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Reduction in parvalbumin expression in the zona incerta after 6OHDA lesion in rats

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Abstract In an effort to understand better the neurochemical changes that occur in Parkinson disease, we have examined the expression patterns of the calcium-binding protein parvalbumin in the zona incerta in parkinsonian rats. Sprague-Dawley rats had small volumes of either saline (control) or 6 hydroxydopamine (6OHDA) injected into the medial forebrain bundle, the major tract carrying dopaminergic nigrostriatal axons. After various post-lesion survival periods, ranging from 2 hrs to 84 days, rats were perfused with formaldehyde and their brains processed for routine tyrosine hydroxylase (TH) or parvalbumin immunocytochemistry. In the 3 to 84 days post-lesion cases, there was an overall 50% reduction in the number of parvalbumin$^+$ cells in the zona incerta on the 6OHDA-lesioned side when compared to control. In the 2 hrs post-lesion cases, there was no substantial loss of parvalbumin$^+$ cells in the zona incerta after 6OHDA lesion, although in these cases (unlike the longer survival periods), there was limited loss of TH$^+$ cells in the midbrain on the lesion side. The loss of parvalbumin$^+$ cells from the zona incerta was due to a loss of antigen expression rather than a loss of the cells themselves, since the number of Nissl-stained cells in the zona incerta was similar on the control and 6OHDA-lesioned sides. In summary, our results indicate that a loss of the midbrain dopaminergic cells induces a major change in parvalbumin expression within the zona incerta. This change may have key functional and clinical implications.

Abbreviations

6OHDA 6 hydroxydopamine
cp cerebral peduncle
CPu caudate-putamen complex
GP globus pallidus
Hb habenular complex
IL intralaminar nuclei
LG lateral geniculate complex
LH lateral hypothalamus
mfb medial forebrain bundle
ml medial lemniscus
PBS phosphate buffered saline
Pf parafascicular nucleus
Po posterior thalamic nucleus
Pv parvalbumin
SC superior colliculus
SNc substantia nigra pars compacta
Sub subthalamus
R red nucleus
TH tyrosine hydroxylase
VP ventral posterior nucleus
VTA ventral tegmental area
ZI zona incerta

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Introduction

The zona incerta forms a distinct cellular zone in the diencephalon of mammals. For the most part, the zone lies ventral to the dorsal thalamus and medial lemniscus, lateral to the hypothalamus and dorsal to the subthalamus (Kawana & Watanabe, 1981; Watanabe & Kawana, 1982; Roger

& Cadusseau, 1985; Romanowski *et al.*, 1985; Shammah-Lagnado *et al.*, 1985). The zona incerta has widespread connections across the neuroaxis, from the spinal cord to the cerebral cortex (Ricardo, 1981; Watanabe & Kawana, 1982; Roger & Cadusseau, 1985, Romanowski *et al*., 1985; Shammah-Lagnado *et al*., 1985; Nicolelis *et al*., 1992, 1995; Wagner *et al*., 1995). In particular, the zona incerta has heavy connections with the intralaminar and higher-order nuclei of the dorsal thalamus (Power & Mitrofanis, 2001) and various nuclei of the brainstem, including the superior colliculus, pedunculopontine tegmental nucleus, substantia nigra and the midbrain and pontine reticular nuclei (Ricardo, 1981; Watanabe & Kawana, 1982; Roger & Cadusseau, 1985, Romanowski *et al*., 1985; Shammah-Lagnado *et al*., 1985; Kolmac *et al*., 1998). The zona incerta has been implicated in four major functions, namely shifting attention (May *et al*., 1997), maintaining posture and locomotion (Skinner *et al*., 1990), controlling visceral activity (Mok & Mogenson, 1986) and generating arousal (Kolmac *et al*., 1998; Power & Mitrofanis, 2001). The precise role of the zona incerta in each of these functions is not clear, however (Mitrofanis, 2004).

Several lines of evidence indicate that abnormal activity of the zona incerta contributes to the debilitating symptoms of Parkinson disease. Pèrier *et al.* (2000) have shown that, using both a metabolic marker (cytochrome oxidase) and electrophysiological recordings, the zona incerta is hyperactive in 6 hydroxydopamine (6OHDA)-lesioned rats, a well-accepted animal model of Parkinson disease. Further, the zona incerta has distinct glutamatergic projections to the substantia nigra pars compacta (henceforth known as substantia nigra) and the pedunculopontine tegmental nucleus (Heise & Mitrofanis, 2004), two nuclei of the basal ganglia that show degeneration in Parkinson disease or parkinsonian animals (Parent, 1996; Blandini *et al*., 2000; Pahapill & Lozano, 2000). In clinical studies, surgical lesion of the zona incerta alleviates the motor symptoms of Parkinson disease, although these results probably included damage to many fibres of passage (e.g., pallidothalamic, dentatothalamic) passing through or near the zona incerta (Mundinger, 1965). However, more recently, deep brain stimulation at high frequency (thought to suppress functional activity) of the incertal region has been shown to improve proximal tremor in multiple sclerosis patients (Alusi *et al*., 2001; Nandi *et al*., 2002a, b), as well as akinesia and bradykinesia in Parkinson patients (Henderson *et al*., 2002; Voges *et al*., 2002). Overall, these results indicate that the abnormal activity of the region of the zona incerta contributes to the symptoms of Parkinson disease (Nandi *et al*., 2002b).

The major aim of this study is to examine whether there are any changes in neurochemical expression in the zona incerta of 6OHDA-lesioned rats. We focus on the parvalbumin$^+$ cells of the zona incerta (Nicolelis *et al*., 1992; Kolmac & Mitrofanis, 1999). Parvalbumin is a calcium-binding protein implicated in the control of intracellular free calcium in particular neuronal populations. It is expressed within cells that often also contain the major inhibitory neurotransmitter, γaminobutyric acid (GABA; Celio, 1990). Such cells are associated with high-frequency, nonadapting firing rates (Celio, 1990). The high-frequency firing cells are activated by agonists of N-methyl-D-aspartate glutamate receptors, that increase substantially the levels of intracellular calcium (Feldman *et al*., 1990). We chose to concentrate on the parvalbumin$^+$ cells of the zona incerta for several reasons. First, they are the dominant cell type in the zona incerta (Nicolelis *et al*., 1992; Kolmac & Mitrofanis, 1999; Mitrofanis, 2004), and as such, are the most likely to show any neurochemical changes in response to 6OHDA lesion. Second, parvalbumin expression has been shown in other systems to be affected greatly once an afferent source is lesioned (Barker & Dreher, 1996; Dekkers *et al*., 2002); a similar circumstance could be evident after disruption to the dopaminergic pathways. All in all, our results on the expression of parvalbumin in the zona incerta after 6OHDA lesion will hopefully provide a better understanding of the abnormal circuitry in Parkinson disease.

Material and methods

Subjects

Adult male (~8 weeks old) Sprague-Dawley (250–300 g) rats were used ($n = 29$). Animals were kept in a 12-hrs light-dark cycle and had unrestricted access to food and water. All of the experiments were approved by the Animal Ethics Committee of the University of Sydney.

