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Retina as a Model to Study In Vivo Transmission of α -Synuclein in the A53T Mouse Model of Parkinson's Disease

Najiba Mammadova, Thierry Baron, Jérémy Verchère, Justin J. Greenlee, and M. Heather West Greenlee

Abstract

Parkinson's disease is a neurodegenerative disorder characterized by accumulation of misfolded α -synuclein within the central nervous system (CNS). Retinal manifestations have been widely described as a prodromal symptom; however, we have a limited understanding of the retinal pathology associated with Parkinson's disease. The strong similarities between the retina and the brain and the accessibility of the retina has potentiated studies to investigate retinal pathology in an effort to identify biomarkers for early detection, as well as for monitoring the progression of disease and efficacy of therapies as they become available. Here, we discuss a study conducted using a transgenic mouse model of Parkinson's disease (TgM83, expressing human α -synuclein containing the familial PD-associated A53T mutation) to demonstrate the effect of the A53T α -synuclein mutation on the retina. Additionally, we show that "seeding" with brain homogenates from clinically ill TgM83 mice accelerates the accumulation of retinal α -synuclein. The work described in this chapter provides insight into retinal changes associated with Parkinson's disease and identifies retinal indicators of Parkinson's disease pathogenesis that could serve as potential biomarkers for early detection.

Key words Parkinson's disease, Retina, Mouse model, A53T, α -synuclein

1 Introduction

Parkinson's disease (PD) is a degenerative disorder of the nervous system, characterized by two prominent disease processes: progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the midbrain resulting in motor deficits (e.g., tremors, bradykinesia, and rigidity), and the accumulation of intraneuronal Lewy bodies, that contain misfolded α -synuclein [1–3]. Epidemiological data over the past several decades has reported a dramatic increase in the occurrence of Parkinson's disease, concluding that the worldwide burden of PD has more than doubled from 2.5 million patients in 1990 to 6.1 million patients in 2016 [4]. This has motivated researchers to identify sensitive and reliable

methods to screen individuals at risk prior to permanent damage. Although classically considered a movement disorder, emerging evidence reports a broad spectrum of non-motor manifestations associated with PD (e.g., cognitive dysfunction and hallucinations, sleep and mood disorders, gastrointestinal dysfunction, hyposmia, visual impairments, etc.) that occur decades before the onset of the hallmark motor symptoms [5–8]. Among these, hyposmia and visual dysfunction (impaired visual acuity, contrast sensitivity, and deficits in color vision) have been most widely reported in PD [9–12], with ~96% of PD patients reporting a reduced ability to smell or detect odors, and ~80% of PD patients reporting some extent of visual deficit [10, 13–15]. In the present study, we used a transgenic mouse model (TgM83) to investigate the underlying retinal changes that may contribute to the visual manifestations experienced by PD patients. Moreover, we aimed to identify retinal indicators of PD pathogenesis that could serve as potential biomarkers for early detection as well as for monitoring the effect of therapeutic interventions as they become available.

Visual manifestations have been widely reported as a preclinical phase in several neurodegenerative processes including Parkinson's disease and other synucleinopathies (dementia with Lewy bodies (DLB) and multiple system atrophy (MSA)), described to occur at least 10 years before the cardinal motor symptoms [9–19]. Additionally, several reports describe the accumulation of misfolded α -synuclein aggregates in the retinas of postmortem PD patients [20–24]. However, there is a scarcity of information on the distinguishing events that cause retinal change. The strong similarities between the retina and the brain has led to an increase of studies investigating retinal pathology resulting from PD, particularly using existing mouse models, both toxin-induced and genetic, that can recapitulate varying aspects of the human disease including the progressive accumulation of α -synuclein throughout the central nervous system (reviewed in [25]). Specifically, several studies have described α -synuclein phosphorylated at serine-129 (pSer129) [23, 24] in the retinas of PD patients. Phosphorylation of α -synuclein at serine 129 is one of the several post-translational modifications to α -synuclein known to occur in PD. Studies attribute this post-translational modification to the increased formation and self-propagation of α -synuclein aggregates, and Lewy body formation [26–30]. While the self-propagating ability of pathological α -synuclein (pSer129) has been widely postulated [1, 26, 31–47], more conclusive evidence for the presence and spread of α -synuclein in the retina and its correlation to the cerebral α -synuclein burden is lacking. In a recent report, we described retinal pathology in a transgenic mouse model (TgM83) expressing the human Ala53Thr α -synuclein mutation. Specifically, we demonstrated that the accumulation of α -synuclein (pSer129) in retinas of

