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Blood cell mitochondrial dysfunction as early indicator of alpha-synuclein pathology in rats

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Abstract

Parkinson's disease (PD) is characterized by the progressive degeneration of dopaminergic neurons in the Substantia nigra pars compacta (SNpc) and the accumulation of α-synuclein (α-syn)-containing inclusions. Although the precise mechanisms underlying this process remain incompletely understood, mitochondrial dysfunction is a critical factor in disease pathogenesis. PD is typically diagnosed after substantial neurodegeneration has occurred, when available therapies remain symptomatic and disease-modifying treatments are lacking. This underscores an urgent need for biomarkers that detect PD during its prodromal stages, prior to overt motor symptoms. In this study, we developed an early-stage rat model of PD via viral vector-induced overexpression of human α-syn in the SNpc and investigated whether peripheral mitochondrial alterations reflect early central pathology at 1 and 3 months after vector injection. We evaluated motor and non-motor behaviour, dopaminergic cell loss, brain expression of α-syn and peripheral cytokine levels. Brain tissue and blood cells were collected, and activities of mitochondrial electron transport chain complex activities were quantified using custom-made microarray-based assays. Overexpression of α-syn induced mild dopaminergic degeneration in absence of motor impairment and was associated with anxiety-like behaviour, consistent with prodromal PD. Systemic inflammatory markers were unchanged; however, mitochondrial activity in brain and peripheral blood cells showed significant alterations. Complex I activity was significantly reduced in both SNpc and platelets, showing a non-significant trend in peripheral blood mononuclear cells (PBMCs). In contrast, complex IV activity increased in platelets but decreased in PBMCs. Activities of the remaining complexes were unaffected. A logistic regression model based on complex I and IV activities accurately discriminated α-syn-overexpressing animals from controls. These findings demonstrate that peripheral mitochondrial electron transport chain dysfunction mirrors early α-syn-induced brain pathology and identifies blood-based mitochondrial profiling as a promising non-invasive strategy for early PD detection.

Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease, after Alzheimer’s disease, affecting more than 8.5 million people worldwide [73]. It is characterised by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and by the accumulation of misfolded α-synuclein (α-syn) aggregates forming Lewy bodies [5], the pathological hallmark of the disease. Motor symptoms emerge only after approximately 50-60% of SNpc dopaminergic neurons have been lost, highlighting the late appearance of clinical signs relative to the underlying pathology [72].

Under pathological conditions, α-syn undergoes conformational changes that promote aggregation [10], alter dopamine metabolism and contribute to neuronal degeneration [35]. In addition, α-syn impairs mitochondrial function, a central mechanism in PD pathogenesis, by disrupting the import of nuclear-encoded mitochondrial proteins and increasing oxidative stress [16]. Dopaminergic neurons of the SNpc are particularly vulnerable to these alterations because of their high energetic demands and limited antioxidant capacity [4, 15, 42]. This mechanistic link is further supported by genetic studies showing that mutations in PINK1, DJ-1, and LRRK2 impair mitochondrial homeostasis [12, 49, 75]. Likewise, experimental models based on mitochondrial complex I inhibition, including rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrabydropyridine (MPTP) reproduce key pathological features of PD.

Because motor symptoms emerge only after substantial nigral neurodegeneration, PD is typically diagnosed beyond the stage at which disease-modifying therapies are most likely to be effective [55]. Current neuroimaging methods, including dopamine transporter imaging, also lack sufficient sensitivity to reliably detect PD during its prodromal stages. These limitations have driven the search for biomarkers capable of identifying PD at earlier stages. Given the pivotal role of α-syn in PD pathogenesis, numerous studies have investigated α-syn levels in biological fluids as potential biomarkers. However, measurements in CSF [48], serum and plasma [7, 22], and saliva [70], have demonstrated limited diagnostic accuracy. More recently, phosphorylated α-syn (α-synP) detection in skin biopsies has provided a promising approach for identifying synucleinopathies, including PD and dementia with Lewy bodies [25, 26]. Likewise, α-syn detected in blood-derived exosomes has also shown promise for discriminating PD from other neurodegenerative disorders [18]. Circulating inflammatory markers have also been explored but lack the disease specificity required for PD diagnosis [6, 59]. Beyond α-syn-based biomarkers, mitochondrial dysfunction represents an attractive alternative because it occurs early in PD pathogenesis and can be measured in peripheral blood cells. However, whether mitochondrial dysfunction in peripheral blood faithfully mirrors early nigral pathology remains unknown.

To address this question, we used a rat model of early-stage PD based on viral vector-mediated overexpression of wild-type human α-syn in the SNpc. Mitochondrial electron transport chain (mETC) activity was quantified in brain tissue and peripheral blood cells using custom-made membrane microarray assays previously validated in animal tissues and human cell lines [21, 24, 60]. We hypothesised that peripheral mitochondrial alterations would mirror early nigral pathology and therefore represent candidate biomarkers for prodromal PD. Together, these findings provide a preclinical rationale for the clinical evaluation of blood-based mitochondrial biomarkers for early PD detection.

Materials and methods

The methodology employed in this study is described in detail in the Supplementary Material section.

Study design

A rat model of prodromal PD was generated by bilateral overexpressing of human α-syn in the SNpc using and adeno-associated viral vector (AAV) encoding human SNCA under the synapsin promoter. Control (SHAM) animals received bilateral injections of an empty AAV vector. Behavioural assessments were performed at 1 and

3 months after injection to evaluate motor and non-motor phenotypes. Animals were subsequently sacrificed, and brain and blood samples were collected for downstream analyses.

Brain tissue was analysed to assess α-syn distribution, dopaminergic neurodegeneration and mETC activity in the SNpc and associated regions. Peripheral mitochondrial function was evaluated in platelets and peripheral mononuclear cells (PBMCs), whereas serum samples were analysed for inflammatory cytokine levels. Mitochondrial activity was quantified using custom membrane microarray-bases assays in isolated brain membranes and blood-derived cells (Fig. 1). The primary outcome measures were mitochondrial electron transport chain (mETC) activity in blood cells and their association with neuropathological changes.

Experimental animals

Forty-eight-8-week old Adult male Sprague–Dawley wild-type rats (approximately 250g; Envigo, Spain) were used in this study. All experimental procedures were approved by the Local Committee for Animal Experimentation of the University of the Basque Country (UPV/EHU) (Leioa, Spain; #M20-2021 and #M30-2021-242) and performed in accordance with European Directive 2010/63/EU and Spanish legislation (RD 53/2013) and ARRIVE guidelines.

Stereotaxic surgery

Rats were bilaterally injected into the SNpc of an AAV encoding human SNCA gene to induce α-syn overexpression. SHAM animals received bilateral injections of the corresponding empty AAV vector. Injections where performed using stereotaxic frame (David Kopf® Instruments, U.S.A.) on coordinates (relative to Bregma): AP=5.3, ML=1.8, DV=7.2 to target SNpc. Following surgery, animals were allocated to two experimental groups: a SHAM group (n=24), which received bilateral injections of an empty AAV (rAAV9-CMVie/SynP_WPRE), and an α-syn group (n=24), which received bilateral injections of an AAV encoding human α-syn (rAAV9-CMVie/SynP-wtsyn-WPRE).

Behavioural evaluation

Behavioural assessment was performed at 1 and 3 months post injection using a battery of tests evaluating general activity, motor and non-motor behaviour. The open field test assessed spontaneous locomotor activity, whereas the rotarod and bar test evaluated motor performance and coordination. Anxiety-like behaviour was assessed using elevated plus maze (EPM) and marble burying test (MBT).

