Journal of Biological Regulators & Homeostatic Agents
Volume 27, Number 2, April - June 2013
(Supplement)
CONTENTS

Biological regulators of synaptic dysfunction and neuronal death in Alzheimer's disease: A tribute to Rita Levi Montalcini

IN THIS SUPPLEMENT ALL AUTHORS REPORT NO CONFLICT OF INTEREST

INTRODUCTION

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As the population in developed countries ages, Alzheimer's disease (AD) will become an increasing public health problem. Anticipated health care costs, in addition to the economic and social burden are enormous. Therapeutic interventions that could delay disease onset even modestly would have a major public health impact and decrease the burden on patients and their families.

In this issue, Journal of Biological Regulators & Homeostatic Agents decided to publish a set of reviews summarizing the current state of knowledge of the molecular and synaptic mechanisms of neurodegeneration in AD, focusing on novel targets for therapeutic intervention.

The first three articles of this special issue deal with different —but otherwise interconnected— preclinical aspects of the molecular mechanisms involved in AD. In the first review, Cavallucci et al. highlight the role of mitochondria in either neuronal function or dysfunction leading to progressive synapse and memory deterioration in AD. Characterization of the molecular players involved in mitochondria alterations will hopefully provide new opportunities to identify pharmacological targets for new mitochondria-based drugs aimed at slowing down pathological processes and/or ameliorating symptoms of AD (1). Among the key signaling molecules involved in cellular processes leading to either survival or death are nitric oxide (NO) and sphingolipids that might act in concert to control apoptosis and autophagy with a significant impact on AD pathogenesis. Cervia et al. suggest that the targeting of NO and sphingolipid-dependent pathways are worth exploiting in therapeutic perspectives (2). In this context, another review deals with the role of altered cholesterol homeostasis and hypercholesterolemia in APP processing and Aβ generation. Indeed, Fiorenza and colleagues present an extensive overview on the role played by specific proteins involved in cholesterol metabolism associated with the development of late-onset AD (3).

The review presented by Nisticò et al. describes how both synaptic dysfunction and neuronal loss are highly correlated with cognitive impairment in AD. Evaluating the efficacy of novel therapeutic strategies by electrophysiological studies, such as hippocampal synaptic transmission and long-term potentiation (LTP) analyses, remains an important task in the field. Authors highlight the intrinsic limitations in the use of experimental systems, especially when translating preclinical studies into human clinical trials (4). Among the molecules capable of modulating LTP in animal models are D-amino acids, namely D-aspartate and D-serine. The review by Errico et al. suggests how D-aspartate, in light of its ability to enhance plasticity mechanisms, can be taken into account to counteract age-dependent processes related to physiological or pathological reduction of NMDAR signaling (5). The endocannabinoid system and the heme oxygenase/biliverdin reductase (HO/BVR) pathway represent other mechanisms implicated in pathological plasticity processes related to AD. Indeed, D'Addario et al. extensively review current knowledge on endocannabinoid system (ECS) regulation both in animal models of AD and in human tissues, suggesting how ECS might represent a promising approach to halt or slow down disease progression (6). Along a similar line, the review by Mancuso et al. proposes an exhaustive overview of the recent results regarding HO/BVR-A system involvement in AD, clearly supporting the

0393-974X (2013)

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view of its neuroprotective role, thus representing a potential target for newly developed anti-dementia drugs (7). Another challenging therapeutic approach is based on recombinant antibody domains exploited as intracellular antibodies (intrabodies). In this issue, Meli and colleagues discuss several applications and new promising developments of the intrabody approach for protein interference, especially in the field of AD research (8).

According to increasing evidence from epidemiologic and experimental data, the last two reviews deal with original yet intriguing aspects linking neurodegeneration to cognitive deterioration. Specifically, Scaccianoce and colleagues discuss the neurodegenerative potential of anabolic androgenic steroids (AASs) occurring through complex mechanisms ranging from neurotrophin unbalance to increased neuronal susceptibility to apoptotic stimuli. Hence, exposure to AASs might also predispose to enhanced risk of diseases not usually linked to drug abuse, especially neurodegenerative disorders (9). The last review focuses on the contribution given to neurodegeneration by infectious agents. In this frame, HIV-related cognitive disorders are one of the major complications of chronic HIV-infected patients. Here, Surdo and colleagues highlight how HIV-1 could promote the neurodegenerative process through inflammatory mediators released from infected cells (10).

We hope you will find this special issue informative, interesting and stimulating.

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EMERGING ROLE OF MITOCHONDRIA DYSFUNCTION IN THE ONSET OF NEURODEGENERATIVE DISEASES

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Mitochondria play a pivotal role in a number of biochemical processes in the neuron including energy metabolism and ATP production, intracellular Ca^{2+} homeostasis and cell signalling which are all implicated in the regulation of neuronal excitability. For this reason, it is not surprising that alterations in mitochondrial function have emerged as a hallmark of aging and various age-related neurodegenerative diseases in which a progressive functional decline of mitochondria has been described. The evidence that mitochondria are concentrated in synapses, together with the observation that synaptic dysfunction identifies an early forerunner of a later neurodegeneration, strongly suggests that significant alterations to synaptic mitochondrial localization, number, morphology, or function can be detrimental to synaptic transmission and might characterize the early stages of many neurological diseases. Thus, the characterization of both molecular players and pathway involved in mitochondria dysfunction will provide new chances to identify pharmacological target for new mitochondria-based drugs aimed at interrupting or slowing down pathological processes and/or ameliorating symptoms of neurological disorders. In this review we provide a current view on the role of mitochondria for neuronal function and how mitochondrial functions impinge on neurological diseases.

Mitochondria are key organelles for the life and death of the neuron and several neurodegenerative diseases that include amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, Parkinson's disease, stroke, brain trauma and spinal cord injury, have been associated with mitochondria dysfunction leading to an inappropriate activation of a neuronal cell-suicide program. (1,2). Mitochondria serve as platforms that sense damage and amplify it by releasing cytochrome *c* and other cofactors in the cytoplasm in order to activate effector caspases that accomplish the demise of the cell (3). The cytochrome *c* release is tightly controlled by proteins of the Bcl-2 family, is sustained by the permeabilization of the outer mitochondrial membrane and is accompanied by changes in the morphology and ultrastructure of mitochondria (4). Remodelling of the mitochondrial cristae, with widening of their narrow tubular junctions (5), and fragmentation of the mitochondrial network (6), both required for the complete release of cytochrome *c* and the progression of apoptosis, cross-talk. A growing family of mitochondria-shaping proteins controls mitochondrial morphology in living and dying cells. The core components of this machinery include both pro-fission (the cytoplasmic dynamin related protein 1, Drp1, and its mitochondrial receptor fission-1, Fis1) and pro-fusion (the large GTPases Optic Atrophy 1, Opa1, in the inner membrane and Mitofusin, Mfn, 1 and 2 in the outer mitochondrial membrane) proteins (7).

In many degenerative diseases, mitochondria are more susceptible to apoptotic stimuli (8). This is particularly evident in neuronal tissues, characterized by high energy demands to maintain proper functions and unable to switch to glycolysis when mitochondrial oxidative phosphorylation is impaired. In keeping with the importance of mitochondrial shape regulation for the progression of cell death, several neurodegenerative diseases are associated with mutations in the genes coding for mitochondria shaping proteins. Mutations in Opa1 cause dominant optic atrophy (9), and mutations in other accessory mitochondria-shaping proteins like GDAP1

Key words: Alzheimer's disease, Parkinson's disease, Huntington's disease, synaptic plasticity, LTP, LTD, dendritic spine loss, mitochondrial dynamics.

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0393-974X (2013)

are associated with other neurodegenerative diseases. Considerable interest was recently captured by the potential role of mitochondrial morphology changes as the pathogenic mechanism for familial forms of Parkinson's disease (PD) caused by mutations in the *Pink1* and *Parkin* genes, albeit it is unclear whether the defect is primary (10) or a consequence of mitochondrial dysfunction (11). Altogether, there is a general consensus that mitochondrial dynamics is a key process for neurons, where it controls not only survival, but also synaptogenesis and formation of dendritic spines (12). Thus, mitochondrial alterations are intensely studied as potential key components in the natural history of neurodegenerative conditions, including familial Alzheimer's disease (FAD). Along this line, mitochondrial shape changes have been retrieved in fibroblasts from patients with sporadic AD; moreover, in AD brains the levels of Drp1, OPA1, Mfn1, and Mfn2 were reduced, whereas the levels of Fis1 were increased. However, the mechanism by which mitochondrial morphology is altered in AD is unclear, as well as the contribution of morphology changes to the loss of dendritic spines.

A pathway for selective degradation of mitochondria by autophagy, known as mitophagy, has been described, and is of particular importance in neurons (13). Although much remains to be learned about mitophagy, it appears that the regulation of this process shares key steps with the macroautophagy pathway, while exhibiting distinct regulatory steps specific for mitochondrial autophagic turnover. Mitophagy is emerging as an important pathway linked to the pathogenesis of neurodegenerative disease (14). The involvement of mitochondrial dynamics and mitophagy in the neurodegenerative-related synaptic dysfunction, especially in the context of early-stages of the disease, is under intense investigation.

Mitochondria and neuronal function

The most attractive property of neurons is to modify their own structure and function in response to physiological inputs or pathological alterations. These adaptive changes occur in the nervous system and are defined as neuroplasticity phenomena that are involved in learning and memory. From morphological point of view, examples of neuroplasticity include formation of new synapses or synaptic sprouting, growth of axons and all these morphological changes are dependent on cellular and molecular mechanisms, which occur in pre- and post-synaptic sites of neurons. A relevant role in synaptic plasticity is also played by glial cells, which produce and release neuronal factors influencing synaptic function and remodelling.

The evidence that mitochondria are distributed in the axons, in pre-synaptic terminal and in dendritic shafts, draws attention to the crucial role of these organelles in synaptic plasticity and in the function of neuronal circuits. Emerging studies recognize mitochondria as not only an energetic source of synapses but also a signalling platform involved in synaptic function.

Long-term potentiation (LTP) and long-term depression (LTD) are two main forms of synaptic plasticity, which can occur at the same synapse in response to different patterns of activation of NMDA (N-methyl-D-aspartate) glutamatergic receptors (NMDAR).

