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NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - REVIEW ARTICLE

Autophagy in trimethyltin-induced neurodegeneration

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Abstract
Autophagy is a degradative process playing an important role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria and endoplasmic reticulum, as well as eliminating intracellular pathogens. The autophagic process is important for balancing sources of energy at critical developmental stages and in response to nutrient stress. Recently, autophagy has been involved in the pathophysiology of neurodegenerative diseases although its beneficial (pro-survival) or detrimental (pro-death) role remains controversial. In the present review, we discuss the role of autophagy following intoxication with trimethyltin (TMT), an organotin compound that induces severe hippocampal neurodegeneration associated with astrocyte and microglia activation. TMT is considered a useful tool to study the molecular mechanisms occurring in human neurodegenerative diseases such as Alzheimer's disease and temporal lobe epilepsy. This is also relevant in the field of environmental safety, since organotin compounds are used as heat stabilizers in polyvinyl chloride polymers, industrial and agricultural biocides, and as industrial chemical catalysts.

Keywords Autophagy · Trimethyltin · TMT · Neurodegeneration · Lithium

Trimethyltin and its relevance to environmental health

Trimethyltin chloride (TMT; $(CH_3)_3SnCl$) belongs to the family of organotin compounds. These molecules bear several commercial applications although some of them are known to produce neurotoxicity. Organotins are employed in the plastic industry to inhibit the dissociation of hydrochloric acid from polyvinylchloride (PVC) which is widely utilized in packaging, piping, coating, and window frames (Gomez et al. 2007). Dimethyltin (DMT) is the main methyltin compound used to synthesize PVC, but during DMT synthesis the more toxic TMT is produced as a by-product (Besser et al. 1987; Fent 1996; Al-Malack and Sheikheldin 2001; Hoch 2001; Gomez et al. 2007). In the past, TMT was used as agricultural pesticide, wood preservative, and antifouling paint on ships (Bennett 1996).

Fortemps et al. (1978) first reported two TMT poisoning cases in Belgium that occurred in 1974–1975. Since that date and up to 2008, 1849 cases of poisoning and 23 deaths have been reported in 67 incidents that occurred throughout the world (Tang et al. 2008, 2010). Concerns about the toxicity of the organotins led to progressive global restrictions in many countries in Europe and North America in the use of the most toxic compounds. Most recent cases of occupational poisoning occurred in China (Xiao and Zou 2018; Zhu et al. 2019), where organotins have wide industrial applications and are found in many environmental contexts (Cao et al. 2009).

Other than acute accidental exposure, specific workers and the entire population may be chronically exposed to methyltins. In fact, considering its widespread use, TMT has been shown to contaminate soil and water systems via different routes (Hoch 2001). In this connection leakage of organotins from PVC material has been proposed to be one of the main sources of environmental contamination (Richardson and Edwards 2009). It has been reported that the concentration of TMT in the environment is 0.98–20 ng/L in seawater, and 415 ng/L in microlayers of estuarine water.

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In bulk water, concentrations up to 508 ng/L have been detected, and in sediments the amount was found to be 750 ng/g (Hamasaki et al. 1995).

In humans, exposure to TMT mainly occurs by ingestion, as a consequence of intake of TMT contaminated food and/or drinks. TMT exposure can also occur via endogenous methylation of DMT. In fact, there are reports of workers who were exposed to DMT and suffered severe hypokalemia and neurological disturbances such as ataxia, and memory loss similarly to what occurs following TMT exposure (Piver 1973; Mushak et al. 1982; Yoo et al. 2007; Tang et al. 2010). Moreover, urine and blood samples from these workers contained both TMT and DMT (Jiang and Zhou 2000; Yoo et al. 2007; Ichihara et al. 2019). This has also been confirmed by experiments in rodents, where i.p. injection of DMT chloride produces a detectable amount of TMT in blood and urine (Furuhashi et al. 2008).

In human bodies, when urine samples of six individuals working with organotin compounds were analyzed, TMT was detected at concentrations of 445–1580 parts per billion (2.23–7.93 μM) (Feldman et al. 1993). Another case report detected 3.75–13.31 μM TMT in workers exposed to TMT stabilizers (Wang et al. 2008).

More recently, Tang et al. (2013) showed that TMT has a 15-day half-life in rats, suggesting that methyltins could accumulate in the human body after chronic exposure.

The impact of methyltin compounds on human health is primarily focused on its neurotoxic effects (Caito and Aschner 2015; Ferraz da Silva et al. 2018). TMT exposure produces the so-called "limbic-cerebellar" syndrome. Symptoms include: hearing loss, disorientation, confabulation, amnesia, aggressiveness, hyperphagia, disturbed sexual behavior, complex partial and tonic–clonic seizures, nystagmus, ataxia, and mild sensory neuropathy. Syndrome severity parallels the amount of urinary organotin levels (Besser et al. 1987). Magnetic resonance imaging (MRI) shows symmetrical hyperintensities in the brain stem, cerebellum, corpus callosum, internal capsule, and subcortical spaces (Wang et al. 2017). Pathologic studies following TMT poisoning in humans provided evidence for neuronal necrosis in the hippocampus, cerebral cortex, basal ganglia, Purkinje cell layer of the cerebellum, and spinal cord. Electron microscopy evidenced abundant lysosomal dense bodies and disorganization of the endoplasmic reticulum within neurons (Kreyberg et al. 1992).

