Altered M_1 muscarinic acetylcholine receptor (CHRM1)-$G\alpha_{q/11}$ coupling in a schizophrenia endophenotype

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Abstract

Alterations in muscarinic acetylcholine receptor (CHRM) populations have been implicated in the pathology of schizophrenia. Here we have assessed whether receptor function of the M_1 subtype (CHRM1) is altered in a sub-population of subjects with schizophrenia, defined by marked (60-80%) reductions in cortical [^{3}H]-pirenzepine (PZP) binding, and termed 'muscarinic receptor-deficit schizophrenia' (MRDS). Using a [^{35}S]-GTPγS-$G\alpha_{q/11}$ immunocapture method we have assessed whether CHRM1 signalling in human cortex (BA9) is altered in post-mortem tissue from a MRDS group compared with a subgroup of subjects with schizophrenia displaying normal PZP binding, and control subjects with no known history of psychiatric or neurological disorders. The CHRM agonist (oxotremorine-M) and a CHRM1-selective agonist (AC-42) increased $G\alpha_{q/11}$-[^{35}S]-GTPγS binding, with AC-42 producing responses that were ~50% of those maximally evoked by the full agonist oxotremorine-M in control and sub-groups of subjects with schizophrenia. However, the potency of oxotremorine-M to stimulate $G\alpha_{q/11}$-[^{35}S]-GTPγS binding was significantly decreased in the MRDS group (pEC_{50} (M) = 5.69 ± 0.16) compared to the control group (6.17 ± 0.10) and the non-MRDS group (6.05 ± 0.07). The levels of $G\alpha_{q/11}$ protein present in BA9 did not vary with diagnosis. Maximal oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in BA9 membranes was significantly increased in the MRDS group compared to the control group. Similar, though non-statistically significant trends were observed for AC-42. These data provide evidence that both orthosterically- and allosterically-acting CHRM agonists can stimulate a receptor-driven functional response ([^{35}S]-GTPγS binding to $G\alpha_{q/11}$) in membranes prepared from *post-mortem* human dorsolateral prefrontal cortex of subjects with schizophrenia and control subjects. Furthermore, in sub-group of subjects with schizophrenia displaying markedly decreased PZP binding (MRDS) we have demonstrated that while agonist potency may decrease, the efficacy of CHRM1-$G\alpha_{q/11}$ coupling is increased, suggesting an adaptative change in receptor-G protein coupling efficiency in this endophenotype of subjects with schizophrenia.

Keywords: Muscarinic acetylcholine receptor (CHRM); $G\alpha_{q/11}$ protein; schizophrenia; [^{3}H]-pirenzepine binding; human brain tissue

INTRODUCTION

Schizophrenia is a complex syndrome defined by the presence of positive and negative symptoms, as well as cognitive dysfunction (Pantelis *et al*., 1999; Raedler *et al*., 2007). Post-mortem studies (Dean *et al*., 1996; Crook *et al*., 2000; Zavitsanou *et al*., 2004; Deng and Huang, 2005; Scarr *et al*., 2007), and a recent neuroimaging study (Raedler, 2007), have consistently shown that widespread decreases in the levels of muscarinic acetylcholine receptors (CHRM) occur in the CNS of subjects with schizophrenia. Significantly, protein and mRNA levels for the M_1 (CHRM1; Dean *et al*., 2002; Mancama *et al*., 2003), but not the M_4 (CHRM4; Dean *et al*., 2002) or M_2/M_3 (CHRM2/CHRM3; Scarr *et al*., 2006) subtypes, have been shown to be decreased in the frontal cortex of subjects with schizophrenia. These data support the hypothesis that decreases in the binding of the CHRM subtype-selective antagonist [^{3}H]-pirenzepine reflect decreases in the CHRM1 in the frontal cortex of subjects with the disorder.

In other syndromes, such as diabetes (Gale, 2001), the ability to sub-divide subjects into biologically more homogenous groups using biological markers has often underpinned the beginnings of defining the pathologies of different diseases within the syndrome. Therefore, it is highly significant that it has recently been reported that a distinct sub-population of subjects with schizophrenia can be defined that have a 60-80% reduction in cortical [^{3}H]-pirenzepine binding (Scarr *et al* 2008). This low [^{3}H]-pirenzepine binding sub-group has been defined as having 'muscarinic receptor-deficit schizophrenia' (MRDS) (Scarr *et al*., 2008). To date, it has not been possible to determine if changes in CHRM1 density in the CNS of subjects with schizophrenia is associated with a change in receptor function; something that might be predicted to occur in MRDS subjects, given the marked down-regulation of CHRM1 protein within this sub-group.

CHRM1 couples preferentially to heterotrimeric G proteins of the $G_{q/11}$ sub-family to exert the majority of its cellular actions (Caulfield and Birdsall, 1998). This involves the activated, ligand-bound receptor interacting with a $G_{q/11}$ protein to facilitate GTP-for-GDP exchange on the $G\alpha_{q/11}$ subunit. In the presence of a radiolabelled, non-hydrolysable GTP analogue [^{35}S]guanosine-5'-O-(3-thio)triphosphate ([^{35}S]-GTPγS), the receptor will facilitate the binding of [^{35}S]-GTPγS to

$G_{q/11}$ proteins and by immunoprecipitating $G\alpha_{q/11}$ subunits using specific antibodies it is possible to quantify the transduction of receptor activation to a proximal downstream step in the signal transduction pathway (DeLapp *et al*., 1999). By using a novel adaptation of this method, [^{35}S]-GTPγS-$G\alpha_{q/11}$ immunocapture (Salah-Uddin *et al*., 2008), it is now possible to measure G protein-coupled receptor-$G\alpha_{q/11}$ coupling in membranes prepared from human post-mortem tissue to assess agonist potency and efficacy. Here, this technique has been used to determine whether agonist-dependent CHRM1 signalling in human cortex (Brodmann Area 9) is altered in tissue from subjects with MRDS compared to that in other forms of schizophrenia (non-MRDS), and subjects with no known history of psychiatric or neurological disorders (control subjects). Our data indicate that while CHRM agonist potency is decreased in MRDS, there is an increase in the efficacy of CHRM1-$G_{q/11}$ coupling. These new data indicate that despite marked declines in CHRM1 expression in some subjects with schizophrenia, the ability of this receptor to initiate signal transduction via $G_{q/11}$ proteins is undiminished.

MATERIALS AND METHODS

Materials

[^{35}S]-GTPγS (1000–1200 Ci mmol^{-1}) and anti-rabbit-IgG-coated SPA beads (RPNQ0016) were obtained from GE Healthcare. Complete protease inhibitor cocktail was purchased from Roche Applied Science. All other chemicals and reagents were obtained from Sigma-Aldrich. The $G_{q/11}\alpha$ antiserum was generated (against the C-terminal sequence (C)LQLNLKEYNLV) as previously described (Akam *et al*., 2001). AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride) was synthesized by GlaxoSmithKline, Harlow, U.K.

Tissue Collection

Approval was granted for tissue acquisition and use in this study by the Ethics Committee of the Victorian Institute of Forensic Medicine and the North Western Mental Health Programme Behavioural and Psychiatric Research and Ethics Committee, and consent for use of the samples for research purposes was approved by the donor's relatives. Blocks of tissue containing BA9 were excised from the left hemisphere of subjects, who were retrospectively defined as having MRDS or non-MRDS, as well as age/sex-matched control subjects (See Table 1). BA9 was taken as the region of the CNS on the lateral surface of the frontal lobe and includes the middle frontal gyrus superior to the inferior frontal sulcus.

