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Striatal Neurotransmitter Release-related Presynaptic Proteins in L-dopa Induced Dyskinesia in a Model of Parkinsonism

Parkinsonizm Modelinde L-dopa Diskinezisine Eşlik Eden Striatal Presinaptik Nörotransmitter Salıverilmesi ile ilişkili Proteinler

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ABSTRACT

Introduction: In Parkinson's disease, L-dopa-induced dyskinesia (LID) and motor fluctuations incapacitate patients as much as the disease itself. Many studies demonstrated that postsynaptic alterations and striatal synaptic plasticity changes play a role in LID development. Here, we aimed to study the role of striatal presynaptic proteins in LID pathogenesis.

Methods: For this purpose, 6-hydroxydopamine model of parkinsonism was used. To induce LID, these rats were treated with intraperitoneal injections of L-dopa 25 mg/kg with benserazide 6.25 mg/kg b.i.d for 21 days. Rats with parkinsonism treated with saline and control rats treated with saline or L-dopa/ benserazide were also included. Behaviors of rats were videotaped and scored according to dyskinesia scale. Striatal tissue was analysed with immunofluorescence staining and immunoblotting to confirm loss of tyrosine hydroxylase (TH) expression due to dopaminergic denervation and to explore the alterations in the expression of presynaptic proteins, secretogranin 2 (SG2), synaptophysin (Syp) and synaptotagmin 7 (Syt7).

Result: LID developed only in rats with parkinsonism treated with chronic L-dopa. Immunofluorescence and immunoblotting studies for TH confirmed depletion of dopaminergic neurons, which was also strongly and negatively correlated with severity of LID. Striatal SG2 and Syp levels were found increased in parkinsonian rats. Chronic L-dopa treatment further increased SG2 levels in denervated striatum. Striatal SG 2 level showed a significant moderate, positive correlation with LID severity. Immunofluorescence studies also demonstrated increased expression of these presynaptic proteins in the denervated striatum.

Conclusion: As, severity of LID was clearly correlated with striatal SG2 expression; there is supposedly a functional relationship between striatal SG2 and LID. Further studies are needed to find out molecular mechanisms linking increased SG2 expression and LID.

Keywords: Parkinson's disease, motor fluctuations, synaptic plasticity, secretogranin 2, striatum

ÖZ

Amaç: Parkinson hastalığında, L-dopa'ya bağlı gelişen diskineziler (LID) ve motor dalgalanmalar hastalar için hastalığın kendisi kadar zorluk oluştururlar. Çok sayıda çalışmada LID'ye postsinaptik değişikliklerin ve striatal sinaptik plastisite değişikliklerinin neden olduğu gösterilmiştir. Bu çalışmada LID patogenezine dair yeni bilgilerin ortaya konması ve striatal presinaptik proteinlerin patogeneze katkısının incelenmesi hedeflendi.

Yöntem: Bu amaçla, sıçanlarda 6-hidroksidopamin ile oluşturulan deneysel parkinsonizm modeli kullanıldı. Bu sıçanlara 21 gün boyunca günde 2 kez L-dopa 25 mg/kg ve 6.25 mg/kg benserazid tedavisi intraperitoneal olarak uygulanarak LID gelişimi sağlandı. Çalışmaya ayrıca parkinsonizmi olan ve kronik serum fizyolojik (SF) tedavisi verilen ve lezyon oluşturulmamış olan ve kronik L-dopa veya SF tedavisi verilen sıçanlar alındı. Sıçanların tedavi sonrası davranışları video kayıtlarıyla incelenerek diskinezi skalasına göre skorlandı. Deneylerin bitiminde striatal doku örneklerinde, dopaminerjik deplesyonu değerlendirmek için tirozin hidroksilaz (TH), presinaptik proteinlerden sekretogranin 2 (SG2), sinaptofizin (Syp) ve sinaptotagmin 7 (Syt7) ifadelerindeki değişiklikler immünoflöresan ve immünoblotlama yöntemleriyle incelendi.

