

Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging

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Keywords: 6-OHDA, inflammation, Parkinson's disease, PET imaging, PK11195, Sprague–Dawley rats

Abstract

We investigated the microglial response to progressive dopamine neuron degeneration using *in vivo* positron emission tomography (PET) imaging and postmortem analyses in a Parkinson's disease (PD) rat model induced by unilateral (right side) intra-striatal administration of 6-hydroxydopamine (6-OHDA). Degeneration of the dopamine system was monitored by PET imaging of presynaptic dopamine transporters using a specific ligand ¹¹C-CFT (2 β -carbomethoxy-3 β -(4-fluorophenyl) tropane). Binding of ¹¹C-CFT was markedly reduced in the striatum indicating dopaminergic degeneration. Parallel PET studies of ¹¹C-PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3 isoquinoline carboxamide) (specific ligand for activated microglia) showed increased binding in the striatum and substantia nigra indicative of a microglial response. Postmortem immunohistochemical analyses were performed with antibodies against CR3 for microglia/macrophage activation. Using a qualitative postmortem index for microglial activation we found an initially focal, then widespread microglial response at striatal and nigral levels at 4 weeks postlesion. These data support the hypothesis that inflammation is a significant component of progressive dopaminergic degeneration that can be monitored by PET imaging.

Introduction

Microglia are the resident immune-competent cells of the brain. Their expression of proinflammatory mediators and increased numbers in Alzheimer's disease brains led to the suggestion that immune mechanisms may play an important role in the pathogenesis of neurodegenerative diseases (McGeer *et al.*, 1987; Cagnin *et al.*, 2001). Furthermore, microglial activation has also been observed in Parkinson's disease (PD) (Brooks, 1999). Recent findings by Langston *et al.* (1999), in postmortem analysis of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxicated patients and by Brownell *et al.* (1999) in primates pointed to the possibility of an inflammatory and glial response in the progress of nigral cell death (also see review by McGeer *et al.*, 2001).

Neuronal death activates and instructs microglia to remove cellular debris at the site of injury, but this activation may also be a source of additional neuronal death. Earlier studies have documented the presence of an inflammatory response in pathological processes reminiscent of PD (Akiyama & McGeer, 1989; Czlonkowska *et al.*, 1999; Kurkowska-Jastrzebska *et al.*, 1999). It has been suggested that this inflammatory response, propagated by

the cyclooxygenases, is one of the factors in neural degeneration leading to the induction of apoptosis. Inhibition of the inflammatory response could thus lead to a relative sparing of neuronal systems undergoing marked degeneration.

Positron emission tomography (PET) imaging has been used as a research tool to determine alteration of brain dopamine (DA) function and glucose metabolism associated with movement disorders. Recently, high resolution PET imaging has been applied in studies of animal models of PD (Brownell *et al.*, 1998a, 1999), allowing us to study such phenomena as the inflammatory response in the brains of living animals. Microglial activation using PET imaging of PK11195 (a peripheral benzodiazepine receptor ligand) has been identified and investigated in several neurological conditions (Junck *et al.*, 1989; Vowinckel *et al.*, 1997; Banati *et al.*, 1999; Brooks, 1999; Pappata *et al.*, 2000). Intra-striatal administration of 6-hydroxydopamine (6-OHDA) develops a slow and progressive degeneration of the DA system (Sauer & Oertel, 1994; Lee *et al.*, 1996; Bjorklund & Stromberg, 1997). 6-OHDA toxicity induces retrograde degenerative changes in DA neurons of the substantia nigra through mitochondrial damage (Bjorklund *et al.*, 1997; Costantini *et al.*, 2001). To explore inflammation during neurodegeneration, we chose this particular PD model to conduct imaging studies of DA transporters and microglial activation in parallel with post-mortem analysis of the DA degeneration and inflammatory response.

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Received 31 October 2001, revised 20 January 2002, accepted 30 January 2002

