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COORDINATORE: PROF. EMANUELE ALBANO

STUDENT: **VIDONI CHIARA** CYCLE: **XXIX** YEAR: **II**

TUTOR: PROF. CIRO ISIDORO

"MUTANT Q113 FL HTT SENSITIZES DOPAMINERGIC NEURONS TO DOPAMINE – INDUCED TOXICITY"

1. **Project aim/objectives**

The aim of our project is to investigate whether and how the dopamine stimulation causes toxic effects in "*in vitro*" model of Huntigton's disease, in particular in human neuroblastoma SH-SY5Y cell line expressing mutant Q113 FL Htt.

Huntington's Disease (HD) is a devastating neurodegenerative condition caused by abnormal expansions (>37) of a polyglutamine (PolyQ) tract in the huntingtin protein (Htt) (Rubinzstein, 2002). This proteinopathy is associated with the progressive loss of neurons, especially the striatal neurons. Wild-type Htt is a large protein widely expressed in neurons, and its functions include anti-apoptotic, vesicles trafficking/endocytosis and scaffold protein for selective macroautophagy. The mutant polyQ Htt leads to the formation of toxic cytoplasmatic and nuclear inclusions and may affect the endocytic traffic and autophagy. The length of polyQ influences the toxicity of mutant htt (DiFiglia et al., 1997). Protection against a number of pathologies such as cancer, neurodegeneration, cardiac disease and infections could be achieved by upregulation of autophagy (Lavallard et al., 2012). Autophagy is a catabolic process that plays a crucial role in cell homeostasis through the lysosomal digestion of dysfunctional organelles and protein aggregates. Autophagy exerts beneficial effect to neurons enabling the removal of toxic protein aggregates and oxidized mitochondria, though it may be detrimental if hyper-activated by multiple triggers (Kepp et al. 2011). Oxidative stress is a well known cause of protein aggregation. Dopamine (DA) is reported to induce oxidative stress in neurons and to trigger the endosomal-lysosomal system (Cagnin et al., 2012). Moreover, alteration in DA balance in the striatum leads to pathological conditions, such as abnormal movements and cognitive deficits in HD (Chen et al., 2013).

We will test the hypothesis that HD dopaminergic neurons are sensitized to oxidative stress, which is induced by dopamine stimulation, leading to the formation of toxic Htt aggregates through autophagy inhibition.

2. Background

Huntington's Disease is a devastating autosomal dominant neurodegenerative condition caused by abnormal expansions of a polyglutamine (polyQ) tract to more than 37 Qs in the huntingtin protein (Rubinzstein, 2002). HD affects approximately 5-10 individuals per 100000. Individuals typically suffer from progressive motor and cognitive impairments, loss of self and spatial awareness, depression, dementia and increased anxiety over the course of 10-20 years before death. Currently, treatment is limited to suppressing chorea: the involuntary and irregular movements of the arms and legs that accompanies HD (Munoz-Sanjuan and Bates, 2011). The principal histopathological feature of HD is the loss of medium-sized spiny neurons (MSNs) and, to a lesser degree, neuronal loss in cerebral cortex, thalamus, hippocampus and hypothalamus. Prevalence of HD is equally distributed between both sexes (Sarfarazi et al., 1987; Pearson et al., 1955). Onset is generally in the midlife, but can occur in childhood and old age. At the molecular level, HD is caused by a CAG trinucleotide repeated expansion within exon 1 of the HTT gene. This gives rise to an elongated polyglutamine tract at the amino terminus of the translated huntingtin (Htt) protein that is associated with protein aggregation and gain-of-function toxicity (Davies et al., 1997). In HD, the degeneration preferentially occurs in striatum, deep layers of the cortex and, during the later stages of the disease, extends to a variety of brain regions, including hypothalamus and hippocampus (Vonsattel et al., 1985).

The Htt protein is widely expressed within the CNS, more intensely in neurons than in glial cells, and in extraneural tissues. Normal Htt has been shown to be anti-apoptotic (Dragatsis et al., 2000; Ramaswamy et al., 2007; Rigamonti et al., 2000) and essential for normal embryonic development (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). From immunohistochemistry, electron microscopy, and subcellular fractionation studies of the molecule, it has been found that Htt is primarily associated with vesicles and microtubules (Hoffner et al., 2002; DiFiglia, 1995). These appear to indicate a functional role in cytoskeletal anchoring or transport of mitochondria. The Htt protein is involved in vesicle trafficking as it interacts with HIP1, a clathrin binding protein, to mediate endocytosis (Velier et al., 1998; Waelter et al., 2001). At cellular and molecular levels, mutant Huntingtin (mHtt) is highly aggregation prone and the formation of cytoplasmatic aggregates and nuclear inclusions throughout the brain is one of the most striking hallmarks of HD (DiFiglia at al., 1997; Soto, 2003). Htt protein can be degraded within lysosomes by cathepsin D

(Qin et al., 2003). Mutant Htt toxicity is thought to be exposed after a series of clevange events by proteasomes, calpain and aspartyl proteases, resulting in formation of an N-terminal fragments of around 150 residues containing the polyQ stretch (Sarkar et al., 2008). The length of polyQ influences the toxicity of mHtt. The expanded protein can be cleaved into N-terminal fragments that are more toxic than full-length Htt (DiFiglia et al., 1997; Ratovitski et al., 2007; Ratovitski et al., 2009).

