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Dopamine exacerbates mutant Huntingtin toxicity via oxidative-mediated inhibition of autophagy in SH-SY5Y neuroblastoma cells: Beneficial effects of anti-oxidant therapeutics

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ABSTRACT

Neuronal cell death in Huntington's Disease (HD) is associated with the abnormal expansions of a polyglutamine (polyQ) tract in the huntingtin protein (Htt) at the N-terminus that causes the misfolding and aggregation of the mutated protein (mHtt). Autophagy-lysosomal degradation of Htt aggregates may protect the neurons in HD. HD patients eventually manifest parkinsonian-like symptoms, which underlie defects in the dopaminergic system. We hypothesized that dopamine (DA) exacerbates the toxicity in affected neurons by over-inducing an oxidative stress that negatively impinges on the autophagy clearance of mHtt and thus precipitating neuronal cell death. Here we show that the hyper-expression of mutant (>113/150) polyQ Htt is *per se* toxic to dopaminergic human neuroblastoma SH-SY5Y cells, and that DA exacerbates this toxicity leading to apoptosis and secondary necrosis. DA toxicity is mediated by ROS production (mainly anion superoxide) that elicits a block in the formation of autophagosomes. We found that the pre-incubation with N-Acetyl-L-Cysteine (a quinone reductase inducer) or Deferoxamine (an iron chelator) prevents the generation of ROS, restores the autophagy degradation of mHtt and preserves the cell viability in SH-SY5Y cells expressing the polyQ Htt and exposed to DA.

The present findings suggest that DA-induced impairment of autophagy underlies the parkinsonism in HD patients. Our data provide a mechanistic explanation of the DA toxicity in dopaminergic neurons expressing the mHtt and support the use of anti-oxidative stress therapeutics to restore protective autophagy in order to slow down the neurodegeneration in HD patients.

1. Introduction

Huntington's Disease (HD) is a devastating autosomal dominant neurodegenerative condition characterized by neuronal loss in striatum (particularly of medium-sized spiny neurons (MSNs)), deep layers of the cortex and, when disease progresses, in hypothalamus and hippocampus and other brain regions (Vonsattel et al., 1985). Pathogenesis and progression of HD are strictly correlated with the presence in the affected neurons of cytoplasmatic aggregates and nuclear inclusions of the mutated form of the protein Huntingtin (Htt) and of its N-terminal fragments (DiFiglia et al., 1997; Soto, 2003; Melone et al., 2005). Mutant Htt (mHtt) is characterized by abnormal expansions of a polyglutamine (polyQ) tract to more than 37 Qs (Rubinsztein, 2002). While normal Htt has anti-apoptotic function, mHtt is neurotoxic. The N-terminal fragments of around 150 residues containing the polyQ stretch arising from the proteolytic processing by proteasomes, calpain and aspartyl proteases are even more toxic than full-length Htt (DiFiglia et al., 1997; Ratovitski et al., 2007, 2009; Rossetti et al., 2008). HD patients typically suffer from progressive motor and cognitive impairments, loss of self and spatial awareness, depression, dementia and anxiety over the course of 10–20 years before death. Alteration in dopamine (DA) neurotransmission is clearly involved in motor

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and cognitive symptoms of HD patients (Cepeda et al., 2014). Striatal and cortical loss of DA receptors in early stage of HD patients has been correlated with early cognitive decline, such as attention, executive function, learning and memory (Bäckman and Farde, 2001). The level of DA is elevated in the early stage of the disease (characterized by the chorea), while it decreases in the late stage when Parkinson-like symptoms (akinesia) become apparent (Garrett and Soares-da-Silva, 1992; Kish et al., 1987; Chen et al., 2013). Indeed, the level of DA in HD patients with parkinsonism resembles that of Parkinson Disease patients (Chen et al., 2013). DA is normally present in the striatum at elevated concentration, and is not harmful to normal neurons. However, DA is *per se* an excitotoxic neurotransmitter that triggers oxidative stress and may cause neuronal cell death (Jakel and Maragos, 2000). *In vitro*, DA-mediated oxidative stress was shown to induce apoptosis of striatal MSNs derived from transgenic R6/2 mice, an animal model of HD (Petersén et al., 2001a). It is possible that DA and glutamate synergize for the production of reactive oxygen species (ROS), so enhancing the toxicity of mHtt in MSNs (Cepeda et al., 2014).

The autophagy-lysosomal proteolytic system plays a protective role in HD by removing Htt aggregates (Sarkar and Rubinsztein, 2008). Dysfunctional regulation of this proteolytic system is consistently found in neurodegenerative disorders (Vidoni et al., 2016). Drugs able to increase the level of autophagy promote the clearance of Htt aggregates and relief the clinical symptom in '*in vivo*' model of HD (Rubinsztein, 2006; Sarkar et al., 2007, 2008; Roscic et al., 2011). Autophagy has been reported to be up-regulated *in post mortem* striatum regions of HD patients (Cherra et al., 2010). Abnormal expression of autophagy-related (ATG) proteins in the neurons of a knock-in HD mouse model indicates that alteration of the autophagic flux is an early stress response to mHtt (Heng et al., 2010). The Htt protein itself can affect autophagy by directly interacting with SQSTM1/p62 (which tags the autophagy substrates) (Martinez-Vicente et al., 2010; Bjørkøy et al., 2005) and with ULK1 (which activates the BECLIN 1-PI3KC3 complex that triggers the autophagosome formation) (Rui et al., 2015), or by sequestering in the protein aggregate mTOR, a kinase that negatively regulates ULK1 (Ravikumar et al., 2004). Interestingly, autophagosomes accumulate in primary striatal neurons from HD mice expressing truncated mutant Htt following dopamine-induced oxidative stress (Petersén et al., 2001b).

What is the functional role of autophagy in dopaminergic neurons expressing the mHtt and what is the cytotoxic mechanism of DA that could underlie the onset of parkinsonism in HD patients remain however obscure. As an attempt to fill in this gap of knowledge, we investigated the molecular link between DA-induced oxidative stress and mHtt toxicity in relation to the activation of the autophagy pathway in an '*in vitro*' model of parkinsonian HD. We found that DA-induced ROS production causes the death of dopaminergic human neuroblastoma SH-SY5Y cells expressing transgenic mHtt following the inhibition of autophagosome formation. Preventing ROS generation by N-Acetyl-L-Cysteine (an inducer of quinone reductase) or Deferoxamine (an iron chelator) restores the autophagy flux and the clearance of mHtt, and saves the mHtt-expressing neuronal-like cells from DA toxicity. Thus, *anti*-ROS drugs able to restore autophagy could slow down the progression of HD and prevent the onset of parkinsonian-like symptoms in HD patients.

2. Materials and methods

2.1. Cell culture and treatment

Human dopaminergic neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured under standard culture condition (37 °C; 95 v/v% air: 5 CO_2 v/v%) in 50% Minimum Essential Medium (MEM, cod. M2279, Sigma-Aldrich Corp. St. Luis, MO, USA), 50% Ham's F12 Nutrient Mixture (HAM, cod. N4888, Sigma-Aldrich Corp.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, cod. ECS0180L, EuroClone S. p.A. Milan, Italy), 1% w/v of a penicillin-streptomycin solution (cod. P0781, Sigma-Aldrich Corp.) and 2 mM di L-glutamine (cod. G7513, Sigma-Aldrich Corp.). The cells were seeded (starting density 70.000/cm^2) on sterile Petri dishes or glass coverslip, as indicated, and allowed to adhere for 24–36 h prior to start any treatment. Excitotoxic treatment was performed with 100 μM Dopamine (DA, cod. H8502, Sigma-Aldrich Corp. dissolved in sterilized H_20). Apoptosis was inhibited with 20 μM of the pancaspase inhibitor z-VAD (OMe)-fmk (z-VAD-fmk, cod. 260-020-M005, Alexis Laboratories, San Diego, CA). The cells were pre-incubated 2 h with 2 mM N-Acetyl-L-Cysteine (NAC, cod. A9165, Sigma-Aldrich Corp.) or 1 mM Deferoxamine mesylate salt (DFO, D9533, Sigma-Aldrich Corp.) to prevent ROS generation. Chloroquine (ClQ, 30 μM, cod. C6628, Sigma-Aldrich Corp.) was used to inhibit autophagosome degradation. Pepstatin A (PstA, 100 μM, cod. P 5318, Sigma-Aldrich Corp.) was used as inhibitor of Cathepsin D.

