



Modelling Non-motor Symptoms of Parkinson's Disease: AAV Mediated Overexpression of Alpha-synuclein in Rat Hippocampus and Basal Ganglia

Parkinson Hastalığında Motor Dışı Semptomların Modellenmesi: Sıçan Hipokampüsünde ve Bazal Ganglionlarında Alfa-sinükleinin AAV Aracılı Aşırı İfadesi

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Abstract

Objective: Our aim was to investigate the relation between unilateral hippocampal and/or nigral alpha-synucleinopathy and cognitive dysfunction, anxiety and anhedonia.

Materials and Methods: Female Sprague-Dawley rats were stereotactically injected adeno-associated viruses carrying alpha-synuclein (α -syn) into unilateral dentate gyrus (DG), substantia nigra (SN) or both SN + DG. The animals were tested for motor functions and memory, spatial learning, anxiety and hedony. Levels of α -syn and synaptophysin were evaluated by Western blot (WB) analysis.

Results: In apomorphine-induced rotation test, a mild motor dysfunction was found in SN- α -syn group compared to control. DG- α -syn group showed memory impairment in novel object recognition test. All the α -syn injected groups spent more time to find the platform compared to controls in Morris water maze but this difference did not reach statistical significance. DG- α -syn group consumed more sucrose solution in sucrose consumption test and spent more time on the open arm in elevated plus maze, while the opposite was observed in SN- α -syn group compared to controls. We showed α -syn protein expression in the injected areas of all α -syn groups by WB and immunohistochemical staining. In WB analysis, both hippocampal and striatal synaptophysin expression levels were lower in the α -syn groups compared to controls.

Conclusion: Parkinson's disease (PD) is characterized by both motor and non-motor symptoms (NMS). However, an animal model recapitulating NMS with the background of dopaminergic denervation is still lacking. This model may help to investigate hippocampal α -syn pathology correlated especially with cognitive dysfunction and other NMS of PD.

Keywords: Parkinson's disease, adeno-associated viral vector, behavioral tests, hippocampus, substantia nigra

Öz

Amaç: Amacımız, oluşturulan unilateral hipokampal ve/veya nigral alfa-sinükleinopati ile bilişsel disfonksiyon, anksiyete ve anhedoni arasındaki ilişkiyi araştırmaktır.

Gereç ve Yöntem: Dişi Sprague-Dawley sıçanların dentat girus (DG) ve substantia nigra (SN) veya her ikisine DG + SN unilateral olarak adeno-ilişkili virüs- aracılı alfa-sinüklein (α -sin) stereotaksik olarak enjekte edildi. Hayvanlar, motor işlevlerin yanısıra hafıza, uzaysal öğrenme, anksiyete ve hedoninin incelenmesi amacı ile test edildiler. α -sin ve sinaptofizin düzeyleri Western blot (WB) analizleri ile değerlendirildi.

Bulgular: Apomorfın ile indüklenen dönme testinde, SN- α -sin grubunda kontrol grubuna kıyasla hafif motor bozukluk olduğu gözlemlendi. DG-a-sin grubunda yeni nesne tanıma testi ile hafıza bozukluğu olduğu görüldü. α -sin enjeksiyonu gerçekleştirilmiş bütün gruplarda Morris su labirenti testinde platform bulma süresi kontrol grubuna kıyasla daha uzun olsa da gruplar arasındaki bu fark istatistiksel anlamlılığa ulaşmadı. DG- α -sin grubunun sükröz tercih testinde sükröz çözeltisini daha çok tercih ettiği ve yükseltilmiş artı labirenti testinde açık kolda daha çok vakit geçirdiği gözlenirken, SN-a-sin grubunun ise bu testlerde DG- α -sin grubunun tam tersi yönde davranış gösterdiği görüldü. α -sin proteininin bütün enjeksiyon bölgelerinde ifade edildiği, WB yöntemi ve immünohistokimyasal boyama ile gösterildi. Yine WB analizi ile α -sin enjekte edilmiş grupların hipokampus ve striatularında sinaptofizin düzeylerinin kontrollere göre daha az olduğu bulundu.

Sonuç: Parkinson hastalığı (PH) motor ve motor-dışı semptomlarla (NMS) karakterize bir hastalıktır, fakat dopaminergic denervasyon zemininde NMS'lerin gösterildiği bir hayvan modeli henüz geliştirilememiştir. Oluşturulan bu model, PH'de bilişsel disfonksiyon ve NMS'lerin özellikle hipokampal α -sin patolojisi temelinde araştırılmasına yardım edebilir.

Anahtar Kelimeler: Parkinson hastalığı, adeno-ilişkili viral vektör, davranış deneyleri, hipokampus, substantia nigra

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of dopaminergic neurons in substantia nigra (SN) pars compacta as well as widespread expression of Lewy bodies (LB) throughout the brain. Clinical diagnosis is mainly based on the motor symptoms such as tremor, rigidity, postural instability, and bradykinesia. However, there are also non-motor symptoms (NMS) that can be disabling as much as motor symptoms such as neuropsychiatric, sensorial and autonomic disorders. NMS may precede the onset of motor symptoms or occur at any stage of the disease. Hippocampus is thought to be implicated in cognitive decline, anxiety, depression and anhedonia observed in PD (1). Alpha-synuclein (α -syn) is recognized as the key element in pathogenesis of PD. Misfolding and aggregation of the protein lead to formation of toxic oligomers or fibril species and finally Lewy inclusions (2).

