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**Modulation of neuronal mitochondrial
dynamics, autophagy and huntingtin proteostasis by
HDAC inhibitors: Insights for Huntington's disease**

**Thesis for Doctor Degree in Pharmaceutical Sciences
Pharmacology and Pharmacotherapy**

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Scientific communications

Articles:

1. **Guedes-Dias, P.** and Oliveira, J.M. (2013) Lysine deacetylases and mitochondrial dynamics in neurodegeneration. *Biochimica et Biophysica Acta – Molecular Basis of Disease* 1832(8):1345-59
2. **Guedes-Dias, P.**, de Proença, J., Soares, T.R., Leitão-Rocha, A., Duchen, M.R., Oliveira, J.M. (2015) HDAC6 inhibition induces mitochondrial fusion, autophagic flux and reduces diffuse mutant huntingtin in striatal neurons. *Submitted*

Selected communications by the candidate at scientific conferences:

- **Guedes-Dias, P.**, Leitão-Rocha, A., de Proença, J., Soares., T.R., Duchen, M.R. and Oliveira, J.M. (2015) HDAC6 inhibition allows neuronal autophagic turnover and mutant huntingtin clearance @ XVIII Congress of the Portuguese Biochemical Society – Coimbra, Portugal (poster communication) – Awarded both the Best Poster Prize and the FEBS Journal Best Poster Prize
- **Guedes-Dias, P.**, Duchen, M.R. and Oliveira, J.M. (2014) Mitochondrial dynamics, autophagy and mutant huntingtin aggregation are differentially regulated in cortical and striatal neurons: effects of HDAC6 inhibition @ Neuroscience 2014, Society for Neuroscience Annual Meeting – Washington D.C., U.S.A. (poster communication)
- **Guedes-Dias, P.**, Duchen, M.R. and Oliveira, J.M. (2013) Differential mitochondrial dynamics in cortical and striatal neurons – an epigenetic approach @ FinMIT Nordic Symposium – Vaaksy, Finland (poster communication)
- **Guedes-Dias, P.** and Oliveira, J.M. (2012) Epigenetic drugs differentially modulate mitochondrial dynamics and metabolism in cortical and striatal neurons @ 8th FENS Forum of Neuroscience – Barcelona, Spain (poster communication)

Abstract

Histone deacetylase (HDAC) inhibitors are protective in several models of neurodegenerative diseases. Specifically, HDAC inhibition enhanced mitochondrial Ca^{2+} handling in Huntington's disease (HD) cell models – which may prove beneficial for striatal neurons, given their sensitivity to Ca^{2+} -loads. HD is associated with mitochondrial dysfunction, mitochondrial fragmentation and trafficking impairment. Pharmacological modulation of mitochondrial dynamics is considered a promising strategy to counteract mitochondrial dysfunction and modify HD progression. Inhibition of the α -tubulin deacetylase HDAC6 is known to increase mitochondrial trafficking, but whether inhibiting HDACs in neurons modulates mitochondrial biogenesis or fission-fusion balance remains to be addressed. Additionally, increasing α -tubulin acetylation by inhibiting HDAC6 may also promote transport of autophagic vesicles and consequently elevate neuronal autophagic flux. However, given that HDAC6 activity is reportedly required for autophagosome-lysosome fusion in cell-lines, the impact of HDAC6 inhibition for the autophagic pathway in neurons is unclear. In this thesis, we assessed the effects of HDAC1 inhibitor MS-275, and HDAC6 inhibitor tubastatin, on mitochondrial dynamics in cortical and striatal neurons. We also tested whether HDAC6 inhibition blocks autophagy and mutant huntingtin (mHtt) clearance in neurons. Our results show that neither HDAC1 nor HDAC6 inhibition altered mitochondrial biogenesis. HDAC6 inhibition increased α -tubulin acetylation, which promoted mitochondrial motility and fusion in HD-vulnerable striatal neurons – a promising finding, since striatal mitochondria were smaller and less motile than mitochondria in the less HD-vulnerable cortical neurons. HDAC6 inhibition did not block neuronal autophagosome-lysosome fusion or mHtt clearance but instead, increased LC3-vesicle retrograde flux and reduced diffuse mHtt levels in striatal neurons. Results from this thesis suggest that HDAC6 inhibition may be a promising pharmacological strategy to reduce striatal neurons vulnerability in HD.

Keywords: HDACs, mitochondria, autophagy, neurons, huntingtin

Resumo

Os inibidores das desacetilases de histonas (HDACs) são protetores em vários modelos de doenças neurodegenerativas. Especificamente, a inibição das HDACs melhorou o controlo mitocondrial de Ca^{2+} em modelos celulares da doença de Huntington (DH) – o que poderá ser benéfico para os neurónios estriatais, devido à sua sensibilidade a cargas de Ca^{2+} . A DH está associada a disfunção mitocondrial, fragmentação e distúrbios no tráfego mitocondrial. A modulação farmacológica da dinâmica mitocondrial é considerada uma estratégia promissora para contrariar a disfunção mitocondrial e modificar a progressão da DH. Sabe-se que a inibição da α -tubulina desacetilase HDAC6 aumenta o tráfego mitocondrial, mas continua por ser testado em neurónios se a biogénese mitocondrial ou o equilíbrio fissão-fusão são modulados pela inibição das HDACs. Adicionalmente, o aumento da acetilação da α -tubulina através da inibição da HDAC6 poderá também promover o transporte das vesículas autofágicas e consequentemente elevar o fluxo autofágico neuronal. No entanto, visto que se encontra documentado que a atividade da HDAC6 é necessária para a fusão autofagossoma-lisossoma em linhas-celulares, o impacto da inibição da HDAC6 para a via autofágica em neurónios não é claro. Nesta tese, abordámos os efeitos do inibidor da HDAC1 MS-275, e do inibidor da HDAC6 tubastatina, sobre a dinâmica mitocondrial em neurónios corticais e estriatais. Testámos também se a inibição da HDAC6 bloqueia a autofagia e a degradação de huntingtina mutante (mHtt) em neurónios. Os nossos resultados mostram que nem a inibição da HDAC1 ou da HDAC6 alteraram a biogénese mitocondrial. A inibição da HDAC6 levou a um aumento da acetilação da α -tubulina, o que estimulou a motilidade e fusão mitocondrial em neurónios estriatais sensíveis à DH – um resultado promissor, uma vez que as mitocôndrias estriatais eram mais pequenas e menos móveis que as mitocôndrias em neurónios corticais, menos sensíveis à DH. A inibição da HDAC6 não bloqueou a fusão autofagossoma-lisossoma ou a degradação da mHtt mas, ao invés disso, aumentou o fluxo retrógrado das vesículas LC3-positivas e reduziu os níveis de mHtt difusa em neurónios estriatais. Os resultados desta tese sugerem que a inibição da HDAC6 poderá ser uma estratégia farmacológica promissora para reduzir a vulnerabilidade dos neurónios estriatais na DH.

Palavras-chave: HDACs, mitocôndria, autofagia, neurónios, huntingtina

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List of abbreviations

List of abbreviations

Atg	Autophagy-related protein
ATP	Adenosine-5'-triphosphate
CAG	Glutamine(-encoding trinucleotide)
DD	Deacetylase domain
DFCP1	Double FYVE-containing protein 1
DNA	Deoxyribonucleic acid
DMB	Dynein motor binding
Drp1	Dynamic-related protein 1
FEZ1	Fasciculation and elongation protein zeta 1
Fis1	Fission 1 protein
GABA	γ -aminobutyric acid
GTP	Guanosine triphosphate
HD	Huntington's disease
HDAC	Histone deacetylase
HDAC1	Histone deacetylase isoform 1
HDAC2	Histone deacetylase isoform 2
HDAC6	Histone deacetylase isoform 6
HDAC8	Histone deacetylase isoform 8
Hsp90	Heat shock protein 90
htt	huntingtin
mHtt	mutant huntingtin
IC50	Half maximal inhibitory concentration
JIP1	JNK-interacting protein 1
JNK	c-Jun N-terminal kinase
LC3	Microtubule-associated protein 1A/1B-light chain 3
Mff	Mitochondrial fission factor

List of abbreviations

Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MiD49	Mitochondrial dynamic protein of 49 kDa
MiD51	Mitochondrial dynamic protein of 51 kDa
mRNA	messenger Ribonucleic acid
MS-275	Entinostat
MSN	Medium spiny neurons
NAD ⁺	Nicotinamide adenine dinucleotide
NES	Nuclear export signal
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
OPA1	Optic atrophy 1
p62	Sequestosome 1 (SQSTM1) of 62 kDa
PGC-1 α	Peroxisome-proliferator activator receptor- γ (PPAR- γ) co-activator 1 α
RanBP2	Ran-binding protein 2
SAHA	Vorinostat
SE14	Ser-Glu-containing tetradecapeptide
SIRT1	Sirtuin isoform 1
TBA	Tubastatin A
TSA	Trichostatin A
TRAK	Trafficking kinesin protein
ZnF-UBP	Zinc-finger motif

Thesis outline

This thesis is divided in four main sections:

Section 1 – Background and hypotheses

Brief literature review to contextualize this thesis research objectives.

Section 2 – Research

The two articles developed within the scope of this thesis.

1 – (Review article) Guedes-Dias, P. and Oliveira, J.M. (2013) Lysine deacetylases and mitochondrial dynamics in neurodegeneration. *Biochimica et Biophysica Acta – Molecular Basis of Disease* 1832(8):1345-59

2 – (Original research article) Guedes-Dias, P., de Proença, J., Soares, T.R., Leitão-Rocha, A., Duchen, M.R., Oliveira, J.M. (2015) *Submitted*

Section 3 – Discussion and future directions

Discussion of this thesis experimental findings, highlighting open questions for future investigation.

Section 4 – References

List of studies cited in Section 1 and Section 3.

Studies cited in Section 2 are listed at the end of the respective article.

Section 1

Background and hypotheses

HISTONE DEACETYLASES (HDACs)

Acetylation is a post-translational modification regulated by the opposing activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation was first described in 1964 (Allfrey et al., 1964; Murray, 1964) and histone deacetylase activity a few years later (Inoue and Fujimoto, 1969, 1970). Currently, over 1750 proteins distributed across the nucleus, cytoplasm and mitochondria are known to be acetylated in human cells (Choudhary et al., 2009). In mammals, the deacetylation of such diverse range of proteins is carried out by 18 HDAC isoforms. These may be either divided in two groups, based on yeast HDAC homology ('classical HDACs' – Zn^{2+} -dependent, and 'sirtuins' – NAD^+ -dependent), or four classes, based on phylogenetic analysis and sequence homology ('classical HDACs' comprise class I, class IIa/IIb and IV; 'sirtuins' are included in class III) (Yang and Seto, 2008).

HDAC1 is a class I isoform that is ubiquitously expressed across tissues. Typically, HDAC1 resides in the nucleus and is found associated with HDAC2 in co-repressor complexes that associate with DNA and regulate gene transcription (Sengupta and Seto, 2004). Although initially the predominant model was that histone deacetylation induced states of transcriptional repression, the current understanding is that there is an interplay between different epigenetic mechanisms which render transcription regulation rather more complex and difficult to predict (Falkenberg and Johnstone, 2014). Studies in yeast and mammalian cells revealed that HDAC1/2 knockouts were associated with the down-regulation of a significant portion of genes, indeed suggesting that HDAC1/2 activity are involved in the activation of some genes (Kelly and Cowley, 2013). Additionally, HDAC1 pharmacological inhibition was reported to reduce expression levels of fission protein Fis1 in cell-lines (Lee et al., 2012).

Constitutive HDAC1 knockout mice are embryonically lethal (~E10.5; Lager et al., 2002). In neurons, HDAC1 is reportedly involved in DNA damage repair (Dobbin et al., 2013; Wang et al., 2013) and synaptogenesis (Akhtar et al., 2009; Yamada et al., 2014), but conditional HDAC1 deletion neither alters neuronal development (Montgomery et al., 2009) nor learning and memory performances (Morris et al., 2013). Some studies have implicated HDAC1 activity as a contributing factor for neurodegeneration. In a Huntington's disease (HD) mouse model, HDAC1 was reported to be upregulated in the striatum, a particularly vulnerable brain region in HD (Bardai et al., 2012), whereas in a neuroinflammation model, HDAC1 was detected to impair mitochondrial trafficking by shuttling to the cytosol and reducing α -tubulin acetylation (Kim et al., 2010).

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HDAC6 is a class IIb isoform, highly expressed in neurons and primarily localized in the cytoplasm (Southwood et al., 2007). In humans, the HDAC6 cytoplasmic localization is achieved by two nuclear export signals (NES1 and 2) and maintained by a cytoplasmic retention Ser-Glu-containing tetradecapeptide (SE14) domain (Bertos et al., 2004). Murine HDAC6 do not present a SE14 motif and albeit containing NES1, the region corresponding to NES2 is less conserved and reported to be nonfunctional (Verdel et al., 2000; Bertos et al., 2004). The C-terminal zinc finger motif (ZnF-UBP) in HDAC6 is a unique feature among the HDAC superfamily (Simões-Pires et al., 2013) and allows binding to free ubiquitin and both mono- and polyubiquitinated proteins (Li et al., 2013). A dynein motor binding (DMB) domain is thought to assist the microtubule-based retrograde transport of ubiquitinated proteins to the perinuclear region via HDAC6 (Kawaguchi et al., 2003). Unlike other known members of the HDAC superfamily, HDAC6 possesses two active deacetylase domains, DD1 and DD2 (Simões-Pires et al., 2013). The relative contribution of each deacetylase domain to the overall HDAC6 activity remains unclear, but is possibly substrate-dependent (Li et al., 2011; Li et al., 2013). HDAC6 deacetylates a number of non-histones proteins, including α -tubulin, cortactin and Hsp90, and is thus involved in the regulation of microtubule-based transport, cell motility and chaperone activity (Guedes-Dias and Oliveira, 2013).

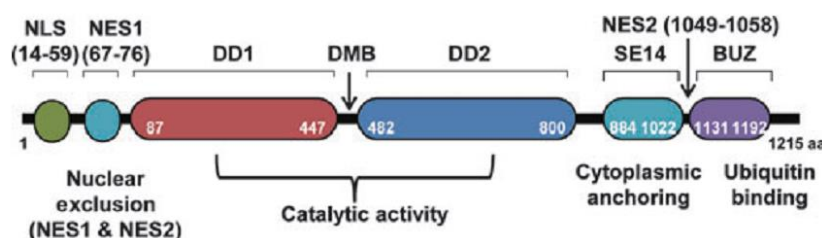


Figure 1. Schematic representation and functional domains of human HDAC6. Original image from Li et al., (2013) FEBS Letters.

Several neurodegenerative diseases are associated with intracellular transport defects (Sheng and Cai, 2012; Maday et al., 2014). In particular, brains of HD patients show reduced levels of acetylated α -tubulin and neurons expressing mutant huntingtin present impaired axonal transport (Dompierre et al., 2007). Acetylated α -tubulin promotes recruitment of motor proteins to microtubules and microtubule-mediated transport (Reed et al., 2006; Dompierre et al., 2007). Inhibiting the α -tubulin deacetylase HDAC6 is, therefore, considered a promising approach to rescue transport defects in neurodegeneration. Indeed, HDAC6 activity inhibition has been shown to improve neuronal mitochondrial trafficking *in vitro* (Chen et al., 2010; d'Ydewalle et al., 2011) and showed beneficial effects in *in vivo* models of Alzheimer's disease (Govindarajan et al., 2013), amyotrophic lateral sclerosis (Taes et al., 2013) and Charcot-Marie-Tooth disease (d'Ydewalle et al., 2011).

HDAC inhibitors are generally divided in three structural components: the metal-binding moiety, which coordinates the catalytic Zn^{2+} ion within the active site; the capping group moiety, which interacts with aminoacid residues surrounding the entrance of the active site; and the linker region, which connects the two (Bieliauskas and Pflum, 2008). Selective pharmacological inhibition among class I, II and IV HDAC isoforms has been difficult to achieve due to high catalytic domain homology (Bieliauskas and Pflum, 2008). First generation HDAC inhibitors, such as trichostatin A (TSA) and vorinostat (SAHA), do not show isoform or class selectivity and are usually referred to as broad spectrum or pan-HDAC inhibitors. In recent years, however, greater understanding of the structural biology and molecular modeling of HDACs has led to the design and synthesis of small molecules with significantly improved selectivity profiles (Thaler and Mercurio, 2014). Examples of such molecules are the two inhibitors used in this thesis: MS-275 and tubastatin A, which preferentially inhibit HDAC1 and HDAC6, respectively (Figure 2).

Background and hypotheses

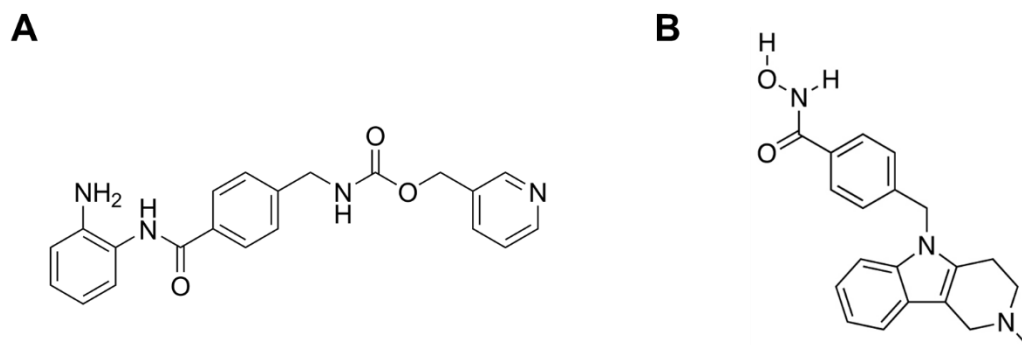


Figure 2. The two HDAC inhibitors used in this study: **(A)** MS-275, also known as entinostat, is considered a class I inhibitor with preferential selectivity towards HDAC1 (IC_{50} 181 nM and >6-fold selectivity over HDAC2; Khan et al., 2008; Bradner et al., 2010); **(B)** Tubastatin A (TBA), is a selective HDAC6 inhibitor (IC_{50} 15 nM and >50-fold selectivity over HDAC8; Butler et al., 2010).

HUNTINGTON'S DISEASE

Huntington's disease (HD) is a monogenic and dominantly inherited neurodegenerative disease characterized by motor dysfunction, cognitive decline and psychiatric disturbances (Ross and Tabrizi, 2011). HD is caused by an expansion of the CAG trinucleotide repeat in the gene encoding the protein huntingtin (Htt) (The Huntington's Disease Collaborative Research Group, 1993). Expansions containing between 36 and 40 CAG-encoded glutamines have incomplete penetrance, whereas expansions of 41 or more glutamines are fully penetrant (Walker, 2007). Mutant Htt (mHtt) is expressed during the individual whole lifetime, but disease onset is typically around 40 years. Significantly, despite widespread distribution of mHtt, preferential atrophy of the striatum is observed even prior to HD onset (Paulsen et al., 2010; Aylward et al., 2011) – consequence of substantial degeneration of GABAergic medium spiny neurons (MSNs; Ross and Tabrizi, 2011). Why are the striatal MSNs particularly vulnerable to mHtt is an outstanding question in the field and significant efforts to unveil specific cellular and molecular mechanisms that might contribute for such vulnerability continue to be made.

Several factors including mitochondrial dysfunction (Johri et al., 2013) and altered proteostasis pathways (Margulis and Finkbeiner, 2014) are thought to be involved in HD pathology. Importantly, they have been implicated as likely factors that render striatal neurons preferential vulnerability in HD: both mitochondria isolated from the striatum (Brustovetsky et al., 2003) and mitochondria in cultured striatal neurons (Oliveira and Gonçalves, 2009) are more vulnerable to Ca^{2+} -loads than their cortical counterparts; diffuse mHtt disrupts mitochondrial trafficking in striatal (Orr et al., 2008) but not cortical neurons (Chang et al., 2006), and diffuse mHtt is less efficiently cleared in striatal compared to cortical or cerebellar neurons (Tsvetkov et al., 2013). Thus, strategies to prevent mitochondrial dysfunction and to improve mHtt clearance are considered potentially useful in HD (Costa and Scorrano, 2012; Nixon, 2013). Given the role of protein acetylation in autophagy (True and Matthias, 2012; Bánrétí et al., 2013) and mitochondrial dynamics (Schon and Przedborski, 2011; Guedes-Dias and Oliveira, 2013), we were interested in evaluating HDAC inhibition as a potential modifier of striatal neurons vulnerability in HD.

MITOCHONDRIAL BIOGENESIS

The mammalian mitochondrial proteome is estimated to comprise between 1100 and 1500 proteins, of which 13 are encoded by the mitochondrial DNA and the remainder by the nuclear DNA (Meisinger et al., 2008; Pagliarini et al., 2008). Since mitochondria cannot be generated *de novo*, pre-existing mitochondria are used as templates to generate more mitochondrial mass in a process involving a tight orchestration between the nuclear, cytosolic and mitochondrial compartments. In the nucleus, expression of genes encoding for mitochondrial proteins is regulated by a family of transcription coactivators, of which PGC-1 α is considered the main member, and further coordinated by a group of transcription factors, which include the nuclear respiratory factor 1 (NRF1) and 2 (NRF2; Scarpulla, 2011). Following translation in the cytosol, proteins are imported into mitochondria and targeted to specific mitochondrial subcompartments, where they may be further assembled into functional complexes (DiMauro et al., 2013). Coordination of mitochondrial biogenesis is established by a circuit which integrates external and internal signals into genetic programs (Battersby and Richter, 2013). Specifically, cellular growth and physiological states, such as long-term cold exposure, nutrient deprivation and exercise, alter the energy demands of particular cells or tissues and activate signaling pathways that regulate gene transcription and mitochondrial biogenesis (Scarpulla et al., 2012). Indeed, several studies have addressed strategies to modulate those signaling pathways and rescue levels of mitochondrial biogenesis in neurodegenerative diseases (Schapira et al., 2014; Corona and Duchon, 2015).

In developing neurons, mitochondria are distributed from the cell body to emerging axons (Ruthel and Hollenbeck, 2003) and concentrate in active growth cones, where ATP consumption is elevated (Morris and Hollenbeck, 1993; Ruthel and Hollenbeck, 2003). Mitochondrial energy production is essential for neurite outgrowth and morphogenesis both *in vitro* and *in vivo* (Oruganty-Das et al., 2012) thus, significant levels of mitochondrial biogenesis support neurite extension. Moreover, in mice dentate gyrus, considerable mitochondrial biogenesis takes place in adult-born granule neurons even beyond the period of maximal dendritic arbor extension, underscoring the reliance of mature neurons on mitochondrial metabolism (Steib et al., 2014). The paradigm of neurite extension during neuronal maturation led some authors to favor a model in which mitochondrial biogenesis occurs mainly in the cell body (O'Toole et al., 2008). Interestingly, however, detection of axonal mRNA for nuclear-encoded mitochondrial proteins (Gioio et al., 2001) was followed by the observation of mitochondrial biogenesis occurring in axons separated from their cell bodies (Amiri and Hollenbeck, 2008).

