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Retinal Degeneration Following Chronic Administration of the Parkinsonism-Inducing Neurotoxin MPTP.

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ABSTRACT.

During late stages retinal degenerative disorders affecting photoreceptors progress independently from the specific disease trigger. In fact, a number of detrimental consequences occur downstream of photoreceptors, which are triggered by the loss of photoreceptors themselves. Such a downstream anatomical alterations were originally thought to be compensatory events aimed to restore retinal function. At present, these phenomena are deciphered as detrimental effects and the term retinal degeneration is used to indicate the loss of cells and architecture within the inner retina as a consequence of a damage to photoreceptors. In the process of testing a photoreceptor-dependent downstream spreading of neurodegeneration we applied a neurotoxin mimicking Parkinson's disease (PD), 1-methyl, 4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP). Chronic MPTP administration produces degeneration within the mouse retina. This is evident by apoptosis quite circumscribed to photoreceptors, which is reminiscent of most phenotypes of retinal degeneration. Retinal pathology following plain H&E histochemistry, is more widespread with delamination and loss of neuronal packaging in the inner retina. The retinal damage is characterized by a marked synucleinopathy mostly within retinal ganglion cells. In contrast dopamine-containing structures are intact while norepinephrine is significantly reduced. Despite the involvement of retina in PD is documented, no study so far analyzed the onset of a synucleinopathy and a degenerative process mimicking what is now recognized in typical retinal degeneration. The present data provides a novel vista on the reciprocal role of retina in neurodegenerative disorders.

Key words.

Ganglion cells • Synucleinopathy • Amber light • IPRCs • Cyanide light • Extra-geniculate nuclei • Brainstem reticular formation • Efferent retinal innervation • Retinal synucleinopathy • Parkinson's Disease • Experimental parkinsonism

Introduction.

A number of studies indicate that various types of retinal disorders, when progressing to a late stage, converge into a multiple degeneration, thus mimicking what occurs in classic degenerative disorders affecting the central nervous system (CNS, Pfeiffer et al., 2020a). .

In particular, the retinal placement confers specific features to these disorders, at an early stage, which allow a specific diagnosis. In this way, age-related macular degeneration (AMD), retinitis pigmentosa and other chronic degenerative phenomena of the nervous tier of the eye are clearly distinct at onset and during disease progression. Nonetheless, a late stage occurs, in which retinal alterations merge to

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recapitulate the spreading of neurodegeneration (Pfeiffer et al., 2020a, 2020b, 2020c; Pinelli et al., 2021). At this point, early features, which allow a clear-cut syndrome distinction and pathological diversity, are lost and a merged disease is evident throughout the retinal layers. This is analogous to various disease phenotypes and disease spreading occurring in Parkinson's disease (Fornai and Ruggieri, 2013; Gambardella et al., 2017), Alzheimer's disease (Raunio et al., 2019; Sanchez et al., 2021), and even amyotrophic lateral sclerosis (Silani et al., 2017; Corcia et al., 2019; Bede et al., 2020). Even the latter, despite being considered a disorder which exclusively alters motor neurons, now is described in its whole pathological and clinical spectrum, which develops beyond motor neurons to produce extrapyramidal deficits and extending to extra-motor functions. These include cognitive-behavioral disturbances and multi-system neuropathology (Silani et al., 2017; Verde et al., 2017; Gorges et al., 2018; Kassubek et al., 2018; Limanaqi et al., 2017, 2019). In all these cases, when advanced disease stages occur, a dramatic spreading of pathology, towards a variety of brain areas occurs. Advanced symptoms become similar across various disorders to cast a multi-system syndrome and neuropathology where a corticofugal axonal spreading drives the transmission of the disorder along brain anatomy (Fornai et al., 2008; 2017; Pasquali et al., 2009, 2014; Braak et al., 2013; Brettschneider et al., 2014; Fornai et al., 2014; Eisen et al., 2017; Ruffoli et al., 2017; Kassubek et al., 2018). Similarly, at advanced stages of degeneration, a retinal pathology is described, which starting from photoreceptors follows analogous axonal diffusion to spread pathology to various of cell types belonging to various retinal layers including glial cells ultimately reaching retinal ganglion cells (RGCs). Thus, in a similar way to the corticofugal pattern of degeneration within motor systems, in the retina an early damage is supposed to involve photoreceptors to proceed downstream along axons according to a time-related and site-dependent progression. This process includes a variety of disorders such as retinitis pigmentosa (RP) and photoreceptors dystrophies, as well as diseases affecting the retinal pigment epithelium (RPE) such as Lebers Congenital Amaurosis (LCA) and AMD, or physical trauma leading to retinal detachment (Pfeiffer et al., 2020a). Independently of the primary trigger, which induces the loss of photoreceptors, the lack of synaptic enrichment from photoreceptors towards the internal retina produces synaptic and neuronal plasticity within retinal layers, placed downstream to photoreceptors. In the retina, changes in synaptic connectivity and neuronal shape were originally thought to be compensatory attempts, finalized to produce a sort of steady recovery from the original retinal disorder. Instead, data from the last decades indicate that such a maturation leads to maladaptive plasticity evidenced by a self-sustaining degeneration within neuronal layers of the inner retina. Thus, maladaptive plasticity or extreme retinal remodeling (Fisher et al., 2005; Marc et al., 2008) discloses a novel scenario, in retinal pathology, which explains the onset of a self-sustaining degenerative condition, where multiple disorders converge once the damage to photoreceptors provides a chronic de-afferentation of the inner retina. Nowadays, as reported in a paper published in the previous issue of this journal (Pinelli et al., 2021) this phenomenon is thought to rely on abnormal spreading of an aggregate-prone prionoid protein known as α-synuclein (α-syn). This protein takes a center stage to spread across various retinal layers as elegantly hypothesized by Pfeiffer et al (2020a, 2020b, 2020c). Thus, the occurrence of a later stage for various retinal degenerative conditions implicates abnormal cell-to-cell spreading of α-syn and the concomitant recruitment of abnormal maturation phenomena, which modify the retinal neuronal phenotypes in various retinal layers. Where such a cascade starts is likely to depend on the specific nature of each specific degenerative disorder, which early affects the retina. It is likely that early on, at the onset of a specific degenerative disorder, or later on, when degeneration is spreading, the involvement of RPE or Muller cells play a significant role. In fact, RPE, along with glial Muller cells (Pfeiffer et al., 2020b) represents the weak point in the course of retinal degeneration being sometimes also the starting point of early pathological events, as shown for AMD. At this level, a defect in the autophagy machinery as well as a dysfunction in other clearing system starts the disease, which then progresses towards converging final pathways (Pinelli et al., 2020a). Thus, it is not surprising that, the clearance of α-syn, which is strongly dependent on the effective

