Effect of A53T-α-synuclein expression on DRG neurons and on tissues of the peripheral nervous system

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Μελέτη της επίδρασης της έκφρασης της A53T-α-συνουκλεϊνης στους DRG νευρώνες και σε ιστούς του περιφερικού νευρικού συστήματος

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Abstract

Parkinson's disease (PD) is a common progressive neurodegenerative disorder that affects 1% of the population above 60 years and, among neurological disorders, it is the fastest growing in prevalence, disability and deaths. PD affects predominantly dopamine-producing neurons in the brain area called substantia nigra and disrupts the facilitation of voluntary movements, thus disability in patients with PD is primarily due to impaired motor function. The hallmark of the PD pathology is the aggregation of the protein α-synuclein into Lewy bodies, a pathology that adopts a prion-like spreading pattern. Point mutations in the α-synuclein gene can cause heritable early-onset PD in rare pedigrees, an example is A53T missense point mutation, which increases the kinetics of α-synuclein fibrillization. Although the mostly considered PD symptoms are motor-related, up to 85% of PD cases experience pain, even before motor symptoms are prominent, while one third of cases develop sensory neuropathies. PD patients display altered pain thresholds and changes in sensory perception early in the disease progression, which suggests alterations in stimuli processing along the neuronal axis. Studies have also confirmed the correlation between axonal degeneration and α-synuclein deposition. Altered pain thresholds of sensory neurons could imply alterations in excitability at the level of voltage gated sodium channels (Na_Vs), which are critically important for electrogenesis. The expression of $Na_V1.8$ subtype, that is highly expressed in nociceptors, is said to be altered upon axotomy and inflammation.

Currently, this PD-related sensory system dysregulation is insufficiently investigated, thus the present study aims to study aspects of sensory processing in the transgenic A53T mouse model, which expresses the mutated form of human α-synuclein. Characterization of primary dorsal root ganglion (DRG) cultures derived from A53T homozygous mice confirms the deposition of A53T α-synuclein in DRG neurons, while expression of the transgene is correlated with larger DRG's soma. A53T DRGs exhibit numerous areas of axonal degeneration and they are susceptible to stress, induced by the proteasome's inhibitor, epoxomicin. Also, calcium transients of A53T DRGs seem to be inefficiently evoked by KCl. $Na_V1.8$ expression is elevated affected by the A53T α-synuclein in DRG neurons, while it is reduced in the sciatic nerve tissue, where loss of axonal material is observed. A53T and wild type mice have a similar pattern of epidermal footpad innervation and no significant differences are observed in terms of behavioral evaluation of cold-induced pain. These findings suggest that the differences in $Na_V1.8$ expression detected in A53T DRG primary cultures and A53T sciatic nerve tissue could be part of the remodelling process following axonopathy. Further electrophysiological and behavioral evaluation of sensory processing in A53T mice would be a desirable research plan in the future.

Περίληψη

Η νόσος του Πάρκινσον (PD) είναι μια προοδευτική νευροεκφυλιστική νόσος που προσβάλλει το 1% του πληθυσμού άνω των 60 ετών και συγκριτικά με άλλες νευρολογικές διαταραχές, είναι η ταχύτερα αναπτυσσόμενη σε επικράτηση, ποσοστό αναπηρίας και θάνατο. Το PD επηρεάζει κυρίως νευρώνες που παράγουν ντοπαμίνη στην περιοχή του εγκεφάλου που ονομάζεται περιοχή του μίσχου μέλαινα ουσία (substantia nigra) και με τον τρόπο αυτό διαταράσσει την κινησιολογία των ασθενών. Το χαρακτηριστικό γνώρισμα της PD παθολογίας είναι η συσσωμάτωση της πρωτεΐνης α-συνουκλεΐνης, η οποία στη μορφή αυτή καθίσταται τοξική για τα κύτταρα. Οι σημειακές μεταλλάξεις στο γονίδιο της α-συνουκλεΐνης μπορούν να οδηγήσουν σε PD κληρονομικού τύπου, ένα παράδειγμα είναι η μετάλλαξη A53T, η οποία αυξάνει την τάση συσσωμάτωσης της α-συνουκλεΐνης. Τα συμπτώματα του PD αφορούν ως επί το πλείστον την κίνηση, παρ'όλα αυτά η συμπτωματολογία αφορά και μη κινητικά συμπτώματα. Για παράδειγμα, έως και το 85% των περιπτώσεων με PD παρουσιάζει πόνο, ενώ το ένα τρίτο αναπτύσσει αισθητηριακές περιφερικές νευροπάθειες. Η διαφορετική αισθητηριακή αντίληψη σε πρώιμα στάδια της νόσου υποδηλώνει μεταβολές στην επεξεργασία των ερεθισμάτων, γεγονός που πιθανόν υποδηλώνει μεταβολές στη διεγερσιμότητα των νευρώνων στο επίπεδο των τασοελεγχόμενων καναλιών νατρίου (NaVs), οι οποίοι είναι καθοριστές της ηλεκτρογένεσης. Συγκεκριμένα, έχει μελετηθεί ότι η έκφραση του υποτύπου NaV1.8, που εκφράζεται σε νευρώνες - αισθητήρες του πόνου μεταβάλλεται έπειτα από εκφυλισμό των νευραξόνων.

Επί του παρόντος, η αισθητηριακή δυσλειτουργία που εμφανίζεται στο PD δεν έχει διερευνηθεί επαρκώς, επομένως η παρούσα εργασία στοχεύει στη μελέτη της επίδρασης της έκφρασης της παθολογικής α-συνουκλείνης σε νευρώνες γαγγλίων ραχιαίας ρίζας (DRG) και σε ιστούς του περιφερικού νευρικού συστήματος. Για τον σκοπό αυτό, χρησιμοποιήθηκε το διαγονιδιακό μοντέλο ποντικού A53T το οποίο εκφράζει την παθολογική ανθρώπινη α-συνουκλεΐνη. Ο χαρακτηρισμός των πρωτογενών DRG νευρώνων που προήλθαν από ομόζυγους A53T μύες επιβεβαίωσε την εναπόθεση της A53T α-συνουκλεΐνης σε DRG νευρώνες, ενώ η έκφραση του διαγονιδίου συσχετίστηκε με το μέγεθος του σώματος των DRG νευρώνων. Οι A53T νευρώνες ήταν επιρεπείς στην πρόκληση στρες από αναστολέα του πρωτεασώματος, ενώ παρατηρήθηκαν στους άξονες αυτών πολυάριθμες περιοχές εκφυλισμού. Ο εντοπισμός του τασοελεγχόμενου καναλιού NaV1.8 στους άξονες των νευρώνων, μέσω της ανοσοϊστοχημικής μεθόδου, παρατηρήθηκε αυξημένος στους A53T νευρώνες. Μελετήθηκε ακόμη η ηλεκτρική δραστηριότητα των νευρώνων μέσω της απεικόνισης ασβεστίου. Στον ιστό του ισχιακού νεύρου των A53T μυών παρατηρήθηκε απώλεια αξονικού υλικού και μειωμένη έκφραση του NaV1.8. Δεν παρατηρήθηκαν στατιστικά σημαντικές διαφορές στην επιδερμική νεύρωση του πέλματος μεταξύ των διαγονιδιακών και μη διαγονιδιακών μυών, όπως επίσης και στην αισθητικότητα του πόνου που προκαλείται από το κρύο. Η διαφορά στην έκφραση NaV1.8 που εντοπίστηκε στις πρωτογενείς καλλιέργειες A53T DRG και στον ιστό του ισχιακού νεύρου, καθώς και ο νευροεκφυλισμός των νευρώνων και των ιστών, υποδηλώνουν πιθανή αναδιαμόρφωση στο επίπεδο των καναλιών νατρίου έπειτα από αξονοπάθεια. Η περαιτέρω ηλεκτροφυσιολογική μελέτων των A53T νευρώνων και συμπεριφορική αξιολόγηση της αισθητηριακής επεξεργασίας A53T μυών αποτελεί ένα επιθυμητό μελλοντικό ερευνητικό σχέδιο.

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1. Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is a common progressive neurodegenerative disorder that affects 1% of the population above 60 years. Recently, a 10-fold increasing risk of PD between the ages of 50 and 81 has been demonstrated (Collier, Kanaan and Kordower 2017). The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2015 noted that the number of people affected by the condition worldwide had more than doubled from 1990 to 2015 and, among neurological disorders, PD was the fastest growing in prevalence, disability and deaths. This could be a result of an increasing number of elder people, longer disease duration and environmental factors (Collaborators 2018). The incidence of PD is linked to risk and protective factors, such as age and pollutants (de Lau and Breteler 2006, Bellou et al. 2016, Pezzoli and Cereda 2013, Weisskopf et al. 2010, Vlaar et al. 2018), whereas longer disease duration could be affected by increased longevity (Pringsheim et al. 2014, Wanneveich et al. 2018). Therefore, as ageing and industrialisation increase, the prevalence of PD is rising as well (Rossi et al. 2018).

There are certain diagnostic criteria for PD that are described in different sets of criteria, such as The United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) and the Gelb Diagnostic Criteria, which have been widely used in clinical practice (Jankovic et al. 2000, Gibb and Lees 1988, Gelb, Oliver and Gilman 1999). In general, disability in patients with PD is primarily due to impaired motor function, characterised by resting tremor, rigidity and bradykinesia. In a clinical cohort study, baseline characteristics of 1244 individuals diagnosed with PD revealed that individuals were predominantly right-handed (92%), male (67%), with a mean age of 70 years and symptomatic disease duration of about 7 years (Uitti et al. 2005). About 25% of the sample had a family history of a neurodegenerative disorder, whereas tremor (47%) and bradykinesia (29%) were the two most common initial symptoms, both occurring primarily in the upper extremities (68%). Eventhough PD is diagnosed on the basis of the motor signs described above, non-motor features such as autonomic dysfunction, peripheral neuropathies, cognitive dysfunction, sleep disturbances and psychiatric disorders are also present. A recent study found 122 significant associations of 832 phenotypes and PD, such as trouble in walking, history of brain surgery or less able to clap to a beat (Heilbron et al. 2019). Other associated phenotypes, such as REM sleep behavior disorder and olfactory loss, are used in the International Parkinson and Movement Disorder Society research criteria for prodromal PD.

PD motor symptoms result from predominantly affected dopamine-producing neurons in the nigrostriatal system, which originates in the zona compacta of the substantia nigra (SNc) (McRitchie, Cartwright and Halliday 1997, Gibb 1991). Dopaminergic neurons in the midbrain are the main source of the neurotransmitter dopamine in the mammalian central nervous system and, even though they correspond to less than 1% of the total number of neurons, they control multiple brain functions including voluntary movement and behavioral processes, such as mood,

motivation and reward (Calabresi et al. 2007). Specifically, loss of dopaminergic neurons in SNc results in severe dopamine depletion in the striatum, responsible for the motor symptoms, especially bradykinesia, tremor, rigidity and loss of postural control (Chase 2004). Loss of non-nigral areas, such as the dopaminergic ventral tegmental area, the noradrenergic locus coeruleus and the ascending cholinergic pathway from the Meynert basalis nucleus lead to cognitive and psychological impairments (Candy et al. 1983, Aarsland et al. 1996). Dopaminergic neurons are prone to degeneration and to oxidative stress, due to their high rate of oxygen metabolism, low levels of antioxidants, extensive branching and the large amounts of energy required to send nerve signals along their network (Surmeier, Obeso and Halliday 2017, Matsuda et al. 2009, Pacelli et al. 2015). It is estimated that 31% of the dopaminergic neurons are lost at time of symptom onset, adjusted for age (Fearnley and Lees 1991).

A commonly prescribed drug for PD is L-dopa (levodopa), an uncharged natural precursor for the metabolism of dopamine used to replace lost midbrain dopamine (Cotzias, Van Woert and Schiffer 1967). Even though it is widely used to control some of the PD symptoms, it is associated with significant complications such as levodopa-induced dyskinesias and other motor complications. Also, other symptoms, especially non-motor related, are relatively unaffected by current levodopa treatments (Curtze et al. 2015). Other tactics of treatment are physical therapy and motor training (Yitayeh and Teshome 2016, Smania et al. 2010, Klamroth et al. 2016), however, degradation of motor learning ability could limit the benefits of this therapy (Roemmich et al. 2014, Pendt, Reuter and Muller 2011, Frith, Bloxham and Carpenter 1986). The implication of other affected neurological systems, including cholinergic, the noradrenergic, the glutaminergic and GABAergic pathways has been reported (Francis and Perry 2007, Perez-Lloret and Barrantes 2016). Based on this, catechol-o-methyl-transferase inhibitors, dopamine agonists and non dopaminergic therapy, surgical techniques and deep brain stimulation represent other alternatives therapies that remain palliative and do not affect disease courses (Hausdorff et al. 2009). More recently, transplantation of neural stem cells from fetal tissue into the PD striatum has been developed and seems to be a promising approach considering the capability of progenitor cells to replace the function of the damaged dopaminergic neurons (Storch et al. 2004).

Dopaminergic cell loss is associated with the presence of eosinophilic intraneuronal inclusions, called Lewy bodies, composed of alpha-synuclein (α-synuclein), neurofilament and ubiquitin (Goldman et al. 1983). The aggregation of the protein α-synuclein into Lewy bodies is considered as the hallmark of PD initiation. A physiological role of α-synuclein, which can be found in an equilibrium between α-helical multimers and a natively unfolded monomeric state, is among others, the regulation of synaptic vesicle clustering (Pineda and Burre 2017). In PD, these α-synuclein forms aggregate into fibrils and engage a toxic status by adopting a prion-like spreading pattern (Spillantini et al. 1997, Poewe et al. 2017, Braak et al. 2006, Makin 2016). The relationship of α-synuclein aggregation and dopaminergic degeneration is unclear, however, it is considered that when the native state of α-synuclein is disturbed due to genetic mutations, increased expression levels or age-related deterioration of the protein, it may interact with oxidized dopamine in the cytosol resulting in particularly neurotoxic conformations.

1.2 Generation of the A53T Parkinson's Disease model

Most cases of PD are sporadic, however, point mutations in the α-synuclein gene can cause heritable early-onset PD in rare pedigrees. Mutations in the gene of α-synuclein (SNCA) usually induce early-onset PD, under 50 years of age, with an initially good response to levodopa treatment. However, the disease has a rapid progression, often accompanied by cognitive decline and sometimes with atypical features, such as central hypoventilation and myoclonus. Lewy bodies are present and spread through the substantia nigra, locus coeruleus, hypothalamus and cerebral cortex (Polymeropoulos et al. 1996). Meta-analysis of genome-wide association studies has identified 28 independent risk variants across 24 SNCA loci (Nalls et al. 2014). Increased copy number of SNCA is associated with early-onset and increased severity of the clinical phenotype, indicating that elevated expression of wild type α-synuclein is also detrimental (Singleton et al. 2003, Ferese et al. 2015). The first mutation in the SNCA gene identified is the Alanine to Threonine (A53T) missense point mutation, which has been linked to an autosomal dominant and early-onset PD, documented in families of Italian and Greek descent, as well as in a Korean and a Swedish family (Polymeropoulos et al. 1997, Athanassiadou et al. 1999, Ki et al. 2007). Penetrance of this missense mutation appears to be as high as 85% (Polymeropoulos et al. 1996). Today it remains unclear how the mutated protein has such potent toxicity in the central nervous system, however it seems that there are increased α-synuclein kinetics of fibrilization compared to the wild-type protein (Conway, Harper and Lansbury 1998).

A-synuclein consists of 140 amino acids containing three functional domains (Chen et al. 1995). The N-terminal domain is frequently acetylated and is characterized by lysine-rich repeats that form amphipathic α-helices upon lipid interaction (Dikiy and Eliezer 2014, Maroteaux, Campanelli and Scheller 1988). All α-synuclein point mutations that cause autosomal dominant forms of PD, including A53T, occur in the N-terminal domain, suggesting that disruption of this region is pathogenic (Polymeropoulos et al. 1997, Kruger et al. 1998, Pasanen et al. 2014, Appel-Cresswell et al. 2013). Amino acids 61-95 compose the hydrophobic non-amyloid-β component of amyloid plaques domain, containing a sequence that is necessary and sufficient for α-synuclein aggregation into β-sheet-rich fibrils (Giasson et al. 2001b). The C-terminal domain is at the most part unstructured when α-synuclein is bound to lipids, and when aggregated into fibrils. This domain increases the rate of α-synuclein aggregation and is the site of many post translational modifications, including phosphorylation at Serine 129, which was first detected in Lewy bodies and is used as a marker of pathological α-synuclein (Fujiwara et al. 2002). It is considered that α-synuclein remains in a dynamic equilibrium between disordered monomers and metastable multimers and this, among others, regulates its physiological role in the synapse (Burre, Sharma and Sudhof 2014, Wang et al. 2014). In particular, α-synuclein modulates vesicular trafficking during neurotransmission, a function that is disrupted by PD-related mutations (Logan et al. 2017, Larsen et al. 2006). The A53T familial mutation has been shown to reduce the levels of physiological multimers, suggesting that disruption of this equilibrium may promote α-synuclein aggregation (Dettmer et al. 2015).

