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NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - ORIGINAL ARTICLE

Muscarinic receptor binding changes in postmortem Parkinson's disease

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Abstract Parkinson's disease (PD) is a devastating disorder, affecting approximately 2% of people aged 60 and above. It is marked by progressive neurodegeneration that has long been known to impact dopaminergic cells and circuits, but more recently the acetylcholine system has also been implicated in the complex aetiology and symptomatology of the disease. While broad changes in cholinergic markers have been described, insight into the contribution of specific acetylcholine receptors is less clear. To address this important unknown, in this study we performed [^{3}H] pirenzepine, [^{3}H] 4DAMP, and [^{3}H] AF-DX 384 in situ radioligand binding on postmortem tissues from Brodmann's area 6, 9, 46, and the caudate putamen, from PD and matched controls to detect muscarinic M1, M3, and M1/2/4 receptors, respectively. We found no difference in [^{3}H] pirenzepine binding between PD and controls across all regions assessed. [^{3}H] 4DAMP binding was found to be higher in PD CPu and BA9 than in controls. [^{3}H] AF-DX 384 was higher in BA9 of PD compared with controls. In sum, we show selective increase in M3 receptors in cortical and subcortical regions, as well as increased M2/M4 in cortical area BA9, which together support a role for cholinergic dysfunction in PD.

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Introduction

Parkinson's disease (PD) is a progressive degenerative disorder of the central nervous system (CNS), characterized by motor, psychiatric, and cognitive impairment. While an impaired dopaminergic system explains some aspects of the symptomatology, the pathophysiology of the full spectrum is not yet delineated and therefore, there is increasing interest in the role of other neurotransmitter systems in the genesis of the symptoms of the disorder. Both cognition and motor perseveration are affected in early PD, and it is notable that both are modulated by the cholinergic system (Stoffers et al. 2001). Hence, there have been efforts to identify changes in markers of the cholinergic system in PD as a step toward understanding how cholinergic function may be affected.

Postmortem studies in the CNS from subjects with PD have investigated the presynaptic marker choline acetyltransferase (ChAT) as well as the two families of receptors that are targeted by acetylcholine; the nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). The nAChRs are ligand gated ion channels found throughout the brain in homomeric or heteromeric conformations. Studies have reported lower levels of nAChRs in cortex, hippocampus, substantia nigra, and striatum of PD subjects (Aubert et al. 1992; Perry et al. 1990; Pimlott et al. 2004). These decreases have been attributed to a loss of neuronal integrity and are most pronounced for α6β2* nAChRs (reviewed in Quik et al. 2011). The other family of cholinergic receptors is the muscarinic acetylcholine

receptors (mAChRs), made up of five G protein-coupled receptors (M1–M5). Significantly, neuroimaging studies using a non-selective radioligands have reported higher mAChRs in frontal cortex (Asahina et al. 1998) and occipital lobe (Colloby et al. 2006) in PD. These data are supported by some studies in postmortem CNS (Lange et al. 1989, 1993; Perry et al. 1990; Ruberg et al. 1982). However, the contribution of the specific mAChRs subtypes is less clear, as relatively few investigations have attempted to determine whether all mAChR are differentially expressed in PD, or whether select subtypes underlie these changes (Aubert et al. 1992; Joyce 1993; Perry et al. 1990; Rinne et al. 1989). Importantly, muscarinic antagonists can be clinically effective in PD (Fahn et al. 1990), highlighting the relevance of further investigations into this receptor (Dencker et al. 2012).

We have carried out extensive studies on muscarinic receptors in the CNS from subjects with schizophrenia and have used varying assay conditions and tissue from muscarinic receptor knock-out mice to increase the specificity of binding for commonly used muscarinic receptor ligands. Thus, in the assays we use we have been able to increase the specificity of [^{3}H]pirenzepine binding to the muscarinic M1 receptor in human CNS tissue to ~80% (Gibbons et al. 2013; Scarr and Dean 2008) (Crook et al. 2001) and to make [^{3}H]4DAMP binding very selective for the muscarinic M3 receptor (Jeon et al. 2013). In the present study, we used these methodologies along with a non-modified [3H]AF-DX protocol to determine if there are subtype specific changes in levels of M1, M2/M4, and M3 subtypes of mAChRs in brain regions associated with PD pathology and symptomatology.

Methods

Human tissue collection

Following consent from the Ethics Committee of the Victorian Institute of Forensic Medicine and the Mental Health Research and Ethics Committee of Melbourne Health, postmortem samples were obtained from the Victorian Brain Bank Network (VBBN). The left hemispheres of human CNS were collected postmortem, rapidly sliced, and frozen to −80 °C as described previously (Dean et al. 1999).

