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Letter

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Abstract: Pathological accumulation of misfolded α-synuclein (α-syn) in the brain plays a key role in the pathogenesis of Parkinson's disease, leading to neuronal dysfunction and motor disorders. The underlying mechanisms linking α-syn aggregations with neurotransmitter disturbance in Parkinson's brains are not well characterized. In the present study, we investigated transgenic mice expressing an aggregation-prone form of full-length human α-syn (h-α-synL62) linked to a signal sequence. These mice display dopamine depletion and progressive motor deficits. We detected accumulation of α-syn in cholinergic interneurons where they are colocalized with choline acetyltransferase. Using microdialysis, we measured acetylcholine levels in the striatum at baseline and during stimulation in the open field and with scopolamine. While no difference between wild-type and transgenic mice was detected in 3 month old mice, striatal acetylcholine levels at 9 months of age were significantly higher in transgenic mice. Concomitantly, high-affinity choline uptake was also increased while choline acetyltransferase and acetylcholine esterase activities were unchanged. The results suggest a disinhibition of acetylcholine release in α-syn transgenic mice.

Keywords: alpha-synuclein, acetylcholine, microdialysis, cholinergic interneurons, scopolamine, muscarinic receptors

Introduction

Parkinson`s disease (PD) is a common neurodegenerative disorder in humans with typical motor symptoms such as bradykinesia, rigidity and tremor. The pathophysiological process in the brain is characterized by a high load of the protein α-syn, which gradually deposits to form intracellular aggregates and fibrils known as Lewy bodies and Lewy neurites.[1]

The physiological function of α-syn is not fully understood, but there is clear evidence for it playing a role in vesicular function at the synaptic terminal.[2] Monomeric α-syn interacts with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, which are directly involved in vesicle fusion and exocytosis.[3] Therefore, a change in the structure, due to oligomerization and aggregation, can

contribute to degenerative processes and altered synaptic function. Misfolded α-syn is known to impair axonal transport,[4] and increase neuroinflammation.[5]

It is proposed that motor symptoms in PD are caused by a progressive loss of dopamine (DA) neurons projecting from the midbrain to the striatum and therefore influence basal ganglia function.[6] However, DA interacts with a variety of other neurotransmitters to control motor behavior. It is well known that the balance between acetylcholine (ACh) and DA in the striatum is necessary for normal motor function. The striatum is composed mainly of GABAergic projection neurons (medium spiny neurons, MSNs) and interneurons.[7] Striatal cholinergic interneurons (ChIs) represent only about 2% of the total striatal neuronal population,[8] but have large arborizing axonal varicosities within the striatum. This suggests that these neurons may have an important role in modulating striatal activity.[9] ChIs receive inputs from cortical and thalamic glutamatergic neurons and from dopaminergic projections from the Substantia Nigra pars Compacta (SNpC).[10] Release of ACh from ChIs is regulated by both dopaminergic receptors (D1/D5 and D2) and muscarinic ACh receptors (M2/M4) on somatodendritic and axonal sites.[11] D2 receptors inhibit striatal ACh efflux by lowering both synaptic activation and autonomous action potential firing. D1 receptors are located mainly at the somatodendritic sites where they depolarize the cell and enhance ACh release. The muscarinic M2/M4 receptors both reduce ACh release by either reducing exocytosis on the axonal site or inducing hyperpolarization on the somatodendritic site. DA depletion in the striatum can therefore lead to a disturbed function of ChIs, contributing to the emergence of motor symptoms.

Several genetic mouse models of PD have been developed that reflect many aspects of the disease and the underlying mechanisms of α-syn pathology contributing to motor symptoms and neurodegeneration.[12] Most of the transgenic synuclein models overexpress human wild-type α-syn or α-syn carrying A53T or A30P mutations.[12] Overexpression of α-syn has been shown to reduce dopamine release in transgenic mice.[13] However, not all a-syn-overexpressing transgenic mice form intracellular inclusions or cell loss in the substantia nigra.[14] The vulnerability of dopamine neurons to α-syn overexpression has been well studied, but several reports have shown that noradrenergic, serotonergic or cholinergic neurotransmitter systems are also affected.[15]

We have recently reported on transgenic h-α-synL62 (L62) mice, which show high levels of expression of human α-syn (h-α-syn); aggregates were already observed by 3 months of age.[16] L62 showed a hypoactive phenotype and age progression was associated with more severe impairments in motor activity and coordination. In microdialysis experiments, a progressive lowering of amphetamine-induced dopamine (DA) release in the striatum appeared in L62 between 3 and 9 months of age.[16] In the present study, we used h-α-synL62 mice at the age of 3 and 9 months to investigate the impact of h-α-syn aggregation on cholinergic functions in the striatum.

Results and Discussion

Cholinergic neurons are affected by h-α-syn inclusions. The pathologic hallmark of PD is a degeneration of dopaminergic neurons of the Substantia nigra pars

compacta and of their striatal terminals. However, there are also reports of neuronal loss and alterations in cholinergic neurotransmission in the basal forebrain and in cortical structures.[17,18] Cholinergic neurons are characterized by expression of the enzyme choline acetyltransferase (ChAT), which is localized to the soma and presynaptic endings. Alpha-synuclein plays a major role in the presynaptic cytoskeleton and accumulates specifically in presynaptic terminals. In histological staining experiments from L62, h-α-syn inclusions are pronounced in ChAT-positive neurons in several brain areas including striatum, olfactory nucleus, spinal cord and nucleus basalis magnocellularis (Fig. 1A) that are all known for their role in PD pathology. Cell counts of ChAT-positive neurons in the striatum confirmed a significant reduction of cells in L62 mice at 3 and 8 months of age relative to WT (Fig. 1B). Our results suggest that accumulation of h-α-syn in cholinergic neurons causes a loss of ChIs in the striatum, a brain region particularly important for the control of movements. These findings could be confirmed in another study with a model of widespread progressive synucleinopathy throuought the forebrain. Animals displayed progressive reduction in cortical and striatal ChAT positive interneurons, indicating that cholinergic interneurons may be more vulnerable to h-α-syn toxicity.[19] However, in our model there is a lack of progression in the loss of ChAT positive neurons between 3 and 9 months of age. In L62 mice, h-α-syn aggregates reached their maximum aggregation state also with 3 months and showed no age progression in aggregation, but in severity of motor symptoms.[16] Possibly there are other mechanisms, such as changes in neurotransmission, which contribute to the progression of motor symptoms.
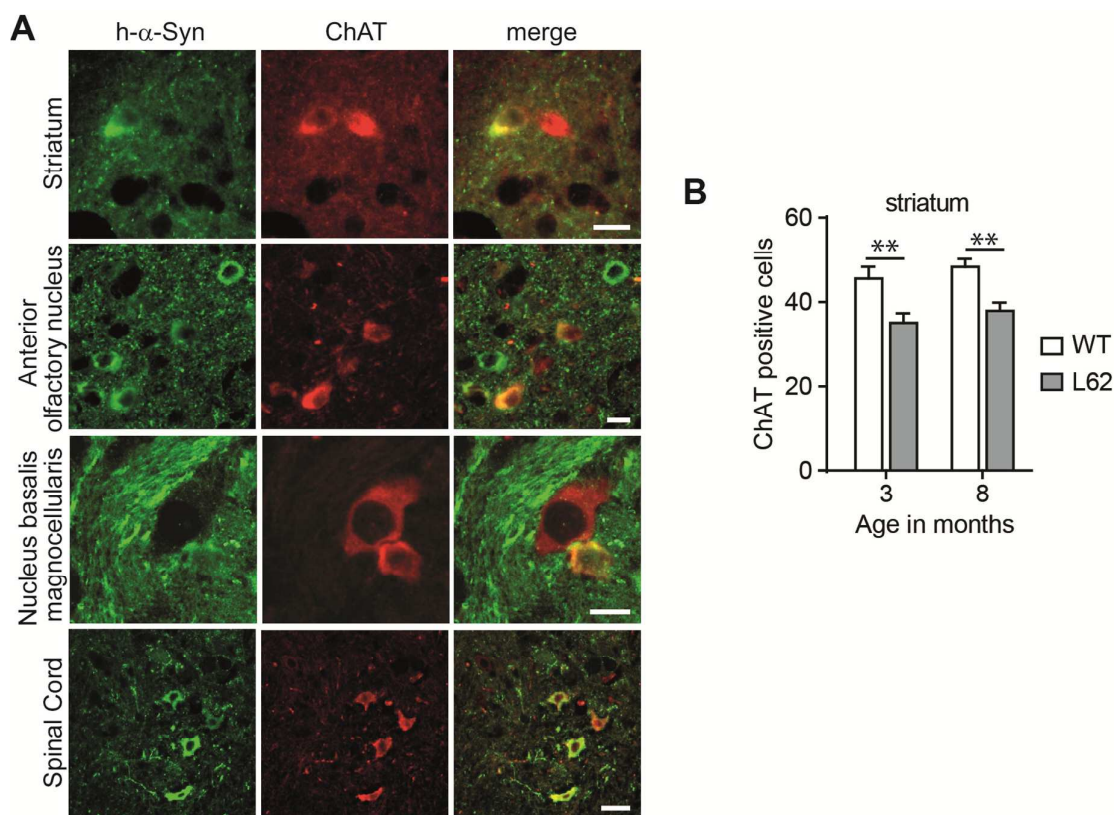

