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Reduced *Chrna7* expression in mice is associated with decreases in hippocampal markers of inhibitory function: implications for neuropsychiatric diseases

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Abstract

The $\alpha 7^*$ nicotinic acetylcholine receptor encoded by *CHRNA7* (human)/*Chrna7* (mice) regulates the release of both the inhibitory neurotransmitter γ -aminobutyric acid (GABA) and the excitatory neurotransmitter glutamate in the hippocampal formation. A heterozygous deletion at 15q13.3 containing *CHRNA7* is associated with increased risk for schizophrenia, autism and epilepsy. Each of these diseases is characterized by abnormalities in excitatory and inhibitory hippocampal circuit function. Reduced *Chrna7* expression results in decreased hippocampal $\alpha 7^*$ receptor density, abnormal hippocampal auditory sensory processing and increased hippocampal CA3 pyramidal neuron activity in C3H mice heterozygous for a null mutation in *Chrna7*. These abnormalities demonstrate that decreased *Chrna7* expression alters hippocampal inhibitory circuit function. The current study examined the specific impact of reduced *Chrna7* expression on hippocampal inhibitory circuits by measuring the levels of GABA, GABA_A receptors, the GABA synthetic enzyme glutamate decarboxylase-65 (GAD-65) and the vesicular GABA transporter GAT-1 in wild type (*Chrna7*^{+/+}) and heterozygous (*Chrna7*^{+/-}) C3H $\alpha 7$ mice of both genders. GAD-65 levels were significantly decreased in male and female heterozygous C3H $\alpha 7$ mice while GABA_A receptors were significantly reduced only in male heterozygous C3H $\alpha 7$ mice. No changes in GABA and GAT-1 levels were detected. These data suggest that reduced *CHRNA7* expression may contribute to the abnormalities in hippocampal inhibitory circuits observed in schizophrenia, autism and/or epilepsy.

Keywords

Chrna7; λ -aminobutyric acid (GABA); GABA_A receptors; hippocampus; nicotinic receptors; GAT-1

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1.0

The $\alpha 7^*$ nicotinic acetylcholine receptor encoded by *CHRNA7* (humans)/*Chrna7* (mice) is a rapidly desensitizing, ligand-gated ion channel that fluxes calcium (Albuquerque et al., 2009, Bertrand, 2010). In hippocampus, the receptor is localized to neurons containing the inhibitory neurotransmitter γ -aminobutyric acid (GABA) as well as to those containing the excitatory neurotransmitter glutamate (Fabian-Fine et al., 2001, Albuquerque et al., 2009). Hippocampal $\alpha 7^*$ receptor activation leads to the release of both GABA and glutamate (Albuquerque et al., 2009), indicating that the receptor can modulate the inhibitory/excitatory balance of the hippocampus.

CHRNA7 expression appears to be abnormal in several neuropsychiatric disorders. A heterozygous microdeletion of chromosome 15q13.3 that encompasses several genes, including *CHRNA7*, is associated with increased risk for schizophrenia, autism and epilepsy (Helbig et al., 2009). Individuals with the microdeletion may exhibit no abnormalities or may exhibit a variety of clinical features, including cognitive deficits (developmental delay, learning disabilities, mild to severe mental retardation), behavioral problems (poor attention span, aggressive behavior, impulsivity, hyperactivity, psychosis, rage), neurologic anomalies (seizures, abnormal electroencephalograms, hypotonia), facial dysmorphism (large ears, bulbous nasal tip, prominent philtrum, everted upper lip, upslanting palpebral fissures), abnormal extremities (tapered fingers, 5th finger clinodactyly, lax thumb joint, brachydactyly) and/or cardiac defects (tetralogy of Fallot, cardiac hypoplasia, tricuspid stenosis, mitral valve prolapse) (van Bon et al., 2009, Masurel-Paulet et al., 2010, Cubells et al., 2011). *CHRNA7* itself has been associated with the P50 gating and memory deficits observed in schizophrenic individuals as well as with schizophrenia in many, but not all, studies (Stephens et al., 2012). In addition, *CHRNA7* has been associated with bipolar disease (Ancin et al., 2010) and with juvenile myoclonic epilepsy (Taske et al., 2002).

Schizophrenia, autism and epilepsy are characterized by abnormal excitatory and inhibitory circuits in numerous brain regions (Benes et al., 1996, Benes and Berretta, 2001, Volk and Lewis, 2002, Schleimer et al., 2004, Lee et al., 2006, Page et al., 2006, Eid et al., 2008, Sperk et al., 2009, Heckers and Konradi, 2010, Beneyto et al., 2011, Blatt and Fatemi, 2011). The density of $\alpha 7^*$ receptors is significantly decreased in several regions of postmortem brain from schizophrenic patients (Adams and Stevens, 2007). Similarly, $\alpha 7^*$ receptor mRNA is significantly lower in postmortem cortical samples from autistic individuals (Yasui et al., 2011). We hypothesize that reduced *CHRNA7* expression contributes to the alterations in brain function observed in these disorders.

This hypothesis is supported by data from an animal model of reduced *Chrna7* expression. Heterozygous (Het - *Chrna7*^{+/-}) C3H $\alpha 7$ mice exhibit significant decreases in hippocampal $\alpha 7^*$ receptors, significant increases in hippocampal CA3 pyramidal neuron activity and abnormal hippocampal auditory sensory processing as compared to wild type (WT - *Chrna7*^{+/+}) C3H $\alpha 7$ mice (Adams et al., 2008). These abnormalities demonstrate that decreased *Chrna7* expression alters hippocampal inhibitory circuit function.

