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Autophagy in neurodegeneration: New insights underpinning therapy for neurological diseases

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Abbreviations used:
ACD, autophagic cell death
AR, androgen receptor
ATFS1, Activated Transcription Factor Associated with Stress 1
Atg5, autophagy related protein 5
Atg7, autophagy related 7
BAG3, Bcl-2-associated athanogene 3
Bcl-2, B-cell lymphoma 2
BECN1, Beclin1
CASA, chaperone-assisted selective autophagy
CCCP, carbonyl cyanide m-chlorophenyl hydrazone
CHIP, carboxyl terminus of Hsc70-interacting protein
CMA, chaperone-mediated autophagy
DA, dopaminergic
EHNA, erythro-9-[3-(2-hydroxynonyl)]adenine
ER, endoplasmic reticulum
FUS, fused in sarcoma protein
HI, hypoxia-ischemia
HIE, hypoxic-ischemic encephalopathy
HSD17B10, mitochondrial matrix hydroxysteroid dehydrogenase;
HSP, heat shock protein
LC3, microtubule-associated protein light chain 3

MND, Motor neuron disease

MDV, mitochondria-derived vesicles

MiT/TFE, transcription factors of the microphtalmia/transcription factor E family

Mfn2, Mitofusin 2

NDs, neurodegenerative diseases

NEF, nucleotide exchange factor

NLRP3, nucleotide-binding domain-like receptor protein 3

OMM, outer mitochondrial membrane

PCD, programmed cell death

PD, Parkinson's disease

polyQ, elongated glutamine repeats

SBMA, spinal bulbar and muscular atrophy

siRNA, short interfering RNA

SOD1, superoxide dismutase 1

TDP-43, TAR DNA binding protein of 43 kDa

TFEB, transcription factor EB

TOM, translocase of outer mitochondrial membrane

UPR^{mt}, mitochondrial unfolded protein response

Abstract

In autophagy long-lived proteins, protein aggregates or damaged organelles are engulfed by vesicles called autophagosomes prior to lysosomal degradation. Autophagy dysfunction is a hallmark of several neurodegenerative diseases in which misfolded proteins or dysfunctional mitochondria accumulate. Excessive autophagy can also exacerbate brain injury under certain conditions. In this review, we provide specific examples to illustrate the critical role played by autophagy in pathological conditions affecting the brain and discuss potential therapeutic implications. We show how a singular type of autophagy-dependent cell death termed autosis has attracted attention as a promising target for improving outcomes in perinatal asphyxia and hypoxic-ischaemic injury to the immature brain. We provide evidence that autophagy inhibition may be protective against radiotherapy-induced damage to the young brain. We describe a specialized form of macroautophagy of therapeutic relevance for motoneuron and neuromuscular diseases, known as chaperone-assisted selective autophagy, in which heat shock protein B8 is used to deliver aberrant proteins to autophagosomes. We summarize studies pinpointing mitophagy mediated by the serine/threonine kinase PINK1 and the ubiquitin-protein ligase Parkin as a mechanism potentially relevant to Parkinson's disease, despite debate over the physiological conditions in which it is activated in organisms. Finally, with the example of the autophagy-inducing agent rilmenidine and its discrepant effects in cell culture and mouse models of motor neuron disorders, we illustrate the importance of considering aspects such a disease stage and aggressiveness, type of insult and load of damaged or toxic cellular components, when choosing the appropriate drug, timepoint and duration of treatment.

Autophagy involves an intracellular homeostatic process wherein newly formed double-membrane vesicles termed autophagosomes, engulf long-lived proteins or organelles such as mitochondria, and transfer them to lysosomes for degradation (Ravikumar *et al.* 2010). The autophagy machinery is conserved across species, but because the brain possesses precise mechanisms regulating its nutrient and energetic supply, basal autophagic flux was a relatively late discovery in healthy neurons (Boland & Nixon 2006). Indeed perhaps the first demonstration of the relevance of autophagy in brain were findings in the autophagy related protein 5 and 7 (Atg5, Atg7) knockout mice (Komatsu *et al.* 2006, Hara *et al.* 2006). Here Atg7 deficiency caused massive neuronal loss in the cerebral and cerebellar cortices with notable behavioral deficits and accumulation of polyubiquitinated proteins in neurons (Komatsu *et al*. 2006). There is now a rapidly proliferating literature about the importance of autophagy in brain, with autophagic and associated lysosomal function considered determinant for the maintenance of neuronal health. Autophagy dysfunction caused by gene mutations and/or potentially toxic aggregation-prone proteins is a hallmark of several human neurodegenerative diseases. Autophagic organelle clearance, particulary that of damaged mitochondria (mitophagy), currently attracts appreciable attention because of the brain's unique energy dependence and because the process is suspected to be dysfunctional in various pathologies (e.g. Van Laar & Berman 2013). It is therefore not surprising that drugs that aid the clearance of potentially toxic debris or damaged organelles are now the focus of escalating attention as potential therapeutics (Menzies *et al.* 2017, Boland *et al.* 2018).

On the other hand, more than 2,000 articles in PubMed link autophagy and cell death in neuron or brain, with a number of studies pointing at a causative role of autophagy in cell death. This topic is somewhat controversial, as evidence for such a causative link has often been partial. There has been debate over the ambiguous usage of the terminology "autophagic cell death" (ACD) to define cell death modalities associated with autophagy or triggered by autophagy and occurring in the presence or absence of other standard cell death mechanisms of death, such as apoptosis or necrosis. The use of more precise expressions, such as autophagy-associated cell death, autophagy-mediated cell death and autophagy-dependent cell death, accompanied by more rigorous analyses to determine the level of involvement of the process, has been recommended (Klionsky *et al.* 2016). Despite these reservations, several studies have demonstrated that autophagy can indeed cause neuronal death, indicating that under specific circumstances autophagy inhibition should considered as a therapeutic option (Liu & Levine 2015). This review follows from a symposium entitled "*Autophagy in Neurodegeneration: New Insights Underpinning Therapy for Neurological Diseases*" at the 19th International Neuroscience Winter Conference held at Sölden, Austria, in early 2017. Based on the topics covered by the presentations from the symposium, we here propose to use specific examples to illustrate the critical role of autophagy in pathological conditions affecting the brain, and discuss its potential as a target for therapy. The first and second sections of the review illustrate

the contribution of autophagy to hypoxic-ischaemic injury in the adult brain and in the vulnerable immature neonatal brain, and highlight the emergence of autophagy inhibition as a promising approach for treating perinatal asphyxia. The third section describes a type of macroautophagy called chaperone-assisted selective autophagy (CASA), which uses the HSPB8-BAG3 chaperone complex to target misfolded proteins for autophagic degradation and is considered a candidate target for the treatment of neuromuscular and motoneuron diseases (MND). The fourth section is dedicated to the multifunctional proteins PINK1 and Parkin and their role in the regulation of a stress-induced mitophagy program suspected to be central to the physiopathology of certain forms of Parkinson's disease (PD). Finally, the fifth section illustrates the complexity associated with therapeutic approaches targeting autophagy in brain diseases with the concrete example of the autophagy activator rilmenidine and its discordant effects in neuronal and mouse models of MND. The key points addressed in the review and associated take-home messages are summarized schematically in Figure 1.

I. Insult severity, dysfunctional autophagy and hypoxic-ischaemic injury.

There is an existent literature that patterns of neuronal injury are highly dependent upon the type, intensity and duration of insult, especially oxidative stress and excitotoxicity, and presumably load of toxic protein aggregates (Galluzzi *et al.* 2016). Indeed, we found in cultured cortical neurons that caspase-independent PCD induced by the oxidative stressor hydrogen peroxide was greatly attenuated by 3-methyladenine and knockdown with short interfering RNA (siRNA) directed at Atg7 and Beclin1 (BECN1) as shown by use of specific autophagic and cell death morphological markers. This diverse evidence supports the involvement of autophagy-mediated neuronal death, and also caspase-independent PCD, in a model with different interactive cell death pathways (Higgins *et al.* 2011). Consistent with the concept for a determinant role in neuronal of insult intensity, in severe oxidative stress there was even more extensive crosstalk between different forms of cell death with siRNA directed at Atg7 being less effective versus injury progression, even though autophagy-associated cell death was found (Higgins *et al.* 2012). Additionally, we found in a comparative analysis of our extensive microarray data that there are many commonalities in autophagic-lysosomal genes regulated downstream of oxidative stress and excitotoxicity, and that these events lead to downstream inhibition of autophagy and the autophagic-lysosomal pathway (Yap *et al.* 2016).

