Studies on the binding of [^{3}H]NMS in human cortex from subjects with schizophrenia and age sex matched controls: Different outcomes with the allosteric modulator BQCA.

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

[1]The Florey Institute for Neuroscience and Mental Health, Parkville, Victoria, Australia,

[2]The Department of Pharmacology, [4]The Department of Psychiatry, The University of Melbourne, Victoria, Australia.

[3]Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN, USA,

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

Telephone: 614-8344-3786; Email: brian.dean@florey.edu.au

Abstract

Introduction

Studies using postmortem CNS (see (Dean et al, 2003; Scarr and Dean, 2008) for reviews), and a neuroimaging study (Raedler et al, 2003), have reported lower levels of muscarinic receptors (CHRMs) in the cortex of people with schizophrenia (Sz). There are 5 CHRMs in human CNS (Bymaster *et al*, 2002), it is therefore significant that current data suggests it is levels of the muscarinic M1 receptor (CHRM1) that are lower in the cortex from people with Sz (Dean *et al*, 2002; Mancama *et al*, 2003; Scarr *et al*, 2006). The growing recognition that Sz is a syndrome (Schwarz *et al*, 2013) is relevant to our findings that lower levels of cortical CHRM1 are restricted to a sub-group of people with Sz who have widespread deficits in that receptor in their cortex (Gibbons et al, 2012; Scarr et al, 2009); raising the possibility low cortical CHRM1 defines a sub-group of subjects (muscarinic receptor deficit schizophrenia: MRDS) within the syndrome. The notion that MRDS may have a distinct aetiology is supported by data showing from these subjects have altered cortical 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) compared to that in other forms of Sz (Sz_{other}) and age / sex matched controls.

The growing body of data suggesting muscarinic receptors are altered in the CNS of people with Sz, along with data from a number of pre-clinical pharmacological studies, underpins the hypotheses that stimulating cortical CHRM1s would alleviate some symptoms of the disorder (Bymaster et al, 2002; Dean, 2004; Dean et al, 2003). This hypothesis gained support from the

finding that xanomeline, a CHRM1 / CHRM4 agonist, reduced the severity of positive, negative and cognitive deficit symptoms in a small group of people with treatment resistant Sz (Shekhar et al, 2008); outcomes not detected in the placebo group. Unfortunately, despite showing clinical promise for the treatment of Sz (Shekhar et al, 2008), the severe peripheral side effects of xanomeline (Bodick et al, 1997) in addition to its complex pharmacology (Shannon et al, 1994), metabolic instability and a high hepatic first pass effect (Mirza *et al*, 2003) prevented further clinical development of xanomeline . That being acknowledged it is of interest that pre-clinical findings using xanomeline in CHRM knock out mice suggested that the its effects on cognition, attention and learning were mediated through the CHRM1 (Bymaster et al, 2003) whilst the antipsychotic effects observe in people with Sz are likely mediated by CHRM4 indirectly modulating dopamine levels in the CNS (Tzavara *et al*, 2004; Woolley *et al*, 2009).

Despite being an attractive target for the treatment of Sz, the conserved amino-acid sequence around the orthosteric binding site across CHRMs (Wess et al, 2007) has thwarted attempts to synthesize compounds specific to the orthosteric site of individual CHRMs. The discovery of allosteric binding sites, that have not been conserved across all CHRMs (Lazareno and Birdsall, 1995), offered an alternate drug target site on the CHRM1. Subsequently, screening of over 1,000,000 compounds against human cloned receptors led to the identification of [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] (BQCA), which is a positive allosteric modulator at the CHRM1 (Ma et al, 2009). An allosteric modulator acts at a non-orthosteric site on a receptor to change the affinity of the orthosteric site for a ligand or increase the efficacy of the physiological response to the ligand, in the case of muscarinic receptors the endogenous ligand is acetylcholine (ACh). Subsequent studies using cloned human receptors,

behavioral studies in wild type and *Chrm*$^{-/-}$ mice, pharmacology at the rat Chrm1 and effects on rat behavior strongly suggest that BCQA is highly selective, if not specific, for the CHRM1 (Ma et al, 2009; Shirey et al, 2009). Importantly, a similar set of data suggested that N-desmethylclozapine, a biologically active metabolite of clozapine, was a CHRM1 selective allosteric agonist (Sur et al, 2003) but subsequent studies using human native CHRM1 showed that N-desmethylclozapine was an antagonist at that receptor (Thomas et al, 2010). This highlights the importance of showing that any drug retains its predicted pharmacology across species, particularly in human CNS in this instance.

Given the promising data on the CHRM1 specificity of BQCA we decided to determine if it acted as a positive allosteric modulator at that receptor in human cortex. A reliable method of quantifying the effect of BQCA at the CHRM1 is to measure the ability of the drug to decrease the concentration of ACh required to displace [^{3}H]n-methylscopolamine ([^{3}H]NMS) from the CHRM1 (Ma et al, 2009; Shirey et al, 2009). Hence, we determined if BCQA could decrease the concentration of ACh required to displace [^{3}H]NMS from human cortical membrane and whether this effect was proportional to the density of CHRM1 in the tissue. In addition, as [^{3}H]NMS binding has been reported to be higher in the cortex of people with Sz (Watanabe et al, 1983) we measured the binding parameters for that radioligand to determine if they were altered in the cortex of people with Sz. We then investigated whether the ability of BQCA to modulate ACh displacement of [^{3}H]NMS was altered in cortex from people with Sz; tissue was used from subjects with MRDS and Sz_{other} (Scarr et al, 2009). Finally, to allow us to better understand the potential impact of differing methods used for [^{3}H]NMS and [^{3}H]pirenzapine binding , we investigated whether the binding of these radioligands was ion dependent.

METHODS

Materials

[^{3}H] N-methyl scopolamine ([^{3}H] NMS) and UltimateGold® scintillation cocktail were purchased from PerkinElmer (Waltham, Massachusetts, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), acetylcholine chloride (ACh), dimethyl sulfoxide (DMSO), $MgCl_2$, NaCl and polyethylenimine (PEI) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Glass fibre (GF/B) filter paper was from GE Healthcare Life Sciences (Buckinghamshire, England). Benzyl quinolone carboxylic acid (BQCA) was a generous gift from Prof. P. Jeffery Conn (Vanderbilt University, Nashville, Tennessee, USA). The J2-HS centrifuge and JA 20.1 rotor were from Beckman Coulter Inc., Indianapolis, Indiana, USA.