5-month-old transgenic mice is accelerated upon “seeding” or intracerebral inoculation with brain homogenate from clinically ill transgenic mice [48]. This study is based on *in vivo* experiments using the TgM84 mouse model that demonstrated that “seeding” with an inoculum derived from the brain of clinically ill mice results in acceleration of α -synuclein-associated disease and shortening of survival time [36]. Our studies use the TgM83 mouse model to demonstrate the effect of α -synuclein propagation in the central nervous system, on the progression of retinal changes associated with the A53T mutation, specifically the accumulation of misfolded α -synuclein. This *in vivo* model provides insight into the effect of Parkinson's disease on the retina and warrants further exploration into the potential use of retinal α -synuclein as a biomarker for early detection.

2 Methods

2.1 *In Vivo Animal Experiments*

1. For these studies, we used TgM83 transgenic mice, that express the A53T-mutated human α -synuclein protein (B6; C3H-Tg[SNCA]83Vle/J, RRID:MGI:3603036, The Jackson Laboratory, Bar Harbor, ME, USA), and spontaneously develop motor deficits between 8 and 16 months of age.

2.2 *Animals*

1. Animals include: B6C3H mice (genetic background of the TgM83 mouse line) at 5 and 8 months of age; TgM83 mice inoculated with brain homogenate obtained from a 2-month-old healthy TgM83 mouse; C57Bl/6S mice, presenting a deletion of the α -synuclein locus [49] inoculated with brain homogenate obtained from a clinically ill TgM83 mouse to gauge for unspecific toxicity of the inoculum; homozygous TgM83 mice at 5 months of age (corresponding to a preclinical stage), 8 months of age, and clinically ill animals (~12–18 months of age); homozygous TgM83 mice inoculated at 2 months of age with brain homogenate obtained from two clinically ill (12 and 18 months) TgM83 mice, and euthanized 3 months after inoculation (5 months of age). *Eyes are only collected from the following animals:* B6C3H mice at 5 and 8 months of age; TgM83 mice at 5 months of age, 8 months of age, and clinically ill animals; and TgM83 mice inoculated at 2 months of age with brain homogenate obtained from two clinically ill (12 and 18 months) TgM83 mice, and euthanized 3 months after inoculation (5 months of age).

3 Methods

3.1 Preparation and Inoculation of Brain Homogenates

1. Mice are euthanized by an intraperitoneal injection of lethal dose of sodium pentobarbital. All homogenates are prepared from half sagittally sectioned brains. Each half is weighed and placed in a ribolysis tube containing grinding balls. High Salt (HS) buffer [50 mM Tris-HCl, pH 7.5, 750 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% phosphatase and protease inhibitor cocktails] is added to the brain halves to obtain 20% (wt/vol) homogenates. Brain extract inoculation samples are prepared using a mechanical homogenizer at 6.0 m/s for 23 sec twice. After the first 23 sec homogenization, the tubes contained the homogenates are placed on ice for 2 min before the second 23 s homogenization cycle.
2. Before brain extract inoculation, mice are anesthetized with a xylazine (10 mg/kg) and ketamine (100 mg/kg) mixture. Mice are subjected to intracerebral inoculation (IC) with 20 μ L of 1% (wt/vol in glucose 5%) brain homogenates obtained from half of the brain of a clinically ill TgM83 mouse. Mouse brains are stored at -80°C for Western blot (WB) analyses, or fixed in buffered 10% formalin for immunohistochemical (IHC) studies [50, 51].

