Dopamine \(D_1\), \(D_2\), \(D_3\) Receptors, Vesicular Monoamine Transporter Type-2 (VMAT2) and Dopamine Transporter (DAT) Densities in Aged Human Brain

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Abstract

The dopamine \(D_1\), \(D_2\), \(D_3\) receptors, vesicular monoamine transporter type-2 (VMAT2), and dopamine transporter (DAT) densities were measured in 11 aged human brains (aged 77–107.8, mean: 91 years) by quantitative autoradiography. The density of \(D_1\) receptors, VMAT2, and DAT was measured using \(^{3}H\)SCH23390, \(^{3}H\)dihydrotetrabenazine, and \(^{3}H\)WIN35428, respectively. The density of \(D_2\) and \(D_3\) receptors was calculated using the \(D_2\)-preferring radioligand, \(^{3}H\)WC-10 and the \(D_3\)-preferring radioligand \(^{3}H\)raclopride using a mathematical model developed previously by our group. Dopamine \(D_1\), \(D_2\), and \(D_3\) receptors are extensively distributed throughout striatum; the highest density of \(D_1\) receptors occurred in the nucleus accumbens (NAc). The density of the DAT is 10–20-fold lower than that of VMAT2 in striatal regions. Dopamine \(D_3\) receptor density exceeded \(D_2\) receptor densities in extrastriatal regions, and thalamus contained a high level of \(D_3\) receptors with negligible \(D_2\) receptors. The density of dopamine \(D_1\) linearly correlated with \(D_2\) receptor density in the thalamus. The density of the DAT was negligible in the extrastriatal regions whereas the VMAT2 was expressed in moderate density. \(D_3\) receptor and VMAT2 densities were in similar level between the aged human and aged rhesus brain samples, whereas aged human brain samples had lower range of densities of \(D_1\) and \(D_2\) receptors and DAT compared with the aged rhesus monkey brain. The differential density of \(D_3\) and \(D_2\) receptors in human brain will be useful in the interpretation of PET imaging studies in human subjects with existing radiotracers, and assist in the validation of newer PET radiotracers having a higher selectivity for dopamine \(D_3\) or \(D_2\) receptors.

Introduction

The dopaminergic system is involved in neurological disorders such as Parkinson disease, drug addiction and schizophrenia [1–4]. Dopamine receptors have been classified into two subtypes: \(D_1\)-like and \(D_2\)-like receptors. Stimulation of \(D_1\)-like (\(D_1\) and \(D_3\)) receptors activates adenylate cyclase and increases cAMP (cyclic adenosine monophosphate) production. Stimulation of \(D_2\)-like (\(D_2\), \(D_3\) and \(D_4\)) receptors inhibits adenylate cyclase activity, increases arachadonic acid release and phosphatidylinositol hydrolysis [5,6]. The dopamine transporter (DAT) is a presynaptic membrane protein which is responsible for the reuptake of dopamine into dopaminergic nerve terminals. The vesicular monoamine transporter type-2 (VMAT2) is a vesicular membrane protein that transport monoamines from the cytosol into synaptic vesicles [7]. Both have been used as dopamine presynaptic markers for nigrostriatal neuronal integrity.

Since radioligands for PET imaging dopamine \(D_2\)-like receptors, such as the antagonists \(^{11}C\)raclopride [8], \(^{18}F\)fallypride [9] and the full agonist \(^{11}C\)[+]PHNO [10], bind to both the dopamine \(D_2\) and \(D_3\) receptors, PET studies can only measure the composite density of these receptors, the dopamine \(D_2\)/\(D_3\) receptor binding potential. Quantitative autoradiography measuring dopamine \(D_2\) and \(D_3\) receptor densities have yielded equivocal receptor density values and distribution patterns in human and monkey brain [11–18]. This can be attributed to the low \(D_2\)/\(D_3\) selectivity of all radioligands used in these studies. Some studies have attempted to quantify dopamine \(D_3\) receptors using “selective” radiolabeled dopamine \(D_3\) agonists (7-OH-DPAT, PI2AP and PD128947), but these ligands also bind to the high affinity agonist binding state of the \(D_2\) receptor and require first

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decoupling the D2 receptor from G proteins to image the D3 receptor. Studies using radiolabeled selective dopamine D1 versus D3 receptor antagonists are not well documented [5,18,19].

