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Role of AMBRA1 in nervous tissue homeostasis and in neurodegeneration.

Ruolo di AMBRA1 nell'omeostasi del tessuto nervoso e in processi neurodegenerativi.

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Contents

Abstract

My PhD project focused on the role of AMBRA1 in mouse brain in physiological and pathological conditions. *Ambra1* ("Activating Molecule in Beclin 1 Regulated-Autophagy") is a recently identified gene encoding for a large protein (around 130 kDa), with an N-terminal WD40 domain (Fimia et al., 2007). *Ambra1* gene is highly conserved among vertebrates, and is expressed in different splicing isoforms. In the developing mouse, AMBRA1 protein shows abundant expression in the central and peripheral nervous system. At embryonic day 8.5 (E8.5), AMBRA1 is present in the neuroepithelium, while at E11.5 protein expression is localized to the ventral-most part of the spinal cord, the encephalic vesicles, the neural retina, and the dorsal root ganglia. This distribution pattern suggests that AMBRA1 is centrally involved in the proper development of the nervous system. Indeed, *Ambra1*$^{gt/gt}$ mice, obtained by gene trapping technique, and displaying a deficient expression of the protein, show early and severe neuropathological features, including neuroepithelial hyperplasia and defective neural tube closure, leading to embryonic death (Fimia et al., 2007; Cecconi et al., 2008). These disturbances appear to derive from dysfunctions in the autophagic process, also resulting in an imbalance of apoptotic cell death and cell proliferation. These data, together with *in vitro* evidence, allowed proving a regulatory role of AMBRA1 in autophagy activation in vertebrates (Fimia et a., 2007). Autophagy is involved in the intracellular turnover of proteins and cell organelles, and has an important role in regulating cell fate in response to stress (Levine, 2005). Three types of autophagy have been described: microautophagy, chaperone-mediated autophagy and macroautophagy. The last one is a bulk degradation pathway and the only intracellular mechanism potentially capable of degrading large protein aggregates or damaged organelles. A cup-shaped isolation membrane forms around cytosolic components, eventually fusing to form a double membrane bound vesicle, the so-called "autophagic vacuole" (AV) (Mizushima et al., 2002). In thi respect AMBRA1 is essential in the induction of AV formation (Di Bartolomeo et al, 2010). Recent studies show that macroautophagy is constitutively active in healthy neurons and is vital to cell survival (Bolland and Nixon, 2006). Mice lacking either Atg5 or Atg7 genes exhibit motor and behavioral deficits as well as degeneration of specific neuronal subtypes (Hara et al., 2006; Komatsu et al., 2006). Diffuse protein aggregates appear in surviving neurons within several brain regions, culminating in the formation of toxic inclusion bodies. This supports an essential role of autophagy is for neuronal health. The evidence so far presented and the severe neuropathological phenotype of *Ambra1*$^{gt/gt}$ mice prompted us to investigate the expression of AMBRA1 protein in adult mouse CNS, in physiological and pathological conditions.

The main results obtained from the research and described in this thesis can be summarized as follows:

1. I provided the first neuroanatomical/histological/ ultrastructural map of the distribution of AMBRA1 expression in mouse brain taking advantage of immunohistochemical, immunofluorescence and immunoelectron microscopy approaches. Wide presence of the protein in the forebrain, midbrain, and hindbrain was observed, demonstrating prevalent expression in neurons, even though astrocytes and microglial cells also contain moderate levels of AMBRA1. This suggests that in physiological conditions AMBRA1 is crucial for neural tissue homeostasis. Ultrastructural analysis revealing association of with the endoplasmic reticulum strongly supports AMBRA1 activity in regulating basal autophagy, essential for neuronal survival. Detailed examination of different brain territories along the rostro-caudal axis allowed me to show that AMBRA1 content varies among brain regions, neuronal populations, and subtypes. The concentration of neuronal AMBRA1 appears at least partially related to cell volume. Neurons featuring a large soma, highly brached dendrites and long axons generally display higher immunoreactivity, compared to smaller cells. Among these, mitral cells in the olfactory bulb, giant pyramidal neurons of the neocortex, motor neurons of the brainstem, Purkinje cells of the cerebellar cortex are paradigmatic. Some of these giant neurons have also been described to contain other pro-autophagic molecules (Tamura et al., 2010), suggesting that their high metabolic and turnover rates are likely to involve large amounts of autophagy regulators, such as AMBRA1. Even more interestingly, we also found some correlation between AMBRA1 content and other parameters, namely susceptibility to damage. In particular, some neuronal subsets, which are selectively spared by neurodegenerative insults, demonstrably contain high levels of the protein. Representative examples of this concept are cholinergic interneurons in the corpus striatum, virtually unaffected by Huntington's Disease (HD), and CA3 pyramidal neurons of the hippocampus, resistant to Alzheimer's Disease (AD) and to ischemic injury. Importantly, this pathology is suggested to involve inefficient regulation of autophagic processes (Jaeger et al., 2009)). However, in some cases, neurons showing relatively high AMBRA1 content are target of specific neurodegenerative disease. For instance, Purkinje and mitral cells are both affected by the so-called "Purkinje cell death" (pcd) pathology. Remarkably, in this syndrome neuronal cell loss which probably occurs through the autophagic, rather than apoptotic, pathway, indicating that dysregulation of autophagy, possibly involving also AMBRA1 expression, may participate to some neuropathologies.

2. I highlighted important age-related variations in AMBRA1 expression in regions prone to neurodegeneration, such as the neocortex and hippocampus during normal and Alzheimer-like ageing. For this part of the project, I used a WT and transgenic strain for AD (Tg2576) at different time points.

In the neocortex, the high levels of AMBRA1 in normal young mouse led me to hypothesize that AMBRA1 is essential for a faultless maturation of neocortical neurons. Interestingly, Tg2576 mice show lower AMBRA1 content at the very

onset of disease, when most of the histopathological hallmarks are still undetectable, thus suggesting the possible involvement of AMBRA1 down-regulation in the impaired plasticity of neuronal circuits. During normal adulthood, we found an overall decrease of AMBRA1 content which may reflect a relatively balanced cell activity, with a stable regulation of biosynthesis and degradation pathways. The novel increase of the protein in the aged neocortex, which is independent of the genotype, may instead suggest that AMBRA1 is up-regulated in this critical period, since brain ageing likely requires a higher rate of basal autophagy also to counteract oxidative stress and consequent accumulation of damaged organelles. Differences in AMBRA1 expression are not only related to age, but even to the specific brain region, since the the neocortex and the hippocampus show different behavior during ageing and pathological progression. In particular, overall levels in young mouse hippocampal formation are similar in the two genotypes. However, a different distribution in hippocampal subregions of Tg2576 animals compared to WT was detected. In fact, the neurogenic region of dentate gyrus is more intensely AMBRA1 immunoreactive in the transgenic animals than in WT, thus suggesting that in the pathological condition an early response involving enhanced neurogenesis may occur to cope the first deficits. Then we found a decrease in AMBRA1 level during adulthood and ageing in both genotypes, even though, at 18 months, the transgenic hippocampus shows a further drop in AMBRA1 reactivity, possibly contributing to neurodegeneration.

3. I showed that AMBRA1 expression can be induced in dopaminergic neurons by a mild 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment, producing a pharmacological model of early PD. Importantly, similar effects are also observed in a genetic model of genomic instability (ERCC1 mutants) which is associated with an early PD-like phenotype. These findings support the involvement of AMBRA1 in cellular response against oxidative imbalance in dopaminergic neurons that are closely related to PD onset. Surprisingly, AMBRA1 levels increase in both the substantia nigra (SN) and ventral tegmental area (VTA), the former of which turns to cell death in PD while the latter is spared. One could envision that the activation of the autophagic pathway by AMBRA1 up-regulation, can be defensive in some cases and detrimental in others, strictly depending on the specific neuronal population. To this respect, it is worth noting that the features of neuronal cell death in PD are still debated, because it seems to bring back to autophagic cell death. In conclusion, the results obtained in my PhD project suggest that AMBRA1 is a fundamental molecule in the nervous tissue, given the abundant content in neurons, although to different degree with respect to the brain area and neuronal population. In addition, modulation of AMBRA1 expression may be critical for establishing, promoting or counteracting neurodegenerating processes. Thus, our study opens the way to further investigations aimed to defining the precise contribution of AMBRA1 to nervous tissue development, homeostasis and response to acute or chronic injury.

Riassunto

L'attività di ricerca svolta nei tre anni di dottorato si è focalizzata sul ruolo della proteina AMBRA1 nel cervello di topo in condizioni fisiologiche e patologiche. Il gene *Ambra1*, recentemente identificato, codifica per una proteina di grandi dimensioni (circa 130 kDa), contente un dominio WD40 nella porzione ammino-terminale (Fimia *et al*., 2007). Durante lo sviluppo embrionale AMBRA1 è altamente espressa nel sistema nervoso centrale e periferico. Inoltre gli embrioni omozigoti per la mutazione nel gene *Ambra1* muoiono durante lo sviluppo embrionale prima del sedicesimo giorno e mostrano una severa compromissione nella chiusura del tubo neurale caratterizzata da iperplasia del neuroepitelio ed esencefalia (Fimia *et al*., 2007; Cecconi *et al*., 2008). Questo fenotipo sembra associato a un mancato equilibrio tra apoptosi e proliferazione cellulare ed a una disfunzione nella regolazione dell'autofagia. Il coinvolgimento di AMBRA1 nella regolazione dell'autofagia è suggerito dalla sua interazione con Beclin1, il cui ruolo nel meccanismo autofagico è stato ben caratterizzato. In particolare, AMBRA1 sembra svolgere un ruolo essenziale per stabilizzare il legame molecolare tra Beclin1 and VPS34, interazione fondamentale affinché venga indotta la formazione dell'vacuolo autofagico (o autofagosoma). Quest'ultimo è costituito da una doppia membrana all'interno della quale si accumula il materiale che verrà degradato dagli enzimi lisosomiali, in seguito alla fusione dell'autofagosoma con il lisosoma (Fimia *et al*., 2007, Mizushima *et al*., 2002). L'autofagia è un sistema di degradazione in cui porzioni di citoplasma e organelli danneggiati vengono degradati all'interno dei lisosomi (Wang e Klionsky, 2004). Le vie autofagiche maggiormente caratterizzate nel sistema nervoso sono l'autofagia mediata da chaperoni e la macroautofagia. Quest'ultima è attiva costitutivamente nei neuroni e, studi recenti, hanno dimostrato che è un meccanismo cellulare essenziale per l'omeostasi neuronale. Infatti topi mutanti per geni autofagici del gruppo degli *Atg*, come ad esempio *Atg5* o *Atg7*, mostrano disturbi nel comportamento e dell'attività motoria associati a neurodegenerazione, con perdita delle cellule del Purkinje nel cervelletto e dei neuroni piramidali dell'ippocampo. I neuroni che sopravvivono nei mutanti presentano aggregati proteici con formazione di inclusioni tossiche (Hara *et al*., 2006; Komatzu *et al*., 2006). Il ruolo essenziale del meccanismo autofagico per la sopravvivenza neuronale, e dunque il ruolo di AMBRA1 nella regolazione di tale meccanismo, ci hanno spinto ad analizzare l'espressione di AMBRA1 nel cervello di topo adulto in condizioni fisiologiche e patologiche.

I principali risultati ottenuti durante la mia attività di ricerca e descritti in questa tesi possono essere riassunti come segue:

1. Con questo studio è stata ottenuta la prima mappa neuronanatomica, istologica e ultrastrutturale dell'espressione della proteina AMBRA1 nel cervello di topo. Per raggiungere tale risultato ci siamo avvalsi di un approccio morfologico utilizzando tecniche di immunoistochimica, immunofluorescenza e immunolocalizzazione

ultrastrutturale. Dallo studio effettuato è emerso che AMBRA1 è ampiamente espressa in tutto il cervello, mostrando una localizzazione prevalentemente neuronale, infatti astrociti e microglia mostrano livelli più bassi della proteina, suggerendo che AMBRA1 sia essenziale per l'omeostasi neuronale. L'analisi della localizzazione intracellulare ha mostrato come all'interno del neurone la proteina sia associata al reticolo endoplasmatico, coerentemente con i dati descritti in letteratura (Di Bartolomeo *et al*., 2010). L'analisi dettagliata delle differenti regioni cerebrali ha rivelato che l'espressione di AMBRA1 può variare nelle diverse regioni cerebrali, nelle popolazioni e nei sottotipi neuronali. La concentrazione della proteina può essere correlata parzialmente al volume cellulare, infatti i neuroni caratterizzati da un corpo cellulare grande, dendriti ramificati e lunghi assoni mostrano alti livelli di AMBRA1 rispetto a neuroni di dimensioni inferiori. Tra le cellule di grandi dimensioni, le cellule mitrali nel bulbo olfattivo, i neuroni piramidali giganti nella corteccia, i neuroni motori del tronco encefalico e le cellule di Purkinje rappresentano chiari esempi. Inoltre diversi studi hanno dimostrato un'elevata espressione di molecole pro-autofagiche nei su citati tipi cellulari (Tamura *et al*., 2010), suggerendo che le loro richieste metaboliche implichino alti livelli di autofagia. Inoltre possiamo ipotizzare che il contenuto di AMBRA1 sia correlato ad altri parametri come la suscettibilità dei neuroni ad insulti cronici e acuti. In particolare alcune popolazioni neuronali che vengono risparmiate durante certi insulti neurodegenerativi contengono alti livelli di AMBRA1 in condizioni fisiologiche. Esempi rappresentativi possono essere considerati gli interneuroni colinergici nel corpo striato, preservati nella malattia di Huntington (HD) e i neuroni dello strati piramidale del CA3 nell'ippocampo resistenti alla malattia di Alzheimer (AD) e al danno ischemico. E' importante notare come in queste patologie sia stato descritto un'inefficiente regolazione del meccanismo autofagico. Comunque in alcuni casi neuroni contenenti alti livelli di AMBRA1 vengono danneggiati durante alcune patologie neurodegenerative, come ad esempio le cellule mitrali e le cellule di Purkinje negli animali *pcd* (Purkinje cell death). In questa sindrome si assiste ad una morte cellulare caratterizzata da figure autofagiche, probabilmente legata ad una disfunzione dell'autofagia che coinvolge direttamente AMBRA1.

2. Nella seconda parte della mia attività di ricerca mi sono focalizzata sullo studio dell'espressione di AMBRA1 durante l'invecchiamento fisiologico e patologico, utilizzando un modello transgenico per la malattia di Alzheimer (Tg2576), considerando in particolare le aree della neocorteccia e della formazione ippocampale, zone fortemente suscettibili agli insulti neurodegenerativi.

I risultati ottenuti hanno evidenziato delle differenze nell'espressione di AMBRA1 correlate all'età considerata, al genotipo e alla regione cerebrale. In dettaglio nella neocorteccia i livelli di proteina osservati a 3 mesi di età nell'animale WT sono più alti rispetto alla controparte transgenica. Questo dato fa pensare che AMBRA1 sia essenziale per la corretta maturazione dei neuroni neocorticali e che AMBRA1 possa essere rilevante all'esordio della patologia, dati i livelli relativamente più

bassi nella neocorteccia dell'animale Tg2576 in questa fase in cui sopraggiungono i primi deficit neuronali, legati ad una *down-regolazione* della plasticità sinaptica.
Nelle età successive nel genotipo transgenico i livelli di AMBRA1 si mantengono piuttosto stabili, mentre nella condizione fisiologica, si assiste ad una diminuzione della proteina, che riflette una attività cellulare bilanciata, caratterizzata da una stabile regolazione dei meccanismi di sintesi e di degradazione. Il successivo incremento nei soggetti anziani, indipendentemente dal genotipo, indica un induzione dell'espressione di AMBRA1 durante l'invecchiamento cerebrale caratterizzato da modificazioni cellulari legate all'aumentare dello stress ossidativo e al conseguente accumulo di organelli danneggiati. Inoltre sono state individuate differenze nell'espressione di AMBRA1 relative alla regione considerata. Infatti nell'ippocampo si assiste ad un generale decremento con l'età in tutti e due i genotipi, anche se nell'ippocampo dell'animale transgenico anziano si assiste ad un decremento più accentuato rispetto alla controparte WT, suggerendo una maggiore suscettibilità di questa zona durante l'invecchiamento patologico. Un dato rilevante risulta dall'analisi morfologica della formazione ippocampale degli animali giovani, in cui nonostante l'analisi biochimica non abbia mostrato differenze tra i livelli di espressione nei due genotipi, la distribuzione di AMBRA1 è differente nelle sottoregioni ippocampali, in particolare la regione neurogenica del giro dentato è più immunoreattiva nel Tg2576 rispetto al WT, suggerendo un coinvolgimento di AMBRA1 nella proliferazione e nel differenziamento cellulare come risposta ai deficit iniziali.
3. Lo studio è stato esteso all'analisi dell'espressione di AMBRA1 mediante approccio morfologico-quantitaitivo in modelli di malattia di Parkinson presintomatico. In particolare sono stati utilizzati due modelli: i) un modello farmacologico in cui abbiamo utilizzato una molecola tossica (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) specifica per i neuroni dopaminergici; ii) un modello genetico che presenta una mutazione in un gene appartenente al sistema di riparo del DNA definito Nuclear Excition Repair (NER). In entrambi i modelli l'insulto tossico induce un aumento dell'espressione di AMBRA1 nei neuroni dopaminergici. Interessante è che l'induzione avviene nei neuroni dopaminergici sia nelle aree colpite dalla patologia (Substantia Nigra, SN) sia in quelle risparmiate (Ventral tegmental area). Questo dato suggerisce che l'induzione dell'autofagia nelle due zone porta a risoluzioni diverse, dal momento che la morte cellulare dei neuroni dopaminergici nella zona affetta (SN) sembra, secondo recenti studi, avere caratteristiche autofagiche.
In conclusione i risultati ottenuti suggeriscono che AMBRA1 sia una molecola fondamentale nell'omeostasi del tessuto nervoso, data la sua abbondante espressione nelle aree cerebrali. Inoltre possiamo concludere che la modulazione dell'espressione può essere critica per l'*onset*, la progressione e la risposta cellulare durante i processi neurodegenerativi.