Figure 1: Cholinergic neurons are affected by h-α-syn inclusions in L62 mice. (A) A subset of cholinergic neurons, positive for ChAT (red), in the striatum, the olfactory

nucleus, the nucleus basalis magnocellularis and the spinal cord were also stained for h-α-syn (green) inclusions in L62 mice. The yellow color in the merged images indicates colocalization of ChAT and h-α-syn. Scale bars: 20 µm. (B) Manual cell counting of striatal interneurons at Bregma 0.74 ± 0.25 revealed loss of striatal cholinergic interneurons in transgenic L62 compared to WT mice at 3 and 8 months of age. Values are given as mean ± SEM (N = 8-12 mice per group). Statistics: two-way ANOVA for genotype and age as variables: Genotype $p < 0.0001$, age and interaction ns. Bonferroni posttest: **, $p < 0.01$ vs WT.

High-affinity choline uptake is upregulated in 9 months old transgenic mice. To gain a better understanding of the overall effects of h-α-syn on cholinergic synapses, the activity of three enzymes and transporters obligatory for ACh synthesis was measured (Fig. 2 A–C). ChAT is localized in presynaptic terminals and is responsible for the synthesis of ACh. When ChAT activities were determined, we found an age-dependent increase in ChAT activity ($p<0.01$), but no genotype effect (Fig. 2A). Hence, ChAT activity was unchanged in spite of reduced cholinergic cell counts (Fig. 1B). However, ChAT activity is not limiting for ACh synthesis.

The activity of high-affinity choline uptake (HACU) reflects the transport of choline (Ch) back into the presynaptic compartment and is considered a rate-limiting step for the synthesis of ACh.[20] HACU activity is controlled by the presence of the choline transporter-1 (CHT-1) in the plasma membrane which is internalized depending on the neuronal firing rate.[21] Hence, HACU measured *ex vivo* provides a measure of *in vivo* turnover of ACh prior to sacrifice.[22] The activity of HACU in 9 month old mice was significantly greater for L62 mice relative to WT (Fig. 2B, $p=0.02$). This suggests that degeneration of cholinergic cells in the striatum can lead to compensatory up-regulation of HACU activity which maintains cholinergic function. This finding is reminiscent of a study with mice, which were heterozygous for a null mutation in the ChAT gene. In these mice, ChAT activity was reduced by 50%, but brain ACh levels were normal due to an increased expression of CHT1.[23]

Ex vivo-data of the activity of the acetylcholinesterase (AChE) reflect the rate of breakdown of ACh in the synaptic cleft because AChE terminates the action of ACh. There was no difference in AChE activity between the two mouse strains at 9 months of age (Fig. 2C). Hence, the rate of breakdown of ACh is not affected by h-α-syn.
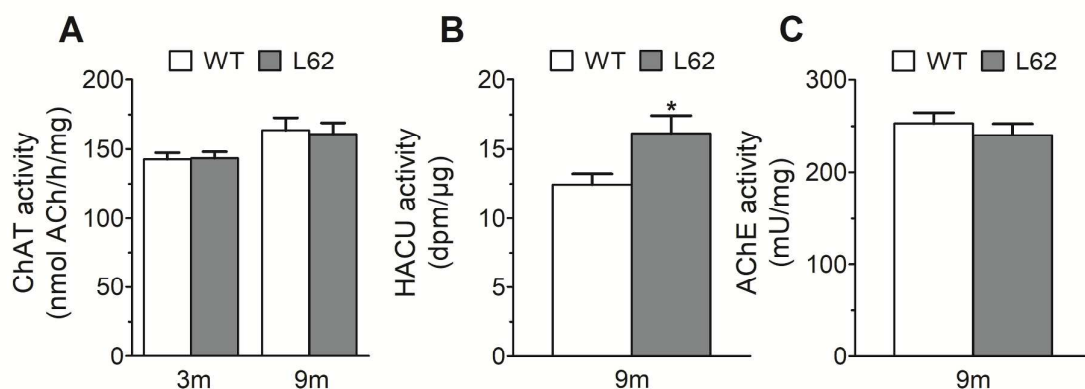

Figure 2. Activity of ChAT (A) in 3 and 9 month old mice, HACU (B) and AChE (C) in 9 month old mice. Data were obtained in hemibrain homogenates. Values are given

as mean ± SEM (N = 6-10 mice per group). Statistics: two-way ANOVA for genotype and age as variables: (A) Age $F_{1,36}$=7.5, p < 0.01, genotype and interaction ns. (B) Student's t-test, two-tailed: p=0.02; (C) p=0.48.

9 month old L62 mice display increased striatal ACh release during the open field experiment. To confirm a possible cholinergic phenotype caused by h-α-syn accumulation, microdialysis measurements of the neurotransmitter ACh were performed. The striatum was chosen as region of interest, since previous experiments in L62 mice revealed that striatal dopamine levels are reduced in 9 month old mice upon amphetamine challenge (2 mg/kg). Baseline levels of DA in the striatum of 9 month old WT and L62 mice were about 1 nM and during amphetamine stimulation they increased 400% from baseline in WT and 250% from baseline in L62 mice.[16] This difference in DA release was significant and shows that, despite of lack of neuronal loss in the substantia nigra, striatal DA neurons are impaired in transgenic L62 mice. As mentioned above, ChIs densely innervate the entire striatal complex and receive input from extrinsic DA neurons of the mesencephalic tegmentum.[19] The output targets of the ChIs are the MSNs, which are important for initiating and controlling movements.

