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Rôle of Autophagy in Mediating Neuronal Cell Death in Preterm Hypoxic- Ischemic Injuries, Literature Review and Expérimental Study

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Role of Autophagy in Mediating Neuronal Cell Death in Preterm Hypoxic-Ischemic Injuries, Literature Review and Experimental Study

THESE

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"Neuronal death after perinatal cerebral hypoxia-ischemia: Focus on autophagy-mediated cell death"

L'encéphalopathie hypoxique-ischémique néonatale est une condition critique pouvant survenir à la naissance, ainsi qu'une cause importante de mortalité et de morbidité neurologique en lien avec des séquelles invalidantes à long-terme. Elle touche 1-2 pour 1000 naissances vivantes en Suisse. Considérant les importantes conséquences cliniques et le manque de stratégies neuroprotectrices efficaces, il est urgent de mieux comprendre les différents mécanismes de mort cellulaire impliqués ainsi que leurs interconnections, dans le but de développer de nouvelles approches thérapeutiques. Les caractéristiques morphologiques des trois différents mécanismes principaux de mort cellulaire peuvent toutes être observées dans des modèles animaux de lésion cérébrale hypoxique-ischémique périnatale : mort cellulaire nécrotique, apoptotique et autophagique. Ces différents types de morts cellulaires sont le plus souvent retrouvés de manière combinée dans le même neurone agonique. Dans cette revue, nous discutons les différents mécanismes de mort cellulaire impliqués dans l'hypoxie-ischémie cérébrale néonatale, avec une attention particulière portée à la manière dont l'autophagie pourrait être impliquée dans la mort neuronale. Nous nous basons à la fois sur des modèles expérimentaux d'hypoxie-ischémie périnatale et d'accident vasculaire cérébral ainsi que sur des études autoptiques menées sur des cerveaux de nouveau-nés humains ayant souffert d'hypoxie-ischémie néonatale.

Neuronal death after perinatal cerebral hypoxia-ischemia: Focus on autophagy—mediated cell death

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ABSTRACT

Neonatal hypoxic-ischemic encephalopathy is a critical cerebral event occurring around birth with high mortality and neurological morbidity associated with long-term invalidating sequelae. In view of the great clinical importance of this condition and the lack of very efficacious neuroprotective strategies, it is urgent to better understand the different cell death mechanisms involved with the ultimate aim of developing new therapeutic approaches. The morphological features of three different cell death types can be observed in models of perinatal cerebral hypoxia-ischemia: necrotic, apoptotic and autophagic cell death. They may be combined in the same dying neuron. In the present review, we discuss the different cell death mechanisms involved in neonatal cerebral hypoxia-ischemia with a special focus on how autophagy may be involved in neuronal death, based: (1) on experimental models of perinatal hypoxia-ischemia and stroke, and (2) on the brains of human neonates who suffered from neonatal hypoxia-ischemia.

1. Neonatal hypoxic-ischemic encephalopathy

Neonatal asphyxia is one of the most prominent long term invalidating insults occurring around birth. The cerebral injury after neonatal hypoxia-ischemia (HI) appears to be responsible for nearly 25% of all cases of developmental disabilities in children such as cerebral palsy, mental retardation, learning disturbances and neurosensorial deficits (Vannucci and Hagberg, 2004). The known etiologies are multiple intrapartum events including placental abruption, maternal infection, umbilical cord prolapse or vasa praevia rupture (Rennie et al., 2008) all inducing an insufficient supply of oxygen and blood to the central nervous system, thus potentially leading to cerebral tissue lesions. The pathophysiological mechanisms of perinatal stroke, as another prominent perinatal cerebral pathology, are similar to those of neonatal asphyxia, but in this brief review we will mainly focus on the latter.

A severe global asphyxia may affect numerous brain regions, especially the perirolandic cortex, basal ganglia, thalamus, hippocampus and rarely the cerebral pons (Johnston et al., 2005). The clinical cerebral entity observed is termed hypoxic-ischemic encephalopathy (HIE), with loss of tonus and archaic reflexes, epileptic seizures, diminished levels of consciousness and impaired breathing (Ferriero, 2004).

To understand the different cellular mechanisms leading to the neurological sequelae later in life, it is crucial to ask at least three questions: why does a neuron die after hypoxic-ischemic events, when does it die and how or by which cell death mechanisms does it die?

In the following paragraphs, we will attempt to give partial answers to these three questions, based on animal models of perinatal cerebral hypoxia-ischemia as well as human data and focusing on autophagy-mediated cell death mechanisms.

2. Why and when does a neuron die after hypoxia-ischemia?

2.1. Pathophysiology

2.1.1. Primary versus secondary energy failure

The concept of the biphasic evolution of high energy phosphates such as phosphocreatine and ATP after an hypoxic-ischemic event

was developed in the late 1980s and consolidated in the early 1990s, opening the way for new protective approaches and defining more precisely the therapeutical window, thought to be between 6 and 48 h according to the species and depth of asphyxia (Lorek et al., 1994; Nedelcu et al., 1999; Thornton et al., 2012).

Primary energy failure takes place immediately after the hypoxic event, very abruptly, and corresponds at the cellular level to necrotic cell death, which is irreversible, and on MRI is noted as cytotoxic edema. This first energy decrease is thus hard to prevent other than through optimizing the training and skills of the people taking care of pregnant women and their newborns.

A second energy failure (associated with reperfusion injury) starts after 24 h, with a peak around 48 h, after which the brain energy level returns to near-normal values. This reperfusion stage is associated with the production of toxic substances such as free radicals, and the activation of multiple cascades and transcription factors, with subsequent programmed cell death. On MRI, it is also known as vasogenic edema (on diffusion-weighted imaging) (Vannucci and Hagberg, 2004; Yager and Ashwal, 2009). In attempts at neuroprotection, it is the reperfusion injury phase that is the most targeted nowadays.

Since 2006, moderate hypothermia has been acknowledged as evidence based medicine for application in moderate to severe cerebral hypoxic-ischemic encephalopathy (HIE), and is now widely used as a therapeutic approach (Edwards et al., 2010; Gluckman et al., 2005; Gunn, 2000; Xiong et al., 2013) in the first 72 h after the event. While mortality was reduced in several large studies (Azzopardi et al., 2014; Gluckman et al., 2005; Shankaran et al., 2005), the neurological outcome benefit at 18 months was lost at 6–7 years of age in one study and maintained in another study (Shankaran et al., 2012).

2.1.2. Excitotoxicity

One of the central deleterious processes implicated in neuronal damage after hypoxic-ischemic brain injuries is *excitotoxicity* which is also involved in other cerebral pathologies such as epilepsy, traumatic brain injuries or neurodegenerative diseases (Sattler and Tymianski, 2000). It represents an excessive and prolonged activation of excitatory amino acid receptors, especially those for glutamate and in particular N-methyl-D-aspartate (NMDA) receptors (Olney and Ho, 1970; Vannucci and Hagberg, 2004). Failure in glutamate reuptake combined with excessive release at the synaptic level lead to glutamate accumulation, overactivation of glutamate receptors and massive entry of calcium (Lau and Tymianski, 2010). The increased intracellular calcium concentration triggers the activation of numerous downstream enzymes (proteases, lipases, kinases. . .) leading to cell damage by numerous mechanisms including oxygen and nitrogen reactive species formation, membrane destruction, impairment of organelles and/or DNA damages (Sattler and Tymianski, 2000).

The immature brain is particularly susceptible to excitotoxicity. During brain development NMDA receptors show a higher density (McDonald et al., 1988) and are more readily activated due to their higher permeability to calcium, greater responsiveness to glycine activation and lower sensitivity to Mg^{2+} blockade in comparison to mature receptors (Gurd et al., 2002; Johnston et al., 2005). This is crucial for synaptic plasticity and brain maturation (Herlenius and Lagercrantz, 2001), but renders neurons more vulnerable to excitotoxic stimuli. Moreover α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are usually impermeable to calcium, except when the GluR2 subunit is lacking, which is mainly the case in immature brains. In both white and gray matter, the periods of high GluR2 deficiency correspond to those of regional susceptibility to hypoxic/ischemic injury in young rats (Talos et al., 2006).

2.1.3. Reactive oxygen species (ROS)

Oxidative stress is another important damaging mechanism leading to HI cerebral damage. It is closely related to excitotoxicity since excessive calcium influx can activate different enzymes involved in ROS production such as neuronal nitric oxide synthase or xanthine and NADPH oxidases. Dysfunction in mitochondrial oxidative phosphorylation is a main cause of excessive formation of free radicals (superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radical (OH^-) and peroxynitrite (NOO^-)). ROS are extremely toxic for the surviving cells since natural defense systems are overwhelmed in HI conditions. ROS accumulation triggers a cascade of cytotoxic events including protein nitrosylation and oxidation, lipid peroxidation, membrane destruction and DNA damage. It increases blood-brain-barrier permeability (Baburamani et al., 2012; Pun et al., 2009) and activates inflammation through the release of cytokines and chemokines (from reactive astrocytes, activated microglia, neurons and endothelial cells) that are involved in leukocyte recruitment. Oxidative stress can lead to a massive secondary phase of tissue loss over the next few days or even weeks after the primary injury (Ferriero, 2001). Immature brains are particularly sensitive to oxidative stress, as their antioxidant enzymatic capacities are lower than in adults and as they have higher concentrations of free iron and unsaturated fatty acids (Haynes et al., 2005; Vannucci and Hagberg, 2004).

2.1.4. Inflammation

Finally a third central factor that can play a role in long term cerebral injury of the newborn is inflammation, *i.e.* mainly microglial activation and leukocyte recruitment. It acts via cytokines like TNF-α, IL-1β and IL-6 that are able to act locally but also to spread the inflammatory reaction and thus increase the vulnerability of the rest of the brain tissue (Fleiss et al., 2015; Hagberg et al., 2015). This thematic is discussed elsewhere in this issue and is therefore not further developed here.

3. How does a neuron die after perinatal asphyxia?

3.1. Necrosis and apoptosis in neonatal HI

The morphological features of three different cell death types can be observed following HI, even in the same dying neuron (Northington et al., 2011): necrotic, apoptotic and autophagic cell death (Fig. 1). Whereas apoptotic and necrotic cell death are well-known cell death types that have been the most studied in perinatal HI models (Northington et al., 2011), the importance of autophagy-mediated cell death is a recent field of interest that is starting to be elucidated (Ginet et al., 2014a; Ginet et al., 2014b; Puyal et al., 2013).

3.2. Necrotic cell death

Necrotic cell death (or type 3) represents the earliest death mechanism in the context of cerebral ischemia and excitotoxicity since it is associated with the initial massive influx of calcium into the neuron's cytoplasm (Northington et al., 2011). Necrotic cell death, which is characterized by a cellular swelling that can lead to cell lysis, is already seen as early as 30 min after the insult. It is triggered by abrupt energy failure with notably ATP depletion. No specific markers of necrotic cell death are known, but calcium-dependent activation of calpains is suggestive of it and can be detected through the presence of calpain-dependent α-fodrin fragments around 150 kDa in immunoblot (Neumar et al., 2001). Electron microscopy is the only definitive method for diagnosing necrotic death, whose specific features include organelle dilatation, vacuolization of the cytoplasm and nuclear fragmentation (Clarke, 1990; Puyal et al., 2013). Necrotic cell death plays a
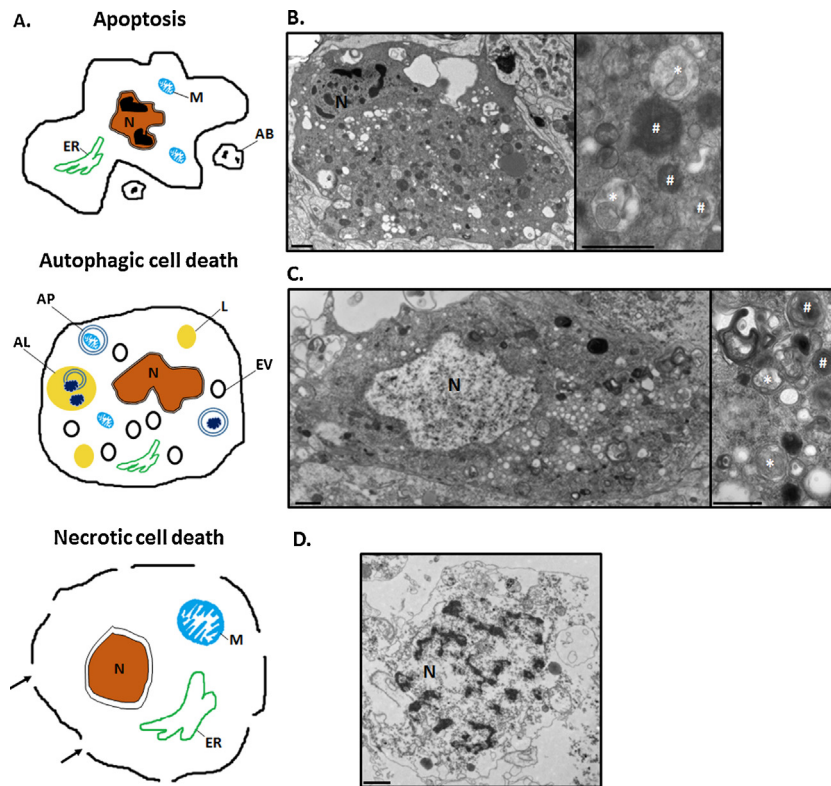
Fig. 1. Main morphological features of the 3 different cell death pathways: apoptosis, necrosis and autophagic cell death. (A) Apoptosis is characterized by nuclear and chromatin condensation, cytoplasmic shrinkage and the presence of apoptotic bodies. Necrotic cell death shows swollen organelles (mitochondria, endoplasmic reticulum. . .) and lysis of the plasma membrane. Autophagic cell death displays numerous autophagosomes and autolysosomes, and often also endosomes and empty vacuoles. (B–D) Electron micrographs illustrate the different cell death features that could be observed in dying neurons after neonatal HI (p7 rat). (B) Dying neuron in the thalamus at 24 h after neonatal HI showing mixed features of both apoptosis and enhanced autophagy. (C) Dying neuron in the CA3 region of the hippocampus at 24 h after neonatal HI displaying strong autophagic features. (D) Dying neuron in the CA1 of the hippocampus at 24 h after neonatal HI with mixed morphological characteristics of apoptosis and necrosis. *: autophagosome; #: autolysosome. N: nucleus; ER: endoplasmic reticulum; M: mitochondrion; AP: autophagosome; AL: autolysosome; EV: empty vacuole; L: lysosome. Scale bars: 1 μm.

central role in acute brain lesions, as has been shown in several adult models (Mehta et al., 2007), but also in models of neonatal HI (Carloni et al., 2007; Nakajima et al., 2000; Northington et al., 2001). Some post-mortem neuropathological studies have indicated the presence of necrotic-like cells in human perinatal brain injuries (Northington et al., 2011; Takizawa et al., 2006). Since necrosis occurs at the very beginning of the hypoxic-ischemic event, when the levels of oxygen and nutrients fall below critical values, it might be thought an unpromising target for neuroprotection as the lesion could often be constituted by the time the diagnosis is made. However, even with necrosis, the therapeutic window seems to be relatively large in immature brains (up to 6 h), thus the clinical potential of necrosis inhibition remains interesting (Degterev et al., 2005; Markgraf et al., 1998; Mehta et al., 2007; Puyal et al., 2013). While the original descriptions of necrosis in the 1980s pointed to an unprogrammed and passive process, active mechanisms are now known to be involved in many cases of it, including hypoxic and/or ischemic neurons, whose necrotic death pathways can involve calpains (Yamashima et al., 2003), c-jun N-terminal kinases (Arthur et al., 2007) or even caspases (Niquet et al., 2003). One of the best studied form of active (regulated) necrosis is necroptosis (Yuan and Kroemer, 2010), which has been shown to occur in neonatal HI (Thornton et al., 2012). The molecular pathways mediating necroptosis can be triggered by death receptors whose ligands can also be involved in apoptosis (such as TNFα, FasL and TRAIL) (Vandenabeele et al., 2010; Yuan and Kroemer, 2010) and by toll-like receptors (Galluzzi et al., 2014). After death receptor stimulation and when caspase-8 is inhibited, reduced or absent, RIPK1 and 3 (receptor-interacting protein kinase) and MLKL (mixed

lineage kinase domain-like) form the necrosome, a signaling complex mediating necroptosis (Galluzzi et al., 2014; Linkermann and Green, 2014). The regulated forms of necrosis seem to be relatively delayed and could represent an interesting therapeutic target. Neuroprotection against perinatal HI has been achieved with inhibitors of calpains (Kawamura et al., 2005) or of necroptosis up to 6 h after the injury (Degterev et al., 2005; Northington et al., 2011).