2.2. HD expression constructs and plasmid transfection

The plasmids encoding for Htt (either wild-type or mutant) and for the polyQ tract (see Supplementary Fig. S1A) were generously provided by Prof. F. Persichetti (Università del Piemonte Orientale, Italy). (HD)N1-171Q21 and (HD)N1-171Q150 mammalian expression constructs in pcDNA3/Zeo (+) encode the amino-terminal 171 amino acids of human Htt protein (Accession No. L12392, bp 314–823), with 21 and 150 glutamines, respectively. (HD)N1-171Q21GFP and (HD)N1-171Q150GFP were created by inserting a fragment of pGreen Lantern-1, encoding a GFP-tag, in frame with the carboxy-terminus of the Htt sequence (Persichetti et al., 1999). Full length HD constructs Q21-FL (pC_3F_7HD Q21) and Q113-FL (FL 113Q HD CMV) encode the full length of human huntingtin protein, with 21 and 113 glutamines, respectively. The construct Q21-FL was created by inserting the FLAG-tag in frame with the amino-terminus of the full length human huntingtin protein.

The cells were transfected with the plasmids using the Lipofectamine 3000 Reagent (cod. L3000-015, Life Technologies Ltd, Paisley, UK) as indicated by the purchaser. Briefly, SH-SY5Y were plated in P35 Petri dish at 70.000/cm^2 and let adhere 24 h before transfection. The DNA–Lipofectamine complexes were prepared in 500 μl of Opti-MEM I Reduced Serum Medium (cod. 11058021, Life Technologies Ltd) with 6 μg of plasmid, 5 μl of P3000 reagent and 7.5 μl of Lipofectamine. After 6 h of incubation, the transfection medium was replaced with a serum-containing culture medium (10% FBS), and the cells were cultivated for further 21 h to allow for maximal expression of the transgenic protein prior to any treatment.

2.3. Assessment of cell proliferation, cell viability and cell toxicity

Cell culture growth was assessed by cell counting of adherent viable (trypan blue-excluding) cells, and the doubling time (Dt) was calculated using the software Doubling Time Online Calculator (http://www.doubling-time.com/compute.php).

To test cell viability, the cells adherent on coverslips were labeled with CellTracker TM (CellTracker TMBlue-CMAC 7-amino-4-chloromethylcoumarin; cod. C2110, Life Technologies Ltd), and the blue fluorescence, an indicator of the mitochondrial respiratory activity, was immediately imaged under the fluorescence microscope Leica DMI6000 (Ekkapongpisit et al., 2012; Cagnin et al., 2012).

At least five fields randomly chosen in each coverslip were examined in three separate experiments and quantitated with ImageJ by two independent investigators. Representative images are shown.

Necrotic cells were identified on the basis of trypan blue staining or propidium iodide (PI; cod. P4170, Sigma-Aldrich Corp.) labeling (Castino et al., 2007, 2010). Hypodiploid (Sub-G1 peak) PI-labeled cells were evaluated by cytofluorimetry and assumed as apoptotic. At least 5.000 cells were analyzed using a FacScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser. Data were elaborated with the winMDI software.

Mitochondrial membrane integrity was assayed by Rhodamine 123 (cod. R8004, Sigma-Aldrich Corp.; 50 nM, 10 min in the dark at 37 °C) fluorescence retention test. Mitochondrial ROS (essentially anion superoxide) were assayed by staining living cells with MitoSOX (cod. M36008, Life Technologies Ltd; 5 μM) fluorescence staining (Castino et al., 2011).

2.4. Protein expression analysis

Cell homogenates were collected in RIPA-buffer containing phosphatase and protease inhibitors. Protein extracts were mixed with 5× sample loading buffer and boiled for 5 min at 95 °C. Protein content was determined using the Bradford reagent (Cod. B6916, Sigma-Aldrich Corp.), as previously (Follo et al., 2011). About 30 μg of protein was separated by electrophoresis on a 15% and 6% (depending on the molecular size of the protein of interest) SDS-PAGE gel and then transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). PageRuler Prestained Protein Ladder (Cod. 26616, Thermo Fisher Scientific Inc. Waltham, MA, USA) and Spectra Multicolor High Range Protein Ladder (cod. 26625, Thermo Fisher Scientific Inc.) were used as Molecular weight markers. The membranes were blocked with 5% non-fat milk in Phosphate Buffer Saline (PBS) with 0.2% Tween-20 for 1 h at room temperature and incubated with specific primary antibodies overnight at 4 °C. β-tubulin was used as homogenate protein loading control. Immunocomplexes were revealed by incubation with an appropriate peroxidase-conjugated secondary antibody (cod. 170515, 1706516, Bio-Rad), followed by peroxidase-induced chemiluminescence reaction (cod. NEL103E001 EA, PerkinElmer, Waltham, MA, USA). Intensity of the bands was estimated by densitometry (Quantity One Software, Bio-Rad or ImageJ software).

2.5. Immunofluorescence staining

Cells plated and let adhere on glass coverslip were fixed in cold methanol, permeabilized with 0.2% Triton-PBS and then re-fixed with methanol. After washing with PBS, the coverslips were incubated with the specific primary antibodies dissolved in 0.1% Triton-PBS plus 4% FBS overnight at 4 °C. The following day, the immunocomplexes were revealed by secondary antibodies, either IRIS-2 (green fluorescence) - or IRIS-3 (red fluorescence)-conjugated goat-*anti*-rabbit IgG or goat-*anti*-mouse IgG (cod. 2W5-08, 2W5-07, 3W5-08, 3W5-07, Cyanine Technology SpA, Turin, I) dissolved in 0.1% Triton-PBS plus 4% FBS for 1 h in the dark. Fluorescence counterstaining of the nuclei was performed with DAPI (4',6-diamidino-2-phenylindole, cod. 32670, Sigma-Aldrich Corp.). Finally, the coverslips were washed with 0.1% Triton-PBS and mounted onto glasses using SlowFade (cod. S36936, Life Technologies Ltd), and the images were acquired under the fluorescence microscope Leica DMI6000.

2.6. Primary antibodies used for western blotting and immunofluorescence

The following primary antibodies were used to identify Htt (see Supplementary Fig. S1B for the epitope recognized): monoclonal mouse *anti*-HTT (1:1000 for immunoblotting and 1:500 for immunofluorescence, cod. MAB2166, EMD Millipore Corporation, Billerica, MA, USA); monoclonal mouse *anti*-HTT1C2 Ms X Polyglutamine (1:1000 for immunoblotting, cod. MAB1574, EMD Millipore Corporation). Other antibodies used were: rabbit *anti*-SQTM1/p62 (1:500, cod. 8025, Cell Signaling Technology, Danvers, MA U.S.A.), rabbit *anti*-LC3B (1:1000, cod. L7543, Sigma Aldrich Corp.), rabbit anti-Cathepsin D (1:100 for immunofluorescence, produced in our laboratory (Erdmann et al., 2007); mouse anti-LAMP1 (1:1000 for immunofluorescence, cod. 555798, Becton, Dickinson and Company, New Jersey, NJ, USA), mouse *anti*-β-tubulin (1:1000 for immunoblotting, cod. T5201, Sigma Aldrich Corp.), mouse *anti*-p21 (1:100, cod. sc-817, Santa Cruz Biotechnology Inc. Dallas, TX, USA), polyclonal rabbit *anti*-MKI67 (Ki-67, 1:100 for immunostaining, cod. HPA001164, ATLAS-Sigma Prestige Antibodies, Sigma-Aldrich Corp.), mouse *anti*-GFP (1:1000, cod. 632381, Living Color Monoclonal Antibody (JL-8), Clontech Laboratories Inc. Mountain View, CA U.S.A.).