Histochemical procedures

Coronal brain sections were used to confirm α-syn overexpression and assess dopaminergic neurodegeneration. Sections were immunolabelled for α-syn or tyrosine hydroxylase (TH). Striatal TH and α-syn immunoreactivity were quantified by optical density measurements using ImageJ/Fiji, with background correction performed using TH or α-syn negative cortical regions. TH-positive neurons in the SNpc were quantified using unbiased stereology with the optical fractionator method, and SNpc volume was estimated using Cavalieri principle. Detailed staining procedures and stereological parameters are provided in the Supplementary Methods.

Membrane isolation

Brain membrane fractions were isolated as previously described with minor modifications [21]. Briefly, the brain regions of interest were dissected, homogenized, and subjected to differential centrifugation to isolate the membrane fraction. The resulting membrane pellets were washed and stored at −80°C until further analysis.

Platelets and PBMCs isolation

Peripheral blood was collected by cardiac puncture under deep anesthesia into acid-citrate-dextrose anticoagulant tubes. Platelets and PBMCs were isolated using Ficoll-Paque density gradient with balanced saline solution (BSS), according to manufacturer´s instructions (Cytiva, U.S.A.). Following separation, platelet and PBMC fractions were collected and stored at -80°C until microarray fabrication.

Serum collection and ELISA multiplex assay

Serum samples were collected from peripheral blood and analysed using a multiplex ELISA assay to quantify inflammatory cytokines (IL-1β, IL-2, IL-6, IL-10, TNF-α and IFN-γ). The assay kit was obtained from Sigma-Aldrich and performed according to the manufacturer's instructions.

Microarrays fabrication

Cell membrane microarrays (CMMAs) and cell microarrays (CMAs) were designed for distinct purposes and fabricated using collected samples. Microarray fabrication was performed as previously described (IMG Pharma Biotech S.L., Spain) [21, 45]. Membrane fractions derived from the cortex, striatum and SNpc were used for the generation of CMMAs, whereas isolated platelets and PBMCs were used to fabricate CMAs. These microarrays were subsequently used to assess mETC activity.

Determination of mitochondrial activity in microarrays

mETC activity was assessed in brain CMMAs and blood cell CMAs. Enzymatic activity of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified by measuring enzyme-dependent superoxide production using nitro blue tetrazolium (NBT)-based detection. Cytochrome c oxidase (CCo, complex IV) activity was determined by 3,3´-diaminobenzidine (DAB)-based histochemical detection [21, 24]. Signal intensity was quantified by optical density and normalized to immobilized protein content.

Predictive model

A supervised classification approach was used to evaluate whether peripheral mitochondrial biomarkers could discriminate between SHAM and α-syn animals. Model performance was assessed using five-fold cross-validation. Following feature selection to remove non-informative variables, the four peripheral biomarkers with the greatest discriminatory power were used to train and evaluate logistic regression and neural network classifiers.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.2.0 (GraphPad Software, U.S.A.). Data distribution was assessed using the Shapiro-Wilk normality test. Results are presented as mean ± SEM. Behavioural, immunohistochemical, mitochondrial, and ELISA data were analysed using two-way analysis of variance (ANOVA), followed by Tukey´s multiple-comparisons test when appropriate. Statictical significance was set at $p < 0.05$.

Results

Behavioural evaluation

Behavioural testing revealed no evidence of motor deficit in α-syn-overexpressing rats, either 1 or 3 months after AAV injection, consistent with an early stage of PD. In the open field, overall locomotor activity was comparable between SHAM and α-syn animals (Fig. 2A–D). Both groups showed an age-dependent decline in travelled distance and rearing activity at 3 months, whereas no group differences were detected in either parameter (Fig. 2A–D). Similarly, rotarod performance and bar test latency did not differ between groups (Fig. 2E,F), although rotarod performance declined over time in both SHAM and α-syn animals (Fig. 2E).

As for non-motor behaviour, no differences between groups were observed in the open field or EPM, as indicated by similar time spent in the centre of the arena, open-arm entries and open-arm exploration (Fig. 2G–I). However, the MBT revealed a modest increase in anxiety-like behaviour in α-syn animals at 3 months compared with 1 month, suggesting a subtle increase in anxiety-like behaviour over time (Fig. 2J).

Overall, these findings indicate that α-syn-overexpressing rats display minimal behavioural abnormalities, according with a prodromal stage of PD.

Immunohistochemical verification

Human α-syn expression was detected throughout the rostrocaudal axis exclusively in α-syn animals (Fig. 3A, B). Striatal expression remained stable between 1 and 3 months. Although expression was widespread, the highest density of α-syn positive axons was observed in regions connected to the nigrostriatal system, including the striatum, nucleus accumbens, zona incerta, subthalamic nucleus, substantia nigra pars reticulata, ventral tegmental area and dorsal raphe nucleus, together with reticular formation of the submedius thalamic nucleus (Supplementary Fig. 1; Supplementary Table 3).

Overexpression of α-syn induced mild nigrostriatal degeneration. At 3 months, α-syn animals showed approximately 20% reduction in TH-positive striatal fibres compared with SHAM animals (Fig. 3C), predominantly affecting the central and caudal striatum (Fig. 3E). Stereological analysis further revealed a 23% reduction of TH-positive neurons in the SNpc, with degeneration concentrated in its central region (Fig. 3D,F).

Double immunofluorescence confirmed extensive colocalization of human α-syn with TH in the SNpc, striatum and nigrostriatal fibres (Fig. 4A,B). In contrast, α-synP was detected exclusively in the SNpc despite the

widespread distribution of total α-syn, indicating that pathological α-syn accumulation was confined to nigral cells (Fig. 4C).

These findings confirm stable human α-syn overexpression, accompanied by limited nigrostriatal degeneration and restricted α-syn phosphorylation within the SNpc.

Inflammatory markers in serum

Systemic inflammation was assessed by multiplex ELISA in serum samples from SHAM and α-syn animals at 1 and 3 months. No differences were detected in circulating levels of pro- and anti-inflammatory cytokines, including IL-1β, IL-2, IL-6, IL-10, TNF-α and IFN-γ, between experimental groups at either time point (Supplementary Fig.2). These findings indicate that α-syn overexpression was not associated to systemic inflammation.

Measurement of mitochondrial activity

Using custom-made microarrays, we investigated the impact of α-syn overexpression on mitochondrial activity in the brain and blood cells.

Mitochondrial activity in brain

Overexpression of α-syn selectively impaired mitochondrial complex I activity in the nigrostriatal system. At 3 months, complex I activity was significantly reduced in the SNpc of α-syn animals compared with SHAM controls, whereas the striatum showed a similar, although non-significant, trend (Fig. 5A,B). In contrast, cortical complex I activity was increased in α-syn animals at 3 months (Fig. 5C). Complex II activity remained unchanged in the SNpc and cortex throughout the study. In the striatum, however, complex II activity showed an age-dependent reduction in SHAM animals, which was not observed in the α-syn group (Fig. 5D-F). Similarly, age-related decreases in GAPDH and complex IV activity were observed in the striatum of both groups at 3 months (Fig. 5G-L).

Overall, mitochondrial alterations associated with α-syn overexpression were predominantly observed at the level of complex I within the nigrostriatal system.

Mitochondrial activity in platelets and PBMCs

We next examined whether mitochondrial alterations were also detectable in peripheral blood cells.