As part of the clinical assessment using the Diagnostic Instrument for Brain Studies (DIBS) (Hill *et al*., 1999), a number of parameters were calculated. PMI was calculated as the time from death to autopsy when deaths were witnessed. In cases where death was witnessed, the time between death and autopsy was taken as the *post-mortem* interval (PMI). In the latter case, tissue was only taken from individuals who had been seen alive up to 5 h before being found dead, and PMI was taken as the interval halfway between the donor being found dead and last being seen alive. In all cases, cadavers were refrigerated within 5 h of being found and tissue was rapidly frozen to -70°C within 30 min of autopsy. The pH of the CNS tissue was measured as described previously (Kingsbury *et al*., 1995). When available, *post-mortem* toxicology was reviewed to exclude recent substance misuse and levels of antipsychotic and anti-cholinergic drugs in blood

were recorded. Duration of illness (DOI) was calculated as the time from first contact with a psychiatric clinical service to death. The most recently prescribed anti-psychotic and anti-cholinergic drugs and their final recorded prescribed doses were recorded and converted to standardized drug doses (see Supplementary Table).

Diagnostic Evaluation

For a subject to be included in this study, sufficient information needed to be available from clinical case records to enable a psychologist and psychiatrist to reach a diagnostic consensus using the DIBS and thus be able to make a diagnoses according to DSM-IV criteria (American Psychiatric Association.**,** 1994). Subjects with schizoaffective disorder were excluded.

[^{3}H]-Pirenzepine Binding

In situ radioligand binding and autoradiography were performed using five 20 μm frozen tissue sections from each block of BA9. The binding of [^{3}H]-pirenzepine (15 nM) was measured in the presence (non-specific binding (NSB): two sections) or absence (total binding: three sections) of 1 μM quinuclidinyl xanthene-9-carboxylate hemioxalate (Dean *et al*., 1996) after incubation in 10 mM KH_2PO_4, 10 mM Na_2HPO_4; pH 7.4 (buffer A) at 25 °C for 30 min. Sections were washed twice for 2 min in ice-cold buffer A, dipped in ice-cold water and thoroughly dried prior to being fixed overnight in paraformaldehyde fumes in a desiccator. The sections, and a set of [^{3}H]-micro-scales™, were apposed against a BAS-TR2025 imaging plate until an image of appropriate intensity was obtained for scanning in the BAS 5000 phosphoimager. Exposure time related to both the density of binding sites and the specific activity of the radioligand were utilized. The intensity of the phosphoimages was then measured by comparison to the intensity of the blocks of radioactivity on the [^{3}H]-microscales using AIS image analysis software, with results being expressed as d.p.m. mg^{-1} estimated wet weight tissue equivalents (ETE) and then converted to fmol mg^{-1} ETE. In this way, [^{3}H]-pirenzepine binding was measured using a single point saturation analysis, which provides a good approximation of the density of radioligand binding sites in tissue sections (Dean *et al*., 2002).

Western Blotting

Tissue samples from Brodmann area 9 (BA9) were solubilized in sample buffer containing 1% SDS, 1 mM Na_3VO_4 and 10 mM Tris/HCl, pH 7.5. Proteins were loaded (25 µg $well^{-1}$) in duplicate on to 10% SDS polyacrylamide gels and resolved by gel electrophoresis for 1 h at 150 V. Gels were then equilibrated in Towbin's transfer buffer (Tris/glycine/methanol) for 15 min. Proteins were then transferred to Hybond nitrocellulose membranes (GE Healthcare) for 1 h at 100 V in Towbin's buffer. Membranes were blocked for 1 h at room temperature in Tris-buffered saline/0.1% Tween 20 (TBS-T) containing 5% non-fat milk powder and then incubated overnight at 4 °C in 7.5 mL TBS-T containing rabbit anti-$G\alpha_{q/11}$ (1:500 dilution). The next day, membranes were washed 3 x 5 min in TBS-T at room temperature and incubated for 2 h at room temperature in TBS-T containing Dako goat anti-rabbit IgG:HRP-conjugated secondary antibody diluted 1:2000. Membranes were washed 3 x 5 min in TBS-T at room temperature and incubated for a further 5 min at room temperature in SuperSignal ECL solution (Pierce). Excess solution was drained and blotted and a single 5 min exposure was captured using a 440CF Kodak imaging station. Band (~43 KDa) intensity is reported as a ratio to an internal control. A representative Western Blot of relative $G\alpha_{q/11}$ levels in a control subject and a subject with schizophrenia is shown in Figure 5b. Prior to measuring Gαq/11 in the cases, sufficient protein homogenate (Internal Control: IC) was prepared from the frontal cortex of a subject with no history of psychiatric or neurological illness. Aliquots of this homogenate were run in each of 12 wells on 2 gels (24 samples over all), and the OD of each immunopositive Gαq/11 band was measured as described above. These experiments revealed that the anti-human Gαq/11 antibody bound to a CNS protein of appropriate molecular weight. Moreover, using data from the IC, both the inter- and intra-gel variation for the measurement of Gαq/11 was shown < 15%. Subsequently, a sample of IC was included in two lanes of each subsequent gel on which protein from samples were separated and the OD of each sample was expressed as a ratio of the IC to control for gel to gel variation in our analyses (Dean *et al*., 2002).

Membrane Preparation

BA9 of each individual donor, was homogenized using a Polytron in 10 volumes of 10 mM HEPES, pH 7.4, containing 1 mM EGTA, 1 mM dithiothreitol (DTT), 10% sucrose and complete

protease inhibitor cocktail. The resultant homogenate was diluted 10-fold and centrifuged at 1,000 x*g* for 10 min at 4°C, the supernatant saved and the pellet re-homogenized and centrifuged as above. The combined supernatants were then centrifuged at 11,000 x*g* for 20 min at 4°C. The resulting pellet was re-homogenized in 40 volumes of 10 mM HEPES, 1 mM EGTA, 1 mM DTT, 1 mM $MgCl_2$, pH 7.4, and centrifuged at 27,000 x*g* for 20 min at 4°C. The resulting pellet was re-suspended in the same buffer at a protein concentration of 1 mg mL^{-1}, aliquots snap-frozen in liquid nitrogen and stored at -80°C. To minimize membrane degradation, assays were conducted within 24 h of membrane preparation.

[^{35}S]-GTPγS Binding/Immunocapture Assay

[^{35}S]-GTPγS-G$\alpha_{q/11}$ immuno-specific binding using a 96 well SPA-based method was performed using the method described by Salah-Uddin *et al*. (2008). Assays were performed blind to subject diagnosis, and extracts from each cohort of matched subjects with schizophrenia and control subjects were run together in the same set of experiments. Membranes were pre-treated with 10 mM *N*-ethylmaleimide (NEM) for 60 min on ice and subsequently diluted in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) at a final protein concentration of 25 μg per assay point. GDP (0.1 μM) was added at 55 min into the NEM-containing incubation medium. Experimental reactions were performed in a final volume of 100 μL in 96-well Optiplates™. To ensure equilibrium [^{35}S]-GTPγS binding, 60 μL of membranes were added to each well containing agonist and incubated at 25°C for 20 min. Nucleotide exchange was initiated through the addition of 20 μL [^{35}S]-GTPγS to each well to give a final concentration of 500 pM and membranes were incubated for 60 min at 25°C. Ice-cold 0.27% Igepal-CA630 was used to terminate the reaction. Rabbit anti-G$\alpha_{q/11}$ antibody (1:300) was then added to each well and the plate agitated at 4°C for 60 min. Finally, Anti-IgG-coated PVT-SPA beads were added to each well, the plate agitated once again for a further 30 min at 4°C after which it was centrifuged. Bound radioactivity was measured using a TopCount detector.

Data Analysis

Concentration-response curves were fitted by non-linear regression analysis with variable slope using GraphPad Prism 4 (GraphPad Prism Software Inc., San Diego, CA). The residuals of all

the experimental responses were analyzed to identify the distribution. G protein and [^{3}H]-pirenzepine were normally distributed and signal window (c.p.m.), Hill slope and pEC_{50} were log normally distributed. A one-way analysis of variance (ANOVA) was used to analyze the G protein, [^{3}H]- pirenzepine, Hill slope and pEC_{50}, and Analysis of Covariance (ANCOVA) was used to analyze binding-over-basal with age being fitted as the covariate. Planned comparisons using Student's *t* test were then investigated and adjusted for multiplicity (using the Tukey's test) to identify if there were significant differences in the responses between the 3 diagnostic groups.

