Lower levels of cortical [^{3}H]pirenzepine binding to postmortem tissue defines a sub-group of older people with schizophrenia with less severe cognitive deficits.

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Abstract

Multiple lines of evidence argue for lower levels of cortical muscarinic M1 receptors (CHRM1) in people with schizophrenia due to a sub-group within the disorder who have a marked loss of CHRM1 (muscarinic receptor deficit sub-group (MRDS)). In this study we sought to determine if the lower levels of CHRM1 was apparent in older people with schizophrenia and whether the loss of CHRM1 was associated with symptom severity by measuring levels of cortical [^{3}H]pirenzepine binding to CHRM1 from 56 people with schizophrenia and 43 controls. Compared to controls (173 ± 6.3 fmol / mg protein), there were lower levels of cortical [^{3}H]pirenzepine binding in the people with schizophrenia (mean ± SEM: 153 ± 6.0 fmol / mg protein; $p = 0.02$; Cohen's $d = -0.46$). [^{3}H]pirenzepine binding in the people with schizophrenia, but not controls, was not normally distributed and best fitted a two-population solution. The nadir of binding separating the two groups of people with schizophrenia was 121 fmol / mg protein and levels of [^{3}H]pirenzepine binding below this value had a 97.5 % specificity for the disorder. Compared to controls, Clinical Dementia Rating Scale (CDR) did not differ in MRDS but were significantly higher in the sub-group with normal radioligand binding. Positive and Negative Syndrome Scale scores did not differ between the two sub-groups with schizophrenia. Our current study replicates and earlier finding showing a MRDS within schizophrenia and, for the first time, suggest this sub-group have less severe cognitive deficits others with schizophrenia.

Keywords: schizophrenia, muscarinic M1 receptor, sub-group, symptom ratings, cortex

1.0 Introduction

A growing body of evidence from pre-clinical (Dean and Scarr, 2021; Miyauchi et al., 2017), postmortem CNS (Crook et al., 2001; Mancama et al., 2003; Zavitsanou et al., 2004) and neuroimaging (Bakker et al., 2018; Raedler et al., 2003b) studies support a role for muscarinic receptors (CHRM) in the molecular pathology of schizophrenia. In humans, there are five (CHRM1 to CHRM5) muscarinic receptors that influence various physiological pathways due to differences in their signalling systems and tissue / cellular distribution (van der Westhuizen et al., 2020) however, current data show it is the CHRM1 and CHRM4 that are predominantly implicated in the molecular pathology of schizophrenia (Dean and Scarr, 2020). Significantly, studies using postmortem CNS suggest there are lower levels of the CHRM1 (Dean et al., 2016; Dean et al., 2002; Mancama et al., 2003; Scarr et al., 2006), but not CHRM 2, 3 (Scarr et al., 2006) or 4 (Dean et al., 2002), in the cortex from people with the disorder.

It is now argued that understanding the cause(s) of complex disorders such as schizophrenia will require the study of more biologically homogenous sub-groups within what is a syndrome of disorders (Tamminga, 2008). Thus, it is significant that it has been shown using cortical [^{3}H]pirenzepine binding that there is a sub-group (~ 25%) within schizophrenia who can be identified because of a marked loss (~ 75%) of cortical CHRM1 (muscarinic receptor deficit schizophrenia (MRDS)) (Scarr et al., 2009). Since the discovery of the sub-group of people within schizophrenia it has been shown they have widespread losses of the CHRM1 in many regions of the cortex (Gibbons et al., 2013), in most regions of the hippocampus (Hopper et al., 2019) and in the striatum (Hopper et al., 2019). Data also indicates the molecular pathology of MRDS involves changes in CHRM1 gene promoter methylation (Scarr et al., 2013), gene expression (Scarr et al., 2018b), a microRNA that affects the level of expression of the CHRM1 (Scarr et al., 2013) and cortical levels of the α7 nicotinic receptor

(Dean et al., 2020). These data show that people with MRDS have some unique disruptions in their cortical biochemical homeostasis.

It has recently been reported that the level of cortical CHRM1 in the cortex of people with schizophrenia are inversely related to their cognitive ability (Bakker et al., 2018). Hence, the extremely low levels of CHRM1 in people with MRDS would predict the sub-group would have severe cognitive deficits. In addition, it has been reported that there are low levels of cortical CHRM1 mRNA in the cortex of individuals with single nucleotide polymorphism (SNPs) in the catechol-O-methyltransferase (COMT) gene at rs4818 (CC vs GG) and rs4680 ((Val158Met) AA vs GG)) which was not apparent at SNPs rs737865 and rs165599 and was not dependent on the diagnosis of schizophrenia (Dean and Scarr, 2016). These findings are significant because genotype at rs4818 and rs4680 are suggested to be associated with levels of cognitive ability whilst rs737865 and rs165599 do not show such a relationship (Roussos et al., 2008). However, understanding the exact nature of the association between levels of cortical CHRM1 expression and cognition is complex because, as an example, a recent metanalysis has suggest that for the Val158Met genotype the Val allele is associated with improved cognitive flexibility whilst the Met allele allows improved sustained attention and target neural processing (Morris et al., 2020). It has also been reported that the 267C/C SNP in the CHRM1 is associated with higher levels of perseverative errors in people with schizophrenia (Scarr et al., 2012), a behaviour that is inversely related to the capacity to utilise cognitive ability (Crider, 1997). Thus, despite some need for further studies, from current data it can be hypothesised that people with MRDS would have severe cognitive deficits.