TMT-treated rodents as experimental model of neurodegeneration

To understand the pathophysiology of TMT-induced neuronal damage in humans, TMT has been used to provide an in vivo rodent model of neurodegeneration. In fact, acute TMT neuropathology in rodents is similar with that observed in humans with comparable behavioral disorders. Besides, TMT is also considered a useful tool to study the molecular mechanisms occurring in human neurodegenerative diseases such as Alzheimer's disease and temporal lobe epilepsy (Lee et al. 2016).

The multi-faced behavioral syndrome recorded following rodent exposure was named originally as "the trimethyltin syndrome" (Dyer et al. 1982). Animals intoxicated with TMT show behavioral abnormalities, including ataxia, aggressiveness, tail mutilation, vocalization, and seizures (Perretta et al. 1993). They also exhibit hyperactivity in an open field test, depression-like behavior in a forced swimming test, increased anxiety in a complex maze, and impairments in learning and memory in Morris water maze tests (Park et al. 2011a; Tamburella et al. 2012; Kim et al. 2013; Yoneyama et al. 2014).

In the light of multiple brain areas being involved it has been useful to identify regions which appear mostly vulnerable to TMT. In fact, differing from other chemicals, which produce global brain injuries, TMT primarily damages the limbic system, particularly the hippocampus, with an intact blood–brain barrier. Thus, TMT allows hippocampus-specific neurodegeneration without effects mediated by blood-borne factors (Little et al. 2002). TMT also affects a variety of extra-limbic regions. In TMT-intoxicated rats, Bouldin et al. (1981) demonstrated a damage in the allocortical piriform area which also extends to the isocortex, basal ganglia, brainstem, spinal cord, dorsal root ganglia. In keeping with a primary involvement of the limbic system, Balaban et al. (1988) using silver staining techniques provided evidence for the implication of specific limbic regions. These include a number of limbic nuclei such as the intermediate and ventral divisions of the lateral septal nucleus, the septohippocampal nucleus, the septohypothalamic nucleus, the anterior olfactory nucleus, the bed nucleus of the stria terminalis, the endopiriform nucleus, the parafascicular nucleus. The limbic recruitment extends to allocortical hippocampal regions including toxicity to the granule cells of the dentate gyrus and pyramidal cells within various sub-fields of the Cornu Ammonis (CA). The hippocampal allocortical damage extends towards continuous allocortical regions of the mesial temporal lobe such as the subiculum and piriform cortex and the entorhinal mesocortex. In contrast, the main limbic amygdala complex is damaged later on, at days 5–7 following TMT exposure. At the first stage TMT toxicity also involves specific layers of contiguous isocortical regions (mainly layer Vb and VI). Proceeding caudally, the damage extends to the diencephalon at the level of ventral posterolateral and ventral posteromedial thalamic nuclei and the interstitial nucleus of the posterior commissure. The effects of TMT extend to the brainstem starting from the superior and inferior colliculi and the periaqueductal gray, the pontine

nuclei, the raphe nuclei, the spinal trigeminal nucleus (pars caudalis), the caudal aspect of nucleus tractus solitarius, the dorsal vagal motor nucleus. These brain regions are involved variably depending on the animal species. In fact, both mice and rats are sensitive to TMT-induced toxicity, although discrepancies exist between these species, the rat being the most studied. It is noticeable that mice are intoxicated by doses of TMT lower than rats (Ishikura et al. 2001) and mice develop clinical signs earlier than rats (Lee et al. 2014); similarly, hippocampal neuropathology is more severe in mice than rats (Chang et al. 1983). Apart from damage severity, there is a discrepancy in zonal vulnerability within the hippocampus of mice and rats. In detail, while in the mouse, the main target of TMT is the granule cell in the dentate gyrus, the preferred target of TMT in the rat corresponds to the pyramidal cell of CA subfields (Lattanzi et al. 2013). Indeed, some studies report that TMT toxicity in rats also damages the dentate gyrus (Bouldin et al. 1981; Johnson et al. 2014). Trabucco et al. (2009) hypothesized that the distinct neuropathological effects observed in different animal species may be not related to species differences in TMT sensitivity but rather to a different susceptibility to secondary effects produced by TMT. In fact, apart from a primary neurotoxic damage induced by TMT at neuronal level, this compound promotes the onset of limbic and generalized seizures, which in turn add a secondary damage to that induced immediately by TMT. Thus, the different neuropathology observed in different animal species could be produced by a different sensitivity to epilepsy-induced brain damage.

Autophagy machinery

Autophagy is a highly conserved process in all eukaryotes that plays important roles in cell survival and maintenance. The dysfunction of this process contributes to the onset of many human diseases. Autophagy consists in the degradation of cytoplasmic organelles, proteins, and macromolecules, and the recycling of the breakdown products by the cell in biosynthetic processes or to generate energy. The autophagic pathway is activated during starvation to restore intracellular nutrient supply but also following different stress conditions allowing the removal of protein aggregates or damaged organelles (for a recent review see Noda and Inagaki 2015).

Autophagic machinery consists of a variety of membrane structures, which organize to form vesicles, where abnormal material is segregated and delivered to lysosomal degradation.

In mammalian cells, there are three primary types of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) (Fig. 1).

