An investigation of the factors that regulate muscarinic receptor expression in schizophrenia

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Abstract

We previously identified a group of subjects with schizophrenia who, on average, have a 75% decrease in cholinergic receptor, muscarinic 1 (CHRM1) in Brodmann's area (BA) 9. To extend this finding, we determined i) if the decrease in CHRM1 was present in another related CNS region (BA6) ii) whether the marked decrease in CHRM1 was accompanied by changes in levels of other CHRMs and iii) potential factors responsible for the decreased CHRM1 expression. We measured CHRM1 and CHRM3 using in situ radioligand binding with [^{3}H]pirenzepine and [^{3}H]4-DAMP respectively in BA6 from 20 subjects with schizophrenia who had low levels of CHRM1 in BA9 (SzLow[^{3}H]PZP), 18 subjects with schizophrenia whose levels of CHRM1 were similar to controls (SzNormal[^{3}H]PZP) and 20 control subjects. Levels of CHRM1, 3 and 4 mRNA were measured using qPCR and levels of the transcription factors, Sp1 and Sp3, determined using Western blots. In BA6, the density of [^{3}H]pirenzepine binding was decreased in subjects with SzLow[^{3}H]PZP ($p < 0.001$) compared to controls. The density of [^{3}H]4-DAMP binding, levels of CHRM1, 3 and 4 mRNA and levels of Sp1 and Sp3 were not significantly different between the three groups. This study shows that the previously identified decrease in CHRM1 expression is not confined to the dorsolateral prefrontal cortex but is present in other cortical areas. The effect shows some specificity to CHRM1, with no change in levels of binding to CHRM3. Furthermore, this decrease in CHRM1 does not appear to be associated with low levels of CHRM1 mRNA or to simply be regulated by the transcription factors, Sp1 and Sp3, suggesting that other mechanisms are responsible for the decreased CHRM1 in these subjects.

1. Introduction

Schizophrenia is a neuropsychiatric disorder that most likely encompasses a syndrome of diseases with multiple underlying pathologies (Jablensky, 2006). Therefore, one approach to better understand the syndrome of schizophrenia is to study its component disorders (Raedler et al., 2007). We have reported a sub-group (25%) of people with schizophrenia that can be separated into a separate population within schizophrenia because they have a marked loss (~70%) of the levels of [^{3}H]pirenzepine binding in BA9 (Scarr et al., 2009), opening up the opportunity to separate people with schizophrenia into two populations based on cortical [^{3}H]pirenzepine binding.

We have shown that, under the assay conditions we use, [^{3}H]pirenzepine predominately binds to the CHRM1 (Gibbons et al., 2013; Scarr and Dean, 2008). Importantly, clinical and preclinical studies suggest that cortical CHRM1 is important in cognitive functions (Anagnostaras et al., 2003; Nathan et al., 2012). Thus, in this study, we choose to expand our studies on cortical CHRM1 to BA6 because this region of the cortex has been shown to act in concert with BA9 when cognitive tasks such as attention, working memory, episodic memory retrieval and visual awareness are being undertaken (Naghavi and Nyberg, 2005). This is of particular interest because these processes are often affected in people with schizophrenia (Blasi et al., 2010; Ranganath et al., 2003). In addition, decreased levels of [^{3}H]pirenzepine binding and CHRM1 mRNA in BA6 have been reported in people with schizophrenia (Dean et al., 2008; Mancama et al., 2003). Thus, in our current studies, we measured levels of [^{3}H]pirenzepine binding in BA6 from people with schizophrenia who had low (SzLow[^{3}H]PZP) and relatively unchanged (SzNormal[^{3}H]PZP) levels of [^{3}H]pirenzepine binding in BA9 and in age sex matched controls. In addition, we determined if changes in CHRM1 may be somewhat selective by measuring levels of CHRM3 using a modified radioligand binding technique (Jeon et al., 2013) and of mRNA for CHRM1, 3 and 4.

Notably, we have shown that there was no change in CHRM1 gene sequence associated with a change in levels of CHRM1 gene expression (Scarr et al., 2009), suggesting changes in levels of CHRM1 expression could be due to changes in transcriptional regulation. Importantly, assessing the putative promoter region of the human CHRM1 gene with Transcription Element Search System (TESS), which searches putative transcription binding sites in DNA sequence (http://www.cbil.upenn.edu/cgi-bin/tess/tess), identified a Sp1 binding site. Furthermore, this Sp1 site is reported to be capable of recruiting Sp1 in vitro (Wood et al., 1999) and, by interacting with Sp3, regulating levels of CHRM1 expression. One marker of a change in control of CHRM1 expression by this transcriptional regulation pathway would be changes in cellular localisation and ratio of Sp1 and Sp3 (Chuang et al., 2008; Li et al., 2004; Suske, 1999; Tan and Khachigian, 2009). Therefore, we measured the nuclear and cytosolic levels of Sp1 and Sp3 and their ratios as a surrogate measurement of whether changes in translocation of the transcription factors had occurred in SzLow[^{3}H]PZP group.