Extracellular baseline concentrations of ACh were slightly higher in L62 but not significantly different to WT at 3 and 9 months of age at the beginning of day 1 dialysis (Fig. 3A). The findings of baseline levels of ACh in the striatum are in accordance with concentrations stated in the literature, which are about 1-5 nM.[24,25] In comparison to ACh, choline, the precursor of ACh, was lower in 9 month old mice, an effect that was significant in L62 mice (Fig. 3B). This is possibly caused by an increased turnover of ACh and heightened HACU activity of L62 mice at 9 months of age (Fig. 2B), resulting in an increased uptake of extracellular choline into the presynapse. It should be noted, however, that choline levels are much higher than ACh levels, and choline metabolism mostly reflects phospholipid metabolism and not ACh release.[20]

When mice were placed into a novel environment (open field), ACh release was stimulated by approx. 2-fold (Figs. 3C, D). This increase of ACh in the dorsal striatum is probably due to motor activity during exploration in the open field.[26] There are no data for open-field induced increases of ACh in the striatum of mice in the literature. However, in experiments with Wistar rats, increased motor activity during the dark phase of the rats' day-night cycle led to an increase in striatal ACh of 58%.[27]

The time course in 3 month old mice was identical between L62 and WT (Fig. 3C). In 9 month old (Fig. 3D) mice, however, the movement stimulated rise in ACh was higher in L62 mice during exploration and remained elevated over a longer period after placing mice back into the home cage (two-way ANOVA: Genotype $F_{1, 22}$=7.32, p=0.01). In similar experiments, Frahm et al. monitored the distance moved in an open field, in which L62 mice showed a reduced activity[16], but this was not recorded here. Both reduced locomotor activity and elevated ACh levels can be explained by an impaired regulation of firing of ChIs. Striatal cholinergic interneurons express both D1 and D2 DA receptors.[28,29] Dopamine modulates striatal cholinergic tone via both excitatory and inhibitory actions. D2 receptor stimulation slows the discharge rate of striatal ChIs by modulating sodium ion currents,[30] and inhibits striatal ACh release.[31]

Conversely, D1 receptor stimulation facilitates ACh release.[32] Therefore, impairment in dopaminergic D2 receptor regulation of ChIs would explain an increased ACh release after stimulation.
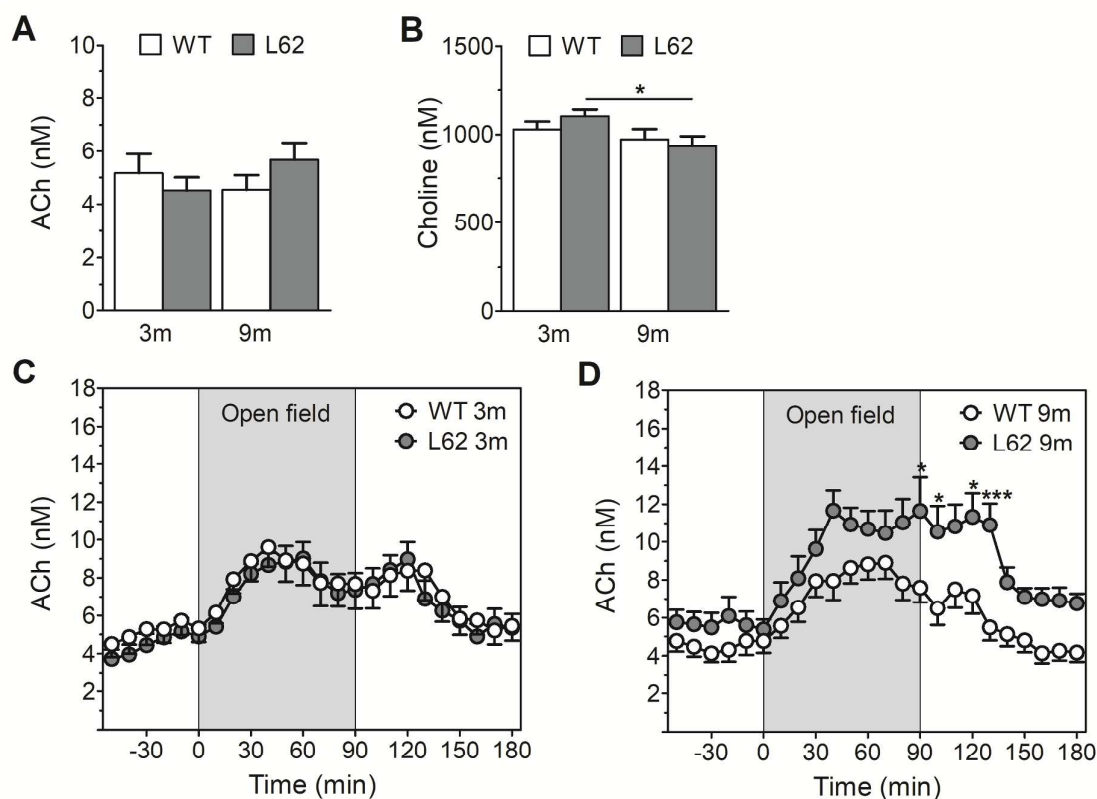

Figure 3. Baseline ACh and Ch levels before stimulation (A and B). Statistics: two-way ANOVA for genotype and age as variables: (A) Genotype, age and interaction ns. (B) Age $F_{1, 53}$=5.6, p=0.02, genotype and interaction ns. Bonferroni post-test: *, p<0.05 vs L62. Extracellular striatal ACh levels in 3 and 9 month old mice before, during and after exposure to the open field (C and D). Statistics: two-way ANOVA for genotype and time as variables: (C) Time $F_{23, 690}$=20.47, p<0.0001, genotype and interaction ns. (D) Time $F_{23, 506}$=22.35, p<0.0001, genotype $F_{1, 22}$=7.32, p=0.01, interaction $F_{23, 506}$= 2.25, p=0.0008. Bonferroni post-test: *, p<0.05; **, p<0.01; ***, p<0.001 for L62 9m vs WT 9m. Values are presented as mean ± SEM, derived from absolute values, not corrected for *in vitro* recovery (N=12-18 mice per group). Data for (A) and (B) were obtained for individual animals and averaged from 5-6 baseline samples for each mouse.

9 month old L62 mice display increased striatal ACh release during infusion of scopolamine. Besides DA receptors, muscarinic AChRs modulate the release of ACh in the striatum. ChIs express M2 and M4 receptors, which are present on the somatodendritic areas and the axon terminals.[33] At the somatodendritic site, mAChR are proposed to inhibit striatal ChIs by inducing hyperpolarization mediated by an outward K^+ current. mAChRs on the axon terminals cause presynaptic autoinhibition of ACh release by modulating K^+ and Ca^{2+} channels.[34] Scopolamine acts as a muscarinic antagonist; it blocks inhibitory M2/M4 receptors and causes an increase of extracellular ACh.[35] In 3 month old mice, scopolamine led to a 3-fold increase of

