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Reduced BDNF mRNA Expression in the Parkinson's Disease Substantia Nigra

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Brain-derived neurotrophic factor (BDNF) has potent effects on survival and morphology of dopaminergic neurons and thus its loss could contribute to death of these cells in Parkinson's disease (PD). *In situ* hybridization revealed that BDNF mRNA is strongly expressed by dopaminergic neurons in control substantia nigra pars compacta (SNpc). In clinically and neuropathologically typical PD, SNpc BDNF mRNA expression is reduced by 70% ($P = 0.001$). This reduction is due, in part, to loss of dopaminergic neurons which express BDNF. However, surviving dopaminergic neurons in the PD SNpc also expressed less BDNF mRNA (20%, $P = 0.02$) than their normal counterparts. Moreover, while 15% of control neurons had BDNF mRNA expression >1 SD below the control mean, twice as many (28%) of the surviving PD SNpc dopaminergic neurons had BDNF mRNA expression below this value. This 13% difference in proportions (95% CI 8–17%, $P \leq 0.000001$) indicates the presence of a subset of neurons in PD with particularly low BDNF mRNA expression. Moreover, both control and PD neurons displayed a direct relationship between the density of BDNF mRNA expression per square micrometer of cell surface and neuronal size ($r^2 = 0.93$, $P \leq 0.00001$) which was lost only in PD neurons expressing the lowest levels of BDNF mRNA. If BDNF is an autocrine/paracrine factor for SNpc dopaminergic neurons, loss of BDNF-expressing neurons may compromise the well-being of their surviving neighbors. Moreover, neurons expressing particularly low levels of BDNF mRNA may be those at greatest risk of injury in PD and possibly the trigger for the degeneration itself.

Key Words: **Parkinson's disease; brain-derived neurotrophic factor; dopaminergic; *in situ* hybridization.**

INTRODUCTION

Idiopathic Parkinson's disease (PD) is one of the most common neurodegenerative disorders and affects 1% of people over the age of 65 years. While the pathological changes are well described (11) and consist of a defined pattern of Lewy body formation, neuronal loss and gliosis in the mesodopaminergic nuclear complex, and, most noticeably, loss of pigmented dopaminergic substantia nigra pars compacta (SNpc) neurons, little is known about the disease etiology.

One hypothesis that remains largely untested is that reduced expression of one or a combination of the neurotrophic factors known to support the survival and neurite outgrowth of dopaminergic neurons might contribute to the degeneration and death of these neurons (2).

There are several neurotrophins and growth factors with dopaminergic activity (30), most notably brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). However, while both have potent effects on survival and neurite outgrowth from dopaminergic neurons (10, 13, 19, 23, 38–41, 44), GDNF is normally only expressed at very low levels in adult brain (24) and no alteration of expression has been detected in Parkinson's disease (18). Of all the factors with dopaminergic activity, only BDNF is both a potent dopaminergic neurotrophin (19, 23, 38–41) and normally expressed in the adult nigrostriatal dopamine system (8, 43).

The potency of BDNF's action on dopaminergic neurons is illustrated by examining its effects on animal models of Parkinson's disease. In cell culture, BDNF enhances the survival of dopaminergic neurons and protects them against the neurotoxic effects of both MPP^+ and 6-OHDA (19, 39). Similarly, intrastriatal injections of BDNF improve the apomorphine-induced rotations that follow loss of nigral dopamine neurons after 6-OHDA toxicity in rats (38) while nigral implantation of BDNF-secreting fibroblasts prevents the loss of these neurons (23) and increases substantia nigra dopamine levels after MPP^+ toxicity (9). Importantly, intrathecal infusion of BDNF reduces loss of dopamine neurons and ameliorates parkinsonism in the best available model of Parkinson's disease, the MPTP-treated monkey (41).

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Induction of dopaminergic sprouting contributes to these effects. BDNF promotes sprouting and neurite outgrowth from cultures of human fetal dopaminergic neurons (40), induces sprouting of a halo of dopaminergic axons when BDNF is injected into the striatum (38), and enhances graft-derived reinnervation of denervated striatum (44). We have shown that dopaminergic sprouting also occurs after surgical injury to mouse striatum (14) and that this sprouting is accompanied by induction of periwound expression of mRNA for BDNF (43) by microglia which accumulate around the wound (3).

