Research Article

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Motor Dysfunction and Related Synaptic Organisation in AVV-Mediated Alpha-Synuclein Overexpression Model of Parkinson's Disease

AAV-aracılı Alfa-Sinüklein Aşırı İfadesi ile Sağlanan Parkinson Hastalığı Modelinde Motor Bozukluklar ve İlişkili Sinaptik Organizasyon

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ABSTRACT

In Parkinson's disease (PD), pathological intracellular aggregation of alpha-synuclein plays a key role in the neurodegenerative process. In this study, we aimed to investigate the progression of motor dysfunction and related changes in synaptic organization in an alpha-synuclein overexpressing viral vector model of Parkinson's disease (PD) in rats.

Adeno-associated viral vectors (AAV) were stereotaxically injected bilaterally into substantia nigra (SN) together with dentate gyrus (DG). Further 5 animals were used as naïve controls. All animals were tested for locomotor activity for an hour from 3rd to 15th week. After that rats striati were analyzed by Western blotting for alpha-synuclein, tyrosine hydroxylase and synaptophysin expression.

Alpha-synuclein injected group moved less distance compare to control and their movement decreased by time till the 7th week then start to increase but yet slightly lower than the controls. Synaptophysin level was 13% and TH level was 25% decreased in alpha-synuclein group compare to controls.

Due to compensation mechanisms to protect neurons from neuronal death or alpha-synuclein accumulation in DG, alpha-synuclein group sustained to move in open field locomotor activity test. Although the model is open for improvement, it is useful to study early stage of PD and motor dysfunctions that occur due to alpha-synuclein overexpression.

Keywords: Parkinson's disease, alpha-synuclein, locomotor activity, substantia nigra, dentate gyrus

ÖZET

Parkinson hastalığında (PH) hücre içi patolojik alfa-sinüklein (α-sin) agregatları nörodejeneratik süreçte önemli bir rol oynamaktadır. Amacımız α-sin aşırı ifadesi oluşturulmuş PH hayvan modelinde, zaman içerisinde meydana gelen motor fonksiyon değişikliklerinin izlenmesi ve sinaptik düzeydeki etkilerinin gösterilmesidir.

Sıçanlarda bilateral olarak substantia nigra (SN) ve dentat girus (DG)'da birlikte stereotaksik olarak adeno ilişkili viral vektör (AAV)-aracılı α-sin enjeksiyonu gerçekleştirildi. Ayrıca, hiçbir işleme tabi tutulmamış 5 sıçan kontrol olarak kullanıldı. Bütün hayvanların motor hareketleri, 3. ile 15. haftalar arasında lokomo-

tor aktivite kafesinde test edildi. Davranış deneylerinden sonra, sıçanların striatumlarında α-sin aşırı ifadesi ile tirozin hidroksilaz ve sinaptofizin seviyeleri Western blot yöntemi ile semi-kantitatif olarak analiz edildi.
Lokomotor aktivite testinde, α-sin grubu kontrol grubuna göre daha az mesafe kat etti ve kat ettikleri mesafe 7. haftaya kadar azalma eğilimi gösterdi. Daha sonra ise harekette meydana gelen azalmanın hafif bir artış göstererek, kontrol grubuna yaklaştığı gözlendi. Kontrol grubuna göre sinaptofizin seviyesinde %13, TH seviyesinde ise %25 azalma olduğu gözlendi.
Olası kompansasyon mekanizmalarının devreye girmesi ya da DG'de α-sin yükünün artmasından, α-sin grubunun hareketlerinde kontrol grubunda meydana gelen azalma gözlenmemiştir. Bu model α-sin aşırı ifadesine bağlı olarak meydana gelen motor fonksiyon bozukluklarının çalışılmasında erken dönem araştırmalar için geliştirilerek kullanılabilecek bir yöntem oluşturmaktadır.

