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Failure of NMDA receptor hypofunction to induce a pathological reduction in PV-positive GABAergic cell markers

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Abstract

Reduction in cortical presynaptic markers, notably parvalbumin (PV), for the chandelier subtype of inhibitory γ-amino-butyric acid (GABA) interneurons is a highly replicated post-mortem finding in schizophrenia. Evidence from genetic and pharmacological studies implicates hypofunction of N-methyl-D-aspartate receptor (NMDAR)-mediated glutamatergic signaling as a critical component of the pathophysiology of schizophrenia. Serine racemase (SR) produces the endogenous NMDAR co-agonist D-serine, and disruption of the SR gene results in reduced NMDAR signaling. SR null mutant (−/−) mice were used to study the link between NMDAR hypofunction and decreased PV expression, assessed by immunoreactive (IR) cell density in the medial prefrontal cortex and hippocampus and protein levels in brain homogenates from the frontal cortex and hippocampus. Contrary to expectations, SR −/− mice showed modest elevations in PV-IR cell density and no difference in PV expression in brain homogenate. To control for these surprising results, we investigated PV expression in mice and rats following subchronic phencyclidine or ketamine treatments in adulthood. PV expression was not affected by drug these treatment in either species, failing to reproduce previously published findings. Our findings challenge the hypothesis that pathological deficits in PV expression are simply a consequence of NMDAR hypofunction.

Keywords

NMDA receptor; D-serine; parvalbumin; GAD67; phencyclidine; ketamine

Introduction

A lack of understanding of the etiology and pathophysiology of schizophrenia has limited the development of therapeutics that improve upon current neuroleptic therapy. Research over the last two decades has begun to link deficits in γ-amino-butyric acid (GABA)-expressing interneuron signaling in the neocortex and hippocampus to schizophrenia pathophysiology ([8], [26]). Replicated post-mortem findings include decreases in the expression of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD; [2], [18]), the calcium-binding protein parvalbumin (PV; [4], [20]) and subtype 1 of the GABA

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transporter ([32], [38]). These findings point towards a deficit in chandelier cells, a subtype GABAergic interneurons. Chandelier cells are PV-containing, fast-spiking neurons that synapse upon the axon initial segment of pyramidal neurons (for review see [8]). These characteristics position them to serve as critical regulators of action potential initiation and signal propagation in cortical microcircuitry. Furthermore, deficits in chandelier cell activity are hypothesized to underlie functional deficits in dorsal lateral prefrontal cortex (PFC) activity and dysregulation of working memory and executive function, prominent aspects of the cognitive symptoms of schizophrenia ([26]).

Hypofunction of the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors is hypothesized to contribute to the pathophysiology of schizophrenia, promoting deficits in GABAergic signaling ([13], [27]). One prominent experimental model of NMDA receptor (NMDAR) hypofunction involves subchronic treatment with non-competitive NMDAR blockers, e.g. phencyclidine (PCP), ketamine or MK801 ([24]). This model bears a phenotypic resemblance in rodents to many symptomatic features of schizophrenia, including deficits in social interaction ([11]), working memory ([1], [33]), and executive function ([15]). Repeated treatment with NMDAR blockers are reported to reduce the density of PV-expressing interneurons, as shown in selective investigations of the hippocampus ([10],[23],[25]) or medial PFC ([1]). The work of Behrens and colleagues suggests that ketamine-induced GABAergic deficits are downstream of increased superoxide production resulting from an interleukin-6 mediated enhancement in nicotinamide adenine dinucleotide phosphate ([5], [6]). Repeated ketamine treatment produces a physiological decrease in inhibitory neurotransmission in rat cortical slices ([39]). These findings support a circuit-based framework, whereby NMDAR hypofunction causes decreased inhibitory tone of PV-positive cells on cortical pyramidal neurons and resultant dysfunctional neurotransmission to subcortical regions ([27]).