Given that both oxidative stress and excitotoxicity are documented to contribute to hypoxic-ischaemic injury (HI) (Khoshnam *et al.* 2017), studies of the possible recruitment of autophagy to stroke were a logical extension of our work. Indeed, in post-mortem human brain tissue from patients with a history of stroke we found abundant microtubule-associated protein light chain 3 (LC3)-immunolabelled autophagic vacuoles (Frugier *et al.* 2016). Dense Sequestosome 1 (SQSTM1) labelling was also found, although immunopositive neurons were further away from the penumbral area unlike those found after LC3 labelling – both SQSTM1 mRNA and protein levels were also increased. This increased level of SQTM1 may be

indicative of inhibition of autophagy, however our current understanding of the recruitment of the autophagic-lysosomal system in HI is very much in its infancy with existent evidence suggestive that the "load" of cellular debris and damaged proteins may determine the mode of recruitment of autophagy (Wen *et al.* 2008, Li *et al.* 2010, Shi *et al.* 2012). Clearly, given the dearth of drugs for the clinical management of HI, autophagy and its management represents an attractive target (*vide infra*).

II. Selective neuronal deletion of Atg7 is protective in neonatal brain injury.

The neonatal brain differs from the adult brain.

Autophagy is crucial for maintaining homeostasis in response to stress in most eukaryotic cells, including neurons, mediating neuroprotection following several forms of brain damage. Involvement of autophagy in neuronal cell death has therefore been controversial, and efforts have been made to distinguish cell death-promoting autophagy from autophagic responses that occur parallel to cell death, or even counteracting degenerative mechanisms. Emerging evidence indicates that inappropriate activation of autophagy can directly mediate neuronal cell death (Piras *et al.* 2017, Ginet *et al.* 2014b, Ginet *et al.* 2014a, Clarke & Puyal 2012). In the immature, neonatal brain, multiple mechanisms of cell death are often activated simultaneously, even in the same neuron (Puka-Sundvall *et al.* 2000, Blomgren *et al.* 2001), and the relative contributions of the various mechanisms are strikingly different from the adult brain (Zhu *et al.* 2005, Hu *et al.* 2000). In mice and rats, mixed morphological phenotypes indicative of necrotic, apoptotic, and autophagic features are present following HI-induced neuronal cell death in the immature brain, each confirmed by specific biochemical and morphological criteria (Li et al. 2010, Northington *et al.* 2011, Blomgren *et al.* 2007, Puyal & Clarke 2009). Levels of caspase-dependent, caspase-independent cell death and lipidation of LC3 provided evidence for greater activity of the apoptotic and autophagic machinery in the immature brain than in the adult brain (Zhu *et al.* 2005). This is reasonable, given the massive growth and continuous remodelling, including removal of redundant cells and synapses, in the immature brain, where autophagy plays an integral role in maintaining cellular and tissue homeostasis (Oppenheim 1991, Semple *et al.* 2013). Neuronal autophagy has been reported to be enhanced in rodent models of perinatal cerebral ischemia or HI (Zhu et al. 2005, Liu *et al.* 2008, Koike *et al.* 2008, Carloni *et al.* 2010). Neuroprotective measures to treat injury in the immature brain should most likely be quite different from those applied in the adult brain (Wang *et al.* 2009, Zhu *et al.* 2005), and autophagy has evolved as a particularly promising strategy in the immature brain

Methodological aspects.

The role of autophagy in neonatal brain injury is controversial, with independent studies showing protective effects in brain injury in rodents following inhibition or induction of autophagy by pharmacological agents (Vaslin *et al.* 2009, Carloni *et al.* 2010). Confounding aspects are linked to the fact that pharmacological autophagy-modulating agents have poor specificity and that they have been used intraperitoneally or intravenously, although they may not readily cross the blood-brain barrier (Galluzzi et al. 2016). In addition, the methodologies used to monitor autophagic responses *in vitro* and *in vivo*, have not always been discriminative. Cellular markers used include expression of LC3, ATG5, BECN1; phosphorylation of AMP-activated protein kinase, Unc-51 Like Autophagy Activating Kinase 1 or substrates of mammalian target of rapamycin complex 1; interaction of BECN1 with B-cell lymphoma 2 (BCL-2) family members; LC3 lipidation; lysosomal acidification; and the degradation of p62 or autophagic substrates. Cytoplasmic accumulation of autophagosomes and autolysosomes, LC3 lipidation, and other morphological or biochemical features of autophagy have been exploited to imply increased autophagy in neurons and other cells in brain tissue (Klionsky et al. 2016). However, cytoplasmic vacuolization and LC3 lipidation can also increase when lysosomal function is impaired, or when autophagosomes cannot fuse with lysosomes. To distinguish between active autophagic responses and autophagy blockade, it is necessary to take into account autophagic flux (Klionsky *et al.* 2016), an aspect that has not been considered in many of the reports in the literature. Genetic approaches are more specific, but also have disadvantages. Genetically targeting a component of the autophagic machinery, for example *Atg5* or *Atg7*, is more specific than pharmacological agents, but the targeted protein may also play additional roles unrelated to autophagy. Furthermore, the genetic deletion is often constitutive and life-long, leading to adaptive changes of the tissue and its metabolism. Making the deletion inducible, not only in terms of which cells that will be targeted, but also when the deletion will occur, will make the interpretations of the results more accurate. Autophagy is also involved in the release of danger signals and inflammation, both systemically and locally in the central nervous system, and these contribute to the overall short and long term outcomes of any brain injury. Future studies taking these aspects into consideration are needed to more accurately dissect the effects of autophagy in brain injuries.

Transient and specific inhibition of autosis reduces injury to the immature brain.

In 2013, a study was published demonstrating that selective over-activation of autophagy could cause a pattern of cell death with morphological characteristics different from apoptosis or necrosis, termed "autosis" (Liu *et al.* 2013) (Table 1). Autosis occurs *in vitro* and *in vivo* in cerebral HI of the immature brain, and is inhibited by Na^+/K^+-ATPase antagonists approved for clinical use. Lithium has been demonstrated to induce autophagy (Sarkar *et al.* 2005). However, we found it to be neuroprotective in a rat

model of neonatal HI, where it reduced the levels of LC3 lipidation, consistent with autophagy inhibition (Li *et al.* 2011, Li *et al.* 2010, Xie *et al.* 2014). To circumvent the limited specificity of pharmacological agents, we investigated the effect of HI in mice with neuron-specific deletion of the essential autophagy gene, *Atg7*. Tissue loss was decreased by more than 40%, and caspase-dependent and -independent cell death was attenuated in several brain regions compared to wild-type mice (Xie *et al.* 2016). These findings suggest that autophagy inhibition may be a promising therapeutic avenue for the treatment of human neonates developing severe hypoxic-ischemic encephalopathy (HIE). These results extend similar observations in the hippocampus of a milder HI model (Koike *et al.* 2008), supporting a lethal role of autophagy in brain regions highly vulnerable to HI in neonates, such as cortex, thalamus and striatum. Moreover, investigation of the abundance of LC3 II- and cathepsin D-positive cells in susceptible areas of the brain of human term newborns who died from severe asphyxia with HIE revealed increased neuronal autophagy compared to brains from neonates who died from other causes, without brain injury (Xie *et al.* 2016).