3.2 Clinical Monitoring of Mice

1. Care and housing of mice, as well as ethical approval is described as per Mougenot et al., 2011 [36]. Mice are monitored daily and clinically examined individually three times a week to detect any symptoms of TgM83 disease. Indications of clinical motor disease include: reduced mobility and/or persistent immobility, partial paralysis of the hind leg (e.g., freezing of a hind limb during spontaneous walking that lasts a few seconds), and balance impairment. Balance impairment can be examined by observing the spontaneous mobility of the mice (e.g., falling after rearing up, gently pushing the mouse on its side to see if the mouse can recover). These clinical signs progress to feeding difficulty and subsequent weight loss, prostration and/or hunched back, and general paralysis rapidly requiring sacrifice of the mouse for ethical reasons. A mouse is thus considered “clinically ill” as soon as the hind limb paralysis is detected by two independent observers [50].

3.3 In Vitro Animal Experiments

1. After mice are euthanized, eyes are enucleated before the brain has been removed. The eyelids are pulled apart to improve access to the eyeball surface. Curved forceps are placed under the globe in the orbit and is used to gently push the eye out of its socket and grip the globe from underneath without squeezing the globe. The globe is gently moved from left to right and upward until released from the socket, with the optic nerve still

attached (*see* **Notes 1** and **2**). The eye globe is transferred into a Petri dish containing phosphate-buffered saline, after which the extraocular muscles are gently removed, and a single puncture wound is made into the cornea allowing fixative to penetrate the eye tissue. The entire eye is immediately placed into and fully immersed in 10% formalin for 24 h, then 70% ethanol. Eye globes are embedded in paraffin and sectioned sagittally at 4- μ m onto Superfrost plus glass slides.

2. In the brain and the retina, phosphorylated α -synuclein (pSer129) was detected by WB and IHC analyses as previously described [48, 52]. For WB and IHC analyses, pSer129 was detected with anti-rabbit pSer129 α -synuclein monoclonal antibody (mAB) (WB 1:1000, IHC 1:300) (*see* **Notes 3–5**).

3.4 Representative Results

1. Survival curves were obtained by Kaplan–Meier method [52]. There was no statistical significance between survival times of uninoculated TgM83 mice (435 days old), and TgM83 mice inoculated with a brain homogenate from a healthy 2-month-old TgM83 mouse (359 days old) [36]. There was a significant difference between survival times of the aforementioned mice and TgM83 mice inoculated with brain homogenate obtained from two clinically ill (12 and 18 months) TgM83 mice (193 and 182 days old, respectively) (*see* Fig. 1a) [36].
2. Western blot analysis of brains from TgM83 mice inoculated with brain homogenate obtained from two clinically ill (12 and 18 months) TgM83 mice, revealed pSer129 protein expression as early as 97 dpi (161 days old), while Western blot analysis of brains from age-matched uninoculated TgM83 mice revealed no pSer129 (*see* Fig. 1c) [36]. IHC analysis revealed accumulation of pSer129 in the dorsal raphe nucleus and lateral vestibular nucleus of 1) M83 mouse (339 days old) inoculated with brain homogenate obtained from a healthy mouse (2 months of age), 2) M83 mouse (198 days old) inoculated with brain homogenate obtained from a clinically ill mouse (12 months of age) and 3) M83 mouse (328 days old) inoculated with brain homogenate obtained from a clinically ill mouse (18 months of age) (*see* Fig. 1d) [36].
3. Immunohistochemistry and Western blot analysis were used to detect α -synuclein (pSer129) and total α -synuclein in retinas of transgenic and control mice (*see* Fig. 2) [48]. Immunohistochemical analysis of retinas from B6C3H mice at 5 and 8 months of age revealed no pSer129 or total α -synuclein immunolabeling. In retinas of 5-month-old transgenic mice, pSer129 immunolabeling was evident only in the outer nuclear layer. However, pSer129 immunoreactivity was minimal, with two to three patches of labeling per retinal section. Compared