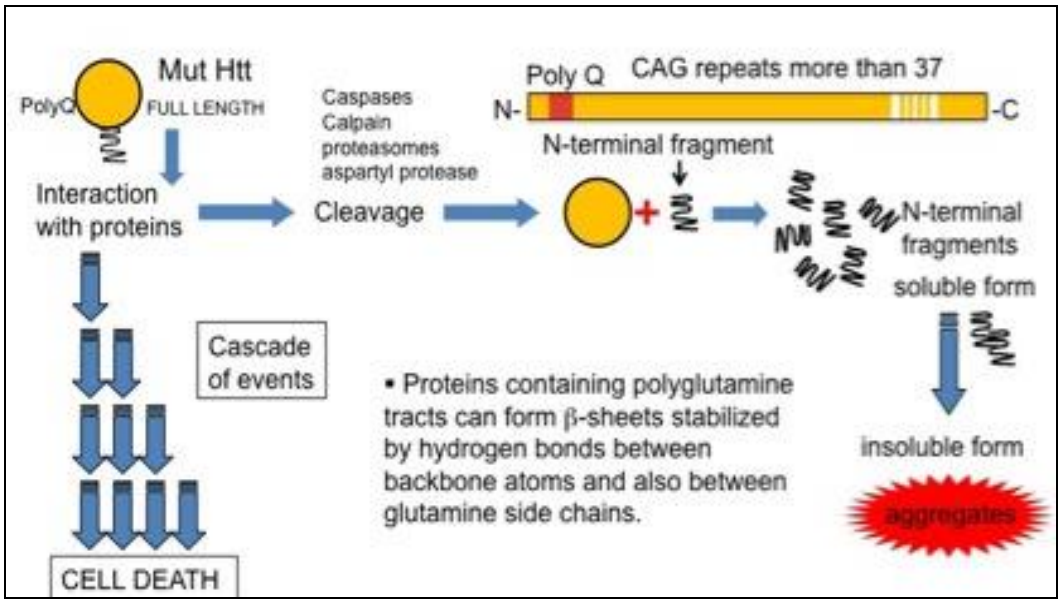

Fig. 1: Scheme of the events leading to cell death in model of Huntington's disease (Rossetti et al., 2008)

The principal HD pathological feature is the loss of striatal and cortical projection neurons. This mechanism remains unclear but may involve excessive glutamate release from cortical and thalamic terminals. Alterations in dopamine (DA) function and neurotransmission have a significant role in motor and cognitive symptoms of HD, as it is well known that glutamate receptor function is modulated by activation of DA receptors (Cepeda et al., 2014). Dopamine plays an essential role in the control of coordinated movements. Modulatory function of DA is disrupted early in disease progression, leading to aberrant glutamate transmission and consequent excitotoxic cascades (Richfield et al., 1991; Van Oostrom et al., 2009). Changes in brain DA content and receptor number contribute to abnormal movements and cognitive deficits in HD. However, later neurochemical studies of HD patients suggested that increased DA occurs in the early stages of the disease (Garrett and Soares da Silva, 1992), while post mortem studies of late stage HD patients showed a reduced levels of caudate DA (Kish et al., 1987). Striatal and cortical loss of DA receptor in early stage of HD patients has been correlated with early cognitive decline, such as attention, executive function, learning and memory (Backman and Farde, 2001). Thus, it was thought that DA levels in HD may show biphasic, time-dependent changes, with early increases followed by late decreases associated with biphasic movement symptoms of early HD (chorea) and late HD

(akinesia), where in the latter DA levels resemble those of a Parkinsonian-like state (Chen et al., 2012). Although DA exists in high concentration in the striatum, studies also suggest a toxic role for DA in which cell death is accelerated through increases in free radical production (Jakel and Maragos, 2000).This has been demonstrated in striatal cultured derived from R6/2 mice, where MSNs undergo DA-mediated oxidative stress and apoptosis (Petersèn et al., 2001 a). DA and glutamate signaling pathways can synergistically enhance MSNs sensitivity to mHtt toxicity (Cepeda et al., 2014).

Cagnin et colleagues reported that dopamine induces oxidative stress in neurons and triggers the endosomal-lysosomal system (Cagnin et al., 2012). Dysfunctional autophagy compromises the clearance of aggregates-prone mutant proteins that are potentially harmful to neurons, and is therefore considered an important factor in neurodegeneration (Castino et al., 2010). Autophagy exerts beneficial effect to neurons enabling the removal of toxic protein aggregates and oxidized mitochondria, but it may be detrimental if hyper-activated by multiple triggers (Kepp et al., 2011).