A different injury model, genotoxic stress induced through ionizing radiation, was used to model the effects of cranial radiotherapy in children treated for brain malignancies. Irradiation typically targets actively proliferating cells, making it effective in the treatment of high grade brain tumours. Very few cells in the brain proliferate under normal conditions, making the brain relatively radioresistant, but the neurogenic niches in the hippocampus and the subventricular zone, but also in the cerebellum, harbouring neural stem and progenitor cells, display overt cell death after irradiation (Naylor *et al.* 2008, Roughton *et al.* 2012, Fukuda *et al.* 2004, Zhou *et al.* 2017). The loss of neurogenic capacity observed after a single moderate dose of irradiation increased over time, such that the difference compared to controls was bigger the longer we waited, up to more than one year (Kalm *et al.* 2013, Bostrom *et al.* 2013). In paediatric patients, damage to normal brain tissue adjacent to the tumour is a major concern associated with adverse side effects (Kahalley *et al.* 2016). The young developing brain is more sensitive to irradiation than the adult brain (Fukuda *et al.* 2005, Duffner 2010). Radiotherapy can cause long-term cognitive impairment, secondary malignancies, and perturb growth and puberty. Irradiation-induced depletion of neural stem and progenitor cells may underlie some of the cognitive deficits. Using the same *Atg7* deletion mouse strain as above, we subjected 10-day-old mice to a single dose of 6 Gy whole brain irradiation and found that the number of dying stem and progenitor cells in the dentate gyrus of the hippocampus was reduced by 60% (Wang *et al.* 2017). The ensuing inflammatory reaction, as judged by microglial activation and cytokine levels, was also reduced to a similar extent, but since *Atg7* was deleted only in neurons it is likely that the reduced inflammation observed was due to the lower number of dying cells rather than autophagy inhibition. The large reduction of neural stem and progenitor cell death was somewhat surprising, given that *Atg7* was only deleted in neurons, and the progenitors commit to a neuronal fate approximately 3 days after the stem cells

start proliferating (Steiner *et al.* 2006, Brandt *et al.* 2003), and require 4 weeks to be mature neurons (Esposito *et al.* 2005, van Praag *et al.* 2002). The deletion of *Atg7* was driven by a neuron-specific nestin promoter (Komatsu *et al.* 2006), and it is unclear when this promoter was activated and hence when *Atg7* was deleted in the neural stem and progenitor cells. Dying stem and progenitor cells display primarily signs of apoptosis (Fukuda *et al.* 2004) and it remains to determine to what extent autophagy is involved in this type of genotoxic cell death, which is very different from the type of neuronal cell death observed after HI. Furthermore, a recent study suggests that *Atg7* deletion merely delayed neural progenitor/neuronal death, since tissue "loss" (lack of growth) and levels of neurogenesis-related markers were similar to WT brains 5 days after irradiation, on postnatal day 15 (Wang *et al.* 2019). However, prior to irradiation, *Atg7*-deficient brains displayed a regenerative state, as judged by the higher levels of transcripts related to neurogenesis and oligodendrocyte precursors, indicating that loss of *Atg7* in neurons is stressful to the brain tissue, a foreboding of the neurological symptoms that inevitably will develop several weeks later. Importantly, 5 days after irradiation, myelin development was less impaired in the *Atg7*-deficient animals than in WT mice (Wang *et al.* 2019). In summary, this relatively recent publication suggests that autophagy inhibition in neurons has indirect protective effects also in other cell types. Autophagy may thus be considered a target for preventing brain injury triggered by irradiation.

Together, these findings point to the importance of elucidating in further detail the mechanisms through which *Atg7* deletion renders neurons resistant to ischaemic injury. It also remains to investigate if autosis is an important mechanism of cell death in all types of neurons in the brain, and if it is less important in the adult brain. The role of autophagy in the demise of neural stem and progenitor cells in the neurogenic niches and oligodendrocyte progenitor cells after genotoxic stress through ionizing radiation also requires further investigation, since these cells typically display signs of classic apoptosis rather than the mixed morphologies observed in neurons dying after HI. Prolonged overall inhibition of autophagy, though, would not be a fruitful strategy, since autophagy is of fundamental importance in the homeostasis of the developing brain. This is evidenced by the reduced lifespan of neuronally *Atg7*-deficient mice; their brains degenerate and they usually die at 6-10 weeks of age (Komatsu *et al.* 2006). In addition to direct inhibition of cell death-related mechanisms, the lack of autophagic capacity, even if restricted to one cell type, may cause tissue stress, which in turn elicits protective, regenerative responses. In summary, transient and specific autophagy inhibition strategies should be considered to improve clinical outcome after perinatal asphyxia and HIE, and possibly also after radiotherapy-induced brain damage.

III. Modulation of the selective, chaperone assisted type of autophagy to protect against neuronal death.

As mentioned above, the term autophagy covers several types of degradative processes, but usually refers to macroautophagy, a pathway generally characterized by high capacity, but lower specificity in the removal of misfolded proteins compared to proteasomal degradation, and also capable of clearing aggresomes or aggregates (aggrephagy) (Cuervo 2011).

The CASA complex.

Macroautophagy involves the formation of autophagosomes engulfing organelles/aggregate, and their subsequent fusion to lysosomes for content degradation (Klionsky et al. 2016). However, autophagosomes can also receive cargoes of aberrant damaged, oxidize or misfolded proteins utilizing more selective pathways. Aberrant proteins that must be cleared from cells can be selectively delivered by specific chaperones (Arndt *et al.* 2010). These aberrant proteins, particularly in their misfolded forms are potentially neurotoxic and chaperones facilitate their degradation in their monomeric/oligomeric form, generally (and possibly) prior to their aggregation (Arndt *et al.* 2010). This type of macroautophagy assisted by specific chaperones has been named chaperone-assisted selective autophagy (CASA) (Arndt *et al.* 2010, Cuervo 2011, Rusmini *et al.* 2017). CASA differs from other forms of autophagy, like chaperone-mediated autophagy (CMA). In CASA misfolded proteins are delivered to autophagosomes, which then fuse with lysosomes, while in CMA a specific set of chaperones deliver misfolded proteins directly to lysosomes, thus escaping the autophagosome pathway. CASA takes place in muscle and brain, as well as in cancer cells (Crippa *et al.* 2010b, Crippa *et al.* 2010a, Piccolella *et al.* 2017, Arndt *et al.* 2010, Cuervo 2011, Rusmini *et al.* 2017) and CASA involves a peculiar chaperone of the family of the small HSPs, known as HSPB8 (Rusmini *et al.* 2017). In this pathway, HSPB8 dimerizes and form a stable complex with its co-chaperone BAG3, a nucleotide exchange factor (NEF) for HSP70s. Of note, mutation found in HSPB8 or in BAG3 have been associated with motor neuronal or neuromuscular diseases (Fontaine *et al.* 2006, Irobi *et al.* 2010, Ghaoui *et al.* 2016, Adriaenssens *et al.* 2017, Bouhy *et al.* 2018, Guilbert *et al.* 2018, Fang *et al.* 2017, Konersman *et al.* 2015, Selcen *et al.* 2009), suggesting they play an essential role in the protein quality control system (PQC) in these two tissues. Together, HSPB8 and BAG3 specifically interact with the ATP-dependent chaperone HSP70 conjugated to the E3 ubiquitin ligase carboxyl terminus of Hsc70-interacting protein (CHIP). HSPB8 is the limiting factor of the complex and recognizes misfolded protein, while BAG3, via its PxxP, allows the interaction with dynein (and the protein 14-3-3) for delivery of the entire complex to the microtubule organizing center (MTOC) where autophagosomes are assembled. Because of its function, the complex HSPB8-BAG3-HSP70-CHIP has been named CASA complex (Arndt *et al.* 2010). The CASA complex component CHIP ubiquittes the misfolded protein cargo for the autophagosomal receptor (SQSTM1/p62) recognition and insertion into autophagosomes (Rusmini *et al.* 2017, Cristofani *et al.* 2017).

The role of HSPB8 in the CASA complex and in protective activity in NDs.

