International Society for Neurochemistry
Committee for Aid and Education in Neurochemistry
(ISN-CAEN)

Final Report
Award Category 1A
August, 2016 Round

Awardee from category 1A: Stella Célio Junqueira
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Supervisor: Prof. Helena Cimarosti
Host laboratory: University of Bristol
Principal investigator of the host laboratory: Prof. Jeremy M. Henley

1. Overview and Outcomes

In October 2016 I was awarded by the Committee for Aid and Education in Neurochemistry, Category 1A (Visit by the applicant to another laboratory). The initial project proposal comprised the period from February to May 2017. However, I was contemplated with a further scholarship from the Brazilian government (CAPES), so I could extend my stay at the host laboratory until September 2017. I greatly appreciate the grant money that I used to purchase flight tickets, the visa and health insurance, while the rent and food I've paid with my Brazilian scholarship.

My visit to the laboratory of Prof. Jeremy M. Henley, at the University of Bristol, UK was a wonderful opportunity that contributed both to my professional and personal growth (Fig. 1). There I could perform experiments related to my PhD thesis and learn new technics, which I could bring to my laboratory in Brazil. There are several different and interesting projects in the field of biochemistry being carried out in the Henley group. The difficulties presented by the students during the development of their projects are shared in the weekly group meeting, where every participant is invited to collaborate with new ideas and viable solutions. During my stay there I could also attend departmental seminars of distinct groups in the university and lectures from several professors in molecular biology, providing me new professional contacts.

Part of the results obtained with this work were presented by Izi Izumi, an undergraduate that I co-supervise with Prof. Helena Cimarosti at UFSC, Brazil, at the Neurotoxicity Society & International Neurotoxicology Association (NTS-INA) meeting in Florianopolis, Brazil (May 2017). The poster was awarded as one of the five best presented at the entire conference. I am currently finalizing the data analysis and preparing a manuscript to submit to a peer-reviewed scientific journal. Prof. Henley has also invited me to write a scientific review and I have no doubts that this visit certainly strengthened my professional relationship with him.

Figure 1: Royal Fort - University of Bristol, Enaam (international student) and me in Henley's laboratory - Biomedical Sciences building.

2. Synopsis of the background and aims of the project

SUMOylation is a posttranslational modification of one or more lysine residues in target proteins by the covalent attachment of small ubiquitin-like modifier (SUMO), which acts as a biochemical switch in many pathways. There are two main SUMO paralogues, SUMO-1 and SUMO-2/3, and the key SUMOylation machinery proteins include Ubc9, the sole E2 conjugating enzyme, and the SENP family of SUMO specific isopeptidases[1]. The underlying mode of action of SUMOylation is to alter the molecular interactions of substrate proteins and thereby modulate their activity, stability, and/or subcellular localization[2,3]. To date, most attention has focused on the nuclear roles of SUMOylation in regulating gene transcription. However, the functional consequences of SUMOylation can be extremely diverse and in neurons it plays fundamentally important roles in regulating synaptic transmission, synaptic plasticity, and cell stress pathways[4]. We are particularly interested in the observations that SUMOylation can be neuroprotective against damage induced by brain ischemia[5-7] and neurodegeneration[8,9] including Parkinson's disease (PD).

Several genes associated with the hereditary forms of PD including α-synuclein, DJ-1 and parkin are subject to SUMO modification[10,11]. Furthermore, defects in SUMOylation have been strongly implicated in α-synuclein aggregation[12], and enhanced SUMOylation has been linked to a protective response in a unilateral rotenone PD model[13]. Taken together, this emerging evidence points to an important and potentially therapeutically manipulatable role for SUMOylation in the molecular mechanisms underlying PD.

Mitochondrial dysfunction has emerged as a critical contributing factor in the damage to dopaminergic neurons in sporadic PD[14,15]. Mitochondria are dynamic organelles that undergo fission and fusion that maintains normal mitochondrial function and neuronal viability[16]. In PD aberrant mitochondrial dynamics and consequent dysfunction leads to synaptic pathology[17], which occurs before neurodegeneration[18]. Thus, the processes involved in mitochondrial dysfunction provide an attractive target for therapeutic intervention. Under normal conditions the GTPase dynamin-related protein 1 (Drp1) is recruited to the mitochondrial outer membrane (MOM) to mediate fission[19]. However, Drp1 has dual roles because under stressed conditions it causes mitochondrial release of cytochrome *c* and activates caspases leading to apoptosis[20]. Consistent with this, inhibiting Drp1 function reduces neurotoxicity in cell culture[21,22] and transgenic mice models of PD[20].