Methods

6-OHDA lesion model

Intrastratial administration of 6-OHDA creates progressive and retrograde degenerative changes in DA neurons of the substantia nigra (Bjorklund *et al.*, 1997; Costantini *et al.*, 2001). To investigate the progression of an inflammatory response, 15 Sprague–Dawley rats were lesioned using Kopf Instruments stereotactic frames. Animals were kept under ketamine/xylazine anaesthesia (60 mg/kg and 3 mg/kg, respectively). 6-OHDA infusions were made as described previously (Costantini *et al.*, 2001) using a 10- μ L Hamilton microsyringe, fitted with a 22-gauge bevelled (45°) Hamilton needle into four striatal sites from Bregma (site 1, AP + 1.4, ML –2.6, DV –5.5/–5.0; site 2, AP + 0.4, ML –3.0, DV –6.0/–5.5; site 3, AP –0.4, ML –4.2, DV –6.5/–6.0; and site 4, AP –1.3, ML –4.5, DV –6.5/–6.0). The incisor bar was set at 0.0. The 6-OHDA solutions were kept on ice (4 °C) and protected from light to minimize oxidation. Volumes of 2 μ L were injected into each striatal site (6 μ g/site; total of 24 μ g) with an infusion rate of 1 μ L/2 min. After infusion, the needle remained *in situ* for 4 min. Using the same method, three control rats received striatal infusions of saline +0.2% ascorbic acid.

Animals used in this study were maintained according to the guidelines of the Committee on Animals of the Harvard Medical School and Massachusetts General Hospital and those of the *Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources*, National Research Council, Department of Health, Education and Welfare. All efforts were made to reduce the number of animals used.

PET imaging

A total of three saline-injected and five 6-OHDA lesioned rats were imaged by PET using ^{11}C -CFT (2 β -carbomethoxy-3 β -(4-fluorophenyl) tropane), a specific ligand for presynaptic DA transporters (Hantraye *et al.*, 1992; Brownell *et al.*, 1998b). To explore activation of microglia/macrophage function, imaging studies were conducted in the same rats with ^{11}C -PK11195, a specific ligand for activated microglia (Banati *et al.*, 1999). Imaging studies were performed prior to 6-OHDA lesioning and 3 weeks after 6-OHDA injections using an in-house-built, dedicated PET system (PCR-I) (Brownell *et al.*, 1985). This later time-point was determined by immunohistochemistry performed at 3, 5, 10, 21 or 28 days, pointing to the 3-week time-point as a peak in the inflammatory response. The radiopharmaceuticals, ^{11}C -CFT and ^{11}C -PK11195, used in imaging studies, were prepared according to previously published procedures (Camsonne *et al.*, 1984; Hantraye *et al.*, 1992). Binding ratios were calculated as described previously (Brownell *et al.*, 1998b).

For PET imaging studies, animals were anaesthetized with halothane (1–1.5%) using an oxygen flow rate of 3 L/min. Tail vein and artery were catheterized for infusion of the labelled ligands and drawing of blood samples needed for quantification. The animal was placed in the imaging position and the head was adjusted into an in-house-built stereotactic head-holder with the ear-bar at the origin. Sequential dynamic imaging data were acquired at seven different coronal brain levels. Calibration of the positron tomograph was performed in each study session using a cylindrical plastic phantom (diameter 6 cm) and 18F-solution. Imaging data were corrected for uniformity, sensitivity, attenuation, decay and acquisition time (Brownell *et al.*, 1998b). PET images were reconstructed using Hanning-weighted convolution backprojection and overlaid on MR templates to confirm anatomical location. Regions of interest, including the striatum, midbrain and cerebellum areas on both sides

of the brain, were drawn and activity per unit volume, percentage activity of injected dose and ligand concentration were calculated (Brownell *et al.*, 1998b).

Immunohistochemical evaluation

Animals were killed at 3, 5, 10, 21 or 28 days after intrastratial 6-OHDA injection under deep anaesthesia with sodium pentobarbital (300 mg/kg intraperitoneally). Transcardiac perfusion with heparinized saline was followed by 4% paraformaldehyde. Brains were postfixed in paraformaldehyde 4% for 6–8 h and sectioned on a freezing microtome (40 μ m thick sections) after overnight cryoprotection in 20% sucrose. Immunohistochemical staining to investigate DA loss and brain macrophage activation was performed using the tyrosine hydroxylase (TH) (Pel-Freez, Rogers, AR, USA) and OX42 antibody recognizing CR3 (microglia/macrophages) (Sera-lab/Accurate Chemical, Westbury, NY, USA). The sections were preincubated for 30 min in a solution containing 5% normal goat serum and 0.1% triton X-100. Sections were incubated overnight at 4 °C in a solution containing 5% normal goat serum, 0.1% triton X-100 and anti-TH (1 : 500) or anti-OX42 (1 : 100). As a control for specificity of antibodies, some sections were treated as described except that the primary antibody was omitted from the incubation medium.

