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The decrease of dopamine D_2/D_3 receptor densities in the putamen and nucleus caudatus goes parallel with maintained levels of CB_1 cannabinoid receptors in Parkinson's disease: A preliminary autoradiographic study with the selective dopamine D_2/D_3 antagonist [^{3}H]raclopride and the novel CB_1 inverse agonist [^{125}I]SD7015

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Abstract

Cannabinoid type-1 receptors (CB_1Rs) modulate synaptic neurotransmission by participating in retrograde signaling in the adult brain. Increasing evidence suggests that cannabinoids through CB_1Rs play an important role in the regulation of motor activities in the striatum. In the present study, we used human brain samples to examine the relationship between CB_1R and dopamine receptor density in case of Parkinson's disease (PD).

Post mortem putamen, nucleus caudatus and medial frontal gyrus samples obtained from PD patients were used for CB_1R and dopamine D_2/D_3 receptor autoradiography. [^{125}I]SD7015, a novel selective CB_1R inverse agonist, developed by a number of the present co-authors, and [^{3}H]raclopride, a dopamine D_2/D_3 antagonist, were used as radioligands. Our results demonstrate

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unchanged CB_1R density in the putamen and nucleus caudatus of deceased PD patients, treated with levodopa (L-DOPA). At the same time dopamine D_2/D_3 receptors displayed significantly decreased density levels in case of PD putamen (control: 47.97 ± 10.00 fmol/g, PD: 3.73 ± 0.07 fmol/g (mean ± SEM), $p < 0.05$) and nucleus caudatus (control: 30.26 ± 2.48 fmol/g, PD: 12.84 ± 5.49 fmol/g, $p < 0.0005$) samples. In contrast to the putamen and the nucleus caudatus, in the medial frontal gyrus neither receptor densities were affected.

Our data suggest the presence of an unaltered CB_1R population even in late stages of levodopa treated PD. This further supports the presence of an intact CB_1R population which, in line with the conclusion of earlier publications, may be utilized as a pharmacological target in the treatment of PD. Furthermore we found discrepancy between a maintained CB_1R population and a decreased dopamine D_2/D_3 receptor population in PD striatum. The precise explanation of this conundrum requires further studies with simultaneous examination of the central cannabinoid and dopaminergic systems in PD using higher sample size.

Keywords

Parkinson’s disease; Endocannabinoid CB1 receptor; Dopamine D_2/D_3 receptor; Molecular imaging biomarker; Human brain autoradiography; Striatum

1. Introduction

The endocannabinoid (EC) system is commonly described as a neuromodulatory system that interacts with and regulates the functions of many neurotransmitter systems, including cholinergic (Ach), dopaminergic (DA), serotoninergic, adrenergic, opiate, glutamatergic and GABAergic systems [29,58,62]. The main contribution of ECs to the control of synaptic neurotransmission is to act as retrograde messengers through type 1 cannabinoid receptors (CB_1R) [44,105]. Presynaptic CB_1Rs are abundant in the adult mammalian brain [29]. CB_1Rs are coupled to Gi/o proteins and, under specific conditions to Gs proteins [35,48,69]. CB_1Rs regulate the activity of various plasma membrane proteins and signal transduction pathways, including ion channels, context-dependent recruitment of second messengers (Erks, STATs, etc.) and various kinases. In addition, CB_1Rs activate G protein-independent pathways, as well [12,23,75]. Among various other functions, endocannabinoids have neuromodulatory functions, as well, [47] and play an important role in long term potentiation (e.g. [66]).

Multiple levels of evidence suggest that ECs have a potential to protect neurons under chronic degenerative conditions via CB_1R-dependent and -independent mechanisms [19,35,73,78,95]. An increasing number of studies have demonstrated that CB_1R density and binding is altered in the extrapyramidal system of humans in e.g., Huntington disease and PD [16,20,36,50,63,70,89,95].

