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Alterations of mGluR5 and its endogenous regulators Norbin, Tamalin and Preso1 in schizophrenia: towards a model of mGluR5 dysregulation

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Abstract
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Keywords
Metabotropic glutamate receptor, GRM5, immunoblot, human brain, schizophrenia, antipsychotic

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ORIGINAL PAPER

Alterations of mGluR5 and its endogenous regulators Norbin, Tamalin and Preso1 in schizophrenia: towards a model of mGluR5 dysregulation

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Abstract

Knockout of genes encoding metabotropic glutamate receptor 5 (mGluR5) or its endogenous regulators, such as Norbin, induce a schizophrenia-like phenotype in rodents, suggesting dysregulation of mGluR5 in schizophrenia. Human genetic and pharmacological animal studies support this hypothesis, but no studies have explored mGluR5 dysfunction at the molecular level in the postmortem schizophrenia brain. We assessed mGluR5 mRNA and protein levels in the dorsolateral prefrontal cortex (DLPFC) using a large cohort of schizophrenia and control subjects (n=37/group), and additionally measured protein levels of recently discovered mGluR5 endogenous regulators, Norbin (neurochondrin), Tamalin (GRASP-1), and Preso1 (FRMPD4), which regulate mGluR5 localization, internalization and signaling. Whilst mGluR5 mRNA expression was unchanged, mGluR5 protein levels were significantly higher in schizophrenia subjects compared to controls (total: +22%; dimer: +54%; $p<0.001$). Conversely, mGluR5 regulatory proteins were expressed at lower levels in schizophrenia subjects compared to controls (Norbin -37%, $p<0.001$; Tamalin -30%, $p=0.084$; Preso1 -29%, $p=0.001$). mGluR5 protein was significantly associated with mGluR5 mRNA and mGluR5 endogenous regulators in control subjects, but these associations were lost in schizophrenia subjects. Lastly, there were no associations between protein measures and lifetime antipsychotic history in schizophrenia subjects. To confirm no antipsychotic influence, all proteins were measured in the prefrontal cortex of rats exposed to haloperidol or olanzapine; there were no effects of antipsychotic drug treatment on mGluR5, Norbin, Tamalin or Preso1. The results from our study provide compelling evidence that mGluR5 regulation is altered in schizophrenia, likely contributing to the altered glutamatergic signaling that is associated with the disorder.

Keywords metabotropic glutamate receptor, *GRM5*, immunoblot, human brain, schizophrenia, antipsychotic

Introduction

Deficits in executive function, reasoning abilities and working memory seen in patients with schizophrenia are largely attributable to altered glutamate signaling in cortical regions [13]. These neurocognitive deficits are arguably the most debilitating aspects of the disorder, and the most difficult to treat [33]. While we and others have found changes in the main cortical ionotropic glutamate receptor, the *N*-methyl-D-aspartate receptor (NMDAR) in schizophrenia [1, 39], other glutamate receptors are likely to be involved. Accumulating evidence strongly suggests the involvement of metabotropic glutamate receptor subtype 5 (mGluR5) in the emergence of glutamate deficits in schizophrenia. Firstly, mGluR5 antagonism in rodents produces a range of schizophrenia-related behaviors, such as deficits in social interaction, working and spatial memory, instrumental learning, and amplification of deficits induced by NMDAR antagonists. Secondly, *mGluR5* knockout mice display schizophrenia-like behaviors, including reduced sensorimotor gating, impaired spatial memory, and sensitivity to deficits induced by NMDAR antagonists or amphetamine. Finally, mGluR5 positive allosteric modulators (PAMs) have therapeutic potential in the treatment of schizophrenia-like behaviors, particularly the cognitive-like symptoms which are induced in both glutamatergic (NMDAR antagonists) and dopaminergic (amphetamine) animal models [see 18].

Based on these studies, a role of mGluR5 in schizophrenia pathophysiology has been hypothesized. Ohnuma and colleagues first reported that mGluR5 mRNA expression was increased in pyramidal cell layers of the superior frontal cortex (Brodmann Area 9; BA9) of schizophrenia subjects [25], although more recently, no change was reported in a larger cohort examining the same region [8]. Conversely, mGluR5 monomer protein levels were reported to be significantly reduced in the same region [8]. However, other postmortem studies in cortical regions of patients with schizophrenia have reported that mGluR5 mRNA, protein and binding levels are unaltered [see 18, 23]. These inconsistent results might be due to region-specific differences, small sample sizes and/or other methodological differences in protein detection, such as the examination of monomer versus dimer protein expression [23]. A potential explanation for pathological disturbances in mGluR5 is abnormal regulation, caused by alterations to key proteins that regulate mGluR5 signaling, localization and trafficking as previously suggested by us [23] and others [4]. These type of disturbances have been previously reported for other aspects of the glutamatergic system [19]. In support of this hypothesis, proteins that regulate glutamatergic receptors (e.g. NMDAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and mGluRs), including Homer1, are reportedly altered in some schizophrenia studies [see 35]. However, additional proteins that critically modulate mGluR5 activity have also been identified [12, 14, 37], but have not yet been directly examined in the brains of people with schizophrenia.

Of the known mGluR5 endogenous regulators, Norbin, Tamalin and Preso1 have particularly distinctive roles in mGluR5 modulation. The cytoplasmic protein Norbin (neurochondrin) is reported to increase mGluR5 cell surface localization and positively regulate mGluR5 signaling in *in vitro* systems [38]. Norbin is also critically involved in neurite outgrowth, and thus neuronal plasticity and cognitive functioning [22, 30]. Cortical Norbin knock-out mice have reduced mGluR5 cell surface expression and function, and display strong schizophrenia-like behaviors [38]. Tamalin (also named GRP1-associated scaffold protein 1 or GRASP-1), is a 95 kDa postsynaptic density protein (PSD-95)/discs-large/ ZO-1 or PDZ) domain-containing scaffold, which plays a fundamental role in the localization of mGluR5 from the soma to the neuritic processes in cultured hippocampal neurons [14]. In accordance, disruption of Tamalin/mGluR5 couplings causes reduced mGluR5 cell surface expression and increased agonist-induced internalization of mGluR5 in cellular assays [34].

Furthermore, the scaffold protein Preso1 (FRMPD4) was also reported to be essential to mGluR5 signaling, with Preso1-dependent phosphorylation of mGluR5 serving to down-regulate mGluR5 signaling under control conditions in cellular assays, whilst *Preso1*$^{-/-}$ mice exhibit characteristics reminiscent of enhanced mGluR5 signaling [12]. Preso1 additionally modulates mGluR5 coupling to the scaffold protein Homer [12], which has been previously associated with schizophrenia [6, 31]. However, the role of Preso1 in schizophrenia-relevant paradigms has not been assessed. Together, Norbin, Tamalin and Preso1 have been shown to critically modulate mGluR5 localization, trafficking and signaling, and may thus serve as markers of these functions in postmortem studies where manipulation of the signaling system is not possible. Furthermore, considering that mGluR5 activity could be impaired should these endogenous regulators be altered, it is crucial that they are considered in the context of schizophrenia.

We therefore explored in the present study whether patients with schizophrenia express altered mRNA and protein levels of mGluR5 compared to controls, in a well-powered postmortem cohort. We specifically examined the dorsolateral prefrontal cortex (DLPFC; BA46) as this region is highly implicated in schizophrenia pathology due to its role in the development of cognitive deficits [2, 39]. For the first time, we also assessed the protein levels of novel mGluR5 endogenous regulators, Norbin, Tamalin and Preso1, as markers of mGluR5 localization, trafficking and signaling. Based on the extensive evidence that reduction of mGluR5 activity causes behavioral phenotypes associated with schizophrenia [see 18], our hypotheses were that levels of mGluR5 and endogenous modulators would be decreased in the DLPFC of people with schizophrenia. Considering that premortem medication history is a confounding factor in human postmortem studies, we also investigated whether the influences of commonly used antipsychotic drug treatments extend to the mGluR5 system. We therefore conducted the same protein analyses in a pharmacological animal model exposed to typical (haloperidol) or atypical (olanzapine) antipsychotic medication.

Materials and Methods

Human postmortem brain samples

Human DLPFC tissues were obtained from the New South Wales Brain Bank Network (Sydney, Australia). The complete cohort consisted of 37 schizophrenia (including 7 schizoaffective) postmortem brain samples and 37 controls (with no history of psychiatric diagnosis), which were matched according to tissue pH, age at death, postmortem interval and RNA integrity number (RIN) (Table 1). Schizophrenia subjects were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders IV. Antipsychotic drug treatment premortem was standardized to lifetime chlorpromazine equivalent for each patient. Antidepressant drug treatment history was also specified on a qualitative scale (i.e. yes/no). Further details regarding clinical, demographic and tissue characterization of the cohort has been described previously [40]. This study was approved by the Human Research Ethics Committees at the University of Wollongong (HE99/222) and the University of New South Wales (HREC07261).

