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Reduced *Chrna7* expression in C3H mice is associated with increases in hippocampal parvalbumin and glutamate decarboxylase-67 (GAD67) as well as altered levels of GABA_A receptor subunits

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Abstract

Decreased expression of *CHRNA7*, the gene encoding the $\alpha 7^*$ subtype of nicotinic receptor, may contribute to the cognitive dysfunction observed in schizophrenia by disrupting the inhibitory/excitatory balance in the hippocampus. C3H mice with reduced *Chrna7* expression have significant reductions in hippocampal $\alpha 7^*$ receptor density, deficits in hippocampal auditory gating, increased hippocampal activity as well as significant decreases in hippocampal glutamate decarboxylase-65 (GAD65) and γ -aminobutyric acid-A (GABA_A) receptor levels. The current study investigated whether altered *Chrna7* expression is associated with changes in the levels of parvalbumin, GAD67 and/or GABA_A receptor subunits in hippocampus from male and female C3H *Chrna7* wildtype, C3H *Chrna7* heterozygous and C3H *Chrna7* knockout mice using quantitative western immunoblotting. Reduced *Chrna7* expression was associated with significant increases in hippocampal parvalbumin and GAD67 and with complex alterations in GABA_A receptor subunits. A decrease in $\alpha 3$ subunit protein was seen in both female C3H *Chrna7* Het and KO mice while a decrease in $\alpha 4$ subunit protein was also detected in C3H *Chrna7* KO mice with no sex difference. In contrast, an increase in δ subunit protein was observed in C3H *Chrna7* Het mice while a decrease in this subunit was observed in C3H *Chrna7* KO mice, with δ subunit protein levels being greater in males than in females. Finally, an increase in $\gamma 2$ subunit protein was found in C3H *Chrna7* KO mice with the levels of this subunit again being greater in males than in females. The increases in hippocampal parvalbumin and GAD67 observed in C3H *Chrna7* mice are contrary to reports of reductions in these proteins in postmortem hippocampus from

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schizophrenic individuals. We hypothesize that the disparate results may occur because of the influence of factors other than *CHRNA7* that have been found to be abnormal in schizophrenia.

Keywords

Chrna7; λ -aminobutyric acid (GABA); GABA_A receptors; parvalbumin; glutamate decarboxylase 67 (GAD67); schizophrenia

Cognitive dysfunction is a core feature of schizophrenia (Barch and Ceaser, 2012, Minzenberg and Carter, 2012). Deficits have been observed in a variety of cognitive domains, including sensory filtering (gating), attention, spatial working memory, executive function, context processing and episodic memory (Piskulic et al., 2007, Barch and Ceaser, 2012, Minzenberg and Carter, 2012). Cognitive abnormalities are detected at the first presentation of the disease, but are also present prior to illness onset (Kalkstein et al., 2010).

Normal cognitive function depends upon a number of brain regions, including the hippocampus (Kroes and Fernandez, 2012). Schizophrenia-associated cognitive deficits may occur, at least in part, because of abnormalities found in the hippocampus of schizophrenic individuals (Todtenkopf and Benes, 1998, Tregellas et al., 2004, Tregellas et al., 2007, Heckers and Konradi, 2010, Konradi et al., 2011, Williams et al., 2011). The factors contributing to schizophrenia-associated hippocampal abnormalities are currently ill-defined.

Schizophrenia is thought to arise from a complex interaction between genetic and environmental risk factors (Pickard, 2011, Hosak, 2013). A large number of genes have been proposed as potential risk factors for schizophrenia, including the gene encoding the $\alpha 7^*$ subtype of nicotinic receptor, *CHRNA7*(human)/*Chrna7*(mouse) (Pickard, 2011, Hosak, 2013, Mowry and Gratten, 2013). *CHRNA7* is found in the q13-q14 region of chromosome 15 in humans (Leonard and Freedman, 2006). The 15q13-q14 region has been linked to a P50 auditory gating deficit observed in schizophrenic patients as well as to schizophrenia in a number of studies, although not all (Stephens et al., 2009). A recurrent microdeletion at 15q13.3, which includes *CHRNA7*, has been shown to significantly associate with schizophrenia (Stefansson et al., 2008). In addition, the *CHRNA7* gene has been associated with schizophrenia and with the memory and P50 auditory sensory gating deficits observed in schizophrenic individuals (Leonard and Freedman, 2006, Stephens et al., 2012).

The density of $\alpha 7^*$ receptors is significantly reduced in postmortem hippocampus from schizophrenic individuals (Heckers and Konradi, 2010). The reduction in hippocampal $\alpha 7^*$ receptor levels may contribute to schizophrenia-associated cognitive dysfunction as hippocampal $\alpha 7^*$ receptors have been implicated in several cognitive domains impaired in schizophrenia, including sensory gating, spatial working memory, context processing and episodic memory (Thomsen et al., 2010, Graef et al., 2011, Wallace and Porter, 2011). Treatment with $\alpha 7^*$ receptor agonists has been shown to improve cognitive abnormalities in both animals and humans. The selective $\alpha 7^*$ receptor agonist TC-5619 corrected abnormalities in social behavior as well as deficits in paired-pulse inhibition (PPI), a measure of sensory gating, in transgenic mice and Sprague-Dawley rats (Hauser et al.,

