Decreased Muscarinic Receptor Binding in Subjects with Schizophrenia: A Study of the Human Hippocampal Formation

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Background: *Acetylcholine is important to hippocampal function, including the processes of learning and memory. Patients with schizophrenia show impaired learning and memory and hippocampal dysfunction. Thus, acetylcholinergic systems may be primarily or secondarily disrupted in the hippocampal formation of schizophrenic patients. The present study tested the hypothesis that [^{3}H]pirenzepine-labeled muscarinic cholinergic receptor levels are altered in the hippocampal formation of patients with schizophrenia.*

Methods: *We have used quantitative autoradiography to measure [^{3}H]pirenzepine binding to M_1 and M_4 receptors in the hippocampal formation from 15 schizophrenic and 18 nonschizophrenic subjects.*

Results: *The mean density of [^{3}H]pirenzepine binding was reduced in all regions studied, including the dentate gyrus, subdivisions of Ammon's Horn (CA1–CA4), subiculum, and the parahippocampal gyrus, of the schizophrenic cohort. Moreover, unlike controls, there was no significant variation between the mean levels of [^{3}H]pirenzepine binding across the subregions of the hippocampal formation from schizophrenic subjects.*

Conclusions: *These findings provide support for a possible involvement of the muscarinic cholinergic system in the pathology and/or treatment of schizophrenia.* Biol Psychiatry 2000;48:381–388 © *2000 Society of Biological Psychiatry*

Key Words: Schizophrenia, muscarinic receptors, human hippocampus, [^{3}H]pirenzepine

Introduction

The hippocampal formation (HF; including the dentate gyrus, subdivisions of Ammon's Horn [CA1–CA4], subiculum, and parahippocampal gyrus) is a focus of schizophrenia research (Torrey and Peterson 1974; Weinberger 1991, 1999). Cholinergic afferents from the medial septum and diagonal band of Broca project to all layers of the hippocampus (Frotscher and Leranth 1985). Moreover, muscarinic cholinergic receptors are important to hippocampal function, including the processes of learning and memory (Fadda et al 1996; Frotscher and Leranth 1985; McAlonan et al 1995). Alterations to the muscarinic cholinergic system of the HF may be primarily or secondarily involved in the pathophysiology of schizophrenia. Importantly, pre- and postsynaptic muscarinic receptors appear to modulate both cholinergic and noncholinergic activity in the hippocampus. Noncholinergic systems modulated by acetylcholine (ACh) include glutamate, γ-aminobutyric acid (GABA), noradrenalin, and serotonin, all of which have been implicated in schizophrenia (Umbriaco et al 1995; Vizi and Kiss 1998). Conversely, there is evidence to suggest that noncholinergic systems including dopaminergic (Imperato et al 1994) and serotonergic (Fujii et al 1997; Koyama et al 1999; Vizi and Kiss 1998) modulate hippocampal ACh release and associated muscarinic receptor activity. Interestingly, we have previously reported that the serotonin transporter is altered in the hippocampus of subjects with schizophrenia (Dean et al 1996; Naylor et al 1996). The altered serotonin transporter activity may be associated with increased levels of serotonin. As it has been shown that increased serotonergic activity in the hippocampus increases ACh efflux in the hippocampus (Fujii et al 1997; Koyama et al 1999), we hypothesize that a down-regulation in the levels of hippocampal muscarinic receptors may occur in schizophrenia.

To test our hypothesis, we have measured the binding of [^{3}H]pirenzepine ([^{3}H]Pz) using quantitative autoradiography, in regions of the HF from subjects with and without schizophrenia. The muscarinic antagonist [^{3}H]Pz was employed, as it binds selectively to M_1 and M_4 receptors (Doods et al 1987; Hulme et al 1990),[1] both of which are important for hippocampal neurochemistry (Flynn et al 1995; Levey et al 1995; Vizi and Kiss 1998). Moreover, in

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[1] The selective binding of [^{3}H]Pz to both M_1 and M_4 receptors is also supported by saturation binding experiments using cloned human receptors, performed in our laboratory but not presently detailed. Dose-response curve studies demonstrated that the affinity of [^{3}H]Pz binding to cloned human M_1 (K_d = 3 nmol/L) receptors was similar to that for cloned human M_4 (K_d = 7 nmol/L) receptors.

