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BRIEF REPORT

Early expression of monomeric and oligomeric alpha-synuclein and reduction of tyrosine hydroxylase following intranigral injection of lipopolysaccharide

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Abstract

Background The insoluble tangles of alpha-synuclein (α-syn) protein in the nigrostriatal circuit, characteristic of synucleinopathy, originate from low molecular weight oligomers, whose appearance and dissemination are related to neuroinflammation. These oligomeric forms of α-syn are considered highly cytotoxic but transient, so knowing the timing in which they appear remains challenging. Therefore, this study aimed to analyze the abundance of oligomeric forms of α-syn and tyrosine hydroxylase (TH) between 3 and 7 days after inducing neuroinflammation with lipopolysaccharide **(**LPS).

Methods and results LPS (2.5 µg/2.5 µL) was stereotaxically injected in the substantia nigra (SN) of adult male Wistar rats, which were sacrificed 3, 5 and 7 days after this intervention. The brains were processed for semi quantitative Western blot, along with brains from control and sham animals. Our results show an increased expression of α-syn monomer (15 kDa) only 3 days after LPS infusion, and the formation of 50 KDa and 60 kDa α-syn oligomers in the SN and striatum (STR) between 3 and 7 days after LPS infusion. Furthermore, the presence of these oligomers was accompanied by a decrease in the expression of nigral TH.

Conclusion These findings highlight the rapidity with which potentially toxic forms of α-syn appear in the nigrostriatal circuit after a neuroinflammatory challenge, in addition to allowing us to identify specific oligomers and a temporal relation with neurodegeneration of TH-positive cells. Knowledge of the timing and location in which these small oligomers appear is essential to developing therapeutic strategies to prevent its formation.

Keywords α-syn · Oligomers · Nigrostriatal circuit · Synucleinopathies · Tyrosine hydroxylase

Introduction

Synucleinopathies constitute a group of neurodegenerative diseases that share the misfolding and aggregation of the alpha-synuclein protein (α-syn) as a major pathological hallmark, from monomeric α-syn in its native form (15 kDa) to various, dynamic oligomeric forms, ultimately evolving into a fibrillar form [1–3]. Although the specific causes that promote the aggregation of native α-syn to higher-order oligomers are unknown, the notion that the initial stages of α-syn aggregation are higly toxic, at least for nigral dopaminergic neurons, has gained attention in recent years [4]. Determining the exact size of these early forms of α-syn is challenging, as they likely emerge early in the disease process and exist for short periods, before evolving into increasingly complex aggregates.

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Tyrosine hydroxylase (TH) positive dopaminergic neurons located in the substantia nigra (SN) are particularly vulnerable to degeneration due to accumulation of oligomeric and fibrillar forms of α-syn [5]. These neurons send inputs to the striatum (STR), forming the nigrostriatal circuit. Owing to the structure of this circuit, the gradual degeneration of nigral neurons during synucleinopathies development leads to a dopaminergic deficit in the STR [6].

Although the neuropathological substrate where α-syn aggregation and accumulation occur remains incompletely understood, evidence points to two fundamental and relatively common processes: neuroinflammation and oxidative stress. Lipopolysaccharide (LPS), a bacterial endotoxin that induces an inflammatory response by activating glial cells [7, 8], also induces α-syn aggregation in rodents [9]. Consequently, LPS has been widely utilized to investigate the involvement of different cytotoxic effectors in α-syn aggregation and dopaminergic cell neurodegeneration.

Intending to evaluate the presence of the native form of α-syn (15 kDa), the closest oligomeric forms (37, 50, and 60 kDa), and the expression of TH in the SN and STR in a neuroinflammation environment, we conducted this study in adult male rat brain.

Materials and methods

Ethical considerations, experimental design, and treatments

The experiments described here received the approval of the bioethics committee of the Research Coordination of the University Campus of Biological and Agricultural Science (CUCBA) of the University of Guadalajara, under the agreement CINV.104/12. The experiments were aligned with the Mexican Official Norms (NOM-062-ZOO-1999 and NOM-033-ZOO-1995) and Directive 2010/63/EU. Every effort was made to minimize the number of animals and the distress experienced by the animals during the experiment.