**WC-10**, a weak partial agonist/antagonist at the D3 receptor, binds with a 66-fold higher affinity to human HEK D3 than HEK D2L receptors, with a dissociation constant (Kd) of 1.2 nM at HEK D2 receptors [19,20]. By using [3H]WC-10 and a D2L/D3 ligand [3H]raclopride, we have developed a quantitative autoradiography assay for measuring the absolute densities of dopamine D2 and D3 receptors in the striatal regions of rat and rhesus monkey brain [18]. In this study, the absolute densities of dopamine D2 and D3 receptors were determined by using the same autoradiography assay in the striatal and extrastriatal regions of an aged monkey (25 years old) and aged human brains (average age = 91, range = 77–107.8 years old). The dopamine D1 receptor, DAT, and VMAT2 densities were also measured by quantitative autoradiography. The results of this study provide a unique measurement of the density of D1, D2, and D3 receptors, and DAT and VMAT2 levels, in the same human brain samples.

**Materials and Methods**

**Ethics Statement**

After death, the written consent of the next of kin was obtained for brain removal, following local Ethical Committee procedures (Human Studies Committee, Washington University School of Medicine). Postmortem receptor autoradiography study has been approved by the Alzheimer’s disease Research Center (ADRC) Committee; the approval letter is submitted as a supplement.

The monkey used in this study belongs to our group and was euthanized using pentobarbital 100 mg/kg i.v. due to age-related health decline. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. These studies have been approved by the IACUC at Washington University (approval #20110161). Washington University is fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

**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 detailed synthesis scheme for [3H]WC-10 has been previously described [19].

**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/mm) and [3H]WIN35428 (76 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). [3H]dihydrotetrobenzene ([3H]DTBZ) (20 Ci/mm) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA).

**Tissue collection**

Clinically and neuropathologically well-characterized human brain tissues were obtained from the Knight Alzheimer’s Disease Research Center, Washington University School of Medicine. All cases were longitudinally assessed, healthy elderly individuals without neurological or psychiatric disease and included 4 males and 7 females, aged 77–107.8 years (mean: 91) years. Table 1 shows the demographic case variables. Brains were removed at autopsy and the right hemibrain was coronally sliced and snap-frozen by contact with Teflon-coated aluminum plates cooled in liquid nitrogen vapor, subsequently stored in zip-lock airtight plastic bags and stored at −80°C until used. Microscopy was performed using established rating scales. Alzheimer’s disease pathological changes were assessed using Braak staging [21,22]. For autoradiography studies, frozen frontal sections (20 μm) were cut with a Microm cryotome and mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) from following brain regions: precommissural striatal regions containing the caudate, putamen and nucleus accumbens (NAc); globus pallidus (GP) containing the internal and external part (GPI and GPe); thalamus containing postcommissural striatal regions; and middle brain containing substantia nigra (SN) and red nucleus (RN). For the determination of total binding, data from 2–4 sections were averaged and nonspecific binding was defined by average of 1–2 adjacent sections for all the radioligands. Another set of adjacent sections used for cresyl violet staining to identify related anatomical structures.

**Quantitative autoradiography protocol**

Sections for dopamine D1, D2, and D3 receptor binding were pre incubated for 20 min at room temperature in buffer (50 mM Tris buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl) to remove endogenous dopamine. After incubation with the respective radiotracer, slides were then rinsed five times at 1 min intervals with ice-cold buffer. Slides were incubated in an open staining jar, with the free radioligand concentration loss at less than 5% as previously described [18,19].

**Dopamine D1 receptor binding.** D1 receptors were labeled with [3H]SCH23390 using the procedure described by Savasta [23] with minor modifications. Briefly, after preincubation to remove endogenous dopamine, sections were incubated for 60 min at room temperature in a similar buffer solution with the addition of 1.44 nM [3H]SCH23390 and 30 nM ketanserin tetrartre (Tocris Bioscience, Ellisville, Missouri, USA) to block 5-HT2 receptors. Nonspecific binding was determined in the presence of 1 μM (+)-butaclamol as described previously [24,25].

**Dopamine D2 receptor binding.** D2 receptors were labeled with [3H]raclopride using the previously described procedure for
rat and monkey tissue [18]. Brain sections were incubated for 60 min in buffer solution at room temperature with the addition of 2.50 nM [3H]raclopride. Non-specific binding was determined from the slides in the presence of 1μM S(-)-eticlopride [18].

**Dopamine D3 receptor binding.**  D3 receptors were labeled with [3H]WC-10 using the previously described procedure for rat and monkey tissue [18]. Brain sections were incubated for 60 min in buffer solution at room temperature with the addition of 3.54 nM [3H]WC-10. 10 nM WAY-100635 was added to solution to block 5-HT1A receptors. Non-specific binding was determined in the presence of 1μM S(-)-eticlopride [18].

**DAT binding.**  DAT were labeled with [3H]WIN35428. Brain sections were incubated for 60 min in buffer solution at room temperature with the addition of 2.19 nM [3H]WIN35428. Non-specific binding was determined from the slides in the presence of 1μM nomifensine.