metabolism operated by the proteasome and autophagy (Fornai et al., 2006; Ebrahim-Fakhari et al., 2011, 2012 Engelender, 2012; Petroi et al., 2012; Tanik et al., 2013; Yang et al., 2013; Yan et al., 2018; Langston and Cookson, 2020; Limanaqi et al., 2020), is occluded. Such a metabolic dysfunction is supposed to take place within RPE and Muller cells (Pinelli et al., 2020b; Pinelli et al., 2021). This might explain why a failure in the clearance of α-syn fosters its persistency and diffusion throughout retinal layers to produce the common late retinal degenerative stage (Pinelli et al., 2020b, 2020c, 2021). In the process of testing the hypothesis whether a photoreceptor-dependent downstream spreading of inappropriate maturation leads to neurodegeneration we applied a classic neurotoxin mimicking Parkinson's disease. In fact, the well-known neurotoxin, 1-methyl, 4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP) is responsible to produce a number of pathological effects occurring in early and advanced stages of Parkinson's disease. When applied chronically, MPTP is capable to reproduce a multi-system pathology, which involves a damage to the orthosympathetic innervation of the hearth (Fornai et al., 2007; Ruffoli et al., 2008a), gut pathology (Natale et al., 2008, 2010), spinal cord pathology (Vivacqua et al., 2012, 2020), genitourinary dysfunction (Ruffoli et al., 2008b), all of which appear in the course of PD patients, when PD switches towards a multi-system disease. Remarkably, chronic and continuous (Fornai et al., 2005; Jackson-Lewis and Przedborski, 2007; Meredith et al., 2008; Gibrat et al., 2009), and even acute (Purisai et al., 2005; McCormack et al., 2008) or probenecid-adjunct (Petroske et al., 2001), MPTP administration increases α-syn levels and chronically, may produce α-syn aggregates. This is also related to a strong detrimental effect of MPTP both on autophagy and proteasome machinery (Fornai et al., 2005). Therefore, in the present study we analyzed the retina of C57 Black mice challenged with chronic (twice daily, for 21 days) MPTP administration to assess whether, concomitantly to toxic parkinsonism, a substantial damage to photoreceptors occurs, which in turn, may lead to abnormal deposition of α-syn down, within RGCs. For this purpose, the catecholamine neurotoxin MPTP is administered to C57/Black mice chronically, for 21 days. Mice are sacrificed at 7 days following the last MPTP dose and occurrence of retinal alterations was measured by checking a potential damage to retinal photoreceptors as counted by caspase activation. The general alterations in the morphology of the retina were detected by plain hematoxylin & eosin (H&E) staining, while being MPTP a catecholamine neurotoxin the potential occurrence of a loss of tyrosine hydroxylase (TH) in various retinal layers was measured. Since MPTP damages mostly dopamine (DA) containing neurons of the mesencephalon, the occurrence of MPTP-dependent decrease in the dopamine transporter (DAT) was evaluated, while the potential spreading of α-syn was counted within RGCs by immunohistochemistry. .

Methods.

Animals and MPTP treatment.

Eight C57/BL6 male mice (Harlan, S. Pietro al Natisone, UD, Italy), weighting 22-25g were used in the present study to analyse a total of 16 retina. Mice were housed for one week, four per cage, at light/dark cycles of 12 hours with free access to food and water under the observance of adequate measures to minimize animal pain and discomfort. The European rules (CEE 86/609) were followed for what concerned animal housing, health and experimentation. The retinal samples used for this study were dissected out in the course of experiments carried out between years 2011 and 2013, which were harvested in our bank of mouse tissues previously used under a project approved by the Italian Ministry of Health (authorization number 267/2011-B). .

At the time of treatment, mice were housed for one week, four per cage, at 12 hours light/dark cycles with free access to food and water. After seven days, mice were divided into two groups of four mice each and treated as follows: (i) The first group was treated intra-peritoneally (i.p.) with MPTP hydrochloride (purchased from Sigma, Milan, Italy), at the dose of 6 mg/Kg (corresponding to 5 mg/Kg of MPTP free-base), dissolved in saline. MPTP was administered twice daily, at 6 h interval, for 21 days (cumulative dose, 210 mg/Kg of MPTP free-base). (ii) The second group was administered saline, using the same schedule of administrations. After treatments,

mice were housed for one week, two per cage, in the same environmental conditions. Mice were then sacrificed under chloral hydrate anesthesia, i.p. (360 mg/Kg) (Sigma, Milan, Italy). All animals were processed for immunohistochemical analysis. .

Tissue preparation.

Sixteen eyes (eight from MPTP-treated mice and eight from saline-treated mice) were dissected out and immediately placed into a 4% paraformaldehyde in 0.1 M phosphate buffered solution. Twenty h later, eyes were placed into a 70% ethanol solution until they were included in paraffin. The eyes were cut at microtome (Leica Microsystem, RM2125, Milan, Italy) into 10 µm-thick sagittal sections for immunohistochemical procedures.

Histology and immunohistochemistry.

Paraffin embedded sections of eyes (10 µm), were used for immunohistochemical analysis. The retina was cut into 10 µm serial sagittal sections (through the medio-lateral extent) and used for histochemistry and immunohistochemistry analysis. Sections were de-waxed and processed for staining with H&E. .