ACh in both lines (Fig 4C). In 9 month old mice (Fig. 4D), however, L62 mice displayed much higher increases in extracellular ACh levels than wild-type mice (p<0.01). Most likely, the amplified effect of scopolamine in L62 mice can be explained by a disinhibition of ChIs in the striatum due to DA depletion and loss of inhibition by D2 receptors. Furthermore, DA denervation has been shown previously to reduce M4 mRNA which resulted in a loss of negative feedback inhibition and increased ACh release and turnover.[36] This mechanism may explain the small but significant increase of basal ACh in L62 vs. wild-type mice before scopolamine infusion (Fig. 4A). This increase in baseline levels of ACh is also reflected in the increased HACU activity measured in 9 month old mice (Fig. 2B).
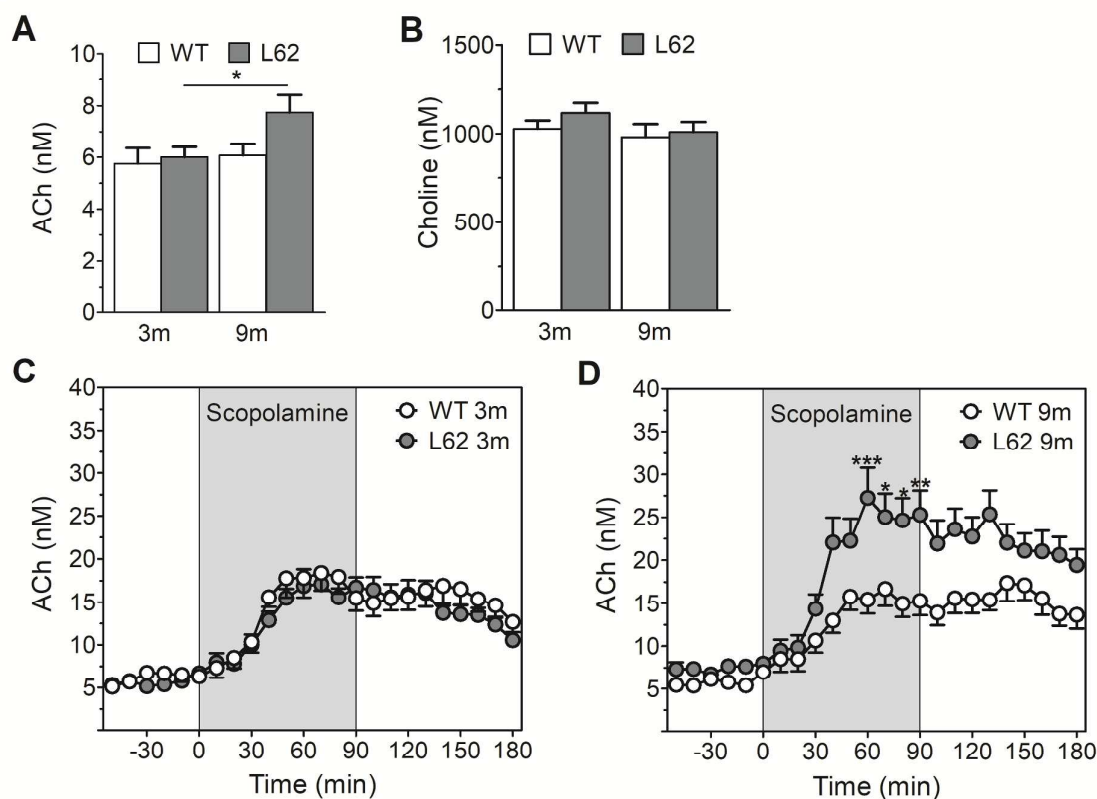

Figure 4. Baseline ACh and Ch levels before stimulation (A and B). Statistics: two-way ANOVA for genotype and age as variables: (A) Genotype $F_{1, 54}$ = 3.06, p = 0.09, age $F_{1, 54}$ = 3.5, p = 0.07, interaction ns. Bonferroni post-test: *, p < 0.05 vs L62. (B) Genotype, age and interaction ns. Extracellular striatal ACh levels in 3 and 9 month old mice before, during and after scopolamine exposure (shaded area) (C and D). Statistics: two-way ANOVA for genotype and time as variables: (C) Genotype, time and interaction ns. (D) Genotype $F_{1, 24}$ = 8.69, p = 0.007, time $F_{23, 552}$ = 36.13, p < 0.0001, interaction $F_{23, 552}$ = 3.19, p < 0.0001. Bonferroni post-test: *, p < 0.05; **, p < 0.01; ***, p < 0.001 for L62 9m vs WT 9m. Values are presented as mean ± SEM, derived from absolute values, not corrected for *in vitro* recovery (N = 12-18 mice per group). Data for (A) and (B) were obtained from individual animals and averaged from 5-6 baseline samples for each mouse.

Collectively, our results show that L62 at 9 months of age display higher striatal ACh release from challenged ChIs. The difference in extracellular ACh release seems to

be independent of the impact of α-syn on ChAT neurons in the striatum. Although there is a reduction in ChAT positive cells in L62 mice, the cell counts did not decline with age. Furthermore, there was no change in the activity of ChAT. There is evidence that both phosphorylation and dephosphorylation of ChAT can alter its catalytic activity and thereby regulate ACh synthesis.[37] α-syn may also have a chaperone-like function and transgenic overexpression of this protein can lead to changes in phosphorylation of ChAT.[38] However, it is generally considered, that the activity of the high affinity choline transporter (HACU) is the rate-limiting step in ACh synthesis and release, and measurements confirmed an increased activity of HACU in L62 mice.

We suggest that the impairment of dopamine neurons leads to a disturbance in the normal DA-ACh balance and that the increased cholinergic activity in this model may arise from the reduced inhibition of dopaminergic neurons ending upon cholinergic cells in the dorsal striatum.[39] The plastic alterations of striatal cholinergic interneurons by DA denervation have never been investigated in α-syn overexpressing mouse models. A change in activity of ChIs might have consequences on movement and contribute to the progression of motor symptoms in PD.

Glucose and lactate levels increase in the open field but not during scopolamine challenge. Brains of PD patients showed glucose hypometabolism,[40] and α-syn aggregation in transgenic mouse models led to mitochondrial dysfunction.[41] We investigated the influence of α-syn aggregation on brain energy metabolism by measuring extracellular glucose and lactate levels in microdialysate of L62 and WT mice. Results were obtained from 3-4 consecutive baseline samples, and 3-4 consecutive samples after placing mice into the open field and stimulating with scopolamine, respectively.
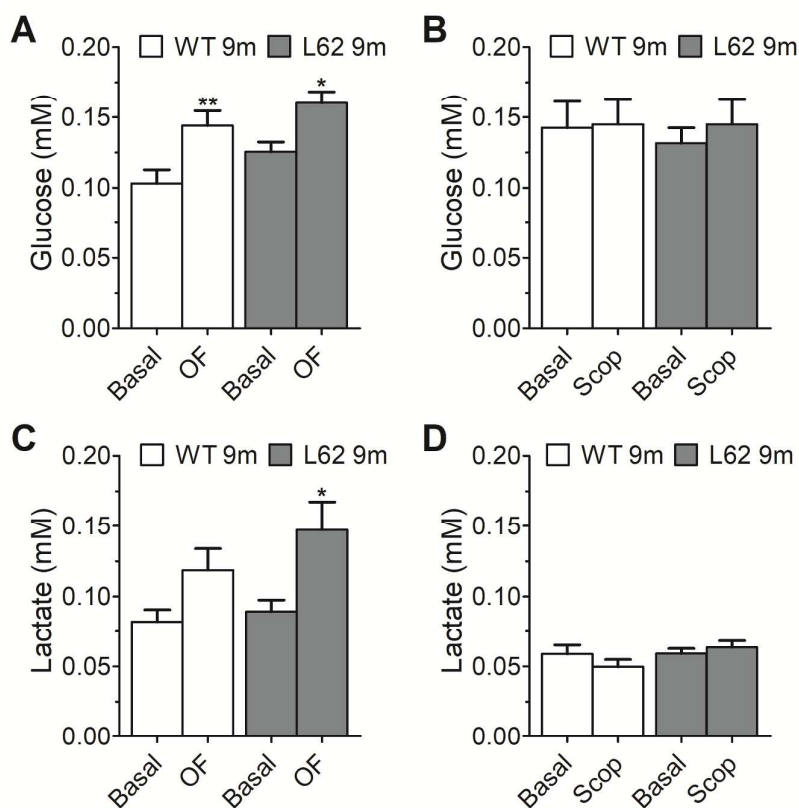
Figure 5: Glucose levels on day 1 (A) and day 2 (B) and lactate levels on day 1 (C) and day 2 (D) in 9 month old mice before and after stimulation measured in microdialysates. Statistics: two-way ANOVA for genotype and challenge as variables: (A) Genotype $F_{1,40}$= 4.7, p=0.04, challenge $F_{1,40}$=18. 15, p=0.0001, interaction ns. Bonferroni post-test: **, p<0.01 vs WT, *, p<0.05 vs L62. (B) Genotype, challenge and interaction ns. (C) Challenge $F_{1,28}$=14.03, p=0.0008, genotype and interaction ns. Bonferroni post-test: *, p<0.05 vs L62. (D) Genotype, challenge and interaction ns. Values are presented as means ± SEM. Data were obtained from individual animals and averaged from 3-4 consecutive samples. (A/C) N=9-11, (B/D) N=8-9 mice per group.

