SYNAPSE 63:836–846 (2009)

Increased Expression of the NR2A NMDA Receptor Subunit in the Prefrontal Cortex of Rats Reared in Isolation

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KEY WORDS animal model; prepulse inhibition; schizophrenia; isolate

ABSTRACT A hypofunction of the *N*-methyl-D-aspartate (NMDA) receptor has been implicated in the pathophysiology of schizophrenia. Compelling evidence of altered NMDA receptor subunit expression in the schizophrenic brain has not, however, so far emerged. Rats reared in isolation exhibit several characteristics, including disturbed sensory gating, which resemble those seen in schizophrenia. To explore the possibility that NMDA receptor dysfunction may contribute to the behavioral and neurochemical consequences of rearing rats in isolation, we compared NMDA receptor subunit expression in brains of rats which were housed in isolation and which displayed a deficit in prepulse inhibition of the acoustic startle response with that of socially housed controls. An initial microarray analysis revealed a 1.26-fold increase in NR2A transcript in the prefrontal cortex, but not in the nucleus accumbens, of rats reared in isolation compared with those housed socially. In contrast, NR1, NR2B, NR2C, NR2D, NR3A, and NR3B subunit expression was unchanged in either brain area. In a second cohort of animals, in situ hybridization revealed increased NR2A mRNA expression in the medial prefrontal cortex, an observation that was substantiated by increased [^{3}H]CGP39653 binding suggesting that NR2A receptor subunit protein expression was also elevated in the medial prefrontal cortex of the same animals. No changes in expression of NR1 or NR2B subunits were observed at both mRNA and protein level. Altered NR2A subunit expression in the medial prefrontal cortex of rats reared in isolation suggests that NMDA receptor dysfunction may contribute to the underlying pathophysiology of this preclinical model of aspects of schizophrenia. **Synapse 63:836–846, 2009.**

INTRODUCTION

A hypofunction of the *N*-methyl-D-aspartate (NMDA) receptor has been implicated in the pathophysiology of schizophrenia (see Coyle, 2006; Stone et al., 2007 for recent reviews). This hypothesis originated with the observation that pharmacological blockade of the NMDA receptor ion channel by low doses of dissociative anesthetics such as phencyclidine (PCP) and ketamine exacerbates symptoms in stable schizophrenic patients and induces a syndrome in healthy individuals that bears many of the hallmarks of schizophrenia symptomatology (see Javitt, 2007 for a recent review). The concept was further supported by indications that the efficacy of antipsychotic drugs, particularly versus negative and cognitive symptoms, is enhanced when coadministered with agonists of the NMDA receptor glycine site (Javitt, 2007). In addition to this pharmacological evidence, genetic evidence has highlighted several schizophrenia susceptibility genes that converge on NMDA receptor-mediated signaling (Harrison and West, 2006). Furthermore, it has recently been demonstrated that enhanced signaling of the susceptibility gene neuregulin 1 via the

Contract grant sponsor: MRC Industrial Collaborative Studentship (to J.J.T.-J.) with GlaxoSmithKline.

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Received 24 September 2008; Accepted 23 December 2008

DOI 10.1002/syn.20665

Published online in Wiley InterScience (www.interscience.wiley.com).

receptor tyrosine kinase erbB4 may directly suppress NMDA receptor function (Hahn et al., 2006).

Although these strands of evidence are strongly indicative of a hypofunction of NMDA receptor-mediated neurotransmission in the schizophrenic brain, postmortem evidence of a direct effect of the disease on NMDA receptor subunit expression which may explain the deficit is less compelling. For example, mRNA and protein expression of the obligatory NR1 subunit, which contains the binding site for the coagonist glycine, is reported to be increased, decreased, or unchanged (see Kristiansen et al., 2007 for recent review) in different regions of the cerebral cortex. While postmortem studies have generally yielded modest and contradictory results, it is important to note that patient and symptom heterogeneity, drug-induced effects, methodological differences including postmortem delay, and the effects of drug treatment may all conspire to obscure subtle but potentially meaningful alterations in NMDA receptor subunit expression.

A complementary approach to gain a better understanding of the role of NMDA receptor subunit expression in schizophrenia is via the utilization of animal models of the disease. Of course, no animal model recapitulates the entire symptomatology of schizophrenia but certain restricted aspects of the disease may be expressed in rodents. Rearing rats from weaning in isolation induces certain behavioral, neurochemical, and anatomical changes that parallel those observed in schizophrenia. Behaviorally, isolation-reared rats exhibit sensorimotor gating deficits, as determined by prepulse inhibition (PPI) of the acoustic startle response (Cilia et al., 2001; Geyer et al., 1993; Weiss et al., 2001; Wilkinson et al., 1994). Also, in light of the recognition that cognitive impairment is a core symptom domain in schizophrenia, it is significant that isolation-reared rats exhibit deficits in a number of cognitive paradigms (Bianchi et al., 2006; Dalley et al., 2002; Li et al., 2007; McClean et al., in press; Weiss et al., 2001). Importantly, isolation rearing induced behavioral changes are developmental in origin (Bakshi and Geyer, 1999; Ellenbroek and Cools, 1990; Wilkinson et al., 1994) and are ameliorated by antipsychotic drugs (Cilia et al., 2001; Li et al., 2007; Varty and Higgins, 1995). Neurochemically, monoamine neurotransmitter systems are altered in the brains of isolation-reared rats, including the elevation of extracellular dopamine (DA) concentrations in the nucleus accumbens (Acb) that resemble changes observed in schizophrenia (Swerdlow and Geyer, 1998). Finally, the anatomical consequences of isolation-rearing that mimic schizophrenia include decreased volume (Day-Wilson et al., 2006), lower dendritic spine density (Silva-Gomez et al., 2003), and a loss of GAT-1 immunopositive GABA-ergic chandelier cartridges (Bloomfield et al., 2008) in the prefrontal cortex (PFC). Thus, with this model exhibiting construct, predictive and face validities of certain aspects of schizophrenia, we utilized the multiple approaches of microarray analysis, in situ hybridization, and radioligand autoradiography to examine NMDA receptor subunit expression in brains of rats reared in isolation with the aims of further validating isolation rearing as a relevant model of schizophrenia and, indirectly, to gain greater insight into the possible role of altered NMDA receptor subunit expression in the disease.