Mitochondrial functions in both form of synaptic plasticity have been largely studied at glutamatergic synapses in the hippocampus that represents a key brain region mainly involved in memory function. The mitochondria are functional to the expression of these forms of synaptic plasticity, since they *a)* are the major source of energy (NAD^+ and ATP) that is required for neurotransmitter release, maintenance and restoration of ion gradients in both pre- and post-synaptic terminals and *b)* are also involved in Ca^{2+} homeostasis and Ca^{2+}-dependent cellular signalling.

On the pre-synaptic side it has been demonstrated that the distribution and motility of mitochondrial pool is associated with neurotransmitter release and synaptic short-term facilitation (15,16). A first pioneristic study on the functional role of mitochondria transport in synaptic transmission has been carried out in *Drosophila* (16). Genetic screen for *Drosophila* mutations that affect the function of axon and its synaptic terminal has identified a protein, called Milton, expressed in photoreceptors. Milton is crucial to the localization of mitochondria within neurons, as demonstrated by the observation that mutated photoreceptors show a reduced number of mitochondria in both axons and synaptic terminals, even though mitochondria are numerous in neuronal cell bodies. Similarly, Guo and co-workers (17) demonstrated that mutant *Drosophila* in the mitochondrial Rho-GTPase (dMiro) gene exhibits defects in locomotion, and microscopical analysis revealed the lack of pre-synaptic mitochondria in neuromuscular junctions. The role of mitochondria distribution in pre-synaptic terminal of mammals neurons has been established by Kang and co-workers (15) by means of genetic syntaphilin deletion, a neuron-specific protein initially identified as a candidate inhibitor of pre-synaptic function (18). The ablation of this gene leaded to the discovery of a novel role for syntaphilin as a docking receptor of axonal mitochondria, and the syntaphilin mutant neurons exhibit enhanced short-term facilitation during prolonged stimulation, probably by affecting calcium signalling at pre-synaptic buttons. Notably, this neuronal phenotype is fully rescued when syntaphilin is reintroduced into the mutant neurons. This study demonstrates that the function of mitochondria

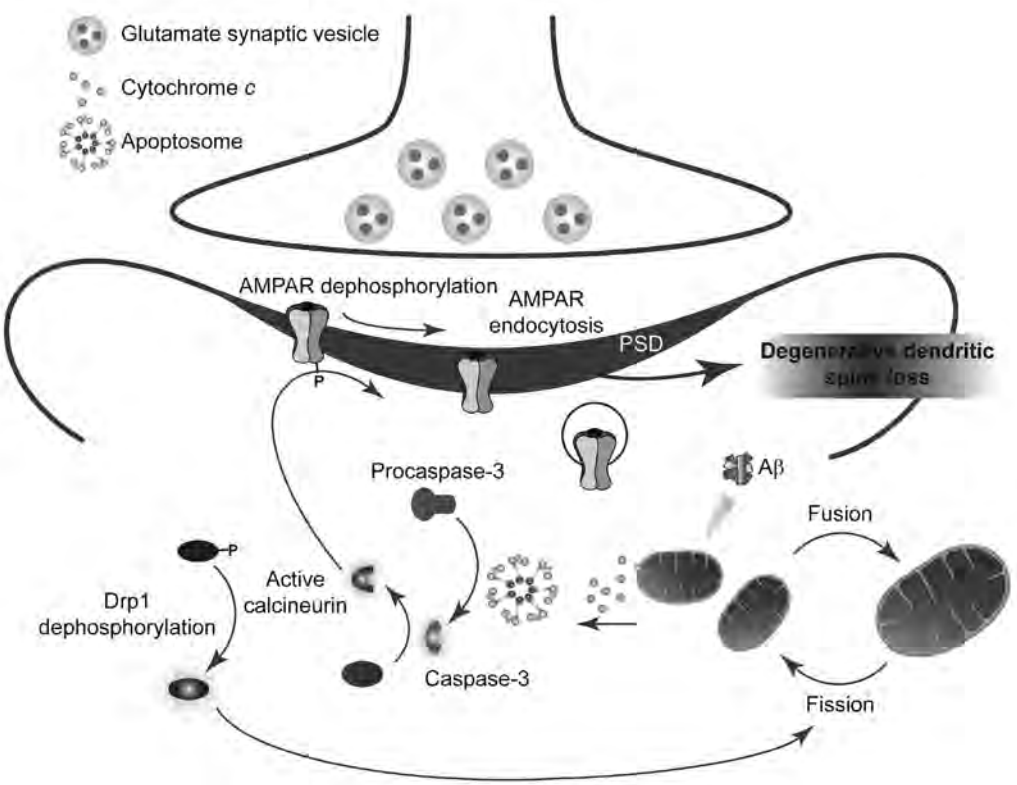

Fig. 1. *Schematic representation of mitochondria-dependent degenerative synapse loss. The amyloid-beta accumulation induces mitochondrial stress and an imbalance of mitochondrial fission/fusion process, causing mitochondrial dysfunction and consequent cytochrome c release into the cytosol. Once released, cytochrome c triggers the formation of active apoptosome leading to the activation of caspase-3. Active caspase-3 cleaves and activates calcineurin which in turn dephosphorylates GluR1 AMPAR subunit leading to the AMPAR removal from synaptic sites. AMPAR internalization leads to synaptic dysfunction and, consequently, to synapse degeneration (63). In addition, active calcineurin can dephosporylate Drp1 inducing its translocation to the mitochondria and increasing mitochondrial fission (72).*

is strictly associated with their distribution, which is elicited by neuron stimulation.

On the post-synaptic side, it has been demonstrated that distribution of mitochondria is regulated by neuronal activity and by proteins regulating the balance of mitochondria fusion/fission (12). In particular, the Authors demonstrated that in cultured hippocampal slices a local synaptic stimulation induces extension of mitochondria into dendritic spines for at least 90 min after the electrical stimulus. Moreover, the surprising finding that over-expression of Drp1 (a primarily cytoplasmic protein that regulates mitochondrial morphology) increases the density of spines and synapses implies that mitochondria are not only required but even limiting for the formation and/or maintenance of synapses (19,20). Thus, Drp1 is critical for distribution of mitochondria to dendrites, possibly by facilitating mitochondrial fission in the neuronal soma.

It has recently been demonstrated that the mitochondrial pathway of apoptosis is necessary for NMDAR-dependent LTD and internalization of AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid) receptors (AMPAR) (21,22). Specifically, the release of cytochrome *c* from mitochondria, the activation of the apoptosome with the consequent activation of caspase-3, and cleavage of the protein kinase Akt are all required for LTD induction. Indeed, LTD is abolished in knockout mice lacking caspase-3, or BAD and Bax (regulators of mitochondrial outer membrane permeabilization and hence cytochrome *c* release). Moreover, LTD-like stimulation of cultured hippocampal or cortical neurons is coupled with a fast and transient modest activation of caspase-3 in dendrites (as well as in cell bodies) that does not induce neuronal death (21,22). LTD is believed to show some degree of synapse specificity, and these findings imply that caspase-3 activation serves as a localized non-apoptotic function in the vicinity of synapses during LTD. Indeed, activated caspases can be detected in dendrites, axons, and pre- and post-synaptic compartments of non-

apoptotic neurons (23-25).

Emerging findings suggest a role for mitochondria as mediators of neurotrophic factors, which have been shown to modify synaptic plasticity (26). For example, it has been demonstrated that brain-derived neurotrophic factor (BDNF) promotes, in part, neuronal differentiation and synaptic plasticity by enhancing mitochondrial energy production as consequence of BDNF-mediated increase of glucose utilization (27) and by increasing mitochondrial respiratory coupling at complex I. Therefore, in addition to modifying neuronal plasticity, BDNF can modify brain metabolism and the efficiency of oxygen utilization (28,29). Very recently (30) it has been proven evidence of nerve growth factor (NGF)-receptor expression at mitochondrial level and their interaction with specific proteins. Collectively these findings suggest that, at least in part, neurotrophins can alter neuronal function by acting on mitochondrial function. This is particularly relevant in the aetiology and progression of the more common degenerative diseases which are known to be associated with heavy mitochondrial dysfunction.

Mitochondria failure and neurodegenerative disorders

As discussed in the first section of this mini-review, mitochondria play a crucial role in neuronal-death decision, but sub-lethal mitochondrial stress might alter mitochondrial energy metabolism leading to reduced ATP production, impaired calcium buffering, and accumulation of reactive oxygen species (ROS). These early mitochondrial alterations might severely affect the release of neurotransmitters and/or the molecular mechanisms, which regulate the neuronal response in post-synaptic terminals: it is evident that the clinical phenotype of these synaptic alterations depends on the disease-specific brain area in which this alteration occurs. Here we review examples of progressive neurodegenerative disorders in which alteration of both mitochondria and synaptic function has been characterized.

Parkinson's Disease

Parkinson's disease (PD) is a progressive and irreversible movement neurodegenerative disorder characterized by bradykinesia, resting tremor, rigidity, and postural instability. The clinical feature of PD principally results from the massive and selective loss of dopaminergic neurons in the *substantia nigra pars compacta* (31). Another histopathological feature of PD is the presence of degenerating ubiquitin-positive neuronal processes (Lewy neuritis) and intra-cytoplasmic insoluble inclusions (Lewy bodies) in surviving neurons (32). The role of mitochondria in early-PD is sustained by *1)* the evidence that the activities of enzymes in mitochondria metabolism are reduced in early phase of disease; *2)* the administration of mitochondrial toxins induce PD-like pathology in rodent and monkeys (33); *3)* genetic forms of PD result from mutations in gene codifying for proteins involved in mitochondrial function (e.g. Parkin, DJ-1 and PINK1).

Electrophysiological studies performed on nigrostrial neurons from Parkin-knockout mice provided evidence that Parkin is strictly associated with defects in both dopamine release from nigral neurons and synaptic excitability of medium-sized spiny striatal neurons, which are the major target of nigral dopaminergic projections (34). Similarly, depletion of Parkin in hippocampal neurons enhances the release of glutamate and causes an increase of glutamatergic synapses that is associated with increased vulnerability to synaptic excitotoxicity (35). Studies performed in *Drosophila* neurons further confirm the synaptic role of mitochondria-related PINK1 (PTEN-induced putative kinase 1) protein. In fact, its deficiency affects synaptic function, as the reserve pool of synaptic vesicles is not mobilized during rapid stimulation (11). Interestingly, in the same work the Authors demonstrated that synaptic deficits are rescued by adding ATP proving the importance of PINK1 for energy supply under increased demand during neuronal stimulation.