Anahtar Kelimeler: Parkinson hastalığı, alfa-sinüklein, lokomotor aktivite, substantia nigra, dentat girus

1. Introduction

Parkinson's disease (PD) is mainly characterized by motor symptoms such as resting tremor, bradykinesia, rigidity and postural instability, primarily resulting from progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and depletion of dopamine levels in the striatum due to terminal loss in the projection areas (1). In the time course of PD progression, there is a long prodromal or premotor period around 20 years before the classical motor symptoms present. In this period, patients may have constipation, hyposmia, REM sleep behavior disorder and depression followed by anhedonia, apathy, mild cognitive changes and attention problems as premotor symptoms due to on-going pathological process of alpha-synucleinopathy in the enteric plexus, olfactory bulb, medulla and midbrain which effects the quality of patient's life standards as much as motor symptoms. Due to that, various animal models trying to recapitulate NMS are increasing in number in order to characterize the timing and development of pathology (2-7).

The toxin-mediated models of PD have long been used successfully to mimic the motor symptoms such as motor learning and coordination, balanced and impaired locomotor activity and they were also used to explore cognitive aspects of the disease based on classical nigra-centric pathogenetic approach, but gave only limited information in relation to mesensephalic-striatal-frontal networks of cognition (memory and learning deficiencies) (4, 8-10). Although the underlying mechanism of the Parkinson's disease is not known precisely, intracellular Lewy body (LB) and Lewy neurites, which contains mainly the alpha-synuclein (α-syn) protein, believed to have a key role in the pathogenesis, are the pathologic and histological hallmark of the disease (11-15). Even the toxin-mediated models are useful the study motor deficiency and dopaminergic cell loss in the SN, Lewy body and Lewy neurites formations are not seen in these models and not sufficient to mimic the progressive underlying mechanism of the disease exactly (16, 17).

Misfolding α-syn protein is believed to be one of the key factors of the underlying mechanism of the PD (11, 18, 19). Because of that many α-syn overexpression or mutation based animal models were used to study PD pathogenesis (20-26). The advantages of using the viral vector to deliver the α-syn, overexpression and accumulation of α-syn protein can be achieved in the targeted area with higher effiency and progressive nature of the disease can be recapitulated more precisely in different animal species (21, 22, 26).

In the present study, our aim was isolating ongoing motor impairment in PD after α-syn injection and to monitor changes in motor function within weeks compared to naïve controls, beyond that to examine how early neuronal or synaptic loss could begin. We generated an experimental animal model with co-existing midbrain dopaminergic and hippocampal α-syn pathology. We have planned to target both substantia nigra pars compacta (SNpc) and hippocampus dentate gyrus (DG) together with bilateral injections of recombinant adeno-associated viral vectors (rAAV) encoding for human wild type α-syn, to explore the motor impairment in relation to Lewy pathology and the cognitive dysfunction simultaneously.

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2. Material and Methods

200-250 g female Sprague-Dawley (SD) rats (n=10) were used in this experiments. Rats were kept under a stable room temperature with a 12/12 h light/dark cycle. All animal procedures were performed in accordance with the Institutional Guidelines for Care and Use of Laboratory Animals, and were approved by the Hacettepe University Animal Ethics Committee (2014/51-08). Before starting experimentation, animals were acclimatized to the experimental room for 1 an hour.

2.1. Streotaxic Surgery

The surgical procedures were performed under anesthesia using a mix of 90 mg/kg ketamine and 10mg/kg xylazine i.p. injection. The coordinates for subtantia nigra (SN) injection and dentate gyrus (DG) were as follows; AP=-5.2 mm, ML=±2.0 mm (SN); AP=-5 mm, ML=±3.5 (DG) from the bregma, DV=-7.2 mm (SN); DV=-2.6 mm (DG) from the dural surface. The viral vector solution (*AAV5-CBA-aSyn,* $1.0X10^{13}$ vg/ml or *AAV5-CBA-eGFP,* $9.5X10^{12}$ vg/ml) was kindly obtained from Michael J. Fox Foundation. 2 µl of vector suspension containing r*AAV5-α-syn* (n=5) were injected slowly to bilateral SN and DG. Further n=5 animal used as naïve controls.