Glucose is the standard fuel for brain energy production and a precursor of ACh synthesis. It is taken into cholinergic presynaptic terminals and transformed into acetyl-Coenzyme A, which is used subsequently to synthesize ACh. ACh levels depend on glucose oxidation in periods of increased cholinergic activity.[42] Extracellular glucose levels reflect a balance between supply from the blood and uptake into neurons. Behavioral stimulation in the open field caused glucose levels to increase, likely indicating an increased flux of glucose from blood during motor activity (Fig. 5A). Blood glucose, blood flow, and glucose uptake in the brain are coupled,[43] and thus an increase of glucose can reflect an increase of blood sugar or of blood flow in the striatum. Glucose levels were slightly greater in L62 at baseline (Fig. 5A), but the extent of glucose mobilization in the open field was almost identical in WT and L62 mice indicating that h-α-syn does not cause a generised impairment in the ability of the brain to respond adequately to a physiological stimulus. Scopolamine perfusion did not change glucose levels in either WT or L62 (Fig. 5B).

In the absence of ischemia, extracellular lactate concentrations in the brain reflect formation by astrocytes which support neuronal function in periods of high neuronal demand.[44] Increase of ACh during the open field correlates with higher neuronal activity shown by lactate increases in both L62 and WT to a similar extent (Fig. 5C). Lactate levels in L62 mice were slightly higher in the open field experiment, which may be explained by increased firing of the ChIs (Fig. 5C, Bonferroni posttest: *: p < 0.05 vs L62). Mice did not respond with an increase of lactate during stimulation with scopolamine (Fig. 5D), probably because scopolamine blocks cholinergic excitation of downstream neurons.

Conclusion

In this study we investigated the cholinergic system in the striatum in a mouse model of synucleinopathy. L62 mice at 9 months of age displayed a cholinergic phenotype which was characterized by strong increases of acetylcholine levels under stimulated conditions, i.e. in the open field and during scopolamine perfusion. Direct effects of synuclein on ChI are possibly due to the co-localization of h-α-syn and ChAT in striatal cholinergic interneurons. However, the presynaptic function of cholinergic interneurons does not seem to be affected by α-syn, as ChAT, HACU and AChE activities are not reduced. In fact, although ChAT positive cells were reduced in the striatum, the total activity of ChAT was unchanged and HACU showed an increased activity, evidently to maintain the cholinergic function of the synapses. Glucose and lactate responded normally, but the higher concentrations of lactate in L62 seen during locomotor activity are in agreement with a greater neuronal activity during exploration, which is proposed to be due to a disinhibition of cholinergic interneurons.

Methods

Chemicals. Neostigmine bromide, scopolamine and chemicals for the artificial cerebrospinal fluid (aCSF) were purchased from Sigma-Aldrich (Munich, Germany). The chemicals for the mobile phase of the HPLC analysis were obtained from Merck, Darmstadt, Germany ($KHCO_3$), from VWR, Darmstadt, Germany (EDTA-2Na), from Alfa Aesar, Karlsruhe, Germany (sodium decane-1-sulfonate) and from Sigma-Aldrich, Munich, Germany (RotisolV HPLC grade water). Chemicals for AChE, ChAT and HACU assays were purchased from Biotrend, Köln, Germany ([³H]-choline, [³H]-acetyl coenzyme A), Sigma-Aldrich, Munich, Germany (acetylthiocholine iodide, dithionitrobenzoic acid, Triton X-100, tetraisopropyl pyrophosphoramide, hemicholinium-3 and sodium tetraphenylboron) and from Merck, Darmstadt, Germany (HEPES sodium salt). CMA 600 Microdialysis analyser reagents (Glucose, Lactate and Calibrator reagents) were obtained from M Dialysis AB, Stockholm, Sweden.

Animals. Transgenic mice were generated by GenOway (Lyon, France). Mice were previously described and characterized by Frahm et al.[16] They were bred in the University facility of Charite Berlin and shipped by truck to the animal house in Frankfurt. After delivery of the mice they were habituated to the animal facilities for at least two weeks. Male and female homozygous L62 mice and WT litters were housed in small colonies in a facility with controlled temperature and humidity and a day/night cycle of 12/12 h. They had free access to food and water. All experiments were done

according to the German Law for Animal Protection (Tierschutzgesetz) and the European Community Directive 63/2010/EU.

ChAT-h-α-syn double labeling and ChI counting. Formalin-fixed brain tissue was embedded in paraffin, cut into 5-µm coronal sections, deparaffinised and boiled in 10mM citric buffer.

For immunofluorescence, sections were blocked for 1 h in incubation buffer ((5% v/v) normal goat serum in PBS containing 0.3% (v/v) Triton X-100) and incubated overnight at 4° C in a primary antibody cocktail of mAb 204 (Santa Cruz Biotechnology USA, diluted 1:200) and ChAT (H-95, Santa Cruz Biotechnology USA, diluted 1:200), diluted in incubation buffer. The next day sections were incubated for 1.5 h with fluorochrome-conjugated secondary antibodies (Alexa Fluor® 488-conjugated donkey anti-mouse IgG and Alexa Fluor® 568-conjugated goat anti-rabbit IgG, Life Technologies, USA; both diluted 1:500 in incubation buffer) and examined using a microscope equipped for fluorescence (Carl Zeiss, Germany).

For immunohistochemistry, sections were incubated in 0.3% (v/v) hydrogen peroxidase solution and blocked for 20 min in blocking buffer (0.1% w/v BSA in PBS). Afterwards, ChAT antibody (H-95, diluted 1:50) was added, followed by incubation with corresponding biotinylated secondary antibody diluted 1:100 (Dako, Denmark). Sections were developed with diaminobenzidine solution (Dako, Denmark), counterstained with Ehrlich haematoxylin solution (Carl Roth, Germany), embedded in Neo-Mount® (Merck Millipore, Germany) and images taken using a light microscope (Carl Zeiss, Jena, Germany). Primary and secondary antibodies were diluted in blocking buffer. Cholinergic interneurons were counted manually for the entire striatum at Bregma +0.74 ± 0.25 mm (according to Franklin and Paxinos)[45] for 3 consecutive brain sections. The mean value for each animal was used for analyses.

Activity of cholinergic enzymes and transporters. Brain hemispheres were homogenized with a 10-fold volume of isotonic HEPES-sucrose buffer (HEPES sodium salt 10 mM, Sucrose 0.32 M, pH 7.4) using a tissue grinder (Potter S, B. Braun, Melsungen, Germany) at 800 rpm and 15 strokes.