All patients came to the clinic with a diagnosis of PD. Postmortem neuropathological diagnosis of PD was made using the standard criteria of neuronal loss, pigment incontinence, and the presence of α-synuclein reactive Lewy Bodies in the substania nigra. All patients came to VBBN with a clinical diagnosis of PD. None of the subjects were diagnosed with associated Alzheimer's disease, but most had scattered Amyloid beta plaques, as is commonly observed with aging. 8 of the 11 cases also presented with Lewy bodies in the cortex. These were limited in number, and the clinical significance is difficult to predict.

Brodmann's Area 6 (BA6), BA9, BA46, and Caudate Putamen (CPu) were selected for study based upon their known involvement in PD, their anatomy, or involvement in perseverative, cognitive behavior.

BA6 was defined as the caudal superior frontal gyrus and the middle frontal gyrus extending from the cingulate sulcus on the medial surface to the lateral sulcus on the lateral surface. BA9 (bridging the dorsolateral prefrontal cortex and the medial prefrontal cortex) was bounded by the lateral surface of the frontal lobe, comprising the middle frontal gyrus superior to the inferior frontal sulcus. Dorsolateral prefrontal cortical BA46 was defined as within the lateral surface of the frontal lobe approximately constrained to the middle third of the middle frontal gyrus and the most rostral inferior frontal gyrus. Caudate putamen is a distinct region.

Cadavers were stored at 4 °C within 5 h of death. During case history, review age at death, gender, post mortem interval (PMI), and brain pH (Kingsbury et al. 1995) were determined (see Table 1). With regard to PMI, where death was witnessed, PMI was from time of death to autopsy, but if the death was not witnessed, time of death was taken as the mid-point between the time the donor was last observed alive (maximum of 5 h was allowed), and the time found dead. Cohorts were matched as closely as possible for age, PMI, and brain pH.

Autoradiographic ligand binding

Autoradiography was performed upon 20 µm sections cut using a Leica cryostat onto gelatinised slides. Frozen sections were stored at −70 °C. On the day of the experiment, sections were removed from −70 °C and air-dried at room

Table 1 Cohort demographics

	CTL ($N = 10$)	PD ($N = 10$)	P value	T value
Age (years)	76.76 (1.89)	76.55 (1.74)	0.94	0.02
Male:female	5:5	5:5		
PMI (h)	32.85 (5.81)	35.85 (5.10)	0.70	0.39
pH	6.22 (0.06)	6.52 (0.06)	<0.01	3.49

Our experimental design meant that PD patients were matched with control subjects, according to age, sex, and PMI. pH was found to be significantly higher in PD subjects than in controls. All continuous variables are presented as mean (standard error of the mean)

Control (CTL), Parkinson's disease (PD), postmortem interval (PMI)

temperature for 1 h prior to radioligand specific protocols (described below). Following the final wash, slides were dried under a stream of cool air, and fixed overnight in a desiccator containing paraformaldehyde powder. Slides were then apposed to phosphorimaging plates. Microscales were used to provide a frame of reference for measuring radioactivity, and allowing us to normalize values across plates and across experiments. After exposure, plates were scanned with a BAS5000, and density was established using AIS software.

[^{3}H]pirenzepine autoradiography

The methodology use for [^{3}H]pirenzepine binding was modified to increase selectivity for the muscarinic M1 receptor to ~80% (Gibbons et al. 2013; Scarr and Dean 2008); thus, frozen tissue sections were incubated in 15 nM [^{3}H]Pirenzepine ([^{3}H]PZP) in assay buffer (10 mM KH_2PO_4, 10 mM Na_2PO_4, pH 7.4) in the absence [total binding (TB)] or presence [non-specific binding (NSB)] of 1 mM 3-quinuclidinyl xanthene-9-carboxylate hemioxalate hydrate (QNX) (a broad spectrum muscarinic receptor binding ligand to block non-specific binding). Following this incubation, sections were washed twice for 2 min in ice-cold assay buffer, dipped in ice-cold ddH_2O. Slides were apposed to phosphorimaging plates with microscales for 3 days.

[^{3}H]4DAMP

The [^{3}H]-DAMP methodology used in this study was developed to increase selectivity of the radioligand for the muscarinic M3 receptor (Jeon et al. 2013). Hence, sections were preincubated for 15 min in assay buffer (50 mM Tris–HCl, pH7.4), rinsed in ddH_2O, and air-dried at room temperature for 1 h. Subsequently, sections were incubated in assay buffer containing 6 nM [^{3}H]4DAMP + 1uM PZP (to block non-specific binding to M1/M4) in the absence (TB) or presence (NSB) of 10 μM 4DAMP mustard for 60 min at room temperature. Slides were then washed twice for 5 min in ice-cold assay buffer, and dipped in ice-cold ddH_2O for 15 s. Slides were apposed to phosphorimaging plates for 3 days.