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HDAC class III isoform SIRT1 is reported to deacetylate and activate PGC-1 α , increasing mitochondrial biogenesis (Canto and Auwerx, 2009). Both HDAC6 and HDAC1 inhibition have been reported to promote neurite extension in cortical (Rivieccio et al., 2009) and dorsal root ganglion neurons (Finelli et al., 2013), respectively. However, whether HDAC1 and HDAC6 inhibition modify neuronal mitochondrial biogenesis levels remains unknown. In this thesis, we thus assessed whether altered neurite outgrowth caused by HDAC1 and HDAC6 inhibition could be associated with changes on mitochondrial biogenesis levels in developing cortical and striatal neurons.

MITOCHONDRIAL TRANSPORT

A single cortical neuron in the resting brain is estimated to consume about 4.7 billion ATP molecules per second (Zhu et al., 2012). A large part of this energy is produced in mitochondria and is used far away from the cell body in synapses and conducting action potentials (Schwarz, 2013). Proper mitochondrial distribution throughout the neuritic arbor is thus essential for neuronal function and it is achieved by a highly complex and specialized intracellular transport system.

Mitochondrial transport in neurons is mainly mediated along microtubules by two superfamilies of opposing motors: kinesins and dyneins. Whereas kinesins process cargo transport towards the plus end of microtubules (anterograde), minus end-directed (retrograde) cargo transport is regulated by dyneins. The kinesin-1 family is the main mediator of mitochondrial anterograde transport and, in mammals, attaches to mitochondria through the Miro/TRAK1-2 adaptor complex (MacAskill et al., 2009a). Ca^{2+} binds to EF-hand domains in Miro, altering its conformation and arresting kinesin-1 processing – a mechanism thought to underlie mitochondrial immobilization in areas of locally high Ca^{2+} concentration (Macaskill et al., 2009b; Wang and Schwarz, 2009). Other protein adaptors that assist kinesin-1 recruitment to mitochondria include FEZ1, RanBP2 and syntabulin (Maday et al., 2014). Dynein requires binding to dynactin to process minus-end directed cargo transport (Moughamian and Holzbaur, 2012). Evidence suggest that the dynein-dynactin motor complex also attaches to mitochondria through the Miro/TRAKs complex: dynein-dynactin was found to interact with TRAK proteins (van Spronsen et al., 2013) and loss of Miro in *Drosophila* affects mitochondrial movement in both anterograde and retrograde directions (Russo et al., 2009). Mechanisms to immobilize mitochondria in sites of high Ca^{2+} concentration and energy consumption are fundamental to assure proper mitochondrial distribution (Sheng, 2014). One important mediator for mitochondrial docking in axons is syntaphilin, which immobilizes mitochondria by anchoring them to microtubules (Chen and Sheng, 2013). Interestingly, the absence of syntaphilin in dendrites (Kang et al., 2008) and the different mitochondrial-arrest mechanisms mediated by Miro/ Ca^{2+} in axons (Wang and Schwarz, 2009) and dendrites (Macaskill et al., 2009b), suggest that mitochondrial docking is differentially regulated in distinct neuronal regions.

Mitochondrial transport defects have been implicated in a number of neurodegenerative diseases and particularly in HD (Sheng and Cai, 2012). Diffuse mutant huntingtin impaired mitochondrial trafficking in striatal neurons (Orr et al., 2008), whereas in cortical neurons, mitochondria tended to stop and accumulate only next to aggregates (Chang et al., 2006). This suggests that mutant huntingtin preferentially affects mitochondrial transport in striatal neurons and that this factor might be an important

Background and hypotheses

contributor for striatal neurons vulnerability in HD. Why is there a more striking inhibition of mitochondrial trafficking in striatal than in cortical neurons is unclear. To address this issue, we used sister cultures of cortical and striatal neurons to analyze whether basal mitochondrial trafficking parameters differed between the two neuronal populations.

MITOCHONDRIAL FISSION-FUSION

Mitochondrial fission and fusion events are mainly mediated by a group of conserved GTPases and permit the modulation of mitochondrial morphology, number and size. Mitochondrial fission allows for the isolation of defective mitochondria, the partition of mitochondria to daughter cells during mitosis, and the distribution of mitochondria along neuronal processes (Otera and Mihara, 2011). The cytosolic dynamin-related protein 1 (Drp1) is the main mediator of mitochondrial fission, forming spirals around mitochondria which constrict and ultimately split both outer and inner mitochondrial membranes (Smirnova et al., 2001). In mammals, recruitment and assembly of Drp1 on the mitochondrial outer membrane are mediated by a group of effector proteins, which include Fis1, Mff, MiD49 and MiD51 (DuBoff et al., 2013). Mitochondrial fusion is thought to render functional complementation between mitochondria by allowing the distribution of mtDNA, RNA, proteins and lipids among them (Youle and van der Bliek, 2012). In mammals, membrane-anchored Mitofusin 1 (Mfn1) and 2 (Mfn2) mediate the fusion of mitochondrial outer membranes, while Optic Atrophy 1 (OPA1) mediates the fusion of mitochondrial inner membranes (Chan, 2006). At a mechanistic level, it is thought that fusion-GTPases anchored on two opposing membranes form homo- or hetero-complexes that allow mitochondrial tethering and consequent fusion (Chan, 2006).

Neurons are particularly sensitive to defective mitochondrial fission-fusion balance. Mutations in OPA1 cause autosomal dominant optic atrophy, which is characterized by retinal ganglion cell degeneration and visual loss, while mutations in Mfn2 cause Charcot-Marie-Tooth disease type 2A neuropathy (Itoh et al., 2013). Mounting evidence suggest that altered mitochondrial fission-fusion balance is also a pathophysiological trait in other neurodegenerative diseases (DuBoff et al., 2013; Itoh et al., 2013). HD, in particular, has been associated with increased mitochondrial fragmentation: Drp1 and Fis1 expression levels were reported to be increased in brains of HD patients (Shirendeb et al., 2011), and additionally, mutant huntingtin was described to interact and increase Drp1 enzymatic activity (Song et al., 2011; Shirendeb et al., 2012). Concomitantly, there has been a growing interest in researching strategies to correct mitochondrial structural defects in HD (Costa and Scorrano, 2012). A recent study reported that pharmacological inhibition of Drp1 prevented excessive mitochondrial fragmentation and improved survival of both *in vitro* and *in vivo* HD models (Guo et al., 2013). Even so, evidence indicating that inhibition of mitochondrial fission is not beneficial for neurons in the long-term (Li et al., 2004; Kageyama et al., 2012; Sheng and Cai, 2012), suggests that strategies aiming at promoting mitochondrial fusion might be more promising for HD.

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Neuronal mitochondria frequently go through fission and fusion cycles (Cagalinec et al., 2013). The number of contacts between mitochondria predicts fusion events in neurons and is mostly dependent on two factors: 1) the number of mitochondria available and 2) mitochondrial movement (Cagalinec et al., 2013). Modulation of one of these factors is therefore likely to alter mitochondrial fission-fusion balance. HDAC6 inhibition increases α -tubulin acetylation, which in turn promotes motor proteins recruitment to microtubules (Reed et al., 2006; Dompierre et al., 2007) and mitochondrial motility in neurons (Chen et al., 2010; d'Ydewalle et al., 2011). We thus hypothesized that enhancing mitochondrial motility by inhibiting HDAC6 would increase the number of contacts between mitochondria and facilitate mitochondrial fusion. This hypothesis was particularly appealing since HD is not only associated with mitochondrial fragmentation, but also with decreased levels of α -tubulin acetylation (Dompierre et al., 2007) and defective mitochondrial transport (Chang et al., 2006; Orr et al., 2008) – which could be potentially rescued by inhibiting HDAC6.

AUTOPHAGIC DYNAMICS

Autophagy and the ubiquitin-proteasome system (UPS) are the two main mechanisms in the cell for disposing toxic and defective components. The importance of autophagy as a mechanism which confers efficient degradation of defective organelles and misfolded protein aggregates is most evident in neurons (Nixon, 2013). Whereas cell-lines may divide to segregate and dilute toxic components (Eden et al., 2011), such mechanisms are precluded from post-mitotic and long-lived neurons. Moreover, neurons are highly polarized, which further compels a unique degree of autophagy spatial regulation. Indeed, neuron-specific knockdown of essential components of the autophagic pathway resulted in severe neurodegenerative phenotypes (Hara et al., 2006; Komatsu et al., 2006) and mounting evidence suggest that autophagy defects are associated with several neurodegenerative diseases, including HD (Martin et al., 2015).

The autophagic machinery has been largely identified through yeast genetic studies (Reggiori and Klionsky, 2013) and it consists of approximately 35 autophagy-related proteins (Atg) which mostly function as multiprotein complexes (Mizushima and Komatsu, 2011). In mammalian cells, core Atg proteins are highly conserved and act in a similar hierarchical manner as in yeast (Mizushima and Komatsu, 2011). The autophagic pathway involves the formation of an isolation membrane (or phagophore) which nucleates from the endoplasmic reticulum (other cellular compartments, such as the Golgi complex, mitochondria and plasma membrane, are thought to contribute for the isolation membrane expansion; Lamb et al., 2013). The isolation membrane grows, engulfing a portion of the cytoplasm and eventually taking a fishbowl-like shape (Hurley and Schulman, 2014). When the isolation membrane is sealed and detaches from the endoplasmic reticulum, the newly formed vesicle is referred to as the autophagosome – this structure ultimately fuses with a lysosome, resulting in the degradation of its contents (Hurley and Schulman, 2014). Significantly, this pathway has been extensively studied in yeast and mammalian cell-lines, however, only in recent years has the neuronal autophagic pathway been addressed in further detail.

Background and hypotheses

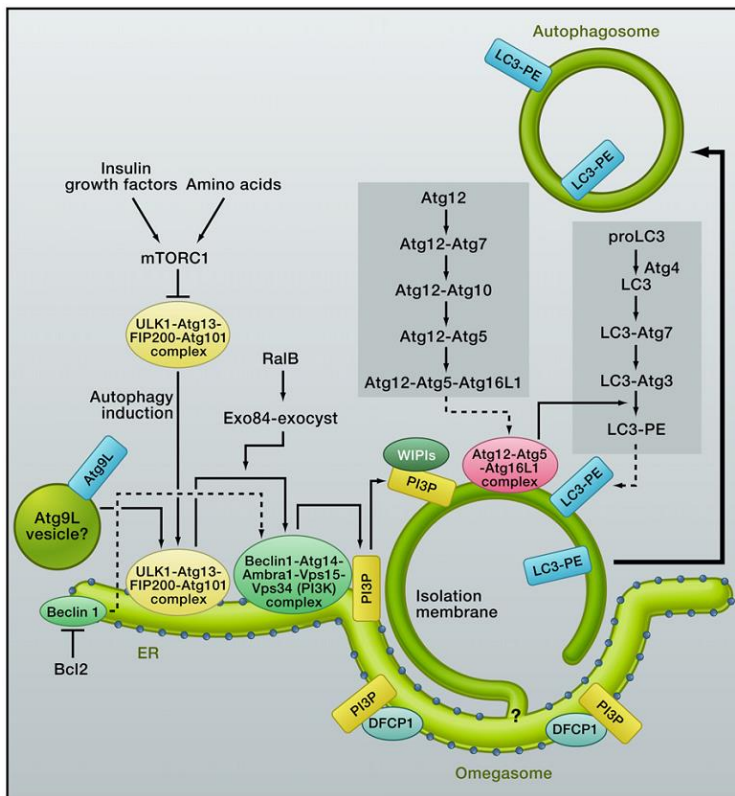


Figure 2. Autophagosome Formation and Atg Proteins in Mammalian Cells

mTOR complex 1 (mTORC1) suppresses the ULK1 (Atg1 homolog) complex under nutrient-rich conditions. Upon autophagy induction, the ULK1 complex (including ULK1, Atg13, FIP200, and Atg101) is activated and translocates to a certain domain of the endoplasmic reticulum (ER). Once in the ER, the ULK1 complex regulates the class III phosphatidylinositol (PtdIns) 3-kinase complex (including Beclin 1, Atg14(L)/barkor, Vps15, Vps34, and Ambra1), and this regulation is promoted by RalB and an Exo84-containing exocyst complex. Recruitment of Beclin 1 to the PtdIns3-kinase complex is also sensitive to starvation; Beclin 1 forms a complex with ER-associated Bcl-2 under nutrient-rich conditions and is released upon phosphorylation of Bcl-2 by JNK1. Atg9L, a multimembrane-spanning protein, is also involved in an early stage of autophagosome formation (Atg9L is recruited likely on vesicles). Formation of PtdIns(3)P recruits double FYVE-containing protein 1 (DFCP1) and promotes the formation of the omegasome, from which autophagosomes appear to be generated. Other PtdIns(3)P-binding WIPI proteins (WD-repeat protein interacting with phosphoinoside) (Atg18 homologs) are also crucial for the maturation of the omegasome/isolation membrane. The Atg12-Atg5-Atg16L1 complex and the LC3 (Atg8 homolog)-phosphatidylethanolamine (PE) conjugate play important roles in the elongation and closure of the isolation membrane. The Atg12-Atg5-Atg16L1 complex is also required for formation of the covalent bond between LC3 and PE.

Figure 3. Original image and legend from Mizushima and Komatsu (2011) Cell.

In neurons, the autophagic pathway is highly compartmentalized – over 80% of autophagosomes generate at the axon tip (Maday and Holzbaur, 2014) and are retrogradely transported along the axon towards the somatodendritic compartment, where most of the cargo degradation is thought to take place (Lee et al., 2011; Maday et al., 2012). Similarly to nonpolarized cells, Atg13 recruitment to nascent autophagosomes occurs prior to double FYVE-containing protein 1 (DFCP1) and LC3 recruitment (Maday and Holzbaur, 2014). Although studies have positioned Atg5 downstream of Atg13 and DFCP1 (Itakura and Mizushima, 2010), Atg5 recruitment was unexpectedly observed to occur simultaneously with Atg13, which could have been due to the relatively low time resolution used in the reported experimental context (2 sec; Maday and Holzbaur, 2014)). The disassembly of proteins at the nucleation site also takes place in an orderly fashion, with Atg5 exit being followed by loss of Atg13 and ultimately, DFCP1 decay (Maday and Holzbaur, 2014). Autophagosomal biogenesis is followed by the highly processive retrograde transport of autophagosomes towards the somatodendritic region. The motor proteins kinesin and dynein bind to autophagosomes in the axonal tip, leading to a tug-of-war between the two motors in which autophagosomes are observed moving bidirectionally with frequent direction switching (Fu et al., 2014). The tug-of-war is resolved when JIP1 is recruited to autophagosomes and binds to LC3 – the binding of JIP1 to LC3 prevents kinesin processing

and sustains dynein-mediated retrograde transport, allowing autophagosomes to exit the distal axon and travel towards the soma (Fu et al., 2014). Along the axon, autophagosomes display robust retrograde movement and go through a maturation process in which they increasingly acidify by fusing with late endosomes and lysosomes, ultimately forming autolysosomes (Lee et al., 2011; Maday et al., 2012). Fully acidified autolysosomes show bidirectional movement and concentrate in the somatodendritic compartment where recycling of proteins and lipids is considered more efficient (Maday et al., 2012).

Autophagosome-lysosome fusion facilitates cargo degradation and is a key step for autophagic clearance. A study suggested that F-actin filaments assemble around mature autophagosomes and mediate fusion with lysosomes by tethering the two vesicles into close contact (Lee et al., 2010). Significantly, the same study reported that the F-actin network recruitment was triggered after cortactin deacetylation by HDAC6 and that autophagosome-lysosome fusion was blocked in HDAC6 knockout fibroblasts (Lee et al., 2010). However, a key role for HDAC6 in autophagosome-lysosome fusion in neurons conflicted with the fact that HDAC6 knockout mice present increased acetylated α -tubulin levels in the brain (Bobrowska et al., 2011), are viable and fertile (Zhang et al., 2008), and do not develop neurodegenerative phenotypes similar to mice with impaired neuronal autophagy (Hara et al., 2006; Komatsu et al., 2006). We thus tested whether HDAC6 pharmacological inhibition could increase α -tubulin acetylation and promote intracellular trafficking without blocking neuronal autophagosome-lysosome fusion. Moreover, subsequent studies showed that both autophagosomal (Maday et al., 2012) and lysosomal (Hendricks et al., 2010) transport in neurons is mediated by the microtubule-based motors dynein and kinesin. We thus hypothesized that if HDAC6 inhibition increased α -tubulin acetylation, then it could promote neuronal autophagic vesicle flux and facilitate mutant huntingtin clearance.

Objectives

Our main interests in this study were two-fold:

- 1) Assess whether potential differences in mitochondrial dynamics, autophagy and mutant huntingtin proteostasis may assist in elucidating differential vulnerability of striatal and cortical neurons in HD;
- 2) Investigate whether HDAC inhibition could modulate mitochondrial dynamics, autophagy and mutant huntingtin proteostasis and assess its potential as experimental therapeutics to reduce vulnerability of striatal neurons in HD.

The study was performed by addressing the following points:

Mitochondrial dynamics:

- Assessment of mitochondrial biogenesis, fission-fusion balance and motility in cortical and striatal neurons;
- Assessment of whether HDAC1 or HDAC6 inhibition modulate mitochondrial dynamics in cortical and striatal neurons.

Autophagy:

- Assessment of whether HDAC6 pharmacological inhibition blocks neuronal autophagosome-lysosome fusion;
- Assessment of whether HDAC6 inhibition promotes neuronal autophagic flux;
- Comparison of basal autophagic flux in cortical and striatal neurons.

Mutant huntingtin proteostasis (mHtt):

- Comparison of mHtt aggregation profile in cortical and striatal neurons;
- Comparison of diffuse mHtt proteostasis in cortical and striatal neurons;
- Assessment of whether HDAC6 inhibition modulates mHtt aggregation profile or diffuse mHtt levels in cortical and striatal neurons.

Section 2

Research

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Lysine deacetylases and mitochondrial dynamics in neurodegeneration.

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Competing interests

The authors declare they have no competing interests.

ABSTRACT

Lysine acetylation is a key post-translational modification known to regulate gene transcription, signal transduction, cellular transport and metabolism. Lysine deacetylases (KDACs), including classical KDACs (a.k.a. HDACs) and sirtuins (SIRT1, SIRT2, SIRT3, SIRT6, SIRT7), are emerging therapeutic targets in neurodegeneration. Given the strong link between abnormal mitochondrial dynamics and neurodegenerative disorders (e.g. in Alzheimer, Parkinson and Huntington diseases), here we examine the evidence for KDAC-mediated regulation of mitochondrial biogenesis, fission-fusion, movement and mitophagy. Mitochondrial biogenesis regulation was reported for SIRT1, SIRT3, and class IIa KDACs, mainly via PGC-1 α modulation. SIRT1 or SIRT3 overexpression rescued mitochondrial density and fission-fusion balance in neurodegeneration models. Mitochondrial fission decreased with pan-classical-KDAC inhibitors and increased with nicotinamide (pan-sirtuin-inhibitor/activator depending on concentration and NAD⁺ conversion). Mitochondrial movement increased with HDAC6 inhibition, but this is not yet reported for the other tubulin deacetylase SIRT2. Inhibition of HDAC6 or SIRT2 was reported neuroprotective. Mitophagy is assisted by the HDAC6 ubiquitin-binding and autophagosome-lysosome fusion promoting activities, and was also associated with SIRT1 activation. In summary, KDACs can potentially modulate multiple components of mitochondrial dynamics, however, several key points require clarification. The SIRT1-biogenesis connection relies heavily in controversial caloric restriction (CR) regimes or CR-mimetic drugs, and appears cell-type dependent, recommending caution before linking SIRT1 activation with general neuroprotection. Future studies should clarify mitochondrial fission-fusion regulation by KDACs, and the interplay between HDAC6 and SIRT1 in mitophagy. Also, further studies are required to ascertain whether HDAC6 inhibition to enhance mitochondrial trafficking does not compromise autophagy or clearance of misfolded proteins in neurodegenerative disorders.

Abbreviations:

AD, Alzheimer's disease; CR, caloric restriction; DRG, dorsal root ganglion; Drp1, dynamin-related protein 1; HAT, histone acetyltransferase; HD, Huntington's disease; HDAC, histone deacetylase; Hsp90, heat shock protein 90; KAT, lysine acetyltransferases; KDAC, lysine deacetylase; Mff, mitochondrial fission factor; Mfn, mitofusin; MIEF1, mitochondrial elongation factor 1; mtDNA, mitochondrial DNA; NRF, nuclear respiratory factor; OPA1, optic atrophy 1; PD, Parkinson's disease; POMC, proopiomelanocortin; PTM, post-translational modification; Tfam, mitochondrial transcription factor A.

Introduction

1.1 An extended phenotype for lysine acetylation

Acetylation at the ϵ -amino group of lysines is a reversible post-translational modification (PTM), crucial for regulating the function of multiple proteins (Yang and Seto, 2008b; Choudhary et al., 2009). Lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) act in concert to modulate the acetylation status of their targets. Since lysine acetylation prevents positive charges from forming on the amino group, this PTM strongly influences protein electrostatic properties (Glozak et al., 2005). The functional consequences vary with the relative position of specific lysine residues within the target protein, and may manifest as increases as well as decreases in activity, affinity, stability, or in protein-protein interaction (Kouzarides, 2000; Glozak et al., 2005).

Histones were the first substrates identified for eukaryotic KATs and KDACs, explaining their common designation as histone acetyltransferases (HATs) and deacetylases (HDACs), respectively (Yang and Seto, 2008b). Histone acetylation strongly correlates with gene transcription, likely by relaxing chromatin and facilitating access to the transcription machinery. Moreover, acetylation of specific lysine residues may directly serve as recognition sites for transcription factors (Shahbazian and Grunstein, 2007). Still, when considering KDACs non-histone targets, particularly transcription factors, it can no longer be generalized that increased acetylation stimulates transcription. Indeed, acetylation of transcription factors may increase or decrease their DNA binding affinity depending on whether the specific acetylation sites fall directly adjacent or within the DNA-binding domain, respectively (Kouzarides, 2000). Thus, KATs and KDACs effects on acetylation and transcription provide a versatile mechanism for coupling extracellular signals with the genome.

Non-histone targets regulated by lysine acetylation are mostly comprised by transcription factors, but the growing list also includes other key cellular proteins, such as α -tubulin, importin α , heat shock protein 90 (Hsp90), and cortactin, among others (Glozak et al., 2005; Zhang et al., 2007). Thus, lysine acetylation plays import regulatory roles not only in transcription, but also in signal transduction and cellular transport processes. Further, lysine acetylation is a key metabolic regulatory signal, particularly at the level of mitochondria (He et al., 2012).

KDACs in particular have received much attention not only for their physiological roles, but also for their involvement in disease states and, consequently, for being a therapeutic target (Haberland et al., 2009). In addition to cancer, neurodegenerative disorders are also under the scope of possible therapy with drugs targeting KDACs (Kazantsev and Thompson, 2008; True and Matthias, 2012). Thus, considering the emerging role of

abnormal mitochondrial dynamics in the pathogenesis of neurodegenerative disorders (Schon and Przedborski, 2011), in this review we examine the hypothesis that the extended phenotype of KDACs activity involves a modulation of mitochondrial dynamics that may have therapeutic implications in neurodegeneration. We start by briefly addressing key aspects of KDACs, followed by focused analyses on how KDAC modulation impacts 4 divisions of mitochondrial dynamics: biogenesis, fission-fusion, movement, and mitophagy; and how such dynamics are affected in neurodegenerative diseases.