Others have shown that mRNA for BDNF and its receptor trkB are also both expressed in striatum (35, 43), substantia nigra, and ventral tegmental area (8, 27, 37) of adult rodents. BDNF immunohistochemistry after colchicine treatment to inhibit axonal transport causes accumulation of BDNF in the substantia nigra and depletion in the striatum revealing that BDNF produced in the substantia nigra is anterogradely transported to the striatum (1). Moreover, in adult life dopaminergic stimulation induced by oral administration of L-DOPA directly promotes the expression of BDNF in the striatum (31). Therefore, one role for BDNF may be to act as an autocrine/paracrine regulator of both striatal dopaminergic innervation and substantia nigra dopaminergic neuron survival.

A pilot study in 1998 first hinted that reduced BDNF mRNA concentrations in substantia nigra might contribute to development of Parkinson's disease (16). More recently ELISA has demonstrated that the concentration of BDNF itself is reduced in the Parkinson's disease substantia nigra (29). Similarly, BDNF immunohistochemistry has shown that most mesencephalic pigmented neurons are immunoreactive for BDNF (32). However, neither study tells us how much BDNF is being produced by each neuron. The aim of this study was to use *in situ* hybridization to determine how much BDNF mRNA is expressed by individual neurons in the Parkinson's disease substantia nigra and determine whether reduced expression was likely to compromise their well-being.

SUBJECTS AND METHODS

Patients

We have used *in situ* hybridization to examine the expression of BDNF mRNA in the substantia nigra of postmortem tissue from five patients with idiopathic PD and five controls with no known neurological or psychiatric disease. The two groups were not significantly different for age at death (PD, mean age 75 years, range 56–88 years; controls, mean age 73.5 years, range 58–90 years) or postmortem (PM) delay between death and tissue collection (PD, mean PM delay 16.1 h, range 3.5–32.5 h; controls, mean PM delay 14.6 h, range 3.5–23 h). Since tissue pH is believed to be critical for the success of *in situ* hybridization (21), 2-g blocks of tissue from the lateral cerebellar hemisphere were collected from each patient and homogenized in 20 ml of distilled H_2O (pH 7.0) before reading the pH at room temperature using a standard pH meter. Cerebellar tissue was used because it is thought to be unaffected by Parkinson's disease and provides a consistent proportion of gray and white matter (21). There was no difference in the tissue pH between the control (mean 6.3, range 6.1–6.6) and Parkinson's disease groups (mean 6.3, range 6.0–6.54).

The mean disease duration in the PD patients was 17.6 years (range 13–20 years) and L-DOPA treatment had been given for an average of 11.5 years (range 7–16 years). The mean Hoehn and Yahr score at death was 3.6 (range 3–4). All the PD patients satisfied accepted clinical and neuropathological criteria for idiopathic Parkinson's diseases (11, 17, 42), diagnosed on the basis of a progressive disorder with rigidity, bradykinesia and rest tremor, L-DOPA responsiveness, asymmetry of signs, and the absence of clinical features characteristic of other causes of parkinsonism. All PD patients had 40–60% loss of dopaminergic substantia nigra neurons and Lewy bodies in the SNpc, locus coeruleus, pedunculopontine tegmental nucleus, dorsal motor nucleus of the vagus, and nucleus basalis of Meynert. None of the patients had changes which would satisfy diagnostic criteria for Alzheimer's disease or diffuse Lewy body disease.

Tissue Preparation

At autopsy, l-cm-thick coronal blocks of midbrain were snap-frozen in isopentane cooled in liquid nitrogen and stored at −80°C. Serial 20-μm sections were then cut through the substantia nigra and sections at the level of the red nucleus showing exiting third nerve fibers were selected to allow analysis of a consistent anatomical region.

In Situ Hybridization

In situ hybridization for dry film autoradiography was performed on three to five replicate sections from four of the PD and three of the control subjects using a single 50-base oligonucleotide (43) end labeled with [γ-^{33}P]ATP. Hybridization occurred at 42°C for 18 h in 350 μl of hybridization buffer placed on the section. After washing in 1× SSC at 55°C and drying, the sections were exposed to Amersham Hyperfilm for 4 days. After autoradiography all sections were stained with thionine for anatomical alignment. To allow quantitation of images on Amersham Hyperfilm, a series of [γ-^{33}P]ATP standards were exposed to film together with the brain sections and used as standards for densitometry. To estimate the extent of BDNF mRNA expression over individual dopaminergic neurons in

the substantia nigra, *in situ* hybridization was performed on a single demelanized and counterstained section from five PD and five control subjects using a pair of [γ-^{33}P]ATP-labeled oligonucleotides corresponding to sequences 731–685 and 575–527 of the human BDNF sequence (20) for oligonucleotides 1 and 2, respectively: Oligonucleotide 1, 5′-ATAGTAAGGGCCCGAACATACGATTGGGTAGTTCGGCATTGCGAGTTCCA-3′; and Oligonucleotide 2, 5′-TAGGACTGTGACCGTCCCGCCAGACATGTCCACTGCAGTCTTTTTATCTG-3′.