MATERIALS AND METHODS

Animals and rearing conditions

All behavioral work was conducted in compliance with the Home Office Guidance under the UK Animals (Scientific Procedures) Act 1986. As previously described (Cilia et al., 2001), male Lister Hooded rats (Charles River) arrived with foster mothers at 8–9 days old. At 28 days of age, rats were rehoused in isolation or in groups of five using a pseudorandom schedule to balance for weight and litter. All animals were housed in the same room under constant temperature (21°C) and humidity (50–58%), a 12-hour light/dark cycle (lights on 0600 hours), and free access to food (Harlan) and water. Rats were maintained in these conditions for at least 8 weeks before testing during which time handling associated with husbandry and all noise was kept to a minimum. A total of 44 rats in two cohorts, each consisting of 12 isolation and 10 socially reared animals, were employed in these studies.

Testing of prepulse inhibition (PPI)

PPI was measured using computer-controlled startle chambers housed in sound-attenuated boxes (Instrument Design Technologies IDT, GlaxoSmithKline) fitted with a sprung metal grid floor and a loudspeaker capable of delivering 115 decibels (dB). Socially and isolation-reared rats were tested within the same period. The startle response of the rat was measured as the magnitude of deflection of the grid floor, recorded by an accelerometer. Rats were placed in the startle chambers with 70 dB of background noise and, after 10 minutes habituation, were subjected to one pulse alone trial (110 dB for 50 ms) followed by 24 trials of either pulse alone or prepulse (75 dB white noise for 30 ms) followed by pulse with an interstimulus interval (ISI) of 100 ms. Trials were separated by 15 seconds. PPI, defined as the percentage reduction in startle amplitude when the pulse was preceded by a prepulse compared with that elicited by pulse alone, was calculated as follows: % PPI = 100 − (prepulse + pulse/pulse alone) × 100. PPI data were analyzed using Student's t test. To ensure

that any deficit was stable, PPI was evaluated up to three times, with each test session separated by at least 10 days. For a more detailed description of the isolation rearing and PPI determination protocols, see Cilia et al. (2001, 2005).

Microarray

After the completion of PPI testing, animals comprising the first cohort of socially ($n = 10$) and isolation-reared ($n = 12$) animals (Cohort 1) were decapitated, their brains removed, and the PFC (anterior portion of the frontal cortex up to 2.15 mm rostral from bregma, Paxinos and Watson, 1997) and nucleus accumbens (Acb, punched from 2 mm slice approximately 2.15 mm − 0.10 mm rostral from bregma) dissected from both hemispheres. All samples were stabilized in RNA Later reagent (Qiagen, Valencia, CA) overnight at 4°C before removal of reagent and storage at −80°C. Total RNA from approximately 5–15 mg Acb and 50–90 mg PFC tissue was extracted into TriZol reagent (Invitrogen, Paisley, UK; 1 ml TriZol per 20 mg tissue) with the aid of a mechanical homogenizer (YellowLine D1 25 Basic) and then purified using RNeasy mini-columns (Qiagen, Valencia, CA) including on-column DNAse-1 step and elution in water. The quantity of extracted RNA was determined by spectrophotometry and quality was assessed using an Agilent 2100 Bioanalyzer (South Plainfield, NJ). All samples showed clear 28S and 18S rRNA peaks and limited degradation. Three micrograms (Acb) or 5 μg (PFC) of total RNA was processed to biotin-labeled cRNA and hybridized to Rat230_2.0 GeneChips® (containing 31,099 probe sets) in accordance with the supplier's protocol (Affymetrix, Santa Clara, CA). Arrays were scanned on a GeneChip Scanner 3000s and fluorescence intensity for each feature of the array was obtained by using GeneChip® Operating Software (Affymetrix). Samples from individual rats were hybridized to individual Rat230_2.0 arrays, giving a total of 44 arrays for the study. Quality control criteria, as defined by Affymetrix, were assessed. Background intensities for the microarray study were in the range 40–70 intensity units, the average percentage of probe sets on the array defined as detected (percent present) was 56% for PFC and 52% for Acb, and the average 3′/5′ ratios for GAPDH were 1.18 for PFC and 1.85 for Acb.

The raw signal intensities for each scan were imported into the gene expression analysis software (Resolver version 5.0, Rosetta Biosoftware, Seattle, Washington). Signal extraction was performed by application of the Resolver Affymetrix-specific error model (Weng et al., 2006). The differential expression of genes in isolation versus group-reared rats was determined by one-way ANOVA. Fold changes and corresponding P values were calculated for each probe set. Probe sets were excluded if representing potential alternative or antisense transcription or assigned to UTR sequences.

In situ hybridization

After the completion of PPI testing, animals comprising the second cohort of socially and isolation-reared animals (Cohort 2) were decapitated and their brains removed and snap-frozen in isopentane cooled to −50°C. Coronal sections (14 μm) between bregma +3.70 mm and −3.30 mm (Paxinos and Watson, 1997) were prepared using a cryostat (Leica, Wetzlar, Germany) and sequentially mounted onto electrostatically charged microscope slides. Sections from the brains of the same isolation and socially reared rats were used for both in situ hybridization and radioligand binding autoradiography.