2.7. Statistical analysis

Image processing and data quantification of the intensity of fluorescence (INT. DEN) images were performed with the software ImageJ 1.48v (http://imagej.nih.gov/ij/). At least five randomly chosen fields were analyzed. Fluorescence Intensity (INT. DEN) is given in arbitrary units as an average value per cell in the selected representative fields. Quantification of fluorescence was performed on single channel (either red or blue) in the case of MitoSOX and Cell Tracker staining, or on the two-channels merge (green + red = yellow) in the case of double-staining to estimate the co-localization of two markers.

Densitometry of Western Blot bands was performed with the Quantity One-4.5.0 software (Bio-Rad).

Statistical analysis was performed with the GraphPad Prism 5 software. Tukey's test after one-way ANOVA analysis and *t*-test (paired, two-tailed) were employed. Significance was taken as follow: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

All data have been reproduced at least three times in separate and independent experiments, and data are reported as average ± S.D.

3. Results

3.1. The polyQ tract negatively affects cell viability and cell proliferation of dopaminergic SH-SY5Y cells

SH-SY5Y cells transfected with the plasmid empty (SHAM) or encoding the wild-type full length Htt (Q21-FL) or the mutant polyQ Htt (Q113-FL, mHtt) expressed the protein at relatively high level for up to 72 h (Supplementary Fig. S2A). However, the transgenic expression of the chimeric GFP-tagged N-terminal fragments bearing the Q21 tract or the mutant Q150 tract (N171Q21-GFP and N171Q150-GFP, respectively) was maximal at 24 h post-transfection and thereafter declined (Supplementary Fig. S2A). Therefore, in the next experiments, the phenomena were described at 24–48 h post-transfection. The transgenic expression of Q113-FL Htt, but not that of the Q21-FL Htt (considered as the normal wild-type Htt), affected cell viability and cell reproduction (Dt was increased) of dopaminergic human neuroblastoma SH-SY5Y cells, as assayed by counting the viable cells, the trypan blu-stained cells

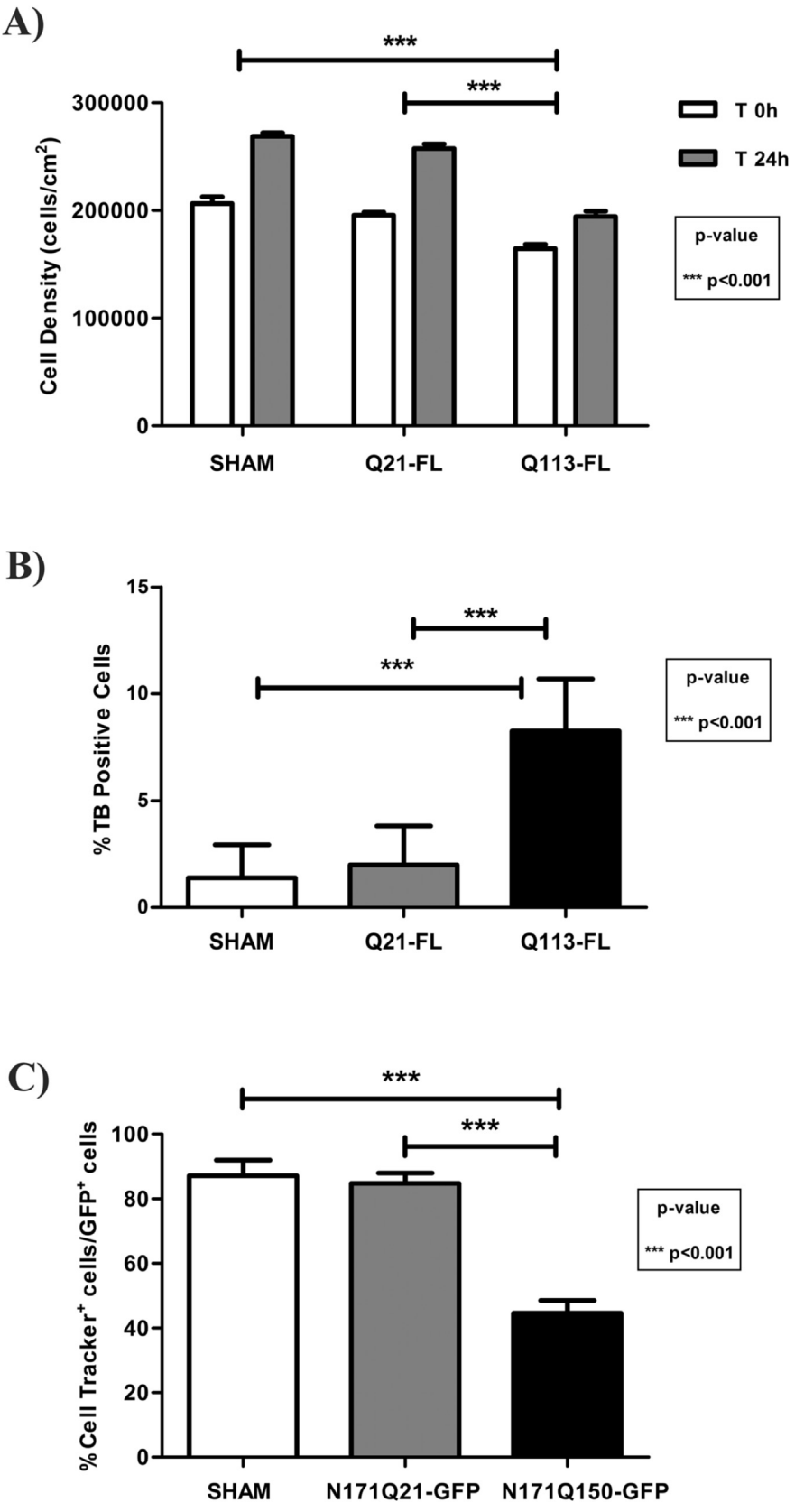

Fig. 1. Ectopic expression of mutant Q113-FL Htt affects the growth and viability of dopaminergic neuroblastoma SH-SY5Y cells. SH-SY5Y cells were plated on sterile plastic dishes, transfected with pcDNA3 (SHAM), Q21-FL, or Q113-FL encoding plasmids, then further incubated with complete medium for 24 h. (A) At the end of the incubation, cell culture growth was determined by cell counting. Cell growth (measured as the difference between cell density at T24 and T0) is much lower in Q113-FL transfected cultures than in sham and Q21-FL transfected cultures. (B) Cell death (necrosis) was assessed by trypan blue staining. (C) At the end of treatment, cells were labeled with CellTracker to assess cell viability. Data in this figure are from three separate experiments. Data are given as average ± S.D. Statistical analysis demonstrates that the effect of mHtt expression, compared to that of sham and normal Htt, on cell culture growth, necrosis and cell survival is significant.

and the CellTracker-positive cells (Fig. 1). At 48 h post-transfection, the doubling time of SH-SY5Y cultures expressing the Q113-FL Htt was two-folds longer than that of SHAM or Q21-FL Htt transfected counterparts, and the final cell density was reduced by some 30% (data not shown). Consistently, immunofluorescence staining showed an increased expression of p21 and a decreased expression of Ki-67 in the cells expressing the Q113-FL (data not shown). To see whether the polyQ tract was responsible for such toxic effect, we assayed cell viability upon transfection with the N-terminal portion bearing the Q21 or the Q150 tract. As assayed by CellTracker staining, the cells expressing the N171Q150-GFP protein showed a reduced vitality, while SHAM-GFP and N171Q21-GFP transfected cells were fully viable (Figs. S2B and S2C). Taken together, we conclude that the over-expression of mHtt with a polyQ tract is *per se* toxic to dopaminergic neuroblastoma cells.

