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Regulation of dopamine D₃ receptor in the striatal regions and substantia nigra in diffuse Lewy body disease (DLBD)

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Abstract

The regulation of D₃ receptor has not been well documented in diffuse Lewy body disease (DLBD). In this study, a novel D₃ preferring radioligand [³H]WC-10 and a D₂-preferring radioligand [³H]raclopride were used and the absolute densities of the dopamine D₃ and D₂ receptors were determined in the striatal regions and substantia nigra (SN) from postmortem brains from 5 cases DLBD, which included dementia with Lewy bodies (DLB, n=4) and Parkinson disease dementia (PDD, n=1). The densities of the dopamine D₁ receptor, vesicular monoamine transporter 2 (VMAT2), and dopamine transporter (DAT) were also measured by quantitative autoradiography using [³H]SCH23390, [³H]dihydrotetrabenazine, and [³H]WIN35428, respectively. The densities of these dopaminergic markers were also measured in

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the same brain regions in 10 age-matched control cases. Dopamine D₃ receptor density was significantly increased in the striatal regions including caudate, putamen and nucleus accumbens (NAc). There were no significant changes in the dopamine D₁ and D₂ receptor densities in any brain regions measured. VMAT2 and DAT densities were reduced in all the brain regions measured in DLB/PDD, however the significant reduction was found in putamen for DAT and in the NAc and SN for VMAT2. The decrease of dopamine pre-synaptic markers implies neuronal loss in the substantia nigra pars compacta (SNpc) in these DLB/PDD cases, while the increase of D₃ receptors in striatal regions could be attributed to dopaminergic medication history and psychiatric state such as hallucinations. Whether it also reflects compensatory regulation upon dopaminergic denervation warrants further confirmations on larger populations.

Introduction

Diffuse Lewy body disease (DLBD) encompasses two clinicopathologic entities: dementia with Lewy bodies (DLB) and Parkinson disease dementia (PDD) (McKeith et al., 1996). DLB/PDD has been reported to be the second most common subtype of dementia, accounting for 15–25% of all elderly cases (McKeith et al., 1996). Both entities share the same neuropathological and clinical features. The signature lesions of DLB or PDD are Lewy bodies (LBs) and Lewy neurites (LNs) in specific regions throughout the brain (Duda et al., 2002). Postmortem analysis of brains from DLB/PDD patients commonly reveals additional Alzheimer's disease-type changes including amyloid-beta (A β) plaques and neurofibrillary tangles (McKeith et al., 2004, Kotzbauer et al., 2012). The clinical phenotype of DLB/PDD includes: progressive cognitive impairment, motor features of parkinsonism, visual hallucinations, and fluctuating cognition (Hanson and Lippa, 2009). Dopaminergic medications commonly used to treat the motor symptoms may precipitate dyskinesia as well as cause or worsen hallucinations and psychosis in DLB/PDD (Goldman et al., 2008). Furthermore, a poor response to L-dopa appears to be more frequently occurred in DLB/PDD (Duda, 2004).

Dopamine D₃ receptor is involved in the pathophysiology of Parkinson's disease (PD) and DLB/PDD. However the effect of dopamine deficiency on the D₃ receptor is not well established. In neurotoxin induced rat model of PD, D₃ receptor mRNA level and receptor density are consistently decreased in the striatal region and SNpc (Sokoloff et al., 1990, Levesque et al., 1995, Bordet et al., 1997, Bordet et al., 2000, Stanwood et al., 2000, Guillin et al., 2001), indicating that D₃ receptor is down regulated upon dopaminergic depletion in rodent brain. However in nonhuman primate model of PD and PD patient, there is no decrease of striatal D₃ mRNA (Hurley et al., 1996b, Quik et al., 2000, Joyce et al., 2002), and two studies reported an upregulation of D₃ mRNA in MPTP treated monkey and cat model of PD (Todd et al., 1996, Wade et al., 2001). Receptor density measured by D₃ "selective" agonist such as [3H]7-OH-DPAT, [125I]7-OH-PIPAT and [11C]-(+)-PHNO usually revealed a decreased binding in selective regions of PD (Ryoo et al., 1998, Piggott et al., 1999, Boileau et al., 2009) or nonhuman primate PD model (Morissette et al., 1998, Quik et al., 2000, Bezard et al., 2003). On the other hand unchanged (Hurley et al., 1996a, Hurley et al., 1996b) or increased D₃ receptor density (Joyce et al., 2002) was also reported. In a cat model of PD D₃ receptor was found first decrease and then time dependent increase

in recovered cats (Wade et al., 2001). Until now two studies measured the striatal D₃ receptor density in DLB/PDD cases and showed either not changed (Piggott et al., 1999) or decreased (Joyce et al., 2002) receptor binding.

