Neuropsychopharmacology (2016) 41, 1620–1628

www.neuropsychopharmacology.org

# Changes in BQCA Allosteric Modulation of [$^3$H]NMS Binding to Human Cortex within Schizophrenia and by Divalent Cations

**Brian Dean*[,1,2,3], Shaun Hopper[1,2,3], P Jeffrey Conn[4] and Elizabeth Scarr[1,2,3]**

*[1]The Florey Institute for Neuroscience and Mental Health, Parkville, VIC, Australia; [2]The CRC for Mental Health, Parkville, VIC, Australia; [3]Department of Psychiatry, The University of Melbourne, Parkville, VIC, Australia; [4]Vanderbilt Center for Neuroscience Drug Discovery, Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN, USA*

Stimulation of the cortical muscarinic M1 receptor (CHRM1) is proposed as a treatment for schizophrenia, a hypothesis testable using CHRM1 allosteric modulators. Allosteric modulators have been shown to change the activity of CHRMs using cloned human CHRMs and CHRM knockout mice but not human CNS, a prerequisite for them working in humans. Here we show *in vitro* that BQCA, a positive allosteric CHRM1 modulator, brings about the expected change in affinity of the CHRM1 orthosteric site for acetylcholine in human cortex. Moreover, this effect of BQCA is reduced in the cortex of a subset of subjects with schizophrenia, separated into a discrete population because of a profound loss of cortical [$^3$H]pirenzepine binding. Surprisingly, there was no change in [$^3$H]NMS binding to the cortex from this subset or those with schizophrenia but without a marked loss of cortical CHRM1. Hence, we explored the nature of [$^3$H]pirenzepine and [$^3$H]NMS binding to human cortex and showed total [$^3$H]pirenzepine and [$^3$H]NMS binding was reduced by $Zn^{2+}$, acetylcholine displacement of [$^3$H]NMS binding was enhanced by $Mg^{2+}$ and $Zn^{2+}$, acetylcholine displacement of [$^3$H]pirenzepine was reduced by $Mg^{2+}$ and enhanced by $Zn^{2+}$, whereas BQCA effects on [$^3$H]NMS, but not [$^3$H]pirenzepine, binding was enhanced by $Mg^{2+}$ and $Zn^{2+}$. These data suggest the orthosteric and allosteric sites on CHRMs respond differently to divalent cations and the effects of allosteric modulation of the cortical CHRM1 is reduced in a subset of people with schizophrenia, a finding that may have ramifications for the use of CHRM1 allosteric modulators in the treatment of schizophrenia.
*Neuropsychopharmacology* (2016) **41,** 1620–1628; doi:10.1038/npp.2015.330; published online 2 December 2015

## INTRODUCTION

Post-mortem CNS studies and a neuroimaging study (see Raedler *et al*, 2007) have reported lower levels of cortical muscarinic receptors (CHRMs) in people with schizophrenia due, at least in part, to lower levels of CHRM1 (Dean *et al*, 2002; Mancama *et al*, 2003; Scarr *et al*, 2006). The recognition that schizophrenia is a syndrome (Schwarz *et al*, 2013) is relevant to these findings because it has been shown that lower levels of cortical CHRM1 are restricted to a subgroup of people with schizophrenia (Scarr *et al*, 2009) (muscarinic receptor-deficit schizophrenia (MRDS)) who may have a different etiology that includes altered agonist-induced CHRM1-mediated G-protein recruitment (Salah-Uddin *et al*, 2009), differentiating CHRM1 promoter methylation (Scarr *et al*, 2013), and increased levels of a CHRM1 targeting microRNA (Scarr *et al*, 2013).

*Correspondence: Professor B Dean, The Molecular Psychiatry Laboratory, The Florey Institute for Neuroscience and Mental Health, 30 Royal Parade, Parkville, VIC 3052, Australia, Tel: +61 3 8344 3786, Fax: +61 3 9348 1707, E-mail: brian.dean@florey.edu.au
Received 23 July 2015; revised 4 October 2015; accepted 20 October 2015; accepted article preview online 29 October 2015

Based on data from human and preclinical pharmacological studies, it was suggested that stimulating cortical CHRM1 would alleviate some symptoms of schizophrenia (Bymaster *et al*, 2002). This hypothesis gained support from the finding that, compared with placebo, xanomeline, a CHRM1/CHRM4 agonist, reduced the severity of positive, negative, and cognitive deficit symptoms in treatment-resistant schizophrenia (Shekhar *et al*, 2008). Unfortunately, despite this finding, the severe peripheral side effects of xanomeline (Bodick *et al*, 1997), its complex pharmacology (Shannon *et al*, 1994) and metabolic instability, as well as a high hepatic first-pass effect (Mirza *et al*, 2003) prevented further clinical development. Notably, preclinical findings suggests xanomeline's effects on cognition, attention, and learning were mediated through CHRM1 (Bymaster *et al*, 2003) and the antipsychotic effects were mediated by CHRM4 modulating dopamine levels (Woolley *et al*, 2009).

Structural homology at the orthosteric binding site across CHRMs (Wess *et al*, 2007) has thus far thwarted attempts to synthesize compounds specific to individual CHRMs. However, the discovery of allosteric binding sites specific to each CHRM (Lazareno and Birdsall, 1995) now offers an opportunity to independently modulate the activity of each

**Table 1** Demographic, Diagnostic, and Tissue Collection Data for Cases Used in This Study (Mean ± SEM)

| | M/F | Age (yr) | Sui (yes/no) | DI (yr) | FRADD | LEAP | FRBD | FRAcD | PMI (h) | pH |
|---|---|---|---|---|---|---|---|---|---|---|
| Controls | 16/4 | 46 ± 3.8 | 0/20 | | | | | | 43 ± 3.6 | 6.32 ± 0.05 |
| Schizophrenia | 32/8 | 48 ± 3.7 | 13/27 | 21 ± 3.4 | 603 ± 93 | 12 ± 2.7 | 6.1 ± 1.11 | 1.8 ± 0.08 | 41 ± 3.0 | 6.19 ± 0.06 |
| *P* | 1.00 | 0.75 | <0.005 | | | | | | 0.41 | 0.09 |
| MRDS | 16/4 | 47 ± 2.6 | 6/14 | 21 ± 2.4 | 617 ± 134 | 11 ± 3.7 | 6.6 ± 0.08 | 1.8 ± 0.13 | 40 ± 2.0 | 6.22 ± 0.04 |
| Sz other | 16/4 | 47 ± 3.8 | 7/13 | 20 ± 3.5 | 593 ± 124 | 12 ± 4.0 | 5.8 ± 1.99 | 1.9 ± 0.09 | 39 ± 2.7 | 6.24 ± 0.04 |
| F | | 0.08 | | | | | | | 0.49 | 1.87 |
| df | | 2, 57 | | | | | | | 2, 57 | 2, 57 |
| *P* | 1.00 | 0.92 | <0.05 | 0.74 | 0.94 | 0.83 | 0.72 | 0.49 | 0.61 | 0.16 |