Bulgular: LID gelişimi parkinsonizmi olan ve kronik L-dopa tedavisi verilen sıçanlarda görüldü. TH immünoflöresan ve immünoblotlama çalışmaları ile dopaminerjik deplesyonun varlığı doğrulandı ve deplesyon düzeyinin LID şiddetiyle yüksek düzeyde ve negatif yönde korelasyon gösterdiği izlendi. Parkinsonizmi olan sıçanlarda striatal SG2 ve Syp düzeylerinin arttığı, SG2 düzeylerindeki artışın kronik L-dopa tedavisi alan sıçanlarda daha da belirgin hale geldiği izlendi. LID şiddetiyle striatal SG2 düzeyleri arasında orta düzeyde, anlamlı pozitif korelasyon saptandı. İmmünoflöresan çalışmalarda da bu presinaptik proteinlerin ifadelerinin denerve striatumda artış gösterdiği izlendi.

Sonuç: LID şiddetiyle striatal SG2 düzeyleri arasında saptanan anlamlı korelasyon, striatal SG2 ile LID gelişimi arasında fonksiyonel bir ilişki bulunabileceğini ortaya koymuştur. SG2 ifadesindeki artış ile LID arasındaki bağlantının moleküler mekanizmalarının aydınlatılması için ileri çalışmalar planlanmalıdır.

Anahtar kelimeler: Parkinson hastalığı, motor dalgalanmalar, sinaptik plastisite, sekretogranin 2, striatum

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INTRODUCTION

Loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) being the major pathology, oral levodopa is still the most effective treatment for Parkinson's disease (PD), it replenishes the deficit in the dopamine pool providing symptomatic relief (1). Unfortunately, this relief does not last long and 50% of patients end up suffering from levodopa-induced dyskinesia (LID) following 5 years of therapy (2) and almost 95% of patients have LID inevitably after 15 years (3). These involuntary movements are as much incapacitating as the disease itself for the patients. Thus, understanding the underlying pathophysiology of LID will be helpful for both prevention and the treatment of this unwanted condition.

Before 90s presynaptic mechanisms, namely all processes determining the striatal dopamine levels like production, storage, release and reuptake by the dopaminergic terminals, were more commonly accused for LID, as nigral dopaminergic cell loss is the initial and the most prominent pathological finding in PD. Due to this loss, exogeneous L-dopa cannot be metabolized properly leading to non-physiological dopamine release and LID eventually. However, in the following years, many studies demonstrated that postsynaptic alterations and striatal synaptic plasticity changes, affecting mostly dopamine (D1) and glutamate (NMDA and AMPA) receptor subunits, are the main events leading to LID (4,5). Presynaptic hypothesis regained its popularity with the findings of human imaging studies showing that LID are more common in patients with larger fluctuations of dopamine levels in the striatum with standard L-dopa treatment and other experimental studies showing that presynaptic dopaminergic compartment plays a major role in determining the susceptibility to LID (6,7). With this view, all factors affecting the striatal dopamine levels can be classified as presynaptic. Additionally alterations of corticostriatal, thalamostriatal afferents to striatum may further influence striatal projecting neuronal activity leading to LID.

In this study, we aimed to extend this knowledge and study the role of striatal presynaptic proteins, with regard to the quantitative changes in their levels, in LID pathogenesis. For this aim, three different presynaptic proteins, namely synaptophysin (Syp), synaptotagmin 7 (Syt7) and secretogranin 2 (SG2), were selected considering their roles in different parts of the presynaptic compartment. Syp is a specific synaptic vesicular protein consisting 7% of total vesicular protein. It does not have a role in synaptic release of neurotransmitters but it is a structural component of the vesicle, giving indirect information about the number of synaptic vesicles and synapses (8). Previously, in a study using 6-hydroxydopamine (6-OHDA) parkinsonism model to study LID in rats, striatal expression of synapsin, another structural synaptic vesicle protein, was found increased both in the rats with LID and the rats which received bromocriptine treatment and did not develop dyskinesia. The treatment period was 3 weeks for both L-dopa and bromocriptine (9). Two different studies looking for any changes in the expression of different proteins in the striati of PD patients, found no difference for synaptophysin levels compared to controls (10,11). Synaptotagmin 7 is a Ca2+ sensor located on the active zone of the plasma membrane and plays a role in Ca2+-mediated vesicular fusion (12). It has been shown that striatal Syt7 expression increases with chronic intermittent L-dopa/carbidopa treatment (in total 6 injections each containing 5mg/kg L-dopa in 24 days) in 6-OHDA injected hemiparkinsonian rats (13). Whereas SG2 is a component of large dense-core vesicles that are less abundant than the synaptic vesicles and are located at a distance from the active zone. These vesicles usually contain hormones, neuroactive peptides, amines and growth factors (14). Similarly, striatal SG2 mRNA level was found to be higher in the 6-OHDA lesioned rats compared to controls and after 3 weeks of L-dopa 50 mg/kg and carbidopa (12.5 mg/kg) treatment, this increase became more evident (15).