In the microautophagy, cytoplasmic contents (including intact organelles) enter the lysosome through invaginations or protrusions of the lysosomal membrane (Mijaljica et al. 2011). Recently, it has been demonstrated that invagination of the endosomal membrane also contributes to incorporation of cytoplasmic proteins into the lysosome suggesting the existence of an alternative pathway of microautophagy. This latter process is dependent on endosomal sorting complexes required for transport (ESCRT) and chaperonine Hsc70 (Sahu et al. 2011; Oku and Sakai 2018).

Macroautophagy, more commonly called autophagy, relies on de novo formation of cytosolic double-membrane vesicles, termed autophagosomes, to sequester and transport cargoes to the lysosomes. Several studies suggest that the formation of autophagosomes begins at endoplasmic reticulum (ER)-associated structures called omegasomes (Hayashi-Nishino et al. 2009; Yla-Anttila et al. 2009). Following initiation, the membrane begins to expand and at this stage it is called phagophore. Phagophore represents the primary double-membrane sequestering compartment, where the materials start to be hosted. Furthermore, the expanding membrane closes around its cargo to seal a spherical autophagosome, which delivers its cargo to the lysosome. As it reaches its destination, the outer membrane of the autophagosome fuses with the lysosomal membrane to form an autophagolysosome. The content of the autophagolysosome is then degraded by resident hydrolases of the lysosome and, subsequently, the component parts are exported back into the cytoplasm for being used by the cell (Yorimitsu and Klionsky 2005; Yang and Klionsky 2009). In some instances, the autophagosome may also associate with an endosome, forming an amphisome, before fusing with the lysosome to become autolysosome (Tooze et al. 1990; de Duve et al. 1995; Berg et al. 1998; Klionsky 2008; Ciechanover and Kwon 2015; Fujiwara et al. 2017). In some cases, the fusion between the autophagosome with proteasome components produces a merging organelle named autophagoproteasome (Lenzi et al. 2016; Klionsky et al. 2016).

During the autophagy process each step is highly regulated by various kinases, phosphatases, and guanosine triphosphatases (GTPases) (Klionsky and Emr 2000). In mammalian cells, the autophagosome formation is initially regulated by the induction complex that consists of Unc-51 like kinase (ULK) 1/2, autophagy-related protein (ATG) 13, retinoblastoma coiled coil protein 1 (RB1CC1), and chromosome 12 open reading frame 44 (C12orf44). Under nutrient-rich conditions, the mechanistic target of rapamycin complex 1 (mTORC1) associates with the induction complex and inactivates ULK1/2 and ATG13 through phosphorylation. However, when cells are treated with rapamycin or during starvation, mTORC1 dissociates from the complex and ATG13 and ULK1/2 become partially dephosphorylated, resulting in induction of macroautophagy (Hosokawa et al.