6 Hydroxydopamine (6OHDA) lesions

Rats were anaesthetised after an intraperitoneal injection of ketamine (100 mg/kg) and rompun (10 mg/kg). Thirty minutes prior to surgery, rats were given intraperitoneal injections of desipramine hydrochloride (25 mg/kg; to protect noradrenergic cells) and pargyline (50 mg/kg; to help stop peripheral breakdown of 6OHDA). Rats were placed in a stereotactic apparatus and had 6OHDA (4 μg/μl saline with addition of 0.1–1% ascorbic acid) injected by pressure (~5 μl injected total) into the medial forebrain bundle (2 separate injections; coordinates 4.5–4.8 mm caudal to bregma, 1.1–1.2 mm lateral, 8.2–8.4 mm ventral; Paxinos & Watson, 1986). The right hand side was injected with 6OHDA, while the left hand side was injected with saline (with addition of 0.1–1% ascorbic acid; controls). Rats were allowed to recover for either 2 hrs ($n = 3$), 3 days ($n = 3$), 7 days ($n = 6$), 14 days ($n = 3$), 28 days ($n = 3$) or 84 days ($n = 3$). In three

other cases, saline (with addition of 0.1–1% ascorbic acid) was injected into the medial forebrain bundle of the right hand side as described above (the left hand side was not injected); the rats were allowed to survive for 7 days. These cases were used as a further control. In five more cases, we injected 6OHDA into the caudate-putamen complex (the major target area of the dopaminergic cells of the substantia nigra). Four injections (coordinates 1.7 mm caudal to bregma, 2 mm lateral, 5 mm ventral; 0.5 mm rostral to bregma, 3 mm lateral, 6 mm ventral; 1.4 mm rostral to bregma, 4 mm lateral, 6 mm ventral; 2.5 mm rostral to bregma, 4.5 mm lateral, 6 mm ventral; Paxinos & Watson, 1986) were made along the rostrocaudal axis of the caudate-putamen complex (16 μl injected total). These rats were allowed to survive for 14 days. After the end of each survival period in all cases, rats were perfused transcardially with 0.1M phosphate-buffered saline (PBS; pH 7.4), followed by 4% buffered formaldehyde. The brains were removed and post-fixed overnight in the same solution. Next, brains were placed in PBS with the addition of 20% sucrose until the block sank. Coronal sections were cut using a freezing microtome at a thickness of 50μm. Every section was collected (1:5 series) and stored in PBS. Analysis of the 6OHDA lesions was carried out by post-mortem tyrosine hydroxylase (TH) immunohistochemistry (see below) and by apomorphine (dopaminergic agonist; Sigma) injections (0.05 mg/kg). For the latter, rats were given a subcutaneous injection of apomorphine (after 3–7 days post-lesion) and they were monitored for any rotational behaviour. If the lesion was successful, rats would start rotating (>20 times per 5 min) contralateral to the lesion soon after each. In three cases, rats were not given apomorphine injections, as to ensure that this drug did not influence the expression of parvalbumin in the zona incerta (these rats had 6OHDA injections into the medial forebrain bundle and 7 days post-lesion survival).

Immunocytochemistry

Sections were immersed in a solution of 0.1% Triton (Sigma) and 10% normal horse serum in PBS at room temperature for 1 hr. Sections were then incubated in either anti-TH (Chemicon, 1:1000; midbrain sections) or anti-parvalbumin (Sigma; 1:1000; diencephalic sections) for 24–48 hrs. Next, sections were incubated in biotinylated anti-mouse IgG (Sigma; 1:200) for 2–3 hrs at room temperature. Finally, sections were incubated in avidin-biotin-complex (Vector; 1:100) at room temperature for 3 hrs. To visualise the bound antibody, sections were reacted in nickel-Tris-buffered saline (pH 7.4)–3,3'- diaminobenzidine tetrahydrochloride (Sigma) solution. Between each incubation sections were washed in three changes of PBS. Sections were mounted onto gelatinised slides, air dried overnight, dehydrated in ascending alcohols, cleared in Histoclear and coverslipped using DPX. Some sections were counterstained in neutral red before coverslipping. For controls, sections were processed as described above, except that there was no primary antibody used. Control sections were immunonegative.

Analysis

The number of TH^+ cells in the substantia nigra and $parvalbumin^+$ cells in the zona incerta were counted in each case. This analysis of each nucleus was performed from at least six sections per animal. Since stereological methods were not used in this study, it was important that closely matched sections in the different cases were analysed. Counts were made from comparable sections across the full rostrocaudal extent of the substantia nigra and zona incerta (250–350 μm apart). Each section analysed was matched as closely as possible to the sections depicted in Fig. 1, which correspond to the plates illustrated in the rat brain atlas of Paxinos and Watson (1986). A mean number of cells per section on the 6OHDA and control sides were generated at each survival period. A statistical comparison of samples was made using the Student *t*-test. In three cases (at 84 days survival post-lesion), the number of Nissl-stained cells was counted in the zona incerta (as described above), after counterstaining with neutral red, on the 6OHDA-lesioned and control sides. For the schematic diagrams, coronal sections were drawn with reference to Paxinos and Watson (1986) and the $parvalbumin^+$ cells in the zona incerta on the 6OHDA and control side were plotted at each survival period. Schematic diagrams and digital images were constructed using Microsoft PowerPoint programme.

Results

The section that follows will be presented in three parts (i) Controls (ii) 6OHDA lesion and (iii) Patterns of parvalbumin immunoreactivity in the zona incerta.

Controls

The main control used in this study was saline injections into medial forebrain bundle (or caudate-putamen complex) of the side contralateral to the 6OHDA injections in the same animal ("saline*"; classical hemiparkinsonian model; Fig. 2). This is a well-accepted control for these sorts of experiments because most nigral pathways are ipsilateral and saline has no major effect on antigen expression in neural cells (Schober, 2004). Nonetheless, in three cases, we made saline injections into the medial forebrain bundle of one side ("$saline^{\#}$"; Fig. 2). In these cases, the number of TH^+ cells in the substantia nigra (Fig. 2A) and $parvalbumin^+$ cells in the zona incerta

