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Differential response of the central noradrenergic nervous system to the loss of locus coeruleus neurons in Parkinson's disease and Alzheimer's disease

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Abstract

In Parkinson's disease (PD), there is a significant loss of noradrenergic neurons in the locus coeruleus (LC) in addition to the loss of dopaminergic neurons in the substantia nigra (SN). The goal of this study was to determine if the surviving LC noradrenergic neurons in PD demonstrate compensatory changes in response to the neuronal loss, as observed in Alzheimer's disease (AD). Tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) mRNA expression in postmortem LC tissue of control and age-matched PD subjects demonstrated a significant reduction in the number of noradrenergic neurons in the LC of PD subjects. TH mRNA expression/neuron did not differ between control and PD subjects, but DBH mRNA expression/neuron was significantly elevated in PD subjects compared to control. This increase in DBH mRNA expression in PD subjects is not a response to neuronal loss because the amount of DBH mRNA expression/neuron in AD subjects was not significantly different from control. Norepinephrine transporter (NET) binding site concentration in the LC of PD subjects was significantly reduced over the cell body region as well as the peri-LC dendritic zone. In PD subjects, the loss of dendrites from surviving noradrenergic neurons was also apparent with TH-immunoreactivity (IR). This loss of LC dendritic innervation in PD subjects as measured by TH-IR was not due to LC neuronal loss because TH-IR in AD subjects was robust, despite a similar loss of LC neurons. These data suggest that there is a differential response of the noradrenergic nervous system in PD compared to AD in response to the loss of LC neurons.

Keywords

Parkinson's disease; Alzheimer's disease; locus coeruleus; tyrosine hydroxylase; dopamine β-hydroxylase; norepinephrine transporter; mRNA; immunohistochemistry

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1. Introduction

Parkinson's Disease (PD) is a progressive and debilitating neurodegenerative disorder that currently affects 2% of the population over the age of 65 years and will only become more prevalent as the younger generations age. The hallmark symptom of PD, motor dysfunction, is the result of a significant loss of dopaminergic neurons in the substantia nigra (SN) (Damier et al., 1999; Gibb, 1991; Gibb and Lees, 1991). However, the SN is not the only region that demonstrates significant neuronal loss in PD. There is also a significant loss of noradrenergic neurons in the locus coeruleus (LC), which is equal to or greater than the neuronal loss observed in the SN (Bertrand et al., 1997; Cash et al., 1987; Chan-Palay V and Asan E, 1989; Hornykiewicz O and Kish SJ, 1987; Marien et al., 2004; Patt and Gerhard, 1993; Zarow et al., 2003). The consequence of this neuronal loss on the surviving noradrenergic nervous system is unknown. In PD, there is a significant reduction in DBH, the synthetic enzyme specific to noradrenergic neurons, in the cortex, suggesting a reduction in noradrenergic innervation (Gasper et al., 1991). However, a loss of noradrenergic neurons in the LC does not necessarily result in reduced noradrenergic function: in Alzheimer's disease (AD), another neurodegenerative disorder where there is a significant loss of noradrenergic neurons in the LC, the surviving LC noradrenergic neurons demonstrate several compensatory changes. The content of NE in terminal regions in postmortem AD subjects is reduced, but the reduction does not correspond to the degree of neuronal loss (Adolfsson et al., 1979; Hoogendijk et al., 1999; Mann et al., 1981; Palmer et al., 1987; Reinikainen et al., 1988; Tomlinson et al., 1981). Similar results were observed for NE-synthesizing enzymes in the forebrain of postmortem AD subjects (Cross et al., 1981; Palmer et al., 1987; Perry et al., 1981; Russo-Neustadt et al., 1998). Recently, our laboratory showed that the remaining noradrenergic neurons in the LC of AD and a related dementing disorder, dementia with Lewy body (DLB), showed three different compensatory changes: (1) increase in the expression of tyrosine hydroxylase (TH) mRNA; (2) sprouting of dendrites into the peri-LC dendritic zone; and (3) sprouting of axonal projections into the hippocampus and prefrontal cortex (Szot et al., 2006, 2007).

In this study we determined if the surviving LC noradrenergic neurons in PD subjects compensate for the neuronal loss: mRNA expression for the key synthetic enzymes, TH and DBH, was measured by *in situ* hybridization in the LC of PD subjects and age-matched control subjects. In addition, DBH mRNA expression was measured in AD subjects to determine the response of the surviving noradrenergic neurons in AD and to compare these results with an earlier study measuring TH mRNA expression (Szot et al., 2006). These enzymes are important in the synthetic pathway for NE: TH converts tyrosine to L-DOPA; L-DOPA is converted to dopamine (DA) by aromatic amino acid decarboxylase, and then dopamine is converted to NE by DBH, which is specific to adrenergic neurons. NE transporter (NET) binding sites were measured in the LC of PD subjects and age-matched control subjects as a marker of dendritic innervation, to determine if it is altered in the LC of PD subjects. In addition, TH-immunoreactivity (IR) was measured in the LC of PD, AD, DLB and control subjects to support data generated with TH mRNA and NET binding. These measurements would indicate if the surviving LC noradrenergic neurons in PD subjects result in a similar response as observed in AD subjects (Szot et al., 2006).

2. Results

2.1 In the LC of PD subjects there is a significant loss of TH-positively labeled neurons with no compensatory response

TH is considered the rate-limiting enzyme in the synthesis of NE; changes in the expression of this protein will alter the level of NE at terminal regions. Atlas matching of tissue specimens of PD and control subjects indicated that many of the PD subjects lacked the

rostral portion of the LC (30% level). This portion of the LC may be missing from PD subjects because the most caudal portion of the SN overlaps anatomically with the most rostral portion of the LC. Since the SN is such an important region in PD, studies would require the entire SN. Therefore, TH mRNA expression was analyzed only at the 50 and 70% levels of the LC in both control and PD subjects (Figure 1). Although limited, these measurements are critical and illuminating because the mid to caudal portion of the LC is thought to innervate regions in the midbrain area, including the SN/VTA (Fallon and Loughlin, 1982;Loughlin et al., 1982). TH-positive labeled cell numbers from control subjects in this study yielded values at each level within the range that was observed in the control subjects of previously published work (Szot et al., 2006). Figure 1A (and photomicrographs) shows that in the LC there was a significant loss of TH-positive neurons in PD subjects at both the 50% level (88% reduced)($p=0.009$) and 70% level (71% reduced) ($p=0.008$). Figure 1B (and photomicrographs) shows that in the surviving LC neurons in PD subjects TH mRNA expression (grain count)/neuron did not differ between control and PD subjects at the two different levels.