3.3. Apoptosis

Apoptotic features are often observed in excitotoxic injuries, such as cerebral ischemia and neonatal asphyxia (Northington et al., 2011; Puyal et al., 2013), including in human HIE (Northington et al., 2011; Taniguchi et al., 2007). As has been well described, apoptosis is a programmed and delayed death mechanism that can be mediated by either of two intracellular signaling pathways, the intrinsic and the extrinsic pathways.

a The intrinsic pathway is mediated by a release of apoptogenic factors (cytochrome-c, Smac/Diablo, apoptosis inducing factor (AIF), endonuclease G (endoG), Omi/HtrA2) from the mitochondria after membrane permeabilization that trigger caspase-dependent and -independent cell death processes. Bcl-2 family proteins including the pro-apoptotic Bax, Bim and Bak and the anti-apoptotic Bcl-2, Bcl-XL and Mcl-1 are implicated in the regulation of mitochondrial membrane permeabilization (Thornton et al., 2012). Cytochrome-c release will result in initiator caspase-9 activation that activates the main effector caspases, caspase-3, -6 and -7 (Broughton et al., 2009; Fan et al., 2005;

Thornton et al., 2012). Smac/Diablo and Omi/HtrrA2 contribute to caspases activation by preventing IAP (Inhibitor of Apoptosis Protein) family of proteins to interact and to inhibit effector caspases. Other molecules released from mitochondria can activate caspase-independent pathways; these include AIF (apoptosis inducing factor) and the endonuclease endoG, which are both translocated to the nucleus and mediate DNA damage.

b The extrinsic pathway depends on the activation of death receptors whose ligands include cytokines such as tumor necrosis factor-alpha (TNFα). It involves the action of the initiator caspases-8 and -10 that activate effector caspases directly and/or by interacting with the intrinsic pathway after the caspase-mediated cleavage of Bid (a BH3-only Bcl-2 family member) and subsequent mitochondrial membrane permeabilization.

All of these apoptotic mechanisms are known to be more prominent in immature brains than in adult ones (Zhu et al., 2005). After HI and especially in immature brains, the apoptotic pathways are strongly activated and seem to be involved especially in the secondary phase of the insult described under point 2.1. (Nakajima et al., 2000; Northington et al., 2011). Apoptosis thus represents a potential target for neuroprotective interventions within the therapeutic window. Indeed several experimental studies found that treatment with pan-caspase inhibitors or specific ones led to a reduction of the lesion and a better neurologic outcome in both adult cerebral ischemia (Akpan and Troy, 2013; Mehta et al., 2007) and neonatal HI (Adachi et al., 2001; Carlsson et al., 2011; Chauvier et al., 2011; Feng et al., 2003) models. However, some of these authors applied the anti-apoptotic drugs before the injury, which is clinically less relevant, and different models of perinatal HI showed controversial results (Gill et al., 2002; Joly et al., 2004; Koike et al., 2008; Puyal et al., 2009). The neuroprotection afforded by caspase inhibitors depends on the type and severity of the ischemic insult, as also shown in adult models (Li et al., 2000) and caspase inhibitors remain rather unspecific, which restricts the interpretation of the results (Northington et al., 2011). Until now, no caspase inhibitor has been tested in a clinical trial.

4. Autophagy and cell death

We will first briefly introduce the molecular mechanisms involved in autophagy and then discuss the role of autophagy in neuronal death specifically in neonatal cerebral HI (see Section 5).

4.1. Overview of the molecular mechanisms of autophagy

Autophagy is an essential intracellular catabolic pathway that functions in parallel with the ubiquitine-proteasome system (Feng et al., 2014). There are three types of autophagy that all lead to degradation in lysosomes and recycling (Feng et al., 2014; He and Klionsky, 2009). (1) *Microautophagy* implicates the engulfment of part of the cytoplasm directly by the lysosomal membrane. (2) *Chaperon-mediated autophagy* is a more specific mechanism that targets proteins bearing a precise amino-acid sequence in order to target them directly to the lysosome. (3) *Macroautophagy* (hereafter called autophagy) involves the participation of an intermediate multimembranous compartment, the autophagosome, which then fuses with a lysosome for degradation of its content. During autophagosome formation, part of the cytosoplasm is engulfed, containing long-lived proteins and/or defective organelles (mitochondria, endoplasmic reticulum, etc). Autophagy is not as nonspecific as it was originally thought, since some proteins on the autophagosomal membrane (such as SQSTM1/p62, NBR1, optineurin. . .) serve as adaptor proteins and target some ubiquitinated substrates including mitochondria (mitophagy) or misfolded proteins (Johansen and Lamark, 2011).

Autophagy can be divided into three main steps: *nucleation* (induction), *autophagosome formation* (elongation, incurvation, closure) and *maturation* (fusion with a lysosome and formation of an autolysosome) (Feng et al., 2014; He and Klionsky, 2009). An overview of the molecular mechanisms is described in Figure 2. Over 35 autophagic genes (named Atg: autophagic related genes) have been discovered in yeast and most of them have homologues in mammals.

4.1.1. Nucleation (induction)

This first step is the formation of the pre-autophagosomal membrane (also called *phagophore* or *isolation membrane*), which can be derived from different organelles (mitochondria, endoplasmic reticulum and/or Golgi apparatus) and possibly from the plasma membrane. The pre-autophagosomal membrane serves as a platform for recruiting most of the Atg proteins necessary to the formation of an autophagosome. Its isolation can be induced in different conditions including under stress stimuli such as deprivation of nutrients/energy (Hosokawa et al., 2009), hypoxia/ROS exposure (Chen et al., 2009; Scherz-Shouval et al., 2007) or intracellular calcium increase (Gao et al., 2008; Hoyer-Hansen et al., 2007). Among the downstream molecular regulators, the serine/threonine kinase mTor (mammalian target of rapamycin) is a central player and its inhibition (*e.g.* by rapamycin) activates autophagy. Complex molecular mechanisms involving interactions between several Atg proteins are implicated in autophagy. Briefly, the ULK1/2 (mammalian homolog of Atg1) complex, whose activation depends on mTor inhibition and AMPK phosphorylation (Shang and Wang, 2011), plays an important role in autophagy induction and probably in Atg protein recruitment. The class III PI3K/Beclin1 (Atg6) complex produces phosphatidylinositol 3-phosphate (PI3P) favoring Atg protein recruitment especially when the complex contains Atg14L. It can also contain UVRAG, which is important for autophagosomal membrane deformation and curvation (Itakura et al., 2008).

4.1.2. Autophagosome formation

The autophagosome formation involves the elongation and incurvation of the pre-autophagosomal membrane followed by its closure to form the double-membraned autophagosome. The ubiquitin-like conjugation system of Atg12-Atg5-Atg16L, whose formation depends on Atg7 and Atg10, is important for the elongation process and for the recruitment and formation of a second ubiquitin-like conjugation system containing a microtubule-associated light chain 3 protein (LC3) (Fujita et al., 2008). This LC3 (mammalian homolog of Atg8) conjugation system acts on the lengthening and closure of the autophagosome (Nakatogawa et al., 2007; Sou et al., 2008). Similarly to ubiquitination, the cytosolic Atg4-dependent cleaved form of LC3 (LC3-I form) is conjugated by Atg7 and Atg3 to phosphatidylethanolamine (PE), an abundant membrane phospholipid, that allows LC3 to be incorporated into the autophagosomal membrane (LC3-II form) (Geng and Klionsky, 2008).

4.1.3. Autophagosome maturation

Whereas most of the known Atg proteins are implicated in the early phases of autophagy, a few of them have been shown to act in autophagosome maturation. Since autophagosomes sometimes fuse with endosomal compartments (early endosomes, multivesicular bodies, late endosomes) prior to their fusion with a lysosome (Simonsen and Tooze, 2009), some endocytic proteins (Rab, COPs, ESCRT, LAMP and SNARE proteins) occur in autophagosomal membranes and are involved in their maturation (Mehrpour et al., 2010). The resulting acidification allows lysosomal enzymes (such
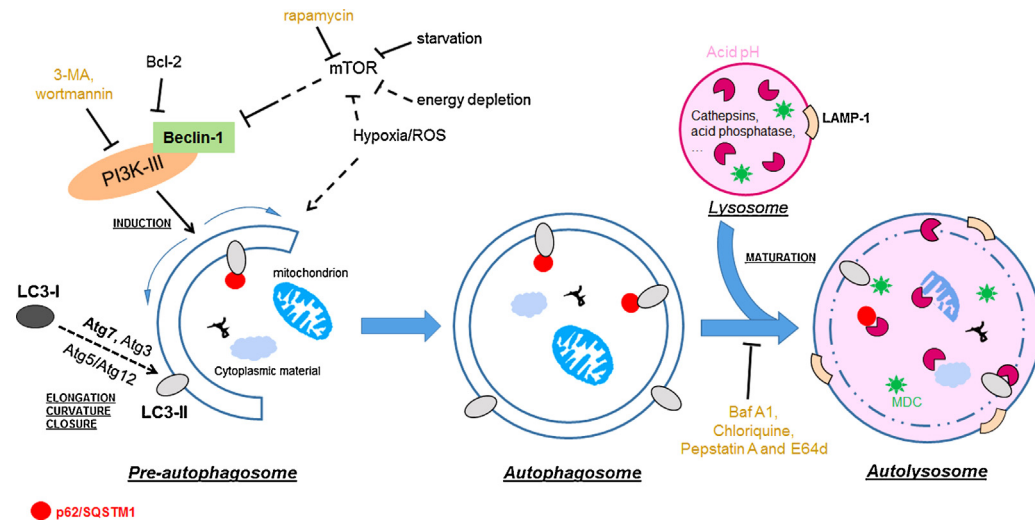
Fig. 2. Overview of the main molecular pathways involved in macroautophagy. This scheme highlights the main markers that can be used *in vivo* to monitor autophagy. First the nucleation is induced by the complex Beclin-1(Atg6)/PI3K-III. The progress of the incurvation and elongation phase is notably controlled by LC3-II (Atg8) that determines the sizes of the autophagosomes. The transformation of the cytosolic LC3-I into the membrane-bound form LC3-II is mediated by different complexes such as Atg7 or Atg5/Atg12. The compartment is then acidified and hydrolases such as cathepsins are recruited by fusion with a lysosome. Monodansylcadaverine (MDC), an acidotropic dye, is incorporated into acidic endosomes, lysosomes and autophagosomes and can be used as a marker of late autophagosomes.

as cathepsins, acid phosphatases or β-hexosaminidase) to degrade and recycle the autophagosome content.

In order to demonstrate that autophagy is enhanced, it is important to investigate the whole autophagic pathway, not only the increase in autophagosomal markers such as LC3-positive dots in immunohistochemistry and LC3-II level in immunoblots. Increased autophagic degradation must also be shown since an accumulation of autophagosomes might also be due to a defect in fusion with lysosomes. For this reason, it is important to obtain evidence for an increase in autophagic flux. To demonstrate increased degradation *in vivo* is often challenging, but possible approaches include: measuring the expression level of p62/SQSTM1, a long-lived protein preferentially degraded by autophagy, evaluating lysosomal enzyme activities (such as acid phosphatase), quantifying the numbers and sizes of lysosomes (labelled with MDC, LAMP1, cathepsins. . .), using lysosomal enzyme inhibitors or measuring the expression of an autophagy flux reporter such as Cherry-GFP-LC3 (Castillo et al., 2013; Puyal et al., 2013).

4.2. Dual role of autophagy

As mentioned above autophagy is a very complex molecular mechanism that plays an important role in physiological and vital functions including cellular homeostasis, and cell growth and differentiation during the developmental period. Whereas a basal level of autophagy is essential for the well-being of cells especially neurons, its induction can lead to opposite effects depending on the level of activation, the stimulus and the cell type (Wang et al., 2008). The best-known role of enhanced autophagy is a protective reaction to promote cell survival by providing an alternative source of energy in cells deprived of nutrients. It also promotes survival by its capacity to eliminate toxic metabolites or intracellular pathogens (He and Klionsky, 2009; Levine and Klionsky, 2004; Uchiyama et al., 2008). However, in pathological situations autophagy can also promote cell death (see Section 4.3) (Clarke and Puyal, 2012; Edinger and Thompson, 2004; Galluzzi et al., 2007; Lockshin and Zakeri, 2004; Maiuri et al., 2007b; Northington et al., 2011).

This dual role of autophagy is particularly important in neurons. In the central nervous system, an excessively low basal autophagic activity leading to decreased turnover of proteins is associated with the development of neurodegenerative diseases (Komatsu et al.,

2006; Puyal et al., 2012). In contrast, excessive activity of autophagy has been involved in cell death occurring naturally during developmental morphogenesis (Boland and Nixon, 2006) or in acute neurological disorders like cerebral HI (Ginet et al., 2014b; Liu and Levine, 2015; Koike et al., 2008; Puyal and Clarke, 2009; Xing et al., 2012).

4.3. Autophagy in cell death

The life promoting roles of autophagy are well known and accepted but a consensus about its pro-death roles has been hard to find. An increase in autophagy in dying cells could be interpreted as a last attempt for survival. Ambiguities due to the dual role of autophagy (see above) and inconsistent use of the term "autophagic cell death" have fueled the controversy (Clarke and Puyal, 2012; Kroemer and Levine, 2008; Yuan and Kroemer, 2010). The discovery of *atg* genes and the possibility of manipulating them (by knock-out or knockdown) strongly contributed to determining specifically the roles of autophagy, and this has led to evidence that autophagy can be involved in cell death in one of two ways: as a trigger leading to apoptotic or necrotic cell death (autophagy-mediated cell death), or less frequently, as an independent molecular mechanism of cell death morphologically different from apoptosis and necrosis (autophagic cell death or type 2). In this latter case, the autophagy seems to be abnormally enhanced, leading to degradation of important parts of the cell or even its nucleus (Clarke, 1990). Enhanced autophagy can be considered as an autonomous cell death pathway if its inhibition reduces or delays the cell death in the absence of signs of apoptosis or necrosis (Clarke and Puyal, 2012; Puyal et al., 2013). To date, whereas autophagy-mediated cell death and autophagic cell death could easily be recognized by using morphological criteria, the molecular pathways triggering both are poorly understood. Beside the fact that the autophagic flux should be increased in both cases, it is not clear whether similar or different Atg–Atg protein interactions are involved. It remains essential to elucidate which molecular mechanisms can shift autophagic machinery towards one of these two cell death pathways. However, it has been shown recently that cardiac glycosides, known inhibitors of Na$^+$/K$^+$-ATPase, are able to prevent a form of autophagic cell death called autosis both *in vitro* and *in vivo* (Liu et al., 2013).

Survival promotion

Growth, differentiation
Alternative source of energy
Recycling of damaged organelles / proteins
Elimination of toxic metabolites or intracellular pathogens

Autophagy

Cell death

Energy depletion

Apoptosis
(Caspase dependent / independent)

Energy depletion

Necrosis
Including Necroptosis
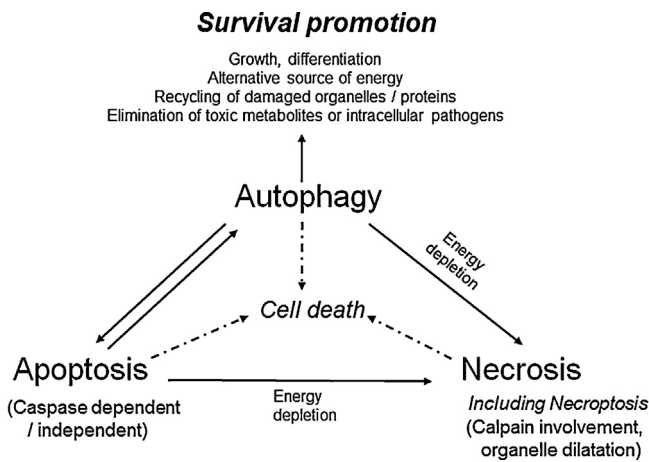(Calpain involvement, organelle dilatation)

Fig. 3. Interconnections between cell death mechanisms in neonatal cerebral HI.

