

Bidirectional Regulation of Aggression in Mice by Hippocampal Alpha-7 Nicotinic Acetylcholine Receptors

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Humans with 15q13.3 microdeletion syndrome (15q13.3DS) are typically hemizygous for *CHRNA7*, the gene coding for the $\alpha 7$ nicotinic acetylcholine receptor (nAChR), and manifest a variable neuropsychiatric phenotype that frequently includes persistent aggression. In mice, nAChR activation by nicotine is anti-aggressive, or 'serenic,' an effect which requires $\alpha 7$ nAChRs and is recapitulated by GTS-21, an $\alpha 7$ nAChR partial agonist. Pharmacotherapies potentiating $\alpha 7$ nAChR signaling have also been shown to reduce aggression in human 15q13.3DS. These findings identify the $\alpha 7$ nAChR as an important regulator of aggressive behavior, but the underlying neurobiological substrates remain to be determined. We therefore investigated the brain regions and potential neural circuits in which $\alpha 7$ nAChRs regulate aggressive behavior in male mice. As in 15q13.3DS, mice heterozygous for *Chma7* were significantly more aggressive compared to wild-type controls in the resident-intruder test. We subsequently examined the hippocampus, where $\alpha 7$ nAChRs are highly expressed, particularly in GABAergic interneurons. Resident-intruder interactions strongly activated granule cells in the dentate gyrus (DG). In contrast, GTS-21, which reduces aggression in mice, reduced DG granule cell activity during resident-intruder interactions. Short hairpin RNA knockdown of *Chma7* in the DG enhanced baseline aggression and eliminated the serenic effects of both nicotine and GTS-21 on attack latency. These data further implicate $\alpha 7$ nAChRs in regulation of aggression, and demonstrate that hippocampal $\alpha 7$ nAChR signaling is necessary and sufficient to limit aggression. These findings suggest that nAChR-mediated regulation of hippocampal excitatory-inhibitory balance could be a promising therapeutic intervention for aggression arising in certain forms of neuropsychiatric disease.

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INTRODUCTION

Improved understanding of neural circuits contributing to aggression is necessary to develop more effective therapeutic options for patients, including those with neuropsychiatric disorders. Syndromes associated with well-defined abnormalities at the genetic level can provide an important starting point to understand fundamental pathophysiological mechanisms. 15q13.3 microdeletion syndrome (15q13.3DS; OMIM #612001) is a rare condition that results from the loss of part of the proximal portion of chromosome 15, an unstable genetic region prone to deletions and duplications (Sharp *et al*, 2008; Shinawi *et al*, 2009). The most common form of 15q13.3DS results from an ~1.5 megabase deletion that encompasses roughly six genes, including *CHRNA7*, the gene encoding the excitatory ligand-gated $\alpha 7$ nicotinic acetylcholine receptor (nAChR). This deletion is highly penetrant and results in a variable phenotype that frequently includes intellectual disability, seizures, neuropsychiatric disorders including autism spectrum disorder (ASD) or

schizophrenia, and behavioral problems including aggression (Sharp *et al*, 2008; Shinawi *et al*, 2009). Similar phenotypes are reported from smaller deletions encompassing only *CHRNA7* and in at least some cases a portion of a neighboring gene, *OTUD7A* (Hoppman-Chaney *et al*, 2013; Masurel-Paulet *et al*, 2010; Mikhail *et al*, 2011; Shinawi *et al*, 2009). Furthermore, children and adolescents with aggressive behaviors severe enough to warrant antipsychotic treatment were recently reported to be far more likely to harbor deletions of *CHRNA7* than the general population, with *CHRNA7* deletion conferring an odds ratio of 184 for aggression (Gillentine *et al*, 2017). Mouse models of 15q13.3DS recapitulate multiple phenotypic aspects of the disorder, including abnormal electroencephalography and neuronal excitability, abnormal spatial memory and attention, and aggressive behavior following mild stressors (Fejgin *et al*, 2014; Nilsson *et al*, 2016). Taken together, evidence from human and animal studies suggests that abnormal genetic dosage of *CHRNA7* and related neuronal signaling disturbance likely plays a causal role in some neurobehavioral aspects of the 15q13.3DS, including aggression regulation.

In a mouse model of aggression, we recently found the $\alpha 7$ nAChR was necessary for the anti-aggressive, or 'serenic' effects of systemic administration of the pan-nAChR agonist nicotine, and that GTS-21, an $\alpha 7$ nAChR partial agonist,

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recapitulated the serenic effects of nicotine (Lewis *et al*, 2015). In humans, galantamine, a positive allosteric modulator at the $\alpha 7$ nAChR and an acetylcholinesterase inhibitor, was reported to successfully reduce persistent, severe rage outbursts in a man with 15q13.3DS (Cubells *et al*, 2011), while transdermal nicotine successfully reduced persistent aggressive behavior in an adolescent with idiopathic ASD (Van Schalkwyk *et al*, 2015). Despite genetic and pharmacological evidence linking *CHRNA7* to pathological aggressivity, the brain regions and neural circuitry important for aggression regulated by $\alpha 7$ nAChRs are not well understood. The $\alpha 7$ nAChR is highly expressed in the hippocampus of both mice (Baddick and Marks, 2011) and humans (Rubboli *et al*, 1994), where it is preferentially expressed on GABAergic interneurons and can inhibit activity of principal cells in multiple hippocampal regions (Frazier *et al*, 2003; Freedman *et al*, 1993; Ji and Dani, 2000). This organization suggests $\alpha 7$ nAChR signaling might regulate excitatory–inhibitory (E/I) balance in the hippocampus, which is commonly altered in neurodevelopmental conditions (Gao and Penzes, 2015) and directly linked to aggressive behavior (Kohl *et al*, 2013; van der Kooij *et al*, 2014).