To determine the specific changes in hippocampal inhibitory circuits associated with reduced *Chrna7* expression, we investigated the effects of reduced *Chrna7* expression on hippocampal levels of GABA, the GABA synthetic enzyme L-glutamic acid decarboxylase-65 (GAD-65), the vesicular GABA transporter GAT-1 and GABA_A receptors in adult WT and Het C3H $\alpha 7$ mice of both sexes. Examining WT C3H $\alpha 7$ mice allows for characterization of normal hippocampal levels of the GABAergic markers, whereas Het C3H $\alpha 7$ mice provide a model of the reduced *CHRNA7* expression observed in some human diseases (e.g. schizophrenia and autism). Hippocampal GABA levels were measured

to determine whether reduced *Chrna7* expression alters baseline GABA production. The levels of hippocampal GAD-65, GAT-1 and GABA_A receptors were examined because these markers are changed in individuals with schizophrenia, epilepsy and/or autism (Benes et al., 1996, Benes and Berretta, 2001, Volk and Lewis, 2002, Schleimer et al., 2004, Lee et al., 2006, Sperk et al., 2009, Blatt and Fatemi, 2011). Data from the present study indicate that reduced *Chrna7* expression alters hippocampal GABAergic markers in a gender-specific manner.

2.0 Experimental Procedures

2.1 Animals

Mice were originally obtained from the Institute for Behavioral Genetics breeding colony at the University of Colorado, Boulder, CO. The C3H/Ibg (C3H) $\alpha 7$ null mutant (knockout - KO) mice were generated by backcrossing $\alpha 7$ KO mixed background (129 x C57BL/6) mice (Orr-Urtreger et al., 1997) to C3H mice (both sexes used) for 10 generations (maintaining 6 families). Mice from the last generation were used to establish a breeding colony in the animal facility of the Denver Veterans Administration Medical Center, Denver, CO. A recent genome diversity analysis (The Jackson Laboratory, Bar Harbor, Maine) indicates that the KO C3H $\alpha 7$ mice are at least 99.7% identical to the C3H parent strain.

Male and female WT and Het C3H $\alpha 7$ mice used in the current experiments were obtained from Het X Het matings at the VA animal facility. The pregnant dams were housed in large, low-air-flow cages with microisolator tops, Nestlets (Ancare, Bellmore, NY) and plastic igloos. The pups were weaned at 21 days and maintained in small low-air-flow cages with microisolator tops (5 mice/cage or less) and plastic igloos until collection at 5 – 6 months of age. Cage changes occurred twice weekly for all animals. All animals were provided with *ad libitum* water and Teklad rodent chow (Harlan Teklad, Indianapolis, ID) and a 12 hr light/dark cycle (lights on at 6 AM).

2.2 Tissue collection for GABA_A receptor autoradiography

Adult (5 – 6 months of age) male and female WT and Het C3H $\alpha 7$ mice ($n = 6$ /group) were rapidly decapitated without anesthesia, the brains were removed, frozen in dry ice snow and stored at -80°C until processing. Sequential cryostat sections (12 μm) through the hippocampus from animals of each genotype and gender were cut, thaw-mounted onto gelatin-coated slides and stored at -80°C before binding with the convulsant [^{35}S]-tert-butylbicyclophosphorothionate ([^{35}S]-TBPS, Perkin Elmer, Waltham, MA), an antagonist that binds to the chloride ionophore of GABA_A receptors (Olsen et al., 1990).

2.3 Radioligand binding

The tissue, subdivided into total- and nonspecific-binding groups, was incubated in a solution containing 50 mM Tris-HCl and 0.9% sodium chloride (TBS buffer, pH 7.4, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 4°C . Nonspecific binding was determined by adding 100 μM picrotoxin (Sigma-Aldrich, St. Louis, MO) to the TBS buffer. The two tissue sets were then incubated in the TBS buffer containing [^{35}S]-TBPS (10 nM) at room temperature for 2 hours. The buffer for the nonspecific group again contained 100 μM picrotoxin. Following incubation with the ligand, the tissue was rinsed in TBS buffer for 3×5 min at 4°C . Subsequently, the slides were dipped briefly in cold distilled water, dried quickly under a stream of cool air and exposed to radiation-sensitive Hyperfilm (Amersham, Piscataway, NJ) for 72 hours with [^{14}C] standards (Amersham, Piscataway, NJ) of known radioactivity in order to generate autoradiograms for quantitative analysis. Digital images were captured using a Chroma-Pro 45 light box and Retiga CCD camera (Qimaging, Surrey, BC, Canada) using Simple PCI software (Hamamatsu Corp., Sewickley, PA).

Autoradiograms were quantified with a computer-based image analysis system (ImageJ, NIH) using calibrated standards of reference. A calibration curve of gray value against radioligand concentration (nCi/gm tissue) was constructed using standards of known radioactivity. Specific radioligand binding was calculated by subtracting values obtained in the presence of an excess of competing ligand (nonspecific binding) from those in the absence (total binding), and was expressed as nCi/g tissue. For each animal, measurements were taken from both left and right hemispheres of 15 hippocampal sections. [³⁵S]-TBPS binding was minimal in stratum pyramidale and lucidum, so measurements of GABA_A receptor density were not made in these layers.