After three rinses of 10 min in phosphate-buffered saline (PBS), the sections were incubated for 1 h at room temperature in secondary antibodies (biotinylated goat IgG) (goat antirabbit for TH or goat antimouse that was preabsorbed against rat tissues for OX-42) (Vector Laboratories, Burlingame, CA, USA) (dilution 1 : 200). After three more rinses in PBS, the sections were reincubated for 1 h at room temperature in avidin-biotin complex (Vectastain, ABC kit Elite, Vector Laboratories). The sections were then washed twice in PBS and once in Tris-buffered saline. The bound peroxidase was revealed by placing the sections in a medium containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA) and 0.04% hydrogen peroxide (H₂O₂, 30%) in Tris-buffered saline at room temperature.

Photomicrographs were taken using a spot camera (Diagnostic Instruments) linked to the imaging software Adobe Photoshop 5.5.

Rating and statistical analysis of the immune-activated response

Due to the essential qualitative determination of activated microglia morphology and significant problems in assessing total numbers of activated microglia on thick (40 μ m) sections, brain macrophage/microglia-activation was graded in the following manner.

(i) None: no OX42-immunopositive microglia display the morphology of activated cells.

(ii) Focal: the majority of OX42-immunopositive microglia display a typical morphology of activated cells. The OX42-positive staining is side-specific and confined to the striatum and substantia nigra on the ipsilateral side of the lesion.

(iii) Widespread: the majority of OX42-immunopositive microglia display a morphology typical of activated cells. The OX42 staining is widespread and detectable in areas other than the striatum and the substantia nigra and also observed contralateral to the lesion side.

All TH cells present in the substantia nigra were counted under brightfield illumination at high magnification. Abercrombie correction was performed as described previously (Abercrombie, 1946). TH cell loss was quantitatively expressed by an exponential function $f(t) = (96.99 \pm 2.36) \times \exp[-(0.062 \pm 0.002) \times t]$ using a least square fitting technique with the SAAM II program package (SAAM Institute, Seattle, WA, USA).

