Published in final edited form as:

Neurobiol Dis. 2014 March ; 63: 129–140. doi:10.1016/j.nbd.2013.11.021.

Cortical synaptic NMDA receptor deficits in $\alpha 7$ nicotinic acetylcholine receptor gene deletion models: Implications for neuropsychiatric diseases

Hong Lin¹, Fu-Chun Hsu¹, Bailey H. Baumann¹, Douglas A. Coulter^{1,2}, and David R. Lynch^{1,2}

¹Departments of Pediatrics and Neurology, The Children's Hospital of Philadelphia, Philadelphia PA, 19104

²Perelman School of Medicine, University of Pennsylvania, Philadelphia PA, 19104

Abstract

Microdeletion of the human *CHRNA7* gene ($\alpha 7$ nicotinic acetylcholine receptor, nAChR) as well as dysfunction in N-methyl-D-aspartate receptors (NMDARs) have been associated with cortical dysfunction in a broad spectrum of neurodevelopmental and neuropsychiatric disorders including schizophrenia. However, the pathophysiological roles of synaptic vs. extrasynaptic NMDARs and their interactions with $\alpha 7$ nAChRs in cortical dysfunction remain largely uncharacterized. Using a combination of *in vivo* and *in vitro* models, we demonstrate that $\alpha 7$ nAChR gene deletion leads to specific loss of synaptic NMDARs and their coagonist, D-serine, as well as glutamatergic synaptic deficits in mouse cortex. $\alpha 7$ nAChR null mice had decreased cortical NMDAR expression and glutamatergic synapse formation during postnatal development. Similar reductions in NMDAR expression and glutamatergic synapse formation were revealed in cortical cultures lacking $\alpha 7$ nAChRs. Interestingly, synaptic, but not extrasynaptic, NMDAR currents were specifically diminished in cultured cortical pyramidal neurons as well as in acute prefrontal cortical slices of $\alpha 7$ nAChR null mice. Moreover, D-serine responsive synaptic NMDAR-mediated currents and levels of the D-serine synthetic enzyme serine racemase were both reduced in $\alpha 7$ nAChR null cortical pyramidal neurons. Our findings thus identify specific loss of synaptic NMDARs and their coagonist, D-serine, as well as glutamatergic synaptic deficits in $\alpha 7$ nAChR gene deletion models of cortical dysfunction, thereby implicating $\alpha 7$ nAChR-mediated control of synaptic NMDARs and serine racemase/D-serine pathways in cortical dysfunction underlying many neuropsychiatric and neurodevelopmental disorders, particularly those associated with deletion of human *CHRNA7*.

© 2013 Elsevier Inc. All rights reserved.

CORRESPONDING AUTHOR: David R. Lynch, M.D., Ph.D., Division of Neurology, The Children's Hospital of Philadelphia, 502 Abramson Research Center, 3615 Civic Center Blvd, Philadelphia, Pennsylvania 19104-4318, Tel: 215-590-2242, Fax: 215-590-3779, lynchd@mail.med.upenn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST: No conflict of Interest exists in this paper for all authors.

Keywords

$\alpha 7$ nicotinic acetylcholine receptor; synaptic NMDA receptor; extrasynaptic NMDA receptor; D-serine; serine racemase; cortex; glutamatergic synaptic deficits; neuropsychiatric diseases; schizophrenia

Introduction

As the major excitatory neurotransmitters in the CNS, the nicotinic and glutamatergic systems have been implicated in many neurological, neurodevelopmental and neuropsychiatric disorders (Waxman and Lynch, 2005; Dani and Bertrand, 2007; Traynelis et al., 2010). Some of the most powerful evidence for the role of nicotinic receptors comes from genetic studies in which a homozygous microdeletion of human 15q13.3, including the $\alpha 7$ nAChR gene, is associated with disorders including schizophrenia, autism, mental retardation, epilepsy, and intellectual disability (Sharp et al., 2008; Helbig et al., 2009; Miller et al., 2009; Shinawi et al., 2009; Endris et al., 2010; Lepichon et al., 2010; Ancin et al., 2011; Liao et al., 2011; Spielmann et al., 2011; Yasui et al., 2011; Hoppman-Chaney et al., 2012). Based on the size and the overlap of the deletions in different individuals, loss of the $\alpha 7$ nAChR gene alone appears sufficient to cause the majority of clinical features in such patients (Shinawi et al., 2009; Endris et al., 2010; Liao et al., 2011; Hoppman-Chaney et al., 2012). Deletion of the $\alpha 7$ nAChR gene in mice causes analogous abnormalities including impaired attention, working-memory and learning (Fernandes et al., 2006; Young et al., 2007; Levin et al., 2009; Brown et al., 2010; Young et al., 2011; Hellier et al., 2012).

Dysfunction in NMDARs has also been implicated in many neurological, neurodevelopmental and neuropsychiatric disorders including schizophrenia. In particular, NMDAR hypofunction has been suggested to underlie features of schizophrenia, and the NMDAR coagonist D-serine has been particularly implicated in models of this disorder. Targeted deletion or pathogenic perturbation of serine racemase (SR), which synthesizes D-serine in the brain, reduces D-serine production and glutamatergic transmission in the forebrain and leads to schizophrenia-like behavior in mice (Basu et al., 2009; Labrie et al., 2012; Ma et al., 2012; Balu et al., 2013), suggesting that synaptic NMDAR hypofunction due to D-serine depletion may underlie functional glutamatergic deficits in schizophrenia.

Interestingly, $\alpha 7$ nAChRs and NMDARs interact in physiological and pathological events. Presynaptic $\alpha 7$ nAChR stimulation facilitates glutamatergic transmission and enhances synaptic plasticity at selected CNS glutamatergic synapses (Girod et al., 2000). $\alpha 7$ nAChR/NMDAR interactions in working memory have been implicated in the cognitive impairment of schizophrenia (Timofeeva and Levin, 2011). Our previous studies demonstrated that axonal $\alpha 7$ nAChRs modulate presynaptic NMDAR expression and presynaptic and postsynaptic maturation of glutamatergic synapses during early synaptic development of cortical neurons (Lin et al., 2010). We thus hypothesize that $\alpha 7$ nAChR/NMDAR interactions modulate cortical glutamatergic development and that abnormal interactions due to $\alpha 7$ nAChR gene deletion may produce permanent changes that contribute to cortical dysfunction in many neuropsychiatric and neurodevelopmental disorders, such as those associated with deletion of human CHRNA7. Although glutamatergic synaptic deficits have been found in the hippocampus of $\alpha 7$ nAChR null mice (Lozada et al., 2012), the pathophysiological interactions among synaptic NMDARs and $\alpha 7$ nAChRs in cortical dysfunction remain largely uncharacterized. Such interactions are important, as synaptic NMDARs are crucial for plasticity and cell survival, but extrasynaptic NMDARs may trigger neurodegeneration (Karpova et al., 2013). In the present study, we identify specific loss of cortical synaptic NMDARs and the D-serine synthetic enzyme, SR, as well as

glutamatergic synaptic deficits in $\alpha 7$ nAChR gene deletion models, implicating $\alpha 7$ nAChR-mediated control of synaptic NMDARs and SR/D-serine pathways in cortical dysfunction underlying many neuropsychiatric and neurodevelopmental disorders, particularly those associated with deletion of human CHRNA7.

Materials and Methods

Materials

Timed-pregnant C57BL/6 mice were purchased from Charles River Laboratories and bred from $\alpha 7$ nAChR *knockout* mice (B6.129S7-Chrna7^{tm1Bay/J}, Jackson Laboratory). Biochemicals included D-AP5, bicuculline, NMDA, D-serine, picrotoxin, MK-801 and strychnine (Sigma). Antibodies included α -NR1 (BD Pharmagen, mouse monoclonal), α -NR2A, α -NR2B (Alomone Labs, rabbit polyclonal), α -NR3A (Tocris Bioscience, rabbit polyclonal), α -PSD95 (BD Transduction Laboratories, mouse monoclonal; NeuroMab, mouse monoclonal), α -VGLUT1 (Synaptic Systems, polyclonal), α -Serine Racemase (Abcam, rabbit polyclonal), α -GAPDH (Novus Biologicals, mouse monoclonal), α -actin (Sigma, rabbit polyclonal).