2.2 In the LC of PD subjects there is a significant loss of DBH-positively labeled neurons but the surviving neurons demonstrate a compensatory increase

DBH is the synthetic enzyme responsible for the conversion of dopamine (DA) to NE and is localized to noradrenergic neurons. DBH mRNA expression serves as another marker to examine the noradrenergic neuronal loss in PD subjects. The number of DBH-positive neurons in both control and PD subjects (Figure 2A and photomicrographs) was very similar to the number of TH-positive neurons (Figure 1A), supporting a significant loss of noradrenergic neurons in the LC of PD subjects at the 50 ($p=0.007$) and 70% ($p=0.03$) level. However, in PD subjects, the surviving noradrenergic neurons showed increased DBH mRNA expression (grain count)/neuron at the 50% ($p=0.03$) level with a trend toward an increase at the 70% level ($p=0.18$)(Figure 2B and photomicrographs).

2.3 In the LC of AD subjects there is a significant loss of DBH-positive neurons and no compensatory response

Since DBH is not considered to be the rate-limiting enzyme in the synthesis of NE, it is unclear if the increase in DBH mRNA expression (grain count)/neuron in PD subjects described above is an adequate compensatory functional response to neuronal loss. We reasoned that any such compensatory increase in DBH mRNA expression in response to the loss of LC neurons should also be detectable in AD subjects, who also exhibit a severe loss of LC neurons and compensatory adaptations in response to this loss (Szot et al., 2006). Thus, we measured DBH mRNA expression in the LC of the AD subjects, plus DLB, that were previously studied (Szot et al., 2006). Quantitation of the number of DBH mRNA-labeled neurons in AD and DLB subjects indicate a significant reduction from control subjects at the 30% ($F_{(2, 38, 40)} = 17.1$, $p<0.0001$), 50% ($F_{(2,37,39)} = 38.0$; $p<0.0001$) and 70% ($F_{(2,39,41)} = 29.7$; $p<0.0001$) levels of the LC (Figure 3A). Two-way ANOVA indicated a significant difference in TH positive-labeled neurons between the 30% level to the 50% ($F_{(4,114)} = 67.9$, $p<0.001$)and 70% level ($F_{(4,114)} = 86.2$, $p <0.001$) in control subjects; between the 30% level and 70% level ($F_{(4,114)} = 46.0$, $p<0.001$) and between the 50% level and 70% level ($F_{(4,114)} = 31.5$, $p<0.05$) in AD subjects; and between 30% level and the 70% level ($F_{(4,114)} = 30.4$, $p<0.05$) in DLB subjects. The loss of DBH mRNA-positive labeled neurons in AD subjects was similar to DLB subjects at the all three levels of the LC (approximately 70-80%). This assessment of LC noradrenergic neuronal loss in AD and DLB subjects with DBH mRNA is similar to what was observed using TH-positive labeled cells (Szot et al., 2006). However, DBH mRNA expression (grain count)/neuron was not significantly different between control, AD and DLB subjects at any level of the LC,

indicating that DBH mRNA expression does not demonstrate compensatory changes in response to LC neuronal loss (Figure 3B).

2.4 In the LC and peri-LC of PD subjects there is a significant loss of NET binding sites

NET is the main transporter for the removal of NE from the synapse and is found exclusively on noradrenergic neurons and terminals. NET binding sites, as measured by ^{3}H-nisoxetine binding, are found over the cell bodies in the LC and on the dendrites that innervate the peri-LC dendritic region. NET binding sites over the LC in PD subjects were significantly reduced in both the cell body region (50% level p=0.006; 70% level p=0.03) and peri-LC dendritic (50% level p=0.012; 70% level p=0.015) region. At the 50% level of the LC PD subjects had ~70% reduction in both regions; and at the 70% level, about a 65% reduction in both regions (Figure 4). At the 50 and 70% level in the LC, a significant correlation was observed between the number of TH mRNA-positive labeled neurons and NET binding over cell body region (50% level: r = 0.61, p= 0.028; 70% level: r = 0.87, p= 0.02) for control and PD combined, but this correlation was not evident with respect to NET binding over the peri-LC dendritic region (50% level: r = 0.31, p=0.31; 70% level: r = 0.46, p=0.10). This suggests that the surviving LC neurons in PD are not demonstrating dendritic sprouting.

2.5 TH-immunoreactivity (IR) in the LC of PD and AD subjects supports a lack of dendritic sprouting of the surviving LC neurons in PD subjects and the presence of dendritic sprouting of the surviving LC neurons in AD

TH-IR labels noradrenergic cell bodies and fibers in the LC. The number of TH-IR-positive neurons in AD and DLB subjects at the 70% level of the LC were significantly reduced (50% for AD and 70% for DLB) as compared to control ($F_{(2, 32, 34)}$ =11.8, p=0.0001)(Figure 5A-left histogram, Figure 6A and B). The reduction in noradrenergic neurons, as assessed by TH-IR is similar to our previously published analysis using TH mRNA (Szot et al., 2006). The number of TH-IR-positive neurons in PD subjects at the 70% level of the LC was also significantly reduced (~75%) as compared to control (p=0.008)(Figure 5B-left histogram, Figure 6C and D), and the degree of loss was comparable to the reduction observed with TH mRNA (Figure 1;~71%). The amount of TH-IR-positive fibers in AD and DLB subjects as compared to controls was not different (Figure 5A-right histogram, Figure 6A and B) even though there was a significant loss of TH-IR-positive neurons, suggesting that the surviving noradrenergic neurons are compensating for the loss, supporting the NET binding data previously published (Szot et al., 2006). In contrast, the amount of TH-IR labeling in fibers of PD subjects was reduced as compared to controls (p=0.004)(Figure 5B-right histogram, Figure 6C and D). This would suggest that the surviving LC noradrenergic neurons in PD subjects are not compensating, a conclusion supported by the NET binding in the LC of PD subjects (Figure 4).

3. Discussion

This study demonstrates a significant loss of LC noradrenergic neurons in PD subjects and that the surviving PD noradrenergic neurons do not appear to demonstrate compensatory changes as to TH mRNA expression/neuron and NET binding. The response of the surviving LC noradrenergic neurons in PD subjects is different from what is observed in the surviving LC noradrenergic neurons in subjects with dementia (AD and DLB), suggesting that the different pathologies associated with these two neurodegenerative disorders may affect the response of the surviving LC neurons to neuronal loss.

Neuronal loss in PD subjects, as determined in this study by counting the number of TH and DBH mRNA expressing neurons in the LC, is similar using the two mRNAs as markers and

was in agreement with TH-IR and equivalent to that reported in previously published works (Bertrand et al., 1997; Cash et al., 1987; Chan-Palay V and Asan E, 1989; Hornykiewicz O and Kish SJ, 1987; Marien et al., 2004; Patt and Gerhard, 1993). The loss of LC noradrenergic neurons in subjects with dementia (AD and DLB) was also verified by DBH mRNA expression and TH-IR, and corresponds to our previously published work assessing neuronal loss with TH mRNA expression (Szot et al., 2006) and to other published studies (Marcyniuk et al., 1986; Chan-Palay and Asan, 1989).