However, the observed alterations in CB_1R’s in various neurodegenerative diseases, such as HD or PD, may be of diverse origins. The GABAergic spiny neurons (MSNs) are the most populous neuronal cell type of the striatum (90–95% in rats and over 85% in humans), along with several small populations of interneurons [53,104]. CB_1Rs are primarily expressed my

MSNs. The HD brain is characterized by loss of the MSNs of the striatum, which results in robust down-regulation of CB_1Rs. A severe loss of CB_1R's in the striatum has, consequently, been described as a landmark of HD [8,82]. On the other hand, the alteration of striatal CB_1R population in PD is full of controversies and the most important striatal cells expressing the CB_1R are affected in a lesser extent compared to HD and the EC systems shows a strong tendency for reorganization [18,84].

It is well known that a progressive degeneration of the dopaminergic system, especially the dopaminergic neurons of the substantia nigra pars compacta (SNc) [17,24], underlies the pathogenesis and clinical manifestations of PD. The decrease in striatal dopamine (DA) alters the regulation of synaptic dopamine levels, and dopamine receptor density and functional state [6,7,11,14,27,65,76,86,94,96]. Alterations in basal ganglia CB_1R density or EC levels have been described in rat models of PD on the basis of which a strong functional connection between the striatal dopamine and endocannabinoid systems has been hypothesized [63,107]. However, experimental models in small animals are inconclusive regarding the direction of changes of CB_1R density in Parkinson models: whereas there is evidence for the decrease of CB_1Rs in the striatum, as a consequence of 6-hydroxydopamine-induced nigrostriatal terminal lesion in rats [101]; studies using postmortem human PD brain samples, 6-hydroxydopamine (6-OHDA) or reserpine-treated rat models of PD, MPTP-lesioned marmoset and mouse mutant models of PD indicate an up-regulation [10,30,63,70,80,89], no change [45,68,89,107] or down-regulation [50,95] of CB_1Rs in Parkinson's disease.

In order to investigate changes in CB_1R: D_2/D_3 balance in PD in the human basal ganglia, we explore correlative alterations in dopamine D_2/D_3 receptors, key players in the disease process [52,61,93], and the alteration in CB_1R using selective radioligands – [^{3}H]raclopride [39,59], [^{125}I]SD7015 [22] – in brain tissues obtained from PD and age-matched control subjects.

2. Material and methods

2.1. Radioligand and chemicals

The detailed synthesis procedure of [^{125}I]SD7015 has been recently described elsewhere [22,21]. The specific radioactivity of the ligand was 2175 Ci/mmol. [^{3}H]raclopride was obtained commercially (specific activity 87 Ci/mmol, PerkinElmer Life Sciences Inc.). TRIS-HCl, bovine serum albumin (BSA), pargyline hydrochloride (selective MAO-B inhibitor), GBX Developer and Fixer Twin Pack were from Sigma–Aldrich (Budapest, Hungary), rimonabant (CB_1R antagonist) was from Cayman Chemicals (Michigan, USA). Other chemicals were from commercial suppliers and at the analytical grade.

2.2. Brain tissue and processing

Putamen tissues (n = 2) were collected and stored in the human brain bank of the Department of Anatomy, Semmelweis University, Budapest (age: 72 and 74, post mortem time: 2 and 4 h). Nucleus caudatus (n = 3) and medial frontal gyrus (n = 3) specimens were obtained from The Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience,

Amsterdam (age of death: age: 68, 74 and 77, postmortem delay: 3, 7 and 8 h). The causes of death were dehydration, pneumonia leading to septic shock, uremic coma, terminal circulatory and respiratory failure. All materials have been collected from donors for or from whom written informed consent for brain autopsy had been obtained. The study was approved by the Local Ethics Committee of the University of Debrecen (protocol number: DEOEC RKEB/IKEB M2547a-2006).

