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Short communication

Dopaminergic drug-induced modulation of the expression of the dopamine transporter in peripheral blood lymphocytes in Parkinson's disease

Alessandra Fanciulli[1,2], Roberta Misasi[3], Dario Campanelli[2], Francesca R. Buttarelli[2], Francesco E. Pontieri[1,2]

[1]Movement Disorder Unit, "Sant'Andrea" Hospital, [2]Department of Neurology and Psychiatry, "Sapienza" University, [3]Department of Experimental Medicine, "Sapienza" University, Rome, Italy

Correspondence: Francesco E. Pontieri, e-mail fe.pontieri@gmail.com

Abstract:
The modulation of expression of the dopamine transporter by dopaminergic drugs was investigated by flow cytometry in peripheral blood lymphocytes from patients suffering Parkinson's disease. An 8-week *in vivo* exposure to pramipexole (0.7 mg free base, 3 times a day) or ropinirole (12 mg, once daily), but not levodopa/carbidopa (100/25 mg, 3 times a day), significantly reduced the mean fluorescence intensity of the dopamine transporter in peripheral blood lymphocytes. These results demonstrate that levodopa differs from dopamine agonists in its regulation of dopamine transporter expression in peripheral blood lymphocytes.

Key words:
dopamine transporter, levodopa, Parkinson's disease, peripheral blood lymphocytes, pramipexole, ropinirole

Abbreviations: DAT – dopamine transporter, LD – levodopa, PBL – peripheral blood lymphocytes, PD – Parkinson's disease, PPX – pramipexole, RP – ropinirole

Introduction

The dopamine transporter (DAT) is an 80-kD plasma membrane glycoprotein located at presynaptic dopaminergic terminals in the nervous system that re-uptakes dopamine from the extracellular matrix [8]. The progressive degeneration of dopaminergic neurons in the brain is the pathological landmark of Parkinson's disease (PD) [12], and experimental models of parkinsonism [21]. Functional neuroimaging methods using radioligands to DAT allow *in vivo* identification of the dopaminergic nigrostriatal damage in PD [22] and have been applied as instrumental surrogate markers of disease progression in clinico-pharmacological trials [15, 16].

In recent years, a number of studies have focused on the modulation of DAT expression during PD progression. In the prodromal and early stages of PD, the down-regulation of DAT occurs as a compensatory mechanism aimed at increasing extracellular dopamine concentrations [12]. With disease progression, however, dopamine replacement therapy with dopamine agonists or levodopa (LD) becomes mandatory [12]. Despite the symptomatic nature of these drugs, it is unclear if chronic exogenous dopaminergic stimu-

lation influences disease progression by affecting synaptic dynamics in terms of receptor sensitivity, endogenous dopamine release, or dopamine re-uptake by DAT. Several studies have dealt with the regulation of DAT expression by dopaminergic drugs. Recent findings from animal studies show that chronic or sub-chronic exposure to dopaminergic drugs may alter DAT levels [20]. Studies conducted with functional neuroimaging techniques in humans, however, led to rather contradictory conclusions depending on the methodology and radioligand applied [9, 19].

In addition to its action on the nervous system, dopamine is involved in neural-immune interactions. A complete dopaminergic system has been characterized in human peripheral blood lymphocytes (PBL) [3]. Previous studies from our group helped to define the alterations of the PBL dopaminergic system in PD. Interestingly, reduced DAT immunoreactivity has been measured in PBL from PD patients compared to that of healthy subjects [4, 17], or patients suffering essential tremor [17]. These previous reports suggested that measurement of DAT expression in PBL may provide a useful, easily accessible, and cost-effective tool to investigate *in vivo* the effects of dopaminergic drugs on DAT expression. Given these premises, we measured DAT mean fluorescence intensity by flow cytometry in PBL from PD patients before and 8 weeks after initiation of dopamine replacement therapy with either LD, pramipexole (PPX), or ropinirole (RP).

Materials and Methods

Twenty-five drug-naive PD patients were consecutively recruited at the movement disorder outpatient service of our institution between March 2008 and September 2009. The demographic and clinical features of the included subjects are summarized in Table 1. All patients fulfilled the international diagnostic criteria for probable PD [6]. In all cases, the positive response to dopaminergic therapy, measured by a > 20% reduction of the Unified Parkinson's Disease Rating Scale (UPDRS) – part III score [5], was verified during follow-up. All patients were on stage 1–2 of the modified Hoehn and Yahr scale [10] at the time of enrollment. The exclusion criteria were as follows: *i*. Age < 40 or > 80 years; *ii*. Mini Mental State Examination score < 26; *iii*. Laboratory values outside the appropriate reference interval; *iv*. Major medical illnesses (diabetes, obstructive pulmonary disease or asthma, hematologic disorders, neoplasms, and clinically significant and unstable active gastrointestinal, renal, hepatic, endocrine, or cardiovascular diseases); *v*. Known or suspected history of immunologic disorders; *vi*. Co-morbidity with primary psychiatric or neurological disorders, such as schizophrenia, major depression, stroke, Alzheimer's disease, and head trauma with loss of consciousness, *vii*. Known or suspected history of alcoholism, drug dependence or abuse; *viii*. MRI evidence of significant focal abnormalities; *ix*. Ongoing chemo- or radiotherapy; or *x*. Peripheral or central dopamine antagonist therapy. The study was approved by the local ethical committee, and each subject signed an informed consent before inclusion.