2.2. Experimental procedure

Following the surgery animals housed in their home cage for three weeks through healing period. After that, ones in every two weeks both α-syn injected group and naïve controls (control) locomotor activity were examined until sixteenth week (Figure 1).

2.3. Open Field Locomotor Activity Test

Animals were tested for motor function in locomotor activity cage as well. Plexiglass boxes (40 x 40 x 40 cm) equipped with infrared photo beam emitters which senses both vertical and horizontal activity and receptors were used for this test. Animals were placed in these boxes by one by and their baseline activity was recorded for one hour. Total movement (*stereotypic, ambulatory* ve *vertical),* horizontal activity and total distance moved (cm) were recorded automatically by locomotor activity system. Changes occurred in these activities for α-syn group were analyzed compared to controls.

2.4. Tissue Preparation and Immunoblotting

With a help of RIPA lysis buffer containing protease and phosphatase inhibitor cocktails striati tissue samples were homogenized. After homogenization the supernatants were collected and protein concentration was measured with the help of BCA protein assay kit (Pierce, Thermo Fisher Scientific). Samples are prepared with loading buffer and loaded to the polyacrylamide gels and electrophoresed. Then proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Transfer membranes were blocked with 5% non-fat dry milk for one hour and after blockage all membranes were incubated with one of the specific primer antibodies on Table 1. All the antibodies were probed with appropriate secondary antibodies (HRP conjugated anti-rabbit, anti-mouse) on room temperature at 1:5000 dilutions (Table 1). Chemiluminescence was recorded by Image Station 4000MM (Kodak) and intensities of bands were measured by using Image J 1.37v (NIH, USA). The results were expressed as the ratio of optical densities of each band which standardized through tubulin III.

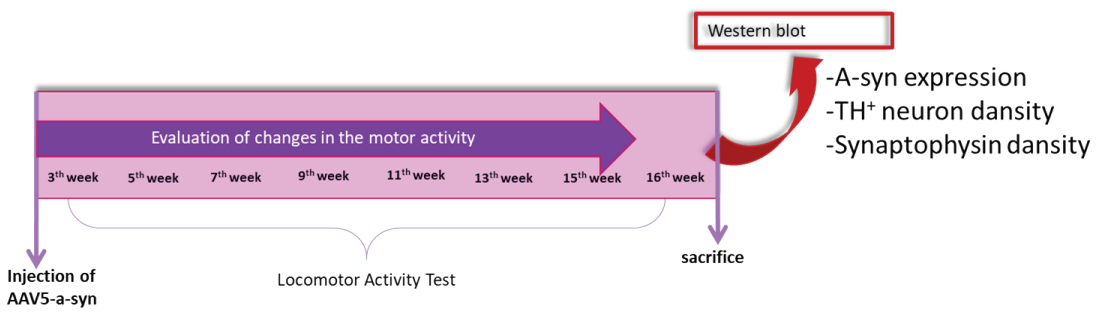

Figure 1: Experimental procedure of AAV-mediated α-syn overexpression animal model

Tablo 1. Antibodies used for immunoblotting in this experiments

	Primer antibody	Antibody concentration	Firm	Seconder Antibody
Alpha-synuclein	Monoclonal anti-alpha-sinuclein (α-syn, ab80627)	1:1000	Abcam	1:5000 Anti-mouse IgG-HRP linked antibody (7076, Cell Signaling Technology Inc)
Synaptophysin	Monoclonal anti-synaptophysin (S 5768)	1:300	Sigma Aldrich Inc.	
Tyrosine hydroxylase	Monoclonal anti- tyrosine hydroxylase (TH, ab75875)	1:1000	Abcam	
Tubulin III	Polyclonal anti-β-Tubulin III (A2200)	1:7500	Sigma Aldrich Inc.	1:5000 Anti-rabbit IgG-HRP (7074, Cell Signaling Technology Inc)