METHODS

Animals: Thirty-five adult male Wistar rats, weighing 250-300 grams at the beginning of the experiment, were used in the study. All animals, 3 per cage, were kept under stable temperature (18-20 °C) and humidity conditions, 12 h light/12 h dark cycle with ad libitum access to food and water. Experiments were performed at least 2 days after the animals had been brought to their final cages. All experiments, except for the surgical procedures were performed in their home-cage. The local ethical committee for animal studies approved the study.

Surgical Procedure and Evaluation of the Nigrostriatal Lesion: Rats were anesthetized with chloral hydrate (0.35 mg/kg) and given nasal oxygen (2 l/min) together with isoflurane anesthesia during the procedure. After placing the animal in the stereotaxic frame, a unilateral 6-OHDA hydrobromide (12.5 mg in 5 ml 0.02 % ascorbic acid in saline) microinjection was performed targeting right medial forebrain bundle (MFB) rostral to substantia nigra according to Pellegrino stereotaxic atlas at coordinates as follows (mm): (A - 2.2, L + 1.5 V - 8.0) (16). The efficacy of the 6-OHDA lesion was evaluated with apomorphine test (0.05 mg/kg, 1 ml/kg, S.C.) in all rats 3 weeks after the surgery. Animals that showed more than 6/min contralateral rotations were accepted to have more than 90 % dopaminergic denervation at the lesion site (17); and recruited to "severe dopaminergic denervation" experimental groups. Rats that showed contralateral rotations less than 6 /min were accepted to have partial dopaminergic denervation and recruited to partial dopaminergic denervation experimental group. The degree of dopaminergic denervation was confirmed by immunoblotting of striatum of the 6-OHDA-lesion site for TH at the end of the experiment.

Experimental Groups and Treatment: All the animals received chronic intermittent intraperitoneal (ip) injections of either L-dopa or saline twice daily (at 9 a.m. and 5 p.m.). For L-dopa treatment, 25 mg/kg L-dopa (L-dopa methyl ester hydrochloride-Sigma-Aldrich, USA) was dissolved in saline together with 6.25 mg/kg benserazide (benserazide hydrochloride-Sigma-Aldrich, USA) as a peripheral dopa-decarboxylase inhibitor. The volume of the injection solution was adjusted to be equal to 1 ml/kg.

Experimental groups were as follows:

1. 6-OHDA lesioned rats which had severe dopaminergic denervation-treated with L-dopa (Complete/L-dopa) (n=6)
2. 6-OHDA lesioned rats which had severe dopaminergic denervation-treated with saline (Complete/saline) (n=6)
3. 6-OHDA lesioned rats which had partial dopaminergic denervation-treated with L-dopa (Partial/L-dopa) (n=6)
4. Intact rats-treated with L-dopa (Control/L-dopa) (n=6)
5. Intact rats-treated with saline (Control/saline) (n=5)

At the end of 21 days treatment period, all of the animals were decapitated under high dose chloral hydrate anesthesia, 12-14 hours after the last injection; and the brain tissue was quickly taken out of the cranium. The striati were dissected out and frozen in dry ice to be used in the immunoblotting experiments.

Evaluation and Analysis of Dyskinetic Behaviors: The rats were observed in their home-cage for one hour following the morning injections starting from the first day and every third day thence forth (1,4,7,...19) and their behaviors were videotaped for 2 minutes, 30 and 60 minutes after the injection and these recordings were analyzed and rated according to the below scale for rating L-dopa induced dyskinesia in rats (Table 1) (18).