Interestingly, HSPB8 has been found dramatically increased in spinal cord motor neurons that survive at end stage of disease in amyotrophic lateral sclerosis (ALS) mouse models (Crippa et al. 2010b, Crippa et al. 2010a), as well as in the spinal cord of ALS patients (Anagnostou *et al.* 2010). In the skeletal muscle, another tissue typically affected in motor neuron diseases, like ALS or the polyglutamine disease spinal bulbar and muscular atrophy (SBMA), HSPB8 is robustly upregulated during the course of disease (Crippa *et al.* 2013a, Crippa *et al.* 2013b, Marino *et al.* 2014, Rusmini *et al.* 2015). Studies in cellular models of neurodegenerative diseases involving different neuropathogenic proteins, like proteins with elongated glutamine repeat (polyQ) tracts (polyQ-huntingtin, polyQ-ataxin-3 or the SBMA-linked androgen receptor, polyQ-AR), beta-amyloid, alpha-synuclein, superoxide dismutase 1 (SOD1), TAR DNA binding protein of 43 kDa (TDP-43), repeat-associated non-AUG translated dipeptides from the chromosome 9 open reading frame 72 (C9ORF72) gene (Chavez Zobel *et al.* 2003, Wilhelmus *et al.* 2006, Carra *et al.* 2008a, Carra *et al.* 2008b, Crippa et al. 2010b, Bruinsma *et al.* 2011, Seidel *et al.* 2011, Rusmini *et al.* 2013, Crippa *et al.* 2016a, Cristofani et al. 2017), demonstrated that HSPB8 possesses a potent anti-aggregant activity via CASA (Crippa et al. 2016a, Rusmini *et al.* 2016, Giorgetti *et al.* 2015, Rusmini et al. 2013).

Indeed, HSPB8 restores a normal autophagy flux which is found to be blocked in several NDs. Conversely, in most cases, HSPB8 down-regulation resulted in increased accumulation of these mutant proteins (SOD1, TDP-43, dipeptide repeats coded by C9ORF72) supporting its role in the clearance of misfolded proteins (Crippa et al. 2010b, Crippa et al. 2016a, Rusmini et al. 2013, Cristofani et al. 2017). HspB8 also efficiently removes misfolded proteins containing PolyQ tracts, such as polyQ-AR (Carra *et al.* 2005, Carra *et al.* 2008a, Carra *et al.* 2009), or those involved in Alzheimer's and PD (Wilhelmus *et al.* 2006, Bruinsma *et al.* 2011, Seidel *et al.* 2011). In most neurodegenerative diseases with alterations of the autophagic pathway the pro-autophagic role of HSPB8 mediated by the enhanced activity of the CASA complex appears to be crucial to prevent misfolded protein accumulation in affected neurons (or surrounding cells).

HSPB8, with BAG3 in the CASA complex mediate the interplay between autophagy and proteasome systems.

It is of note that the CASA complex also serves as routing system of misfolded proteins to autophagy to prevent possible proteasome overwhelming and /or impairment (Figure 2). When dynein mediated retrograde transport is blocked, a condition present in a number of NDs (Sau *et al.* 2011) (and that we reproduced genetically using siRNAs against dynein or pharmacologically using the selective dynein inhibitor erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA)), the complex HSP70-CHIP cannot bind the HSPB8-BAG3 complex, and the CASA activity is perturbed and cannot dispose of misfolded proteins via autophagy (Cristofani *et al.* 2017). Under these conditions, specific transcription factors (still unknown)

activate the *de novo* transcription of another nucleotide exchange factor (NEF/BAG) called BAG1. Also BAG1 is capable of selectively binding the complex HSP70/CHIP; but in this case, misfolded proteins are routed to the proteasome instead of autophagy (Behl 2016, Gamerdinger *et al.* 2011, Cristofani *et al.* 2017). Indeed, BAG1 overexpression facilitates proteasomal removal of ARpolyQ, mutant SOD1 and mutant TDP-43 (Cristofani *et al*. 2017), and this BAG1 activity is fully counteracted by the inhibition of the proteasome activity, but not by inhibition of autophagy (Cristofani *et al.* 2017). Since, the inhibition of the proteasome also results in the upreguation of both HSPB8 and of BAG3 (Crippa *et al.* 2010b), while blockage of the delivery of misfolded protein to autophagy results in upregulation of BAG1(Cristofani *et al.* 2017), this gives rise to a very nice equilibrium between the proteasome and autophagy selective degradation. The equilibrium is maintained by the ratio BAG3:BAG1, which determines the relative amount of the proteins BAG3 (associated with HSPB8) and BAG1 capable of associating with the dimer HSP70/CHIP and of selecting the proper degradative pathway for a misfolded protein in a given neuron.

HSPB8 protects against misfolded protein toxicity in animal models of ALS.

Few animal model studies have been performed to study the effect of HSPB8 overexpression in NDs. However, by enhancing the expression of the fly functional ortholog of *HSPB8* (*HSP67Bc)* in two *Drosophila melanogaster* models of ALS, we found that HSP67Bc prevents the mislocalization of a neurotoxic mutant TDP-43 protein (Ritson *et al.* 2010). As a proof of principle, HSP67Bc downregulation resulted in TDP-43 and polyubiquitinated proteins accumulation and worsened the eye phenotype of mutant TDP-43 flies (Crippa *et al.* 2016a). The HSPB8 fly ortholog also extended survival of the fly ALS models, since it rescued from pupae lethality the flies expressing an ALS-associated 35 kDa TDP-43 fragment (TDP-35) (Crippa *et al.* 2016a). Surprisingly, no major effects of HSPB8 silencing were observed in a transgenic mouse model with a functional knock-out of HSPB8 which showed motor behaviour performances similar to those of wild-type animals (Bouhy *et al.* 2018), thus suggesting that the pro-autophagic activity of HSPB8 may be relevant specifically when aberrant proteins are generated in affected cells. Conversely, mice expressing a Charcot-Marie-Tooth disease related mutation of HSPB8 showed clear sign of motor deficits due to degeneration of peripheral nerves, which were accompanied by severe muscle atrophy and protein inclusions (Bouhy *et al.* 2018).

On these bases, a large screen aimed to identify commercially available drugs that are able to enhance HSPB8 expression in motor neuronal cells for therapeutic purposes (Crippa *et al.* 2016b) led to the identification of the drug colchicine as a potent inducer of HSPB8 together with a series of other genes involved in autophagy activation (transcription factor EB, p62, LC3) suggesting that colchicine could represent a useful candidate to be tested in misfolded protein associated NDs (Crippa *et al.* 2016b). Of note, estrogens are also potent inducers of HSPB8 expression (Piccolella *et al.* 2017), confirming previous

published data (Sun *et al.* 2007), and this might in part explain some of the gender differences described in misfolded protein associated NDs neurodegenerative diseases, including ALS.

IV. Mitochondrial autophagy and beyond in Parkinson's disease.

Parkinson's disease-linked proteins promote mitophagy at the endoplasmic reticulum-mitochondria interface.

Mitophagy is a relatively recent term describing the selective autophagy of mitochondria under specific stress conditions, including during apoptosis induction in the presence of caspase inhibitors, following nutrient deprivation or toxin-induced mitochondrial damage (Tolkovsky *et al.* 2002, Elmore *et al.* 2001, Kissova *et al.* 2004, Lemasters 2005), or during specific developmental programs, such as metabolic reshuffling in the developing heart (Gong *et al.* 2015) or red blood cell maturation (Sandoval *et al.* 2008, Schweers *et al.* 2007). Mitophagy became relevant to neurodegeneration when the mitochondrial serine/threonine kinase PINK1 and the ubiquitin-protein ligase Parkin, the products of two genes (*PINK1* and *PARK2*) mutated in autosomal recessive forms of PD, were found to jointly promote the clearance of depolarized mitochondria in cell lines treated with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Narendra *et al.* 2008, Narendra *et al.* 2010, Geisler *et al.* 2010, Matsuda *et al.* 2010). We now know that a number of stress-related stimuli activate this program, including specific inhibitors of the respiratory chain, unfolded protein stress in the mitochondrial matrix, and impairment of the mitochondrial protein import pathway (Jin & Youle 2013, Greene *et al.* 2012, Bertolin *et al.* 2013, Wang *et al.* 2011, Barini *et al.* 2018, Jin *et al.* 2010, Fiesel *et al.* 2017). These conditions impair PINK1 import into mitochondria, promoting its accumulation on the outer mitochondrial membrane (OMM), in proximity of the translocase of the outer mitochondrial membrane (TOM) through which the majority of the mitochondrial proteins enter the organelle (Lazarou *et al.* 2012, Hasson *et al.* 2013, Okatsu *et al.* 2013). This triggers a cascade of events, including the PINK1-dependent recruitment and activation of Parkin, the ubiquitylation of a number of proteins of the OMM, and the recruitment of autophagy receptors and upstream autophagy-related protein to prime mitochondria for mitophagy (reviewed by (Sekine & Youle 2018, Truban *et al.* 2017, McWilliams & Muqit 2017). These events occur in proximity of the interface between mitochondria and the endoplasmic reticulum (ER) (Yang & Yang 2013, Gelmetti *et al.* 2017), a subcellular compartment increasingly involved in neurodegeneration (reviewed by Erpapazoglou *et al.* 2017). This interface is perturbed in cells from Parkin-deficient mice and patients with *PARK2* mutations, due to accumulation of the Parkin substrate and ER-mitochondria tethering protein Mitofusin 2 (Mfn2) (Gautier *et al.* 2016). Ubiquitylation of Mfn2 by Parkin, disassembly of Mfn2 complexes and dissociation of mitochondria from the ER are instrumental for the initiation of mitophagy (McLelland *et al.* 2018). These events are probably concomitant to the separation of the damaged mitochondrion from the rest of the