We therefore investigated the brain regions critical to the regulation of aggressive behavior by $\alpha 7$ nAChR signaling in mice, focusing on the hippocampus. Our results have important implications for better understanding how neuromodulatory systems might influence brain regions within larger networks important for aggression. Furthermore, our results lend additional support to targeting E/I imbalance for treatment of pathological aggression, including pharmacological agents active at $\alpha 7$ nAChRs, which are well tolerated in human studies (Freedman, 2014).

MATERIALS AND METHODS

Animals

Wild-type (WT) male C57BL/6 (B6) and Balb/c (B/c) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and habituated to the animal facility for at least 1 week. Male mice heterozygous for a null mutation of the $\alpha 7$ subunit (HET) (Orr-Urtreger *et al*, 1997) maintained on the B6 background for over 30 generations were bred with female B/c mice to generate F1 WT and HET littermates. As B/c mice are significantly more aggressive than B6 mice, (Schneider *et al*, 1992), the enhanced baseline aggressivity of these F1 crosses leads to an increased number of bouts of aggressive behavior, and therefore greater power to detect small or medium effect sizes of *Chrna7* heterozygosity using moderate sample sizes. Expression of $\alpha 7$ nAChRs was previously shown not to differ significantly between B6 and B/c in multiple brain regions (Marks *et al*, 1983). Mice were housed under standard conditions with temperature maintained at $21 \pm 2^\circ\text{C}$ and a 12 h light–dark cycle with food and water available *ad libitum*. The Yale University Animal Care and Use Committee approved all procedures.

Drugs

All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS, 0.1 M, pH 7.3) + 0.2% DMSO for picrotoxin experiments or

PBS only for all other experiments. Nicotine hydrogen bitartrate was used for nicotine solutions, which were adjusted to pH 7.0 ± 0.5 with 1 M sodium hydroxide and doses expressed as free base. All solutions were injected intraperitoneally (i.p.) 10–15 min prior to the start of resident–intruder tests at a volume of 10 ml/kg body mass. Drug administration was counterbalanced across animals for repeated-measures experiments.

Resident–Intruder Test

Resident–intruder tests were performed exactly as described previously (Lewis *et al*, 2015) and detailed in the Supplementary Materials and Methods.

Locomotor Activity

Mice were habituated to the test room for 1 h, then placed in a clean, clear plexiglass rat cage for 30 min. Activity was measured by horizontal and vertical infrared sensors (Fusion HC version 1.5, AccuScan Instruments, Inc., Columbus, OH, USA).

Hippocampal Infusion of Adeno-Associated Viral Vectors Expressing Small Hairpin RNA (shRNA) Constructs

Design, purification, and validation of viral vectors are described in Supplementary Materials and Methods. Viral vectors were infused bilaterally into the hippocampus using a stereotaxic frame mounted with 5 μl Hamilton syringes under 2% isoflurane anesthesia. An aliquot of 2 μl of purified virus was infused into each side of the intermediate hippocampus (coordinates: anterior/posterior = -3.08 mm, lateral = ± 3.0 mm, dorsal/ventral = -3.2 mm) over the course of 10 min, and the syringe left in place for an additional 5 min to reduce backflow. Mice were subsequently single housed and allowed to recover for 3 weeks prior to behavioral testing. After testing, viral infusion sites were identified by GFP fluorescence in 40 μm brain slices from paraformaldehyde (PFA)-perfused animals. Mice with evidence of GFP-positive cells in dentate gyrus (DG; either unilateral or bilateral) were included in behavioral analysis.

Binding and Immunofluorescence (IF)

α -bungarotoxin binding was performed as previously described (Mineur *et al*, 2016). Cardiac perfusion, tissue sectioning, and IF staining for c-Fos were performed as previously described (Lewis *et al*, 2015). Staining for Arc (guinea pig, 1 : 1000, Synaptic Systems 156005, Goettingen, Germany), CaMKII (mouse, 1 : 1000, Abcam ab22609 [6G9], Cambridge, MA, USA), GABA (rabbit, 1 : 500, Sigma-Aldrich A2052), or GFP (rabbit, 1 : 1000, Life Technologies 11122, Carlsbad, CA, USA) was performed as detailed in the Supplementary Materials and Methods. For Arc-staining experiments, mice were perfused 90 min following the initiation of resident–intruder tests.

Microscopy and Image Analysis

Images were acquired with an FV10i confocal microscope (Olympus, Tokyo, Japan) equipped with UPLSAP $\times 10$ (numerical aperture (NA) = 0.4) and $\times 60$ (NA = 1.2) objectives. Acquisition software was Olympus Fluoview Version 4.1a. Image analysis of *c-Fos*-stained sections was performed as previously described (Lewis *et al*, 2015). For Arc IF analysis, area of the DG upper blade was quantified by thresholding the DAPI image using Fiji (Schindelin *et al*, 2012), then Arc-positive cells were manually counted blind to condition and normalized to area. Only the DG upper blade was quantified as Arc expression in this region is substantially more dynamic than in the lower blade (Snyder *et al*, 2009). Data from left and right sides of two tissue sections were then averaged to obtain a single value per mouse from rostral or caudal DG. A single confocal z-slice was used for Arc quantification as well as for co-labeling experiments. Image adjustments were limited to changes in contrast, which were applied equally to the entire image and equally to experimental and control images for immunofluorescent target proteins.

