THE ROLE OF AUTOPHAGY IN EPILEPTOGENESIS AND IN EPILEPSY-INDUCEDNEURONAL ALTERATIONS

Filippo Sean Giorgi • Francesca Biagioni • Paola Lenzi • Alessandro Frati • Francesco Fornai

Abstract Recent evidence suggests that autophagy alterations are present in a variety of neurological disor- ders. These range from neurodegenerative diseases to acuteneurological insults. Thus, despite a role of autophagy was investigated in a variety of neurological diseases, only recently these studies included epilepsy. This was fostered by the evidence that rapamycin, a powerful autophagy inducer, strongly modulates a variety of seizure models andepilepsies. These findings were originally interpreted as theresults of the inhibition exerted by rapamycin on the molecular complex named ‘‘mammalian Target of Rapa- mycin’’ (mTOR). Recently, an increasing number of papers demonstrated that mTOR inhibition produces a strong activation of the autophagy machinery. In this way, it is now increasingly recognized that what was once defined asmTORpathy in epileptogenesis may be partially explained by abnormalities in the autophagy machinery. The present review features a brief introductory statement about the autophagy machinery and discusses the involvement ofautophagy in seizures and epilepsies. An emphasis is posedon evidence addressing both pros and cons making it sometime puzzling and sometime evident, the role ofautophagy in the epileptic brain.

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Cell clearing pathways and their involvement in neurological disorders

In eukaryotic cells, two main clearing mechanisms are well described: one is known as ubiquitin proteasome system (UPS); the second one consists in the autophagy machinery. The UPS clears short-lived protein substrates when they are bound to a poly-ubiquitin chain which allows the delivery to the proteasome 20S subunit. The 20S subunit possesses three main enzymatic activities within a pore which is normally closed at both extremities by two 19S subunits, each one forming a cap on each side of the 20S subunit. The poly-ubiquitinated substrate opens the 19S cap allowing the entry of the substrate within the pore to be metabolized as single amino acids or short amino acid chains. There are two specific conditions which limit the range of clearing activities by UPS: (1) the need of a poly-ubiquitin chain binding the substrate; (2) the size and shape of the substrate which needs to fit into the narrow pore allowing only pro- tein but not organelles to be delivered to the 20S proteasome catalytic core (Fornai et al. 2003, 2004, 2005a, b). In con- trast, the autophagy pathway is more flexible in both respects since: (1) both mono- and poly-ubiquitin chains enter the autophagy machinery; (2) there are neither size nor shape limitations for the substrate. This is allowed by the large gateway to autophagy structures made up by two layered membrane which encircle the substrates to seal them into a vacuole known as autophagosome.

Autophagosomes recruit even large organelles such as mitochondria (leading to a specialized type of autophagy, known as mitophagy) as well as long-lived proteins. Being more specific, autophagy is the generic term which defines three specific clearing activities: namely, macroautophagy, microautophagy, and chaperone-mediated autophagy (Pas- quali et al. 2009). Macroautophagy, from now on simply referred as autophagy, the major subtype of autophagy. This pathway starts from enwrapping selected substrates within a double-membrane vacuole, which upon merging with lysosome provides the digestion of the substrate by hydro- lases. Microautophagy is limited to small quantities of proteins which merge with late endosomes and multive- sicular bodies.

Chaperone-mediated autophagy (CMA), features the degradation of proteins containing a consensus pentapep- tide motif, in their primary sequence. This is mediated by the activity of a chaperone such as heat shock protein 70, which escorts substrate proteins to lysosome by targeting the membrane protein LAMP2A (Nixon 2013, for a recent review). All three activities are involved in neurological disorders, however, in the present review evidence will be provided on the role played by macroautophagy, simply referred to as autophagy. Autophagy is characterized by the presence of autophagosomes which are commonly defined as LC3- and beclin-1-positive vacuoles limited by a double membrane and deriving from a nascent structure named phagophore (Klionsky et al. 2014; Bernard and Klionsky 2014) (Fig. 1). Autophagosomes may be empty in resting conditions or they may contain a variety of substrates at various stages of degradation, such as misfolded proteins and disrupted mitochondria. Other organelles, including the endoplasmic reticulum, are also removed by large autophagy vacuoles. Apart from cell organelles which are removed by autophagy only, a few protein substrates can be removed alternatively by UPS and autophagy. Thus, it may be difficult to establish for each specific protein which is the pivotal clearing pathway. This is well exemplified by misfolded alpha synuclein which may be a substrate for both pathways (Fornai et al. 2006a; Giorgi et al. 2006; Ebrahimi-Fakhari et al. 2012; Engelender 2012; Xilouri et al. 2013; Yang et al. 2013). Depending on the specific substrate UPS and autophagy take a leading role in protein degradation (Petroi et al. 2012). If one considers specific autophagy-preferring substrates, upon autophagy inhibition the over- activity of the UPS cannot really provide com- pensation, whereas autophagy can compensate for a failure of UPS (Qiao and Zhang 2009; Cecarini et al. 2012; Viiri et al. 2013). Thus, as far as protein degradation is con- cerned, UPS and autophagy may synergize differently, depending on the specific substrate. In contrast, the autophagy machinery is the sole clearing pathway for cell organelles including mitochondria.

So far, we described, autophagy and proteasome as two separate pathways and classic literature reports these deg- radative complex as distinct clearing systems. However, we recently provided morphological ultrastructural evi- dence that these pathways may co-localize in newly described organelles (Pasquali et al. 2010) (Fig. 2), which are expressed in baseline conditions and increase upon autophagy stimulation (Castino et al. 2008; Pasquali et al. 2010). These organelles are named autophagoproteasomes, consisting in vacuoles limited by double or multiple cell membranes where autophagy markers such as LC3 and beclin-1 co-localize with P20S and PA 700. In this way, the complementary roles of these cell clearing systems