Thirty-five adult male Wistar rats aged 8–10 weeks and weighing between 200 and 250 g each were housed within a CUCBA vivarium, two per cage, under a 12:12 h light: dark cycle and *ad libitum* access to food and water. To start the experimental process, the animals were randomly assigned to the following groups: the control group (which was not treated), the sham group (which received only the vehicle: 0.9% saline solution), or the LPS group (which received LPS from *Escherichia coli*, O111:B4, Sigma–Aldrich, USA) at a dosage of 2.5 µg/2.5 µl, diluted in vehicle. This solution was kept at 4 °C and protected from light during the entire procedure. Each group consisted of 5 animals.

On day 1, rats in the sham and LPS groups were anesthetized with 4% isoflurane in a ventilated anesthesia chamber and mounted in a stereotaxic frame with the head immobilized using ear and incisor bars. A small hole was created in the skull at the level of the SN in the right hemisphere, following the coordinates of Paxinos and Watson [10]: AP -5.8 mm, L -1.0 mm, V -7.8 mm, as is shown in the supplementary material (Figure S1). Vehicle or LPS was delivered into the SN using a 50 µL microsyringe (Hamilton Company, Nevada, USA) connected to a microinfusion pump (Fusion 200, Chemyx Inc., USA) at a controlled flow rate of 1 µL/min for 2.5 min. Then, the needle was held in place for 3 min before being slowly retracted. Once this procedure was completed, the hole was sealed using cyanoacrylate, and the skin incision was sutured, cleaned, and disinfected. Once the animals recovered from anesthesia, they were returned to their respective cages until sacrifice.

Measurement of α-syn and tyrosine hydroxylase expression by western blot

To assess early α-syn expression in response to intranigral LPS injection, animals were euthanized at 3, 5, and 7 days post-injury (DPI) by an intraperitoneal overdose of sodium pentobarbital (60 mg/kg). The brains were rapidly removed and then placed on an ice-cold plate to dissect ipsilateral and contralateral SN and STR tissues, as is shown in the supplementary material (Figure S2), which were weighed, and frozen at -20 °C until processing. Afterward, the tissue samples were thawed but kept at 4 °C, homogenized by sonication in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM NaF, 0.5 mM Na_3VO_4, and 1% Tergitol type NP-40) and supplemented with a protease inhibitor cocktail, according to manufacturer's instructions (*cOmplete™ Protease Inhibitor Cocktail*, cat. 04693116001, Roche, Germany). Homogenates were centrifuged at 13,000×*g* for 30 min at 4 °C, in a Sorvall Legend Micro 21R centrifuge (Thermo Scientific, Germany). The supernatant was collected, aliquoted, and frozen at -20 °C until protein analysis.

The protein concentration was determined according to the Lowry method [11] with a DC Protein Assay Kit (cat. 5000116; Bio-Rad Laboratories, USA) on a Multiskan Go spectrophotometer (Thermo Scientific, Finland), using bovine serum albumin (cat. 500-0007, Bio-Rad Laboratories, USA) as an external standard. Briefly: twenty micrograms of total protein from each supernatant was denatured in 5 µL of Laemmli buffer (500 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue) at 95 °C for 5 min. Denatured samples were then loaded on a 12% SDS-polyacrylamide (SDS–PAGE) gel for electrophoretic separation, using the following running buffer: 25

mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3), and subsequently transferred onto a nitrocellulose membrane (Protean Premium 0.45 µm, Amersham, Germany) with the following transfer buffer: 25 mM Tris, 250 mM glycine, and 20% methanol (pH 8.3). Afterward, the membranes were blocked in a 5% blocker solution (QuickBlocker, EMD Millipore, USA) dissolved in 0.1 M PBS with 0.1% Tween-20 at 4 °C for 1 h. The membrane was then immunolabeled using commercially available antibodies against total α-syn (1:1000-1:2000; Cat. ab212184, Abcam, Cambridge, UK), and TH (1:1000; Cat. ab112, Abcam, Cambridge, UK). The membranes were incubated with these primary antibodies at 4 °C overnight and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Cat. 926–8000, LI-COR Bioscience, USA) at 4 °C for 2 h. Subsequently, the membranes were exposed to a chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific, USA). The signals corresponding to the proteins of interest were acquired using a C-DiGit Blot Scanner (LI-COR Bioscience, USA), and the bands were analyzed using Image Studio Lite 3.1.4 software (LI-COR Bioscience, USA).

