice, expressing only 50% nAChRs relative to WT, indicates that only partial expression is required for nicotine to improve performance. Although the initial learning deficits of α7 nAChR KO mice could underlie the lack of nicotine-induced improvement in these mice - possibly due to compensatory mechanisms - the genotype-dependent slowing of initial learning [WT<HT<KO mice as seen in other tasks (Young et al., 2007; Young et al., 2011)], and nicotine-induced improvement in HT mice, supports a potential a7 nAChR mediation. Hence, α7 nAChRs could mediate the reward learning improvement seen with nicotine treatment. Treatment with α7 nAChR agonists improved learning in mice and rats that were pharmacologically impaired (McLean et al., 2011; Redrobe et al., 2009), but to date it remains unclear whether α7 nAChR agonists could improve learning in normal mice similarly to nicotine treatment.

4.2. Effects of α7 nAChR agonists and modulators on probabilistic reversal learning in C57BL/6 mice.

In contrast to our main hypothesis, α7 nAChR agonists did not affect PRLT in C57BL/6 mice. We first tested the orthosteric selective full agonists, PNU282987 and AR-R-17779, in the PRLT following reports of PNU282987 reversal of MK-801-induced deficits in attentional set-shifting (Jones et al., 2014) and AR-R-17779 improving social recognition memory in rats (Van Kampen et al., 2004). PNU282987 also improved memory in rhesus monkeys, although the same doses disrupted reversal learning (Gould et al., 2014). Neither drug improved initial or reversal learning of mice in the PRLT. The indirect dopamine agonist amphetamine was used as a positive control and significantly improved performance in the PRLT, indicating that performance could have been improved and hence, full selective α7 nAChR agonists did not improve performance. Unlike nicotine therefore, it is possible that chronic administration may be required to observe pro-learning effects of selective α7 nAChR agonists. For example, while acute administration of the selective α7 nAChR agonist, A-582941, led to the rapid up-regulation of α7 nAChR in the medial prefrontal cortex and ventrolateral orbitofrontal cortex while repeated administration of the compound over seven days resulted in additional up-regulation in the parietal cortex and hippocampus (Christensen et al., 2010). Alternatively however, full selective α7 nAChR agonists both activate and desensitize receptors as the α7 nAChR becomes permeable to calcium but has a low probability of opening and rapidly desensitize *in vitro* (Williams et al., 2011). This dual action may explain why neither PNU282987 nor AR-R-17779 improved PRLT performance in this study as they may have induced desensitization of the α7 nAChRs preventing downstream signaling that would improve reward learning.

Partial agonists for the α7 nAChRs do not induce the same long-term desensitization of these receptors. Therefore, the partial α7 nAChR agonist, SSR180711, was also tested in the

PRLT, given that it attenuated phencyclidine-induced deficits of mice in the novel object recognition task (Hashimoto et al., 2008). In the present study, SSR180711 did not improve initial or reversal learning in the PRLT in mice however. It is possible that subchronic administration of SSR180711 would be required to improve cognition (Hashimoto et al., 2008), and/or that improvements would only be observed after phencyclidine-induced deficits. While these studies would be useful, importantly SSR180711 did not improve reward-learning in mice unlike acute nicotine treatment.

Finally, positive allosteric modulators (PAMs) bypass the limitations of full selective and partial α7 nAChR agonist as the type I PAM increases the probability of the α7 nAChR channel opening. CCMI, a type I PAM, was then tested in the PRLT at doses comparable to those reported as having a pro-cognitive effect in impaired rats (Nikiforuk et al., 2015), wherein methyllycaconitine blocked CCMI effects. Currently however, none of the doses of CCMI tested resulted in a better initial or reversal learning of mice in the PRLT compared to vehicle- treated mice. PAMs do not directly activate α7 nAChR but facilitate activation of those receptors by endogenous α7 nAChR ligands such as acetylcholine. Naïve C57BL/6J were utilized; limiting the likelihood that they would exhibit altered acetylcholine homeostasis. It therefore remains unclear as to why CCMI did not improve learning in the PRLT. The lack of efficacy of these compounds relative to acute nicotine-induced improvement remains unclear.