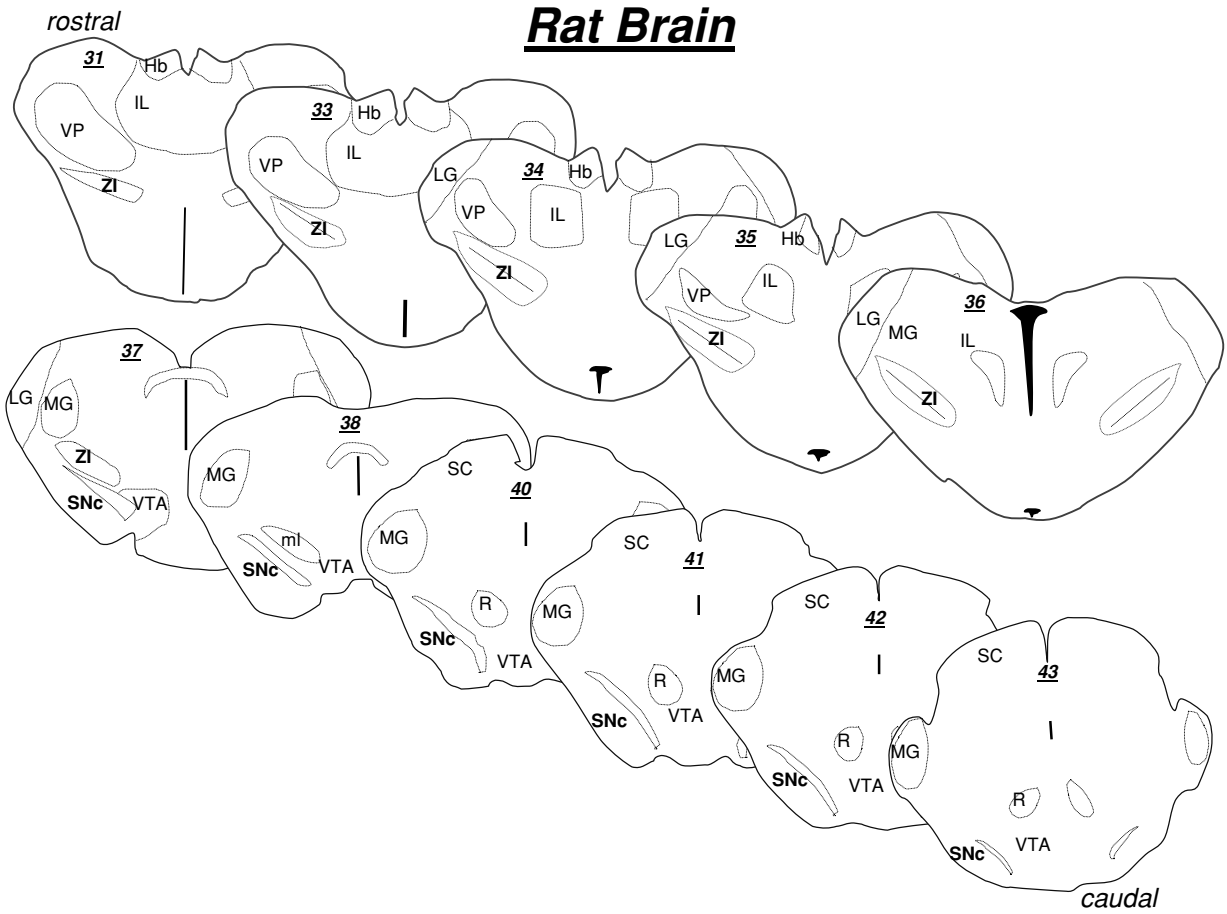

Fig. 1 Schematic diagrams of the rat brain. Coronal sections, dorsal to top. The underlined numbers corresponds to the plates in the rat brain atlas of Paxinos and Watson (1986). The lower the number, then the more rostral the section. Counts of labelled cells in the zona incerta and substantia nigra were made from comparable sections across the full rostrocaudal extent of each nucleus (250–350 μm apart)

(Fig. 2B) cells was similar to "normal" (cases where no injections were made; Fig. 2) and comparable to the number of cells in these nuclei on the saline side contralateral to the 6OHDA side ("saline*", Fig. 2). Thus, in terms of TH^+ and $parvalbumin^+$ cell number, the latter cases were considered satisfactory controls.

6 Hydroxydopamine (6OHDA) lesion

We have examined the patterns of parvalbumin expression in the zona incerta after 6OHDA lesion of the midbrain. The survival period after lesion was staged, from 2 h to 84 days. This was done as to explore the acute and chronic effects of lesion on the expression of parvalbumin. In most cases, there was a small electrode tract marking the location of the injection site in the vicinity of the medial forebrain bundle (arrows Fig. 3; or caudate-putamen complex). In this study, none of the rats developed any observable motor and/or visceral deficit post-lesion and they were eating and grooming soon after surgery.

The efficacy of the 6OHDA lesion was measured in terms of TH immunoreactivity and apomorphine rotational behaviour. All rats that had a large loss of TH^+ cells (>70%; 6OHDA-lesioned side compared to control) after 6OHDA lesion (see below) displayed contralateral rotational behaviour after injection of apomorphine (>20 rotations per 5 min). Rats that did not have a substantial loss of TH^+ cells (<70%) after 6OHDA lesion generally showed marked hyperkinetic movements, but no rotation.

Figure 4 shows photomicrographs of TH-immunostained sections of the midbrain at various survival periods after 6OHDA lesion. The 2 hrs post-lesion cases (Fig. 4A and B) showed no observable loss in TH immunoreactivity on the 6OHDA lesion side when compared to control. However, in each of the 3, 7 (Fig. 4C and D), 14 (Fig. 4E and F), 28 and 84 day post-lesion cases, a clear loss of immunoreactivity in the substantia nigra was evident on the 6OHDA lesion side. A similar pattern of loss in TH immunoreactivity was apparent in the caudate-putamen complex, the major target area of the TH^+ cells of the substantia nigra. In the 2 hr cases (Fig. 5A and B), no substantial difference was

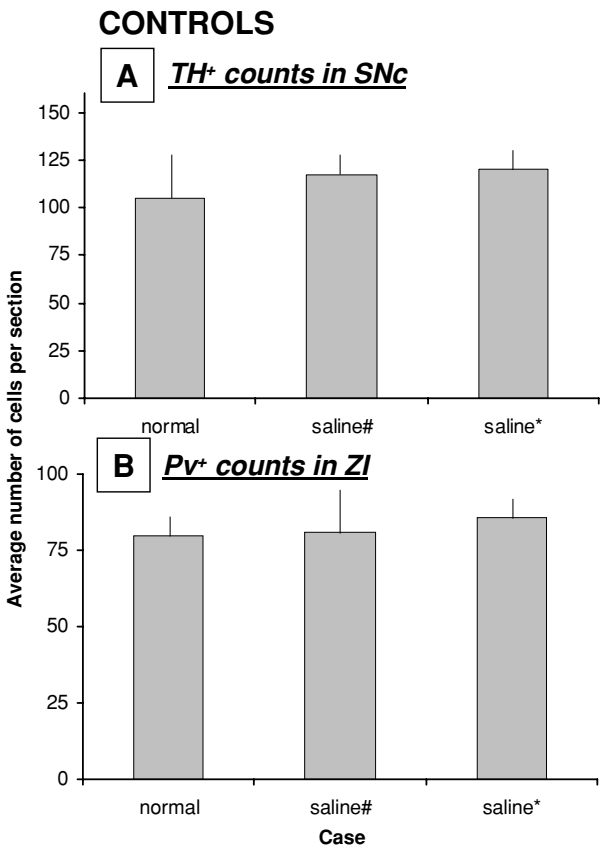

Fig. 2 Graph showing the number of TH^+ cells in the substantia nigra pars compacta (A) and parvalbumin$^+$ cells in the zona incerta in control cases. "Normal" cases had no injections; "Saline#" cases had injections on one side only; "Saline*" had injections on the side contralateral to the 6OHDA lesion. In each case, rats were allowed to survive for 7 days. Note that the number of TH^+ and parvalbumin$^+$ cells was similar in the different cases

apparent in the TH immunolabelling in the caudate-putamen complex of control and 6OHDA-lesioned sides, while after 14 days (as in after 3, 7, 28, and 84 days), a clear reduction in caudate-putamen complex immunolabelling was evident in the 6OHDA lesion side (Fig. 5C and D).

The loss of TH^+ cells in the substantia nigra after 6OHDA lesion was quantified and the results are shown in the graph of Fig. 6. From the cases examined at 2 hrs post-lesion, there was only a small reduction (~20%) in the number of TH^+ cells on the 6OHDA lesion side when compared to control; this reduction was not significant ($p = 0.3$). In the 3 day cases, a larger 58% reduction was evident between 6OHDA lesion and control sides, but this was also not significant ($p = 0.2$). In the longer survival cases, 7, 14, 28, and 84 days, the reduction of TH^+ cells on the 6OHDA lesion side was more substantial, being 72%, 86%, 87% and 96% respectively. In each case, the differences in TH^+ cell number in the 6OHDA-lesioned and control sides were significant ($p < 0.005$; Fig. 6). In all of these longer survival cases, rats showed apomorphine-induced rotational behaviour.