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light of the actions of the atypical antipsychotic drugs clozapine and olanzapine, which bind with high affinity to M_1 and M_4 receptors, we propose that of the five known types of muscarinic receptors, M_1 and M_4 receptors in particular may be important to the pathology and/or treatment of schizophrenia (Bolden et al 1992, 1991; Bymaster et al 1999; Zorn et al 1994).

Methods and Materials

Tissue Collection

After gaining ethical approval from the Human Ethics Committee of the Mental Health Research Institute of Victoria, human HF (dentate gyrus, CA1–CA4, subiculum, and parahippocampal gyrus) was collected at autopsy from the left-brain hemispheres of 15 subjects with a provisional diagnosis of schizophrenia. Tissue was also collected from the same brain region of the left-brain hemispheres of 18 subjects with no clinical history of psychiatric illness, or histopathological evidence of neurological disease (control subjects). Wherever possible, control subjects were matched for age, postmortem interval (PMI), and gender to subjects who had schizophrenia (Tables 1 and 2). Where death was not witnessed, PMI was taken as the interval halfway between the last sighting of a subject while still alive and being found dead, to autopsy. Tissue was only collected where the interval between a person being found dead and last seen alive was less than 5 hours. All cadavers were stored at 4°C, within 5 hours of death or discovery, until autopsy. Following autopsy, tissue was rapidly frozen to −70°C until required (freezer time, FT; Tables 1 and 2).

In an attempt to control for the effects of agonal state on tissue collected, radioligand binding studies were preceded by determining the pH of the brain tissue (Kingsbury et al 1995). Tissue was only used when the pH was greater than 6.0 (Tables 1 and 2). Finally, selection of tissue blocks was standardized using a set of standard landmarks (Duvernoy 1991; Vonsattel et al 1995). Thus, serial sections (4 × 20 μm per subject) of HF were coronally cut within a region of the hippocampus and parahippocampal gyrus lying proximal to the rostro-caudal boundaries of the lateral geniculate body. Sections were cut using a cryomicrotome (CM 1800, Leica Microsystems, Bannockburn, IL) and thaw-mounted onto chrome-alum/gelatin coated glass slides, ready for further study.

Diagnostic Evaluation

An extensive review of the case histories of subjects with a provisional diagnosis of schizophrenia was undertaken by a senior psychiatrist and psychologist using a structured instrument (Hill et al 1996a, 1996b). Confirmation of each provisional diagnosis of schizophrenia was made according to DSM-III-R criteria (American Psychiatric Association 1987). From the case histories, the duration of illness (DOI), defined as the time from first hospital admission to death, was determined for each schizophrenic subject (Table 1). Also, the final recorded antipsychotic drug dose administered prior to death was obtained and converted to chlorpromazine equivalents (Foster 1998; Table 1).

In Situ [^{3}H]Pirenzepine Binding with Autoradiography

For in vitro radioligand binding studies with autoradiography, mounted tissue sections were thoroughly dried and incubated for 30 min with 15 nmol/L (greater than $2 \times K_d$)[2] [^{3}H]Pz in the absence (2 sections: TB) or presence (2 sections: NSB) of 1 μmol/L quinuclidinyl xanthene-9-carboxylate hemioxilate tetraoxilate (QNX). All incubations were carried out in 10 mmol/L sodium-potassium phosphate buffer (assay buffer; 10 mmol/L KH_2PO_4, 10 mmol/L Na_2HPO_4; pH 7.4) at 25°C. Following this, sections were washed twice for 2 min in ice-cold assay buffer, dipped in ice-cold water and air-dried. The dried sections were apposed to Amersham [^{3}H]-Hyperfilm (Amersham International, Little Chalfont, UK) with Amersham [^{3}H] micro-scales for up to 5 weeks. Images produced were subsequently analyzed using a Microcomputer Imaging Device image analysis system (Imaging Research, St Catherine's, Canada). Results were initially expressed as dpm per milligram of estimated tissue equivalents (ETE) and converted to femtomoles per milligram of ETE. Specific radioligand binding was calculated as TB minus NSB.