Irrespective of the type of α7 nAChR ligand (antagonist, full agonist, partial agonist, PAM), none significantly improved initial or reversal learning. The contrast of positive reports of α7 nAChR agents on cognition and the current studies could result from differing cognitive domains being measured, or that models of impaired cognition were used (McLean et al., 2011; McLean et al., 2012; Pichat et al., 2007; Redrobe et al., 2009). Nicotinic stimulation on cognitive performance has been proposed to follow an inverted U-shape pattern where similar levels of nicotinic stimulation can result in opposite effects and where an optimal level of performance can no longer be further improved (Knott et al., 2015). Pro-cognitive effects for α7 nAChR ligands could work similarly and explain why impairments would first be needed. Learning in the PRLT is likely already at an optimal level in C57BL/6 mice treated with vehicle and therefore any further activation of α7 nAChR would not result in improved learning. In contrast with this hypothesis however, we observed that nicotine improved learning in the PRLT in α7 nAChR WT and HT mice, hence improvements could be observed in normal performance. Moreover, mice lacking α7 nAChRs did not exhibit the same improvement. The mechanism(s) underlying these observations remain unclear.

4.3. Pro-cognitive effects of nicotine may not only be mediated by the α7 nAChRs.

Nicotine acts on several different types of nAChR including the α4β2 and α7 nAChRs (the most documented), and α5, α4α6β2β4, or α7β2 nAChRs (Jackson et al., 2010) with varying degrees of sensitivity (Barik and Wonnacott, 2009). It is possible therefore, that nicotine exerted its procognitive effects by acting on other or several nAChRs simultaneously. Nicotine can switch dopamine neurons from a resting to an excited state by activating the β2* nAChR. Further activation of α7 nAChR by nicotine fine-tunes the excited state of dopamine neurons to act specifically on dopamine D_1 receptors (Mameli-Engvall et al.,

2006; Wonnacott et al., 2005). This tuning could indicate that both β2-containing and β7 nAChRs are required for the subsequent activation of dopamine neurons linked with reward learning. Hence, nicotine (at 0.3 mg/kg) exerted some, but not significant, positive effects in α7 nAChR KO mice and a lower dose of nicotine (0.03 mg/kg) did not improve performance in WT or HT mice.

Alternatively, methyllycaconitine-sensitive currents in dopaminergic neurons - thought to represent α7 nAChR activation of dopamine receptors - may not all be due to α7 nAChR, since this type of current was still present in α7 nAChR KO mice but absent in β2 nAChR KO mice (Klink et al., 2001; Picciotto et al., 2001). Alternatively, compensatory mechanisms in α7 nAChR KO mice may result in altered dopaminergic neuronal activation following nicotine administration (Yu et al., 2007). α7 nAChR KO mice exhibited impaired learning as seen in the PRLT compared to WT mice, consistent with previous reports (Keller et al., 2005; Levin et al., 2009; Young et al., 2004). Nevertheless, nicotine partially attenuated the learning deficits of α7 nAChR KO mice possibly by activating α4β2 nAChRs. Follow-up studies using other selective nAChR agonists - and their potential interactive effects with α7 nAChR agonists - would prove beneficial.

To conclude, nicotine improved probabilistic reversal learning in α7 nAChR WT (and HT) mice and partially - but not significantly - attenuated the learning deficits of α7 nAChR KO mice. The pro-cognitive properties of nicotine were unlikely to mediated by α7 nAChRs however, as none of the ligands tested significantly improved reward learning in unimpaired mice. It therefore remains to be elucidated by what mechanisms nicotine exerted its pro-cognitive effects in mice.