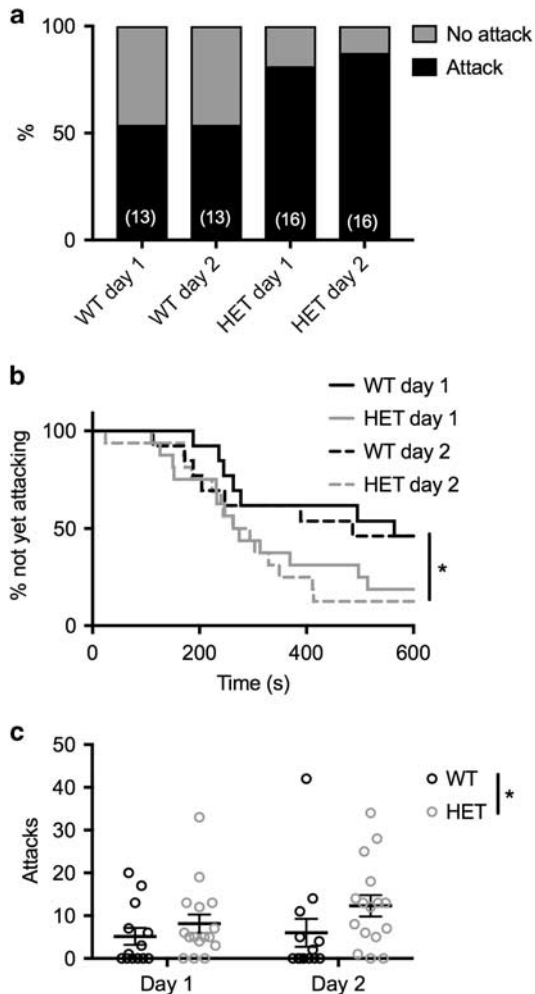


Figure 1 $\alpha 7$ nAChR HET mice on F1 C57BL/6 \times BALB/c background initiate attack in a greater percentage of bouts (a), initiate attack faster (b), and attack more (c) than WT littermates in resident–intruder tests. For all panels, $N = 13$, WT; $N = 16$, HET. Data in c are expressed as mean \pm SEM. * $p \leq 0.05$ WT vs HET.

Statistical Analysis

Ordinary or repeated-measures (RM) analysis of variance (ANOVA) was used, as appropriate, to analyze the number of attacks and immediate early gene (IEG) staining. Between subject factors included ‘genotype,’ ‘intruder,’ and ‘shRNA virus.’ Within-subject factors included ‘drug,’ ‘day,’ and ‘rostrocaudal location.’ For violations of normality of error residuals in number of attack analysis, a $y = \log(y+1)$ transformation was used. Multiple comparisons to a control group were corrected using Dunnett’s test. Analysis of attack latency was performed using parametric multi-level survival analysis exactly as previously described (Lewis *et al*, 2015). When low variability of data precluded appropriate use of survival analysis, RM-ANOVA on ranked dependent variable was performed (Zimmerman and Zumbo, 1993). Correlations were performed using Pearson correlation. All tests were two-tailed. Graphical results depict mean \pm standard error of the mean (SEM) unless otherwise specified. Values of $p \leq 0.05$ were considered significant. Survival analyses were conducted in Stata version 13.1, all other analyses were conducted using Graphpad Prism7 and SPSS (IBM, Version 24) for Mac.

RESULTS

$\alpha 7$ HET Mice Demonstrate Shorter Attack Latency and More Attacks than WT Littermates

Resident–intruder tests were performed to determine the effects of *Chrna7* dose on aggressive behavior. HET mice rather than *Chrna7* homozygous knockout mice were studied because most humans with 15q13.3DS are hemizygous at *CHRNA7*. While *Chrna7* homozygous knockout mice are viable, humans with homozygous loss at 15q13.3 demonstrate profound neurological impairments, including encephalopathy and hypotonia (Spielmann *et al*, 2011), thus, it is unclear if aggressivity is a component of this condition. HET mice initiated attack in more bouts than did WT littermates on both test days (Figure 1a). HET mice also attacked significantly faster than WT littermates (HR = 5.11; 95% confidence interval (CI) = 1.03, 25.48; $p = 0.046$) (Figure 1b). Analysis of variance revealed an effect of genotype on number of attacks ($F(1, 27) = 5.93$; $p = 0.022$), no significant effect of day ($F(1, 27) = 0.46$, $p = 0.50$), and no significant attack \times day interaction ($F(1, 27) = 1.19$, $p = 0.29$) (Figure 1c). No significant differences in locomotor properties were observed between WT and HET mice (Supplementary Figure S1). These data suggest that altering $\alpha 7$ nAChR gene dose is associated with an enhanced aggression phenotype, as observed in both mouse models and humans with 15q13.3DS.