Abbreviations: DI, duration of illness; F, female; FRAcD, final recorded anticholinergic drug dose as mg benztropine equivalents per day; FRADD, final recorded antipsychotic drug dose as mg chlorpromazine equivalents per day; FRBD, final recorded benzodiazepine dose as mg diazepam equivalents per day; LEAP, lifetime exposure to antipsychotic drugs as chlorpromazine equivalents per year × $10^{-3}$; M, male; MRDS, muscarinic receptor-deficit schizophrenia; PMI, post-mortem interval; Sui, suicide completer; Sz other, schizophrenia without a marked loss of cortical [3H]pirenzepine binding.

of these five receptors. For example, using cloned human receptors, behavioral studies in wild-type and *Chrm*$^{-/-}$ mice, pharmacology at the rat Chrm1, and effects on rat behavior strongly suggests that the positive allosteric modulator [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] (BQCA) is highly selective, if not specific, for the CHRM1 (Ma *et al*, 2009; Shirey *et al*, 2009). Notably, a reliable method is now available for quantifying the effect of BQCA at the CHRM1 that involves measuring the ability of the drug to decrease the concentration of acetylcholine required to displace [3H]n-methylscopolamine ([3H]NMS) from the cloned CHRM1 or CNS membranes (Ma *et al*, 2009; Shirey *et al*, 2009).

We have previously shown that the pharmacology of a compound established using models such as human cloned receptors does not necessarily correspond with the pharmacology of the drug in human CNS tissue (Dean *et al*, 2006). Therefore, because of our interest in CHRM1 as a drug target, we wanted to show the defining pharmacological response to BQCA established using cloned receptors was detectable using human cortex. In addition, our interest in CHRM1 in schizophrenia led us to determine whether the action of BQCA on [3H]NMS binding might be altered using cortical membranes from subjects with the disorder, particularly in subjects with MRDS compared with non-MRDS (Scarr *et al*, 2009). In addition, as [3H]NMS binding has been reported to be higher in the cortex of people with schizophrenia (Watanabe *et al*, 1983) we measured the binding parameters for that radioligand to cortical membranes from subjects with the disorder. Finally, to allow us to better understand the potential impact of differing methods used for [3H]NMS and [3H]pirenzepine binding, we investigated whether the binding of these radioligands was ion dependent. Our studies were completed using Brodmann's area (BA) 6 because we confirmed that the marked decrease in [3H]pirenzepine binding in subjects with MRDS, which was first identified in BA 9, was detectable in BA 6 (Seo *et al*, 2014) and because we had shown that this region of the cortex had decreased levels of *neuregulin 1* (*NRG1*) (Barakat *et al*, 2010). As changes in *NRG1* gene sequence has been associated with an increased risk of schizophrenia (Stefansson *et al*, 2002), we postulated molecular changes in BA 6 may reflect the impact of a biology dictated by an increased genetic predisposition for the disorder.

## MATERIALS AND METHODS

### Tissue Collection, Diagnostic Validation, and Membrane Preparation

After approval from the Ethics Committee of the Victorian Institute of Forensic Medicine, tissue was collected post-mortem and diagnostic evaluation completed on a series of subjects with a likely history of psychiatric illness and age/sex-matched subjects who appeared to have no history of a psychiatric disorder as described previously (Scarr *et al*, 2009) (Supplementary Methods). The left cerebral hemisphere was sliced according to a standardized protocol and then rapidly frozen to −80 °C for storage until required (Dean *et al*, 1999).

Following approval from the Tissue Access Committee of the Victorian Brain Bank Network, tissue was obtained from BA 6 (a cortical region that includes the anterior part of the paracentral lobule and the adjacent superior gyrus, the dorsal bank of the callosomarginal sulcus (minus its posterior third), and the bases of superior and middle frontal gyrus and pre-central gyrus not included in BA 4 (Garey, 1994)) from five subjects for methodological optimization. In addition, tissue was obtained from BA 6 from 40 subjects with schizophrenia and 20 age/sex-matched controls (demographics summarized in Table 1, complete detail in Supplementary Table 1) who had essentially been used in a previous study of [3H]pirenzepine binding in that cortical region (Seo *et al*, 2014). The subjects with schizophrenia comprised 20 MRDS and 20 non-MRDS cases; the 20 subjects with MRDS were selected from the cases that we have previously shown separated into a subgroup within the syndrome of schizophrenia because of a marked loss of [3H]pirenzepine binding in BA 9 (Scarr *et al*, 2009).

### Membrane Preparations for [3H]NMS Binding

Membranes were prepared using methodologies similar to that used for human cloned CHRMs (Shirey *et al*, 2009). Human cortices were thawed on ice and homogenized into

10 vol of 20 mM HEPES containing 100 mM NaCl and 10 mM $MgCl_2$, pH 7.4 (Assay Buffer) by 10 strokes of Glass/PTFE Potter Elvehjem tissue grinders. The resulting homogenates were centrifuged at 21 500 *g* for 20 min at 4 °C and the supernatant was discarded. The pellet was suspended in 10 vol Assay Buffer and washed twice by repeating the centrifugation process. The washed membranes were stored at − 80 °C until required, at which time their protein concentrations were determined using the Bio-Rad protein assay.

## [3H]NMS Binding: Methodological Optimization

*[3H]NMS binding: binding parameters.* The binding of [3H]NMS (0.01 to 2 nM: triplicate) to washed human cortical membranes ($n = 3$; 0.1 mg protein) was measured in the absence (total binding (TB)) and presence (nonspecific binding (NSB)) of 1 mM acetylcholine, and this and all subsequent experiments were completed in a final vol of 1 ml made up with Assay Buffer. After incubating for 2 h at room temperature (RT), 5 ml of ice cold Assay Buffer was added to each tube and the bound radioactivity filtered onto a Whatman GF/B filter. Each filter was washed thrice with ice-cold 0.9% saline and placed into 5 ml of UltimateGold scintillation cocktail. After 1 h, the radioactivity on each filter paper was counted using a Tri-Carb 2910 TR (PerkinElmer) and corrected for radioactive decay and counting efficiency before being converted from counts per min to specific binding of fmol/mg protein; TB and NSB at each concentration of [3H]NMS was used in a Scatchard analysis (GraphPad Prism 6.0) to calculate the affinity (Kd) and maximal binding (Bmax) of [3H]NMS.

*[3H] NMS binding: effects of acetylcholine and BQCA.* The ability of acetylcholine (0 to $10^{-1}$ M) to displace [3H]NMS (0.13 nM; Kd) from cortical membranes (0.1 mg protein) was measured after incubating for 2 h at RT. Bound and free radioligand were separated as described previously. Then, the binding of [3H]NMS (0.13 nM) in the absence or presence of BQCA (0.3 and 3 μM dissolved in 0.1% DMSO) and acetylcholine (0 to $10^{-3}$ M) was measured (NB 0.1% DMSO meant final concentration of 0.01% in Assay Buffer; at this concentration DMSO did not affect [3H]NMS binding (data not shown)). Finally, [3H]NMS binding (0.13 nM) to cortical membrane in the absence and presence of BQCA (3 μM) and acetylcholine (0 to $10^{-3}$ M) was measured at varying protein concentrations (0.01 to 0.10 mg/ml protein).