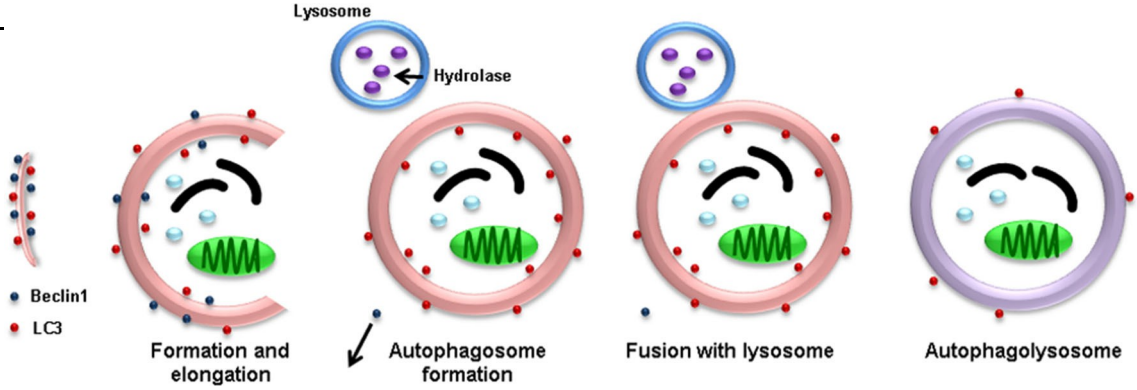

Fig. 1 The autophagy pathway. The *cartoon* reports the main steps in the formation of the autophagosome and the merging with the lysosome to produce the autophagolysosome. Upon mono or poly- ubiquitination a variety of substrates (including long-lived proteins and organelles such as mitochondria) are surrounded by a nascent structure, the phagophore, which seals and forms a double membrane limited vacuole which stains for LC3 and beclin-1 named autophagosome. The clearance of both proteins and organelles is promoted by protease activity of the lysosome. In some cases, before merging with lysosomes the autophagosome converges with endo- somal compartment including late endosomes known as multivesic- ular bodies (MVB) to produce a hybrid structure named amphisome which further merge with lysosome to produce an enriched catabolic activity

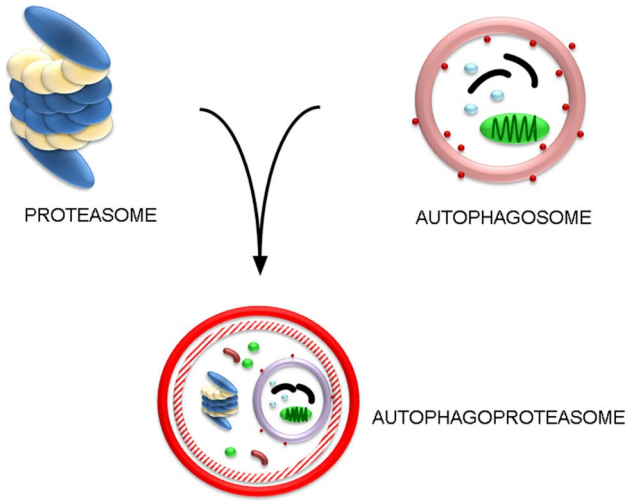

Fig. 2 The autophagoproteasome as the richest clearing structure. In baseline conditions and mostly upon autophagy stimulation, the autophagosome or its merging with the endosome (the amphisome) further converge with classic component of the ubiquitin proteasome system UPS. In this case, the various subunits of UPS are surrounded and included in the double layered membrane to produce the most effective clearing apparatus which is known as autophagoproteasome (Pasquali et al. 2010). In the autophagoproteasome enzymatic activities typical of autophagy, endosome and proteasome pathways are clustered in single morphological entities

previously thought as distinct physical entities converge indeed in time and space providing a unified, energy sparing, clearing apparatus. Morphological evidence of autophagoproteasomes is confirmed by biochemical find- ings showing either co-localization or functional interplay of UPS and autophagy enzymatic activities (Kraft et al. 2010; Cecarini et al. 2012; Aronson et al. 2013; Yang et al. 2013). In keeping with this, a recent paper identified the presence of UPS components in the proteomic analysis of autophagosomes (Dengjel et al. 2012).

These novel vistas on protein clearing systems now start to apply to the field of epileptology, where changes of either autophagy (McMahon et al. 2012) or UPS (Zhao et al. 2011) were described. No study so far systematically addressed the specific contribution of each molecular pathway. Now we may look at this *scenario* as a whole clearing system where autophagy and UPS play a syner- gistic effect. This may explain puzzling results obtained either for autophagy or UPS modulators in the field of epilepsy.

The same concept explains conflicting reports concern- ing other neurological disorders such as amyotrophic lat- eral sclerosis (Urushitani et al. 2002; Fornai et al. 2008a, b; Ferrucci et al. 2011; Pasquali et al. 2009, 2010; Madeo et al. 2009); Parkinson's disease (Fornai et al. 2003; Fer- rucci et al. 2008; Pasquali et al. 2009; Anglade et al. 1997; Jellinger and Stadelmann 2000; Pan et al. 2008; Lenzi et al.

2012), Huntington's disease (Wyttenbach et al. 2000; Ra- vikumar et al. 2002); prion disorders (Liberski et al. 2004; Fornai et al. 2006b; Natale et al. 2008; Chen et al. 2011; Orzi et al. 2012).

The complexity of cell clearing pathways consists in a number of steps, each one requiring appropriate modula- tion to provide homeostasis. For instance a mere failure of cell clearing systems does not tell very much if alterations of specific steps are not detailed. This explains why in a variety of neurological conditions clearing systems are impaired but the clinical phenotypes are different leading to multiple neurological disorders. Therefore, we feel it as mandatory to finely identify the kind of autophagy impairment. In keeping with these concepts, the present review aims to dissect evidence connecting the field of epilepsy with alterations of the autophagy pathway. This short paper is built up to dissect those conflicting details which leaves the role of autophagy in epilepsy under debate rather than offering a generalized or oversimplified solution. In fact, the spirit of this paper is to foster research on the fine tuning of those specific critical steps involving cell clearing systems which may differ in various epilepsies emphasizing pros and cons concerning the various effects of autophagy modulation.

Experimental evidence for a role of autophagy in epilepsy (Which links between mTOR activationand autophagy impairment?)

