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Developmental Regulation of the NMDA Receptor Subunits, NR3A and NR1, in Human Prefrontal Cortex

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Subunit composition of *N*-methyl-D-aspartate–type glutamate receptors (NMDARs) dictates their function, yet the ontogenic profiles of human NMDAR subunits from gestation to adulthood have not been determined. We examined NMDAR mRNA and protein development in human dorsolateral prefrontal cortex (DLPFC), an area in which NMDARs are critical for higher cognitive processing and NMDAR hypofunction is hypothesized in schizophrenia. Using quantitative reverse transcriptase–polymerase chain reaction and western blotting, we found NR1 expression begins low prenatally, peaks in adolescence, yet remains high throughout life, suggesting lifelong importance of NMDAR function. In contrast, NR3A levels are low during gestation, surge soon after birth, and decline progressively through adolescence and into adulthood. Because NR3A subunits uniquely attenuate NMDAR-mediated currents, limit calcium influx, and suppress dendritic spine formation, high levels during early childhood may be important for regulating neuroprotection and activity-dependent sculpting of synapses. We also examined whether subunit changes underlie reduced NMDAR activity in schizophrenia. Our results reveal normal NR1 and NR3A protein levels in DLPFC from schizophrenic patients, indicating that NMDAR hypofunction is unlikely to be maintained by gross changes in NR3A-containing NMDARs or overall NMDAR numbers. These data provide insights into NMDAR functions in the developing CNS and will contribute to designing pharmacotherapies for neurological disorders.

Keywords: antipsychotic, DLPFC, glutamate, postmortem, schizophrenia

Introduction

N-methyl-D-aspartate (NMDA)–type ionotropic glutamate receptors (NMDARs) are involved in a wide array of biological processes crucial for brain development and function. In addition to modulating neuronal and synapse maturation in development (Perez-Otano and Ehlers 2004), NMDARs are responsible for short-term and long-term memory storage through mechanisms of synaptic plasticity (Malenka and Nicoll 1999). For example, many executive functions and working memory tasks require NMDAR activity in the prefrontal cortex (PFC) (Lisman et al. 1998; Durstewitz and Gabriel 2007), suggesting that disruption of these receptors could cause profound cognitive deficits (Goldman-Rakic 1995; Lewis 1997). Given the diverse roles of NMDARs, it is not surprising that NMDAR dysfunction is thought to underlie several neurological and psychiatric disorders. Accordingly, a major goal in neuroscience is to understand how NMDARs normally change during human development and how this might be altered in disease states.

NMDAR function is dictated by its subunit composition (Monyer et al. 1992). NMDARs are tetramers consisting of essential NR1 subunits in combination with NR2 (A-D) or NR3 (A-B) subunits that provide functional molecular diversity (Perez-Otano et al. 2001; Al-Hallaq et al. 2002; Matsuda et al. 2003). However, unlike the conventional NR1/NR2 receptors, those containing the recently identified NR3 subunits exhibit decreased single-channel conductance, insensitivity to magnesium blockade, and reduced calcium (Ca^{2+}) permeability (Das et al. 1998; Perez-Otano et al. 2001; Chatterton et al. 2002; Sasaki et al. 2002). Because Ca^{2+} influx is responsible for many forms of synaptic plasticity (Malenka and Bear 2004) and excitotoxic cell death (Choi 1988), the inclusion of NR3 subunits into NMDARs is likely to critically regulate the properties of NMDAR-mediated plasticity and may also serve a neuroprotective role.

Developmental regulation of human NMDAR subunits is poorly understood. Such knowledge is important for the rational design of pharmacotherapies for diseases involving NMDAR subunit dysfunction, such as neurodegenerative conditions, stroke, epilepsy, neuropathic pain, and schizophrenia (reviewed in Cull-Candy et al. 2001; Waxman and Lynch 2005; Kristiansen et al. 2007). For example, existing treatments for schizophrenia are very limited in their specificity and efficacy (Ross et al. 2006). This disease is thought to arise from decreased NMDAR activity that disrupts normal synaptic connectivity and plasticity, especially within the dorsolateral prefrontal cortex (DLPFC) (Javitt and Zukin 1991; Olney et al. 1999; Lewis and Levitt 2002; Coyle et al. 2003; Frankle et al. 2003). Although there is little consistent evidence that overall NR1 and NR2 levels differ between schizophrenic patients and controls (Kristiansen et al. 2007), a recent study suggests that NR3A may be elevated within the schizophrenic DLPFC (Mueller and Meador-Woodruff 2004). Therefore, NR3A's unique ability to suppress NMDAR function makes it an extremely attractive candidate both as an endogenous contributor to the NMDAR hypofunction observed in schizophrenia and as a potential target for pharmacological interventions.

To reveal how NMDAR subunit composition changes through the course of brain development and to begin to understand whether NMDAR subunits are altered in schizophrenia, we used postmortem tissue to examine NMDAR subunits, NR3A and NR1, during normal human DLPFC development and in the DLPFC of schizophrenic patients. Additionally, we demonstrate the effects of antipsychotic medications on NMDAR subunits. To our knowledge, we provide the first systematic evaluation of the normal developmental profiles of NMDAR expression in maturation of the human PFC from midgestation to early adulthood.

Materials and Methods

Postmortem Human Samples

Postmortem human tissue samples were deidentified to protect personal health information. Samples from the developmental cohort contained uniform 1-cm coronal sections of PFC (Brodmann areas 9/46) from 45 subjects ranging in age from 18 weeks gestation through 25 years (Table 1). Frozen tissue was obtained from the National Institute of Child Health and Human Development–University of Maryland (UMD) Brain and Tissue Bank for Developmental Disorders under contracts N01-HD-4-3368 and N01-HD-4-3383. Individuals died from non-CNS causes and had no known history of substance abuse or major psychiatric disorders. A priori, samples were divided into 7 age groups established in a previous study (Glantz et al. 2007): prenatal (n = 6), birth to 12 months of age (n = 5), 1–5 years (n = 6), 6–10 years (n = 6), 11–15 years (n = 11), 16–20 years (n = 4), and 21–25 years (n = 7). The schizophrenia cohort consisted of Brodmann area 9 tissue samples from 35 subjects (control n = 20; schizophrenia n = 15) obtained from the Harvard Brain Tissue Resource Center (McLean MA; supported in part by PHS grant number R24 MH068855), with ages ranging from 21 to >80 years (Table 2). To protect personal health information, exact ages were used for statistical purposes, but only age ranges of subjects can be published. PFC samples in the schizophrenia and control groups were group matched for age, gender, ethnicity, side-of-brain, brain pH, and postmortem interval (PMI). All fresh-frozen tissue blocks from the UMD and Harvard collections were chipped from larger frozen cortical slabs and consisted primarily of gray matter with small amounts of underlying white matter. The samples from the UMD tissue collection were harvested as 1-cm coronal slabs. Cuts were made with guidance from a neuroanatomical atlas to select Brodmann areas 9 and 46 and immediately frozen at –80 °C. Cytoarchitectonic localizations of Brodmann areas 9 and 46 were ascertained using Nissl staining of sections cut immediately adjacent to tissue blocks used for this study (see Glantz et al. 2007 for details). Samples of Brodmann area 9 from the Harvard tissue were obtained through uniform dissection from the superior prefrontal gyrus according to the Brodmann map. All tissues were stored frozen at –80 °C until use. Tissue pH was measured as described (Salimi et al. forthcoming) and samples with pH <5.8 were excluded from analysis. This study was approved by the Biomedical Institutional Review Board of the University of North Carolina at Chapel Hill (UNC-CH).

Animal Use

The animal use in this study was approved by the Institutional Animal Care and Use Committee of the UNC-CH. All rodents were maintained and sacrificed according to protocol guidelines.

Postmortem Stability of NMDAR Proteins

Eighteen C57BL/6 mice at age postnatal day 10 (P10) were divided into 3 equal groups, anesthetized with a lethal dose of sodium pentobarbital, and decapitated upon disappearance of corneal reflexes. In the first group, frontal cortex was dissected and immediately frozen at –80 °C (PMI = 0 h). To approximate the human PMI and simulate morgue conditions, heads of the second and third groups were kept at room temperature for 6 h, then at 4 °C for 6 h and 18 h, respectively, after which time frontal cortices were dissected (PMI = 12 h and PMI = 24 h) and frozen at –80 °C until use in immunoblotting analysis (Jarskog et al. 2004).

Antipsychotic Treatment

Subchronic drug treatments were administered to 36 singly housed, male Sprague–Dawley rats (150–200 g, Charles River) as described previously (Jarskog et al. 2007). Briefly, animals received daily intraperitoneal injections of haloperidol (1 mg/kg/day, n = 12), clozapine (10 mg/kg/day, n = 12), or saline (0.9%, n = 12) for 4 weeks. One hour after the final dose, rats were killed and anterior right medial frontal cortices were dissected. All tissue was kept frozen at –80 °C until use in immunoblotting analysis.

