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NADPH oxidases in Huntington's Disease?

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Introduction

Huntington's disease (HD) is characterized by an expansion of a CAG repeat which encodes for a polyglutamine tract within the Huntingtin protein. The neuropathological signature of HD is major striatal neuron loss and the presence of intranuclear inclusion bodies consisting of mutant HTT proteins (Roze et al., 2011). Before neuronal cell death is observed in HD, a dysfunctional interaction between cortical glutamatergic neurons and striatal medium spiny neurons (MSNs) occurs. Moreover, there is aberrant synaptic function and plasticity long before the onset of classical disease indicators (Raymond and Milnerwood,2010). Interestingly, in HD, the contribution of the family of NADPH oxidases (NOXs) to the latter has not been thoroughly investigated; this despite the fact that NOXs have been shown to have a role in long term potentiation (LTP), long term depression (LTD) (Yi et al., 2018); (Massaad and Klann, 2011), excitotoxicity (Brennan-Minnella et al., 2013), and neuronal arborization (Terzi and Suter, 2020); processes which are all somehow compromised in HD pathology.

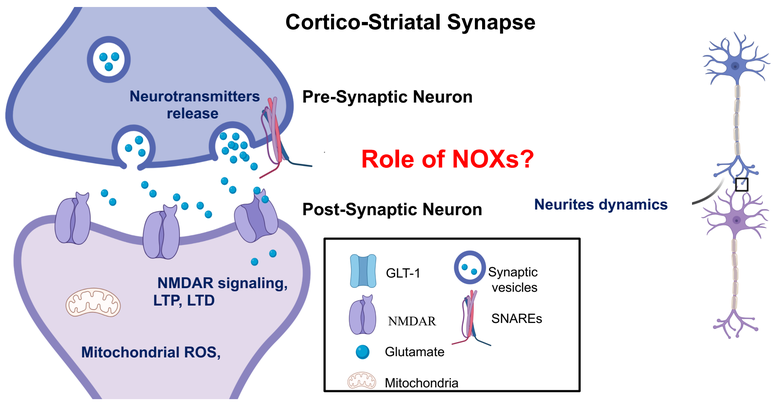

Fig. 1. Putative Roles of NADPH oxidases (NOXs) in the HD cortico-striatal synapse. In different neuronal populations NOXs are involved in processes such as NMDAR related excitotoxicity, LTP, LTD, enhanced mitochondrial ROS production and possible oxidation of the machinery for neurotransmitters release, such as SNAREs. All of the latter with a disrupted functionality in HD. Moreover, neuronal arborisation is regulated by NOXs. Hence, NOX malfunction could lead to abnormal synaptic connectivity in HD.

Synaptic dysfunction in HD has not been studied in terms of oxidative stress. Reactive Oxygen Species (ROS) are mainly produced in the cells by NOXs or by dysfunctional mitochondria. Knockout mice of NOX show defects in synaptic structure and memory consolidation (Kishida et al. 2006) but the contribution of NOX to HD pathology has not been studied in detail, and it may play a key role in the loss of synaptic connectivity. For this reason, our group focuses in understanding the role of NOX in neurite arborization and synaptic function, to later understand the putative role of NOX in HD.

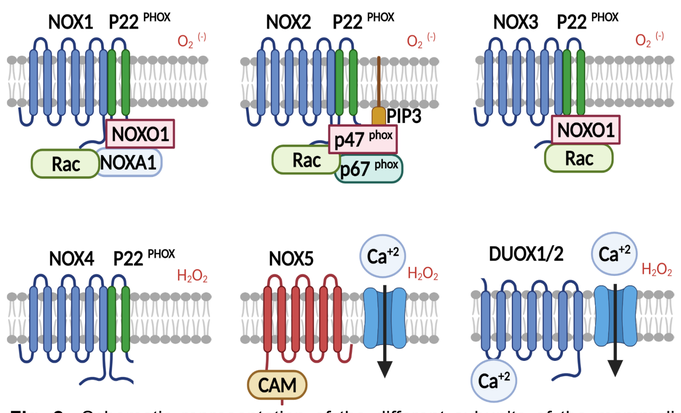

Fig. 2 Schematic representation of the different subunits of the mammalian family of NADPH oxidases. NOX1-3, activated in the presence of cytosolic factor, PIP3 serves as a membrane anchor for cytosolic subunit p47^{phox}. NOX4 is constitutively active. NOX5 and DUOX1/2 are calcium dependent.