Hippocampal Immediate Early Gene (IEG) Expression is Upregulated by Resident–Intruder Interactions and Reduced by $\alpha 7$ Agonist

In situ hybridization for *Chrna7* from the Allen Brain Atlas (Lein *et al*, 2007) and α -bungarotoxin binding to assembled $\alpha 7$ nAChRs verified a high level of hippocampal expression of this nAChR subtype in WT B6 mice (Figure 2a), consistent with previous studies (Baddick and Marks,

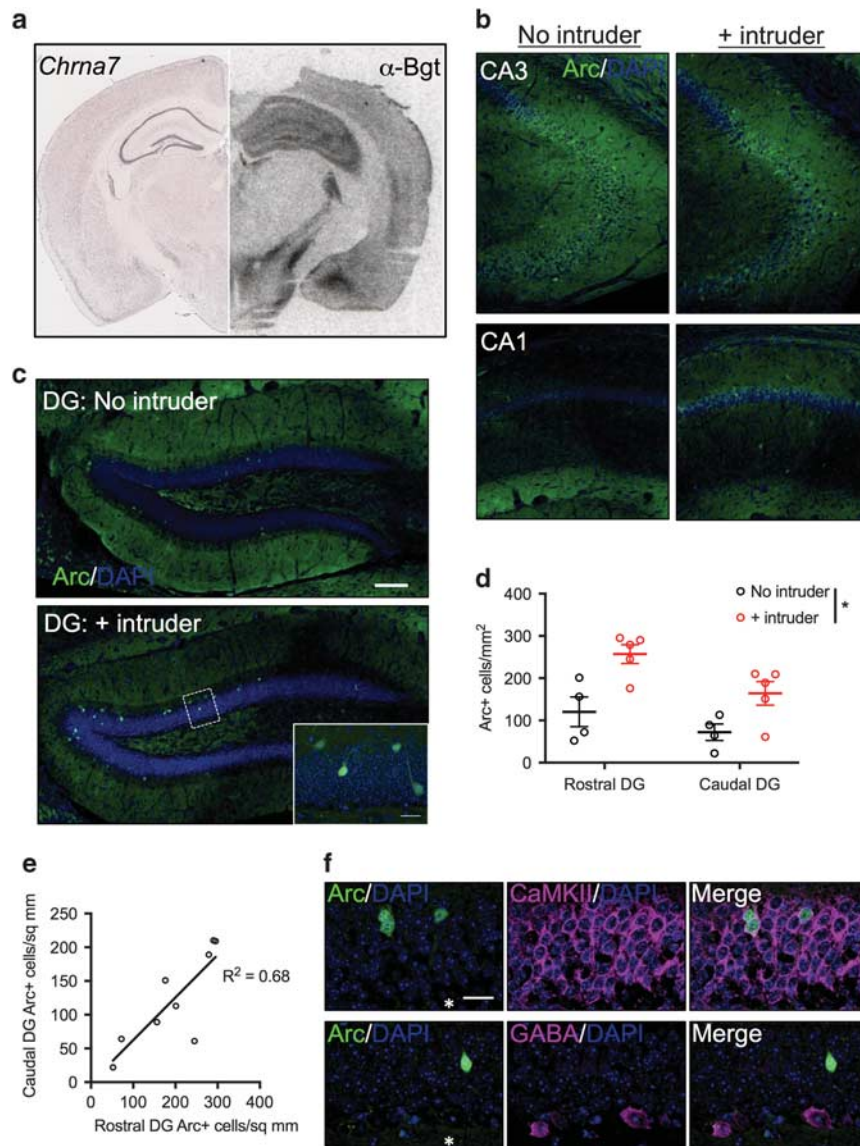


Figure 2 Resident–intruder interaction upregulates Arc immunoreactivity in rostral and caudal DG GCs of resident mice. *In situ* hybridization (ISH) for *Chrna7* (a, left) and α -bungarotoxin (α -Bgt) binding (a, right) demonstrate the enrichment of mRNA and assembled $\alpha 7$ nAChRs, respectively, in mouse hippocampus. Immunostaining for the immediate early gene Arc following resident–intruder tests in subregions of hippocampus reveals limited upregulation in CA3 and CA1 (b), with strong upregulation in DG (c, d, $N = 4$, no intruder; $N = 5$, + intruder). Inset in lower panel of c is collapsed Z-stack of outlined region. Arc upregulation was similar in both rostral and caudal regions of the DG (e, $N = 9$). Arc staining in the DG GC layer colocalizes with CaMKII but not with GABA, suggesting the activated cells are excitatory DG GCs (f). Data are expressed as mean \pm SEM. * $p \leq 0.05$. ISH in a is reproduced from the Allen Mouse Brain Atlas (Lein *et al*, 2007), *Chrna7*-74511738, image 254 (<http://mouse.brain-map.org/experiment/siv?id=74511738&imageId=74362605&initImage=ish&coordSystem=pixel&x=5064.5&y=3744.5&z=1>). Asterisk in f denotes DG hilus. Scale bar in c (also applies to b): 100 microns. Scale bar in c inset and f: 20 microns.

2011). We thus focused on identifying hippocampal subregions strongly activated by aggressive interactions to determine whether these regions are regulated by $\alpha 7$ agonists with serenic effects. Staining for the IEG Arc in WT B6 mice did not appear qualitatively different in areas CA1 and CA3 following aggressive resident–intruder interactions in the home cage compared to home cage controls (Figure 2b). However, Arc was strongly upregulated in the granule cell (GC) layer of the DG in both rostral (dorsal) and caudal (ventral) regions under these conditions (Figure 2c and d and Supplementary Figure S2). Analysis of variance revealed main effects of intruder introduction ($F(1, 7) = 11.28$,

$p = 0.012$) and rostrocaudal location ($F(1, 7) = 18.24$, $p = 0.0037$). The intruder \times location interaction was not significant ($F(1, 7) = 1.83$, $p = 0.22$). A correlation between rostral and caudal DG Arc staining was observed ($R^2 = 0.68$, $p = 0.0064$), illustrating that DG GCs are activated across the rostrocaudal axis by aggressive encounters (Figure 2e).