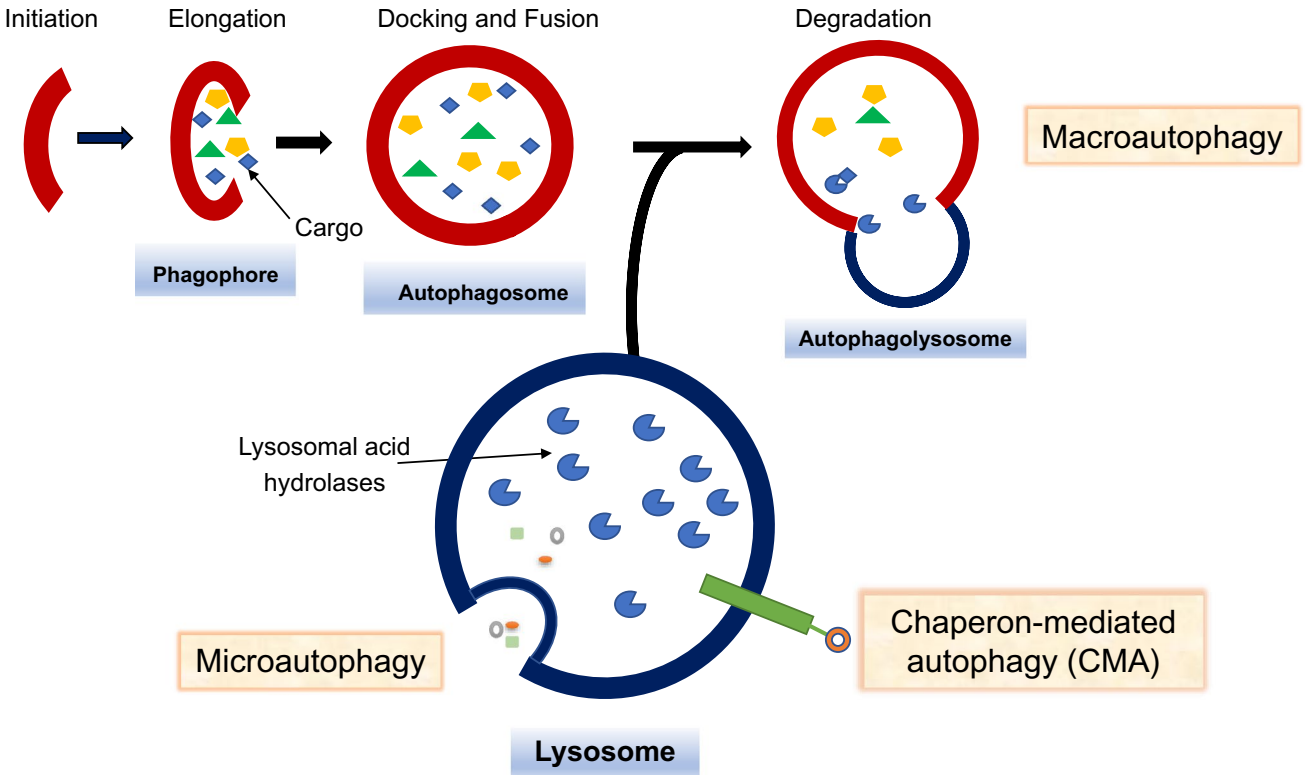

Fig. 1 Primary types of autophagy. There are three main types of autophagy including macroautophagy, microautophagy and chaperon-mediated autophagy (CMA). During macroautophagy, an isolation membrane (known as the phagophore) encloses a portion of cytoplasm, forming a characteristic double-membraned organelle named autophagosome. Autophagosome then fuses with lysosome to form autophagolysosome and the cytoplasmic components are subsequently degraded by lysosomal enzymes. During microautophagy, cargo delivery occurs upon the direct invagination of the lysosomal membrane. CMA involves the recognition of autophagic cargoes bearing a KFERQ motif by heat shock proteins (HSPs), which is followed by the lysosomal-associated membrane protein 2 (LAMP2)-dependent translocation of chaperoned autophagic cargoes across the lysosomal membrane

2009; Jung et al. 2009; Parzych and Klionsky 2014). The phosphatases responsible of this process are still unknown.

In mammalian cells the nucleation and assembly of the initial phagophore membrane requires Beclin 1 inserted in its membrane. Beclin 1 binds to phosphatidylinositol-3-kinase class III (PI3K-III)/VPS34 (vacuolar protein sorting 34), forming the active complex PI3K-III, which produces a focal increase of phosphatidylinositol-3-phosphate (PI3P). The function of Beclin 1 in autophagy is regulated by Bcl-2 (B cell lymphoma/leukemia-2), an antiapoptotic protein that inhibits autophagy by binding and sequestering Beclin 1 under nutrient-rich conditions; dissociation of Beclin 1 from Bcl-2 is required for autophagy induction. The PI3K-III complex leads to the Atg9-mediated recruitment of endosomes and multivesicular bodies; moreover, it recruits two interrelated ubiquitin-like (Ubl) conjugation systems to the phagophore (Wang et al. 2001; Webber and Tooze 2010), which play an essential role in regulating the membrane elongation and expansion of the forming autophagosome. These conjugation systems consist in the formation of the Atg5/Atg12/Atg16 complex (Harada et al. 2019) and the conjugation of LC3 (microtubule-associated proteins 1A/1B light chain 3B) to the phosphatidyletanolamine (PE), thus forming LC3-II (also called LC3 lipidation). LC3 lipidation is mediated by E1-like enzyme Atg7 and E2-like enzyme Atg3. Meanwhile, the late phagophore folds to enwrap the aged or damaged cell components, such as the ubiquitinated mitochondria and p62-bound poly-ubiquitinated proteins, which are sequestered within a mature autophagosome. The fusion between the autophagosome and the lysosome involves proteins belonging to the soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNARE) complex.

Chaperone-mediated autophagy (CMA), unlike microautophagy and macroautophagy, is highly specific. The unique feature of this type of autophagy consists on the protein-selective of specific proteins to be degraded, which is achieved by the specific shuttling of these proteins across the lysosomal membrane without requiring additional vesicles. Common to all CMA substrates is a pentapeptide motif biochemically related to KFERQ. Target proteins containing this KFERQ consensus motif are recognized by a cytosolic

chaperone, such as the heat shock 70 kDa protein 8 (HSPA8/HSC70), and are transported to the lysosomal surface, where they interact with monomers of the CMA substrate receptor, lysosomal-associated membrane protein 2A (LAMP2A) (Cuervo and Dice 1996). This substrate-receptor binding leads to the multimerization of LAMP2A (Cuervo and Dice 1996; Bandyopadhyay et al. 2008; Parzych and Klionsky 2014). By the help of HSP90, the substrate is then unfolded and translocated through LAMP-2A-enriched, directly across the lysosomal membrane. Following translocation of the substrate into the lysosomal lumen, the translocation complex is actively disassembled by cytosolic HSPA8. Substrate proteins (including certain glycolytic enzymes, transcription factors and their inhibitors, calcium and lipid binding proteins, proteasome subunits, and proteins involved in vesicular trafficking) are degraded in the lysosomal lumen (Orenstein and Cuervo 2010), whereas LAMP2A returns to a monomeric state, where it can bind new substrate and initiate a new round of translocation.

It is suggested that HSPA8 and LAMP2A also participate in a type of macroautophagy called chaperone-assisted selective autophagy. During this process, chaperones aid in the clearance of selectively ubiquitinated organelles and protein complexes (Kirkin et al. 2009). Association of these ubiquitinated targets with receptors, such as SQSTM1/p62 and NBR1 (neighbor of BRCA1 gene 1), and with enzymes, including HDAC6 (histone deacetylase 6), allows for recognition by the macroautophagy machinery, delivery to the lysosome, and degradation (Kirkin et al. 2009; Lamark et al. 2009).

The most common and well-known autophagy is macroautophagy, hereafter referred to simply as autophagy.