Tissue Collection, Diagnostic Validation and Membrane Preparation

After obtaining approval from the Ethics Committee of the Victorian Institute of Forensic Medicine tissue was collected postmortem from subjects with a likely history of psychiatric illness and age /sex matched subjects who appeared to have no history of a psychiatric disorder. The left cerebral hemisphere was sliced according to a standardised protocol and then rapidly frozen to – 80^{o}C for storage until required (Dean et al, 1999).

For all subjects an extensive case history review was undertaken. For potential psychiatric cases, this involved a review of the donor's clinical history and, where possible, consultation with treating clinical staff and family interviews. For the non-psychiatric cases there was consultation with treating physician and family interviews to discover any potential episodes of psychiatric disorders, clinical histories were reviewed where available. In addition, non-psychiatric cases had blood toxicology screens that showed they were free of any prescription drugs at death. The presence of over the counter drugs such as aspirin or codeine were not an exclusion criteria. During examination of case histories any pharmacological treatments were noted.

All information was collated using the Diagnostic Instrument for Brain Studies (DIBS) which could then be used to arrive at a consensus diagnosis (Hill et al, 1996) according to DSM-IV criteria (Roberts et al, 1998) by 2 senior psychiatrists and a senior psychologist. In completing the DIBS, duration of illness (DI) was calculated as time from first contact with a psychiatric service to death. In addition, the final recorded dose of antipsychotic drug (FRADD) was recorded and converted to chlorpromazine equivalents (Foster, 1989; Woods, 2003) and total lifetime exposure to such drugs was estimated (LEAP) (Hashimoto et al, 2004). Similarly, the final recorded dose of benzodiazepine and anticholinergic drugs were recorded and converted to mg diazepam equivalents / day (FRBD) (Ashton, 2002) or mg benztropine equivalents / day (Minzenberg *et al*, 2004), respectively. Post-mortem interval was calculated in two ways; when death was witnessed, post-mortem interval (PMI) was calculated as the time from death to autopsy. Where death was not witnessed, tissue was only collected from subjects who had been

seen alive up to 5 hr. prior to being found dead; here the PMI was taken as the midpoint between the person being found and being last seen alive. Importantly, all cadavers were stored at 4^{o}C after reaching the VIFM; meaning they were at low temperatures for most of their PMI.

After autopsy, tissue was rapidly processed and frozen to -80^{o}C within 30 min, processing tissue in this way significantly slows autolytic changes (Ferrer et al, 2007). CNS pH was measured as this provides a good measure of overall tissue preservation for the study of the molecular cytoarchitecture of the CNS (Stan et al, 2006).

Following approval from the Tissue Access Committee of the Victorian Brain Bank Network tissue was obtained from Brodmann's area (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), the bases of superior and middle frontal gyrus and pre-central gyrus not included in BA 4 (Garey, 1994)) from 5 subjects for methodological optimisation. In addition, tissue was obtained from BA 6 from 40 subjects with Sz and 20 age sex matched controls (demographics summarized in Table 1, complete details in Supplementary Table 1) who had essentially been used in a previous study of [^{3}H]pirenzepine binding in that cortical region (Seo *et al*, 2014). The subjects with Sz were made up of 20 MRDS and 20 Sz_{other}.

Membrane Preparations for [^{3}H]NMS Binding

Membranes were prepared from each case using methodologies similar to that used for human cloned CHRMs (Shirey et al, 2009).Thus human cortices were thawed, on ice and homogenised into 10 vols of 20mM HEPES containing 100mM NaCl and 10mM MgCl2, pH7.4 (Assay Buffer) by ten strokes of a Glass/PTFE Potter Elvehjem tissue grinders. The resulting homogenates were centrifuged at 21,500 g for 20 minutes at 4^{o}C, after which the supernatant was discarded. The pellet was suspended in 10 vols. assay buffer and washed twice by repeating the centrifugation process. The washed membranes were stored at -80^{o}C until required, at which time their protein concentrations s were determined using the Biorad protein assay, adapted for the microplate, immediately prior to use.

[^{3}H]NMS binding: Methodological optimisation

[^{3}H]NMS binding: Binding parameters

The binding of [^{3}H]NMS, at a range of concentrations (0.01nM to 2nM), (triplicate) to washed human cortical membranes (n = 3; 0.1 mg protein) was measured in the absence (Total Binding: TB) and presence (Non-Specific Binding: NSB) of 1mM ACh in a final vol. of 1 ml made up with Assay Buffer. After incubating for 2 hr at room temperature (rt) 5 ml of ice cold Assay Buffer was added to each tube and the bound radioactivity separated by filtering the cell membrane onto a Whatman GF/B filter. Each filter was washed thrice with ice cold 0.9% saline and then placed into 5 ml of UltimateGold® scintillation cocktail and, after 1 hr., the

radioactivity on each filter paper was counted using a Tri-Carb 2910 TR (PerkinElmer). Radioactivity was corrected for radioactive decay and machine counting efficiency before being converted from counts per minute to disintegrations per minute (d.p.m). The specific activity of the radioligand was used to convert these data to fmol/mg protein and TB and NSB at each concentration of [^{3}H]NMS was used to generate a Scatchard analysis (GraphPad Prism 6.0) to calculate the affinity (Kd) and maximal binding (Bmax) of [^{3}H]NMS to each membrane.

[^{3}H] NMS Binding: Effects of acetylcholine and BQCA

The ability of ACh (0 to 10^{-1}M) to displace [^{3}H]NMS (0.13 nM) from washed cortical membranes (n = 3; 0.1 mg protein) from 3 cases made to a final volume of 1 ml with Assay Buffer was measured after incubating for 2 hr at rt. Bound and free radioligand were separated as described previously. Subsequently, the binding of [^{3}H]NMS (n = 3; 0.13 nM) in the absence or presence of BQCA (0.3µM and 3µM dissolved in 0.1% DMSO) at a range of ACh concentrations (0 to 10^{-3}M) made up to 1 ml made up with Assay Buffer was measured (N.B. 0.1% DMSO meant final concentration of 0.01% in Assay Buffer; at this concentration DMSO did not affect [^{3}H]NMS binding (data not shown)). Finally, [^{3}H]NMS binding (n = 3; 0.13 nM) in the absence and presence of BQCA (3µM) and ACh (0 to 10^{-3}M) was measured, in triplicate, to washed cortical membrane at varying protein concentrations (0.01 to 0.10 mg / ml protein) made up to a final volume of 1 ml with Assay Buffer.