These controversial results of D₃ receptor regulation in PD or PD animal models may be attributed to several factors. First, D₃ receptor measurements are thought to represent a mixed population including both pre- and post-synaptic fractions which may be differently regulated upon dopaminergic neuronal loss; second, dopaminergic (Bordet et al., 1997, Morissette et al., 1998, Quik et al., 2000, Bezard et al., 2003) and other medications, such as neuroleptics (Wang et al., 1996, Sweet et al., 2001) may alter D₃ receptor binding level or binding affinity; third, dopamine D₂-like receptors undergo a time-dependent change upon dopamine denervation (Todd et al., 1996, Wade et al., 2001); and most importantly, previous *in vitro* autoradiography or PET studies were performed with radioligands not selective for the D₃ receptor, because such radioligands were not available. “Selective” radiolabeled dopamine D₃ agonists used in autoradiography studies also bind to the high affinity agonist binding state of the D₂ receptor and require first decoupling the D₂ receptor from G proteins to measure the D₃ receptor density. Recently a novel dopamine D₃ receptor preferring ligand [³H]WC-10 which is a weak partial agonist/antagonist at the D₃ receptor has been developed and showed a 66-fold higher affinity for human HEK D₃ than HEK D_{2L} receptors, with a dissociation constant (K_d) of 1.2 nM at HEK D₃ receptors (Chu et al., 2005, Xu et al., 2009). We have developed a quantitative autoradiography assay capable of measuring the absolute densities of dopamine D₂ and D₃ receptors using [³H]WC-10 and the D₂/D₃ ligand [³H]raclopride in rat, monkey (Xu et al., 2010) and human brain (Sun et al., 2012). In the current study, brain tissue from a group of DLB/PDD patients and age-matched controls was selected and the absolute densities of D₂ and D₃ receptors were measured in the striatal regions as well as the substantia nigra (SN). Densities of the D₁ receptor, DAT and VMAT2 in the same regions were also measured by quantitative autoradiography. The results of our study reveal a differential regulation of presynaptic and postsynaptic dopaminergic markers in DLB/PDD.

Materials and Methods

Cases

Postmortem human brain tissue samples from 5 DLBD cases (DLB, n = 4; PDD, n = 1) (2 male, 3 female) aged 79–91 (mean: 84±4) years and 10 age-matched cognitively intact (Clinical Dementia Rating (CDR) 0) control subjects (4 male, 6 female) aged 77–92 years were obtained from participants who were longitudinally assessed clinically and neuropathologically at the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (Knight ADRC), Department of Neurology, Washington University School of Medicine (Tables 1 and 2). The clinical diagnosis of DLB/PDD was made using the McKeith et al. criteria (McKeith et al., 1996, McKeith et al., 2005). After death, the written consent of the next-of-kin was obtained for brain removal, following local Institutional Review Board policies and procedures at Washington University in St. Louis.

Tissue collection

Briefly, the left hemibrains were fixed in 10% neutral buffered formalin for 2 weeks, paraffin-embedded, and sections cut at 6 μ m. Blocks were taken from frontal, temporal, parietal, and occipital lobes, thalamus, striatum including the nucleus basalis of Meynert, amygdala, hippocampus, midbrain, pons, medulla oblongata, and the cervical spinal cord. Histologic stains included hematoxylin and eosin and a modified Bielschowsky silver impregnation. Immunohistochemistry was performed using the following antibodies: A β (10D5, Elan Pharmaceuticals, San Francisco, CA), phosphorylated tau (PHF-1, supplied by Dr. Peter Davies, Albert Einstein Medical School, Bronx, NY), phosphorylated TDP-43 (Cosmo Bio USA Inc., Carlsbad, CA) and phosphorylated α -synuclein (Wako Chemicals USA Inc., Richmond, VA). The severity of Lewy body pathology was assessed using the Braak et al. Parkinson's disease staging scale (Braak et al., 2003). Alzheimer's disease pathological changes were assessed using the Braak staging method (Braak and Braak, 1991, Braak et al., 2006).

The right hemibrains were coronally sliced and snap-frozen by contact with Teflon-coated aluminum plates cooled in liquid nitrogen (N₂) vapor, slices were subsequently placed in airtight plastic bags and stored at -80°C using a modification of the method described by Vonsattel et al. (Vonsattel et al., 2008). For autoradiography studies, frozen coronal sections (20 μ m) from the basal ganglia including the caudate nucleus and putamen and from the midbrain to include the substantia nigra (SN) were cut with a Microm cryotome and mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). The slices for all the brain employed in the study were roughly from the same rostrocaudal striatal level in striatal regions. Data from 2–4 sections were averaged for determination of total binding, while nonspecific binding was defined by an average of 1–2 adjacent sections for all the radioligands.

Precursor synthesis and radiolabeling

[³H]WC-10 (Figure 1) was synthesized by American Radiolabeled Chemicals (St Louis, Missouri, USA) by alkylation of the desmethyl precursor with [³H]methyl iodide. The specific activity of the radioligand was 80 Ci/mmol. The synthesis of [³H]WC-10 has been previously described (Xu et al., 2009).

Drugs

Chemical reagents and the standard compounds were purchased from Sigma (St. Louis, MO) and Tocris (Ellisville, MO). [³H]Raclopride (76 Ci/mmol), [³H]SCH23390 (85 Ci/mmol) and [³H]WIN35428 (76 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). [³H]Dihydrotetrabenazine ([³H]DTBZ) (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA).

Quantitative autoradiography protocol

Sections for dopamine D₁, D₂, and D₃ receptor binding were preincubated to remove endogenous dopamine for 20 min at room temperature in buffer (50 mM Tris buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl). After a 30 min incubation in an open staining jar

with the respective radiotracer, slides were then rinsed five times at 1 min intervals with ice-cold buffer. The free radioligand concentration loss was determined to be < 5% as previously described (Xu et al., 2009, Xu et al., 2010).