Many studies demonstrated that autophagy is also involved in neuronal injury in several neurological disorders, such as cerebral ischemia (Gabryel et al. 2012), traumatic brain injury (Chen et al. 2007; Liu et al. 2008), and epilepsy (Li et al. 2018). However, its role is still controversial. In fact, in some models of neurodegeneration, autophagy contributes to non-apoptotic cell death (Koike et al. 2008; Ginet et al. 2014; Descloux et al. 2018), while in other cases, it plays a protective role (Spencer et al. 2009; Lee et al. 2010; Hochfeld et al. 2013; Ruffoli et al. 2015) (for a recent review see Corti et al. 2020).

Autophagy in TMT-induced neurotoxicity

Autophagy has been suggested to be related with TMT intoxication by several authors. Ultrastructural studies performed in human and rodent brains exposed to TMT show an increased number of lysosomes and vacuoles, suggesting the presence of an altered autophagy (Bouldin et al. 1981; Chang and Dyer 1985; Besser et al. 1987). In particular, Bouldin et al. (1981) found that TMT intoxication in rats produces neuronal necrosis as evidenced by shrunken neurons with dense, eosinophilic cytoplasm and pyknotic nuclei. The numerous necrotic neurons were detected in the hippocampal formation and piriform cortex, being characterized by cytoplasmic accumulations of dense-core vesicles and tubules, and polymorphic dense bodies. Many dense bodies feature a double limiting membrane and strongly resemble autophagy vacuoles. Occasionally, authors described morphological patterns witnessing the various steps in the autophagy process. In particular, a few nascent autophagy vacuoles possessed mitochondria-like structures, while other vacuoles contained normal appearing cytoplasm or moderately electrondense cytoplasm. Most autophagy vacuoles contained only amorphous electron-dense granular material or fragments of membranes.

Besides, Besser et al. (1987) reported in humans exposed to TMT the presence in the amygdala of multilamellar bodies made up of concentric membranes named "zebra bodies", which are likely to correspond to the presence of tin molecules within non-effective, stagnant autophagy vacuoles.

In line with this, ultrastructural studies of TMT-treated neurons show the presence of a great number of lysosomes and vacuoles, some of which being typical autophagic vacuoles. Besides, TMT-treated neurons also exhibit a decrease in mitochondrial mass and the presence of mitochondria with an evident altered morphology (Fabrizi et al. 2012). Mitochondria represent one of the main target of TMT cytotoxicity (Thompson et al. 1996; Misiti et al. 2008) and it is known that the clearing of mitochondria that contain damaged components is accomplished via autophagic degradation (mitophagy).

Whether autophagy is associated with beneficial (pro-survival) or detrimental (pro-death) functions in TMT-induced neurotoxicity is controversial, as it remains debatable in many neurological disorders. This is the case of cerebral ischemia, traumatic brain injury and epilepsy (Koike et al. 2008; Spencer et al. 2009; Lee et al. 2010; Hochfeld et al. 2013; Ginet et al. 2014; Ruffoli et al. 2015; Descloux et al. 2018; Ferrucci et al. 2018).

Bouldin et al. (1981) suggested that autophagy could contribute to cell death, showing accumulation of autophagic vacuoles in TMT-treated rat hippocampal neurons. Additionally, Lattanzi et al. (2007) reported an increase in autophagy-associated genes using microarray analysis in TMT-treated PC12 cells with respect to untreated controls. Very recently Ceccariglia et al. (2019) investigated the autophagy status through the analysis of LC3, p62, and Beclin 1 as main regulatory markers in the rat hippocampus at different time points after TMT administration. Authors concomitantly evaluated the main apoptosis pathways to check for a potential correlation between the autophagy status and apoptosis machinery. These data indicate that, after TMT intoxication

in the rat hippocampus, an activation of both autophagy and apoptosis takes place. In addition, the expression of classic autophagic and apoptotic markers indicate that autophagy is activated just before apoptotic neuronal death, already at early time intervals after treatment, though persisting at later time points. The authors speculate that, at early stages after treatment, increased autophagy can be a compensatory response within neurons following the stressful exposure to TMT. On the other hand, at later stages, when cellular loss is increased, autophagy can be involved directly as a deleterious mechanism to promote neuronal death even by triggering apoptosis. This multi-faced significance may coexist in the same and/or in different neuronal populations.

In addition, Fabrizi et al. (2012) found that autophagy protects cultured hippocampal and cortical neurons from TMT toxicity. In neuronal cultures TMT at low doses does not produce evident toxicity (Fig. 2). This is likely to depend on beneficial effects induced by autophagy activation, which clears misfolded proteins and damaged mitochondria from the cells. In line with this, when neurons are challenged with TMT in combination with autophagy blockers such as 3-methyladenine (3-MA) and L-asparagine (L-Asn), the threshold dose for TMT to produce toxicity is reduced. In fact, in these experimental conditions even low doses of TMT become frankly toxic, being such an effect more evident in hippocampal than iso-cortical neurons. Conversely, when a frankly toxic dose of TMT is administered to neuronal cultures a great number of acidic vacuoles appear, configuring a pathological scenario of autophagy-related cell death (Fig. 2). This is likely due to weakened autophagy vacuoles when engulfed by organotins. Since organotins derivatives cannot be metabolized they impair the autophagy machinery producing a defect in autophagy progression which may be responsible for TMT-induced cell death. This neurotoxicity induced by high doses of TMT could be prevented by autophagy inducers, even owing a different mechanism of action, such as rapamycin and lithium. Again this confirms the seminal role of the autophagic pathway in preventing TMT neurotoxicity. Apart from removal of misfolded proteins, it is likely that autophagy inducers protect neurons from TMT toxicity because of their prompt removal of damaged mitochondria which occludes the release of free radicals triggering apoptosis.

