3-NITROPROPIONIC ACID INDUCES AUTOPHAGY BY MITOCHONDRIAL PERMEABILITY TRANSITION PORE RATHER THAN ACTIVATION OF THE MITOCHONDRIAL FISSION PATHWAY.

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Huntington's disease is a neurodegenerative process that has been associated with mitochondrial alterations including depletion in complex I activity. Drug inhibitors of complex I, such as 3-nitropropionic acid (3NP), are frequently used as pharmacological model to study the molecular and cellular pathways that are involved in this disease. Mitochondria are considered multifunctional organelles of changing morphology. Participation of the intrinsic apoptosis pathway, which is critically dependent on mitochondrial outer membrane permeabilization (MOMP), and the consequent release of cell-death mediating mitochondrial intermembrane space proteins, such as cytochrome c, has been described in this model. The knowledge of upstream modulators of mitochondrial dynamics is still not complete. Consistent with this, autophagy contributes to mitochondrial dysfunction-induced neurodegeneration and in vivo administration of 3NP activates autophagy. How 3NP regulates autophagy, and how this is related to mitochondrial morphology is an important question. Therefore, in the present study, we investigated the contribution of the mitochondrial-morphology pathway to autophagy activation as induced by 3-NP.

1 3NP induces autophagy

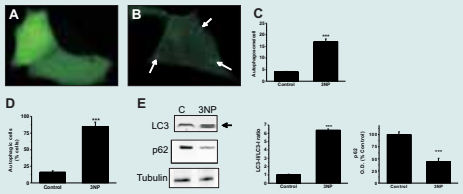

Fig 1. A-B. Confocal images of SH-SY5Y cells 24h after transfection with GFP-LC3. Representative non-treated cells (control, A) or incubated for 3h in 5mM 3NP (B).**C.** Number of cytosolic GFP-LC3 vacuoles per cell were determined in at least six different cultures under basal conditions (Control), and after 3 h treatment with 5mM 3NP. **D.** Proportions of autophagic cells in cell cultures challenged or not with 5mM 3NP cells for 3h. **E.** Left representative western blot showing LC3-I and II, p62 and tubulin protein bands from total cellular extracts, challenged or not with 5mM 3NP for 3h. Histograms: Quantitative analysis of the ratio of LC3-II/LC3-I. Histogram represents p62 levels normalized to tubulin.

2 3NP disrupts mitochondrial morphology

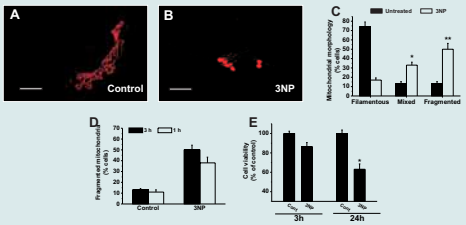

Fig 2. A-B. Representative mitochondrial morphology in non-treated cells (**A**) or incubated for 3h in 5mM 3NP (**B**).**C.** Proportions of cells with filamentous, mixed or fragmented mitochondrial patterns were determined. **D.** 3NP does not induce reversible morphological changes. Proportions of cells with fragmented mitochondrial patterns were determined after 3h (Untreated, black columns), or 1h and 2h after removal of the inhibitor from cell cultures challenged for 1h with 3NP (white columns). **E.** Cell viability studies of cultures that were exposed for 3 or 24h to 5mM 3NP.

3 Mitochondrial permeability transition pore formation due to 3NP

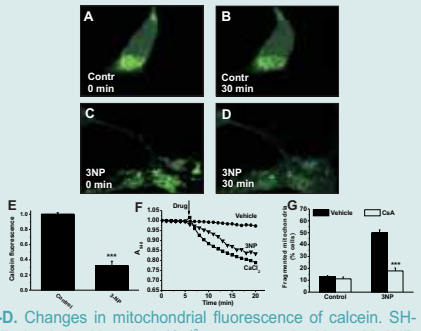

Fig 3. A-D. Changes in mitochondrial fluorescence of calcein. SH-SY5Y cells loaded with calcein/Co^{+2} were treated with 5mM 3NP. SH-SY5Y cells, before (left) or 30min after (right) treatments. **E.** Fluorescence intensity normalized to the initial value. **F.** 3NP induces mitochondrial swelling. Mitochondrial suspensions were exposed to 5mM 3NP and A540 was recorded. Ca^{+2} (75μM) was used as a positive control for mitochondrial swelling. **G.** Effect of CsA in mitochondrial morphology. Cells were terated with CsA 1μM or with CsA 1μMand 3NP 5mM.