Patterns of parvalbumin immunoreactivity in the zona incerta

Many previous studies have described four main cytoarchitectonic sectors in the zona incerta, namely rostral, dorsal, ventral and caudal (Nicolelis *et al*., 1992; Kolmac & Mitrofanis, 1999; Mitrofanis, 2004). Parvalbumin immunoreactivity is found mainly among cells in the ventral sector of the zona incerta. In fact, all of the cells of this sector have been reported to express parvalbumin. There are few parvalbumin$^+$ cells found in the other sectors of the zone (Kolmac & Mitrofanis, 1999).

Figure 7 shows a schematic diagram of the distribution of parvalbumin$^+$ cells in the zona incerta after 6OHDA lesion at the various survival periods. After 2 hrs, there was little change in the pattern of parvalbumin immunoreactivity in the zona incerta of the 6OHDA lesion side when compared to control. However, in the 3 to 84 day post-lesion cases, parvalbumin immunoreactivity was reduced dramatically in the zona incerta of the 6OHDA-lesioned side (Fig. 7). The loss was most evident within the ventral sector of the zone, the region where the bulk of the parvalbumin$^+$ cells are located in normal cases (Kolmac & Mitrofanis, 1999; Mitrofanis, 2004). Within this sector, the pattern of loss was rather uniform, in that no particular region of the sector was more affected than any other (eg, lateral or medial).

Figure 8 shows photomicrographs of parvalbumin immunoreactivity in the zona incerta of two cases, one at 3 (Fig. 8A and B) and the other at 14 (Fig. 8C and D) days post-lesion. The two sides shown were from the same section in each case. The reduction in parvalbumin immunoreactivity on the 6OHDA lesion side was striking.

The graph in Fig. 9 quantifies this phenomenon. From the cases examined at 2 hrs post-lesion, there were slightly fewer (~3%) parvalbumin$^+$ cells on the 6OHDA lesion side when compared to control; this difference was not significant ($p = 0.8$). By 3 days post-lesion, the reduction in parvalbumin$^+$ cell number on the 6OHDA lesion side was more substantial (~50%), but it still did not reach significance ($p = 0.09$ at 3 days). At 7, 14, 28 and 84 days, the reduction in parvalbumin$^+$ cell number on the 6OHDA lesion side was still substantial (~50%, 65%, 48% and 42% respectively) and these reductions were significant ($p < 0.05$; Fig. 9). When pooled together (3–84 days), there was a mean parvalbumin$^+$ cell loss of ~50% after 6OHDA lesion. These data also indicate that there was no major recovery of parvalbumin$^+$ cell number with increasing survival period. The loss of parvalbumin$^+$ cells was substantial from very

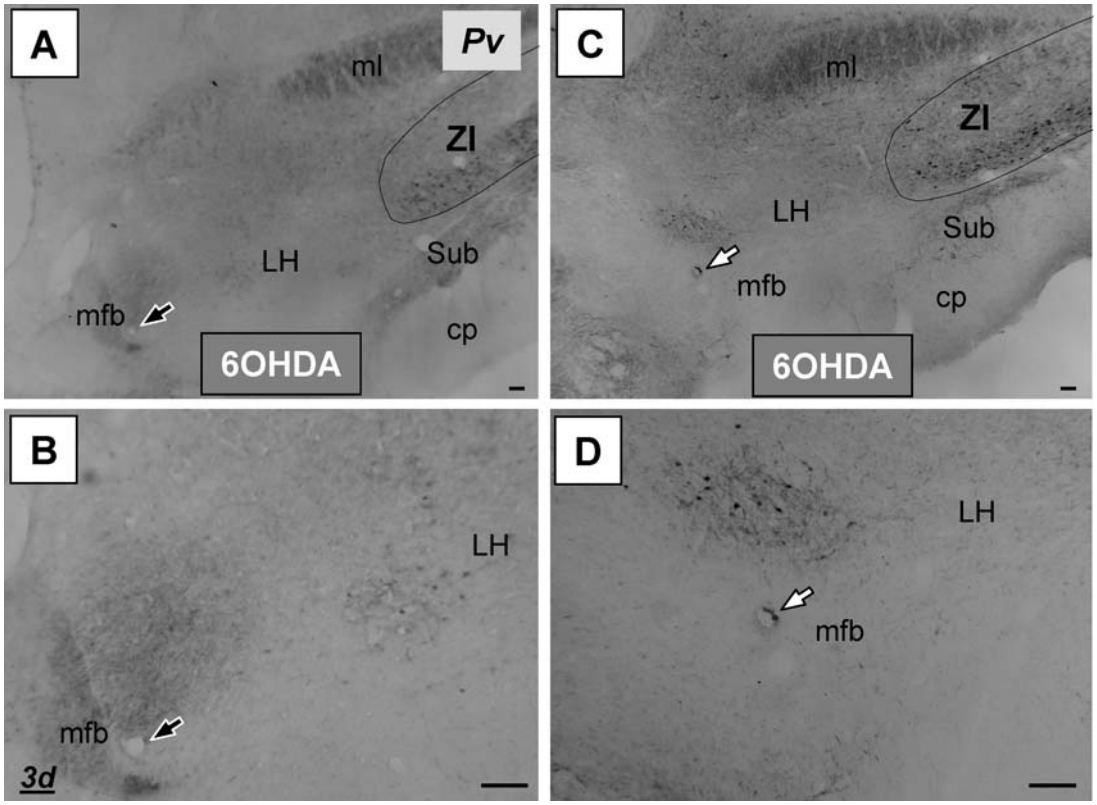

Fig. 3 Photomicrographs indicating the location of the electrode site in the medial forebrain bundle. Figure A and B are of the same case, while Fig. C and D are of a different case. Figure B and D are higher magnifications of the electrode site shown in Fig. A and C respectively. Both cases are of the side that was injected with 6 hydroxydopamine and were after 3 days survival. The corresponding shaded arrows indicate the same spot in the sections. These sections were immunostained for parvalbumin. Scale bars = 100 μm.

early (3 days) and remained so until our last survival period (84 days) (Fig. 9). The reductions in parvalbumin$^+$ cell number on the 6OHDA-lesioned side from 2 hrs to 3 days and from 2 hrs to 84 days were significant ($p < 0.005$).

The administration of apomorphine, used in this study as a test of effectiveness of lesion, had no major effect on the overall number of parvalbumin$^+$ cells in the zona incerta. In the three cases where apomorphine was not given (and had 7 days post-lesion survival), the reduction in the number of parvalbumin$^+$ cells was 41%, 62% and 63%; these values were within the range of the reductions described for the cases that were given apomorphine beforehand (see above).

In summary, there was a broad relationship between the extent of the 6OHDA lesion in the midbrain—as measured TH$^+$ cell number—and the number of parvalbumin$^+$ cells in the zona incerta. When there was a loss of TH$^+$ cells of more than ∼60% after 6OHDA lesion (3–84 day post-lesion), then there was a substantial (∼50%) reduction in the number of parvalbumin$^+$ cells in these cases as well. In the cases where the TH$^+$ cell loss was ∼70% (7, 14, 28 and 84 days), then the parvalbumin$^+$ cell reduction was significant ($p < 0.05$). By contrast, in cases where the reduction of TH$^+$ cells was smaller (∼20% at 2 hrs post-lesion), then the reduction of parvalbumin+ cells was correspondingly smaller (∼3%).