RNA Extraction, cDNA Preparation, and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from pulverized human developmental PFC tissue samples using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentrations and quality (mean RNA Integrity Number = 8.75 ± 1.0) were determined using Series II RNA 6000 Nano Assay in the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA was reverse transcribed using the High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Relative expression levels of NMDAR mRNAs were measured by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using prevalidated assays for NR1, NR3A, and β-glucuronidase (GUSB), the latter of which served as the endogenous control (Applied Biosystems Assays-on-Demand: Hs00609557_m1 for NR1, Hs00370290_m1 for NR3A, and 4333767F for GUSB). All probes had 6-carboxy-fluorescein phosphoramidite fluorescent reporter dye and minor groove binder (MGB) quencher. The NR1 primer–probe combination spanned exon boundary 1–2, covering all 8 splice variants, and the NR3A assay covered exon boundary 3–4. Pilot experiments using cDNA dilutions were used to define the dynamic range of each assay. GUSB was selected from several candidate reference genes as the endogenous control most closely matching in expression level to the target NMDAR genes. Reactions

Table 1
Developmental study demographics

Age group (years)	Age (years)	Subjects	PMI (h)	pH	Storage time (years)	Race	Sex
<0	−0.41 ± 0.0	6	1.0 ± 0.0	6.1 ± 0.2	13.6 ± 0.8	1 C, 5 AA	3 M, 3 F
0–1	0.1 ± 0.1	5	7 ± 3.2	6.3 ± 0.4	10.3 ± 2.9	2 C, 3 AA	3 M, 2 F
1–5	3.4 ± 1.6	6	15.2 ± 4.8	6.1 ± 0.3	6.1 ± 1.1	2 C, 4 AA	2 M, 4 F
6–10	8.3 ± 0.8	6	19.8 ± 9.8	6.3 ± 0.3	5.0 ± 0.8	3 C, 3 AA	2 M, 4 F
11–15	13.3 ± 1.0	11	18.3 ± 6.1	6.4 ± 0.3	7.3 ± 3.7	5 C, 3 AA, 3 U	9 M, 2 F
16–20	18.1 ± 1.7	4	14.8 ± 5.4	6.1 ± 0.1	9.3 ± 4.0	1 C, 3 AA	3 M, 1 F
21–25	23.1 ± 1.6	7	14.7 ± 5.9	6.2 ± 0.2	6.5 ± 1.5	2 C, 4 AA	6 M, 1 F

Note: Characteristics of normal subjects. Means are reported as ±standard deviation. C, Caucasian; AA, African-American; U, unknown; M, male; and F, female.

Table 2
Schizophrenia study demographics

Diagnosis	Subjects	Age (years)	PMI (h)	pH	Storage time (years)	Race	Sex
Control	20	56.7 ± 18.3	21.2 ± 6.0	6.4 ± 0.3	6.1 ± 1.5	9 C, 11 U	15 M, 5 F
Schizophrenia	15	54.3 ± 17.0	21.7 ± 5.1	6.4 ± 0.3	7.8 ± 1.2	14 C, 1 U	10 M, 5 F

Note: Characteristics of normal control and schizophrenic subjects. Means are reported as ±standard deviation. C, Caucasian; AA, African-American; U, unknown; M, male; and F, female.

were run in a 384-well plate format on an ABI 7900HT Fast RT-PCR sequence detection system (Applied Biosystems). Each 20-μl reaction contained 9 μl diluted cDNA (NR1: 6.25 ng; NR3A: 12.5 ng; GUSB: 6.25 ng or 12.5 ng), 0.9 μM each primer, 0.25 μM probe, and 10 μl TaqMan Universal PCR 2× MasterMix with AmpErase UNG (Applied Biosystems) with MGB/TAMRA 5′ endonuclease and ROX passive reference dye. PCR cycle parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s. Each examined mRNA was quantified from a single plate, and C_T values were within the linear range of the standard curve. All samples were loaded in triplicates. RT was omitted from control reactions for each tissue sample to verify the absence of amplified genomic DNA. The same threshold and baseline were used for all samples. Three of the 45 human brain samples did not meet our inclusion criteria for the mRNA quantification and were thus omitted from analysis.

Cortical Brain Extracts

Homogenates were prepared from postmortem human, monkey, mouse, and rat frontal cortices. Tissues were homogenized (1:10, w/v) on ice for 30 s (PowerGen 125, Fisher Scientific, Pittsburgh, PA) and sonicated for 10 s at 10 mV (Sonic Dismembrator 60, Fisher Scientific) in ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 0.6 M NaCl, 0.2% Triton X-100, 1 mM benzamidine, 0.1 mM benzethonium chloride, and 0.1 mM phenylmethylsulfonyl fluoride (Sigma, St Louis, MO). Samples were cleared of debris by centrifugation at 4 °C for 15 min at 15 000 × *g*, and supernatants were assayed for total protein in triplicate using the bicinchoninic acid method (Micro BCA Protein Assay Kit, Pierce Chemical, Rockford, IL). Aliquots were stored at –80 °C until use.

Quantitative Immunoblotting

Equal amounts of total protein (25–50 μg) were heated for 10 min at 70 °C in sample buffer and applied to 10-lane 4–12% gradient NuPAGE Novex Tris-glycine mini-gels (Invitrogen, Carlsbad, CA), along with molecular weight markers, a pooled sample homogenate, and a mouse frontal cortex sample as positive control. Coded samples were loaded randomly onto gels to preserve the integrity of blinded, unbiased data analyses. Samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 125 V for 2 h. Separated proteins were then electrophoretically transferred (Bio-Rad, Hercules, CA) to nitrocellulose membranes (Millipore, Billerica, MA) at 25 V overnight at 4 °C. After eliminating nonspecific protein binding to membranes with blocking buffer (Odyssey, LI-COR, Lincoln, NE) for 1 h at room temperature, membranes were cut into 3 strips with the aid of molecular size markers. Because the Odyssey Imaging System uses 2 near-infrared channels to detect fluorescent signals, 2 proteins can be simultaneously probed on each blot strip. Thus, 6 proteins were able to be detected from the same gel (upper blot strip, NR2B/NR2A; middle, NR3A/NR1; lower, β-tubulin/GAPDH). This approach allowed us to analyze multiple proteins while avoiding complications arising from stripping and reprobing membranes. The following primary antibodies were incubated overnight at 4 °C in 1:1 blocking buffer:phosphate-buffered saline (PBS), at optimized concentrations: rabbit anti-pan NR1 (#SC9058, 1:6000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-NR1 (#556308, 1:5000, BD/Pharmingen, San Jose, CA), rabbit anti-NR2A (#SC9056, 1:500, Santa Cruz), goat anti-NR2B (#SC1469, 1:10,000, Santa Cruz), mouse anti-NR3A (#MAB5388, 1:1000, Chemicon, Temecula, CA), rabbit anti-NR3A (#07-356, 1:500, Millipore/Upstate), goat anti-GAPDH (#IMG3073, 1:40,000, Imgenex, San Diego, CA), and mouse anti-β-tubulin (#MAB3408, 1:100,000, Millipore/Chemicon). Membranes were washed extensively in PBS-0.1% Tween 20 and then incubated at room temperature for 1 h (shielded from light) with appropriate secondary antibodies diluted in 1:1 blocking buffer/PBS-0.1% Tween 20: Alexa Fluor 680-labeled anti-goat IgG or anti-mouse IgG (1:5000, Molecular Probes/Invitrogen), and IRDye 800-labeled anti-rabbit IgG (1:3000, Rockland Immunochemicals, Gilbertsville, PA). Membranes were washed and fluorescent signals measured directly on the Odyssey Infrared Imaging System (LI-COR). Band density analysis was performed using Odyssey software (v2.1) supplied by the manufacturer. Antibody specificities for the following have been previously established: rabbit anti-pan NR1 (Fernandez-Monreal et al; Miyamoto et al. 2005; Offenhauser et al. 2006; Talbot et al. 2006; Li et al. 2007), mouse anti-NR1 (Siegel et al. 1994; 1995; Wood et al. 1995; Lack et al. 2005; Stepulak et al. 2005; Perez-Otano et al. 2006; Welch et al. 2007), rabbit anti-NR3A (Ishihama and Turman 2006), rabbit anti-NR2A (Miyamoto et al. 2005; Yashiro et al. 2005), and goat anti-NR2B (May et al. 2004; Pawlak et al. 2005; Yashiro et al. 2005; Czaja et al. 2006). Mouse anti-NR3 antibody produces no band at the expected molecular weight (~130 kD) in NR3A knockout versus wild-type cortical homogenates (data not shown). Antibody signal intensities were collected from 3 separate experiments for each sample. A standard curve of increasing protein amounts of pooled sample was immunoblotted for each protein to ensure a linear relationship between increasing total protein amounts and density of the respective bands (data not shown).