There is now evidence that the CHRM1 and 4 are targets for the treatment of schizophrenia as studies have reported that the drug xanomeline (Shekhar et al., 2008) and xanomeline in a formulation with a peripherally active CHRN1 antagonist (Brannan et al., 2021) decrease the severity of positive and negative symptoms in people with the disorder. Moreover, the earlier study found that treatment with

xanomeline lessoned cognitive deficits in people with schizophrenia (Shekhar et al., 2008). As xanomeline activates CHRM1 and CHRM4, the data from these clinical trials validate the hypothesis that activating CHRM1 and / or CHRM4 (Dean et al., 2003; Felder et al., 2001) would reduce the severity of a broad spectrum of symptoms in people with schizophrenia. Importantly, we have shown that activating the CHRM1 in post mortem tissue from people with MRDS at either the orthosteric (Salah-Uddin et al., 2009) or allosteric (Dean et al., 2016; Hopper et al., 2019) binding sites does not cause the same level of drug-responsive effects in MRDS compared to what is observed using tissue from controls or people with non-MRDS.

Our current data suggests that people with MRDS have distinct changes in cortical biochemical pathways and, based on our molecular findings, may be resistant to treatment with drugs that activate the CHRM1. One factor we have yet to fully address is suggested by data showing that age-related changes in CHRM1 signalling can exacerbate disease-related cognitive decline, as occurs in Alzheimer's disease (Medeiros et al., 2011). This could be relevant to people with MRDS as there are age-related changes in gene expression in the cortex from people with schizophrenia that appear to be part of its molecular pathology (Tang et al., 2012). Thus, even though there was no relationship between age and a patient being part of the MRDS sub-group (Scarr et al., 2009), we decided to compare levels of cortical CHRM1 in a population of older people with schizophrenia to older controls. Moreover, in using these cohorts we, for the first time, would be able to compare levels of cortical CHRM1 to ratings of symptom severity recorded pre-mortem.

2.0 Materials and Methods

2.1 Postmortem Tissue

Samples of dorsolateral prefrontal cortex (Brodmann's area (BA) 9) were obtained from the Mount Sinai Medical Center NIH Neurobiobank. Twenty-three of the donors with schizophrenia were antemortem assessed by research clinicians as part of a longitudinal study cohort and were diagnosed with schizophrenia according to DSM-III-R - IV criteria. The medical records diagnosis of schizophrenia for the remaining donors was confirmed by research clinicians after extensive medical records review and semi-structured interviews with donor-knowledgeable informants and family members (Powchik et al., 1998). In this latter group, for the diagnosis of schizophrenia, the symptoms associated with the disorder must have been documented to be present before 40 years of age, medical records collected during hospitalisation must have shown evidence of psychotic symptoms for at least 10 years. In addition, neuropathological examination must not have revealed any evidence of the presence of Alzheimer's disease or any other degenerative disorder. Finally, the data collected during the case history review had to allow two experienced clinicians to agree on a diagnosis of schizophrenia according to the DSM-III-R criteria was appropriate at time of death.

It is notable for this study that the tissue used was from donors that, during the pre-agonal 6 months proximal to death (Haroutunian et al., 1994), underwent the Clinical Dementia Rating Scale (CDR) (Hughes et al., 1982). The CDR is a global rating device that distinguishes a wide range of cognitive functioning, from healthy to severely impaired, in older people (Hughes et al., 1982) with each domain being rated given a score of 0 = healthy, 0.5 = Questionable Dementia, 1 = mild dementia, 2 = moderate dementia and 3 = severe dementia (Hughes et al., 1982). However, in this study we used the community-based version of the CDR scale which extends the original CDR scale to include scores of 4=profound and 5=terminal (Dooneief et al., 1996) . Importantly, the information collected using the CDR allows cognitive performance to be assessed in six domains, which are memory,

orientation, judgment and problem solving, community affairs, home and hobbies as well as personal care (Morris, 1997) which is why, in this study, the CDR ratings were used as an indicator of the severity of cognitive impairment of each donor which is an approach validated in other studies (Davidson et al., 1996; Kincaid et al., 1995). Measures of symptom severity using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) at the antemortem assessment visit closest to the time death were used for the current study.

As smoking is highly prevalent in subjects with schizophrenia (Hughes et al., 1986; Strand and Nybäck, 2005) efforts were made to qualitative the smoking status of each tissue donor into one of four categories (Y = smoker, N = non-smoker, E = ex-smoker and U = unknown).

CNS pH was also measured for all donors as this is the best measure of the quality of tissue preservation (Stan et al., 2006).

2.2 Preparation of membrane enriched cortex

Samples of BA 9 from each case was supplied as 50 mg of tissue that had been pulverised in liquid nitrogen and then stored at -80^{o}C prior to being freighted on dry ice. [^{3}H]pirenzepine binding to cortical membranes was measured as described previously (Dean et al., 2008b), hence each tissue sample was first reconstituted into 5.0 ml of ice-cold 10 mM potassium dihydrogen phosphate (KH_2PO_4), 10mM disodium hydrogen phosphate (Na_2PO_4) adjusted to pH 7.4 (assay buffer). The reconstituted tissue was then centrifuged at 21,500 x g in a Beckmann J2-HS centrifuge, using a JA 20.1 rotor, for 10 min at 4 °C. The resulting supernatant was discarded and the pellet suspended in 2.0 ml of assay buffer. The pellet containing the cortical enriched membrane fraction (CEMF) was then centrifuged under identical conditions, the resulting supernatant discarded and the pellet re-suspended in 1.0 ml of assay buffer. The protein concentration of the CEMF were determined using

the BioRad protein assay adapted for a microplate reader.