The role of autophagy in epilepsy emerged due to the evidence that rapamycin, a powerful autophagy inducer, strongly modulates a variety of seizure models and epi- lepsies. These findings were originally interpreted as the results of the inhibition exerted by rapamycin on the molecular complex named ''mammalian Target of Rapa- mycin'' (mTOR). Despite the close relationship between activation of mammalian target of rapamycin (mTOR) and autophagy impairment is automatically validated by most studies, this is unlikely to be a general role given the multiple effects of mTOR. The overemphasized effects of mTOR on autophagy compared with a number of other metabolic activities may mislead interpretation of data obtained using rapamycin. In fact, mTOR plays multiple roles in neuronal and non-neuronal cells development, neuronal plasticity, as well as in the expression of different neuronal molecules involved in cell excitability. Most of these effects may be independent from autophagy (Lasarge and Danzer 2014), although they contribute to the mTOR- induced modulation of epilepsy. Thus, arbitrarily attribu- tion of mTOR-related epilepsy to autophagy impairment remains a mere speculation. Only those studies which assess directly the autophagy status in relationship with

epileptogenesis and epilepsy-induced brain alterations should be primarily considered. Again, even in keeping with these studies, the autophagy status should be carefully monitored. In fact, it is well known that in several cases an increase in autophagy markers is automatically considered as synonymous of autophagy activation (Pasquali et al. 2009). However, when a blockage of the autophagy flux occurs, autophagy markers increase despite an impairment of autophagy progression. This condition brings to a par- adoxical increase in autophagy markers in the presence of autophagy inhibition. This is the case of mTOR unrelated autophagy inhibition which should be considered as well in monitoring the autophagy status in epilepsy. Thus, there are studies showing the occurrence of epilepsy as a direct consequence of autophagy failure depending on mTOR over-activation (Garyali et al. 2014), while other studies demonstrate that the occurrence of epilepsy as a conse- quence of autophagy impairment is due to mechanisms independent from mTOR activity such as the lack of ATG 18 (Saitsu et al. 2013) or ATG7 (McMahon et al. 2012). In these conditions, a blockage of the autophagy flux occurs along with encephalopathy and epilepsy. This results in the accumulation of aberrant autophagy structures. In keeping with this, Shacka et al. (2007) described the accumulation of LC3 positive autophagy vacuoles in the hippocampus of mice after repeated seizures induced by kainate. This is likely to reflect a blockage of autophagy flux; in fact, chronic over-activation of glutamate receptor leads to autophagy impairment due to the blockage of autophagy progression (Fulceri et al. 2011). Consistently, the neuronal damage induced by kainate can be worsened by autophagy blockers while it is prevented by autophagy inducers (Fornai et al. 2008a; Calderó et al. 2010).

Despite these clarifications, most data claiming the involvement of autophagy in seizures remain indirect. In fact, these conclusions represent an inference from data merely assessing the status of mTOR, but neglecting the relevance of the autophagy pathway compared with other mTOR-related activities. In fact, it is well known that mTOR activation induces neurogenesis including aberrant neuronogenesis in specific cortical areas. This may induce aberrant neuronal circuitries leading to epileptogenesis independently from autophagy alterations. In contrast, the evidence of a direct effect of autophagy in epileptogenesis has been shown by altering directly the autophagy path- way, bypassing mTOR modulation. This was obtained in ATG7 KO mice, which possess a normal mTOR signaling but disrupted autophagy activity (McMahon et al. 2012). When exploring the role of autophagy in seizure, one is naturally led to focus on autophagy status in neuronal cells; nonetheless it should be considered that abnormal autophagy activity may alter astrocytes and abnormalities in astrocytes may be produced by seizure as well. This is

the case of clasmatodendrosis, which consists in autoph- agy-related glial cell death, appearing as a consequence of prolonged seizure activity (status epilepticus) (Ryu et al. 2011a, b). This is relevant, since glia plays a multi-faceted role in those mechanisms which regulates seizures and epileptogenesis (De Lanerolle et al. 2010; Seifert and Steinhäuser 2013).

Before discussing in depth the specific studies it is rel- evant here to define the clinical and experimental settings of epilepsy and seizures.

General considerations about epilepsy

Epilepsy is featured by the recurrence of spontaneous epileptic seizures, which are the clinical manifestation of ‘‘abnormal excessive or synchronous neuronal activity in the brain’’ (Fisher et al. 2005). Actually, the term epilepsy includes different types of syndromes. (1) ‘‘Hydiopathic epilepsies’’ (either generalized or focal) are characterized by normal neuroimaging and brain development, while electroencephalography (EEG) may show typical abnor- malities, and a genetic background is hypothesized. (2) ‘‘Probably symptomatic epilepsies’’ (either focal or sec- ondarily generalized) are hypothesized to be due to focal structural abnormalities, which cannot be found by routine neuroimaging. (3) ‘‘Symptomatic epilepsies’’, are related to evident brain damage, either of known iatrogenic origin (e.g., post-traumatic or post-stroke), or due to abnormal development or genetic mutations. The latter ones include epilepsies due to malformations during cortical develop- ment (such as cortical dysplasia or tuberous sclerosis) and they will be extensively discussed in the present review. (4) ‘‘Severe symptomatic epilepsies’’, can be associated with severe brain damage or marked alterations of brain devel- opment. These include: ‘‘progressive myoclonic epilep- sies’’, and ‘‘epileptic encephalopathies’’, including Infantile spasms/West, and Lennox–Gastaut syndromes.

This brief outline provides a plethora of epileptic syn- dromes making it difficult to model epilepsy as a single disorder. Most research efforts focused on limbic or tem- poral lobe epilepsy due to its high prevalence among epi- lepsy patients. Thus, most models are designed to mimic limbic (temporal lobe) epilepsy by producing seizures originating from the hippocampus and/or limbic cortical areas. Among these models, the most common consists in the systemic administration of kainic acid (KA) (an agonist of glutamatergic non-NMDA receptors), or pilocarpine (PILO) (an acetylcholine muscarinic receptor agonist) in rodents (Ben-Ari 1985; Turski et al. 1989).