Age matched control samples of the same regions were obtained from deceased subjects (*n* = 5) with no documented history of neurological or psychiatric disorders. Neuropathological examination excluded any pathological finding in case of controls whereas all PD cases show advanced Lewy body disease pathology with severe neocortical involvement, consistent with Braak alpha-synuclein stage 5 or 6. The PD patients underwent long-time levodopa treatment (14 ± 6 years), started between 3 and 5 years after first diagnosis. Out of five at two patients we do not have exact data about disease duration and/or about treatment duration. Despite this fact, since these specimens were showing results concordant with those obtained in the specimens from patients with well know medical records, we included them in the final analysis.

Frozen brain samples were sectioned at 20 μm thickness using a cryomicrotome (Leica, CM 1850) at −20 °C. Sections were thaw mounted onto glass slides, air dried and stored at −20 °C for later use.

2.3. CB_1R autoradiography with [^{125}I]SD7015

Consecutive tissue sections in duplicates were used from the nucleus caudatus, putamen and medial frontal gyrus. Tissue were incubated with [^{125}I]SD7015 (40 pM) using 0.174 mCi/ml RA concentration for 60 min in a TRIS buffer (50 mM, pH 7.4) containing sodium chloride (120 mM), potassium chloride (5 mM), calcium chloride (2 mM), magnesium chloride (1 mM), ascorbic acide (0.1% w/v), 10 μM pargyline and bovine serum albumin (BSA 0.1%). Non-specific binding was determined in the presence of 10 μM rimonabant (CB_1R antagonist) [22]. The sections were then washed in the same buffer three times for thirty minutes each time and briefly dipped in ice cold distilled water. The sections were dried on a warm plate and afterwards exposed to γ-radiation sensitive film (Kodak Biomax MS, Sigma–Aldrich, Budapest, Hungary) for 24 h. Autoradiograms were digitized using a high-resolution scanner (Epson Perfection V750 Pro). Adobe Photoshop CS2 software was used for measurements and image processing. ^{14}C-calibration scales (American Radiolabelled Chemicals Inc, St Louis, MO, USA) were used for quantification as described by Baskin and Wimpy [5]. Briefly, radioactivity of the [^{14}C] plastic standards, supplied by the manufacturer, was transformed in tissue equivalent concentrations of [^{125}I] expressed as disintegrations per minute per mm^2 (DPM/mm^2). The transformation was based on the following quadratic polynomial equation:

$$y([^{125}\mathrm{I}]\mathrm{DPM/mm}^2) = -26.798 + 104.261x - 1.295x^2$$

where *x* values represent [^{14}C] radioactivity in μCi/g plastic. Mean pixel values were converted to DPM/mm^2 applying the obtained [^{125}I] DPM/mm^2 values of the scale. Taking

into consideration the standard specific radioactivity value of the radioligand (2200 Ci/mM), the 1 DPM = 451 fCi conversion factor and the slide thickness (20 μm), DPM/mm^2 values were converted into fmol/g_tissue concentration of the radioligand bound to the receptors, i.e. the radioligand's specific binding value. Statistical analysis was performed with Student's *t*-test (two tailed, unequal variance). A $p < 0.05$ value was considered statistically significant.

2.4. Dopamine D_2/D_3 receptor autoradiography with [^{3}H]raclopride

Autoradiography with [^{3}H]raclopride was performed as described earlier [41,42]. [^{3}H]raclopride was obtained commercially (specific activity 87 Ci/mmol, PerkinElmer Life Sciences Inc.). After 30 min of pre-incubation at room temperature in TRIS buffer containing 20 mM Hepes (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid), pH 7.4, 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM $MgSO_4$, and 10 mM NaOH, the sections were incubated for 15 min at room temperature in Krebs-Ringer-HEPES (KRH) buffer (HEPES 10 mM, pH 7.4, KCl 4.7 mM, $CaCl_2$ 2.2 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, NaCl 120 mM, dextrose 10 mM), supplemented with 0.05 mg/mL BSA (bovine serum albumin) and 1 nM [^{3}H]raclopride. *Nonspecific binding* was determined in the presence of 10 μM *cold raclopride* [40]. The measurements were made in duplicates. The washing of labeled sections was carried out for 20-min in the ice-cold TRIS buffer (without ligand), followed by a brief dipping in ice-cold distilled water to remove salts. Finally, the sections were dried under a stream of cold air.