Human brain tissue preparation

Anatomical identification and preparation of the tissue has been previously described in detail [40]. Briefly, samples corresponding to BA46 were dissected from coronal blocks from the middle frontal gyrus, anterior to the genu of the corpus callosum. For gene expression analyses, 300mg of frozen tissue was pulverized on dry ice. Trizol was used to extract total RNA, and the quality was analyzed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, USA). For immunoblot studies, 40mg of frozen tissue was homogenized in 400µL of homogenizing buffer, containing 50mM Tris pH 7.5, 50% glycerol and 1:20 (by volume) protease inhibitor cocktail (Sigma, Sydney, Australia). Homogenates were diluted to a total protein concentration of 2µg/µl, and concentration was verified by Bradford and bicinchoninic acid assays.

Animal housing and treatment

This study was designed to assess whether commonly used antipsychotic medications affect the protein expression of mGluR5 and mGluR5 endogenous regulators. Adult male Sprague-Dawley rats at 10-weeks of age were purchased from the Animal Resource Centre (Perth, Australia) and single housed under standard conditions with food and water available ad libitum. It should be noted that although animals were singly housed to ensure accurate dosage of APDs, measures were taken to ensure animals were not socially isolated: wire cages were stored in an adjoining manner to allow for social interaction between cages, and animals were given pipes for hiding and wooden toys to chew, to reduce any stress caused by separation.

After one week of acclimatization, rats were distributed into 9 treatment groups (3 treatments x 3 treatment-durations; n=6/group), as described previously [17]. Animals were fed prepared food pellets 3 times/day at 8 hour intervals, containing either: (1) typical antipsychotic, haloperidol (0.1mg/kg body weight; daily total 0.3mg/kg), (2) atypical antipsychotic, olanzapine (1mg/kg body weight; daily total 3mg/kg), (3) vehicle control (empty food pellets), for short term (8 days), medium term (15 days) and long term (35 days) durations. Consumption of food pellets was visually validated. Antipsychotic doses and treatment durations were chosen to model a clinical setting [11, 41]. All animal experiments in this study were approved by the University of Wollongong Animal Ethics Committee (AE10/18) and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animal brain tissue preparation

Rats were euthanized 48 hours following the final treatment using carbon dioxide asphyxiation. Brains were rapidly removed, and the prefrontal cortex (PFC; prelimbic area, Bregma 3.7-2.7) was dissected on ice and snap frozen in liquid nitrogen, and stored at -80°C. Identification of rat brain regions for quantification was based on a standard rat brain atlas [26]. Tissue was homogenized in NP-40 lysis buffer (Invitrogen), containing beta-glycerophosphate and pheylmethanesulfonylfluoride protease cocktail inhibitor cocktail (Sigma). Protein concentration was determined by detergent-compatible colorimetric assay (BioRad, Gladesville, Australia), according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Transcript levels of mGluR5 mRNA were measured by qRT-PCR as previously described [40]. Briefly, total RNA was extracted using Trizol as per the manufacturer's protocol (Invitrogen) and RNA quality was determined using Agilent Bioanalyzer. Complementary DNA synthesis was performed using Superscript III (Invitrogen), in triplicate and using 3µg total RNA per sample (pooled). qRT-PCR for mGluR5 mRNA was subsequently performed using TaqMan Gene Expression Assays (Applied Biosystems, Hs00168275_m1) and the ABI Prism 7900HT system. Quantity mean for expression was normalized to four housekeeping genes - ubiquitin C (Hs00824723-m1), ß-actin (Hs99999903-m1), glyceraldehyde-3-phosphate dehydrogenase (Hs99999905-m1), and TATA box binding protein (Hs00427620- m1) - that have been reported not to differ between the diagnostic groups in the same brain region, same cohort and same cDNA synthesis [40].

Immunoblot

Relative protein densities were determined by immunoblot. The following polyclonal antibodies were used: mGluR5 (ABCAM ab27190 [17]), Tamalin (ABCAM ab30576 [32]), Preso1 (Santa Cruz sc-242862 [16, 27]) and Norbin (ABCAM ab130507). Immunoblotting was performed as described previously [17], with minor modifications optimized for the measurement of each protein of interest (Table 1). Samples were loaded at 5µg and 10µg for all proteins assessed in human and rat brain samples respectively. Samples were visualized using an enhanced chemiluminescent detection kit (BioRad). Band density was detected by the Gel Doc 2200 Pro (Carestream Molecular Imaging, USA) and quantified with Carestream MI software (v 5.0.4.44, Carestream Molecular Imaging). All bands were within the linear range of detection. Protein measures were subsequently normalized to the respective ß-actin density (i.e. [band intensity of protein] ÷ [band intensity of respective ß-actin]), which was unaltered in the present study and has also been reported as a reliable standard in this postmortem cohort [40]. Actin-normalised values were then renormalised to a pooled sample, which was run on each gel to control for any gel-to-gel variability. Each sample was run in duplicate and the average value of both samples was then used in the statistical analyses. Experiments and quantification were performed blind to diagnosis (human) or treatment group (rat).

Statistical Analysis of Human Data

Statistical analyses were performed with SPSS version 19. Significance was set to $p<0.05$ and data are presented as mean ± standard error of the mean. mGluR5 mRNA expression was normally distributed. As distributions for all protein measures were skewed to the right (Kolmogorov-Smirnov: d=0.161-0.224, $p<0.001$), normalized distribution for these proteins was achieved by transforming to the natural logarithm of the relative protein values. Outliers were screened as mean ± 2 standard deviations and were removed. No subjects were removed from the mRNA analysis, although 2 subjects were removed on average from each protein analysis.

Analyses of variance (ANOVA) were used to detect differences in protein expression between diagnostic groups (schizophrenia/control) as well as gender (male/female), hemisphere (left/right) and antidepressant history (yes/no). Spearman's correlations were used to determine whether sample characteristics (age at death, pH, postmortem interval, RIN, brain weight and freezer storage time) were associated with protein measures. Analyses of covariance (ANCOVA) for diagnostic effects on protein expression were subsequently performed, accounting for factors that were associated with protein measures, as determined by the Spearman's correlations. Additional measures of disease characteristics were correlated specifically with the schizophrenia group (lifetime antipsychotic drug history, age of disease onset and duration of illness).

Statistical Analysis of Animal Data

For analysis of animal data, statistics were performed with SPSS, significance was set to $p<0.05$ and data presented as the mean ± standard error of the mean. Differences between treatment (haloperidol/olanzapine/vehicle) x duration (short term/medium term/long term) were analyzed by two-way ANOVA.

Results

Detection of mRNA and proteins

For qRT-PCR of mGluR5 mRNA, control samples (no template and no reverse transcriptase enzyme) did not produce a signal during measurement of mGluR5 mRNA expression, where we obtained robust signal from all samples and a reliable standard curve (slope=-3.42, y=31.27). For protein measurements, mGluR5 monomer was detected as a single band at 135kDa whilst the mGluR5 dimer was detected at 270kDa. As mGluR5 was detected at two molecular weights, mGluR5 dimer and total (sum of monomer and dimer) levels are reported. We have previously reported only mGluR5 monomer levels [17]; total and dimer measures in this study were analyzed from the same experimental run. Norbin (79kDa) and Tamalin (96kDa) proteins were identified as single bands at the expected molecular weights in the human DLPFC (Fig. 1a, Fig. S1) as well as in the rat PFC. Preso1 probing resulted in several bands; a single specific band was verified and quantified at 144kDa by peptide pre-absorption/neutralization, and all other non-specific bands thus were excluded (Fig. S1; see Supplementary Methods). ß-Actin was reliably detected at 45kDa (Fig. 1a).

mGluR5 mRNA is unaltered and mGluR5 protein is increased in the DLPFC in schizophrenia

mGluR5 mRNA expression was not altered in DLPFC of schizophrenia subjects compared to controls after co-varying for brain pH, age at death, RIN and brain weight ($F_{1,68}$=0.009, p=0.924; Fig. 1b), which were variables that correlated with mRNA expression (Table S2). There was no effect of gender or hemisphere on mGluR5 mRNA expression. We have previously reported no change in mGluR5 monomer levels in this cohort in DLPFC [17]. However, mGluR5 dimer expression was 53.5% higher in schizophrenia subjects compared to controls ($F_{1,70}$=8.688, p<0.001; co-varying for freezer storage time, which correlated with dimer levels [Table S2]: $F_{1,69}$=44.808, p<0.001). Total (monomer and dimer) mGluR5 protein was significantly increased by 21.9% in schizophrenia subjects compared to controls ($F_{1,68}$=19.090, p<0.001; Fig. 1b). Significance was maintained after co-varying for freezer storage time ($F_{1,67}$=17.45, p<0.001 [correlation values in Table S2]). There were no effects of gender and hemisphere on mGluR5.

