

Multiple Nicotinic Acetylcholine Receptor Subtypes in the Mouse Amygdala Regulate Affective Behaviors and Response to Social Stress

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Electrophysiological and neurochemical studies implicate cholinergic signaling in the basolateral amygdala (BLA) in behaviors related to stress. Both animal studies and human clinical trials suggest that drugs that alter nicotinic acetylcholine receptor (nAChR) activity can affect behaviors related to mood and anxiety. Clinical studies also suggest that abnormalities in cholinergic signaling are associated with major depressive disorder, whereas pre-clinical studies have implicated both $\beta 2$ subunit-containing ($\beta 2^*$) and $\alpha 7$ nAChRs in the effects of nicotine in models of anxiety- and depression-like behaviors. We therefore investigated whether nAChR signaling in the amygdala contributes to stress-mediated behaviors in mice. Local infusion of the non-competitive non-selective nAChR antagonist mecamylamine or viral-mediated downregulation of the $\beta 2$ or $\alpha 7$ nAChR subunit in the amygdala all induced robust anxiolytic- and antidepressant-like effects in several mouse behavioral models. Further, whereas $\alpha 7$ nAChR subunit knockdown was somewhat more effective at decreasing anxiety-like behavior, only $\beta 2$ subunit knockdown decreased resilience to social defeat stress and c-fos immunoreactivity in the BLA. In contrast, $\alpha 7$, but not $\beta 2$, subunit knockdown effectively reversed the effect of increased ACh signaling in a mouse model of depression. These results suggest that signaling through $\beta 2^*$ nAChRs is essential for baseline excitability of the BLA, and a decrease in signaling through $\beta 2$ nAChRs alters anxiety- and depression-like behaviors even in unstressed animals. In contrast, stimulation of $\alpha 7$ nAChRs by acetylcholine may mediate the increased depression-like behaviors observed during the hypercholinergic state observed in depressed individuals.

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INTRODUCTION

Major depressive disorder affects hundreds of millions of individuals worldwide, but its etiology is not fully elucidated because it is highly heterogeneous. Although there are available treatments, there is still a large proportion of patients that do not respond satisfactorily to traditional antidepressant therapies (Khan *et al*, 2012). Many etiological factors could underlie depressive disorders, including alterations in monoaminergic function, but several studies have implicated dysregulation of the cholinergic system in depression in human subjects (Janowsky *et al*, 1972; Risch *et al*, 1981). Recent human SPECT imaging studies show that depressed unipolar and bipolar subjects have increased levels of ACh throughout the brain (Saricicek *et al*, 2012; Hannestad *et al*, 2013). In humans, blocking the degradation of ACh can induce depressive symptoms even in individuals with no history of illness (Janowsky *et al*, 1972; Risch *et al*, 1981), whereas in mice, blocking ACh breakdown in the

hippocampus alone is sufficient to induce depression- and anxiety-like behaviors (Mineur *et al*, 2013). Finally, activity of the medial habenula-IPN pathway (a region rich in $\alpha 3\beta 4$ nAChRs; Gotti *et al*, 2006) is also critical for the control of anxiety induced by nicotine withdrawal (Zhao-Shea *et al*, 2015) and contributes to the aversive properties of self-administered nicotine through nAChRs containing the $\alpha 5$ subunit (Fowler *et al*, 2011).

The cholinergic system has been a recent focus for development of novel antidepressant medications (Drevets *et al*, 2013; Mineur and Picciotto, 2010; Picciotto *et al*, 2012; Saricicek *et al*, 2012). Acetylcholine signals through muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively) and many studies of depression have focused on mAChR signaling (Cannon *et al*, 2011; Riemann *et al*, 1994); however, blockade of either mAChRs or nAChRs can reverse the depression-like effects of increased cholinergic signaling in mice (Mineur *et al*, 2013) and it is likely that multiple receptors can mediate the effects of cholinergic signaling on depression (Drevets *et al*, 2013). The recent failure of a large clinical trial (Vieta *et al*, 2013) suggests that global antagonism of nAChRs will not be a viable strategy for antidepressant development; however, small human (Philip *et al*, 2009), and a number of rodent studies (Mineur *et al*, 2009; Rollema *et al*, 2009) suggest that targeting specific

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Table 1 Experimental Groups and Design

Figure	Manipulation	Treatment	Regimen	Assays performed	Other assays
1	Amygdala infusion	Mecamylamine (1 ng @ 2 µg/ml)	Acute (30 min before test)	Tail suspension	
2, 3	β2 amygdala knockdown α7 amygdala knockdown		Chronic (~5 weeks before test)	Light/dark, tail suspension, forced swim, social defeat	c-fos, locomotion
4	Amygdala knockdown (β2 or α7)	Mecamylamine (1 mg/kg)	Acute (30 min before each test)	Tail suspension, social defeat	Locomotion
5	Amygdala knockdown (β2 or α7)	Physostigmine (0.15 mg/kg)	Acute (60 min before test)	Tail suspension, forced swim	Locomotion

nAChR subtypes may be a potential strategy for treating depression. Numerous studies suggest that nAChRs are important for cellular and behavioral responses to stress, and that modulation of corticolimbic circuits by nAChRs may be particularly critical (Mansvelder *et al*, 2009). Thus, studies identifying the locus for nAChR signaling could be important for unraveling the mechanisms underlying cholinergic control of stress-related behaviors.

