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Central Muscarinic Acetylcholine Receptor Availability in Patients Treated with Clozapine

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Clozapine is the prototypical atypical antipsychotic. *In vitro*, clozapine antagonizes a broad range of receptors, including dopamine, serotonin and muscarinic acetylcholine receptors. *In vivo*, receptor occupancy studies have shown moderate dopamine D_2 receptor blockade as well as high serotonin $5HT_2$ receptor blockade for clozapine. Using [I-123]IQNB SPECT, we explored the influence of clozapine on muscarinic receptors *in vivo*. Eight schizophrenia patients underwent a total of 12 [I-123]IQNB SPECT scans after treatment with low to moderate doses of clozapine (mean 210 mg/day, range 50–450 mg/day). Muscarinic receptor availability was determined for basal ganglia, cortex, thalamus, and pons. A group of 12 age- and sex-matched unmedicated schizophrenia patients was used for comparison. Compared to unmedicated patients, [I-123]IQNB binding was lower in all regions in subjects treated with clozapine and decreased with increasing dose. In patients treated with a daily clozapine dose of at least 200 mg (mean 275 $\pm$ 88 mg/day), these differences were highly significant ($p < 0.003$) with mean reductions of muscarinic receptor availability of 45% for basal ganglia, 58% for cortex, 66% for pons, and 79% for thalamus. These preliminary data indicate that reduction of muscarinic receptor availability by clozapine can be measured *in vivo* and that moderate daily doses are associated with moderate to high reductions of muscarinic receptor availability. These results may explain, at least in part, the lack of extrapyramidal side effects as well as some side effects seen with clozapine.

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INTRODUCTION

Clozapine is a tetracyclic dibenzodiazepine that was synthesized in 1958 and has been available for clinical use since the 1970s. After being withdrawn for its potential to cause agranulocytosis, clozapine was reintroduced in the United States in 1990 with the requirement of weekly leukocyte counts. Due to its lack of extrapyramidal side effects and superior clinical efficacy, particularly for treatment of refractory schizophrenic patients (Kane *et al*, 1988; Rosenheck *et al*, 1997), clozapine is regarded as the prototypical atypical antipsychotic.

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In vitro, clozapine has a high affinity for a large variety of neurotransmitter receptors, including $5HT_{2A}$, $5HT_{2C}$, $5HT_6$, $5HT_7$, D_4, α_1 and all five subtypes of muscarinic receptors, but has only moderate affinity at dopamine D_1 and D_2 receptors (Bolden *et al*, 1992; Bymaster *et al*, 1996; Schotte *et al*, 1996). *In vivo* PET and SPECT studies of the receptor occupancy of clozapine have shown that clozapine combines moderate D_2 receptor occupancy with high $5HT_2$ receptor occupancy (Farde *et al*, 1992; Kapur *et al*, 1999). So far, no imaging studies of muscarinic receptors have been performed in subjects treated with clozapine.

Cholinergic projections are ubiquitous in the brain and may be involved in the modulation of several processes thought to be dysfunctional in schizophrenia, such as memory (Goldberg *et al*, 1993), sleep (Jus *et al*, 1973), eye movements (Holzman *et al*, 1973), and motor control (Manschreck, 1986). Striatal cholinergic interneurons may also be involved in the pathophysiology and treatment of motor disturbances in schizophrenia. In addition to their beneficial effect on motor side effects of antipsychotics, anticholinergic medications have been associated with worsening of positive symptoms and improvement of negative symptoms in schizophrenia (Tandon *et al*, 1991). Neuropathologic studies reveal a reduction of muscarinic

receptors in schizophrenia (Dean *et al*, 1996; Crook *et al*, 1999, 2000, 2001).

Quinuclidinyl benzilate (QNB) has been used for years as an *in vitro* marker of muscarinic receptors and has subnanomolar affinity at all five muscarinic receptor subtypes (Bolden *et al*, 1992). [I-123]IQNB, the iodinated form of QNB, has been used in SPECT studies for *in vivo* measurements of muscarinic receptor availability (Eckelman *et al*, 1984; Weinberger *et al*, 1991; Sunderland *et al*, 1995). Using [I-123]IQNB SPECT, our group has previously reported reduced muscarinic receptor availability in unmedicated schizophrenic patients *in vivo* (Raedler *et al*, 2003), a finding similar to that of the post-mortem studies. We also have shown that treatment with the atypical antipsychotic olanzapine results in dose-dependent moderate muscarinic occupancy in the cortex and the basal ganglia and high muscarinic occupancy in the thalamus and the pons (Raedler *et al*, 2000).