Neuronal cultures

Primary cortical cultures from E17-19 *wild-type* or $\alpha 7$ nAChR *knockout* mice were prepared as described (Dong et al., 2004). Briefly, the cortex was dissected, gently minced, trypsinized (0.027%, 37 °C; 7% CO₂ for 20 min), and then washed with 1× HBSS. Neurons were seeded to a density of 3×10^5 viable cells in 35-mm culture dish with five 12-mm glass coverslips or a density of 1.6×10^6 viable cells in 60-mm culture dishes. The culture dishes were coated with poly-D-Lysine (100 µg/ml) prior to seeding neurons. Neurons were maintained at 37°C with 5% CO₂ in Neurobasal medium with B27 supplement. Neurobasal medium contains choline chloride, a selective agonist at $\alpha 7$ nAChR (Zhang and Warren, 2002). In addition, cholinergic neurons are present in cortical cultures as identified by ChAT immunostaining as described by Abcam in their manufacturer's instructions. At 21-28 *days in vitro* (DIV), cultures were subject to western blotting analysis, immunocytochemistry or patch clamp recording.

For cell lysate preparation, cultures were lysed in lysis buffer (150 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, 1% Triton X-100, 1% sodium deoxycholate and 1% SDS, pH 7.4, supplemented the day of use with 1:500 EDTA-free protease inhibitor cocktail III (Calbiochem) for 1 hr at 4°C and collected. Debris was cleared by centrifugation at $16,100 \times g$ for 20 mins at 4°C. Supernatants were stored at -80°C until use.

Tissue preparation

For tissue homogenate preparation, the age-matched *wild-type* (WT) and $\alpha 7$ nAChR *null* ($\alpha 7$ -KO) mouse littermates at postnatal day (P1-P56) and 9 months older of either sex were anesthetized with isoflurane before decapitation in accordance with protocols approved by The Children's Hospital of Philadelphia Animal Care and Use Committee. The mouse brain was rapidly removed, and the cortex was dissected under Leica EZ4 stereomicroscope and immediately transferred to dry ice. The cortex was homogenized in 20ml lysis buffer per 1 g weight, and lysed for 1 hr at 4°C. Lysis buffer contained 150 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, 1% Triton X-100, and 1% sodium deoxycholate, pH 7.4, supplemented the day of use with 1:500 EDTA-free protease inhibitor cocktail III (Calbiochem). Debris was cleared by centrifugation at $39,000 \times g$ for 1 hr at 4°C. Supernatants were stored at -80°C until use.

For immunohistochemical studies, the age-matched male WT and $\alpha 7$ -KO mice at P56-P90 were anesthetized with isoflurane and cardiac perfused with 10 ml of PBS, followed by 20 ml of PBS containing 4% paraformaldehyde in accordance with protocols approved by The Children's Hospital of Philadelphia Animal Care and Use Committee. Brains were excised and immersed overnight in 4% paraformaldehyde, washed in PBS, dehydrated and embedded in paraffin. A series of brain coronal sections (5 μ m thick) were obtained using microtome at The Children's Hospital of Philadelphia Pathology Core Facility.

Western blotting analysis

Western blotting was performed as described previously (Dong et al., 2004). Protein content was determined using BCA Protein Assay (Thermo Scientific). Equal amounts of total protein (5 μ g cell lysate or 30 μ g cortex homogenate per lane) was subjected to 4-12% NuPAGE Gel for electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 3% nonfat milk and incubated with primary antibody overnight at 4 °C. Blots were then incubated with appropriate horseradish peroxidase, HRP-conjugated secondary antibodies (Cell Signaling) for 2 hr at room temperature and then washed; Reaction bands were visualized using a luminol-enhanced chemiluminescence (ECL) HRP substrate (Thermo Scientific). Each blot was then incubated with stripping buffer (2% SDS, 50 mM Tris, pH 6.8, and 100 mM μ -mercaptoethanol) for 45 mins at room temperature to remove the signals and reprobed for other proteins including actin and GAPDH as internal controls. Reaction product levels were quantified by scanning densitometry and normalized by the levels of actin or GAPDH using NIH Image J software. Since the cortical actin and GAPDH levels alter during the time course of postnatal development, the protein levels were normalized by internal control at the same time-point between WT and KO mice.

Immunocytochemistry and immunohistochemistry

Primary cultured cortical neurons were fixed for 20 min at 4°C with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4), and then subject to immunostaining procedure. The paraffin-embedded brain sections were deparaffined, rehydrated, and antigen-retrieved in antigen unmasking solution (Vector Laboratories) and then subject to immunostaining procedure. For immunostaining procedure, after blocking with 5% normal goat serum and 1 % bovine serum albumin in combination with 0.3% (vol/vol) Triton X-100 in PBS at room temperature for 1 hr, the coverslips or slides were incubated with primary antibodies at 4°C overnight and then secondary antibodies conjugated to Alexa fluor 488 or 594 (Invitrogen) at room temperature for 60-90 mins. Following several washes with PBS, cells or slides were mounted with Vectashield with DAPI (Vector Laboratories).

Fluorescence imaging

Fluorescence images were obtained with Olympus FluoView laser scanning confocal microscope. For mouse cortex, the brain sections were stained for glutamatergic synaptic markers (VGLUT1 and PSD95). The infralimbic, prelimbic, or anterior cingulate regions of the mouse medial frontal cortex are collectively referred to as mouse prefrontal cortex (Rotaru et al., 2011). Confocal scans were performed in layer 2/3 or layer 5/6 of mouse prefrontal cortex, and the imaging parameters were identical for *wild-type* and $\alpha 7$ nAChR *knockout* mice. *Wild-type* controls were always included in all experiments to normalize for expected variations in antibody staining intensity performed on different days. The confocal images were acquired at the focal plane with maximal number of synapses from at least 3 sections per animal and from at least 3 animals per group. NIH Image J software was used to quantify VGLUT1-positive glutamatergic synapses in the acquired confocal images. Thresholds were set at 3 SDs above the mean staining intensity of six nearby regions in the same visual field. Thresholded images present a fixed intensity for all pixels above threshold

after having removed all of those below and VGLUT1-labeled puncta in the thresholded images were quantified (Lozada et al., 2012). For cortical cultures, neurons were stained for NR1, glutamatergic synaptic markers (VGLUT1 and PSD95). For quantification analysis, the confocal images were acquired under 63 $\times$ objectives with zoom x6 from the dendrites of 5 neurons and 3 different cultures for quantification of VGLUT1-positive puncta on the dendrites of *wild-type* and $\alpha 7$ nAChR *knockout* cortical neurons. NIH Image J software and the thresholded images were used to quantify the number of glutamatergic synapses on primary and secondary dendrites of cortical neurons. Since 100% of VGLUT1-positive puncta were colocalized with or adjacent to one or more PSD95-positive puncta in the dendrites of cortical neurons, the number of VGLUT1-positive puncta best represents the number of glutamatergic synapses on the dendrites.

Whole-cell patch-clamp recording

Spontaneous excitatory postsynaptic current (sEPSC) recording was performed using standard whole-cell voltage clamp techniques in cultured mouse cortical neurons grown on coverslips for 21–28 DIV. Cells were transferred to a recording chamber at room temperature ($23 \pm 2^\circ\text{C}$) containing an extracellular solution composed of (mM): 155 NaCl, 3 KCl, 3 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4. Recording electrodes were pulled on a Flaming-Brown micropipette puller (Model P-97, Sutter Instrument Co.) and filled with a solution containing (mM): 100 Trizma phosphate (dibasic), 28 Trizma base, 0.5 CaCl₂, 2 MgCl₂, 11 EGTA, 4 Mg-ATP, pH 7.35, 300–310 mOsm. In order to increase synaptic neuronal activity, the activity of sEPSCs was enhanced by bath application of the GABA_A receptor antagonist bicuculline (10 μM) or picrotoxin (100 μM) in extracellular solution. The holding potential for sEPSC recording was -60 mV. Recorded signals were amplified using an Axopatch-1D amplifier, filtered at 5 kHz and then saved as data files using pCLAMP 9.01 software (Axon Instrument Inc., Hamden, CT) for off-line analysis. Whole-cell NMDA receptor-mediated currents were recorded in the similar extracellular solution as used in sEPSC recordings but without Mg²⁺. The holding potential was also -60 mV. Currents were elicited by 100 μM NMDA along with 10 μM glycine and 1 μM strychnine. Drugs were applied for 2 sec using a combination of a solenoid-activated system (Warner Instruments) and a step-perfusion device (Warner Instruments). To isolate the extrasynaptic NMDA receptor-mediated effect, synaptic NMDA receptors were blocked using the treatment of 100 μM picrotoxin and 20 μM MK-801 for 5–10 min. After thorough washout, NMDA receptor-mediated current was elicited by NMDA application in Mg²⁺-free extracellular solution. NMDAR-mediated currents were expressed as current density, which was calculated as the ratio of the maximal response generated by the application of 100 μM NMDA, divided by the cell membrane capacitance.