Drp1 function is very tightly regulated by multiple post-translational modifications, including phosphorylation, ubiquitination, and SUMOylation[23]. SUMOylation of Drp1 by SUMO-1 enhances the mitochondrial recruitment and promotes fragmentation and apoptosis[24], whereas SUMOylation of Drp1 by SUMO-2/3 decreases mitochondrial localization and prevents cytochrome *c* release and caspase-mediated cell death[25]. Although it is well established that disruption of mitochondrial dynamics is a central feature of PD pathogenesis[26], the roles of Drp1 SUMOylation in PD remain to be defined.

SUMO conjugation may have different outcomes depending on the modified protein(s) and the functional consequences of manipulation of SUMO levels in terms of PD symptoms are still unknown. Previous findings from our lab have indicated that enhancing Drp1 SUMOylation acts as a regulatory mechanism to minimize cell damage caused by extreme metabolic stress[25].

In summary, this project aimed to study the neuronal SUMOylation in an *in vitro* model of PD with the aim of characterize the SUMOylation proteins (SUMO2/3, SUMO1 and Senp3) and target proteins, focusing on mitochondrial fission proteins (Drp1 and Mff).

3. Experimental methods

3.1 Neuronal cell culture

Embryonic cortical and hippocampal neurons were isolated from E18 Wistar embryos. Brain areas were dissected and trypsinised before being plated either PLL-coated 25 mm glass coverslips (for hippocampal cells) or PLL-coated 6-well plates (for cortical cells). Cells were initially plated in plating media (Neurobasal media + 10% horse serum, B27 supplement, 2 mM Glutamax, 1× penicillin/streptomycin), which after 24 hours was replaced with feeding media (Neurobasal media, B27 supplement, 1.2 mM Glutamax, 1× penicillin/streptomycin)[27]. For all biochemistry and imaging experiments, cortical and hippocampus neurons were used at DIV14-18. For the *in vitro* PD model, cell cultures were exposed to 50 µM 1-methyl-4-phenylpyridinium (MPP^+) diluted in PBS for 12, 24 and 48 h[22].

3.2 Western blotting

Samples were resolved by SDS-PAGE (10-12% gels), and analysed by western blotting[28]. Primary antibodies were used to detect SUMO 2/3 (1:1000, Cell Signaling), Senp3 (1:1000, Cell Signaling), Drp1 (1:1000, BD Biosciences), Mff (1:1000, Proteintech Sigma) p-AMPK (1:1000, Cell Signaling), AMPK (1:1000, Cell Signaling), β-tubulin (1:10.000 Sigma-Aldrich), GAPDH (1:10.000 Abcam), VDAC (1:5000, Cell Signaling).

3.3 Subcellular fractionation

Cells were harvested by cooling the plates on ice, suspending the adherent cells with a cell scraper in buffer A (NaCl 150 mM, Hepes 50 mM pH 7.4, digitonin, protease inhibitor, phosphatase inhibitor, NEM). Then 200 µL were collected into an Eppendorf tube for total lysate analysis and the remainder sample was collected in a glass homogenizer. Cells were homogenized with a pistil and centrifugated at 4° C and 16.200 g for 30 min. The supernatant (non-nuclear fraction) was collected in falcon tubes and added with ice-cold acetone, stored in a freezer for 1 h, centrifugated at 4°C 5000rpm for 30min, the acetone was discarded. Buffer C (NaCl 150 mM, Hepes 50mM, sodium deoxycholate, SDS 0,1%, triton 0,5%, protease inhibitor, phosphatase inhibitor, NEM) was added to the pellets non-nuclear and nuclear fractions and sonicated every 10 min

3 times. Aliquots of both subcellular fractions were collected for BCA protein assay and all samples were stored in a freezer until the day of the Western blotting.