Huntington's Disease

Huntington's disease (HD), is an autosomal dominant inherited neurodegenerative disorder characterized by involuntary choreiform movements, rapid, irregular, and jerky motor actions associated with progressive dementia and psychiatric manifestations – including depression, psychosis, apathy, irritability. The main pathological change in HD brains is the selective neuron loss occurring in the striatum and cortex (36). HD is caused by a triplet repeat expansion in the huntingtin gene encoding an enlarged polyglutamine sequence in the mature protein. Mitochondrial defects have been described in patients with HD *in vivo*, in affected brains *post-mortem*, and in cell and animal models of the disease (37).

The mitochondrial defects in HD are associated with abnormalities of calcium handling, increased susceptibility to calcium-induced opening of the mitochondrial permeability pore, and reduced respiration (38). Evidence shows that mutant huntingtin protein associates with mitochondrial membranes and can impair axonal trafficking of mitochondria and reduce synaptic ATP concentrations (39).

Alteration in mitochondrial function is also implicated in dysfunction of synaptic plasticity as demonstrated in experimental model of HD.

Synaptic abnormalities at striatal synapses and alterations in long-term plasticity at hippocampal synapses have been reported previously in several mouse

models of HD (40-42). One of these studies (43), using R6/2 mice, demonstrated that a conspicuous feature of transgenic CA1 synapses was the expression of an NMDAR-dependent form of LTD, a form of plasticity that was absent in age-matched controls. More recently it has been demonstrated that layer II/III neurons from perirhinal cortex of R6/1 HD mouse model, display impaired LTP associated with progressive loss of membrane integrity (44). These accumulating data support that mutant huntingtin can impair mitochondrial function contributing to impair neuronal plasticity.

Alzheimer's Disease

Alzheimer's disease (AD) represents the most common cause of dementia, accounting for 50–60% of all cases (45), characterized by an age-related brain degeneration leading to progressive cognitive and behavioural impairments.

One of the most frightening aspects is that the cohort of initial symptoms heralding AD condition is preceded for a long time by a pre-symptomatic stage during which the disease hallmarks are already operative in destroying synapses and connections.

New findings have brought a different perspective to mitochondrial involvement in AD pathogenesis (46). Polymorphism of the TOMM40 gene seems to be an important risk factor for AD and age of onset (47). TOMM40 (translocase of outer mitochondrial membrane 40) is an outer mitochondrial membrane protein that forms part of a pore that serves as the import site for cytoplasmic proteins to enter the mitochondrion. Interestingly, it has been demonstrated that amyloid-β precursor protein (AβPP) accumulates in this pore (48). Presenilins 1 and 2 and γ-secretase are associated with the mitochondria-associated membrane (49), a connection site between the endoplasmic reticulum and the mitochondrion that is dependent on Mfn2 function (50). The mitochondria-associated membrane plays an important role in lipid metabolism, including the synthesis of phosphatidyl-ethanolamine, which is transported into mitochondria and has a modulator role in tau phosphorylation.

Centaurin-1 (CentA1), a GTPase-activating protein, is a brain-specific ADP-ribosylation factor localized to dendrites, dendritic spines, post-synaptic density, and axons (51-54). CentA1 interacts with the mitochondrial permeability transition pore complex and regulates its function (55). Moreover, CentA1 is up-regulated in AD brain (56) and Aβ-dependent mitochondrial permeability transition pore dysfunction contributes to Aβ-induced neuronal dysfunction (57). Recently, it has been demonstrated that Aβ increases the expression of CentA1 in rat hippocampal neurons and organotypic hippocampal slices. This Aβ-dependent upregulation of CentA1 activates the Ras-Elk-1 pathway at mitochondria, which impairs mitochondrial activity. Furthermore, downregulation of CentA1-Ras-Elk-1 signaling restores normal mitochondrial activity, synaptic function, and spine density in Aβ-treated neurons (58).

Accumulating evidence indicates that disruption of connectivity within neural circuits in key brain regions (59), loss of synapses, and impairment of synaptic plasticity precede the death of neurons (60-62) and involve mitochondria pathway (60,63). For instance, studies in the Tg2576 AD mouse model, in which the human AβPP gene harbouring the Swedish mutation associated with familial AD is expressed, have demonstrated that mitochondrial-dependent caspase-3 activation in hippocampal dendritic spines correlates with the onset of memory decline and dendritic spine degeneration accumulating both active caspase-3 and its substrates (63). Activation of calcineurin, a substrate of caspase-3, results in dephosphorylation of GluR1 (glutamate receptor 1) AMPAR subunit, causing removal of AMPAR from synaptic sites and increase of LTD expression. Pharmacological inhibition of calcineurin activity in Tg2576 hippocampus rescues AMPAR levels at post-synaptic sites and LTD to wild-type levels (64).

The crucial role of caspase-3 in synaptic plasticity was also shown in an independent study in which Aβ inhibited LTP through activation of mitochondria-dependent caspase-3 (65). The caspase-3-dependent Akt cleavage removes tonic inhibition of glycogen synthase kinase-3 (GSK-3). The subsequent increase in GSK-3 interferes with synaptic plasticity (66), besides promoting tau phosphorylation and neurofibrillary tangle formation (67).

In recent years, it has been demonstrated that different familiar AD mouse model show *a)* early biochemical and morphological modifications in the hippocampus (68); *b)* loss of the integrity of synaptic mitochondria and energy production prior to the onset of memory and neurological phenotype and before the formation of amyloid deposits (69); *c)* amyloid-beta overproduction causing an imbalance of mitochondrial fission/fusion that results in mitochondrial fragmentation and abnormal distribution, which might contributes to mitochondrial and neuronal dysfunction (70); *d)* alterations in the expression levels of mitochondrial proteins and metabolic enzymes without neuronal loss (71).

Collectively, these findings suggest that changes in the levels of metabolites reflecting altered energy metabolism and mitochondrial dysfunction, might be involved in early stage of disease and might contribute to the neurofibrillary tangle formation and to the alteration of synaptic plasticity leading to degenerative synapse loss (Fig.1).

CONCLUSIONS

There is increasing evidence that mitochondrial

dysfunction occurs in a number of major neurodegenerative diseases, including Huntington's disease, Parkinson's disease and Alzheimer's disease. Moreover, at onset of clinical symptoms, the disease process has already been at work in the patient for many years and possibly even decades, and high percentage of neurons in vulnerable areas are already dead. Therefore, finding synaptic markers that can help researchers identify patients prior to symptoms will provide more information on both therapeutic target and efficacy of agents aimed at improving mitochondrial health and will implement better interventions for these debilitating diseases.

ACKNOWLEDGMENTS

MDA is financially supported by PRIN 2009 (Projects for Research of National Interest) and by a grant from the Alzheimer's Association (NIRG-11-204588).

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NITRIC OXIDE AND SPHINGOLIPIDS CONTROL APOPTOSIS AND AUTOPHAGY WITH A SIGNIFICANT IMPACT ON ALZHEIMER'S DISEASE

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Aberrant regulation of signalling pathways promoting and regulating apoptosis and autophagy contributes to the development of most human neurodegenerative diseases characterised by progressive dysfunction and death of neuronal and glial cells. Both in central and peripheral nervous systems cell death is either apoptotic or autophagic, depending on the cellular setting and the initial pathogenic cue. While some mixed phenotypes have been reported, apoptosis and autophagy tend to develop into mutually exclusive ways to such an extent that they inhibit each other. The sphingolipid ceramide is a key intracellular signalling molecule involved in many cellular processes leading to either survival or death; in most of these processes also the short-lived gaseous messenger nitric oxide (NO) plays a crucial role. The crosstalk between these two messengers and their downstream mediators has been thus extensively investigated and we now have a deep understanding of it and of its multiple feedback controls. What we provide here are details on how NO- and sphingolipid-dependent signalling and their crosstalk impact on degenerative brain diseases, in particular Alzheimer's disease; we also describe how the ability of these molecules to regulate autophagy and apoptosis plays a significant role in determining the pathogenic evolution of these diseases. The evidence reported in this review suggests that targeting the NO and sphingolipid-dependent signalling pathways is worth exploiting in therapeutic perspective. In order to pursue these strategies, however, we still need to understand conclusively how the crosstalk between the NO and ceramide/sphingolipid pathways balances towards beneficial vs. toxic effects. In view of the nature of the signalling pathways involved and their multiple roles, the type of crosstalk involved is complex and intermingled with other signalling pathways.

INTRODUCTORY NOTE ON APOPOTOSIS AND AUTOPHAGY IN NEURODEGENERATIVE DISEASES

Cell fate is determined by the balance of survival signals that mediate the maintenance of cell homeostasis with signals that induce cell proliferation, differentiation, transformation or apoptosis. The natural occurrence of cell death has been appreciated since long time and widely studied in the twentieth century. While multiple modes of cell death have been defined, undoubtedly the most known and widespread process is the one called programmed cell death or apoptosis. Apoptosis is characterised by distinctive stereotyped morphological and biochemical alterations, among which the most notable are exposure of phosphatidylserine on the outer leaflet of the plasma membrane, membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (1-2) ultimately resulting in formation of apoptotic bodies and their clearance via phagocytosis by phagocytosis-competent cells. A key event in apoptosis initiation and progression is the activation of caspases, a family of cysteinyl aspartate-specific proteases (3). They are constitutive enzymes, expressed in almost all cell types in the form of inactive

Key words: Alzheimer's disease, apoptosis, autophagy, cell death, nervous system, neuroinflammation, nitric oxide, sphingolipids.

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0393-974X (2013)

proenzymes (zymogens) that are cleaved and thus activated in response to a variety of pro-apoptotic *stimuli*. Caspases activation is a sequential process: during apoptosis, apoptogenic *stimuli* induce the autocatalytic activation of initiator caspases; subsequently they cleave and thereby activate downstream effector caspases that finally cleave specific proteins and allow "dismantling" of the cell (3). The induction of apoptosis is mediated by two pathways acting alone or in concert: these are the death receptor-dependent and the mitochondria-dependent pathways, also known as the extrinsic and intrinsic apoptotic pathways, respectively (1-2). The extrinsic pathway is activated by the death receptors via the interaction with their ligands leading to receptor clusterisation (4). The intrinsic pathway is instead primarily activated by *stimuli* such as hypoxia, nutrient deprivation, radiation, heat, cellular stress, all of them inducing mitochondrial damage (5). Generally, activation of the intrinsic pathway requires the direct activation of members of the Bcl-2 family endowed with proapoptotic roles, such as Bid and Bax that translocate to the mitochondria, thus shifting the balance with the antiapoptotic protein Bcl2/Bcl-xL, and disrupting the membrane integrity to induce opening of the mitochondrial permeability transition pore (5-6). Stimulation of the extrinsic pathway may also trigger the intrinsic pathway, mostly via the sequential caspase activation, in a coordinated synergic action (7-8).

