

Appendix 1 to Scarr E, Hopper S, Vos V, et al. Low levels of muscarinic M1 receptor-positive neurons in cortical layers III and V in Brodmann's areas 9 and 17 from individuals with schizophrenia. *J Psychiatry Neurosci* 2018.

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Supplementary Methods

Collection and processing of human postmortem CNS

All tissue collected at VIFM was from cadavers that were refrigerated within 5 hours of being found and brain tissue was rapidly processed and frozen to -70°C using a standardised procedure (1). Following these two processes is important as they help to minimise autolytic effects (2), which was confirmed by measuring CNS pH for each case as described previously (3) because this provides a good measure of overall tissue preservation (4).

Case Reviews

Data was collected during case histories reviews using the Diagnostic Instrument for Brain Studies (DIBS) (5, 6) which is a clinical assessment tool which enables a diagnostic consensus to be reached, postmortem, according to DSM-IV criteria. (6) Tissue was only collected where the donor had been seen alive within 5 hours of being found dead. From the data gathered using the DIBS, post-mortem interval (PMI) was calculated as the time from death to autopsy except when death was not witnessed, in which case PMI was taken as the midpoint between the person being found and being last seen alive. Duration of illness (DI) was calculated as the time from first hospital admission to death and the final recorded dose of antipsychotic drugs (FRADD) was noted and then converted to a standardized drug dose (chlorpromazine equivalents per day; Chlor. Eqs.). Given the focus of our studies on muscarinic receptors it was noted whether donors had been prescribed muscarinic receptor antagonists (anticholinergic drugs) to control extrapyramidal side effects.

Immunohistochemistry of muscarinic M1 receptors

Free-floating sections from BA 9, BA 17, hippocampus and thalamus ($15\ \mu\text{M}$) were removed from storage solution then washed in PBS before being

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incubated in 1% hydrogen peroxide in methanol to quench endogenous peroxidase activity. The sections were then washed in deionised water followed by PBS before being microwaved in citrate buffer (pH 6.0) (Abcam, Melbourne, VIC, Australia; Cat# 64236; Lot# GR80382-1) to retrieve antigens. The microwaved sections were washed in PBS before blocking with PBS containing 1% normal goat serum, 5% non-fat milk powder (NFMP) and 0.1% Triton x-100 (antibody diluent) for two hours at room temperature (r/t). The blocked tissue sections were then incubated with 1/67 dilution of anti-muscarinic M1 receptor antibody (Frontier Institute Co. Ltd, Hokkaido, Japan; mAChR-M1-Rb; batch Af340) in antibody diluent, overnight at 4° C. After washing in PBS the sections were incubated with a 1/200 dilution of biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) in PBS containing 0.1% Triton x-100 for two hours at r/t. After washes in PBS, the sections were incubated in PBS containing 1: 100 dilution of Avidin-DH (Vector Laboratories, Inc., Burlingame, CA, USA; Cat #: A-3100) and a 1:100 dilution of biotinylated peroxidase horse radish peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA; Cat #: B-2004) for two hours at r/t. Sections were washed in PBS before being incubated with 1 : 33 dilution of 3, 30-diaminobenzidine in the supplied diluent (Vector Laboratories, Inc., Burlingame, CA, USA; Vector ImmPACTTM, SK4105) for 90 s at r/t, followed by washes in deionised water and PBS. Sections were mounted on slides, air-dried and then counterstained with haematoxylin (Vector 1 Hematoxylin Nuclear Counterstain (Gill's Formula); Vector Laboratories, Inc., Burlingame, CA, USA: H-3401; Lot #Z0916) for 210 s at r/t, washed and then dehydrated through graded alcohols, cleared in histolene and permanently mounted in DPX slide mounting media (Sigma-Aldrich, Castle Hill, NSW, Australia).

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References

1. 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, editors. *Using CNS tissue in psychiatric research: A practical guide*. Amsterdam: Harwood Academic Press, pp 67-83.
2. 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*. 66:35-46.
3. Kingsbury AE, Foster OJ, Nisbet AP, Cairns N, Bray L, Eve DJ, et al. (1995): Tissue pH as an indicator of mRNA preservation in human post-mortem brain. *Brain ResMolBrain Res*. 28:311-318.
4. Stan AD, Ghose S, Gao XM, Roberts RC, Lewis-Amezcu K, Hatanpaa KJ, et al. (2006): Human postmortem tissue: what quality markers matter? *Brain Res*. 1123:1-11.
5. Hill C, Keks N, Roberts S, Opeskin K, Dean B, Mackinnon A, et al. (1996): Problem of diagnosis in postmortem brain studies of schizophrenia. *AmJPsychiatr*. 153:533-537.
6. 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*. 32:73-76.