**VMAT2 binding.**  VMAT2 binding sites were labeled with [3H]DTBZ. Brain sections were incubated for 60 min in buffer solution at room temperature with the addition of 4.53 nM [3H]DTBZ. Non-specific binding was determined from the slides in the presence of 1μM S(-)-tetrabenazine.

**Quantification of total radioactivity.**  Slides were air dried and made conductive by coating the free side with a copper foil tape. Slides were then placed into a gas chamber containing a gaseous detector system, the Beta Imager 2000Z Digital Beta scanner. Slides were then placed into a gas chamber containing a gaseous detector system, the Beta Imager 2000Z Digital Beta

**Cresyl violet staining.**  A set of adjacent sections was fixed with 4% paraformaldehyde for 10 min, washed with PBS for 1 min, then dipped in 100% ethanol for 20 seconds to remove fat and fixation chemicals. Sections were then stained with 0.5% cresyl violet solution for 3 min, washed in running tap water 10 min, dehydrated by a series of alcohol baths, and made transparent by xylene (2×4 min) and scanned with an Epson scanner.

**Determination of absolute densities of D2 and D3 receptors.**  Measurement of the absolute densities of dopamine D2 and D3 receptors using the D3 selective radioligand [3H]WC-10 and the D2/D3 ligand, [3H]raclopride was described previously [18]. Briefly, the receptor fractional occupancy of [3H]WC-10 and [3H]raclopride to human dopamine D2 and D3 receptors can be calculated by the saturation binding isotherm:

\[ \text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{ligand}] + K_d} \]

The total amount of receptor bound for [3H]WC-10 and [3H]raclopride can be expressed by formula:

\[ [3H]WC-10: a_1D_2 + b_1D_3 = B_1 \]
\[ [3H]Raclopride: a_2D_2 + b_2D_3 = B_2 \]

Where \( a_1 \) and \( b_1 \) are the fractional occupancies of [3H]WC-10 to D2 and D3 receptors; \( B_1 \) is the total receptor density (D2/D3) directly measured from autoradiography studies of [3H]WC-10; \( a_2, b_2 \), and \( B_2 \) are the same parameters for [3H]raclopride; \( D_2 \) and \( D_3 \) is the absolute density of D2 and D3 receptors. The absolute densities of D2 and D3 receptors can be calculated by solving the simultaneous equations:

\[ D_2 = \frac{b_2B_1-b_1B_2}{a_1b_2-a_2b_1} \]
\[ D_3 = \frac{a_1B_2-a_2B_1}{a_1b_2-a_2b_1} \]

**Statistical analysis.**  The receptor-bound radioligand binding apparent densities were calculated using the specific activity of each radioligand expressed as Bq/mg tissue as previously described [18]. The experimenter was blinded to all conditions during the analysis. Comparison of receptor densities was analyzed by an unpaired Student’s t test. Assessment of correlation between different receptors binding was calculated using Pearson product moment correlation coefficient.

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Table 1. Demographic details of human brains.

| Autopsy number | Age(y) | Gender | Brain Weight(g) | PMI(h) | Braak NFT | Braak Amyloid | CDR |
|----------------|--------|--------|-----------------|--------|-----------|--------------|-----|
| 105.460        | 77     | F      | 1410            | 10     | 1         | A            | 0   |
| 107.050        | 96     | M      | 1165            | 12     | 1         | 0            | 0   |
| 100.060        | 91.6   | F      | 1310            | 16     | 2         | 0            | 0   |
| 105.210        | 107.8  | F      | 1080            | 5      | 2         | A            | 0   |
| 101.200        | 92.1   | F      | 1120            | 6      | 0         | 0            | 0   |
| 104.300        | 91.6   | F      | 1220            | 16     | 2         | A            | 0   |
| 11.027         | 79     | F      | 1100            | 25     | 1         | A            | 0   |
| 11.041         | 100    | F      | 1450            | 21     | 2         | C            | 0   |
| 10.740         | 90     | M      | 1150            | 10     | 4         | A            | 0   |
| 10.150         | 84     | M      | 1010            | 5.5    | 1         | B            | 0   |
| 9.255          | 91     | M      | 1170            | 8.5    | 1         | A            | 0   |

PMI: Post-mortem interval; CDR: Clinical dementia rating.
Results

Quantitative autoradiography

The sensitivity limit of Beta Imager 2000Z Digital Beta Imaging System is 0.07 dpm/mm². A tritium standard [3H]Microscale with a known amount of radioactivity (ranging from 0 to 36.3 nCi/mg) was counted with each section and used to create a standard curve; in each case the standard curve had a correlation coefficient (R) greater than 0.99. On the basis of the saturation binding analysis and the in vitro binding data of [3H]WC-10 and [3H]raclopride to cloned human D2 and D3 receptors [19], Kd value and fractions of D2 and D3 receptor occupancies with 3.54 nM [3H]WC-10 and 2.50 nM [3H]raclopride binding in human brain can be readily determined. The values of Kd and receptors occupancies fractions are summarized in Figure 1.