The loss of LC noradrenergic neurons in PD subjects does not appear to elicit a compensatory response of increased TH mRNA in the surviving neurons, as observed in AD and DLB subjects (Szot et al., 2006). Lack of a compensatory response is also observed in the SN dopaminergic neurons of PD subjects where TH mRNA expression (grain count)/neuron is significantly reduced in the surviving dopaminergic neurons (Javoy-Agid et al., 1990). However, we found that DBH mRNA expression (grain count)/neuron was significantly elevated at the 50% level of the LC (a tendency at the 70% level) in PD subjects. The increased DBH mRNA expression (grain count)/neuron in the surviving LC neurons may not solely be a response to neuronal loss because DBH mRNA expression (grain count)/neuron in AD/DLB subjects is not different from control subjects. Therefore, the increase in DBH mRNA expression (grain count)/neuron observed in the surviving LC neurons in PD subjects may be a response to some other factor particular to PD. Classically, DBH is not considered to be the rate-limiting enzyme in the synthesis of NE, so it is unclear how this increase would affect NE levels. However, there is evidence to indicate that DBH activity can affect NE levels in the CNS when DBH levels are altered genetically (Thomas et al., 1995, 1998; Bourdelat-Parks et al., 2005), with excessive stimulation of noradrenergic neurons (Scatton et al., 1984) or when DBH inhibitors such as disulfiram or nepicastat are administered (Goldstein, 1966; Musacchio et al., 1966; Bourdelat-Parks et al., 2005; Beliaev et al., 2006; Schroeder et al., 2010). These data suggests that under certain circumstances DBH levels can regulate NE levels, therefore NE levels could be affected in PD subjects when there is a significant loss of noradrenergic neurons with increased DBH mRNA expression (grain count)/neuron.

Particular to PD subjects is the chronic treatment of L-DOPA to relieve motor symptoms (Cotzias et al., 1969) by increasing central DA levels. Relief of motor symptoms is associated with a significant increase in CSF DA levels of PD subjects as compared to control subjects (Personal communication). Since DBH is the enzyme responsible for the conversion of DA to NE, L-DOPA-induced elevation of DA levels may be responsible for the increase in DBH mRNA expression (grain count)/neuron in noradrenergic neurons in PD subjects. When dopaminergic neurons are reduced, noradrenergic terminals in the striatum appear to be responsible for removing DA from the synapse (Arai et al., 2008). While, CSF DBH activity in un-medicated PD subjects is significantly reduced as compared to controls (Hurst et al., 1985). However, L-DOPA therapy does not appear to relieve PD subjects of their non-motor symptoms, which are associated with reductions in NE (Sethi, 2008), and NE levels and its metabolites in CSF of PD subjects on L-DOPA therapy are not significantly different from control subjects (Mann et al., 1983; Turkka et al., 1987; Chia et al., 1995).

NET binding site concentrations in the LC cell body region and peri-LC region of PD subjects are significantly reduced as compared to control subjects. NET binding site concentration over the cell body region correlated to the number of TH mRNA-positive neurons in combined PD and control subjects. These data indicate that the surviving LC neurons in PD subjects are not increasing NET levels to make up for the loss of surrounding neurons at the cell body region. In AD/DLB subjects, there is evidence that the surviving LC neurons are sprouting. NET binding sites over the cell body region are reduced in AD and

DLB subjects, but these numbers do not consistently correlate with TH-positive neurons: this disparity is even more apparent when examining NET binding over the peri-LC dendritic zone as compared to TH-positive neurons (Szot et al., 2006). This data would indicate that the surviving LC neurons in AD and DLB subjects are exhibiting dendritic sprouting to the peri-LC dendritic zone.

TH-IR quantitation in PD and AD/DLB supports the conclusions described above concerning sprouting in AD/DLB subjects but not in PD subjects. TH-IHC in AD/DLB subjects demonstrates extensive TH-IR in the region surrounding surviving LC neurons, which is comparable to controls; while in PD subjects, TH-IR in the surrounding region of surviving neurons is reduced as compared to control.

Studies in postmortem human tissue do not indicate the order of dopaminergic and noradrenergic neuronal loss in PD. However, clinical studies of PD subjects indicate that the noradrenergic system may be affected before the dopaminergic system. In the progression of this disorder, the loss of dopaminergic neurons is not observed until Stage 3 (out of 6 Stages) (Braak et al., 2003a, 2003b, 2006), and approximately a 70% loss of dopaminergic neurons is required to observe PD symptoms. During the earlier stages of the disorder, non-motor preclinical symptoms of PD are observed. These preclinical PD symptoms include hyposmia (Berendse et al., 2001; Ponsen et al., 2004), REM-sleep disorder (Boeve et al., 2003; Scheneck et al., 1994) or autonomic dysfunction (Boeve et al., 2003), which can be attributed to neuropathological changes in the noradrenergic nervous system.

There is no clear evidence in human subjects to indicate a role for the CNS noradrenergic nervous system in the support of the dopaminergic system. Human studies suggest that the noradrenergic nervous system is affected in the early stages of PD, as indicated by the non-dopaminergic symptoms described above, but the consequence for dopaminergic neurons is unknown. Increasing synaptic NE with an α_2-adrenoreceptor antagonist reduces the detrimental side effects of L-DOPA treatment without affecting improved motor function (Gomez-Mancilla and Bedard, 1993; Henry et L., 1999; Grondin et al., 2000; Rascol et al., 2001; Savola et al., 2003; Fornai et al., 2007; Fulceri et al., 2007). Animal research suggests that the noradrenergic nervous system may protect dopaminergic neurons from damage by neurotoxins (Gesi et al., 2000). For example, when NE levels are reduced, the neurotoxic damage of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and methamphetamine on dopaminergic neurons in the SNpc is enhanced (Bing et al., 1994; Fornai et al., 1995, 1996, 1997, 1999; Marien et al., 1993; Mavridis et al., 1991). In dopamine β-hydroxylase (DBH) knockout mice that cannot synthesize NE from DA, motor deficits develop with age that mimic PD (Rommelfanger et al., 2007). Conversely, increased NE levels reduce the neurotoxic effect but do not completely protect the dopaminergic neurons (Rommelfanger et al., 2004; Srinivasan and Schmidt, 2004). These animal studies suggest that reduced NE input results in a greater loss of dopaminergic neurons to neurotoxic damage; reduction in NE input after a dopaminergic neurotoxin does not affect the recovery of the dopaminergic system (Bing et al., 1994; Fornai et al., 1997), suggesting that a loss of noradrenergic innervation can enhance the loss of dopaminergic neurons.

