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RESEARCH PAPER

Hippocampal α7 nicotinic ACh receptors contribute to modulation of depression-like behaviour in C57BL/6J mice

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BACKGROUND AND PURPOSE
Clinical studies have identified links between cholinergic signalling and depression in human subjects. Increased cholinergic signalling in hippocampus also increases behaviours related to anxiety and depression in mice, which can be reversed by ACh receptor antagonists.

EXPERIMENTAL APPROACH
As the α7 subunit of the nicotinic ACh receptor (nAChR) is highly expressed in hippocampus, we determined whether blocking α7 nAChRs could reverse the effects of increased ACh signalling in anxiety- and depression-related behaviours in mice.

KEY RESULTS
Administration of the α7 nAChR agonist GTS-21 had no effect in tail suspension or forced swim tests. Conversely, the α7 nAChR antagonist methyllycaconitine (MLA) induced significant antidepressant-like effects in male mice in these paradigms, consistent with previous studies, but this was not observed in female mice. MLA also decreased physostigmine-induced c-fos immunoreactivity (a marker of neuronal activity) in hippocampus. Local knockdown of α7 nAChRs in hippocampus had no effect on its own but decreased a subset of depression-like phenotypes induced by physostigmine in male mice. Few effects of α7 nAChR knockdown were observed in depression-like behaviors in female mice, possibly due to a limited response to physostigmine. There was no significant effect of hippocampal α7 nAChR knockdown on anxiety-like phenotypes in male mice. However, a modest increase in anxiety-like behavior was observed in female mice infused with a scrambled control vector in response to physostigmine administration, that was not seen after a7 nAChR knockdown in the hippocampus.

CONCLUSIONS AND IMPLICATIONS
These results suggest that ACh signalling through α7 nAChRs in the hippocampus contributes to regulation of a subset of depression-like behaviours when ACh is increased, as can occur under stressful conditions. These studies also provide evidence for sex differences that may be relevant for treatments of mood disorders based on cholinergic signalling.

LINKED ARTICLES
This article is part of a themed section on Nicotinic Acetylcholine Receptors. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v175.11/issuetoc

Abbreviations
AAV, adeno-associated virus; DG, dentate gyrus of the hippocampus; MLA, methyllycaconitine; nAChRs, nicotinic ACh receptors; SSRI, serotonin-specific reuptake inhibitor

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Tables of Links

TARGETS	
Ligand-gated ion channels[a]	**Enzymes**[c]
nAChRs, nicotinic ACh receptors	Acetylcholinesterase
α7 nAChRs	
GPCRs[b]	
Muscarinic receptors	

LIGANDS	
ACh	Mecamylamine
Amitriptyline	Methyllycaconitine
Fluoxetine	Nicotine
Imipramine	Physostigmine
Isoflurane	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015a,b,c).

Introduction

The cholinergic hypothesis of depression proposed that depressed mood is induced by increased activity of the cholinergic system (Janowsky *et al.*, 1972). Consistent with this hypothesis, human studies have shown that administration of the reversible AChE inhibitor physostigmine and other compounds that increase ACh levels can induce symptoms of depression (Risch *et al.*, 1980; Risch *et al.*, 1981; Steinberg *et al.*, 1997). Similarly, a genetic animal model of depression selectively bred for its sensitivity to cholinergic agents (the Flinders sensitive rat strain) shows increased ACh levels and exhibits behaviours resembling symptoms of depression (Overstreet, 1993). This rat model also shows greater stress-induced complement response (Ayensu *et al.*, 1995) and abnormal expression of 5-HT receptors (Osterlund *et al.*, 1999), suggesting that changes in cholinergic signalling have farther reaching effects on several brain systems involved in depression and antidepressant response. Clinical studies show that human depressed subjects have decreased occupancy of high-affinity β2-containing (β2*) nicotinic ACh receptors (nAChRs) in many brain areas, with no change in the number of these nAChRs (Saricicek *et al.*, 2012; Esterlis *et al.*, 2014), which is interpreted as an increase in ACh levels in depressed subjects (Esterlis *et al.*, 2013). Patients in remission exhibit an intermediary phenotype with ACh levels greater than control patients, but lower than actively depressed patients (Saricicek *et al.*, 2012). These observations demonstrate a strong and dynamic correlation between depressive states and cholinergic signalling in the brain.

We have shown that blocking AChE activity in mice can also induce phenotypes that model depression-like symptoms, and these can be reversed by administration of an antidepressant effective in depressed individuals (fluoxetine) but also by cholinergic receptor antagonists (Mineur *et al.*, 2013). The effects of AChE blockade were reproduced by altering ACh signalling in the hippocampus, identifying this brain area as a critical node in the cholinergic regulation of behaviours related to depression. Stress, which can contribute to the development of depressive symptoms, modulates ACh levels in many brain areas. For instance, restraint stress in rats increases ACh levels acutely in specific brain regions, but also increases AChE levels chronically, potentially leading to a decrease in ACh levels over time (Kaufer *et al.*, 1998; Sternfeld *et al.*, 2000; Shaked *et al.*, 2008). The valence and effects of ACh depend on the brain region, and stress-induced ACh release is greatest in the prefrontal cortex and the hippocampus of animals subjected to restraint stress (Mark *et al.*, 1996), making it a challenge to determine the brain areas most critical for ACh-dependent changes in behaviours related to depression.