Figure 10 examines whether the loss of parvalbumin$^+$ cells after 6OHDA lesion reflects a loss parvalbumin expression from the cells or a loss of the cells themselves. Fig. 10A and B show photomicrographs of parvalbumin immunoreactivity in the zona incerta on the control and 6OHDA-lesioned side of the same section. The section was then counterstained with neutral red to reveal all of the cell bodies within the zona incerta (Fig. 10C and D; arrows indicate the same cells in each region). On the control side, most, if not all, cells in this region of the zona incerta were parvalbumin$^+$; counterstaining did not reveal large numbers of cells that were not immunostained already. On 6OHDA-lesioned side however, there were many cells in this region that were Nissl-stained, but not parvalbumin$^+$. Such cells are immunostained in normal cases (Kolmac & Mitrofanis, 1999). We counted the number of Nissl-stained cells in the zona incerta, on control and 6OHDA-lesioned sides, in three cases (after 84 day survival post-lesion). Overall, the values on the two sides matched closely. On the control side, the number of cells averaged 277 (SE 10) while on the 6OHDA-lesioned, it was 278 (SE 9). Hence, this result indicates that rather than a

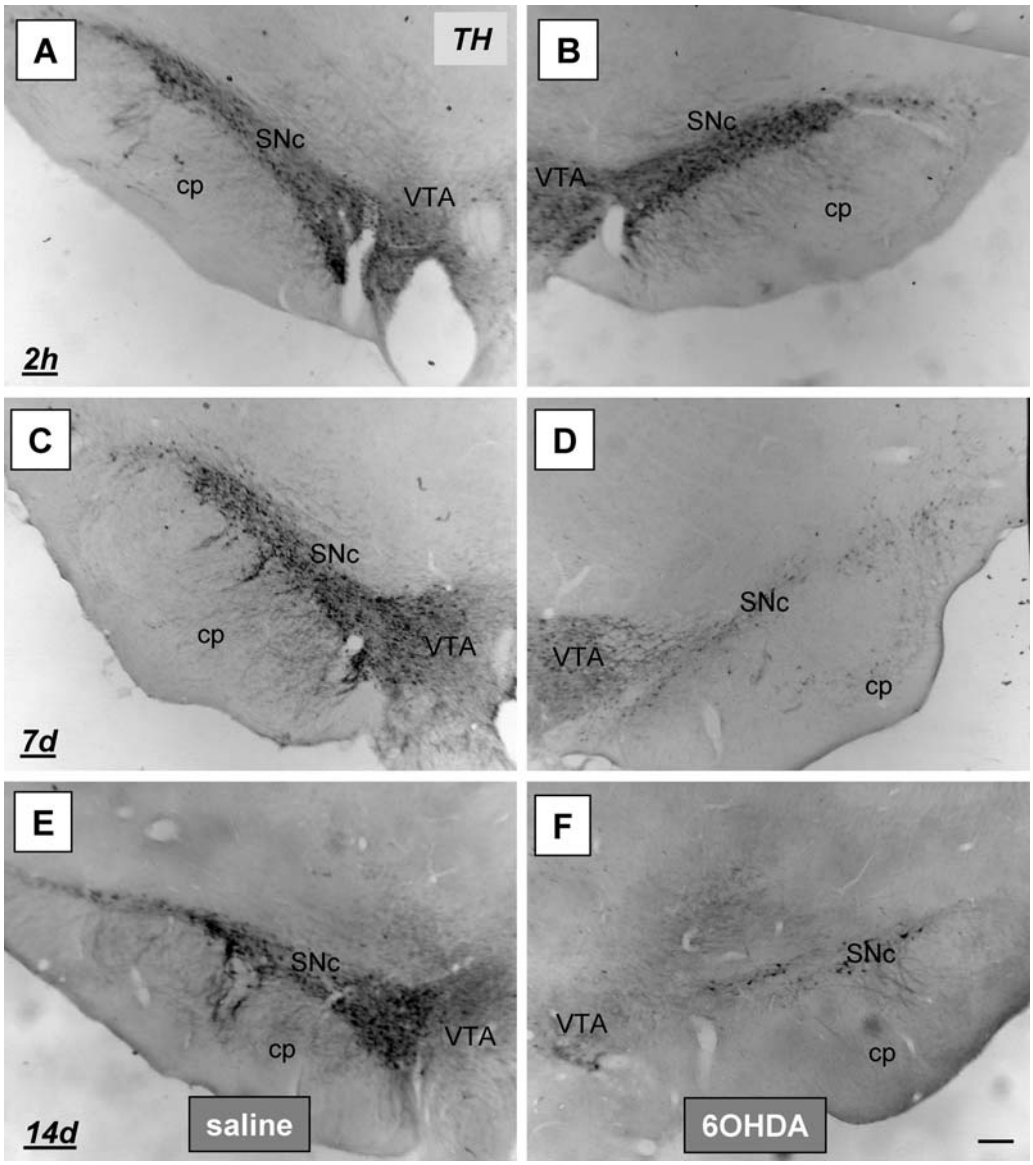

Fig. 4 Photomicrographs of tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta after saline (A, C, E) or 6 hydroxydopamine (B, D, F) injections into the medial forebrain bundle. Right and left adjacent photomicrographs are of the same section. Three survival periods post-lesion are shown, 2 h (A, B), 7 days (C, D) and 14 days (E, F). Note the substantial loss of immunostained cells on the 6 hydroxydopamine lesioned side in the 7 and 14 day cases. There was no such loss after 2 h. Coronal sections; dorsal to top, midline to middle of plate. Scale bar = 100 μm

loss of the cells themselves, there is a loss of parvalbumin expression after 6OHDA lesion.

In three cases, the patterns of parvalbumin expression in the zona incerta was examined after 6OHDA injections into the caudate-putamen complex, rather than the medial forebrain bundle. From these cases, the patterns of parvalbumin immunoreactivity were similar to those described for the medial forebrain injections. After 6OHDA injections into the caudate-putamen complex, there was an overall reduction of ~50% (reductions of 27%, 36%, 28%, 63% and 75% in each case) in the number of parvalbumin$^+$ cells in the zona incerta of the 6OHDA-lesioned side when compared to control (in these cases, there was an overall reduction of TH$^+$ cell number of ~75% in the substantia nigra). These results indicate that the reduction of parvalbumin$^+$ cells in the zona incerta is independent of the site of the 6OHDA injection site, whether in the medial forebrain bundle or caudate-putamen complex.