2. Materials and Methods

2.1 Human tissue collection, clinical follow up and diagnosis

All human tissues were sourced though the Victorian Brain Bank Network, held at the Mental Health Research Institute. Approval for the study was obtained from both the Ethics Committee of the Victorian Institute of Forensic Medicine and the Mental Health Research and Ethics Committee of Melbourne Health. In order for the diagnosis of a psychiatric illness to be accepted, clinical case histories were used to reach a diagnostic consensus using the Diagnostic Instrument for Brain Studies (DIBS) (Hill et al., 1999), according to DSM-IV criteria (Roberts et al., 1998). Duration of illness (DOI), the final recorded antipsychotic drug dose (FRADD), lifetime exposure to antipsychotic drugs (LTEA), post-mortem interval (PMI) and CNS pH was recorded as described previously (Dean et al., 2008).

2.2 Tissue preparation

BA6, defined as the caudal portion of the superior frontal gyrus and the middle frontal gyrus extending from the cingulate sulcus on the medial surface to the lateral sulcus on the lateral surface, was collected from the left hemisphere of 58 subjects; 38 subjects with schizophrenia and 20 subjects with no history of psychiatric disorders. The 38 subjects with schizophrenia consisted of 18 subjects with SzNormal[³H]PZP and 20 subjects with SzLow[³H]PZP, all matched as closely as possible for donor age, PMI, brain pH, sex, and DOI, death by suicide, FRADD, LTEA or prescription of anti-cholinergics and benzodiazepines in the schizophrenia groups (Table 1).

2.3 In Situ radioligand binding with autoradiography

Radioligand binding was performed using single-point saturation methods as described previously (Dean et al., 1996), which give a good approximation of the density of radioligand binding sites (Pavey et al., 2002) in tissue sections. The use of autoradiography provides information regarding the anatomical distribution of binding.

Tissue sections (20 µm) were incubated with radioligand in the presence (non-specific binding (NSB): 2 sections) or absence (total binding (TB): 3 sections) of a competing displacing agent. [³H]pirenzepine binding was performed as described previously (Dean et al., 1996). For [³H]4-DAMP binding, the sections were pre-incubated in assay buffer (50 mM Tris-HCl (pH 7.4)) for 15 min at room temperature and then [³H]4-DAMP was incubated for 1 hr in assay buffer containing the radioligand (6 nM) (PerkinElmer, MA, USA) and 1 µM pirenzepine (Sigma-Aldrich, MO,USA) in the presence (2 sections: NSB) or absence (3 sections: TB) of 10 µM 4-DAMP mustard (Sigma-Aldrich, MO,USA) (Jeon et al., 2013). The sections were apposed to BAS-TR2025 phosphoimage plates (Fujifilm, Tokyo, Japan) with [³H]microscales™ (G.E. Healthcare, UK) for 3 days. The plates were scanned in a

BAS5000 high resolution phosphoimager (Fujifilm, Tokyo, Japan). The resulting images were analysed using analytical imaging station (AIS) imaging software (Imaging Research, St. Catharines, ON, Canada), and the total minus non-specific binding (specific binding) was expressed as fmol / mg estimated tissue equivalents (ETE) (Pavey et al., 2002).

2.4 RNA purification and first strand cDNA synthesis

Total RNA was extracted from 100 mg frozen tissue using 1 ml of Trizol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quantity and quality were determined by spectrophotometer readings and RNA integrity numbers (RINs). DNA contamination was checked by PCR using primers specific for genomic DNA. Total RNA was reverse transcribed into cDNA as previously described (Udawela et al., 2011).

2.5 Real-time PCR assay

Real-time PCR was performed using a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad, Laboratories, CA, USA) as previously described (Udawela et al., 2011). Oligonucleotides were designed using Beacon Designer 6.00 software (premier Biosoft, Palo Alto, CA, USA; see table 2). The identities of the amplicons were confirmed by sequencing. The cycling conditions for all genes in this study were 95°C for 3 min then 40 cycles of 95°C for 10 sec, 57°C for 30 sec and 72°C for 30 sec. The mRNA expression was determined using the Pfaffl method (Pfaffl, 2001), employing the standard curve efficiency. Expression was normalised against the geometric mean of three reference genes: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Cyclophillin A (PPIA) and Alpha-synuclein (SNCA), selected as the most stable from 9 reference genes in post-mortem CNS tissue using geNorm software (M=1.398, 1.386 and 1.407, respectively) (Vandesompele, geNorm.).