These models produce limbic seizures which are sec- ondarily generalized, and can be easily scored in terms of behavior (through specific behavioral scales),

electrographic features (through EEG) and duration. Moreover, through these models, a neuropathological alteration often found in patients suffering from temporal lobe epilepsy, i.e. the so-called mesial temporal sclerosis or Ammon's Horn Sclerosis, can be studied experimentally (Ben-Ari 1985; Turski et al. 1989; Dudek et al. 2006). Ammon's Horn Sclerosis consists in the loss of pyramidal cells belonging to the Cornu Ammonis (CA) areas CA1 and CA3–CA4, together with the loss of interneurons of the hilus of the Dentate Gyrus, and aberrant proliferation of mossy fibers originating from granule cells of the dentate gyrus (mossy fiber sprouting) (Sadler 2006). Experimental data obtained by inducing limbic seizures by KA or PILO strengthened the ''two-hit'' hypothesis of epileptogenesis. According to this hypothesis, an early strong insult (1 severe long-lasting seizure named status epilepticus) can trigger progressive changes in the brain, which can be documented following a smoldering asymptomatic time interval, after which spontaneous recurrent seizures along with Ammon's Horn Sclerosis occur (Sloviter 2008). Some authors challenged this model, claiming to be unusual the severity of the early insult (Sloviter 2005), and debating the occurrence of a cause-effect relationship between Ammon's Horn Sclerosis and development of spontaneous recurrent seizures (Sloviter 2005; Silva and Mello 2000). Other models of limbic seizures are: stimulation of the perforant path (Mazarati et al. 2002), intra-hippocampal infusion of KA (Rattka et al. 2013), focal injection of chemoconvulsants into the anterior extent of the rat piri- form cortex (Piredda and Gale 1985). All these models confirm the occurrence of spontaneous recurrent seizures and Ammon's Horn Sclerosis after a silent, smoldering time interval ranging from the early epileptogenic insult to the first spontaneous seizure (Mazarati et al. 2002; Rattka et al. 2013; Giorgi et al. 2003, 2006).

Other epilepsy models exist as well, which bear inter- esting features for studying epileptogenesis. Some of them are based on reproducing in rodents those genetic altera- tions which are known to cause epilepsy in humans: this is the case of models reproducing severe epilepsy such as tuberous sclerosis complex or Lafora disease.

Lafora disease as a prototype of autophagy-relatedepilepsy (Figs. 3, 4)

Lafora disease (LD) is a catastrophic form of autosomal recessive epilepsy featured by seizures, progressive myoclonus, cognitive impairment, and typical glycogen- like inclusions named Lafora bodies (LB) (Ramachandran et al. 2009). LD is the most frequent form of a group of epilepsies named progressive myoclonic epilepsies (PME). The disease is devastating, leading to death within the

second–third decade of life. The mutation causing Lafora disease affects either laforin (encoded by the PME2A gene) or malin (encoded by PME2B gene). Malin is an E3 ligase (an enzyme which binds covalently ubiquitin to the sub- strate), while laforin is a glycogen 6 phosphatase (an enzyme which degrades glycogen chains). LD has been modeled in mice by knocking out either the malin gene, thelaforin gene, or both.

The progressive accumulation of LB, along with the devastating effects of seizures themselves, causes massive brain damage and lethality. The occurrence of inclusion bodies within different tissues indicates altered cell clear- ing pathways. Accordingly, it is well established that nor- mal laforin is critical to promote autophagy progression (Aguado et al. 2010), while the laforin–malin complex promotes degradation of intracellular misfolded proteins also by activating the UPS (Garyali et al. 2009). Since autophagy and UPS often work in single functional and morphological units as discovered very recently and reported in the first paragraph (Fig. 2), it is not surprising that autophagy and UPS impairment were both reported in LD generating a trivial debate. In 2012, it was definitely shown that in double knock-out mice for laforin and malin, a phenotype highly reminiscent of Lafora disease occurred. Accordingly, knock-out mice possess severe motor and cognitive impairment which associates with severe motor seizures witnessed by marked EEG abnormalities. These symptoms are accompanied by progressive and diffuse accumulation of LB (Garc´ıa-Cabrero et al. 2012). These data confirm the occurrence of widespread neurodegener- ation along with electrographic and behavioral seizures and with accumulation of LB in mice featuring disruption of laforin synthesizing gene (Ganesh et al. 2002).

More recently, the chains of events linking one to each other laforin, malin, and LB formation has been further clarified, since in the present year two different research groups showed that glycogen accumulation is responsible for neurodegeneration in LD. In fact, malin knock-out mice when lacking glycogen synthesis are protected from behavioral and pathological alterations as well as altered EEG and seizure susceptibility (Duran et al. 2014; Turnbull et al. 2014). Remarkably, neurodegeneration, occurring in mice lacking malin is reversed in malin ? glycogen syn- thetase double KO mice (Duran et al. 2014).

Laforin–malin complex might suppress glycogen syn- thesis but the chance that this complex promotes glycogen degradation via mTOR inhibition should be considered as well (Singh et al. 2012).

The tight connection between LD and autophagic impairment has been proposed by several authors through different studies (for a review, see Polajnar and Zerovnik 2011); this connection has been clearly shown in mice lacking laforin by Puri et al. (2012) and Criado et al.