In platelets, complex I activity was reduced in α-syn animals, particularly at 1 month, consistent with the impairment observed in the SNpc (Fig. 6A). In contrast, complex II activity increased at 3 months in both experimental groups, while GAPDH activity remained unchanged (Fig. 6B,C). Complex IV activity was increased in α-syn animals at 3 months, displayed faster reaction kinetics than in SHAM controls (Fig. 6D-F).

In PBMCs, complex I activity showed a similar pattern with a non-significant reduction in α-syn animals (Fig. 7A). Complex II activity increased at 3 months, whereas GAPDH activity remained unchanged (Fig. 7B,C). In contrast, complex IV activity was reduced in α-syn animals compared with SHAM controls (Fig. 7D).

These findings indicate that α-syn overexpression induces peripheral mitochondrial alterations that partially mirror central bioenergetic deficits.

Predictive model

Peripheral mitochondrial parameters were used to develop supervised classification models to discriminate between SHAM and α-syn animals. Complex I and IV activities in platelets and PBMCs were selected as input variables based on their discriminative performance in the preceding analyses. The neural network model achieved an accuracy of 0.75 and an AUC of 0.82, whereas logistic regression showed slightly higher predictive performance, with an accuracy of 0.78 and an AUC of 0.82 (Fig. 8A,B; Supplementary Table 4). Accordingly, logistic regression was selected as the best-performing model. However, model performance should be interpreted with caution due to the limited sample size and the use of the same dataset for model training and evaluation.

The logistic regression model correctly classified 78% of animals, which, under the experimental conditions of this study, supports the ability of peripheral mitochondrial parameters to discriminate between control animals and those with prodromal PD.

Discussion

The present study demonstrates that selective human α-syn overexpression in the SNpc recapitulates key features of prodromal PD, including minimal behavioural abnormalities, early nigrostriatal degeneration and selective mitochondrial dysfunction within the nigrostriatal system. Using this model, we further demonstrated that mitochondrial alterations are also detectable in peripheral blood cells and that these measures discriminate α-syn-overexpressing animals from controls. Together, these findings identify a distinct peripheral mitochondrial signature that supports the investigation of blood-based mitochondrial function as a source of accessible biomarkers for the early detection of PD.

The absence of motor deficits despite early nigrostriatal degeneration supports the prodromal nature of this model. This finding is consistent with previous AAV-mediated α-syn overexpression models, in which motor impairment typically emerges only after prolonged expression of wild-type α-syn, often beyond one year [51, 53], whereas α-syn preformed fibril models generally develop motor dysfunction at earlier stages [66]. The age-dependent decline in locomotor performance observed in both experimental groups is therefore more likely to reflect normal increases in body size and weight than an effect of α-syn overexpression, as previously reported in transgenic models [47]. In contrast, α-syn-overexpressing rats displayed a modest increase in anxiety-like behaviour, one of the earliest non-motor manifestations of PD according to Braak´s hypothesis [5]. Although this effect was only detected in the MBT, subtle anxiety-like alterations have also been reported in other transgenic models using different behavioural paradigms [47, 51]. These findings indicate that behavioural abnormalities remain limited at this stage of pathology, with subtle non-motor alterations preceding the onset of overt motor dysfunction.

Human α-syn expression remained stable throughout the study and extended beyond the nigrostriatal pathway to multiple interconnected brain regions, in accordance with previous AAV-mediated models [14, 27, 30, 50, 68]. A similar pattern of α-syn spreading has also been reported following selective α-syn overexpression in other brain regions, including the locus coeruleus [58], suggesting that this phenomenon is not restricted to the nigrostriatal system. Although this widespread distribution was initially attributed to anatomical connectivity [5], increasing evidence supports a prion-like mechanism underlying α-syn propagation to more distant brain regions [44].

Notably, a similarly widespread pattern of regional involvement has been described in PD patients, including the caudate, nucleus accumbens and cingulate cortex [65], supporting the translational relevance of this model. In contrast to the widespread distribution of total α-syn, phosphorylated α-syn remained largely restricted to the SNpc, in line with evidence that soluble α-syn propagates more extensively than aggregated species during the early stages of pathology [31]. Aligned with previous AAV-mediated models [9, 14, 27, 30, 38, 50, 68, 74], α-syn overexpression induced mild dopaminergic degeneration in both the SNpc and striatum, resulting in an approximately 20% reduction in TH immunoreactivity. Unlike many studies reporting more severe degeneration in the SNpc than the striatum, reflecting a degeneration process starting in the injection site [9, 14, 27, 37, 39] both regions were similarly affected in our model, likely reflecting the early stage of pathology. Colocalization of TH and human α-syn confirmed that the use of a neuron promoter effectively targets dopaminergic cells [14, 27, 39, 68]. Taken together, these findings indicate that α-syn pathology is stablished while neurodegeneration remains limited and insufficient to produce motor deficits.

No evidence of systemic inflammation was detected in α-syn-overexpressing animals, as circulating levels of IL-1β, IL-2, IL-6, IL-10, TNF-α and IFN-γ remained unchanged throughout the study. Although systemic inflammatory responses have been reported in several PD models, including toxin- and α-syn-based paradigms [23, 32, 52, 54, 57, 63], these findings are highly variable, and circulating cytokines lack disease specificity, limiting their utility as PD biomarkers. Alternatively, transient inflammatory responses may have occurred at earlier stages, as increased serum levels of IL-1β, IL-6 and TNF-α have been reported shortly after MPTP administration [63], suggesting that earlier sampling time points may be required to detect systemic inflammatory changes in this model.

The present findings identify mitochondrial dysfunction as one of the earliest detectable consequences of α-syn overexpression, with complex I emerging as the most consistently affected component of the electron transport chain. Importantly, complex I impairment was detected not only within the nigrostriatal system but also in peripheral blood cells, suggesting that central bioenergetic alterations are accompanied by a distinct peripheral mitochondrial signature. This observation is particularly relevant because mitochondrial dysfunction and oxidative stress are widely recognised as central mechanisms in PD pathogenesis and are thought to precede neurodegeneration and the onset of motor symptoms [67].

The contribution of mitochondrial dysfunction to PD is supported by converging evidence from both genetic and experimental models. Mutations in PINK1, DJ-1 and LRRK2 disrupt mitochondrial homeostasis [12, 49, 75], while experimental models, such as Mitopark [71] and MCI-Park mice [28], reproduce key pathological and behavioural features of PD through primary mitochondrial impairment. Although Mitopark mice develop a progressive parkinsonian phenotype as a consequence of selective mitochondrial dysfunction in dopaminergic neurons [71], the more specific conditional complex I disruption in MCI-Park mice results in prodromal alterations as sleep-wake regulation and arousal [64], accompanied by only subtle motor deficits, suggesting that mitochondrial complex I dysfunction perturbs distributed neural circuits before the onset of parkinsonian motor symptoms [28].

The selective vulnerability of SNpc dopaminergic neurons provides a plausible biological explanation for the mitochondrial alterations observed in our model [8, 17]. These neurons have exceptionally high energetic demands, making mitochondrial homeostasis essential for maintaining neuronal function [34, 36]. In addition,

dopamine metabolism promotes oxidative stress through dopamine oxidation and the generation of reactive species [42], while the relatively limited antioxidant capacity of SNpc neurons further enhances their susceptibility to mitochondrial dysfunction. This selective vulnerability is even more pronounced within the SNpc itself, where ALDH1A1-positive neurons located in the ventral tier are particularly susceptible to degeneration [11, 43]. Importantly, the concomitant detection of similar bioenergetic alterations in circulating blood cells suggests that peripheral mitochondrial dysfunction may reflect early nigral pathology rather than secondary consequences of advanced neurodegeneration.