Beyond causing neuronal toxicity, TMT massively alters glial cells by producing in vivo an extensive microgliosis and astrogliosis (Haga et al. 2002; Pompili et al. 2004, 2006, 2011, 2019). This poses the question whether an alteration in the glial compartment plays a role in TMT toxicity and, in turn whether the autophagic pathway modulates these phenomena as well. When astrocytes and microglia are exposed

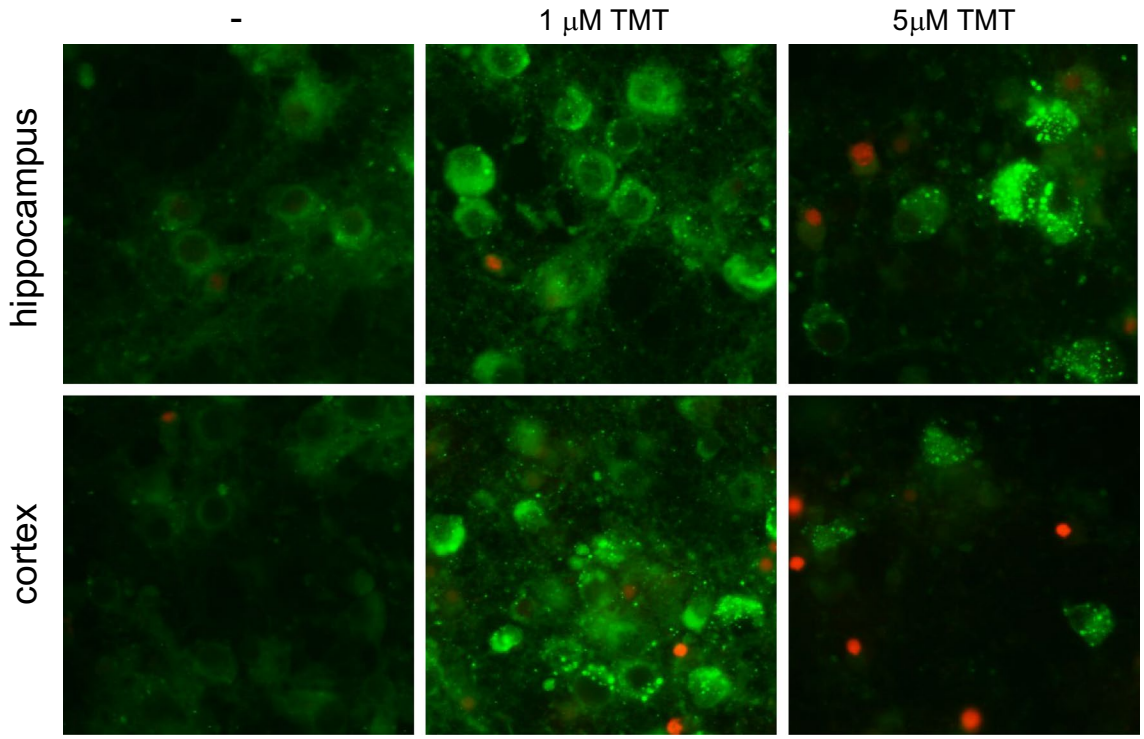

Fig. 2 Acidic vacuoles positive for monodansylcadaverine (MDC) in neuronal cultures after TMT treatment. Hippocampal and cortical neurons were treated with 1 and 5 μM TMT for 24 h and then labelled with MDC. Many MDC-positive vacuoles appear in neurons after TMT treatment. Dead cells are labelled with sytox orange. *Scale bar* 20 μm (from Fabrizi et al. 2012)

to TMT, marked subcellular alterations are described reminiscent of what observed in neurons (Fabrizi et al. 2012). In detail, the ultrastructural analysis by transmission electron microscopy (TEM) of TMT-treated astrocytes indicates an extensive cytosolic vacuolization with stagnant vesicles resembling autophagic vacuoles. In particular, these vesicles appeared to contain flocculated and precipitated material or part of the cytoplasm with various levels of electron density. Alteration of the mitochondrial morphology is also detected in TMT-treated astrocytes (Fabrizi et al. 2016). The autophagic flux in TMT-treated astrocytes is impaired at late autophagy stages leading to accumulation of autophagosomes. When time-lapse recording is carried out following TMT, autophagosomes within astrocytes keep their intracellular placement for time interval exceeding 1 h. Such an arrest in autophagosomes dynamics impairs their merging with other compartments and occludes the occurrence of autophagolysosomes (Fabrizi et al. 2016). It is likely that, such a loss of movement of autophagic vacuoles following TMT is due to the inhibition exerted by TMT on microtubule assembly (Jensen et al. 1991). Such an impairment in autophagosome motility takes place already at 3 h after TMT exposure, earlier than the occurrence of gross morphological changes at the level of the cytoskeleton or the lysosomal compartment. Such a phenomenon anticipates several hours the occurrence of glial cell death. In fact, in these experimental conditions apoptotic cell number increases significantly compared with control only at 8 h following TMT exposure. Consistently with a delay in the progression of autophagy, a marked accumulation of p62 is described in TMT-treated astrocytes. In fact, p62 is a protein which is specifically degraded through autophagy. Thus, increased p62 levels further witness for a block in autophagy progression (Liu et al. 2017). In line with this, when we checked ex vivo the expression of p62 in the brain of TMT intoxicated rats, high protein levels were detected within hippocampal reactive astrocytes concomitantly with hippocampal damage (Fabrizi et al. 2016).