To normalize the expression of monomeric α-syn and TH, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as a loading control (1:5000; Cat. ab125247, Abcam, Cambridge, UK), while the oligomeric forms of α-syn (35–60 kDa) were normalized to monomeric α-syn (15 kDa). All samples were analyzed in duplicate.

Statistical analysis

The data are expressed as the mean ± SD for each measured parameter. For data analysis, two-way ANOVA with Sidak *post hoc* test was applied. Statistical significance was considered when $p < 0.5$. Statistical analyses and graphical representations were performed using GraphPad Prism v.8 software.

Results and discussion

Expression of monomeric and oligomeric α-syn, and TH following LPS injection in the SN

At 7 days post-lesion (DPI), there was a slight increase in monomeric α-syn (15 kDa) ipsilateral to the lesion, compared to immediately preceding day (SHAM 5D and LPS 5D) (Fig. 1a). Also, we identified bands corresponding to oligomeric forms of α-syn at 37 y 50 on both ipsi and contralateral sides, although without significant statistical changes in its expression level (Fig. 1b, c and e). Notably, the 60 kDa oligomeric form showed an increase on the ipsilateral side at 5 DPI, compared to SHAM 7D (Fig. 1d and e).

Firstly, the application of LPS in the SN results in an increase in the expression of monomeric α-syn, followed by a rapid appearance of low molecular weight oligomers forms within a few days (5 DPI) after the stimulus, indicating a dynamic process of α-syn aggregation. Future studies are needed to assess the role of glial activation and the release of cytokines or other molecules in this process. The results in this region suggest that the monomers begin to increase soon after the application of LPS, but quickly move through molecular weights of 30 and 50, and stabilize at 60 kDa. This is relevant because the oligomer of 60 kDa potentially corresponds to α-syn tetrameric, a more stable form than the previous ones [12], which could facilitate the mobility of the oligomers towards the STR.

To our knowledge, this is the first report of the early presence of low molecular weight α-syn oligomers in the SN in rat brain after a pro-inflammatory stimulus.

The degeneration of dopaminergic neurons in the SN stands as a defining characteristic of synucleinopathies. In line with the early increase in the expression of α-syn in the SN, we found a decrease in TH expression ipsilateral to the LPS injection at 5 DPI compared with the SHAM 5D and 7D groups (Fig. 2a), suggesting a loss of dopaminergic neurons in the SN, in a temporal association with the increase in oligomers of α-syn.

This decrease in TH expression levels, which is observed at 5 DPI in the ipsilateral SN, suggests a progressive loss in the TH mark as the oligomers appear. Previous evidence has indicated that α-syn overexpression leads to reduced TH content in the SN [13, 14], with the involvement of Ser40, whose phosphorylation significantly contributes to TH activation and dopamine synthesis [15, 16]. This result supports the hypothesis that the conditions induced by LPS injection might be toxic to TH-positive dopaminergic neurons. However, our approach does not allow us to distinguish whether the observed effects are due to neurodegeneration or a detrimental impact on the expression of the TH protein specifically caused by slightly elevated levels of α-syn. Further studies are necessary to separate the effects of endotoxin exposure from α-syn overexpression to clarify the exact cause of TH neuron vulnerability.

