Increases in [3H]Muscimol and [3H]Flumazenil Binding in the Dorsolateral Prefrontal Cortex in Schizophrenia Are Linked to α4 and γ2S mRNA Levels Respectively

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Abstract

Background: GABA$_A$ receptors (GABA$_A$R) are composed of several subunits that determine sensitivity to drugs, synaptic localisation and function. Recent studies suggest that agonists targeting selective GABA$_A$R subunits may have therapeutic value against the cognitive impairments observed in schizophrenia. In this study, we determined whether GABA$_A$R binding deficits exist in the dorsolateral prefrontal cortex (DLPFC) of people with schizophrenia and tested if changes in GABA$_A$R binding are related to the changes in subunit mRNAs. The GABA orthosteric and the benzodiazepine allosteric binding sites were assessed autoradiographically using [3H]Muscimol and [3H]Flumazenil, respectively, in a large cohort of individuals with schizophrenia (n = 37) and their matched controls (n = 37). We measured, using qPCR, mRNA of β (β1, β2, β3), γ (γ1, γ2, γ2S for short and γ2L for long isoform, γ3) and δ subunits and used our previous measurements of GABA$_A$R α subunit mRNAs in order to relate mRNAs and binding through correlation and regression analysis.

Results: Significant increases in both [3H]Muscimol (p = 0.016) and [3H]Flumazenil (p = 0.012) binding were found in the DLPFC of schizophrenia patients. Expression levels of mRNA subunits measured did not show any significant difference in schizophrenia compared to controls. Regression analysis revealed that in schizophrenia, the [3H]Muscimol binding variance was most related to α4 mRNA levels and the [3H]Flumazenil binding variance was most related to γ2S subunit mRNA levels. [3H]Muscimol and [3H]Flumazenil binding were not affected by the lifetime anti-psychotics dose (chlorpromazine equivalent).

Conclusions: We report parallel increases in orthosteric and allosteric GABA$_A$R binding sites in the DLPFC in schizophrenia that may be related to a "shift" in subunit composition towards α4 and γ2S respectively, which may compromise normal GABAergic modulation and function. Our results may have implications for the development of treatment strategies that target specific GABA$_A$R receptor subunits.

Introduction

The majority of inhibition in the cerebral cortex of the mammalian brain is mediated by the interaction of the neurotransmitter γ-aminobutyric acid (GABA) with ionotropic GABA$_A$ receptors (GABA$_A$R). These ligand-gated channels allow chloride ions to enter neurons, hyperpolarising their membrane and attenuating their activity. Dysfunctional cortical inhibition has been suggested as a mechanism through which symptoms of schizophrenia are mediated [1].

The functional GABA$_A$R is composed of five subunits, typically two α's, two β's and a single γ/δ or ε subunit, assembled into a pentamer. Nineteen subunits of the GABA$_A$R can be grouped together in 8 families through sequence homology (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) [2]. The prevalent γ2 subunit has a well-known splice variant in which exon 10 (24 base pairs) is removed producing the γ2 short isoform (γ2S) [3,4]. Multiple binding sites are found on the GABA$_A$R with an orthosteric binding site for GABA at the interface of the α and β subunits and an allosteric binding site for benzodiazepines at the junction of an α (α1, α2, α3 or α5) and a γ subunit [2,5–9]. The specific subunits present determine the pharmacological sensitivity, synaptic localisation and function of the GABA$_A$R [10] and these subunits change during normal human cortical development as well as in disease states [5,11–14].

Changes in the expression and function of GABA$_A$R have been strongly implicated in schizophrenia. Binding studies targeting the orthosteric site of GABA$_A$R have robustly showed an increase in [³H]Muscimol binding particularly in the dorsolateral prefrontal cortex (DLPFC), and also in the caudate nucleus, posterior and anterior cingulate cortices, superior temporal gyrus, and hippo-campal formation of post-mortem schizophrenic brains compared to controls [15–22]. This finding has been reinforced by the observation of increased GABA$_A$R protein subunit α2 in the prefrontal cortex in schizophrenia [23]. However, studies on benzodiazepine binding (to the allosteric GABA$_A$ receptor site) in schizophrenia show conflicting results: Increased [³H]Flunitraze-pam binding has been reported across a variety of cortical areas including the medial frontal cortex, orbitofrontal cortex, temporal gyrus, and the parahippocampal cortex with the DLPFC yet to be assessed [24,25]. Other post-mortem [26] or imaging [27–29] studies have reported no changes or decreased [30] benzodiaze-pine binding in schizophrenia.

At the mRNA level, results have also been inconsistent with studies reporting an increase in α1 and α5 [31], a decrease in α1, α5 and β2 [12], a decrease in γ2S [32] and our own group observing no diagnostic change in α1–4 and a decrease in α5 in people with schizophrenia [11]. Presumably, a substantial heterogeneity in the pathophysiology of schizophrenia coupled with small cohort sizes contributes to the lack of consistency amongst the above findings. Clarification on the alterations in the expression of GABA$_A$R subunits is important as they may contribute to changes in both the orthosteric and allosteric binding sites in schizophrenia [5,33,34] affecting pharmacological receptor properties and response to treatment. However, these parameters have not been studied together in the same cohort.

Therefore, in the present study, we used a fairly large cohort of 37 schizophrenia cases and their matched controls to examine whether deficits in the GABA orthosteric and benzodiazepine allosteric binding site of the GABA$_A$R exist in the DLPFC in schizophrenia. An important aspect of our study was to determine, in the same cohort, if mRNA expression of the subunits involved in the formation of these binding sites (α and β for GABA binding site and α and γ or δ for benzodiazepine binding site) were also different in schizophrenia and to determine how they may relate to changes in binding levels.

Materials and Methods

2.1. Human post-mortem brain samples and ethics statement

All research was conducted in accordance with the latest version of the declaration of Helsinki and approved by the Human Research Ethics Committees at the University of Wollongong (#HE99/22) and at the University of New South Wales (#HREC0761) that follow the guidelines set out in the National Statement on Ethical Conduct in Research involving humans (http://www.nhmrc.gov.au). Written consent for use of tissues in the study was obtained from next of kin. Characterization and tissue preparation for this Australian schizophrenia cohort has been described in detail previously [35]. Tissue samples and frozen cryostat sections were prepared from the large cohort of schizophrenia (n = 37) and controls (n = 37) cases matched for age, gender, pH, and post-mortem interval (see Table 1 for demographics information) [35].

2.2. Tissue dissection and section preparation

Tissue dissection has been described in detail previously [35]. Briefly, at autopsy, brain weight and volume were determined

Table 1. Summary of Cohort Demographics.

	n	Age at death (yrs)	pH	PMI (hrs)	Freezer Storage (Months)	Brain Volume (ml)	Brain Weight (g)	RIN	Age of Onset	Duration of Illness	Lifetime chlorpromazine (mg)
Control	37	51.1±14.6	6.7±0.3	24.8±11.0	69.6±42.7	1438.2±121.7	1446.4±127.1	7.3±0.6	NA	NA	NA
Schizophrenia	37	51.3±14.1	6.6±0.3	28.5±13.8	79.9±37.2	1408.1±165.5	1394.3±164.0	7.3±0.6	23.7±6.1	27.6±13.8	7.9×8.0×10⁶
Depressive§	4	52.3±24.0	6.9±0.1	27.5±15.8	46.8±20.7	1517.3±125.3	1490.0±94.2	7.4±0.5	24.5±9.3	27.8±21.3	4.4±5.5×10⁶
Bipolar§	3	50.7±14.6	6.6±0.3	23.7±5.9	71.7±29.7	1449.7±95.7	1396.7±105.0	7.2±0.3	29.3±2.1	21.3±12.5	1.1±1.0×10⁶
Paranoid	16	51.6±13.0	6.6±0.3	32.2±16.0	83.6±27.5	1379.4±184.1	1364.4±177.7	7.2±0.6	23.6±6.4	28.1±12.8	7.7±7.8×10⁶
Disorganized	5	54.0±24.2	6.7±0.3	25.7±6.8	74.8±42.8	1406.6±190.5	1385.2±184.2	7.2±0.7	19.8±1.8	34.2±12.5	8.9±4.3×10⁶
Undifferentiated	7	48.6±16.9	6.5±0.2	28.0±14.9	82.0±53.2	1420.3±147.0	1433.0±175.1	8.0±0.5	21.9±3.9	27.0±16.4	9.9±1.2×10⁶
Residual	2	51.0±0.0	6.1±0.6	16.5±6.4	136.0±4.2	1317.0±231.9	1325.0±233.3	6.6±0.5	31.0±5.7	20.0±5.7	3.4±1.8×10⁶

Abbreviations: yrs, years; hrs, hours; PMI, post-mortem interval; RIN, RNA integrity number.
Average values ± SD for continuous variables are shown.
§Schizoaffective.
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[36]. The fresh tissue was cut into ~1 cm coronal slices and various anatomical areas were dissected for separate freezing. For the DLPFC (Brodmann's area 46, Figure 1A) dissections, frozen tissue was dissected on a dry ice platform using a dental drill (Cat# UP500-UG33, Brasseler, USA) for homogenates. DLPFC tissue (average weight of tissue ~0.5 g grey matter tissue from the crown of the middle frontal gyrus) was obtained from an adjacent coronal slab corresponding to the middle one-third (rostral caudally) found anterior to the genu of the corpus callosum. Coronal tissue sections of the DLPFC containing the superior and inferior frontal sulcus were cut (14 μm) on a cryostat, thaw mounted onto microscope slides and stored at −80°C until use.