The following antisense oligonucleotide probes (two per subunit, each with a GC basepair content of 50–65%) were designed to rat NR1, NR2A, and NR2B subunits: NR1 (Ref.Seq ID NM_0170 10), CTGGGT CAAACTGCAGCACCTTCTCTGCCTTGGACT (bases 828–863), ATTGTAGATGCCCACTTGCACCAGCTTG CGGTTCT (bases 1335–1369); NR2A (Ref.Seq ID NM_0125 73), TCTGTCACGTCGTGGCTGTGACC-CAGCAGCACCGCAAT (bases 195–232), GCTTTCCC TTTGGCTAAGTTTCTGTTGTATCCAACAGG (bases 1830–1867); NR2B (Ref.Seq ID NM_0125 74), ATCTC AGGGTGTGGAGAGCAGCTCACAATGCAGAA (bases 82–116), TCTGCACAGGTACGGAGTTGTTAAACAC-CAGACCCCAGAG (bases 2175–2214). Lyophilized oligonucleotide probes (Sigma Genosys, Haverhill, UK) were rehydrated in Tris-EDTA buffer and 3′ end labeled with [α-^{35}S]dATP (GE Healthcare, Little Chalfont, UK) using terminal deoxytransferase (Roche, Burgess Hill, UK). Unincorporated nucleotides were removed from the labeled oligonucleotide probes using Quick Spin columns (Roche). Tissue sections were initially fixed in 4% paraformaldehyde, rinsed in 0.1 M phosphate buffered saline followed by 0.1 M triethanolamine (TEA) containing 0.25% acetic anhydride, then incubated with 1 ng ^{35}S-labeled oligonucleotide probe in 100 μl hybridization buffer (4× sodium chloride-sodium citrate (SSC), 1× Denhardt's solution, 10% dextran sulfate, 50% deionized formamide, 0.1 mg/ml denatured salmon testis DNA, 100 mM fresh dithiothreitol) at 43°C for 16 hours. Control sections were hybridized with 1 ng labeled probe in the presence of a 100-fold excess of unlabeled probe (100 ng/μl). Slides were washed in 4×, 2×, and 1× SSC at 50°C followed by two rinses in 0.1 M PBS at room temperature. Sections were then dehydrated for 1 minute each in 70%, 90%, and 100% ethanol. Dried sections were exposed to BioMax MR film (Kodak) alongside a set of ^{14}C plastic standards (American Radiolabeled Chemicals, St. Louis). Com-

TABLE I. Radioligand binding autoradiography protocols[a]

Radioligand (NMDA receptor binding site)	Preincubation conditions	Incubation conditions	Unlabelled competing ligand	Postincubation washes
[^{3}H]MK801 (channel pore)	50 mM Tris-HCl, pH 7.5, 30 minutes, RT	75 nM [^{3}H]MK801 in preincubation buffer, 60 minutes, RT	100 μM MK801	3 × 10 minutes in preincubation buffer
[^{3}H]MDL105,519, (glycine site, NR1 subunit)	50 mM Tris-HCl, pH 6.0, 30 minutes, RT	50 mM [^{3}H]MDL105,519 in pre-incubation buffer, 60 minutes, RT	1 mM gavestinel	3 × 1 minutes in ice-cold preincubation buffer
[^{3}H]CGP39653 (NR2A subunit-preferring)	50 mM tris-acetate, pH 7.6, 30 minutes, 0°C, then 20 minutes, 30°C	100 nM [^{3}H]CGP39653 in preincubation buffer, 30 minutes, 0°C	100 μM glutamate	3 × 30 seconds in ice-cold preincubation buffer
[^{3}H]Ro25-6981 (NR2B subunit)	50 mM Tris-HCl + 10 mM EDTA, pH 6.0, 30 minutes, RT	30 nM [^{3}H]Ro25-6981 in 50 mM Tris-HCl + 10 μM 1,3-di-tolylguanidine, pH 7.5, 90 minutes, 4°C	10 μM CP101606	3 × 5 minutes in ice-cold incubation buffer (minus EDTA)

RT, room temperature.
[a][^{3}H]MK801 (17.1 Ci/mmol), [^{3}H]CGP39653 50 Ci/mmol), and [^{3}H]Ro25-6981 (50 Ci/mmol) were obtained from Perkin Elmer and [^{3}H]MDL105,519 (71 Ci/mmol) from GE Healthcare.

puter-assisted image analysis of digitized autoradiographic images was performed using MicroComputer Imaging Device software (MCID elite 6.0, MCID) and expression (nCi/g) within predefined areas calculated from relative optical density via a standard curve. Total hybridization signal for each region was the average of four determinations performed in both hemispheres of two adjacent sections with nonspecific signal determined in both hemispheres of a third section and subtracted from total signal to reveal specific hybridization.

Radioligand receptor autoradiography

Radioligand receptor autoradiography was performed on cryosections of isolation and socially reared rats as described using protocols described in Table I. Binding of each radioligand was quantified by direct beta particle imaging (BetaImager™, Biospace Measures, Paris). Total radioligand binding (expressed as cpm/mm^2) for each radioligand was the average of four determinations performed in the region of interest (ROI) in both hemispheres of two sections per animal. Nonspecific binding, the average of (two) determinations performed in a third adjacent section, was subtracted from total binding to yield an estimate of specific binding for each region of each animal.

In situ hybridization and radioligand autoradiography data were analyzed using a repeated measures ANOVA, with repeated factor brain subregions, followed by planned comparisons on the predicted (least square) means using SAS PROC MIXED (version 9.1, SAS Institute) software. Significance (set at 5%) was confirmed using the Hochberg adjustment in order to account for the risk of false positive results when making multiple comparisons.

RESULTS

Prepulse inhibition (PPI)

Tissues from two cohorts of isolation and socially reared rats were used in these studies. In the first cohort (Cohort 1), employed for the analysis of NMDA receptor subunit transcript expression by microarray, the PPI of rats reared in isolation (5.8 ± 8.2%, n = 12) was significantly lower (P = 0.008) than that of socially housed controls (40.9 ± 6.3%, n = 10) (Fig. 1A). Similarly, in the second cohort (Cohort 2), employed for both in situ hybridization and radioligand autoradiography analyses, the PPI of rats reared in isolation (20.7 ± 7.0%, n = 12) was significantly lower (P = 0.004) than that of group-housed rats (42.6 ± 8.2%, n = 10) (Fig. 1C). Isolation rearing had no effect on the startle response in either case (Figs. 1B and 1C).

Microarray

The quality of RNA recovered from the prefrontal cortex (PFC) and nucleus accumbens (Acb) of Cohort 1 was excellent with average recoveries of 1.43 and 0.82 μg RNA/mg tissue, respectively. With a statistical threshold of $P < 0.05$, 1051 (PFC) and 939 (Acb) probe sets differentiated between animals of Cohort 1 that were housed socially or in isolation. Focusing only on NMDA receptor subunits, and excluding probe sets representing potential alternative or antisense transcription or designed to UTR sequence, eight GeneChip® probe sets were identified that represented known NR1, NR2, and NR3 subunits (see Table II). A significant (P = 0.034) 1.26-fold increase in expression of NR2A transcript was detected in the PFC but not Acb of rats reared in isolation compared with those housed socially (Table II). No significant differences in probe sets representing NR1, NR2B, NR2C, NR2D, NR3A, or NR3B subunits were detected in either brain region as a result of isolation rearing (Table II).