Data Analysis

A Mann–Whitney test was used to compare radioligand binding, age, PMI, FT, and pH between schizophrenic and control groups. A one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test, was used for comparisons between the levels of radioligand binding in different HF regions per study cohort. The relationships between radioligand binding and age, PMI, FT, DOI, pH, and final recorded antipsychotic drug dose were assessed using Pearson product moment correlation coefficients, derived using an assumed straight-line fit. Analysis of covariance was carried out to determine if age, PMI, or FT were confounding variables influencing the apparent relationship between radioligand binding data from the schizophrenic and control subjects.

Materials

[^{3}H]Pz (specific activity: 72 Ci/mmol) was purchased from Du Pont (Australia), Sydney. [^{3}H] microscale standards for autoradiography were obtained from Amersham. QNX was obtained from Research Biochemicals International (Natick, MA). Other chemicals were purchased from BDH Laboratory Supplies (Poole, UK).

Results

The specific binding of [^{3}H]Pz to sections of HF was >95% of TB (Figure 1). There was a significant decrease in the mean levels of [^{3}H]Pz binding in all regions of the HF studied from schizophrenic compared to control subjects (Tables 1 and 2 and Figure 2). In addition, although there were regional differences in the mean levels of

[2] The radioligand concentration chosen for single-point saturation binding studies is supported by saturation binding experiments performed in our laboratory but not presently detailed. K_d values obtained from dose-response curve studies using cortical membrane were equal to 7 nmol/L. Furthermore, temporal equilibrium was achieved within the time span selected.

Table 1. Demographic Data for Schizophrenic Subjects Studied and the Binding of [^{3}H]Pirenzepine in Regions of the Hippocampal Formation

										Hippocampal formation [^{3}H]pirenzepine binding[b]						
Subject	Gender	Age at death (years)	PMI (hours)	DOI (years)	pH	Freezing time (months)	Cause of death	Antipsychotic drug treatment	Final recorded antipsychotic drug dose[a]	Dentate gyrus	CA1	CA2	CA3	CA4	Subiculum	Para-hippo-campal gyrus
1	M	51	20	32	6.0	39	Ischemic heart disease	Fluphenazine Thioridazine	2000	99	140	111	78	102	123	96
2	M	47	33	27	6.4	33	Ischemic heart disease	Fluphenazine Thioridazine	800	107	129	112	96	129	97	115
3	M	27	22	8	6.3	31	Suicide: burning	Chlorpromazine Pimozide	1200	175	226	226	162	201	174	204
4	M	30	53	9	6.3	30	Suicide: burning	Fluphenazine	300	220	274	252	158	220	202	256
5	F	72	59	37	6.5	29	Pneumonia	Chlorpromazine	25	192	216	203	174	194	160	160
6	M	53	37	30	6.0	29	Intestinal ischemia	Fluphenazine Chlorpromazine	1200	45	58	47	37	40	43	34
7	M	38	36	11	6.4	22	Suicide: hanging	Fluphenazine	200	157	167	180	122	137	116	144
8	M	27	46	8	6.4	21	Suicide: hanging	Chlorpromazine	600	122	135	143	103	113	98	121
9	M	67	21	23	6.5	20	Pneumonia	Fluphenazine	75	51	59	68	39	45	39	45
10	M	47	42	21	6.5	18	Suicide: multiple injuries	Chlorpromazine Haloperidol	825	197	261	256	171	214	158	202
11	M	32	17	15	6.1	11	Suicide: CO poisoning	Haloperidol	285	92	159	107	71	95	87	120
12	M	63	73	44	6.1	27	Chronic cardiac failure	Chlorpromazine Stelazine	300	186	85	180	170	205	166	212
13	M	35	47	17	6.3	24	Perforated gastric ulcer	Fluphenazine	400	167	195	184	133	136	181	152
14	F	35	15	7	6.3	5	Carotid arterial thrombosis	Haloperidol	300	225	216	223	174	216	203	150
15	F	48	53	22	6.2	3	Pulmonary thromboemboli	Fluphenazine Chlorpromazine	700	171	132	164	139	175	146	155
Mean ± SE		45 ± 3.7	38 ± 4.4	21 ± 2.9	6.3 ± 0.04	23 ± 2.6				147 ± 15	164 ± 17	164 ± 17	122 ± 13	148 ± 16	133 ± 13	144 ± 16

PMI, postmortem interval; DOI, duration of illness; M, male; F, female.
[a]Chlorpromazine equivalents (mg/day).
[b]fmol mg^{-1} estimated tissue equivalents.