Data Collection and Statistical Analyses

To assess mRNA levels for each human target gene, GUSB was used as the internal reference gene and relative quantities were obtained by the $\Delta\Delta C_T$ method using Sequence Detection Software (SDS v.2.2.2, Applied Biosystems). Western blot experiments measured antibody signal intensities in postmortem tissue, interleaving control and experimental conditions where appropriate. A pooled sample was run on each gel, and the band density of each experimental sample was measured relative to this reference standard to permit intergel comparisons. Samples were run in triplicate experiments, and individual antibody values for each sample were averaged.

Developmental age group or diagnosis group means were calculated from individual averages. Group means were normalized either to the mean level of expression for the maximally expressing age group (developmental studies) or to the control group (schizophrenia and antipsychotic drug studies). For multiple group comparisons, 1-way analyses of variance (ANOVAs) were performed, followed by between-group comparisons with Tukey–Kramer tests. Unpaired Student's *t*-tests were used to compare diagnosis between schizophrenic subjects and normal controls with NMDAR proteins as variables. Two-way ANOVAs were performed post hoc to probe interactions between diagnosis and sex. All levels of significance represent 2-tailed values. Statistical analyses were conducted using Graphpad Instat (San Diego, CA) and SAS (Cary, NC). Developmental brain tissues binned into 7 groups based on age were not significantly different with regard to pH or sex; however, significant differences were observed between the groups due to PMI (ANOVA, $F_{6,38} = 8.11$, $P < 0.0001$) and storage time (ANOVA, $F_{6,38} = 8.08$, $P < 0.0001$) (see Table 1). Therefore, we included PMI and storage time in secondary analysis of covariance (ANCOVA) analyses with these variables as covariates. Although brain tissues from normal controls and schizophrenic patients had no significant differences between groups with regard to age, pH, or PMI, a significant difference was detected between the groups due to storage time. No secondary analyses were performed for the schizophrenia study.

Results

NR1 and NR3A, but not NR2A and NR2B, Protein Levels Can Be Studied Effectively in Postmortem Tissue

A difficulty of studying human tissue is that protein degradation occurs during the PMI after death and prior to tissue preservation. Therefore, it is necessary to assess how PMI affects antibodies' recognition of target sites. To test this, we used a mouse model to mimic the decay of human tissue under simulated morgue conditions (Jarskog et al. 2004). Protein integrity was measured by immunoblotting of cortical homogenates run in triplicate (Fig. 1). Antibodies directed against NMDA receptor subunits NR3A (125 kD), NR2A (165 kD), and NR2B (170 kD), as well as loading controls, β-tubulin (55 kD) and GAPDH (36 kD), all detected single bands at the appropriate molecular weights, consistent with predicted sizes. The double band for NR1 (116 kD) may be the result of splice variants, deglycosylation, or proteolytic cleavage products (Monyer et al. 1992;

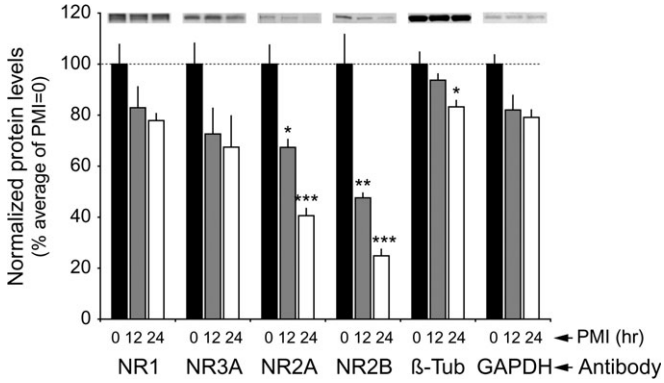

Figure 1. Protein stability of NMDA receptor subunits (NR1, NR3A, NR2A, and NR2B) and loading controls (β-Tubulin and GAPDH) in postmortem mouse frontal cortex, normalized to the average of PMI = 0 (n = 4/group except n = 2/group for GAPDH). Data are presented as means ± standard error of the mean. Significance from PMI = 0 h: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Representative immunoblots are shown in addition to averaged data.

Brose et al. 1993; Sheng et al. 1994; Zukin and Bennett 1995; Luo et al. 1996).

Immunoblotting revealed that NR1 and NR3A proteins displayed modest and predictable decreases in band densities, with no main effects noted by ANOVA (NR1, n = 4/group, $F_{2,9}$ = 3.01, P = 0.10; NR3A, n = 4/group, $F_{2,9}$ = 2.89, P = 0.11). Moderate protein degradation was also observed for the loading controls, although the decrease in β-tubulin reached statistical significance (β-tubulin, n = 4/group, $F_{2,9}$ = 6.27, P = 0.02; GAPDH, n = 2/group, $F_{2,3}$ = 7.06, P = 0.07). Unlike NR1 and NR3A, however, degradation of both NR2A and NR2B subunits was substantial and progressive, with total decreases in signal intensities of 69% and 75%, respectively. ANOVA demonstrated that there was a main effect of PMI on NR2A and NR2B levels (NR2A, n = 4, $F_{2,9}$ = 18.91, $P < 0.001$; NR2B, n = 4, $F_{2,9}$ = 30.74, $P < 0.0001$), and post hoc analyses revealed that protein levels were significantly reduced at both 12 and 24 h PMI compared with baseline (PMI = 0 h) values. This effect of PMI on NR2A and NR2B is consistent with the rapid proteolysis of NR2 subunits soon after death in human tissue (Wang et al. 2000) and is also consistent with difficulties in reliably measuring NR2A and NR2B levels in rhesus monkeys (O'Connor et al. 2006) and in human tissue (current study, data not shown). For these reasons, we performed no further analyses of NR2A and NR2B in human tissue. Furthermore, to minimize problems arising from studying tissue taken after a long PMI, we limited our analysis to tissue with a relatively low PMI (mean PMI ~13 and 21 h for the developmental and schizophrenia studies, respectively).

Our mouse data indicate that NR1 and NR3A subunits exhibit relatively high postmortem stability (>65%) for at least 24 h, suggesting that they are also likely to be more stable in human tissue than NR2 subunits. Importantly, our pilot experiments demonstrated that in both mouse and human tissue, the NR1 and NR3A antibodies recognized bands consistent with predicted sizes at 116 kD and ~130 kD, respectively. This indicates that these antibodies similarly recognize mouse and human NMDAR subunit homologues at their expected molecular weights. Assuming the postmortem stability of these proteins is similar in mice and humans, our data indicate that the NR1 and NR3A antibodies used in this study are appropriate to study these NMDAR subunits in human postmortem tissue.

In addition to PMI, another important consideration in western blotting of postmortem tissue is accurate protein band measurement. Typically, signal intensity for the protein of interest is measured relative to an in-lane reference, a loading control such as GAPDH or β-tubulin. However, as both of these common proteins varied considerably in their expression levels across development (data not shown), they were inappropriate for our studies. To overcome this limitation, we instead standardized protein levels of each sample to a homogenate pool that was included on every gel as a reference (see Materials and Methods) and allowed for intergel comparisons. Other studies have found similar methods to be reliable alternatives for quantification (Quinlan et al. 1999; Folkerth et al. 2004; Haynes et al. 2005; Murphy et al. 2005; Glantz et al. 2007; Salimi et al. forthcoming). GAPDH and β-tubulin normalizations were then used only for confirmatory analyses of adult samples in the schizophrenia study (Supplementary Figs S4 and S5), which had similar levels of these loading control proteins across groups. Thus, unless otherwise noted, all analyses were performed on protein levels standardized to the pooled sample that was run on each gel. Moreover, protein data from each sample was averaged from 3 independent western blotting experiments, as this further eliminated the possibility of loading errors.

Developmental Expression of NR3A Peaks in Early Childhood in Human DLPFC

Studies in rodents indicate that expression of the nonconventional NMDAR subunit, NR3A, is upregulated soon after birth, peaks during early postnatal life (around postnatal day 7; P7), and then decreases through the subsequent weeks in many regions of the brain (Ciabarra et al. 1995; Sucher et al. 1995; Das et al. 1998; Sun et al. 1998; Al-Hallaq et al. 2002; Sasaki et al. 2002; Wong et al. 2002; Ishihama and Turman 2006; Perez-Otano et al. 2006). We hypothesized that NR3A expression in humans is also upregulated early in development and downregulated in adolescence. To test this, we determined NR3A transcript levels by quantitative RT-PCR of postmortem human tissue from individuals aged 18 weeks gestation to 25 years (see Table 1 and Materials and Methods). Our studies focused on the DLPFC, an area implicated in higher cognitive function and in which NMDAR hypofunction has been hypothesized in the pathophysiology of schizophrenia.

