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REVIEW ARTICLE

Autophagy and amino acid metabolism in the brain: implications for epilepsy

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Abstract Autophagy is a catabolic pathway responsible for the maintenance of the tissue and organism homeostasis. Several amino acids regulate autophagic activity in different tissues, such as liver and muscle, but much less is known about this regulation in the brain. The lack of autophagy in neurons leads to a strong neurodegenerative phenotype and epileptic disorders. We summarize the current knowledge about the regulation of autophagy mediated by amino acids and how macroautophagy could serve as source of amino acids. We review the contribution of macroautophagy in the brain physiology and pathology emphasizing the relevancy of the proper control of amino acid levels such as glutamate and GABA in the brain due to its role as neurotransmitters and energy source. Furthermore, we discuss how malfunction in autophagy may result in pathological consequences, because many genetic epileptic disorders are related to signaling or metabolic pathways controlling both macroautophagy and amino acid metabolism in the brain.

Keywords Macroautophagy · Leucine · Glutamine · Epilepsy · Ketogenic diet · mTOR

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Introduction

An appropriate equilibrium between protein synthesis and proteolysis is essential to maintain the physiology of the tissues and cells. Both processes must be tightly regulated to assure the adequate turnover of components. Two pathways are involved in the hydrolysis of proteins to provide free amino acids (AAs) to the cellular metabolism: ubiquitin–proteasome system and autophagic-lysosomal system.

The term *autophagy* was derived from the Greek mean self-eating. The hallmark of macroautophagy (hereafter called autophagy) is the formation of a double-membrane structure called autophagosome where cytoplasmic components are engulfed to be degraded. Research in yeast revealed a group of highly conserved proteins called Atg proteins (autophagy-related proteins) involved in the mechanism of autophagosomal biogenesis. The molecular core of autophagy is conserved from yeast to humans and the induction of the autophagic process has been broadly reported in most of the tissues in mammals (Mizushima et al. 2008; Boya et al. 2013).

Autophagy is a catabolic pathway associated with different physiological processes such as recycling of cellular component, degradation of aggregates, cell survival, differentiation, development and lifespan extension (Singh and Cuervo 2013; Wong and Cuervo 2011; Bergamini et al. 2007). For many years, the regulation of the autophagic activity by changes in the nutritional status constituted the center of attention, and protein catabolism and AAs release after starvation were the first defined functions for autophagy (Mortimore and Schworer 1977; Mortimore and Poso 1987). In the last decade, this autophagic route has captured the attention in research because autophagy was clearly linked to several pathological conditions and, in

this context, a renewed attention to the interplay between autophagy and cellular metabolism has been raised.

In the brain, autophagy has been mainly studied in the context of clearance of aggregates preventing neurodegenerative diseases and, more recently, the autophagy function in the hypothalamus has been revealed to control the food consumption (Nixon et al. 2005; Kaushik et al. 2011; Nixon 2014). Nevertheless, much less attention has been given to its possible contribution to the AA metabolism despite the alterations observed in essential and branched-chain AAs in the brain in mice with genetically compromised macroautophagy (Kuma et al. 2004).

In this review, we summarize the most recent evidences of autophagy roles in the physiology and pathology of the brain. We focus in the autophagic role on metabolism of AAs and the metabolic coupling required for a proper homeostasis and neural transmission in brain and its implications in epilepsy.

Modulation of autophagic activity mediated by amino acids

Although both ubiquitin–proteasome system and autophagic-lysosomal system generate free AAs as final product, only the autophagy pathway has been described to be controlled by the levels of AAs. The modulation of autophagic through AAs offers a feedback regulatory mechanism to assure that the metabolic homeostasis and an enormous effort have been undertaken to clarify how the AAs exert the regulation and what is the physiological significance.

Several AAs (Leu, Tyr, Gln, His, Trp, Pro, Met and Ala) have been associated with suppression of proteolysis in the liver (Mortimore and Poso 1987), but not all of them are equally effective in the autophagy regulation (Bergamini et al. 1994). It is unclear how different AAs impact on autophagy and it has been also reported that the modulation mediated by AAs varies among tissues (Kadowaki and Kanazawa 2003; Naito et al. 2013). In fact, little is known about the regulation of autophagy specifically in the brain, given that most of the knowledge has been originated from studies in liver and muscle.

In the past, it was hypothesized the existence of putative receptors at the plasma membrane sensing the levels of AAs in plasma and triggering the intracellular response (Kadowaki and Kanazawa 2003). Different groups focused the energies on identifying proteins in the cell surface responsible for the extracellular AAs sensing. The existence of a leucine-binding protein was reported in the membrane of hepatocytes, which might act as sensor of AAs triggering a cascade of intracellular signaling a posteriori (Mortimore et al. 1994). Despite the possibility of sensing AAs levels directly in the plasma membrane, the consensus model in the field has established that the AAs sensing takes place in the cytoplasm of the cells after the uptake through membrane transporters (Hyde et al. 2003). Intracellular mediators would be acting after the AAs import through sodium-dependent or independent transporters. It has been demonstrated that the coupling between the transporters SLC1A5 and SLC7A5-SLC3A2 [that together form the large neutral AA tansporter (LAT1)] is essential for the modulation of intracellular Leu levels (Nicklin et al. 2009). In fact, knockdown of any of these transporters leads to induction of autophagy supporting the relevance of the AAs availability in the modulation of autophagic activity and the key role of AAs transporter in the uptake into the cellular cytoplasm.

Undoubtedly, the molecular aspect that has mainly captured the interest of researchers was the intracellular signaling scenario modulating the autophagic process. Despite the remarkable progress in the understanding of the modulation of autophagy mediated by AAs, only a few candidates for the targeting of AAs have been proposed. Actually, the molecular targets of AAs do not seem to be members of the Atg proteins family responsible for the formation of the autophagosomes but proteins involved in the phosphorylation signaling upstream of autophagosomal biogenesis. Intense research has uncovered that the response of different signaling cascades modulates the triggering and the duration of autophagic activity. Early studies revealed that the ability of phosphorylation of mTOR was dependent on different energetic inputs including growth factors, ATP concentration and AAs so mTOR complex 1 (mTORC1) was identified as the master regulator of autophagy (Blommaart et al. 1995; Hara et al. 1998; Patti et al. 1998).