Quantification of total radioactivity

Slides were air-dried and made conductive by covering the free side with copper foil tape. Slides were then placed into a gas chamber containing a mixture of argon and triethylamine (Sigma-Aldrich, USA) as part of a gaseous detector system, the Beta Imager 2000Z Digital Beta Imaging System (Biospace, France), whose sensitivity limit is 0.07dpm/mm². After the gas was well mixed and a homogenous state achieved, further exposure for 20 h yielded high-quality images. A [³H]microscale with a known amount of radioactivity (ranging from 0 to 36.3nCi/mg) was counted with each section and used to create a standard curve; in each case the standard curve had a correlation coefficient (R) > 0.99. Quantitative analysis was performed with the program Beta-VisionPlus (BioSpace, France) for each anatomical region of interest.

DAT binding—DAT were labeled with [³H]WIN35428. Brain sections were incubated at room temperature for 30 min in buffer solution containing 2.19 nM [³H]WIN35428. Nonspecific binding was determined in the presence of 1 μM nomifensine.

VMAT2 binding—VMAT2 binding sites were labeled with [³H]DTBZ. Brain sections were incubated at room temperature for 30 min in buffer solution containing 4.53 nM [³H]DTBZ. Nonspecific binding was determined in the presence of 1 μM S(-) tetrabenazine.

Dopamine D₁ receptor binding—D₁ receptors were labeled with [³H]SCH23390 using the procedure described by Savasta with minor modifications (Savasta et al., 1986). Briefly, following preincubation to remove endogenous dopamine, sections were incubated for 30 min at room temperature in a buffer solution containing 1.44 nM [³H]SCH23390 and 30 nM ketanserin tartrate (Tocris Bioscience, Ellisville, Missouri, USA) to block 5-HT₂ receptors. Nonspecific binding was determined in the presence of 1 μM (+)-butaclamol as described previously (Novick et al., 2008, Lim et al., 2011).

Determination of absolute densities of D₂ and D₃ receptors

[³H]raclopride autoradiography—[³H]raclopride autoradiography used the procedure previously described for rat and nonhuman primate tissue (Xu et al., 2010). Brain sections were incubated at room temperature for 30 min in buffer solution containing 2.50 nM [³H]raclopride. Nonspecific binding was determined in the presence of 1 μM S(-)-eticlopride (Xu et al., 2010).

[³H]WC-10 autoradiography—[³H]WC-10 autoradiography used the previously described procedure for rat and nonhuman primate brain sections (Xu et al., 2010). Brain sections were incubated at room temperature for 30 min in buffer solution containing 3.54 nM [³H]WC-10; 10 nM WAY-100635 was added to block 5-HT_{1A} receptors. Nonspecific binding was determined in the presence of 1 μM S(-)-eticlopride (Xu et al., 2010).

K_d values and fractional occupancy of [^3H] raclopride and [^3H] WC-10 to human dopamine D_2 D_3 receptors—The K_d values of [^3H]raclopride and [^3H]WC-10 to human dopamine D_2 D_3 receptors were determined through saturation binding analysis of [^3H]WC-10 and [^3H]raclopride to cloned human D_2 and D_3 receptors (Xu et al., 2009). The receptor fractional occupancy of [^3H]WC-10 and [^3H] raclopride to human dopamine D_2 and D_3 receptors can be calculated with 3.54 nM [^3H]WC-10 and 2.50 nM [^3H]raclopride by the saturation binding isotherm:

$$\text{Fractional occupancy} = [\text{Ligand}] / ([\text{Ligand}] + K_d)$$

[Ligand] represents radioligand concentration. The calculated values for K_d and receptor occupancy fractions are summarized in Figure 1.

Calculation of absolute densities of D_2 and D_3 receptors—The absolute densities of dopamine D_2 and D_3 receptors using the D_3 selective radioligand [^3H]WC-10 and the D_2/D_3 ligand, [^3H]raclopride have been previously described (Xu et al., 2010). Briefly, the total amount of receptor bound for [^3H]WC-10 and [^3H]raclopride can be expressed by formula:

$$\begin{aligned} [^3\text{H}]\text{WC-10}: a_1 D_2 + b_1 D_3 &= B_1 \\ [^3\text{H}]\text{raclopride}: a_2 D_2 + b_2 D_3 &= B_2 \end{aligned}$$

where a_1 and b_1 are the fractional occupancies of [^3H]WC-10 to D_2 and D_3 receptors; B_1 is the total receptor density (D_2/D_3) directly measured from autoradiography studies of [^3H]WC-10; a_2 , b_2 , and B_2 are the same parameters for [^3H]raclopride; D_2 , D_3 is the absolute density of D_2 and D_3 receptors. The absolute densities of D_2 and D_3 receptors can be calculated by solving the simultaneous equations:

$$D_2 = \frac{b_2 B_1 - b_1 B_2}{a_1 b_2 - a_2 b_1} \quad D_3 = \frac{a_1 B_2 - a_2 B_1}{a_1 b_2 - a_2 b_1}$$

Statistical analysis

The apparent binding density for each receptor-bound radioligand was calculated using the specific activity of the radioligand expressed as fmol/mg tissue, as previously described (Xu et al., 2010). The experimenter was blinded to all conditions during the analysis. For the regional differences of receptors binding in the striatum (caudate, putamen and NAc), one-way ANOVA was used to estimate overall significance followed by post hoc tests with for multiple comparisons. Comparisons of receptor densities and demographic and neuropathological parameters between age matched control and DLB/PDD cases were conducted using the Mann–Whitney non-parametric U-test. All the receptor binding data was expressed as mean $\pm$ S.E.M. Results were considered significant if $P < 0.05$. All analyses were completed using the IBM SPSS Statistics 21.