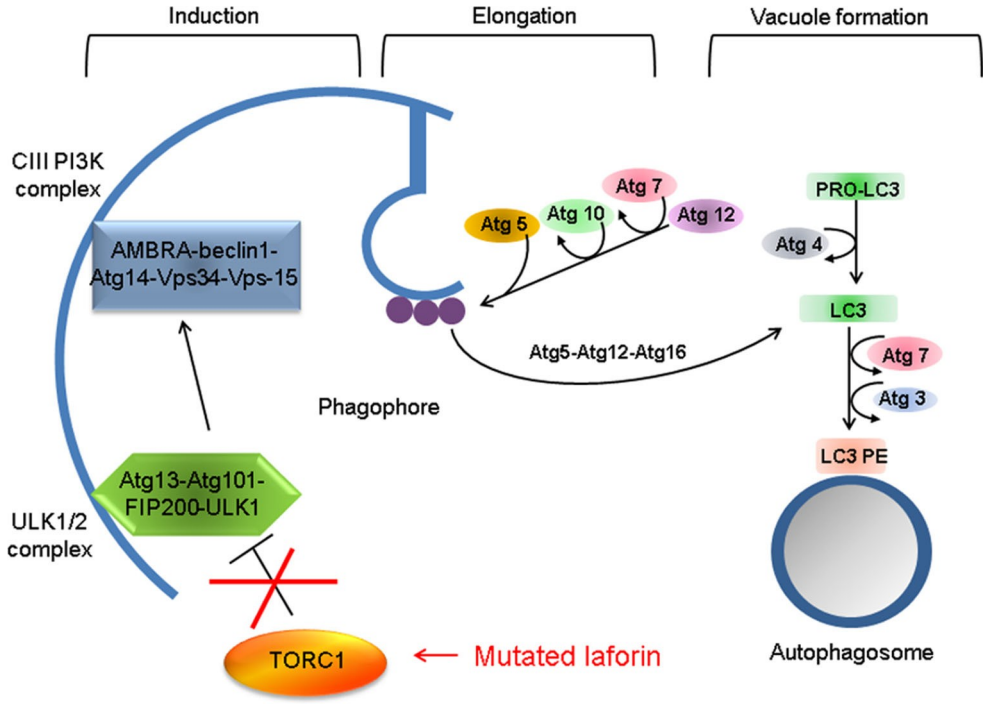

Fig. 3 The autophagy status in Lafora disease. In the *cartoon,* the regulation of the autophagy machinery is reported detailing the inhibitory effects which are induced by the mTOR complex. In normal conditions, the mTOR inhibits the autophagy machinery by inhibiting early steps thereby occluding induction elongation and formation of the autophagy vacuole. The mutation causing Lafora disease affects either laforin or malin (see text). In any case, these mutations lead to the activation of the mTOR complex. Normal laforin or malin (here simply reported as laforin) inhibits the mTOR complex thus relieving the autophagy machinery from a powerful and constant inhibitory activity. In contrast, when laforin is mutated the autophagy pathway undergoes a strong inhibition under the influence of hyperactive mTOR

(2012), who consider autophagy impairment as the primary trigger in LD. In line with this, it has been shown that impairment of hippocampal and cortical GABAergic neu- rons anticipates the appearance of LB (Valles-Ortega et al. 2011; Ortolano et al. 2014).

Experimental temporal lobe epilepsy and autophagy

Concerning the role of autophagy in temporal lobe epilepsy models, an early observation dating back 7 years ago showed in mice an increase of autophagy markers, such as LC3-II, p-mTOR/m-TOR ratio, and phospho-Akt/Akt ratios, after KA administration (Shacka et al. 2007). Two years later, a sudden increase of autophagy markers (LC3II/LC3I, beclin-1) was found also after PILO admin- istration in rats (Cao et al. 2009). In the following years, the potential role of autophagy creped back again as a side observation in studies evaluating the role of mTOR and its gold standard inhibitor, rapamycin, in two classic temporal lobe epilepsy models, PILO or KA systemic administra- tion. These studies provided a fair convergency showing that: (a) acute seizures induce hyperactivation of mTOR;

(b) mTOR activation induces acute neurotoxicity after status epilepticus; (c) persistent mTOR activation associ- ates with delayed limbic neurodegeneration.

In detail, after KA and PILO-induced status epilepticus,mTOR is activated both acutely and chronically. Thisactivation is prominent in the hippocampus and othercortical areas (Macias et al. 2013). Such an activation isprolonged, since it is present up to 5 days after a seizure episode (Sha et al. 2012), and it persists several weeks afterstatus epilepticus (Huang et al. 2010). Further data madethe *scenario* more complex than initially expected: Maciaset al. (2013) showed at least two ‘‘waves’’ of mTOR acti-vation after KA-induced status epilepticus: an initial onemainly involves the neurons, while the second occurswithin glial cells. This confirms preliminary observationsprovided by Sha et al. (2012). To further address the effects of mTOR on temporal lobe epilepsy, many authors haveassessed the effects of rapamycin in this kind of seizures.The earlier studies tested the effects of rapamycin administration after status epilepticus showing a reductionof EEG alterations and hippocampal damage such as mossyfiber sprouting (Buckmaster et al. 2009; Buckmaster andLew 2011; Buckmaster and Wen 2011; Heng et al. 2013).

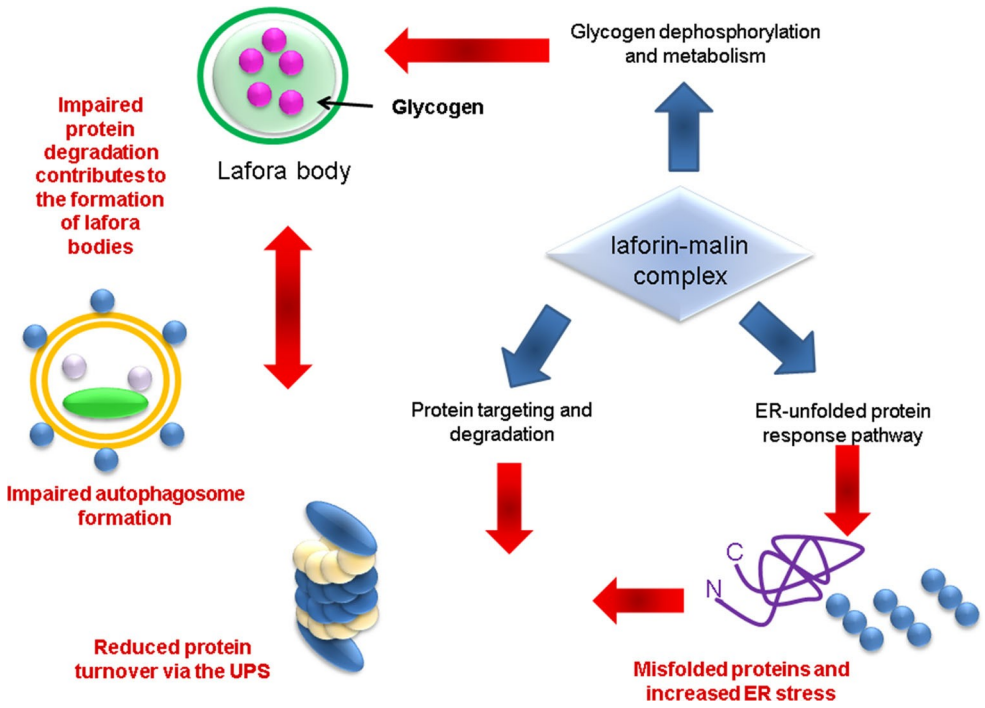

Fig. 4 Formation of Lafora bodies as a consequence of autophagy inhibition. The mutation causing Lafora disease affects either laforin (encoded by the PME2A gene) or malin (encoded by PME2B gene). Malin is an E3 ligase (an enzyme which binds covalently ubiquitin to the substrate), while laforin is a glycogen 6 phosphatase (an enzyme which degrades glycogen chains). The combined effects of laforin and malin leads to degradation of glycogen chains and to the activation of both autophagy and proteasome. When mutations occur in laforin–malin, complex glycogen chains are no longer disrupted and they accumulate within Lafora bodies (LB). At the same time, the impaired clearance of both proteins and organelles leads to neuronal dysfunc- tion which causes massive brain damage and lethality. Since autophagy and UPS often work in single functional and morpholog- ical units (Fig. 2), it is not surprising that autophagy and UPS impairment were both reported in Lafora disease