Much of the research on the cholinergic receptors involved in antidepressant action has focused on muscarinic receptors, particularly the muscarinic agonist scopolamine, which can induce rapid antidepressant effects (Drevets *et al.*, 2013). These effects appear to be mediated by muscarinic M1 receptors located on interneurons in the prefrontal cortex (Wohleb *et al.*, 2016). Nicotinic receptors have also been suggested as targets for antidepressant development (Tizabi *et al.*, 2000; Picciotto *et al.*, 2002; Picciotto *et al.*, 2008; Khadrawy *et al.*, 2011). Nicotine-containing patches can induce antidepressant effects in non-smokers (Salin-Pascual *et al.*, 1995), and there are numerous studies showing co-morbidity between smoking and depression. Several classes of antidepressants, including tricyclic antidepressants, serotonin-specific reuptake inhibitors (SSRIs) and atypical antidepressants, can act as non-competitive antagonists of nAChRs (Shytle *et al.*, 2002a). Some clinical studies have shown that the non-selective nAChR antagonist mecamylamine exerts antidepressant effects when added to an SSRI in depressed patients unresponsive to SSRI alone (George *et al.*, 2008). Studies in mice have also shown that mecamylamine has antidepressant-like effect in several behavioural paradigms and can potentiate the effect of classical antidepressants such as amitriptyline and imipramine (Popik *et al.*, 2003). Several pharmacological studies have demonstrated that compounds that target α7 or β2 nAChRs have antidepressant-like profiles (Andreasen *et al.*, 2009). We have shown that decreasing expression of the β2 and α7 nAChR subunits in amygdala can have antidepressant-like effects on their own and increase resilience to social stress (Mineur *et al.*, 2016). While nAChR signalling in the amygdala is sufficient to mediate some

antidepressant-like effects, this does not identify the targets for ACh in the hippocampus that mediate the local effects of increased ACh signalling following AChE knockdown in this structure.

Several nAChR subtypes are expressed in the hippocampus, but the α7 subunit is the most highly expressed receptor subtype in this structure, and the majority of electrophysiological responses to nicotine in the CA fields are mediated through α7 nAChRs (Seguela *et al.*, 1993; Orr-Urtreger *et al.*, 1997). Many antagonists, agonists and allosteric modulators have been developed to target α7 nAChRs in an attempt to improve cognitive function (Lieberman *et al.*, 2013; Dineley *et al.*, 2015), providing a large number of potential tools for pharmacological targeting and modulation of α7 nAChR function. We therefore investigated the effects of compounds targeting α7 nAChRs on behaviours related to anxiety and depression in male and female C57BL/6J mice. We further examined whether these compounds could alter a neurochemical marker of hippocampal activity in response to hypercholinergic stimulation. We then investigated the effects of selectively knocking down α7 nAChRs in the hippocampus on behaviours related to anxiety and depression at baseline or in response to increased ACh signalling induced by physostigmine.

Methods

Animals

All animal care and experimental procedures were in accord with the Guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Yale University Committee on the Care and Use of Animals. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

C57BL/6J male and female mice were purchased from Jackson Laboratory (Bar Harbour, ME, USA) and were 10–12 weeks of age at the beginning of the study. Mice were group-housed (five per cage) on a 12:12 light/dark cycle (07:00 to 19:00 h) at 22.1 ± 1°C with *ad libitum* access to food and water. Cages were then randomly assigned to a specific treatment group. We performed power analyses based on similar experiments carried out previously in the laboratory and determined that using five animals for histological analyses and 10 to 15 animals for behavioural experiments would provide sufficient power to detect a significant difference with 95% confidence. A small number of animals were excluded due to off-target stereotaxic surgery; final numbers are detailed in each figure. Experimental design and analyses were carried out in accord with the guidelines of the BJP (Curtis *et al.*, 2015).

Adeno-associated viral vectors and shRNA constructs

Adeno-associated viruses (AAV) carrying small hairpin RNAs (shRNAs) were used to target the α7 nAChR subunit for knockdown. AAVs were purified and the efficiency of the knockdown was validated as previously described (Mineur *et al.*, 2016). Two shRNAs targeting the mRNA encoding the α7 nAChR subunit were designed and ligated into pAAV-eGFP–shRNA as described previously (Hommel *et al.*, 2003; Mineur *et al.*, 2011). shRNA-containing plasmids were then packaged into AAV-2 by calcium-based triple transfection of HEK 293 cells with 135 μg each of pAAV–shRNA, pHelper and pAAV-RC plasmids (Stratagene, La Jolla, CA, USA). Cells were recovered after 5 days, suspended in [0.15 M NaCl, 50 mM Tris, pH 8.0], lysed by freeze–thaw cycles and incubated for 30 min at 37°C with benzonase at 50 U·mL^{-1}. Clarified lysate was applied to a 15, 25, 40 and 60% iodoxanol step gradient and centrifuged at 50 000× *g* for 3.5 h at 10°C. The bottom of the 40% fraction was removed, diluted in [1× PBS, 1 mM MgCl2, 2.5 mM KCl], concentrated, washed and purified with Amicon 100 K filter columns and PBS-MK. Purified high-titre virus (100–200 μL) was stored at 4°C until use. Each experiment was performed with viruses generated from a single preparation. Following purification, we routinely evaluate infectivity of each batch by *in vivo* injection and discard any batches of virus that do not result in GFP expression consistent with our standard preparations.

Surgical procedures

Infusions of viral vectors into the hippocampus were done with a stereotaxic frame mounted with 5 μL Hamilton syringes and under 2% isoflurane anaesthesia. The infusions were bilateral and care was taken to minimize potential tissue damage. Each side of the hippocampus (Anterior/Posterior = −2.5 mm, Lateral = ±1.5 mm, Dorsal/Ventral = −2.8 mm) received 1.5 μL of the AAV suspension over the course of 7 min per side, and the syringe was left in for a further 5 min to reduce the chance of backflow upon removal. All animals were carefully monitored during and after surgery to ensure vital signs were satisfactory. Infectivity and location of the virus were visualized by GFP expression. We targeted the dorsal hippocampus, although diffusion is also visible in the ventral part of the hippocampus, but to a lesser extent. After AAV infusion, animals were allowed to recover for 3 weeks, to allow sufficient time for infection and knockdown to take place before testing. Following surgery, mice did not exhibit any obvious changes in demeanour or behaviour.