An important difficulty is that autophagy-mediated cell death often involves mixed features of autophagy and apoptosis/necrosis. While crosstalk between necrosis and apoptosis exists (Galluzzi et al., 2007; Northington et al., 2011), there is also evidence that autophagy interacts with both of them (see Fig. 3). In some specific situations, as when cells are unable to perform apoptosis, autophagy can promote death by necrosis (Ullman et al., 2008). In excitotoxic and hypoxic-ischemic conditions, mixed forms of cell death sharing autophagic and apoptotic features are more often observed than pure autophagic cell death (see Section 5.2). This can be partly explained by the fact that the autophagic and apoptotic pathways share numerous common mediators and molecular interconnections, creating a complex cross-talk. Some autophagic proteins (Beclin1, Atg5, Atg4D, Atg1, Atg7...) and apoptotic molecules (Bcl-2, Flip proteins, Puma, Bax, p53, FoxO1...) have been shown to be implicated in the regulation of both autophagy and apoptosis (Delgado et al., 2014; Marino et al., 2014; Nikoletopoulou et al., 2013). One of the best known regulatory interconnections is the formation of complexes between the anti-apoptotic proteins Bcl-2, Bcl-XL or Mcl-1 with the autophagic protein Beclin1, which inhibit autophagy by sequestering Beclin1. Beclin1 release from these complexes by competitive interaction with different pro-apoptotic BH3-only proteins such as Bax, Bim, Nip3, Nix, Noxa and Puma promotes autophagy (Maiuri et al., 2007a). Among other examples, the protein p53, a strong apoptosis inducer, is able to regulate autophagy (Tasdemir et al., 2008), whereas it has been proposed that Atg7 can affect p53 activity (Lee et al., 2012). It has also been suggested that autophagosomal membranes can serve as a platform for caspase-8 activation (Young et al., 2012).

5. Autophagy mediated cell death in neonatal HI

It is now commonly accepted that autophagy is enhanced in dying neurons in both *in vitro* (Borsello et al., 2003; Dong et al., 2012; Matyja et al., 2005; Sadasivan et al., 2010), and *in vivo* models of excitotoxicity, including intracerebral kainate (Ka) injection (Chang et al., 2012; Shacka et al., 2007; Wang et al., 2006; Wang et al., 2008) as well as in adult models (Adhami et al., 2006; Cui et al., 2012; Degterev et al., 2005; Nitatori et al., 1995; Tian et al., 2010; Xu et al., 2013) and in models of perinatal cerebral HI (Ginet et al., 2009). However, as discussed above (see Section 4.3), the functional role of enhanced neuronal autophagy remains a subject of debate. Part of the problem is that autophagic flux is rarely correctly examined and/or that the role of autophagy is deduced from the use

of pharmacological inhibitors with often questionable specificity (Klionsky, 2012).

5.1. Lessons from *in vitro studies*

We here highlight briefly some important insights from *in vitro* studies in order to give a complementary view of the role that autophagy can play in hypoxic/excitotoxic conditions before addressing the subject in more relevant *in vivo* models in which the study of autophagic flux is more difficult (see Sectiont 5.2 and Table 1).

Oxygen-glucose deprivation (OGD) and exposure to an excito-toxin (NMDA, glutamate, Ka), are the most widely used *in vitro* models of cerebral HI. In both models, increased autophagosome presence has been observed (Borsello et al., 2003; Dong et al., 2012; Shi et al., 2012). Borsello et al. observed the appearance of numerous double membrane vacuoles and an increase in acid phosphatase in the same dying neurons in organotypic hippocampal cultures of P5 rats exposed to NMDA, suggesting an increased autophagic flux (Borsello et al., 2003).

Concerning the functional role of enhanced autophagy in *in vitro* models, there have been discrepancies between studies arguing for a protective (Kulbe et al., 2014; Perez-Carrion et al., 2012; Zhang et al., 2013) or deleterious (Dong et al., 2012; Ginet et al., 2014b; Kim et al., 2009; Kubota et al., 2010; Meloni et al., 2011; Puyal et al., 2013) effect of enhanced autophagy. However, very few investigated the autophagic flux or used genetic methods to inhibit or enhance autophagy. Other factors that may have contributed to these discrepancies are differences in the time points assessed, the stimuli used, the cell types (primary neuronal cultures *versus* neuronal cell lines), the culture conditions and the dose and specificity of the pharmacological inhibitors/inducers of autophagy. As an *in vitro* model of cerebral HI, our group developed a model involving the cytotoxic combination of Ka and hypoxia (KaHx) in primary cortical neurons cultures (Ginet et al., 2014b). We demonstrated that neuronal death occurs in the presence of enhanced autophagic flux by different methods (enhanced SQSTM1/p62 degradation, increased LC3-II levels in the presence of lysosomal inhibitors and increased autophagosome maturation shown using an RFP-GFP-LC3 reporter). We also demonstrated that KaHx-enhanced autophagy was involved in neuronal death since inhibition of autophagy, either by pharmacological inhibitors (3-methyladenine or pepstatinA/E64) or by silencing of Atg7 or Beclin1, was neuroprotective, whereas overexpression of Atg7 or Beclin1 increased the vulnerability of the neurons to KaHx.

5.2. Hypoxia-ischemia and autophagy *in vivo*

As research on *in vivo* models of adult focal cerebral ischemia had shown enhanced autophagy in the affected neurons (Adhami et al., 2006; Cui et al., 2012; Degterev et al., 2005; Nitatori et al., 1995; Rami et al., 2008; Tian et al., 2010; Xu et al., 2013), this question was also addressed in less mature brains. In the field of perinatal brain lesions, different animal models have been developed in order to define the molecular mechanisms implicated in hypoxic-ischemic brain lesions. Many of the studies were performed on rodents, with the most widely used and well characterized model being a unilateral common carotid artery occlusion followed by a period of global hypoxia (Rice et al., 1981) (the Rice-Vannucci model). To our knowledge, only rodent models have been used until now to study the autophagic pathways in neonatal brain injury, which are reviewed in Table 1. Zhu et al were the first to investigate LC3-II level following cerebral HI. They subjected mice of different postnatal ages (from P5 to P60) to the Rice-Vannucci model and concluded that there was a significant increase in LC3-II at all ages even though it was more pronounced

Table 1

Autophagy in *in vivo* models of neonatal cerebral hypoxia-ischemia and human newborn autoptic studies. AIF: apoptosis inducing factor; CathB/CathD: cathepsin B/D; r/l/t CCAO: right/left/transient common carotid artery occlusion; O2: oxygen, EM: electron microscopy (presence of autophagosomes and autolysosomes); ER; endoplasmic reticulum; GA: gestational age; hipp: hippocampus; HI: hypoxia-ischemia; HIE: hypoxic-ischemic encephalopathy; IC: internal capsule; icv: intra-cerebro-ventricular; ip: intra-peritoneal; MCAO: middle cerebral artery occlusion; MDC: monodansylcadaverine; nd: not determined; ROIs: regions of interest; SD: Sprague Dawley; striat: striatum; thal: thalamus; WMI: white matter injury; WT: wortmannin, shRNA: small or short hairpin RNA.

Model	Age	ROIs	Autophagy markers	Auto-phagicflux	Relationship with other cell death	Neuroprotective strategies targeting autophagy	References
MOUSE l CCAO, 40–60 min 10% O$_2$	P5, 9,21, 60	Cortex, hipp, thal., striat.	LC3 ↗	nd.	nd.	nd.	(Zhu et al., 2005)
RAT SD r CCAO, 90 min 8% O$_2$	P7	Cortex	LC3, Beclin1 ↗, EM	nd.	nd.	nd.	(Shi et al., 2012)
RAT SD r CCAO 90 min 8% O$_2$	P7	Cortex, hipp	LC3, Beclin1, lyso-trackers ↗	nd.	nd.	nd.	(Weis et al., 2014)
RAT SD r CCAO, 2 h 8% O$_2$	P7	Thal. (ventrolat. nucleus)	LC3, LAMP1, CathD, ↗ lysosomal activity ↗, p62 ↘		colocalization caspase 3 and LAMP-1. EM: mixed features autophagy and apoptosis	nd.	(Ginet et al., 2014a)
RAT SD r CCAO, 2 h 8% O$_2$	P7	Cortex, hipp	LC3, LAMP1, CathD, ↗ lysosomal activity ↗, EM		colocalization caspase 3 and LAMP-1. EM: mixed features autophagy and apoptosis in the cortex	nd.	(Ginet et al., 2009)
RAT SD r CCAO, 2 h 8% O$_2$	P7	Thal	LC3 ↗	nd.	nd.	nd.	(Ginet et al., 2009)
Neuroprotection by autophagy inhibition							
MOUSE l CCAO, 40 min 8% O$_2$	P7	Hipp.	LC3 ↗ and EM	nd.	EM: mixed features autophagy and apoptosis.	Atg7 deficient mice: ↘ lesion volume	(Koike et al., 2008)
RAT SD r CCAO, 2 h 8% O$_2$	P7	Striat	LC3, LAMP1, CathB, ↗ lysosomal activity ↗ p62 ↘		nd.	Intrastriatal injections of lentiviral vectors transducing Beclin-1 shRNA: ↘ lesion volume	(Ginet et al., 2014b)
RAT SD l MCAO + 90 min t CCAO	P12	Cortex	LC3, LAMP1, lysosomal activity ↗ EM	↗	3-MA ↘ cleaved caspase-3 and -9 and AIF nuclear translocation.	3-MA icv after MCAO: ↘ lesion volume	(Puyal et al., 2009)
RAT SD r CCAO, 2 h 8% O$_2$	P7	Hipp. (CA3)	LC3, LAMP1 ↗	nd.	nd.	Neriifolin ip before HI: ↘ lesion volume.	(Liu et al., 2013)
RAT Wistar r CCAO, 50 min 7.7% O$_2$	P9	Cortex, hipp, thal, striat	LC3 ↗	nd.	nd.	Lithium chloride ip after HI: ↘ lesion volume	(Li et al., 2000)
Neuroprotection by autophagy induction							
RAT SD r CCAO, 2,5 h 8% O$_2$	P7	Cortex, hipp	Beclin1 ↗	nd.	Colocalization of Beclin-1 and TUNEL staining. Rapamycin and 3-MA ↘ cleaved caspase-3.	Rapamycin icv 20 min before HI: ↘ lesion volume. 3-MA or WT icv 20 min before HI: no ↘ lesion volume.	(Carloni et al., 2008)
RAT SD r CCAO, 2.5 h 8% O$_2$	P7	Cortex, hipp	LC3, Beclin1, MDC ↗	↗	nd.	Rapamycin icv 30min before HI: ↘ lesion volume	(Carloni et al., 2010)
RAT SD r CCAO, 2,5 h 8% O$_2$	P7	Cortex, hipp	LC3, Beclin1, MDC ↗	↗	Rapamycin ↘ ER stress and unfolded protein response.	Rapamycin icv 30min before HI: ↘ lesion volume 3-MA 20min before HI: no ↘ lesion volume.	(Carloni et al., 2014)
Human autoptic studies							
Premature HUMAN (WMI)	<32 GA	Thal and IC	LC3, LAMP1 ↗	nd.	nd.	nd.	(Vontell et al., 2015)
Newborn HUMAN (HIE)	35–42 GA	Thal (ventrolat n)	LC3, LAMP1, p62, CathD ↗	↗	Colocalization caspase 3/LAMP-1 and CathD/TUNEL staining.	nd.	(Ginet et al., 2009)

in adult brains. Later, in both rat and mouse models of perinatal HI, ultrastructural studies revealed numerous autophagosomes and autolysosomes in the cytosol of dying neurons (Ginet et al., 2009; Koike et al., 2008; Zhu et al., 2005). In P7 hypoxic-ischemic rat pups, our group showed that an LC3-II increase was already detectable as soon as 6 h after the insult and peaked at around 24 h (Ginet et al., 2014a; Ginet et al., 2009). Autophagic flux enhancement was suggested by an increase in both LC3-positive dots and lysosomal markers (LAMP1, cathepsins) and activity (acid phosphatase, β-hexosaminidase) that can occur in the same dying neurons (Ginet et al., 2009). A decrease in p62/SQSTM1 was also observed (Ginet et al., 2014a; Ginet et al., 2014b). Depending on the cerebral region, the pattern of HI-induced autophagy can vary (Ginet et al., 2009; Weis et al., 2014) with for instance an earlier activation in the rat cortex and hippocampal CA3 than in the thalamus or striatum (Ginet et al., 2009).

Few studies have addressed the functional role of enhanced autophagy in neonatal cerebral HI models until now. All the studies suggest that enhanced autophagy plays a role in the apoptotic pathway since dying neurons can express both autophagic and apoptotic features (Carloni et al., 2008; Ginet et al., 2014a; Ginet et al., 2009; Koike et al., 2008) and since autophagy inhibition decreases apoptotic markers such as caspase-3 activation (Carloni et al., 2008; Koike et al., 2008). Interestingly, we showed that autophagy could mediate apoptosis in primary cortical neurons cultures treated with classical apoptotic stimuli (Grishchuk et al., 2011). However this interaction between autophagy and apoptosis may depend on the cerebral region. In the rat cortex our results indicate that enhanced autophagy is related to apoptosis since some highly autophagic neurons expressed also cleaved caspase-3 and most of the dying cortical neurons presented a mixture of apoptotic (chromatin condensation, cytoplasmic shrinkage) and autophagic (autophagosomes, autolysosomes) morphological characteristics by electron microscopy. However, in the hippocampus, there was a region-specific dichotomy in cell death type: CA3 neurons displayed a strong increase in autophagy without apoptosis (probably a type of autophagic cell death), whereas CA1 neurons were highly apoptotic but not autophagic (Ginet et al., 2009).

Most of the studies investigating the role of autophagy in neonatal cerebral HI employed pharmacological inhibitors of limited specificity, so prudence is required concerning the conclusions (Hughes and Kennedy Cell biology, 2012; Wu et al., 2010). In a rat cerebral HI model the studies of Carloni and collaborators reported a beneficial effect of autophagy stimulation with rapamycin on apoptotic cell death, endoplasmic reticulum stress and brain damage (Carloni et al., 2014; Carloni et al., 2008; Carloni et al., 2010). Since rapamycin was administrated in pre-treatment, the possible benefit of a sort of pre-conditioning could not be excluded (Sheng and Qin, 2015). Autophagy inhibition with 3-MA in the same model showed no significant difference in the size of the brain lesion volume (Carloni et al., 2008). However the dose of 3-MA used in this study was weaker than those necessary to provide neuroprotection in other studies (Puyal et al., 2009; Wen et al., 2008). 3-MA was also injected before HI whereas post-treatment with 3-MA in a rat model of neonatal stroke showed strong neuroprotective effect (Puyal et al., 2009). Intraperitoneal injection of the cardiac glycoside neriifolin, a highly potent Na$^+$/K$^+$ ATPase inhibitor that has been shown to prevent autosis (a type of autophagic cell death), attenuated the autophagosome increase and drastically reduced brain damage one week after perinatal cerebral HI (Liu et al., 2013).

However, to date, the stronger evidence for a death-promoting role of enhanced autophagy after perinatal HI comes from studies using genetic methodologies to inhibit autophagy such as neuron-specific knockout (KO) of Atg7 (Koike et al., 2008) or knockdown of Beclin1 (Ginet et al., 2014b). Thus, the hippocampus was resistant to HI (compared to WT) in KO mice deficient for Atg7 specifically in neurons (Koike et al., 2008) and knockdown of Beclin-1 by intrastriatal injection of lentiviral vectors transducing shRNA was likewise neuroprotective against HI (Ginet et al., 2014b).

More in vivo studies investigating the autophagic flux and specifically manipulating the autophagic pathway at a genetic level are still needed to provide solid data on the role of autophagy in neonatal HI.

5.3. Autophagy in human neonates with cerebral HI

Human studies investigating the molecular pathophysiological mechanisms of perinatal brain injuries are still too sparse to clearly identify the time course and clinical implication. However, it is known that classical apoptosis is rarely seen in the human neonatal brain after HIE, highlighting the importance of better understanding the crosstalk between the different cellular death pathways (Northington et al., 2011).