For the last several decades there have been many efforts to develop experimental models of PD. Toxin-based models represent end-stage PD, yet they are far from demonstrating the progressive nature of the disease and LB pathology (3). Transgenic models enable the widespread expression of α -syn in the brain. However, results from transgenic mice models are controversial and, most importantly, the selective and progressive nigrostriatal neuron death cannot be achieved (3). Fibril models of α -syn were developed mainly to explore the mechanisms of α -syn propagation (4). Although neurodegeneration and neuroinflammation were also observed in fibril models (5), the behavioral component of the disease has not been investigated hence the controversy exists over results and further validation of the model is necessary.

Viral vectors are effective tools to create the pathology like progressive death of nigral dopaminergic neurons and in many studies, it has been shown that α -syn inclusions in the desired brain region accompanies motor dysfunction (6,7,8,9,10,11,12,13). All of these models have expanded our knowledge about PD etiology, underlying molecular mechanisms and its relation with genetics, while none of them has been capable of replicating all aspects of the disease collectively.

To date, in studies using adeno-associated viruses (AAV), the target has mainly been the nigrostriatal system, which is not likely to lead to development of NMS (14). More recently, there have been attempts to investigate the effects of α -syn overexpression in different brain regions other than nigrostriatal system (15,16,17,18,19,20). However, number of those studies is limited and hippocampal synucleinopathy is yet to be elaborated. Therefore, in this study, we aimed to develop an animal model that represented both motor and NMS together with pathological changes at the cellular level. For this aim, unilateral stereotactic injections of AAV virus type (AAV6) carrying α -syn were performed into dentate gyrus (DG) of hippocampus, SN pars compacta or both areas together. Motor and non-motor impairments, as well as pattern of α -syn expression in target structures, levels of synaptic and neuronal markers were evaluated.

Materials and Methods

Experimental Procedures

Animals

Female Sprague-Dawley rats (aged 8-10 weeks, weighing 220-280 g) were used (n=50). Rats were brought to laboratory at least 2 days before the experiments and kept under standard conditions with *ad libitum* access to food and water, except during sucrose preference test. All experimental procedures were approved by Hacettepe University Local Animal Ethics Committee (decision number: 2014/44-10) and performed in compliance with national and local animal care and use guidelines.

AAV-carrying α -syn was unilaterally injected into DG (n=12), SN (n=12) or SN + DG (n=12). Further, 14 animals were used as naïve controls. Fourteen weeks after the injection, animals were tested for motor and behavioral changes. *In vivo* experiments lasted three weeks (Figure 1A), right after that, at the 17th week, in each group, animals were divided into 2 subgroups to be analyzed by either immunohistochemistry [(IHC), n=25] or Western blot [(WB), n=25].

Surgery

Animals were deeply anesthetized with a mix of ketamine and xylazine and placed in a stereotaxic frame (Stoelting, USA) with the toothbar set to -2.3 mm (Paxinos and Watson) (21). Animals received unilateral injections in either SN or DG alone, or SN and DG together. Coordinates for SN injections were anteroposterior (AP): -5.2 mm and mediolateral (ML): -2.0 mm relative to bregma and dorsoventral [(DV): -7.2 mm] from dura, and for DG injections were AP: -5 mm, ML: -3.5 mm relative to bregma and DV: -2.6 mm from dura. Ten μ l Hamilton syringe fitted with a glass capillary was used for injections. Viral vectors were purchased from Prof. Kirik's Laboratory (BRAINS Unit, Lund University). 2 μ l of vector suspension containing rAAV6- α -syn (5.9E13 vg/ml) was injected slowly at a rate of 0.2 μ l/minute as described previously (22).

Behavioral Tests

Behavioral tests were conducted starting from 14th week after the injection, that was required amount of time for gene expression in target areas, with 2-day intervals between each test (Figure 1A). Animals were handled for 2 days before starting behavioral tests, to reduce the anxiety. Each behavioral test was performed at the same time period of the day (Figure 1B).

Cylinder Test (CT)

For evaluating motor asymmetry expected due to unilateral injection, CT was performed and number of contralateral forelimb contacts on the cylinder wall was analyzed. A glass cylinder (25 cm in diameter) and three mirrors to see touches from all angles were used. The room was kept dim. Rats were put in the cylinder and recorded for ten minutes with a video camera (Sony Handycam DCR-HC37). Recordings were analyzed by a blinded observer. If both limbs touched the wall at the same time, it was counted for both sides. The ipsilateral and contralateral contacts were expressed as the percentage of total number of contacts.

Open Field Apomorphine-induced Rotation Test (AIRT)

In unilateral dopaminergic denervation, dopamine receptors become supersensitive and apomorphine causes contralateral turning behavior in the animals. This was assessed by putting

animals in a black, open box (40x40x30 cm) after subcutaneous injection of 1 mg/kg apomorphine and recorded for 40 minutes with a video camera tracking system. Partial turning behavior (45°) of the rats were analyzed. Results were given as average number of contralateral partial (45°) rotations per each group.