Further studies, on the effects of rapamycin administration after status epilepticus led to conflicting results concerning the development of spontaneous recurrent seizures. In fact, Huang et al. (2010) reported that chronic rapamycin treatment after PILO-induced status epilepticus in mice led to reduction in spontaneous recurrent seizures, while Buckmaster and Lew (2011) could not confirm this effect. To make the *scenario* even more complex, rapamycin administration induces an increase in p-S6 (an index of mTOR activity, Chen et al. 2012), which might explain the paradoxical exacerbation of mTOR activation by KA when rapamycin is administered 1 h before (Chen et al. 2012). This effect is no longer present either after rapamycin alone or rapamycin ? KA, when mTOR activity is assessed at later time intervals. Thus, it is very likely that the increase in p-S6 represents a compensatory effect operating to counteract the inhibition of mTOR.

Remarkably, rapamycin suppresses the occurrence of mossy fiber sprouting after PILO-induced status epilepti- cus, both in rats (Buckmaster et al. 2009), and mice (Buckmaster and Lew 2011), even though such an effect appears to be transient

and quickly reversible after rapa- mycin withdrawal (Buckmaster and Lew 2011).

Interestingly, the observation by Buckmaster and Lew (2011) that chronic rapamycin administration did not affect spontaneous recurrent seizures after PILO-induced status epilepticus, added a further element to debate whether mossy fiber sprouting plays a crucial role in spontaneous recurrent seizures. Previously, Zeng et al. (2009) had shown that rapamycin administration before KA-induced status epilepticus reduces the occurrence of mossy fiber sprouting, cell loss in limbic structures, and spontaneous recurrent seizures. Surprisingly enough, rapamycin administered after KA is still able to reduce mossy fiber sprouting and spontaneous recurrent seizures, without affecting, neither cell loss nor neurogenesis. These con- flicting findings may be explained as follows: (a) different rodent species (mice vs. rats) and strain differences;
(b) different cellular and molecular substrates of epilepto- genesis between two models of limbic status epilepticus (PILO vs. KA); (c) different timing, dosing, route of administration and duration of rapamycin administration. In surgical human specimens, it has been shown that mTOR pathway is increased within Ammon's Horn Scle- rosis (Sha et al. 2012). In particular, Sha and co-workers found mTOR activation within reactive astrocytes, as well

as within granule cells. In the same study, these authors described a time sequence of mTOR expression following experimental Ammon's Horn Sclerosis due to KA, where at later time points the higher expression was within astrocytes and dispersed granule cells. Detailed analysis of specimens from patients with Ammon's Horn Sclerosis (Sosunov et al. 2012) showed a marked p-S6 immuno- staining in reactive astrocytes, but not in CA1 pyramidal cells. A detailed time-course study by Macias et al. (2013) showed hyper-expression of p-S6 within astrocytes in Ammon's Horn Sclerosis after KA-induced seizures at 24 h after status epilepticus. This effect shifts from neu- ronal to glial p-S6 from 2 to 24 h after KA (Macias et al. 2013).

Another issue which has been tested in models of limbic status epilepticus is whether rapamycin, apart from chronic, plastic changes, also modulates seizure severity, either acutely following systemic KA administration or chronically during spontaneous recurrent seizures. Briefly, it has been shown that single rapamycin administration does not reduce seizure severity by KA neither in adult mice (Hartman et al. 2012) nor in developing rats (Chachua et al. 2012). In adult rats, rapamycin pre-treatment 1 h before KA administration worsens seizure severity and duration (Chen et al. 2012). Similarly, in immature rats, rapamycin pre-administration worsens seizures induced by PILO (Huang et al. 2010). Only one study assessed chronically administered rapamycin after PILO-induced status epilepticus in rat, on the development of spontaneous recurrent seizures. The authors found a significant reduction of spontaneous recurrent seizures, however, when rapamycin was withdrawn seizures recurred again (Huang et al. 2010).

In their extensive and detailed study, Macias et al. (2013) found that both short (i.e. 3 treatments/week during the previous week) or long (i.e. 3 treatments/week during the previous 4 weeks) pre-treatment with rapamycin reduced latency to acute seizures induced by KA and increased the number of animals with acute seizures. They also found that long-lasting rapamycin pre-treatment induces marked increase in lethality after acute KA sei- zures. They further showed an increase of frequency of epileptic discharges caused by high dose KA in hippo-campal slices from rats following short and long-term rapamycin pretreatment (Macias et al. 2013).

The role of autophagy in epilepsy due to malformation of cortical development

Malformations of cortical development are frequently linked to epilepsy. Among them, focal cortical dysplasia accounts for a high percentage of refractory focal

epilepsies, especially in children. In fact, focal cortical dysplasia occurs in up to one-fourth of patients undergoing epilepsy surgery for resistance to antiepileptic drugs; among focal cortical dysplasia occurring in these specimens, type II focal cortical dysplasia (FCDII) is present in almost one-third of cases (Gaitanis and Donahue 2013;Sisodiya et al. 2009).

Another malformation of cortical development which is associated with refractory seizures is tuberous sclerosis complex (TSC). TSC is a congenital disease affecting almost 1/6,000 of births, and it is one of the most common forms of cortical dysplasia. It is a multi-organ disease, whose main pathological features are hamartomas. Brain malformations consist of cortical tubers (mainly), or astrocytomas or sub ependymal nodules. Tubers typically contain large balloon cells indistinguishable from those found in FCDII (for a review, see Crino et al. 2002). Patients affected by TSC often experience seizures, and significant behavioral, and cognitive impairment.

Both for FCD and TSC, a tight correlation between hyperactivation of mTOR and cortical alterations has been clearly established. More recently, alteration in autophagy mechanisms in these disorders was investigated, too.