Norbin, Tamalin and Preso1 proteins are reduced in the DLPFC in schizophrenia

Protein levels of all three mGluR5 endogenous regulators examined were significantly decreased in schizophrenia subjects (Fig. 1c). Specifically, Norbin was decreased by 37.4% ($F_{1,70}$=34.497, p<0.001), Tamalin by 30.4% ($F_{1,70}$=4.428, p=0.039), and Preso1 by 21.5% ($F_{1,70}$=5.607, p=0.021). Significance was maintained for Norbin and Preso1 after co-varying for freezer storage time, which was significantly correlated with these protein levels (Table S2); however Tamalin was not significantly different after co-varying for freezer storage time and age at death ($F_{1,69}$=3.075, p=0.084), due to a strong effect of freezer storage time (r=-4.75, p<0.001) and age at death in control subjects (r=0.353, p=0.032; correlation values in Table S2). There were no effects of gender or hemisphere on Norbin, Tamalin or Preso1 protein levels (p>0.050).

Correlations with demographic measures, disease characteristics and medication estimates

Spearman's correlations were performed to assess any relationships between mGluR5 mRNA expression and protein measures of mGluR5, Norbin, Tamalin and Preso1 with age at death, RIN, freezer storage time, brain pH, postmortem interval and brain weight (Table S2).

mGluR5 mRNA expression was significantly associated with brain pH, age at death, RIN and brain weight overall in all subjects. Specifically in control subjects, mGluR5 mRNA was negatively correlated with age at death and positively correlated with brain weight. In schizophrenia subjects,

mGluR5 mRNA expression was weakly associated with pH, RIN, and brain weight. There were no effects of illness duration, age of disease onset or lifetime antipsychotic drug history on mGluR5 mRNA in schizophrenia subjects, nor were there any effects of antidepressant medication (yes/no: t_{35}=0.299, p=0.588).

There was a significant interaction between mGluR5 total protein with freezer storage time in both control and schizophrenia subjects when analyzed separately, but not overall. mGluR5 dimer was also significantly correlated with freezer storage time an RIN in schizophrenia subjects. An effect of freezer storage time was seen on Norbin, Tamalin and Preso1. There were no other significant correlations with protein measures of mGluR5 endogenous modulators in all subjects. Specifically in control subjects, Tamalin, but not Norbin or Preso1, was negatively associated with age at death, while all proteins were associated with freezer storage time. In the schizophrenia group, there was a negative association of age of disease onset with measures of Norbin and Preso1 only, and an effect of freezer storage time on Norbin. There was no effect of illness duration or lifetime antipsychotic drug history on mGluR5 (total or dimer), Norbin, Tamalin or Preso1.

Relationships of mGluR5 protein with mGluR5 mRNA, Norbin, Tamalin and Preso1 proteins in the DLPFC

A distinct relationship was seen between mGluR5 total protein and mGluR5 mRNA expression, as well as protein measures of mGluR5 with Norbin, Tamalin and Preso1 (Fig. 2). While mGluR5 total protein was positively associated with mGluR5 mRNA expression in control subjects (r=0.361, p=0.030), this correlation was lost in schizophrenia subjects. Similarly, mGluR5 protein was strongly and negatively associated with all endogenous regulators in the control group (Norbin: r=-0.657, p<0.001; Tamalin: r=-0.560, p<0.001; Preso1: r=-0.428, p=0.009), but this association was not present in the schizophrenia group (Norbin: r=0.096, p=0.578; Tamalin: r=0.202, p=0.238; Preso1 r=0.080, p=0.644). An extensive list of correlation values is included in Table S3.

Effects of antipsychotic drug treatment on mGluR5 and its endogenous regulators in the prefrontal cortex

To assess the effects of antipsychotic medications on the mGluR5 system, two-way ANOVA was used to compare the effects of haloperidol, olanzapine and vehicle/control treatment (8, 16 or 36 days) on protein levels of mGluR5 (total, monomer, dimer), Norbin, Tamalin and Preso1 in the PFC of rats. There were no significant main effects of treatment or duration on protein expression, nor were there any interactions between treatment and duration on protein expression (F>0.050, p>0.961; Fig. 3). This is in line with the aforementioned human studies, in which there were no effects of lifetime antipsychotic drug medication or antidepressant history on protein measures.

Discussion

Here, we provide compelling evidence that mGluR5 is dysregulated in the DLPFC (BA46) of patients with schizophrenia. We found that whilst mGluR5 mRNA expression levels were not different, mGluR5 protein levels were significantly higher in schizophrenia subjects compared to controls (total: +22%; dimer: +54%). Conversely, in the same samples, mGluR5 endogenous regulators Norbin (-37%), Tamalin (-30%; p=0.084 after co-varying for confounding factors) and Preso1 (-29%) were expressed at substantially lower levels in the DLPFC of schizophrenia subjects compared to control subjects. mGluR5 mRNA, Norbin, Tamalin and Preso1 proteins were significantly associated with mGluR5 protein levels in control subjects, but these associations were lost in schizophrenia subjects. Lastly, there was no effect of current antipsychotic drug treatment on mGluR5 or mGluR5 endogenous regulators in either human or animal studies. Thus, the present findings suggest that mGluR5 regulation is altered in the DLPFC in schizophrenia, but not influenced by current therapeutics.

In this study, higher mGluR5 protein expression was detected in schizophrenia subjects compared to controls, but there was no difference in mGluR5 mRNA expression in these same cases. Previous studies have similarly reported no change in mGluR5 mRNA expression in the adjacent BA9 region of schizophrenia subjects [8, 36] accompanied by altered mGluR5 protein levels [8]. These findings support the existence of dissociation between mGluR5 mRNA and protein levels in schizophrenia subjects. Our results also revealed that mGluR5 mRNA expression was significantly and positively associated with mGluR5 protein in control subjects, but not schizophrenia subjects. mRNA and protein have differing half-lives due to variations in protein synthesis and degradation, so that the change in protein concentration is equal to the “rate of translation” minus the “rate of degradation” [10]. As we report no change in the rate of translation (as measured by mGluR5 mRNA expression), the loss of association between mGluR5 mRNA and protein in schizophrenia is likely indicative of reduced receptor degradation and a prolonged lifespan of mGluR5 proteins. This may be owing to slowed mGluR5 trafficking, as we further report reduced levels of the mGluR5 trafficking proteins Norbin and Tamalin in this study; we however caution that Tamalin did not reach significance after covarying for confounding factors (p=0.084), but due to the large magnitude of change (30%) and significant correlations of Tamalin with the other measures (Fig 4c, Table S3, and discussed below), we will further discuss this finding.

Norbin and Tamalin bind to amino acid residues on the intracellular terminus of mGluR5, and critically regulate mGluR5 cell surface localization and intracellular trafficking [14, 15, 34, 38]. Accordingly, in cultures derived from cortical Norbin knockout mice, mGluR5 cell surface localization was reduced [38]. Kitano and colleagues also determined that Tamalin was required for transport of mGluR5 from the soma to neuritic processes [14], whilst Timms et al. demonstrated that deletion of the Tamalin binding site on mGluR5 resulted in increased agonist-dependent internalization of mGluR5 [34]. This evidence suggests reductions in Norbin and Tamalin simultaneously cause reduced trafficking of mGluR5 protein, decreased cell surface expression, and intracellular retention of mGluR5 (Fig. 4a-b). In view of the fact that G-protein coupled receptor dimerization occurs soon after biosynthesis [20], it is also likely that these captive mGluR5 are in the dimer form. A growing body of evidence suggests that mGluR5 proteins are only functional in the form of disulfide-linked dimers [5, 29], suggesting an increase in mGluR5 dimers represents an increase in functional mGluR5 units in the present study. However, considering *reduced* mGluR5 signaling (by mGluR5 knockout or pharmacological antagonism) produces schizophrenia-like behaviors, and mGluR5 PAMs have therapeutic efficacy in preclinical rodent models [see 18], we

hypothesize a model of mGluR5 dysregulation in which higher protein expression and dimer units does not represent increased activity and/or function in the schizophrenia brain, but rather, alterations to mGluR5 localization (Fig 4a and b) and also signaling (Fig. 4a and c).

In addition to a trafficking deficit, our results further indicate altered mGluR5 signaling as substantial reductions were observed in Norbin and Preso1 proteins, which are critical regulators of mGluR5 signaling [12, 38]. Firstly, Norbin was demonstrated to enhance mGluR5 signaling in cell-based assays, by increasing inositol phosphates, intracellular calcium release, and ERK1/2 phosphorylation compared to mGluR5 expression alone [38], indicating decreased mGluR5 signaling due to reduced Norbin expression in the present study (Fig. 4a). In contrast, Preso1 reportedly inhibits mGluR5-dependent intracellular release and ERK1/2 phosphorylation, via Homer1/2/3 under control conditions [12], indicating disinhibition of mGluR5 signaling due to reduced Preso1 in the present study (Fig. 4c). Notably, Preso1 and Norbin levels are positively correlated in control subjects ($r=0.559$, $p=0.003$) but not schizophrenia subjects ($r=0.152$, $p=0.368$), supporting the notion of opposing functions and thus a balance of Norbin:Preso1 under control conditions. Although further studies will be required to determine whether reductions in Norbin and Preso1 are causal or compensatory to mGluR5 dysregulation, this finding suggests that the ratio of Norbin:Preso1 and thus positive and negative modulation of mGluR5 is altered in schizophrenia, strongly supporting the hypothesis that mGluR5 signaling is affected in the DLPFC in the schizophrenia brain.

