Manuscript Number: SCHRES-D-20-00023R1

Title: Higher levels of $\alpha 7$ nicotinic receptors, but not choline acetyltransferase, in the dorsolateral prefrontal cortex from a sub-group of patients with schizophrenia

Article Type: Full Length Article

Keywords: $\alpha 7$ nicotinic receptor; choline acetyltransferase; cotinine; cholinergic load; cortex; schizophrenia

Corresponding Author: Professor Brian Dean, PhD

Corresponding Author's Institution: Florey Institute for Neuroscience and Mental Health

First Author: Brian Dean, PhD

Order of Authors: Brian Dean, PhD; Geoffrey Pavey, BSc (Hons); Elizabeth Scarr, PhD

Abstract: It has been suggested the study of sub-groups within the syndrome of schizophrenia will assist in elucidating the complex pathophysiology of the syndrome. Hence, we have studied a number of cholinergic markers in the cortex from a sub-group of subjects with schizophrenia that have a marked decrease in levels of muscarinic M1 receptors (MRDS). The displacement of [3H]NMS by cortical extracts was used to measure tissue anticholinergic load, [125I] α bungarotoxin binding was used to measure levels of the $\alpha 7$ nicotinic receptor (CHRNA7) and western blotting was used to measure levels of choline acetyltransferase (ChAT) 68 and 82 as well as synaptosome nerve-associated protein 25 (SNAP25). In comparing schizophrenia, MRDS and non-MRDS to controls, there were no differences in levels of ChAT 68 or 82, SNAP 25 or cholinergic load in BA 9. However, levels of CHRNA7 were higher in BA 9, but not BA 6 or 44, from subjects with MRDS. These data argue that there is no change in cholinergic innervation (measured using ChAT), presynaptic neurons (measured using SNAP25) or cholinergic load in schizophrenia, MRDS or non-MRDS. However, increased levels of CHRNA7 may be contributing to a breakdown in cholinergic homeostasis in BA 9, but not BA 6 or 44, in subjects with MRDS.

30 Royal Parade,
Parkville,
Victoria 3052,
Australia.

Telephone: 613-8344-3786
Facsimile: 613-9348-1707
Email: anddali@unimelb.edu.au

16th April 2020

Dr Matcheri Kashavan
Editor-in-Chief
Schizophrenia Research

Dear Dr Kashavan

We hereby submit a revised version of our manuscript entitled “Higher levels of $\alpha 7$ nicotinic receptors, but not choline acetyltransferase, in the dorsolateral prefrontal cortex from a sub-group of patients with schizophrenia” (SCHRES-D-20-00023) for consideration for publication in Schizophrenia Research.

We look forward to hearing from you on this matter.

Yours sincerely,

On Behalf of the Authors

Higher levels of $\alpha 7$ nicotinic receptors, but not choline acetyltransferase, in the dorsolateral prefrontal cortex from a sub-group of patients with schizophrenia.

Brian Dean^{1,2,4}, Geoffrey Pavey¹ and Elizabeth Scarr^{1,3}

¹ The Molecular Psychiatry Laboratory, The Florey Institute for Neuroscience and Mental Health, Victoria, Australia.

² The Centre for Mental Health, Swinburne University of Technology, Hawthorn, Victoria, Australia.

³ Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Victoria, Australia.

⁴ Florey Department of Neuroscience and Mental Health, The University of Melbourne, Victoria, Australia.

Corresponding author: Professor Brian Dean, Head, the Molecular Psychiatry Laboratory,
The Florey Institute for Neuroscience and Mental Health, 30 Royal
Parade, Parkville, Victoria 3052, Australia.

Email: brian.dean@florey.edu.au, Telephone: 613-8344-3786

Declarations of interest: none

Abstract

It has been suggested the study of sub-groups within the syndrome of schizophrenia will assist in elucidating the complex pathophysiology of the syndrome. Hence, we have studied a number of cholinergic markers in the cortex from a sub-group of subjects with schizophrenia that have a marked decrease in levels of muscarinic M1 receptors (MRDS). The displacement of [^3H]NMS by cortical extracts was used to measure tissue anticholinergic load, [^{125}I] α bungarotoxin binding was used to measure levels of the $\alpha 7$ nicotinic receptor (CHRNA7) and western blotting was used to measure levels of choline acetyltransferase (ChAT) 68 and 82 as well as synaptosome nerve-associated protein 25 (SNAP25). In comparing schizophrenia, MRDS and non-MRDS to controls, there were no differences in levels of ChAT 68 or 82, SNAP 25 or cholinergic load in BA 9. However, levels of CHRNA7 were higher in BA 9, but not BA 6 or 44, from subjects with MRDS. These data argue that there is no change in cholinergic innervation (measured using ChAT), presynaptic neurons (measured using SNAP25) or cholinergic load in schizophrenia, MRDS or non-MRDS. However, increased levels of CHRNA7 may be contributing to a breakdown in cholinergic homeostasis in BA 9, but not BA 6 or 44, in subjects with MRDS.

Keywords: $\alpha 7$ nicotinic receptor; choline acetyltransferase; cotinine; cholinergic load; cortex; schizophrenia

1.0 Introduction

There is considerable evidence to suggest dysfunctional cholinergic systems contribute to the pathophysiology of schizophrenia (Gibbons and Dean, 2016), at least in part through its extensive interactions with other neurotransmitter systems such as the dopaminergic, serotonergic, glutamatergic and GABAergic systems (Scarr et al., 2013b). Acetylcholine exerts its actions through two families of receptors, the G-protein coupled muscarinic receptors and the ion-gated nicotinic receptors (Racke et al., 2006). Data from post-mortem studies (Crook et al., 2001; Mancama et al., 2003), neuroimaging studies (Bakker et al., 2018; Raedler et al., 2003) and a drug trial (Shekhar et al., 2008) suggest muscarinic receptors are important in the pathophysiology and treatment of schizophrenia.

We have reported that it is possible to separate approximately 25 % of subjects with schizophrenia into a sub-group because they have a marked loss of cortical muscarinic M1 receptors (CHRM1), as shown by levels of [³H]pirenzepine binding < 100 fmol / mg estimate tissue equivalents (Scarr et al., 2009). The study of a muscarinic receptor deficit sub-group in schizophrenia (MRDS) is important as it is now accepted studying such biological-based sub-groups, or intermediate phenotypes, within the syndrome of schizophrenia will be required to advance understanding of the pathophysiology of the disorder (Tamminga, 2008). Relevant to this notion, we have now shown that patients with MRDS have lower levels of CHRM1 in a number of cortical regions (Gibbons et al., 2013; Seo et al., 2014). Our data suggests these lower receptor levels have resulted from changes in CHRM1 promoter methylation, higher levels of CHRM1 targeting micro RNAs (Scarr et al., 2013a) and changes in the activity of a specific biochemical pathway (Scarr et al., 2018b) that are acting separately or in concert to decrease CHRM1 expression. Thus, the mechanisms causing lower levels of CHRM1 in

Formatted: Superscript

MRDS are being elucidated and potential downstream changes in cortical function are being identified confirming studying sub-groups within the syndrome of schizophrenia can help in unravelling the complex pathophysiologies of disorders within the syndrome.

One mechanism that could reduce levels of CHRM1 in the cortex of patients with MRDS could be agonist induced receptor downregulation (Vaughn et al., 1999) in response to high levels of acetylcholine due to changes in cholinergic innervation. Choline acetyltransferase (ChAT), is a marker of innervating cholinergic neurons (Mesulam et al., 1992). It is therefore significant that studies have failed to show changes in the levels of ChAT (Bird et al., 1977; Karson et al., 1993) or ChAT activity (Haroutunian et al., 1994; Powchik et al., 1998) in the cortex of patients with schizophrenia. However, we postulated that changes in ChAT may only be present in MRDS where altered cholinergic innervation, resulting in changes in acetylcholine levels, could be down regulating CHRM1.