In situ hybridization

Preliminary studies revealed that, when hybridized to control rat brain sections, the two oligonucleotide probes complementary to different parts of each sub-

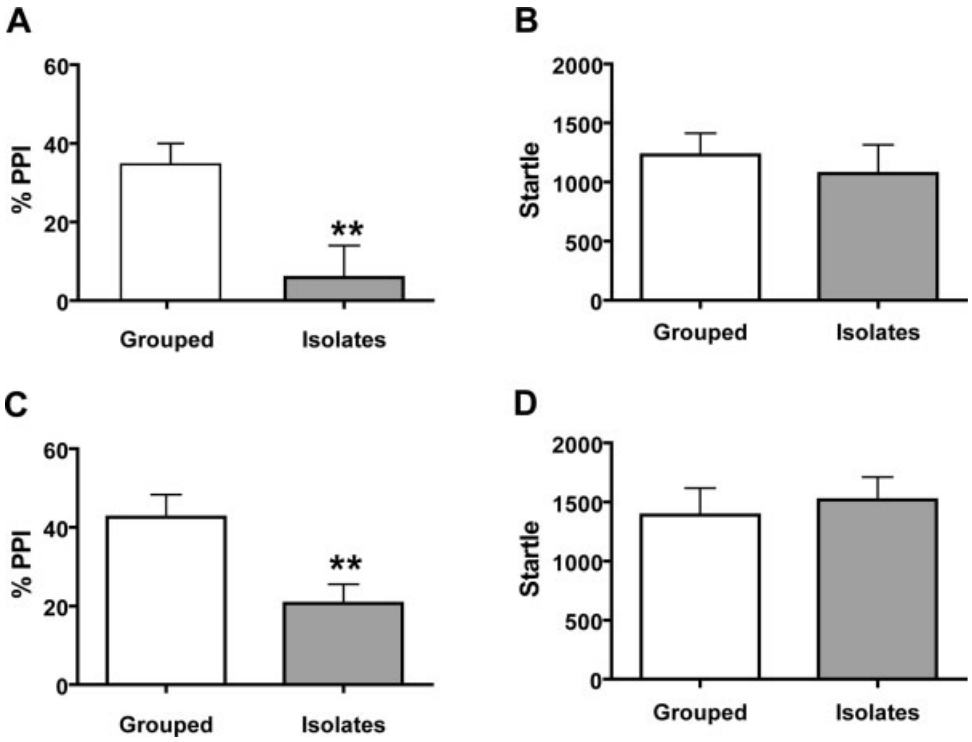

Fig. 1. PPI responses and startle reactivity of two cohorts of rats reared in isolation and socially. PPI (**A**) and startle response (**B**) of Cohort 1 isolation ($n = 12$) and socially ($n = 10$) housed animals (used for microarray analysis). PPI (**C**) and startle response (**D**) of Cohort 2 isolation ($n = 12$) and socially ($n = 10$) housed animals (used for in situ hybridization and radioligand autoradiography analyses). Data are presented as mean ± SEM % PPI or startle amplitude. $^{**}P < 0.01$.

TABLE II. Affymetrix GeneChip® microarray comparison of NMDA subunit transcript expression in brains of rats reared socially and in isolation[a]

			PFC		Acb	
Subunit	Gene name	Sequence code	Fold change	*P* value	Fold change	*P* value
NR1	GRIN1_RAT	1368572_a_at	1.01	0.847	−1.05	0.581
NR2A	GRIN2A_RAT	1368985_at	**1.26**	**0.034**	−1.03	0.931
NR2B	GRIN2B_RAT	1369745_at	1.04	0.536	−1.02	0.863
NR2C	GRIN2C_RAT	1368306_at	−1.14	0.147	1.02	0.799
NR2D	GRIN2D_RAT	1368950_a_at	−1.10	0.633	−1.09	0.446
NR2D	GRIN2D_RAT	1368951_at	−1.05	0.287	1.04	0.759
NR3A	GRIN3A_RAT	1370601_a_at	1.07	0.558	−1.05	0.794
NR3B	GRIN3B_RAT	1387559_at	−1.13	0.363	1.05	0.549

PFC, prefrontal cortex; Acb, nucleus accumbens.
[a]Data are presented as mean fold change in hybridization signal in PFC and Acb of rats reared in isolation ($n = 12$) compared with rats reared socially ($n = 10$). Significant ($P < 0.05$) change is highlighted in bold text.

unit transcript produced autoradiographic distribution patterns that were essentially the same and in good agreement with published distribution patterns in rat brain (Wisden et al., 2000). Therefore, each pair of oligonucleotide probes was used in tandem in in situ hybridization experiments performed on cryosections of Cohort 2 rat brains.

NR1 mRNA expression was widespread in cortical and subcortical regions with high levels of hybridization in the hippocampal CA1-3 pyramidal cell layer, the granule cell layer of the dentate gyrus (DG), striatal regions, and all areas of the cerebral cortex (Figs. 2A–2C). No difference in NR1 subunit mRNA expression was detected between isolation reared and group housed animals of Cohort 2 in any of the 14 separate regional analyses (Table III). NR2A expression was highest in the hippocampal pyramidal (CA1-3) cell and DG granule cell layers with more modest expression levels in regions of the cerebral cortex and low levels of expression in striatal regions (Figs. 2D–2F). A comparison of hybridization signal in tissues obtained from rats of Cohort 2 (Table III) revealed that NR2A subunit mRNA expression was significantly greater ($P < 0.01$) in the infralimbic, prelimbic,