4 3NP did not activate mitochondrial Drp1 translocation

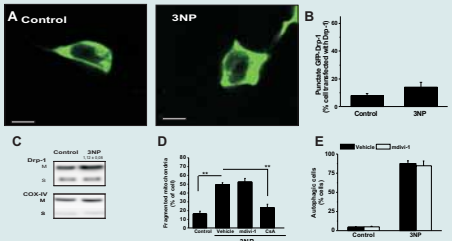

Fig 4. A. GFP-Drp1 demonstrated primarily diffuse staining in control (left), and after 3h treatment with 3NP (right). **B.** Number of cells with punctuate Drp1-GFP distribution were counted and expressed as percentage of the total number of cells expressing GFP-Drp1. **C.** Drp-1 levels were determined in mitochondrial fraction (upper panel) and soluble extracts (lower panel) from SH-SY5Y control cells and cells treated with 5mM 3NP. **D.** SH-SY5Y cells were co-treated with either 10μM mdivi-1 or CsA and 5mM 3NP. Proportions of cells with fragmented mitochondrial patterns were determined. **E.** Mdivi-1effects on 3NP-induced autophagy. Cells were transfected with GFP-LC3 and co-treated with 10μM mdivi-1 and 5mM 3NP. Proportions ofautophagic cells challenged or not with the drugs.

5 Bax translocates to the mitochondria after 3NP addition

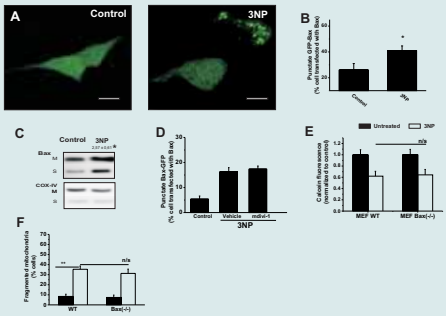

Fig 5. A. Confocal imaging of SH-SY5Y cells transfected with GFP-Bax, incubated for 24h to allow sufficient GFP-Bax expression, and treated with 5mM 3NP. Bax-GFP demonstrated primarily diffuse staining in control, whereas 3h after 3NP treatment a punctuate pattern is evident. **B.** The numbers of cells with punctuate GFP-Bax distribution were expressed as percentage of the total number of cells. **C.** Levels of Bax were determined in mitochondrial fraction (M) and soluble extracts (S) from SH-SY5Y cells treated with or without 3NP. **D.** Cells transfected with GFP-Bax were co-treated with mdivi-1 10μM and with 3NP 5mM after 3h of treatment. **E.** Confocal calcein fluorescence intensity in MEF Bax -/- cells 45min after 5mM 3NP treatments. **F.** MEF Bax -/- cells were trasnfected with pDsRed2Mito and treated with 3NP 5mM. Proportions of cells with fragmented mitochondrial pattern were determined under basal conditions and after 3h of treatment with 3NP 5mM.

6 Role of ROS

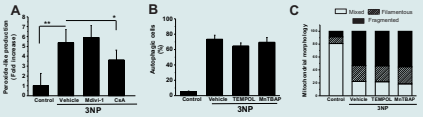

Fig 6. A. 3NP induces peroxides production. Cell cultures were treated with mdivi-1 10μM, CsA 1μM or vehicel for 30min before the addition of 3NP 5mM. 3h later, production of peroxide-like ROS was measured. **B.** SH-SY5Y cells were transfected with GFP-LC3 and pre-incubated with TEMPOL 0.2μM or with MnTBAP 10nM for 30min prior the treatment with 3NP 5mM. 3h later, proportion of autophagiuc cells was measured. **C.** Confocal microscopy analysis of mitochondrial morphology of cell cultures overexpressiong DsRed2-Mito protein and pre-incubated with TEMPOL 0.2μM or with MnTBAP 10nM 30min before the treatment with 3NP 5mM. Proportions of cells with each kind of mitochiondrial morphology were determined under basal conditions and after the different treatments.

Statistics

All data represent mean ± SEM of four independent experiments (*p < 0.05, ** p<0.001 and ***p < 0.001; t test versus control or one way ANOVA post hoc Tukey. Scale bars, 10 μm.

Conclusions

SH-SY5Y cells presented a long, tubular and filamentous net of mitochondria. Upon 3NP treatment, mitochondria became dramatically shorter and rounder. Furthermore, 3NP induces the formation of mitochondrial permeability transition pore, both in cell cultures and in isolated liver mitochondria, and this process is sensitive to the presence of its inhibitor, cyclosporine A. Participationof the mitochondrial fission pathway is excluded because 3NP did not induce translocation of the DRP1 to the mitochondria. Also, inhibition of this GTPase, using mdivi-1, did not abrogate the observed mitochondrial alteration morphology. Finally, we ascertaine the participation of reactive oxygen species in this neurodegenerative model, observing that scavenger drugs failed to prevent mitochondrial alterations while the presence of cyclosporine A, and not mdiv1, prevented ROS generation.

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