Immunoblotting and Data Analysis: Right striati dissected from the whole brain, were homogenized in radioimmunoprecipitation

Table 1. Scale for rating LID in rats

Behaviour	Severity	Score
Contralateral turning behaviour	Absent	0
	<6/minute	1
	≥6/minute	2
Contralateral involuntary repetitive forelimb movement	Absent	0
	Mild	1
	Moderate	2
	Severe	3
Hindlimb dystonia	Absent	0
	Present	1
Oral stereotypy	Absent	0
	Present	1
Twisted posture of head and/or body according to contralateral side	Absent	0
	Mild	1
	Moderate	2
	Severe	3

*According to the scale for rating L-dopa induced dyskinesia, a highest dyskinetic behavior is rated as 10.

assay (RIPA) buffer that contained 2% protease inhibitor cocktail. The homogenates were then centrifuged for 15 minutes at +4°C and 14000 rpm. Aliquots from striatal homogenates were separated by SDS-PAGE in 10% Novex Bis-Tris (Invitrogen) gel and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. To prevent non-specific binding, blocking solution (Tris Buffered Saline, 0.1% Tween-20, 5% casein) was applied and the membranes were incubated overnight at + 4 °C with primary antibodies for TH (tyrosine hydroxylase) (mouse, monoclonal, 1:1000; T2928, Sigma Aldrich, USA), synaptotagmin 7 (rabbit, polyclonal, 1:1000; 105-173 Synaptic systems, Germany), synaptophysin (mouse, monoclonal, 1:1000; S5768, Sigma Aldrich, USA) and secretogranin 2 (mouse, monoclonal, 1:1000; SG-II 6B 1/3, ab20245, Abcam, USA) at different sessions. Second day, to show the primary antibody binding, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies anti-mouse (horse, 7076, Cell Signaling Technology Inc, USA) and anti-rabbit (goat, 7074, Cell Signaling Technology Inc, USA), accordingly.

To identify the immunoreactive bands, a chemiluminescent assay (WesternBreeze Chemiluminescent Kit, Invitrogen) was used. Chemoluminescence was recorded by Image Station 4000MM (Kodak) and intensities of bands were measured by using Image J 1.37v (NIH, USA). The density of the beta- actin bands (mouse, monoclonal, 1:3000; A5441, Sigma Aldrich, USA) was used to control and correct the unequal protein loading. The results were expressed as the ratio of the optical densities of each band obtained with the specific antibody to that of beta-actin.

Immunofluorescent Staining: For this procedure, animals were perfused transcardially with 4% paraformaldehyde solution under high dose chloral hydrate anesthesia and following cryoprotection in 30% sucrose solution; brains were cut in the coronal plane at 20 µm using a cryostat (Cryostat-Leica CM 1100). Then, selected striatal and SN sections were blocked by 10% normal goat serum to prevent non-specific staining and incubated with anti-TH (1:1000) to demonstrate dopaminergic denervation and anti-synaptotagmin 7 (1:200), anti-synaptophysin (1:200) and anti-secretogranin (1:200) antibodies to evaluate any possible change in the amount and pattern of expression. For anti-TH and anti-SG 2, cy2 goat anti-mouse (118, Jackson ImmunoResearch Laboratories Inc, USA), for anti-Syp, cy3 goat anti-mouse (120, Jackson ImmunoResearch Laboratories Inc, USA) and for anti-Syt 7, cy2 goat anti-rabbit (119, Jackson ImmunoResearch Laboratories Inc, USA) secondary antibodies were used. Hoechst 33258 was used for the counter-staining. The results were evaluated with the help of NIS-Elements AR 2.30 software (Nikon Eclipse E600, Ex 450-560 nm). The immunofluorescent staining results were not analyzed by any quantitative method, as the number of animals was limited for statistics, so only some representative images were obtained to further direct the immunoblotting studies.

Statistical Analysis

Statistical Package for Social Sciences, 16.0 (SPSS Inc.; Chicago, USA) was used for statistical analysis. As data distribution was not normal, non-parametric tests (Kruskal-Wallis, Mann-Whitney U and Spearman correlation coefficient) were selected for comparisons among groups. z test proposed by Nemenyi- Dunn was performed for post-hoc pair-wise comparisons. Wilcoxon and Friedman tests were used for the analysis of dyskinetic behavior scores.

RESULTS

Evaluation of LID Severity: All of the 6-OHDA-lesioned rats with severe dopaminergic denervation and treated with chronic intermittent L-dopa-benserazide injections developed dyskinesia and demonstrated increased number of contralateral turning behavior during the 21 days of L-dopa treatment period. The dyskinesia score of the last treatment day (4.08±0.63) was significantly higher than that of the first day (1.5±0.94) (p=0.007, Wilcoxon test) and the increment of dyskinesia score in between days of observation and measurement was also statistically significant (p<0.01, Friedman test). Gradual accentuation of dyskinesia scores (e.g. behavioral sensitization) was evident only in 6-OHDA-lesioned rats treated with L-dopa-benserazide combination, but not the other treatment groups (e.g 6-OHDA-lesioned rats treated with saline or intact rats treated with L-dopa-benserazide) (Figure 1). On the other hand, partial dopaminergic denervation group treated with L-dopa had very low dyskinesia scores throughout the treatment period (Figure 1).