2.4 Immunohistochemistry

Adult (5 – 6 months of age) male and female WT and Het C3H α 7 mice ($n = 6$ /group) were deeply anesthetized with Isoflurane and transcardially perfused with saline followed by a 4% paraformaldehyde/0.1M phosphate buffer (pH 7.4) fixative solution. The brains were removed, postfixed in the fixative for 6 hours, cryoprotected overnight in 30% sucrose/0.1 M phosphate buffer (pH 7.4), embedded in mounting media (Neg 50, Fisher Scientific, Pittsburg, PA), frozen in liquid nitrogen-chilled isopentane and stored at -80°C until processing. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.5 Antibodies

GAD-65: primary antibody (rabbit, 1:2000 dilution, Millipore Corp., Billerica, MA), donkey anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch, West Grove, PA). GABA: primary antibody (guinea pig, 1:2000, Protos Biotech Corp., New York, NY), donkey anti-guinea pig secondary antibody (1:500, Jackson ImmunoResearch, West Grove, PA). GAT-1: primary antibody (rabbit, 1:100 dilution, Millipore Corp., Billerica, MA), donkey anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch, West Grove, PA).

2.6 General immunohistochemistry procedure

Sequential cryostat sections (40 μm) through the hippocampus from animals of each genotype and gender were cut and collected in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The tissue sections were rinsed 3×5 minutes in PBS, quenched in 0.3% hydrogen peroxide/PBS solution at room temperature (RT) for 30 minutes, rinsed 3×5 minutes in PBS and incubated in blocking solution (PBS-X, 5.0% normal donkey serum in PBS containing 0.3% Triton-X 100) for 1 hour at RT. The tissue was then placed in incubation solution (PBS-X containing 0.5% normal donkey serum) containing the primary antibody overnight at 4°C . The tissue was rinsed 3×5 minutes in PBS at RT and placed in incubation solution containing the secondary antibody for 1 hour at RT. The tissue was rinsed 3×5 minutes in PBS at RT and placed in ABC solution (Vector Laboratories, Burlingame, CA) for 1 hour at RT. The tissue was rinsed 3×5 minutes in PBS at RT and reacted in a solution containing 0.0004% 3,3'-diaminobenzidine, 0.002% hydrogen peroxide in PBS for 20 minutes followed by 3×5 minute rinses in PBS at RT. The tissue sections were mounted on gelatin-coated slides, dried under a stream of cool air and coverslipped with Permount (Fisher Scientific, Pittsburg, PA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Deletion of the primary antibody was used as a control. Digital images were captured and analyzed as described previously (2.3). A Kodak No. 2 photographic step-tablet (Kodak, Rochester, NY) was used to generate a relative optical density curve. The background level of immunostaining (defined as the gray value in the corpus callosum) was subtracted from the level of immunostaining (gray values) in different hippocampal layers to give a final gray value for each layer. The final gray value was converted to a relative optical density value using the equation generated from the step-tablet. For each animal, measurements were taken from both left and right hemispheres of 15 hippocampal sections.

2.7 Statistical analysis

Data were analyzed by a 3-way ANOVA assessing differences in binding or immunohistochemical staining across hippocampal layers, gender and genotype, with Fisher's Protected Least Significant Differences *a posteriori* analyses where appropriate.

3.0 Results

3.1 Reduced *Chrna7* expression is associated with decreased hippocampal GAD-65

Immunostaining for GAD-65 was found predominately in neuronal terminals, although a few scattered GAD-65-positive cells were also observed (Figure 1A1–A4, Figure 3, Table 1). GAD-65 labeling was greatest in the outer molecular layer of the dentate gyrus, in stratum lacunosum/moleculare of hippocampal area CA3 and in stratum oriens of hippocampal areas CA3 and CA1 in both male and female WT and Het C3H α 7 mice. Despite a similar pattern of staining, GAD-65 was generally decreased in the hippocampal formation of male and female Het mice compared to their WT counterparts (Table 1), although significant reductions were detected only in CA3 stratum lucidum/radiatum ($F(1,20) = 3.855, p=0.042$) and in CA3 stratum lacunosum/moleculare ($F(1,20) = 3.855, p=0.043$) (Figure 1B,C). Overall, even though no statistical differences in gender were detected, the Het-associated decrease in GAD-65 was greater in female (decreased 39 – 49%) than in male (decreased 17 – 30%) mice.

3.2 Reduced *Chrna7* expression is associated with decreased hippocampal GABA_A receptors in males

Binding of [³⁵S]-TBPS was greatest in the outer molecular layer of the dentate gyrus, in stratum oriens of hippocampal areas CA3 and CA1 and in stratum lacunosum/moleculare of hippocampal area CA1 in all mice (Figure 1B1–B4, Figure 4, Table 2). Lower levels of [³⁵S]-TBPS binding were observed in stratum radiatum of areas CA3 and CA1 and in the inner molecular layers and hilus of the dentate gyrus. Binding was virtually absent in the granule cell layer of the dentate gyrus, in strata pyramidale and lucidum of hippocampal area CA3 and in stratum pyramidale of hippocampal area CA1. Again, although the pattern of binding was comparable, GABA_A receptor density was significantly decreased in the molecular layer of the dentate gyrus ($F(1,20) = 5.165, p=0.034$), in CA3 stratum radiatum ($F(1,20) = 4.426, p=0.048$) and stratum lacunosum/moleculare ($F(1,20) = 6.314, p=0.021$) and in CA1 stratum radiatum ($F(1,20) = 5.485, p=0.030$) and stratum lacunosum/moleculare ($F(1,20) = 5.245, p=0.033$) of male Het compared to male WT mice (Table 2). The decrease in binding in the male Het mice ranged from 7 – 17%. In contrast, GABA_A receptor density was comparable in the hippocampal formation of female WT and Het C3H α 7 mice. The subunit composition and location (presynaptic, postsynaptic and/or extrasynaptic) of the affected GABA_A receptors is currently unknown.

