

NITRIC OXIDE AND SPHINGOLIPIDS AS MODULATORS OF APOPTOSIS AND AUTOPHAGY: FUNCTIONAL IMPLICATIONS IN NEURODEGENERATIVE CHRONIC BRAIN DISORDERS

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Aberrant regulation of the apoptosis and autophagy machineries is a central abnormality in most human neurodegenerative disorders characterized by progressive dysfunction and death of neuronal and glial cells. Both in central and peripheral nervous systems, cell death can be either apoptotic or autophagic, depending on the cellular setting and inducing stressor. However, while some mixed phenotypes have been reported, apoptosis and autophagy ultimately may develop in mutually exclusive ways and appear to inhibit each other. Generation of the pleiotropic sphingolipid mediator ceramide is a key event in many cellular processes including survival and death, in which also the short-lived gaseous messenger nitric oxide (NO) plays a crucial role. Much progress has been made in understanding the crosstalk among the NO and the sphingolipid pathway, with its multiple feedback controls which have important implications in neurophysiological and neuropathological processes. Strikingly these mediators impact on both apoptosis and autophagy. What we provide here are details on how NO- and sphingolipid-dependent signaling impact on chronic brain disorders, i.e., Alzheimer's, Parkinson's, and Huntington's diseases; we also describe how their crosstalk and regulation of autophagy and apoptosis may play a significant role in determining the pathogenic evolution. The evidence we report suggest that targeting the NO and sphingolipid signalling pathways may ultimately be exploited in therapeutic perspective. However, defining how this integrated pathway balances towards beneficial vs. toxic effects appears to be complex and needs being resolved to identify suitable therapeutic targets and strategies.

The fate of a cell is determined by a balance of survival and promoting signals. While survival signals mediate cell maintenance, promoting signals induce cells to proliferate, differentiate, transform or apoptose. The natural occurrence of cell death has long been appreciated and was widely studied by twentieth century biologists. While multiple modes of cell death have been described, undoubtedly the most renowned process is the programmed form

of cell death known as apoptosis. Apoptosis, also known as programmed cell death, is characterized by distinctive stereotyped morphological and biochemical alterations, such as exposure of phosphatidylserine on the outer leaflet of the plasma membrane and blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation (Jin and El-Deiry, 2005; Munoz-Pinedo, 2012). This process leads to the formation of apoptotic bodies that are

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subsequently eliminated by phagocytosis. Key event in the apoptotic process is the activation of caspases, a family of cysteinyl aspartate-specific proteases (Kurokawa and Kornbluth, 2009). They are constitutively expressed in almost all cell types as inactive proenzymes (zymogens) that become processed and activated in response to a variety of pro-apoptotic stimuli. Evidence for the sequential activation of caspases leads to the concept of a caspase cascade: during apoptosis, apoptogenic stimuli induce the autocatalytically activation of initiator caspases; subsequently they cleave and thereby activate downstream effector caspases that carry on the cleavage of specific proteins in order to "dismantle" the cell (Kurokawa and Kornbluth, 2009). The induction of apoptosis can be mediated by two pathways: the death receptor-dependent or the mitochondria-dependent pathways, also known as the extrinsic and intrinsic apoptotic pathways, respectively (Jin and El-Deiry, 2005; Munoz-Pinedo, 2012). Extrinsic pathway is activated by the death receptors through the interaction with their ligands or by inducing receptor clusterization (Tchikov et al, 2011). On the other hand, the intrinsic pathway of apoptosis activated by stimuli such as hypoxia, nutrient deprivation, radiation, heat, cellular stress is proposed to be a consequence of a mitochondrial damage (Martinou and Youle, 2011). Generally, this event follows the direct activation of the members of the Bcl-2 family with proapoptotic roles, such as Bax and Bid, that translocate to the mitochondria, deregulating the balance with the antiapoptotic protein Bcl2/Bcl-xL, and disrupt the membrane integrity to induce mitochondrial permeability transition pore (Kroemer and Reed, 2000; Martinou and Youle, 2011).

In the last decade increasing attention has been focused on alternative signaling pathways leading to cell remodeling, as for instance autophagy (Mizushima and Komatsu, 2011). Autophagy is an evolutionarily conserved lysosomal pathway which is important for the maintenance of cytoplasmic homeostasis. In particular, macroautophagy is a homeostatic self-eating process, in normal condition it allows cells to break down long-lived proteins complementing the proteasome action (Wong et al, 2011). Autophagy is upregulated when cells require nutrients and energy, such as during starvation, in

condition of high bioenergetic demand or under other stresses (Mizushima and Komatsu, 2011). Autophagy involves the formation of double membrane bound structures that are known as autophagosomes; these vesicles assemble around and entrap damaged organelles or cellular debris and then fuse with lysosomes to degrade their content (Pattingre et al, 2008). Autophagy process requires different steps for autophagic vesicles (AV) formation and turnover, including initiation, nucleation and maturation of AV, followed by fusion and degradation of AV contents in lysosomes. Any steps are regulated by specific molecules; initiation requires ULK1 kinase complex consisting of ULK1, Atg13 and FIP200 (Atg17) and the newly identified Atg101 (Hosokawa et al, 2009; Mercer et al, 2009; Mizushima, 2010). Nucleation and assembling of the initial phagophore membrane is dependent on generation of phosphoinositide signals by the multiprotein complex including PI3 kinases, Vps34 and Beclin1. The Vps-complex is found on the phagophore and thought to facilitate recruitment of other Atgs to the developing vesicles. The identification of incipient AVs depends on Atg8, also named LC3 (microtubules-associated protein 1 light chain), conjugation to phosphoethanolamine on the surface of these membranes, in a mechanism defined LC3 lipidation (Tanida et al, 2004). Once LC3 is integrated into the bilayer, it binds cargo adaptors protein like p62 and NIX, that in turn recruit cargo from the cytoplasm to promote AV closure (Pankiv et al, 2007; Schweers et al, 2007). AVs are delivered to lysosomes where the AVs content is finally degraded by lysosomal hydrolases and released into the cytosol for reuse.

In many diseases, aberrant regulation of the apoptosis and autophagy machineries is the central abnormality. In central and peripheral nervous system the criteria for these cell remodeling processes are fulfilled in many neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's diseases (HD), Amyotrophic lateral sclerosis, Frontotemporal dementia, Multiple sclerosis and in acute neurodegenerative conditions such as stroke, trauma and severe epileptic seizures, in which neuronal death is a central feature (Mattson, 2000; Jaeger and Wyss-Coray, 2009; Rosello et al, 2012). Of interest, the resulting cell death can be either apoptotic or autophagic, depending on

the cellular setting and inducing stressor (Jaeger and Wyss-Coray, 2009). This dichotomous role is the result of a complex relationship between the apoptotic and autophagic pathways. While some mixed phenotypes have been reported, apoptosis and autophagy in the nervous system ultimately develop in mutually exclusive ways and appear to inhibit each other (Jaeger and Wyss-Coray, 2009). In this review, we will consider recent insights achieved on apoptosis and autophagy in the context of neurodegenerative chronic brain disorders considering exclusively their regulation exerted by nitric oxide (NO) and/or sphingolipid pathway.

NITRIC OXIDE/SPHINGOLIPID PATHWAY IN APOPTOSIS AND AUTOPHAGY

Generation of the pleiotropic sphingolipid mediator ceramide by Acid and Neutral Sphingomyelinases (A-SMase and N-SMase) is a key event in many cellular pathophysiological processes including survival, death, proliferation and differentiation, in which also the short-lived gaseous messenger NO plays a crucial role.

Nitric oxide

NO is generated in cells by specific enzymes, the NO synthases (NOS). Of these, the neuronal NOS (nNOS or NOS I) and the endothelial NOS (eNOS or NOS III) isoforms are expressed constitutively, operate under the control of second messengers and lead to generation of physiological concentrations of NO (Alderton et al, 2001). A third enzyme, inducible NOS (iNOS or NOS II), is inducible by a variety of stimuli, including cytokines and bacterial products, and often yields high NO concentrations that participate to the immune responses that lead to cell damage (Alderton et al, 2001). Because of its chemical reactivity and high diffusibility, NO production by NOS is under a complex and tight control, designed to dictate specificity of its signaling and to limit toxicity to other cellular components. Studies in recent years have uncovered an increasingly important role of physical association of the NOS isoforms with a variety of regulatory and structural proteins (Kone et al, 2003). Of importance, these protein-protein interactions, as well as regulating NOS activity often target them

to cellular membranes. The N-terminus of nNOS contains a PDZ (post-synaptic density protein-95, discs-large, ZO-1) domain that allows interactions of the enzyme with other PDZ-containing proteins at the plasma membrane including α 1-syntrophin, PSD-95 and PSD-93 (Brenman et al, 1996). eNOS is localized at both the plasma membrane, and the Golgi complex through its ability to be myristoylated and palmitoylated (Fulton et al, 2001). Several other interactions, especially between eNOS and various proteins such as calmodulin, HSP70, NOSIP and NOSTRIN have been described to play important structural and functional roles (Fulton et al, 2001). In addition, both iNOS and eNOS may interact with caveolin 1 and/or 3, proteins responsible not only of the localization of these enzymes at the plasma membrane but also of the regulation of their activity (eNOS) and expression (iNOS) in an inhibitory fashion (Felley-Bosco et al, 2000; Fulton et al, 2001). Indeed the localization at cellular membranes is a key aspect of NOS and their signaling and constitutes the structural basis of the functional interaction among NO, sphingolipids and their generating enzymes.

Sphingolipids

The role of lipids as merely structural components was a tenet until the late 1970's: the discovery of the phosphoinositide cycle (Berridge, 1984) represented a major breakthrough, pointing for the first time to lipids as key players also in signal transduction events. From that point onwards, other lipid molecules found acceptance as biological mediators and modulators; among these a class of lipids has emerged as a group of important signaling molecules: the sphingolipids. Lipids of the sphingolipid class contain a long-chain sphingoid base backbone (such as sphingosine), an amide-linked, long-chain fatty acid and one of various polar head groups. The structure of these head groups defines the various classes of sphingolipid subtypes, with a hydroxyl group found in ceramide, phosphorylcholine in sphingomyelin (SM) and carbohydrates in glycosphingolipids. Their synthesis begins in the endoplasmic reticulum and continues in the Golgi apparatus, and they are thereafter found in all intracellular membrane structures (for a review see Futerman, 2006).

Richard Kolesnick's and Yussuf Hannun's groups were the first to point attention to SM-

based signaling pathway: the former showed in 1987 the rapid activation of SMases in response to 1,2 diacylglycerol treatment (Kolesnick, 1987) and proposed the existence of a SMase-generated signaling pathway (Kolesnick, 1989); the latter showed that this pathway could be activated by receptor-mediated mechanisms and provided first evidence for ceramide as a cellular mediator (Okazaki et al, 1990). Despite the possible *de novo* formation of ceramide, in the vast majority of cells SM appears to be the primary sphingolipid source for bioactive ceramide, thereby emphasising the critical role for SMases, i.e., the lysosomal phosphodiesterase A-SMase and the membrane-bound N-SMase, in initiating ceramide-activated signalling.

Nitric oxide/sphingolipid crosstalk in apoptosis

Generation of NO by the constitutive nNOS or eNOS, at low, physiological concentration is one of the mechanism of inhibition of apoptosis induced by the activation of death receptors (the TNF- α receptor (TNF-RI)/CD95 superfamily) (Sciorati et al, 1997; Liu and Stamler, 1999). On the contrary, at high concentrations, such as those generated by iNOS in inflammation, NO can induce apoptosis *per se*. Various mechanisms have been proposed to account for these two, apparently conflicting effects of NO (Liu and Stamler, 1999; Brune, 2003). One of these is the ability of NO to regulate ceramide cell levels. In studies carried out in cells of the monocytic lineage and in clones of $\gamma\delta$ T lymphocytes it has been shown that NO inhibits apoptosis induced by the stimulation of CD95 or TNF-RI by impairing the ability of these receptors to generate ceramide (Sciorati et al, 1997; Sciorati et al, 1999; De Nadai et al, 2000; Bulotta et al, 2001; Barsacchi et al, 2003). Although both A-SMase and N-SMase are inhibited by NO, protection from apoptosis is due to the inhibition of A-SMase; this mechanism of apoptosis protection appears to be general since it has been shown to be active also in human and murine dendritic cells (DCs) as well as in cancer cells. In particular, NO generation inhibited apoptosis of dendritic cells exposed to apoptogenic concentrations of lipopolysaccharide (LPS) *in vitro*, and in a model of LPS-induced sepsis *in vivo* (Falcone et al, 2004); in addition it protected DCs from the toxic effect of the chemotherapeutic drug, cisplatin, *in vitro* and *in vivo* in a model of tumor

chemotherapy (Perrotta et al, 2007). Also in DCs the primary target of NO action appears to be A-SMase, activated by both LPS, acting via the Toll-like receptor 4 (Falcone et al, 2004) and cisplatin, acting via CD95 activation (Lacour et al, 2004; Perrotta et al, 2007). Similar results were observed in studies on glioma cell in which A-SMase is activated by CD95 stimulation (Perrotta et al, 2010). Of importance, inhibition of activity of A-SMase, and N-SMase and protection from apoptosis by NO appear to be mediated through activation of soluble guanylyl cyclase, cGMP generation and activation of cGMP-dependent protein kinase (Bulotta et al, 2001; Barsacchi et al, 2003; Falcone et al, 2004; Perrotta et al, 2007).

The finding outlined above open an important question about the mechanism of SMases inhibition by NO. A- and N-SMases are quite different in terms of their intracellular distribution, localisation-activity relationship and second messenger regulation. Thus, it is unlikely that NO operates to control them through the same mechanism. While no studies have so far addressed this question specifically, we have evidence that regulation of activity of A-SMase is mediated through regulation of its intracellular localization (Perrotta et al, 2010; C. Perrotta and E. Clementi, unpublished results); no information is available about the mechanism of N-SMase inhibition by NO.

The exposure to high levels of NO have effects opposite to those of physiological NO concentrations. Indeed, it has been demonstrated in different cell lines that high concentration of NO increase the generation of ceramide through the activation of both A- and N-SMase and the ensuing death for apoptosis (Huwiler et al, 1999; Takeda et al, 1999; Pilane and LaBelle, 2004; Castillo et al, 2007). The mechanisms involved in SMases activation by NO have not been investigated; however, they are clearly distinct from those involved in SMase inhibition, since the former are independent of cGMP and require a caspase-3-dependent step (Huwiler et al, 1999; Takeda et al, 1999; Castillo et al, 2007) possibly involving arachidonic acid-derived eicosanoids (Pilane and Labelle, 2005). Finally, it's worth mentioning the existence of a loop of ceramide on NO to potentiate its apoptotic effect. In this context ceramide can operate through the activation of NOS and the

increase of NO levels, thus inducing the damage of mitochondrial membranes and initiating caspase cascades in various types of cells (Shupik et al, 2011).

Autophagy: possible implications of nitric oxide and sphingolipids

Autophagy is emerging as an important mediator of pathological responses and engages in cross-talk with ROS (reactive oxygen species) and RNS (reactive nitrogen species) in both cell signaling and protein damage (Lee et al, 2012). A role for the sphingolipids ceramide/sphingosine 1 phosphate (S1P), has been shown to contribute to autophagy regulation (Lavieau et al, 2007). Ceramide is able to induce autophagy with multiple mechanisms. For instance, it can inhibit Akt phosphorylation and upregulate Beclin1 (a key regulatory protein in autophagy pathway). In addition, ceramide can activate the protein kinase JNK1, inducing Bcl2 phosphorylation which results in its dissociation from Beclin1 complex (Wei et al, 2008; Pattingre et al, 2009). Furthermore, ceramide enhances accumulation of the cell death factor BNIP3 and trigger BH3-dependent dissociation of the Beclin1-Bcl2 inhibitory complex (Levine et al, 2008). A role of this mechanism is significant in cancer: C2-ceramide induces autophagic cell death in malignant glioma cells enhancing BNIP3 expression associated with mitochondrial dysfunction (Daido et al, 2004). Finally, ceramide interferes with aminoacids transport and induces autophagy by disregulating nutrient transporters (Guenther et al, 2008) and by inhibiting the mammalian target of rapamycin (mTOR) signal pathway as revealed by p70S6 kinase inhibition. S1P appears to work in the same direction as ceramide. Increasing S1P levels after sphingosine kinase 1 (SK1) overexpression induces autophagy by inhibition of the mTOR activity, without affecting Akt and with a moderate increase of Beclin1 (Lavieau et al, 2006). In addition during starvation SK1 activity is increased and in presence of siRNA for SK1 autophagy is blocked with the appearance of apoptotic hallmarks. The picture is even more complex if S1P lyase (SPL) is also taken into account. SPL activity, although increasing intracellular S1P levels by preventing its degradation, is not accompanied by autophagy induction. Murine embryonic fibroblasts derived from SPL-deficient mouse (Sgpl1^{-/-}) show resistance

to apoptosis induced by chemotherapy or starvation, with a blockade of the apoptotic cascade upstream to mitochondrial damage. SPL deficiency is associated with upregulation of Bcl2 and Bcl-xL but autophagy is not altered in these cells compared to Sgpl1^{-/-} cells during starvation, suggesting that autophagy does not account for the Sgpl1^{-/-} cells resistance to apoptosis (Colie et al, 2009). According to this we can hypothesize a different regulatory role for S1P produced by SK1 induction or generated by SPL deficiency, which might be related to the different subcellular localization of the two enzymes. SK1 is in the cytosol and, when activated, translocates to the plasma membrane to produce S1P near its membrane receptors that might be involved in the process. Otherwise SPL is an endoplasmic reticulum (ER) integral membrane protein and controlling S1P levels near the ER leading to different signaling pathway. Overall, both ceramide and S1P can enhance autophagy, but with some key differences that could account for cell decision to survive or die (Lavieau et al, 2007). Ceramide acts on Akt levels whereas S1P acts only on mTOR, independently of PI3 kinase; only ceramide induces a strong accumulation of Beclin1 and more likely, this strong accumulation of Beclin1 changes the ratio Beclin1/Bcl2 leading to cell death. It means that autophagic response to S1P is milder compared to ceramide response and probably it is the only one compatible with cell survival. Also the nature of stress is important to begin the autophagic response. During starvation the ceramide levels are unchanged, suggesting that ceramide is not a mediator of this autophagic response. Conversely chemotherapy enhances ceramide levels and induces autophagic cell death. A Carefully balanced view would therefore envisage S1P as mediator of starvation-induced autophagy (which is a well established survival mechanism), and ceramide is the mediator of autophagic cell death.

Autophagy is also regulated by NO. NO blocks autophagosomes synthesis via two mechanisms (Sarkar et al, 2011), both independent of the cGMP pathway. NO S-nitrosylates and inactivates JNK1, preventing Beclin1-hVps34 association. In addition, it activates mTOR complex 1 leading to decreased AMP activated protein kinase phosphorylation. It has been shown that pharmacological or genetic inhibition

of NOS increases the clearance of mutant huntingtin (Ravikumar et al, 2004), providing the evidence that inhibition of autophagy by NO contribute to excitotoxicity phenomenon, increasing the levels of aggregate-prone proteins. According to this, in glioma cells, when combined with hypothermia, NO blocks the completion of autophagy process, as is evident by LC3-II accumulation, prompting cells to die (Janjetovic et al, 2008). The latter result is consistent with recent microarray analysis, showing inability of NO to induce autophagy-related genes (Rabkin and Klassen, 2007). Conversely in neurons NO induces nitrosylation of Drp1, enhancing massive mitochondrial fission, which in turn causes mitophagy (Barsoum et al, 2006). As mentioned before the NO and sphingolipid pathway regulate each other via interactions at various levels including on S1P (De Palma et al, 2006; Perrotta and Clementi, 2010). How and to what extent such crosstalk influences autophagy remains to be investigated.

CHRONIC NEURODEGENERATIVE BRAIN DISORDERS

NO production from inflammation and subsequent oxidative stress, nitrated proteins, RNS and NOS activity are significant factors in several neuropathologies (Steinert et al, 2010). Sphingolipid metabolism is regulated along the differentiation and development of the nervous system, and the expression of a peculiar spatially and temporarily regulated sphingolipid pattern is essential for the maintenance of the functional integrity of the nervous system. As recently reviewed (Jana et al, 2009; Haughey, 2010; Piccinini et al, 2010), the study of the roles that sphingolipids play in regulating normal neural activity is an emerging field and we are beginning to understand how perturbations in sphingolipid metabolism contribute to the pathogenesis of a variety of neurodegenerative conditions. Indeed, in several neurodegenerative diseases, sphingolipid metabolism is deeply deregulated, leading to the expression of abnormal sphingolipid patterns and altered membrane organization that participate to several events related to the pathogenesis of these diseases. Of importance, there is general agreement that excessive death of one or more populations of neurons and glial cells is the result of disease

or injury. In this respect, neuronal NOS is tightly coupled to the activation of NMDA receptors which precedes neuronal cell death (Nakagomi et al, 2008), and hence excitotoxicity-related neuronal injury could have a nitrenergic component. Noteworthy, a key role of sphingolipid pathway in the modulation of neurotoxic modulators such as NO has been recently reported in microglia (Nayak et al, 2010), thus suggesting that the crosstalk of the NO/sphingolipid pathway has important implications in neurophysiological and neuropathological processes.

In contrast to the rapid turnover of cells in proliferative tissues, neurons commonly survive for the entire lifetime of the organism. This enduring nature of neurons is necessary for maintaining the function of those cells within neuronal circuits. In addition, during development of the nervous system, many neurons undergo apoptosis during a time window that coincides with the process of synaptogenesis. Initial overproduction of neurons, followed by death of some, is probably an adaptive process that provides enough neurons to form nerve cell circuits that are precisely matched to their functional specifications. Accordingly, the decision as to which neurons die is made by cellular signal transduction pathways that are 'tuned' to the functionality of neuronal circuits. While the role of autophagy in neurodegenerative diseases is far from being understood, the available data indicate it plays an integral role in the cellular response to intracellular protein aggregation common to these diseases. Several excellent reviews have recently covered the emerging relationship between apoptosis, autophagy and various neurodegenerative diseases (Mattson, 2000; Martinez-Vicente and Cuervo, 2007; Cherra and Chu, 2008; Nixon et al, 2008; Jaeger and Wyss-Coray, 2009; Mizushima and Komatsu, 2011; Lee et al, 2012; Rosello et al, 2012). What we provide here are details on the most prevalent chronic brain diseases associated with NO and/or sphingolipid pathway and in which their regulation of autophagy and apoptosis may play a significant role in determining the pathogenic evolution.

Alzheimer's disease

AD is the most common form of dementia and is characterized by the accumulation of amyloid neuritic plaques, neurofibrillary tangles, inflammation,

oxidative stress, loss of synapses, impairment of memory and severe dementia. Laboratory and animal studies together with post-mortem human studies and analysis of the cerebrospinal fluid (CSF) of patients have indicated a role of sphingolipids in the development of amyloid-beta plaques, as well as neurodegenerative process, both critical features of AD. Of importance, it has been reported that ceramide and sphingosine, the most important pro-apoptotic sphingolipids, are elevated in AD brain and CSF, as a consequence of the high activity of A-SMase and acid and neutral Ceramidases (Voloshin et al, 2010). In this contest, ceramide may induce apoptosis by the reorganization of the plasma membrane but may also be involved in the production of Amyloid β (A β) peptide by stabilizing the Amyloid precursor protein (APP)-cleaving enzyme 1 (Sawamura et al, 2004; Patil et al, 2007). In particular, while under normal circumstances ceramide content is maintained at low levels to modulate neuronal cell homeostasis, upon aging, various stress factors, including the formation of fibrillar aggregates of the A β peptide, that may activate SMases, become elevated and induce the production of huge amount of ceramide. Ceramide, in turn, may enhance A β production further by promoting APP processing, leading to a positive feedback regulatory pathway that promotes cell death (Mielke and Lyketsos, 2010). Moreover, in a cellular model of AD, it has been shown that one of the mechanism through which ceramide potentiates the apoptotic signal cascade is by transcriptionally activating iNOS with the ensuing generation of elevated, toxic levels of NO, leading to generation of RNS thus contributing to the pathogenesis of AD (Ayasolla et al, 2004). Accordingly, the deficiency of NOS cofactor tetrahydrobiopterin BH4 was found to be associated with AD further implying a role of RNS in AD pathogenesis (Kuiper et al, 1994). Interestingly in senescent brains it has been observed also an increase of the activity of cGMP hydrolyzing phosphodiesterases (PDEs) leading to a decrease in the levels of cGMP. This would conceivably remove the protective effect of the physiological NO signaling thus contributing to enhance NO pathogenic effects (Domek-Lopacinska and Strosznajder, 2010). Puzzo et al indeed reported that the PDE5-selective inhibitor sildenafil, through elevation of cGMP levels, is beneficial against the AD phenotype in a

mouse model of amyloid deposition (Puzzo et al, 2009).

In the last few years autophagy has been showed as a key player in the pathogenesis of AD and its role has been defined as having a 'double-edged sword' role in the homeostasis of A β peptide production in neurons. Plenty of evidence reveals that A β is an autophagic substrate and is subject to autophagy-mediated clearance supporting the model in which autophagy is essential for the removal of detrimental A β peptides and aggregates (Lunemann et al, 2007; Pickford et al, 2008; Tung et al, 2012). Conversely, A β has been implicated in the modulation of autophagy, despite being an autophagy substrate, suggesting that A β may create a feedback loop to promote its own degradation and constitute an internal checkpoint for the homeostasis of its own production (Hung et al, 2009; Tung et al, 2012). Recently Tamboli and co-worker (Tamboli et al, 2011) have demonstrated a very interesting link between the storage of sphingolipids, the promotion of autophagy and the pathogenesis of AD, starting from the evidence that the presence of autophagosomes in dystrophic neurites is characteristic for AD brains as well as lipid storage diseases such as Niemann-Pick type C disease. The authors indicate that the accumulation of sphingolipids plays a dual role in autophagy; while promoting the induction of autophagy, sphingolipid may also impair the turnover of autophagic vesicles, leading to in their accumulation and consequently to the accumulation of APP. This might be very important for the etiology of AD, since the accumulation of membrane lipids is an age-related event and might contribute to both major neuropathologic events in AD, neurofibrillary tangles and amyloid plaques.

Parkinson's disease

PD is a chronic progressive neurodegenerative disease that is clinically manifested by a triad of cardinal motor symptoms - rigidity, bradykinesia and tremor - due to loss of dopaminergic neurons in their substantia nigra. The elucidation of the mechanisms coupled to the degeneration of dopaminergic neurons is still elusive; however several factors which interact each other, inducing a vicious cycle of toxicity causing neuronal dysfunction, atrophy and finally cell death have been proposed (Yuan et

al, 2010). From these studies a role for NO clearly emerges.

As reviewed by Steinert and coll. (Steinert et al, 2010), changes in NO release and increased expression of iNOS were reported in neutrophils obtained from PD patients and in 6-Hydroxydopamine and LPS-induced experimental models of PD, respectively. In addition, iNOS expression in humans appears to occur in astrocytes, and raised astroglial iNOS immunoreactivity is reported in postmortem brain tissue from patients with PD. The deficiency of NOS cofactor tetrahydrobiopterin BH4 is associated with PD (Foxton et al, 2007) further implying a contribution of NO to this neurodegenerative disease. Accordingly, compromised metabolism in substantia nigra following 1Methyl4phenyl1,2,3,6-tetrahydropyridine (MPTP) treatment in this PD model was slowed by competitive nNOS antagonists (Hantraye et al, 1996), and nNOS inhibition also blocked MPTP-mediated decrease in striatal dopamine in mice (Yokoyama et al, 2008). In addition, NO inhibits mitochondrial complex I activity persistently thus mimicking some of MPTP effects (Clementi et al, 1998). Recent results also show that NOS inhibition reduces L-DOPA-induced dyskinesia in rats and mice. The effect is dose-dependent, does not suffer tolerance nor interferes with L-DOPA positive motor effects. These preclinical findings suggest that NOS inhibition is a promising therapeutic target for the reduction of L-DOPA-induced dyskinesia (Del-Bel et al, 2011).

Several lines of evidence have implicated the protein α -synuclein and its modification as having important roles in PD pathogenesis (Lee et al, 2006). Evidence suggests an involvement of α -synuclein in apoptosis, autophagy, mitochondrial function and ROS-induced cellular damage. It is tempting to note that α -synuclein can be modified by nitrate stress, which increases its propensity to aggregate (Paxinou et al, 2001). Moreover, that a redox imbalance of oxidation and nitration (which is known to promote autophagy) is a prominent feature in brains of individuals with PD is supported by a number of findings (Lee et al, 2006). It has been also reported that recessively inherited forms of PD can be caused by loss-of-function mutations in genes encoding proteins that target to the mitochondria and mediate autophagy, including parkin (Valente et al, 2004).

Parkin can be inhibited by S-nitrosylation, which provides an important link to the generation of RNS prevalent in neurodegenerative disease (Yao et al, 2004).

The most impressive consequence of sphingolipid deregulation in PD is represented by anomalous sphingolipid-protein interactions that are at least, in part, responsible for the misfolding events that cause the aggregation of α -synuclein followed by the formation of fibrils (intracellularly accumulated in PD) (Piccinini et al, 2010). Of interest, the treatment with the monosialoganglioside GM1 had a beneficial effect restoring neurochemical, pharmacological, histological, and behavioral parameters in different animal models of PD and reversing the dopaminergic deficits in nigrostriatal neurons of aged rats, whereas trisialoganglioside GT1b, that is abundantly expressed in neurons, induced in vivo degeneration of nigral dopaminergic neurons in rats with a synuclein-independent mechanism (Piccinini et al, 2010). Targeting sphingolipid metabolism may thus represent today an underexploited but realistic opportunity to design novel therapeutic strategies for the intervention in this disease.

Huntington's disease

HD is an inherited disorder in which neurons in the striatum degenerate, resulting in uncontrolled body movements. HD is an autosomal dominant neurodegenerative disease that is caused by an expansion of the CAG trinucleotide repeat of the htt gene. This results in the expression of mutant htt with a long polyglutamine stretch at the N-terminus. HD is characterized by the presence of inclusion bodies composed of the mutant htt. Studies of patients with HD, and of rodents, indicate that impaired mitochondrial function and excitotoxic death may be central to the disease. In addition, the activation of an apoptotic program is implicated (Mattson, 2000). Also in this pathological setting the sphingolipid and NO signaling appear to play a role involving control of autophagy and apoptosis.

Several early studies suggested that altered sphingolipid metabolism is associated with HD (Piccinini et al, 2010). Noteworthy, a disrupted patterns of glycolipids (acidic and neutral lipids) and/or ganglioside levels was reported in both the forebrain of the R6/1 transgenic mice (a mouse

model of HD) and caudate samples from human HD subjects (Desplats et al, 2007). However, although R6/1 transgenic mice have severe cerebellar glycosphingolipid abnormalities that may account, in part, for their abnormal motor behavior, the same abnormalities were not found in the cerebellum of human HD subjects (Denny et al, 2010). The potential benefits of using gangliosides for treating the behavioral deficits associated with HD have also been described (Dunbar et al, 2006). In particular, the administration of GM1 restores ganglioside levels in HD cells and promotes activation of Akt and phosphorylation of mutant htt, leading to decreased mutant htt toxicity and increased survival of HD cells (Maglione et al, 2010). More recently, in vivo experiments demonstrated that intraventricular infusion of ganglioside GM1 induces phosphorylation of mutant huntingtin at specific serine amino acid residues leading to attenuated huntingtin toxicity, and restored motor function in already symptomatic HD mice (Di Pardo et al, 2012).

An increased autophagy, increased oxidative stress, and polyU aggregates in cultured striatal neurons from transgenic mice expressing mutant human huntingtin in response to a single exposure of a neurotoxic concentration of dopamine was indeed reported, suggesting that dopamine triggered free radical-mediated oxidation of macromolecules and stimulated autophagy (Petersen et al, 2001). Conversely, autophagy induction was also demonstrated as a protective mechanism in neurodegeneration in a drosophila model of HD, degrading both soluble and aggregated form of cytosolic mutant htt (Sarkar and Rubinsztein, 2008), suggesting that mutant huntingtin, unlike wt protein, is dependent on autophagy for its clearance. Recently it has been found out a strong correlation between autophagy and NO in HD. Treatment with NO inhibitor L-NAME prevented neurodegeneration in a fly model of HD (Sarkar et al, 2011), and also in a zebrafish model of HD, L-NAME had a positive effect on mutant huntingtin aggregates clearance (Sarkar et al, 2009). Consistent with this data, decreasing NO levels appear to have a protective effects in HD through multiple mechanisms. NO is involved in excitotoxicity resulting from NMDA receptor stimulation, indeed it is one of the mediators of this phenomenon (Gu et al, 2010); moreover NO

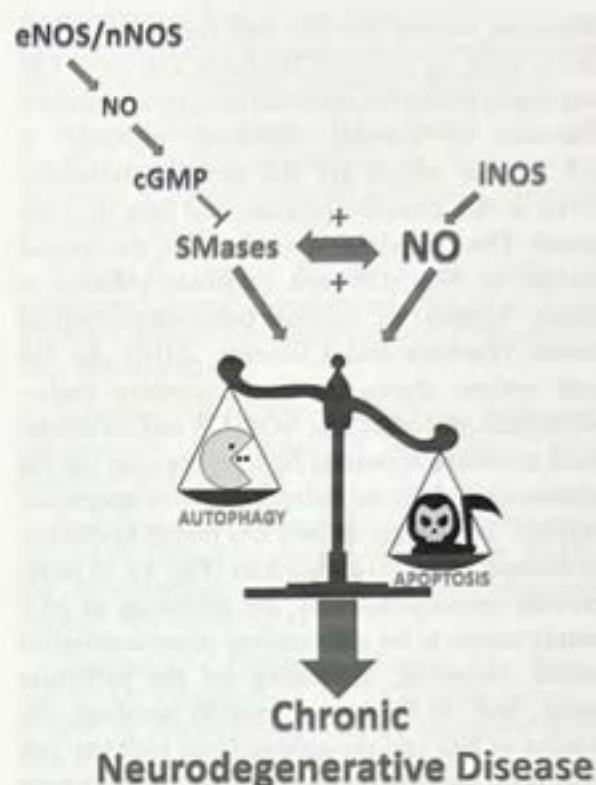


Fig. 1. The schematic diagram represents a pictorial view of the NO/NOS and ceramide/SMases crosstalk and its relevance in the regulation of the cellular balance between apoptosis/autophagy in neuronal cells. Depending on the balance between apoptosis or autophagy, pathological events concurring to the pathogenesis of neurodegenerative disorders may be promoted or inhibited.

is a potent inhibitor of autophagy, contributing to accumulation of aggregate-prone protein. Thereby NO inhibition may have beneficial effects in neurodegenerative disease, where nitrosative stress has a prominent role in the pathogenesis. Conversely NO, when derived from nNOS, could instead be important and necessary for cells. In support to this hypothesis are observations that nNOS is absent such as in models of amyotrophic lateral sclerosis and catabolic stress and this loss has been associated with pathogenic events in these pathologies (Suzuki et al, 2010; Finanger Hedderick et al, 2011).