Novel Object Recognition (NOR)

For evaluating short-term memory, NOR test was applied as described previously (22). In short, a black, open box was used. After 2 days of habituation, on the third day, two identical objects were placed in the box and rats were allowed to familiarize with these for 10 minutes. One hour later one of the objects was replaced with a new one and behaviors of rats were recorded for 5 minutes using a video tracking system (EthoVision®XT, Noldus IT, Holland). Ratio of the time spent with the novel object to the total time spent with the novel and the familiar objects was used. Additionally, ratio of the frequency of visits to the novel object to the frequency of total visits to the novel and familiar objects was used as another measure of short-term memory.

Morris Water Maze (MWM)

Animals were tested for spatial learning and memory in MWM as previously described (22). The maze consisted of a pool, and a hidden platform at northeast direction of the theoretically defined four quadrants. The training was conducted on first four consecutive days and each day in 4 sessions. At each session, rats were put into the maze at different starting points (N, W, S, E) and expected to find the hidden platform following the external cues. Cut-off time was 120 seconds. Latency to find the platform and time spent in quadrants were calculated as averages. On day 5, the platform was removed and a probe trial was performed. All rats were put into the maze from south and expected to find the former place of the hidden platform in 90 seconds. Trials were recorded using a video camera tracking system (VideoMot2, TSE Systems, Germany). Latencies to find the former place of platform and time spent in each quadrant were analyzed.

Sucrose Preference Test (SPT)

For assessing hedonia, SPT was performed; as sucrose solution consumption was reduced in rats exposed to chronic stressors and this represented anhedonia. For this test, rats were placed in separate cages as per their groups. For the first two days, rats were given both tap water and 2% sucrose solution for them to habituate to sucrose solution. At the end of day 1, the sides of the bottles were switched to prevent side-preference bias. At the end of day 2, all bottles were removed from the cages and rats were deprived of water for 8 hours. Pre-weighed bottles, containing tap water or 2% sucrose solution, were placed on the cages for 12 hours then their sides were switched for another 12 hours. At the end of 24 hour, bottles were weighed. Results were given as percentage of the 2% sucrose solution consumption to total liquid consumption.

Elevated Plus Maze (EPM)

To assess anxiety-like behavior, EPM test was performed as described earlier (22). Plus-shaped maze consisted of 2 closed and 2 open arms, all accessible from the centre. Rats were put in the centre facing the closed arm and then were recorded for 5 minutes with the video tracking system. Time spent in centre, open and closed arms were analyzed. Results were given as ratio of time spent on open arms to total time and time spent in closed arms to total time.

Western Blot

Animals were asphyxiated with carbon dioxide, decapitated and the brains were rapidly extracted. Both right and left hippocampi and striata were dissected. Basic procedure described in earlier was followed (22). In short; tissues were homogenized with lysis buffer and protease inhibitor cocktail. Protein concentration was determined using BCA protein assay kit (23225, Thermo Fisher Scientific). Samples were centrifuged with LDS Sample buffer (NP0008, Thermo Fisher Scientific, USA) and β -mercaptoethanol at 2,500 rpm, then boiled for 5 minutes at 90 °C and rapidly cooled down. Protein separation was performed at 90 V for 120 minutes, on 4-12% sodium dodecyl sulfate-polyacrylamide gels (Nu-PAGE™ 4-12% bis-tris, Thermo Fisher Scientific, USA) using proper running buffer. Then proteins were electrotransferred to polyvinylidene fluoride membranes. Blocking was performed for 1 hour with 5% non-fat dry milk containing buffer solution. After blocking, membranes were incubated either with anti- α -syn primary antibody (1:1000, ab80627, Abcam) or anti-synapthophysin primary antibody (1:1500, S 5768, Sigma Aldrich) overnight or as a loading control with anti-beta-tubulin-III for 40 minutes at +4 °C. After washing in tris-buffered saline (TBS)-tween solution, membranes were incubated with one of the following secondary anti-bodies; anti-mouse or anti-rabbit (7076 and 7074, Cell signalling technology Inc.) at room temperature (RT) for 1h. Membranes were washed gently in TBS-tween. Signal was detected with a ready-to-use kit (34095, Thermo Scientific) and with the imaging device Image Station 4000MM (Kodak). Band intensities were measured by Image J 1.49v (NIH, USA). The results were given as the ratio of optical densities of each band to that of beta-tubulin III.