From these experiments, two binding parameters could be measured that were the change in ΔlogEC50 for the displacement of [3H]NMS by acetylcholine at different concentrations of BQCA and the area encompassed between the acetylcholine displaceable [3H]NMS binding curves at two concentrations of BQCA (Area BQCA Curves: see methodological optimization in Results).

## Studies in Schizophrenia

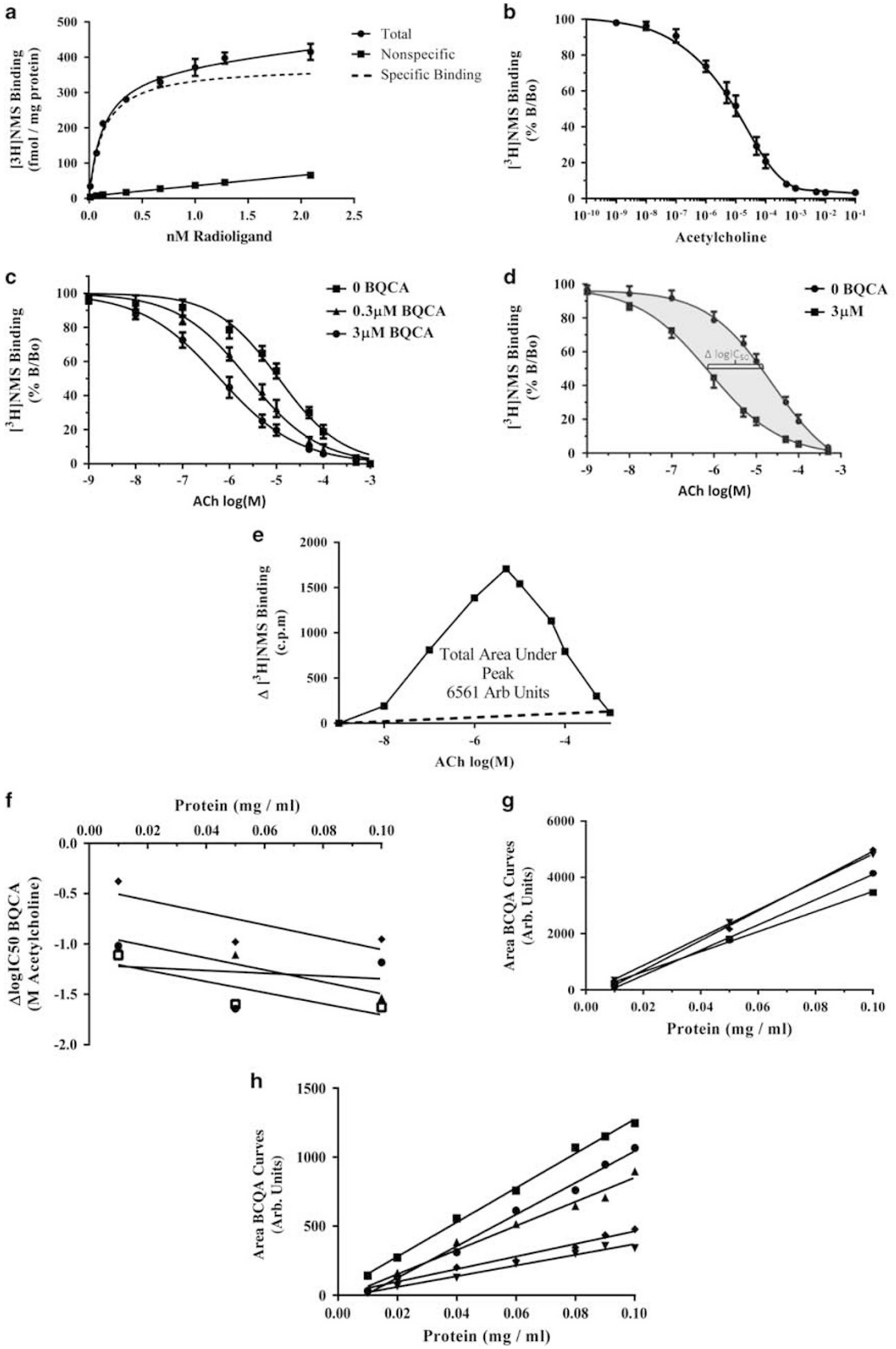
Using the described methodology, [3H]NMS binding parameters for human cortical membranes from BA 6 from the 40 subjects with schizophrenia and the 20 age- and sex-marched controls were measured. In addition, the Area BQCA Curves were measured for all cases.

## Statistical Analysis

Because of difficulties in determining data distribution on small sample sizes (D'Agostino *et al*, 1990), all statistical analyses were completed using parametric analyses and effect size determined using Cohen's *d* values (Cohen, 1988). Again, because of small sample sizes, only linear regression analyses with $r^2 > 0.49$ were taken as reflecting strong enough relationships between two variables to warrant acceptance as potential covariates (Cook and Weisberg, 1999) and in such cases an ANCOVA was used to determine the impact of such factors on our analyses.

Donor demographic, drug, and CNS collection data were assessed using GraphPad Prism and either one-way ANOVA followed by *post hoc* Tukey's multiple comparison test (Motulsky, 1999) comparing data across cohorts or Student *t*-tests (if 2 cohorts). The sex ratios and rates of suicide in the different cohorts were compared using Fisher's exact test. Variation in experimental data was assessed using Student's *t*-tests or one-way ANOVA followed by *post hoc* Dunnett's comparison with control. Relationships between experimental variables and demographic, tissue collection, and pharmacological data were assessed using linear regression. If strong relationships were revealed, and there was no variation in the nonexperimental data with group (Miller and Chapman, 2001), the variation in the experimental data was reassessed using the ANCOVA with the appropriate nonexperimental data as covariant(s) using Minitab 15. Data are presented as mean ± SEM unless otherwise stated.

**Figure 1** (a) The binding (mean ± SEM) of [3H]NMS at varying concentrations in the absence (total binding (TB)) or presence (nonspecific binding (NSB)) of cortical membranes from three subjects with no history of psychiatric illness. Specific binding (SB) was calculated as TB − NSB at each concentration of [3H]NMS. (b) The binding (mean ± SEM) of [3H]NMS (0.13 nM) to cortical membranes from three subjects with no history of psychiatric illness in the presence of increasing concentrations of acetylcholine. (c) The binding (mean ± SEM) of [3H]NMS (0.13 nM) to cortical membranes from three subjects with no history of psychiatric illness in the presence of increasing concentrations of acetylcholine and increasing concentrations of [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] (BQCA). (d) A schematic diagram showing the two possible measures of the effects of BQCA on the ability of increasing concentrations of acetylcholine to displace [3H]NMS (0.13 nM). One measure is the difference between the $\log IC_{50}$ values for the displacement of [3H]NMS by acetylcholine in the absence and presence of BQCA ($\Delta \log IC_{50}$), the second is an integrated measure of the area within the acetylcholine displacement curves in the absence or presence of 3 μM BQCA. (e) An example of the area between the two displacement curves being derived as the area under the curve defined by the difference in [3H]NMS in the absence or presence of BQCA at each concentration of acetylcholine corrected for variation in baseline. (f) The relationship between the $\Delta \log IC_{50}$ caused by BQCA (3 μM) on the ability of acetylcholine to displace [3H]NMS from cortical membranes from three individuals added at differing levels of protein. (g) The relationship between Area BQCA Curves and cortical membranes from three individuals added at three different levels of protein. (h) The relationship between Area BQCA Curves for cortical membranes from three individuals added at five different levels of protein.