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Supplementary Table 1: Demographic, antipsychotic and anticholinergic drug treatment as well as CNS collection data on cases with a muscarinic deficit form of schizophrenia (MRDS), schizophrenia with no loss of cortical muscarinic receptors (non-MRDS) and controls used in this study of muscarinic M1 receptors.

	Age (yr)	Sex	PMI (hr)	pH	DI (yr)	Sui	Cause of Death	FRAD	FRADD*	Anticholinergic drug	Hippo
Controls											
	75	M	69.4	6.19		N	Cardiogenic shock			N	
	52	M	31.8	6.75		N	Ventricular fibrillation			N	
	52	M	33.75	6.52		N	Cardiomegaly			N	
	66	F	49.25	6.44		N	Infrarenal atherosclerosis			N	
	52	M	50	6.78		N	Ischaemic heart disease			N	
	66	M	71.75	6.47		N	Coronary artery atheroma			N	
	36	M	42	6.46		N	Crush accident			N	✓
	68	F	38	6.32		N	Acute asthma			N	
	25	M	24	6.42		N	Electrocution			N	✓
	42	M	63	6.34		N	Cardiomegaly			N	✓
	21	F	58	6.03		N	Myocarditis			N	✓
	21	M	40	5.82		N	Acute epiglottitis			N	✓

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MRDS

21	F	56	6.24	2	Y	Carbon monoxide poisoning	Haloperidol	NR	Y	✓
25	M	49	6.38	2	Y	Overdose	Trifluoperazine	200	N	
53	M	37	5.98	30	N	Intestinal ischemia	Fluphenazine Chlorpromazine	1700	Y	✓
44	M	32	6.28	23	N	Ischaemic heart disease	Thioridazine	600	N	
71	M	48	6.45	53	N	Aspiration: food	Thioridazine	150	Y	✓
53	M	43	6.23	7	N	Aspiration: food	Chlorpromazine	200	N	
69	M	44.5	6.38	47	N	Ischaemic heart disease	Trifluoperazine	100	Y	
68	F	42	5.73	40	N	Ischaemic heart disease	Trifluoperazine	400	Y	
22	M	37	6.17	3	Y	Overdose	Pimozide	200	N	
65	F	50	6.35	18	N	Ruptured abdominal aneurysm	Fluphenazine, haloperidol	550	Y	✓
41	M	31	6.2	11	Y	Combined drug toxicity	Fluphenazine Trifluoperazine	500	N	✓
53	M	9	6.29	9	N	Coronary artery atheroma	Trifluoperazine Chlorpromazine	300	N	✓

Non-MRDS

47	M	32.5	6.41	27	N	Ischaemic heart disease	Fluphenazine Thioridazine	530	N	
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27	M	22	6.28	8	Y	Burning	Chlorpromazine Pimozide	1200	N	
72	F	58.5	6.48	37	N	Aspiration: Pneumonia	Chlorpromazine	25	N	
23	M	42.5	6.4	6	Y	Hanging	Haloperidol	1750	Y	✓
48	M	41.5	6.52	21	Y	Multiple injuries	Chlorpromazine Haloperidol	1400	N	✓
38	M	50	6.02	4	N	Meningoencephalitis	Clozapine	100	Y	
35	F	15	6.26	7	N	Coronary artery thrombosis	Haloperidol	300	Y	✓
55	M	25	6.1	33	N	Coronary artery atheroma	Thioridazine	400	Y	✓
48	F	52.5	6.21	22	N	Pulmonary thromboembolism	Fluphenazine Chlorpromazine	700	N	✓
65	M	41	6.57	35	N	Ischaemic heart disease	Fluphenazine	150	N	✓
65	M	42	6.29	36	N	Bronchopneumonia	Trifluoperazine Haloperidol	460	Y	
53	M	42	6.17	11	N	Metastatic cancer			N	✓