It should be noted that the increase in mGluR5 protein levels in the present study was specific to mGluR5 dimers, as we previously reported no change in mGluR5 monomers in the same samples in the same experimental run [17]. As discussed previously by Corti et al. [3], who similarly reported altered dimerization of mGluR3 in the DLPFC in schizophrenia and hypothesized the same of mGluR5 [4], there are several possible interpretations of altered monomer and dimer densities. Firstly, the composition of heterodimeric complexes involving mGluR5 with structurally similar G-protein coupled receptors (such as mGluR1a, adenosine or gamma-aminobutyric acid (GABA)-B receptors) has been proposed [9, 29], presenting the possibility of alterations to the ratio of heterodimers:homodimers, and thus mGluR5 function and activity in schizophrenia. Furthermore, as hypothesized for mGluR3, the presence of mutations within *mGluR5* and/or altered splicing of *mGluR5* might affect the formation, affinity and activity of mGluR5 dimers [3, 34]. Lastly, it remains unclear whether dimerization might be affected during the perimortem period, or whether dimers measured under reducing immunoblot conditions is reflective of physiological levels [7, 23]. Even so, considering we found an increase rather than a decrease in mGluR5 dimer levels, it is unlikely that these factors impact on the overall results of this study. Further, the mechanisms or molecules responsible for regulating mGluR5 dimerization are not known but would be useful for interpreting these findings. It is additionally interesting that in the present study, the levels of all measured mGluR5 endogenous regulators were negatively correlated with protein levels of mGluR5 in control patients, and this association was lost in patients with schizophrenia (Supplementary Table 3). Although this finding supports the dissociation of mGluR5 with endogenous regulatory processes in the schizophrenia brain, the relationship between mGluR5 and its endogenous regulators under control conditions is counter-intuitive and requires further investigation.

We found no associations between mGluR5 and its endogenous regulators with lifetime antipsychotic drug treatment in our postmortem human study, and no effects of haloperidol or olanzapine treatment on the expression of the mGluR5 system proteins in our animal study. Our results thus strongly suggest that the aspects of the mGluR5 system measured in this report are unaffected by commonly used antipsychotic drugs. This is in line with our previous work, in which we found that mGluR5

binding in the PFC was also unaffected in the same animal model and treatment regime [17]. It is notable, however, that these results are reflective of "control" conditions, which would be enhanced by follow-up studies of antipsychotic drug treatment in an animal model of schizophrenia. Drugs targeting mGluR5 have therapeutic efficacy against cognitive and negative schizophrenia-like deficits in animal models [24], while current therapeutics have little efficacy for these symptoms [21]. mGluR5-targeting drugs therefore present a compelling adjunct treatment strategy for schizophrenia, specifically in relation to cognitive deficits associated with glutamatergic dysfunction. However, it should be specifically determined whether mGluR5 target availability is altered in people with schizophrenia due to intracellular retention, and whether this will impair the efficacy of novel therapeutics aimed at mGluR5.

Using one of the largest postmortem cohorts to date to study mGluR5 and its endogenous regulators, we have for the first time established that mGluR5 is dysregulated in schizophrenia. Due to the prominent role of cortical glutamate function in the emergence of emotional and cognitive deficits associated with schizophrenia, we further suggest that dysregulation of mGluR5 might contribute to the cognitive dysfunctions that are observed in these patients. Whilst this study supports the presence of mGluR5 dysregulation and/or signaling deficits in schizophrenia, it is important to determine whether mGluR5 target availability is altered, and whether this will impair the efficacy of novel therapeutics aimed directly at mGluR5. Future postmortem studies focusing on functional assays and analyses of protein-protein interactions in specific neuronal populations and/or subcellular compartments are thus required to examine the nature of functional alterations to mGluR5 (for example, altered phosphorylation, protein misfolding, reduced protein interactions, and/or sequence mutations). Further studies investigating the basic processes involved in mGluR5 translation, dimerization and regulation (under control conditions) will also be helpful in determining the exact processes underlying mGluR5 regulation, the role of mGluR5 in schizophrenia pathology, and how this may impact on novel therapeutics aimed at mGluR5.

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References

1. Beneyto M, Meador-Woodruff JH (2007) Lamina-Specific Abnormalities of NMDA Receptor-Associated Postsynaptic Protein Transcripts in the Prefrontal Cortex in Schizophrenia and Bipolar Disorder. Neuropsychopharmacology 33:2175–2186. doi: 10.1038/sj.npp.1301604

2. Catts VS, Fung SJ, Long LE, Joshi D, Vercammen A, Allen KM, Fillman SG, Rothmond DA, Sinclair D, Tiwari Y, Tsai S-Y, Weickert TW, Shannon Weickert C (2013) Rethinking schizophrenia in the context of normal neurodevelopment. Front Cell Neurosci. doi: 10.3389/fncel.2013.00060

3. Corti C, Crepaldi L, Mion S, Roth AL, Xuereb JH, Ferraguti F (2007) Altered dimerization of metabotropic glutamate receptor 3 in schizophrenia. Biol Psychiatry 62:747–755. doi: 10.1016/j.biopsych.2006.12.005

4. Corti C, Xuereb JH, Crepaldi L, Corsi M, Michielin F, Ferraguti F (2011) Altered levels of glutamatergic receptors and Na+/K+ ATPase-α1 in the prefrontal cortex of subjects with schizophrenia. Schizophr Res 128:7–14. doi: 10.1016/j.schres.2011.01.021

5. El Moustaine D, Granier S, Doumazane E, Scholler P, Rahmeh R, Bron P, Mouillac B, Banères J-L, Rondard P, Pin J-P (2012) Distinct roles of metabotropic glutamate receptor dimerization in agonist activation and G-protein coupling. Proc Natl Acad Sci U S A 109:16342–16347. doi: 10.1073/pnas.1205838109

6. Engmann O, Hortobágyi T, Pidsley R, Troakes C, Bernstein H-G, Kreutz MR, Mill J, Nikolic M, Giese KP (2011) Schizophrenia is associated with dysregulation of a Cdk5 activator that regulates synaptic protein expression and cognition. Brain 134:2408–2421. doi: 10.1093/brain/awr155

7. Fatemi SH, Folsom TD (2014) Existence of monomer and dimer forms of mGluR5, under reducing conditions in studies of postmortem brain in various psychiatric disorders. Schizophr Res 158:270–271.

8. Fatemi SH, Folsom TD, Rooney RJ, Thuras PD (2013) mRNA and protein expression for novel GABAA receptors θ and ρ2 are altered in schizophrenia and mood disorders; relevance to FMRP-mGluR5 signaling pathway. Transl Psychiatry 3:e271. doi: 10.1038/tp.2013.46

9. Fuxe K, Marcellino D, Rivera A, Diaz-Cabiale Z, Filip M, Gago B, Roberts DCS, Langel U, Genedani S, Ferraro L, de la Calle A, Narvaez J, Tanganelli S, Woods A, Agnati LF (2008) Receptor-receptor interactions within receptor mosaics. Impact on neuropsychopharmacology. Brain Res Rev 58:415–452.

10. Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 4:117.

11. Han M, Newell K, Zavitsanou K, Deng C, Huang XF (2008) Effects of antipsychotic medication on muscarinic M1 receptor mRNA expression in the rat brain. J Neurosci Res 86:457–464.

12. Hu J-H, Yang L, Kammermeier PJ, Moore CG, Brakeman PR, Tu J, Yu S, Petralia RS, Li Z, Zhang P-W, Park JM, Dong X, Xiao B, Worley PF (2012) Preso1 dynamically regulates group I metabotropic glutamate receptors. Nat Neurosci 15:836–844. doi: 10.1038/nn.3103

13. Javitt DC (2012) Twenty-five years of glutamate in schizophrenia: Are we there yet? Schizophr Bull 38:911–913.

14. Kitano J, Kimura K, Yamazaki Y, Soda T, Shigemoto R, Nakajima Y, Nakanishi S (2002) Tamalin, a PDZ Domain-Containing Protein, Links a Protein Complex Formation of Group 1 Metabotropic Glutamate Receptors and the Guanine Nucleotide Exchange Factor Cytohesins. J Neurosci 22:1280–1289.

15. Kitano J, Yamazaki Y, Kimura K, Masukado T, Nakajima Y, Nakanishi S (2003) Tamalin is a scaffold protein that interacts with multiple neuronal proteins in distinct modes of protein-protein association. J Biol Chem 278:14762–14768. doi: 10.1074/jbc.M300184200

16. Lee HW, Choi J, Shin H, Kim K, Yang J, Na M, Choi SY, Kang GB, Eom SH, Kim H, others (2008) Preso, a novel PSD-95-interacting FERM and PDZ domain protein that regulates dendritic spine morphogenesis. J Neurosci 28:14546–14556.