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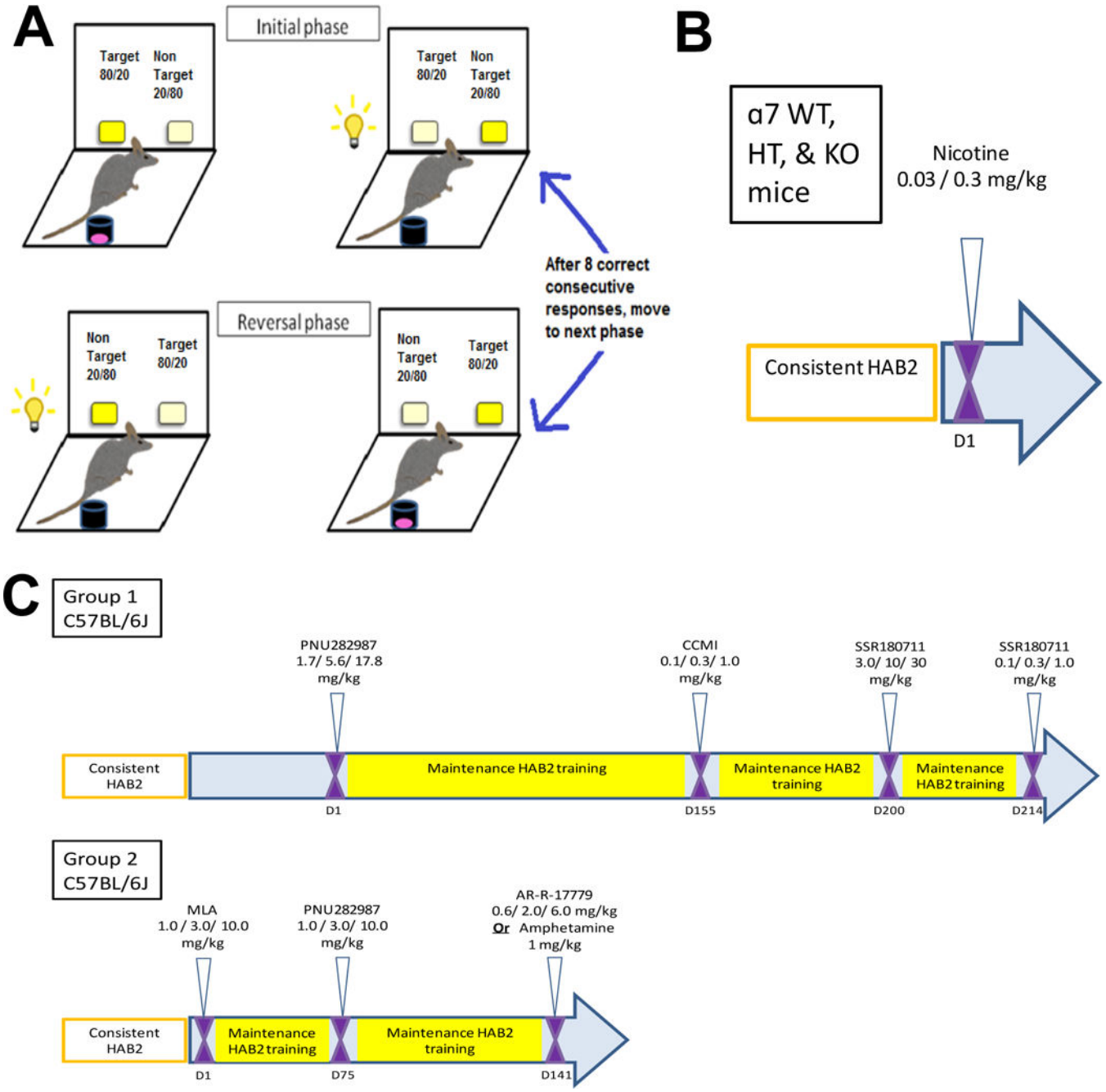

Figure 1. Task schematic for Probabilistic Reversal Learning Task (PRLT) and timeline of testing.

Schematic representation of the probabilistic reversal learning task (PRLT) (**A**). Mice can poke in one of two illuminated holes and will either be rewarded (strawberry milkshake) or punished (time-out period of 4 sec with the house light on). The timeline of the nicotine challenge using α7 WT, HT, and KO mice is also presented (**B**). Additionally, one group of C57BL/6J mice (n=60; Group 1) was tested multiple times on the PRLT after receiving treatment with PNU282987 once, CCMI once, and SSR180711 twice with different doses. A second group of C57BL/6J mice (n=60; Group 2) was tested twice on the PRLT after

receiving treatment with PNU282987 at the same time as group 1, and AR-R-17779 or amphetamine once. Mice were trained on HAB2 in between the indicated testing days to respond to either of the two lit holes for reward (**C**).