As stated, acetylcholine regulates the activity of the cholinergic system by binding to two families of receptors, the muscarinic and nicotinic receptors. At least in the hippocampus, CHRM1 regulates the activity of the $\alpha 7$ nicotinic receptor (CHRNA7) (Shen et al., 2009). This raises the possibility that changes in levels of CHRM1 in the cortex from patients with schizophrenia could be altering CHRNA7 levels or activity. Notably, one [postmortem](#) study has reported lower levels of [125 I] α bungarotoxin binding in the cingulate (BA 24), but not orbitofrontal (BA 11, 12 and 47) and temporal (BA 20, 21 and 26), cortices from patients with schizophrenia when compared to that in controls with a history of smoking (Marutle et al., 2001); as [125 I] α bungarotoxin almost exclusively binds to the CHRNA7 (Breese et al., 1997), these data would be consistent with regional-specific changes in levels of CHRNA7 in the cortex of patients with the disorder. These data differ from a study measuring levels of

CHRNA7 protein using Western blotting, which reported lower levels of the CHRNA7 sub-unit in the prefrontal, but not parietal, cortices from patients with schizophrenia (Guan et al., 1999). By contrast, levels of CHRNA7 sub-unit mRNA were reported as unchanged in the prefrontal cortex (BA 46) from subjects with schizophrenia compared to controls (De Luca et al., 2006). Thus, findings on cortical CHRNA7 in schizophrenia are not uniform and there is no data on levels of that receptor in patients with MRDS. In addition, whilst this study is focussed on the cortex, it is notable that lower levels of CHRNA7 have been reported in the hippocampus (Freedman et al., 2000) and thalamic reticular nucleus (Court et al., 1999) from patients with schizophrenia.

One limitation in any study in schizophrenia that does not use drug naïve patients is that treatments for the disorder become confounds. This is significant with regards to the cholinergic system in schizophrenia as drugs such as some atypical antipsychotic drugs (Miyamoto et al., 2005) and , such as benztropine, used to treat the extrapyramidal side effects associated with treatment with ~~typical~~ antipsychotic drugs (Tune and Coyle, 1981) are CHRM antagonists. Hence, it could be that the low levels of CHRM1 in subjects with MRDS is because of residual anti-cholinergic load in their central nervous system (CNS).

In this study, designed to give a further understanding of changes in the cortical cholinergic system in MRDS, we decided to measure levels of cortical ChAT to determine if that subgroup had altered levels of cholinergic innervation. In addition, we measured levels of synaptosome nerve-associated protein 25 (SNAP25), a surrogate marker of the pre-synaptic bouton (Geddes et al., 1990) in the same cases. We also decided to measure levels of [¹²⁵I]α bungarotoxin binding to the CHRNA7 in the same cohorts to see if changes in CHRM1 signalling in MRDS could have resulted in changed levels of that receptor as well as the

“anticholinergic load” in MRDS, non-MRDS and controls to determine if increased levels of residual drugs targeting CHRMs were present in MRDS. Finally, it has been suggested that smoking close to death could be a confound when studying nicotinic receptors (Saricicek et al., 2012) and that levels of cotinine in tissue can provide an indication of the time since the last exposure to nicotine (Strand and Nybäck, 2005). We therefore assessed the suitability of quantifying levels of cotinine in brain using the NicAlert cotinine test strip (Cooke et al., 2008).

2.0 Materials and Methods

2.1 *Materials*

See Supplementary Methods

2.2 *CNS Collection and Case History Review*

2.2.1 Ethical Approval

Approval to collect human tissue was obtained from the Ethics Committee of the Victorian Institute of Forensic Medicine. Subsequently, written permission to collect tissue was obtained from the closest next of kin, as dictated by State law.

2.2.2 Tissue Collection

The left CNS hemisphere was collected from each donor and processed in a standardized manner allowing tissue to be frozen to -80°C within 30 min of autopsy (Dean et al., 1999). CNS pH was measured as described previously (Kingsbury et al., 1995) as an indicator of the quality of tissue preservation (Stan et al., 2006).

2.2.3 Case History Reviews

For each psychiatric case, relevant data from clinical histories and interviews with treating clinicians and relatives were obtained using a standardised instrument, the Diagnostic

Instrument for Brain Studies (Hill et al., 1996). The information collected allowed the diagnosis of schizophrenia to be made by consensus using DSM-IV criteria (Roberts et al., 1998) and data to be collated as describe previously (Scarr et al., 2018b) (Supplementary Methods).

2.2.4 CNS Processing

All tissue used in this study was provided by the Victorian Brain Bank Network at the Florey Institute for Neuroscience and Mental Health. For radioligand binding studies, tissue was collected from BA 6, 9 and 44 in the left hemisphere, the definitions of each region being as described in detail elsewhere (Garey, 1994). Western blot analyses and cotinine measurements were completed using only tissue from BA 9.

Drugs that could contribute to the anticholinergic load in CNS tissue were extracted as described previously (Jindal et al., 1981) (Supplementary Methods).

2.3 *Cotinine Levels in Human Cortex*

Having established the validity of use (Supplementary Methods), the NicAlert™ test strip was used to measure cotinine levels in human CNS cortex of the cases to be used in this study.

2.4 *Radioligand Binding*

2.4.1 [³H]N-methylscopolamine ([³H]NMS) Binding

The cortical anticholinergic load was measured as the ability of the hexane extracted material to displace [³H]NMS from cortical membrane (Dean et al., 2016). Thus, as established during method optimisation, the 5 µl of reconstituted anticholinergic load tissue extract was diluted 1:16 in distilled water to prevent residual salts interfering with the binding of [³H]NMS. Then, 50 µl of diluted pellet was incubated in triplicate with [³H]NMS (0.13 nM) in the presence of 0.01 mg / ml cortical membrane homogenate in a final volume made up to 1 ml with 20 mM HEPES, 100 mM NaCl, 10 mM MgCl; pH 7.4. After incubating for 2 hr at room temperature each sample was filtered through GF/B filters, that had been soaked in polyethyleneimine, and washed thrice with ice cold 0.9% saline. 5 ml of Perkin Elmer Ultima Gold Liquid Scintillation Fluid was added to each filter paper and the radioactivity on each filter paper counted in a Perkin Elmer Tri Carb 2910TR liquid scintillation analyser. Anticholinergic load was expressed as a percent of another case with no history of any psychiatric or neurological disorder which had been processed identically with the cases of interest in each batched extraction run.

2.4.2 [¹²⁵I]α bungarotoxin Binding

As described previously (Marutle et al., 2001)(Supplementary Methods) except that image capture was achieved using a rather than on X-ray film.

Western Blot Analyses

The methodology to measure levels of ChAT68, ChAT82, SNAP25 and β-Actin were

optimised so that each protein could be measured after a single transfer onto nitrocellulose (Supplementary Table 1). Between the measurement of ChAT68 with ChAT 82, SNAP25 and β -Actin each nitrocellulose membrane was washed 3 x in distilled water, placed in 0.1 M sodium hydroxide for 5 mins and washed 2 x with 50 mM Tris-Cl, pH 7.5. 150 mM NaCl + 0.1% Tween 20. The chemiluminescence of the immunogenic band for each protein was measured using a UVP BioSpectrum 810.