RESULTS

Clinical and neuropathologic features of DLBD cases

Although the subjects were relatively elderly, reflecting the subject recruitment population at our center, there was no significant difference in the average age at death or postmortem interval between DLB/PDD and age-matched controls. All the DLB cases had severe dementia (CDR 3) while the PDD cases showed only mild dementia (CDR 1). None of the control cases had parkinsonism, hallucination or had been treated with L-dopa. Three DLB/PDD cases developed parkinsonism and two were treated with L-dopa treatment with good motor response. Three DLB/PDD cases had hallucinations but none of these five cases developed dyskinesia (Table 2). Pathologically, none of control cases had Lewy body pathology, while in addition to widespread Lewy body pathology (brainstem, limbic, and neocortical), the five cases of DLB/PDD also had some AD neuropathological changes typically found in this age cohort (Table 1). The AD changes were prominent in the DLB/PDD group compared to the age-matched controls (Tables 1). Significant neurodegeneration was also evident in the DLB/PDD cases: the brain weight of the DLB/PDD cases was significantly lower than that of the age-matched control subjects. Detailed information regarding clinical and pathological features is summarized in Tables 1 and 2.

Quantitative analysis of dopamine D₁, D₂, D₃ receptors, DAT and VMAT2 densities in aged DLB/PDD and age-matched control cases

Dopamine D₁ receptor—In the striatal regions, the dopamine D₁ receptor was abundantly distributed and no regional differences of receptor binding were found in the putamen, caudate and the NAc in both age-matched controls and DLB/PDD (Figure 2A, C, F; Table 3). There was no significant change in dopamine D₁ receptor density in the striatal regions, while DLB/PDD cases showed a decreased trend (−22%, $p=0.073$, Mann-Whitney Test) of D₁ receptor density in the caudate compared to age-matched controls (Figure 2F). In the SN, the dopamine D₁ receptor density was at a moderate level, which was 26% of that in putamen both in DLB/PDD and age-matched controls. There was no significant difference in the dopamine D₁ receptor density between DLB/PDD and age-matched controls in the SN (Figure 2G; Table 3).

Dopamine D₂ receptor—Similar as dopamine D₁ receptor, the dopamine D₂ receptor was abundantly distributed (receptor density less than D₁ receptor) and no regional differences of receptor binding were found in the putamen, caudate and the NAc in both age-matched controls and DLB/PDD (Figure 3A,C,F; Table 3). Dopamine D₂ receptor density was not significantly different between DLB/PDD and age-matched controls in the putamen, caudate and NAc. In the SN, D₂ receptor density was lower, which was 15% of that in putamen both in DLB/PDD and age-matched controls (Figure 3B, D, G; Table 3). No significant difference in D₂ receptor density was found between DLB/PDD and age-matched controls in the SN (Figure 3G; Table 3).

Dopamine D₃ receptor—The dopamine D₃ receptors was widely distributed in the striatal regions, however the receptor density is much lower than those of dopamine D₂

receptors (Table 3). Regional analysis of D₃ receptor revealed significant differences in the striatum in both control ($p=0.04$, one way ANNOVA) and DLBD ($p=0.03$, one way ANNOVA) cases. Post hoc comparisons revealed that the D₃ receptor density was significantly higher in the NAc compared to the putamen ($p=0.005$) and caudate ($p=0.005$) in control cases and similar to putamen ($p=0.05$) in DLB/PDD cases (Figure 4A, C, F; Table 3). There is no differences of receptor densities between putamen and caudate. Dopamine D₃ receptor density was significantly higher in the putamen (+32%, $p=0.04$, Mann-Whitney Test), caudate (+38%, $p=0.03$, Mann-Whitney Test) and NAc (+27%, $p=0.04$, Mann-Whitney Test) in DLB/PDD cases compared to age-matched controls (Figure 4F, Table 3). Dopamine D₃ receptor is also abundantly distributed in the SN in which the receptor density was lower than that in NAc but higher than that in putamen in control cases (Figure 4B, D, G; Table 3). Although a 38% reduction of dopamine D₃ receptor density was found in the SN in DLB/PDD compared to age-matched control cases, it did not reach statistical significance ($p=0.14$, Mann-Whitney Test) (Figure 4G; Table 3).

The effect of L-Dopa on dopamine D₃ receptor density in the striatal regions and SN was minimal (Figure 5); also, DLB/PDD cases with hallucinations did not have increased D₃ receptors (Figure 6).

VMAT2—DLB/PDD cases had a significant reduction of VMAT2 density in the SN (−39%, $P=0.02$, Mann-Whitney Test) and NAc (−35%, $p=0.03$, Mann-Whitney Test) compared to age matched controls; 29% and 14% reduction of VMAT2 density was found in putamen and caudate respectively, without statistical significance (Figure 7; Table 3).