2. KDACs: classes and functions

The mammalian KDAC superfamily currently holds eighteen members. A core division based on homology with yeast KDACs separates ‘classical HDACs’ (zinc-dependent, Rpd3/Hda1 homologues) from ‘sirtuins’ (NAD⁺-dependent, Sir2 homologues). Another division establishes four classes based on phylogenetic analysis and sequence homology. Classes I, II and IV comprise classical HDACs (with class II further divided into IIa and IIb), while class III comprises sirtuins (Yang and Seto, 2008a).

Class I KDACs (HDAC1, 2, 3, and 8) are predominantly nuclear and widely expressed in most tissues, except for HDAC8 that is confined to smooth muscle where it associates with α -actin and is essential for contractility (Waltregny et al., 2005). HDAC1 and 2 are highly homologous and act together as the catalytic subunits of major transcriptional repressor complexes such as Sin3, NuRD/NRD/Mi2, and CoREST (Sengupta and Seto, 2004). This collaborative spirit of KDACs (Yang and Seto, 2003) also applies to HDAC3, which is responsible for the deacetylase activities associated with Class II KDACs, working together in a repressor complex with SMRT/N-CoR (Fischle et al., 2002). Class II KDACs display tissue-specific expression patterns, being highly expressed in the brain, heart, and muscle. Class IIa contains HDAC4, 5, 7, and 9. While full-length HDAC9 remains in the nucleus, HDAC4, 5, 7 and a splice variant of HDAC9 (MITR) shuttle between the nucleus and cytosol. Phosphorylation and binding to 14-3-3 proteins anchors these HDACs in the cytosol, whereas dephosphorylation releases them to return to the nucleus (Kazantsev and Thompson, 2008). Class IIb consists of HDAC6 and 10, both primarily cytosolic. HDAC6 is unique in containing a C-terminal ubiquitin-binding domain and two functional deacetylase domains. Cytosolic HDAC6 deacetylates tubulin, cortactin and HSP90, regulating axonal trafficking, cell motility and degradation of misfolded proteins. HDAC6 can also shuttle to the nucleus to regulate transcription, with its activity and subcellular localization being regulated by acetylation (Liu et al., 2012b). HDAC10 possesses a unique leucine-rich domain, interacts with HDAC3, and represses transcription when tethered to a promoter

(Tong et al., 2002). Class IV consists only of HDAC11, a predominantly nuclear KDAC that regulates immune tolerance (Villagra et al., 2009).

Sirtuins (Class III KDACs) comprise seven mammalian enzymes, SIRT1-7. Their deacetylation reaction consumes the cofactor NAD⁺ while generating nicotinamide plus a mixture of 2' and 3'-O-acetyl-ADP-ribose (Lawson et al., 2010). SIRT1 is present in the nucleus, deacetylating histones and several transcription factors. SIRT1 deacetylates and activates the transcriptional co-activator PGC-1 α , a master regulator of mitochondrial biogenesis (Nemoto et al., 2005). SIRT1 was reported predominantly cytosolic in the adult brain (Li et al., 2008), while PGC1- α may also reside in the cytosol being directed to the nucleus by stimuli-associated PTMs (Cowell et al., 2007; Little et al., 2010). SIRT2 is primarily cytosolic but may also occur in the nucleus where it preferentially deacetylates histone H4K16 (Vaquero et al., 2006). SIRT2 shares α -tubulin deacetylase activity with HDAC6 (Southwood et al., 2007), and has been suggested as the main microtubule deacetylase in mature neurons (Maxwell et al., 2011), although it was also reported that SIRT2 genetic reduction or ablation has no effect on the acetylation of α -tubulin or H4K16 in mouse brain (Bobrowska et al., 2012). Additionally, SIRT2 is reported to deacetylate the transcription factor p65 in the cytosol, thus regulating expression of NF- κ B-dependent genes (Rothgiesser et al., 2010). SIRT3, 4 and 5 are also called 'mitochondrial sirtuins' given their subcellular location. SIRT3 is the main deacetylase in the mitochondria, where it regulates oxidative phosphorylation, protein synthesis and multiple metabolic pathways. The other two mitochondrial sirtuins show weak deacetylase activity, with SIRT5 exhibiting pronounced demalonylase and dessuccinylase activity, and the primary activity of SIRT4 remaining elusive (He et al., 2012). SIRT6 is a nuclear histone H3K9 deacetylase with a key role in telomere maintenance and DNA repair (Jia et al., 2012). SIRT7 is a selective histone H3K18 deacetylase (Barber et al., 2012) and an activator of RNA polymerase I transcription (Ford et al., 2006).

3. The modulation of KDAC activity

Physiological modulation of KDACs may occur at multiple steps of their life cycle, including transcription, pos-transcriptional and proteolytic processing. Additionally, protein-protein interactions, co-factor availability, and several PTMs allow dynamic activity control. Most KDACs require integration in multi-protein complexes and nuclear localization in order to repress transcription. Thus, the activity of such KDACs predictably decreases upon interference with complex assembly/stability, reduced availability of interacting proteins (e.g. N-CoR for HDAC3), or enhanced nuclear export plus cytosolic retention (e.g. class II

KDACs). Conversely, for KDACs acting primarily in the cytosol (e.g. HDAC6 and SIRT2), cytosolic retention can increase their activity (Sengupta and Seto, 2004).

PTM by phosphorylation may either increase (e.g. HDAC1) or decrease (e.g. HDAC8) class I enzymatic activity, while regulating class II subcellular localization via binding to 14-3-3 proteins (Sengupta and Seto, 2004). In turn, PTM by acetylation of the catalytic domain and C-terminal region strongly diminishes HDAC1 enzymatic activity (Qiu et al., 2006) and, similarly, p300-mediated SIRT2 acetylation reduces its deacetylase activity (Han et al., 2008). Also, 'site B'-acetylation decreases HDAC6 tubulin- but not histone-deacetylase activity (*in vitro*), although *in situ* histone acetylation should decrease since the same PTM reduces HDAC6 nuclear import (Liu et al., 2012b). Concerning co-factor availability, the NAD⁺:NADH ratio links cellular metabolic status to class III KDACs' activity (Sengupta and Seto, 2004), whereas zinc is critical for other KDACs' catalytic activity. Finally, while proteolytic degradation of KDACs or their co-activators predictably diminishes their activity, specific proteolytic processing may be required for full deacetylase activity (e.g. mitochondrial SIRT3; Huang et al., 2010). Thus, multiple physiological processes modulate KDAC activity, and some have already assisted drug development.

Pharmacological KDAC modulators are predominantly inhibitors, with different structural requirements for acting on classical KDACs vs. sirtuins. Few activators have been reported, except for SIRT1 where several presumed activators have been synthesized (inc. SRT1720, SRT2183 and SRT1460), but there is now evidence that these compounds and resveratrol are not direct SIRT1 activators (Pacholec et al., 2010), albeit recent data suggests that at least some of the physiological effects of these type of compounds may occur by "assisted allosteric activation" (Hubbard et al., 2013). Most classical KDACs' inhibitors lack isoform-selectivity as they act by chelating zinc in the catalytic domain. Differential interaction with amino acid residues at the entry of the KDAC active site, and the mimicking of natural substrates may account for isoform-selective inhibitors, but currently little is known about their structure-activity relationships (Bieliauskas and Pflum, 2008; Marson, 2009; Bertrand, 2010). Most sirtuin inhibitors prevent NAD⁺ from binding the catalytic domain by blocking the required nicotinamide binding site. Alternatively, some sirtuin inhibitors compete with the acetylated peptide substrate for its binding site in the catalytic domain (Lawson et al., 2010).

Examples of commonly used KDAC inhibitors of particular relevance for this review are as follows. Pan-(classical)-KDAC inhibitors with nanomolar IC₅₀ include the hydroxamate derivatives trichostatin A and vorinostat (SAHA; Khan et al., 2008). The short chain fatty acids butyrate and valproate are class I and IIa inhibitors in the micro to millimolar range (Bieliauskas and Pflum, 2008). Entinostat (MS-275) is an HDAC1-selective inhibitor

(Khan et al., 2008), whereas tubacin and tubastatin A are HDAC6-selective inhibitors, respectively, with ~350- and 1,000-fold selectivity over HDAC1 (Butler et al., 2010). Concerning sirtuins, nicotinamide is often used as a pan-sirtuin inhibitor, whereas EX527 and AGK2 are described as selective SIRT1 and SIRT2 inhibitors, respectively (Lawson et al., 2010).

4. Biogenesis

Mitochondria are continuously renewed by the physiological equilibrium between biological generation (biogenesis) and selective degradation through autophagy (mitophagy). Increasing the cellular mitochondrial mass is biologically expensive and a long-term adaptive response. As such, transient energy demands are met by changes in expression of subsets of genes, regulators, or increases in mitochondrial function. Physiological states such as endurance training, caloric restriction, and long-term cold exposure (leading to adaptive thermogenesis) are reported to promote mitochondrial biogenesis (Hock and Kralli, 2009; Onyango et al., 2010).

A complex network of nuclear and mitochondrial transcription factors orchestrate mitochondrial biogenesis, a process in which the PGC-1 family of transcriptional coactivators plays master regulatory roles. PGC-1 coactivators integrate signals and coordinate biological responses allowing cellular adaptation to changes in energy demand, including increases in mitochondrial biogenesis, respiration and metabolism (Finck and Kelly, 2006). PGC-1 α coactivates nuclear respiratory factors (NRFs), which control expression of nuclear-encoded mitochondrial structural proteins (Wu et al., 1999). The resulting preproteins must be imported, processed and correctly assembled in mitochondria, a complex process involving finely tuned posttranscriptional mechanisms and the target of rapamycin (TOR) signalling pathway (Devaux et al., 2010). PGC-1 α -coactivated NRFs also regulate expression of mitochondrial transcription factor A (Tfam), a nuclear-encoded transcription factor crucial for replication, transcription, and maintenance of mitochondrial DNA (mtDNA; Kang and Hamasaki, 2005).

4.1 KDACs and mitochondrial biogenesis

The link between KDACs and mitochondrial biogenesis stems primarily from the modulation of PGC-1 α by transcriptional or posttranslational mechanisms (Figure 1; Table 1). PGC-1 α transcription is promoted by myocyte-enhancer factor-2 (MEF2) and diminished by class IIa KDACs that repress MEF2 activity. Both class IIa KDACs and MEF2 transcription factors are highly expressed in muscle and brain, and phosphorylation of these KDACs promotes their nuclear export, releasing MEF2 to activate PGC-1 α transcription

(Czubryt et al., 2003). Consistently, pan-KDAC inhibitors (trichostatin A and valproate) upregulated PGC-1 α in neuroblastoma cells (Cowell et al., 2009). Concerning posttranslational mechanisms, PGC-1 α acetylation by the GCN5 acetyltransferase reduces its transcriptional activity (Lerin et al., 2006), whereas deacetylation by SIRT1 activates PGC-1 α (Nemoto et al., 2005). Interestingly, SIRT1 converges with AMP-activated kinase (AMPK) to activate PGC-1 α ; AMPK increases levels of the SIRT1 cofactor NAD⁺ (Canto et al., 2009) and activates PGC-1 α by phosphorylation (Jager et al., 2007). Further, these posttranslational modifications of PGC-1 α activity also promote its own transcription via an autoregulatory feedforward loop (Handschin et al., 2003).

The relationship between SIRT1 and mitochondrial biogenesis has been mostly explored in the context of caloric restriction (CR) – a regime reportedly capable of extending life span by inducing SIRT1 expression (Cohen et al., 2004). However, studies reporting increased mitochondrial biogenesis following CR (Nisoli et al., 2005; Lopez-Lluch et al., 2006; Civitarese et al., 2007) were challenged by a recent study showing no increases in mitochondrial structural proteins in several rat tissues including the brain (Hancock et al., 2011). Notwithstanding, CR lacks the selectivity required to scrutinize the pathways linking SIRT1 and mitochondrial biogenesis. Alternatively, compounds designated as SIRT1 activators or “CR mimetic drugs” have been tested and reported to improve mitochondrial function and enhance mitochondrial biogenesis and function (Lagouge et al., 2006; Csiszar et al., 2009; Funk et al., 2010). Still, there is now evidence that such compounds (inc. resveratrol, SRT1720, SRT2183, and SRT1460) do not directly activate SIRT1 (Pacholec et al., 2010), but see also the recently suggested “assisted allosteric activation” in (Hubbard et al., 2013). Attention has thus turned to AMPK, the SIRT1 partner in activating PGC-1 α (Canto et al., 2009). Some authors argue that AMPK is upstream of SIRT1 in the cascade of resveratrol metabolic effects, including mitochondrial biogenesis. Accordingly, AMPK-deficient mice presented a faulty response to resveratrol and AMPK was considered the main resveratrol target (Um et al., 2010). More indirectly, resveratrol was reported to inhibit cAMP-degrading phosphodiesterases, increasing cAMP levels and igniting a cascade that activates AMPK (Park et al., 2012). In both studies, upstream AMPK activation led to NAD⁺ increases explaining indirect SIRT1 activation by resveratrol (Um et al., 2010; Park et al., 2012). In contrast, a recent study positions AMPK downstream of SIRT1, provided that resveratrol is used in “moderate” doses (Price et al., 2012). In that study, authors argue that while high doses of resveratrol may activate AMPK directly, moderate doses increase mitochondrial biogenesis in a SIRT1-dependent manner, upstream of AMPK activation. Thus, resveratrol indirect activation of AMPK was reported SIRT1-dependent, via deacetylation of the AMPK kinase LKB1. Significantly, both high and moderate doses of

resveratrol failed to increase mitochondrial biogenesis in SIRT1 knockouts (Price et al., 2012).

4.2 Neuronal mitochondrial biogenesis and KDAC modulation

The effects of KDAC modulation on neuronal mitochondrial biogenesis have been scarcely explored (the wealth of current data on these signalling pathways pertains to non-neuronal cells). As far as we could find, KDAC activation in neurons (or neuroblastoma cells) has been tested only for SIRT1, by means of overexpression or using compounds such as resveratrol (with doubtful SIRT1 specificity; Pacholec et al., 2010). Conversely, the effects of KDAC inhibition have been tested using SIRT1 deletion, the SIRT1 inhibitor EX-527, and pan-KDAC inhibitors. Such data are reviewed below, identifying the models and with the proviso that neuroblastoma cell lines can behave quite differently from post-mitotic neurons.

In neuroblastoma cells (Neuro2a), resveratrol was reported to increase mitochondrial biogenesis markers through AMPK activation (Dasgupta and Milbrandt, 2007). Authors excluded SIRT1 involvement in AMPK activation in neuroblastoma or primary neurons since neither SIRT1 inhibitors (sirtinol, splitomycin, and nicotinamide) nor SIRT1 deletion attenuated AMPK activation. Interestingly, their study suggests that resveratrol effects are neuron-type-dependent. According to their results, AMPK phosphorylation in dorsal root ganglion (DRG) neurons is primarily dependent on LKB1 activity, whereas in cortical neurons it also requires CamKKB (Dasgupta and Milbrandt, 2007). Still, their study suggests that LKB1 activity is SIRT1 independent, which is compatible with some studies (Park et al., 2012), but contrasts with others reporting SIRT1-dependence (Lan et al., 2008; Price et al., 2012). Such diverse findings might be explained by cell-type-dependent variations on these signalling pathways. Still, regardless of whether resveratrol has multiple targets that might modulate mitochondrial biogenesis, it is important to verify if directly targeting SIRT1 activity suffices for altering mitochondrial biogenesis in neurons.

In primary cortical neurons, SIRT1 overexpression or GCN5 acetyltransferase silencing increased mitochondrial density in cell bodies and axons (Wareski et al., 2009). Further, increased mitochondrial biogenesis by SIRT1 was critically dependent on PGC-1 α , but independent from AMPK. Also, authors showed that SIRT1 effects resulted from deacetylating PGC-1 α and increasing its transcriptional activity (Wareski et al., 2009).

In vivo SIRT1 inhibition (with EX-527) increased mitochondrial density in hypothalamic proopiomelanocortin (POMC) neurons, without affecting indexes of mitochondrial morphology (Dietrich et al., 2010). This was interpreted as an adaptive response to decreased inhibitory tone on POMC neurons (Dietrich et al., 2010). Conceivably, the

heightened activity of uninhibited POMC neurons consumes ATP, increasing the AMP:ATP ratio and activating AMPK-dependent mitochondrial biogenesis via PGC-1 α phosphorylation.

Taken together, these findings suggest that increased SIRT1 activity promotes neuronal mitochondrial biogenesis (Wareski et al., 2009), but this may also occur without SIRT1 involvement (Dasgupta and Milbrandt, 2007), and even following SIRT1 inhibition, at least in specific neuronal populations (Dietrich et al., 2010). Significantly, pan-inhibition of KDACs (trichostatin A and valproate) in neuroblastoma cells was reported to upregulate PGC-1 α (Cowell et al., 2009), and thus may evoke mitochondrial biogenesis. Clearly, more studies are required to elucidate how the AMPK-SIRT1-PGC-1 α axis, and other KDACs work in neurons to modulate mitochondrial biogenesis. Current evidence suggests that SIRT1 activity and signalling are highly cell-type dependent, thus advising caution before establishing links between SIRT1 activation and general neuroprotection.

4.3. Mitochondrial biogenesis in neurodegeneration

Multiple neurodegenerative disorders have been associated with abnormal mitochondrial biogenesis. Decreases in PGC-1 α levels were reported in the context of Huntington (HD), Alzheimer (AD), and Parkinson's (PD) diseases as well as in spinal and bulbar muscular atrophy, whereas PGC-1 α overexpression was protective in several *in vitro* and *in vivo* disease models (Jones et al., 2012), although a recent study evidenced that sustained PGC-1 α overexpression was deleterious to dopaminergic neurons *in vivo* (Ciron et al., 2012). Mitochondria number was found decreased in HD patients' brains, together with decreased levels of PGC-1 α , Tfam and mitochondrial cytochrome c oxidase subunit II (Kim et al., 2010a). Moreover, PGC-1 α null mice presented neurodegenerative lesions predominantly in the striatum (Lin et al., 2004), a particularly vulnerable region in HD (Oliveira, 2010). Mitochondria number was also decreased in primary neurons cultured from AD mice (Calkins et al., 2011) and in AD patients' brains (Hirai et al., 2001) together with decreased expression of PGC-1 α , NRF1, NRF2a/2b and Tfam (Sheng et al., 2012). In PD patients, both PGC-1 α and NRF-1 mRNA were decreased in the *substantia nigra* and striatum (Shin et al., 2011). Furthermore, some PGC-1 α polymorphisms have been tentatively associated with risk or age of onset of PD (Clark et al., 2011) and HD (Taherzadeh-Fard et al., 2009; Weydt et al., 2009; Che et al., 2011), although population stratification may have influenced result interpretation (Ramos et al., 2012).

PGC-1 α thus represents an interesting target to rescue mitochondrial biogenesis in neurodegeneration models, yet relatively unexplored in what concerns KDAC modulation. Significantly, one study reported that SIRT1 overexpression restored mitochondrial density

and increased survival both in PD and HD neuronal models, respectively, expressing A53T α -synuclein and 120Q huntingtin (Wareski et al., 2009). Further studies are required to clarify SIRT1 and other KDACs potential as mitochondrial biogenesis modulators in neurons and more specifically in neurodegenerative disorders models.

Recently, another sirtuin (SIRT3) was reported to stimulate mitochondrial biogenesis (Kong et al., 2010; Figure 1). Silencing of the mitochondrial sirtuin SIRT3 in myotubes decreased PGC-1 α -mediated mitochondrial biogenesis, and authors proposed that SIRT3 might regulate NRF-1 and Tfam activities (Kong et al., 2010). Interestingly, another study reported that SIRT3 deacetylates the ribosomal protein MRPL10 down-regulating the synthesis of mitochondrial proteins. Consistently, SIRT3 knockout mice presented increased expression of mitochondrially-encoded components of oxidative phosphorylation (Yang et al., 2010). Thus, whether SIRT3 positively or negatively modulates mitochondrial biogenesis remains uncertain and, as far as we could find, unaddressed in neurons. Nevertheless, it was recently shown that expression of either SIRT3 or PGC-1 α was neuroprotective in an amyotrophic lateral sclerosis model, rescuing defects in mitochondrial dynamics (Song et al., 2012), specifically fission-fusion dynamics, which is the subject of the next section.

5. Fission-Fusion

Mitochondria are a highly dynamic organelle population that changes size and morphology by fusing together or dividing through fission. Mitochondrial fusion enables the exchange of mtDNA and other matrix components between mitochondria, rendering protection against mtDNA mutations by allowing functional complementation and thus maintaining a healthy oxidative phosphorylation system (Chan, 2006; Chen et al., 2007). Mitochondrial fission permits mitochondrial separation to daughter cells during mitosis (Taguchi et al., 2007), allows segregation of dysfunctional mitochondria to be targeted for mitophagy (Twig et al., 2008), and enables the mitochondrial size and shape adaptations required for distribution in neuronal ramifications (Li et al., 2004; Ishihara et al., 2009; Kageyama et al., 2012; Figure 1).

Mitochondrial fusion involves merging of the outer as well the inner mitochondrial membranes, a coordinated process assisted by different proteins (Song et al., 2009). Mitofusins (Mfn1 and Mfn2) promote outer membrane fusion. These are highly homologous GTPases anchored to the outer membrane and able to form homo- or hetero-protein complexes (Chen et al., 2003), which allows mitochondrial tethering and fusion in a GTP hydrolysis-dependent manner (Ingelman et al., 2005). Significantly, a dominant-negative mutation in Drp1 was reported in a newborn with lethal neurodevelopmental abnormalities,

exhibiting defects in both mitochondrial and peroxisomal fission (Waterham et al., 2007). Consistently, Drp1 knockout causes abnormal brain development and embryonic death in mice (Ishihara et al., 2009). In yeast, Drp1 attach to mitochondria by binding to Fis1, a protein anchored to the mitochondrial outer membrane. In mammals, however, other mitochondrial outer membrane proteins seem to take over the Drp1 receptor role. Thus, mitochondrial fission factor (Mff) was proposed as an essential factor for Drp1 recruitment, mediating fission independently from Fis1 (Otera et al., 2010). Alternatively, mitochondrial elongation factor 1 (MIEF1) (Zhao et al., 2011), also identified as MiD49/51 (Palmer et al., 2011), can bind Drp1 and inhibit its GTP hydrolysis thus promoting fusion instead of fission (Oettinghaus et al., 2012). According to such model, Fis1 could promote fission by sequestering MIEF1 and consequently unblocking Drp1 GTP hydrolysis (Zhao et al., 2011; Oettinghaus et al., 2012). It is also possible that MIEF1 levels determine the outcome, with elevated levels compromising selective Drp1 recruitment to constriction sites, leading to its uniform distribution, preventing formation of active scission complexes, and thus causing fusion instead of fission (Palmer et al., 2011). Recently, Fis1 was also proposed to modulate mitochondrial morphology by recruiting the GTPase regulator protein TBC1D15, in a Drp1-independent manner (Onoue et al., 2012). Thus, while both Drp1 and Fis1 seem to be key players in the regulation of mammalian mitochondrial dynamics, whether and how they interplay remains to be unravelled.