ChAT was measured by a modification of the Fonnum method.[46] Brain homogenate containing 0.5 mg/ml protein was added to a total volume of 250 µl reaction mix (0.5% Triton X-100, 0.3 M NaCl, 0.02 M EDTA, 0.05 M Na_3PO_4 pH 7.4, 2 mM choline chloride, 1 mM neostigmine bromide, 0.5 mCi [^{3}H]-acetyl coenzyme A). Following incubation at 37°C for 15 min, the reaction was stopped by addition of 250 µl ice-cold phosphate buffer (1 mM, pH 7.4). ACh was extracted with 1 ml 0.5 % sodium tetraphenylboron in 85% toluene-15% acetonitrile. After centrifugation, aliquots of 0.8 ml were used for tritium quantification with a scintillation counter (Wallac system 1409, Perkin Elmer). ChAT activity was expressed as nmol of ACh formed per hour per mg of protein (nmol/h/mg). Blank values were obtained by omitting the brain homogenate.

HACU activity was determined in synaptosomal (P2) fractions obtained from one hemisphere as previously described.[47] Brain hemispheres were homogenized in 10 mM HEPES-sucrose solution and centrifuged at 1,000 g for 10 min at 4°C and, the

resulting supernatant centrifuged again at 17,000 g for 10 min. The pellets from the last centrifugation step (P2 fraction, containing the synaptosomes) were used for HACU determination. Aliquots were incubated at 30°C in the presence of 0.5 µM [3H]-choline (diluted to 0.5 Ci/mmol; Biotrend, Cologne, Germany) in Krebs-Henseleit buffer (KHB; containing NaCl 115 mM, KCl 7.1 mM, $CaCl_2$ 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, Na_2HPO_4 1.5 mM, glucose 12.8 mM, and saturated with carbogen adjusting to pH 7.2-7.4). Incubations were done both, in the presence and absence of 1 µM hemicholinium-3 (HC-3). Choline uptake was stopped after 5 min by placing the reaction mix on ice and by adding ice-cold KHB. After three centrifugation steps (14,000 g, 10 min) and washing with KHB buffer, the pellets were solubilized in 0.5 ml methanol and 4 ml scintillation fluid (IRGA-SAFE PLUS, Perkin Elmer) and used for tritium quantification by liquid scintillation counting. The HC-3-sensitive, high-affinity choline uptake was calculated as the difference between uptake in the absence and presence of HC-3 and expressed as dpm/mg protein. Protein content was determined by the Bradford method.

AChE activity was measured by a modified Ellman method.[48,49] Brain homogenate (50 µl) was mixed with 0.5% Triton X-100 (Sigma-Aldrich, Munich, Germany) to dissolve membranes. Samples were centrifuged for 10 min. at 12,000 g and 4°C. 10 µl of the supernatant was mixed with Ellman buffer and iso-OMPA (final concentration 100 µM). Acetylthiocholine and dithionitrobenzoic acid (1 mM and 500 µM final concentrations, respectively) were added before measuring the absorbance at 405 nm using a Victor multi-label plate reader (Perkin Elmer, Bedford, USA). Enzyme activity was calculated using a standard curve prepared with each assay and expressed in relation to protein amount (mU/mg protein). Protein determination was carried out using the Bradford method.[50]

Probe implantation. Self-made, I-shaped, concentric dialysis probes with an exchange length of 2 mm and a molecular cut-off of 10,000 Da were constructed as previously described.[51] The in vitro recovery of the self-built probes amounted to 18.5 ± 2.7 % for ACh and 23.2 ± 2.8 % for Ch. After at least one week of acclimatization to the housing conditions, the microdialysis probes were implanted. Mice were anaesthetized with isoflurane (Forene®, Abbvie, Ludwigshafen, Germany) in concentrations (v/v) of 4% isoflurane in air for induction and 1.5-2% isoflurane for maintenance of anesthesia by a vaporisator (Kent Scientific, USA). The skull was exposed and a small hole was drilled in the skull of one hemisphere. By means of a stereotaxic apparatus (Stoelting, Chicago, USA), the probes were implanted into the dorsal striatum using the following coordinates from bregma: AP: +0.5 mm, L: +2.2 mm, DV: -2.3 mm, according to the atlas of Franklin and Paxinos.[45] Glass ionomer eluting cement (Micron® i-Cem, PrevestDenPro, Heidelberg, Germany) was used to fix the implanted probe on the skull. All animals were allowed to recover at least 18 hours after surgery before starting the microdialysis experiments.

In vivo **microdialysis procedures.** Microdialysis experiments were carried out for two consecutive days after probe implantation. Artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$) was pumped through the probe at a constant rate of 2 µl/min with a microinjection pump. Dialysate samples (20 µl) were collected every 10 min and immediately stored on ice. After collection, samples were frozen at -80°C until analysis. For detection of extracellular ACh,

neostigmine (0.1 µM) was added to the perfusion liquid to stabilize basal extracellular ACh levels for the following stimulating experiments. On day one of microdialysis, after collection of basal levels for 60 min, mice were placed in a novel environment (open field box, 35x32x20 cm) for 90 min to physiologically stimulate the ACh release due to exploration and increased locomotor activity. After the open field stimulation, mice were placed back into their home cage and dialysis was continued for another 90 min. On day two, baseline levels were collected for 60 min and then scopolamine (1 µM) was infused for 90 min through the microdialysis probe to increase ACh release pharmacologically. After stimulation, dialysis was continued without scopolamine for another 90 min. Subsequently, mice were sacrificed and brains were sectioned to verify the correct location of the probe in the dorsal striatum. The hemisphere without probe implantation was used to prepare homogenates (see above) for the measurement of acetylcholinesterase (AChE) activity, choline acetyltransferase (ChAT) activity and high-affinity choline uptake (HACU).

Determination of Acetylcholine, Choline, Glucose and Lactate. ACh and choline (Ch) were analyzed by directly injecting dialysates into a high performance liquid chromatography (HPLC) system (Eicom HTEC-500, Kyoto, Japan) consisting of degasser, low-speed pump, pre- and separation column, enzyme reactor carrying immobilized AChE and choline oxidase, and an electrochemical detector with a platinum electrode operating at 500 mV relative to an Ag/AgCl reference electrode. The mobile phase contained 50 mM $KHCO_3$, 134 µM Na_2EDTA, and 1.6 mM sodium decane-1-sulfonate dissolved in RotisolV® HPLC gradient water (pH 8.4). The flow rate was set to 150 µl/min and the injection volume was 10 µl. In this condition the sensitivity of the assay for ACh is about 1-3 fmol per sample. Data acquisition was performed using EPC-500 PowerChrom® software. After detection of ACh and Ch, the dialysates were analyzed in the CMA-600 microdialysis analyser (CMA Microdialysis AB, Stockholm, Sweden) to determine the concentrations of glucose and lactate by an enzymatic reaction and colorimetric detection at 530 nm.

Data analysis and statistical evaluation. Data for the in vitro recovery of microdialysis probes were given as means ± standard deviation (SD). Concentrations of ACh, Ch, energy metabolites and enzyme activities were expressed as means ± standard error of the mean (SEM) for the respective group with the number of experiments indicated in figure legends. AChE and ChAT activity was analyzed with Student's t-test using Prism 5 (GraphPad® Software, San Diego, USA). Two-way ANOVA and Bonferroni post-test was used to analyze baseline concentrations of ACh, Ch and manual ChI counting (with age and genotype as variables) and for glucose and lactate levels (with challenge and genotype as variables). ACh and Ch time courses as obtained by microdialysis were also analyzed using two-way ANOVA for repeated measures (time and genotype as variables) and Bonferroni post-test. Significance of data was assumed when statements could be made with 95% confidence.

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Notes

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References

(1)   George, S.; Rey, N. L.; Reichenbach, N.; Steiner, J. A.; Brundin, P. α-Synuclein: The Long Distance Runner. *Brain Pathology*. 2013, Vol. 23, 350–357.

(2)   Jensen, P. H.; Nielsen, M. S.; Jakes, R.; Dotti, G.; Goedert, M. Binding of Alpha-Synuclein to Brain Vesicles Is Abolished by Familial Parkinsons-Disease Mutation. *J. Biol. Chem.* 1998, *273* (41), 26292–26294.