2.3. In vitro autoradiography

[³H]Muscimol autoradiography was carried out based on the method described previously [17,21,30] with minor modifications. All sections were processed simultaneously to minimize experimental variance. On the day of the experiment, sections were pre-incubated three times for 5 min at 4°C in 50 mM Tris citrate buffer (pH 7.0). Sections were then incubated for 60 min at RT in the same buffer with the addition of 20 nM [³H]Muscimol (specific activity 35.6 Ci/mmol, Perkin Elmer, USA). Non-specific binding was determined by incubating adjacent sections in the same solution with the addition of 100 μM GABA (Sigma). The concentration of [³H]Muscimol was measured in 10 μl aliquots taken from the incubation mixture. After the incubation, sections were washed two times for 1 minute in cold (4°C) 50 mM Tris citrate buffer (pH 7.0). Sections were then dipped briefly in cold distilled water and then air dried.

[³H]Flumazenil (Ro-15-1788) autoradiography was carried out based on the method described in Hand et al., 1997 [37] with minor modifications. On the day of the experiment, sections were pre-incubated for 20 min at room temperature in 50 mM Tris

(pH 7.4). Sections were then incubated for 80 min at room temperature in the same buffer with the addition of 5 nM [³H]Flumazenil (specific activity 83.4 Ci/mmol, Perkin Elmer, USA). Non-specific binding was determined by incubating adjacent sections in the same solution but in the presence of 10 μM flumazenil (Sigma). The concentration of [³H]Flumazenil was measured in 10 μl aliquots taken from the incubation mixture. After the incubation, sections were washed two times for 1 minute in cold 50 mM Tris buffer (pH 7.4). Sections were then dipped briefly in cold distilled water and then dried.

Following the assays, dried sections were apposed to Kodak Biomax MR films together with autoradiographic standards ([³H]microscales from Amersham), in x-ray film cassettes. Films were developed after 8 weeks ([³H]Muscimol) and 4 weeks ([³H]Flumazenil) using Kodak GBX developer and fixed with Kodak GBX fixer.

2.4. Quantitative analysis of autoradiographic images

All films were analyzed by using a computer-assisted image analysis system, Multi-Analyst, connected to a GS-690 Imaging Densitometer (Bio-Rad, USA). Quantification of receptor binding in DLPFC was performed by measuring the average optical density in adjacent brain sections. Both ligands presented a similar distribution binding pattern across the grey matter of the DLPFC with "superficial layers" (I, II and III) having higher binding compared to "deep layers" (IV, V and VI) (Figure 1B). Non-specific binding was found to be negligible (<5%) for both [³H]Muscimol and [³H]Flumazenil binding. Optical density measurements for specific binding were then converted into fmoles of radioligand per mg of tissue equivalent (fmol/mg TE), according to the calibration curve obtained from the [³H]microscales standards.
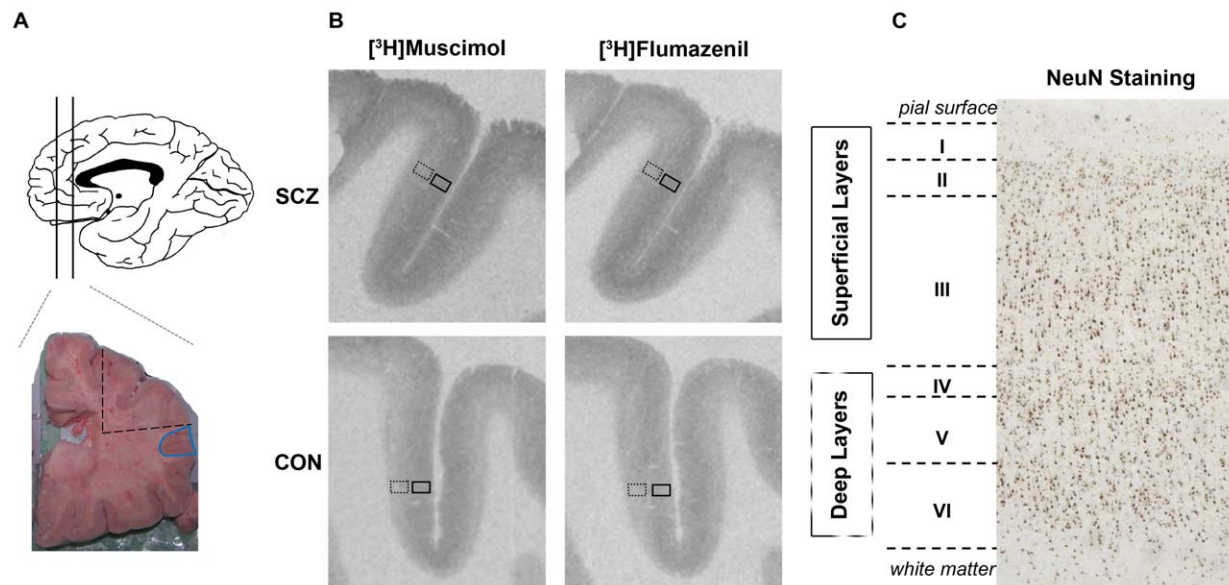
Figure 1. An overview of the experimental procedures used in the autoradiography experiments. A Sagittal view of brain with lines indicating area of coronal section with the blue outlined area is where homogenate tissue was isolated with the dashed lines indicating the bounds of tissue slices used for autoradiography (A). Typical autoradiographs (B) obtained with [³H]Muscimol (GABA orthosteric binding site agonist) and [³H]Flumazenil (benzodiazepine allosteric binding site antagonist) in the DLPFC of control (CON) and schizophrenia (SCZ) cases. NeuN staining (C) that allowed identification of the cortical layers in the DLPFC is presented on the right side of the figure, along with the drawing methodology used to quantify binding in the "superficial" (layers I, II, and III) and "deep" layers (layers IV, V and VI) of the DLPFC. The superficial layers quantification "box" is indicated in solid line, while the deep layers quantification "box" is indicated in dotted line.
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2.5. Total RNA isolation and RNA quality assessment

Total RNA was extracted from ~300 mg of frozen tissue per subject using Trizol (Invitrogen, Carlsbad, California) according to the manufacturer's instructions (Kozlovsky et al., 2004). The quality of extracted total RNA was determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California). A volume containing 100–200 ng RNA was applied to an RNA 6000 Nano LabChip, without heating before loading. The RNA integrity number (RIN) was used as an indicator of RNA quality, ranging from 1 (lowest quality) to 10 (highest quality). The cDNA was synthesized in three reactions of 3 µg of total RNA in a 26.25 µl reaction using the Superscript First-Strand Synthesis Kit (Invitrogen) according to the manufacturer's protocol.