In order to investigate whether the toxic effects of chronic MPTP treatment could induce retinal cell apoptosis, tissue sections underwent TdT-mediated dUTP Nick-End Labelling (TUNEL test) using a kit for *in situ* cell death detection with fluorescein to be visualized at fluorescence microscopy (Promega Corporation, Madison, USA).

To detect a potential loss of retinal cells and derangement of retinal layers immunohistochemistry was carried out. De-waxed tissue slices were incubated overnight with monoclonal mouse anti-tyrosine hydroxylase (TH), monoclonal rat anti-dopamine transporter (DAT), polyclonal rabbit anti-α-syn (Sigma Aldrich, Milano, Italy). .

Slices were exposed to normal sera for 1 h (10% in PBS). Then, they were incubated overnight with primary antibody (see Table I) and then for 1 h with secondary biotin-conjugated anti-mouse or anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, USA). Negative methodological control sections were processed by omitting primary antibodies.

Tab. I - Types, sources and code for antibodies used in the study.

Antibody and Kit	Distributor	Catalog Number	RRID	Concentration
Monoclonal mouse anti-TH	Sigma Aldrich, Milan, Italy	Cod.T1299	AB_477560	1:100
Biotinylated horse anti-mouse IgG (H+L)	Vector lab. Burlingame, CA, U.S.A.	Cod. BA-2000	AB_2313581	1:200
Polyclonal rabbit anti-a-syn	Sigma Aldrich, Milan, Italy	Cod. SAB4502828	AB_10746104;	1:100
Biotinylated Goat anti-rabbit (H+L)	Merck Millipore, Burlington, MA, U.S.A.	Cod. 401393	AB_437797	1:2000
Monoclonal rat anti-DAT	Sigma Aldrich, Milan, Italy	Cod. MAB369	AB_2190413	1:200
Biotinylated rabbit anti-rat (H+L)	Vector lab. Burlingame, CA, U.S.A.	Cod. BA-4000	AB_2336206	1:200
DeadEnd™ Fluorometric TUNEL System	Promega, Milan, Italy	Cod. G3250		

Densitometric Analysis of TUNEL and TH immunoreactivity in the retina.

Retinal TUNEL and TH immunoreactivity was quantified by measuring relative optical densities of fluorescence and immunoperoxidase. The retina was sectioned for the entire medio-lateral extent in 280 µm distant medio-lateral serial sections. Each section was analyzed by measuring optical density for five microscopic field for each animal. Images were acquired at × 20, and the analysis was performed by assessing the intensity of the background values (i.e., the optical density measured in unlabelled areas present in the section, sclera) by using Zeiss Axiophot Imager microscope with a digital video camera. Results are expressed as mean ± S.E.M. for each animal. .

Counts of α-syn positive retina ganglion cells (RGCs).

The RGC layer was analysed, and the number of α-syn positive RGCs in a linear segment of the slice was counted. The number of α-syn-positive cells identified in a 700 μm sagittal linear extent was counted. Each section was analysed by measuring the number of α-syn positive RGCs. Images were acquired at × 20, from 5 distinct medio-lateral sagittal fields per eye. Results are expressed as the number of α-syn per surface unit (1 mm) and number are given as the mean ± S.E.M. of 5 counts for each retina, from 2 retina from four mice per group (5X2X4=40 counts). .

Results .

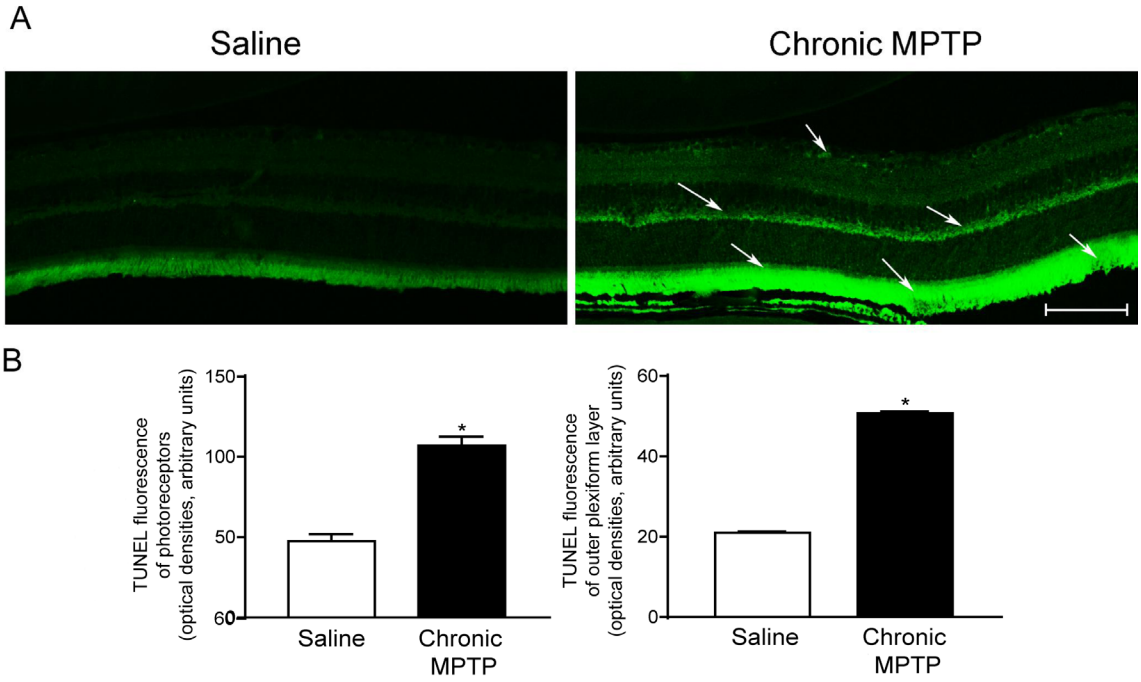
TdT-mediated dUTP Nick-End Labelling (TUNEL staining).