In the most accepted model of autophagic signaling, several energy-related inputs converge in mTORC1 determining the ability of phosphorylation of the complex. The kinase activity of mTORC1 leads to a blockage of the autophagic initiation through the direct phosphorylation of Atg13 and ULK1 (putative mammalian homolog of Atg1) (Lee et al. 2007; Egan et al. 2011a). In the presence of AAs, mTORC1 inhibits the activity of the complex ULK1-Atg13-FIP200 that is involved in the early steps of autophagosomal biogenesis. By contrast, when AAs are scarce (or under biochemical blockade using rapamycin treatment), the ability of mTOR phosphorylation is diminished and the complex ULK1-Atg13-FIP200 is quickly dephosphorylated and released to activate the events of autophagosomal formation (Fig. 1). More recently, it has been also shown that mTOR complex contributes to the control of nuclear/cytoplasmic shutting of TFEB, a transcription factor regulating autophagy and lysosomal biogenesis (Settembre et al. 2012).

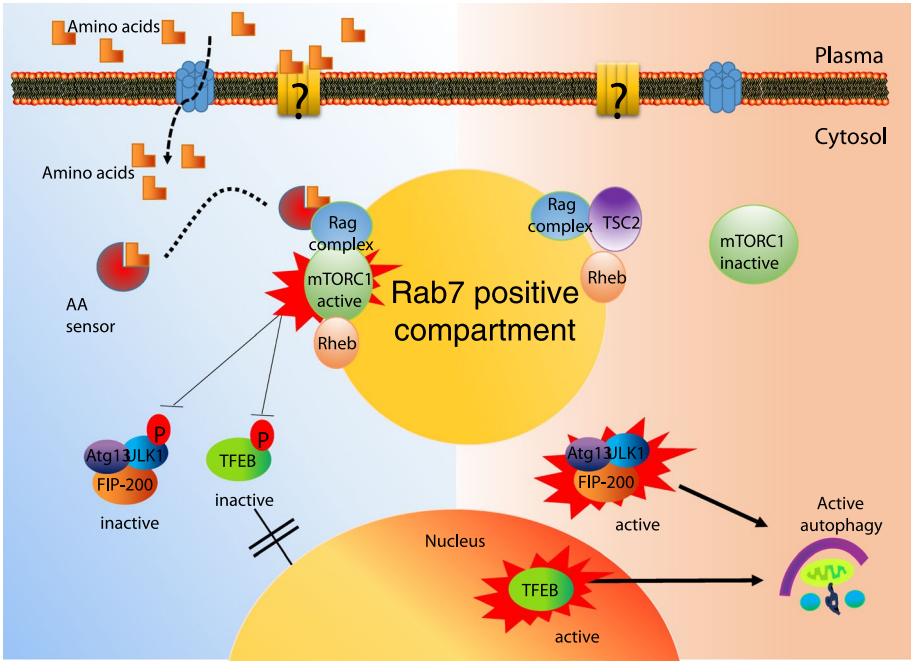

Fig. 1 Role of AAs modulating mTOR activity on autophagy. Under high level of AAs in plasma (*left*), the AAs are either sensed by unknown AAs-sensors in plasma membrane or internalized through transmembrane transporters. Once the AAs are imported into the cytoplasm, the AAs bind to cytosolic AA sensor (i.e., leucyl-tRNA synthetase) targeting the mTORC1 to the surface of Rab7-positive compartment where the activator Rheb switches on the mTORC1 activity. By phosphorylation, the complex ULK1-Atg13-FIP200 and the transcription factor TFEB are inhibited by suppressing macroautophagy activity. By contrary, when amino acids are scarce (*right*), RagGTPases complex is not able to bind to mTORC1 leading to a cytoplasmic distribution of the complex and inhibition of its activity. The loss of phosphorylation of ULK1 triggers the autophagosomal biogenesis and the TFEB enters into the nucleus activating the autophagic transcriptional program

In the last years, other molecular players were identified linking the activation of mTOR complex and the AAs intracellular levels but this modulation does not take place through the direct modification of mTOR complex elements but by modifying the localization of the complex from cytoplasm to the surface of Rab-7 positive subcellular compartments (Fig. 1). A genetic screening uncovered a group of small GTPases as activators of mTORC1 in response to AAs called Rag GTPases. These GTPases promote the localization of mTOR complex to a Rab7-positive compartment where the activator ras homolog enriched in brain (Rheb) resides, stimulating the kinase activity of the mTOR complex and, consequently, inactivating autophagy (Kim et al. 2008; Sancak et al. 2008). The key burning question in the field was to identify the sensor of AAs in the cytoplasm triggering the process. A recent breakthrough identified a leucyl-tRNA-synthetase as the sensor of intracellular Leu concentration, which determines the mTOR functionality through Rag GTPase activation. Upon AAs withdrawal, mTOR complex displays a diffuse distribution; however, when high levels of intracellular leucine are present, the leucyl-tRNA synthetase directly binds to Rag GTPase which promotes the targeting of the mTORC1 to the lysosomal–endosomal compartment for its stimulation through the activator Rheb (Han et al. 2012) (Fig. 1). Besides, upon AAs withdrawal the tuberous sclerosis complex 2 (TSC2) is recruited to the Rab7 positive location to act on the protein Rheb facilitating the release of mTORC1 to the cytosol (Demetriades et al. 2014) triggering the autophagic activity although the AA-sensing mechanism of TSC2 remains unclear.