2.5 *Statistics*

All numeric data were initially analysed using the D'Agostino-Pearson normality test as this is better at identifying the distribution of data in small sample sizes (D'Agostino et al., 1990). In the case of parametric data, any outliers (maximum 1 per data set) were identified with a Grubb's test (Grubbs, 1969).

Variation in demographic, CNS collection, pharmacological and experimental data between patients with schizophrenia and controls was assessed using Student's t-test. When comparing these data between MRDS, non-MRDS and controls a one-way ANOVA with a post hoc Dunnett's comparison to controls was used. For non-numeric data, differences in the frequency of variables was compared using the χ^2 test. Relationships between demographic, CNS collection and pharmacological data with experimental data and between the levels of binding of different radioligands was assessed with a linear regression assuming a straight line best fit. All analyses were completed using GraphPad Prism vs 7.00 for Windows (GraphPad Software, La Jolla California USA).

If covariates were identified, their impact on the primary analyses of variance was assessed

using an ANCOVA, including any identified covariates, using Minitab 18[®] Statistical Software (Minitab Pty Ltd, Sydney, NSW, Australia). If data were found to be not normally distributed an equivalent non-parametric test was used to identify variance between groups.

3.0 Results

3.1 Demographic, case history and CNS collection

We wished to complete our study on cases we have included in previous studies (Gibbons et al., 2013; Scarr et al., 2009) to provide continuity in our findings. Unfortunately, limitations on the amount of available tissue meant we were not able to complete all measurements in every case. Hence, measurements of total cholinergic load were measured using cortical tissue from 5 patients with MRDS, 5 patients with non-MRS and 5 controls (Table 1A: Supplementary Table 2). In these cohorts there

There were no significant differences in gender ratio, suicide rates, age, CNS pH, postmortem interval (PMI) or CNS weight between the groups used for the measurement of anticholinergic load (Table 1A; Supplementary Table 2). There were no significant differences between duration of illness (DI), final recorded antipsychotic drug dose expressed as chlorpromazine equivalents (FRADD) and lifetime exposure to antipsychotic drugs expressed as grams chlorpromazine equivalents $\times 10^{-3}$ (LEAP) between MRDS and non-MRDS.

All remaining measures were made using cortical tissue from ~~Due to limited tissue being available from previously studied cases, this study was completed using tissue from~~ 27 patients with schizophrenia and 12 controls (Table 1B; Supplementary Table 3). There were no significant differences in age, PMI, CNS weight or gender ratio with diagnoses. CNS pH was significantly lower in the patients with schizophrenia and the rate of suicide was significantly higher.

The patients with schizophrenia consisted of 14 patients that had markedly reduced levels of [³H]pirenzepine binding to the CHRM1 in BA 9 (MRDS: Figure 2+D) which we have shown allows them to be separated from the remaining patients that were non-MRDS. Notably, there were no significant differences in age, PMI, CNS weight or gender ratios in patients with MRDS and non-MRDS compared to the controls (Table 1B). Whilst, compared to controls, both groups had significantly lower levels of CNS pH only in the MRDS groups did rates of suicide differ significantly. There were no significant differences in age, CNS pH, PMI, CNS weight, ~~duration of illness (DI), final recorded antipsychotic drug dose (FRADD), lifetime exposure to antipsychotic drugs (LEAP)~~, gender ratio and frequency of suicide between MRDS and non-MRDS.

3.2 *Radioligand binding*

3.2.1 Anticholinergic Load

There was a significant variation in anticholinergic load, as measured using [³H]NMS binding displacement, between patients with MRDS, non-MRDS and controls (Figure 1A: $F = 5.78$, d.f. = 2,12, $p = 0.02$). However, post-hoc analyses showed that the anticholinergic load in tissue from MRDS ($p = 0.22$) and non-MRDS ($p = 0.17$) did not differ from that in controls. In addition, the extract of homogenate that had 1 μ M benztropine added before processing cause an 80% reduction in the level of [³H]NMS (Figure 1A), showing the extraction process does remove muscarinic receptor antagonists from cortical homogenates. By contrast, there was a significant variation in the binding of [³H]pirenzepine with group (F

= 42.3, d.f. = 2,12, $p < 0.0001$) due to markedly lower levels of binding in subjects with MRDS compared to controls (Figure 1B).

3.2.2 $[^{125}\text{I}]\alpha$ bungarotoxin Binding

As described previously (Marutle et al., 2001), $[^{125}\text{I}]\alpha$ bungarotoxin binding was distributed uniformly across all cortical laminae and therefore an integrated measure across all laminae was taken as the measure of cortical radioligand binding.

Compared to controls, there were no significant variation in levels of $[^{125}\text{I}]\alpha$ bungarotoxin binding to BA 6, 9 or 44 from subjects with schizophrenia (Figure 2A-C). However, levels of $[^{125}\text{I}]\alpha$ bungarotoxin binding to BA 9, but not 6 or 44, from patients with MRDS were significantly higher than that in controls (Figure 2B) with a large effect size (Cohen's $d = 1.33$; Hedges' $g = 1.30$: <https://www.socscistatistics.com/effectsize/default3.aspx>: (Lakens, 2013)).

Levels of $[^{125}\text{I}]\alpha$ bungarotoxin binding did not vary with gender in any of the three cortical regions (Supplementary Table 4). Levels of $[^{125}\text{I}]\alpha$ bungarotoxin binding was significantly higher in BA 44, but not BA 6 or 9, from suicide completers compared to that in those who died of other causes. Notably, compared to deaths by other causes, the suicide completers were younger but did not differ significantly with regards to gender ratio, pH, PMI, NicAlert readings, CNS weight, FRADD or LEAP (Supplementary Table 5).

Levels of [125 I] α bungarotoxin binding in BA 6, 9 and 44 did not show any significant relationship with age, CNS pH, PMI, CNS Weight, levels of cotinine, DI, FRADD or LEAP (Supplementary Table 6).

Despite there being no significant correlation between [125 I] α bungarotoxin binding and CNS pH, in acknowledgment CNS pH varied significantly between controls, MRDS and non-MRDS, we reanalysed our radioligand binding data using an ANCOVA with pH as a covariate. Notably, the difference in [125 I] α bungarotoxin binding with group in BA 9 from subjects with MRDS remained significant ($p < 0.001$) after accounting for the potential effects of CNS pH.

Overall, there was a moderate inverse relationship between [125 I] α bungarotoxin binding and [3 H]pirenzepine binding in BA 9 from subjects with schizophrenia (Supplementary Figure 1). However, this may be somewhat artifactual as there were no significant relationships between the binding of these two radioligands in the controls ($r^2 = 0.03$; $p = 0.58$), MRDS ($r^2 = 0.02$; $p = 0.63$) or non-MRDS ($r^2 = 0.23$, $p = 0.1$) when each group was analysed separately.

3.2.3 Western blot analyses

We have previously shown that β -actin can be problematic when used as a house-keeping protein in human cortex (Parkin et al., 2018) but in the cases used in this study levels of that protein did not vary significantly with group (Chemiluminescence Controls: Mean $\pm$ SEM = 182710 ± 19909 ; schizophrenia 175210 ± 15067 ; $p = 0.76$, MRDS = 173452 ± 17793 , $p = 0.93$; non-MRDS = 176969 ± 25018 ; $p = 0.97$) and therefore all Western blot analyses were normalised to levels of β -actin.

Compared to controls, levels of ChAT68, ChAT82 and SNAP25 did not differ in BA 9 from patients with schizophrenia, MRDS or non-MRDS (Figure 3). Levels of ChAT68 and ChAT82, but not SNAP25, tended to be higher in males (Supplementary Table 4). Levels of these three proteins did not differ between suicide completers and subjects who died by other causes (Supplementary Table 4). There were no significant relationships between the levels of ChAT68, ChAT82 or SNAP25 with age, CNS pH, PMI, CNS Weight, levels of cotinine, DI, FRADD or LEAP (Supplementary Table 6).