Nicotinic antagonists at both β2* and α7 nAChRs can have antidepressant-like effects in mice (Andreasen *et al*, 2012, 2013; Mineur *et al*, 2009; Rollema *et al*, 2009). In addition, nAChR subtypes containing the α5, α2, α3, and β4 subunits have all been implicated in the aversive properties of nicotine, symptoms related to nicotine withdrawal and behaviors related to anxiety and depression (Fowler *et al*, 2011; Frahm *et al*, 2011; Salas *et al*, 2003; 2004; Upton and Lotfipour, 2015). The non-competitive, non-selective antagonist mecamylamine and the partial agonist cytisine are both effective in mouse models of antidepressant efficacy and converge to decrease neuronal activity in the basolateral amygdala (BLA) (Mineur *et al*, 2007), a brain region that is often hyperactivated in depressed patients (Drevets *et al*, 2008). In addition, nicotine can facilitate glutamatergic signaling in the amygdala through activity of α7 nAChRs (Jiang and Role, 2008) and these nAChRs are particularly critical for synaptic plasticity in this brain region (Jiang *et al*, 2013). Although many nAChR subtypes may contribute to anxiety- and depression-like behaviors, based on the critical role of the amygdala in emotional behaviors, the current study investigated whether altering cholinergic signaling through β2* or α7 nAChRs in the amygdala could affect neuronal activity and performance in behaviors related to stress and depression. We also investigated whether limiting the activity of these nAChRs in the amygdala was sufficient to reverse the effects of increasing cholinergic activity in a mouse model of depression to identify a causal link between amygdala cholinergic signaling and stress-induced behaviors.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (10–12-week-old upon arrival) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were maintained in a temperature-controlled vivarium (21 ± 2 °C) under a 12 h light–dark cycle with lights on at 7:00 AM. Unless separated for humane concerns or due

to cannulation for local infusion, animals were housed five per cage. Food and water were available *ad libitum*. Ten to 12 animals were initially assigned to each treatment group. A summary of the different experimental groups and the experimental design is outlined in Table 1. All procedures were approved by the Yale University Animal Care and Use Committee.

Drugs

All drugs were purchased from Sigma-Aldrich. For i.p. injections, mecamylamine was dissolved in phosphate-buffered saline (saline; 1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄, pH = 7.4) at 0.1 mg/ml and physostigmine was dissolved at 0.15 mg/ml. Solutions were injected i.p. at 10 ml/kg of body weight unless otherwise described.

Cannulation and Local Infusion

Mecamylamine was infused into the amygdala at a concentration of 2 µg/ml, through bi-lateral cannulae. Placement was checked by injecting blue dye through the cannula (Figure 1a) and subjects with off-target placements were not analyzed further. Knockdown was verified *in vivo* by equilibrium binding (see Supplementary Materials and Supplementary Figure S1).

AAV-shRNA Infusions into the Amygdala

Details of design, purification, and validation of viral vectors can be found in the Supplementary Materials. Infusion of viral vectors into the amygdala was performed with a stereotaxic frame mounted with 5 µl Hamilton syringes under 2% Isoflurane anesthesia. In total, 0.5 µl per side of a mix of the different AAV-shRNA for each gene was infused into the amygdala with the following coordinates (from Bregma): anterior/posterior = −1.8 mm; lateral = ± 3.4 mm; dorsal/ventral = −5.2 mm. The infusion rate was ~0.1 µl/min and the needle was then left in place for an additional 5 min to allow for diffusion. Control animals received a scrambled shRNA (Scr), ie, a sequence that does not have a target in the mouse genome/transcriptome. Infusion sites were assessed by detection of GFP in brain slices from paraformaldehyde (PFA)-perfused animals (see immunohistochemistry for details). Subjects with off-target cannula placement (0–3 per group) were removed from further analyses.