In agreement with the central role of mitochondrial dysfunction in PD, α-syn overexpression induced an early reduction in complex I activity within the nigrostriatal system, reaching significance in the SNpc and showing similar trend in the striatum. By contrast, the increased cortical activity may reflect compensatory metabolic adaptations previously described in unilateral lesion models [13]. Age-related reductions in mitochondrial activity were observed in the brain but not in peripheral blood cells, suggesting that brain mitochondria may be more susceptible to ageing, consistent with previous experimental and clinical studies [1, 20, 33]. Importantly, similar bioenergetic alterations were also detected in peripheral blood cells of α-syn-overexpressing animals, with platelets showing a marked reduction in complex I activity and PBMCs displaying a similar, although non-significant, trend. These findings are in line with previous reports of reduced complex I activity in the SNpc of PD patients and MPTP-treated monkeys [24, 29, 61]. Evidence from human studies supports the notion that peripheral mitochondrial alterations mirror central PD-related pathology. Mitochondrial dysfunction and oxidative stress are well-established features of both monogenic and idiopathic PD, with reduced complex I activity and increased oxidative damage consistently reported in post-mortem substantia nigra and other affected regions. Importantly, similar alterations have been detected in accessible peripheral tissues. Several studies describe impaired mitochondrial respiration, a shift towards glycolysis, and reduced complex I activity in PBMCs from PD patients. In platelets, reduced complex I activity is one of the most reproducible peripheral mitochondrial signatures of PD and has been replicated across independent cohorts [2, 29, 40, 69]. These observations are in line with the robust complex I deficit we observed in rat platelets and the trend towards reduced activity in PBMCs, supporting the translational relevance of this peripheral bioenergetic profile.

Complex IV activity showed distinct responses in peripheral blood cell populations, increasing in platelets while decreasing in PBMCs following α-syn overexpression. Although the increase in platelet complex IV activity appears counterintuitive in the presence of reduced complex I activity, mitochondrial complex I dysfunction can be partially compensated by increased electron entry through complex II [19, 56], providing a plausible explanation for the concomitant increase in complex II and IV activities observed in platelets. The divergent responses of platelets and PBMCs likely reflect their distinct biological properties, as platelets lack a nucleus and therefore have limited capacity for mitochondrial biogenesis and transcriptional adaptation [46]. Consistent with these observations, previous studies have reported heterogeneous changes in platelet complex IV activity in PD, ranging from reduced activity to no detectable differences [3, 40, 41, 69]. Evidence regarding PBMC mitochondrial function remains limited, although an overall reduction in mitochondrial activity has been described in patients with PD [62].

Together, these findings demonstrate that α-syn overexpression induces early mitochondrial alterations affecting both the nigrostriatal system and peripheral tissues, with complex I dysfunction representing a prominent feature. The detection of similar mitochondrial alterations in blood-derived cells highlights their potential translational

value as accessible indicators of early α-syn-related pathology. Although recent studies have reported that platelet complex I and IV activities alone are insufficient to stratify PD patients [41], this does not necessarily preclude the utility of peripheral mitochondrial biomarkers. Indeed, this study was performed in clinically diagnosed PD patients with established disease, whereas our model was designed to capture early α-syn-driven pathology. Furthermore, by integrating mitochondrial parameters from both platelets and PBMCs, we captured complementary aspects of peripheral bioenergetic dysfunction that may be missed when assessing a single cell type. Consistent with this, a supervised classification model based on complex I and IV activities achieved an accuracy and precision of 78%, supporting the potential of this peripheral bioenergetic signature to discriminate between SHAM and α-syn-overexpressing animals. Although these findings require validation in larger cohorts and independent datasets, they provide proof-of-concept that combining mitochondrial measurements from multiple peripheral blood cell populations may represent a promising strategy for the development of non-invasive biomarkers of early PD.

Overall, this study shows that these early central bioenergetic alterations are paralleled by a distinct peripheral mitochondrial signature that discriminates α-syn-overexpressing animals from controls. Although validation in human cohorts will be required, these findings provide experimental support for the development of blood-based mitochondrial biomarkers capable of detecting PD during its prodromal stages.

Data availability

The data that support the findings of this study are openly available in Zenodo at http://doi.org/10.5281/zenodo.22673531

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Competing interests

‘The authors report no competing interests.’

Supplementary material

Supplementary material is available at Acta Neuropathologica’.

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Figure legends

Fig. 1 Experimental design followed in the study**.** After surgery, behavioural assessments were performed and animals were sacrificed at 1 and 3 months, when brain and blood samples were collected. Brain samples were used for histochemical verification, whereas brain and blood samples were used to fabricate membrane microarrays for mitochondrial activity assays. Serum samples were analysed using a multiplex ELISA to quantify circulating inflammatory cytokines.

Fig. 2 Behavioural assessment of motor and non-motor function. (**A**) Representative locomotor tracks obtained during the open field test. (**B**) General activity in the open field test. (**C**) Total distance travelled in the open field test. (**D**) Number of rearings during the open field test. (**E**) Motor performance assessed using the rotarod test and expressed as latency to fall. (**F**) Catalepsy assessed using the bar test and expressed as latency to remove both forepaws from the bar. (**G**) Time spent in the centre of the open field arena. (**H**) Anxiety-like behaviour in the elevated plus maze (EPM) expressed as number of entries in the open arms. (**I**) Time spent in the open arms of the EPM. (**J**) Anxiety-like behaviour assessed using the marble burying test and expressed as the number of buried marbles. Data are presented as mean ± SEM of n= 8-24 animals per group: *P < 0.05, **P < 0.01, two-way ANOVA followed by Tukey´s post hoc test.

Fig. 3 Immunohistochemical validation of human α-syn expression and dopaminergic degeneration**.** (**A**) Distribution of human α-syn expression along the rostrocaudal axis of the brain. (**B**) Quantification of human α-syn expression in the striatum. (**C**) Optical density of TH-positive fibres in the striatum. (**D**) Stereological quantification of TH-positive neurons in the SNpc. (**E**) Representative TH immunostaining in the striatum. (**F**) Representative TH immunostaining in the SNpc. Data are presented as mean ± SEM of n= 6-10 animals per group: *P < 0.05, ****P < 0.0001, two-way ANOVA followed by Tukey´s post hoc test. Scale bar: 500 µm.

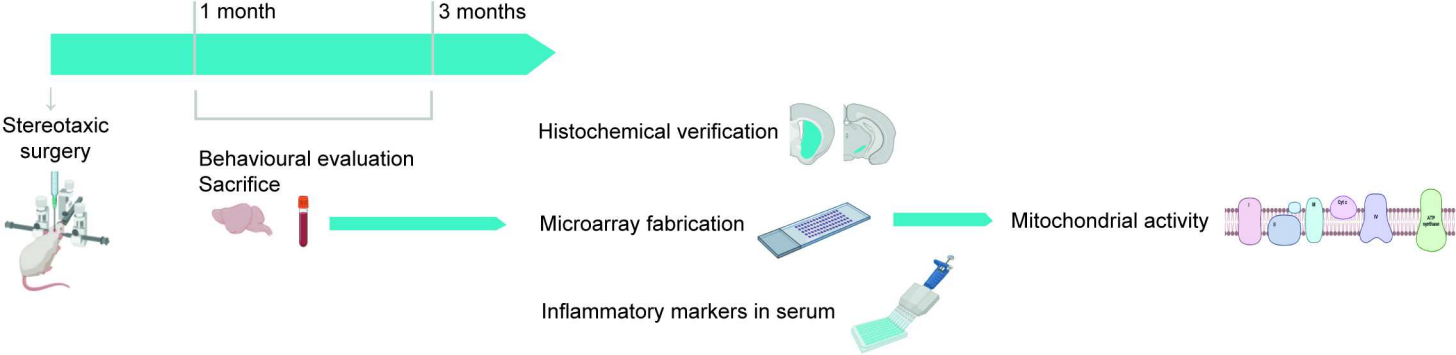
Fig. 4 Double immunofluorescence for tyrosine hydroxylase and α-synuclein in the nigrostriatal system**.** (**A**) Representative images showing co-localization of TH and human α-syn in the SNpc. Scale bar: 100 µm. (**B**) Representative image illustrating TH and human α-syn distribution throughout the nigrostriatal pathway in an α-syn-overexpressing animal. Scale bar: 2000 µm. (**C**) Representative images showing TH and α-synP immunoreactivity in the SNpc. Scale bar: 100 µm.