Consistent with previous rodent studies, we found highly regulated expression of NR3A during prenatal and postnatal cortical development for both mRNA and protein levels (Fig. 2). NR3A mRNA expression was shown to be significantly regulated across the various age groups (ANOVA: $F_{6,35}$ = 8.60, $P < 0.0001$). Post hoc analyses indicate that NR3A transcript levels are significantly reduced prenatally compared with the 0- to 1-year group ($P < 0.0001$), the 1- to 5-year group ($P < 0.05$), and the 11- to 15-year group ($P < 0.01$). Furthermore, we observed a statistically significant decline in NR3A from the peak expression of NR3A mRNA (0- to 1-year group) compared with the 1- to 5-year group ($P < 0.02$), the 6- to 10-year group ($P < 0.001$), the 11- to 15-year group ($P < 0.02$), the 16- to 20-year group ($P < 0.01$), and the 21- to 25-year group ($P < 0.0001$). These data demonstrate that NR3A transcription is weak embryonically, increases dramatically in the first year of life, and then declines progressively into adulthood. NR3A mRNA expression levels increased 10-fold from the fetal samples as compared with the age group with peak expression.

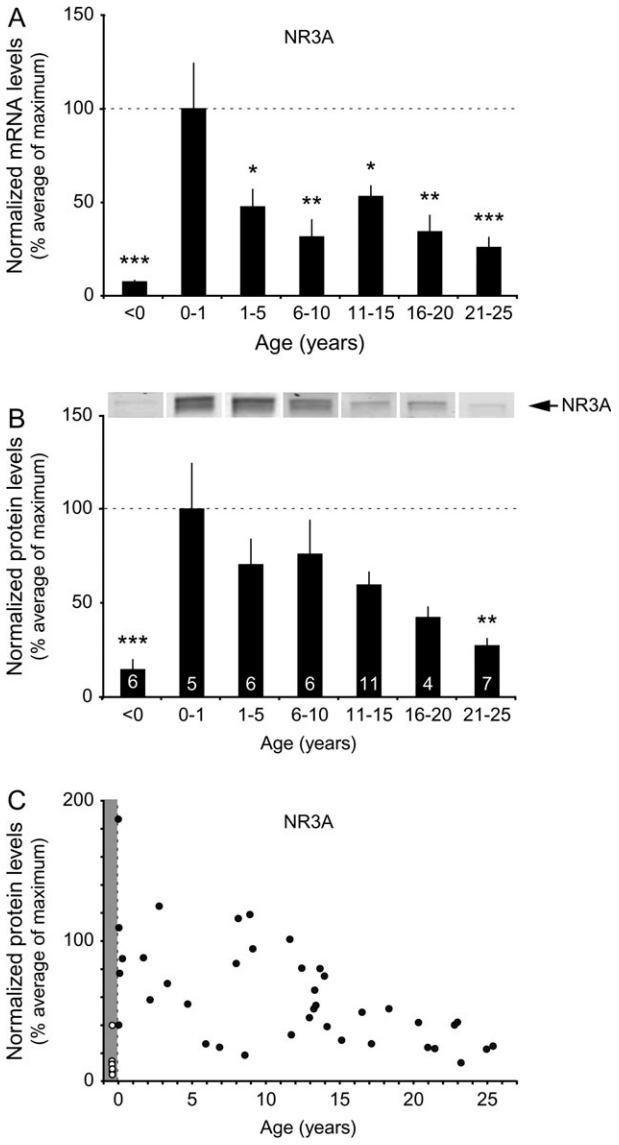

Figure 2. NR3A mRNA and protein expression surges in DLPFC after birth and then declines progressively. Quantification of NR3A levels, normalized to the average value in the age group where NR3A expression was maximal (0–1 year). (*A*) Normalized and averaged NR3A transcript levels binned into developmental age groups. (*B*) Normalized and averaged NR3A protein expression data. Values within bars represent sample sizes. Representative immunoblots are shown in addition to averaged data. Data are presented as means ± standard error of the mean. Significance from age group 0–1 year: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*C*) Scatter plot depicting NR3A expression over development. The dashed line and gray shading divide prenatal and postnatal tissue. Open circles and closed circles represent prenatal and postnatal tissue, respectively. Some points are obscured by overlying points with similar values.

In a secondary analysis, we performed an ANCOVA covarying PMI and storage time across the different age groups (ANOVA: $F_{6,33} = 6.84$, $P < 0.0001$). The results were largely consistent with the previous analysis run without covariates, as post hoc tests indicated that fetal tissue was significantly reduced compared with the same older age groups as described above ($P < 0.05$). Moreover, the peak expression of NR3A mRNA (0–1 year) was significantly greater than that at 16–20 years ($P < 0.02$) and 21–25 years ($P < 0.01$). Unlike the primary analysis, peak expression compared with 1- to 5-year, 6- to 10-year, and 11- to 15-year age groups was not significant.

Strikingly, developmental changes in NR3A protein abundance were qualitatively similar to that of NR3A mRNA levels. After low prenatal expression, a sharp postnatal increase in band densities was followed by a progressive reduction in NR3A protein levels through childhood and adolescence (Fig. 2). A 1-way ANOVA detected a significant effect of age on NR3A expression ($F_{6,38} = 5.60$, $P<0.001$). Subsequent Tukey–Kramer post hoc tests revealed that prenatal and young adult (ages 21–25 years) tissues expressed significantly less NR3A than infants aged 0–1 year ($P<0.001$ and $P<0.01$, respectively). Also, prenatal values differed significantly from age groups 1–5 and 6–10 (both $P<0.05$). The results of an ANCOVA covarying PMI and storage time across the different age groups (ANOVA: $F_{6,36} = 4.72$, $P < 0.002$) and post hoc tests were consistent with the previous analysis, indicating that even with PMI and storage time taken into account, the developmental differences we observed were robust and still significant.

To examine NMDAR expression changes in another relevant model system, where postmortem protein decay was not a potential confound, we immunoblotted NMDAR subunit proteins in developing postnatal macaque temporal cortex (Supplementary Fig. S1A). Postmortem frozen cortical brain tissue from 17 rhesus monkeys (*Macaca mulatta*) ages 3 months to 8 years were obtained from D.A. Lewis (University of Pittsburgh). Although we were unable to acquire fetal macaque tissue, our findings in monkeys show similar developmental changes to those observed in human postnatal NR3A protein expression (Fig. 2), with high expression in infancy that tapers off into adulthood.

NR1 Levels Are Relatively High in the Developing Human DLPFC

As the obligatory subunit of the NMDAR (Monyer et al. 1992; Perez-Otano et al. 2001; Matsuda et al. 2003), NR1 serves as an accurate gauge of the total number of NMDARs. Transcript and protein levels of NR1 have been reported in various brain regions in humans (Zhong et al. 1995; Akbarian et al. 1996; Scherzer et al. 1998; Law et al. 2003; Clinton et al. 2006; Kristiansen et al. 2006) and in rodents (Monyer et al. 1994; Laurie et al. 1997; Goebel and Poosch 1999; Prybylowski and Wolfe 2000; Sun et al. 2000; Ritter et al. 2002; Babb et al. 2005). However, to our knowledge, no study has examined NR1 expression over development in human PFC.

To determine the normal developmental profile of NR1 mRNA transcript and protein levels in the DLPFC, we again turned to quantitative RT-PCR and immunoblotting (Fig. 3). NR1 mRNA expression was shown to be significantly regulated across the various ages (ANOVA: $F_{6,35} = 8.26$, $P < 0.0001$). As in the NR3A quantitative PCR assays, there was a 10-fold increase in NR1 mRNA expression from prenatal to maximal postnatal levels. Post hoc analyses revealed that in the group with the lowest mRNA content (fetal samples), NR1 was significantly ($P < 0.001$) reduced compared with samples from the age group in which transcript levels were maximal, the 11- to 15-year group. Furthermore, samples from the 0- to 1-year group ($P < 0.01$), the 1- to 5-year group ($P < 0.05$), and the 6- to 10-year group ($P < 0.05$) also were significantly reduced compared with the 11- to 15-year group. In a secondary analysis, we performed an ANCOVA covarying PMI and storage time across the binned age groups (ANOVA: $F_{6,33} = 6.73$, $P < 0.0001$). As with the NR3A secondary analyses, these results were consistent with the