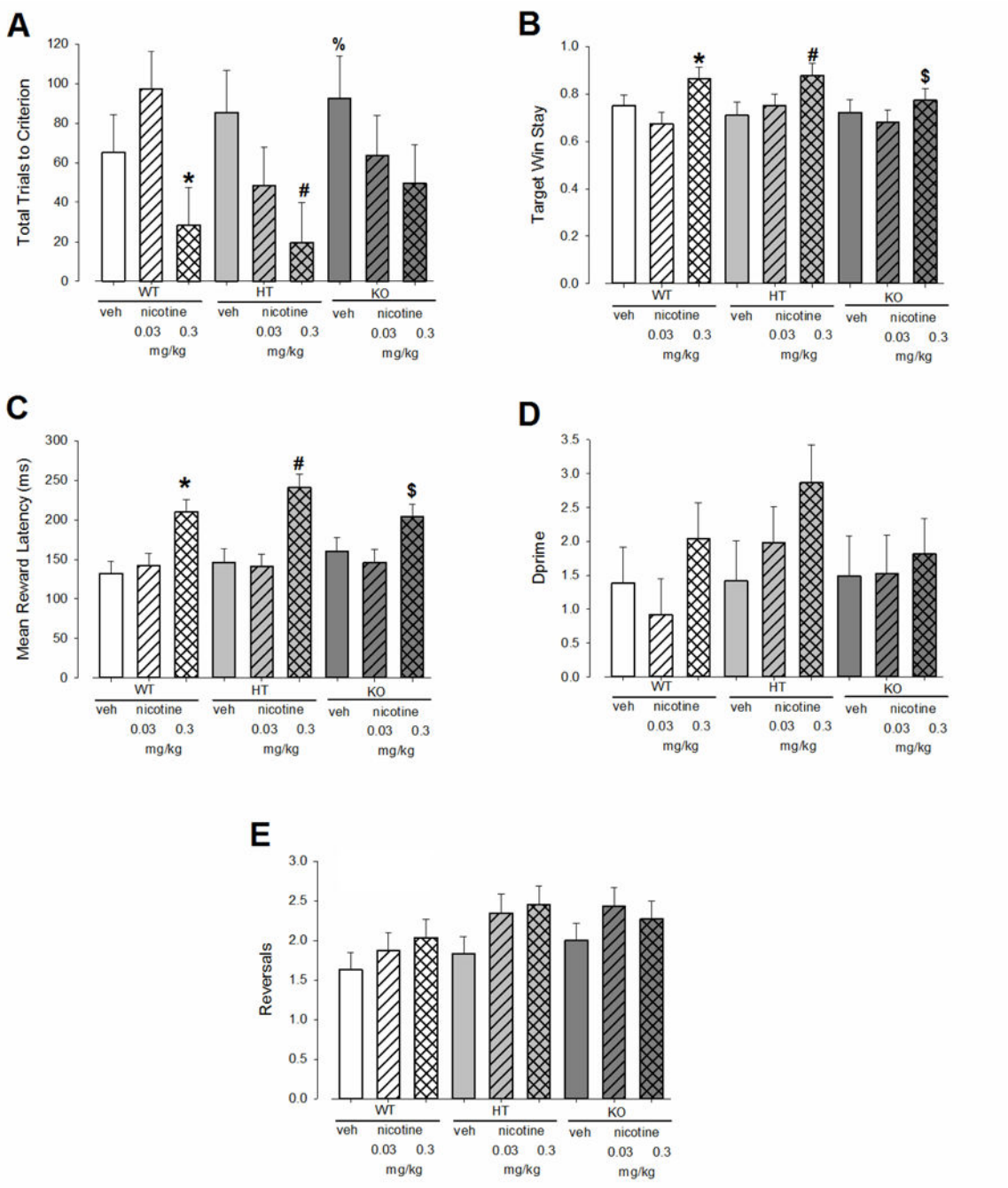

Figure 2. Effects of nicotine in α7 nAChR KO, HT, & WT mice on probabilistic reversal learning.

The number of trials needed to reach initial criterion (initial learning) during a 60 min session is displayed (**A**). Nicotine significantly decreased the total trials to criterion in WT and HT mice at 0.3 mg/kg. This effect was not observed in KO mice. Additionally, the target win stay ratio is displayed here (**B**). The highest dose of nicotine (0.3 mg/kg) significantly increased the target win stay ratio irrespective of genotype. Similarly, 0.3 mg/kg of nicotine significantly increased the latency to collect a reward in all genotypes (**C**). There was no

effect of genotype or nicotine treatment on Dprime (**D**). Nicotine improved reversal learning at both doses irrespective of genotype (**E**). Data presented as mean + S.E.M. *$p<0.05$ c.f. WT mice treated with vehicle (veh); $^{\#}p<0.05$ c.f. HT mice treated with vehicle; $^{\%}p<0.05$ c.f. WT mice; $^{\$}p<0.05$ c.f. KO mice treated with vehicle, $^{!!}$ $p<0.01$ c.f. vehicle treatment at both doses.