network, operated by the dynamin-related GTPase Drp1 and regulated by the ER-mitochondria interface (Twig *et al.* 2008, Tanaka *et al.* 2010, Buhlman *et al.* 2014, Friedman *et al.* 2011). Drp1 is cooperatively recruited by PINK1 and Parkin on depolarized mitochondria, suggesting that mitochondrial fission and mitophagy are orchestrated at the ER-mitochondria interface (Buhlman et al. 2014, Erpapazoglou & Corti 2015).

PINK1 and Parkin keep mitochondria functional by multiple mechanisms.

PINK1 and Parkin jointly regulate various mitochondrial quality control mechanisms in addition to mitophagy: the delivery of damaged mitochondrial components to the lysosome by mitochondria-derived vesicles (MDV), mitochondrial biogenesis and local translation on the OMM of transcripts for nuclear-encoded respiratory chain components (McLelland *et al.* 2014, Sugiura *et al.* 2014, Gehrke *et al.* 2015, Shin *et al.* 2011, Lee *et al.* 2017) (Figure 3). This latter mechanism requires functional interaction between PINK1 and the TOM complex to target the transcripts to the OMM during cotranslational import (Gehrke *et al.* 2015). Considering the proximity of the activated PINK1/Parkin system to the TOM complex (Lazarou et al. 2012, Hasson et al. 2013, Okatsu et al. 2013, Bertolin et al. 2013), and the increasing importance gained by local translation on the mitochondrial surface (Golani-Armon & Arava 2016), this mechanism may be of broader relevance. Supporting this hypothesis, we found that Parkin participates in maintaining mitochondrial levels of the multifunctional mitochondrial matrix hydroxysteroid dehydrogenase, HSD17B10 (Bertolin *et al.* 2015), shown to be depleted in a PD mouse model and PD patients (Tieu *et al.* 2004). HSD17B10 was co-recruited with Parkin near the TOM complex and PINK1, suggesting regulation of its mitochondrial import by a PINK1/Parkin-dependent mechanism (Bertolin *et al.* 2015). Using an original biosensor for exploring the presequence-mediated protein import pathway in living cells, we recently showed that this process is more generally facilitated by the PINK1/Parkin system (Jacoupy *et al.* 2019).

Dissecting the various mechanisms by which the PINK1/Parkin sensor/effector system maintains mitochondrial quality has highlighted the central role played by mitochondrial protein import in monitoring the mitochondrial functional state. There is a remarkable parallel between the characteristics of the PINK1/Parkin system and a key regulatory component of the mitochondrial unfolded protein response (UPR^{mt}) in the small invertebrate organism *C. elegans*, Activated Transcription Factor Associated with Stress 1 (ATFS-1). ATFS-1 is constitutively imported into functional mitochondria by an N-terminal mitochondrial targeting signal, but under mitochondrial stress it translocates to the nucleus to activate a protective transcriptional response mediated by hundreds of genes with various functions, including coping with the accumulation of unfolded proteins in mitochondria, fighting against an excess of reactive oxygen species, and remodelling of cellular metabolism (Nargund *et al.* 2012). By analogy, it is likely that PINK1

and Parkin are more broadly involved in the modulation of the mitochondrial stress response in mammalian cells, considering the well-known cell-protective properties of Parkin (Imai *et al.* 2000, Darios *et al.* 2003, Johnson *et al.* 2012, Bouman *et al.* 2011), and the fact the PINK1/Parkin system is induced during the UPR^{mt} (Jin & Youle 2013). Establishing the consequence of PINK1 and Parkin dysfunction on the mitochondrial stress response, and investigating the possibility that these proteins coactivate transcription factors near dysfunctional mitochondria, are major challenges for future research. Activating Transcription Factor 5 (ATF5) may mediate the UPR^{mt} response in mammals, but whether this transcription factor interacts with the PINK1/Parkin system is unknown (Fiorese *et al.* 2016). Notably, PINK1 and Parkin contribute to the activation of transcription factors of the microphtalmia/transcription factor E (MiT/TFE) family during mitophagy, including the master regulator of lysosomal biogenesis, transcription factor EB (TFEB) (Nezich *et al.* 2015). Morover Parkin acts as transcription factor itself, although the precise mechanisms regulating its nuclear translocation and transcriptional activity remain to be determined (da Costa *et al.* 2009, Alves da Costa & Checler 2012).

Fluorescent reporters with pH-sensitive components illuminate PINK1/Parkin-dependent mitophagy in cultured neurons and in vivo.

We still lack sufficient evidence for a role of PINK1/Parkin-dependent mitophagy in mitochondrial maintenance and neuronal survival *in vivo* and in the context of PD. The question of whether mitophagy is relevant to neurons has been intensively debated (Grenier *et al.* 2013, Van Laar *et al.* 2011). Studies based on the simultaneous expression in primary neurons of fluorescent reporters targeted to the mitochondrial compartment, the autophagosome and the lysosome have, however, provided evidence in neurons for events of engulfment of mitochondria into autophagosomes, or of fusion of autophagosomes containing mitochondria with lysosomes (Cai *et al.* 2012, Ashrafi *et al.* 2014, Hsieh *et al.* 2016). These events were observed to a lesser degree in neurons from Parkin- and PINK1-deficient mice, supporting their relation to PINK1/Parkin-dependent mitophagy (Ashrafi *et al.* 2014). More convincingly, mitophagy has been investigated in cultured primary cells, including neurons and *in vivo*, in *Drosophila* and mice, using ratiometric or dual-fluorescence reporters with pH-sensitive components for direct tracking of mitochondria in lysosomes (Bingol *et al.* 2014, Bonello *et al*. 2019, McWilliams *et al.* 2016, McWilliams *et al.* 2018, Sun *et al.* 2015, Lee *et al.* 2018, Cornelissen *et al.* 2018, Shin *et al*., 2019). These studies have demonstrated the occurrence of mitophagy under physiological conditions in neurons, including the highly vulnerable dopaminergic (DA) neuron in the mouse (McWilliams *et al.* 2018) and fly brains (Lee *et al*. 2018, Cornelissen *et al*. 2018). However, it remains controversial whether PINK1 and Parkin coregulate this type of physiological mitophagy detected in the absence of exogenous insults, particularly during aging (Cornelissen *et al.* 2018, Lee *et al*. 2018, McWilliams *et al*. 2018), which is recognized today as the major risk factor for PD (Collier *et al.* 2017). Notably, a previous study reported strong immunostaining for

phosphorylated ubiquitin partially colocalizing with mitochondria and lysosomes in the aged and PD-affected human brain, but it remains to be clarified whether this reflects impaired or increased mitophagy (Fiesel *et al*., 2015).

Beyond neurons: a role in innate immunity.