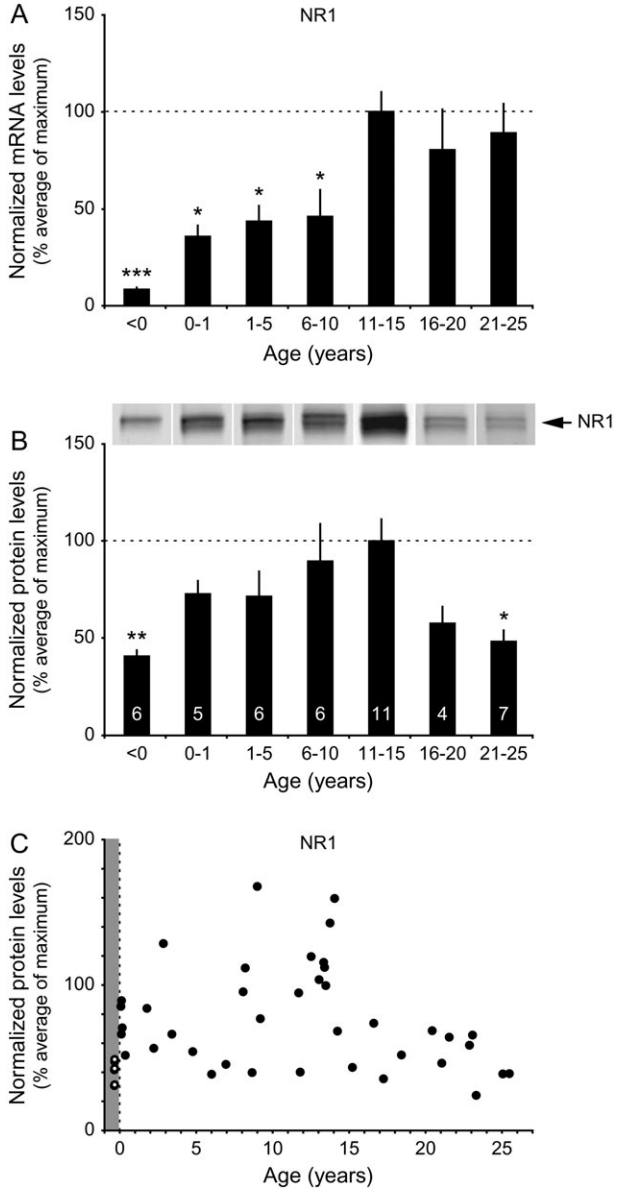

Figure 3. NR1 levels in human DLPFC change modestly over development. (*A*) Normalized and averaged NR1 mRNA expression levels. (*B*) Normalized and averaged NR1 protein expression data. Data are presented as means ± standard error of the mean. Values within bars represent sample sizes. Representative immunoblots are shown in addition to averaged data. Significance from age group 11–15 years: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*C*) Scatter plot depicting NR1 levels over human development. The dashed line and gray shading divide prenatal and postnatal tissue. Open circles and closed circles represent prenatal and postnatal tissue, respectively. Some points are obscured by overlying symbols with similar values.

primary analysis as well as subsequent post hoc analyses, suggesting that PMI and storage time were independent from the developmental differences we observed.

Western blot analyses of frontal cortical homogenates prepared from the developmental samples demonstrate robust expression of NR1. Consistent with NR1 mRNA, protein levels are low prenatally and rise gradually to early adolescence and then decline modestly into adulthood (Fig. 3). Protein expression of NR1 was found to be significantly regulated across developmental groups (ANOVA: $F_{6,38} = 3.98$, $P < 0.005$). Post hoc tests indicate that the prenatal group was significantly ($P < 0.01$) reduced compared with the 11- to 15-year group and that there is a significant reduction ($P < 0.02$) of NR1 protein levels when comparing the 11- to 15-year group to the 21- to 25-year group. This reduction in adult tissue was not observed with NR1 mRNA, suggesting that protein expression may be regulated differently in maturity. An ANCOVA covarying PMI and storage time across the different age groups found that NR1 mRNA expression was significantly regulated across developmental groups (ANOVA: $F_{6,36} = 2.86$, $P < 0.05$). However, post hoc tests revealed that, unlike the previous analysis, fetal tissue failed to differ significantly from the 11- to 15-year group. The observed significant ($P < 0.01$) decline in protein levels comparing the 11- to 15-year group to the 21- to 25-year group was maintained when including PMI and storage time as covariates.

In a parallel study of cortical development, we probed fresh-frozen postmortem tissue from developing postnatal macaque cortex for NR1 protein (generous gift from D.A. Lewis, University of Pittsburgh) (Supplementary Fig. S1B). Although not statistically significant, developmental expression levels of NR1 in monkeys appeared similar to that in humans, with high levels exhibited until a drop during late puberty.

Because NR1 is essential for all functional NMDARs (Monyer et al. 1992; Perez-Otano et al. 2001; Matsuda et al. 2003), the ratio of NR3A to NR1 provides an estimate for the proportion of NR3A-containing receptors out of the total pool of NMDARs (Supplementary Fig. S2). By this measure, the NR3A/NR1 mRNA ratio was shown to be significantly regulated across the various age groups (ANOVA: $F_{6,35} = 14.66$, $P < 0.0001$). Post hoc analyses indicate the peak NR3A/NR1 ratio of transcript levels (0–1 year of age) was significantly different from all other age groups. NR3A/NR1 levels are significantly reduced prenatally compared with the 0- to 1-year group ($P < 0.0001$). Furthermore, we observed a statistically significant decline in NR3A/NR1 ratio from peak expression (0- to 1-year group) compared with the all other age groups ($P < 0.0001$). In a secondary analysis, we performed an ANCOVA with PMI and storage time as covariates across the different age groups (ANOVA: $F_{6,33} = 13.09$, $P < 0.0001$). The results of this analysis were consistent with the previous analysis run without covariates.

The ratio of NR3A/NR1 protein similarly changed over development (Supplementary Fig. S2). A 1-way ANOVA detected a significant effect of age on NR3A/NR1 ($F_{6,38} = 7.81$, $P < 0.0001$). Subsequent Tukey–Kramer post hoc tests revealed that prenatal tissue had a significantly lower NR3A/NR1 ratio than infants aged 0–1 year and ages 1–5 ($P < 0.0001$ and $P < 0.01$, respectively). Furthermore, the maximum ratio of NR3A/NR1 (0–1 year) differed significantly from all other age groups ($P < 0.01$) except 1–5 years. From an ANCOVA covarying PMI and storage time across the different age groups (ANOVA: $F_{6,36} = 7.27$, $P < 0.0001$), our results were largely consistent with the previous analysis as well as subsequent post hoc analyses. Of the differences we observed, fetal expression was no longer significantly different from the 1- to 5-year age group and maximal expression did not differ substantially from the 6- to 10-year age group. Overall, this impressive age-dependent regulation of NR3A/NR1 mRNA and protein levels underscores how vital the understanding of the ontogeny of these subunits will be to investigations of how their dysregulation contributes to neurodevelopmental disorders, such as schizophrenia or bipolar disorder (Mueller and Meador-Woodruff 2004).

NR1 and NR3A Protein Levels Are Unchanged in Schizophrenic DLPFC

A prominent theory of schizophrenia suggests that the disease may arise from NMDAR hypofunction in the PFC (Javitt and Zukin 1991; Tamminga 1998; Weickert and Weinberger 1998; Olney et al. 1999; Lewis and Levitt 2002; Coyle et al. 2003; Frankle et al. 2003). However, studies examining NR1 levels in schizophrenia have found conflicting results (reviewed in Kristiansen et al. 2007), suggesting that possible changes in NR1 levels need further evaluation. To test whether protein expression of NR1 is abnormal in the schizophrenic DLPFC compared with tissue from comparison subjects (Table 2), we probed immunoblots of human tissue homogenates to measure NR1 protein (Fig. 4). A 2-way ANOVA (diagnosis × sex) revealed no significant main effects on NR1 expression (diagnosis $F_{1,31} = 2.68$, $P = 0.11$; sex $F_{1,31} = 0.08$; $P = 0.78$). However, there was an interesting trend for an interaction between diagnosis and sex ($F_{1,31} = 3.32$, $P = 0.08$) (Supplementary Fig. S3), indicating that it might be worthwhile for future studies to investigate sex differences in NR1 expression in schizophrenic and control subjects.