4.0 Discussion

In this study we show that, compared to controls, [125 I] α bungarotoxin binding is higher in BA 9 from subjects with MRDS. Given [125 I] α bungarotoxin binds with a high level of specificity to CHRNA7 (Breese et al., 1997) our data are consistent with there being higher levels of CHRNA7 in BA 9, but not BA 6 or 44, from subjects with MRDS. Our radioligand binding data contrasts to our data from our expression microarray study that did not show a change in mRNA for CHRNA7 in BA 9 from subjects with MRDS (Scarr et al., 2018b). Hence, it is possible the higher levels of CHRNA7 in MRDS are due to decreased degradation of the receptor. CHRNA7 degradation occurs through the ubiquitin–proteasome system (Colombo et al., 2013) which is suggested to be affected in the CNS in patients with schizophrenia (Andrews et al., 2017). However, it has also been shown that increased levels of CHRNA7 are linked to anti-apoptotic processes (Dawe et al., 2019) raising the possibility that the higher levels of CHRNA7 may be associated with a protective response to pathophysiological processes occurring in the cortex of patients with MRDS. It has been shown that CHRM1 regulates the activity of CHRNA7 (Shen et al., 2009) and therefore the higher levels of CHRNA7 in BA 9 could be connected to the extremely low levels of CHRM1 in that cortical region from subjects with MRDS (Scarr et al., 2009). However, we have shown that there are equally low levels of CHRM1 in BA 6 (Seo et al., 2014) and BA 44 (Gibbons et al., 2013) from patients with MRDS which means low levels of CHRM1 are not universally associated with high CHRNA7 in the cortex of subjects with MRDS.

Comparing our data to other studies of the syndrome of schizophrenia, we did not find changes in [125 I] α bungarotoxin binding in BA 6, 9 and 44 from patients with the disorder. An earlier study reported lower levels of [125 I] α bungarotoxin binding was only present in the

anterior cingulate (BA 24), but not the orbitofrontal (BA 11, 12 + 47) or temporal (BA 20, 21 + 26) cortices from patients with schizophrenia (Marutle et al., 2001). Another study in the adjacent cortical region of the dorsolateral prefrontal cortex (BA 46) to BA 9 reported no change in the level of CHRNA7 protein in patients with schizophrenia (De Luca et al., 2006). These data contrast those in a study reporting lower levels of CHRNA7 in the prefrontal, but not parietal, cortex from subjects with schizophrenia (Guan et al., 1999). Thus, our data on CHRNA7 are consistent with there being cortical-region specific changes in levels of that receptor in patients with schizophrenia.

In this study we did not show any significant changes in levels of ChAT68 or ChAT82, the two predominant forms of ChAT in the CNS (Oda, 1999), in BA 9 from patients with MRDS or non-MRDS. ChAT has long been regarded as a definitive marker for cholinergic neurons (Wu and Hersh, 1994) and therefore our data suggest that there is no significant change in levels of innervating cholinergic neurons in a region of the cortex where there is a highly significant decrease in levels of CHRM1 in patients with MRDS (Scarr et al., 2009). This finding strengthens the argument that levels of acetylcholine are not higher in patients with MRDS and would therefore not be causing agonist induced down-regulation of CHRM1 in those patients. We have previously shown that levels of SNAP25 were not altered in the BA 9 from patients with schizophrenia (Scarr et al., 2006) and here we show that is the case for subjects with MRDS and non-MRDS. Levels of SNAP25 have been suggested to be a surrogate marker of levels of presynaptic boutons (Geddes et al., 1990) and therefore our current data on ChAT and SNAP25 would be consistent with the hypothesis that there are no major changes in levels of neural number in BA 9 from subjects with MRDS and non-MRDS. This conclusion is consistent with our recent data showing, that whilst there are lower levels

of CHRM1 positive neurons in laminae III and V in both MRDS and non MRDS there is no change in the total number of neurons in those patients (Scarr et al., 2018a).

Muscarinic receptor antagonists have been used to treat the extra-pyramidal side effects of some antipsychotic drugs (Desmarais et al., 2014) and thus residual muscarinic receptor antagonists in the cortex could be acting to reduce available CHRM1 binding sites in tissue from subjects with schizophrenia. The combined levels of muscarinic receptor targeting drugs in CNS tissue, termed the anticholinergic CNS load (Jindal et al., 1981), can be measured as a displacement of pan-muscarinic receptor radioligands following the extraction of drugs from tissue. Here we show that there is no significant difference in levels of anticholinergic load between MRDS, non-MRDS and controls. This argues that the markedly lower levels of [³H]pirenzepine binding we have reported in many areas of the cortex in subjects with MRDS (Gibbons et al., 2013; Scarr et al., 2009; Seo et al., 2014) were not due to a higher anticholinergic load in the cortex of those patients.

It has long been known that smoking close to death can affect levels of nicotinic receptors in human CNS (Perry et al., 1999) and that levels of cotinine can be used to assess whether nicotine had been ingested in the 5 days before tissue collection (Cooke et al., 2008). Here we have shown that NicAlert, a strip-based test, can be used to measure levels of cotinine in post-mortem CNS. Using this approach, we have shown that there were no significant differences in cortical cotinine levels between subjects with MRDS, non-MRDS and controls. These data argue that the change in levels of CHRNA7 we report in BA 9 from subjects with MRDS are not due to those patients ingesting more nicotine close to death.

There are a number of limitations to our study. First, whilst not dissimilar to other studies using tissue obtained post-mortem from patients with schizophrenia, our cohort sizes are relatively small. In addition, like all studies in schizophrenia that do not use drug naïve patients, antipsychotic drug treatment is a confound. However, in this study we report differences between two groups of patients with schizophrenia, MRDS and non-MRDS, who would appear to have been treated similarly with antipsychotic drugs, making drug treatment less of a confound.

This study now raises the likelihood that patients with MRDS do not have changes in cholinergic innervation to their cortex or altered levels of cortical acetylcholine. By contrast, subjects with MRDS have very low levels of cortical CHRM1 along with increased levels of CHRNA7. Caution must be observed in trying to translate these findings to have some clinical relevance, however when it was first recognised that patients with schizophrenia had low levels of CHRM 1 and 4 it was suggested that treatment with CHRM 1 and / or 4 agonists may alleviate the symptoms of the disorder (Dean et al., 2003; Felder et al., 2001). This suggestion has gained credence by the demonstration that treating patients with schizophrenia with the muscarinic M1 / M4 agonist, xanomeline, and a coformulation of xanomeline and the peripheral muscarinic receptor antagonist, trospium, does reduce the symptoms of the disorder (for review see (Dean and Scarr, In Press)). However, there is some data using CHRM1 orthosteric (Salah-Uddin et al., 2009) and allosteric agonists (Dean et al., 2016; Hopper et al., 2019) to activate that receptor in human cortex, that suggests patients with MRDS may not respond optimally to such treatments. It is therefore intriguing to postulate whether treating patients with MRDS with CHRNA7 antagonists to reduce the increased signalling through the high levels of that receptor could have therapeutic benefits for MRDS. In considering this posit it is of interest that some pre-clinical data suggests that

low doses of the selective CHRNA7 antagonist, methyllycaconitine, improves memory acquisition (van Goethem et al., 2019) and that memory acquisition is impaired in schizophrenia (Moritz et al., 2006). Moreover, reducing the activity of CHRM1s with CHRM1 antagonists has long been recognised to worsen memory acquisition in patients with schizophrenia (McEvoy et al., 1987). Thus, if patients with MRDS have decreased CHRM1 activity because of a lack of CHRM1, the use of a CHRNA7 antagonist to reverse this deficit could be useful in treating patients with MRDS. In testing this hypothesis it will first be necessary to identify patients with MRDS which may now be possible using CHRM1 preferring neuroimaging ligands (Bakker et al., 2015; Rowe et al., 2019). It is important to stress the use of a CHRNA7 antagonist may only be useful in MRDS, a sub-group within the syndrome of schizophrenia, because of the large body of evidence that suggests that at the level of the syndrome treating with drugs that can activate the CHRNA7 should have therapeutic benefits (Terry and Callahan, 2020). Unfortunately, this approach to treating patients with schizophrenia is now under question because of a failure to show a clear clinical benefit from treating patients with the disorder with the CHRNA7 positive allosteric modulator AVL-3228 (Kantrowitz et al., 2020).