Concerning TSC, the genes most frequently involved are TSC1 and TSC2, encoding for hamartin and tuberin, respectively (Fig. 5). Both proteins are connected with mTOR function. In particular, they form a complex acti- vating Rheb, a G protein inhibiting mTOR (Tee et al. 2003; Manning and Cantley 2003). In TSC, targets of mTOR are hyperphosphorylated compared with control tissue (Baybis et al. 2004; Yasin et al. 2013). Recently, Yasin et al. (2013) studied in detail the features of balloon cells both in TSC and in FCDII. In particular, they showed that balloon cells from TSC feature the followings: (a) a significant accu- mulation of lysosomes, as shown by LAMP1 and LAMP2 immunohistochemistry, as well as by LysoTracker; (b) a significant expression of ATG5, LC3/ATG8, ATG12, and beclin-1/ATG6; (c) a significant expression of a co-factor involved in autophagy initiation, DOR; (d) a rise in p62 immunostaining, which is a marker autophagy vacuoles. When rapamycin was administered to the balloon cells which were cultured from TCS specimens, all the markers of impaired autophagy progression disappeared. Thus, they showed that the inhibition of autophagy in balloon cells from TSC is associated with mTOR hyperactivity. In the same year, another group (Miyahara et al. 2013) found autophagy suppression in balloon cells and dysmorphic cells from hamartomas of TSC patients, with a normal expression in intermingled normal-appearing neurons.

In human FCD type IIb, which is often associated with epilepsy the autophagy status has been debated (Baybis et al. 2004). However, Yasin et al. (2013) recently dem- onstrated that autophagy is selectively impaired in balloon

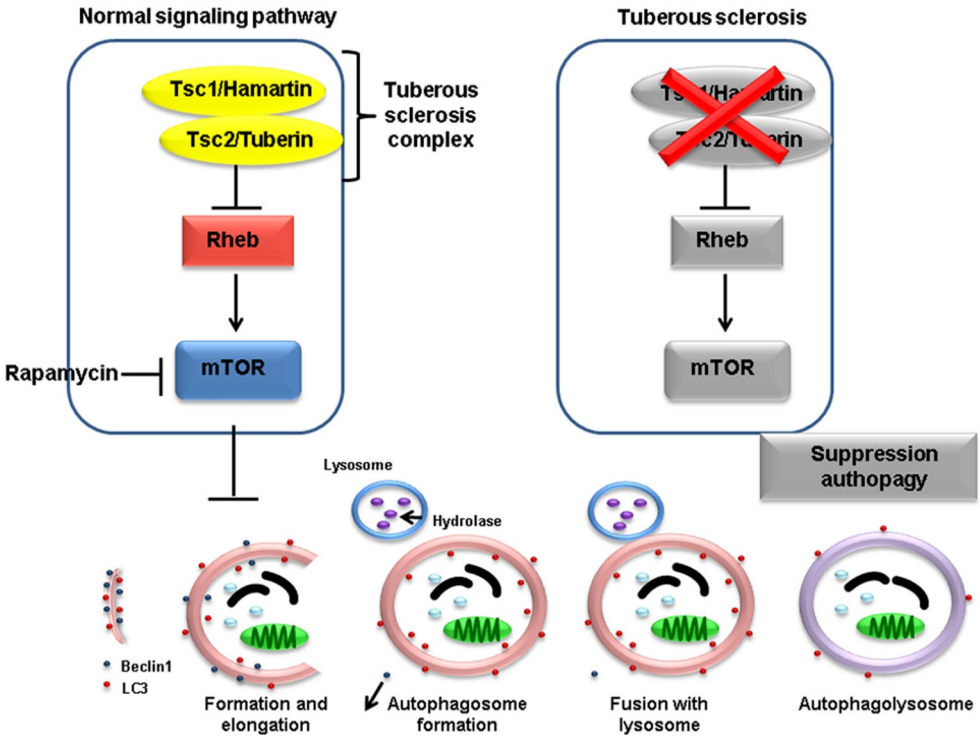

Fig. 5 The autophagy status in tuberous sclerosis. Tuberous sclerosis leads to altered cortical development which is associated with refractory seizures. In the *cartoon,* the physiological inhibition of the mTOR complex by hamartin and tuberin is reported. This inhibition removes the block produced by mTOR on the autophagy machinery. When hamartin and tuberin are mutated (genes known as TSC1 and TSC2, respectively) their inhibition of mTOR is lost. This generates autophagy inhibition. In these pathological conditions, the powerful mTOR inhibitor rapamycin may rescue autophagy activity

cells from FCD type IIb possessing the same features reported in TSC (see above), thus disclosing a significant autophagy inhibition in FCD type IIb, which is strongly associated with mTOR activation.

A glance on channelopaties

Among several functions which are known to be supported by the mTOR pathways it is relevant in the field of epilepsy to mention the chance to

modify ion channels. The mTOR complex has been related to presynaptic efficacy and ionic channel expression on neuronal membrane are known to be critical targets in epilepsy. In vitro, increased mTOR sig- naling induces a marked increase of the evoked synaptic response both in GABAergic and glutamatergic neurons (Weston et al. 2012). The latter effects have been interpreted to be due to an increase in miniature event size, number of active synapses, and vesicles available per synapse, even though same authors showed also a potential inhibition of synaptic vesicles fusion mechanisms by mTOR activation (Weston et al. 2012). Raab-Graham et al. (2006) showed that mTOR inhibition promotes cell mem- brane expression of voltage-gated potassium (Kv1.1) channel in neuronal dendrites. Even more interestingly, cultures of astrocytes bearing a conditional inactivation of Tsc1 gene, i.e. bearing an hyperactivity of mTOR, show a reduced expression of specific inward rectifier potassium channels (Kir) subunits, and, accordingly, reduced Kir currents (Jansen et al. 2005). The role of autophagy in these events bound to mTOR modulation remains to be investigated.