a
[3H]NMS Binding (fmol / mg protein)
nM Radioligand
Total
Nonspecific
Specific Binding
b
[3H]NMS Binding (% B/Bo)
Acetylcholine
c
[3H]NMS Binding (% B/Bo)
ACh log(M)
0 BQCA
0.3μM BQCA
3μM BQCA
d
[3H]NMS Binding (% B/Bo)
ACh log(M)
0 BQCA
3μM
e
Δ [3H]NMS Binding (c.p.m)
Total Area Under Peak
6561 Arb Units
ACh log(M)
f
Protein (mg / ml)
ΔlogIC50 BQCA (M Acetylcholine)
g
Area BCQA Curves (Arb. Units)
Protein (mg / ml)
h
Area BCQA Curves (Arb. Units)
Protein (mg / ml)

## RESULTS AND DISCUSSION

Where we describe results on subjects with schizophrenia, these data related to the combined MRDS and non-MRDS data.

### [3H]NMS Binding: Methodological Optimization

[3H]NMS bound to human cortical membranes with a Kd = 0.13 ± 0.02 nM and a Bmax = 342 ± 85 fmol/mg protein (Figure 1a); for subsequent experiments [3H]NMS was standardized to 0.13 nM. Acetylcholine displaced [3H]NMS binding from human cortical membranes with nonspecific binding at $10^{-3}$ M (Figure 1b).

The ability of acetylcholine (0 to $10^{-3}$ M) to displace [3H]NMS binding to cortical membranes increased with increasing concentrations of BQCA ($logIC_{50}$: 0 BQCA = − 4.95 M, 0.3 μM BQCA = − 5.50 M, and 3.0 μM BQCA = − 6.21 M: Figure 1c). It was possible to measure the effects of BQCA in the absence or presence of 3.0 μM drug by measuring either $\Delta logIC_{50}$ or the area between BQCA curves (Area BQCA Curves) (Figure 1d). The Area BQCA Curves was calculated by subtracting [3H]NMS binding in the absence of BQCA from that in the presence of the drug at each concentration of acetylcholine, plotting the differences against concentration of acetylcholine and then calculating the area under the curve, correcting for any variation in baseline (Figure 1e). Subsequent experiments showed there was no strong correlation between $\Delta$ $logIC_{50}$ BQCA and level of protein (membrane) in each assay tube ($r^2$ from 0.04 to 0.92, $p$ from 0.18 to 0.88: Figure 1f) but there were strong correlations between Area BQCA Curve and protein levels ($r^2$ from 0.979 to 0.999, $p$ from $<0.0001$ to 0.03: Figure 1g and h (all $p<0.0001$)).

### Studies on Schizophrenia

There were no significant differences in sex ratio, age, PMI, or CNS pH between the subjects with schizophrenia and the age/sex-matched controls (Table 1 and Supplementary Table 1). The frequency of suicide completion was significantly higher in subjects with schizophrenia. There were no significant differences in sex ratio, age, PMI, or CNS pH between MRDS, non-MRDS, and controls. There were no differences in FRADD, LEAP, FRBD, or FRAcD between MRDS and controls. The frequency of suicide completion was higher in non-MRDS compared with MRDS. The frequency of antipsychotic ($p=0.18$), benzodiazepine ($p=0.74$), or anticholinergic ($p=0.52$) drug prescription at death did not vary between MRDS and non-MRDS.

Compared with controls, the affinity (Kd; nM: schizophrenia = 0.26 ± 0.02 *vs* Controls = 0.13 ± 0.007; Cohen's $d=0.87$; Figure 2a) of [3H]NMS binding was lower, but density of binding was not changed, in schizophrenia. Compared with controls, the affinity, but not density (MRDS $p=0.51$; Cohen's $d=0.21$ and non-MRDS $p=0.64$; Cohen's $d=0.14$), of [3H]NMS binding was lower in MRDS (Figure 2a: $p<0.0001$; Cohen's $d=-1.66$) but not non-MRDS ($p=0.13$; Cohen's $d=-0.62$).

The Area BQCA Curve was not different in schizophrenia compared with controls ($p=0.16$; Figure 3). However, the Area BQCA Curve was lower in subjects with MRDS (1460 ± 225 Arbitrary (Arb) Units) compared with non-MRDS (2710 ± 236 Arb Units) and controls (2572 ± 295 Arb Units; $p<0.01$; Cohen's $d=-0.948$).

The only strong relationship ascertained was between the affinity for [3H]NMS binding and the density of [3H]pirenzepine binding in human cortex (Supplementary Table 2); there is no clear explanation why there was such a relationship, especially given the absence of correlation between the maximal number of binding sites measured using the same radioligands.

There were no strong or significant correlations between any experimental measure and age, PMI, CNS pH, DI, FRADD, LEAP, or FRBD (Supplementary Table 3A). Neither the affinity ($p=0.82$; $p=0.11$) nor density ($p=0.13$, $p=0.19$) of [3H]NMS binding nor the Area BQCA Curves ($p=0.55$; $p=0.47$) varied with gender or suicide completion, respectively (Supplementary Table 3B). These data show that the different rates of suicide completion in MRDS *versus* non-MRDS was not a significant confound for this study. There were no significant differences in the affinity ($p=0.92$) or density ($p=0.07$) of [3H]NMS binding or Area BQCA Curves ($p=0.56$) in subjects with schizophrenia whether or not they were being prescribed anticholinergic agents close to death.

The lack of change in density of [3H]NMS binding was significant because the selection of subjects with schizophrenia for this study was dictated by their levels of cortical

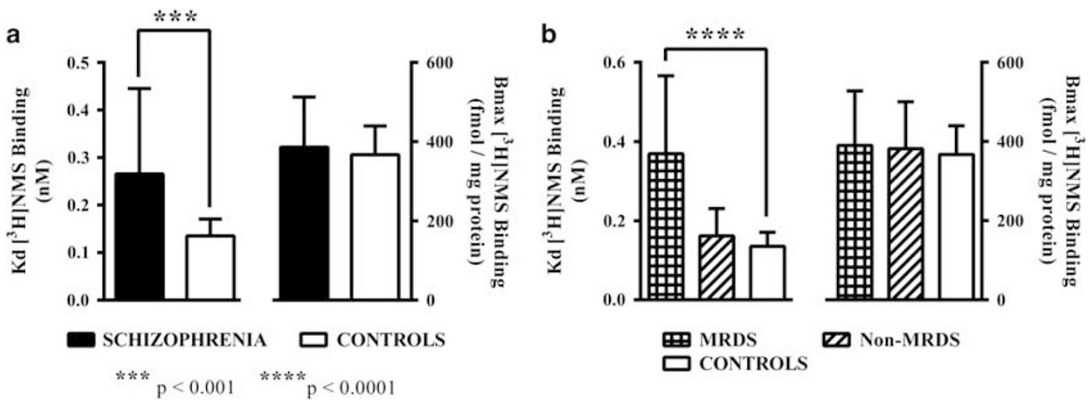



**Figure 2** The affinity (a) and density (b) of [3H]NMS to cortical membranes from subjects with schizophrenia and a deficit of cortical muscarinic receptors measured using [3H]pirenzepine binding (MRDS), subjects with schizophrenia without that deficit (Non-MRDS), and age- and sex-matched controls.