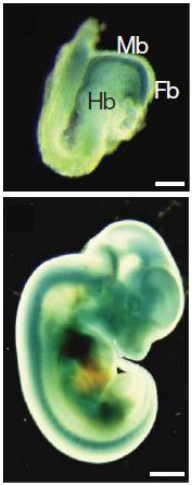
Section I: Introduction and Objectives

Chapter 1

Ambra1, autophagy and the nervous system

1.1 Ambra1

Ambra1 ("Activating Molecule in Beclin 1 Regulated-Autophagy") is a recently identified gene encoding for a large protein (around 130 kDa), with an N-terminal WD40 domain (Fimia et al., 2007). *Ambra1* is localized on chromosome 1 in mouse and on chromosome 11 in humans, it is highly conserved among vertebrates, and is expressed in different splicing isoforms.



In the developing mouse, AMBRA1 protein shows abundant expression in the central and peripheral nervous system. At embryonic day 8.5 (E8.5), AMBRA1 is present in the neuroepithelium, while at E11.5 protein expression is localized to the ventral-most part of the spinal cord, the encephalic vesicles, the neural retina, the limbs and the dorsal root ganglia (Fig.1.1). This distribution pattern suggests that AMBRA1 is centrally involved in cell proliferation and differentiation in the developing nervous system. Indeed, Ambra1$^{gt/gt}$ mice, which was obtained by gene trapping technique and displays a deficient expression of the protein, show early and severe neuropathological features, including neuroepithelial hyperplasia and defective neural tube closure, leading to embryonic death (Fig.1.2) (Fimia et al., 2007; Cecconi et al., 2008).

Fig. 1.1 Expression of Ambra1 in the mouse embryonic midbrain (Mb), forebrain (Fb) and hidebrain (Hb). β-gal staining on whole-mount Ambra1$^{+/gt}$ mouse embryos at E8.5 (upper picture) and at E11.5 (lower picture) (Fimia et al., 2007). Bar 10 μm.

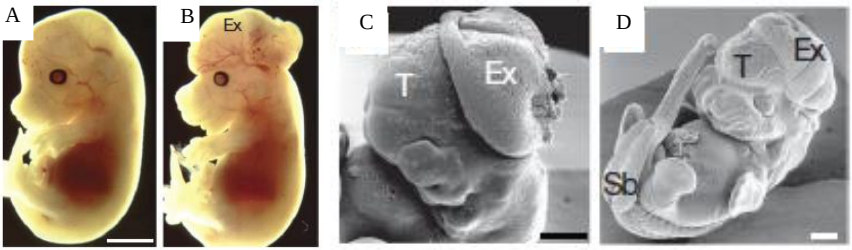


Fig.1.2 Anatomical features of Ambra1 deficient mice. A, B, Wild-type (A) and Ambra1gt/gt (B) embryos at E14.5 are characterized by prominent exencephaly (Ex) C, D, Scanning electron microscopic analysis of E11.5 (C) and E12.5 (D) Ambra1$^{gt/gt}$ embryos. Note the failure of the neural tube closure, the extensive midbrain/hindbrain exencephaly (Ex) with a closed telencephalon (T), and the lumbosacral spina bifida (Sb) (Fimia et al., 2007).(A), (B) 2 mm; (C), (D) 500 μm.

The reported disturbances to the nervous system in Ambra1$^{gt/gt}$ mice appear to derive to dysfunctions in the autophagic process, also resulting in an imbalance of apoptotic cell death and cell proliferation. These data, together with other *in vitro* evidence, allowed proving a regulatory role of AMBRA1 in autophagy activation in vertebrates (Fimia et a., 2007).
It has been demonstrated that AMBRA1 interacts with Beclin1 (Bcl2 intarcting protein), promoting its binding to lipid kinase Vps34, thus mediating autophagosome nucleation (Fimia et al, 2007). Taken together, AMBRA1, Beclin1, and Vps34 have been defined as the autophagy core-complex (He and Levine, 2010).
Autophagosome formation is primed by AMBRA1 release from the cytoskeleton. In fact, upon autophagy induction, AMBRA1-"dynein light chain 1" (DLC1) complex translocates from the microtubules to the endoplasmic reticulum (ER), thus enabling autophagosome nucleation (Di Bartolomeo et al, 2010). Dissociation of AMBRA1-DLC1 from the dynein complex requires phosphorylation of AMBRA1 by the serine/threonine kinase ULK1 (Di Bartolomeo et al., 2010) (Fig.1.3).

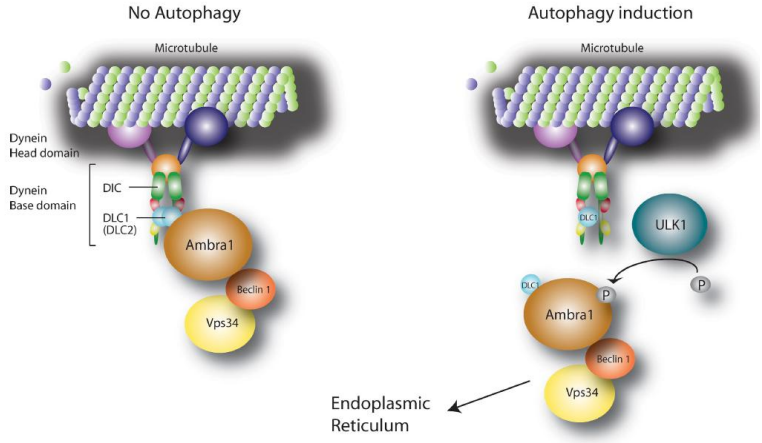


Fig.1.3 Proposed model of AMBRA1 dynamic interaction with the dynein motor complex during autophagy induction (Di Bartolomeo et al., 2010).

Beclin1/Vps34-mediated autophagy is negatively regulated through a interaction between Beclin1 and BCL-2. In this context, it is worth mentioning that recent studies showed that AMBRA1 is a new partner for BCL-2 in mammals and that their binding is independent of Beclin1 (Strapazzon et al., 2011).
As AMBRA1 and BCL-2 bind Beclin1 on the same site, the two proteins could be competitors. In addition, after autophagy induction, AMBRA1/Beclin1 interaction

increases, whereas the AMBRA1/BCL-2 interaction is disrupted. Altogether, these results led to propose a model in which, under normal conditions, a pool of AMBRA1 associates with BCL-2 in proximity of mitochondria, inhibiting its autophagic function; after autophagy induction, this mitochondrial pool of AMBRA1 separates from mito-BCL-2 and increases its binding to Beclin1 in order to favor the autophagic program (Strapazzon et al., 2011).
To this respect, it is worth mentioning that a recent study demonstrates an interaction between AMBRA1 and Parkin in dopaminergic neurons, occurring on the mitochondrial surface and leading to mitophagy induction (Van Humbeeck et al., 2011).

1.2 Autophagy

Cells have a constant need for the building blocks of life: amino acids, lipids, carbohydrates, and nucleic acids. To sustain this catabolic and anabolic needs, they rely on uptake and recycling. While nutrient uptake is important, different degradation systems are in place to efficiently degrade recyclable intracellular material and provide quality control.
The main pathways for protein degradation and recycling are the ubiquitin/proteasome pathway (for degrading short-lived cytosolic and nuclear proteins), the lysosomal pathway (for cytosolic proteolysis), and autophagy (for bulk cytosolic degradation and organelle recycling) (Rubinsztein et al., 2006). Deficits in any of these recycling routes can result in uncontrolled accumulation of cellular debris or severe deficiencies in metabolic productivity, ultimately causing cell death. The importance of these pathways is emphasized by a group of human metabolic disorders, often referred to as accumulation diseases, caused by impaired degradation machineries (Bi et al., 2010).
Autophagy is involved in the intracellular turnover of proteins and cell organelles, and has an important role in regulating cell fate in response to stress (Levine, 2005). It is a highly conserved process that occurs in all species and cell types studied thus far.
The term autophagy was coined in 1963 by Christian de Duve to establish a nomenclature for different cellular pathways and compartments in the endosomal-lysosomal pathway (Klionsky, 2008). Early studies performed in rat liver allowed to define the autophagic process as a physiological response to starvation, in order to degrade and recycle non-essential intracellular macromolecules (Deter et al., 1969). Later, the molecular mechanisms underlying autophagy were identified in yeast (Takeshige et al., 1992; Tsukada et al., 1993). Subsequent identification of the mammalian homologues enhanced the interest of several research groups towards the investigation of the role of autophagy in tissue and organ homeostasis.
Two main types of mammalian autophagy have been identified: macroautophagy and chaperone-mediated autophagy (CMA) (Fig. 1.4).

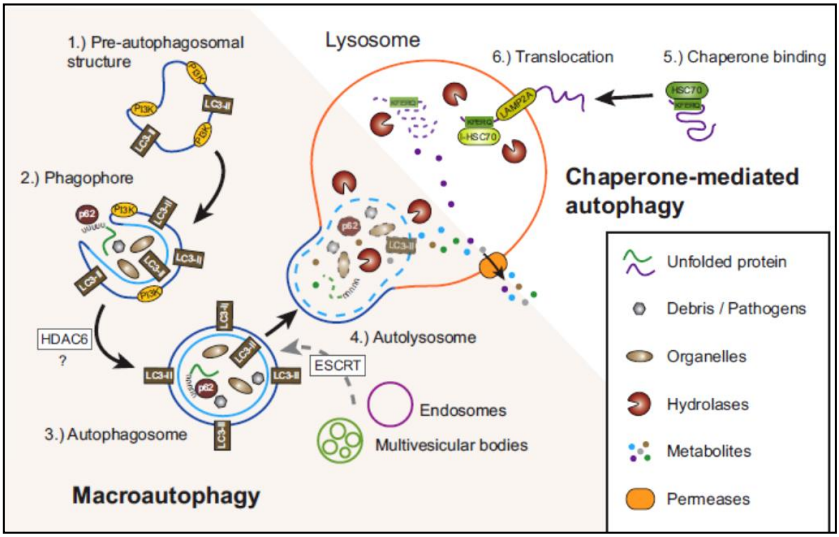


Fig.1.4 Steps in macroautophagy and chaperone-mediated autophagy.
Macroautophagy: 1.) Nucleation. A class III PI3K complex consisting of at least BECN1, PIK3C3, PIK3R4, UVRAG, and AMBRA1 is required for PAS (Pre-autophagosomal structure) formation and MAP1LC3 is anchored to the membrane via a phosphoethanolamine (PE) anchor (LC3-II). 2.) Expansion. This stage is also called "isolation membrane". More membrane and LC3-II is being recruited to the developing vacuole. 3.) Maturation. The exact nature and sequence of this maturation, and whether these steps are always required is currently unknown. The autophagosomal lumen becomes more acidified during this maturation. 4.) Docking and fusion. During docking and fusion the inner membrane compartment together with its content gets released into the lysosome/autolysosome and is being degraded by lysosomal hydrolases.
Chaperone-mediated autophagy: 5.) Recognition and binding. The HSC70 chaperone complex recognizes unfolded proteins with the KFERQ sequence and moves them to the lysosome. 6.) Translocation. LAMP2A and a lysosomal form of HSC70 (l-HSC70) translocate the substrate protein across the lysosomal membrane into the lumen for degradation. (Jaeger and Wyss-Coray, 2008).

Macroautophagy is a bulk degradation pathway and the only intracellular mechanism potentially capable of degrading large protein aggregates or damaged organelles. It is a well-understood process in yeast, but details about the exact sequence of events and the proteins involved are still uncertain in mammals. A cup-shaped isolation membrane forms around cytosolic components, eventually fusing to form a double membrane bound vesicle, the so-called “autophagic vacuole” (AV) (Mizushima et al., 2002).
The origin of the membrane material for the formation of the isolation membrane is still under investigation, but recent evidence suggests that it might derive from the ER (Axe et al., 2008). The protein MAP1LC3 is docked via conjugated phosphatidyl ethanolamine (MAP1LC3-II) to the isolation membrane and is a specific marker for the so-called autophagosome (Mizushima et al., 2004). The autophagosome undergoes several microtubule (Jahreiss et al., 2008) and dynein-dependent maturation events (Kimura et al., 2008). For successful degradation of

the autophagosomal content, autophagosomes need to migrate from their site of formation to lysosome rich perinuclear regions (Fass et al., 2006). After fusion with the lysosome, the outer autophagosome membrane can be reused, while lysosomal enzymes degrade the inner membrane and its cytosolic content, enabling the recycling of macromolecules (Kimura, 2007).
Large number of autophagy-related genes (ATG) have been identified over time and their nomenclature has been unified (Klionsky et al., 2003). Most of the gene products function at the step of autophagosome formation. Since the membrane dynamics of autophagy in yeast is quite similar to that in mammalian cells, these discoveries prompted the researchers to look for mammalian homologues of yeast ATG genes (Fig. 1.5).

Yeast	Human	Yeast	Human
Atg1	-	Atg15	Lipase
Atg2	-	Atg16	Atg16L1
Atg3	Atg3	Atg18	WIPI1
Atg4	-	Pep4	Proteinase A
Atg5	Atg5	PRB1	Proteinase B
Atg6	Beclin1	SEC18	NSF
Atg7	Atg7	VAM3	Sintassyne
Atg8	MAPLC3	VAM7P	SNAP-25
Atg9	Atg9A	VPS15	P150
Atg12	Atg12	VPS34	PI3KC3
Atg13	-	VPS38	UVRAG
Atg14	-	YPT7	Rab7

Fig. 1.5. Homologue genes in Homo sapiens of autophagic genes discovered in yeast (modified from Levine and Klionsky 2005).

This allowed manipulations of autophagy (by knocking down or overexpressing ATG genes) to elucidate its contribution to different physiological and pathological processes. These molecules can be used as specific probes for autophagy (Mizushima, 2004).
Chaperone-mediated autophagy (CMA) differs from macroautophagy in that no vesicular trafficking is involved (Fig. 1.4). Instead, a pentapeptide motif in substrate proteins allows their specific translocation through the lysosome membrane (Majeski and Dice, 2004). Thus, CMA degrades only proteins with the motif KFERQ or a biochemically related sequence, which is present in about 30% of all cytosolic proteins (Chiang and Dice, 1988).

1.3 Autophagy in the nervous system.

The two main types of autophagic processes, i.e., CMA and macroautophagy, importantly contribute to degradation of cytosolic components in the nervous tissue. The selective nature of CMA makes it the ideal system for removing misfolded proteins when refolding is not possible; in fact oxidized and misfolded CMA substrates are more readily degraded through this autophagic pathway (Finkbeiner et al., 2006). CMA declines with age because of a decrease in the levels of lysosome-associated membrane protein (LAMP) type 2A, a lysosomal receptor for this pathway. This favours the accumulation of CMA substrates, possibly causing damage to the neural tissue (Massey et al., 2006).
Macroautophagy components are expressed in neurons and neuronal cell lines. While the function of autophagy-related proteins has been described for some, it is still unknown for others. (Jaeger et al, 2009).
Recent studies show that macroautophagy is constitutively active in healthy neurons and is vital to cell survival (Bolland and Nixon, 2006). For this reason and for the relatively non-specific nature of this process, responsible for bulk cytoplasmic turnover, the term macroautophagy will be referred to as autophagy.