Abbreviations: DI = duration of illness, FRAD = final recorded antipsychotic drug, FRADD* = final recorded drug dose as chlorpromazine equivalents per day,

✓ = cases with hippocampal tissue, hr = hour, PMI = postmortem interval, N = no, Sui = suicide completion, Y = yes, yr = year

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Supplementary Table 2: Full statistical analyses comparing numbers of muscarinic receptor positive neurons (CHRM+), total number of neurons and total number of glia in Brodmann's area (BA) 9 and 17 as well as the medial dorsal nucleus of the thalamus and regions of the hippocampus from subjects with schizophrenia (Sz), muscarinic receptor deficit schizophrenia (MRDS) and non-muscarinic receptor deficit schizophrenia (non-MRDS) compared to controls.

Measurement	CNS Region	Sz vs Controls			ANOVA			MRDS vs Non-MRDS vs Controls				
		t	d.f.	p	F	d.f.	p	Dunnet's				
CHRM1+	BA 9	Layer 3	3.71	34	0.00	8.66	2,33	0.009	MRDS	4.13	33	0.0005
			non-MRDS	2.46	33	0.04						
	Layer 5	4.48	34	< 0.0001	13.59	2,33	<0.0001	MRDS	5.19	33	0.0001	
		non-MRDS	2.99	33	0.0099							
	BA 17	Layer 3	3.06	34	0.00	7.10	2,33	0.003	MRDS	3.76	33	0.001
			non-MRDS	1.77	33	0.15						
		Layer 5	4.00	34	0.00	8.81	2,33	0.0009	MRDS	4.98	33	0.0005
			non-MRDS	2.89	33	0.013						

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	Thalamus		0.58	34	0.56	0.17	2,33	0.84
	Hippocampus	Dentate Gyrus	1.67	16	0.11	1.74	2,15	0.21
		CA1	1.28	16	0.22	1.30	2,15	0.3
		CA2	1.35	16	0.20	0.96	2,15	0.4
		CA3	1.62	16	0.16	2.31	2,15	0.13
Total	BA 9	Layer 3	1.10	34	0.28	0.78	2,33	0.46
Neurons		Layer 5	1.17	34	0.25	1.48	2,33	0.24
	BA 17	Layer 3	0.04	34	0.97	2.39	2,33	0.11
		Layer 5	0.72	34	0.47	0.26	2,33	0.77
	Thalamus		0.30	34	0.76	0.24	2,33	0.79
	Hippocampus	Dentate Gyrus	0.69	16	0.5	0.29	2,15	0.75
		CA 1	2.54	16	0.02	3.02	2,15	0.08
		CA2	1.64	16	0.12	1.27	2,15	0.31
		CA 3	0.76	16	0.46	0.30	2,15	0.74

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Total Glia	BA 9	Layer 3	0.17	34	0.86	0.55	2,33	0.58				
		Layer 5	0.14	34	0.89	0.53	2,33	0.54				
	BA 17	Layer 3	0.36	34	0.72	3.56	2,33	0.04	MRDS	1.66	33	n.s.
		Layer 5	0.16	34	0.88	0.74	2,33	0.48	non-MRDS	0.98	33	n.s.
	Thalamus		0.79	34	0.43	1.86	2,33	0.17				
	Hippocampus	Dentate Gyrus	0.72	16	0.48	0.29	2,15	0.75				
		CA1	1.10	16	0.29	0.98	2,15	0.39				
		CA2	0.05	16	0.96	0.33	2,15	0.72				
		CA 3	0.74	16	0.47	0.32	2,15	0.73				

Abbreviation: CA = Cornu Ammonis

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Supplementary Table 3: Relationship between demographic, antipsychotic drug treatment and CNS collection data to the number of muscarinic M1 receptor positive neurons, total neurons and total glia in Brodmann's areas 9 and 17, the medial dorsal nucleus and hippocampus.