Other epilepsy models: West syndrome

Recently the role of mTOR pathway has been tested also in a model of epileptic encephalopathy, Infantile Spams/West syndrome (IS). IS is a catastrophic form of epilepsy affecting infants, with an onset in the early infancy, fea- tured by a marked diffuse EEG alteration (hypsarrhyth- mia), associated with typical seizures (infantile spasms), and a marked impairment of brain development: it accounts for 25 % of patients with epilepsy onset in the first year of life. It has a symptomatic etiology (either metabolic or organic) in the vast majority of cases, and only a few cases are linked to a genetic cause. Only a few models are available so far. The multiple-hit protocol is often used, which consists in multiple intra-cerebral injections of doxorubicin and lipopolysaccharide, to postnatal day 3 rats. This treatment produces brain damage and generates some pathological, EEG, and seizure features reminiscent of symptomatic form of Infantile Spasms (Scantlebury et al. 2010). Using this rat model, Raffo et al. (2011) showed that rapamycin normalizes pS6 levels and occurrence of spasms in a dose-dependent manner. Furthermore, such an effect was persistent even after withdrawal of rapamycin in the high dosing schedule. The protective effects of rapamycin

were not reproduced by Chachua et al. (2012) using another IS model, the prenatal betamethasone/postnatal *N*- methyl-D-aspartate (NMDA) (Vel´ısek et al. 2007). How- ever, in the latter case, rapamycin was given before the induction of seizures; several other methodological differ- ences could explain these differences (reported in Gala- nopoulou et al. 2012). Unfortunately, no detailed study on autophagy markers in these two experimental models was performed so far.

An attempt to distinguish between mTOR activationand autophagy impairment in seizures

As discussed in the second paragraph, most of the evidence on the involvement of autophagy in epilepsy derives from studies assessing the state of mTOR in experimental models of seizures or in surgical specimens from epileptic patients. An exception is the direct assessment of a link between mTOR activity and autophagy impairment within balloon cells from TSC and FCD patients performed by Yasin et al. (2013). In most cases, the link between mTOR and autophagy impairment remains to be clearly estab- lished. Thus, the role of autophagy as a critical step in the process of epileptogenesis or in seizures occurrence, sometime is evident, while other times is only speculated based on the effects of rapamycin. This drug was repeat- edly used to modulate seizures or epileptogenesis due to its inhibitory activity on mTOR, however, mTOR plays mul- tiple roles in neuronal/non-neuronal cells development, plasticity, as well as in the expression of different neuronal molecules involved in cell excitability, which are inde- pendent from its role in modulating autophagy (Lasarge and Danzer 2014).

Upon mTOR hyperactivity aberrant connections of newly generated cells take place, and eventually generates abnormally synchronously firing cells. This mechanism which may lead to seizure onset is reminiscent to what occurs for epilepsy induced by frank cortical heterotopia. In line with this, mTOR activation and aberrant granule cell proliferation has been described in transgenic mice over-expressing mTOR (Amiri et al. 2012). In fact, in transgenic mice carrying over-activation of the mTOR pathway, subtle alterations, in otherwise normally appear- ing neurons, have been described which might underlie the development of hyper-excitable neuronal networks. These features consist in hypertrophy and abnormal axonal length, abnormal, and hypertrophic dendrites, as well as abnormal synaptic contacts between neighboring neurons. These network alterations are expected to affect signifi- cantly, though unpredictably, neuronal excitability. These phenomena are likely to be an important cause of decreased threshold for a variety of epileptic stimuli.

Recently, McMahon et al. (2012), performed an elegant study to test directly the hypothesis whether inactivation of autophagy by mTOR modulation is sufficient per se to cause seizures. They showed that mice with conditional lack of brain TSC1, experience spontaneous seizures and autophagy impairment. Most importantly, the evidence of a direct effect of autophagy in epileptogenesis was obtained bypassing mTOR modulation to directly manipulate the autophagy pathway. This was obtained in ATG7 KO mice, which possess a normal mTOR signaling but disrupted autophagy activity. These mice possess spontaneous epi- leptogenesis making it certain the link between autophagy impairment and epileptogenesis. This is so far the most stringent demonstration for a direct role for autophagy impairment in epileptogenesis in vivo.

Final remarks

In this short review, we critically analyzed evidence about the involvement of the autophagy pathway in epilepsy. In this process, we faced increasing data showing a direct link between the mutation of specific genes inducing severe epilepsy and mTOR alterations. Although mTOR complex is a powerful inhibitor of autophagy, the up-regulation of this system does not necessarily imply the involvement of autophagy, being innumerous the neuronal pathways under mTOR modulation. This analysis led to tone down some enthusiastic viewpoints lending to include autophagy in all mTOR alterations. Nonetheless, when examining evidence which demonstrates a direct relationship between autophagy and epileptogenesis it becomes clear that autophagy is seminal indeed for epileptogenesis and epi- lepsy-inducedbrain damage. For instance, transgenic mice reproducing the genetic alterations of human epilepsies allowed to dissect the molecular phenomena occurring downstream to mTOR alteration, showing that, along with mTOR activation a significant impairment of autophagy occurs. This anticipates the development of typical brain damage (e.g. the tuberi in TSC). Remarkably, during acquired epilepsy mTOR up-regulation, along with autophagy suppression has been recently demonstrated. This is the case of Ammon's Horn Sclerosis and temporal lobe epilepsy, both in human specimens and in brain tissues from animal models. Finally, in light of data obtained in transgenic mice, it is becoming clear that autophagy depression, during mTOR upregulation can trigger per se spontaneous seizures (McMahon et al. 2012). Likewise, autophagy alteration independently of mTOR modulation leads to epilepsy as well.

Thus, it might be speculated that seizures caused by each classic mechanisms can trigger pathological altera- tions associated with autophagy impairment which, in turn,

can trigger further epileptogenesis in a feed-forward loop leading to higher seizure frequency and treatment refractoriness.

This suggests the potential use of drugs activating autophagy at different steps including powerful mTOR inhibitors such as rapamycin in those cases where severe epilepsy needs to be faced. On the other hand, the use of autophagy modulators independently of mTOR, either as drugs or dietary components, may become a useful tool to be used early in the course of epilepsy, to prevent the onset of seizure relapse and drug refractoriness. Nowadays, mTOR inhibitors are being tested early in children affected by devastating epilepsy, in which a connection between brain degeneration and mTOR activation has been shown, as in TSC (Cabrera-López et al. 2012; Franz et al. 2013). Recent exciting data raise the possibility that mTOR pathway could be a therapeutic target also in another cat- astrophic epilepsy, such as IS/West syndrome (Galanop- oulou et al. 2012; Riikonen 2014), even though in this case only very few models have been developed so far, and mTOR inhibition does not provide univocal results (Chachua et al. 2012).

Interestingly, the ketogenic diet is effective in some patients with multi-resistant catastrophic epilepsies. It is likely that this is based on starvation-induced autophagy activation. In line with this, it was recently proposed an intermittent caloric restriction as an additional treat- ment in pharmacoresistant epilepsy (Yuen and Sander 2014).

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