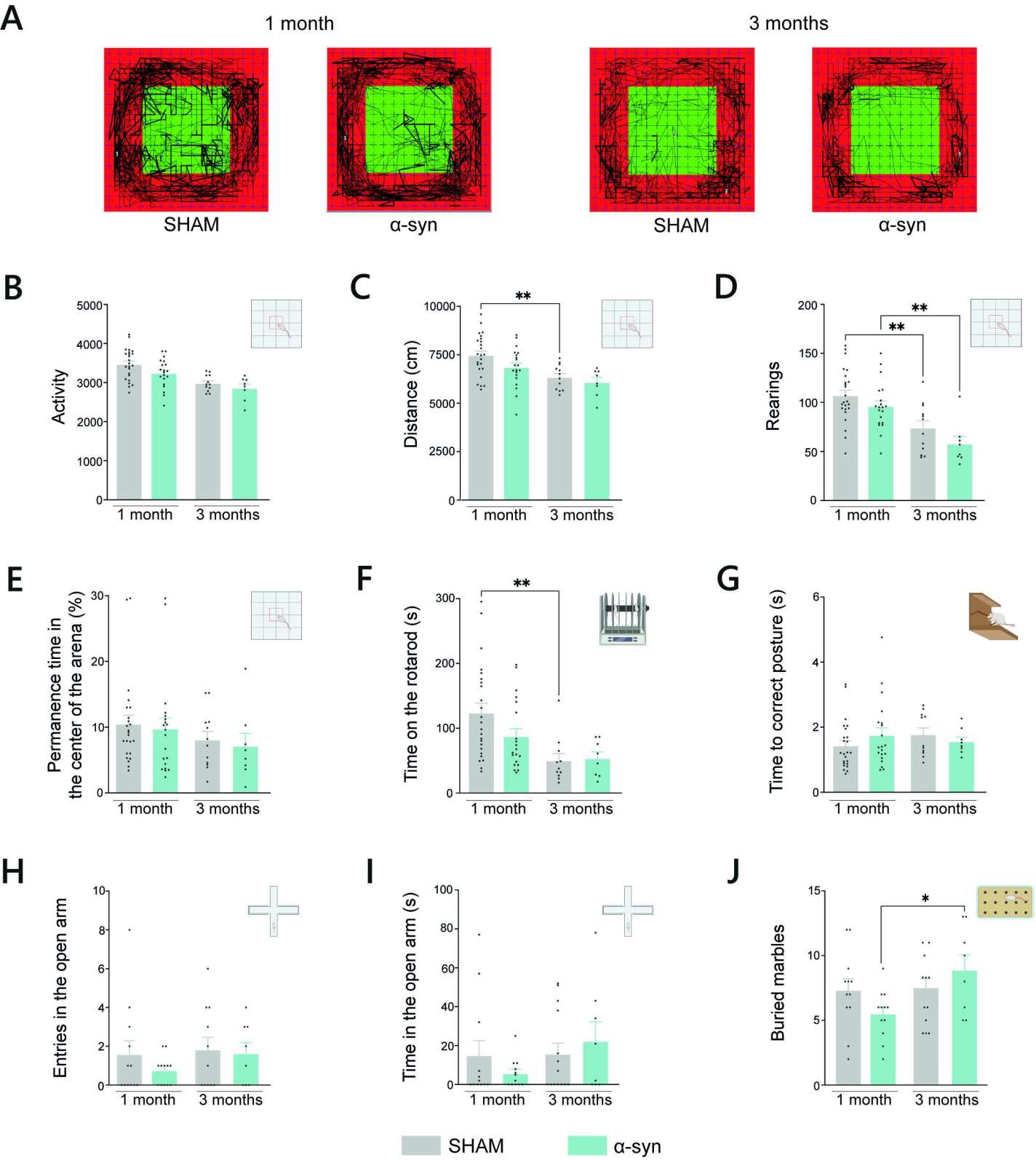
Fig. 5 Mitochondrial electron transport chain activity in the sustantia nigra compacta, striatum and cortex**.** (**A-C**) Maximum superoxide production mediated by mitochondrial complex I. (**D-F**) Maximum superoxide formation mediated by complex II. (**G-I**) Maximum superoxide formation mediated by GAPDH. (**J-L**) Mitochondrial complex IV activity. Data are presented as mean ± SEM of n= 8-12 animals per group: *P < 0.05, two-way ANOVA followed by Tukey´s post hoc test. #P < 0.05, two-way ANOVA.