CONCLUSIONS

Much progress has been made in understanding

the crosstalk among the NO and the sphingolipid pathway, with its multiple feedback controls. The system shares properties common to most modulatory mechanisms of cellular signaling, although it is still unclear which are the specific molecules involved in this complex process, and how they are regulated. The best characterized so far, the mutual regulation of NO/NOS and ceramide/SMases, is a tuning system in crucial patho-physiological processes (Perrotta and Clementi, 2010). As the present review shows, in such complex patho-physiological processes, the NO/NOS and ceramide/SMases crosstalk appear to be relevant also via the regulation of the cellular balance between apoptosis/autophagy, thus appearing as a key player in chronic brain neurodegenerative disorders (Fig. 1). In order to prevent neurocytotoxicity the inhibition of NO/ceramide seems to be a promising pharmacological approach. However, depending on the particular situation, and on the specific signal involved, the activation of NO and the sphingolipid pathway can be also good for brain cells thus eliciting responses to adapt to detrimental environmental situations. Definition of whether this integrated pathway balances towards beneficial vs. toxic effects appears to be more complex than expected, also because the elucidation in further detail on how the signaling processes involved are relevant for cell homeostasis is far from being understood. Resolving this issue, especially in response to specific neurodegenerative signals, will be an area of future focus that deserves attention also in therapeutic perspective.

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PROTEIN SILENCING WITH INTRACELLULAR ANTIBODIES: TARGETING ALZHEIMER'S DISEASE PROTEIN

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Despite intensive research, no generally accepted mechanism has yet been formulated causally linking the Alzheimer's disease (AD) triad (cholinergic deficit, amyloid A β and tau pathologies) into one unified conceptual scheme. A major current obstacle in the AD field is the lack of techniques to reliably validate targets relevant for the pathogenic mechanism. Indeed, a validated target is not just a well identified molecule. In order to be validated, and to become the object of a pharmacological intervention, targets need to be defined in their protein interactions, cellular context, post translational modifications, including quaternary structure and oligomerization state or conformers. This is true in general, for most human diseases, but even more so for neurodegenerative diseases. There is, therefore, the need for new approaches for target discovery and validation relevant for AD. Currently, much of the target discovery and validation arena exploits nucleic acid based approaches, such as transgenic approaches for gain of function studies, and gene knock-out or RNA interference for loss of function. The latter represent powerful technologies but, from the point of view of target validation, their predictive value is intrinsically limited, as they cannot access selectively the diversity of protein targets. This review article describes the so called intrabody technology (intracellular antibodies), whereby antibodies are used as genes, rather than as proteins, to achieve protein silencing in a spatially, temporally and molecularly defined manner. Antibodies represent a particularly promising class of reagents, because of their ability of potentially recognizing, in a highly specific manner, a virtually unlimited repertoire of protein antigens, including, for instance, the different pathological conformation intermediates of misfolding-prone proteins involved in neurodegenerative diseases or post-translationally modified proteins. The intrabody technology, which combines the molecular richness and selectivity of antibodies with the subtleties and power offered by gene transfer and precise subcellular targeting, shows a great potential for target validation in AD and other neurodegenerative diseases and promises to become a main weapon in the quest to find new treatments for these devastating diseases.

Despite an unprecedented research effort worldwide, in the past two decades, there are still no effective treatments in sight to prevent, halt or reverse Alzheimer's disease (AD) (Huang and Mucke, 2012; Selkoe, 2011), and the industry pipeline for drug development seems to provide frustrating and dismal prospects for the future. The current lack of accepted

biomarkers for an early diagnosis represents one major problem (Hampel et al, 2010 and Hampel, 2012). Thus, despite intensive research, no generally accepted mechanism has yet been formulated causally linking the AD triad (cholinergic deficit, amyloid A β and tau pathologies) into one unified conceptual scheme. Genetic studies of rare monogenic forms of

Key words: intracellular antibodies, Alzheimer's disease, protein silencing, neurodegenerative disorders, Amyloid beta

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the disease (early onset AD, EOAD) provided the main driving force in the debate for the mechanisms leading to AD neurodegeneration, and for bridging the knowledge from the genetic forms to the sporadic late onset forms of AD (LOAD). These studies formed the basis for a serial model of causality in AD, with elevation of A β as the prime pathogenic driver of AD, the "amyloid hypothesis" (Selkoe, 2002; Karran et al, 2011). The amyloid hypothesis has been the driving force in guiding pharmaceutical efforts towards the development of new treatments. Accordingly, pharmacological agents reducing brain A β levels should act as effective drugs for AD. However, it is noteworthy that, to date, the result of many clinical studies testing new amyloid-lowering treatments failed to deliver the expected results and have been largely disappointing (Golde et al, 2011). This gloomy picture calls for the need for a paradigm shift in the current views on the pathogenic mechanisms leading to AD, with the search for upstream drivers of the neurodegeneration cascade (such as neurotrophic deficits (Cattaneo et al, 2008) and the focus on the multifactorial basis of AD pathogenesis (Small and Duff, 2008; Capsoni et al, 2011, Huang and Mucke, 2012) being two main directions for investigation.

TARGET VALIDATION AND ALZHEIMER'S DISEASE

In general, a major current obstacle in the AD field is the lack of techniques to reliably validate targets that are indeed relevant for the pathogenic mechanism. Indeed, a validated target is not just a well identified molecule. In order to be validated, and to become the object of a pharmacological intervention, targets need to be defined in their protein interactions, cellular context, post translational modifications, including quaternary structure and oligomerization state or conformers. This is true in general, for most human diseases, but even more for neurodegenerative diseases. Even two targets whose relevance for AD is robust and unquestionable, namely the A β peptide and the microtubule associated protein tau, are far from representing unequivocally validated targets. A number of key questions in AD still need to receive convincing answers (Hampel, 2012), including: i) are any of the drug targets, currently considered

"validated", to be of clinical relevance? ii) do targets change over the disease course or a patient's lifespan? Indeed, a major obstacle in the field is the lack of techniques to reliably quantify the abundance and the assembly state of the soluble non fibrillar assemblies of A β such as dimers, trimers or larger oligomers (Benilova et al, 2012). Like A β , tau also can exist in different assembly states, but little is known about which forms of tau represent the most important target to hit.

Besides quaternary structure, the definition of a validated target needs to take into consideration a number of other properties and parameters, such as the cellular context and subcellular location, the network of protein interaction partners, the post translational modifications of the protein target itself etc. In the light of all these considerations, it is fair to say that despite intense research, the lack of full validation of targets for drug discovery and development has been one of the major difficulties the Alzheimer's field is facing.

There is, therefore, the need for new approaches for target discovery and validation, particularly in the Alzheimer's disease field. Currently, much of the target discovery and validation arena exploits nucleic acid based approaches, such as gene knock-out or RNA interference. Both approaches represent, undoubtedly, powerful technologies, however, it must be clear that, from the point of view of target validation, their predictive value is intrinsically limited. Indeed, nucleic acid-centered target validation approaches, such as gene knock-out or RNA interference, by definition, cannot capture the complexities of the protein-state diversity generated from a single gene or an individual mRNA species. Thus, the diversity of different protein isoforms, protein states, protein interactions, protein locations, or protein modifications, that can be achieved postrationally, from the same gene or mRNA, is huge. For this reason, unraveling the complexity of what really is a disease target and fully validating a disease target is a daunting task.

In this respect, antibodies represent a particularly promising class of reagents, because of their ability of potentially recognizing, in a highly specific manner, a virtually unlimited repertoire of antigens, including, for instance, a number of different pathological conformation intermediates of misfolding-prone

proteins involved in neurodegenerative diseases. For this reason, ever since Rita Levi-Montalcini's seminal immunosympathectomy experiment (Levi-Montalcini, 1964), which represents the first example of a knock out experiment (albeit a protein knock-out), antibodies have had a long and successful history as a tool to selectively interfere with the function of proteins in cells and in organisms and antibody technologies represent a major weapon in the set of target validation techniques. This article describes a technology, pioneered by our group, based on recombinant antibody domains, the so called intrabody technology (Intrabody stands for intracellular antibodies), whereby antibodies are used as genes, rather than as proteins. The intrabody technology allows to interfere with a protein target with a high spatio-temporal precision.

NEUROANTIBODIES: PROTEIN SILENCING WITH SECRETED RECOMBINANT ANTIBODIES IN TRANSGENIC MICE

Antibodies are normally used as binding proteins, for research, diagnostic and therapeutic purposes. Antigens targeted by antibodies are most often protein antigens, hence the diversity of antibodies is well matched to the huge diversity of the protein universe.

Our group pioneered the idea that antibodies can be used as genes, isolated from different sources (hybridoma cells secreting specific monoclonal antibodies or phage-display libraries of recombinant antibody domains) and ectopically expressed via gene-transfer techniques (reviewed in Cattaneo and Biocca, 1997). Depending on the localization of target protein of interest (extra- or intra-cellular), the antibody, suitably engineered, is expressed as a secreted or as an intracellular protein, targeted to different subcellular compartments.

Following the first demonstration that antibodies could be ectopically expressed in non-lymphoid cells, and secreted with particular efficiency by neuronal cells (Cattaneo and Neuberger, 1987), the concept of achieving a phenotypic knock-out in the nervous system (neuroantibody approach) by recombinant antibodies was demonstrated by targeting of the neurokinin substance P neuropeptide with a recombinant antibody expressed in the adult

brain of transgenic mice (Piccioli et al, 1995). The neuroantibody approach was instrumental to derive the AD11 mouse model, in which the postnatal expression of the anti NGF recombinant antibody α D11 in transgenic mice determines a progressive AD-related neurodegeneration, characterized by cholinergic deficit, tau and amyloid related pathology, and synaptic plasticity and behavioural deficits (Capsoni et al, 2000; 2011). This antibody-based transgenic model has been instrumental to validate the NGF/NGF receptor system as a target for Alzheimer's disease, located upstream of the Alzheimer's Ab and tau endpoints, in the neurodegeneration cascade. More specifically, the α D11 antiNGF antibody binds mature NGF with an affinity 2000-fold higher than proNGF. Thus, the α D11 antiNGF antibody leads to an effective and selective neutralization of mature NGF in the mouse brain, while leaving its unprocessed form proNGF free to act (Cattaneo et al, 2008; Covaceuszach et al, 2008), experimentally creating a proNGF to NGF imbalance. This has allowed to validate proNGF/NGF dysequilibrium as an upstream driver for Alzheimer's neurodegeneration and as a target for the design of therapies aimed at re-establishing the proNGF/NGF balance and the neurotrophic equilibrium (Cattaneo et al, 2008). The causal links between neurotrophic signalling imbalance and Alzheimer's neurodegeneration has been confirmed in a transgenic mouse expressing the neutralizing mAb MNAC13 anti TrkA antibody, which recapitulates the neurodegenerative phenotype of the AD11 model (Capsoni et al, 2010). From the experimental point of view, the selective binding properties of the anti NGF antibody, carefully characterized from the biochemical and biophysical point of view (Covaceuszach et al, 2008), have been crucial to achieve a highly selective protein interference, specifically targeting proNGF versus mature NGF, that would not have been possible with gene- or mRNA-based approaches.

In the context of another neurodegenerative disease, this antibody protein silencing approach, based on the expression in transgenic mice of the genes coding for an antibody directed to a protein antigen of interest, was exploited for immunological inhibition of prion disease *in vivo* (Heppner et al, 2001). Expression of anti-prion protein antibodies

in transgenic mice prevented pathogenesis of prions introduced by intraperitoneal injections in spleen or brain, demonstrating the feasibility of immunological inhibition of prion disease *in vivo*, and validating passive immunization therapeutic approaches for prion diseases.

These considerations highlight the potential of using transgenic expression of secreted forms of antibody genes in transgenic mice for effective and highly specific protein interference studies.

THE INTRACELLULAR ANTIBODY (INTRABODY) APPROACH

Having established that antibodies could be ectopically expressed, as secreted proteins (Cattaneo and Neuberger, 1987), we extended the "antibody protein silencing" concept to the intracellular targeting of antibodies to different compartments of mammalian cells (Biocca et al, 1990; Biocca and Cattaneo, 1995). The intracellular antibody (intrabody) approach is a gene-based strategy that relies on the expression of recombinant antibodies (or antibody domains) directed to subcellular compartments, to block or modulate the function of target molecules. Thus, by exploiting targeting sequences that normally direct the subcellular localization of proteins inside the cell, antibodies have been targeted to a number of cellular compartments, including the endoplasmic reticulum, Golgi, plasma membrane, cytoplasmic face of the membrane, nucleus, mitochondria (Biocca and Cattaneo, 1995). The antigen-recognition portion of an antibody is mediated by its Variable (V) regions. A full immunoglobulin, made of two heavy and two light chains, linked by inter-chain disulphide bonds, is not practical in the reducing environment of the cell cytoplasm or nucleus. Also, the effector functions, carried by the Fc portions of immunoglobulins, are not required, nor useful, inside the cell. For this reason, the fine specificity for protein recognition afforded by the antibody combining site, comprising three complementarity determining regions (CDRs) on each variable region has led to antibody fragments being employed for intracellular use, based on variable V regions only (for a review see Cattaneo and Biocca, 1997). The most widely used intracellular antibody fragment is

the single chain Fv format (scFv, single chain variable fragment), consisting of a heavy chain (VH) and a light chain (VL) variable region linked by a flexible linker peptide. One clear advantage of the scFv is that it is a single polypeptide and can be expressed *in vivo* from a single vector. An even simpler format is the single V region domain (domain antibody or Dab), made of an isolated VH or VL domain. These minimal recognition units do not require invariant intradomain disulphide bond formation for protein folding and stability (Tanaka and Rabbitts, 2008).

In the two decades following the first description of the use of intrabodies in mammalian cells (Biocca et al, 1990), and following the initial proof of concept functional studies by us and others (Biocca et al, 1993; 1994; Marasco et al, 1993; Tavladoraki et al, 1993,) several examples of intracellular antibodies effectively inhibiting the function of intracellular targets have been published (recent reviews in Lobato and Rabbitts, 2003; Miller and Messer, 2005; Lo et al, 2008), mostly, but not exclusively, related to the fields of cancer, viral and neurodegenerative diseases. From these studies, it can be concluded that intrabodies can provide very effective inhibition of protein function, in widely diverse cellular contexts subcellular compartments and intracellular processes (signalling or transcription pathways, protein trafficking, viral assembly and replication).

Intrabody studies have been performed mostly in cultured cells, but their effectiveness *in vivo*, after delivery with viral vectors, or in transgenic animals, has also been demonstrated. Thus, a single domain antibody specifically recognizing GTP-bound RAS, neutralizing its oncogenic effect in human cancerous cells, was expressed in developing mouse lungs of transgenic mice, without detectable changes to lung structure and function, but with effective suppression of RAS-dependent lung tumors (Tanaka et al, 2007; Tanaka and Rabbitts, 2010).

Thus, intracellular antibody fragments exploit the virtually unlimited diversity repertoire of antibodies to target proteins inside cells and achieve effective protein silencing. Compared to RNA based interference methods, such as antisense oligonucleotides and short interfering RNA (siRNA), intrabodies can, in principle, address the diversity of the protein space, including quaternary states and misfolding states of a given protein, which RNA

targeting methods cannot. Moreover, intrabodies can target proteins in a subcellular compartment while not affecting the pool in another compartment, a property which can be very useful in highly polarized cells such as neurons. Finally, intrabodies appear to be a versatile and general method to interfere with intracellular protein networks, as discussed below. In conclusion, intrabodies can mediate effective protein silencing, addressing questions that gene- or mRNA-targeting approaches cannot deal with.

USER-FRIENDLY LIBRARIES TO ISOLATE FUNCTIONAL INTRABODIES

The theoretical and practical advantages of the intrabody approach, have been somewhat offset, in the initial development stages of the technology, by the fact that the isolation of functional intrabodies was, initially, somewhat laborious and prone to failure. New methods have now been developed, that allow the fast, effective and user-friendly isolation of functional intrabodies, greatly reducing the time and labour required.

Initially, intracellular antibodies were derived from hybridomas (Winter and Milstein, 1991) by a labour intensive cloning of the antibody VH and VL domains into the scFv format. With the advent of phage-display technology (McCafferty et al, 1990; Hoogenboom, 2005), intrabodies were derived from these highly diverse antibody domain libraries. When displayed on phage, antibodies are folded in the periplasmic space of *E. coli* cells, which is oxidizing, similarly to the secretory pathway of mammalian cells. However, the intracellular expression requires that antibody domains are stable enough and fold properly as functional proteins in the reducing environment of the cytoplasm and nucleus. Indeed, all antibody domains contain two universally conserved disulphide linked cysteine residues, which provide folding stability. This intrachain disulphide bond cannot usually form in a reducing environment (Biocca et al, 1995). Most antibody domains do not tolerate the absence of this bond and, as a consequence, cannot fold in the cell cytoplasm, and will not work as intrabodies. Yet, some antibodies, that are intrinsically more stable, fold even without the additional stability contribution by this intrachain disulfide bond. These are therefore the antibodies

that have the ideal folding and stability properties to function as intrabodies. In the attempt to enrich for stable antibodies, selection strategies have been developed. In particular, phage display libraries have been generated based on a single framework derived from a stable intrabody, or optimized for intracellular expression (Philibert et al, 2007). Conversely ribosome display antibody libraries have been used for isolating antibody domains that are stable under reducing conditions (Contreras-Martinez and DeLisa, 2007). However, the diversity of these ad hoc libraries is anecdotal and their generality not proven. A breakthrough for the isolation of functional intrabodies came from schemes whereby antibodies are selected on the basis of their ability to bind antigen *in vivo* (Visintin et al, 1999 and 2002). The two hybrid method (Fields and Song, 1989) for protein-protein interactions was adapted to the selection of intracellular antibodies binding a given protein antigen, resulting in the selection of functional antigen binding scFv intrabody fragments (Visintin et al, 1999 and 2002; Tse et al, 2002) (IAC or Intracellular antibody capture technology). The initial IAC method required a first round of selection of scFv from phage display antibody libraries (Visintin et al, 2002; Tse et al, 2002), but was superseded by methods allowing the direct library screening in yeast cells, expressing synthetic scFv libraries made from intracellular stable consensus scFv frameworks (Visintin et al, 2002; Tanaka and Rabbitts, 2003), from natural immunoglobulins (Visintin et al, 2004) or from immunized mice (Meli et al, 2009). These "single-pot libraries of intrabodies" (SPLINT) (Visintin et al, 2004) allow direct-in-cell screening and since the interaction between antibody member of the library and the antigen-bait occurs in the reducing conditions of the cell cytoplasm, the selected antibody binders are guaranteed to be functional intrabodies, when expressed in the relevant cellular system (Fig. 1). Thus, SPLINT libraries provide the ideal and accessible resource for functional studies with intrabodies, circumventing the tedious and laborious trial-and-error process, necessary when isolating intracellular antibodies from hybridomas or phage display libraries. The advent of SPLINT libraries has greatly facilitated the selection of antibody fragments for downstream use as intrabodies in functional studies, providing a user-

friendly and robust source of stable antibodies. A large number of antibodies against a diverse set of protein antigens have been derived from direct screening of SPLINT/IAC libraries and successfully used for functional studies in mammalian cells, including the Alzheimers proteins microtubule associated protein tau (Visintin et al, 2002), and Amyloid β peptide (Meli et al, 2009), the proNGF precursor of NGF (Paoletti et al, 2012), the synaptic protein gephyrin (Zacchi et al, 2008) and the cancer related proteins RAS (Tanaka and Rabbitts, 2003) and transcription factor LMO2 (Nam et al 2008). A major advantage of SPLINT as a source of intracellular antibodies is that the only requirement is the cDNA for the target antigen. Thus, isolating antibodies from SPLINT libraries is the only procedure allowing the direct isolation of antibodies directly from gene sequences, with no manipulation whatsoever of the protein antigen. This represents a significant saving of time and effort, allowing to streamline the isolation of intrabodies for large scale proteomic studies, scaling up antibody isolation to a high throughput,

overcoming the severe protein-expression bottleneck (Duebel et al, 2010). An additional advantage of SPLINT selections is that isolated antibodies, when expressed as secreted proteins and allowed to form their intrachain disulphide bond, have an additional stability bonus and represent therefore superior quality antibodies. For these reasons, SPLINT /IAC have the potential of becoming the best and more convenient source of antibodies in the future. For intrabody selection, SPLINT/IAC is the only real option available (Fig 1).

TARGETING THE INTERACTOME WITH INTRABODIES

Cells are complex webs of macromolecular interactions and systems biology experimental approaches are generating data on global protein-protein interaction maps (Vidal et al, 2011). The collection of all protein interactions of a cell is defined as its "interactome". The interactome and the cell-specific protein networks are key elements of normal

3HY-SPLINT: targeting protein-protein interactions

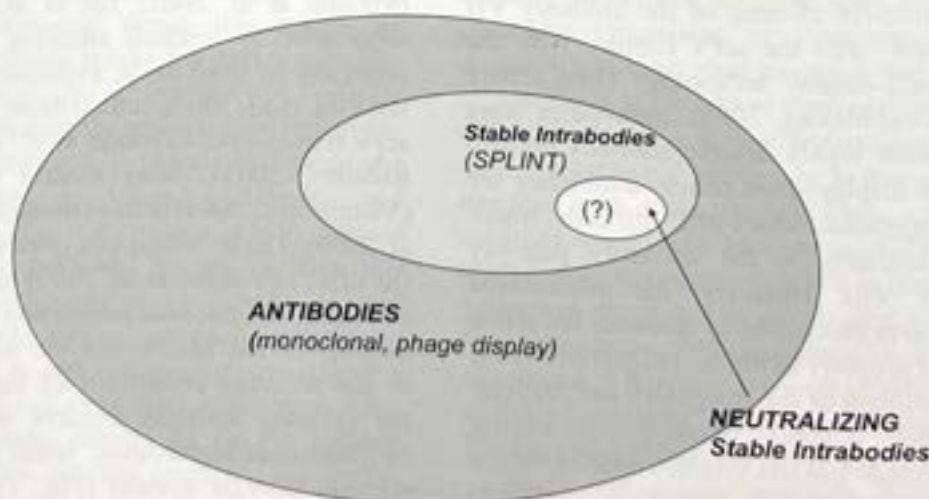


Fig. 1 The universe of stable antibodies, which tolerate the absence of the intrachain disulphide bond, and hence are functional intrabodies, is a subset of all natural antibodies. This subset can be isolated and selected from the SPLINT libraries. SPLINT libraries provide antibodies that bind a given target antigen bait, but do not ensure that the antibody will be neutralizing. In the SPLINT format, neutralization is a property that can be verified a posteriori, after the selection. Intrinsically neutralizing, stable antibodies are a subset of SPLINT antibodies. An important class of intrinsically neutralizing intrabodies is represented by those intrabodies that inhibit protein-protein interactions. In order to provide a direct selection for intrabodies targeting protein-protein interaction domains, 3HY-SPLINT libraries have been engineered (see Fig. 2).

cell function and of disease states. Any given protein is inserted as a "node" in the cellular protein network, and its interactions are the "edges". A different state of a given protein (a different folding, a post translationally modified form(s), etc.) is a different node. This is why defining any given protein as a disease target, even if validated by human genetics, can be grossly oversimplifying. Disease states arise from perturbations of cellular interactome networks. These alterations can range from the complete loss of a gene product (equivalent to "node" removal in the network, with loss of all its interactions), through the loss of some but not all the interactions, to the specific perturbation of a single molecular interaction, while retaining all others ("edge"-specific perturbation). The consequences on cellular network function are expected to be radically dissimilar, for node removal, versus edge-specific (or "edgetic") perturbations (Fig 2). Node removal not only disables the function of a node, but also disables all the interactions of that node with other nodes, disrupting the function of all of the neighbouring nodes. An edgetic disruption, removing one or a few interactions, but leaving the rest intact and functioning, has subtler effects on the network and on the resulting phenotype (Fig 2). The distinction between node removal and edgetic perturbation provides important clues on mechanisms underlying human disease. This is particular true for misfolding proteins, whose different folding states can be engaged in entirely different sets of interactions. From the point of view of target validation techniques, the distinction between nodes and edge removal is even more important. Indeed, nucleic acid based approaches (gene knock-out or RNA interference) are typically node-removal approaches. Target validation has relied heavily on these node-interfering techniques, also because no general technique was readily available to specifically interfere with edges in a protein network of interest. The lack of such techniques is also the reason why experimental models for many human diseases are still very poor mimics of the disease process (and Alzheimer's disease mouse models are certainly no exception (Zahs and Ashe, 2010)). Given that the disruption of specific protein interactions can be the molecular basis for many human diseases, it is clear that there is the need for experimental approaches tailored for edgetic perturbations.

In principle, intracellular antibodies might indeed

provide such an edge-perturbing platform, and individual cases of intrabody silencing do indeed demonstrate inhibition of protein-protein interactions as the key mechanism of action (Tanaka et al, 2007), an important development has been the design of approaches to accelerate the specific isolation of antibodies directed against protein interaction sites (Visintin et al, 2008). In one approach, intrabody libraries were first screened with a target antigen that has known protein interaction partners and the resultant antigen-specific antibody domains were, subsequently and downstream, individually assessed in a three-hybrid competition assay (Triplex assay) (Tanaka and Rabbitts, 2010). In a more direct and general approach (Fig 3), scFv libraries of intracellular antibodies were screened directly *in vivo* to select those that could block the interaction of a target protein with a binding partner (Visintin et al, 2008). In the so-called 3-SPLINT approach (Fig 3), the interacting protein-protein pair is expressed in yeast cells, respectively fused to a DNA-binding (DBD-A) and an Activation- domain (AD-B) of the two hybrid transactivator, controlling the expression of a tetracycline repressor gene controlling the HIS3 gene. When the tetracycline repressor is activated, by the interaction between protein antigens A and B, it binds to TET operator and suppresses the transcription of the HIS3 gene, preventing yeast from growing in the absence of histidine. If these yeast cells are transformed with a scFv library, as a third partner, and scFv are present that bind either protein partners A or B, blocking their interaction, the production of tetracycline repressor is stopped and the HIS3 gene will be expressed, allowing yeast to grow in the absence of histidine and selection of the cells carrying the specific scFv.

The 3-SPLINT platform allows the direct selection of intrinsically neutralizing intrabodies, targeting specific protein-protein interactions, and opens an enormous potential for a pipeline of drug target validation of great therapeutic importance.

MODES OF ACTION OF INTRABODIES: ADDING EFFECTOR FUNCTIONS

Normally, antibodies carry effector functions, coupled to antigen binding, through their constant regions (e.g. complement fixation). Intracellular

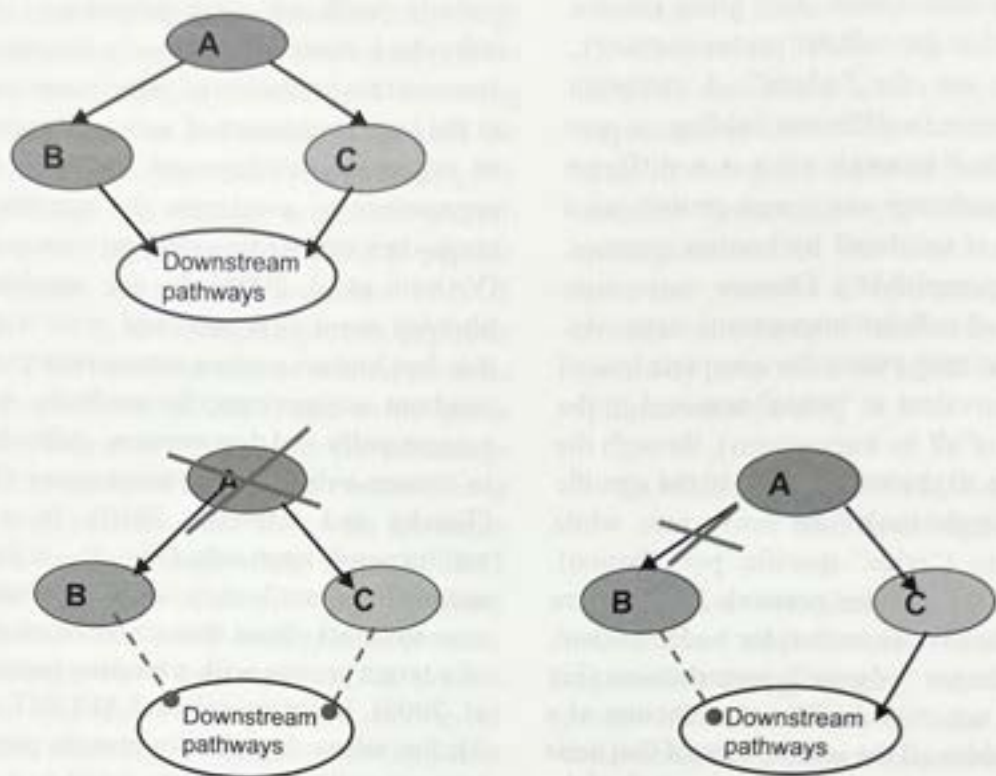


Fig. 2 Edgetic perturbation versus node removal. Schematic illustration of distinct outcomes in a protein network from complete loss of gene product (node removal) versus perturbation of specific molecular interactions (edgetic perturbation). Intrabodies allow edgetic perturbations, while gene- or mRNA- centered silencing approaches determine node removal.

3-SPLINT screen

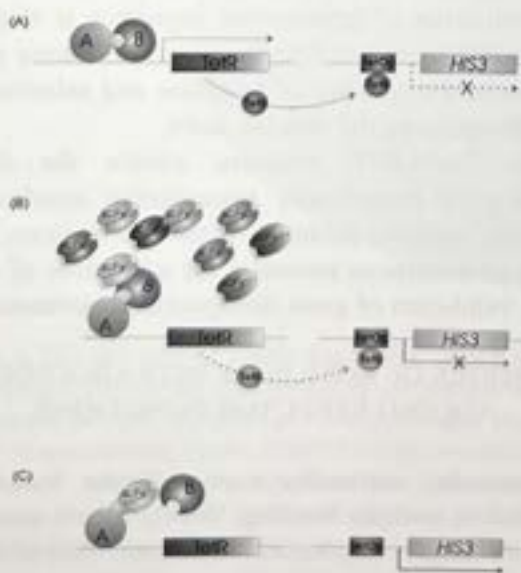


Fig. 3. 3-SPLINT screen, a direct method of library screening, for neutralizing intracellular antibodies. A) In this approach, a complex of two interacting proteins A and B is expressed in yeast, as DNA binding domain-protein A (A) and Activation domain-protein B (B) directing the transcription of a tetracycline repressor gene controlling the HIS3 gene. When the tetracycline repressor is activated by interaction of proteins A and B, it binds to the TET operator and suppresses activation of the HIS3 gene, preventing yeast from growing in the absence of histidine. B) If these yeast are transformed with a scFv SPLINT library and some scFv in the library bind either protein A or protein B without inhibiting their interaction, the TetR will be expressed, HIS3 gene will not be expressed and no scFv will be selected. C) If these yeast are transformed with a scFv SPLINT library and scFv are present that bind to either of the protein partners A or B, blocking their interaction, the production of tetracycline repressor (TetR) will be stopped and the HIS3 gene will be expressed allowing growth in the absence of histidine and selection of the yeast carrying the scFv neutralizing the interaction between protein A and protein B.

antibodies do not require such immune effector functions, and, also for this reason, do not carry constant immunoglobulin regions. Past work with chimaeric antibodies (Neuberger et al, 1985) showed that linking Variable V regions to other protein entities can produce hybrid molecules that specifically bind to target proteins and can carry other payloads.

The mode of action of an intrabody, upon binding to its target protein in the cell, may be any of several possibilities (reviewed in Cattaneo and Biocca, 1997). The intrabody may be intrinsically neutralizing, such as for instance if it binds the active site of an enzyme, or it may act as a retargeting agent, that redirects the target away from the subcellular compartment where it is acting. For membrane or secreted proteins, intrabodies equipped with a SEKDEL C-terminal sequence can act as intracellular anchoring agents, sequestering the target protein in the endoplasmic reticulum.

Besides targeting sequences for different subcellular compartments, effector functions have been added to the antigen binding variable domains, that either cause the induction of cell death upon antigen binding (antibody-antigen interaction-dependent apoptosis (AIDA)) (Tse and Rabbitts, 2000), or proteolysis of the target protein (suicide (or silencing) intrabody technology (SIT) (Melchionna and Cattaneo, 2007)). The AIDA strategy is based on the fusion of pro-caspase to a single domain intrabody and its proximity-induced dimerization and activation. Dual targeting of two proximal antigenic epitopes (such as may occur on two interacting proteins, or on an intracellular fusion protein resulting from a chromosomal translocation) with two antibody fragments linked to pro-caspase will result in proximity induced dimerization of pro-caspase and self-activation of caspase through proteolysis and apoptosis induction (Tse and Rabbitts, 2000). This approach might be particularly useful with fusion oncogenes, such as those occurring in cancer, or with oligomeric antigens, such as those occurring in many neurodegenerative diseases.

A second strategy for adding effector functions to intracellular antibodies was aimed at achieving an intrabody-mediated protein degradation (Fig 4). Proteolysis is a regulated activity in the cell, carried out through the ubiquitin/proteasome pathway. In the SIT approach (silencing intrabody technology),

we harness the cellular machinery and signalling that regulates proteolysis to mediate the degradation of cellular proteins, upon intrabody binding. An antigen-specific intracellular antibody is expressed in cells as a fusion with a known ubiquitin-proteasome pathway substrate, activated by a ligand-receptor pair. The intracellularly expressed scFv is non neutralizing, so that in the absence of the ligand, the scFv is long-lived, and the function of the protein is not inhibited. Upon activation of the degradation pathway by the extracellular ligand, the complex between the intracellular antibody and the antigen will be recruited to the E2/E3 ubiquitination complex via the specific substrate-binding domain of a member of the complex (e.g F-box protein). Once ubiquitinated, the intrabody-antigen complex is degraded by proteasome (Fig 4). Among the antigens tested in this work is the microtubule associated protein tau, which was thus silenced in 15 minutes in a conditional way, upon addition of the TNF ligand to the cells. The possibility of effectively silencing the tau protein in a conditional way in neurons, provides an important and innovative experimental tool, in combination with the possibility of targeting the amyloid beta oligomers (see below), to dissect the causal relationships between these crucial players of the Alzheimer's neurodegeneration process.

This protein switch for degradation provides a unique tool for rapid and reversible protein silencing on a fast time scale, something that cannot be achieved on such a time scale with RNA interference methods, that require much longer times (24-36 hours for knock-down).