2009). Improved performance on a social recognition task was observed in Wistar rats following treatment with the selective $\alpha 7^*$ receptor agonist AR-R 17779 (Van Kampen et al., 2004). Another selective $\alpha 7^*$ receptor agonist, ABT-107, improved auditory gating abnormalities in DBA/2 mice (Radek et al., 2012). The P50 gating deficit observed in schizophrenic individuals was significantly improved by administration of tropisetron, a partial agonist at $\alpha 7^*$ receptors and an antagonist at 5-HT₃ receptors (Koike et al., 2005). DMXB-A, a partial agonist at the $\alpha 7^*$ receptor and an antagonist at $\alpha 4\beta 2^*$ receptors, improved attention, working memory, and negative symptoms in nonsmoking patients with schizophrenia (Olinco and Freedman, 2012). Improvement in both cognitive deficits and negative symptoms was also reported in smoking and nonsmoking schizophrenic patients following treatment with EVP-6124, an $\alpha 7$ agonist and 5-HT₃ antagonist (Wallace and Bertrand, 2013). Finally, the full $\alpha 7$ agonist TC-5619 improved cognitive dysfunction as well as negative symptoms in a 12-week study of both smoking and nonsmoking schizophrenic individuals (Wallace and Bertrand, 2013). These data suggest that treatment with $\alpha 7^*$ receptor agonists may be a viable approach for improving schizophrenia-associated cognitive deficits.

Hippocampal $\alpha 7^*$ receptors are expressed by neurons containing the inhibitory neurotransmitter λ -aminobutyric acid (GABA) as well as by neurons containing the excitatory neurotransmitter glutamate (Thomsen et al., 2010, Wallace and Porter, 2011). Activation of the receptor modulates the release of both hippocampal GABA and glutamate (Thomsen et al., 2010, Wallace and Porter, 2011). Therefore, the $\alpha 7^*$ receptor can influence the inhibitory/excitatory balance in the hippocampus, an important factor underlying normal cognition (Leiser et al., 2009, Morellini et al., 2010). A decrease in $\alpha 7^*$ receptor density could disrupt hippocampal inhibitory/excitatory homeostasis, thereby altering hippocampal-mediated cognitive function.

We hypothesize that reduced *CHRNA7* expression is one of many factors contributing to schizophrenia-associated hippocampal abnormalities. To test this hypothesis, we are using a mouse model of reduced *Chrna7* expression (C3H *Chrna7* mice) to examine to what extent a decrease in $\alpha 7^*$ receptor density may, or may not, be associated with specific hippocampal abnormalities observed in schizophrenia. Compared to wildtype (C3H *Chrna7* WT) mice, C3H mice heterozygous for *Chrna7* (C3H *Chrna7* Het mice) have significant reductions in hippocampal $\alpha 7^*$ receptor density, deficits in hippocampal auditory gating, increased hippocampal activity (Adams et al., 2008) and significant decreases in hippocampal glutamate decarboxylase-65 (GAD65) and GABA_A receptor levels (Adams et al., 2012). With the exception of the decreased GABA_A receptor levels, the hippocampal alterations observed in the C3H *Chrna7* Het mice are comparable to those reported in hippocampus of schizophrenic individuals (Todtenkopf and Benes, 1998, Tregellas et al., 2004, Tregellas et al., 2007, Heckers and Konradi, 2010, Konradi et al., 2011, Williams et al., 2011). These data suggest that decreased *CHRNA7* expression may be a primary factor underlying some, but not all, of the hippocampal abnormalities observed in schizophrenia.

The current study expanded our investigation by examining protein levels of parvalbumin, GAD67 and GABA_A receptor subunits in hippocampus from male and female C3H *Chrna7* WT, C3H *Chrna7* Het and C3H *Chrna7* KO (knockout) mice. We investigated parvalbumin

and GAD67 because the $\alpha 7^*$ receptor is expressed by many subtypes of GABAergic neurons (Freedman et al., 1993, Thomsen et al., 2010, Wallace and Porter, 2011), including a subpopulation which contains parvalbumin (Arevalo-Serrano et al., 2008) and each of these proteins has been reported to be decreased in postmortem hippocampus from schizophrenic patients (Zhang and Reynolds, 2002, Benes et al., 2007, Heckers and Konradi, 2010, Konradi et al., 2011, Thompson Ray et al., 2011). Parvalbumin is a calcium binding protein that plays an important role in regulating pyramidal neuron activity (Freund, 2003), while GAD67 is one of two enzymes that synthesize GABA (Martin and Tobin, 2000). We examined the levels of GABA_A receptor subunit proteins to determine whether decreases in these subunits could account for the reduction in hippocampal GABA_A receptor binding observed in male C3H *Chrna7* Het mice (Adams et al., 2012).

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 *Chrna7* KO mice were derived by mating $\alpha 7$ mixed background (129xC57BL/6) mice with C3H mice (both sexes used). The progeny that resulted from this crossing were +/+ and +/- at the $\alpha 7$ locus. The +/- mice were crossed with C3H mice. The resulting +/- progeny were used to create the next generation. This process was continued 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 Affairs Medical Center, Denver, CO. Genome diversity analysis (The Jackson Laboratory, Bar Harbor, Maine) indicates that the C3H *Chrna7* KO mice are at least 99.7% identical to the C3H parent strain. C3H *Chrna7* Het mice were bred together to obtain 25% C3H *Chrna7* WT, 50% C3H *Chrna7* Het and 25% C3H *Chrna7* KO from each litter. Mice used in the current study were housed by sex in shoe-box cages (maximum 5 mice/cage) with filter tops and passive air flow conditions. The cages were changed twice a week (Aspen chip bedding) and the mice were provided with *ad libitum* dry food (Teklab 2018) and water.