3.2. The ectopic hyper-expression of Q113-FL mutant Huntingtin sensitizes dopaminergic neuroblastoma cells to Dopamine toxicity

We then tested the hypothesis that dopaminergic cells expressing the mutant Htt are more frail and sensitive to DA excitotoxicity. The SH-SY5Y cells were transfected with the plasmids coding for the wild-type or the mutant polyQ Htt and their viability was assessed after a 24 h exposure to DA. A parallel set of cells was pre-incubated with the pan-caspase inhibitor z-VAD-fmk in order to assess the possible onset of apoptosis. Based on cytofluorometry analysis, upon exposure to DA the fraction of SubG1 cells (considered as apoptotic) was increased in the cultures expressing the Q113-FL mHtt, compared to SHAM and Q21-FL Htt cultures, and this toxic effect was not observed when the caspases were inhibited (data not shown). Consistently, cleaved active caspase 3 was detected in the cells expressing the Q113-FL mHtt exposed to DA (not shown). Upon exposure to DA, the proportion of necrotic (trypan blu positive) cells increased, and in parallel the number of adherent viable cells decreased, in the cultures transfected with the Q113-FL mHtt plasmid compared to that of SHAM or Q21-FL transfected cultures (Fig. 2). To see whether the polyQ tract is itself toxic and to ensure that indeed necrosis occurred in the transfected cells, the SH-SY5Y cells were transfected with the plasmid encoding for the GFP-tagged N-terminal Htt bearing the Q21 or the Q150 and then necrotic cells were revealed by PI staining (the cells being not fixed). Representative images of this experiment are shown Fig. S3, and its quantitation is illustrated in the graph of Fig. 2C. It is apparent that cell necrosis occurs in the cells expressing the N-terminal polyQ tract of Htt upon exposure to DA, and that such death can be prevented by the pan-caspase inhibitor, indicating that necrosis is secondary to apoptosis.

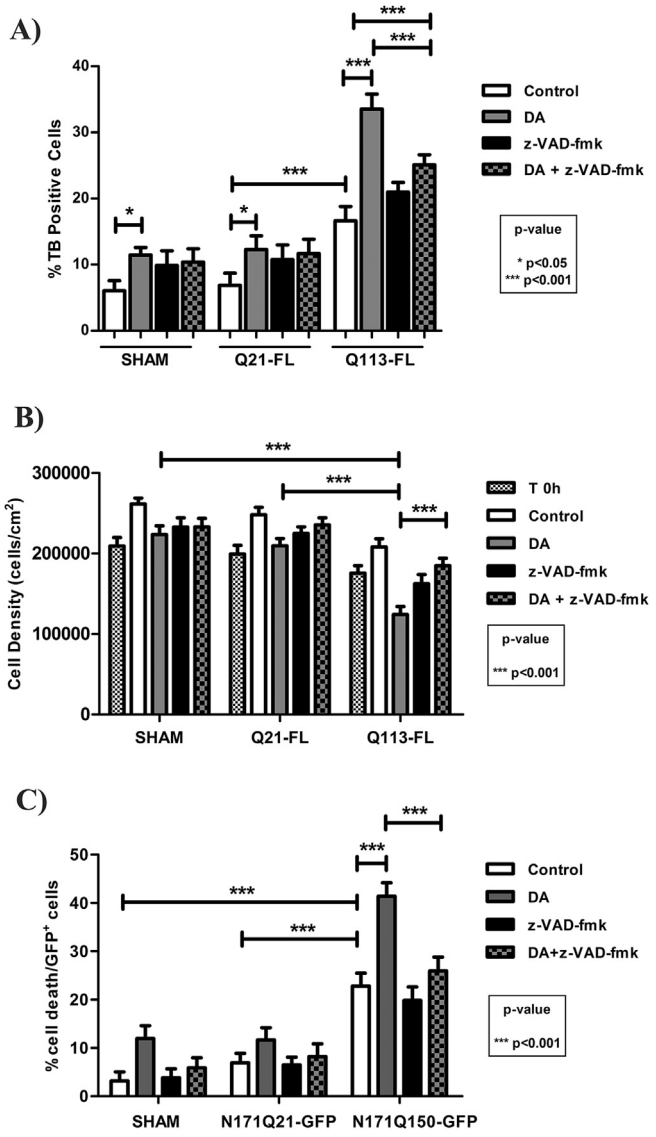

Fig. 2. Dopamine induces cell apoptosis in polyQ Htt expressing SH-SY5Y neuroblastoma cells. SH-SY5Y cells adherent on sterile plastic dishes, were transfected with pcDNA3 (SHAM), Q21-FL, Q113-FL, then incubated with 100 μM DA in the presence or absence of the pancaspase inhibitor z-VAD-fmk for 24 h. At the end of the treatment, cell necrosis was assessed by trypan blue staining (A) and cell density in the cultures was determined by cell counting (B). (C) SH-SY5Y cells were plated on coverslip, transfected with pEGFP-C2 (SHAM-GFP), N171Q21-GFP, N171Q150-GFP, then incubated with 100 μM DA in the presence or absence of the pancaspase inhibitor z-VAD-fmk for 24 h. At the end of the treatment, cell death (necrosis) was assessed by PI staining in not-fixed cells. Only transfected cells (GFP positive) were considered. Data in this figure arise from four separate replicates and are presented as average ± S.D. Statistical analysis demonstrates that the deleterious effect of DA and the protective effect of z-VAD-fmk effect on cell culture growth, necrosis and cell survival in mHtt expressing cultures is significant.

3.3. Dopamine impairs autophagy and mutant Huntingtin degradation

Autophagy-mediated clearance of aggregates-prone mutant proteins preserves the neuronal vitality and function (Castino et al., 2005a; Sarkar et al., 2007; Heiseke et al., 2009). The mHtt itself has been reported to negatively affect the autophagy process (Martinez-Vicente et al., 2010; Rui et al., 2015). First, we checked whether in our experimental model Htt was indeed targeted to the autophagy-lysosomal system. The lysosomal protease cathepsin D (CD) that has been shown to effect the proteolysis of Htt (Qin et al., 2003). In the experiment, we have therefore included Pepstatin A, its specific inhibitor. The images in Fig. 3A and B show that both the 'wild-type' and the mutant Htt co-localize with LC3 and with CD, markers of autophagosomes and lysosomes, respectively. It is also apparent that the fluorescence staining of the mutant Htt (Q113-FL) increases in the cells exposed to Pepstatin A. Taken together, these data indicate that Htt is translocated in the autophagy-lysosomal compartments. Next, we investigated whether and how DA could interfere with the autophagy flux in the cells expressing the mHtt. To this end, the cells expressing either the wild-type (Q21-FL) or the mutant (Q113-FL) Htt and exposed (or not) to DA were stained for LC3 and LAMP1 (the latter is a marker of endosomes and lysosomes). The images in Fig. 3C prove that upon exposure to DA the co-localization of these two markers (indicative of autolysosome formation) is drastically reduced, along with the single staining of