5.1 KDACs and mitochondrial fission-fusion

Treatment with different KDAC inhibitors, including pan-, class I- and isoform-selective inhibitors, induced mitochondrial elongation in several cell lines, including primary cultures, untransformed and cancer cell lines (Lee et al., 2012). Mitochondrial elongation occurred at both subtoxic and toxic concentrations, indicating that mitochondrial structural integrity *per se* was not sufficient to protect cells against apoptotic stimuli. Further, the KDAC inhibitors increased histone H3 acetylation, decreased Fis1 expression levels, and decreased Drp1 recruitment to the mitochondria, without altering the levels or acetylation status of the Drp1, Mff, Mfn1, Mfn2, and OPA1 proteins (Lee et al., 2012). These findings suggest that mitochondrial elongation by KDAC inhibitors resulted from the down-regulation of an essential mitochondrial fission mediator, Fis1, which was recently reported as a mitochondrial fusion preventer via MIEF1 sequestration (Zhao et al., 2011; Figure 1).

The concept that KDAC inhibitors decrease Fis1 levels may, at first, seem unexpected given the general view that increased histone acetylation should promote transcription and, consequently, protein expression. Still, KDACs act on multiple non-histone targets, and changes in the lysine acetylation status of transcription factors may either increase or

decrease their activity (Kouzarides, 2000). Also, KDAC inhibition has been shown to down-regulate proteins by promoting their ubiquitin-dependent degradation; e.g. DNA methyltransferase 1 (DNMT1) is polyubiquitinated when KDAC inhibition hyperacetylates the Hsp90 chaperone preventing its interaction with DNMT1 (Zhou et al., 2008). Thus, the reported decrease in Fis1 protein levels (Lee et al., 2012) might partly result from increased degradation, and not necessarily from decreased transcription.

Increased mitochondrial fragmentation was reported for human fibroblasts treated with nicotinamide (Kang and Hwang, 2009). Similar findings were reported for SIRT1 activators, SRT1720 or fisetin, only when SIRT1 expression was intact (Jang et al., 2012). Nicotinamide, one of the final products of sirtuin-catalized deacetylation, is frequently used as a sirtuin inhibitor. However, it is reported to activate SIRT1 when used in lower concentrations (5 mM), since nicotinamide readily converts into the SIRT1 cofactor NAD⁺ via the “NAD⁺ salvage pathway” (Jang et al., 2012). Together with increased mitochondrial fragmentation, nicotinamide reduced the mitochondrial mass, increased mitochondrial membrane potential ($\Delta\psi_m$), and evoked a time-dependent increase in the levels of Drp1, Fis1 and Mfn1. Thus, it was suggested that nicotinamide enhances mitochondrial quality, with optimized levels of fission and fusion mediators facilitating separation of defective mitochondria for mitophagy (Kang and Hwang, 2009), this being mediated by high NAD⁺:NADH ratio and SIRT1 activation (Jang et al., 2012; Figure 1; Table 2).

5.2 Neuronal mitochondrial fission-fusion and KDAC modulation

In spite of accumulating evidence for abnormal mitochondrial fission-fusion dynamics in neurodegenerative diseases, there is limited data on the regulation of neuronal mitochondrial morphology by KDAC modulation. Impaired mitochondrial fusion and smaller mitochondrial size was recently reported for motor neurons expressing mutant superoxide dismutase (SOD1; Magrane et al., 2012). Accordingly, previous studies reported decreased mitochondrial length and disrupted mitochondrial distribution in cell and animal models of amyotrophic lateral sclerosis (ALS; Magrane et al., 2009; Tradewell et al., 2011; Vande Velde et al., 2011), which may stem from decreased OPA1 and increased Drp1 levels in mitochondria (Ferri et al., 2010). Concerning KDACs in this context, it was recently reported that SIRT3 overexpression rescued mitochondrial fragmentation in cortical neurons expressing SOD1^{G93A} (Song et al., 2012). While the mechanisms by which SIRT3 corrects mitochondrial morphology remain uncertain, it was proposed that SIRT3 deacetylation of cyclophilin D and resulting inhibition of mitochondrial permeability transition may play a neuroprotective role (Song et al., 2012).

5.3. Mitochondrial fission-fusion in neurodegeneration

Abnormal mitochondrial fission-fusion dynamics, with disequilibrium towards fission has been described for multiple neurodegenerative disorders. In the AD brain, reductions in Mfn1, Mfn2, OPA1 and Drp1 levels were reported together with increased Fis1 levels (Wang et al., 2009). In the context of PD, mutations in the serine/threonine kinase PINK1 or in the E3 ubiquitin ligase Parkin are major causes of familial PD, and these two proteins seem to functionally interact in the control of mitochondrial dynamics, albeit not in a simple linear pathway (Chen and Chan, 2009). Mutant PINK1 is reported to promote mitochondrial fission or decrease fusion in mammalian cells. Possible mechanisms are that mutant PINK1 promotes Drp1 mitochondrial translocation, and interferes with wild-type PINK1 pro-fusion effect of increasing the fusion/fission protein ratio (Cui et al., 2010). Parkin acts downstream of PINK1, thus mutations in either protein may promote fission by reducing wild-type Parkin-promoted degradation of Drp1 (Wang et al., 2011a) or Fis1 (Cui et al., 2010).

HD is also associated with increased mitochondrial fission, with a study in patients brain samples reporting increased expression of Drp1 and Fis1, and decreased expression of Mfn1, Mfn2, and OPA1 (Shirendeb et al., 2011). This contrasts with findings in several HD cell lines reporting no relevant changes in pro-fission or pro-fusion protein levels (Costa et al., 2010). Alternatively, the pro-fission phenotype observed in HD cells may stem from abnormal Ca^{2+} homeostasis activating calcineurin, which dephosphorylates Drp1 promoting its translocation onto mitochondria (Cereghetti et al., 2008; Costa et al., 2010; Oliveira and Lightowlers, 2010). Consistently, a recent study proposes that phosphorylation hinders Drp1 oligomerization, reducing its recruitment by Mff or preventing completion of fission-competent Drp1 spirals, thus inhibiting mitochondrial fission (Strack and Cribbs, 2012). Alternatively, increased mitochondrial fission in HD may stem from an abnormal interaction between mutant huntingtin and Drp1, which is proposed to increase Drp1 enzymatic activity and thus promote mitochondrial fragmentation (Song et al., 2011; Shirendeb et al., 2012). Similarly, in AD context, beta amyloid was reported to abnormally interact with Drp1 (Manczak et al., 2011).

The growing association of mitochondrial fission and neurodegeneration has sprouted the interest in compounds capable of inhibiting mitochondrial fission. Still, while inhibiting mitochondrial fission may afford protection against acute injury (Grohm et al., 2012), it is becoming clearer that in the long run, inhibiting fission is not beneficial to neurons (Li et al., 2004; Kageyama et al., 2012; Sheng and Cai, 2012). Thus, decreasing fission probability by epigenetic modulation, namely with KDAC inhibitors (Lee et al., 2012), is worth further examination as an alternative to direct fission inhibition.

6. Movement

Mitochondrial movement allows their efficient distribution throughout the cell. This is particularly relevant in the highly polarized neurons, where ATP diffusion *per se* would be inefficient, and thus mitochondria must travel to supply distant and metabolically demanding sites such as synapses, nodes of Ranvier, and active growth cones (Hollenbeck and Saxton, 2005). In mammalian cells, mitochondrial transport relies heavily on microtubules, motor and adaptor proteins. The kinesin-1 motor family (KIF5) drives anterograde transport assisted by adaptors such as Milton orthologues (TRAK1 and TRAK2) linked to mitochondrial rho (MIRO), an outer membrane RHO family GTPase and Ca^{2+} sensor. Syntabulin and FEZ1 are other KIF5-mitochondria adaptors, possibly allowing directed responses to different physiological signals (Sheng and Cai, 2012). The motor dynein typically drives retrograde mitochondrial movement, but may also be involved in bidirectional transport. Also, presence of both KIF5 and dynein in the same single mitochondrion allows for complex bidirectional movement, possibly coordinated by dynactin, which enhances dynein processivity (Cai et al., 2011; Sheng and Cai, 2012). A key element for microtubule-based docking of mitochondria in sites of need is syntaphilin, which acts as a 'static anchor' for axonal mitochondria thus regulating their mobility (Kang et al., 2008).

6.1 Microtubule deacetylases and mitochondrial movement

Microtubules are key cytoskeletal elements involved in neuronal mitochondrial trafficking. They are polymers of α/β -tubulin heterodimers and their functional diversity can be regulated by PTMs (Hammond et al., 2008; Janke and Kneussel, 2010). Acetylation of α -tubulin at lysine 40 was reported as a specific PTM that enhances recruitment of kinesin-1 and dynein/dynactin motor complexes to microtubules, and stimulates anterograde and retrograde transport (Reed et al., 2006; Dompierre et al., 2007). KATs such as the ARD1-NAT1 (ADP-ribosylation factor domain protein1 in complex with N-terminal acetyltransferase), and the Elongator complex, were shown capable of acetylating α -tubulin (Janke and Kneussel, 2010; Creppe and Buschbeck, 2011). Subsequently, α TAT1 was proposed as the major and possibly the sole α -tubulin K40 acetyltransferase in mammals and nematodes (Shida et al., 2010). Conversely, two KDACs, specifically HDAC6 and SIRT2, were found to interact and deacetylate α -tubulin *in vitro* and *in vivo* (Hubbert et al., 2002; North et al., 2003; Zhang et al., 2003; Figure 1; Table 3).

HDAC6 and SIRT2 were reported to co-localise along the microtubule network and coimmunoprecipitate. Also, silencing of HDAC6 or SIRT2 alone sufficed to evoke tubulin hyperacetylation (North et al., 2003). Taken together with the report that tubulin does not

bind HDAC6 or SIRT2 individually (Nahhas et al., 2007), these data supported the hypothesis that HDAC6 and SIRT2 act interdependently in a protein complex. There are other studies, however, suggesting they are unlikely binding partners *in vivo* given their different expression profiles in brain cells, with HDAC6 predominating in neurons (esp. Purkinje cells) and SIRT2 in oligodendrocytes (Li et al., 2007; Southwood et al., 2007). Still, there is also evidence for SIRT2 expression in hippocampal, cortical and striatal neurons *in vitro* (Pandithage et al., 2008; Luthi-Carter et al., 2010), and a study reporting abundant neuronal expression of SIRT2, particularly in the adult brain (Maxwell et al., 2011). In such study, authors allude to a previous observation that tubulin is “not hyperacetylated” in the brains of HDAC6-deficient mice, and explain it with the possibility that abundant SIRT2 compensates for lack of HDAC6 (Maxwell et al., 2011). Such allusion, however, contrasts with the original publication in HDAC6-deficient mice, where the respective authors state that no significant increase in tubulin acetylation was found because, in the brain, tubulin is “already highly acetylated” in wild-type animals, and therefore, HDAC6 inactivation has no visible impact on acetylation levels (Zhang et al., 2008). Still, a recent study reports significant increases in α -tubulin K40 acetylation in HDAC6^{-/-} mice (Govindarajan et al., 2013).

SIRT2-mediated modulation of mitochondrial trafficking has not been reported, as far as we could find in the literature. Nevertheless, there are reports that SIRT2 inhibition does modulate neuronal physiology, being neuroprotective in disease models highly associated with mitochondrial dysfunction, such as PD and HD. Specifically, SIRT2 inhibition protected against α -synuclein toxicity, decreasing dopaminergic neuron death in both *in vitro* and *in vivo* (*Drosophila*) PD models, with the suggested mechanisms being that increased α -tubulin acetylation promotes coalescence of misfolded proteins into larger protective inclusions (Outeiro et al., 2007). SIRT2 inhibition was also found protective in a striatal neuron model of HD, by a mechanism involving decreased sterol biosynthesis (Luthi-Carter et al., 2010). Such mechanism has been questioned partly due to contrasting evidence that low sterol/cholesterol levels are associated with HD neurodegeneration (Valenza and Cattaneo, 2010). Thus, further studies are required to elucidate the putative neuroprotective role of SIRT2 inhibition and its effects on mitochondrial dynamics.

HDAC6 inhibition promoted both retrograde and anterograde mitochondrial movement in hippocampal neurons, together with increased tubulin acetylation and KIF5-mitochondria association. Further, Glycogen Synthase Kinase 3 β (GSK3 β) inhibition: reduced HDAC6 phosphorylation at serine 22; increased tubulin acetylation; and enhanced mitochondrial movement. Thus, leading to the proposal that GSK3 β may regulate HDAC6 activity by phosphorylation (Chen et al., 2010). The implications are that

misregulation of HDAC6, presumably overactivated by GSK3 β -mediated phosphorylation, might underlie impaired mitochondrial transport. Significantly, mitochondrial and vesicular trafficking impairment in AD models was associated with abnormal GSK3 β activation (Rui et al., 2006; Decker et al., 2010). Taken together, these data suggest that HDAC6 might be involved in linking GSK3 β to the trafficking impairment in AD. In fact, evidence suggests that HDAC6 is involved in trafficking abnormalities in several neurodegenerative disorders, as addressed below.

6.2. KDAC modulation of trafficking and neurodegeneration

In neurodegenerative diseases such as Huntington and Alzheimer's, current evidence suggest a beneficial role for HDAC6 inhibition. Indeed, HD patients' brain samples exhibit decreased tubulin acetylation; and cellular HD models present compromised microtubule-dependent transport, suggesting that transport might be restored by tubulin deacetylase inhibition (Dompierre et al., 2007). Consistently, selective HDAC6 inhibition with tubacin, but not HDAC1 inhibition with MS275, increased α -tubulin acetylation at lysine 40; thus enhancing KIF5 and dynein recruitment to microtubules, and promoting bidirectional transport in striatal cell lines. Further, KDAC inhibitors capable of inhibiting HDAC6 were shown to enhance transport-dependent BDNF release in cortical neurons expressing either wild type or mutant N-terminal huntingtin constructs (Dompierre et al., 2007). Similarly, decreased α -tubulin acetylation (Hempfen and Brion, 1996) and increased HDAC6 levels (Ding et al., 2008) were reported for the AD brain, suggesting a role in abnormal mitochondrial trafficking in this disease. Also, in hippocampal neurons challenged with amyloid- β , HDAC6 inhibition with tubastatin A enhanced bidirectional mitochondrial motility, rescuing transport and reducing mitochondrial fragmentation (Kim et al., 2012). More recently, HDAC6 deletion was reported to improve memory function in AD mice without affecting amyloid- β plaque load (Govindarajan et al., 2013). Significantly, HDAC6 deletion protected primary neurons from mitochondrial trafficking defects induced by amyloid- β derived diffusible ligands, and enhanced mitochondrial distribution in the hippocampi of AD mice (Govindarajan et al., 2013).

Degeneration of the peripheral nervous system may also benefit from HDAC6 inhibition, as shown for Charcot-Marie-Tooth disease models with altered mitochondrial transport. In this context, mice expressing mutant heat-shock protein HSPB1 presented decreased acetylated tubulin and severe axonal transport deficits. Significantly, DRG neurons from symptomatic HSPB1^{S135F} mice exhibited decreased mitochondrial number and motility in their neurites. Pharmacological HDAC6 inhibition *in vitro* and *in vivo* rescued the mitochondrial number and motility phenotype in DRG neurons. Further, *in vivo* HDAC6

inhibition improved motor performance, together with improved electrophysiological/histological parameters, suggesting that it might be a useful therapeutic approach in peripheral neuropathies (d'Ydewalle et al., 2011).

In spite of the above evidence, HDAC6 inhibition is unlikely a universal solution for abnormal axonal transport. Interestingly, evidence from neuroinflammation models points towards another HDAC – the normally nuclear-located HDAC1 - as playing a critical role in the onset of axonal damage and mitochondrial transport abnormalities, not ameliorated by HDAC6 inhibition. Neuroinflammatory stimuli (glutamate plus $\text{TNF}\alpha$) were reported to evoke a Ca^{2+} -dependent nuclear export of HDAC1 (Kim et al., 2010b). Interestingly, pan-classical-KDAC inhibition improved neuronal Ca^{2+} recovery following glutamate receptor (NMDAR) activation (Oliveira et al., 2006). Ca^{2+} -dependent nuclear export appears to confer a cytosolic gain of function to HDAC1, namely, binding α -tubulin and motor proteins (KIF5 and KIF2A), thus impairing their ability to transport cargo such as mitochondria, leading to localized neurite swelling and degeneration (Kim et al., 2010b). These toxic effects were partly rescued by preventing HDAC1 nuclear export, or by pharmacological inhibition of HDAC1 with MS275, but not by HDAC6 inhibition with tubacin (Kim et al., 2010b). Hence, it seems that different KDACs may impair mitochondrial transport as a function of different pathological triggers, explaining the opposite findings of inhibiting HDAC6 vs. HDAC1 in models of HD (Dompiere et al., 2007) vs. neuroinflammation (Kim et al., 2010b; Figure 1). Still, inhibiting KDACs for rescuing mitochondrial transport must be balanced against putative interference with other roles, such as the role of HDAC6 in mitophagy.

7. Mitophagy

The selective degradation of defective mitochondria prevents them from releasing oxidants and apoptosis triggers, thus being critical for neuronal health and survival. The mitophagy machinery engulfs and digests small fusion-deficient mitochondria exhibiting sustained depolarization (Twig and Shirihai, 2011). A key mitophagy regulator is the PINK1-Parkin signalling pathway. PINK1 acts as $\Delta\psi_m$ sensor, recruiting Parkin to depolarized mitochondria, thus triggering the mitophagy machinery. Mechanistically, PINK1 is normally imported into polarized mitochondria and constitutively degraded by PARL (presenilin-associated rhomboid-like protein). In bioenergetic incompetent mitochondria, however, PINK1 is no longer degraded and accumulates in the outer membrane where it can recruit Parkin (Jin et al., 2010). Through its E3 ubiquitin ligase activity, Parkin ubiquitinates mitochondrial proteins like VDAC1 (Geisler et al., 2010) and MIRO (via an interplay with Pink1 that arrests damaged mitochondria; Wang et al., 2011b; Liu et al., 2012a), and also ubiquitinates fusion mediators like mitofusins (Gegg et al., 2010). Thus, depolarized

mitochondria are rendered fusion-deficient and with a coating that attracts the ubiquitin-binding autophagic components, p62 and HDAC6 (Lee et al., 2010a; Youle and Narendra, 2011; Ding and Yin, 2012; Figure 1).

7.1 KDACs and mitophagy

HDAC6 is reported to play a key role in the quality control autophagy of protein aggregates and mitochondria (Lee et al., 2010b; Lee et al., 2010a). HDAC6 has the capacity to bind polyubiquitinated proteins and also dynein motors, thus linking target recognition with its transport to aggresomes (Kawaguchi et al., 2003; Ouyang et al., 2012). Moreover, HDAC6 facilitates aggresome clearance (Iwata et al., 2005; Pandey et al., 2007) by controlling autophagosome-lysosome fusion (Lee et al., 2010b; Figure 1; Table 3).

The HDAC6 ubiquitin- and dynein-binding motifs are distinct from the tubulin deacetylase domain that is selectively targeted by HDAC6 inhibitors (Haggarty et al., 2003), which were neuroprotective in several disease models (Dompierre et al., 2007; d'Ydewalle et al., 2011; Kim et al., 2012). Nevertheless, there is evidence that a functional HDAC6 deacetylase domain is required for aggresome formation, autophagosome-lysosome fusion and autophagic turnover (Kawaguchi et al., 2003; Iwata et al., 2005; Pandey et al., 2007; Lee et al., 2010b). Thus, although HDAC6 inhibition enhances mitochondrial and vesicle trafficking, it may hinder mitophagy as well as the turnover of misfolded proteins. Still, when reduced axonal trafficking is the main problem for a given disease state, HDAC6 inhibition might be beneficial if compensatory mechanisms allow for adequate protein and mitochondria turnover.

SIRT1 may partly compensate for HDAC6 inhibition. Autophagosome-lysosome fusion requires cytosolic HDAC6 catalytic activity to deacetylate cortactin, which mediates the necessary F-actin remodelling (Lee et al., 2010b). Meaningfully, in the adult brain, SIRT1 is predominantly located in the cytosol (Li et al., 2008), thus being in a position to interact with cortactin. In fact, HDAC6 and SIRT1 were both shown to bind and deacetylate cortactin independently, but may also work cooperatively or competitively, with their relative dominance being cell type dependent (Zhang et al., 2009; Figure 1). Further, although HDAC6 knockout mice are reported to develop ubiquitin-positive brain aggregates (Lee et al., 2010b), they are also described as developing normally, being fertile and viable, without obvious brain and spinal cord abnormalities (Zhang et al., 2008). Thus, it is conceivable that increased activity of SIRT1 might compensate for the consequences of HDAC6 knockout/inhibition on cortactin acetylation, and consequently on autophagosome-lysosome fusion.

SIRT1 activation was associated with mitophagy induction in human fibroblasts. Indeed, treatment with SIRT1 activators or with nicotinamide (5 mM - a concentration that converts to NAD⁺ and activates SIRT1; Jang et al., 2012) was reported to accelerate mitophagy at least in part by inducing mitochondrial fragmentation. Consistently with quality control mitophagy activation, treated cells exhibited a decreased mitochondrial mass but increased $\Delta\Psi_m$; together with increased levels of the autophagosomal marker LC3-II, and higher number of mitochondria-associated LC3 puncta and lysosomes (Kang and Hwang, 2009; Jang et al., 2012). While the exact mechanisms downstream of SIRT1 activation remain uncertain, findings are consistent with the ongoing degradation of small depolarized mitochondria, which are preferential targets for mitophagy (Twig and Shirihai, 2011).

Further studies are required to clarify the interplay between HDAC6 and SIRT1 in the regulation of mitophagy. While HDAC6 modulation may have therapeutic potential in neurodegeneration (Li et al., 2011), the effects of HDAC6 inhibition on axonal trafficking must be balanced against the fact that misfolded proteins aggregates are a hallmark of several neurodegenerative diseases (Ross and Poirier, 2004). Therefore, HDAC6 inhibition aimed at promoting neuronal mitochondrial trafficking should be further explored to test for implications in autophagolysosome formation and clearance in neurons.

7.2 Mitophagy in Neurodegeneration

Parkinson's disease has taken the lead in the research on mitophagy impairment, partly due to the links between the PINK1-Parkin pathway and familial forms of this neurodegenerative disorder (Vives-Bauza and Przedborski, 2011). Wild-type Parkin is selectively recruited to dysfunctional mitochondria and promotes their autophagy (Narendra et al., 2008). Parkin recruitment depends on functional PINK1, and loss of function mutations in PINK1 or Parkin can block mitophagy (Geisler et al., 2010). Thus, PD neurodegeneration may at least partly stem from impairment in selective mitochondrial clearance, leading to the accumulation of dysfunctional organelles.