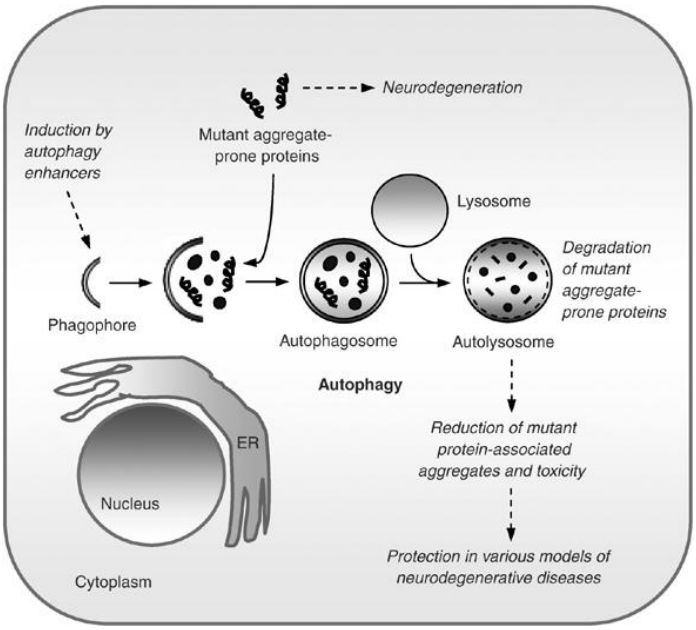

Fig. 2: Autophagy as a protective pathway for the clearance of aggregate-prone proteins. Autophagy is one of the primary degradation pathways for various aggregate-prone proteins associated with neurodegenerative diseases (Sarkar et al., 2009)

The Htt protein itself can act as scaffold to form aggregates that sequester signaling proteins, among which mTOR, a kinase that regulates several pro-survival pathways including autophagy and protein synthesis (Ravikumar et al., 2004). Htt is a scaffold protein that promotes selective autophagy through its ability to simultaneously interact with components involved in two major autophagy steps, p62 and ULK1, and thus modulate both cargo recognition efficiency and autophagosome initiation (Rui et al., 2015). Induction of autophagy reduces both aggregated and soluble Htt species, resulting in a decreased toxicity in HD cell models of fly and mouse

(Rubinsztein, 2006; Sarkar et al., 2008; Roscic et al., 2011). Of note, enhancing autophagy confers protection in HD animal models (Sarkar et al. 2007). Autophagy has been reported to be up-regulated in post mortem striatum regions of HD patients (Cherra et al., 2010) and autophagosomes accumulate in primary striatal neurons from HD mice expressing truncated mutant huntingtin following dopamine-stimulated oxidative stress (Petersén et al., 2001 b). More recently, it has been demonstrated that mHtt protein negatively interferes with p62-mediated cargo recognition, thus leading to frustrated autophagy in HD neurons (Martinez-Vicente et al., 2010). Furthermore, early and sustained expression of autophagy-related proteins in a novel knock-in HD mouse model suggests that the autophagic flux alteration is an early and important component of neuronal response to mHtt (Heng et al., 2010).

Based on the knowledge reported in literature, we investigated whether dopamine-induced toxicity is exacerbated in human dopaminergic neuroblastoma cells expressing the human mutant Q113 FL Huntingtin. The hypothesis to be tested is that DA interferes with the clearance of Htt aggregates through inhibition of autophagy, thus leading to neuronal cell death.

3. Experimental plan and methods

For our study, the human dopaminergic neuroblastoma SH-SY5Y cell line was employed.
SH-SY5Y were cultivated under standard culture condition (37°C; 95 v/v% air: 5 v/v% CO_2) in 50% Minimum Essential Medium (MEM) (SIGMA, St. Louis), 50% Ham's F12 Nutrient Mixture (HAM) (SIGMA, St. Louis) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL), 1w/v% of a penicillin-streptomycin solution (SIGMA, St. Louis), 2mM di L-glutamine (SIGMA, St. Louis).
Cells were transfected with different HD expression constructs in pcDNA3 through the reagent Lipofectamine 3000.
Cells have been transfected with an empty plasmid (sham) or with plasmids harbouring either of Htt different forms (subcloned into pcDNAzeo (-)). Constructs N171 Q21 GFP, N171 Q150 GFP, N171 Q21 N/X, N171 Q150 N/X encode the amino-terminal 171 amino acids fragment NcoI/XhoI of human Htt protein, with 21 glutamines, which is the wild-type form, and 150 glutamines, which is the mutant form, respectively. Two of these plasmids generated by inserting a fragment of pGreen Latern, encoding a GFP-tag in frame with the C-terminal of the Htt sequence. Moreover we have other two constructs, Q21 FL and Q113 FL, which encode the full length human Htt protein, with 21 glutamines (wild-type Htt), and 113 glutamines (mHtt), respectively. Q21 FL has FLAG-tag in

frame with the N-terminal of Htt. All these plasmids were kindly provided by Prof.ssa Francesca Persichetti, (DSS, UPO, Italy).

To mimic the situation in the dopaminergic brain region subjected to oxidative stress by physiological catecholamine neurotransmitters, , the cells were incubated with 100 μM Dopamine (DA). To test if apoptosis were induced, the cells were also incubated with the pan-caspase inhibitor z-VAD-fmk (20 μM).

The autophagy system and the fate of endogenous and transgenic Htt, both fragments and full length protein, were studied by Western blotting and Immunofluorescence analyses.

Cell death was assessed by counting Trypan blue-stained cells, SubG1 analysis and by Propidium Iodide (PI) staining in not-fixed cells (necrotic cells).