2.6. Quantitative real-time PCR (qPCR)

GABA$_A$R subunits mRNA levels were measured using a TaqMan Gene Expression Assays (Applied Biosystems) for GABRB1 (Hs00181306_m1), GABRB2 (Hs00241451_m1), GABRB3 (Hs00241459_m1), GABRG1 (Hs00381554_m1), GABRG2 (Long and Short isoforms, Forward Primer: 5′-CCAAGCAAGGACAAA-GATAAAAGAA-3′, Reverse Primer: 5′-TGCTGATCTTGG GCGGATAT-3′, Probe Short: AAAACCCTGCCCCTACC, Probe Long: ATGTTTTCCTTCAAGGCC, Figure S1), and GABRG3 (Hs00264276_m1). Each 10 µl qPCR reaction contained FAM-labeled probe (250 nmol/l), primers (900 noml/l), and 1.14 ng cDNA in 1× TaqMan Universal Mastermix containing AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates, uracil-N-glycosy-lase, and passive reference. The PCR protocol used involved incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 consecutive cycles of 95°C for 15 sec and 60°C for 1 min. Serial dilutions of pooled cDNA (combined from all cases) were included on every qPCR plate and used by Sequence Detection Software (SDS; Applied Biosystems) to quantify sample expression through the relative standard curve method. Control wells containing no cDNA template displayed no amplification in any assay. Efficiencies of the qPCR reactions ranged from 77 to 100%, with r^2 values of between 0.95 and 1.00. All reactions were performed in triplicate. Expression levels were normalized to the geometric mean of four "housekeeper" genes that did not change expression with diagnosis (all genes t's(>69)<0.86, p's>0.4): ACTB (Hs99999903_m1), GAPDH (Hs99999905_m1), UBC (Hs00824723_m1), and TBP (Hs00427620_m1) [35,38]. Calculations of housekeeper stability were performed using geNorm (medgen.ugent.be/genorm) with resulting M-values of 0.15, 0.19, 0.25 and 0.30 for ACTB, GAPDH, UBC, and TBP respectively. For further detail on "housekeeper" gene selection, variance and stability please refer to Weickert et al. 2010 [35].

2.7. Statistical analysis

Statistical analyses were conducted using PASW Statistics 18.0 (SPSS, IBM) and GraphPad Prism (CA, USA) statistical packages. The data were normally distributed. All cases were included in the analyses. Mean values for binding and mRNA expression are reported ± standard error mean (SEM). Student's t-tests were used to compare the mean brain pH, age at death, PMI, freezer storage time, brain weight, brain volume and RIN between the schizophrenia and control groups. We tested the continuous variables (brain pH, age at death, PMI, freezer storage time, brain weight, brain volume, RIN, age of illness onset, illness duration, and estimated lifetime exposure to antipsychotics) for significant Pearson's correlations with binding and mRNA subunits expression. Non-continuous variables such as gender (male/female), hemisphere (right/left), cause of death (suicide/other), daily alcohol intake (none 0, low: 1, moderate: 2, and high: 3), and tobacco smoking (moderate: 1, and heavy: 2) were used as

grouping variables with t-tests or one-way ANOVA to evaluate their effects on binding and mRNA expression.

GABA$_A$R bindings between diagnostic groups (schizophrenia and control) were analysed using two-way analysis of covariance (ANCOVA), with diagnosis (schizophrenia and control) and layers (superficial and deep) as independent variables and co-varying for continuous variables that were significantly correlated with binding. GABA$_A$R subunit mRNA expression levels between diagnostic groups (schizophrenia and control) were analysed using ANCOVA, co-varying for continuous variables that were significantly correlated with mRNA subunit expression.

To assess the relative implication of GABA$_A$R mRNA subunits on binding measures in patients with schizophrenia and controls, we used forward regression analysis with all α and β mRNA subunits as predictors of the GABA orthosteric binding site targeted with [³H]Muscimol, and all α, γ and δ mRNA subunits as predicators of the benzodiazepine allosteric binding site, in both layers of the DLPFC. The criterion probability of F for predicators to enter the regression model was set at ≤0.05.

Results

The mean age, pH, PMI, freezer storage time, brain weight, brain volume and RIN did not differ between the schizophrenia and control groups as shown by non-significant Student t-tests (age at death: t(72) = 0.057, p = 0.955; pH: t(72) = −0.644, p = 0.521; PMI: t(72) = 1.264, p = 0.21; freezer storage time t(72) = 1.102, p = 0.274; brain weight t(72) = −1.525, p = 0.132; brain volume t(72) = −0.893, p = 0.375; and RIN t(72) = −0.243, p = 0.809).

3.1. Disease related effects on [³H]Muscimol and [³H]Flumazenil binding

Significant associations were found between [³H]Muscimol binding and age and freezer storage time in both superficial and deep layers (age: −0.523<r<−0.524, p<0.001, freezer storage: 0.403<r<0.425, p<0.001, see Table S1 for details and section 3.4). In the whole cohort, these associations accounted for less than 20% of the variance and were co-varied for by performing two-way ANCOVA. This analysis revealed [³H]Muscimol binding varied with diagnosis (mean ± SEM: superficial layers control = 251.5±9.1 vs. schizophrenia = 272.9±10.0; deep layers control = 161.5±6.3 vs. schizophrenia = 180.4±7.5 fmol/mg TE, F(1,142) = 5.98, p = 0.016). [³H]Muscimol binding also varied with layer (F(1,142) = 184.5, p<0.001) with no interaction between the variables (F(1,142) = 0.033, p = 0.885). We found higher levels of [³H]Muscimol binding in DLPFC of people with schizophrenia compared to controls and the variation with layer was due to higher levels of [³H]Muscimol binding in superficial layers of the DLPFC compared to deep layers (see Figure 2).

For [³H]Flumazenil binding, significant associations were observed between and continuous variables in both superficial and deep layers (pH: 0.289<r<0.411, p<0.01, freezer storage: 0.434<r<0.526, p<0.001, see Table S1 for details and section 3.4), that accounted for less than 28% of the variance and were co-varied for in the ANCOVA. [³H]Flumazenil binding (mean ± SEM: superficial layers control = 246.2±13.0 vs. schizophrenia = 280.8±14.0; deep layers control = 168.5±10.1 vs. schizophrenia = 197.2±9.7 fmol/mg TE) varied with diagnosis (F(1,140) = 6.5, p = 0.012) and with layers (F(1,140) = 70.5, p<0.001) but no significant interaction between diagnosis and layers was found (F(1,140) = 0.095, p = 0.758). [³H]Flumazenil binding was higher in schizophrenia compared to controls and higher in the superficial layers of the DLPFC compared to deep layers (see Figure 2).
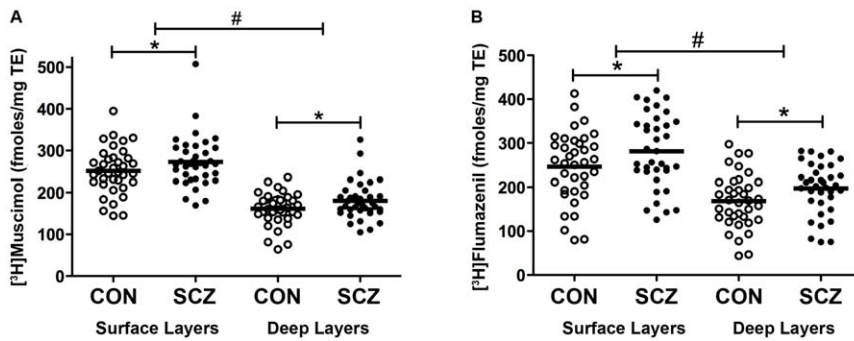
Figure 2. Scatter plots presenting the binding values (fmoles/mg tissue equivalent) of [³H]Muscimol (A) and [³H]Flumazenil (B) in control (CON) (n = 37) and schizophrenia (SCZ) (n = 37), in the superficial (surface) and deep layers of the DLPFC. * = p<0.05 and refers to a significant main effect of diagnosis (schizophrenia>control); # = p<0.001 and refers to a significant main effect of layers (superficial>deep) after two-way ANCOVA. Bars represent the means.
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In schizophrenia, [³H]Muscimol and [³H]Flumazenil binding were significantly correlated in both the superficial (r = 0.434, p = 0.008) and deep layers (r = 0.399, p = 0.016). In the control group, these correlations approached significance (r = 0.299, p = 0.073 in superficial and r = 0.310, p = 0.062 in deep layers).

3.2. Disease related effects on GABA$_A$R mRNA subunits expression (βs and γs)

One-way ANCOVA (controlling for demographic variables significantly correlated to GABA$_A$R mRNA subunit expression) revealed no significant effect of diagnosis (Figure 3) in any of the nine transcripts examined (β1–β3, γ1, γ2, γ2S, γ2L, γ3 and δ) (all F's<3.4, all p's>0.05).