A critical step for NMDAR activation is the binding of a co-agonist to the glycine modulatory site (GMS) on the NR1 subunit of the receptor. While glycine is the namesake, D-serine is the more prominent GMS agonist in the forebrain ([19]). D-serine concentrations are regulated by the synthetic enzyme serine racemase (SR) and the catabolic enzyme D-amino acid oxidase ([34,37]). Constitutive SR deletion reduces NMDAR signaling and causes behavioral and neurostructural changes relevant to schizophrenia ([3],[14]). Using the SR mutant mouse line, the present studies investigated the effects of genetically-induced NMDAR hypofunction on the expression of markers of GABAergic interneurons. Expression was determined in intact tissue as well as brain homogenate. Given the methodological variability and restricted scope in prior work, subchronic PCP and ketamine treatments in adult mice and rats were also examined to serve as positive controls for the SR mutant.

Methods

Experiments were conducted on three groups of subjects: 1) SR homozygous null mutant (−/−) mice and wild-type (WT) littermates, 2) WT mice, and 3) Sprague-Dawley rats. The SR mutant construct consists of a targeted deletion of the first coding exon of the murine SR gene, which encodes the catalytic domain of the enzyme ([3]). SR −/− and WT littermates were derived from intercrosses of SR −/+ parents (backcrossed for >10 generations onto C57BL/6J background). WT mice were ≤ 2 generations separated from commercially derived C57BL/6J mice (Jackson Laboratories; Bar Habor, ME). 98 male mice (age 9–13 weeks of age) were used for these studies (18 SR −/−, 18 WT littermate, and 62 WT mice). 36 male Sprague-Dawley rats (250–300 g; Charles River Laboratories; Willmington, MA) were used. Animals were housed in a facility with a 12/12 hr light/dark cycle with food and water provided *ad libitum.* Principles of humane laboratory animal care were followed and

studies were performed with the approval of the McLean Hospital Institutional Animal Care and Use Committee.

Subchronic drug treatment experiments followed similar protocols. In a dose-finding experiment mice (n=42) were injected with PCP (1.0, 3.0 or 6.0 mg/kg, s.c.), ketamine (7.5, 15 or 30 mg/kg, s.c.) or vehicle once per day for 5 days. In the second mouse study, subjects (n=20) were administered PCP (6.0 mg/kg, s.c.), ketamine (30.0 mg/kg, s.c.) or vehicle once per day for 5 days. In the rat studies, subjects (n=36) were treated with either ketamine (30.0 mg/kg, i.p.) or vehicle once per day for 5 days. In all experiments, subjects were sacrificed 72 hours after the last drug treatment. PCP (Phencyclidine hydrochloride, Sigma Aldrich; St. Louis, MO) and ketamine (Sigma-Aldrich) were dissolved in sterile water. All mouse injections were administered at a volume of 10 ml/kg, while rats were injected at a volume of 2 ml/kg.

Tissue for immunohistochemical studies was obtained from transcardially perfused subjects. Subjects were deeply anesthetized and perfused with 4.0% paraformaldehyde (in 0.1M phosphate buffer, pH 7.4). Brains were post-fixed for 24 hrs in 4.0% paraformaldehyde and cryoprotected in increasing concentrations of sucrose (15% and 30%). Immunohistochemistry was performed on 40 μm free-floating sections. PV antibody (1:20K; MsαPV, P3088, Sigma-Aldrich) and GAD67 antibody (1:2500; RbαGAD67, AB9706, Millipore; Billerica, MA) was diluted in phosphate-buffered saline with 3.0% bovine serum albumin. GAD67 antibody was pre-absorbed against mouse brain tissue prior to usage. Sections were incubated with biotinylated secondary antibody raised against the primary antibody host species [1:1000; HsαMs (BA-2000) or GtαRb (BA-1000), Vector Laboratories; Burlingame, CA] and ABC reagent (ABC Elite kit, Vector Laboratories). Colorimetric detection was performed with 3,3-diaminobenzidine (DAB, 0.02%) enhanced with nickel (II) sulfate (0.08%). Experimental and respective control samples were processed in parallel.

Immunoreactive (IR) cells density was determined bilaterally in sections containing medial PFC [infralimbic (IL) and prelimbic (PL) cortices] and hippocampus [CA1, CA2, CA3, and dentate gyrus (DG)]. For each subject, three sections containing the medial PFC (mouse: +1.98 to +1.54 Bregma, rat: +2.70 to +2.20 Bregma) and four sections containing the hippocampus (mouse: −1.46 to −2.18 Bregma, rat: −2.80 to −3.60 Bregma) were analyzed. Subregion identification was guided by landmarks and coordinates detailed in a mouse or rat brain atlases ([30],[31]). Sections were pseudo-randomly chosen from the regions of interest, with an average intersection distance of 160 μm. IR cell counting and planimetric determination of counting area was performed by an investigator blinded to experimental condition using StereoInvestigator (MBF Bioscience; Welliston, VT). The IR cell density was determined for each subject within a given sub-region as (total number of IR cells counted/total area analyzed). Data were analyzed using two-way ANOVA, with brain region and either genotype or drug treatment as the main effects. *Post hoc* comparisons were performed within brain regions using Bonferroni-corrected t-tests.

