

Calcium Ca_v1 channel subtype mRNA expression in Parkinson's disease examined by *in situ* hybridization

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Abstract

The factors which make some neurons vulnerable to neurodegeneration in Parkinson's disease while others remain resistant are not fully understood. Studies in animal models of Parkinson's disease suggest that preferential use of Ca_v1.3 subtypes by neurons may contribute to the neurodegenerative process by increasing mitochondrial oxidant stress. This study quantified the level of mRNA for the Ca_v1 subtypes found in brain by *in situ* hybridization using Ca_v1 subtype specific [³⁵S]-radiolabelled oligonucleotide probes. In normal brain the greatest amount of mRNA for each Ca_v1 subtype was found in the midbrain (substantia nigra), with a moderate level in the pons (locus coeruleus) and lower quantities in cerebral cortex (cingulate and primary motor). In Parkinson's disease the level of Ca_v1 subtype mRNA was maintained in the midbrain and pons, despite cell loss in these areas. In cingulate cortex, Ca_v1.2 and Ca_v1.3 mRNA increased in cases with late stage Parkinson's disease. In primary motor cortex the level of Ca_v1.2 mRNA increased in late stage Parkinson's disease. The level of Ca_v1.3 mRNA increased in primary motor cortex of cases with early stage Parkinson's disease and normalized to near the control level in cases from late stage Parkinson's disease. The finding of elevated Ca_v1 subtype expression in cortical brain regions supports the view that disturbed calcium homeostasis is a feature of Parkinson's

disease throughout brain and not only a compensatory consequence to the neurodegenerative process in areas of cell loss.

Keywords: Parkinson's disease, calcium channel, brain, dihydropyridines, *in situ* hybridization

Introduction

Parkinson's disease is a neurodegenerative disorder which affects approximately 1% of people aged over 60 in the UK and up to 4% of individuals aged over 80 years old. Except for 10-20% of cases where a genetic mutation is linked to neurodegeneration, the cause of Parkinson's disease remains unknown, but it is believed to be multifactorial and a number of cellular deficits or mechanisms have been implicated; namely mitochondrial dysfunction, oxidative stress, neuroinflammation and decreased proteasome function (Lees et al., 2009).

Long-lasting current voltage-gated calcium channels (Ca_v1) have been implicated in the aetiology of Parkinson's disease in a number of different ways: 1) Electrophysiological experiments show that $\text{Ca}_v1.3$ contributes to the autonomous firing (pacemaking) of midbrain (substantia nigra) and medulla (dorsal nucleus ten of the motor vagus nerve) neurons, but they are not necessary for cellular pacemaking since other ion channels can sustain electrical oscillations when $\text{Ca}_v1.3$ are blocked pharmacologically (Chan et al., 2007, 2011; Fujimura and Matsuda, 1989; Goldberg et al., 2012; Guzman et al., 2010; Khaliq and Bean, 2010; Mercuri et al., 1994; Nedergaard et al., 1993; Puopolo et al., 2007; Wilson and Callaway, 2000); 2) Blockade of Ca_v1 channels with the dihydropyridine nimodipine mitigated the neurotoxic effects of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in non-human primates and had a limited effect against 6-hydroxydopamine toxicity in rodent models of Parkinson's disease (Kupsch et al., 1996; Sautter et al., 1997). However, more recent studies found isradipine (which has the highest ratio of affinity for $\text{Ca}_v1.3$ versus $\text{Ca}_v1.2$ of available Ca_v1 ligands) could prevent 6-hydroxydopamine toxicity in mice (Ilijic et al., 2011). This suggested that the earlier studies using 6-hydroxydopamine-lesioned animals failed due to insufficient blockade of $\text{Ca}_v1.3$ channels; 3) Guzman and colleagues (2010) demonstrated a link between calcium entry through the plasma membrane and mitochondrial oxidant stress. The resulting metabolic burden that use of calcium for creation of electrochemical gradients (as opposed to sodium or potassium) places upon neurons has been posited to explain the susceptibility of brainstem (medulla, pons and midbrain) neurons to the detrimental effects of mitochondrial dysfunction and oxidative stress (whether innate or due to an as yet unidentified environmental toxin) when compared to neurons that do not rely on $\text{Ca}_v1.3$ for autonomous firing (e.g. cortical neurons), which are less vulnerable to neurodegeneration in Parkinson's disease (Surmeier et al., 2012).

The apparent link between Ca_v1 subtype channels and susceptibility to neurodegeneration prompted a number of retrospective epidemiological studies of patients treated with dihydropyridines to determine whether their use affected the risk of Parkinson's disease. These studies reported conflicting results with some finding a modest decreased risk for Parkinson's disease while others have found no effect (Becker et al., 2008; Marras et al., 2012; Pasternak et al., 2012; Rees et al., 2011; Ritz et al., 2010; Ton et al., 2007). However, the comparatively low affinity of clinically used dihydropyridines for Ca_v1.3 versus Ca_v1.2 combined with the problems associated with collecting and analysing such data confounds the results of these studies. Fortunately, the situation should change soon since novel compounds with greater Ca_v1.3 selectivity are becoming available and isradipine is moving into phase III trials in early Parkinson's disease following success of phase II safety, tolerability and dose selection studies (Kang et al., 2013; Parkinson study group, 2013).