Expression of monomeric and oligomeric α-syn, and TH following LPS injection in the STR

An early increase in the expression of monomeric α-syn (15 kDa) at 3 and 5 DPI on ipsilateral sides was observed in STR, in comparison to the SHAM 7D group (Fig. 3a). However, no changes were observed in the 37 kDa (Fig. 3b) or 50 kDa (Fig. 3c) oligomers in this region. Interestingly, the

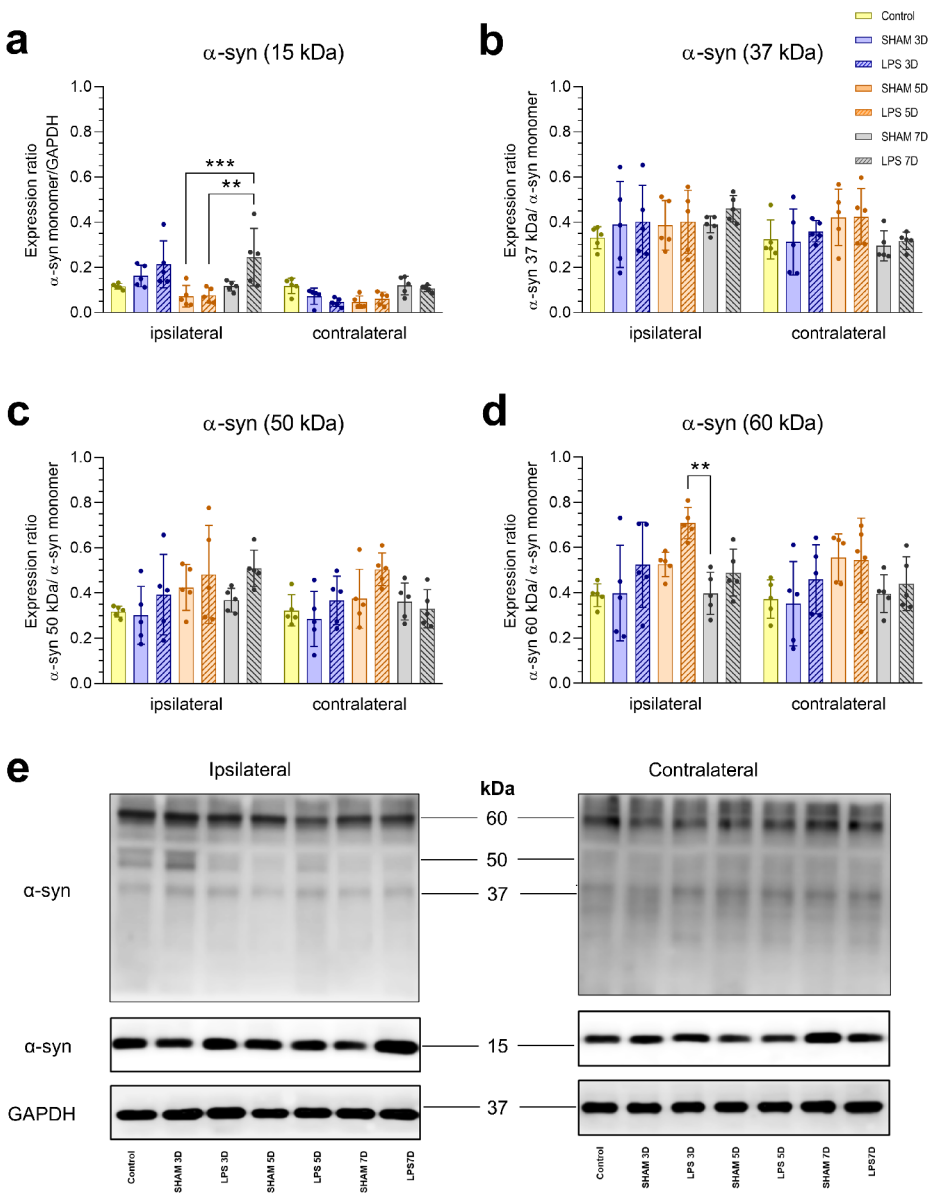

Fig. 1 Semiquantitative analysis of α-syn in the SN was performed using western blotting. (**a-d**) The expression of α-syn in monomeric (15 kDa) and oligomeric forms (37, 50, and 60 kDa) respectively. (**e**) Representative western blot images of α-syn monomers (15 kDa) and oligomers (37, 50, and 60 kDa), with GAPDH as the loading control. The data are presented as the mean ± SD. Data were analyzed by two-way ANOVA with a *post hoc* Sidak test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to SHAM and LPS groups ($n = 5$ per group)

60 kDa form was increased at 5 and 7 DPI, in both SHAM and LPS groups, on this region's ipsilateral side compared to SHAM 3D, and interestingly, we also found a significative increase of tetrameric α-syn in contralateral STR in the LPS 7D group compared with the SHAM 3D group (Fig. 3d).