Notwithstanding it has been molecularly dissected the modulation of autophagy mediated by Leu, it is not the only AA shown to inhibit the process. Although the molecular mechanisms of inhibition have not been defined yet, several reports have suggested that the non-essential AA Gln also suppresses the mTORC1 activity by preventing the targeting to Rab7-positive compartment (Duran et al. 2012; van der Vos et al. 2012).

Another crucial modulator of the autophagic process in response to energy restriction is the AMP-activated protein kinase (AMPK). AMPK acts over the process in two different ways, directly phosphorylating the ULK1 complex (Gwinn et al. 2008; Lee et al. 2010) and also negatively regulating the mTOR complex (Egan et al. 2011b; Kim et al. 2011). Thus, the levels of energy sensed by the ATP/AMP and the GTP/GDP ratios seem to play a crucial role as metabolic checkpoint in the modulation of autophagy

through the AMPK-mTORC1 and TSC-Rheb-mTORC1 axis.

The understanding of the molecular scenario and signaling networks governing the autophagic pathways by AAs remains a crucial aspect in the field of autophagy. Advances in the knowledge of the relationship AAs—autophagy are a challenge to translate the modulation of autophagy to the therapy of diseases, especially in the brain.

Roles of autophagy in brain physiology and pathology

It is known that autophagy takes place in most of the tissues and the modulation of autophagic activity has been suggested to be organ-specific because the level of activity and induction were not uniform in all the tissues (Mizushima et al. 2004). In spite of the high expression of Atg proteins in brain if compared with other organs, during years it was thought that autophagy did not play a key role in proteolysis in brain based on different evidences: low levels of conversion of the autophagosomal marker LC3 and low level of autophagic vacuoles detected in morphological assays. However, a growing number of reports have clearly demonstrated the essentiality of autophagy in brain. Two seminal articles demonstrated that macroautophagy occurs in neurons and it is essential for the maintenance of cellular homeostasis (Hara et al. 2006; Komatsu et al. 2006). The low levels of autophagic vacuoles present at a given time in brain would not be consequence of the absence of autophagic activity but the result of the rapid clearance of autophagosomes through the fusion with lysosomes (Boland et al. 2008).

Furthermore, it was questioned if the inducible upregulation of autophagy in brain tissue takes place because the analysis of the active form of autophagosomal marker LC3 did not reveal dramatic changes upon prolonged starvation treatment (Mizushima et al. 2004). Upon fasting, other organs might provide energy to the brain tissue and the adaptation to starvation would be very different in brain compared to other organs. In addition to LC3, other members of the LC3 family (human homologs of Atg8 in *Saccharomyces cerevisiae*) such as GABARAP and GABARAPL1 could be acting instead of LC3 upon a prolonged nutritional deficiency. These proteins show levels higher than LC3 in brain and have been also involved in neurodegenerative diseases (Mansuy-Schlick et al. 2006; Tanji et al. 2011) and both of them were described as interacting proteins of the GABA receptor in the brain (Chen et al. 2000).

Nowadays, the autophagy activation in brain is unquestionable and it has been broadly shown that different stimuli such as short-term fasting or exercise lead to the induction of brain autophagy (Alirezaei et al. 2010; He et al. 2012). Imaged-based assays showed the autophagosomal induction in cortical neurons and Purkinje cells by nutrient deprivation suggesting that sporadic low caloric diet could be used as neuroprotective therapy (Alirezaei et al. 2010). Additionally, acute stressors such hypoxia, ischemia or oxidative stress have been shown to induce higher levels of autophagy proteins and autophagosomal structures in brain (Uchiyama et al. 2008; Ginet et al. 2014). It has also been reported a remarkable pharmacological upregulation of the autophagy activity in brain using drugs such as rapamycin or trehalose to improve the phenotype of different neurodegenerative disease models (Sarkar et al. 2007; Rodriguez-Navarro et al. 2010; Nixon 2014).

Under physiological conditions, a role of autophagy in food intake has been demonstrated. In hypothalamic AgRP neurons as well as in POMC neurons, the genetic elimination of autophagy causes opposite changes in body weight and food intake, modulating the α-MSH production and causing metabolic dysregulation in the body (Kaushik et al. 2011; Coupe et al. 2012; Kaushik et al. 2013). This role of autophagy in food intake could possibly be related to the classic Leu-mediated control of ingest (Blouet et al. 2009; Schwartz 2013).

Besides, a huge number of evidences support the key role of autophagy in the physiology of the brain and a major role in quality control has been associated with this proteolytic system. For example, the most impacting proofs of the essential role of autophagy in brain have come from the studies in knockout mice for essential Atg proteins. Independent knockouts mice for key factors in the autophagosomal formation (neuron-specific deletion of Atg7 and deletion of Atg5) evoked dramatic neurodegenerative defects (Hara et al. 2006; Komatsu et al. 2006). The loss of autophagy per se leads to a progressive neurodegeneration even when the proteasomal function was not impaired suggesting a pivotal role of macroautophagy in the brain physiology. Measurement and control of the activity of autophagy in the brain are difficult, but different reported methods have been developed for cell cultures and for the brain of mice in vivo (Castillo et al. 2013). Pathological accumulation of autophagosomal structures has been documented in postmortem brain tissues of patients suffering from different neurodegenerative diseases (Yu et al. 2004; Nixon et al. 2005) and defects of autophagy have been associated with an impaired clearance of aggregates in many neurodegenerative diseases. In fact, malfunction of autophagy has been widely characterized in diseases such as Alzheimer's disease, amyotrophic lateral sclerosis or Parkinson's diseases; however, the defect arises in different stages of the autophagic process with differential implications in pathogenesis and therapy (Nixon 2014).

Autophagy as therapeutic target for epilepsy treatment

A growing number of evidences associated altered autophagic activity with different epileptic disorders. The loss of Atg7, key component of the autophagy machinery, in a transgenic mouse model results in spontaneous seizures indicating that inactivation of autophagy is sufficient to cause epilepsy (McMahon et al. 2012). Additional evidences have come from established models of Lafora Disease, a genetic form of epilepsy characterized by the accumulation of polyglucosan inclusion bodies called Lafora bodies. Lafora disease is an autosomal recessive progressive myoclonus epilepsy that can be caused by either deficient activity of laforin phosphatase or malin ubiquitin ligase. Independent transgenic mouse losing one of these enzymes shows similar clinical symptoms and prominent defects in autophagy, suggesting an important role of autophagy in the pathogenesis of Lafora disease (Aguado et al. 2010; Criado et al. 2012).