In the last decade increasing attention has been attracted by alternative signalling pathways leading to cell remodelling, among which autophagy is of recognised significant impact (9). Autophagy is a lysosomal pathway, evolutionarily conserved, involved in the maintenance of cytoplasmic homeostasis. In particular macroautophagy acts as a homeostatic self-eating process that in physiological conditions allows cells to break down slow-turnover proteins, thus complementing the action of the proteasome (10). Autophagy is upregulated when cells are in need of nutrients and energy, such as during starvation, in situations of increased bioenergetic demand or under stress conditions (9). Autophagy requires the formation of double membrane bound structures, termed autophagosomes; these vesicles assemble around, and entrap within them, damaged organelles or cellular debris to then fuse with lysosomes allowing degradation of their content (11). Autophagy requires sequential steps for autophagic vesicles (AV) formation and turnover; this includes initiation, nucleation and maturation of AV, followed by fusion to lysosomes. All steps are regulated by the intervention of specific molecules. Initiation requires the ULK1 kinase complex consisting of ULK1, Atg13, FIP200 (Atg17) and Atg101 (12-14). Nucleation and assembling of the initial phagophore membrane depends on the stimulation of phosphoinositide signals by a multiprotein complex consisting of PI3 kinases, Vps34 and Beclin1 also known as the Vps-complex. This complex is localised at the phagophore and facilitates recruitment of other Atgs to the developing vesicles. The identification of incipient AVs formation depends on Atg8, also named LC3 (microtubules-associated protein 1 light chain) and its conjugation to phosphoethanolamine on the surface of AV membranes. This mechanism is defined as LC3 lipidation (15). Once LC3 is integrated into the membrane bilayer, it binds to the specific cargo adaptors protein p62 and NIX, that in turn recruit cargoes from the cytoplasm and promote the closure of AV (16-17). AVs are then delivered to lysosomes where the AVs content is finally degraded by the lysosomal hydrolases and released into the cytosol for its reuse.

In various diseases aberrant regulation of apoptogenic and autophagic machineries is the central abnormality. This occurs in many neurological diseases of the central and peripheral nervous systems, including Alzheimer's disease (AD), Huntington's disease, Parkinson's disease, Frontotemporal dementia, Amyotrophic lateral sclerosis, Multiple sclerosis and in specific forms of spastic paresis dependent on mutations in the spataxin (SPG15) protein. It also occurs in acute neurodegenerative conditions such as stroke, trauma and severe epileptic seizures, where neuronal death is a central feature (18-21). Of interest, the resulting cell death can be apoptotic or autophagic depending on the cellular setting and the pathogenic cue (20). The dichotomous role of these two cell death processes results from complex relationship between the apoptotic and autophagic pathways. While some mixed phenotypes have been reported, in the nervous system apoptosis and autophagy ultimately develop into mutually exclusive ways that inhibit each other initiation and progress (20).

ROLE OF THE NITRIC OXIDE/SPHINGOLIPID PATHWAYS IN APOPTOSIS AND AUTOPHAGY

Generation of ceramide by Acid and Neutral Sphingomyelinases (A-SMase and N-SMase) and of nitric oxide (NO) following activation of NO synthases (NOS) act in concert to regulate several cellular pathophysiological processes including those leading to cell death via apoptosis or autophagy.

Nitric oxide

NO is generated in cells by specific enzymes, the NOS of which three isoforms exist: 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 generate NO at physiological concentrations (22). A third enzyme, the

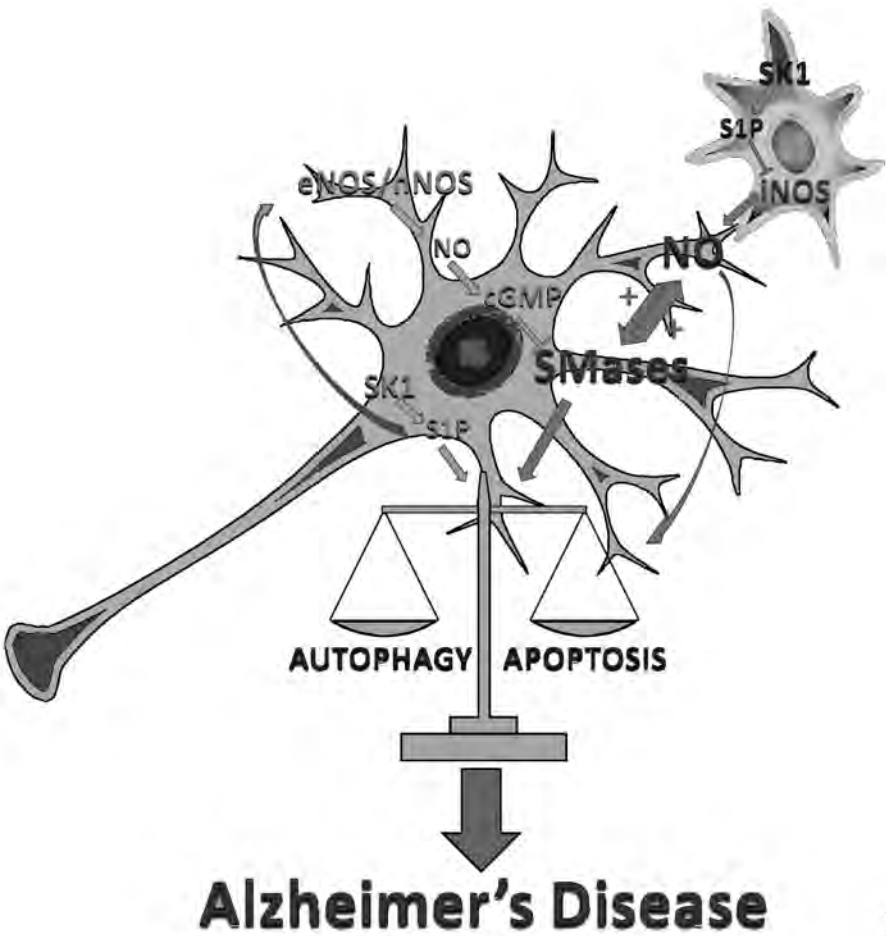

Fig. 1. *The schematic diagram shows how neuronal cells are affected by the NO/NOS and ceramide/SMases crosstalk and the relevance of this crosstalk in the regulation of the cellular balance between apoptosis and autophagy in neuronal cells. Depending on the status of the balance, i.e. whether it is inclined towards apoptosis or autophagy, pathological events concurring to the pathogenesis of neurodegenerative diseases may be promoted or inhibited.*

inducible NOS (iNOS or NOS II), is expressed following cell exposure to various pro-inflammatory *stimuli*, including cytokines and bacterial products. It generates high concentrations of NO that participate to the regulation of immune responses and contribute to cell damage (23). Because of its chemical reactivity and high diffusion properties, NO production by NOS is under tight control, designed to dictate specificity of NO signalling and to limit its toxicity for cells and tissues. A first important level of control is mediated by the physical association of the NOS proteins with several regulatory and structural proteins (24). Of importance, these protein-protein interactions regulate the activity of NOS and often position these enzymes to cellular membranes such that they are in close contact with the *stimuli* triggering their activation. The N-terminus of nNOS contains a domain, termed PDZ. This domain allows protein-protein interactions of nNOS with other PDZ-containing proteins at the plasma membrane including PSD-95, PSD-93 and α1-syntrophin (25). eNOS is localised mostly at the plasma membrane, but may localise also at the Golgi complex through its ability to be myristoylated and palmitoylated (26). eNOS may also interact with several regulatory proteins such as calmodulin, HSP70, NOSIP and NOSTRIN to contribute to important functional roles (26). Finally, both iNOS and eNOS may interact with caveolin 1 and/or 3 proteins, leading to the localisation of these enzymes at the plasma membrane and to the regulation of their activity (eNOS) and expression (iNOS) in an inhibitory fashion (26-27). The localisation at cellular membranes of NOS isoforms not only is a key aspect of NO-dependent signalling but constitutes the structural basis of the functional interaction described below among NO, sphingolipids, their generating enzymes and their downstream effectors.

Sphingolipids

For long time lipids were considered merely as structural components of cells, up to the discovery, in the late 1970's, of the phosphoinositide cycle (28). This discovery represented a major breakthrough indicating that lipids are also key players in signal transduction events. From that moment onwards, several lipid molecules were identified to act as biological mediators and modulators; among these are sphingolipids, *i.e.* lipids containing a long-chain sphingoid base backbone (for instance sphingosine), an amide-linked, long-chain fatty acid and various different polar head groups. The structure of these head groups is the basis of the classification of sphingolipid subtypes: a hydroxyl group characterises ceramide, phosphorylcoline characterises sphingomyelin (SM) and carbohydrates glycosphingolipids. The synthesis of sphingolipids begins in the endoplasmic reticulum and continues in the Golgi apparatus in a complex array of intermingled enzymatic reactions. Sphingolipids are thereafter found as relevant components of all intracellular membrane structures (for a detailed review on these pathways see (29)).

In the last 20 years great interest has attracted the SM-based signalling pathway, after the seminal work of Richard Kolesnick's and Yussuf Hannun's groups. Richard Kolesnick's group showed in 1987 that SMases are rapidly activated in response to 1,2 diacylglycerol treatment (30) and proposed the existence of a SMase-dependent signalling pathway (31); the Yussuf Hannun's group showed that SMases are activated by receptor-mediated mechanisms and provided the first evidence that the ceramide they generate acts as a cellular mediator (32). Indeed, in the vast majority of cells SM appears to be the primary sphingolipid source for bioactive ceramide, although more recent studies showed that ceramide is formed also *de novo*. This emphasises the critical role of SMases, both the lysosomal phosphodiesterase A-SMase and the two membrane-bound N-SMases (termed 1 and 2), in promoting ceramide-activated signalling (33-34).