Arc-positive cells consistently colocalized with CaMKII, a marker of DG GCs, but did not colocalize with GABA-positive cells, which were frequently found in the subgranular region of the DG (Figure 2f).

Dentate gyrus GC activation as a result of aggressive interactions suggests the DG may contribute to a network of

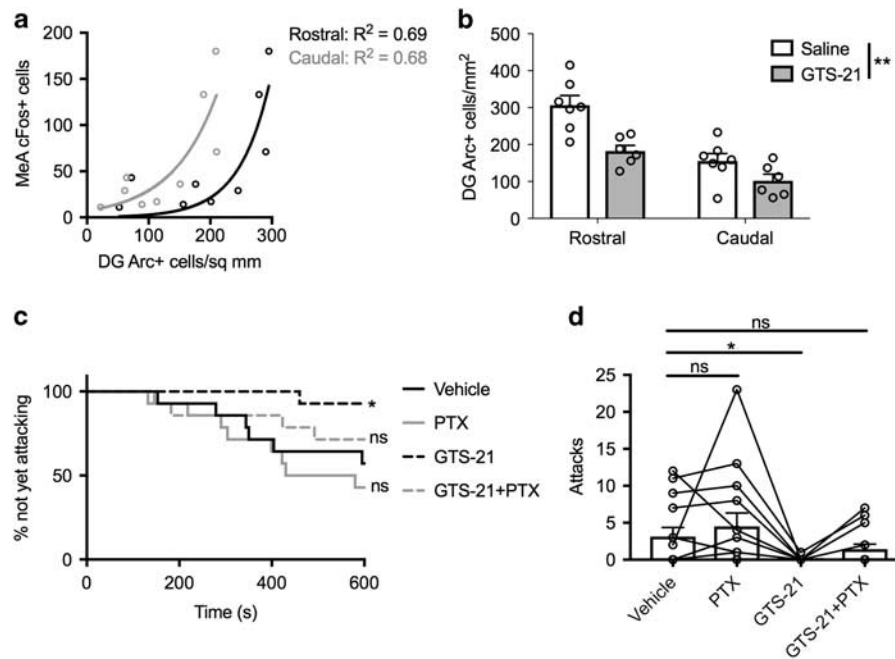


Figure 3 DG GC activity correlates with activity in other brain regions important for aggressive behavior, and the $\alpha 7$ nAChR partial agonist GTS-21 reduces DG GC activity. Arc immunostaining in rostral or caudal DG GCs and cFos immunostaining in medial amygdala (MeA) is fit by an exponential model (a, $N = 9$). GTS-21 (17 mg/kg) significantly reduced both rostral and caudal DG GC activity compared to saline, as measured by the number of Arc-positive neurons in mice undergoing resident-intruder tests that did not culminate in attack (b, $N = 7$, saline; $N = 6$, GTS-21). The GABA-A antagonist picrotoxin (PTX, 0.1 mg/kg) blunted the ability of GTS-21 (17 mg/kg) to delay attack initiation (c) and to reduce total number of attacks (d) (c, d, $N = 14$ for all treatments, within-subject). Data are expressed as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$ vs vehicle treated group; ns, not significant.

brain regions regulating aggressive behavior. We therefore investigated the relationship between IEG staining in the rostral and caudal DG, and IEG staining in the posterior part of the medial amygdala, which contains populations of neurons critically important and highly specific for aggression in male mice (Unger *et al*, 2015). This within-brain correlation was fit by an exponential curve in both rostral and caudal DG, suggesting a threshold of DG activity may be permissive for further activation of brain regions involved in aggression initiation (Figure 3a). In this case, $\alpha 7$ agonists such as GTS-21, which delay attack onset (Lewis *et al*, 2015), might inhibit DG activation through enhancement of fast GABA-A-mediated inhibitory neurotransmission onto DG GCs (Frazier *et al*, 2003). To test this, we treated WT B6 mice undergoing resident-intruder tests with saline or GTS-21 (17 mg/kg), followed by Arc staining in rostral and caudal DG. We performed Arc staining in non-attacking animals, which allowed isolation of neural activity during a period when the resident is appraising the social environment and not confounded by motor or other processes related to attack initiation itself. Consistent with our hypothesis, ANOVA revealed a main effect of GTS-21 to reduce DG GC Arc staining ($F(1, 11) = 14.82$, $p = 0.0027$), a main effect of rostrocaudal location ($F(1, 11) = 37.36$, $p < 0.0001$), and no significant drug \times location interaction ($F(1, 11) = 3.52$, $p = 0.088$) (Figure 3b and Supplementary Figure S3). To further test the necessity of GABA-A signaling for the serenic effects of GTS-21, we systemically co-administered a non-epileptogenic low dose of the GABA-A antagonist picrotoxin (PTX, 0.1 mg/kg) with GTS-21 (17 mg/kg) in WT B6 mice (Figure 3c and d). GTS-21 significantly delayed time to

attack and reduced number of attacks in B6 mice compared to vehicle. However, the addition of PTX to GTS-21 blunted its serenic effects, despite PTX itself not significantly altering aggressive behavior compared to vehicle (attack latency: $F(3, 39) = 6.13$, $p = 0.0016$; post hoc vs vehicle (Dunnett corrected): PTX, $p = 0.53$; GTS-21, $p = 0.014$; GTS-21 + PTX, $p = 0.57$; number of attacks: $F(3, 39) = 6.24$, $p = 0.0014$; post hoc vs vehicle (Dunnett corrected): PTX, $p = 0.64$; GTS-21, $p = 0.010$; GTS-21 + PTX, $p = 0.38$). Taken together, these results demonstrate DG GC activity is strongly upregulated by aggressive interactions, and suggest that $\alpha 7$ nAChR agonists such as GTS-21 and nicotine may lengthen the period before aggression initiation through enhancement of inhibitory GABAergic signaling in the $\alpha 7$ nAChR-rich DG.