To demelanize the sections, the slides were first soaked in 1% aqueous $KMnO_4$ for 5 min, rinsed in dH_2O 1 min, and then incubated in 5% aqueous oxalic acid (5–10 min) until only a faint brown stain was left to delineate the cells when examined microscopically.

Autoradiography was then performed by coating the sections in photographic emulsion (Amersham, LM-1 emulsion) and exposing for 3 weeks. The outline of each neuron was defined manually and the circumference was used to determine the cross-sectional area of each cell using the Spatial Area option of the MCID suite of software (Imaging Research Inc.) after calibration of the system in the *X* and *Y* dimensions using a stage micrometer. Silver grains were then counted automatically within these manually defined regions using the MCID counting function after setting the target acceptance criteria to ignore objects larger or smaller than the silver grains. To minimize the effects of variation in emulsion thickness, a background grain count was made one cell diameter away from each neuron using each neurons own outline as a template. This background count was made either 0°, 90°, 180°, or 270° from each neuron depending on where the first space not occupied by a neighboring neuron was found. Silver grains were counted over every SNpc neuron visible in each section. The specificity of probe binding was assessed by examining the signal produced in the presence of an excess of unlabeled antisense oligonucleotides, when corresponding sense oligonucleotides were used as the probes, and after RNase digestion of mRNA in the sections. Statistical analysis was performed using Simstat for Windows (Provalis Research) and confidence interval analysis (BMJ).

RESULTS

Dry Film Autoradiography

On the dry film autoradiograms, a dark band indicating BDNF mRNA expression was found in the substantia nigra of control brains and was particularly intense over the SNpc (Fig. lA). The expression of BDNF in the control SNpc (434 ± 42 cpm/mm^2, mean ± SEM) was more than three times greater (P = 0.003) than expression in the red nucleus (130 ± 37cpm/mm^2) (Figs. lA and 1C).

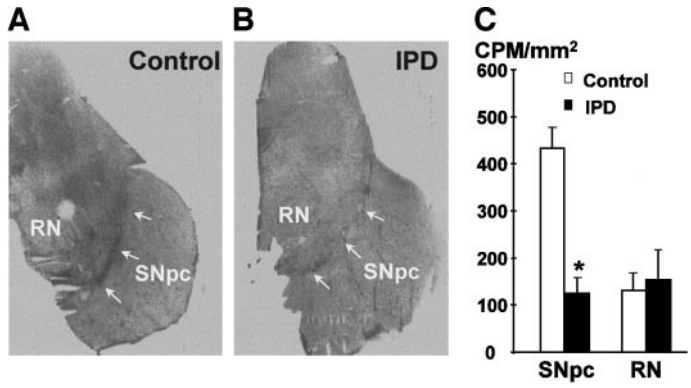

FIG. 1. BDNF mRNA expression in control (n = 3) and PD (n = 4) substantia nigra pars compacta (SNpc) and red nucleus (RN). (A) Dry film autoradiograph of control SNpc. (B) Dry film autoradiograph of PD SNpc. (C) Densitometry showing reduced BDNF mRNA expression in PD SNpc but no change in red nucleus. All values are means ± SEM, *P = 0.001, t test assuming equal variance.

In patients with PD, the density of BDNF mRNA expression in the red nucleus was the same as in the controls (154 ± 63 cpm/mm^2). However, while some BDNF expression was still detected over the SNpc (Fig. 1B), the level of expression (125 ± 33 cpm/mm^2) was approximately 30% (P = 0.001) of that seen in the controls (434 ± 42 cpm/mm^2) (Fig. 1C).

Corresponding sense oligonucleotides produced no specific signal over the SNpc. Performing the *in situ* hybridization in the presence of an excess of unlabeled antisense oligonucleotides inhibited binding of the radiolabeled probes to dopaminergic neurons in the SNpc while RNase digestion of mRNA in the section abolished the signal.

Emulsion Dipped Autoradiography

Qualitative emulsion dipped autoradiography revealed that all pigmented neurons in the substantia nigra of both controls and Parkinson's disease patients expressed BDNF mRNA (Figs. 2A and 2B). Because the neuromelanin present in the pigmented dopaminergic neurons interfered with counting silver grains on both bight field (Fig. 2C) and dark field microscopy (Fig. 2 B), subsequent quantitative analysis was performed on demelanized sections (Fig. 2D).

