Neuropsychopharmacology (2015) 40, 938–946

www.neuropsychopharmacology.org

Expression of the 5-HT1A Serotonin Receptor in the Hippocampus Is Required for Social Stress Resilience and the Antidepressant-Like Effects Induced by the Nicotinic Partial Agonist Cytisine

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Nicotinic acetylcholine receptor (nAChR) blockers potentiate the effects of selective serotonin reuptake inhibitors (SSRIs) in some treatment-resistant patients; however, it is not known whether these effects are independent, or whether the two neurotransmitter systems act synergistically. We first determined that the SSRI fluoxetine and the nicotinic partial agonist cytisine have synergistic effects in a mouse model of antidepressant efficacy, whereas serotonin depletion blocked the effects of cytisine. Using a pharmacological approach, we found that the 5-HT1A agonist 8-OH-DPAT also potentiated the antidepressant-like effects of cytisine, suggesting that this subtype might mediate the interaction between the serotonergic and cholinergic systems. The 5-HT1A receptors are located both presynaptically and postsynaptically. We therefore knocked down 5-HT1A receptors in either the dorsal raphe (presynaptic autoreceptors) or the hippocampus (a brain area with high expression of 5-HT1A heteroreceptors sensitive to cholinergic effects on affective behaviors). Knockdown of 5-HT1A receptors in hippocampus, but not dorsal raphe, significantly decreased the antidepressant-like effect of cytisine. This study suggests that serotonin signaling through postsynaptic 5-HT1A receptors in the hippocampus is critical for the antidepressant-like effects of a cholinergic drug and begins to elucidate the molecular mechanisms underlying interactions between the serotonergic and cholinergic systems related to mood disorders.
Neuropsychopharmacology (2015) **40,** 938–946; doi:10.1038/npp.2014.269; published online 29 October 2014

INTRODUCTION

Major depressive disorder (MDD) is one of the most common psychiatric illnesses, with a lifetime prevalence of ~15%. The most widely prescribed class of antidepressant medications is the selective serotonin reuptake inhibitors (SSRIs) that increase levels of serotonin at the synapse. Although current antidepressant therapies are effective in ~60% of depressed individuals, remission is only observed in approximately a third of subjects (Han *et al*, 2013; Preston and Shelton, 2013). The incomplete response of patients, combined with side effects of current treatments (Ginsberg, 2009), has led to the hope that investigation of other targets or the development of an augmentation strategy to potentiate the effects of commonly used pharmacotherapies (for a review, see Duric and Duman, 2013) could result in remission for a greater proportion of patients. For example, the NMDA receptor antagonist ketamine induces rapid-onset antidepressant effects, opening new possibilities for alternative treatment (Duman *et al*, 2012). Similarly, targeting the cholinergic system by altering signaling through its muscarinic and nicotinic receptor families (muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR), respectively) has shown some promising results (Drevets *et al*, 2012; Furey and Drevets, 2006; Shytle *et al*, 2002b). Despite the recent failure of one clinical trial of a nicotinic antagonist, several studies have demonstrated that SSRI-resistant patients show improvement of depressive symptoms when a nicotinic antagonist or partial agonist is added (George *et al*, 2008; Philip *et al*, 2009). The idea that limiting cholinergic signaling could be antidepressant is in line with human studies indicating that blocking the breakdown of acetylcholine (ACh), thereby *increasing* ACh levels, can induce symptoms of depression. More recently, human imaging studies have suggested that ACh levels are high throughout the brain in patients who are actively depressed (Saricicek *et al*, 2012; Hannestad *et al*, 2013). Thus, although targeting the monoamine system is effective for treating depression in a significant subset of patients, emerging data suggest that abnormalities in the cholinergic system could be one of

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Received 26 March 2014; revised 30 September 2014; accepted 1 October 2014; accepted article preview online 7 October 2014

the etiological factors underlying the development of depression.

Although the nicotinic antagonist mecamylamine has been effective in relieving the symptoms of some human depressed subjects, full blockade of nAChRs can result in side effects, limiting the tolerability of the drug. Therefore, decreasing ACh signaling through these receptors while preserving some function, as can be achieved by treating with a nicotinic partial agonist such as cytisine or varenicline, may be a more effective strategy for targeting the nicotinic cholinergic system in depressed individuals. The combination of a nicotinic partial agonist with an SSRI antidepressant is therefore one augmentation strategy that could be effective in patients refractory to current antidepressant treatments (Rollema *et al*, 2009); however, the mechanisms underlying the interaction between cholinergic and serotonergic medications are unknown. Most antidepressant drugs can also antagonize nAChRs in the nM μM range (Shytle *et al*, 2002a). It is therefore possible that cholinergic compounds and current antidepressant medications could converge to decrease activity of nAChRs. Alternatively, these drugs could affect serotoninergic and cholinergic pathways independently, or changes in cholinergic signaling could alter serotonin neurotransmission directly. We therefore investigated the nature and site of interaction between the cholinergic and monoaminergic neurotransmitter systems in the regulation of behavior in rodent tests of antidepressant efficacy using a combined pharmacological and molecular genetic approach.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), allowed to acclimate to the animal facility for at least 14 days before testing, and housed under standard laboratory conditions (temperature $21 \pm 2\,^{\circ}C$, 12:12 light/dark cycle, lights on at 0700 h). Food and water were available *ad libitum* throughout the studies. All procedures were approved by the Yale University Animal Care and Use Committee and conformed to the standards of the NIH Guide for the Care and Use of Laboratory Animals.