2.6 Tissue preparations for Western blots

Whole tissue homogenates were prepared as previously described (Dean et al., 2008). To prepare subcellular fractions, tissue was homogenised in 1 ml homogenisation buffer (1 mM $CaCl_2$, 5 mM EDTA, 10 mM HEPES, 0.5 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 10 mM NaCl). Subsequently, 150 µl of 2.5 M sucrose was added to each sample, which was then centrifuged at 6,300 g for 10 min at 4°C. The supernatant was collected and stored as the membrane, organelle and cytoplasmic fraction. The pellet, containing the nuclei, was resuspended in 500 µl of Tris-Sucrose-EDTA buffer (TSE buffer; 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 300 mM Sucrose, 0.1% IGEPAL-CA 630), centrifuged at 4,000 g for 5 min at 4°C and the supernatant discarded. The pellet was then resuspended in 100 µl of whole tissue homogenisation buffer (10 mM Tris-HCl (pH 7.4), 1% SDS, 1 mM Na_3VO_4). The supernatant containing the membrane, organelle and cytoplasmic fractions was

centrifuged at 107,000 g for 30 min at 4°C. This final supernatant fraction was used for cytosolic protein.

2.7 Western blots

Western blot analyses of Sp1 and Sp3 were performed using optimised protocols. Protein was separated on a SDS-PAGE and then transferred onto nitrocellulose membranes. For Sp1, the process was as previously described (Dean et al., 2008). For Sp3, the membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) with 0.1% Tween 20 (Sigma-Aldrich, MO, USA) (0.1% TTBS) for 1 hr at room temperature and incubated with a 1:1,000 dilution of rabbit anti-human Sp3 antibody (D-20) (Santa Cruz, CA, USA) in blocking buffer overnight at 4°C. In order to show that the process produced clean fractions with no cross-over of the subcellular fraction, a Western blot was conducted on nuclear and cytosol fractions using the subcellular markers, histone deacetylase 1 (HDAC1; nuclear marker) and beta-actin (cytosol marker) in each fraction. The membranes were incubated with a 1:1,000 dilution of rabbit anti-human HDAC1 antibody (Upstate, MA, USA) in 0.1% TTBS overnight at 4°C or a 1:100,000 dilution of mouse anti-human beta-actin antibody (Chemicon, MA, USA) in 0.1% TTBS for 1 hr at room temperature. Secondary antibodies (DAKO, Glostrup, Denmark) were incubated at a 1:2,000 dilution of the goat anti-rabbit antibody IgG conjugated to HRP for HDAC1, Sp1 and Sp3 or a 1:2,000 dilution of goat anti-mouse antibody for beta-actin for 1 hr at room temperature.

Antigenic bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, MA, USA). To control for intra and inter-blot variation, an internal control (IC) was employed as described previously (Dean et al., 2007). The sum intensity of the desired band was then determined from the image for each subject in the cohort. The each nuclear and cytosolic level of Sp1 and Sp3 were calculated by dividing by the sum intensity of total cellular Sp1 and Sp3 on that blot to obtain a distribution ratio in the levels of nuclear and cytosol fractions.

2.8 Statistics

The first series of analyses initially involved comparing data from all subjects with schizophrenia to control subjects. Subsequently, the subjects with schizophrenia were divided according to level of [3H]pirenzepine binding and analysis was performed comparing SzNormal[3H]PZP, SzLow[3H]PZP and control subjects. Numerical demographic and all experimental measurement data were assessed for differences using a Generalized Linear Model (GLM) and the source of any variation was determined using Bonferroni Simultaneous Tests. The non-numerical demographic variables were compared using a Fisher's exact test between subjects with schizophrenia and control subjects and a chi squared test in SzNormal[3H]PZP and SzLow[3H]PZP and control subjects. To assess whether a relationship existed between experimental measurements

and numerical demographic factors or RNA integrity numbers (RINs) for mRNA, Pearson's product moment correlations assuming a straight line best fit were performed. Due to small cohort sizes only strong relationships ($r^2 > 0.49$) (Cook and Weisberg, 2011) where the regression deviated significantly were considered as being of interest. Where necessary, the impact of non-numerical demographic variables were analysed using an analysis of co-variance (ANCOVA). Statistics were conducted using Prism 5.01 (Graphpad Software, CA, USA) and Minitab version 16.1 (Minitab Inc. PA, USA) software.

3. Results

3.1 CNS collection, clinical and pharmacological data

Due to our experimental design, there were no significant differences between subjects with schizophrenia and control subjects for age (F=0.02, d.f.=1,56, p=0.88), brain pH (F=2.83, d.f.=1,56, p=0.10), PMI (F=0.45, d.f.=1,56, p=0.50) or gender (P=1, Fisher's exact test). When subjects with schizophrenia were divided into groups according to their levels of [³H]pirenzepine binding, Age (F=0.22, d.f.=2,55, p=0.98), PMI (F=0.37, d.f.=2,55, p=0.69), brain pH (F=1.95, d.f.=2,55, p=0.15) and gender ratio (Chi square=0.04, d.f.=2, p=0.98) did not differ across three groups. There was no significant difference between the schizophrenia groups in DOI (F=0.00, d.f.=1,36, p=0.98), FRADD (F=0.08, d.f.=1,32, p=0.76), LTEA (F=0.11, d.f.=1,32, p=0.74), the frequency of administration of anti-cholinergics (p=0.755, Fisher's exact test), benzodiazepines (p=0.76, Fisher's exact test) or death by suicide (p=0.73, Fisher's exact test).