3.4 Mitochondrial Imaging

The media from coverslips was aspirated and the cells were treated with Mito Tracker Red (final concentration of 100 nM) in pre-warmed neuronal feeding media (2 mL/dish) for 30 min prior to fixation. After that, cells were washed carefully 3 times in cold PBS, fixed with cold acetone (1 mL/dish) and placed in freezer for 5 min. Carefully, cells were washed 3 times in cold PBS then placed face down in glass slides with Dako containing DAPI avoiding air bubbles.

4. Results

SUMO conjugation may have different outcomes depending on the modified protein(s) and the functional effects of manipulation of SUMO levels in terms of PD molecular pathophysiology are still under investigation. This study started by investigating the neuronal SUMOylation in the MPP^+ *in vitro* model of PD. The main aim was to characterize the SUMOylation proteins (SUMO-2/3, SUMO-1 and Senp3) and target proteins, focusing on mitochondrial fission proteins (Drp1 and Mff) in total lysate (Fig. 2).

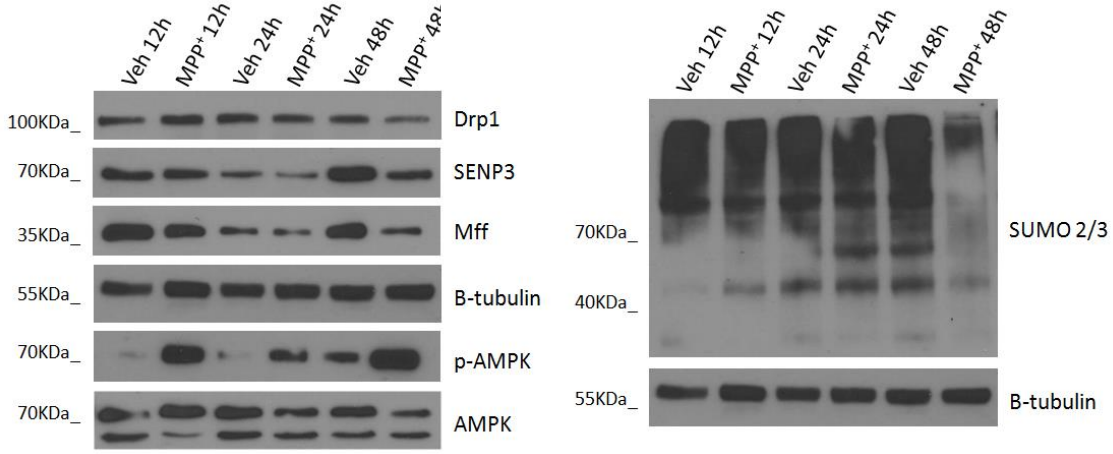

Figure 2: Representative western blotting of primary cortical culture (total lysate) exposed to MPP^+ to analyse SUMOylation (SUMO 2/3) and targets proteins (Drp1, Mff, Senp3). Cortical neurons were exposed to MPP^+ 50 µM or vehicle (H_2O sterile) for 12, 24 or 48 hours and total lysate followed by western blotting. B-tubulin was used as a loading control. MPP^+ toxicity is confirmed by increased in AMPK phosphorylation. The representative western blotting suggests that after 48h, MPP^+ reduces Senp3, Mff and SUMO2/3.

The next step was to use subcellular fractionation protocols to separate nuclear from non-nuclear compartments and investigate SUMOylation in each. Most protein SUMOylation events occur in the nucleus where, among other things, it is a key regulator of transcription factor activity and function. However, it is now clear that SUMOylation is also fundamental in regulating proteins at mitochondria and at synapses[4,11]. We expected that both nuclear and extranuclear SUMOylation will be affected in PD, but we had intended to focus predominantly on extranuclear targets because we have a successful track record in defining the triggers, mechanisms and consequences of extranuclear protein SUMOylation in other systems (*e.g.* ischemia, AD) and already have extensive tools, expertise and technical know-how[5-9] (Fig. 3 and 4).