Drugs

All drugs (8-OH-DPAT, cytisine, *p*-chlorophenylalanine (PCPA)) were purchased from Sigma Aldrich and were diluted in phosphate-buffered saline at 10 mg/ml (pH = 7.4) and injected i.p. Serotonin depletion was performed as described in Lucki and O'Leary (2004) by injecting mice i.p. with PCPA (300 mg/kg) twice daily for 3 days and testing ~18 h after the last treatment.

Design of Small Hairpin RNAs (shRNAs)

Two shRNAs directed against the htr1A mRNA encoding the 5-HT1A receptor were constructed by selecting unique 24 base sequences (5′ to 3′: **1.** GCCAGTTGGACAGCGAC AAAGTGA; **2.** GCTAGACAGGTACTGGGCAATCAC; GenBank accession NM_008308.4) as previously described (Mineur *et al*, 2011). As a control, a random sequence of 24 bases without similarities to any known transcripts in mice was selected as a scrambled shRNA. Synthetic oligonucleotide duplexes were designed by adding antisense sequences directed against the selected mRNA region, followed by a miR23 loop of 10 nucleotides (CTTCC TGTCA) to the 5′ end of the sequences above, and by adding overhanging ends identical to those created by *Sap*I and *Xba*I restriction enzyme digestion. Annealed oligonucleotides were ligated into a pAAV-EGFP-shRNA vector as described previously (Benavides *et al*, 2007; Hommel *et al*, 2003; Mineur *et al*, 2011) and positive clones were confirmed by sequencing.

Viral Production

Viruses were produced by transfecting HEK 293 cells with 135 μg each of pAAV2-shRNA, pHelper, and pAAV-RC plasmids using calcium phosphate (Hommel *et al*, 2003; Zolotukhin *et al*, 1999). Cells were harvested 96 to 120 h later, suspended in freezing buffer (0.15 M NaCl and 50 mM Tris, pH 8.0), freeze/thawed several times, and treated with 50 U/ml of benzonase for 30 min at 37 °C. The lysate was then centrifuged (3700 *g* for 20 min) and the supernatant was added to a centrifuge tube containing a 15, 25, 40, and 60% iodixanol step gradient. The gradient with lysate was then centrifuged (50 000 *g* for 200 min, 10 °C) before the 4/5 bottom of the 40% fraction was recovered. This extract was then diluted in PBS-MK (1 × PBS, 1 mM $MgCl_2$, 2.5 mM KCl), and concentrated and purified with a Centricon Plus-20 (100K) filter device. Purified viruses were stored at 4 °C.

Efficacy of infectivity was first evaluated by qualitative assessment of GFP-positive cells (Figure 1a) following infection and observation of 5-HT1A-R immunostaining in mice that received AAV-sh5-HT1A compared with AAV-Scr in the hippocampus (Figure 1b). The efficacy of shRNA-mediated 5-HT1A knockdown *in vivo* was further confirmed by quantitative RT-PCR that identified >30% decrease in htr1A mRNA in hippocampal tissue (see 'Quantitative real-time (q)-PCR and mRNA quantitation' below). Because of the potential mismatch between mRNA levels and protein function (Vogel and Marcotte, 2012), functional evaluation of 5-HT1A KD was determined by measuring the reduction of 8-OH-DPAT-induced hypothermia in mice with knockdown of the 5-HT1A receptor in the dorsal raphe (Figure 1c). This assay is highly dependent on function of 5-HT1A autoreceptors (Ginefri-Gayet and Gayet, 1993).

Stereotaxic Surgery

C57BL/6J mice, 10–12 weeks old, were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) under isoflurane anesthesia. Purified high-titer AAV2 constructs were injected bilaterally into the dorsal raphe nucleus (from bregma: −2.5 mm anteroposterior, ±1.5 mm lateral, 2.7 mm dorsoventral) or the hippocampus (from bregma: −2 mm anteroposterior, ±1.5 mm lateral, 2.8 mm dorsoventral) over 15 min using a 26-s beveled-tipped Hamilton syringe (Hamilton, Reno, NE). For the raphe experiments, injection volume was 0.5 μl of AAV (knockdown or scrambled); for hippocampus, the volume was 1.5 μl per side. At the end of the infusion, the needle was kept in place for an additional 5 min before it was slowly withdrawn.