From these experiments, two binding parameters could be measured which were the change in Δ logEC50 for the displacement of [^{3}H]NMS by ACh at different concentrations of BCQA and the area encompassed between the ACh displaceable [^{3}H]NMS binding curves at two concentrations of BCQA (Area BCQA Curves: See below). The Δ logEC50 was determined by first calculating the [^{3}H]NMS bound at each concentration of ACh (B) as a percent of that bound in the absence of ACh (B0) in the absence and presence of BQCA.

Studies in Schizophrenia

Using the described methodology, [^{3}H]NMS binding parameters for human cortical membranes from BA 6 from the 40 subjects with Sz and the 20 age and sex marched controls were measured. In addition, the Δ logEC50 and the Area BCQA Curves were measured for all cases.

Statistical analysis

There has been growing concern over the focus on p values alone as an interpretation of significance (Hulme *et al*, 1983) and it has been long recognized that determining data distribution on small sample sizes is extremely difficult (D'Agostino et al, 1990). We have attempted to minimise the impact of these two issues on our data analyses by first accepting that most biological variables tend to be normally distributed (McKillup, 2006) and thus all statistical analyses were completed using parametric analyses. In addition, to give an indication of effect

size, Cohen's D values (Cohen, 1988) are provided where appropriate. In assessing relationships between variables when using small sample sizes there needs to be a strong relationship between variables that is apparent using linear regression analyses. It has been shown that in small sample sizes an $r^2 > 0.49$ is required to reflect a strong relationship between two variables (Cook and Weisberg, 1999) and only where such a strong relationship exists should the variables be considered covariates and the potential impact of such a relationship be investigated. 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 a 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 a Fisher's exact test. Variation in experimental data was assessed using student t-tests or one-way ANOVA followed by a post hoc Dunnett's comparison to 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 non-experimental data with group (Miller and Chapman, 2001), the variation in the experimental data was re-assessed using the ANCOVA with the appropriate non-experimental data as a covariant(s) using Minitab 15. Data is presented as mean ± SEM unless otherwise stated.

RESULTS

[^{3}H]NMS binding: Methodological optimisation

[^{3}H]NMS binding to human cortical membranes from 3 cases was saturable with a Kd = 0.13 ± 0.02 nM and a Bmax = 342 ± 85 fmol / mg protein (Figure 1A). For subsequent analyses of the effects of BQCA the concentration of [^{3}H]NMS was standardized to 0.13 nM.

[^{3}H]NMS binding to human cortical membranes from the same 3 cases could be displaced by increasing concentrations of ACh (Figure 1B) with non-specific binding being obtained in the presence of 10^{-3}M ACh. Moreover, the ability of ACh (0 to to 10^{-3}M) displace [^{3}H]NMS binding to human cortical membranes increased with increasing concentrations of BQCA ($logIC_{50}$: 0 BQCA = - 4.95 M, 0.3µM BQCA = -5.50 M and 3.0 µM BQCA = -6.21 M: Figure 1C). The effects of BQCA in the absence or presence of 3.0 µM BQCA was measured by quantifying either the Δ $logIC_{50}$ BQCA or Area BCQA Curves (Figure 1D). To measure Area BCQA Curves, the difference between [^{3}H]NMS binding in the absence or presence of BQCA at each concentration of ACh was plotted against the concentration of ACh, the area under that curve was calculated after correcting for any variation in baseline (Figure 1E). To determine if either measure related to levels of cortical CHRM1 we then measured the Δ $logIC_{50}$ and Area BCQA Curves at varying concentrations of protein. Significantly, there was no strong relationship between Δ $logIC_{50}$ BQCA and level of protein added to each assay tube (r^2 from 0.04 to 0.92, p from 0.18 to 0.88: Figure 1F). By contrast, Area BCQA Curves consistently correlated with level of protein added (r^2 from 0.997 to 0.999, p from 0.01 to 0.03: Figure 1G). Moreover, this relationship was retained when an increased number of membrane protein dilutions were used to

challenge the validity of using a linear regression to describe the relationship (r^2 from 0.979 to 0.992, p all < 0.001: Figure 1H).

Studies on Schizophrenia

There were no significant differences in sex ratio, age, PMI or CNS pH between the subjects with Sz and the age / sex matched controls (Table 1: Supplementary Table 1). As there were no suicide completers in the control group, the frequency of suicide completion was significantly higher in subjects with Sz. There were no significant differences in sex ratio, age, PMI or CNS pH between MRDS, Sz_{other} and controls. There were no differences in FRADD, LEAP, FRBD, or FRAcD between MRDS and controls. The frequency of suicide completion was higher in Sz_{other} compared to 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 SZ_{other}.

The affinity (p = 0.002), but not density (p = 0.54), of [^{3}H]NMS binding varied with diagnosis (Figure 2A) with binding affinity being lower in Sz (Kd (nM:; Sz = 0.26 ± 0.02 vs. Controls = 0.13 ± 0.007; Cohen's d = 0.87; Figure 2A). When the subjects with Sz were divided into MRDS and Sz_{other} there was a significant variation in the affinity ($F_{d.f.} = 22_{2,57}$, p < 0.0001), but not density ($F_{d.f.} = 22_{2,57}$, p = 0.81), of [^{3}H]NMS binding with group (Figure 2B). Post-hoc

analyses showed this variation was due to the affinity of [^{3}H]NMS binding being lower in tissue from subjects with MRDS compared to controls ($p < 0.0001$; Cohen's $d = 1.66$).