As shown in representative pictures of Figure 1, chronic MPTP administration produces a dramatic increase in TUNEL fluorescence, which was way in excess within photoreceptor layer. A slight signal was also detected from external and internal plexiform layers and some fluorescent spots were evident from the RGC layer. This indicates a degenerative effect of the neurotoxin MPTP at the level of photoreceptors, with a downstream slighter degeneration, which is consistent with a synaptopathy, which progresses throughout the synapses between bipolar and amacrine cells to reach down RGCs. However, TUNEL positivity at the level of photoreceptors compared with other layers remains quite remarkable. These data indicate that photoreceptors are the primary target of the toxicity induced by the parkinsonian neurotoxin MPTP as much as these cells represent the first culprit in a variety of retinal insults, which trigger neurodegeneration. .

H&E staining.

As shown in representative Figure 2, chronic MPTP administration produces a dramatic alteration in the trim of most retinal layers. This is mostly evident

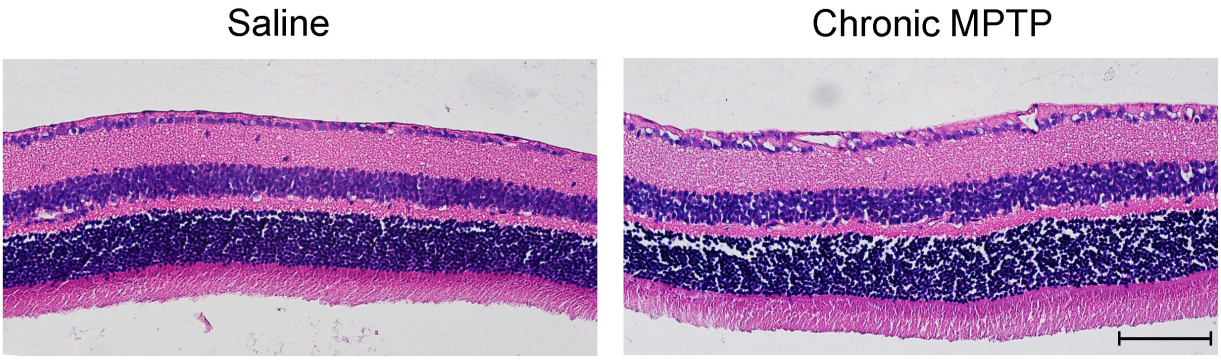
Fig. 1- TUNEL staining.
In (**A**) representative pictures of TUNEL fluorescent staining from a Control retina and a retina from a mouse administered chronically MPTP 5 mg/Kg twice a day for 21 days being sacrificed at 7 days following the last MPTP dose. The fluorescence (arrows) is evident following MPTP mostly at photoreceptor level with some fluorescence detected within external plexiform and retinal ganglion cell layers. In (**B**) the count of fluorescence indicates a significant increase both in the photoreceptor layer and within outer plexiform layer. Data are given as the Mean +S.E.M. from 8 retinas per group from 4 mice per group.



*P<0.05 compared with control retinas.
Scale bar=100 μm.

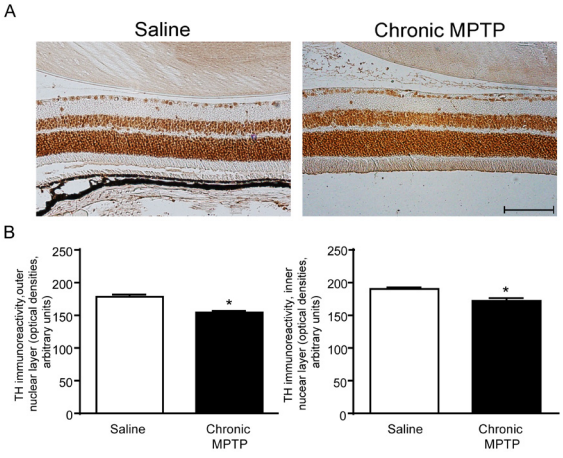
within the inner and outer segment of photoreceptors, which is compatible with findings obtained at TUNEL, as well as within inner and outer nuclear layer. An altered cell density is evident also within ganglionic cell layer. In detail, the hematoxilin staining is disrupted in these neurons and the retina loses its integrity with wide spaces between otherwise densely packed neurons, segments and nuclei.

Fig. 2 - Histological evaluation of mouse retina with H&E. .
H&E staining provides a less specific though more analytical vision of MPTP-induced retinal degeneration. The chronic administration of MPTP induces a loss of architecture evident as a delamination and loss of neuronal packaging throughout the retina with clear areas of cell loss and derangement of structure typical of each layer.



Scale bar=100 µm.

Fig. 3 - Representative pictures and graphs of Tyrosine Hydroxylase. .
In (A) representative pictures of TH immunostaining show the classic clusters of the pigmented mammalian retina, where TH is abundant within outer and inner nuclear layer with a noticeable staining of the retinal pigment epithelium and retinal ganglion cells. Following MPTP a slight decrease in TH immunostaining is visible within inner and outer nuclear layers. In (B) graphs measure the amount of TH immunostaining, which in both nuclear layers is significantly decreased.
Data are given as the Mean +S.E.M. from 8 retinas per group from 4 mice per group.
*P<0.05 compared with control retinas.



Scale bar=100 µm.

Catecholamine (TH and DAT) immunostaining .