Knockdown of $\alpha 7$ nAChRs in the DG Speeds Attack Onset and Blunts Serenic Effects of Nicotine and GTS-21

To determine more directly whether $\alpha 7$ nAChRs in the DG regulate aggressive behavior and are necessary for the serenic effects of $\alpha 7$ agonists, we infused adeno-associated virus 2 (AAV2) expressing shRNAs targeting $\alpha 7$ nAChRs, or a scrambled control, bilaterally into DG of WT male B/c mice (Figure 4a). The B/c strain was used in these experiments to enhance the number of bouts resulting in aggressive behavior (see 'Methods' section). Knockdown of $\alpha 7$ significantly shortened the latency to attack across 3 days of testing (HR = 3.18; 95% CI = 1.01, 9.97; $p = 0.048$) (Figure 4b–e). In contrast, there was no significant effect of virus on number of attacks ($F(1, 13) = 0.55$, $p = 0.47$) (Figure 4f). We next tested whether $\alpha 7$ in the DG was necessary for the effects of

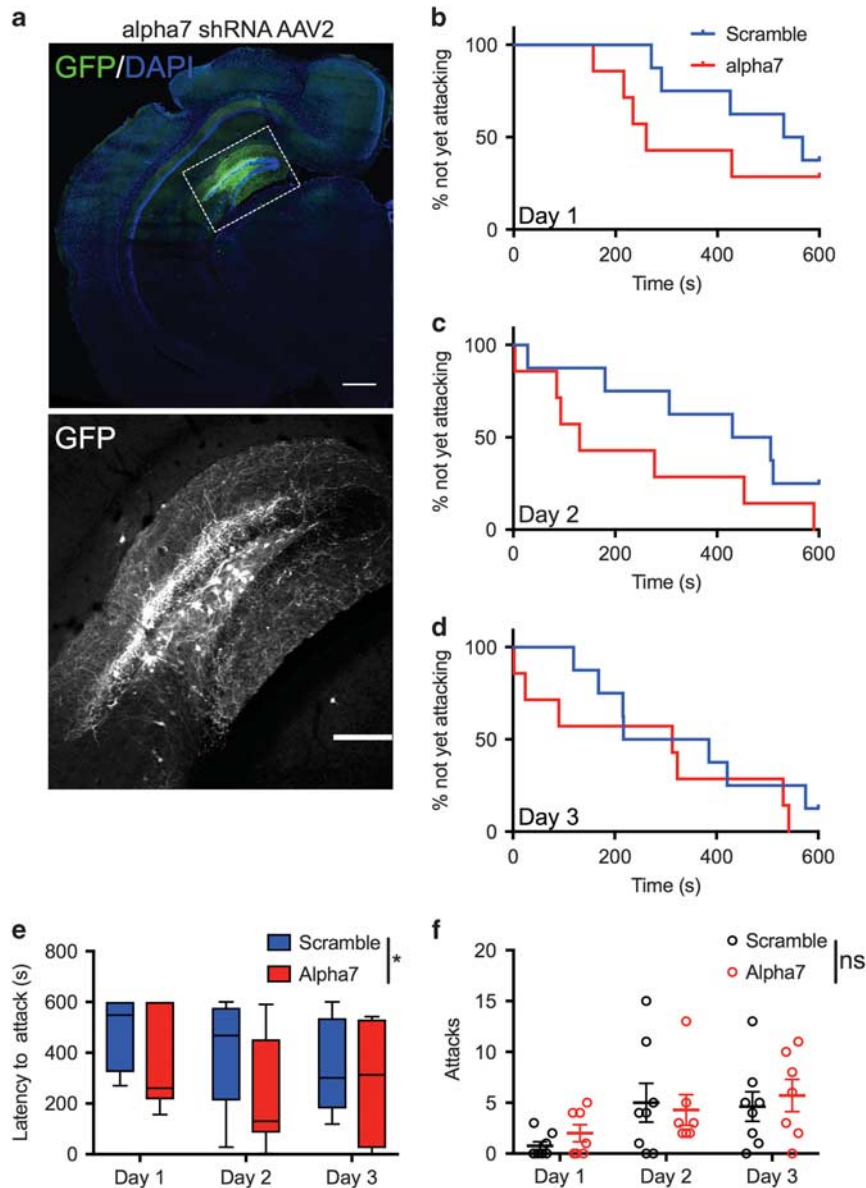


Figure 4 Delivery of shRNA targeting $\alpha 7$ nAChRs into the DG shortens attack latency, but does not influence number of attacks. AAV2-expressing shRNA was infused into the DG (a, lower panel is increased magnification of outlined region in upper panel). 'Time to attack' survival plots over 3 days of resident-intruder testing in mice receiving scrambled shRNA ($N = 8$) or $\alpha 7$ shRNA ($N = 7$) (b–d), and Tukey box plot summarizing attack latency across days of testing (e), demonstrate shortened attack latency in mice receiving $\alpha 7$ shRNA. There was no significant effect of shRNA on number of attacks (f, $N = 8$, scrambled shRNA; $N = 7$, $\alpha 7$ shRNA). In e, center line depicts median latency, box depicts interquartile distance, whiskers extend to furthest data point. Data in f are expressed as mean \pm SEM. * $p \leq 0.05$; ns, not significant. Scale bar in a: upper panel, 500 microns; lower panel, 200 microns.