Quantitative analysis of dopamine D1, D2, D3 receptors, DAT and VMAT2 densities in aged human brain

The binding densities of dopamine D1 receptor, DAT and VMAT2 were determined by quantitative autoradiography using 1.44 nM [3H]SCH23390, 2.19 nM [3H]WIN355428 and 4.5 nM [3H]DTBZ, respectively. The apparent receptor binding densities (B1 and B2) of D2 plus D3 receptors were measured by using 3.54 nM [3H]WC-10 and 2.50 nM [3H]raclopride respectively, and the absolute D2 and D3 receptors densities were determined as described above. The nonspecific binding was determined by using different high affinity cold compounds (Figures 2B, 3B, 4B, 5B). The receptor density values are summarized in Table 2.

Precommissural striatal regions. Dopamine D1, D2 and D3 receptors were found to be extensively distributed throughout the precommissural striatal regions. The dopamine D3 receptor density was much lower than that of the D1 and D2 receptors (Table 2; Figure 2). The dopamine D3 receptor density was significantly lower in the putamen (p = 0.001) and caudate (p = 0.0001) than that of the NAc (Figure 1E). No difference in the D3 receptor density was found between the putamen and caudate. The dopamine D2:D3 receptor density ratio was significantly higher in the putamen (p = 0.04) and caudate (p = 0.04) compared to that of the NAc, but was not different between the caudate and putamen (Figure 1F). The VMAT2 density was found to be ~10-fold higher than that of DAT in this region. Densities of DAT and VMAT2 were similar among the three sections of the precommissural striatal regions; an exception was the putamen, which showed significant increase in VMAT2 density versus that of the NAc (P = 0.01) (Figure 1E).

Globus pallidus. The density of dopamine D1 and D2 receptors, and DAT and VMAT2 were dramatically lower in the GP, whereas the density of the dopamine D3 receptor was just slightly lower when compared to those of the striatal regions (Table 2; Figure 3). The distribution of dopamine receptors was different between the GP and GPe; the dopamine D1, D2, and D3 receptor densities were similar in the GPe, while the dopamine D1 receptor density was significantly higher (p = 0.02) and the D2 receptor density was significantly lower (p = 0.03) in the GP compared to that of GPe (Figure 3E). Because of the lower density of dopamine D2 receptors in the GP, the dopamine D2:D3 receptor density ratio was significantly lower than that of GPe (Figure 3F). A lower level of VMAT2 density was distributed in both regions of the GP, whereas the density of the DAT was negligible compared to that in the striatal regions (Table 2; Figure 3E).

Thalamus. Dopamine D1 receptor density was much lower and the D2 receptor was negligible in the thalamus compared to those of the striatal regions (Table 2; Figure 4A, E). In contrast, the dopamine D3 receptor density exceeded that observed in striatal regions, resulting in a low D2:D3 receptor density ratio in the thalamus (0.11±0.05) compared to that of the striatal regions (Figure 4F). A strong linear correlation (R² = 0.78) between the average density of dopamine D1 and D3 receptors was found in the thalamus (Figure 4G). A lower level of VMAT2 was found in the thalamus, whereas DAT density was nearly zero (Table 2; Figure 4A, E).

Postcommissural striatal regions. There were no significant differences in dopamine D2 and D3 receptor densities, and the D2:D3 receptor density ratio, between the pre- and postcommissural striatal regions. However, the dopamine D3 receptor density was found to be significantly lower in the postcommissural putamen (p = 0.01) and caudate (p = 0.01) compared to their precommissural counterparts. The DAT density was found to be significantly decreased in the post- versus precommissural putamen (p = 0.04), while the VMAT level did not change.

Substantia nigra. Dopamine D1 and D2 receptor densities were much lower in the SN compared to those of the striatal regions. In contrast, the dopamine D3 receptor density in the SN was the highest among the extrastriatal regions, and is only slightly lower than that of NAc (Table 2; Figure 5D). Consequently, the dopamine D2:D3 receptor density ratio in the SN was very low (Figure 5E). There was a moderate density of VMAT2 in the SN, while DAT density was negligible in this region (Figure 5D).

Red nucleus. Receptor densities in red nucleus (RN) were extremely low except for the dopamine D1 receptor, which showed a relatively high density in this area (Table 2; Figure 5D). The dopamine D2:D3 receptor density ratio in the RN was similar as that of SN (Figure 5E).