2.2 Tissue Collection for Western Immunoblotting

Brains ($n = 6$ /group, 3 – 4 months of age) from male and female C3H *Chrna7* WT, C3H *Chrna7* Het and C3H *Chrna7* KO mice were removed, the overlying cortices were peeled back and the entire hippocampus from each hemisphere was rapidly dissected. The two hippocampi were placed in a plastic tube, frozen in dry ice snow and stored at -80°C in a CoolRack M90 (BioCision; BCS-117; Mill Valley, CA) until processing.

2.3 Sample lysate preparation

Hippocampi from each animal were brought out individually and added to lysis buffer composed of 2% SDS (sodium dodecyl sulfate), 40 mM DTT(dithiothreitol), 20 mM Tris-HCl (pH 6.5), 1% (v/v) phosphatase inhibitors (Sigma-Aldrich; P2850; P5726; St. Louis, MO), 3x Protease Arrest (Cambiochem/EMD; 539124; Gibbstown, NJ), 20 mM EDTA (ethylenediaminetetraacetic acid) and then homogenized in a Potter-Elvehjem tissue homogenizer. Lithium dodecyl sulfate (LDS) buffer (Invitrogen; NP0008; Carlsbad,

California) was added to samples, vortexed, and stored at -20°C until use in the Western blot studies.

2.4 Western immunoblotting

Hippocampal lysates were thawed at room temperature and loaded into an electrophoresis chamber (Invitrogen, Midi-Cell Runner WR0100, Carlsbad, California) with NuPAGE Novex 4-12% Bis-Tris Midi gels (Invitrogen, WG1403 Carlsbad, California). The gels were run for 2 hours at 70 volts and were then transferred (Invitrogen, Novex Semi-Dry Blotter SD1000 Carlsbad, California) to a fluorescence-checked PVDF (polyvinylidene fluoride membrane) (Millipore, Immobilon-FL IPFL07810 Billerica, MA) for 1 hour and 45 minutes at 22 volts. The membrane was then incubated in undiluted blocking buffer (LI-COR Biosciences; 927-40000; Lincoln, NE) for 1 hour on a rotator (Stovall; The Belly Dancer Lab Shaker) at room temperature. Primary antibodies were added to undiluted blocking buffer and poured over the blot, then allowed to rotate overnight at 4°C. Following two quick washes and one 20-minute wash, each with TBS (Tris-buffered saline) containing 0.05% Tween-20 (TBST), the blot was incubated for 1 hour at room temperature with IRDye 800CW goat anti-rabbit pAb antibody (1:10,000; LI-COR Biosciences; 926-32211; Lincoln, NE) and/or IRDye 680LT goat anti-mouse pAb antibody (1:20,000; LI-COR Biosciences; 926-68020; Lincoln, NE). The blot again received two quick washes and then one 20-minute wash in TBST. After washing, it was placed in cold PBS (phosphate buffered saline) buffer (pH 7.4) until arranged on the Odyssey glass bed (LI-COR Biosciences; Odyssey Infrared Imager System; Lincoln, NE). A silicone mat was placed on top to provide a low contrast background around the blots. Fluorescent immunocomplexes were detected with the LI-COR Odyssey (700 nm and/or 800 nm channel, 42 µm resolution, and highest quality). Channel sensitivity was optimized for each blot. Typical laser intensity settings ranged from 5.0 to 6.0 and integrated intensities were determined with the Odyssey system software. After scanning with both lasers, target protein density values were normalized by the actin value for each sample.

2.5 Primary Antibodies

Parvalbumin (rabbit, 0.1 µg/ml, ab11427), GABA_A α1 (rabbit, 0.5 µg/ml, ab33299), GABA_A α2 (rabbit, 1:1000, ab72445), GABA_A α3 (rabbit, 1:1000, ab72446), GABA_A α4 (rabbit, 1:1000, ab78384), GABA_A α5 (rabbit, 1:1000, ab10098), GABA_A λ2 (rabbit, 1 µg/ml, ab87328), GABA_A δ (rabbit, 1:1000, ab111048), GABA_A β1 (mouse, 1 µg/ml, ab93612), GABA_A β2 (rabbit, 1:1000, ab8340) and GAD67 (mouse, 1:1000, ab26116). All primary antibodies were purchased from Abcam (Cambridge, MA).

2.6 Statistical Analysis

Data were analyzed by a two-way ANOVA assessing differences in hippocampal protein levels across sex and genotype, with Holm-Sidak *a posteriori* analysis where appropriate (SigmaPlot 12).

3.0 Results

3.1 Hippocampal parvalbumin levels are altered across *Chrna7* genotype and sex

Hippocampal levels of parvalbumin protein varied in a Genotype X Sex manner. Significantly higher levels of the calcium binding protein were observed in male C3H *Chrna7* Het and C3H *Chrna7* KO mice (39% and 52%, respectively) than in male C3H *Chrna7* WT mice. In addition, parvalbumin levels were significantly greater (20%) in male C3H *Chrna7* KO mice than in male C3H *Chrna7* Het mice. Female C3H *Chrna7* Het mice also exhibited significantly higher (41%) levels of hippocampal parvalbumin than female C3H *Chrna7* WT mice. Finally, parvalbumin levels were significantly greater (29%) in male C3H *Chrna7* KO mice than in female C3H *Chrna7* KO mice (Genotype: $F(2,30) = 17.767$, $p < 0.001$, Sex: $F(1,30) = 2.321$, $p = 0.138$, Genotype X Sex: $F(2,30) = 3.481$, $p = 0.044$). (Figure 1).