17. Matosin N, Frank E, Deng C, Huang X-F, Newell KA (2013) Metabotropic glutamate receptor 5 binding and protein expression in schizophrenia and following antipsychotic drug treatment. Schizophr Res 146:170–176. doi: 10.1016/j.schres.2013.01.018

18. Matosin N, Newell KA (2013) Metabotropic glutamate receptor 5 in the pathology and treatment of schizophrenia. Neurosci Biobehav Rev 37:256–268. doi: 10.1016/j.neubiorev.2012.12.005

19. McCullumsmith RE, Hammond JH, Shan D, Meador-Woodruff JH (2014) Postmortem Brain: An Underutilized Substrate for Studying Severe Mental Illness. Neuropsychopharmacology 39:65–87. doi: 10.1038/npp.2013.239

20. Milligan G (2004) G Protein-Coupled Receptor Dimerization: Function and Ligand Pharmacology. Mol Pharmacol 66:1–7. doi: 10.1124/mol.104.000497

21. Miyamoto S, Miyake N, Jarskog LF, Fleischhacker WW, Lieberman JA (2012) Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. Mol Psychiatry 17:1206–1227. doi: 10.1038/mp.2012.47

22. Mochizuki R, Ishizuka Y, Yanai K, Koga Y, Fukamizu A (1999) Molecular cloning and expression of human neurochondrin-1 and -2. Biochim Biophys Acta BBA - Gene Struct Expr 1446:397–402. doi: 10.1016/S0167-4781(99)00120-7

23. Newell KA, Matosin N (2014) Rethinking metabotropic glutamate receptor 5 pathological findings in psychiatric disorders: implications for the future of novel therapeutics. BMC Psychiatry 14:23. doi: 10.1186/1471-244X-14-23

24. Newell KA, Matosin N, Lum JS (2014) Metabotropic Glutamate Receptors in the Pathophysiology and Treatment of Schizophrenia and Major Depression. In: Olive MF (ed) Metabotropic Glutamate Recept. Mol. Mech. Role Neurol. Disord. Pharmacol. Eff. Nova Science Publishers Inc, New York, pp 73–106

25. Ohnuma T, Augood SJ, Arai H, McKenna PJ, Emson PC (1998) Expression of the human excitatory amino acid transporter 2 and metabotropic glutamate receptors 3 and 5 in the prefrontal cortex from normal individuals and patients with schizophrenia. Mol Brain Res 56:207–217. doi: 10.1016/S0169-328X(98)00063-1

26. Paxinos G, Watson C (2007) The Rat Brain in Stereotaxic Coordinates: Hard Cover Edition, 6th ed. Academic press

27. Piton A, Gauthier J, Hamdan FF, Lafrenière RG, Yang Y, Henrion E, Laurent S, Noreau A, Thibodeau P, Karemera L, Spiegelman D, Kuku F, Duguay J, Destroismaisons L, Jolivet P,

Côté M, Lachapelle K, Diallo O, Raymond A, Marineau C, Champagne N, Xiong L, Gaspar C, Rivière J-B, Tarabeux J, Cossette P, Krebs M-O, Rapoport JL, Addington A, DeLisi LE, Mottron L, Joober R, Fombonne E, Drapeau P, Rouleau GA (2010) Systematic resequencing of X-chromosome synaptic genes in autism spectrum disorder and schizophrenia. Mol Psychiatry 16:867–880. doi: 10.1038/mp.2010.54

28. Radulovic J, Tronson NC (2012) Preso1, mGluR5 and the machinery of pain. Nat Neurosci 15:805–807. doi: 10.1038/nn.3118

29. Romano C, Yang W-L, O'Malley KL (1996) Metabotropic Glutamate Receptor 5 Is a Disulfide-linked Dimer. J Biol Chem 271:28612–28616. doi: 10.1074/jbc.271.45.28612

30. Shinozaki K, Maruyama K, Kume H, Kuzume H, Obata K (1997) A Novel Brain Gene, Norbin, Induced by Treatment of Tetraethylammonium in Rat Hippocampal Slice and Accompanied with Neurite-Outgrowth in Neuro 2a Cells. Biochem Biophys Res Commun 240:766–771. doi: 10.1006/bbrc.1997.7660

31. Spellmann I, Rujescu D, Musil R, Mayr A, Giegling I, Genius J, Zill P, Dehning S, Opgen-Rhein M, Cerovecki A, Hartmann AM, Schäfer M, Bondy B, Müller N, Möller H-J, Riedel M (2011) Homer-1 polymorphisms are associated with psychopathology and response to treatment in schizophrenic patients. J Psychiatr Res 45:234–241. doi: 10.1016/j.jpsychires.2010.06.004

32. Tai H-C, Besche H, Goldberg AL, Schuman EM (2010) Characterization of the Brain 26S Proteasome and its Interacting Proteins. Front Mol Neurosci 3:1–19. doi: 10.3389/fnmol.2010.00012

33. Tandon R, Nasrallah HA, Keshavan MS (2010) Schizophrenia, "just the facts" 5. Treatment and prevention. Past, present, and future. Schizophr Res 122:1–23. doi: 10.1016/j.schres.2010.05.025

34. Timms AE, Dorschner MO, Wechsler J, Choi KY, Kirkwood R, Girirajan S, Baker C, Eichler EE, Korvatska O, Roche KW, Horwitz MS, Tsuang DW (2013) Support for the N-Methyl-D-Aspartate Receptor Hypofunction Hypothesis of Schizophrenia From Exome Sequencing in Multiplex Families. JAMA Psychiatry Chic Ill 70:582–590. doi: 10.1001/jamapsychiatry.2013.1195

35. Ting JT, Peça J, Feng G (2012) Functional Consequences of Mutations in Postsynaptic Scaffolding Proteins and Relevance to Psychiatric Disorders. Annu Rev Neurosci 35:49–71. doi: 10.1146/annurev-neuro-062111-150442

36. Volk DW, Eggan SM, Lewis DA (2010) Alterations in metabotropic glutamate receptor 1a and regulator of G protein signaling 4 in the prefrontal cortex in schizophrenia. Am J Psychiatry 167:1489–1498. doi: 10.1176/appi.ajp.2010.10030318

37. Wang H, Nong Y, Bazan F, Greengard P, Flajolet M (2010) Norbin: A promising central nervous system regulator. Commun Integr Biol 3:487.

38. Wang H, Westin L, Nong Y, Birnbaum S, Bendor J, Brismar H, Nestler E, Aperia A, Flajolet M, Greengard P (2009) Norbin Is an Endogenous Regulator of Metabotropic Glutamate Receptor 5 Signaling. Science 326:1554–1557. doi: 10.1126/science.1178496

39. Weickert CS, Fung SJ, Catts VS, Schofield PR, Allen KM, Moore LT, Newell KA, Pellen D, Huang X-F, Catts SV, Weickert TW (2012) Molecular evidence of N-methyl-D-aspartate receptor hypofunction in schizophrenia. Mol Psychiatry. doi: 10.1038/mp.2012.137

40. Weickert CS, Sheedy D, Rothmond DA, Dedova I, Fung S, Garrick T, Wong J, Harding AJ, Sivagnanansundaram S, Hunt C, Duncan C, Sundqvist N, Tsai SY, Anand J, Draganic D, Harper C (2010) Selection of reference gene expression in a schizophrenia brain cohort. Aust N Z J Psychiatry 44:59–70. doi: 10.3109/00048670903393662

41. Weston-Green K, Huang XF, Lian J, Deng C (2012) Effects of olanzapine on muscarinic M3 receptor binding density in the brain relates to weight gain, plasma insulin and metabolic hormone levels. Eur Neuropsychopharmacol 22:364–373.

Tables

Table 1 Summary of postmortem subject demography for control and schizophrenia groups.