		Age		PMI		pH		DI		FRADD	
CNS Region		r ²	p	r ²	p	r ²	p	r ²	p	r ²	p
BA 9	CHRM1+ neurons	< 0.01	0.61	< 0.01	0.72	0.17	0.01	0.02	0.54	0.07	0.23
Layer III	total neurons	< 0.01	0.74	< 0.01	0.58	< 0.01	0.92	< 0.01	0.92	0.05	0.30
	total glia	< 0.01	0.85	< 0.01	0.70	< 0.01	1.00	0.07	0.22	0.02	0.48
BA 9	CHRM1+ neurons	0.03	0.25	< 0.01	0.87	0.09	0.07	0.03	0.43	0.02	0.50
Layer V	total neurons	0.05	0.20	0.02	0.45	0.03	0.30	0.05	0.31	< 0.01	0.94
	total glia	0.03	0.30	0.01	0.48	< 0.01	0.94	< 0.01	0.94	0.07	0.23
BA 17	CHRM1+ neurons	0.01	0.55	< 0.01	0.74	0.41	< 0.0001	0.01	0.61	0.02	0.57
Layer III	total neurons	< 0.01	0.78	< 0.01	0.80	< 0.01	0.60	0.05	0.30	< 0.01	0.99
	total glia	< 0.01	0.77	< 0.01	0.84	< 0.01	0.58	0.02	0.52	< 0.01	0.90
BA 17	CHRM1+ neurons	< 0.01	0.71	< 0.01	0.99	0.44	< 0.0001	< 0.01	0.90	< 0.01	0.67
Layer V	total neurons	< 0.01	0.68	0.01	0.56	0.45	0.21	0.01	0.62	< 0.01	0.90
	total glia	< 0.01	1.00	0.02	0.39	< 0.01	0.65	0.02	0.50	< 0.01	0.85
MDN	CHRM1+ neurons	0.31	< 0.001	< 0.01	0.94	0.11	0.05	0.06	0.23	0.22	< 0.05

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	total neurons	0.21	< 0.01	0.02	0.43	0.04	0.24	0.02	0.48	0.24	< 0.05
	total glia	< 0.01	0.63	< 0.01	0.62	< 0.01	0.59	< 0.01	0.73	0.02	0.55
Hippocampus	CHRM1+ neurons	0.42	0.42	< 0.01	0.79	< 0.01	0.87	< 0.01	0.82	0.09	0.37
CA1	total neurons	< 0.01	0.93	0.05	0.36	< 0.01	0.78	0.08	0.33	< 0.01	0.87
	total glia	0.99	0.20	0.21	0.05	0.04	0.43	0.05	0.45	< 0.01	0.86
Hippocampus	CHRM1+ neurons	0.09	0.22	0.01	0.63	0.03	0.46	0.08	0.35	< 0.01	0.92
CA2	total neurons	0.09	0.23	0.03	0.46	< 0.01	0.89	0.18	0.15	0.15	0.25
	total glia	< 0.01	0.94	0.12	0.17	0.01	0.66	< 0.01	0.78	0.05	0.51
Hippocampus	CHRM1+ neurons	0.14	0.12	< 0.01	0.95	< 0.01	0.94	0.19	0.14	0.05	0.51
CA3	total neurons	0.03	0.51	0.09	0.24	< 0.01	0.86	0.01	0.70	0.04	0.56
	total glia	0.02	0.54	0.21	0.05	0.02	0.54	0.02	0.65	0.09	0.38
Hippocampus	CHRM1+ neurons	0.05	0.40	< 0.01	0.77	0.01	0.68	0.07	0.38	0.08	0.40
Poly	total neurons	< 0.01	0.98	< 0.01	0.94	0.03	0.50	< 0.01	0.96	0.25	0.12
	total glia	0.03	0.48	0.17	0.09	0.06	0.34	0.04	0.52	0.20	0.17

Abbreviations: BA = Brodmann's area, CA = Cornu Amonis, CHRM1+ neurons = anti-muscarinic receptor 1 positively stained neurons, FRADD = final recorded antipsychotic drug dose as mg clorpromazine equivalents per day, DI = duration of illness, MDN = medial dorsal nucleus of the thalamus, PMI = postmortem interval, Poly = polymorphic layer of the dentate gyrus.

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Supplementary Table 4: Cohort sizes required to give a significant difference of $p < 0.05$ in CA1, 2, 3 and the polymorphic layer (POLY) of the dentate gyrus from subjects with MRDS and non-MRDS compared to controls

	Controls	MRDS	non-MRDS
CA1	151	25	151
CA2	27	15	27
CA3	76	14	76
POLY	30	11	30

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