The cross talk of the Nitric Oxide and sphingolipid signalling pathways in apoptosis

Generation of NO at physiological concentrations, such as the one yielded by the constitutive nNOS or eNOS, is a mechanism of inhibition of apoptosis induced by the activation of death receptors (the TNF-α receptor (TNF-RI)/CD95 superfamily) (35-37). On the contrary, at high concentrations, which typically occur during inflammatory states following activation of iNOS, NO may directly induce apoptosis, also acting in concert with other cell death inducing signals (38-39). Various mechanisms account for these two effects of NO, only apparently conflicting (35, 40). One of these is the ability of NO to regulate cellular levels of ceramide. Studies carried out in the U937 monocytic cell line and in clones of γδ T lymphocytes demonstrated that NO inhibits apoptosis induced by CD95 or TNF-RI by impairing the ability of these cells to trigger the generation of ceramide (36, 41-44). Both A-SMase and N-SMase are inhibited by NO, although the protective effect of NO is due solely to the inhibition of A-SMase. This mechanism appears to be general since NO protects from A-SMase-induced apoptosis also in human and murine dendritic cells (DCs) as well as in cancer cells. Of importance, NO inhibits apoptosis of DCs exposed to high concentrations of lipopolysaccharide (LPS) in a model of LPS-induced sepsis, both *in vitro* and *in vivo* (45); moreover NO protects DCs from the lethal effect of the chemotherapeutic drug cisplatin, in a model of tumour chemotherapy (46). The primary target of NO in DCs appears to be A-SMase, activated by LPS acting on the Toll-like receptor 4 (45) and cisplatin, acting on CD95 (46-47). Studies carried out in glioma cells demonstrating that A-SMase is activated by CD95 stimulation confirm these results (48). The inhibitory effect of NO on the activity of A-SMase and N-SMase and the ensuing protection from apoptosis is due to the activation of soluble guanylyl cyclase, the generation of cGMP, and the activation of cGMP-dependent protein kinase, a physiological pathway of NO signalling (41-42, 45-46). It has been demonstrated that A-SMase activation depends on its translocation from the intracellular compartments to the plasma membrane (48-49). A- and N-SMase do differ in terms of intracellular regulation (50) and localisation (33-34). We have evidence that the regulation of A-SMase activity is a consequence of the regulation of its intracellular localisation mediated by NO (C. Perrotta and E. Clementi, unpublished results); no information is instead available on the molecular mechanism by which NO/cGMP inhibits N-SMase.

When produced at high concentrations, NO has effects opposite to those of physiological NO concentrations on ceramide metabolism. Indeed high concentrations of NO activate both A- and N-SMase, increase the generation of ceramide and this triggers signalling pathways leading to apoptosis (51-54). The molecular mechanisms of activation of SMases by NO have not been yet investigated; however, since they are independent of cGMP and require a caspase-3-dependent step (51-52, 54) possibly involving arachidonic acid-derived eicosanoids (55), it is not improper to assume that they are distinct from those involved in SMase inhibition. Finally, it is worth pointing out the existence of a potentiating loop operated by ceramide on the pro-apoptotic effect of NO. In this contest ceramide, through the activation of NOS, increases NO levels, thus resulting in the loss of the mitochondrial transmembrane potential and the initiation of the caspase activation in various types of cells (56).

Nitric oxide, sphingolipids and their role in autophagy

Generation of ROS (reactive oxygen species) and RNS (reactive nitrogen species) plays a role in the autophagic responses both in physiological cell signalling and when they induce protein damage (57). In this contest, the ceramide/sphingosine 1 phosphate (S1P) rheostat has been shown to contribute to regulation of autophagy (58). Ceramide can induce autophagy via multiple mechanisms: *i*) it can upregulate Beclin1 (a key regulatory protein in autophagy pathway) and at the same time inhibit Akt phosphorylation; *ii)* it can activate the protein kinase JNK1, thus inducing the phosphorylation of Bcl2, leading to its dissociation from Beclin1 complex (59-60); *iii)* it can enhance the accumulation of BNIP3 (a cell death factor), also in this case triggering the dissociation of the Beclin1-Bcl2 inhibitory complex (61-62); finally, *iv*) it can induce autophagy by downregulating nutrient transporters (63) and by inhibiting p70S6 kinase and thus the mammalian target of rapamycin (mTOR) signalling pathway. S1P seems to work in inducing autophagy in the same direction as ceramide. For instance, overexpression of sphingosine kinase 1 (SK1) in such a way that S1P levels are increased triggers autophagy via the inhibition of the mTOR activity, with a moderate increase of Beclin1 but without changes in Akt (64). In support of the role of S1P in autophagy is the fact that SK1 activity is increased during autophagy-inducing starvation while silencing of SK1 leads to autophagy blockade and induction of apoptotosis. A further level of complexity is due to the involvement of S1P lyase (SPL). Although SPL activity increases intracellular S1P levels by preventing its degradation, this does not lead to induction of autophagy. In particular, it has been demonstrated that murine embryonic fibroblasts derived from SPL-deficient mouse ($Sgpl1^{-/-}$) are resistant to apoptosis induced by chemotherapeutic drugs or starvation and that this event is accompanied by the upregulation of Bcl2 and Bcl-xL, but not by an increase in the autophagic flux with respect to $Sgpl1^{+/+}$ cell. This suggests that autophagy does not account for the resistance to apoptosis observed in the $Sgpl1^{-/-}$ cells (65). From the results outlined above it is therefore possible to hypothesise different roles for S1P when produced by SK1 induction or when generated by SPL deficiency in the regulation of autophagy/apoptosis. A possibility to explain these different roles is the difference in the cellular localisation of the two enzymes. SK1 is localised in the cytosol and, upon activation, translocates onto the outer leaflet of the plasma membrane producing S1P close to its membrane receptors that are involved in autophagy stimulation. SPL is instead an endoplasmic reticulum integral membrane protein and controls S1P levels near the endoplasmic reticulum. Generation of S1P at this level is likely to activate signalling pathways differing from those triggered at the plasma membrane, thus leading to different signalling events (66-67).

The evidence reported above indicates that both ceramide and S1P may enhance autophagy, but with some key differences accounting for different cell fates in terms of survival or death (58). To summarise, ceramide acts on Akt phosphorylation levels and induces a marked accumulation of Beclin1 that can modify the ratio of Beclin1 to Bcl2 leading to cell death; by contrast S1P acts only on mTOR, and its downstream pathway. This explains why the autophagic response to S1P is milder compared to that induced by ceramide and, because of this, why it is compatible with cell survival. Another key factor to be considered is the nature of the stressor leading to autophagy. Starvation does not alter ceramide levels, suggesting that ceramide does not mediate this autophagic response. Conversely, chemotherapy increases ceramide levels that lead to autophagic cell death. Thus we can conclude that S1P is the mediator of starvation-induced autophagy a well known survival mechanism, whereas ceramide is the mediator of autophagic cell death.

Also NO is involved in the regulation of autophagy. It is able to block autophagosomes formation via two mechanisms (68), both independent of the cGMP pathway: *i*) the *S*-nitrosylation and ensuing inactivation of JNK1, which prevents the association of Beclin1 with hVps34; and *ii)* the activation of the mTOR complex 1 with the decrease of the AMP-activated protein kinase phosphorylation. This phenomenon appears to contribute significantly to the pathophysiology of diseases in which regulation of autophagy is of primary importance. For instance, genetic ablation or pharmacological inhibition of NOS enhance the clearance of mutant huntingtin (68-69), providing evidence that inhibition of autophagy by NO increases the levels of aggregate-prone proteins and thus contributes to excitotoxicity related to Huntington's disease. In accordance with this are results in glioma cells where NO blocks the autophagic process prompting cells to die in the presence of hypothermia (70). These results are consistent with recent microarray analyses showing the inability of NO to induce the expression of autophagy-related genes (71). Conversely in neurones NO may induce S-nitrosylation of the GTPase dynamin-related protein-1 (Drp1) leading to massive mitochondrial fission and then mitophagy (72). As previously explained NO and sphingolipids regulate each other at various levels (73-74); how and to what extent such interactions affect autophagy remains to be examined.

THE CROSS TALK OF NITRIC OXIDE AND SPHINGOLIPIDS IN ALZHEIMER'S DISEASE

The role of autophagy and apoptosis in AD is gaining

momentum. We provide here information on how these pathways are affected by the NO and/or sphingolipid pathways in the aetiology and development of AD.

Evidence on a role of sphingolipids and nitric oxide in neurodegenerative diseases

Publications in the last decades of the twentieth century showed how perturbation in brain lipid metabolism is connected with the progression of AD. These studies showed that sphingolipid metabolism is tightly regulated during the development and differentiation of the nervous system, and that the expression of peculiar sphingolipid patterns in specific time windows is essential for the maintenance of the structural and functional integrity of the nervous system. Thus, alterations in sphingolipid metabolism conceivably contribute to the pathogenesis of neurodegenerations (75-78). Increasing evidence indicates that in many neuronal degenerative diseases sphingolipid metabolism is deeply dysregulated, resulting into an altered expression of sphingolipids and thus into a modified membrane organisation. The consequent alterations in membrane structure and biophysical properties account for events related to the pathogenesis of central nervous system diseases, especially those in which inflammatory conditions play a role (78-80). The role for sphingolipids as signalling molecules in inflammatory responses such as those present in neurodegenerative diseases has been extensively investigated in the last few years (81-83). Of interest, it has been demonstrated that sphingolipids are involved in inflammation both by regulating a pre-existing inflammatory response, and by directly initiating it (83). This may explain why controversial reports have been published on the beneficial vs. detrimental role of sphingolipids in inflammation and points to the need of further studies in this area (84-92).

The contribution of NO to the pathogenesis of neurodegenerative diseases has been somehow more extensively characterised; for a comprehensive review see (93). NO production is largely recognised to occur frequently in neurodegenerative diseases and contribute to death of neuronal and glial cells (93). In this respect, nNOS is tightly coupled to the activation of NMDA receptors, a mechanism involved in excitotoxicity, *i.e.* the pathological process that in most cases precedes neuronal cell death (94). This indicates the existence of a nitrergic component in excitotoxicity-related neuronal injury. NO plays a role also in the context of inflammation related to neurodegeneration (95). NO production and release by inflammatory cells in the nervous systems (*i.e.* microglial cells and macrophages) and the ensuing oxidative stress, with S-nitrosylation of proteins and generation of RNS are significant factors in Parkinson's disease and AD (93).