Comparison of dopamine D1, D2, and D3 receptors, and DAT and VMAT2 densities in the striatal regions between aged rhesus monkey and aged human brain

To investigate the species differences of dopamine receptors and presynaptic markers, we compared the density of dopamine D1, D2, and D3 receptors and DAT and VMAT2 in the striatal regions of an aged rhesus monkey (25 years old) to those of aged human brain (average age: 91 years old). The densities of dopamine D1 and D2 receptors and DAT were found to be lower in aged human brain compared to those of rhesus monkey, whereas the dopamine D3 receptor and VMAT2 densities were similar between these two species (Table 3; Figure 6).

Different regulation of VMAT2 and DAT in the striatal regions and substantia nigra of aged human brain

In all brain regions measured, the VMAT2 density was found to be significantly higher than that of the DAT (Table 2). The VMAT2:DAT density ratio was regionally-dependent: the VMAT2 density was 30-fold higher than that of the DAT in the SN but only 10-fold higher in the precommissural striatal regions (Figure 7D). The average VMAT2 density strongly linearly correlated with DAT densities in the precommissural putamen (r² = 0.68) and caudate (r² = 0.73), but not in the SN (r²<0.01) (Figure 7A). The VMAT2 density in the SN significantly correlated with those in the precommissural putamen (r² = 0.60) (Figure 7B) and caudate (r² = 0.50), but no such correlation was found for the DAT either in the precommissural putamen (r² = 0.10) or caudate (r² = 0.11) (Figure 7C).

Discussion

Our group had previously reported the density of dopamine D2 and D3 receptors in rat and rhesus monkey brain using a novel
Dopamine Receptors in Aged Human Brain

Figure 2. Quantitative autoradiographic analysis of dopamine receptors, DAT and DTBZ densities in the precommissural striatal regions. Autoradiograms show total binding of 1.44 nM [3H]SCH23390, 2.50 nM [3H]raclopride, 3.54 nM [3H]WC-10, 2.19 nM [3H]WIN35428, and 4.53 nM [3H]DBZ (A), and nonspecific binding in presence of 1 μM (+) butaclamol (for [3H]SCH23390), 1 μM St-eticlopride (for [3H]raclopride and [3H]WC-10), 1 μM nomifensine (for WIN35428) and 1 μM St-tetabenazine (for DTBZ) (B) in the precommissural striatal regions of human brain sections. The adjacent section shows cresyl violet staining to identify related anatomical structures (C). [3H]Microscale standards (ranging from 0 to 36.3 nCi/mg) were also counted (D). Quantitative analysis of dopamine D1, D2, and D3 receptors, and DAT and DTBZ densities (fmol/mg) and the dopamine D2/D3 receptor density ratio in human striatal regions are shown in E and F respectively. The numbers 1 through 4 designate the following CNS anatomical regions: 1: Precommissural Putamen (PrePu); 2: Precommissural caudate (PreCd); 3: Nucleus accumbens (NAc); 4: Internal capsule (IC).

*p<0.05, #p<0.01 compared to NAc.

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The autoradiography method involving the use of two different radioligands, the D1-preferring ligand [3H]WC-10 and the D2/D3 nonselective ligand [3H]raclopride [18]. Here we report first measurements of D2 and D3 specific receptors in aged human postmortem brain. We also included measurements of the density of dopamine D1 receptors, DAT and VMAT2 using well-established tritiated ligands and quantitative autoradiography. Some noteworthy findings include: 1) D1 receptors were widely distributed throughout the striatal and extrastriatal regions in the aged human brain; 2) in the striatal regions, D3 receptors were more enriched in the NAc than in the caudate and putamen; 3) in the extrastriatal regions, dopamine D1 receptor density exceeded D2 receptors; 4) DAT density in aged human brain was more than 10-fold lower than that of VMAT2 in the striatal regions, and was negligible in the SN, whereas VMAT2 density was relatively high; 4) receptor densities of dopamine D1, D2 and DAT was lower in human versus monkey brain, but D3 and VMAT2 densities appeared to be similar.

Quantitative autoradiography to measure dopamine D3 receptor density have previously been conducted using radiolabeled agonists such as [3H]7-OH-DPAT, [3H]PIPAT, and [3H]PD 128907. Since these ligands bind to both the D3 receptor and the dopamine “high affinity binding site” of the D2 receptor [26], the D2 receptor must first be “decoupled” to form the dopamine low affinity agonist binding state in order to measure D3 receptors with these radioligands [13–15,27,28]. Other studies have used radiolabeled D2/D3 antagonist in the presence of a D2-preferring blocking agent. Recently our lab has developed a new radiolabeled D3...
receptor antagonist/partial agonist [3H]WC-10, which has high binding affinity and selectivity to D3 versus D2 receptors [19,20]. By combining autoradiography studies with [3H]WC-10 with the D2/D3 nonselective ligand [3H]raclopride, the density of dopamine D2 and D3 receptors can be easily determined using the mathematical model [18].