Only two studies have investigated autophagy in autopsied brains of human neonates presenting cerebral lesions. In a recent study focused mainly on the toll-like receptor 3, (Vontell et al., 2015) examined LC3 immunolabelling in post-mortem brain sections of autopsied preterm newborns (<32 weeks of gestational age) with diagnosed white matter injury. They showed an increase in LC3-positive dots (autophagosomes) in the injured thalamus. The LC3 dots colocalized with a mitochondrial marker suggesting increased mitophagy. However their main inclusion criterion was the presence of white matter injury and they thus did not specifically address HIE.

Our group recently showed for the first time that autophagy was enhanced in the brains of human term newborns (35–7 weeks gestational age) who died from perinatal asphyxia and severe HIE (Ginet et al., 2014a). We analyzed the ventrolateral nucleus of the thalamus since this cerebral region is known to be highly sensitive to cerebral asphyxia. We showed an increase in both autophagosomal (punctate LC3) and lysosomal (LAMP1, Cathepsin D) markers occurring in dying neurons of HIE human newborns. Moreover, p62/SQSTM1 labelling was strongly decreased arguing for an increase in the autophagic flux. We also showed that autophagic and apoptotic markers were expressed in the same dying neurons. Altogether these data indicate that autophagy is increased in the brains of hypoxic-ischemic newborns and associated with neuronal death.

5.4. Summary

To summarize, until now 15 studies have investigated the role of autophagy in in vivo rodent models of neonatal cerebral HI, but only half of them examined its neuroprotective potential by inhibition or enhancement of autophagy. The majority of the studies favors the concept of a death-promoting effect of autophagy-mediated cell death in neonatal HIE.

6. Conclusion

The existence of multiple types of cell death, and of different death-mediating pathways even in a single neuron, may explain the difficulty up until now of developing effective techniques of neuroprotection, because attempts at neuroprotection have often relied on targeting just a single pathway. But current understanding of the different death pathways, and of the crosstalk between them, is still limited and much more research is needed. In particular, the relatively new concept of autophagy-mediated cell death deserves considerable attention in the coming years.

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"Enhanced autophagy contributes to excitotoxic lesions in a rat model of preterm brain injury"

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La leucomalacie périventriculaire est une lésion cérébrale soit diffuse, soit kystique, qui se manifeste souvent chez des enfants prématurés ayant présenté une condition systémique ischémique de type sepsis, entérocolite nécrosante ou asphyxie néonatale, résultant de lésions hypoxiques-ischémiques sévères de la matière blanche cérébrale et impliquant également des dommages au niveau de la matière grise. Les voies de signalisation menant aux différents mécanismes de mort cellulaire dans ce type de lésion cérébrale chez le prématuré restent encore très peu élucidées. L'excitotoxicité est un mécanisme prépondérant impliqué dans la formation de lésions hypoxiques-ischémiques dans le cerveau en développement. Il a été récemment démontré que l'autophagie pouvait être excessivement augmentée dans des conditions d'excitotoxicité, transformant ce mécanisme physiologique de dégradation intracellulaire en un processus délétère pour la cellule. Dans la présente étude, nous avons investigué le rôle de l'autophagie dans un modèle reconnu de lésion excitotoxique périnatale chez le rat, reproduisant les lésions de type « leucomalacie kystique périventriculaire » retrouvée chez l'humain prématuré. Une lésion de type excitotoxique affectant les matières grise et blanche a été induite par l'injection d'ibotenate, un analogue du glutamate, au niveau de la matière blanche sous-corticale (aire sous-cingulaire) chez des ratons âgés de 5 jours. L'injection d'ibotenate a causé une augmentation de l'autophagie dans les neurones en voie de mort cellulaire après 6h, comme démontré par la présence augmentée d'autophagosomes (augmentation de l'expression de LC3-II et du marquage ponctiforme LC3-positif) ainsi que l'augmentation de la dégradation autophagique (diminution de l'expression de SQSTM1 et augmentation du nombre et de la taille des lysosomes (vésicules positives pour LAMP1 et CATHEPSIN-B)). La co-injection de 3-méthyladenine, inhibiteur pharmacologique de l'autophagie, a montré une diminution non seulement de l'induction de l'autophagie, mais également de l'activation de CASPASE-3 et du clivage calpaine- dépendant de SPECTRIN 24h après la lésion, induisant ainsi une diminution significative des lésions cérébrales à long terme (16 jours après l'injection d'ibotenate). Notre étude a démontré une réduction significative de la dilatation des ventricules latéraux, de la diminution du volume du tissu cérébral ainsi que de la diminution de l'épaisseur de la matière blanche sous-corticale causées par l'injection d'ibotenate seul. L'effet neuroprotecteur « autophagie-dépendant » de la 3-méthyladénine a également été confirmé dans des cultures primaires de neurones corticaux par le moyen d'une inhibition pharmacologique mais également génétique de l'autophagie excessive induite par le traitement à l'ibotenate. Des stratégies basées sur l'inhibition de l'autophagie pourraient donc représenter une approche neuroprotectrice prometteuse dans le contexte des lésions cérébrales rencontrées chez les enfants prématurés.

Cell Death & Disease

ARTICLE

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Enhanced autophagy contributes to excitotoxic lesions in a rat model of preterm brain injury

Céline Descloux[1,2], Vanessa Ginet[1], Coralie Rummel[1], Anita C. Truttmann[2] and Julien Puyal[1]

Abstract

Cystic periventricular leukomalacia is commonly diagnosed in premature infants, resulting from severe hypoxic-ischemic white matter injury, and also involving some grey matter damage. Very few is known concerning the cell death pathways involved in these types of premature cerebral lesions. Excitotoxicity is a predominant mechanism of hypoxic-ischemic injury in the developing brain. Concomitantly, it has been recently shown that autophagy could be enhanced in excitotoxic conditions switching this physiological intracellular degradation system to a deleterious process. We here investigated the role of autophagy in a validated rodent model of preterm excitotoxic brain damage mimicking in some aspects cystic periventricular leukomalacia. An excitotoxic lesion affecting periventricular white and grey matter was induced by injecting ibotenate, a glutamate analogue, in the subcortical white matter (subcingulum area) of five-day old rat pups. Ibotenate enhanced autophagy in rat brain dying neurons at 24 h as shown by increased presence of autophagosomes (increased LC3-II and LC3-positive dots) and enhanced autophagic degradation (SQSTM1 reduction and increased number and size of lysosomes (LAMP1- and CATHEPSIN B-positive vesicles)). Co-injection of the pharmacological autophagy inhibitor 3-methyladenine prevented not only autophagy induction but also CASPASE-3 activation and calpain-dependent cleavage of SPECTRIN 24 h after the insult, thus providing a strong reduction of the long term brain injury (16 days after ibotenate injection) including lateral ventricle dilatation, decreases in cerebral tissue volume and in subcortical white matter thickness. The autophagy-dependent neuroprotective effect of 3-methyladenine was confirmed in primary cortical neuronal cultures using not only pharmacological but also genetic autophagy inhibition of the ibotenate-induced autophagy. Strategies inhibiting autophagy could then represent a promising neuroprotective approach in the context of severe preterm brain injuries.

Introduction

The important progress done in neonatal care constantly increases the survival rates of premature infants. Conversely, the proportion of neurological disabilities developed by survivors is hardly reduced especially for

those with severe impairment. One of them is the diplegic cerebral palsy, called also spastic diplegia of Little[1], affecting still between 3–7% of very low birth weight (VLBW) infants[2,3]. The strongest predictor of this form of cerebral palsy in VLBW infants is cystic periventricular leukomalacia (cPVL)[4], a form of preterm white matter (WM) injury adjacent to the lateral ventricles, which occurs either from a hypoxic-ischemic (HI) event around birth or after infectious events such as septic shock, necrotizing enterocolitis or even reported after viral infections[5,6]. Improving the outcomes for

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these severely affected babies remains a challenging health issue.

Beside inflammation and reactive oxygen species formation, excitotoxicity seems to be crucial in the pathophysiology of many preterm brain injuries such as PVL[3,7,8]. Excitotoxicity consists in an excessive or prolonged activation of excitatory amino acid receptors (especially those of glutamate) due to a failure of sufficient reuptake and/or excessive release at the synaptic level. It induces a massive increase in intracellular calcium concentration and thus activates numerous intracellular cascades potentially leading to neuronal cell death[9,10]. Glutamate homeostasis is highly important for human brain development (proliferation, migration, differentiation, survival processes and synapses refinement)[11]. However it also confers to immature brain a vulnerability to excitotoxic injuries since a higher level of ionotropic glutamate receptors are expressed in developing brain compared to that of adult[8,12–15]. These receptors are in addition more readily activated. Excitotoxic lesions can occur following a panel of deleterious events (that can be related) such as infection/inflammation, hypoxia and/or ischemia. Excitotoxicity is then a common pathological mechanism of various perinatal brain injuries. In neurons the peak of expression of NMDA receptors appears to occur at term in which grey matter (GM) damage is predominant than in preterm[13]. In human WM, this peak occurs in preterm brain glial cells, especially in pre-oligodendrocytes O4[+], and contributes to the high sensitivity of preterm WM. PVL was mainly thought to be associated to WM injury but it is clearly shown now that GM damage is also often involved in a "neuronal-axonal disease"[16,17].

Experimental research has revealed the complexity of the pathophysiology of excitotoxic death showing multiple and interrelating cell death mechanisms reflected by mixed features of neuronal death including not only the well-known "apoptotic-necrotic continuum" with features of apoptosis and necrosis in the same dying neurons[18] but also characteristics of enhanced macroautophagy[10,19–21]. Autophagy is a physiological cellular mechanism of degradation and recycling of dysfunctioning long lived proteins and organelles[22]. Its main form (macroautophagy, hereafter called autophagy), consists in the formation of a multimembrane intermediate compartment, named autophagosome, that engulfs part of the cytosol containing proteins and organelles to be degraded. The autophagosome then fuses with a lysosome, forming an autolysosome, to degrade its content through lysosomal hydrolases activity[22]. Autophagy is thus essential for cellular homeostasis and could be used as a survival response to different stresses such as nutrients deprivation, accumulation of toxic proteins or pathogen invasion[23]. However, dysregulated increase in autophagic

process has been also implicated in cell death as an independent mechanism (termed "autophagic cell death") or more frequently as a mediator of other types of cell death, mainly apoptosis, and then designed as "autophagy-mediated cell death"[10,24–28]. Abnormal high level of autophagosomes and autolysosomes with increased lysosomal enzyme activity were often observed in dying neurons in models of excitotoxicity including perinatal cerebral HI[19,20,29–31]. Interestingly, we also recently demonstrated excessive autophagic features in postmortem brains of human term newborns presenting severe hypoxic-ischemic encephalopathy (HIE)[29,31]. Although controversies remain concerning the role of autophagic activation[24,32–35], most of the studies using autophagy inhibition, either through pharmacological inhibitors such as 3-methyladenine (3-MA)[30,36–40] or through specific and genetic inhibition of autophagy-related genes (*atg*)[20,29,31,41], have revealed a pro-death role of autophagy in perinatal and adult cerebral HI models.

The present study aims to determine the role of autophagy in excitotoxic lesions of the premature brain using a widely recognized rodent model that mimics some features of cPVL[42]. Autophagy flux and the neuroprotective effect of autophagy inhibition either pharmacologically with 3-MA or genetically in neuronal cultures was investigated in the context of an excitotoxic insult induced by an injection of the glutamate analogue ibotenate. An involvement of autophagy in excitotoxic preterm brain damage would reveal a new cellular death pathway and open the way for new neuroprotective strategies in severe preterm brain injuries.

Results
Ibotenate injection in subcortical WM of rat pups induces brain injury

As a model mimicking some aspects of cPVL, we selected a widely recognized model of preterm excitotoxic brain injury consisting in applying an intracerebral injection of ibotenate in rat pups[42]. The injection of ibotenate (10 µg) in the subcortical WM at the level of the right cingulum of 5-days-old rat pups causes a severe brain damage of the WM and GM resulting in ventricular enlargement as illustrated by cresyl-violet-stained coronal sections in Fig. 1a 24 h and 16 days after the insult. Immunoblot analysis of two death markers, the calpain-dependent 150–145 kDa spectrin/fodrin (SPTAN) fragment and cleaved CASPASE-3 (CASP3), indicated an activation of both calcium-dependent necrotic cell death and caspase-dependent apoptosis (Fig. 1b) at 6 and 24 h after the insult (~5 and 14 fold increases respectively at 24 h). Although the profile of CASP3 activation was similar between females and males, we observed 24 h after the insult a greater activation (~2 times more) in females. Double immunolabeling showed that CASP3 was
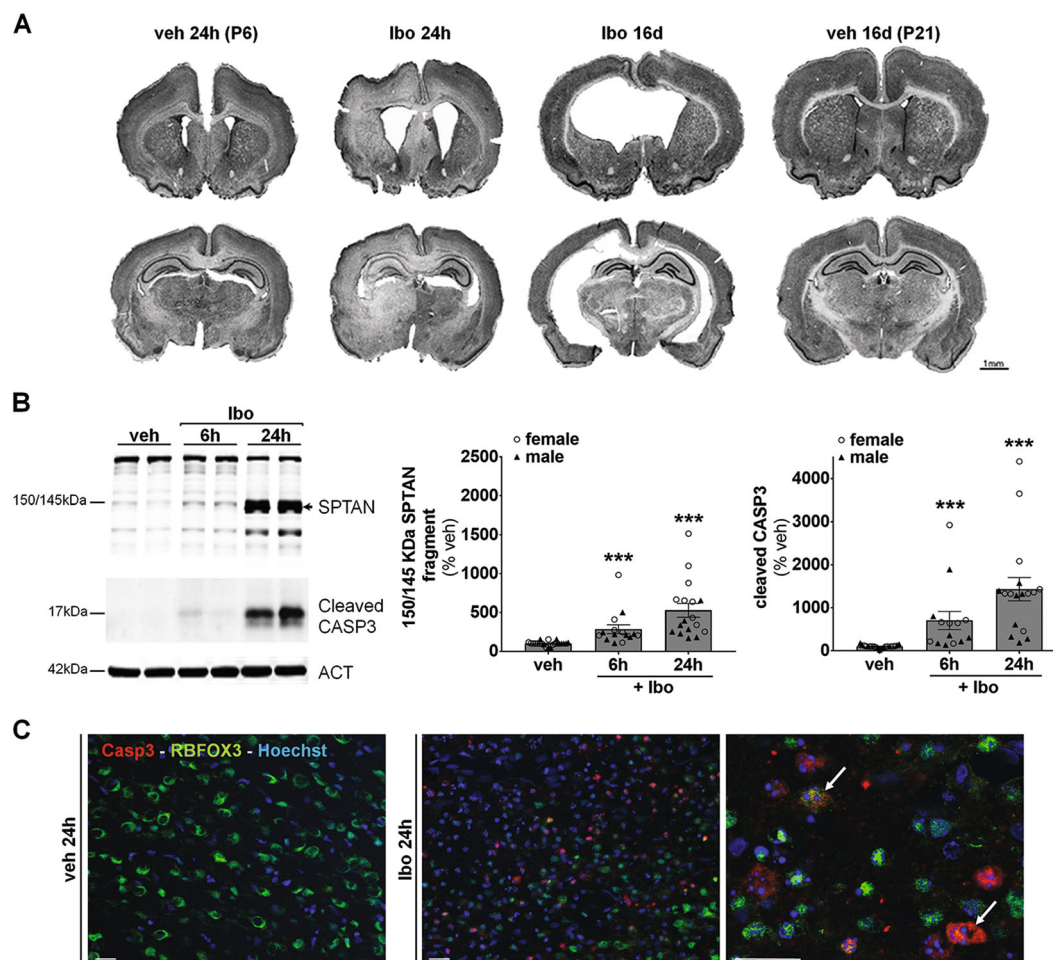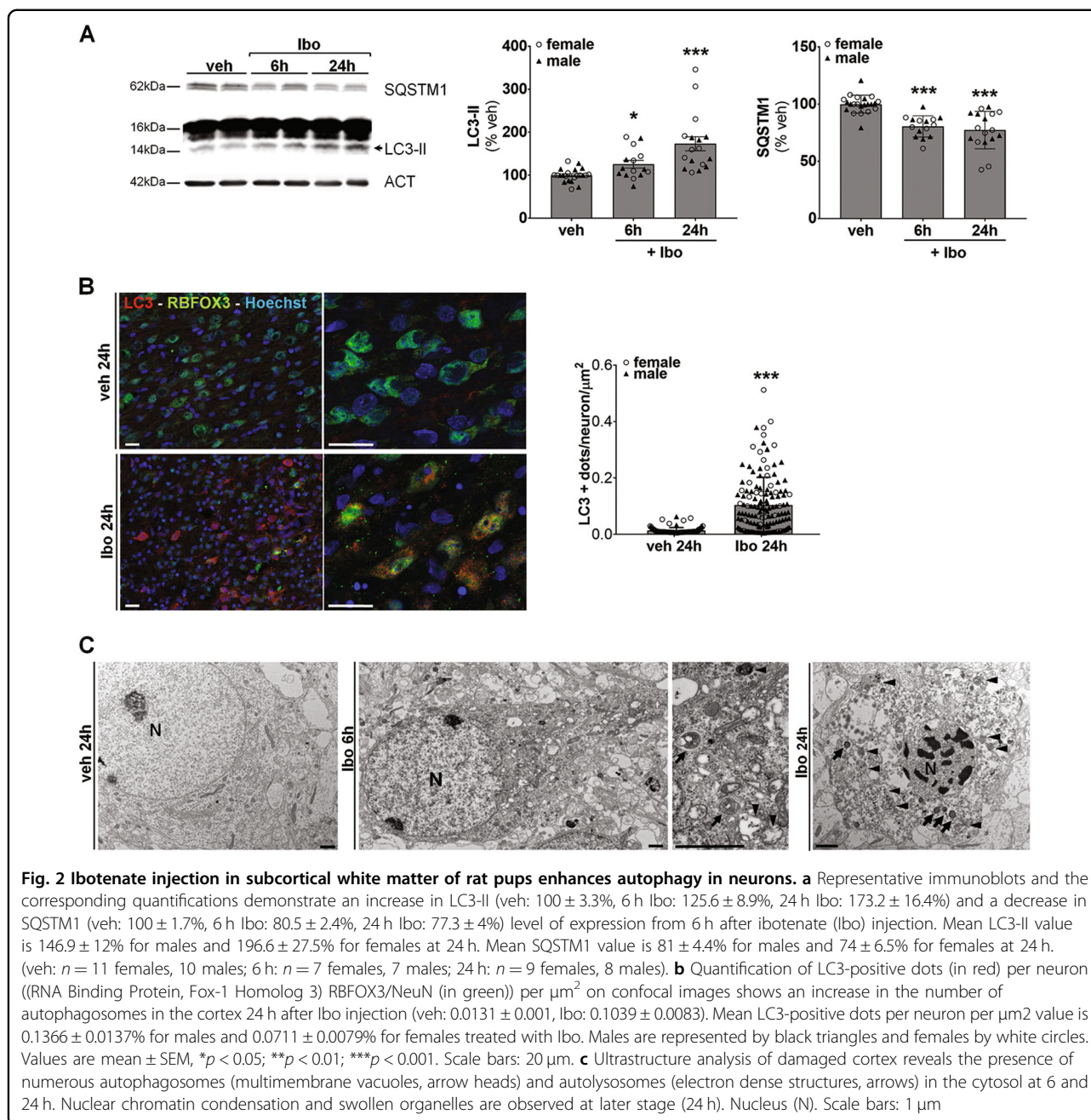
Fig. 1 Ibotenate injection in subcortical white matter of rat pups induces brain injury. a Representative cresyl violet-stained coronal sections illustrating the brain damage 24 h (at 6 postnatal days (P6)) and 16 days (P21) after ibotenate (Ibo) injection. Scale bar: 1 mm. **b** Ibo injection activates both 150–145 kDa calpain-dependent cleavage of spectrin/fodrin (SPTAN) (veh: 100 ± 6.3%, 6 h Ibo: 284.4 ± 56.6%, 24 h Ibo: 528.1 ± 88.8%) and those of caspase-3 (CASP3) (veh: 100 ± 7.13%, 6 h Ibo: 702.6 ± 210%, 24 h Ibo: 1430.9 ± 271.1%) as shown by representative immunoblots and the corresponding quantifications. Mean CASP3 value is 870.7 ± 204.1% for males and 1929 ± 423.5% for females at 24 h. Mean SPTAN value is 312.1 ± 55.9% for males and 720.1 ± 132.5% for females at 24 h. (veh: n = 11 females, 10 males; 6 h: n = 7 females, 7 males; 24 h: n = 9 females, 8 males). Males are represented by black triangles and females by white circles. **c** CASP3 is activated in neurons as revealed by double immunolabeling against cleaved CASP3 (in red) and the neuronal marker RNA Binding Protein, Fox-1 Homolog 3 (RBFOX3)/NeuN (in green) 24 h after Ibo injection in neurons. Scale bar: 20 μm. Values are mean ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