NO may also have a protective effect against a variety of toxic *stimuli* involved in neurodegeneration, including on NMDA activation, DNA damage, endoplasmic reticulum stress, and generation of ROS. In a neuroblastoma cell line it has been demonstrated that the protective role of TNF-α during inflammation involves the activation of SK1, the ensuing generation of S1P, the stimulation of S1P receptors, and the activation of eNOS (37). Thus, the crosstalk between sphingolipids and NO appears to have important implications in neurophysiological and neuropathological processes. In support of this it has recently reported in microglial cells a regulatory action of S1P on neurotoxic mediators through the transcriptional regulation of iNOS (96-97).

Implications of nitric oxide and sphingolipids in autophagy and apoptosis in the context of Alzheimer's disease

AD is an irreversible, progressive brain disease characterised by the accumulation of amyloid neuritic plaques, neurofibrillary tangles, loss of synapses, oxidative stress, inflammation, impairment of memory and severe dementia (98-99). Studies *in vitro* and *in vivo*, together with post-mortem studies carried out on human specimens and analysis of the cerebrospinal fluid of patients have evidenced a key role of sphingolipid metabolism in the formation of amyloid β (Aβ) plaques, as well as in the neurodegenerative process, both critical features of AD (100). Noteworthy, the post-mortem studies have revealed the presence of very high levels of ceramide in AD brains, even in the early stages of the pathology (101). These high levels of ceramide may lead not only to neuronal dysfunctions (102) but also promote inflammatory processes (103). Generation of ceramide in AD has been studied in primary oligodendrocyte and in cultures derived from neonatal rat brains and found to be due to activation of A-SMase by Aβ peptides (101, 103). Also sphingosine, the pro-apoptotic ceramide metabolite, is elevated in AD brain and cerebrospinal fluid as a consequence of the high activity of acid and neutral ceramidases (101). Ceramide induces apoptosis in AD by two different mechanisms: *i)* the modification of the biophysical properties of the plasma membrane; and *ii)* the production of Aβ peptide by stabilising the Amyloid precursor protein (APP)-cleaving enzyme 1 (104-106). It appears therefore that there is a positive loop between ceramide and Aβ peptide that promotes cell death in AD (100). The accumulation of Aβ peptide is also responsible for the activation of microglia and the subsequent release of pro-inflammatory cytokines and ROS that contribute to neuroinflammation and neurodegeneration (107). In this context also NO plays a relevant role (108-109). In a cellular model of AD ceramide generated by Aβ peptide was found to induce the generation of elevated toxic levels of NO through the transcriptional activation of iNOS (110). Accordingly, it

has been shown that the deficiency of the NOS cofactor tetrahydrobiopterin BH4 is associated with AD, further highlighting a role for NO and RNS in AD pathogenesis (111). Of interest, an increase in the activity of the cGMP hydrolysing phosphodiesterases (PDEs) and the ensuing decrease of cGMP have been observed in senescent brains. This event conceivably contributes to enhance NO pathogenic effects by removing the protective effect of the physiological NO signalling (112). This evidence is confirmed by the observation that that the PDE5-selective inhibitor sildenafil, which increases cGMP levels, is beneficial against the AD phenotype in a mouse model of amyloid deposition (113).

Recently autophagy has been demonstrated as having a 'double-edged sword' role in the homeostasis of Aβ peptide production in neurones, leading to its recognition as a key mechanism in the pathogenesis of AD. Indeed Aβ peptide undergoes autophagy-mediated clearance thus supporting the current model in which autophagy is required for the removal of detrimental Aβ peptides and aggregates (114-116). At the same time, Aβ peptide appears to be involved in the enhancement of autophagy (116-117). This suggests that Aβ peptide generates a feedback positive loop that promotes its own degradation, thus triggering an internal checkpoint for the control of its own production (116-117). Autophagosomes are abundant in brains from patients affected by AD and lipid storage diseases such as the Niemann-Pick type C disease; based on these observations Tamboli and co-workers have defined an intriguing link between the storage of sphingolipids, the promotion of autophagy and the pathogenesis of AD (118). This research has revealed that sphingolipids accumulation plays a dual role in autophagy: the promotion of autophagy, but also the impairment of the turnover of autophagic vesicles, resulting in their accumulation and then in the accumulation of APP. This phenomenon might be crucial for the aetiology of AD, since the cellular accumulation of lipids might contribute to both major neuropathological events in AD, the formation of neurofibrillary tangles and amyloid plaques.

CONCLUDING REMARKS

In the last decades significant progress has been made in understanding how the NO and sphingolipid pathways interact with each other and which are the mutual feedback controls although not all molecular players have been elucidated, nor are clear all the regulatory steps in these pathways. So far, the mutual regulation of NO/NOS and ceramide/SMases has been well characterised in terms of its action as a tuning system in pathophysiological processes ranging from the control of calcium homeostasis neurotransmitter release and secretion (74). As discussed in the present review, the NO/NOS and ceramide/SMases crosstalk appear now to be relevant also because of its modulatory effect on the cellular balance between apoptosis/autophagy, thus appearing as a key player in AD (**Fig. 1**). This is of importance in therapeutic perspective for neurodegenerative diseases, where the quest for valuable therapy is still in full swing (119). In order to prevent the neurocytotoxicity typical of AD the inhibition of the NO and ceramide-dependent signals appear a promising pharmacological approach that might be combined with the already established treatments (120). The activation of the NO/sphingolipid pathway might also play a positive anabolic role for brain cells, by eliciting adaptive responses.

ACKNOWLEDGEMENTS

This work was supported by grants from the "Associazione Italiana Ricerca sul Cancro" (AIRC, IG11365) to E.C., from the "Ministero della Salute" (RC2013) to E.C., and from the "Ministero dell'Istruzione, Università e Ricerca" (MIUR, PRIN2010-2011) to E.C. and D.C.

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CHOLESTEROL METABOLISM-ASSOCIATED MOLECULES IN LATE ONSET ALZHEIMER DISEASE

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Alzheimer's disease (AD) is the most common cause of dementia and, with an aging population, poses a huge public health problem. Although a small per cent is caused by single gene changes, most AD is sporadic and unexplained. Of many modifying factors, changes in brain cholesterol homeostasis are the best studied. We present a review of the role of altered cholesterol metabolism and hypercholesterolemia in APP processing and Aβ generation. We also provide an overview of the potential pharmacological modulation of cholesterol homeostasis in the brain by cholesterol-lowering agents and β-cyclodextrins.

Alzheimer's disease (AD) neurodegeneration is the most common cause of dementia affecting more than 11% of individuals aged 65 years and older and 32% of individuals aged 85 years and older. The number of individuals aged 65 years and older is predicted to triple by 2050 [1, 2]. Genetic components of highly penetrant and autosomal-dominantly inherited forms have been identified. These include mutations in the amyloid precursor protein (*APP*) and Presenilins 1 and 2 (*PSEN1* and *PSEN2*) genes, which were found to be associated with inherited early-onset AD (EOAD) forms [3, 4, 5, 6]. However, these dominant familial forms only account for approximately 5% of patients with AD, and most so-called sporadic late onset AD (LOAD) forms are non-familial [7].

The strongest susceptibility gene that consistently confers an increased LOAD risk is the Apolipoprotein E gene (*APOE*). *APOE* encodes a lipoprotein highly expressed in the brain that plays a major role in the extracellular cholesterol transport [8]. The *APOE* locus is polymorphic with the three different variant alleles: *APOEε2*, *APOEε3*, and *APOEε4*. *APOEε2* is protective against AD and cortical atrophy in some populations [9, 10]. *APOEε3* occurs with the highest frequency (64% of *APOEε* alleles [11]) and is considered the ''neutral'' APOE genotype. By contrast, *APOEε4* is associated with increased risk of LOAD [12] and impaired cognitive function [13]. A single *APOEε4* allele and homozygosity for this allele increase the risk by three- and twelve-fold, respectively. Using DNA markers for *APOEε4* even higher relative risks were found [14].

Although the molecular basis linking *APOE* genotype and AD are poorly understood, recent data suggest that the clearance of amyloid β (Aβ) deposits depends on the isoform of APOE. On one hand, APOEε3 binds to Aβ peptides more strongly than APOEε4 [15, 16, 17]; on the other hand, APOEε2 and APOEε3, but not APOEε4, form dimers that might contribute to the regulation of Aβ degradation [18], and APOE levels in the plasma and brain of humans carrying the *APOEε4* allele are lower than in *APOEε3* carriers [19]. The concept that Aβ deposits depend on the APOE isoform is further supported by data showing higher levels of amyloid deposits in the brain of mice expressing human *APOEε4* compared with those expressing *APOEε3* or *APOEε2* [20, 15].

Because APOE variation only accounts for 10-20% of total LOAD genetic risk, additional loci likely contribute to LOAD susceptibility. Accordingly, large

Key words: LOAD susceptibility genes; Niemann-Pick C1; β-cyclodextrins

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0393-974X (2013)

genome-wide association studies (GWAS) have identified several candidate genes for LOAD risk, including several polymorphic loci encoding proteins involved in cholesterol metabolism [21, 22]. However, the real contribution of these loci still appears controversial because of the likely effect of rare variation(s) to be still identified, and/or of epistatic (gene–gene) interactions which again could well remain undetected using conventional GWAS approaches. Among these are Niemann-Pick C1 (*NPC1*) and ATP-binding cassette transporter A1 (*ABCA1*). Indeed, an association between single nucleotide polymorphisms (SNPs) in *NPC1* and LOAD has been recently found [23]. However, this association depends on the use of centenarians not affected by LOAD as a control group. Interestingly, another group confirmed the association but only in the presence of particular *ABCA1* alleles [24]. This is not surprising since their controls were younger individuals, not yet affected but who could eventually become affected by the disease. Furthermore, it was found that heterozygosity for *Npc1* deficiency accelerated amyloid plaque accumulation in a mouse model of AD [25].