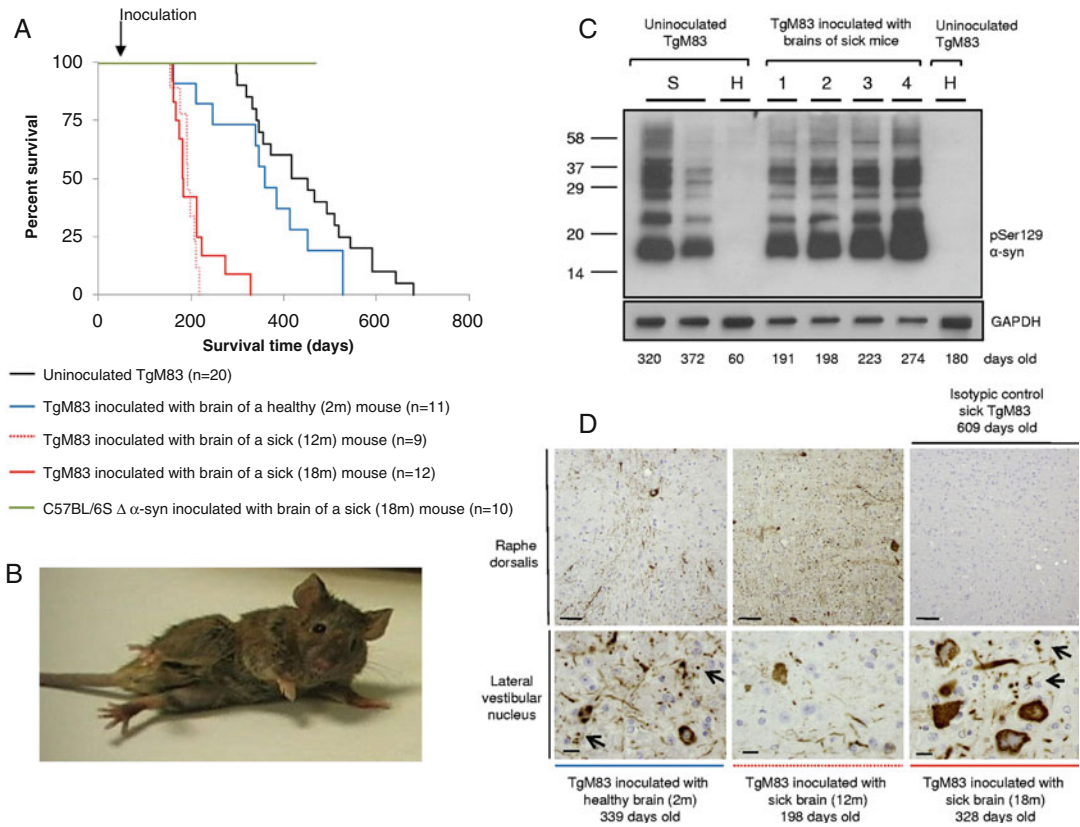


Fig. 1 (a) Survival times of TgM83 mice inoculated with brain homogenates from sick, 12-month-old (red dotted line) or 18-month-old (red line) TgM83 mice, compared with uninoculated TgM83 (black line) or TgM83 inoculated with a brain homogenate from a 2-month-old healthy TgM83 mouse (blue line). (b) A 161-day-old TgM83 mouse inoculated with a brain homogenate from a sick TgM83 mouse shows (97 days post-inoculation [d.p.i.]) paralysis of the hind limbs and impaired rotation to upright posture. (c) Western blot detection of insoluble pSer129 α -synuclein (α -syn) in brains of uninoculated sick (S) TgM83 mice and in brains of TgM83 mice inoculated with brain homogenates from sick TgM83 mice (1–4). pSer129 α -syn was not detected in brains of uninoculated healthy (H) (2- or 6-month-old) TgM83 mice. Below each lane, immunodetection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using a mouse monoclonal antibody (mAb) [1:10,000] (Millipore, Molsheim, France) is shown, as well as a figure indicating the age at death of the corresponding mouse. (d) Immunohistochemical detection of pSer129 α -syn. The right upper photomicrograph attests the specificity of the labeling. The α -syn pathology is indicated by dystrophic neuritis and labeling of the neuronal perikarya (upper lane in the raphe nucleus of TgM83 mice which died when 339 or 198 days old). Bars: 16 μ m. Lower panel: diffuse perikaryal inclusions as well as spheroid-like inclusions (arrows) in the gray matter of the lateral vestibular nucleus were also detected in a TgM83 mouse that died at 328 days old after inoculation with a brain homogenate from a sick TgM83 mouse or in a TgM83 mouse which died when 339 days old after inoculation with a brain homogenate from a healthy 2-month-old TgM83 mouse. Bars: 64 μ m

to 5-month-old non-inoculated transgenic mice, retinas of inoculated 5-month-old transgenic mice revealed marked pSer129 immunoreactivity detected throughout the inner and outer retina. Retinas of 8-month-old and clinically ill transgenic mice had pSer129 immunolabeling similar to that of