Cell Model: PC12 cell line which is derived from a rat pheochromocytoma. When stimulated with nerve growth factor (NGF),they differentiate into neuron-like cells. The cell line has been further modified through lentiviral transduction to stably express a tetracycline controlled transcriptional activation (TET-ON) of WT huntingtin (18Q, cell line name: D491) and mutant huntingtin (115Q, cell line named D493).

Study Goal: Loss of synapses in HD could be explained by an exacerbated oxidative stress due to an imbalance of free radicals produced by NOXs and dysfunction of NOXs normal function.

Results

Studies from our lab demonstrate the presence in the synapses of one of the activating cytosolic subunit of NOX2. The latter has been confirmed by western blot of cortical and striatal synaptosome fractions of both WT and R6/2 mice, a mouse model for HD **(Figure 3).** Moreover, p22^{PHOX,} a common subunit for NOX isoforms 1-4 has been shown to be downregulated in HD cell lines **(Figure 4).**

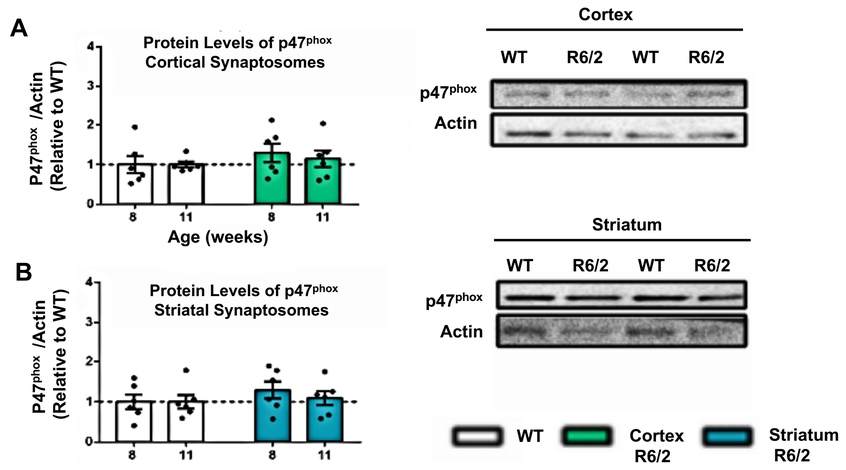

Fig. 3. Bar plots represent the level of p47phox in (A) cortical synaptosome samples and (B) striatal synaptosome samples from R6/2 mice (colored bars) and WT littermates (white bars) at 8 and 11 weeks of age.

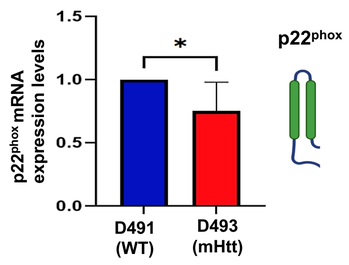

Fig. 4. Relative p22^{phox} mRNA expression levels of a WT PC12 cell line carrying a normal length polyQ huntingtin stretch (D491, WT, blue); and a PC12 cell line carrying a mutant polyQ huntingtin stretch (118Q, D493, mHtt)

Function of different NADPH oxidases

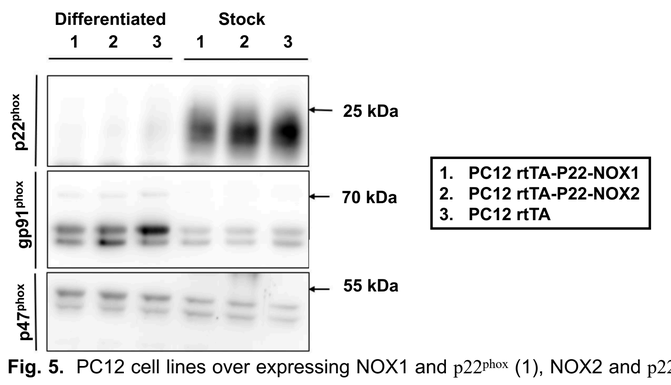

Fig. 5. PC12 cell lines over expressing NOX1 and p22^{phox} (1), NOX2 and p22^{phox} (2), and regular PC12 rtTA (3). p22^{phox} could not be detected at protein levels in NGF-differentiated cells in any of the mentioned lines, despite p22^{phox} overexpression .