In Alzheimer's disease brains, the area of intact mitochondria was found decreased in vulnerable neurons, together with increased mtDNA and proteins in vacuoles associated with lipofuscin. This increase in mitochondrial degradation products suggested either increased mitophagy or decreased proteolytic turnover (Hirai et al., 2001). Indeed, it has been proposed that AD mitochondria are susceptible to increased autophagic degradation (Moreira et al., 2007a, b), but it is still uncertain whether increased mitophagy is a protective response or contributing to pathology, possibly in a synergistic manner with dysfunctional fission-fusion dynamics (Santos et al., 2010). Interestingly, increased Parkin expression in AD mice was shown to decrease intracellular amyloid- β levels and extracellular plaque

deposition, while also promoting autophagic clearance of defective mitochondria (Khandelwal et al., 2011).

Huntington's disease cellular models were reported to exhibit defects in cargo recognition by autophagic vacuoles, preferentially affecting organelle sequestration, and leading to the accumulation of abnormal mitochondria. Further, an abnormal interaction between mutant huntingtin and the autophagic adaptor p62 was proposed to cause the cargo recognition failure (Martinez-Vicente et al., 2010). Interestingly, it has been reported that HDAC6 is required for efficient autophagic degradation of aggregated huntingtin (Iwata et al., 2005). Thus, the modulation of HDAC6 activity might be an interesting strategy to improve the clearance of both mutant huntingtin and abnormal mitochondria in HD.

8. Concluding Remarks

Lysine deacetylases are emerging therapeutic targets in neurodegeneration. Current evidence suggests that their modulation, namely with epigenetic drugs such as KDAC inhibitors, may assist correction of abnormal mitochondrial dynamics in neurodegenerative diseases. The enhancement of mitochondrial biogenesis, movement, quality control mitophagy, and the restoration of fission-fusion balance have all been proposed as neuroprotective strategies. Concerning mitochondrial biogenesis, it is predominantly reported enhanced by SIRT1 activation. Still, recent findings suggest caution in interpreting data generated with uncertain SIRT1 activators. Future studies should help clarify the SIRT1-biogenesis connection and provide further mechanistic data for the role of SIRT3 and other KDACs in this process. Excessive mitochondrial fission is consistently reported for multiple neurodegenerative diseases, but arresting this crucial physiological event is also detrimental to neurons. Thus, decreasing fission probability with KDAC inhibitors is worth further examination as an alternative to direct fission inhibition. Abnormal mitochondrial transport has been associated with both HDAC6 and HDAC1 activities depending on the pathological trigger. In different neurodegenerative disease models, HDAC6 inhibition was shown to rescue trafficking abnormalities. Still, given the ubiquitin-binding and autophagosome-lysosome fusion promoting activities of HDAC6, the consequences of HDAC6 inhibition upon autophagy and clearance of misfolded proteins require further exploration. Conceivably, when reduced axonal trafficking is the main problem for a given disease state, HDAC6 inhibition might be beneficial if compensatory mechanisms allow for adequate autophagy and protein turnover.

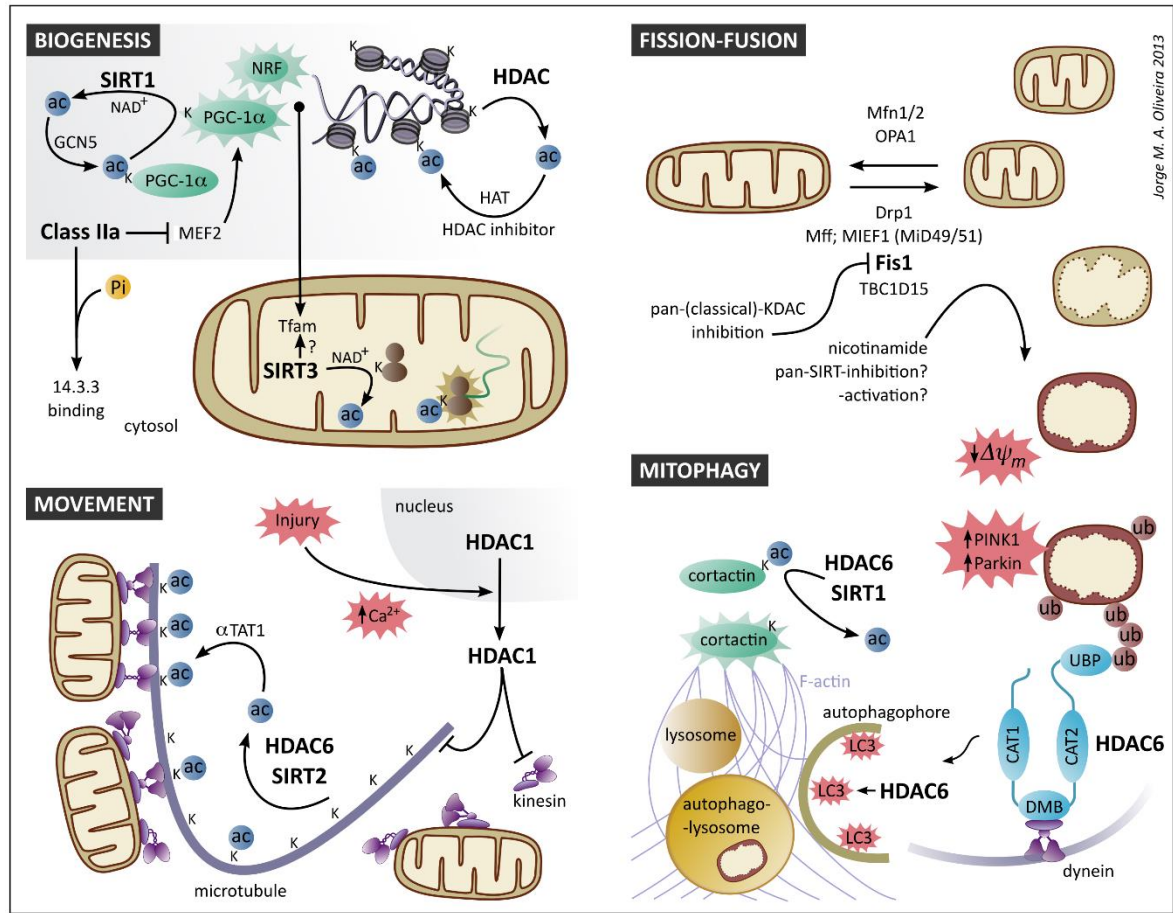


Figure 1. Modulation of mitochondrial dynamics by KDACs. *Biogenesis*: Involvement of classical KDACs and sirtuins on mitochondrial biogenesis pathways. *Movement*: Role of tubulin deacetylases HDAC6 and SIRT2 in regulating mitochondrial trafficking. Also, nuclear export and trafficking impairment by HDAC1 following injury. *Fission-Fusion*: Mediators of mitochondrial fission and fusion, and putative roles of KDAC modulation. *Mitophagy*: HDAC6 recognizing ubiquitinated mitochondria, assisting transport and promoting autophagosome-lysosome fusion via cortactin deacetylation together with SIRT1.

KDAC isoform	Model	Activity modulation method	Main findings	Comments	Ref.
SIRT1	C2C12 myoblasts	10 μ M EX-527 (24h)	Decreased mtDNA	Suggests that SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function, including enhanced mitochondrial biogenesis.	[65]
	SIRT1-Tg mice skeletal muscle	shSIRT1	Decreased mtDNA. Unaltered PGC-1 α , NRF-1, NRF-2, COX5 β and ATP5omRNA.		
SIRT1	Mice with heart-specific SIRT1 overexpression	Constitutive SIRT1 expression	Increased mtDNA. Increased PGC-1 α , NRF-1 and Tfam mRNA.	Reports that constitutive SIRT1 overexpression in the heart impairs mitochondria and reduces cardiac function.	[186]
		Low SIRT1 expression	Unaltered mitochondria number per cardiomyocyte area. Unaltered PGC-1 α , NRF-1 and Tfam mRNA.		
		Moderate SIRT1 expression	40% decrease in NRF-1 mRNA.		
SIRT1	Rabbit primary renal proximal tubule cells	High SIRT1 expression	50% decrease in NRF-1 mRNA.	Suggests that SIRT1 activation induces mitochondrial biogenesis through an AMPK-independent pathway, protecting mitochondria and renal proximal tubule cells against acute oxidant injury.	[61]
		10 μ M SIRT1720 (24h)	Increased PGC-1 α expression. 50% decrease in acetylated-PGC-1 α . Unaltered PGC-1 α mRNA levels. Increased (3.5x) mtDNA content.		
SIRT1	Mouse POMC neurons	100 μ M Nicotinamide	Unaltered mtDNA content. Nicotinamide pre-treatment prevents SIRT1720-induced mtDNA content increase.	SIRT1 blocked the recruitment of inhibitory synapses onto POMC neurons, increasing their activity. Suggests that SIRT1 activation in NPY/AgRP neurons is essential for physiological adaptation to negative energy balance.	[69]
		1.5 nmol/mouse EX-527 (l.c.v)	Increased mitochondrial density and area in the soma.		
SIRT1	Rat primary cortical neurons	SIRT1 overexpression	Increased mitochondrial density. SIRT1 increased PGC-1 α transcriptional activity but not PGC-1 α transcription.	Reports the regulation of mitochondrial density in neurons by PGC-1 α and PGC-1 β , and that increased PGC-1 α or SIRT1 expression protects against mutant-synuclein or huntingtin.	[68]
	Cortical neurons expressing mutant-synuclein	SIRT1 ^{C281A} expression	Unaltered mitochondrial density.		
	Cortical neurons expressing mutant huntingtin	SIRT1 overexpression	Reverts mitochondrial density decrease.		
SIRT1	C2C12 myotubes	shSIRT1	Unaltered PGC-1 α protein levels.	Reports that resveratrol benefits mice with diet-induced obesity; SIRT1 activation by resveratrol and SIRT1-dependent PGC-1 α activation.	[60]
SIRT3	C2C12 cells	SIRT3 overexpression	Decreased mitochondrial protein synthesis	Proposes that matrix SIRT3 deacetylates mitochondrial ribosomes controlling their activity.	[85]
		SIRT3 ^{MTA} expression	Unaltered mitochondrial protein synthesis		
		shSIRT3	Increased mitochondrial protein synthesis		
	Liver mitochondria from SIRT3 ^{-/-} mice	SIRT3 ^{-/-}	Higher translational activity of mitochondrial ribosomes		
SIRT3	Bovine liver mitochondria	Isolation with 10 mM Nicotinamide		Proposes SIRT3 as a PGC-1 α downstream target gene and a regulator of PGC-1 α effects on mitochondrial metabolism. SIRT3 suggested to suppress ROS levels and regulate mitochondrial biogenesis.	[84]
		SIRT3 overexpression	mtDNA copy number increased by 1.7-fold		
Class I, IIa and IIb	SH-SY5Y neuroblastoma	shSIRT3	Unaltered mtDNA copy number	Reports that PGC-1 α overexpression protects SH-SY5Y cells and that HDAC inhibition upregulates PGC-1 α .	[50]
		0.4 μ M TSA (16-18h)	Increased PGC-1 α mRNA		
HDAC5	Mice with doxycycline (DOX)-inducible cardiac-specific HDAC5/A signal-resistant mutant	10 mM Valproate (16-18h)		Identifies HDAC5 as an indirect PGC-1 α transcription regulator via MEF2 activity control.	[49]
		(+) vs. (-) DOX: represses vs. induces HDAC5/A transcription	(+) DOX decreased mitochondria number. (-) DOX decreased PGC-1 α expression		
HDAC3	COS cells	HDAC5/A expression	Prevents PGC-1 α promoter activation by MEF2	Does not alter PGC-1 α promoter activation by MEF2	
		HDAC3 overexpression			

Table 1. KDACs and mitochondrial biogenesis

Corresponding references:

[65] – Price et al., 2012

[186] – Kawashima et al., 2011

[61] – Csiszar et al., 2009

[69] – Dietrich et al., 2010

[68] – Wareski et al., 2009

[60] – Lagouge et al., 2006

[85] – Yang et al., 2010

[84] – Kong et al., 2010

[50] – Cowell et al., 2009

[49] – Czubryt et al., 2003

KDAC isoform	Model	Activity Modulation Method	Main Findings	Comments	Ref.
SIRT1	Normal human fibroblasts	5 mM Nicotinamide	Mitochondrial fragmentation and decreased mitochondrial content.	Reports that 5mM nicotinamide converts in NAD ⁺ through the "NAD ⁺ salvage pathway", increasing the NAD ⁺ /NADH ratio and activating SIRT1, which leads to mitochondrial fragmentation and autophagy activation.	[112]
		20 mM Nicotinamide	Increased mitochondrial content.		
		1 μ M - 5 mM NAD ⁺	Mitochondrial fragmentation and decreased mitochondrial content.		
		Resveratrol	Mitochondrial fragmentation and decreased mitochondrial content.		
		10 μ M Fisetin + siSIRT1	Mitochondrial fragmentation and decreased mitochondrial content rescued by SIRT1 KO.		
SIRT1	Mice with heart-specific SIRT1 overexpression	Low, moderate, and high SIRT1 expression	Unaltered mitochondrial size	Reports that constitutive SIRT1 overexpression in the heart impairs mitochondria and reduces cardiac function.	[186]
SIRT1	Mouse POMC neurons	1.5 nmol/mouse EX-527 (i.c.v.)	Unaltered mitochondrial morphology. Unaltered soma mitochondria cross-sectional area and circularity.	SIRT1 blocked recruitment of inhibitory synapses onto POMC neurons, increasing their activity. Suggests that SIRT1 activation in NPV/AgRP neurons is essential for the physiological adaptations to negative energy balance.	[69]
SIRT1	Normal human fibroblasts	5 mM Nicotinamide	Induced mitochondrial fragmentation and reduced mitochondrial mass. Increased Drp1, Fis1 and Mfn1 expression levels.	Proposes that nicotinamide optimises mitochondrial quality control by promoting fragmentation and mitophagy.	[111]
SIRT2-3	Spinal cord tissue from SOD1 ^{G93A} mice	SIRT2 Fluorimetric Drug Discovery Kit (Enzo Life Science) - buffer containing 10 mM nicotinamide and 1 μ M TSA	Decreased deacetylase activity	SOD1 ^{G93A} mutation increases mitochondria fragmentation and impairs movement. Non-functional Drp1 decreases mitochondria fragmentation, restores movement and diminishes cell death in SOD1 ^{G93A} motor neurons. SIRT3 or PGC-1 α transfection rescued mitochondrial fragmentation improving neuronal	[86]
SIRT3	Mice cortical neurons expressing SOD1 ^{G93A}	SIRT3 overexpression	SIRT3 expression rescued mitochondrial fragmentation		
Class I, IIa and IIb	Hep3B (hepatoma cell line)	8 μ M SAHA (48h) - induces 50% cell death	Mitochondrial elongation. Decreased Fis1 expression and Drp1 translocation to the mitochondria. Unaltered Drp1, Mfn1, Mfn2 and OPA1 expression. Increased H2A, H3 and H4 acetylation. Unaltered Drp1, Fis1, Mfn1 and OPA1 acetylation.	Reports that small molecule KDAC inhibition elongates mitochondria even at concentrations causing 50% cell death. Decreased Fis1 expression might be involved.	[109]
Class I, IIa and IIb (TSA), Class I and IIa (VPA and NaBut), Class I (Trapoxin A), HDAC2, 3 and 8 (Apicidin A), HDAC1 (MS-275)	ARPE19 (retinal pigment epithelium cell line)	40 μ M TSA; 30 mM VPA; 10 mM Sodium Butyrate; 10 nM Trapoxin A; 1 μ g Apicidin A; 15 μ M MS-275 (48h) - These concentrations induce apoptosis in ~50% cells after 48h	Mitochondrial elongation. Increased H3 acetylation.		
	Primary rat articular chondrocytes		Mitochondrial elongation		
	Cancer cell lines (Hep3B, T98G, U118MG, U87MG, U373MG, PC3, ZR-75-1, and KAT18)				
Class I, IIa and IIb	DRG neurons from 10 months-old HSPB1 ^{S135F} mice	0.4 μ M TSA (12h)	Increased mitochondria number	Mutant HSPB1 mice presented impaired mitochondria movement and decreased acetylated-tubulin in peripheral nerves. HDAC6 inhibition reverted the phenotype.	[158]
HDAC6		2 μ M Tubacin or 1 μ M Tubastatin A (12h)			
Class I, IIa and IIb	DRG neurons from 8 months-old HSPB1 ^{S135F} mice	10 mg/kg TSA (3 weeks <i>in vivo</i>)	Unaltered mitochondria number		
HDAC6		25 mg/kg Tubastatin A (3 weeks <i>in vivo</i>)	Increased mitochondria number		
HDAC6	Rat primary hippocampal neurons	5 μ M Tubastatin A (3h)	Unaltered mitochondria length or density	Reports that HDAC6 inhibition increases α -tubulin acetylation, preventing axonal mitochondrial trafficking impairment and disrupted morphology caused by Δ 1-42.	[157]
Class I, IIa and IIb	SH-SY5Y neuroblastoma	0.4 μ M TSA (16-18h)	Unaltered Mfn1 and Mfn2 gene expression	Reports that PGC-1 α overexpression protects SH-SY5Y cells and that HDAC inhibition upregulates PGC-1 α .	[50]
Class I and IIa		10 mM Valproate (16-18h)			
HDAC5	Mice with doxycycline (DOX)-inducible cardiac-specific HDAC5 Δ signal-resistant mutant	(+)DOX - represses HDAC5 Δ transcription (-)DOX - induces HDAC5 Δ transcription	(-)DOX mitochondria were swollen with disrupted cristae	Identifies HDAC5 as an indirect PGC-1 α transcription regulator via MEF2 activity control.	[49]

Table 2. KDACs and mitochondrial fission-fusion

Corresponding references:

[112] – Jang et al., 2012

[186] – Kawashima et al., 2011

[69] – Dietrich et al., 2010

[111] – Kang and Hwang, 2009

[86] – Song et al., 2012

[109] – Lee et al., 2012

[158] – d'Ydewalle et al., 2011

[157] – Kim et al., 2011

[50] – Cowell et al., 2009

[49] – Czubryt et al., 2003

KDAC isoform	Model	Activity Modulation Method	Main Findings	Comments	Ref.
HDAC6	Rat primary hippocampal neurons	5 μ M Tubastatin A (3h)	Increased anterograde/retrograde mitochondrial velocity and % motility	Reports that HDAC6 inhibition increases α -tubulin acetylation, preventing axonal mitochondrial trafficking impairment and disrupted morphology caused by A β 1-42	[157]
Class I, IIa and IIb	DRG neurons from 10 months-old HSPB1 ^{S135F} mice	0.4 μ M TSA (12h)	Increased number of motile mitochondria	Mutant HSPB1 mice presented impaired mitochondria movement and decreased acetylated-tubulin in peripheral nerves. HDAC6 inhibition reverted the phenotype.	[158]
HDAC6		2 μ M Tubacin (12h)			
HDAC6		1 μ M Tubastatin A (12h)			
Class I, IIa and IIb	DRG neurons from 8 months-old HSPB1 ^{S135F} mice	10 mg/kg TSA (3 weeks in vivo)	25 mg/kg Tubastatin A (3 weeks in vivo)	HDAC1 inhibition prevents mitochondrial trafficking impairment caused by glutamate and TNF- α treatment	[159]
HDAC6		25 mg/kg Tubastatin A (3 weeks in vivo)			
HDAC1	Rat primary cortical and hippocampal neurons	MS-275	HDAC6 inhibition does not prevent mitochondrial trafficking impairment caused by glutamate and TNF- α treatment	Reports that, in neurons exposed to excitotoxic or inflammatory injury, HDAC1 exits the nucleus and binds motor proteins impairing mitochondrial trafficking.	[159]
HDAC6		Tubacin	HDAC6 inhibition does not prevent mitochondrial trafficking impairment caused by glutamate and TNF- α treatment		
Class I, IIa and IIb	Rat primary hippocampal neurons	10 μ M TSA (2h)	Increased axonal mitochondria motility and velocity.	This study shows that HDAC6 inhibition increases axonal mitochondria trafficking and reports HDAC6 activity is modulated by GSK3 β . HDAC6 inhibition increased motor proteins recruitment to the mitochondria.	[152]
HDAC6		20 μ M Tubacin (2h)			
HDAC6	Primary hippocampal neurons from HDAC6 ^{-/-} mice	HDAC6 KO	Prevented abnormal mitochondrial trafficking induced by A β -derived diffusible ligands	Reports that reducing HDAC6 endogenous levels improves memory function in AD mice, and suggests that this may stem from improved mitochondrial trafficking	[149]
	Hippocampi from APPPS1-21_HDAC6 ^{-/-} mice		Promoted neuronal mitochondrial distribution		
SIRT1	Mice with heart-specific SIRT1 overexpression	Low SIRT1 expression	Unaltered degenerated or collapsed mitochondria number. Unaltered LC3b gene expression and LC3-II/LC3-I ratio.	Reports that constitutive SIRT1 overexpression in the heart impairs mitochondria and reduces cardiac function.	[186]
		Moderate SIRT1 expression	Increased (2x) degenerated or collapsed mitochondria number. Decreased LC3b gene expression and LC3-II/LC3-I ratio.		
		High SIRT1 expression	Increased (4x) degenerated or collapsed mitochondria number. Decreased LC3b gene expression and LC3-II/LC3-I ratio.		
SIRT1	Normal human fibroblasts	5 mM Nicotinamide	Induced mitochondrial fragmentation and reduced mass. Increased LC3 puncta and LC3-II/I ratio. Decreased Complex V levels.	Proposes that nicotinamide optimises mitochondrial quality control by promoting fragmentation and mitophagy.	[111]
HDAC6	Parkin-overexpressing mouse fibroblasts treated with CCCP for 16h to induce mitophagy	HDAC6 KO	Impaired mitophagy	Proposes that the ubiquitin-binding HDAC6 assists mitophagy, being recruited to mitochondria ubiquitinated by Parkin. HDAC6 was also reported to deacetylate cortactin promoting F-actin remodelling and autophagosome-lysosome fusion [170].	[167]
		HDAC6 KO followed by reintroduction of human HDAC6	Restored mitophagy		

Table 3. KDACs and mitochondrial movement or mitophagy.

Corresponding references:

[157] – Kim et al., 2012

[158] – d'Ydewalle et al., 2011

[159] – Kim et al., 2010

[152] – Chen et al., 2010

[149] – Govindarajan et al., 2013

[186] – Kawashima et al., 2011

[111] – Kang and Hwang, 2011

[167] – Lee et al., 2010

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Submitted

HDAC6 inhibition induces mitochondrial fusion, autophagic flux and reduces diffuse mutant huntingtin in striatal neurons

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Competing interests

The authors declare they have no competing interests.