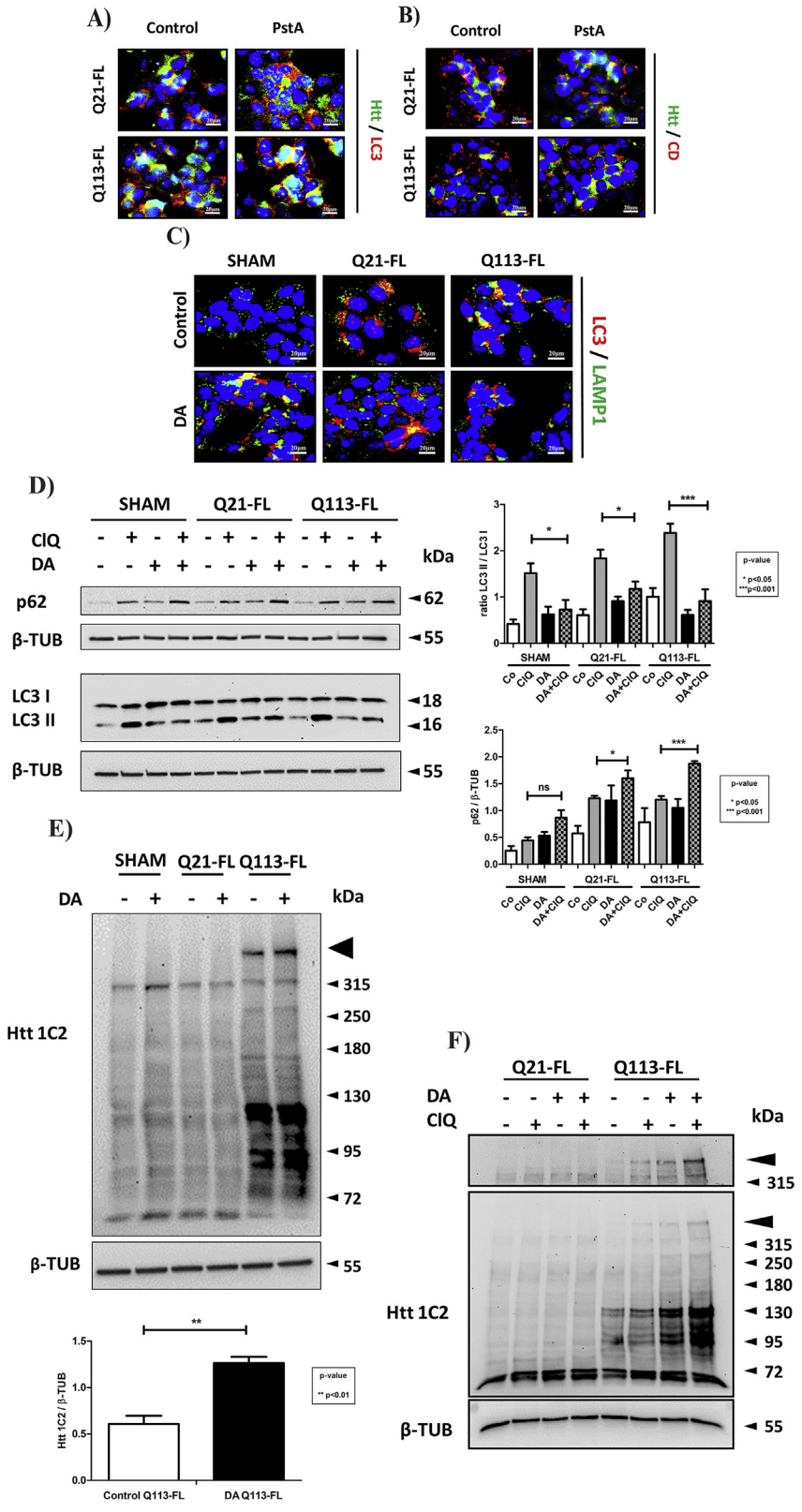

Fig. 3. Dopamine impairs autophagosome formation and Htt clearance in dopaminergic cells expressing the polyQ Htt. A-B-C) SH-SY5Y cells plated on coverslips were transfected with Q21-FL or Q113-FL plasmids, then exposed (or not) for 24 h to 100 μM Pepstatin A (PstA) or to 100 μM DA as indicated. The cells were then stained for immunofluorescence labeling of Htt, LC3, Cathepsin D (CD) and LAMP1, as indicated. The images demonstrate that: 1. Htt co-localizes with autophagy-lysosomal markers (LC3, CD); 2. Inhibition of CD results in increased labeling of Htt; 3. DA treatment result in a decreased number of LC3-stained vacuoles. Representative images of three separate experiments are shown. D-E-F) SH-SY5Y cells plated on petri dishes were transfected with SHAM, Q21-FL and Q113-FL plasmids, and exposed to 100 μM DA in the presence or absence of 30 μM ClQ for 24 h, as indicated. At the end of the incubation, the cell homogenates were separated by SDS-PAGE and probed by western blotting for the protein indicated. D) Expression of the autophagic markers LC3 and p62. Densitometric analysis of four separate experiments is included. Data are presented as average ± S.D. Statistical analysis demonstrates that the inhibitory effect of DA on autophagosome formation (LC3-II/LC3-I) and autophagy flux (p62/Tubulin) is significant, especially in the cells expressing the Q113-FL mutant Htt. E) The accumulation of Htt related peptides was also revealed by western blotting using the antibody HTT1C2. Densitometry analysis of three separate experiments indicates that the level of Q113-FL (Htt/Tubulin) doubles in the cells exposed to DA. F) A parallel set of Q21-FL and of Q113-FL transfected cells was incubated with Chloroquine (ClQ) and the cell homogenates was assayed for Htt peptides accumulation by western blotting with the antibody HTT1C2. Data indicate that ClQ synergizes with DA in causing the accumulation of undegraded mHtt (a darker exposure of the blot is shown in the upper part of the panel).