Behavioural testing

All behavioural assays were conducted in a battery used extensively in our laboratory (see Table 1). The tests were conducted in the order shown, with the least stressful first and the most stressful last as has been recommended (Crawley, 2008). A range of 10 to 15 animals were initially assigned to the different experimental groups. Following evaluation of the site of viral infusion, 8 to 13 animals were used for the final analyses.

Light/dark box - A rectangular box was split 50–50 into two compartments; one side was covered and therefore dark, while the other had a light (60 W, ~700 lx) shining downwards onto it. Mice were placed into the farthest point of the light side, and the time taken to cross over to the dark side was recorded. After this had occurred, the time in the dark side and the number of crossings were recorded over a 6 min period.

Table 1

Experimental design for pharmacological and molecular genetic manipulations

	Pharmacological experiment				α7 nAChR knockdown in hippocampus							
	Group 1 males	Group 2 females	Group 3 males	Group 4 females	Group 5 males				Group 6 females			
	Saline GTS-21 MLA	Saline GTS-21 MLA	Saline MLA	Saline MLA	Scrambled shRNA		α7 shRNA		Scrambled shRNA		α7 shRNA	
					Saline	Physo	Saline	Physo	Saline	Physo	Saline	Physo
Light/dark	x	x			x	x	x	x	x	x	x	x
Marble burying	x	x										
Tail suspension	x	x			x	x	x	x	x	x	x	x
Forced swim	x	x			x	x	x	x	x	x	x	x
Social defeat	x				x	x	x	x				
Locomotion	x	x			x	x	x	x	x	x	x	x
c-fos			x	x								

Marble burying - Mice were placed in an open field evenly covered with 4 cm of bedding. Twenty marbles were placed in a grid pattern (5 × 4), and a small space was preserved at one end of the arena where each subject was placed at the start of a trial. The open field was covered with a lid to prevent the animal from jumping out and to dim the field. The number of marbles buried over the 30 min trial was counted. A marble was considered 'buried' when more than 75% was submerged in bedding.

Tail suspension test - Mice were suspended gently by the tail for 6 min, and time spent immobile was recorded.

Forced swim test - On day 1 of the test, each mouse was placed in a 4 L beaker of room temperature water for 15 min. On day 2, mice were placed back in the beaker and time spent immobile was recorded for 5 min (pharmacological experiments) or 10 min (knockdown experiments).

Social defeat paradigm (males only) - Each male C57BL/6J subject was introduced into the home cage of a male, single-housed CD1 mouse that had been screened to be aggressive. The time taken for the first fight was recorded, and then the two mice were separated using a metal mesh, with the CD1 mouse receiving two thirds of the home cage. After 10 min, the tested 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 tested mouse exposed to a different CD1 aggressor for each session. On the fourth day, social interaction was assessed. Each tested mouse was placed in an open field and allowed to explore. The time spent inside the interaction area (7 cm in width) around the holding rectangle was recorded for the first 2.5 min, after which a CD1 mouse was added to the holding rectangle. The time spent in the interaction area was then recorded again for 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 - Ambulatory activity was measured once the battery of tests was completed in a 48 cm × 22 cm × 18 cm enclosure for 30 min.

Immunohistochemistry

Mice, 90 min after the locomotor activity test, were anaesthetised and quickly perfused intracardiacally with chilled PBS (0.1 M, pH 7.3) followed by chilled 4% paraformaldehyde for 10 min each (~100 mL of each solution per animal). Brains were subsequently removed and post-fixed for 24 h in paraformaldehyde at 4°C. After fixation, samples were placed in PBS (0.1 M, pH 7.3) with 30% sucrose for cryoprotection. Brains were stored in sucrose at 4°C until slicing, and 40 μm sections were cut with a microtome. Immuno-staining was conducted in five animals per treatment group, with two representative dorsal hippocampus sections (counted on the left and right and matched across all groups) per animal. 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, San Diego, CA, USA SC-52) for 48 h at 4°C. Positively identified cells were counted within the dentate gyrus (DG) or CA1 in the dorsal part of the hippocampus. The coordinates for infusion were chosen based on previous work showing that blocking AChE activity or knocking down AChE with viral infusion to the same site resulted in increased anxiety- and depression-like behaviours (Mineur *et al.*, 2013). Because CA2/3 showed little c-fos expression, this brain region was excluded.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are presented as means ± SEM. One-way ANOVA was used to investigate the differences between groups with pharmacological treatments, and *t*-tests with Bonferroni corrections were

performed when relevant. Two-way ANOVA, with 'virus' and 'physostigmine treatment' as between subject factors, was used to evaluate data in the knockdown experiments. This test was preferred because Kahn and Rayner (2003) have demonstrated that ANOVA is as robust as Kruskal–Wallis tests even for non-normal data and that ANOVAs are preferable for samples with limited '*n*' size. *Post hoc* analyses with partial least squares differences were performed when relevant and if F achieved $P < 0.05$. Because males and females were not tested at the same time to prevent unforeseen confounds during testing, data were analysed individually for each sex. $P < 0.05$ was taken to show a significant difference between group means. There were few significant behavioural effects of pharmacological or molecular genetic manipulations of α7 nAChRs in female mice; therefore, all data for tests in female animals are reported in Table 2, along with non-significant changes in tests with male mice.

Materials

All drugs were dissolved in PBS (0.1 M, pH 7.4) and were injected i.p. in volumes of 10 $mL{\cdot}kg^{-1}$. Optimal doses were based on previous studies in C57BL/6 male and female mice (Mineur *et al.*, 2013; Mineur *et al.*, 2015). Physostigmine (Sigma Pharmaceuticals, North Liberty, IA, USA) was administered at a dose of 0.15 $mg{\cdot}kg^{-1}$, 45 to 60 min before testing. Methyllycaconitine (MLA; Sigma) and GTS-21-OH (Tocris Bioscience, Bristol, UK) were injected 30 min before testing at doses of 5 and 17 $mg{\cdot}kg^{-1}$, respectively, and as previously described (Lewis *et al.*, 2015). This concentration of MLA was chosen because in vivo studies suggest that a higher dose (7.5 $mg{\cdot}kg^{-1}$) may have non-specific effects (Franceschini *et al.*, 2002) independent of α7 nAChRs (Klink *et al.*, 2001; Mogg *et al.*, 2002).