3.2 [³H]pirenzepine binding

[³H]pirenzepine binding showed differential distribution across the cortical layers (Figure 1 A). A previous study from our laboratory, comparing [³H]pirenzepine binding to cresyl violet stained sections, has shown that the outer binding layer overlays cortical laminae I to III whilst the inner layer covers laminae IV to VI (Dean et al., 2008). However, as the densities of [³H]pirenzepine binding showed strong correlations between outer and inner layers in each group (Con: r^2=0.60, p<0.0001; Sz: r^2=0.96, p<0.0001; SzNormal[³H]PZP: r^2=0.79, p<0.0001; SzLow[³H]PZP: r^2=0.98, p<0.0001), the intensities were measured across the entire gray matter including outer and inner layers because the density within each layer was not an independent variable.

The GLM analysis revealed that [³H]pirenzepine binding showed significant variations with diagnosis (F=34.80, d.f.=1,56, p<0.001 (Figure 1 B). Bonferroni Simultaneous Tests revealed that the variation with diagnosis was due to significant decreases in [³H]pirenzepine binding density in tissue from people with schizophrenia (p<0.001) compared to that from controls.

When subjects with schizophrenia were divided into groups according to their levels of [³H]pirenzepine binding, the levels of [³H]pirenzepine binding varied significantly with groups (F=90.70,

d.f.=2,55, p<0.001; Figure 1 C). Bonferroni Simultaneous Tests revealed that the variance with groups was due to [³H]pirenzepine binding density being lower in both subjects with SzLow[³H]PZP (p<0.0001) and SzNormal[³H]PZP (p<0.01) compared to controls. [³H]pirenzepine binding density was also lower in subjects with SzLow[³H]PZP compared to SzNormal[³H]PZP (p<0.0001).

3.2.1 Potential Biological Relationships

As the levels of [³H]pirenzepine binding were decreased in subjects with SzLow[³H]PZP levels of binding in BA6 were compared to that in BA9. There were strong correlations between binding in BA6 and BA9 in the group with SzLow[³H]PZP (r²=0.80, p<0.0001) that did not exist for SzNormal[³H]PZP (r²=0.33, p=0.01) or control subjects (r²<0.01, p=0.97).

3.3 [³H]4-DAMP binding

[³H]4-DAMP binding was homogenous across all cortical layers in BA6 (Figure 2 A). Therefore, an integrated measure was taken across the gray matter. Densities of [³H]4-DAMP binding were not different between schizophrenia and control subjects (F=0.01, d.f.=1,56, p=0.94, Figure 2 B) or between SzNormal[³H]PZP, SzLow[³H]PZP and control subjects (F=0.05, d.f.=2,55, p=0.95, Figure 2 C).

3.4 Real time-PCR

In the RNA experiment, one of samples from a subject with schizophrenia (specifically, SzNormal[³H]PZP) was not given RIN value indicating degradation and low quality. Since it was not appropriate for gene expression analysis, this sample was excluded from the qPCR study.

There were no variations in levels of CHRM1, 3 and 4 mRNA in BA6 from subjects with schizophrenia compared to controls (CHRM1: F=1.80, d.f.=1,55, p=0.19; CHRM3: F=0.98, d.f.=1,55, p=0.33; CHRM4: F=3.52, d.f.=1,51, p=0.07) (Figure 3 A. C. and E) or in low- and normal-[³H]PZP binding groups compared to control (CHMR1: F=1.19, d.f.=2,54, p=0.31; CHRM3: F=0.49, d.f. =2,54, p=0.62; CHRM4: F=1.73, d.f.=2,50, p=0.19; Figure 3 B. D. and F). In 4 cases (1 sample for CON, 2 samples for SzNormal[³H]PZP, 1 sample for SzLow[³H]PZP), levels of mRNA for CHRM4 were below the sensitivity of the assay and these samples were excluded in the CHRM4 analysis.

3.5 Western blots

Importantly, we showed the subcellular protein markers, HDAC1 (nuclear) and beta-actin (cytosolic) were localised in the appropriate cellular fraction with negligible crossover (Figure 4 A)

indicating a good separation of nuclei and cytosol. Although Sp1 and Sp3 have been reported to be distributed in nuclear and cytosol fractions (Van Wijnen et al., 1993), in human cortex we showed that Sp3 was mainly present in the nucleus whilst Sp1 was present in both fractions (Figure 4 B).