3.3 Reduced *Chrna7* expression does not alter hippocampal GABA levels

Scattered GABA-positive neurons were observed throughout the hippocampal formation (Figure 2A1–A2). In addition, somewhat higher levels of diffuse staining were seen in the hilus of the dentate gyrus, in stratum lacunosum/moleculare of hippocampal area CA3, at the border of stratum radiatum and lacunosum/moleculare of hippocampal area CA1 and in stratum pyramidale of areas CA3 and CA1. No significant differences in GABA levels were found between genotypes or genders (data not shown).

3.4 Reduced *Chrna7* expression does not alter hippocampal GAT-1 levels

GAT-1 labeling was restricted to neuronal terminals and was highest in the outer molecular layer of the dentate gyrus, in stratum lacunosum/moleculare of hippocampal area CA3 and

in stratum pyramidale of hippocampal areas CA3 and CA1 (Figure 2B1–B2). Somewhat lower levels of GAT-1 were seen in strata oriens and radiatum of hippocampal area CA3 and in stratum lacunosum/moleculare of hippocampal area CA1. The lowest levels of GAT-1 were observed in strata oriens and radiatum of hippocampal area CA1. No significant differences in GAT-1 levels were observed between genotypes or genders (data not shown).

4.0 Discussion

Hippocampal GAD-65 was significantly reduced in Het C3H $\alpha 7$ mice of both genders while hippocampal GABA_A receptors were significantly reduced in male Het mice only. No significant differences in GABA or GAT-1 levels were detected. These data suggest that reduced *Chrna7* expression may alter some, but not all, hippocampal inhibitory circuit components in a gender-specific manner.

4.1 Reduced GAD-65

GAD-65 is primarily located in inhibitory nerve terminals (Martin and Tobin, 2000). Therefore, the lower GAD-65 levels observed in Het C3H $\alpha 7$ mice could indicate that reduced *Chrna7* expression leads to a decrease in hippocampal inhibitory terminal density. However, the lack of change in hippocampal GAT-1, also an inhibitory terminal marker (Zahniser and Doolen, 2001), does not support this supposition.

Another possibility is that altered kinase function may underlie the observed decrease in GAD-65. Expression of *Gad2*, the gene encoding GAD-65, is regulated by the phosphorylated form of cAMP response element binding protein (pCREB) (Bitner et al., 2007, Gubbins et al., 2010). In addition, GAD-65 activity is increased by phosphorylation via protein kinase C (PKC) and possibly by Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) and mitogen-activated protein kinase (MAPK) (Wei et al., 2004, Wei and Wu, 2008). Stimulation of the $\alpha 7^*$ receptor activates CaMKII, MAPK and PKC (Zhang and Berg, 2007; Wanaverbecq et al., 2007) and CaMKII and MAPK activation secondary to $\alpha 7^*$ receptor stimulation increases pCREB levels in hippocampal neurons (Hu et al., 2002). Therefore, $\alpha 7^*$ receptor stimulation may modulate hippocampal *Gad2* expression and/or GAD-65 activation by increasing kinase function. If so, a decrease in hippocampal $\alpha 7^*$ receptor density could lead to reduced kinase activation and/or decreased *Gad2* expression, resulting in lower GAD-65 levels.

GAD-65 levels were reduced 20 – 47% in postmortem hippocampus from unmedicated schizophrenic individuals relative to controls (Benes and Berretta, 2001), a reduction comparable to the 17 – 49% decrease in GAD-65 observed in Het C3H $\alpha 7$ mice in the present study. GAD-65 contributes primarily to the activity-regulated synthesis and release of vesicular GABA (Martin and Tobin, 2000). A disruption of activity-dependent GABA release might shift the inhibitory/excitatory balance within the hippocampus to a more excited state. Such a shift could contribute to both the auditory sensory processing deficits and the hyperactivity observed in hippocampus from Het C3H $\alpha 7$ mice (Adams et al., 2008) and schizophrenic patients (Tregellas et al., 2007).

4.2 Reduced GABA_A receptor density

Reduced *Chrna7* expression was associated with decreased hippocampal GABA_A receptor density in males. Although the anatomical (pre/postsynaptic) location of the decreased binding is not known, modest (5 – 35%) reductions in postsynaptic GABA_A receptors are associated with significant behavioral alterations (Luscher et al., 2011). This suggests that the 7 – 17% decrease in hippocampal GABA_A receptors observed in male Het mice in the current study may have negative functional consequences.

GABA_A receptor density is regulated by a variety of mechanisms (Barnes, 1996, Luscher et al., 2011). Prolonged exposure to GABA or GABAergic agonists can cause a decrease in GABA_A receptor levels (Barnes, 1996). However, as baseline GABA levels were not significantly different between Het and WT mice, it is unlikely that the decrease in GABA_A receptor density observed in male Het mice was due to a ligand-mediated downregulation.