(3)   Burré, J.; Sharma, M.; Tsetsenis, T.; Buchman, V.; Etherton, M. R.; Südhof, T. C. Alpha-Synuclein Promotes SNARE-Complex Assembly in Vivo and in Vitro. *Science.* 2010, *329* (5999), 1663–1667.

(4)   Volpicelli-Daley, L. A. Effects of α-Synuclein on Axonal Transport. *Neurobiol. Dis.* 2017, *105*, 321–327.

(5)   Wong, Y. C.; Krainc, D. α-Synuclein Toxicity in Neurodegeneration: Mechanism and Therapeutic Strategies. *Nat. Med.* 2017, *23* (2), 1–13.

(6)   Obeso, J. A.; Rodriguez-Oroz, M. C.; Rodriguez, M.; Lanciego, J. L.; Artieda, J.; Gonzalo, N.; Olanow, C. W. Pathophysiology of the Basal Ganglia in Parkinson's Disease. *Trends Neurosci.* 2000, *23* (10 Suppl), S8–S19.

(7)   Kawaguchi, Y.; Wilson, C. J.; Augood, S. J.; Emson, P. C. Striatal Interneurones: Chemical, Physiological and Morphological Characterization. *Trends in Neurosciences*. 1995, 527–535.

(8)   Zhou, F.-M.; Wilson, C. J.; Dani, J. A. Cholinergic Interneuron Characteristics and Nicotinic Properties in the Striatum. *J. Neurobiol.* 2002, *53* (4), 590–605.

(9)   Pisani, A.; Bernardi, G.; Ding, J.; Surmeier, D. J. Re-Emergence of Striatal Cholinergic Interneurons in Movement Disorders. *Trends Neurosci.* 2007, *30* (10), 545–553.

(10) Smith, Y.; Villalba, R. Striatal and Extrastriatal Dopamine in the Basal Ganglia:

An Overview of Its Anatomical Organization in Normal and Parkinsonian Brains. *Mov. Disord.* 2008, *23 Suppl 3*, S534-47.

(11) Bonsi, P.; Cuomo, D.; Martella, G.; Madeo, G.; Schirinzi, T.; Puglisi, F.; Ponterio, G.; Pisani, A. Centrality of Striatal Cholinergic Transmission in Basal Ganglia Function. *Front. Neuroanat.* 2011, *5*, 6.

(12) Koprich, J. B.; Kalia, L. V.; Brotchie, J. M. Animal Models of α-Synucleinopathy for Parkinson Disease Drug Development. *Nature Reviews Neuroscience*. 2017, 515–529.

(13) Lundblad, M.; Decressac, M.; Mattsson, B.; Bjorklund, A. Impaired Neurotransmission Caused by Overexpression of -Synuclein in Nigral Dopamine Neurons. *Proc. Natl. Acad. Sci.* 2012, *109* (9), 3213–3219.

(14) Daher, J. P. L.; Ying, M.; Banerjee, R.; McDonald, R. S.; Hahn, M. D.; Yang, L.; Beal, M. F.; Thomas, B.; Dawson, V. L.; Dawson, T. M.; et al. Conditional Transgenic Mice Expressing C-Terminally Truncated Human α-Synuclein (ASyn119) Exhibit Reduced Striatal Dopamine without Loss of Nigrostriatal Pathway Dopaminergic Neurons. *Mol. Neurodegener.* 2009, *4* (1).

(15) Lim, S. Y.; Fox, S. H.; Lang, A. E. Overview of the Extranigral Aspects of Parkinson Disease. *Arch. Neurol.* 2009, *66* (2), 167–172.

(16) Frahm, S.; Melis, V.; Horsley, D.; Rickard, J. E.; Riedel, G.; Fadda, P.; Scherma, M.; Harrington, C. R.; Wischik, C. M.; Theuring, F.; et al. Alpha-Synuclein Transgenic Mice, h-α-SynL62, Display α-Syn Aggregation and a Dopaminergic Phenotype Reminiscent of Parkinson's Disease. *Behav. Brain Res.* 2018, *339* (August 2017), 153–168.

(17) Braak, H.; Del Tredici, K.; Rüb, U.; De Vos, R. A. I.; Jansen Steur, E. N. H.; Braak, E. Staging of Brain Pathology Related to Sporadic Parkinson's Disease. *Neurobiol. Aging* 2003, *24* (2), 197–211.

(18) Mattila, P. M.; Röyttä, M.; Lönnberg, P.; Marjamäki, P.; Helenius, H.; Rinne, J. O. Choline Acetytransferase Activity and Striatal Dopamine Receptors in Parkinson's Disease in Relation to Cognitive Impairment. *Acta Neuropathol.* 2001, *102* (2), 160–166.

(19) Aldrin-Kirk, P.; Davidsson, M.; Holmqvist, S.; Li, J.-Y.; Björklund, T. Novel AAV-Based Rat Model of Forebrain Synucleinopathy Shows Extensive Pathologies and Progressive Loss of Cholinergic Interneurons. *PLoS One* 2014, *9* (7), e100869.

(20) Jope, R. S. High Affinity Choline Transport and AcetylCoA Production in Brain and Their Roles in the Regulation of Acetylcholine Synthesis. *Brain Research Reviews*. 1979, 313–344.

(21) A.G. Black, S.; Jane Rylett, R. Choline Transporter CHT Regulation and Function in Cholinergic Neurons. *Cent. Nerv. Syst. Agents Med. Chem.* 2012, *12* (2), 114–121.

(22) Ferguson, S. M.; Savchenko, V.; Apparsundaram, S.; Zwick, M.; Wright, J.; Heilman, C. J.; Yi, H.; Levey, A. I.; Blakely, R. D. Vesicular Localization and Activity-Dependent Trafficking of Presynaptic Choline Transporters. *J. Neurosci.* 2003, *23* (30), 9697–9709.

(23) Brandon, E. P.; Mellott, T.; Pizzo, D. P.; Coufal, N.; D'Amour, K. A.; Gobeske, K.; Lortie, M.; López-Coviella, I.; Berse, B.; Thal, L. J.; et al. Choline Transporter 1 Maintains Cholinergic Function in Choline Acetyltransferase Haploinsufficiency. *J. Neurosci.* 2004, *24* (24), 5459–5466.

(24) Farrar, A. M.; Callahan, J. W.; Abercrombie, E. D. Reduced Striatal Acetylcholine Efflux in the R6/2 Mouse Model of Huntington's Disease: An Examination of the Role of Altered Inhibitory and Excitatory Mechanisms. *Exp. Neurol.* 2011, *232* (2), 119–125.

(25) Mohr, F.; Krejci, E.; Zimmermann, M.; Klein, J. Dysfunctional Presynaptic M2 Receptors in the Presence of Chronically High Acetylcholine Levels: Data from the PRiMA Knockout Mouse. *PLoS One* 2015, *10* (10), e0141136.

(26) Watanabe, H.; Shimizu, H.; Matsumoto, K. Acetylcholine Release Detected by Trans-Striatal Dialysis in Freely Moving Rats Correlates with Spontaneous Motor Activity. *Life Sci.* 1990, *47* (9), 829–832.

(27) Day, J.; Damsma, G.; Fibiger, H. C. Cholinergic Activity in the Rat Hippocampus, Cortex and Striatum Correlates with Locomotor Activity: An in Vivo Microdialysis Study. *Pharm. Biochem. Behav.* 1991, *38*, 723–729.