The brain slices of prefrontal cortex were prepared as described below. Mice at 8–10 weeks of age were anesthetized with isoflurane before decapitation in accordance with protocols approved by The Children's Hospital of Philadelphia Animal Care and Use Committee. The mouse brain was rapidly removed, and the coronal prefrontal cortical slices (350 μm) were prepared using a vibratome (VT1200S; Leica, Deerfield, IL) in ice-cold artificial CSF (aCSF). The aCSF composition was (in mM): 130 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 glucose (saturated with 95% O₂ and 5% CO₂). Slices were then allowed to recover for at least 1 hr at room temperature before recording. The whole cell patch clamp recordings of sEPSCs in acute brain slice were obtained from prefrontal cortex layer II/III principal neurons identified by a fixed stage upright microscope (Olympus, BX51W1) with a 63 $\times$ water immersion objective, equipped with a CCD camera (#5985; Hamamatsu, Shizuoka, Japan). In order to properly compare the results with the cultured cells, all recordings were also performed at room temperature ($23 \pm 2^\circ\text{C}$) using glass pipettes that were pulled on a Flaming-Brown micropipette puller (Model P-97, Sutter

Instrument Co.). The electrodes were 4–6 M filled with potassium gluconate-based intrapipette solution (in mM: 145 K-gluconate, 2 MgCl₂, 2 ATP-Mg, 0.5 GTP-Tris, 0.1 BAPTA, 2.5 KCl, 2.5 NaCl, and 10 HEPES). The recording chamber was continuously perfused by gravity with aCSF solution. sEPSCs were recorded at holding of –70 mV with GABA_A receptor blockage by 10 μM bicuculline. Synaptic currents were recorded using a Multiclamp 700B amplifier (Molecular Device Co., Sunnyvale CA, USA), filtered at 2 kHz, sampled at 20 kHz, digitized (Digidata 1320A; Molecular Devices), and stored for off-line analysis using MiniAnalysis software (Synaptosoft Inc., Fort Lee, NJ). Curve fitting and raw trace figure preparation were performed using Clampfit 10 software (Molecular Devices). Frequency of sEPSCs was measured from identified events with template-based analysis software in Clampfit 10 as well as MiniAnalysis software. Access resistance (10–15 M Ω ; 80% compensation) was monitored and the results were discarded if access resistance was changed more than 20% during recording.

Statistical analysis

Data were shown as the mean $\pm$ S.E.M. Experiments were analyzed using Student's *t*-test to compare two conditions or ANOVA followed by planned comparisons of multiple conditions. Significance was set at $P < 0.05$.

Results

Cortical NR1, NR2A and NR2B expression levels were decreased in $\alpha 7$ nAChR null mice during early postnatal development.

We first determined whether cortical NMDAR expression levels were altered in $\alpha 7$ nAChR null mice during early postnatal life by examining NMDAR subunit (NR1, NR2A, NR2B and NR3A) expression in cortical homogenates of *wild-type* (WT) and $\alpha 7$ nAChR null ($\alpha 7$ -KO) mice at postnatal day P1–P56 and P9M (> 9 months old). Both NR1 and NR2B levels in the WT cortex increased from P1 to P8, peaked at P14–P21, and then declined from P21 to P56 (Fig. 1A, 1B). NR2A expression was low through P8, increased to a peak level at P14–P21, but remained high from P21 to P56 (Fig. 1C). The developmental switch of NR2B and NR2A expression at P14–P21 in the mouse cortex has been noted before (Sanz-Clemente et al., 2013). NR3A expression increased from P1, peaked at P8 and then declined from P8 to P56 (Fig. 1D). The developmental pattern was the same in $\alpha 7$ -KO mice; however, levels of NR1, NR2A and NR2B were reduced during early postnatal days at P1–P21 compared with WT (Fig. 1), while NR3A expression was not altered in the cortex of $\alpha 7$ -KO mice at P1–P21 (Fig. 1D). These findings indicate that NR1, NR2A and NR2B expression was particularly reduced in the mouse cortex of $\alpha 7$ nAChR null mice during early postnatal life, with no effect on an NMDAR subunit, NR3, that is less implicated in synaptic development during this time period.

Glutamatergic synapse formation was decreased in the prefrontal cortex of $\alpha 7$ nAChR null mice during late postnatal development and adulthood

We then examined the expression of glutamatergic synaptic markers, including the glutamatergic presynaptic terminal marker vesicular glutamate transporter 1 (VGLUT1), and the postsynaptic marker PSD95 in the cortex of $\alpha 7$ -KO mice and WT littermates at P1–P56 and P9M. In WT mice, the expression level of VGLUT1 remained low at P1–14, then substantially increased by P21 and reached a peak at P56 (Fig. 2A). Levels of PSD95 gradually increased from P1 to P8, then increased from P14–P21 and reached a peak at P56 (Fig. 2B). The results suggest that the maturation of mouse cortical glutamatergic synapses occurs from P21–P56, similar to development of glutamatergic circuitry in monkeys (Hoftman and Lewis, 2011) and other species (Zheng et al., 2011). In $\alpha 7$ -KO mice, expression levels of VGLUT1 were significantly decreased at P21–P56 (Fig. 2A), while

those of PSD95 were significantly reduced slightly later at P56 and adulthood compared with WT littermates (Fig. 2B). Thus, deletion of the $\alpha 7$ nAChR gene disrupts development of glutamatergic synapses during late postnatal development and adulthood, with effects on glutamatergic presynaptic terminals possibly occurring prior to effects on postsynaptic terminals.

We specifically examined VGLUT1 and PSD95 immunoreactivities microscopically in the prefrontal cortex of $\alpha 7$ -KO mice and WT littermates at P56-P90. In WT mice, VGLUT1- and PSD95-positive glutamatergic synapses were widely and extensively distributed in the neuropil surrounding the neurons within all layers of prefrontal cortex (Fig. 3A-3C and 3G-3I), while the overall levels of the VGLUT1 and PSD95 immunoreactivities were decreased in the neuropil of the prefrontal cortex of $\alpha 7$ -KO mice (Fig. 3D-3F and 3J-3L), consistent with reduction of cortical VGLUT1 and PSD95 expression levels in $\alpha 7$ -KO mice (Fig. 2). Quantification of synapse number showed a significant decrease in the number of VGLUT1-positive glutamatergic synapses in the prefrontal cortex layer 2/3 of $\alpha 7$ -KO mice compared with WT littermates (Fig. 3M). Thus, our findings demonstrate that glutamatergic synapse formation was decreased in the prefrontal cortex of $\alpha 7$ nAChR null mice during late postnatal development and adulthood.

NMDAR expression, glutamatergic synapse formation and synaptic NMDAR localization were reduced in cortical cultures lacking $\alpha 7$ nAChRs

To further test our hypothesis, we examined NMDAR expression and localization as well as glutamatergic synapse formation in cortical cultures from WT and $\alpha 7$ -KO embryonic (E17-19) mice at 21-28 *days in vitro* (DIV). Double immunostaining with α -VGLUT1 and α -PSD95 antibodies identified presynaptic and postsynaptic components of glutamatergic synapses in cortical neurons (Fig. 4A-4L). A marked reduction of VGLUT1- and PSD95-positive glutamatergic synapses was observed in $\alpha 7$ -KO cortical cultures (Fig. 4D-4F and 4J-4L) compared with WT cultures (Fig. 4A-4C and 4G-4I). High magnification of confocal images further showed that 100% of VGLUT1-positive puncta were colocalized with or adjacent to one or more PSD95-positive puncta in the dendrites of cortical neurons (Fig. 4G-4L), the number of VGLUT1-positive puncta thus best represents the number of glutamatergic synapses on the dendrites. Quantification of the number of VGLUT1-positive puncta demonstrated a significant reduction in the number of glutamatergic synapses on the dendrites of $\alpha 7$ -KO cortical neurons compared with WT neurons (Fig. 4M).