Pearson product-moment correlations, assuming a straight-line best fit, were used to analyze relationships between experimental parameters, and relationships between the continuous clinincal parameters and the experimental parameters. The clinical responses, age, PMI, brain weight and brain pH were analysed using one-way ANOVA, and planned comparisons using Student's *t* test were then investigated and adjusted for multiplicity to identify if there were significant differences in the responses between the 3 diagnostic groups; DOI and last recorded drug dose were analysed using Student's t test, and sex and suicide were analysed using Fisher's exact test. Any significant differences observed in the experimental dataset were not attributable to demographics.

RESULTS

Demographic Data

Dorsolateral prefrontal cortex (BA9) tissue was obtained from 14 male and 6 female subjects with schizophrenia, and 8 male and 2 female control subjects. There was no statistical difference in the mean ages and gender of the subjects with schizophrenia and control subjects ($F(2, 27) = 0.008$, $P = 0.992$ and $P = 0.153$, respectively). Likewise, there were no significant differences in CNS pH ($F(2, 27) = 0.929$, $P = 0.407$), brain weight ($F(2, 20) = 2.083$, $P = 0.151$), PMI ($F(2, 27) = 0.441$, $P = 0.648$), duration of illness ($F(1, 18) = 0.302$, $P = 0.589$), or last recorded drug doses ($F(1,18) = 0.493$, $P = 0.492$). An increased suicide rate was observed in the combined patient group of subjects with schizophrenia, but was not significantly different between the sub-groups of subjects with schizophrenia ($P = 0.076$).

[^{3}H]-Pirenzepine Binding

[^{3}H]-PZP binding was significantly decreased in tissue sections prepared from BA9 from subjects with schizophrenia compared to control brains (105 ± 17 versus 183 ± 12 fmol mg^{-1} ETE; ($F(2, 27) = 53.618$, $P < 0.0001$). Subjects with schizophrenia were divided into two sub-groups based on [^{3}H]-PZP binding: "low" [^{3}H]-PZP (MRDS; <100 fmol mg^{-1} ETE) and "normal" [^{3}H]-PZP (non-MRDS; >100 fmol mg^{-1} ETE) binding groups; [^{3}H]-PZP binding in these sub-groups was 38 ± 9 and 174 ± 12 fmol mg^{-1} ETE, respectively (Figure 1). [^{3}H]-PZP binding in the MRDS group was significantly different from both the control and non-MRDS groups ($P < 0.001$), while there was no difference between the control and non-MRDS groups ($P = 0.806$).

Western Blot Analysis

Immunoblotting for $G\alpha_{q/11}$ proteins in BA9 of all subjects revealed no differences between controls, MRDS or non-MRDS groups ($F(2,27) = 0.164$, $P = 0.850$). The ratio of band intensity compared to the internal control was 1.09 ± 0.17 and 1.00 ± 0.15 for the MRDS and non-MRDS groups, respectively, and 0.98 ± 0.06 for the control group. No correlation was found between $G\alpha_{q/11}$ immunoreactivity and [^{3}H]-pirenzepine binding ($P = 0.667$).

[^{35}S]-GTPγS Binding/Immunocapture Assay

To assess CHRM1 function in membranes prepared from BA9, agonist-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS immunospecific binding was assessed in controls, MRDS and non-MRDS groups. The CHRM full agonist oxotremorine-M and the CHRM1-selective allosteric partial agonist, AC-42 (Spalding *et al*., 2002; Langmead *et al*., 2006), were used to stimulate receptors in all membrane preparations. Representative concentration-dependent responses for oxotremorine-M- and AC-42-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in the different groups are shown in Figure 2. The potency of oxotremorine-M differed between groups ($F(2,27) = 4.234$, $P = 0.025$) and was significantly decreased in MRDS group (pEC_{50} (M), 5.69 ± 0.16) compared to the control group (pEC_{50} = 6.17 ± 0.10; $P = 0.024$). No difference in potency was observed between control and the non-MRDS group (pEC_{50} = 6.05 ± 0.07; $P = 0.091$). Scatter graphs of these data are shown in Figure 3. Similar trends were also seen with respect to AC-42-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in the different groups (pEC_{50} (M) values: control, 5.31 ± 0.19; MRDS, 4.72 ± 0.11; non-MRDS, 5.14 ± 0.14; $F(2, 27) = 2.222$, $P = 0.127$) with a significantly decreased potency in the MRDS group ($P = 0.024$).

No differences were detected in basal levels of $G\alpha_{q/11}$-[^{35}S]-GTPγS binding between the groups (basal values (c.p.m. per 25 μg membrane protein): control, 2147 ± 96; MRDS, 2138 ± 93; non-MRDS, 2384 ± 152; $F(2,27) = 2.074$, $P = 0.145$). We therefore chose to analyze relative agonist efficacy by expressing agonist-stimulated increases in $G\alpha_{q/11}$-[^{35}S]-GTPγS binding as the magnitude of the signal window generated in c.p.m. (Figure 4). Maximal oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in BA9 membranes (measured as concentration-response curve maxima – basal calculated by GraphPad Prism) was significantly greater in the MRDS group (signal window, 4703 ± 296 c.p.m.) compared to the controls (3240 ± 190 c.p.m.; P=0.003). In contrast, no significant difference was observed between the control and non-MRDS groups ($P = 0.464$). Similar, but not statistically significantly different, trends were seen with respect to AC-42 relative efficacy differences between the groups with respect to the increase in $G\alpha_{q/11}$-[^{35}S]-GTPγS binding-over-basal (c.p.m): control, 1964 ± 173; MRDS, 2664 ± 228; non-MRDS, 2234 ± 176; $F(2, 27) = 2.183$, $P = 0.062$). The intrinsic activity of AC-42 relative to oxotremorine-M ([maximal AC-42 response/maximal oxotremorine-M response] x

100) remained unchanged in all sub-groups (control, 53 ± 3%; MRDS, 54 ± 4%; non-MRDS, 50 ± 3%).

Representative concentration-response curves from the control sub-groups of subjects with schizophrenia are shown in Figure 5. As well as illustrating the dextral-shift and increase in maximal response observed for the MRDS group, it can also be seen that there are apparent differences in the slope factors (Hill coefficients) for the three curves ($F(2, 27) = 14.835$, $P < 0.001$). We therefore determined Hill coefficients for oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding responses (Figure 6) in the control and sub-groups subjects with schizophrenia. Hill coefficients for oxotremorine-M concentration-response curves for the MRDS (nH = 0.82 ± 0.02) were significantly greater than those determined for control (0.64 ± 0.01; $P < 0.001$) and non-MRDS (0.68 ± 0.02; $P = 0.0013$) groups. There was no significant difference between the latter two groups.

When potencies, relative efficacies and Hill coefficients for oxotremorine-M-stimulated receptor-$G\alpha_{q/11}$-[^{35}S]-GTPγS binding were plotted against [^{3}H]-pirenzepine binding values for all subjects, significant correlations were obtained for all parameters; pEC_{50} ($P < 0.001$, $r^2 = 0.325$; Figure 7A), maximal $G\alpha_{q/11}$-[^{35}S]-GTPγS binding-over-basal ($P = 0.046$, $r^2 = 0.134$; Figure 7B) and Hill coefficient ($P < 0.001$, $r^2 = 0.551$; Figure 7C). Although, the observed correlation between receptor expression and pEC_{50} value was consistent with receptor theory (i.e. a decrease in pEC_{50} as receptor expression increases), the *increasing* maximal responsiveness with *decreasing* receptor expression was not.