More recently the impairment of autophagy progression at later stages was also observed in microglia which are considered the resident macrophages of the central nervous system (Fabrizi et al. 2017). The occurrence of an autophagic block in microglial primary cultures following TMT treatment was inferred by considering the accumulation of LC3-II and p62. This effect was less pronounced with respect to what previously observed in astrocytes. Confirming these data obtained in vitro with less potential experimental bias, analogous findings were reported in the brain of TMT intoxicated rats, where the abnormal increase of p62 was mostly associated to astrocytes being less evident in microglia (Fabrizi et al. 2016, 2017).

Such a susceptibility of the autophagic pathway to organotins may be inherent to the evolutionary complexity of the autophagic machinery in multicellular organisms. In fact, some bacteria (i.e., Pseudomonas) and phytoplankton retain the ability to degrade organotin compounds under certain conditions (Hoch 2001; Sampath et al. 2012). This calls for dissecting which divergence may be present in these organisms making their autophagic machinery more resistant to the deleterious effects of organotins. The molecular mechanisms underlying the impairment of autophagy progression during TMT intoxication remain to be established. When observing TMT-intoxicated astrocytes it is striking the observation of the engulfment of autophagic vesicles with undigested materials, which cells are unable to process further. Still, the relented vesicle movement due to TMT-induced impairment in microtubule assembly should be considered as well. In this connection, a very recent article identified the kinesin family member 5A (KIF5A) as a key target in the blockage of the autophagic flux induced by TMT (Liu et al. 2020). KIF5A was reported to be involved in the transport of lysosomes, synaptic vesicle precursors, other diverse vesicles, and mitochondria (Morfini et al. 2016). This kinesin also regulates the intracellular transport of cargo, such as autophagosomes, in cardiomyocytes (Blakeslee et al. 2017). To note, the deficiency in KIF5A induces epilepsy in both humans (Dixit et al. 2016) and mice (Nakajima et al. 2012) and is also associated with myoclonic seizures in humans (Rydzanicz et al. 2017). Liu et al. (2020) observed a decreased KIF5A expression in the hippocampus of TMT-treated mice; the overexpression of KIF5A alleviated TMT-induced seizures, suppressed histomorphological signs of injury of the hippocampus, and restored the autophagic flux.

In complex organisms organotins are not the solely neurotoxicant capable of blocking autophagy, in fact the inhibition of the autophagic flux and the p62-dependent activation of the Keap1-Nrf2 [Kelch-like ECH-associated protein 1-nuclear factor (erythroid-derived 2)-like 2] pathway also occur following exposure to other toxicants, such as arsenic acid (Lau et al. 2013). Besides, the Nrf2/p62 signaling pathway is involved in apoptosis resistance from cadmium exposure (Son et al. 2014). Manganese activates autophagy at early time points, but inhibited autophagy in the long term (Zhang et al. 2013). Thus, we should consider the combined action of different neurotoxicants which potentially act on the autophagic pathway each contributing to its impairment.

Lithium-mediated neuroprotection from TMT toxicity

Our results show that TMT neurotoxicity can be dramatically modified, at least in vitro, by lithium administration. Indeed, this may rely on different mechanisms even depending on a brief or a prolonged exposure. In fact, lithium, which is well

known as a mood stabilizer, has a variety of mechanisms of action, which include autophagy activation, VEGF (vascular endothelial growth factor) and BDNF (brain-derived neurotrophic factor) production, stem cells stimulation, biogenesis of mitochondria and others extensively reviewed by Pasquali et al. (2010) and Kerr et al. (2018). Differently from rapamycin (an mTOR inhibitor), a number of enzymes have been proposed as potential targets of lithium action, including inositol monophosphatase (IMPase), a family of structurally related phosphomonoesterases, and the protein kinase glycogen synthase kinase (GSK) 3b (Fig. 3). In particular, when administrated at low doses, lithium is known to induce autophagy via IMPase inhibition leading to free inositol depletion and reduced myo-inositol-1,4,5-triphosphate (IP3) levels (Sarkar and Rubinsztein 2006). At higher doses, lithium directly inhibits the activity of GSK3b acting as a competitive inhibitor of Mg^{++} at its catalytic site and indirectly by increasing Ser21/Ser9 phosphorylation of GSK3a/b (Jope 2003).

Lithium administered before TMT, according to an acute treatment protocol (2 h pre-treatment), was able to counteract TMT toxicity in hippocampal but not in cortical neurons. This neuroprotective effect of lithium acutely administered against TMT in hippocampal neurons could be completely reverted by an excess of inositol and it is possibly related to the inactivation of IMPase. Conversely, when lithium was administered according to a chronic treatment protocol (starting at 6 days before TMT administration) it was able to rescue both hippocampal and cortical neurons from TMT neurotoxicity. This effect was concomitant by increased phosphorylation of GSK3b (Fabrizi et al. 2012).