There was no significant difference in Area BCQA Curves between Sz and controls ($p = 0.16$; Figure 3). However, when comparing MRDS, Sz_{other} and controls, there was significant variation in Area BCQA Curves with group ($F_{d.f.} = 7.28_{2,57}$, $p = p < 0.002$). Post hoc analyses showed this variation was due to lower Area BCQA Curves in subjects with MRDS (1460 ± 225 Arbitary (Arb) Units), but not Sz_{other} (2710 ± 236 Arb Units), compared to controls (2572 ± 295 Arb Units; $p < 0.01$; Cohen's $d = -0.948$).

There was a strong relationship between the affinity for [^{3}H]NMS binding and the density of [^{3}H]pirenzepine binding in human cortex. There were no other strong correlations between parameters for radioligand binding to human cortex (Supplementary Table 2). There is no clear explanation as to why there could be a relationship between the affinity of binding of one radioligand to the density of binding of another, 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 ($p = 0.82$) and density ($p= 0.13$) of [^{3}H]NMS binding nor the Area BCQA Curves ($p = 0.55$) varied with gender (Supplementary Table 3B). Nor did the affinity ($p = 0.11$), density ($p= 0.19$) of

[^{3}H]NMS binding nor Area BCQA Curves (p = 0.47) differ between suicide completers and those who died by other causes (Supplementary Table 3C). These data show that the different rates of suicide completion in MRDS versus SZ_{other} was not a significant confound for this study. There was no significant differences in the affinity (p = 0.92) or density (p = 0.07) of [^{3}H]NMS binding or Area BCQA Curves (p = 0.56) in subjects with SZ whether or not they were being prescribed anticholinergic agents close to death.

Ion Dependency of Radioligand Binding

The selection of subjects with Sz for this study was dictated by their levels of cortical [^{3}H]pirenzepine binding in BA 6 (Seo *et al*, 2014). This meant, compared to control, the binding of that radioligand was lower in subjects with Sz (mean ± SEM: Sz = 47 ± 5.9 vs. Controls = 98 ± 4.0 fmol / mg ETE, p <0.0001; Cohen's d = -1.631). In addition, whilst 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), the binding of the radioligand was also lower in Sz_{other} (mean ± SEM: 79 ± 5.2 fmol / mg ETE, p <0.01; Cohen's d = -0.945) compared to controls. By contrast, in this study [^{3}H]NMS binding to tissue from the same subjects did not vary in Sz, MRDS or Sz_{other} whilst the Area BCQA Curves was lower in MRDS. Here we showed that the ability of ACh 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 ACh displacement of [^{3}H]NMS binding was Mg^{2+} dependent ($\Delta logIC_{50}$: 0 mg / ml Mg^{2+} = -0.193 vs. 10 mg / ml Mg^{2+} -1.082) and that these changes occurred without changing maximal specific binding of [^{3}H]NMS binding to cortical membranes

(Figure 4A). These data were significant as in our studies [^{3}H]NMS, but not [^{3}H]pirenzepine, were measured in the presence of Mg^{2+}.

In a further series of experiments, we confirmed that the ability of ACh to displace [^{3}H]NMS binding was enhanced by both Mg^{2+} and Zn^{2+} ($logIC_{50}$: 0 mg/ml Mg^{2+} or Zn^{2+} = -4.486 vs. 10 mg / ml Mg^{2+} = -5.688 vs. 10 mg / ml Zn^{2+} = -7.294). Significantly, the effects of Mg^{2+} and Zn^{2+} differed because Zn^{2+}, but not Mg^{2+}, reduced the maximal specific binding of [^{3}H]NMS binding to cortical membranes (Figure 4B). BQCA enhancement of ACh displacement of [^{3}H]NMS binding was greater in the presence of both Mg^{2+} and Zn^{2+} ($\Delta logIC_{50}$: 0 mg / ml Mg^{2+} or Zn^{2+} = -0.227 vs. 10 mg / ml Mg^{2+} -2.009 vs. 10 mg / ml Zn^{2+} = -1.694). Finally, we showed that the ability of ACh to displace [^{3}H]pirenzepine binding was reduced 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 enhanced by Zn^{2+} (10 mg / ml Zn^{2+} = -5.029). Zn^{2+}, but not Mg^{2+}, reduced the specific maximal binding of [^{3}H]pirenzepine (Figure 4C). BQCA enhanced ACh displacement of [^{3}H]pirenzepine binding was not affected by Mg^{2+} or Zn^{2+} ($\Delta logIC_{50}$: 0 mg / ml Mg^{2+} or Zn^{2+} = -0.227 vs. 10 mg / ml Mg^{2+} -0.256 vs. 10 mg / ml Zn^{2+} = -0.229).

Discussions

Here we report that, as shown with cloned CHRM1s (Shirey *et al*, 2009), BQCA modulates the ability of ACh to displace [^{3}H]NMS bound to human cortical membranes. BQCA has been

shown to be highly selective for the allosteric site on the CHRM1 (Ma *et al*, 2009), one of 5 CHRMs in human CNS (Wess *et al*, 2003). Therefore the ability of BQCA to modulate the effects of ACh on [^{3}H]NMS binding to cortical membranes would probably be mediated by CHRM1. We also showed that the ability of BQCA to alter ACh displacement of [^{3}H]NMS is strongly correlated to the amount of cortical membrane available for radioligand binding. As the amount of cortical membrane is a surrogate measure of the amount of available CHRM1, we argue that the effect of BQCA on the ability of ACh to displace [^{3}H]NMS is related to the level of CHRM1 in human cortex.