2.5. Statistical Analysis

All statistical analysis were performed with the help of GraphPad Prism version 7.00 (GraphPad Software Inc., La Jolla CA, USA). One-way analysis of variance, followed by Tukey's post hoc analysis, was applied to compare results in-group and unpaired *Student's t* test was applied to compare results of α-syn and naïve control group. $P<0.05$ was considered to indicate a statistically significant difference. Experimental data were presented as the mean ± standard error.

3. Results and Discussion

This bilateral SN and DG, adeno associated viral vector (AAV)-mediated α-syn overexpression rat model was chosen to examine the timeline of the pathological changes and to characterize the time point of the behavioral changes due to pathologic α-syn overexpression.

3.1. Changes of motor functions in open field locomotor activity test

Assessing motor behavioral changes, which are occurred due to α-syn pathology in the basal ganglia, is important to analyze the role of α-syn accumulation for the PD pathogenesis. All injections were made bilaterally into SN and DG. The locomotor activity was tested starting from the third week after the AAV-mediated α-syn injection following once in every two weeks until sixteenth week. Animals' total movements, which comprised of vertical, ambulatory and stereotypic movements, were recorded for an hour by the open field locomotor activity system and changes between the groups were analyzed.

Total movement was decreased in α-syn injected group until seventh week but then, α-syn injected group was showed a tendency to increase for the total movement (Figure 2A). This decrease which was seen in α-syn group in the fifth week was statistically significant compared to sixteenth week ($p=0.0174$, 5^{th} week vs 16^{th} week of α-syn group; Figure 2B). On the other hand, naïve control group showed an increase in total movement until ninth week (Figure 2B). Decrease of total movement in α-syn group was statistically significant compared to naïve controls for ninth week ($p=0.0374$, $\alpha\text{-syn}_{9th\ week}$ vs $control_{9th\ week}$; Figure 2B).

Horizontal activity was also examined with the same protocol once in a two week starting from the third week following the AAV-mediated α-syn injection. α-syn group showed an aim to decrease until seventh week and this decrease was statistically significant compared to sixteenth week ($p=0.03$, $\alpha\text{-syn}_{3rd\ week}$ (5241±1037) vs $\alpha\text{-syn}_{16th\ week}$ (10261±1713); $p=0.0108$, $\alpha\text{-syn}_{5th\ week}$ (4370±817) vs $\alpha\text{-syn}_{16th\ week}$ (10261±1713); Figure 3A). But the naive control group's horizontal activity shows a tendency to increase until eleventh week similar to total distance movement (Figure 3B).

In locomotor activity test, total distanced moved by animals was also recorded once in a two week starting from the third week following the AAV-mediated α-syn injection. Similar to total movement results, α-syn group showed an aim to decrease in distanced moved until seventh week and this decrease seen between weeks were statistically significant compared third week and fifth week to sixteenth week ($p=0.03$, $\alpha\text{-syn}_{3rd\ week}$ (3168±1007) vs $\alpha\text{-syn}_{16th\ week}$ (8030±1741); $p=0.019$, $\alpha\text{-syn}_{5th\ week}$ (2865±704) vs $\alpha\text{-syn}_{16th\ week}$ (8030±1741); Figure 4A). Surprisingly,