activated in neurons (RBFOX3-positive cells) 24 h after ibotenate injection (Fig. 1c).

Ibotenate injection in subcortical WM of rat pups enhances autophagic flux in cortical neurons

To study the effect of intracerebral injection of ibotenate on autophagy, the expression levels of LC3-II (the autophagosomal membrane bound form of LC3) and Sequestosome-1 (SQSTM1, selectively degraded by autophagy) in ipsilateral cortical extracts was first analyzed at 6 and 24 h after the ibotenate injection (Fig. 2a). LC3-II was significantly enhanced by ibotenate compared to vehicle treated-rats (increase of 72% at 24 h)

whereas SQSTM1 was decreased (reduction of 23% at 24 h) suggesting an increase in the autophagy flux in females as well as in males. A quantification of the number of LC3-positive dots per neuron (RBFOX3-positive cells) confirmed a significant increase of 7.9 fold in the number of autophagosomes in cortical neurons 24 h after the insult (Fig. 2b). Moreover, strong morphological features of enhanced autophagy could be observed at the ultrastructural level (Fig. 2c). At earlier stage (6 h after ibotenate injection), electron microscopy revealed numerous multimembrane structures containing undigested cellular contents (autophagosomes) and electron dense vacuoles containing cytoplasmic materials

Fig. 2 Ibotenate injection in subcortical white matter of rat pups enhances autophagy in neurons. a Representative immunoblots and the corresponding quantifications demonstrate an increase in LC3-II (veh: $100 \pm 3.3\%$, 6 h Ibo: $125.6 \pm 8.9\%$, 24 h Ibo: $173.2 \pm 16.4\%$) and a decrease in SQSTM1 (veh: $100 \pm 1.7\%$, 6 h Ibo: $80.5 \pm 2.4\%$, 24 h Ibo: $77.3 \pm 4\%$) level of expression from 6 h after ibotenate (Ibo) injection. Mean LC3-II value is $146.9 \pm 12\%$ for males and $196.6 \pm 27.5\%$ for females at 24 h. Mean SQSTM1 value is $81 \pm 4.4\%$ for males and $74 \pm 6.5\%$ for females at 24 h. (veh: $n = 11$ females, 10 males; 6 h: $n = 7$ females, 7 males; 24 h: $n = 9$ females, 8 males). **b** Quantification of LC3-positive dots (in red) per neuron ((RNA Binding Protein, Fox-1 Homolog 3) RBFOX3/NeuN (in green)) per μm^2 on confocal images shows an increase in the number of autophagosomes in the cortex 24 h after Ibo injection (veh: 0.0131 ± 0.001, Ibo: 0.1039 ± 0.0083). Mean LC3-positive dots per neuron per µm2 value is $0.1366 \pm 0.0137\%$ for males and $0.0711 \pm 0.0079\%$ for females treated with Ibo. Males are represented by black triangles and females by white circles. Values are mean $\pm$ SEM, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Scale bars: 20 µm. **c** Ultrastructure analysis of damaged cortex reveals the presence of numerous autophagosomes (multimembrane vacuoles, arrow heads) and autolysosomes (electron dense structures, arrows) in the cytosol at 6 and 24 h. Nuclear chromatin condensation and swollen organelles are observed at later stage (24 h). Nucleus (N). Scale bars: 1 µm

at different stages of degradation (autolysomes) whereas later on (at 24 h) hybrid phenotypes of cell death are observed in dying neurons including apoptosis-like morphological features (chromatin condensation), enhanced autophagy (numerous autophagosomes and autolysosomes) and swollen organelles.

Finally, in addition to a higher number of autophagosomes, autolysosomes were also more numerous in neurons of ibotenate-treated rat pups than in vehicle-treated animals. Immunohistochemistry and quantification of the number and the size of vesicles labeled with antibodies against either a lysosomal membrane bound protein, lysosomal-associated membrane protein 1 (LAMP1) (Fig. 3a), or a lysosomal enzyme (CATHEPSIN B) (Fig. 3b) demonstrated an increase not only in thenumber but also in the size of either LAMP1 (~4 fold) or CATHEPSIN B (~3 fold) positive-dots, and especially those larger than $0.5 \, \mu m^2$ (increase of 29 and 23%, respectively) that are presumably autolysosomes. These results showed that ibotenate resulted in enhancement of neuronal autophagy in the damaged cortex of rat pups.
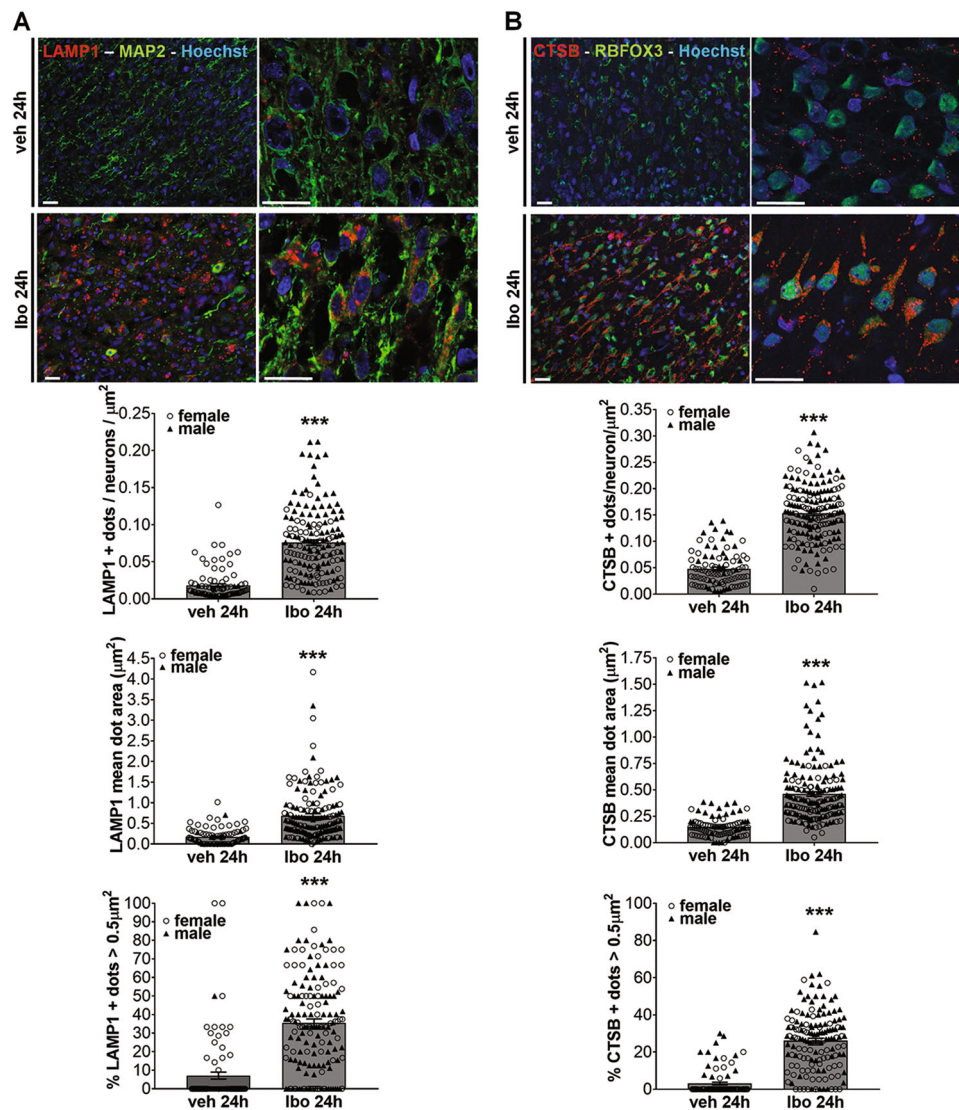
Fig. 3 Ibotenate injection in subcortical white matter of rat pups increases autolysosomes formation in neurons. a Quantification of LAMP1-positive dots (in red) per neuron (MAP2 (in green)) per μm^2 on confocal images shows an increase in the number of LAMP1-positive lysosomes in the cortex 24 h after Ibo injection (number of dots: veh: 0.018 ± 0.002, Ibo: 0.075 ± 0.003/neurons/μm^2). Mean value is $0.089 \pm 0.005\%$ for males and $0.057 \pm 0.004\%$ for females treated with Ibo. On average, the measure of their size reveals the presence of larger LAMP1-positive dots following Ibo injection (mean dot area: veh: 0.171 ± 0.002, Ibo: $0.685 \pm 0.049 \ \mu m^2$) resulting from more dots larger than $0.5 \ \mu m^2$ (veh: $7.08 \pm 1.90\%$, Ibo: $35.56 \pm 2.08\%$). Mean value is $0.571 \pm 0.051 \ \mu m^2 / 38.3 \pm 3.4\%$ for males and $0.849 \pm 0.091 \ \mu m^2/38.3 \pm 3.4\%$ for females treated with Ibo. **b** Quantification of CATHEPSIN B (CTSB)-positive dots (in red) per neuron ((RNA Binding Protein, Fox-1 Homolog 3) RBFOX3/NeuN (in green)) confirms an increase not only in the number (number of dots: veh$_{M+F}$: 0.047 ± 0.003, Ibo$_{M+F}$: 0.153 ± 0.004, Ibo$_M$: 0.153 ± 0.005, Ibo$_F$: 0.153 ± 0.007/neurons/μm^2) but also in the size of lysosome (mean dot area: veh$_{M+F}$: 0.146 ± 0.009, Ibo$_{M+F}$: $0.460 \pm 0.020 \ \mu m^2$, Ibo$_M$: $0.490 \pm 0.025 \ \mu m^2$, Ibo$_F$: $0.343 \pm 0.021 \ \mu m^2$; dots $> 0.5 \ \mu m^2$: veh$_{M+F}$: $3.033 \pm 0.723\%$, Ibo$_{M+F}$: $26.10 \pm 1.274\%$, Ibo$_M$: $26.8 \pm 1.3\%$, Ibo$_F$: $18.6 \pm 2.1\%$). Scale bars: 20 μm. Values are mean $\pm$ SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (veh: $n = 63$ females, 30 males; Ibo: $n = 35$ females, 140 males). Males are represented by black triangles and females by white circles

Ibotenate induced-autophagy and -cell death are reduced by pharmacological inhibition of autophagy