Although PD is rarely inherited and familial forms represent only 10% of all PD cases, the identification of familial genetic mutations has permitted the development of animal and cellular models that are used to understand the molecular mechanisms underlying sporadic PD. For this purpose, transgenic mice that overexpress the wild type and mutant A53T human α-synuclein have been generated as models of α-synucleinopathies and PD (Giasson et al. 2002). In these models, the transgene was expressed under the mouse prion protein (PrP) gene promoter, in order to drive a high expression in neurons. Up to 7 months of age, homozygous transgenic mice expressing wild-type or A53T homozygous A53T mutant α-synuclein remained, exhibited no signs of muscular weakness and by 8 months of age, a few homozygous mice began to develop a particular phenotype. This phenotype mostly included neglect of grooming, weight loss and reduced ambulation, followed by hunched back morphology, severe movement impairment with resistance to passive movement and partial paralysis of limbs, which usually began at the hindlimbs, but within a few days, all four limbs were affected. A-synuclein inclusions were also abundant and widely distributed in the somatodendritic compartment and throughout the neuronal axis of homozygous A53T mutant α-synuclein animals ranging between 8 to 16 months of age. A high density of inclusions was also observed in the spinal cord, brainstem, deep cerebellar nuclei, deep cerebellar white matter and in some thalamic regions. A gradient of neuronal pathology was also found in the striatum and sparse pathology detected in the motor cortex. Other regions of the cerebral cortex, the olfactory bulb and the hippocampus did not show α-synuclein pathology. Purkinje cells and granular cells in the cerebellum and tyrosine hydroxylase (TH)-positive neurons of the substantia nigra did not show α-synuclein pathology as well. Overall, this model exhibits neurodegenerative phenotypes, features - typical traits of human inclusions and reveal the differences between wild-type and mutant α-synuclein, demonstrating that this amino acid substitution leads to neurodegeneration by promoting the formation of filamentous inclusions.

1.3 Nociception and sensory neuropathy in Parkinson's disease

Even though the most considered PD symptoms are motor-related, up to 85% of PD patients experience pain, which is often undiagnosed and untreated despite its high prevalence and impact on daily life (Defazio et al. 2008, Beiske et al. 2009). 43% of PD patients have pain in the early stages of PD, when motor symptoms are not yet conspicuous (Giuffrida et al. 2005). This suggests that pain at a premotor stage of PD may imply sensory impairments in either peripheral or central nociceptive pathways. Patients with PD experience various types of acute or chronic pain, including musculoskeletal, dystonic, neuropathic, nocturnal and visceral (Pont-Sunyer et al. 2015). King's Parkinson's disease pain scale allows exploring the distribution of the different pain types and its association with life quality (Martinez-Martin et al. 2017). Studies have shown that PD patients display altered sensory and pain thresholds during early-stage PD, as well as, overly activated brain areas involved in pain processing, which suggests alterations in stimuli processing along the neuronal axis (Spanagel, Herz and Shippenberg 1992, Mylius et al. 2009). Loss of dopamine in the basal ganglia could possibly explain the changes in pain thresholds, however, the unfeasible restoration of these symptoms, after dopaminergic replacement, suggests that non-dopaminergic mechanisms are involved in the initiation and maintenance of pain symptoms (Brefel-Courbon et al. 2005). Neurodegeneration that induces changes in nociceptive processing could be due to alterations in cholinergic, noradrenergic and serotonergic pathways (Gandolfi et al. 2017, Rukavina et al. 2019), however, it is mostly considered that in PD, both peripheral pain transmission and sensory perception and interpretation are disrupted.

Quantitative sensory testing shows that one third of PD patients develop progressive sensory neuropathies that affect somatosensory and autonomic nerves (Gierthmuhlen et al. 2009, Nolano et al. 2017). This results in polyneuropathic pain that contributes to musculoskeletal or widespread pain (Uceyler et al. 2018). In PD, polyneuropathic pain does not respond efficiently to levodopa medication, in fact, it has been shown that peripheral dopamine overload by levodopa treatment merely contributes to the development of sensory and autonomic neuropathies (Silverdale et al. 2018, Nolano et al. 2017, Zis et al. 2017). The predominant phenotype of sensory neuropathy consists of loss of thermal perception, pathological heat pain and mechanical hypersensitivity (Gierthmuhlen et al. 2009), which mostly agrees with the denervation pattern observed in PD, diabetes and other metabolic diseases (Vollert et al. 2018, Vollert et al. 2017, Vollert et al. 2015). Reports of reduced thermal, electrical, cold or mechanical pain thresholds in PD patients are not reported in all cases, however, numerous clinical studies indicate their existence. This seems to be independent of the presence of a clinical pain syndrome, indicating subclinical alterations to pain-related pathways in some patients (Petschow et al. 2016, Brefel-Courbon et al. 2013). The progressive loss of sensory functions parallels the progression of the disease, but it may precede motor symptoms for more than 10 years, suggesting that sensory neurons are particularly vulnerable to PD pathophysiology (Pont-Sunyer et al. 2015, Silverdale et al. 2018). Specifically, due to the long distances of axonal transport of organelles and vesicles, peripheral neurons are vulnerable to alterations of microtubule dynamics. Autophagosomes are constantly built at the peripheral terminals of sensory nerves that need to be transported towards the soma in order to remove synaptic protein waste (Maday and Holzbaur 2012, Maday and Holzbaur 2014, Colvin 2019). Thus, sensory neurons, having long axons, are easily affected by

axonal transport defects, as it is also shown by chemotherapy-induced neuropathies caused by microtubule disrupting molecules (Colvin 2019, Zheng, Xiao and Bennett 2012).

Pathological α-synuclein imposes several toxic effects, including disruption of microtubule and membrane dynamics (Bodner et al. 2010, Bendor, Logan and Edwards 2013, Ludtmann et al. 2018), inhibition of the proteasome's activity (Zondler et al. 2017) and disruption of autophagolysosomal pathways, such as autophagy, mitophagy and chaperone-mediated autophagy (Poehler et al. 2014, Cuervo et al. 2004, Orenstein et al. 2013, Pan et al. 2008, Vives-Bauza et al. 2010, Wang et al. 2016). Consequently, susceptible neurons accumulate organelle, protein and lipid waste, and are unable to maintain transport to and from the synapse (Sulzer 2010). Post-mortem findings of 256 patients with α-synucleinopathies revealed that Lewy bodies were found in the spinal cord, dorsal root ganglia and brainstem of PD patients, partly supporting the hypothesis that α-synuclein depositions initiate in the peripheral cutaneous nerves and follow a caudal to rostral direction to the brainstem (Sumikura et al. 2015, Tseng and Lin 2017). The first skin biopsy study in PD patients examined cutaneous free and encapsulated sensory nerve endings in 18 patients and 30 controls (Nolano et al. 2008). A significant loss of epidermal nerve fibers and Meissner corpuscles (MCs) was observed, as well as, increase in tactile and thermal thresholds and reduction in mechanical pain perception. Increased branching, was also observed, as well as, sprouting of nerves and enlargement of the vascular bed, suggesting attempts that counteract degenerative processes (Nolano et al. 2008). The histopathology of other skin biopsies, also showed swollen axons and loss of terminal fiber density, compensatory fiber sprouting, and α-synuclein deposits in cutaneous sensory and autonomic nerves (Nolano et al. 2017, Podgorny et al. 2016, Donadio et al. 2017, Wang et al. 2013). Another study, in which the quantity of epidermal nerves of the more affected side of PD patients was examined, revealed that up to 94.1% of patients expressed an abnormal epidermal nerve density (Lin et al. 2016). Studies also confirmed the degeneration of sensory nerves responsible for pain perception, as well as, the deposition of phosphorylated α-synuclein in cutaneous sensory nerves early on in PD (Donadio et al. 2014). The inverse correlation between the deposition of a-synuclein in peripheral nerve fibers and the extent of skin denervation and nerve function suggests that the characteristic pathology of PD may affect pain processing in the peripheral nerve terminals (Donadio et al. 2014, Wang et al. 2013). Another study aiming to characterize *in vivo* α-synuclein aggregates in skin nerves underlined the importance of α-synuclein localization and differences in aggregate loads in order to identify specific diagnostic traits among synucleinopathies (Donadio et al. 2018).

1.4 Sensory neurons

Peripheral sensory information can be detected through the wide population of sensory neurons known as first-order neurons, the cell bodies of whom are located in the dorsal root ganglion (DRG), a cluster of cell bodies in the dorsal root of the spinal cord. The axons of DRGs, known as afferents, relay sensory information into the central nervous system. Sensory neurons are pseudounipolar, meaning that one process attached to the cell body separates into two processes (Matsuda et al. 1998). The distal process projects to cutaneous or deep peripheral tissues that terminate either in specialized end organs or as free terminals, whereas the proximal process terminates in the dorsal horn of the spinal cord or sensory nuclei of the brainstem (Ha 1970). Internal or external stimuli that can be chemical, thermal or mechanical induce the release of specific neurotransmitters and neuropeptides, which leads to depolarization and action potential propagation to the central nervous system (Fundytus 2001, Ban, Brassai and Vizi 2020, Chery-Croze 1983).

Sensory neurons are a very heterogeneous population that differs in size, level of myelination and neurochemical characteristics (Li et al. 2016, Haberberger et al. 2019). Differences in the degree of myelination results in neuronal populations with distinct axonal conduction velocities. In particular, they can be found as heavily myelinated Aα-fibers, moderately myelinated Aβ-fibers, thinly myelinated Aδ-fibers, as well as unmyelinated C-fibres (Image 1). These differences in conduction velocity have been associated with discrete functions. Specifically, Aα and Aβ-fibers are largely associated with the detection of light touch and proprioception, Aδ-fibers are associated with the detection of a variety of innocuous and noxious stimuli, while free C-fibers are mostly involved in detecting nociceptive stimuli and merely for relaying innocuous light touch stimulation (Dubin and Patapoutian 2010, Djouhri and Lawson 2004). Neurons of smaller size usually correspond to Aδ and C fibre conductivity. Sensory neurons are organized in a basal structure of three cold-sensitive neuron types, five mechano-heat sensitive nociceptor types, four A-Low threshold mechanoreceptor types, five itch-mechano-heat–sensitive nociceptor types and a single C–low-threshold mechanoreceptor type with a strong relation between molecular neuron types and functional types (Abraira and Ginty 2013). It is mostly the unmyelinated neurons with free nerve endings in the skin that initiate and transduce noxious stimuli, thermoception and itch sensation, although some myelinated Aδ and Aβ-fibers are also involved in the transduction of noxious stimuli (Djouhri and Lawson 2004). Neurons that respond preferentially to noxious stimuli are termed nociceptors and typically they have high thresholds of activation (Woolf and Ma 2007).

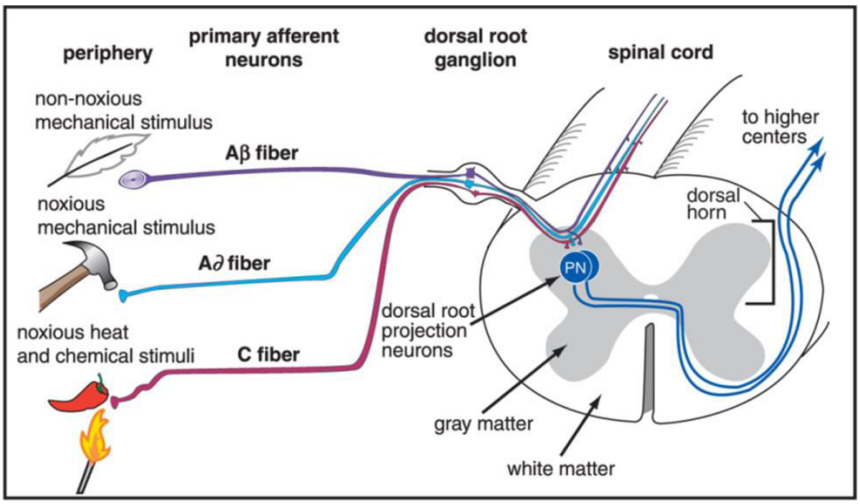


Image 1. Sensory neurons are a very heterogeneous population. Differences in the degree of myelination results in neuronal populations with distinct axonal conduction velocities. In particular, they can be found as heavily myelinated Aα-fibers, moderately myelinated Aβ-fibers, thinly myelinated Aδ-fibers, as well as unmyelinated C-fibres. These differences in conduction velocity have been associated with discrete functions. Aα and Aβ-fibers are largely associated with the detection of light touch and proprioception, Aδ-fibers are associated with the detection of a variety of innocuous and noxious stimuli, while free C-fibers are mostly involved in detecting nociceptive stimuli and merely for relaying innocuous light touch stimulation.

1.5 Voltage gated sodium channels and nociception

The correlation between PD pain-related pathophysiology and altered neuronal thresholds of sensory neurons could imply alterations in neuronal physiology and excitability. It is well known, that voltage-gated sodium channels (Na_V), that conduct sodium ions through the cell's plasma membrane, maintain neuronal signaling and viability via initiation and propagation of action potentials (Hodgkin and Huxley 1990, Catterall 2000). Specifically, Na_V provide an inward current that underlies the depolarization of the membrane for an action potential to occur. Na_V contain a pore-forming α-subunit and one or two auxiliary β-subunits. Each α-subunit constitutes a polypeptide of four domains that are connected with cytoplasmic loops and are organised in a circular fashion around the ion channel pore (Image 2). The ion channel domains are highly conserved within the sodium channel family and consist of two pore-forming transmembrane helices and four voltage sensor transmembrane ones (Catterall 1995). Nine Na_V α-subunits have been identified and are grouped on the basis of sequence homology and their sensitivity to tetrodotoxin (TTX). There are six TTX-sensitive (TTX-s), $Na_V1.6$, $Na_V1.1$, $Na_V1.7$ and $Na_V1.2$ and three TTX-resistant (TTX-r), $Na_V1.8$, $Na_V1.9$ and $Na_V1.7$ α-subunit genes. Each subtype differs in biophysical properties, including voltage dependence, rate of activation, rate of inactivation and distinct contributions to membrane excitability (Pinto, Derkach and Safronov 2008). The different subtypes have also a distinct pattern of expression in different cell types and tissues.

The development of pain states involve dynamic plastic changes in peripheral sensitization and specific Na_V channels are involved in these changes and therefore the generation and maintenance of abnormal neuronal electrogenesis and hyper excitability. Among different Na_V subtypes, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are highly expressed in nociceptors and have been the centre of research in order to develop blockers that are specific for these channel isoforms in order to treat pathological pain states (Dib-Hajj et al. 2010). In nociceptors TTX–s, $Na_V1.1$, $Na_V1.6$, and $Na_V1.2$ are present, however the individual contribution of these isoforms to nociceptor function remains largely unknown. However, electrophysiological evidence indicates that both TTX-s and TTX-r sodium currents are present in nociceptive fibers (Elliott and Elliott 1993). The major TTX-r current has several unique biophysical properties including high thresholds for activation, high thresholds for steady state-inactivation, rapid recovery from inactivation and a slow rate of inactivation. These properties correlate with nociceptor characteristics, such as high activation thresholds in response to noxious stimuli, persistent activation in the presence of prolonged depolarization and wide action potentials (Gold 1999). Within the population of neurons with small diameter cell bodies that merely represent the nociceptor population, distinct TTX-r sodium currents are observed that are represented mostly by $Na_V1.8$ and $Na_V1.9$ channels (Akopian, Sivilotti and Wood 1996). $Na_V1.8$, in particular, generates a slowly-inactivating sodium current in small diameter DRG neurons, which is resistant to TTX (Akopian et al. 1999). $Na_V1.8$ is expressed in nociceptors with C and Aδ-fibre conductivity and conveys some unique excitability traits to the cells in which it is expressed. There is a positive correlation between expression of $Na_V1.8$ and action potential rise time and action potential overshoot suggesting that $Na_V1.8$ contributes to these aspects of the action potential. Also, C-fibers derived from knock down animals for $Na_V1.8$ show a reduced peak action potential response and a slower rate of depolarization than wild type neurons. Expression of $Na_V1.8$ channel in Purkinje cells, which normally lack this Na_V isoform, alters the action potential activity of these neurons (Renganathan et al. 2003). These findings suggest that $Na_V1.8$ is a major C-fiber related channel isoform that affects the firing pattern of nociceptors.