Further support for an involvement of Ca_v1.3 in the pathogenesis of Parkinson's disease comes from the recent finding using immunohistochemical staining techniques that Ca_v1 subtype immunoreactivity was elevated in brain regions affected in Parkinson's disease, resulting in a generally higher level of Ca_v1.3 subtype expression relative to that of the Ca_v1.2 subtype (Hurley et al., 2013). The increased expression and therefore presumed cellular use of Ca_v1 subtypes, could lead to increased metabolic stress on neurons and thereby contribute to or cause their death.

The purpose of this study was to further explore changes in Ca_v1 subtype gene expression in brain in areas vulnerable and resistant to neurodegeneration in Parkinson's disease at the mRNA level by *in situ* hybridization using Ca_v1 subtype specific [³⁵S]-radiolabelled oligonucleotide probes. Since DNA probes can be more accurately quantified and do not have the same specificity concerns that are associated with antibodies when used for immunohistochemistry.

Methods

Post-mortem human brain

Slide-mounted frozen brain sections (10 μm) from 8 control individuals (4 males and 4 females) and 11 patients (8 males and 3 females) dying with Parkinson's disease were obtained from the Parkinson's UK Tissue Bank at Imperial College via a prospective donor scheme with full ethical approval (80/MRE09/31+5) (Table 1). Note that frozen sections for each brain region were not available from all cases. All subjects had consented whilst living

to brain donation upon death. The average age of control subjects was 83.1 years (range 75-91 years) and the average age of Parkinson's disease cases was 79.5 (range 75-89). The clinical diagnosis of Parkinson's disease was confirmed post-mortem by neuropathological analysis and cases were classified according to the degree of α -synuclein inclusions into early (Braak α -synuclein stage 3 and 4) or late (Braak α -synuclein stage 5 and 6) stage Parkinson's disease (Alafuzoff et al., 2009; Braak et al., 2003). Control cases were from individuals with no clinical diagnosis of a neurological or psychiatric disorder during life, nor any neuropathological abnormality evident post-mortem other than normal age related changes (e.g. blood vessel calcification). The brain regions chosen consisted of two regions with early involvement in the disease process which undergo cell loss and had extensive α -synuclein pathology (substantia nigra and locus coeruleus) and two regions which do not undergo significant cell loss and only contain α -synuclein pathology at the late-stage of Parkinson's disease (anterior cingulate cortex and primary motor cortex). All Parkinson's disease cases were receiving treatment with L-DOPA and/or dopamine agonists prior to death and some individuals had received treatment with dihydropyridines.

In situ hybridization

The level of Ca_v1.2 and Ca_v1.3 mRNA was quantified in human brain sections by *in situ* hybridization using Ca_v1 subtype specific [³⁵S]-labelled oligonucleotide probes as previously described (Hurley et al., 1996). To increase the intensity of the hybridization signal, a mixture of three (Ca_v1.3) or two (Ca_v1.2) oligonucleotide probes was used to hybridize to the calcium channel mRNA transcripts. The level of Ca_v1 subtype mRNA was quantified in units of amol/mg tissue with ¹⁴C microscale standards (American Radiolabelled Chemicals Inc.) in a way analogous to quantitative receptor autoradiography (Nunez et al. 1989). Unless stated otherwise, all experimental reagents were purchased from Sigma-Aldrich (www.sigmaaldrich.com).

Probe design: Oligonucleotide probes were selected by computer analysis of nucleotide sequences using the nucleotide Basic Local Alignment Search Tool (Blastn) program (Altschul et al. 1990) on the National Centre for Biotechnology Information website. For Ca_v1.2 the reference mRNA sequence used in the database had accession number NM_199460.2 and the two probes were complementary to bases 1898-1945 and 3638-3685. For Ca_v1.3 the reference mRNA sequence used in the database had accession number NM_000720.3 and the three probes were complementary to bases 1111-1158, 5836-5883 and

6355-6404. No probe showed significant homology with any sequence (other than the intended target) within the non-redundant GenBank nucleotide sequence databases. The probes did not distinguish between alternatively spliced variants of either Ca_v1 subtype.

Oligonucleotide labelling: To label each probe, 5 pmol oligonucleotide was incubated in 40 µl labelling buffer [100 mM cacodylate buffer (pH 6.8), 1 mM CoCl₂, 0.1 mM dithiothreitol (DTT), 100 mg/ml bovine serum albumin (BSA)] containing 40 µCi [³⁵S]-dATP (1250 Ci/mmol, Perkin-Elmer) and 30 units of terminal deoxynucleotidyl transferase (Promega) for 1 h at 37°C. Probes were purified by size exclusion chromatography through NICK columns (GE Healthcare) and stored in the eluent at -20°C until use. The average specific activity of the Ca_v1.2 and Ca_v1.3 probes was 1.9 x 10⁹ cpm/µg and 1.5 x 10⁹ cpm/µg respectively.

Prehybridization treatments: Slides were removed from storage (-80°C) and the sections were immediately fixed by immersion in ice-cold PBS (pH 7.4) containing 4% paraformaldehyde, for 10 min at room temperature. The sections were rinsed three times in PBS for 5 min at room temperature, then acetylated in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min with agitation. Sections were then rinsed in PBS for 5 min followed by dehydration through a graded series of ethanols (50% for 5 min, 70% for 5 min, 95% for 5 min and 100% for 2 x 5 min), delipidation with chloroform (two 5 min immersions) and rehydration to 95% ethanol. Finally the sections were fully dried under a stream of cool air.