In conclusion, our current study suggests that cholinergic innervation is not altered in the in the cortex from subjects MRDS. It has been suggested that low levels of CHRM1 may be pro-apoptotic (Shant et al., 2009) whilst higher levels of CHRNA7 would have an anti-apoptotic effect (Dawe et al., 2019), thus the changes in CHRM1 and CHRNA7 we report in MRDS may reflect an attempt to preserve cholinergic apoptotic homeostasis in the cortex of patients with MRDS. Whatever the outcomes, the presence of changes in both muscarinic and nicotinic receptors in BA 9 from subjects with MRDS suggests a breakdown in

cholinergic innervation must be an important contributor to the pathophysiology of those with that disorder in that cortical region.

Acknowledgements

The authors thank Madhara Udawela and Clare Duncan for the technical assistance that contributed to the data included in this manuscript. This research was funded in part by NHMRC Project Grant # APP1105332 and the CRC for Mental Health.

References

- Andrews, J.L., Goodfellow, F.J., Matosin, N., Snelling, M.K., Newell, K.A., Huang, X.-F., Fernandez-Enright, F., 2017. Alterations of ubiquitin related proteins in the pathology and development of schizophrenia: Evidence from human and animal studies. *J. Psychiatr. Res.* 90, 31-39.
- Bakker, G., Vingerhoets, C., Boucherie, D., Caan, M., Bloemen, O., Eersels, J., Booij, J., van Amelsvoort, T., 2018. Relationship between muscarinic M1 receptor binding and cognition in medication-free subjects with psychosis. *Neuroimage Clin* 18, 713-719.
- Bakker, G., Vingerhoets, W.A., van Wieringen, J.P., de Bruin, K., Eersels, J., de Jong, J., Chahid, Y., Rutten, B.P., DuBois, S., Watson, M., Mogg, A.J., Xiao, H., Crabtree, M., Collier, D.A., Felder, C.C., Barth, V.N., Broad, L.M., Bloemen, O.J., van Amelsvoort, T.A., Booij, J., 2015. 123I-iododexetimide preferentially binds to the muscarinic receptor subtype M1 in vivo. *J. Nucl. Med.* 56(2), 317-322.
- Bird, E.D., Spokes, E.G., Barnes, J., Mackay, A.V., Iversen, L.L., Shepherd, M., 1977. Increased brain dopamine and reduced glutamic acid decarboxylase and choline acetyl transferase activity in schizophrenia and related psychoses. *Lancet* 2(8049), 1157-1158.
- Breese, C.R., Adams, C., Logel, J., Drebing, C., Rollins, Y., Barnhart, M., Sullivan, B., DeMasters, B.K., Freedman, R., Leonard, S., 1997. Comparison of the regional expression of nicotinic acetylcholine receptor $\alpha 7$ mRNA and [125I]- α -bungarotoxin binding in human postmortem brain. *J. Comp. Neurol.* 387(3), 385-398.

Colombo, S.F., Mazzo, F., Pistillo, F., Gotti, C., 2013. Biogenesis, trafficking and up-regulation of nicotinic ACh receptors. *Biochem. Pharmacol.* 86(8), 1063-1073.

Cooke, F., Bullen, C., Whittaker, R., McRobbie, H., Chen, M.H., Walker, N., 2008. Diagnostic accuracy of NicAlert cotinine test strips in saliva for verifying smoking status. *Nicotine Tob Res* 10(4), 607-612.

Court, J., Spurden, D., Lloyd, S., McKeith, I., Ballard, C., Cairns, N., Kerwin, R., Perry, R., Perry, E., 1999. Neuronal nicotinic receptors in dementia with Lewy bodies and schizophrenia: alpha-bungarotoxin and nicotine binding in the thalamus. *J. Neurochem.* 73(4), 1590-1597.

Crook, J.M., Tomaskovic-Crook, E., Copolov, D.L., Dean, B., 2001. Low muscarinic receptor binding in prefrontal cortex from subjects with schizophrenia: a study of Brodmann's areas 8, 9, 10, and 46 and the effects of neuroleptic drug treatment. *Psychiatry Res.* 158(6), 918-925.

D'Agostino, R.B., Belanger, A., D'Agostino Jnr, R.B., 1990. A suggestion for using powerful and informative tests of normality. *Psychiatry Res.* 44, 316-321.

Dawe, G.B., Yu, H., Gu, S., Blackler, A.N., Matta, J.A., Siuda, E.R., Rex, E.B., Bredt, D.S., 2019. $\alpha 7$ nicotinic acetylcholine receptor upregulation by anti-apoptotic Bcl-2 proteins. *Nature Communications* 10(1), 2746.

- De Luca, V., Likhodi, O., Van Tol, H.H., Kennedy, J.L., Wong, A.H., 2006. Regulation of alpha7-nicotinic receptor subunit and alpha7-like gene expression in the prefrontal cortex of patients with bipolar disorder and schizophrenia. *Psychiatry Res.* 114(3), 211-215.
- Dean, B., Bymaster, F.P., Scarr, E., 2003. Muscarinic receptors in schizophrenia. *Curr. Mol. Med.* 3(5), 419-426.
- Dean, B., Hopper, S., Conn, P.J., Scarr, E., 2016. Changes in BQCA allosteric modulation of [3H]NMS binding to human cortex within schizophrenia and by divalent cations. *Neuropsychopharmacol.* 41, 1620-1628.
- Dean, B., Hussain, T., Hayes, W., Scarr, E., Kitsoulis, S., Hill, C., Opeskin, K., Copolov, D.L., 1999. Changes in serotonin2A and GABA(A) receptors in schizophrenia: studies on the human dorsolateral prefrontal cortex. *J. Neurochem.* 72(4), 1593-1599.
- Dean, B., Scarr, E., In Press. Muscarinic M1 and M4 receptors: Hypothesis driven drug development for schizophrenia. *Psychiatry Res.*
- Desmarais, J.E., Beauclair, L., Annable, L., Bélanger, M.-C., Kolivakis, T.T., Margoless, H.C., 2014. Effects of discontinuing anticholinergic treatment on movement disorders, cognition and psychopathology in patients with schizophrenia. *Therapeutic advances in psychopharmacology* 4(6), 257-267.
- Felder, C.C., Porter, A.C., Skillman, T.L., Zhang, L., Bymaster, F.P., Nathanson, N.M., Hamilton, S.E., Gomez, J., Wess, J., McKinzie, D.L., 2001. Elucidating the role of muscarinic receptors in psychosis. *Life Sci.* 68(22-23), 2605-2613.