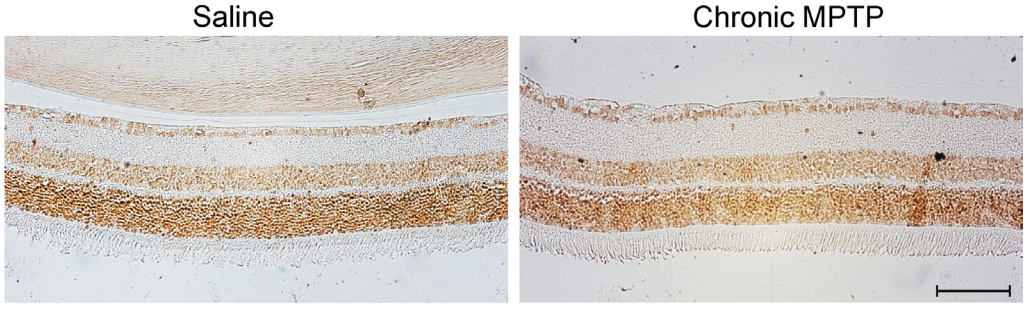
As shown in Figure 3, TH immunostaining was slightly prevalent in Control retina. This is consistent with a significant loss of TH immunostaining, which is produced by MPTP administration. The difference was significant as reported in the graphs of Figure 3 both in the external and internal nuclear layers. This is compatible with the primary mechanism of action of MPTP, which targets catecholamine containing cells. In contrast, when the DAT was visualized no difference in the density of immunostaining was detected (Figure 4), which contradicts the hypothesis that MPTP in the retina targets DA-containing structures, as it does instead produce DA cell loss at the level of mesencephalon. Thus, a decrease in TH immunostaining should be rather attributed to a loss of noradrenergic (NA) component within the retina. Nonetheless, the presence of well represented DAT molecules provides an important finding to justify the entry of MPTP within sensitive neurons. In fact, the neurotoxin MPTP, which is transformed into the active metabolite MPP^+ by monoamine oxidase (MAO)-B, which are expected to occur within Muller glial cells, is then extruded in the extra-cellular space, where MPP^+ is selectively taken up by the DAT to

reach the intracellular target to exert its mitochondrial toxicity. The presence of DAT immunostaining, similarly to TH, was detected throughout the retina being abundant in the outer and inner nuclear levels as well as in the ganglionic cell layer (Figure 4).

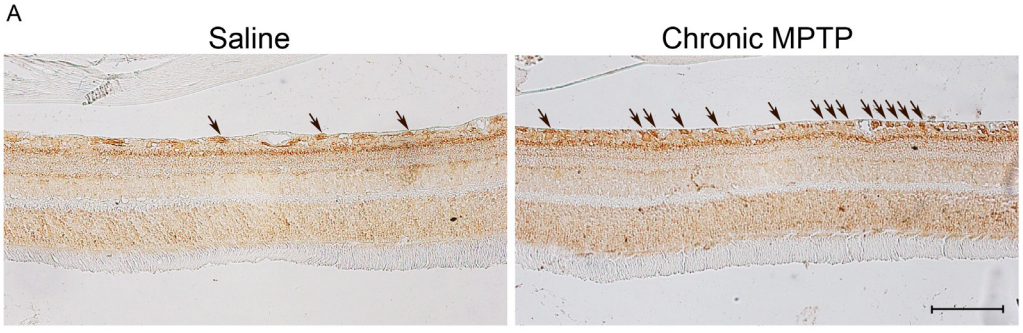
α-Syn immunostaining.

α-Syn immunostaining was really ubiquitous in the retina (Figure 5), with a variety in the intensity being detectable in the outer nuclear and plexiform layers as well as in the inner nuclear and plexiform layers. At this level, the staining was more evident within cell bodies which were sometimes clearly evident by the staining. As reported in the representative pictures of Figure 5, such a staining was more evident and demarked the cell bodies of RGCs with an excess of roughly 90% in MPTP-treated retina compared with controls (graph at Figure 5). In detail, RGCs from MPTP-treated mice were mostly clearly stained and shaped by α-syn antibodies compared with a faint scattered staining which was detected in some RGC from controls. The counts of α-syn-positive RGSs carried out in a sagittal linear tract of 0.7 mm provided an amount of RGCs which was almost the whole population. This is compatible with a synucleinopathy downstream to photoreceptors to finally reach the RGC as reported in the Introduction section for most retinal degenerative disorders at late stages.

Fig. 4 - Representative immunostaining for DAT. .
Representative pictures show a similar amount of DAT immunostaining, which is visible mostly in the outer nuclear layer and, with a lower intensity within inner nuclear and retinal ganglion cell layer. No effect is produced by chronic MPTP administration.



Scale bar=100 µm.



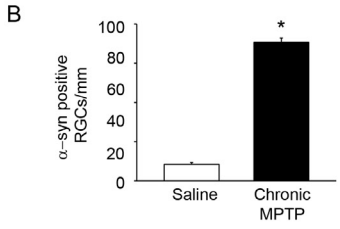


Fig. 5 - Representative pictures and graph for a-syn.
In (**A**) representative pictures report the effects of a-syn immunostaining. It is evident how ubiquitous the staining is, being present with various intensity in the outer nuclear and plexiform layer as well as in the inner nuclear and plexiform layers. The RGC layer was also stained. In detail, RGCs from a MPTP treated mouse were clearly stained and shaped by a-syn antibodies compared with a faint scattered staining which was detected in some RGC from a control retina. This was analysed in the graph reported in (**B**), where the number of a-syn positive RGC in a linear segment of the slice was counted. In MPTP-treated mice the amount of a-syn-stained RGCs almost correspond to the whole population. This is compatible with a synucleinopathy downstream to photoreceptors.
The number of a-syn-positive cells identified in a 700 µm sagittal linear extent was counted. Each section was analysed by measuring the number of a-syn positive RGC cells. Images were acquired at × 20, from 5 distinct medio-lateral sagittal fields per eye. Results are expressed as the number of a-syn per surface unit (1 mm) and numbers are given as the mean ± S.E.M. of 5 counts from each retina, from 2 retinas from four mice per group (5X2X4=40 counts).

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*P<0.05 compared with control retinas.
Scale bar=100 µm.

Discussion.