The lack of compensatory changes in the surviving LC neurons of PD subjects as shown by TH mRNA, NET binding sites and TH-IR in the LC region are in contrast to what was observed in the surviving LC neurons of AD (Szot et al., 2006). In AD, there is a significant loss of LC noradrenergic neurons; however, the surviving LC neurons demonstrate a significant increase in the expression of TH mRNA (grain count)/neuron and dendritic sprouting to the surrounding peri-LC dendritic zone (Szot et al., 2006). Also as indicated above, the response of DBH mRNA expression (grain count)/neuron in PD subjects is in contrast to what is observed in the surviving LC neurons in subjects with dementia. These

results indicate that noradrenergic neuronal loss alone does not necessarily result in a compensatory response of the surviving neurons. Mechanisms of neuronal loss (either directly at the LC or degeneration from terminals) may have a major role in the response of the surviving neurons. However, in PD subjects, the data indicate that the surviving LC noradrenergic neurons do not appear to be compensating as measured by immunohistochemistry for synthesizing enzymes for norepinephrine (NE) (Gasper et al., 1991). Our data would suggest noradrenergic function is reduced in PD.

In summary, our studies indicate that surviving noradrenergic neurons in the LC of PD subjects respond differently from those in AD subjects. This suggests that external circumstances surrounding each of these disorders may affect how surviving LC noradrenergic neurons respond to neuronal loss. Chan-Palan (1989) and German et al., (1992) drew similar conclusions when examining the pattern of LC neuronal loss in PD and AD subjects.

4. Experimental Procedures

4.1 PD subjects

Postmortem tissue was obtained from several sources: NICHD Brain and Tissue Bank for Developmental Disorders (BTBDD), Alzheimer's disease Research Center (ADRC) at the University of Washington, Emory Tissue Bank and University of California San Diego (SD), where permission for use of tissue in scientific experiments was obtained. PD is commonly characterized by various motor deficits including tremor, rigidity and bradykinesia (Singh et al., 2007). All PD subjects (n=7) in this study had exhibited these symptoms, though it is unknown if any PD subjects in this study received pharmacological intervention (L-DOPA) to alleviate the symptoms. Medical records of control subjects (n=8) indicated no clinical, neurological, or psychiatric illness or evidence of cognitive or functional decline and had no obvious neuropathology during autopsy. Table 1 contains information on age, sex, postmortem interval (PMI) and duration of PD (when known). Subjects (control and PD) that had been diagnosed with depression before death or had taken any antidepressant medication were excluded because depression and antidepressants can alter the noradrenergic nervous system (Bauer and Tejani-Butt, 1992; Lacroix et al., 1991; Harro and Oreland, 2001; Ordway et al., 2003). Subjects with a history of alcohol or drug abuse were also excluded. The original goal was to exclude PD subjects with impaired cognition (Boller et a., 1980; Gasper and Gray, 1984; Hakim and Mathieson, 1979; Mayeux et al., 1992), but because of the high prevalence of cognitive impairment in PD, some of the PD subjects used exhibited mild cognitive impairment (MCI) prior to death (Table 1).

4.2 PD Tissue

The LC tissue blocks obtained from the sources described above were stored at -70°C until sectioned. Serial coronal sections (20 μm) were cut on a cryostat, thaw mounted onto Fisher Super Frost slides, and stored at -70°C. The rostral-to-caudal distance of the LC was determined for each case by examining sections stained with thionin every 500 μm. The rostral (or 0%) was defined as the beginning of the trochlear nucleus, and the caudal pole (100%) ended at the rostral level of the trigeminal motor nucleus (Hoogendijk et al., 1999). After the rostral-to-caudal distance of the LC was determined for each case, sections were taken, if possible, to include the 30, 50 and 70% levels of the LC. Multiple levels of LC were chosen because the rostral portion of the LC innervates forebrain structures such as the hippocampus, whereas the mid to caudal portion of the LC innervates hindbrain structures such as the substantia nigra and ventral tegmental region (Fallon and Loughlin, 1982; Loughlin et al., 1982).

4.3 AD/DLB subjects

All postmortem tissue was obtained from the University of Washington Alzheimer's Disease Research Center (Seattle, WA, USA), where permission for use of tissue in scientific experiments was obtained. AD is characterized by the insidious onset and gradual progression of impaired memory, language, and executive function. Psychosis, agitation, and other behavioral disturbances characteristically appear late in the disease course. Dementia with Lewy bodies (DLB), which accounts for ~20% of patients with late-life dementia, presents early in its course with psychotic symptoms such as visual hallucinations and with fluctuating cognition and pronounced attentional deficits and often bradykinesia and increased muscle tone (Ballard et al., 1999; Barber et al., 2001; McKeith et al., 1996). The subjects used in this study were the same subjects used in a previous study measuring TH mRNA and NET binding sites in the LC and were described in detail in a previous publication (Szot et al., 2006). AD subjects (n=15) met the National Institute on Aging Reagan criteria for AD (Braak stage IV/C or higher with no vascular dementia, frontotemporal dementia, or Lewy body pathology (McKhann et al., 1984). DLB subjects (n= 13) met the same neuropathological diagnostic criteria for AD plus had the presence of Lewy body pathology in the brainstem and limbic regions, confirmed by α-synuclein immunohistochemistry. Medical records of control subjects (n= 15) indicated no clinical, neurological, or psychiatric illness or evidence of cognitive or functional decline and had no obvious neuropathology during autopsy.

4.4 TH mRNA In-situ Hybridization

Tissue preparation and labeling of the TH oligonucleotide probes was performed as described previously for oligonucleotide labeling (Szot et al., 1997). For each subject, three consecutive slides at each level of the LC of PD subjects were labeled with TH mRNA oligonucleotide probe. The TH probe consisted of three separate oligonucleotide probes to the following nucleotides of the published human sequence (O'Malley et al., 1987) (GenBank accession number X05290): 326-377, 520-571, and 1309-1360. The oligonucleotide probes were 3′ end labeled with [^{33}P]dATP (PerkinElmer, Boston, MA) using terminal deoxyribonucleotidyl transferase (Invitrogen, Piscataway, NJ). The TH probe contained 1.04×10^6 cpm/50 μl and was washed as described in detail in previously published work with oligonucleotides (Szot et al., 1997). Slides were apposed to film (Eastman Kodak, Rochester, NY) for 5 d at room temperature, and then the slides were coated with NTB2 Nuclear Track Emulsion (undiluted) (Eastman Kodak) and stored at -20°C for 7 d. Films and slides were developed as described previously (Szot et al., 1997).