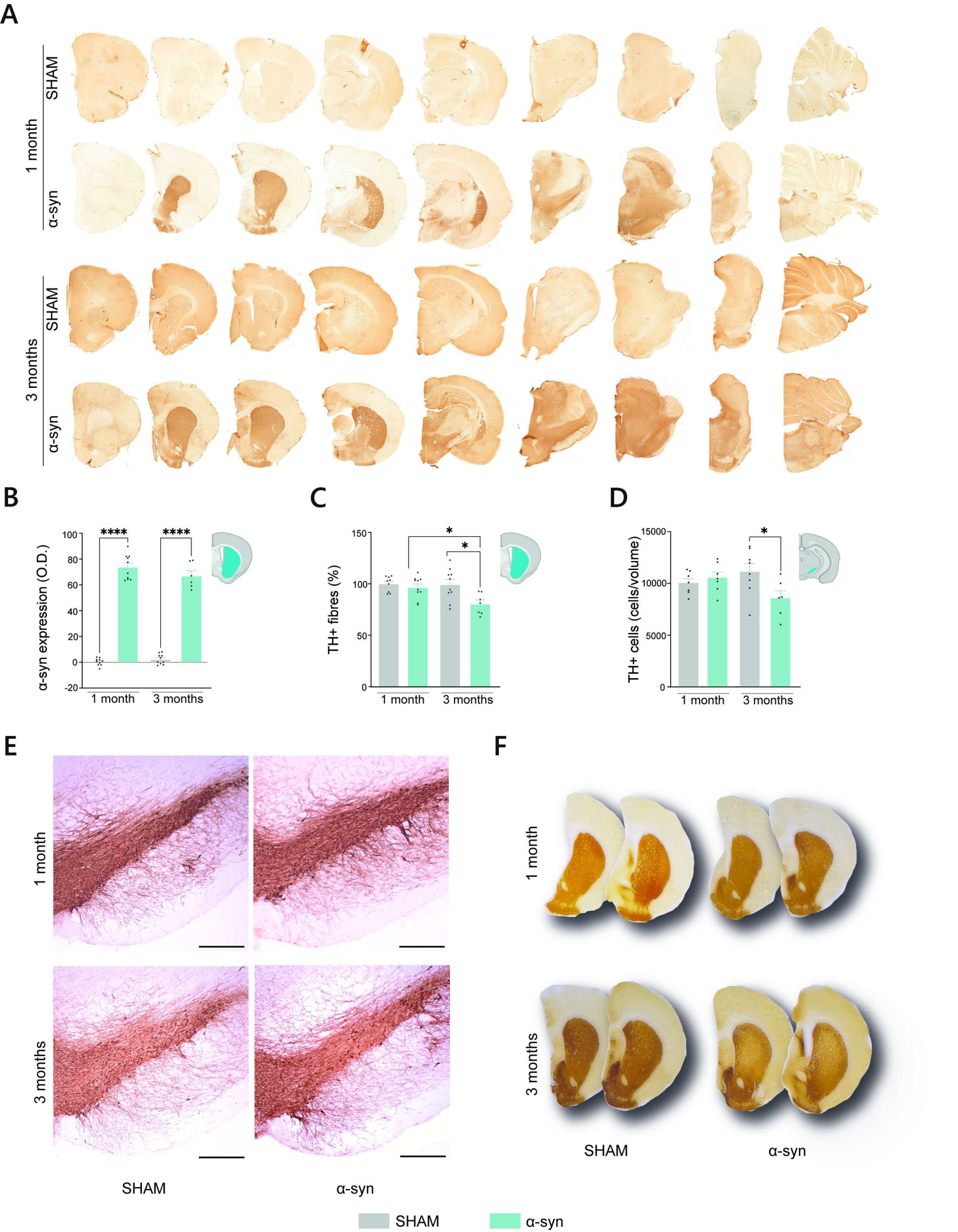
Fig. 6 Mitochondrial electron transport chain activity in platelets**.** (**A**) Maximum superoxide formation mediated by complex I. (**B**) Maximum superoxide formation mediated by complex II. (**C**) Maximum superoxide formation mediated by GAPDH. (**D**) Complex IV activity. (**E**) Representative complex IV reaction kinetics. (**F**) Complex IV kinetic slope. Data are presented as mean ± SEM of n= 8-12 animals per group: *P < 0.05, **P < 0.01, two-way ANOVA followed by Tukey´s post hoc test.

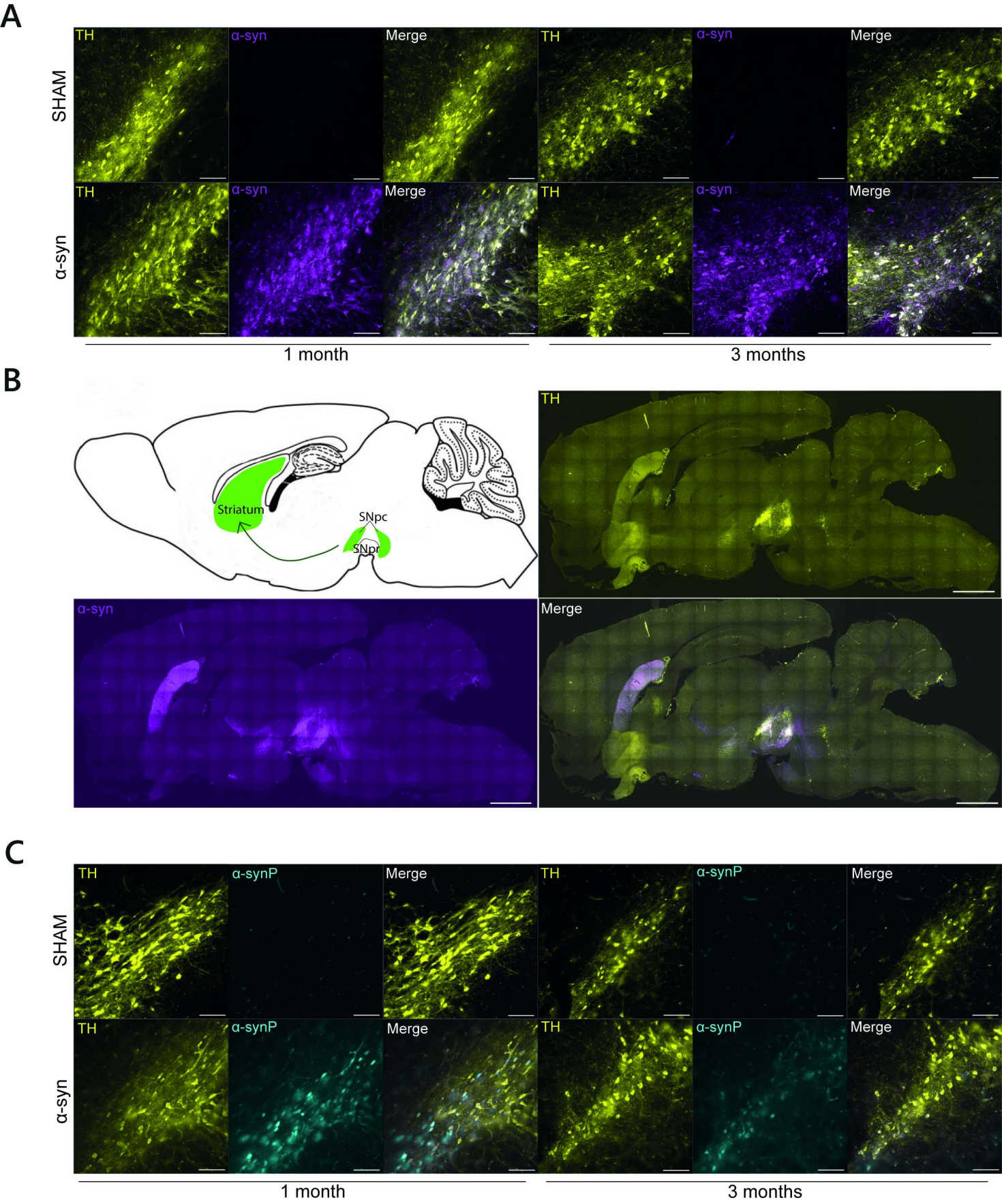
Fig. 7 Mitochondrial electron transport chain activity in PBMCs**.** (**A**) Maximum superoxide production mediated by complex I. (**B**) Maximum superoxide production mediated by complex II. (**C**) Maximum superoxide production mediated by GAPDH. (**D**) Mitochondrial complex IV. Data are presented as mean ± SEM of n= 8-12 animals per group: *P < 0.05, two-way ANOVA followed by Tukey´s post hoc test.