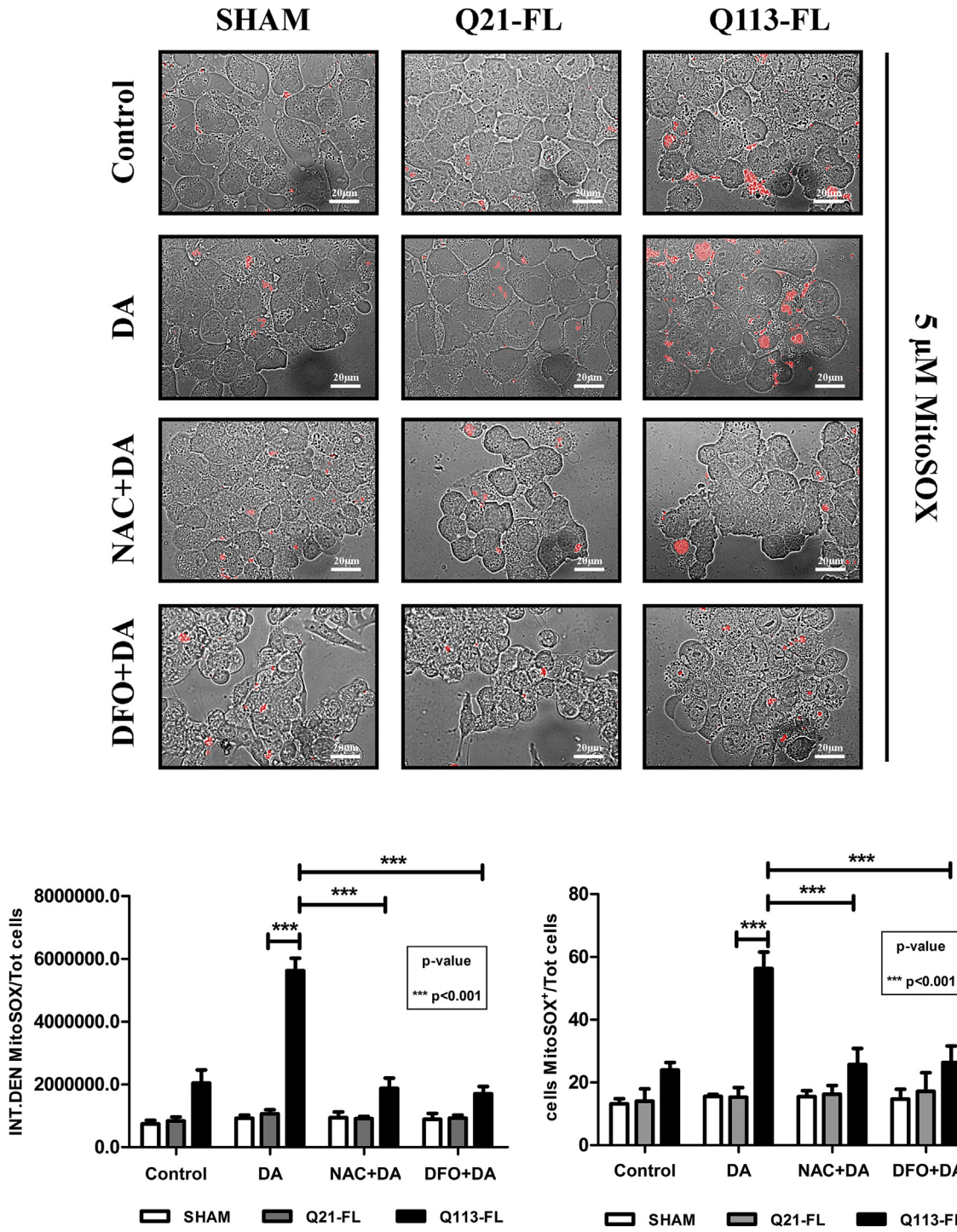

Fig. 4. NAC and DFO prevent the generation of mitochondrial anion superoxide by Dopamine in Q113-FL Htt expressing SH-SY5Y cells. The cells adherent on coverslip were transfected with the SHAM, Q21-FL and Q113-FL plasmids, then exposed to 100 μM DA in the presence or absence of 2 mM NAC or 1 mM DFO for 24 h. At the end, the cells were stained with MitoSox, which reveals the presence of anion superoxide. Representative images shown (from four separate experiments) were taken under the fluorescence microscope. The ImageJ quantification of fluorescence (average ± S.D.) is reported. Statistical analysis demonstrates that the effect of DA on the induction of oxidative stress, and the protective effect of NAC and of DFO, in Q113-FL transfected cells are significant.

LC3 (marker of autophagosomes), in the cells expressing the mHtt. To better assess the potential inhibitory effect of DA on the autophagy process, we analyzed the rate of conversion of LC3-I (localized in the cytoplasm) into LC3-II (the lipidated isoform that localizes on the autophagosomal membranes). To follow the net production of autophagosome in the various experimental conditions, in parallel samples we included Chloroquine (ClQ) that prevents the degradation of autophagosomes, thus allowing the accumulation of all the autophagosomes produced in the incubation time (Klionsky et al., 2016). Assuming the ratio LC3II/LC3I as a measure of autophagosomes (Klionsky et al., 2016), it appears that the expression of the Q113-FL induces the autophagy process, at least at the first step of autophagosome formation (compare the samples with ClQ in Fig. 3D). The increased production of

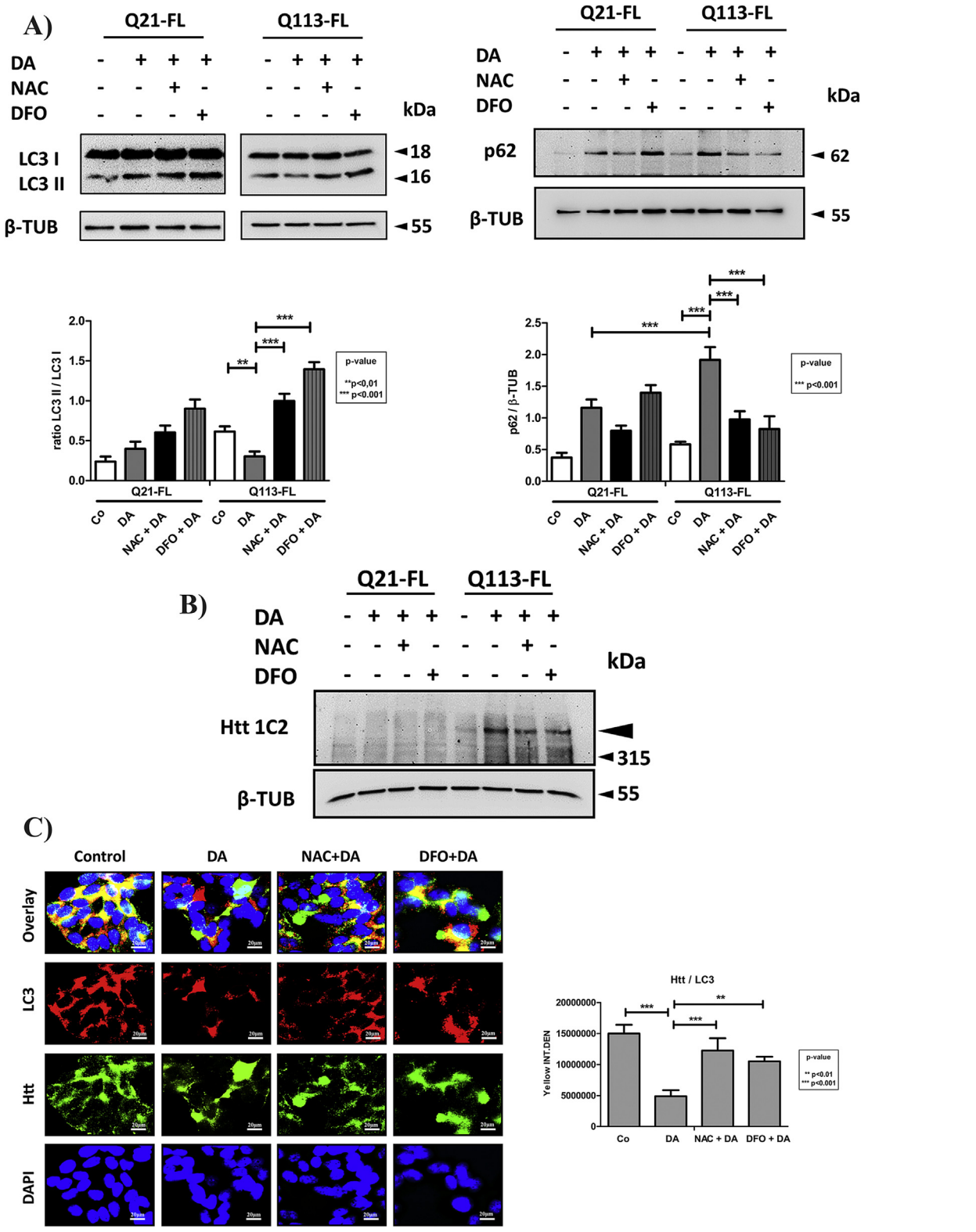

Fig. 5. NAC and DFO restore autophagy and Htt clearance in Dopamine-treated cells expressing the Q113-FL Htt. SH-SY5Y cells were transfected with the plasmid encoding for the Q21-FL or Q113-FL Htt and then incubated with 100 μM DA in the presence or absence of 2 mM NAC or 1 mM DFO for 24 h. At the end, the cell homogenate was assayed by western blotting for LC3 and p62 (A) and for Htt peptides (B). A) Representative immunoblots of LC3 and of p62 showing that DA indeed impairs the autophagosome formation and autophagy flux, while NAC and DFO exert a protective action. Densitometry of three separate experiments is included. Data are presented as average ± S.D. Statistical analysis demonstrates that the effects of DA, NAC and DFO described above are significant, and occur more specifically in cells expressing the Q113-FL Htt. B) Representative immunoblot of Htt showing that NAC and DFO treatments can promote the degradation of mHtt even in the presence of DA. (C) The cells adherent on coverslip were transfected with the Q113-FL plasmid and treated with DA, NAC and DFO as above, and then processed for immunofluorescence staining of LC3 and Htt. Images representative of three separate experiments as shown. The ImageJ quantification of fluorescence co-staining (average ± S.D.) is reported. Statistical analysis demonstrates that the negative effect of DA on the co-localization of Htt and LC3, and the counter effect of NAC and of DFO, in Q113-FL transfected cells are significant.