Authors' contributions

PGD participated in the design of the study, performed the majority of experiments and data analysis, and drafted the manuscript. JP and TS contributed to image acquisition and analysis of mitochondrial dynamics. ALR performed and analyzed qPCR experiments. MRD contributed to imaging experiments and edited the manuscript. JMAO conceived and coordinated the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

ABSTRACT

Striatal neurons are vulnerable to Huntington's disease (HD). Impaired mitochondrial dynamics and decreased levels of acetylated alpha-tubulin are associated with HD, but it is unclear how these might contribute to the preferential degeneration of striatal neurons. Both HDAC1 and HDAC6 inhibitors rescued mitochondrial trafficking in neurological disease models, but their effects on neuronal mitochondrial fission-fusion or biogenesis remain undetermined. Also, inhibition of the alpha-tubulin deacetylase HDAC6 as a therapeutic strategy for HD is controversial – studies in neurons suggest it may compensate intracellular transport deficits, whereas data from cell-lines suggest it may impair autophagosome-lysosome fusion and mutant huntingtin (mHtt) degradation. Using primary cultures of rat striatal and cortical neurons, we show that striatal mitochondria are less motile and more balanced towards fission than cortical mitochondria, and that striatal neurons present lower autophagic flux compared to the less HD-vulnerable cortical neurons. The HDAC1 inhibitor MS-275 decreased mitochondrial fission and levels of the fission-associated Fis1 protein. The HDAC6 inhibitor tubastatin (TBA) increased acetylated alpha-tubulin levels, inducing mitochondrial motility and fusion in striatal neurons to levels observed in cortical neurons. Neither MS-275 nor TBA modified mitochondrial biogenesis. Importantly, TBA did not block neuronal autophagosome-lysosome fusion. Instead, TBA increased autophagic flux and reduced diffuse mHtt in striatal neurons, possibly by promoting transport of initiation factors to sites of autophagosomal biogenesis. This study identifies pharmacological HDAC6 inhibition as a potential strategy to reduce the vulnerability of striatal neurons to HD.

INTRODUCTION

Huntington's disease (HD) is a fatal neurodegenerative disorder caused by polyglutamine (polyQ) expansion mutations in the huntingtin protein. Although ubiquitously expressed, mutant huntingtin (mHtt) induces selective neurodegeneration that is most harmful to striatal neurons (Ross and Tabrizi, 2011). There are several reports suggesting that mHtt can induce mitochondrial dysfunction (Oliveira, 2010a,b Costa and Scorrano, 2012; Reddy and Shirendeb, 2012), but it is not clear how that might contribute to preferential striatal neurodegeneration. One hypothesis is that striatal neurons are intrinsically vulnerable to mitochondrial dysfunction. Indeed, even without mHtt, striatal neurons show increased susceptibility to oxidative phosphorylation defects (Pickrell et al., 2011), and to calcium-induced mitochondrial permeability transition (Oliveira and Goncalves, 2009) when compared to cortical neurons. It remains an open question whether striatal and cortical neurons also present intrinsic differences in mitochondrial dynamics that might influence their differential vulnerability.

Histone deacetylase (HDAC) inhibitors are experimental therapeutics for neurological disorders, primarily intended to increase histone acetylation and rescue transcriptional dysregulation (Kazantsev and Thompson, 2008), or to increase α -tubulin acetylation and rescue impaired intracellular transport (Hinckelmann et al., 2013). Pan-HDAC inhibitors and, particularly, selective HDAC1 or HDAC6 inhibitors, also seem capable of rescuing mitochondrial dysfunction (Guedes-Dias and Oliveira, 2013). Pan-HDAC inhibitors rescued mitochondrial calcium-handling in neurons from HD mice (Oliveira et al., 2006), whereas HDAC1 (Kim et al., 2010) and HDAC6 inhibitors (d'Ydewalle et al., 2011; Kim et al., 2012) rescued mitochondrial trafficking in different neurological disease models. In non-neuronal cells, HDAC1 inhibitors increased mitochondrial biogenesis (Galmozzi et al., 2013) and reduced mitochondrial fission (Lee et al., 2012). These properties might be therapeutically useful for neurological disorders, but since HDAC inhibitor effects are often cell-type dependent (Dietz and Casaccia, 2010) it remains unknown whether HDAC1 or HDAC6 inhibitors modulate mitochondrial biogenesis and fission-fusion balance in neurons.

Use of HDAC6 inhibition as a therapeutic strategy for HD has been controversial. HDAC6 is unique for its primary tubulin-deacetylase and ubiquitin-binding activities (Simões-Pires et al., 2013). Studies in neuronal HD models support HDAC6 inhibition as a neuroprotective strategy capable of compensating intracellular transport deficits (Dompierre et al., 2007), whereas studies in cell-lines indicate that HDAC6 inhibition/knockout may disrupt autophagosome-lysosome fusion (Lee et al., 2010) and prevent mHtt degradation (Iwata et al., 2005), predicting detrimental effects in HD. However, HDAC6-knockout mice show no evidence of brain abnormalities (Zhang et al., 2008; Govindarajan et al., 2013),

failing to support a role for HDAC6 in autophagosome-lysosome fusion, which would be expected to impair autophagy and cause neurodegeneration (Nixon, 2013). It is therefore important to clarify whether pharmacological HDAC6 inhibition affects neuronal autophagy and mHtt proteostasis.

Primary cultures of striatal and cortical neurons are frequently used to model differential vulnerability in HD (Oliveira and Goncalves, 2009; Tsvetkov et al., 2013). Here we investigated whether these neurons present intrinsic differences in mitochondrial biogenesis, trafficking and fission-fusion dynamics, and whether such dynamics are modulated by treatment with HDAC1 or HDAC6 inhibitors. We also used live-imaging to determine whether pharmacological HDAC6 inhibition affects autophagosome-lysosome fusion in neurons, and how it impacts mHtt proteostasis in striatal and cortical neurons.

MATERIALS AND METHODS

Plasmids and antibodies. Plasmids: mito-DsRed2 (mtDsRed; Michael Ryan, La Trobe University, Australia), mCherry-EGFP-LC3B (Jayanta Debnath, University of California, USA - Addgene 22418; N'Diaye *et al.*, 2009), EGFP-Htt^{ex1}Q74 (David Rubinsztein, University of Cambridge, UK - Addgene 40262; Narain *et al.*, 1999) and pmaxGFP (GFP; Amara). Primary antibodies and dilutions for Western blotting: anti-acetylated-histone-H3K9 (ab10812; 1:500), anti-acetylated- α -tubulin [6-11B-1] (ab24610; 1:5000), anti- β -actin [mAbcam 8226] (ab8226; 1:2000), anti-Fis1 (ab71498; 1:250), anti-mitofusin2 [NIAR164] (ab124773; 1:1000), anti-succinate dehydrogenase complex subunit A [2E3GC12FB2AE2] (SDHA; ab14715; 1:1000) were from Abcam; anti- α -tubulin [11H10] (#2125; 1:1000), anti-histone-H3 [96C10] (#3638; 1:1000), anti-LC3A/B (#4108; 1:1000) and anti-SQSTM1/p62 (#5114; 1:500) were from Cell Signaling; anti-OPA1 (612606; 1:1000) was from BD Biosciences; anti-acetylated-cortactin (09-881, 1:400) was from Merck-Millipore.

Drugs and reagents. The HDAC1 inhibitor MS-275 and the HDAC6 inhibitor tubastatin A (TBA) (Selleck Chemicals) were dissolved in dimethyl sulfoxide (DMSO), present at 0.1% in all treatment and control conditions ('solvent'). With the proviso that studies in cells typically require higher concentrations, studies with the isolated enzymes suggest that 1 μ M MS-275 preferentially inhibits HDAC1 (IC_{50} = 0.2 μ M), but may also inhibit HDAC9 (IC_{50} = 0.5 μ M) and partly HDAC2 (IC_{50} = 1.2 μ M) (Khan *et al.*, 2008). Still, other studies suggest that 1 μ M MS-275 has no inhibitory activity over HDAC9 (Bradner *et al.*, 2010). TBA is HDAC6 selective – isolated enzyme IC_{50} values are 0.015 μ M for HDAC6, 0.9 μ M for HDAC8, 16.4 μ M for HDAC1 and > 30 μ M for other HDAC isoforms (Butler *et al.*, 2010). Here we use MS-275 and TBA always at 1 μ M, as previously described for neurons (Baltan *et al.*, 2011; d'Ydewalle *et al.*, 2011). Fura-2 AM, MitoTracker Green FM and cell culture reagents were from Invitrogen. All other reagents were from Sigma-Aldrich, unless otherwise stated.

Neuronal culture and transfection. Sister cortical and striatal primary cultures were generated from Wistar rat embryos as previously described (Oliveira *et al.*, 2006; Oliveira and Goncalves, 2009), in full compliance with European Union directive 2010/63/EU. Cortical and striatal cells were plated at 10^3 cells per mm^2 on polyethylenimine coated glass coverslips and maintained in culture medium (Neurobasal supplemented with 2% B27, 1% fetal bovine serum, 1% penicillin/streptomycin and 1% GlutaMAX) at 37°C, 5% CO₂. Cytosine arabinoside (10 μ M) was added 48 h after plating to prevent glial proliferation. For neuronal transfection, culture medium was replaced with a mixture of 450 μ l Neurobasal

(with 1% Glutamax) and 50 µl Opti-MEM (containing 0.3-0.5 µg DNA, 0.5 µl Lipofectamine LTX and 0.5 µl Plus Reagent; Invitrogen). Following 30-45 min incubation (37°C, 5% CO₂) neurons were washed twice with Dulbecco's modified Eagle medium prior to restoring the conditioned culture medium. The average transfection efficiency was 5%, allowing single neuron identification for analyzing neurites, mitochondria, LC3-vesicles, or mHtt levels, without excessive overlap between cells.

Gene transcription and mitochondrial DNA (mtDNA) levels. For mRNA quantification, total RNA was isolated with illustra RNAspin Mini kit (GE Healthcare) and cDNA prepared with iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed with iQ5 MyiQ System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). GAP-43 primers: forward 5'-CCATGCTGTGCTGTATGAGAAG, reverse 5'-TCCTCCGGTTTGACACCATC. RPL13A primers: forward 5'-GGATCCCTCCACCCTATGACA, reverse 5'-CTGGTACTTCCACCCGACCTC. For mtDNA levels, total DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen). Primers for mtDNA (MTND1 gene): forward 5'-AATACGCCGCAGGACCATTC, reverse 5'-GGGGTAGGATGCTCGGATTC. Primers for nuclear DNA (nDNA; eEF1A gene): forward 5'-AGCCAAGTGCTAATGTAAGTGAC, reverse 5'-CCCTTGAACCACGGCATCTA. Relative quantifications were performed with the Pfaffl method (correcting for measured efficiencies) (Pfaffl, 2001).

Western blotting. Neurons were rinsed with ice-cold phosphate-buffered saline (PBS), lysed in buffer containing 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris (pH 8.0), and protease inhibitors. Protein was quantified by Bradford assay (Bio-Rad). Samples were boiled in Laemmli buffer, loaded at 20-25 µg per lane in polyacrylamide gels, electrophoresed under reducing conditions, and electroblotted to polyvinylidene difluoride membranes (PVDF; Millipore). Membranes were blocked in PBS with 0.05% Tween 20 (PBST) containing 5% non-fat dry milk, then incubated overnight at 4°C with primary antibodies (diluted in PBST with 5% BSA – bovine serum albumin), followed by washing in PBST and incubation with respective horseradish peroxidase conjugated antibodies for detection by enhanced chemiluminescence.

Neurite outgrowth in young neurons. Neurons with 3 days *in vitro* (DIV) were imaged 24 h post-transfection with GFP. Imaging (488 nm excitation) was performed at 37°C, 5% CO₂, using an inverted microscope equipped with LSM700 confocal scanner, 20× Plan-Apochromat 0.8 NA air objective, and Definite Focus (Axio Observer.Z1 system; Zeiss). Sholl analysis (Sholl, 1953; ImageJ) with 1 µm spaced concentric circles, was performed

on binary images (following background correction and thresholding), to calculate branching peak (intersection maxima) and total outgrowth (summed intersections).

Dendrite outgrowth, mitochondrial occupancy, size and number, in mature neurons.

Neurons at 10 DIV were fixed (4% paraformaldehyde, 37°C for 15 min) after 24 h transfection with GFP and mtDsRed, and imaged with an inverted Eclipse TE300 microscope system (Nikon; 60× PlanFluor 0.85 NA air objective; Polychrome II monochromator, TILL Photonics; C6790 CCD camera and Aquacosmos 2.5 software, Hamamatsu). At 10 DIV, the extensive axonal sizes, branching and inter-neuronal overlaps precluded full single-neuron tracings, focusing our quantitative analyses in dendrites and proximal axon. Sholl analysis with 1 μm -spaced circles was used to calculate dendrite branching peaks (intersection maxima), outgrowth (summed intersections), and mitochondrial fractional occupancy (mitochondrial intersections divided by dendrite intersections). Particle analysis (ImageJ) was used to calculate mitochondrial number and size (Feret diameter) in neurons divided into 'mitochondrial regions', each comprising the area between two circles of increasing radii: α -region (15-30 μm); β -region (30-80 μm); γ -region (80-130 μm).

Mitochondrial motility. Neurons at 10 DIV were loaded with Fura-2 AM (4 μM ; for labeling neurites; 380nm excitation) and MitoTracker Green (50 nM; for labeling mitochondria; 488nm excitation; Oliveira, 2011) for 30 min, washed twice and live imaged (5s intervals for 10 min) in recording media (133 mM NaCl, 5 mM KCl, 1.3 mM CaCl_2 , 1 mM MgCl_2 , 1 mM Na_2SO_4 , 0.4 mM KH_2PO_4 , 15 mM glucose, 20 mM HEPES, pH 7.4) at 37°C. The proportion of motile mitochondria was assessed via kymographs (20 representative lines per video; Multi Kymograph – J. Rietdorf and A. Seitz).

LC3-vesicle dynamics. Neurons expressing mCherry-EGFP-LC3B were live imaged at 8-10 DIV (48 h post-transfection) in culture media at 37°C, 5% CO_2 , using an Axiovert 200M, with LSM510 and a 63× Plan-Apochromat 1.4 NA oil objective (Zeiss). Combining acid-sensitive EGFP (488 nm excitation) with acid-insensitive mCherry (543 nm excitation) allows distinction of autophagosomes (EGFP + mCherry signal) from autolysosomes (mCherry signal only) (Pankiv et al., 2007). LC3-positive vesicles were counted in whole somata Z-stacks. Cytoplasmic volume was measured with 3D Objects Counter (F. Cordelières), deleting nuclei and using diffuse EGFP-LC3 fluorescence to define somatic boundaries. Vesicle motility parameters were analyzed with MTrackJ in 10 min videos at 3 s intervals, acquiring on the mCherry channel only (minimum velocity threshold was 0.1

$\mu\text{m/s}$; Lee et al., 2011). Vesicles crossing the first axonal branch towards the soma were expressed as events per 5 min (retrograde flux).

Immunofluorescence. Neurons were fixed with 4% paraformaldehyde for 15 min at 37°C, washed 3 times in PBS, permeabilized and blocked with 0.1% Triton X-100 and 3% BSA in PBS (Abdil) for 30 min. Neurons were then incubated for 1 h with primary antibody (anti-acetylated-cortactin, 1:150 in Abdil), and washed 3 times with 0.1% Triton X-100 in PBS, followed by 1 h incubation with Alexa Fluor 488 conjugated secondary antibody (Invitrogen; A-11034; 1:200 in Abdil). After assembly in fluorescent mounting medium (Dako), neurons were imaged with the aforementioned Eclipse TE300 system, ensuring non-saturating identical equipment settings for intensity comparisons between treatments.

Huntingtin proteostasis. Neurons were transfected with mHtt encoding plasmid at 5 DIV (Tsvetkov et al., 2013), and live imaged at 24 and 48 h post-transfection in culture medium at 37°C, 5% CO₂. Fluorescently tagged mHtt exon 1 (EGFP-Htt^{ex1}Q74) was excited at 488 nm and imaged at 20 $\times$ with the aforementioned Axio Observer.Z1 system, ensuring non-saturating identical equipment settings for fluorescence intensity comparisons. After imaging at 24 h post-transfection (6 DIV), neurons were treated with either solvent or TBA and re-imaged 24 h later (7 DIV; 48h post-transfection). Transfected neurons were screened for presence of aggregates and their location (soma and neurites), and their counts expressed in percentage of EGFP-positive neurons. Diffuse mHtt levels were measured by the average somatic EGFP fluorescence in neurons without visible aggregates.

Image processing and data analysis. Image processing was performed with ImageJ (<http://rsbweb.nih.gov/ij/>; National Institutes of Health) using the indicated plugins. Numerical data calculations were automated in Excel spreadsheets (Microsoft). Other data analyses and statistical calculations were performed using Prism 6.0 (GraphPad Software). Two-tailed Student's *t* test was used when comparing two groups, one-way ANOVAs with Dunnet's post-hoc when comparing three or more groups, and two-way ANOVA with Sidák's post-hoc when testing the interaction plus the main effects of region (cortical $\times$ striatal) and treatment (solvent $\times$ drugs). Curve fit comparisons in nonlinear regression analyses were performed with extra sum-of-squares *F* test. Unless otherwise stated, data are mean $\pm$ SEM of the *n* specified in figure legends.

RESULTS

Striatal and cortical neurons show intrinsic differences in mitochondrial dynamics

Neurons are highly polarized post-mitotic cells that distribute their mitochondria throughout neurites (Ruthel and Hollenbeck, 2003). To investigate whether mitochondrial dynamics differ between striatal and cortical neurons, we compared their neurite outgrowth and mitochondrial biogenesis along development. Young striatal and cortical neurons presented similar dendrites and branching peaks (Fig. 1A,B at 3 DIV), but total outgrowth was lower in striatal neurons as their axons were shorter (Fig. 1F – white boxes). Consistently, growth-associated protein 43 (GAP-43) mRNA levels were also lower in young striatal neurons, reaching cortical levels during maturation (Fig. 1C). Mature cortical neurons were larger and more branched than striatal neurons, but the average dendrite outgrowth of the two populations was similar (Fig. 1A,B at 10 DIV; Fig. 2A,B – white bars).

To determine whether mitochondrial biogenesis differed between the two cell types, we measured changes in mitochondrial DNA relative to nuclear DNA, which is predicted to remain stable in post-mitotic cells. Mitochondrial DNA increased during maturation of both neuronal populations, with differences matching their neurite outgrowth (Fig. 1D). The proportion of neurite length occupied by mitochondria was not significantly different in striatal and cortical neurons (Fig. 2Ci), while mitochondrial motility was significantly reduced in striatal neurons (Fig. 2D – white bars). These data therefore suggest that mitochondrial biogenesis and trafficking are modulated to maintain a constant mitochondrial volume occupancy in neurons.

To compare the mitochondrial fission-fusion balance, we measured mitochondrial size and number in three concentric ‘mitochondrial regions’ progressively away from the soma (α -, β -, and γ -regions; Fig. 3A). Mitochondrial size was significantly affected by distance from soma, decreasing from the α - towards the γ -region (Fig. 3B). Mitochondria in striatal neurons were smaller and more numerous than in cortical neurons (Fig. 3B,C – white bars). These data, together with identical mitochondrial occupancy (Fig. 2Ci) and higher levels of fission-associated protein in striatal neurons (Fis1/SDHA, Fig. 3D, Table 1), indicate that the striatal mitochondria population is intrinsically more balanced towards fission.

The HDAC inhibitors MS-275 and TBA modulate the fission-fusion balance of neuronal mitochondria without altering its biogenesis

To assess the effects of HDAC inhibitors on mitochondrial dynamics we measured changes in neuronal outgrowth and mitochondrial biogenesis. We confirmed the efficacy of 72 h treatment with the HDAC1 inhibitor MS-275 (1 μ M; Baltan et al., 2011) the HDAC6 inhibitor TBA (1 μ M; d'Ydewalle et al., 2011) by detecting increased acetylation, respectively, of the HDAC1-substrate histone-H3K9 or the HDAC6-substrate α -tubulin-K40, in comparison to the solvent control (0.1% DMSO; Fig. 1E). Early cortical outgrowth was significantly reduced by MS-275 treatment and increased by TBA treatment; early striatal outgrowth was not significantly modified by these treatments (3 DIV; Fig. 1F). In mature cultures, MS-275 had no significant effect on dendrite branching, reducing outgrowth only in striatal neurons; TBA had no significant effect on the branching or outgrowth of mature neurons (10 DIV; Fig. 2A,B). These results suggest that the effects of HDAC1 and HDAC6 inhibitors on neuronal outgrowth are developmental- and neuron-type dependent.

The level of mitochondrial biogenesis of striatal or cortical neurons was not affected by treatment with either MS-275 or TBA, as judged from unaltered mtDNA/nDNA ratios at 3 or 10 DIV (Fig. 1G). Treatment with MS-275 increased mitochondrial volume occupancy near cortical somata (Fig. 2Cii), without significantly affecting mitochondrial motility (Fig. 2D). Moreover, MS-275 increased mitochondrial size in cortical and striatal neurons (Fig. 3B), and reduced mitochondrial number in striatal neurons (Fig. 3C). Taken together with the observation that MS-275 decreases Fis1 levels in cortical and striatal neurons, and also the levels of the fusion-associated OPA1 and Mfn2 in cortical neurons (Table 1), these results suggest that MS-275 shifts the mitochondrial fission-fusion balance by limiting fission and not by promoting fusion.

Treatment with TBA significantly increased the proportion of motile mitochondria in cortical and striatal neurons (Fig. 2D), without modifying fractional occupancy (Fig. 2Cii,iii). In cortical neurons, TBA had no significant effect on mitochondrial size and number (Fig. 3B,C). In striatal neurons, however, TBA increased the size and reduced the number of mitochondria (Fig. 3B,C). Since TBA did not alter mitochondrial fission-fusion protein levels (Table 1), these results suggest that increasing the low mitochondrial motility of striatal neurons with TBA treatment increases the probability of mitochondrial contact and fusion.