2.3 [^{3}H]pirenzepine binding

This study of [^{3}H]pirenzepine binding used single point saturation analysis, where the concentration of radioligand is 3 x Kd (15 nM), this approach gives a good estimation of the total number of binding sites in tissue (Dean et al., 1999; Rodbard, 1981). Given our approach, it was important that we have shown, using both CEMF's (Dean et al., 2008b) and tissue sections (Dean et al., 1996), that under our assay conditions the Kd for [^{3}H]pirenzepine binding to human cortical tissue is 5 nM. Whilst [^{3}H]pirenzepine binds to both the CHRM1 and CHRM4, in our experimental design we used a short incubation time (30 min) which means the more rapid on-rate of [^{3}H]pirenzepine to the CHRM1 increases the specificity of the radioligand for that receptor to >80% which has been confirmed using both human cloned receptors (Scarr and Dean, 2008) and cortex from muscarinic receptor knockout mice (Gibbons et al., 2013).

To measure levels of [^{3}H]pirenzepine binding to BA 9, CEMFs (in 0.2 mg total protein) was incubated with [^{3}H]pirenzepine (15 nM) in assay buffer in the absence (triplicate, total binding (TB)) or presence (duplicate, non-specific binding (NSB)) of 1.0μM 3-Quinuclidinyl 8-xanthenecarboxylate (QNX) for 30 min at room temperature. The incubation was stopped by placing the tubes in an ice bath and separating the bound and non-bound radioligand by filtration through Whatman GF/B filter paper using a Brandel Cell-harvester. After washing the filters ×3 with 3.0 ml ice-cold saline each filter paper was placed into a scintillation vial, 4.0ml of Beckman Ready-Protein® scintillation cocktail added and the radioactivity on each filter paper counted using a Packard Tricarb 2100 TR liquid scintillation analyser. The specific binding of [^{3}H]pirenzepine was calculated as TB – NSB and expressed as fmol / mg protein.

2.4 Statistics

Unless otherwise stated all statistics were completed using GraphPad Prism version 9.0.

The distribution of continuous numerical data was tested for normality using the D'Agostino and Pearson test. Then, Student t-tests (schizophrenia vs controls) or one-way ANOVA (MRDS vs non-MRDS vs Controls) were used to identify variation in age, postmortem interval (PMI after $\log_{10}$ transformation), final recorded antipsychotic drug dose (FRADD) and PANNS ratings across groups with $p < 0.05$ taken indicating the presence of significant variation. Mann-Whitney test (schizophrenia vs controls) or Kruskal-Wallis test and a post hoc Dunn's multiple comparison test (MRDS vs non-MRDS vs Controls) were used to identify variation in CDR scores with $p < 0.05$ taken indicating the presence of significant variation. Differences for non-numerical data across groups were identified using either the Fisher's exact test or the χ^2 test. Pearson Product-Moment Correlations were used to identify relationships between [^{3}H]pirenzepine binding with age, sex, PMI (for PMI after $\log_{10}$ transformation) and FRADD. As weak to medium correlations are not reflective of relationships of biological significance (Cook and Weisberg, 1999); only strong correlations ($r^2 \geq 0.49$) were considered to be potential confounds in relation to variations in [^{3}H]pirenzepine binding with diagnosis.

This study was particularly designed to identify the presence of a sub-group withing the syndrome of schizophrenia that could be defined by low levels of [^{3}H]pirenzepine binding (Scarr et al., 2009). Hence, given the problems in correctly assessing the distribution of data in small cohorts (D'Agostino et al., 1990), further analyses of the distribution of [^{3}H]pirenzepine binding was completed using Kernel density estimations (Rosenblatt, 1956) and Q-Q plots (Wilk and Gnanadesikan, 1968)(Free Statistics Software (v1.2.1) (Wessa, 2015)) to more closely assess any variation in the distribution of data. Where there was evidence of multiple populations within a diagnostic group, variation in

[^{3}H]pirenzepine binding between diagnostic groups and controls was assessed using a one-way ANOVA.

3.0 Results

3.1 Demographic, race, CNS collection and symptom ratings: Variation with diagnosis and impact on [^{3}H]pirenzepine binding

Dorsolateral prefrontal cortex was obtained from 56 people with schizophrenia and 43 controls (Table 1; Supplementary Table 1). There was no significant variation in age, CNS pH or racial mix with diagnosis. There was variation in the ratio of females to males and, compared to controls and PMI was longer in the schizophrenia cohort. Smoking status was not obtained for 13 controls and 15 people with schizophrenia (Supplementary Table 1). There was a significant variation in the frequency of people in each smoking category in controls compared to people with schizophrenia (Table 1) due to a higher proportion of people with schizophrenia being categorised as smokers (Supplementary Figure 1A). In addition, the CDR score was significantly higher in people with schizophrenia.

Levels of [^{3}H]pirenzepine binding did not vary with sex (mean ± SEM: female = 168 ± 7.1 vs. male = 157 ± 5.7; p = 0.25) or race (p = 0.2: Table 1; Supplementary Table 1) which meant the difference in sex ratio with diagnosis would not be a major confound when analysing [^{3}H]pirenzepine binding. There were no correlations between the levels of [^{3}H]pirenzepine binding to the dorsolateral prefrontal cortex and age (r^2 = 0.004, p = 0.54) or PMI (r^2 = 0.003, p = 0.32) and the levels of [^{3}H]pirenzepine binding did not vary with CDR scores (Figure 1A). These findings show that differences in age, PMI or variation in CDR scores with diagnosis would not be confounds in the analyses of [^{3}H]pirenzepine binding.