3.3. Correlation and regression analysis

Pearson's correlation between [³H]Muscimol binding and α (measured in our previous study [11]) and β mRNA subunits are presented in Table 2 with separate analyses in the whole cohort, in patients with schizophrenia only, and in controls only. Pearson's correlation between [³H]Flumazenil binding and α, γ and δ mRNA subunits are presented in Table 3 with separate analyses in the whole cohort, in patients with schizophrenia only, and in controls only.

In patients with schizophrenia, we used forward regression analysis with all α and β mRNA subunits (constituting the GABA orthosteric binding site) as predictors for [³H]Muscimol binding in both layers of the DLPFC. Interestingly, the α4 subunit mRNA was most strongly associated with binding and alone could account for 34% and 23% of the variance in [³H]Muscimol binding in superficial and deep layers of the DLPFC respectively (see Figure 4 for correlation plot and Table 4 for regression results). In contrast, in the superficial layers of the control group, no predictors (mRNA subunits) passed the criterion probability of F to enter the regression model. In the deep layers however, β2 mRNA subunit contributed the most (14%) to [³H]Muscimol variance in controls (Table 4).

In patients with schizophrenia, we used forward regression analysis with all α, γ and δ mRNA subunits (constituting the benzodiazepine allosteric binding site) as predictors of [³H]Flumazenil binding in both layers of the DLPFC. Interestingly, the γ2S subunit mRNA was most strongly associated with binding and alone could account for 31% and 13% of the variance in [³H]Flumazenil binding in superficial and deep layers of the DLPFC respectively (see Figure 5 for correlation plot and Table 5 for regression results). In contrast, when applying the same regression method in the control group, several mRNA subunits contributed to [³H]Flumazenil variance, including γ1, γ2 pan and γ2L in the superficial layers and α1, α2, γ2 pan and γ2S in the deep layers of the DLPFC (Table 5).

3.4. Effect of continuous and non-continuous variables

In the whole cohort and in each diagnostic group, age at death was negatively correlated with [³H]Muscimol binding in both layers of the DLPFC (all r<−0.3, all p<0.05), freezer storage was positively correlated with [³H]Flumazenil in both layers (all r>0.3, all p<0.04) and with [³H]Muscimol in deep layers (all r>0.35, all p<0.02), and pH was positively correlated with [³H]Flumazenil in superficial layers only (all r>0.4, all p<0.01). Other significant correlations between GABA$_A$R binding and continuous variables were found and are presented in Table S1, along with significant correlations between GABA$_A$R mRNA subunit expression and continuous variables.

The t-tests for gender showed no significant male/female variation in binding measures (data not shown). Concerning mRNA subunits, only α4 showed a significant increase in males compared to females (+15.2%, t(71) = 2.003, p = 0.049). All the other subunits did not show a statistically significant change according to gender (data not shown).

The hemisphere analysed, right or left, did not have any significant effect on binding or mRNA subunits measures (data not shown). Cases who died by suicide in the schizophrenia group were characterised by an increase in [³H]Muscimol binding in the superficial (+19.5%, t(35) = 2.2, p = 0.035) and in the deep (+23.9%, t(35) = 2.4, p = 0.021) layers of the DLPFC compared to cases who died by natural means, whereas [³H]Flumazenil binding did not differ according to manner of death (superficial: t(34) = 0.2, p = 0.824; deep: t(34) = 0.1, p = 0.932). Concerning the mRNA subunits expression, all α1, α4 and α5 were significantly increased in the DLPFC of schizophrenia cases who committed suicide compared to the ones who did not (α1:+37.3%, t(35) = 2.6, p = 0.013), α4: +23.9%, t(34) = 2.1, p = 0.047 α5: +29.5%, t(35) = 2.6, p = 0.013).

In the schizophrenia group the amount of daily alcohol intake (none 0, low: 1, moderate: 2, and high: 3) resulted in greater [³H]Flumazenil binding in the deep layers of cases with high alcohol intake compared to those with no alcohol intake (ANOVA: F(3,29) = 2.871, p = 0.053, post-hoc: high alcohol and no alcohol, p = 0.018), but had no significant effect on [³H]Muscimol measures or mRNA subunit expression. The tobacco smoking habits during life of the individuals with schizophrenia (moderate:
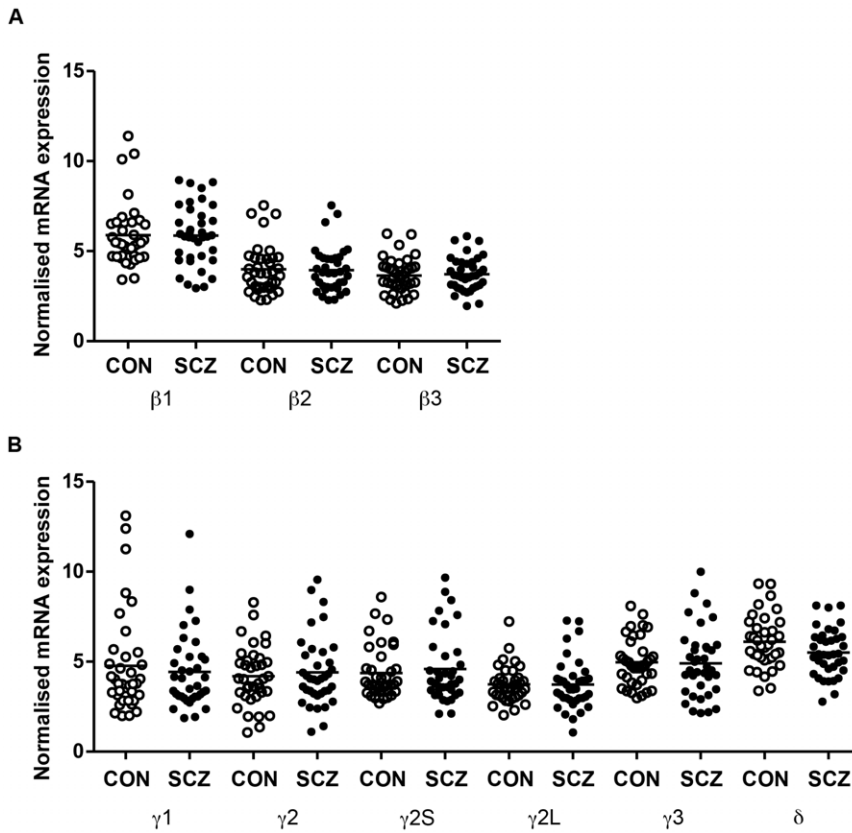
Figure 3. Scatter plots of the normalised expression level of GABA$_A$R mRNA subunits (qRT-PCR) in the DLPFC of control (CON) (n = 37) and schizophrenia (SCZ) (n = 37) cases. (A) β mRNA subunits and (B) γ and δ mRNA subunits. Bars represent the means.
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Table 2. Correlations between [³H]Muscimol binding and α-β GABA$_A$R subunit mRNAs.

mRNA		All individuals (n = 74)		Schizophrenia (n = 37)		Control (n = 37)	
		Superficial Layers	Deep Layers	Superficial Layers	Deep Layers	Superficial Layers	Deep Layers
α1	r	0.380	0.323	0.455	0.381	0.306	0.273
	p	0.001	0.005	0.005	0.020	0.065	0.102
α2	r	0.239	0.213	0.342	0.310	0.140	0.117
	p	0.043	0.072	0.041	0.066	0.415	0.498
α3	r	0.191	0.239	0.236	0.295	0.119	0.148
	p	0.106	0.041	0.160	0.077	0.490	0.390
α4	r	0.396	0.354	0.582	0.474	0.172	0.211
	p	0.001	0.002	<0.001	0.004	0.309	0.210
α5	r	0.085	0.130	0.199	0.252	0.078	0.144
	p	0.472	0.269	0.238	0.133	0.646	0.395
β1	r	0.076	0.139	0.164	0.224	−0.013	0.056
	p	0.522	0.236	0.331	0.183	0.941	0.742
β2	r	0.327	0.372	0.384	0.387	0.249	0.376
	p	0.004	0.001	0.019	0.018	0.137	0.022
β3	r	0.343	0.306	0.377	0.304	0.306	0.310
	p	0.003	0.008	0.021	0.068	0.066	0.062