Immunohistochemistry

Under terminal anesthesia, animals were perfused transcardially with heparine followed by 4% paraformaldehyde infusion. Brains were cut into 35 μ m-thick coronal slices with microtome (Leica SM2000 R, Leica Biosystems). Immunohistochemical staining was performed with Acu-Stain HRP Kit (52-0003, Genemed) on free-floating slices. All steps were carried out at RT as described earlier (22). In short, slices were rinsed with Tris-HCl solution then quenched for 20 minutes and rinsed again with Tris buffer. Then they were pre-incubated with blocking solution from the kit for 10 minutes. After blocking, slices were incubated with monoclonal anti- α -syn (1:1000, ab80627, Abcam) or anti-tyrosine hydroxylase (TH) (1:1000, ab75875, Abcam) antibodies overnight, rinsed 3 times and incubated with biotinylated secondary anti-body solution from the kit for 10 minutes. After rinse, slices were incubated with streptavidin-peroxidase complex for 10 minutes and rinsed for the last time, then they were developed by 3,3-diaminobenzidine as a chromogen and H₂O₂ as a catalyst (Power-Stain™ 1.0 Poly HRP DAB Kit, Genemed), mounted on slides and cover slipped with DPX mounting medium.

Statistical analysis

Data were presented as mean \pm standard error of the mean. The significance threshold was set at 0.05. One-Way ANOVA was used for multiple comparisons. Post hoc analyses were made either by Tukey's or Sidak test. All data were analyzed in Graphpad PRISM v6.0 software.

Results

Motor and Cognitive Behavioral Changes Due to Alfa-synuclein Overexpression in SN and/or DG

Open field locomotor activity test showed no difference between the groups in terms of basal locomotor activity (Figure 1C, D). In CT, the ratio of contralateral forelimb contacts to total contacts were lower in all α -syn groups compared to naïve controls however, it did not reach statistical significance (Figure 1E). AIRT, the number of partial rotations were higher in SN α -syn group and this difference was statistically significant compared to naïve control and SN + DG α -syn groups (* $p < 0.05$ SN α -syn vs naïve, # $p < 0.05$ SN α -syn vs SN + DG α -syn; Figure 1F). Thus, unilateral α -syn overexpression in SN caused only mild unilateral motor impairment.

We tested the short-term memory by NOR test. The recognition ability of DG α -syn group which was shown by spending less time with the novel object, was significantly lower than SN + DG α -syn group but not than controls (* $p < 0.05$ DG α -syn vs SN + DG α -syn; Figure 2A). In addition, this decrease in cognition was not observed in SN α -syn group ($p > 0.05$; Figure 2A), so α -syn injection directly to DG could be essential for cognitive impairment.

The learning curves of MWM showed that all groups achieved the learning phase, which was finding the platform less than 120 seconds at the end of fourth day (Figure 2B). In the probe trial,

latency to find the platform was highest in DG α -syn group and SN α -syn and SN + DG α -syn groups slightly spent more time compared to controls, however, without statistical significance (Figure 2C). The time spent in the platform area was similar between groups (Figure 2D) confirming that there was no motor impairment which may have had an impact on the swimming ability of the animals. DG α -syn pathology could be important for spatial learning.

In sucrose preference test, sucrose solution consumption of SN α -syn group was significantly lower than SN + DG α -syn group, whereas it was significantly higher in DG α -syn group compared to naïve controls (* $p < 0.05$ SN α -syn vs SN + DG α -syn, # $p < 0.05$ DG α -syn vs controls; Figure 2E), thus SN α -syn pathology could be more related to anhedonia.

In EPM test, DG α -syn animals spent the most time in the open arms but it was only significant compared to SN + DG α -syn animals ($p < 0.05$ DG α -syn vs SN + DG α -syn; Figure 2F). Even though SN α -syn group spent less time than controls and SN + DG α -syn group, it did not reach statistical significance ($p > 0.05$; Figure 2F), thus DG α -syn pathology could be more related to anxiety.

Morphological Changes due to Alfa-synuclein Overexpression in SN and/or DG

Immunoblot analysis revealed 14 kDa bands in striata of SN and SN + DG groups and hippocampi of DG and SN + DG groups