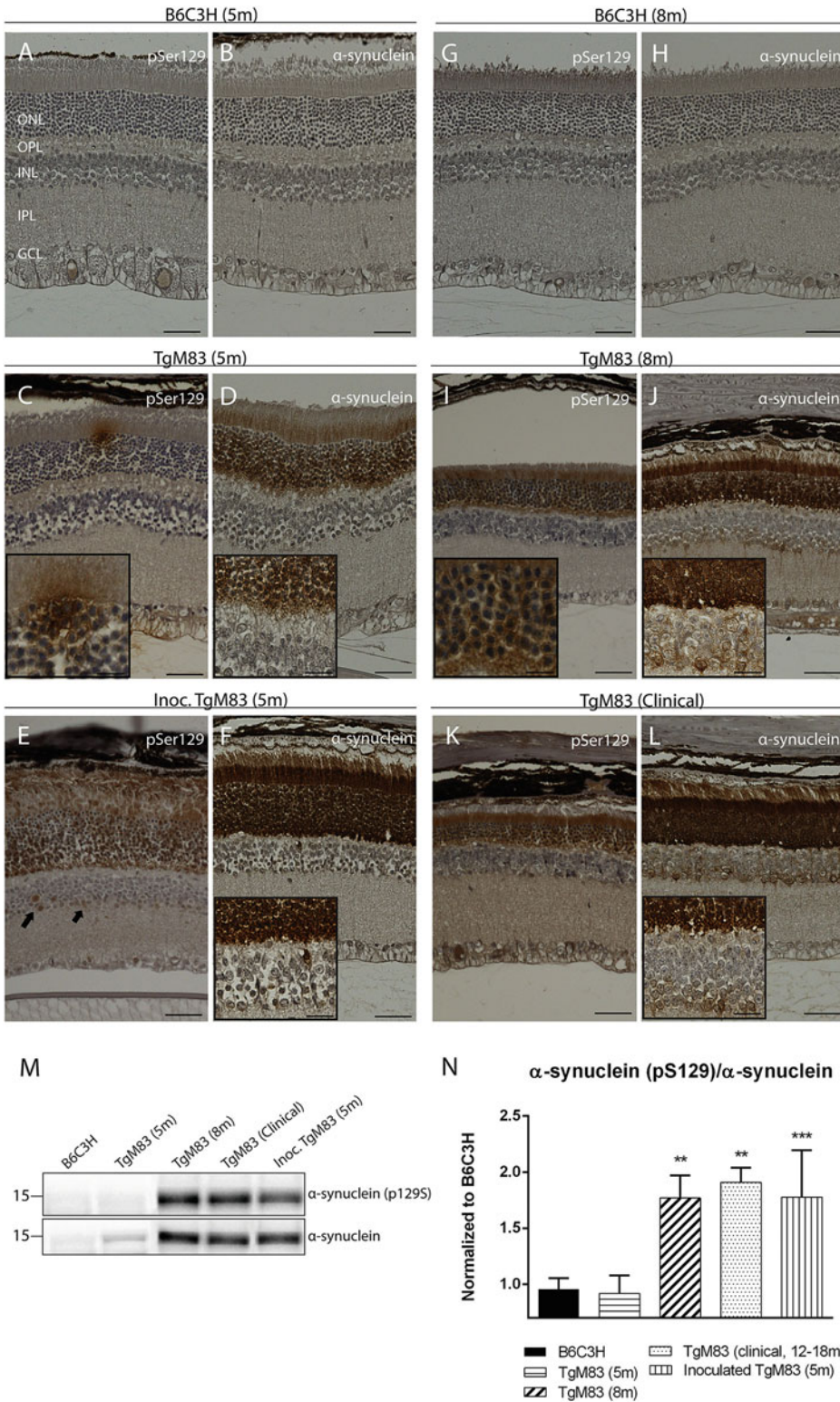


Fig. 2 Accumulation of α -synuclein. (a, b) Retinas of B6C3H mice had no phospho- α -synuclein (p129S) immunoreactivity. (c-f) Retinas of transgenic mice showed a similar trend, with sparse perinuclear and