3.2 Hippocampal GAD67 protein levels are altered across *Chrna7* genotype and sex

Hippocampal GAD67 protein levels also varied in a Genotype X Sex manner. The levels of this enzyme were significantly higher (36%) in male C3H *Chrna7* KO mice than in male C3H *Chrna7* WT and C3H *Chrna7* Het mice. In addition, GAD67 protein was significantly higher in female C3H *Chrna7* Het and C3H *Chrna7* KO (36% and 24%, respectively) mice than in female C3H *Chrna7* WT mice. Lastly, significantly greater (47%) levels of hippocampal GAD67 were observed in female C3H *Chrna7* Het mice than in male C3H *Chrna7* Het mice (Genotype: $F(2,30) = 7.016$, $p = 0.003$, Sex: $F(1,30) = 12.078$, $p = 0.002$, Genotype X Sex: $F(2,30) = 3.810$, $p = 0.034$) (Figure 2).

3.3 A subset of hippocampal GABA_A receptor subunit protein levels are altered across *Chrna7* genotype and sex

Hippocampal protein levels of nine GABA_A receptor subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, δ , $\gamma 2$) were examined. A Genotype X Sex interaction was seen for hippocampal levels of the $\alpha 3$ subunit protein. Reductions in *Chrna7* expression had no significant effect on $\alpha 3$ protein levels in males. The level of $\alpha 3$ subunit protein was altered by reduced *Chrna7* expression in female mice, however, as significantly lower levels of the subunit were seen in female C3H *Chrna7* Het mice (54%) and female C3H *Chrna7* KO (39%) mice compared to female C3H *Chrna7* WT mice. In addition, $\alpha 3$ subunit protein levels were significantly lower in male C3H *Chrna7* WT (42%) mice relative to female C3H *Chrna7* WT mice (Genotype: $F(2,30) = 2.036$, $p = 0.148$, Sex: $F(1,30) = 0.0246$, $p = 0.877$, Genotype X Sex: $F(2,30) = 8.10$, $p = 0.002$) (Figure 3). There was no effect of sex for the GABA_A receptor $\alpha 4$ subunit so the data was pooled across genotype. Hippocampal levels of the $\alpha 4$ subunit protein were significantly lower in C3H *Chrna7* KO mice (12% and 14%, respectively) than in C3H *Chrna7* WT and C3H *Chrna7* Het mice. (Genotype: $F(2,30) = 4.815$, $p = 0.015$, Sex: $F(1,30) = 0.0736$, $p = 0.788$, Genotype X Sex: $F(2,30) = 0.163$, $p = 0.850$) (Figure 4). Separate genotype (Figure 5, left panel) and sex (Figure 5, right panel) effects were observed for the GABA_A receptor δ subunit, with no interaction between the two factors. Hippocampal levels of the δ subunit protein were significantly greater (18%) in C3H *Chrna7* Het mice than in C3H *Chrna7* WT mice. In contrast, δ subunit protein levels were significantly lower in C3H *Chrna7* KO mice (24% and 38%, respectively) than in C3H *Chrna7* WT and C3H *Chrna7*

Het mice. With regard to sex, δ subunit protein was significantly lower (14%) in females than in males (Genotype: $F(2,30) = 22,863$, $p < 0.001$, Sex: $F(1,30) = 7.357$, $p = 0.011$, Genotype X Sex: $F(2,30) = 7.65$, $p = 0.474$). Distinct genotype (Figure 6, left panel) and sex (Figure 6, right panel) effects, with no interaction, were also observed for the GABA_A receptor $\gamma 2$ subunit. A significant increase (24%) in $\gamma 2$ subunit protein was observed in C3H *Chrna7* KO mice relative to C3H *Chrna7* WT mice, while $\gamma 2$ subunit protein was significantly lower (23%) in females than in the males (Genotype: $F(2,30) = 22,863$, $p < 0.001$, Sex: $F(1,30) = 7.357$, $p = 0.011$, Genotype X Sex: $F(2,30) = 7.65$, $p = 0.474$). No significant changes were observed across genotype or sex for the $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 1$ or $\beta 2$ subunits (data not shown).

4.0 Discussion

Reduced *Chrna7* expression was associated with increases in hippocampal levels of the calcium binding protein parvalbumin and the GABA-synthesizing enzyme GAD67 as well as with complex changes in a subset of GABA_A receptor subunits in the present study. These data provide additional evidence that a decrease in $\alpha 7^*$ receptor density disrupts normal hippocampal inhibitory processes.

4.1 Increased parvalbumin

Significant increases in hippocampal parvalbumin were observed in male C3H *Chrna7* Het and KO mice and in female C3H *Chrna7* Het mice in the current study. Parvalbumin is a calcium-binding protein found in GABAergic interneurons (basket and chandelier cells) that innervate the perisomatic region of hippocampal pyramidal cells (Freund, 2003). Parvalbumin both binds to and dissociates from calcium at a relatively slow rate (Collin et al., 2005). A functional consequence of these binding kinetics is that parvalbumin slows the decay of intracellular calcium that enters when the neuron is depolarized by action potentials, resulting in a prolonged release of neurotransmitter (GABA) that can last for seconds, increasing inhibition (Collin et al., 2005, Lisman et al., 2008).