[^{3}H]AF-DX 384

The [^{3}H]AF-DX 384 protocol used in this study detects M1, M2, and M4 receptors. Sections were preincubated for 30 min in Assay Buffer (10 mM KH_2PO_4, 10 mM Na_2PO_4, pH 7.4) at room temperature, rinsed in ddH_2O and air-dried. Sections were incubated in assay buffer containing 7 nM [^{3}H]AF-DX 384 in the absence (TB) or presence of 1 μM tropicamide (NSB) for 60 min at room temperature, washed twice for 2 min each in ice-cold assay buffer, and dipped in ice-cold water (15 s). Slides were apposed to plates for 7 days.

Statistical analyses

Data were analysed using the D'Agostino Pearson normality test, to determine Gaussian distribution. Pearson's correlation analysis was performed to determine whether binding in different brain regions was independent, and to identify covariates (PMI, age, and pH). An R^2 value greater than 0.49 was considered a strong relationship (Cook and Weisberg 1999). *T* tests were used to determine whether gender had a significant association with binding levels. A *P* value of <0.05 was considered significant.

Significant correlations were shown between regions with each ligand. Given that regions were not independent variables, *T* tests were thus performed for each region, to determine the influence of diagnosis over binding. If a group failed the normality test, diagnostic differences were analysed by Mann–Whitney test. Where an overall effect was detected, Cohen's *d* effect size was calculated. Data are represented as mean ± SEM. With the exception of Cohen's d, all statistical analyses were performed using Prism 6.0. Cohen's d was calculated using an online tool available at http://www.cognitiveflexibility.org, and ANCOVA was performed in minitab.

Results

Cohort demographics

The cohort comprised of ten control subjects (five male and five female) and ten subjects who had a diagnosis of PD (five male and five female) (Tables 1, 2). Age at death did not differ according to diagnosis, or did PMI. Variance also did not differ according to diagnosis (Age $F = 1.181$, $P = 0.8087$, PMI $F = 1.298$, $P = 0.7042$).

pH was significantly higher ($P < 0.01$, $t = 3.494$, $df = 18$) in the PD cohort (6.52 ± 0.06) than in controls (6.22 ± 0.06). Variance did not differ between groups ($P = 0.76$, $F = 1.23$).

[^{3}H]PZP

In BA6, [^{3}H]PZP binding did not vary between controls and PD ($t = 0.7085$, $df = 18$, $P = 0.4428$)(Fig. 1a); *F* tests to compare variance showed no significant

Table 2 Clinical scores for Parkinson's disease patients

#	LED	Cog	MMSE	H&Y	Fluc	DK	AT
1	800	N	29/30	IV	+	+	–
2	1250	N	26/30v	V	+	+	+
3	600	Ab	21/30	III	++	–	–
4	800	N	–	III	+	–	–
5	400	Ab	20/30	IV	++	–	–
6	1900	Ab	17/30	V	++	+	–
7	600	N	–	IV	+	+	–
8	800	N	28/30	III	+	+	–
9	1100	Ab	19/30	V	++	+	Apo
10	525	N	–	IV	+	+	–

LED levodopa equivalent dose; Cog, *N* normal; *Ab* abnormal; *MMSE* mini mental state examination (where available >24 = normal cognition); *H&Y* Hoehn and Yahr Scale, Fluct-Fluctuation; *DK* dyskinesia; + mild; ++++ severe; *AT* advanced therapy; *DBS* deep brain stimulators; *Apo* Apomorphine infusion

difference ($F = 1.009$, DFn = 9, Dfd = 9, $P = 0.99$). In BA9, [^{3}H]PZP binding was similar in PD and controls (Fig. 1b, Mann–Whitney test: $P = 0.28$). In BA46, [^{3}H]PZP binding was similar in PD and controls (Fig. 1c, $t = 1.062$, $df = 18$, $P = 0.30$). Variance also did not differ between diagnostic groups ($F = 1.559$, DFn = 9, Dfd = 9, $P = 0.52$). [^{3}H]PZP binding in the CPu was similar in PD and controls (Fig. 1d, Mann–Whitney: $P = 0.89$) (Table 3).

Sex, age, pH, or PMI and did not influence [^{3}H]PZP binding in any of the regions assessed (Table 4).