DISCUSSION

In this study we have shown that a sub-group of subjects with schizophrenia defined as MRDS (Scarr *et al*., 2008), display distinct functional changes with respect to the proximal downstream signalling consequences of CHRM activation. In particular, despite a marked reduction in CHRM1 expression (down by 75-80% compared to both a control group and a sub-group of subjects with schizophrenia displaying normal [^{3}H]-pirenzepine binding), agonist-stimulated [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ proteins was increased in MRDS membranes prepared from dorsolateral cortex (BA9), relative to both the other non-MRDS and control groups.

Changes in CHRM expression in the brains of subjects with schizophrenia have been widely reported, with the majority of studies reporting subtype-specific deficits (Dean *et al*., 1996; 2002; Crook *et al*., 2000; 2001; Raedler *et al*., 2003). Based on the criteria used recently to define a CHRM-deficit endophenotype (Scarr *et al*., 2008), we have performed a pharmacological analysis of CHRM function in membranes prepared from BA9 of MRDS and non-MRDS subjects, and control subjects. We have utilized a $G\alpha_{q/11}$-[^{35}S]-GTPγS binding immunocapture assay (Salah-Uddin *et al*., 2008), which allows us to assess productive receptor-G protein coupling in membrane preparations and hence report a proximal functional readout of relative efficacy, as well as expression level of CHRM, within human dorsolateral cortex for the different subject groups. In addition, we have previously shown in human cortical (BA23/25) membranes that the oxotremorine-M-mediated signal is wholly attributable to CHRM1 stimulation, as agonist-stimulated [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ proteins is completely prevented by pre-incubation with the selective CHRM1 toxin, MT-7 (Salah-Uddin *et al*., 2008).

Initial experiments demonstrated that the full agonist oxotremorine-M and the CHRM1-selective allosteric partial agonist, AC-42 (Spalding *et al*., 2002; Langmead *et al*., 2006), each caused concentration-dependent increases in $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in control and both sub-groups subjects with schizophrenia. This is of particular relevance in the MRDS endophenotype as it might have been predicted that CHRM1 function would be diminished due to the observed decrease in receptor number, and that therapeutic strategies targeting the CHRM1 in this group might not be effective. However, although receptor number was decreased in the MRDS group, the residual CHRM1 population coupled with greater efficiency to this key functional readout

such that maximal responses were undiminished. Data were generated using oxotremorine-M for the comparison of potency, efficacy and receptor/G protein cooperativity in healthy and diseased tissue. This is because full agonist oxotremorine-M provides a larger signal window compared to the partial agonist AC-42 that allows for subtle differences in pharmacology to be measured. This is very important when investigating pathological mechanisms. Furthermore, the intrinsic activity of AC-42 relative to oxotremorine-M was similar in all groups. The selectivity and novel binding site of AC-42 at the CHRM1 affords it, and similarly acting compounds, potential therapeutic advantages (Langmead and Christopoulos, 2006) and therefore our observation is important in showing that AC-42 is effective in functionally activating the CHRM1 sub-type with similar relative efficacy in brain tissue from both normal donors and subjects with schizophrenia. Effects of AC-42 followed similar trends to oxotremorine-M, but were less pronounced in subject groups due to the smaller signal window.

Given the marked decrease in [^{3}H]-pirenzepine binding sites it is not surprising that the potency of oxotremorine-M to stimulate G$\alpha_{q/11}$-[^{35}S]-GTPγS binding was reduced 2-3 fold in the MRDS sub-group compared to controls and the normal PZP schizophrenia sub-group. Classical studies in which receptor expression levels are incrementally decreased by irreversible alkylation have demonstrated that concentration-effect curves become increasingly right-shifted and then collapse once all 'spare' receptors have been eliminated (see Kenakin, 2006).

In contrast, the relative efficacy of oxotremorine-M was significantly greater in the MRDS sub-group compared to the control and non-MRDS groups, despite the marked decrease in [^{3}H]-pirenzepine binding observed in this sub-group. This difference cannot be explained or predicted simply by receptor number as efficacy is generally determined by multiple components in the signal transduction system measured. An alternative way of showing this between sub-groups efficacy difference is presented in Figure 8. Here we have calculated the increase in [^{35}S]-GTPγS bound to G$\alpha_{q/11}$ proteins (as fmol mg^{-1} membrane protein) stimulated by a maximally-effective oxotremorine-M concentration and compared this to the receptor density (assuming that 15 nM [^{3}H]-pirenzepine provides an estimate of the receptor density in each BA9 membrane preparation). In membranes prepared from control and non-MRDS groups this yields a stoichiometry of <1, however, for the MRDS sub-group this value rises to ~4, representing an 8-10 fold change in the CHRM1-to-G$\alpha_{q/11}$-[^{35}S]-GTPγS stoichiometry in this schizophrenia

endophenotype, and suggesting a fundamentally altered receptor-G protein coupling in this group. Given that the intrinsic activity of AC-42 does not vary between sub-groups we can conclude that a comparable stoichiometry change also occurs for this allosteric partial agonist in MRDS.

Another potentially important observation made in this study is that the slope factor (Hill coefficient) of the concentration-response curves for oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding varies between groups. The Hill coefficient can be used to indicate cooperativity between receptor and G protein and in turn, be used as a measure of receptor-G protein coupling efficiency. Hill coefficients of less than one were consistently observed in all subject groups, however significantly greater values were observed in the MRDS group. There are a number of possible explanations why Hill coefficients within this range are observed with respect to receptor-G protein coupling. These include the possibility of receptor populations existing in different affinity states for the agonist (e.g. "free" receptors versus pre-coupled receptor-G protein ternary complexes (De Lean *et al*., 1980)), and/or the compartmentalization of receptors and/or G proteins constraining productive coupling. Irrespective of the precise explanation, the observed increase in Hill coefficient for oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding concentration-response curves in the MRDS group may be a further indication of an adaptation to decreased CHRM1 number in cortical neuronal populations of subjects with schizophrenia categorized within the MRDS endophenotype. This suggests an enhanced CHRM1/$G\alpha_{q/11}$ coupling and supports the altered stoichiometry of receptor-facilitated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding observed in this sub-group (Figure 8).

The stoichiometry of productive receptor-G protein coupling is likely to be influenced by a variety of factors. Here we have shown that levels of $G\alpha_{q/11}$ protein expression within BA9 are not altered in schizophrenia. A previous study, which undertook quantitative analysis of $G\alpha_{q/11}$ protein expression in human cerebral cortex, indicated that this G protein subtype is expressed at a level of 20-30 pmol mg^{-1} protein (López de Jesús *et al*., 2006). This indicates that $G\alpha_{q/11}$ proteins are likely to be expressed in considerable (>10 fold) excess compared to the CHRM1 in the membrane preparations used here, and thus $G\alpha_{q/11}$ protein availability for productive coupling to agonist-occupied CHRM1 is unlikely to be rate-limiting. Therefore, it may be possible for

each activated CHRM1 to recruit multiple $G_{q/11}$ proteins and in the MRDS endophenotype the data would suggest that an increased receptor/G protein ratio may manifest itself as a greater cooperativity between receptor and G protein (higher Hill coefficient) along with increased [^{35}S]-GTPγS binding per CHRM1.