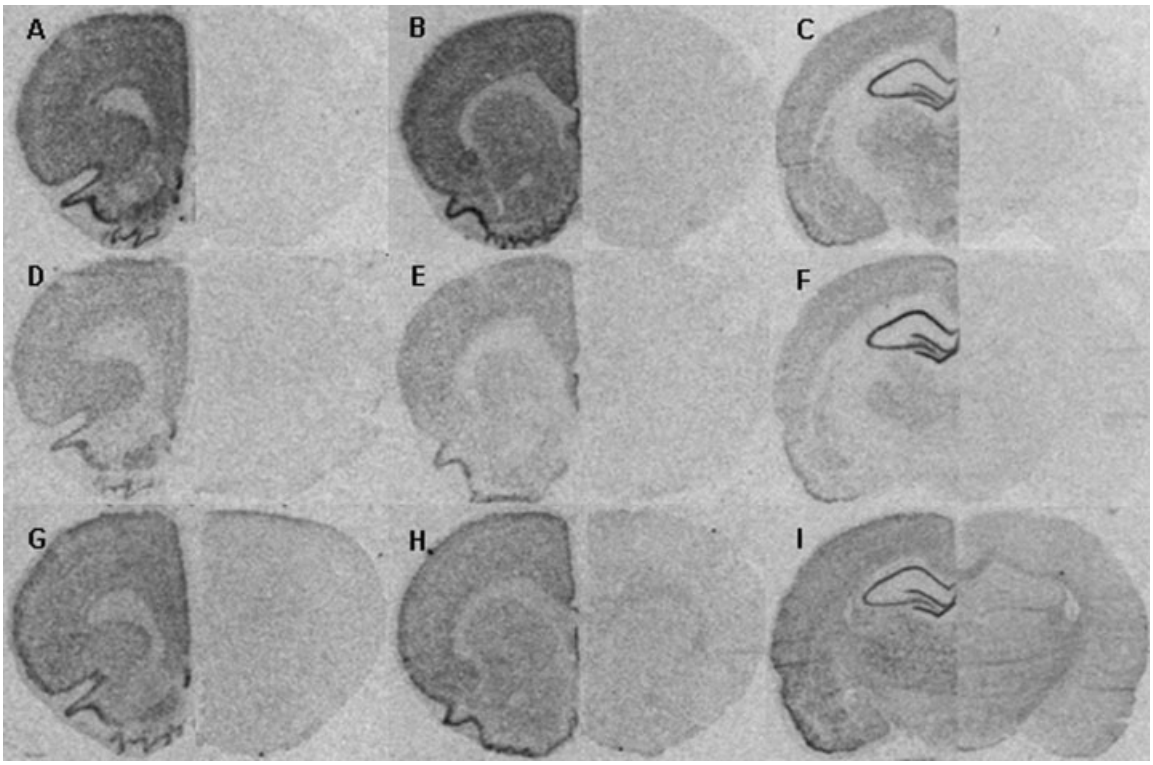

Fig. 2. NMDA receptor subunit mRNA expression in rat brain. Representative in situ hybridization autoradiographs of coronal rat brain sections prepared at bregma +3.00 mm (**A**, **D**, **G**), +1.70 mm (**B**, **E**, **H**), and −3.14 mm (**C**, **F**, **I**) hybridized with [α^{35}S]dATP labeled oligoprobes to NR1 (A–C), NR2A (D–F), and NR2B (G–I) subunits. Sections in the right half of each image were incubated with radiolablelled oligoprobe in the presence of an excess of unlabeled probe.

TABLE III. NR1, NR2A, and NR2B subunit mRNA expression in brains of isolation-, and socially-reared rats[a]

	NMDA receptor subunit mRNA expression (nCi/g)					
	NR1		NR2A		NR2B	
Region	Social	Isolate	Social	Isolate	Social	Isolate
CC	107.0 ± 4.6	109.1 ± 3.4	13.7 ± 1.1	18.8 ± 1.3*	30.8 ± 2.1	33.7 ± 1.1
PrL	113.2 ± 4.8	113.1 ± 3.2	13.4 ± 1.0	16.0 ± 1.2*	35.2 ± 1.6	37.5 ± 1.5
IL	110.5 ± 5.3	111.0 ± 3.4	10.9 ± 0.9	14.8 ± 1.0*	36.9 ± 1.8	40.9 ± 1.7
RS	82.4 ± 3.3	73.9 ± 4.4	13.9 ± 1.1	16.3 ± 0.5	12.6 ± 1.0	12.3 ± 1.4
CP	82.1 ± 4.0	79.1 ± 3.6	6.0 ± 0.3	7.2 ± 0.7	19.3 ± 2.0	21.7 ± 1.2
Acb	99.2 ± 4.3	96.5 ± 4.1	5.6 ± 0.6	7.3 ± 0.7	26.1 ± 1.2	27.4 ± 1.3
LS	86.9 ± 4.2	90.9 ± 4.3	3.2 ± 0.6	6.2 ± 0.7*	14.8 ± 1.7	16.1 ± 1.4
Hippocampus	188.5 ± 7.6	181.9 ± 10.5	201.9 ± 4.8	193.2 ± 5.7	61.2 ± 2.4	59.0 ± 2.5
CA1	194.4 ± 7.5	190.6 ± 14.1	296.8 ± 12.9	278.8 ± 16.3	70.4 ± 2.5	70.2 ± 2.8
CA3	223.5 ± 8.8	213.7 ± 13.1	181.0 ± 6.9	172.4 ± 4.9	50.7 ± 1.1	49.9 ± 1.5
DG	203.2 ± 8.6	185.7 ± 12.8	263 ± 6.9	239.9 ± 9.1	91.3 ± 3.6	89 ± 3.8
LD/LP thalamus	56.4 ± 2.2	57.3 ± 2.5	8.31 ± 0.8	8.3 ± 0.5	14.8 ± 1.5	16.4 ± 1.3
VP/PM thalamus	73.5 ± 3.5	72.5 ± 5.2	14.9 ± 0.9	13.5 ± 0.8	22.6 ± 1.7	19.3 ± 1.5
Amygdala	73.6 ± 4.6	73.3 ± 3.6	11.2 ± 1.1	11.4 ± 0.9	20 ± 1.6	15.3 ± 1.7

[a]Estimations of NMDA receptor subunit mRNA expression, determined by in situ hybridization, were made in coronal brain sections prepared at bregma +3.00 mm (CC, PrL, and IL), bregma +1.70 mm (CP, Acb, LS), and bregma −3.14 mm (hippocampus, CA1, CA3, DG, LD/LP thalamus, VPL/PM thalamus, amygdala). Data are expressed as mean ± SEM nCi/g,
*$P < 0.01$.
CC, cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex; RS, retrosplenial cortex; CP, caudate putamen; Acb, nucleus accumbens; LS, lateral septum; DG, dentate gyrus; LD/LP thalamus, laterodorsal/lateral posterior thalamus; VP/PM, ventral posterolateral/posteromedial thalamus.

and cingulate subregions of the medial PFC (mPFC) of the animals reared in isolation. A significant ($P < 0.01$) increase in NR2A subunit hybridization was also observed in the lateral septum (LS) of rats reared in isolation. NR2A subunit mRNA expression was unchanged in any of the striatal, hippocampal, thalamic, or amygdala regions examined. With the exception of the hippocampus and DG, NR2B subunit hybridization signal was modest and relatively even throughout the brain regions examined (Figs. 2G–2I). No differences in NR2B subunit hybridization signal was detected between the animals of Cohort 2 that were housed socially or in isolation (Table III).