Hybridization: Radiolabelled oligonucleotide probes were diluted in hybridization buffer (5 pmol/ml/probe) and 50 µl was spread over the sections and covered with parafilm. Sections were hybridized at 37°C for 20 h in hybridization buffer [50% deionized formamide, 4 x SSC (1 x SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), 4 x Denhardt's solution (0.08% BSA, 0.08% Ficoll, 0.08% polyvinyl-pyrrolidone), 500 µg/ml denatured salmon sperm DNA, 100 µg/ml yeast tRNA, 1 µM dATP, 10% (w/v) dextran sulphate, 100 mM DTT and 0.1% sodium dodecyl sulphate].

Post-hybridization washing: Sections were immersed in 1 x SSC for 5–10 min and the parafilm was removed, followed by four 15-min washes at 55°C in 1 x SSC and two 30-min washes at room temperature in 1 x SSC. Sections were then briefly dipped into deionized water to remove buffer salts, dried under a stream of cool air and then apposed to Hyperfilm MP (GE Healthcare).

Quantification of autoradiographs: Images were digitized and quantified using ImageJ (NIH, v1.48d) image analysis software and computer generated curves derived from the ¹⁴C standards. Specific hybridization values were derived by subtraction of a background

measurement from each image and the amount of probe hybridized calculated from the specific activity and standard curve. Cresyl-violet and immunohistochemically stained sections were used to assist with identification of the region of interest on autoradiograms.

Controls for hybridization specificity: The specificity of the *in situ* hybridization signal was determined by the following: 1) elimination of hybridization signal by pretreatment of sections with RNase (20 mg/ml RNase A, in PBS for 30 min at 37°C); 2) elimination of hybridization signal by the presence of a 100-fold excess of unlabelled probes. In addition, preliminary experiments (data not shown) used the probes labelled with digoxigenin to establish: 1) a correlation between the predicted hybrid melting temperature and the signal seen/abolished at different washing stringencies; 2) the use of multiple probes directed towards the same target mRNA yielding identical patterns of hybridization when used individually; 3) the observed pattern of hybridization corresponded to that previously published for Cav1 subtypes mRNA (reviewed by Hurley and Dexter, 2012).

Data analysis

Data (mean $\pm$ standard error of the mean (SEM)) were analysed by full factorial one-way analysis of variance (*F*) using the univariate general linear model followed, where appropriate, by a Dunnett multiple comparison test, using SPSS™ statistics software v.19 (IBM). Prism™ V (Graphpad) was used to plots graphs of means $\pm$ SEM. For analysis, Parkinson's disease cases were divided into either, early (α -synuclein Braak stage 3 or 4) or late (α -synuclein Braak stage 5 or 6) stage Parkinson's disease (Table 1). The level of statistical significance for all tests was $P < 0.05$.

Results

The expression Cav1.2 and Cav1.3 was investigated by quantitative *in situ* hybridization in normal and parkinsonian human brain using [³⁵S]-labelled antisense oligonucleotide probes specific for the alpha subunit of each channel. The probes did not distinguish between alternatively spliced variants.

Cav1 subtype mRNA expression in normal brain

In normal brain the highest level of Cav1.2 mRNA was detected in the substantia nigra (1.63 $\pm$ 0.17 amol/mg). Moderate levels of Cav1.2 mRNA were found in the locus coeruleus (0.86

± 0.17 amol/mg), while a low amount of $\text{Ca}_v1.2$ mRNA was present in primary motor cortex (0.26 ± 0.10 amol/mg) and cingulate cortex (0.13 ± 0.08 amol/mg). Similarly, the highest level of $\text{Ca}_v1.3$ mRNA was also found in the substantia nigra (0.93 ± 0.17 amol/mg), with a moderate amount detected in the locus coeruleus (0.57 ± 0.17 amol/mg). Like the $\text{Ca}_v1.2$ mRNA, $\text{Ca}_v1.3$ mRNA was also present at a lower level in the cortical areas examined (cingulate cortex, 0.30 ± 0.06 amol/mg; primary motor cortex, 0.12 ± 0.02 amol/mg).

With the exception of cingulate cortex, $\text{Ca}_v1.2$ mRNA was expressed at a higher level than $\text{Ca}_v1.3$ mRNA in all brain regions examined; such that in the midbrain and pons the percentage ratio was 60:40 and 64:36 for the locus coeruleus and substantia nigra respectively. Hence, the ratio favoured expression of the $\text{Ca}_v1.2$ channel in the brainstem of control brain. In the cortical areas examined this difference in relative mRNA levels was similar in primary motor cortex (68:32). That is, $\text{Ca}_v1.2$ mRNA was expressed at a higher level than $\text{Ca}_v1.3$ mRNA. But, the situation reversed in cingulate cortex so that the percentage ratio of $\text{Ca}_v1.2$ mRNA to $\text{Ca}_v1.3$ mRNA was 30:70 in this brain region (Table 2, Figure 1 and 2).

Ca_v1 subtype mRNA expression in Parkinson's disease

In tissue from patients dying with Parkinson's disease there was no significant alteration in the level of either Ca_v1 subtype mRNA in either of the brainstem regions examined. That is, the level of mRNA was maintained (Table 2, Figure 3) despite significant loss of catecholamine neurons in both these brain regions in late stage (Braak α -synuclein stage 5 and 6) Parkinson's disease (Hurley et al., 2013).

In the two cortical regions examined there were significant differences in Ca_v1 subtype expression in Parkinson's disease cases when compared to control tissue. Specifically, in cingulate cortex $\text{Ca}_v1.2$ ($F(2,9) = 5.953$, $P = 0.028$) and $\text{Ca}_v1.3$ ($F(2,10) = 5.279$, $P = 0.015$) mRNA was significantly increased in cases with late Parkinson's disease.