At the time of enrollment (i.e., before starting dopaminergic drug therapy), a venous blood sample (approximately 15 ml) was drawn from a peripheral vein into glass tubes containing EDTA. A second blood sample was drawn after 8 weeks (56 days) of therapy with either LD/carbidopa (100/25 mg, 3 times a day, n = 8), PPX (0.7 mg free base, 3 times a day, immediate release formulation, n = 8) or RP (12 mg, once daily, prolonged release formulation, n = 9). For each subject, the treatment choice was based on the international therapeutic guidelines for the early stages of PD [11].

PBL were isolated from each blood sample by Ficoll-Hystopaque density gradient centrifugation, washed twice with Gibco AIM-V medium (Invitrogen, Italy), frozen in freezing medium containing 90% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO), and cryo-preserved in liquid nitrogen until use. Frozen isolated PBL were cultured (1×10^6 cells/ml) in 25 cm^2 flasks with AIM-V medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine, and activated with lipopolysaccharide (LPS) (10 mg/ml) (Sigma, USA) and interleukin (IL)-2 (0.2 ng/ml = 2 U/ml) (Peprotech, USA) for 18 h before staining.

PBL were then fixed in 4% paraformaldehyde for 25 min at RT, followed by permeabilization in digitonin 60 μM (in PBS, 5% FCS) for 10 min at RT. DAT expression was determined by double indirect immunostaining using a rat anti-DAT N-terminus primary antibody (MAB369 Chemicon International, USA, 1:400 diluted), a rabbit anti-rat secondary antibody (R3756 Sigma, USA, 1:500 diluted) and a FITC-conjugated goat anti-rabbit antibody (F0382 Sigma-Aldrich, USA, 1:50 diluted). The mean fluorescence

intensity was measured by flow cytometric analysis (EPICS XL-MCL cytometer, Coulter Electronics, USA) by subtracting the cellular auto fluorescence and the primary antibody's non-specific binding.

Differences in the demographic or clinical parameters among the three groups were analyzed for significance by a one-way analysis of variance followed by a *t*-test using the Bonferroni correction for multiple comparisons. For the statistical analysis of DAT fluorescence intensity, the data were normalized and expressed as the percent variation of the mean fluorescence intensity of the second sample compared to the first one. Student's *t*-test was performed to compare pre- and post-treatment values in each group independently. All statistical tests were conducted using MedCalc 9.3.7.0.

Results

Patients from the 3 treatment groups had similar demographic features, with the exception of the significantly higher age in the LD-treated group (Tab. 1). Such a difference reflects the current international guidelines for therapy of early PD [11], in particular the indication to preferentially use dopamine agonists in patients younger than 70 years.

The results of the study are summarized in Table 1. Briefly, the basal levels of DAT in PBL, measured as mean fluorescence intensity, were lower in the LD-treated group with respect to the dopamine agonist-treated group.

Tab. 1. Demographics of subjects enrolled and DAT immunoreactivity in PBL

Feature	LD (n = 8)	PPX (n = 8)	RP (n = 9)
Sex (M/F)	6/2	5/3	6/3
Age (years)	71 ± 2	62 ± 2#	59 ± 2#
Disease Duration (months)	17 ± 5	16 ± 5	16 ± 4
H&Y score	1.5 ± 0.2	1.6 ± 0.1	1.6 ± 0.1
DAT – Pre	1.47 ± 0.31	1.81 ± 0.41	1.72 ± 0.27
DAT – Post	1.39 ± 0.29	1.06 ± 0.34**	1.32 ± 0.22*

Data are represented as the mean ± SEM. H&Y = Hoehn and Yahr. # $p < 0.05$ different from LD, Bonferroni-corrected *t*-test; * $p < 0.05$ different from DAT – Pre; ** $p < 0.01$ different from DAT – Pre, Student's *t*-test statistic

An 8-week treatment with LD did not produce a significant difference in the mean fluorescence intensity of DAT in PBL (mean Δ = –5%, range –16/+13%). Conversely, an 8-week treatment with the dopamine agonists was accompanied by a significant reduction in the mean fluorescence intensity of DAT in PBL (mean Δ = –33%, range –8/–85%, $p < 0.05$ Student's *t*-test). PPX caused a more robust reduction of the mean fluorescence intensity of DAT in PBL (mean Δ = –39%, range –10/–85%, $p < 0.01$, Student's *t*-test) than RP (mean Δ = –26%, range –8/–71%, $p < 0.05$, Student's *t*-test).