Results

GTS-21 and MLA had no effects on anxiety-like behaviour in male or female mice

Mice received an injection of either GTS-21 (an α7 nAChR agonist) or MLA (an α7 antagonist) and were tested in behavioural paradigms responsive to anxiolytic treatment. In the light–dark box, there were no significant effects on the time spent in the dark side in either female or male mice (Table 2). In the marble burying assay, none of the results reached significance in female or male mice (Table 2).

MLA has an antidepressant-like effect in the tail suspension and forced swim tests but does not affect response to social defeat stress

In the tail suspension test, there was an overall effect of treatment in female ($F_{2, 27} = 15.8$) and male mice ($F_{2, 27} = 4.8$). In female mice, *post hoc* analyses revealed that GTS-21 had no effect compared with saline, but that MLA significantly decreased the time spent immobile (Table 2). In male mice, there was no significant effect on immobility following GTS-21 treatment, whereas MLA treatment resulted in a significant decrease in time spent immobile (Figure 1A).

Table 2

Summary of quantitative results of behavioral studies

		Treatment		
Test	**Sex**	**Saline**	**GTS-21**	**MLA**
Light–dark box (s)	Females	229.5 ± 5.5	221.5 ± 10.5	238.1 ± 8.0
Light–dark box (s)	Males	227.8 ± 5.7	233.9 ± 11.4	213.6 ± 8.0
Marble burying (number buried)	Females	6.6 ± 0.8	5.8 ± 1.1	7 ± 1
Marble burying (number buried)	Males	6.9 ± 1	4.5 ± 0.7	4.4 ± 1.2
TST (s)	Females	164.4 ± 11.6	164.2 ± 8.9	54.9 ± 23.27
FST (s)	Females	118.6 ± 6.7	139.1 ± 7.3	117.6 ± 12
Social defeat (%)	Males	139.2 ± 12.9	123.3 ± 12.1	121.4 ± 10.1

		Treatment			
Test	**Sex**	**Saline/saline**	**Physo/saline**	**Saline/MLA**	**Physo/MLA**
c-fos DG (counted cells)	Females	53.2 ± 4.4	60.8 ± 2.9	51.6 ± 1.4	40.1 ± 2.4
c-fos CA1 (counted cells)	Females	35.4 ± 2.4	48 ± 2.2	44 ± 1.9	39.6 ± 1.2

		Treatment			
Test	**Sex**	**Scr ShRNA/saline**	**Scr ShRNA/physo**	**a7 KD/saline**	**a7 KD/physo**
Light–dark box (s)	Females	219.2 ± 6.9	249.5 ± 8.1	220 ± 5.4	218.6 ± 4.9
TST (s)	Females	144.1 ± 14	173.4 ± 12.3	161.1 ± 14	169.9 ± 11.3
FST (s)	Females	130.7 ± 8.7	139 ± 9.3	147.7 ± 13.7	123 ± 11.8
Social defeat (%)	Males	122.3 ± 21.5	192. ± 121.6	98.6 ± 7.6	225.6 ± 43.9
Locomotion (beam breaks)	Females	3632 ± 716	3354 ± 187	2368 ± 521	1948 ± 308

±SEM. FST, forced swim test; TST, tail suspension test.

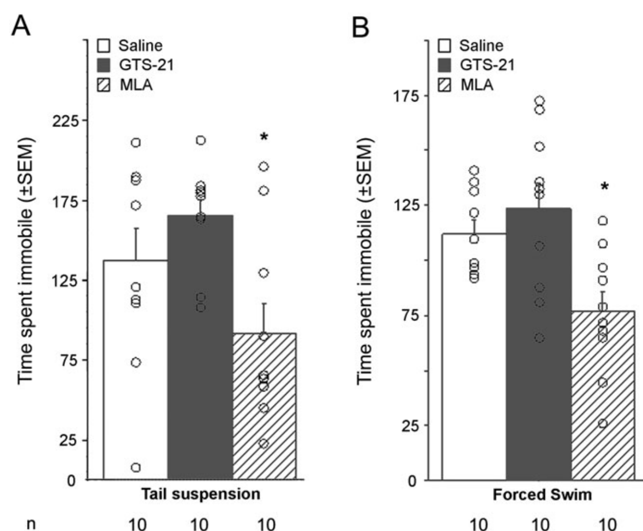

Figure 1

Effect of GTS-21 and MLA injection in tests of depression-like phenotypes. Time spent immobile in the (A) tail suspension and (B) forced swim tests following systemic injection of GTS-21 (17 mg·kg^{-1}) or MLA (5 mg·kg^{-1}) (i.p.) in C57BL/6J male mice. Data are expressed as means ± SEM, and dots represent individual data points. $n = 10$ animals per treatment group. *$P < 0.05$, significant effect of MLA; ANOVA and *post hoc* *t*-test with Bonferroni correction.