The STR is the main terminal field of nigral dopaminergic neurons, so it is also affected in synucleinopathies due to its direct connections. The increase in monomers at 3 and 5 DPI in the STR but not in the SN was surprising; it is possible that in the SN the abundance of monomers between the moment of the stimulus and three days later is still close to normal, sufficient to maintain cellular functions of α-syn, but not enough to be detected by WB. Besides, it is known that the expression of this small protein is generally higher in the STR than in the SN, probably due to its affinity for small-sized vesicles, such as presynaptic vesicles [17]. In such a way, the monomers may begin to accumulate faster in the terminals of the STR than in those of the SN.

Also, in STR we found a high expression of the 60 kDa oligomer, which could reflect the dissemination of those previously formed in the SN, or those that were formed locally in the STR, or both. The presence of this oligomer ipsi- and contralateral to the stimulus indicates its rapid movement from one side of the brain to the other.

In association with the increase in the expression of monomeric α-syn at 3 DPI on ipsilateral STR, a reduction in TH expression compared to SHAM and LPS 7D groups was observed on the same side and same day (Fig. 2c). This

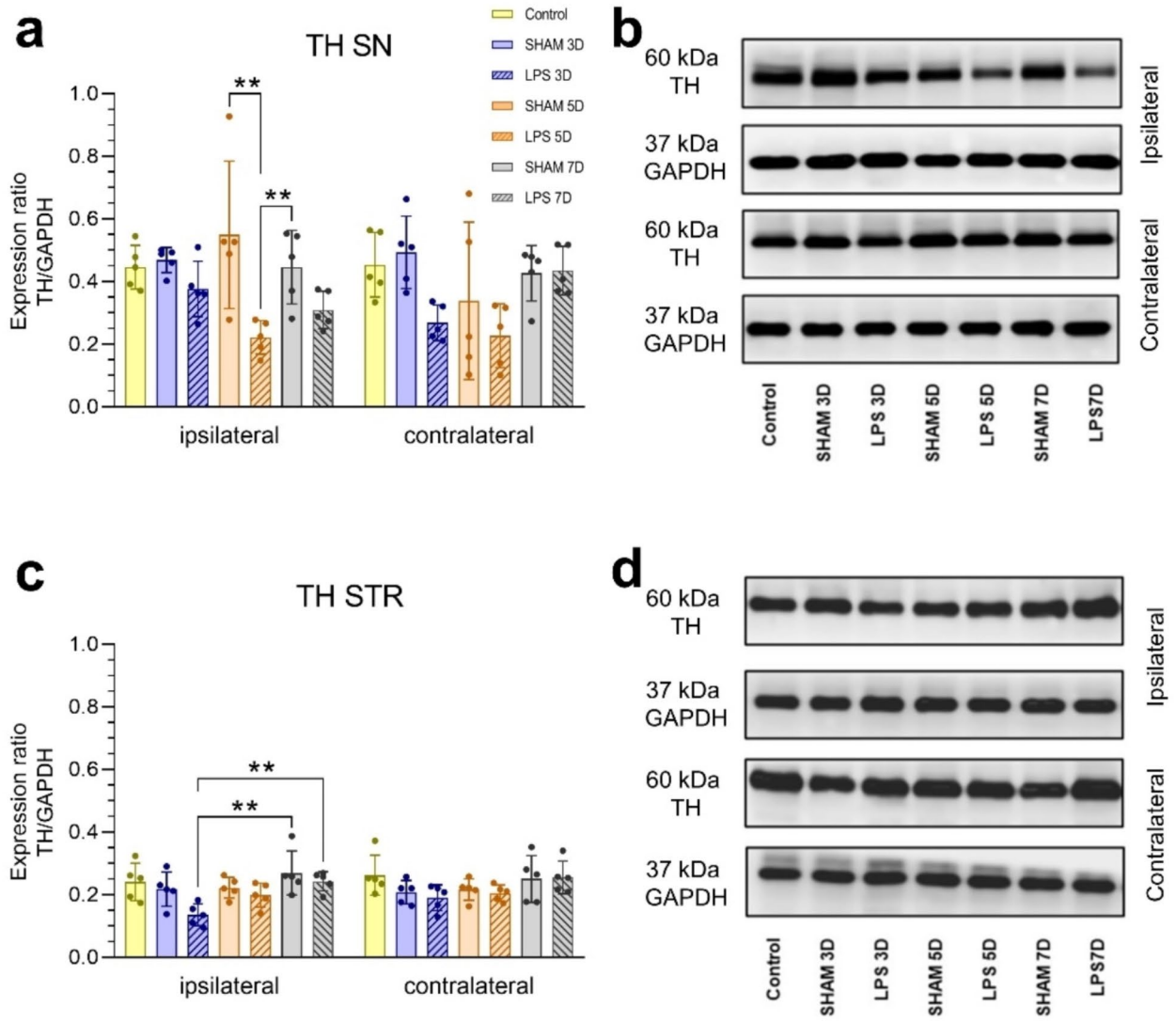