The SIT strategy was initially demonstrated for antigens located in the cytosol. A similar silencing strategy can be also applied to the specific degradation of proteins located in the secretory pathway, by exploiting the endoplasmic reticulum associated degradation (ERAD) pathway. ERAD is a cellular quality control mechanism to dispose of misfolded proteins of the secretory pathway via proteasomal degradation. ER resident proteins identify misfolded proteins in the ER as ERAD substrates, inducing their ER-to-cytosol retro-translocation and degradation. By fusing a target-specific intrabody in the luminal side of the ER to proteins involved in the ERAD pathway, it will be possible to promote intrabody-mediated degradation also of proteins in

Protein silencing with intrabodies targeted to degradation

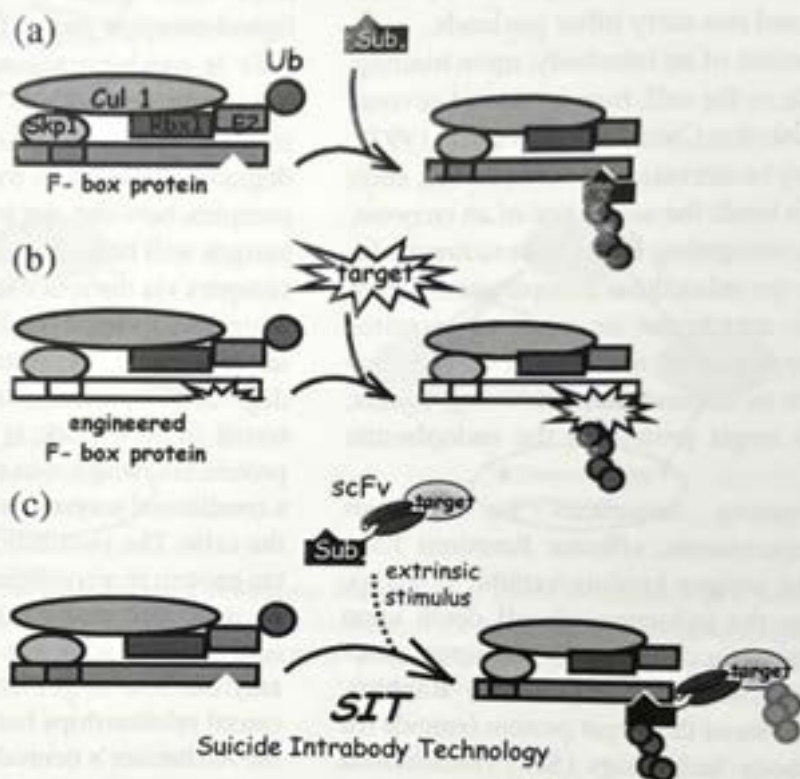


Fig. 4 *SIT: suicide (or silencing) intrabody technology.* Protein silencing with intrabodies targeted to degradation can be achieved by engineering an antigen-specific intracellular antibody as a fusion with a known ubiquitin-proteasome pathway substrate, activated by an extracellular ligand through interaction with a membrane receptor (TNF and TNF receptor ligand-receptor pair). *A)* Normally, cellular protein substrates for degradation are recognized by a binding domain on one F-box protein, that recruits the substrate for degradation *B)* Targeted degradation can in principle be achieved by modifying an F-box protein, so that its substrate recognition domain is suitably modified. This requires, however, to tailor specific F-box proteins for each target of interest *C)* The SIT approach relies on fusing an intrabody against a target cellular protein of interest to a naturally short lived protein, substrate for degradation (even a substrate for regulated, ligand-dependent, degradation). The intrabody-antigen complex is then recognized by the ubiquitin-proteasome system, escorting the antigen to degradation.

the secretory pathway.

INTRABODY APPLICATIONS IN NEURODEGENERATIVE DISORDERS

In several neurological disorders, specific proteins can accumulate within cells as a result of changes in protein conformation (misfolding) that render the molecules prone to self-aggregation and resistant to clearance. These conformational diseases

are marked by the build-up of characteristic proteins in the brain, such as the Amyloid beta ($A\beta$) peptide and tau in Alzheimer's disease (AD), huntingtin (HTT) in Huntington's disease (HD), α -synuclein in Parkinson's disease (PD), and the PrP in prion diseases. Targeting these proteins selectively, in their pathology-related conformations, while sparing the non-pathological conformations, is a scientific and therapeutic objective whose realization would represent a true breakthrough. Antibodies represent

the class of molecules of choice, to this aim, and their expression *in vivo*, in cells or the nervous tissue, provides a unique opportunity. For this reason, the intrabody approach is emerging as a very competitive and rather unique experimental platform to target selectively neurological disease proteins and to provide tools to understand disease mechanisms and validate targets for drug discovery.

Several studies support the use of intrabodies with the aim of targeting epitopes of all the above mentioned neurological disease proteins (recently reviewed by Messer et al, 2009; Zhou and Przedborski, 2009). Here we briefly summarize some intrabody-based targeting applications against HTT in HD, and against APP/A β in AD.

A variety of recombinant antibodies against the translation product of HTT exon 1 have been derived, based on the fact that expression of only HTT exon 1 with an expanded polyQ stretch (mHTT exon 1) is sufficient to cause HD-like pathology in several models of HD. Intrabodies, derived from different sources, have been directed toward 3 separate regions of HTT exon 1: i. the N-17 aminoacids which form a highly conserved amphipathic alpha helix; ii. the polyQ tract, which is the site of HD mutation; iii. the proline rich region that is C-terminal to the polyQ. The intrabodies targeting protein domains adjacent to the polyQ may alter the context for the misfolding, blocking post-translational modifications or changing the folding dynamics. Different intrabodies either counteract *in situ* length-dependent HTT aggregation (Lecerf et al, 2001) or increase its turnover and reduce neurotoxicity in cellular models (Southwell et al, 2008), but depending on the bound epitopes, the cellular system or other parameters, some intrabodies may accelerate cell death and aggregation, when assayed in similar HD cell culture models (Khoshnan et al, 2002), confirming the complexity of the regulation and the need for subtle, selective and finely tuned targeting systems.

Several intrabodies that bind on either side of the expanded polyglutamine tract of mutant HTT have been reported to improve the mutant phenotype in cell and organotypic cultures, in *Drosophila* fruit flies, and in mice (reviewed in Messer et al, 2009 and in Butler et al, 2012).

Altogether, these studies have shown that conditions can be found to achieve effective inhibition of the toxic and detrimental effects of

mutant huntingtin, in some cases with selective binding of the mutant versus wild type protein, and are teaching us the influence of antibody properties (epitope specificity, stability, *in vivo* half-life, cellular targeting etc.) on the final outcome. However, these studies are also showing the complexity of the neurodegeneration process triggered by mHTT and the need for a fine regulation and tuning of the interfering system with intrabodies. In any event, a mutant HTT-selective protein interference would be much harder with approaches targeting HTT mRNA.

Among the intrabody studies in AD research, both the A β peptide and its precursor (APP) have been targeted with intracellular antibodies. There is indeed a great need to understand the cell biology and trafficking of APP, in relation to the cellular site(s) and timing of its processing to A β (Lichtenthaler et al, 2012; Sannerud and Annaert, 2009), and its oligomerization. Indeed, while A β oligomers (A β O) have recently been recognized as the main toxic A β assemblies in AD, almost nothing is known about the cellular sites and mechanisms of the oligomerization of A β (Benilova et al, 2012). This is largely because no convincing A β O-specific probe has been generated yet, selectively recognizing specific, biologically relevant oligomeric forms of A β , with respect to A β monomeric or fibrillar forms. For this reason, we have decided to focus our efforts on the generation of A β O-specific recombinant antibody domains, to be used as intrabodies to selectively target A β O s in different cellular compartments of neurons.

A β is generated by a complex proteolytic processing of β -amyloid precursor protein (APP), through sequential cleavages by β -secretase and γ -secretase. Of note, APP is an extremely complex protein, functionally important in its full-length configuration, as well as being the source of numerous fragments with varying effects on neural function. The subcellular traffic and localization of APP biosynthesis and processing in neurons is a crucial aspect of its (mis)regulation, and its study requires specific cell biology methods, coupled to specific molecular probes. Thus, intrabody-based interference selective for A β or some of its pathological assemblies should be an extremely powerful approach.

Paganetti et al (2005) generated intrabodies directed to the the β -secretase cleavage site of human APP.

Intracellular expression of scFv intrabody along the secretory pathway of human embryonic kidney cells shields the β -secretase cleavage site and inhibits the formation of toxic A β . The KDEL version of the same intrabody is more effective because it retains APP in the ER, preventing its appearance on the plasma membrane. This study shows how intrabodies targeting a specific site on APP, perturbing its traffic and its processing, can be used to modulate the formation of the A β processing product.

An independent targeting of APP processing was obtained by the expression of an anti-nicastrin scFv intrabody; this abolished the proteolytic activity on APP, by the destabilization of the γ -secretase complex and the inappropriate glycosylation of nicastrin (Hayashi et al, 2009).

These studies show that targeting the APP substrate complex with intrabodies can be used to modulate its processing along the amyloidogenic pathway, but do not tell us how to interfere directly with A β O s , the toxic forms of A β .

In vivo intrabody approaches directly targeting A β (either its intracellular- or the extracellular pool) with antibody domains have also been reported. Several groups have recently tested a gene therapy modality, where adeno-associated virus (AAV) encoding secretory (Ryan et al, 2010; Fukuchi et al, 2006; Levites et al, 2006) or ER-retained (Desai et al, 2010; Sudol et al, 2009) anti-A β scFvs were intracranially injected in AD mouse models. AD mouse models subjected to AAV injection showed a reduced amyloid pathology. However, these studies do not use conformational and oligomeric-specific scFvs and the therapeutic mechanisms by which scFvs act *in vivo* are completely unknown and not addressed (e.g. if acting or not through APP processing interference). Indeed, it must be underlined that most anti-A β antibodies also recognize the A β peptide in the context of APP, and the studies need to be interpreted taking this APP binding into account, unless specifically addressed, which is rarely the case.

DIRECT INTRACELLULAR SELECTION OF CONFORMATION-SENSITIVE ANTIBODY DOMAINS TARGETING ALZHEIMER'S AMYLOID BETA OLIGOMERS

A β oligomers (A β O s), are considered the

most synaptotoxic A β species linked to the AD pathogenesis. Although increasing evidence supports the role of intracellular A β oligomerization and accumulation, as an early event in AD pathogenesis (LaFerla et al, 2007), little is known about the intracellular processing and trafficking events of the different forms of A β O s . Targeting the pathological assemblies of A β with specific probes, for mechanistic studies, for intracellular imaging or for therapeutic purposes, is therefore very important. Moreover, the intracellular targeting of A β O s would require the availability of antibody domains suitable for intracellular expression.

We recently generated a large panel of anti-A β O s recombinant scFv antibodies (Meli et al, 2009), exploiting the "Intracellular Antibody Capture Technology" (IACT). Their direct availability for intra- or extra-cellular "genetic delivery" make them ideally suited for new experimental approaches for studying and imaging the intracellular processing and trafficking of A β oligomers.

The selected anti-A β O s scFvs show unique properties in terms of sequence, epitope recognition, conformational selectivity for A β oligomers *in vitro*, immunoreactivity towards naturally-produced A β deposits in AD brains, inhibition of synaptic binding of A β oligomers (ADDLs) and neutralization of their-induced cyto-toxicity (Meli et al, 2009).

A human A β 1-42 bait was the target antigen chosen, to challenge two SPLINT (Single Pot Library of Intracellular Antibodies) antibody domain libraries: a naïve SPLINT library, derived from non immune repertoires of natural variable (V) regions of immunoglobulins (Visintin et al, 2004), and an A β 1-42 immune SPLINT library, derived from V regions isolated from A β 1-42-immunized mice.

Interestingly, antibody domains derived from the naïve and immune libraries showed complementary epitope recognition patterns, with a large percentage of immune SPLINT antibody domains (>90% of total) recognizing the N-terminus of A β . This result reproduces what was found for the immunization protocols performed in humans and in animals (Schenk et al, 2004), suggesting that A β immunization provides a "directed evolution" of V regions, biasing their binding specificity toward N-terminal A β residues. IACT selections preserve such a bias.

The sequence analysis confirm high similarities between scFvs selected from the immune SPLINT. Thus, SPLINT selection is able to capture the mechanism of oligoclonal response of the immune system to the A β antigen, but it is also able to select new VL/VH pairings, on the basis of its own selection for A β binding.

It was quite unexpected to see the large proportion of anti-A β scFvs selected from SPLINT libraries showing conformation sensitivity, with a preferential binding ability versus A β oligomers. It is likely that the A β conformation sensitivity of the antibody domains was favored by the intracellular selection and binding conditions. The most straightforward explanation would be that the A β bait displays, in yeast, a conformation that mimics that one found in pathological A β assemblies.

The panel of anti-A β O antibody domains selected has rather unique properties, displaying both conformational-sensitivity and sequence/epitope specificity, a property which is the reason for their specificity and potency in immunostaining and neutralization assays in cells.

The conformation specificity of anti-A β antibodies is most often not associated with sequence specificity for the epitope recognized on A β (O'Nuallain and Wetzel, 2002; Kaye et al, 2003; 2007) and the coexistence of conformation sensitivity, together with sequence specificity is a relatively rare property of anti-A β antibodies. For immunotherapy applications, the sequence specificity of anti-A β antibodies, is an essential property to be considered, besides their conformation specificity, due to *in vivo* mechanistic and safety reasons. Indeed, the A β O scFvs were mentioned as potential tool for new generation A β immunotherapies to overcome safety and efficacy concerns associated with the current approaches (Lemere and Masliah, 2010). Moreover, as intrabody domains the anti-A β O scFvs are intrinsically suited for intracellular expression and targeting, allowing new experimental strategies of imaging and selective functional knock-down also in AD animal models.

We are currently exploiting the intrabody approach to dissect the cellular pathways leading to Alzheimer's A β O formation and actions in cellular models, by using conformational anti-A β O scFvs as intracellular antibodies (intrabodies). Remarkably, the anti-A β O scFvs show the peculiar conformation

selectivity for A β O even when expressed as intrabodies (Meli et al, 2011), essential prerequisite for *in vivo* applications. This provides the unique opportunity to study the detailed genesis and traffic of A β O in living neurons.

CONCLUSIONS

Protein silencing with subcellular precise targeting of recombinant antibody domains is emerging as a powerful technology that can help filling the gap of target validation in the field of AD and other neurodegenerative diseases. A number of crucial questions, in search of adequate answers, are posing serious problems to the development of disease modifying therapies for AD: what is a validated target for drug development? when, in the disease progression, is this target acting? where in the cell is a given target exerting its disease promoting actions? what is the most toxic folding state or aggregation state of that target? what protein interactions is the target engaged in?

These questions are ideally addressed by the intrabody approach, which exploits the molecular binding diversity of the antibody repertoire with the precision of subcellular targeting. The availability of user-friendly antibody libraries for the isolation of functional intrabodies provides an unlimited source of antibodies of superior stability and binding properties. Effector functions added to the binding moiety of the antibody can be tailored to the particular experimental needs, including live imaging, and provide further strength to the technology. In particular, we envisage three aspects of the intrabody approach as being very promising: i) the possibility of targeting specific protein-protein interactions, while sparing other interactions engaged by the same protein; ii) the possibility of targeting post-translationally modified proteins, selectively with respect to the unmodified protein; iii) the possibility of targeting a subcellular pool of a given protein. These experimental approaches would not be possible with gene- or mRNA-centered silencing approaches and highlight the uniqueness and the potential of intrabody technology.

With the growing evidence for trans-cellular propagation of tau and of amyloid beta misfolding and the recognition of neurodegeneration as a

spreading pathology from initially defined sites (Aguzzi and Rajendran, 2009; Best and Diamond, 2010; Goedert et al., 2010), the availability of recombinant antibodies against tau (Visintin et al., 2002; Melchionna and Cattaneo, 2007) and A β O (Meli et al., 2009) will allow their expression in the nervous system of transgenic animals (Piccioli et al., 1995) or with viral vectors, the best approach to test the feasibility and efficacy of therapeutic approaches based on tau or A β O vaccination.

Following the initial proof of concept studies in the early 90's (Biocca et al., 1990; Cattaneo and Biocca, 1997), intrabodies have been, so far, mostly applied in the field of cancer and viral diseases. Given the great need for new target validation technologies in the field of Alzheimer's and other neurodegenerative diseases, we anticipate that the growing application of intrabody technology to this field will deliver important results in the near future. The resulting improvement of our understanding of the basic cell biology of neurodegeneration will pave the way for the therapeutic uses of intrabodies.

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PATHOLOGY OF TAU PROTEIN IN ALZHEIMER'S DISEASE

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The progression of tau pathology through different brain regions resembles in some aspects the progression found in Alzheimer disease. Tau pathology starts in the hippocampal region and afterwards it spreads to the cortex. During that process tau itself, in extracellular form, appears to be the agent that propagates the degeneration from neuron to neuron. In this review we will mainly comment on the possible mechanisms for the development of tau pathology in the brain of Alzheimer disease patients. Two major mechanisms have been proposed for the transmission of tau protein from a neuron to neuron. One of these mechanisms suggests that upon neuron death, taking place in Alzheimer disease, the released tau could be toxic for the surrounding neurons. The other one suggests a prion-like transmission, where intracellular aggregated tau is released through membrane vesicles that could be further incorporated into the surrounding neurons by endocytosis.

Alzheimer's disease (AD) is characterized by a huge neuron death and the presence, in the brain of the patients, of two histopathological aberrant structures, senile plaques and neurofibrillary tangles (Alzheimer, 1907). After the pioneer work of Glenner and Wong (1984) and Master et al (1985), we know that beta amyloid peptides (A β) are the main component of senile plaques. We also know that beta amyloid peptides of different size with different characteristics could regulate the formation of senile plaques (for a review see Walsh and Selkoe, 2007).

On the other hand, it is known that the main component of neurofibrillary tangles (NFT) is tau, a microtubule associated protein (Grundke-Iqbal et al, 1986). Tau binds to microtubule through a microtubule binding region consisting of three (3R) or four (4R) with similar but not identical sequences. These sequences are present close to the C-terminal region of the molecule (Lee et al, 1988). Those

sequences are involved in tau self interaction that results in the aggregation of the protein (Avila et al, 2004).

Neurofibrillary tangles are large complexes of fibrillary polymers, the paired helical filaments (PHFs) (Gonatas et al, 1963; Kidd, 1963) that are tau aggregates (Grundke-Iqbal et al, 1986). The shape of PHF and the possible origin of their helicity have been analyzed by electron microscopy, x-ray diffraction, or atomic force microscopy (Moreno-Herrero et al, 2004; Wischik et al, 1985; Wisniewski et al, 1976). The differences in their morphology could be determined by the relative proportion of tau isoforms containing three or four tubulin binding motif (Goedert et al, 1996), by the association of tau polymers with sulfoaminoglycans (Arrasate et al, 1997), or by the presence of some clusters of charged residues in tau isoforms (DeTure et al, 2002).

The presence of the aberrant beta amyloid peptide or tau aggregates could be followed *in vivo* in AD

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patients by the use of some compounds in positron emission tomography (PET). These compounds are Pittsburgh compound B (PIB) (Klunk et al, 2004), for beta amyloid aggregates, or 18F-THK23 (Fodero-Tavoletti et al, 2011) for tau imagine radiotracers.

TAU ASSEMBLY

Tau self-assembly could result in the formation of dimers, tetramers, higher-order oligomers or fibrillary polymers (Ramachandran and Udgaonkar, 2011; Sahara et al, 2007). It has been suggested that dimers could be formed with two antiparallel tau molecules (Ksiazek-Reding and Yen, 1991). In that interaction, the third tubulin binding repeat plays an important role (Perez et al, 1996; Von Bergen et al, 2000). *In vitro*, several products, quinones, heparin, polyanions, etc. (Goedert et al, 1996; Perez et al, 2000; Perez et al, 1996; Santa-Maria et al, 2004; Wilson and Binder, 1997) could facilitate tau aggregation.

Tau dimers could assembly in higher-order oligomers (Sahara et al, 2007), being some granular tau oligomers possible intermediates to build tau filaments (Maeda et al, 2007). In addition, tau filaments, that could have a straight or helical (PHF) morphology, could be assembled into larger complexes (NFT), a process that appears to be facilitated by tau glycation (Ledesma et al, 1995; Ledesma et al, 1994). Also, extracellular NFT, known as "ghost tangles" have been also described (Augustinack et al, 2002). These extracellular tau aggregates could arise from neuron degeneration (Alonso et al, 2008; Gomez-Ramos et al, 2006).

POST-TRANSLATIONAL MODIFICATIONS AND TAU ASSEMBLY

In vitro studies have indicated that unmodified tau is able to self assemble in aggregates (Perez et al, 2001). Tau is, mainly, an unstructured molecule, but it may adopt, in some conditions, a paperclip conformation (Jeganathan et al, 2006). In this conformation, the C-terminal end of tau binds to the microtubule binding region, and the N-terminal end could bind to the C-terminal region of tau molecule. The addition of some compounds, like polyanions, could open this conformation to facilitate tau self-

assembly (Fig.1A). Also, there are some post-translational modifications that could regulate tau-tau interaction; among those posttranslational modifications are oxidation, phosphorylation, acetylation or truncation. Once, tau-tau interaction takes place the formation of dimers, oligomers, fibrillar polymers and neurofibrillary tangles may occur (Fig.1B).

Oxidation

There is a single cysteine residue in tau 3R and two cysteines in tau 4R. In the first case, the intermolecular interaction to form a tau dimer could take place. In the second case inter and intra molecular interactions could compete, decreasing the probability to form tau dimers (Schweers et al, 1995).

Phosphorylation

Depending on the modified site, tau phosphorylation may facilitate or prevent tau-tau interaction, if the modification is at the microtubule binding region or, a decrease in tau aggregation takes place (Noble et al, 2005; Perez et al, 2003; Schneider et al, 1999). However, tau phosphorylation at other sites (mainly at the C-terminal region) of the molecule may facilitate tau assembly, probably by opening the paperclip conformation (Noble et al, 2005; Perez et al, 2000; Perez et al, 2002; Perez et al, 2003). Tau phosphorylation at threonine 231, by proline directed kinases, like GSK3, may result in the appearance of *cis*-trans-phospho protein isomers (Lu et al, 1999). *Cis*-phosphotau appears to be more prone to aggregation than trans-phosphotau (Nakamura et al, 2012). Thus, the presence of that *cis*-isomerization of phosphotau could be one of the first steps to start tau aggregation.

Acetylation

Another posttranslational modification, acetylation (Min et al, 2010) may facilitate tau aggregation (Cohen et al, 2011). A possible mechanism to explain how tau acetylation facilitates tau aggregation is based in the fact that acetylation and ubiquitination could take place at the same lysine residues in tau molecule. Thus, acetylation may prevent ubiquitination (and degradation) of tau molecules and these molecules could accumulate

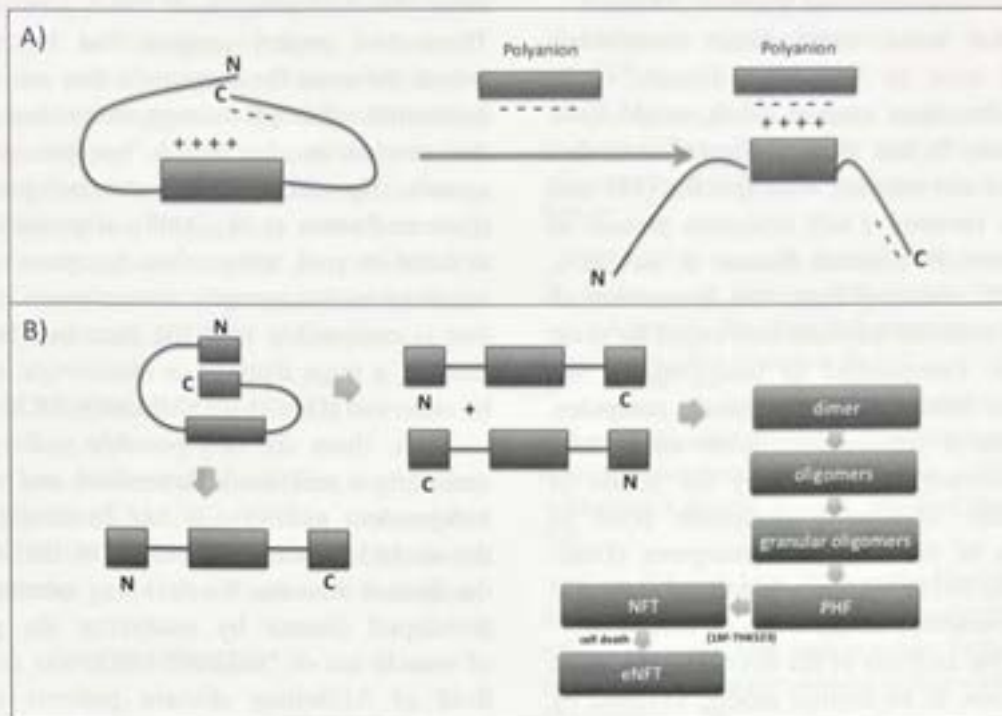


Fig. 1. Tau aggregation. *A) Paper-clip tau conformation could be open, for example, by the presence of polyanions. B) Once tau shows an open conformation it can form dimers, oligomers, paired helical filaments (PHF) and neurofibrillary tangles (NFT) that could be detected by positron emission tomography by the compound 18F-THK23. Upon cell death, NFT could become extracellular NFT (eNFT).*

inside the cell and this accumulation could facilitate tau aggregation.

Truncation

Finally, another post-translational modification, truncation (Abraha et al, 2000; Mena et al, 1996), or removal of C-terminal region, may facilitate tau assembly. On the other hand, caspase-cleaved N-terminal region of tau may not affect to tau assembly although it may yield a toxic tau fragment (Amadoro et al, 2011; Corsetti et al, 2008).

Spreading of tau pathology

In Alzheimer disease, tau pathology follows a reproducible pattern, in which phosphorylated and/or aggregated tau first appears in the entorhinal cortex/hippocampal region and it spreads from that region to the surrounding areas (Braak and Braak, 1991). During the development of the disease, neuron death takes place and intracellular tau could be released to the extracellular space. Both monomeric and

aggregated tau can be found outside the cell. In aggregated form, tau could be present in extracellular NFT or "ghost tangles" (Bondareff et al, 1989; Cras et al, 1995).

A different behavior has been reported for extracellular monomeric or aggregated tau (Fig. 2). For aggregated tau, it has been indicated that it could be incorporated, by endocytosis, into the surrounding cells, inducing the aggregation of intracellular tau (Clavaguera et al, 2009; Frost et al, 2009). In this way is not needed cell death for tau spreading and intracellular tau could pass from cell to cell through membrane vesicles, when tau is overexpressed (see below).

Recently, tau secretion in membrane vesicles has been reported (Saman et al, 2011; Simon et al, 2012). An explanation for this secretion has been given, it has been suggested that tau secretion through membrane vesicles could facilitate its discharge when its concentration inside the cell increases, a process that has been reported for other proteins

(Simon et al, 2012; Simons and Raposo, 2009).

On the other hand, extracellular monomeric tau that could arise in Alzheimer disease, or in other tauopathies, upon neuron death, could have a toxic function. It has been indicated that this extracellular tau can interact with specific (M1 and M3 muscarinic receptors) cell receptors present in surrounding neurons (Gomez-Ramos et al, 2006; 2008 and 2009), resulting from that interaction an increase in intracellular calcium that could be toxic for the neuron. Unmodified or unaggregated tau seems to react better with muscarinic receptors. For phosphotau, a dephosphorylation could take place in the extracellular space by the action of tissue-nonspecific alkaline phosphatase prior to the interaction of tau with cell receptors (Diaz-Hernandez et al, 2010). This dephosphorylation will facilitate the interaction of tau with cell receptors.

Following, the analysis of the development of tau pathology *in vivo*, in an animal model, initiated by Clavaguera et al (2009), two papers have recently reported how takes place the propagation of tau pathology from the entorhinal cortex to surrounding

areas (De Calignon et al, 2012; Liu et al, 2012). These two papers suggest that tau propagation occurs between those neurons that are synaptically connected, although no explanation was given about the mechanism by which synaptic transmission occurs. By taking into account previous data (Gomez-Ramos et al, 2008), a possibility is that, at least in part, muscarinic receptors seem to be involved in that synaptic transmission, a possibility that is compatible with the fact that, in Alzheimer disease, a huge damage in cholinergic neurons can be observed (Davies and Maloney, 1976).

Thus, there are two possible pathways for tau spreading a cell death dependent and a cell death independent pathway. It has been suggested that the second way mainly occurs at earlier stages of the disease whereas the first way takes place in the developed disease by analyzing the propagation of vesicle tau or "naked" tau in the cerebrospinal fluid of Alzheimer disease patients at different stages of the disease (Saman et al, 2011). It cannot be discarded that both ways for tau spreading could simultaneously occur although from the possible

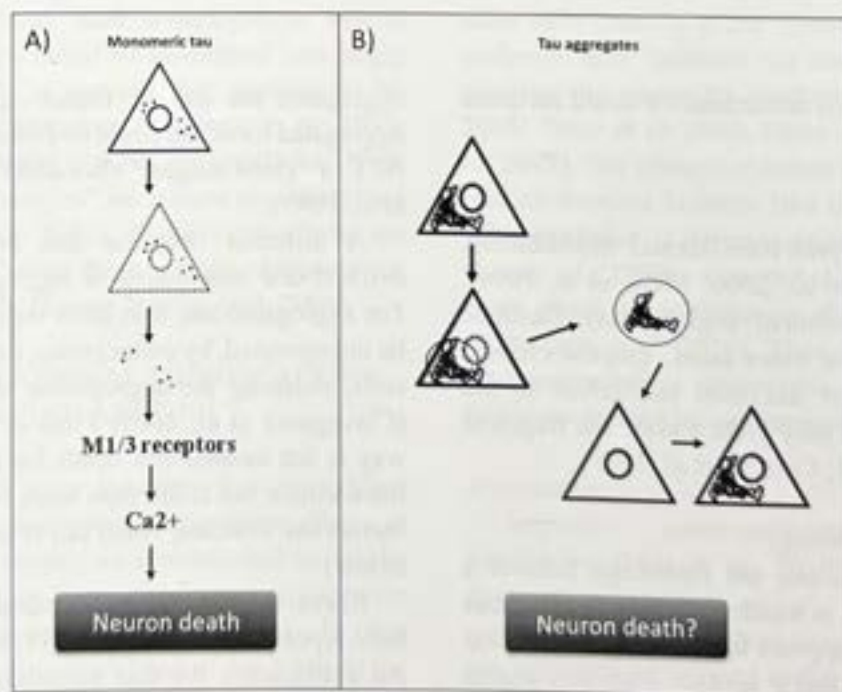


Fig. 2. Intercellular tau spreading. **A)** Extracellular tau could raise by neuron death. This extracellular (monomeric) tau could interact with cellular receptors (muscarinic M1 and M3 receptors) resulting in an increase of intracellular calcium (Ca^{2+}) level that may end in neuron death. **B)** Alternatively, intracellular tau aggregates could be exocytosed in membrane vesicles and endocytosed by surrounding neurons. This type of extracellular tau may be also toxic.

therapeutic actions, it will be different if extracellular tau is in "naked" or "enveloped" form, because in the first case the use of tau antibodies to block the progression could be suggested, but this will not be possible for "enveloped" tau. In a recent report it has been suggested that an antitau monoclonal antibody blocks tau aggregation, suggesting that there is a population of extracellular tau that is not included in membrane vesicles and that can interact with antibodies (Kfoury et al, 2012).

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MITOCHONDRIAL BIOGENESIS IN THE ISCHEMIC BRAIN: A NOVEL TARGET FOR NEUROPROTECTION

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Ischemic stroke is a serious public health problem. Despite many efforts have been devoted in preclinical research with successful results, no neuroprotective treatment has been translated from bench to bedside so far. Enhancement of endogenous brain neuroprotective mechanisms is regarded as a major research possibility at this crossroad, and mitochondria are emerging among the key regulators of these phenomena. In particular, regulatory processes of mitochondrial biogenesis take part to the adaptive responses that occur after preconditioning hypoxic-ischemic stimuli or transient global ischemia in rodent models. This could possibly contribute to neuroprotection or to augment brain tolerance to further ischemic episodes. However, in the case of severe focal brain ischemia there is evidence for a lack of adaptive mitochondrial biogenesis. Strategies to implement mitochondrial biogenesis are currently investigated as possible novel therapies directed at neuroprotection and/or neurorepair in cerebral ischemia.

Stroke is among the most serious and debilitating neurological disorders. More than 80% of all strokes are ischemic in nature, being due to a thrombosis, an embolism, or a systemic hypoperfusion that lead to the sudden reduction or cessation of blood flow in a cerebral artery, thereby decreasing the amounts of oxygen and glucose reaching the brain. The clinical management of stroke - based on administration of the thrombolytic tissue plasminogen activator (tPA) to induce clot lysis and restore blood flow - is at present unsatisfactory. In fact, the drug has been demonstrated to improve clinical outcome only if administered intravenously within the first 3 hours of stroke onset. More recently, the time window has been expanded to 4.5 hours with intravenous t-PA (Hacke et al, 2008). Anyway, this narrow time window greatly limits the clinical use of tPA, and less than 5% of patients in the community receive thrombolysis (Iadecola & Anrather, 2011). Further,

the thrombolytic therapy is associated with an increased risk of intracranial hemorrhage.

Our knowledge regarding the cellular and molecular processes underlying neuronal death following ischemic stroke (including excitotoxicity, oxidative stress, inflammation or apoptosis) have prompted the study of alternative therapeutic strategies aimed at neuroprotection. Experiments in rodent models demonstrate that the ischemic brain can be protected pharmacologically, reducing infarction and improving functional outcome. However, so far every attempt to translate this preclinical success into clinically effective therapies has failed (Ginsberg, 2008; Dirnagl & Fisher, 2012), fueling a debate about the promise of neuroprotection in stroke therapy. At this crossroad, experts in translational stroke research have suggested that understanding how brain protects itself might provide useful lessons on how to best counteract ischemic brain injury.

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Therefore, major efforts are at present focusing on the enhancement of endogenous brain neuroprotective mechanisms (Iadecola & Anrather, 2011). The potential for brain self-protection is exemplified by the phenomenon of ischemic tolerance or preconditioning, whereby a mild cerebral ischemic insult not sufficient to produce extensive damage (or a variety of mild stressful stimuli) protects the brain from subsequent damaging ischemia. Endogenous adaptive phenomena that confer brain protection are seemingly active also in human individuals with a history of preceding transient ischemic attack (TIA), which experience less severe strokes (Weber et al, 2011). Most recently, mitochondria have emerged among the key regulators of preconditioning-triggered endogenous neuroprotection (Correia et al, 2011) and are considered attractive targets for stroke therapy (Perez-Pinzon et al, 2012).

Mitochondria orchestrate an extensive repertoire of cellular functions, ranging from the biosynthesis of amino acids to the beta-oxidation of fatty acids. However, the main mitochondrial function is the production of ATP through the tricarboxylic acid cycle and the oxidative phosphorylation system in the respiratory chain. As neurons are highly metabolically active cells with energy demands at locations distant from the cell body, they are particularly dependent on mitochondrial function (Correia et al, 2011). Mitochondrial dysfunction contributes to the pathophysiology of acute neurologic deficit in cerebral ischemia. The lack of oxygen and glucose supply to the brain results in a drastic reduction in mitochondrial ATP production – i.e., a severe bioenergetics dysfunction – with subsequent necrosis. Further, mitochondrial dysfunction contributes to oxidative stress and apoptosis (Perez-Pinzon et al, 2012).

BASIC CONCEPTS ON MITOCHONDRIAL PHYSIOLOGY AND IMPLICATIONS IN CEREBRAL ISCHEMIA

Mitochondria are highly dynamic organelles, which move throughout the cell and undergo structural transitions, changing their morphology (length, shape, and size) according to metabolic activity and energy needs (Michel et al, 2012). These changes are tightly regulated by the balance between

fusion and fission phenomena. Interestingly, a cross-talk does exist between dynamics and function of mitochondria as dynamics affect the activity of the organelle while mitochondrial dysfunction can also impact on its morphology and dynamics (Michel et al, 2012).

