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 hedged beam test.
• α-synuclein overexpression in VTA impaired emotional memory in the passive avoidance test.

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.

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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 adenovirus-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 ($2.3 \times 10^{14}$ 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 accordance with the European Council Directive (86/609/EEC) and approved by the local Animal Ethics Committee (Stockholms Norra Djurförsökssetiskta Nämnd, ethical permits NS24/11 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 (Stoeling 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 $-5.3 \text{ mm (antero-posterior), } -/+/0.5 \text{ mm (mediolateral) and } -7 \text{ mm (dorso-ventral) relative to bregma (flat skull position) [8].}$ 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% H2O2, 70% EtOH and dH2O. 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% H2O2 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 H2O2 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
paws the door was closed, and a weak electrical stimulus (0.5 mA, 2 s scrambled current) was delivered through the grid floor. The rat was removed from the dark compartment 30 s after step-through. Twenty-four hours later, the rat was again placed into the light compartment. The sliding door was opened after 10 s, and the step-through latency to return to the dark compartment was measured during a 540 s time period. The retention latency, i.e. the time duration before the animal entered the dark compartment during the PA retention test, was used as a measure of emotional contextual memory. The number of entries into the dark compartment and the total time spent in the dark compartment during the test phase were manually measured. All behavioural data was analysed using repeated measures analyses of variance (ANOVA) followed by Fisher’s least significant difference (LSD) post hoc test.

During the PA training session the step-through latencies did not differ between AAV-α-synuclein and AAV-GFP-injected control animals (Fig. 2A). However, AAV-α-synuclein-injected animals displayed alterations in emotional memory compared with AAV-GFP-injected animals during the PA memory test (treatment: $F_{(1,14)} = 6.10, p < 0.05$; time: $F_{(1,14)} = 14.23, p < 0.01$; interaction: $F_{(1,14)} = 0.90, p = 0.36$) (Fig. 2A). The AAV-α-synuclein group displayed a significantly shorter step-through latency 8 weeks after injection compared with the AAV-GFP control group (Fisher’s LSD post hoc test: $p < 0.05$). Whereas the AAV-GFP group had a significantly longer step-through latency at week 8 compared with at week 3 after AAV-injection ($p < 0.01$), AAV-α-synuclein-injected animals did not display a significantly longer step-through latency at the later time point. The AAV-GFP group spent a significantly shorter time in the dark compartment at week 8 compared with at week 3, indicating avoidance of the dark compartment (treatment: $F_{(1,15)} = 9.14, p < 0.05$; time: $F_{(1,15)} = 8.76, p < 0.01$; interaction: $F_{(1,15)} = 0.56, p = 0.47$) (Fisher’s LSD post hoc test: $p < 0.05$) (Fig. 2B). The AAV-α-synuclein group spent a significantly longer time in the dark compartment at week 8 compared with the AAV-GFP group ($p < 0.05$), consistent with the shorter step-through latency seen at this time point, indicating an impairment of emotional contextual memory (Fig. 2B).

To make a basic assessment of sensorimotor functions, animals were trained on a tapered/ledged beam-walking test, adapted from a previously described procedure [11]. Rats were trained to walk along a progressively narrowing Plexiglas beam, elevated above the floor with an incline of 15°, to reach their home cage (beam length: 165 cm; width: 6.5 cm at the wide end, 1.5 cm at the narrow end). The surface of the beam was covered in rubber matting to provide traction. Two cm below the beam was a 2.5 cm-wide Plexiglas ledge providing a platform to prevent the rats from falling off the beam. Taking a step with only one or two toes placed on the main surface of the beam and the other toes overhanging the ledge was scored as a half-foot-fault. Stepping with the entire foot on the ledge rather than on the main surface of the beam was scored as a full-foot-fault. Before testing, each animal was allowed one refresher trial which was not videotaped. One test consisted of 3 consecutive trials videotaped from the rear to allow a clear observation of the hindlimbs. The AAV-α-synuclein and AAV-GFP groups required the same amount of time to cross the beam at both time points (treatment: $F_{(1,14)} = 0.007, p = 0.94$; time: $F_{(1,14)} = 2.03, p = 0.18$; interaction: $F_{(1,14)} = 2.18, p = 0.16$) (Fig. 3A). However, animals injected with the AAV-α-synuclein construct displayed a higher number of errors per step compared to the AAV-GFP group (treatment: $F_{(1,14)} = 10.47, p < 0.01$; time: $F_{(1,14)} = 3.59, p = 0.079$; interaction: $F_{(1,14)} = 0.003, p = 0.24$), both at week 3 (Fisher’s LSD post hoc test: $p < 0.05$) and at week 8 ($p < 0.01$) (Fig. 3B). AAV-α-synuclein-injected animals also performed more errors per step at week 8 compared with at week 3 after AAV-injection ($p < 0.05$), suggesting a progressive motor impairment. There was no difference between the groups in the total number of steps (data not shown). These findings indicate that α-synuclein overexpression in the VTA was associated with a mild early onset coordination impairment expressed as an increased number of stepping errors.