To our knowledge, the NR3A subunit has been evaluated for a role in schizophrenia in only one study, which demonstrated that NR3A mRNA levels are significantly increased by 32% within subregions of the DLPFC in schizophrenic patients (Mueller and Meador-Woodruff 2004). We hypothesized that schizophrenia could arise in part because of a failure of NR3A to downregulate during development. Because NR3A suppresses calcium entry and NMDAR-mediated currents, higher than normal NR3A levels would be expected to cause NMDAR hypofunction. Thus, we sought to determine whether NR3A levels were increased at the protein level in the DLPFC of the schizophrenic brain (Fig. 4). Contrary to our hypothesis, there was no main effect of diagnosis by a 2-way ANOVA ($F_{1,31} = 0.53$, $P = 0.47$), indicating that NR3A levels are similar in control and schizophrenic DLPFC. However, there was a significant main effect of sex ($F_{1,31} = 4.21$, $P < 0.05$) (Supplementary Fig. S3), suggesting that NR3A expression in the DLPFC is lower in females compared with males. Storage time and PMI were not statistically controlled because no significant differences were observed between men and women regarding these variables. Although these findings are intriguing, limited tissue availability precluded our ability to further explore the possible gender-related regulation of NR3A. To demonstrate that the lack of effect of NR1 and NR3A on schizophrenia was not a consequence of our standardization procedure, we also show similar results by standardizing band densities to loading controls, β-tubulin and GAPDH (see Supplementary Figs S4 and S5).

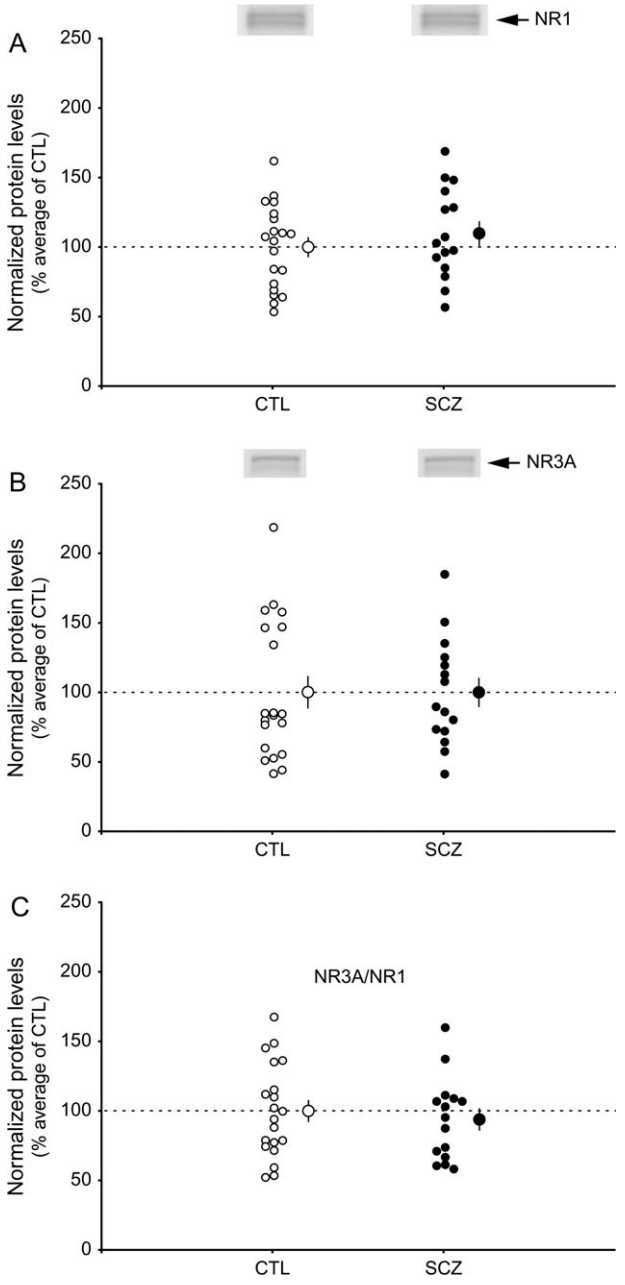

Figure 4. NMDAR subunit protein levels in control and schizophrenic subjects. Scatter plots showing (*A*) NR1, (*B*) NR3A, and (*C*) NR3A/NR1. Small circles represent data from individuals; large circles represent group means ± standard error of the mean. Representative immunoblots are shown in addition to averaged data. CTL, control (open circles); SCZ, schizophrenia (filled circles).

Antipsychotic Drugs Fail to Alter NMDAR Subunit Expression

Antipsychotic drugs are standard treatments for schizophrenic patients, primarily producing direct antagonistic effects on the dopamine and serotonin systems. For example, haloperidol, a typical antipsychotic, is a potent dopamine D2-like receptor antagonist, whereas clozapine, an atypical antipsychotic, blocks not only D2 but also serotonin receptors. Many drugs also interact directly with the glutamate system, binding to NMDARs (Ilyin et al. 1996; Gallagher et al. 1998) and affecting NMDAR expression and activity (Ossowska et al. 1999; 2000; Leveque et al. 2000; Schmitt et al. 2003; Bressan et al. 2005; O'Connor et al. 2006). It was therefore important that we examine how antipsychotics affect the expression of NMDAR subunits.

To evaluate the possibility that differences in NMDAR subunits between controls and schizophrenics might have been "normalized" by antipsychotic drug usage, we measured NMDAR subunit proteins in frontal cortical tissue from subchronic drug-treated rats (Jarskog et al. 2007) (Fig. 5). Adult rats were injected for 4 weeks with saline, haloperidol, or clozapine, and immunoblotting was used to establish protein levels from cortical homogenates. In our study, the drug

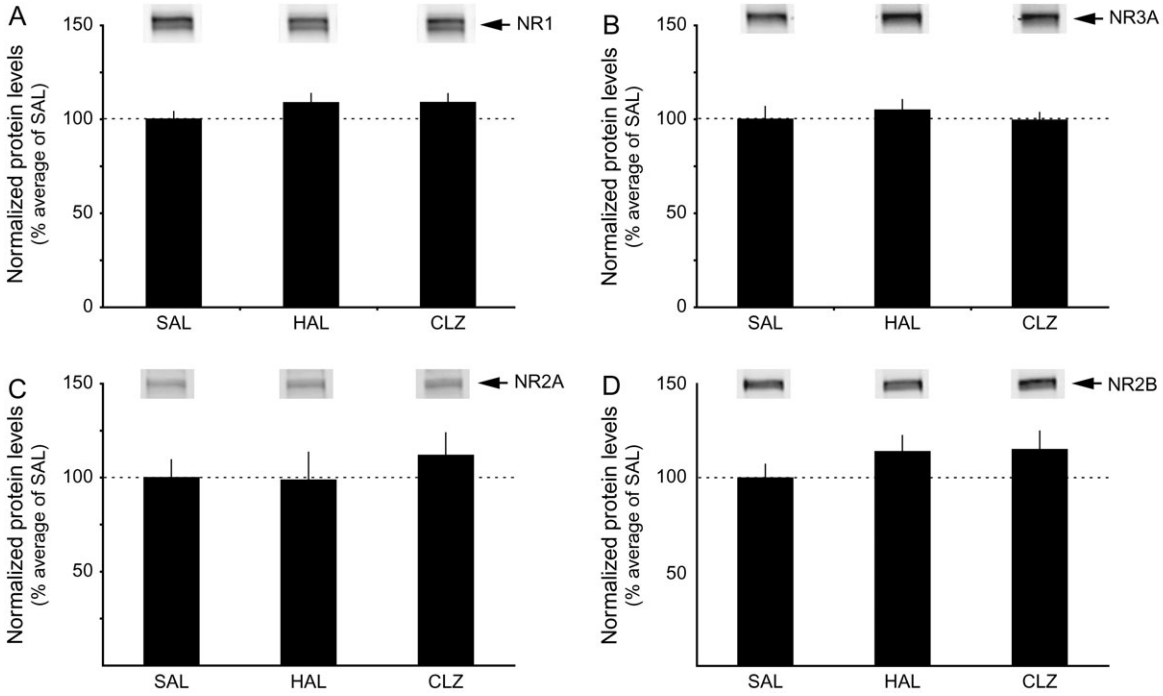

Figure 5. Antipsychotic drugs fail to alter NMDAR subunit levels, shown normalized to saline control values. Bar graphs depicting quantification of (*A*) NR1, (*B*) NR3A, (*C*) NR2A, and (*D*) NR2B in frontal cortical tissue from antipsychotic-treated rats. Data are presented as means ± standard error of the mean. SAL, saline; HAL, haloperidol; and CLZ, clozapine.

treatments failed to modify NMDAR subunit levels, as indicated by ANOVA (Fig. 5; $0.29 < F_{2,32} < 1.16$, $P > 0.32$ for all subunits), suggesting that antipsychotic treatments are unlikely to have affected NR1 and NR3A protein levels observed in schizophrenic patients.