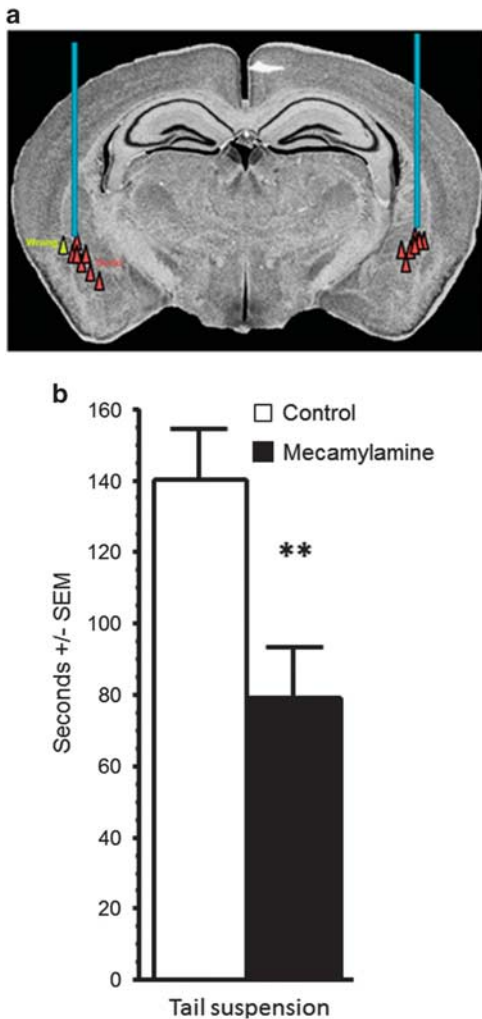


Figure 1 Mecamylamine infusion into the amygdala has an antidepressant-like effect. (a) Placement of all on-target infusions into the amygdala. The yellow triangle represents an example of an off-target infusion. (b) Time spent immobile in the tail suspension test following acute infusion of mecamylamine. Data are expressed as means \pm SEM. ** $p < 0.01$.

Behavioral Testing

Behavioral tests were conducted in sequence, with 24–48 h between each assay, as previously described (Mineur *et al*, 2013) and based on published recommendations. For local infusion experiments, only the tail suspension test was performed as the protruding cannula was incompatible with other behavioral tests. In knockdown experiments combining shRNA infusion with injections of mecamylamine or physostigmine, mice were tested in the tail suspension test and the social defeat paradigm only. This choice was made to avoid repeated exposure to these pharmacological agents that could lead to adaptation. Animals that only received AAV-shRNA infusion were tested in sequence in the light–dark exploration box, the tail suspension test, the forced swim test, the social defeat paradigm, and a final evaluation of locomotor activity. Each group of nAChR knockdown animals (KD) was tested side-by-side with an equivalent control group (Scr) that underwent surgery at the same time

to avoid any between-experiment differences that might bias outcomes. Data are expressed as percent control within each group to normalize the data across experiments.

Light–dark exploration box. A rectangular box was split evenly into two compartments: one covered and the other open and illuminated by a 60 W light. Mice were placed into the farthest point of the light side and the time taken to cross to the dark compartment was recorded. After the first crossing, time in the dark side and the number of crossings was recorded over a 6 min period.

Tail suspension test. Mice were suspended gently by the tail for a period of 6 min, behavior was videotaped, and the number of bouts and time spent immobile were scored manually.

Forced swim test. Each mouse was placed in a 4-l beaker of room temperature water for 15 min. For experiments with physostigmine, a second forced swim test was performed 24 h later for 5 min to limit confounds due to hypothermia. Behavior was videotaped and time spent immobile was scored manually.

Social defeat paradigm. CD1 mice were selected for aggressive behavior as described previously (Mineur *et al*, 2013, 2015). On the initial defeat day, each C57BL/6J mouse was placed into a CD1 aggressor mouse home cage. The time taken until the first fight was recorded and the two mice were then separated using a metal mesh, with the CD1 mouse receiving two thirds of the home cage. After 10 min, the C57BL/6J mouse was returned to its home cage and the metal mesh was removed. This process occurred twice a day for 3 days, with the C57BL/6J mouse exposed to a different CD1 aggressor mouse each day. On the fourth day, social interaction was assessed. Initially, each tested mouse was placed in the open field and allowed to roam freely. The time spent in the area surrounding the holding square was recorded for the first 2.5 min, after which a CD1 mouse was placed inside this square. The time spent in the area around the holding square was then recorded for another 2.5 min. Data are expressed as a ratio of time spent in the area around the holding square with or without the CD1 mouse.

Locomotor activity. Locomotor activity was measured once the battery of tests was completed in a 48 cm \times 22 cm \times 18 cm plastic cage for 30 min. None of the treatments evaluated induced a significant change in locomotion compared with animals from the comparable control group.

C-fos Immunohistochemistry

Following a 15-min forced swim stress, mice were irreversibly anesthetized with pentobarbital (50 mg/kg) and perfused intracardially with 4% PFA in PBS. Brains were post-fixed in PFA overnight, cryoprotected in 30% PBS/sucrose solution and sliced at 40 μ m with a sliding microtome. Immunostaining was conducted on one in six amygdala slices (4–6 slices per animal) for 6–8 animals per treatment group. Tissue was pre-incubated with 0.3%

Triton/1% normal goat serum/PBS (pH = 7.4) for 30 min and was then incubated with the same solution combined to a pre-conjugated anti-c-fos antibody (1:1000; Santa Cruz SC-52) for 48 h at 4 °C. Positively identified cells were counted within a defined area (expressed in pixels) of the BLA, and number of counted cell was then normalized to area. For each brain, both the left and right amygdala was sampled and an average per amygdala was taken of these two values for statistical analysis.

Statistical Analysis

Results of behavioral and c-fos assays were analyzed by ANOVA with 'drug' and/or 'virus' when relevant, as between-factors. *Post hoc* analyses were then performed by *t*-test with Bonferroni corrections. Significance was set at $p < 0.05$.