Quantitation of TH mRNA expression in control and PD subjects was similar to that performed by Szot et al., (2000). All quantitation was done blind to the subject's condition and by a single individual to reduce variability. The number of cells that achieved labeling threefold higher than background were counted on the three slides at each level of the LC for each subject and expressed as TH-positively labeled cell ± SEM. Experimental data were analyzed using the computer program GraphPad Prism (v. 5.0, GraphPad Software Inc.). The density of TH mRNA expression (grain count)/neuron in PD subjects was determined by measuring the amount of silver grains over the cell bodies of labeled neurons that were threefold higher than background under 20× dark-field illumination with a side-mounted light using the MicroComputer Imaging Device system (MCID) (Imaging Research, St. Catharines, Ontario, Canada). Therefore, all labeled neurons that were counted as positively labeled were also quantitated for the amount of TH mRNA expression (grain count)/neuron. The data are expressed as the average of grains per cell ± SEM at each level of the LC for each PD subject.

4.5 DBH mRNA

Tissue preparation and labeling of the DBH oligonucleotide probes was performed as described previously for oligonucleotide labeling (Szot et al., 1997). For each subject, three consecutive slides at each level of the LC were labeled with DBH mRNA oligonucleotide probes. Three different oligonucleotides (regions 478-529, 928-979 and 1339-1390) of the human DBH sequence (Kobayashi et al., 1989) were used and labeled as described above for TH mRNA. The oligonucleotide probes were 3′ end labeled with [^{33}P]dATP (PerkinElmer, Boston, MA) using terminal deoxyribonucleotidyl transferase (Invitrogen, Piscataway, NJ). The DBH probe contained 1.38×10^6 cpm/50 μl for the PD subjects and 1.17×10^6 cpm/50 μl for the AD subjects. Slides were washed as described in detail in previously published work for oligonucleotides (Szot et al., 1997) and apposed to film (Eastman Kodak, Rochester, NY) for 4 d at room temperature, and then the slides were coated with NTB2 Nuclear Track Emulsion (undiluted) (Eastman Kodak) and stored at -20°C for 7 d. Films and slides were developed as described previously (Szot et al., 1997). Quantitation of DBH mRNA expression was similar to that performed by Szot et al., (2000) and described above for TH mRNA.

Due to the variability of labeling of the oligonucleotide probes and parameter for analysis of the labeling, the quantitative amount of DBH mRNA expression (grain count)/neuron in the control group for AD/DLB subjects cannot be compared to the control group for PD subjects. Because these variables can affect labeling, all experimental groups are run in the same assay and analyzed at the same time. DBH mRNA expression in PD subjects was a separate experiment from DBH mRNA expression in AD/DLB subjects; comparison of expression/neuron cannot be made between control subjects.

4.6 NET binding sites

NET binding sites were measured in the LC of PD subjects with ^{3}H-nisoxetine (American Radiolabeled Chemicals, St. Louis, MO). From each subject, four consecutive slides from each LC level were run: three slides for total binding and the fourth slide for nonspecific binding. Slides were thawed at room temperature for 10 min, and then 600 μl/slide of incubation buffer (~3 nM ^{3}H-nisoxetine in 50 mM Tris buffer with 300 mM NaCl and 5mM KCl, pH 7.7) was placed over the tissue. Slides were incubated for 90 min at room temperature and then washed twice for 2 min in ice-cold 50 mM Tris buffer, pH 7.4, dipped in ice-cold distilled water to remove salts, and then rapidly dried under a stream of cool air. Nonspecific binding was defined in the presence of 1 μM mazindol. Slides were apposed to Biomax MR film (Eastman Kodak) for 8 weeks. Films were developed and analyzed as described previously (Szot et al., 1997). Specific binding was obtained by taking the total average value minus nonspecific value in the same region. Specific binding for ^{3}H-nisoxetine constituted 60-70% of total binding.

Quantitation of NET binding sites was performed over the cell body region and over the peri-LC dendritic zone of each subject and expressed as Optical Density as determined by MCID. All quantitation was done blind to the subject's condition and by a single individual to reduce variability. Cell body region was defined by comparing NET binding labeling on film to TH and DBH mRNA labeling on each section, while labeling beside the cell body region was considered the peri-LC dendritic zone. The peri-LC dendritic zone is the region where the vast majority of LC afferents affect the activity of LC neurons; LC afferents rarely go directly to the LC cell bodies (Aston-Jones et al., 2004). This method has been used previously to demonstrate compensatory changes in the surviving LC noradrenergic neurons in AD subjects (Szot et al., 2006).

4.7 TH-Immunohistochemistry (IHC)

TH-IHC was performed in each PD (n= 7) and age-matched control (n= 8) subject, as well as in control subjects (n=15) age-matched for AD (n=15) and DBL (n=13) subjects that were used to measure DBH mRNA expression. For PD and control subjects, two consecutive frozen LC sections from the 70% level were used, while a single frozen LC section from each control, AD and DLB subjects at the 70% level were used. The slides were air dried and fixed in 4% paraformaldehyde/PBS for 8 minutes at room temperature. Sections were permeabilized in 1% Triton X-100/PBS for 30 minutes before the melanin in LC was bleached by incubating slides in 10% H_2O_2/PBS for 22 hours. Sections were blocked in 5% non-fat milk in PBS supplemented with 10% normal goat serum and 1% BSA for 1 hour at room temperature, followed by incubation with rabbit polyclonal anti-tyrosine hydroxylase (AB152, Millipore, Temecula, CA) at 1:500 for 2 hours and biotinylated anti-rabbit secondary antibody at 1:200 for 30 minutes at room temperature. Finally, sections were incubated in an avidin-biotin complex (Vector's Vectastain Elite ABC kit, Burlingame, CA), and the reaction product was visualized with 0.05% diaminobenzidine (DAB)/0.01% hydrogen peroxide in PBS. Negative controls with secondary antibody alone did not immunostain tissue sections (data not shown).

The number of TH-IR positively labeled cell bodies were counted in the LC of control, AD, DLB and PD subjects and expressed as the average ± SEM. TH-IR labeled fibers in the LC was assessed by a semi-qualitative method, the amount of TH-IR fibers was scored as light (1), moderate (2) or intense (3) for each subject. Scoring was done blind to the subjects condition and a single individual to reduce variability. Scores were averaged for each group and presented as the mean ± SEM. Photomicrographs were taken with a digital camera and imported into Adobe Photoshop for mounting. To optimize visualization of staining, photomicrographs were modified, when necessary, by adjusting brightness and contrast.