3.2 [^{3}H]pirenzepine binding: schizophrenia compared to controls

The level of [^{3}H]pirenzepine binding to the DLPFC of people with schizophrenia (mean ± SEM: 153 ± 6.0 fmol / mg protein) was lower than that in controls (173 ± 6.3 fmol / mg protein; p = 0.02; Cohen's d = - 0.46)) (Figure 1B). Notably, for people with schizophrenia, there was no correlation between levels of [^{3}H]pirenzepine binding with FRADD, PANSS Positive, PANSS Negative, PANSS General or PANSS Total scores (Supplementary Table 2).

To further probe the nature of cortical [^{3}H]pirenzepine binding, Kernel density estimations were used to analyse radioligand binding. These analyses argued that the radioligand binding data from the controls was symmetrical and best fitted single population (Figure 2A), a conclusion supported by the normal Q-Q plot closely fitting a straight line (Figure 2B). In addition, all tests for normality suggested the data had a binomial distribution (Supplementary Table 3). By contrast, Kernel density estimation showed the distribution of [^{3}H]pirenzepine binding in subjects with schizophrenia was not symmetrical and bested fitted a two-population model with the nadir of separation at [^{3}H]pirenzepine binding of 121 fmol / mg protein (Figure 2C). The hypothesis that the data from the people with schizophrenia was not normally distributed was supported by a Q-Q plot that did not fit a straight line (Figure 2D). By contrast, normality testing indicated the data from people with schizophrenia did not deviate significantly from a binomial distribution (Supplementary Table 3) which highlights problems with normality tests which are not optimum in testing small datasets for deviations from a binomial distribution (D'Agostino et al., 1990).

Notably, 17 (30 %) of the people with schizophrenia, compared to 4 (9 %) controls, had levels of [^{3}H]pirenzepine binding ≤ 121 fmol / mg protein (Supplementary Table 1), meaning [^{3}H]pirenzepine binding ≤ 121 fmol / mg protein had a 90.7 % specificity for schizophrenia.

3.3 Outcomes from a two-population model of [^{3}H]pirenzepine binding in people with schizophrenia

To further analyse our data we divided the diagnostic groups into controls, people in the MRDS sub-group and those in the non-MRDS sub-group.

In comparing the three groups, age, sex, CNS pH and race did not vary with diagnosic category (Table 1). Smoking status was not available for 5 people with MRDS and 10 people with non-MRDS. There was significant variation in the frequency of smoking categorisation across the three diagnostic categories (Supplementary Figure 1B) due to a higher frequency of people with MRDS and non-MRDS being allocated to the category of smoker. There was significant variance in the frequency of allocating people with MRDS and non-MRDS to smoking category ($p = 0.007$). However, as there was no significant difference between the frequency of people with MRDS and non-MRDS being allocated to the category of smoker or non-smoker did not differ significantly ($p = 0.33$), this variance was due to the allocation of the frequency of the small number of ex-smokers in the non-MRDS group.

PMI did vary with diagnosis, being longer in the non-MRDS group. CDR scores varied with diagnosis (Figure 3, Table 1), with those with non-MRDS having a significantly higher CDR score compared to controls and MRDS. Analysing these data further, the median and 75% percentile score for the controls was 0 and 0, respectively. This means the majority of controls would be rated as being cognitively "healthy" (Hughes et al., 1982). For the MRDS group the median and 75% percentile scores were 0.5 and 1 respectively. CDR scores are a combined measure that is inversely related to levels of memory, cognition and the ability for independent living expected in healthy elderly people (Hughes et al., 1982) but, in the context of schizophrenia, CDR scores have been used as an indication of cognitive impairment (Haroutunian et al., 1994). Thus, although CDR scores did

not significantly differ from controls in the MRDS group, our data suggests that group has a mild level of cognitive impairment. The non-MRDS group had median and 75% percentile scores of 0.5 and 2, respectively indicating the group had a higher number of people with significant cognitive impairment. Finally, as would be expected, levels of [^{3}H]pirenzepine binding varied between the groups because of lower levels of binding in the people with MRDS; levels of binding did not differ between the controls and non-MRDS.

Comparing data relative to the people with schizophrenia, FRADD and none of the PANSS rating scores differed between MRDS and non-MRDS (Table 1; Supplementary Table 1). To further explore the potential for drugs targeting the CHRM1 being a significant confound in this study, we divided cases according to the available data of the affinity of the antipsychotic drug(s) they were receiving (Supplementary Table 1) for CHRM1 (1 = < 10 nM; 2 = > 10 < 100 nM; 3 = >100 < 1000 nM; 4 = > 1000 < 10000 nM and 5 = > 10,000 nM (Bolden et al., 1992; Bymaster et al., 1996; Snyder et al., 1974). Notably, the level of [^{3}H]pirenzepine binding did not vary significantly across the five groups of people with schizophrenia (Supplementary Figure 1: $F = 2.04$, d.f. = 4, 38, $p = 0.11$). In addition, the frequency of people with MRDS and non-MRDS being prescribed an antipsychotic drug with different levels of affinity for the CHRM1 did not vary (Supplementary Figure 2: $\chi^2 = 6.74$, d.f. = 4, $p = 0.15$).

4.0 Discussion

Here we report, for the first time, lower levels of cortical [^{3}H]pirenzepine binding in an older population of people with schizophrenia extending previous studies using cortical tissue from younger donors (Crook et al., 2001; Dean et al., 2002; Mancama et al., 2003; Zavitsanou et al., 2004). In addition, as reported in a younger cohort of people (Scarr et al., 2009), this study shows that older people with schizophrenia can be divided into sub-groups, MRDS and non-MRDS, based on levels of cortical [^{3}H]pirenzepine binding. Uniquely, in this study we show that CDR scores in the non-MRDS group, but not the MRDS, are significantly higher than in controls. This differs to the findings from PANSS ratings which shows there are no differences in the severity of positive and negative symptoms between the MRDS and non-MRDS sub-groups within schizophrenia. In interpreting these data it is significant that, using cloned human receptors and CHRM knockout mice (Scarr and Dean, 2008), we have shown that the method we use to measure [^{3}H]pirenzepine binding gives the radioligand a > 80 % specificity for the CHRM1. Hence, our current data suggests that there are lower levels of cortical CHRM1 in elderly people with schizophrenia, an hypothesis supported by our studies using Western blotting (Dean et al., 2002) and immunohistochemistry (Scarr et al., 2018a) with cortical tissue from younger people with schizophrenia.