Discussion and Conclusions

The results of the present study demonstrate the different effects of dopamine agonists and LD on DAT expression, measured as mean fluorescence intensity, in PBL from PD patients. In particular, despite the small number of subjects enrolled, an 8-week exposure to either PPX or RP significantly decreased the DAT mean fluorescence intensity in PBL, whereas exposure to LD did not modify the PBL DAT mean fluorescence intensity (Tab. 1).

The lack of effect of LD on the DAT mean fluorescence intensity in PBL from PD patients measured herein is consistent with previous observations using functional neuroimaging methods [19] to investigate the expression of DAT in the striatum of PD patients. Together with those previous findings, therefore, our results indicate that sub-chronic exposure to LD does not modify DAT expression at central as well as peripheral level in PD patients.

The reduction of DAT expression in PBL from subjects treated with dopamine agonists is partially consistent with the previous report of the slight reduction of the ^{11}C-RTI-32 PET signal following a 6-week treatment with PPX in early PD patients [9]. The specificity of the effect of PPX and RP with respect to LD suggests that the down-regulation of DAT at the peripheral level measured here is a direct consequence of exposure to dopamine agonists rather than test-retest or inter-test variability. Moreover, the more robust reduction of PBL DAT expression by PPX with respect to RP might be related to the higher affinity of PPX to D_3 receptors [7], and to the high density of D_3 receptors on PBL plasma membrane [14, 18].

Moreover, the significant difference in age among the groups might have contributed to the results. However, the DAT mean fluorescence intensity in the LD group was slightly, but not significantly, lower than that measured in PPX or RP groups (Tab. 1), further suggesting that age-dependent reduction of dopaminergic function occurs in human PBL, as demonstrated previously for dopamine receptors [1]. In order to limit the influence of age on the results, the data were normalized for each subject. However, further studies with larger sample size are needed to define this issue.

The mechanisms underlying the down-regulation of DAT expression in PBL by dopamine agonists are currently unknown. Indeed, previous studies have suggested that there is differential pathophysiological regulation of DAT expression at the central rather than the peripheral level in PD [2]. Given the previous evidence that PBL synthesize dopamine and express dopamine receptors and DAT on their plasma membrane [3], however, one can hypothesize that stimulation of the PBL dopamine receptors by PPX or RP might down-regulate dopamine synthesis, liberation, and re-uptake. Consequently, this would cause the down-regulation of DAT expression by negative feed-back in the PBL in the same manner as occurs in dopaminergic neurons [13]. Conversely, stimulation of the PBL dopamine receptors by exogenous LD or dopamine synthesized from exogenous LD might be coupled with the active re-uptake mechanism of the transmitter in PBL, thus requiring the physiological expression of DAT. If this were the case, despite different physiological mechanisms of the regulation of DAT expression in PBL compared to the nervous system, the processes underlying drug-induced modulation of DAT expression in PBL might resemble those occurring at presynaptic terminals in the central nervous system [13]. Alternatively, the different effects of the dopamine agonists and LD might depend on the pharmacokinetic behavior of the drugs. In particular, LD has a much shorter plasma half-life than that of the dopamine agonists, therefore producing a pulsatile stimulation of the PBL dopamine receptors that might contribute to differential modulation of DAT expression. However, further studies are advised to define this issue.

This study has some limitations: the lack of a control (non-PD) group did not allow confirmation of the reduction of DAT immunoreactivity with the current technique (flow cytometry) in PBL from PD patients identified previously by immunocytochemistry [2, 3, 17]. However, we would like to emphasize that the primary aim of the present study was to identify the modulation of DAT expression in PBL from PD patients by dopaminergic drugs, not to confirm the reduction of DAT expression measured previously. With regard to the latter issue, preliminary findings from ongoing studies in our laboratory suggest that the reduction in the DAT expression in PBL from PD patients may also be identified using flow cytometry. Furthermore, the significant difference in age among the groups might have contributed to the present results. However, the DAT mean fluorescence intensity in the LD group was slightly, but not significantly, lower than those measured in the PPX or RP groups (Tab. 1). This suggests that age-dependent reduction of dopaminergic function occurs in human PBL, as was demonstrated previously for dopamine receptors [1]. To limit the influence of age on the results, the data were normalized for each subject. However, further studies with a larger sample size and comparable age are needed to define this issue.

In conclusion, the dopaminergic-drug induced modulation of DAT expression in PBL measured herein suggests that flow cytometric measurement of DAT immunoreactivity may represent a useful and manageable approach to study the effects of anti-parkinsonian drugs on a dopaminergic marker in peripheral cells. Moreover, the present evidence of the modification of DAT expression in PBL following sub-chronic exposure to dopamine agonists suggests that caution is necessary in applying functional neuroimaging methods with striatal DAT as an endogenous target of radioligands as an instrumental marker for disease progression in PD. Indeed, the present results suggest that changes in expression of striatal DAT might occur with dopaminergic treatment, thus interfering with the reliability of DAT binding imaging studies in neuroprotection.

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