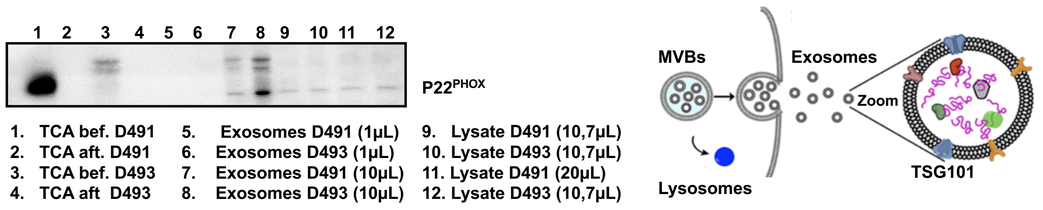

Fig. 6. Exosomes isolation was performed on D491 and D493 cells after 44 hours of NGF treatment and 72 hours of WT Htt and mHtt expression, respectively. P22^{PHOX} was mainly detected in the exosome's fractions (7 and 8) and in the cell culture supernatants (1 and 3) before centrifugation. In the schematic picture, is relevant to mention that TSG101 is a protein component of the ESCRT-I complex, which is key for exosome biogenesis.

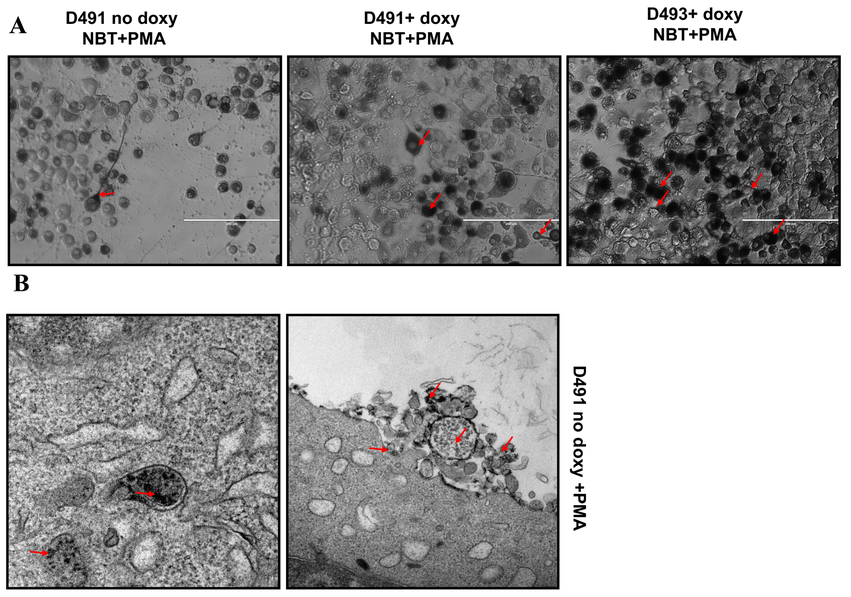

Fig. 7. A) NitroBlue Tetrazolium (NBT) assay of D491 cells without induction of Htt expression (D491 no doxy), D491 cells with WT Htt expression (D491+ doxy) and D493 cells expressing mutant Htt (D493+ Doxy). Within the cells, NBT reacts with superoxide to form water insoluble blue formazan crystals. Blue crystals formation was higher in the D493+ cells as depicted in the pictures, all cell lines were treated with PMA (100ng/mL), a known activator of NOX **B)** EM pictures of D491 cells stimulated with PMA. Method that involves the formation of an electron dense precipitate when H202 (A bi-product of superoxide) reacts with cerium chloride. The precipitate was mainly seen inside multivesicular bodies, MVBs (left picture) and in exosomes in the extracellular space (right picture).