Freedman, R., Adams, C.E., Leonard, S., 2000. The $\alpha 7$ -nicotinic acetylcholine receptor and the pathology of hippocampal interneurons in schizophrenia. *J.Chem.Neuroanat.* 20(3-4), 299-306.

Garey, L.J., 1994. *Bordmann's 'Localisation in the cerebral cortex'*. Smith-Gordon, London.

Geddes, J.W., Hess, E.J., Hart, R.A., Kesslak, J.P., Cotman, C.W., Wilson, M.C., 1990. Lesions of hippocampal circuitry define synaptosomal-associated protein-25 (SNAP-25) as a novel presynaptic marker. *Neurosci.* 38(2), 515-525.

Gibbons, A., Dean, B., 2016. The Cholinergic System: An Emerging Drug Target for Schizophrenia. *Curr. Pharm. Des.* 22(14), 2124-2133.

Gibbons, A., Scarr, E., Boer, S., Money, T., Jeon, W.J., Felder, C., Dean, B., 2013. Widespread decreases in cortical muscarinic receptors in a subset of people with schizophrenia. *Int. J. Neuropsychopharmacol.* 16, 37-46.

Grubbs, F., 1969. Procedures for Detecting Outlying Observations in Samples. *Technometrics* 11(1), 1-21.

Guan, Z.Z., Zhang, X., Blennow, K., Nordberg, A., 1999. Decreased protein level of nicotinic receptor $\alpha 7$ subunit in the frontal cortex from schizophrenic brain. *Neuroreport* 10(8), 1779-1782.

- Haroutunian, V., Davidson, M., Kanof, P.D., Perl, D.P., Powchik, P., Losonczy, M., McCrystal, J., Purohit, D.P., Bierer, L.M., Davis, K.L., 1994. Cortical cholinergic markers in schizophrenia. *Schizophr. Res.* 12(2), 137-144.
- Hill, C., Keks, N., Roberts, S., Opeskin, K., Dean, B., Mackinnon, A., Copolov, D., 1996. Problem of diagnosis in postmortem brain studies of schizophrenia. *Psychiatry Res.* 153(4), 533-537.
- Hopper, S., Pavey, G.M., Gogos, A., Dean, B., 2019. Widespread Changes in Positive Allosteric Modulation of the Muscarinic M1 Receptor in Some Participants With Schizophrenia. *Int. J. Neuropsychopharmacol.* 22(10), 640-650.
- Jindal, S.P., Lutz, T., Hallstrom, C., Vestergaard, P., 1981. A stable isotope dilution assay for the antiparkinsonian drug bentspiron in biological fluids. *Clin.Chim.Acta* 112(3), 267-273.
- Kantrowitz, J.T., Javitt, D.C., Freedman, R., Sehatpour, P., Kegeles, L.S., Carlson, M., Sobeih, T., Wall, M.M., Choo, T.-H., Vail, B., Grinband, J., Lieberman, J.A., 2020. Double blind, two dose, randomized, placebo-controlled, cross-over clinical trial of the positive allosteric modulator at the $\alpha 7$ nicotinic cholinergic receptor AVL-3288 in schizophrenia patients. *Neuropsychopharmacol.*
- Karson, C.N., Casanova, M.F., Kleinman, J.E., Griffin, W.S., 1993. Choline acetyltransferase in schizophrenia. *Psychiatry Res.* 150(3), 454-459.

Kingsbury, A.E., Foster, O.J., Nisbet, A.P., Cairns, N., Bray, L., Eve, D.J., Lees, A.J., Marsden, C.D., 1995. Tissue pH as an indicator of mRNA preservation in human post-mortem brain. *Brain Res.Mol.Brain Res.* 28(2), 311-318.

Lakens, D., 2013. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. *Front. Psychol.* 4, 863-863.

Mancama, D., Arranz, M.J., Landau, S., Kerwin, R., 2003. Reduced expression of the muscarinic 1 receptor cortical subtype in schizophrenia. *Psychiatry Res.* 119(1), 2-6.

Marutle, A., Zhang, X., Court, J., Piggott, M., Johnson, M., Perry, R., Perry, E., Nordberg, A., 2001. Laminar distribution of nicotinic receptor subtypes in cortical regions in schizophrenia. *J.Chem.Neuroanat.* 22(1-2), 115-126.

McEvoy, J.P., McCue, M., Freter, S., 1987. Replacement of chronically administered anticholinergic drugs by amantadine in outpatient management of chronic schizophrenia. *Clin. Ther.* 9(4), 429-433.

Mesulam, M.-M., Hersh, L.B., Mash, D.C., Geula, C., 1992. Differential cholinergic innervation within functional subdivisions of the human cerebral cortex: A choline acetyltransferase study. *J. Comp. Neurol.* 318(3), 316-328.

Miyamoto, S., Duncan, G.E., Marx, C.E., Lieberman, J.A., 2005. Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Mol. Psychiatr.* 10(1), 79-104.

Moritz, S., Woodward, T.S., Rodriguez-Raecke, R.E.A., 2006. Patients with schizophrenia do not produce more false memories than controls but are more confident in them. *Psychol. Med.* 36(5), 659-667.

Oda, Y., 1999. Choline acetyltransferase: The structure, distribution and pathologic changes in the central nervous system. *Pathol. Int.* 49(11), 921-937.

Parkin, G.M., Udawela, M., Gibbons, A., Dean, B., 2018. Beta-actin does not show the characteristics of a reference protein in human cortex. *Electrophoresis*.

Perry, D.C., Dávila-García, M.I., Stockmeier, C.A., Kellar, K.J., 1999. Increased Nicotinic Receptors in Brains from Smokers: Membrane Binding and Autoradiography Studies. *J. Pharmacol. Exp. Ther.* 289(3), 1545-1552.

Powchik, P., Davidson, M., Haroutunian, V., Gabriel, S.M., Purohit, D.P., Perl, D.P., Harvey, P.D., Davis, K.L., 1998. Postmortem studies in schizophrenia. *Schizophr. Bull.* 24(3), 325-341.

Racke, K., Juergens, U.R., Matthiesen, S., 2006. Control by cholinergic mechanisms. *Eur.J.Pharmacol.* 533(1-3), 57-68.

Raedler, T.J., Knable, M.B., Jones, D.W., Urbina, R.A., Gorey, J.G., Lee, K.S., Egan, M.F., Coppola, R., Weinberger, D.R., 2003. In vivo determination of muscarinic acetylcholine receptor availability in schizophrenia. *Psychiatry Res.* 160(1), 118-127.

Roberts, S.B., Hill, C.A., Dean, B., Keks, N.A., Opeskin, K., Copolov, D.L., 1998.

Confirmation of the diagnosis of schizophrenia after death using DSM-IV: a Victorian experience. *Psychiatry Res.* 32(1), 73-76.

Rowe, C., Dean, B., Ackermann, U., Goh, R., Guzman, R., Kanaan, R., Chong, L., Dore, V., Bozinovski, S., Masters, C., Villemagne, V., 2019. In vivo imaging of brain muscarinic receptors with 18F-Fluorobenzyl Dextetimide: First in human studies. *J. Nucl. Med.* 60(supplement 1), 1470.

Salah-Uddin, H., Scarr, E., Pavey, G., Harris, K., Hagan, J.J., Dean, B., Challiss, R.A., Watson, J.M., 2009. Altered M(1) muscarinic acetylcholine receptor (CHRM1)-Galpha(q/11) coupling in a schizophrenia endophenotype. *Neuropsychopharmacol.* 34(9), 2156-2166.