Fig. 2 Semiquantitative analysis of TH in the SN and STR was conducted using western blotting analysis. (**a, c**) The expression of TH on the ipsilateral and contralateral sides. (**b, d**) Representative western blot images of TH and the loading control GAPDH. The data are presented as the mean ± SD. Data were analyzed by two-way ANOVA with a post hoc Sidak test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to SHAM and LPS groups ($n = 5$ per group)

suggests an impact on dopamine content in STR, contributing to the initial pathophysiology of synucleinopathies.

Taken together, the results of this study highlight the instability of the α-syn monomer (15 kDa) under conditions of neuroinflammation, and its rapid transformation into more stable forms, such as the 60 kDa tetramer [12], in interconnected brain regions such as the dopaminergic circuit between the SN and the STR. Also, suggests a potential mechanism for spreading α-syn oligomers beyond the initial site of insult, through low molecular weight oligomers associated with dopamine-releasing vesicles in the STR.

Conclusions

Despite differences in the way neuroinflammation is induced, dosage, species, age, and administration method [18–20], our results underscore the rapid response of α-syn and TH to brain inflammation, in this case triggered by LPS, and align with the general hypothesis that inflammatory processes play a pivotal role in synucleinopathies, offering insight into the early molecular events and the apparent first stabilization of the oligomers in the 60 kDa form.

This vulnerability becomes evident when potentially toxic forms of α-syn form rapidly following a minimal dose of LPS, impacting both the SN and the STR in a few days,