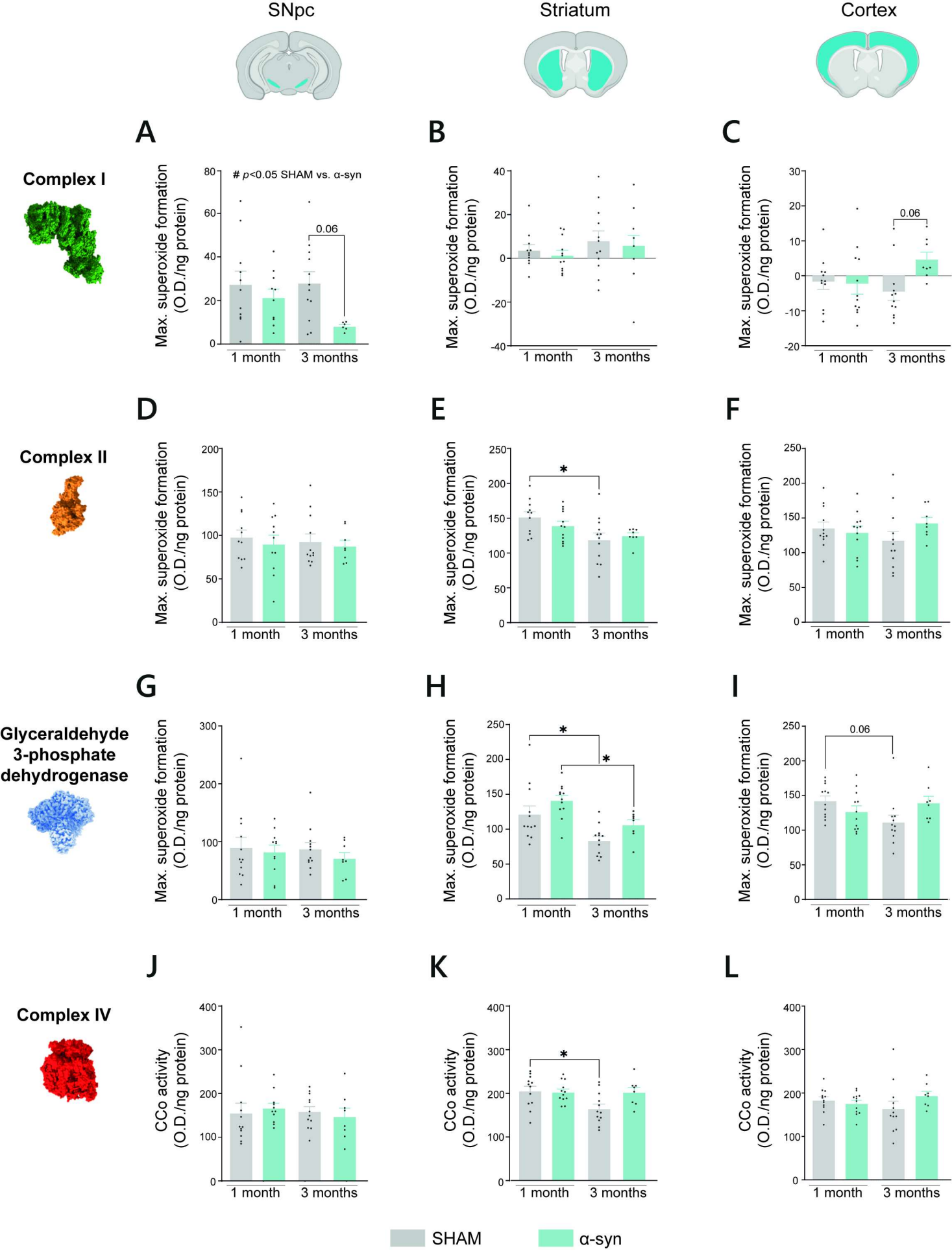
Fig. 8 Predictive model based peripheral mitochondrial parameters. A logistic regression model was trained using mitochondrial complex I and IV activities measured in platelets and PBMCs. (**A**) Confusion matrix showed that the trained model can discriminate between SHAM and α-syn animals with an accuracy between 75% and 80%. (**B**) ROC curve representing specificity of the model.

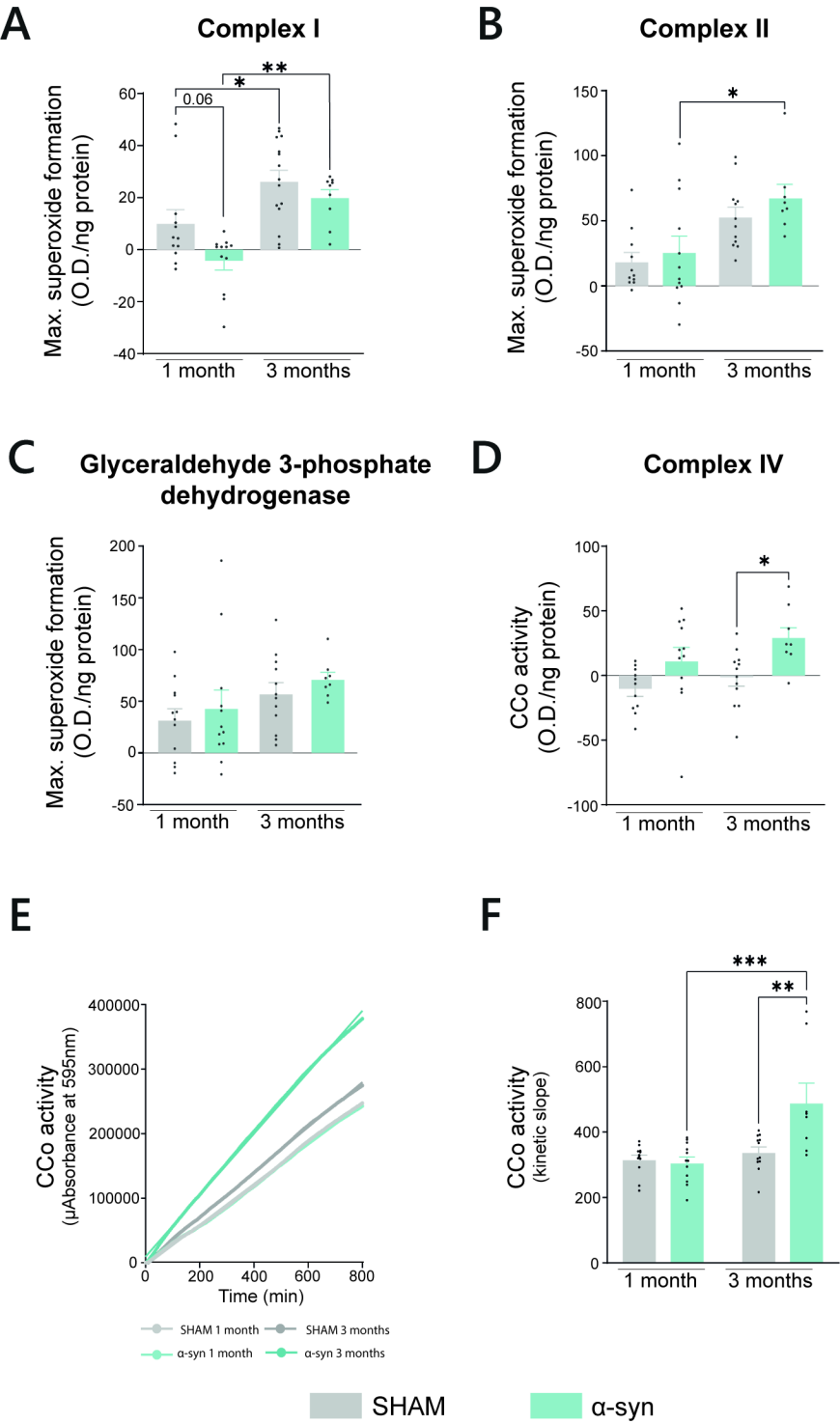

1 month
3 months
Stereotaxic surgery
Behavioural evaluation
Sacrifice
Histochemical verification
Microarray fabrication
Inflammatory markers in serum
Mitochondrial activity