Cell viability was assessed by counting the vital Trypan blue-excluding cells and by CellTracker fluorescence staining in not-fixed cells. Cell proliferation was assessed by immunofluorescence double-staining of Ki67 and p21.

Data and statistical analysis of electrophoresis band densitometry have been performed using VersaDOC Imaging System (Bio-Rad) apparatus, equipped with Quantity One software (Bio-Rad). Microscope observation has been performed with Leica DMI 600 fluorescence microscope (Leica Microsystems AG), equipped with Leica Application Suite software. Imaging processing and quantitation have been made using the ImageJ software.

4. Results

1) At first, we have checked the transfection efficiency of Htt plasmids. (A) Transgenic Q21 FL and Q113 FL Htt expression is higher at 24h of transfection compared to 48h and 72h. However, their expression is maintained in each time point considered. SH-SY5Y cells were transfected with pcDNA3 (Sham), Q21 FL, Q113 FL for 21h, then incubated with complete medium for 24h. (B,C,D) To determine if the presence of mutant Q113 FL Htt could affect cell growth, we assessed cell viability by cell counting and trypan blue staining. Counting of viable cells revealed that cell loss, amounting to approximately 10% in Q113 transfected cells, occurred at 24h of treatment with complete medium. Whereas in control conditions, both Sham and Q21 FL expressing cells, the amount of trypan blue stained cells decreases, involving around 2% of the cells. The interference on cell growth is confirmed by doubling time analysis. In Q113 FL Htt expressing cells, the doubling time is two-folds longer than in Sham and Q21 FL expressing cells.(E,F). Cell cycle analysis showed that in mutant Q113 FL Htt expressing cells cell death population, which is represented by SubG1 peak,

increases to 27%, while the cell replication, represented by G0/G1 phase, decreases to 10% compared to cells transfected with the empty plasmid.

These data suggest that the ectopic expression of mutant Q113 FL Htt, which encodes the human full length protein, interferes with cell growth.

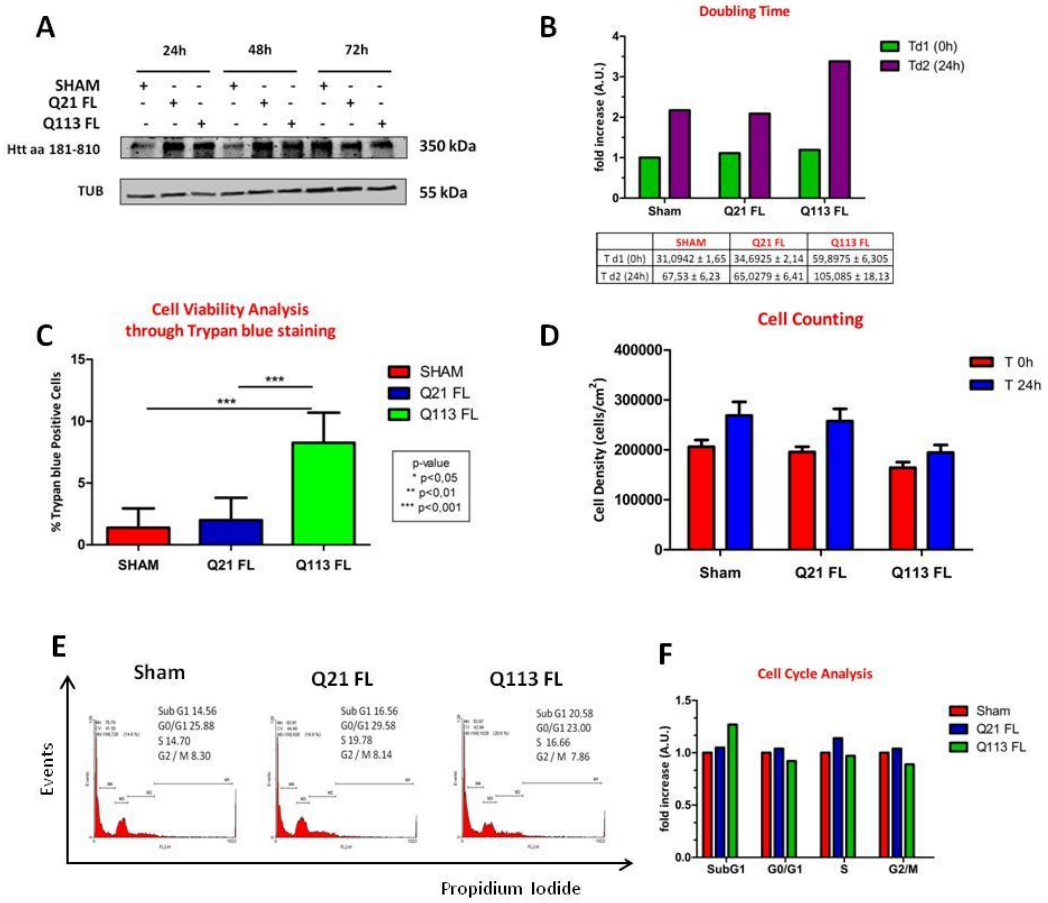

Fig.1: Ectopic expression of mutant Q113 FL Htt affects cell growth in human neuroblastoma cells