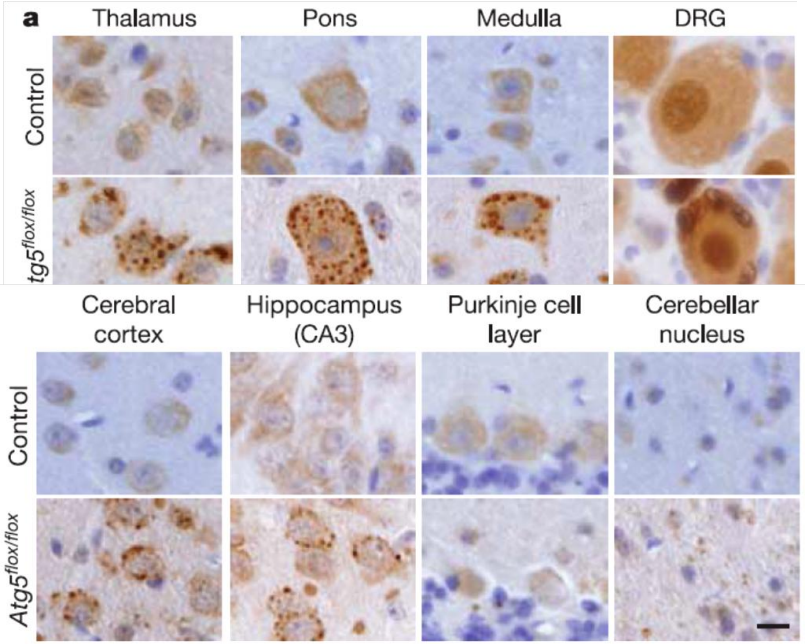


Fig.1.6 Ubiquitin-positive inclusion in Atg5-deficient neurons. Immunoistochemistry of brain sections from control ($Atg5^{flox/+}$; nestin-Cre) and $Atg5^{flox/flox}$; nestin-Cre ($Atg5^{flox/flox}$) mice at six weeks of age (Hara et a., 2006).

Mice lacking either *Atg5* or *Atg7* genes exhibit motor and behavioral deficits as well as degeneration and loss of Purkinje cell neurons in the cerebellum and pyramidal neurons in the hippocampus (Hara et al., 2006; Komatsu et al., 2006).
Diffuse protein aggregates appear in surviving neurons within these brain regions and others, culminating in the formation of toxic inclusion bodies.
Because neuronal proteasome activity is not reduced in these mouse models, the results imply that autophagy is normally responsible for clearing aggregated proteins that are not degraded by the proteasome (Rideout et al., 2004). Moreover, constitutive clearance of cytosolic proteins by low-level basal autophagy has an important cytoprotective function, particularly in neurons, as evidenced by accumulation of ubiquitinated proteinaceous deposits (Fig. 1.6) and development of neurodegeneration in mice deficient for basal autophagy (Hara et al., 2006; Komatsu et al., 2006).
Further insights into the dynamic and essential mechanisms of autophagic process in neurons came when several studies characterized autophagosome-related compartments, their retrograde transport, and progressive maturation and fusion with lysosomes along neuritic processes (Hollenbeck, 1993). These investigations led to robust evidence showing that constitutive neuronal autophagy may serve as both a key mechanism for remodeling neurite and growth cone structure during neurite extension, as well as a neuroprotective mechanism by removing damaged proteins and organelles that may otherwise accumulate within axons (Hollenbeck, 1993). More recent papers suggest that autophagy might contribute to the homeostasis and maintenance of the axons (Gumy et al., 2010). Neuronal homeostasis essentially depends on balanced, bidirectional trafficking of intracellular constituents between distal neurites and the cell soma. In neurons, autophagosomes and endosomes that fuse in the distal axon must be retrogradely transported to the soma, often over great distances, in order to fuse with lysosomes and degrade their contents (Yue, 2007). Thus, subtle disruptions of autophagosome formation, maturation, or trafficking would be predicted to have dire consequences for autophagic flux and neuronal homeostasis.
The fundamental role of autophagy in cell homeostasis explains its importance in development. Ambra1$^{gt/gt}$ mutants show letal neural tube defects, as mentioned earlier (Section 1.1). These phenotypes have been linked to defective neural progenitor death, supporting the function of autophagy as a cell death pathway (Clarke, 1990).
During ageing, the autophagy-lysosome system undergoes striking changes. Ageing leads to reduction in autophagosome formation and autophagosome-lysosome fusion, both of which are consistent with decreased autophagy. There are also notable changes in lysosomes, such as increased volume, decreased stability, altered activity of hydrolases, and accumulation of the indigested material in the form of lipofuscin (Terman and Brunk, 2004). The precise molecular defects remain unknown, but these changes correlate with a decrease in the total capacity

for degradation of long-lived proteins in nervous tissue of aged animals (Donati et al., 2001).
Autophagic dysfunction may also lead to defective turnover of mitochondria, which results in the accumulation of older mitochondria, which generate increased levels of ROS, especially in the microglia. In turn, ROS activate redox-dependent transduction cascades and transcription factors, which induce the expression of inflammatory genes and exacerabate the consequences. Therefore, "microglia-ageing" could function as a major driver for brain ageing. These evidence led to hypothesize that prevention of lysosomal autophagic dysfunction and mitochondrial DNA damage in microglia may be a potential novel therapeutic targets against brain ageing (Nakanishi and Wu, 2009).
In neurons, the consequences of age-related decline in autophagy are diminished turnover of intracellular components and reduced ability of cells to adapt to changes in the extracellular environment (Ward, 2002). Compromised clearance of old and/or damaged organelles (as mitochondria or peroxisomes) by autophagy coupled with a reduced turnover of long-lived proteins likely contribute to the intracellular accumulation of oxidized proteins in aged organisms (Kim et al., 2007). This age-related decline in autophagy may be particularly detrimental to the nervous system, as postmitotic cells such as neurons are vulnerable to the accumulation of undegraded metabolic products over the lifetime of the organism (Terman, 1995). Indeed, evidence is mounting that integrity of the autophagosomal-lysosomal network appears to be critical in the progression of ageing, indeed it is now largely accepted that autophagy affects several cellular activities crucial for longevity and healthy ageing (eulongevity), particularly in the context of neurodegenerative disease states.

1.3.1 Autophagy and neurodegenerative disorders.

Abnormal autophagy may be involved in the pathology of both acute brain injuries and chronic nervous system disorders, including proteinopathies, as Alzheimer's and Parkinson's diseases (Fig.1.7).
Alzheimer's disease (AD) is one of the most common age-related neurodegenerative disorders. It is the prevalent form of dementia, characterized by progressive cognitive dysfunction, together with behavioral and neuro-psychiatric disturbances. The pathogenesis of AD is highly complex, involving both genetic and environmental factors. From a neuropathological point of view, AD features progressive loss of neurons and synapses, intracellular neurofibrillary tangles, composed of hyperphosphorylated Tau protein, extracellular deposition of of senile plaques and cerebral amyloid angiopathy. The main constituents of senile plaques are amyloid β peptides (Aβ), which are generated from amyloid precursor protein (APP) by sequential proteolytic cleavage, mediated by β- and γ-secretases (Bianchi et al., 2011).

Recent evidence has shown that Aβ is generated during autophagic turnover of APP-rich organelles supplied by both autophagy and endocytosis.
Aβ generated during normal autophagy is subsequently degraded by lysosomes. In AD, the maturation of autophagolysosomes and their retrograde transport are impeded, leading to a massive accumulation of 'autophagy intermediates (namely, AVs, see section 1.2) within large swellings along dystrophic and degenerating neuritis (Fig.1.7). The combination of increased autophagy induction and defective clearance of Aβ–containing AVs creates conditions favorable for Aβ accumulation in AD (Nixon, 2007).
PD is the most common neurodegenerative movement disorder and the second most common neurodegenerative disease. Most of the PD cases are sporadic, although familial PD with autosomal dominant or autosomal recessive mutations also account for about 5% of all PD cases.

Disease	Autophagosomal phenotype
Alzheimer disease	Autophagy appears impaired, autophagosomes accumulate, endosomal-lysosomal abnormalities, increased mitophagy, reduction of macroautophagy enhances pathology, pharmacological activation of macroautophagy can promote the clearance of Aβ/APP and reduces tau pathology, autophagosomes contain APP/Aβ/secretases.
Parkinson disease	Autophagy/mitophagy appears impaired, autophagosome-like structures accumulate, pharmacological activation of macroautophagy enhances α-synuclein clearance and is neuroprotective, α-synuclein is a target of CMA and macroautophagy and the proteasome, dopamine-modified/mutated α-synuclein blocks CMA and dopamine induces autophagic cell death and α-synuclein accumulation, mutant UCH-L1 binds to LAMP2A and inhibits CMA.
Huntington diseases	Impaired sorting/degradation of autophagosomes, autophagosomes accumulate, BECN1 is recruited to htt inclusions and BECN1 reduction causes enhanced htt accumulation, pharmacological or signaling mediated activation of macroautophagy reduces htt toxicity, mTOR is sequestered into htt inclusions, which causes macroautophagy activation.
Frontotemporal dementia	Impaired endosome maturation, enlarged autophagosome accumulation, mutant CHMP2B disturbs the ESCRT-III complex for endosomal sorting which results in polyU/SQSTM1 aggregates.
Amyotrophic lateral sclerosis	Impaired early endosomes, impaired sorting/degradation of autophagosomes, CHMP2B disturbs the ESCRT-III complex for endosomal/MVB sorting which results in polyU/SQSTM1 aggregates, MVBs are required for TDP-43 clearance, Lithium activates protective autophagy.

Fig.1.7 Autophagy in common chronic neurodegenerative diseases (modified from Jaeger et al., 2009).

PD patients suffer from resting tremor, bradykinesia, muscle rigidity and postural instability. The deterioration of motor functions observed in PD patients is predominantly attributable to the degeneration of dopaminergic neurons in the substantia nigra, showing that the nigrostriatal circuit is involved in neurodegeneration (Zelda and Nancy, 2009).

On a cellular level, neuronal loss is accompanied by neurite degeneration and the presence of cytoplasmic inclusions known as Lewy bodies, involving α-synuclein aggregation. In PD, the autophagic pathway participates in the degradation of α-synuclein, as well as in the turnover of damaged mitochondria, a hallmark of disease (Zelda and Nancy, 2009). Indeed, a striking increase of oxidative stress related to dysfunction of mitochondrial metabolism is known to occur. This last feature is crucial for dopaminergic neurons, because these cells have distinct redox properties, that make them particularly keen to oxidation, even in normal conditions, compared to other neuronal subtypes (Horowitz et al., 2011).

Despite mounting observations that are in support of a protective role of autophagy in various models of PD, some essential issues remain unsolved. While it is perceivable that insufficient autophagy activation would impair clearance of protein aggregates and dysfunctional mitochondria, whether excessive activation of autophagy occurs in PD, and whether it plays a role in PD pathology remains unknown. This question is particularly important since excessive activation of autophagy is associated with neuronal loss (Bredesen et al., 2006; Zelda and Nancy, 2009).

Huntington's disease (HD) is an age-related neurodegenerative disorder, characterized by motor and cognitive impairment, and caused by mutations in the gene encoding for huntingtin protein (htt). The mutated protein contains abnormally long sequences of polyglutamine, resulting in the formation of intranuclear ubiquitinated inclusions. Mutated htt also accumulates in autophagic compartments, in amounts proportionate to the length of its polyglutamine tract, suggesting that its degradation by autophagy is impeded (Kegel et al., 2000). Importantly, stimulating autophagy by rapamycin treatment reduces htt accumulation and neurodegeneration in cell and fly models of polyglutamine disease and reduces neurological deficits and htt aggregation in a mouse model of HD (Ravikumar et al., 2004). By contrast, inhibiting the formation of autophagosomes or impeding their fusion with lysosomes increases htt aggregation in cells *in vitro* and *in vivo* (Ravikumar et al., 2005).

Under certain conditions, induction of autophagy can be even detrimental in neurons. In some paradigms, massive activation of autophagy can result in self-digestion of the whole cell, leading to cell death. This process has been termed type II programmed cell death, in order for it to be distinguished from apoptotic pathways (Bursch, 2001; Gozuacik and Kimchi, 2004).

In the Lurcher mouse model of cerebellar degeneration, autophagic neuronal death was implicated as mediating the pathological effects of mutations in the glutamate receptor subunit GluR2 (Fig. 1.9; Florez-McClure et al., 2004). These findings may have broad implications regarding the possibility that autophagy may be a common

mediator of cell death initiated by excitotoxicity, but this remains to be established (Orr, 2002).

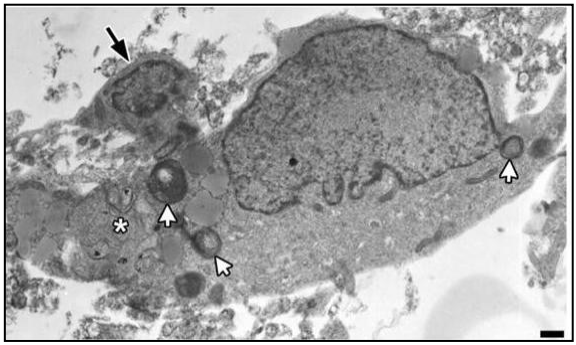


Fig.1.9 Trasmission electron microscopy of Purkinje dying neurons, showing ultrastructural features of autophagy. Autophagic vacuoles are indicated by open arrows (Florez-McClure et al., 2004).

However, a general role for autophagy in neuronal death remains speculative. In most instances in which autophagic morphology has been found to accompany neuronal cell death, it remains indeterminate whether autophagy is the culprit, or is induced secondarily to facilitate the removal of cellular components, or is induced as a cytoprotective response to cellular stress. It is quite possible that autophagic induction has the potential to be either protective or destructive, and the influence of autophagy depends on the type, degree and duration of the inciting cellular stress. (McCray and Taylor, 2008).

Chapter 2

Objectives

AMBRA1 is a recently discovered protein, centrally involved in nervous system development, regulating cell proliferation and autophagy (Fimia et al., 2007). Indeed *Ambra1* deficient embryos shows severe abnormalities, including neural tube defects, leading to early embryonic death.

These findings prompted us to investigate the expression of AMBRA1 in adult mouse CNS, in physiological and pathological conditions.

The primary aim of this study was to analyse the distribution of AMBRA1 protein in the brain, with special reference to the cerebral region considered, to the cell type, to the neuronal population, and to the intracellular compartment. These issues were addressed by morphological approaches, namely immunohistochemistry, immunofluorescence, and immunoelectron microscopy, which in our view are especially suitable to the study of the nervous tissue, for its highly complex organization and heterogeneous composition. As a preliminary step, we screened a panel of custom and commercial antibodies against AMBRA1 protein, to identify the most efficient one for morphological localization purposes. We then performed a systematic histological examination of AMBRA1 immunoreactivity in serial, coronal and sagittal sections of normal adult mouse brain. This approach allowed us to obtain a detailed map of the protein in the different brain territories along the rostro-caudal axis.

Based on this overview, showing some highly immunoreactive areas and others weakly AMBRA1-positive, our next aim was to examine, within each region, protein expression in the different neuronal populations, in the attempt to correlate AMBRA1 content with special features of specific neural cells. To this purpose, different neuronal markers, including tyrosine hydroxylase (TH) for dopaminergic neurons, glutamic acid decarboxylase (GAD) for GABAergic neurons, choline acetyl transferase (ChAT) for cholinergic neurons, were used in combination with AMBRA1, to perform double immunofluorescence experiments. In addition to these light and confocal microscopy studies we selected performed immunoelectron microscopy to characterize the intraneuronal localization of AMBRA1 with ultrastructural resolution to gather crucial information about the function of this protein.

Parallel to this scrutiny, the presence of AMBRA1-immunoreactive glial cells was investigated in different brain areas, using glial fibrillary acidic protein (GFAP), as an astroglial marker, and ionized calcium-binding adaptor molecule1 (Iba1), as a microglial marker.

According to our preliminary interpretation of the above results, heterogeneous expression of AMBRA1 in the various areas of forebrain, midbrain, and hindbrain could be related to the different cell metabolic and turnover request, as well as to susceptibility/resistance of specific neuronal subsets to chronic or acute injury. To

further investigate this hypothesis, we chose a number of neurodegeneration paradigms, including normal ageing, Alzheimer's disease (AD), Parkinson's disease (PD), and genomic instability mouse models. Importantly, in all these physiological and pathological conditions, autophagy, which is demonstrably regulated by AMBRA1, acts as a protective or detrimental process.

Concerning the study of possible age-related variations in AMBRA1 levels, we focused on the neocortex and hippocampal formation, i.e., the primarily affected areas in the ageing brain. To this aim, we analyzed 3-, 6-, 12- and 18-month-old mouse brain by immunoblotting and immunohistochemistry, as means for obtaining both quantitative data and analytical information on protein expression.

Parallel to this investigation, we analyzed by the same approaches the AD-like ageing model, at the same time points. For this part of the project, we chose a transgenic mouse strain (Tg2576), overexpressing the human isoform of amyloid precursor protein, carrying the Swedish mutation. This model was generated by Hsiao et al (1996) and was selected because it closely recapitulates human pathology in time-dependent manner (Jacobsen et al., 2006). In this model, neuronal dysfunction occurs before the accumulation of β-amyloid-containing plaques and patent neurodegeneration. Indeed neuronal deficits in Tg2576 mice can be temporally clustered into early deficits observed in 3 to 5-month-old animal and late deficits observed in animals older than 12 months (D'Amelio et al., 2010; Jacobsen et al., 2006). For a further understanding of age-, genotype-, and brain region-dependent variations in AMBRA1 levels, we extended the study by analyzing the expression pattern of its main interactor, i.e. Beclin1, whose function in autophagy regulation is well established.