After demelanization and lightly counterstaining, every neuron detected in a single section through the substantia nigra at the level of the red nucleus and exiting third nerve of the five PD and five control subjects was examined. Silver grains were counted over a total of 539 and 906 neurons from PD and control subjects, respectively. There was no difference in the mean cross-sectional neuronal areas sampled from each group of subjects (mean ± SEM; PD 946 ± 9 μm^2, control 965 ± 8 μm^2, P = 0.23). In the surviving dopaminergic neurons in the PD brains, the mean number of silver grains due to BDNF mRNA expres-

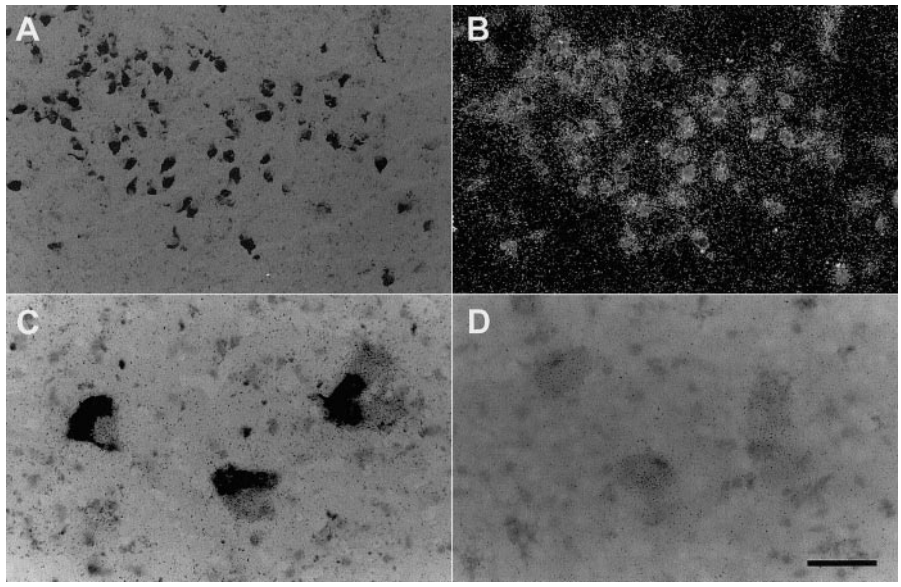

FIG. 2. Photomicrographs showing BDNF mRNA expression by pigmented neurons in the human substantia nigra. (A) Bright field Nissl-stained section of control substantia nigra showing neuromelanin-containing dopaminergic neurons. (B) Dark field image of A showing clustering of silver grains dopaminergic neurons. (C) High magnification image of pigmented dopaminergic neurons showing clustering of silver grains around cells and masking of silver grains by neuromelanin. (D) Sliver grains due to BDNF mRNA expression over demelanized dopaminergic neurons. Bar: 500 μm for A and B; 50 μm for C and D.

sion (mean ± SEM, 61 ± 2.6) was 20% lower (P = 0.02, two-sample t test assuming unequal variances) than in the control brains (76.1 ± 5.6) (Fig. 3B).

Examination of the distribution of BDNF mRNA expression in every dopaminergic neuron sampled revealed the presence of a population of dopaminergic neurons in the PD group with particularly low levels of BDNF mRNA expression. Twenty-eight percent of PD neurons but only 15% of the control neurons had BDNF mRNA expression more than 1 SD below the mean of the control group (42 grains/neuron). This difference in proportions 13% (CI 7.8–16.7%), which cannot merely be attributed to the presence of a population of particularly small neurons, is highly significant (Z = 6.12, $P \leq 0.000001$) (Figs. 4A and 4B).

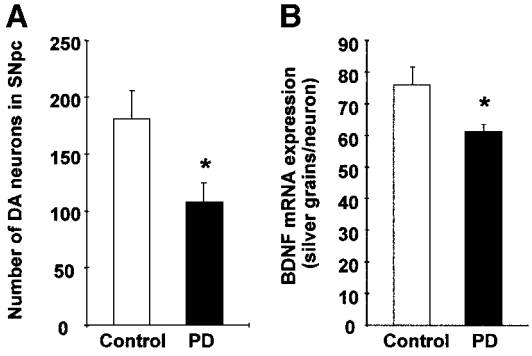

FIG. 3. Number of pigmented neurons and BDNF mRNA expression by pigmented neurons in a single section from each of five control and five PD brains. (A) Mean number of neurons/section examined in each group. (B) Mean BDNF mRNA expression (silver grains/neuron). All values are means ± SEM, *P = 0.02, t test assuming equal variance.