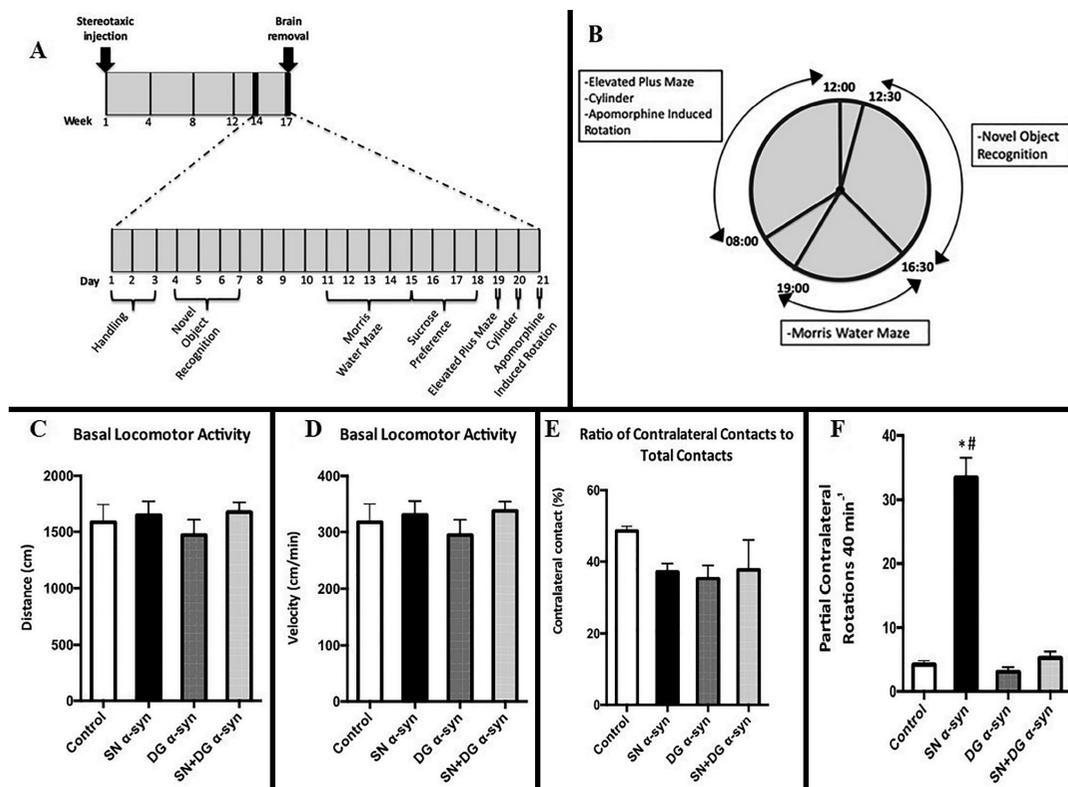


Figure 1. Study timeline and motor tests results. A) Experimental design, B) time slots for behavioral and motor tests, C) distance moved and D) velocity in Basal Locomotor Activity Test did not differ across groups, n (control)=10, n (SN α -syn)=11, n (DG α -syn)=4, n (SN + DG α -syn)=5; $p > 0.05$, E) in cylinder test; decreased contralateral forelimb use in α -syn injected animals was not significant compared to the control. $n=12$, n (SN α -syn)=10, n (DG α -syn)=9, n (SN + DG α -syn)=9; $p > 0.05$. F) α -syn injection to solely SN induces a robust partial turning behavior, while injections to SN and DG in combination lack the same outcome $n=14$, n (SN α -syn)=11, n (DG α -syn)=10, n (SN + DG α -syn)=11; $p < 0.05$ vs. control, $p < 0.05$ vs. SN + DG α -syn, All analysis were performed using One-Way ANOVA followed by Sidak's multiple comparison test. Data were presented as mean \pm SEM. SN: Substantia nigra, DG: Dentate gyrus, α -syn: Alpha-synuclein, SEM: Standard error of the mean

confirming that α -syn overexpression was successful in the target areas (Figure 3A). Immunohistochemical analysis showed distinct patterns of α -syn overexpression. In SN and striatum, α -syn labelling was widespread and unilateral, restricted only to the side of injection, whereas in hippocampus it was bilateral, being more prominent on the injection side (Figure 3B).

In order to assess the degree of neuronal loss; nigral and striatal sections from SN and SN + DG groups were stained with anti-TH and hippocampal sections from DG and SN + DG groups, with anti-NeuN anti-bodies. In α -syn animals, unilateral loss of TH immunoreactivity was observed both in SN and striatum (Figure 4) while in the hippocampus no such difference in NeuN labelling was detected between two sides (data not shown).

For the assessment of synaptic integrity, synaptophysin levels in striatum and/or hippocampus were measured by Western blotting. There was a decrease in synaptophysin levels in all α -syn groups compared to naïve controls although this difference reached statistical significance only for DG α -syn group (* p <0.05 DG α -syn vs. SN+DG α -syn, # p <0.05 DG α -syn vs. control; Figure 5).

Discussion

Our primary aim was to establish an animal model that mimicked both motor and NMS of PD. We chose AAV approach to induce α -syn pathology in SN and/or DG and then conducted

a set of behavioral tests. We further assessed whether molecular changes were related to motor and/or non-motor impairments.

In PD NMS affect patient's life standards as much as motor symptoms. After stage IV, PD patients start to have cognitive problems (23). Dementia, which is seen in the late-stage of PD, is known to be related to the involvement of hippocampal subregions. The relation between hippocampal pathology and neuropsychiatric non-motor aspects of PD such as cognitive dysfunction, psychosis and anxiety have been shown in several studies (24,25,26,27). In a study about α -syn pathology in the hippocampus, DG and specifically CA2 region were shown to be more vulnerable to α -syn involvement in both human and transgenic animals (25). In other studies it has been shown that, especially D1 receptor mediated dopaminergic pathways in the hippocampus are important in spatial learning and memory (24,28). In fact, when we planned targeting hippocampal complex for AAV-mediated α -syn overexpression, the first injection site we had chosen was entorhinal cortex (EC), depending on the postmortem and experimental data. But unfortunately, despite our rigorous attempts, we had failed to target EC consistently because of technical difficulties. Since our aim was to study the impact of induced α -syn pathology in hippocampus proper, we had changed our target to DG, as being the main projection area of EC in hippocampal circuitry.