For immunoblot analysis, brains were sub-dissected as previously described ([16]). Frontal cortex and hippocampal tissue were disrupted via brief sonication in 60mM Tris buffer (pH 6.8) with 2% SDS. Protein samples (20 μg/lane, resulting in the linear range for the proteins of interest) were separated via electrophoresis on 15% acrylamide gels under denatured and reduced conditions, and transferred to nitrocellulose membranes. RbαPV (1:10K; ab11427, Abcam; Cambridge, MA) and Rbαβ-actin (1:8K; ab8227; Abcam), RbαGAD67 (1:10K; AB9706; Millipore), RbαCalretinin (1:10K; 7699/4, Swant; Bellinzona, Switzerland) and MsαCalbindin-D28K (1:10K; 300, Swant) were used for primary antibodies. Secondary antibodies used were raised against the primary antibody host species [1:5K; GtαRb

(ab6721) or RbαMs (ab6728), Abcam]. Proteins were visualized with chemiluminescence (Western Lightning Reagent, PerkinElmer; Waltham, MA). Protein band intensity was determined by densitometry (Quantity One, BioRad; Hercules, CA). PV expression was expressed relative to β-actin for each sample, normalized to the control condition, and averaged across replicate experiments. Data was statistically analyzed using Student's t-test comparisons (SR −/− mice or ketamine-treated rats vs control) or one-way analysis of variance (ANOVA) with Dunnet's *post hoc* comparisons (PCP and ketamine dose-response in WT mice).

Results

The study of the translational relevance of genetically-modified mouse strains to schizophrenia has begun to turn towards pathological endpoints, specifically PV and GAD67 expression. PV expression in SR −/− mice was determined in the medial PFC (IL and PL) and hippocampus (CA1, CA2, CA3, and DG). SR −/− mice displayed a modest elevation in PV-IR cell density (Table 1), shown to be significant by a main effect of genotype in a two-way ANOVA [$F(1,70) = 6.361$, $p<0.05$]. However, *post hoc* tests showed no significant difference between SR −/− and WT mice in any single brain region examined. There was no difference in the total counting area between genotypes. PV-immunoreactivity in all samples showed the expected pattern of distribution, predominately in the subgranular and infragranular layers of the PFC and on the periphery of the principal cell layer of the hippocampus with sporadic staining in the *stratum oriens* and *stratum radiatum*. GAD67-immunoreactivity was also analyzed in the SR −/− mice. No difference between SR −/− and WT littermates in GAD67-IR cell density was observed in the medial PFC or the hippocampus (data not shown).

Prior investigations of subchronic NMDA blocker treatment have demonstrated PV deficits in the PFC of mice ([5]) and the hippocampus of rats ([1], [10], [33]). Both rats and mice were investigated in the present study as positive controls for the SR mutant mouse experiment. WT mice were treated daily for 5 days with PCP (6.0 mg/kg, s.c.), ketamine (30.0 mg/kg, s.c.) or vehicle. Regions of the medial PFC and hippocampus were examined for PV-IR cell density (Table 1). There was no statistically significant main effect of NMDAR blocker treatment. PV immunoreactivity was likewise examined in rats treated daily for 5 days with either ketamine (30.0 mg/kg, i.p.) or vehicle (Table 1). There was no significant main effect of ketamine treatment on the density of PV-IR cells. There was no significant difference in the total counting area due to drug treatment using within species comparisons.

Fractional changes in protein expression that occur in all IR cells may not be detected when performing cell counting. Analysis of protein expression in brain homogenate samples allows for detection of changes in average cell expression that occur across a brain region. The frontal cortex and hippocampus of SR −/− and WT littermate mice were analyzed for PV expression (Table 2). Expression of PV in SR −/− mice was similar to that of WT littermate in homogenate samples in both brain regions. Statistical comparisons of each brain region did not find any significant differences between the genotypes. Expression of other GABAergic interneuron markers (GAD67, calbindin, calretinin) was similarly unaffected by SR deletion (data not shown).