We then addressed the issue of a putative role of AMBRA1 in PD, given the close relationship between the disease and autophagy mechanisms, particularly in the degradation of α-synuclein and the turnover of mitochondria. We were particularly interested in early PD stages, to get an insight into the contribution of the protein at the onset of disease. To this aim, we utilized a pharmacological murine model of PD based on the administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Khun et al., 2003). To analyze AMBRA1 expression in relation to the pathological phenotype, we took advantage of a quantitative-morphological approach to measure TH and AMBRA1 levels in nigrostriatal circuits.

To obtain further insights into the role of AMBRA1 during the neurodegenerative processes in the dopaminergic system associated with ageing – which constitutes the major risk factor for PD - we extended our investigations to mouse models with defective DNA Nucleotide Excision Repair (NER), which corrects a broad spectrum of distortion in the DNA double helix. Defects in the NER repair system have been associated with accelerated ageing, with a phenotype that correlates with the severity of the mutation. In our studies, we used a mutant strain with a truncation in the gene coding for the Excision Repair Cross Complementation Group 1 (ERCC1) protein. We demonstrated that in this particular model, which

exhibits only a mild phenotype, dopaminergic neurons of the nigrostriatal circuits show alterations resembling those observed in the MPTP model during the early stages of the pathogenesis. For these reasons, we examined expression of AMBRA1 protein in dopaminergic neurons from ERCC1$^{\Delta/+}$ mouse mutants.

Section II: Results

Chapter 3

Distribution of AMBRA1 in mouse brain

In order to examine AMBRA1 protein distribution in adult mouse brain we took advantage of several morphological techniques, including immunohistochemistry (IHC), immunofluorescence (IF) and pre-embedding immunoelectron microscopy (IEM) on the brain sections obtained from adult mouse

3.1Immunohistochemical and immunofluorescence analysis.

As a first step, we investigated AMBRA1 presence in neuronal and glial cell populations. Double IF experiments, performed in different brain areas, using anti-AMBRA1 in combination with either anti-neuronal nuclei (NeuN, neuronal marker), or glial fibrillar acid protein (GFAP, astrocytic marker), or ionized calcium-binding adaptor molecule 1 (Iba1, microglia marker), show that AMBRA is more abundant in neurons, with respect to glial cells (Fig. 3.1). Interestingly, while the vast majority of neurons demonstrably contains AMBRA1, the protein is not distributed at the same levels in NeuN positive cells, prompting us to further investigate its distribution in different brain areas and neuronal subsets.

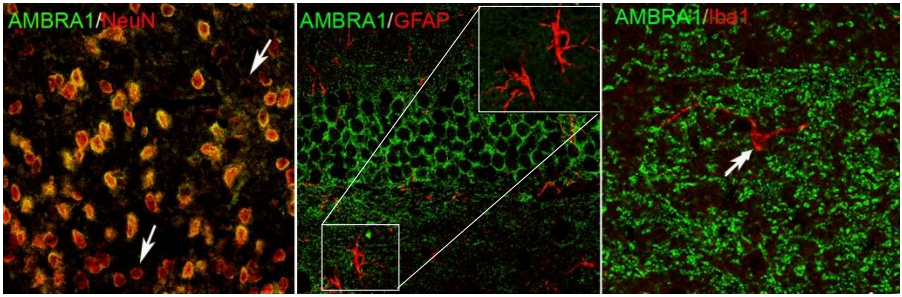


Fig.3.1 Double IF using anti-AMBRA1 (green signal) in combination with neural cell markers (red signal). The protein is abundantly expressed in neurons, although few NeuN-positive cells appear as AMBRA1-negative (arrows). GFAP-positive astrocytes and Iba1-positive microglia are mostly devoid of AMBRA1. (A) Neocortex; (B) Hippocampus; (C) Cerebellar cortex.

AMBRA1 is widely expressed in the forebrain, midbrain and hindbrain, with a generally higher concentration in the cortical area and in the caudal-most nuclei. The protein is present in most neurons of the archicortex (hippocampal formation), paleocortex (piriform cortex) and neocortex, even though the signal intensity varies among different brain regions, and within each area. A detailed description of AMBRA1 IHC distribution along the brain rostro-caudal axis is given below.
In the olfactory bulb, mitral cells, representing the major source of afferent input to the olfactory cortex, are strongly immunoreactive in their cytoplasm (Fig. 3.2A).

Other output neurons, namely tufted cells in the external plexiform layer (EPL), considered the smaller version of mitral cell (Shepherd, 1990) show a lower immunostaining degree. Interestingly, the targets of olfactory bulb, including the piriform cortex (especially pyramidal layer II), the olfactory tubercle and the insular cortex show remarkable staining (Fig. 3.2B-D).

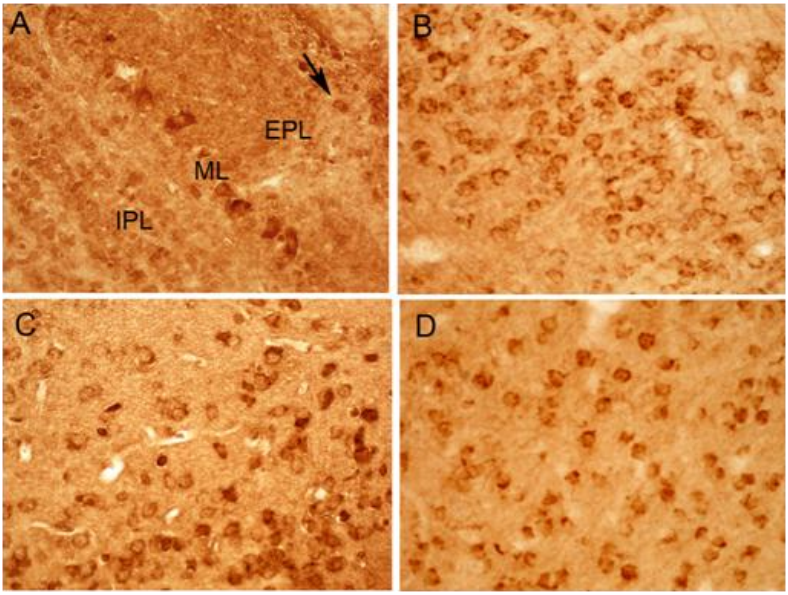


Fig.3.2 AMBRA1 IHC in coronal sections of mouse rhinencephalon. (A) Olfactory bulb, showing intense staining in mitral cell layer (ML) and weaker immunoreactivity in tufted cells (arrows). (B) Layer II of the piriform cortex, showing positive pyramidal neurons.
(C) Olfactory tubercle. (D) Insular cortex.

In the neocortex, pyramidal cells of layer V of the motor cortex show the strongest immunoreactivity (Fig. 3.3A-B). AMBRA1 richness in these giant spiny neurons, which are excitatory in function, could account for the presumed high organelle turnover, due to their size and plasticity. In the basal ganglia, the caudate putamen shows a differential AMBRA1 positivity in its various neuronal populations (Fig. 3.3C). While giant, aspiny interneurons are densely stained in their somata, medium-sized spiny projection neurons show faint immunoreactivity (Fig.3.3D).

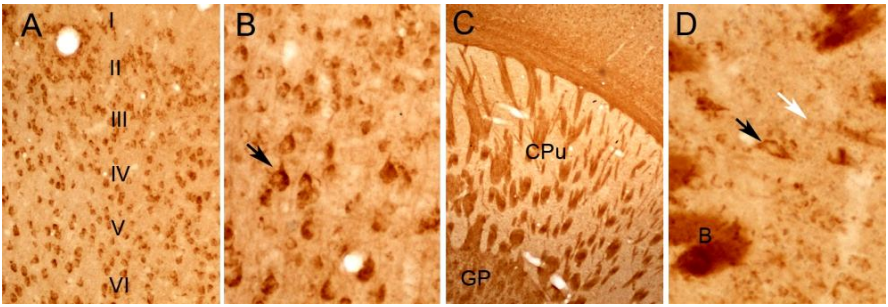


Fig. 3.3 AMBRA1 IHC in coronal sections of mouse telencephalon. (A) Overview of the neocortex, showing numerous positive neurons throughout the layers (I-VI). (B) Pyramidal cells of layer V of the motor cortex are strongly immunoreactive (arrows). (C) Differential staining in the caudate-putamen (CP) and globus pallidus (GP), showing immunoreactivity in both neurons and fiber bundles. (D) Giant aspiny neurons (black arrow) are intensely positive, while medium-sized spiny neurons are weakly immunostained (white arrow).

Even in these neurons, the presence of high protein levels is likely linked to their intense cytoplasmic turnover. Interestingly, axon bundles crossing the whole corpus striatum, representing part of the corticostriatal circuit, are intensely stained, suggesting association of a pool of AMBRA1 protein with axonal cytoskeletal components.

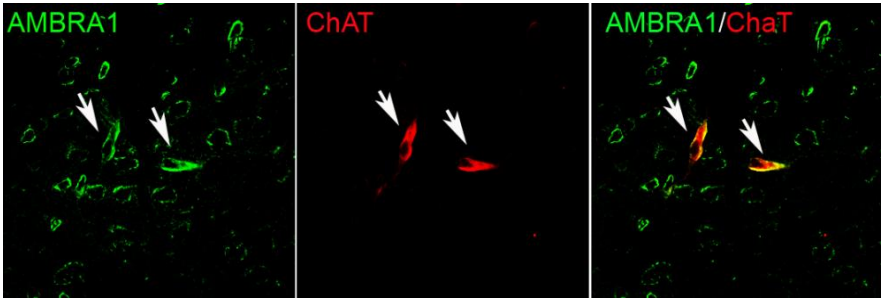


Fig.3.4 AMBRA1(green)/ChAT (red) double IF in the caudate putamen. AMBRA1 signal is especially intense in ChAT positive interneurons (arrows), while ChAT negative spiny neurons are only mildly AMBRA1-positive.

IF analysis of caudate putamen using choline acetyltransferase (ChAT), as a marker for cholinergic neurons, demonstrates high AMBRA1 expression in ChAT positive interneurons (Fig. 3.4), suggesting that in physiological conditions AMBRA1 regulated processes are fundamental for the health of these cells.
The hippocampal formation appears dyshomogeneously AMBRA1-positive (Fig. 3.5A). Interest in this structure arises from its involvement in major cognitive functions, and from its susceptibility to neurodegeneration.

IHC analysis demonstrates moderate staining levels in the pyramidal layer of the hippocampus proper. AMBRA1 pattern seems to follow gradual changes in morphology of pyramidal layers around the hippocampus. Indeed, proceeding from region CA1, where cell bodies are relatively small, to region CA3, where giant neurons are found, AMBRA1 immunoreactivity in pyramidal cells becomes more and more intense (Fig. 3.5B-C). Concerning interneurons, basket cells located in the stratum pyramidale/stratum oriens border are weakly positive, while other cells dispersed in the stratum radiatum and stratum lacunosum-moleculare are more densely labelled. Interestingly, the dentate gyrus (DG) is characterized by a gradient of AMBRA1 immunostaining, moving from the polymorphic layer/ granular layer border to the granular layer/molecular layer border (Fig. 3.5D).

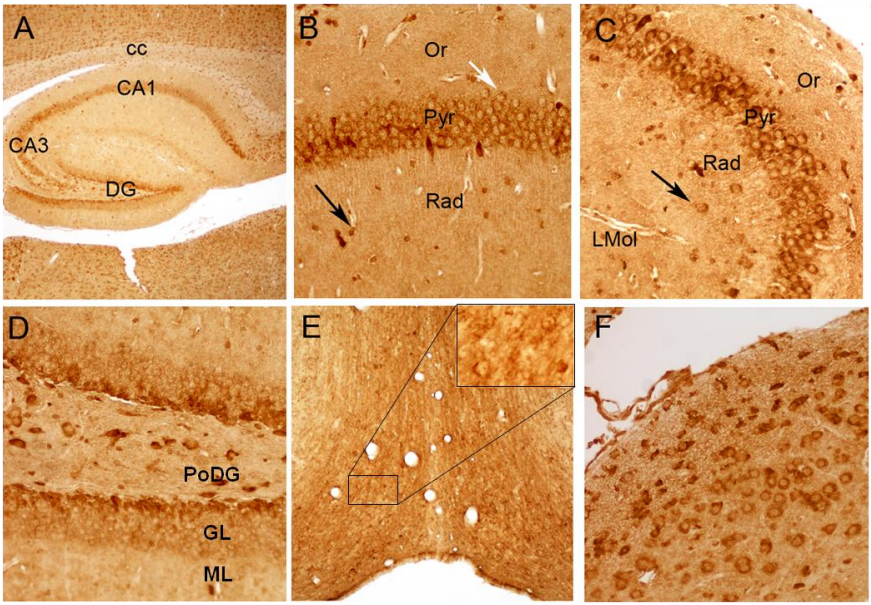


Fig. 3.5 AMBRA1 IHC in coronal sections of mouse telencephalon and diencephalon. (A) Overview of the hippocampal formation showing varying immunreactivity degrees in different fields and layers. (B) CA1 hippocampal region. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum. (C) CA3 hippocampal region. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum, LMol, stratum lacunosum-moleculare. (D) DG of the hippocampal formation. PoDG, polymorphic layer; GL, granule cell layer; ML, molecular layer. Arrow indicated a positive mossy cell. (E) Septal complex; inset shows AMBRA1 positive neurons and fibers. (F) Anterior thalamic nucleus.

The richest population may include neural stem cells, based on their morphology and distribution. In this view, the observed gradual changes in AMBRA1 content may reflect the cell differentiation status, that in this

neurogenic region is known to proceed in an inner-to-outer manner, from neural progenitors, to neuroblasts, to mature neurons (Bonfanti et al., 2011).
Since inputs of the hippocampal formation include terminations of septum and diagonal band (which preferentially project to CA3 and DG), we examined AMBRA1 distribution in the septal complex. Indeed, neurons in this area are stained in both their somata and their processes (Fig. 3.5E). Another central structure involved in learning and memory processes is the anterior thalamic nucleus. It is worth noting that this region receives inputs from the hippocampus, so being part of the hippocampo-diencephalic circuit. Interestingly, neurons belonging to the thalamic region and, markedly to anterior nucleus express high levels of AMBRA1 (Fig 3.5F).
The results of IF in the thalamus, using the glutamic acid decarboxylase (GAD67) as a marker for gabaergic neurons, shows colocalization with AMBRA1, even though the pro-autophagic protein is more widely expressed (Fig. 3.6).

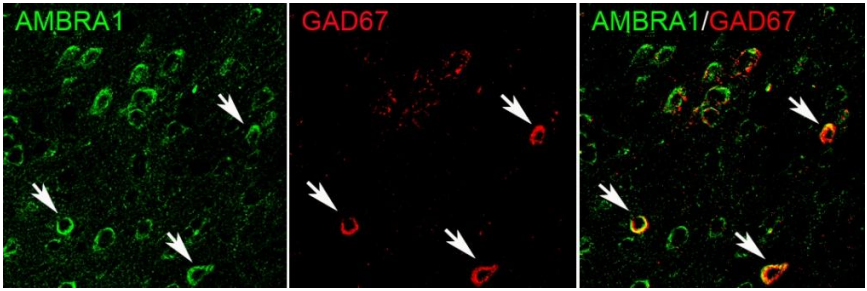


Fig.3.6 AMBRA1(green)/GAD67(red) double IF in the thalamus. Several AMBRA1-positive cells are observed, some of which are also GAD67-positive (arrows).

In the mesencephalon, select regions contain several immunoreactive neurons (Fig. 3.7A). Among these, the deep gray layer of the superior colliculus, red nucleus, locus ceruleus, and oculomotor nucleus (Fig. 3.7B-E).

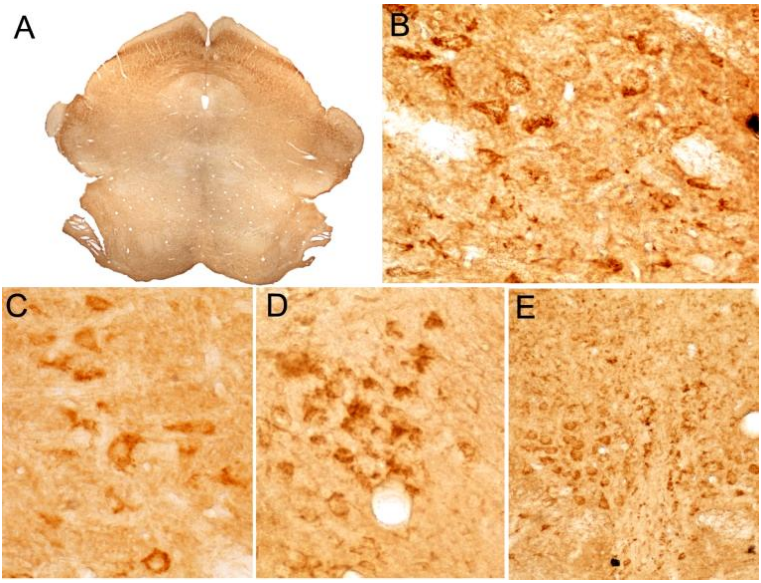


Fig.3.7AMBRA1 IHC in coronal sections of mouse mesencephalon. (A) Overview of the distribution of the protein in mesencephalic territories. (B) Superior colliculus, showing intense staining in the deep gray layer (DpG). (C) Red nucleus, magnocellular part (D)Locus coeruleus, (E) Oculomotor nucleus.