As the read out of the effects of BQCA in the human cortex involved [^{3}H]NMS binding, we measured [^{3}H]NMS binding in the cortex from subjects with schizophrenia and the controls; there was no change in the density of [^{3}H]NMS binding in the cortex of subjects with schizophrenia. These data agree with those from a study of [^{3}H]QNB binding that reported no change in muscarinic receptors in medial frontal cortex from subjects with schizophrenia (Watanabe *et al*, 1983), although that study did report an increase in radioligand binding in the orbito-frontal cortex from subjects with schizophrenia. Our data on [^{3}H]NMS binding disagrees with the majority of studies on [^{3}H]pirenzepine binding which report decreased levels of muscarinic receptors in the cortex of subjects with schizophrenia (Crook *et al*, 2001; Dean *et al*, 2002; Dean *et al*, 2008; Deng and Huang, 2005; Gibbons *et al*, 2012; Newell *et al*, 2007; Scarr *et al*, 2009; Scarr *et al*, 2013; Zavitsanou *et al*, 2004) but agrees with one 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). There was a decrease in the affinity of [^{3}H]NMS binding to the cortex of subjects with schizophrenia, a finding that has been reported for both [^{3}H]QNB (Watanabe *et*

al, 1983) and [^{3}H]pirenzepine (Dean *et al*, 2008) binding. These data raise the possibility that changes to the orthosteric site of CHRMs in the cortex from subjects with SZ is affecting their affinity for CHRM antagonists. Although it is also possible that the different findings on the maximal binding of [^{3}H]NMS and [^{3}H]pirenzepine to cortical membranes from subjects with SZ could be due to the different binding properties of these 2 radioligands for CHRMs (Gillard *et al*, 1986).

We previously reported that it was possible to delineate a sub-group (25%: MRDS) of people with schizophrenia because they had a marked loss of [^{3}H]pirenzepine binding in Brodmann's area (BA) 9 (Scarr *et al*, 2009). In this study we used tissue from subjects MRDS who had a marked loss of [^{3}H]pirenzepine binding in BA 6 as well as BA 9, subjects with schizophrenia that had a small but significant loss of radioligand binding in both CNS regions and age /sex matched controls (Scarr *et al*, 2009; Scarr *et al*, 2013). Using this approach we were able to show the decrease in the affinity of [^{3}H]NMS binding was limited to tissue from subjects with MRDS, further suggesting there might be lower levels of M1 receptors in this subgroup.

BQCA modulates the ability of ACh to displace [^{3}H]NMS binding site to CHRM1 (Abdul-Ridha *et al*, 2014) and in this study we showed this effect of BQCA was reduced in membranes from subjects with MRDS. We have shown that, under the conditions we measured [^{3}H]pirenzepine binding, the radioligand is selective for the CHRM1 (Gibbons *et al*, 2012; Scarr *et al*, 2008) and here we show that the ability of BQCA to modify ACh displacement of [^{3}H]NMS binding is likely related to the number of CHRM1 in the cortical tissue. Thus, the reduced ability of BQCA to modify ACh displacement of [^{3}H]NMS in the cortex of subjects with MRDS could be related

to lower levels of CHRM1 in their cortex. This may have ramifications for using CHRM1 allosteric modulators to alleviate the symptoms associated with the SZ (Melancon *et al*, 2013), as some people with SZ may not have enough CHRM1 to respond to such therapies. However, studies in rodents suggest receptor reserves for the CHRM1 are as high as 85% (Porter *et al*, 2002), if this proves true in the human cortex then our observation that those subjects with SZ with a marked loss of cortical CHRM1 retain around 25% of the CHRM1 in controls suggests they will respond to CHRM1 allosteric modulators.

The different outcomes from measuring [^{3}H]NMS and [^{3}H]pirenzepine binding is a confound we have investigated further. Here we show that the ability of BQCA to alter ACh displaceable [^{3}H]NMS binding is Mg^{2+} dependent, this ion has previously been reported to affect the agonist binding site on muscarinic receptors (Vickroy *et al*, 1984). These data raise the possibility that the BQCA-sensitive allosteric binding site on the CHRM1 may be particularly dependent on the presence of Mg^{2+}, a phenomenon already reported for an allosteric site on a pharmacologically defined muscarinic M2 receptor (Burgmer *et al*, 1998). Zn^{2+} is a known CNS active divalent cation (Marger *et al*, 2014) and here we show it reduces the binding of [^{3}H]NMS in the absence of acetylcholine, affects the ability of acetylcholine to displace [^{3}H]NMS and enhances the effects of BQCA. However, the effects of Zn^{2+} and Mg^{2+} on the cortical binding of radioligands is complex, we have shown that Zn^{2+} increased, whilst Mg^{2+} decreased, the ability of ACh to displace [^{3}H]pirenzepine. Furthermore, Zn^{2+}, but not Mg^{2+}, reduced the binding of [^{3}H]pirenzepine to cortical membranes in the absence of ACh. Finally, the ability of BQCA to enhance ACh to displace [^{3}H]pirenzepine from cortical membranes was shown not to be dependent on Mg^{2+} or Zn^{2+}. Overall, these data suggest that Zn^{2+} can affect the binding of

radioligands to the orthosteric site of at least the CHRM1, and possibly other CHRMs, on human cortical membrane. By contrast, Mg^{2+} but not Zn^{2+} can facilitate the binding of BQCA to the allosteric site on the muscarinic M1 receptor to facilitate a change in the efficacy of ACh binding to the orthosteric site .

As with any study on SZ this study has the potential confound of the effects of treatment on cortical cytoarchitecture. Significantly, in this study changes in [^{3}H]NMS binding and Area BCQA Curves are restricted to subjects with MRDS and according to their medical records, these subjects do not appear to have been treated in a distinct manner to $SZ_{other,}$ 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. We have shown that treating with antipsychotic drugs (Crook *et al*, 2001; McLeod *et al*, 2010), 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) do not affect levels of [^{3}H]pirenzepine binding in the rat cortex. Our data on antipsychotic drug treatments are in line with other studies that fail to show any major changes in levels of cortical [^{3}H]pirenzepine binding after drug treatment (Terry *et al*, 2006; Zavitsanou *et al*, 2007). In addition, antipsychotic drug treatment has been reported not to change the affinity or density of [^{3}H]QNB binding to rat cortex (Boyson *et al*, 1988; Hietala *et al*, 1989). One study has reported that although treatment with antipsychotic drugs can change in the levels of *Chrm1* expression in some regions of the rat CNS they do not affect cortical expression of that receptor (Han *et al*, 2008); these data are significant as *CHRM1* mRNA are reported as decreased in the cortex of subjects with SZ (Mancama *et al*, 2003). Thus, available data do not support the notion that changes in CHRM levels in the CNS of subjects with SZ are

likely to be due to drug treatment prior to death but at present there is no data as to whether treatment with such drugs may affect the ability of BQCA to modulate ACh displacement of [^{3}H]NMS.