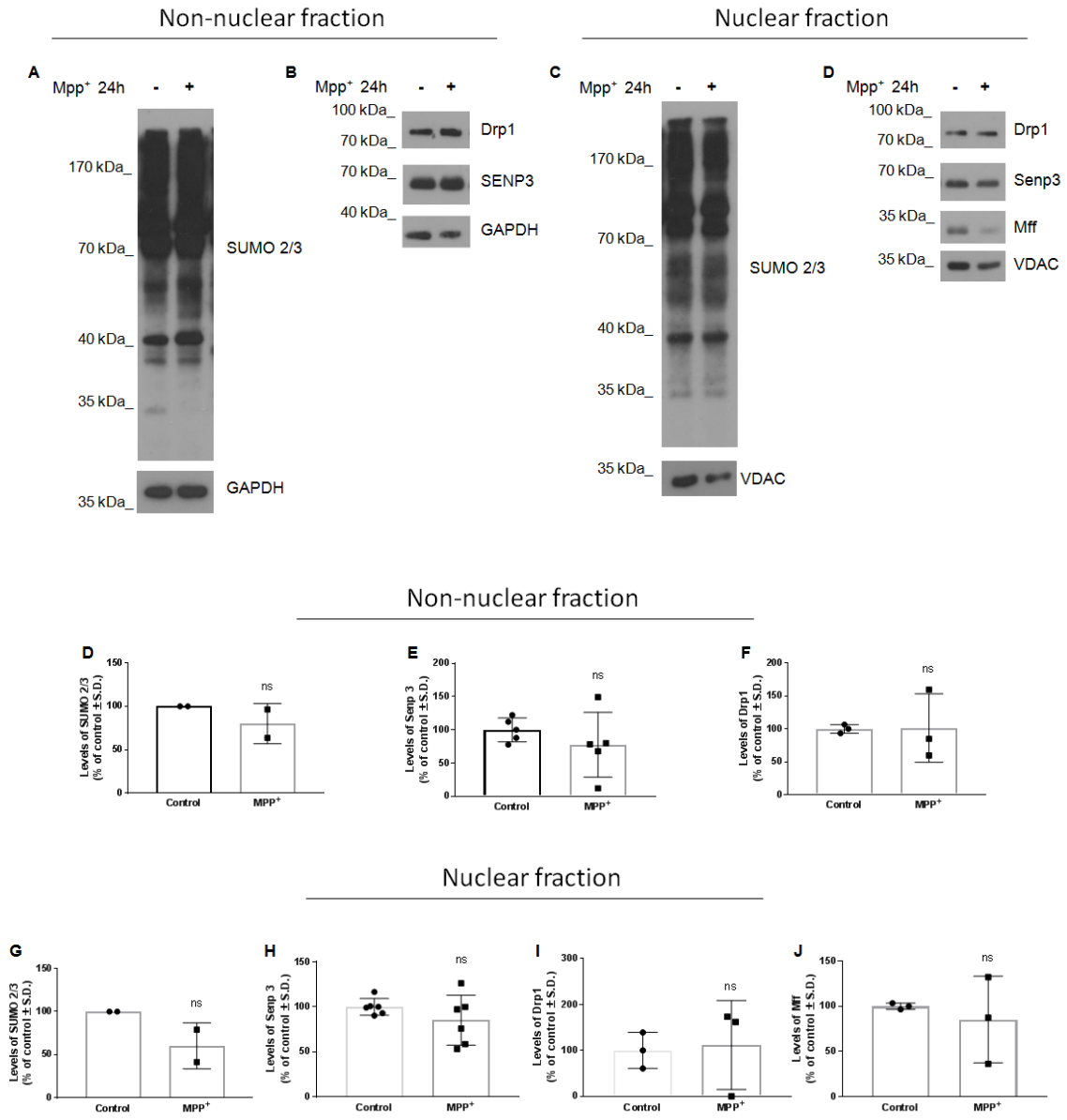

Figure 3: Representative western blotting of primary cortical culture (subcellular fractions) exposed to MPP^+ to analyse SUMOylation (SUMO 2/3) (3A and 3C) and targets proteins (Drp1, Mff, Senp3) (Fig 3 B and 3D). Cortical neurons were exposed to MPP^+ 50 µM or vehicle (H_2O sterile) for 24 hours followed by western blotting of non-nuclear and nuclear fractions. GAPDH was used as a loading control from non-nuclear fraction, VDAC was used as a loading control from nuclear fraction. The initial results suggest that MPP^+ doesn´t alter SUMO 2/3 and target proteins levels (3D-J). (n=2-6; Student's t-test).