Table 2. Demographic Data for Control Subjects Studied and the Binding of [^{3}H]Pirenzepine in Regions of the Hippocampal Formation

							Hippocampal formation [^{3}H]pirenzepine binding[a]						
Subject	Gender	Age at death (years)	PMI (hours)	pH	Freezing time (months)	Cause of death	Dentate gyrus	CA1	CA2	CA3	CA4	Subiculum	Parahippocampal gyrus
1	M	51	48	6.6	31	Suicide: gunshot	177	213	196	166	190	135	118
2	M	26	41	6.4	31	Accidental drowning	183	265	192	212	245	181	197
3	F	59	21	6.6	22	Chronic cardiac failure	233	224	229	208	134	215	193
4	M	31	41	6.2	22	Ischemic heart disease	187	206	207	168	206	175	170
5	M	34	16	6.4	20	Ischemic heart disease	166	206	216	156	189	212	193
6	M	65	41	6.6	19	Ischemic heart disease	184	263	214	141	197	144	154
7	F	38	51	6.1	18	Ischemic heart disease	219	278	238	156	207	225	194
8	M	29	44	6.5	17	Electrocution	177	278	249	160	208	191	206
9	M	33	39	6.5	17	Ruptured thoracic aorta	162	250	230	172	198	124	203
10	M	37	47	6.4	18	Cardiomyopathy	199	241	231	197	233	187	160
11	M	50	69	6.4	16	Ischemic heart disease	203	225	193	178	198	180	216
12	M	65	21	6.5	16	Acute myocardial infarct	226	276	328	178	194	182	143
13	M	53	45	6.6	15	Ischemic heart disease	209	256	210	185	212	164	186
14	F	62	40	6.5	14	Ischemic heart disease	224	245	237	208	225	204	169
15	M	27	31	6.4	12	Asthma	197	284	238	185	241	163	187
16	M	27	14	6.4	12	Ischemic heart disease	231	261	271	199	208	181	249
17	M	50	65	6.4	21	Ischemic heart disease	199	227	229	200	224	229	174
18	F	66	43	6.4	18	Acute myocardial infarct	200	227	221	171	199	210	191
Mean ± SE		45 ± 3.5	40 ± 3.5	6.4 ± 0.03	19 ± 1.3		199 ± 5	246 ± 6	229 ± 8	180 ± 5	206 ± 6	183 ± 7	184 ± 7

PMI, postmortem interval; M, male; F, female.
[a]fmol mg^{-1} estimated tissue equivalents.

radioligand binding in the HF from control subjects (dentate gyrus < CA1 [$p < .001$] and CA2 [$p < .05$]; CA1 > CA3 [$p < .001$], CA4 [$p < .001$], subiculum [$p < .001$], and the parahippocampal gyrus [$p < .001$]; CA2 > CA3 [$p < .001$], subiculum [$p < .001$], and the parahippocampal gyrus [$p < .001$]; Table 2), the mean levels of radioligand binding did not differ between regions for the schizophrenic subjects (Table 1).

There was no significant difference between the mean age or PMI for tissue of the schizophrenic and control groups ($p = .94$ and $p = .86$, respectively; Tables 1 and 2). Although the mean FT of tissue from the schizophrenic cohort tended toward being longer compared to the control cohort, this was not significant ($p = .08$; Tables 1 and 2). The mean pH of tissue from the schizophrenic cohort was significantly lower compared to control subjects ($p = .01$; Tables 1 and 2). For both schizophrenic ($-.13 \geq r \geq -.46$, $p \geq .08$) and control ($.38 \geq r \geq -.42$, $p \geq .08$) subjects, no relationship was demonstrated between the binding of [^{3}H]Pz in all regions of the HF, except for the parahippocampal gyrus from controls ($r = -.48$, $p = .05$; Figure 3A), and age at death. There were significant negative correlations between the density of [^{3}H]Pz binding to area CA2 from control subjects and PMI ($r = -.49$, $p = .03$; Figure 3B) and FT ($r = -.51$, $p = .03$; Figure 3C). There were, however, no other correlations between radioligand binding in HF from schizophrenic or control subjects and PMI ($.47 \geq r \geq .02$, $p \geq .07$ and $.32 \geq r \geq -.19$, $p \geq .20$, respectively), FT of tissue ($-.01 \geq r \geq$