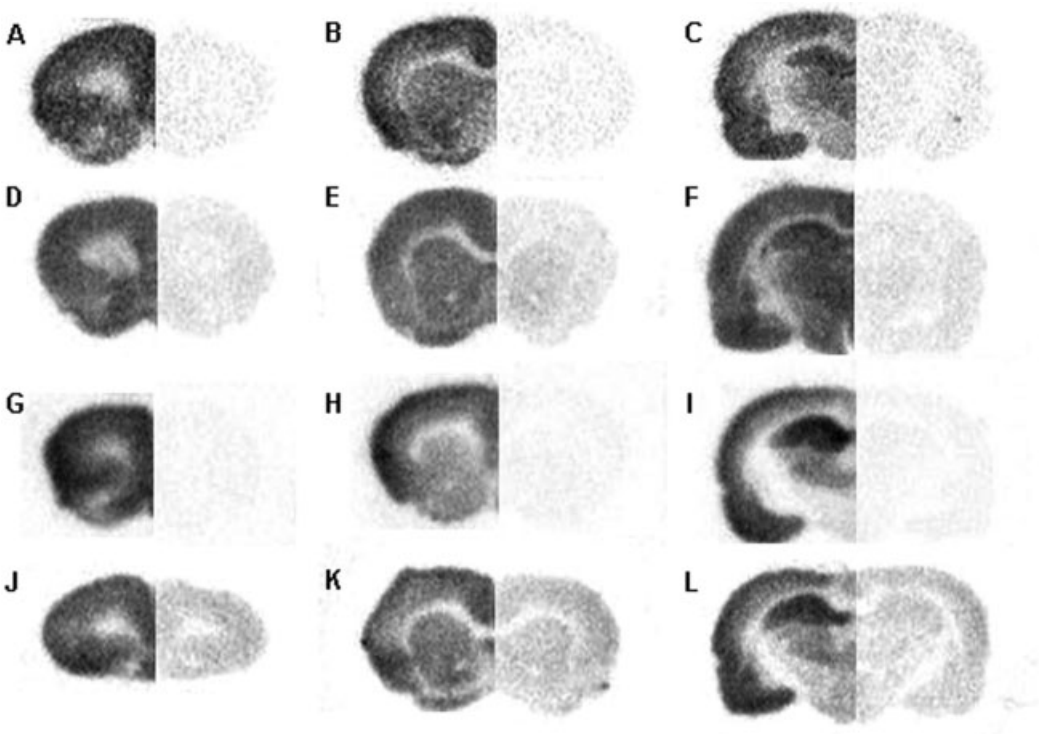

Fig. 3. NMDA receptor subunit radioligand autoradiography. Representative digital autoradiographs illustrating binding of [^{3}H]MK801 (**A–C**), [^{3}H]MDL105519 (**D–F**), [^{3}H]CGP39653 (**G–I**), and [^{3}H]Ro25-6981 (**J–M**) to coronal sections of rat brain prepared at bregma +2.70 mm (A, D, G, J), +1.50 mm (B, E, H, L), and −3.30 mm (C, F, I, M) in the absence (left side) or presence (right side) of excess unlabeled ligand.

TABLE IV. Binding of NMDA receptor radioligands in brain regions of rats reared socially and in isolation[a]

	Radioligand binding (cpm/mm^2)							
	[^{3}H]MK801		[^{3}H]MDL105519		[^{3}H]CGP39653		[^{3}H]Ro 256981	
Region	Social	Isolate	Social	Isolate	Social	Isolate	Social	Isolate
PM	8.4 ± 0.3	8.6 ± 0.2	22.7 ± 0.6	23.9 ± 0.7	19.8 ± 0.7	21.9 ± 0.5	15.1 ± 1.2	15.8 ± 1.3
mPFC	9.5 ± 0.3	9.8 ± 0.2	20.1 ± 0.9	21.9 ± 0.7	16.5 ± 0.6	19 ± 0.7*	22.4 ± 1.0	22.5 ± 0.8
CC	8.0 ± 0.4	7.8 ± 0.2	19.6 ± 0.5	20.7 ± 0.4	16.2 ± 0.7	18.0 ± 0.5	18.3 ± 0.6	17.9 ± 0.8
RS	4.3 ± 0.1	4.5 ± 0.1	18.6 ± 0.8	20.1 ± 0.8	10.1 ± 0.5	11.0 ± 0.4	8.5 ± 0.5	7.8 ± 0.9
CP	4.8 ± 0.2	4.9 ± 0.2	21.0 ± 0.7	22.9 ± 0.5*	4.8 ± 0.3	6.0 ± 0.7	10.1 ± 0.5	10.3 ± 0.6
Acb	6.0 ± 0.2	6.4 ± 0.1	18.0 ± 0.4	19.5 ± 0.4*	6.2 ± 0.3	6.9 ± 0.3	16.8 ± 0.4	17.0 ± 0.8
LS	5.1 ± 0.4	4.8 ± 0.3	17.7 ± 0.7	19.9 ± 0.3**	5.2 ± 0.4	6.4 ± 0.5	10.8 ± 0.9	11.6 ± 0.6
Hippocampus	9.7 ± 0.5	10.3 ± 0.3	22.3 ± 0.6	23.9 ± 0.6	30.2 ± 0.8	31.8 ± 0.9	22.5 ± 0.7	22.6 ± 0.9
LD/LP thalamus	5.1 ± 0.4	5.4 ± 0.2	18.8 ± 0.9	20.2 ± 0.4	12.8 ±0.7	13.7 ± 0.6	11.0 ± 0.6	12.2 ± 0.9
Amygdala	8.2 ± 0.4	8.9 ± 0.4	20.9 ± 0.7	22.5 ± 0.4	21.1 ± 1.2	22.3 ± 0.6	26.3 ± 0.9	24.4 ± 1.3

[a]Estimations of NMDA receptor radioligand binding were made in coronal brain sections prepared at bregma +2.70 mm (medial prefrontal and primary motor cortices), bregma +1.70 mm (CC, CP, Acb, LS), and bregma −3.30 mm (hippocampus, LD/LP thalamus, amygdala).
Data are expressed as mean ± SEM cpm/mm^2.
*$P < 0.05$; **$P < 0.01$.
PM, primary motor cortex; mPFC, medial prefrontal cortex; CC, cingulate cortex; RS, retrosplenial cortex; CP, caudate putamen; Acb, nucleus accumbens; LS, lateral septum; LD/LP thalamus, laterodorsal/lateral posterior thalamus.