RESULTS

Amygdala Infusion of the Non-Competitive, Non-Selective Antagonist Mecamylamine has an Antidepressant-Like Effect in the Tail Suspension Test

An increase in cholinergic tone can be observed in depressed patients (Hannestad *et al*, 2013; Saricicek *et al*, 2012). In contrast, treatment with the non-competitive non-selective antagonist mecamylamine can have an antidepressant-like effect in mice, which is correlated with a decrease in amygdala activity (Mineur *et al*, 2007). Thus, we first determined whether decreasing cholinergic signaling through nAChRs in the amygdala with mecamylamine could recapitulate the antidepressant-like effect observed when this drug is administered systemically. Mice infused with mecamylamine showed a significant decrease in immobility in the tail suspension test, suggesting that limiting nicotinic transmission in the amygdala is sufficient to induce an antidepressant-like effect (Figure 1b; $F(1, 17) = 7.97$, $p = 0.01$). Because mecamylamine is a non-competitive non-selective antagonist, we then investigated whether specific nAChR subtypes contribute to this change in behavior.

Downregulation of $\alpha 7$ and $\beta 2^*$ nAChRs in the Amygdala has Anxiolytic- and Antidepressant-Like Effects

To determine the consequences of altering nicotinic signaling through specific nAChR subtypes in tests of antidepressant and anxiolytic efficacy and in response to social stress, AAV-shRNAs targeting either the $\alpha 7$ or the $\beta 2$ nAChR subunit were infused into the amygdala of C57BL6/J mice. In the light–dark box, knockdown of either the $\alpha 7$ or the $\beta 2$ subunit decreased the time spent in the dark compartment, suggesting an anxiolytic-like effect mediated by the blockade of either nAChR subtype, although the effect of $\alpha 7$ knockdown was somewhat more pronounced ($F(1, 19) = 4.26$, $p = 0.04$ and $F(1, 18) = 10.51$, $p = 0.004$, respectively; Figure 2a and b). None of the treatments had a significant effect on the overall number of transitions between chambers ($F_s < 1$); Supplementary Figure S2A and B). In the tail suspension test, shRNAs targeting either the $\alpha 7$ or the $\beta 2$ nAChR subunit decreased the time spent immobile ($F(1, 18) = 6.71$, $p = 0.02$ and $F(1, 19) = 16.03$, $p = 0.001$;

Figure 2c and d), suggesting an antidepressant-like effect; a similar pattern was also observed in the forced swim test in mice following $\beta 2$ subunit knockdown in the amygdala ($F(1, 18) = 5.59$, $p = 0.03$; Figure 2e), but there was only a non-significant trend following $\alpha 7$ knockdown in the amygdala ($F < 1$; Figure 2f). In the social defeat paradigm, $\beta 2$ subunit knockdown resulted in a significant increase in social interaction compared with control mice ($F(1, 21) = 10.33$, $p = 0.004$ Figure 2g), whereas $\alpha 7$ knockdown did not alter the interaction score ($F < 1$; Figure 2h). These results suggest that both $\alpha 7$ and $\beta 2^*$ nAChRs in the amygdala contribute to behavior in tests of anxiolytic and antidepressant efficacy in the absence of stress, but only blockade of $\beta 2$ signaling in the amygdala increases resilience to social stress.

Downregulation of $\beta 2^*$, but not $\alpha 7$, nAChRs in the Amygdala, Significantly Decreases C-Fos Expression

Mice were challenged with 15 min of forced swim and were killed 90 min later. After checking for GFP expression to ensure proper targeting of the BLA (Figure 3a), GFP-positive brain slices were processed for c-fos immunoreactivity, a marker of neuronal activation (Figure 3b). Following $\beta 2$ subunit knockdown in the amygdala, there was a highly significant ~50% decrease in c-fos expression ($F(1, 30) = 13.92$, $p = 0.0008$); Figure 3c), similar to what had been described previously following systemic injection of mecamylamine (Mineur *et al*, 2007). Conversely, knocking down the $\alpha 7$ subunit had little effect on c-fos immunoreactivity ($F < 1$; Figure 3d), suggesting that blockade of $\beta 2^*$ nAChRs in the amygdala was critical for regulation of overall activity of BLA neurons.

Knockdown of the $\alpha 7$ or $\beta 2$ nAChR Subunit in the Amygdala Occludes the Effect of Mecamylamine in the Tail Suspension Test