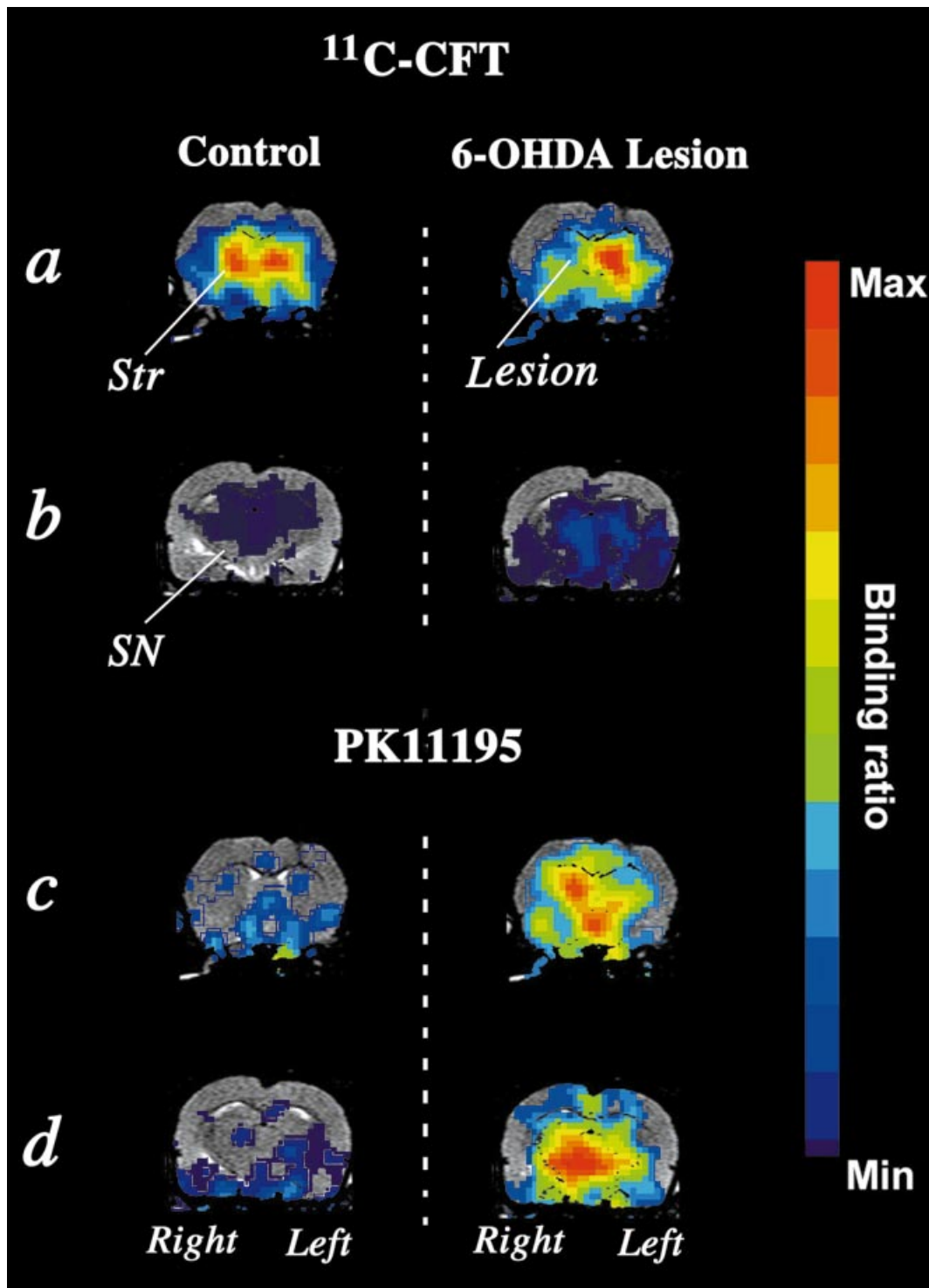


FIG. 1. Neuroinflammation of the nigrostriatal pathway during progressive retrograde dopamine degeneration induced from striatal location. Colour-coded images, overlaid on magnetic resonance images for anatomical precision, showing a significant decrease in binding parameters of $^{11}\text{C-CFT}$ (dopamine uptake sites located on presynaptic terminals) after unilateral intrastratial infusion of the toxin 6-hydroxydopamine (6-OHDA) (on the right side of the brain) (a). Because of size and reduced number of binding sites, it is not possible to accurately detect and estimate binding potential in the substantia nigra (b). The process of selective dopamine axonal degeneration was paralleled by a significantly increased striatal binding of the peripheral benzodiazepine receptor $^{11}\text{C-PK11195}$, which is expressed primarily on reactive microglia during central nervous system inflammation (c). A dramatic increase in $^{11}\text{C-PK11195}$ binding was also seen in the ventral mesencephalon at the level of the substantia nigra (SN) of the striatally (Str) infused side (d). (a) to (d) are all at 21 days after 6-OHDA lesion.