Radioligand binding autoradiography

Radioligand binding autoradiography was performed on cryosections of brains obtained from the same isolation and socially housed rats used for the above in situ hybridization study (Cohort 2). Representative digital autoradiographs of the NMDA receptor ion channel radioligand [^{3}H]MK801, the NR1 subunit glycine site radioligand [^{3}H]MDL105519, the NR2A subunit preferring radioligand [^{3}H]CGP39653, and the NR2B subunit radioligand [^{3}H]Ro-256981 are shown in Figure 3.

No differences in binding of [^{3}H]MK801 or [^{3}H]Ro-256981 were detected between isolation-reared and group housed rats in any of the 10 regions examined (Table IV). In contrast, binding of [^{3}H]CGP39653 was significantly ($P < 0.05$) elevated in the mPFC of rats reared in isolation (Table IV). This region was equivalent to that in which an increase in the NR2A subunit

in situ hybridization signal was detected (in the same Cohort 2 animals) but the inferior resolution of digital autoradiographs compared with film-based in situ hybridization autoradiographs restricts the capacity to quantify radioligand binding within subregions of the medial prefrontal cortex. Significant ($P < 0.05$) increases in [^{3}H]MDL105519 binding were also detected in three subcortical regions (the caudate putamen (CP), Acb, and LS) without corresponding increases in NR1 subunit hybridization signal.

DISCUSSION

Rearing rats in isolation induces several behavioral characteristics, including deficits of PPI, hyperlocomotion in a novel environment, and increased perseverative behavior, which resemble some of the symptomatology of schizophrenia suggesting that the model has face validity for certain aspects of the illness (Braff et al., 1978; Cilia et al., 2001; Dalley et al., 2002; Geyer et al., 1993; Weiss et al., 2001; Wilkinson et al., 1994). Isolation rearing is also considered to be a developmental alternative to pharmacologically or lesion-induced models of schizophrenia (Bakshi and Geyer, 1999; Ellenbroek and Cools, 1990; Wilkinson et al., 1994). The PPI deficit seen in isolation-reared rats mirrors the disrupted sensory gating considered characteristic of schizophrenia (Braff et al., 1978; Cilia et al., 2001; Geyer et al., 1993; Weiss et al., 2001; Wilkinson et al., 1994) and the neural substrates involved are implicated in the underlying pathophysiology of the illness (Harrison, 1999). The amelioration of the behavioral effects of rearing rats in isolation by antipsychotic drugs (Cilia et al., 2001; Li et al., 2007; Varty and Higgins, 1995) argues for the predictive validity of the model. In both cohorts of animals used in the present study, PPI levels were reduced to less than half of those of their grouped housed controls, thus comparing favorably the PPI deficits previously observed in our laboratory in 29 different cohorts (Cilia et al., 2005) and providing clear evidence of impaired sensory gating and, by implication, a disruption in the underlying neural substrates. To discover the impact of isolation rearing on the brain expression of NMDA receptor subunit mRNA and protein, we conducted a series of microarray, in situ hybridization, and radioligand binding experiments.

The most robust change in NMDA receptor subunit expression produced in brains of rats reared in isolation was an increase in NR2A expression in the PFC that was detected at both the mRNA (Cohorts 1 and 2) and protein level (Cohort 2). Microarray revealed a 1.26-fold increase in NR2A subunit mRNA expression, limited to the PFC, of the rats of Cohort 1 reared in isolation whereas expression of the other NR2 subunits and NR1 and NR3 subunits were unaffected by housing conditions. In situ hybridization performed on tissue obtained from Cohort 2 revealed significant increases of NR2A subunit expression of up to 37% in the cingulate, prelimbic, and infralimbic regions of mPFC in rats reared in isolation, an observation that was substantiated at the protein level by increased binding of [^{3}H]CGP39653 in the mPFC in parallel sections from the same animals. Although exhibiting only modest (2–6-fold) selectivity for the NR2A versus the NR2B subunit (Laurie and Seeburg, 1994), the unchanged level of binding of [^{3}H]Ro-256981 suggests that the increase in [^{3}H]CGP39653 binding did reflect altered NR2A expression. A marked increase (92%) in NR2A subunit mRNA was also observed by in situ hybridization in the LS although this was not corroborated at the protein level by significantly elevated [^{3}H]CGP39653 binding. Unlike the regionally specific elevations in NR2A subunit expression observed by in situ hybridization in Cohort 2 of isolation-reared rats, no changes in expression of NR1 or NR2B subunits were observed at both mRNA and protein level in the same tissue. The significance of the modest increases in [^{3}H]MDL105,519 binding detected in the striatum (CP and Acb) and LS of the second cohort of isolation-reared rats, without any evidence of an increase in NR1 subunit mRNA expression in sections from the same animals, is uncertain. Of course, receptor function is a consequence of the expression and assembly of its constitutive proteins rather than mRNA expression per se, but the absence of any change in binding of the open channel radioligand [^{3}H]MK801 in sections from the same animals suggests that the overall receptor number of active receptors is not altered. In this regard, it may be significant that [^{3}H]MDL105,519 is reported to bind not only to the NR1 subunits forming heteromeric NMDA receptors but also to unassembled intracellular NR1 subunits (Chazot et al., 1998).