Irrespective of what underlies the relative efficacy difference, we have clearly shown using a $G\alpha_{q/11}$ protein-specific [^{35}S]-GTPγS binding assay that while a sub-population of subjects with schizophrenia show a marked decrease in dorsolateral prefrontal cortical CHRM1 this is (super-) compensated for by an adaptative change in receptor-G protein coupling efficiency. Whether this adaptation represents a manifestation of the underlying disease process, or is a secondary process mitigating (or militating) the decline in CHRM1 expression remains to be determined. The CHRM1 subtype is predominant among CHRMs in the cortex, striatum and hippocampus (Weiner *et al*., 1990; Levey *et al*., 1991), where it is expressed on the majority of neuronal post-synaptic nerve terminals (Hersch and Levey, 1995). The CHRM1 subtype has been shown to be involved in cognitive processes, most recently through the use of knockout mice (Hamilton *et al*., 1997; Anagnostaras *et al*., 2003; Wess *et al*., 2007). An array of clinical and basic science evidence has implicated dysfunctional prefrontal cortical circuitry in the pathophysiology of schizophrenia (Perlstein *et al*., 2001; Ragland *et al*., 2007), with cholinergic deficits being specifically implicated (Hyde and Crook, 2001). To date, it has not been possible to separate the MRDS endophenotype from other subjects with schizophrenia by CHRM1 sequence, gender, age, suicide, duration of illness or any particular drug treatment (Scarr *et al*., 2008). However, our observation of altered CHRM1 number and efficacy (assessed as receptor-stimulated [^{35}S]-GTPγS-for-GDP exchange on $G\alpha_{q/11}$ proteins) provides new insight into how the prefrontal cortical cholinergic circuitry may change and adapt in different endophenotypes of subjects with schizophrenia. Interestingly, very recent data from a small clinical trial of patients with schizophrenia (Shekhar *et al*., 2008) demonstrated that the CHRM agonist xanomeline, which shows some degree of selectivity for CHRM1, separated from placebo for verbal learning and short term memory indices. These data suggest that this therapeutic intervention may be effective in the treatment of cognitive deficits in schizophrenia and it will be important to establish the relative effectiveness of CHRM1 agonists in the MRDS and non-MRDS sub-groups of subjects with schizophrenia.

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DISCLOSURE/CONFLICTS OF INTEREST

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Figure Legends

Figure 1 [^{3}H]-Pirenzepine binding in dorsolateral prefrontal cortex (BA9) sections from low PZP (MRDS) and normal PZP (non-MRDS) sub-groups of subjects with schizophrenia and control subjects. Specific [^{3}H]-pirenzepine binding was determined as described in the Methods section and expressed as d.p.m. mg^{-1} estimated wet weight equivalents (ETE). The horizontal line indicates the mean value for each group. Statistically significant differences between groups are indicated as $^{***}P<0.001$.

Figure 2 Representative concentration-response curves for oxotremorine-M- and AC-42-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in membranes prepared from dorsolateral prefrontal cortex (BA9) tissue of control and low and normal [^{3}H]-pirenzepine binding in sub-groups of subjects with schizophrenia. Basal and agonist-stimulated specific [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ proteins was determined as described in the Methods section. Curves for 10 donors in each sub-group were constructed and analyzed to provide the data shown in Figs. 3-5

Figure 3 Potency estimates (pEC_{50}) in dorsolateral prefrontal cortex (BA9) sections from MRDS and non-MRDS groups and control subjects. Oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding concentration-effect curves were analyzed for membranes prepared from dorsolateral prefrontal cortex (BA9) tissue of control and low (MRDS) and normal (non-MRDS) [^{3}H]-pirenzepine binding sub-groups of subjects with schizophrenia. A pEC_{50} (M) value was determined for each concentration-effect curve as described in the Methods section. A statistically significant difference between groups is indicated as $^{**}P<0.01$.

Figure 4 Relative efficacy estimates in dorsolateral prefrontal cortex (BA9) sections from MRDS and non-MRDS groups and control subjects. Oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding concentration-effect curves were analyzed for membranes prepared from dorsolateral prefrontal cortex (BA9) tissue of control, MRDS and non-MRDS groups. The signal window (in c.p.m.) was determined as an increase in [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ proteins over

basal as described in the Methods section. A statistically significant difference between groups is indicated as $^{**}P<0.01$.

Figure 5 Representative concentration-response curves for oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding in membranes prepared from dorsolateral prefrontal cortex (BA9) tissue of control and MRDS and non-MRDS groups (panel A). Basal and agonist-stimulated specific [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ proteins was determined as described in the Methods section. Data for one individual from each sub-group is shown to illustrate the rightward curve shift, increase in maximal response and steepened slope in the MRDS group relative to non-MRDS and control subjects. Panel B shows a representative western blot of relative $G\alpha_{q/11}$ levels in the dorsolateral prefrontal cortex (BA9) of a control subject and a subject with schizophrenia. The lanes show images of the band in the Internal Control (IC), a control subject (C) and a subject with schizophrenia (S).

Figure 6 Hill coefficients from analysis of oxotremorine-M concentration-response $G\alpha_{q/11}$-[^{35}S]-GTPγS binding curves in dorsolateral prefrontal cortex (BA9) sections from MRDS, non-MRDS groups and control subjects. Statistically significant differences between groups are indicated as $^{***}P<0.001$.

Figure 7 Correlation analysis of pEC_{50} (**A**), oxotremorine-M-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding signal window (**B**) and Hill coefficients (**C**) with [^{3}H]-pirenzepine binding in dorsolateral prefrontal cortex (BA9) sections from MRDS, non-MRDS groups and control subjects.

Figure 8 Assessing the stoichiometry of agonist-stimulated $G\alpha_{q/11}$-[^{35}S]-GTPγS binding relative to CHRM expression in dorsolateral prefrontal cortex (BA9). Individual values for the net increase in $G\alpha_{q/11}$-[^{35}S]-GTPγS binding stimulated by oxotremorine-M (100 μM) were calculated as fmol mg^{-1} protein. This allowed a direct comparison with CHRM1 expression, assessed by [^{3}H]-pirenzepine binding (see Methods). The stoichiometry (the molar ratio of $G\alpha_{q/11}$-[^{35}S]-GTPγS binding to receptor level) is shown for controls, MRDS and non-MRDS groups (n=10 per group).

Table 1 Demographic, *post-mortem* and pharmacological data for sub-groups of subjects with schizophrenia and control subjects. For each individual donor the sex, age, and cause of death, *post-mortem* interval (PMI, in hours), CNS pH, duration of illness (in years), brain weight (in grams), last recorded anti-psychotic(s) drug and calculated last recorded drug dose are given. Where information is missing from datasets, this is indicated as 'NA'.

Sex	Age	Cause of death	Suicide	PMI	CNS pH	DOI	Brain Weight	Last Recorded Antipscyhotic Drug	Last Recorded Drug Dose
	(yr)			(h)		(yr)	(g)		
Schizophrenia - Low Muscarinic Receptors (MRDS)									
M	71	aspiration / food	N	48.0	6.45	53	1505	Thioridazine	150
M	53	aspiration / food	N	43.0	6.23	7	1575	Chlorpromazine	200
M	69	ischaemic heart disease	N	44.5	6.38	47	1407	Trifluoperazine HCl	100
F	65	ruptured abdominal aneursysm	N	50.0	6.35	18	1170	Fluphenazine decanoate, Haloperidol	550
M	41	combined drug toxicity	Y	31.0	6.20	11	1440	Fluphenazine decanoate, Trifluoperazine HCl	500
M	19	unascertained	Y	43.0	6.22	3	1440	Haloperidol, Chlopromazine	750
M	42	coronary artery atheroma	N	47.0	6.26	22	1530	Fluphenazine decanoate	1000
M	26	carbon monoxide poisoning	Y	52.0	6.39	2	NA	Haloperidol decanoate	500
F	47	pneumonia	U	50.0	6.31	20	1570	Risperidone	600
M	48	bronchopneumonia	U	30.0	6.62	24	NA	Flupenthixol, Thioridazine	1250
Mean ± SEM	48 ± 6			43.9 ± 2.4	6.34 ± 0.04	21 ± 6	1293 ± 44		560 ± 117

Schizophrenia - High Muscarinic Receptors (non-MRDS)