α -syn overexpression in target areas was confirmed by immunoblotting and IHC. Variable expression levels were

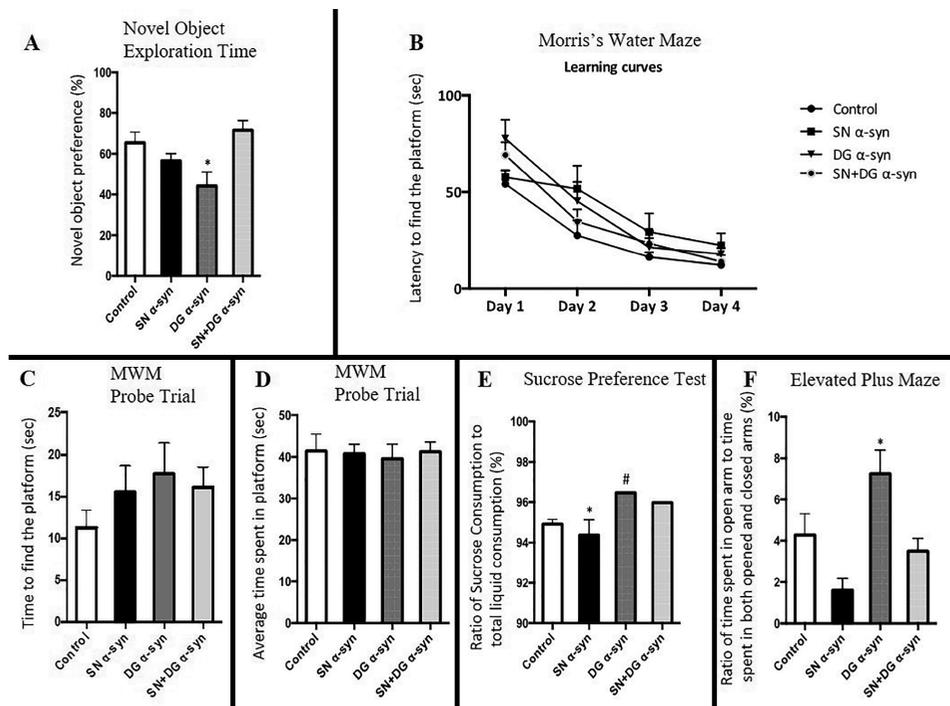


Figure 2. Behavioral test results. A) Short-term memory was found to be slightly impaired in SN- and DG-alpha-synuclein groups. n (control)=12, n (SN α -syn)=11, n (DG α -syn)=4, n (SN + DG α -syn)=11; p *<0.05 vs SN + DG α -syn. B) Average latencies of all groups on the training days. α -syn expression did not cause significant alterations in Morris Water Maze performance. C) Latency to find the platform on the 5th day. n (control)=12, n (SN α -syn)=11, n (DG α -syn)=10, n (SN + DG α -syn)=12. D) Time spent in the platform area on the 5th day. n (control)=8, n (SN α -syn)=5, n (DG α -syn)=4, n (SN + DG α -syn)=6. p >0.05. E) Effect of α -syn overexpression on sucrose consumption. n (control)=14, n (SN α -syn)=6, n (DG α -syn)=6, n (SN + DG α -syn)=6; p *<0.05 vs. SN + DG α -syn, p #<0.05 vs control. F) Effect of α -syn overexpression on anxiety-like behavior n (control)=13, n (SN α -syn)=9, n (DG α -syn)=9, n (SN + DG α -syn)=11; p *<0.05 DG α -syn vs SN + DG α -syn. All analysis were performed using One-Way ANOVA followed by Sidak's multiple comparison test. Data were presented as mean \pm SEM

SN: Substantia nigra, DG: Dentate gyrus, MWM: Morris water maze

observed in striata and hippocampi. As previously reported, this could be due to variable transduction characteristics of the AAV6 serotype in distinct brain regions (29).

Motor impairment was assessed by CT and AIRT. In CT, contralateral forelimb use did not show significant difference in any of the groups compared to controls. This result might be due to insufficient striatal dopaminergic denervation leading to just subclinical changes (9,10). In AIRT, only SN α -syn animals displayed circling behaviour, but the number of turns were found to be low. Again, this might be due to subthreshold striatal denervation which did not cause significant dopaminergic receptor supersensitivity. To our surprise, apomorphine did not induce rotational behaviour in SN + DG α -syn animals. As it was previously put forth, hippocampus and striatum interact with each other, but the molecular mechanisms were not yet clear (30,31,32). This interaction might be responsible for the suppression of denervation supersensitivity.

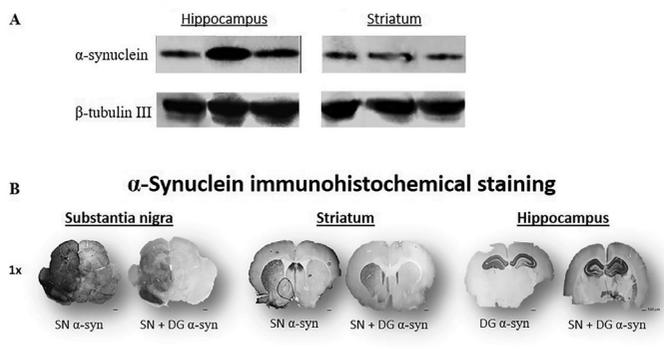


Figure 3. Alpha-synuclein (α -syn) overexpression in all targeted areas. A) Representative Western blots showing expression in hippocampal and striatal tissues. B) Immunohistochemistry staining showed that the α -syn was present unilaterally in striatum and SN and bilaterally in hippocampus (images taken at 1x magnification, scalebars: 500 μ m) SN: Substantia nigra, DG: Dentate gyrus