Relationship Between LID Severity and the Degree of Dopaminergic Denervation: Immunoblotting of the striatal tissues from 6-OHDA lesioned animals for TH showed almost total dopaminergic denervation compared to controls (p<0.001) (Figure 2). Immunofluorescence staining for TH also confirmed the depletion of dopaminergic terminals. The

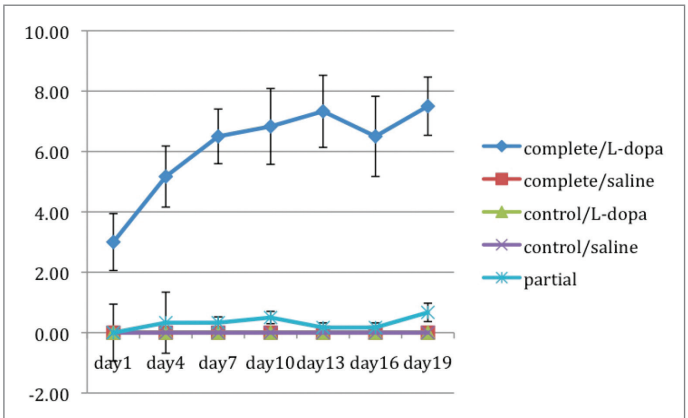

Figure 1. LID scale score follow-up throughout the 3-week treatment period in experiment groups as indicated. The values are the mean ± standard error of the mean (SEM) of the scores of all the animals in each group. Average of the scores obtained at 2 behavioral observations following L-dopa or saline injections (20 and 50 minutes) for each animal is included for analysis

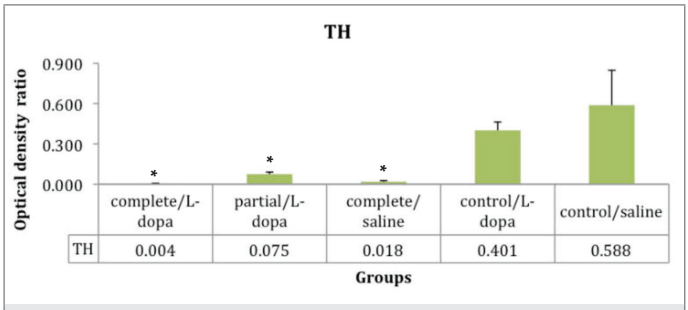

Figure 2. Immunoblotting results for TH (tyrosine hydroxylase); optical density values of TH/actin are presented and compared in different groups (*: p<0.05)

degree of dopaminergic denervation strongly correlated with the dyskinesia scores of the Parkinsonian rats (-r=0,79, p<0,01).

Immunofluorescent Staining for Evaluation of Possible Changes in the Striatal Expression Levels and Patterns of Presynaptic Proteins: First, immunofluorescent staining was performed to visually demonstrate if there is any obvious change in the amount and pattern of striatal labeling of selected presynaptic proteins, Syp, Syt7 and SG2 in the 6-OHDA-lesioned (right) side compared to the intact side (left). For Syp, a homogeneous and general increase was observed in the striatum with dopaminergic denervation (Figure 3a). For Syt7, the increase was specifically found in the dorsolateral region of the striatum, i.e. motor striatum, on the lesion side (Figure 3b). Finally, a heterogeneous increase in the fluorescent staining with SG2, with a dorsolateral to ventrolateral gradient and an interesting islet-style appearance, reminiscent of striosomal organization in the striatum, was noted again on the lesion side compared to the intact side (Figure 3c).

Immunoblotting Study to Observe the Possible Changes in Striatal Expression of Presynaptic Proteins: Then, we employed immunoblotting to compare the whole striatal expression levels of these presynaptic proteins on the 6-OHDA-lesioned side (right) between experiment groups. The results are shown in Figure 4. Multiple group comparison analysis revealed that striatal SG 2 and Syp levels were significantly different between groups (p<0.05), whereas Syt 7 levels were similar. For non-parametric post-hoc analysis, Nemenyi Dunn's Z test was used. This analysis showed that striatal SG 2 and Syp levels were higher in the 6-OHDA-lesioned group treated with saline compared to intact group treated with saline (p<0.05). This significant difference was still present when all groups were compared according to their denervation status.