Subsequently the readings, using tritium sensitive phosphorimager plates (Fujifilm Plate BAS-TR2025) were made in a Fujifilm BAS-500 phosphorimager (90 min) (Fujifilm, Tokyo). Quantitative densitometry was performed using Multi Gauge 3.2 phosphorimager software (Fujifilm, Tokyo). Autoradiographic [^{3}H] microscales (RPA510, Batch 18, GE Bioscience), placed alongside the brain tissue sections, were used to quantify the data and render the quantitative values in fmol/g_tissue.

Results of multiple measurements in the same region were averaged for each subject. These values were then used to calculate the mean specific binding of the radioligand. Specific binding of radioligands ([^{125}I]SD7015 and [^{3}H]raclopride) was calculated as difference between mean total (specific and non-specific) and mean blocked (non-specific) binding. Student's *t*-test (two-tailed, unequal variance) has been applied for statistical comparison between disease and control groups. A $p < 0.05$ value was considered statistically significant.

3. Results

In the present study we have performed autoradiography in control and PD brain specimens, with special regard to the nucleus caudatus and the putamen, using [^{125}I]SD7015 and [^{3}H]raclopride.

In Fig. 1, CB_1R autoradiography is shown in the three regions under investigation. We did not find a significant difference between specific binding values (total binding − non-

specific binding after block, expressed as fmol/g_tissue in the three regions of interest in PD and age matched control brains (Table 1).

In Fig. 2 dopamine D_2/D_3 autoradiographic images are shown in the three anatomical brain regions. We found significant differences between specific binding values measured in the putamen and nucleus caudatus in PD brains, relative to age matched controls. However, there was no significant difference between the specific binding values in the medial frontal gyrus (Table 2).

4. Discussion

We investigated the relationship of CB_1R and D_2/D_3 receptor densities in PD human brains by means of receptor autoradiography. [^{125}I]SD7015, a novel CB_1R agonist, [22] and [^{3}H]raclopride, a dopamine D_2/D_3 receptor antagonist [59], were applied as radioligands.

CB_1R densities in putamen, nucleus caudatus and frontal cortex samples seem to be unchanged in PD while in contrast, dopamine D_2/D_3 receptor density in PD putamen and nucleus caudatus decreases. The latter is in line with previous findings, namely this decrease of D_2/D_3 receptor density in PD putamen and nucleus caudatus could be the consequence of longterm antiparkinsonian treatment. It is generally agreed that dopaminergic denervation leads to striatal D_2 dopamine receptor up-regulation as postsynaptic compensatory mechanism in response to deficiencies in synaptic dopamine signaling [1,3,24,67,85]. Treatment of PD patients with dopaminergic drugs returns the striatal dopamine D_2 receptor expression to near normal levels [1,67,98]. Frontal cortex samples presented no difference between the subject cohorts.

The CB system in experimental PD models and PD patients has been extensively studied, yet with contradictory conclusions. In rat Parkinson models (reserpine treatment or 6-OHDA-lesion models) an increase in endogenous endocannabinoid levels was observed in the striatum [19,26,38,68] as it was also observed in the CNS of 16 untreated PD patients [83]. Other authors found significantly altered CB_1R mRNA expression in animal models of PD or in postmortem human PD brain specimens. [50,89,95,107]. Finally, changes in CB_1R binding sites [30,63,80] and activation of GTP-binding proteins in the basal ganglia of PD patients and of MPTP-treated marmosets were also reported [63]. On the other hand, using the 6-OHDA rat model Romero et al. [89] did not find significant changes in CB_1 receptor binding, measured by [^{3}H]WIN-55,212,2 autoradiography, or in the activation of signal transduction mechanisms, measured by WIN-55,212,2-stimulated [^{35}S]GTPgammaS binding autoradiography, between the lesioned and non-lesioned sides at the level of the lateral caudate-putamen, globus pallidus and substantia nigra.