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Table 3. Correlations between [³H]Flumazenil binding and α-γ/δ GABA$_A$R subunit mRNAs.

mRNA		All individuals (n = 74)		Schizophrenia (n = 37)		Control (n = 37)	
		Superficial Layers	Deep Layers	Superficial Layers	Deep Layers	Superficial Layers	Deep Layers
α1	r	0.382	0.300	0.390	0.175	0.406	0.475
	p	0.001	0.010	0.019	0.309	0.013	0.003
α2	r	−0.233	−0.227	−0.025	−0.079	−0.447	−0.372
	p	0.050	0.057	0.888	0.653	0.006	0.025
α3	r	0.111	0.140	0.113	0.061	0.107	0.248
	p	0.352	0.240	0.510	0.722	0.534	0.144
α4	r	0.210	0.160	0.333	0.156	0.078	0.178
	p	0.076	0.180	0.050	0.370	0.644	0.291
α5	r	−0.038	−0.029	0.056	0.016	−0.017	0.054
	p	0.753	0.807	0.746	0.925	0.921	0.750
γ1	r	−0.347	−0.270	−0.199	−0.062	−0.462	−0.407
	p	0.003	0.021	0.245	0.718	0.004	0.013
γ2	r	−0.103	−0.149	0.058	−0.038	−0.337	−0.314
	p	0.387	0.210	0.736	0.824	0.041	0.058
γ2S	r	0.441	0.393	0.553	0.364	0.280	0.426
	p	<0.001	0.001	<0.001	0.029	0.094	0.009
γ2L	r	0.270	0.248	0.352	0.287	0.167	0.223
	p	0.021	0.034	0.035	0.089	0.322	0.185
γ3	r	0.046	0.069	0.268	0.298	−0.274	−0.222
	p	0.699	0.559	0.114	0.077	0.101	0.186
δ	r	−0.114	−0.140	0.012	0.032	−0.155	−0.204
	p	0.337	0.237	0.942	0.855	0.359	0.227

doi:10.1371/journal.pone.0052724.t003

0, and heavy: 1) had no significant impact on either the binding measures or mRNA subunit expression, with the exception of γ1 subunit that was higher in heavy smokers compared to moderate smokers (t(21) = 2.729, p = 0.013).

The lifetime anti-psychotic dose (chlorpromazine equivalent) did not have any significant impact on either binding measures (r's: −0.3<r<−0.2 and all p's>0.07) or mRNA subunits expression (r's: −0.3<r<0.1, all p's>0.08). The age of onset of schizophrenia did not have any significant effect on either binding measures (r's: −0.3<r<0.1 and all p's>0.08) or mRNA subunits

expression (r's: −0.3<r<0.1, all p's>0.07). The duration of illness (in years) was negatively correlated with [³H]Muscimol binding measures in the superficial (r = −0.632, p<0.001) and deep layers (r = −0.584, p's<0.001) of schizophrenia patients but not with [³H]Flumazenil binding (r's: −0.3<r<−0.1, and all p's>0.1). The mRNA subunits expression for α1 (r = −0.374, p = 0.023) and α4 (r = −0.462, p = 0.005) were negatively correlated with duration of illness whereas the other subunits were not (r's: −0.3<r<0.2, all p>0.07).
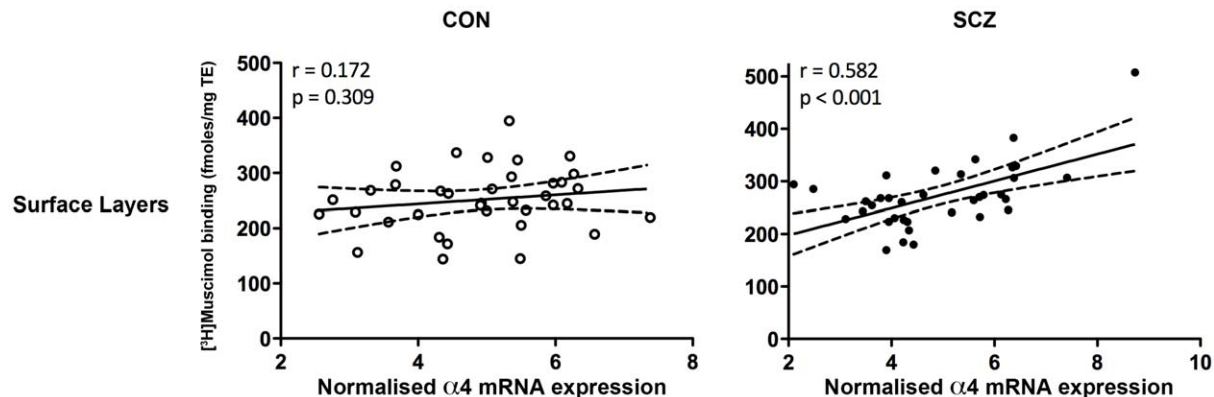

Figure 4. Correlations of [³H]Muscimol binding and α4 mRNA subunit expression level in the superficial layers of the DLPFC of control (CON) (n = 37) and schizophrenia (SCZ) (n = 37) cases.
doi:10.1371/journal.pone.0052724.g004

Table 4. Regression modelling of the interaction between relevant GABA$_A$R subunits (α and β) and [³H]Muscimol binding.

Group	Area	Method	Model	Variable (F-to-enter≤0.05)	B	Std Error B	β	p value	R^2	ΔR^2
Schizophrenia (n = 37)	Superficial Layers	Forward (α1,2,3,4,5,β1,2,3)	1	Constant	143.21	32.29		<0.001	.34	.34
				α4 mRNA	26.19	6.30	.59	<0.001	.34	.34
	Deep Layers	Forward (α1,2,3,4,5,β1,2,3)	1	Constant	101.74	25.97		<0.001	.23	.23
				α4 mRNA	15.83	5.07	.48	0.004	.23	.23
Control (n = 37)	Superficial Layers	Forward (α1,2,3,4,5,β1,2,3)		none with F≤0.050	na	na	na	na	na	na
	Deep Layers	Forward (α1,2,3,4,5,β1,2,3)	1	Constant	88.45	31.04		0.007	.14	.14
				β2 mRNA	18.31	7.65	.38	0.022	.14	.14

doi:10.1371/journal.pone.0052724.t004

Discussion

4.1. Disease-related effects

We report increases of both the GABA and benzodiazepine binding sites of the GABA$_A$R in the DLPFC in schizophrenia that are linked to α4 and γ2S mRNA subunits respectively. Our findings are in line with studies reporting increases in [³H]Muscimol binding (GABA binding site) in the DLPFC [18] and other cortical regions [16,21]. The increase we observed in the benzodiazepine binding site in the DLPFC in schizophrenia confirms previous studies which report increases across a variety of cortical areas including the medial frontal cortex, orbitofrontal cortex, temporal gyrus, and the parahippocampal cortex [24,25] but contradicts other studies that found no changes [26] or decreased binding in individuals with schizophrenia [30].

It is known that many demographic and peri-mortem factors including medication before death can influence binding and/or mRNA expression in post-mortem studies. Although in the present study several associations between these factors and binding/ mRNA expression were identified, the sizes of these associations in the whole cohort were small and taken into account in the ANCOVA analysis. Moreover, no significant correlations between [³H]Muscimol and [³H]Flumazenil binding and antipsychotic medication (lifetime chlorpromazine) were found in the schizophrenia group. This suggests that the observed increases in GABA$_A$R binding are not secondary to medication or demographic and/or peri-mortem factors.

We found significant correlations between [³H]Muscimol and [³H]Flumazenil binding in the whole cohort and in the schizophrenia group, but not in the control group. While the association between [³H]Flumazenil and [³H]Muscimol binding was statistically significant only in the patient group it cannot be discounted that a similar, though weaker, association was present in controls. This suggests that in general there is a positive association between the orthosteric GABA and allosteric benzodiazepine binding site but this is stronger in the disease state. The increases in [³H]Muscimol and [³H]Flumazenil binding we observed in the present study were not accompanied by cohort-wide changes in any of GABA$_A$R subunit mRNA measured here. Despite this lack of overall change in primary transcript levels in schizophrenia, regression analysis revealed that, in the schizophrenia group, the α4 subunit mRNA levels contributed the most to the [³H]Muscimol binding variance in both layers of the DLPFC. In contrast in the control group, no mRNA reached a significant level of correlation with binding to enter the regression model in the superficial layers, whereas in the deep layers, β2 mRNA was the one mainly contributing to [³H]Muscimol binding variance.