Another possibility is that $\alpha 7^*$ receptor-dependent changes in BDNF are responsible for reduced GABA_A in male Het mice. Activation of tropomyosin related kinase-B (TrkB) receptors by BDNF leads to a decrease in hippocampal GABA_A receptor density (Brunig et al., 2001, Wardle and Poo, 2003, Lund et al., 2008, Mou et al., 2011). Blockade of hippocampal $\alpha 7^*$ receptors by intracerebroventricular infusion of the $\alpha 7^*$ receptor-selective antagonist α -bungarotoxin increased BDNF mRNA throughout the hippocampal formation (Freedman et al., 1993). Therefore, reduced *Chrna7* expression may set off a BDNF-mediated signaling cascade that results in reduced GABA_A receptor levels.

A third possibility is that reduced GABA_A receptor levels in the male Het mice may be mediated by changes in kinase function. Activation of CaMKII results in a rapid increase in GABA_A receptor density (Marsden et al., 2007). As described above (4.2), $\alpha 7^*$ receptor stimulation activates CaMKII (Zhang and Berg, 2007). Therefore, the reduction in GABA_A receptors observed in male Het C3H $\alpha 7$ mice could also occur secondary to reduced $\alpha 7^*$ receptor-mediated CaMKII activation.

Finally, we do not know to what extent gender differences in hippocampal GABA_A receptor density might be due to gonadal steroid influences. As female mice were collected over a period of months, it seems likely that any estrus cycle influence on GABA_A receptors would average out. However, if this was not the case, estrogen has been found to regulate hippocampal BDNF levels (Murphy et al., 1998, Scharfman et al., 2003, Spencer et al., 2008). Estrogen also modulates hippocampal acetylcholine release in females, but not males (Gabor et al., 2003). These two factors could contribute to the observed gender differences in hippocampal GABA_A receptor density. In addition, the gender difference in GABA_A receptor density could result from the action of neurosteroid metabolites of progesterone and/or testosterone as these metabolites act as positive allosteric modulators of GABA_A receptors (Majewska, 1992, Gonzalez-Flores et al., 2004, Reddy and Jian, 2010).

In contrast to the current study, an increase in GABA_A receptor binding density was observed in postmortem hippocampus from schizophrenic patients (Benes and Berretta, 2001). However, recent studies have reported significant decreases in mRNA for the $\alpha 1$ subunit of the GABA_A receptor in prefrontal cortex of individuals with schizophrenia (Beneyto et al., 2011, Glausier and Lewis, 2011). An examination of potential GABA_A receptor subunit alterations in the Het C3H $\alpha 7$ mice may help clarify this issue.

4.3 GABA and GAT-1 regulation

Baseline hippocampal GABA levels were not significantly different across mouse groups in the present study. Although not examined, this might be due to the maintenance of GABA levels by L-glutamic acid decarboxylase-67 (GAD-67) (Martin and Tobin, 2000). Normal hippocampal GABA levels in all mouse groups could account, at least in part, for the absence of change in hippocampal GAT-1 levels as GAT-1 is regulated by GABA (Bernstein and Quick, 1999).

4.4 Conclusion

In summary, reduced *Chrna7* expression is associated with decreased hippocampal GAD-65 and GABA_A receptor levels in C3H $\alpha 7$ mice. Lower GAD-65 may result in decreased activity-dependent GABA release which could, in turn, contribute to the hippocampal

disinhibition and deficits in hippocampal auditory sensory processing observed in Het C3H $\alpha 7$ mice. The impact of reduced hippocampal GABA_A receptor density in male Het mice is less clear. We also do not know when the alterations in hippocampal GABA_A receptors and GAD-65 occur in the Het mice. The $\alpha 7^*$ receptor modulates a number of mechanisms important for hippocampal development (Maggi et al., 2001, Maggi et al., 2003, Liu et al., 2006) and does not reach mature levels in rodents until two months of age (Adams et al., 2002), raising the possibility that the GABAergic marker changes may occur prior to the adult age examined in this study. Although a great deal remains to be determined, these data suggest that reduced *Chrna7* expression can alter hippocampal inhibitory function, perhaps contributing to the inhibitory deficits observed in schizophrenia, epilepsy and autism.

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Abbreviations

ADNFLE	autosomal dominant nocturnal frontal lobe epilepsy
BDNF	brain derived neurotrophic factor
CaMKII	Ca ²⁺ -calmodulin-dependent protein kinase II
CHRNA7/Chrna7	gene (human/mouse) encoding the $\alpha 7$ nicotinic acetylcholine receptor
CREB	cAMP response element binding protein
GABA	γ -aminobutyric acid
Gad2	gene encoding GAD-65
GAD-65	L-glutamic acid decarboxylase-65
GAD-67	L-glutamic acid decarboxylase-67
GAT-1	vesicular GABA transporter 1
[³H]	tritiated
Het	heterozygous
KO	knockout
MAPK	mitogen-activated protein kinase
PBS	phosphate-buffered saline
PBS-X	phosphate-buffered saline containing Triton-X 100
PKC	protein kinase C
RT	room temperature
[³⁵S]-TBPS	[³⁵ S]-tert-butylbicyclophosphorothionate
TrkB	tropomyosin related kinase-B
TBS	Tris-HCl/sodium chloride buffer
WT	wildtype

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- Hippocampal GAD-65 is significantly reduced in male and female heterozygous C3H $\alpha 7$ mice.
- Hippocampal GABA_A receptor density is significantly reduced in male heterozygous C3H $\alpha 7$ mice.
- Hippocampal GABA is comparable between wildtype and heterozygous C3H $\alpha 7$ mice.
- Hippocampal GAT-1 is comparable between wildtype and heterozygous C3H $\alpha 7$ mice.