(28) Le Moine, C.; Normand, E.; Bloch, B. Phenotypical Characterization of the Rat Striatal Neurons Expressing the D1 Dopamine Receptor Gene. *Proc. Natl. Acad. Sci. U. S. A.* 1991, *88* (10), 4205–4209.

(29) Le Moine, C.; Tison, F.; Bloch, B. D2 Dopamine Receptor Gene Expression by Cholinergic Neurons in the Rat Striatum. *Neurosci. Lett.* 1990, *117* (3), 248–252.

(30) Maurice, N. D2 Dopamine Receptor-Mediated Modulation of Voltage-Dependent Na+ Channels Reduces Autonomous Activity in Striatal Cholinergic Interneurons. *J. Neurosci.* 2004, *24* (46), 10289–10301.

(31) Bertorelli, R.; Consolo, S. D1 and D2 Dopaminergic Regulation of Acetylcholine Release from Striata of Freely Moving Rats. *J. Neurochem.* 1990, *54* (6), 2145–2148.

(32) Aosaki, T.; Kiuchi, K.; Kawaguchi, Y. Dopamine D1-like Receptor Activation Excites Rat Striatal Large Aspiny Neurons in Vitro. *J. Neurosci.* 1998, *18* (14), 5180–5190.

(33) Zhang, W.; Yamada, M.; Gomeza, J.; Basile, A. S.; Wess, J. Multiple Muscarinic Acetylcholine Receptor Subtypes Modulate Striatal Dopamine Release, as Studied with M1-M5 Muscarinic Receptor Knock-out Mice. *J. Neurosci.* 2002, *22* (15), 6347–6352.

(34) Calabresi, P.; Centonze, D.; Gubellini, P.; Pisani, A.; Bernardi, G. Acetylcholine-Mediated Modulation of Striatal Function. *Trends in Neurosciences*. 2000, 120–126.

(35) Hartmann, J.; Kiewert, C.; Klein, J. Neurotransmitters and Energy Metabolites in Amyloid-Bearing APP SWE XPSEN1dE9 Mouse Brain. *J. Pharmacol. Exp. Ther.* 2010, *332* (2), 364–370.

(36) Ding, J.; Guzman, J. N.; Tkatch, T.; Chen, S.; Goldberg, J. A.; Ebert, P. J.; Levitt, P.; Wilson, C. J.; Hamm, H. E.; Surmeier, D. J. RGS4-Dependent

Attenuation of M4 Autoreceptor Function in Striatal Cholinergic Interneurons Following Dopamine Depletion. *Nat. Neurosci.* 2006, *9* (6), 832–842.

(37) Dobransky, T.; Rylett, R. J. A Model for Dynamic Regulation of Choline Acetyltransferase by Phosphorylation. *J. Neurochem.* 2005, *95* (2), 305–313.

(38) Ostrerova, N.; Petrucelli, L.; Farrer, M.; Mehta, N.; Choi, P.; Hardy, J.; Wolozin, B. Alpha-Synuclein Shares Physical and Functional Homology with 14-3-3 Proteins. *J. Neurosci.* 1999, *19* (14), 5782–5791.

(39) Stadler, H.; Lloyd, K. G.; Gadea-Ciria, M.; Bartholini, G. Enhanced Striatal Acetylcholine Release by Chlorpromazine and Its Reversal by Apomorphine. *Brain Res.* 1973, *55* (2), 476–480.

(40) Borghammer, P.; Chakravarty, M.; Jonsdottir, K. Y.; Sato, N.; Matsuda, H.; Ito, K.; Arahata, Y.; Kato, T.; Gjedde, A. Cortical Hypometabolism and Hypoperfusion in Parkinson's Disease Is Extensive: Probably Even at Early Disease Stages. *Brain Struct. Funct.* 2010, *214* (4), 303–317.

(41) Pickrell, A. M.; Youle, R. J. The Roles of PINK1, Parkin, and Mitochondrial Fidelity in Parkinson's Disease. *Neuron* 2015, *85* (2), 257–273.

(42) Ragozzino, M. E.; Unick, K. E.; Gold, P. E. Hippocampal Acetylcholine Release during Memory Testing in Rats: Augmentation by Glucose. *Proc. Natl. Acad. Sci. U. S. A.* 1996, *93* (10), 4693–4698.

(43) Messier, C.; Gagnon, M. Glucose Regulation and Cognitive Functions: Relation to Alzheimer's Disease and Diabetes. *Behav. Brain Res.* 1996, *75* (1–2), 1–11.

(44) Schurr, A. Lactate: The Ultimate Cerebral Oxidative Energy Substrate? *J. Cereb. Blood Flow Metab.* 2006, *26* (1), 142–152.

(45) Paxinos, G.; Franklin, K. B. The Mouse Brain in Stereotaxic Coordinates. *3rd ed.* 2001, 827–828.

(46) Fonnum, F. Radiochemical Micro Assays for the Determination of Choline Acetyltransferase and Acetylcholinesterase Activities. *Biochem. J.* 1969, *115* (3), 465–472.

(47) Erb, C.; Troost, J.; Kopf, S.; Schmitt, U.; Löffelholz, K.; Soreq, H.; Klein, J. Compensatory Mechanisms Enhance Hippocampal Acetylcholine Release in Transgenic Mice Expressing Human Acetylcholinesterase. *J. Neurochem.* 2001, *77* (2), 638–646.

(48) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol.* 1961, *7* (2), 88–95.

(49) Zimmermann, M.; Westwell, M. S.; Greenfield, S. A. Impact of Detergents on the Activity of Acetylcholinesterase and on the Effectiveness of Its Inhibitors. *Biol. Chem.* 2009, *390* (1), 19–26.

(50) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* 1976, *72* (1–2), 248–254.

(51) Lietsche, J.; Gorka, J.; Hardt, S.; Karas, M.; Klein, J. Self-Built Microdialysis Probes with Improved Recoveries of ATP and Neuropeptides. *J. Neurosci.*

Methods 2014, *237*, 1–8.
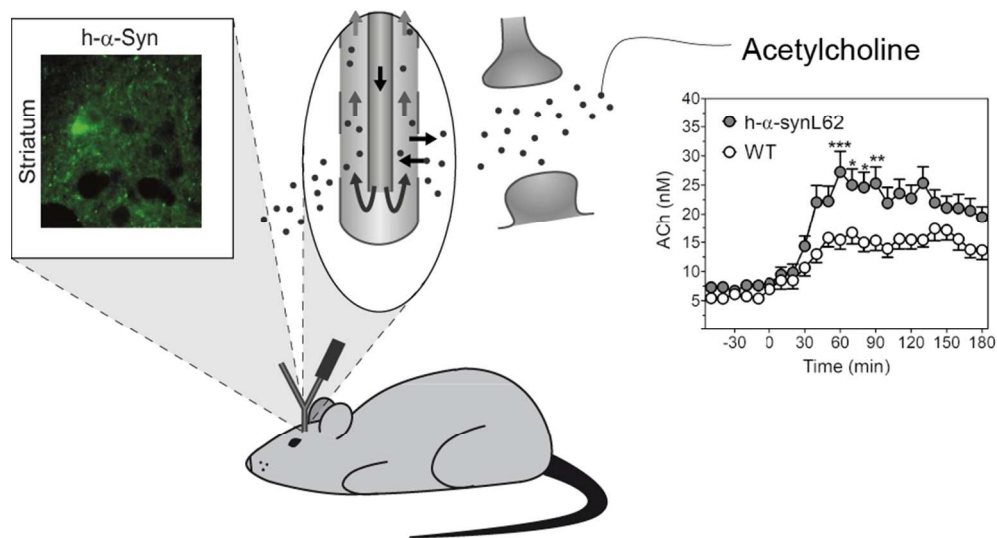
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