On the other hand, important mesencephalic centers, such as the substantia nigra and ventral tegmental area (VTA), are weakly AMBRA1 immunoreactive. Indeed double immunofluorescence confirms low levels of AMBRA1 signal in dopaminergic neurons, identified by anti-tyrosine hydroxylase (TH), (Fig. 3.8)

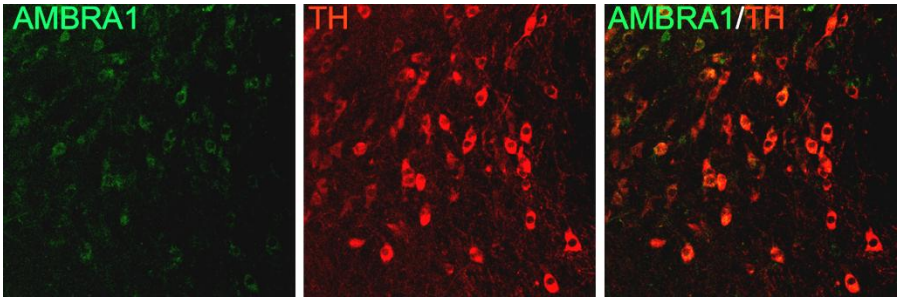


Fig.3.8AMBRA1(green)/TH(red) IF in VTA. Scarce colocalization of the markers is detected.

The brainstem is especially rich in AMBRA1, as shown in Fig. 3.9, illustrating the distribution of the protein in several sensory and motor nuclei.
AMBRA1 is widely expressed in cerebellum (Fig. 3.10A) at different levels depending on the considered area and neuronal subtype.

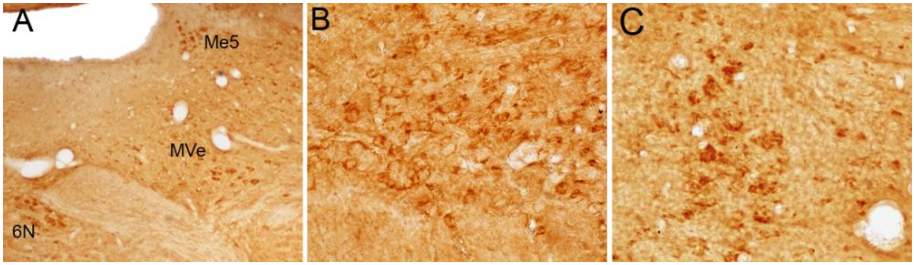


Fig.3.9 AMBRA IHC in coronal sections of mouse brainstem. (A) Overview of positive centers, including abducens nucleus (6N), mesencephalic trigeminal nucleus (Me5), and medial vestibular nucleus (MVe). (B) Pontine reticular nucleus (C) Raphe nucleus.

In the deep cerebellar nuclei, the large excitatory glutamatergic neurons are strongly immunoreactive (Fig. 3.10A). The magnocellular projections of these nuclei reach the red nucleus and the thalamic region in which AMBRA1 is strongly expressed.
IHC analysis of the cerebellar cortex shows a remarkable immunoreactivity in Purkinje cell layer (PL), while the granule cell layer (GL) and stellate and basket neurons in the molecular layer (ML) are only mildly stained (Fig. 3.10B).
Close-up view of the intracellular distribution of AMBRA1 signal in Purkinje cells demonstrates polarized and particulate immunoreaction product, suggesting association of the protein to specific cytoplasmic compartments (Fig. 3.10C). Notably, even within the PL, different degrees of immunoreactivity can be recognised. An inner-to-outer gradient of immunoreactivity in each cerebellar lobule is observed. Even more intriguingly, Purkinje neurons of the whole lobule 10 and of the ventral part of lobule 9 are strikingly negative.

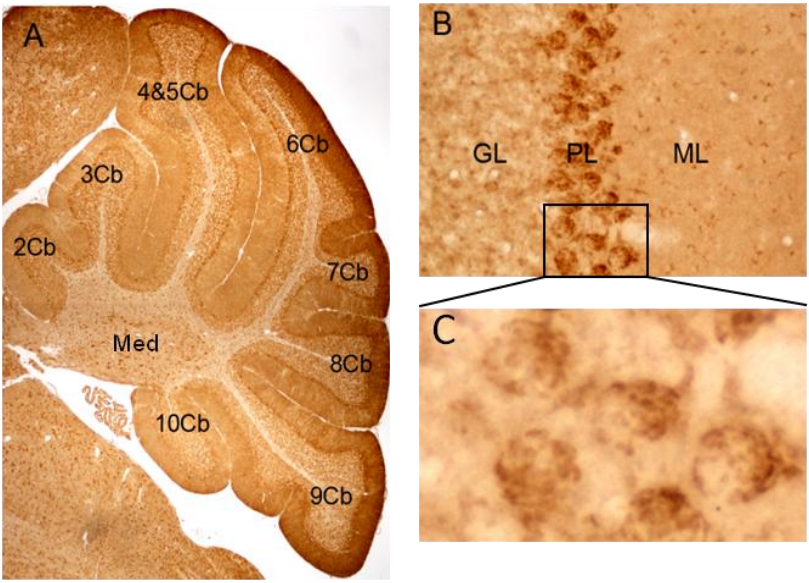


Fig. 3.10 AMBRA1 IHC in sagittal sections of mouse cerebellum.
(A) Overview of the cerebellum showing a different expression in the lobules (2-10 Cb) and staining in the medial cerebellar nucleus (Med); (B) Purkinje cell layer (PL) shows a stronger immunoreactivity than granule (GL) and molecular (ML) layers; (C) detail of the PL showing particulate staining concentrated at the apical cell pole.

3.2 Ultrastructural analysis of AMBRA1

To the aim of investigating the intracellular distribution of AMBRA1 we performed pre-embbedding immunolabelling of brain sections. The IEM analysis revealed an exclusively cytoplasmic localization of the protein (Fig 3.11A), that results to be mostly associated to the endoplasmic reticulum (Fig. 3.11B-C), consistent with its role in regulating autophagosome formation.

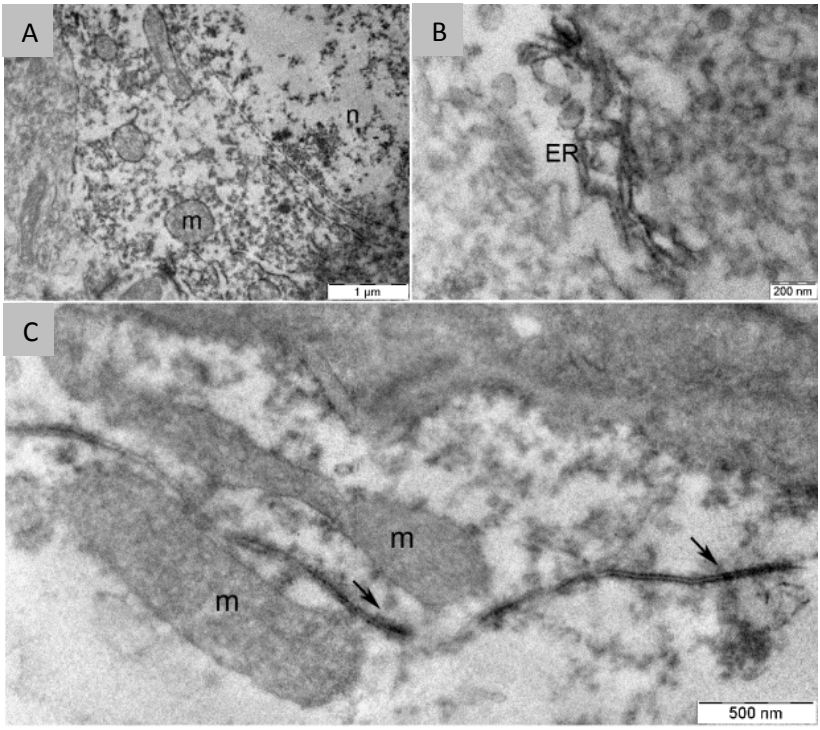


Fig. 3.11. AMBRA1 Pre-embedding IEM of pyramidal hippocampal neurons.
(A) Low-power micrograph showing that immunodeposits are present in the cytoplasm, while absent from the nucleus (n). (B) Higher magnification showing association of immunoprecipitates with ER. (C) AMBRA1- immunoreactivity is found around and inside ER cisternae (arrows), and, to a lesser extent close to mitochondria (m).

Chapter 4

AMBRA1 expression in physiological and Alzheimer-like ageing

The suggested correlation between brain ageing and decreased autophagic efficiency prompted us to analyze age-dependent variations in AMBRA1 expression, focussing on the neocortex and hippocampus, i.e., the most damaged areas during ageing (Moreno et al, 2011)
AMBRA1 expression was investigated in normal and pathological ageing, utilizing a transgenic mouse model (Tg2576, Hsiao et al., 1996) of Alzheimer's disease (AD). The ages selected for this study, i.e., 3, 6, 12 and 18 months, correspond to the onset and progression of disease in the transgenic strain (Jacobsen et al., 2006; D'Amelio et al.,2011).
Moreover, in order to evaluate and interpret our data in view of the role of AMBRA1 in regulating the autophagic process, we also analysed the expression of Beclin1, which is centrally involved in autophagy and considered the main interacting molecule of AMBRA1 (Fimia et al., 2007).

4.1 Expression of AMBRA1 in the ageing neocortex

In the neocortex, western blotting (WB) analysis in wild-type animals (WT) (Fig. 4.1, black columns) shows a progressive decrease in AMBRA1 levels until 12 months of age, followed by an increase at the oldest age considered. While the general trend of AMBRA1 expression during ageing appears similar in the two genotypes, Tg2576 neocortex differs in some respects to its WT counterpart (Fig. 4.1, gray columns). At the starting time point (3 months), AMBRA1 levels are lower in transgenic than in WT mice, but remain relatively stable at subsequent ages. Indeed, the only age-related variation that reaches statistical significance is observed at 18 months, when an increase of AMBRA1 levels is detected. The morphological analysis of the neocortex shows a predominantly neuronal immunostaining and confirms WB results, as age- and genotype-dependent differences in AMBRA1 immunoreactivity are

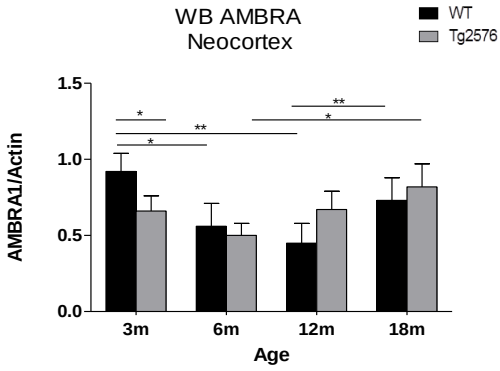


Fig.4.1 Densitometric analysis of AMBRA1WB assay of neocortex from WT and Tg2576 mice, ageing 3, 6, 12 and 18 months. *, $p<0.05$; **, $p<0.01$.

observed (Fig.4.2). Interestingly, in Tg2576 neocortex, a higher heterogeneity degree of AMBRA1 expression is detected, compared to its WT counterpart. Specifically, in 3-month-old transgenic brain, most pyramidal neurons express hardly detectable AMBRA1 levels, while only few display more intense immunoreactivity. This situation dramatically changes at 18 months, when Tg2576 neocortex shows most pyramidal neurons remarkably stained in their somata and apical dendrites. In addition, at this advanced AD stage, the neocortex appears partially disrupted in its cytoarchitecture, due to the appearance of amyloid plaques, surrounded by dystrophic neurites and AMBRA1-positive glial cells (Fig. 4.2, inset). By contrast, in the WT neocortex rather uniform AMBRA1 staining is found throughout the neocortex. In particular, both 3- and 18-month-old animals show high levels of AMBRA1 immunostaining in the vast majority of pyramidal neurons.

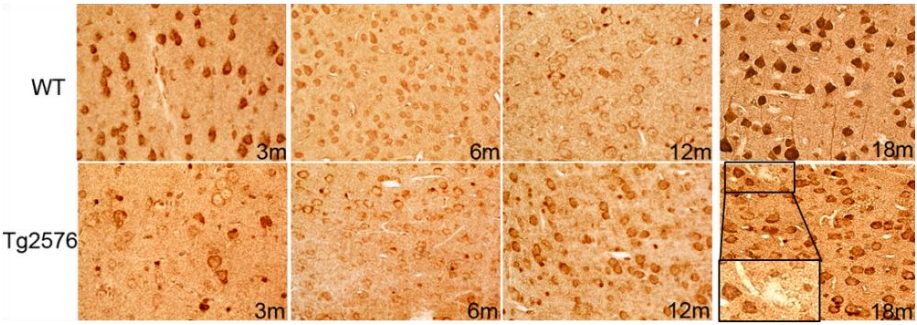


Fig.4.2AMBRA1 IHC in the neocortex of WT and Tg2576 mice ageing 3, 6, 12 and 18 months. Inset shows immunoreactive glial cell bodies surrounding an amyloid plaque.fare inset

Fig. 4.3 shows Beclin1 protein levels in the neocortex during normal and Alzheimer-like ageing. Fairly stable expression during the earliest time points is observed, independent of the genotype. However, a significant increase of protein levels at 12 months of age is detected in both WT and Tg2576 animals. The pathological genotype shows further increment of Beclin1 at 18 months, suggesting autophagy induction.

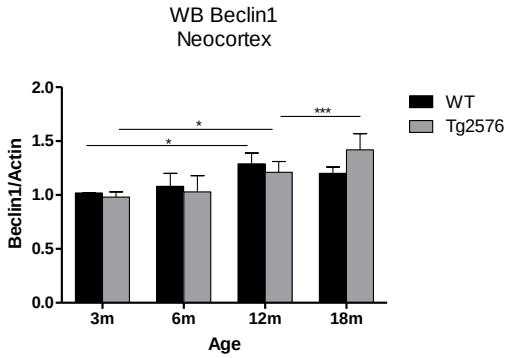


Fig.4.3 Densitometric analysis of Beclin1 WB assay of neocortex from WT and Tg2576 mice, ageing 3, 6, 12 and 18 months. *, $p<0.05$; ***, $p<0.001$

4.2 Expression of AMBRA1 in the ageing hippocampus

In the hippocampus, AMBRA1 expression undergoes a general decrease during ageing (Fig. 4.4). In the WT, this decrease becomes significant at 12 months, and levels remain stably low thereafter. In Tg2576 mice, differences are already significant at 6 months of age and AMBRA1 expression further decreases between 12 and 18 months.

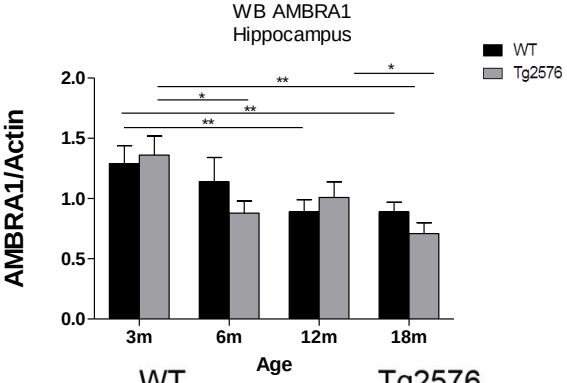


Fig. 4.4 (A) Densitometric analysis of AMBRA1 WB assay of hippocampus from WT and Tg2576 mice, ageing 3, 6, 12 and 18 months. *, p<0,05; **p<0,01.

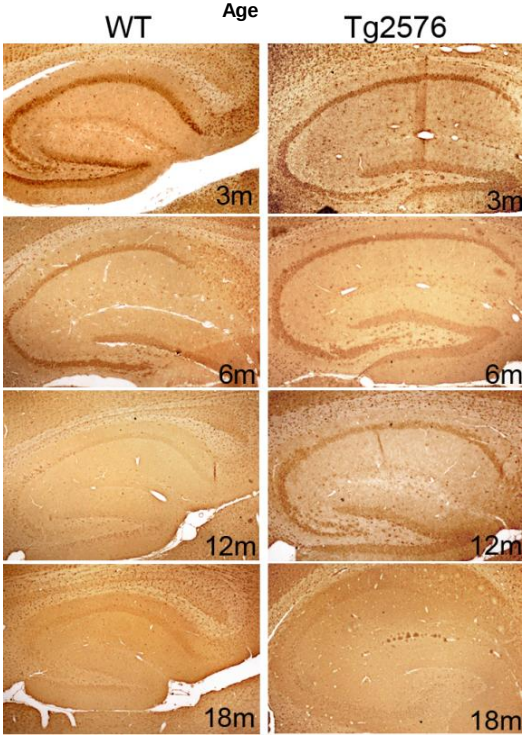


Fig. 4.5 AMBRA1 IHC in the hippocamal formation of 3-,6-,12- and 18 month-old WT and Tg2576 mice.