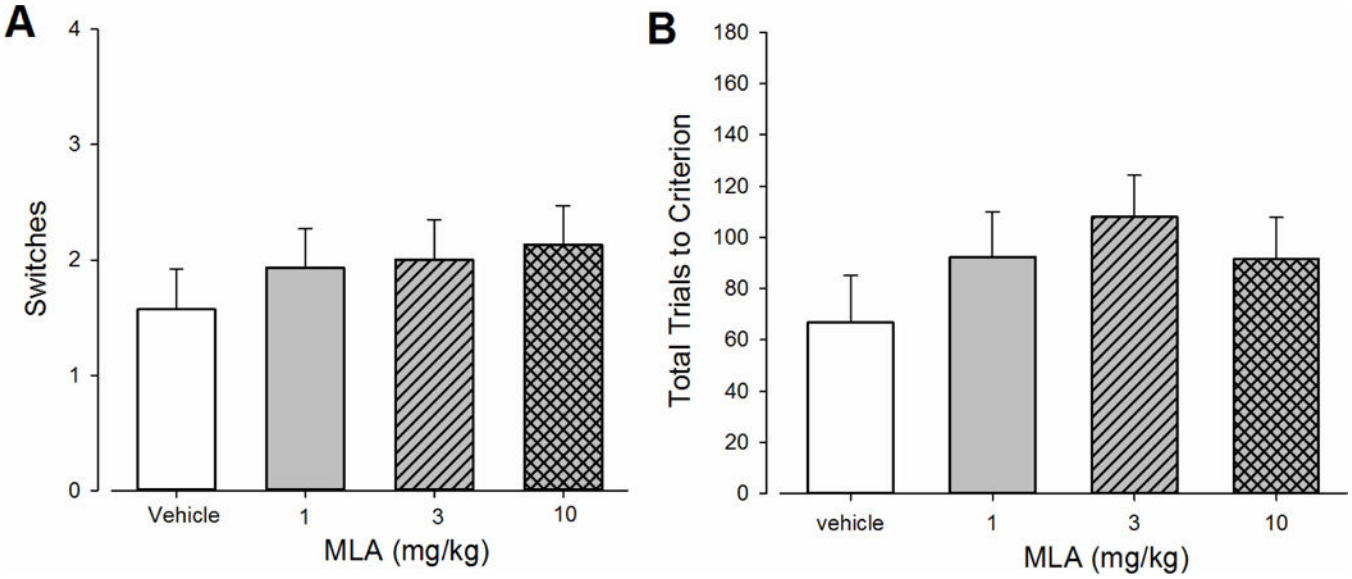

Figure 3. Effects of the α7 nAChR antagonist methyllycaconitine (MLA) on probabilistic reversal learning in C57BL/6 mice.

The number of reversals made by the mice after treatment with increasing doses of methyllycaconitine (MLA; **A**). There was no significant effect of MLA on the number of reversals (reversal learning). There was no significant effect of MLA treatment on initial learning (**B**). Data presented as mean + S.E.M