Parvalbumin levels are modulated through the ERK1/2/CREB (extracellular signal-regulated kinase 1/2/cAMP response element-binding protein) signal transduction pathway in an activity-dependent manner through activation of NMDA receptors (Kinney et al., 2006, Lisman et al., 2008). Blockade of NR2A-containing NMDA receptors results in a decrease in parvalbumin levels (Kinney et al., 2006). The decrease in NMDA receptor activation is hypothesized to be a signal to parvalbumin-containing interneurons that pyramidal neuron activity is too low, leading to a decrease in parvalbumin levels and inhibitory output as a compensatory mechanism (Lisman et al., 2008). An increase in activation of NR2A-containing NMDA receptors would be expected to increase parvalbumin levels, resulting in enhanced inhibition.

C3H *Chrna7* Het mice exhibit hippocampal disinhibition (Adams et al., 2008). We postulate that the increase in parvalbumin observed in male C3H *Chrna7* Het and KO mice and in female C3H *Chrna7* Het mice in the present study is a compensatory response to potentiate inhibition in the face of increased pyramidal neuron activity, at least in Het mice, as we do not know how complete loss of *Chrna7* expression affects pyramidal neuron firing. The

differential responses of the two sexes could result from a small sample size (6/group). Alternately, it is possible that male and female C3H *Chrna7* KO mice have different compensatory responses to a complete loss of *Chrna7* expression, as a number of studies have reported sex differences in hippocampal-related measures in knockout mice (Sakata et al., 2009, Moore et al., 2010, Berry et al., 2012, Wu et al., 2012, Pimentel-Coelho et al., 2013, Procaccini et al., 2013).

The hippocampus of schizophrenic individuals is characterized by elevated levels of activity during rest (Heckers and Konradi, 2010), during a smooth pursuit eye movement task (Tregellas et al., 2004) and during an auditory sensory gating task (Tregellas et al., 2007). In addition, schizophrenic patients exhibit significantly higher absolute concentrations of hippocampal glutamate compared to controls in a magnetic resonance spectroscopy study (van Elst et al., 2005). Despite the increase in activity, however, a decrease in the number of parvalbumin-containing interneurons has been reported in postmortem hippocampus from schizophrenic individuals (Zhang and Reynolds, 2002, Konradi et al., 2011), although the data are somewhat contradictory. In one study, (Zhang and Reynolds, 2002), fewer parvalbumin-positive neurons were observed in all areas of the hippocampal formation in both males and females, but was significantly lower only in males. Postmortem interval, subject age, brain pH, time in formalin and drug exposure (antipsychotic or other) was not significantly correlated with the number of parvalbumin-positive neurons in this study. In contrast, gender differences were not detected in a second study reporting significant schizophrenia-associated decreases in hippocampal parvalbumin-immunoreactive neurons as a main effect. However, post-hoc analysis (subject age, gender, brain hemisphere and postmortem interval as covariates) failed to find a significant decrease in parvalbumin-labeled neurons in hippocampal subregions, although significance was approached in areas CA1 and CA4 (Konradi et al., 2011). Together, these studies suggest that the number of parvalbumin neurons either decreases or remains the same in the hippocampus of schizophrenic patients, depending upon the specific area and gender.

Schizophrenic individuals have been reported to exhibit decreased hippocampal glutamate receptor-1 (GluR1), GluR2, GluR6, kainate receptor-1 (KA1) and NMDA receptor-1 (NR1) subunit expression, decreased hippocampal AMPA and kainate receptor binding as well as reduced hippocampal vesicular glutamate transporter (VLGUT 1 and 2) mRNA (Heckers and Konradi, 2010). These data suggest that compensatory adjustments in excitatory proteins may occur in the hippocampus of schizophrenic individuals in response to excess glutamatergic tone. A decrease in the obligate NR1 subunit (Wyllie et al., 2013) could result in decreased activation of hippocampal NMDA receptors, including those containing the NR2A subunit, potentially leading to a decrease in hippocampal parvalbumin in schizophrenic patients (Lisman et al., 2008).

4.2 Increased GAD67

Significant increases were also observed in hippocampal GAD67 in male C3H *Chrna7* KO mice as well as in female C3H *Chrna7* Het and KO mice. GAD67 is predominantly found in neuronal cell bodies and dendrites where it produces a cytosolic pool of GABA that is

postulated to provide a more slowly acting, diffuse inhibitory signal (Martin and Tobin, 2000).

GAD67 is regulated by at least two mechanisms – phosphorylation and neural activity. Phosphorylation by protein kinase A (PKA) decreases GAD67 activity while dephosphorylation by calcineurin increases GAD67 activity (Martin and Tobin, 2000, Wei et al., 2004). Stimulation of the $\alpha 7^*$ receptor increases activation of PKA (Thomsen et al., 2010). Therefore, reduced *Chrna7* expression could lead to decreased PKA activation and a subsequent increase in GAD67 activity.

Upregulation of both GAD67 mRNA and protein has been observed with increased excitatory activity (Esclapez and Houser, 1999, Ramirez and Gutierrez, 2001, Lau and Murthy, 2012). The increase in GAD67 may be mediated by brain-derived neurotrophic factor (BDNF), as administration of BDNF increases GAD67 mRNA and protein (Mizuno et al., 1994, Yamada et al., 2002) while loss of the tyrosine kinase B (TrkB) receptor for BDNF leads to decreases in GAD67 mRNA (Hashimoto et al., 2005). Blockade of hippocampal $\alpha 7^*$ receptors with the selective antagonist α -bungarotoxin significantly increased hippocampal BDNF expression (Freedman et al., 1993), suggesting that a reduction in *Chrna7* could lead to increased BDNF which, in turn, could upregulate GAD67. The observed disparity in upregulation of hippocampal GAD67 in males and females in the current study could result from a small sample size, from differential sex-dependent regulation of BDNF (Mizuno and Giese, 2010) and/or from sex-specific responses to a complete loss of *Chrna7* expression in the case of C3H *Chrna7* KO mice.