The levels of Sp1 (nuclear: F=0.35, d.f.=1,56, p=0.55; cytosolic: F=0.01, d.f.=1,56, p=0.94), Sp3 (nuclear: F=0.00, d.f.=1,56, p=0.97; cytosolic: F=0.39, d.f.=1,56, p=0.54) and the ratio of Sp1/Sp3 (nuclear: F=0.00, d.f.=1,56, p=0.98; cytosolic: F=0.80, d.f.=1,56, p=0.38) did not vary between subjects with schizophrenia and controls (Figure 4 C. E). When schizophrenia was divided according to [3H]pirenzepine binding levels, the levels of Sp1 (nuclear: F=0.19, d.f.=2,55, p=0.83; cytosolic: F=0.07, d.f.=2,55, p=0.93), Sp3 (nuclear: F=0.33, d.f.=2,55, p=0.72; cytosolic: F=0.30, d.f.=2,55, p=0.74) and the ratio of Sp1/Sp3 (nuclear: F=0.02, d.f.=2,55, p=0.98; cytosolic: F=0.43, d.f.=2,55, p=0.65) were not different in either nuclear or cytosolic fractions when compared between groups (Figure 4 D. F).

3.6 Potential confounds

There were no strong relationships with age, PMI, DOI, brain pH, FRADD or LTEA for any other of experimental data. In addition, an ANCOVA revealed that the levels of experimental measurements were not significantly affected by any categorical demographic data such as gender, death by suicide or prescription of benzodiazepines or anti-cholinergic drugs (see Supplementary Tables 1 and 2).

4. Discussion

This study shows that the density of [3H]pirenzepine binding is decreased in subjects with schizophrenia compared to control subjects and that the loss of radioligand binding that defined SzLow[3H]PZP in BA9 is present in BA6. Although the group with schizophrenia that have normal levels of CHRM1 in BA9 also showed a decrease in CHRM1 in BA6, the magnitude of the decrease was much smaller. In additionImportantly, there was no relationship strong association between experimental data and any potential confounding factor examined in this study. However, there are factors that could not be fully addressed using the data we have available following our postmortem review, such as the full impact of while the data showed that potential confounding factors, like diagnosis, final doses and lifetime exposure equivalents to antipsychotic drugs, did not different in SzLow[3H]PZP, we could not address etiopathobiology and other possible illness confounds, such as types of medications and their combinations, other non-pharmacological treatments, substance abuse, illness effects including decreased stimulation and institutionalization, medical comorbidity, and other comorbid psychiatric conditions. However, these are limitations associated with most, if not all, studies using postmortem CNS tissue in psychiatry as there was no reliable information about etiopathobiology and these possible illness confounds. It will be important to seek to However, to

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more clarify any such relationships in the finding of a sub-groups of people with in SzLow[3H]PZP but this will likely need to be done in living individuals identified using neuroimaging, a future study could include assessing the etiopathobiology and other possible illness confounds. This finding will clarify the relationship whether levels of [3H]pirenzepine binding expression are likely to be mediating an effect on potential confounding factors. Furthermore, levels of [3H]pirenzepine binding in BA6 and BA9 were strongly correlated in the SzLow[3H]PZP, but not SzNormal[3H]PZP or control groups. This strong relationship between levels of [3H]pirenzepine binding in the different brain regions from subjects with SzLow[3H]PZP is possibly the result of altered regulation of CHRM1 expression in the brain and is consistent with our findings in other cortical regions (Gibbons et al., 2013) and in some individuals in a neuroimaging study of muscarinic receptor levels in people with schizophrenia (add Raedler reference here).

Levels of radioligand binding to CHRM3 and levels of CHRM 1, 3 and 4 mRNA were not changed in schizophrenia or in SzLow[3H]PZP or SzNormal[3H]PZP. Notably, studies of radioligand binding to the five human CHRMs (M1-5) showed that anticholinergic drugs, such as benztropine, bound with high affinity to all CHRMs subtypes (Larson et al., 1991). Thus, our finding that there is not decrease in binding of cortical [3H]4-DAMP despite reductions in [3H]pirenzepine argue that residual anticholinergic drugs are not present in the tissue used in this study. Interestingly, this study showed that the level of CHRM1 mRNA was not changed in either group with schizophrenia. This result is contrary to the previous report of reduced expression of CHRM1 mRNA in BA6 from subjects with schizophrenia (Mancama et al., 2003). It is possible that the discrepancy between the studies arises because in this study we used 3 reference genes (GAPDH, PPIA, SCNA) compared to one reference gene (beta-actin) in the earlier study. This could be a significant difference given data suggesting that at least two or three reference genes should be used for normalization purposes to ensure variation in tissue processing are negated (Vandesompele et al., 2002).