[^{3}H]4DAMP

[^{3}H]4DAMP binding did not differ by diagnosis in BA6 ($t = 1.67$, $df = 18$, $P = 0.11$ (Fig. 2a). F tests to compare variance showed no significant difference ($F = 1.02$, DFn = 9, Dfd = 9, $P = 0.97$). In BA9, [^{3}H]4DAMP binding was significantly higher in PD than in controls (Fig. 2b, $t = 3.37$, $df = 18$, $P < 0.01$, Cohen's $d = -4.77$). Variances did not differ between groups ($F = 1.06$, DFn = 9, Dfd = 9, $P = 0.93$). In BA46, [^{3}H]4DAMP binding was similar in controls and PD subjects (Fig. 2c, $t = 1.35$, $df = 18$, $P = 0.19$). Variance did not differ between diagnostic groups ($F = 1.55$, DFn = 9, Dfd = 9, $P = 0.53$). In the CPu (Fig. 2d), [^{3}H]4DAMP binding was significant higher in PD compared to controls ($t = 2.30$, $df = 18$, $P < 0.05$, Cohen's $d = -3.28$). No difference in variance was detected ($F = 1.71$, Dfn = 9, Dfd = 9, $P = 0.44$) (Table 3).

There were no strong correlations between [^{3}H]4DAMP binding and sex, age, pH, or PMI in any region (Table 4).

[^{3}H]AF-DX 384

BA6 [^{3}H]AF-DX 384 binding was similar in PD and controls (Fig. 3a, $t = 1.90$, $df = 18$, $P = 0.07$). F tests to compare variance showed no significant difference ($F = 1.09$, DFn = 9, Dfd = 9, $P = 0.89$). In BA9, AF-DX 384 binding levels were significantly higher in the PD diagnostic group than in control subjects (Fig. 3b, $t = 2.60$, $df = 18$, $P = 0.02$, Cohen's $d = -3.67$). Variances did not differ between groups ($F = 1.06$, DFn = 9,

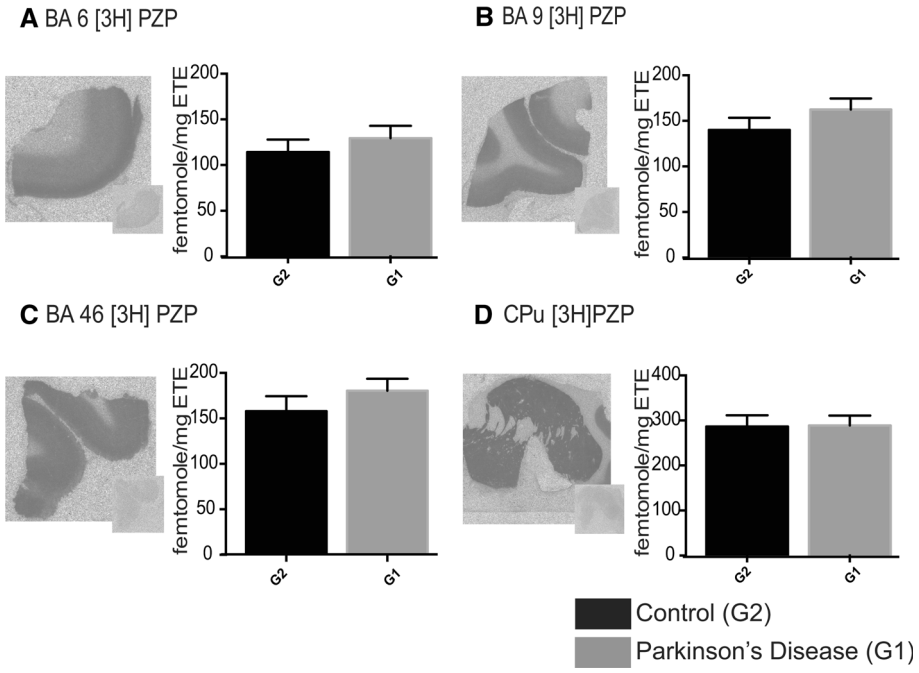

Fig. 1 [^{3}H]Pirenzepine binding in Parkinson's disease and controls. [^{3}H]Pirenzepine (PZP) binding was calculated by subtracting non-specific binding (*inset images*) from total binding. [^{3}H]PZP binding was not different in Parkinson's disease compared with controls in BA 6 (**a**), BA 9 (**b**), BA 46 (**c**), or CPu (**d**)

Table 3 [^{3}H]PZP, [^{3}H]AF-DX 384, and [^{3}H]4DAMP binding in Parkinson's disease and controls