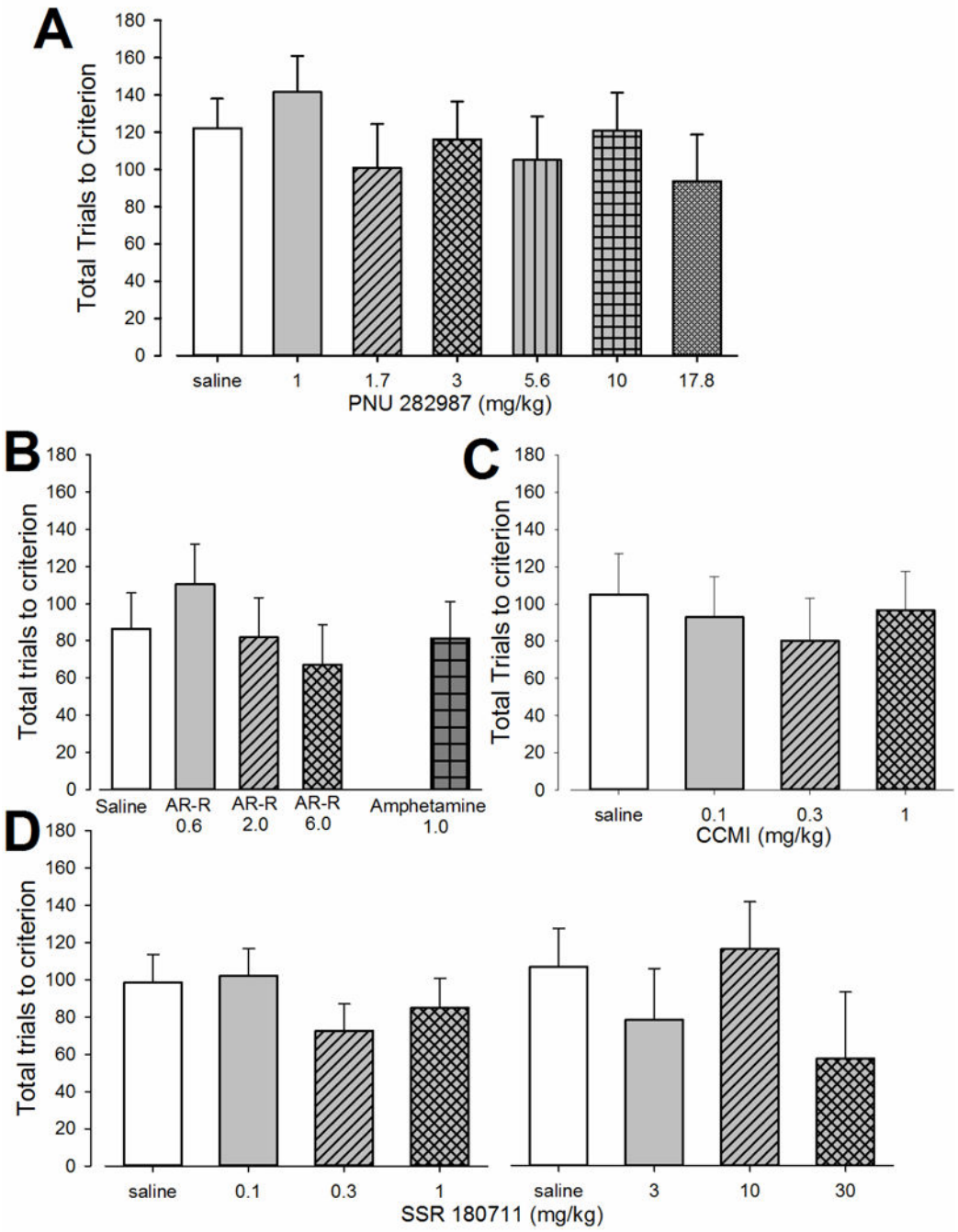

Figure 4. Effects of α7 nAChR agonists or modulators on initial probabilistic reversal learning in C57BL/6 mice.

The selective α7 nAChR agonists PNU282987 (**A**) and AR-R-17779 (**B**) did not significantly affect learning in mice. The positive control amphetamine (**B**) also did not significantly reduce the number of trials needed to reach initial criterion compared to saline treatment. The positive allosteric modulator for α7 nAChR, CCMI (**C**) did not improve initial learning in normal mice. Similarly, the non-selective α7 nAChR agonist, SSR180711

(**D**) did not improve initial learning compared to vehicle treatment. Data presented as mean + S.E.M.