We show that the levels and localisation of Sp1 and Sp3, or the ratio of these two proteins, are not different between subjects with schizophrenia and controls or schizophrenia divided according to [3H]pirenzepine status. These findings are consistent with an earlier study in BA6 which reported that levels of total Sp1 in crude homogenates were not changed in subjects with schizophrenia compared to controls (Dean et al., 2008) and suggest that the decrease in CHRM1 in schizophrenia cannot be simply related to changes in levels or localisation of Sp1 or Sp3. However, there are multiple layers of gene regulation including cell signaling, protein localization, modification and degradation, alterations in which could be responsible for the reduced CHRM1 expression seen in schizophrenia. More recently, a post-mortem study reported a significant upregulation of miR-107, which is predicted to target CHRM1, in BA9 samples from people with schizophrenia (Beveridge et al., 2010), and a study from our laboratory validated that miR-107 expression was increased in a subgroup of people with schizophrenia who have decreased CHRM1 expression in BA9 (Scarr et al., 2013). These data suggest that important factors, other than SP1 and SP3, may be driving the changes in levels of [3H]pirenzepine in BA6 from people with schizophrenia.

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Figure legends.

Figure 1. **A:** A representative autoradiograph of [³H]pirenzepine binding to human BA6 with non-specific binding shown as an inset. The image shows two layers of radioligand binding (layer 1 and layer 2) and white matter. **B:** Graphical depiction (Mean ± s.e.m.) of [³H]pirenzepine binding BA6 from subjects with schizophrenia and control subjects. **C:** [³H]pirenzepine binding in control, SzNormal[³H]PZP group and SzLow[³H]PZP group. **p < 0.01, and ***p < 0.001.

Figure 2. A: Representative autoradiographs of otal and non-specific (insert) binding of [³H]4-DAMP in BA6. **B:** Graphical representation (Mean ± s.e.m) of [³H]4-DAMP binding in subjects with schizophrenia and control subjects. **C:** [³H]4-DAMP binding in control, SzNormal[³H]PZP group and SzLow[³H]PZP group.

Figure 3. Relative mRNA expression of CHRM1 (Mean ± s.e.m. ratio of 3 reference genes: **A:** tissue from control subjects and subjects with schizophrenia and **B:** control, SzNormal[³H]PZP group and SzLow[³H]PZP group). Relative mRNA expression of CHRM3 (Mean ± s.e.m. ratio of 3 reference genes: **C:** control subjects and subjects with schizophrenia and **D:**

control, SzNormal[³H]PZP group and SzLow[³H]PZP group). Relative mRNA expression of CHRM4 (Mean ± s.e.m. ratio of 3 reference genes: **E:** control subjects and subject with schizophrenia and **F:** control, SzNormal[³H]PZP group and SzLow[³H]PZP group).

Figure 4. A: Representative images of HDAC1 (N: nuclear protein), beta-actin (C: cytosol protein) and **B:** Sp1 and Sp3 in subcellular fractions using Western blotting. Sp1 and Sp3 in nuclear and cytosol protein (Mean ± s.e.m. ratio of whole protein: **C:** control subjects and subjects with schizophrenia and **D:** control, SzNormal[³H]PZP group and SzLow[³H]PZP group. Ratio of Sp1 to Sp3 in nuclear protein (Mean ± s.e.m. ratio of Sp1 to Sp3: **E:** control subjects and subjects with schizophrenia and **F:** control, SzNormal[³H]PZP group and SzLow[³H]PZP group.

Figure 1. [³H]pirenzepine binding assay

Fig. 1

A.

Layer 2

Layer 1

B.

C.

☐ Control
■ Schizophrenia

▨ SzNormal[^{3}H]PZP
▨ SzLow[^{3}H]PZP
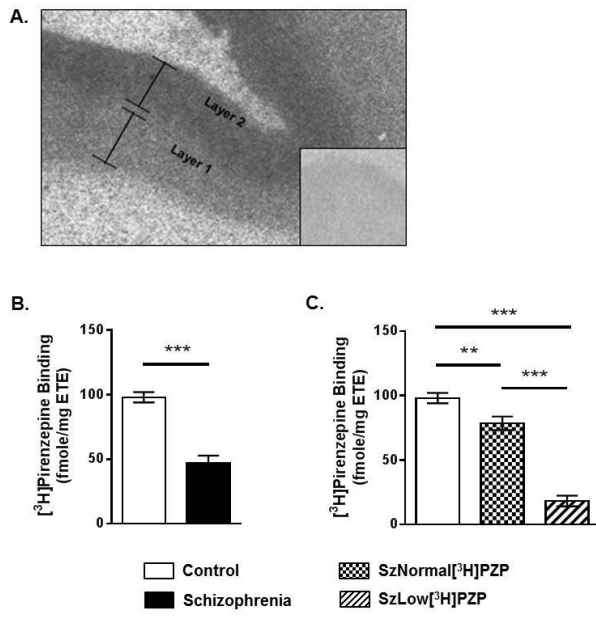
Figure 2. [^{3}H]4-DAMP binding assay

Fig. 2

A.