Examining the proportion of neurons expressing different ranges of BDNF mRNA revealed a consistent loss of neurons expressing moderate to high levels of BDNF mRNA in the PD SNpc (Fig. 5). However, there was relatively little change in the number of neurons expressing lower levels of BDNF mRNA (25–49 silver grains/neuron, Z = 4.49, $P \leq 0.001$) and an increase in the number expressing the lowest levels of BDNF mRNA (0–24 silver grains/neuron, Z = 2.86, $P \leq 0.01$).

Plotting the level of BDNF mRNA expression against the cross-sectional area of each neuron (Fig. 6A) revealed that PD neurons expressing a particular level of BDNF mRNA were generally the same size as their control counterparts. This was not true for the PD neurons expressing particularly low levels of BDNF mRNA; these neurons were significantly larger than expected ($P \leq 0.05$). Correcting the silver grain counts for sampling area (Fig. 6B) exposed a direct relationship between the density of BDNF mRNA expression per square micrometer of cell surface and the size of dopaminergic neurons in both the control and the PD SNpc (r^2 = 0.93, $P \leq 0.00001$). This relationship was lost for the PD neurons expressing the lowest levels of BDNF mRNA (0–24 silver grains/neuron) which had a BDNF mRNA density (silver grains/μm^2) 30% lower than the equivalent neurons in the control group ($P \leq 0.0001$).

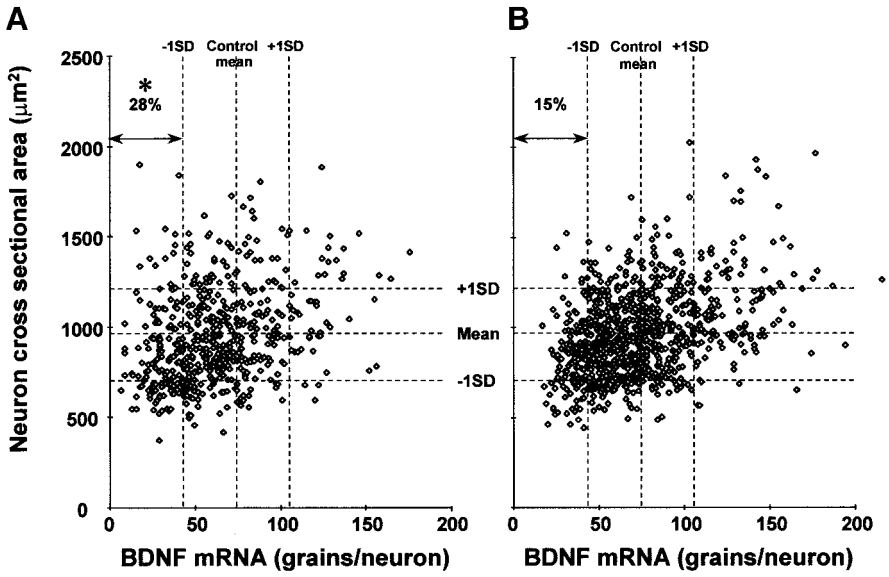

FIG. 4. BDNF mRNA expression (silver grains/neuron) plotted against sample area (μm^2) for (A) PD and (B) control neurons. Means ± 1 SD for control grain number and area are overlaid on both graphs. The proportion of neurons with grain number lower than 1 SD below control mean are indicated, $*P \leq 0.000001$, comparison of proportions.

DISCUSSION

Brain-derived neurotrophic factor has potent effects of the survival and neurite outgrowth from mesencephalic dopaminergic neurons (9, 19, 23, 38–40, 43, 44). Moreover, BDNF is believed to act as an paracrine/autocrine factor for these neurons since in animals it is both produced and used by nigral dopaminergic neurons (8, 31, 27, 35, 37, 43) with the BDNF protein actively transported to dopaminergic terminals in the striatum (1). Loss of BDNF mRNA expression might therefore reduce the function and survival of nigral dopaminergic neurons and contribute to the etiology of PD. The reduced neurite outgrowth and later cell death that would be expected in a BDNF deficiency fits well with the observations that loss of striatal dopamine (~70–80%) (5) is frequently more pronounced than loss of nigral dopamine neurons (~50%) (26). These observations also suggest that in PD, surviving substantia nigra dopaminergic neurons are not mounting the exuberant collateral sprouting response that animal models of PD and striatal injury suggest they are capable of (6, 7, 14, 15, 33, 34). However these surviving dopaminergic neurons can sprout after the injury associated with striatal transplantation of adrenal medullary cells even in the late stages of PD (12, 22). In animals, this injury-induced sprouting response is associated with increased striatal production of BDNF (43). In this context it is worth noting that expression of trkB, the BDNF receptor, is intact in the Parkinson's disease substantia nigra (4). This may explain the continued sprouting capacity of nigral dopaminergic neurons late in Parkinson's disease (12, 22) and offers the hope that application of BDNF might constitute an effective treatment for Parkinson's disease.