Less conclusive are published data on the role of plasma cholesterol levels in AD development. Studies performed in animal models support the idea that increased levels of cholesterol in plasma represent a risk factor for AD. For example, feeding APP transgenic mice a high-fat and high-cholesterol diet increased the number and/or size of amyloid plaques [26, 27] and led to higher Aβ levels in formic acid extracts of brain. A high-cholesterol diet doubled Aβ levels in the hippocampal cortex and also damaged the blood brain barrier (BBB) in rabbit [28]. Since plasma lipoproteins do not cross the BBB, the mechanism by which high levels of plasma cholesterol influence Aβ levels remains unclear. In humans, many epidemiological studies have been performed. However, the results obtained are inconsistent: while some studies reported that increased levels of plasma cholesterol are a risk factor for AD, others did not find an association between high cholesterol levels in plasma and an increased risk for AD [reviewed in 29].

Cholesterol dyshomeostasis alters APP processing: bridging Alzheimer and Niemann Pick C diseases

Cholesterol plays essential roles in cell architecture and function. In the brain, it is involved in neuronal development, maintenance of synaptic plasticity, formation of synapses, neurite outgrowth, synaptic vesicle transport and regulation of neurotransmitter release. As an essential plasma membrane component, it is engaged in the formation and maintenance of lipid rafts, which are implicated in many aspects of brain function such as growth factor signaling and synaptic transmission. In addition, cytoplasmic cholesterol serves as precursor for the synthesis of steroid hormones, oxysterols and myelin [30, 31, 32]. Due to such pleiotropic functions, an imbalance of cholesterol homeostasis may severely affect brain functions.

In the central nervous system (CNS), cholesterol is mainly synthesized "*in situ*" by oligodendrocytes, astrocytes and neurons, without a significant contribution from circulation because plasma lipoproteins cannot cross an intact BBB. Therefore cholesterol homeostasis in the CNS is tightly regulated independently of that of peripheral circulation [33].

The brain is highly enriched in cholesterol compared with other mammalian tissues and most of it is present in myelin. In fact the synthesis of cholesterol is very active in oligodendrocytes during myelinization and decreases over time. A schematic representation of cholesterol metabolism in the CNS is shown in Figure 1. During development, neurons synthesize most of the cholesterol required for growth and synaptogenesis, while in the mature brain cholesterol is synthesized at a lower rate and mainly in astrocytes, which supply cholesterol to neurons. To be shuttled to neurons, cholesterol binds to APOE, the most prevalent lipoprotein in the CNS, which is then secreted by a mechanism involving one or more ATP-binding cassette transporters such as ABCA1, ABCG1 [34, 35, 36]. The APOE-cholesterol complex is internalized by neurons via a receptor-mediated mechanism [37] and then delivered to endosomes/lysosomes. Here the complex is unesterified by the action of acid lipase. The resulting free cholesterol then exits lysosomes by a mechanism dependent on the activity of NPC1 and NPC2 proteins, is transported to endoplasmic reticulum (ER) and/or recycled to plasma membrane [38, 39]. In the ER, cholesterol is re-esterified by the action of cholesterol acyltransferase (ACAT) and stored as cholesterol esters or in the form of lipid droplets. Alternatively, it can be liberated from neurons through the complex APOA1-ABCA1. In mitochondria, cholesterol is hydrolyzed to 24-hydroxy cholesterol (24-OHC) via the cytochrome P450 (CYP) family member (CYP46). 24-OHC sterol can now freely cross the blood-brain barrier or be delivered to the plasma *via* CSF.

Several studies have shown that cholesterol homeostasis has a strong impact on APP processing and Aβ generation in the brain. APP can be processed by non-amyloidogenic α-secretase or amyloidogenic b-secretase pathways. α-Secretase cleaves the Aβ domain of APP precluding the formation of full length Aβ peptide. This pathway yields a soluble N-terminal APPα and a membrane-bound C-terminal fragment (α-CTF or C83) that can be further processed by γ-secretase [40, 41]. The amyloidogenic pathway results in the formation of intact Aβ peptide and is mediated by the activity of β-secretase,

an aspartyl protease called β-site APP-cleaving enzyme 1 (BACE1). BACE1 cleaves APP, generating a soluble APPβ and a membrane bound Aβ-containing C-terminal fragment (β-CTF, also named C99). Further β-CTF proteolysis by γ-secretase, presenilin 1(PS1) or presenilin 2 (PS2) yields the full-length Aβ peptides of 40 or 42 amino acids [42, 43, 44, 45, 46, 47]. Cell culture studies have shown that α-secretase cleavage occurs mostly at the cell surface [48, 49, 50], while the majority of Aβ is generated in the endosomal recycling pathway and a minority of the peptide is produced in the secretory pathway within ER and Golgi apparatus [51, 52, 53, 54, 55].

α-Secretase is mainly located in low-cholesterol non-raft domains [56], while BACE1 and γ-secretase components are associated with lipid raft membrane domains [35, 57, 58, 59]. On the other hand, APP is believed to exist in two separate membrane pools, of which one is associated with lipid rafts and the other with phospholipid-rich domains [60]. These findings suggest that amyloidogenic APP processing occurs in cholesterol-rich lipid rafts and that the non-amyloidogenic processing of APP occurs mainly outside lipid rafts. In light of these findings, it is expected that cholesterol homeostasis alterations strongly affect APP processing. In fact, *in vitro* experiments performed using neuronal cells in culture showed that an increase in cellular cholesterol levels results in increased Aβ production and decreased APP cleavage by α-secretase [61, 62]. By contrast, cholesterol level reduction resulted in decreased Aβ production and increased APP cleavage by α-secretase [63, 62, 56, 64, 65].

Further evidences linking cholesterol dis-homeostasis and APP processing and Aβ generation have been provided by studies performed using *in vivo* and *in vitro* models of Niemann Pick C (NPC) disease [66, 67, 68, 69, 70, 71, 72, 73]. NPC disease is an autosomal recessive disorder having a carrier frequency of approximately 1:300. The majority of NPC cases arise from mutations in the *NPC1* gene on human (and, by chance, mouse) chromosome 18 [74, 75], while the remainder result from mutations in the *NPC2* (formerly *HE1*) gene on human chromosome 14. Lipid and vesicular trafficking alterations due to the absence of NPC1 have been extensively studied [76, 77, 78]. A major biochemical finding in this disorder is the intracellular accumulation of unesterified cholesterol within late endosomes/lysosomes. These findings prompted the conclusion that NPC is a disorder of intracellular cholesterol trafficking, although there are alternative views [79]. In fact, even though NPC1 is a distinctly different neurodegenerative disease compared to AD, the relationship between Alzheimer's and NPC, which led to the latter being termed "juvenile Alzheimer's", is the dementia associated with the presence of neurofibrillary tangles [80, 81]. Common pathological processes appear to contribute to both disorders. These include abnormal cholesterol metabolism [82], neurofibrillary tangles [83] and increased levels of Aβ [84, 72]. Moreover, compared to tangle-free, neurons, those bearing tangles also display a higher level of unesterified cholesterol [85], suggesting that the cholesterol accumulation typical of NPC disease may influence tangle formation. By contrast, amyloid plaques typical of AD are not observed in NPC patients. However, Saito and colleagues [86] reported the presence of diffuse plaques in 3 out of 9 NPC patients that had a

APOEε4/APOEε4 genotype.

Since the amyloidogenic processing of APP occurs in cholesterol-rich lipid rafts within the endosomal pathway, it is possible that cholesterol accumulation in the endocytic compartment, typical of NPC1 disease, may lead to an increase of the lipid rafts-associated APP pool and an increase in the production of Aβ peptides. This hypothesis is supported by experiments performed by Kosicek and colleagues, showing that NPC1-deficient CHO cells display an increased distribution of APP and CTFs towards lipid rafts [69]. Furthermore, the cholesterol accumulation of NPC-deficient cells causes an increase in APP internalization and a decrease in cell surface recycling, resulting in the sequestration of APP and BACE1 within the endocytic compartment, enhancing the accessibility of BACE1 to APP [70]. BACE1 was not detected in late endosomes/lysosomes, suggesting that BACE1-generated APP-CTFs in early endosomes may be either cleaved by γ-secretase there, or shifted to late endosomes and then cleaved to Aβ peptides by γ-secretase [73]. In addition, retention of cholesterol in endosomal/lysosomal compartments, both in NPC1-deficient CHO cells and in neuronal cells exposed to the cholesterol transport-inhibiting agent U18666A, induces PS1 and PS2 and Aβ42 accumulation in Rab7-positive vesicular organelles that are involved in cholesterol sorting [67, 68]. In line with these findings, increased levels of Aβ peptides and unaltered levels of β-cleaved soluble APP have been found in CSF from NPC patients, suggesting increased γ-secretase-dependent Aβ release in the brains of these patients [72].

Besides the role exerted by APP cleavage products, also the full-length APP appears to control cholesterol homeostasis. In fact it was demonstrated that APP directly regulates the activity of sterol regulatory element binding protein (SREBP) in neurons, but not in astrocytes. SREBP belongs to a family of transcription factors controlling several genes involved in cholesterol and fatty acid metabolism [87], such as hydroxymethyl glutaryl-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS), low density lipoprotein receptor (LDLR) and SREBP1/2 itself. Normally retained in endoplasmic reticulum membranes,

SREBP leaves the ER upon cellular cholesterol decrease and undergoes a maturation process in the Golgi apparatus, including two sequential protease cleavages [88], allowing its release in the cytosol and translocation to the nucleus of the mature form. APP and SREBP co-localization and interaction in the Golgi apparatus level prevents SREBP cleavage and mobilization to cytosol/nucleus [89] and to transcriptionally activate downstream genes, including HMGCR and SREBP. These findings indicate that cholesterol biosynthesis is strictly dependent on APP levels as well as other factors.

Cholesterol metabolism-related proteins and AD: ABCA1, LRP1, Clusterin

ABCA1 is the first identified member of the "A" subfamily of ABC family of transporters, the largest group of transmembrane transporters, including 48 known members in humans [90]. All ABC transporters share homology in their ATP-binding domain and use ATP as an energy source to predominantly mobilize lipids and other lipophilic molecules across both intracellular and plasma membranes [91]. For instance, ABCA1 is crucial for HDL biogenesis, because it transports intracellular cholesterol and phospholipids to lipid-free apolipoproteins [92]. APOE and APOJ, also known as Clusterin, are the two major apolipoproteins produced by astrocytes [93]. However, APOE is assembled in particles containing approximately an equal mass of itself, cholesterol, and phospholipids [94], whereas a very limited amount of cholesterol or phospholipids is associated with APOJ.