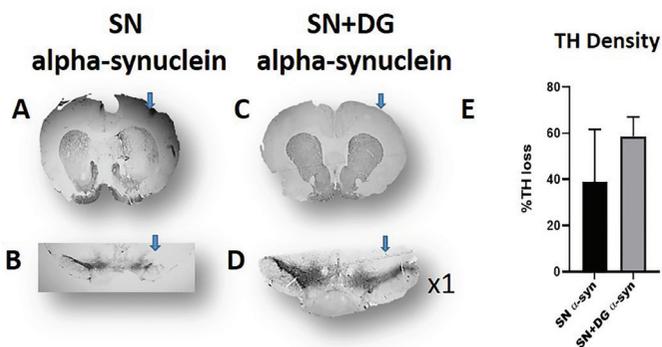


Figure 4. Immunohistochemical tyrosine hydroxylase staining of SN α -syn injected groups A) striatum, and B) substantia nigra in which indicated TH terminal loss in the pointed side of arrow. SN + DG α -syn injected groups, C) striatum, and D) substantia nigra in which contrary to only SN injection, detectable TH terminal loss was not seen qualitatively in these sections, E) quantitative analysis of TH density and % TH density loss compared to intact side by ImageJ SN: Substantia nigra, DG: Dentate gyrus, α -syn: Alpha-synuclein, TH: Tyrosine hydroxylase

Memory function was evaluated in NOR test. Short-term memory performances of α -syn animals did not show significant difference compared to controls. At present, neurobiological basis of cognitive impairment in PD is not fully known. Preclinical and clinical data indicate involvement of several brain regions including hippocampus (33,34), prefrontal cortex (35,36) and basal ganglia (24). We thought that although unilateral AAV- α -syn injection led to bilateral and widespread α -syn overexpression in the hippocampus, it did not induce sufficient damage in memory related networks in the medial temporal lobe structures and frontostriatal circuits in our study. Besides, it was also possible that compensation mechanisms which took over in time, played a role preserving the memory functions.

Visuospatial learning and memory were tested by MWM. In probe trial, latencies to find the platform were found to be increased in all α -syn groups compared to controls but this did not reach statistical significance. Besides, average time spent in the platform area was similar between groups. It is known that both hippocampal and striatal memory systems have a role in spatial navigation (37). But, unlike cued version of MWM, SN lesions do not affect test performance in the spatial version. Thus, our results from SN α -syn animals were consistent with the available data. Regarding the preserved memory in DG α -syn and SN + DG α -syn groups, a plausible explanation could be that, bilateral and widespread α -syn expression in the hippocampus did not lead to functional consequences in spatial learning and memory (38).

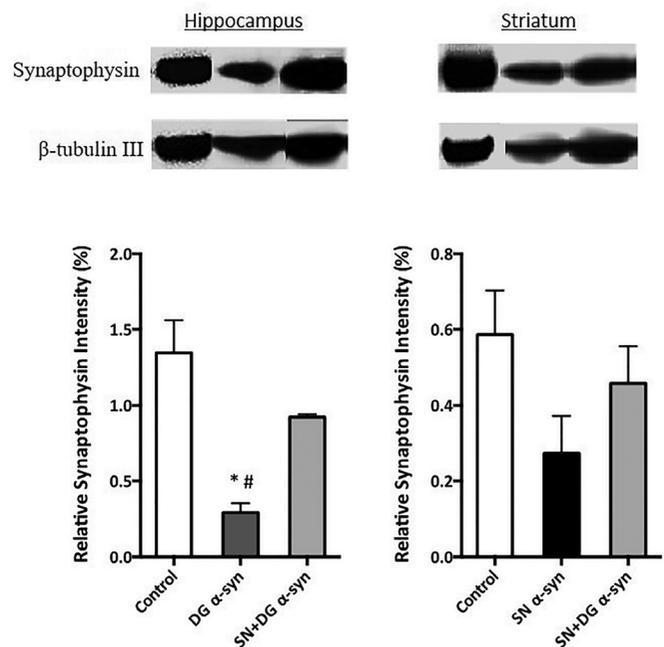


Figure 5. Effect of alpha-synuclein (α -syn) overexpression on synaptophysin levels. AAV- α -syn injection to DG or SN alone resulted in greater decrease of synaptophysin levels compared to injection to both SN and DG. Hippocampus; n (control)=5, n (DG α -syn)=4, n (SN + DG α -syn)=5, striatum; n (control)=5, n (DG α -syn)=5, n (SN + DG α -syn)=5; $p^* < 0.05$ DG α -syn vs SN + DG α -syn, $p^{\#} < 0.05$ DG α -syn vs control. One-Way ANOVA followed by Tukey's multiple comparison test. The data were presented as mean \pm SEM SN: Substantia nigra, DG: Dentate gyrus, SEM: Standard error of the mean