Double immunostaining with α -NR1 and α -VGLUT1 antibodies showed VGLUT1-positive presynaptic glutamatergic terminals and NR1-positive puncta in the soma and dendrites of cortical pyramidal neurons in WT and $\alpha 7$ -KO cultures (Fig. 5A-5L). The levels of NR1 immunoreactivity in the soma and dendrites of cortical neurons were reduced in $\alpha 7$ -KO cultures (Fig. 5D-5F and 5J-5L) compared with WT cultures (Fig. 5A-5C and 5G-5I), suggesting a reduction in NMDAR expression in cortical neurons. The VGLUT1-positive glutamatergic presynaptic terminals localized closely with some NR1-positive puncta in the dendrites of cortical neurons (Fig. 5G-5L), suggesting colocalization of synaptic NR1 with glutamatergic synapses. Because the number of VGLUT1-positive glutamatergic synapses on the dendrites of cortical neurons were decreased in $\alpha 7$ -KO cultures (Fig. 5E and 5K) compared with WT cultures (Fig. 5B and 5H), as demonstrated in Fig. 4, the synaptic NR1 that colocalized with glutamatergic synapses was also reduced in $\alpha 7$ -KO cultures (Fig. 5D-5E and 5J-5L) compared with WT cultures (Fig. 5A-5C and 5G-5I). Furthermore, Western blot analysis demonstrated that the expression levels of NR1, NR2A, VGLUT1 and PSD95 were significantly decreased in $\alpha 7$ -KO cortical cultures compared with WT cultures (Fig. 5M and 5N). Thus, NMDAR expression, glutamatergic synapse formation and synaptic NMDAR localization were reduced in cortical cultures lacking $\alpha 7$ nAChRs.

Synaptic, but not extrasynaptic, NMDAR currents were specifically diminished in cultured cortical pyramidal neurons as well as in acute prefrontal cortical slices of $\alpha 7$ nAChR null mice

Since our findings demonstrated that cortical NMDAR levels were reduced in $\alpha 7$ nAChR null models, we assessed the effects on NMDAR function in cultured cortical pyramidal neurons and in acute prefrontal cortical slices of $\alpha 7$ nAChR null mice. We first recorded spontaneous excitatory postsynaptic currents (sEPSCs) on cultured cortical pyramidal neurons at 21–28 DIV. sEPSC decay time was significantly shorter in cultured $\alpha 7$ -KO pyramidal neurons than in WT neurons (Fig. 6A and 6B). There was also a significant decrease in sEPSC frequency in $\alpha 7$ -KO pyramidal neurons compared with WT neurons (Fig. 6C), while the peak amplitude of sEPSCs was not significantly altered (Fig. 6D), consistent with fewer glutamatergic presynaptic terminals in $\alpha 7$ -KO cortical cultures (Fig. 4). Since NMDAR-mediated EPSCs are longer than AMPAR-mediated EPSCs, we used the NMDAR specific antagonist D-AP5 to determine if the shorter decay time of sEPSCs in $\alpha 7$ -KO pyramidal neurons was associated with lack of synaptic NMDARs. Application of D-AP5 decreased sEPSC decay time in WT pyramidal neurons, but not in $\alpha 7$ -KO neurons (Fig. 6E–6F), showing that the synaptic NMDARs were reduced in $\alpha 7$ -KO neurons.

We further differentiated synaptic vs. extrasynaptic NMDAR activity by recording whole-cell NMDAR-mediated currents in cultured cortical pyramidal neurons. NMDAR-mediated currents were elicited by 100 μ M NMDA with 10 μ M glycine and 1 μ M strychnine in Mg^{2+} -free recording solution. The results show that whole-cell NMDAR-mediated currents were similar in $\alpha 7$ -KO pyramidal neurons compared with WT pyramidal neurons (Fig. 6G and 6H). This suggests that surface expression of NMDAR was similar in both groups, although the total level of NMDAR, which includes intracellular receptors, was decreased in the $\alpha 7$ -KO group (Fig. 5). To isolate the extrasynaptic NMDAR-mediated currents, synaptic NMDARs were blocked with picrotoxin (GABA_A receptor antagonist) and MK-801 (NMDAR open channel blocker) for 5 min in Mg^{2+} -containing recording solution (Monnerie et al., 2010). After thorough washout, NMDAR-mediated current was elicited by NMDA application in Mg^{2+} -free recording solution. The results show that extrasynaptic NMDAR-mediated currents were significantly increased and synaptic NMDAR currents decreased in $\alpha 7$ -KO pyramidal neurons compared with WT neurons (Fig. 6H and 6I), indicating that absence of $\alpha 7$ nAChRs specifically diminished synaptic, but not extrasynaptic, NMDARs in cortical pyramidal neurons.

We then recorded sEPSCs in cortical layer 2/3 pyramidal neurons from acute brain slices of mouse prefrontal cortex. The decay time of sEPSCs was shorter in layer 2/3 pyramidal neurons of $\alpha 7$ -KO mouse than in WT cortex (Fig. 7A and 7B), as it was in culture (Fig. 6A and 6B), suggesting that synaptic NMDARs were reduced in $\alpha 7$ -KO cortical neurons. Interestingly, sEPSC frequency in layer 2/3 pyramidal neurons of $\alpha 7$ -KO mouse prefrontal cortex was also decreased compared with those in layer 2/3 pyramidal neurons of WT mouse prefrontal cortex (Fig. 7C), while the peak amplitude of sEPSCs was not altered (Fig. 7D), further confirming fewer glutamatergic presynaptic terminals in $\alpha 7$ -KO mouse prefrontal cortex (Fig. 3). Application of the NMDAR specific antagonist, D-AP5, significantly decreased sEPSC decay time and frequency in layer 2/3 pyramidal neurons of WT cortex, but not in layer 2/3 pyramidal neurons of $\alpha 7$ -KO cortex (Fig. 7E and 7F), consistent with synaptic NMDAR reduction and hypofunction in such neurons. Overall, our findings demonstrated that $\alpha 7$ nAChR gene deletion led to specific loss of synaptic NMDARs in cortical pyramidal neurons.

The D-serine synthetic enzyme serine racemase and D-serine responsive synaptic NMDAR-mediated activity were reduced in cortical pyramidal neurons lacking $\alpha 7$ nAChRs

D-serine is an endogenous coagonist of synaptic NMDARs, which is synthesized by the enzyme serine racemase (SR) through the conversion of L-serine to D-serine (Papouin et al., 2012). To determine if the level of D-serine was altered in $\alpha 7$ -KO mouse cortex, we examined the expression level of SR in the mouse cortex. In the cortex of WT mice, expression levels of SR were low at P1-P14, increased by P21, reached a peak at P56 and remained high in adulthood (Fig. 8A), matching the development of synaptic NMDAR neurotransmission. In $\alpha 7$ -KO mice, the expression of SR was decreased at P21-P56 and adulthood compared with those of WT littermates (Fig. 8A). Double immunostaining with α -SR and α -NR1 antibodies in the cortex of $\alpha 7$ -KO mice and WT littermates at P56-P90 showed prominent SR and NR1 colocalization in cortical pyramidal neurons, particularly in layer 2/3 pyramidal neurons of prefrontal cortex (Fig. 8B-8G). The levels of SR and NR1 immunoreactivities in the soma of cortical pyramidal neurons were reduced in $\alpha 7$ -KO mice (Fig. 8E-8G) compared with WT mice (Fig. 8B-8D), suggesting that SR/D-serine reduction in the cortical pyramidal neurons of $\alpha 7$ -KO mice.

Furthermore, we assessed the D-serine responsive synaptic NMDAR-mediated currents in cultured WT and $\alpha 7$ -KO pyramidal neurons. Acute application of exogenous D-serine increases the decay time constant of sEPSCs in WT neurons (Fig. 8H, 8I), confirming that D-serine is a coagonist of synaptic NMDARs in cortical pyramidal neurons. However, application of D-serine had no effect on the decay time constant of sEPSCs in $\alpha 7$ -KO neurons (Fig. 8H, 8I), matching the effect of D-AP5 on sEPSCs. This suggests an absence of D-serine responsive receptors contributing to sEPSCs in the $\alpha 7$ -KO neurons, again consistent with an absence of D-serine responsive synaptic NMDARs in $\alpha 7$ -KO neurons. Taken together, our findings demonstrate both D-serine loss and synaptic NMDAR reduction in cortical pyramidal neurons lacking $\alpha 7$ nAChRs.