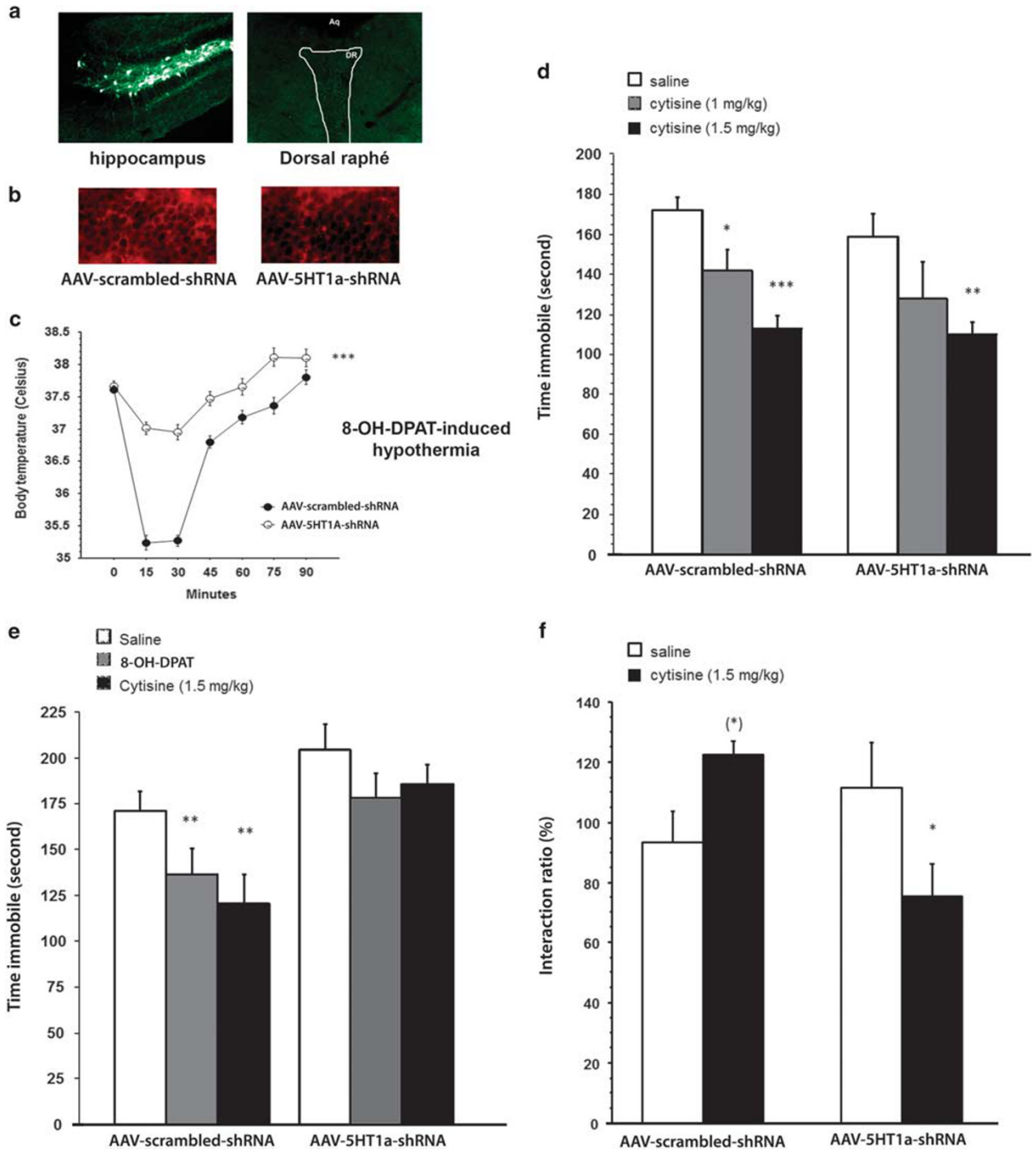

Figure 1 (a) GFP expression in hippocampus and dorsal raphe after AAV infusion and shRNA expression. (b) Qualitative evaluation of 5-HT1A receptor expression following infusion of AAV-scrambled-shRNA or AAV-5-HT1A-shRNA into the hippocampus. (c) Change in body temperature following injection of 8-OH-DPAT (1 mg/kg) in mice with AAV-scrambled-shRNA or AAV-5-HT1A-shRNA infusion into the dorsal raphe. $N = 10$ per group. (d) Time spent immobile in the tail suspension test following treatment with cytisine at subthreshold (1 mg/kg) or suprathreshold (1.5 mg/kg) doses in mice with AAV-scrambled-shRNA or AAV-5-HT1A-shRNA infusion into the dorsal raphe. $N = 7–9$ per group after checking placements. (e) Time spent immobile in the tail suspension test following treatment with cytisine (1.5 mg/kg) or 8-OH-DPAT (1 mg/kg) in mice with AAV-scrambled-shRNA or AAV-5-HT1A-shRNA infusion into the hippocampus. $N = 9–10$ per group after checking placements. (f) Social interaction following treatment with cytisine (1.5 mg/kg) in mice with AAV-scrambled-shRNA or AAV-5-HT1A-shRNA infusion into the hippocampus. $N = 10$ per group after checking placements. Data are presented as means ± SEM; (*)$p < 0.06$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Following stereotaxic surgery, mice were allowed to recover for at least 2–4 weeks before behavioral assays to allow for decrease in 5-HT1A expression.

Validation of Injection and Infection

Mice were anesthetized and perfused intracardially with PBS (~50 ml) followed by 4% PFA in PBS (~75 ml) for 5 min each. Brains were removed, postfixed for 24 h in 4% PFA at 4 °C, and then placed in 30% sucrose in PBS for cryoprotection and stored at 4 °C. Then, 40 μm brain sections were cut with a sliding microtome and placed in PBS before injection sites were examined by visual confirmation of EGFP expression by direct visualization with an actinic flashlight or using a Nikon fluorescence microscope after mounting the tissue on slides. For some of the animals used to determine knockdown efficacy by qPCR, we also ran experiments to detect GFP expression to confirm viral infection.

Quantitative Real-Time (q)-PCR and mRNA Quantitation

Mice infused with AAV-sh5-HT1A or AAV-Scr in the hippocampus were killed 3 weeks after viral infusion. Following rapid decapitation, the hippocampus was removed, and RNA was extracted using a RNeasy Lipid Tissue Mini Kit (Qiagen). Reverse transcription was performed and cDNA was quantified by quantitative PCR with SYBR Green using a StepOnePlus Thermal Cycler (Applied Biosystems). Primer efficiency was determined to be optimal and sequences were as follows: forward, 5′-TACTCCACTTTCG GCGCTTT-3′; reverse, 5′-GGCTGACCATTCAGGCTCTT-3′. Loading control was the TATAbox bing protein 1 (TBP) gene: forward, 5′-AAAGGGAGAATCATGGACCAGAACAA-3′; reverse, 5′-TGGACTAAAGATGGGAATTCCAGGAG-3′.