Above we have pointed out that the mTORC1 as the master regulator of autophagy and different approaches to inhibit its activity might lead to a therapeutic activation of autophagy. For example, rapamycin (canonical mTOR inhibitor and autophagy activator) prevents epilepsy in different mouse models of tuberous sclerosis complex (TSC) deficiency, which represent one of the most common genetic causes of epilepsy (Abs et al. 2013; Meng et al. 2013). Rapamycin has been successfully used for the treatment of subependymal giant cell astrocytomas associated with TSC in humans in a multinational, randomized, placebo-controlled clinical trial (Franz et al. 2013). It has been also reported that rapamycin inhibits recurrent seizures after status epilepticus induced by kainic acid or pilocarpine in rats that do not harbor mutations in the mTOR pathway (Zeng et al. 2008, 2009a, b; Huang et al. 2010; Zeng et al. 2011). Additionally, rapamycin treatment also suppressed behavioral spasms in the model of infantile spasms and decreased susceptibility to kainic acid-induced seizures in a juvenile rat hypoxia model (Zeng et al. 2009b; Raffo et al. 2011). Although rapamycin does not prevent seizures in all the models of epilepsy studied, given that it is a multifactorial disorder (Buckmaster and Lew 2011), the experimental data raise the possibility that mTOR plays a wide role in epilepsy and the modulation of its activity could be useful for clinical interventions (Hartman and Stafstrom 2013).

One of the most striking characteristics of autophagy is the ability to be induced under different conditions, such as nutritional starvation. Based on this feature, the induction of autophagy has been proposed as potential therapy and physiological ways of autophagy stimulation and caloric restriction has been already suggested for ameliorating age-related cognition deficits in normal brain (Mattson 2010; Yang et al. 2014). Although, pharmacological activation of autophagy could be a useful approach, physiological upregulation of the process might take place through diet or exercise. A plethora of metabolic interventions for the treatment of patients with epilepsy are widely used including ketogenic diets, intermittent fasting, calorie restriction or specific diets (Hartman and Stafstrom 2013). Seizure control by fasting is known since the Hippocrates times and the beneficial effect on the brain activity could be mediated by autophagy.

Since the beginning of the twentieth century, variations of ketogenic diets have been used. This diet, a high-fat, adequate-protein, low-carbohydrate diet, is used to treat epilepsy patients who are refractory to other drug treatments due to its clear clinical success (Neal et al. 2008). Ketonic diets decrease mTOR activity in rats leading to an autophagy activation (McDaniel et al. 2011). Interestingly, ketogenic diets differ from rapamycin treatment in their acute antiseizure test profiles but it is unknown whether they share mechanisms in models of chronic epilepsy (Hartman and Stafstrom 2013). It is known that high levels of ketonic bodies increase the synthesis of glutamine through the astrocytic glutamine synthetase, favoring the "buffering" of synaptic glutamate that is taken up by the glia. Then, more glutamine becomes accessible to GABA-ergic neurons for the synthesis of GABA, the major inhibitory neurotransmitter of the brain (Erecinska et al. 1996). Accordingly, it has been proposed that alteration in glutamate, glutamine and GABA metabolism in the brain would be the main mechanism of antiepileptic action of the ketogenic diets (Yudkoff et al. 2005, 2008), but it is also possible that ketogenic diet activates autophagy through mTOR inhibition (McDaniel et al. 2011). Actually, it has been reported that ketone bodies stimulate a special kind of autophagy, chaperone-mediated autophagy (Finn and Dice 2005). This is a selective lysosomal protein degradative process in which proteins are recognized by chaperones and delivered to the lysosomes for degradation that is activated under conditions of prolonged starvation and is modified by different diets (Rodriguez-Navarro and Cuervo 2010; Rodriguez-Navarro et al. 2012). All these evidences suggest a critical role of autophagy in epilepsy disorders. Chemical modulation of autophagy in seizure prevention and treatment deserve further study.

Role of autophagy as source of AAs and in the metabolic coupling between neurons and glial cells

The most broadly known role of autophagy is the ability to provide essential metabolites under nutritional stress. The brain is usually preserved from nutrient deprivation and adapts for consuming ketonic bodies released from

the liver. Upon nutrient deprivation, autophagy accelerates the degradation of intracellular components in different organs releasing essential metabolites (AAs, sugars and triglycerides) which can be used to maintain crucial cellular functions and ATP production in the brain. In this way, autophagy in other organs could be defined as a survival mechanism delivering products in the tricarboxylic acid cycle preserving the cellular viability in this tissue (Singh and Cuervo 2013).

For a long time, changes in the rate of degradation of long-lived proteins were used to monitor autophagy in liver and muscle (Deter et al. 1967) and autophagy was shown to be responsible for up to 70 % of intracellular protein breakdown in liver after starvation, which was later confirmed in mouse models knocked-out for essential autophagy genes (Komatsu et al. 2005). There is a double purpose in the protein breakdown: use of AAs for cellular fueling and replenishment of the pool of AAs to maintain proteins synthesis. In the brain, cytosolic content of AAs is essential because they not only act exclusively as protein building blocks but also are required for neurotransmission.

Bulk protein synthesis has been shown to be substantially reduced in autophagy-deficient cellular and animal models (Onodera and Ohsumi 2005) and different phenotypes due to impaired autophagy could be restored by addition of the cell-permeable tricarboxylic acid cycle substrate methylpyruvate meaning that the function of autophagy is to contribute to energy (Lum et al. 2005). It is also clear that autophagy contributes to the supply of AAs after starvation because neonates with genetically compromised macroautophagy showed marked reduction of different AAs in circulating blood and, more importantly, also in the brain (Kuma et al. 2004). This reduction of supply of AAs in the brain tissue may be indispensable for a proper neurotransmission, because different AAs are neurotransmitters and substrates for its synthesis.