Chronic administration of MPTP produces degeneration within the mouse retina. This is evident by apoptosis activation, which was detected at TUNEL staining. Such a phenomenon appears to be quite circumscribed to photoreceptors. This suggests that, the onset of degeneration induced by MPTP within mammalian retina starts at the level of photoreceptors, which is reminiscent of most phenotypes of retinal degeneration. In fact, photoreceptors take a center stage in most types of specific retinal disorders including traumatic, metabolic and age-related retinal degeneration. In each disorder the damage to photoreceptors occurs quite specifically, although at later stages all these disorders may lead to widespread retinal degeneration which involves the inner retina and appears quite homogenous in spite of various triggers all leading to an overlapping pathology, which is evident as a proteinopathy. The positive TUNEL staining is demonstrated for decades following MPTP neurotoxicity. In fact, detection of apoptosis following MPTP administration was validated by the study of Tatton and Kish (1997), who carried out a TUNEL-like detection with inner validation with acridine orange to witness the occurrence of MPTP-induced apoptosis in experimental parkinsonism. In detail, despite a faint background of TUNEL immunofluorescence is detected in the outer photoreceptor layer of controls, this becomes very intense following MPTP administration. This TUNEL-induced fluorescence of photoreceptors, which correspond roughly to the outer photoreceptor segment, surpasses the TUNEL staining induced by MPTP in all retinal layers for the amount of intensity even when compared with the outer plexiform layer. At this latter level, intense fluorescence is detected following MPTP, although this is limited to a thin layer, which is compatible with the thickness of such a part of the retina. Thus, when comparing MPTP-induced TUNEL fluorescence with controls a marked signal is detected mostly in the outer retina with a slighter though significant increase which is evident also in the outer plexiform layer and some RGCs. In sharp contrast, no signal is detectable in the inner retina from controls. The fluorescence induced by TUNEL within the RGC layer staining involves some cells only from the MPTP-treated group. .

TUNEL fluorescence is substantiated by plain staining with H&E, where pathology appears more widespread being evident as a derangement of the whole inner retinal architecture, which also involves the photoreceptor layer. In detail, following MPTP administration, derangement of retinal structure as evident by H&E staining as a widespread delamination and loss of neuronal packaging involving mostly the inner retina compared with TUNEL fluorescence. In fact, while TUNEL positivity stains just specific markers in the process of apoptotic degeneration, the whole scenario provided by the classic H&E indicates an altered structure in the whole retina, which, as common for retinal degeneration, is likely to begin at photoreceptor levels and proceeding downstream to recruit basically all inner retinal layers. This is evident both in the cell bodies, and even nerve fibers, which is compatible with an axonal spreading of the disease throughout serial synaptopathy as it is reported in late stages of retinal degenerative disorders (Pfeiffer et al., 2020a, 2020b, 2020c). Independently from the specific disease trigger, when a loss of photoreceptors takes place, there are a number of detrimental consequences, which occur downstream of photoreceptors, which are triggered by the loss of photoreceptors itself. These downstream anatomical alterations were originally thought to be compensatory events aimed to restore retinal function, a sort of compensatory plasticity as observed and postulated by Kolb and Gouras (1974), who provided electron microscopic evidence of these changes. Nonetheless, in subsequent studies carried out at later stages of various phenotypes of retinal degeneration spanning from AMD to retinitis pigmentosa to include events following traumatic retinal injury, such a phenomenon was further defined as a maladaptive plasticity, and at present it is considered as a frank spreading of neurodegeneration (Jones et al., 2016a, 2016b, 2016c). Confirming such a vision, in the present manuscript we document that MPTP in fact induces apoptosis in the photoreceptors chronically leading to deranged architecture of the inner retina accompanied by a proteinopathy. In detail, as stated in the elegant review of Pfeiffer et al., (2020a), retinas engaged in photoreceptor damage, progress to further stage, where remodeling likely occurs in excess to produce indeed a sort of inner retina degeneration. This common disorder of the inner retina, which may be defined as true

retinal degeneration is characterized by a marked proteinopathy, which was described to recruit α-syn as the main protein involved. In this way, retinal degeneration is now referring specifically to inner retinal neurodegeneration, which indeed is a common outcome of various types of retinal disorders, which produce at early stage a disease-specific degeneration of photoreceptors. The loss of photoreceptor keeps distinct features, which belong to the specific inherited or acquired metabolic disorder, ageing, trauma, toxin, which may all produces a loss of photoreceptor, while the inner retinal degeneration is a common consequence of these specific disorders at advanced stages, independently by the cause photoreceptor loss. Indeed, is the loss of photoreceptors itself, which trigger degeneration of the inner retina commonly defined as retinal degeneration. This scenario re-establishes the significance of retinal remodeling, in the context of a downstream degeneration induced by inappropriate feeding of inner retinal neurons from altered photoreceptors. This generates cell pathology in the inner retina. This is evident also in the present study where a loss of inner retinal architecture and lamination accompanies the MPTP-induced apoptosis of photoreceptors. The ongoing degeneration of the inner retina may be induced by the progression along axonal processes of a specific proteinopathy involving α-syn. In fact, while H&E provides evidence for a wide-spread retinal neurodegeneration, the occurrence of α-syn proteinopathy is well evident here through the external plexiform layer presumably involving horizontal cells down to the RGCs layer. These layers of the inner retina, despite being deranged in their architecture and owing α-syn proteinopathy do not necessarily stain for the presence of apoptosis with TUNEL. This might be due to alternative cell death pathways during the synaptopathy of the inner retina, or alternatively, this might be the consequence of the specific time window, which makes an apoptotic cell visible by TUNEL staining. In fact, it should be considered that, the present treatment protocol was carried out chronically by administering MPTP twice a day for 21 consecutive days, which may impact various cell layers at different time frames, making the TUNEL positivity dispersed over various time intervals. This may explain why only a few neurons in a few inner retinal layers, placed downstream of photoreceptors, show apoptosis (outer plexiform layer and RGCs). As an apparent paradox the derangement of retinal architecture, which appears following H&E staining is more pronounced downstream of photoreceptors compared with TUNEL, thus confirming the onset of a common pathway of non-specific retinal degeneration which here takes place all over retinal layers. In fact, the onset of cell pathology is better witnessed by H&E since this technique allow to detect irreversible cell pathology independently of the time at onset being steady and persistent the cell alterations and cell loss, independently by when it was produced. Remarkably, α-syn accumulation, which is already slightly evident at the level of the pigment epithelium, proceeds all over the retinal route to become mostly evident in the RGCs, where single α-syn staining shapes positive cell bodies, which can be well defined and counted way in excess compared with controls. .