B.

C.

☐ **Control**

■ **Schizophrenia**

▨ **SzNormal[³H]PZP**

▨ **SzLow[³H]PZP**
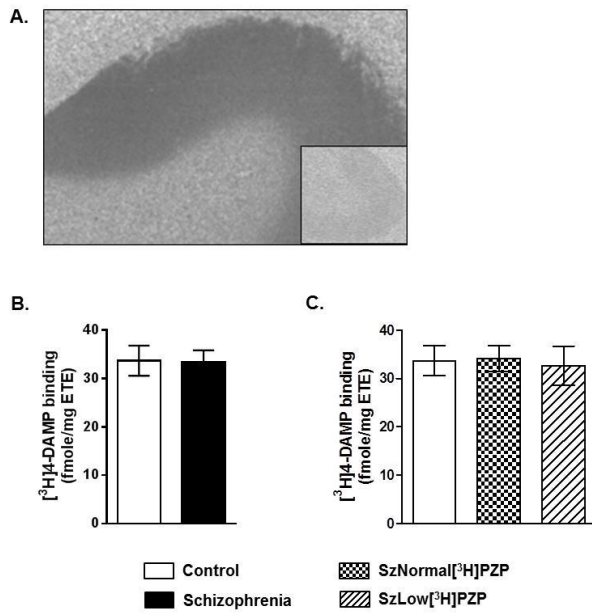
Figure 3. The levels of CHRMs mRNA

Fig. 3

A. CHRM1 mRNA

B. CHRM1 mRNA

C. CHRM3 mRNA

D. CHRM3 mRNA

E. CHRM4 mRNA

F. CHRM4 mRNA

☐ Control   ▨ SzNormal[^{3}H]PZP
■ Schizophrenia   ▨ SzLow[^{3}H]PZP
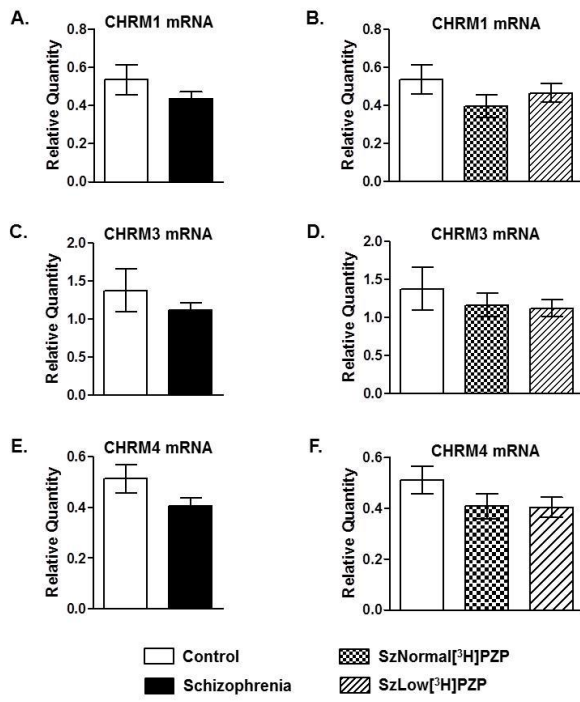
Figure 4. The levels of Sp1 and Sp3 in subcellular fractions

A Post-mortem human brain

HDAC1   beta-Actin

N N C C   N N C C

B Post-mortem human brain

Sp1   Sp3

N N C C   N N C C

97.4 —   ← Sp1

116 —   ← Sp3
97.5 —   ← Sp3
66 —

C.

Ratio of whole protein

1.5
1.0
0.5
0.0

Nuclear Cytosol   Nuclear Cytosol

Sp1   Sp3

D.

Ratio of whole protein

1.5
1.0
0.5
0.0

Nuclear Cytosol   Nuclear Cytosol

Sp1   Sp3

E.

Ratio of Sp1 to Sp3

1.2
0.8
0.4
0.0

Nuclear   Cytosol

F.