Ligand	Region	CTL fmole/ETE (mean ± SEM)	PD fmole/ETE (mean ± SEM)	*P* value	*t* value
PZP	BA6	114.3 ± 13.57	129.4 ± 13.63	0.44	0.78
	BA9	140.0 ± 13.30	162.3 ± 12.17	0.28	(Mann–Whitney)
	BA46	157.90 ± 16.56	180.4 ± 13.26	0.30	1.06
	CPu	286.4 ± 25.09	289.0 ± 21.83	0.89	(Mann–Whitney)
AF-DX 384	BA6	62.08 ± (5.873)	77.54 ± (5.61)	0.07	1.90
	BA9	69.84 (±4.97)	88.35 ± 5.11	0.02*	2.60
	BA46	77.68 ± (6.41)	85.39 ± 4.66	0.34	0.97
	CPu	125.1 ± 9.510	137.9 ± 7.92	0.31	1.03
4DAMP	BA6	30.21 (2.576)	36.35 (2.609)	0.11	1.67
	BA9	43.24 (2.749)	56.15 (2.669)	0.003**	3.37
	BA46	35.04 (2.391)	40.20 (2.974)	0.19	1.35
	CPu	48.03 (3.048)	59.56 (3.988)	0.034*	2.30

In situ radioligand binding was performed in BA6, BA9, BA46, and CPu of Parkinson's disease (PD) and control (CTL) subjects. No differences were observed between groups in [^{3}H]PZP binding. [^{3}H]AF-DX 384 was found to be higher in BA9 of PD subjects, than in controls ($P < 0.05$, $t = 2.59$, Cohen's $d = -3.67$). [^{3}H]4DAMP binding was higher for the PD group in both BA9 ($P < 0.01$, $t = 3.37$, Cohen's $d = -4.8$) and CPu ($P < 0.05$, $t = 2.30$, Cohen's $d = -3.30$)

* $P < 0.05$, ** $P < 0.01$

Table 4 Correlation analysis for [^{3}H]PZP, [^{3}H]AF-DX 384, and [^{3}H]4DAMP binding in Parkinson's disease and controls

Ligand	Region	Age	pH	PMI	Sex
PZP	BA6	$R^2 = 0.01$	$R^2 = 0.25$	$R^2 = 0.06$	$P = 0.28$
		$P = 0.80$	$P = 0.03$	$P = 0.28$	
	BA9	$R^2 = 0.07$	$R^2 = 0.25$	$R^2 = 0.0903$	$P = 0.76$
		$P = 0.26$	$P = 0.03$	$P = 0.1980$	
	BA46	$R^2 = 0.05$	$R^2 = 0.1672$	$R^2 = 3.289e\text{-}005$	$P = 0.61$
		$P = 0.34$	$P = 0.07$	$P = 0.9809$	
	CPu	$R^2 = 0.07$	$R^2 = 0.18$	$R^2 = 0.06$	$P = 0.95$
		$P = 0.25$	$P = 0.06$	$P = 0.28$	
AF-DX 384	BA6	$R^2 = 0.01$	**$R^2 = 0.42$**	$R^2 = 0.05$	$P = 0.37$
		$P = 0.94$	**$P = 0.0018$**	$P = 0.34$	
	BA9	$R^2 = 0.04$	**$R^2 = 0.49$**	$R^2 = 0.08$	$P = 0.76$
		$P = 0.41$	**$P = 0.0005$**	$P = 0.22$	
	BA46	$R^2 = 0.01$	$R^2 = 0.24$	$R^2 = 0.02$	$P = 0.42$
		$P = 0.68$	$P = 0.03$	$P = 0.53$	
	CPu	$R^2 = 0.02$	$R^2 = 0.22$	$R^2 = 0.02$	$P = 0.72$
		$P = 0.56$	$P = 0.04$	$P = 0.57$	
4-DAMP	BA6	$R^2 = 0.04$	$R^2 = 0.26$	$R^2 = 0.001$	$P = 0.12$
		$P = 0.42$	$P = 0.02$	$P = 0.88$	
	BA9	$R^2 = 0.001$	$R^2 = 0.15$	$R^2 = 0.002$	$P = 0.69$
		$P = 0.88$	$P = 0.09$	$P = 0.84$	
	BA46	$R^2 = 0.02$	$R^2 = 0.14$	$R^2 = 0.001$	$P = 0.71$
		$P = 0.56$	$P = 0.10$	$P = 0.89$	
	CPu	$R^2 = 0.007$	$R^2 = 0.11$	$R^2 = 0.04$	$P = 0.93$
		$P = 0.73$	$P = 0.16$	$P = 0.41$	

Pearson's correlation analysis to identify covariates showed that no significant relationship existed between [^{3}H]PZP, or [^{3}H]4DAMP and PMI, age, and pH. [^{3}H]AF-DX 384 binding showed a significant relationship, as defined by and $r^2 > 0.49$ and $P < 0.05$, with pH in BA9. A trend towards a relationship was also detected in BA6