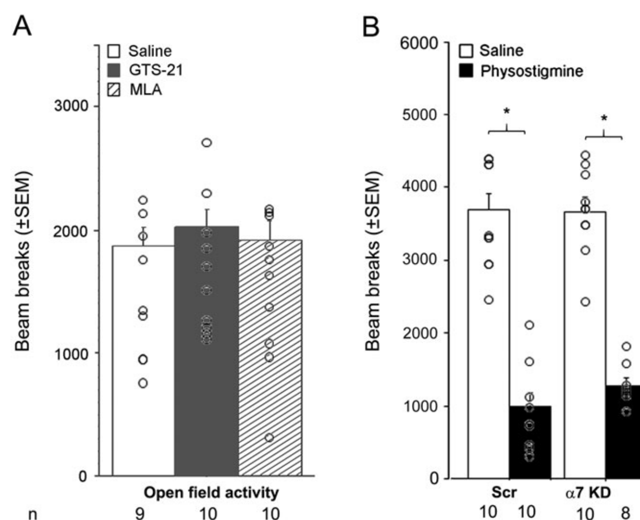

Figure 2

Locomotor activity in an open field following pharmacological manipulation or hippocampal knockdown of α7 nAChRs. (A) Number of beam breaks in an open field (30 min) following systemic injection of GTS-21 (17 mg·kg^{-1}) or MLA (5 mg·kg^{-1}) (i.p.). (B) Number of beam breaks in an open field (30 min) following α7 knockdown in the hippocampus of C57BL/6J male mice; 8 to 13 animals were used per treatment group in the final analyses (final animal numbers are represented at the bottom of the figure). Data are expressed as means ± SEM. *$P < 0.05$; significantly different as indicated; ANOVA and *post hoc* *t*-test with Bonferroni correction.

In the forced swim test, a similar pattern was observed in male mice with an overall effect of treatment ($F_{2, 27} = 7.0$). *Post hoc* analyses showed that GTS-21 did not change the time spent immobile compared with saline-treated male mice, whereas a significant decrease in immobility was observed following MLA administration (Figure 1B). None of the results reached significance in female mice (Table 2).

In the social defeat test, male mice did not show changes in social interaction at baseline or following 4 days of defeat stress whether they received GTS-21 or MLA compared with saline (Table 2).

Locomotor activity measured in an open field in males was not different between the three treatment groups ($F < 1$; Figure 2A); thus, the effects of the drugs in the tail suspension and forced swim tests are not likely to be due to changes in activity.

MLA limits the increase in c-fos immunoreactivity in the hippocampus following physostigmine treatment in male, but not female, mice

Immunoreactivity for c-fos in the DG and CA1 regions of the dorsal hippocampus was used as an indicator of neuronal activity following treatment with the α7 nAChR antagonist MLA, with or without pretreatment with physostigmine, an AChE blocker that increases extracellular ACh levels.

There was an overall pretreatment (physostigmine) by treatment (MLA) interaction ($F(1, 16) = 9.9$), in male mice, indicating that MLA had different effects on c-fos immunoreactivity if mice were pretreated with physostigmine or saline. *Post hoc* analyses showed that treatment with MLA alone did not induce a significant change in c-fos immunoreactivity in DG compared with control (Figure 3A). Following physostigmine administration, male mice showed an increase (about 50%) in c-fos staining in DG, which was decreased to baseline level following MLA administration. Surprisingly, female mice did not show a significant effect of physostigmine on c-fos expression, but MLA still decreased c-fos immunoreactivity below baseline by ~30% (Table 2).

In the CA1 region of the hippocampus, there was an overall pretreatment by treatment interaction ($F(1, 32) = 9.9$; Figure 3B), but no interaction with sex. *Post hoc* analyses revealed that this effect was driven by an increase in c-fos immunoreactivity in female mice in response to both physostigmine and MLA. MLA decreased physostigmine-induced c-fos expression in female CA1 (Table 2). None of the *post hoc* analyses showed a significant effect on c-fos expression in male CA1, regardless of treatment.

Hippocampal knockdown of α7 nAChR has modest effects on anxiety-like phenotypes induced by physostigmine in female but not male mice

In the light/dark box test, there was an interaction between physostigmine treatment and α7 nAChR knockdown in female mice ($F(1, 44) = 5.59$) that was not observed in male mice. *Post hoc* analyses indicated that a modest increase in the time spent in the dark side was induced by physostigmine administration in control animals (Table 2), which was

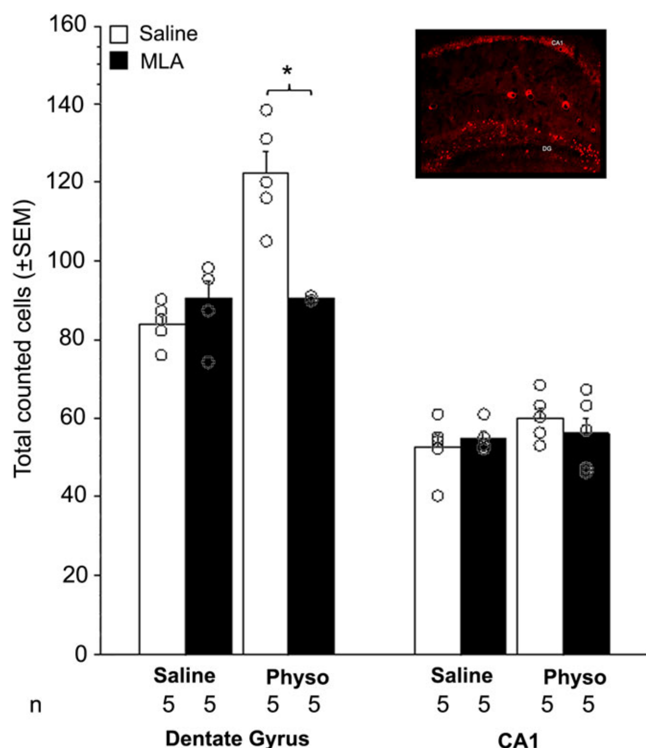

Figure 3

c-fos immunohistochemistry in the hippocampus after systemic MLA treatment. Total number of counted cells in the DG (left) and the CA1 field (right) of the hippocampus in C57BL/6J male mice following injection of MLA (5 mg·kg^{-1}, i.p.) with or without physostigmine (Physo: 0.15 mg·kg^{-1}). Data are expressed as means ± SEM, and dots represent each individual animal; n = 5 mice per treatment group (sum of left and right hippocampi combined). Inset represents an example of c-fos immunohistochemistry in the hippocampus. $^*P < 0.05$, significantly different as indicated; ANOVA and *post hoc t*-test with Bonferroni correction.

absent following knockdown of the α7 nAChR subunit (Table 2). In male mice, there was an overall increase in the time spent in the dark compartment following physostigmine administration (F(1, 36) = 8.4), but α7 knockdown had no effect on its own or following physostigmine treatment (F < 1).