A detailed morphological analysis, while confirming WB data, reveals intriguing genotype-based differences in the intrahippocampal distribution of AMBRA1 protein (Fig. 4.5, 4.6). In particular, CA1 region in 3-month-old Tg2576 hippocampus displays less intensely stained neurons, than its WT counterpart. By contrast, in the dentate gyrus of the same animal, AMBRA1-positive cells are more abundant than in WT, thus suggesting ongoing neurogenesis.

Beclin1 expression in the

normal hippocampus, while following a generally similar trend compared to AMBRA1, shows an earlier decrease (Fig. 4.7). At difference, in the transgenic hippocampus a significant increase in protein levels between 6 and 18 months.

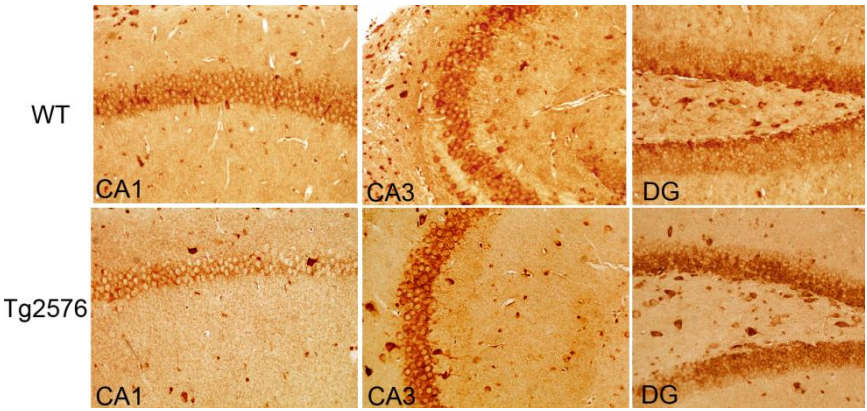


Fig.4.6 AMBRA IHC in the CA1, CA3 and DG fields of 3 month-old-mice WT and Tg2576 mice.

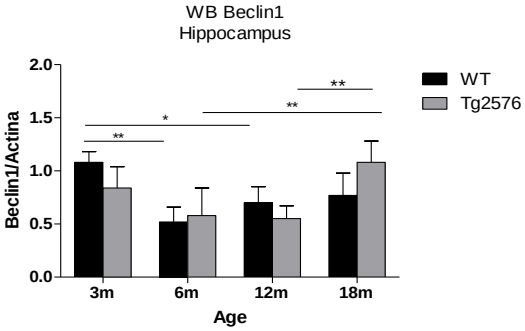


Fig. 4.7 Densitometric analysis of Beclin1 WB assay of hippocampus from WT and Tg2576 mice, ageing 3, 6, 12 and 18 months. *, $p<0,05$; **$p<0,01$.

Chapter 5

AMBRA1 in parkinsonian neurodegeneration

5.1 AMBRA1 in a pharmacological model of pre-symptomatic PD

To address the issue of a putative role of AMBRA1 in PD, we utilized a protocol, based on the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as a toxic agent for dopaminergic neuron degeneration (Kuhn et al 2003). This neurotoxin crosses the blood-brain barrier and is metabolized by glial cell monoamine oxidase-B (MAO-B) to 1-methyl-4-phenylpyridinium (MPP+). MPP+ is uptaken by dopaminergic (DA) neurons, through the dopamine transporter, thus interfering with complex I of the electron transport chain, a component of mitochondrial metabolism. This causes free radical production, contributing to cell destruction. Noteworthy, the toxic effect is limited to the nigrostriatal circuit, involving the substantia nigra (SN) and its projection to the corpus striatum, while the dopaminergic neurons of the ventral tegmental area (VTA) and their projections to the neocortex are not damaged.

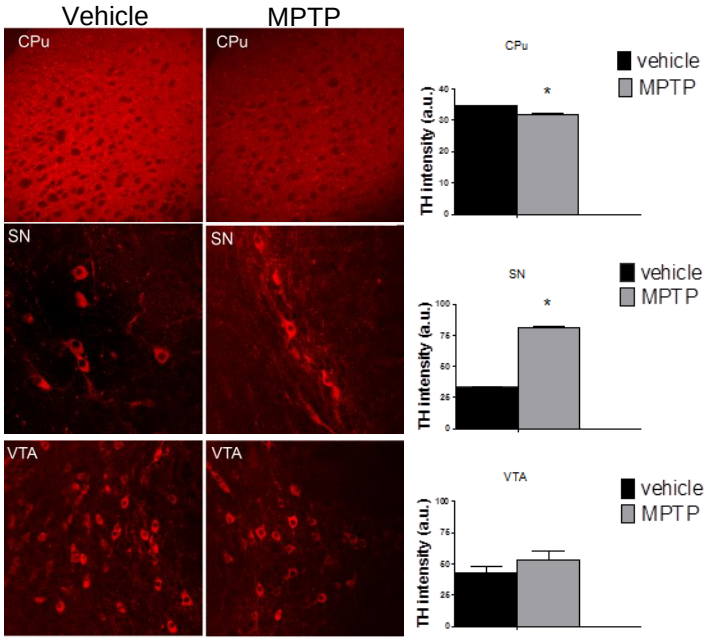


Fig.5.1 TH-IF and relative densitometric analysis of caudate-putamen, substantia nigra (SN), ventral tegmental area (VTA) in MPTP-treated animals compared to vehicle-treated animals. *, $p<0.05$.

Because we are interested in the early stages of PD pathogenesis, in our studies we used a low dose of toxin which does not induce nigral cell death and leads to minimal loss of TH immunoreactivity in the striatum (Kuhn et al 2003). To analyze AMBRA1 expression in relation to the pathological phenotype, we took advantage of a quantitative-morphological approach to measure TH and AMBRA1 levels in nigrostriatal circuit.

The densitometric analysis of TH immunofluorescence signal demonstrates a small but significant decrease in the caudate-putamen of MPTP-treated animals, compared to controls (Fig 5.1, CPu). By contrast, TH immunofluorescence levels are significantly higher in the SN, suggesting an activation of cellular response against the toxic insult (Fig 5.1, SN). In order to make sure that only nigrostriatal circuit is affected, we analyzed TH levels in VTA (Fig 5.1,VTA) These data confirm that the chosen treatment produces a phenotype typical of the early phase of dopaminergic neuron degeneration.

As showed in Chapter 1, dopaminergic neurons in the SN and in VTA express low AMBRA1 levels. Interestingly, after MPTP toxic insult, the protein content strikingly increases in the SN (Fig.5.2), suggesting a important role of AMBRA1in the cellular response in the early PD stage.

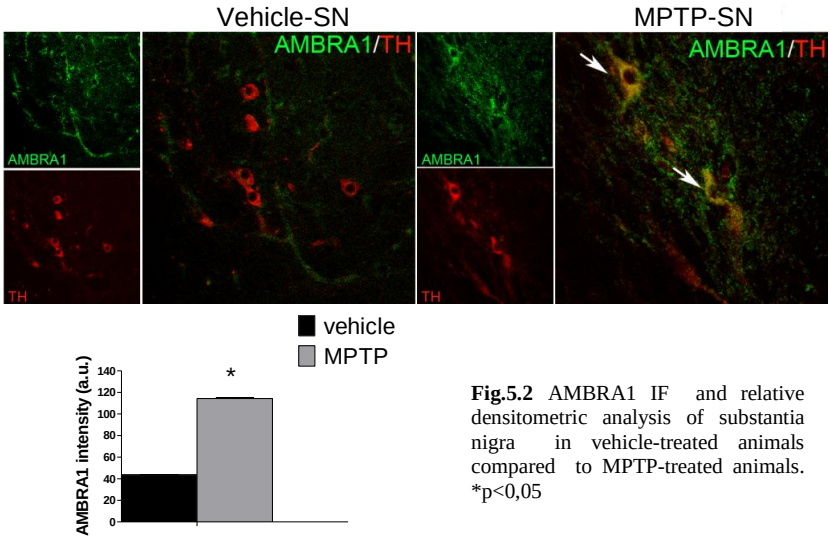


Fig.5.2 AMBRA1 IF and relative densitometric analysis of substantia nigra in vehicle-treated animals compared to MPTP-treated animals. *p<0,05

Similarly, dopaminergic neurons in the VTA show significantly increased AMBRA1 levels (Fig. 5.3), indicating that also in these neurons an autophagic response is activated.

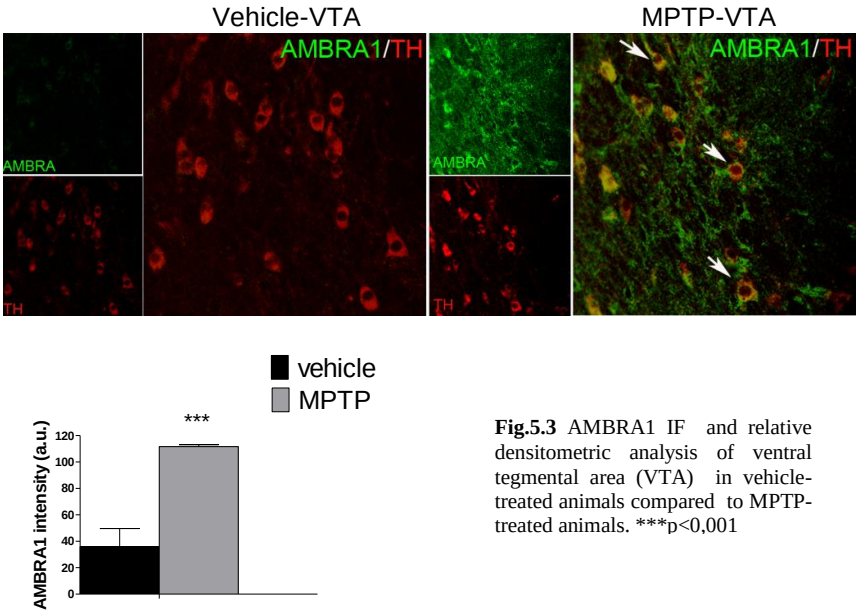


Fig.5.3 AMBRA1 IF and relative densitometric analysis of ventral tegmental area (VTA) in vehicle-treated animals compared to MPTP-treated animals. ***p<0,001

5.2 AMBRA1 in a genetic model of pre-symptomatic PD

The process of neurodegeneration is favoured by ageing -the major risk factor for PD- during which cells lose their ability to properly control redox homeostasis and genome quality. Mutations in NER genes induce accelerated ageing associated with neurological defects. Indeed we demonstrated that a mild ERCC1 mutant model (ERRC1Δ/+) might share some pre-symptomatic features of PD.
Accordingly, we show in this model decreased TH levels in the striatum (Fig.5.4A, CPu), increased TH levels in the SN (Fig. 5.4A, SN), confirming that there is no neuronal loss, but activation of cellular response, typical of early PD. By contrast, TH levels in VTA dopaminergic neurons are unchanged (Fig. 5.4A, VTA), consistent with the specificity of circuits affected by PD. Since during PD pathogenesis, pronounced and irreversible redox imbalance in dopaminergic neurons culminates in oxidative stress, we addressed the question of the intracellular redox state in dopaminergic neurons from ERRC1$^{\Delta/+}$ animals. To this purpose we developed a novel experimental protocol, whereby the actual redox status at the level of single cells can be measured (Horowitz et al 2011). Applying this redox histochemistry (RHC) technique, we could show that the ratio between the oxidized and reduced cysteins in dopaminergic neurons of the substantia nigra is significantly higher in ERRC1$^{\Delta/+}$ than in WT (Fig. 5.4 B).

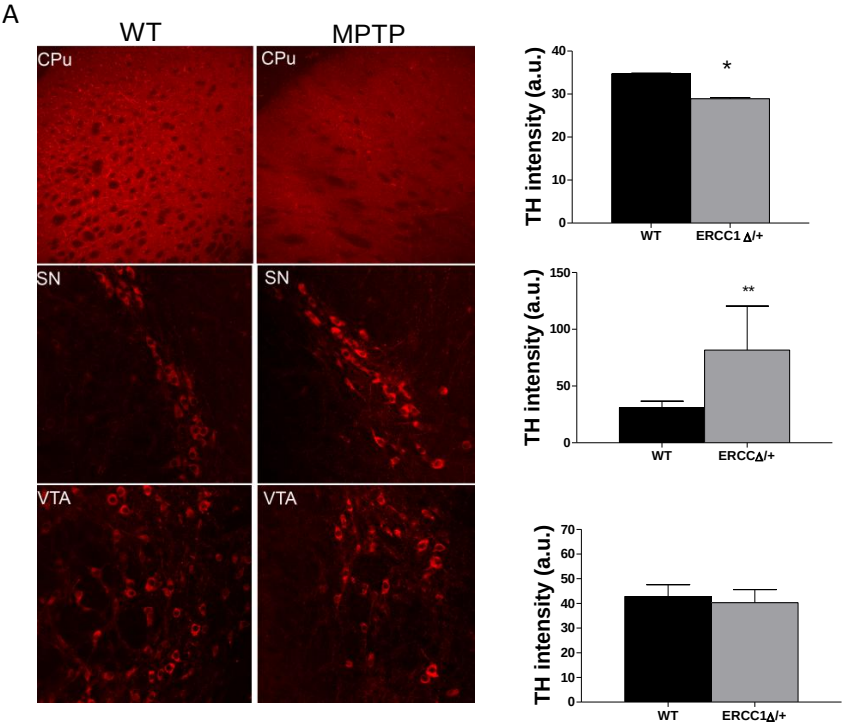


Fig.5.4 (A) Th IF and relative densitometric analysis of caudato putamen (CPu), substantia nigra (SN), ventral tegmental area (VTA) in WT mouse and ERRC1$^{\Delta/+}$. (B)RHC analysis of dopaminergic neurons WT and ERCC1$^{\Delta/+}$ in SN shows an increase of the ratio SS/SH presenting an oxidation state of this neurons.*p<0,05.*p<0.01

After confirming that the ERCC1$^{\Delta/+}$ can be considered a good candidate as a pre-syntomatic PD model, we analyzed AMBRA1 levels by a morphological-quantitative approach. AMBRA1 IF in DA neurons of SN shows an increase of protein levels in the ERCC $^{\Delta/+}$ compared to WT (Fig. 5.5), suggesting an up-regulation of AMBRA1 in the context of cellular defense response.

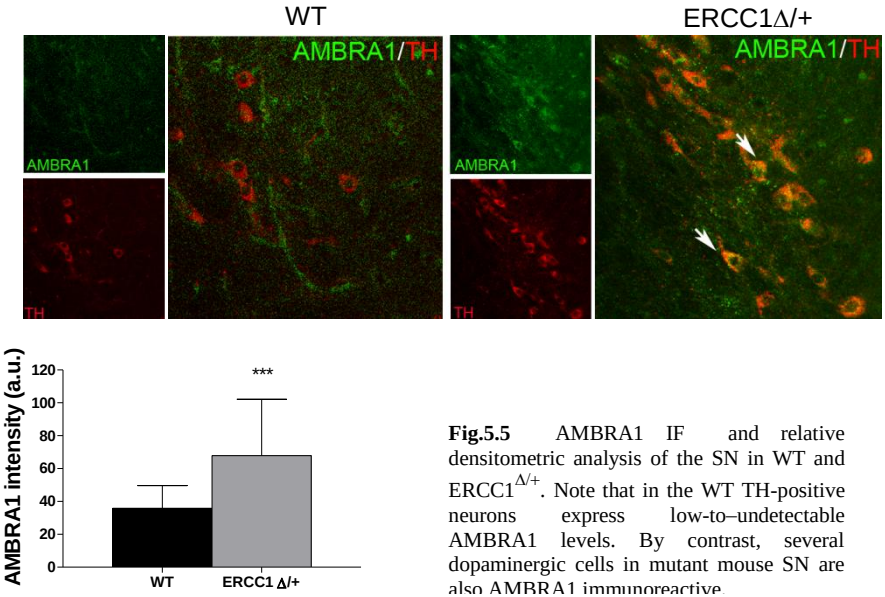


Fig.5.5 AMBRA1 IF and relative densitometric analysis of the SN in WT and ERCC1$^{\Delta/+}$. Note that in the WT TH-positive neurons express low-to–undetectable AMBRA1 levels. By contrast, several dopaminergic cells in mutant mouse SN are also AMBRA1 immunoreactive.