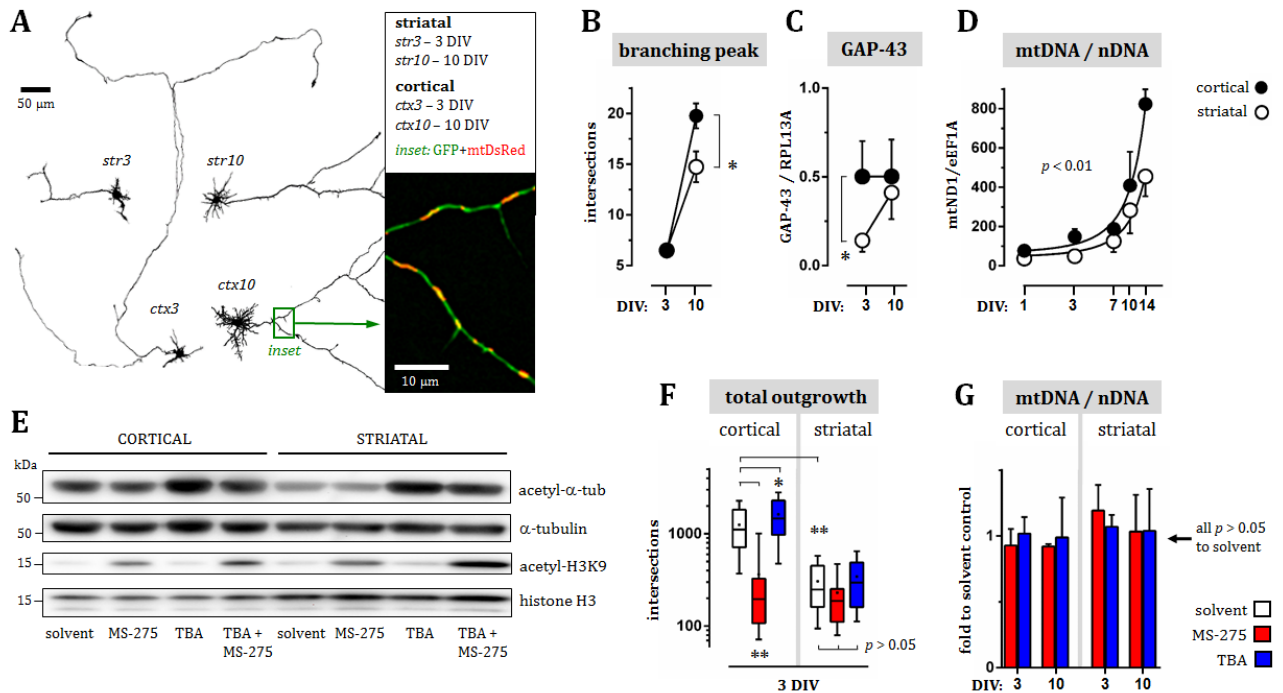
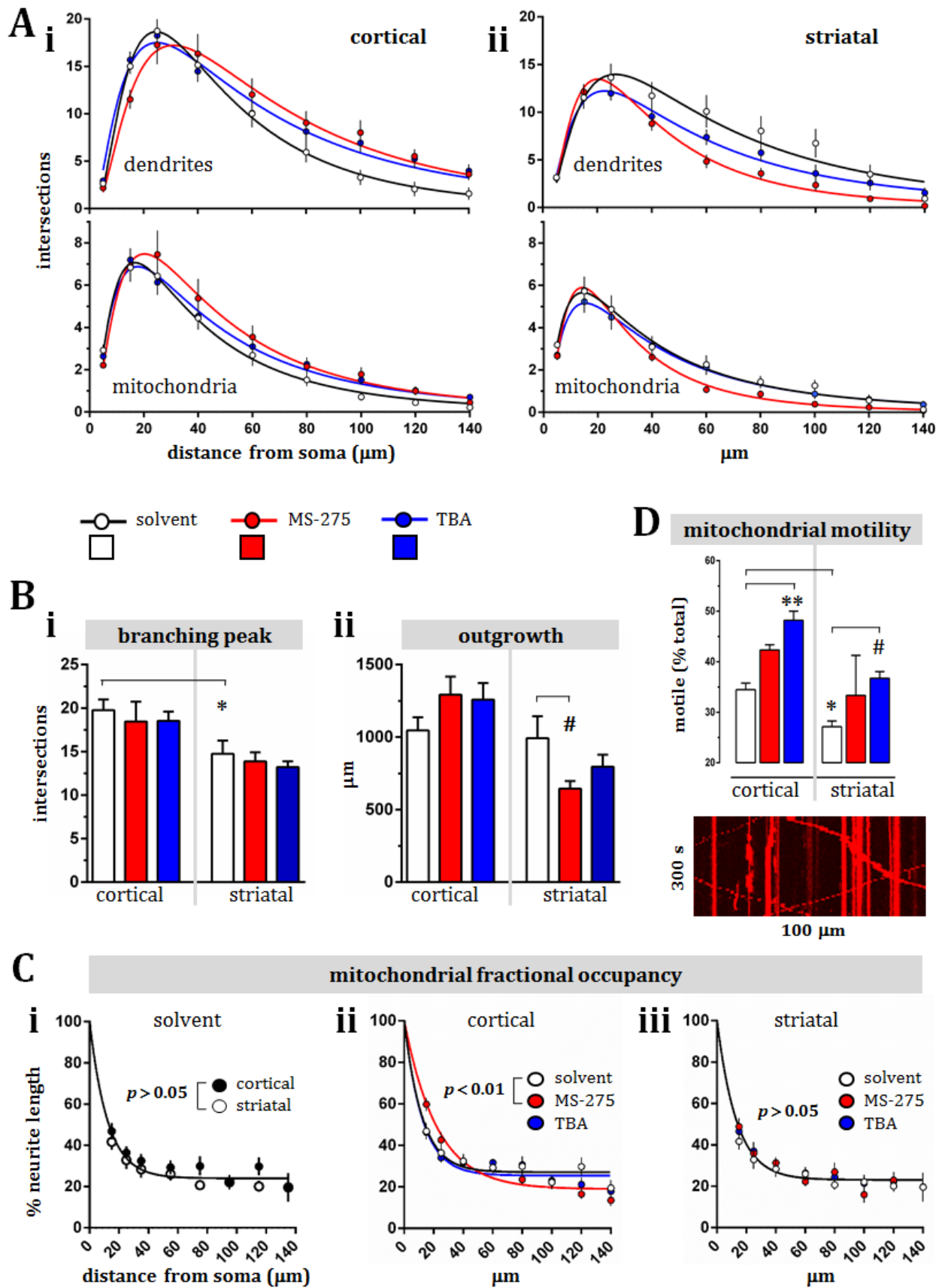


Figure 1. Neuronal outgrowth and mitochondrial biogenesis during maturation of cortical and striatal neurons. (A) Representative neurons at 3 DIV are shown whole; 10 DIV neurons are truncated at $\sim 200 \mu$ m from soma. Inset shows neurite and mitochondrial labeling. (B) Branching peak in maturing cortical and striatal neurons; $n = 10$ -20 neurons from 3-5 independent cultures. (C) GAP-43 mRNA and (D) mitochondrial DNA levels in maturing neurons; $n = 3$ independent cultures. (E) Immunoblot showing α -tubulin and histone-H3 acetylation in 3 DIV neurons treated with 1μ M TBA and/or 1μ M MS-275 for 72 h. (F) Sholl analysis at 3 DIV, total intersections with 1μ m-spaced circles; data are shown as box-plots with median and interquartile distance of $n = 42$ -50 neurons from 5 independent cultures, per treatment condition; * $p < 0.05$, ** $p < 0.01$ to solvent-treated cortical neurons. (G) Mitochondrial DNA levels in fold to respective solvent control (0.1% DMSO) at 3 or 10 DIV; $n = 3$ independent cultures.

Figure 2. Dendrite morphology and mitochondrial dynamics in mature neurons. (A-D) Cortical and striatal neurons at 10 DIV, following treatment with solvent, 1 μ M MS-275, or 1 μ M TBA for 72 h. (A) Intersections with dendrites (*top*) or mitochondria (*bottom*) as a function of distance from soma (Sholl analysis with log-normal curve fits); (B) Dendrite branching peak and outgrowth; (C) mitochondrial fractional occupancy with distance from soma (one-phase decay curve fit); $n = 11-17$ neurons from 3 independent cultures, per treatment condition. (D) Mitochondrial motility and representative kymograph; $n = 3-10$ independent cultures (296-417 individual mitochondria analyzed per treatment condition in each culture). * $p < 0.05$, ** $p < 0.01$ to solvent-treated cortical neurons; # $p < 0.05$ to solvent-treated striatal neurons.



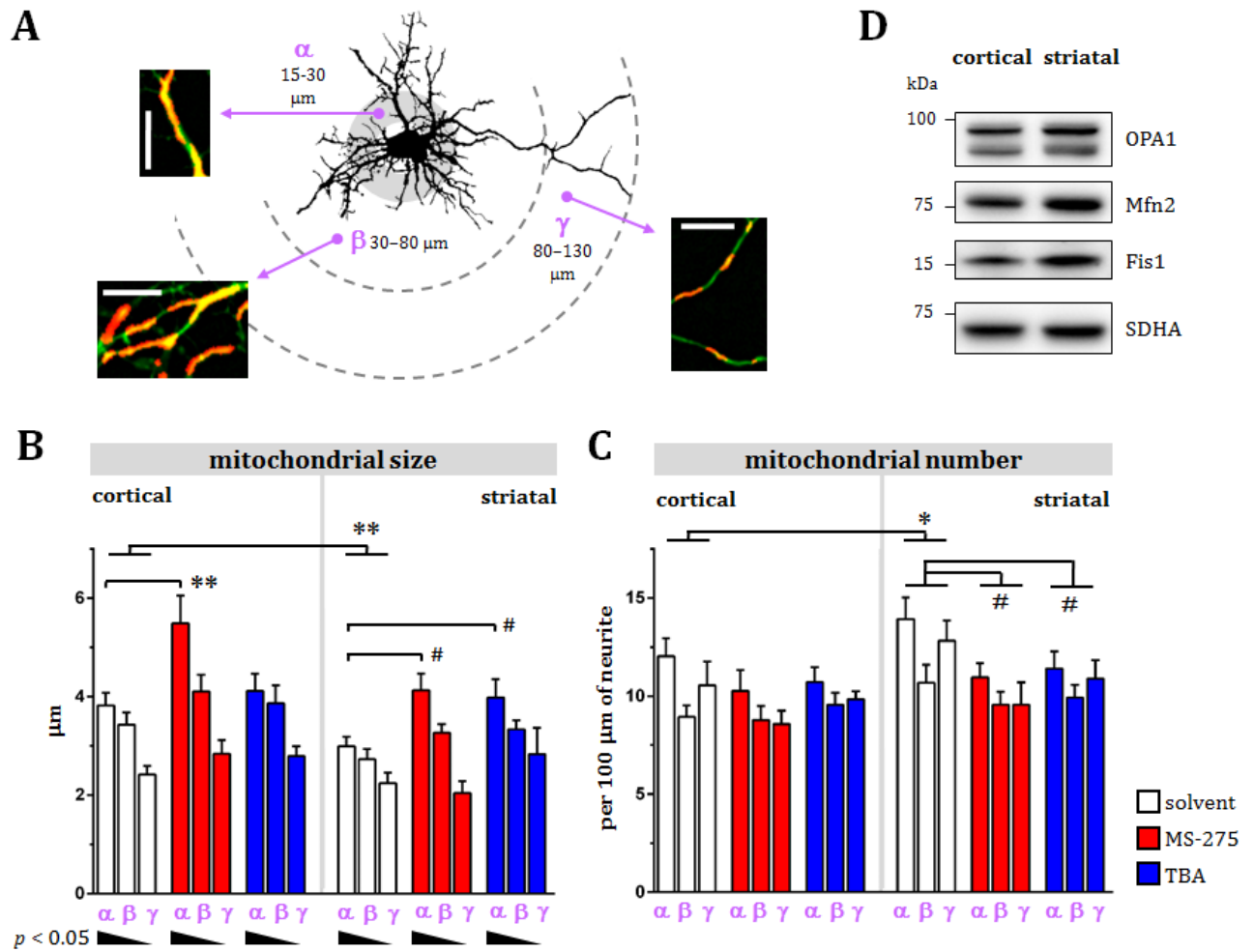


Figure 3. Mitochondrial fission-fusion balance in cortical and striatal neurons. (A) Representative neuron divided in encircled ‘mitochondrial regions’ α , β , γ , and respective radii. Insets show mitochondria (mtDsRed) and neurites (GFP) with 10 μm scale bars. (B, C) Mitochondrial size and number quantification within the α , β , and γ regions of 10 DIV neurons, treated with solvent, 1 μM MS-275 or 1 μM TBA for 72 h. Black triangles denote significant effects of region (α towards γ) on mitochondrial size ($p < 0.05$, ANOVA linear trend). $n = 13$ -21 neurons from 3 independent cultures, per treatment condition. (D) Immunoblot for mitochondrial fission-fusion proteins (see Table 1 for quantifications).

Table 1. Mitochondrial fission-fusion indicators

	Striatal / Cortical	Cortical		Striatal	
	solvent / solvent	MS-275 / solvent	TBA / solvent	MS-275 / solvent	TBA / solvent
Fis1 / SDHA	↑ 1.35 ± 0.07, $p < 0.01$	↓ 0.71 ± 0.09, $p = 0.08$	0.85 ± 0.11, $p = 0.23$	↓ 0.79 ± 0.08, $p = 0.10$	0.91 ± 0.13, $p = 0.46$
OPA1 / SDHA	1.11 ± 0.04, $p = 0.07$	↓ 0.68 ± 0.02, $p < 0.01$	0.97 ± 0.08, $p = 0.66$	0.84 ± 0.09, $p = 0.14$	0.96 ± 0.17, $p = 0.64$
Mfn2 / SDHA	1.11 ± 0.07, $p = 0.20$	↓ 0.77 ± 0.08, $p = 0.10$	1.03 ± 0.11, $p = 0.87$	0.93 ± 0.04, $p = 0.19$	1.05 ± 0.07, $p = 0.48$

Quantification of protein levels by immunoblot densitometry. Arrows indicate direction of differences greater than 20% ($p \leq 0.10$, ratio paired t test). Data are mean ± SEM of the ratios to solvent; $n = 3-7$ independent cultures.

The HDAC6 inhibitor TBA does not block neuronal autophagosome-lysosome fusion, while increasing LC3-vesicle retrograde flux and autophagic turnover

To investigate the effects of HDAC6 inhibition of neuronal autophagosome-lysosome fusion, we imaged live neurons expressing mCherry-EGFP-LC3. Under control conditions (solvent), the vast majority of somatodendritic LC3-vesicles were autolysosomes (acidified, with loss of EGFP-fluorescence; Fig. 4Ai), indicating that constitutive autophagosome-lysosome fusion is highly efficient in neurons (Boland et al., 2008). Inhibition of the lysosomal proton pump with bafilomycin A1 (BAF) impaired LC3-vesicle acidification (EGFP fluorescence retained; Fig. 4Aiii) and lysosomal digestion (p62 accumulation; BAF: Fig. 4B, Table 2), as predicted for impaired autophagosome-lysosome fusion (Klionsky et al., 2012). Treatment with TBA increased tubulin acetylation (Fig. 4D) without impairing LC3-vesicle acidification (Fig. 4Aiv,v) or p62 digestion (Fig. 4B, Table 2). These results show that TBA inhibits HDAC6-mediated tubulin deacetylation without blocking neuronal autophagosome-lysosome fusion.

Since cortactin deacetylation by HDAC6 was found necessary for constitutive autophagosome-lysosome fusion in fibroblasts (Lee et al., 2010) we monitored the levels of acetylated cortactin in neurons, where its function is mostly unknown (Catarino et al., 2013). In cortical and striatal neurons, in situ acetyl-cortactin immunoreactivity presented a punctate distribution in dendrites (Fig. 4C), with some neurons presenting immunoreactivity in the nuclear region as previously reported for hippocampal neurons (Catarino et al., 2013). Treatment with TBA increased in situ acetyl-cortactin immunoreactivity in neurons (Fig. 4C). No changes were detected by immunoblotting for acetyl-cortactin although acetyl-tubulin levels were clearly increased (Fig. 4D).

To investigate the dynamics of neuronal LC3-vesicles, we monitored their direction and velocity in distal axons (>400 µm from soma), their retrograde flux through the first axonal branch (a critical converging point for vesicles moving towards the soma), and we also quantified somatic autolysosomes (Fig. 5). Under control conditions, most LC3-vesicles in distal axons were autophagosomes (mCherry- and EGFP-positive; Fig. 5A) and exhibited robust retrograde movement (Fig. 5B,C), as previously described (Lee et al., 2011; Maday et al., 2012). Treatment with TBA modified neither LC3-vesicle direction (Fig. 5C) nor velocity (Fig. 5C,D), but significantly increased the retrograde flux through the first axonal branch (Fig. 5E,F), and the number of somatic autolysosomes (Fig. 5G,H), suggesting that more autophagosomes are being formed and converted into autolysosomes while moving towards the soma. TBA treatment also increased LC3-II, while decreasing p62 levels (Fig. 4B, Table 2), as predicted for increased formation of autophagosomes together with enhanced lysosomal digestion (Klionsky et al., 2012), indicating that TBA increases

autophagic flux. We next investigated whether these effects of TBA treatment had significant consequences for the proteostasis of mHtt in neurons.

The HDAC6 inhibitor TBA promotes clearance of diffuse mHtt in striatal neurons, which show a lower autophagic flux than cortical neurons

We compared mHtt proteostasis in neurons expressing an EGFP-tagged mHtt construct with 74Q (Fig. 6A). The overall proportion of neurons with mHtt aggregates at 24 h was higher for cortical than for striatal neurons, increasing at identical rates for both populations towards 48 h (Fig. 6Bi). Within aggregate-containing neurons, the proportion with neuritic aggregates at 24h was similar and increased over time without significant differences between cortical and striatal neurons (Fig. 6Ci). In neurons without aggregates, diffuse mHtt levels were higher at 24 h and decreased over time for cortical neurons, whereas the opposite pattern was observed for striatal neurons where diffuse mHtt continued to increase towards 48 h (Fig. 6Di).

To test possible mechanisms behind this differential mHtt proteostasis, we probed striatal and cortical neurons for intrinsic differences in autophagic flux. Following interruption of digestion with bafilomycin, more p62 accumulated in cortical than in striatal neurons (Table 2 – BAF), indicating that autophagic flux is lower in striatal neurons. Induction of autophagy with rapamycin evoked more pronounced increases in LC3-II and decreases in p62 levels in striatal neurons (Table 2 – RAP), respectively indicating proportionally higher increases in autophagosome formation and in lysosomal digestion (Klionsky et al., 2012), which is consistent with a lower autophagic flux in striatal neurons prior to treatment with rapamycin.

Treatment with the HDAC6 inhibitor TBA did not modify the overall proportion of cortical or striatal neurons with mHtt aggregates (Fig. 6Bii), while showing a trend for reducing mHtt aggregates in striatal neurites (Fig. 6Cii). Moreover, treatment with TBA significantly reduced diffuse mHtt levels in striatal neurons (Fig. 6Dii). These data suggest that pharmacological HDAC6 inhibition in neurons increases the clearance of diffuse mHtt.

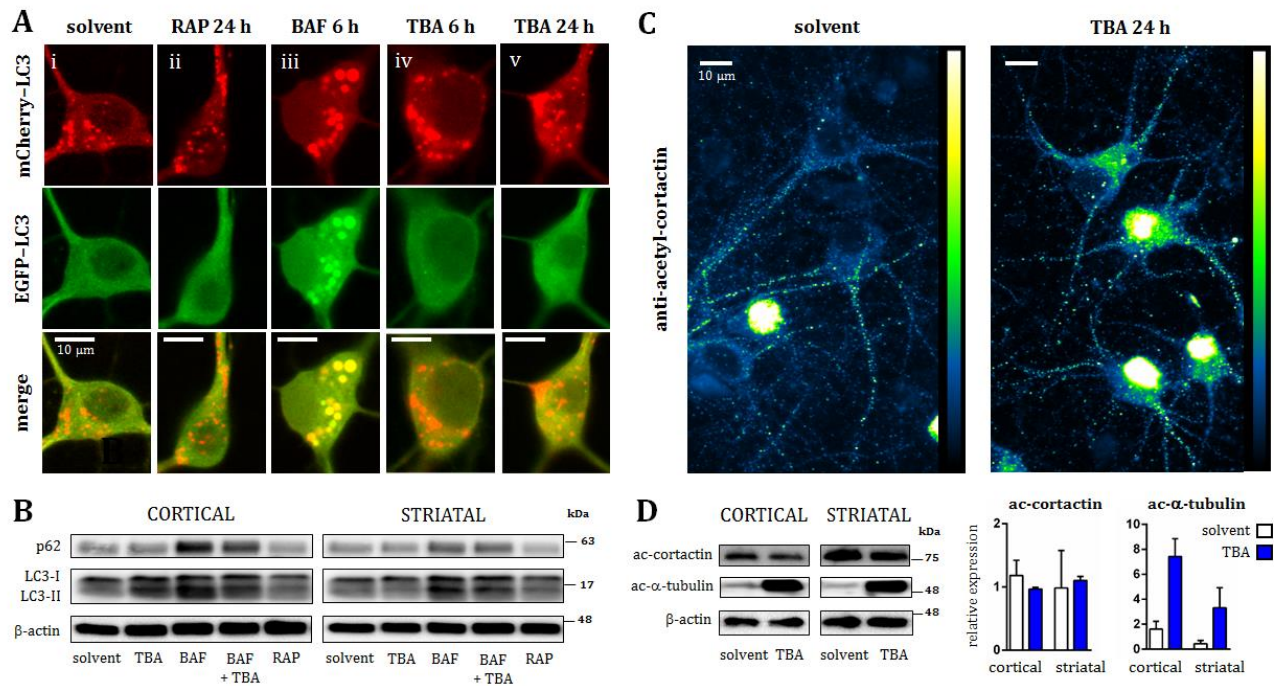
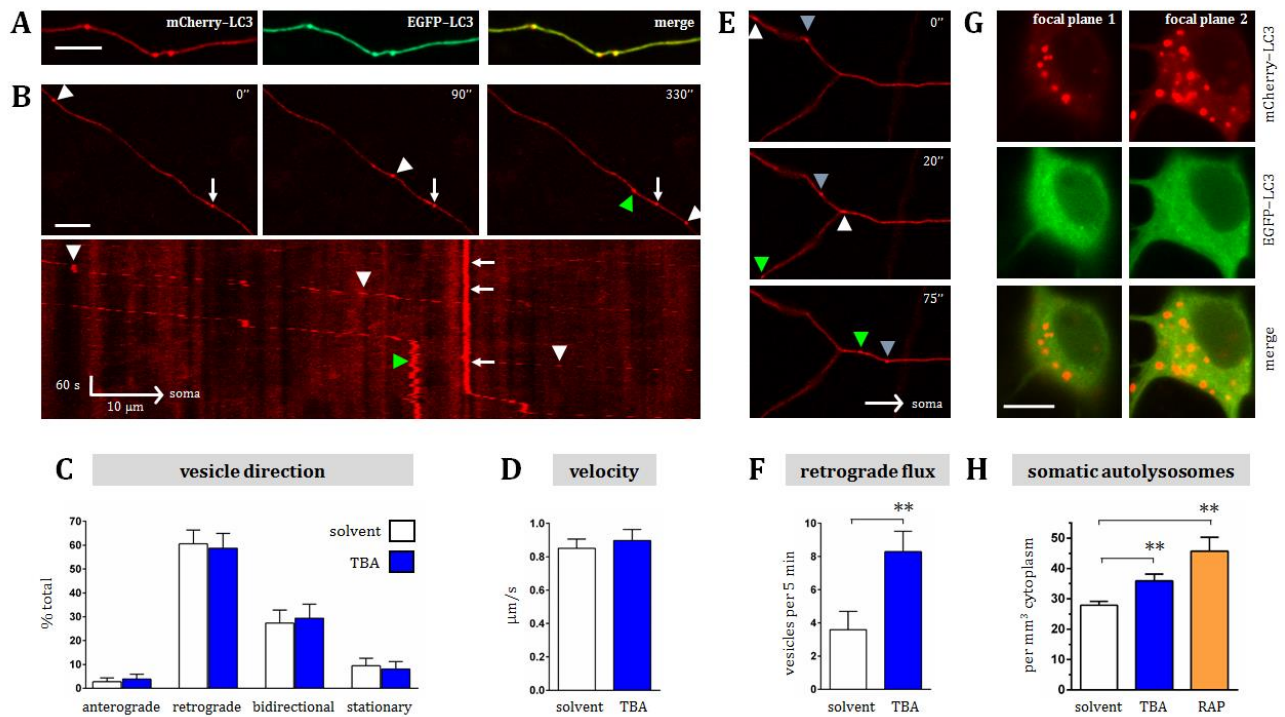


Figure 4. Neuronal autophagosome-lysosome fusion and autophagy markers. (A) Representative somata of cortical neurons expressing mCherry-EGFP-LC3, imaged live following the indicated treatments: RAP – 10 nM rapamycin, BAF – 25 nM bafilomycin A1, TBA – 1 μ M tubastatin A. (B) Immunoblot for autophagy markers: neurons were incubated with solvent, TBA or RAP for 24 h before protein extraction; BAF was present only for the last 6 h of incubation (see Table 2 for quantifications). (C) Representative immunofluorescence images of acetyl-cortactin in cortical neurons treated with solvent or TBA for 24 h. (D) Immunoblot for acetyl-cortactin and acetyl- α -tubulin with respective quantifications relative to β -actin; $n = 2$ independent cultures.