In the present study we did not find changes in CB_1R densities in the striatum and frontal cortex of PD subjects. One of the explanations for the unaltered CB_1R density found by us could be the unchanged density of high affinity CB_1Rs in the investigated PD brain regions [63,89]. This may be possible due to the presence of the large reserve of CB_1Rs and their likely inter-conversion between low and high affinity states [4,94]. Due to low sample size results are only suggestive in the aspect of an intact CB_1R density. This statement requires

further justification in the future by studies using more specimens and performing the detailed investigation of this problem.

On the other hand, functional relationship have been reported between CB_1Rs and both dopamine D_1 and D_2 receptors [33,34,46,51,60,63,70,75,89, 99]. For instance, Giuffrida et al. [33] proved that striatal administration of D_2 agonist results in release of endocannabinoids. Furthermore, activation of CB_1 or dopamine D_2 receptors alone resulted in inhibition of cAMP accumulation whereas simultaneous activation of both receptors increased cAMP levels [34]. Kreitzer and Malenka [60] reported in animal models of Parkinson's disease that DA depletion blocked the generation of endocannabinoid-mediated long-term depression (eCB-LTD) in indirect striatal pathway but administration of dopamine D_2 receptor agonist together with inhibitors of endocannabinoid degradation rescued indirect-pathway eCB-LTDs and in vivo reduced parkinsonian motor deficits. Dopamine receptor antagonists inhibited cannabinoid induced striatal mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) activation [99].

Administration of CB_1R agonists increased dopamine turnover and release [15,31,32,87], excited dopaminergic neurons in the ventral tegmental area and substantia nigra [28], decreased the tremor associated with overactivity of the subthalamic nucleus and improved motor impairment [2,72,92], at the same time a CB_1 antagonist (rimonabant) alleviated hypokinesia in animal models of Parkinson's disease [37,54], probably effecting on the lateral globus pallidus. It has been demonstrated that glutamatergic and GABAergic terminals strongly express CB_1Rs and CB_1R agonists significantly inhibited glutamate and GABA release, however, cannabinoids in vitro, directly did not affect the release of dopamine [29,58,97]. Yin et al. [106] observed that presynaptic reduction in glutamate release was the consequence of a retrograde signal through eCBs; this eCB synthesis and release from the postsynaptic cell results from cooperating, convergent glutamate and dopamine inputs. A potential indirect dopamine–CB_1R interaction through the cannabinoid induced regulation of the upper mentioned neurotransmitters (GABA, glutamate) of striatal neuronal pathways could be the basis of cannabinoid effect on motor activity. However, the findings of [79] that dopaminergic cells also express CB_1R as well as observations about functional interactions between CB_1Rs and both dopamine D_1 and D_2 receptors [33,34,46,51,60,63,70,74,89,99]. could contribute to the upper mentioned effect as well. Due to the complexity of this cannabinoid–dopamine receptor conundrum further researches are required with well designed study protocols. Although our results base on relatively small sample size, they refer to the presence of an apparently intact CB_1 receptor population may be usable in PD therapy, even in advanced PD.

On the other hand, it is accepted that classical neuroinflammatory diseases such as multiple sclerosis present aspects of neurodegeneration, while classical degenerative disorders such as Alzheimer's disease, Parkinson's disease are demonstrably affected by inflammation [90]. In CNS CB_1 receptors exist in all types of neural cells, in astrocytes [9,91], microglia [100,103], and oligodendrocytes [77] whereas CB_2 receptors are expressed on cells of immune system and microglia [103]. Studies report neuroprotective [43,71,88] and anti-inflammatory effects through CB receptors [13,55,56,57,81,102]; CBs protected against dopaminergic cell death, as well [64]. Thus CB_1 and CB_2 receptors could provide substrate

for neuroprotective and anti-inflammatory actions of cannabinoids in neurodegenerative diseases, however, the effects through CB_1Rs are more relevant to neuroprotection, whereas CB_2Rs modulate the immune response primarily, although a potential overlap as well as non CB_1/CB_2-mediated mechanisms may exist [90].