In conclusion, we have shown that the ability of BQCA to modulate the displacement of [^{3}H]NMS by ACh is reduced in a sub-set of people with SZ who have a deficit in cortical CHRM1. We also present data that suggests that the ability of BQCA to modulate the displacement of [^{3}H]NMS by ACh in human cortex is likely related to prevailing levels of CHRM1. Therefore we posit our current data is supportive of the hypotheses that there is a sub-group of subjects with SZ that have a major deficit in cortical CHRM1. Notably, 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). 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 the response to modulation of the allosteric site on CHRM1 may be limited to a sub-group of people with SZ who have a major deficit in that receptor in their cortex (Scarr *et al*, 2009). If that proves to be the case, carefully constructed drug trials may be required to ensure that people with SZ who are non-responders to allosteric modulation at CHRM1 do not mask significant reductions in symptoms in those people who do show improvement on receiving such drugs.

Figure Legends:

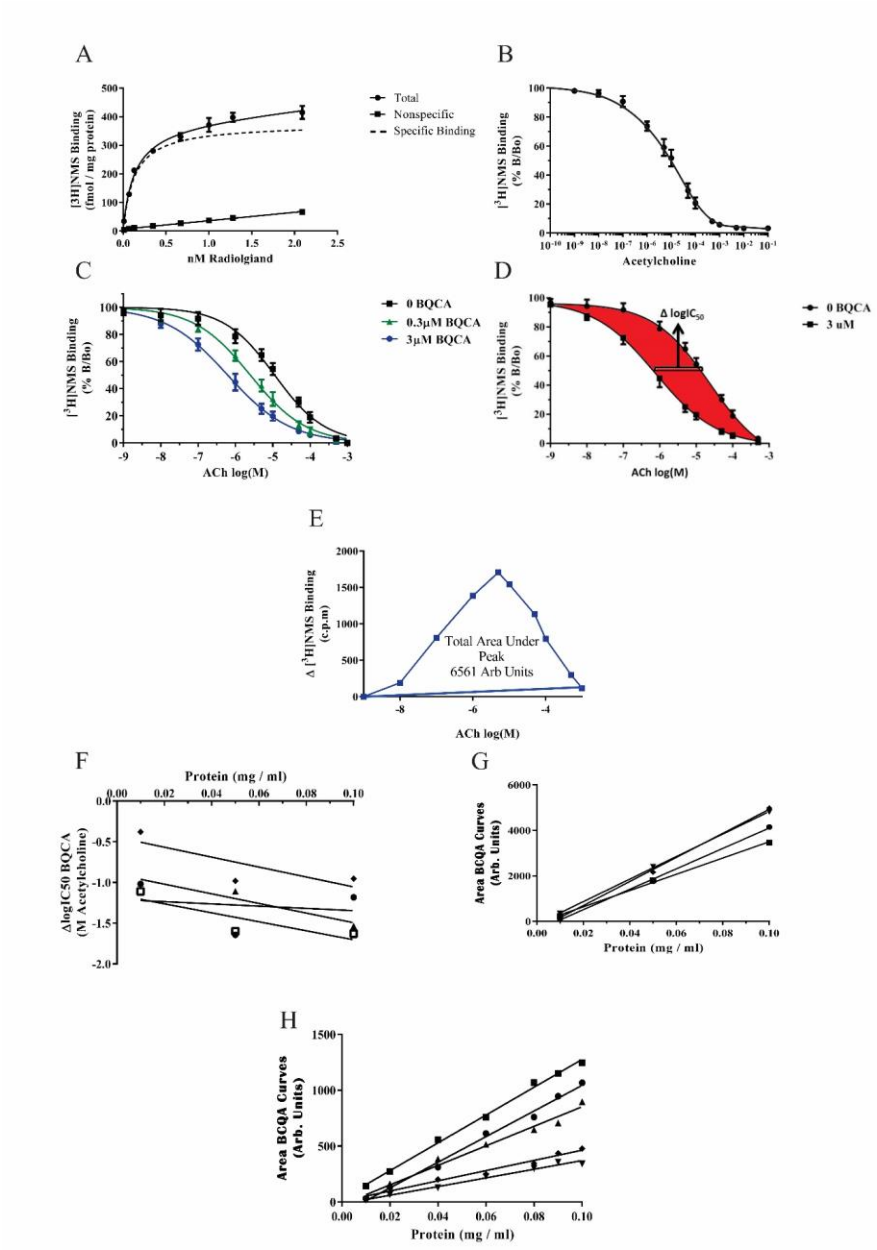

A
[3H]NMS Binding (fmol / mg protein)
nM Radiolgland
Total
Nonspecific
Specific Binding
B
[3H]NMS Binding (% B/Bo)
Acetylcholine
C
[3H]NMS Binding (% B/Bo)
ACh log(M)
0 BQCA
0.3μM BQCA
3μM BQCA
D
[3H]NMS Binding (% B/Bo)
ACh log(M)
Δ logIC50
0 BQCA
3 uM
E
Δ [3H]NMS Binding (c.p.m)
Total Area Under Peak
6561 Arb Units
ACh log(M)
F
Protein (mg / ml)
AlogIC50 BQCA (M Acetylcholine)
G
Area BCQA Curves (Arb. Units)
Protein (mg / ml)
H
Area BCQA Curves (Arb. Units)
Protein (mg / ml)

Figure 1A: The binding (mean ± SEM) of [^{3}H]NMS at varying concentrations in the absence (total binding: TB) or presence (non-specific binding (NSB) to cortical membranes from 3 subjects with no history of psychiatric illness. Specific binding (SB) was calculated as TB – NSB at each concentration of [^{3}H]NMS.

B: The binding (mean ± SEM) of [^{3}H]NMS (0.13 nM) to cortical membranes from 3 subjects no history of psychiatric illness in the presence of increasing concentrations of acetylcholine.

C: The binding (mean ± SEM) of [^{3}H]NMS (0.13 nM) to cortical membranes from 3 subjects 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).