To determine whether the effects of mecamylamine were mediated through activity at $\alpha 7$ or $\beta 2^*$ nAChRs in the amygdala, and to eliminate the possibility of off-target effects (including, among others, at NMDA receptors (Gonzales *et al*, 1993; O'Dell and Christensen, 1988)), we infused shRNAs targeting each subunit into the amygdala and challenged the animals with mecamylamine or vehicle. Following knockdown of either the $\alpha 7$ or the $\beta 2$ subunit in the amygdala, we observed the expected antidepressant-like effect at baseline (ANOVA: $F(2, 26) = 17.91$, $p < 0.0001$; *post hoc*: Scr vs $\alpha 7$ KD: $p < 0.001$; Scr vs $\beta 2$ KD, $p = 0.0001$; Figure 4a, left). Following mecamylamine injection, control mice displayed an antidepressant-like response in the tail suspension test, and this effect was occluded in mice that received shRNAs targeting the $\alpha 7$ (Figure 4a, middle) or the $\beta 2$ subunit (Figure 4a, right; all $F_s < 1$) in the amygdala. Mice infused with shRNAs targeting the $\alpha 7$ subunit showed no change in social behavior, whereas $\beta 2$ subunit knockdown in the amygdala significantly increased interaction after social defeat (ANOVA: $F(2, 26) = 6.58$, $p = 0.004$; *post hoc*: Scr vs $\alpha 7$ KD: $p = 0.29$; Scr vs $\beta 2$ KD, $p = 0.001$; Figure 4b, left). Unexpectedly, the administration of mecamylamine modestly, but significantly, decreased social interaction in mice following knockdown of the $\beta 2$ nAChR subunit in the

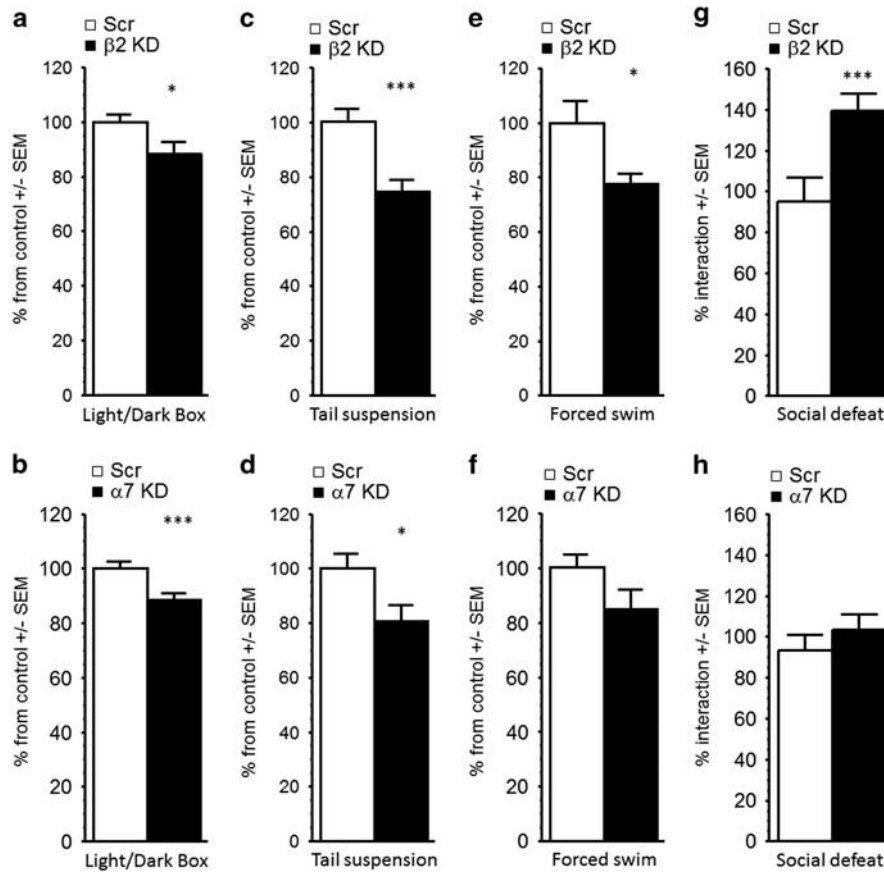


Figure 2 Effects of $\beta 2$ and $\alpha 7$ nAChR subunit knockdown in the amygdala in tests of anxiety- and depression-like phenotypes. Time spent in the dark side of the light/dark box following (a) $\beta 2$ or (b) $\alpha 7$ nAChR subunit knockdown in the amygdala. Time spent immobile in the tail suspension test following (c) $\beta 2$ or (d) $\alpha 7$ nAChR subunit knockdown in the amygdala. Time spent immobile in the forced swim test following (e) $\beta 2$ or (f) $\alpha 7$ nAChR subunit knockdown in the amygdala. Time spent interacting with a CD1 mouse after 4 days of social defeat following (g) $\beta 2$ or (h) $\alpha 7$ nAChR subunit knockdown in the amygdala. Data are expressed as % means \pm SEM normalized to each control group. * $p < 0.05$; *** $p < 0.001$.

amygdala ($F(1, 25) = 4.77$, $p = 0.04$; Figure 4b, middle), whereas there was no effect of mecamylamine following infusion of the Scr or knockdown of the $\alpha 7$ subunit in the amygdala ($F < 1$; Figure 4b, right). Thus, a depressant-like effect of mecamylamine in the social defeat paradigm is unmasked following knockdown of the $\beta 2$ subunit in the amygdala. These data suggest that mecamylamine can promote stress-induced social avoidance through a mechanism independent of $\beta 2^*$ or $\alpha 7$ nAChR signaling in the amygdala.