0.5 mg/kg nicotine and 20 mg/kg GTS-21 to lengthen time to first attack (Lewis *et al*, 2015). In the group of mice treated with vehicle or nicotine, we identified a significant drug \times virus interaction (HR = 8.92; 95% CI = 1.12, 71.06; $p = 0.039$). Nicotine treatment significantly delayed attack latency in mice receiving scrambled shRNA (HR = 0.10; 95% CI = 0.02, 0.47; $p = 0.004$), but not in mice receiving $\alpha 7$ shRNA (HR = 0.88, 95% CI = 0.22, 3.61; $p = 0.86$; Figure 5a). In mice treated with vehicle or GTS-21 (20 mg/kg), the drug \times virus interaction did not reach significance (HR = 2.58; 95% CI = 0.43, 15.38; $p = 0.297$). However, consistent with the results of nicotine treatment, planned comparisons revealed GTS-21 treatment significantly delayed attack

latency in mice receiving scrambled shRNA (HR = 0.25; 95% CI = 0.06, 1.00; $p = 0.05$) but not in mice receiving $\alpha 7$ shRNA (HR = 0.65; 95% CI = 0.18, 2.28; $p = 0.50$; Figure 5b). Taken together, these data suggest $\alpha 7$ nAChRs in the DG can affect attack latency bidirectionally.

DISCUSSION

Nicotine reduces aspects of aggressive behavior across diverse animal models of aggression (Picciotto *et al*, 2015), with recent studies suggesting these effects in mice require activation of $\alpha 7$ nAChRs (Lewis *et al*, 2015). Correspondingly, loss of *CHRNA7* in humans confers substantial risk for

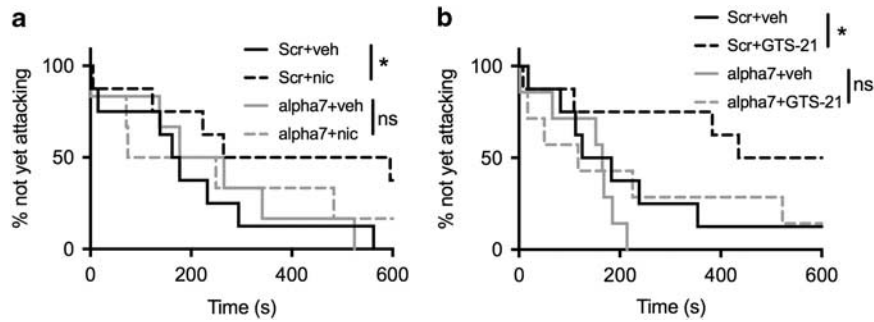


Figure 5 shRNA targeting $\alpha 7$ nAChRs in the DG blunts the effects of 0.5 mg/kg nicotine (a, $N = 8$, scrambled shRNA; $N = 6$, $\alpha 7$ shRNA) or 20 mg/kg GTS-21 (b, $N = 8$, scramble shRNA; $N = 7$, $\alpha 7$ shRNA) to lengthen attack latency. * $p \leq 0.05$; ns, not significant.

aggressive behaviors (Gillentine *et al*, 2017), as well as other neuropsychiatric symptoms, with preliminary evidence that pharmacotherapy targeting the $\alpha 7$ nAChR may be effective for reducing aggression (Cubells *et al*, 2011). However, a definitive understanding of the relationship between $\alpha 7$ nAChRs and aggressive behavior remains elusive, especially given the genetic and behavioral heterogeneity of the 15q13.3DS in humans and behavioral studies in mice with *Chrna7* deletion (Yin *et al*, 2017). Here we sought to further investigate the effects of $\alpha 7$ nAChRs on aggressive behavior in mice using a common behavioral test of aggression, and to identify important brain regions mediating the ability of $\alpha 7$ nAChRs to modulate aggression.

In our study, we found that $\alpha 7$ nAChR HET mice demonstrate faster attack latency and more attacks than WT littermates. Excitatory granule cells in the DG, a brain region with high expression of $\alpha 7$ nAChRs on inhibitory interneurons, are activated during aggressive interactions and the $\alpha 7$ partial agonist GTS-21, which delays attack latency, reduces GC activation. Finally, we demonstrated that knockdown of $\alpha 7$ nAChRs in the DG reduces time to first attack and blocks the ability of nicotine and GTS-21 treatment to delay attack. Together, these findings support a relationship between $\alpha 7$ nAChR signaling and aggressive behavior, and implicate $\alpha 7$ nAChRs expressed in the DG in the underlying mechanism.

A comprehensive behavioral battery recently reported no effect of $\alpha 7$ nAChR deletion in B6 mice, including in the tube test of social dominance in group-housed mice (Yin *et al*, 2017). Aggression in our study was measured by resident-intruder tests following social isolation-induced aggression (Koolhaas *et al*, 2013). Previous work suggests these two tests measure different behavioral constructs (Lindzey *et al*, 1966). Furthermore, an effect of *Chrna7* deficiency on aggression in B6 mice might be masked by this strain's limited propensity to initiate aggression (Schneider *et al*, 1992), which may have been improved by the use of B6 \times B/c F1 mice in the current study. Finally, aggression in the Df(h15q13)/+ mouse model of 15q13.3DS was observed in response to stress (Fejgin *et al*, 2014), which might have been engaged by the use of social isolation in our study.