Discussion

The present study uses a combination of *in vivo* and *in vitro* models to show specific loss of synaptic NMDARs and their coagonist, D-serine, as well as glutamatergic synaptic deficits in mouse cortex with $\alpha 7$ nAChR deletion. Loss of $\alpha 7$ nAChR decreases cortical NMDAR expression during early postnatal life, and alters glutamatergic synaptic development during late postnatal life and adulthood. Moreover, synaptic NMDARs are specifically diminished, and especially D-serine responsive synaptic NMDARs are affected physiologically, along with serine racemase (SR), the synthetic enzyme for D-serine. This implicates $\alpha 7$ nAChR-mediated control of synaptic NMDARs and SR/D-serine pathways as crucial components in cortical glutamatergic development, and thus provides a rationale for the association of $\alpha 7$ nAChR deletion in humans with a spectrum of neurodevelopmental and neuropsychiatric disorders. Specific loss of synaptic NMDARs and their coagonist, D-serine, due to relative loss of $\alpha 7$ nicotinic receptors could represent fundamental pathogenic mechanisms underlying such disorders.

Physiologically, deletion of the $\alpha 7$ nAChR gene specifically diminishes synaptic NMDARs and altered synaptic NMDAR function in cortical pyramidal neurons both *in vivo* and *in vitro*. Synaptic NMDARs play crucial roles in many forms of synaptic plasticity, such as LTP and LTD. Extrasynaptic and synaptic NMDARs are associated with differential gene expression and have different roles in synaptic plasticity and cell death (Hardingham and Bading, 2010; Adams et al., 2012; Kaufman et al., 2012; Papouin et al., 2012; Karpova et al., 2013). Consequently, the selective control of synaptic NMDARs provides a mechanism by which $\alpha 7$ nAChR may regulate postsynaptic events in glutamatergic neurons. The potential role of SR/D-serine is particularly intriguing. Synaptic NMDARs are preferentially

gated by the endogenous coagonist, D-serine, rather than glycine which preferentially gates extrasynaptic NMDARs (Papouin et al., 2012). Indeed, D-serine responsive synaptic NMDAR-mediated activity is diminished in $\alpha 7$ -KO pyramidal neurons. While initial studies suggested that astrocytes were the major sites of SR and resources for D-serine in the brain (Wolosker et al., 1999), our results along with other studies suggest a more prominent neuronal SR expression in the forebrain (Coyle et al., 2012). The reduced levels of SR in cortical pyramidal neurons could directly lead to at least some of the decrease in synaptic NMDAR activity observed here, although acute application of exogenous D-serine did not lead to an increase in the sEPSC decay time constant in $\alpha 7$ -KO neurons. This finding is more consistent with a loss of synaptic receptors rather than loss of agonist itself. However, D-serine availability at synaptic vs. extrasynaptic sites modulates the lateral diffusion of NMDARs in and out of synapses. Thus, the impaired SR/D-serine pathway in $\alpha 7$ -KO mice may reduce D-serine production in the cortex and initially lead to decreased activation of synaptic NMDARs along with maintenance of this hypofunction state by loss of synaptic NMDARs. Additionally, $\alpha 7$ nAChR associates with EphB2 that controls synaptic localization of NMDARs (Liu et al., 2008; Nolt et al., 2011), $\alpha 7$ deletion may affect its associations with EphB2 and thereby contributing to specific loss of synaptic NMDARs.

$\alpha 7$ nAChR null mice show many characteristic neurochemical features of schizophrenia. This is consistent with specific pathophysiological connections among $\alpha 7$ nAChR dysfunction, NMDAR hypofunction, D-serine deficiency and glutamatergic deficits in cognitive dysfunction of schizophrenia. $\alpha 7$ nAChR selective agonists have demonstrated efficacy in clinical trials of schizophrenia patients and in animal models of positive and negative symptoms and cognitive deficits of schizophrenia (Hajos and Rogers, 2010; Tregellas et al., 2011; Wallace and Porter, 2011; Cannon et al., 2013). Moreover, $\alpha 7$ nAChR levels are reduced in multiple brain regions including the prefrontal cortex in schizophrenia patients (Mathew et al., 2007; Olincy and Freedman, 2012). Glutamatergic hypofunction, especially NMDAR hypofunction, has been suggested to underlie the core symptoms of schizophrenia, including psychosis, negative symptoms and cognitive impairment (Bitanhirwe et al., 2009; Belforte et al., 2010; Korotkova et al., 2010; Carlen et al., 2012; Coyle, 2012; Javitt, 2012; Rotaru et al., 2012; Schwartz et al., 2012). Administration of NMDAR antagonists in healthy human subjects and in schizophrenia patients mimics or exacerbates the major symptoms of schizophrenia (Malhotra et al., 1996; Malhotra et al., 1997). Disruption of SR by targeted deletion or pathogenic perturbation reduces D-serine production and glutamatergic transmission in the forebrain and leads to schizophrenia-like behavior in mice (Basu et al., 2009; Labrie et al., 2012; Ma et al., 2012; Balu et al., 2013), suggesting that synaptic NMDAR hypofunction due to D-serine depletion may underlie functional glutamatergic deficits in schizophrenia. Additionally, postmortem studies in schizophrenia patients have shown fewer axon terminals, lower levels of presynaptic proteins and lower dendritic spine density of pyramidal neurons in the prefrontal cortex (Lewis et al., 2012). Thus, the reduced numbers of glutamatergic presynaptic terminals, synaptic NMDAR hypofunction as well as D-serine deficiency in $\alpha 7$ -KO mouse prefrontal cortex mimic the pathological alterations in glutamatergic circuit of schizophrenia patients.

In summary, our findings identify specific loss of synaptic NMDARs and their coagonist, D-serine, as well as glutamatergic synaptic deficits in mice with homozygous deletion of the $\alpha 7$ nAChR gene. These mice share many characteristic neurochemical features of schizophrenia patients. Our findings thus suggest that specific loss of synaptic NMDARs and their coagonist, D-serine, as well as glutamatergic synaptic deficits due to $\alpha 7$ nAChR gene deletion may contribute to cortical dysfunction in many neuropsychiatric and neurodevelopmental disorders, particularly those associated with deletion of human CHRNA7.

Acknowledgments

This work is supported by CHOP Foerderer Grant and NIH grants R21NS072842, R01 NS45986 and P30HD026979 to Drs. Hong Lin and David R. Lynch. We thank Ms. Margaret Maronski for cortical neuronal cultures, Dr. Hajime Takano for confocal imaging, and Dr. Jessica Panzer for helpful discussion.

Abbreviation

nAChR	nicotinic acetylcholine receptor
NMDAR	N-methyl-D-aspartate receptor
SR	serine racemase
VGLUT1	vesicular glutamate transporter 1
WT	wild-type
$\alpha 7$-KO	$\alpha 7$ nAChR <i>null</i>

References

- Adams CE, Yonchek JC, Schulz KM, Graw SL, Stitzel J, Teschke PU, Stevens KE. Reduced ChRNA7 expression in mice is associated with decreases in hippocampal markers of inhibitory function: implications for neuropsychiatric diseases. *Neuroscience*. 2012; 207:274–282. [PubMed: 22314319]
- Ancin I, Cabranes JA, Santos JL, Sanchez-Morla E, Vazquez-Alvarez B, Rodriguez-Moya L, Pousada-Casal A, Fernandez C, Aparicio A, Barabash A. CHRNA7 haplotypes are associated with impaired attention in euthymic bipolar disorder. *J Affect Disord*. 2011; 133:340–345. [PubMed: 21550667]
- Balu DT, Li Y, Puhl MD, Benneyworth MA, Basu AC, Takagi S, Bolshakov VY, Coyle JT. Multiple risk pathways for schizophrenia converge in serine racemase knockout mice, a mouse model of NMDA receptor hypofunction. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:E2400–2409. [PubMed: 23729812]
- Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L, Jiang ZI, Benneyworth MA, Froimowitz MP, Lange N, Snyder SH, Bergeron R, Coyle JT. Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. *Mol Psychiatry*. 2009; 14:719–727. [PubMed: 19065142]
- Belforte JE, Zsiros V, Sklar ER, Jiang Z, Yu G, Li Y, Quinlan EM, Nakazawa K. Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat Neurosci*. 2010; 13:76–83. [PubMed: 19915563]
- Bitanirwe BK, Lim MP, Kelley JF, Kaneko T, Woo TU. Glutamatergic deficits and parvalbumin-containing inhibitory neurons in the prefrontal cortex in schizophrenia. *BMC Psychiatry*. 2009; 9:71. [PubMed: 19917116]
- Brown KL, Comalli DM, De Biasi M, Woodruff-Pak DS. Trace eyeblink conditioning is impaired in $\alpha 7$ but not in $\beta 2$ nicotinic acetylcholine receptor knockout mice. *Front Behav Neurosci*. 2010; 4:166. [PubMed: 20976039]
- Cannon CE, Puri V, Vivian JA, Egbertson MS, Eddins D, Uslaner JM. The nicotinic $\alpha 7$ receptor agonist GTS-21 improves cognitive performance in ketamine impaired rhesus monkeys. *Neuropharmacology*. 2013; 64:191–196. [PubMed: 22659472]
- Carlen M, Meletis K, Siegle JH, Cardin JA, Futai K, Vierling-Claassen D, Ruhlmann C, Jones SR, Deisseroth K, Sheng M, Moore CI, Tsai LH. A critical role for NMDA receptors in parvalbumin interneurons for gamma rhythm induction and behavior. *Mol Psychiatry*. 2012; 17:537–548. [PubMed: 21468034]
- Coyle JT. NMDA receptor and schizophrenia: a brief history. *Schizophr Bull*. 2012; 38:920–926. [PubMed: 22987850]
- Coyle JT, Basu A, Benneyworth M, Balu D, Konopaske G. Glutamatergic synaptic dysregulation in schizophrenia: therapeutic implications. *Handb Exp Pharmacol*. 2012:267–295. [PubMed: 23027419]

- Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol.* 2007; 47:699–729. [PubMed: 17009926]
- Dong YN, Waxman EA, Lynch DR. Interactions of postsynaptic density-95 and the NMDA receptor 2 subunit control calpain-mediated cleavage of the NMDA receptor. *J Neurosci.* 2004; 24:11035–11045. [PubMed: 15590920]
- Endris V, Hackmann K, Neuhaus TM, Grasshoff U, Bonin M, Haug U, Hahn G, Schallner JC, Schrock E, Tinschert S, Rappold G, Moog U. Homozygous loss of *CHRNA7* on chromosome 15q13.3 causes severe encephalopathy with seizures and hypotonia. *Am J Med Genet A.* 2010; 152A:2908–2911. [PubMed: 20979196]
- Fernandes C, Hoyle E, Dempster E, Schalkwyk LC, Collier DA. Performance deficit of alpha7 nicotinic receptor knockout mice in a delayed matching-to-place task suggests a mild impairment of working/episodic-like memory. *Genes Brain Behav.* 2006; 5:433–440. [PubMed: 16923147]
- Girod R, Barazangi N, McGehee D, Role LW. Facilitation of glutamatergic neurotransmission by presynaptic nicotinic acetylcholine receptors. *Neuropharmacology.* 2000; 39:2715–2725. [PubMed: 11044742]
- Hajos M, Rogers BN. Targeting alpha7 nicotinic acetylcholine receptors in the treatment of schizophrenia. *Curr Pharm Des.* 2010; 16:538–554. [PubMed: 19909231]
- Hardingham GE, Bading H. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci.* 2010; 11:682–696. [PubMed: 20842175]
- Helbig I, et al. 15q13.3 microdeletions increase risk of idiopathic generalized epilepsy. *Nat Genet.* 2009; 41:160–162. [PubMed: 19136953]
- Hellier JL, Arevalo NL, Smith L, Xiong KN, Restrepo D. alpha7-Nicotinic acetylcholine receptor: role in early odor learning preference in mice. *PLoS One.* 2012; 7:e35251. [PubMed: 22514723]
- Hoftman GD, Lewis DA. Postnatal developmental trajectories of neural circuits in the primate prefrontal cortex: identifying sensitive periods for vulnerability to schizophrenia. *Schizophr Bull.* 2011; 37:493–503. [PubMed: 21505116]
- Hoppman-Chaney N, Wain K, Seger P, Superneau D, Hodge J. Identification of single gene deletions at 15q13.3: further evidence that *CHRNA7* causes the 15q13.3 microdeletion syndrome phenotype. *Clin Genet.* 2012
- Javitt DC. Twenty-five years of glutamate in schizophrenia: are we there yet? *Schizophr Bull.* 2012; 38:911–913. [PubMed: 22987849]
- Karpova A, Mikhaylova M, Bera S, Bar J, Reddy PP, Behnisch T, Rankovic V, Spilker C, Bethge P, Sahin J, Kaushik R, Zschratte W, Kahne T, Naumann M, Gundelfinger ED, Kreutz MR. Encoding and transducing the synaptic or extrasynaptic origin of NMDA receptor signals to the nucleus. *Cell.* 2013; 152:1119–1133. [PubMed: 23452857]
- Kaufman AM, Milnerwood AJ, Sepers MD, Coquinco A, She K, Wang L, Lee H, Craig AM, Cynader M, Raymond LA. Opposing roles of synaptic and extrasynaptic NMDA receptor signaling in cocultured striatal and cortical neurons. *J Neurosci.* 2012; 32:3992–4003. [PubMed: 22442066]
- Korotkova T, Fuchs EC, Ponomarenko A, von Engelhardt J, Monyer H. NMDA receptor ablation on parvalbumin-positive interneurons impairs hippocampal synchrony, spatial representations, and working memory. *Neuron.* 2010; 68:557–569. [PubMed: 21040854]
- Labrie V, Wong AH, Roder JC. Contributions of the D-serine pathway to schizophrenia. *Neuropharmacology.* 2012; 62:1484–1503. [PubMed: 21295046]
- Lepichon JB, Bittel DC, Graf WD, Yu S. A 15q13.3 homozygous microdeletion associated with a severe neurodevelopmental disorder suggests putative functions of the *TRPM1*, *CHRNA7*, and other homozygously deleted genes. *Am J Med Genet A.* 2010; 152A:1300–1304. [PubMed: 20425840]
- Levin ED, Petro A, Rezvani AH, Pollard N, Christopher NC, Strauss M, Avery J, Nicholson J, Rose JE. Nicotinic alpha7- or beta2-containing receptor knockout: effects on radial-arm maze learning and long-term nicotine consumption in mice. *Behav Brain Res.* 2009; 196:207–213. [PubMed: 18831991]
- Lewis DA, Curley AA, Glausier JR, Volk DW. Cortical parvalbumin interneurons and cognitive dysfunction in schizophrenia. *Trends Neurosci.* 2012; 35:57–67. [PubMed: 22154068]