M	27	burning	Y	22.0	6.28	8	1200	Chlorpromazine, Pimozide	1310
F	72	aspiration pneumonia	N	58.5	6.48	37	NA	Chlorpromazine	25
M	47	multiple injuries	Y	41.5	6.52	21	1420	Chlorpromazine, Haloperidol decanoate	1400
M	22	pericarditis	N	37.0	6.07	3	1500	Trifluoperazine HCl, Flupenthixol	450
M	38	meningoencephalitis	N	50.0	6.02	4	NA		50
F	48	pulmonary thromboembolism	N	52.5	6.21	22	1200	Fluphenazine decanoate, Chlorpromazine	700
M	65	bronchopneumonia	N	42.0	6.29	36	NA	Trifluoperazine, Haloperidol decanoate	460
M	56	metastatic cancer	Y	42.0	6.17	11	1391		NA
M	42	hanging	Y	47.0	6.44	8	NA	Haloperidol decanoate	128
M	70	bronchopneumonia	Y	46.0	5.80	20	1190		NA
Mean ± SEM	49 ± 5			43.9 ± 3.1	6.23 ± 0.07	17 ± 4	1317 ± 56		324 ± 177

Controls

M	42	cardiomegaly		63.0	6.34		1385		
F	21	myocarditis		58.0	6.03		1180		
M	21	acute epiglottitis		40.0	5.82		1420		
M	26	electrocution		24.0	6.42		1501		

	M	48	coronary artery atheroma	24.0	6.37	1240
	M	68	aortic stenosis	41.0	6.06	1510
	M	43	coronary artery atheroma	51.0	6.43	726
	M	53	pulmonary thromboembolism	12.0	6.34	1405
	F	39	acute myocardial infarction	52.0	6.24	NA
	M	42	coronary artery atheroma	26.0	6.32	1350
Mean ± SEM		40 ± 5		39.1 ± 5.3	6.24 ± 0.06	1302 ± 81

Figure 1 (Salah-Uddin et al.)

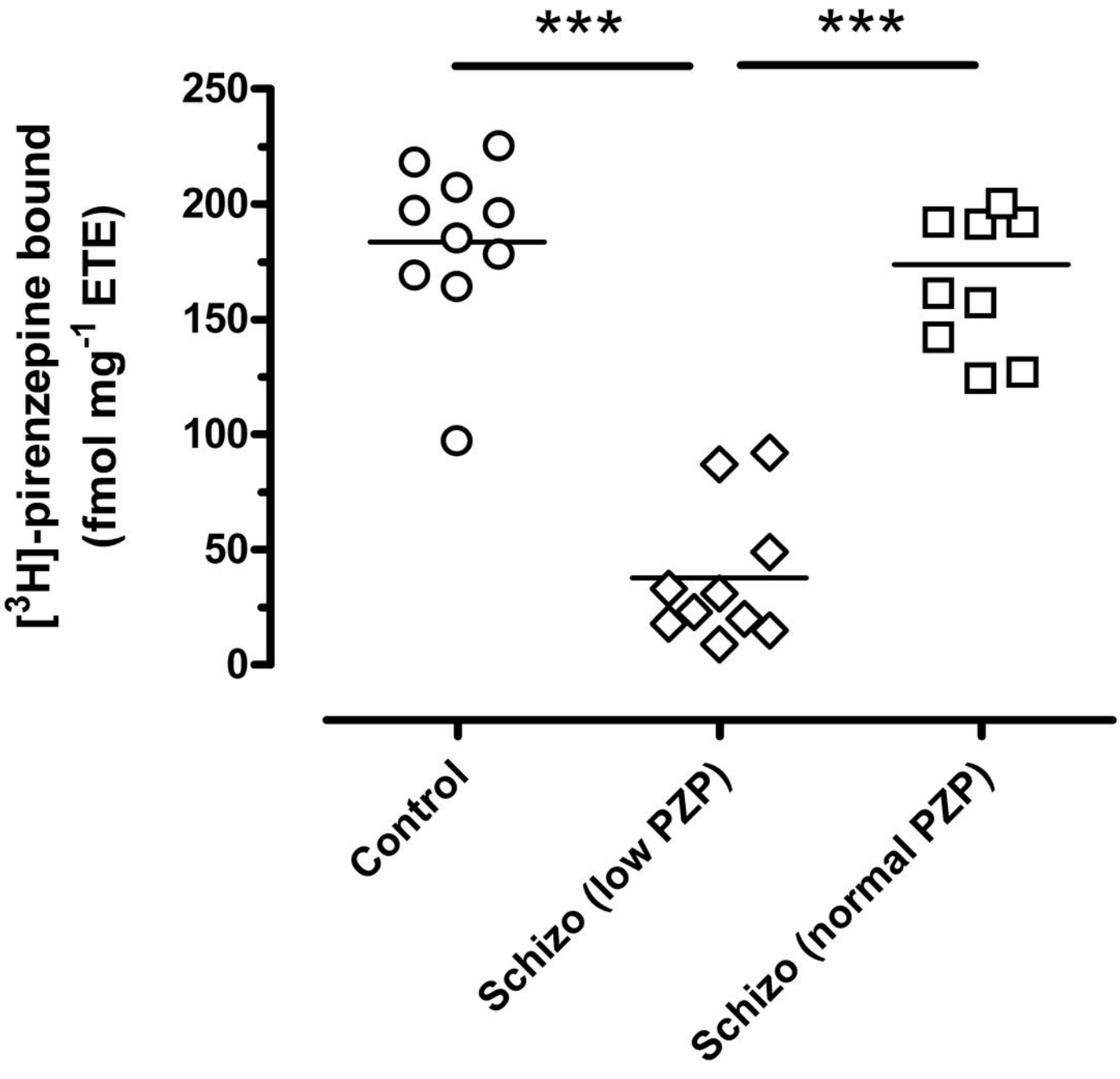

Figure 2 (Salah-Uddin et al.)

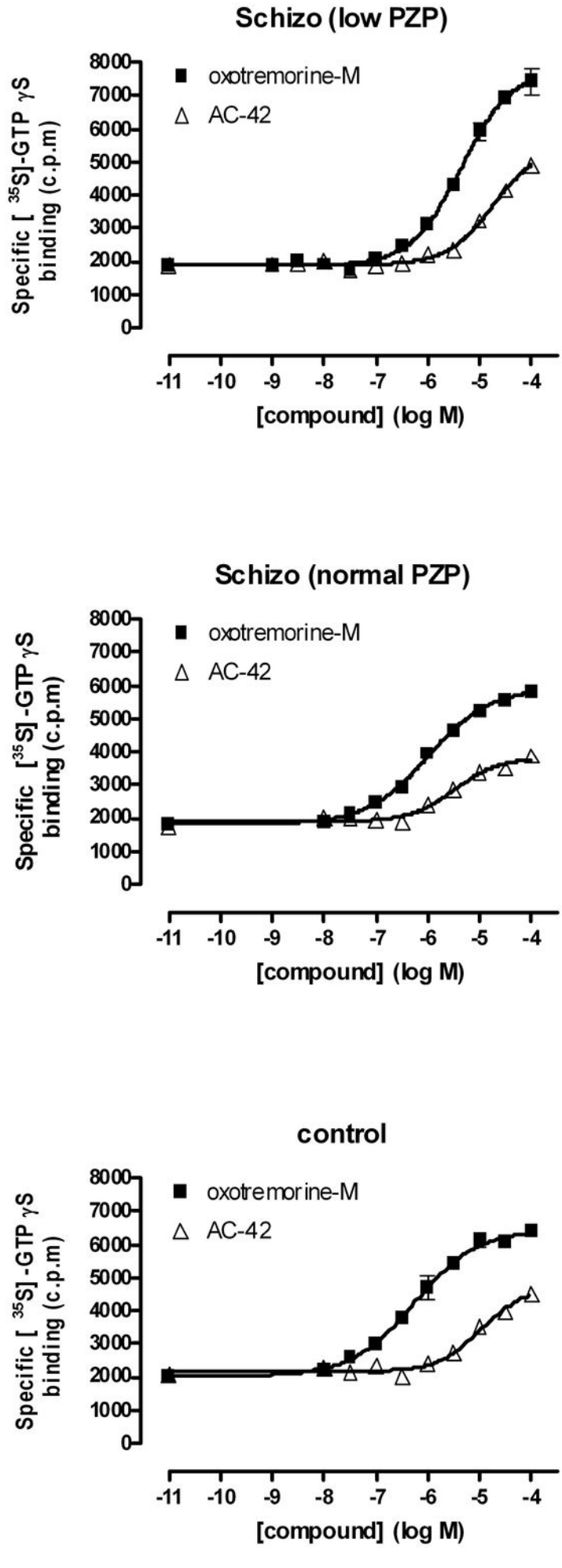

Figure 3 (Salah-Uddin et al.)