Studies of hippocampal GAD67 mRNA levels suggest that GAD67 protein may be decreased in postmortem hippocampus from schizophrenic patients (Benes et al., 2007, Heckers and Konradi, 2010, Konradi et al., 2011, Thompson Ray et al., 2011), though again the data is variable. Decreased GAD67 expression was observed in stratum radiatum, pyramidal and oriens of hippocampal area CA2/3 and in stratum oriens of area CA1 in one study (Benes et al., 2007). The subjects in this study were matched for age, postmortem interval, gender, brain pH, brain laterality and 18S/28S ratios, but the potential influence of drug treatment did not appear to be specifically assessed. In contrast, significant decreases in GAD67 mRNA were seen in the dentate gyrus and in hippocampal area CA4 in another study (Thompson Ray et al., 2011). No correlation was observed between the decreases in hippocampal GAD67 mRNA and sex, age, race, postmortem interval, brain pH, brain hemisphere, suicide, history of substance abuse, smoking history or lifetime neuroleptic use in this study. Collectively, these data suggest that GAD67 mRNA is decreased in at least some regions of postmortem schizophrenic hippocampus, although to our knowledge, GAD67 protein has not been directly measured in this tissue. A schizophrenia-associated decrease in hippocampal GAD67 could result from reduced NMDA receptor activation (Lisman et al., 2008) and/or from reduced levels of hippocampal BDNF and TrkB, as decreased expression of both BDNF and TrkB has been observed in postmortem hippocampus from schizophrenic individuals (Thompson Ray et al., 2011).

4.3 Factors that may contribute to the contradictory results observed between C3H *Chrna7* mice and schizophrenic individuals

The current study observed sex- and genotype-specific increases in hippocampal parvalbumin and GAD67 in C3H *Chrna7* mice. This is in contrast to reports of decreases in the number of parvalbumin-positive neurons and in GAD67 mRNA in postmortem hippocampus from schizophrenic patients. One factor that might contribute to the disparate results is species differences. The human hippocampus is much more complex than that of the mouse. In addition, the density of $\alpha 7^*$ receptors appears to be lower in human than in rodent hippocampus (Breese et al., 1997). Therefore, a reduction in hippocampal $\alpha 7^*$ receptor density might have very different consequences in humans than in mice.

Differences in experimental design might also be a contributing factor. The present study utilized Western immunoblot analysis of fresh-frozen whole hippocampus to quantitatively measure levels of parvalbumin and GAD67 protein. In contrast, the human parvalbumin studies utilized immunohistochemical identification of parvalbumin-labeled neurons in fixed tissue. Variations in tissue fixation and antibody penetration could affect estimates of the number of parvalbumin-positive neurons. In addition, the human studies measured hippocampal levels of GAD67 mRNA as opposed to levels of GAD67 protein. Although the decreases in GAD67 mRNA observed in some hippocampal regions of schizophrenic subjects suggests that GAD67 protein may also be reduced, this has not as yet been directly tested. Another contributing factor could be differences in sample size between the various studies. This study used 6 mice/group, fewer than the number of subjects used in the human studies, which ranged from 7 – 18 control subjects and 7 – 15 schizophrenic subjects/study. However, despite the greater number of subjects in the human studies, only one study utilized stereological techniques for assessing the number of parvalbumin-positive neurons throughout the entire extent of the hippocampus (Konradi et al., 2011). In contrast, Zhang et al. (Zhang and Reynolds, 2002) counted parvalbumin-positive neurons in only two sections/subject. GAD67 mRNA levels were also measured in only two sections/subject by Thompson Ray et al. (Thompson Ray et al., 2011) while Benes et al. (Benes et al., 2007) measured hippocampal GAD67 mRNA in seven sections/subject. Thus, variations in the amount of tissue examined could be another factor underlying the dissimilar results.

Finally, it is possible that the observed differences occur because the effect of reduced *Chrna7* expression on hippocampal levels of parvalbumin and GAD67 is more readily observable in C3H *Chrna7* mice than in schizophrenic individuals. C3H *Chrna7* mice are primarily a model of reduced *Chrna7* expression. Schizophrenia, on the other hand, is characterized by alterations in a large number of genes (Pickard, 2011, Hosak, 2013, Mowry and Gratten, 2013). Although speculative, it is possible that the effect of reduced *CHRNA7* expression on hippocampal parvalbumin and GAD67 in schizophrenic patients may be overshadowed by influences from other factors that have also been found to be abnormal in the disease, especially those affecting NMDA receptor function (Lisman and Buzsaki, 2008, Gonzalez-Burgos and Lewis, 2012).