Glutamate (Glu) is the most widely utilized excitatory neurotransmitter in the nervous system, but it is also used as a key metabolic fuel. Therefore, it is critical to understand how neurons replenish the Glu that they release during neurotransmission. The Glu level must be kept controlled to maintain the signal-to-noise ratio upon the Glu release from neurons and to minimize the risk of excitotoxicity due to excessive glutamatergic stimulation. Under normal conditions, extracellular concentrations of Glu are between 25 nM and 5 μM (Benveniste et al. 1984; Lerma et al. 1986), and intracellular Glu concentrations are approximately 10 mM and in synaptic vesicles around 100 mM (Burger et al. 1989). The constant metabolic supply of Glu, which both neurons and glia utilize also as source of energy, and the Glu regulation and compartmentalization are crucial for the brain function. Gln and the branched-chain AAs, particularly Leu, play an important role in this regard. Importantly, Gln and Leu are the AAs more implicated in the control of autophagic activity as described previously.

The brain is a complex tissue where an orchestrated metabolism between different cell types is required and, interestingly, autophagy has been directly linked in this process called metabolic coupling (Martinez-Outschoorn et al. 2011; Boya et al. 2013). The brain maintains a low level of external Glu and a high intra-neuronal concentration by assigning to the glia (primarily astrocytes) the task of removing synaptic Glu. A proper exchange of essential metabolites between glia and neurons is required for the functional homeostasis of the tissue. For example, glial cells release lactate that the neurons utilize to generate energy for the neural transmission. Autophagy may play a role in this crosstalk and, for example, dysfunctional lysosomal and autophagic degradation in astrocytes lead to degeneration of cortical neurons in vivo (Di Malta et al. 2012).

Astrocytes are responsible for converting Glu released by neurons to Gln via glutamine synthetase (GS), an exclusively glial enzyme, and then export glutamine to neurons that hydrolyze Gln to Glu via the action of phosphate-dependent glutaminase (GA), a mitochondrial enzyme that yields ammonia as well as Glu (Fig. 2) Such shuttling of Glu and Gln between astrocytes and neurons is termed the "*Glutamate–Glutamine Cycle*", and the existence has been recently electrophysiologically corroborated (Albrecht et al. 2007; Tani et al. 2014). The glutamate–glutamine cycle leads to a net transfer of nitrogen from the astrocytes to the neuronal compartment. To operate stoichiometrically the cycle, the glutamate dehydrogenase reaction has to operate in opposite directions in neurons and astrocytes. It is possible, if the ammonium levels in the neurons are high enough, but deamination seems to be favored in normal conditions in both cell types (Zaganas et al. 2001). Then, to maintain nitrogen homoeostasis, this nitrogen must be transferred back to the astrocyte. This may be accomplished by diffusion of NH_3 or by transferring an AA (like Ala, Asp or Leu). These AAs are thought to be transaminated generating Glu from which the amino group may be liberated by the action of glutamate dehydrogenase (GDH). The amino group may subsequently take part in the Glu synthesis reaction for the synthesis of Gln in glial cells (Fig. 2) (Schousboe et al. 1993; Waagepetersen et al. 2003; Bak et al. 2006; Patel et al. 2014).

This local exchange of AAs between neurons and glial cells happens mainly through specific transporters for the different AAs and neurotransmitters but it also relevant the role of different channels and hemichannels for the exchange of ions and small metabolites. Previously, we have mentioned the importance of the AA transporters in the regulation of autophagy and brain physiology but the