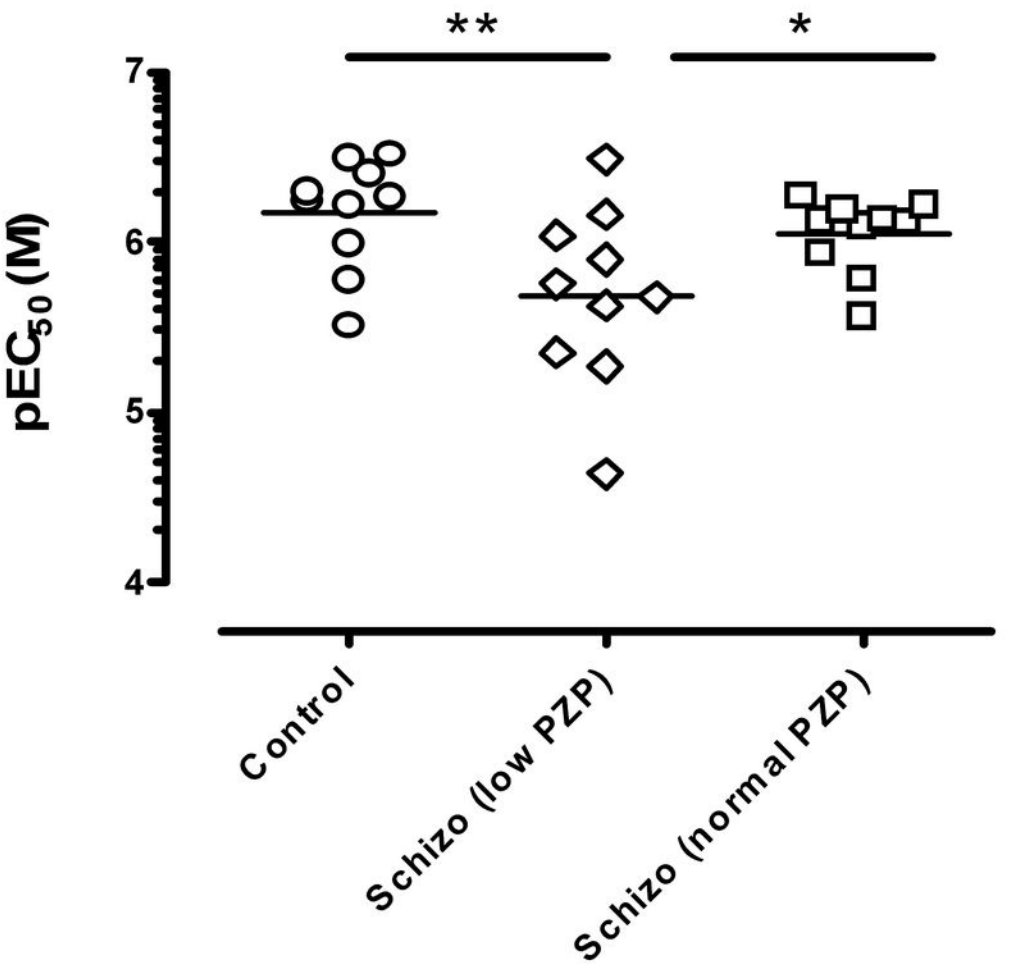

Figure 4 (Salah-Uddin et al.)

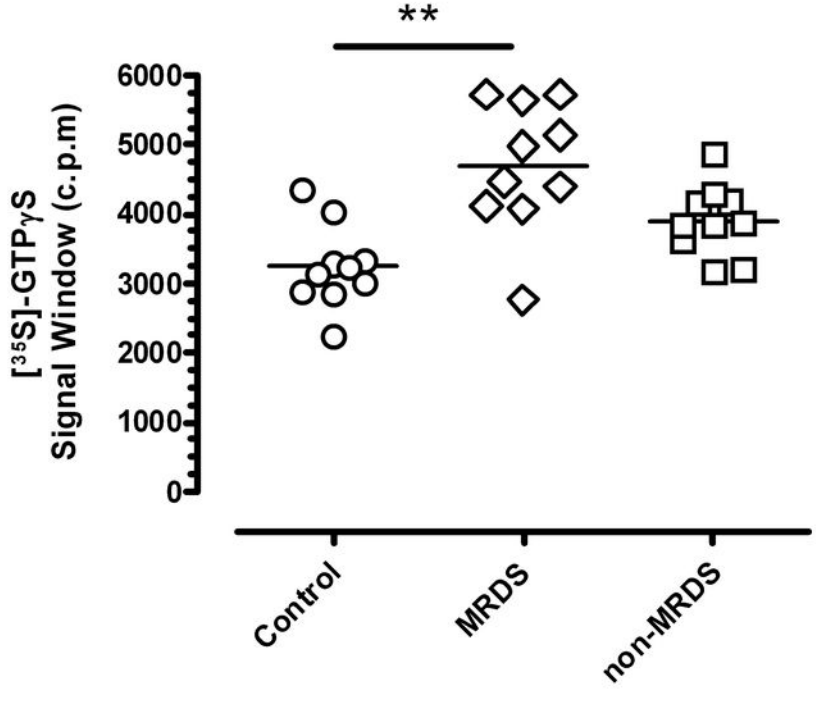

Figure 5 (Salah-Uddin et al.)