- Liao J, DeWard SJ, Madan-Khetarpal S, Surti U, Hu J. A small homozygous microdeletion of 15q13.3 including the CHRNA7 gene in a girl with a spectrum of severe neurodevelopmental features. *Am J Med Genet A*. 2011; 155A:2795–2800. [PubMed: 21990074]
- Lin H, Vicini S, Hsu FC, Doshi S, Takano H, Coulter DA, Lynch DR. Axonal alpha7 nicotinic ACh receptors modulate presynaptic NMDA receptor expression and structural plasticity of glutamatergic presynaptic boutons. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:16661–16666. [PubMed: 20817852]
- Liu Z, Conroy WG, Stawicki TM, Nai Q, Neff RA, Berg DK. EphB receptors co-distribute with a nicotinic receptor subtype and regulate nicotinic downstream signaling in neurons. *Mol Cell Neurosci*. 2008; 38:236–244. [PubMed: 18403216]
- Lozada AF, Wang X, Goukko NV, Massey KA, Duan J, Liu Z, Berg DK. Glutamatergic synapse formation is promoted by alpha7-containing nicotinic acetylcholine receptors. *J Neurosci*. 2012; 32:7651–7661. [PubMed: 22649244]
- Ma TM, Abazyan S, Abazyan B, Nomura J, Yang C, Seshadri S, Sawa A, Snyder SH, Pletnikov MV. Pathogenic disruption of DISC1-serine racemase binding elicits schizophrenia-like behavior via D-serine depletion. *Mol Psychiatry*. 2012
- Malhotra AK, Pinals DA, Weingartner H, Sirocco K, Missar CD, Pickar D, Breier A. NMDA receptor function and human cognition: the effects of ketamine in healthy volunteers. *Neuropsychopharmacology*. 1996; 14:301–307. [PubMed: 8703299]
- Malhotra AK, Pinals DA, Adler CM, Elman I, Clifton A, Pickar D, Breier A. Ketamine-induced exacerbation of psychotic symptoms and cognitive impairment in neuroleptic-free schizophrenics. *Neuropsychopharmacology*. 1997; 17:141–150. [PubMed: 9272481]
- Mathew SV, Law AJ, Lipska BK, Davila-Garcia MI, Zamora ED, Mitkus SN, Vakkalanka R, Straub RE, Weinberger DR, Kleinman JE, Hyde TM. Alpha7 nicotinic acetylcholine receptor mRNA expression and binding in postmortem human brain are associated with genetic variation in neuregulin 1. *Hum Mol Genet*. 2007; 16:2921–2932. [PubMed: 17884806]
- Miller DT, et al. Microdeletion/duplication at 15q13.2q13.3 among individuals with features of autism and other neuropsychiatric disorders. *J Med Genet*. 2009; 46:242–248. [PubMed: 18805830]
- Monnerie H, Hsu FC, Coulter DA, Le Roux PD. Role of the NR2A/2B subunits of the N-methyl-D-aspartate receptor in glutamate-induced glutamic acid decarboxylase alteration in cortical GABAergic neurons in vitro. *Neuroscience*. 2010; 171:1075–1090. [PubMed: 20923697]
- Nolt MJ, Lin Y, Hruska M, Murphy J, Sheffler-Colins SI, Kayser MS, Passer J, Bennett MV, Zukin RS, Dalva MB. EphB controls NMDA receptor function and synaptic targeting in a subunit-specific manner. *J Neurosci*. 2011; 31:5353–5364. [PubMed: 21471370]
- Olincy A, Freedman R. Nicotinic mechanisms in the treatment of psychotic disorders: a focus on the alpha7 nicotinic receptor. *Handb Exp Pharmacol*. 2012:211–232. [PubMed: 23027417]
- Papouin T, Ladepeche L, Ruel J, Sacchi S, Labasque M, Hanini M, Groc L, Pollegioni L, Mothet JP, Oliet SH. Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. *Cell*. 2012; 150:633–646. [PubMed: 22863013]
- Rotaru DC, Lewis DA, Gonzalez-Burgos G. The role of glutamatergic inputs onto parvalbumin-positive interneurons: relevance for schizophrenia. *Rev Neurosci*. 2012; 23:97–109. [PubMed: 22718616]
- Rotaru DC, Yoshino H, Lewis DA, Ermentrout GB, Gonzalez-Burgos G. Glutamate receptor subtypes mediating synaptic activation of prefrontal cortex neurons: relevance for schizophrenia. *J Neurosci*. 2011; 31:142–156. [PubMed: 21209199]
- Sanz-Clemente A, Nicoll RA, Roche KW. Diversity in NMDA Receptor Composition: Many Regulators, Many Consequences. *Neuroscientist*. 2013; 19:62–75. [PubMed: 22343826]
- Schwartz TL, Sachdeva S, Stahl SM. Genetic data supporting the NMDA glutamate receptor hypothesis for schizophrenia. *Curr Pharm Des*. 2012; 18:1580–1592. [PubMed: 22280435]
- Sharp AJ, et al. A recurrent 15q13.3 microdeletion syndrome associated with mental retardation and seizures. *Nat Genet*. 2008; 40:322–328. [PubMed: 18278044]
- Shinawi M, Schaaf CP, Bhatt SS, Xia Z, Patel A, Cheung SW, Lanpher B, Nagl S, Herding HS, Nevinsky-Stickel C, Immken LL, Patel GS, German JR, Beaudet AL, Stankiewicz P. A small

- recurrent deletion within 15q13.3 is associated with a range of neurodevelopmental phenotypes. *Nat Genet.* 2009; 41:1269–1271. [PubMed: 19898479]
- Spielmann M, Reichelt G, Hertzberg C, Trimborn M, Mundlos S, Horn D, Klopocki E. Homozygous deletion of chromosome 15q13.3 including CHRNA7 causes severe mental retardation, seizures, muscular hypotonia, and the loss of KLF13 and TRPM1 potentially cause macrocytosis and congenital retinal dysfunction in siblings. *Eur J Med Genet.* 2011; 54:e441–445. [PubMed: 21596161]
- Timofeeva OA, Levin ED. Glutamate and nicotinic receptor interactions in working memory: importance for the cognitive impairment of schizophrenia. *Neuroscience.* 2011; 195:21–36. [PubMed: 21884762]
- Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev.* 2010; 62:405–496. [PubMed: 20716669]
- Tregellas JR, Tanabe J, Rojas DC, Shatti S, Olincy A, Johnson L, Martin LF, Soti F, Kem WR, Leonard S, Freedman R. Effects of an alpha 7-nicotinic agonist on default network activity in schizophrenia. *Biol Psychiatry.* 2011; 69:7–11. [PubMed: 20728875]
- Wallace TL, Porter RH. Targeting the nicotinic alpha7 acetylcholine receptor to enhance cognition in disease. *Biochem Pharmacol.* 2011; 82:891–903. [PubMed: 21741954]
- Waxman EA, Lynch DR. N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. *Neuroscientist.* 2005; 11:37–49. [PubMed: 15632277]
- Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proceedings of the National Academy of Sciences of the United States of America.* 1999; 96:13409–13414. [PubMed: 10557334]
- Yasui DH, Scoles HA, Horike S, Meguro-Horike M, Dunaway KW, Schroeder DI, Lasalle JM. 15q11.2-13.3 chromatin analysis reveals epigenetic regulation of CHRNA7 with deficiencies in Rett and autism brain. *Hum Mol Genet.* 2011; 20:4311–4323. [PubMed: 21840925]
- Young JW, Meves JM, Tarantino IS, Caldwell S, Geyer MA. Delayed procedural learning in alpha7-nicotinic acetylcholine receptor knockout mice. *Genes Brain Behav.* 2011; 10:720–733. [PubMed: 21679297]
- Young JW, Crawford N, Kelly JS, Kerr LE, Marston HM, Spratt C, Finlayson K, Sharkey J. Impaired attention is central to the cognitive deficits observed in alpha 7 deficient mice. *Eur Neuropsychopharmacol.* 2007; 17:145–155. [PubMed: 16650968]
- Zhang L, Warren RA. Muscarinic and nicotinic presynaptic modulation of EPSCs in the nucleus accumbens during postnatal development. *J Neurophysiol.* 2002; 88:3315–3330. [PubMed: 12466449]
- Zheng CY, Seabold GK, Horak M, Petralia RS. MAGUKs, synaptic development, and synaptic plasticity. *Neuroscientist.* 2011; 17:493–512. [PubMed: 21498811]

Highlights

- Deletion of the human $\alpha 7$ nAChR gene is associated with neuropsychiatric diseases.
- Cortical NMDARs and glutamatergic synapses are reduced in $\alpha 7$ nAChR null models.
- Synaptic, but not extrasynaptic, NMDAR currents are diminished in these models.
- The synthetic enzyme for D-serine (coagonist of synaptic NMDARs) is also decreased.
- Additionally, D-serine responsive synaptic NMDAR currents are reduced.

Figure 1. Cortical NR1, NR2A and NR2B expression levels were decreased in $\alpha 7$ nAChR null mice during early postnatal development

Western blotting analysis of whole cortex homogenates (30 μ g per lane) showing NR1 (A), NR2B (B), NR2A (C) and NR3A (D) expression as well as actin or GAPDH as internal control in the cortex of *wild-type* (WT) and $\alpha 7$ nAChR *null* (KO) mice at postnatal days P1, P4, P8, P14, P21, P56 and P9M (>9 months old) (* $p < 0.05$ ** $p < 0.01$ KO vs. WT; $n = 6-11$; t -test). Since the cortical actin and GAPDH levels alter during the time course of postnatal development, the protein levels were normalized by internal control at the same time-point between WT and KO mice.

Figure 2. Cortical glutamatergic synaptic marker expression levels were decreased in $\alpha 7$ nAChR null mice during late postnatal development and adulthood

A, B. Western blotting analysis of whole cortex homogenates (30 μ g per lane) showing glutamatergic presynaptic marker, VGLUT1 (**A**) and postsynaptic marker, PSD95 (**B**) expression as well as actin or GAPDH as internal control in the cortex of *wild-type* (WT) and $\alpha 7$ nAChR *null* mice (KO) at postnatal days P1-P56 and P9M (* $p < 0.05$ KO vs. WT; $n = 6-11$; t -test).