Significant and trending relationships are indicated in bold

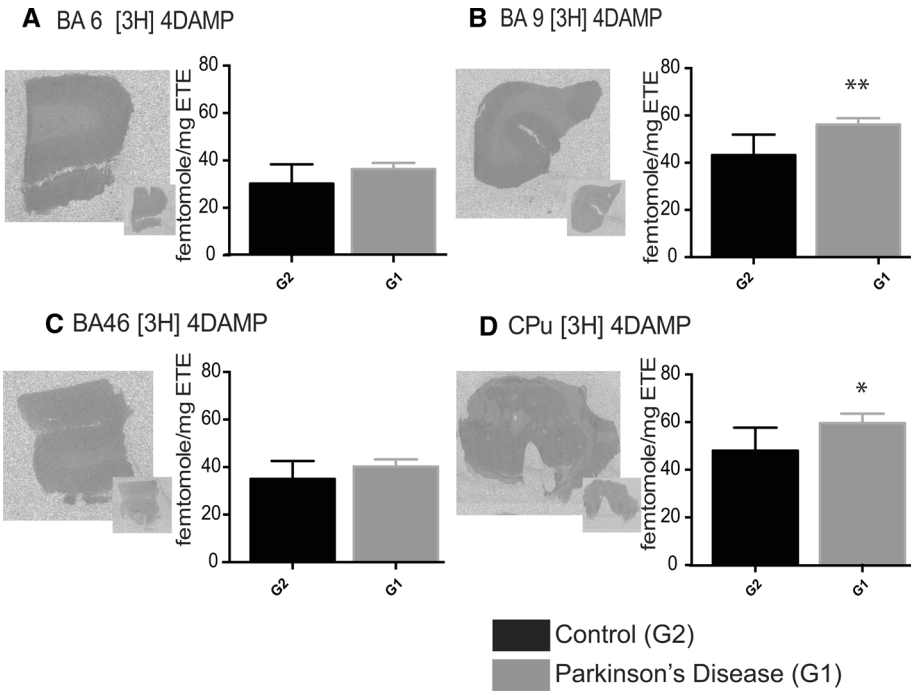

Fig. 2 [^{3}H]4DAMP binding in Parkinson's disease and controls. [^{3}H]4DAMP binding was calculated by subtracting non-specific binding (*inset images*) from total binding. Binding was significantly higher in Parkinson's disease (PD) group in BA 9 relative to control (**b**, $P < 0.01$). Higher binding was also detected in the caudate putamen (**d**, $P < 0.05$) of PD, compared to controls. No changes were observed in BA 6 (**a**), or in BA 46 (**c**)

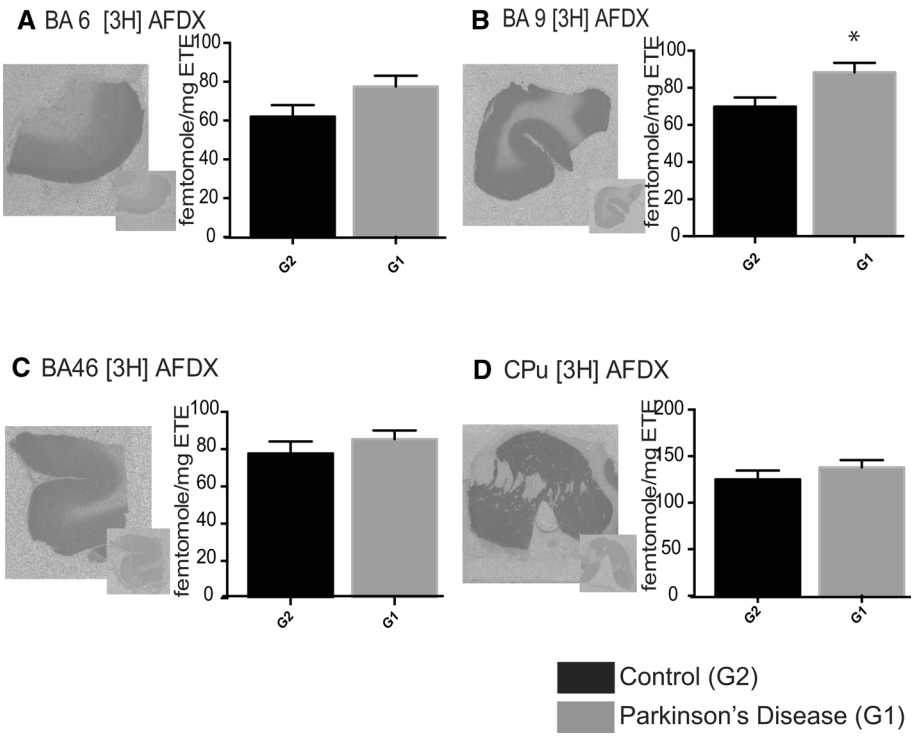

Fig. 3 [^{3}H]AF-DX 384 binding in Parkinson's disease and controls. [^{3}H]AF-DX 384 binding was calculated by subtracting non-specific binding (*inset images*) from total binding. We observed significantly higher [^{3}H]AF-DX 384 binding in BA 9 in Parkinson's disease compared to controls (**b**, $P < 0.05$). No differences were observed in BA 6 (**a**), BA 46 (**c**), or the caudate putamen (CPu) (**d**)