5-HT1A Immunohistochemistry

Free-floating brain slices obtained as described above were washed in PBS 0.1 M (pH = 7.4), incubated for 1 h with Triton-X100 0.3%/Normal Goat Serum/PBS, and incubated overnight at 4 °C with a 5-HT1A primary antibody in PBS (1 : 1000; Abcam ab101914, Cambridge, MA), followed by a fluorophore-conjugated anti-goat secondary antibody (1 : 2000; Alexafluor 647) for 2 h at room temperature. One of every six slices of hippocampus was then mounted onto gelatin-coated slides, covered with mounting media (Vectashield, Vector Laboratories, Burlingame, CA), sealed with a coverslip, and observed under a Zeiss ApoTome fluorescence microscope.

Behavioral Assays

Following AAV infusions, mice were allowed to recover for 21–28 days to achieve viral infection and 5-HT1A receptor knockdown. Mice were then tested in the tail suspension test (TST) and subsequently evaluated for any changes in locomotor activity 48 h later. Another cohort of animals was tested in the social defeat paradigm, following the same timeline.

Mice were habituated to testing rooms for at least 30 min before behavioral testing, and testing took place between 1000 and 1800 h.

Tail suspension test. The TST is used to screen antidepressant compounds or assess depression-like phenotypes (Cryan *et al*, 2005; Steru *et al*, 1985). This test was chosen particularly because it is not confounded by changes in body temperature that could result from manipulations of the 5-HT system (Abdel-Fattah *et al*, 1997; Sheard and Aghajanian, 1967). It is also not affected by changes in appetite that can result from treatment with cholinergic compounds (Mineur *et al*, 2011).

Mice were gently suspended by the tail, filmed and subsequently scored, or observed live, depending on the number of animals tested. The time spent immobile over a 6-min period was determined as described previously (Mineur *et al*, 2007a). Immobility was defined as absence of movement except for respiration. Subjects were returned to their home cage at the end of the test.

Social interaction test following social defeat. We screened CD1 mice (retired breeders) for aggressive behavior, and only the CD1 mice with attack latencies 1 min were used in defeat episodes (Wilkinson *et al*, 2011). Tested animals were subjected to two defeat episodes per day for 4 days and then tested for social interaction. On day 5, experimental mice were placed in a novel open field (39 × 39 cm) in dim light for two consecutive sessions of 2.5 min. During the first session (no target), the open field contained an empty metal grid cage (10 × 6 cm) at one end of the arena. In the second session (target present), an unfamiliar CD1 mouse was introduced in the metal grid. Interaction score was defined as the ratio of time spent in the arena around the grid cage with or without the CD1 mouse.