4.8 Statistical Analysis

Experimental data were analyzed using the computer program GraphPad Prism (v. 5.0, GraphPad Software Inc.). Statistical difference between control and PD subjects was assessed by two-tailed unpaired Student's t-test. Statistical difference between control, AD and DLB subjects was assessed by one-way ANOVA followed by a *post hoc* Tukey's test. Two-way ANOVA was used to compare the effect of different LC levels in the different subject groups. Bonferroni tests were used to compare the different subject groups. P value less than 0.05 was considered significant.

Simple linear regressions were performed between NET binding in the cell body region and peri-LC dendritic zone to the number of TH positively-labeled neurons in the LC of control and PD subjects using GraphPad Prism, with significance at $p<0.05$. This analysis determined if the surviving LC neurons in PD are sprouting.

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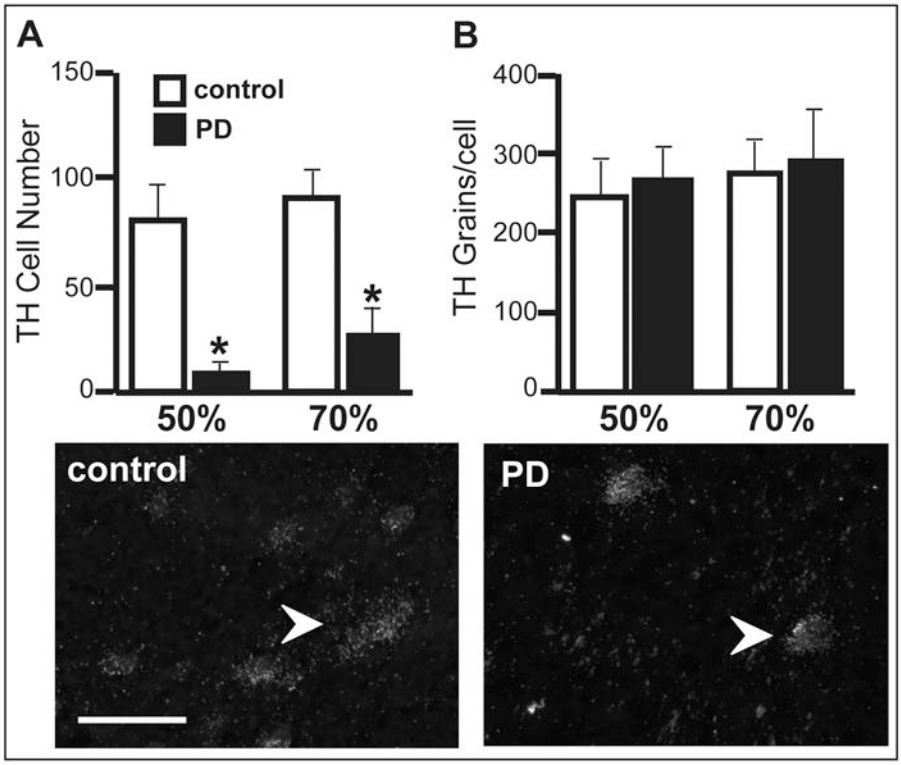

Figure 1.

TH mRNA expression in the LC of age-matched control (n=8) and PD (n=7) subjects. (A) The number of TH positively labeled neurons at the 50 and 70% level of the LC of age-matched control and PD subjects. In PD subjects, there is a significant decrease in the number of TH positively labeled neurons at both levels of the LC (50% level p=0.009, 70% level p=0.008). (B) Expression of TH mRNA/neuron in the LC of control and PD subjects at the 50 and 70% levels. All labeled neurons that were counted as positively labeled were also quantitated for the amount of TH mRNA expression/neuron. There is not a difference in the amount of TH mRNA expression/neuron in PD subjects as compared to control age-matched subjects. The bottom two images are dark-field photomicrographs of TH mRNA labeled neurons in age-matched control (bottom left) and PD (bottom right) at the 50% level of LC. * Indicates significant difference from age-matched control subjects. Arrow indicates a TH mRNA labeled neuron. Scale bar = 100μm.

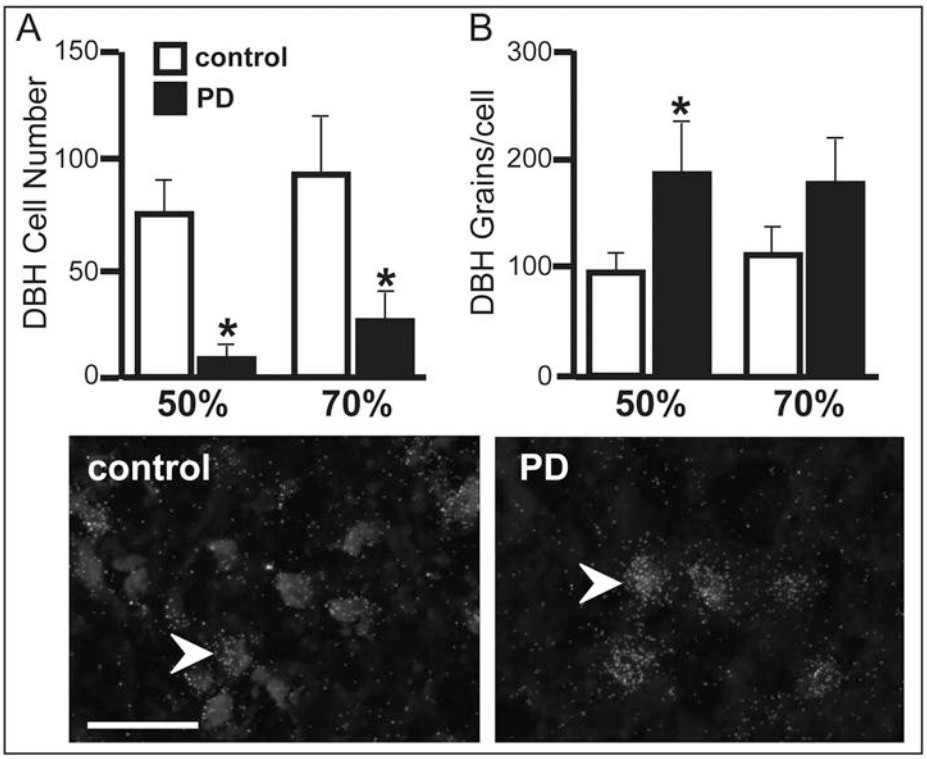

Figure 2.