Dfd = 9, $P = 0.93$). In BA46, no significant differences were detected for AF-DX 384 binding (Fig. 3c, $t = 0.97$, $df = 18$, $P = 0.34$). Variance did not differ between diagnostic groups ($F = 1.90$, DFn = 9, Dfd = 9, $P = 0.38$). No difference in AF-DX 384 binding was observed in the CPu (Fig. 3d), and no difference in variance was detected ($F = 1.43$, Dfn = 9, Dfd = 9, $P = 0.60$) (Table 3).

Again, there were no strong correlations between sex, age, and PMI and [^{3}H]AF-DX 384 binding in any CNS region. There was a significant relationship between AF-DX 384 binding and pH in BA9 (Table 4). ANCOVA was

performed and significance by diagnosis was lost but still was a strong trend ($P = 0.054$).

Discussion

Dysregulation of the cholinergic system is emerging as a potential pathophysiological mechanism in PD. In this study, we showed higher levels of [^{3}H]4-DAMP and [^{3}H]AF-DX 384 binding in BA9 and higher [^{3}H]4-DAMP binding in CPu. We have shown that [^{3}H]4DAMP binding under the conditions used in our study is selective for the muscarinic M3 receptor (Jeon et al. 2013). We have also shown that under the conditions used this study [^{3}H]AF-DX 384 would bind to the muscarinic M1 as well as the M2 and M4 receptors (Gibbons et al. 2013). Thus, together with the absence of change in [^{3}H]PZP binding to M1, these findings suggest higher M2, M3 and/or M4 in BA9, and higher M3 in CPu.

This conclusion can be further refined, with reference to the literature. Decreased ChAT has been reported in PD postmortem tissue in many regions (Hall et al. 2014; Mufson et al. 1991; Perry et al. 1986; Rinne et al. 1989) suggesting a loss of cholinergic neurons, accompanied by a loss of presynaptic muscarinic receptors. M2 and M4 receptors are both considered to localize presynaptically, acting as autoreceptors; however, M2 appears to predominate in this role in the cortex, while M4 does so in the CPu (Zhang et al. 2002). Our finding of increased [^{3}H]AF-DX 384 binding in BA9 may indicate an increase in M2, M4, or M2 and M4 in an attempt to compensate for lower levels of ACh linked to lower levels of innervating cholinergic neurons, as suggested by decreased ChAT.

Considerable evidence for changes in cholinergic function in PD has been generated in patients both with and without dementia; however, a consistent picture is yet to emerge (Asahina et al. 1998; Colloby et al. 2006; Hilker et al. 2005; Kuhl et al. 1996; Lange et al. 1989, 1993; Perry et al. 1990; Ruberg et al. 1982; Shinotoh et al. 1999). Aligning with our findings, studies using radioligand binding to tissue homogenates found M1/3/5 and M2/4 receptors to be increased in various brain regions, accompanied by a decrease in ChAT activity, and that these changes may depend upon the cognitive state of the patient (Perry et al. 1986) (Rinne et al. 1989). [^{3}H]PZP (Aubert et al. 1992) binding in cortical and subcortical regions has been shown to be similar in PD compared with controls (Aubert et al. 1992). In contrast, Aubert et al. (1992) showed decreased [^{3}H]AF-DX 384 binding in the cortex of PD (but comparable levels in the striatum). Studies performed using in situ radioligand binding, have reported unchanged [^{3}H]AF-DX 384 binding in cortical and subcortical regions in PD compared with controls (Piggott et al. 2003), as well as lower [^{3}H]PZP and [^{3}H]NMS binding in the CPu (Joyce 1993).