[$^3$H]pirenzepine binding in BA 6 (Seo *et al*, 2014) and thus, compared with control, [$^3$H]pirenzepine binding was lower in subjects with schizophrenia (mean ± SEM: schizophrenia = 47 ± 5.9 *vs* Controls = 98 ± 4.0 fmol/mg ETE, $p < 0.0001$; Cohen's $d = -1.631$). Levels of [$^3$H]pirenzepine were lowest in MRDS (mean ± SEM: 18 ± 4.1 fmol/mg ETE, $p < 0.0001$; Cohen's d = − 4.444) but binding in non-MRDS (mean ± SEM: 79 ± 5.2 fmol/mg ETE, $p < 0.01$; Cohen's $d = -0.945$) was lower than controls.

## Ion Dependency of Radioligand Binding

We postulated the different outcomes from using [$^3$H]pirenzepine and [$^3$H]NMS binding in BA 6 could have been due to variation in binding characteristics of the radioligands. Pursuing this hypothesis, we showed that although maximal [$^3$H]NMS binding was not $Mg^{2+}$ dependent (Figure 4a) the ability of acetylcholine to displace [$^3$H]NMS binding ($logIC_{50}$: 0 mg/ml $Mg^{2+} = -4.091$ *vs* 10 mg/ml $Mg^{2+} = -4.436$) and to enhance BQCA-mediated acetylcholine displacement of [$^3$H]NMS binding ($\Delta logIC_{50}$: 0 mg/ml $Mg^{2+} = -0.193$ *vs* 10 mg/ml $Mg^{2+} = -1.082$) was $Mg^{2+}$ dependent.

Exploring the effects of bivalent cations further, we showed that $Zn^{2+}$ reduced total [$^3$H]NMS binding independent of BQCA levels (Figure 4a). In contrast, the ability of acetylcholine to displace [$^3$H]NMS binding was enhanced by $Zn^{2+}$ ($logIC_{50}$: 0 mg/ml $Zn^{2+} = -4.486$ *vs* 10 mg/ml $Zn^{2+} = -7.294$). Moreover, BQCA-enhanced acetylcholine displacement of [$^3$H]NMS binding was greater in the presence of $Zn^{2+}$ ($\Delta logIC_{50}$: 0 mg/ml $Zn^{2+} = -0.227$ *vs* 10 mg/ml $Zn^{2+} = -1.694$).

We showed that $Zn^{2+}$, but not $Mg^{2+}$, reduced the specific maximal binding of [$^3$H]pirenzepine independent of BQCA (Figure 4b). In addition, acetylcholine displacement of [$^3$H]pirenzepine binding was slightly less potent in the presence of $Mg^{2+}$ ($logIC_{50}$: 0 mg/ml $Mg^{2+}$ or $Zn^{2+} = -4.656$ *vs* 10 mg/ml $Mg^{2+} = -4.380$) but was enhanced by $Zn^{2+}$ (10 mg/ml $Zn^{2+} = -5.029$). In contrast, BQCA-enhanced acetylcholine displacement of [$^3$H]pirenzepine binding was not affected by $Mg^{2+}$ ($\Delta logIC_{50}$: 0 mg/ml $Mg^{2+}$ or $Zn^{2+} = -0.227$ *vs* 10 mg/ml $Mg^{2+} = -0.256$) or $Zn^{2+}$ (10 mg/ml $Zn^{2+} = -0.229$).

Here we report that BQCA modulates the ability of acetylcholine to displace [$^3$H]NMS bound to human cortical membranes, and this is significant because we have shown that not all findings with cloned receptors can be confirmed using human cortical membranes (Dean *et al*, 2006). BQCA has been shown to be highly selective for the allosteric site on the CHRM1 (Ma *et al*, 2009), one of the five CHRMs in human CNS (Wess *et al*, 2003). Therefore, the ability of BQCA to modulate the effects of acetylcholine on [$^3$H]NMS binding to cortical membranes is likely mediated by CHRM1. Moreover, we have shown the Area BQCA Curves

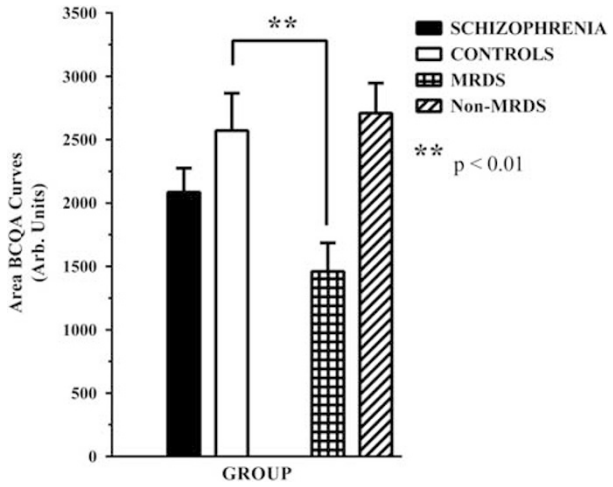



**Figure 3** The area between the BQCA-shifted [$^3$H]NMS binding curves (mean ± SEM) using cortical membranes from subjects with schizophrenia, subjects with schizophrenia and a deficit of cortical muscarinic receptors measured using [$^3$H]pirenzepine binding (MRDS), subjects with schizophrenia without that deficit (Non-MRDS), and age- and sex-matched controls.