Ratio of Sp1 to Sp3

1.2
0.8
0.4
0.0

Nuclear   Cytosol

☐ Control   ▨ SzNormal[³H]PZP
■ Schizophrenia   ▨ SzLow[³H]PZP
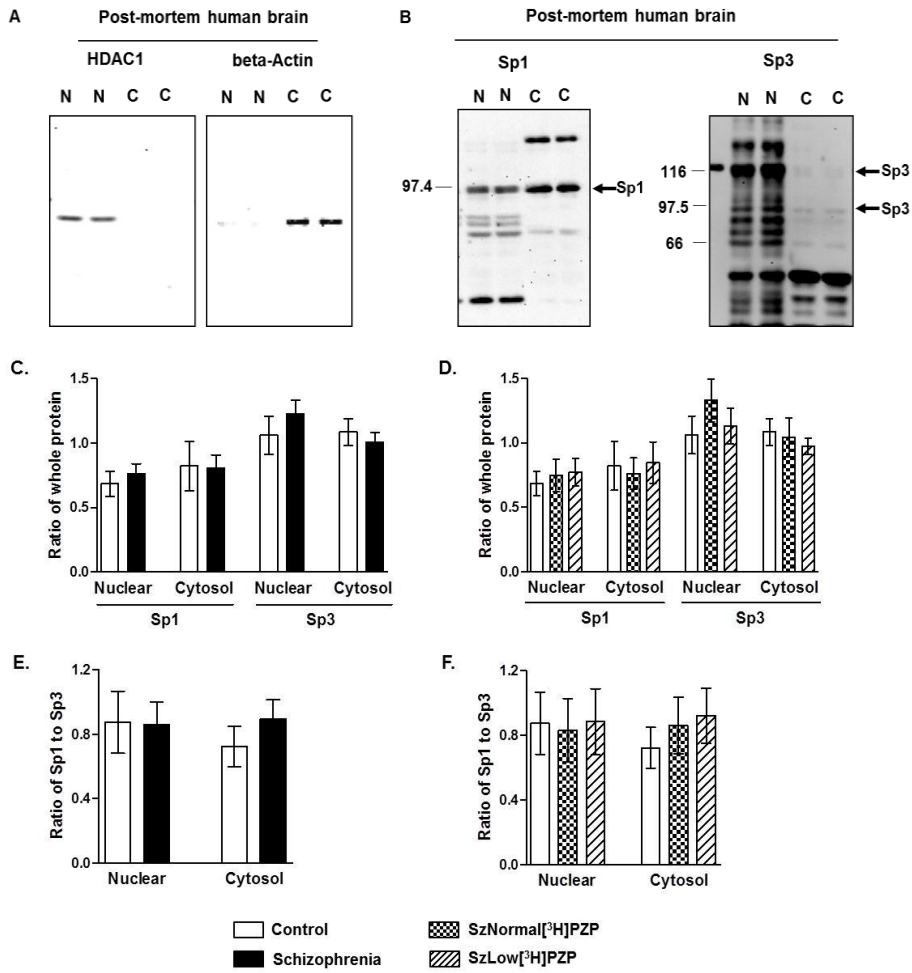
Table 1: A summary of the demographic and collection data (Mean ± s.e.m) for the cohort used in this study in post-mortem brain tissue from BA6.

Diagnosis	Control	Sz	SzNormal[^{3}H]PZP	SzLow[^{3}H]PZP
Age (yr)	46 ± 4	46 ± 3	47 ± 4	46 ± 4
PMI (h)	43 ± 3.4	40 ± 2.1	42 ± 3.0	39 ± 2.7
Brain pH	6.32 ± 0.05	6.21 ± 0.04	6.18 ± 0.06	6.25 ± 0.04
Sex (M/F)	16M/4F	30M/8F	14M/4F	16M/4F
DOI (yr)	NA	20 ± 2	20 ± 4	20 ± 4
Suicide	NA	26N/12Y	13N/5Y	13N/7Y
FRADD	NA	634 ± 98	670 ± 163	593 ± 124
LTEA	NA	12.3 ± 2.8	11 ± 4.0	13.2 ± 4.1
Anti-cholinergics	NA	19N/19Y	10N/8Y	9N/11Y
Benzodiazepines	NA	14N/17Y	13N/8Y	11N/9Y

Abbreviations: PMI: Post-mortem interval, DOI: Duration of Illness, FRADD: Final recorded antpsychotic drug dose, LTEA: Lifetime exposure to antpsychotic drugs, NA: Not applicable

Table 2: A summary of the oligonucleotide primers used for an analysis of CHRMs expression.

Gene	Oligonucleotide sequence
CHRM1	F 5'-GCTCTACTGGCGCATCTACC-3' R 5'-GCCTTCGTCCTCTTCCTCTT-3'
CHRM3	F 5'- AGCAGCAGTGACAGTTGG-3' R 5'-GAGCACGATGGAGTAGATGG-3'
CHRM4	F 5'-GTCCTGGTGAACACCTTCTG-3' R 5'-TCTTTTTAAAGGTGGCGTTG-3'
GAPDH	F 5'-TGCACCACCAACTGCTTAGC-3' R 5'-GGCATGGACTGTGGTCATGAG-3'
PPIA	F 5'-ATGGTCAACCCCACCGTGTTCTTCG-3' R 5'-CGTGTGAAGTCACCACCCTGACACA-3'
SNCA	F 5'-CTGCTGCTGAGAAACCAAA-3' R 5'-CTGCTCCCTCCACTGTCTT-3'