Discussion

This study shows a striking reduction in the expression of the calcium-binding protein parvalbumin, among cells in the

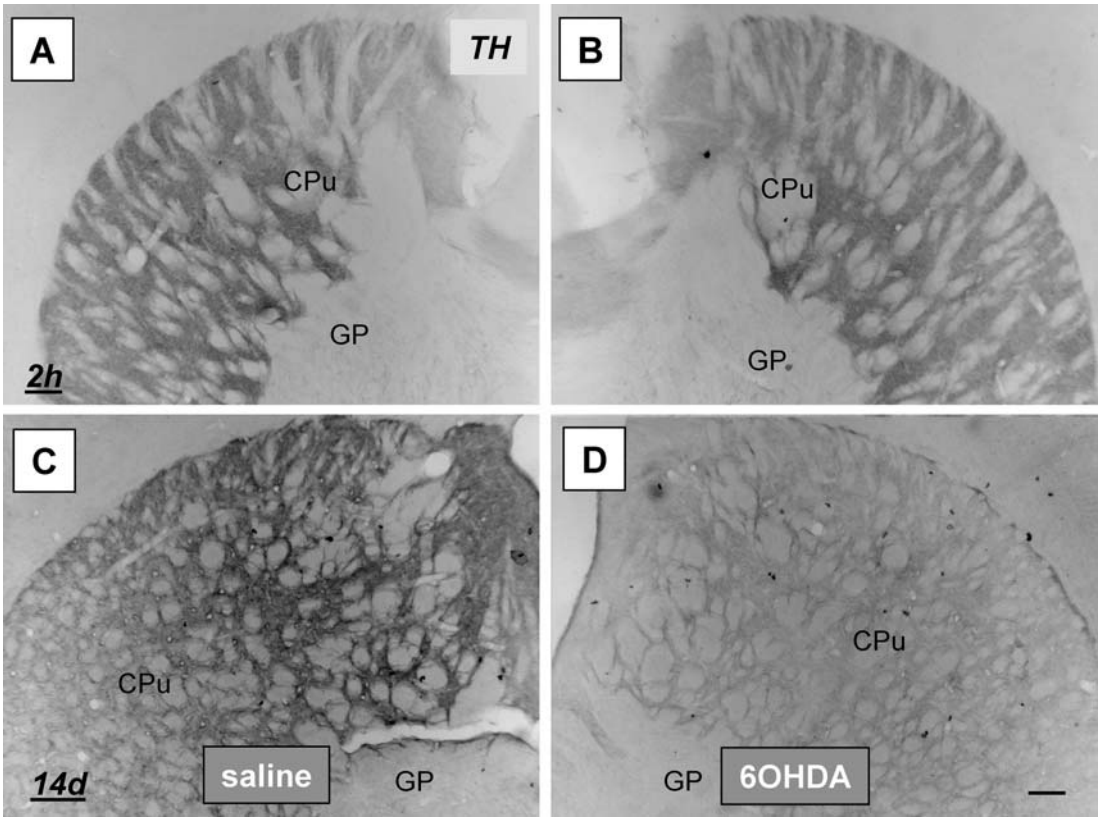

Fig. 5 Photomicrographs of tyrosine hydroxylase immunoreactivity in the caudate-putamen complex after saline (A, C) or 6 hydroxydopamine (B, D) injections into the medial forebrain bundle. Right and left adjacent photomicrographs are of the same section. Two survival periods post-lesion are shown, 2 h (A, B) and 14 days (C, D). Note the substantial loss of immunostained terminals on the 6 hydroxydopamine lesioned side in the 14 day case. There was no such loss after 2 h. Coronal sections; dorsal to top, midline to middle of plate. Scale bar = 100 μm

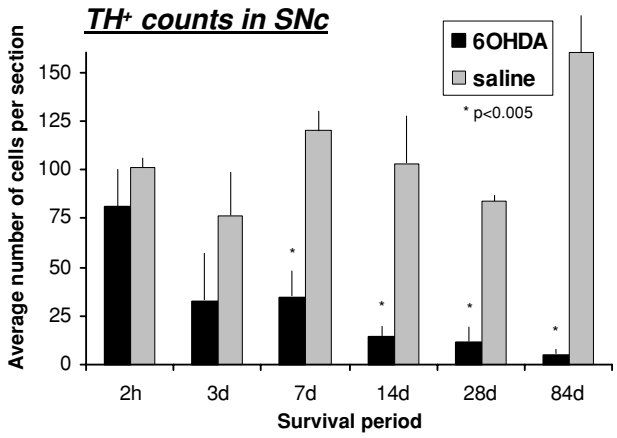

Fig. 6 Graph showing the number of tyrosine hydroxylase$^+$cells in the substantia nigra pars compacta. The average number of cells per section is plotted against the different survival periods post-lesion. The number of cells in the saline and 6 hydroxydopamine injected side are compared for each period. Standard error bars are indicated.

zona incerta of 6OHDA-lesioned rats. This phenomenon is chronic and may well contribute to the abnormal circuitry and symptoms that is evident in Parkinson disease.

Changes in neurochemical expression in the basal ganglia

Previous studies have reported substantial changes in the expression of various neurochemicals in the basal ganglia of parkinsonian animals. For example, in 6OHDA-lesioned rats, there is an upregulation of mRNAs encoding glutamic acid decarboxylase (GAD), preproenkephalin and D_2 receptors within the caudate-putamen complex (Chritin *et al*., 1993; Delfs *et al*., 1995; Soghomonian & Laprade, 1997; Schuller & Marshall, 2000), together with an increase in expression of GAD mRNA (Kincaid *et al*., 1992; Delfs *et al*., 1995; Soghomonian & Chesselet, 1992), substance P, neurotensin and enkephalin in the globus pallidus (Schuller *et al.*, 1999; Martorana *et al.*, 2003). Further, in MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-treated monkeys and cats, there is an upregulation of GAD mRNA in the globus pallidus (and entopeduncular nucleus; rodent/carnivore equivalent of internal globus pallidus; Pedneault *et al.*, 1994; Soghomonian *et al.*, 1994; Schroeder & Schneider, 2001).

To the best of our knowledge, a change in the expression of parvalbumin after 6OHDA lesion has not been reported previously for any region of the brain. Further, a change in

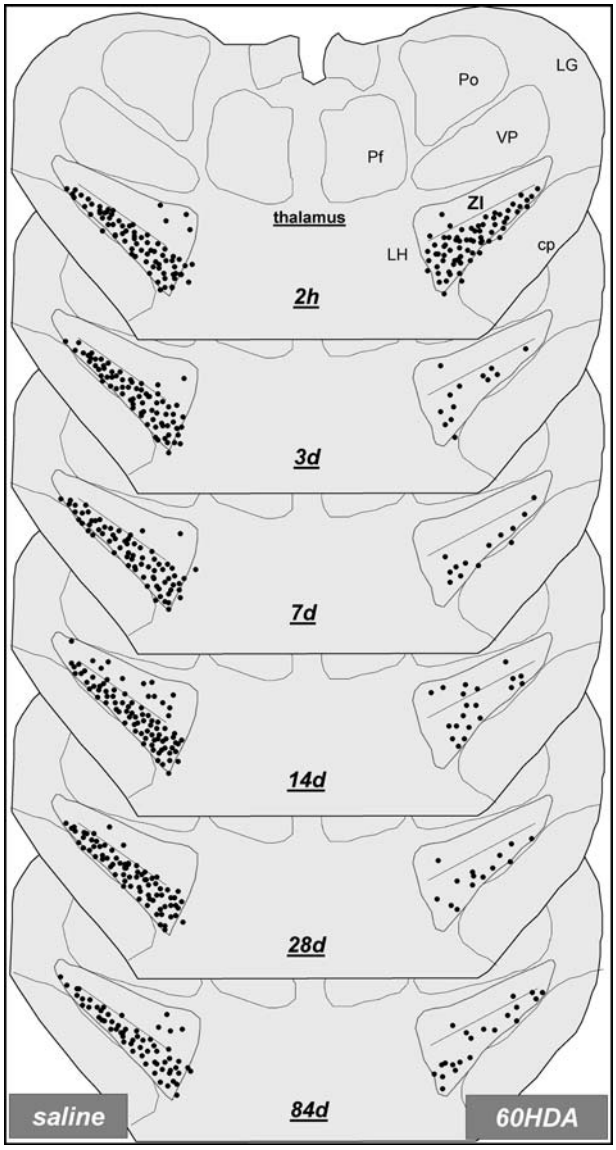

Fig. 7 Schematic diagram of the distribution of parvalbumin$^+$ cells in the zona incerta within a section through the mid-thalamus. The distribution of cells is shown for the six survival periods on the saline (left hand side) and 6 hydroxydopamine (right hand side) injected sides. Each circle indicates one immunolabelled cell. The dashed line within the zona incerta indicates the border between the dorsal and ventral sector of the zone

expression of any neurochemical within the zona incerta has not been reported by previous studies. Our results assume increasing importance given the recent results indicating that abnormal incertal activity contributes to the parkinsonian condition (see Introduction).