Locomotor activity. Locomotion was evaluated in an open field to control for any changes resulting from manipulation of the serotonergic or cholinergic system. Mice were placed in a clean Plexiglas cage (48 × 22 × 18 cm) for 30 min and locomotor activity was recorded using the OptiMax system (Columbus Instruments, Columbus, OH). Subjects were returned to their home cage at the end of the test.

Statistical Analysis

Results of behavioral assays following drug treatments were analyzed by Fisher's protected least square deviation (PLSD). Results of behavioral assays following 5-HT1A knockdown 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

A Low Dose of the SSRI Fluoxetine Potentiates the Antidepressant-Like Effects of Cytisine

Serotonin is one of the primary neurotransmitters targeted by commonly used antidepressants. To determine whether increasing serotonin signaling could potentiate the effects of a cholinergic drug in the TST, a test of antidepressant efficacy, we first identified a subthreshold dose of fluoxetine (7.5 mg/kg) that had no behavioral effects on its own (Figure 2a). Combining the subthreshold dose of fluoxetine

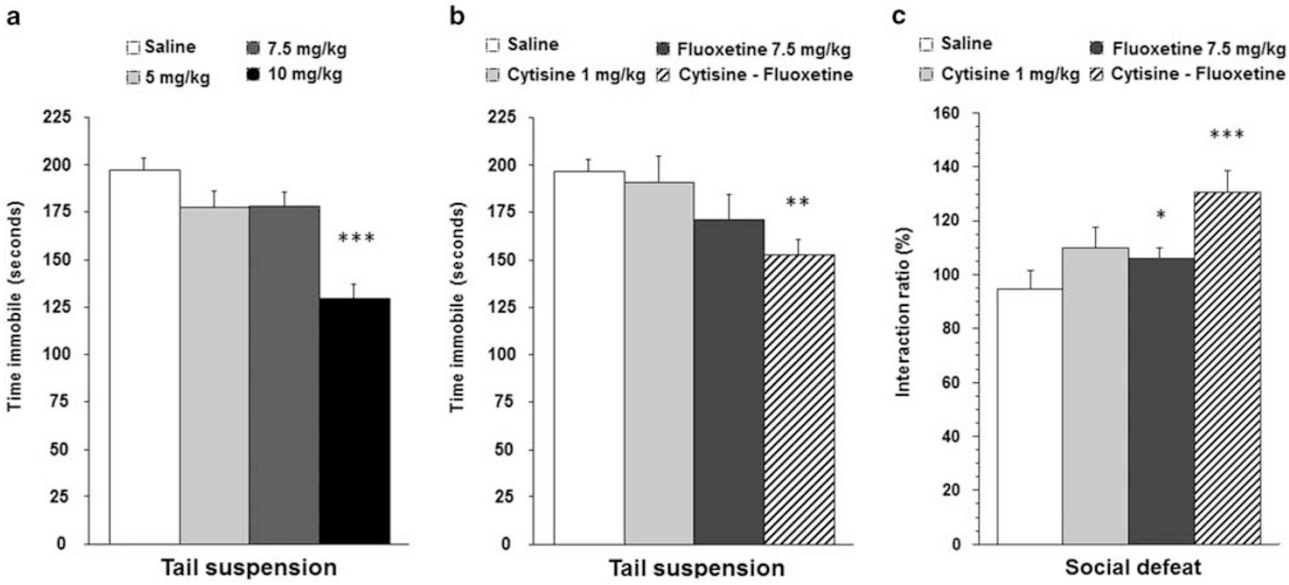

Figure 2 (a) Time spent immobile in the tail suspension test following treatment with different doses of fluoxetine. $N = 10$–11 per group. (b) Time spent immobile in the tail suspension test following treatment with subthreshold doses of cytisine (1 mg/kg) and fluoxetine (7.5 mg/kg). $N = 10$–12 per group. (c) Social interaction following social defeat. Treatment with subthreshold doses of cytisine (1 mg/kg) and fluoxetine (7.5 mg/kg). $N = 10$ per group. Data are means ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(7.5 mg/kg) with a subthreshold dose of the nicotinic partial agonist cytisine (1 mg/kg; Mineur *et al*, 2007b) induced a significant decrease in immobility in the TST (Figure 2b; PLSD, $p < 0.001$), and increased interaction with a novel mouse following social defeat (Figure 2c; PLSD, $p < 0.001$). The combination of the two compounds did not alter locomotor activity significantly ($F < 1$).