Mitochondrial integrity is essential to cellular homeostasis. Fusion and fission events mediate a partial mitochondrial complementation by determining intramitochondrial exchange of mtDNA, proteins and lipid membranes, allowing the segregation of damaged components. To provide the highly efficient energy transducing machinery necessary for cell survival, mitochondria further undergo a strictly regulated process of quality control. Old or damaged mitochondria, which display reduced metabolic capacity and produce the most reactive oxygen species, are to be substituted with renewed mitochondria endowed with high metabolic capacity. Removal of damaged mitochondria is accomplished via mitochondrial autophagy or mitophagy. To provide the highly efficient energy transducing machinery necessary for cell survival, mitophagy must be balanced by the proliferation of mitochondria, a process called mitochondrial biogenesis. The process of mitochondrial renewal is of greatest importance in long-lived cells including neurons (Gottlieb & Carreira, 2010).

The prevailing theory indicates that mitochondria are not synthesized *de novo*, but arise by growth and division of pre-existing organelles. The mechanisms to increase the mitochondrial mass are highly complex and require orchestration of two genomes, since only 13 subunits of respiratory chain complexes are encoded by the mitochondrial DNA (mtDNA). The remaining mitochondrial proteins (approximately 1,500) are encoded by the nuclear DNA (nDNA), translated in the cytoplasm and transported into mitochondria. So, mitochondrial biogenesis is the result of a complex cross-talk between the nucleus and mitochondria that involves the choreographed expression of a network of nuclear DNA-binding transcription factors and co-regulators. Among the transcriptional co-activators of the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family [PGC-1 α , PGC-1 β and PGC-1-related co-activator (PRC)], a major role is played by PGC-1 α , which is considered the master

regulator of mitochondrial biogenesis (Wu et al, 1999). PGC-1 α is involved in the activation of the nuclear respiratory factors (NRF) 1 and 2 (Scarpulla, 2008). NRF-1 (originally identified as a transcription factor binding to a conserved regulatory site of the cytochrome c promoter) is implicated in the transcription of nuclear genes encoding subunits of all five respiratory complexes and other mitochondrial proteins. Further, NRF-1 regulates the expression of the mitochondrial transcription factor A (Tfam), which is critical for the initiation of mitochondrial DNA (mtDNA) replication (Scarpulla, 2008).

A controlled regulation of mitochondrial mass through a delicate balance between mitophagy and mitochondrial biogenesis is a crucial step to ensure the necessary neuronal energetic supplies. The deep implications of changes in mitochondrial dynamics, quality control, and mitochondrial biogenesis in cerebral ischemia are largely unknown, and are the focus of current research (Vosler et al, 2009). In particular, growing but somewhat contradictory evidence is implicating the regulatory processes of mitochondrial biogenesis in the pathophysiology of cerebral ischemia. In the next sections, we will discuss how this burgeoning area of investigation could possibly lead to novel therapeutics.

ADAPTIVE MITOCHONDRIAL BIOGENESIS AND TOLERANCE AGAINST CEREBRAL ISCHEMIA

Evidence for an increase in mitochondrial biogenesis markers has been reported in neonate rodents exposed to hypoxic conditions. A sustained mild hypoxia (9.5% O₂) lasting from 11 days from P3 through P14 (but not a 24 h hypoxia) produced an elevation in NRF-1 and Tfam expression with no change in PGC-1 α , as well as an increase in mtDNA content in the hypoxic compared to the normoxic P14 pup brain (Lee et al, 2008). The increased mitochondrial biogenesis might plausibly act as a compensatory mechanism designed to diminish the impact of compromised oxygenation on neural development. Other groups subjected postnatal day 7 rats to unilateral carotid artery ligation followed by 2.5 hours of hypoxia (8% O₂) (Yin et al, 2008). They observed an increase in mtDNA content in the cortical infarct border zone 6 to 24 hours after

this hypoxic/ischemic (H-I) brain injury. This was paralleled by upregulated NRF-1 and Tfam expression, and increased levels of mitochondrial proteins [cytochrome c oxidase subunit IV (COX-IV) and heat shock protein-60 HSP60] and citrate synthase activity (Yin et al, 2008). PGC-1 α mRNA and protein levels were unchanged after neonatal H-I. Interestingly, mitochondrial number was increased in surviving cells, while damaged cells showed markedly swollen mitochondria with broken or disrupted cristae (Yin et al, 2008). Most recently, diverse and sex-related changes in brain mitochondria have been described in a neonatal rat model of HI. Two h after HI, a reduction of cells with high mitochondrial mass (as measured by fluorescent staining with MitoTracker Green) was observed in the cortex and hippocampus of both sexes. At 18 h, only females displayed a decreased mitochondrial mass, suggesting a persistent decreased intracellular number of mitochondria (Weis et al, 2012).

Much of the existing evidence for an adaptive mitochondrial biogenesis further comes from studies on ischemic preconditioning models in adult rodents (Fig. 1). Gutsaeva and coworkers investigated the effects of acute transient hypoxia in mice subcortex. They found that exposure to hypobaric hypoxia (equivalent to 8% O₂ normobaric hypoxia) for 6 h increased PGC-1 α , NRF-1 and Tfam mRNA levels, which remained significantly elevated at 24 h, and returned to control levels by 48 h (Gutsaeva et al, 2008). This transcriptional program led to increased mtDNA content in brain subcortex. Further, transgenic mice with GFP targeting to mitochondria displayed increased perinuclear neuronal fluorescence in focal areas of the hippocampus, but not in cerebral cortex, 24 h after hypobaric hypoxia (Gutsaeva et al, 2008). The induction of mitochondrial biogenesis by hypoxic preconditioning in brain subcortex was found to be nitric oxide (NO)-dependent and mediated by the neuronal nitric oxide synthase (nNOS) (Gutsaeva et al, 2008). Overall, these results suggest that a regional adaptive program might be activated by preconditioning stimuli, especially in hypoxia-vulnerable areas such as the hippocampus.

Moreover, the effects of a 10-min period of forebrain global ischemia (TGI) (which result in delayed damage in the CA1 hippocampus subfield) have been studied in rats by Chen and collaborators.

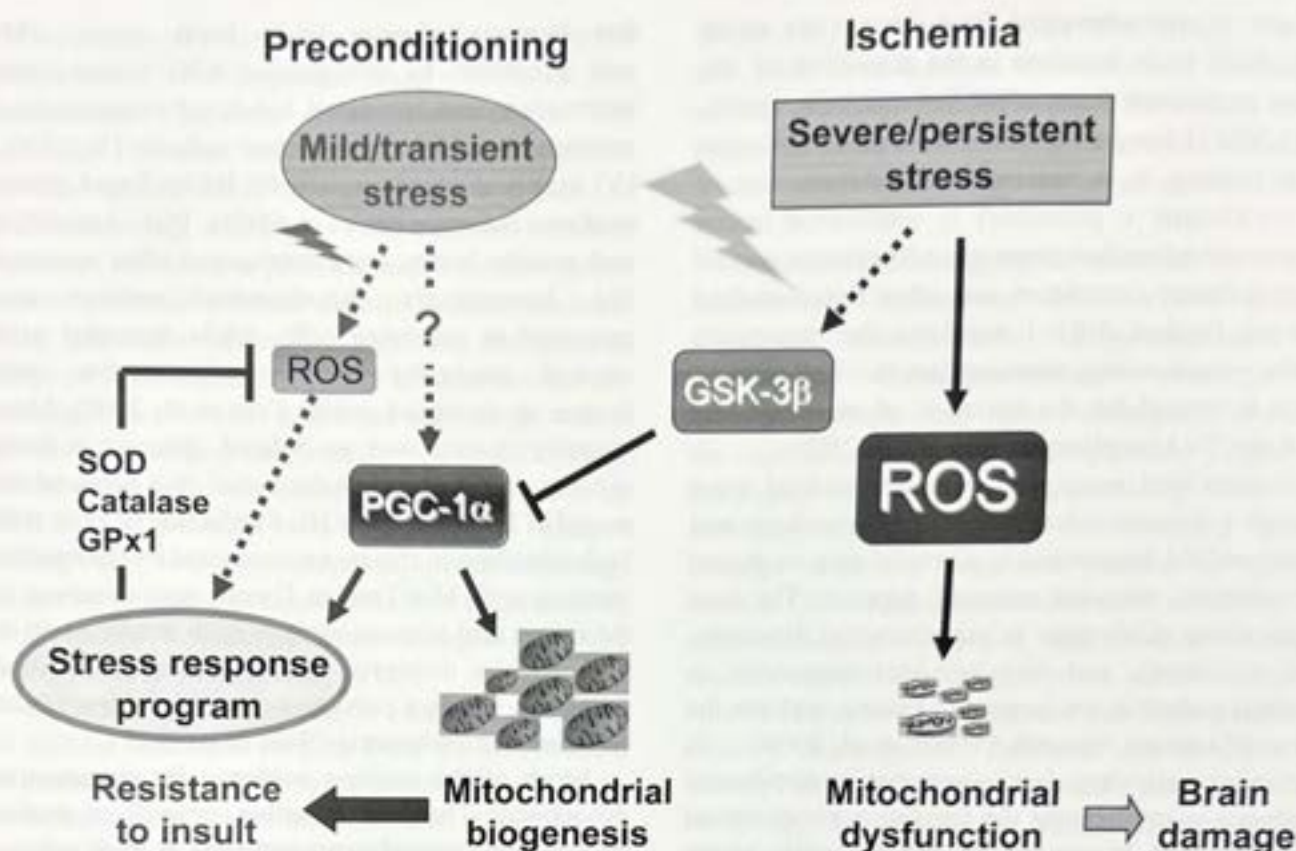


Fig. 1. Putative signaling pathways involved in ischemic preconditioning stimuli and ischemic insults in brain. Ischemic preconditioning elicits protection through a complex signaling cascade that converges on the mitochondrion. Evidence obtained over the past two decades shows that reactive oxygen species (ROS) are involved in brain lesions, including those due to cerebral ischemia-reperfusion. The mitochondria are the primary intracellular source of ROS, as they generate huge numbers of oxidative-reduction reactions and use massive amounts of oxygen. When anoxia is followed promptly by reperfusion, the resulting increase in oxygen supply leads to overproduction of ROS. In ischemic tissues, numerous studies have established a direct role for ROS in oxidative damage to lipids, proteins, and nucleic acids. See text for details on the signaling pathways.

They observed an early induction of PGC-1 α protein levels (as well as of the protein levels of the PGC-1 α target genes UCP2 and SOD2) 1-4 h after TGI, that returned to basal levels 24 h after the insult (Chen et al, 2010). Conversely, knock-down of PGC-1 α expression by pretreatment with a specific antisense oligodeoxynucleotide in the hippocampal CA1 subfield exacerbated oxidative stress and enhanced delayed neuronal cell death in the hippocampus after TGI (Chen et al, 2010).

Altogether, this evidence suggest that PGC-1 α and/or other members of the mitochondrial biogenesis transcriptional program can be activated in the course of an adaptive response, as novel components of the endogenous brain repair mechanisms (Figure

1). This has been found to occur: i) in the context of cerebral hypoxic preconditioning or transient ischemia; ii) in global hypoxia or ischemia; iii) in rodent neonates. However, it has been reasoned that the activation of mitochondrial biogenesis after acute transient hypoxia does not necessarily predict the response to prolonged hypoxia (Gutsaeva et al, 2008). It has been also observed that the endogenous mitochondrial biogenic capacity is reduced with aging (López-Lluch et al, 2008), so that it hardly could achieve an efficient adaptive response in adult or aged individuals. It is also worth making some observations about the different rodent models of cerebral ischemia. Global ischemia models are relevant for the study of anoxic brain damage

due to cardiac arrest (Macrae, 2011). Hypoxia/reoxygenation models can be applicable to human conditions such as sleep apnea, asthma or chronic obstructive pulmonary disease (Banks, 2010). On the other hand, ischemic stroke is studied in animal models by permanently or transiently occluding a cerebral artery, thus causing a severe reduction in cerebral blood flow in its territory. This approach induces a focal ischemic insult in a defined region of brain tissue, as in human cerebral infarction. It is worth mentioning that the majority of human ischemic strokes are not amenable of tPA therapy, resulting in permanent occlusion.

EVIDENCE FOR IMPAIRED MITOCHONDRIAL BIOGENESIS IN FOCAL CEREBRAL ISCHEMIA

After a severe ischemic insult, mitochondria may undergo oxidative mtDNA damage (Dirnagl & Meisel, 2008), changes in mitochondrial dynamics with augmented fission (Barsoum et al, 2006) and uncontrolled autophagy (Vosler et al, 2009) (Figure 1). Chen and colleagues studied the changes in mtDNA content in the ischemic brain in a rat stroke model featuring transient severe ischemia confined to the cerebral cortex of the right middle cerebral artery territory for 30 or 90 min. They found a reduction in mtDNA content after 30- or 90-min ischemia (Chen et al, 2001). The mtDNA was restored to near nonischemic levels 24 h after 30-min ischemia, suggesting the possibility that mtDNA repair or repletion occurs after brief ischemia. On the contrary, 90-min ischemia led to a profound reduction of mtDNA content, which failed to be restored within the 24-h time course of the study (Chen et al, 2001). Cerebral ischemia has been shown to cause mitochondrial swelling, which may lead to the rupture of mitochondrial membranes, with resultant release of mtDNA that is susceptible to endonuclease digestion (Figure 1).

Whatever the mechanism(s) underlying the reduction of mtDNA content in transient focal cerebral ischemia (including severe mitochondrial swelling, with rupture of mitochondrial membranes, and release of mtDNA susceptible to endonuclease digestion), such a reduction of mtDNA contents could only be replenished with regeneration of

mitochondria (Chen et al, 2001). The results at 90-min ischemia attested the inadequacy of adaptive brain mitochondrial biogenesis in condition of severe ischemia.

With this situation in mind, we conducted a study to evaluate the efficiency of the mitochondrial biogenic program in the context of cerebral ischemia. We exposed primary mouse cortical neurons to oxygen-glucose deprivation (OGD, with $O_2 < 0.4\%$ in glucose-free conditions for 3 h) and observed that indexes of mitochondrial biogenesis were reduced by OGD (Valerio et al, 2011). In particular, PGC-1 α protein levels were reduced 3 h after OGD. Further, NRF-1, Tfam, cyt c and COX IV expression were reduced 3 to 24 h after OGD. We further found early and persistent reduction of mtDNA content in neurons exposed to ischemia-reoxygenation in vitro. Interestingly, all of these changes preceded the detectable signs of OGD-mediated neuronal death (Valerio et al, 2011). OGD also reduced neuronal mitochondrial function, determined by citrate synthase activity (Valerio et al, 2011). Finally, as previously observed in transient focal ischemia models (Chen et al, 2001), a profound loss of mtDNA content was observed in the infarcted area 24 h after permanent middle cerebral artery occlusion (pMCAO) in mice (Valerio et al, 2011). These in vivo experiments, suggesting that inefficient mitochondrial renewal occurs after permanent cerebral artery occlusion, are in line with results obtained in rodent model of myocardial infarction (Forini et al, 2011).

The molecular and cellular pathways leading to the down-regulation of PGC-1 α and downstream targets by severe cerebral ischemia deserve to be investigated. Different hypotheses can be put forward. Post-ischemic actions of calpain proteases (Bever & Neumar, 2008) might cause PGC-1 α degradation (Rasbach et al, 2008). Further mechanism(s) might involve the modulation of glycogen synthase kinase-3 β (GSK-3 β), an enzyme known to regulate PGC-1 α turnover (Anderson & Prolla, 2009). GSK-3 β inhibition has been linked to PGC-1 α stabilization and increased PGC-1 α levels in primary neurons (Olson et al, 2008). Aberrant GSK-3 β hyperactivation due to GSK-3 β -Tyr216 phosphorylation has been reported 3-6 h after MCAO in mice (Bhat et al, 2000). Further, ischemia might trigger calpain-mediated

GSK-3 β N-terminal cleavage, that augments its kinase activity (Goñi-Oliver et al, 2007). Thus, pathologically hyperactivated GSK-3 β might reduce PGC-1 α levels in ischemic neurons (Figure 1). The length and strength of the hypoxic-ischemic metabolic stress might differently modulate the PGC-1 α /mitochondrial biogenic response. In fact, it has been recently demonstrated that, contrary to low/transient stress (such as short glucose or serum starvation), high/prolonged stress triggers PGC-1 α degradation via ubiquitin-proteasome pathway (Sen et al, 2011) (Figure 1).

INTERVENTIONS TO IMPROVE MITOCHONDRIAL BIOGENESIS IN STROKE THERAPY

GSK-3 β inhibition has been considered as an attractive strategy for the therapy of myocardial (Tong et al, 2002; Juhaszova et al, 2009) and cerebral ischemia (Koh et al, 2008; Cowper-Smith et al, 2008; Valerio et al, 2009; 2011). Given the GSK-3 β -mediated control of PGC-1 α protein levels and further the ability of GSK-3 β inactivation to augment NRF-1 cell content via NF-E2-related factor (Nrf2)-mediated transcription (Piantadosi et al, 2008), we conducted an in-depth investigation of the possible role of GSK-3 β inhibition in neuronal mitochondrial biogenesis. We demonstrated that the GSK-3 inhibitor SB216763 activated an efficient PGC-1 α -mediated mitochondrial biogenesis program in mouse cortical neurons (Valerio et al, 2011). SB216763 exerted neuroprotective effects against cerebral ischemia both *in vitro* and *in vivo*. Most interestingly, SB216763 counteracted the OGD-mediated impairment in neuronal mitochondrial biogenesis (Valerio et al, 2011). Further, when systemically administered at the onset of pMCAO in mice, the drug restored mtDNA content to non-ischemic levels (Valerio et al, 2011).

Further sparse evidence exist for a possible contribution of mitochondrial biogenesis to neuroprotection. Leptin is an adipose-derived hormone that displays multifaceted properties as a neuroprotectant. It takes part to the endogenous neuroprotective response during cerebral ischemia and, when exogenously administered, protects against ischemic neuronal injury *in vitro* and *in vivo*

through signals converging on GSK-3 β inhibition (Valerio et al, 2009). Interestingly, leptin is able to phosphorylate and activate eNOS (Vecchione et al, 2002), a potent inducer of PGC-1 α -mediated mitochondrial biogenesis in numerous tissues including brain (Nisoli et al, 2003). Compelling data from animal models demonstrate that eNOS activation improves the outcome of cerebral ischemia (Endres et al, 2004; Atochin et al, 2007). Thus, eNOS/PGC-1 α mediated mitochondrial biogenesis might take part to the protective effects of leptin against cerebral ischemia. Leptin was also found to improve neurological disability and reduced infarct volume along with increasing the expression of sirtuin 1 (SIRT1) (Avraham et al, 2010), which deacetylates and activates PGC-1 α . Similarly, administration of resveratrol (a SIRT1 activator and inducer of mitochondrial biogenesis) is neuroprotective against cerebral ischemia (Della-Morte et al, 2009; Sakata et al, 2010). Other interventions known to improve mitochondrial biogenesis and function, such as calorie restriction or physical activity, have been shown to reduce cerebral ischemic damage (Yu & Mattson, 1999; Ploughman et al, 2007).

Recently, it has been reported that treadmill exercise initiated early after transient MCAO in the rat induces a time-dependent increase in the expression of PGC-1 α , NRF-1, Tfam and COX IV as well as increased mtDNA content in brain, together with significant changes in behavioral scores and cerebral infarct volume (Zhang et al, 2012), suggesting that exercise may benefit the brain recovery through regulation of mitochondrial biogenesis.

CONCLUSIONS

Due to the bioenergetics failure in ischemic conditions, there is a rationale for preserving or improving mitochondrial function to re-establish normal ATP production. The biological programs regulating mitochondrial biogenesis have been found to augment adaptive processes and tolerance to cardiac ischemia and have been suggested as new targets for therapeutic interventions to treat ischemic heart disease (McLeod et al, 2005; Forini et al, 2011). We have reviewed recent evidence suggesting that improved mitochondrial biogenesis can be helpful to reduce the effects of ischemia-induced cerebral

damage. Owing to the fact that mitochondria are responsible for generating energy, a larger pool of functional mitochondria also plays important roles in diverse brain processes, including neurogenesis, neural proliferation, neural differentiation, neurite outgrowth, and dendritic remodeling (Cheng et al, 2010). Thus, mitochondrial biogenesis might take part in several aspects of neurorestoration after cerebral ischemia. The example of molecules targeting GSK-3 β is paradigmatic, since besides favouring neuroprotection at least in part through improved mitochondrial biogenesis (Valerio et al, 2011), they can promote neurogenesis (Mao et al, 2009) and axonal growth (Valerio et al, 2006; Dill et al, 2008). Further, GSK-3 β inhibitors enhance angiogenesis after ischemia (Kaga et al, 2006) a phenomenon that could be PGC-1 α -related and exert additional benefit in ischemic disease (Arany et al, 2008). Given the disappointing results of clinical trials in stroke patients, it is unlikely that a pharmacological intervention targeting a single pathway will translate into clinical improvements. Further research on mitochondrial biogenesis in ischemia models is needed to solve many open questions. The possibility to identify therapeutic strategies mimicking endogenous neuroprotection and possibly engaging multiple programs directed at brain restoration is worth the effort.

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NEURODEGENERATION ABIDES BY THE LAWS OF MITOCHONDRIAL BIOLOGY

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Tuned mitochondrial physiology is fundamental for qualitative cellular function. This is true for every cell but it is particularly important for those with delicate bio-energetic equilibrium such as neurons whose pathology is frequently associated with mitochondrial dysfunction. Defects in mitochondria are key features in most neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's diseases (PD), Huntington's disease (HD) and Amyotrophic Lateral Sclerosis (ALS). When mitochondrial coupling impairs, so does cellular metabolism, trafficking of mitochondria and the cell signalling depending on this. Moreover, the quality control of mitochondria –mitophagy- results biased in neurodegeneration. In this short review, our aim is to highlight the most notable and acknowledged deficiencies of mitochondrial function and their relationship with diseases of the neurons and neuronal transmission. We shall discuss the physiological aspects of mitochondrial biology in relation to: i) bio-energy, ii) handling of the mitochondrial driven ATP consumption; iii) dynamics and iv) quality control. This with the finality to form a comprehensive picture of mitochondrial contribution to the pathophysiology of neurodegenerative conditions and conceive strategies to better diagnose and tackle these highly debilitating diseases.

Mammalian cells and mitochondria have had an indispensable symbiosis ongoing for several million years. Maternally inherited, mitochondria are the locus for many of the body's "housekeeping" functions such as the biosynthesis of amino acids and steroids, the beta-oxidation of fatty acids and of course of their "iconic" function of cellular fuel producers (Henze and Martin, 2003; McBride et al, 2006). They are therefore central to normal cell physiology but become increasingly relevant in cells in which the balanced bio-energy is pivotal to their function: in the case of this review, the neurons.

Neurons are very sensitive cells, highly susceptible to deterioration and death. They have a low regenerative and self-repair capability and are characterized by high metabolism that requires

a great amount of energy. The brain consumes the 20% of body's energy although it makes up 2% of the body's weight. Hence, neuronal demand for glucose and oxygen is massive and neurons are heavily dependent on internal sources of energy and consequently on mitochondria. Lack of physiological energy supply leads to deterioration of neurons and leads to a range of neurodegenerative diseases with different characteristics.

Although the exact molecular mechanisms are far from being exhaustively explained, there is increasing evidence for mitochondria as important contributors to the aetiology of neurodegenerative pathologies, especially in those that are age-related.

Neurodegeneration (ND) that leads to the death of neurons is a condition that arises with age and the

Key words: mitochondria, neurons, energy, dynamics, mitophagy

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number of patients afflicted by dramatic alterations of the cognitive or motor capacities is expected to increase substantially in the years ahead in line with increasing life expectancies. In spite of this, the treatments available are few and exclusively for symptomatic relief not for definitive cure, and in most cases, are associated with unpleasant side effects.

The development of effective therapies is therefore paramount and our limited knowledge of the pathways underlying these conditions restrains the possibility of tangible improvements in curative protocols. Understanding exhaustively the contribution of mitochondria to this plague of modern era may identify mitochondrial dysfunction as a recurring pathogenic theme in ND and consequently facilitate their targeting (please see Di Mauro and Schon, 2008 and references therein).

Quite remarkably, alterations in mitochondrial physiology go well beyond defects in energy production, embracing the regulation of mitochondrial dynamics, cell signalling and selective removal by quality-devoted processes: autophagy above all (Rubinsztein, 2006).

This review pinpoints the available literature on the subject highlighting the link between ND and mitochondria by citing and discussing examples in which mitochondrial dysfunction associates with degeneration and death of neurons. It is our aim to furnish the reader with a comprehensive picture of the mitochondrial role in neurodegenerative diseases, to stimulate novel angles of investigation and reason on what extent degeneration of neuron is consequent an overruling of the laws commanding mitochondrial biology.

IS THE NEURONAL FRAGILITY CAUSING ND A CONSEQUENCE OF GENETIC DEFICIENCIES IN RESPIRATION?

Mitochondria are the "powerhouses of the cell" and their most acknowledged activity is the production of adenosine triphosphate (ATP). This occurs via the combined efforts of the tricarboxylic acid cycle and the respiratory chain/oxidative phosphorylation system (OxPhos). The electron respiratory chain (ERC) is a set of biochemically linked multisubunit complexes (complexes I, II, III, and IV) and two

electron carriers (ubiquinone/coenzyme Q and cytochrome c). It has the unique capacity to couple O_2 to generate a H^+ gradient across the mitochondrial inner membrane, which drives ATP synthesis via the F_1F_0 -ATP synthase (or complex V), producing water (H_2O) at the same time. Although mitochondria have their own DNA (mtDNA), the ERC is the product of a joint effort between mitochondrial and nuclear genomes (Mitchell, 1967).

In their seminal work, DiMauro and Schon have described numerous syndromes caused by defects in respiration associated with impairment of the OxPhos (see for example DiMauro and Schon, 2003). Most of these syndromes are caused by either mtDNA or nuclear DNA mutations and display a variety of neurological features that arise during infancy. However, of these, just few associate with the adult-onset neurodegenerative diseases, i.e. the Parkinsonism, mitochondrial encephalo-myopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome (De Coo et al, 1999) and the Spinocerebellar ataxia (SCA) (Silvestri et al, 2000). Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's Disease (AD) have all been associated with mtDNA polymorphisms but with a yet ill-defined pathogenicity. Exception seems the Friedrich's ataxia (FA), a condition that still exhibits a severe neurological phenotype, but seems caused by defects in the Frataxin protein that leads to a defective synthesis of iron-sulfur proteins embedded within mitochondria (Rötig A et al 1997). Other examples of ND caused by mtDNA mutations include the recessive form of SCA caused by inefficient synthesis of coenzyme Q in the respiratory chain (Gerards et al, 2010) and rare forms of cerebellar degeneration caused by mutations in mtDNA polymerase γ and in the helicase Twinkle (Hakonen et al, 2008).

The link between PD and mtDNA alterations lies in the genes encoding the ERC's subunits (complex I) and the enzymes involved in glucose metabolism, all of which are regulated by peroxisome proliferator-activated receptor gamma co-activator 1 α (PGC-1 α) (Zheng et al, 2010; Nishioka et al, 2010). Relevant for this pathogenic interaction was also the identification of Parkin-interacting substrate (PARIS) (Shin et al, 2011). Parkin is a cytosolic ubiquitinating protein responsible for selective removal of defective mitochondria (to be discussed

below), and is mutated in some cases of hereditary PD. Inactivation of Parkin, either by mutation or environmental stress, leads to accumulation of PARIS and consequent inhibition of PGC-1 α transcription, which in turn may reduce mitochondrial biogenesis and cause deficiency in respiration (Shin et al, 2011). Accumulation of large-scale deletions of mtDNA were found in the substantia nigra of sporadic forms of PD (Bender et al, 2006; Kravtsov et al, 2006; Dauer and Przedborski, 2003) although in these forms of the disease a precise evidence for a link with mtDNA mutations are still missing (Simon et al, 2010).

In spite of this many are the features that distinguish the adult-onset neurodegenerative diseases from the primary diseases of mitochondrial complexes, the so-called "mitochondrial cytopathies" (Ackrell, 2002) which present several neurological features at relatively young age (please refer to DiMauro and Schon, 2003; Schon and Przedborski, 2011 and references therein). Whether late-onset ND is contributed to by i) mitochondrial respiratory defects unleashed by events such as increased oxidative stress [with increased production of Reactive Oxygen Species (ROS) and O₂^{•-}], ii) reduced efficiency of the repairing mechanisms and/or iii) evasion of quality control is still unclear and not adequately investigated; even though remains a real and fascinating possibility.

IS THE CONSUMPTION OF ENERGY (ATP) BY MITOCHONDRIA AN UNDERLYING AND NEGLECTED CONTRIBUTOR OF ND?

Increased free radical damage and oxidative stress associated to ageing, are among the risk factors in the majority of neurodegenerative pathologies (Lin and Beal, 2006). Mitochondria are the primary consumers of oxygen among the organelles and although the electron flux is terminated in a four-electron reduction of molecular oxygen to water (catalized by Complex IV, the Cytochrome c oxidase), numerous are the redox enzymes, contained within the organelle, capable of transferring single electrons to oxygen, to generate a vast number of ROS, like superoxide (O₂^{•-}). This renders mitochondria the most prominent source of ROS in the cell despite their antioxidant defence systems focused to protect their enzymes and

mtDNA from oxidative stress (Lin and Beal, 2006).

Oxidative stress and impairment of cell respiration are commonly detectable in ND, and are associated with an often under considered organelle pathology: the shifting of mitochondria toward ATP consumers rather than ATP producers. This is caused by the reversal of F₁F_o-ATP synthase activity and controlled by the enzyme's regulator the Inhibitor Factor 1 (IF₁) (please refer to Campanella et al, 2008; 2009a; 2009b).

Both primary defects in ETC complexes and those recorded in ND (see precedent paragraph) that affect the organelle's respiration, trigger the reversal of F₁F_o-ATP synthase and transform mitochondria into ATP consumers (Campanella et al, 2009b): a condition that worsens cellular damage and accelerates death.

Neurons are highly oxidative cells and repercussions on bio-energetic insufficiency caused by mitochondrial ATP hydrolysis result in a progressive deregulation of Ca²⁺ and Na⁺ homeostasis, with enhanced glutamate release and excitotoxicity (Childs et al, 2007). Moreover, the increased ROS production occurring with the reversal of the F₁F_o-ATP synthase, induces the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), resulting in a further limitation of ATP generation by glycolysis.

The IF₁, acting as a molecular regulator of the F₁F_o-ATP synthase, inhibits the reversal of the enzyme and protects the cell from damage by reducing both mitochondrial ATP consumption and ROS generation. When the respiratory chain is slowed down, or when $\Delta\psi_m$ increases over the value for maximal rates of F₁F_o-ATP synthase function (>120-140mV) and the enzyme reverses its activity, hydrolyzing ATP to pump H⁺ into the intermembrane space, more electrons are diverted from their normal pathway, resulting in elevated ROS production (DiMauro et al, 1976; Carrozzo et al, 2001).

We have demonstrated that ROS generation is limited in IF₁ over-expressing HeLa cells and that the cellular level of the protein affects the morphology and dynamics of the mitochondria (Campanella et al, 2009a).

Notably, the protein is highly expressed in neurons compared to glia and it plays a relevant role in modulating respiration and autophagy (Campanella

et al, 2009b). Interestingly, mitochondrial dynamics, including fusion, fission, transport and mitophagy are altered in several ND thus rendering the cells more susceptible to apoptotic stimuli (Chen and Chan, 2009). Proteins involved in the regulation of these processes are therefore the focal point of studies aimed at unravelling the molecular bases of ND.

DO DEFECTS IN MITOCHONDRIAL DYNAMICS CONTRIBUTE TO ND?

Mitochondria are dynamically transported on microtubules and actin cables (Jung et al, 2004) and a great deal of literature on the subject has emerged in recent years. This is core to consent the indispensable processes of fusion and fission and the preservation of mitochondrial network shape. Movement on actin, which is mostly short-range, requires myosin motors, whereas long-range movement on microtubules requires dynein/dynactin and kinesins respectively for retrograde and anterograde transport (Hollenbeck, 1996). Although direct evidence for a correlation between defects in mitochondrial trafficking/dynamics and ND does not exist -since the actual mitochondrial capacity for movement cannot be assessed in post-mortem samples of human patients- hints on this are anyway still retrievable in the literature.

The efficiency of mitochondrial transportation is instrumental to neuronal health and as such the "toolkit" of proteins associated with mitochondrial quality (see Fig. 1) seems to cooperate with those associated with mitochondrial movements on microtubules (see Wang et al, 2011 and references within).

Additionally, data exist for disorders in mitochondrial network's morphology in AD that associates with the reduced expression of a microtubule motor kinesin-1 (Stokin et al, 2005). AD, a disorder targeting primarily the "short" neurons within cortex and hippocampus, displays features of aberrant axonal trafficking of cargo (Stokin et al, 2005), altered mitochondrial movements (Wang et al, 2009a) and dynamics (Wang et al, 2008; 2009b).

Huntington's disease (HD), a fatal adult-onset chorea targeting the relatively short striato-pallidal neurons, may also be classified as a disorder of mitochondrial trafficking. It is caused by mutations

in huntingtin (HTT), that, if transfected in primary rat cortical neurons, blocks mitochondrial movement (Chang et al, 2006) and impairs trafficking of the organelles (Trushina et al, 2004) in both the anterograde and retrograde directions (Orr et al, 2008). Other interesting aspects are that the HTT binding partner huntingtin-associated protein 1 (HAP1) takes contact with-membranous organelles, including mitochondria (Gutekunst et al, 1998) and interacts with both kinesin and dynein/dynactin to regulate the transport of cargo on microtubules (Bossy-Wetzel et al, 2008; Sack, 2010).

Further, Milton, a mitochondrial microtubule adaptor (Glater et al, 2006) binds HTT and dynactin (Stowers et al, 2002) suggesting that altered mitochondrial trafficking might contribute to neuronal damage in HD (Bossy-Wetzel et al, 2008; Kim et al, 2010; Oliveira, 2010).

Quite notably, the expression of mitochondrial fission-related proteins, such as FIS1 and DRP1 (Costa et al, 2010), which interact with HTT (Song et al, 2011) are increased in striatum and frontal cortex of HD patients, whereas fusion-related proteins, including MFN1, MFN2, and OPA1, are decreased (Shirendeb et al, 2011). The bottom line is that fragmented mitochondria and deregulation of proteins that dictate the network physiological dimension may contribute to the pathogenesis of ND kind (Pandey et al, 2010; Shirendeb et al, 2011).

While the above examples refer to pathologies targeting short neurons, in the case of ALS, which affects instead motor neurons -i.e., long neurons in which mitochondria must travel for longer distances to sustain the cellular activity- the use of genetically engineered mice has provided a greater amount of data to study defects in mitochondrial trafficking. The mutant superoxide dismutase-1 (SOD-1) as well as the misfolded isoform tackle both the anterograde (De Vos et al, 2007) and retrograde (Shi et al, 2010) mitochondrial transport (Bosco et al, 2010). Moreover, impaired mitochondrial trafficking has also been observed upon overexpression of at least two other proteins whose mutations cause familial forms of ALS (Millecamps et al, 2005; Shan et al, 2010).