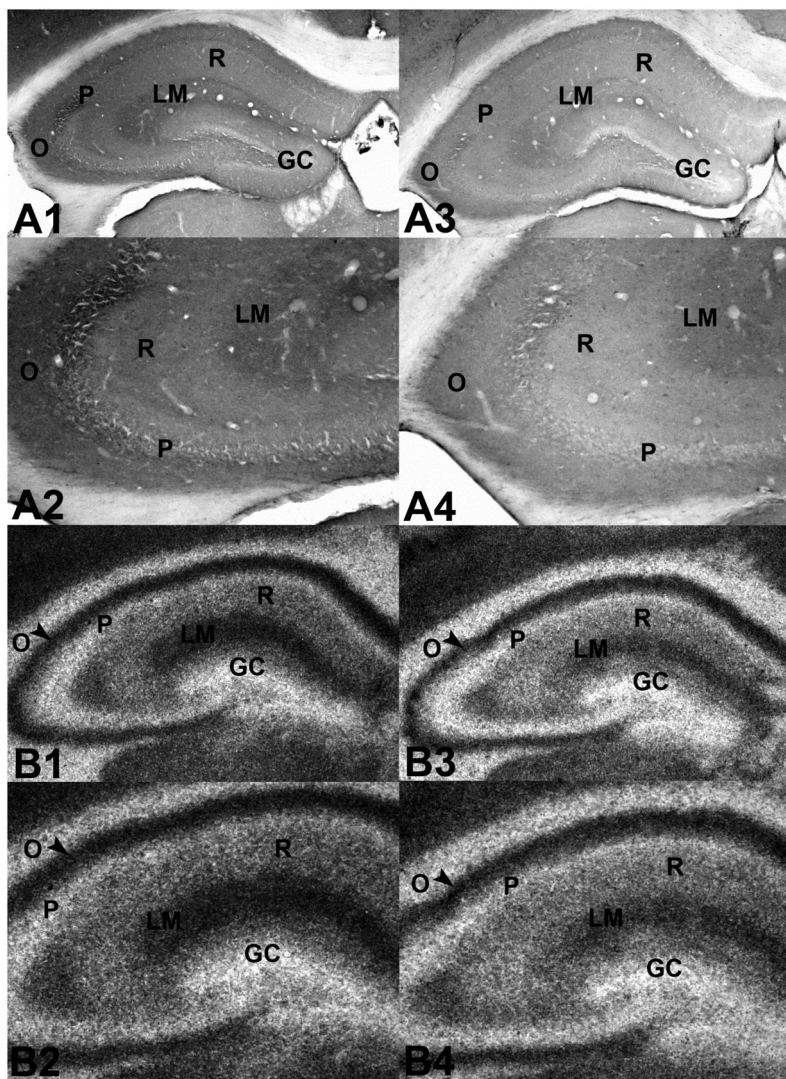


Figure 1. Photomicrographs of transverse sections through the hippocampus of adult C3H $\alpha 7$ mice labeled for glutamate decarboxylase-65 (GAD-65) (A1 – A4) or the GABA_A receptor antagonist [³⁵S]-tert-butylbicyclophosphorothionate ([³⁵S]-TBPS) (B1 – B4). **A1.** Transverse section through the hippocampal formation of a female wild type C3H $\alpha 7$ mouse immunolabeled for GAD-65. **A2.** Higher magnification of GAD-65 labeling in hippocampal area CA3 of the same section as in A1. **A3.** Transverse section through the hippocampal formation of a female heterozygous C3H $\alpha 7$ mouse immunolabeled for GAD-65. **A4.** Higher magnification of GAD-65 labeling in hippocampal area CA3 of the same section as in A3. Note the decrease in GAD-65 labeling in strata oriens, pyramidale, radiatum and lacunosum/moleculare of the heterozygous mouse (A3, A4) relative to the same regions in the wild type mouse (A1, A2). **B1.** Transverse section through the hippocampal formation of a male wild type C3H $\alpha 7$ mouse bound with the GABA_A antagonist [³⁵S]-TBPS. **B2.** Higher magnification of [³⁵S]-TBPS binding in the same section as in B1. **B3.** [³⁵S]-TBPS binding in a transverse section through the hippocampal formation of a male heterozygous C3H $\alpha 7$ mouse. **B4.** Higher magnification of [³⁵S]-TBPS binding in the same section as in B3. Note the decrease in [³⁵S]-TBPS binding in strata radiatum and lacunosum/moleculare of the heterozygous mouse (B3, B4) compared to the same regions in the wild type mouse

(B1, B2). *Abbreviations:* GC - granule cell layer of the dentate gyrus, O - stratum oriens, P - stratum pyramidale, R - stratum radiatum, LM - stratum lacunosum/moleculare.