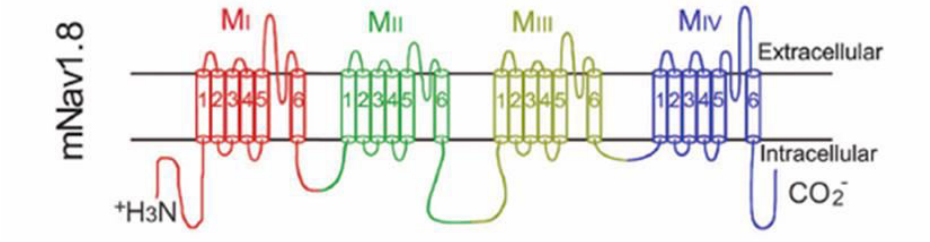


Image 2. Schematic diagram of sodium channel α subunit Nav1.8 from M. musculus (mNav1.8). MI to MIV represent four different domains of mNav1.8 (Rowe et al., 2013, Science).

1.6 Axonopathy and Nav1.8

Neural plasticity is the ability of the neural system to undergo structural or physiological changes in order to optimize the neural networks during procedures, such as learning. Adaptive changes in ion channel expression provide a response to changes in the neuronal environment and this is considered as a mechanism of neural plasticity (Birch et al. 2004). Remodeling of channel expression, however, is also a feature of pathology associated with aging, chronic pain and other pathological factors, such as injury (Toro et al. 2002, Waxman et al. 2002). Dysregulation of ion channel expression and activity can be a major contributing factor to the development and maintenance of a pathology. For instance, changes in the ion conductance of sodium can lead to either a raised or lowered value of threshold. The diameter of the axon, as well as, the density of Na_V and their properties within the axon significantly affect the threshold value (Trautwein 1963). In electrophysiology, the threshold potential is the critical level to which a membrane potential should be depolarized in order to initiate an action potential.

After nerve injury, affected neurons undergo membrane remodeling, which includes shifts in the pain threshold and subsequent hyperexcitability (Hameed 2019) (Image 3). This post-injury change is referred to as ectopic electrogenesis and leads to spontaneous ectopic activity, reduction of threshold and elevated response to suprathreshold stimuli. Persistent electrical hyperexcitability and abnormal impulse generation in DRG neurons is essential for the peripheral and central sensitization, that leads to the development and maintenance of chronic neuropathic pain. Neurons of the central nervous system become more excitable, receptive fields expand and pain is perceived in response to low threshold mechanoreceptive Aβ-fibers, leading to allodynia. It is considered that neuropathic changes that cause spontaneous abnormal activity in peripheral neurons involve Na_V subtype-specific alterations in density, distribution and function (Hameed 2019). Following peripheral nerve transection in animal models of neuropathy, $Na_V1.8$ channel expression in sensory neurons has been found altered. In human patients with chronic neuropathic pain and in animal models of inflammatory pain, $Na_V1.8$ expression is upregulated, however in axotomized DRG neurons $Na_V1.8$ is downregulated (Akopian et al. 1996, Gold et al. 2003, Kort et al. 2010).

There are indications that render the role of $Na_V1.8$ in neuropathic pain significant in adjacent uninjured sensitized neurons, rather than injured neurons. In particular, $Na_V1.8$ expression and its slowly inactivating current is significantly reduced in injured DRG neurons of a transected peripheral fibers (Dib-Hajj et al. 1996), whereas it is upregulated in adjacent uninjured sensitized neurons (Gold et al. 2003). It is considered that peripheral upregulation of Nav1.8 may occur in the end of uninjured axons adjacent to injured axons, a hypothesis supported by the detection of increased $Na_V1.8$ in patients dealing with chronic pain. It has been suggested, however, that $Na_V1.8$ response to neuropathic pain may change overtime (Coward et al. 2000). Specifically, two weeks after nerve injury, $Na_V1.8$ was found upregulated in intact nociceptors, overtime however, $Na_V1.8$ was found upregulated in injured nociceptors. Therefore, there might be a time-dependent shift in the mechanism underlying $Na_V1.8$ action in the pathophysiology of neuropathic pain. This

shift may involve the cross-talk between injured and non-injured neurons, the exact mechanism of which is unknown, but could be mediated by products of Wallerian degeneration (Wu et al. 2001).

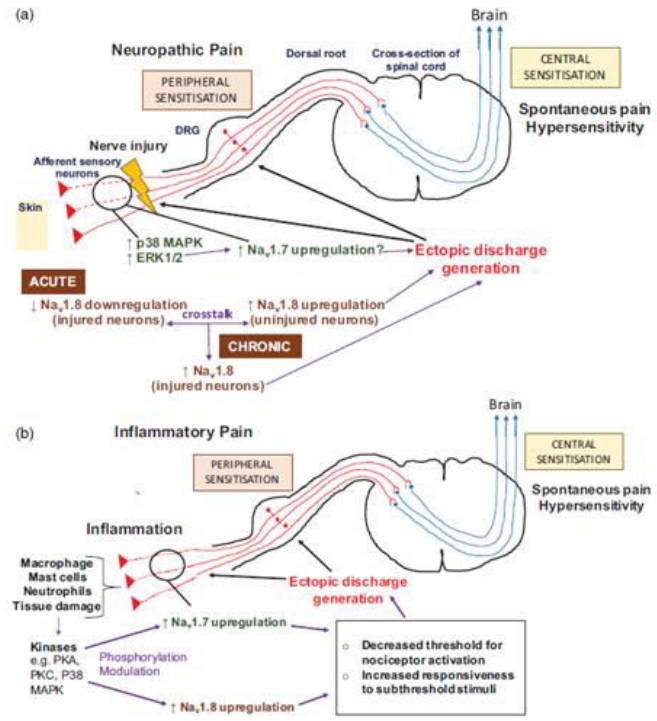


Image 3. Proposed mechanisms of the role of Nav1.7 and Nav1.8 in the pathophysiology of neuropathic pain and inflammatory pain.(a) Nerve injury leads to the modulation of Nav1.7 channels which are upregulated as a result of nerve injury. This contributes to increased generation of ectopic discharge. In the acute phase following nerve damage, Nav1.8 is downregulated in injured neurons and upregulated in neighbouring uninjured neurons, which contributes to increased spontaneous discharge. In the chronic phase via a form crosstalk between injured and intact neurons Nav1.8 is alsoupregulated in injured neurons resulting in a further increase and maintenance of the ectopic discharge. This leads to spontaneous pain andhypersensitivity. (b) Inflammatory cells and mediators are present at increased numbers at the site of tissue injury and inflammation. This results in an increased number of kinases that phosphorylate and modulate the Nav1.7 and Nav1.8 channels, which are upregulated innociceptors innervating the damaged tissue. This leads to an increase in ectopic action potentials. Together, these mechanisms result inspontaneous pain and hypersensitivity, that is, hyperalgesia and allodynia (Hameed et al., 2019, Mol Pain).

Objectives

Consideration of pain as an early feature of PD might imply a mechanism-based sensory system dysregulation, which for the time being, is insufficiently investigated. Findings suggest that pain in early PD could derive from atypical sensory inputs of degenerated peripheral primary afferents. In terms of pain assessment in early PD, this study focuses on the pain processing, approached by 1) the *in vitro* molecular and morphological characterization of primary DRG neuron cultures, derived from the transgenic A53T-α-synuclein mouse model and corresponding control mice, 2) the evaluation of $Na_V1.8$ channel expression affected by the A53T α-synuclein in DRG neurons, 3) the assessment of electrical activity of DRGs by calcium imaging analysis, 4) the evaluation of Nav1.8 channel expression in sciatic nerve and footpad tissue of A53T and wild type mice and 5) the assessment of cold-induced pain in A53T and wild type mice.

2. Materials

2.1 Consumables

Corning Vaccume Filter (Fischer Scientific)

Cover Glasses (VWR®, 24x50mm)

13mm round Coverslips

Falcon tubes (15ml, 50ml)

Glass Graduated Pipettes

Liquid Blocker Super Pap Pen

Microscope slides (VWR® Superfrost® Plus)

Microplates (96-well)

Parafilm M PM996

Pipette tips

Tissue Freezing Medium (Leica)

Tubes (1,5, 2 ml) (Eppendorf)

Well plates (Costar® 24-well Clear TC-treated)

2.2 Reagents

Calcium Chloride (Sigma)

Cytotoxicity Detection KitPLUS (LDH) (Sigma)

Disodium hydrogen phosphate (Applichem)

Dulbecco's Modified Eagle's Medium (DMEM) - high glucose (Gibco)

Ethanol (Pan Reac and Merck)

Epoxomicin (Sigma-Aldrich)

Fetal Bovine Serum (Gibco)

F12 (Gibco)

D+ -Glucose (Sigma)

Glutamate

HBSS - Hank's Balanced Salt Solution, no calcium, no magnesium, no phenol red (ThermoFischer Scientific)

HEPES Buffer Saline (FLUKA)

Imipramine

Isoflurane (Iso-Vet)

Laminine

Magnesium Chloride (Sigma)

MG-132 (Calbiochem, Merck-Millipore)

N-Methyl-D-aspartic acid (NMDA)

Normal Goat Serum (S30 Merck)

Paraformaldehyde (Sigma-Aldrich)

Penicillin/Streptomycin (Gibco)

Poly-D-lysine (Sigma)

Potassium Chloride (Applichem)

Potassium dihydrogen phosphate (FLUKA)

ProLong™ Gold Antifade Mountant with DAPI (Biotium)

PROTEOSTAT® Aggresome Detection Kit (Enzo Life Sciences, Inc.)

Silicone

Sodium azide (Sigma)

Sodium Chloride (Sigma)

Triton-X 100 (FLUKA)

2.3 Enzymes

Collagenase type XI (Sigma Aldrich, C7657)

Trypsin

2.4 Viruses

pAAV.Syn.GCaMP6f.WPRE.SV40

2.5 Antibodies

β-tubulin III (TUJ1) (rabbit, Cell signaling 5568 & mouse, Biolegend 801202)

Caspase-3 (rabbit, Cell Signalling)

Fibronectin (rabbit, Sigma F-3648)

Human α-synuclein (sc 12767)

MAP-2 (mouse, MAB3418 & chicken, abcam ab5392)

Mouse α-synuclein (mouse BD, 610787)

$Na_v1.8$ (mouse, ab93616, N134/12)

2.6 Buffers and Solutions

ACSF

145 mM NaCl, 2,5 mM KCl, 1mM MgCl2, 2mM CaCl2, 20mM HEPES, Glucose 10mM, pH=7.4

Coating solution

0,2mg/ml laminin and 0,02 mg/ml PDL in HBSS

Culture medium

DMEM high glucose : F12, 10% FBS, 1% P/S

Enzyme solutions

0,1% Trypsin in DMEM (-FBS)

0,2% Collagenase type XI and 0,05% Trypsin in DMEM (-FBS)

Normal Goat Serum Buffer solution

5% v/v NGS in 0.01M PBS azide

PBS 10x 1L

80g NaCl, 2g KCl, 14,4g Na_2HPO_4, 2,4g KH_2PO_4, pH=7.1]

2.7 Lab instruments

CO_2 incubator (Heracell 150, Marshall Scientific)

Cryostat (Leica Biosystems)

Freezers -20, -80 ^{o}C

Heraeus Function Line B6 (Thermo Fischer Scientific)

Laboratory Balance (METTLER TOLEDO)

Laminar hood (Thermo Fischer Scientific)

Microsurgery instruments (Dissecting scissors, spring scissors & forceps)

Milli-Q (M) Direct 8 (Millipore)

Perfusion pump (LKB Bromma)

pH-Meter (Thermo Scientific™ Orion™ 3-Star)

Pipettes 2-20µl, 20-200µl, 100-1000µl (Gilson)

Spectrophotometer

Water Bath (JULABO GmbH)

2.8 Microscopes

Leica TCS SP8 Confocal Laser Scanning Microscope – LASX

Olympus Time lapse IX81 CellR Microscope

Bausch & Lomb Stereozoom 3 Stereo/Dissecting Microscope

Axio Vert.A1 Bio - Inverted Microscope

3 Methods

3.1 Animals

All animal procedures were performed in strict compliance with the European and National Laws for Laboratory Animal Use (Directive 2010/63/EU and Greek Law 56/2013), according to FELASA recommendations for euthanasia and the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the Hellenic Pasteur Institute (Animal House Establishment Code: EL 25 BIO 013) and the License No 5677/25-09-2012 for experimentation was issued by the Greek authorities (Veterinary Department of Athens Prefecture).

Heterozygous transgenic mice (B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J), derived from Jackson Laboratory, were intercrossed in order to generate transgenic and non-transgenic mice. Specifically, heterozygous females were mated with either heterozygous or wild type males. All mice were housed at controlled temperature and humidity conditions, in a 12-hour light/dark cycle and fed an ad libitum diet of standard mouse chow. C57BL/6JolaHsd α-synuclein KO mice were also housed at controlled temperature and humidity conditions, in a 12-hour light/dark cycle and fed an ad libitum diet of standard mouse chow.

Genotyping procedure was performed in order to discriminate transgenic and non-transgenic mice, as well as, homozygous and heterozygous transgenic A53T mice.

3.2 Primary culture of Dorsal Root Ganglion neurons

Glass coverslips (13mm) were coated with ice-cold PDL/laminin solution, placed in 4^0C for 3 hours. Coated coverslips were rinsed with distilled water prior to cell plating. Primary DRG neuron cultures derived from homozygous transgenic A53T-α-synuclein mice and wild-type littermates. Mice, six to eight weeks postpartum, were euthanized by an overdose of isoflurane and were perfused intracardially with cold HBSS ($-Ca^{2+}$, $-Mg^{2+}$). DRG dissection and tissue dissociation was performed following a modified version of Malin et al. protocol (Malin, Davis and Molliver 2007). After perfusion, back fur was sprayed with 70% ethanol and a transverse cut was made in the middle of the back skin, using iris scissors. Muscle and adipose tissue was removed in order to expose cervical, thoracic and lumbar spinal regions. Lengthwise laminectomy of the posterior segment of the spine (spinous process, inferior articular process and superior articular process) was performed in order to expose the spinal cord. Spinal cord was

removed, while ventral and dorsal roots were cut along both sides of the spinal cord. Ganglia remained in between each vertebrae, in the neural foramina. Approximately 40 ganglia were removed within 1 hour and were stored in ice-cold HBSS (-Ca^{2+}, -Mg^{2+}). After dissection ganglia were incubated in collagenase type XI and trypsin solution for 30 minutes in 37^{o}C, flicking the tube every 10 minutes. Ganglia were then incubated in trypsin solution for 30 minutes in 37^{o}C, flicking the tube every 10 minutes. For a single cell suspension in culture medium, trituration was performed using a serum-coated glass pipette. Single DRG neurons were plated in drops on

3.3 Immunocytochemistry

Cells on coverslips were fixed with 4% paraformaldehyde for 20 minutes at room temperature. Cells were blocked and permeabilized with 5% NGS and 0.1% Triton X-100 in 1x PBS for one hour and were incubated with primary antibodies diluted in 2% NGS and 0,05% Triton X-100 at 4oC overnight. Coverslips were rinsed with 1x PBS and were incubated with secondary antibodies conjugated to AlexaFluor 488 (green), 546 (red) or 638 (blue) and diluted in 2% NGS for two hours at room temperature. Coverslips were then mounted with ProLong Gold Antifade Mountant with DAPI (Biotium). DRGs were labelled by anti-TUJ1 (1:4000), anti-NF200 (1:500), anti-CGRP (1:2000) and anti-MAP-2 (1:2000). Schwann cells were labelled by anti-GFAP (1:500) and fibroblasts by anti-fibronectin (1:500). Human and mouse α-synuclein were labelled by anti-human α-synuclein (1:2000) and anti-mouse α-synuclein (1:1000). All voltage-gated sodium channels were labelled by anti-Nav-PAN (1:1500) and Nav1.8 channel by anti-Nav1.8 (1:100), while caspase-3 was labelled by anti-caspase-3 (1:600). Protein aggregates were detected with the PROTEOSTAT Aggresome Detection Kit (Enzo) followed by immunolabeling for human α-synuclein.

3.4 Immunohistochemistry

Whole hind-footpad tissue and sciatic nerves were fixed with 4% paraformaldehyde for two hours at 4^{0}C. Tissues were then washed overnight with 1x PBS at 4^{0}C and then stored in 25% sucrose at 4^{0}C overnight. Tissues were then stored in Tissue Freezing Medium at -80^{0}C. Footpad tissue was cut in a cryotome in 20μm thick coronal sections, while sciatic nerve was cut in sagittal sections of 10μm thickness. Sections were blocked and permeabilized with 10% NGS and 0.3% Triton X-100 in 1x PBS for two hours at 4^{0}C and were incubated with primary antibodies diluted in 2% NGS and 0,05% Triton X-100 at 4^{0}C overnight. Sections were incubated with secondary

antibodies conjugated to AlexaFluor 488 (green), 546 (red) or 638 (blue) for two hours at room temperature and slides were mounted with ProLong Gold Antifade Mountant with DAPI (Biotium). Sciatic nerve axons and footpad neurons were labelled by anti-TUJ1 (1:4000), human α-synuclein was labelled by anti-human α-synuclein (2:000) and Nav1.8 channel by anti-Nav1.8 (1:300).