DAT—DAT density was significantly reduced in the putamen (−53%, $p=0.02$, Mann-Whitney Test); 39%, 23%, and 20% reduction of DAT density was found in NAc, caudate and SN respectively in DLB/PDD compared to age-matched controls, without statistical significance (Figure 8; Table 3).

Discussion

In this study, the absolute density of dopamine D₂ and D₃ receptors was determined in the striatal regions and the SN of a group of DLB/PDD cases and age-matched cognitively intact subjects using the novel D₃ preferring ligand [³H]WC-10 and the D₂/D₃ nonselective ligand [³H]raclopride (Xu et al., 2010). The densities of the dopamine D₁ receptor, DAT and VMAT2 in the same samples were also measured using well-validated tritiated ligands and quantitative autoradiography. The densities of the presynaptic markers DAT and VMAT were found to be decreased indicating dopaminergic neuronal loss. Surprisingly the dopamine D₃ receptor density was significantly increased in the striatal regions, while the D₁ and D₂ receptors densities appeared to be unchanged in both striatum and SN in DLB/PDD.

The universal reduction of presynaptic markers VMAT2 and DAT was observed in the striatum and SN of DLB/PDD, however the statistical significance was found in putamen for DAT and VMAT2 in NAc and SN when using non-parametric Mann–Whitney U-test. This is partly because of small sample size in this study. Previously DAT density was found to be

decreased in DLB/PDD by in vivo PET (Walker et al., 2002, Tabet et al., 2003, Ceravolo et al., 2004) or in vitro autoradiography (Joyce et al., 2002) studies, and one study showed a decreased binding of VMAT2 in DLB/PDD (Koeppe et al., 2008). Piggott et al reported 57% and 75% reduction in DAT density in the post mortem putamen of DLB/PDD and PD respectively, indicating less extent of dopaminergic neuronal loss in DLB/PDD. This is consistent with our finding showing 53% reduction of DAT in putamen in DLB/PDD. Two of DLB/PDD cases did not present with parkinsonism, even though they had a Braak PD stage 6 (Table.2), indicating that neuronal loss in the SN of these cases didn't reach a stage that causes motor parkinsonism. It is notable that the reductions in DAT and VMAT2 densities in the caudate are less than the reductions observed in the putamen and NAc, which is in line with previous reports (Piggott et al., 1999, Joyce et al., 2002, Koeppe et al., 2008) and may reflect the regional differences of neuronal loss in the nigrostriatal system of DLB/PDD. The similar result was also reported previously in PD (Kumakura et al., 2006, Nikolaus et al., 2009).

The finding of no difference in D₁ receptor density in the striatal regions of DLB/PDD compared to that of age-matched controls is in agreement with previous report (Piggott et al., 1999), and is also consistent with that in PD (Shinotoh et al., 1993, Shinotoh, 1996, Cropley et al., 2008). The lack of change in D₁ receptor density in the SN of DLB/PDD is unsurprising because dopamine D₁ receptors are predominately located in GABAergic striatonigral projection neurons (Barone et al., 1987a, Barone et al., 1987b, Freneau et al., 1991, Mengod et al., 1991, Yung et al., 1995) which regulate GABA release (Kliem et al., 2007, Kliem et al., 2009). GABAergic striatonigral projection neurons are not affected in DLB/PDD.

No significant change in the striatal dopamine D₂ receptor density was found in this study. Some previous reports have shown either decrease (Piggott et al., 1999, Sweet et al., 2001) or increase (Walker et al., 1997) of the D₂ receptor density in DLB/PDD. In animal models of PD (Joyce et al., 1986, Graham et al., 1990, Joyce, 1991, LaHoste and Marshall, 1991, Gagnon et al., 1995) and in early stage PD patients (Rinne et al., 1990, Sawle et al., 1993), an increase of the D₂ receptor density was reported, which may reflect an adaptive regulation of D₂ receptor upon denervation. However, D₂ receptor density was found to be down regulated with increased disease duration (Hagglund et al., 1987, Antonini et al., 1995, Antonini et al., 1997) and with L-dopa treatment (Guttman et al., 1986, Brooks et al., 1992, Pizzolato et al., 1995). These findings could explain the D₂ receptor density found in this study, for DLB/PDD cases in this study represent patients with long-standing disease duration, with some cases having received antiparkinsonism treatment.

Dopamine D₂ receptors are located in dopaminergic cell bodies in the SN (Sokoloff et al., 1990, Bowery et al., 1996, Piercey et al., 1996, Mercuri et al., 1997). However, a difference in D₂ receptor density in this area between DLB/PDD and control cases was not detected in the current study. This may be due to the less extent of dopaminergic neuronal loss in DLBD, and may also attributed to the low density of D₂ receptors in the human SN. This has been observed in recent PET imaging (Rabiner et al., 2009, Graff-Guerrero et al., 2010, Searle et al., 2010, Tziortzi et al., 2011, Boileau et al., 2012) and a recent post mortem

autoradiography study (Sun et al., 2012) showing a high density of D₃ receptors and negligible D₂ receptors in the human SN.