Saricicek, A., Esterlis, I., Maloney, K.H., Mineur, Y.S., Ruf, B.M., Muralidharan, A., Chen, J.I., Cosgrove, K.P., Kerestes, R., Ghose, S., Tamminga, C.A., Pittman, B., Bois, F., Tamagnan, G., Seibyl, J., Picciotto, M.R., Staley, J.K., Bhagwagar, Z., 2012. Persistent beta2*-nicotinic acetylcholinergic receptor dysfunction in major depressive disorder. *Am J Psychiatry* 169(8), 851-859.

Scarr, E., Cowie, T.F., Kanellakis, S., Sundram, S., Pantelis, C., Dean, B., 2009. Decreased cortical muscarinic receptors define a subgroup of subjects with schizophrenia. *Mol. Psychiatr.* 14(11), 1017-1023.

Scarr, E., Craig, J.M., Cairns, M.J., Seo, M.S., Galati, J.C., Beveridge, N.J., Gibbons, A., Juzva, S., Weinrich, B., Parkinson-Bates, M., Carroll, A.P., Saffery, R., Dean, B., 2013a.

Decreased cortical muscarinic M1 receptors in schizophrenia are associated with changes in gene promoter methylation, mRNA and gene targeting microRNA. *Trans.Psychiatr.* 3, e230.

Scarr, E., Gibbons, A.S., Neo, J., Udawela, M., Dean, B., 2013b. Cholinergic connectivity: it's implications for psychiatric disorders. *Front Cell Neurosci.* 7, 55.

Scarr, E., Gray, L., Keriakous, D., Robinson, P.J., Dean, B., 2006. Increased levels of SNAP-25 and synaptophysin in the dorsolateral prefrontal cortex in bipolar I disorder. *Bipolar Disord.* 8(2), 133-143.

Scarr, E., Hopper, S., Vos, V., Seo, M.S., Everall, I.P., Aumann, T.D., Chunam, G., Dean, B., 2018a. Low levels of muscarinic M1 receptor positive neurons in cortical layers III and V in Brodmann's areas 9 and 17 from individuals with schizophrenia. *J. Psychiatry Neurosci.* 43, 338-346.

Scarr, E., Udawela, M., Thomas, E.A., Dean, B., 2018b. Changed gene expression in subjects with schizophrenia and low cortical muscarinic M1 receptors predicts disrupted upstream pathways interacting with that receptor. *Mol. Psychiatr.* 23, 295-303.

Seo, M.S., Scarr, E., Dean, B., 2014. An investigation of the factors that regulate muscarinic receptor expression in schizophrenia. *Schizophr. Res.* 158(1-3), 247-254.

Shant, J., Cheng, K., Marasa, B.S., Wang, J.-Y., Raufman, J.-P., 2009. Akt-dependent NF-kappaB activation is required for bile acids to rescue colon cancer cells from stress-induced apoptosis. *Exp. Cell Res.* 315(3), 432-450.

Shekhar, A., Potter, W.Z., Lightfoot, J., Lienemann, J., Dube, S., Mallinckrodt, C., Bymaster, F.P., McKinzie, D.L., Felder, C.C., 2008. Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. *Am. J. Psychiatr.* 165(8), 1033-1039.

Shen, J.-x., Tu, B., Yakel, J.L., 2009. Inhibition of $\alpha 7$ -containing nicotinic ACh receptors by muscarinic M1 ACh receptors in rat hippocampal CA1 interneurons in slices. *The Journal of Physiology* 587(5), 1033-1042.

Stan, A.D., Ghose, S., Gao, X.M., Roberts, R.C., Lewis-Amezcu, K., Hatanpaa, K.J., Tamminga, C.A., 2006. Human postmortem tissue: what quality markers matter? *Brain Res.* 1123(1), 1-11.

Strand, J.-E., Nybäck, H., 2005. Tobacco use in schizophrenia: a study of cotinine concentrations in the saliva of patients and controls. *Eur. Psychiatry* 20(1), 50-54.

Tamminga, C.A., 2008. Accelerating new knowledge in schizophrenia. *Am. J. Psychiatr.* 165(8), 949-951.

Terry, A.V., Callahan, P.M., 2020. $\alpha 7$ nicotinic acetylcholine receptors as therapeutic targets in schizophrenia: Update on animal and clinical studies and strategies for the future. *Neuropharmacol.* 170, 108053.

Tune, L., Coyle, J.T., 1981. Acute extrapyramidal side effects: serum levels of neuroleptics and anticholinergics. *Psychopharmacol. (Berlin)* 75(1), 9-15.

van Goethem, N.P., Paes, D., Puzzo, D., Fedele, E., Rebosio, C., Gulisano, W., Palmeri, A., Wennogle, L.P., Peng, Y., Bertrand, D., Prickaerts, J., 2019. Antagonizing $\alpha 7$ nicotinic receptors with methyllycaconitine (MLA) potentiates receptor activity and memory acquisition. *Cell. Signal.* 62, 109338.

Waugh, M.G., Challiss, R.A., Berstein, G., Nahorski, S.R., Tobin, A.B., 1999. Agonist-induced desensitization and phosphorylation of m1-muscarinic receptors. *Psychiatry Res.* 338 (Pt 1), 175-183.

Wu, D., Hersh, L.B., 1994. Choline Acetyltransferase: Celebrating Its Fiftieth Year. *J. Neurochem.* 62(5), 1653-1663.

5.0 Figure Legends

Figure 1A: The ability of cortical extracts from Brodmann's (BA) area 9 from subjects with MRDS, non-MRDS and controls to displace [^3H]NMS binding (mean $\pm$ SEM) from a homogenate prepared from BA 9 of a reference case.

1B: Levels (mean $\pm$ SEM) of [^3H]pirenzepine to BA 9 homogenates from the same cases.

Figure 2: Levels (mean $\pm$ SEM) of [^{125}I] α bungarotoxin (A-C) or [^3H]pirenzepine binding (D) to Brodmann's areas 6, 9 and 44 ([^3H]pirenzepine BA 9 only) from patients with schizophrenia (MRDS + non-MRDS), MRDS, non-MRDS and controls.

Figure 3: Levels (mean $\pm$ SEM) of choline acetyltransferase (ChAT) isoforms (molecular weight) 68 (A) and 82 (B) as well as synaptosome nerve-associated protein 25 (SNAP 25) (C) in BA 9 from patients with schizophrenia (MRDS + non-MRDS), MRDS, non-MRDS and controls.

30 Royal Parade,
Parkville,
Victoria 3052,
Australia.

Telephone: 613-8344-3786
Facsimile: 613-9348-1707
Email: anddali@unimelb.edu.au

16 April 2020

Dr Matcheri Kashavan
Editor-in-Chief
Schizophrenia Research

Dear Dr Kashavan,

We thank the Reviewers for providing comments on our manuscript entitled “Higher levels of $\alpha 7$ nicotinic receptors, but not choline acetyltransferase, in the dorsolateral prefrontal cortex from a sub-group of patients with schizophrenia” (SCHRES-D-20-00023). We now detail how these comments have allowed us to revise specific aspects of our original manuscript:

Reviewer #1:

1. Table 1 suggests that the authors were apparently studying two different samples (A and B). This is confusing and should be clarified in the manuscript.

Response: We have changed the beginning of the results section of our revised manuscript to emphasise that two different cohorts of subjects were used in our studies.

2. The authors found no differences in cotinine-levels between subjects with schizophrenia and healthy controls. This finding is surprising as subjects with schizophrenia generally have higher rates of smoking than the general population.

Response: This Reviewer is correct in asserting that patients with schizophrenia smoke at higher rates than do members of the general population. Importantly, as we state in our original and revised manuscript, cotinine levels provide a measure of the ingestion of nicotine over 5 days and hence our cotinine data would suggest that the patients with schizophrenia and controls had been smoking at similar rates for 5 days before death. Our cotinine measures do not address long term smoking practices.