Systemic, but not Local, Blockade of Acetylcholine Esterase Increases Depression-Like Behavior and this is Partially Reversed by $\alpha 7$ or $\beta 2$ nAChR Knockdown in the Amygdala

Blocking or knocking down nAChRs in the amygdala has antidepressant-like effects, so we then determined whether increasing ACh levels by infusing shRNAs targeting acetylcholinesterase (AChE: the main enzyme degrading ACh) into the amygdala could have the opposite effect in tests of antidepressant efficacy. Contrary to what was observed when AChE was knocked down in the hippocampus (Mineur *et al*, 2013), knockdown in the amygdala had no effect on behavior in the tail suspension test (control vs AChE knockdown: 175 ± 24.75 vs 195 ± 25.24 s, $F < 1$), nor

did it affect c-fos expression (control vs AChE knockdown: 110 ± 34.4 vs 87 ± 37.4 counted cells per section, $F < 1$). We then determined whether knockdown of the $\alpha 7$ or $\beta 2$ nAChR subunit in the amygdala could reverse the effect of systemic antagonism of AChE activity by physostigmine. ANOVAs reveal an overall physostigmine and knockdown effect ($F(1, 63) = 66.38$, $p < 0.0001$ and $F(2, 63) = 22.46$, $p < 0.0001$, respectively), but no interaction ($F < 1$), suggesting that the nAChR knockdown had a similar effect on immobility in the tail suspension test regardless of the degree of AChE activity (Figure 5a). *Post hoc* analyses revealed that infusion of shRNAs targeting the $\alpha 7$ or $\beta 2$ nAChR subunit into the amygdala significantly reduced time spent immobile in the tail suspension test compared with saline treated, scrambled shRNA-infused controls (all $p < 0.001$). A similar effect was observed in the forced swim test (Figure 5b) with an overall physostigmine ($F(1, 61) = 29.65$, $p < 0.0001$) and knockdown ($F(1, 61) = 17.59$, $p < 0.0001$) effect, but no interaction ($F < 1$). *Post hoc* analyses showed that $\beta 2$ and $\alpha 7$ subunit knockdown in the amygdala decreased immobility at baseline in the forced swim test (all $p < 0.0001$) but only marginally decreased immobility following physostigmine administration ($p = 0.022$). It is possible that confounding factors could have affected these results as cholinesterase

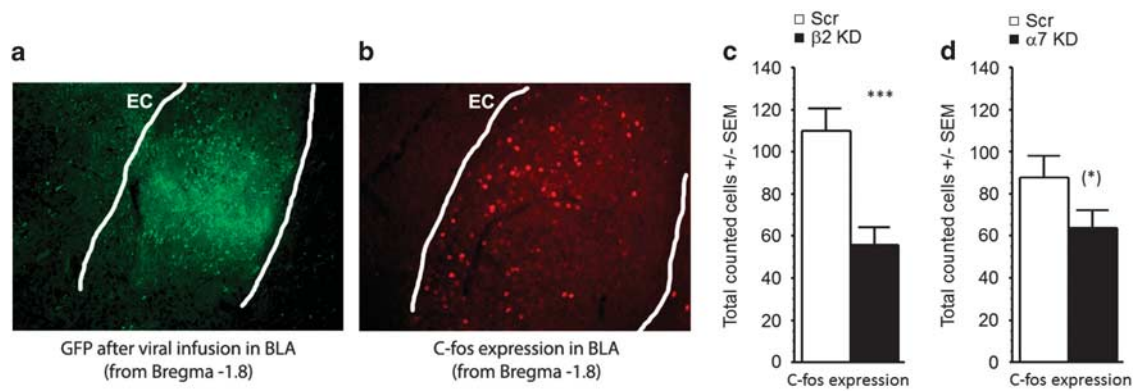


Figure 3 Immunohistochemistry for GFP and c-fos in the amygdala after $\beta 2$ or $\alpha 7$ knockdown. (a) Representative photomicrograph of GFP expression in the BLA following viral infusion. (b) Representative photomicrograph of c-fos immunohistochemistry in the amygdala following viral infusion. EC, external capsule. (c, d) Total number of c-fos immunoreactive cells in the amygdala following (c) $\beta 2$ or (d) $\alpha 7$ nAChR subunit knockdown. Data are expressed as means \pm SEM. * $p < 0.06$; *** $p < 0.001$.