Our results show that dopamine D₃ receptor density is significantly increased in the striatal regions in DLB/PDD compared to cognitively intact elderly controls. This is different from previous reports showing either no change or decrease of D₃ receptor density in DLB/PDD (Piggott et al., 1999, Joyce et al., 2002) or PD (Hurley et al., 1996b, Ryoo et al., 1998, Piggott et al., 1999, Boileau et al., 2009). Since the expression of dopamine D₃ receptor can be induced by dopaminergic medication, such as L-dopa and dopamine agonist treatment in rodent (Bordet et al., 1997, Bordet et al., 2000) and nonhuman primate PD model (Morissette et al., 1998, Quik et al., 2000, Bezard et al., 2003); the increase of D₃ receptor density found in this study could be attributed to drug history since some patient had history of L-dopa treatment, and it is likely that other dopaminergic medication was also used in this group patient. It has been reported that AD+LB patient with psychosis (Sweet et al., 2001) had a significant higher level of D₃ receptor, so that the increase of D₃ receptor density may also be related hallucinations which was found in this group patient.

A possible explanation is that the increased D₃ receptor density reflects compensatory regulation upon dopaminergic neuronal loss in DLB/PDD. Previously a significant increase of D₃ receptor mRNA was found in MPTP-induced PD models of monkey (Todd et al., 1996) and cat (Wade et al., 2001), and one study showed a increased trend of D₃ receptor mRNA in PD (Hurley et al., 1996b). The striatal dopamine D₃ receptor mRNA level reflects the transcriptional regulation of this receptor in the post synaptic neurons since it is only express in cell bodies but not in dopaminergic terminals. Although the reduction of D₃ receptor density was usually found in nonhuman primate PD model and PD patient, this decrease seems to be time and region dependent. For example, one postmortem human study showed the reduction of D₃ receptor in PD but it was only found in patients with a disease history >10 years (Ryoo et al., 1998). Another study showed that D₃ receptor was decreased in caudate striatum but increased in rostral striatum in PD (Joyce et al., 2002). In MPTP treated monkey, in which 70 to 99% depletion of the dopamine transporter was found in the basal ganglia, the decline of D₃ receptor was only found in caudate but not in putamen (Quik et al., 2000, Bezard et al., 2003). Interestingly the D₃ receptor density in globus pallidus measured by autoradiography was increased (Joyce et al., 2002) or no changed in PD (Ryoo et al., 1998) and MPTP treated monkey (Bezard et al., 2003). Globus pallidus is the only region where the D₃ receptor is abundant distributed but no D₃ receptor mRNA expression (Quik et al., 2000), so that it stands for D₃ receptor regulation in the single population of postsynaptic striatal neurons. The compensatory regulation of D₃ receptor was also observed in neurotoxin induced rat model of PD. For example, BDNF receptor was found to be up regulated in the striatum upon dopaminergic depletion (Guillin et al., 2001). BDNF from dopamine neurons is responsible for inducing normal expression of the dopamine D₃ receptor both during development and in adulthood. Recently an electrophysiological study showed that D₂ like receptors supersensitivity following DA depletion can in part be attributed to an enhanced D₃ receptor activity in striatal neurons (Prieto et al., 2011). Take together, all this data indicate that the reduction of D₃ receptor in PD may be attributed to the dopaminergic neuronal loss and a compensatory upregulation of the D₃ receptor in post

synaptic neurons might be involved in the D₃ receptor regulation in PD or nonhuman PD models. However this needs further investigation in future large cohort study.

Dopamine D₃ receptor density was no significantly changed in the SN in DLBD. This is in line with previous report in nonhuman primate PD model (Quik et al., 2000) and PD (Boileau et al., 2009), but it is different from rodent PD model, in which D₃ receptor mRNA and receptor density is significantly reduced in SN (Sokoloff et al., 1990, Bordet et al., 2000, Stanwood et al., 2000). In this study we measured the D₃ density in SN which includes SNpc and substantia nigra Pars reticulata (SNpr), where dopamine D₃ receptor is also abundantly distributed (Bordet et al., 2000, Diaz et al., 2000, Stanwood et al., 2000) but without neuronal loss in PD and DLBD. No change of D₃ receptor density may also be attributed the less extent of dopaminergic neuronal loss in DLBD, which can be compensated by upregulation of the receptor in the remaining neurons. Importantly the small sample size in this study can lead to no significant result although 38% reduction of D₃ receptor density was found.

A weak partial agonist/antagonist [³H] **WC-10** was used to measure dopamine D₃ density in this study, which is different from previous reports, in which agonist radioligand such as [³H]7-OH-DPAT, [¹²⁵I]7-OH-PIPAT were often used and this approach requires first decoupling the D₂ receptor from G proteins to measure the D₃ receptor density. Recently a study using [³H]7-OH-DPAT binding in membrane preparations from the rat caudate-putamen suggested a coupling of endogenous D₃ receptors to G proteins and multiple agonist binding states of dopamine D₃ receptors in rat brain (Hillefors and von Euler, 2001). Whether some low affinity state D₃ receptors were missed in the measurement by radiolabeled dopamine D₃ agonists such as [³H]7-OH-DPAT should be considered when using agonist binding. In fact, a significant increase in the density of low affinity D₂-like receptors (D₂ mRNA unchanged while D₃ mRNA increased) was observed in the brain of MPTP treated monkey (Todd et al., 1996), where it was proposed that low affinity state D₂ receptors were up regulated, however we think it is very likely that an upregulation of low affinity state of D₃ receptor occurred in this PD model, which would be worth further investigation. The current study used a weak partial agonist/antagonist [³H] **WC-10** to measure dopamine D₃ density, therefore our findings may reflect the regulation of D₃ receptors including both high and low affinity state upon dopaminergic denervation. It has been reported that the binding affinity of agonist to dopamine D₃ receptor increased in AD + LB case (Sweet et al., 2001). It is not clear if such change occurred for antagonist, [³H] **WC-10** in this study, which needs further investigation.