We then evaluated the role of this enhanced neuronal autophagy on brain damage. Since long term autophagy inhibition could be potentially deleterious particularly for neurons[43], we decided to manage the transient inhibition of autophagy with the widely used pharmacological inhibitor of autophagy 3-MA. 3-MA was administered in the ipsilateral ventricle just before ibotenate injection as previously done in ischemic rat pups[30]. As shown by immunoblots, ibotenate-induced increase in LC3-II expression levels at 24 h was reduced by
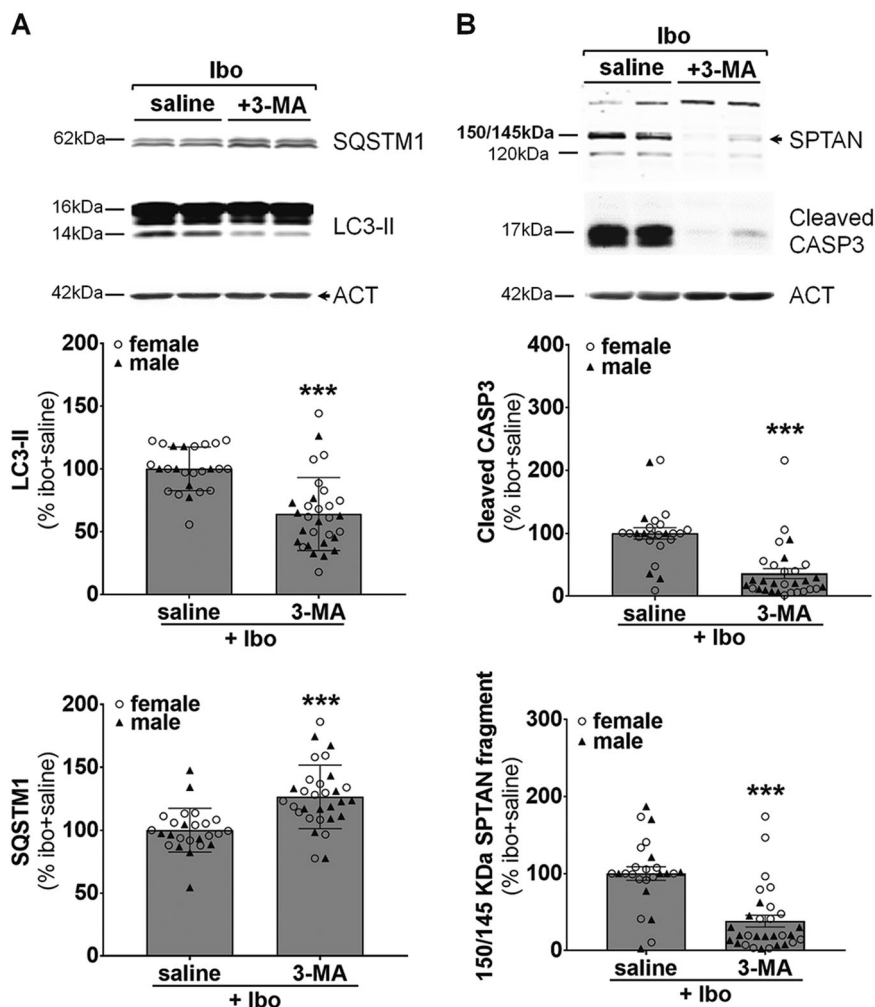
Fig. 4 Ibotenate induced-autophagy and cell death is reduced by pharmacological inhibition of autophagy. a Intracerebroventricular injection of the autophagy inhibitor 3-methyladenine (3-MA) just before ibotenate (Ibo) treatment decreases LC3-II (Ibo + 3-MA: 64.1 ± 5.3%) and increases SQSTM1 (Ibo + 3-MA: 126.5 ± 4.6%) levels 24 h after Ibo injection compared to rat pups treated with saline (Ibo + saline: LC3, 100 ± 3.5%; SQSTM1, 100 ± 3.5%) as shown by representative immunoblots of cortical extracts and the corresponding quantifications. Mean LC3-II value is 55.6 ± 6.7% for males and 71.5 ± 7.7% for females at 24 h. Mean SQSTM1 value is 127.4 ± 7.2% for males and 127.5 ± 7.07% for females at 24 h. **b** 3-MA prevents both 150–145 kDa calpain-dependent cleavage of spectrin/fodrin (SPTAN) (Ibo + saline: 100 ± 8.8%, Ibo + 3-MA: 51.5 ± 12.9%) and those of caspase-3 (CASP3) (Ibo + saline: 100 ± 9.1%, Ibo + 3-MA: 55.8 ± 14.4%). Mean SPTAN value is 21.7 ± 4.3% for males and 52.7 ± 12.9% for females at 24 h. Mean CASP3 value is 25.8 ± 6.1% for males and 44.8 ± 13.7% for females at 24 h. Values are mean ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (Ibo + saline: $n = 15$ females, 10 males; Ibo + 3-MA: $n = 16$ females, 14 males). Males are represented by black triangles and females by white circles

36% in 3-MA-treated rats compared to saline-injected rats (Fig. 4a). Moreover, SQSTM1 expression levels were also significantly increased by 26% with 3-MA treatment confirming an efficient inhibition of autophagy by 3-MA (Fig. 4a). We then evaluated the effect of autophagy inhibition on cell death markers at 24 h. The expression levels of both the 150–145 kDa SPTAN fragment and cleaved CASP3 were significantly decreased by 64 and 62% respectively with 3-MA treatment (Fig. 4b) demonstrating that autophagy inhibition by 3-MA could be neuroprotective 24 h after the induction of the

excitotoxic brain lesion. Moreover 3-MA treatment was as efficient in females as well as in males.

Long term ibotenate-induced brain damage is strongly reduced by pharmacological inhibition of autophagy

We then evaluated the long term benefit of 3-MA-mediated autophagy inhibition on brain injury at P21 (16 days after ibotenate injection). As shown in Figs. 5a, 3-MA treated-brains were less sensitive to ibotenate-induced damage. The ventricle enlargement of ~20 fold produced by ibotenate injection was strongly prevented by
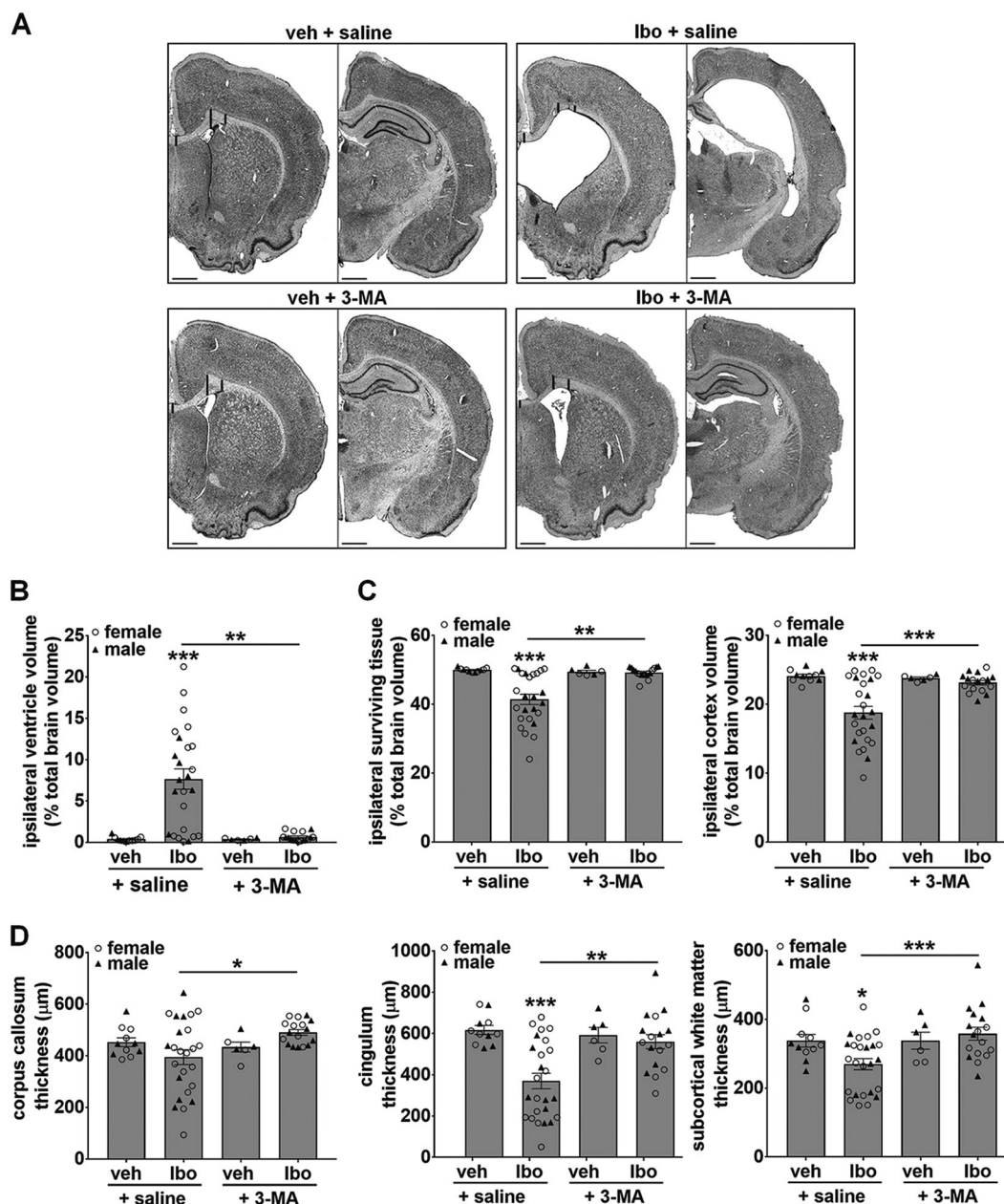
Fig. 5 Long term ibotenate-induced brain damage is strongly reduced by pharmacological inhibition of autophagy. a Representative cresyl violet-stained coronal sections of brain 16 days (P21) after injury illustrating the protective effect of the autophagy inhibitor 3-methyladenine (3-MA) injected in the ipsilateral ventricle just before ibotenate (Ibo). Scale bar: 1 mm. **b** 3-MA treatment reduces Ibo-induced ventricle dilatation (veh + saline: 0.386 ± 0.086%, Ibo + saline: 7.655 ± 1.233%, Ibo + 3-MA: 0.646 ± 0.112%, veh + 3MA: 0.366 ± 0.056%). Mean values for Ibo + saline treatment are 6.88 ± 1.38% for males and 8.10 ± 1.79% for females whereas those for Ibo + 3-MA are 0.53 ± 0.16% for males and 0.78 ± 0.21% for females. **c** Quantification of ipsilateral surviving tissue volume (veh + saline: 49.94 ± 0.167%, Ibo + saline: 41.41 ± 1.486%, Ibo + 3-MA: 49.22 ± 0.379%, veh + 3MA: 49.43 ± 0.428%) relatively to total brain volume and more specifically those of the ipsilateral cortex (veh + saline: 24.09 ± 0.258%, Ibo + saline: 18.76 ± 0.926%, Ibo + 3-MA: 23.13 ± 0.329%, veh + 3MA: 23.78 ± 0.170%) shows that Ibo-induced decrease in brain tissue volume is prevented by 3-MA. Mean surviving tissue volumes for Ibo + saline treatment are 42.11 ± 1.68% for males and 41.02 ± 2.16% for females whereas those for Ibo + 3-MA are 49.92 ± 0.34% for males and 48.45 ± 0.59% for females. **d.** 3-MA attenuates the Ibo-induced decrease in the thickness of white matter located at three different levels: corpus callosum (veh + saline: 452.61 ± 17.16 μm, Ibo + saline: 394.72 ± 28.76 μm, Ibo + 3-MA: 490.73 ± 11.08 μm, veh + 3MA: 433.67 ± 19.19 μm), cingulum region (veh + saline: 617.54 ± 21.96 μm, Ibo + saline: 369.76 ± 37.63 μm, Ibo + 3-MA: 560.37 ± 35.46 μm, veh + 3MA: 592.40 ± 37.53 μm) and at the beginning of the external capsule (veh + saline: 337.89 ± 18.44 μm, Ibo + saline: 269.80 ± 16.23 μm, Ibo + 3-MA: 358.28 ± 19.20 μm, veh + 3MA: 338.05 ± 24.81 μm). 3-MA tends to have a similar protective effect on white matter thickness in males and females. Values are mean ± SEM, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. (veh + saline: $n = 6$ females, 5 males, Ibo + saline: $n = 16$ females, 9 males, Ibo + 3-MA: $n = 8$ females, 9 males, veh + 3MA: $n = 3$ females, 3 males). Males are represented by black triangles and females by white circles

3-MA treatment (reduced to only 1.7 fold) (Fig. 5b). Moreover, ibotenate-induced tissue loss of 8.54% (total ipsilateral WM and GM), with a decrease of 5.33% specifically for cortex (total ipsilateral intact cortex), was also, on average, significantly prevented in 3-MA-treated rats compared to saline-treated rats (Fig. 5c).

To evaluate the effect of ibotenate on WM, the thickness of the subcortical WM was measured at three different levels around the ibotenate injection site. Reduced thickness could reflect an indirect effect of the GM lesion with secondary axonal degeneration and/or a direct excitotoxic effect on immature oligodendrocytes. A significant decrease in the thickness of subcortical WM of 13% for corpus callosum, 40% for the WM located at the level of cingulum and 20% for external capsule was detected in ibotenate-treated brains (Fig. 5d). These WM alterations were not observed when 3-MA was co-injected with ibotenate (Fig. 5d).

Taken together, these data showed a long term beneficial effect of pharmacological autophagy inhibition against ibotenate-induced brain damage that was moreover observed both in males and females.

An excitotoxic dose of ibotenate enhances autophagic flux in primary cortical neuronal cultures

In order to confirm that ibotenate could enhance autophagy and that specific autophagy inhibition could be neuroprotective against ibotenate-mediated excitotoxicity, we then decided to evaluate the effect of an excitotoxic dose of ibotenate on autophagy in primary cortical neuronal cultures. In general, 50 μM of ibotenate promoted neuronal death as suggested by a 3 and 14 fold increase in lactate deshydrogenase (LDH) release in the culture medium 3 and 6 h, respectively after ibotenate treatment (Fig. 6a). This LDH release was abolished by MK801 or EGTA pretreatment (data not shown) confirming excitotoxic mechanisms (NMDA receptors over-activation and calcium overload).

As shown in Fig. 6b, activation of autophagy occurred along with neuronal death since both LC3-II expression and SQSTM1 degradation were increased at 3 and 6 h. In order to confirm an enhanced autophagic flux, ibotenate was first applied in the presence of lysosomal enzymes inhibitors (Fig. 6c). A combination of E64 and pepstatin A1 (PepA) (10 μg/ml) induced an accumulation of both LC3-II (of 123%) and SQSTM1 (of 13%) reflecting the failure in the autophagy degradation step. When ibotenate was applied 4 h after E64/PepA, LC3-II and SQSTM1 accumulations were even greater (174 and 21% respectively), demonstrating that ibotenate treatment triggered the new formation of autophagosomes and thus confirming that ibotenate treatment increased the autophagosome biogenesis. Second, autophagic flux was monitored using the tandem mRFP-GFP-LC3 plasmid

that allows to discriminate between LC3 expressed in neutral compartments (GFP + RFP+; early autophagosomes: i.e. autophagosomes before fusion with lysosomes) and in acidic vesicles (GFP-RFP+; i.e., late autophagosomes: autophagosomes after fusion with lysosomes (autolysosomes)) thanks to the pH sensitivity differences exhibited by the two fluorescent proteins (Fig. 6e). A quantification of the different LC3-positive dots per neuron per $μm^2$ clearly demonstrated that both autophagosomes formation (~5 fold increase of GFP + RFP + dots) and their fusion with the lysosomes (~13 fold increase of GFP−RFP + dots) were enhanced by ibotenate treatment. Taken together, these results on cortical neuronal cultures allow to conclude that an excitotoxic dose of ibotenate was efficient to induce a boost of neuronal autophagic flux.

Pharmacological and genetic inhibition of autophagy is protective against ibotenate-induced excitotoxicity in primary cortical neuronal cultures

We then assessed the functional role of the ibotenate-enhanced autophagy in primary cortical neuronal cultures. We first used 3-MA. Pre-treatment with 3-MA (10 mM) prevented the increases in both LC3-II expression and SQSTM1 degradation at 6 h after ibotenate treatment (Fig. 7a). 3-MA displayed a significant neuroprotective effect as shown by a decrease of 26% in LDH release (Fig. 7b). Interestingly, blocking lysosomal degradation with E64/PepA also reduced neuronal death (Fig. 7c) suggesting a role of autophagy degradation step in ibotenate-induced neurotoxicity.

Then, in order to inhibit more specifically autophagy, downregulation of the expression of two important autophagy proteins (ATG7 and BECLIN1 (BECN1)) was performed using lentiviral vectors transducing short hairpin RNAs (shRNAs) (Fig. 8a). Transduction of cultured primary cortical neurons with *Atg7* and *Becn1* shRNAs resulted in an efficient inhibition of autophagy as demonstrated by a decrease in both LC3-II expression and SQSTM1 degradation (Figs. 8b, c). The death-promoting role of enhanced autophagy in ibotenate-induced neuronal death was confirmed by a reduction of ~30% of LDH release when ATG7 and BECN1 were downregulated (Fig. 8d).

In conclusion, these in vitro data confirmed that an excitotoxic dose of ibotenate could induce autophagy-mediated neuronal death and that targeting autophagy inhibition could lead to neuroprotection.