Serotonin Depletion Prevents the Antidepressant-Like Effects Induced by the Nicotinic Partial Agonist Cytisine

In order to determine whether serotonin signaling is critical for the ability of a cholinergic drug to induce an antidepressant-like effect, we evaluated the effect of cytisine after serotonin depletion with PCPA. Treatment with cytisine (1.5 mg/kg) resulted in an antidepressant-like effect in C57BL/6J male mice (Figure 3; PLSD $p < 0.05$), as has been published previously (Mineur *et al*, 2009; Mineur *et al*, 2007b). Pretreatment with PCPA did not induce an increase in time spent immobile on its own, suggesting that 5-HT depletion did not induce a change in depression-like phenotype (PLSD, $p < 0.5$). However, PCPA-mediated serotonin depletion abolished the response to cytisine (1.5 mg/kg) in the TST, suggesting that serotonin signaling is required for its antidepressant-like effects.

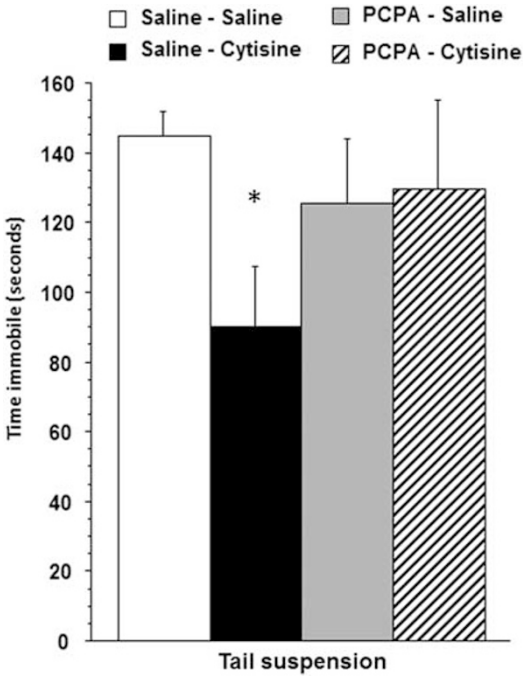

Figure 3 Time spent immobile in the tail suspension test following serotonin depletion by PCPA without or with treatment with cytisine (1.5 mg/kg). $N = 7$–8 per group (some animals had to be removed because of wounding). Data are means ± SEM; $^{*}p < 0.05$.

The 5-HT1A Receptor Agonist 8-OH-DPAT Potentiates the Effects of Cytisine in the TST

To identify the receptor subtypes involved in the interaction between serotonin and cholinergic signaling, we determined whether stimulation of 5-HT1A receptors could augment the effects of cytisine in the TST by using 8-OH-DPAT, a 5-HT1A agonist that also has effects at the 5-HT7 subtype. The 8-OH-DPAT has antidepressant-like effects and is highly selective for 5-HT1A and 5-HT7 receptors (Lucki and Wieland, 1990; Wieland and Lucki, 1990). Using a subthreshold dose identified previously (Lucki and O'Leary, 2004; Wieland and Lucki, 1990), mice were administered a low dose of 8-OH-DPAT (0.1 mg/kg) combined with a subthreshold dose of cytisine (1 mg/kg). As expected, the subthreshold doses of each drug had no effect on their own,

but the combination of 8-OH-DPAT (0.1 mg/kg) and cytisine (1 mg/kg) resulted in a significant decrease in the time spent immobile in the TST ($p<0.01$; Figure 4). No significant differences were observed in locomotor activity ($F<1$).

Postsynaptic 5-HT1A in the Hippocampus Is Required for the Antidepressant-Like Effects of Cytisine

The 8-OH-DPAT is highly selective for 5-HT1A and 5-HT7 receptors, but cannot distinguish between the receptor subtypes. In addition, 5-HT1A receptors serve as inhibitory autoreceptors that decrease 5-HT release in the raphe, and also as postsynaptic 5-HT receptors, with high expression in the hippocampus. To determine whether the effect of cytisine in the TST is because of a decrease in 5-HT neuron activation due to 5-HT1A autoreceptors or postsynaptic effects in hippocampus, we designed AAV2 vectors carrying shRNAs targeting the 5-HT1A receptor. The AAV-5-HT1A-shRNA constructs are efficiently expressed in the hippocampus and raphe nuclei (Figure 1a) and decrease 5-HT1A receptor expression *in vivo* (Figure 1b). Note that sections were taken from perfused tissue for the hippocampus *vs* fresh frozen tissue for the dorsal raphe, and hence there is a difference in the intensity of the amplified immunocytochemistry signal as compared with the intrinsic fluorescence of GFP. In addition, functional knockdown of 5-HT1A was confirmed by measuring the hypothermic response to 8-OH-DPAT (1 mg/kg) challenge following infusion of the AAV-5-HT1A-shRNA construct into the dorsal raphe (Figure 1c).