3.5 Cytotoxicity assays

A53T- and wild type-derived DRG neurons on day 10 were subjected to stress by adding 10μM imipramine that inhibits exocytosis, or 1μM epoxomicin which is a proteasome inhibitors, or 100μM glutamate that acts as an excitotoxin, for 24 hours. Quantification of cell cytotoxicity was performed by the colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. Cell cytotoxicity (Cytotoxicity DetectionKit, LDH; Roche) was determined in cultures in 24 hours after stressor addition according to the manufacturer's protocol and LDH activity was measured in 450 nm by a microplate spectrophotometer.

3.6 Calcium imaging

DRG neurons were incubated with a culture medium containing 3 μM Fluo-4 acetoxymethyl Ca^{2+} dye (Molecular Probes) for 30 minutes and washed with a fresh culture medium at 37 °C for 30 minutes. Each coverslip was then placed in an ACSF solution on a glass-bottom petri dish with a 10 mm hole and was sealed with silicone. Cells were excited at 488 nm with a fluorescein isothiocyanate (FITC) filter and Ca^{2+} images were recorded before, during, and after a chemical stimulation by high KCl concentration (80mM). Fluorescence signals were recorded at 10 frames per second (40ms exposure time) using a fluorescence Olympus Time lapse IX81 CellR microscope. From each field, cells were selected for analysis of Ca^{2+} responses using ImageJ software. The amplitude of fluorescence signal for each region of interest (ROI) was presented as relative change in fluorescence ($\Delta F/F_0$) after background subtraction (F^0 stands for basal fluorescence and $\Delta F = F - F_0$).

3.7 Cold Allodynia Assay

In order to measure the aversive behavior caused by the cold sensation, a cold allodynia assay was performed. Three homozygous A53T male mice six heterozygous A53T male mice and six wild type male littermates of 9-12 months of age were handled and habituated in a 14 x 9 x 6 cm conductive metallic surface surrounded by a 20 cm tall transparent film which prevented mice from escaping. On the day of testing, dry ice was used to cool the metallic surface to 15^0C. Mice paw movement was recorded and paw response was measured within 1 minute after the placement of mice on the surface. Paw lift was considered as a positive response.

3.8 Image acquisition

Images were acquired using a Leica TCSSP5II confocal microscope (LEICA Microsystems) using ×40 oil-immersion objective lens and were analyzed using Fiji-ImageJ software. For the assessment of axonal degeneration and Nav1.8 density, spots and puncta respectively were counted manually in randomly selected fields. Threshold levels were chosen manually and were applied to all images. In order to evaluate colocalization of Nav1.8 and TUJ1 in footpad tissues, a single slice of z-stack that included a TUJ1 positive nerve fiber was used. The region of interest was cut-out from the image and Image J's Coloc2 plugin was used. Threshold applied on the two channels was auto-generated by Coloc2 in the Costes regression threshold.

3.9 Statistical analysis

All *in vitro* experiments were replicated at least three times and data from parallel wild type- and A53T- derived cultures were acquired. All data represent mean±standard error of the mean (SEM). Statistical analysis was performed in GraphPad Prism and IBM SPSS Statistics 25 and comparisons between groups were performed using unpaired t test, one-way ANOVA and Tukey's multiple comparisons test. Probability values less than 0.05 ($p < .05$) were considered significant. All graphs were created in GraphPad Prism.

4 Results

4.1 Characterization of p.A53T DRG neuronal cultures

Mouse α-synuclein and human A53T α-synuclein expression in DRG neurons

Endogenous α-synuclein expression in murine DRGs has been previously described by Giasson et al. (Giasson et al. 2001a), as well, as pathological α-synuclein accumulation in DRGs of Lewy body-related α-synucleinopathy (LBAS) subjects (Sumikura et al. 2015). In the current study, DRGs derived from wild type mice, as well as homozygous A53T mice, exhibited endogenous α-synuclein expression both in somata and axons (Figure 1). As it was anticipated, DRGs derived from α-synuclein knock-out mice did not exhibit expression of endogenous α-synuclein.

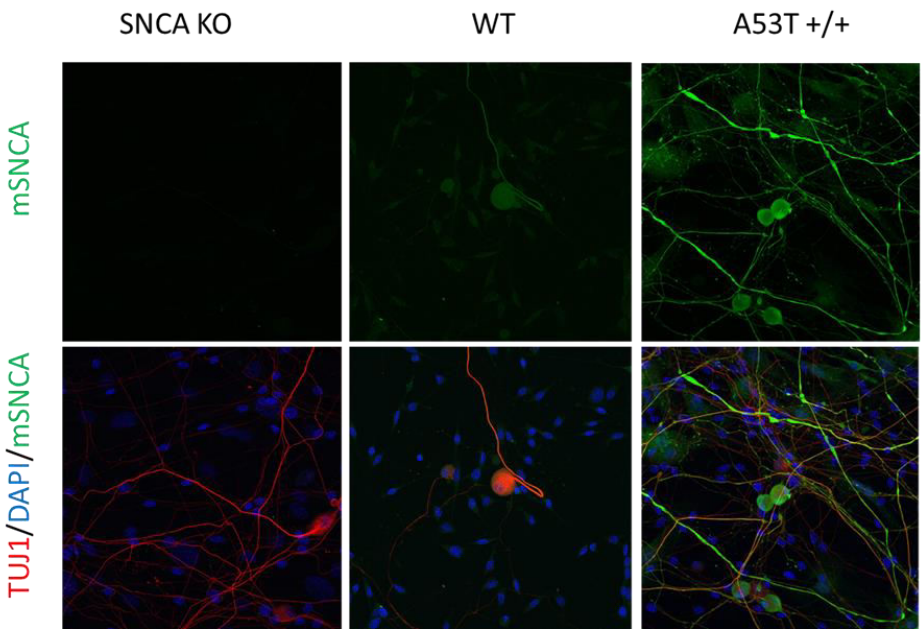


Figure 1. Mouse α-synuclein (mSNCA, green) is expressed in wildtype and A53T +/+ DRGs, while its expression is absent in SNCA KO DRGs (N=2).

Inclusions of human α-synuclein with the PD-associated mutation A53T have been previously detected in the spinal cord of A53T mouse model (Giasson et al. 2002), however their presence in DRGs in the A53T mouse model has not been studied. In the current study, only DRGs derived from homozygous A53T mice express human α-synuclein p.A53T, compared to wild type and α-synuclein KO mice that do not exhibit expression of this gene (Figure 2). A53T +/+ DRGs, hSNCA appeared in a typical punctate pattern, both in axons and somata, and in the nucleus as well (Figure 3).

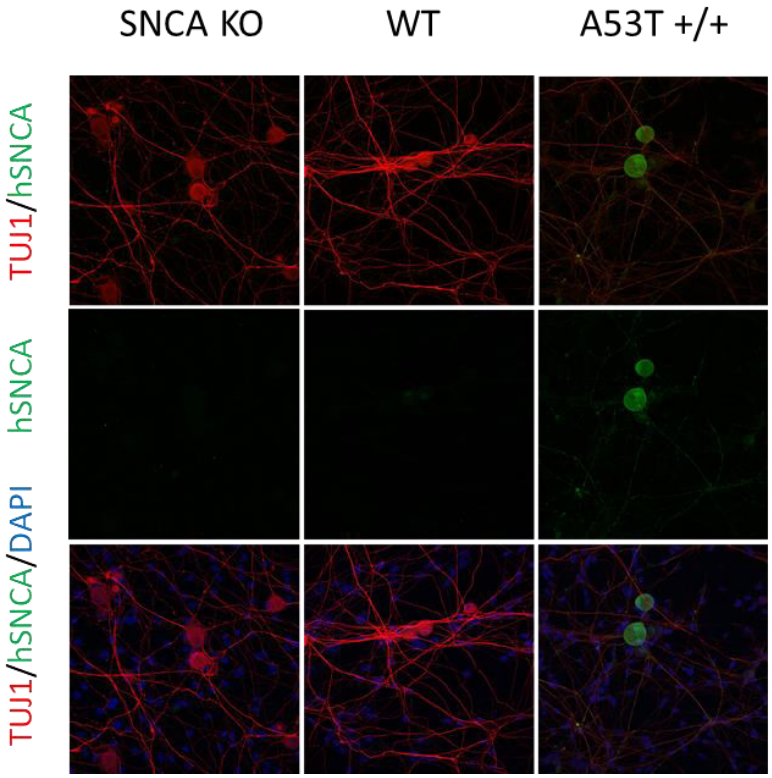


Figure 2. Human p.A53T α-synuclein (hSNCA, green) is expressed in A53T +/+ derived DRG neurons, while its expression is absent in wild type and SNCA KO derived DRGs (N=4).

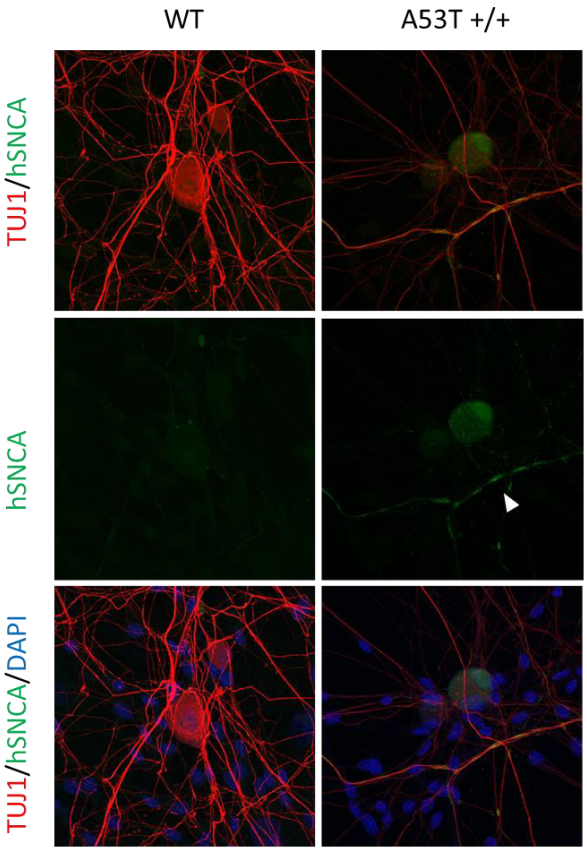


Figure 3. Axons in A53T +/+ derived DRGs present typical α-synuclein depositions (white arrow) (N=4).

Co-culture of DRGs, fibroblasts and Schwann cells

DRGs are normally surrounded by supportive cells, such as glial satellite cells (SGCs), Schwann cells and fibroblasts. In the current study, a typical DRG culture is positive for fibroblasts and Schwann cells (Figure 4).

P0 is a major structural component of the myelin sheath in the peripheral nervous system and is expressed by Schwann cells, however, neither DRG axons on day 12, nor Schwann cells were positive for Myelin protein zero (P0, MPZ).

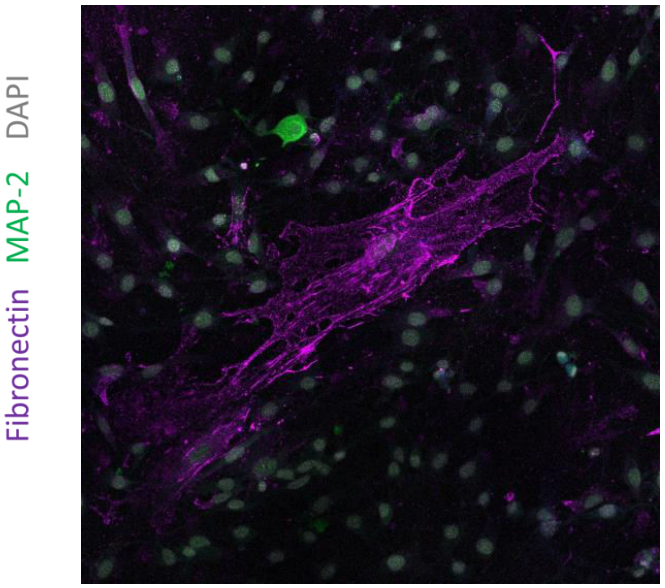


Figure 4. DRG primary culture includes DRGs (MAP-2, green) and fibroblasts (fibronectin, purple) (N=1).

Correlation of soma size and hSNCA expression in A53T +/+ DRGs

DRGs are a heterogeneous population of cells that differs in size, level of myelination and neurochemical characteristics (Li et al. 2016, Haberberger et al. 2019). DRG neurons in primary cultures are classified in the literature with respect to soma-size diameter in three main groups: small (<25 µm), medium (25–40 µm), or large (>40 µm) neurons (Hiruma et al. 2000; Ren et al. 2012). The current study investigated the correlation between A53T +/+ derived DRG soma size and hSNCA expression. In particular, hSNCA intensity (Mean) was measured in DRG somata of different size (large, intermediate and small) (Figure 5,6). Out of the z-stack, only the z-image with the highest intensity for each cell was measured in order to avoid the volume coefficient. The statistical analysis was performed using a one way ANOVA with post hoc Tukey's multiple-comparisons test in IMB SPSS Statistics 25. Significance was measured as follows, F (2, 234) = 67,32, **** p <,0001 (One-way ANOVA); LARGE vs. INTERMEDIATE, **** p <,0001; LARGE vs. SMALL *** p <,000; INTERMEDIATE vs. SMALL, *** p = ,0007 (Tukey's multiple comparisons test). Pearson's correlation coefficient was also measured (r = 0,6872, r^2 = 0,4723, ****, <0,0001). DRGs were characterized as positive or negative by setting a threshold value under which intensity was considered as background. 74% of A53T +/+ DRGs with a large soma size were considered positive, 18% of A53T +/+ DRGs with an intermediate soma size were considered positive, while only 4% of A53T +/+ DRGs with a small soma were considered positive (N=1, 236 neurons) (Figure 7).

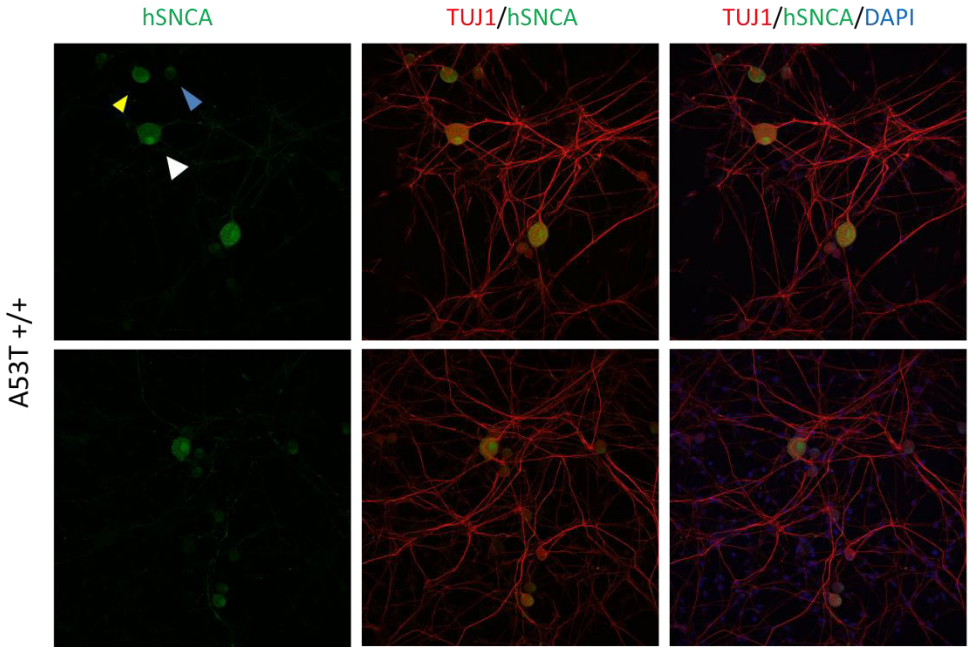


Figure 5. Based on DRG soma size, DRGs are characterized as large (white arrow), intermediate (yellow arrow) and small (blue arrow).