Loss of expression, not cell death

Our results indicate that the loss of parvalbumin$^+$ cells from the zona incerta reflects a loss of antigen expression, rather than a loss of the cells themselves. The number of Nissl-stained cells in the zona incerta of the control side matched closely the number of Nissl-stained cells on the 6OHDA-lesioned side (Fig. 10), indicating little or no cell death. This effect is long-lasting, being evident 84 days post-lesion. It is of course possible that these cells undergo death at a later stage post-lesion, because cells that lose calcium buffering proteins have been shown to become more susceptible to excitotoxicity and death (see Eyles *et al.*, 2002). However, one would assume that if these cells were going to undergo cell death as a result of a 6OHDA lesion then they would have done so well within 84 days post-lesion period (e.g., Mattson *et al.*, 1991).

Stimulus for change in expression

There are several factors that might trigger the change in parvalbumin expression in the zona incerta after 6OHDA lesion. First, the loss of the direct dopaminergic projection from the substantia nigra to the zona incerta (Heise & Mitrofanis, 2004). It is well established that destroying an afferent source to a nucleus results in changes—either an increase or decrease—in the number of cells expressing a particular antigen. For example, Gunluk *et al.* (1994) have shown an increase in NADPH-diaphorase cell number in the lateral geniculate nucleus after eyelid suture, which effectively generates a lesion to the retinofugal tract. Further, Dreher and colleagues report that there is a reduction in the number of parvalbumin$^+$ and calbindin$^+$ cells in the tectum after retinofugal or corticofugal tract lesions (Dreher *et al.*, 1996; Barker & Dreher 1998). Along similar lines, Dekkers *et al.* (2002) have shown recently that nerve injury results in a drop in parvalbumin immunoreactivity in the spinal cord.

Second, the loss of parvalbumin could be secondary to the loss of the dopaminergic cells after 6OHDA lesion. The dopaminergic cell loss generates an abnormal activity of many brains centres, for example the cerebral cortex (Steiner & Kitai, 2001; Pelled *et al.*, 2002; Orieux *et al.*, 2002) and the pedunculopontine nucleus of the brainstem (Crossman *et al.*, 1985; Orieux *et al.*, 2000; Breit *et al.*, 2001; Jeon *et al.*, 2003). These two centres in particular, project heavily to the zona incerta, and it follows that the abnormal activity of their inputs may trigger the loss of parvalbumin expression.

The latter possibility appears the most likely because the cortical (Mitrofanis & Mikuletic, 1999) and pedunculopontine (Kolmac *et al.*, 1998) projections are rather widespread across the zona incerta, potentially affecting most incertal cells. This would match the topography of parvalbumin$^+$ cell loss seen in the zona incerta, which is across the mediolateral extent of the zone. The dopaminergic projections to the zona incerta however, terminate exclusively within the medial edge of the zone (Heise & Mitrofanis, 2004) and this

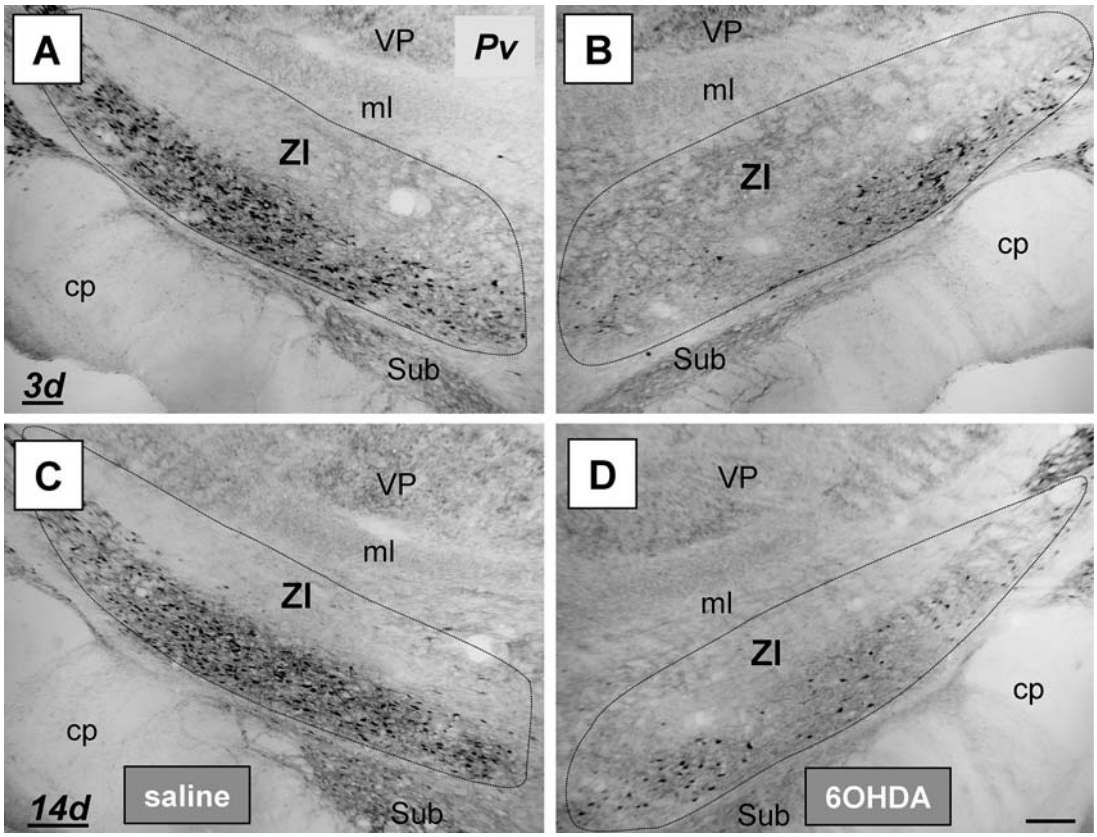

Fig. 8 Photomicrographs of parvalbumin immunoreactivity in the zona incerta after saline (A, C) or 6 hydroxydopamine (B, D) injections into the medial forebrain bundle. Right and left adjacent photomicrographs are of the same section. Two survival periods post-lesion are shown, 3 (A, B) and 14 days (C, D). Note the substantial loss of immunostained cells on the 6 hydroxydopamine lesioned side in both cases. Coronal sections; dorsal to top, midline to middle of plate. Scale bar = 100 μm.

