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# Emotional memory impairments induced by AAV-mediated overexpression of human α-synuclein in dopaminergic neurons of the ventral tegmental area

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## HIGHLIGHTS

- α-synuclein overexpression in the VTA caused a 22% reduction of TH+ neurons in VTA.
- α-synuclein overexpression in VTA increased the number of stepping errors in the ledged beam test.
- α-synuclein overexpression in VTA impaired emotional memory in the passive avoidance test.

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## ABSTRACT

Parkinson's disease (PD) is associated with extensive degeneration of dopaminergic neurons originating in the substantia nigra pars compacta, but neuronal loss is also found in the ventral tegmental area (VTA). The VTA projects to areas involved in cognitive and emotional processes, including hippocampus, amygdala, nucleus accumbens and prefrontal cortex, and has thus been proposed to play a role in emotional memory impairments in PD. Since the formation of α-synuclein inclusions throughout the central nervous system is a pathological hallmark of PD, we studied the progressive effects of α-synuclein overexpression in the VTA on motor functions, emotional behaviour and emotional memory. Adeno-associated viral (AAV) vectors encoding either human α-synuclein or green fluorescent protein (GFP) were injected stereotactically into the VTA, and behaviour was monitored 3 and 8 weeks following AAV injection. At week 8, there was a 22% reduction of TH+ neurons in the VTA. We demonstrate that α-synuclein overexpression in dopaminergic neurons of the VTA induced mild motor deficits that appeared 3 weeks following AAV-α-synuclein injection and were aggravated at week 8. No depressive- or anxiety-like behaviours were found. To address emotional memory, we used the passive avoidance test, a one-trial associative learning paradigm based on contextual conditioning which requires minimal training. Interestingly, emotional memory impairments were found in α-synuclein overexpressing animals at week 8. These findings indicate that α-synuclein overexpression induces progressive memory impairments likely caused by a loss of function of mesolimbic dopaminergic projections.



Parkinson's disease (PD) is a movement disorder characterized by the accumulation of α-synuclein-enriched Lewy bodies and loss of dopaminergic neurons originating in the substantia nigra (SN) pars compacta projecting to the striatum [1]. However, neurodegeneration in PD is not restricted to SN, but also affects the ventral tegmental area (VTA) where α-synuclein aggregates are found also in early stages of the disease [1]. The VTA projects to several brain regions involved in emotional and cognitive processes, including hippocampus [2], nucleus accumbens, amygdala and prefrontal cortex [3]. Although neurons of the VTA are less prone to degenerate in PD compared with neurons of the SN [4], pathological lesions of the mesohippocampal and mesocortical projections have been implicated in the emotional and cognitive symptoms of PD [5,6].

*Abbreviations:* AAV, adeno-associated virus; GFP, green fluorescent protein; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area; WT, wild-type.

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Indeed, both emotional and cognitive impairments are common in PD, appear early, and are often not responsive to antiparkinsonian treatments [5,6].

Since the role of the VTA in the symptomatology of PD remains elusive, the effects of α-synuclein overexpression in this region may provide specific insights into mechanisms underlying PD. We here overexpressed α-synuclein specifically in the VTA using an adeno-associated virus (AAV) vector construct, aiming to evaluate the pathological and behavioural impact. Motor functions, emotional behaviour and emotional memory were assessed at two time points; at 3 and 8 weeks after the bilateral vector injections. Animals were sacrificed after the last behavioural test, 8 weeks after AAV injections, and midbrain and caudal sections were stained for TH for assessment of TH+ neuron loss.

We used an AAV6-α-synuclein vector construct to overexpress human α-synuclein or green fluorescent protein (GFP). Transgenic expression was driven by the human synapsin-1 promoter and enhanced using a woodchuck hepatitis virus posttranscriptional regulatory element, as described previously [7]. Genome copy titers were $7.7 \times 10^{14}$ genome copies/ml, as determined using real time quantitative PCR. An equivalent number of genome copies ($2.3 \times 10^{11}$ genome copies/3 μl) were injected in both groups.

Adult female Sprague Dawley rats were housed 4 per cage under a 12 h light/dark cycle with access to food and water *ad libitum*. Experiments were performed in agreement with the European Council Directive (86/609/EEC) and approved by the local Animal Ethics Committee (Stockholms Norra Djurförsöksetiska Nämnd, ethical permits N524/11 and N62/13). Surgical procedures were adapted from previous protocols [7]. A mix of ketamine/xylazine (90/10 mg/kg, i.p.) (Apoteket, Solna, Sweden), diluted in saline, was used for general anaesthesia. Rats were placed in a stereotaxic frame (Stoelting co., Wood Dale, IL, USA), and vector solutions were injected bilaterally using a 10 μl Hamilton syringe fitted with a glass capillary (outer diameter: 250 μm). The coordinates for the injections were −5.3 mm (antero-posterior), −/+0.5 mm (medio-lateral) and −7 mm (dorso-ventral) relative to bregma (flat skull position) [8]. 3 μl of viral vector solution (AAV6-GFP or AAV6-α-synuclein) was infused at a rate of 0.2 μl per minute. The capillary was left in place for one minute, slowly moved 1 mm upwards and left in place for 1 additional minute. The capillary was cleaned between injections with 30% $H_2O_2$, 70% EtOH and $dH_2O$. A combination of atipamezole (0.264 mg/kg) and buprenorphine (0.036 mg/kg) (Apoteket) was injected immediately after surgery to reverse anaesthesia and provide pain relief.