This study is significant because it is the first to show that the MRDS sub-group (Scarr et al., 2009) can be detected in old age. Based on CDR scores, this study also shows that people with MRDS have less severe cognitive deficits than people with non-MRDS. This is a surprising finding because a neuroimaging study has shown that low levels of cortical CHRM1 in medication-free people with schizophrenia are associated with worsening cognitive deficits (Bakker et al., 2018). One explanation for this apparent dichotomy is that our radioligand binding studies using [^{125}I]α bungarotoxin, which bindings specifically to the α7 nicotinic receptor (Marutle et al., 2001), show that younger people with MRDS have higher levels of that receptor in their cortex when compared to controls and those

with non-MRDS (Dean et al., 2020). This is significant because pre-clinical models suggest the α7 nicotinic receptor is an important modulator of cognition (Medeiros et al., 2014) and that the receptor is a promising drug target for treating cognitive deficits in people with schizophrenia (Terry and Callahan, 2020). Thus, our new data may indicate that changes in CHRM1 and the α7 nicotinic receptor may be protective against the poorer cognitive functioning that should be associated with very low levels of cortical CHRM1 present in the MRDS sub-group. Significantly, in the rat, it has been shown that that the α7 nicotinic receptor is located on glutamatergic neurons (Levy and Aoki, 2002) with glutamatergic neurons in the human cortex being a component of cortical pyramidal cells (Bekkers, 2011). This is significant because we have shown that the CHRM1 is localised to pyramidal cells in the human cortex (Scarr et al., 2018a) which means the increase in α7 nicotinic receptors in MRDS (Dean et al., 2020) may be compensating for the decrease in CHRM1 on those neurons in people with MRDS (Scarr et al., 2018a).

Following the demonstration of low levels of cortical CHRM1 in people with schizophrenia using postmortem CNS (Crook et al., 2001) and cortical CHRMs using neuroimaging (Raedler et al., 2003b) it was suggested treating the disorder with CHRM1 and / or CHRM4 agonist could bring therapeutic benefits (Dean, 2004; Dean et al., 2003; Felder et al., 2001). This hypothesis has now been supported by two drug trials showing that treating people with the CHRM1/4 agonist, xanomeline, or a coformulation of xanomeline and the peripheral CHRM antagonist tropsium, reduces the severity of the psychotic (Brannan et al., 2021; Shekhar et al., 2008), negative (Brannan et al., 2021; Shekhar et al., 2008) and cognitive (Shekhar et al., 2008) symptoms of schizophrenia. These two approaches to using the drug xanomeline were necessary because the first study using xanomeline alone reported unacceptable drug effects associated with the use of that drug (Shekhar et al., 2008). By contrast, the use of xanomeline in a coformulation with a drug that blocks its peripheral effects has reported a side effect profile of the coformulation that does not differ significantly from placebo (Brannan et al., 2021). Interestingly, studies measuring drug responsiveness to activation

with CHRM1 orthosteric (Salah-Uddin et al., 2009) or allosteric (Dean et al., 2016; Hopper et al., 2019) using postmortem cortical tissue suggest people in the MRDS sub-group may not respond optimally to CHRM1 activation. Our new data suggests that sub-optimal response to CHRM1 agonists may remain an issue in treating older people within the MRDS sub-group.

It has been argued that CHRM4 receptor specific allosteric agonists could be used to treat the syndrome of schizophrenia (van der Westhuizen et al., 2020). This approach could be effective in people with schizophrenia and low levels of CHRM1 as available data suggests they do not have altered levels of cortical CHRM4 (Dean et al., 2002), especially if the symptoms of schizophrenia were caused by a breakdown in the homeostasis of signalling pathways common to both receptors. However, people with schizophrenia and low levels of cortical CHRM1 have sub-group specific changes in gene expression (Scarr et al., 2018b), mechanisms that regulate levels of gene expression and translation (Scarr et al., 2013) and higher levels of α7 nicotinic receptors (Dean et al., 2020). Thus, these data suggest it would be worthwhile to search for other potential drug targets beyond CHRM1 and CHRM4 that may target pathways that appear to be uniquely affected in MRDS.

This study has limitations. Whilst the cohort sizes are relatively large for a postmortem CNS study they are small compared to cohort sizes in many studies of schizophrenia, especially once the people with schizophrenia were divided into two groups. However, this shortfall is countered by some of the findings in this study replicating those in other studies of cortical CHRM1 in schizophrenia. In addition, whilst the use of the CDR to give some indication of the cognitive status of each donor provides invaluable insight the scale was designed for use in dementia and therefore may not provide data of sufficient granularity to identify more subtle cognitive deficits present in people with schizophrenia (Dean et al., 2022). Another limitation of the study is that PANNS ratings were not available for all cases with schizophrenia. In addition, as with any study involving treated people,

the effects of drug treatment remain a potential confound. Importantly, this is less so when comparing MRDS and non-MRDS as both groups were treated similarly during life.