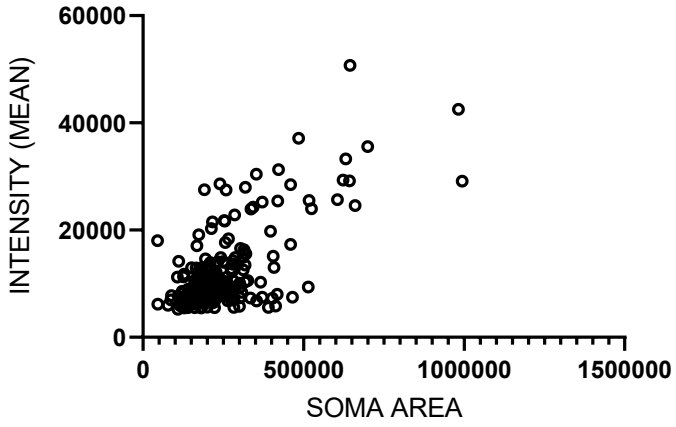


Figure 6. Detailed presentation of fluorescence intensity against soma area. Each open circle plotted in the diagram corresponds to a neuron, its intensity being represented on ordinate and its soma area on the horizontal axis.

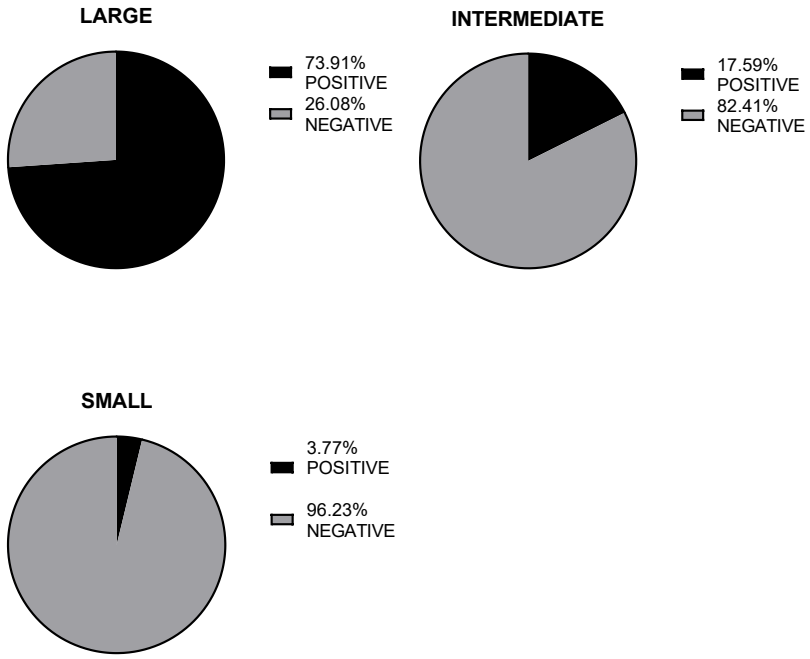


Figure 7. 74% of A53T +/+ DRGs with a large soma size are considered positive, 18% of A53T +/+ DRGs with an intermediate soma size are considered positive, while only 4% of A53T +/+ DRGs with a small soma are considered positive (N=1, 237 neurons).

Evaluation of Axonal Degeneration Index

Since DRGs develop long axons and complex neural networks, they are more susceptible to axonal degeneration. Axons of both wild type and A53T +/+ DRGs showed a degree of degeneration, however, A53T +/+ axons had numerous areas of neuritic pathology, such as breaks, grain-like inclusions, swellings and knotted spheroids (N=3, 45 fields total) (Figure 8). For the assessment of axonal degeneration, the number of TUJ1 positive areas of blebbed, fragmented and dystrophic axons was counted manually on randomly selected fields from immunostained cultures (N=3). The ratio between the number of spots and the total TUJ1 positive staining area was defined as axon degeneration index (Figure 9). Statistical analysis was performed using unpaired t test on GraphPad Prism for three experimental repeats (experiment 1, t=5,311, df=27, **** p <,0001; experiment 2, t=3,417, df=24, ** p = ,0023; experiment 3, t=4,283, df=34, *** p =,0001) (Figure 9).

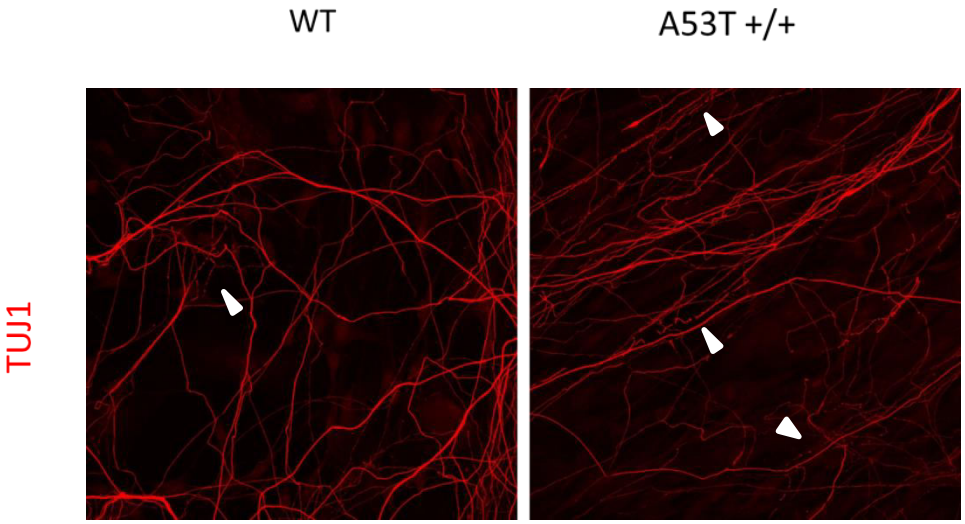


Figure 8. Axons of A53T+/+ DRG neurons are susceptible to degeneration. Axons of both wild type and A53T +/+ DRGs show a degree of degeneration, however, A53T +/+ axons have numerous areas of neuritic pathology, such as breaks, grain-like inclusions, swellings and knotted spheroids (N=3, 45 fields total).

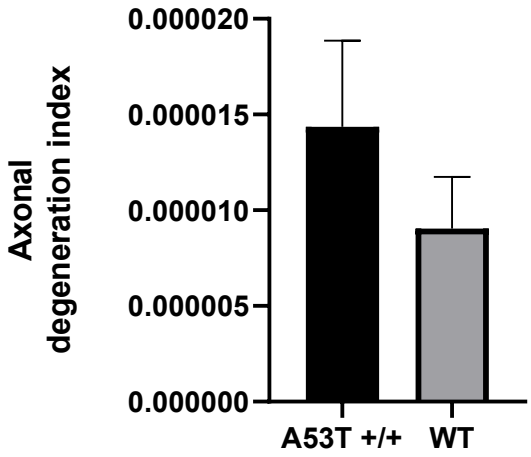


Figure 9. Axon degeneration index, defined as the ratio between the number of spots and the total TUJ1+ staining. Statistical analysis was performed using unpaired t-test for three experimental repeats (experiment shown in graph, t=4,283, df=34, *** p =,0001).

Stress-induced cytotoxicity in DRG cultures

In order to assess DRG susceptibility to stressors, neuronal degeneration and cell death was accelerated by treatment with the proteasome inhibitor epoxomicin (1μM) that interferes with α-synuclein clearance via the proteasome, or by treatment with imipramine (10μM) that inhibits exocytosis, or by glutamate (100μM) that acts as an excitotoxin. Cell death was induced by epoxomicin as assessed by lactate dehydrogenase (LDH) release (Figure 10). Quantification of LDH release also revealed that glutamate- and imipramine- treated A53T +/+ and wild type DRGs were not particularly susceptible to death compared to untreated A53T +/+ and wild type DRGs. Epoxomicin (1 μM) treatment evoked a significant increase in cleaved caspase-3 immunoreactivity and disruption of the MAP-2 positive network (Figure 11).

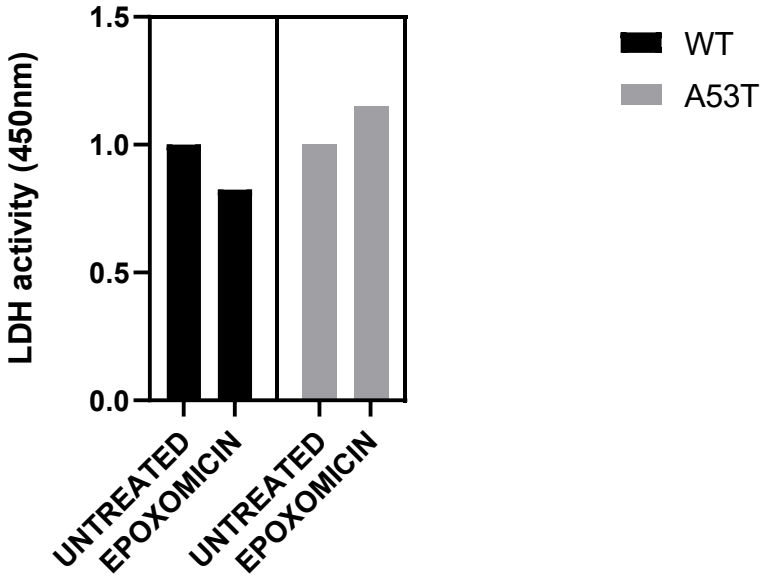


Figure 10. Quantification of LDH activity (450 nm) in culture supernatant, as a measure of cytotoxicity, in wild type and A53T+/+ derived DRGs after treatment with epoxomicin for 24 hours (N=1).

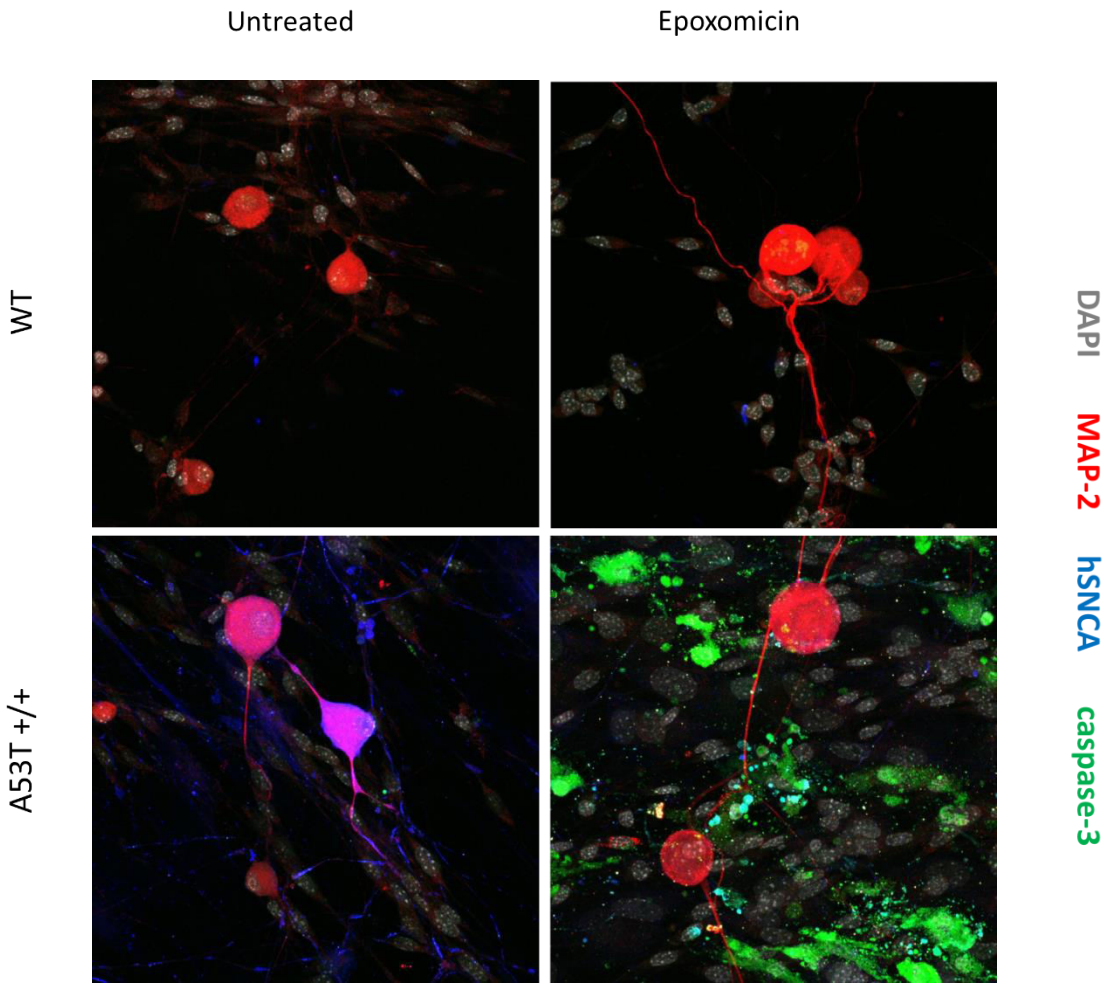


Figure 11. Epoxomicin treatment evoked a significant increase in cleaved caspase-3 immunoreactivity and disruption of the MAP-2 network (N=2).

4.2 Expression of $Na_V1.8$ *in vitro*

$Na_V1.8$ is a major C-fiber related channel isoform that affects the firing pattern of nociceptors. Adaptive changes in ion channel expression provide a response to changes in the neuronal environment and this is considered as a mechanism of neural plasticity. Based on A53T +/+ DRG's susceptibility to axonal degeneration, $Na_V1.8$ protein's density on DRG axons was assessed (Figure 12). Hippocampal neurons were used as negative controls of anti-NaV1.8 immunostaining (Figure 13). The number of $Na_V1.8$ puncta on TUJ1 positive axons was counted manually on randomly selected fields from immunostained cultures and the ratio between the number of puncta and the total TUJ1 positive staining area was defined as $Na_V1.8$ density (N=5, 76 fields total) (Figure 14,15). Statistical analysis was performed using GraphPad Prism unpaired t test for five experimental repeats (experiment 1, t=5,505, df=47 **** p <0,0001; experiment 2, t=2,372, df=24 * p = ,0260 ; experiment 3, t=4,761, df=14 *** p = ,0003; experiment 4, t=1,513, df=26 p = ,1425 ; experiment 5, t=3,924, df=28 ***p = ,0005) (Figure 9).

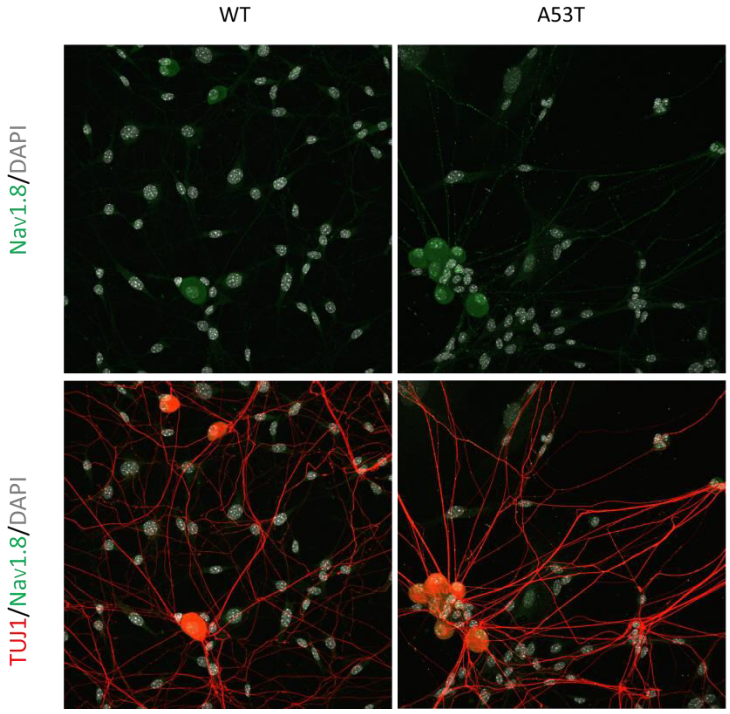


Figure 12. NaV1.8 is expressed in somata and axons of both wild type and A53T +/+ derived DRGs (N=5, 75 fields).

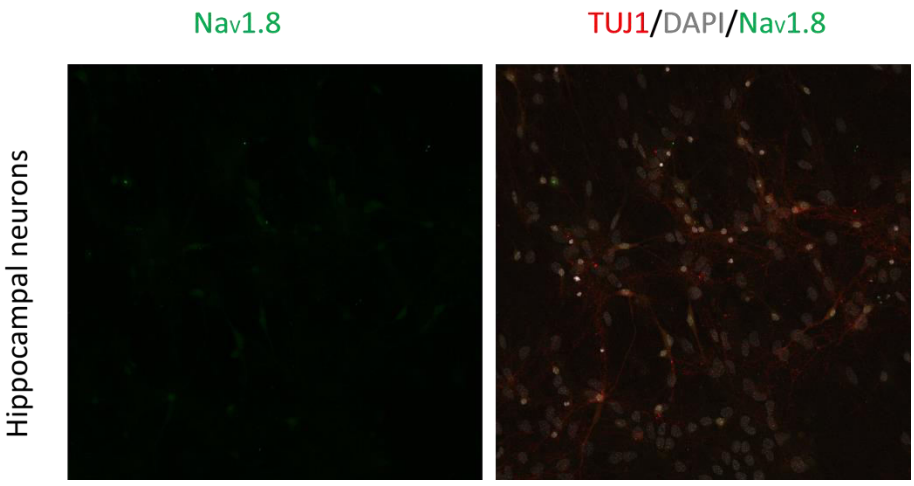


Figure 13. $Na_V1.8$ expression is absent in hippocampal neurons.