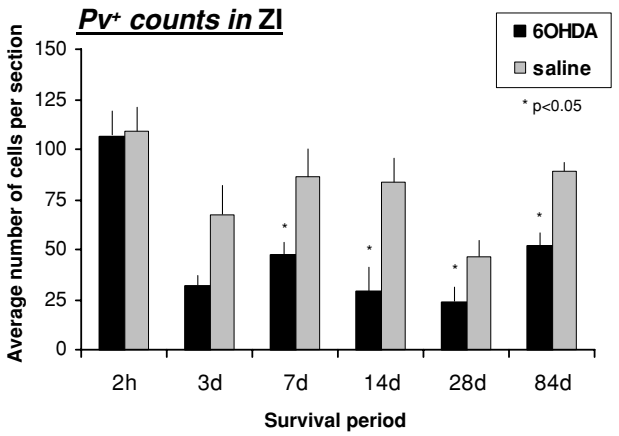

Fig. 9 Graph showing the number of parvalbumin$^+$cells in the zona incerta. The average number of cells per section is plotted against the different survival periods post-lesion. The number of cells in the saline and 6 hydroxydopamine injected side are compared for each period. Standard error bars are indicated.

topography does not match the pattern of loss reported in this present study.

The loss of parvalbumin expression by incertal cells might be argued, on the other hand, to be due to their uptake of 6OHDA, effectively destroying the protein. There are several reasons why this is unlikely, however. First, there are very few instances of 6OHDA pick up by non-monoaminergic cells, particularly in vivo (Decker *et al.*, 1993). There is no reason to suspect that the non-catecholaminergic cells of the zona incerta are any different. Second, the regions that were injected in this study, namely the medial forebrain bundle and the caudate-putamen complex, are well removed from the zona incerta, particularly the latter (see Paxinos & Watson, 1986; Fig. 3). It would be unlikely that the toxin spread into the zona incerta from the injection site and affected the parvalbumin expression of the incertal cells.

Functional and clinical implications: A speculation

The parvalbumin$^+$ cells of the zona incerta are located principally in the ventral sector of the zone. They lie in a region of very dense cytochrome oxidase immunoreactivity and are reported to also contain GABA (Nicolelis *et al.*, 1992; Kolmac & Mitrofanis, 1999). The GABAergic parvalbumin$^+$ cells have been shown to project to the superior colliculus (Nicolelis *et al.*, 1992) and are considered important in generating head and eye orientating movements towards a novel

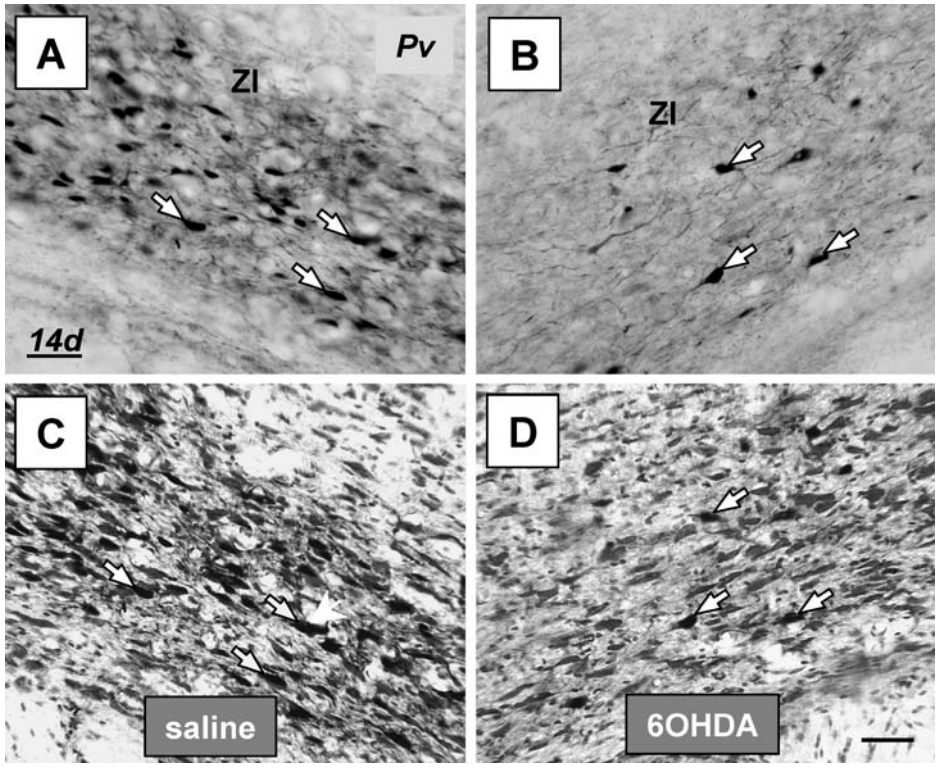

Fig. 10 Parvalbumin immunoreactivity and Nissl staining sections in the zona incerta. Photomicrographs in (A) and (B) show parvalbumin$^+$ cells in the zona incerta after saline (A) and 6 hydroxydopamine (B) injections into the medial forebrain bundle. Photomicrographs in (C) and (D) show the same section as the one immediately above it, but after counterstaining with neutral red. The corresponding arrows in (A) and (C) and those in (B) and (D) indicate the same cells. Scale bar = 100 μm.

stimulus (Schall, 1995; Moschovakis, 1996). In this context, Ma (1996) has shown that the ongoing cell activity in the zona incerta of monkeys "pauses" before the start of a saccade and resumes at the end of a saccade, suggesting the incertal cells inhibit collicular cells. This pause in activity of incertal cells could permit triggering of collicular activity generating head and eye movements.

The results of this study indicate that many incertal cells that presumably project to the superior colliculus, although do not degenerate after 6OHDA lesion, are abnormal in terms of their loss of parvalbumin expression. Previous studies have reported that cells with lowered levels of intraneuronal calcium-binding content become less excitable (Kohr *et al.*, 1991). This is thought to be due to a reduced ability to buffer intracellular calcium levels, potentially altering depolarising events (Chard *et al.*, 1995). Such a circumstance could lead to these cells becoming dormant (Eyles *et al.*, 2002). Hence, in the present context, a loss of parvalbumin in the GABAergic cells of the zona incerta could lead to these cells becoming less excitable and lead subsequently to a disinhibition of the superior colliculus. This would result in excessive head and orientative movements. However, this is not the case in parkinsonian cases. In MPTP-treated monkeys (Brooks *et al.*, 1986; Schultz *et al.*, 1989; Kato *et al.*, 1995; Kori *et al.*, 1995; Tereshchenko *et al.*, 2002) and parkinsonian patients (Shibasaki *et al.*, 1979), such orientative movements are suppressed greatly. Hence, in this light, one may speculate that a loss of parvalbumin in the zona incerta renders the cells more excitable. Without parvalbumin in the incertal cells, intracellular calcium levels may escalate and stimulate excessive release of GABA onto their postsynaptic targets in the superior colliculus. The cellular overactivity in the zona incerta of 6OHDA-lesioned rats reported by Pèrier *et al.*, (2000) lends some support to this notion. This overactivity may well involve the GABAergic incertal projections to the superior colliculus, which would compliment the other overactive collicular inputs from the GABAergic basal ganglia output nuclei (Pan & Walters, 1988; Robledo & Feager, 1991; Burbaud *et al.*, 1995; Hassani *et al.*, 1996; Ni *et al.*, 2000; Murer *et al.*, 1997). This would manifest in an overinhibition of the superior colliculus and result in a suppression of head and eye orientative movements.

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