	Control	Schizophrenia
Brain pH	6.66 ± 0.29	6.61 ± 0.30
Postmortem interval (hours)	24.8 ±10.97	28.8 ± 14.07
RNA integrity number	7.3 ± 0.57	7.3 ± 0.58
Age at Death (years)	51.1 ± 2.40 (range: 18–78)	51.3 ± 2.32 (range: 27-75)
Gender	7 F, 30 M	13 F, 24 M
Hemisphere	23 L, 14 R	17 L, 20 R
Freezer Storage Time (months)	81.6 ± 42.7	91.9 ± 37.2
Age of disease onset (years)	-	23.7 ± 6.1
Duration of illness (years)	-	27.62 ± 13.81
Number patients with antidepressant medication history	-	19
Lifetime antipsychotic drug medication (standardized to chlorpromazine equivalent: mg)	-	702 ± 90

Abbreviations: F: female; M: male; L: left; R: right. Data are expressed as mean ± standard deviation

Figure Legends

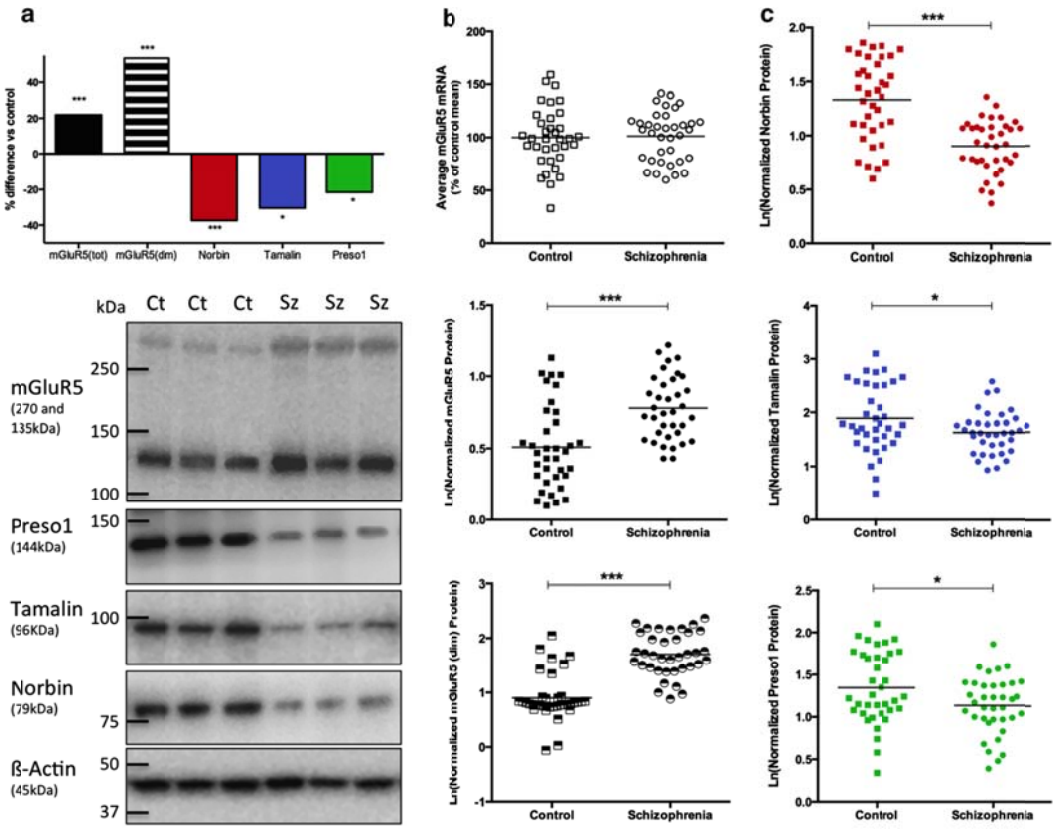


Fig. 1 Protein levels of mGluR5 and mGluR5 signaling partners Norbin, Tamalin and Preso1 in the DLPFC of subjects with schizophrenia and matched controls. Representative ß-actin are shown from mGluR5 membrane only **a.** Graphical depiction of percentage differences between protein measures in patients with schizophrenia compared to comparison subjects, as seen in representative immunoblots. **b.** Normalized mGluR5 mRNA, and protein levels of mGluR5 (total and dimer) of schizophrenia subjects. mGluR5 mRNA expression was unaltered, whilst mGluR5 protein expression (total and dimer) was significantly increased. **c.** Normalized protein expression of mGluR5 endogenous regulators, Norbin, Tamalin and Preso1. All mGluR5 endogenous regulators were significantly decreased in schizophrenia subjects compared to controls. **Abbreviations:** Ct: control; Dim: dimer; DLPFC: dorsolateral prefrontal cortex; Ln: natural logarithm; kDa: kilodaltons; mGluR5: metabotropic glutamate receptor 5; Sz: schizophrenia; Tot: total. ***P<0.05, **P<0.01, ***P<0.001.**

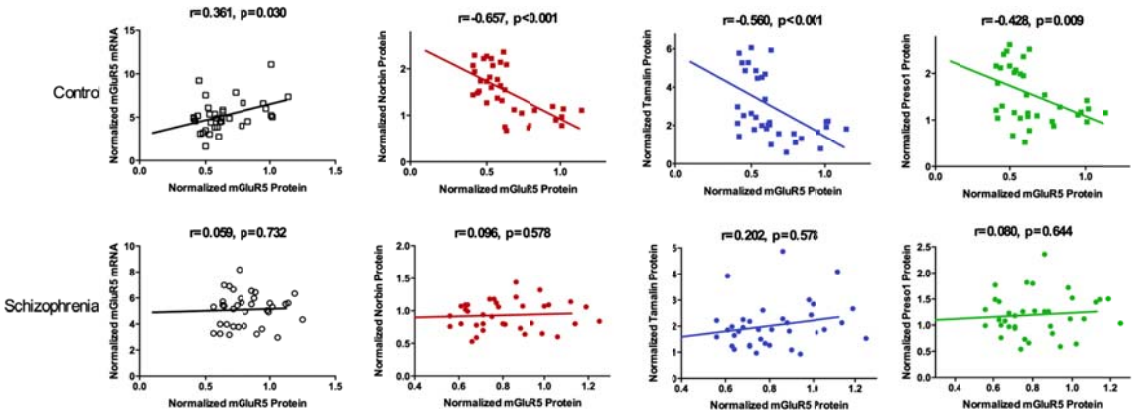


Fig. 2 Correlation plots depicting the relationship between mGluR5 protein and mGluR5 mRNA, and protein levels of mGluR5 endogenous regulators. mGluR5 protein was positively associated with mGluR5 mRNA in the control group, but not the schizophrenia group. Furthermore, protein measures of mGluR5 and its endogenous regulators were strongly and negatively associated in the control group, however, this association was lost in the schizophrenia group.

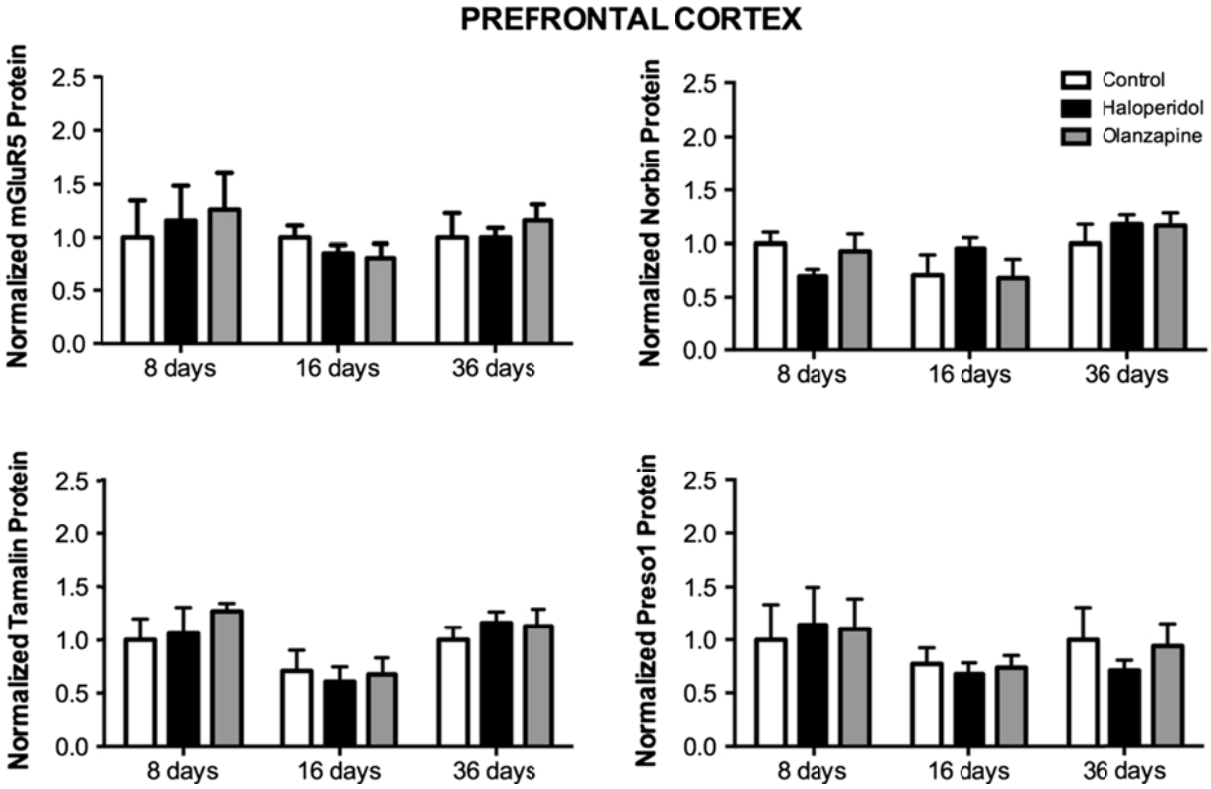


Fig. 3 Effects of current antipsychotic drug treatment on mGluR5, Norbin, Tamalin and Preso1. Protein levels in the prefrontal cortex of rats treated for 8, 16 or 36 days with haloperidol (0.3mg/kg/day) or olanzapine (3mg/kg/day). There were no significant effects of antipsychotic drug treatment on levels of mGluR5 or its endogenous regulators.