Given the limited number of studies in mice employing a subchronic PCP or ketamine treatment regimen, we investigated the effects of multiple doses of each drug on PV expression in brain homogenate. WT mice were administered PCP (1.0, 3.0, or 6.0 mg/kg s.c.), ketamine (7.5, 15, or 30 mg/kg s.c.), or vehicle once per day for 5 days and were subsequently analyzed for expression levels of PV in frontal cortex and hippocampus.

Neither PCP nor ketamine significantly altered PV expression levels in the frontal cortex, producing only modest non-significant decreases at the highest treatment doses (16.0 % and 14.8% respectively) as compared to vehicle treatment (Table 2). We have observed these doses of PCP to alter behavior acutely ([9]). Similarly, no significant effect was found in the hippocampus (Table 2). The effect of subchronic ketamine treatment (30 mg/kg daily for 5 days) on PV expression in rats was also studied. No reduction in PV expression was observed in either the frontal cortex or hippocampus (Table 2).

Discussion

The primary aim of the present studies was to use a SR null mutant mouse line to test whether constitutive NMDAR hypofunction would give rise to a decrease in PV expression. Our previous work in this mutant strain showed electrophysiologically that occupancy of the GMS was lower and that NMDAR mediated long-term potentiation was markedly reduced in a D-serine reversible manner, and biochemically that NMDAR-mediated neurotransmission was decreased as measured by changes in S-nitrosylation of proteins [3]. SR deletion also resulted in a reduction in dendritic arborization and spine density in the PFC ([14]). Nonetheless, we did not observe a decrease in PV expression, or that of other GABAergic markers, using multiple methods of protein expression analysis. These findings suggest that NMDAR hypofunction alone does not result in decreases in PV and GAD67 protein levels, or that compensation for the constitutive NMDAR hypofunction abrogates the GABAergic neuronal changes.

A recent study by Belforte *et al*. reported that the expression of GAD67 and PV was decreased in the interneurons of mice in which NMDAR expression was selectively ablated ([7]). One possible explanation for the discrepancy is the severity of the NMDAR hypofunction. The aforementioned study used a deletion of the NR1 subunit of the NMDAR in PV-expressing interneurons, while our SR mutant has reduced cortical D-serine content, thereby causing a reduction in NMDAR signaling as opposed to outright receptor inactivation. In addition to the difficulty of directly comparing different mutant lines, the Belforte *et al*. study diverges methodologically from our work as well. The prior study measured protein expression using mean cell fluorescence, whereas our analysis involved counting of IR cells and immunoblot analysis of brain homogenate. Our methods are less sensitive to changes in expression that occur in a regionally selective pattern or in an average per-cell decrease, but are consistent with those used in human post-mortem studies that demonstrate pathological changes in GABAergic protein expression ([4], [17], [18]).

The lack of reduced PV expression in the SR −/− mouse alone is not enough to conclude that NMDAR hypofunction does not cause the pathology of interest. This led us to analyze PV expression following subchronic NMDAR blocker treatment, the experimental model that is the foundation of the link between altered NMDAR activity and pathological PV deficiency. Contrasting published findings of decreased PV expression, we observed no changes in mice following subchronic PCP or ketamine treatment or in rats following subchronic ketamine treatment. Table 3 outlines the published studies reporting that subchronic treatment with a NMDAR blocker reduces PV expression along with the present findings, illustrating differences in drug treatment regimen (drug, dose and duration), post-treatment interval of analysis, and means of quantifying PV expression (*e.g.* protein vs. mRNA). Many studies examined a single brain region, limiting the ability to determine the broader distribution of the observed effect. Other studies examined multiple brain regions, finding region-specific differences; one study found an increase in the medial prefrontal cortex but not hippocampus ([36]), while another found the opposite effect ([10]). Several reports analyzed tissue obtained ≤3days after last treatment, but one study found that a 42 day post treatment interval was necessary to see decreases in PV expression ([22]), with