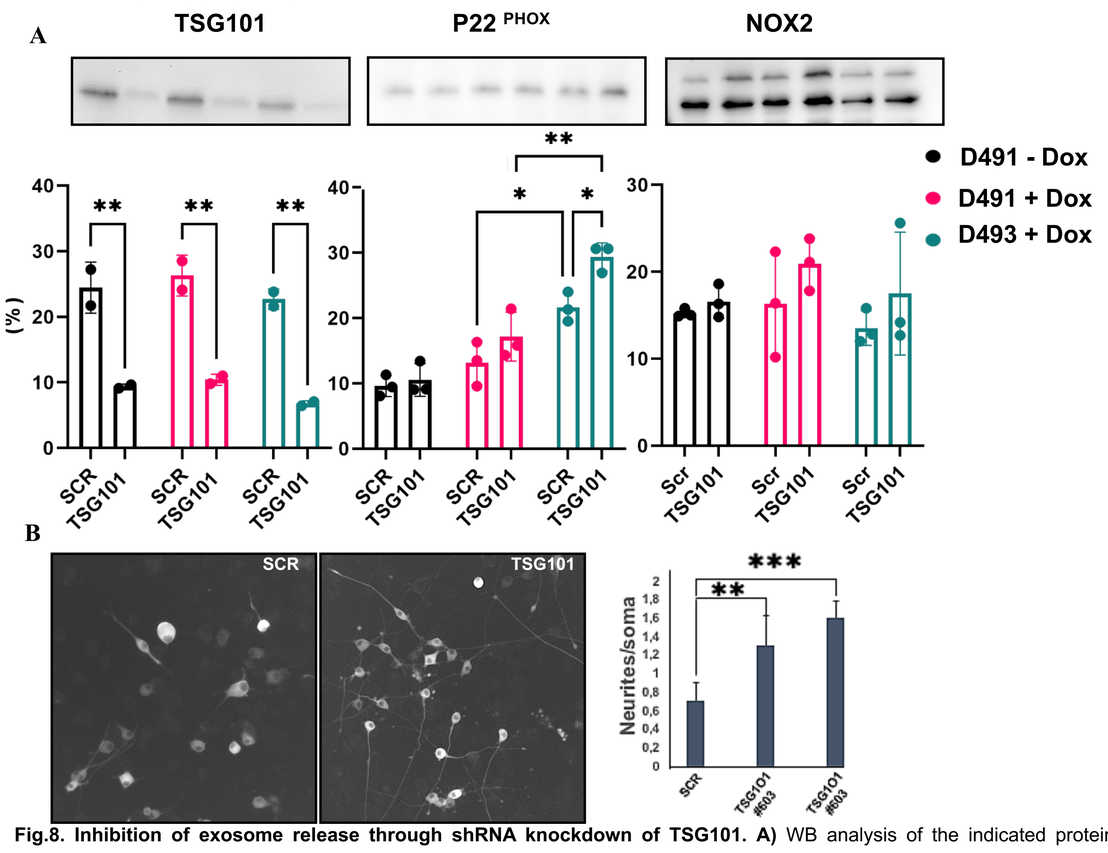

Fig.8. Inhibition of exosome release through shRNA knockdown of TSG101. A) WB analysis of the indicated proteins (TSG101, P22^{phox} and NOX2) after transduction with pLKO.1 scrambled (Denoted as SCR) and pLKO.1 TSG101 shRNA (denoted as TSG101). Knockdown (KD) of TSG101 was significant for all the three cell lines used (D491 without doxycycline induction of Huntingtin, D491 treated with doxycycline, and D493 treated with doxycycline: black, pink and green respectively). KD of TSG101 increased levels of P22^{phox} intracellularly; a similar pattern occurred for NOX2, and NOX1 (data not shown) but with no significant effect. **B)** KD of TSG101, increased number of neurites per soma and neurite length (data quantification not shown), suggesting that, after inhibition of exosomes release, NOX2 is retained if the cells, given this particular isoform role in neurite extension. Further analysis needed, also in relation to mHtt.

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