To examine the effect of L-dopa treatment on expression patterns of these presynaptic proteins, we first compared the results of those without 6-OHDA lesion, according to their treatment group (L-dopa or saline). There was no significant difference, showing that L-dopa treatment alone without dopaminergic denervation, does not lead to any change in striatal levels of these proteins (Figure 4). For the next step, animals, which have the same level of dopaminergic denervation, were compared according to their treatment group. Striatal expression level of SG-2 was significantly higher in the L-dopa group compared to saline group (p<0.05). On the other hand, Syp levels, which were shown to increase with dopaminegic denervation, were similar between these groups.

Relationship Between Striatal SG2 Levels and LID Severity: Interestingly, we also detected a significant moderate, positive correlation between striatal SG 2 levels and LID severity, when total and partial denervation groups were analyzed altogether (Spearman rho=0.43, p<0.05). Moreover, striatal SG 2 levels were highly and negatively correlated with TH levels (Spearman rho=-0.66, p<0.01); implicating that striatal expression of SG 2 increases in parallel with the denervation severity.

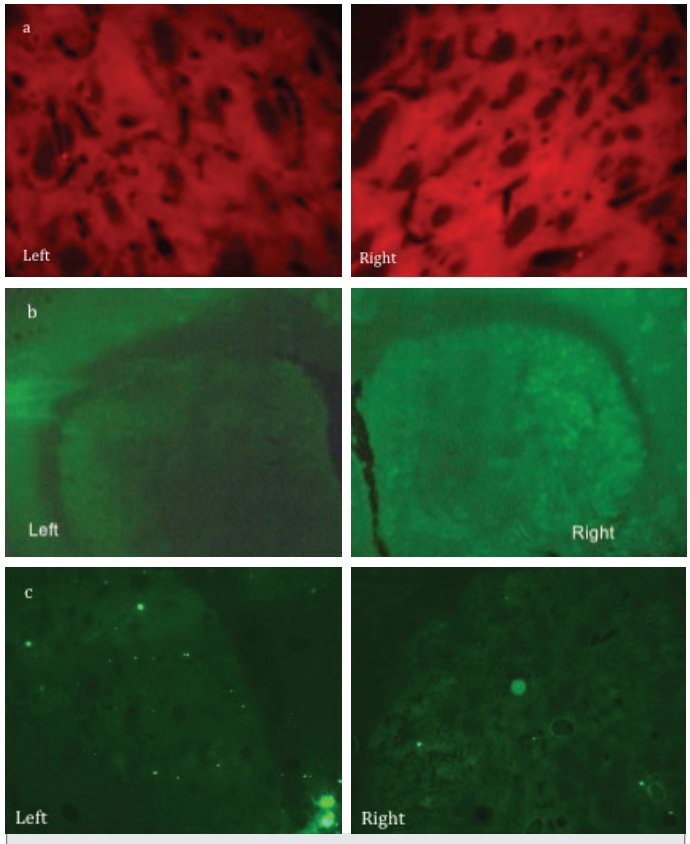

Figure 3. a-c. Representative images showing immunofluorescent staining of striatal tissue with (a) anti-Syp (x20 magnification) (b) anti-Syt7 (x4 magnification) and (c) anti-SG2 (x10 magnification) antibodies (from an animal with complete denervation and LID. Striatum on the lesion side (right) is compared to the contralateral intact side (left)

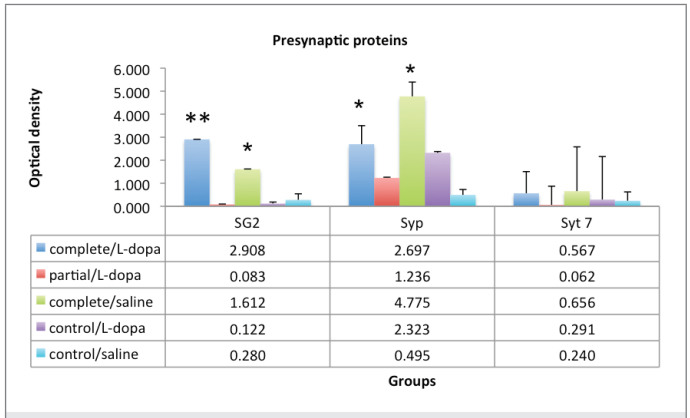

Figure 4. Immunoblotting results for secretogranin 2 (SG2), synaptophysin (Syp) and synaptotagmin 7 (Syt 7); optical density ratios of each synaptic protein to actin are compared between groups (*:p<0.05; **:p<0.05-p value for comparison between complete/L-dopa and complete/saline groups)