Finally, there is mounting evidence that PINK1 and Parkin jointly regulate immune-related mechanisms, which is of particular interest, considering the neuroinflammatory component in PD (Greene *et al.* 2005, Manzanillo *et al.* 2013, Matheoud *et al.* 2016, Torres-Odio *et al.* 2017, Sun *et al.* 2018, Kim *et al.* 2014, Kang *et al.* 2016, Zhong *et al.* 2016, Sumpter *et al.* 2016, Mouton-Liger *et al.* 2017, Sliter *et al.* 2018). In some cases, these regulatory roles of PINK1 and Parkin have been clearly linked to modulation of mitophagy (Kim et al. 2014, Zhong et al. 2016, Sumpter et al. 2016). Specifically, PINK1/Parkin-dependent mitophagy has been recognized to counterbalance the action of the nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome, a proinflammatory multiprotein complex activated in response to pathogens as well as endogenous signals associated with cell and tissue damage (Sumpter et al. 2016, Zhong et al. 2016). This complex plays a central role in a number of inflammatory/autoimmune disorders, and is emerging as key player in neurodegenerative diseases, including PD (Labzin *et al.* 2018). Specifically, the neurotransmitter DA has been shown to attenuate NLRP3 inflammasome signalling in immune cells, whereas mitochondrial toxins, such as MPTP or rotenone, exacerbate NLRP3-dependent inflammation, leading to DA neurodegeneration in cell and animal models (Yan *et al.* 2015, Sarkar *et al.* 2017). We have recently reported exacerbation of the NLRP3 inflammasome pathway in bone-marrow-derived macrophages and microglial cells from Parkin- and PINK1-deficient mouse models, and confirmed this defect in PD patients with *PARK2* mutations (Mouton-Liger *et al.* 2018).

V. Focus on autophagy as therapeutic target in brain pathologies.

With our improved understanding of the regulation of autophagy, its diverse regulatory mechanisms and a greater awareness that it represents a unique target with multiple therapeutic options for the clearance of toxic aggregates and damaged organelles, there has been an extraordinary rise of interest in its clinical applicability and options for translation (e.g. (Feng *et al.* 2017, Tramutola *et al.* 2017) (Lipinski *et al.* 2015, Rubinsztein *et al.* 2015, Maiese 2016, Boland *et al*. 2018). Indeed these reviews are pertinent to topics as different in pathology as Alzheimer's disease and traumatic brain injury, reflecting the wide applicability of such therapeutic options. A seminal publication that initiated cognisance of this area, indeed a veritable tour de force, overviewed multiple classes of drugs and potential molecular targets regulating autophagic mechanisms (Rubinsztein *et al.* 2007).

We became aware of the FDA approved drug rilmenidine, considered to act via a mTOR-independent mechanism, after it was reported to have beneficial actions in a mouse model of Huntington's disease (Rose *et al.* 2010). At the time we were working on 3,4-methylene-dioxymethamphetamine (ecstasy) induced injury of serotonin (5-HT) neurons, and (i) since autophagy was considered to be involved in the brain's response to amphetamines (Galluzzi et al. 2016), and (ii) rilmenidine binding sites exist in 5-HT raphe nuclei (King *et al.* 1995), the inclusion of rilmenidine in our study was an obvious step forward. Indeed ecstasy-induced neurotoxicity of 5-HT neurons involved an autophagic component and rilmenidine completely blocked the toxicity of ecstasy (Mercer *et al.* 2017). Thus this FDA-approved molecule, subsequently documented to be well-tolerated and suitable for human usage (Underwood *et al.* 2017), likely also possesses a profile suitable for use in drug abuse. Success here led to our further studies exploring the potential of rilmenidine in cellular and animal models of MND. We found beneficial effects on rilmenidine in a MN cell line and in MNs derived from human stem cells expressing mutant superoxide dismutase 1 (SOD1): there was an increase in autophagic flux following treatment, as assessed by monitoring LC3 lipidation levels and abundance of autolysosomes, using a tandem mCherry-GFP-LC3B reporter construct, accompanied by a 20% reduction in the load of mutant SOD1 (Perera *et al.* 2018). However, we were disappointed at its lack of effectiveness in the $SOD1^{G93A}$ mouse, where it actually worsened the pathological and functional outcome (Perera *et al.* 2018). What our *in vivo* study does highlight is that rilmenidine was certainly efficacious enhancing autophagic flux, but that there was a concurrent accumulation of misfolded SOD1, and mitochondrial depletion with autophagosome accumulation in MNs. In ongoing work in a transgenic mouse model overexpressing the normal (WT) and ALS-linked Q331K variants of the human TDP-43 protein in neurons ($TDP\text{-}43^{WTxQ331K}$ mice) (Mitchell *et al*., 2015), we have found similar findings with rilmenidine with reduced TDP-43 immunoreactivity in MNs (Supplementary Fig. 1), so moving forward it will be interesting to determine whether the enhanced auto-/mitophagic flux found with rilmenidine (Perera *et al*. 2017) will prove useful in other TDP-43 models. Given the positive outcomes here rilmenidine may prove effective in less aggressive forms of MND and certainly a key issue is that the timepoint of drug intervention may well be critical relative to toxic aggregate load and stage of disease progression. Indeed, our evidence for activation of autophagy in TARDBP in cell culture models, when taken the finding that rapamycin produces benefits in a mouse TARDBP model (Wang *et al.* 2012), strongly suggests that autophagy is likely to be a valid target in MND. Although toxic aggregates and dysfunctional proteostasis (Yerbury *et al.* 2016) have been linked to diverse proteins in MND, emergent reports increasingly suggest that successful management may be possible by targeting autophagic signalling (Deng *et al.* 2017). For example, recent elegant work screening clinically approved drugs revealed a number of small molecules with beneficial actions using FUS-expressing stem cells and a *Drosophila* model (Marrone *et al.* 2018).

In addition to the current great interest in potentially clinically effective small molecules, numerous strategies, including gene therapy, RNA interference or antisense oligonucleotides, continue to attract attention focusing on for example mTOR-dependent and-independent pathways, and broadly on signalling linked to Beclin1, Atg5, AMP-dependent protein kinase, PICALM and histone deacetylases (Shoji-Kawata *et al.* 2013, Hu *et al.* 2017, Menzies *et al*. 2017, Rahman & Rhim 2017). Although at the present time subsets of such published data are relatively small as they relate to neurodegeneration and human brain pathologies, there continue to be rapid developments in this area with new advances in our understanding of the regulation and dynamics of autophagic flux, and its interface with the ubiquitin proteasome, as highlighted by the preceding sections.

Conclusions and perspectives.

The examples discussed in this review are representative of a growing body of literature showing how autophagy has not only physiological roles in neurodevelopment and the maintenance of neuronal homeostasis, but also different levels of involvement in pathological processes affecting the brain, including neurodegenerative disorders and stroke. As we have seen here, a set of observations involve dysfunction of selective types of autophagy in specific neurodegenerative disease. For example, defective CASA has been linked to motor neuron disorders such as ALS and SBMA and mitophagy is suspected to be impaired in specific forms of Parkinson's disease, as well as in other neurodegenerative diseases, according to studies not discussed in this review (Wong & Holzbaur 2014, Cai & Jeong 2020). In other pathological conditions of the brain, autophagy appears to be uncontrollably activated and to cause neuronal death, as in the case of perinatal asphyxia. From an assessment of the current state of knowledge, it is clear that we are still some distance from a holistic understanding of how autophagy is recruited in different brain diseases and in which cases it is merely associated with cell death rather than mediating or even causing cell death (Klionsky *et al.* 2016). Thus, we still need appropriate *in vivo* documentation of the various forms of autophagy in different disease contexts and across different disease stages, as well as their relationship to different forms of cell death

to fully appreciate whether and when they play beneficial or detrimental roles. Findings presented herein argue that steps to take advantage of the beneficial modulation of autophagy by pharmacological strategies, and the specific modalities of such strategies, are likely to be quite different depending on the age of the affected individual, the type of injury/disease, the specific stage of progression, as well as the overall duration and severity of the disease. For instance, it has already been reported in the $SOD1^{G93A}$ mouse model of MND that short-term treatment with clemastine is beneficial, but long-term treatment fails to alleviate disease (Apolloni *et al.* 2016). As an additional take-home message, we need to better understand

the complex interplay between autophagy and other intracellular processes that may also at some point be recruited by the pathological process, either as contributing factors or protective responses. As illustrated with PINK1 and Parkin, many proteins involved in autophagy and its regulation have multiple functions in the cell, and these have to be taken into consideration if we want to explore meaningful therapeutic avenues for neurological diseases. The case of CASA, on the other hand, illustrates the interplay between autophagy and the proteasome system, leaving room for envisaging strategies aimed at promoting alternative degradation routes when autophagy is impaired. Finally, while autophagy and mitophagy are emerging as central modulators of inflammatory and immune-related mechanisms, which are recognized as a component of the pathological process in various brain diseases, work discussed here also shows that the selective modulation of autophagy in neurons has broader impact on brain tissue homeostasis. Therefore, a better knowledge of the different cell types affected by the disease process and their complex interactions will provide invaluable insight into the most appropriate ways of using autophagy modulation for therapeutic purposes in the brain.