In primary motor cortex the level of $\text{Ca}_v1.3$ mRNA was increased in the early Parkinson's disease group ($F(2,11) = 9.318$, $P = 0.003$) and the level of $\text{Ca}_v1.2$ mRNA was increased ($F(2,11) = 3.336$, $P = 0.047$) in the late Parkinson's disease group (Table 2, Figure 3).

The ratio of the percentage expression level of $\text{Ca}_v1.2$ mRNA to $\text{Ca}_v1.3$ mRNA in the locus coeruleus remained unchanged in early stage Parkinson's disease cases (61:39) but changed

in favour of increased levels of Ca_v1.2 subtype mRNA (74:26) in late stage Parkinson's disease cases. In contrast, in the substantia nigra the ratio became more even in early Parkinson's disease cases (56:44), which suggested more use of the Ca_v1.3 channel, while in the late stage cases the ratio changed back to favour the Ca_v1.2 channel (76:24), which mirrored the ratio found in the locus coeruleus. In cingulate cortex the ratio changed to be even more in favour of the Ca_v1.3 subtype mRNA in early stage Parkinson's disease (19:81), but in late stage cases the expression of each subtype was even (48:52). Conversely, in primary motor cortex, the ratio of each Ca_v1 subtype became more even in the early Parkinson's disease cases (56:44) but changed to greatly favour the Ca_v1.2 subtype mRNA (83:17) in late stage Parkinson's disease cases (Table 2).

Discussion

This study used [³⁵S]-radiolabelled oligonucleotide probes and densitometry to quantify the mRNA for Ca_v1 subtypes in post-mortem brain from patients dying with Parkinson's disease at different α -synuclein Braak stages and age-matched control brain. An increased expression of both isoforms was found in cortical regions of brain from patients dying with Parkinson's disease. In contrast, no change in the level of mRNA for either subtype occurred in the substantia nigra or locus coeruleus, despite catecholamine cell loss in these regions in Parkinson's disease.

The data do not indicate which cells alter expression in cortex, though they must be cells intrinsic to the region since *in situ* hybridization labels mRNA in cell bodies and not afferent terminals. The increase in cortical regions occurred in the absence of pathological changes (i.e. cell loss) which suggested the alteration was intrinsic to the disease process and not a compensatory change in response to the loss of cells through neurodegeneration. The increased expression may be the result of altered activity of neural circuits consequent to neurodegeneration elsewhere, or could result from the chronic treatment with L-DOPA and/or dopamine agonists that all Parkinson's disease patients were receiving prior to death. In this regard, chronic neuroleptic treatment acting via D₂ dopamine receptors has been shown to lower the level of mRNA for Ca_v1.3 without affecting Ca_v1.2 mRNA levels in rat pituitary (Fass et al., 1999). This indicated that dopaminergic tone could regulate Ca_v1.3 expression. Decreased dopamine D₂ receptor activation due to reduced levels of endogenous dopamine might therefore explain alterations in the relative expression of Ca_v1 subtypes in parkinsonian brain and it would therefore be of interest to examine the status of Ca_v1

subtypes and dopamine D₂ receptors in untreated parkinsonian brain, should any become available. However, the predominant dopamine receptor in cerebral cortex is of the D₁ subtype, so this is unlikely to be a factor in the current study (Hurley and Jenner, 2006). Furthermore, the absence of a reduction of Ca_v1.3 mRNA in the midbrain, despite chronic dopaminergic drug treatment also supports the view that altered dopaminergic tone is not responsible for the altered Ca_v1 subtype expression found in Parkinson's disease.

The changes in Ca_v1 subtype mRNA expression mirrored the elevated Ca_v1 subtype protein levels found in a previous immunohistochemistry study and indicate that changes observed in total protein levels measured by densitometry arise from Ca_v1 expressed on perikarya in these areas rather than Ca_v1 present on presynaptic terminals in the neuropil (Hurley et al., 2013). Although a contribution of mRNA for Ca_v1.2 or Ca_v1.3 expressed by cells other than large principal projection neurons (e.g. microglia, interneurons) cannot be excluded from the densitometry data of this study. In the case of non-neuronal cells such channels presumably serve functions other than the regulation of electrical activity (e.g. control of exocytosis and gene transcription etc.). The absence of a reduction in the amount of mRNA for Ca_v1 subtype in the substantia nigra and locus coeruleus, where catecholaminergic cell loss occurs in Parkinson's disease, could represent an upregulation of mRNA in the surviving cells in these areas. [These data are in keeping with a DNA microarray study from our laboratory which also found no change in Ca_v1.2 or Ca_v1.3 mRNA levels in substantia nigra from patients dying with Parkinson's disease compared to control cases \(Durrenberger et al., 2012\).](#)

Although there is no way of knowing from these experiments whether this presumed increase occurred in [surviving dopamine](#) neurons or in other cell types which express Ca_v1 subtypes (e.g. GABAergic, glutamatergic and microglia) in the brainstem. The presence of Ca_v1 subtypes on non-neuronal cells in brain is debatable (e.g. Hashioka et al., 2013; Moller, 2002), though we support the view that microglia do express both subtypes of Ca_v1 based on double-labelling experiments with a microglia marker and Ca_v1 subtype antibodies (Hurley et al., 2013) [and quantitative real time PCR using mRNA extracted from N9 microglia cell cultures \(unpublished observations\)](#). Where mRNA was not significantly increased, yet an increase in Ca_v1 protein was found, the increased protein expression most likely occurred in the neuropil (i.e. the terminals of neurons afferent to the region). This reasoning assumes that more mRNA in a cell equates to increased expression of the cognate protein for the mRNA, which is generally accepted to be the case. Though protein expression levels may also be regulated in many different ways (e.g. reduced degradation and recycling) rather than control of transcription and mRNA and protein levels are therefore not necessarily stoichiometric.