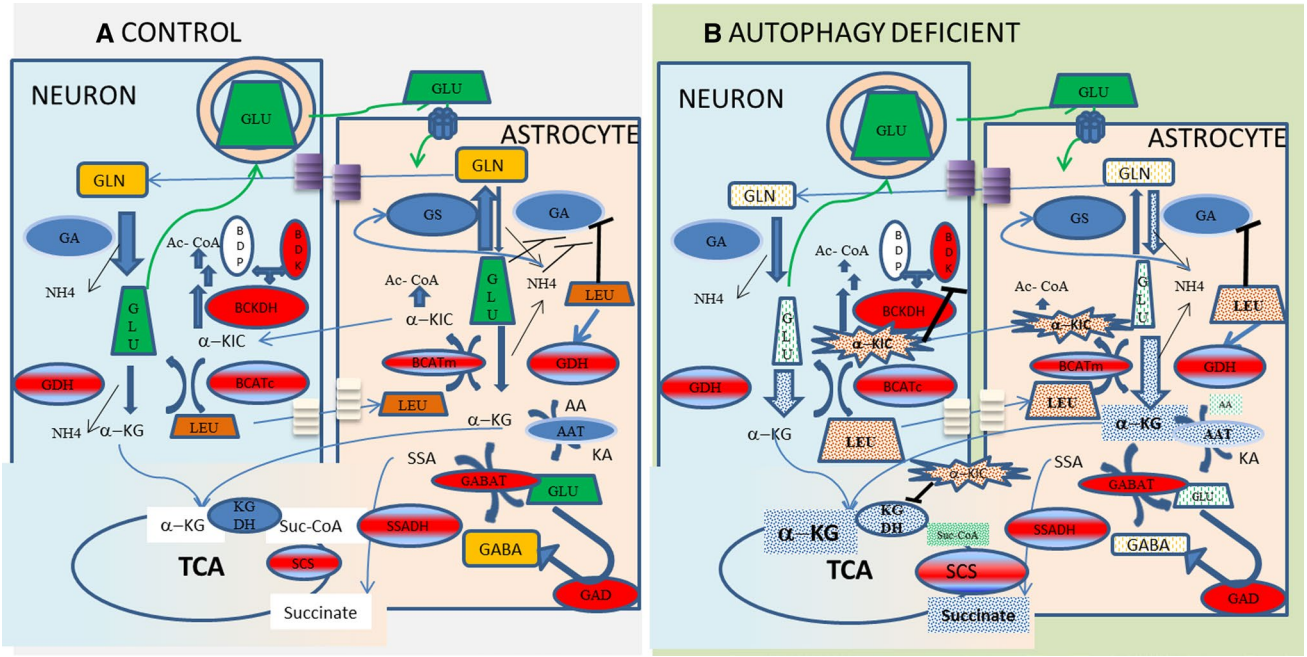

Fig. 2 Schematic diagrams indicating the pathways of Gln, Glu, GABA and Leu metabolism coupled in neurons and astrocytes in normal conditions (**a**, *left*) and in a hypothetical model of macroautophagy deficiency (**b**, *right*). The enzymes in *blue* have been related to macroautophagy, the ones in *red* are involved in epilepsy, and the enzymes related to autophagy and epilepsy are in *blue* and *red*. The Gln–Glu cycle is depicted coupled with Leu transamination, but Ala or other amino acids might work in the same way. The different size and the punctate pattern of the metabolites in the *panel B* in the right reflect and highlight the changes observed in these metabolites in macroautophagy-deficient cells taking into account the metabolic compartmentalization between neurons and glial cells. The TCA cycle is represented and shared by both cell types. In absence of macroautophagy, with a constant supply of nutrients, the levels of Leu and KIC metabolites are elevated, meanwhile Gln, Glu, Asp and Ala are decreased. α-KIC levels would inhibit the α-KGDH promoting the energy utilization through the GABA shunt. *Glu* glutamate, *α-KG* α-ketoglutarate, *AA* amino acid, *GDH* glutamate dehydrogenase, *GS* glutamine synthetase, *GA* glutaminase, *GLN* glutamine, *AAT* aspartate aminotransferase, *BCAT* branched-chain aminotransferase m mitochondrial and c cytosolic, *BCKDH* branched-chain ketoacid dehydrogenase complex, *BCK* branched-chain ketoacid dehydrogenase complex kinase, *BCP* branched-chain ketoacid dehydrogenase complex phosphatase, *α-KGDH* α-ketoglutarate dehydrogenase, *LEU* leucine, *α-KIC* keto isocaproate

role of these hemichannels and channels could be relevant in the AAs homeostasis. Multicellular glial networks are formed by intercellular gap junctions to transport neurotransmitter metabolites long distances for recycling the neurotransmitters (Chaturvedi et al. 2014). Interestingly, autophagy contributes to the turnover of these gap junction channels in basal conditions but it plays a critical role under nutrient deficient conditions accelerating their degradation (Bejarano et al. 2012; Lichtenstein et al. 2012). In addition, a bidirectional crosstalk between autophagy and gap junction intercellular communication happens given that the connexins, blocks of gap junctions, negatively modulate the autophagic function (Bejarano et al. 2014).

Importance of leucine in the cerebral metabolism of amino acids

Leu, the best-characterized AA inhibiting the autophagy process, has been involved in the control of Glu levels coupled with the Glu-Gln cycle between glia and neurons through transamination (Fig. 2) (Hutson et al. 2001). Leucine has also been proposed to provide the amino group in the transamination reaction for de novo synthesis of Glu and, indeed, around 30 % of all α-amino groups in brain Glu and Gln are derived from Leu alone (Yudkoff et al. 2005). In addition to modulate autophagy, it serves as a metabolic precursor of fuel molecules in the nervous system acting as master regulator of the activity of some enzymes important for brain energy metabolism. Therefore, the levels of Leu are a key aspect in the physiology of the brain, and these levels are regulated both by its uptake through the blood brain barrier and its degradation.

Leu enters the brain from the blood more rapidly, with higher affinity than any other AA through the LAT1 transporter, which is highly expressed in the blood brain barrier (Boado et al. 1999; Kanai et al. 2000). Astrocytes, which are close to brain capillaries, probably are the initial site of catabolism of Leu after uptake. A mitochondrial branched-chain aminotransferase (BCATm) is very

active in astrocytes. They release the product of the reaction, α-ketoisocaproiate (α-KIC) to neurons, which have a cytosolic branched-chain aminotransferase (BCATc) that reaminates the α-KIC to Leu. The last reaction consumes Glu by providing a mechanism for Glu "buffering" if concentrations become excessive. In the reverse way, when needed, Glu could be synthesized using Leu as donor of the amino group. Inhibition of the shuttle in the direction of Glu synthesis can be achieved by inhibiting BCATc using the antiepileptic drug gabapentin (Hutson et al. 2001). Leu transamination by these enzymes is faster than the decarboxylation of α-KIC in normal conditions, but low levels of energy in the neurons can drive the oxidation of both Leu and α-KIC to CO_2 generating energy. This irreversible oxidative decarboxylation is catalyzed by an enzyme complex, the branched-chain ketoacid dehydrogenase (BCKDH) complex (Fig. 2). In maple syrup urine disease, a congenital deficiency of BCKDH, the brain concentration of α-KIC and other branched-chain ketoacids increase till 20-fold. This leads to a depletion of Glu and a consequent reduction in the concentration of brain Gln, Asp, Ala and other AAs (Hutson et al. 2001; Yudkoff et al. 2008; Cole et al. 2012). If left untreated, most patients experience epileptic seizures, changes in muscle tone, and coma. Pathological concentrations of branched-chain keto-acids, such as those that occur in maple syrup urine disease, inhibit oxygen consumption in brain slices and the enzymatic activity of alpha-ketoglutarate- and pyruvate dehydrogenase complexes altering the whole metabolism (Shestopalov and Kristal 2007). In cells lacking macroautophagy, the levels of α-KIC and other branched-chain keto-acids reach those pathological levels and this causes defects in the regulation of the response to nutrient status (Fig. 2) (Lin et al. 2012).