Eight weeks after the AAV-α-synuclein injection, and subsequent to the last behavioural test, rats were deeply anaesthetised with a mix of ketamine/xylazine (90/10 mg/kg, i.p.) (Apoteket) and perfused through the ascending aorta with 40 ml 0.1 M phosphate buffer and 80 ml ice-cold paraformaldehyde (4% w/v in 0.1 M sodium phosphate buffer). Brains were removed, post-fixed for 24 h in 4% paraformaldehyde, cryoprotected in sucrose (30% w/v in 0.1 M phosphate buffered saline) for 72 h, and sectioned on a freezing microtome (Leica, Wetzlar, Germany). 35 μm-thick coronal sections were collected in 6 different series. Immunohistochemical stainings were performed on free-floating sections using antibodies raised against tyrosine hydroxylase (TH) (rabbit 1:2000; Chemicon AB 152), GFP (chicken 1:1000; AbCam ab13970) and human α-synuclein (mouse 1:1000; Santa Cruz Biotechnology sc12767). The sections were rinsed three times in phosphate buffered saline containing potassium and 0.25% Triton X-100 between incubations. Sections were quenched for 10 min in 3% $H_2O_2$ and 10% methanol. Pre-incubation for one hour with 5% normal goat, horse and rabbit serum was followed by incubation with the primary antibody in 2% serum (room temperature, overnight), incubation with 1:200 dilutions of biotinylated goat anti-rabbit (BA 1000, for TH), goat anti-chicken (BA9010, for GFP) or horse anti-mouse (BA 2001, for α-synuclein) antibodies (Vector Laboratories, Peterborough, UK), followed by avidin–biotin–peroxidase complex (ABC Elite; Vector Laboratories). Stainings were visualised using 3,3-diaminobenzidine as a chromogen and $H_2O_2$ as a catalyst, mounted and cover-slipped with DPX mounting medium. Every 6 sections were included in the TH+ cell counting, which was made by hand. Data were analysed using Student's unpaired *t*-test. We observed a decrease in the number of TH+ neurons in the VTA (22% degeneration, $p < 0.05$), but not in the SN ($p = 0.79$) (Fig. 1).

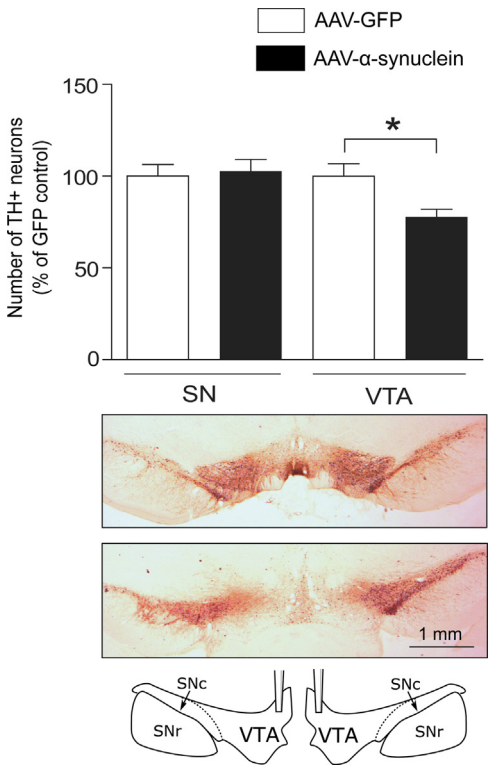


**Fig. 1.** Number of TH+ neurons in SN and VTA following bilateral AAV-α-synuclein injections into the VTA. Animals were sacrificed after the last behaviour test 8 weeks after vector injection. AAV-α-synuclein injections into the VTA did not affect the number of TH+ cell bodies in the substantia nigra, but caused a reduction in TH+ cell bodies in the VTA. Data are expressed as a percentage of corresponding AAV-GFP control animals ± s.e.m. $^*P < 0.05$ (Student's unpaired *t*-test).

To assess the functional consequences of human α-synuclein overexpression in VTA dopaminergic neurons, we performed behavioural studies at two time points, 3 and 8 weeks after the bilateral vector injections, corresponding to the pre-symptomatic and symptomatic stages of PD, respectively [7].

The VTA sends dopaminergic projections to the amygdala, nucleus accumbens, medial prefrontal cortex and hippocampus which are required for the formation and expression of emotional contextual memories [2,3]. To evaluate the impact of human α-synuclein overexpression in VTA on emotional memory, we performed the passive avoidance (PA) test and assessed the step-through latency and place preference. The PA paradigm is based on Pavlovian fear-conditioning [9], and highly dependent on the amygdala and hippocampus [10]. The PA apparatus consisted of a brightly lit compartment with white walls and a dark compartment with black walls connected via a sliding door. By coupling the innately preferred dark compartment with an aversive stimulus, the animal can be taught to prefer the light compartment to the dark compartment. During the PA training phase, rats explored the bright compartment (light intensity: 1000 lux) for 60 s before the door to the dark compartment was opened remotely. When the rat stepped through into the dark compartment with all four