As in the previously examined pharmacological model of early PD, also in this PD-like condition, TH levels in the VTA increase (Fig. 5.6), suggesting an involvment of AMBRA1 in the dopaminergic defense response, upregulating the autophagic process.

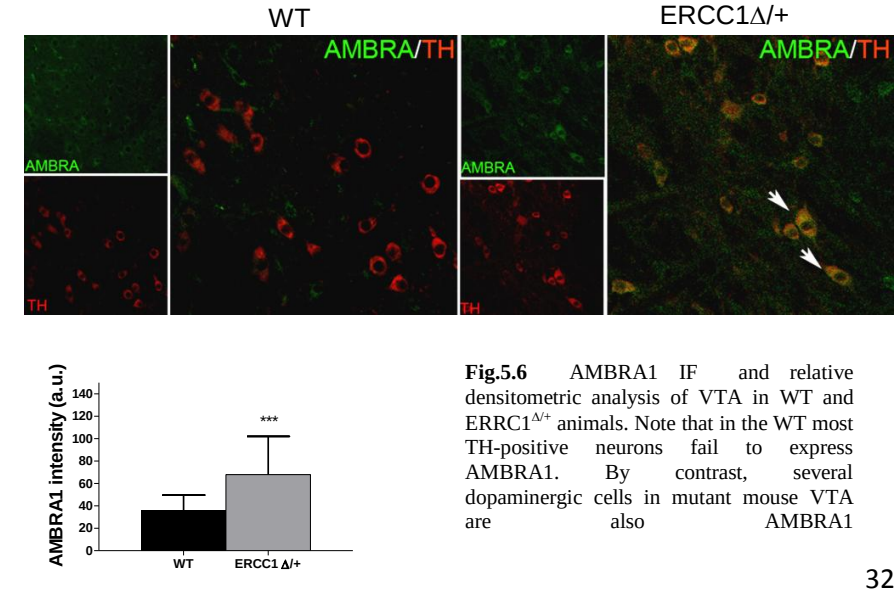


Fig.5.6 AMBRA1 IF and relative densitometric analysis of VTA in WT and ERRC1$^{\Delta/+}$ animals. Note that in the WT most TH-positive neurons fail to express AMBRA1. By contrast, several dopaminergic cells in mutant mouse VTA are also AMBRA1

Section III: Discussion and Conclusion

Chapter 6

Discussion

6.1 Distribution of AMBRA1 in mouse brain

In the present study we accomplished the first complete map of AMBRA1 protein distribution in adult mouse brain. This represents an important step towards the understanding of the role of this recently identified actor in nervous tissue development and homeostasis. In fact, while a crucial function of AMBRA1 gene has been established since its discovery, as mutant mice show a dramatic phenotype characterized by neural tube defects (Fimia et al.,2007), the precise localization in the CNS had not been investigated so far. The relevance of our findings also relate to the role AMBRA1 is thought to play in the autophagic process, which acts as a quality control system in all eukaryotic cells. Specifically, in the nervous tissue, which is mainly composed of post-mitotic cells (a "perennial tissue", according to Bizzozero's classification), the macroautophagic pathway and its proper regulation are especially important for the high turnover rate of cell components. Indeed, electron microscopy of human and mouse brain tissue shows the presence of lysosomes and autophagosomes in neurons, further supporting a basal level of autophagy during normal neuronal homeostasis. Accordingly, a number of autophagy related genes (Atg) are expressed in neuronal tissues of humans, rodents, and insects (Jaeger et al., 2008). On the other hand, their deletion results in profound nervous tissue abnormalities, including intracellular accumulation of ubiquitinated-positive aggregates (Hara et al., 2006). It should noted, however, that these mutants fail to show early developmental disturbances, such as defects to neural tube closure, suggesting that AMBRA1 may play additional and specific roles in the nervous tissue, other than regulating autophagosome initiation.

The first general assessment that can be made, based on our data, is that AMBRA1 in the CNS is highly expressed in neuronal cells. The particulate cytoplasmic perinuclear staining observed after light microscopic immunolocalization of AMBRA1 prompted us to further investigate the intracellular distribution of AMBRA1 by immunoelectron microscopy. The results obtained applying pre-embedding immunolabelling in several brain regions showing association of AMBRA1 to the endoplasmic reticulum at the apical pole of neuronal cells are consistent with recent data suggesting that AMBRA1 translocates to the perinuclear region upon autophagy induction, given its role in regulating autophagosome formation (Di Bartolomeo et al., 2010). This evidence strongly supports the contribution of our protein to the maintenance of basal levels of autophagy in neurons, essential for their homeostasis.

Glial cells appear poorer in this protein, compared to neuronal cells. Double IF experiments, performed in different brain areas and using general neuronal (NeuN) and glial (GFAP, Iba1) markers give strong support to this statement. The high AMBRA1 expression in neurons, which are terminally differentiated cells and often have large cytoplasmic volumes, could be explained in view of the basal autophagy as a crucial mechanism for neuronal metabolism, to recycle cellular constituents. Indeed, neuronal metabolic request is particularly high, also considering that neurons ensure maximum glucose utilization by having superior glucose uptake and higher expression of the glucose-degrading enzyme, hexokinase, compared to glial cells (Zukor et al., 2009). Moreover, neurons use macroautophagy as a defense response, to degrade damaged and/or senescent organelles and cytosolic components, which progressively accumulate due to oxidative stress. On the other hand, astrocytes are likely to be less active in the autophagic pathways, since these cells are considered more stable than neurons. For example, they are more resistant to oxidative damage, since they are endowed with more efficient antioxidant defenses, such as the glutathione system (Dringen et al., 1999). Even microglial cells is supposed to undergo a low-rate of autophagy, not only for their small size, but also because in physiological conditions the brain is well protected by the blood-brain barrier and phagocytes are in a rather quiescent state.

Our immunohistochemical analysis on serial coronal brain sections allowed us to demonstrate the presence of AMBRA1 in virtually all the brain regions along the rostro-caudal axis. The protein is widely expressed in the rhinencephalon, telencephalon, mesencephalon, metencephalon, and myelencephalon. However, the immunosignal intensity is not uniform within each brain territory, showing region-, population-, and cell-specificity. This general observation suggests that neurons have different requirements as to the abundance of AMBRA1, in relation to its known function as a pro-autophagic protein and, possibly, to other, undefined roles in cell maintenance.

In order to gain a further insight into possible relationships between AMBRA1 content and specific neuronal features, we performed double IF experiments in the different brain areas, using anti-AMBRA1 in combination with antibodies to select markers, to detect cholinergic, GABAergic, and dopaminergic neurons.

Overall, our data suggest a correlation between the abundance of AMBRA1 and the expression of choline acetyl transferase, the biosynthetic enzyme of acetylcholine. Indeed, principal neurons of the septal complex, giant aspiny interneurons of the caudate-putamen, and neurons of virtually all the motor nuclei of the brainstem would favor this hypothesis. Concerning the septohippocampal cholinergic system, the meaning of this finding could relate to the importance of this type of neurotransmission in learning and memory function, thus supporting the participation of autophagic processes in neuronal plasticity, which is modulated by mTOR. This is consistent with the reported beneficial effect of rapamycin, an

inducer of autophagy through mTOR inhibition, on synaptic plasticity and memory formation (Santos et al., 2011).

While cholinergic neurons consistently appeared as strongly immunoreactive, it should be stressed that several other neuronal subtypes are also rich in AMBRA1. Among these, the thalamic region, which is largely composed of GABAergic neurons, is especially AMBRA1immunoreactive. For example, the anterior thalamic nucleus, shows a good colocalization of the two markers. Interestingly, this thalamic area is closely connected to the septohippocampal system, and therefore involved in learning and memory processes. However, colocalization of AMBRA1with glutamic acid decarboxylase (converting glutamate to GABA) fails to occur consistently. For example, GABAergic spiny neurons of the corpus striatum are faintly AMBRA1-positive, suggesting that these medium-sized neurons rely on cellular mechanisms other than autophagy, for their homeostasis.

Hypothesizing a correlation between AMBRA1 and dopamine is in our view more critical than that with other neurotransmitters. In fact, little overlapping of the two markers can be detected by double IF for AMBRA1 and tyrosine hydroxylase -the biosynthetic enzyme for dopamine- in the different brain regions examined, mostly belonging to the mesencephalon. Specifically, in the substantia nigra and ventral tegmental area AMBRA1 levels are low, indicating that in physiological conditions these regions display tightly modulated autophagic activity. Indeed some studies showed that autophagic increase in dopaminergic neurons can be detrimental especially in SN (Cheng et al., 2011).

Based on the above results, showing that AMBRA1 immunoreactivity pattern can only partially be correlated with the expression of certain neurotransmitters, we searched for other neuronal features that could imply high turnover rate by autophagic mechanisms. Generally, intense immunoreactivity was found in large-sized neurons, including mitral cells of the olfactory bulb, neocortical pyramidal neurons, giant aspiny interneurons of caudate-putamen, neurons of magnocellular part of red nucleus and of medial vestibular nucleus, motor nuclei neurons, and Purkinje cells of the cerebellar cortex. In these highly heterogeneous neuronal subsets, AMBRA1 abundance is presumably linked to high organelle turnover, due to the size and consequent metabolic request. AMBRA1 content not only appears to correlate with the volume of cell somata, but may also depend on the extension of neuronal processes. To this respect, it is worth noting that in the corpus striatum, axon bundles crossing the whole corpus striatum, representing part of the corticostriatal circuit, are intensely AMBRA1-positive, suggesting association of a pool of protein with axonal cytoskeleton components. Our data are consistent with the demonstrated association of AMBRA1 with dynein light chain, suggesting that neurons contain an AMBRA1 pool in their axons which translocates to the endoplasmic reticulum when autophagy is up-regulated (Di Bartolomeo et al., 2010).

In addition the presence of AMBRA1 in axon compartment could be related to evidence that constitutive neuronal autophagy may serve as both a key mechanism for remodeling neurites and growth cone structure during neurite extension and as a neuroprotective mechanism by removing damaged proteins and organelles that may otherwise accumulate within axons (Gumy et al., 2010). Thus, subtle disruptions of autophagosome formation, maturation or trafficking are predicted to have dire consequences for autophagic flux and neuronal homeostasis (Boland and Nixon, 2006).

Consistent with the above hypothesis of a link between cell size and AMBRA1-mediated autophagy requirements, several neuronal populations characterized by small-to-medium cell size display relatively low immunoreactivity levels. Tufted cells in the olfactory bulb (OB), spiny neurons of the caudate putamen, granule cells of the dentate gyrus, parvicellular parts of the red nucleus and vestibular nuclei, and granule cells of the cerebellar cortex are paradigmatic examples substantiating this concept. Nevertheless, some small neurons, e.g., those located in the *locus coeruleus*, express high AMBRA1 levels, allowing to envision a correlation to other neuronal properties.

Interestingly, we collected some circumstantial evidence that certain neuronal populations containing high levels of the protein are more protected against neurotoxic insults of diverse origin. In the following paragraphs, examples supporting this putative correlation are given.

The *locus coeruleus*, even though involved in neurodegeneration during Parkinson's disease (PD), is affected in the final steps of the disease, being spared in early stages, (Zelda and Nancy 2009). By contrast, the deterioration of motor functions observed in PD patients is predominantly attributable to the degeneration of dopaminergic neurons in the substantia nigra that displays weak AMBRA1 immunostaining.

Even in the basal ganglia, and particularly in the caudate putamen, AMBRA1 expression pattern shows a differential AMBRA1 positivity in its various neuronal populations, possibly reflecting different susceptibility to neurodegeneration. The corpus striatum is known to be selectively affected by Huntington's disease (HD). Morphological changes in the striatum are probably primed initially by alterations in the intrinsic functional properties of medium-sized spiny projection neurons, which in our experiments were found to contain low AMBRA1 levels. By contrast, giant, aspiny interneurons, which are densely stained in their somata, appear to be less affected in HD (Cepeda et al., 2007). This observation may be relevant, in view of the demonstration that autophagy is centrally involved in degradation of mutated hungtigtin, indeed experimental induction of autophagy enhances the mutant huntingtin clearance and decreased the levels of soluble proteins and aggregates (Sarkar et al., 2008).

Being the hippocampal formation among the most sensitive regions of the brain to chronic and acute neurodegeneration events, we also focused on this area, which

appears dyshomogeneously AMBRA1-positive. IHC analysis demonstrates low-to-moderate staining levels in the pyramidal layer of the hippocampus proper, with a pattern following gradual changes in morphology of pyramidal cells. Indeed, proceeding from region CA1, where cell bodies are relatively small, to region CA3, where giant neurons are found, AMBRA1 immunoreactivity in pyramidal cells becomes more and more intense. The autophagic process in hippocampal formation seems to have a relevant, though controversial, role in neuronal homeostasis, in either physiological or pathological conditions. The pyramidal layer in the CA1 region of the hippocampus, is extremely sensitive to ischemic damage. On the other hand, it is worth noting that many of the AMBRA1-rich cells, such as pyramidal cells of the CA3 region of the hippocampus and interneurons of the hippocampal formation, appear highly resistant to ischemia-reperfusion injury (Nakatomi et al., 2001). It is generally accepted that ischemic damage is mediated by reactive oxygen species (ROS); it therefore seems possible that high AMBRA1 levels, present in some neurons, may protect these cells against oxidative stress, related to a more efficient autophagic induction against the oxidative insult. Besides the speculation about its protective role, excessive autophagy could be even detrimental in certain conditions. In fact, an electron microscopic study of hypoxic/ischemic neonatal hippocampus shows the presence of damaged pyramidal neurons containing abundant autophagic vacuoles, some of which are nascent double membrane-limited autophagosomes. Importantly, pyramidal neuron death in this type of injury is largely prevented by Atg7 deficiency, supporting a role of neuronal autophagy in ischemia-induced cell death (Uchyama, 2009). Our findings suggest that AMBRA1 can play a important role regulating finely the induction of autophagosome formation and maintain the balance in autophagic flux.

Hippocampal region is also primary affected by chronic neurodegeneration, during Alzheimer disease (AD), in which the autophagic mechanism seems to play a pathogenetic role. Indeed in AD, the maturation of autophagolysosomes and their retrograde transport are impeded, which leads to a massive accumulation of 'autophagy intermediates' within large swellings along dystrophic and degenerating neurites. The combination of increased autophagy induction, detected in AD brain, and defective clearance of autophagic vacuoles (AVs) in which Aβ is generated creates conditions favorable for toxic AV accumulation in AD (Nixon 2007). Correlating these findings with AMBRA1 expression, we suggest that in this region the fine regulation of autophagy, including expression of autophagy regulating protein, is crucial during the pathological insult.

Another clue to elucidate the autophagic involvement and AMBRA1 function in neurodegeneration may come from the distribution of the protein in select neuronal populations, sharing specific involvement in certain degeneration phenotypes. In particular, mitral cells of the OB and Purkinje cells in the cerebellar cortex, even though located in distant brain regions, are similarly affected in the so-called

"Purkinje cell degeneration" (*pcd*) (Valero et al., 2007). This mouse model is a recessive mutant characterized by complete and dramatic post-natal, cell autonomous Purkinje neuron degeneration and death. As the basis of Purkinje cell death in *pcd* is unresolved, and contradictory data has emerged for the role of autophagy in Purkinje cell degeneration. La Spada's group studies demonstrated that apoptosis is not responsible for Purkinje cell loss. Indeed, the association of a autophagic features within dying cells led to the designation of a type of cell death (type II or autophagic cell death) that became viewed as independent from the classic apoptotic (or type I) pathway (Chakrabarti et al., 2009). Notably, in the cerebellar cortex, not all the neurons are equally affected by *pcd*, some neuronal types, namely stellate, basket and granule cells, being spared. Even in the OB, *pcd* mice suffer from a selective degeneration of mitral cells with no direct alterations in other neuronal types, such as tufted cells (Valero et al., 2007). Interestingly, all the neurons resistant to *pcd* in either cerebellum or OB are found to contain remarkably lower levels of AMBRA1 immunostaining, when compared to Purkinje and mitral cells. These data allow to envision a correlation between dysregulation of autophagy in *pcd*, as aberrant mitophagy, and neurodegeneration in *pcd* mice. The high AMBRA1 levels showed by mitral and Purkinje cells may indicate high basal autophagic activity, that under a toxic stimulus may result in improper activation of the process. In this specific model, the culprit is represented by a mutated form of cytosolic carboxypeptidase 1 (CCP1/Nna1), which leads to reduced degradation of the peptides downstream of the proteasome, and consequent autophagic massive induction.