To investigate anxiety-like and depressive-like behaviour, animals were tested in the elevated plus maze and Porsolt swim test, respectively. The percentage of time spent in the closed arms of the elevated plus maze did not significantly differ between AAV-GFP and AAV-α-synuclein injected animals at week 3 or week 8 following AAV injection (treatment: $F_{(1,15)} = 0.08, p = 0.79$; time: $F_{(1,15)} = 2.75, p = 0.12$; interaction: $F_{(1,15)} = 1.02, p = 0.33$) (Fig. 3C). No differences were found when comparing the distance travelled in the elevated plus maze, suggesting that locomotor behaviour did not affect the outcome of the behavioural assessment (week 3: AAV-GFP: 2152 ± 92.97 cm, AAV-α-synuclein: 2313 ± 117.6 cm; week 8: AAV-GFP: 3400 ± 1373 cm, AAV-α-synuclein: 3717 ± 943.8 cm; treatment: $F_{(1,13)} = 0.08, p = 0.78$; time: $F_{(1,13)} = 2.74, p = 0.12$; interaction: $F_{(1,13)} = 0.01, p = 0.922$).

The Porsolt swim test did not reveal any significant differences in immobility (data not shown) or climbing time (treatment: $F_{(1,15)} = 0.02, p = 0.89$, time: $F_{(1,15)} = 0.27, p = 0.61$; interaction: **P < 0.01** (Repeated measures ANOVA followed by Fisher’s LSD post hoc test).
**Fig. 3.** Motor performance in the ledged beam test in animals injected bilaterally with AAV-GFP or AAV-α-synuclein into the VTA. (A) AAV-α-synuclein injections into the VTA did not affect the total time required to cross the beam in the ledged beam test. (B) AAV-α-synuclein-injected animals performed more errors per step compared with AAV-GFP-injected animals both at 3 weeks and 8 weeks after viral vector injection in the ledged beam test. AAV-α-synuclein-injected animals also performed more errors per step at 8 weeks compared with at 3 weeks. (C) AAV-α-synuclein injections into the VTA did not affect the percentage of time spent in the closed arms of the elevated plus maze. (D) AAV-α-synuclein injections into the VTA did not affect the percentage of time spent climbing in the Parsol swim test. Data are presented as the mean ± s.e.m.

\[ F_{1,15} = 0.12, \ p = 0.73 \] between AAV-GFP and AAV-α-synuclein injected animals at week 3 or 8 following the AAV injection (Fig 3D).

These findings indicate that AAV-mediated α-synuclein overexpression into the VTA did not induce any prominent depressive- or anxiety-like phenotype.

The impaired PA performance seen here is consistent with the role of the VTA and its projection areas in contextual emotional learning and memory. Since a functional dopaminergic connection between the dorsal hippocampus and VTA has been shown to modulate PA learning [12], we propose a hippocampal involvement in the effects reported here. Indeed, reversible inactivation of the VTA by local injections of lidocaine impaired the acquisition and consolidation in an inhibitory avoidance task [13], whereas 6-OHDA injections into the VTA were shown to induce deficits in PA learning as well as locomotor hyperactivity [14]. The mesohippocampal system has previously also been shown to play a pivotal role in spatial learning [15,16]. Consistently, rats injected with α-synuclein containing viral vectors into both the VTA and medial septum/vertical limb of the diagonal band of Broca displayed spatial learning and memory deficits in the Morris water maze compared with control animals [17].

It is also possible that α-synuclein-induced lesions in dopaminergic projections from VTA to other limbic structures play a role in the cognitive phenotype observed here. For instance, the mesoamygdaloid dopamine pathway plays a crucial role in Pavlovian learning, including emotional learning [18]. Dopaminergic projections to the prefrontal cortex are mainly mediated via the VTA [3], and dopaminergic signaling in the prefrontal cortex play a key role in the storage of fear-related memories [19]. Indeed, dopamine release is enhanced in the VTA, prefrontal cortex and amygdala in response to aversive stimuli including tail-shock stress [20].

The observed motor impairment defined as an increased number of stepping errors in the ledged beam test may represent a loss of coordination. Whereas the stepping mechanism for gait is controlled by brainstem and spinal mechanisms, the timing and amplitude of consecutive footsteps required for adjusting the gait to environmental cues is affected by brain regions including the cerebellum, basal ganglia and cortex [21]. Indeed, the rat primary motor cortex receives dopaminergic innervation from the VTA, and this pathway is required for motor skill learning [22]. However, rats with partial bilateral 6-OHDA lesions of the VTA did not display any motor impairments [23]. Two previous studies, using vectors of different serotypes and other coordinates for injection to overexpress wild-type or A53T mutated α-synuclein in the VTA, did not observe any changes in basal locomotor behaviour, nor any dopamine neuron cell loss, but found dystrophic mesocorticollimbic dopaminergic projections and mild dopamine-induced locomotor impairments in the open field test [17,24].

The lack of depressive- and anxiety-like behaviour in this model could be related to the α-synuclein pathology being modest and/or restricted to the dopaminergic neurons of the VTA. Emotional symptoms are associated with Lewy pathology in the locus coeruleus and raphe nuclei [1], which harbor noradrenergic and serotonergic cell bodies, respectively, and these regions likely remain unaffected in this model.
In conclusion, these experiments demonstrated that α-synuclein overexpression in the VTA caused mild motor impairments 3 weeks after injection, and emotional memory impairments paralleled by a loss of TH+ neurons in the VTA 8 weeks after injection. The emotional impairments are likely caused by a loss of dopaminergic projections from VTA to limbic areas related to Pavlovian learning, including the amygdala, hippocampus and prefrontal cortex. These findings suggest that α-synuclein overexpression in VTA can be used to address emotional learning impairments in PD.

Conflict of interest

The authors declare no conflict of interest.

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References