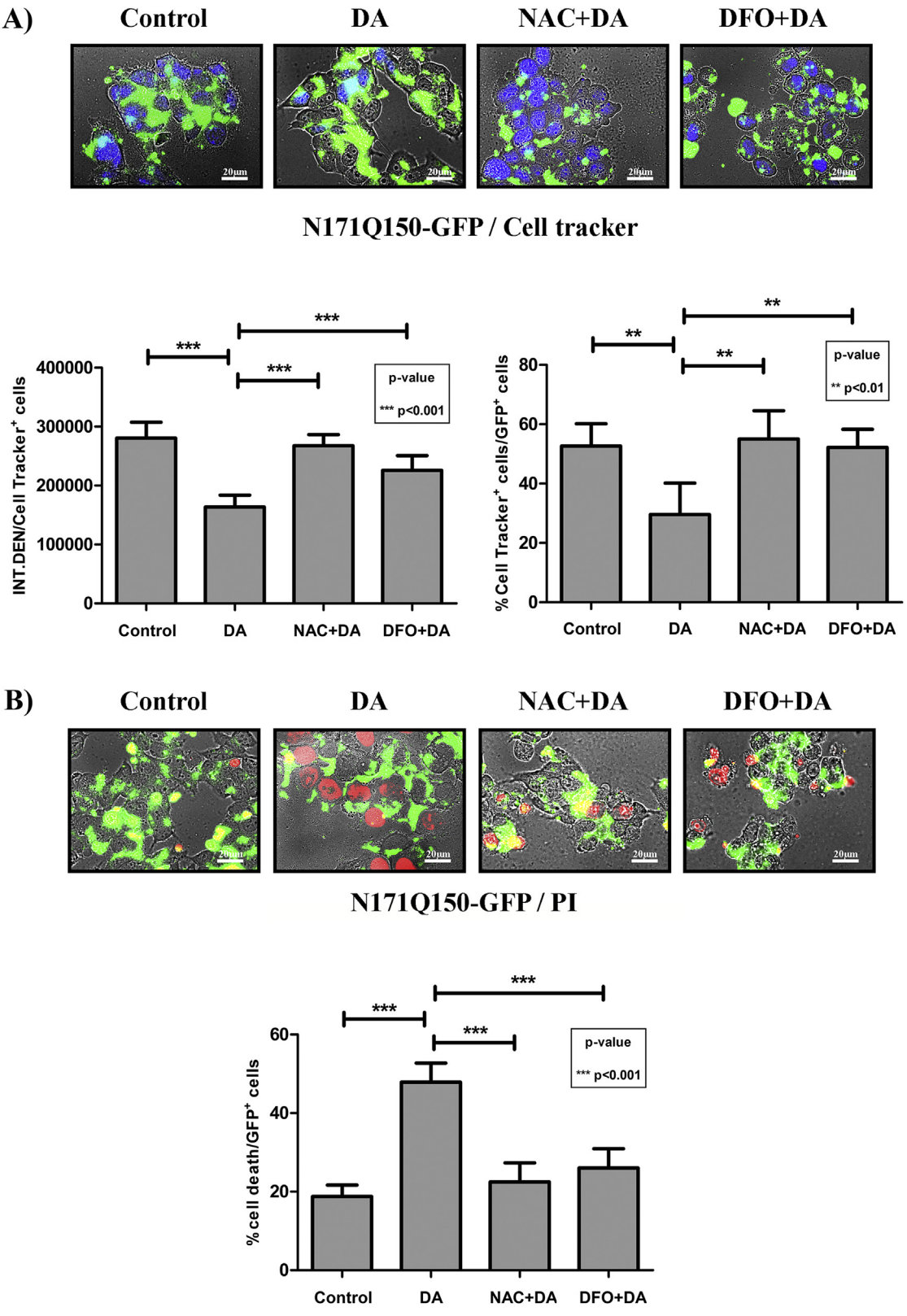

Fig. 6. NAC and DFO protect from dopamine toxicity the dopaminergic cells expressing the Q150 N-fragment of Htt. The cells adherent on coverslip were transfected with the plasmid encoding the GFP-tagged Q150N-fragment of Htt and incubated with 100 μM DA in the presence or absence of 2 mM NAC or 1 mM DFO for 24 h. At the end, the cells were stained with CellTracker (A) or Propidium Iodide (B) to check for cell viability or necrosis, respectively. Images representative of four separate experiments are shown. The quantitative analysis of fluorescence (average ± S.D.) performed with ImageJ is included. Only transfected cells (GFP positive) were considered. Statistical analysis demonstrates that the deleterious effect of DA on cell survival, and the protective effect of NAC and of DFO, are significant.

autophagosomes is however not observed in the cultures exposed to DA (Fig. 3D). In all conditions DA precludes the consumption of LC3II, suggesting a block of the autophagy flux. Monitoring the autophagy flux as ratio of SQSTM1/p62 degradation leads to the same conclusion (Fig. 3D). Finally, we investigated the functional consequences of DA effects on the autophagy clearance of Htt aggregates. As shown by western blotting, DA treatment led to an accumulation of the high molecular weight Htt-related products in the cells transfected with the Q113-FL plasmid (Fig. 3E). This effect was additive to that of ClQ, confirming that degradation of the mHtt occurs in acidic compartments, namely the lysosomes (Fig. 3F). These data strongly indicate that DA negatively affect the autophagy clearance of mHtt.

3.4. Reactive oxygen species mediate the Dopamine impairment of the autophagy process

The relationship between oxidative stress and autophagy is complex, being cell context and ROS type dependent. While low level of hydrogen peroxide has been shown to induce autophagy (Scherz-Shouval et al., 2007; Castino et al., 2010, 2011; Higgins et al., 2011; Oh et al., 2012), high level of anion superoxide may elicit the opposite effect (Janda et al., 2012, 2015). Anion superoxide is formed at mitochondrial level and can be monitored with the fluorescent dye MitoSOX (Castino et al., 2010). We first labeled the mitochondria with Rhodamine 123, which accumulates in the intermembrane space of healthy mitochondria and fluoresces in red if their membrane potential is preserved. We found that the expression of the Q113-FL mHtt *per se* was toxic to mitochondria, and the toxicity in these cells was exacerbated by DA (Supplementary Fig. S4). Mitochondrial function in these cultures was rescued when the cells had been pre-incubated with NAC or DFO, which are well known to prevent the formation of ROS (Papa and Rockwell, 2008; Castino et al., 2007, 2011; Zhang et al., 2009; Janda et al., 2015; Jiao et al., 2016). To confirm the involvement of anion superoxide in the toxic effect of DA, we stained the cultures with MitoSOX. The images in Fig. 4 clearly demonstrate that in the cells expressing the mutant form of Htt (Q113-FL) DA indeed stimulates the production of anion superoxide and that this could be prevented by NAC or DFO. Next we checked if DA-induced ROS are directly responsible for the impairment in autophagosome formation. To this end, we assayed the production and accumulation of LC3II and measured the degradation of p62 in the cells transfected with the Q21-FL (wild type) or the Q113-FL (mutant) Htt and exposed to DA in the absence or the presence of NAC or DFO. The western blotting shown in Fig. 5A demonstrates that in the cells DA indeed impairs the conversion of LC3I into LC3II (index of impaired formation of autophagosomes) and causes the accumulation of p62 (index of reduced degradation of autophagy substrates). Of note, the inhibitory effect of DA was especially evident in the cells expressing the mHtt. Remarkably, when the cells were pre-incubated with NAC or DFO the deleterious effects of DA on the autophagy process were completely abrogated (Fig. 5A). These effects reflected on the degradation of mHtt in that DA led to an accumulation of un-degraded Htt, while both NAC and DFO could rescue the ability to degrade this molecule (Fig. 5B and Supplementary Fig. S5B). To confirm that degradation of mHtt occurred via autophagy, we double stained the cells for the transgenic mHtt and for LC3. The images in Fig. 5C shows that in untreated cells mHtt and LC3 co-localize, suggesting that Q113-FL mutant Htt can be sequestered within the autophagy-lysosomal compartments and that in DA-treated cells this co-localization is less apparent and mHtt accumulates in the cytoplasm (consistent with data in Fig. 3A–C). To be noted, the pre-incubation with either NAC or DFO counteracts the negative effects of DA on autophagy (Fig. 5C).