The hereditary spastic paraplegia (HSP) is due to mutations in proteins associated with the cytoskeleton such as Spartin and the receptor

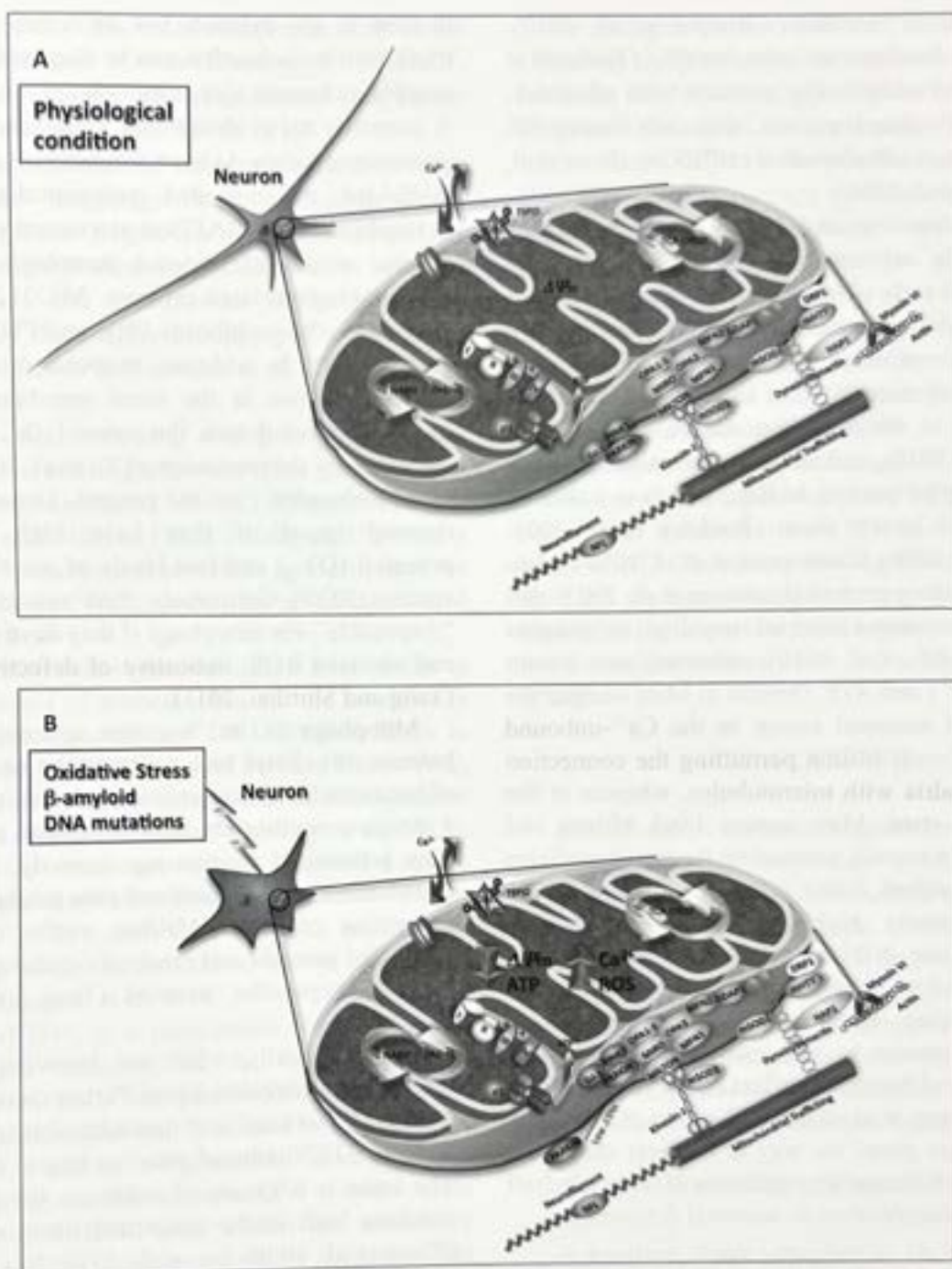


Fig. 1. Mitochondrial Pathways Targeted in Neuronal Pathology **A.** Mitochondrial bio-energy and proteins interaction at resting physiology; **B.** Neurodegenerative stress conditions that promote cause mitochondrial bioenergetic dysregulation (Ca^{2+} , ROS, \sim ATP), aberrant mitochondrial trafficking, altered interorganellar communication, and impaired mitochondrial quality control.

expression-enhancing protein 1 (REEP1). These are mutations that promote ND without affecting mitodynamics. Spartin is localized on microtubules and mitochondria via sites of the N- and C-terminal

regions of the protein, respectively (Lu et al, 2006). The proteomic analysis implies a role in protein folding and turnover both in mitochondria and ER (Milewska et al, 2009), and a possible involvement

in lipid droplet formation (Hooper et al, 2010). REEP1 also localizes on mitochondria (Zuchner et al, 2006), and additionally interacts with atlastin-1, another HSP-related protein, that coordinates ER shaping but not mitochondrial trafficking (Bian et al, 2011; Park et al, 2010).

More exhaustive and highly relevant for the understanding of neuronal physiology are the evidences recently obtained on the adaptor proteins of mitochondria on cellular skeleton. In neurons, two cargo-adaptor proteins, Miro and Milton, regulate the linkage of mitochondria to kinesin-1. The first is anchored to the mitochondrial outer membrane (Guo et al, 2005), and binds to the mitochondrial-specific adaptor protein Milton, which is linked to the kinesin-1 heavy chain (Brickley et al, 2005; Glater et al, 2006; Koutsopoulos et al, 2010). Miro is a Ca^{2+} -binding protein (Fransson et al, 2003) that regulates the mitochondrial motility in neurons (MacAskill AF et al, 2010), operating as a sensor of local $[Ca^{2+}]$ and ATP. Defects in Miro hamper the extension of neuronal axons: in the Ca^{2+} -unbound state, Miro binds Milton permitting the connection of mitochondria with microtubules, whereas in the Ca^{2+} -bound state, Miro cannot bind Milton and mitochondria remain uncoupled from microtubules (Rice and Gelfand, 2006).

These results highlight the evidences that possibly relate ND to defects in mitochondrial dynamics and mitochondrial anchoring mechanisms to cytoskeleton. Finally, the PD-related protein α -synuclein seems to influence the mitochondrial quality control through its effect on the fusion/fission balance (Kamp et al, 2010; Nakamura et al, 2011). This evidence paves the way to the next chapter in which we will discuss the regulation of mitochondrial quality and contribution to neuronal deficiency.

HOW MUCH IS IMPORTANT THE MITOCHONDRIAL QUALITY CONTROL TO AVOID ND?

If the right positioning of mitochondria is paramount to health of neuronal cells, the preservation of their quality possibly more. Cells have surveillance mechanisms to eliminate mutated, unfolded, and otherwise unwanted proteins, via autophagic and ubiquitin-proteasome systems

located in the cytosol. Via the same mechanism, inefficient mitochondria can be disposed by targeted autophagy known also as mitophagy. Although there is currently no evidence that mitochondria contain proteasomes, they do have mechanisms to eliminate misfolded or unneeded polypeptides, via, for example, the AAA (ATPase associated with diverse cellular activities) protease paraplegin/SPG7 and the paraplegin-related protease AFG3L2, and their regulators, the prohibitins PHB and PHB2 (Osman et al, 2009). In addition, mitochondrial proteins, especially those in the outer membrane, can be retro-translocated into the cytosol for subsequent clearance by the proteasome (Xu et al, 2011).

Mitochondria, as in general knowledge, are deemed 'good' if they have high membrane potential ($ΔY_m$) and low levels of reactive oxygen species (ROS). Conversely, 'bad' mitochondria are "disposable" via mitophagy if they have a low $ΔY_m$ and elevated ROS, indicative of defective OxPhos (Twig and Shirihai, 2011).

Mitophagy is an "extreme approach" in the balance of cellular biology since the accumulation of bad mitochondria is limited by the maintenance of a dynamic equilibrium between fission and fusion. This process, if functioning correctly, allows the complementation of genes and gene products thereby preventing protein misfolding and/or eliminating misfolded proteins and randomly mutated mtDNAs from the organelles' network (Twig and Shirihai, 2011).

Mechanistically what we know is that the cytosolic E3 ubiquitin ligase Parkin co-operates via another PD-related and mitochondrially localized protein, PTEN-induced putative kinase 1 (PINK1). The latter is a kinase of unknown specificity that localizes both in the inner and outer membranes (Zhou et al, 2008; Jin et al, 2010; Silvestri et al, 2005). PINK1 presents two forms, the long (64 kDa) and the short (52kDa), with the latter cleaved within the mitochondrial inner membrane by presenilin-associated rhomboid-like protein (PARL) (Deas et al, 2011; Jin et al, 2010).

The model states that, upon loss of $ΔY_m$ in damaged mitochondria, PINK1 residing in the outer membrane triggers the recruitment of cytosolic Parkin to the mitochondria (Jin et al, 2010; Narendra et al, 2010b; Vives-Bauza et al, 2010). Mitochondrial

proteins located in the outer membrane, such as the Voltage-Dependent Anion Channel 1 (VDAC1; also called porin) are then ubiquitinated in a Parkin dependent manner (Geisler et al, 2010).

The ubiquitination of outer membrane proteins recruits the autophagy molecule microtubule-associated protein-1 light chain-3 (LC3) to build the autophagosome around the damaged mitochondrion (Vives-Bauza and Przedborski, 2011): a process mediated by the adaptor proteins p62 and HDAC6 (Geisler et al, 2010; Narendra et al, 2010a; Okatsu et al, 2010).

How Parkin, following a drop in DY_m , induces ubiquitination and degradation of other mitochondrial proteins [e.g. mitofusins MNF1/2 (Gegg et al, 2010; Ziviani et al, 2010), or DRP1 (Wang et al, 2011)] is not yet fully understood as well as how critical is the fragmentation of mitochondria for timed removal of the organelles.

Other PD related proteins with a relationship to quality control of mitochondria are the α -synuclein –as mentioned above- and DJ-1. DJ-1 is likely to interact with both PINK1 and Parkin (Moore et al, 2005) and to modulate mitochondrial fission/fusion in a ROS-dependent manner (Irrcher et al, 2010). This effect is consistent with its proposed function as scavenger of mitochondrial H_2O_2 (Andres-Mateos et al, 2007). Moreover, DJ-1 seems to regulate the expression of the mitochondrial uncoupling (UCP) proteins (Guzman et al, 2010).

Loss of DY_m is a prerequisite for the disposal of damaged mitochondria, the loss- of-function mutations in DJ-1 that causes PD may therefore impair mitochondrial quality control by distorting the relationships among mitochondrial damage, DY_m , and mitophagy. In this scenario, DJ-1 would operate upstream of PINK1/Parkin within the mitophagy pathway, an idea consistent with the demonstration that silencing DJ-1 in human cell lines does not affect PINK1- dependent recruitment of Parkin and ensuing mitophagy in response to collapse by membrane uncoupling (Vives-Bauza et al, 2010). Clearly, further work must determine how the activity of PD-related proteins intersects with mitodynamics and contributes the pathogenesis of the disease.

A mitophagy model for pathogenesis in PD is absolutely appealing but future studies are needed to assess it properly and overcome the criticisms over:

i) the endogenous low level of PINK1 and Parkin (Kitada et al, 2009) and ii) the experimental protocols currently employed to trigger Parkin recruitment. The latter sees the use of the uncoupler m-chlorophenyl hydrazone (CCCP) or the p-trifluoromethoxy form (FCCP) that may damage proteins not necessarily involved in the process besides representing per se a non-physiological stimulus that acts too indiscriminately on the bio-energy of the organelle.

However, a few aspects of the current model require improvements and further experimental endeavours to map its actual hierarchy and efficiency in brain tissues look indispensable.

The impaired mitochondrial quality control due to inefficiency of its regulation is one of the underlying pathogenic mechanisms in PD likewise of other kinds of ND. That many leading laboratories in the field are working diligently to unravel this process highlights the importance this regulatory mechanism has in modern neuroscience and pathologies of the brain.

CONCLUSIONS

Mitochondrial dysfunction undoubtedly -although not always directly- contributes to ND. Alterations in: i) quality control, iii) metabolism regulation and iii) susceptibility to death are all mito-associated phenomena that contribute the making of neuronal pathology. This remarks the necessity for innovative and continuous efforts to comprehend exhaustively the origin and pattern of brain's conditions via deep exploitation of mitochondrial function that stands up as a reliable read out of neuronal homeostasis.

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INTERLEUKIN-1 β IN CENTRAL NERVOUS SYSTEM INJURY AND REPAIRS.J. HEWETT¹, N. A. JACKMAN^{2*} AND R. J. CLAYCOMB^{3*}¹*Department of Biology, Program In Neuroscience, Syracuse University, USA*²*Department of Neuroscience, University of Connecticut Health Center, Farmington, USA*³*Department of Neurology, Yale University, New Haven, USA***Both authors contributed equally to this work*

Acute inflammation is a self-limiting, complex biological response mounted to combat pathogen invasion, to protect against tissue damage, and to promote tissue repair should it occur. However, unabated inflammation can be deleterious and contribute to injury and pathology. Interleukin-1 β (IL-1 β), a prototypical "pro-inflammatory" cytokine, is essential to cellular defense and tissue repair in nearly all tissues. With respect to brain, however, studies suggest that IL-1 β has pleiotropic effects. It acts as a neuromodulator in the healthy central nervous system (CNS), has been implicated in the pathogenic processes associated with a number of CNS maladies, but may also provide protection to the injured CNS. Here, we will review the physiological and pathophysiological functions of IL-1 β in the central nervous system with regard to synaptic plasticity. With respect to disease, emphasis will be placed on stroke, epilepsy, Parkinson's disease and Alzheimer's disease where the ultimate injurious or reparative effects of IL-1 β appear to depend on time, concentration and environmental milieu.

INTERLEUKIN 1 IN THE CNS

Interleukin-1 is a cytokine released by many cell types that acts in autocrine and/or paracrine fashion, thereby stimulating a variety of signaling pathways (for a more detailed review see Dunne and O'Neill, 2003; O'Neill and Greene, 1998; Weber et al, 2010). Although since expanded, the canonical family members consist of two agonists, IL-1 α and IL-1 β , an endogenous antagonist, IL-1 α , and two receptors (IL-1RI and IL-1RII) [reviewed in (Dinarello, 2009a; Weber et al, 2010)]. IL-1R accessory protein (IL-1RAcP), which complexes with IL-1RI following binding IL-1, is a required receptor partner in signaling (Cullinan et al, 1998). All ligands and receptors are expressed in the healthy CNS at low

levels (Breder et al, 1988; Lechan et al, 1990; Molenaar et al, 1993), though data on distribution of the signaling receptor IL-1RI (Greenfeder et al, 1995) suggest that distinct brain regions may depend differentially on the IL-1 system, at least under basal conditions (Ban et al, 1991; French et al, 1999; Gayle et al, 1997). IL-1RII cannot transduce signals due to a short cytoplasmic tail, effectively rendering it a decoy receptor (Colotta et al, 1994; McMahan et al, 1991). IL-1 α binds to IL-1RI and thereby prevents IL-1 binding and subsequent signal transduction (Dinarello, 1998; Dripps et al, 1991). Interestingly, proteolytic cleavage of the IL-1RI and RII extracellular domains produces soluble receptors whose binding to IL-1 α and IL-1 β , respectively, lead to either an enhanced (sILR1) or diminished

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(sIL-RII) inflammatory response (Arend et al, 1994; Preas et al, 1996). The presence of two inhibitors, IL-1ra and IL-1RII (both membrane bound and soluble forms) suggest that this system is tightly controlled for the maintenance of cellular health.

At first viewed as merely a peripheral messenger that communicated with the CNS via passage across the blood brain barrier (Banks and Kastin, 1991; Banks et al, 1991), IL-1 is now known to be produced directly by cells of the CNS including microglial cells (Giulian et al, 1986; Hetier et al, 1988; Yao et al, 1992), astrocytes (Knerlich et al, 1999; Lieberman et al, 1989; Zhang et al, 2000), oligodendrocytes (Blasi et al, 1999), and neurons (Lechan et al, 1990; Takao et al, 1990; Watt and Hobbs, 2000). These same cell types are also capable of responding to the cytokine (Ban et al, 1991; Ban et al, 1993; Blasi et al, 1999; Cunningham and De Souza, 1993; French et al, 1999; Friedman, 2001; Hammond et al, 1999; Pinteaux et al, 2002; Tomozawa et al, 1995; Wang et al, 2006; Wong and Licinio, 1994). Interestingly, neurons express a novel functional isoform of IL-1RAcP that mediates an alternative signaling pathway (Huang et al, 2011). Further, a yet-to-be-identified IL-1 signaling receptor within the CNS has been postulated. Touzani and colleagues report that exogenously administered IL-1 β significantly increases cerebral ischemic damage in IL-1RI null mice compared to vehicle-treated control mice, in a manner that could not be obviated by co-administration of IL-1ra (Touzani et al, 2002). IL-1 β treatment increases the expression of approximately 400 genes in mixed glial cultures derived from IL-1RI-null mice (Andre et al, 2006). Finally, outside-out patched membranes from retinal ganglion cells — which lack all intra-cytoplasmic signaling machinery — respond to exogenously applied IL-1b (5 ng/mL for 150 sec) with decreased sodium and potassium currents that could not be reversed by the co-application of IL-1ra (Diem et al, 2003). Although these studies suggest that IL-1 β can signal in an IL-1RI-independent manner, no identification of this putative, alternative CNS IL-1 signaling receptor has been proffered.

With respect to the two agonists, IL-1 α is translated as a 31-kD pro-peptide, although, prior to cleavage, this molecule still has full biological activity; that is, pro-IL-1 α initiates signal transduction when bound to its receptor (Mosley et al, 1987). Interestingly,

IL-1 α does not contain a leader sequence, therefore, it cannot be released by the cell via normal Golgi apparatus-mediated vesicular exocytosis. Instead, after translation it remains within the cytoplasm where it becomes myristoylated and inserted into the plasma membrane. The majority of pro-IL-1 α remains within the cell (Endres et al, 1989; Lonnemann et al, 1989; Schindler et al, 1990); however, upon cell injury/death, it can be released into the extracellular space where cleavage by extracellular proteases can occur. Therefore, the current literature supports the hypothesis that the major function of IL-1 α is that of an autocrine growth factor and/or a mediator of local inflammation (Dinarello, 1996; 2009a).

Unlike IL-1 α , IL-1b has a TATA box within its promoter region in addition to a cAMP responsive element (Shirakawa et al, 1993; Tsukada et al, 1994), an NF- κ B binding site, an AP-1 site and an Sp-1/PU.1 binding site (Shirakawa et al, 1993). These transcriptional elements allow for the induction of IL-1b mRNA by a variety of microbial (e.g., lipopolysaccharide [LPS] and teichoic acid) and non-microbial stimuli (e.g., hypoxia, hyperosmolarity, thermal injury and gamma radiation). Translation occurs upon activation of MAP kinases (Lee et al, 1994) yielding a 31-kD pro-peptide, pro-IL-1b, which must be cleaved by the cysteine protease caspase-1 (a.k.a. interleukin-1 converting enzyme or ICE) to adopt biological activity (Cerretti et al, 1992; Thornberry et al 1992). Like IL-1 α , absence of a signaling sequence indicates that a classical pathway of exocytosis is not utilized for IL-1 β release. While the exact mechanism by which IL-1b is released from cells is unknown, there is some consensus that — at least from cells of the monocyte/macrophage lineage — IL-1 β release is dependent on ATP, its purinergic receptor P2X, (Bianco et al, 2005; Clark et al, 2010; Sanz and Di Virgilio, 2000; Solle et al, 2001), and calcium (Andrei et al, 2004; MacKenzie et al, 2001). However, while macrophages lacking P2X, receptors fail to secrete IL-1 β following ATP exposure, secretion induced by the K⁺ ionophore, nigericin, is not altered in the P2X₂-deficient cells indicating the involvement of an additional regulatory pathway(s) (Solle et al 2001). A few major hypotheses have been put forth (Singer et al, 1995; Andrei et al, 2004; MacKenzie et al, 2001; Brough and Rothwell, 2007; Qu et al, 2007).

Since most studies to elucidate release mechanism utilize cells of the macrophage lineage, further study in different cell types is warranted (Yazdi et al, 2010). To wit: there is evidence that IL-1 β can be released exocytotically from neurons (Tringali et al, 1996; Tringali et al 1997; Watt and Hobbs, 2000). Hypothalamic explant cultures release IL-1 β when incubated with high K⁺ via a process blocked by tetrodotoxin and voltage-gated Ca²⁺ blockers (ω -conotoxin and verapamil), implicating calcium-dependent exocytosis (Tringali et al, 1996; Tringali et al, 1997). Loss of IL-1 β immunoreactivity from nerve terminals of vasopressin and oxytocin positive neurons within the neurohypophysis upon sustained lactation (facilitating oxytocin release) or a hyperosmotic challenge (facilitating vasopressin release) provides further support for a neuronal release mechanism (Watt and Hobbs, 2000). Whether calcium-dependent exocytotic release occurs from neurons from other areas of the brain seems likely, but remains to be experimentally established.

IL-1 β IN CNS PHYSIOLOGY: POSITIVE OR NEGATIVE MODULATORY FUNCTION?

The presence of IL-1RI and IL-1 β under basal conditions in the CNS suggests a normal physiologic role for IL-1 β . Indeed, IL-1 β has important functions in the regulation of core body temperature, which has been extensively studied (for a detailed review see Dantzer and Kelley, 2007; Dinarello, 2004). Additionally, compelling evidence suggests a physiological role for IL-1 β in sleep (De Sarro et al, 1997; Imeri and Opp, 2009; Krueger et al, 2001; Taishi et al, 1997). Of particular interest to this review is the neuromodulatory effects of IL-1 β on synaptic transmission.

Synaptic Plasticity

The first study to investigate a role for IL-1 β in synaptic plasticity did so using a long term potentiation (LTP) paradigm. LTP is defined as a persistent increase in synaptic efficacy and is thought to be a biological correlate to learning and memory (Bliss and Collingridge, 1993). Exogenous application of IL-1 β substantially reduced the magnitude of long term potentiation (LTP) when applied 20 min prior to tetanic stimulation of the rat mossy fiber path (Katsuki

et al, 1990). Furthermore, this effect was blocked using the synthetic tripeptide Lys-D-Pro-Thr, a reagent previously shown to antagonize the peripheral hyperalgesic effects of IL-1 β (Ferreira et al, 1988). Interestingly, the tripeptide alone, which presumably antagonized any endogenous IL-1 β released, did not affect LTP acquisition, suggesting that endogenous IL-1 signaling did not contribute to this form of synaptic plasticity under these experimental conditions (Katsuki et al, 1990). Although antagonism of LTP in the mossy fiber pathway is highly correlative with amnesia (Sato et al, 1986; Sato et al, 1988), it is not a model of classical Hebbian NMDA-receptor dependent LTP (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990). Bellinger and colleagues were the first to investigate the effects of IL-1 β on this form of LTP (Bellinger et al, 1993). IL-1 β applied both 10 and 60 min prior to tetanus-induced LTP also significantly attenuated the induction of LTP within the rat Schaffer collateral pathway (Bellinger et al, 1993).

Surprisingly, experiments designed to examine the effects of endogenous IL-1 β on LTP found opposite results. IL-1 β mRNA expression is enhanced one hour after LTP induction in rat hippocampal slices *in vitro* and *in vivo* in the ipsilateral hippocampus of rats that had robust potentiation lasting at least 8 hr (Schneider et al, 1998). Blockade of endogenous IL-1 β by acute application of recombinant IL-1ra abolishes Schaffer collateral LTP in mouse (Ross et al, 2003) and prevents LTP maintenance (but not initiation) in rat (Schneider et al, 1998). Additionally, attempts to induce *in vitro* (Schaffer collateral) and *in vivo* (mossy fiber path) LTP in IL-1RI null mice either completely failed or the potentiation was drastically reduced (Avital et al, 2003).

These seemingly paradoxical effects of IL-1 β are observed in behavioral experiments as well. Mice completely lacking endogenous IL-1 signaling (IL-1RI null mice) demonstrate hippocampus-dependent learning deficits as measured by poorer performance in the Morris water maze (Avital et al, 2003), suggesting that endogenous IL-1 β is required for normal learning. In support, animals with chronic blockade of IL-1RI signaling in the CNS via transgenic overexpression of human IL-1ra also demonstrate impaired learning (Oprica et al, 2005; Spulber et al, 2009). Recombinant IL-1 β applied either into cerebral ventricles or directly

into the hippocampus of normal mice results in the impairment of memory (Barrientos et al, 2002; Hein et al, 2007; Pugh et al, 1999). And finally, animals engineered to overexpress IL-1 β chronically in hippocampus also demonstrate impaired spatial and contextual memory development (Matousek et al, 2010; Moore et al, 2009)

Altogether, these experiments highlight the complexity of IL-1 signaling on experimental LTP and ultimately in learning and memory animal behavioral paradigms. What the data suggest is that physiological levels of IL-1 β , presumably released from neurons, function as a neuromodulator to promote experimental LTP and hence memory acquisition and retention. In contrast, pathophysiological or "inflammatory levels" of IL-1 β — in the case of the overexpression studies from astrocytes but also potentially from resident microglial cells or infiltrating myeloid cells — antagonize the synaptic responses associated with LTP leading to failure of memory acquisition or its recall. Indeed, in an elegant study, Goshen and colleagues confirm that intra-hippocampal injection of a high concentration of IL-1 β or administration of rIL-1ra produce memory deficits, whereas infusion of a low concentration of IL-1 β facilitates memory formation in rat (Goshen et al, 2007). Interestingly, the hypothesis that an enhanced pro-inflammatory phenotype in brain plays a role in age-related cognitive decline has recently been posited (Viviani and Boraso, 2011). Possible mechanisms underlying the plasticity-modulating properties of IL-1 β involve its ability to positively and/or negatively regulate voltage- and ligand-gated neuronal ion channel excitability (Gardoni et al, 2011; Huang et al, 2011; Lynch, 1998; Miller et al, 1991; Viviani et al, 2003; Viviani and Boraso, 2011; Wang et al, 2000a; Yang et al, 2005; Zhang et al, 2008; Zhou et al, 2011). Overall, the ultimate effect of IL-1 β on LTP and associated learning and memory paradigms appear to be cell and target specific, as well as, concentration-dependent.

IL-1 β IN CNS PATHOPHYSIOLOGY: DELETERIOUS OR PROTECTIVE?

IL-1 is rapidly induced in brain tissue following acute brain injury and has been shown to be

upregulated in more classical neurodegenerative diseases. The predominate view-point is that IL-1 β contributes to and/or sustains the pathophysiological processes. However, studies also point to its potential role in protection and repair. Below, the more complex and contextual actions of IL-1 β in the CNS will be discussed.

Seizures and Epilepsy

The hippocampus, a brain structure implicated in the generation of seizures, has been shown to express both IL-1 β and IL-1RI (Ban et al, 1991; Breder et al, 1988; Farrar et al, 1987; French et al, 1999; Gayle et al, 1999; Huitinga et al, 2000; Lechan et al, 1990; Plata-Salaman et al, 2000; Takao et al, 1990; Wang et al, 2000a). Evidence from several studies suggests that IL-1 β may influence acute seizure development and activity and/or epileptogenesis, the process by which the brain becomes prone to spontaneous seizure activity. However, the nature of its role in these processes remains controversial. A comprehensive review has recently appeared (Rijkers et al, 2009).

Three single nucleotide polymorphisms (SNP) of the human IL-1 β gene have been identified and all involve C-to-T switches (Huynh-Ba et al, 2007; Shirts et al, 2006; Wang et al, 2007). Of the three, the IL-1 β -511T polymorphism has been associated with an increased susceptibility to seizures or epilepsy (Kauffman et al, 2008; Kanemoto et al, 2000; Kira et al, 2005; Ozkara et al, 2006). Interestingly, leukocytes taken from patients bearing the -511T haplotype produce less IL-1 β following LPS stimulation than those of haplotype -511C (Wen et al, 2006). Given the ability of IL-1 β to modulate synaptic currents (*vide supra*), it is interesting to speculate that an increased susceptibility to seizures — defined as a transient disturbance of normal cerebral function caused by abnormal neuronal discharges (Victor and Ropper, 2002) — might result from a deficit of IL-1 β production.

Recent data from our lab supports this supposition. Using transgenic mice harboring targeted deletions in the genes for IL-1 β or its signaling receptor, IL-1RI, — which addresses the role of endogenous IL-1 β production — we find that the incidence of convulsive seizures induced by the chemoconvulsant pentetate (PTZ)

increases in both null mutant mouse lines compared to their respective wild-type littermate controls (Claycomb et al, 2012). Hence, the lack of IL-1 β signaling reduced PTZ seizure threshold suggesting that IL-1 β functions to suppress or dampen neuronal excitability (Claycomb et al, 2012). This differs from a previously published study using IL-1RI null mutant mice, which demonstrated that motor seizures are delayed following intrahippocampal bicuculline administration with no change in severity or incidence of seizures (Vezzani et al, 2000). Moreover, the incidence of seizures induced by intrahippocampal bicuculline injection in mice genetically engineered to overexpress recombinant IL-1ra (rIL-1ra) in the CNS was reported to be reduced, supporting a pro-convulsant role for endogenous IL-1 β (Vezzani et al, 2000). Differences in the chemoconvulsant (bicuculline vs. PTZ) and its route of administration (intrahippocampal vs systemic) and the background strain (129/SV vs. C57Bl/6) might explain these discrepant results. However, a higher incidence of sustained generalized convulsive seizure behavior and mortality following systemic kainic acid administration was observed in IL-1 β null mice in a B6/B10 background, as compared to their wild-type littermate controls (Claycomb et al, 2012), indicating that the potential anti-seizure actions of endogenous IL-1 β is neither model nor background specific.

When IL-1 β is administered exogenously, most (De Simoni et al, 2000; Dube et al, 2005; Ravizza et al, 2008b; Ravizza et al, 2006; Ravizza and Vezzani, 2006; Vezzani et al, 1999; Vezzani et al, 2000; Vezzani et al, 2002; Vezzani et al, 2004) but not all (Miller et al, 1991; Sayyah et al, 2005) studies report a pro-convulsive phenotype. Although the reasons for this discrepancy are not immediately evident, differences in experimental paradigms here too exist. For example, the route of administration of convulsant differed. When convulsant stimuli were administered locally into the hippocampus, exogenous IL-1 β promoted seizures (De Simoni et al, 2000; Vezzani et al, 1999; Vezzani et al, 2000), whereas it suppressed seizure activity generated when the convulsant was administered systemically (Miller et al 1991). Additionally, intracerebroventricular administration of IL-1 β exhibited anti-convulsant actions elicited by electrical stimulation of the amygdala (Sayyah et al, 2005) but pro-convulsant properties when electrical

seizures were initiated in the hippocampus (De Simoni et al, 2000).

The conclusion that IL-1 β contributes to the process of epileptogenesis must also be approached with caution. Although levels of IL-1 β in the hippocampi and cortex of epileptic EL mice are elevated during the time of presumed epileptogenesis (Murashima et al, 2008) and expression of both IL-1b and IL-1RI are up-regulated in the kindled CNS (Plata-Salaman et al, 2000), this is merely an association. Given our results, it seems plausible that the increases of endogenous IL-1 β and IL-1RI may serve as compensatory response geared toward dampening seizure activity associated with epileptogenesis (Claycomb et al, 2012). Indeed, administration of IL-1 β antagonized electrical-kindling of the amygdala (Sayyah et al, 2005), although pharmacological antagonism of ICE suppressed electrical-kindling of the hippocampus (Ravizza et al, 2008).

Overall, available literature with respect to the role of IL-1 β in seizure generation and epilepsy formation is contradictory and could potentially be due to the variations in experimental models employed. It is clear that the effects of IL-1 β differ when produced endogenously or offered exogenously, vary by brain region, and may even be dependent on the type of convulsant utilized. While no model is perfect, the question as to which, if any, most effectively mimics the human condition is, at present, unanswerable. Thus, testing the validity of the experimental findings via demonstration that neutralization of IL-1 β signaling provides a positive — and not negative outcome as some data might predict — will only be determined via clinical testing.

Parkinson's Disease

Substantia nigral dopaminergic neuronal cell loss is pathoneumonic of Parkinson's disease (PD). Although the exact causes are not known, the disease is associated with a profound inflammatory response evident at the histological level by the presence of microgliosis and astrocytosis. The prevalent view is that inflammatory processes play an important role in pathogenesis of this disease (Tansey et al, 2007). Increased IL-1 β levels have been detected in the cerebrospinal fluid and in the striatum post-mortem of PD patients (Mogi et al, 1994) as compared

to control patients and tissues. Single-nucleotide polymorphisms (SNPs) in the IL-1 α and β genes have been reported to be more frequent in some (McGeer et al, 2002; Wahner et al, 2007) but not all PD cohorts (Pascale et al, 2011; Moller et al, 2004). However, experimental data points to a role for IL-1 in both injury and repair.

In the MPTP mouse model of PD, treatment with minocycline prevents activation of microglia, IL-1 β release, and dopaminergic neuronal cell death (Wu et al, 2002), suggesting a possible role for IL-1 β in neuronal cell death. Supporting this supposition, Ferrari and colleagues report that chronic expression of IL-1 β in the rat substantia nigra (using recombinant adenovirus) elicited most of the characteristics of PD, including progressive dopaminergic cell death, akinesia and glial cell activation (Ferrari et al, 2006). Taking the opposite approach, and coming to the same conclusion, Klevenyi and colleagues demonstrate that mice deficient in ICE are less susceptible to MPTP toxicity *in vivo* (Klevenyi et al, 1999).

In contrast, in the 6-hydroxydopamine (6-OHDA) model of PD, mice deficient in IL-1RI, show a worse disease outcome — defined as lack of dopaminergic neuron sprouting after lesioning — compared to the control cohort, implying that IL-1 imparts a protective role (Parish et al 2002). Remarkably, IL-1RI $-/-$ animals completely lacked microgliosis and astrogliosis, leading the authors to speculate that decreased neuronal sprouting was due to a lack of trophic support from activated glial cells (Parish et al, 2002). Indeed, several studies demonstrate that IL-1 β -stimulated astrocytes support neuronal survival via production of neurotrophic factors (Albrecht et al, 2002; John et al, 2005; Saavedra et al, 2007). However, others demonstrate that IL-1 β can interfere with neurotrophin signaling (Soiampornkul et al, 2008; Tong et al, 2008). Regardless of the exact mechanisms by which this might occur, Parish's study corroborates the results of a much earlier study performed in 6-OHDA lesioned rats (Wang et al, 1994). In this study, both histological (tyrosine hydroxylase immunoreactive (TH-IR) fibers) and behavioral outcomes (amphetamine-induced turning) are improved when IL-1 pellets are implanted directly into the caudate nucleus when compared to placebo-treated animals assessed 8 weeks after the

lesion (Wang et al, 1994). Hence, in these 6-OHDA studies, IL-1 β appears to encourage repair.