In addition, both lithium and rapamycin were effective in reducing TMT neurotoxicity in PC12 cells confirming that a prompt activation of the autophagy machinery can protect from TMT-induced cell death (Fabrizi et al. 2014). In rodents, lithium improves the symptoms of TMT intoxication. In fact, this cation can rescue seizures and ameliorate memory deficits in TMT-intoxicated rodents, increasing at the same time the inhibitory phosphorylation of GSK3b in the hippocampus (Kim et al. 2013). Moreover, lithium enhances neurogenesis ameliorating the depression-like behavior observed in mice treated with TMT (Yoneyama et al. 2014). Altogether, various data, including that from our lab, converge in establishing a beneficial effect of lithium administration in TMT-intoxicated rodents (Kim et al. 2013; Yoneyama et al. 2014; Fabrizi et al. 2016, 2017).

In vitro experiments indicate that in microglia lithium limits the release of a pro-inflammatory mediator, such as TNF-α and potentiates the production of the anti-inflammatory cytokine IL-10 (Fabrizi et al. 2017). As mentioned above, lithium inhibits GSK3b, both indirectly and directly, competing with magnesium at its catalytic site. GSK3b is known to promote inflammation (Martin et al. 2005) and lithium, as well as other GSK3 inhibitors, limits the

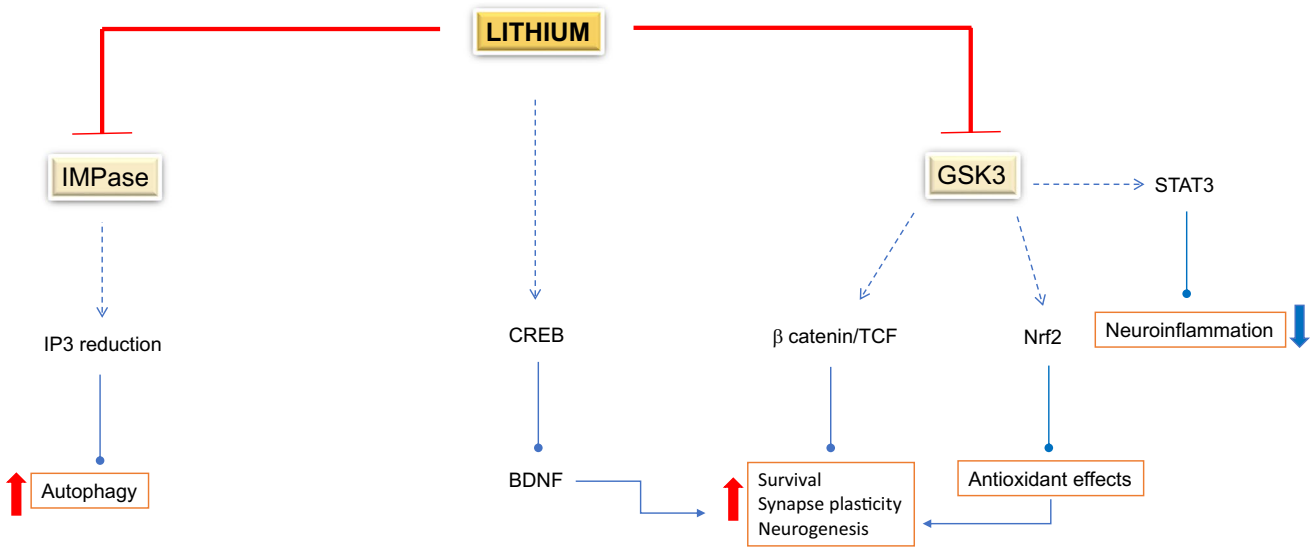

Fig. 3 Multiple mechanisms of lithium-mediated prevention of toxicity. Most of the activities of lithium can be mainly reconducted to inositol monophosphatase (IMPase) and glycogen synthase kinase 3 (GSK3) inhibition. The inhibition of IMPase determines the induction of autophagy through the depletion of free inositol and the reduction of inositol 1,4,5 triphosphate (IP3) levels. GSK3 inhibition generates pleiotropic effects: (1) translocation of β catenin to the nucleus in association with transcription factors of the T-cell family (TCF) favoring neuronal survival, synapse plasticity and neurogenesis; (2) block of the signal transducer and activator of transcription 3 (STAT3) and reduction of neuroinflammation; (3) antioxidant effects through the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). The lithium-induced BDNF transcription through CREB (cAMP response element binding protein) is independent from GSK3 and IMPase

production of inflammatory cytokines in macrophages (Park et al. 2011b), dendritic cells (Rodionova et al. 2007) and microglia (Huang et al. 2009; Yuskaitis and Jope 2009). It remains to be explained how lithium in microglial cultures could exert a pro-survival and immunomodulatory action without rescuing TMT-induced autophagic block. This is crucial since lithium, alone or in combination with valproic acid, is considered a promising agent for treating neurodegeneration (Chiu et al. 2013; Ge and Jakobsson 2018), which is characterized by protein misfolding, aggregation and deposition, all reminiscent of autophagy dysfunction (Vidal et al. 2014).

In conclusion, the present review suggests that studies about the roles of autophagy are still insufficient, and more evidence is needed to determine the precise role of autophagy in TMT-induced neurodegeneration. Similarly, the molecular mechanisms leading to beneficial effects of lithium in different cell populations to counteract TMT intoxication need further investigation. This emerges to be relevant beyond the field of environmental safety related to organotin compounds, since the very same mechanisms appear to be effective in a variety of neurodegenerative disorders.

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