2) Next, we investigated further on the effects on cell viability driven by mutated full length Htt protein. SH-SY5Y cells were transfected with pEGFP-C2 (Sham-GFP), HD-N171 Q21-GFP, HD-N171 Q150-GFP for 21h, then incubated with complete medium for 24h. Cell viability was assessed by Cell Tracker. In Figure 2, the (A, B) images display that the intensity of blue fluorescent signal, obtained by quantification with the ImageJ software, is decreased in cells expressing mutant Htt (N171 Q150-GFP) compared to control condition, such as cells expressing Sham-GFP and N171 Q21-GFP. Accordingly, metabolically active mitochondria are not detectable in the presence of mutated Htt protein but only in Sham-GFP and Q21-GFP expressing cells. The data obtained show that the proportion of viable cells is reduced, approximately around 50%, in cells transfected with mutated Htt. We also quantified the cell viability impairment by the ectopic expression of mutated htt by cells-

associated blue fluorescence counting (Figure 2C). The mutant Htt causes a reduction of number of total cells, among these around 55% are metabolically inactivated cells. We have also checked whether cell replication is affected by immunofluorescence double staining, using cell the cycle markers Ki67 and p21 (Figure 2D). The images show a reduction of Ki67 expression and an increment of p21 levels in mutated Htt expressing neuroblastoma cells.

These data suggest that the ectopic expression of mutant Htt interferes with cell survival and cellular replication.

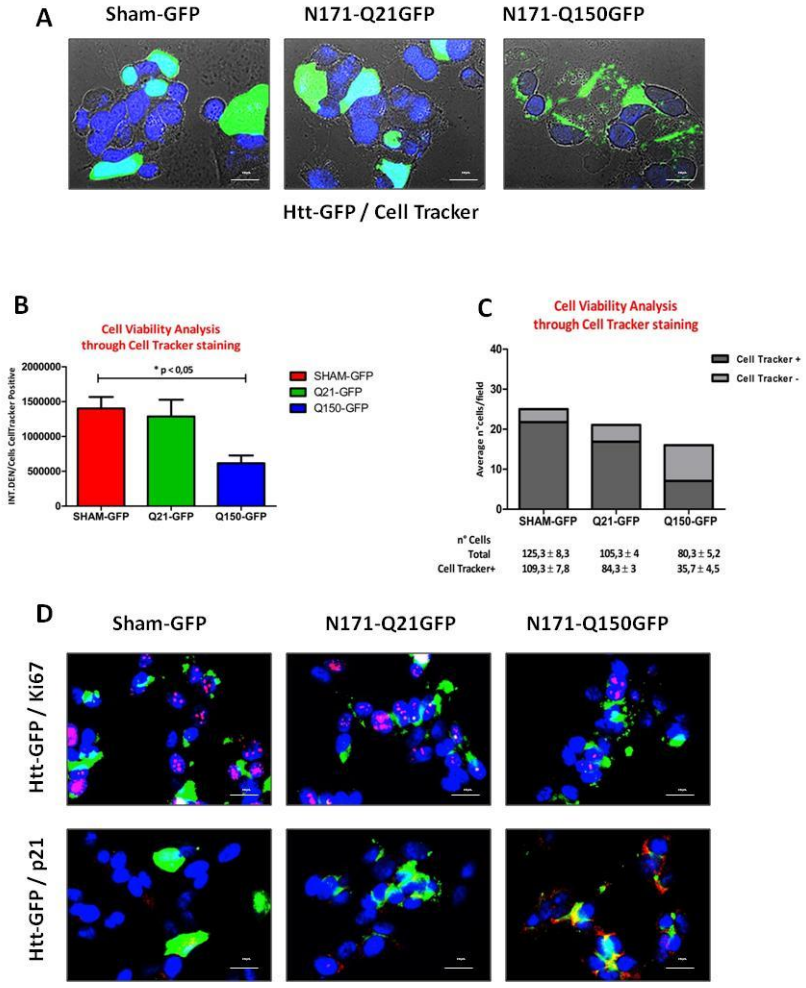

Fig. 2: Cell viability is impaired by the presence of mutant Q113 FL Htt

3) To verify the efficiency of transfection (A), we performed a Western blotting to detect GFP, which could recognize GFP protein in Sham-GFP transfected cells, whereas in HD-N171 Q21GFP and HD-N171 Q150GFP expressing cells, we could detect the Htt-GFP fusion protein at 59 KDa and 67 KDa, respectively Q21-GFP and Q150-GFP proteins. Accordingly, Htt N-terminal fragments, both HD-N171 Q21GFP and HD-N171 Q150GFP, display a peak of htt expression at 24h, which is significantly decreases at 72h. (B) To

investigate the dopamine induced toxicity in Htt expressing cells, we performed the nuclei staining with Propidium Iodide (PI) in not fixed cells. SH-SY5Y cells were transfected with pEGFP-C2 (Sham-GFP), HD-N171 Q21GFP, HD-N171 Q150GFP and exposed to DA until 72h. Images obtained representing 24h of transfection, along the 18 h of DA incubation, show that the PI staining is higher in mutated Q150 Htt expressing cells compared to control condition, in which PI is not detectable.