While most research concerning the unique properties of clozapine has focused on antagonism at serotonin and dopamine receptors, it is possible that some of the beneficial and deleterious effects of clozapine can be explained by its actions on muscarinic receptors. Based on our experience in previous [I-123]IQNB SPECT studies as well as the *in vitro* binding of clozapine to muscarinic receptors, we predicted that the effects of clozapine on muscarinic receptor availability could be measured *in vivo* by this noninvasive technique.

METHODS

Subjects

We studied eight in-patients at the National Institute of Mental Health Neuropsychiatric Research Hospital at St Elizabeths in Washington, DC. All subjects gave written informed consent to participate in the [I-123]IQNB SPECT protocol, as approved by the NIMH Institutional Review Board. All subjects received a medical, neurological and psychiatric evaluation prior to enrollment. This included a brain MRI to rule out structural disease and for coregistration with SPECT images. The subjects were free of comorbid disorders, including substance abuse during the 6 months preceding this study. All subjects were chronically ill and were diagnosed with schizophrenia ($n=6$) or schizoaffective disorder ($n=2$) (DSM IV, American Psychiatric Association, 1994). All subjects had received other antipsychotics and supplemental anticholinergic treatment (benztropine = 6; trihexyphenidyl = 2) before treatment with clozapine. Prior to clozapine treatment initiation, other medications with known antimuscarinic properties were tapered and discontinued over a 2-week period. Other medication dosages were held constant for at least 2 weeks prior to SPECT scanning.

In order to study the effects of low doses of clozapine on muscarinic receptors, four subjects underwent [I-123]IQNB SPECT scans during the initiation of clozapine treatment. These subjects received treatment with daily doses of 50, 75 (two patients), or 150 mg for 3 days prior to SPECT scanning. Subsequently, clozapine dosage was optimized according to the clinical impression of the research ward team. Optimal clozapine dosage (range 200–450 mg/day) was held constant for 2 weeks prior to [I-123]IQNB SPECT. Four subjects were studied on two doses of clozapine. Of the eight subjects, seven were studied on clinically optimal clozapine doses. The [I-123]IQNB SPECT scan on the higher clozapine dose was used for the group analysis of muscarinic receptor availability. Table 1 summarizes the demographic data.

On the day of the [I-123]IQNB SPECT scan, the patients were rated with the Positive and Negative Symptom Assessment Scale (PANSS, Kay *et al*, 1986), Modified Abnormal Involuntary Movement Scale (AIMS, Wyatt, 1993) and the Wechsler Visual Memory Scale (WVMS, Wechsler, 1987). In 10 subjects, the National Psychopharmacology Laboratory (Knoxville, Tennessee) assessed clozapine blood levels on the day of SPECT scan with estimated coefficient of variation less than $\pm$ 10% (Pickar *et al*, 1996).

Contemporaneously, 12 unmedicated schizophrenia patients were scanned identically as the comparison group. Prior medication histories of comparison subjects were similar to those of treatment subjects: seven had received typical antipsychotics and four had been treated with

Table 1 Subject Demographics

Subject	Age (years)	Sex	Diagnosis[a]	Illness duration (years)	Anticholinergic medication prior to this study	Concomitant medication	Clozapine dose (mg/day)	Clozapine blood level (ng/ml)
1	34	M	SA	14	Benztropine	None	**225**	246
2	44	M	SA	21	Benztropine	Valproic acid	50	40
						Lithium	**200**	193
3	37	M	CUS	19	Benztropine	None	250	110
							300	150
4	32	F	CPS	9	Trihexyphenidyl	None	75	77
5	46	M	CPS	21	Benztropine	Ranitidine	75	133
							200	396
6	25	M	CUS	4	Trihexyphenidyl	Propranolol	150	NA
							450	215
7	38	M	CPS		Benztropine	Ranitidine	**250**	NA
8	48	M	CPS	21	Benztropine	None	**300**	207

[a]SA = Schizoaffective disorder; CUS = chronic undifferentiated schizophrenia; CPS = chronic paranoid schizophrenia.
Clinically optimal doses of clozapine in each patient, which were used in determining muscarinic acetylcholine receptor occupancy from the [I-123]IQNB SPECT data, are shown in bold.