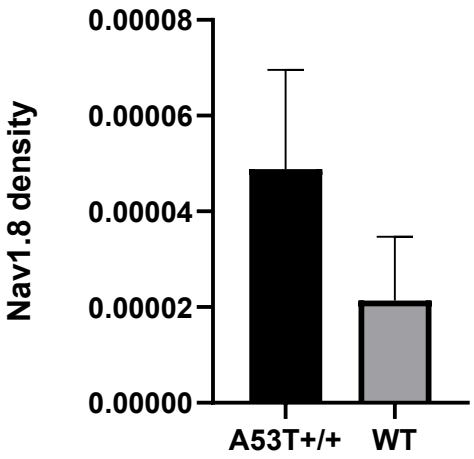


Figure 14. Quantification of Nav1.8 density on A53T+/+ and wild type DRG axons (puncta per axon area). Statistical analysis was performed using unpaired t-test for five experimental repeats (experiment shown in graph, t=5,505, df=47 **** p< ,0001).

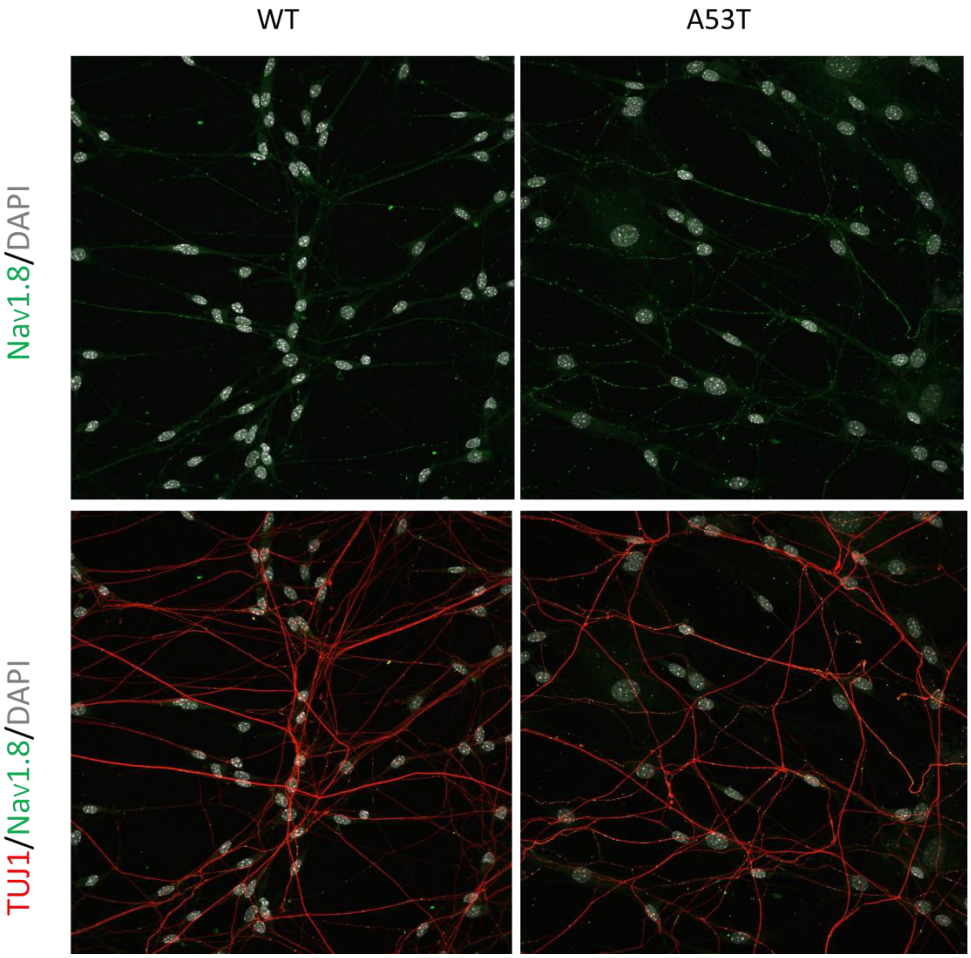


Figure 15. $Na_V1.8$ puncta are more prominent in axons of A53T +/+ derived DRGs compared to wild type.

4.3 Calcium activity of DRG neurons

During electrical activity, intracellular concentration of calcium (50–100 nM) can rise transiently to levels 10 to 100 times higher and at any given moment, the cytosolic calcium concentration is determined by the balance between calcium influx and efflux and by the exchange of calcium with internal stores or mitochondria (Berridge, Lipp and Bootman 2000). A-synuclein is said to modulate ER-mitochondria interface and mitochondrial Ca^{2+} transients, suggesting that, at mild levels of expression, α-synuclein sustains cell metabolism. It has been recently shown that overexpression of A53T α-synuclein enhances mitochondrial Ca^{2+} transients in HeLa cells (Cali et al. 2019). Other findings suggest that in some neuronal populations, the reduction of basal mitochondrial oxidative stress predicts reduced activity-dependent calcium levels, suggesting a homeostatic remodeling capacity (Lasser-Katz et al. 2017). Specifically, in contrast to dopamine neurons in the substantia nigra, cholinergic vagal motoneurons did not enhance their excitability and oxidative load in response to chronic A53T α-synuclein overexpression. This reduction in oxidative stress resulted from a transcriptional downregulation of voltage-activated calcium channels, which led to a reduction in activity-dependent calcium influx.

Even tthough $Na_V1.8$s are the main determinants of action potential activity, recording of somatic calcium signals is often used to monitor action potential activity *in vitro*. Thus, in the current study, calcium imaging was performed, in order to assess electric activity of wild type and A53T DRG neurons (Figure 16). DRGs were incubated with Fluo-4 acetoxymethyl Ca^{2+} dye, which is often used to measure calcium concentrations inside living cells, and they were excited at 488 nm with a fluorescein isothiocyanate (FITC) filter. The Ca^{2+} images were recorded before, during, and after a chemical stimulation by high KCl concentration (80 mM). KCl is known to reliably depolarize neurons causing Ca^{2+} influx into functional neurons (Estacion et al. 2015). Fluorescence was recorded at 10 frames per second (40ms exposure time) and from each field, cells were selected for analysis of Ca^{2+} responses using ImageJ software. The amplitude of fluorescence signal for each region of interest (ROI) was presented as relative change in fluorescence ($\Delta F/F_0$) after background subtraction (F_0 stands for basal fluorescence and $\Delta F = F - F_0$) (Figure 17).

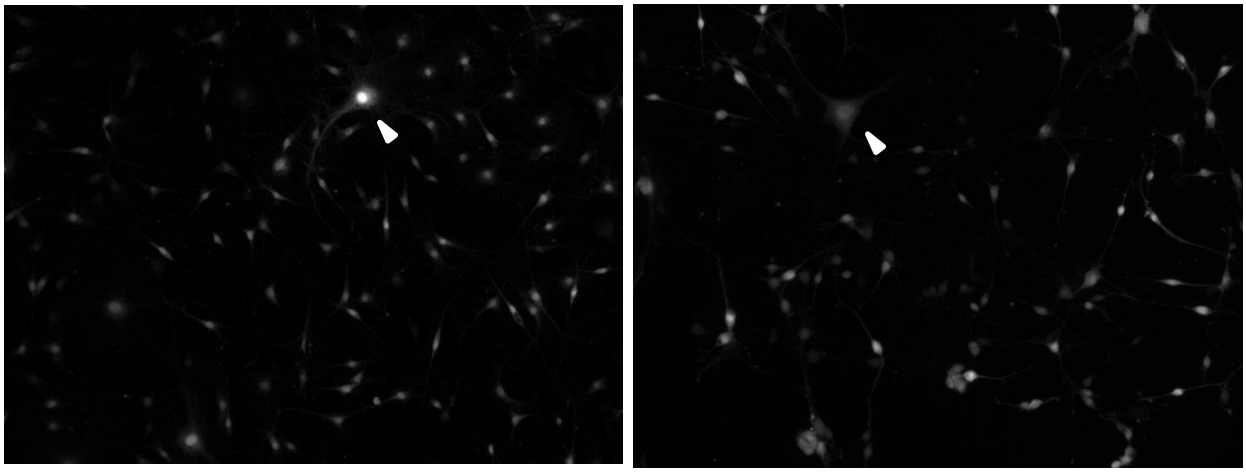
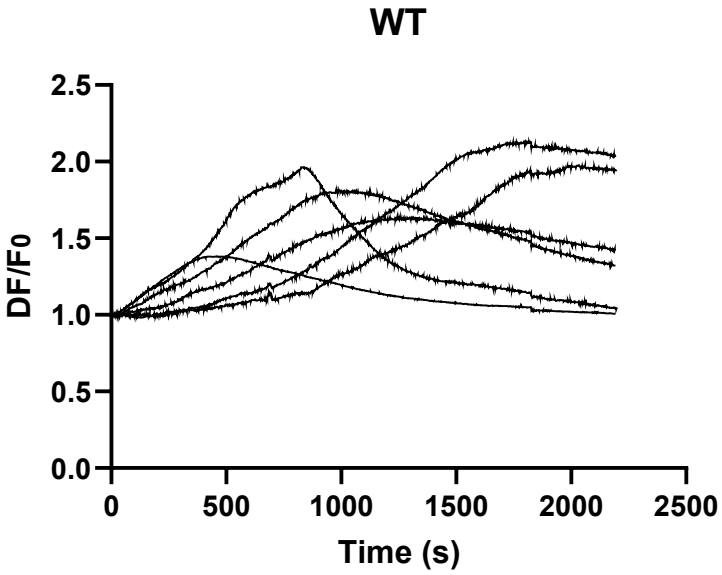
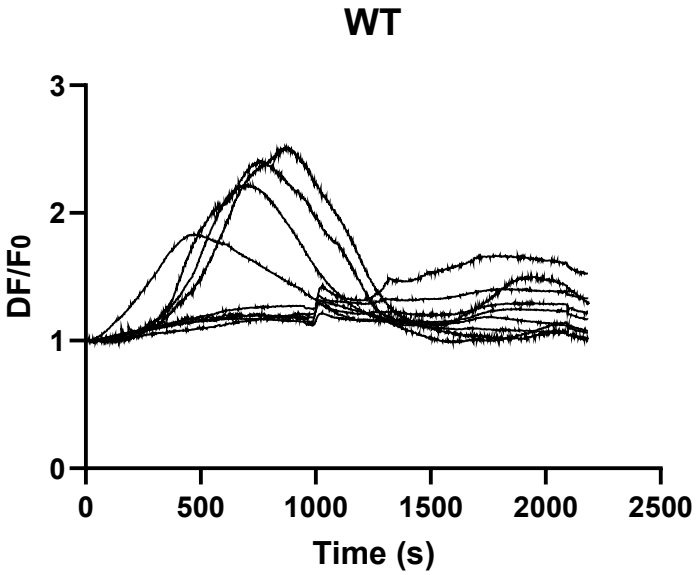


Figure 16. DRGs stained with Fluo-4 acetoxymethyl Ca^{2+} dye, which is used to measure calcium concentrations inside living cells. White arrows indicate wild type (left) and A53T (right) large DRG neurons at 13 days of culture.


WT
DF/F0
2.5
2.0
1.5
1.0
0.5
0.0
0
500
1000
1500
2000
2500
Time (s)


WT
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3
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Time (s)

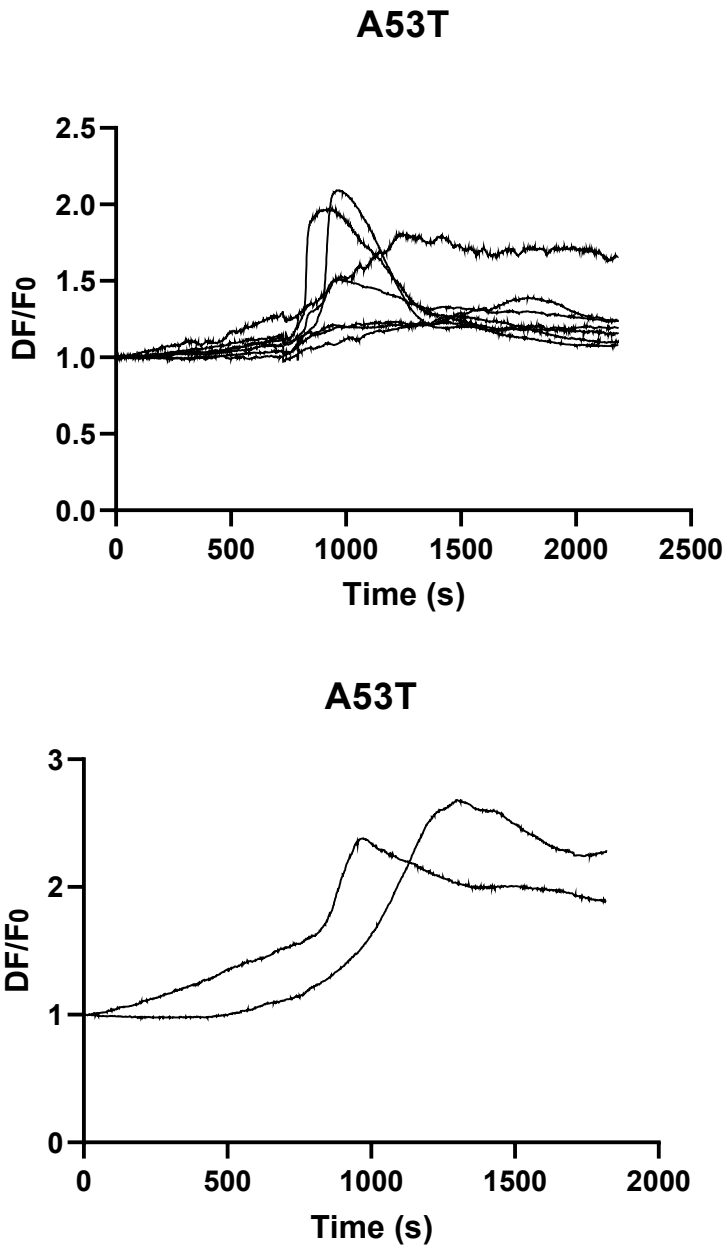

Figure 17. Calcium transients of wild type and A53T derived DRGs evoked by KCl (N=2).

4.4 Expression of $Na_V1.8$ *in vivo*

Behavioral characterization of A53T mice has revealed gross motor alterations both in forelimbs and hindlimbs (Paumier et al. 2013, Gu et al. 2010). Some studies reveal that hindlimbs are most often the first affected (Cabin et al. 2005) or more severely affected (Giasson et al. 2002). +. For the time being, skin biopsy studies in PD patients have revealed α-synuclein deposits in cutaneous sensory and autonomic nerves, loss of epidermal nerve fibers, swollen axons and increase in tactile and thermal thresholds (Nolano et al. 2008, Nolano et al. 2017, Podgorny et al. 2016, Donadio et al. 2017, Wang et al. 2013, Lin et al. 2016, Donadio et al. 2014). A-synuclein deposition in sensory neurons and integrity of sensory epidermal fibers of A53T mice have not yet been investigated. However, α-synuclein ovoids have been detected in the sciatic nerve of transgenic p.A53T mice by Giasson et al. (Giasson et al. 2002), while affected sciatic nerve axons were also observed in A53T / mouse α-synuclein KO and A53T mice (Cabin et al. 2005). In particular, heavy SNCA staining was observed, as well as loss of axonal material and increased immunoreactivity (Cabin et al. 2005). Electron microscopy images showed that the sciatic nerve of A53T / mouse α-synuclein KO mice were subjected to severe degeneration, while sciatic nerves of wild type mice showed healthy myelinated axons and Schwann cells. Considering that expression of p.SNCA could affect peripheral neural integrity, sciatic nerve and footpad tissue was obtained from wild type and A53T +/+ mice.

$Na_V1.8$ expression in the sciatic nerve and foot pad tissue

In order to assess NaV1.8 expression in vivo, tissues were stained with NaV1.8 and TUJ1 antibodies. In the sciatic nerve tissue, the axonal integrity was investigated, as well as, the level of NaV1.8 expression. Both conditions exhibited TUJ1 positive axon bundles, however A53T +/+ axons were less defined compared to wild type axons (Figure 18, 20). In both conditions, expression of NaV1.8 was present, however, A53T +/+ axons exhibited lower immunofluorescence intensity (Figure 19, 20, 21).