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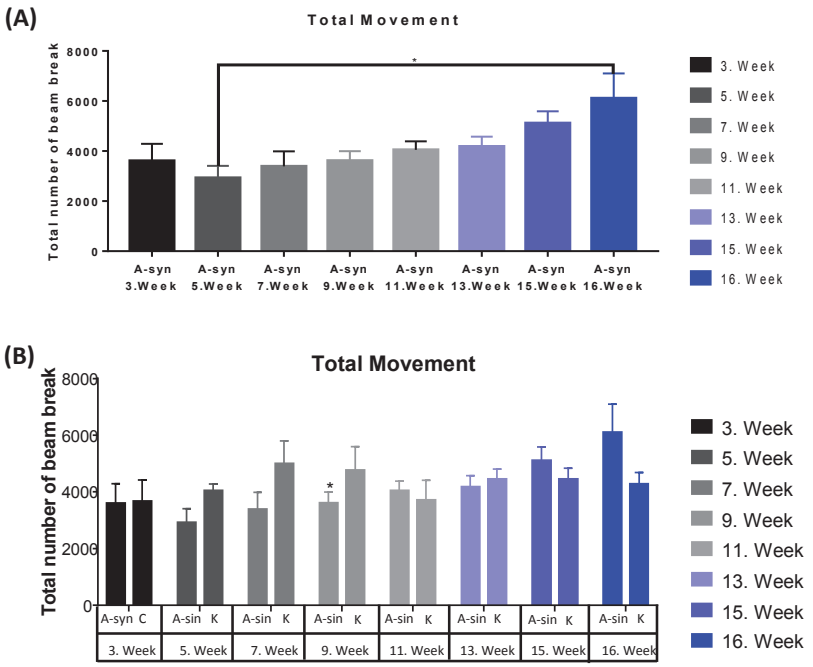

Figure 2: Open field locomotor activity results: **(A)** Changes of α-syn group total movements between weeks (One way ANOVA followed by post hoc Tukey's multiple comparison test was applied, *p<0.05, α-syn$_{5th\ week}$ vs α-syn$_{16th\ week}$), **(B)** Changes of total movement by weeks between α-syn and naïve control groups (Unpaired *Student's t* test was applied, *p<0.05, α-syn$_{9th\ week}$ vs control$_{9th\ week;}$ graphics are shown as mean±SEM, n=4 α-syn group, n=4 naive control group).