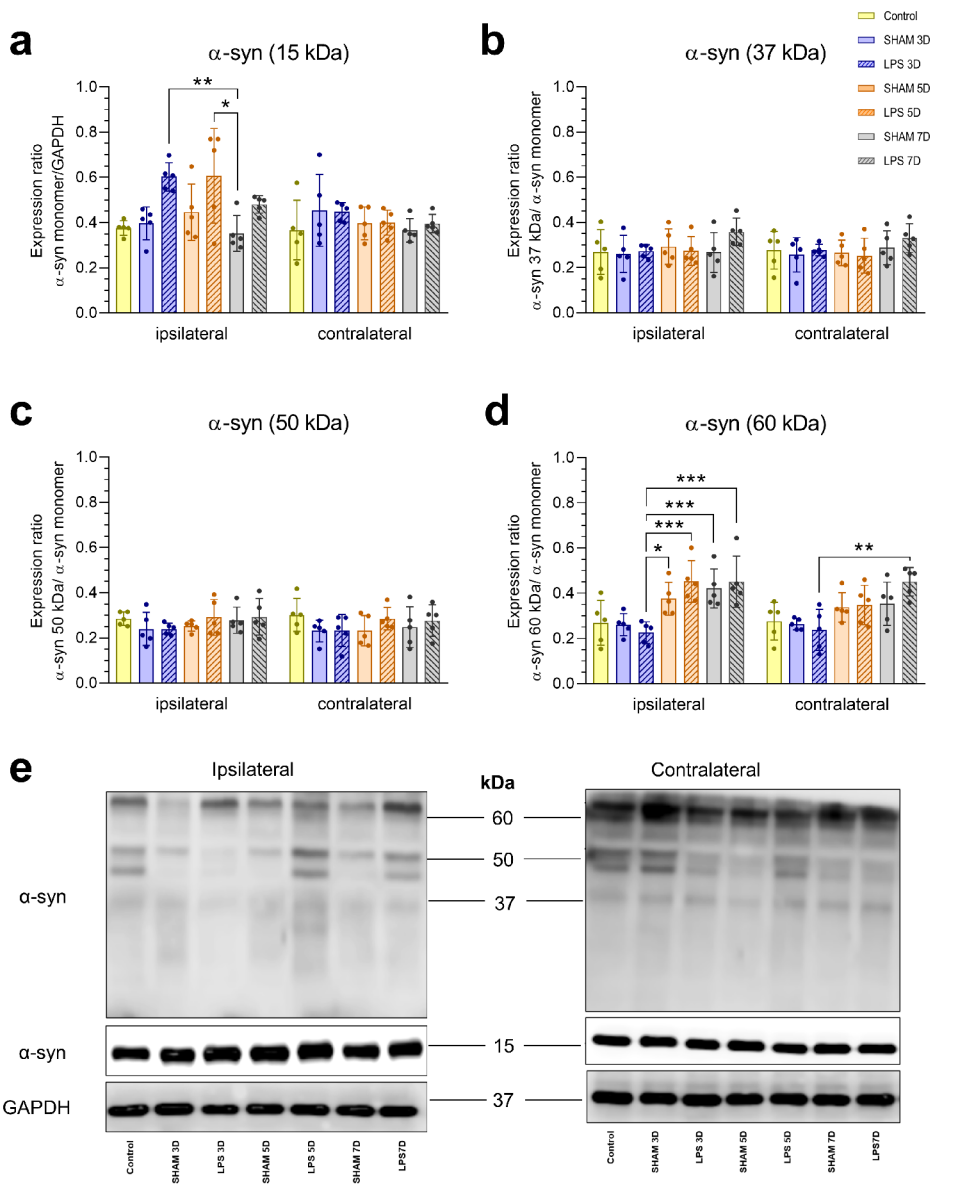

Fig. 3 Semiquantitative analysis of α-syn in the STR was performed using western blotting. (**a-d**) The abundance of α-syn in monomeric (15 kDa) and oligomeric forms (37, 50, and 60 kDa). (**e**) Representative western blot images of α-syn monomers (15 kDa) and oligomers (37, 50, and 60 kDa), along with the loading control GAPDH. The data are presented as the mean ± SD. Data were analyzed by two-way ANOVA with a post hoc Sidak test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to SHAM and LPS groups ($n = 5$ per group)

placing a seed that can compromise future brain functioning. Enhancing our comprehension of the cellular and molecular mechanisms driving the onset of α-synucleinopathies will undoubtedly contribute to the future development of therapeutic strategies to mitigate this pathology's progression.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-024-09935-2.

Author contributions All authors reviewed and authorized the final manuscript and agreed to publish it. AKLL did the experimental work and wrote the first draft under the supervision of SJLP. JLCC helped in data collection and data analysis. SJLP and MEUG critically review the final manuscript. All authors read and approved the final manuscript.

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Data availability The data presented in this study are available on request from the corresponding author.

Declarations

Ethical approval Ethical approval was taken from the Research Coordination of the University Campus of Biological and Agricultural Science (CUCBA) of the University of Guadalajara, under the agreement CINV.104/12.

Competing interests The authors declare no competing interests.

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