For example, Gegg and colleagues (2012) found no change in mRNA for glucocerebrosidase levels in putamen, despite measuring a reduction in glucocerebrosidase protein levels in that region in Parkinson's disease, which indicated that the alteration was a post-translational effect on protein levels.

An unexpected finding which did not tally with the immunohistochemistry was the reversal of the ratio of Cav1 subtype expression in cingulate cortex of control cases. The reason for this is unknown. However, tissue was only available from a small number of fresh frozen cases for use in this study and this was particularly the case for the cingulate cortex. Since there is a high degree of inherent variation between samples of human tissue and other factors such as agonal state drug history, lifestyle etc. could all influence the results, a non-representative subset of cases could have inadvertently been used. Consequently, we have included the individual data for each case in the manuscript to enable the reader to decide upon the validity of the findings. While we are not aware of any other study which has examined mRNA for cingulate or motor cortex in Parkinson's disease, a microarray study by Vogt and colleagues (2006) found increased mRNA for Cav1.2 and decreased mRNA for Cav1.3 in occipital cortex in Parkinson's disease. In studies using animal brain tissue and quantitative real-time PCR analysis, Schlick and colleagues (2010) found that Cav1.2 and Cav1.3 were expressed at similar levels in mouse cerebral cortex. Whereas, Sinnegger-Brauns and colleagues (2009) found the ratio of Cav1.2 to Cav1.3 to be 2:1 in mouse cerebral cortex and 1:2 in rat cerebral cortex. Similarly, one must question whether a many-fold increase in mRNA has biological significance even when deemed statistically not significant (Nuzzo, 2014). Especially when one considers that cell death occurs in idiopathic Parkinson's disease over decades and at any particular time the physiological conditions responsible for stressing neurons that die may not be measurably different (significantly) to those found in neurons which survive until death of the individual and post-mortem tissue analysis (Przedborski et al., 2003).

Novel short splice variants of the Cav1.3 have been described which show differential expression in mouse brain and have different kinetics or/and signalling pathways to the full length channel (Bock et al., 2011; Tan et al., 2011; Huang et al., 2013). The oligonucleotides used in this work did not distinguish between splice variants of the Cav1 subtypes, hence the relative contribution of alternative transcripts of the Cav1 subtypes in Parkinson's disease remains unknown and further studies which examine expression of splice variants of Cav1 subtypes in Parkinson's disease are warranted.

In summary this study provides further evidence of increased Ca_v1 subtype expression in cerebral cortex in Parkinson's disease before the appearance of neurodegeneration. In addition the relative expression of Ca_v1.3 to Ca_v1.2 increased in areas where cell loss occurred which supports the theory that increased calcium flux and hence increased metabolic stress occurs in neurons vulnerable to neurodegeneration in Parkinson's disease and supports the case for further research into neuroprotective strategies based upon modulation of Ca_v1.3 channel function (Surmeier, 2007).

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Disclosure statement

The authors declare that there are no conflicts of interest associated with this work.

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Table 1 Details of patients and control individuals who donated brain used in this study

Case	Age	Gender	Onset Age	PMI	NPD	aSYN score	Tau score	Clinical diagnosis	Complications motor	Complications psychiatric	Drugs for PD at death	DHP exposure	Cause of death from clinical notes
<i>Control</i>													
C01	91	F	-	22	-	0	1	-	none	none	-	-	bronchopneumonia
C02	75	M	-	12	-	0	1	-	none	none	-	-	lung cancer
C03	78	F	-	23	-	0	2	-	none	none	-	-	not recorded
C04	80	F	-	23	-	0	1	-	none	none	-	amlodipine	metastatic cancer
C05	84	F	-	11	-	0	2	-	none	none	-	amlodipine	pancreatic cancer
C06	82	M	-	48	-	0	1	-	none	none	-	nifedipine	metastatic cancer
C07	90	M	-	12	-	0	1	-	none	none	-	amlodipine	lung cancer
C08	85	M	-	34	-	0	1	-	none	none	-	-	not recorded
<i>Early Parkinson's disease</i>													
PE01	78	M	68	22	LBDBS	3	2	PD	high	low	Sinemet/amantadine-	-	bronchopneumonia
PE02	79	M	67	21	LBDBS	3	2	PD	none	High	Madopar/ropinirole	nifedipine	Parkinson's disease
PE03	77	M	67	10	LBDBS	3	2	PD	none	None	Madopar	amlopidine	not recorded
PE04	86	F	18	5	LBDBS	3	5	PD	low	High	Madopar/ropinirole	-	not recorded
<i>Late Parkinson's disease</i>													
PL01	75	M	57	22	LBDBS	6	2	PD	high	High	Sinemet/ pergolide	-	chest infection
PL02	89	M	86	16	LBDBS	5	1	PD	low	None	Madopar	-	septicaemia
PL03	83	F	72	15	LBDBS	6	3	PD	low	High	Sinemet CR	-	not recorded
PL04	80	M	61	16	LBDBS	6	2	PD	high	High	Sinemet/ropinirole	-	not recorded
PL05	76	F	58	7	LBDBS	6	3	PD	high	High	Sinemet/ropinirole	-	not recorded
PL06	70	M	69	13	LBDN	5	2	DLB	low	High	Madopar	-	bronchopneumonia
PL07	80	M	60	22	LBDBS	6	2	PD	high	High	Sinemet/ pergolide	nifedipine	not recorded