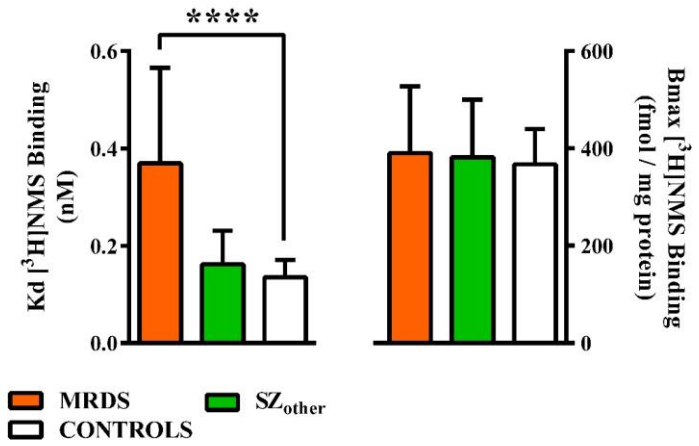
D: A schematic diagram showing the two possible measures of the effects of BQCA on the ability of increasing concentrations of acetylcholine to displace [^{3}H]NMS (0.13 nM). One measure is the difference between the logIC_{50} values for the displacement of [^{3}H]NMS by acetylcholine in the absence and presence of BQCA (ΔlogIC_{50}), the second is an integrated measure of the area within the ACh displacement curves in the absence or presence of 3µM BQCA.

E: An example of the area between the 2 displacement curves being derived as the area under the curve defined by the difference in [^{3}H]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 [^{3}H]NMS from cortical membranes from 3 individuals added at differing levels of protein.

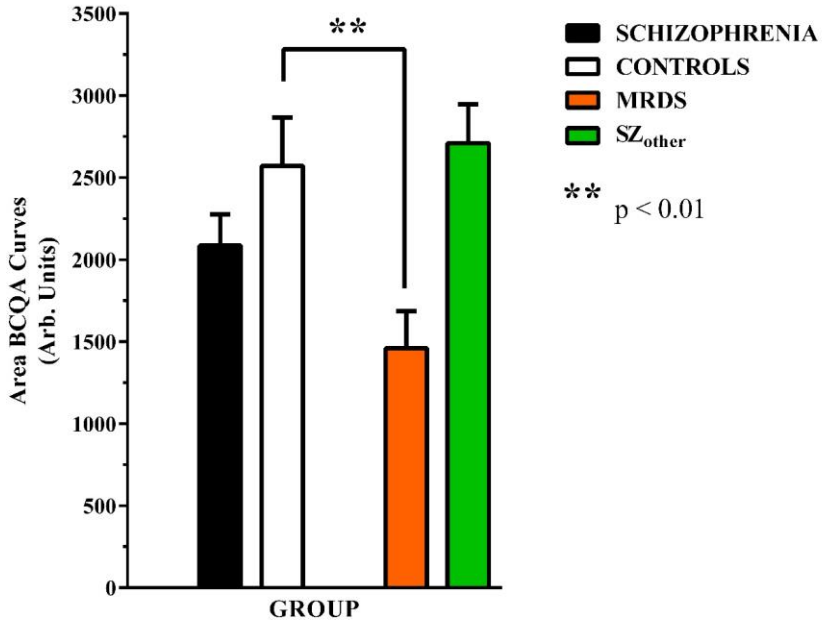
G: The relationship between Area BCQA Curves and cortical membranes from 3 individuals added at differing levels of protein.

H: The relationship between Area BCQA Curves for cortical membranes from 3 individuals added at differing levels of protein.



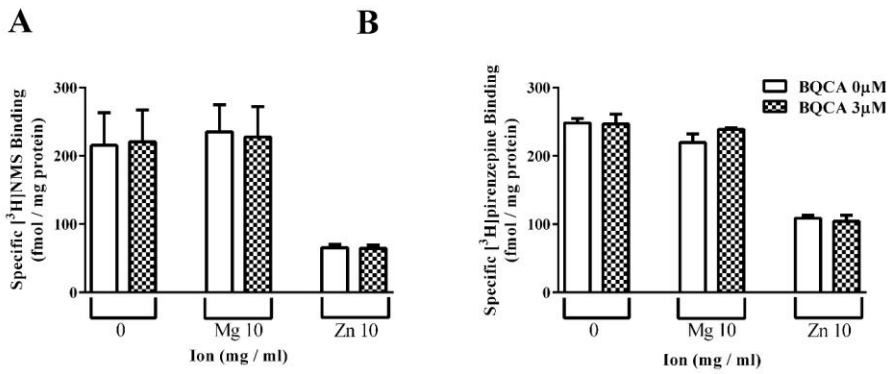
Formatted: Centered

Figure 2: The affinity (A) and density (B) of [^{3}H]NMS to cortical membranes from subjects with schizophrenia and a deficit of cortical muscarinic receptors measured using [^{3}H]pirenzepine binding (MRDS), subjects with schizophrenia without that deficit (SZ_{other}) and age and sex matched controls.



Formatted: Centered

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 (schizophrenia – other) and age and sex matched controls.



Formatted: Centered

Figure 4: The specific binding of [^{3}H]NMS (A and B) or [^{3}H]pirenzepine (C) in the absence of acetylcholine but in the present of 10 mg / ml Mg or Zn.

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**(48): 33701-33711.

Ashton H (2002). Benzodiazepines: How they work and how to withdraw.

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. *ArchNeurol* **54**(4): 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. *JPharmacolExpTher* **244**(3): 987-993.

Burgmer U, Schulz U, Trankle C, Mohr K (1998). Interaction of Mg2+ with the allosteric site of muscarinic M2 receptors. *Naunyn Schmiedebergs Arch Pharmacol* **357**(4): 363-370.

Bymaster FP, Felder C, Ahmed S, McKinzie D (2002). Muscarinic receptors as a target for drugs treating schizophrenia. *CurrDrug TargetsCNSNeurolDisord* **1**(2): 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. *NeurochemRes* **28**(3-4): 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. *AmJPsychiatry* **158**(6): 918-925.

D'Agostino RB, Belanger A, D'Agostino Jnr RB (1990). A suggestion for using powerful and informative tests of normality. *The American Statistician* **44**: 316-321.

Dean B (2004). M1 receptor agonism, a possible treatment for cognitive deficits in schizophrenia. *Neuropsychopharmacology* **29**(8): 1583-1584.

Dean B, Bymaster FP, Scarr E (2003). Muscarinic receptors in schizophrenia. *CurrMolMed* **3**(5): 419-426.