Hippocampal knockdown of α7 nAChR induces some antidepressant-like phenotypes induced by physostigmine but does not alter resilience to social stress

ANOVA revealed a treatment by knockdown interaction in the time spent immobile in male (F(1, 36 = 7.09; Figure 4A), but not female, mice (Table 2). *Post hoc* analyses of the behaviour in male mice indicated that physostigmine significantly increased the time spent immobile, which was reduced to control level in the animals with knockdown of α7 nAChR in the hippocampus. α7 knockdown also modestly increased the time spent immobile in male mice.

In the forced swim test, there was no main effect of physostigmine treatment, knockdown of α7 nAChR or interaction between treatment and knockdown in female mice (Table 2). In male mice, there was an overall increase in time spent immobile induced by physostigmine, which was blunted by α7 knockdown in hippocampus, as shown by the interaction between treatment and knockdown (F(1, 36) = 3.9; Figure 4B). The effect of physostigmine at 5 min was still significantly greater than that of saline, in control mice or with α7 knockdown, as revealed by *post hoc* analyses. At 10 min, further analyses showed that there was complete reversal of the physostigmine effect (F(1, 36) = 17.1) and there was no difference between animals with α7 knockdown in hippocampus and baseline, whether they received saline or physostigmine pretreatment, while the difference was still observed in control animals.

In the social defeat test, knockdown of α7 nAChR in the hippocampus had no effect regardless of physostigmine treatment. This may suggest that these receptors do not contribute to effects of ACh in this behaviour, but this may also be due to a large effect of physostigmine that decreased time spent in the interaction zone by nearly 80%, with or without a CD1 mouse. Indeed, physostigmine-treated mice exhibited thigmotaxis, demonstrating an increase in anxiety-like behaviour which would confound measurement of exploration of the open field and the interaction zone.

Physostigmine significantly reduced ambulatory activity in female (F(1, 36) = 7.4: Table 2) and in male mice (F(1, 41) = 200.1; Figure 2B), potentially due to increased freezing behaviour. However, α7 knockdown did not alter physostigmine's effect on locomotion in an open field in either sex (treatment x knockdown: F < 1), making it unlikely to explain the changes observed in the tail suspension or forced swim test.

Discussion

A number of studies have suggested that elevated ACh signalling contributes to behaviours related to depression, at least in part through actions in the hippocampus, but the role of specific ACh receptor subtypes in hippocampus has not been as well studied. This study focused on the role of α7 nAChRs in the hippocampus and how signalling through this receptor subtype could modulate anxiety- and depression-like phenotypes. Previous studies have highlighted the relevance of targeting the nicotinic cholinergic system to improve depression-related symptoms (Shytle *et al.*, 2002a; Shytle *et al.*, 2002b), most recently by limiting nicotinic signalling through subsets of nAChRs including the α7 nAChR subtype in the amygdala (Mineur *et al.*, 2016). The results presented here indicate that α7 nAChRs in the hippocampus are likely to contribute to some of the depression-like effects of AChE blockade, but not to endophenotypes related to anxiety-like behaviour in male mice. Further, the results suggest that there are sex differences in the role of α7 nAChR signalling, such that male mice are more susceptible to alterations in cholinergic signalling on depression-like in hippocampus, than female mice.

Systemic administration of the α7 nAChR antagonist MLA resulted in acute, anxiolytic- and antidepressant-like effects, as has been observed previously in male mice (Andreasen *et al.*, 2009). MLA administration decreased immobility in

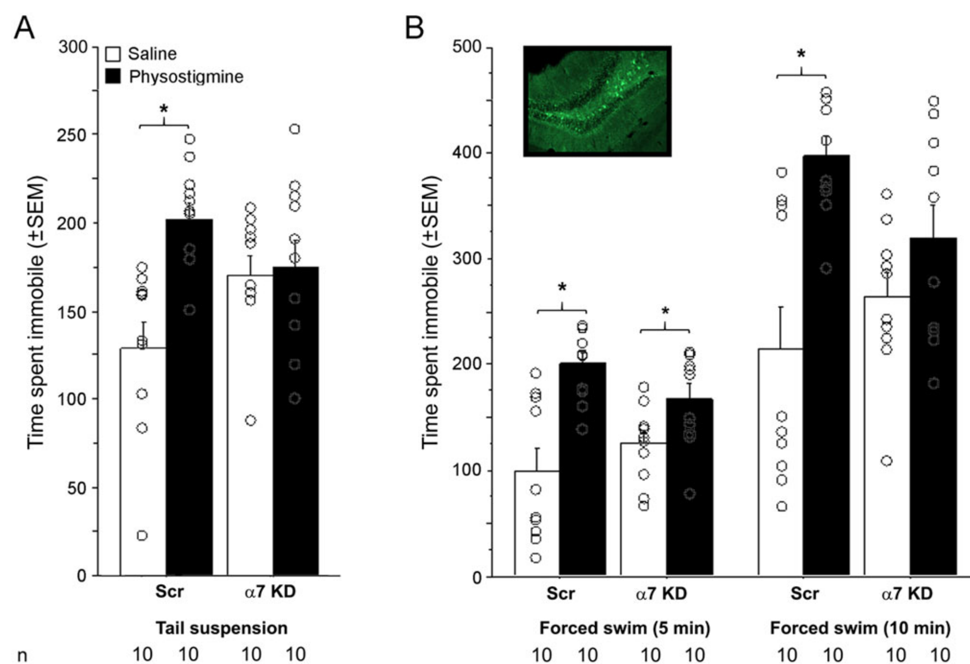

Figure 4

Effect of α7 nAChR knockdown on behaviour in tests of depression-like phenotypes. Time spent immobile in (A) the tail suspension and (B) forced swim tests following α7 nAChR knockdown in the hippocampus of C57BL/6J male mice. In the forced swim test, data are split into 5- and 10-min time bins and represent the same assay; 10 animals were used per treatment group in the final analyses. Data are expressed as means ± SEM, and dots represent individual data points. Inset represents an example of viral infection (GFP-positive cells in the dorsal hippocampus/DG). $^{*}P < 0.05$, significantly different as indicated; ANOVA and partial least squares differences *post hoc* tests, for each time point.