Table 2 Quantification of Cav1 subtype mRNA in human brain (amol/mg)

Control

	Locus coeruleus		Substantia nigra		Cingulate cortex		Primary motor cortex	
	Cav1.2	Cav1.3	Cav1.2	Cav1.3	Cav1.2	Cav1.3	Cav1.2	Cav1.3
C01	1.37	0.41	1.92	1.43	0.29	0.19	0.03	0.08
C02	0.51	0.38	-	-	0.06	0.32	-	-
C03	0.75	0.21	-	-	0.03	0.40	0.53	0.16
C04	0.52	1.09	1.18	0.41	-	-	0.39	0.15
C05	0.58	0.22	2.12	0.55	-	-	0.08	0.13
C06	1.40	1.13	1.14	1.28	-	-	0.28	0.07
C07	-	-	1.58	1.09	-	-	-	-
C08	-	-	1.88	0.84	-	-	-	-
mean	0.86	0.57	1.63	0.93	0.13	0.30	0.26	0.12
sem	0.17	0.17	0.17	0.17	0.08	0.06	0.10	0.02
ratio 1.2:1.3	60:40		64:36		30:70		68:32	

Early Parkinson's disease

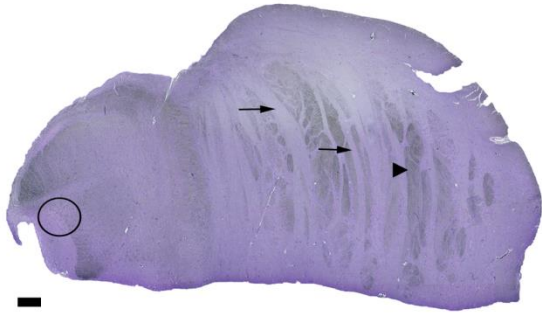
	Locus coeruleus		Substantia nigra		Cingulate cortex		Primary motor cortex	
	Cav1.2	Cav1.3	Cav1.2	Cav1.3	Cav1.2	Cav1.3	Cav1.2	Cav1.3
PE01	1.99	0.71	1.65	1.39	-	1.07	-	0.84
PE02	1.26	1.93	1.44	1.71	0.17	0.63	0.48	0.25
PE03	0.77	0.76	1.56	0.83	0.08	0.70	0.77	0.89
PE04	2.07	0.44	1.55	0.99	0.49	1.79	1.86	1.28
mean	1.53	0.96	1.55	1.23	0.25	1.05	1.04	0.81**
sem	0.31	0.33	0.04	0.20	0.12	0.26	0.42	0.21
ratio 1.2:1.3	61:39		56:44		19:81		56:44	

Late Parkinson's disease

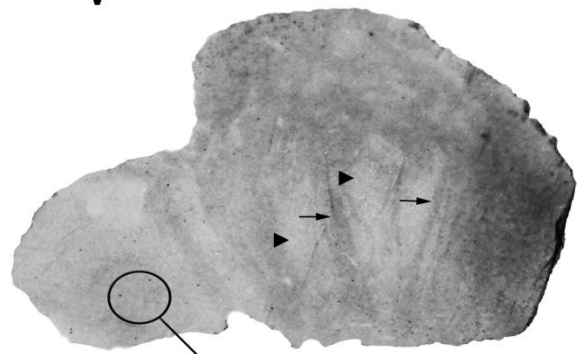
	Locus coeruleus		Substantia nigra		Cingulate cortex		Primary motor cortex	
	Cav1.2	Cav1.3	Cav1.2	Cav1.3	Cav1.2	Cav1.3	Cav1.2	Cav1.3
PL01	1.81	0.73	1.72	0.99	1.28	1.96	1.52	0.50
PL02	1.17	0.19	1.83	0.32	2.09	-	2.03	0.25
PL03	-	-	-	0.58	1.05	2.02	-	-
PL04	0.60	0.39	1.01	-	2.00	0.91	1.79	0.46
PL05	-	0.37	-	0.14	-	0.89	0.21	-
PL06	1.86	0.39	1.44	0.39	0.96	1.56	2.98	0.18
PL07	0.42	-	1.23	0.35	0.19	1.01	0.36	0.13
mean	1.17	0.42	1.45	0.46	1.26*	1.39*	1.48*	0.31
sem	0.30	0.09	0.15	0.12	0.29	0.21	0.43	0.08
ratio 1.2:1.3	74:26		76:24		48:52		83:17	

Figure 1 Representative transverse sections through the pons of normal human brain