As BCKDH is the rate-limiting step of the pathway that commits branched-chain AAs to degradation, it is tightly regulated by a phosphorylation/dephosphorylation cycle. BCKDH is phosphorylated and inactivated by BDK (BCKDH kinase) and dephosphorylated and activated by BDP (BCKDH phosphatase). BDK is inhibited by α-KIC produced by transamination of Leu. When BCAAs are present in excess, the amount of BDK bound to the complex is reduced and α-KIC inhibits BDK activity, resulting in a highly dephosphorylated and active BCKDH that disposes the excess BCAAs. When there is a danger of AAs becoming limited for protein synthesis, as in the case of dietary protein starvation, the concentration of α-KIC falls below the concentration and inhibits the BCKDH by BDK phosphorylation (Obayashi et al. 2001). Recently, mutations in BDK gene in humans that lead to a potentially treatable form of autism with epilepsy have been described (Garcia-Cazorla et al. 2014) and the mice deficient in BDK show hind limb clasping throughout life and epileptic seizures (Joshi et al. 2006) similar to that observed in the macroautophagy-deficient mice (Hara et al. 2006; McMahon et al. 2012). Therefore, alterations in the different enzymes involved in the catabolism of Leu lead to epileptic disorders. In conditions of macroautophagy inhibition, the levels of Leu and its metabolites are elevated in a basal metabolic state (Fig. 2), but when the nutrients are scarce, they are used as metabolic substrates and the regulation of the Glu levels by Leu is probably lost.

Molecular players related to autophagy in the control of glutamate levels

Leu, in addition to serve as a source of Glu by transamination, tightly controls its levels because it inhibits the main enzyme that synthesizes it from Gln and activates the main enzyme that degrades it to alpha-ketoglutarate (α-KG). GA is the most important Gln-metabolizing enzyme for the synthesis of Glu in neurons (Kvamme et al. 2000) and this enzyme is regulated by neuronal activity, and is strongly inhibited by its reaction products Glu and ammonia.

Cytosolic variations of Leu can inhibit the glutaminase activity even tenfold (Lellos et al. 1991) and glutaminolysis controls the autophagic activity in apparently contradictory ways. Enhanced glutaminolysis by GA and production of α-KG by GDH stimulate mTORC1 activity through Rag GTPases inhibiting autophagy (Duran et al. 2012), meanwhile the ammonia generated by this reaction induced autophagy in proliferating cells (Eng et al. 2010). This opposing effects could be due to the existence of different GA isoforms, but can be reconciled if the inhibition of GA by ammonium is taken into account. An increase in GS (expressed in glial cells and producing Gln from Glu) inhibits mTORC1 and activates autophagy (van der Vos et al. 2012). Thus, mTORC1 appears to sense the flux between Gln–Glu and αKG in both directions.

The α-KG/Glu ratio can be controlled by aspartate or alanine aminotransferases leading to enhanced consumption of Asp and Ala, or by the activity of the GDH. The aspartate aminotransferase would lead to enhanced consumption of Asp, and without it, the malate-aspartate shuttle no longer can effectively transfer reducing equivalents from cytosol to mitochondrion. In autophagy-deficient cells, Asp and Ala levels are decreased (Lin et al. 2012) and, in addition, the aspartate amino transferase and the mitochondrial aspartate shuttles have been described to interact with key components of the macroautophagy machinery (Behrends et al. 2010). If α-KIC accumulates, as it does in BCKDH deficiency or in macroautophagy-deficient cells, there is increased consumption of Glu and increased production of both Leu and α-KG via BCAT (Lin et al. 2012). Intracellular Glu is diminished and α-KG is increased under these conditions (Fig. 2). Usually α-KG

would enter in the Krebs cycle to produce energy, but the elevated levels of α-KIC inhibit the KGDH further increasing the α-KG/Glu ratio (Shestopalov and Kristal 2007). In the hypothetical scenario presented here, with elevated KIC levels, lactate acidosis and energy failure would be the consequences. In cells lacking macroautophagy, this process is avoided by the transcriptional downregulation of lactate dehydrogenase, and a more than fourfold increase in KGDH, the alanine aminotransferase, and other enzymes of the citric acid cycle (Lin et al. 2012). If this adaptation happens in the glial cells, the supply of lactate from the astrocytes to neurons would be decreased and the energetic balance in neurons compromised.

An alternative to the transamination reactions to control the fate of glutamate is the cerebral GDH reaction, which acts normally in the direction of Glu oxidation. GDH activity is reversible and subject to complex regulation by negative (GTP, palmitoyl-coenzyme A) and positive (ADP, leucine) allosteric effectors (Fig. 2). This complex regulation allows GDH activity to be modulated by changes in energy state and AA availability (McKenna 2011). The knock down of GDH impairs the regulation of autophagy by nutrients and mTOR and it has been proposed to contribute to Leu sensing in the regulation of autophagy (Lorin et al. 2013).

In addition to the widely expressed GDH in all the tissues (GDH1), primates have acquired a GDH2 isoenzyme losing the GTP regulation, which is exclusively expressed in glial and Sertoli cells (Spanaki and Plaitakis 2012). Astrocytes and Sertoli cells are known to support neurons and germ cells, respectively, providing them metabolic substrates derived from the tricarboxylic acid cycle. As GDH2 is not subject to GTP control, the enzyme is able to metabolize glutamate even when the tricarboxylic acid cycle generates GTP amount sufficient to inactivate the housekeeping GDH1 protein. The importance of GDH regulation in humans has been highlighted by the discovery of a disorder in children, the hyperinsulinism-hyperammonemia syndrome, caused by dominantly expressed, activating mutations of GDH1 that impair its inhibition by GTP. Affected children show hypoglycemic epileptic seizures after brief periods of fasting or the ingestion of a high-protein meal.