the tail suspension test, but variability in the behaviour was observed, making the results difficult to interpret. In the forced swim test, however a similar behavioural pattern was observed with greater homogeneity, suggesting that MLA had an antidepressant-like effect. Although these paradigms have been validated with acute antidepressant administration, further studies using chronic administration regimens would also be of interest to determine whether long-term blockade of α7 nAChRs results in differential effects in these behaviours.

Interestingly, most of the effects of knocking down α7 nAChR expression on depression-like behaviours were not seen at baseline and were only observed when mice were injected with physostigmine to block AChE activity and increase ACh tone. There is, however, a significant increase in immobility in the tail suspension test at baseline following knockdown of α7 nAChRs in hippocampus. This may be due to the fact that α7 nAChRs are expressed by both GABA and glutamate neurons in the hippocampus and can therefore regulate excitatory/inhibitory balance in this brain structure (Siok *et al.*, 2006; Stoiljkovic *et al.*, 2015a; Stoiljkovic *et al.*, 2015b). It is therefore possible that α7 nAChRs have effects on the two opposing classes of neurons that both have behavioural consequences. It is also possible that knockdown of the subunit alters the dynamics of signalling in the hippocampus and results in a baseline change in behaviour in the tail suspension test. These issues will be of interest for future studies. These data also suggest that limiting α7 nAChR signalling in the hippocampus is only relevant when ACh levels are increased, as can occur in this structure following stress (Gilad, 1987; Mark *et al.*, 1996). Conversely, this also indicates that when cholinergic signalling is 'normal', α7 nAChR signalling does not induce depressive-like phenotypes.

The forced swim test was initially validated as a two-day paradigm in rats to potentiate immobility that was not significant on day 1 (Porsolt *et al.*, 1977). Thus, plasticity mediated through α7 nAChRs may contribute to the change in immobility across days and could explain the difference in immobility seen in following α7 nAChR knockdown in hippocampus. While we cannot exclude a learning component that would account for some of the results observed, the 'learning' in the forced swim test across days is thought to be related to the process of behavioural despair, so the plasticity component would be relevant to the action of antidepressant medications. In addition, hippocampal α7 nAChR knockdown recapitulated only a subset of behaviours altered by systemic MLA administration. Interestingly, the effects of hippocampal α7 nAChR knockdown were limited to behaviours responsive to acute injection with antidepressants (forced swim and tail suspension) and were not observed in a stress-induced behaviour that is sensitive to chronic antidepressant administration in mice (social defeat). Forced swim and tail suspension are responsive to acute administration of a broad spectrum of antidepressant medications effective in human depressed individuals. It is therefore possible that these two tests measure changes in brain activity that result early in the antidepressant response, before the full effects are instantiated in human patients. The current experiments

showing that α7 nAChR knockdown in hippocampus is only effective in altering behaviour in these tests following increased cholinergic signalling could be an important step toward identifying the initial changes in neuronal activity necessary for later antidepressant efficacy following chronic administration.

MLA also had significant effects in the absence of physostigmine administration, while knockdown of α7 nAChRs in the hippocampus did not. These data provide hints that hippocampal cell body α7 nAChRs regulate neuronal circuits that are responsible for the initial effects of antidepressants and that MLA exerts some of its antidepressant-like effects through nAChRs in other brain areas in addition to the hippocampus. This could include α7 nAChRs on neuronal terminals in hippocampus from other brain areas, which would not be affected by AAV-2-mediated shRNA delivery to hippocampal cell bodies. We have previously shown that knockdown of α7 nAChRs in the amygdala has anxiolytic and antidepressant-like effects that are distinct, though overlapping, with those observed following hippocampal α7 nAChR knockdown (Mineur *et al.*, 2016). It is therefore likely that the combined effect of systemic MLA in the hippocampus, the amygdala, and potentially other brain areas may synergize to induce a stronger antidepressant-like effect in the forced swim test. We should note that at higher concentrations, MLA can block numerous nAChR subtypes. While studies have shown that MLA penetrates the blood–brain barrier, it is also cleared rapidly and has limited bioavailability (Ballesta *et al.*, 2012). As a result, it is difficult to evaluate the local concentration in brain following peripheral administration.

As stimulation of α7 nAChRs with GTS-21 did not alter behaviours related to anxiety, depression or social stress resilience, contrary to blockade of AChE, stimulation of the α7 nAChR subtype alone is likely not sufficient to mediate the effects of increased cholinergic signalling. This suggests that ACh signalling through multiple receptor subtypes, including heteromeric nAChRs and muscarinic AChRs, is necessary to induce a depression-like state. This may explain why antagonism of either nAChRs or muscarinic AChRs is sufficient to reverse the effects of physostigmine in a mouse model (Mineur *et al.*, 2013). Future studies should focus on determining how multiple nAChR subtypes mediate the coordinated effect of ACh signalling on these complex behaviours. Another possibility could be that knockdown of the α7 nAChR results in altered expression of other nAChR subtypes or synaptic remodelling in hippocampal neurons sensitive to ACh. We do not believe this is likely, however, since previous experiments have not identified any changes in expression of any other nAChR subunits in mice lacking specific subunits of the nAChR (Picciotto *et al.*, 2001; Champtiaux and Changeux, 2004). Furthermore, α7 knockout mice do not show any gross abnormalities that would suggest a compensatory mechanism.