Discussion

In the present study, we investigated for the first time the role of autophagy in a preclinical model of premature brain damage related to cPVL[42]. The effect of ibotenate injection on rodent brain development is highly
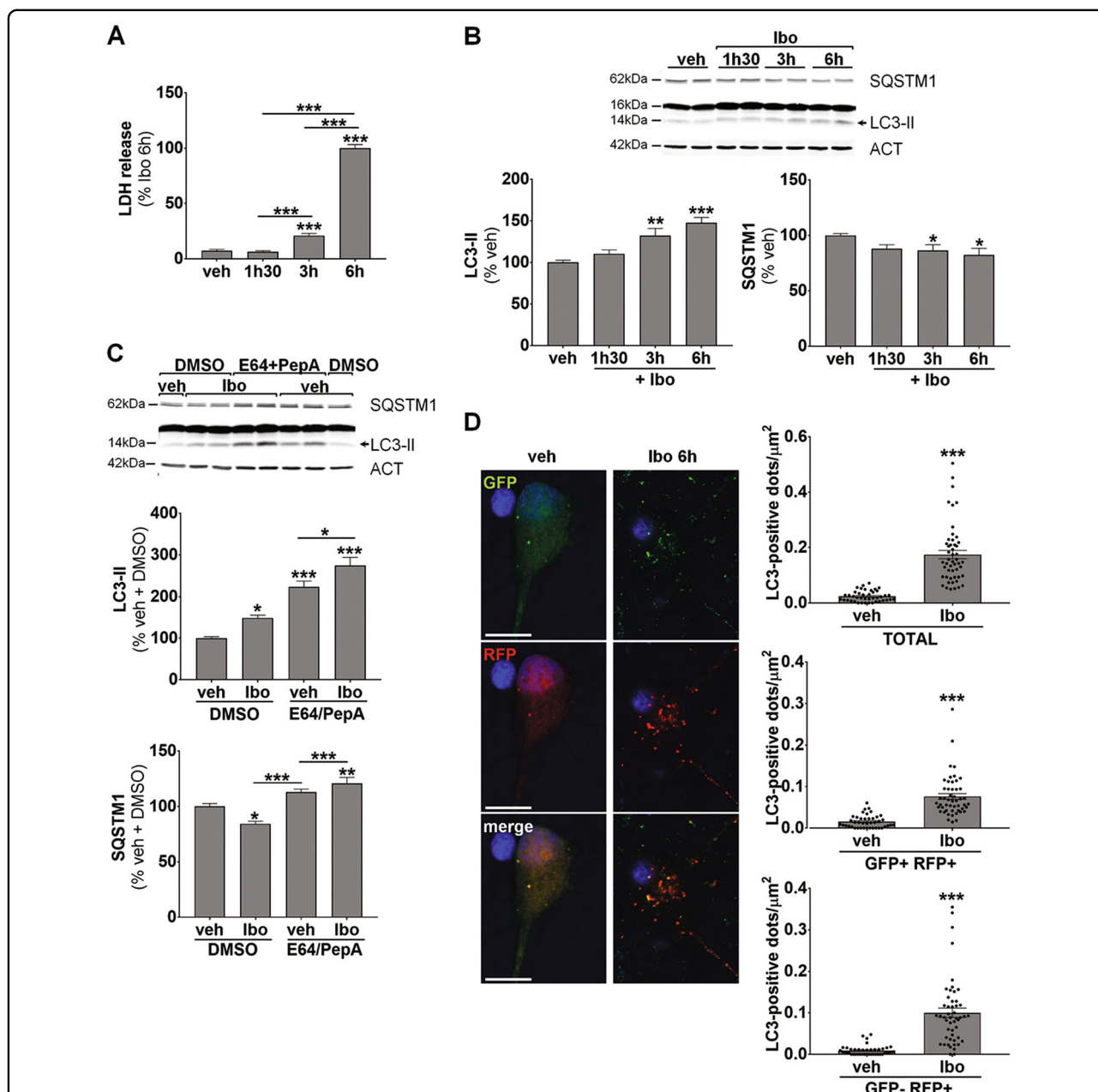
Fig. 6 Excitotoxic dose of ibotenate enhances autophagic flux in primary cortical neuronal cultures. a Ibotenate (Ibo, 50 μM) is neurotoxic as shown by lactate deshydrogenase (LDH) release in the culture medium of cultured neurons (veh: 7.4 ± 1.1%, 1h30: 6.4 ± 0.9%, 3 h: 21 ± 1.9%, 6 h: 100 ± 3.2%). **b** Representative immunoblots and the corresponding quantifications demonstrate an increase in LC3-II (veh: 100 ± 2.7%, 1h30: 110.4 ± 4.6%, 3 h: 132.3 ± 8.5%, 6 h: 147.6 ± 6.6%) and a decrease in SQSTM1 (veh: 100 ± 1.5%, 1h30: 89.7 ± 3.3%, 3 h: 88.1 ± 4.4%, 6 h: 84.2 ± 4.4%) level of expression following Ibo treatment. **c** Addition of pepstatin A (PepA) and E64 prevents lysosomal degradation as shown by increases in the level of LC3-II (veh Pep/E64: 223.2 ± 14.3%) and SQSTM1 (veh Pep/E64: 113 ± 2.8%) relative to neurons treated with DMSO (veh DMSO; LC3-II: 100 ± 3.3%, SQSTM1: 100 ± 2.6%). When added 4 h before Ibo (3 h), PepA/E64 treatment results in a greater increase in LC3-II (Ibo E64/PepA: 274.1 ± 19.9%) than treatment with E64/PepA or Ibo alone (Ibo DMSO: 148.7 ± 6.7%). E64/PepA pretreatment inhibits the Ibo-induced degradation of SQSTM1 (Ibo DMSO: 84.4 ± 2.2%; Ibo E64/PepA: 120.8 ± 5.5%). **d** Representative confocal images of cultured neurons transfected with the tandem mRFP-GFP-LC3–expressing plasmid 6 h after Ibo addition. Quantification of the number of LC3-positive dots per neuron per μm^2 demonstrates an enhanced functional autophagic flux (Total = GFP + RFP+ and GFP−RFP+ : veh: 0.024 ± 0.003, Ibo: 0.174 ± 0.016; GFP + RFP + (early autophagosomes): veh: 0.015 ± 0.002, Ibo: 0.076 ± 0.007; GFP−RFP + (autolysosomes) veh: 0.008 ± 0.001, Ibo: 0.100 ± 0.011). Scale bar: 10 μm. Values are mean ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$
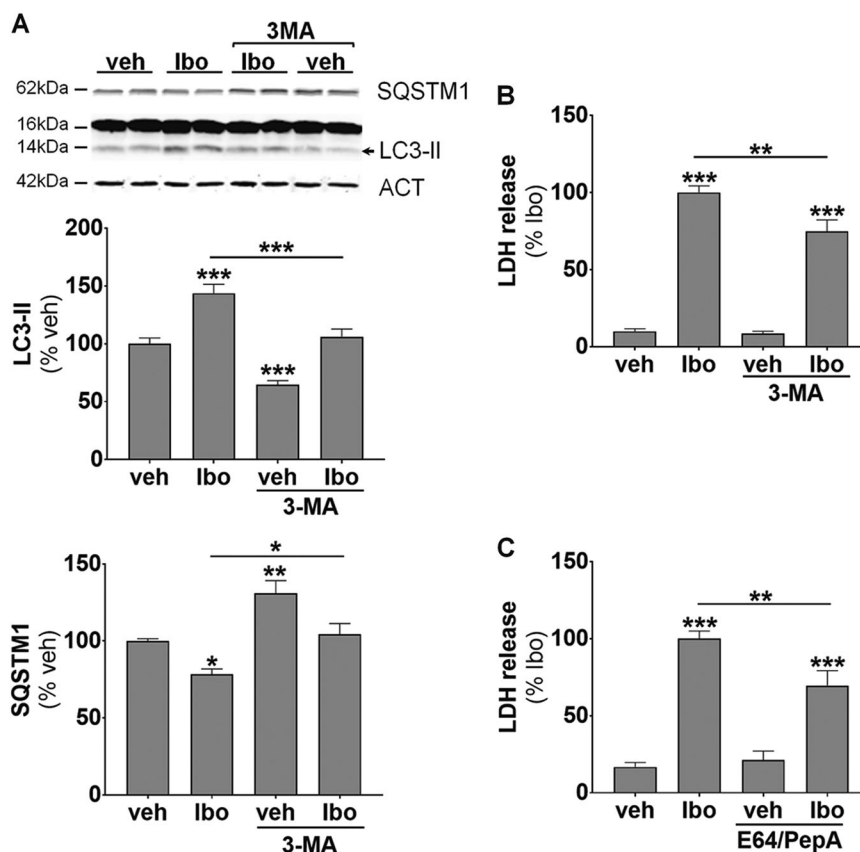
Fig. 7 Pharmacological inhibition of autophagy is protective against ibotenate-induced excitotoxicity in primary cortical neuronal cultures. a Pretreatment with 3-methyadenine (3-MA) 1 h before ibotenate (Ibo) addition for 6 h prevents LC3-II increase (veh: $100 \pm 5\%$; Ibo: $143.7 \pm 7.8\%$; veh + 3-MA: $64.6 \pm 3.4\%$; Ibo + 3-MA: $106.1 \pm 6.7\%$) and SQSTM1 decrease (veh: $100 \pm 1.4\%$; Ibo: $78.4 \pm 3.4\%$; veh + 3-MA: $131 \pm 8.2\%$; Ibo + 3-MA: $104.3 \pm 7.1\%$) induced by Ibo as shown by representative immunoblots and the corresponding quantifications. **b** 3-MA decreases neuronal death as shown by reduced lactate deshydrogenase (LDH) release in the culture medium of cultured neurons (veh: $10 \pm 1.7\%$; Ibo: $100 \pm 4.3\%$; veh + 3-MA: $8.6 \pm 1.4\%$; Ibo + 3-MA: $74.7 \pm 7.5\%$). **c** E64/PepA pretreatment reduces neuronal death as indicated by a decrease in LDH release measured 6 h after Ibo addition (veh DMSO: 16.8 ± 2.9; Ibo DMSO: $100 \pm 5\%$; veh E64/PepA: $21.3 \pm 5.8\%$; Ibo E64/PepA: $69.6 \pm 9.7\%$). Values are mean ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

dependent of the age. If injected around P5, when neuronal migration is completed, the model mimics some of the preterm brain injury features[42]. Human preterm WM injury is often observed with GM developmental alteration and/or damage (neuronal-axonal disease)[2,3,16,17,44]. Persistent cerebral volume reduction and ventriculomegaly are also observed in premature infants compared to full-term[45,46]. The injection of ibotenate in the subcortical WM at the level of the right cingulum caused a severe lesion in rat pups and led to reduced subcortical WM thickness, significant loss of tissue and important lateral ventricle enlargement 16 days after injury. It has been shown that excitotoxicity-induced inflammation through microglial release of cytokines and free radicals played a central deleterious role[47,48] and that apoptotic pathways were implicated in similar models of preterm brain injury[49,50]. However, autophagy-

mediated neuronal death has never been investigated in this context.

Macroautophagy is an important physiological mechanism of degradation present at a basal level complementary to the proteasome system. A "controlled" upregulation of autophagy has been considered for a long time as a survival response, for instance, acting as an alternative source of energy during starvation or as a quality control step eliminating toxic metabolites, defective organelles or intracellular pathogens[23,51,52]. However, this dogma has been challenged about 30 years ago by the description of dying cells without typical morphological hallmarks of apoptosis or necrosis and containing numerous autolysosomes[27]. This new morphological type of cell death was coined "autophagic cell death" and the missuse of the term led to a strong debate on a possible pro-death role of autophagy in some stress
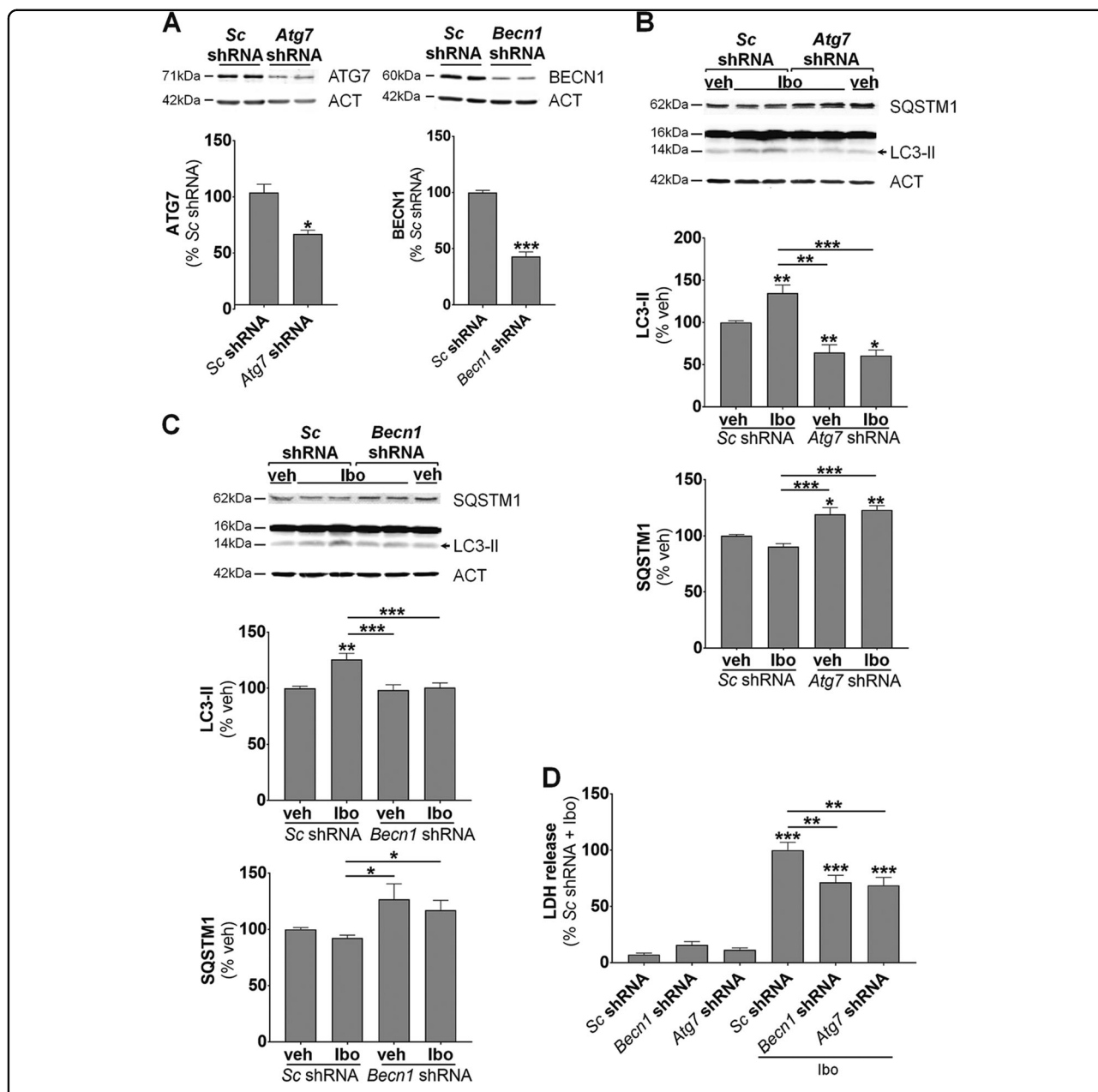
Fig. 8 Genetic inhibition of autophagy is protective against ibotenate-induced excitotoxicity in primary cortical neuronal cultures.
a Lentiviral transduction of shRNA directed against BECLIN1 (BECN1) (sc shRNA: 100 ± 1.9%; *Becn1* shRNA: 43 ± 12.3%) and ATG7 (sc shRNA: 100 ± 7.4%; *Atg7* shRNA: 63 ± 10.9%) proteins efficiently reduced the expression of both proteins. **b, c** Downregulation of both (**b**) BECN1 and (**c**) ATG7 prevents LC3-II increase ((**b**) veh sc shRNA: 100 ± 2.1%; Ibo sc shRNA: 135 ± 9.4%; veh *Atg7* shRNA: 64.4 ± 9.1%; Ibo *Atg7* shRNA: 60.8 ± 6.6%; (**c**) veh sc shRNA: 100 ± 1.8%; Ibo *sc* shRNA: 125.6 ± 5.5%; veh *Becn1* shRNA: 98.4 ± 4.6%; Ibo *Becn1* shRNA: 100.6 ± 4.1%) and SQSTM1 decrease ((**b**) veh sc shRNA: 100 ± 1.4 %; Ibo sc shRNA: 93 ± 3.4%; veh *Atg7* shRNA: 109.5 ± 4%; Ibo *Atg7* shRNA: 117.2 ± 3.6%; (**c**) veh sc shRNA: 100 ± 1.4%; Ibo sc shRNA: 91.3 ± 3.2%; veh *Becn1* shRNA: 132.4 ± 19.3%; Ibo *Becn1* shRNA: 119.4 ± 10.7%) induced by Ibo (6 h) as shown by representative immunoblots and the corresponding quantifications. **d** Downregulation of both ATG7 and BECN1 has a neuroprotective effect as demonstrated by reduced lactate deshydrogenase (LDH) release in the culture medium of cultured neurons (veh sc shRNA: 7.2 ± 1.4 %; veh *Atg7* shRNA: 11.3 ± 1.9%; veh *Becn1* shRNA: 15.9 ± 3%; Ibo sc shRNA: 100 ± 7%; Ibo *Atg7* shRNA: 71.4 ± 6.3%; Ibo *Becn1* shRNA: 68.8 ± 7%). Values are mean ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

conditions[10,34,35]. In fact, "autophagic cell death" is a distinct mechanism of cell death independent of apoptotic and necrotic machinery that is observed in some specific circumstances[27,38,53]. However, there is now evidences and it is well accepted, that autophagy can be more frequently involved in cell death as a trigger leading to

apoptotic or necrotic cell death (autophagy-mediated cell death)[20,21,29,34,38,54–57]. Excitotoxicity and cerebral HI are some of the conditions where autophagy is enhanced[58]. Although some controversies exist[32,33,59], most of the studies using pharmacological inhibitors such as 3-MA[30,37] and especially those using specific and genetic inhibition of *atg*[20,29,31] revealed a deleterious role of autophagy. They also supported the concept of a strong interconnection between autophagic and apoptotic mechanisms in perinatal cerebral HI[19,20,32,60]. The present study is the first to demonstrate a death-promoting role of autophagy in a preterm model of excitotoxic brain lesion.