These data indicate that DA exposure induces necrotic toxicity only in human neuroblastoma cells transfected with mutated Htt.

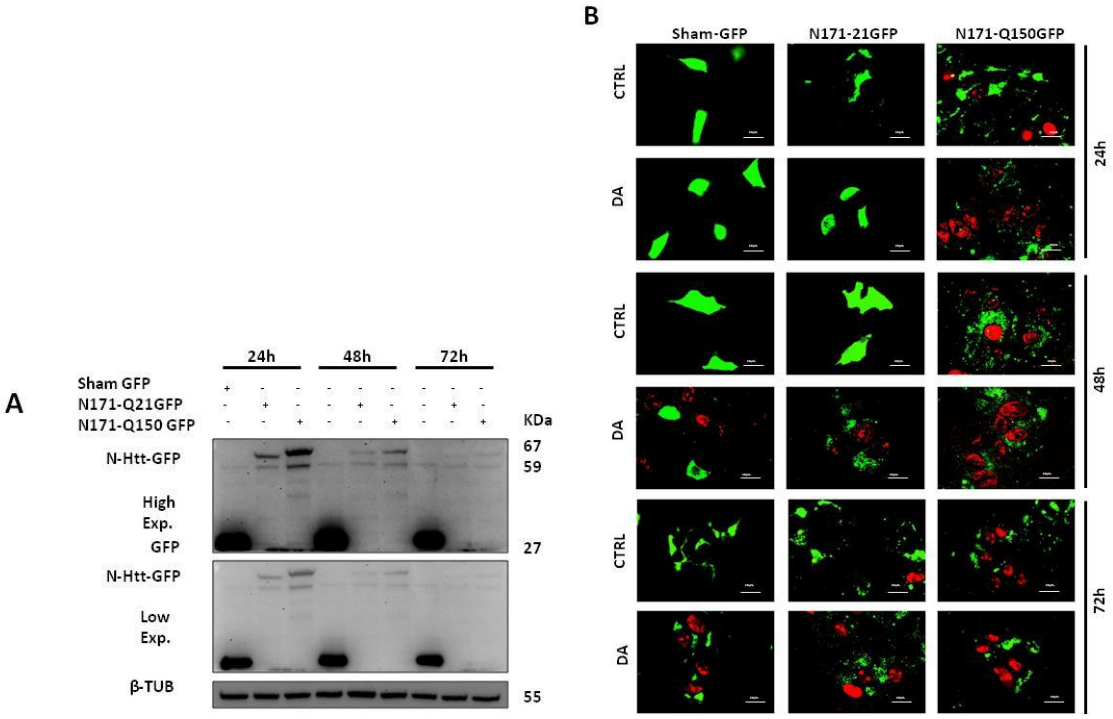

Fig. 3: Dopamine-induced toxicity in HD-N171 Q150 expressing neuroblastoma cells

4) To investigate the relationship between dopamine induced toxicity and mutant Htt expression, SH-SY5Y neuroblastoma cells were transfected with HD constructs encoding the human full length Htt protein for 24 h, a time corresponding to the peak of Htt expression, and incubated for 24 h with DA (100 µM) in presence or absence of pan-caspase inhibitor, z-VAD-fmk (20 µM). (A) Cell cycle cytofluorometry analysis suggests that the mutated Htt expression compromises the cell replication, notably in DA exposure. (B,C) As assessed by counting of Trypan blue stained cells, in Q113 FL expressing cells DA toxicity is exacerbated three-times more than cells transfected with Sham and Q21 FL, approximately 40% Q21 FL treated with DA *versus* Q21 FL Control (p-value<0,05%) and 135% Q113 FL treated with DA *versus* Q113 FL Control (p-value<0,001%). The presence of z-VAD-fmk partially rescues cell viability in Q113 FL transfected cells exposed to DA.

These data, confirmed by cell counting, indicate that ectopic over-expression of mutated Htt sensitizes the dopaminergic neuronal cells to dopamine, leading to secondary necrotic toxicity, that could be prevented by the presence of pan-caspase inhibitor z-VAD-fmk.