The limited number of DLB/PDD cases made it difficult to correlate D₃ receptor changes with L-dopa treatment or hallucinations. There was no apparent effect of L-Dopa on D₃ receptor density and no difference between cases with and without hallucinations in this group of DLB/PDD subjects. However, this needs to be further investigated in a larger patient cohort.

In conclusion, our data show that DLBD patient have significant reduction of the presynaptic dopaminergic markers VMAT2 and DAT and significant increase of striatal D₃ receptor density. Although this is preliminary data because of small size samples, and it is

probably that the increase of D₃ receptor density is due to drug history or psychiatric state. However whether other mechanisms such as post synaptic compensatory upregulation is also involved need to be further investigated on a large population.

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Figure 1. Chemical structures of [³H]WC-10 and [³H]raclopride

K_d values were obtained through saturation binding of [³H]WC-10 and [³H]raclopride to cloned human D₃ and D_{2L} receptors expressed in HEK cells. a₁ and b₁ represent the fractional receptor occupancy to dopamine D₂ and D₃ receptors in human brain at a ligand concentration of 3.54 nM for [³H]WC-10. a₂ and b₂ represent the same parameters at a ligand concentration of 2.50 nM [³H]raclopride. The receptor occupancy fractions were calculated from the saturation binding isotherm using the K_d values. *Data taken from (Xu et al., 2009)

Figure 2. Quantitative autoradiographic analysis of dopamine D₁ receptor density in the striatal regions and substantia nigra (SN) of subjects with DLB/PDD and age-matched controls
 Autoradiograms show total binding of 1.44 nM [³H]SCH23390 (A, B) and nonspecific binding in the presence of 1 μM (+) butaclamol (C,D) in the striatal regions (A, C) and substantia nigra (SN) (B, D) in DLB/PDD and age-matched controls. [³H]Microscale standards (ranging from 0 – 36.3 nCi/mg) were also counted (E). Quantitative analysis of the dopamine D₁ receptor density (fmol/mg) in the striatal regions and SN of DLB/PDD and age-matched control are shown in F and G respectively. The numbers 1 – 4 designate the following regions: Putamen (1); Caudate (2); Nucleus Accumbens (3); Substantia Nigra (4).

Figure 3. Quantitative autoradiographic analysis of dopamine D₂ receptor density in the striatal regions and substantia nigra of subjects with DLB/PDD and age-matched controls
 Autoradiograms show total binding of 2.50 nM [³H]raclopride (A, B) and nonspecific binding in the presence of 1 μM S(-)-eticlopride (C, D) in the striatal regions (A, C) and substantia nigra (SN) (B, D) in DLB/PDD and age-matched controls. [³H]Microscale standards (ranging from 0 – 36.3 nCi/mg) were also counted (E). Quantitative analysis of the dopamine D₂ receptor density (fmol/mg) in the striatal regions and SN of DLB/PDD and age-matched controls are shown in F and G respectively. The numbers 1 – 4 designate the following regions: Putamen (1); Caudate (2); Nucleus Accumbens (3); Substantia Nigra (4).

Figure 4. Quantitative autoradiographic analysis of dopamine D₃ receptor density in the striatal regions and substantia nigra of subjects with DLB/PDD and age-matched controls
 Autoradiograms show total binding of 3.54 nM [³H]WC-10 (A, B) and nonspecific binding in the presence of 1 μ M *S*(-)-eticlopride (C, D) in striatal regions (A, C) and substantia nigra (SN) (B, D) in DLB/PDD and age-matched controls. [³H]Microscale standards (ranging from 0 – 36.3 nCi/mg) were also counted (E). Quantitative analysis of the dopamine D₃ receptor density (fmol/mg) in the striatal regions and SN of DLB/PDD and age-matched controls are shown in F and G respectively. The numbers 1 – 4 designate the following regions: Putamen (1); Caudate (2); Nucleus Accumbens (3); Substantia Nigra (4). *p<0.05 for DLB/PDD vs. control

Figure 5. L-Dopa effect on dopamine D₃ receptor density in the striatal regions and substantia nigra

Two of DLBD cases had history of L-Dopa treatment and showed as open scale. L-Dopa effect on D₃ receptor density is minimal in all the brain regions, with an exception in nucleus accumbens, where an increased trend of D₃ receptor density in L-Dopa treatment cases was found. DLBD: Diffuse Lewy body disease.

Figure 6. Hallucinations and dopamine D₃ receptor density in the striatal regions and substantia nigra

Three of DLBD cases presented with hallucinations and showed as open scale. DLBD cases with hallucinations didn't show increased level of dopamine D₃ receptor density in any brain regions. DLBD: Diffuse Lewy body disease; Ha: Hallucinations.