Another potential confound that is relevant to all studies that do not use drug naïve people with schizophrenia is that prescribed drugs may be a confounding factor. In particular, in our study the prescription of drugs that target muscarinic receptors would be particularly relevant and this could include antipsychotic drugs, such as chlorpromazine, clozapine and olanzapine (Bymaster et al., 1999), and anticholinergic drugs prescribed to control extra-pyramidal side effects (Bolden et al., 1992). In considering such confounds with regards to this study, an important methodological consideration is that we used tissue that had been extensively washed before being used to measure [^{3}H]pirenzepine binding, a process that would remove any residual antipsychotic drugs from the tissue preparation (Dean et al., 2008a; Mita et al., 1986). Moreover, we have shown that there is no difference in the cortical antimuscarinic receptor cholinergic load, which is a measure of the amount of soluble extractable drugs that bind to muscarinic receptors (Jindal et al., 1981), in tissue from people with MRDS compared to those with MRDS (Dean et al., 2020). Again arguing the change in [3H]pirenzepine binding in MRDS is not dues to residual drugs binding to CHRMs. Hence, it is more likely that it is the long-term effects of drug treatment that may be a study confound rather than the apparent decrease in CHRMs observed after the intake of drugs that target those receptors due to the occupancy of receptors by unable drugs (Raedler, 2007; Raedler et al., 2003a).

Focussing on antipsychotic drugs, data from this study shows that the FRADD for MRDS and non-MRDS, that the level of cortical [^{3}H]pirenzepine binding was not associated with the affinity of the prescribed antipsychotic for the CHRM1 and that the frequency of drugs with varying affinity for the CHRM1 did not differ between MRDS and non-MRDS. We have also addressed the effects of chronic treatments with antipsychotic drugs and the anticholinergic drug benztropine in well-established rat models and have shown that chronic treatment with the antipsychotic drugs

haloperidol, clozapine, chlorpromazine, or thioridazine does not alter levels of cortical [^{3}H]pirenzepine binding (Crook et al., 2001; McLeod et al., 2010). Supporting this finding, we have recently reported that chronic treatment with haloperidol or chlorpromazine does not affect the level of expression of any of the 5 Chrms in rat cortex (Dean and Scarr, 2022). Together, these data argue that assigning a person to the MRDS sub-group is not due to the antipsychotic drug prescribed close to death.

In addition we have shown that chronic treatment with the anticholinergic drug benztropine does not alter levels of ^{3}H]pirenzepine binding in rat cortex (Crook et al., 2001).

Any study in schizophrenia could be confounded by people with the disorder using non-prescription drugs. In schizophrenia it is well established that there are much higher rates of nicotine (Leonard et al., 2000) and alcohol (Winklbaur et al., 2006) as well as a higher use of cannabis (Linszen et al., 1994) and cocaine (Chambers et al., 2001) compared to that in the general population. In considering these factors in relation to identifying the sub-group with MRDS in this study we show the rate of smoking did not vary between MRDS and non-MRDS. In addition, there appears to be no data to suggest that smoking is associated with changes in levels of either CHRM1 or CHRM 4 (Scarr, 2012). These findings argue that it is unlikely that the differentiation of the MRDS sub-group is related to the increased rates of smoking in schizophrenia. Notably, studies in rats has shown that alcohol increases levels of radioligand binding ([^{3}H]quinuclidinyl benzilate: [^{3}H]QNB) binding to muscarinic receptors which contrasts markedly to the lower levels of [^{3}H]pirenzepine binding that differentiates people with MRDS. However, by contrast, levels of binding of the same radioligand are reported as decreased by 40 % in the cortex of persons with alcohol use disorder (Freund and Ballinger, 1989) and it could be people with MRDS are all persons with alcohol use disorder whilst those with non-MRDS are not. Recently, it has been reported that the impact of alcohol abuse on the cholinergic system involves a loss of cholinergic neurons as indicated by a marked decrease in cortical choline acetyltransferase (Chat) (Crews et al., 2021). As people with MRDS and non-MRDS have levels of Chat that does not differ from controls (Dean et al., 2020), it would therefore seem unlikely that all the people with MRDS and none of the people with non-MRDS are ~~alcohol abusers~~persons with alcohol use disorder. We (Dean et al., 2001), and others (Newell et al., 2006), have shown that the cannabinoid system is perturbed in the cortex of patients with schizophrenia but there is no data to show cannabis use is related to levels of [^{3}H]pirenzepine binding in the human cortex. One study has reported that treating adolescent mice with levels of tetrahydrocannabinol expected in the CNS of humans using cannabis reduces the levels of CHRM1 by < 10% (Garzon et al., 2021); these data do not support the notion that the large decrease in [^{3}H]pirenzepine binding that characterises people with MRDS is due to cannabis use. Finally, there appears to be no data on levels of [^{3}H]pirenzepine

binding or levels of CHRM1 and 4 levels in the CNS of persons with cocaine use disorders. One study in rats has reported that 5 days treatment with cocaine caused an increase in [^{3}H]QNB binding to muscarinic receptors in the hippocampus and a decrease in binding of that radioligand in the striatum with no data being obtained using cortex(Lipton et al., 1995). Thus, it is not possible to exclude the possibility that people in the non-MRDS group are not ~~cocaine abuser~~persons with cocaine use disorder whereas those in the MRDS sub-group are all ~~cocaine abusers~~persons with cocaine use disorder and that the change in [^{3}H]pirenzepine binding is related to the use of cocaine, even though this scenario would seem unlikely.