inoculated 5-month-old mice (*see* Fig. 2g–l). Western blot analysis of pSer129 and total α -synuclein protein revealed a similar trend. Retinas of inoculated 5-month-old mice, 8-month-old, and clinically ill mice had significantly more pSer129 (~two fold compared to control and 5-month-old transgenic mice; Fig. 2m, n). This result suggests accelerated accumulation of the pathogenic form of α -synuclein, pSer129, in the seeded model.

4 Notes

1. In order for the retina to remain intact, mouse eyes must be enucleated before the brain has been removed. Care must be taken to ensure that the optic nerve is not damaged during enucleation. The use of curved dressing forceps makes this easier. When the curved forceps are placed under the globe in the orbit to push the eye out of its socket, it is best to grasp the extraocular muscles/connective tissue behind the globe to avoid squeezing the globe and damaging the retina [53]. A 30-gauge needle can be used to make a single puncture wound 1–2 mm into the eyeball [53]. Curved Westcott dissection scissors can be used to remove extraocular muscles and other connective tissues from the eye globe prior to fixation.
2. For optimum results, eye globes should be fixed in 10% formalin or Bouin's fixative for 12–24 h.
3. Western blot analysis of pSer129 protein expression in retinas of transgenic mice was carried out using paraffin-embedded tissues as per Mammadova et al. 2019 [48]. However, similar results are anticipated with frozen tissues.
4. For protein extraction from paraffin-embedded retinas for Western blot analysis, retinal tissues are collected into 1.5 mL microcentrifuge tubes, and then deparaffinized and rehydrated

Fig. 2 (continued) extracellular phospho α -synuclein (p129S) immunoreactivity at 5 months of age, evident in the inner retina at the stage of clinical disease. **(e)** Retinas of inoculated mice showed increased α -synuclein (p129S) immunoreactivity, localized to ONL as well as the inner retina. **(g, h)** Retinas of B6C3H mice had no α -synuclein immunoreactivity. **(i)** Perinuclear and extracellular α -synuclein accumulation first evident at 5 months of age, localized to ONL. **(j–l)** As disease progressed, α -synuclein accumulation increased in intensity, distributed throughout the inner retina. **(k)** Retinas of inoculated TgM83 mice are comparable to retinas of clinically ill TgM83 mice, with α -synuclein localized to ONL and inner retina. Abbreviations: *GCL* ganglion cell layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer. Insets: High magnification images of α -synuclein immunoreactivity. Scale bars: 40 μ m; insets 15 μ m. **(m)** α -synuclein (pS129) (15 kDA), α -synuclein (15 kDA), immunoreactive bands. **(n)** Representative bar graph showing quantitative densitometric analysis of α -synuclein (pS129)/ α -synuclein \pm SD $**P < 0.01$ vs. B6C3H and TgM83 (5 m); $***P < 0.001$ vs. B6C3H and TgM83 (5 m)

using xylene, followed by a decreasing ethanol concentration gradient (100%, 95%, 70%) by incubation at room temperature in each solution for 10 min. After each incubation, tissue is pelleted at $14000 \times g$ for 3 min, and incubation/centrifugation is repeated two times. For optimum results, 100 microns of retinal tissue and at least 500 μL of solution (e.g., xylene or various concentrations of ethanol) must be used each time to ensure proper deparaffinization and rehydration. Further detail has been described by Guo et al. 2012 [54]. Alternatively, the Qproteome FFPE Tissue kit (Cat No./ID: 37623 Qiagen, Germantown, Maryland, USA) can potentially be used.

5. Besides immunohistochemistry and Western blot analysis, detection of disease-associated α -synuclein (pSer129) has been previously described in the TgM83 mouse model using an enhanced ELISA technique as per Bétemps et al., 2015 [51].

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