subsequent studies from that laboratory only using that extended time point ([1], [23], [28]). Regarding whether the deficit occurs as a reduction in the number of PV expressing cells or a decrease in the average expression level per cell, the work of Behrens *et al*. contends that there is no loss of PV-IR cells, but a decrease in average cellular PV expression ([5], [6]). However, as stated previously, the post-mortem findings in schizophrenia were based on PV-IR cell density changes. While methodological variability is expected wherever similar lines of investigation are pursued by multiple research groups, the inconsistencies here make it very difficult to evaluate whether subchronic NMDAR blockade does indeed decrease PV expression. Many studies, the present studies included, do not demonstrate that any post-mortem changes correlate with functional or behavioral abnormalities. This lack of tethering to an *in vivo* phenotype raises questions of validity and standardization across laboratories.

A growing body of scientific literature supports the notion that reduced expression of GABAergic interneuron markers, particularly those of the chandelier cell subtype, is a pathological hallmark of schizophrenia ([17]). Such deficits seem to reflect diminished interneuron activity, which has been linked to working memory and executive function deficits, prominent components of the cognitive symptoms of schizophrenia ([26]). Since cognitive symptoms in schizophrenia remain unaffected by current medications, discovering the root cause of the pathophysiology linked to cognitive dysfunction is of critical interest in defining risk factors and the development of therapeutics. To this end, genetically-modified mouse models are particularly useful tools. For example, in a model of genetic susceptibility for schizophrenia, decreased PV expression was observed in mice expressing a dominant-negative form of Disrupted-in-schizophrenia-1 ([21]). The initial aim of the present study was to exploit the SR mutant in a similar way, using a targeted genetic manipulation to test for a link between diminished NMDAR signaling and the pathological reduction of GABAergic cell markers. The absence of a PV deficiency phenotype in the SR −/− mice, failure of any of our “positive control” studies to reproduce previous findings, and conflict among the results of published studies support the conclusion that PV deficits are not caused simply by NMDAR hypofunction.

Research highlights

- Parvalbumin expression is unaffected by a serine racemase null mutation
- Parvalbumin expression is unaffected by subchronic dosing of PCP and ketamine
- NMDAR hypofunction may not be a cause for PV-positive interneuron pathologies

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Table 1

NMDA receptor hypofunction causes minimal or no differences in PV immunoreactivity in mPFC and Hippocampus

		mPFC		Hippocampus			
Species	**Test groups**	**PL**	**IL**	**CA1**	**CA2**	**CA3**	**DG**
Mouse (C57BL/6J)	SR −/− (n=8)	127.4 (5.0)	146.3 (7.4)	57.3 (2.6)	97.6 (3.1)	70.7 (3.4)	30.7 (1.8)
	WT (n=8)	123.3 (4.4)	132.2 (9.3)	45.1 (2.9)	82.7 (3.8)	61.2 (4.3)	24.2 (3.1)
Mouse(C57BL/6J)	PCP 6.0 mg/kg (n=7)	100.6 (7.6)	112.2 (4.7)	33.6 (2.4)	61.6 (5.1)	46.2 (4.1)	21.0 (2.4)
	Ketamine 30 mg/kg (n=7)	91.9 (13.9)	101.7 (8.7)	31.8 (2.6)	67.8 (9.4)	46.0 (4.0)	19.6 (2.2)
	Control (n=6)	88.3 (6.0)	107.8 (7.4)	30.1 (5.0)	57.3 (11.5)	42.5 (6.8)	18.6 (3.6)
Rat (Sprague-Dawley)	Ketamine 30 mg/kg (n=8)	123.2 (10.8)	113.4 (4.1)	44.7 (1.6)	60.0 (3.7)	40.0 (1.4)	30.0 (1.9)
	Control (n=8)	118.9 (6.3)	105.9 (5.4)	45.9 (1.9)	62.7 (1.8)	41.2 (1.6)	30.4 (2.2)

IR cell density is shown for each indicated brain region and experimental group. Cell density is in units of cells/mm^2 (±SEM). No region-specific differences were found between experimental groups and the respective control.