Cytisine induced a dose-dependent decrease in immobility in the TST following infusion of the AAV-5-HT1A-shRNA construct into the dorsal raphe. ANOVA revealed that there was no interaction between knockdown in the dorsal raphe and the time spent immobile ($F<1$), suggesting that the 5-HT1A autoreceptors in the raphe do not contribute significantly to the antidepressant-like properties of cytisine (Figure 1d). The hippocampus receives a significant serotoninergic projection, and has one of the highest levels of 5-HT1A receptor expression in the brain (Hensler *et al*, 2007). In addition, recent studies have suggested that increased ACh signaling in the hippocampus exacerbates anxiety- and depression-like behaviors in mice (Mineur *et al*, 2013). We therefore infused the AAV-5-HT1A-shRNA construct into the hippocampus (Figure 1a) and measured immobility in the TST following cytisine treatment. Cytisine decreased immobility significantly in control mice ($p<0.05$), but had no significant effect in animals with 5-HT1A knockdown in the hippocampus (Figure 1e). The 8-OH-DPAT is an agonist at both 5-HT1A and 5-HT7 receptors, and hence the lack of behavioral effects of the compound in 5-HT1A knockdown mice confirms the specificity of the receptor subtype mediating the nicotinic interaction, and validates the efficacy of 5-HT1A knockdown. A similar pattern was observed in the social defeat paradigm: whereas cytisine increased social interaction in control animals, this effect was completely reversed in animals with the knockdown (Figure 1f; $F(1,36)=9.48$, $p<0.004$). None of the treatment groups exhibited significant changes in locomotion ($F<1$).

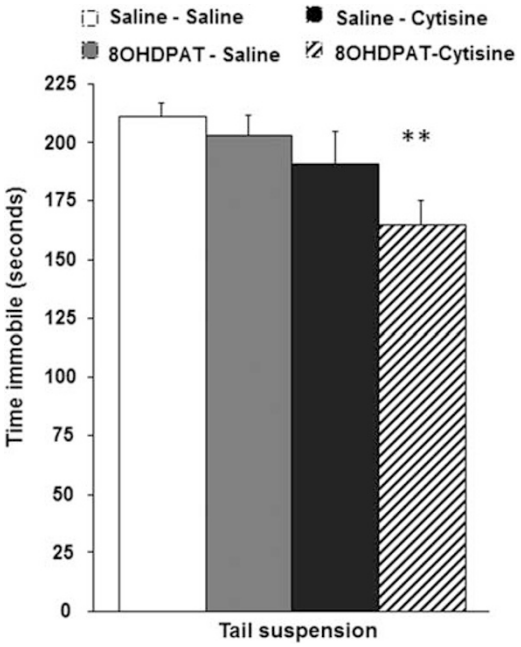

Figure 4 Time spent immobile in the tail suspension test following treatment with subthreshold doses of cytisine (1 mg/kg) and 8-OH-DPAT (0.1 mg/kg). $N=10$–11 per group. Data are means ± SEM; $^{**}p<0.01$.

DISCUSSION

Although there are several avenues for treatment of MDD, a significant subset of patients do not respond to current treatments, and many others receive some relief of symptoms, but not full remission. There is clear evidence demonstrating that serotonin signaling can be dysregulated in depressed subjects (for a review, see Araragi and Lesch, 2013) and SSRIs are currently the treatment of choice for mood disorders. Accumulating evidence also suggests that alteration of the cholinergic system may contribute to depression and be a target for antidepressant therapies. For example, human imaging studies of individuals with MDD or bipolar disorder show that ACh levels are high when individuals are actively depressed (Hannestad *et al*, 2013; Saricicek *et al*, 2012). These recent imaging studies are consistent with experiments showing that increasing ACh levels in human subjects by challenging with physostigmine, a drug that prevents breakdown of ACh, results in symptoms of anxiety and depression (Risch *et al*, 1981). Furthermore, recent rodent studies have shown that decreasing breakdown of ACh in the hippocampus is sufficient to cause anxiety- and depression-like behaviors in mice (Mineur *et al*, 2013).

Preclinical studies and clinical trials have demonstrated the possibility that decreasing cholinergic signaling through nAChRs using nicotinic antagonists or partial agonists can have antidepressant-like effects on their own and can also augment the efficacy of SSRIs (Andreasen *et al*, 2012; George *et al*, 2008; Philip *et al*, 2009; Rollema *et al*, 2009). A number of questions remain to be answered, however. A recent large clinical trial failed to show a significant effect of a nicotinic antagonist as an antidepressant in patients who were nonresponsive to SSRI administration (Vieta *et al*,

2013). The results of this trial contradict other smaller clinical trials that showed a significant improvement in depressive symptoms following the addition of nicotinic antagonist mecamylamine to patients taking an SSRI (Bacher *et al*, 2009; George *et al*, 2008). It is possible that complete blockade of nAChRs is not well tolerated, and hence the dose of the drug could not be increased sufficiently to achieve clinical efficacy. Thus, partial agonists of nAChRs that decrease ACh signaling, but do not have broad effects on their own, may be a better avenue for therapeutic development as antidepressant medications. Along this line, an intriguing study showed that the nicotinic partial agonist varenicline could also be antidepressant in subjects resistant to an SSRI in a similar add-on design (Philip *et al*, 2009).