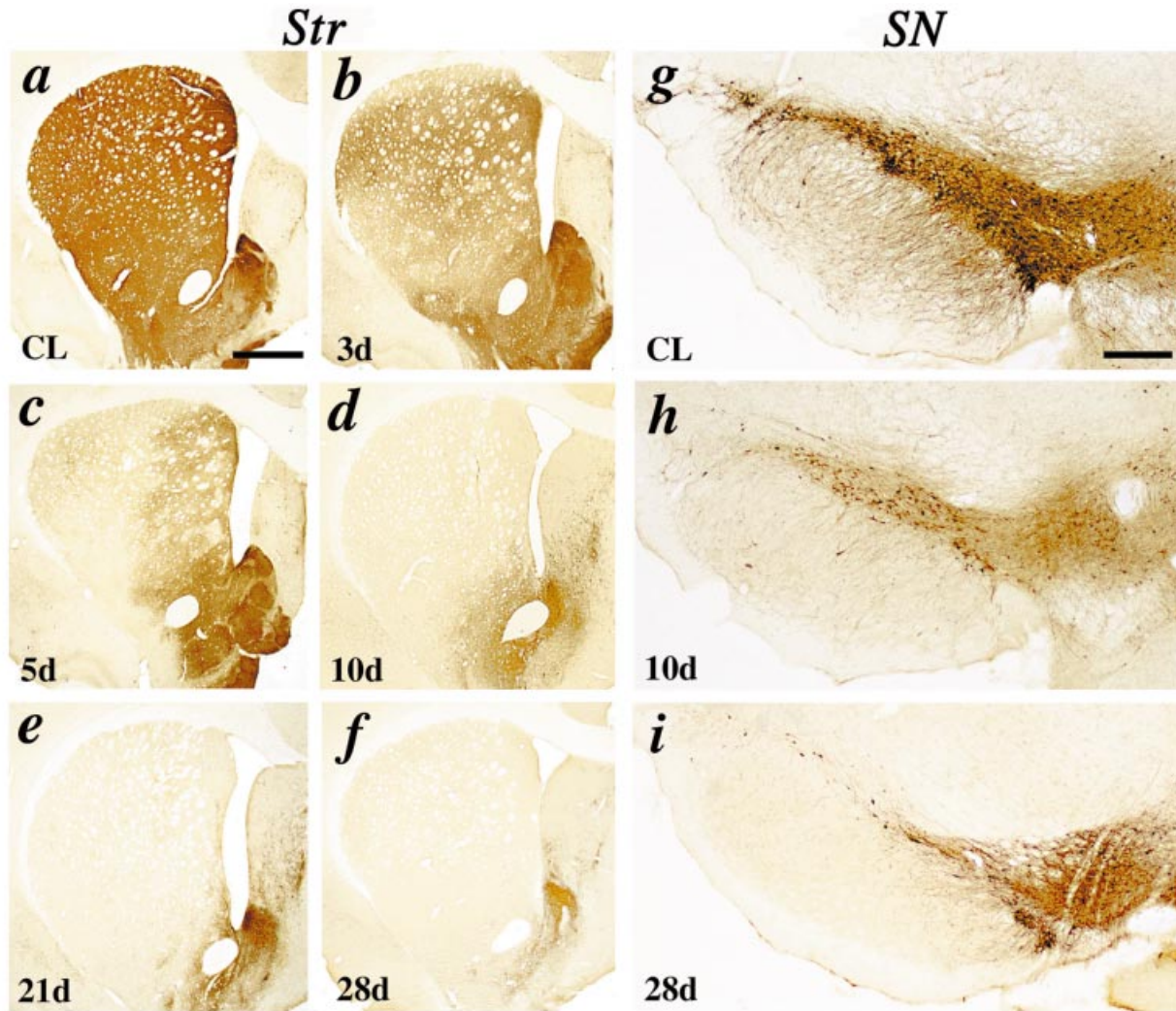


FIG. 2. Striatal tyrosine hydroxylase (TH) fibre loss and nigral TH cell loss. Low power photomicrographs of TH immunostaining in the striatum of five animals killed at 3 (a), 5 (b), 10 (c), 21 (d) and 28 days (f) after intrastriatal 6-hydroxydopamine (6-OHDA) lesions, illustrating the progressive striatal TH fibre loss. Note the normal innervation (part a has been flipped for comparison purposes) of the contralateral side of the lesion to a fully denervated striatum 28 days after 6-OHDA striatal lesion (f). Low power photomicrographs of TH immunostaining in the substantia nigra of animals killed at 10 days or 28 days after intrastriatal 6-OHDA injection (h and i). Note the progressive loss of dopaminergic neurons on the lesioned side at 10 (b) and 28 days (c) compared with the unlesioned side (g). Scale bar in a, 120 μ m (for a–i).

Results

Imaging studies

Imaging studies of the DA system showed in 6-OHDA-lesioned rats a significant decrease in the binding parameters of ^{11}C -CFT in the striatum (Fig. 1a). The binding ratio in the striatum using the cerebellum as a reference tissue was $35.0 \pm 4.2\%$ ($n = 5$) of the original value 3 weeks postlesion. Based on imaging studies, however, it is not possible to accurately estimate the binding potential in the substantia nigra (Fig. 1b) because the size of the site is small and the binding sites are much fewer than in the striatum. However, ^{11}C -PK11195, a ligand of the mitochondrial benzodiazepine receptor (Cappelli *et al.*, 1997) and a sensitive marker of activated microglia, showed significantly increased uptake in the striatum (Fig. 1c), the substantia nigra and in the whole mesencephalon area on the lesion side (Fig. 1d). The binding ratio of ^{11}C -PK11195 using cerebellum as a reference tissue was increased $45 \pm 17\%$ in the substantia nigra and $67 \pm 23\%$ ($n = 5$) in the

striatum after 3 weeks. The progressive decrease of ^{11}C -CFT binding in the striatum indicates degeneration of the DA system, and parallel increases of ^{11}C -PK11195 binding indicate microglia activation both in the right striatum and substantia nigra. ^{11}C -CFT and ^{11}C -PK11195 did not show any left-right side difference in the binding parameters after sham lesion induced by saline.

Postmortem analysis

6-OHDA progressive lesion model

After intrastriatal 6-OHDA injection, there was a time progression of DA fibre loss in the striatum (Fig. 2a–f) as well as neuronal loss in the substantia nigra (Fig. 2g–i). Striatal TH fibre staining intensity decreased progressively such that at 10 days postlesion there was a complete loss of fibre staining indicating a full denervation (Fig. 2d). Quantitative analysis of TH-positive neurons in the pars compacta of the substantia nigra revealed that the decrease in TH cell number was distinguishable as early as 3 days postlesion (Fig. 4). From this time-