atypical antipsychotics; five subjects had also received adjunctive treatment with anticholinergic medication (benztropine). At the time of their [I-123]IQNB scans, comparison subjects had been unmedicated for a median 17 days (range 7–180 days) (Raedler *et al*, 2003). The average age of the comparison group (34.6 $\pm$ 6.8 years, range: 24–44 years) did not differ significantly ($t = 1.20$, df = 22, $p = 0.24$) from the treatment group (38.2 $\pm$ 7.8 years, range: 25–48 years). Similarly, the gender ratio of the comparison group (8M : 4F) did not differ significantly ($\chi^2 = 2.27$, df = 1, $p = 0.13$) from the treatment group (8M : 1F).

SPECT Procedure

(*R*,*S*)-[I-123]IQNB synthesis followed previously described procedures (Lee *et al*, 1996). Each subject received an intravenous injection of about 7 mCi of [I-123]IQNB (mean: 6.9 $\pm$ 1.6 mCi, range: 4.9–10.0 mCi) at 13.00 h. The subjects received five drops of Lugol's solution on the day prior to injection and for 3 subsequent days to reduce radiation exposure to the thyroid. The average injected dose for comparison subjects (6.5 $\pm$ 1.5 mCi) did not differ significantly ($t = 1.23$; df = 22; $p = 0.23$) from the treatment group (7.3 $\pm$ 1.7 mCi). A 60-min SPECT scan was performed 21 h after injection when nonspecific binding in the cerebellum was indistinguishable from the scanner background count rate. For the scan, the subjects reclined into the CERASPECT (Digital Scintigraphics, Waltham, MA) dedicated brain SPECT camera. A laser beam was used to align the canthomeatal line to the transverse plane of the camera. The SPECT camera was calibrated for each scan session by imaging a 1-l uniform flood phantom of known radioactivity.

[I-123]IQNB scans were acquired in a step-and-shoot mode over 120 projections with a high-resolution collimator (7.5 mm full width at half maximum) A photopeak window (143–175 keV) and windows for scatter and septal penetration correction (175–191, 127–143 keV) were recorded. SPECT data were reconstructed with a Butterworth filter (cutoff = 1 cm, power factor = 10) as a 64 slice $\times$ 128 $\times$ 128 volume of isotropic 1.67-mm voxels.

Image Analysis

The image analysis has been previously described in detail (Raedler *et al*, 2000; Raedler *et al*, 2003). An MRI was obtained on each subject with a 1.5 T Signa (General Electric Medical Systems, Milwaukee, WI) scanner, using a spoiled GRASS sequence (TR = 24 ms, TE = 5 mse that generated 124 contiguous sagittal slices (thickness: 1.5 mm, field of view: 240 mm, matrix: 256 $\times$ 256 matrix). Using the public domain NIH Image software, the MRI volume was rotated so that a line connecting the anterior and posterior commissures in a midsagittal slice was horizontal. The MRI was then scaled to the dimensions of the SPECT scans and imported to the CERASPECT console. Based on gross anatomical features, regions of interest (ROIs) were drawn on central slices of the SPECT scan in three orthogonal planes. These ROIs were then superimposed onto the MRI and aligned for optimal fit to the MRI. Subsequently, these reoriented ROIs were transferred back to the SPECT scan and the SPECT scans rotated and translated to fit into the alignment ROIs. If necessary, this process was repeated to assure optimal coregistration. Using standard atlases (Aquilonius and Eckernas, 1980; Talairach and Tournoux, 1988; Duvernoy, 1991), anatomical ROIs for the cerebellum, pons, thalamus, caudate, and putamen as well as medial frontal, lateral frontal, temporal, parietal, and occipital cortex were drawn on five contiguous slices of the MRI scan to form a volume of interest (VOI) for each area. These ROIs were then transferred onto the coregistered SPECT scans. Data from right- and left-sided structures were averaged together. The average concentration of activity in each VOI was determined by dividing the counts per minute by the volume of the VOI; these data were decay corrected and converted to nCi/ml tissue using data from the calibration phantom. Finally, data were normalized to the injected dose to yield units of nCi/ml tissue per mCi injected dose, and cerebellum data, assumed to estimate nonspecific binding, were subtracted from each VOI value. These values serve as our measure of muscarinic receptor availability and to evaluate the percent reduction when comparing groups.