DBH mRNA expression in the LC of age-matched control (n=8) and PD (n=7) subjects. (A) The number of DBH positively labeled neurons at the 50 and 70% level of the LC of age-matched control and PD subjects. In PD subjects, there is a significant decrease in the number of DBH positively labeled neurons at both levels of the LC (50% level p=0.007, 70% level p=0.03). (B) Expression of DBH mRNA/neuron in the LC of age-matched control and PD subjects at the 50 and 70% levels. All labeled neurons that were counted as positively labeled were also quantitated for the amount of DBH mRNA expression/neuron. There is a significant increase in the amount of DBH mRNA expression/neuron at the 50% (p=0.03) level of the LC in PD subjects, as compared to control age-matched subjects. The bottom two images are dark-field photomicrographs of DBH mRNA labeled neurons in age-matched control (bottom left) and PD (bottom right) at the 50% level of LC. * Indicates significant difference from age-matched control subjects. Arrow indicates a DBH mRNA labeled neuron. Scale bar = 100μm.

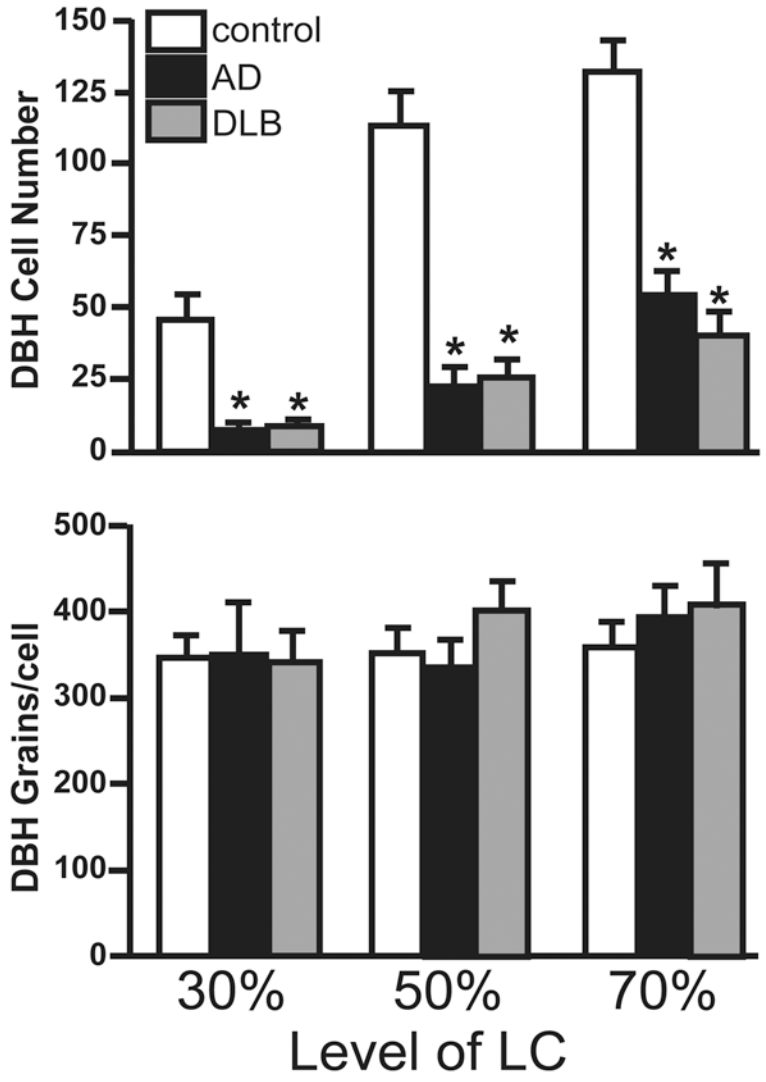

Figure 3.

DBH mRNA expression in the LC of age-matched control (n=15), AD (n=15) and DLB (n=13) subjects. (A) The number of DBH positively labeled neurons at the 30 ($F_{(2,38,40)}$ =17.1, p<0.0001), 50 ($F_{(2,37,39)}$ =38.0, p<0.0001) and 70% ($F_{(2,39,41)}$ =29.7, p<0.0001) level of the LC of age-matched control, AD, and DLB subjects. In AD and DLB subjects, there is a significant decrease in the number of DBH positively labeled neurons at all three levels of the LC. (B) Expression of DBH mRNA/neuron in the LC of age-matched control, AD, and DLB subjects at the 30, 50 and 70% levels. All labeled neurons that were counted as positively labeled were also quantitated for the amount of DBH mRNA expression/neuron. There is not a difference in the amount of DBH mRNA expression/neuron in the surviving LC neurons in AD and DLB subjects as compared to control age-matched subjects. * Indicates significant difference from age-matched control subjects.

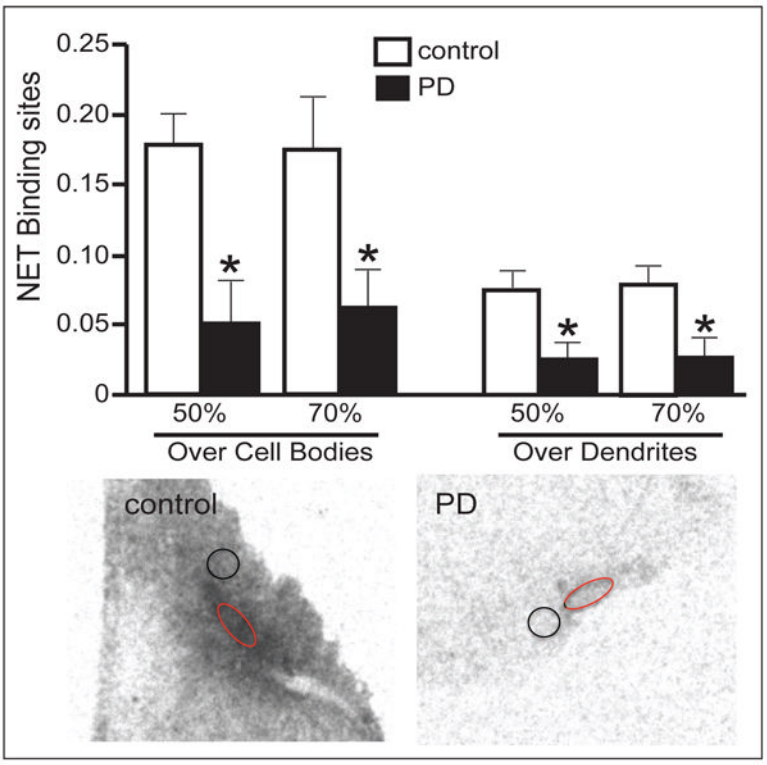

Figure 4.

NET binding site concentrations over the LC region in age-matched control (n=8) and PD (n=7) subjects. Top panel: NET binding site concentrations over LC cell body region and over the peri-LC dendritic zone at the 50 and 70% level of the LC in age-matched control and PD subjects. NET binding site concentrations are significantly reduced in PD subjects at both levels of the LC and in both regions of the LC as compared to age-matched control subjects (cell body region 50% level p=0.006, 70% level p=0.03; dendritic region 50% level p=0.012, 70% level p=0.015). Bottom panel: Autoradiographic image of NET in the LC of age-matched control and PD subjects. * Indicates significant difference from age-matched control subjects. The dark circle represents NET binding over the peri-LC dendritic zone and the grey oblong represents NET binding over LC body region.