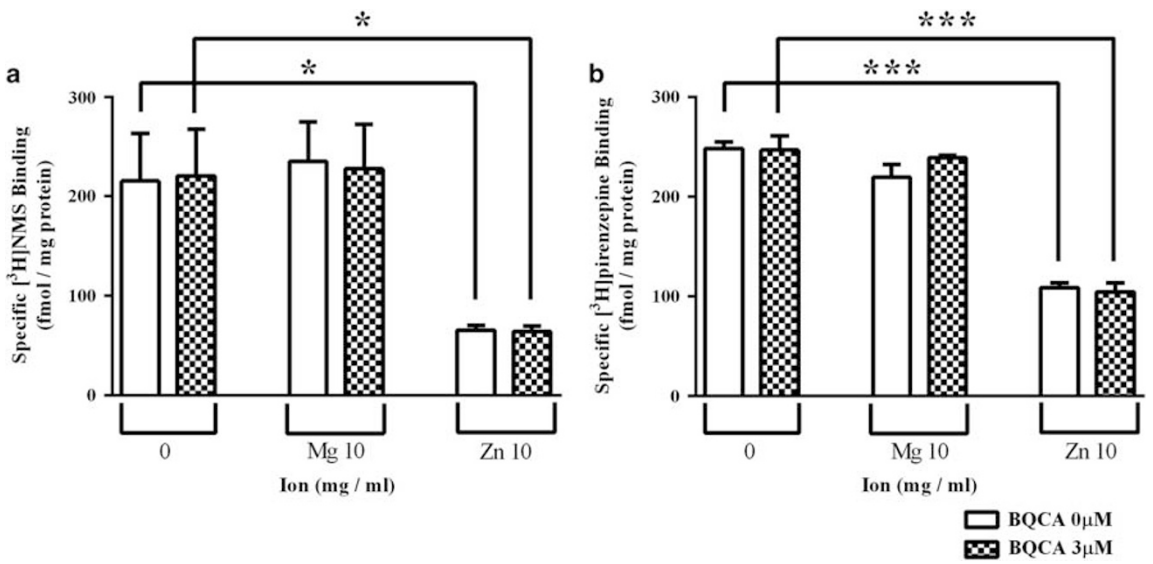



**Figure 4** The specific binding of [$^3$H]NMS (a) or [$^3$H]pirenzepine (b) in the absence of acetylcholine but in the presence of 10 mg/ml Mg or Zn. *$P < 0.05$; ***$p < 0.001$.

strongly correlates with the amount of cortical membrane available for radioligand binding, suggesting this measure would appear to be a novel and specific measure of cortical CHRM1 levels.

In addition to measuring the effects of BQCA Curves we showed that there was no change in the density of [3H]NMS binding in the cortex of subjects with schizophrenia. These data agree with a study using another pan-muscarinic receptor antagonist, [3H]QNB, that reported no change in muscarinic receptor density in medial frontal cortex from subjects with schizophrenia (Watanabe *et al*, 1983); this study did report increased radioligand binding in the orbitofrontal cortex from subjects with the disorder. Our data on [3H]NMS binding do not agree with the majority of studies on [3H]pirenzepine binding that report lower CHRMs in the cortex of subjects with schizophrenia (Crook *et al*, 2001; Dean *et al*, 2002, 2008; Deng and Huang, 2005; Gibbons *et al*, 2012; Newell *et al*, 2007; Scarr *et al*, 2009, 2013; Zavitsanou *et al*, 2004) but agrees with a study that showed no change in the levels of binding of that radioligand in the cortex of subjects with the disorder (Matsumoto *et al*, 2005). The decrease in the affinity of [3H]NMS binding to the cortex of subjects with schizophrenia agrees with findings using [3H]QNB (Watanabe *et al*, 1983) and [3H]pirenzepine (Dean *et al*, 2008). It would seem likely that these variable outcomes could be associated with the different binding properties of radioligands used to study CHRMs (Gillard *et al*, 1986). However, the data from the study of [3H]pirenzepine binding may better reflect the status of CHRM1 because the levels of that protein (Dean *et al*, 2002), but not CHRM2, CHRM3, or CHRM4 proteins (Dean *et al*, 2002; Scarr *et al*, 2006), have been shown to be decreased in the cortex of subjects with schizophrenia.

We previously reported a subgroup (25%: MRDS) of people with schizophrenia who have a marked loss of [3H]pirenzepine binding in BA 9 (Scarr *et al*, 2009). In this study we used tissue from subjects with MRDS as well as non-MRDS (Scarr *et al*, 2009, 2013) and age- and sex-matched controls. This study design was fortuitous because we showed the decrease in the affinity of [3H]NMS binding was limited to tissue from subjects with MRDS.

Our study design has allowed us to show that Area BQCA Curves was decreased in MRDS, a measure that we and others (Abdul-Ridha *et al*, 2014) propose is mediated by CHRM1. We have also shown that, under the conditions we measured [3H]pirenzepine binding, the radioligand is selective for the CHRM1 (Gibbons *et al*, 2012; Scarr and Dean, 2008). Thus, two lines of evidence argue for markedly lower cortical CHRM1 in MRDS. This may have ramifications for using CHRM1 allosteric modulators to alleviate the symptoms associated with schizophrenia (Melancon *et al*, 2013), as some people with schizophrenia may not have enough CHRM1 to respond to such therapies. However, studies in rodents suggest that receptor reserves for the CHRM1 are as high as 85% (Porter *et al*, 2002), and if this proves true in the human cortex, then our observation that subjects with MRDS retain ~25% of the CHRM1 compared with controls suggests they will respond to CHRM1 allosteric modulators.

In addition, we showed that total [3H]NMS and [3H]pirenzepine binding were not dependent on $Mg^{2+}$. However, the ability of acetylcholine to displace [3H]NMS was enhanced by $Mg^{2+}$ but its ability to displace [3H]pirenzepine was reduced by that ion. This is consistent with $Mg^{2+}$ affecting the availability of the agonist orthosteric binding site on CHRMs in a way that discriminates between the ability of acetylcholine to displace [3H]NMS and [3H]pirenzepine. In contrast, $Zn^{2+}$ decreased total [3H]NMS and [3H]pirenzepine binding, suggesting it affects the availability of the orthosteric site to a broader range of antagonist. Moreover, unlike $Mg^{2+}$, $Zn^{2+}$ enhanced the ability of acetylcholine to displace both [3H]NMS and [3H]pirenzepine. These data highlight the complexities of radioligand binding to CHRMs and increase the likelihood that the use of different radioligands is contributing to different outcomes in schizophrenia.

With regard to BQCA, and hence CHRM1, we showed that the ability of acetylcholine to displace [3H]NMS, but not [3H]pirenzepine, was enhanced by both $Mg^{2+}$ and $Zn^{2+}$. These data may indicate that [3H]NMS, but not [3H]pirenzepine, binds to a site on the CHRM1 that is directly modulated by the effects of the allosteric modulation or is closer to the orthosteric agonist site, hence allowing increased displacement when acetylcholine increasingly occupies that site due to the allosteric modulation of CHRM1. These issues may be addressed once the structure of the CHRM1 is elucidated.

As with any study on schizophrenia, this study has the potential confound of the effects of treatment but the changes in [3H]NMS binding and Area BQCA Curves are restricted to subjects with MRDS. According to their medical records, these subjects do not appear to have been treated in a distinct manner to non-MRDS, who do not have changes in any measured parameter. This argues against the changes we report using cortical tissue are due to a straightforward drug effect. In addition, treating rats with antipsychotic drugs (Crook *et al*, 2001; McLeod *et al*, 2010; Terry *et al*, 2006; Zavitsanou *et al*, 2007), the anticholinergic drug benztropine (Crook *et al*, 2001) and benzodiazepines (McLeod *et al*, 2010), or a combination of antipsychotic drugs and benzodiazepines (McLeod *et al*, 2010), does not affect levels of cortical [3H]pirenzepine binding. In addition, antipsychotic drug treatment has been reported not to change the affinity or density of [3H]QNB binding to rat cortex (Boyson *et al*, 1988; Hietala *et al*, 1989). Thus, it would seem unlikely that the changes in [3H]NMS or Area BQCA Curves in MRDS are simply because of drug treatments before death.