5.0 Conclusions

Our study suggests MRDS persists into old age and that people with MRDS may have less severe cognitive deficits than do those with non-MRDS. This finding also argue that efforts should be made to study sub-groups within psychiatric disorders based on biologically defined criteria as this will provide insights into their molecular and behavioural pathologies (Tamminga et al., 2017). In addition, our findings in older people with schizophrenia contrasts to our data showing no changes in levels of cortical CHRM1 in people with Alzheimer's disease (Scarr et al., 2017). This finding is pertinent because of a recent report of increased rates of dementia in people with schizophrenia in the United States of America (Stroup et al., 2021). Thus our current data could argue for differing roles for the CHRM1 in the cholinergic pathophysiologies, including the onset of dementia, of schizophrenia (Gibbons and Dean, 2016) and Alzheimer's disease (Hampel et al., 2018). Finally, our study adds to the argument that it would be worthwhile to use recently developed CHRM receptor-selective neuroimaging ligands (Rowe et al., 2021; Tiepolt et al., 2022) to measure levels of cortical CHRM1 in people with schizophrenia to identify the MRDS sub-group and show they have mild cognitive impairments compared to those in the non-MRDS sub-group.Figure Legends

Figure 1: A comparison of the levels of [^{3}H]pirenzepine binding to Brodmann's area 9 from people with varying CDR scores (A) and in people with schizophrenia and non-psychiatric controls (B).

Figure 2: Kernel Density Estimations of [^{3}H]pirenzepine binding to Brodmann's area 9 from people non-psychiatric controls (A) and people with schizophrenia (C) and Q-Q plots of [^{3}H]pirenzepine binding to Brodmann's area 9 from non-psychiatric control (B) and people with schizophrenia (C).

Figure 3: A violin plot showing the distribution of CDR scores in controls and people with MRDS or non-MRDS.

Declaration of conflicting interests

BD, VF and ES have no conflicts of interest.

Contributors

BD and ES conceptualised the study. VH supplied the tissue for the study. ES completed all experiments. BD analysed the data. BD, VH and ES prepared the manuscript for publication.

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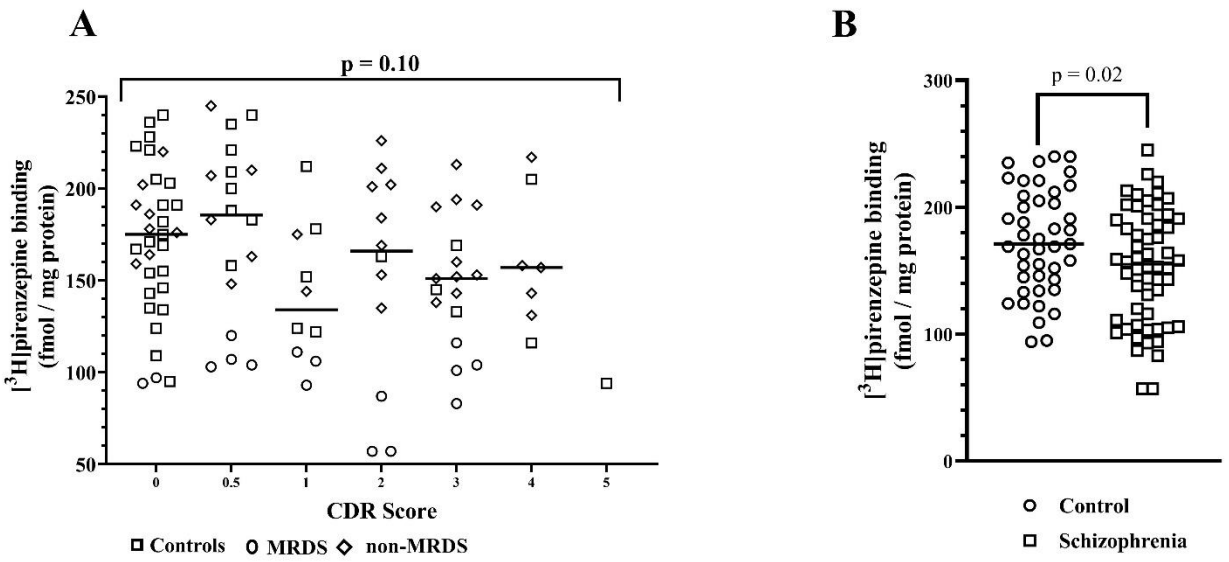
Figure 1


A
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250
200
150
100
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1
2
3
4
5
CDR Score
Controls
MRDS
non-MRDS
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200
100
0
Control
Schizophrenia