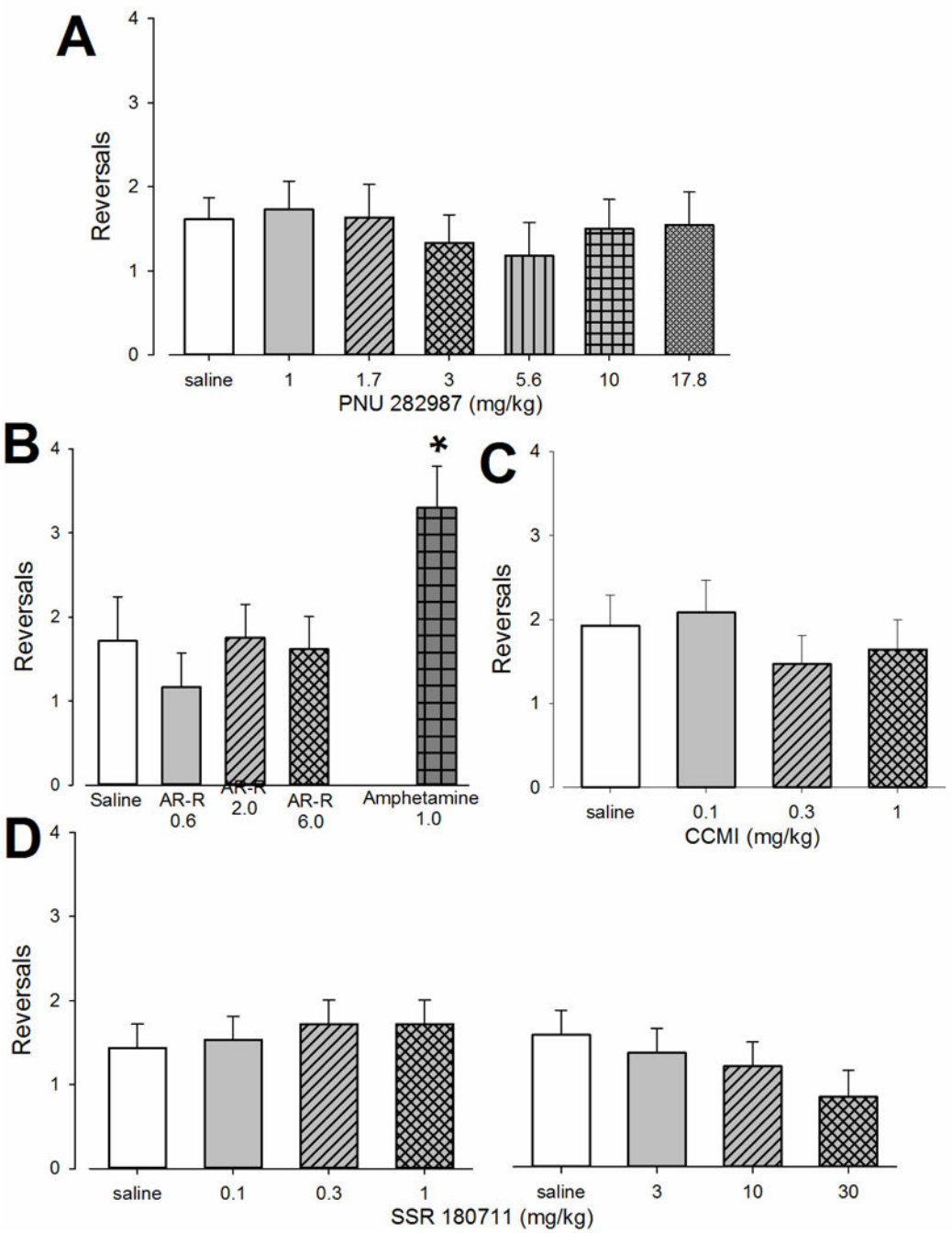

Figure 5. Effects of α7 nAChR agonists or modulators on probabilistic reversal learning in C57BL/6 mice.

The selective α7 nAChR agonists PNU282987 (**A**) and AR-R-17779 (**B**) did not significantly improve reversal learning in mice. The positive control amphetamine significantly increased the number of reversals made by the mice compared to saline treatment (**B**). The positive allosteric modulator for α7 nAChR, CCMI (**C**) did not improve reversal learning in normal mice. Similarly, the non-selective α7 nAChR agonist,

SSR180711 (**D**) did not improve reversal learning compared to vehicle treatment. Data presented as mean + S.E.M. *p=0.05 c.f. vehicle treatment.

Table 1.

Overview of drug used and their mechanism of action.

Drug	Mechanism of action
Nicotine	Agonist for various nAChR in humans with different affinities (α4, α7, β2, α2, α3, α5, α6, α9, α10, β3, β4). (Barik and Wonnacott, 2009)
Methyllycaconitine	Antagonist for α7 nAChR Selective and potent (Ki = 1.4 nM) Antagonist for α4β2 and α6β2 nAChR at concentrations >40 nM (Bioscience)
PNU282987	Agonist for α7 nAChR Selective and potent (in rats; Ki = 26 nM) (Hajos et al., 2005)
AR-R-17779	Agonist for α7 nAChR Selective and potent (Ki = 190 nM) (Bioscience)
CCMI	α7 nAChR selective positive allosteric modulator Increases peak current amplitude response following activation of orthosteric binding site of the receptor by endogenous or exogenous α7 nAChR agonists (Hu et al., 2009)
SSR180711	Partial agonist for α7 nAChR Selective (Ki = 22 nM in rat) (Pichat et al., 2007)

Table 2.