In conclusion, we have shown that the ability of BQCA to modulate the displacement of [3H]NMS by acetylcholine is reduced in MRDS, reflecting a decrease in cortical CHRM1 in these subjects. CHRM1 has been shown to play an important role in cognition and allosteric modulators for this receptor have been shown to affect cognition in rodents, primates (Uslaner *et al*, 2013), and humans (Nathan *et al*, 2013). Although these data add weight to the argument that CHRM1 allosteric modulators could improve the cognitive deficits associated with schizophrenia (Melancon *et al*, 2013), our data suggest that such an outcome may be absent in MRDS because CHRM1 levels are too low to allow a full response to CHRM1 allosteric modulators. One provisor on this posit being that, if the high levels of CHRM1 reserves present in rodent cortex (Porter *et al*, 2002) are present in human cortex, the 25% of receptors remaining in the cortex of subjects with MRDS may allow an acceptable therapeutic response to allosteric modulation. However, our data would

argue that carefully constructed drug trials using CHRM1 imaging, to identify subjects with low CHRM1, may be required to ensure the benefits of CHRM1 allosteric modulation in schizophrenia are not masked because of the inclusion of subjects with low cortical CHRM1.

## FUNDING AND DISCLOSURE

BD, SH and ES declare no conflict of interest. PJC receives or has received research support in the past 3 years from NINDS, Bristol Myers-Squibb, Johnson and Johnson, AstraZeneca, Michael J Fox Foundation, Thome Foundation, and the Dystonia Foundation. PJC is an inventor on patents that protect different classes of M1 PAMs. He has consulted over the past 3 years for Pfizer and received compensation. Over the past 3 years, he has served on the Scientific Advisory Boards of Seaside Therapeutics, Michael J Fox Foundation, Stanley Center for Psychiatric Research Broad Institute (MIT/Harvard), Karuna Pharmaceuticals, Lieber Institute for Brain Development Johns Hopkins University, Clinical Mechanism (POCM) and Proof of Concept (POC) Consortium, Brain and Behavior Research Foundation, and Neurobiology Foundation for Schizophrenia and Bipolar Disorder.

## ACKNOWLEDGMENTS

We acknowledge Mr Geoffrey Pavey for his contribution to processing human tissue for our studies. BD is the recipient of an NHMRC Fellowship (APP1002240). ES was supported by the Australian Research Council (Future Fellowship FT100100689). SH is the recipient of an Australian Postgraduate Award. PJC receives research support for this work from National Institute of Mental Health (grants U01 MH087965, R01 MH073676, and 2RO1 MH082867). These studies were funded in part by NHMRC Project Grant APP1045619, the CRC for Mental Health, the Rebecca Cooper Medical Research Foundation, and the Victorian Government's Operational Infrastructure Support. The Victorian Brain Bank Network is supported by the Florey Institute for Neuroscience and Mental Health, the Alfred Hospital, the Victorian Forensic Institute of Medicine, the University of Melbourne, and funded by Australia's National Health & Medical Research Council, Helen Macpherson Smith Trust, Parkinson's Victoria, and Perpetual Philanthropic Services.

## REFERENCES

Abdul-Ridha A, Lane JR, Mistry SN, Lopez L, Sexton PM, Scammells PJ *et al* (2014). Mechanistic insights into allosteric structure-function relationships at the M1 muscarinic acetylcholine receptor. *J Biol Chem* **289**: 33701–33711.

Barakat A, Dean B, Scarr E, Evin G (2010). Decreased Neuregulin 1 C-terminal fragment in Brodmann's area 6 of patients with schizophrenia. *Schizophr Res* **124**: 200–207.

Bodick NC, Offen WW, Levey AI, Cutler NR, Gauthier SG, Satlin A *et al* (1997). Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. *Arch Neurol* **54**: 465–473.

Boyson SJ, McGonigle P, Luthin GR, Wolfe BB, Molinoff PB (1988). Effects of chronic administration of neuroleptic and anticholinergic agents on densities of D2 dopamine and muscarinic cholinergic receptors in rat striatum. *J Pharmacol Exp Ther* **244**: 987–993.

Bymaster FP, Felder C, Ahmed S, McKinzie D (2002). Muscarinic receptors as a target for drugs treating schizophrenia. *Curr Drug Tar CNS Neurolog Dis* **1**: 163–181.

Bymaster FP, McKinzie DL, Felder CC, Wess J (2003). Use of M1-M5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. *Neurochem Res* **28**: 437–442.

Cohen J (1988). *Statistical Power Analysis for the Behavioral Sciences*, Hillsdale: New Jersey.

Cook RD, Weisberg S (1999). *Applied Regression Including Computing and Graphics*. Wiley: Hoboken.

Crook JM, Tomaskovic-Crook E, Copolov DL, 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. *Am J Psychiatry* **158**: 918–925.

D'Agostino RB, Belanger A, D'Agostino RB Jr (1990). A suggestion for using powerful and informative tests of normality. *Am Stat* **44**: 316–321.

Dean B, McLeod M, Keriakous D, McKenzie J, Scarr E (2002). Decreased muscarinic(1) receptors in the dorsolateral prefrontal cortex of subjects with schizophrenia. *Mol Psychiatry* **7**: 1083–1091.

Dean B, Pavey G, Chai SY, Mendelsohn FAO (1999). The localisation and quantification of molecular changes in the human brain using in situ radioligand binding and autoradiography. In: Dean B, Kleinman JE, Hyde TM (eds). *Using CNS Tissue in Psychiatric Research: A Practical Guide*. Harwood Academic Press: Amsterdam, pp 67–83.

Dean B, Pavey G, Thomas D, Scarr E (2006). Cortical serotonin(7, 1D) and (1F) receptors: Effects of schizophrenia, suicide and antipsychotic drug treatment. *Schizophr Res* **88**: 265–274.

Dean B, Soulby A, Evin GM, Scarr E (2008). Levels of [(3)H] pirenzepine binding in Brodmann's area 6 from subjects with schizophrenia is not associated with changes in the transcription factor SP1 or BACE1. *Schizophr Res* **106**: 229–236.

Deng C, Huang XF (2005). Decreased density of muscarinic receptors in the superior temporal gyrus in schizophrenia. *J Neurosci Res* **81**: 883–890.

Garey LJ (1994). *Bordmann's 'Localisation in the Cerebral Cortex'*. Smith-Gordon: London.

Gibbons AS, Scarr E, Boer S, Money T, Jeon WJ, Felder C *et al* (2012). Widespread decreases in cortical muscarinic receptors in a subset of people with schizophrenia. *Int J Neuropsychopharmacol* **16**: 37–46.

Gillard M, Waelbroeck M, Christophe J (1986). In vitro effects of gallamine on dissociation kinetics of (3H)N-methylscopolamine and (3H)pirenzepine from rat brain muscarinic receptors. *J Recept Res* **6**: 47–61.