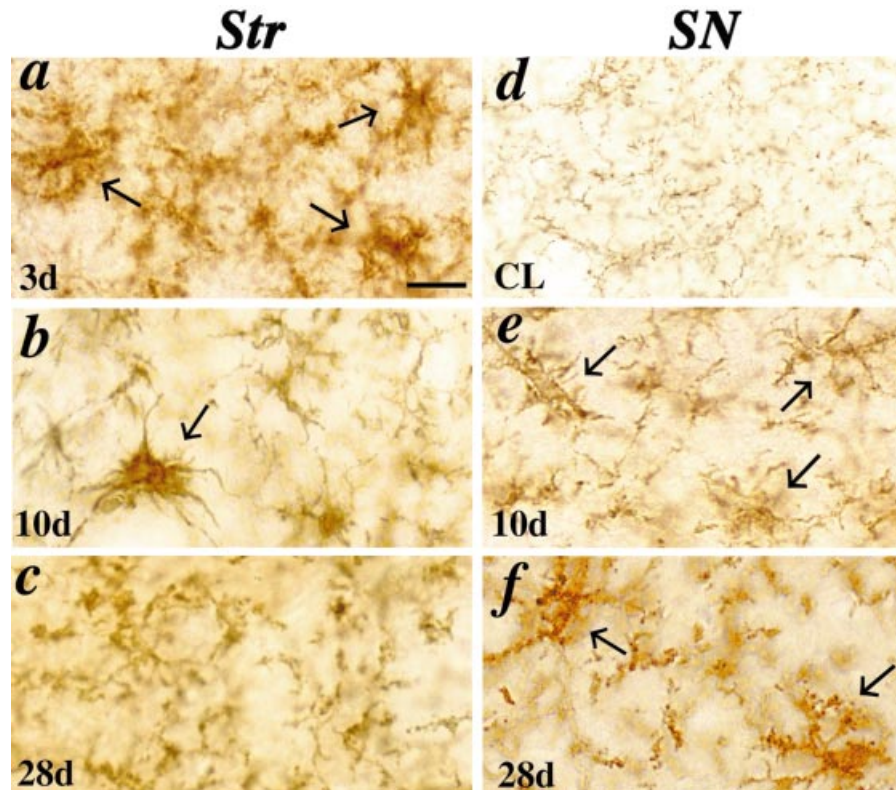


FIG. 3. High power photomicrographs of OX42 immunostaining in the striatum (a–c) and substantia nigra (d–f) of animals killed at 3 (a), 10 (b and e) or 28 (c and f) days after intrastriatal 6-hydroxydopamine (6-OHDA) injection. Note that striatal photomicrographs (a–c) were taken in areas far removed from the needle tract. Activated microglia were identifiable at all time-points (shown by black arrows in parts a and b although photomicrograph in part c shows a striatal area that did not contain activated microglia). Activated microglia were also observable in the substantia nigra at all time-points (e and f), whereas normal animals did not show the response of activated microglia (d). Scale bar in a, 25 μ m (b–f).

point, there was a progressive decline in the number of TH-positive cells. At 28 days postlesion, there were few identifiable TH-positive cells in well lesioned animals (Figs 2i and 4). A survival function of TH neurons in the substantia nigra was derived from cell count data: $f(t) = (96.99 \pm 2.36) \times \exp [-(0.062 \pm 0.002) \times t]$.

Striatal immune-activated response

OX42 immunostaining (microglia and macrophages) was observed in the striatum of all lesioned animals at all time-points (3, 5, 10, 21 and 28 days). Immunostaining was more pronounced (intensity and number of activated cells) within the needle tract but the OX42 immunostaining was also present in striatal areas far removed from the needle tract (see Fig. 3a and b). In areas of ongoing immune activation, the microglia appeared activated, as characterized by larger cell bodies, shorter proximal processes, reduced ramification of the distal processes and increased staining intensity of OX42 (Fig. 3a, b, e and f). In nonactivated areas, microglia showed the typical ramified morphology of resting cells with small cell bodies and numerous processes with extensive ramification (Fig. 3c and d). OX42 staining was absent in the needle tract of saline/ascorbic acid-injected rats.

Nigral immune-activated response

The pattern of OX42 staining in the substantia nigra of lesioned animals paralleled that of the striatum at all time-points. At the early time-point (3 days), the OX42 staining demonstrated nonactivated microglia based on morphology (Fig. 3d). At 5 and 10 days, activated

microglia were evident (Fig. 3e and f) focally in the substantia nigra on the ipsilateral side of the lesion. At 10 days, the intensity of the OX42 staining reached its peak where the majority of cells in the substantia nigra had the morphology of activated cells. The intensity of the staining and the number of activated cells were confined to the substantia nigra ipsilateral to the lesion. At 28 days, while there were still a number of activated microglia in the substantia nigra, there was a more widespread distribution in structures away from the striatum and substantia nigra, as well as contralateral to the lesion (see Fig. 4). All well-lesioned animals, as evaluated by immunohistochemistry (including animals that were previously scanned) showed a widespread inflammatory response, which corresponded to the classification criteria of both PET imaging and immunohistochemistry.