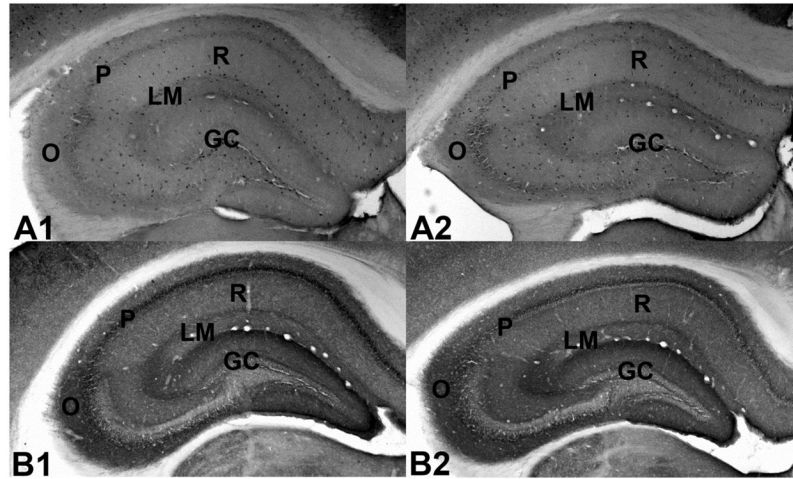


Figure 2. Photomicrographs of transverse sections through the hippocampus of adult C3H $\alpha 7$ mice labeled for γ -aminobutyric acid (GABA) (A1, A2) or the GABA vesicular transporter GAT-1 (B1, B2). **A1.** Transverse section through the hippocampal formation of a male wild type C3H $\alpha 7$ mouse labeled for GABA. **A2.** Transverse section through the hippocampal formation of a male heterozygous C3H $\alpha 7$ mouse labeled for GABA. The level of GABA labeling is comparable between the two genotypes. **B1.** Transverse section through the hippocampal formation of a male wild type C3H $\alpha 7$ mouse labeled for GAT-1. **B2.** Transverse section through the hippocampal formation of a male heterozygous C3H $\alpha 7$ mouse labeled for GAT-1. The level of GAT-1 labeling is comparable between the two genotypes. *Abbreviations:* GC - granule cell layer of the dentate gyrus, O - stratum oriens, P - stratum pyramidale, R - stratum radiatum, LM - stratum lacunosum/moleculare.

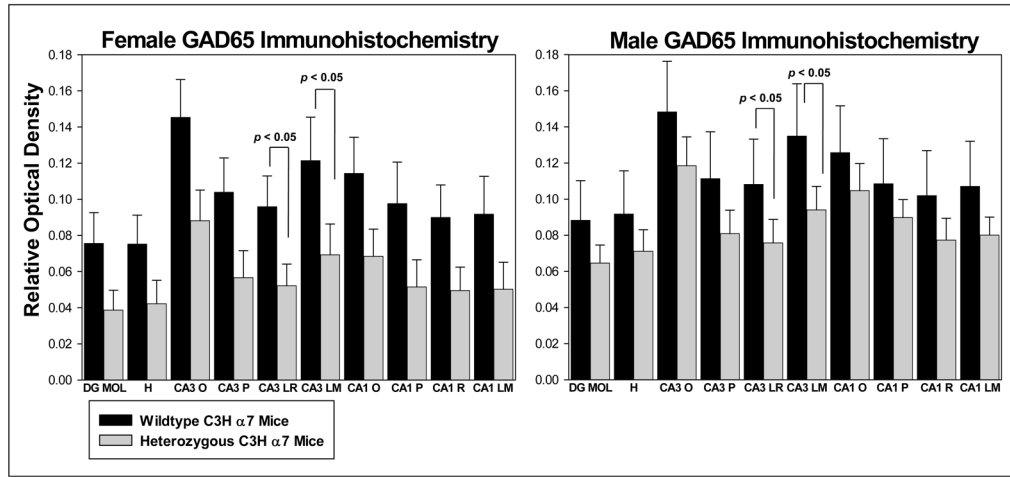


Figure 3.

Qualitative measurement of immunostaining for the GABA synthesizing enzyme GAD-65 in hippocampal sections from female ($n = 6/\text{group}$, left panel) and male ($n = 6/\text{group}$, right panel) wild type and heterozygous C3H $\alpha 7$ mice. Decreased levels of GAD-65 immunostaining were seen in both male and female heterozygous mice, with significant ($p < 0.05$) decreases in strata lucidum/radiatum and lacunosum/moleculare of hippocampal area CA3 in mice of both genders. *Abbreviations:* DG MOL – molecular layer of the dentate gyrus, H – hilus (polymorph layer) of dentate gyrus, CA3 O – oriens layer of hippocampal area CA3, CA3 P – pyramidal cell layer of hippocampal area CA3, CA3 LR – lucidum/radiatum layer of hippocampal area CA3, CA3 LM – lacunosum moleculare layer of hippocampal area CA3, CA1 O – oriens layer of hippocampal area CA1, CA1 P – pyramidal cell layer of hippocampal area CA1, CA1 R – radiatum layer of hippocampal area CA1, CA1 LM – lacunosum moleculare of hippocampal area CA1. Values = mean \pm S.E.M.