Figure 3. Glutamatergic synapse formation was reduced in the prefrontal cortex of $\alpha 7$ nAChR null mice during late postnatal development and adulthood

A-L. Confocal images of VGLUT1 (red), PSD95 (green) immunofluorescence and merged images with DAPI-stained nucleus showing a reduction in the overall level of VGLUT1 and PSD95 immunoreactivities and in the number of VGLUT1- and PSD95-containing glutamatergic synapses in the prefrontal cortex of $\alpha 7$ nAChR null ($\alpha 7$ -KO) mice (**D-F** and **J-L**) compared with *wild-type* (WT) littermates (**A-C** and **G-I**) at P56-P90. **M.**

Quantification of the number of VGLUT1-positive puncta per 1000 μm^2 showing a significant decrease in the number of VGLUT1-positive glutamatergic synaptic terminals in the cortical layer 2/3 prefrontal cortex of $\alpha 7$ nAChR null (KO) mice compared with *wild-type* (WT) littermates at P56-P90 (*** $p < 0.001$ KO vs. WT, 6 sections per animal; 3 animals per group; *t*-test). Scale bars as indicated.

Figure 4. Glutamatergic synapse formation was reduced in $\alpha 7$ nAChR null cortical cultures
A-L. Confocal images of VGLUT1 (red), PSD95 (green) immunofluorescence and merged images showing a marked reduction in VGLUT1- and PSD95-positive glutamatergic synapses on the dendrites of cultured $\alpha 7$ -KO cortical neurons (**D-F** and **J-L**) compared with WT neurons (**A-C** and **G-I**). **G-L.** High magnification of confocal images showing that 100% of VGLUT1-positive puncta (**G, J**) were colocalized with or adjacent to one or more PSD-95 puncta (**H, K**) on the dendrites of cortical neurons in WT and $\alpha 7$ -KO cultures (**I, L**). **M.** Quantification of the number of VGLUT1-positive puncta per 1 μ m length dendrite of cortical neurons showing a significant decrease in the number of glutamatergic synapses in $\alpha 7$ -KO cortical neurons compared with WT neurons (*** $p < 0.001$ KO vs. WT; 5 neurons per culture, 3 different cultures; t -test).

Figure 5. Synaptic NMDAR localization and NMDAR expression were reduced in cultured cortical pyramidal neurons lacking $\alpha 7$ nAChRs

A-L. Confocal images of NR1 (green), VGLUT1 (red) immunofluorescence and merged images showing the reduced NR1-positive puncta in the soma and dendrites of cortical pyramidal neurons in $\alpha 7$ -KO cortical cultures (**D, J**) compared with WT cultures (**A, G**) and the reduced VGLUT1-positive glutamatergic synaptic terminals on the dendrites of cortical pyramidal neurons in $\alpha 7$ -KO cortical cultures (**E, K**) compared with WT cultures (**B, H**). **G-L.** High magnification of confocal images showed that VGLUT1-positive presynaptic terminals (**H, K**) localized closely with some of NR1-positive puncta (**G, J**) in the dendrites of WT and $\alpha 7$ -KO neurons (**I, L**). Scale bars as indicated. **M, N.** Western blotting analysis further showed a significant reduction in the expression levels of NR1, NR2A, VGLUT1 and PSD95 in $\alpha 7$ -KO cortical cultures compared with WT cultures (*** $p < 0.001$ KO vs. WT; 3-6 samples per culture, 4-6 different cultures; t -test). Scale bars as indicated.

Figure 6. Synaptic, but not extrasynaptic, NMDAR currents were specifically diminished in cultured cortical pyramidal neurons lacking $\alpha 7$ nAChR

A-F. Reduction of synaptic NMDAR-mediated spontaneous EPSC (sEPSC) in cultured $\alpha 7$ -KO cortical neurons compared with WT neurons. **A**, Representative sEPSC traces for both WT and $\alpha 7$ -KO cultured cortical neurons and averaged representative traces for both WT (black line) and $\alpha 7$ -KO (red line). **B-D**, sEPSC analysis demonstrated that the decay time constant and the frequency of sEPSC were significantly decreased (**B**, **C**), but the amplitude was not altered (**D**) in $\alpha 7$ -KO cortical neurons compared WT neurons. Graph data are expressed as mean $\pm$ S.E.M. from 7-8 independent experiments. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs. WT; $n = 40-54$; t -test. **E**, Effects of NMDAR specific antagonist D-AP5 (50 μ M) on sEPSCs in cortical neurons. Representative sEPSC traces for both WT and $\alpha 7$ -KO cortical neurons and averaged sEPSC traces before and during D-AP5 treatment were plotted; blue line denotes baseline averaged traces before D-AP5 treatment and red line denotes averaged traces after D-AP5 treatment. **F**, D-AP5 treatment decreases sEPSC decay time constant in cultured WT cortical neurons, but not in cultured $\alpha 7$ -KO neurons. Graph data are expressed as mean $\pm$ S.E.M. from 3-4 independent experiments. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs. baseline control; $n = 7-9$; t -test. **G-I**, Synaptic NMDAR reduction in $\alpha 7$ -KO

cortical neurons. NMDAR-mediated current densities were obtained with or without synaptic blockade of NMDAR using pretreatment with picrotoxin (PTX) and MK-801 for 5 min before recording. **G**, Representative traces of NMDAR-mediated currents for both WT and $\alpha 7$ -KO cortical neurons. *** $p < 0.001$ vs. WT; $n = 9-10$; t -test. **H**, Data analysis revealed no significant difference in the whole-cell surface NMDAR-mediated current density in WT and $\alpha 7$ -KO cortical neurons; However, there was a significant increase in the residual currents generated by extrasynaptic NMDAR activation following PTX/MK-801 pretreatment in $\alpha 7$ -KO vs. WT cortical neurons ($p < 0.001$); **I**, Percentage of synaptic NMDAR was significantly reduced in $\alpha 7$ -KO vs. WT cortical neurons ($p < 0.001$).

Figure 7. Loss of synaptic NMDAR currents in layer 2/3 pyramidal neurons of acute prefrontal cortical slices of $\alpha 7$ nAChR null mice

A. Representative sEPSC traces for both WT and $\alpha 7$ -KO cortical pyramidal neurons recorded from layer 2/3 of prefrontal cortex in acute brain slices and averaged representative traces for both WT (black line) and $\alpha 7$ -KO (red line). **B-D.** sEPSC analysis demonstrated that the decay time and frequency of sEPSC were significantly reduced (**B**, **C**), but the sEPSC amplitude was not altered (**D**) in $\alpha 7$ -KO prefrontal cortex layer 2/3 cortical neurons compared with WT. Graph data are expressed as mean $\pm$ S.E.M. from 4-5 independent experiments. ** $p < 0.01$ and *** $p < 0.001$ vs. WT; $n = 10$ for WT and $n = 15$ for $\alpha 7$ -KO; t -test. **E, F.** Effects of D-AP5 on sEPSCs in prefrontal cortex layer 2/3 cortical neurons in acute brain slices. **E.** Representative sEPSC traces for both WT and $\alpha 7$ -KO cortical pyramidal neurons recorded from layer 2/3 prefrontal cortex and averaged sEPSC traces before and during D-AP5 treatment; blue line for baseline before D-AP5 treatment and red line for during D-AP5 treatment. **F.** D-AP5 treatment was able to decrease sEPSC decay time constant in WT but not in $\alpha 7$ -KO cortical pyramidal neurons. Graph data are expressed as mean $\pm$ S.E.M. from 3-4 independent experiments. ** $p < 0.01$ ** $p < 0.001$ vs. baseline control; $n = 3-6$; t -test.

Figure 8. The D-serine synthetic enzyme serine racemase and D-serine responsive synaptic NMDAR-mediated activity were both reduced in cortical pyramidal neurons lacking α7 nAChRs
A. Western blotting analysis showing that serine racemase (SR) levels as well as actin as internal control in the cortex of WT and α7-KO mice at postnatal days P1-P56 and P9M (*p<0.05 **p<0.01 KO vs. WT; n=6; t-test). **B-G.** Confocal images of SR (red), NR1 (green) immunofluorescence and merged images with DAPI-stained nucleus showing a reduction in the overall level of SR and NR1 immunoreactivities in the prefrontal cortex of α7-KO mice (E-G) compared with WT littermates at P56-P90 (B-D). Scale bars as indicated. **H.** Representative sEPSC traces for D-serine treatment in both WT and α7-KO cortical pyramidal neurons and averaged sEPSC traces for both before (baseline; black line) and during D-serine treatment (D-serine; red line). **I.** D-serine treatment increases sEPSC decay time constant in cultured WT cortical neurons, but not in cultured α7-KO neurons. Graph data are expressed as mean ± S.E.M. from 6-8 independent experiments. # p<0.05, D-serine treatment vs. baseline control; ** p<0.01, baseline decay time constant for α7-KO neurons vs. WT neurons, n = 9-14; t-test.