Therefore, GTP levels are key in normal conditions regulating GDH activity and the activity of autophagy through the GTPases-mTOR pathway. GTP is generated in the mitochondria only by the succinyl-CoA synthetase. Succinyl-CoA synthetase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA. The reverse reaction occurs in the Krebs cycle, while the forward reaction produces succinyl-CoA necessary for the activation of ketone bodies to be used in the generation of energy. Succinyl-CoA synthetase is a heterodimer composed of an invariant alpha subunit (SUCLA) and a beta subunit that determines the enzyme's nucleotide specificity, SUCLG1 for ATP and SUCLG2 for GTP. Interestingly, these three enzymes interact with autophagic components in a seminal work describing the network organization of the autophagic system (Behrends et al. 2010). The direction of the activity of Succinyl-CoA synthetase is dependent on the levels of substrates and products. In conditions of ketosis, prolonged starvation, succinyl CoA is needed to utilize the ketonic bodies in the brain. Some succinyl CoA can be provided by the ketoacids originated from Ile and Val transamination, meanwhile succinate could be generated in astrocytes and GABAergic neurons metabolizing GABA. If macroautophagy is deficient, Ile and Val supply would be compromised and the GABA would be used as energy source, probably altering the inhibitory neurotransmission.

GABA and monoamine metabolism, autophagy and epilepsy

GABA, the major central nervous system inhibitory neurotransmitter, is metabolized into succinic semi-aldehyde in the mitochondria by the GABA-aminotransferase. Succinic semi-aldehyde dehydrogenase catalyzes the subsequent oxidation of succinic semi-aldehyde to succinate, which may be used in the Krebs cycle to generate energy or for Glu and Gln synthesis. Metabolism of GABA appears to be relevant for the functional capacity of GABAergic neurotransmission, since inhibitors of astrocytic GABA transporters as well as GABA-aminotransferase act as anticonvulsants (Sarup et al. 2003). Succinic semi-aldehyde dehydrogenase deficiency is a rare heritable non-progressive neurological disorder in which seizures are prominently displayed (Gupta et al. 2004). Mice losing succinic semi-aldehyde dehydrogenase showed increased levels of GABA and decreased levels of Glu and Gln. The phenotype can be rescued using a ketogenic diet that restores the ATP levels in the brain of this model (Nylen et al. 2008). Although the levels of GABA in conditions of autophagy deficiency are not known, the high KIC levels observed could direct the metabolism of GABA into the Krebs cycle for the production of energy through the GABA shunt (Fig. 2) (Hernandez-Fisac et al. 2006; Pizarro-Delgado et al. 2009). The consequence of this process would be a decreased intracellular GABA level and an imbalance between GABA and Glu-neurotransmission that could be responsible for the epileptic phenotype.

In addition to Glu and GABA, monoamine neurotransmitters use aromatic AAs as precursors. Trp is the substrate for the serotonin, Tyr for catechol-amines (dopamine, norepinephrine, and epinephrine) and His for histamine. The rates of synthesis of these neurotransmitters in the brain

are sensitive to local substrate concentrations, particularly in the physiological ranges in vivo. Consequently, factors that influence on the brain levels of these AAs, such as diet, affect their rates of conversion to neurotransmitter products leading to important functional and pathological consequences (Fernstrom 2013). Trp, Tyr and His are transported into brain by LAT, the competitive carrier system shared with other large neutral AAs such as Phe, Leu, Ile, and Val. Physiologic variations in the plasma neutral AA levels directly alter this competitive process, and thereby modify the uptake of Trp, Tyr and His into the brain, consequently modifying the levels of serotonin, histamine and catecholamines in the different regions of the brain (Fernstrom 2013). The detailed review of the implications of monoamines in epilepsy is out of the scope of this review but all of them have been involved in the epilepsy development and its treatment (Kurian et al. 2011). For example, thioperamide, which is an antagonist of the H3 histamine receptor able to delay seizure response in mice models, has been recently described as an autophagy stimulator in the brain in vivo (Yan et al. 2014).

Whether the activity of autophagy can alter the local levels of these precursors in specialized regions of the brain is unclear but, as happen with Leu, they are increased in cells lacking key autophagy genes, and the levels drop dramatically after Glu starvation (Lin et al. 2012). Therefore, autophagy malfunction could impact on the homeostasis of these neurotransmitters that, although are less important quantitatively from the brain metabolic point of view, are essential in the brain physiology and pathology.

Conclusions

Autophagy is a catabolic pathway that releases free AAs from the degraded proteins and its activity is inhibited when the levels of certain AAs such as Leu and Gln are elevated. In the brain, the homeostasis of the levels and the activity of the AAs metabolism are crucial, because they act as neurotransmitters or precursors of neurotransmitters. Genetic inhibition of autophagy alters brain levels of essential AAs and its inhibition specifically in neurons, leads to epileptic seizures and neurodegeneration in mice models. Mutations in components of the pathways that control autophagic activity are responsible for epileptic-related disorders also in humans. Actually, treatments that stimulate autophagy are used for the treatment of certain epileptic disorders. However, in spite of the importance that the AAs balance has for the neurotransmitter homeostasis and activity in the brain, and the growing interest in the role of autophagy in neurodegenerative diseases, little attention has been paid to the relationship between autophagy and AA metabolism in the brain.

Future perspectives

Although the relationship between autophagy and AAs has been deeply studied for the last 50 years, there are many aspects of the biology of this crosstalk that are not understood, especially in the brain. In the last decade, several reports have shown the crucial role of autophagy in the brain physiology and pathology and modulation of autophagy activity have been already proposed for clinical interventions. Further studies will be required to understand the role of autophagy in the AAs metabolism specifically in the brain, due to the highly specialized metabolism and the cellular complexity of the nervous system. Whether autophagy could serve as a local source of nutrients and neurotransmitter precursors in different physiological and pathological situations remains to be experimentally determined. Deciphering the role of autophagy in neurotransmission and a better molecular characterization of the process in the nervous system will help to design new therapeutic tools, not only for epilepsy, but also for brain tumors, neurodegenerative diseases and brain-related metabolic syndromes.

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