The current finding of the dopamine D3 receptor distribution pattern in the striatal regions is in agreement with some previous reports [13,14,18], but not consistent with other reports demonstrating a restricted distribution in the limbic areas of the striatum [17,29,30]. However, in situ hybridization studies have shown that dopamine D3 receptor mRNA is found in the caudate, putamen and nucleus accumbens in human and monkey brain [13,31,32], which provides additional support for the current observations. The distribution of dopamine D3 receptors in the putamen and caudate, with a higher density in the NAc, suggests that the dopamine D3 receptor may also be involved in the regulation of locomotor function in addition to their well-recognized role in the limbic system.

The measurement of dopamine D3 receptors in the GPi is consistent with previous publications [17]. Interestingly, the dopamine D1 receptor density was found to be significantly higher and the D2 receptor density significantly lower in the GPi versus GPe, which is in agreement with the recent finding showing the similar distribution of dopamine D1 and D2 receptors in the globus pallidus by using bacterial artificial chromosome (BAC) transgenic mice in which expression of enhanced green fluorescent protein (eGFP), is driven by the promoter region of either the D1 or the D2 [33]. The different distribution pattern of the dopamine D1 and D2 receptors in the GPe and GPi found in this study has provided the additional proof that the D1 receptor-mediated direct pathway
Figure 4. Quantitative autoradiographic analysis of dopamine receptors, DAT and DTBZ densities in the thalamus. Autoradiograms show total binding of 1.44 nM [3H]SCH23390, 2.50 nM [3H]raclopride, 3.54 nM [3H]WC-10, 2.19 nM [3H]WIN35428, 4.53 nM [3H]DTBZ (A), and nonspecific binding in presence of 1 μM (+) butaclamol (for [3H]SCH23390), 1 μM S(-)-eticlopride (for [3H]raclopride and [3H]WC-10), 1 μM nomifensine (for WIN35428) and 1 μM S(-)-tetrabenazine (for DTBZ) (B) in the thalamus of human brain sections. The adjacent section shows cresyl violet staining to identify related anatomical structures (C). [3H]Microscale standards (ranging from 0 to 36.3 nCi/mg) were also counted (D). Quantitative analysis of dopamine D1, D2, and D3 receptors, DAT and DTBZ densities (fmol/mg) and the dopamine D2 : D3 receptor density ratio in human brain are shown in E and F, respectively. Linear correlation analysis of the average dopamine D1 and D3 receptor densities in human thalamus is shown in (G). The numbers 1 through 4 designate the following CNS anatomical regions: 1: Postcommissural putamen (PostPu); 2: Postcommissural caudate (PosCd); 3: Thalamus; 4: Internal capsule (IC). #p<0.01 compared to thalamus.

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going from striatum to GPi and the D2 receptor mediated indirect pathway going from striatal to GPe.

The thalamus is another interesting target for brain dopamine [34]. Previous receptor autoradiography studies with the radioligand [125I]epidepride found a modest density of dopamine D2-like receptors in the thalamus [12,35]. On the other hand, dopamine D3 receptor density was found to be very low in human thalamus when [3H]7-OH-DPAT was used as the radioligand [13]. In PET imaging studies, radiotracers such as [18F]fallypride which has a high affinity for both D2 and D3 receptors, and [11C]PHNO which is a D3 preferring ligand, display a high uptake in the thalamus of human and monkey brain [9,36–40]. This is in contrast to [18F]NMB and [11C]raclopride, both tracers have lower uptakes in the thalamus versus the striatum and putamen [41,42]; NMB and raclopride have higher affinities for D2 versus D3 receptors, which could explain their relatively low uptake in the thalamus. In the present study, dopamine D1 receptors were found to be abundant while the D2 receptor was nearly negligible in the thalamus of aged human brain. Consequently, the dopamine D2:D3 receptor density ratio was very low in this area. Although the current autoradiography study was conducted in aged subjects with no sign of neurological disease, it is not likely that the aging process would result in a complete loss of dopamine D2 receptors in lieu of D3 receptors. Therefore, our data indicate that the thalamus can be used as a good region to study dopamine D3 receptor function in PET imaging studies using radiotracers such as [18F]fallypride,
[11C]raclopride, and [11C]PHNO. It should be noted that differences in the D2/D3 binding potential of these PET radiotracers in the thalamus have been reported in a variety of neurological and neuropsychiatric disorders, including schizophrenia [43–46], substance abuse [47,48] and dystonia [49,50] relative to age-matched controls. The finding of the high D3 receptor density and low D2:D3 ratio in the human thalamus indicates that the changes of D2/D3 thalamic binding potential in these patients measured by PET may be attributed to changes in dopamine D3 receptor function, and that dopamine D3 receptors may play a key role in the pathophysiology of these disorders.