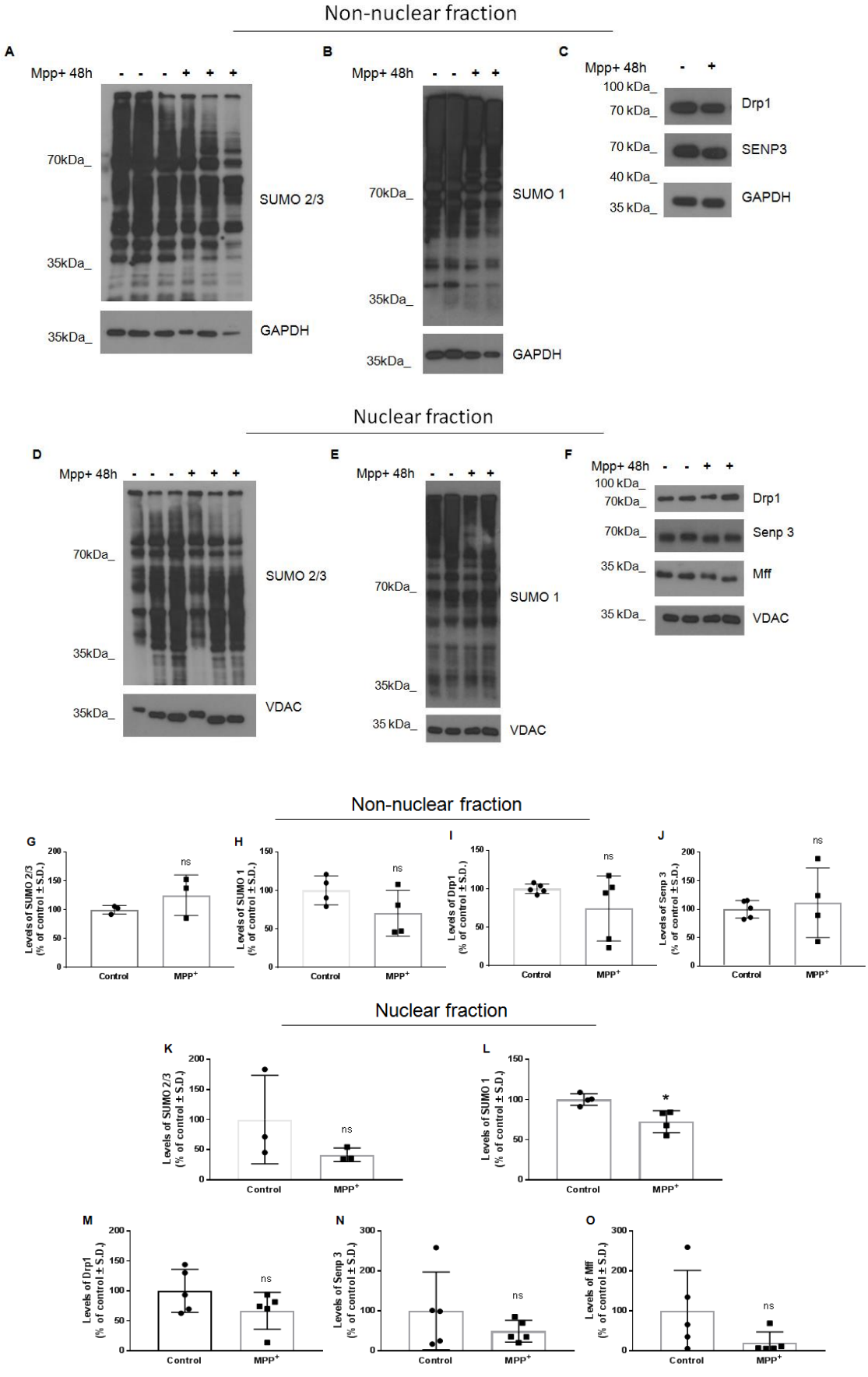
Non-nuclear fraction
A
Mpp+ 48h - - - + + +
70kDa_
35kDa_
SUMO 2/3
35kDa_
GAPDH
B
Mpp+ 48h - - + +
70kDa_
SUMO 1
35kDa_
35kDa_
GAPDH
C
Mpp+ 48h - +
100 kDa_
70 kDa_
Drp1
70 kDa_
SENP3
40 kDa_
35 kDa_
GAPDH
Nuclear fraction
D
Mpp+ 48h - - - + + +
70kDa_
SUMO 2/3
35kDa_
35kDa_
VDAC
E
Mpp+ 48h - - + +
70kDa_
SUMO 1
35kDa_
35 kDa_
VDAC
F
Mpp+ 48h - - + +
100 kDa_
70kDa_
Drp1
70kDa_
Senp 3
35 kDa_
Mff
35 kDa_
VDAC
Non-nuclear fraction
G
Levels of SUMO 2/3 (% of control ± S.D.)
ns
Control
MPP+
H
Levels of SUMO 1 (% of control ± S.D.)
ns
Control
MPP+
I
Levels of Drp1 (% of control ± S.D.)
ns
Control
MPP+
J
Levels of Senp 3 (% of control ± S.D.)
ns
Control
MPP+
Nuclear fraction
K
Levels of SUMO 2/3 (% of control ± S.D.)
ns
Control
MPP+
L
Levels of SUMO 1 (% of control ± S.D.)
*
Control
MPP+
M
Levels of Drp1 (% of control ± S.D.)
ns
Control
MPP+
N
Levels of Senp 3 (% of control ± S.D.)
ns
Control
MPP+
O
Levels of Mff (% of control ± S.D.)
ns
Control
MPP+