Overall, it would appear that the potential mechanisms by which IL-1 β may influence the development, progression or protection from PD requires further exploration. As it is now accepted for eicosanoids (Serhan et al, 2007), and also seems likely for Alzheimer's disease (*vide infra*), it seems plausible that IL-1 β may be detrimental early on via contribution to the pathological environmental milieu, but in later stages or when presented in a pharmacological context, could contribute to regeneration and repair. A better understanding of the timing and duration of the inflammatory vs. repair response could be crucial to devising effective neuroprotective therapies for PD.

Alzheimer's Disease

A role for IL-1 β in amyloid plaque formation in the AD brain was first postulated by Vandenabeele and colleagues (Vandenabeele and Fiers, 1991). Elevated levels of IL-1 were found in post-mortem brain tissue from (Griffin et al, 1989), as well as in CSF (Blum-Degen et al, 1995; Cacabelos et al, 1991) of Alzheimer's disease patients. Thus the hypothesis that excessive expression of IL-1 in brain might represent the driving force for the cascade of events that culminate in the neuropathological changes characteristic of AD, namely neuritic tangles and amyloid plaques, was borne. Indeed, regional relationships between activated IL-1 positive microglia, tau-positive neuritic tangles, β -amyloid plaques, and activated astrocytes seem to support a causal association (Griffin et al, 1995; Sheng et al, 1996; Sheng et al, 1995; 1998). But is this increase harmful or could it be compensatory and beneficial? Perhaps both. An excellent review describing the evolving perspective on the role of IL-1 β in AD has appeared elsewhere (Shaftel et al, 2008); hence only highlights are discussed below.

Human IL-1 gene polymorphisms — associated with increased IL-1 production — have been documented to increase the relative risk for AD and/or promote earlier disease onset (Grimaldi et al, 2000; Licastro et al, 2004; Mrak and Griffin, 2000; Nicoll et al, 2000). In seeming support, IL-1 can increase expression of β -amyloid precursor protein (A β PP) (Goldgaber et al, 1989; Griffin et al, 2006;

Ma et al, 2005; Yang et al, 1998b) and has been tied to formation and exacerbation of neurofibrillary tangles as well (Griffin et al, 2006; Sheng et al, 2000). Interestingly, A β can directly activate processes leading to the secretion of mature IL-1 β (Halle et al, 2008) representing a potential feed forward pro-inflammatory response mechanism. Moreover, mice lacking IL-1ra show enhanced microgliosis and neuronal cell death when human IL-1 β is infused into the cerebral ventricles, suggesting that the lack of this negative regulator to the system can increase AD-like pathology (Craft et al, 2005). Overall, the initiation and propagation of neuroinflammatory changes in AD have been tied to demonstrable changes in CNS IL-1 β levels (for review see Mrak and Griffin, 2005).

Nevertheless, recent data indicate that the story is more complex. Within the hippocampus, chronic overexpression of IL-1 β did indeed trigger a profound neuroinflammatory response (Matousek et al, 2012). Yet, surprisingly, AD pathology in a model of Alzheimer's disease (APP/PS1 transgenic mice) is ameliorated, as measured by a decrease in β -amyloid plaque size and frequency (Shaftel et al, 2007). Unlike chronic expression in the substantia nigra (*vide supra*), sustained hippocampal expression of IL-1 β produced these potentially protective responses without inducing overt neurodegeneration (Matousek et al, 2012), although suppression of adult neurogenesis does occur (Wu et al, 2012). Of note, a recent study in an Tg2576/IL-1R1^{-/-} mouse show no clear alterations in β -amyloid deposition when compared to Tg2576 controls (Das et al, 2006). Clearly more information is needed to determine whether the timing of the response, the nature of the response stimulus, and the differential cellular processes set in motion may affect the ability of IL-1 β to trigger adaptive, reparative responses in the setting of AD.

Cerebral Ischemia (Stroke)

Unlike the other maladies discussed in detail above, there is incontrovertible evidence that supports the conclusion that IL-1 β contributes to the evolution of the infarct (for reviews see also Fogal and Hewett, 2008; Loddick et al, 1998; Rothwell et al, 1997; Rothwell and Luheshi, 2000; Rothwell and Relton, 1993; Rothwell and Strijbos, 1995). Establishing

a cause-and-effect relationship, administration of either an IL-1 β neutralizing antibody (Yamasaki et al, 1995) or IL-1ra (pharmacologically or genetically) markedly reduces subsequent cerebral ischemic damage (Betz et al, 1995; Loddick and Rothwell, 1996; Martin et al, 1994; Mulcahy et al, 2003; Relton and Rothwell, 1992; Yang et al, 1998a; Yang et al, 1999; Yang et al, 1997). Animals deficient in ICE, the enzyme necessary for processing and activation of IL-1 β , also show diminished infarct volumes along with a concomitant reduction in IL-1 β levels (Hara et al, 1997; Liu et al, 1999; Schielke et al, 1998). Mice lacking the ligand IL-1 (α and β) or the signaling receptor, IL-1R1, have less brain injury after middle cerebral artery occlusion (MCAO) than their wild-type counterparts (Boutin et al, 2001; Fogal et al, 2007; Ohtaki et al, 2003) [but see also Touzani et al, 2002]. Interestingly, mice null for the IL-1ra gene have larger infarct volumes and increased mortality after experimental cerebral ischemia indicating that this endogenous protective mechanism is triggered following stroke to limit damage (Pinteaux et al, 2006). Even intraventricular injection of recombinant IL-1 β , while not toxic alone, increases neuronal injury after MCAO in rat (Yamasaki et al, 1995). Finally and most importantly, the concentration of IL-1 β is significantly increased in the cerebrospinal fluid of stroke patients (Gusev and Skvortsova, 2003; Tarkowski et al, 1999) and positive results of a prospective Phase II placebo-controlled study of recombinant human (rh) IL-1ra in patients with acute stroke have been published (Emsley et al, 2005). Thus, the totality of experimental – and human data – provide compelling evidence that IL-1 β is a contributing factor in brain injury that follows cerebral ischemia. While the exact mechanism(s) by which IL-1 β mediates these neurodestructive responses following cerebral ischemia are incompletely defined, plausible theories can be found in these comprehensive reviews (Fogal and Hewett, 2008; Pinteaux et al, 2009).

Although it seems clear cut, we included the discussion of cerebral ischemia here because evidence suggest that the upregulation of IL-1 β following ischemic insult may be a part of a protective response that ultimately goes awry. In support of this idea, IL-1 has been demonstrated to be a mediator of ischemic tolerance (Ohtsuki et al, 1996), highlighting

its ability to effectively mount protective responses. The preconditioning response to sublethal global ischemia was blocked by administration of IL-1ra and mimicked by the addition of either IL-1 α or IL-1 β (Ohtsuki et al, 1996). Further, support for the assertion that IL-1 β can promote positive outcomes is shown by the fact that IL-1 β can reduce excitotoxic neuronal cell death, a main contributor to ischemic injury. Neuron death induced by the addition of ionotropic glutamate receptor agonists NMDA, AMPA, and kainate in primary neuronal cultures and in organotypic slice is effectively ameliorated with treatment with rather high concentrations of IL-1 (Bernardino et al, 2005; Carlson et al, 1999; Ohtsuki et al, 1996; Pringle et al, 2001; Strijbos and Rothwell, 1995; Wang et al, 2000b). This may be due to its ability to protect neurons and promote growth and/or survival via stimulated production of neurotrophic factors (Carlson et al, 1999; Strijbos and Rothwell, 1995). However, when paradigms involving energy deprivation — which might more faithfully mimic the *in vivo* situation associated with cerebral ischemia — are employed, IL-1 β potentiates neuronal injury (Fogal et al, 2005; Jackman et al, 2012; Pringle et al, 2001).

Why these dichotomous results? Previous work from our lab indicate that IL-1 β regulates the expression and activity of a cystine/glutamate antiporter, system x_c⁻, in astrocytes exclusively and that glutamate exported via astrocytic system x_c⁻ directly underlies the neurotoxic propensity of IL-1 β under hypoxic conditions (Fogal et al, 2007; Jackman et al, 2010). Interestingly, increased activity of system x_c⁻ is not inherently injurious as under physiological conditions the accumulation of glutamate exported is prevented by its rapid clearance from the extracellular space; consequently, no neuronal toxicity is observed (Fogal et al, 2005). In contrast, when glutamate uptake is impaired, as occurs under hypoxic conditions, increased system x_c⁻ activity can result in the accumulation of extracellular glutamate and subsequent excitotoxic neuronal cell death (Fogal et al, 2007). A previous study reported that neuronal cell death induced by IL-1 β required astrocyte activation as well (Thornton et al, 2006).

Interestingly, the same transporter fluxing the glutamate which produces excitotoxicity during

periods of energy deprivation has a Janus-face and has a well-characterized role in the synthesis of the antioxidant molecule glutathione (Bannai and Tateishi, 1986; Meister and Anderson, 1983). In this respect, astrocytes function as indispensable support cells by protecting themselves and neurons against oxidative insults (Gegg et al, 2005; Jakel et al, 2007; Shih et al, 2003; Tanaka et al, 1999). Hence, it is intriguing to speculate that under conditions of cerebral ischemia, IL-1 β is released as a protective mechanism to increase glutathione synthesis in efforts to thwart oxidative stress (Jackman et al, 2011).

CONCLUSIONS

It is becoming increasingly clear that IL-1 β is important for normal brain function. An equally impressive amount of literature support its role in pathology. Although, anti-IL-1 therapies have proven to be revolutionary treatments for several human autoinflammatory disorders (reviewed in Dinarello, 2009b), demonstrating a causative role in these diseases, a clear cause-and-effect relationship between the presence of neuroinflammatory processes and CNS damage does not always exist. Indeed, its pleiotropic effects in the brain indicate that despite having potent pro-inflammatory functions, IL-1 β can also participate in neuroprotection, tissue remodeling and repair. So whether IL-1 β initiates damage, results from damage and goes on to promote, halt or repair injury may depend heavily on the context, that is, the local concentration, the prevailing environmental milieu, the cellular target, the presence or absence of negative feedback regulators, and the temporal characteristics of the response. Shaftel and colleagues said it best: "IL-1 can no longer be regarded as simply the villain in the setting of brain injury and disease, but instead must be understood as a factor that can influence the balance between beneficial and detrimental outcomes" (Shaftel et al, 2008).

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ENDOPLASMIC RETICULUM STRESS IN NEURODEGENERATIVE DISEASES: THE ROLE OF RETICULON PROTEINS

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Endoplasmic reticulum stress is characterized by misfolded protein accumulation into the ER lumen, and elicits the activation of molecular mechanisms by which the cell attempts to resolve the stressful condition. However, the failure of this defensive strategy leads the cell towards ER stress-mediated cell death. ER stress has been associated to several pathological conditions among which neurodegenerative diseases, characterized by the common feature of dysfunctional protein aggregates in neuronal cells. Although ER stress occurs in these disorders, its real role in their pathogenesis still remains unclear. ER impairment could have a direct implication in the onset of the neurodegenerative diseases or maybe be a result of other trigger factors. More recently, reticulon proteins, other factors involved in ER stress induction, have been associated to several neurodegenerative disorders such as amyotrophic lateral sclerosis or Alzheimer's disease. Together these indications enforce the linkage between neurodegenerative processes and ER stress.

THE ENDOPLASMIC RETICULUM STRESS

The endoplasmic reticulum (ER) is a large organelle constituted by an articulated membrane network of tubules and sheets (like cisternae), forming smooth ER, rough ER and the outer nuclear envelope. ER represents a crucial source for lipid biogenesis, it regulates calcium homeostasis and redox balance. Importantly, ER is also the main site for the synthesis and folding of membrane-bound and secretory proteins. In the oxidizing environment, the nascent peptides acquire the correct folding via resident protein chaperones and disulphide bond formation (Naidoo, 2009). Perturbation of these functions (energy level, calcium concentration or redox state) results in misfolded and unfolded protein accumulation into the ER lumen, a condition known as ER stress. To prevent ER damages, the cell activates a series of protective mechanisms,

collectively called unfolded protein response (UPR). However, if ER stress is too strong or prolonged and UPR fails to restore ER protein homeostasis, cell death program is activated (Shore et al, 2011).

UPR is a tripartite signalling pathway triggered by the activation of three transmembrane protein-sensors of ER: PKR-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme-1 (IRE1). In normal conditions, these stress-receptors are kept in an inactive state by association with the ER chaperone GRP78. During ER stress, the accumulation of unfolded proteins into the ER causes the dissociation of GRP78 in order to engage unfolded proteins and leads the activation of the three ER-sensors (Ron and Walter, 2007) (fig. 1).

Upon ER stress, PERK phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) which inhibits conventional protein translation. (Harding et al, 1999). This strategies leads to reduce the overload

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of nascent proteins into the ER lumen. However, PERK activation facilitates the cap-independent translation typical of selective mRNA, among which the activating transcription factor 4 (ATF4) (Harding et al, 2000). *ATF4* is known to promote cell survival by inducing genes involved in redox reactions, stress response, amino-acid metabolism, and protein secretion. In addition, PERK promotes the nuclear import of nuclear factor E2 related factor 2 (Nrf2) that induces the expression of different genes involved in antioxidant defense mechanisms (Cullinan and Diehl, 2006).

The dissociation of GRP78 from ATF6 leads its export to the Golgi apparatus, where ATF6 is processed by site-1 and site-2 proteases. The cleaved ATF6 active form translocates to the nucleus to induce the expression of ER stress response genes, such as different ER chaperones, protein disulphide isomerase (PDI), and X box-binding protein-1 (Xbp-1) (Schroder, 2006).

Activated IRE1 specifically cleaves an intron from the Xbp-1 mRNA induced by ATF6. The Xbp-1 spliced product works as an activator of UPR target genes including ER chaperones. (Yoshida et al, 2001; Woehlbier and Hetz, 2011). Moreover, the RNase activity of IRE1 is also involved in the degradation of mRNA encoding for secretory proteins (Hollien and Weissman, 2006).

Beyond the enhancement of ER folding capacity, the restore of ER homeostasis is achieved by ER-associated degradation (ERAD) where the unfolded proteins are targeted for degradation by the ubiquitin-proteasome system (UPS) (Vembar and Brodsky, 2008).

Until now, conversely to pro-survival UPR signalling which are deeply characterized, the molecular mechanisms regulating ER stress-mediated apoptosis still remain elusive. It is known that the same actors of UPR, during prolonged stress conditions, are also involved in UPR switching into

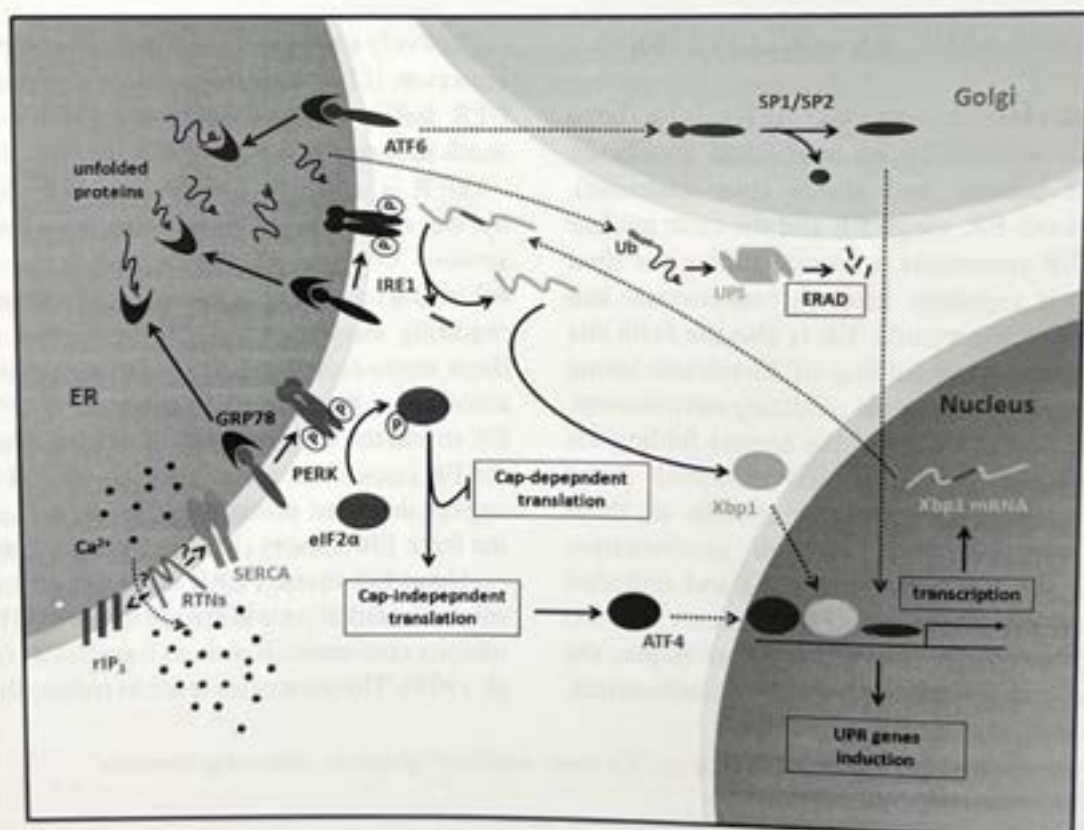


Fig. 1. Molecular mechanism of UPR. Misfolded protein accumulation into the ER as well as ER Ca²⁺ depletion are events due to ER stress induction. The specific ER stress sensors PERK, ATF6, and IRE-1 trigger fine signalling pathway aimed to protein translation inhibition, proteasome degradation of unfolded proteins, and increase of the ER folding ability. RTN proteins might regulate Ca²⁺ efflux from ER by indirectly impairing the activity of SERCA pump or IP₃-receptors.

an alternative pathway promoting apoptosis. (Shore *et al.*, 2011). In fact, the PERK-eIF2 α -ATF4 arm of UPR is required to induce growth arrest- and DNA damage-inducible gene 153 (GADD153) (Woo *et al.*, 2009), which finally alters the expression of genes involved in apoptosis (i.e. downregulation of bcl2) and oxidative stress (i.e. induction of endoplasmic reticulum oxidoreductin 1(ERO1 α)) (McCullough *et al.*, 2001; Woehlbier and Hetz, 2011). Moreover, JNK signalling is activated by IRE1-TRAF2-ASK1 branch of UPR, and it has been reported to regulate the BH3-only proteins PUMA, Noxa and BIM (Puthalakath *et al.*, 2007; Li *et al.*, 2006; Woehlbier and Hetz, 2011). Thus, ER stress-mediated apoptosis would be mediated by the control of the integrity of the outer mitochondrial membrane typical of intrinsic apoptotic pathway. In fact, intramembranous homooligomerization of Bax and Bak play a central role in the initiation of ER stress-mediated apoptosis by BH3-only proteins induction (Kim *et al.*, 2009).

Although apoptosis induction is well documented during prolonged ER stress stimuli, the role of initiator caspases in ER stress-mediated apoptosis still remains controversial. In murine cells Caspase-12 activation has been known to be correlated with UPR signalling (Nakagawa *et al.*, 2000). In particular once activated, Caspase-12, an ER resident protein, translocates to the cytosol where it processes pro-Caspase-9, which then activates downstream Caspases cascade leading to apoptosis (Naidoo, 2009). However, likely Caspase-12 amplifies rather than initiating ER stress-induced apoptosis. In fact, in humans Caspase-12 is produced in an unfunctional truncated protein form (Fisher *et al.*, 2000) suggesting other initiator Caspases involved in ER stress signalling, such as Caspase-2, or Caspase-4 (Matsuzaky *et al.*, 2010; Upton *et al.*, 2008). Nonetheless, their effective essential role remains to be determined.

ER stress signalling pathway has been associated with several physio-pathological conditions among which diabetes, heart disease and neurodegeneration (Shore *et al.*, 2011). In particular, in this review we mainly focus with the ER stress implication in the context of the neurodegenerative disorders.

NEURODEGENERATIVE DISEASES

Neurodegenerative pathologies are devastating

disorders that affect movements, cognition and memory. These diseases have in common the accumulation of misfolded protein aggregates and inclusions into neural cells of central nervous system (CNS) as a consequence of protein homeostasis disturbances. These conditions are caused by genetic mutations, or by indirect mechanisms still unknown. Amyotrophic Lateral Sclerosis (ALS) is characterized by toxic accumulation of mutated superoxide dismutase-1 (SOD1). Huntington's disease (HD) results from accumulation of mutant huntingtin protein containing expanded sequences of glutamine repeats. Alzheimer's disease (AD) is characterized by deposits of β -amyloid aggregates while accumulation of misfolded α -synuclein is linked to Parkinson's disease (PD) (Soto and Estrada, 2008). It is now well accepted that ER stress plays a pivotal role in the development and progression of these neurodegenerative diseases. For instance, induction of PERK-eIF2 α arm and IRE1 activation have been observed in hippocampal neurons of AD patients (Hoozemans *et al.*, 2009). Moreover, ER stress was also demonstrated in α -synuclein-dependent neurodegeneration. In fact, UPR activation was found in *post-mortem* tissue from PD patients (Hoozemans *et al.*, 2007). More recently ER stress induction was also investigated in mutant α -synuclein overexpressing Tg mice. In this studies, although it has been observed an increase in the expression of several ER chaperones, eIF2 α phosphorylation doesn't change in comparison to controls (Colla *et al.*, 2012). As the phosphorylation of eIF2 α is thought to prevent the switch of UPR towards cell death, low levels of phosphorylated eIF2 α may be a favorable condition for ER stress-mediated cell death induction. A close link between ALS and ER stress was also found in a mouse model for familiar ALS (mSOD^{G93A} mice) (Atkin *et al.*, 2006). More importantly, besides the strong increase of the chaperone protein expression, clearly UPR signal transduction is shown to be active in spinal cords of human patients with sporadic ALS. GADD153 gene expression was found upregulated indicating that a prolonged ER stress stimulus and UPR-induced cell death occur in these spinal cord tissues (Atkin *et al.*, 2008).

Moreover, UPS is required for ERAD mechanism and its function has been implicated in the pathogenesis

of neurodegenerative diseases such as ALS and AD. In fact, in the cerebral cortex of AD patients it has been found low levels of HRD1 protein, an ubiquitin ligase involved in the degradation of the unfolded amyloid- β precursor protein (APP) by ERAD system (Kaneko et al, 2010). The reduced level of HRD1 was due to its insolubilization leading its loss of function and a massive amyloid- β accumulation (Kaneko et al, 2012). It would be interesting to understand how HRD1 protein is insolubilized and to elucidate whether a reduction in HRD1 protein causes beta-amyloid production. However, it is not clear whether other unknown factors (i.e. oxidative stress) trigger amyloid- β generation or HRD1 dysfunction represents a downstream event.

Moreover, in a mouse model of ALS (SOD1^{G93A} mice) it has been found that mutant SOD1 interacts with Derlin-1, another component of ERAD machinery, causing the impairment of this specific protein degradation system. Moreover, SOD1-Derlin1 binding may be necessary to trigger ER stress and UPR-mediated cell death through mainly IRE1-TRAF2-ASK1 pathway activation (Nishitoh et al, 2002). Further, as expected, the authors highlight that SOD1^{G93A}-dependent motor neuron death is not only due to ASK1 activation. Despite ASK1 deficiency increases the lifespan of SOD1^{G93A} mice, it is not sufficient to alleviate the onset of ALS disease suggesting the probable involvement of other ER stress-induced pro-apoptotic pathways (Nishitoh et al, 2008). According to these data, PERK^{-/-} deficiency in SOD1^{G93A} mice accelerates ALS progression (Wang et al, 2011). Conversely, it has been reported that Xbp-1^{-/-} deficiency seems to protect against the development of ALS (Hetz et al, 2009). Thus, the UPR role in these pathologies may be difficult to define.

Taken together, it is still not clear the UPR signalling role in the onset and progression of neurodegenerative diseases. The open question is whether the UPR acts to up- or downstream of these pathologies. Notably, it has been reported that ER stress enhances γ -secretase activity, an enzyme involved in APP processing to amyloid- β generation, by ATF4 expression in correlation with a real increase in amyloid- β production (Ohta et al, 2011). These data suggest how UPR signalling may be relevant to promote or to exacerbate the amyloid- β

accumulation typical of AD, allowing to hypothesize ER stress not only as an effect of AD development but having also as a direct role in the onset of this pathology. However, further studies are necessary to test this hypothesis.

It is also known that in neurodegenerative disorders a relevant contribute to cell death is associated to changes in Ca²⁺ homeostasis. In general, the level of Ca²⁺ stores modulate important steps of cell death signalling that can contribute to cellular degeneration and apoptosis. Ca²⁺ release from ER is one of the most immediate and critical effect caused by misfolded proteins accumulation-mediated ER stress (Smaili et al, 2009) (fig. 1). Moreover, Ca²⁺ release channels in the ER, i.e. inositol 1, 4, 5-trisphosphate receptor (IP₃R) may be involved in apoptotic pathway (Hajnoczky et al, 2000). Although the strong linkage between ER Ca²⁺ depletion and ER stress-induced apoptosis is well documented, several aspects of this relationship remain to be investigated.

It has been reported that in various neurodegenerative diseases there is an increase of cytoplasmic Ca²⁺ concentration. In the brain of AD mouse models, it has been observed an overactivation of Ca²⁺-dependent phosphatase calcineurin in correlation with a low phosphorylation level of proapoptotic BH3-only protein BAD (Reese et al, 2008). In normal condition phosphorylated BAD is sequestered to the cytosol by 14-3-3 scaffold-protein, when cytosolic Ca²⁺ concentration is increased calcineurin-dependent dephosphorylation of BAD occurs leading to its release and its proapoptotic activity on the mitochondria (Springer et al, 2000). Then, overactivated calcineurin is also implicated in the aberrant dephosphorylation of huntingtin protein that results in the brain-derived neuronal growth factor (BDNF) transport impairment associated with neurodegeneration in HD (Pineda et al, 2009). According to this, the over-expression of RCAN1-1L, a negative regulator of calcineurin, show a protective effect against mutant huntingtin toxicity (Ermak et al, 2009) indicating calcineurin like a potential therapeutic target for this disease, as suggested by Pineda et al (2009).

Mitochondria are important for Ca²⁺ homeostasis and signalling, and it has been reported that these organelles are in close contact and communication

with ER (Rizzuto and Pozzan, 2006). Intracellular Ca^{2+} flux is under the control of ER and mitochondria and pro-apoptotic members of Bcl2 family regulate Ca stores from these organelles (Nutt et al, 2002). In particular, when Bax resides in the ER may lead Ca^{2+} from this store and activate caspase-12 (Zong et al, 2003). Furthermore, Ca^{2+} signalling during apoptosis correlates with pro-apoptotic Bax activity. In fact, it has been reported that release of ER Ca^{2+} store through various stimuli induce Bax translocation to mitochondria (Smaili et al, 2009). In addition, many neurodegenerative diseases show abnormal morphology and biochemical alterations of mitochondria, and ROS production (Karbowski and Neutzer 2012). Although this growing branch of research is attracting much interest, it is not yet clear whether mitochondria dysfunction is a necessary step in neurodegeneration. However, in neurodegenerative disorders there are alterations in Ca^{2+} homeostasis which may largely contribute to cell death, and may be also amplified by impairing of Ca^{2+} storage ability of mitochondria.

RETICULON PROTEINS AND NEURODEGENERATION

An additional linkage between ER stress and neurodegenerative diseases may be given by the potential involvement of reticulon (RTN) proteins (Yan et al, 2006). Reticulons are an emerging group of ER-membrane proteins that strongly affect ER structure and various ER-associated cellular processes, such as vesicle trafficking, and more importantly ER stress (Yan et al, 2006). In mammal, this protein family comprises eleven isoforms generated through spliced mRNAs encoded by four genes, *rtnl*, 2, 3, and 4. Moreover, an evolutionary conserved carboxyl-terminal region, known as reticulon homology domain (RHD) represents the common structural feature peculiar to all members of RTN family (fig. 2). Instead, the N-terminal part represents the variable part likely responsible for the specific function of each isoform (Teng and Tang, 2008). RHD includes two not canonical transmembrane segments that are presumed to form an harpin in the ER membrane (fig. 2), that more likely result essential to generate and keep the tubular network of the ER, and so to preserve the

functionality of this cellular compartment (Bauer and Pelkmans, 2006; Di Sano et al, 2012). Moreover, to support a role of reticulon proteins in membrane shaping, it has been also reported their involvement in nuclear envelope formation (Dawson et al, 2009). However, while the specific functions of reticulon isoforms remain to be defined, RTN4/Nogo has been demonstrated to be an inhibitor of neurite outgrowth in mammalian central nervous system (Oertle et al, 2003).

More importantly, it has been observed RTN1 and RTN3 implicated in endo- and exocytosis processes (Teng and Tang, 2008). Interesting, RTN1-C isoform was found to physically interact with important components of vesicle trafficking, such as several Syntaxin isoforms and vesicle associated membrane protein-2 (VAMP2) (Steiner et al, 2004). Notably, it has been reported that abnormalities in cellular trafficking may be one of the relevant determinant of neurodegenerative disorders (Chua and Tang, 2011).

Beyond other functional implications of reticulons, there are many evidences about their involvement in ER stress. Notably, it has been reported that several RTN isoforms induce ER stress-mediated apoptosis by UPR activation and ER Ca^{2+} depletion (Di Sano et al, 2012). In particular, in the last years, we demonstrated that RTN1-C-overexpressing cells are sensitized to ER stressors, and RTN1-C *per se* promotes an increase in cytosolic Ca^{2+} concentration and ER stress-induced apoptosis (Di Sano et al, 2007).

The mechanism by which RTN proteins induce Ca^{2+} efflux from the ER is still unknown. It has been hypothesized that the ability of reticulons to oligomerize and form pores in the ER membrane could explain these observations (Oertle et al, 2003). On the other hand RTN proteins could affect the activity of IP_3R or sarcoplasmic/endoplasmic reticulum Ca^{2+} (SERCA) pump (fig. 1), but until now no direct interaction between those proteins has been reported.

It is worth to note that RTN4-B was found to interact with the anti-apoptotic factors Bcl2 and Bcl-X_L. In addition, also the binding between RTN1-C and Bcl-X_L has been demonstrated (Tagami et al, 2000). On the basis on these evidences, and knowing Bak and Bax implication in ER Ca^{2+} regulation, it is possible to hypothesize that reticulons affect

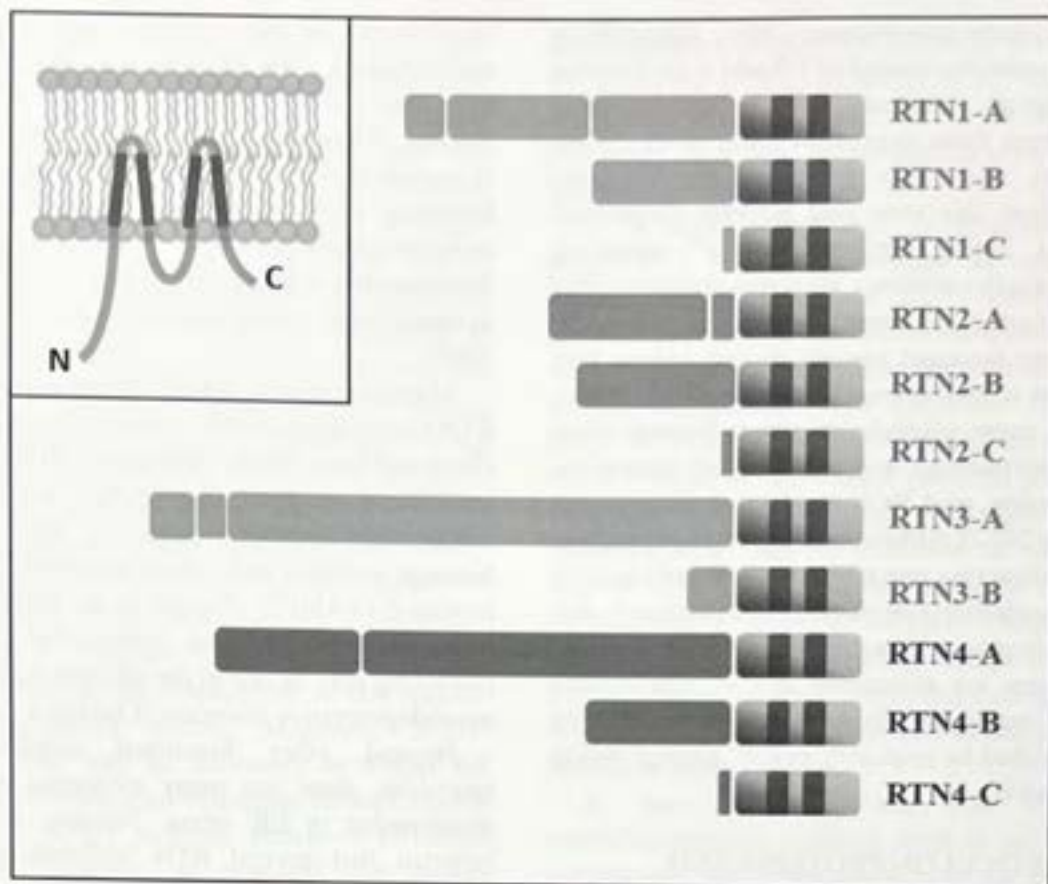


Fig. 2. Mammalian reticulon proteins. RTN family members share a common C-terminus (gray) while the variability of the N-terminal portion is due to alternative splicing. The insert shows the atypical membrane integration of these proteins due to the presence of two unusual hydrophobic segments (dark gray) in the C-terminal region.

intracellular Ca^{2+} homeostasis by reducing inhibitory bonds of Bax and Bak with Bcl2/Bcl-X_L in the ER. However, the molecular mechanisms by which reticulons are really implicated in ER stress and cell death still remain to be elucidated.

A first implication of reticulons in neurodegenerative processes derives from the findings that RTN4-B, as well as RTN3, interact and negatively modulate β -secretase BACE1, an enzyme deeply involved in the APP proteolytic cleavage (Murayama et al, 2006). To support a potential role of reticulons in AD, an increased expression of RTN3 has been observed in cerebral tissues of AD patients (Yokota et al, 2006). Furthermore, in RTN3-overexpressing mice a major retention of BACE1 into the ER is observed in correlation with a reduced level of amyloid- β deposition (Shi et al, 2009).

Recently, it has been suggested a putative

protective role of reticulons in ALS by affecting the cellular localization of protein disulphide isomerase (PDI) (Yang et al, 2009). PDI is an ER resident protein essential for correct protein maturation, and its dysfunction inevitably causes the accumulation of misfolded proteins. Then, it is known that PDI activity is inhibited by S-nitrosylation, and high level of S-nitrosylated PDI has been found in AD, and PD brain tissues (Uehara et al, 2006), as well as in spinal cord tissues of sporadic ALS patients (Walker et al, 2010). These findings could explain the very low defensive ability of PDI against misfolded protein production in these neurodegenerative diseases. In light of this evidence, it should be more interesting to better investigate the functional relationship between reticulons and PDI, indicated by Yang et al (2009). In addition, our microarray analysis indicates that RTN1-C is able to modulate

the expression of human gene clusters involved in neurodegenerative disorders. Further, ER stress induction and abnormal synaptic plasticity has been observed in mouse cerebral cortex overexpressing RTN1-C. Thus, these our evidences enforce the hypothesis about the implication of reticulon proteins in neurodegeneration (Fazi et al, 2010; Di Sano and Piacentini, 2012).