The current data support the idea that decreasing ACh signaling while simultaneously increasing serotonin signaling could be a useful approach for treating symptoms of depression and appears to increase resilience to social stress, a critical factor in the development of depression symptoms (Caspi *et al*, 2003). We show that these neurotransmitter systems can act synergistically to induce an antidepressant-like effect, even when they do not induce any behavioral change individually. The current studies also show that serotonin signaling is necessary for the antidepressant-like effects of cytisine, and indicate that signaling through postsynaptic 5-HT1A receptors of the hippocampus is critical for the antidepressant-like effect of this nicotinic partial agonist. Clinically, this suggests that even when SSRIs do not lead to a full antidepressant effect, potentiation of serotonin signaling may be sufficient to allow a decrease in cholinergic signaling to result in an antidepressant response. Furthermore, the current studies also emphasize that a deficit in serotonergic signaling in patients with depression could also decrease the response to augmentation therapy with cholinergic drugs. Interestingly, a recent study suggests that chronic treatment with nicotine can partially restore alterations induced by monoamine depletion (Khadrawy *et al*, 2011). As nicotine treatment can desensitize high-affinity nAChRs (Fenster *et al*, 1997; Grady *et al*, 1994), this provides some evidence that decreasing nAChR signaling could facilitate 5-HT signaling. These results are consistent with studies showing that treatment with either chronic nicotine or the nicotinic antagonist mecamylamine can increase serotonin release and signaling in the hippocampus (File *et al*, 2000; Kenny *et al*, 2000). Antagonism of muscarinic AChRs can also result in increased 5-HT release (Kenny *et al*, 2000), providing further evidence that decreasing ACh signaling in hippocampus can synergize with serotonin signaling. It should be noted that AAV2 is nonselective and targets many, if not all, neuronal types in the brain, and thus knockdown of 5-HT1A likely occurred in non-5-HT cells of the dorsal raphe as well. However, given the high rate of infectivity of the virus and the significant blunting of 8-OH-DPAT-induced hypothermia after knockdown, the data suggest that among the cell types affected by the 5-HT1A manipulation were ascending 5-HT neurons expressing 5-HT1A autoreceptors.

The precise cellular targets of 5-HT1A activation and nAChR blockade in the hippocampus remain to be elucidated but it seems possible that this interaction leads to decreased signaling in common pathways. 5-HT1A receptors are Gi/o-coupled receptors and will therefore decrease neuronal activity through inhibition of adenylyl cyclase and activation of potassium channels (Colino and Halliwell, 1987; De Vivo and Maayani, 1986). Postsynaptic effects of 5-HT1A stimulation in the hippocampus appear to be dependent on hyperpolarization mediated through GIRK2 potassium channels (Luscher *et al*, 1997). Blockade of nAChRs also decreases neuronal activation by decreasing ACh-mediated depolarization. Mechanistically, we propose the following model: acute stress leads to an increase in 5-HT and ACh release in different brain regions, including the hippocampus (Gartside *et al*, 2000; Grahn *et al*, 1999). Conversely, chronic stress, one of the main factors that can induce depression (Heim *et al*, 1997; Pace *et al*, 2006), decreases 5-HT release and sensitivity in the hippocampus that leads to depression-like symptoms (Kang *et al*, 2005; Luo *et al*, 2008) along with a decrease in 5-HT1A activation (for a broad review, see Mahar *et al*, 2014), but maintains higher ACh levels. SSRI treatment can restore the balance in serotonin signaling and lead to an increase in neurotrophic factor expression and neurogenesis, critical factors underlying antidepressant response (Brezun and Daszuta, 2000; Santarelli *et al*, 2003), mainly through brain 5-HT1A receptor stimulation of the hippocampus (Banasr *et al*, 2004).

This study shows that 5-HT1A signaling in the hippocampus is required for the antidepressant-like effects of the nAChR partial agonist cytisine. These data provide support for the idea that nAChR partial agonists could augment antidepressant effects of serotonergic medications, and highlight the neurobiological mechanisms that could underlie an augmentation strategy.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Grants MH077681, DA14241, and P50 DA033945 from the National Institute on Drug Abuse (NIDA), the Food and Drug Administration (FDA), and the Office of Research on Women's Health (ORWH).

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