Figure 4: Representative western blotting of primary cortical culture (subcellular fractions) exposed to MPP^+ to analyse SUMOylation (SUMO 2/3 and SUMO 1) (Fig 4A, 4B, 4D and 4E) and targets proteins (Drp1, Mff, Senp3) (Fig 4C and 4F). Cortical neurons were exposed to MPP^+ 50 µM or vehicle (H_2O sterile) for 48 hours followed by western blotting of non-nuclear and nuclear fractions. GAPDH was used as a loading control from non-nuclear fraction, VDAC was used as a loading control from nuclear fraction. The initial results suggest that MPP^+ doesn't alter SUMO 2/3 and target proteins levels in non-nuclear fraction (4G-J), however it seems to reduce levels of SUMO 1 in nuclear fraction (4L). (n=3-5; Student's t-test).

As mentioned before, a dysfunction in fission and fusion mitochondrial dynamics is present in PD pathophysiology which implicated in normal mitochondrial function and neuronal viability[16,17]. To observe the mitochondria and analyse fission and fusion dynamics in neurons treated with MPP^+ we used the Mito Tracker Red to stain mitochondria (Fig. 5).

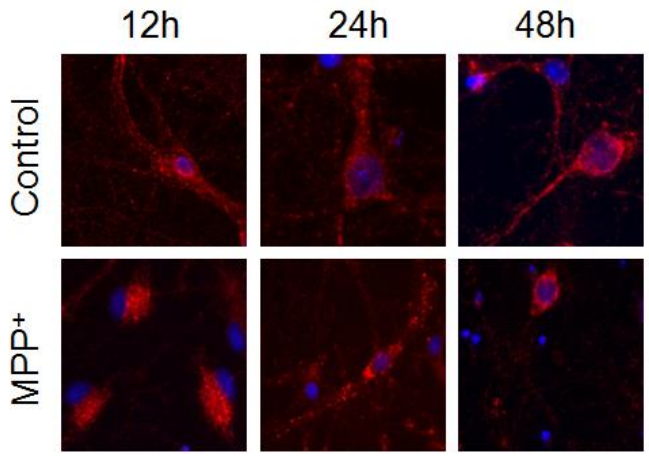

Figure 5: Representative mitochondrial images from neurons culture exposed with MPP^+ to analyse fission and fusion mitochondrial dynamics. Hippocampus neurons were exposed to MPP^+ 50 µM or vehicle (H_2O sterile) for 12, 24 or 48 hours followed by Mito Tracker stain and mitochondrial confocal imaging. The representative images suggest that MPP^+ exposition can increase levels of mitochondria near the nucleus also increase the mitochondrial fission process in axons. Mito Tracker Red – mitochondria, DAPI – nucleus.

The results presented in this report represent the work performed in Prof. Henley's lab. All experimental data are still being analysed for future conclusions. We will complement this work with all further experiments necessary for its publication in a scientific journal.

5. References

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