At chronic stages, 28 days following the neurotoxic double daily administration of MPTP at the dose of 10 mg/Kg daily, the amount of α-syn, which increases compared with controls, is mostly severe at the level of RGCs. This confirms what reported by previous manuscripts about the spreading of the synucleinopathy downstream of photoreceptors to reach down the RGCs (Pinelli et al., 2020b, 2021). In the context of widespread degeneration, with a remarkable amount of apoptosis of photoreceptors, it is surprising that MPTP does not produce any decrease in DAT immunostaining, which suggests that DA-containing structures preserve their integrity in the retina following chronic systemic MPTP administration. Nonetheless, when the amount of TH immunostaining was quantified, mice administered chronic MPTP own a slight though significant decrease of TH immunostaining. This suggests that a chronic regimen of MPTP administration, despite sparing DA-containing neurons, destroys noradrenergic structures within the retina at the level of the inner and outer nuclear layers, where in fact TH-positive immunostaining naturally clusters. .

The occurrence of this kind of retinal degeneration following the parkinsonism-inducing neurotoxin MPTP is not surprising since inner retinal degeneration occurs at late stage both following traumatic retinal disorders as well as metabolic retinal degenerations (Pfeiffer et al., 2020a). In detail, gross changes of retinal lamination as described by Kolb and Gouras, (1974) in the whole inner retina

following outer retina, photoreceptor degeneration occurs in retinitis pigmentosa and AMD, as much as it is visible here following MPTP-induced apoptosis of photoreceptors. Thus, it is not surprising that a neurotoxin may sort similar effects. Nonetheless, MPTP still owns a remarkable degree of specificity when hitting photoreceptors, since it does not damage cells and tissue, which are spared during the course of PD. It is remarkable that TH immunopositive structures are engaged in the retina during MPTP-induced neurotoxicity. In fact, MPTP seems to target TH positive and melanin-containing neurons as much as PD may affect the retina hitting light-absorbing, melanin-containing structures as shown by Willis et al. (2014). These authors suggest that retina and light exposure is involved in the etiology of PD. Nonetheless, it should be considered that they obtained their results by administering DA neurotoxins (including MPTP) to Sprague-Dawley (albino) rats (Willis et al., 2014). In a recent manuscript we could demonstrate that albino vs pigmented retinas possess a pattern of TH immunostaining, which differs dramatically (Pinelli et al., 2021). In detail, albino rats do not possess TH immunostaining in the retina, making albino species unreliable as a model to demonstrate the deleterious effects of light in PD including experimental parkinsonism. For similar reasons albino rats are mostly refractory to parkinsonism induced by the neurotoxin MPTP compared with the high sensitivity of the heavy pigmented C57 Black mice (Giovanni et al., 1994a, 1994b; Fornai et al., 1997; Staal et al., 2000a, 2000b), which rises further questions about the interpretation of findings by Willis et al. (2014). Again, the concept of light in the onset of retinal degeneration as well as in the onset of PD should be treated carefully. In fact, although the retina is reported to be damaged in PD patients (Schneider et al., 2014), one should consider at first, the wavelength, which may contribute to a detrimental or, at opposite, a beneficial effect. In fact, the anatomy of the retina and retinal projection to the CNS are strongly modified depending on the wavelength and the kind of photoreceptors being electively stimulated. For instance, a wavelength in the cyanide spectrum is known to alter the projection from the retina to a number of extra-geniculate regions, which include the catecholamine containing nuclei of the reticular formation (Meng et al., 2020; Pokorny and Smith, 2020; Aranda and Schmidt, 2021; Beier et al., 2021; Dannerfjord et al., 2021). This mainly occurs due to intrinsically photoreceptors retinal ganglion cells (IPRGC) which contain melanopsin and project to photosensitive nuclei of the brain (Stinchcombe et al., 2021; Roecklein et al., 2021; Hannibal, 2021; Grünert et al., 2021; Amorim-de-Souza et al., 2021; Mouland et al., 2021; Mure, 2021). In contrast, an opposite wavelength in the visual range, which falls close to red, like amber is likely to produce opposite effects (Adrian et al., 1977; Pinelli et al., 2021). In line with this, a defect due to a loss/degeneration in melanopsin RGCs of the human retina, which are sensitive to cyanide wavelength, occurs in PD patients (Gaynes et al., 2021). In fact, in a pioneer study Adrian et al. (1977) provided evidence that amber light may sort a protective effect in patients affected by retinitis pigmentosa. This contrast with an abnormal activity, which takes place within dystrophic retinas due to an overdrive of melanopsin-responsive cells which respond to short (cyanide) wavelength (Eleftheriou et al., 2020). These results should be cautiously analyzed when inferring to the role of light in retinal degeneration, similarly this should be put in the right context of specific anatomical connections between the retina and specific brain nuclei, when inferring on PD and other central degenerative disorders. In fact, the effect of light varies depending on which specific wavelength is activating specific photoreceptors in the retina itself. This in turn possess distinct, typical central projecting fields in the visual canonical and non-canonical projecting areas. Among the latter a special emphasis should be posed on the brainstem reticular formation including the substantia nigra and locus coeruleus, which degenerate in PD, (Hornykiewicz, 1975; Fornai et al., 1997; Gesi et al., 2000; Zarow et al., 2003; Lyness et al., 2003; Zecca et al., 2004; Tong et al., 2006). Thus, the concept reported in a very recent paper (Pinelli et al., 2021) describing the retina as a gateway to understand the neurobiology of disease in the CNS should be enlarged by the present study. In fact, these data serve as a compass to understand those potential pathways, which drive either neurodegeneration or neuroprotection within specific areas of the brain. In fact, melatonin projection to Locus Coeruleus seem to protect from degeneration (Jameie et al., 2019), and LC degeneration is a main pathological alteration in PD. An appropriate insight into the fine neuroanatomy

and odology/connectivity operating in the visual system appears to be mandatory to understand the influence of retinal input towards CNS degeneration and vice versa. In fact, due to protective effects of NE in neurodegeneration (Giorgi et al., 2017), one might expect that retinal disorders might be influenced by the integrity and activity of central NE projections within the retina. Such a hypothesis is now more and more likely since NE exerts protective effects in retinal dystrophies and retinitis pigmentosa (Wagner and Wiederholt,1996). Similarly, it is likely that NE, by activating β2 adrenoceptors, may protect retina in diabetes and wet AMD by suppressing the exuberant formation of new vessels induced by VEGF (Jiang et al., 2015). Thus, the present data, despite providing an extension of classic neuropathology of the CNS to a peripheral sensory organ, may be re-used back again to understand how a peripheral sensory organ may drive pathology (or even protection) within the CNS. In this way, the retina serves as a round-trip gateway to understand neurodegenerative disorders. A careful distinction should preliminarily concern the following items: (i) the specificity of the wavelength; (ii) the specificity of wavelength-dependent anatomical pathways; (iii) the specificity of neurotoxic/neurodegenerative insults primarily affecting either the retina or the CNS.