Localization of $\alpha 7$ nAChRs in DG and hippocampus and their functional contributions to these circuits have previously been explored in rodents using α -bungarotoxin binding, *in situ* hybridization, and electrophysiology. In humans, regional effects of $\alpha 7$ nAChR signaling have been

assessed using pharmacology coupled with functional imaging. In the rodent DG, $\alpha 7$ nAChRs are preferentially localized to non-GCs, in particular GABAergic inhibitory neurons of a variety of subtypes, including those expressing neuropeptide-Y, somatostatin, and cholecystokinin (Frazier *et al*, 2003; Freedman *et al*, 1993; Son and Winzer-Serhan, 2008). Functionally, ACh acting on DG interneurons can produce inhibitory post-synaptic potentials on nearby GCs, as well as enhance inhibition on nearby GCs through a disinaptic pathway originating from entorhinal cortex glutamatergic inputs onto interneuron dendrites (Frazier *et al*, 2003). In agreement with physiological effects on inhibitory networks observed in rodents, GTS-21 in humans with schizophrenia normalizes excitation of the hippocampus during a smooth pursuit eye movement task (Tregellas *et al*, 2010). Because individuals with schizophrenia demonstrate abnormal activity of the hippocampus during this task compared to controls (Tregellas *et al*, 2004), the introduction of an $\alpha 7$ nAChR agonist may normalize an existing abnormality in E/I balance.

Consistent with these rodent and human studies, the current data also suggest an inhibitory effect of GTS-21 on DG GCs, presumably through its actions on $\alpha 7$ nAChRs on local inhibitory interneurons. Further, DG $\alpha 7$ nAChRs are necessary for GTS-21 to increase attack latency. Interestingly, HET mice appeared to be qualitatively more aggressive than mice with knockdown of *Chrna7* in the DG, especially in total number of attacks (Figures 1 and 4). Although these mice differ in background strain as well as in surgical manipulation, this suggests that $\alpha 7$ nAChRs in brain regions outside the DG, including candidate regions rich in $\alpha 7$ nAChRs known to be important in aggression regulation such as the amygdala and prefrontal cortex (Unger *et al*, 2015), may contribute to the serene effects of nicotinic drugs targeting $\alpha 7$ nAChRs. Overall, these data support a mechanism whereby the hippocampal E/I balance set point might regulate the likelihood of aggressive behavior. Such a mechanism is supported by previous studies of neuroligin-2, a postsynaptic cell adhesion protein necessary for proper hippocampal inhibitory neurotransmission linked to neuropsychiatric disorders such as ASD and schizophrenia. Viral overexpression of neuroligin-2 in the rat dorsal hippocampus increased expression of inhibitory synaptic markers and correspondingly reduced aggressive behavior (Kohl *et al*, 2013), while local hippocampal infusion of a peptide interfering with neuroligin-2 function increased aggressive

behavior (van der Kooij *et al*, 2014). These studies extend work demonstrating the overall importance of the hippocampus in the regulation of aggression in animal models (Siegel and Flynn, 1968) and in humans with neuropsychiatric disorders (Sala *et al*, 2011; Vermeiren *et al*, 2014). Studies in humans with schizophrenia complicated by pathological impulsive aggression identify abnormal functional connectivity between ventral prefrontal cortex and amygdala (Hoptman *et al*, 2010) or other limbic structures (Hoptman *et al*, 2014). Future experiments might use rodent models of enhanced aggression related to changes in hippocampal E/I balance, such as the *Chrna7* HET mice studied here, *Df(h15q13)/+* mice (Fejgin *et al*, 2014), or rodents with neuroligin-2 manipulations, and perform molecular and physiological techniques to understand long-range regional interactions mediating enhanced aggressive behavior. In turn, results from such experiments might have potential translational importance for designing functional imaging experiments in human populations with neuropsychiatric disorders and co-morbid aggression.

Finally, the current results suggest that systematic investigation of pharmacological agents targeting $\alpha 7$ nAChRs is warranted to reduce pathological aggressive behavior in 15q13.3DS, and potentially other neuropsychiatric disorders with abnormal hippocampal E/I balance, such as idiopathic forms of ASD and schizophrenia (Gao and Penzes, 2015; Nelson and Valakh, 2015), as well as Alzheimer's disease (Rissman and Mobley, 2011). While a placebo-controlled trial demonstrated that nicotine patch can be beneficial for acute aggression in nicotine-dependent individuals with schizophrenia, evidence for efficacy of pharmacotherapies putatively targeting $\alpha 7$ nAChRs in 15q13.3DS (Cubells *et al*, 2011), idiopathic ASD (Van Schalkwyk *et al*, 2015), and dementia (Carmel and Sheitman, 2007; Rosin *et al*, 2001) remains limited to case reports. Importantly, the expression pattern of $\alpha 7$ nAChRs both at the regional level of the hippocampus and microcircuit level of inhibitory networks potentially permits a more specific targeting of neural substrates regulating aggressive behavior by $\alpha 7$ nAChR pharmacotherapy, as opposed to interventions targeting GABA receptors that are more widely expressed throughout the brain. The tolerability and safety of $\alpha 7$ agonists in humans with neuropsychiatric disorders, as well as the availability of biomarkers of response such as improvements in P50 gating deficits in schizophrenia (Olincy *et al*, 2006) or ASD (Olincy *et al*, 2016), would strongly support future evaluation of this therapeutic target for treating pathological aggression.

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