Moreover, recently another further involvement of reticulons has been found in the context of neurodegeneration. In fact, the complete deletion, or a frameshift mutation of RTN2 has been associated with the onset of hereditary spastic paraplegias, a group of neurodegenerative conditions (Montenegro et al, 2012). However, reticulons implication in this axonopathy may be caused by the aberrant impact of reticulon dysfunction on the ER morphology.

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PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY (PML) AND BIOLOGICALS

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Progressive multifocal leukoencephalopathy (PML), is a rare, frequently fatal demyelinating disease caused by the JC virus. It is observed in severely immunosuppressed individuals with HIV infection, lymphoid malignancies and patients with organ- and stem cell transplantations. More recently, PML has been increasingly diagnosed in patients treated with biological therapies like natalizumab, efalizumab or rituximab. Informed clinical decision making as well as effective risk mitigation of medicinal products require understanding the risk of PML associated with these therapies. The main purpose of the article is to review the scientific knowledge concerning PML associated with medicinal products, in particular monoclonal antibodies.

Progressive multifocal leukoencephalopathy (PML) is a potentially fatal, demyelinating disease of the human brain caused by the John Cunningham virus (JC virus), a non-enveloped, double-stranded DNA virus in the Polyomaviridae virus family. PML was first described in 1958 (Astrom et al, 1958). It is named after the two initials of a patient with progressive multifocal leukoencephalopathy (PML). Immunological pathologies that are associated with PML are disorders of cell-mediated immunity either through depletion of lymphocytes or alteration of CNS immunosurveillance such as HIV/AIDS, chronic lymphatic leukaemia and other B-cell malignancies and – less frequently – autoimmune diseases such as rheumatoid arthritis and lupus erythematosus.

JC VIRUS INFECTION AND PATHOLOGY

Asymptomatic initial infection with JCV occurs

during childhood. Seroprevalence rates vary in the literature, depending on the population and ethnical origin investigated. Egli et al (2009) reported a seroprevalence rate of 58% in healthy adults, consistent with recent prevalence data of anti-JCV antibodies determined in multiple sclerosis (MS) patients (Gorelik et al, 2010). A comparable seroprevalence rate has also been detected in MS patients treated with natalizumab (Trampe et al, 2012). The route of primary JCV infection is not definitely known. Primary infection via tonsils (Monaco et al, 1998) or the gastrointestinal tract have been assumed. After primary infection JCV can persist in bone marrow, kidney, spleen, tonsils, lymph nodes and possibly also the gastrointestinal tract (Ricciardiello et al, 2000) for a long time in a latent phase. Transient virus shedding in the urine in approximately 30 % of non-immunocompromised healthy individuals has been detected, indicating that kidney latency is not associated with a pathological

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condition (Major, 2011). JCV isolated from the kidney is referred to as the archetype sequence. In contrast, the neurotropic form of the virus, which is characterized by deletions and re-arrangements in the non-coding control region (NCCR) compared to the archetype, is found in PML brain tissue. Additionally, mutations in the JC virus capsid viral protein 1 (VP1) appear to increase the risk for the development of PML (Sunyaev et al, 2009). Mutations in VP1 may alter the binding to sialic acid receptors, thus favouring the occurrence of PML (Gorelik et al, 2011).

PML most likely results from reactivation of a latent infection rather than occurring as a complication of a primary infection. However, the latter may occasionally also happen. One natalizumab-treated patient developed PML approximately six months following a negative antibody ELISA test result (internal data of the Paul-Ehrlich-Institut). This may be either explained by a false negative assay result or by PML as a result of primary infection. Bone marrow CD34+ haematopoietic progenitor cells are thought to play a key role in JCV pathogenesis as they are considered a site of latency for JCV. Factors which trigger the reactivation of JCV from the latent phase are not known. If reactivated, JCV can be detected in B cells from which it possibly infects the brain parenchyma. Interestingly, viral DNA can be detected within the brain of asymptomatic individuals (White et al, 1992). Thus, there may be latency also in the brain. Apparently, immunosuppression increases latent infection of the brain (Bayliss et al, 2011). Once the brain is infected, JCV starts to replicate. The highest replication measured *in vitro* has been seen in astrocytes and glial cells. Immunity against JCV appears to be mainly performed by CD8+ T-lymphocytes specific for JC virus. Recent publications also indicate that JCV-specific CD4+ T-cell responses appear to play a critical role in the control of JCV infection (Gasnault et al, 2003). Conditions resulting in CD8+ and CD4+ lymphopenias are therefore considered risk factors for PML development.

SYMPTOMS AND DIAGNOSIS

Rapid diagnosis of PML is of critical importance as there is no effective therapy, and prompt

withdrawal of immunosuppressive agents is virtually the only chance for a patient to survive. Therefore, it is of utmost importance to establish standardised protocols for early PML diagnosis based on clinical symptoms and diagnostic means.

Symptoms

The presence of focal neurological deficits that may be sub-acute in onset may point toward PML. Cognitive dysfunction or recent changes in behaviour or personality, motor symptoms, language or speech disturbances/difficulties, visual disturbance, ataxia/loss of motor coordination, and seizures have been reported as first symptoms. Early neurological symptoms may be non-specific or subtle, and dependent on the initial focus of reactivation in the brain. Thus, early symptoms may be easily misdiagnosed or detected too late, for example for cognitive dysfunctions or changes in personality.

Magnetic resonance imaging (MRI)

Single or multifocal PML lesion(s) frequently affect the subcortical hemispheric white matter with involvement of U fibres, but the lesions might also be located periventricularly or in the white matter of the cerebellar hemispheres or peduncles. Although PML lesions typically involve the white matter, grey matter involvement can occur. Normally there is no mass effect in PML. PML lesions are (partially) hypointense on T1-weighted MR imaging and hyperintense on T2-weighted images, FLAIR (fluid attenuated inversion recovery) images, and proton density-weighted images. Newer lesions and the advancing edge of large lesions have a high signal on diffusion-weighted images (DWI), with reduced signal intensity typically being seen in the core of these lesions (Yokoyama et al, 2008; Clifford et al, 2010; Shah et al, 2010). Unlike in classic PML where gadolinium enhancement in MRI is usually minimal or absent, a number of natalizumab-treated patients who developed PML had gadolinium enhancement at diagnosis (Clifford et al, 2010). This can make the differential diagnosis to a newly occurring multiple sclerosis lesion challenging.

Virological and histopathological testing

The confirmation of a suspected PML case usually requires the presence of JCV DNA in the cerebrospinal

fluid (CSF) by polymerase chain reaction (PCR) or a histopathological confirmation in a brain biopsy. Histological PML features include focal areas of demyelination, enlarged oligodendrocytes, and large 'bizarre-looking' astrocytes. In most cases, JCV proteins (large T antigen and VP1 capsid protein) are detectable by immunohistochemistry on brain biopsy samples of a PML lesion. JCV DNA can be detected by *in situ* hybridisation in the brain of individuals without PML (Tan et al, 2010). Thus, the sole detection of JCV DNA in a brain biopsy is not sufficient to provide evidence for a PML.

When the suspicion of PML persists after physical examination and MRI, a PCR for JCV DNA in the CSF is obligatory. Importantly, a negative JCV PCR does not exclude a PML. CSF can be negative for JCV DNA despite clinical and MRI findings, for example in early PML or during IRIS (immune reconstitution inflammatory syndrome, see below) (Kuhle et al, 2011). The presence of JCV DNA in the CSF without clinical symptoms and without MRI features consistent with the diagnosis may be insufficient to confirm the diagnosis of PML (Iacobaeus et al, 2009).

DISEASES ASSOCIATED WITH PML

HIV/AIDS

In the 1980s PML occurred with an incidence of up to 5 % in AIDS patients (Berger et al, 1998), and PML is among the AIDS-defining diseases. The incidence appeared to continuously decrease (Engsig et al, 2009; Khanna et al, 2009) with the introduction of anti-retroviral treatment with a somewhat improving prognosis. However, despite the documented benefits, PML has occurred in individuals being successfully treated with HAART (highly active antiretroviral therapy), and there is evidence that PML may worsen or occur in some individuals who experience immune recovery associated with HAART.

Idiopathic immunodeficiency syndromes

There are a few case reports of PML in patients with idiopathic immunodeficiency syndromes (Hartman et al, 2008). In a few cases, isolated CD4+ T-cell lymphopenia was the only underlying cause identified (Moloney et al, 2012). There are also case

reports of PML in apparently immunocompetent patients (White et al, 1992).

Other diseases associated with PML

Several cases of PML in patients with lymphoproliferative diseases were published from 1958 onwards (summarised in Garcia-Suarez et al, 2005). Identified risk factors for PML included uncontrolled Hodgkin's disease, treatment with purine analogues, and high-dose therapy with haematopoietic stem cell transplantation (HSCT); i.e., conditions that target lymphocytes. A Mayo clinic review from 1957 to 2005 identified 58 cases of PML in non-HIV patients (Askamit, 2006). In this series, 55% of the patients had lymphoreticular malignancies; 15% had connective tissue disease with nearly half of whom suffering from systemic lupus erythematosus (SLE); 9% had sarcoidosis; 7% had organ transplants; 7% had other predisposing conditions; and 7% had no documented immunosuppressive condition. Calabrese et al (2007) identified 36 cases from peer-reviewed literature in patients with rheumatic diseases treated with non-biologic agents. The authors found that nearly two-thirds had SLE as an underlying diagnosis and noted that more than 40% of these patients had minimal or no immunosuppression. The same authors evaluated data from the Nationwide Inpatient Sample database in the US, which is an approximately 20% sample of all US hospital discharges. Based on these data, the rate of PML in SLE patients was 4 in 100000 discharges, compared with the rate of PML in RA patients of 0.4/100000 discharges and 0.2/100000 discharges in the background population, respectively. Their conclusion was that SLE may be associated with a predisposition to develop PML (Molloy and Calabrese, 2009).

Immune Reconstitution Inflammatory Syndrome (IRIS)

Immune Reconstitution Inflammatory Syndrome (IRIS) has been observed in patients with HIV infection treated with combination antiretroviral therapy as well as patients in whom the PML-inducing treatment has been terminated (natalizumab treated patients). Although the syndrome is well described, the pathomechanism is not fully understood. Potential mechanisms for the syndrome which can result in

paradoxical clinical deterioration include a partial recovery of the immune system or exuberant host immunological responses to antigenic stimuli leading to an inflammatory response during reconstitution. Possibly, re-emerging cytotoxic T cells directed against JC virus antigens attack brain tissue hosting JC virus, thus leading to this clinical deterioration.

PML TREATMENT OPTIONS

The treatment options for PML are limited. The mainstay of treatment appears to be to re-establish a functioning immune system in order to fight JC virus. The standard of care for HIV-associated PML is therefore HAART to achieve immunologic recovery by optimal HIV virologic control. Various medicines targeting JC virus itself or its spread such as antiviral and immunomodulatory drugs (e.g. interferon alpha) (Huang et al, 1998), 5-HT antagonists (mirtazapine), or intravenous immunoglobulins have been used, but none has demonstrated clear efficacy. At present the main approach to treat PML associated with immunosuppressive therapies is to reconstitute the immune function of patients by stopping treatment. Because monoclonal antibodies have a long half-life, plasma exchange or immunoadsorption have been used in PML cases associated with natalizumab to eliminate residual drug from plasma. Thus, effective

management of PML, including treatment of IRIS, still needs to be investigated.

IMMUNOSUPPRESSIVE BIOLOGICAL MEDICINAL PRODUCTS AND THE RISK OF PML

The common feature of immunosuppressive drugs is their ability to impair the functions of lymphocytes, either by direct and indirect depletion of lymphocytes or by inhibition of activation or migration of the cells (see Table 1). An important effect is the blockade of cytotoxic T-cell activation. Due to that down-regulation of the function of B- and T-lymphocytes, the administration of immunosuppressants creates favourable conditions for the replication of reactivated DNA viruses otherwise produced in low amounts.

Natalizumab

Natalizumab is an anti- $\alpha 4$ -integrin monoclonal antibody, which disrupts the transmigration of leucocytes across the endothelium into inflamed parenchymal brain tissue. It is indicated for highly active relapsing remitting multiple sclerosis, and in addition for Crohn's disease in the US. Obviously, the interaction of $\alpha 4$ -integrins with their receptors is a major contributor of extravasation of T-cells into the

Table 1. Immunosuppressive drugs (authorized in the EU) grouped by their mode of action and the presence of a statement regarding PML risk in section 4.4 of the EU Summary of Product Characteristics (red shadow)

Inhibitors of IL-2 synthesis	Inhibitors of lymphocyte proliferation		Anti-lymphocytes antibodies and fusion proteins (targets)
	Cytostatics/ antimetabolites	Inhibition of IL-2-induced proliferation signal	
Glucocorticoids	Cyclophosphamide	Sirolimus	Anti-lymphocyte globulin
Ciclosporin	Azathioprin	Everolimus	Alemtuzumab (CD52)
Tacrolimus	Methotrexat	(Basiliximab)	Rituximab (CD20)
	Mycophenolate mofetil	(Daclizumab)	Ofatumumab (CD20)
	Leflunomid		Ibritumomab-Tiuxetan (CD20)
			Basiliximab (CD25=IL-2R)
			Daclizumab (CD25=IL-2R)
			Natalizumab (alpha4-integrin)
			Abatacept (CD80/CD86)
			Belatacept (CD80/CD86)
			Belimumab (BLyS)

CNS tissue for normal immunosurveillance. Stüve et al (2008) showed that natalizumab decreased the number of CD4+ and CD8+ T- lymphocytes as well as CD19+ B-cells and CD138+ plasma cells in the cerebrospinal fluid (CSF) of patients with MS. Thus, the reduced immunosurveillance within the CNS may lead to uncontrolled reactivation of this virus (or potentially also other pathogens). Additionally, natalizumab results in hematopoietic stem cell mobilization, in particular of immature cells of the B cell lineage and CD34+ progenitor cells from the bone marrow. Thus, the particular mode of action of natalizumab appears to create a very favourable condition for the development of PML following reactivation of JC virus, respectively the infection of oligodendrocytes by JCV in the brain (Tan et al 2010).

Natalizumab was voluntarily withdrawn from the US market in 2005 after 3 cases of PML had been identified in clinical trials, representing an incidence of approximately 1 case per 1000 patients (95% confidence interval [CI], 0.2 to 2.8) (Yousry et al, 2006; Bloomgren et al, 2012). After the establishment of an intensive global risk management program, natalizumab was reintroduced to the US market in 2006 and shortly thereafter licensed in the European Union (EU).

Up to May 2012, a total of 242 natalizumab patients with PML have been reported from

spontaneous reporting, post-marketing safety studies and registries with an overall mortality rate of approximately 22 % (Europe/ Rest of World 12 %). As far as clinical outcomes in terms of Karnofsky performance index (Karnofsky and Burchenal, 1949) of PML patients who survived are known, large variability of PML outcomes have been documented (Fig.1). Some patients survive with minimal or no disabilities as reflected by the Karnofsky index, whereas others suffer from permanent and serious disabilities. Risk factors predicting outcome are not known and need to be investigated.

Hazard rates reveal an estimated cumulative likelihood for the development of PML of patients treated with natalizumab for the US population of 11.2/1000 (95%-CI: 8.4/1000 – 15.0/1000) and for the European Union/ Rest of the World population of 9.6/1000 (95%-CI: 7.8/1000 – 11.8/1000) (calculations by the authors). The analysis of the accumulated safety data have identified three risk factors for the development of PML following natalizumab treatment, which are duration of treatment of at least two years, the use of immunosuppressants before the initiation of natalizumab therapy, and positive status with respect to anti-JC virus antibodies, as assessed with the use of a two-step anti-JC virus antibody assay. Patients who cumulate all three risk factor are considered to

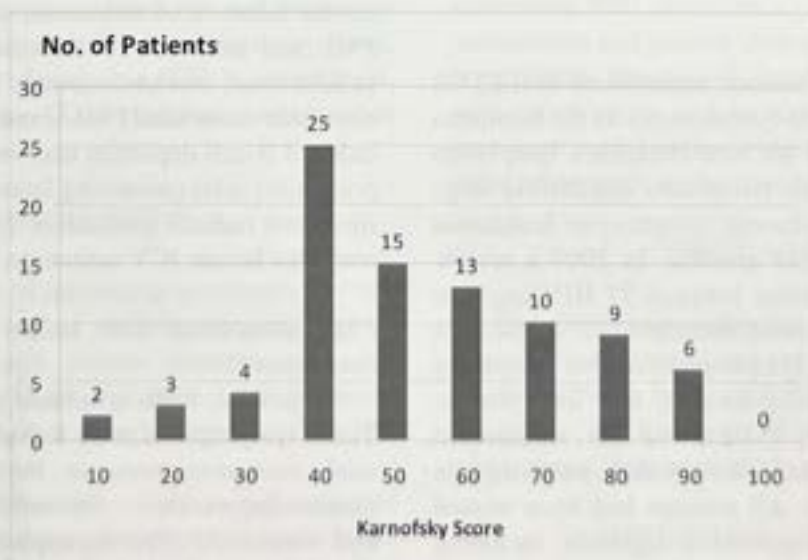


Fig. 1. Karnofsky performance index in 87 PML patients treated with natalizumab, who survived and for whom Karnofsky index was available on follow-up (medium months) Karnofsky score runs from 100 to 0, where 100 is "perfect" health and 0 is death.

have a higher risk (Bloomgren et al, 2012).

Efalizumab

Efalizumab is an anti-CD11a IgG1 antibody which was licensed for moderate to severe plaque psoriasis. Efalizumab acts by inhibiting the initial T-cell activation in lymph nodes, preventing binding of T-cells to endothelial cells and blocking trafficking of T-cells from the circulation into the psoriatic skin preventing their reactivation in the dermal and epidermal layer. More than 6000 patients had been treated with efalizumab before its removal from the European and U.S. markets in February 2009; of these, 166 patients had received more than three years of therapy (Berger et al, 2009). Six suspected cases of PML have been reported from the US (n=3) and Germany (n=3, reported to Paul-Ehrlich-Institut), thereof four with confirmed and two with suspected diagnosis of PML. With the exception of one patient, who was treated for 13 months prior to onset of PML, all were treated for more than three years with efalizumab.

In its mechanism of action, efalizumab resembled natalizumab by also affecting the process of integrin-dependent lymphocyte trafficking by inhibition of LFA-1 binding to ICAM-1, which is involved in the step of firm adhesion of T-cells to endothelial cells. Therefore, it also possessed a mode of action bearing a high risk for PML development.

Rituximab

Rituximab is a chimeric monoclonal anti-CD20 antibody that targets B-lymphocytes. In the European Union, it is licensed for Non-Hodgkin's lymphoma (stage III-IV follicular lymphoma and diffuse large B-cell lymphoma), chronic lymphocytic leukaemia (CLL) and rheumatoid arthritis. In 2009 a review suggested an association between 57 HIV negative PML cases and rituximab treatment. 52/ 57 patients were treated for lymphoproliferative disorders (generally B-cell malignancies), two for systemic lupus erythematosus (SLE), one for rheumatoid arthritis (RA), and one for autoimmune pancytopenia (Carson et al, 2009). All patients had been treated with other immunosuppressive regimens, including hematopoietic stem cell transplantation. In contrast to PML associated with the treatment of natalizumab and efalizumab, the development of PML in

rituximab treated patients may not be unexpected due to a low basal risk for PML in the conditions treated with rituximab. Thus, determination of the possible attributable risk of rituximab is difficult. A retrospective, single-centre cohort study compared rituximab exposed Non-Hodgkin's lymphoma (NHL) patients with rituximab unexposed NHL patients. A significantly higher incidence of PML cases with a rate difference of 2.2/ 1000 patient years (95 % confidence interval 0.1 – 4.3) was detected in rituximab exposed patients. However, the study results need to be interpreted with caution as the study may be affected by several limitations as discussed in (In a recent publication the reporting frequency of PML following rituximab treatment for RA was calculated to be approximately one per 25000 patients. It is likely that the number of cases of PML reported with rituximab is an underestimate of the true incidence. Although any estimation is limited by the small number of five patients, it suggests a somewhat higher frequency of PML in RA patients treated with rituximab compared to the background rate in this population (Molloy and Calabrese, 2009). There are additional spontaneous reports of PML following rituximab treatment in patients with SLE (n=5), vasculitis (n=2) and dermatomyositis (n=1) (Molloy and Calabrese, 2012).

In contrast to PML development after natalizumab and efalizumab, which is apparently dependent on the duration of treatment, the interval between PML and initiation of rituximab therapy appears to be a more stochastic event. The pathogenesis of rituximab-associated PML is not yet clear. Rituximab induced B-cell depletion may result in expansion of progenitor cells containing latent JCV. Furthermore, rituximab reduces qualitative T-cell response which may also favour JCV activation.

PML associated with other immunosuppressive treatments

In general, PML is related to CD4+ and CD8+ T-cell lymphopenia and, linked to this, to a high total immunosuppressive burden. Thus, several immunosuppressive monoclonal antibodies and chemicals like mycophenolate mofetil and ciclosporin may confer to PML development. Consequently, statements on PML development have been introduced into the product information

(Summary of Product Characteristics, which is the prescribing information for the physician) of numerous medicinal products (Tab. 1).

There have been isolated reports of PML after TNF- α inhibitors (Kothary et al, 2011, Molloy and Calabrese, 2012). However, case reports of PML in patients treated with TNF- α inhibitors were confounded by the use of other immunosuppressive therapies and a low baseline rate of PML in the treated condition, or were not confirmed as PML cases after individual case review. It is well known that TNF α affects T-cell mediated effector mechanisms. Considering the high number of patients treated with TNF α -inhibitors and the low number of reported PML cases, a causal association appears to be unlikely.

Until now no cases of PML have been reported to the European Medicines Agency's database EudraVigilance for tocilizumab, a more recently approved monoclonal antibody directed against the human interleukin-6 (IL-6) receptor, indicated for rheumatoid arthritis. By the time of this review, one suspected PML case with questionable diagnostic certainty based on the information submitted has been reported to EudraVigilance for abatacept, and two cases for belatacept in patients with organ transplantation, of which one case report has been published (Grinyó et al, 2010). Both patients with PML following belatacept have also received other immunosuppressive treatment including mycophenolate mofetil for prophylaxis of organ transplant rejection. Abatacept and belatacept are both fusion proteins consisting of human cytotoxic lymphocyte associated antigen 4 (CTLA-4) and IgG1, thereby inhibiting the CD28-mediated co-stimulation of T cells.

CONCLUSIONS

The importance of increasing awareness of PML among physicians and of understanding the risk of PML associated with certain immunosuppressive products is of critical importance for informed decision making of doctors and patients, and for risk mitigation.

Various mechanisms of cell-mediated immunodeficiency may constitute a permissive immune environment that leads to JC virus reactivation. Medicinal products targeting T-cells with the aim of disruption and/or the impairment of

T-cell function, either by inhibition of activation or by inhibition of lymphocyte trafficking may bear the highest risk of creating favourable conditions for development of PML. With regard to a potential risk becoming a manifest PML case, the duration of treatment and confounding factors such as the baseline risk for PML development of the treated condition and concomitant immunosuppressive treatment need to be considered. PML is the most prominent complication resulting from JCV reactivation. Nevertheless it has to be kept in mind that JCV reactivation may also lead to other diseases such as JC virus granule cell neuronopathy (Koralnik et al, 2005) and may also be linked to malignancies. Thus, the more general potential risk of reactivation of JCV and associated diseases need to be investigated.

The development of PML has the potential to limit the benefits of highly effective medicinal products. A lot has been learned in recent years about JC virus biology; however, a lot remains unknown such as predictive markers for development of PML as well as effective prophylaxis and treatment of PML. Therefore, US and EU regulators have demanded a global research agenda (Vinhas de Souza et al, 2012). Regulatory agencies indeed play an important role in handling PML by regulatory measures such as restriction of the indication to a patient population where benefit/risk is, despite PML risk, still favourable, or by issuing warning statements, PML detection algorithms in prescribing information and patient alert cards. PML was, from a regulatory perspective, handled for the first time with natalizumab, and the lessons learned from this case were useful for handling the risk suspected with other biologicals as discussed in this article.

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The views expressed in this article are the personal views of the author(s) and may not be understood or quoted as being made on behalf of or reflecting the position of the European Medicines Agency or one of its committees or working parties.

CONTRIBUTION OF AUTHORS

Karin Weisser and Brigitte Keller-Stanislawski mainly wrote the article, all other authors wrote

parts of the article and/or provided specific analysis of PML cases associated with natalizumab.

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HSV-1 AND CNS: THE LONG TERM EFFECTS OF A LASTING AFFAIR

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Starting from the pioneering studies showing evidence of herpes simplex virus type-1 (HSV-1) genome in Alzheimer's disease (AD) brains, different epidemiological and experimental reports have proposed a possible connection between AD risk and HSV-1 recurrent infections. The main hypothesis is that, beyond massive HSV-1 entry in the brain, resulting in rare, but severe form of herpetic encephalitis, milder cerebral infection may also occur, followed by latency and virus reactivations, whose damages, may accumulate over life and result in pathologic outcomes in the elderly. This paper provides a review of literature supporting HSV-1 as a risk factor for neurodegeneration and showing the possible mechanisms involved.

Accumulating evidence documents that, besides acute illness, infectious agents can produce slowly progressive chronic diseases, including atherosclerosis (Rassu et al, 2001; Saikku, 1999), cardiovascular and metabolic pathologies (Gulcelik et al, 2005; Martin-de-Argila et al, 1995; Mendall et al, 1994; Quatrini et al, 2001) and various neurological and neuropsychiatric disorders (Krause et al, 2010; Rott et al, 1985; Salvetti et al, 2009; Schretlen et al, 2010). Several reports have also suggested the possibility that infections affecting the central nervous system (CNS) may play a role in the induction of neurodegeneration that characterizes highly complex diseases of the elderly including Alzheimer's disease and Parkinson's disease (Bowery et al, 1992; De Chiara et al,

2012; Holmes and Cotterell, 2009; Mattson, 2004; Takahashi and Yamada, 1999). This is confirmed by experiments in which HIV coat protein GP120, given intraventricularly in rats, produced neuronal apoptosis and DNA fragmentation (Bageeta et al, 1995; 1996). In particular, those infectious agents that establish a life-long latent infection in the nervous system and reactivate under different stress stimuli are the best candidates as risk factors for neurodegenerative diseases. Damages that follow each reactivation may accumulate over time, combine with genetic and environmental factors, and drive up favouring conditions for neurodegeneration. In addition, reactivations of neurotropic pathogens during aging may be particularly dangerous for the central nervous system (CNS) on account of

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alterations to the blood-brain barrier (BBB), as well as of age-related increased oxidative stress and impaired energy production (reviewed in Mattson, 2004).

This review focuses on the possible involvement of herpes simplex virus type 1 (HSV-1) infection and reactivations in the pathogenesis of Alzheimer's disease (AD), the most prevalent neurodegenerative disorder in the elderly. AD is a multifactorial disease characterized by memory impairment and progressive cognitive decline. Molecular hallmarks of AD are the overproduction of amyloid- β protein (A β) and the hyperphosphorylation of the microtubule-associated protein tau, followed by their deposition in insoluble aggregates (Selkoe, 2001).

HSV-1 invasion of CNS is known to cause serious neurological disorders during acute infections and has been recently associated with deficits in memory and executive functioning in diverse neurological pathologies (Dickerson et al, 2004; Schretlen et al, 2010; Shirts et al, 2008). These findings, together with the recent epidemiological and experimental evidences correlating HSV-1 infection with AD pathogenesis, support the idea that the virus/host life-long cohabitation, and the long-term effects of virus reactivations in neurons may play a cofactorial role in neurodegeneration.

HSV-1: LIFE-CYCLE AND ENTRY IN THE CNS

HSV-1 is a double-stranded DNA virus with an enveloped icosahedral capsid whose primary infection usually occurs in the oro-facial mucosa during childhood. There the virus undergoes lytic replication, starting within the nucleus of infected epithelia and ending with the production of infectious virus and lysis of the host cell. Newly produced viral particles released from the initial site of infection may enter sensory neurons, travel through retrograde axonal transport toward the trigeminal ganglion or the olfactory bulb, where they may establish a life-long latent infection or rapidly enter the CNS (Baringer and Swoveland, 1973; Salinas et al, 2010). The process regulating the establishment of latency is characterized by the presence of a functional viral genome without production of the infectious virus. During latent infection, the latency-associated transcripts (LATs) are the only prominent transcripts (Stevens et al, 1987). Reactivation stimuli

such as stress, immunosuppression, DNA damaging agents, etc. activate viral gene expression, which ends with lytic release of viral progeny. The latter is organized in a temporal cascade, with the first expression of immediate early gene products (IE), which are required to coordinate the expression of early (E), and late (L) gene products. Newly formed virions proceed by axonal transport back to the sites of primary infection, or onward to the CNS, where they may cause a productive, but usually mild infection, or may establish life-long latent infection, as described for rodents (Kastrukoff et al, 1982; Lewandowski et al, 2002). In particular, viral particles may target the limbic system, as reported for HSV encephalitis (HSE) (Liedtke et al, 1993), through the olfactory bulb, since HSV-1 can infect cells in the nasal endothelium (Boggian et al, 2000; Mori et al, 2005). After entering the CNS, HSV-1 uses the anterograde transport system (from cell body to axon) to reach the axon termini where it is released by exocytosis into the synaptic cleft (Salinas et al, 2010). However, during anterograde transport, the virus can exit via axonal varicosities before reaching the termini and infect neighbouring cells (De Regge et al, 2006; Diefenbach et al, 2008). Spread of HSV-1 in the CNS may cause acute infections, possibly followed by latency and reactivations.

HSV-1, APOE4, AND AD

HSV-1 is a ubiquitous neurotrophic virus that affects about 85% of the world population, and almost one third of the infected population has recurrent clinical HSV-1 manifestations. As described above, the virus may reach the CNS causing HSE. However, animal studies revealed that HSV-1 may also replicate in the brain without producing any neurological signs (Boggian et al, 2000), and the virus has been found to infect human brain latently (Gordon et al, 1996), and to cause milder herpetic encephalitis as documented in Fodor et al (1998) and Klapper et al (1984). Since it has been found that the brain regions primarily targeted by HSV-1 in HSE (i.e., the frontal and temporal cortices and hippocampus) are the same as those most prominently affected by AD, chronic and persistent exposition to HSV-1 has been proposed as a potential risk factor for this neurodegenerative disease (Ball, 1982; Denaro et al, 2003). Interestingly, epidemiological studies have reported the presence of the HSV-1 genome in post-

mortem brain specimens from numerous AD patients, particularly those who carry the type 4 allele of the gene encoding Apolipoprotein E (ApoE) (Corder et al, 1998; Itzhaki et al, 1997; Itzhaki and Wozniak, 2008; Wozniak et al, 2005). ApoE, expressed in three isoforms (designed ApoE2, ApoE3 and ApoE4), is the predominant apolipoprotein of the HDL complex in the brain and is involved in lipid transport and repair of damaged tissues. Among its several functions in brain physiology (Kim et al, 2009), its role in the development of AD has reportedly been associated to its ability to bind A β and to promote its clearance. In particular, the presence of *APOE e4* allele is strongly associated with increased brain A β deposition (Tiraboschi et al, 2004), and its protein product, ApoE4, shows a weaker binding to A β compared to ApoE2 and ApoE3 (Tokuda et al, 2000). The link between ApoE4 and HSV-1 has been somehow controversial: Itabashi et al (1997) confirmed that the presence of HSV-1 in ApoE4 carriers increases the risk of AD, whereas Beffert et al (1998) did not find any significant association among HSV-1, APOE e4 allele and AD. Other studies provide controversial results (Hemling et al, 2003; Marques et al, 2001), and these discrepancy has been attributed to the low detection levels of HSV-1 in the brains analyzed (due to procedural differences) and to the different populations studied (reviewed in Wozniak and Itzhaki, 2010). Interestingly, the APOE e4 genotype has been reported to increase the risk for HSV-1-induced cold sores (Itzhaki et al, 1997) and for infection by the genital herpes virus HSV-2 (Jayasuriya et al, 2008; Koelle et al, 2010). In the same line, some *in vivo* studies support the idea that the ApoE isoform influences brain susceptibility to HSV infections. Burgos et al analyzed HSV-1-infected transgenic mice expressing the APOE3 or 4 allele exclusively, as well as wild type and/or APOE knock out mice, revealing a greater viral load in the brains of ApoE4 transgenic animals during the acute phase of infection, latency and vertical transmission (Burgos et al, 2002; 2003; 2006 and 2007). These authors also suggested that ApoE4 may increase the risk to develop AD by increasing latent HSV-1 viral load in the CNS (2006). By analyzing HSV1-infected transgenic mice expressing specific isoforms of the protein in the brain in a ApoE $-/-$ background, Miller and Federoff (2008) reported that the carriage of ApoE4 leads to an higher expression of the viral early genes and decreased the expression of the latency associated

genes, suggesting that the presence of that specific isoform would promote more frequent reawakening of the virus. Bhattacharjee et al (2008) also reported a possible association between APOE e4 genotype and HSV-1 susceptibility in *in vivo* models of ocular HSV-1 infections and suggested ApoE4 as a risk factor for ocular herpetic related pathologies.

How may ApoE4 act synergically with HSV-1 in driving up AD risk? A possible mechanistic explanation relies on the evidence that the virus and ApoE share the heparan sulfate proteoglycan as receptor on the cell surface. When HSV-1 competes with the ApoE4 isoform, that is the weaker receptor ligand among ApoE isoforms, this may allow the entry of more viruses into cells, as proposed by Itzhaki and Wozniak (2006). The same mechanisms, together with an altered cholesterol composition of lipid rafts in ApoE4 carriers that may enhance viral load in neurons, have been reported to increase also the pathogenicity of human immunodeficiency virus (HIV) infection associated to APOE e4 genotype (Burt et al, 2008; Kuhlmann et al, 2010). Moreover, Apolipoprotein E is associated with neuronal repair, but the latter appears less efficient in E4 carriers, whereas oxidative stress, inflammatory cytokine and nitric oxide production are increased. Therefore, as proposed by Kuhlmann et al (2010), the different pathological effects of an ApoE4 genotype and neuronal impact of HSV-1 infection may contribute to increase AD risk. In this line, some authors proposed that HSV-1 infection alone is not associated with AD, whereas in combination with ApoE4 genotype the risk of AD increases in the elderly (Itabashi et al, 1997).

HSV-1 AND AD: IMMUNOLOGICAL AND GENETIC CORRELATIONS

Genes related to HSV-1 reactivation have been detected in the brain of patients with familial AD, associated with A β deposits (Mori et al, 2004). Moreover, HSV-1 DNA has been found in amyloid plaques from the temporal and frontal cortices of AD sufferers (Wozniak et al, 2009a), supporting the link between HSV-1 infection and AD. Recently, a large prospective population-based study of 512 non-demented elderly subjects also showed a significant association between primary HSV-1 infection or reactivation (anti-HSV-1-IgM-positive status) and the

risk of AD, whereas no association was observed within a life-long HSV-1 infection (anti-HSV-1 IgG positive status) (Letenneur et al, 2008). Within a follow up of 14 years, 99 subjects developed dementia, including 77 cases of AD. No synergic effect was found between APOE e4 genotype and anti-HSV-1 IgM-positive status, likely due to the small number of e4 carriers with HSV-1 infection in the population analyzed. These findings support the view that repeated reactivation of this virus may contribute to the development of AD.