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--Human subjects --
Involves human subjects:
If yes: Informed consent & ethics approval achieved:
=> if yes, please ensure that the info "Informed consent was achieved for
all subjects, and the experiments were approved by the local ethics
committee." is included in the Methods.

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Figure legends

Figure 1

Key points addressed in the review and take-home messages. This figure was drawn using illustrations adapted from the image bank of Servier Medical Art (Cellular Biology; https://smart.servier.com/). Servier

Figure 2

The HSC70/CHIP-mediated routing system between proteasome and autophagy. BAG1 is a nucleotide exchange factor which is capable to complex the chaperone HSC70 and E3 ubiquitine ligase CHIP. This complex recognize monomeric misfolded proteins to be driven to proteasome degradation. Alternatively, misfolded monomeric, oligomeric and/or aggregated misfolded proteins can be recognized by another nucleotide exchange factor BAG3 associated to the small HSP named HSPB8. Once the complex HSPB8/BAG3 recognizes target misfolded proteins, the entire complex can associate to the HSC70/CHIP complex, thus sequestering it from the BAG1 binding. The HSPB8/BAG3/HSC70/CHIP complex has been named the CASA complex, since it mediates a peculiar form of autophagy known as "Chaperone-Assisted Selective Autophagy (CASA)". In this case misfolded aggregating proteins, bound to the CASA complex, are routed by a dynein-dependent transport to the microtubule organization center (MTOC) where aggresomes are formed and inserted into nascent autophagosomes. The HSC70/CHIP-mediated routing system thus generates a delicate equilibrium which is responsible for the choice of two alternative pathways for damaged/misfolded protein degradation. Of note, the blockage of the dynein-mediated transport of the CASA complex correlates which enhanced BAG1 expression. Conversely, proteasome inhibition results in HSPB8 and BAG3 overexpression. This transcriptionally regulated expression of the routing system factors maintains the fine tuned equilibrium between proteasome and autophagy degradation in cells.

Figure 3

Mechanisms by which the PINK1/Parkin system keeps mitochondria functional. In addition to regulating the degradation of damaged mitochondrial components and whole organelles through the MDV and mitophagy pathways, the PINK1/Parkin system promotes mitochondrial biogenesis by at least two distinct mechanisms: the proteasomal degradation of PARIS (Parkin Interacting Substrate/ZNF746), a trascriptional repressor of the master regulator of mitochondrial biogenesis PGC-1α (peroxisome-proliferator-activated receptor γ coactivator-1α); and the displacement of translational repressors, including the Parkin substrate hnRNP-F, from transcripts encoding certain nuclear-encoded respiratory chain components, anchored on the outer mitochondrial membrane in a PINK1-dependent manner. During mitophagy, PINK1 and Parkin also promote the nuclear translocation and activation of transcription factors of the MiT/TFE family, leading to lysosomal biogenesis and facilitation of mitophagy. The mechanism underlying the PINK1/Parkin-dependent activation of these transcription factors remain to be determined. This figure was drawn using illustrations adapted from the image bank of Servier Medical Art (Cellular Biology; https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License. https://creativecommons.org/licenses/by/3.0/.

Tab le 1: Characteristic features of autosis comparatively to other forms of cell death

	Autosis	Autophagy-dependent cell death	Apoptosis	Necrosis
Morphological features	• Nuclear shrinkage; focal separation of inner and outer nuclear membranes with focal expansion of perinuclear space • Extensive cytoplasmic vacuolization • Enhanced cell-substrate adhesion	• Extensive cytoplasmic vacuolization	• Nuclear compaction and fragmentation • Marked chromatin condensation` • Cell shrinkage, membrane blebbing (apoptotic bodies) • Cell rounding up, detachment from substrate	• Swelling of organelles • Cell swelling
Molecular markers			• DNA fragmentation (laddering)	
Mechanism of execution	• Requires core autophagy machinery • Occurs independently of apoptotic pathways (Bax, Bak, caspases…) • Depends on Na^+,K^+-ATPase	• Requires core autophagy machinery • Occurs independently of apoptotic pathways	• Requires apoptotic pathways (extrinsic, intrinsic) • Occurs independently of core autophagy machinery	
Blocked by	• Genetic ablation of key components of core autophagy machinery (beclin1, ATG13, ATG14) • Pharmacological inhibition or genetic ablation of Na^+/K^+-ATPase	• Genetic ablation of key components of core autophagy machinery	• Caspase inhibitors • Genetic ablation of pro-apoptotic factors	
Disposal of cell corpses	• Not determined	• Phagocytic uptake and lyososmal degradation	• Phagocytic uptake and lyososmal degradation	

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Autophagy in human neurological diseases: prospects for therapy

Hypoxic-ischemic injury

- Autophagy may be recruited differently depending on load of cellular debris and maturity of the brain
- In the neonatal brain, overactivation of autophagy leads to autosis

Radiotherapy-induced damage to the young brain

- Leads to depletion of neurogenic niches

➢ Autophagy inhibition
➢ Autophagy stimulation
➢ HSPB8 induction

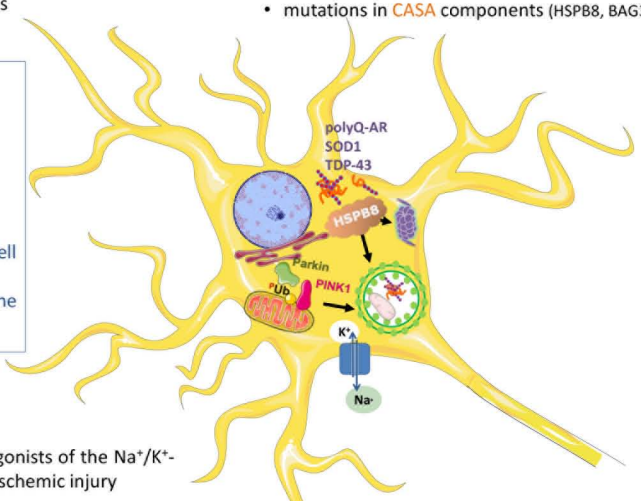
Parkinson's disease

- Autosomal recessive forms caused by dysfunction of the PINK1 and Parkin genes are suspected to be caused by mitophagy defects

Motor neuron and neuromuscular disorders

Are associated with:

- accumulation of misfolded proteins (polyQ-AR, mutant SOD1, TDP-43, C90RF72 dipeptide repeats)
- mutations in CASA components (HSPB8, BAG3)



Autophagy as a therapeutic target

Key issues to be considered

- Intrinsic differences between immature and adult brain
- Crosstalk between different cell death pathways
- Interplay with proteasome
- Multifunctionality of autophagy components
- Broad tissue responses elicited by targeting a single cell type
- Stage, severity and duration of disease may determine protective versus detrimental recruitment

Mechanisms

❖ *Autosis*

- A specific form of autophagic cell death Inhibited by antagonists of the Na^+/K^+-ATPase, observed in the neonatal brain following hypoxic-ischemic injury