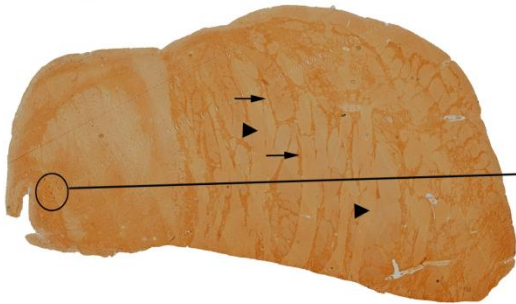
a cresyl violet



c $\text{Ca}_V1.2$ mRNA



b $\text{Ca}_V1.2$ immunoreactivity



d

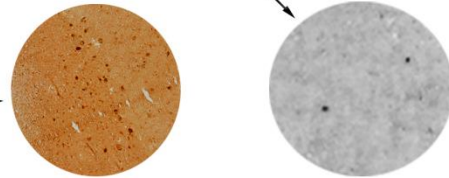


Figure 2 Representative sections through the midbrain (a), anterior cingulate cortex (b) and primary motor cortex (precentral gyrus) of normal human brain

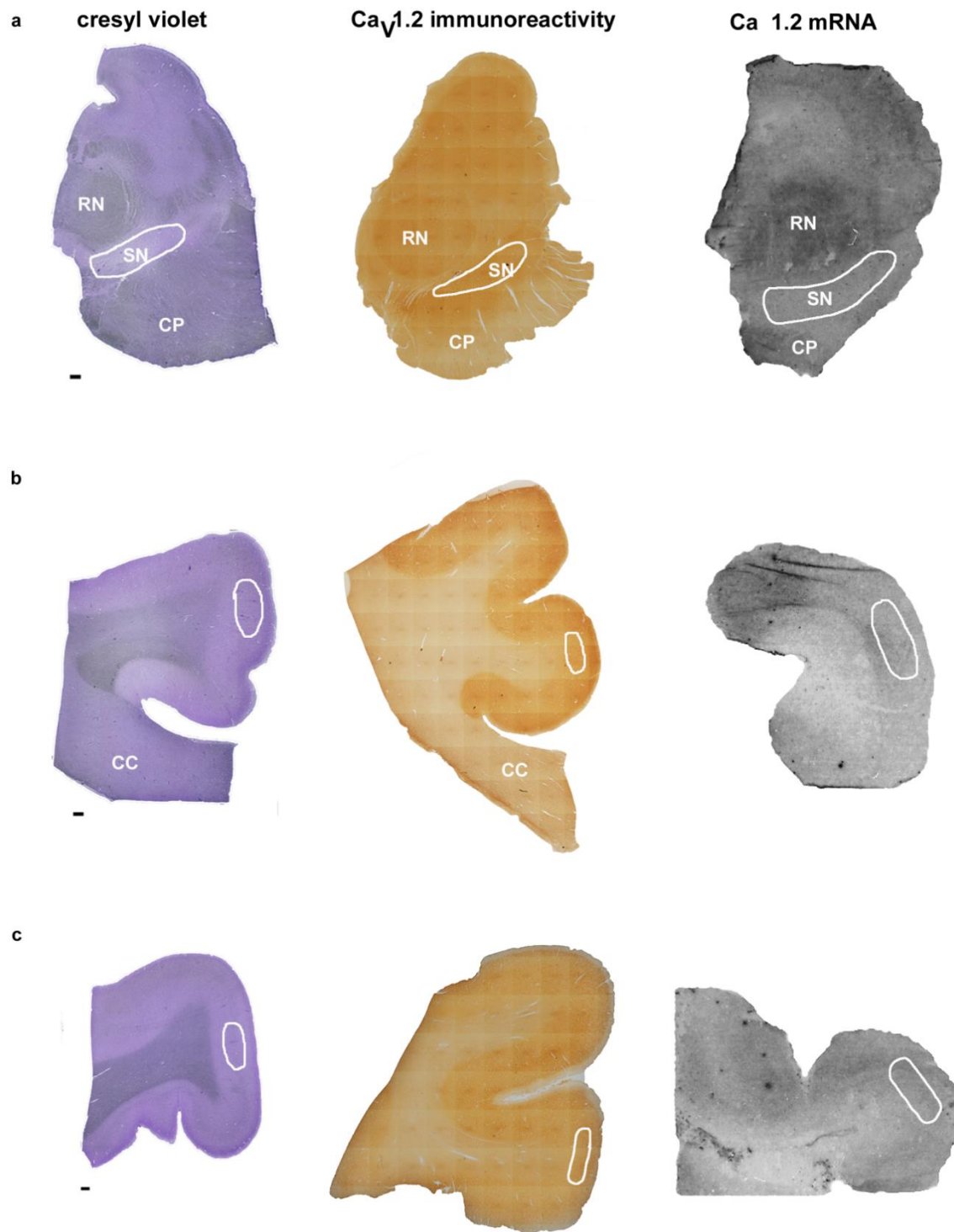


Figure 3 Comparison of Ca_v1 subtype mRNA in normal and parkinsonian brain

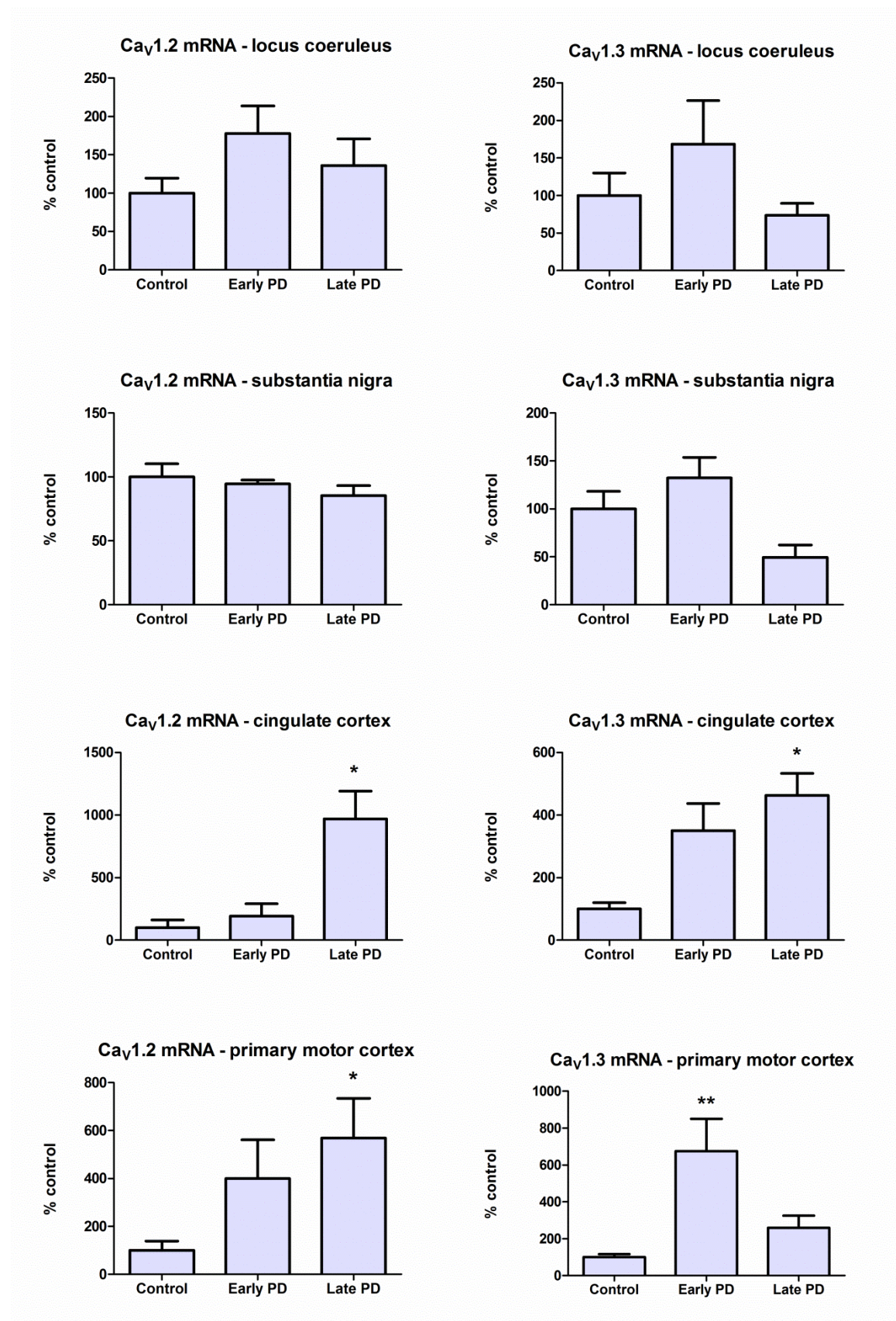


Table 1 legend

Details of patients and control individuals who donated brain used in this study.

Abbreviations: PMI, post-mortem interval; NPD, neuropathological diagnosis; aSYN score, α -synuclein Braak stage; DHP, dihydropyridine (treatment during life); LBDBS, Lewy body disease brainstem variant; LBDN, Lewy body disease neocortical variant; PD, Parkinson's disease; DLB, dementia with Lewy bodies.

Table 2 legend

Quantification of Cav1 subtype mRNA in human brain (amol/mg). * $P < 0.05$; ** $P < 0.01$

Figure 1 legend

Representative transverse sections through the pons of normal human brain stained with cresyl violet (a) an anti-Cav1.2 antibody (b) and a representative autoradiogram (c) from a section hybridized with [35 S]-labelled oligonucleotide probes specific for Cav1.2 mRNA. The circled area in panels a, b and c, which is enlarged in panel d, corresponds to the locus coeruleus and was quantified on autoradiograms by densitometry. The arrows indicate