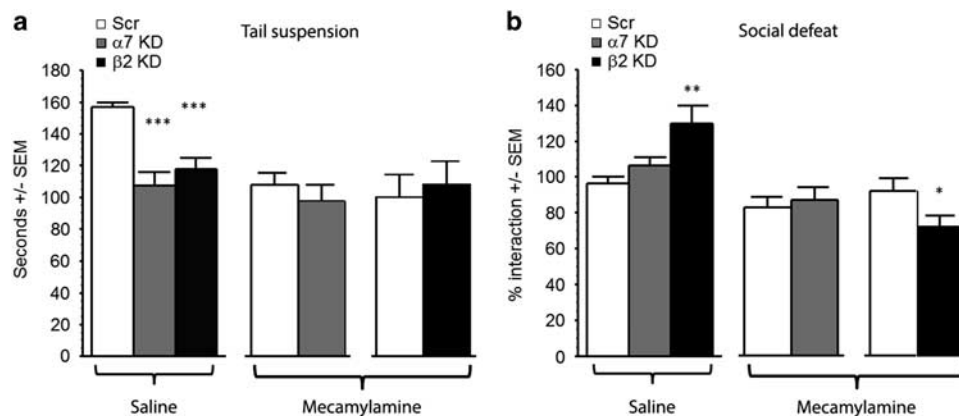


Figure 4 $\alpha 7$ and $\beta 2^*$ nAChRs in the amygdala mediate the antidepressant-like effects of a nicotinic antagonist. Effect of acute mecamylamine injection on (a) the time spent immobile in the tail suspension test and (b) time spent interacting with a CD1 mouse in the social defeat test following $\alpha 7$ or $\beta 2$ nAChR subunit knockdown in the amygdala. Data are expressed as means \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

inhibitors can significantly alter body temperature. Alternatively, *post hoc* corrections for multiple comparisons (where the p cutoff value is 0.017 in this design) could underestimate the effect. These experiments suggest that decreasing signaling through $\alpha 7$ or $\beta 2$ nAChRs in the amygdala results in an antidepressant-like effect that can partially reverse the consequences of increased ACh levels systemically.

DISCUSSION

Depression is a complex, multidimensional disorder, and it is likely that multiple neurotransmitter systems and brain regions contribute to its pathophysiology. This may explain why a significant proportion of depressed individuals do not show complete remission in response to existing antidepressant treatments. Thus, it remains critical to identify the mechanisms underlying the development of mood disorders.

Recent studies have suggested that individuals with uni- or bipolar illness have increased ACh levels throughout the brain when they are acutely depressed (Saricicek *et al*, 2012; Hannestad *et al*, 2013). A number of studies have also

addressed the possibility that nAChRs could be involved in depression, because depressed individuals are twice as likely to smoke, have a harder time quitting, and can develop symptoms of depression during withdrawal (for reviews, see Moylan *et al*, 2012; Picciotto *et al*, 2012). Despite controversy about the connection that may exist between nAChRs and mood regulation, many studies have shown that decreasing ACh signaling through nAChRs can have antidepressant-like properties in mice (Mineur *et al*, 2007, 2009) and can improve symptoms of depression in some clinical studies (George *et al*, 2008). Although both the nicotinic antagonist mecamylamine and the low efficacy partial agonist cytisine can decrease c-fos activity in the amygdala (Mineur *et al*, 2007), it is not known where in the brain nAChRs are required to alter behaviors relevant to stress and depression. Many brain areas that receive significant ACh innervation, including hippocampus, prefrontal cortex, habenula, IPN, ventral tegmental area, and nucleus accumbens/striatum (Leao *et al*, 2015; Mineur *et al*, 2013, 2015; Picciotto and Mineur, 2014; Polter and Kauer, 2014; Zhao-Shea *et al*, 2013, 2015) could be involved in nAChR-related mood regulation. We demonstrate here that the non-selective nAChR antagonist mecamylamine can induce antidepressant-like

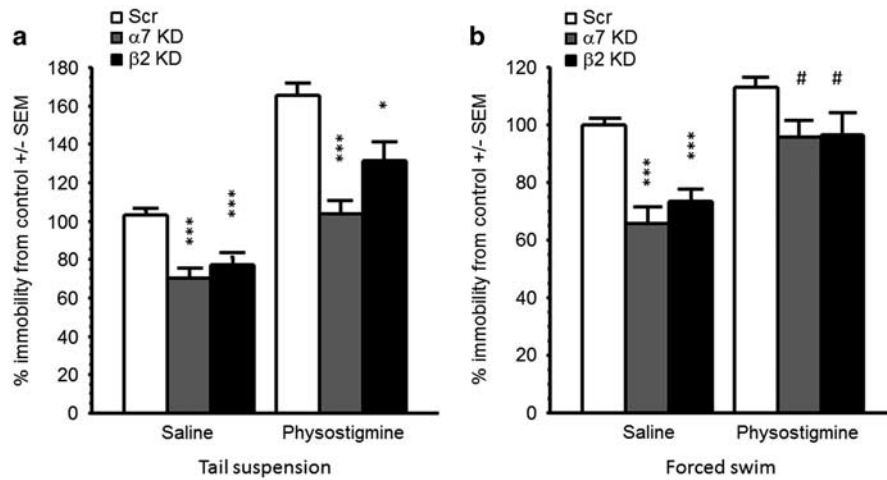


Figure 5 Knockdown of $\alpha 7$ or $\beta 2^*$ nAChRs in the amygdala can oppose the effects of systemic physostigmine injection. (a) Time spent immobile in the tail suspension test. (b) Time spent interacting with a CD1 mouse in the forced swim test. Data are expressed as means \pm SEM. * $p < 0.05$; *** $p < 0.001$; # $p < 0.007$.

effects when infused locally into the amygdala, suggesting that nAChR signaling is critical for baseline neuronal activity in this brain region, and that decreasing nAChR activity in amygdala is sufficient for an antidepressant-like response.