Discussion

In this study, DA neuronal degeneration was induced in the rat by intrastriatal administration of 6-OHDA, causing a progressive striatal fibre loss and neuronal degeneration in the substantia nigra. PET imaging of ^{11}C -PK11195 in lesioned rats showed a marked inflammatory response in both the striatum and the substantia nigra at 3 weeks postlesion. Increases in OX42 immunostaining also indicated a significant inflammatory response in both the striatum and substantia nigra, persisting at 4 weeks postlesion. These observations are consistent with an ongoing inflammatory response resulting from neural injury and/or neuronal death.

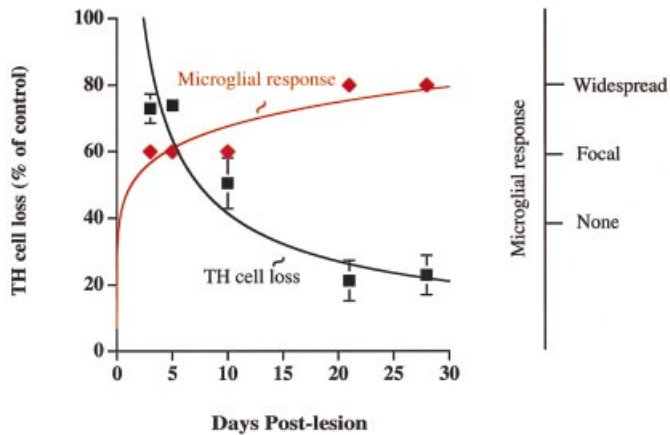


FIG. 4. Graph depicting the tyrosine hydroxylase (TH) cell loss (calculated as a percentage of control) in parallel with a microglial response observed over a 4-week period following unilateral intrastratial 6-hydroxydopamine (6-OHDA) infusion in rats. The TH cell loss function $\{f(t) = (96.99 \pm 2.36) \times \exp[-(0.062 \pm 0.002) \times t]\}$ was calculated using a least square fitting technique. The qualitative microglial response index was based on three main criteria. None: no OX42-immunopositive microglia displayed the morphology of activated cells. Focal: the majority of OX42-immunopositive microglia displayed a typical morphology of activated cells. The OX42-positive staining was side-specific and mostly confined to the striatum and substantia nigra on the ipsilateral side of the lesion. Widespread: the majority of OX42-immunopositive microglia displayed a morphology typical of activated cells. The OX42 staining was widespread and detectable in areas other than the striatum and the substantia nigra and also observed contralateral to the lesion side. Note the widespread distribution and persistence of microglial response as TH cell loss progresses over time, indicative of a continuous inflammatory response.

^{11}C -PK11195 has been successfully used in human studies to investigate microglial activation in multiple sclerosis (Vowinckel *et al.*, 1997), human gliomas (Junck *et al.*, 1989), Rasmussen's encephalitis (Banati *et al.*, 1999), ischaemic stroke (Pappata *et al.*, 2000), and more recently in Alzheimer's disease (Cagnin *et al.*, 2001) and PD (Gerhard *et al.*, 2001). In stroke patients, the persistent increase in PK11195 binding in the thalamus after cortical stroke suggests that an active tissue pathology leading to microglia activation is present in the ipsilateral thalamus many months after the stroke event (Pappata *et al.*, 2000). Experimental models for ischaemic stroke also mimic the increased binding of PK11195 in thalamic regions, which persists beyond a period of 4 weeks (Myers *et al.*, 1991). Parallel immunocytochemical observations corroborate the increase in PK11195 binding linked to the increased expression of peripheral benzodiazepine binding sites by activated microglia, in areas remote from the focal infarct lesion (Banati *et al.*, 1997).

In Alzheimer's disease the accumulation of senile plaques and neurofibrillary tangles are accompanied by an inflammatory response (Lue *et al.*, 1996; Cagnin *et al.*, 2001). Interestingly, a chronic inflammatory response has also been identified in the cortex of patients with dense Lewy bodies (Mackenzie, 2000). However, epilepsy patients with hippocampal sclerosis with a stabilized disease process (nonprogressive) do not show a significant accumulation of PK11195 in PET studies. Recent observations (Brooks, 1999; Gerhard *et al.*, 2001) of increased PK11195 binding in the substantia nigra of PD patients are consistent with our observations in the rat model of PD.