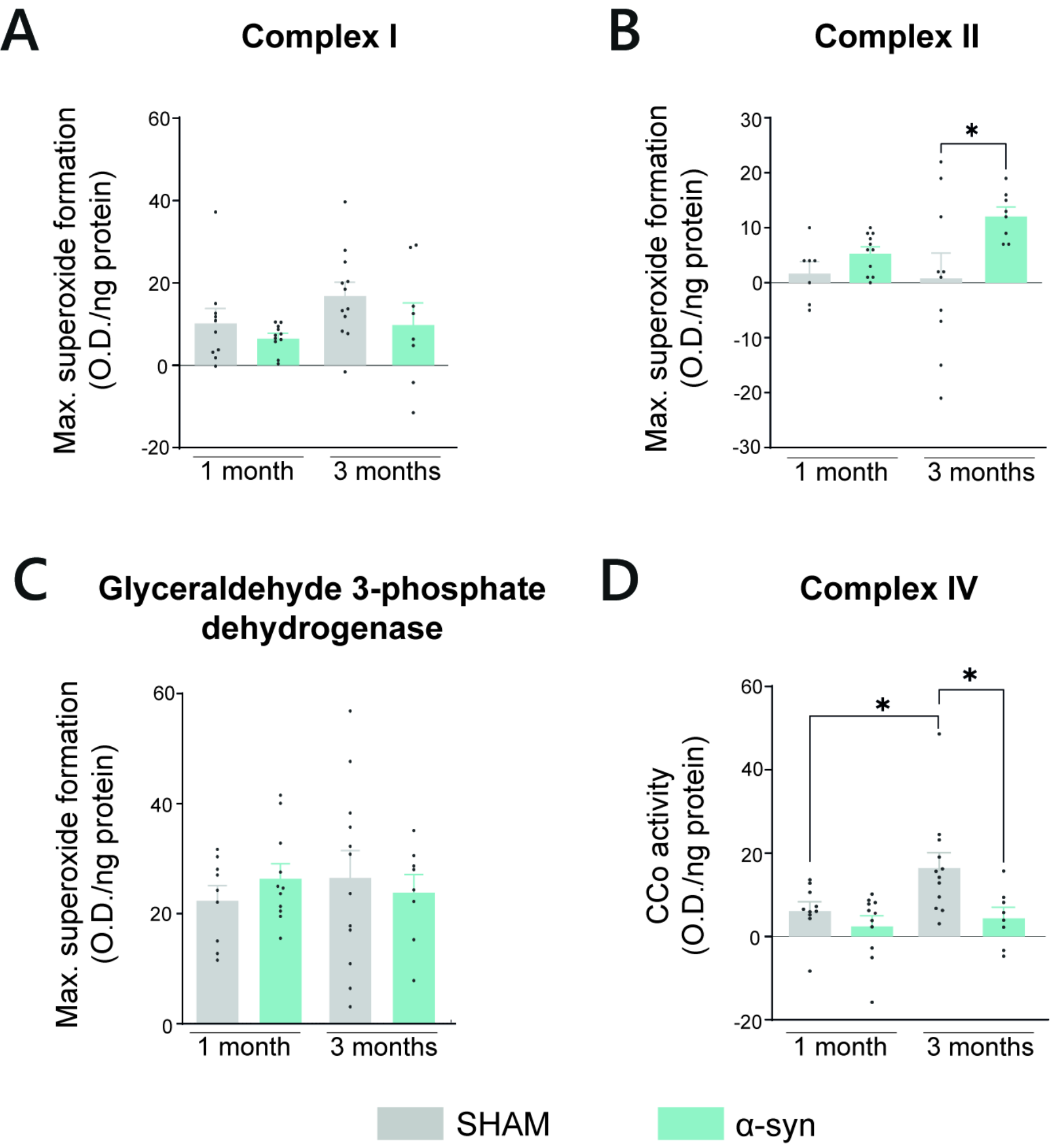

A
1 month
3 months
SHAM
α-syn
SHAM
α-syn
B
Activity
5000
4000
3000
2000
1000
0
1 month
3 months
C
Distance (cm)
10000
7500
5000
2500
0
**
1 month
3 months
D
Rearings
200
150
100
50
0
**
**
1 month
3 months
E
Permanence time in the center of the arena (%)
30
20
10
0
1 month
3 months
F
Time on the rotarod (s)
300
200
100
0
**
1 month
3 months
G
Time to correct posture (s)
6
4
2
0
1 month
3 months
H
Entries in the open arm
10
8
6
4
2
0
1 month
3 months
I
Time in the open arm (s)
100
80
60
40
20
0
1 month
3 months
J
Buried marbles
15
10
5
0
*
1 month
3 months
SHAM
α-syn


A
1 month
SHAM
α-syn
3 months
SHAM
α-syn
B
α-syn expression (O.D.)
100
80
60
40
20
0
-20
****
****
1 month
3 months
C
TH+ fibres (%)
150
100
50
0
*
*
1 month
3 months
D
TH+ cells (cells/volume)
15000
10000
5000
0
*
1 month
3 months
E
1 month
3 months
SHAM
α-syn
F
1 month
3 months
SHAM
α-syn
SHAM
α-syn


A
SHAM
α-syn
TH
α-syn
Merge
1 month
3 months
B
Striatum
SNpc
SNpr
TH
α-syn
Merge
C
SHAM
α-syn
TH
α-synP
Merge
1 month
3 months


SNpc
Striatum
Cortex
A
B
C
Complex I
# p<0.05 SHAM vs. α-syn
0.06
Max. superoxide formation (O.D./ng protein)
1 month
3 months
D
E
F
Complex II
*
G
H
I
Glyceraldehyde 3-phosphate dehydrogenase
J
K
L
Complex IV
CCo activity (O.D./ng protein)
SHAM
α-syn

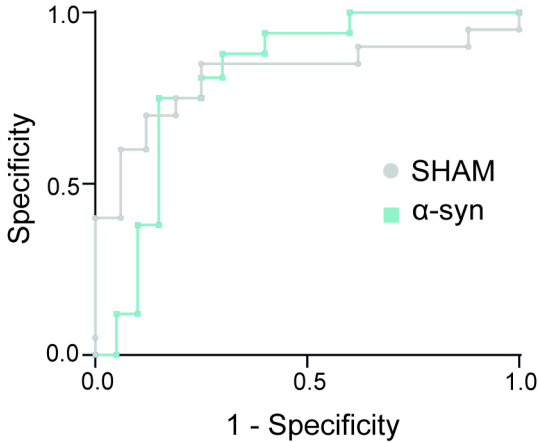

A
Complex I
Max. superoxide formation (O.D./ng protein)
0.06
*
**
1 month
3 months
B
Complex II
Max. superoxide formation (O.D./ng protein)
*
1 month
3 months
C
Glyceraldehyde 3-phosphate dehydrogenase
Max. superoxide formation (O.D./ng protein)
1 month
3 months
D
Complex IV
CCo activity (O.D./ng protein)
*
1 month
3 months
E
CCo activity (µAbsorbance at 595nm)
Time (min)
SHAM 1 month
SHAM 3 months
α-syn 1 month
α-syn 3 months
F
CCo activity (kinetic slope)
***
**
1 month
3 months
SHAM
α-syn


A
Complex I
Max. superoxide formation (O.D./ng protein)
60
40
20
0
-20
1 month
3 months
B
Complex II
Max. superoxide formation (O.D./ng protein)
30
20
10
0
-10
-20
-30
*
1 month
3 months
C
Glyceraldehyde 3-phosphate dehydrogenase
Max. superoxide formation (O.D./ng protein)
60
40
20
0
1 month
3 months
D
Complex IV
CCo activity (O.D./ng protein)
60
40
20
0
-20
*
*
1 month
3 months
SHAM
α-syn

A

Actual \ Predicted	α-syn	SHAM	Σ
α-syn	75 %	20 %	16
SHAM	25 %	80 %	20
Σ	16	20	36

B



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuppmaterialActa.pdf