Discussion

To our knowledge, this report provides the first evidence, in any region of the human brain, for age-dependent differences in NMDAR expression spanning the range from gestation to early adulthood. We performed quantitative analyses of NMDAR subunit mRNA and protein in postmortem human brain sections from the DLPFC. Specifically, we demonstrate 1) robust developmental regulation of NR3A and moderate developmental regulation of NR1, 2) close associations in abundance of NR3A and NR1 transcript and protein levels, and 3) strong parallels to previous findings in developing cortex in other mammalian systems. Our results show that NR3A levels are low prenatally, surge after birth, and then decrease progressively into adulthood. These data indicate that NR3A serves a prominent role in the development of the PFC soon after birth, and its role is likely less prominent prenatally and in adulthood. In contrast, NR1 levels rise from prenatal levels and vary only modestly over development, supporting a lifelong importance of NMDAR-dependent functions, including many forms of learning and memory. The defined expression patterns of these particular subunits will increase our understanding of NMDAR-mediated processes during ontogeny, will aid studies of NMDAR dysfunction, and will guide the rational design of subunit-specific NMDAR pharmacotherapies for neurological disorders.

Importance of Age-Dependent Changes in NMDAR Subunits to Normal Human Development

NMDAR subunits exhibit remarkable heterogeneity of expression. The significance of this molecular diversity is poorly understood in humans, in part due to a lack of knowledge of how these subunits change during development. Compared with the rich literature describing developmental regulation of NMDAR subunits in animal models, human developmental studies have been largely limited to investigations at the mRNA level (Law et al. 2003). Studies measuring mRNA must be interpreted cautiously because protein and mRNA levels are not always well correlated (Luo et al. 1996; Philpot et al. 2001). Thus, we felt it essential to accurately describe both NMDAR mRNA and protein levels in the human brain. By using quantitative RT-PCR (Mimmack et al. 2004) and infrared immunoblot imaging, we obtained highly sensitive detection of mRNA and protein levels from postmortem tissues. Additionally, the samples were binned into developmental age groups based on models of cortical development, which was a hypothesis-driven measure to enable us to overlay the data onto findings from prior investigations (Glantz et al. 2007; Salimi et al. forthcoming).

Although NR1 is obligatory for NMDAR function, the specific properties of NMDARs are shaped by the combination of NR1 with NR2 and/or NR3 subunits. NR3A is the most recently described NMDAR subunit and consequently its influence on NMDAR properties is less well defined compared with the NR2 subunits. However, interest in this unique subunit has grown recently with exciting observations that it acts in a novel, dominant-negative manner to reduce calcium influx and the unitary conductance of NMDAR currents (Ciabarra et al. 1995; Sucher et al. 1995; Perez-Otano et al. 2001; Sasaki et al. 2002;

Matsuda et al. 2003), thereby suppressing NMDAR function. Here, we show that in human PFC NR3A levels peak during early childhood and then decrease into adulthood. Animal studies have established a similar developmental pattern of NR3A expression (Ciabarra et al. 1995; Sucher et al. 1995; Das et al. 1998; Sun et al. 1998; Al-Hallaq et al. 2002; Sasaki et al. 2002; Wong et al. 2002; Ishihama and Turman 2006; Perez-Otano et al. 2006), suggesting that this might be a general feature of mammalian brain development.

What functions might high levels of NR3A have during early development? Based on in vitro and in vivo studies in animal models, at least 6 nonmutually exclusive possibilities exist for the function of NR3A during DLPFC development. 1) Given that genetic deletion of NR3A in mice increases spine density (Das et al. 1998), NR3A may regulate the formation of dendritic spines, the major sites of excitatory synapses. Indeed, massive spinogenesis and synaptogenesis in infancy and early childhood, as well as synapse elimination in adolescence, have been demonstrated in both human (Huttenlocher 1979; Bourgeois et al. 1994; Huttenlocher and Dabholkar 1997; de Graaf-Peters and Hadders-Algra 2006; Glantz et al. 2007) and nonhuman primate PFC (Rakic et al. 1986; Bourgeois et al. 1994; Anderson et al. 1995; Gonzalez-Burgos et al. 2007). 2) Because NR3A limits NMDAR-mediated calcium entry and its expression is elevated during a period of intense programed cell death, NR3A is positioned to actively influence apoptosis by attenuating calcium-mediated excitotoxicity (Lipton and Nakanishi 1999). On the other hand, because NMDAR antagonism can also lead to cell death (Ikonomidou et al. 1999), excessive NR3A levels could actually promote apoptosis. Thus, the relative balance of NR3A expression might serve to control which cells are targeted for cell death versus survival. 3) Calcium is a critical mediator of both long-term depression and potentiation, which are thought to be mechanistic substrates for learning and memory (Malenka and Bear 2004). NR3A-dependent control of calcium entry would be expected to dramatically shape the properties of NMDAR-mediated plasticity, which could be revealed in future studies through mutant mice that either lack or overexpress NR3A (Das et al. 1998; Sucher et al. 2003; Brody et al. 2005). 4) Exciting new data demonstrate that NR3A-containing receptors appear to undergo rapid endocytosis that is regulated in an activity-dependent manner by PACSIN1/syndapin1 (Perez-Otano et al. 2006). Thus, NR3A may be important for clearing immature synaptic NMDARs so that they can be replaced by more mature receptors. 5) NR3A specifically forms a signaling complex with PP2A (Chan and Sucher 2001), a phosphatase that can dephosphorylate NR1 subunits on serine 897. NR3A may indirectly modulate NMDAR function through this interaction, and thus provide bidirectional control of synaptic activity. 6) Uniquely, NR3A-containing receptors lack strong blockade by magnesium at hyperpolarized potentials (Ciabarra et al. 1995; Sucher et al. 1995; Das et al. 1998; Al-Hallaq et al. 2002; Sasaki et al. 2002). Thus, although speculative, a novel role for NR3A-containing NMDARs during early life might be to support synaptic transmission at "silent synapses" before there is an activity-dependent mobilization of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors to the synapse during maturation (Durand et al. 1996; Wu et al. 1996; Isaac et al. 1997; Rumpel et al. 1998; Zhu et al. 2000; Plitzko et al. 2001).

The observation that expression levels of the essential NR1 subunit increase progressively from prenatal stages and remain relatively high throughout life is perhaps not surprising as NMDAR function has been implicated in a variety of vital functions, including learning and memory, neuronal migration, synapse stabilization, pain perception, and neuronal cell death (Komuro and Rakic 1993 and reviewed in Malenka and Nicoll 1999; Cull-Candy and Leszkiewicz 2004). These data are consistent with that seen in other regions of the human brain (Law et al. 2003) and observations in animal models (Watanabe et al. 1992; Luo et al. 1996; Laurie et al. 1997; Chen et al. 2000; Sans et al. 2000; Ritter et al. 2002; Awobuluyi et al. 2003; Ontl et al. 2004; Petralia et al. 2005). NMDAR levels may also be particularly high in the DLPFC as the PFC may contain the highest concentration of NMDARs in the cortex (Scherzer et al. 1998). Notably, NMDAR-mediated functions are apparently crucial even for embryonic life because genetic deletion of NR1 in mice leads to neonatal lethality (Forrest et al. 1994).

Whereas, we observed clear developmental regulation of NR1 mRNA indicating a low prenatal level of expression compared with later developmental age groups, we observed a more modest increase in NR1 protein levels when comparing prenatal to maximum expression (11–15 years). However, when we performed a secondary analysis using PMI and storage time as covariates, this difference in protein levels failed to reach significance. Due to the modest developmental regulation of NR1 compared with other NMDAR subunits, such as NR3A, the detection of subtle differences in NR1 protein expression levels over development will require closer examination in future studies. We largely observed similar trends in mRNA and protein expression in both NR3A and NR1. We did, however, observe one inconsistency. Interestingly, whereas NR1 transcript levels remain high in adult PFC, protein levels drop significantly. Similar discrepancies between mRNA and protein levels have been noted and may result from translational inefficiency or increased rates of protein degradation (Awobuluyi et al. 2003; VanDongen AM and VanDongen HM 2004).

The abundant NR1 protein levels observed during childhood are present during a period of extensive development of neuronal processes and formation of synaptic connections (Webb et al. 2001de Graaf-Peters and Hadders-Algra 2006). Why does NMDAR expression peak around early adolescence in the DLPFC? The elevated NR1 levels may provide a molecular substrate for robust synaptic plasticity in cortical regions at this time. The intriguing reductions in NR1 protein expression after puberty parallel the loss of synapses observed in human PFC and are consistent with a role for NMDARs in synaptic pruning or elimination in humans (Huttenlocher and Dabholkar 1997; Glantz et al. 2007) and in nonhuman primates (Bourgeois et al. 1994; Gonzalez-Burgos et al. 2007). Additionally, this might also be associated with critical period closure for many forms of NMDAR-mediated plasticity (Malenka and Bear 2004).