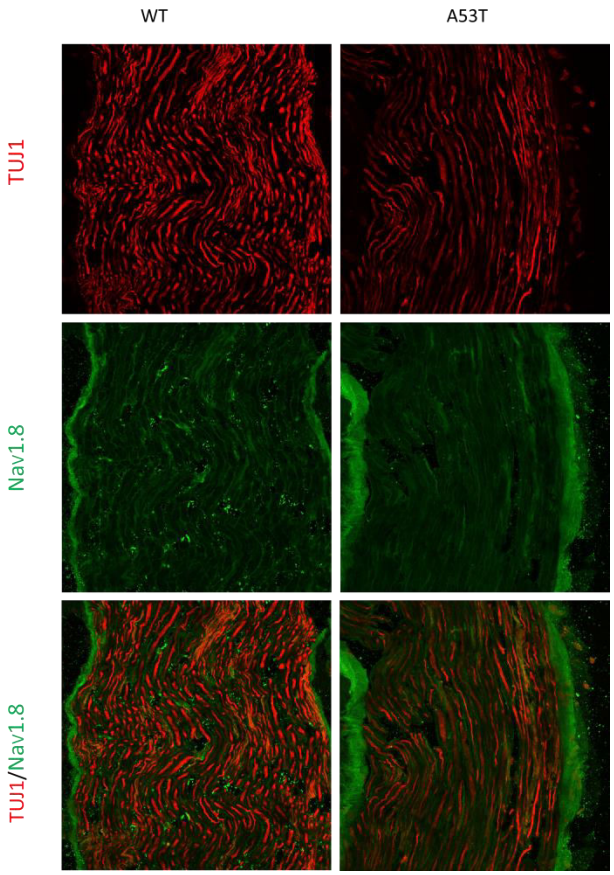

Figure 18. Sciatic nerve axonal bundles of wild type (left) and A53T +/+ (right) mice (N=4).

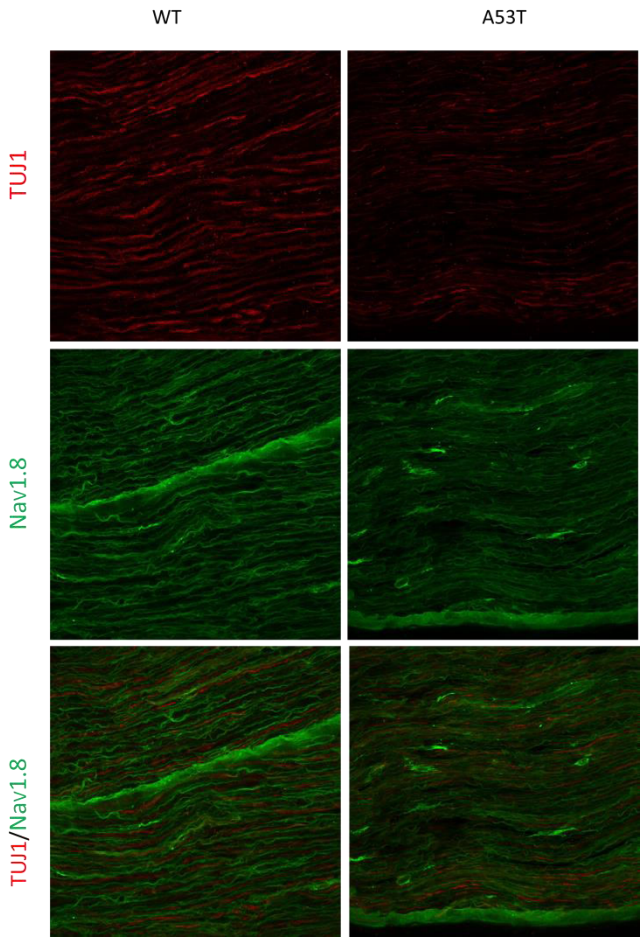

Figure 19. Sciatic nerve axonal bundles of wild type (left) and A53T +/+ (right) mice (N=4).

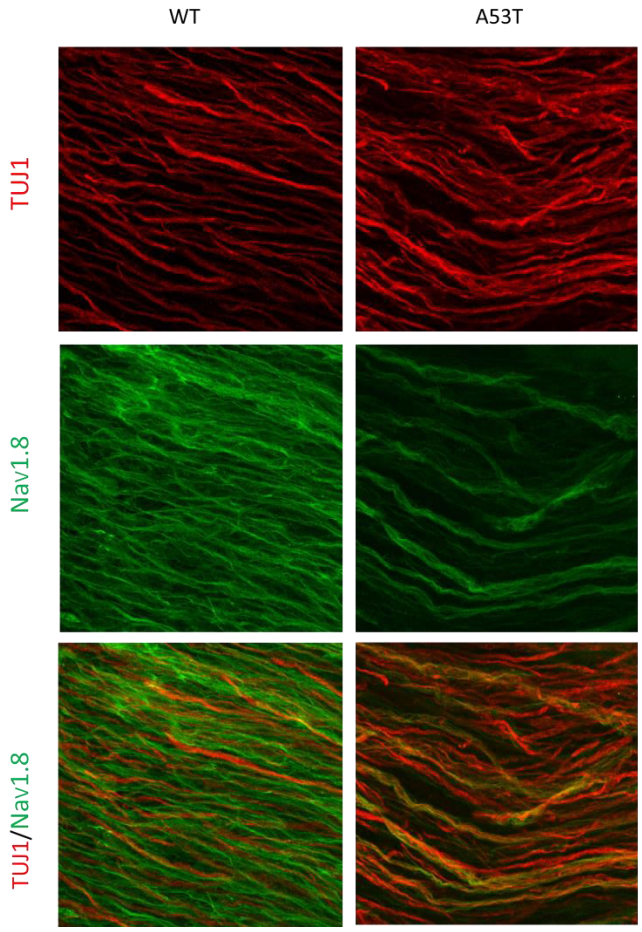

Figure 20. Sciatic nerve axonal bundles of wild type (left) and A53T +/+ (right) mice in larger magnification (N=4).

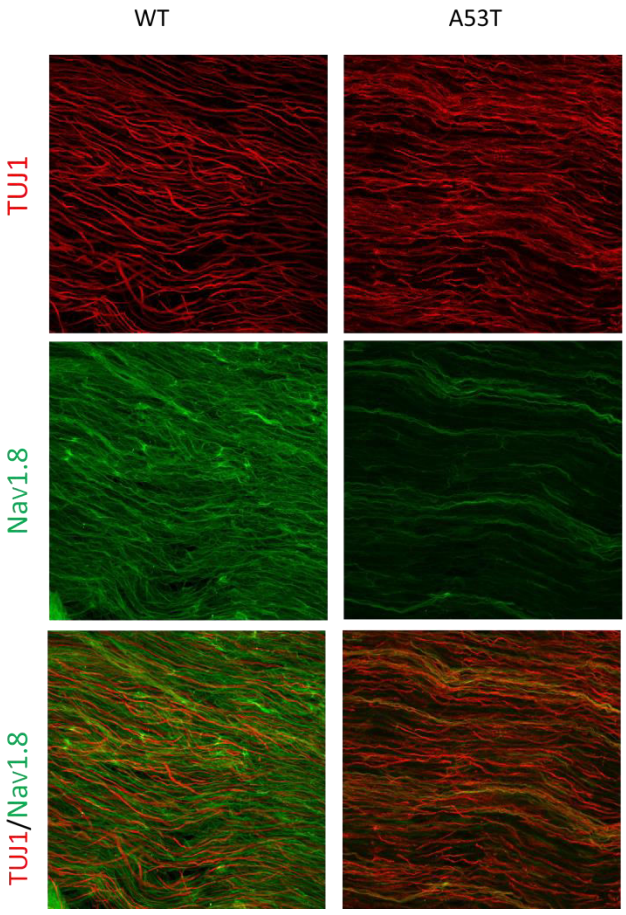

Figure 21. Sciatic nerve axonal bundles of wild type (left) and A53T +/+ (right) mice (N=4).

In the footpad tissue, the integrity of fibers that reach the epidermis was investigated, as well as, their number. Small fibers were present in both wild type and A53T +/+ tissues and no obvious differences were observed among the two conditions (Figure 22). A previous study on cutaneous innervation of PD patients has shown increased branching and sprouting of nerves, as well as enlargement of the vascular bed, suggesting attempts that counteract degenerative processes, however this was not observed in the current study (Nolano et al. 2008, Nolano et al. 2017). Quantification of fibers that reach the epidermis revealed no significant difference between wild type and A53T condition (t=0,8133, df=13 p = ,4307) (N=1, 16 fields) (Figure 23). Statistical analysis was performed using an unpaired t test in GraphPad Prism.

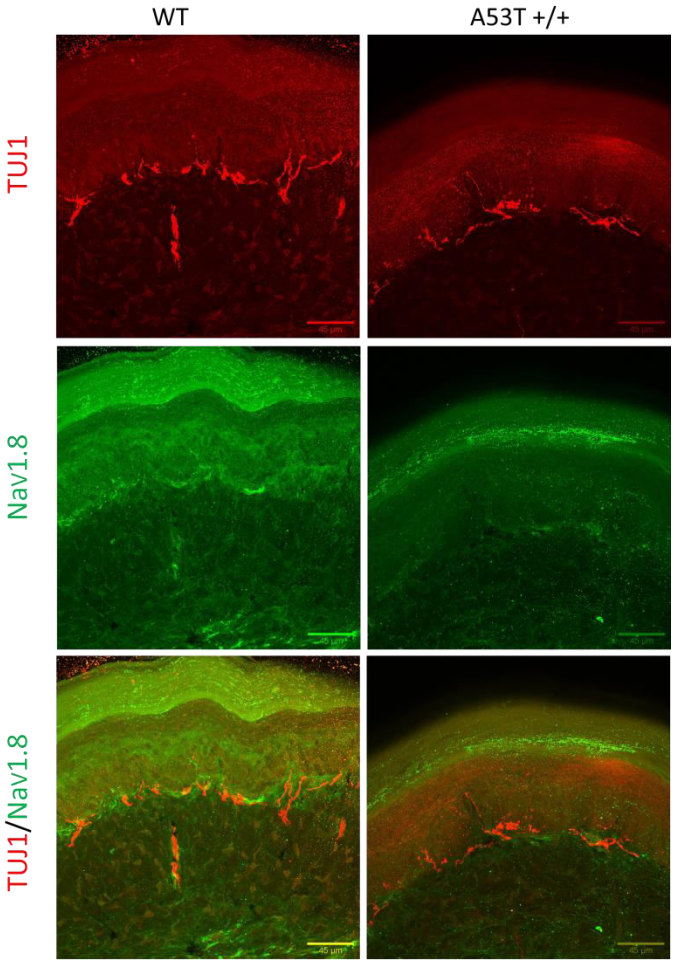

Figure 22. Footpad tissue of wild type and A53T +/+ mice (N=1, 16 fields).

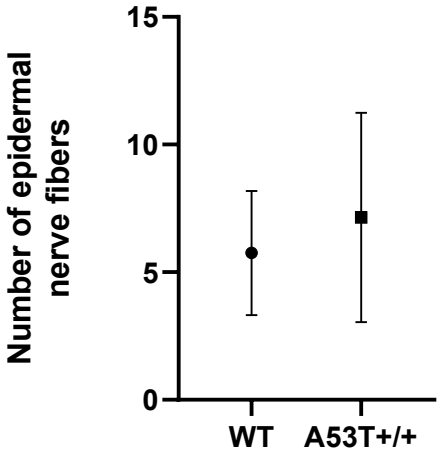

Figure 23. Quantification of fibers that reach the epidermis revealed no significant difference between wild type and A53T condition (t=0,8133, df=13 p = ,4307) (N=1, 16 fields).

The expression levels of $Na_V1.8$ in epidermal neuronal fibers of wild type and A53T+/+ hind footpads was could not be evaluated since co-localization assessment of $Na_V1.8$ and TUJ1 revealed similar but negative values of Pearson's coefficient in both WT and A53T (Figure 24). Pearson's coefficient, as well as Mander's coefficient revealed no colocalization between Nav1.8 and TUJ1 [WT (Pearson's R value (below threshold): 0.71, Pearson's R value (above threshold): -0.69, M1(Above autothreshold of Ch2): 0.025, M2(Above autothreshold of Ch1): 0.019) and A53T (Pearson's R value (below threshold): 0.67, Pearson's R value (above threshold): -0.70, M1 (Above autothreshold of Ch2): 0.054 M2(Above autothreshold of Ch1): 0.022)].

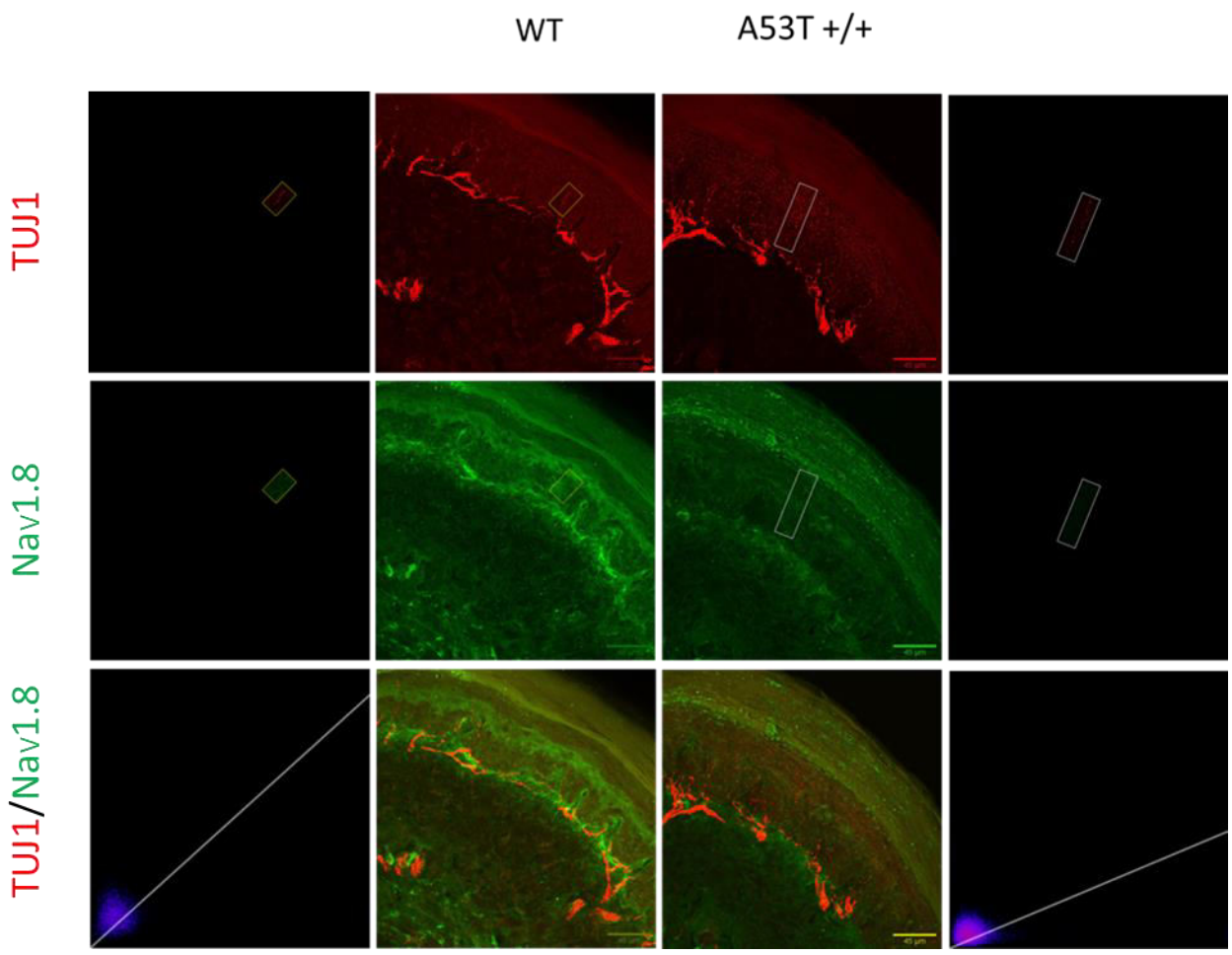

Figure 24. Expression of $Na_V1.8$ in epidermal neuronal fibers of wild type and A53T+/+ hind footpads and evaluation of co-localization of $Na_V1.8$ and TUJ1 expression. 2D intensity histograms depicted at the bottom left and right image.