DISCUSSION

In this study, we used the classical rodent model of PD, realized by unilateral stereotactic injection of 6-OHDA to MFB. LID was induced by chronic daily intraperitoneal injections of L-dopa and as expected it developed only in rats with 6-OHDA lesion and severe dopaminergic denervation but not in controls. Severity of LID increased throughout the treatment days and the dyskinesia score of the last treatment day showed strong negative correlation with striatal TH levels in line with the general knowledge that the severity of dopaminergic degeneration determines

which dose and duration of L-dopa treatment would lead to emergence of LID (19). The high correlation value obtained in our study suggests that severity of striatal dopaminergic denervation can be an important determinant of dyskinesia in PD. In another study using 6-OHDA rat model, it was shown that LID severity is highly correlated with the amount of TH (+) neuron loss in SNpc, in agreement with our study (20). In PD patients, dyskinesia first appear on the side where the Parkinsonian symptoms are more severe, confirming the relationship between the severity of dopaminergic denervation and LID (21). Clinically, it is essential to predict which patients tend to develop severe dyskinesia early in the disease process to be able to take preventative measures.

In the current study, a rather high dose of L-dopa is preferred (25 mg/kg) both to obtain a more robust effect on presynaptic proteins to be able to capture any subtle change due to LID for further evaluation and also as it is the common dosage that has been used by our group previously and some other groups that we have been following (22,23). 3-week treatment scheme was also applied for the same reason. Although it may have some priming effect in the denervated striatum, apomorphine test was used for the evaluation of lesion severity, as again this is the routine and only method that's available to be used for this aim in our lab. Higher number of treatment groups receiving different doses of L-dopa, such as 6 and 12 mg/kg and/or dopamine receptor agonists may have been included in the study to increase the diversity to tease apart more specific details about the relationship between these presynaptic proteins and LID. Another limitation is that further mechanistic studies to explain the moderate positive correlation between striatal SG2 levels and dyskinesia severity are missing from the current study.

The main pathophysiological mechanism accused in the development of LID is the maladaptive structural and synaptic plasticity changes taking place largely in the striatum due to the non-physiological, pulsatile delivery of dopamine via oral L-dopa treatment (24). Early on, it was believed that the main mechanism acting in the formation of dyskinesia is the defect in storage and appropriate release of dopamine from the presynaptic sites. Further studies showed that post-synaptic alterations in MSNs might also play an important role. At the cellular level, a variety of molecular mechanisms have been claimed by different studies. Alterations in dopamine receptors and their downstream signaling pathways as well as changes in striatal glutamatergic signaling have been shown to be responsible in both 6-OHDA rat and MPTP- monkey models (25,26,27,28). More recently, presynaptic hypothesis became more popular again as human imaging studies showed that dopamine handling is deficient in dyskinetic patients when compared to non-dyskinetic patients, leading to higher fluctuations of striatal dopamine levels (6,29). According to presynaptic hypothesis, post-synaptic changes are secondary to this condition rather than being responsible from dyskinesia primarily (5,7).

In this study, we studied the role of presynaptic proteins in relation to possible changes in synaptic plasticity and vesicular dynamics, taking place in the striatum during the process of LID formation. For this aim, three different proteins were selected with regard to their differential functional roles in the presynaptic compartment.