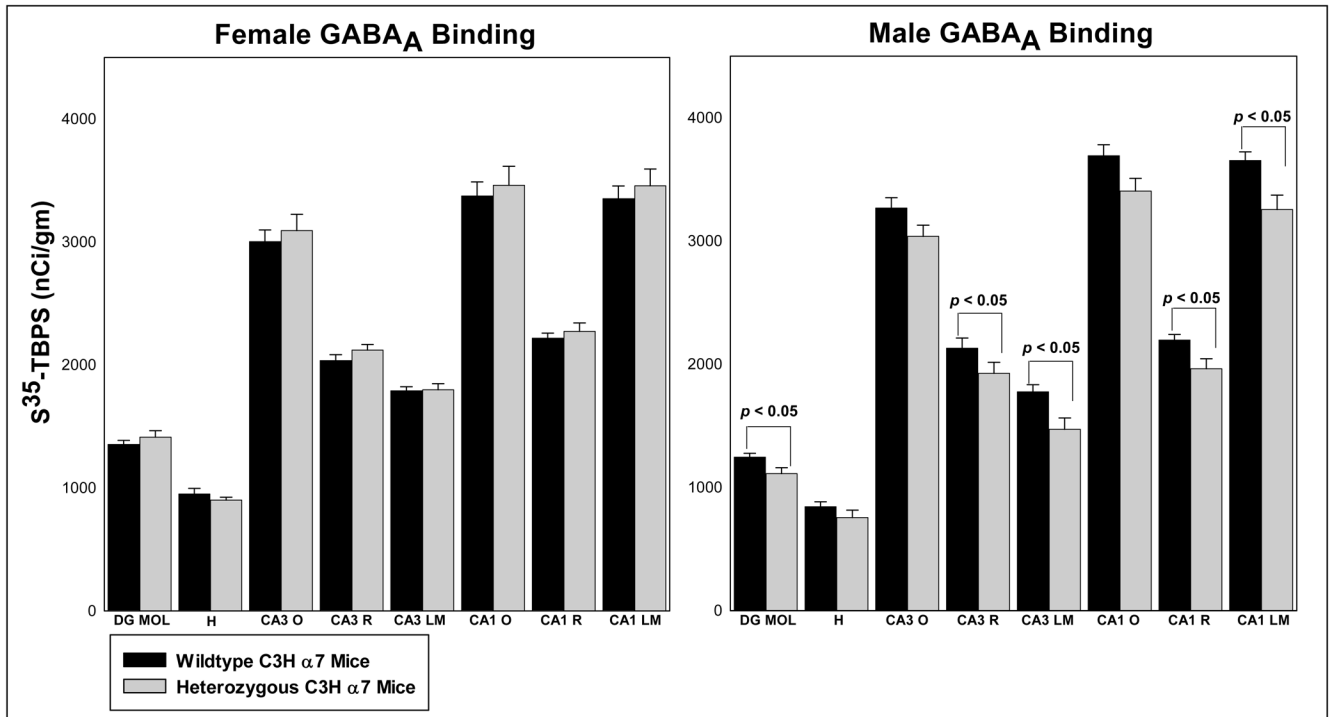


Figure 4.

[35S]-TBPS binding in hippocampal sections from female ($n = 6/\text{group}$, left panel) and male ($n = 6/\text{group}$, right panel) wildtype and heterozygous C3H a7 mice. No significant differences in GABA_A receptor levels were seen in the female mice. In contrast, significant decreases in GABA_A receptor density were seen in the molecular layer of the dentate gyrus and in strata radiatum and lacunosum moleculare of hippocampal areas CA3 and CA1 in male heterozygous C3H a7 mice. *Abbreviations:* DG MOL – molecular layer of the dentate gyrus, H – hilus (polymorph layer) of dentate gyrus, CA3 O – oriens layer of hippocampal area CA3, CA3 R – radiatum layer of hippocampal area CA3, CA3 LM – lacunosum moleculare layer of hippocampal area CA3, CA1 O – oriens layer of hippocampal area CA1, CA1 R – radiatum layer of hippocampal area CA1, CA1 LM – lacunosum moleculare of hippocampal area CA1. Values = mean \pm S.E.M.

Table 1Hippocampal GAD₆₅ Levels in C3H α 7 Wildtype vs Heterozygous Mice

Layer	F(1,20) =	<i>p</i>
Dentate gyrus molecular layer	3.617	0.072 *
Dentate gyrus hilus	2.471	0.132
CA3 Oriens	4.259	0.052 *
CA3 Pyramidale	4.297	0.051 *
CA3 Lucidum/Radiatum	4.698	0.042 *
CA3 Lacunosum moleculare	4.651	0.043 *
CA1 Oriens	2.935	0.102
CA1 Pyramidale	2.874	0.106
CA1 Radiatum	3.439	0.078 *
CA1 Lacunosum moleculare	3.373	0.081

* Statistically significant

* Trend

Table 2
 GABA_A Receptor Density in the Hippocampal Formation of Male and Female C3H α 7 Wildtype and Heterozygous Mice

Layer	Gender F(1,20)=	p	Genotype F(1,20)=	p	Interaction F(1,20)=	p
Dentate gyrus of the molecular layer	23.125	<0.001	0.778	0.388	5.165	0.034
Dentate gyrus hilus	8.270	0.009	2.538	0.127	0.190	0.667
CA3 Oriens	1.048	0.318	0.471	0.500	2.438	0.134
CA3 Radiatum	0.525	0.477	0.731	0.403	4.426	0.048
CA3 Lacunosum moleculare	7.466	0.013	5.676	0.027	6.314	0.021
CA1 Oriens	1.238	0.279	0.737	0.401	2.527	0.128
CA1 Radiatum	7.304	0.014	2.141	0.159	5.485	0.030
CA1 Lacunosum moleculare	0.200	0.660	1.792	0.196	5.245	0.033