Currently little is known about NR3A in humans, but the high level of homology between human and rodent NR3A (93%) (Andersson et al. 2001; Eriksson et al. 2002) and the strong overall relationship between NMDAR subunit mRNA and protein levels found in this study suggest its function is likely similar between mammalian species. To observe NMDAR developmental changes without the issues of postmortem degradation, we ran a parallel study in cortical tissue from rhesus macaque, a close genetic relative of humans (Gibbs et al. 2007), and found broad similarities to the human studies (Supplementary Fig. S1). Most importantly, the early peak in

NR3A protein expression that tapers into maturity coincides with the developmental loss of NR3A in humans and rodents. Thus, despite the unavoidable caveats associated with studying human postmortem tissue, the similarities in the combined human transcript and protein data and monkey protein results produced from this study strengthen our conclusions and support the view that NMDAR subunits are similarly modified across development in rodents, nonhuman primates, and humans.

We initially sought to characterize the subunits in postnatal cortex: NR1, NR2A, NR2B, and NR3A (Watanabe et al. 1993; Monyer et al. 1994; Sheng et al. 1994; Stocca and Vicini 1998). Because all proteins are subject to decay after death, we first established our ability to quantify NMDAR subunit levels in postmortem tissue using immunoblotting (see Materials and Methods). Although >65% of NR1 and NR3A remained intact up to a 24 h PMI, less than 50% of the NR2A and NR2B levels could be detected. This was consistent with our difficulty, and that of others, in reliably measuring NR2A and NR2B levels in human and nonhuman primate tissue (Wang et al. 2000; O'Connor et al. 2006). Although we observed rapid decay of NR2A and NR2B with these particular antibodies (see Materials and Methods), protein degradation may differ among epitopes or tissue preparation techniques. As such, antibodies or approaches may exist that are better suited for detecting NR2A and NR2B levels in tissue with long PMIs (see Murphy et al. 2005; Kristiansen et al. 2006). Due to our difficulties in detecting NR2A and NR2B levels, we focused on NR1 and NR3A.

Relevance of NR1 and NR3A Expression to Schizophrenia

Because NMDAR hypofunction has been hypothesized to underlie some of the cognitive deficits observed in schizophrenia (Javitt and Zukin 1991; Jentsch and Roth 1999; Olney et al. 1999; Lewis and Levitt 2002; Coyle et al. 2003; Frankle et al. 2003), we asked whether this could be due to alterations in NMDAR subunit proteins within the DLPFC. Reports of subunit modifications in schizophrenia are largely conflicting (reviewed in Kristiansen et al. 2007). Our data reach the same conclusions as Kristiansen et al. (2006), indicating that NR1 protein levels are unchanged in the schizophrenic brain. Subtle changes in NR1 splice-variant expression (Prybylowski and Wolfe 2000; Magnusson et al. 2005) might account for alterations of the subunit but may not be detectable in our system.

Recent data raised the intriguing possibility that NMDAR hypofunction in schizophrenia might arise from an aberrant increase in NR3A expression, as NR3A suppresses NMDAR function and NR3A mRNA transcript levels are significantly elevated in the DLPFC of schizophrenic patients (Mueller and Meador-Woodruff 2004). In this same region where NR3A is overexpressed, spine densities are reduced in schizophrenic patients (Glantz and Lewis 2000). Because NR3A likely has a strong influence on limiting spine density (Das et al. 1998), a lack of the normal developmental downregulation of NR3A could result in significant reductions in spine numbers. Therefore, it is appealing to hypothesize that NR3A levels are elevated in schizophrenics, as this could explain both the NMDAR hypofunction and the reductions in dendritic spine density that have been observed.

However, our data provide the first evidence that schizophrenia is not associated with a gross change in NR3A at the protein level within the DLPFC. Given that NR3A expression is normally very low in the adult human brain (this study) and that subregion- and lamina-specific differences in the expression of NR3A exist (Mueller and Meador-Woodruff 2004; 2005; Bendel et al. 2005), we cannot rule out the possibility that there may be subtle or region-specific differences in NR3A proteins that our methods were unable to detect. A more selective analysis of synaptic membranes from different regions and/or laminae have the potential to reveal significant differences in receptor protein expression in the schizophrenic brain, if such differences exist.

Even in the absence of differences in adult NR3A protein levels, other possibilities for NMDAR hypofunction involving NR3A may exist. Genetic variants of NR3A could provide a molecular substrate for abnormal NR3A function (Gallinat et al. 2007), particularly relevant for prefrontal information processing. As schizophrenia is considered a neurodevelopmental disorder, a transient increase of NR3A during development could disturb the normal formation of cortical circuits yet not be apparent in the adult brain (Lewis 1997). Because antipsychotic drugs can alter NMDAR subunit expression in a region-specific manner (Fitzgerald et al. 1995; Hanaoka et al. 2003; Schmitt et al. 2003; O'Connor et al. 2006), we explored the possibility that drug treatments might account for the normal levels of NR3A and NR1 we found in schizophrenic patients. After modeling the effects of subchronic clozapine and haloperidol exposure in rodent frontal cortex, we found that NMDAR subunit expression is unchanged, consistent with previous observations (Hanaoka et al. 2003). Although we cannot preclude the possibility that different antipsychotic treatment regimens might alter NMDAR protein levels, our data suggest that normal NR1 and NR3A protein levels in the DLPFC of schizophrenic patients are unlikely to be consequences of antipsychotic treatments. This suggests either that overall NMDAR hypofunction in schizophrenia is not related to widespread changes in NMDAR proteins or that such deviations fail to be maintained throughout life in this brain region. We argue that the NMDAR hypofunction observed in the disease is not maintained by gross differences in total NMDAR number or the proportion of NR3A-containing NMDARs, although there may be more subtle laminar or regional effects.

In light of reports of gender influences in normal NMDAR expression as well as in schizophrenia, we examined possible gender differences in NR1 and NR3A. We observed marginal significance for sex-based differences suggesting that females have significantly less NR3A and a trend for less NR1 than their male counterparts. The meaning of these results in human DLPFC is unknown. However, there is a precedent for gender-specific differences in NR1 expression within other regions of the brain (Shi and Schlenker 2002; Ontl et al. 2004), suggesting that NR1 levels are differentially regulated by sex hormones (Gazzaley et al. 1996; McEwen 2002; McEwen 2002). Like NR1, NR3A levels might also be regulated by sex hormones, such as estrogen. Indeed, genetic deletion of NR3A impairs prepulse inhibition, a measure of sensorimotor gating, in male but not female mice (Brody et al. 2005). This finding suggests that estrogen might normally downregulate NR3A levels, thus mirroring effects of genetic NR3A deletion in mice. Such an interpretation would be consistent with our observation that NR3A, and possibly NR1, levels are lower in the DLPFC of females compared with males. It might also explain the observation that there is a sex-specific difference in prepulse

inhibition in normal human subjects (Swerdlow et al. 1993). Interestingly, schizophrenics are known to exhibit deficits in prepulse inhibition to startle (reviewed in Braff et al. 2001), suggesting a potential link between sex-specific NMDAR subunit expression and the schizophrenic condition. Although we stress the preliminary nature of these gender-related observations, the possibility for reduced expression of NR3A in females warrants more intense scrutiny, as it could provide a biological basis to explain why female schizophrenic patients tend to manifest symptoms at a later age, are more responsive to medications and exhibit less severe symptoms than males (Castle and Murray 1991).

Although we attempted to carefully address the confounding factors that are inherent to all human postmortem studies, one of the main limitations to such investigations is the relatively small numbers of postmortem brain samples available. However, even after considering such factors as PMI, pH, sex, ethnicity, and storage time on the quality of the mRNA and proteins studied, we still found significant developmental changes in NMDAR subunits, suggesting that the increases and decreases in expression patterns are due to robust developmental regulation. The observed statistical differences in developmental NMDAR subunit levels likely represent the most dramatic and consistent age-dependent differences in subunit levels. Analyses of additional tissue might reveal more subtle developmental effects. It is important to note that the current study represents the first developmental analysis of NMDA receptors in human cortex from gestation to adulthood. Developmental studies in human brain of key regulatory systems—including glutamate—are needed to advance our understanding of normal brain development and provide insights into the pathophysiology of neurodevelopmental disorders such as schizophrenia.

In summary, we report the developmental and schizophrenia profiles of NMDA receptor subunits, NR1 and NR3A, in human PFC. The data are consistent with lifelong functional roles for NR1 and a particularly important role during early human brain development for NR3A. Our results also suggest that there are no gross differences in NR1 and NR3A protein levels between schizophrenic and control DLPFC. Collectively, this study will be relevant in understanding subunit functions in key NMDAR-mediated processes of ontogeny, such as the formation and refinement of cortical circuits. Additionally, these results contribute to understanding subunit roles in disorders of NMDAR dysfunction, such as the basis for NMDAR hypofunction in schizophrenia, as well as subtype-specific targeting in drug development.

Supplementary Material

Supplementary figures S1–S5 can be found at: http://www.cercor.oxford journals.org/.

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Notes

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