A combination of pharmacological and molecular genetic experiments has suggested that $\beta 2^*$ nAChRs contribute to stress-induced behaviors and may contribute to the effects of antidepressant medications effective in human patients (Caldarone *et al*, 2004; Harrist *et al*, 2004). The effect of $\alpha 7$ nAChRs on behaviors related to depression is more complex, however. Although the antidepressant-like effects of the non-competitive, non-selective antagonist mecamylamine are not observed in knockout mice lacking the $\alpha 7$ subunit (Rabenstein *et al*, 2006), $\alpha 7$ nAChR agonists can potentiate the antidepressant-like effects of a monoamine reuptake inhibitor, with no effects on their own (Andreasen *et al*, 2013). The current studies demonstrate that decreasing expression of either the $\alpha 7$ or $\beta 2$ nAChR subunit in the amygdala mimics the effect of classical antidepressants in behavioral assays in mice, and that the effect of either knockdown is similar to local infusion of mecamylamine into the amygdala. It also suggests that there may be distinct effects of nAChR signaling in the amygdala that could account for specific endophenotypes related to depression as compared other with stress-induced behaviors, as social defeat was only different following $\beta 2$ knockdown. Further, $\alpha 7$ and $\beta 2^*$ nAChRs may be expressed in distinct subcellular compartments, cell types or subregions of the amygdala, resulting in differing transduction of ACh signaling in this brain region. This idea is supported by studies of c-fos immunoreactivity performed here, showing that knockdown of the $\beta 2$ nAChR subunit significantly decreased neuronal activity in the BLA, whereas $\alpha 7$ subunit knockdown had much less effect on c-fos expression.

The results presented here are consistent with the role of $\beta 2^*$ nAChRs described in the VTA, in which blockade decreases the reactivity of dopaminergic neurons to a broad range of stimulation (Koranda *et al*, 2014). Conversely, these studies suggest that the role of $\alpha 7$ nAChR signaling in stress-related behaviors is specific to the endophenotype assessed.

$\alpha 7$ nAChRs are highly expressed in BLA and have a critical role in neuronal excitability (Almeida-Suhett *et al*, 2014; Jiang and Role, 2008; Klein and Yakel, 2006). Because the $\alpha 7$ subunit is expressed in both glutamatergic and GABAergic neurons in the amygdala and can modulate both excitatory and inhibitory neurons (Pidoplichko *et al*, 2013), decreasing the activity of this nAChR subtype could have opposite behavioral consequences depending on whether the nucleus is highly active or quiescent (Pandya and Yakel, 2013). It has been suggested that the primary role of $\alpha 7$ nAChRs in BLA is to enhance inhibitory synaptic transmission (Pidoplichko *et al*, 2013), but several studies have demonstrated that signaling through $\alpha 7$ nAChRs can increase excitatory transmission (Barazangi and Role, 2001; Jiang and Role, 2008; Klein and Yakel, 2006). Along these lines, regulation of excitability of the BLA by $\alpha 7$ nAChRs has been associated with increased anxiety-like behavior in a model of traumatic brain injury (Almeida-Suhett *et al*, 2014).

These observations suggest that the net effect of altering $\alpha 7$ signaling is highly dependent on the physiological status of the amygdala but do not explain why knockdown of AChE in the amygdala has no effect on behavior in the tail suspension test or on c-fos expression. Thus, fluctuations in levels of ACh in amygdala may not be the primary mediator of stress-related changes in behavior. Rather, ACh acting through nAChRs at baseline could be required for normal amygdala function, and decreasing cholinergic activity in this structure could be protective against changes in neurotransmitter systems at other brain sites in response to stress, including changes in $\alpha 7$ -regulated glutamatergic projections (Yang *et al*, 2013). This would be consistent with electrophysiological studies demonstrating that cholinergic neurons of the nucleus basalis, a major source of ACh to the amygdala, have a high firing rate when animals are awake, leading to a high level of baseline cholinergic signaling in the amygdala even in the absence of a stressor (Whalen *et al*, 1994). This is also consistent with the observation that inescapable stress increases extracellular ACh levels in the hippocampus as measured by microdialysis, but has little effect on ACh levels in the amygdala (Mark *et al*, 1996).

Signaling through the $\alpha 7$ and $\beta 2^*$ nAChR subtypes in the amygdala is critical for stress-related behaviors, but there are likely differences in the neurobiological substrates underlying the effects of each nAChR subtype. In a model of depression induced by systemic physostigmine administration, knockdown of the $\beta 2$ subunit was not as effective as $\alpha 7$ subunit knockdown, suggesting that $\alpha 7$ has a permissive role in the amygdala that may amplify the effects of stress-induced glutamate release, similar to what has been reported in other brain regions. In conclusion, dysregulation of nAChR activity in amygdala may alter susceptibility to stress and contribute to maladaptive behaviors.

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The authors declare no conflict of interest.

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