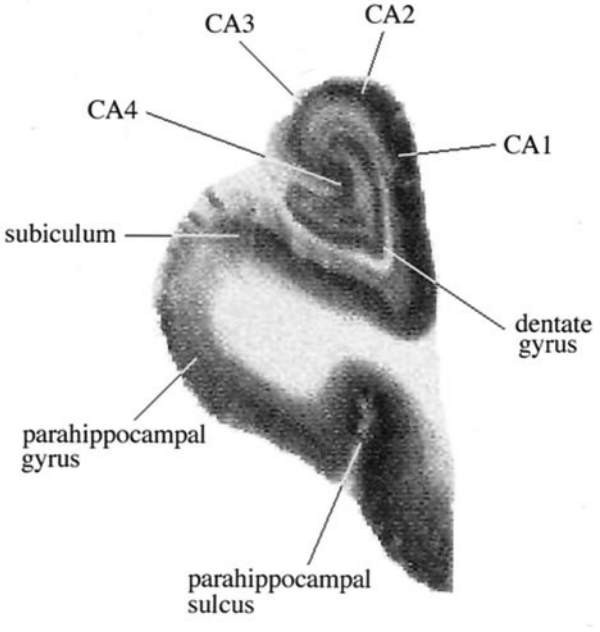

Figure 1. A representative image of the specific binding of [^{3}H]pirenzepine to human hippocampal formation.

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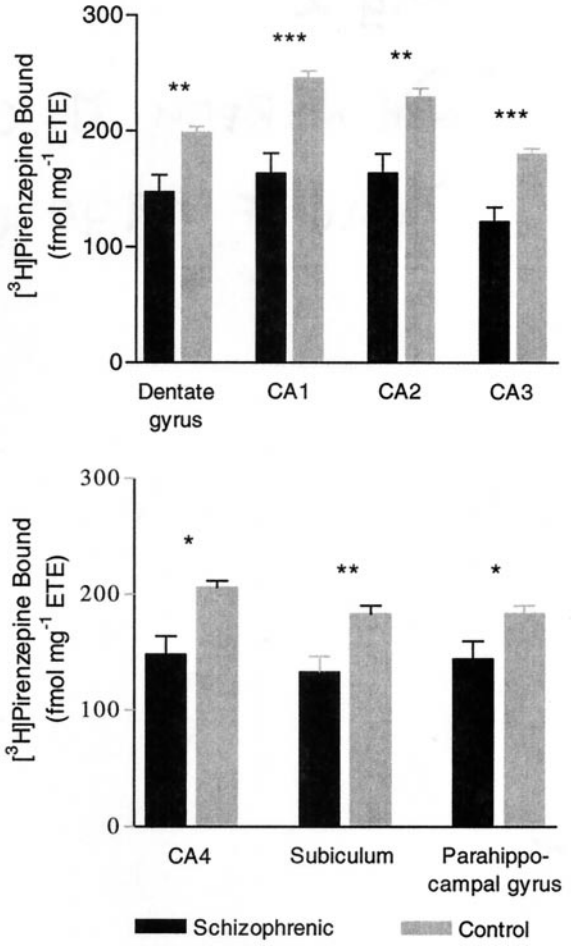

Figure 2. The binding of [^{3}H]pirenzepine in regions of the hippocampal formation from schizophrenic and control subjects. ETE, estimated tissue equivalents. *$p < .05$; **$p < .005$; ***$p < .0005$.