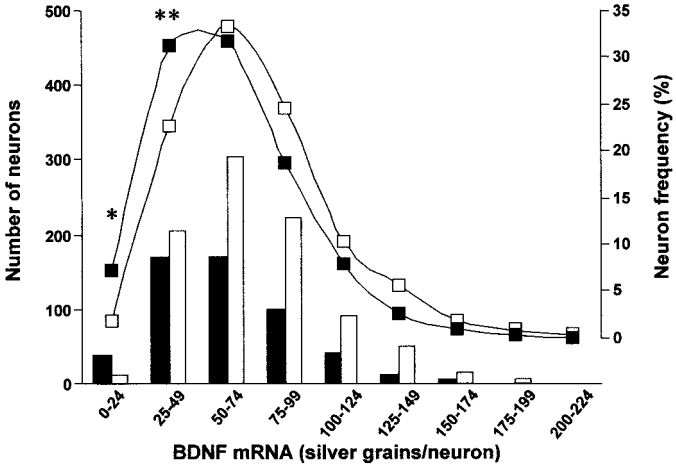

FIG. 5. Number and frequency of pigmented neurons within different ranges of BDNF mRNA expression (silver grains/neuron). Bars indicate total number from all subjects per range; lines indicate frequency. Closed bars and data points, PD; open bars and data points, controls. $*P \leq 0.01$, $**P \leq 0.001$, comparison of proportions.

To be able to play a role in the development of PD it seems reasonable to expect that BDNF be synthesized by human nigral dopaminergic neurons. The substantia nigra contains BDNF protein (29) and the nigral dopaminergic neurons are BDNF immunoreactive (32) but as BDNF is readily moved by anterograde transport (1) they are not necessarily its source. In this study, *in situ* hybridization has demonstrated that BDNF mRNA is expressed by dopaminergic neurons in

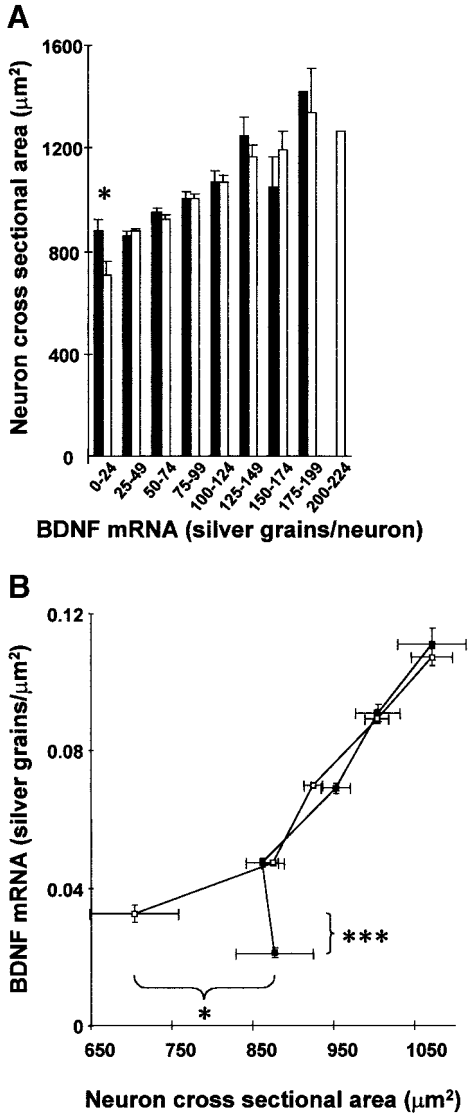

FIG. 6. Relationship between BDNF mRNA expression and cell size. (A) Neuron cross-sectional area plotted against number of silver grains/neuron. (B) Density of BDNF mRNA expression (silver grains/μm^2) plotted against cross-sectional area. Closed bars and data points, PD; open bars and data points, controls. All values are means ± SEM, $*P \leq 0.05$, $***P \leq 0.0001$, *t* test assuming equal variance.

the human substantia nigra. Dry film autoradiograms reveal that in the midbrain, the substantia nigra pars compacta (Fig. lA) is a particularly rich source of BDNF mRNA expression with more than three times ($P \leq 0.003$) the level of background expression seen in surrounding tissues such as the red nucleus (Figs. 1A and 1C). Emulsion dipped autoradiography and microscopy revealed that all large neuromelanin-containing dopaminergic neurons express BDNF mRNA (Fig. 2). These findings are consistent with observations that TH-immunopositive neurons in rat substantia nigra express BDNF mRNA (8, 37) and confirm that, as in other species, BDNF mRNA is expressed by dopaminergic neurons in the human SNpc.