Our results strongly suggest that autophagy is enhanced in neurons after ibotenate injection in the brain of rat pups, as also demonstrated in primary cortical neurons cultures. Autophagosome formation is increased from 6 h as shown by a higher level of LC3-II and more LC3-positive dots. This increase was not due to impaired autophagosome degradation that would have occurred if lysosomal function was defective. In fact, p62/SQSTM1 is also degraded and autolysosomes are increased in neurons as indicated by larger and more numerous LAMP1- and CTSB-positive dots. This result is consistent with EM observations showing the presence of both autophagosome-like multimembrane vesicles and autolysosome-like dense structures in dying neurons of ibotenate-injected rat pups. We provide here, especially in primary neuronal cultures, compiling evidences demonstrating that the autophagy flux is increased by ibotenate treatment. Both Western blots against LC3 and p62/SQSTM1 with or without E64/pepstatinA co-treatments and the use of the GFP-RFP-LC3 construct lead to the same conclusion, ibotenate treatment is increasing autophagosome formation and degradation. Moreover, the GFP-RFP-LC3 construct we used is less sensitive to acidification comparing to other constructs, such as the mKate2-pHluorin-hLC3[61], suggesting that the number of autolysosomes could be theoretically underestimated. Our in vitro data on primary cortical neuronal cultures also strongly argued for the induction of an autophagy-mediated neuronal death by ibotenate. Inhibition of autophagy, not only pharmacologically with 3-MA or E64/PepA, but also genetically by downregulating two important ATG proteins, ATG7 or BECN1, was neuroprotective as previously shown when excitotoxicity was induced in hypoxic conditions[29].

Even if caution is necessary concerning the limited specificity of 3-MA as an autophagy inhibitor[22], we here clearly showed that the dose used in vivo efficiently prevented autophagy (decrease in LC3-II level and SQSTM1/p62 degradation). Furthermore, since it is known that permanent impaired autophagy leads to neurodegeneration (as it would be the case with long term genetic inhibition of autophagy)[43], the use of 3-MA was

appropriate to study long term effect on brain lesion in vivo (16 days after the insult). When ibotenate-enhanced autophagy was prevented by 3-MA treatment, both CASP3 activation and calpain-dependent cleavage of SPTAN (used as an indicator of necrotic cell death characterized by a calcium increase) were significantly decreased. Moreover, ultrastructural observations showed condensed chromatin in nuclei of dying and highly autophagic neurons. Mixed features of apoptosis and autophagy were also observed in rodent models of perinatal cerebral HI[19,20,30,31,60] and we previously demonstrated that autophagy could contribute to apoptosis using widely recognized apoptotic stimuli in primary cortical neuron cultures[62]. The beneficial effect of 3-MA on neuronal cell death, including apoptosis, resulted here in a strong significant neuroprotective effect at long term on both GM and WM, suggesting a crucial role of autophagy in mediating (apoptotic and necrotic) cell death. Studies on perinatal cerebral HI models have also shown that autophagy inhibition could be neuroprotective and reduce apoptosis[20,30,31] suggesting that the transient inhibition of autophagy could be a promising strategy to protect the immature brain against excitotoxic insults.

It has been shown that females are more resistant to perinatal cerebral injury than males, especially in the context of cerebral HI and after moderate lesions, in rodents as well as in humans[63–66]. The reason of this gender difference is still unclear, but sex-dependent cell death pathways have been recognized after perinatal cerebral HI, especially more active caspase-dependent pathways in females[66–68]. In the present study, we also found a stronger CASP3 activation and a more important variability in females in almost all the different parameters investigated. However, mean ibotenate-induced brain lesion volume was similar in both genders. Moreover, the protective effect of 3-MA against both ibotenate-induced autophagy and cerebral lesions was as efficient in males as in females.

In conclusion, we showed for the first time that enhanced autophagy could mediate cell death in a premature model of excitotoxic brain damage. Autophagy inhibition in this severe model is very promising since the protective effect obtained is similar or even better to other previously described neuroprotectants such as caspases inhibitors[69], erythropoietin[70,71], BDNF[72,73], melatonin[74] or magnesium sulfate[75,76]. Interestingly, we recently demonstrated that autophagy is enhanced in dying neurons in the ventrolateral nucleus of the thalamus and the lentiform nucleus of term newborns with severe HIE[31,60]. Apoptotic markers were also expressed in dying highly autophagic neurons, arguing for a possible association between autophagy and apoptosis also in humans. Moreover an increased presence of autophagosomes (LC3-positive dots) was recently demonstrated in WM

injury of extremely preterm infants[77]. These 3 different studies using human newborn brain sections[31,60,77] and the important neuroprotection obtained with 3-MA in the present study in a severe model of cPVL suggest that enhanced neuronal autophagy could be a promising target. The development of strategies transiently inhibiting autophagy could pave the way for new therapies against neonatal severe excitotoxic injuries such as HIE and cPVL.

Material and methods
Primary cortical neuronal cultures

Primary neuronal cultures were prepared according to the Swiss laws for the protection of animals from pieces of cortices of 2-day-old Sprague-Dawley rat pups (Janvier Labs, Mayenne, France). The procedures were approved by the Vaud Cantonal Veterinary Office. After dissection, dissociation and trituration, neurons were plated in neurobasal medium (Gibco, NY, USA; 21103–049) supplemented with 2% B27 (Gibco; 17504044), 0.5 mM L-glutamine (Sigma, MO, USA; 49419) and 100 µg/ml penicillin-streptomycin (Gibco; 15140122) and maintained at 37 °C with a 5% CO_2-containing atmosphere as described previously[78]. Western blot analyses were done on neurons plated at a density of $\sim 7 \times 10^5$ cells/dish (35-mm poly-D-lysine pre-coated dishes (BD Biosciences, NJ, USA; 356467) and at a density of $\sim 3 \times 10^5$ cells on 12-mm glass coverslips coated with 0.01% poly-L-lysine (Sigma; P4832) for immunocytochemistry and imaging. For all the Western blots or imaging results, at least three independent experiments, each involving two or three culture dishes or coverslips, were performed.

Pharmacological treatments

Primary cortical neuronal cultures were pre-treated for 1 h with 10 mM 3-methyladenine (3-MA) (Sigma; M9281), 5 mM EGTA (Sigma; E0396) or 40 µM MK801 (Sigma; M107). For inhibition of lysosomal degradation, a cocktail of 10 µg/ml pepstatin A1 (PepA) (Sigma; P5318) and 10 µg/ml E64 (Sigma; E3132) was applied for 4 h prior to 50 µM ibotenic acid (Enzo Life Sciences, NY, USA; BML-EA120–0001) addition.

Quantification of cell death with lactate dehydrogenase release

Cell death was assayed by measurement of LDH released in the medium using the Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, WI, USA; G1780) as previously described[78]. LDH measurements were normalized with respect to the values of ibotenate-treated neurons 6 h after the ibotenate addition.

Knockdown of ATG using lentiviral vectors

Downregulation of *Atg* genes were performed with pLKO lentiviral vectors (Open Biosystems/Dharmacon, CO, USA) expressing rat-specific shRNA sequences from TRC (the RNAi consortium) library as described previously[62]. A combination of TRCN0000092163 and TRCN0000092166 for *Atg7* (GenBankTM NM_001012097), TRCN0000033552 for *Becn1* (GenBankTM NM_053739.2) and a pLKO vector containing scrambled shRNA (Open Biosystems/Dharmacon) as control vector were used. Primary cortical neuron cultures were infected at DIV7 with 50 ng of the viral capsid protein p24/ml culture medium for each vector.

mRFP-GFP-LC3 plasmid transfection and quantification

Neurons on coverslips were transfected with the tandem mRFP-GFP-LC3–expressing plasmid ptfLC3 (Addgene, MA, USA; 21074)[79] using Lipofectamine 2000 (Invitrogen, CA, USA; 11668-019) as described previously[29]. Coverslips were fixed with 4% paraformaldehyde for 15 min. Confocal images were acquired using a Zeiss LSM 710 Meta confocal laser scanning microscope. Total LC3-positive dots ($GFP^+ RFP^+$ and $GFP^- RFP^+$ dots), early autophagosomes ($GFP^+ RFP^+$ dots) and mature or late autophagosomes ($GFP^- RFP^+$ dots) were analyzed using ImageJ software and expressed as a number of positive dots per neuron per μm^2.

Rat model of preterm excitotoxic brain injury

All experiments were performed in accordance with the Swiss laws for the protection of animals and were approved by the Vaud Cantonal Veterinary Office. Ten µg of ibotenate (diluted 5 µg/µl in acetic acid 0.02%) were stereotaxically injected under isoflurane anesthesia (2.5%) in the subcortical WM at the level of the right cingulum (1 mm posterior and 1 mm right from Bregma and 1.5 mm depth from the skull surface) of 5-day-old male and female Sprague Dawley rats (Janvier Labs) (model adapted from Marret and colleagues (1995)[42]). The control animals received an injection of the same volume of vehicle (acetic acid 0.02%). The pharmacological autophagy inhibitor 3-MA (2 µl of 30 mg/ml in saline) was stereotaxically injected in the right lateral ventricle (0.5 mm posterior and 1 mm right from Bregma and 2.5 mm depth from the skull surface) just before ibotenate injection. Control animals received an injection of the same volume of saline as vehicle. After recovering from anesthesia, rat pups returned to the dam until sacrifice.

Electron microscopy

Electron microscopy (EM) was done on rat brains fixed following intracardiac perfusion with 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (0.1 M, pH7.4) as previously described[80].

Immunoblotting

Immunoblots were performed on extracts from primary neuronal cultures or from cortex collected in lysis buffer (20 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$, 2.5 mM EGTA, 0.1 mM dithiothreitol, 50 mM NaF, 1 mM Na$_3$VO$_4$, 1% Triton X-100 and a protease inhibitor cocktail (Sigma;11873580001)[29]. Protein concentration was determined using a Bradford assay. Proteins (20–40 μg) were separated on 10, 12 or 15% polyacrylamide gels and analyzed by immunoblotting. Antibodies were diluted in the blocking solution containing 0.1% casein (Sigma; C8654). Primary antibodies used were: anti-ATG7 (sc-33211) rabbit polyclonal and anti-BECN1 (sc-11427) rabbit polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-LC3 (ab48394) rabbit polyclonal from Abcam (MA, USA); anti-cleaved CASP3/caspase-3 (9661) rabbit polyclonal from Cell Signalling Technology (MA; USA); anti-SQSTM1 (P0067) rabbit polyclonal from Sigma; anti-FODRIN/SPECTRIN (FG6090) mouse monoclonal from Enzo Life Sciences and anti-ACTA/α-ACTIN (MAB1501) mouse monoclonal from Millipore/Merck (MA, USA). Secondary antibodies were polyclonal goat anti-mouse or anti-rabbit IgG from LiCOR (IRDye 680 or IRDye 800). Protein bands were visualized with the Odyssey Infrared Imaging System (LICOR, NE, USA). Odyssey v1.2 software (LICOR) was used for analysis. Values were normalized with respect to ACTIN.

Immunohistochemistry

Immunostainings were performed on 20μm cryostat brain sections from rat pups perfused transcardially with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4)[29]. PBS with 15% donkey serum and 0.3% Triton X-100 were used for blocking and permeabilization for 30–45 min. Primary antibodies diluted in PBS with 1.5% donkey serum and 0.1% Triton X-100 overnight at 4 °C were: anti-cleaved CASP3/caspase-3 (9661) rabbit polyclonal from Cell Signalling Technology; anti-LC3 (ab48394) rabbit polyclonal from Abcam; anti-CTSB/cathepsin B (06–480), anti-MAP2 (AB5622) rabbit polyclonal antibodies, anti-RBFOX3/NeuN (MAB377) and anti-LAMP1 (428017) mouse monoclonal antibodies from Merk/Millipore. Secondary antibodies were diluted in PBS for 2 h at room temperature.

For immunofluorescence labeling, Alexa Fluor donkey-anti-rabbit or mouse secondary antibodies (Invitrogen; A21202, A21203, A21206, A21207) were used. A LSM 710 Meta confocal microscope (Carl Zeiss) were used for confocal laser microscopy. Images were processed with LSM 510 software and mounted using Adobe Photoshop.

Quantification of autophagic and lysosomal labeling

Confocal images of fluorescent immunostaining against LC3, CTSB and LAMP1 were acquired using the LSM 710 Meta confocal laser scanning microscope (Carl Zeiss) and images were then processed with Adobe Photoshop CC 2015. Positive dots were quantified using ImageJ software and expressed as a number of positive dots per neuron per μm^2 and, for the lysosomal markers CTSB and LAMP1, as a mean dot area per neuron per μm^2.

Cerebral regions volume and WM thickness measurements

Sixteen days after the injury (at P21) brains were perfused, frozen and entirely cut into series of 20 μm coronal sections spaced at 500μm disposed in series. On a cresyl violet-stained series, the ipsilateral volume of the total surviving tissue, the cortex and the lateral ventricle were measured using the Zen Blue software (Zeiss). The volumes were then expressed as a percentage of the total brain volume.

WM thickness were measured on 3 consecutive cresyl violet-stained sections starting from the first on which the genu of the corpus callosum appeared (approx. 0.6–0.8 mm anterior to the Bregma according to the "atlas of the rat brain in stereotaxic coordinates at P21" of Khazipov et al., http://www.ialdevelopmentalneurobiology.com/images/atlases/Atlas-p21.pdf[81]). The thickness of the ipsilateral corpus callosum (on the midline) and the subcortical WM at the level of the cingulum (1.4 mm apart from the midline) and at the beginning of the external capsule (2 mm apart from the midline) were measured parallel to the midline with the Zen Blue software (Zeiss). Values are expressed as a mean of the three measures.

Statistics

Values were expressed as mean values ± standard error of the mean (SEM). Data were analyzed statistically using GraphPad PRISM (version 7.03) software. The normality of the distribution was first tested using Shapiro–Wilk tests. Parametric data were analyzed using a Welch's ANOVA test (one-way ANOVA with unequal variances) followed by a post-hoc Tukey-Kramer test. Non-parametric data were analyzed using a Kruskal–Wallis test (non-parametric analog of the one-way ANOVA) followed by a post-hoc Steel-Dwass. $P < 0.05$ was chosen as threshold for statistical significance.

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