The γ2 subunit is an important functional determinant of GABA$_A$ receptors and is essential for formation of high-affinity benzodiazepine binding sites [39–41]. Using transgenic mice, Baer et al (2000) demonstrated that expression of either the long or the short γ2 splice variant resulted in mice that had indistinguishable [³H]Flumazenil binding, γ2 protein levels and phenotypes [42]. It is interesting that mRNA levels encoding the γ2S subunit was the
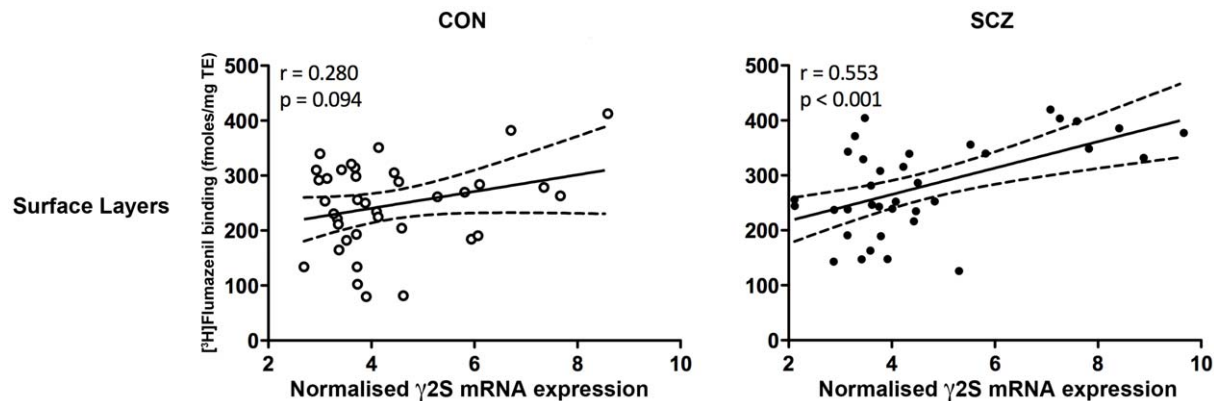

Figure 5. Correlations of [³H]Flumazenil binding and γ2S mRNA subunit expression level in the superficial layers of the DLPFC of control (CON) (n = 37) and schizophrenia (SCZ) (n = 37) cases.
doi:10.1371/journal.pone.0052724.g005

Table 5. Regression modelling of the interaction between relevant GABA$_A$R subunits (α, γ and δ) and [^{3}H]Flumazenil binding.

Group	Area	Method	Model	Variable (F-to-enter≤0.05)	B	Std Error B	β	p value	R^2	ΔR^2
Schizophrenia (n = 37)	Superficial Layers	Forward (α1,2,3,4,5;γ1,2,3;γ2L;γ2S;δ)	1	Constant	170.12	31.39		<0.001	.31	.31
				γ2 mRNA Short	24.08	6.31	.553	0.001	.31	.31
	Deep Layers	Forward (α1,2,3,4,5;γ1,2,3;γ2L;γ2S;δ)	1	Constant	146.85	24.28		<0.001	.13	.13
				γ2 mRNA Short	10.95	4.88	.364	0.032	.13	.13
Control (n = 37)	Superficial Layers	Forward (α1,2,3,4,5;γ1,2,3;γ2L;γ2S;δ)	1	Constant	308.69	23.75		<0.001	.21	.21
				γ1 mRNA	−13.10	4.31	−.462	0.005	.21	.21
			2	Constant	396.66	37.31		<0.001	.37	.16
				γ1 mRNA	−14.53	3.94	−.512	0.001	.37	.16
				γ2 mRNA Pan	−19.33	6.70	−.401	0.007	.37	.16
			3	Constant	318.27	44.96		<0.001	.49	.12
				γ1 mRNA	−15.70	3.64	−.554	<0.001	.49	.12
				γ2 mRNA Pan	−24.57	6.44	−.510	0.001	.49	.12
				γ2 mRNA Long	28.29	10.53	.358	0.011	.49	.12
	Deep Layers	Forward (α1,2,3,4,5;γ1,2,3;γ2L;γ2S;δ)	1	Constant	69.77	32.66		0.040	.23	.23
				α1 mRNA	21.95	6.97	.475	0.003	.23	.23
			2	Constant	118.63	38.59		0.004	.32	.09
				α1 mRNA	21.71	6.64	.470	0.003	.32	.09
				γ2 mRNA Pan	−11.39	5.34	−.307	0.040	.32	.09
			3	Constant	218.49	52.13		<0.001	.44	.12
				α1 mRNA	18.22	6.26	.394	0.006	.44	.12
				γ2 mRNA Pan	−13.94	5.01	−.375	0.009	.44	.12
				α2 mRNA	−14.84	5.66	−.361	0.013	.44	.12
			4	Constant	187.56	49.93		0.001	.53	.09
				α1 mRNA	12.77	6.20	.276	0.048	.53	.09
				γ2 mRNA Pan	−12.86	4.67	−.346	0.010	.53	.09
				α2 mRNA	−16.81	5.31	−.409	0.003	.53	.09
				γ2 mRNA Short	13.88	5.57	.330	0.018	.53	.09

strongest predictor of [³H]Flumazenil variance in both cortical layers of people with schizophrenia but also predicted a relatively small proportion of [³H]Flumazenil binding variance in deep cortical layers of controls. This implies that γ2S may be contributing more to binding in the disease state which would suggest that functional coupling, downstream of receptor activation, using PKC may be diminished in schizophrenia regardless of increased binding. In contrast, in the control group, several mRNA subunits were contributing to the variance in [³H]Flumazenil binding in the superficial and deep layers of the DLPFC. These observations suggest that the relationships between binding and mRNA expression at the benzodiazepine binding site are probably indirect and quite varied amongst the control population.

Overall the results of our regression analysis suggests that there may be a "shift" in the subunit composition of GABA_AR towards inclusion of α4 when studying the GABA orthosteric binding site and γ2S when analysing the benzodiazepine allosteric binding site in schizophrenia.

4.2. Functional and therapeutic implications

Our results have several functional and therapeutic implications. The increases in both [³H]Muscimol and [³H]Flumazenil binding together with the putative "shift" in the subunit composition support the theory of altered GABAergic neurotransmission in the DLPFC. The increases in binding observed in the present study and others [18,20,23,27,28] could be due to compensatory adaptations for reduced GABA synthesis [11,43–47] and reuptake [48] at parvalbumin-positive chandelier neurons that synapse on the axon initial segment of pyramidal cells [26,53,54]. However, compensation at GABA_AR level has been suggested to be insufficient to restore the synchronized oscillatory activity of cortical pyramidal neurons, in the gamma band range, necessary for normal cognitive functioning [49]. If a dysfunctional GABA system in schizophrenia does lead to cognitive impairments, therapeutic strategies to correct or modulate disrupted GABAergic pathways could be used to treat cognitive symptoms of schizophrenia, regardless of whether these GABAergic deficits are primary or compensatory [50].

Our results point to therapeutic potential of drugs that target α4 or γ2S subunits in the treatment of symptoms of schizophrenia. GABA_AR that include α4 subunits are generally insensitive to diazepam, the prototypical benzodiazepine [51]. If, as we propose, schizophrenia patients undergo a "shift" in their subunit composition of GABA_AR towards α4 subunits, this could account for the 50% failure of response to benzodiazepine prescribed as "add-on" therapy in schizophrenia [52,53]. Moreover, GABA_AR that include α4 subunits, assembled with a γ or δ subunit [50], are located at extrasynaptic sites and are responsible for tonic inhibition. Tonic inhibition is defined as the constant activation of extrasynaptic receptors that reduces the probability of generating an action potential [54]. A "shift" in the subunit composition of GABA_AR towards α4 subunits in our schizophrenia cohort might therefore reflect an abnormal shift towards extrasynaptic GABA_AR mediated tonic inhibition, that could compromise normal GABAergic modulation of cortical excitability, contributing to schizophrenia symptoms.