BDNF mRNA expression was also detected over the SNpc in patients with PD (Fig. lB). However, the level of expression was less than one-third of that seen in the controls ($P \leq 0.001$) (Fig. 1C). This was not a global decline of mRNA expression in a group of ill patients but specific to the substantia nigra since expression in the red nucleus, used as an internal control, remained unaltered (Figs. 1A and 1C). While this at first appears to be entirely consistent simply with loss of the nigral dopaminergic neurons which express the BDNF mRNA, BDNF expression in the SNpc fell by approximately 70% (Figs. 1B and 1C) while the number of pigmented neurons fell by only 40% (Fig. 3A). This led us to look more closely at how much BDNF mRNA was expressed by each surviving substantia nigra neuron.

Emulsion dipped autoradiography revealed that in both the normal and the PD substantia nigra, BDNF mRNA appears to be expressed at some level by all the large neuromelanin-containing dopaminergic neurons. There was no difference in the mean cross-sectional neuronal areas sampled from each group of subjects; thus the reduction in BDNF mRNA expression cannot be attributed to a sampling error caused by reduced neuron size in the PD group. Therefore, the reduction of BDNF mRNA expression in PD occurs because of both loss of BDNF mRNA-expressing neurons and reduced BDNF expression by a proportion of those that survive.

The observation that all large neuromelanin-containing substantia nigra neurons express BDNF mRNA is somewhat at odds with the immunohistochemical data which suggest that only 65% of melanized neurons are BDNF immunoreactive (32). One explanation for this discrepancy might be that immunonegative neurons are those expressing lower levels of BDNF mRNA. Alternatively, if the BDNF protein is rapidly transported to dopaminergic terminals in the caudate nucleus or putamen, immunoreactivity in the cell body may be hard to detect even though BDNF mRNA is present and protein synthesis is occurring. On demelanized sections, 20% fewer silver grains ($P \leq 0.02$) were detected on average over the surviving neurons in the PD substantia nigra than in their control counterparts (Fig. 3B). If lack of BDNF immunoreactivity in melanized neurons reflects lower levels of BDNF mRNA expression, this would provide a ready explanation for the apparent sensitivity of BDNF-immunoreactive melanized neurons to Parkinson's disease (32) reflecting changes in detection limits rather than sensitivity to injury. The intensity of BDNF-immunoreactive staining is not an all-or-nothing affair as is illustrated by the observation that intracerebroventricular injections of colchicine to inhibit axonal transport of BDNF in rodents greatly elevates BDNF protein staining in nigral cell bodies (1).

While the mean reduction of BDNF mRNA expression per surviving neuron is relatively small (20%), examination of the distribution of BDNF mRNA expression in every dopaminergic neuron sampled revealed the presence of a population of dopaminergic neurons in the PD group with particularly low levels of BDNF mRNA expression. Twenty-eight percent of PD neurons but only 15% of the control neurons had BDNF mRNA expression more than 1 SD below the mean of the control group (42 grains/neuron). This difference in proportions is highly significant ($P \leq 0.000001$, Fig. 4).