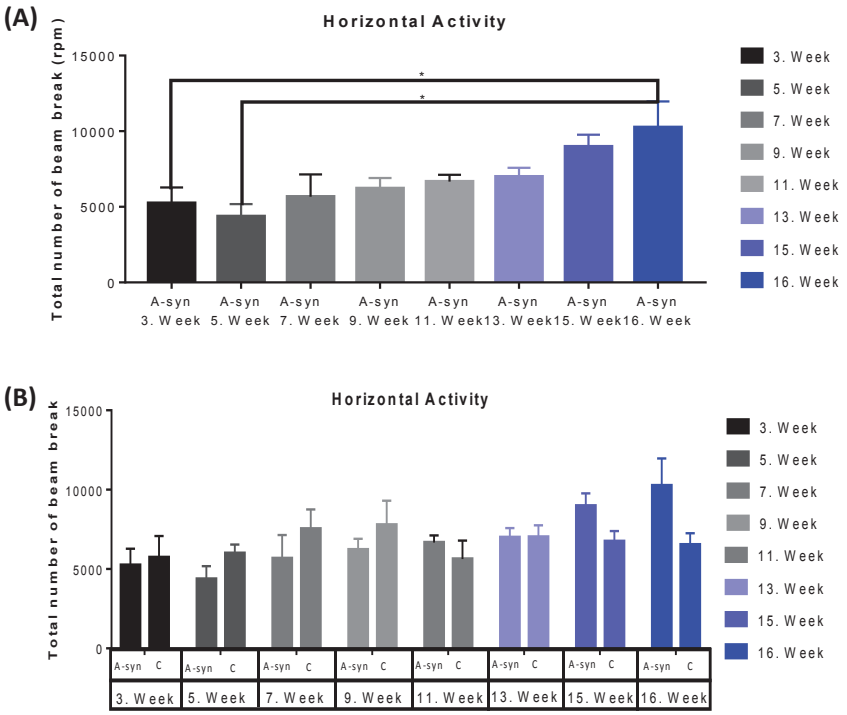

Figure 3: Open field locomotor activity results: (A) Changes of α-syn group total horizontal activity between weeks (One way ANOVA followed by post hoc Tukey's multiple comparison test was applied, *p<0.05, α-syn$_{3rd\ week,}$ and α-syn $_{5th\ week}$ vs α-syn$_{16th\ week}$), (B) Changes of horizontal activity of weeks between α-syn and naïve control groups (Unpaired *Student's t* test was applied, p>0.05, α-syn vs naïve control; graphics are shown as mean±SEM, n=4 α-syn group, n=4 naive control group).

distance moved by naive control group was increased until eleventh week and showed an aim to decrease but this change was not significant neither between weeks nor between groups (Figure 4B). In addition, analysis of total distanced moved per minute within weeks were showed that at third week both groups moved almost equal ($p>0.05$) but in seventh week only the control groups total distanced moved were increased ($p>0.05$; Figure 4C). However, control groups also tend not to move in the following weeks (Figure 4C).

3.2. Morphological changes due α-syn overexpression in bilateral SN and DG

In this model, we aim to recapitulate the Lewy body like pathology due to α-syn accumulation following intracerebral injection of AAV-mediated human α-syn protein bilaterally and simultaneously both into SN and DG. To test the motor complications due to α-syn pathology, open field locomotor activity test was applied once in a two week. After motor functions were tested, molecular changes were measured