Statistical Analyses

Statistics were evaluated using Statistica for Windows 5.1. (StatSoft, Inc., Tulsa, OK) and Microsoft Excel (Microsoft Corp., Redmond, WA). Multivariate Hotelling's T^2 and *post hoc* Student's *t*-tests (independent samples) compared treatment subjects with unmedicated subjects. Dichotomous variables were analyzed with χ^2 tests. Correlations were assessed with Spearman's rank order correlation coefficient (*R*). In a pilot analysis employing nonlinear curve fitting with Solver in Excel, we estimated the *in vivo* muscarinic availability *vs* clozapine dose curve assuming clozapine binding to be in equilibrium with receptors after 2 weeks of fixed dosing.

RESULTS

Figure 1 shows an illustrative scan of a subject at a daily clozapine dose of 150 mg (top row) and 450 mg (middle row). Treatment subjects and comparison subjects showed similar patterns of [I-123]IQNB binding with highest muscarinic receptor availability in the cortex and the basal ganglia and lower availability in the thalamus and the pons.

Overall, we found lower muscarinic receptor availability (Table 2) in patients treated with clozapine than in unmedicated schizophrenics: the seven subjects treated with at least 200 mg/day of clozapine (mean 275 $\pm$ 88 mg/day) exhibited significantly lower [I-123]IQNB binding (multivariate Hotelling's $T^2 = 107.8$; $F(8,10) = 7.92$; $p < 0.002$). These differences reached significance for every VOI (all $t > 2.96$; all $p < 0.008$) in *post hoc* *t*-tests. Table 3 summarizes the percent reduction in muscarinic receptor availability in detail. The reductions ranged between 41 and 49% in the striatum (45% combined) and 57 and 59% in the cortex (58% combined) to higher reductions in the pons (66%) and the thalamus (79%).

Negative correlations between daily clozapine dose and muscarinic availability were significant in all ROIs (all Spearman $R < -0.7$, all $p < 0.02$) except pons ($R = -0.36$, $p = 0.24$). The strongest negative correlation between daily

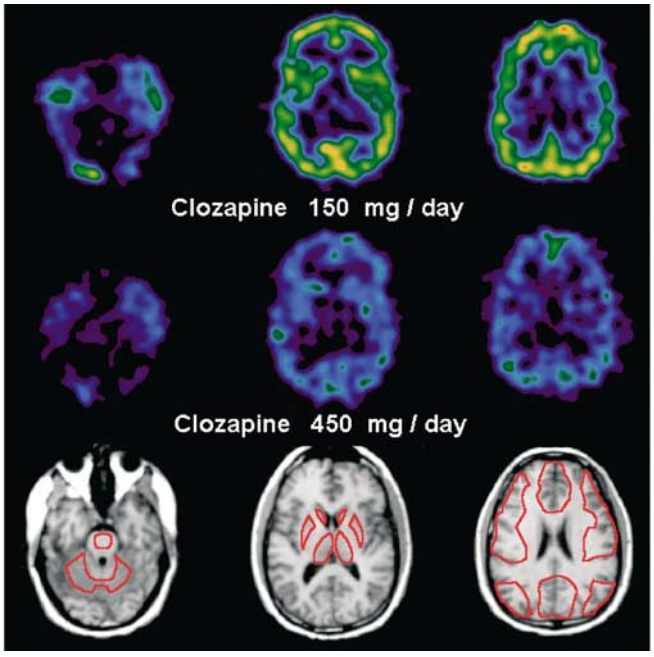

Figure 1 Illustrative [I-123]IQNB SPECT images of a subject scanned at both 150 mg/day of clozapine (top row) and 450 mg/day of clozapine (middle row). The leftmost slices are at the level of the pons and pass through the posterior poles of the occipital lobes, the slices in the center column are through the middle of the thalamus, and the rightmost slices are through the dorsal arch of the corpus callosum. The bottom row shows the corresponding slices of the coregistered MRI and the ROIs for these slices.