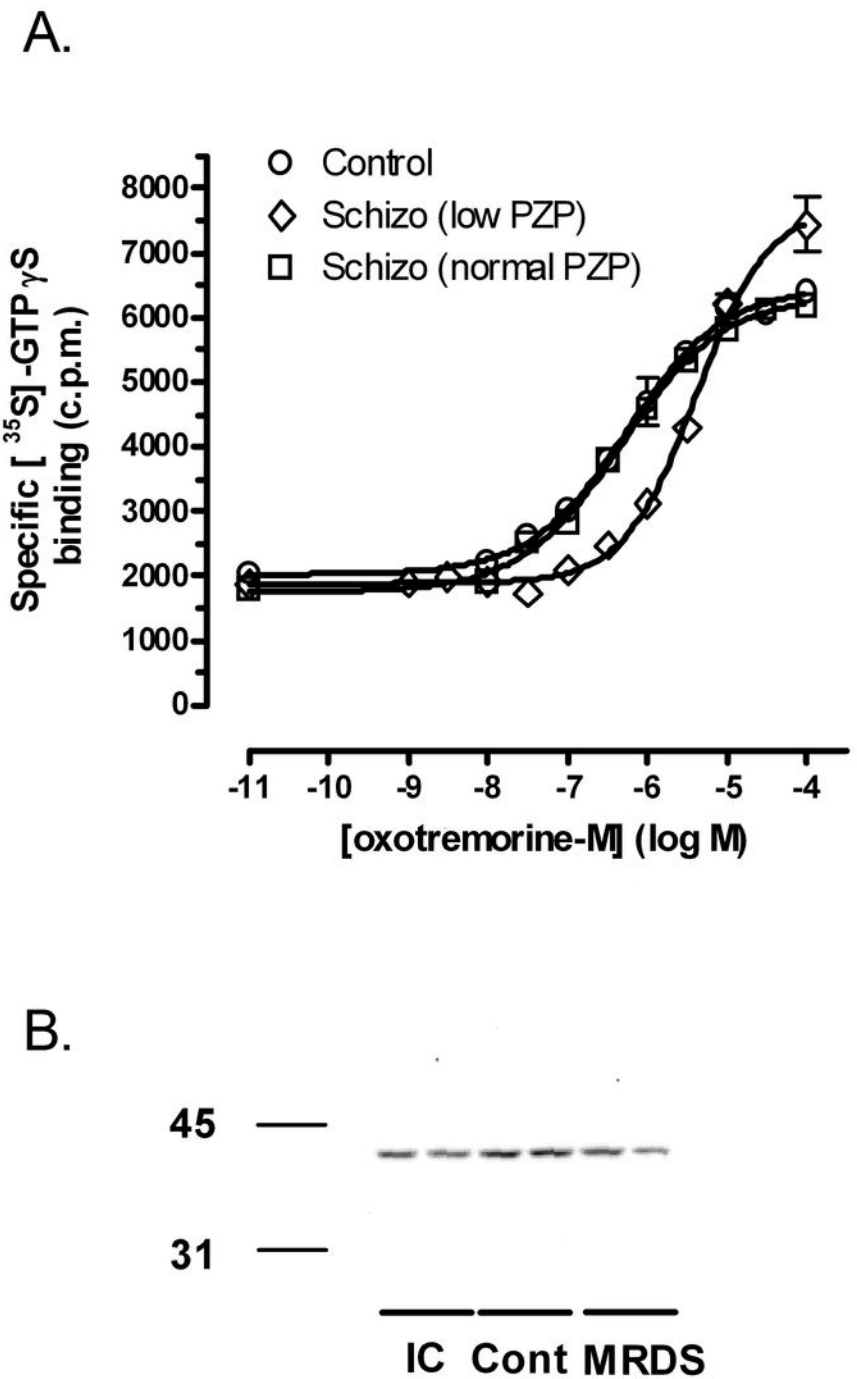

Figure 6 (Salah-Uddin et al.)

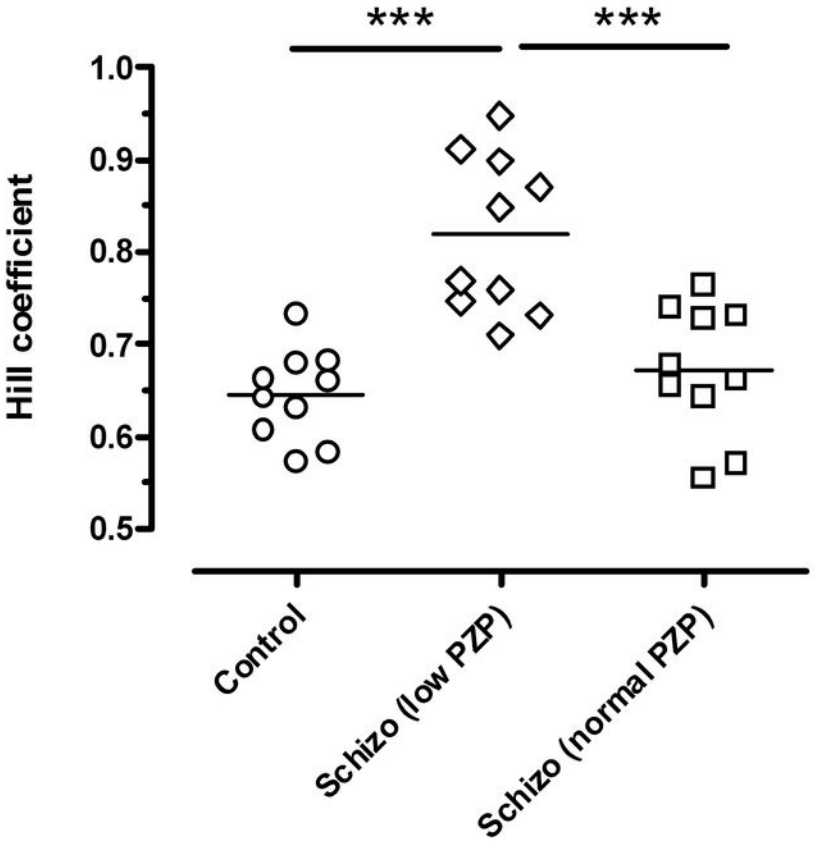

Figure 7 (Salah-Uddin et al.)

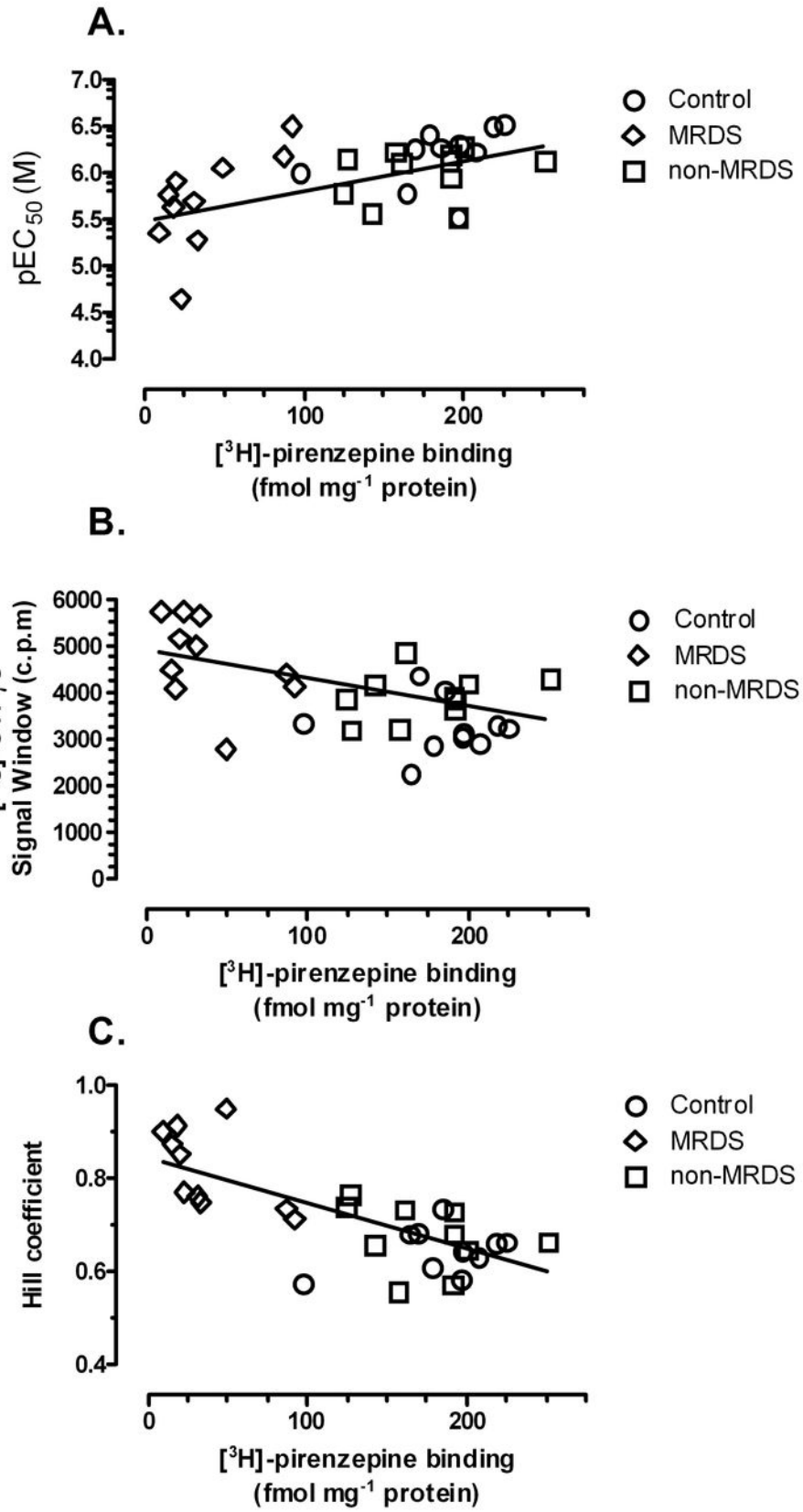

Figure 8 (Salah-Uddin et al.)