$-.21$, $p \geq .45$ and $.02 \geq r \geq -.42$, $p \geq .08$, respectively), or tissue pH ($.39 \geq r \geq .11$, $p \geq .15$ and $.11 \geq r \geq -.43$, $p \geq .07$, respectively). Analysis of covariance showed that there was no significant effect of subject age, PMI, or FT on the comparison of [^{3}H]Pz binding in tissue from the schizophrenic and control subjects.

No relationship was found between radioligand binding in the HF from schizophrenic subjects and the final recorded antipsychotic drug dose ($-.11 \geq r \geq -.30$, $p \geq .28$) or DOI ($-.10 \geq r \geq -.48$, $p \geq .07$).

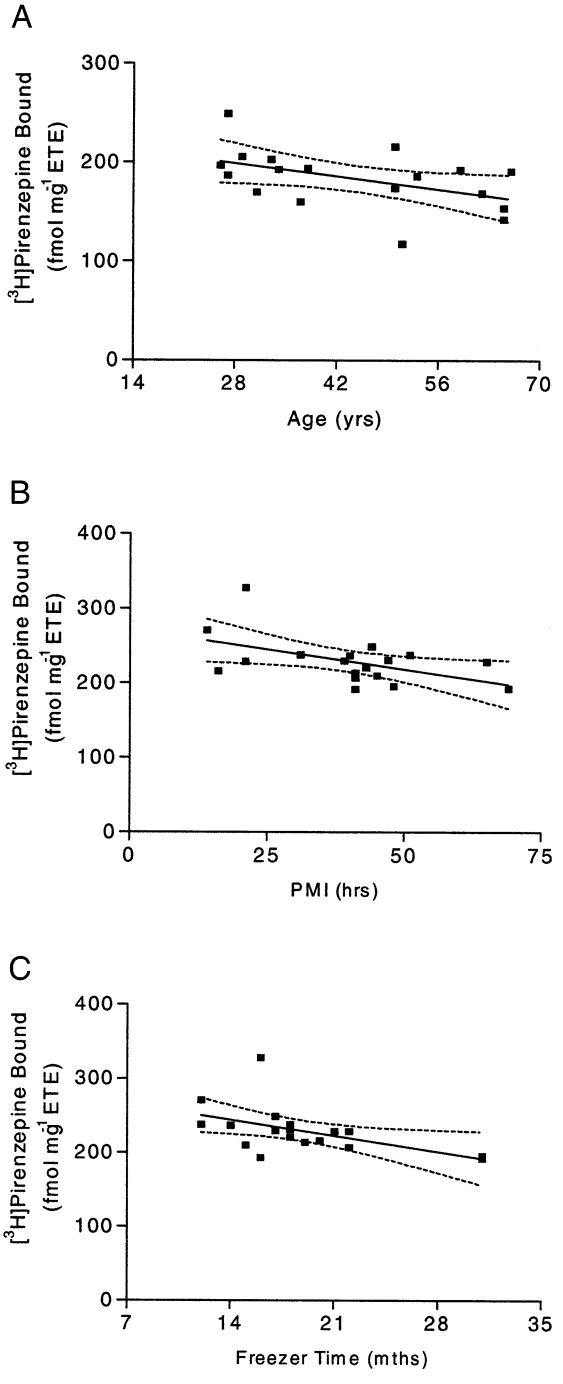

Figure 3. The relationship between the binding of [^{3}H]pirenzepine in **(A)** parahippocampal gyrus of control subjects and age at death; **(B)** CA2 of control subjects and postmortem interval; or **(C)** freezer time. ETE, estimated tissue equivalents.

Discussion

Based on the receptor selectivity of [^{3}H]Pz binding (Doods et al 1987; Hulme et al 1990), and the high and intermediate abundance of M_1 and M_4 receptors respectively in the HF (Levey et al 1995), our study suggests that the density of M_1 and/or M_4 receptors are decreased throughout the HF of subjects who had schizophrenia. Similarly, Perry and Perry (1980) previously measured a decrease in the density of [^{3}H]QNB-labeled muscarinic receptors in the whole hippocampus from subjects who had schizophrenia; however, the research discussed here is

the first study of schizophrenia using postmortem tissue that delineates radioligand binding to muscarinic receptors in the different regions of the HF. Moreover, unlike Perry and Perry (1980), we have been able to collectively measure radioligand binding to M_1 and M_4 receptors only, rather than all known muscarinic receptor subtypes (M_1 through to M_5).