Syp, comprising 7 % of total synaptic protein, does not have a direct role in the neurotransmitter release (8). As a structural component of the synaptic vesicle, it is usually included in various studies as a synapse marker. It is also known to have a role in the activity-dependent synapse formation (30). In our study, immunofluorescent staining showed that there is an increased signal on the denervated side of the striatum (right) (Figure 3a). Immunoblotting further approved that the striatal expression of Syp is significantly increased with dopaminergic denervation ($p<0.05$) but LID development does not lead to an additional change in the expression pattern of Syp (Figure 4). In a similar study using 6-OHDA model to study LID, striatal level of synapsins, structural synaptic vesicle proteins closely related to Syp, was found to be higher in the LID group as well as in the group chronically treated with bromocriptine but did not develop LID. It was suggested that this increase may be associated with locomotor activity increase itself rather than dyskinesia development (9). In our study, increased striatal expression of Syp following dopaminergic denervation may be related to new synapse formation, axonal sprouting and synaptic plasticity changes; as Syp acts as a structural synapse marker and has a role in LTP and activity-dependent synapse formation.

Synaptotagmins have a regulatory role in membrane trafficking; therefore they may be associated with motor complications of chronic L-dopa treatment in PD. This idea led to a study using 6-OHDA model of chronic dopaminergic depletion in rats; these rats were treated with apomorphine for one week and then received one acute dose of either L-dopa or D1 receptor agonist and striatal mRNA levels of Syt4 and 7 were found elevated in both cases compared to controls which received saline (31). On the other hand, striatal Syt4 mRNA levels decreased and Syt7 levels increased following chronic intermittent L-dopa treatment leading to behavioral sensitization (13). There may be two reasons for not obtaining similar results in our study. First, Syt7 is analyzed at the protein level. Second, immunofluorescent staining showed an increase specifically in the dorsolateral striatum, and this difference may have been diluted as whole striatum is homogenized for immunoblotting experiments. Thus, different experimental strategies and more detailed evaluation are required to clarify this issue in further studies.

SG2, third presynaptic protein included in this study, is localized inside large dense core vesicles and is mainly found in brain and endocrine organs (14). More than 90% of SG2 is endoproteolytically converted to a 33 amino acid peptide, named secretoneurin, and is secreted upon depolarization. It's been shown to induce dopamine release in a dose-dependent manner in the rat striatum (32).

The findings of the present study imply that SG2 has a critical functional role in the pathogenesis of LID. In a study performed in 6-OHDA lesioned rats aiming to identify the genes with an altered striatal expression pattern; it's been shown that SG2 mRNA level was significantly higher than controls and this increase became more pronounced with chronic L-dopa treatment, similar to our study (15). Furthermore, secretoneurin, which is a proteolytic product of SG2, immunoreactivity was found increased in the denervated striatum compared to the intact side. Interestingly, SG2 mRNA and protein increase was observed inside the cell bodies in the striatum and this observation led to the idea that pathological changes underlying LID actually take place in the output neurons rather than dopaminergic terminals (15). In the same study, the researchers found a positive correlation between SG2 mRNA levels and number of apomorphine induced contralateral turns, i.e. lesion severity, similar to what is found in the current study. We additionally found that striatal SG2 levels show intermediate, positive correlation with the severity of LID induced by chronic L-dopa treatment. Specific to our study, striatal SG2 increase has been demonstrated at the protein level using both immonufluorescence and immunoblotting methods. Studying two other presynaptic proteins with different functional properties, together with SG2 gives us the opportunity to state that SG2 increase and its correlation with LID are specific findings and this may be related to its specific role in the presynaptic compartment.

In a study searching for the role of secretoneurin, it was demonstrated that local infusion of secretoneurin by microdialysis into the striatum and nigra led to increased release of glutamate, GABA and dynorphin B (33). The fact that, even very small amounts of secretoneurin lead to more

than 20-fold increase in dynorphin B release means this effect is specific for dynorphin B (33).

Dopaminergic denervation has opposite effects on the expression of striatal enkephalin, substance P and dynorphin, as dopaminergic afferents act differentially on direct and indirect pathways. L-dopa treatment restores the expression pattern of these peptides except for dynorphin B, reaching 300% of its original levels in the striatum (34). Another study about LID pathophysiology revealed that there is a huge increase in the prodynorphin and GAD-67 mRNA levels with LID development and prodynorphin levels are highly correlated with severity of dyskinesia (35).

The major finding of the current study; the significant increase in striatal SG2 levels correlating with the severity of dopaminergic denervation and dyskinesia, may be contributing to the LID pathogenesis by increasing dynorphin B release. The significant correlation between LID severity and striatal SG2, at the protein level is demonstrated for the first time in the literature and this may be an important finding to pursue for understanding LID pathogenesis in the future studies. Further studies are required to find out molecular mechanisms linking increased SG 2 expression and LID.

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