Table 2

NMDA receptor hypofunction does not change brain homogenate levels of PV

Species	Test groups	Frontal Cortex	Hippocampus
Mouse (C57BL/6J)	SR −/− (n=10)	1.05 (0.08)	1.09 (0.16)
	WT (n=10)	1.00 (0.11)	1.00 (0.11)
Mouse (C57BL/6J)	PCP 6.0 mg/kg (n=6)	0.84 (0.09)	1.00 (0.10)
	PCP 3.0 mg/kg (n=6)	0.81 (0.07)	1.24 (0.18)
	PCP 1.0 mg/kg (n=6)	0.93 (0.12)	1.02 (0.12)
	Control (n=6)	1.00 (0.08)	1.00 (0.14)
	Ketamine 30 mg/kg (n=6)	0.86 (0.04)	0.95 (0.18)
	Ketamine 15 mg/kg (n=6)	0.91 (0.03)	1.09 (0.09)
	Ketamine 7.5 mg/kg (n=6)	1.01 (0.06)	1.04 (0.11)
	Control (n=6)	1.00 (0.08)	1.00 (0.15)
Rat (Sprague-Dawley)	Ketamine 30 mg/kg (n=9)	1.06 (0.06)	1.03 (0.11)
	Control (n=9)	1.00 (0.05)	1.00 (0.08)

Protein expression shown is expressed as fold of control expression of PV/β-actin (±SEM). No differences were found between experimental groups and the respective control.

Table 3

Studies of GABA pathology following repeated NMDAR blocker treatment

Ref.	Species	Drug	Treatment	Post- treatment interval	Methods	mPFC	Results HP	Other
[5]	Mouse	Ketamine	30 mg/kg, × 2 days	18 hours	IF (protein)	↓		
[6]	Mouse	Ketamine	30 mg/kg, × 1 or 2 days	1, 3 or 10 days	IF (protein)	↓ (2 day) n.c. (1 day)		
[35]	Mouse	PCP	10 mg/kg × 10 days	3 days	ISH (mRNA)	n.c.		↓ VLO
[1]	Rat	PCP	2 mg/kg b.i.d., × 7 days	42 days	IHC (protein)		↓ DG and CA2/3 n.c. CA1	↑ M2 and CG1 ↓ M1
[12]	Rat	PCP	2.58 mg/kg, × 5 days or alternate days for 1 month	3 days	ISH (mRNA)	n.c. (5 day) ↓ (1 month)		
[36]	Rat	PCP	5 mg/kg × 5 days	3 days	ISH (mRNA)	↓	n.c.	↓ VLO
[28]	Rat	PCP	2.0 mg/kg b.i.d. × 7 days	42 days	IHC (protein)		↓	
[23]	Rat	PCP	2.0 mg/kg b.i.d. × 7 days	42 days	IHC (protein)		↓	
[22]	Rat	PCP	2 mg/kg b.i.d. × 7 days	24 hours, 7, 21 or 42 days	IHC (protein)		↓ (42 days only)	
[25]	Rat	Ketamine	30 mg/kg × 5 days	14 days	IHC (protein)		↓	
[33]	Rat	MK801	0.02 mg/kg × 21 days	24 hours	IHC (protein)	n.c. (cell density)		
[10]	Rat	MK801	0.02 mg/kg × 21 days	24 hours	IHC (protein)	n.c.	↓	
[29]	Monkey	PCP	0.3 mg/kg b.i.d. × 14 days	8 days	IHC (protein)	n.c. (cell density) ↓ (IR terminals)		
‡	Mouse	PCP Ketamine	6 mg/kg × 5 days 30 mg/kg × 5 days	3 days	IHC (protein)	n.c.	n.c.	
‡	Mouse	PCP Ketamine	1,3 or 6 mg/kg × 5 days 7.5,15,or 30 mg/kg × 5 days	3 days	WB (protein)		n.c.	n.c in frontal cortex
‡	Rat	Ketamine	30 mg/kg × 5 days	3 days	IHC (protein)	n.c.	n.c.	
‡	Rat	Ketamine	30 mg/kg × 5 days	3 days	WB (protein)		n.c.	n.c in frontal cortex

Abbreviations and symbols: IF, immunofluorescence (mean fluorescence/cell); IHC, immunohistochemistry (IR cell density); ISH, in situ hybridization (densitometry); WB, Western Blot; HP, hippocampus; mPFC, medial prefrontal cortex; CG1, cingulate cortex; VLO, ventrolateral orbitofrontal cortex; M1, primary motor cortex; M2, secondary motor cortex; n.c. no change; ↓, decrease; ↑, increase;

‡present studies