In this study we used PD putamen, nucleus caudatus, medial frontal gyrus samples in order to correlate CB_1 receptor density with dopamine D_2/D_3 receptor density. Our results refer to an unchanged CB_1R and decreased dopamine D_2/D_3 receptor density in nucleus caudatus and putamen of PD patients whereas medial frontal gyrus sections did not show any alteration. Our data suggest that in case of long-term L-DOPA treatment and long disease progression CB_1R density does not fall under control levels, although, dopamine D_2/D_3 receptor density is significantly decreased. Various explanations could exist: (1) neurodegeneration induced affinity or sensitivity increase of 'reserve' CB_1Rs could compensate CB_1R density changes, (2) reactive changes of CB system could go along with PD progression, until more effective compensatory mechanisms come into action, (3) despite the decreased density, dopamine D_2 receptor signal transduction seems functionally intact [25]) and possible physiological interactions with CB_1 receptors could be maintained even in later stages of PD, which could result in unchanged CB_1R density; however, functional receptor crosstalks between these receptor types are, yet, unequivocally unproven. Nevertheless the combination up to various degree of the aforementioned or other, yet, unknown mechanisms is the most probable phenomenon.

We concluded that an intact CB_1R population could represent alternative target for treatment of PD; additionally it could open new perspectives in neuroprotection and anti-inflammatory therapy of this neurodegenerative disease. Better understanding and further exploration of central cannabinoid system and related questions need further in vivo and in vitro detailed designed studies, emphasizing study population/sample group homogeneity regarding to data about PD neuropathological stage, disease duration and L-DOPA substitution/dopamine agonist therapy. This is suggested since one of the limitation of this study is the low sample number and consequently the questionable reliability of statistical calculations on these data. By the parallel investigation of CB_1 and DA $D_{2/3}$ receptors in PD striatum we wished to base and pioneer future researches aiming this field of neuroscience.

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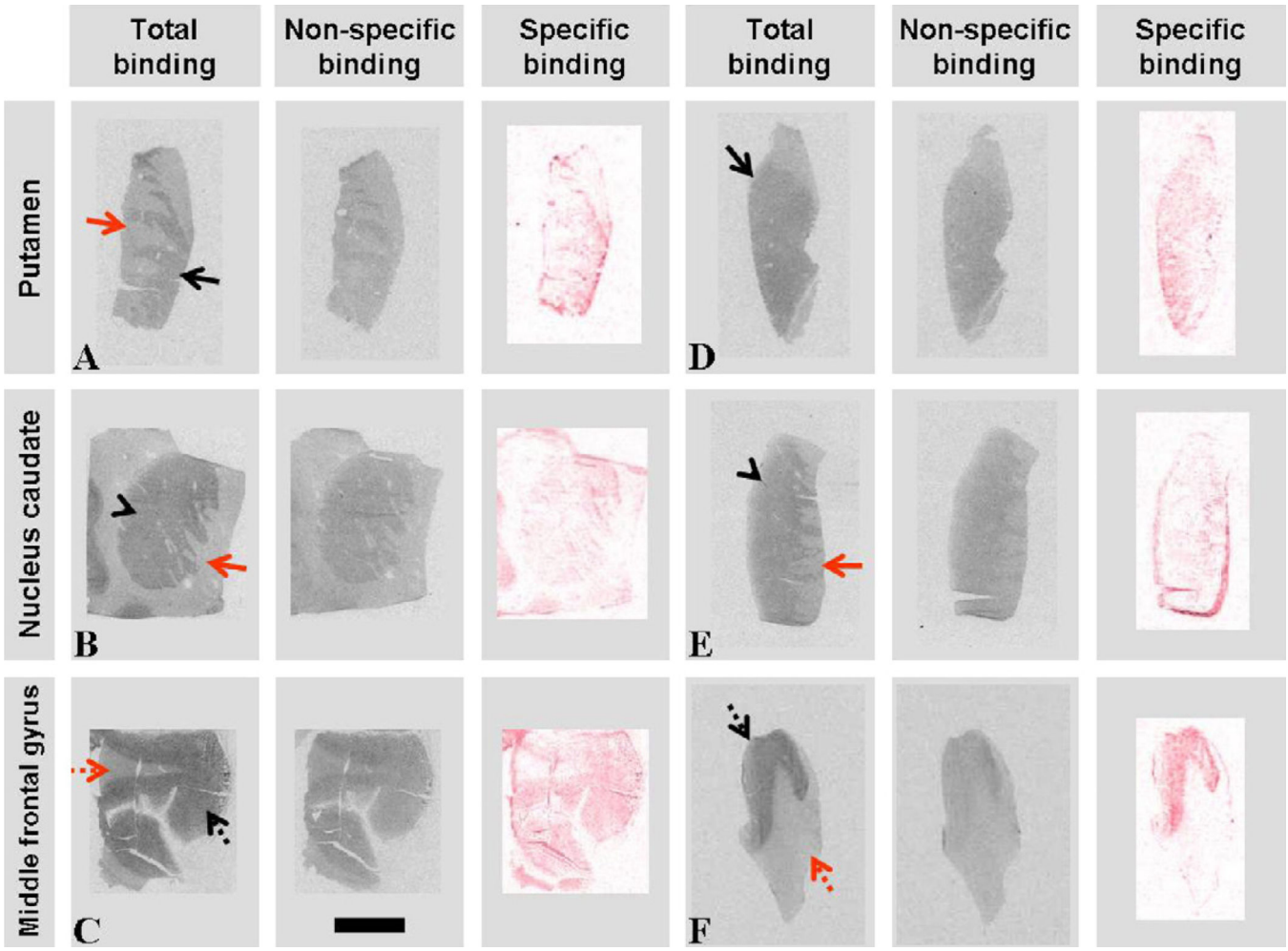