4.4 Alterations in GABA_A receptor subunit proteins

Complex alterations in the protein levels of several hippocampal GABA_A receptor subunits were found in the present study. A decrease in $\alpha 3$ subunit protein was seen in both female C3H *Chrna7* Het and KO mice. A decrease in $\alpha 4$ subunit protein was also detected in C3H *Chrna7* KO mice with no sex difference. In contrast, an increase in δ subunit protein was observed in C3H *Chrna7* Het mice while a decrease in this subunit was observed in C3H *Chrna7* KO mice, with δ subunit protein levels being greater in males than in females. Finally, an increase in $\gamma 2$ subunit protein was found in C3H *Chrna7* KO with the levels of this subunit again being greater in males than in females. The changes in GABA_A receptor subunit levels may contribute to the hippocampal inhibitory abnormalities observed in C3H *Chrna7* mice (Adams et al., 2008, Adams et al., 2012).

None of the subunit changes appear to explain the decrease in hippocampal GABA_A receptor binding found in male C3H *Chrna7* Het mice (Adams et al., 2012), as the only alterations observed in male Het mice in the current study were increases in the δ and $\gamma 2$ subunits. This could be due to a small sample size. Alternatively, the decrease in hippocampal GABA_A receptor binding observed previously in male C3H *Chrna7* Het mice may result primarily from sex-dependent influences of BDNF, kinases and/or gonadal steroids as discussed in the earlier study (Adams et al., 2012).

The mechanisms responsible for the changes in GABA_A receptor subunits observed in the current study are unclear. Activity-dependent increases in the expression of δ and $\gamma 2$ subunits have been reported (Gault and Siegel, 1997, Holopainen and Lauren, 2003, Saliba et al., 2007), although prolonged treatment with NMDA reduced both δ and $\alpha 4$ subunit expression via an ERK1/2-dependent process (Joshi and Kapur, 2013). Reduced *Chrna7* expression results in hippocampal disinhibition (Adams et al., 2008). Thus, an increase in neural activity combined with sex-specific modulation of signal transduction mechanisms (Mizuno and Giese, 2010) may account for some of the observed subunit changes.

Brief GABA exposure was associated with a reduction in $\alpha 3$ subunit-containing GABA_A receptors due to increased internalization (Gutierrez et al., 2013). BDNF has been shown to increase GABA_A receptor internalization (Brunig et al., 2001) and reduced *Chrna7* expression may increase hippocampal BDNF levels (Freedman et al., 1993). Therefore, the decrease in hippocampal $\alpha 3$ subunit protein observed in female C3H *Chrna7* Het and KO mice may result from enhanced GABA exposure secondary to increases in GAD67 and parvalbumin in combination with sex-specific regulation of BDNF (Mizuno and Giese, 2010). Despite these speculations, it must be kept in mind that the changes in GABA_A receptor subunits observed in the present study may have no functional consequences since the majority of GABA_A receptor subunits are not assembled into receptors (Baumgartner et al., 1994, Miranda and Barnes, 1997, Luscher et al., 2011).

Various changes in GABA_A receptor subunit expression have been observed in postmortem schizophrenic neocortex. Significant decreases in $\alpha 5$ subunit mRNA were observed in the dorsal lateral prefrontal cortex (DLPFC) of an Australian cohort with no change in the expression levels of other α subunits (Duncan et al., 2010). In contrast, $\alpha 1$, $\alpha 5$ and $\beta 2$ expression were decreased while $\alpha 2$ expression was increased in various layers of the

DLPFC in another study (Beneyto et al., 2011). To our knowledge, potential alterations in GABA_A receptor subunit expression in postmortem hippocampus from schizophrenic individuals has not been investigated. Data from the present study suggest that reduced *CHRNA7* expression may result in abnormalities in hippocampal GABA_A receptor subunits in schizophrenic individuals.

4.5 Conclusion

Decreased *Chrna7* expression is associated with abnormalities in hippocampal proteins important for inhibitory function and a disruption in normal hippocampal excitatory/inhibitory balance in C3H mice. These data suggest that reduced *CHRNA7* expression may contribute to hippocampal abnormalities observed in schizophrenia, abnormalities that may be a factor in the cognitive dysfunction characteristic of the disease. Decreased *CHRNA7* expression may also play a role in the etiology of epilepsy and autism. A 15q13.3 microdeletion containing 6 - 7 genes, including *CHRNA7*, has been associated with schizophrenia, epilepsy and autism (Sharp et al., 2008, Ben-Shachar et al., 2009, Dibbens et al., 2009, Helbig et al., 2009, Miller et al., 2009, Pagnamenta et al., 2009). Interestingly, a recent study reported that a subset of probands with a 15q13.3 microdeletion restricted to *CHRNA7* exhibited clinical indications of autism and/or epilepsy (Hoppman-Chaney et al., 2013). The probands were too young to assess a potential association with schizophrenia. Both autism and epilepsy are characterized by inhibitory deficits (Fiedler et al., 2006, Briggs and Galanopoulou, 2011, Coghlan et al., 2012) and cognitive impairment (Boucher et al., 2012, Braakman et al., 2012) which could result, at least in part, from abnormal *CHRNA7* expression.

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Abbreviations

5-HT₃	serotonin 3 receptor
BDNF	brain derived neurotrophic factor
<i>CHRNA7/Chrna7</i>	gene (human/mouse) encoding the $\alpha 7$ nicotinic acetylcholine receptor
CREB	cAMP response element binding protein
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ERK1/2	extracellular signal-regulated kinase 1/2
GABA	γ -aminobutyric acid
GAD-65	L-glutamic acid decarboxylase-65
GAD-67	L-glutamic acid decarboxylase-67
Het	heterozygous
KO	knockout
LDS	lithium dodecyl sulfate

mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PKA	protein kinase A
PVDF	polyvinylidene fluoride membrane
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TBST	TBS containing 0.05% Tween-20
TrkB	tropomyosin related kinase-B
VLGUT	vesicular glutamate transporter
WT	wildtype

- Reduced *Chrna7* expression is associated with increased hippocampal parvalbumin
- Reduced *Chrna7* expression is associated with increased hippocampal GAD67
- Reduced *Chrna7* expression alters hippocampal GABA_A receptor subunit levels

Figure 1.