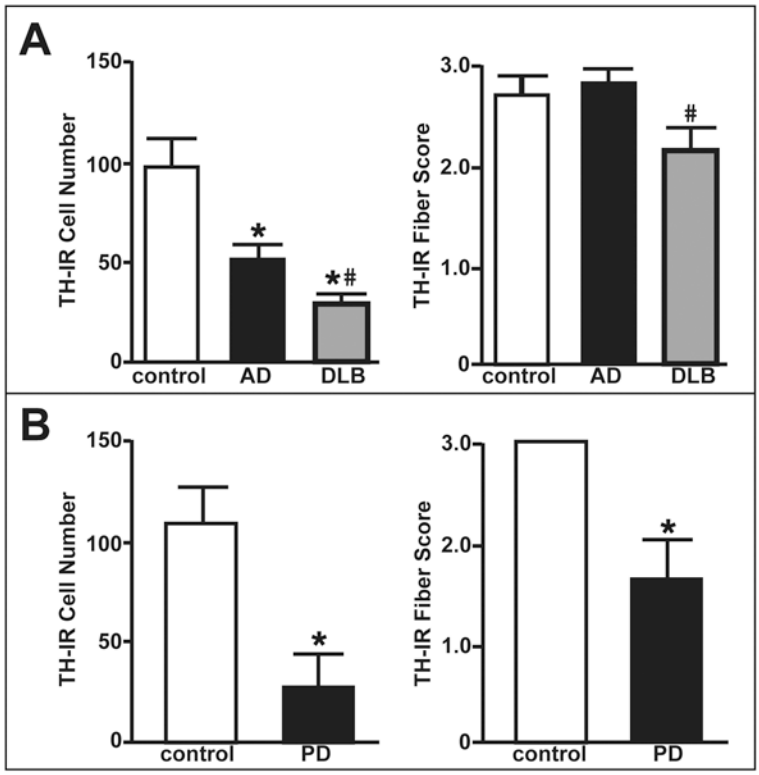

Figure 5.

Comparison of TH-IR at the 70% level of the LC of control, AD/PD and PD subjects. (A) The number of TH-IR positive neurons (left histogram) and the density of TH-IR fibers (right histogram) in control, AD and DLB subjects. The number of TH-IR neurons is significantly decreased in AD and DLB subjects as compared to control ($F_{(2,32,34)}$ =11.8, p=0.0001), while TH-IR fibers in AD and DLB subjects are not significantly different from controls. DLB subjects exhibit a further reduction as compared to AD subjects. (B) The number of TH-IR positive neurons (left histogram) and the density of TH-IR fibers (right histogram) in control and PD subjects. The number of TH-IR neurons (p=0.008) and TH-IR fiber (p=0.004) density are significantly decreased in PD subjects as compared to control subjects. * Indicates significant difference from age-matched control subjects. # Indicates significant difference from AD subjects.

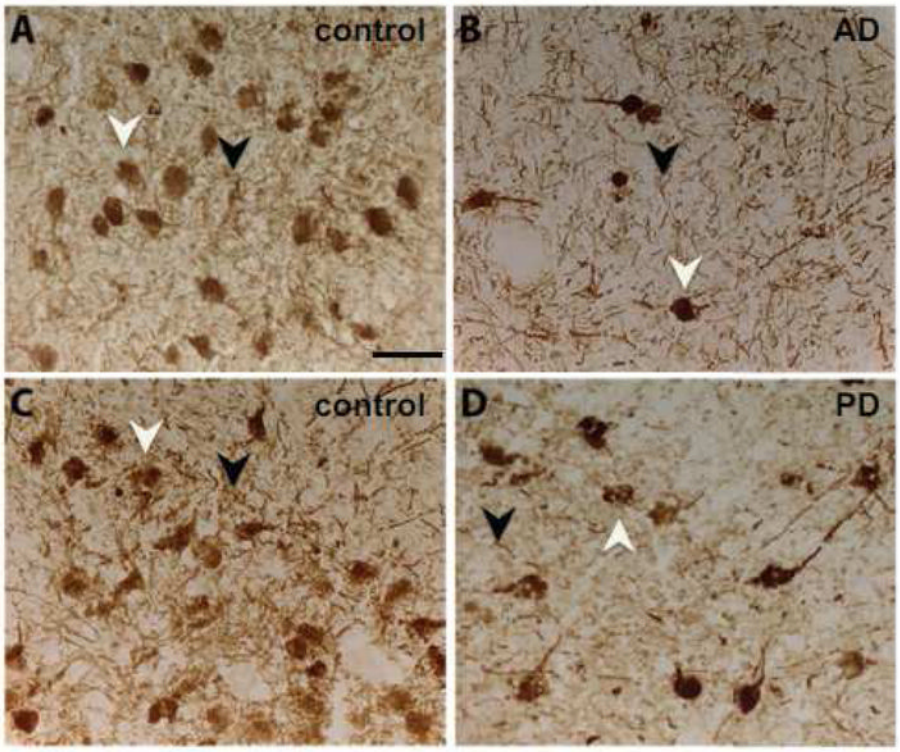

Figure 6.
Photomicrographs of TH-IR labeling in the LC at the 70% level in control (A and C), AD (B) and PD (D) subjects. White arrowhead indicates TH-IR labeled cell body and dark arrowhead indicates a TH-IR labeled fiber tract. Scale Bar = 100 μm.

Table1

Specifics on age, PMI, sex and duration of PD for each subject

Source	Disease	Age/Sex	PMI (h)	Dementia	Duration (y)
BTBDD	control	70/M	28	No	
BTBDD	control	72/F	19	No	
BTBDD	control	69/M	14	No	
ADRC	control	97/M	3	No	
Emory	control	59/M	6	No	
Emory	control	70/M	5	No	
Emory	control	74/F	7	No	
SD	control	unknown	unknown		
BTBDD	PD	78/M	14	No	unknown
BTBDD	PD	68/F	31	No	unknown
Emory	PD	66/M	12	No	25
Emory	PD	67/M	12	No	7
Emory	PD	64/M	13	MCI	12
Emory	PD	74/M	5	MCI	11
SD	PD	unknown	unknown	unknown	unknown

BTBDD=Brain and Tissue Bank for Development Disorder; ADRC=Alzheimer's Disease Research Center at UW; SD= UC San Diego. Tissue from SD has not been disclosed to us until the studies are done.

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