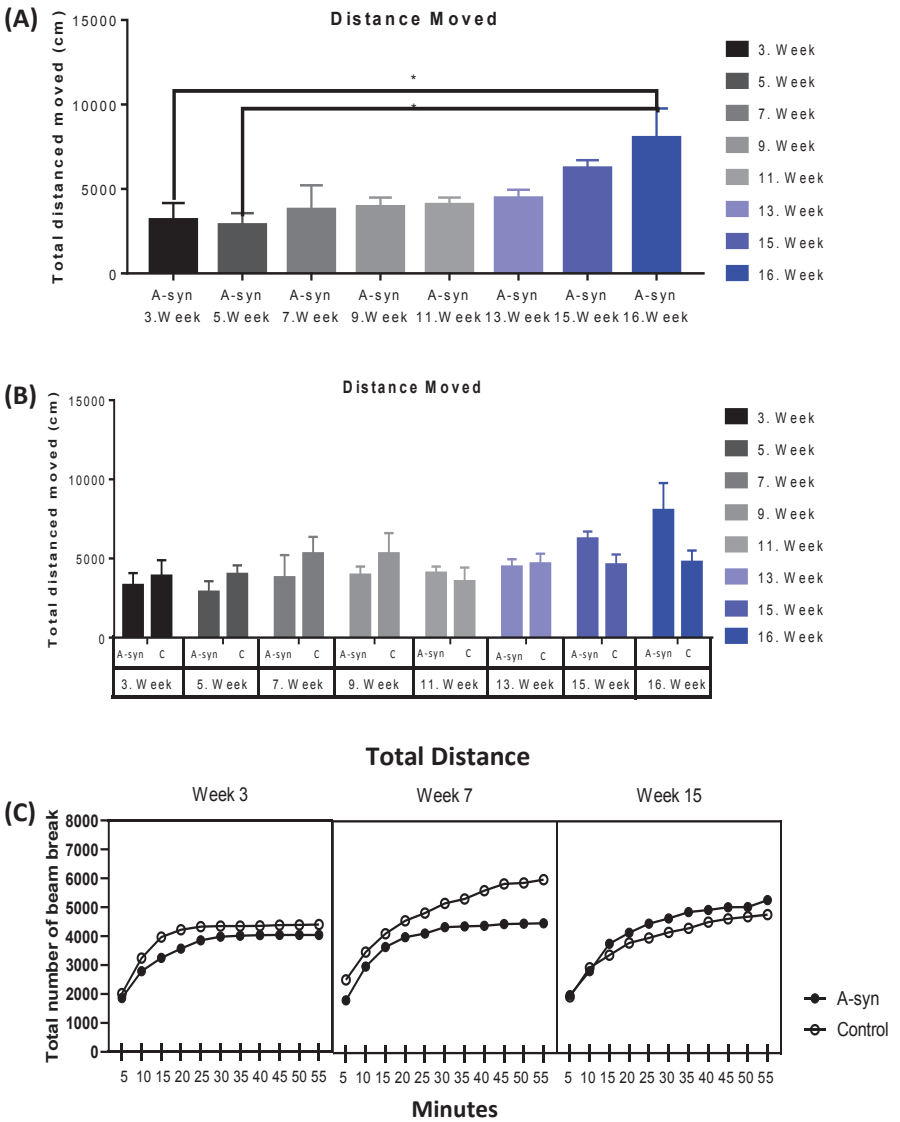

Figure 4: Open field locomotor activity results: (A) Changes of α-syn group total distanced moved between weeks (One way ANOVA followed by post hoc Tukey's multiple comparison test was applied, *$p<0.05$, α-syn$_{3rd\ week}$ and α-syn$_{5th\ week}$ vs α-syn$_{16th\ week}$), (B) Changes of total distanced moved of weeks between α-syn and naïve control groups, (C) Changes in total distances moved in one hour within weeks between groups (Unpaired *Student's t* test was applied, graphics are shown as mean±SEM, n=4 α-syn group, n=4 naive control group).

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by Western blotting. One of the test animal was excluded due to excessive weight loss and another due to lack of α-syn overexpression.

Success of the AAV-mediated α-syn injection bilaterally into SN and DG were confirmed by Western blot analysis in the animals left striatum dissections. α-syn overexpression was showed with representative Western blot band (Figure 5A).

TH-immunoblotting was performed in the striatum to examine the dopaminergic terminal loss. α-syn group, average relative optical density of TH was less than the naïve control group (p=0.02, α-syn (0.70±0.1) vs naive control (1.06±0.9); Figure 5B).

Relative synaptophysine optical density was measured by Western blotting in the striatum to examine the synaptic loss. The analyses were showed that α-syn group relative optical density of synaptophysin were significantly decreased compared to naïve control group (p=0.0172, α-syn (0.88±0.5) vs control (1.08±0.5); Figure 5C).

4.Conclusions

In the present study, we aimed to study reliable early motor dysfunction phenotype proceed within weeks and to investigate functional and morphological correlation of synaptic dysfunction before the neuronal loss in accordance to extensive α-syn burden. Viral vector-mediated α-syn overexpression or recombinant α-syn fibril injection models which give the opportunity to study more slowly and more naturally by self-seeded and propagated α-syn centered pathology in brain regions of interest, even though each model has important limitations of its own (21, 22, 26, 27). Our results show that consistent with the previous studies, α-syn overexpression can induce a progressive neurodegeneration in the striatum, the projection area of the SNpc.

AAV-mediated α-syn expression bilaterally into SN were lead to mild motor dysfunction in open field locomotor activity test. The results of total distanced moved per minute within weeks showed that in the beginning both groups moved almost equal distance but after that, only the control groups total distanced moved showed an aim to increase and not the α-syn