Left graph: Hippocampal levels of parvalbumin protein from adult C3H *Chrna7* wildtype (WT), heterozygous (Het) and knockout (KO) mice ($n = 6$ /group). A sample Western blot for each group is shown above the respective bar for that group. Parvalbumin protein levels were significantly greater in male Het and KO mice and in female Het mice. In addition, parvalbumin protein was significantly greater in male than in female KO mice. Values mean $\pm$ S.E.M. All samples were normalized to actin. Right graph: Scatter plots of hippocampal parvalbumin protein levels for individual mice in each group. Genotype: $F(2,30) = 17.767$, $p < 0.001$, Sex: $F(1,30) = 2.321$, $p = 0.138$, Genotype X Sex: $F(2,30) = 3.481$, $p = 0.044$.

Figure 2.

Left graph: Hippocampal levels of GAD67 protein from adult C3H *Chrna7* wildtype (WT), heterozygous (Het) and knockout (KO) mice ($n = 6$ /group). A sample Western blot for each group is shown above the respective bar for that group. Significant increases in GAD67 protein were observed in male KO mice and in female Het and KO mice. GAD67 protein was also found to be significantly greater in female than in male Het mice. Values mean $\pm$ S.E.M. All samples were normalized to actin. Right graph: Scatter plots of hippocampal GAD67 protein levels for individual mice in each group. Genotype: $F(2,30) = 7.016$, $p = 0.003$, Sex: $F(1,30) = 12.078$, $p = 0.002$, Genotype X Sex: $F(2,30) = 3.810$, $p = 0.034$.

Figure 3.

Left graph: Hippocampal levels of GABA_A receptor $\alpha 3$ subunit protein from adult C3H *Chrna7* wildtype (WT), heterozygous (Het) and knockout (KO) mice ($n = 6$ /group). A sample Western blot for each group is shown above the respective bar for that group. Significant decreases in $\alpha 3$ subunit protein were observed in female Het and KO mice while $\alpha 3$ subunit protein was significantly higher in female WT than in male WT mice. Values mean $\pm$ S.E.M. All samples were normalized to actin. Right graph: Scatter plots of hippocampal GABA_A receptor $\alpha 3$ subunit protein levels for individual mice in each group. Genotype: $F(2,30) = 2.036$, $p = 0.148$, Sex: $F(1,30) = 0.0246$, $p = 0.877$, Genotype X Sex: $F(2,30) = 8.10$, $p = 0.002$.

Figure 4.

Left graph: Hippocampal levels of GABA_A receptor α4 subunit protein from adult C3H *Chrna7* wildtype (WT), heterozygous (Het) and knockout (KO) mice ($n = 6/\text{group}$). A sample Western blot for each group is shown to the right of the graph. A significant decrease in α4 subunit protein was only observed in the KO mice with no effect of sex. Values mean $\pm$ S.E.M. All samples were normalized to actin. **Right graph:** Scatter plots of hippocampal GABA_A receptor α4 subunit protein levels for individual mice in each group. Genotype: $F(2,30) = 4.815$, $p = 0.015$, Sex: $F(1,30) = 0.0736$, $p = 0.788$, Genotype X Sex: $F(2,30) = 0.163$, $p = 0.850$.

Figure 5.

Hippocampal levels of GABA_A receptor δ subunit protein from adult C3H *Chrna7* wildtype (WT), heterozygous (Het) and knockout (KO) mice ($n = 6$ /group). Both genotype (upper left panel) and sex (upper right panel) effects were observed. A sample Western blot for each group is shown at the bottom left of the figure. A significant increase in δ subunit protein was observed in the Het mice while a significant decrease was observed in the KO mice. In addition, δ subunit protein was significantly lower in females than in males. Values mean $\pm$ S.E.M. All samples were normalized to actin. **Bottom graph:** Scatter plots of hippocampal GABA_A receptor δ subunit protein levels for individual mice in each group. Genotype: $F(2,30) = 22,863$, $p < 0.001$, Sex: $F(1,30) = 7.357$, $p = 0.011$, Genotype X Sex: $F(2,30) = 7.65$, $p = 0.004$.

Figure 6.

Hippocampal levels of GABA_A receptor $\gamma 2$ subunit protein from adult C3H *Chrna7* wildtype (WT), heterozygous (Het) and knockout (KO) mice ($n = 6$ /group). Separate genotype (upper left panel) and sex (upper right panel) effects were observed. A sample Western blot for each group is shown at the bottom left of the figure. A significant increase in $\gamma 2$ subunit protein was observed in the KO mice while $\gamma 2$ subunit protein was significantly lower in females than in males. Values mean $\pm$ S.E.M. All samples were normalized to actin. Bottom graph: Scatter plots of hippocampal GABA_A receptor $\gamma 2$ subunit protein levels for individual mice in each group. Genotype: $F(2,30) = 22.863$, $p < 0.001$, Sex: $F(1,30) = 7.357$, $p = 0.011$, Genotype X Sex: $F(2,30) = 7.65$, $p = 0.004$.