Overview of sample sizes by genotype and/or by treatment for each experiment performed.

Study	Group Sizes						
PRLT - methyllycaconitine (MLA) in C57BL/6 mice	**Vehicle**	**MLA- 1.0 mg/kg**	**MLA- 3.0 mg/kg**	**MLA- 10 mg/kg**			
	11	12	14	14			
PRLT - PNU282987 in C57BL/6 mice	**Vehicle**	**PNU - 1.0 mg/kg**	**PNU - 1.7 mg/kg**	**PNU - 3 mg/kg**	**PNU-5.6 mg/kg**	**PNU - 10 mg/kg**	**PNU 17.8 mg/kg**
	15 + 15	14	15	15	15	15	15
PRLT - AR-R-17779 in C57BL/6 mice	**Vehicle**	**AR-R- 0.6 mg/kg**	**AR-R- 2.0 mg/kg**	**AR-R- 6 mg/kg**	**Amphetamine 1 mg/kg**		
	11	12	12	12	11		
PRLT - CCMI in C57BL/6 mice	**Vehicle**	**CCMI - 0.1 mg/kg**	**CCMI - 0.3 mg/kg**	**CCMI - 1 mg/kg**			
	13	12	15	15			
PRLT - SSR180711 in C57BL/6 mice	**Vehicle**	**SSR - 0.1 mg/kg**	**SSR - 0.3 mg/kg**	**SSR - 1 mg/kg**	**SSR-3 mg/kg**	**SSR - 10 mg/kg**	**SSR - 30 mg/kg**
	14 + 14	15	15	14	14	15	15

PRLT: Probabilistic Reversal Learning Task; WT: wildtype mice; HT: heterozygous mice; KO: knockout mice.

For the PRLT study using alpha 7 KO, HT and WT mice, all mice received all treatment options in a within subject design in a counterbalance manner for order of treatment.

Table 3.

Effects of genotype and/or treatment on Dprime.

Study	Treatment – Dprime Mean (S.E.M)						
PRLT – methyllycaconitine in C57BL/6 mice	**Vehicle**	**MLA – 1.0 mg/kg**	**MLA – 3.0 mg/kg**	**MLA – 10 mg/kg**			
	0.26 (0.11)	0.30 (0.11)	0.38 (0.11)	0.25 (0.11)			
PRLT – PNU282987 in C57BL/6 mice	**Vehicle**	**PNU – 1.0 mg/kg**	**PNU – 1.7 mg/kg**	**PNU – 3 mg/kg**	**PNU – 5.6 mg/kg**	**PNU – 10 mg/kg**	**PNU 17.8 mg/kg**
	0.09 (0.08)	0.23 (0.11)	0.05 (0.13)	0.08 (0.11)	0.12 (0.13)	0.28 (0.11)	0.14 (0.13)
PRLT – AR-R-17779 in C57BL/6 mice	**Vehicle**	**AR-R – 0.6 mg/kg**	**AR-R – 2.0 mg/kg**	**AR-R – 6 mg/kg**	**Amphetamine – 1 mg/kg**		
	0.31 (0.22)	0.10 (0.17)	0.05 (0.17)	0.02 (0.16)	0.45 (0.18)		
PRLT – CCMI in C57BL/6 mice	**Vehicle**	**CCMI – 0.1 mg/kg**	**CCMI – 0.3 mg/kg**	**CCMI – 1 mg/kg**			
	0.21 (0.15)	0.30 (0.16)	0.06 (0.14)	0.14 (0.15)			
PRLT – SSR180711 in C57BL/6 mice	**Vehicle**	**SSR – 0.1 mg/kg**	**SSR – 0.3 mg/kg**	**SSR – 1 mg/kg**	**SSR – 3 mg/kg**	**SSR – 10 mg/kg**	**SSR – 30 mg/kg**
	0.20 (0.12) / −0.01 (0.18)	0.23 (0.12)	0.21 (0.12)	0.21 (0.12)	−0.01 (0.18)	−0.21 (0.17)	0.02 (0.19)

There was no effect of any of the drug treatments on Dprime for any of the experiments performed using C57BL/6 mice.