The dopamine D1 receptor was also found to be abundantly distributed in the SN and RN, whereas the density of D2 receptors was lower. Dopamine D2-like receptors were observed in the RN and SN with high and moderate density in a human PET imaging study using [11C]FLB 457 [51]. More recent studies using the dopamine D3-preferring agonist [11C]PHNO [38], reported a high density of dopamine D3 receptors and negligible D2 receptors in the SN [40,52–55], which is consistent with our autoradiography findings. The dopamine D1 receptor was also found to be present in the SN with a density intermediate to D3 and D2 receptors, which agrees with previous reports [56–58]. The abundance of dopamine D3 receptors and lower D2:D3 receptor

| Table 2. Dopamine D1, D2, D3 receptors, dopamine transporter (DAT) and vesicular monoamine transporter type-2 (VMAT2) densities and D2 : D3 receptor density ratio in aged human brain. |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Region          | Region abbreviation | DAT  | VMAT2  | D1  | D2  | D3  | D2:D3 ratio  | n  |
| Precommissural putamen | PrePu            | 35±3 | 336±13 | 82±3 | 39±3 | 2.30±0.28 | 10 |
| Precommissural caudate   | PreCd            | 30±4 | 325±29 | 115±7 | 79±5 | 38±4 | 2.29±0.27 | 10 |
| Nucleus accumbens     | NAc              | 26±5 | 282±16 | 126±7 | 93±6 | 55±2 | 1.69±0.11 | 8  |
| Postcommissural putamen | PosPu           | 24±5 | 373±39 | 90±9 | 76±3 | 38±5 | 2.21±0.27 | 7  |
| Postcommissural caudate | PosCd           | 24±3 | 294±38 | 86±16 | 63±7 | 28±7 | 2.87±0.91 | 4  |
| Globus pallidus external part | GPe         | 2±0.4 | 54±8 | 16±2 | 18±3 | 18±3 | 0.96±0.25 | 6  |
| Globus pallidus internal part | GPI         | 0.6±0.3 | 36±8 | 29±5 | 9±4 | 21±3 | 0.44±0.16 | 6  |
| Thalamus              | Th               | 0.8±0.4 | 44±5 | 14±1 | 4±2 | 33±5 | 0.11±0.05 | 6  |
| Substantia nigra      | SN               | 4±0.4 | 122±9 | 30±3 | 13±1 | 47±6 | 0.30±0.05 | 8  |
| Red nucleus           | RN               | 4±0.3 | 30±5 | 10±0.9 | 8±1 | 29±1 | 0.29±0.05 | 6  |

Receptor densities (fmol/mg) presented as mean value ±SEM.

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Figure 6. Comparison of dopamine D1, D2, and D3 receptors, and DAT and DTBZ densities in the striatal regions between an aged rhesus monkey (25 years old) and aged human brain samples. Autoradiograms show neuroanatomical localization of [1H]SCH23390 for D1, [1H]raclopride for D2, [1H]WC-10 for D3 receptors, [1H]WIN35428 for DAT and [1H]DBZ for VMAT2 in the striatal regions of rhesus monkey (A) and aged human brain (B). [1H]Microscale stnadards (ranging from 0 to 36.3 nCi/mg) (C). The numbers 1 through 8 in panels (A) (B) designate the following CNS anatomical regions: 1: Monkey putamen; 2: Monkey caudate; 3: Monkey globus pallidus; 4: Monkey thalamus; 5: Human putamen; 6: Human caudate; 7: Human globus pallidus; 8: Human thalamus.

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Table 3. Comparison of dopamine D₁, D₂, D₃ receptors, DAT and VMAT2 densities (fmol/mg) in the striatal regions of adult rhesus monkey and aged human brain.

|     | D₁   | D₂   | D₃   | DAT  | VMAT2 |
|-----|------|------|------|------|-------|
|     | Pu   | Cd   | Pu   | Cd   | Pu    | Cd     | Pu   | Cd   | Pu   | Cd   |
| Rhesus monkey | 256±19 | 228±9 | 178±9 | 205±6 | 36±9   | 46±4  | 185±12 | 177±17 | 341±20 | 351±10 |
| Human | 117±23 | 119±16 | 82±11 | 77±15 | 39±11  | 36±11 | 35±10  | 30±11  | 336±40 | 325±86 |

Data were obtained from 10 aged healthy human and a 25 years old rhesus monkey brain and presented as mean value ± stdev. Pu: Putamen; Cd: Caudate. doi:10.1371/journal.pone.0049483.t003

Figure 7. Correlation of DAT with VMAT2 in the striatal regions and substantia nigra. The correlation between the VMAT2 and DAT densities in the precommissural putamen (PrePu), caudate (PreCd) and substantia nigra (SN) (A). Correlation of the VMAT densities between the substantia nigra (SN) and PrePu or PreCd (B). Correlation of the DAT densities between the SN and PrePu or PreCd (C). The average VMAT DAT density ratio in the PrePu, PreCd and SN (D). #p<0.01 compared to SN.