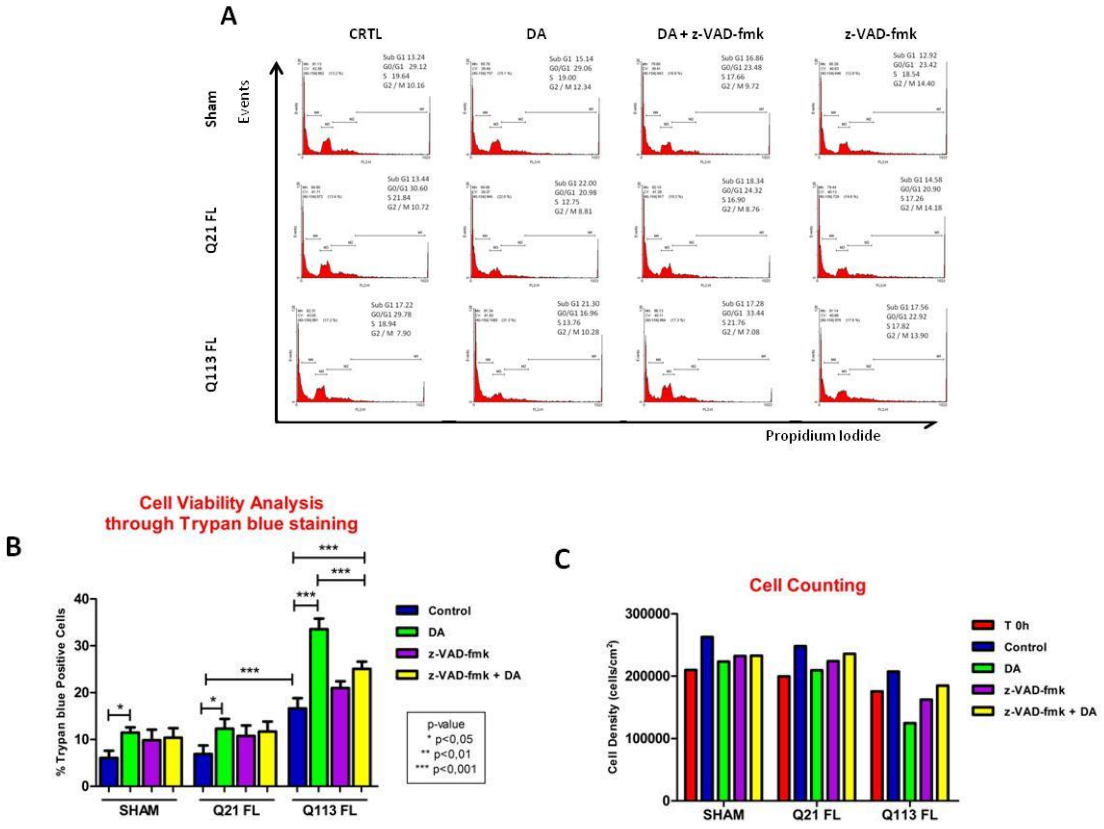

Fig. 4: Expression of Q113 FL Htt sensitizes to dopamine-induced toxicity

5) To confirm that dopamine induces necrotic toxicity in mutant Htt expressing SH-SY5Y cells, we next assessed cell death by PI immunostaining in not fixed cells exposed with DA in presence or absence of z-VAD-fmk. Data obtained (Figures 5A,B) demonstrate that DA-induced necrotic toxicity is exacerbated in HD-N171 Q150 expressing cells after 24 h treatment. Accordingly, PI staining in mutated Htt-GFP transfected cells is higher than in control, being around 40%. Furthermore, the presence of z-VAD-fmk partially prevents necrotic cell death induced by DA, restoring the level observed in control condition.

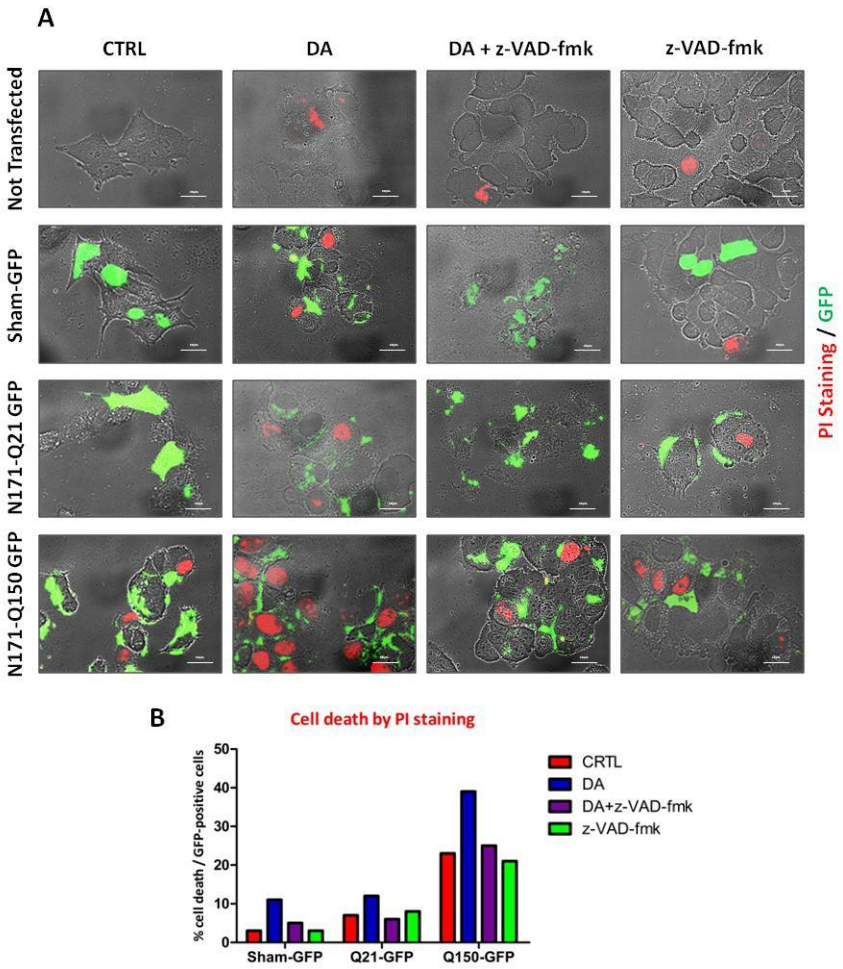

Fig. 5: Dopamine-induces necrosis in HD-N171 Q150 expressing neuroblastoma cells

In conclusion, these results indicate that mutated Htt expression affects cell growth, interfering with cell survival and cell viability. Accordingly, the expression of mutant Htt protein sensitizes human dopaminergic neuroblastoma cells to dopamine-induced toxicity, which leads to secondary necrotic toxicity.