Examining the proportion of neurons expressing increasing ranges of BDNF mRNA revealed a consistent loss of neurons expressing moderate to high levels of BDNF mRNA and confirmed the increase in the proportion of neurons expressing low (25–49 silver grains/neuron, $P \leq 0.001$) and very low (0–24 silver grains/neuron, $P \leq 0.01$) levels of BDNF mRNA (Fig. 5). The observation that the number of neurons expressing the lowest levels of BDNF mRNA in PD exceeds that in controls suggests that this downward shift in the frequency distribution represents a decrease in BDNF mRNA expression across the entire population of PD dopaminergic neurons rather than a relative preservation of neurons less dependent on BDNF. This is consistent with the finding that the PD neurons expressing the lowest levels of BDNF mRNA are larger than expected ($P \leq 0.05$, Fig. 6A). To ensure that these observations were not biased by an unexpected sampling error, the silver grain counts per neuron were corrected for sampling area to give a grain density per square micrometer (Fig. 6B). This revealed a direct and identical relationship between the density of BDNF mRNA expression and neuron cross-sectional area ($r^2 = 0.93$, $P \leq 0.00001$) for all of the control and the majority of PD dopaminergic neurons (Fig. 6B). This is consistent with the observation that application of BDNF increases the soma area of cultured human fetal mesencephalic dopaminergic neurons (40) and provides evidence that the activity of adult human dopaminergic neurons is directly dependent on autocrine/paracrine production of BDNF. Within the group of neurons with the lowest total number of silver grains per neuron (0–24 silver grains/neuron), the density of silver grains per square micrometer was 30% lower in the PD group than in the controls ($P < 0.001$) confirming the presence of a subpopulation of neurons abnormal in both BDNF mRNA expression and size. It is interesting to note that the neurons expressing the lowest levels of BDNF (below the lowest value seen in the controls) comprises approximately 3% of the population of surviving pigmented neurons, a number very close to estimated 5% of neurons lost annually in PD (26). It is therefore conceivable that low levels of BDNF expression in adult life could result in dopaminergic neuronal death in PD. The finding that these neurons are larger than expected for their level of BDNF mRNA expression also suggests that loss of BDNF precedes reduction in size.

It is also possible that depression of BDNF mRNA expression might be an irrelevant consequence of neuronal injury rather than its cause. This is an important issue which is particularly difficult to answer in a study of the level of BDNF mRNA expression by a small subset of individual cells. The traditional approach in most *in situ* hybridization studies would be to "normalize" the quantitation of BDNF mRNA measurements in an area of interest by measuring the expression of an additional "housekeeping gene" such as β-actin or glyceraldehyde-3-phosphate dehydrogenase on adjacent tissue sections. It is theoretically possible to take much thinner sections that allow different probes to be used on adjacent sections but differences in the proportion of an individual cells volume represented in both sections are likely to compound rather than reduce experimental error. The alternative of quantifying the expression of two labeled probes within a single section (and thus in single cells), as would be required for this study, is impractical as all current detection systems would interfere with each others' quantitation. Moreover, the utility of housekeeping genes as internal controls is itself under question. For example, of six different GAPDH isoforms, all are differentially regulated during neuronal apoptosis (36) and a 75% increase of GAPDH mRNA expression can be induced by hypoxia (45). Similar observations also suggest that β-actin mRNA expression is itself regulated by axonal regrowth (25) and that β-actin may be enriched in structures having a high capacity of remodeling in the rat cerebellum (28). The only conclusive way to answer this elusive "chicken-and-egg" question of which comes first, loss of BDNF and then cell injury and death or cell injury and then loss of BDNF, would be to model a long-term deficit of BDNF production in an adult animal (to mimic the time course of development of Parkinson's disease) and determine whether it leads to loss of substantia nigra dopaminergic neurons and a syndrome akin to Parkinson's disease.

Reduced BDNF expression might affect dopaminergic neuron viability by a number of mechanisms. If BDNF is an autocrine/paracrine factor for dopaminergic neurons, loss of dopaminergic neurons due to disease progression with a concomitant local reduction in BDNF production would be predicted to compromise the well-being of their surviving neighbors making them more susceptible to the insult which causes Parkinson's disease. An extreme version of this mechanism would allow a relatively minor neuronal loss to establish a wave of degeneration that spreads through all of the BDNF-dependent nigral neurons. However, as we do not see a secondary wave of degeneration following partial 6-OHDA and MPTP lesions this op-

tion seems unlikely. Another possibility is that failing to synthesize enough BDNF leads directly to the BDNF mRNA-positive cells own demise. Infusing antisense oligonucleotides directed against the BDNF mRNA sequence into mouse striatum greatly diminishes the capacity for neurite outgrowth in the nigrostriatal dopaminergic system (unpublished observation) confirming that diminished BDNF expression can indeed have a profound deleterious effect on the adult nigrostriatal dopaminergic system.

In conclusion, our findings demonstrate that BDNF mRNA is expressed in the human SNpc and that this expression occurs specifically in dopaminergic neurons. In clinically and neuropathologically typical idiopathic Parkinson's disease, BDNF expression in the SNpc is reduced. This reduction is due, in part, to loss of SNpc dopaminergic neurons which express BDNF. However, surviving and apparently normal dopaminergic neurons in the PD SNpc express less BDNF than normal with a subset of neurons expressing particularly low levels of BDNF. These observations suggest the possibility that reduced BDNF expression might contribute directly to loss of nigral dopaminergic neurons and the genesis of Parkinson's disease.

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