Fig. 1.

Images of CB_1R receptor autoradiography obtained in control (A–C) and PD (D–F) putamen (black arrow), nucleus caudatus (black arrow head) and medial frontal gyrus sections. Red arrow, internal capsule; dotted black arrow, cortex; dotted red arrow, white matter. Specific binding represents subtracted, red weighted images of total binding − non-specific binding. Scale bar indicates 10 mm.

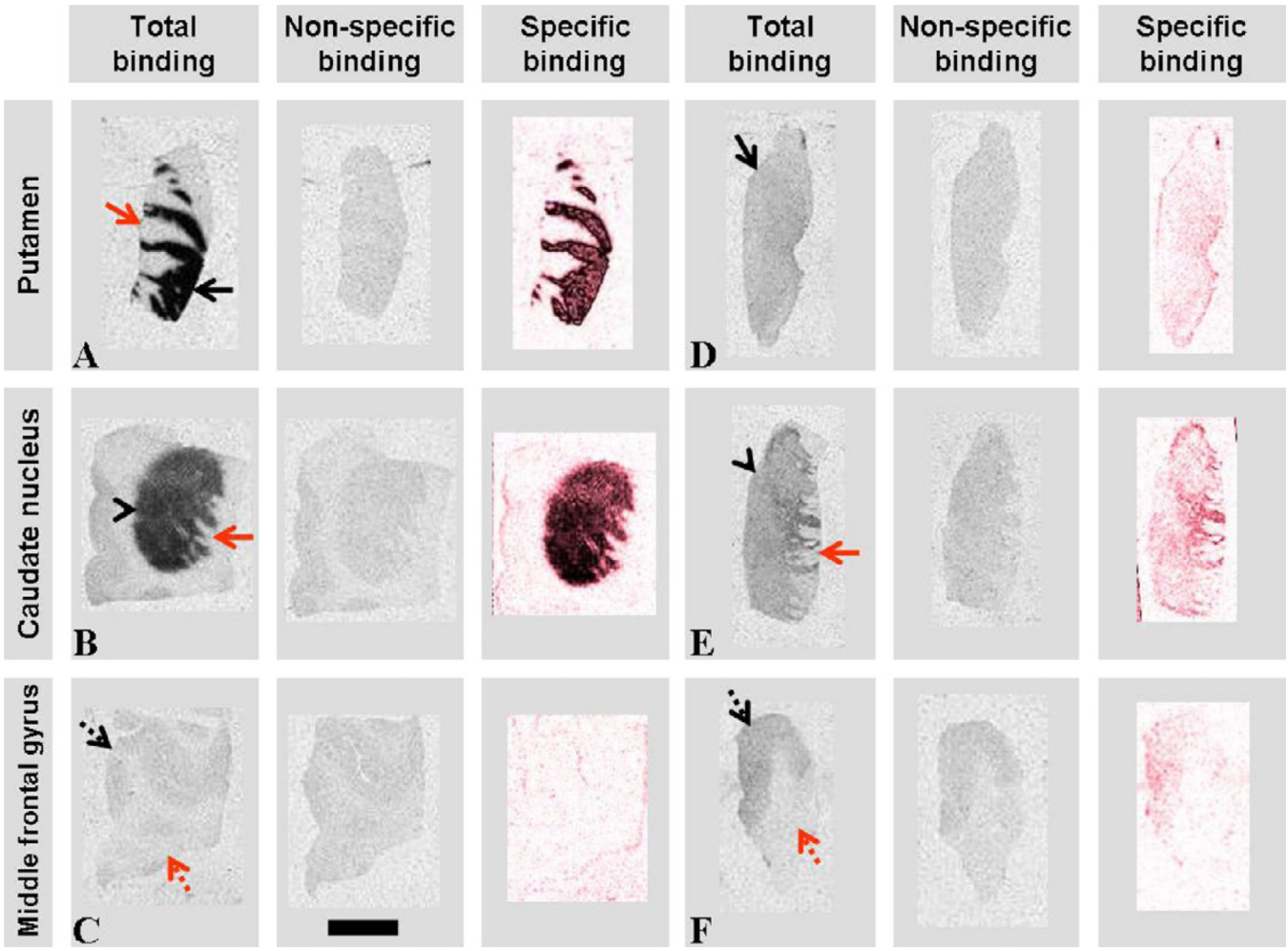

Fig. 2.

Images of D_2/D_3 receptor autoradiography obtained in control (A–C) and PD (D–F) putamen (black arrow), nucleus caudatus (black arrow head) and medial frontal gyrus sections. Red arrow, internal capsule; dotted black arrow, cortex; dotted red arrow, white matter. Specific binding represents subtracted, red weighted images of total binding −non-specific binding. Scale bar indicates 10 mm.

Table 1