For the future experiments, we will analyze the mechanism involved in dopamine-induced toxicity in human neuroblatoma cells expressing mutant Htt plasmids. To this end, we will evaluate the detection of ROS levels by immunofluorescence in not fixed cells stained with Acridine Orange (AO), Rhodamine 123 (Rho 123), Mitosox and H_2-DCF-DA. We will also investigate the involvement of autophagy in cells subjected to oxidative stress by dopamine stimulation through Western blotting and Immunofluorescence analysis.

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B.5b Participation at courses or summer schools organized by others than the PhD program

Course title and location	Organizer(s) and affiliation(s)	Number of hours	
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{add rows if needed}

B.8b

Periods (months) in foreign institutions and laboratories (address)	
Publications (articles, posters, abstracts)	• **Vidoni C**, Savino M., Lapadula A., Janda E., Melone M.A.B., Isidoro C. Toxicity and clearance of mutant Huntingtin in human neuroblastoma cells subjected to oxidative stress: role of the autophagy-lysosomal system. 51° Congresso dell'Associazione di Neuropatologia e Neurobiologia Clinica. 4-6 giugno. Verona. (Poster) • **Vidoni C.**, Follo C., Savino M., Melone M.A.B., Isidoro C. The role of Cathepsin D in the pathogenesis of human neurodegenerative disorders. Progr Neurobiol. Under submission. (articles: review). • Suratchanee Phadngam, **Chiara Vidoni**, Andrea Castiglioni, Miriam Savino, Ciro Isidoro. "Nanotheranostics for cancer diagnosis and therapy: concerns and applications". 20th october *International*

	mini-conference on BASIC TO TRANSLATIONAL MEDICINE 2015. Mahidol University, Bangkok. (Abstract). • Alessandra Ferraresi, Suratchanee Phadngam, **Chiara Vidoni**, Andrea Castiglioni, Ciro Isidoro. "Resveratrol from flowers and fruits: promising application in cancer therapy" 20th october *International mini-conference on* BASIC TO TRANSLATIONAL MEDICINE 2015. Mahidol University, Bangkok. (Abstract).

B.XX Attended seminars

Speaker	**Title**
1. Prof. March Diederich 28/10/14	Natural compounds as modulators of epigenetic events
2. Dr. A. Snow 06/11/14	Dysregulated antigen receptor signaling:molecular lessons from two congenital lymphoproliferative disorders
3. Dott. F. Boccafoschi 14/11/14	Tissue engeneering: the state of the art
4. Prof. M. Prat 22/11/14	Stem cell in the regeneration and repair of the tissues and organs
5. Prof. Laura Baglietto 27/11/14	Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori

6. Prof. Arvind Patel 28/11/14	Humoral responses to HCV infection and clinical outcomes
7. Prof. Girish Patel 04/12/14	Uncovering the role of β-HPV in field cancerization: a collaboration in progress
8. Prof. Rifaat Safadi 05/12/14	Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections"
9. Prof. Antonio Musarò 16/12/14	From the legend of Prometheus to regenerative medicine
10. Prof. Furlan 17/12/14	Microglia microvescicles: messengers from the diseased brain
11. Prof. Giancarlo Agnelli 16/01/15	Pregi e difetti dei nuovi anticoagulanti orali nella pratica clinica
12. Dr. Yong-Sang Song 19/01/15	Anticancer strategy targeting cell metabolism in ovarian cancer
13. Prof. Valeria Poli 21/01/15	Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states
14. Prof. Antonio Sica 27/01/15	Myeloid cells as therapeutic target in cancer
15. Prof. Darko Bosnakovski 11/03/15	Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells
16. Prof. Xiaoping Zhong 09/04/15	Signal control in iNKT cell development and function
17. Prof. Piergiorgio Percipalle 21/04/15	Actin-based mechanisms in the control of gene expression and cell fate
18. Prof. John McDonald 07/05/15	An integrated approach to the diagnosis and treatment of ovarian cancer
19. Prof. Steve Ellis 25/05/15	Ribosomopathies
20. Prof. Mariet C.W. Feltkamp 05/06/15	Recent Developments in (cutaneous) Human Polyomavirus Research

21. Prof. Nicoletta Filigheddu 10/06/15	Basic of scientific research
22. Dr Gwenaël Rolin Thomas Lihoreau – Ingénieur hospitalier 09/07/15	Miniworkshop on “Biotechnology for Dermatology”
23. Dott. Vincenzo Cantaluppi 28/07/15	Le cellule staminali nel danno renale acuto e nel trapianto di rene
24. Prof. Darko Bosnakovski 03/09/15	Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)