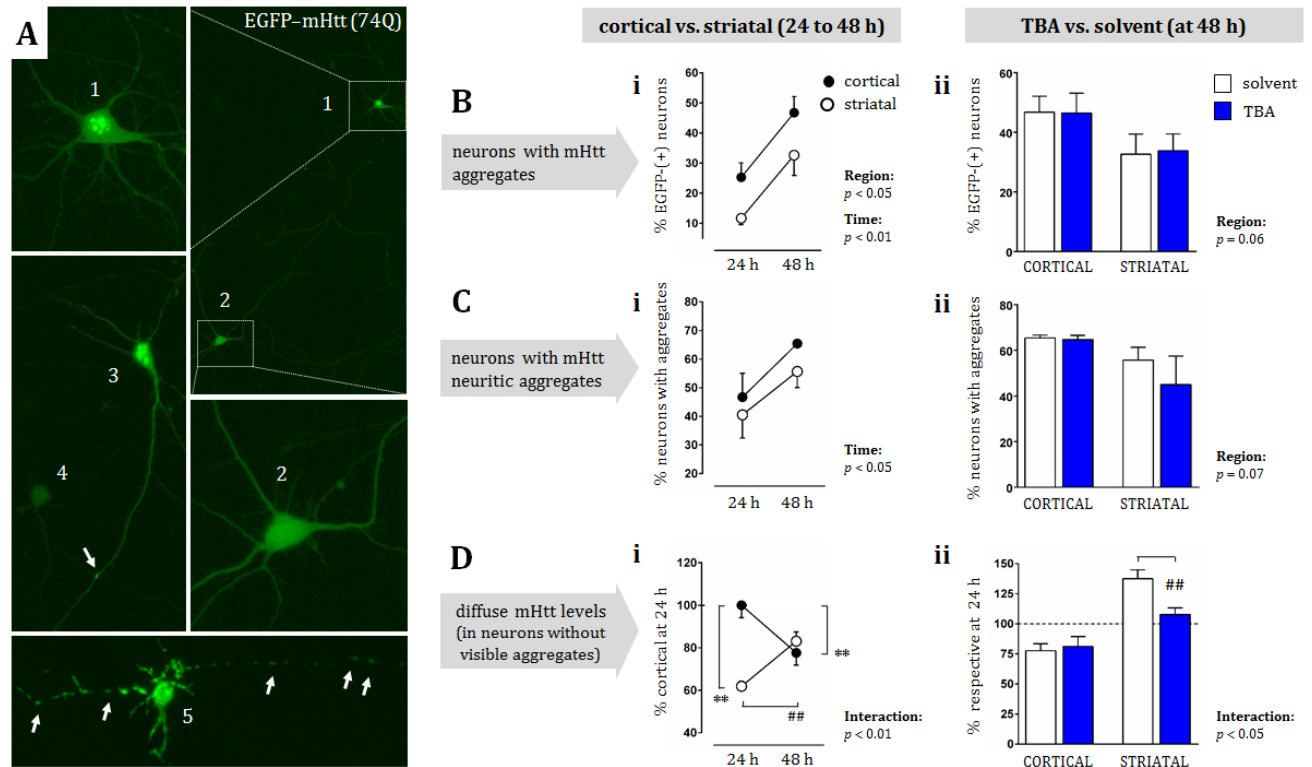


Figure 6. Huntingtin proteostasis in cortical and striatal neurons. (A) Representative mHtt (EGFP-Htt^{ex1}Q74) expression patterns: somatic (1) and neuritic aggregates (3,5); diffuse (2,4). (B-D) (i) region × time: comparison of solvent-treated cortical and striatal neurons from 24 to 48 h; (ii) region × treatment: comparison of cortical and striatal neurons at 48 h, following 24 h treatment with solvent or 1 μM TBA. (B) Neurons with mHtt aggregates in percentage of EGFP-positive neurons; (C) Neurons with neuritic aggregates, in percentage of aggregate-containing neurons; $n = 3$ independent experiments with 599-852 EGFP-positive neurons per experimental group. (D) Diffuse mHtt levels in neurons without visible aggregates; $n = 86$ -220 neurons per experimental group, from 3-4 independent cultures. Interaction, region, and time p values are from two-way ANOVAS; ** $p < 0.01$ to solvent-treated cortical neurons; ## $p < 0.01$ to solvent-treated striatal neurons.

Table 2. Autophagy markers in cortical and striatal neurons

	LC3-II / β -actin		p62 / β -actin	
	Cortical	Striatal	Cortical	Striatal
TBA / solvent	↑ $1.48 \pm 0.10, p < 0.05$	↑ $1.25 \pm 0.04, p < 0.01$	$0.88 \pm 0.20, p = 0.52$	↓ $0.82 \pm 0.02, p < 0.05$
BAF / solvent	↑ $1.79 \pm 0.12, p < 0.05$	↑ $2.43 \pm 0.21, p < 0.01$	↑ $1.90 \pm 0.18, p < 0.05$	$1.22 \pm 0.17, p = 0.34$
RAP / solvent	$1.18 \pm 0.11, p = 0.24$	↑ $1.72 \pm 0.17, p < 0.05$	$0.66 \pm 0.13, p = 0.14$	↓ $0.58 \pm 0.08, p < 0.05$

Quantification of protein levels by immunoblot densitometry. Arrows indicate direction of differences ($p < 0.05$, ratio paired t test). Data are mean $\pm$ SEM of the ratios to solvent; $n = 3-4$ independent cultures.

DISCUSSION

Here we show that striatal and cortical neurons present intrinsic differences in mitochondrial biogenesis, fission-fusion and trafficking dynamics, modulated to maintain identical mitochondrial volume occupancy. Mitochondria in striatal neurons are more balanced towards fission and less motile than those in cortical neurons. The HDAC1 inhibitor MS-275 or the HDAC6 inhibitor TBA did not modify mitochondrial biogenesis, but both altered the mitochondrial fission-fusion balance – MS-275 reduced fission, whereas TBA increased mitochondrial motility and promoted fusion. Pharmacological HDAC6 inhibition with TBA did not block neuronal autophagosome-lysosome fusion, but increased autophagic flux and reduced diffuse mHtt in striatal neurons. These data provide insight into HD striatal vulnerability and experimental therapeutics with HDAC inhibitors, as addressed below.

Intrinsic differences in mitochondrial dynamics may contribute for striatal vulnerability to HD

Mitochondrial dysfunction, including impaired calcium handling, excessive fission and reduced trafficking, are associated with HD pathophysiology (Oliveira, 2010a,b; Costa and Scorrano, 2012; Reddy and Shirendeb, 2012). Although direct interactions with mHtt may cause mitochondrial dysfunction (Panov et al., 2002; Shirendeb et al., 2012; Yano et al., 2014), the widespread distribution of mHtt in the brain argues against such interactions as the basis for the selective striatal vulnerability seen in HD (Han et al., 2010). Here we show that mitochondria in striatal neurons are intrinsically more balanced towards fission and display lower motility than in cortical neurons. This pre-existent higher fission may contribute towards striatal neurons being precociously affected by the increase in mitochondrial fragmentation that occurs in HD (Shirendeb et al., 2011). Similarly, a pre-existent lower motility may contribute to a more striking inhibition of mitochondrial trafficking by diffuse mHtt in striatal (Orr et al., 2008) than in cortical neurons (Chang et al., 2006).

Together with previous findings of increased striatal susceptibility to oxidative phosphorylation defects (Pickrell et al., 2011) and to calcium-induced mitochondrial permeability transition (Brustovetsky et al., 2003; Oliveira and Goncalves, 2009), the present data support the hypothesis that striatal neurons have an intrinsic vulnerability to mitochondrial dysfunction contributing to their preferential degeneration in HD.

HDAC1 and HDAC6 inhibitors modulate mitochondrial fission-fusion balance in neurons

MS-275, an inhibitor of HDAC1, increased cortical and striatal mitochondria size while decreasing Fis1 levels, indicating that HDAC1 inhibition reduces mitochondrial fission in

neurons by the same mechanism reported for cell-lines (Lee et al., 2012). MS-275 concentrated mitochondria near cortical somata, likely by preventing the size adaptation required for distribution and not by increasing biogenesis, as we found no changes in mtDNA/nDNA levels in MS-275-treated neurons. This contrasts with data from cell-lines showing increased mitochondrial biogenesis following MS-275 treatment, presumably because we used an HDAC1 selective concentration of 1 μ M MS-275, whereas cell-lines were treated with 5 μ M MS-275 – also inhibiting HDAC3, thereby increasing PGC-1 α expression and biogenesis (Galmozzi et al., 2013).

TBA, the inhibitor of HDAC6, increased tubulin acetylation and mitochondrial motility in cortical and striatal neurons, consistent with increased motor affinity to acetylated microtubules (Dompierre et al., 2007), and with previous studies in hippocampal (Chen et al., 2010) or dorsal-root-ganglion neurons (d'Ydewalle et al., 2011). We also show that TBA shifts the mitochondrial fission-fusion balance of striatal neurons towards more fusion, possibly because increasing microtubule-dependent movement increases fusion probability (Liu et al., 2009; Cagalinec et al., 2013), particularly when mitochondrial contact probability is otherwise reduced by low motility. Decreasing fission or increasing fusion might be useful pharmacological properties to counteract excessive fission in diseases such as HD (Costa et al., 2010; Guo et al., 2013). However, sufficient fission must remain for suitable mitochondrial distribution and quality-control (Twig et al., 2008; Jahani-Asl et al., 2010; Oliveira and Lightowlers, 2010). HDAC6 inhibition might be a better approach to modulate the mitochondrial fission-fusion balance. Indeed, fission reduction with MS-275 also reduced neurite outgrowth, whereas TBA increased tubulin acetylation (shown decreased in HD brains; Dompierre et al., 2007), and promoted fusion in striatal neurons without affecting neurite outgrowth.

HDAC6 is not essential for autophagosome-lysosome fusion in neurons, and HDAC6 inhibition might stimulate autophagosomal biogenesis

In a previous study using HDAC6-knockout fibroblasts, HDAC6 was found necessary for constitutive autophagosome-lysosome fusion by a mechanism involving cortactin deacetylation (Lee et al., 2010). However, both HDAC6 and cortactin deacetylation were found dispensable for autophagosome-lysosome fusion when the same fibroblasts were starved (Lee et al., 2010). Here we show that TBA treatment increases tubulin acetylation in neurons without impairing LC3-vesicle acidification or p62 digestion, thus indicating that HDAC6 is dispensable for neuronal autophagosome-lysosome fusion.

HDAC6 inhibition with TBA contrasts with HDAC6-knockout by presumably allowing for ubiquitin-binding and for some deacetylase domain 1 (DD1) activity as TBA was

designed against HDAC6 DD2 (Butler et al., 2010). Nevertheless, studies showing no obvious brain abnormalities in HDAC6-knockout mice (Zhang et al., 2008; Govindarajan et al., 2013) are consistent with HDAC6 being dispensable for neuronal autophagosome-lysosome fusion. The contrasting finding in neurons and fibroblasts may also stem from differences in cortactin distribution. Neuronal cortactin concentrates in dendritic spines (Hering and Sheng, 2003) being scarce in axons (Racz and Weinberg, 2004). Since most neuronal autophagosomes initiate distally and fuse with lysosomes along the axon (unlike throughout the cytosol in fibroblasts; Maday et al., 2012; Maday and Holzbaur, 2014), scarce axonal cortactin suggests it is less important for autophagosome-lysosome fusion in neurons than in fibroblasts.

TBA-treated neurons exhibited higher LC3-vesicle retrograde flux, more somatic autolysosomes, and increased LC3-II with decreased p62 levels. These findings are compatible with the stimulation of autophagosomal biogenesis (Klionsky et al., 2012). Consistently, previous studies found that starvation induced a more robust increase in LC3-II conversion and long-lived protein degradation in HDAC6-knockout fibroblasts than in control fibroblasts (Lee et al., 2010). How might HDAC6 inhibition stimulate autophagosomal biogenesis? We hypothesize that the associated increase in microtubule-dependent transport promotes initiating factor arrival to sites of autophagosomal formation. Such factors might include the endoplasmic reticulum subdomains containing DFCP1 (Double-FYVE-Containing-Protein-1; involved in neuronal autophagosomal biogenesis: Maday and Holzbaur, 2014) (Gonzalez and Couve, 2014), the c-Jun-N-terminal-protein-Kinase-1 (JNK1; required for autophagosomal biogenesis: Wei et al., 2008), and the JNK1-interacting protein (JIP1; required for autophagosomal exit from distal axons: Fu et al., 2014) (Geeraert et al., 2010).

Striatal and cortical neurons differ in mHtt proteostasis, and HDAC6 inhibition promotes diffuse mHtt clearance

Here we found higher initial levels of diffuse mHtt and more pronounced aggregation in cortical than striatal neurons, consistent with higher initial levels predicting aggregate formation (Arrasate et al., 2004; Miller et al., 2010), and with more aggregates in cortex than striatum in HD patients (Gutekunst et al., 1999). In the absence of aggregates, our data suggest that diffuse mHtt accumulates over time in striatal but not in cortical neurons, which is consistent with the mean lifetime of mHtt being higher in striatal than cortical neurons (Tsvetkov et al., 2013), and with recent data showing preferential accumulation of mHtt in the striatum (Wade et al., 2014). The correlation of diffuse mHtt levels with the risk of neuronal death suggests that the toxic species reside within the diffuse fraction (Arrasate

et al., 2004; Miller et al., 2010) and, therefore, that treatments capable of reducing diffuse mHtt should hold neuroprotective potential.

The HDAC6 inhibitor TBA reduced diffuse mHtt in striatal neurons, without altering the proportion of cortical or striatal neurons with mHtt aggregates. These present findings in neurons apparently contrast with studies in HDAC6-knockout cell-lines showing reduced clearance of protein aggregates (Kawaguchi et al., 2003) and reduced autophagic degradation of mHtt (Iwata et al., 2005). Our findings agree, however, with other studies in neurons, where HDAC6 inhibition alleviated abnormal tau accumulation (Cook et al., 2012), and with *in vivo* HDAC6-knockout in R6/2 mice showing no increase in mHtt aggregates (Bobrowska et al., 2011). As previously suggested (Bobrowska et al., 2011), some of the effects of HDAC6 in cell-lines may not apply to neurons.

HDAC6-knockout R6/2 mice showed neither symptomatic improvement, nor changes in mHtt aggregate load (Bobrowska et al., 2011). Studies reporting increased mHtt clearance upon induction of autophagy have most frequently used 68-97Q mHtt (Ravikumar et al., 2004; Jeong et al., 2009; Rose et al., 2010; Tsvetkov et al., 2010; Jia et al., 2012), whereas HDAC6-knockout R6/2 had an unusually high mHtt polyQ – 201Q (Bobrowska et al., 2011). Aggregates precede symptom onset in R6/2 (Bobrowska et al., 2011), and data from neuronal models predict that diminishing diffuse mHtt levels should be more beneficial in the ‘pre-aggregate epoch’ (Miller et al., 2010). Although HDAC6-knockout R6/2 showed no changes in global levels of soluble mHtt, their cortex showed decreased soluble mHtt (no data on soluble mHtt was reported for their striatum; Bobrowska et al., 2011). Therefore, it would be valuable to start HDAC6 inhibition at the pre-aggregate epoch and test for delayed symptom onset in HD mice with shorter polyQ and slower disease progression.

Concluding remarks

The intrinsic balance towards fission and lower motility of striatal mitochondria may contribute towards the greater sensitivity of striatal neurons to HD-associated mitochondrial fragmentation and impaired trafficking. Pharmacological HDAC6 inhibition with TBA approximates the mitochondrial fission-fusion balance and motility of striatal neurons to that of the less HD-vulnerable cortical neurons, and also increases neuronal autophagic flux, promoting clearance of diffuse mHtt in striatal neurons. Recent *in vivo* studies support HDAC6 inhibition as a neuroprotective strategy in Alzheimer’s disease (Govindarajan et al., 2013), Charcot-Marie-Tooth (d’Ydewalle et al., 2011), and amyotrophic lateral sclerosis (Taes et al., 2013). The present study supports pharmacological HDAC6 inhibition as a strategy with the potential to reduce striatal vulnerability to HD.

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Section 3

Discussion and future directions

As reviewed in Guedes-Dias and Oliveira (2013), although the association between HDAC6 inhibition and increased mitochondrial trafficking in neurons was well documented, the effects of HDAC activity modulation on mitochondrial biogenesis and fission-fusion balance in neurons were still largely unexplored. Moreover, it remained an open question whether the use of HDAC6 inhibitors to enhance mitochondrial motility, particularly in Huntington's disease (HD), could compromise autophagic clearance and mutant huntingtin (mHtt) degradation. We addressed these points in Guedes-Dias et al. (2015) and our findings showed that: 1) neither exposure to MS-275, an HDAC1 inhibitor, nor to Tubastatin A (TBA), an HDAC6 inhibitor, modified mitochondrial biogenesis in neurons; 2) HDAC1 inhibition reduced mitochondrial fission by decreasing Fis1 expression levels, whereas HDAC6 inhibition increased mitochondrial motility, inducing mitochondrial fusion in striatal neurons; 3) HDAC6 inhibition did not block autophagosome-lysosome fusion in neurons; 4) HDAC6 inhibition increased neuronal autophagic flux; 5) HDAC6 inhibition reduced levels of diffuse mHtt in striatal neurons.

The therapeutic potential of neuronal HDAC1 inhibition is still controversial. HDAC1 depletion reduced mHtt acetylation and promoted mHtt clearance, which could prove effective in HD (Jeong et al., 2009), and HDAC1 inhibition in dorsal root ganglion neurons was reported to facilitate central axon regeneration after peripheral axotomy, which could be potentially useful in treating spinal cord injuries (Finelli et al., 2013). However, HDAC1 inhibition is also associated with inducing aberrant expression of cell-cycle genes, DNA damage and neuronal death both *in vitro* and *in vivo* (Kim et al., 2008). Our results show that HDAC1 inhibition blocked neurite outgrowth in cortical and striatal neurons, without altering mitochondrial biogenesis. One possible explanation is that, since HDAC1 inhibition decreased the expression of mitochondrial fission protein Fis1, reduced mitochondrial fission hindered the distribution of mitochondria to growing neurites, thus impairing their outgrowth (Ishihara et al., 2009).

HDAC6 inhibition promoted neurite outgrowth in developing cortical neurons, which is in line with a previous study (Rivieccio et al., 2009). Although HDAC6 inhibition did not alter mitochondrial biogenesis, increased tubulin acetylation may have promoted mitochondrial distribution to the neuritic arbor, facilitating energy supply to growth cones. Indeed, we detected increased mitochondrial motility in mature neurons after HDAC6 inhibition – an effect that could be especially beneficial for striatal neurons, given the lower motility of their mitochondrial population compared to the less HD-vulnerable cortical neurons.

In this thesis, we show that cortical and striatal neurons present differential mitochondrial dynamics and argue that this may be a contributing factor for striatal neurons preferential vulnerability in HD. Increased mitochondrial fragmentation (Shirendeb et al.,

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2011; Song et al., 2011; Shirendeb et al., 2012), decreased levels of acetylated α -tubulin in patient brains (Dompierre et al., 2007), and impaired mitochondrial trafficking in striatal neurons (Orr et al., 2008) are associated with HD. Our findings show that mitochondria are less motile in striatal than cortical neurons, suggesting that this may underlie the higher susceptibility of striatal (Orr et al., 2008) over cortical neurons (Chang et al., 2006) to mitochondrial trafficking impairment by diffuse mHtt. Mitochondrial fraction per neurite length was similar between cortical and striatal neurons, importantly, however, mitochondria in striatal neurons were smaller and more numerous, suggesting that their dynamics are inherently balanced towards fission. Moreover, the higher expression levels of Fis1 detected in striatal neurons may predispose mitochondria to further fragmentation by overactivated Drp1 in HD (Song et al., 2011). HDAC6 inhibition increased α -tubulin acetylation, promoting mitochondrial motility and fusion in striatal neurons to levels similar to those observed in less HD-vulnerable cortical neurons. This suggests that inhibiting HDAC6 may counteract the HD-associated mitochondrial fragmentation and trafficking impairment in striatal neurons. Future studies, however, should investigate whether HDAC6 inhibition rescues mitochondrial dynamics in striatal neurons expressing mHtt.

HDAC6 inhibition did not block neuronal autophagosome-lysosome fusion or mHtt clearance, but in fact, induced autophagic flux and decreased diffuse mHtt levels in striatal neurons. Interestingly, our results suggest that HDAC6 inhibition induced autophagic flux not by boosting LC3-vesicles retrograde velocity, but instead by evoking autophagosomal biogenesis. Evidence indicates that mHtt proteostasis is, at least in part, mediated by autophagy: a small-molecule autophagy inducer decreased diffuse mHtt levels in neurons (Tsvetkov et al., 2010), whereas low concentrations of an autophagy inhibitor elevated diffuse mHtt lifetime (Tsvetkov et al., 2013). We therefore hypothesize that autophagy induction by HDAC6 inhibition may account for the diffuse mHtt levels decrease we observed in striatal neurons. Nevertheless, future studies are required to elucidate the mechanisms by which HDAC6 inhibition may promote autophagosomal biogenesis. Moreover, since we assessed autophagic flux in wild-type striatal neurons, further studies are needed to confirm whether HDAC6 inhibition increases LC3-vesicle flux towards the soma of striatal neurons expressing mHtt. Finally, performing a longitudinal analysis in individual mHtt-expressing striatal neurons to assess whether HDAC6 inhibition decreases their risk of death, will be critical to confirm the potential of HDAC6 inhibition to reduce striatal vulnerability in HD.

Altered mitochondrial dynamics and defective autophagy are involved in HD pathology. Correcting mitochondrial shape and trafficking defects (Costa and Scorrano, 2012), and stimulating autophagy clearance (Martin et al., 2015) are thus regarded as

potentially beneficial in HD. Here, we show that HDAC6 inhibition promotes mitochondrial motility and induces mitochondrial fusion in HD-vulnerable striatal neurons to levels similar to those found in the less HD-vulnerable cortical neurons. Moreover, we show that HDAC6 pharmacological inhibition does not inhibit neuronal autophagosome-lysosome fusion, but in fact, promotes autophagic flux and reduces the levels of diffuse mHtt in striatal neurons. In conclusion, our findings here support HDAC6 inhibition as a promising strategy to reduce the vulnerability of striatal neurons in HD.

Section 4

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