CB_1R density expressed as specific binding values of [^{125}I]SD7015 to CB_1Rs (fmol/g_tissue). There was no significant difference between the average values belonging to control and PD brains in any region. Data are expressed as mean ± SEM; *n*: number of subjects. Measurements in all brains were made in duplicates.

Region	Control		PD	
Putamen	157.54 ± 25.52	*n* = 5	180.30 ± 36.80	*n* = 2
Nucleus caudatus	170.77 ± 15.89	*n* = 3	187.58 ± 21.22	*n* = 3
Medial frontal gyrus	263.84 ± 41.51	*n* = 3	286.70 ± 25.83	*n* = 3

Table 2

Dopamine D_2/D_3 receptor densities expressed in fmol/g_tissue. There was a significant difference between the average values belonging to control and PD brains in the putamen and the nucleus caudatus, whereas no significant difference was present in the medial frontal gyrus. Data were expressed as means ± SEM; *n*: number of subjects. Measurements in all brains were made in duplicates.

Region	Control		PD	
Putamen	47.97 ± 10.00	*n* = 2	3.73 ± 0.07	*n* = 2
Nucleus caudatus	30.26 ± 2.48	*n* = 3	12.84 ± 5.49	*n* = 3
Medial frontal gyrus	3.29 ± 2.50	*n* = 3	4.33 ± 1.45	*n* = 3