Figure 2

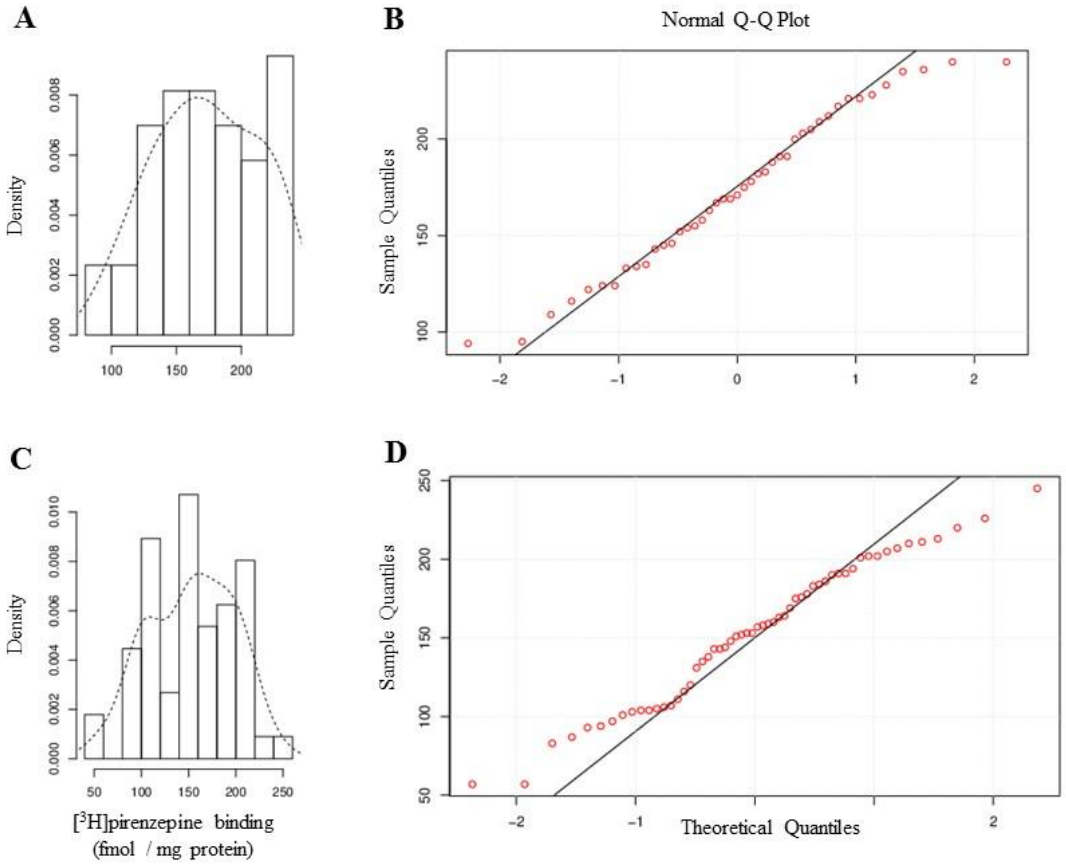
A
Density
0.000
0.002
0.004
0.006
0.008
100
150
200
B
Normal Q-Q Plot
Sample Quantiles
100
150
200
−2
−1
0
1
2
C
Density
0.000
0.002
0.004
0.006
0.008
0.010
50
100
150
200
250
[3H]pirenzepine binding
(fmol / mg protein)
D
Sample Quantiles
50
100
150
200
250
−2
−1
2
Theoretical Quantiles

Figure 3

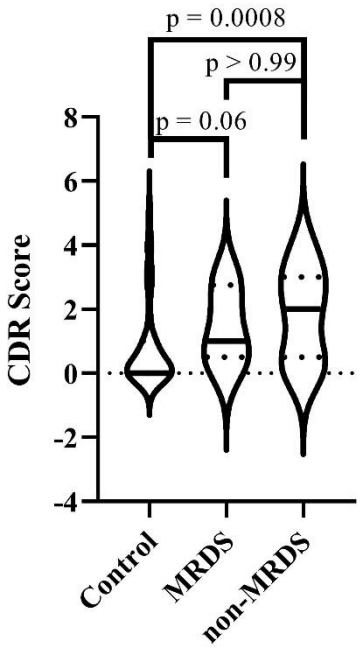

Table 1: Summary of demographic, CNS collection, treatment, symptom rating and experimental data from the cases used in this study.

	Cont.	Schizo.	p	MRDS	Non-MRDS	F: d.f.	p	MRDS vs Cont	Non-MRDS vs Cont	MRDS Vs non-MRDS
n	43	56		17	39					
Age (mean ± SEM, yr)	78 ± 2.0	75 ± 1.5	0.21	73 ± 2.3	76 ± 1.9	1.18: 2,96	0.31			
Sex	**24 F / 19 M**	**19 F / 37 M**	**0.04**	4 F, 13 M	15 F, 24 M		0.06			
Race	3 A, 6 B, 6 H, 28 W	7 B, 6H, 43 W	0.20	2 B, 1 H, 14 W	5 B, 5 H, 29 W		0.52			
PMI (mean ± SEM, min)	**549 ± 65**	**882 ± 66**	**0.0006**	**731 ± 107**	**948 ± 81**	**7.68: 2,96**	**0.0008**	0.36	**0.0005**	0.24
FRADD (mean ± SEM, fmol / mg chor. Eq.)		301 ± 58		244 ± 56	331 ± 84					0.49
Symptom Rating										
CDR Score (median and 25 / 75 Percentile)	**0, 0, 1**	**0.5, 2, 3**	**0.0001**	**0.5, 1, 2.8**	**0.5, 2, 3**	**K-W 14.5**	**0.0007**	0.06	**0.0008**	> 0.9999
PANSS Pos (mean ± SEM)		17.3 ± 1.2		17.8 ± 2.7	17.1 ± 1.4					0.82
PANSS Neg (mean ± SEM)		27.9 ± 1.6		26.0 ± 2.4	28.5 ± 1.9					0.53
PANSS Gen (mean ± SEM)		45.4 ± 2.3		38.7 ± 3.6	47.2 ± 2.6					0.11
PANSS Tot (mean ± SEM)		90.6 ± 4.2		82.5 ± 7.9	92.9 ± 4.9					0.31
[^{3}H]pirenzepine binding (mean ± SEM, fmol / mg prot)	**173 ± 6.3**	**153 ± 6.0**	**< 0.0001**	**96 ± 4.3**	**177 ± 4.6**	**39.1: 2, 96**	**<0.0001**	**<0.0001**	0.86	**<0.0001**

Notes: Race: A = Asian, B = Black, H = Hispanic, W = White

Abbreviations: Cont. = Controls, d.f. = degrees of freedom, MRDS = sub-group with schizophrenia and low radioligand binding, non-MRDS = sub-group with schizophrenia but not low levels of radioligand binding, Schizo. = Schizophrenia, SEM = Standard error of the mean,