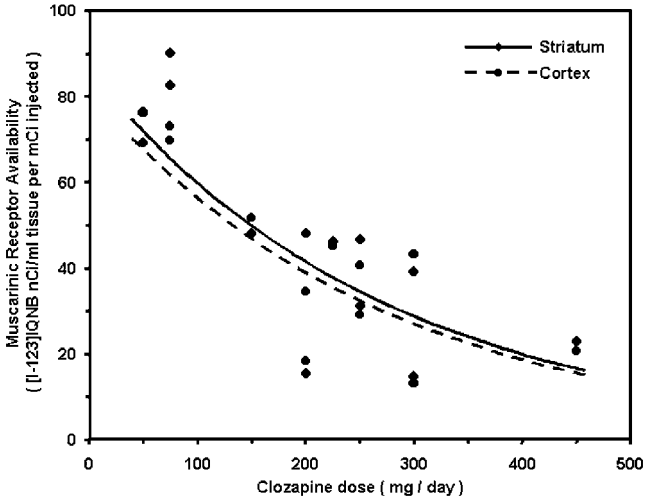

Figure 2 Relation between daily dose of clozapine (mg/day) and muscarinic receptor availability, as measured by [I-123]IQNB binding (nCi/ml tissue per mCi injected), in the striatum (average of caudate and putamen VOIs) and the cortex (average of medial frontal, lateral frontal, temporal, and occipital cortex VOIs).

clozapine dose and muscarinic availability was in the putamen ($R = -0.82$, $p < 0.001$). Negative correlations between clozapine serum levels and muscarinic receptor availability were significant in the lateral and the medial frontal cortex ($R = -0.74$, $p < 0.02$ and $R = -0.65$, $p < 0.05$, respectively). There were no significant correlations with symptom severity on the different psychopathology scales (PANSS), with extrapyramidal motor scales (AIMS) or with the WVMS.

The nonlinear curve fit analysis of muscarinic availability data predicts that maximal clinical doses of clozapine (900 mg/day) may reduce availability by roughly 70% in the striatum, 80% in the cortex, 90% in the thalamus, and 70% in the pons (Figure 2).

Table 3 Mean Muscarinic Acetylcholine Receptor Availability Reduction in Patients Treated with at least 200 mg/day of Clozapine ($n = 7$; Mean Dose 275.0 ± 87.8 mg/day; Range 200–450 mg/day) Relative to Unmedicated Patients ($n = 12$)

VOI	Percent reduction of muscarinic receptor availability (%)
Caudate	41.3
Putamen	48.6
Basal ganglia[a]	45.3
Thalamus	78.9
Pons	65.5
Medial frontal cortex	56.8
Lateral frontal cortex	59.4
Temporal cortex	56.8
Occipital cortex	58.0
Cortex[b]	57.7

[a]Mean of caudate and putamen VOIs.
[b]Mean of all cortical VOIs.

Table 2 Muscarinic Receptor Availability (Mean ± SD) in Schizophrenia Patients Treated with Clozapine at least 200 mg/day (Mean Dose 275.0 ± 87.8 mg/day) Compared to Unmedicated Schizophrenia Patients (Multivariate Hotelling's $T^2 = 107.84$; $F(8,10) = 7.92$; $p < 0.002$)

	[I-123]IQNB binding (nCi/ml tissue per mCi injected)			
VOI	Clozapine ($n = 7$)	Unmedicated ($n = 12$)	t-Value (df = 17)	p-Value
Caudate	31.0 ± 12.1	52.8 ± 17.0	2.96	0.008
Putamen	33.7 ± 13.7	65.6 ± 21.4	3.52	0.003
Thalamus	6.5 ± 3.0	30.6 ± 10.0	6.16	0.0001
Pons	4.5 ± 3.4	12.9 ± 6.9	3.01	0.007
Medial frontal cortex	27.9 ± 11.1	64.8 ± 15.0	5.62	0.0001
Lateral frontal cortex	27.3 ± 11.2	67.2 ± 14.7	6.20	0.0001
Temporal cortex	34.3 ± 12.4	79.5 ± 16.9	6.13	0.0001
Occipital cortex	31.7 ± 12.7	75.5 ± 15.5	6.32	0.0001

DISCUSSION

This pilot study is the first to measure the reduction of central muscarinic receptor availability in schizophrenia patients treated with clozapine. Relative to a matched sample of unmedicated patients, we found significant reductions of 45% (striatum) to 79% (thalamus) of muscarinic availability in patients treated with 200–450 mg/day of clozapine. In both samples, the pattern of [I-123]IQNB binding, our measure of muscarinic availability, was similar to that described in earlier reports (Weinberger *et al*, 1991; Sunderland *et al*, 1995; Raedler *et al*, 2000). In a related [I-123]IQNB SPECT study, we found a significant reduction of muscarinic availability in unmedicated schizophrenia patients (Raedler *et al*, 2003), a finding consistent with several neuropathological studies of muscarinic receptors in schizophrenia subjects (Dean *et al*, 1996; Crook *et al*, 1999, 2000, 2001). Thus, we used unmedicated schizophrenia patients as a comparison group because comparison to normal controls might inappropriately overestimate the reduction of muscarinic availability as a result of clozapine treatment.