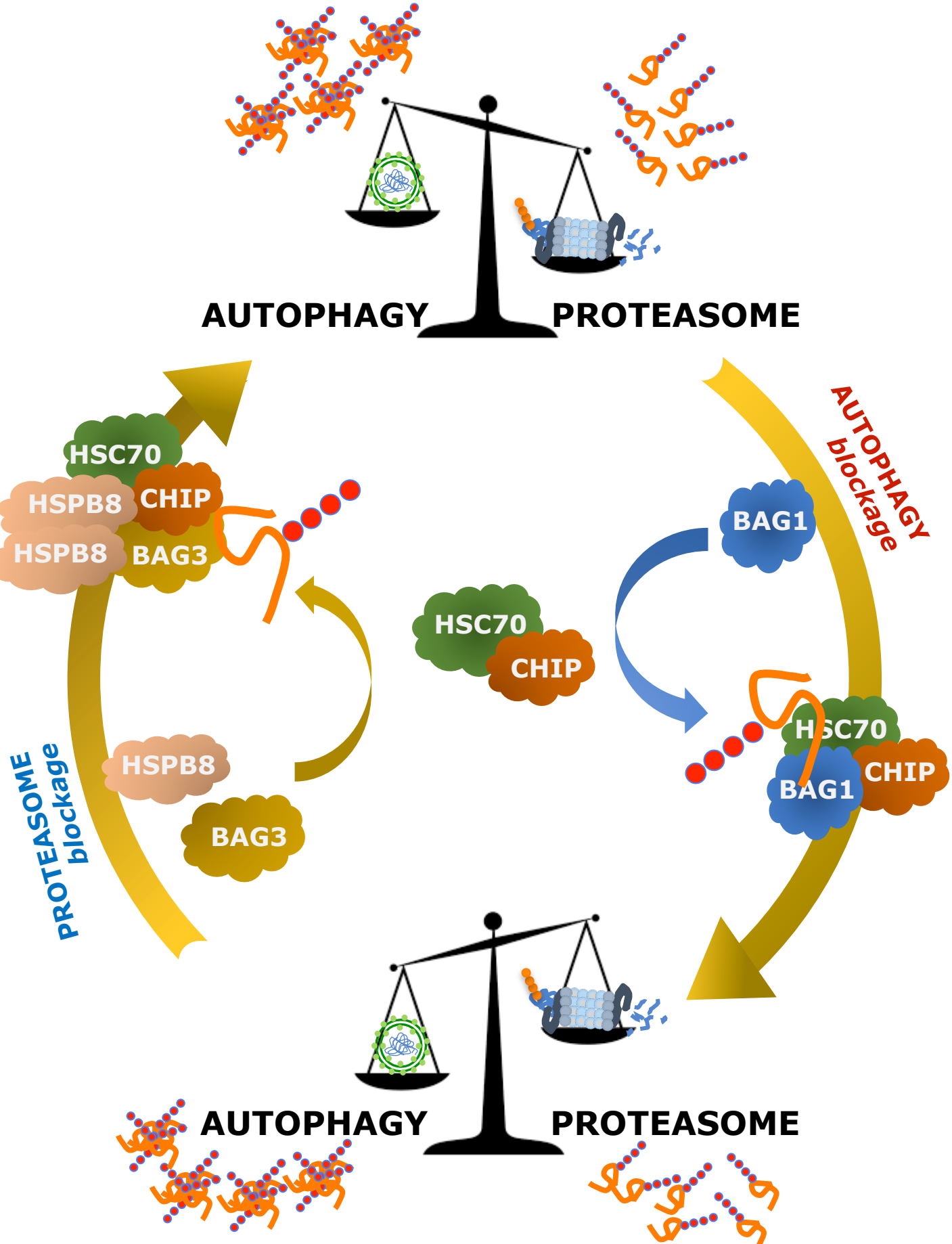
❖ *Chaperone-assisted selective autophagy: the CASA complex*

- Involves the heat-shock protein HSPB8, the nucleotide exchange factor BAG3, Hsp70 and the E3 ubiquitin ligase CHIP
- Ubiquitylates misfolded proteins and delivers them to the autophagosome
- In the absence of BAG3, the Hsp70-CHIP complex routes misfolded proteins to the proteasome

❖ *the mitochondrial PINK1 kinase and Parkin ubiquitin ligase: a multifunctional mitochondrial quality control system*

PINK1 activates Parkin to jointly promote:

- The autophagic degradation of dysfunctional mitochondria (mitophagy)
- The mitochondria-derived vesicle pathway to lysosomal degradation
- The local translation of respiratory chain components
- Mitochondrial biogenesis
- Mitochondrial protein import


AUTOPHAGY
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HSC70
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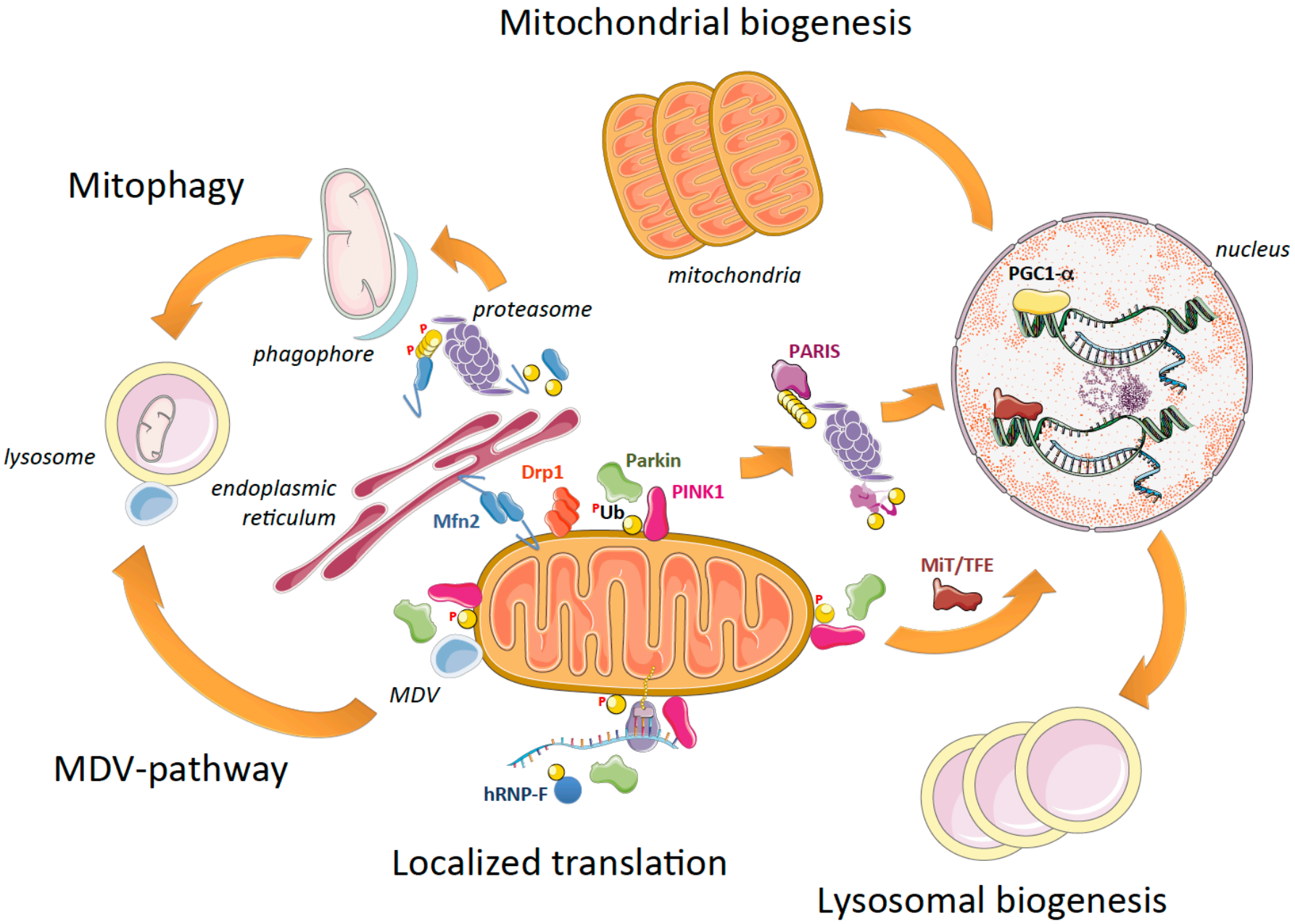
Mitochondrial biogenesis
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Mitophagy
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lysosome
endoplasmic
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Drp1
Parkin
PINK1
Ub
Mfn2
MiT/TFE
MDV
hRNP-F
MDV-pathway
Localized translation
Lysosomal biogenesis