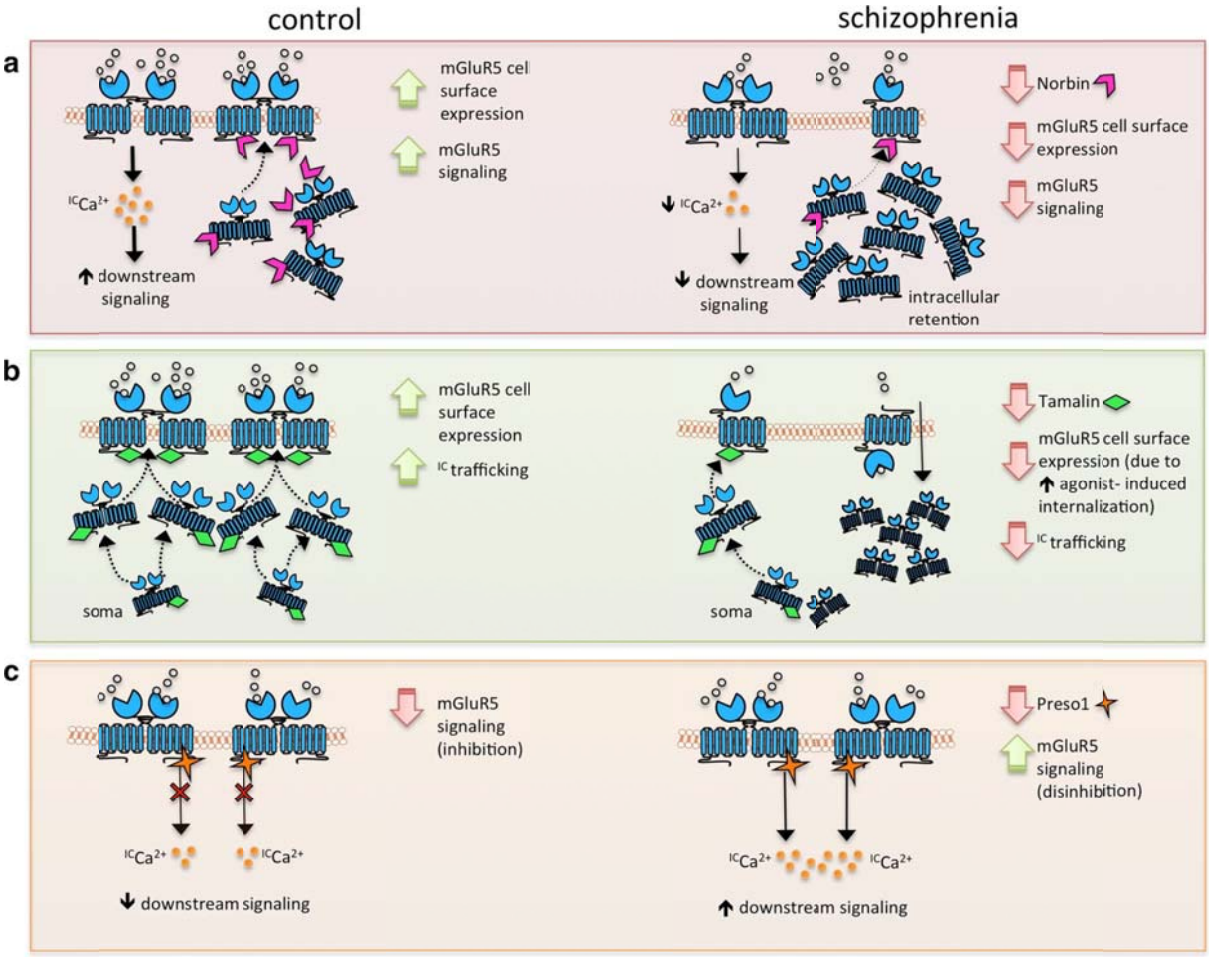
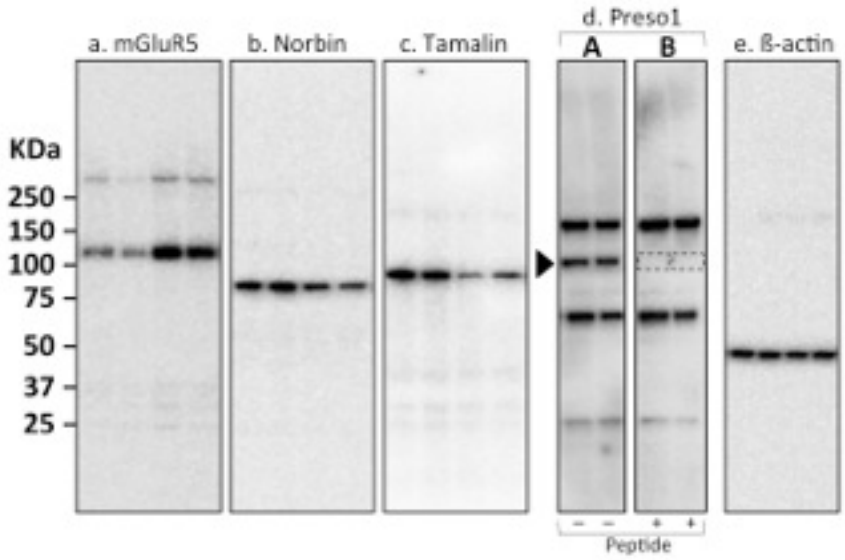


Fig. 4 Proposed model of mGluR5 dysregulation. In all panels (**a-c**), control conditions are depicted on the left, whilst a proposed model of mGluR5 dysregulation is depicted on the right. **a.** Under controlled conditions, Norbin (pink) promotes mGluR5 cell surface expression and positively regulates mGluR5 signaling [38]. In schizophrenia, reduced levels of Norbin are proposed to result in decreased mGluR5 surface expression, resulting in intracellular retention of mGluR5 and reduced mGluR5 signaling. **b.** Tamalin reportedly modulates mGluR5 cell-surface expression [34] as well as trafficking mGluR5 from the soma to the neuritic processes [14]. In schizophrenia, reduced levels of Tamalin protein are hypothesized to cause decreased mGluR5 cell surface expression due to agonist-induced internalization, and impaired intracellular trafficking of mGluR5, leading to intracellular retention. **c.** Under control conditions, Preso1 is an inhibitor of mGluR5 signaling by reducing release of calcium from intracellular stores, inhibiting downstream signaling [12, 28]; thus reduced levels of Preso1 in schizophrenia is proposed to disinhibit mGluR5 signaling and result in increased intracellular calcium concentrations and downstream signaling.

Supplementary Materials

Supplementary Figures:



Supplementary Fig. 1 Full-length immunoblots for (a) mGluR5, (b) Norbin, (c) Tamalin, and (d) Preso1 (A. with no peptide, B. with blocking peptide). mGluR5 (a) appeared as 2 distinct bands at 135kDa (monomer) and 270kDa (dimer) and these bands have previously been shown to be specific for mGluR5 in HEK cells transfected with mGluR5 (www.abcam.com/Metabtropic-Glutamate-Receptor-5-antibody-ab27190). Norbin (b) and Tamalin (c) were reliably detected as single bands at the expected molecular weights (79kDa and 96kDa respectively) with no non-specific binding. The Preso1 antibody (Santa Cruz sc-242862) produced several bands at various molecular weights (d); A single specific band was verified at 144kDa by peptide pre-absorption/neutralization, and this band was quantified. The representation of ß-actin (e); is taken from the mGluR5 immunoblot which was reprobed with ß-actin as a loading control. ß-actin was measured in the same way for all immunoblots in this study.

Supplementary Methods:

Determination of Preso1 specificity by peptide pre-absorption (FRMPD4 Antibody, Santa Cruz: sc-242862 and sc-242862 P)

Peptide pre-absorption/neutralization was performed according to the protocol described by Santa Cruz (http://www.scbt.com/protocols.html?protocol=peptide_neutralization). The highest concentration of antibody dilution for which a consistently positive result was achieved, was determined to be 1:1000. For blocking/competition, 10µL of antibody, 50µL of peptide and 500µL of PBS, was combined and incubated for 2 hours at room temperature. The antibody/peptide solution was subsequently diluted up to 10mL with 0.5% skim milk in PBST, and applied to membranes containing 15µg of electrophoresed protein from the rat prefrontal cortex. These immunoblots were prepared and electrophoresed as described in the main methods.

Supplementary Tables:

Supplementary Table 1 Immunoblot conditions: all immunoblots were run on bis-tris polyacrymalide gels (4-12% Bis-Tris, BioRad), at a loading concentration of 5µg (human) or 10µg (rat) of total protein per lane. Samples were reduced with laemmli buffer containing ß-mercaptoethanol.