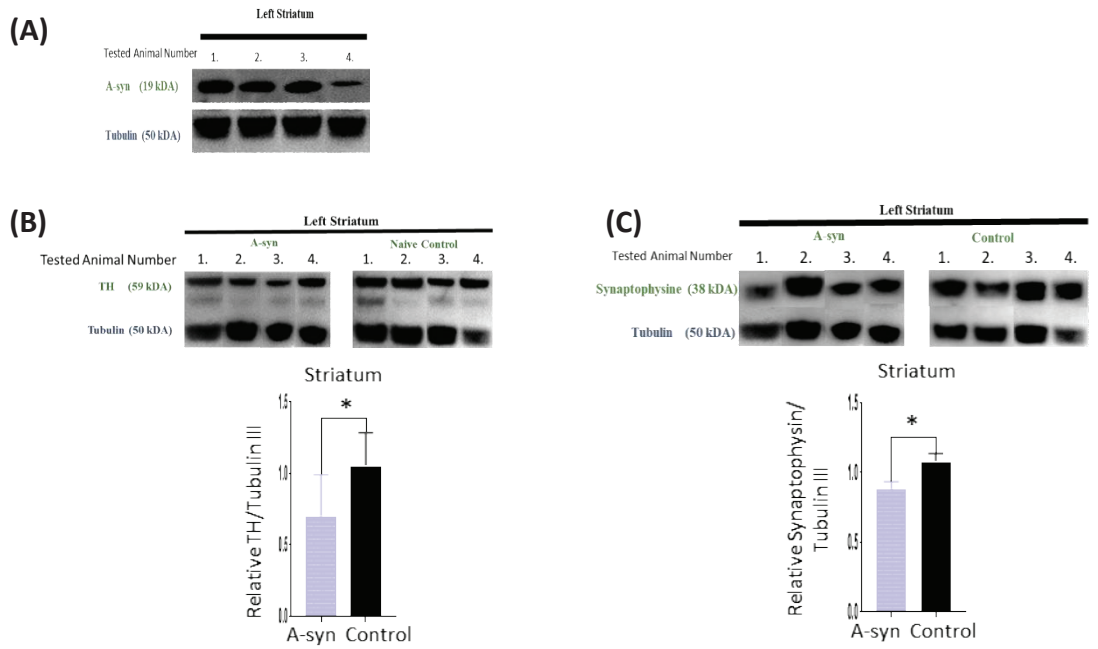

Figure 5: Immunoblotting results: (A) Representative blots confirmed α-syn overexpression in the striatum of each animals, (B) semi-quantitative analysis of TH optical density shows decrease in α-syn group compare to naïve control group (*p<0.05, α-syn vs control; unpaired *Student's t* test was applied), (C) semi-quantitative analysis of synaptophysin optical density shows decrease in α-syn group compare to naïve control group (*p<0.05, α-syn vs control; unpaired *Student's t* test was applied. Graphics are shown as mean±SEM, n=4 α-syn group, n=4 naive control group).

group, which could be due to α-syn accumulation and its effects on synapsis (28, 29). However, this tendency to increase was not seen in the following weeks due to learning of test protocol. In this study, AAV-mediated α-syn expression carried out in DG, too. In our previous study, we showed that, α-syn overexpression bilaterally in SN and DG leads to memory impairment (30). Because of that loss of willingness to move seen in control group, did not observed in α-syn group. Dopaminergic cell loss and in the projection area dopaminergic terminal loss was expecting due to AAV-mediated α-syn accumulation in SN and its projection area in striatum (31-33). In our morphological analysis, α-syn overexpression in the left striatum was confirmed and semi-quantitative analysis of TH and synaptophysin optical density showed that synaptic dysfunction and TH terminal loss in the striatum by Western blotting. With these results, we concluded that partial dopaminergic deprivation leads to movement deficiency in locomotor activity test.

In conclusion, AAV-mediated α-syn injection bilaterally into SN and DG, which is accomplished by our groups for the first time, can cause both motor and cognitive dysfunction besides morphological changes even in the early period. This findings also showed that before the neuronal loss, behavioral changes can be seen and early synaptic dysfunction might be responsible for triggering compensation mechanisms. Therefore, this gives us a prosperous model for studying early stages of Parkinson's disease. On the other hand, behavioral test protocols revision and confirmation of Western blot results by other methods to follow the underlying pathological changes with the time lapse will allow interpreting more precisely.

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