We and colleagues have shown that following MPTP or 6-OHDA *in vivo* toxin exposure, there is an inflammatory response around

degenerating axons and cells (Costantini *et al.*, 2001; Kurkowska-Jastrzebska *et al.*, 1999). The 6-OHDA striatal lesion model used in the present study allows for a slow, progressive and retrograde degeneration through the striatal nerve terminal field that mimics several aspects of idiopathic PD pathogenesis. The dynamic and persistent physiological changes observed by PET and magnetic resonance spectroscopy (MRS) methods (Brownell *et al.*, 1998a) of MPTP-exposed primates with PD signs may reflect similar adaptive striatal responses to those occurring in PD. Evidence of microglial activation and elevated levels of inflammatory cytokines has also been documented in PD patients (McGeer *et al.*, 1988; Banati *et al.*, 1998). Recent findings by Langston *et al.* (1999) in MPTP-induced PD suggest that an active inflammatory process could be associated with the process of nigral cell death (Langston *et al.*, 1999).

In early phases of neuronal injury, microglia express numerous molecules with important immune functions and may generate functions such as the adaptive removal of synapses from injured neurons (Graeber *et al.*, 1990, 1993). The loss of synapses, axons and neurons following a toxic insult may generate an inflammatory response within the central nervous system characterized by activation of microglia towards the site of injury (Hirsch *et al.*, 1998). Similar activation could be elicited by neurotoxins or neuronal inclusions, Lewy bodies, plaques or viral antigens. Microglia take on the role of removing degenerating synaptic terminals and neuronal debris. This inflammatory response around damaged neurons or neurites could induce an indiscriminate response similar to chronic inflammation in cartilage or myelin, as observed during autoimmune disease (for example, rheumatoid arthritis or multiple sclerosis (Vowinckel *et al.*, 1997; Banati *et al.*, 1999). This response may result in nonspecific damage unrelated to the original inflammatory signal. Although inflammation is the first line of defence against injury and infection, under certain conditions this reaction could generate more damage to surrounding tissue and lead to further neuronal degeneration that would have otherwise been avoided (McGeer *et al.*, 1987).

Therefore, inhibition of the inflammatory response could lead to a relative sparing of DA neurons (Lu *et al.*, 2000). There are several nonsteroidal anti-inflammatory drugs (NSAIDs) of which one mechanism of action is the reduction of prostaglandin synthesis, via inhibition of cyclooxygenase enzymes. High doses of indomethacin over long periods of time are associated with reduced incidence of Alzheimer's disease in rheumatoid arthritis patients (for review see McGeer *et al.*, 1997; McGeer & McGeer, 1998).

As documented in several neurological conditions, microglial activation as detected by PK11195 PET can be observed up to several weeks after the initial events (Junck *et al.*, 1989; Vowinckel *et al.*, 1997; Banati *et al.*, 1999; Brooks, 1999; Pappata *et al.*, 2000). The specificity and consistency of this method may potentially be useful in monitoring therapeutic effects on microglial activation (Gerhard *et al.*, 2000; Pappata *et al.*, 2000). Furthermore, elevation of biomarkers for oxidative stress and inflammation seen years after the neurotoxic events leading to DA loss suggests that the striatal neuronal circuitry may be compromised and at risk for subsequent structural and pathological processes (Brownell *et al.*, 1998a, b, 1999). Although the primary damage created by the 6-OHDA striatal infusion was confined to the nigrostriatal system, as is the case with excitotoxins (Isacson *et al.*, 1987) widespread changes in microglial activation were observed at 4 weeks by immunohistochemistry. Taken together, the finding of a high inflammatory response in the striatum and substantia nigra in PD (Chen *et al.*, 1998; Hirsch *et al.*, 1998; McGeer & McGeer, 1998; Brooks, 1999) and a progressive degeneration of DA in a PD model provides an opportunity to

evaluate whether preventing reactive inflammation can reduce the observed DA degeneration.

Acknowledgements

This work was supported by NIH (NS41263-0-02) (O.I.), the Century Foundation of Sarasota Memorial Hospital (O.I.), the Parkinson Alliance (O.I.), NS37654 (K.W.) and the Medical Research Council of Canada (F.C.).

Abbreviations

¹¹C-CFT, 2 β -carbomethoxy-3 β -(4-fluorophenyl) tropane; 6-OHDA, 6-hydroxydopamine; DA, dopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate-buffered saline; PD, Parkinson's disease; PET, positron emission tomography; TH, tyrosine hydroxylase.

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