These divergent findings could result from methodological differences between studies. Aubert et al. performed saturation binding analysis on alternating hemispheres of BA9 and BA10 tissue homogenate to determine receptor level. We did not study BA10 and it is possible that mAChR density differs in the two hemispheres, which might explain the findings. Importantly, in the present study, the protocol for [^{3}H]PZP binding has been optimized for the detection of M1 receptors over other mAChRs and possesses several methodological differences from the older investigation that used fewer subjects, a lower concentration of [^{3}H]PZP, and an unspecified incubation time. Finally, the stage of PD and the presence or absence of dementia and/or neuropsychiatric symptoms at the time of death could very well affect mAChR expression; it has been proposed that increases in mAChRs are a compensatory mechanism that decelerates the development of cognitive symptoms. In this way, the changes described above may capture different symptomatological groups of patients; patients with cognitive impairment may show no changes, or decreases in mAChRs while increases may protect against the development of this symptom subset, at least transiently. Clinically, cognitive impairment has been proposed to arise from impaired ability to maintain a cognitive set, difficulty in switching tasks, and a tendency to perseverate (Alevriadou et al. 1999; Cools et al. 1984; Ebersbach et al. 1994); however, in the present study, the lack of data on cognitive performance of these patients prevents correlation between cognition and neurochemistry.

Interestingly, we found the levels of binding to M2, M3 and M4 receptors to be altered, whereas binding to M1, a receptor heavily implicated in cognition, was not. However, the importance of other cholinergic receptors in cognition should not be ignored. From a neuroanatomical perspective, in the present study, the greatest cumulative difference was observed in BA9, which showed higher levels of M2/M4 and M3 receptors, compared with controls. Consistent with a role for mAChR in the symptomatology of PD, BA9 has been implicated as having a role in many domains of cognition (MacDonald et al. 2000). The emphasis on the association between cholinergic denervation and cognition should not obscure the relationships between the cholinergic system and olfaction, sleep, affect, and motor function all of which are affected in PD. Our finding of increased M3 in the CPu may speak to the motor symptoms that develop as part of Parkinson's disease course, as this region has been convincingly shown to play a key role in this dysfunction (Shadmehr and Krakauer 2008).

Studies in knock-out mouse lines for all five mAChR paint a picture of intricate, regulated control over brain

function and behavior. Both M2 and M4 KO mice show mitigation of pharmacologically induced tremulous jaw movements, an endophenotype of parkinsonian tremor (Bymaster et al. 2001; Salamone et al. 2001). M2 receptors have also been implicated in cognition; M2 KO mice demonstrate significant deficits in behavioral flexibility and impairment in passive avoidance tasks (Tzavara et al. 2003). In contrast, M2 antagonism improved learning performances in both rats with scopolamine-induced amnesia and in aged memory-impaired rats (Doods et al. 1993; Quirion et al. 1995), highlighting the potential for compensatory changes to buffer imbalances in the cholinergic system. Behavioral studies have also implicated M3 in maintaining cognitive function (Fedoce et al. 2016; Poulin et al. 2010) however, little else is known about the role of central M3 receptors. Hence, if animal studies can be extrapolated to the human condition, the higher levels of M2 and/or M4 receptors in BA 9 might be involved in tremor and cognition, whereas changes in M3 are more focused on cognition.

We must also consider the relationship between PD, [^{3}H]AF-DX 384 binding, and pH observed in BA9. ANCOVA analyses suggest that the significance detected in this region may be, at least partly, associated with differences in pH across diagnosis; further experiments will be required to determine if changes in [^{3}H]AF-DX 384 binding relate to diagnosis or CNS pH.

The M3 is the least well characterized of the mAChRs and the present study, showing significant increases in [^{3}H]4-DAMP binding, is the only study that we are aware of describing altered expression of this receptor in PD. The changes in mAChR described in the present study are also likely to directly impact dopaminergic activity, exacerbating disruptions of dopaminergic tone in these regions (Zhang et al. 2002), further aggravating the symptomatology, and underscoring the interconnectedness of these two systems.

The classical hypothesis of acetylcholine's contribution to the pathophysiology of PD posits that change in acetylcholine is secondary to changes in dopamine; decreased dopaminergic tone in the striatum releases inhibition over cholinergic neurons, resulting in cholinergic increases (Emre et al. 2004). More recently, a 'dual syndrome hypothesis' proposed that two distinct streams of dysfunction arise from cholinergic or dopaminergic deficits, contributing to different subsets of symptoms. This latter theory is rapidly gaining ground, as research establishes AChR deficits early in the disorder, distinct from DA dysregulation; however, the relationship between dopaminergic and cholinergic deficits is likely to be much more complicated. Here, we show increased [^{3}H]AF-DX 384 binding to M2/M4 in BA9, and increased [^{3}H]4DAMP binding to M3 in BA9 and CPu, with no changes in [^{3}H]PZP. These findings support an important role for muscarinic acetylcholine receptors in the pathophysiology of PD, underscoring the need for further investigations into their contribution to disease development and progression.

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Compliance with ethical standards

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Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and all procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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