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density ratio in human SN represents a second region to study dopamine D3 versus D2 effects using currently available PET ligands. The functional significance of the abundant existence of D3 versus D2 receptors in human SN is not clear. One possible explanation is that dopamine D3 receptors may be involved in the negative feedback regulation of tonic dopamine release.

A number of biochemical and behavioral studies have suggested that D1 and D3 receptors may functionally interact [59,60]. For example, D1 and D3 mRNAs are co-localized in a large number of neurons in the striatum [61] and the NAc [62–64], and co-activation of D1 and D3 receptors in the shell of the NAc synergistically increases substance P expression [63,64]. D1 and D3 interactions are thought to mediate the rewarding properties of low doses of cocaine [36], and L-DOPA administration to rats receiving a unilateral injection of the neurotoxin 6-OH-dopamine results in an overexpression of D3 receptors in nigrostriatal neurons that constitutively express D1 receptors [59,65,66]. Dopamine D1 and D3 receptors were co-expressed in the renal proximal tubule [67] and in transfected HEK-293 cells [68]. Heterodimerization of these two receptors has been observed by co-immunoprecipitation from striatal protein preparations [59] or by bioluminescence resonance energy transfer technique in transfected mammalian cells [59,68]. It is of interest to note that a linear correlation of dopamine D1 and D3 receptor densities was found in the thalamus, which is consistent with either an anatomical or functional coupling of D1 and D3 dopamine receptor subtypes. D1 receptors are not thought to interact with D3 receptors functioning as autoreceptors [60]; and we found no strong correlation of D1 and D3 receptor density in the caudate, putamen and SN (data not shown), where D3 receptors may function as autoreceptors. The availability of [3H]WC-10, a D2-prefering radioligand, will provide a valuable tool for studying the functional interactions between dopamine D1 and D3 receptors in the CNS.

The DAT and VMAT2 distribution pattern found in this study is consistent with the previous reports [69–72]. The higher density level of both DAT and VMAT2 was found in the putamen and caudate compared to that of nucleus accumbens, which is in line with the recent finding using the same radioligands [73]. Surprisingly the DAT density was 10 fold lower than that of VMAT2 in aged human striatum which is different from previous reports. Furthermore the DAT density was lower while the VMAT2 density was not significantly different in the aged human compared to that of monkey brain. This may reflect the different aging related change patterns of these two dopamine presynaptic markers. In fact aging related decline of DAT but not VMAT2 density in the human brain has been reported [74–76]. In the striatal regions, the DAT density was significantly correlated with that of the VMAT2, indicating the anatomical and functional coupling of these two presynaptic dopamine markers.

The monkey brain had a higher density of D1 and D2 receptors relative to the human brain, but a similar density of D3 receptors. Dopamine D1 [58,77–79] and D2 [46,80–82] receptor densities decline with aging in human brain, but no reports have been published measuring the density of D3 receptors as a function of age.

The main limitation of this study is absence of data from younger subjects. The results of this study may only reflect the densities and distribution of the dopamine receptors and transporters in advanced aged human brain, and may not reflect age-related changes of presynaptic and postsynaptic dopamine markers. Therefore, caution should be given when comparing these data with that of PET imaging studies of the dopaminergic in younger subjects.

Conclusions

This study provides quantitative measurements of the density of presynaptic (VMAT2 and DAT) and postsynaptic dopaminergic markers (dopamine D1, D2, and D3 receptors) in the aged human brain. The correlation between the density of D1 and D2 receptors in the thalamus, and the DAT and VMAT2 in the striatal regions, suggests a functional interaction between these markers. The high density of D3 receptors in the thalamus and SN and low density of D2 receptors in these brain regions could provide valuable information for PET imaging D1 and D2 receptor function using [18F]dalfampridine, [11C]raclopride, [18F]NMIB, and [11C]PHNO. The differential density of D2 and D3 receptors in these brain regions can also be used in determining the in vivo selectivity on newer PET radiotracers which are being developed to discriminate between D3 and D2 receptors and vice versa.

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Author Contributions

Conceived and designed the experiments: JX RHM. Performed the experiments: JS. Analyzed the data: JS JX JSP. Contributed reagents/materials/analysis tools: NJC. Wrote the paper: JS RHM.

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Dopamine Receptors in Aged Human Brain

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