GABA_AR containing the γ2 subunit predominantly mediate phasic inhibition and have a synaptic localisation [50]. Phasic inhibition is defined as the rapid and synchronous opening of synaptic receptors that results in an inhibitory postsynaptic potential [54]. The long isoform of γ2 subunit (γ2L) has been shown to preferentially accumulate at synapses through the protein kinase C (PKC) phosphorylation of Ser343. Conversely, the γ2S isoform lacking Ser343 [7,8] might not be able to "transfer" to synapses and rather localise extrasynaptically. In our schizophrenia cohort, a putative "shift" in the subunit composition of GABA_AR towards γ2S isoforms possibly reflects a similar "shift" in the ratio of extrasynaptic/synaptic GABA_AR as we suggested for α4 subunits. Alternatively, an increase in the γ2S isoform may be linked to down-stream changes in PKC phosphorylation signalling associated with abnormal modulation and function of the GABA_AR in schizophrenia.

Therefore, increased contributions of both α4 and γ2S subunits to the formation of the orthosteric and allosteric GABA_AR binding sites in schizophrenia, may result in an increase of GABA_ARs located extrasynaptically, and thus affect tonic inhibition of cortical neurons. Although the implication of synaptic GABA_AR (controlling phasic inhibition) in the generation of rhythmic activity in neuronal networks (thus cognitive processes) is established [54], the role of extrasynaptic GABA_AR (responsible for tonic inhibition) is still unclear. Mutant mice with a loss of tonic inhibition in the hippocampus exhibited an increase in the power of gamma band oscillations [55] and increased gamma band power at baseline (non-evoked) has been reported for people with schizophrenia [56]. It is possible that a dysfunction in tonic inhibition affects gamma band oscillations resulting in cognitive impairments in schizophrenia. Interestingly, neurosteroids acting on extrasynaptic GABA_AR have been found to improve cognition in schizophrenia patients, suggesting that extrasynaptic receptors could be involved in cognitive impairments in schizophrenia [57,58]. These studies, together with ours, reinforce the interest in developing therapeutics that target extrasynaptic GABA_ARs for the treatment of cognitive symptoms in schizophrenia.

Conclusions

Our study showed parallel up-regulation of the GABA orthosteric and benzodiazepine allosteric GABA_AR binding sites in the DLPFC of a large cohort of schizophrenia patients and controls. We reported associations between α4/γ2S mRNA subunit and binding that may suggest a shift in GABA_AR subunit composition in schizophrenia affecting receptor localization and function and may have implications for the development of novel treatment strategies.

Supporting Information

Figure S1 Design for the custom GABA_AR γ2 long and short variants TaqMan assay. Probes (in grey) span exon-exon junctions in order to eliminate genomic signals. One set of primers was used to amplify transcripts both including and excluding exon 10 (in green).
(TIF)

Table S1 Pearson's correlations between continuous variables, binding, and mRNA expression.
(XLS)

Acknowledgments

Author Contributions

Conceived and designed the experiments: SF MV CSW KZ. Performed the experiments: SF MV. Analyzed the data: SF MV CSW KZ. Contributed reagents/materials/analysis tools: CSW KZ. Wrote the paper: MV SF CSW KZ.

References

1. Daskalakis ZJ, Fitzgerald PB, Christensen BK (2007) The role of cortical inhibition in the pathophysiology and treatment of schizophrenia. Brain Research Reviews 56: 427–442.

2. Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacological Reviews 60: 243–260.

3. Boileau AJ, Pearce RA, Czajkowski C (2010) The short splice variant of the gamma 2 subunit acts as an external modulator of GABA(A) receptor function. Journal of Neuroscience 30: 4895–4903.

4. Connolly CN, Uren JM, Thomas P, Gorrie GH, Gibson A, et al. (1999) Subcellular localization and endocytosis of homomeric gamma2 subunit splice variants of gamma-aminobutyric acid type A receptors. Molecular and Cellular Neurosciences 13: 259–271.

5. Hanson SM, Czajkowski C (2008) Structural mechanisms underlying benzodiazepine modulation of the GABA(A) receptor. Journal of Neuroscience 28: 3490–3499.

6. Smith GB, Olsen RW (1995) Functional domains of GABAA receptors. Trends in Pharmacological Sciences 16: 162–168.

7. Sanna E, Mascia MP, Klein RL, Whiting PJ, Biggio G, et al. (1995) Actions of the general anesthetic propofol on recombinant human GABAA receptors: influence of receptor subunits. The Journal of Pharmacology and Experimental Therapeutics 274: 353–360.

8. Hill-Venning C, Belelli D, Peters JA, Lambert JJ (1997) Subunit-dependent interaction of the general anaesthetic etomidate with the gamma-aminobutyric acid type A receptor. British Journal of Pharmacology 120: 749–756.

9. Hevers W, Lüddens H (1998) The diversity of GABAA receptors. Pharmacological and electrophysiological properties of GABAA channel subtypes. Molecular Neurobiology 18: 35–86.

10. Rudolph U, Knoflach F (2011) Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nature Reviews Drug Discovery 10: 685–697.

11. Duncan CE, Webster MJ, Rothmond DA, Bahn S, Elashoff M, et al. (2010) Prefrontal GABA(A) receptor alpha-subunit expression in normal postnatal human development and schizophrenia. Journal of Psychiatric Research 44: 673–681.

12. Beneyto M, Abbott A, Hashimoto T, Lewis DA (2011) Lamina-specific alterations in cortical GABA(A) receptor subunit expression in schizophrenia. Cerebral Cortex 21: 999–1011.

13. Fillman SG, Duncan CE, Webster MJ, Elashoff M, Weickert CS (2010) Developmental co-regulation of the beta and gamma GABAA receptor subunits with distinct alpha subunits in the human dorsolateral prefrontal cortex. International Journal of Developmental Neuroscience: The Official Journal of the International Society for Developmental Neuroscience 28: 513–519.

14. Fatemi SH, Reutiman TJ, Folsom TD, Rooney RJ, Patel DH, et al. (2010) mRNA and protein levels for GABAAalpha4, alpha5, beta1 and GABABR1 receptors are altered in brains from subjects with autism. Journal of Autism and Developmental Disorders 40: 743–750.

15. Benes FM, Khan Y, Vincent SL, Wickramasinghe R (1996) Differences in the subregional and cellular distribution of GABAA receptor binding in the hippocampal formation of schizophrenic brain. Synapse (New York, NY) 22: 338–349.

16. Benes FM, Vincent SL, Alsterberg G, Bird ED, SanGiovanni JP (1992) Increased GABAA receptor binding in superficial layers of cingulate cortex in schizophrenics. The Journal of neuroscience: the official journal of the Society for Neuroscience 12: 924–929.

17. Benes FM, Vincent SL, Marie A, Khan Y (1996) Up-regulation of GABAA receptor binding on neurons of the prefrontal cortex in schizophrenic subjects. Neuroscience 75: 1021–1031.

18. Dean B, Hussain T, Hayes W, Scarr E, Kitsoulis S, et al. (1999) Changes in serotonin2A and GABA(A) receptors in schizophrenia: studies on the human dorsolateral prefrontal cortex. Journal of Neurochemistry 72: 1593–1599.

19. Deng C, Huang X-F (2006) Increased density of GABAA receptors in the superior temporal gyrus in schizophrenia. Experimental Brain Research (Experimentelle Hirnforschung Expérimentation cérébrale) 168: 587–590.

20. Hanada S, Mita T, Nishino N, Tanaka C (1987) [3H]muscimol binding sites increased in autopsied brains of chronic schizophrenics. Life Sciences 40: 259–266.

21. Newell KA, Zavitsanou K, Jew SK, Huang X-F (2007) Alterations of muscarinic and GABA receptor binding in the posterior cingulate cortex in schizophrenia. Progress in Neuro-Psychopharmacology & Biological Psychiatry 31: 225–233.

22. Owen F, Cross AJ, Crow TJ, Lofthouse R, Poulter M (1981) Neurotransmitter receptors in brain in schizophrenia. Acta Psychiatrica Scandinavica Supplementum 291: 20–28.

23. Volk DW, Pierri JN, Fritschy J-M, Auh S, Sampson AR, et al. (2002) Reciprocal alterations in pre- and postsynaptic inhibitory markers at chandelier cell inputs to pyramidal neurons in schizophrenia. Cerebral Cortex 12: 1063–1070.

24. Benes FM, Wickramasinghe R, Vincent SL, Khan Y, Todtenkopf M (1997) Uncoupling of GABA(A) and benzodiazepine receptor binding activity in the hippocampal formation of schizophrenic brain. Brain Research 755: 121–129.