transverse fibres of the pons which express high levels of Cav1.2 immunoreactivity and mRNA, while arrowheads show cerebrospinal fasciculi which have less Cav1.2 immunoreactivity and mRNA. Paraffin wax embedded sections were stained with cresyl violet to assist interpretation of autoradiograms. A section stained with anti-Cav1.2 antibody is included to show how the pattern of mRNA coincides with protein expression. A similar picture was observed for the Cav1.3 subtype (data not shown). Bar = 1 mm.

Figure 2 legend

Representative sections through the midbrain (a), anterior cingulate cortex (b) and primary motor cortex (precentral gyrus) of normal human brain stained with cresyl violet (left), an anti-Cav1.2 antibody (centre) and a representative autoradiogram (right) from a section hybridized with [35 S]-labelled oligonucleotide probes specific for Cav1.2 mRNA. The area delineated by the white line exemplifies the region quantified on autoradiograms by densitometry. Sections were stained with cresyl violet to assist interpretation of autoradiograms. A section stained with anti-Cav1.2 is included to show how the pattern of mRNA coincides with protein expression. A similar picture was observed for the Cav1.3 subtype (data not shown). Abbreviations: CC, corpus callosum; CP, cerebral peduncle; RN, red nucleus; SN, substantia nigra. Bar = 1 mm.

Figure 3 legend

Comparison of Cav1 subtype mRNA in normal and parkinsonian brain

* $P < 0.05$; ** $P < 0.01$