It is worth mentioning that, even within the Purkinje cell layer, different degrees of AMBRA1 immunoreactivity can be recognised. An inner-to-outer gradient of immunoreactivity in each cerebellar lobule is observed. Even more intriguingly, Purkinje neurons of the ventral lobule IX and the whole lobule X are strikingly negative. This specific distribution can be related to zonal organization of the cerebellar cortex, recognized by their histochemical identity. Indeed, although the cerebellum appears to be histologically uniform, a complex parasagittal banding pattern can be revealed by the use of immunocytochemical markers, namely zebrin I and II, functional mapping, and the terminal field distribution of the afferents (Ozol et al., 1999; Armstrong and Hawkes, 2000). This organization is crucial in some pathologies, characterized by patterned Purkinje cell death. Indeed, in the Niemann-Pick disease, an accumulation disorder characterized by enhanced autophagy, most Purkinje cells progressively die, while lobules IX and X, are resistant to the insult and shows limited cell loss (Sarna et al., 2003; Ishibashi et al., 2009). Therefore, the absence of AMBRA1, a key positive regulator of autophagy, from the above cerebellar zones, indicate also in this case an inverse correlation between AMBRA1 expression and neuronal survival, in pathological conditions involving massive induction of autophagic processes.

Even though AMBRA1 function has mainly be related to its role in autophagosome formation, as its main interactor is Beclin1, a well known inducer of autophagy, further roles of AMBRA1 in the control of other cellular activities, cannot be ruled out. Indeed, growing evidence point to other molecules as putative interactors of AMBRA1, namely dynein, parkin and Bcl2 (Di Bartolomeo et al.,2010; Strapazzon et al., 2011; Van Humbeeck et al., 2011). These proteins are known to play diverse roles in nervous tissue development, homeostasis and pathology. Data reported in the first work identifying AMBRA1 appear to support the concept that other important roles of the protein are yet to be defined. Considering the phenotype shown by AMBRA1 gene-trapping mutants in the nervous system, its participation in regulating the balance between proliferation and differentiation can be envisioned. Our results, demonstrating a specific distribution of AMBRA1 immunostaining, in the neurogenic area of the hippocampal formation, *i.e.*, the dentate gyrus, could support this hypothesis. In fact, AMBRA1 pattern, characterized by a gradient from the subgranular zone to the granular layer/molecular layer border, reflects the distribution of progressively differentiating neuroblasts. The richest population may include neural stem cells, based on their morphology and distribution. In this view, the observed gradual changes in AMBRA1 content may reflect the cell differentiation status, that in this neurogenic region is known to proceed in an inner-to-outer manner, from neural progenitors, to neuroblasts, to mature neurons (Bonfanti et al., 2011). Therefore, these findings, consistent with role of AMBRA1 during neurodevelopment, suggest an active role of AMBRA1 in proliferation and differentiation of neuronal cells. Studies are in progress aimed at investigating possible association of AMBRA1 expression with proliferation and differentiation markers identifying specific maturational stages, to further elucidate the role of the protein in neurogenesis.

6.2 AMBRA1 in neurodegeneration.

6.2.1AMBRA1 in physiological and Alzheimer–like ageing

The debated correlation between brain ageing, neurodegeneration and autophagic efficiency prompted us to analyze age-dependent variations in AMBRA1 expression, focussing on the neocortex and hippocampus, *i.e.*, the most damaged areas during physiological and pathological ageing (Small et al, 2011). As a model for Alzheimer's disease (AD), we utilized a transgenic mouse strain (Tg2576, Hsiao et al., 1996). The ages selected for this study, i.e., 3, 6, 12 and 18 months are representative stages of maturation and senescence in physiological conditions; in the transgenic mice the selected time points correspond to onset and progression of disease (Jacobsen et al., 2006; D'Amelio et al., 2011).

In the normal neocortex, WB and IHC analysis show a progressive decrease in AMBRA1 levels until 12 months of age, followed by an increase at the oldest age

considered. The high AMBRA1 neuronal content in young mice suggests a crucial role in plasticity of the neocortex. Indeed this evolutionarily recent brain region differentiates late in embryonic development and its maturation progresses post-natally. At 3 months of age, neurons still undergo morphological modifications and rearrangement of synaptic activity during their maturation (Yuste and Bonhoeffer et al., 2004). The function of AMBRA1 in neocortical neurons plasticity is not necessarily linked to autophagic regulation, as Beclin1 expression is not especially high in young mice.

The progressive decreas of AMBRA1 observed between in 6- and 12-month-old mouse neocortex likely reflects a stabilization of synaptic connections, concomitant with decreased plasticity. On the other hand, the augmented AMBRA1 levels in the senescent brain (18 months) suggest an involvement of our protein in neuronal response to ageing insult, including oxidative stress and misfolded protein accumulation. These data are not in contrast with other Authors' results, suggesting decreased efficiency of the autophagic program in ageing (Madeo and Kroemer, 2010; Rubinsztein et al., 2011). In fact, it is likely that an induction of regulatory proteins occurs, while the formation, maturation and degradation of autophagic vacuole is impaired, thus resulting in an overall decline of autophagic functionality. Our results on the expression pattern of Beclin1, showing increased levels at 12 and 18 months could support this view.

Comparative biochemical and IHC analyses of the Tg2576 mouse neocortex show that, at 3 months of age, AMBRA1 levels are lower than in WT, suggesting contribution to the onset of pathology, since around this age transgenic mice undergo the first neuronal deficits. Interpretation of this aspect is especially difficult, for several reasons. First of all, this age was considered as a pre-symptomatic stage, until recently (D'Amelio et al., 2011), thus the precise neuronal dysfunctions at this stage are yet to be characterized. Moreover, the D'Amelio's paper deals with the hippocampus and to date only one report describes specific features of the neocortex in this model (Cimini et al., 2009). Finally, the available literature on the role of the autophagic process in AD focuses on mouse models, which, differently from ours, display a fast progression of disease, not recapitulating the time course of human AD pathogenesis, thus preventing an accurate study of the onset of disease.

In our hands, the most advanced stage of Alzheimer-like pathology is characterized by enhanced expression of AMBRA1. At this age (18 months) neuronal dysfunction culminates with a neuropathological phenotype characterized by beta-amyloid plaque deposition and consequent disruption of cytoarchitecture (Jacobsen et al., 2006). An increase of AMBRA1 levels can be interpreted as an attempt to cope neuronal damage, possibly related to oxidative stress (Agostinho et al., 2010). The parallel increase of AMBRA1 and Beclin1 in 18-month old mouse neocortex is also totally consistent with the hypothesis taking that an induction of autophagy, coupled with an impairment of the clearance of autophagosomes, leading to

accumulation of AVs containing beta-amyloid toxic peptide occurs in symptomatic AD (Nixon et al., 2007).
The hippocampus, that is the primary and most profoundly affected area in AD pathology, shows a different trend of AMBRA1 levels, during ageing. In particular AMBRA1 expression undergoes a general decrease during ageing both in WT and transgenic mice. As observed in the neocortex, in physiological conditions the decrease becomes significant around 12 months of age, likely due to a downregulation of protein, concomitant to decreased neuronal plasticity. However, differently from the neocortex, at 18 months we failed to observe an AMBRA1 increase, likely attributable to a higher susceptibility of this area, that is unable to counteract oxidative stress by activating autophagy.
As to the pathological phenotype, it is worth noting that at 3 months of age, even though hippocampal protein levels, as assessed by WB, are similar to WT, the detailed morphological analysis reveals intriguing genotype-based differences in the intrahippocampal distribution of AMBRA1 protein. In particular, CA1 region in 3-month-old Tg2576 hippocampus displays less intensely stained neurons, than its WT counterpart. Interestingly CA1 field is mainly affected by neuronal deficits. By contrast, in the dentate gyrus of the same animal, AMBRA1-positive cells are more abundant than in WT, thus suggesting ongoing neurogenesis, since the immunorectivity increases from the sub-granular zone (SGZ), where the less differentiated cells are located, to the granular/molecular layers border where mature neurons are located. This result can be interpreted, in this neurogenic area, as a cell attempt to cope the insult.
In transgenic animals reduced AMBRA1 levels are observed at 6 months of age when long-term potentiation (LTP) deficits in hippocampal CA1 and DG and spatial memory deficits are detected, concomitant to Aβ oligomerization (Jacobsen et al., 2006). Our data, suggesting that low AMBRA1 levels contribute to the initial neuronal damage, may thus reflect impaired ability of the hippocampus to activate an adequate response to the toxic insult. However AMBRA1 expression further decreases between 12 and 18 months, suggesting that when the neuropahological phenotype occurs hippocampal neurons are subjected to a further dramatic drop of cellular response ability.
Interestingly, AMBRA1 and Beclin1 follow a different pattern, supporting a specific involvement of the former protein in AD pathology, not limited to autophagic regulation.

6.2.1 AMBRA1 in mouse of model of Parkinson disease

There is growing evidence that autophagic processes play important roles in the neuronal degeneration of processes and participate to regulated forms of cell death, particularly in large cells as the dopaminergic neurons of substantia nigra (REF zeldan?). It is unclear, however, whether increased AV content indicates an upregulation of the regulatory mechanisms of autophagy or it rather reflects an

engulfment of the autophagic machinery; in the latter scenario, an imbalance between rates of AV formation and degradation would lead to impaired autophagic turnover and vescicles accumulation. In general, dysregulation of the autophagic machinery has deleterious effects on the overall cellular function and has been referred to as "autophagic stress" (Chu et al., 2006). Previous reports demonstrated that the midbrain dopaminergic neurons of the nigrostriatal projection, which degenerate in PD, develop autophagic stress during genetic and idiopathic form of PD (Chu et al., 2006). In our study, we used the very well established MPTP model of PD as a toxic agent for dopaminergic neurons (Kuhn et al 2003). Administration of MPTP induces increased production of free radicals, which exert toxic effects on the cell. Because we were interested in mechanisms participating in the early stages of PD pathogenesis, we used a mild toxicological paradigm, in which low doses of MPTP do not induce nigral cell death, as reflected in the minimal loss of the enzyme Tyrosine Hydroxylase (TH). In addition, we studied AMBRA1 in a mouse model with defective NER, which recapitulates some important molecular features of DA in early pathogenic stages of PD. The choice of this model stems from the concept that the process of neurodegeneration is favored by ageing, which constitutes the major risk factor for PD. The process of ageing is associated with accumulation of DNA damage and consequent genomic instability as well as with increased levels of oxidized bio-molecules. Mutations in genes with fundamental function in NER have been associated with accelerated ageing and neurological defects; interestingly, NER defective mouse models exhibit a pronounced antioxidant response, which indicates that genome instability elicits alterations in the oxido-reductive homeostasis (Niedernhofer LJ et al., 2006, de Waard et al., 2010). Our results indicate that mild and chronic defects in NER lead to molecular phenotypes in DA neurons that resemble those observed in the MPTP model of PD. It is not surprising that the NER mutant line with a milder phenotype (i.e. ERCC1 Δ/+) recapitulates more accurately the features observed in DA neurons of the MPTP PD model. In fact, severe mutants (i.e. ERCC1 Δ/+) require a stronger cellular response to cope with the defect and the overall phenotype rather recapitulates systemic disorders, such as Cockayne syndrome, which is clinically very different from PD. Our results indicate that the ERCC1 Δ/+ mice might constitute a potential pre-symptomatic model of PD, in which molecular pathogenic cascades are activated without overt neurodegeneration, and it could be very useful to study early stages of the disease.. In fact, the observation

In both the MPTP and the NER defective models, we observed a remarkable increase in AMBRA1 expression. This result suggests an important role for this protein in the cellular response during early stages of PD stage, likely in the induction of the autophagy. AMBRA1 up-regulation was not confined to dopaminergic neurons of the SN, but was extended to those in the VTA, thus indicating that this mechanism is not selective for the anatomical regions. This evidence is in agreement with the modality of action of MPTP, which relies on the

presence of dopamine transporters and thus affects all dopaminergic neurons; in addition, it might indicate that DA neurons in the SN and VTA differ in their ability to tolerate cellular stress, including autophagic-stress. Several reports, in fact, have already demonstrated that dopaminergic neurons in the substantia nigra are more susceptible to oxidative stress (Horowitz et al., Chan et al., 2010); the same concept could apply to autophagy and therefore some cell populations (e.g. DA neurons in the VTA) might better tolerate AV accumulation. Importantly, the involvement of AMBRA1 in the pathogenensis of Parkinson's disease is also highlighted by a recent study demonstrating a direct physical interaction between AMBRA1 and Parkin, a protein associated to recessive forms of genetic Parkinson's disease (Van Humbeek et al., 2011). While this study does not provide details about the interaction in pathogenic conditions, it strongly supports a role for AMBRA1 in PD. The results presented in this thesis reinforce the latter concept, demonstrating that AMBRA1 up-regulation occurs not only in toxicological models of PD, but also in genetic models of ageing.

Chapter 7

Conclusions and future perspectives

In conclusion, the main findings of my PhD research project can be summarized as follows. First of all, the study provided the first neuroanatomical/histological/ultrastructural map of the distribution of AMBRA1 expression in mouse brain. Wide presence of the protein in the forebrain, midbrain, and hindbrain was observed, demonstrating prevalent expression in neurons, compared to glial cells. This suggests that in physiological conditions AMBRA1 is crucial for neuronal homeostasis. Ultrastructural analysis revealing localization of AMBRA1 in specific intracellular compartments, namely the endoplasmic reticulum strongly supports AMBRA1 activity in regulating basal autophagy, essential for neuron health. Further studies, implying colocalization of AMBRA1 with other molecules, namely Beclin1, dynein, and parkin, are required to ascertain the actual interactors of the protein in the nervous tissue, at specific intracellular sites (endoplasmic reticulum, cytoskeleton, mitochondria).

Detailed examination of different brain regions allowed me to show that AMBRA1 content varies among neuronal populations and subtypes. Our findings led us to conclude that the concentration of neuronal AMBRA1 is only partially related to cell volume and neurotransmitter type, and may be primarily correlated with other parameters, such as acute and chronic damage susceptibility. This speculation prompted us to further investigate the expression of AMBRA1 under physiological and pathological conditions involving the nervous tissue. More specifically, we highlighted important age-related variations in AMBRA1 expression in regions prone to neurodegeneration, such as the neocortex and hippocampus during normal and Alzheimer-like ageing.

In the neocortex, the high levels of AMBRA1 in normal young mouse led us to hypothesize that AMBRA1 is essential for a faultless maturation of neocortical neurons. Interestingly, the transgenic mouse model counterpart (Tg2576) shows lower AMBRA1 content at the very onset of disease, when most of the histopathological hallmarks are still undetectable, thus suggesting the possible involvement of AMBRA1 down-regulation in the impaired plasticity of neuronal circuits. The overall decrease of AMBRA1 content during normal adulthood may reflect a relatively balanced cell activity, with a stable regulation of biosynthesis and degradation pathways. The novel increase of the protein in the aged neocortex, which is independent of the genotype, may instead suggest that AMBRA1 is up-regulated in this critical period, since brain ageing likely requires a higher rate of basal autophagy also to counteract oxidative stress and consequent accumulation of damaged organelles. However, this putative response does not necessarily imply enhanced autophagic efficiency, as the events downstream the formation of

autophagosome could be insufficient to successfully complete the clearance process.

It is important to emphasize that differences in AMBRA1 expression are not only related to age, but even to the specific brain region, since the the neocortex and the hippocampus show different behavior during ageing and pathological progression. In particular, overall levels in young mouse hippocampal formation are similar in the two genotypes. However, a different distribution in hippocampal subregions of Tg2576 animals compared to WT was detected. In fact, the neurogenic region of dentate gyrus is more intensely AMBRA1 immunoreactive in the transgenic animals than in WT, thus suggesting that in the pathological condition an early response involving enhanced neurogenesis may occur to cope the first deficits. Then we found a decrease in AMBRA1 level during adulthood and ageing in both genotypes. Remarkably, at 18 months the transgenic hippocampus shows a further drop in AMBRA1 reactivity, possibly contributing to neurodegeneration.

Our study on the expression of AMBRA1 in mouse models of Parkinson's disease demonstrates that AMBRA1 expression can be induced in dopaminergic neurons by a mild MPTP treatment. Importantly, similar effects are also observed in a genetic model of genomic instability (ERCC1 mutants) which is associated with an early PD-like phenotype. These findings support the involvement of AMBRA1 in cellular response against oxidative imbalance in dopaminergic neurons that are closely related to PD onset. Surprisingly, AMBRA1 levels increase in both the substantia nigra (SN) and ventral tegmental area (VTA). Since neurons in SN turn to cell death in PD while VTA dopaminergic neurons are spared, one could envision that the activation of the autophagic pathway by AMBRA1 up-regulation, can be defensive in some cases and detrimental in others, strictly depending on the specific neuronal population. To this respect, it is worth noting that the features of neuronal cell death in PD are still debated, because it seems to bring back to autophagic cell death.

Taken together, the results obtained suggest that AMBRA1 is a fundamental molecule in the nervous tissue, given the abundant content in neurons, although to different degree with respect to the brain area and neuronal population. In addition, modulation of AMBRA1 expression may be critical for establishing, promoting or counteracting neurodegenerating processes. In this view, our study opens the way to further investigations aimed to defining the precise contribution of AMBRA1 to nervous tissue development, homeostasis and response to acute or chronic injury. It will be necessary to develop conditional mutant mouse models to clarify these issues and explore novel functions of AMBRA1 in the central nervous system, related or not to autophagy.

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