In addition, analysis of data from genome-wide association (GWA) studies of several thousand European AD patients and controls have correlated individual brain susceptibility to HSV-1 infection with a genetic risk of AD (Lambert et al, 2009a; Porcellini et al, 2010). In particular, besides the expected APOE e4, these gene variants include nectin-2 (NC2), which mediates the entry of HSV into host cells; translocase of the outer mitochondrial membrane 40 homolog (TOMM40), whose variations might influence mitochondrial damage induced by HSV DNAase such as UL12.5; and other genes. In addition, as described in the following section, results from *in vitro* and *in vivo* studies suggest a possible involvement of HSV-1 in A β production though the amyloidogenic endoproteolytic processing of the transmembrane amyloid precursor protein (APP). This idea is also supported by the results of the Three-City population-based cohort study (Féart et al, 2011) showing that high anti-HSV IgM levels, that are markers of HSV reactivation, are associated with lower plasma A β_{40} and A β_{42} levels, a condition that has been linked to an increased risk for developing AD (Lambert et al, 2009b; Song et al, 2011). However, Féart and coll. also stressed that their results should be interpreted with caution due to some limitations, i.e. the lack of a direct causal correlation between the low plasma A β levels and HSV reactivations, as well as the lack of any cerebrospinal fluid measurement of A β levels that may closely reflect what happens in the brain.

HSV-1 AND AD: DEGENERATIVE EFFECTS OF EXPERIMENTAL INFECTIONS IN NEURONAL CELLS

HSV-1 and A β

Starting from the observation describing some degree of sequence homology between Ab and the HSV-1 glycoprotein B and suggesting that this viral

protein may act as a seed for A β deposits in amyloid plaques (Cribbs et al, 2000), several results from *in vitro* and *in vivo* studies associate HSV-1 to A β production and accumulation in the brain. Newly HSV-1 particles produced in the PNS have been proposed to recruit cell membranes containing APP, possibly during packaging in the Golgi apparatus (Bearer, 2004), and to release APP during transport into the brain, thus contributing to the formation of amyloid deposits. HSV-1 has also been shown to bind APP directly within the axonal transport into neurons (Satpute-Krishnan et al, 2003 and 2006) in *in vitro* studies. More recently, Cheng and colleagues demonstrated that intracellular HSV-1 interacts with APP, and that this interplay enhances viral transport and impairs APP transport and distribution in neurons (2011).

HSV-1 infection has also been reported to alter APP processing: Shipley et al (2005) showed that HSV-1 infection of neuroblastoma cells induced the formation of a 55-kDa C-terminal fragment of APP; later, the same group (Wozniak et al, 2007) reported the accumulation of A β in HSV-infected neurons and mouse brains. Our group demonstrated that in neurons HSV-1 triggers amyloidogenic cleavages of APP that are mediated at least in part by the action of β -secretase, γ -secretase and caspase-3-like enzymes (De Chiara et al, 2010). The virus-induced APP cleavage results in the formation and intracellular accumulation of different APP fragments including A β with established potential for neurotoxicity (De Chiara et al, 2010). Some of these fragments are also secreted in the extraneuronal space, where they are able to trigger apoptosis in the neighbour cells. Moreover, HSV-1 produces marked changes in neuronal excitability and intracellular Ca²⁺ signalling that cause APP phosphorylation and intracellular A β accumulation in rat cortical neurons (Piacentini et al, 2011). How may the virus activate the amyloidogenic processing of APP and induce A β accumulation in the brain? We found that HSV-1-induced amyloidogenic APP processing is prevented by antioxidant agents (De Chiara et al, 2010), suggesting that HSV-1-induced oxidative stress in neuronal cells may trigger β - and γ -secretase activation and, consequently, APP processing and A β formation. This hypothesis is supported by several evidences showing both the occurrence of

oxidative damage in the neuronal cells following HSV-1 infection (Fujii et al, 1999; Kavouras et al, 2007; Valyi-Nagy et al, 2000), and the upregulation of β - and γ -secretase under oxidative stress conditions (Tamagno et al, 2002; 2005; 2008). However, other mechanisms activated by the virus may contribute to trigger this event. Among these, HSV-1-induced the activation of double stranded RNA-activated Protein Kinase (PKR) in neuroblastoma cells and peripheral nervous tissue from infected mice has been suggested to increase the amyloidogenic APP processing and A β production by promoting Beta-site APP cleaving enzyme 1 (BACE1) translation via eIF2a phosphorylation (Ill-Raga et al, 2011). Accumulation of A β in AD neurons has also been associated to defects in autophagy (Martinez-Vicente and Cuervo, 2007; Rubistein et al, 2007; Williams et al, 2006), a highly conserved mechanism for the degradation and recycling of superfluous or damaged organelles and proteins, including the aggregates that characterize neurodegenerative diseases. HSV-1 has been shown to modulate the autophagic machinery through the viral protein ICP34.5 (Talloczy et al, 2002 and 2006), possibly to prevent autophagic degradation of HSV-1. Interestingly, recent papers from Santana et al (2011 and 2012) show that HSV-1 infection in neuroblastoma cells induces intracellular A β accumulation in autophagosomes, the double-membrane vesicles of the autophagic machinery that deliver cytoplasmic cargo to lysosomes, to ensure their complete degradation. In particular, the authors found that the autophagosome fusion with lysosomes was aborted in infected cells, thus possibly contributing to the formation of amyloid aggregates and neurofibrillary tangles (NFTs), another well-known hallmark that characterizes AD.

HSV-1 and tau phosphorylation

HSV-1 has been shown to cause the hyperphosphorylation of the microtubule-associated tau protein, thus impairing its intracellular transport functions. Actually, the native form of tau assembles and stabilizes microtubules (Harada et al, 1994), and this function is strongly compromised when the protein is hyperphosphorylated, resulting in a reduction of material movements along the axons and consequently neuronal death induction (Lovestone and Reynolds, 1997). Zambrano et al (2008) first

reported evidence of tau hyperphosphorylation following HSV-1-infection in cultures of murine cortical neurons. In neuroblastoma and glioblastoma cells HSV-1 infection was shown to induce the phosphorylation of tau at a number of sites that are phosphorylated in AD too (Wozniack et al, 2009b). This effect was associated with an increased expression of the enzymes involved in this event, i.e. glycogen synthase kinase 3 β and protein kinase A. More recently, Lerchundi and colleagues (2011) showed that HSV-1 induces the caspase-3-mediated cleavage of tau protein at its specific site (aspartic acid 421). This event has been associated with an increased kinetics of tau aggregation, observed in neurodegenerative pathologies. Tau has also been observed at sites different from microtubules, such as ribosomes (Papasozomenos and Binder, 1987) and the nucleus (Brady et al, 1995; Lefebvre et al, 2003; Sultan et al, 2011; Thurston et al, 1997). The functional significance of the ribosomal or nuclear location of tau has yet to be clarified. Sultan et al (2011) demonstrated that acute oxidative stress and mild heat stress induce the accumulation of dephosphorylated tau in neuronal nuclei where it interacts with DNA, suggesting a protective role for nuclear tau in stress-induced DNA damage in neurons. In neuroblastoma cells Alvarez et al (2012) recently demonstrated that HSV-1 infection provokes a specific increase in hyperphosphorylated tau in nuclear regions corresponding to the compartments where replication and transcription of viral DNA takes place, thus suggesting a functional role for the protein in the nucleus of HSV-1-infected cells possibly related to neurodegeneration.

HSV-1 and functional impairments of neurons

HSV-1 invasion of CNS is known to cause serious neurological pathologies, such as encephalitis, meningitis, and epilepsy (van den Pol, 2006), and long-term neurological sequelae is common among herpes simplex encephalitis survivors (Hokkanen and Launes, 2000). Moreover, HSV-1 replication has been associated with deficits in memory and executive functioning in diverse neurological pathologies, such as chronic psychiatric disease as schizophrenia and bipolar disorders (Dickerson et al, 2004; Schretlen et al, 2010; Shirts et al, 2008), indicating the viral infection as a causative factor in these neuronal

deficits, that also characterize AD. These associations are supported by several results from *in vitro* and *in vivo* studies. In infected organotypic hippocampal slices Ando and collaborators (2008) demonstrated that the virus primarily infects granule cells in the Dentatus Gyrus (DG) subfield and, afterward, kill them through HSV-1-mediated cytopathic effect. These authors suggested that the virus-induced death of DG granule cells in the hippocampus may contribute to the observed memory deficits in *in vivo* models of infections (Beers et al, 1995; McLean et al, 1993). In addition, a systematic analysis of HSV-1-induced neuropathology in animal models of HSE demonstrated that HSV-1-infected mice surviving the acute phase of infection exhibited sustained inflammation, neuronal loss, and neuropathological sequelae (>60 days p.i.), characterized by severe spatial memory deficit (Armien et al, 2010) which was consistent with the functions associated with inflammatory lesions in the piriform and entorhinal cortical regions (Eichenbaum and Lipton, 2008; Squire et al, 2007). Interestingly, *in vitro* studies on infected primary cortical neurons showed that HSV-1 binding to neuronal membrane markedly affects their electrophysiological properties and enhance their excitability (Piacentini et al, 2011). This effect consisted of persistent Na^+ channel activation and K^+ current inhibition leading to membrane depolarization and increased neuronal firing. Voltage-gated Ca^{2+} channels were consequently activated thus triggering intracellular Ca^{2+} signals raising the basal intracellular Ca^{2+} levels. Calcium signals potently promoted APP phosphorylation and processing with consequent intracellular and extracellular accumulation of several neurotoxic fragments including $\text{A}\beta$ oligomers (De Chiara et al, 2010; Piacentini et al, 2011). These virus-induced APP fragments might induce synaptic dysfunction resembling that underlying the cognitive deficits observed in AD. Moreover, besides all these described effects of acute infection, a very recent paper indicated that also latent HSV-1 infection in PNS is associated with progressive deficits in neuron size, density and number in the nervous system (Dosa et al, 2011), underlining another pathological effect of HSV-1.

The findings described so far support the idea that repeated cycles of HSV-1 replication within

the CNS may produce in infected or neighbouring cells functional and molecular alterations that are reminiscent of neurodegeneration (see Fig. 1). These effects may be favoured by a combination of different factors such as metabolic disorders, genetic alterations and other environmental risk factors, involved in the pathogenesis of neurodegenerative diseases. In this line, very recently, Guzman-Sanchez et al (2012), by analyzing over life mice infected at early age through intraperitoneally HSV-1 inoculation, provided evidence that the modulation of HSV-1 load in mouse brains depends on the combination of aging, ApoE profile, and gender. In these animal models, some of which undergoes to spontaneous virus reactivations, HSV-1 *per se* does not directly cause any significant neurodegeneration. However, the authors hypothesize that HSV-1 brain infection may predispose neurons to neurodegeneration. This may be particularly relevant in patients experiencing frequent HSV-1 reactivations.

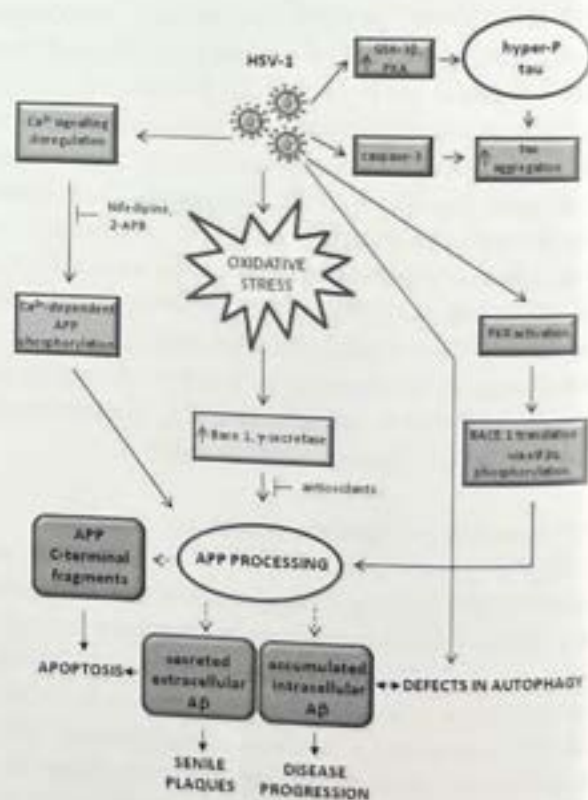


Fig. 1. Schematic representation of the effects of HSV-1 infection on neuronal cells. For details, see text.

CONCLUSIONS

The findings described in this review support the idea that HSV-1 could contribute to neurodegeneration that characterizes AD. Some authors have demonstrated that HSV-1-induced neurodegeneration may be partially inhibited by specific antivirals such as acyclovir or its biodrug valacyclovir (Lukiw et al, 2011; Wozniak et al, 2011; Zambrano et al, 2008) proposing these treatments as a new possible way to prevent AD occurrence in HSV-1 bearing patients, especially those who are ApoE4 carriers. In vivo models of recurrent HSV-1 reactivations are then strongly required both to confirm the long-term neurodegenerative effects caused by repeated cycle of viral replication and to assess the potential preventive effect of antiviral strategies.

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SYNAPSE DISEASES AND INTELLECTUAL DISABILITY SYNDROMES

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Over the last twenty years, numerous mutated genes that code for proteins concerned with synapse function have been identified in patients affected by intellectual disability syndromes. These genes may be functionally involved in synapse formation, the regulation of dendritic spine morphology, the regulation of the synaptic cytoskeleton or the synthesis and degradation of specific synapse proteins. These studies have clearly shown that even mild alterations in synapse morphology and function give rise to mild or severe intellectual disabilities. Interestingly, pharmacological agents that are able to counteract these morphological and functional synaptic anomalies can also improve the symptoms of some of these conditions. This review summarizes recent findings on the functions of some of the genes responsible for intellectual disability syndromes associated with synapse dysfunctions

Several psychiatric and neurological diseases are characterized by the presence of aberrant synaptic formation, signalling and plasticity, or altered spine morphology (Blanpied *et al.*, 2004) and it is now obvious that a precise control of synaptic development is critical for the formation of a normally active neuronal network important for normal brain function.

One of the most common neurodevelopmental disorders is intellectual disability (ID), formerly mental retardation; patients affected by ID have an intelligence quotient of 70 or below and often exhibit deficits in behaviour related to adaptive functioning which includes autism spectrum disorders (ASD).

Over 25% of ID cases are caused by genetic factors (Rauch *et al.*, 2006) but in up to 60% of cases no cause has been identified. Interestingly, a recent study described that ASD patients have a high global burden of rare copy number variants, especially of loci previously known to be associated with this

disease, but also of new genes previously unrelated with ASD (Pinto *et al.*, 2010).

Several single-genes causing syndromic or nonsyndromic ID have been identified over the past 15 years. Most of these genes are located on the chromosome X and are responsible for X-linked intellectual disabilities (XLID). Interestingly more than 50% of the ID-related proteins that are not transcription or chromatin-remodelling factors, are clearly present in the pre- or post-synaptic compartments and appear to be implicated in synaptic functions by regulating actin cytoskeleton rearrangement, synaptic plasticity or synapse formation (Ropers *et al.*, 2005).

Finally, the synapse-related proteins associated with ID can be divided into two groups, one that localizes entirely at synapses and whose mutations directly interfere with synaptic formation, and a second group that regulates neuronal development and synapse formation indirectly by controlling

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synaptic proteins or the synaptic actin cytoskeleton synthesis, degradation or modulation. In this review we will describe the molecular mechanisms by which dysfunctions in some of these proteins contribute to ID.

MUTATIONS IN THE SYNAPTIC SCAFFOLD PROTEINS

The MAGUK family of proteins

DLG4 in humans codifies for PSD-95 the most abundant scaffold protein at the PSD that belongs to the MAGUK family of proteins. Polymorphisms and mutations have been extensively analyzed in *DLG4* in association with neuro-developmental diseases, however only one study suggest an association between a *DLG4* gene variation and ASD and Williams syndrome (Feyder et al, 2010), additionally, a haplotype derived from 2 polymorphic markers at the core promoter has been connected to schizophrenia (Cheng et al, 2010).

By contrast, *DLG3*-the human gene that encodes for synapse-associated protein 102 (SAP102)- is clearly associated with ID (Tarpey et al, 2004; Zanni et al, 2010). The mutations identified in this gene introduce premature stop codons within or before PDZ domain number three. These mutations impair the ability of the possible truncated SAP102 protein to interact with the NMDA receptor and other proteins implicated in NMDA receptor signaling pathways. Interestingly, it has been demonstrated that SAP102 could link NMDA receptor activation to alterations of dendritic spine morphology (Chen et al, 2011).

Mutations in the SHANK/ProSAP gene family

The distal deletion of chromosome 22 involves a critical region that includes *SHANK3* which encodes the Shank3/ProSAP2 postsynaptic scaffold protein. This deletion is causes the Phelan-McDermid syndrome (PMS, also called 22q13.3 deletion syndrome) which is characterized by intellectual impairment, absent or delayed speech, autistic-like behavior, hypotonia, and mild dysmorphic features (Bonaglia et al, 2001; Manning et al, 2004; Phelan et al, 2001; Wilson et al, 2003).

SHANK3 haploinsufficiency is now considered to be the main cause of the neurobehavioral symptoms

of PMS, although other genes may also be lost by the chromosomal deletion (Bonaglia et al, 2001; Delahaye et al, 2009; Durand et al, 2007; Wilson et al, 2003). Indeed, a number of *de novo* mutations in *SHANK3* (Durand et al, 2007; Gauthier et al, 2009; Moessner et al, 2007), *SHANK2* (Berkel et al, 2010), and in *SHANK1* (Sato et al, 2012) have been identified in individuals with ASD and ID.

Several knock out mice have been created for the three *SHANK* genes. The first described mouse, lacking Shank1, has small dendritic spines, weakened synaptic transmission, enhanced learning (Hung et al, 2008), and defects in social communication (Wöhr et al, 2011). More recent studies in mice emphasize the importance of Shank3 haploinsufficiency in the pathogenesis of ID (Bangash et al, 2011; Bozdagi et al, 2010; Peça et al, 2011; Wang et al, 2011). It has been shown that male mice with either heterozygous or homozygous disruption of Shank3 had, compared to wild-type littermates, abnormal behavior, alterations in learning and alterations in memory formation, and defects in synaptic transmission (Bozdagi et al, 2010; Wang et al, 2011). These animals had markedly impaired basal synaptic transmission in CA3-CA1 connections, reduced GluR1 clusters and protein levels in the hippocampus, and an altered activity-dependent AMPAR synaptic plasticity (Bozdagi et al, 2010; Wang et al, 2011).

The *SHANK3* gene has several splice variants and corresponding specific knocks have been made. The genetic deletion of two major Shank3 splice variants causes self-injurious repetitive grooming and deficits in social interaction correlating with major alteration in striatal synapses and cortico-striatal circuits, but not in hippocampal synapses, suggesting that the remaining Shank3 splice variant(s) may be enough to maintain normal synapse structure and function in hippocampus (Peça et al, 2011).

On the contrary, the mutation of the Shank3 protein that eliminates the Homer-binding C terminus region apparently induces a gain-of-function phenotype that greatly impairs its expression at synapses due to its polyubiquitination and degradation. Also, the GluN1 subunit of the NMDA receptor is reduced at synapses by polyubiquitination and degradation, while AMPAR function and number are not modified (Bangash et al, 2011).

In our laboratory we recently knocked down

all major Shank3 splice variants in rodent neuronal cultures by RNA interference (shRNA) and demonstrated that Shank3 absence in hippocampal cells specifically reduced the expression of mGluR5 receptors, and also impaired DHPG-induced phosphorylation of ERK1/2 and CREB (Verpelli et al, 2011). We thus propose that mGluR5-dependent synaptic plasticity is altered in absence of Shank3.

Finally, similarly to Shank3, also the Shank2 knockout mice show abnormalities in behavior tests, impairment in social activities, hyperactivity, and defects in synaptic transmission (Bockers et al, 2004; Schmeisser et al, 2012; Won et al, 2012).

Altogether, these studies show that in mice mutations in the Shank genes cause alterations in both synaptic morphology and signalling and behavior characteristics, therefore, these mice are a good animal model to study ASD and ID.

MUTATIONS IN THE X-LINKED GENES

Most of the XLID are attributable to the Fragile X and Rett syndromes, however mutations of several other genes on chromosome X have been found strongly associated with ID. Among the first to be clearly associated with synaptic function are the mutations in the neuroligin genes: *NLGN3* and *NLGN4* (Jamain et al, 2003).

Neuroligin proteins, first identified as binding partners of neuroligins, are the prototype of all the synaptic adhesion molecules that induce and regulate synaptogenesis in the brain. The Neuroligins/neurexins complex was found to be associated in synapses, forming the trans-synaptic complex which is important for both excitatory and inhibitory synapse formation and function (Südhof, 2008). It has been estimated that about 50% of the XLID gene codify for synaptic proteins (Laumonnier et al, 2007). This is the case also for TSPAN7 protein which is codified by the XLID gene *TM4SF2*. TSPAN7 is a synaptic protein that regulates excitatory synapse development and AMPAR trafficking by interacting with the PDZ domain of protein interacting with C kinase 1 (PICK1) (Bassani et al, 2012).

The IL1RAPL1 gene

Cognitive impairments ranging from nonsyndromic ID to ASD have been found in

patients with mutations in the interleukin-1 receptor accessory protein-like 1 gene (*IL1RAPL1*) (Bhat et al, 2008; Carrie et al, 1999; Franek et al, 2011; Piton et al, 2008). The IL1RAPL1 protein belongs to a new Toll/IL-1 receptor family and shares 52% homology with the IL-1 receptor accessory protein (IL-1RacP) and it is structurally formed by three extracellular Ig-like domains, a transmembrane domain, and an intracellular Toll/IL-1R homology domain (TIR domain). In contrast to the other family members, IL1RAPL1 has 150 additional amino acids at the C-terminus, that interact with the neuronal calcium sensor-1 (Bahi et al, 2003), thus regulating type voltage-gated calcium channel activity in PC12 cells and in neurons (Gambino et al, 2007).

Our laboratory recently showed that IL1RAPL1 binds postsynaptic density protein 95 (PSD-95) and regulates its phosphorylation and synaptic association by activating the c-Jun terminal kinase (JNK) (Pavlovsky et al, 2010).

We also showed that the extracellular domain of IL1RAPL1 induces presynaptic differentiation by binding the receptor tyrosine phosphatase δ (PTP δ), which is localized at the presynaptic terminal, while the TIR domain binds to RhoGAP2 and regulates dendritic spine formation (Valnegri et al, 2011b). It was also demonstrated that the extracellular domain of IL1RAPL1 interacts only with particular splice variants of PTP δ (Yoshida et al, 2011). All these findings suggest that the IL1RAPL1 complex, similarly to the neuroligin/neurexin complex, mediates trans-synaptic signalling that regulates excitatory synapse and dendritic spine formation.

All this data suggests that most of the adhesion molecules found associated with ID regulate excitatory synapse formation and the consequential functional alteration or reduction in number of excitatory synapses arising from their mutations may alter the balance between excitatory and inhibitory synapses. This induces a general disturbance within neuronal circuits and may be the direct cause of ID in humans. Indeed, even small changes in the expression of these synaptic adhesion proteins can induce major changes in synaptic connectivity, resulting in cognitive destruction.

Oligophrenin-1 gene

Some forms of XLID are caused by mutations

or deletions in the synaptic RhoGTPase-activating protein oligophrenin-1 (Nadif Kasri *et al.*, 2008) indicating that signalling involving member A of the Ras homologue gene family (RhoA) is involved in ID.

Oligophrenin-1 is a negative regulator of RhoA, Rac and Cdc42 and also interacts with the postsynaptic adaptor protein Homer (Govek *et al.*, 2004). Knockdown of oligophrenin-1 in CA1 pyramidal neurons significantly reduces spine length and this effect is mimicked by a constitutively active form of RhoA and can be rescued by the presence of constitutively active RhoA which leads to an inhibition of the RhoA effector Rho-kinase (ROCK1) (Govek *et al.*, 2004).

Considering the important role of ROCK1 in actin remodelling, these results strongly suggest that RhoA regulates the actin cytoskeleton of spines, possibly through effects on the LIM kinase, myosin light chain (MLC), or MLC phosphatase (Govek *et al.*, 2004; Nadif Kasri *et al.*, 2008). Thus, loss of repression of RhoA and ROCK1 caused by the absence of oligophrenin-1 leads to alterations of the actin cytoskeleton, resulting in modification of spine morphology.

Khelifaoui *et al.* (Khelifaoui *et al.*, 2007) showed learning impairment in oligophrenin-1-deficient mice. A consequent study demonstrated that oligophrenin-1 localises to dendritic spines after synaptic NMDA activation, where it forms a complex with AMPA receptors and selectively enhances AMPA-receptor-mediated transmission and spine size by stabilising those receptors (Nadif Kasri *et al.*, 2009). The stabilization via oligophrenin-1 of AMPA receptors in synapses is suggested by a reduced number and a reduced activity of such receptors in oligophrenin-1 KO mice. This defect is rescued by blocking AMPA receptor endocytosis which suggests a correlation between oligophrenin-1/RhoA signalling and AMPA receptor endocytosis [18]. AMPA receptor endocytosis at excitatory synapses is also controlled by RhoA/ROCK signalling regulated by Oligophrenin-1 (Khelifaoui *et al.*, 2009).

A new role of oligophrenin-1 in regulating the activity of the circadian clock protein Rev-erba has recently been shown, suggesting that the ethiology of intellectual disability could be related to the interaction between synaptic activity and circadian

oscillators (Valnegri *et al.*, 2011a). In addition to its role at postsynaptic sites, oligophrenin 1 also has a presynaptic function by acting as a modulator of synaptic vesicle availability and by acting as regulator of vesicle pool dynamics. Also, a reduced expression of oligophrenin-1 has been reported to cause synaptic vesicle endocytosis in culture (Nakano-Kobayashi *et al.*, 2009). Very recently Powell *et al.* (2012) showed that oligophrenin-1-deficient mice have changes in the number of vesicles in the readily releasable pool and also have a changed availability of secretory vesicles. Thus, alterations in oligophrenin-1 expression result in multiple deficits of synaptic activity and plasticity that depend on oligophrenin-1 being expressed in both the pre- and postsynaptic terminals.

Fmrp gene and Fragile X

The fragile X syndrome, the most common form of inherited intellectual disability in humans, is caused by the loss of the *fmr1* gene product, fragile X mental retardation protein (FMRP).

FMRP belongs to the heterogeneous nuclear ribonucleoprotein family of RNA-binding proteins; its expression and localization to dendrites increases after synaptic stimulation, suggesting that it is involved in synaptic plasticity, regulating the transport to synapses, and regulating the translation of a subset of neuronal mRNAs (Bagni *et al.*, 2005; Bassell *et al.*, 2008; De Rubeis *et al.*, 2010). Patients with this syndrome have more, longer and thinner dendritic spines than healthy controls (Bagni *et al.*, 2005). The translational dysregulation of target mRNAs that occurs when FMRP is absent appears to be the main cause of the dendritic spine and synapse alterations that characterize fragile X syndrome (Bassell *et al.*, 2008).

In *fmr1* KO mice, DHPG-induced long term depression (LTD) is strongly increased (Huber *et al.*, 2002) and at the same time metabotropic glutamate receptor (mGluR)-dependent local protein synthesis is deregulated (Huber *et al.*, 2002; Lu *et al.*, 2004; Muddashetty *et al.*, 2007; Zalfa *et al.*, 2007). The latter finding has inspired the 'mGluR theory' of fragile X syndrome which proposes that exaggerated consequences of mGlu5-mediated signalling in the absence of FMRP may play a causal role in FXS, so mGluR inhibitors might be useful as treatment (Bear

et al, 2004).

This theory is strongly corroborated by the finding that genetic reduction of mGlu5 expression is sufficient to ameliorate the phenotypes in the *Fmr1* knockout (KO) mouse (Dölen et al, 2007).

Work from several groups showed that the translation rate of several proteins is increased in purified synaptosomes of *fmr1* KO mice (Laggerbauer et al, 2001; Li et al, 2001; Lu et al, 2004; Muddashetty et al, 2007; Napoli et al, 2008; Zalfa et al, 2003).

FMRP is a positive regulator of translation, increasing the levels of the postsynaptic scaffold proteins SAPAP1-3, Shank1, Shank3, and IRSp53, as well as those of the GluN1 and GluN2B subunits of the NMDA receptor and also the levels of the GluA1 subunit of the AMPA receptor in the cortex and hippocampus of *fmr1* KO mice (Schutt et al, 2009). At the same time FMRP is also a negative regulator of transcripts of the GluN2A subunit of the NMDAR. This data suggests that alterations in the NMDA receptor subunit composition due to FMRP absence modify synaptic plasticity in fragile X syndrome (Edbauer et al, 2010).

FMRP also regulates the levels of PSD-95, an important modulator of synaptic signaling and learning, by stabilising PSD-95 mRNA (Zalfa et al, 2007). Also, PSD-95 mRNA and protein levels are lowered in hippocampus but not cortex (Zalfa et al, 2007) in *fmr1* KO mice. Another study showed that in cortex mGluR activation-dependent microRNA translation of PSD-95, CaMKII α , and GluR1/2 is modulated by FMRP (Muddashetty et al, 2007).

In addition, phosphorylated FMRP is associated with the *Argonaute 2* (*AGO2*) miR125a forming an inhibitory complex of PSD-95 mRNA translation; mGluR1 activation leads to dephosphorylation of FMRP and activation of PSD-95 mRNA translation. This data showed that mGluR1 activation regulates FMRP phosphorylation allowing the reversible switch for *AGO2* and microRNA to selectively regulate mRNA translation at synapses (Muddashetty et al, 2011).

Alteration in synaptic plasticity and dendritic spines morphology found in *fmr1* KO mice could, therefore, be related to dysregulation of PSD-95 translation at synapses (Bagni et al, 2005). On the other hand, FMRP regulates the translation of several

other mRNA proteins involved in synapses and neuronal function (see also Bhakar et al, 2012).

MECP2 gene and Rett syndrome

For more than 90% of the patients, Rett syndrome is caused by the mutation of the *MECP2* gene which encodes methyl-CpG-binding protein-2 (MeCP2), a protein that binds methylated DNA and thereby influences gene transcription.

There is evidence suggesting that MeCP2 functions as a molecular link between DNA methylation, chromatin remodelling, and subsequent gene silencing (Jones et al, 1998). However, recent studies indicate also that MeCP2 represses the transcription of certain genes yet promotes the transcription of others (Chahrouh et al, 2008), it may even control the AKT/mTOR signalling pathway and protein translation, suggesting that alterations of the neuronal AKT/mTOR pathway might be responsible for altered protein translational control in MeCP2 knock out neurons (Ricciardi et al, 2011).

Even if MeCP2's function is not completely clear, several experimental models involving either loss or gain of function of the mouse *mecp2* gene show several changes in the function and morphology of synapses and dendrites, associated to severe neurodevelopmental alterations and behavioural defects that recapitulate the human syndrome (Chapleau et al, 2009; Tropea et al, 2009; Zhou et al, 2006; see also Banerjee et al, 2012).

There is evidence from recent post-mortem studies that suggests that MeCP2 contributes to the regulation of synaptic connectivity (Chapleau et al, 2009; Johnston et al, 2005) since hippocampal CA1 pyramidal neurons from Rett syndrome females have a lower spine density than age-matched healthy female (Chapleau et al, 2009; Johnston et al, 2005). Also the syndrome is associated with an altered expression of proteins important for both excitatory and inhibitory synapses (Chapleau et al, 2009; Johnston et al, 2005).

Thus, MeCP2 seems to be important for activity/experience-dependent synaptic plasticity and remodelling. The knock-in mice that lack activity-induced MeCP2 phosphorylation perform better in hippocampus-dependent memory tests, their long-term potentiation is enhanced and excitatory synaptogenesis increased (Li et al, 2011); the

phospho-mutant MeCP2 protein is also able to bind more strongly several MeCP2 target gene promoters, thereby varying gene expression (Cohen *et al.*, 2011).

Interestingly, in the *mecp2*-deficient mice, retinogeniculate synapses develop similarly to wild type littermates between postnatal days 9 and 21, suggesting that the initial phases of synapse formation, elimination, and reinforcement were not affected by the absence of MeCP2 (Noutel *et al.*, 2011).

However, the circuit becomes abnormal and synaptic plasticity in response to visual deprivation is disrupted in MeCP2 knock out mice (Noutel *et al.*, 2011).

These results indicate that MeCP2 is crucially involved in experience-dependent improvement of synaptic circuits, in accordance with the clinical course of patients affected by Rett syndrome who after almost normal initial development later undergo a severe regression in all cognitive functions.

CONCLUSIONS

The discovery of molecular mechanisms contributing to the pathogenesis of various types of genetically determined ASD and ID put forward new possible targets for the development of drugs to ameliorate these pathologies.

Because the ProSAP/Shank mutation in the PMS seems to lead to a hypoglutamatergic state, the up regulation of the glutamatergic system may be a possible therapeutic approach. The use of AMPAkinases, agents that activate synaptic currents mediated by AMPA-type glutamate receptors, are indeed a possible pharmacological approach (Hamdan *et al.*, 2011). It has been shown that these drugs improve the induction of long-term potentiation and exert a positive effect on excitatory transmission by inducing the production of factors like the brain-derived neurotrophic factor (BDNF) which is involved in synaptic potentiation and memory consolidation (Jourdi *et al.*, 2009).

We were able to show that positive allosteric modulators of mGluR5 – like the CDPPB – are able to rescue *in vitro* synaptic defects of Shank3 knockdown neurons and mice (Verpelli *et al.*, 2011). CDPPB was also used to rescue behavioural defects in the *Tsc2* and *Shank2* knock out mice (Auerbach *et al.*, 2011; Won *et al.*, 2012). On the contrary, mGluR5

inhibitors are efficiently used to rescue neurological defects in *fmrp* knock out mice and more recently in Fragile X syndrome affected patients (Levenga *et al.*, 2010). This data suggest that an optimal range of metabotropic glutamate-receptor-mediated signalling is required for normal synaptic plasticity and cognitive functions, and both positive and negative alterations of this pathway might direct to similar ID impairments. Enhanced synapse formation and plasticity and increased BDNF expression have been described in wild type mice reared under environmental enrichment conditions (Sale *et al.*, 2007). Similarly, in the cerebral and cerebellar cortex of MeCP2 null mice, the environmental enrichment promotes synaptic plasticity and regulates synapse (Lonetti *et al.*, 2010).

A cellular replacement for ID syndromes was never considered possible, however it has been recently demonstrated that transplantation of wild type microglia could ameliorate the syndromes in *Mecp2* knock out mice, the animal model of Rett syndrome (Derecki *et al.*, 2012). Finally, the recent developed technology that permits the production of human induced pluripotent stem cells (hiPSC) and derived neurons from ID patients can strongly increase the possibility to test and develop new genetic and pharmacological treatments (Farra *et al.*, 2012; Marchetto *et al.*, 2010).

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