The reduction of muscarinic receptor availability by clozapine implies a potency that differs from its *in vivo* potency at dopamine D_2 and serotonin $5HT_2$ receptors as measured in PET and SPECT studies. While clinical doses of most antipsychotics result in D_2 receptor occupancy by 70% (estimated by IBZM SPECT or raclopride PET), the reduction of D_2 receptor availability does not exceed 65% for clinical doses of clozapine (Farde *et al*, 1992; Nordstrom *et al*, 1993b; Kapur *et al*, 1999), and $5HT_2$ receptors are almost completely occupied by low doses of clozapine (Nordstrom *et al*, 1993a, 1995; Travis *et al*, 1998). Our data indicate that treatment with clozapine reduces muscarinic receptor availability in the striatum and the cortex by an amount similar to the occupancy of striatal D_2 receptors, while it is substantially lower than the occupancy of $5HT_2$ receptors.

The relation between muscarinic receptor occupancy and beneficial or deleterious effects is poorly understood. In studies of the dopaminergic system, a threshold of 60% dopamine D_2 receptor occupancy has been suggested for antipsychotic efficacy, while a threshold of about 75–80% was postulated for the emergence of motor side effects (Nordstrom *et al*, 1993b). Similar thresholds have not been established for the effects of anticholinergic medications on the muscarinic system and the relation between muscarinic receptor occupancy and anticholinergic side effects remains unknown. In clinical practice, treatment with clozapine frequently results in some anticholinergic side effects such as constipation, urinary retention, impaired accommodation, and tachycardia. A quantitative assessment of such side effects was not undertaken in this study and all patients tolerated clozapine well, although minor expected side effects were noted.

Little is known about the direct effects of clozapine on muscarinic receptors in humans. In rodent studies, chronic treatment with clozapine resulted in increased muscarinic receptor densities in the cortex, the striatum, and the hippocampus (Friedman *et al*, 1983; Boyson *et al*, 1988). Similarly, little is known about the possible effects of muscarinic receptor antagonism on other neurotransmitter systems. In PET studies, Dewey *et al* (1990, 1993) have shown that muscarinic antagonists may increase striatal synaptic dopamine.

It has been thought that clozapine acts as a pure antagonist at the muscarinic receptor. However, this concept has been challenged by clinical observations that higher doses of clozapine frequently result in hypersalivation that can be effectively treated with anticholinergics such as pirenzepine. More recent studies suggest that clozapine may act in a dose-dependent manner as a partial agonist at different muscarinic receptor subtypes (Zorn *et al*, 1994; Zeng *et al*, 1997; Olianas *et al*, 1999, Michal *et al*, 1999). In this context, it is important to point out that [I-123]IQNB SPECT assesses muscarinic receptor availability only, but not the function of clozapine at these receptors vis-à-vis agonist or antagonist.

We emphasize the preliminary nature of our findings. This study was carried out in a small sample of subjects, who were treated with low to moderate doses of clozapine. Consistently, the serum levels of clozapine were relatively low. We neither used a fixed dose regimen nor did we employ a within-subject design in which patients were scanned before and after treatment; medicated patients were instead compared to a separate cohort of unmedicated patients. SPECT studies were performed at least 5 weeks after the initiation of washout of other antipsychotic and antimuscarinic medications. We feel that this washout interval is sufficient to insure that the muscarinic receptor availability is not confounded by residual effects of previous medications. This concept is supported by the results from our previous study, where there was no significant correlation between duration of the unmedicated interval and muscarinic receptor availability (Raedler *et al*, 2003).

It is conceivable that medication-induced changes in brain function and subsequent influences on brain physiology, blood flow, or endogenous acetylcholine levels may affect IQNB uptake differently between the treatment group and the unmedicated comparison group. However, it seems unlikely that these effects would reduce IQNB uptake to the extent that we have observed and we ascribe our observations primarily to direct blockade by clozapine. Despite these limitations, we were able to measure the *in vivo* occupancy of muscarinic receptors in patients treated with clozapine. Further evidence for the *in vivo* effects of clozapine on the muscarinic receptor should be gained from additional studies using higher doses of clozapine.

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