4.5 Behavioral Testing - Cold Allodynia

$Na_V1.8$-expressing populations of DRG neurons are essential for noxious cold sensing. In particular, $Na_V1.8$ exhibits a relative insensitivity to cold-induced channel inactivation and the ability to propagate action potentials at temperatures as low as 10 °C (Zimmermann et al. 2007). In order to measure the aversive behavior caused by the cold sensation, a cold allodynia assay was performed. Mice paw movement was recorded and paw response was measured within 1 minute after the placement of mice on the cold or neutral (control) surface (Figure 25). Paw lift was considered as a positive response (Figure 26). Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparisons test on GraphPad Prism (WT control vs. WT cold, *p = .0474; A53T control vs. A53T cold, p = .3921; WT cold vs. A53T cold, p = .6814; WT control vs. A53T cold, p= .3921; WT cold vs. A53T control, *p= .0474; WT control vs. A53T control, p > .9999).

Figure 25. Set-up used in order to assess cold allodynia in wild type and A53T+/+ male mice. Three homozygous A53T male mice, three heterozygous male mice and six wild type male littermates of 9-12 months of age were handled and habituated in a 14 x 9 x 6 cm conductive metallic surface, surrounded by a 20 cm tall transparent film which prevented mice from escaping (right). On the day of testing, dry ice was used to cool the metallic surface to 15^{0}C. Dry ice sticks were placed inside the box holes for efficient cooling (left).

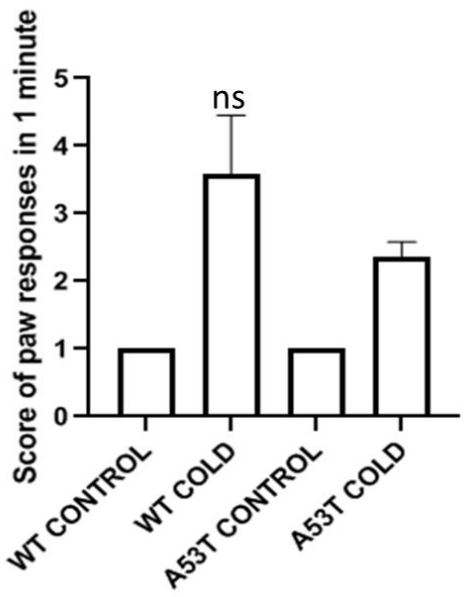

Figure 26. Score of paw responses to cold stimuli in order to assess cold allodynia in mice. Mice paw movement was recorded and paw response was measured within 1 minute after placement of mice on the cold surface or neutral (control). Paw lift was considered as a positive response (N=15). Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparisons test (WT control vs. WT cold, *p = .0474; A53T control vs. A53T cold, p = .3921; WT cold vs. A53T cold, p = .6814; WT control vs. A53T cold, p= .3921; WT cold vs. A53T control, *p= .0474; WT control vs. A53T control, p > .9999).

5 Discussion

The current study focused on the evaluation of the effect of p.A53T-αSynuclein expression on dorsal root ganglion (DRG) neurons and on tissues of the peripheral nervous system.

The A53T mutation of α-synuclein gene has been linked to autosomal dominant, early-onset Parkinson's disease (PD), documented in families of Italian, Greek and Korean descent. A-synuclein in its mutated A53T form is characterized as pathological, since it has higher capacity to fibrillize and form pathological aggregates, closely resembling those found in typical PD conditions. A-synuclein modulates vesicular trafficking during neurotransmission, however, α-synuclein aggregates represent a toxic state that causes neuronal dysfunction and death. The transgenic A53T mouse model has been valuable in studying the mechanisms of α-synuclein aggregation *in vivo* and *in vitro*. This mouse model has been used in the current study in order to assess the effect of α-synuclein's pathological state on DRG neurons, a question that has not been addressed so far. Human p.A53T α-synuclein is abundanlty expressed in A53T +/+ derived DRG neurons, in a typical punctate pattern, found on axons, somata and in the nucleus. Its expression was almost absent in wild type derived DRGs. Human p.A53T expression had a preferential expression in large DRG somata rather than small.

It has been previously shown that in the brain of PD patients carrying the A53T missense point mutation dystrophic neurites are present (Kotzbauer et al. 2004, Duda et al. 2002). This has been recently supported via an iPSC-based model of p.A53T PD where PD neurons exhibited distinct morphological features of extensive neuritic pathology and degeneration (Kouroupi et al. 2017). In particular, PD neuronal processes appeared to have swellings and fragmented areas. In the current study, this has been also observed, since axons of primary DRG neurons derived from the transgenic A53T +/+ mouse model were susceptible to degeneration. In particular, A53T +/+ axons had numerous areas of neuritic pathology, such as breaks, grain-like inclusions, swellings and knotted spheroids. These findings suggest that axonopathy is present in A53T +/+ DRG culture. Signs of degeneration have been also observed in the sciatic nerve tissue of A53T +/+ mice. In particular, axon bundles were less defined than wild type axons, which could reflect an aspect of PD pathology in the periphery.

Even though axonopathy in the peripheral nervous system of PD patients or *in vitro* and *in vivo* PD models has been slightly assessed, evidence from post mortem and imaging studies in the human brain indicates that axon degeneration both in the CNS and PNS is an early feature of PD. Kordower et al., have evaluated the integrity of the nigrostriatal dopamine system in PD patients and showed that fibers in the striatum are only moderately affected at 1 year, but they are absent by 5 years (Kordower et al. 2013). In an earlier study, reduction of axonal terminal structures in the putamen has been reported, indicating an early involvement of axons (Chu et al. 2012). Also, there is evidence from both PD patient studies and genetic animal models that axons are the first site of degeneration, however the primary dysregulation could be firstly induced in the nuclear level and result in transcriptional, translational and trafficking abnormalities that first manifest as axonal dysfunction.

In either case, reports of axonal degeneration in PD cases are essential, since transport maintains neuronal viability. Anterograde axonal transport keeps axons and nerve terminals supplied with proteins, membrane bound-organelles, lipids and mitochondria, while retrograde axonal transport mediates the clearance of misfolded proteins and damaged mitochondria, as well as the transport of signaling molecules (Maday et al. 2014, Millecamps and Julien 2013). Pathological α-synuclein has been proposed to alter axon transport (Saha et al. 2004) and correlation of α-synuclein pathology and axonal dysfunction has been observed at the single fiber level where positive α-synuclein p.Serine 129 expression and loss of the axon transport protein kinesin were combined (Chu et al. 2012). Pathological α-synuclein imposes several toxic effects, including disruption of microtubule and membrane dynamics (Bodner et al. 2010, Bendor, Logan and Edwards 2013, Ludtmann et al. 2018), inhibition of the proteasome's activity (Zondler et al. 2017) and disruption of autophagolysosomal pathways, such as autophagy, mitophagy and chaperone-mediated autophagy (Poehler et al. 2014, Cuervo et al. 2004, Orenstein et al. 2013, Pan et al. 2008, Vives-Bauza et al. 2010, Wang et al. 2016). As it has been shown, the architecture of DRG neurons with long axons makes them especially vulnerable to axonal transport disruption. In the current study, A53T +/+ DRGs were particularly vulnerable to the proteasome inhibitor, epoxomicin. Treatment with epoxomicin evoked a significant increase in cleaved caspase-3 immunoreactivity and disruption of the neuronal network. This indicates that A53T +/+ DRGs are probably already weighted with defective clearance capacity of debris to overcome proteasome inhibition.

In the case of neurological injury or disease, two forms of axon degeneration happen (Raff, Whitmore and Finn 2002, Luo and O'Leary 2005). Anterograde (Wallerian) degeneration occurs after injury to the proximal axon, followed by degeneration of the axon distal to the site of injury. The degenerative process affects the cytoskeletal components, induces swelling of the mitochondria and causes fragmentation of the axon (Griffin, George and Chaudhry 1996). Retrograde degeneration proceeds from the distal axon terminals to the neuron cell body (Luo and O'Leary 2005) and has been identified in neurodegenerative diseases and peripheral neuropathies. In the late stages of retrograde axon degeneration, the axonal fragmentation looks similar to that observed in anterograde degeneration, but the molecular basis of the two forms is likely to be distinct. In PD, there are indications that the disease process begins in the distal axon and proceeds retrograde (Orimo et al. 2008). In the current study, degeneration has been observed along the length of A53T +/+ axons, however defective sites have not been observed proximal to the cell soma. A number of studies of *in vitro* models of injury and both *in vitro* / *in vivo* models of PD, have shown that macroautophagy could be considered as a mediator of retrograde axonal degeneration, which is shown to be suppressed by inhibition of macroautophagy (Yang et al. 2007, Cheng et al. 2011). A study has also shown that PD patients, who either carry the A53T mutation or not, show increased evidence of macroautophagy (Huang et al. 2012). Inhibition of macroautophagy as a suppressor of axonal degeneration observed in A53T +/+ DRG cultures should be assessed in future studies.

The current study also focused on the evaluation $Na_V1.8$ expression levels in p.A53T-α-Synuclein DRG neurons and in tissues of the peripheral nervous system.

85% of people with PD experience pain besides other motor and non-motor features of the disease. 43% of PD patients experience pain in the early stages of PD, when motor symptoms are not yet obvious (Giuffrida et al. 2005). Among many pain types, PD patients develop progressive sensory neuropathies that affect somatosensory and autonomic nerves (Gierthmuhlen et al. 2009, Nolano et al. 2017). The predominant phenotype of sensory neuropathy consists of loss of thermal perception, pathological heat pain and mechanical hypersensitivity (Gierthmuhlen et al. 2009), which mostly agrees with the denervation pattern observed in PD (Vollert et al. 2018, Vollert et al. 2017, Vollert et al. 2015). The progressive loss of sensory functions parallels the progression of the disease, but it may precede motor symptoms for more than 10 years, suggesting that sensory neurons are particularly vulnerable to PD pathophysiology (Pont-Sunyer et al. 2015, Silverdale et al. 2018), which could be due to axonal transport defects described above.

In skin biopsy studies of PD patients a significant loss of epidermal nerve fibers and Meissner corpuscles has been observed, as well as, increase in tactile and thermal thresholds. Increased branching, was also observed, as well as, sprouting of nerves and enlargement of the vascular bed, suggesting attempts that counteract degenerative processes (Nolano et al. 2008, Nolano et al. 2017, Podgorny et al. 2016, Donadio et al. 2017, Wang et al. 2013, Lin et al. 2016). Studies also confirmed the deposition of phosphorylated α-synuclein in cutaneous sensory nerves early on in PD (Donadio et al. 2014). The inverse correlation between the deposition of α-synuclein in peripheral nerve fibers and the extent of skin denervation and nerve function suggests that the characteristic pathology of PD may affect pain processing in the peripheral nerve terminals (Donadio et al. 2014, Wang et al. 2013). In the current study, neuronal fibers were present in the epidermis of both wild type and A53T +/+ footpad tissues, however, no obvious differences in neuronal density were observed among the two conditions. Also, no particular branching or sprouting of nerves and enlargement of the vascular bed has been identified. The behavioural test, performed in order to measure the aversive behavior caused by the cold sensation, did not show differences in cold allodynia between wild type and A53T +/+ mice. Further behavioral evaluation of sensory processing induced by cold or by other stimuli in A53T mice with signs of advanced phenotype would be essential.

Pathological pain is characterized by nerve injury-triggered neuropathic and inflammatory pain states, which induce dynamic plastic changes. These changes consist of peripheral sensitisation, which involves peripheral nociceptive neurons, and central sensitization, which involves dorsal horn and higher order central neurons. Peripheral sensitisation is essential for neuropathic pain state signaling and it is mostly characterized by changes in the voltage gated sodium channel (Na_Vs) dynamics (Hameed 2019). Na_Vs mediate peripheral sensitization due to their capacity to undergo changes with regards to expression levels and ion conductance of sodium, which leads to either raised or lowered value of threshold. Threshold potential is a determinant of the membrane action potential, thus factors that change threshold, also affect the firing pattern of neurons. It has

been shown that after nerve injury, affected neurons undergo membrane remodeling, which includes shifts in the pain threshold and subsequent hyperexcitability (Hameed 2019). Within the population of sensory neurons with small diameter cell bodies that merely represent the nociceptor population, $Na_V1.8$ and $Na_V1.9$ channels are the most functionally dominant Na_V subtypes (Akopian, Sivilotti and Wood 1996). $Na_V1.8$, in particular, generates a slowly-inactivating sodium current in small diameter DRG neurons, which is resistant to TTX (Akopian et al. 1999) and is a major C-fiber related channel isoform that affects the firing pattern of nociceptors. Following peripheral nerve transection in animal models of neuropathy, $Na_V1.8$ channel expression in sensory neurons has been found altered. In human patients with chronic neuropathic pain and in animal models of inflammatory pain, $Na_V1.8$ expression is upregulated, however in axotomized DRG neurons $Na_V1.8$ is downregulated (Akopian et al. 1996, Gold et al. 2003, Kort et al. 2010). In general, it is considered that $Na_V1.8$ is upregulated in intact nociceptors leading to an enhancement in the excitability of the uninjured afferent fibres, but overtime it is also upregulated in injured nociceptors. This time-dependent change in $Na_V1.8$ may involve some form of cross-talk between injured and non-injured neurons, which could be mediated by products of the Wallerian degeneration (Wu et al. 2001).

In the current study, $Na_V1.8$ was expressed in somata and axons of both wild type and A53T +/+ derived DRGs, however, in A53T +/+ axons higher expression levels were observed. This was not true for sciatic nerve tissue, since levels of $Na_V1.8$ expression seemed lower in A53T +/+ condition compared to wild type. Based on *in vitro* findings, $Na_V1.8$'s elevated expression in A53T +/+ DRGs could indicate an aberrant neuronal activity of A53T +/+ DRGs. Even though $Na_V1.8$s are determinants of action potential activity, in order to assess electrical activity of A53T +/+ DRG neurons, calcium imaging was performed. KCl evoked calcium transients in A53T+/+ and wild type DRGs. Neurotransmitter glutamate or capsaicin ,which is the activator of Transient Receptor Potential cation channel subfamily V member 1 (TRPV1), should substitute KCl in future experiments, since high concentration of KCl changes cell osmolarity and inactivation capacity of Na_V channels. Future evaluation of the electrophysiological properties of $Na_V1.8$ channel in A53T and wild type DRGs would be essential.

It has been previously shown that increased levels of nerve growth factors induce hyperalgesia (McMahon 1996). In particular, TNF-α upregulates $Na_V1.8$ in sensory neurons via the accessory protein p11, which binds to the 28-amino-acid fragment at the N terminus of $Na_V1.8$ and promotes its translocation to the plasma membrane (Okuse et al. 2002). Also, p11-null nociceptive sensory neurons have a decrease in $Na_V1.8$'s expression at the plasma membrane (Foulkes et al. 2006). This contributes to the hyperexcitability of sensory neurons during pain. Therefore, the contribution of accessory proteins could be a potential mechanism by which $Na_V1.8$ expression is upregulated or downregulated. These changes could be also due to transcriptional regulation of Na_Vs. It has been recently shown in a bone cancer pain study, that $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ were found to be selectively upregulated in rat DRG neurons treated with Granulocyte-macrophage colony-stimulating factor (GM-CSF), which resulted in enhanced excitability (Zhang et al. 2019). GM-CSF activated the Janus kinase 2 (Jak2)–signal transducer and activator of transcription protein 3 (Stat3) signaling pathway, which promoted the transcription of Na_Vs. Knockdown of either Nav1.7–1.9 or Jak2/Stat3 in DRG neurons *in vivo* alleviated the hyperalgesia in rats.

Future perspectives

In order to investigate a possible correlation between A53T α-synuclein pathology and changes in electrophysiological properties, current clamp recording should be performed. Also, current density alterations should be determined, as well as, changes in spike frequency and membrane depolarization. The use of inhibitors could provide information over the detailed kinetics of $Na_V1.8$. These experiments will indicate any functional changes in $Na_V1.8$ when A53T-α-synuclein is expressed in DRG neurons. Also, in order to study sensory processing in A53T mice, further evaluation of behavioral responses induced by cold or other stimuli should be performed. In that case, behavioral methods will be used to measure pain-like behaviors measuring withdrawal from a nociceptive stimulus, such as heat, cold and mechanical stimuli. Behavior could be evaluated on mice that are positive or negative for signs of motor impairment in order to evaluate whether changes in sensory perception in A53T mice precede motor symptoms or whether they are paired. Another interesting aspect being considered for the future is the investigation of the molecular mechanism underlying the increase in $Na_V1.8$ density on A53T DRGs. This upregulation could be mediated by transcriptional regulation or by accessory proteins that translocate the channel to the plasma membrane. An example of an accessory protein that has been reported to translocate $Na_V1.8$ to the plasma membrane is p11. To examine the effect of p11 on $Na_V1.8$ trafficking, DRGs could be transfected with a GFP–p11 fusion cDNA and co-expression of p11 and $Na_V1.8$ in the plasma membrane could be investigated with immunofluorescence. To test whether p11 binds to the N-terminal intracellular domain of the channel DRGs could be transfected with the N-terminal domain of $Na_V1.8$ as a glutathione S-transferase (GST) fusion protein. Pulled-down assays could be performed in order to assess the interaction between fusion proteins.

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