25. Kiuchi Y, Kobayashi T, Takeuchi J, Shimizu H, Ogata H, et al. (1989) Benzodiazepine receptors increase in post-mortem brain of chronic schizophrenics. European Archives of Psychiatry and Neurological Sciences 239: 71–78.

26. Pandey GN, Conley RR, Pandey SC, Goel S, Roberts RC, et al. (1997) Benzodiazepine receptors in the post-mortem brain of suicide victims and schizophrenic subjects. Psychiatry Research 71: 137–149.

27. Busatto GF, Pilowsky LS, Costa DC, Ell PJ, David AS, et al. (1997) Correlation between reduced in vivo benzodiazepine receptor binding and severity of psychotic symptoms in schizophrenia. The American Journal of Psychiatry 154: 56–63.

28. Ball S, Busatto GF, David AS, Jones SH, Hemsley DR, et al. (1998) Cognitive functioning and GABAA/benzodiazepine receptor binding in schizophrenia: a 123I-iomazenil SPET study. BPS 43: 107–117.

29. Abi-Dargham A, Laruelle M, Krystal J, D'Souza C, Zoghbi S, et al. (1999) No evidence of altered in vivo benzodiazepine receptor binding in schizophrenia. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology 20: 650–661.

30. McLeod MC, Scarr E, Dean B (2010) Effects of benzodiazepine treatment on cortical GABA(A) and muscarinic receptors: studies in schizophrenia and rats. Psychiatry Research 179: 139–146.

31. Impagnatiello F, Guidotti AR, Pesold C, Dwivedi Y, Caruncho H, et al. (1998) A decrease of reelin expression as a putative vulnerability factor in schizophrenia. Proceedings of the National Academy of Sciences of the United States of America 95: 15718–15723.

32. Huntsman MM, Tran BV, Potkin SG, Bunney WE, Jones EG (1998) Altered ratios of alternatively spliced long and short gamma2 subunit mRNAs of the gamma-amino butyrate type A receptor in prefrontal cortex of schizophrenics. Proceedings of the National Academy of Sciences of the United States of America 95: 15066–15071.

33. Hashimoto T, Arion D, Unger T, Maldonado-Avilés JG, Morris HM, et al. (2008) Alterations in GABA-related transcriptome in the dorsolateral prefrontal cortex of subjects with schizophrenia. Molecular Psychiatry 13: 147–161.

34. Lewis DA, Hashimoto T, Morris HM (2008) Cell and receptor type-specific alterations in markers of GABA neurotransmission in the prefrontal cortex of subjects with schizophrenia. Neurotoxicity Research 14: 237–248.

35. Weickert CS, Sheedy D, Rothmond DA, Dedova I, Fung S, et al. (2010) Selection of reference gene expression in a schizophrenia brain cohort. The Australian and New Zealand Journal of Psychiatry 44: 59–70.

36. Harper CG, Kril JJ, Daly JM (1988) Brain shrinkage in alcoholics is not caused by changes in hydration: a pathological study. Journal of Neurology, Neurosurgery, and Psychiatry 51: 124–127.

37. Hand KS, Baird VH, Van Paesschen W, Koepp MJ, Revesz T, et al. (1997) Central benzodiazepine receptor autoradiography in hippocampal sclerosis. British Journal of Pharmacology 122: 358–364.

38. Wong J, Hyde TM, Cassano HL, Deep-Soboslay A, Kleinman JE, et al. (2010) Promoter specific alterations of brain-derived neurotrophic factor mRNA in schizophrenia. Neuroscience 169: 1071–1084.

39. Herb A, Wisden W, Lüddens H, Puia G, Vicini S, et al. (1992) The third gamma subunit of the gamma-aminobutyric acid type A receptor family. Proceedings of the National Academy of Sciences of the United States of America 89: 1433–1437.

40. Lüddens H, Seeburg PH, Korpi ER (1994) Impact of beta and gamma variants on ligand-binding properties of gamma-aminobutyric acid type A receptors. Molecular Pharmacology 45: 810–814.

41. Ymer S, Draguhn A, Wisden W, Werner P, Keinänen K, et al. (1990) Structural and functional characterization of the gamma 1 subunit of GABAA/benzodiazepine receptors. The EMBO Journal 9: 3261–3267.

42. Baer K, Essrich C, Balsiger S, Wick MJ, Harris RA, et al. (2000) Rescue of gamma2 subunit-deficient mice by transgenic overexpression of the GABAA receptor gamma2S or gamma2L subunit isoforms. The European Journal of Neuroscience 12: 2639–2643.

43. Akbarian S, Kim JJ, Potkin SG, Hagman JO, Tafazzoli A, et al. (1995) Gene expression for glutamic acid decarboxylase is reduced without loss of neurons in prefrontal cortex of schizophrenics. Archives of General Psychiatry 52: 258–266.

44. Guidotti A, Auta J, Davis JM, Di-Giorgi-Gerevini V, Dwivedi Y, et al. (2000) Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. Archives of General Psychiatry 57: 1061–1069.

45. Hashimoto T, Bergen SE, Nguyen QL, Xu B, Monteggia LM, et al. (2005) Relationship of brain-derived neurotrophic factor and its receptor TrkB to altered inhibitory prefrontal circuitry in schizophrenia. Journal of Neuroscience 25: 372–383.

46. Straub RE, Lipska BK, Egan MF, Goldberg TE, Callicott JH, et al. (2007) Allelic variation in GAD1 (GAD67) is associated with schizophrenia and influences cortical function and gene expression. Molecular Psychiatry 12: 854–869.

47. Volk DW, Austin MC, Pierri JN, Sampson AR, Lewis DA (2000) Decreased glutamic acid decarboxylase67 messenger RNA expression in a subset of prefrontal cortical gamma-aminobutyric acid neurons in subjects with schizophrenia. Archives of General Psychiatry 57: 237–245.

48. Woo TU, Whitehead RE, Melchitzky DS, Lewis DA (1998) A subclass of prefrontal gamma-aminobutyric acid axon terminals are selectively altered in schizophrenia. Proceedings of the National Academy of Sciences of the United States of America 95: 5341–5346.

49. Whittington MA, Traub RD (2003) Interneuron diversity series: inhibitory interneurons and network oscillations in vitro. Trends in Neurosciences 26: 676–682.

50. Vinkers CH, Mirza NR, Olivier B, Kahn RS (2010) The inhibitory GABA system as a therapeutic target for cognitive symptoms in schizophrenia: investigational agents in the pipeline. Expert Opinion on Investigational Drugs 19: 1217–1233.

51. Knoflach F, Benke D, Wang Y, Scheurer L, Lüddens H, et al. (1996) Pharmacological modulation of the diazepam-insensitive recombinant gamma-aminobutyric acidA receptors alpha 4 beta 2 gamma 2 and alpha 6 beta 2 gamma 2. Molecular Pharmacology 50: 1253–1261.

52. Wolkowitz OM, Pickar D (1991) Benzodiazepines in the treatment of schizophrenia: a review and reappraisal. The American Journal of Psychiatry 148: 714–726.

53. Haw C, Stubbs J (2007) Benzodiazepines–a necessary evil? A survey of prescribing at a specialist UK psychiatric hospital. Journal of Psychopharmacology (Oxford, England) 21: 645–649.

54. Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nature Reviews Neuroscience 6: 215–229.

55. Glykys J, Mann EO, Mody I (2008) Which GABAA receptor subunits are necessary for tonic Inhibition in the hippocampus? Journal of Neuroscience 28: 1421–1426.

56. Flynn G, Alexander D, Harris A, Whitford T, Wong W, et al. (2008) Increased absolute magnitude of gamma synchrony in first-episode psychosis. Schizophrenia Research 105: 262–271.

57. Kellendonk C, Simpson EH, Kandel ER (2009) Modeling cognitive endophenotypes of schizophrenia in mice. Trends in Neurosciences 32: 347–358.

58. Strous RD, Stryjer R, Maayan R, Gal G, Viglin D, et al. (2007) Analysis of clinical symptomatology, extrapyramidal symptoms and neurocognitive dysfunction following dehydroepiandrosterone (DHEA) administration in olanzapine treated schizophrenia patients: a randomized, double-blind placebo controlled trial. Psychoneuroendocrinology 32: 96–105.