3.5. Preventing ROS generation saves the dopaminergic cells expressing mHtt from Dopamine toxicity

The above data showed that DA caused toxicity in dopaminergic neuroblastoma cells expressing the mHtt and concomitantly induced the generation of anion superoxide and inhibited the formation of autophagosomes, which resulted in the accumulation of mHtt. We also showed that NAC and DFO could prevent the ROS generation and relief the inhibition on the autophagy system. We then checked whether the pre-treatment with NAC or DFO could save the dopaminergic cells expressing the polyQ N-terminal fragment of Htt from DA toxicity. To this end, we have transfected SH-SY5Y cells with the plasmid bearing the GFP-tagged N-fragment with Q150 and then checked for cell viability (by CellTracker staining) and necrosis (by PI staining) in non-fixed cultures. The images shown in Fig. 6 clearly demonstrate that both NAC and DFO can preserve cell viability (panel A) and limit necrosis (panel B) in the cultures expressing the polyQ fragment and exposed to DA. Quantification of viability-associated fluorescence with ImageJ software confirmed that the polyQ N-terminal fragment is *per se* toxic and that DA exacerbates this toxicity. However, the pre-incubation with NAC or DFO greatly reduces the toxicity by DA, though cannot completely avoid the intrinsic toxicity due to the expression of the polyQ fragment. The protective effect of NAC and DFO could be evidenced by counting the proportion of viable (blue-stained) cells, as well as the relative intensity of the blue staining in the cells. In numbers, DA treatment caused an increase of cell death from a basal value of approximately 20% up to approximately 50%, and NAC and DFO essentially prevented this increase.

4. Discussion

HD patients present with chorea and Parkinson-like motor symptoms, in addition to cognitive deficits and psychiatric disturbances. Dopamine, an excitotoxic neurotransmitter, is clearly involved in the altered coordination of movement in HD patients. Time-dependent changes in DA transmission parallel biphasic changes in symptomatology. In the early stage of the disease, increased DA stimulation leads to hyperkinetic movements, while in the late stage of the disease a decrease in DA availability causes hypokinesia (Chen et al., 2013; Cepeda et al., 2014). Neurodegeneration in HD is due to the expression of a mutated Htt protein with an abnormal expansion of glutamine (polyQ tract) at the N-terminus (Rubinzstein, 2002), which likely renders the neurons (particularly the MSNs) in the striatum and other brain region frail and susceptible to die upon pro-oxidant injury. Oxidative stress is a well known cause of neurodegeneration (Janda et al., 2012). The continuous generation of ROS within the cells may have various deleterious effects, including the permeabilization of lysosomal and mitochondrial membranes with ensuing apoptosis (Castino et al., 2007; Papa and Rockwell, 2008), the altered folding of proteins with consequent formation of protein macro-aggregates (Castino et al., 2008a; Nakamura et al., 2012; Halloran et al., 2013), and the inhibition of autophagy process (Janda et al., 2015). Effective autophagy allows dopaminergic neuronal cells to face with oxidative stress (Castino et al., 2008a). Neuronal cells expressing mutated aggregate-prone proteins are clearly more susceptible to such damage caused by oxidative stress (Janda et al., 2015; Isidoro et al., 2009). In particular, the impairment of autophagy may have dramatic effects in diseased neurons, since this pathway is devoted to the clearance of unfolded protein aggregates and of leaky mitochondria (Castino et al., 2008b; Cherra et al., 2010). Yet, the sustained hyper-induction of autophagy is deleterious as well to

neurons (Cherra et al., 2010), especially if the neurons are already subjected to an endogenous stress that maintains autophagy at high level. The double-hit hypothesis well illustrates this case (Castino et al., 2005b). It has been shown that neurons expressing a truncated aggregate prone mutant of Vasopressin can survive thanks to the up-regulation of autophagy that continuously clear the aggregates (Castino et al., 2005a). Yet, these cells precipitate into apoptosis when they are stimulated with an excitotoxic stimulus (e.g., DA) that increases further autophagy (Castino et al., 2005b). In HD neurons autophagy appears to be insufficient, since drugs that stimulate autophagy elicit beneficial effects in clearing Htt and thus preventing cell death '*in vitro*' (Bjørkøy et al., 2005; Qin et al., 2003), and in relieving the symptoms in HD animal models (Sarkar and Rubinsztein, 2008; Fusco et al., 2012). Once delivered to lysosomes, Htt is proteolytically processed by lysosomal cathepsins B and D (Qin et al., 2003). Important to note, Htt itself promotes the autophagy sequestration and clearance of aggregates by interacting with p62/SQSTM1 and with ULK1 (Rui et al., 2015). Conversely, mHtt negatively affects the p62-mediated sequestration of autophagy substrates (Martinez-Vicente et al., 2010). Thus, it is reasonable to assume that neuronal cell death in HD neurons is a consequence of an impairment of the autophagy flux. Here we show that DA stimulation can indeed cause an impairment of autophagy and thereafter precipitate cell death in dopaminergic neuroblastoma cells expressing the mutant Htt at high level. This is at variance of what we have previously observed in dopaminergic cells expressing the truncated mutant Vasopressin (Castino et al., 2005b), indicating that the outcome of DA treatment is cell-context, likely depending on the type and subcellular localization of the mutated protein. In fact, mutated Vasopressin forms aggregates within the endoplasmic reticulum and induces a raise of basal autophagy (Castino et al., 2005a), whereas Htt locates in the cytoplasm. Also, different types of ROS may elicit different effects depending on the genetic background of the cell. Oxidative stress could be an effective cause of autophagy impairment. We have previously shown that mitochondrial ROS (particularly anion superoxide) inhibit the conversion of LC3-I into LC3-II, thus impairing autophagosome formation and degradation, despite the concurrent activation of several pro-autophagic signaling pathways (Janda et al., 2015). Similarly, a block of the autophagic flux along with up-regulation of ATG proteins has been reported in neurons expressing mutant Htt (Heng et al., 2010). It was already reported that elevated NADPH oxidase activity increases the production of ROS and favors the onset of cell death in Htt expressing neurons (Valencia et al., 2013). Consistently, we found that DA induces the production of anion superoxide (associated with dysfunctional mitochondria) that eventually cause the impairment of autophagosome formation. In fact, in DA-treated cells expressing the mHtt or the polyQ N-fragment, NAC and DFO could prevent the generation of such ROS and allowed the induction of protective autophagy. DFO was already shown to protect dopaminergic neuroblastoma cells from oxidative stress (Castino et al., 2007, 2011), as well as striatal neuron from dopaminergic toxicity (Haleagrahara et al., 2013). Similarly, NAC has been proven to prevent mitochondrial dysfunction in '*in vitro*' HD models (Sandhir et al., 2012) and to delay the onset of motor deficits in HD animal models (Wright et al., 2015). In conclusion, the present study provides a mechanistic explanation for the dopaminergic cell toxicity observed in HD patients, and gives further support to the potential of anti-oxidant therapeutics to arrest the progression of the disease (Melone et al., 2005).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuint.2016.11.003.

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