The first two issues were just discussed. Concerning the latter issue, several works tested the retinal sensitivity to the gold-standard parkinsonism-inducing neurotoxin MPTP (Wong et al., 1985; Melamed et al., 1985; Ambrosio et al., 1988; Harnois et al., 1989; Reinhardt, 1993; Chen et al., 2003; Nagel et al., 2009; Karlsson and Lindquist, 2016). Nonetheless, only a few of them carefully considered the specificity of such a neurotoxin and the biochemical mechanisms responsible for MPTP-induced neuronal damage. In fact, if one exposes a non-specific cell to MPTP at reasonable doses, no detrimental or even noticeable effect takes place. .

In fact, the mechanism of action of MPTP is based on a high degree of specificity, since MPTP itself is indeed a pro-toxin, which requires to be converted into a toxic metabolite, MPP^+, which in turn occurs only following the presence of oxidative de-amination enzyme called monoamine oxidase (MAO, Chiba et al., 1984; Heikkila et al., 1984). In detail, it was described that, mostly the isoform known as MAO-B (Gessner et al., 1985) is responsible to convert MPTP into MPP^+. The presence of MAO-B in the CNS is abundant within glial cells, where the extra-neuronal conversion is supposed to take place (Youdim, 1985; Da Prada et al., 1985; Uhl et al., 1985), and here again the role of retinal Muller cells seems to be key in fostering the retinal damage, since as typical for astroglia, Muller cells own MAO-B. It is remarkable that the mechanism, which converts MPTP into the active metabolite MPP^+ leads to the inactivation of monoamine oxidase B (Buckman and Eiduson, 1985) through a wavelength, which is compatible with the one stimulating IPRC, which further leads the short wavelength light to play a major role in the mechanism involved to trigger toxic parkinsonism. Once it is generated, the active neurotoxin MPP^+ needs to be taken up by a very selective mechanism, which compete for DA, known as the DAT (Javitch et al., 1985). In this scenario, it is important to include the affinity of MPP^+ for the NE transporter (NET), which appears to take up MPP^+ to a similar potency than the DAT. In this way, MPP^+ may be able to enter both DA- and NE-containing neurons. This explains the results of this study where no effect is evident on DAT containing structures as already shown by the pioneer study of Melamed et al. (1985), although a reduction in TH is produced significantly. When observing these data it is reasonable that NE neurons (including axons) instead of DA-containing neurons are damaged by MPP^+. This is consistent with the uptake of MPP^+ within NE neurons. In line with this, Luthman and Jonsson (1986) demonstrated a damage produced by MPTP within TH-containing structures of the iris, which is compatible with a loss of NE innervation of the retina reported in the present study. In fact, when MPTP is administered chronically at low doses (5 mg/Kg) it is reported that a preferential damage to NE-containing compared with DA-containing neurons takes place (Seniuk et al., 1990; Fornai et al., 2005). In fact, in a dose-dependent study these authors found that low reiterated doses of MPTP, as those administered in the present study, preferentially destroy NE-containing axons compared with DA-containing terminals. Again, the effects of MPTP on retinal DA-containing amacrine cells is only transient (Tatton et al., 1990) and by the time of sacrifice (28 days after starting MPTP administration such an effect is likely to be vanished, since no retinal DA neurons is damaged by MPTP (Melamed et al., 1985). .

In this way, MPP^+ gets an access within neuronal cytosol where it moves towards mitochondria to inhibit complex I activity thereby triggering neurotoxicity along with a marked increase in mitochondrial permeability (Tatton et al., 1999). The preferential target of MPTP within mitochondria leads back the MPTP model and PD to a common toxic mechanism, which operate in primary retinal degeneration, where mitochondrial alterations are taking a center stage. Experiments in our lab are presently under consideration to dissect the commonalities of mitochondrial fine ultrastructural changes in neurodegenerative and retina degenerative disorders. .

Conclusions.

The present research article dissects the neurotoxicity and neurodegeneration exerted by the parkinsonism-inducing neurotoxin MPTP in the retina by analyzing all retinal layers and specific markers to detect apoptosis as well as the integrity of neuronal cells and specifically, catecholamine-containing structures. The kind of retinal degeneration induced by MPTP seems to primarily hit the photoreceptors, where intense TUNEL fluorescence is induced. No comparable effect is observed in other retinal layers despite a significant increase of TUNEL fluorescence was measured within outer plexiform and RGC layers. The comparison of TUNEL analysis with H&E staining was remarkable since, plain histochemistry reveals a whole degeneration throughout inner retina. This picture recapitulates the so-called retinal degeneration, which define the late stage of a variety of retinal disorders hitting primarily the photoreceptors. In line with these conditions, MPTP-induced retinal degeneration produces a proteinopathy driven by the prion-like protein α-syn which accumulates mostly within RGCs. The present study provides a powerful proof of concept on the overlapping between degenerative disorders affecting the CNS and retinal degeneration. In turn, the present data provide novel avenue to approach CNS induced neurodegeneration through an unconventional pathway which exploits the eye as a gateway to modulate the sensitivity of specific brain nuclei to degenerative phenomena. A round-trip gateway connects the retina with extra-geniculate brainstem nuclei, which may spread either degenerative or protective mechanisms all over the CNS through profuse collateral branching of axons arising from brainstem reticular neurons. On the other hand, the altered catecholamine input to the retina may modulate and spread degenerative phenomena which were once considered to begin and terminate within the retina itself.

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