Significant sex differences in response to cholinergic manipulations were observed in the current study. Physostigmine had only a small effect on depression-like behaviour in female mice, limiting the ability to measure the ability of knockdown of α7 nAChRs in the hippocampus to reverse these phenotypes. Studies in human subjects suggest that female smokers may have higher ACh tone at baseline (Zhang *et al.*, 2016). As a result, physostigmine may not increase ACh further in female subjects. However, pharmacological treatment with MLA did not result in significant changes in depression-like behaviours in female mice either. Similar sex differences have been reported for antidepressant-like effects of nicotinic drugs in previous studies using C57BL/6J mice, and specific nicotinic compounds can even induce opposite effects across tests of antidepressant efficacy. For instance, nicotine increases immobility time in female, but not male, C57BL/6J mice, while MLA increases activity in the tail suspension test but decreases it in forced swim test in NMRI female mice (Andreasen and Redrobe, 2009; Andreasen *et al.*, 2009). These results could indicate that female mice are less sensitive to the effects of MLA or that pathways regulating depression-like behaviours in female mice are less sensitive to regulation by ACh or α7 nAChR signalling. It is also possible that oestrus cycle variation could affect behaviour in the tests used here as well as the response to stress, limiting the ability to observe significant effects in female mice. We have, however, not observed greater variability in the behavioural responses of female mice compared with males that would suggest behavioural differences across the oestrus cycle. It would nevertheless be of interest to determine whether hormonal signalling could contribute to the effects of α7 nAChR manipulations performed here.

Measurements of c-fos immunoreactivity in hippocampus are consistent with the sex differences observed in the effects of MLA on behaviour in the tail suspension and forced swim tests. MLA significantly decreased DG c-fos expression in male, but not female, mice. α7 nAChRs are abundantly expressed in the hippocampus, with highest expression in all layers of the CA3 region, the pyramidal layer of the CA1 region and the granule cell layer of the DG (Fabian-Fine *et al.*, 2001). A number of studies have identified physiological effects of α7 nAChR signalling in the DG that could contribute to depression-like behaviours and response to antidepressants. For instance, α7 nAChRs are expressed on immature granule cells and are involved in maturation of dendrites (John *et al.*, 2015), whereas decreased α7 expression reduces cell proliferation in the DG (Koike *et al.*, 2004). Antidepressants increase proliferation and survival of neurons in the adult DG, and this has been shown to contribute to antidepressant response (Chen *et al.*, 2000; Santarelli *et al.*, 2003; Mahar *et al.*, 2014). Prenatal stress, which can induce depression-like phenotypes, decreases α7 nAChR expression, with some of the strongest effects observed in DG (Baier *et al.*, 2015). Finally, stimulation of α7 nAChRs can potentiate glutamate signalling in hippocampus, (Matrisciano *et al.*, 2008; Duric *et al.*, 2013). α7 nAChRs are also expressed on subgranular interneurons of the DG and enhance inhibition of granule cells following glutamate stimulation (Frazier *et al.*, 2003). This mechanism is believed to regulate the activity of glutamatergic afferents from the entorhinal cortex and cholinergic afferents from the medial septum, which could, in turn, regulate hippocampal functions related to depression-like behaviours. Blocking α7 nAChR on glutamatergic terminals would therefore limit the ACh-mediated release of glutamate, which could lead to

similar antidepressant-like effects as that seen following glutamatergic receptor antagonism.

Surprisingly, knockdown of α7 nAChRs in the hippocampus in male mice selectively reversed the effects of physostigmine on a subset of behaviours sensitive to acute treatment with antidepressants, but not behaviours sensitive to chronic antidepressant administration (social defeat) or anxiolytic medications (light–dark test), although a modest anxiolytic effect of knockdown was observed in female mice. This is in contrast to knockdown of AChE in hippocampus, which affects all these behaviours in male mice (Mineur *et al.*, 2013). Unlike effects in hippocampus, decreasing α7 nAChR expression in amygdala reverses both physostigmine-induced anxiety- and depression-like behaviours (Mineur *et al.*, 2016). Anxiety and depression are often co-morbid in human subjects and depend on hippocampal function (Hyman, 1998; de Carvalho *et al.*, 2010), whereas anxiety is more strongly associated with amygdala function (Etkin and Wager, 2007). The experiments reported here suggest that cholinergic signalling in hippocampus can alter both anxiety- and depression-like behaviours, but that α7 nAChRs in this structure are not essential for aspects of cholinergic function related to anxiety-like behaviours in male mice.

The current results suggest that α7 nAChRs in the hippocampus can participate in some of the effects of elevated ACh signalling in hippocampus relevant to depression-like behaviours in male mice. However, additional mechanisms also contribute to the effects of heightened ACh signalling on depression-like behaviours. For example, blocking α7 or β2 nAChRs in the amygdala have effects on these behaviours at baseline and reverse several effects of physostigmine administration as well (Mineur *et al.*, 2016). Furthermore, limiting α7 nAChR signalling can reverse some of the depression-like effects induced by physostigmine, but the primary mechanism of action of physostigmine-induced phenotypes could be unrelated to ACh. It would be of interest to determine whether a similar antidepressant-like effect could be observed in additional models of behaviour related to depression. The results also highlight sex differences in responses to cholinergic manipulations that should be considered when evaluating the contribution of ACh signalling to depression in animal studies and in human subjects. Further exploration of the circuitry modulated by α7 nAChRs could provide insights into the development of depressive disorders and associated neuronal pathways.

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Author contributions

Y.S.M. designed the experiments, conducted the experiments, analysed the data and wrote the manuscript; T.N.M. and S.B. conducted the experiments and analysed the data; M.R.P. designed the experiments, analysed the data and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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