Figure 7. Quantitative autoradiographic analysis of VMAT2 density in the striatal regions and substantia nigra of subjects with DLB/PDD and age-matched controls
 Autoradiograms show total binding of 4.53 nM [^3H]DTBZ (A, B), and nonspecific binding in the presence of 1 μM *S*(-)-tetrabenazine (C, D) in the striatal regions (A, C) and substantia nigra (SN) (B, D) in DLB/PDD and age-matched controls. [^3H]Microscale standards (ranging from 0 to 36.3 nCi/mg) were also counted (E). Quantitative analysis of the VMAT2 density (fmol/mg) in the striatal regions and SN of DLB/PDD and age-matched controls are shown in F and G respectively. The numbers 1 – 4 designate the following regions: Putamen (1); Caudate (2); Nucleus Accumbens (3); Substantia Nigra (4). * $p < 0.05$ for DLB/PDD vs. control

Figure 8. Quantitative autoradiographic analysis of DAT density in the striatal regions and substantia nigra of subjects with DLB/PDD and age-matched controls

Autoradiograms show total binding of 2.19 nM $[^3\text{H}]$ WIN35428 (A, B), and nonspecific binding in the presence of 1 μ M nomifensine (C, D) in the striatal regions (A, C) and substantia nigra (SN) (B, D) in DLB/PDD and age-matched controls. $[^3\text{H}]$ Microscale standards (ranging from 0 – 36.3 nCi/mg) were also counted (E). Quantitative analysis of DAT density (fmol/mg) in the striatal regions and SN of DLB/PDD and age-matched controls are shown in F and G respectively. The numbers 1 – 4 designate the following regions: Putamen (1); Caudate (2); Nucleus Accumbens (3); Substantia Nigra (4). # $p < 0.01$ for DLB/PDD vs. control

Table 1
Demographic and neuropathological features of subjects with DLB/PDD and age-matched controls.

age(years)	Gender	Agonal state	Brain Weight (g)	PMI (hours)	Braak PD	Braak AP	Braak NFT	Other lesions	Diagnosis
84	M	Myocardial infarction	1180	21	5	C	V	TDP-MTL	DLB
82	M	Inanition	1200	16	6	C	V	0	DLB
84	F	Indeterminate	950	5	5	A	III	AGD	PDD
79	F	Inanition	940	21	6	C	VI	TDP-MTL	DLB
91	F	Indeterminate	940	18	6	C	VI	0	DLB
77	F	Respiratory failure	1410	10	0	A	I	0	Control
96	M	Inanition	1165	12	0	0	I	0	Control
91.6	F	Respiratory failure	1310	16	0	0	II	0	Control
92.1	F	Myocardial infarction	1120	6	0	0	0	0	Control
91.6	F	Cardiac arrhythmia	1220	16	0	A	II	0	Control
79	F	Metastatic carcinoma	1100	25	0	A	I	0	Control
100	F	Coronary artery disease	1450	21	0	C	II	0	Control
90	M	Inanition	1150	10	0	A	IV	0	Control
84	M	Bronchopneumonia	1010	5	0	B	I	0	Control
91	M	Myocardial infarction	1170	8	0	A	I	0	Control
0.14			0.04	0.30	0.00000	0.02	0.0001		

PMI, post mortem interval; Braak NFT, Braak neurofibrillary tangle stage (range: I–VI); Braak AP, Braak beta-amyloid plaque stage (range: A–C); Braak PD, Braak Parkinson’s disease stage (range: 1–6); DLB, dementia with Lewy bodies; PDD, Parkinson disease dementia; TDP-MTL, modest TDP-43 proteinopathy in medial temporal lobe; AGD, argyrophilic grain disease. The last row showed P value compared between DLDP and control using Mann–Whitney non-parametric U-test.

Table 2

Clinical features of subjects with DLB/PDD

Onset of dementia(years)	Onset of parkinsonism(years)	Age at death (years)	L-dopa	L-dopa response	Hallucinations	Dyskinesia	Diagnosis
74	79	84	Yes	Yes	None	None	DLB
71	Na	82	No	No	Yes	None	DLB
78	64*	84	Yes	Yes	Yes	None	PDD
69	Na	79	No	No	None	None	DLB
72	86	91	No	No	Yes	None	DLB

CDR, Clinical Dementia Rating at expiration; DLB, dementia with Lewy bodies; PDD, Parkinson disease dementia; Na, parkinsonism not detected;

* Parkinsonism preceded dementia by > 1 year hence this case meets consensus criteria for PDD; other cases meet criteria for DLB (McKeith et al., 2005).

Table 3

Dopamine D₁, D₂, D₃ receptors, VMAT2 and DAT densities in the striatal regions and substantia nigra of subjects with DLB/PDD and age-matched controls.

		Putamen	Caudate	Nac	SN
D1	Control	115±10	119±7	126±9	30±4
	DLB/PDD	119±7	92±12	110±12	31±2
D2	Control	82±5	78±6	89±6	13±1
	DLB/PDD	94±18	68±11	101±13	15±1
D3	Control	36±4	35±5	58±1	47±8
	DLB/PDD	53±4 [*]	57±8 [*]	79±7 [*]	29±8
VMAT2	Control	427±25	371±16	349±20	122±11
	DLB/PDD	282±67	318±55	225±39 [*]	74±11 [#]
DAT	Control	24±2	18±3	18±3	4±0.5
	DLB/PDD	11±3 [#]	14±2	11±2	3±0.4

DLB, dementia with Lewy bodies; PDD, Parkinson disease dementia. Data (in fmol/mg) expressed as mean ± SEM.

^{*} p<0.05,

[#] p<0.01 for DLB/PDD vs. control