Hietala J, Syvalahti E, Roytta M (1989). Dopamine D2 and muscarinic receptor binding characteristics in rat brain after withdrawal of subchronic fluphenazine and sulpiride treatment. *Prog Neuropsychopharmacol Biol Psychiatry* **13**: 247–258.

Lazareno S, Birdsall NJ (1995). Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48**: 362–378.

Ma L, Seager MA, Wittmann M, Jacobson M, Bickel D, Burno M *et al* (2009). Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation. *Proc Natl Acad Sci USA* **106**: 15950–15955.

Mancama D, Arranz MJ, Landau S, Kerwin R (2003). Reduced expression of the muscarinic 1 receptor cortical subtype in schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* **119**: 2–6.

Matsumoto I, Inoue Y, Iwazaki T, Pavey G, Dean B (2005). 5-HT2A and muscarinic receptors in schizophrenia: a postmortem study. *Neurosci Lett* **379**: 164–168.

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

Melancon BJ, Tarr JC, Panarese JD, Wood MR, Lindsley CW (2013). Allosteric modulation of the M1 muscarinic acetylcholine receptor: improving cognition and a potential treatment for schizophrenia and Alzheimer's disease. *Drug Discov Today* **18**: 1185–1199.

Miller GA, Chapman JP (2001). Misunderstanding analysis of covariance. *J Abnorm Psychol* **110**: 40–48.

Mirza NR, Peters D, Sparks RG (2003). Xanomeline and the antipsychotic potential of muscarinic receptor subtype selective agonists. *CNS Drug Rev* **9**: 159–186.

Motulsky H (1999). *Analyzing Data with GraphPad Prism*. Intuitive Software for Science: San Diego, CA.

Nathan PJ, Watson J, Lund J, Davies CH, Peters G, Dodds CM *et al* (2013). The potent M(1) receptor allosteric agonist GSK1034702 improves episodic memory in humans in the nicotine abstinence model of cognitive dysfunction. *Int J Neuropsychopharmacol* **16**: 721–731.

Newell KA, Zavitsanou K, Jew SK, Huang XF (2007). Alterations of muscarinic and GABA receptor binding in the posterior cingulate cortex in schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry* **31**: 225–233.

Porter AC, Bymaster FP, DeLapp NW, Yamada M, Wess J, Hamilton SE *et al* (2002). M1 muscarinic receptor signaling in mouse hippocampus and cortex. *Brain Res* **944**: 82–89.

Raedler TJ, Bymaster FP, Tandon R, Copolov D, Dean B (2007). Towards a muscarinic hypothesis of schizophrenia. *Mol Psychiatry* **12**: 232–246.

Salah-Uddin H, Scarr E, Pavey G, Harris K, Hagan JJ, Dean B *et al* (2009). Altered M(1) muscarinic acetylcholine receptor (CHRM1)-Galpha(q/11) coupling in a schizophrenia endophenotype. *Neuropsychopharmacology* **34**: 2156–2166.

Scarr E, Cowie TF, Kanellakis S, Sundram S, Pantelis C, Dean B (2009). Decreased cortical muscarinic receptors define a subgroup of subjects with schizophrenia. *Mol Psychiatry* **14**: 1017–1023.

Scarr E, Craig JM, Cairns MJ, Seo MS, Galati JC, Beveridge NJ *et al* (2013). Decreased cortical muscarinic M1 receptors in schizophrenia are associated with changes in gene promoter methylation, mRNA and gene targeting microRNA. *Transl Psychiatry* **3**: e230.

Scarr E, Dean B (2008). Muscarinic receptors: do they have a role in the pathology and treatment of schizophrenia? *J Neurochem* **107**: 1188–1195.

Scarr E, Keriakous D, Crossland N, Dean B (2006). No change in cortical muscarinic M2, M3 receptors or [35S]GTPgammaS binding in schizophrenia. *Life Sci* **78**: 1231–1237.

Schwarz E, van Beveren NJ, Ramsey J, Leweke FM, Rothermundt M, Bogerts B *et al* (2013). Identification of subgroups of schizophrenia patients with changes in either immune or growth factor and hormonal pathways. *Schizophr Bull* **40**: 787–795.

Seo MS, Scarr E, Dean B (2014). An investigation of the factors that regulate muscarinic receptor expression in schizophrenia. *Schizophr Res* **158**: 247–254.

Shannon HE, Bymaster FP, Calligaro DO, Greenwood B, Mitch CH, Sawyer BD *et al* (1994). Xanomeline: a novel muscarinic receptor agonist with functional selectivity for M1 receptors. *J Pharmacol Exp Ther* **269**: 271–281.

Shekhar A, Potter WZ, Lightfoot J, Lienemann J, Dube S, Mallinckrodt C *et al* (2008). Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. *Am J Psychiatr* **165**: 1033–1039.

Shirey JK, Brady AE, Jones PJ, Davis AA, Bridges TM, Kennedy JP *et al* (2009). A selective allosteric potentiator of the M1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning. *J Neurosci* **29**: 14271–14286.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S *et al* (2002). Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* **71**: 877–892.

Terry AV Jr, Gearhart DA, Mahadik SP, Warsi S, Waller JL (2006). Chronic treatment with first or second generation antipsychotics in rodents: effects on high affinity nicotinic and muscarinic acetylcholine receptors in the brain. *Neuroscience* **140**: 1277–1287.

Uslaner JM, Eddins D, Puri V, Cannon CE, Sutcliffe J, Chew CS *et al* (2013). The muscarinic M1 receptor positive allosteric modulator PQCA improves cognitive measures in rat, cynomolgus macaque, and rhesus macaque. *Psychopharmacology (Berl)* **225**: 21–30.

Watanabe S, Nishikawa T, Takashima M, Toru M (1983). Increased muscarinic cholinergic receptors in prefrontal cortices of medicated schizophrenics. *Life Sci* **33**: 2187–2196.

Wess J, Duttaroy A, Gomeza J, Zhang W, Yamada M, Felder CC *et al* (2003). Muscarinic receptor subtypes mediating central and peripheral antinociception studied with muscarinic receptor knockout mice: a review. *Life Sci* **72**: 2047–2054.

Wess J, Eglen RM, Gautam D (2007). Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nat Rev Drug Discov* **6**: 721–733.

Woolley ML, Carter HJ, Gartlon JE, Watson JM, Dawson LA (2009). Attenuation of amphetamine-induced activity by the non-selective muscarinic receptor agonist, xanomeline, is absent in muscarinic M4 receptor knockout mice and attenuated in muscarinic M1 receptor knockout mice. *Eur J Pharmacol* **603**: 147–149.

Zavitsanou K, Katsifis A, Filomena M, Xu-Feng H (2004). Investigation of M1/M4 muscarinic receptors in the anterior cingulate cortex in schizophrenia, bipolar disorder, and major depression disorder. *Neuropsychopharmacology* **29**: 619–625.

Zavitsanou K, Nguyen VH, Han M, Huang XF (2007). Effects of typical and atypical antipsychotic drugs on rat brain muscarinic receptors. *Neurochem Res* **32**: 525–532.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)