Protein				1° antibody			2° antibody			
	Buffer	Block	Denaturing	Dilution (human, rat)	Temp.	Time	Type	Dilution	Temp	Time
mGluR5	PBST	Skim milk	95˚C for 5min	1:250, 1:500	4ºC	16 hr	Rabbit	1:3000	RT	1 hr
Tamalin	PBST	BSA	95˚C for 5min	1:100, 1:1000	4ºC	16 hr	Rabbit	1:3000	RT	1 hr
Norbin	PBST	BSA	95˚C for 5min	1:500, 1:500	4ºC	16 hr	Rabbit	1:3000	RT	1 hr
Preso1	PBST	BSA	95˚C for 5min	1:100, 1:1000	4ºC	16 hr	Goat	1:3000	RT	1 hr
ß-actin	As for primary antibody			1:5000, 1:5000	RT	1 hr	Mouse	1:3000	RT	1 hr

Abbreviations: BSA: Bovine serum albumin; mGluR5: metabotropic glutamate receptor 5; PBST: phosphate-buffered saline containing Tween-20; RT: room temperature.

Supplementary Table 2 Spearman's Correlations for continuous clinical and demographic variables and mGluR5 (mRNA, total and dimer), Norbin, Tamalin and Preso1 protein levels in the postmortem dorsolateral prefrontal cortex. Significant values ($p<0.05$) are highlighted in bold.

Variable	All subjects						Controls						Schizophrenia					
	mGluR5 mRNA	**mGluR5 (total)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**Preso1**	**mGluR5 mRNA**	**mGluR5 (total)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**Preso1**	**mGluR5 mRNA**	**mGluR5 (total)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**Preso1**
Brain pH	**r = 0.267**	r = 0.001	r = 0.006	r = 0.123	r = -0.021	r = -0.062	r = 0.137	r = 0.002	r = -0.133	r = -0.028	r = -0.150	r = 0.110	r = 0.351	r = 0.063	r = 0.176	r = 0.175	r = 0.096	r = -0.078
	p = 0.021	p = 0.994	p = 0.960	p = 0.297	p = 0.860	p = 0.598	p = 0.420	p = 0.992	p = 0.431	p = 0.870	p = 0.375	p = 0.517	p = 0.033	p = 0.714	p = 0.303	p = 0.301	p = 0.574	p = 0.645
Age at Death	**r = -0.242**	r = -0.037	r = 0.012	r = -0.009	r = 0.175	r = 0.108	**r = -0.417**	r = -0.110	r = 0.103	r = 0.171	**r = 0.353**	r = 0.218	r = -0.106	r = 0.192	r = 0.095	r = -0.193	r = 0.012	r = 0.009
	p = 0.037	p = 0.758	p = 0.920	p = 0.939	p = 0.136	p = 0.358	**p = 0.010**	p = 0.524	p = 0.543	p = 0.311	**p = 0.032**	p = 0.194	p = 0.531	p = 0.261	p = 0.580	p = 0.253	p = 0.946	p = 0.959
RNA integrity number	**r = 0.229**	r = 0.038	r = 0.134	r = 0.122	r = 0.069	r = 0.098	r = 0.050	r = -0.061	r = 0.047	r = 0.046	r = -0.102	r = -0.020	**r = 0.437**	r = 0.176	**r = 0.355**	r = 0.312	r = 0.305	r = 0.265
	p = 0.050	p = 0.748	p = 0.258	p = 0.301	p = 0.560	p = 0.408	p = 0.767	p = 0.724	p = 0.784	p = 0.788	p = 0.547	p = 0.905	**p = 0.007**	p = 0.306	**p = 0.034**	p = 0.060	p = 0.066	p = 0.113
Post-mortem interval	r = 0.124	r = -0.055	r = 0.174	r = 0.132	r = 0.102	r = 0.002	r = -0.017	r = -0.179	r = -0.081	r = 0.155	r = 0.184	r = 0.119	r = 0.213	r = -0.066	r = 0.273	r = -0.259	r = 0.083	r = -0.027
	p = 0.292	p = 0.649	p = 0.140	p = 0.262	p = 0.385	p = 0.987	p = 0.921	p =0.297	p = 0.636	p = 0.358	p = 0.275	p = 0.483	p = 0.205	p =0.703	p = 0.107	p = 0.122	p = 0.623	p = 0.874
Freezer storage time	r = 0.043	r =0.221	r = 0.146	**r = -0.132**	**r = -0.446**	**r = -0.369**	r = 0.301	**r = 0.501**	r = 0.275	**r = -0.348**	**r = -0.533**	**r = -0.391**	r = -0.254	**r = -0.373**	**r = -0.446**	**r = -0.375**	r = -0.242	r = -0.245
	p = 0.715	p = 0.062	p = 0.217	**p = 0.001**	**p < 0.001**	**p = 0.001**	p = 0.070	**p =0.002**	p = 0.099	**p = 0.035**	**p< 0.001**	**p = 0.017**	p = 0.129	**p =0.025**	**p = 0.006**	**p = 0.022**	p = 0.149	p = 0.145
Brain weight	**r = 0.270**	r = -0.009	r = -0.114	r = 0.120	r = 0.065	r = 0.039	r = 0.181	r = -0.022	r = -0.189	r = 0.055	r = -0.001	r = 0.110	**r = 0.398**	r = 0.057	r = -0.061	r = -0.023	r = 0.084	r = -0.103
	p = 0.020	p = 0.940	p = 0.339	p = 0.309	p = 0.581	p = 0.740	p = 0.282	p =0.899	p = 0.263	p = 0.747	p = 0.993	p = 0.515	**p = 0.015**	p =0.470	p = 0.724	p = 0.895	p = 0.620	p = 0.543
Age of disease onset	-	-	-	-	-	-	-	-	-	-	-	-	r = -0.213	r = 0.130	r = -0.072	**r = -0.362**	r = -0.108	**r = -0.364**
	-	-	-	-	-	-	-	-	-	-	-	-	p = 0.206	p =0.450	p = 0.674	**p = 0.028**	p = 0.524	**p = 0.027**
Duration of illness	-	-	-	-	-	-	-	-	-	-	-	-	r = -0.009	r = 0.120	r = 0.082	r = -0.076	r = -0.079	r = 0.172
	-	-	-	-	-	-	-	-	-	-	-	-	p = 0.959	p =0.486	p = 0.634	p = 0.654	p = 0.641	p = 0.308
Lifetime antipsychotic drug medication	-	-	-	-	-	-	-	-	-	-	-	-	r = 0.038	r = 0.015	p = 0.051	r = 0.043	r = 0.037	r = -0.030
	-	-	-	-	-	-	-	-	-	-	-	-	p = 0.823	p =0.932	p = 0.768	p = 0.802	p = 0.826	p = 0.861

Supplementary Table 3 Spearman's correlations for associations between mGluR5 (total and dimer protein measures, and mRNA) and novel mGluR5 signaling partners, Norbin, Tamalin and Preso1 protein levels in the dorsolateral prefrontal cortex of the postmortem schizophrenia brain. Significant values ($p<0.05$) are highlighted in bold.

CONTROL	mGluR5 total (protein)	mGluR5 dimer (protein)	mGluR5 expression (mRNA)
mGluR5(tot) (protein)	- -	**r = 0.512 p = 0.001**	**r = 0.361 p = 0.030**
mGluR5(dim) (protein)	**r = 0.512 p = 0.001**	- -	r = 0.050 p = 0.770
mGluR5 (mRNA)	**r = 0.361 p = 0.030**	r = 0.050 p = 0.770	- -
Norbin (protein)	**r = -0.657 p < 0.001**	**r = -0.227 p = 0.018**	**r = -0.500 p = 0.002**
Tamalin (protein)	**r = -0.560 p < 0.001**	r = -0.196 p = 0.254	**r = -0.470 p = 0.003**
Preso1 (protein)	**r = -0.428 p = 0.009**	**r = -0.367 p = 0.026**	**r = -0.372 p = 0.023**

SCHIZOPHRENIA	mGluR5 total (protein)	mGluR5 dimer (protein)	mGluR5 expression (mRNA)
mGluR5(tot) (protein)	- -	**r = 0.608 p < 0.001**	r = 0.059 p = 0.732
mGluR5(dim) (protein)	**r = 0.608 p < 0.001**	- -	r = 0.125 p = 0.468
mGluR5 (mRNA)	r = 0.059 p = 0.732	r = 0.125 p = 0.468	- -
Norbin (protein)	r = 0.096 p = 0.578	**r = 0.388 p = 0.019**	**r = 0.466 p = 0.004**
Tamalin (protein)	r = 0.202 p = 0.238	r = 0.175 p = 0.307	r = 0.051 p = 0.764
Preso1 (protein)	r = 0.080 p = 0.644	r = -0.069 p = 0.689	r = -0.009 p = 0.960