Reviewer #2:

Major points:

1. The authors should clarify how to differentiate MRDS from non-MRDS. What is the precise definition of MRDS?

Response: This is a very important point and we now define how patients with MRDS are differentiated in the introduction of our revised manuscript where we first introduce MRDS as a sub-group in patients with schizophrenia.

2. The MRDS group demonstrated marked down-regulation of CHRM1 and upregulation of CHRNA7 in BA9. I think this is the first study that showed higher levels of CHRNA7 in the prefrontal cortex of patients with schizophrenia. If this is true, it is clinically very important, because about 25% of patients with schizophrenia may be a MRDS as described by the authors. Thus, please discuss the

potential clinical consequences of decreased CHRM1 and increased CHRNA7 in schizophrenia and please mention a therapeutic perspective.

Response: Translating findings from postmortem CNS to a more clinical and therapeutic setting is always difficult. However, in our revised manuscript, we have added a section extrapolating our previous suggestion that treating patients with schizophrenia with muscarinic M1 receptor agonists could give therapeutic benefits to discuss a potential for the use of CHRNA7 antagonists to treat MRDS.

Minor points:

1. On page 3 line 5, please insert a comma after "serotonergic".
2. On page 3 line 12, the authors should spell out "CHRM1".
3. On page 4 line 8 from the bottom, please change "one study" to "one post-mortem study".
4. On page 5 line 6, please insert a comma after "cortex".
5. On page 5 line 13, the authors should delete "typical".
6. On page 5 line 15, the authors should spell out "CNS".
7. On page 12 line 3, the authors should spell out "PMI".
8. On page 12 lines 5-6, the authors should spell out "DI", "FRADD" and "LEAP".

Response: Changes 1 to 8 have been made in our revised manuscript.

9. On page 12 line 9 from the bottom, I cannot find Figure 1D. Is this Figure 2D?

Response: The reference to Figure 1D should have been to Figure 2D, this has been changed in our revised manuscript.

10. On page 12 line 3 from the bottom, please change "duration of illness (DI)" to "DI".
11. On page 12 line 3 from the bottom, please change "final recorded antipsychotic drug dose (FRADD)" to "FRADD".
12. On page 12 line 2 from the bottom, please change "lifetime exposure to antipsychotic drugs (LEAP)" to "LEAP".

Response: Changes 10 to 12 have been made in our revised manuscript.

As we have been able to incorporate all the Reviewer's suggestions into our revised manuscript, we hope it will now be acceptable for publication.

We look forward to hearing from you on this matter.

Yours sincerely,

On Behalf of the Authors

Figure 1

[Click here to download high resolution image](#)

Figure 2

[Click here to download high resolution image](#)

Figure 3

[Click here to download high resolution image](#)

Table 1

Table 1: Demographic, CNS collection and case history data for cases used in this study.

			Gender (F / M)	Age (yr.)	Suicide (Y / N)	pH	PMI (hr)	CNS Weight (gms)	DI (yr)	FRADD [†]	LEAP [*]
A	Controls (n = 5)	Mean or Ratio	0 / 5	44	0 / 5	6.38	46	1344			
		SEM		6.5		0.07	9.4	81			
	MRDS (n = 5)	Mean or Ratio	0 / 5	49	2 / 3	6.31	41	1491	24	75	6.6
		SEM		8.6		0.07	5.4	63	8.1	34	3.9
	non MRDS (n = 5)	Mean or Ratio	0 / 5	45	2 / 3	6.21	49	1370	17	197	6.5
		SEM		7.1		0.73	8.6	46	7.1	98	3.4
		F		0.08		1.54	0.25	1.45			
		d.f.		2,12		2,12	2,12	2,9			
		p	1.00	0.88	0.08	0.25	0.78	0.28	0.56	0.28	1.00
B	Controls (n = 12)	Mean or Ratio	3 / 9	45.0	0 / 12	6.44	44	1329			
		SEM		5.0		0.03	4.2	44			
	Schizophrenia (n = 27)	Mean or Ratio	6 / 21	43	11 / 16	6.17	41	1382	15	597	9.2
		SEM		3.3		0.04	2.7	30	2.5	114	2.6
		p vs Control	> 0.999	0.69	0.009	< 0.001	0.59	0.33			
	MRDS (n = 14)	Mean or Ratio	3 / 11	42	7 / 7	6.21	41	1403	16	500	9.2
		SEM		5.0		0.05	3.1	49	3.9	123	4.100
		p vs Control	> 0.999	0.71	0.006	0.006	0.51	0.28			
	non MRDS	Mean or Ratio	3 / 10	43.0	4 / 9	6.13	42	1355	15	726	9.100

(n = 13)	SEM	4.7		0.07	4.5	29	3.1	213	3.100
	p vs Control	> 0.999	0.76	0.10	<0.001	0.78	0.63		
	p vs MRDS	> 0.999	0.94	0.44	0.37	0.75	0.44	0.79	0.34
								0.990	

Abbreviations: DI = duration of illness, F = female, FRADD = final recorded antipsychotic drug dose, gms = grams, hr = hours, LEAP = lifetime exposure to antipsychotic drugs, M = male, MRDS = muscarinic receptor deficit schizophrenia, N = no, non MRDS = not muscarinic receptor deficit schizophrenia, PMI = post-mortem interval, SEM = standard error of the mean, vs = versus, Y = yes, yr = years.

† mg chlorpromazine equivalents per day , * g chlorpromazine equivalents x 10⁻³

Supplementary Figure 1

[Click here to download Supplementary Material for online publication only: Supplementary Figure 2.jpg](#)

Supplementary Methods

[Click here to download Supplementary Material for online publication only: Supplementary Methods.docx](#)

Supplementary Tab e 1

[Click here to download Supplementary Material for online publication only: Supplementary Table 1.docx](#)

Supplementary Table 2

[Click here to download Supplementary Material for online publication only: Supplementary Table 2.docx](#)

Supplementary Table 3

[Click here to download Supplementary Material for online publication only: Supplementary Table 3.docx](#)

Supplementary Table 5

[Click here to download Supplementary Material for online publication only: Supplementary Table 5.docx](#)

***Conflict of Interest**

Conflicts of Interest

The Authors have no conflicts of interest.

Acknowledgements

The authors thank Madhara Udawela and Clare Duncan for the technical assistance that contributed to the data included in this manuscript. This research was funded in part by NHMRC Project Grant # APP1105332 and the CRC for Mental Health.

Role of Funding Source

The organisations that funded this research had no control over the study design, analyses of results or preparation of the submitted manuscript.

Contributors

All authors were involved in the study design and preparation of this manuscript. BD completed the statistical analyses and prepared the first draft of the manuscript. GP complete the experiments described under anticholinergic load. All authors reviewed and approved the final version of the manuscript.

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Dean, B;Pavey, G;Scarr, E

Title:

Higher levels of alpha 7 nicotinic receptors, but not choline acetyltransferase, in the dorsolateral prefrontal cortex from a sub-group of patients with schizophrenia

Date:

2020-08-01

Citation:

Dean, B., Pavey, G. & Scarr, E. (2020). Higher levels of alpha 7 nicotinic receptors, but not choline acetyltransferase, in the dorsolateral prefrontal cortex from a sub-group of patients with schizophrenia. SCHIZOPHRENIA RESEARCH, 222, pp.283-290. <https://doi.org/10.1016/j.schres.2020.05.034>.

Persistent Link:

<http://hdl.handle.net/11343/241391>