Altered expression of NMDA receptor subunits resulting from developmental behavioral manipulations is not unprecedented. Periods of maternal separation at, or prior, to postnatal day 9 is reported to cause reductions in hippocampal NR1 (Bellinger et al., 2006) and NR2A and NR2B (Roceri et al., 2002) subunit expression when rats reach adulthood. Offspring of low licking/grooming rats exhibit a deficit in NR2A and NR2B subunit mRNA expression that is reversed by environmental enrichment (Bredy et al., 2004). Stress induced by immobilization in pregnant mice produces a decrease in synaptic NR1 and NR2B subunit expression in the hippocampus of the offspring (Son et al., 2006). Specifically with regard to rats reared in isolation, a recent microarray study did not identify altered NMDA receptor subunit expression but did observe the altered expression of several immediate early genes in the PFC, some of which may impact on glutamate receptor-mediated signaling

(Levine et al., 2007). An earlier in situ hybridization investigation noted that mRNA of the NR1A subunit splice variant was unaltered in brains of isolation-reared Wistar rats but was elevated in the Acb and decreased in the hippocampus of isolation-reared Fawn-hooded rats compared with their socially housed counterparts (Hall et al., 2002). These strain-dependent differences emphasize the importance of confirming the existence of behavioral abnormalities before searching for underlying pathophysiological changes in central nervous system. The present investigation represents the first demonstration of altered expression of an NMDA receptor subunit in rats reared in isolation and which exhibited a clear sensory gating deficit although it does not provide evidence of a causative link between receptor expression and behavior.

How does the present data, particularly the increase in NR2A subunit expression in the medial prefrontal cortex, compare with NMDA receptor subunit expression in schizophrenia? In fact, although the NMDA receptor hypofunction hypothesis of the pathophysiology of schizophrenia is persuasive, and has been directly linked with enhanced signaling by the schizophrenia susceptibility gene neuregulin 1 in postmortem human brain tissue (Hahn et al., 2006), the numerous examinations of NMDA receptor subunit expression in postmortem human schizophrenic brains have yielded inconsistent results (see Kristiansen et al., 2007 for a recent review) that make comparisons with the present animal model-derived data difficult to make. Certainly, elevated NR2A subunit expression in the cerebral cortex in schizophrenia has been observed but there are also reports of unchanged cortical and subcortical NR2A subunit expression (Kristiansen et al., 2007). NR1 subunit expression is variously reported to be increased, unchanged, and decreased in cortical and subcortical regions including the substantia nigra, hippocampus, thalamus, and striatum, whereas NR2B subunit expression appears not to be altered in the cerebral cortex or, with a few isolated exceptions, in subcortical regions (Kristiansen et al., 2007). Of course, this examination of NMDA receptor subunit expression in isolation-reared rat brains was carefully controlled whereas patient and symptom heterogeneity, drug-induced effects, methodological differences including postmortem delay, and the effects of drug treatment may all conspire to obscure subtle but potentially meaningful alterations in NMDA receptor subunit expression resulting in human postmortem studies.

Although increased detection of NR2A subunit mRNA and protein is not necessarily direct evidence of increased NR2A subunit incorporation into functional NMDA receptors, in the absence of robust evidence of increased NMDA receptor numbers, such as increased [^{3}H]MK801 binding or increased NR1 subunit hybridization signal, it is tempting to speculate that the observed increases in NR2 subunit expression will impact the make up of NMDA receptors in that region. If so, profound changes in channel kinetics may result with NR2A subunits conferring a shorter channel open time (~120 ms) than NR2B/C (~400 ms) or NR2D (~5000 ms) subunits (Monyer et al., 1994). The impact of NR2A subunit composition on NMDA receptor channel function is illustrated by the decrease, or increase, in the offset decay time constant of NMDA receptor-mediated EPSCs in cerebellar granule cells following the overexpression of NR2A or NR2B subunits, respectively (Prybylowski et al., 2002). Similarly, the switch from NMDA receptors at immature synapses, in which NR2B is the major NR2 subunit, to those at mature synapses in which the NR2A subunit predominates results in a marked shortening of channel open time (Flint et al., 1997; Quinlan et al., 1999). Thus, a shift to NR2 subunits that confer shorter channel kinetics might be predicted to produce a relative "hypofunction" of the receptor. Furthermore, in addition to its direct impact on NMDA receptor kinetics, there is increasing evidence that the NR2A subunit is also important in determining the composition of the NMDA receptor. Recent studies suggest that increased NR2A subunit protein is preferentially transported to the synaptic membrane through interactions with PSD-95 and incorporated into the receptor at the synapse (Losi et al., 2003). Furthermore, the number of NR2B-containing receptors was unchanged in the visual cortex of NR2B subunit-overexpressing mice, leading the authors to hypothesize that it is the availability of NR2A subunits that determines the subunit composition of the receptor (Philpot et al., 2001).

In recent years, evidence has accumulated that supports the construct validity of isolation rearing as a model of schizophrenia. Rats reared in isolation reportedly exhibit alterations in monoamine neurotransmitter systems, such as elevated dopamine concentrations in the mPFC and Acb (Hall et al., 1998; Heidbreder et al., 2000; Jones et al., 1992; Swerdlow and Geyer, 1998) that resemble those seen in schizophrenia (Abi-Dargham et al., 1998; Laruelle et al., 1996). Anatomically, isolation-reared rats exhibit a loss of characteristic GAT-1 immunopositive GABAergic terminals in the mPFC (Bloomfield et al., 2008), reduced mPFC volume (Day-Wilson et al., 2006), reduced dendritic spine density in the mPFC and hippocampus (Silva-Gomez et al., 2003), and decreased synaptic density in the DG (Varty et al., 1999) that mimic the loss of GAT-1 cartridges (Pierri et al., 1999; Woo et al., 1998), reduced cortical volume (Selemon and Goldman-Rakic, 1999), and decreased dendritic spine density (Garey et al., 1998; Glantz and Lewis, 2000; Law et al., 2004; Rosoklija et al., 2000) reported in the postmortem schizophrenic brain. As discussed

earlier, a clear picture of altered NMDA receptor subunit expression as a result of schizophrenia has not emerged that would enable direct comparison with the current data. Nevertheless, the present observation of altered NMDA receptor subunit expression in the PFC of isolation-reared rats exhibiting a deficit in PPI is suggestive of NMDA receptor dysfunction in a region of the brain involved in sensory gating (Harrison, 1999) and therefore provides some indirect support for the construct validity of isolation rearing as a preclinical model of schizophrenia.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Simon Andrews (Bioinformatics, Babraham Institute) for assistance with designing oligonucleotide probes and Kriss Harris (Statistics Department, GSK) for performing statistical analysis.

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