In addition to a reduced [^{3}H]Pz binding to the HF of schizophrenic subjects, a regional variation in radioligand binding was absent. Importantly, regional variation of [^{3}H]Pz binding in control HF (including a highest ligand binding to CA1) replicates a previous study of normal HF muscarinic receptor distribution (Perry et al 1993).

All schizophrenic patients studied received antipsychotic drug treatment prior to death. Although there was no relationship between radioligand binding in the HF from schizophrenic subjects and the final recorded antipsychotic drug dose, this does not exclude a causal relationship between drug treatment and altered muscarinic receptor levels. Importantly, as the majority of antipsychotic drugs received by the patients presently studied are putative muscarinic antagonists (i.e., haloperidol, chlorpromazine, thioridazine, fluphenazine; Richelson 1996; Snyder et al 1974), these drugs would not be expected to directly down-regulate the density of any of the muscarinic receptors measured; however, a decrease in receptor levels due to upstream drug activities via associated nonmuscarinic mechanisms cannot be excluded. Evidently further research into the possible effects of antipsychotic drugs on muscarinic receptor levels is necessary.

The measurement of altered [^{3}H]Pz binding in the HF is significant in light of the putative roles of M_1 and M_4 receptors for hippocampal neurochemistry (Vizi and Kiss 1998) and dysfunction in schizophrenia (Tandon and Greden 1989; Tandon et al 1991). It has been proposed that M_1 and M_4 receptors mediate a diversity of post- and presynaptic actions, respectively, in the hippocampus. For example, activation of postsynaptic M_1 receptors appears to enhance glutamate-mediated excitatory neurotransmission in the hippocampus (Halliwell 1990; Markram and Segal 1992; Vizi and Kiss 1998). Since presynaptic M_4 autoreceptors are the major inhibitors of ACh release in the hippocampus, they indirectly influence activation of postsynaptic M_1 receptors and secondarily glutamatergic neurotransmission (Halliwell 1990; Vizi and Kiss 1998). Importantly, glutamatergic cells represent 90% of hippocampal neurons, form the foundation of hippocampal circuitry and function, and may be important to the pathophysiology of schizophrenia (Goff and Wine 1997; Javitt and Zukin 1991; Moghaddam and Adams 1998). Based on the relationship between M_1 and M_4 receptors and other major neurotransmitter systems important to hippocampal function, the muscarinic receptors presently measured are likely to be important to normal hippocampal function and dysfunction in schizophrenia.

The reduced binding of [^{3}H]Pz, and hence possible decrease in M_1 and/or M_4 receptor levels in HF from schizophrenic subjects, may reflect the pathology of schizophrenia. An increase in muscarinic cholinergic neurotransmission might cause a down-regulation in pre- and postsynaptic M_4 and M_1 receptors respectively. This hypothesis is consistent with an increased dopaminergic (Davis et al 1991; Walker and Diforio 1997; Weinberger 1987), serotonergic (Dean et al 1996; Naylor et al 1996) and glutamatergic (Javitt and Zukin 1991; Moghaddam and Adams 1998) tone in the hippocampus of patients with schizophrenia. Specifically, increased hippocampal release of dopamine (Imperato et al 1994) and serotonin (Fujii et al 1997; Koyama et al 1999) may increase the release of ACh in the HF, with a compensatory down-regulation of M_1 and M_4 receptor levels. Furthermore, based on the putative cholinergic regulation of hippocampal glutamate release, an increased glutamate neurotransmission (Javitt and Zukin 1991; Moghaddam and Adams 1998) proposed in schizophrenia is consistent with an increased ACh efflux and the receptor changes presently measured.

In conclusion, there are many possible relationships between muscarinic receptors and other neurotransmitter systems within the HF. Although we can only speculate on the significance of the present findings for hippocampal function and involvement in schizophrenia, it seems reasonable to suggest that muscarinic receptors may be primarily or secondarily involved in the pathology and/or treatment of schizophrenia. Perhaps targeting pre- and postsynaptic muscarinic receptors with selective pharmacological agents will prove to be clinically useful in the treatment of schizophrenia.

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