Dean B, McLeod M, Keriakous D, McKenzie J, Scarr E (2002). Decreased muscarinic(1) receptors in the dorsolateral prefrontal cortex of subjects with schizophrenia. *MolPsychiatry* **7**(10): 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, 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. *SchizophrRes* **106**(2-3): 229-236.

Deng C, Huang XF (2005). Decreased density of muscarinic receptors in the superior temporal gyrus in schizophrenia. *JNeurosciRes* **81**(6): 883-890.

Ferrer I, Santpere G, Arzberger T, Bell J, Blanco R, Boluda S, *et al* (2007). Brain protein preservation largely depends on the postmortem storage temperature: implications for study of proteins in human neurologic diseases and management of brain banks: a BrainNet Europe Study. *JNeuropatholExpNeurol* **66**(1): 35-46.

Foster P (1989). Neuroleptic equivalence. *The Pharmaceutical Journal* **243**: 431-432.

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. *IntJNeuropsychopharmacol*: 1-10.

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**(1): 47-61.

Han M, Newell K, Zavitsanou K, Deng C, Huang XF (2008). Effects of antipsychotic medication on muscarinic M1 receptor mRNA expression in the rat brain. *JNeurosciRes* **86**(2): 457-464.

Hashimoto R, Straub RE, Weickert CS, Hyde TM, Kleinman JE, Weinberger DR (2004). Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. *MolPsychiatry* **9**(3): 299-307.

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**(1-2): 247-258.

Hill C, Keks N, Roberts S, Opeskin K, Dean B, Mackinnon A, *et al* (1996). Problem of diagnosis in postmortem brain studies of schizophrenia. *AmJPsychiatry* **153**(4): 533-537.

Hulme EC, Berrie CP, Birdsall NJ, Jameson M, Stockton JM (1983). Regulation of muscarinic agonist binding by cations and guanine nucleotides. *Eur J Pharmacol* **94**(1-2): 59-72.

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. *MolPharmacol* **48**(2): 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 U S A* **106**(37): 15950-15955.

Mancama D, Arranz MJ, Landau S, Kerwin R (2003). Reduced expression of the muscarinic 1 receptor cortical subtype in schizophrenia. *AmJMedGenetB NeuropsychiatrGenet* **119**(1): 2-6.

Marger L, Schubert CR, Bertrand D (2014). Zinc: an underappreciated modulatory factor of brain function. *Biochem Pharmacol* **91**(4): 426-435.

Matsumoto I, Inoue Y, Iwazaki T, Pavey G, Dean B (2005). 5-HT2A and muscarinic receptors in schizophrenia: a postmortem study. *NeurosciLett* **379**(3): 164-168.

McKillup S (2006). *Statistics explained: An introdutory guide for life sciences* Cambridge University Press: Cambridge.

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**(2): 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**(23-24): 1185-1199.

Miller GA, Chapman JP (2001). Misunderstanding analysis of covariance. *JAbnormPsychol* **110**(1): 40-48.

Minzenberg MJ, Poole JH, Benton C, Vinogradov S (2004). Association of anticholinergic load with impairment of complex attention and memory in schizophrenia. *AmJPsychiatry* **161**(1): 116-124.

Mirza NR, Peters D, Sparks RG (2003). Xanomeline and the antipsychotic potential of muscarinic receptor subtype selective agonists. *CNS drug reviews* **9**(2): 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. *IntJNeuropsychopharmacol* **16**(4): 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. *ProgNeuropsychopharmacolBiolPsychiatr* **31**(1): 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**(1-2): 82-89.

Raedler TJ, Knable MB, Jones DW, Urbina RA, Gorey JG, Lee KS, *et al* (2003). In vivo determination of muscarinic acetylcholine receptor availability in schizophrenia. *AmJPsychiatry* **160**(1): 118-127.

Roberts SB, Hill CA, Dean B, Keks NA, Opeskin K, Copolov DL (1998). Confirmation of the diagnosis of schizophrenia after death using DSM-IV: a Victorian experience. *AustNZJPsychiatry* **32**(1): 73-76.

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**(9): 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**(11): 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? *JNeurochem* **107**(5): 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**(11): 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. *SchizophrBull* **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**(1-3): 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. *JPharmacolExpTher* **269**(1): 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. *AmJPsychiatr* **165**(8): 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. *JNeurosci* **29**(45): 14271-14286.

Stan AD, Ghose S, Gao XM, Roberts RC, Lewis-Amezcua K, Hatanpaa KJ, *et al* (2006). Human postmortem tissue: what quality markers matter? *Brain Res* **1123**(1): 1-11.

Sur C, Mallorga PJ, Wittmann M, Jacobson MA, Pascarella D, Williams JB, *et al* (2003). N-desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates N-methyl-D-aspartate receptor activity. *ProcNatlAcadSciUSA* **100**(23): 13674-13679.

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**(4): 1277-1287.

Thomas DR, Dada A, Jones GA, Deisz RA, Gigout S, Langmead CJ, *et al* (2010). N-desmethylclozapine (NDMC) is an antagonist at the human native muscarinic M(1) receptor. *Neuropharmacology* **58**(8): 1206-1214.

Tzavara ET, Bymaster FP, Davis RJ, Wade MR, Perry KW, Wess J, *et al* (2004). M4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related CNS pathologies. *FASEB J* **18**(12): 1410-1412.

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. *Psychophamacology (Berlin)* **225**(1): 21-30.

Vickroy TW, Watson M, Yamamura HI, Roeske WR (1984). Agonist binding to multiple muscarinic receptors. *Fed Proc* **43**(13): 2785-2790.

Watanabe S, Nishikawa T, Takashima M, Toru M (1983). Increased muscarinic cholinergic receptors in prefrontal cortices of medicated schizophrenics. *Life Sci* **33**(22): 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**(18-19): 2047-2054.

Wess J, Eglen RM, Gautam D (2007). Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *NatRevDrug Discov* **6**(9): 721-733.

Woods SW (2003). Chlorpromazine equivalent doses for the newer atypical antipsychotics. *JClinPsychiatry* **64**(6): 663-667.

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**(1-3): 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**(3): 525-532.