Anhedonia was assessed by SPT. SN $_{\alpha\text{-syn}}$ animals showed a decreased tendency to consume sucrose solution compared to controls. This finding was consistent with the past studies (39) and could be explained by the fact that SPT measured consummatory but not anticipatory anhedonia. Consummatory anhedonia is suggested to be related to μ opioid, endocannabinoid, GABAA receptors, whereas dopamine is more connected with anticipatory anhedonia (40). On the other hand, as we observed a tendency to anhedonic behaviour, it was tempting to suggest that striatal dopaminergic deficit also contributed to consummatory anhedonia. Our analysis also revealed a significantly higher sucrose solution intake in both DG $_{\alpha\text{-syn}}$ and SN + DG $_{\alpha\text{-syn}}$ groups relative to control, being significant in the first one. There is evidence that hippocampal lesions result in higher response rates to rewarding stimuli (41). This finding was suggested to be related to modulation of NAc by hippocampus (41). In support of this view, it is possible that AAV- $\alpha\text{-syn}$ injection to DG causes hedonic behaviour by disrupting glutamatergic modulation of NAc.

Anxiety was measured using EPM test. Anxiety levels of SN $_{\alpha\text{-syn}}$ group tended to be higher than controls. It is clear that dopaminergic dysfunction contributes to anxiety (42). DG $_{\alpha\text{-syn}}$ group displayed reduced anxiety behavior compared to controls. Past studies, including a recent study by our group, reported that bilateral hippocampal lesions resulted in decreased anxiety (22,43). As the reduction we found was not significant, it was likely that unilateral hippocampal $\alpha\text{-syn}$ injection was not sufficient for a complete anxiolytic effect (44). On the other hand, it is intriguing to think that tendency to reduced anxiety might be the result of interneuronal spreading of $\alpha\text{-syn}$ to the contralateral hippocampus leading to a bilateral expression of $\alpha\text{-syn}$ (Figure 3). Considering the opposite effects of $\alpha\text{-syn}$ overexpression in SN and DG, it is possible not to observe any changes with overexpression in both areas.

Synaptic loss was investigated by measuring synaptophysin levels in striata and hippocampi in experimental groups accordingly. $\alpha\text{-syn}$ overexpression led to a decrease in synaptophysin levels in all groups. It was only significant in hippocampus of DG $_{\alpha\text{-syn}}$ animals compared to controls and also compared to SN + DG $_{\alpha\text{-syn}}$ animals. Interestingly, regardless of significant levels, the decrease was lower in both hippocampus and striatum of SN + DG $_{\alpha\text{-syn}}$ animals, compared to DG $_{\alpha\text{-syn}}$ and SN $_{\alpha\text{-syn}}$ animals, respectively. As outlined in the multiple-memory systems theory, hippocampus and basal ganglia are both involved in processing different types of memory. It is known that these structures do not work in isolation, in fact interfere with each other. Evidence shows that there is a bidirectional competition between hippocampal and striatal memory systems, such that damage to one system leads to improvement in another, and vice versa (30,31). Indeed, from the current study, NOR (Figure 2A) and MWM (Figure 2C) data might be the indicators of such interaction. It was not clear yet how these two systems influenced each other at the cellular level, however it was argued that altered synaptic plasticity, more specifically cAMP/CREB levels, in those structures might contribute to bidirectional competition (31,44,45,46). Thus, we may suggest that synaptophysin might be involved in the interplay between hippocampus and striatum, and the pathological changes in one might result in elevated synaptophysin levels in another, due to a compensatory increase in synapse formation.

Study Limitations

There are some limitations of this study. First, unilateral $\alpha\text{-syn}$ injection might not be sufficient to induce neurodegeneration at an adequate level in the target regions. Second, when evaluating NMS, it should be considered that hippocampus and striatum interact with each other and they both might be involved in neuropathology of NMS. Third, brain regions, other than hippocampus and basal ganglia, and non-dopaminergic systems also have an impact on NMS and these may be involved as targets of AAV- $\alpha\text{-syn}$ injections for further combinations. Future research that overcomes above mentioned limitations will give us valuable insights about the pathophysiology of NMS in PD.

Conclusion

In this model, our main objective was to investigate the relation between NMS and unilateral hippocampal and nigral $\alpha\text{-synucleinopathy}$. Although $\alpha\text{-syn}$ overexpression in the target brain regions was successful, the NMS that were tested in detail did not show much significant changes. The data presented here may provide basis for similar studies in the future.

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Ethics

Ethics Committee Approval: All experimental procedures were approved by Hacettepe University Local Animal Ethics Committee (decision number: 2014/44-10) and performed in compliance with national and local animal care and use guidelines.

Informed Consent: Not applicable.

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Authorship Contributions

Surgical and Medical Practices: S.U.M., E.Ç., Concept: A.U., B.E., B.C.T., Design: S.U.M., E.Ç., G.Y.Ç., B.C.T., Data Collection or Processing: S.U.M., E.Ç., Analysis or Interpretation: S.U.M., E.Ç., B.C.T., Literature Search: S.U.M., Writing: S.U.M.

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