Abca1$^{-/-}$ mice display greatly reduced APOE levels both in the cerebral cortex (80% reduction) and the CSF (98% reduction). Moreover, CSF from *Abca1*$^{-/-}$ mice also display significantly reduced cholesterol levels and small APOE-containing lipoproteins, suggesting that ABCA1 regulates both the level of APOE as well as its degree of lipidation [95], which represents an important factor in the ability of APOE to efficiently bind Aβ [96]. As such, highly lipidated APOE more efficiently binds Aβ and diminishes its ability to aggregate by modulating its conformation [95, 97].

By contrast, APOJ particles are not influenced by the deficiency of *Abca1* gene function, likely because of their poor lipidation. In addition to the effect on cholesterol efflux and APOE lipidation, ABCA1 also regulates Aβ secretion [98]. The overexpression of ABCA1 in cell lines that constitutively express human amyloid precursor protein (APP) results in decreased extracellular levels of Aβ peptide [99, 91, 100]. This ABCA1 activity is likely independent of APOE since alterations in ABCA1 expression levels affect Aβ in some cell types that do not express APOE [95]. The activity of ABCA7, which is the closest ABCA1 homolog (54% sequence identity) and is also highly expressed in the brain [101, 102, 91], also appears to regulate cholesterol homeostasis and Aβ production [100].

A functional interaction between ABCA1 and NPC1 in cholesterol transport and in maintaining cell cholesterol homeostasis is suggested by the evidence that the availability of cholesterol for extracellular transport via ABCA1 is at least partly dependent on the intracellular cholesterol transport regulated by NPC1 [103]. Indeed, in NPC1-deficient human fibroblasts the loss of ABCA1 function is accompanied, among others, by a defective lipidation of APO-I [104]. To further strengthen the functional interaction between ABCA1 and NPC1, immunolocalization studies have shown that, besides the plasma membrane, ABCA1 is also localized in late endosomes and lysosomes, where both proteins likely participate in intracellular cholesterol mobilization. Accordingly, NPC1- and ABCA1-deficient cells share the common feature of an excessive storage of unesterified cholesterol in late endosomes/lysosomes. ABCA1 deficiency, also causes a reduction in HDL plasma levels [95]. This occurs because, within late endosomes/lysosomes, a balance exists between the fraction of endocytic cholesterol that is mobilized by NPC1 and the fraction that, associating with ABCA1, moves from the lumen of endocytic vescicles to the cell surface, where it is released to form nascent HDL particles [105].

Coordinated ABCA1 and NPC1 functions in intracellular cholesterol trafficking are in agreement with the coordinate upregulation of these proteins in the hippocampus and cortex of AD patients [106] that probably relies on a similar transcriptional control. For instance, both *NPC1* and *ABCA1* gene promoters are activated by the liver X receptor (LXR) pathway that, through the coordinated regulation of transcriptional programs, controls key aspects of cholesterol metabolism [107]. In line with this observation, it was recently shown that the concentration of at least one of the oxysterols, 27-hydroxycholesterol, which activates LXR, is increased in AD [108]. More recently, a deeper characterization of mechanisms underlying the coordinated transcriptional activation of ABCA1 and NPC1 has led to the identification of miR-33 as a common regulator of *Abca1* and *Npc1* transcript expression in mouse macrophages [109]. MiR-33 is an intronic microRNA (miRNA) located within the gene encoding sterol-regulatory element–binding factor-2 (SREBF-2, a master transcriptional regulator of cholesterol biosynthesis) [109]. MiR-33 binding sites are present in the 3'UTR of both human *ABCA1* and *NPC1* encoding genes and miR-33 expression inversely correlates with that of ABCA1 and NPC1. Besides macrophages, mir-33 is highly expressed in mouse and human hepatic cells, and, among the various

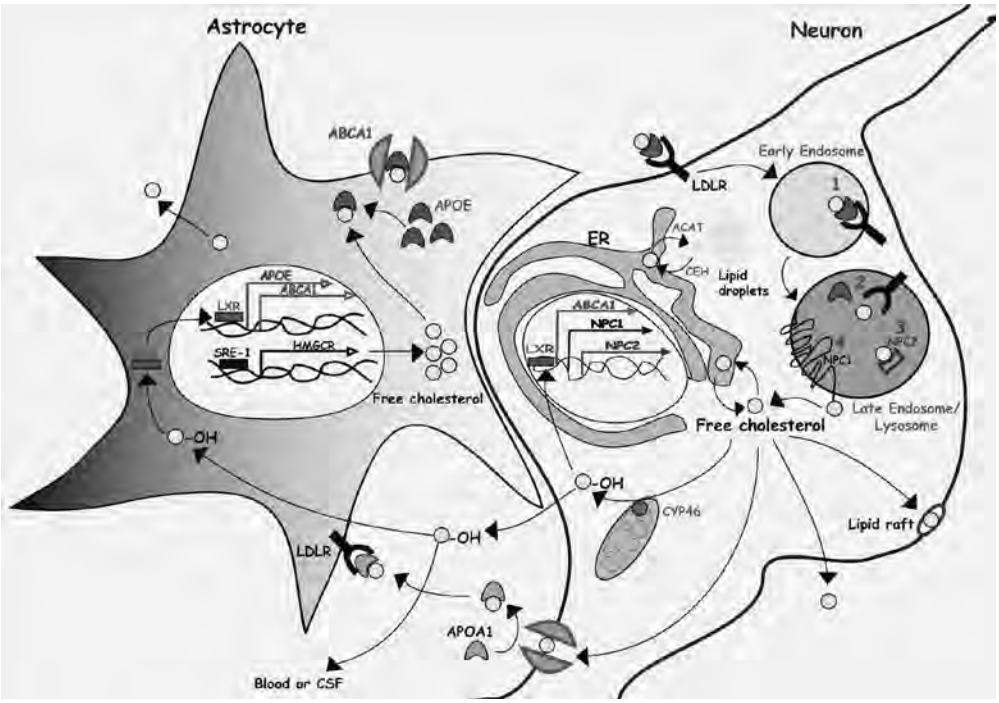

Fig. 1. *Cholesterol metabolism in the CNS. In the adult brain, astrocytes are the main source of cholesterol. Cholesterol is synthesized by the 3-hydroxy-3-methyglutaryl- coenzyme A reductase (HMGR), which is regulated by feedback inhibition via the sterol-regulated element binding protein (SREBP) that binds to the sterol-regulated element-1 (SRE-1), in the HMGR gene promoter. In astrocytes, cholesterol is bound to Apolipoprotein E (APOE) and exported via the adenosine triphosphate (ATP) binding cassette (ABC) transporter protein family member A1 (ABCA1). The APOE-cholesterol complex is taken up by neurons via low-density lipoprotein receptors (LDLR), delivered to endosomes/lysosomes, where cholesterol is unesterified by the action of acid lipase. Niemann-Pick type C (NPC) proteins type 1 (NPC1) and 2 (NPC2) mediate cholesterol lysosomal efflux. Free cholesterol is then delivered to other cellular compartments such as the ER, plasma membrane and mitochondria. In the ER, cholesterol is re-esterified by the action of cholesterol acyltransferase (ACAT) and stored as cholesterol esters in the ER or in the form of lipid droplets, it can be liberated from neurons by the complex APOA1-ABCA1 or free by lipid rafts. At level of mithocondria, cholesterol is hydrolyzed to 24-hydroxy cholesterol (24-OHC) via the cytochrome P450 (CYP) family member (CYP46). 24-OHC sterol can now freely cross the blood–brain barrier or be delivered to the plasma via the CSF. When 24-OHC is internalized by astrocytes or neurons can bind the liver X-activated receptors (LXRs), which translocate to the nucleus and induce expression of both the LXR-regulated gene, APOE and the ABCA1.*

tissues, the brain displays the highest level of expression [109]. However, the role mir-33 actually plays in astrocytes and/or neurons is still unknown. Among other proteins involved in cholesterol metabolism, an important role of the low density lipoprotein-related protein 1 (LRP1) in the pathogenesis of AD has also been reported.

LRP1 belongs to the LDLR family of receptors, including more than ten members sharing the common feature of ligand-binding repeats, EGF-like repeats and β-propeller-like structures with YWTD motifs [110]. These receptors are recognized by a large array of ligands, including APOE, and are involved in their transport and/or signaling [111]. LRP1 is highly expressed in cerebrovascular cells, including astrocytes, microglia [112], neurons [113] and vascular smooth muscle cells [114, 115], and is synthesized as a 600 kDa precursor glycoprotein, which is then cleaved by furin in the trans-Golgi compartment. The resulting 515 kD heavy alpha chain remains non-covalently coupled to the extra-cellular region of the transmembrane and cytoplasmic light beta chain [116, 117]. LRP1 binds about 50 different ligands including: APOE, α-2-macroglobulin, tissue plasminogen activator (tPA), proteinase inhibitors, blood coagulation factors and receptor-associated proteins, Aβ amyloid and prions [116, 118, 119]. Therefore this protein plays a major role in the transport and metabolism of macromolecules, in particular cholesterol-associated and APOE-containing lipoproteins, as well as in the clearance of proteases, proteases inhibitors and toxins, including Aβ amyloid and prion. In fact, Aβ levels in the brain

result from a balance between production and clearance and mounting evidence demonstrates that LPR1 plays an essential role in the maintenance of this balance, mainly by favoring Aβ clearance. Aβ elimination from the brain under physiological conditions involves three distinct steps, LPR1 playing a key role in each of them. First, LRP1 mediates transcytosis of Aβ and tPA to blood across the BBB [120, 118]. In contrast, the receptor for advanced glycation end products (RAGE) mediates Aβ transport across the BBB and its accumulation into brain [121]. In AD patients, LPR1 expression is reduced in both the BBB and vascular smooth muscle cells, while RAGE expression is increased in brain endothelial and vascular smooth muscle cells [122, 120, 121, 123, 124]. These changes in key Aβ transport receptors favor the accumulation of Ab in the brain. Second, circulating plasmatic LRP1 (sLPR1) binds and sequesters Aβ in plasma providing an endogenous peripheral "sink" that promotes the continuous removal of Aβ from the brain [125, 126]. However in AD patients and AD transgenic mice, an increased oxidation of sLRP1 decreases binding affinity for Aβ, resulting in increased plasmatic levels of Aβ [125], which may eventually lead to an increased transport to the brain via RAGE. Third, LRP1 binds and clears circulating Aβ in the liver [127].

In light of the role of LPR1 in the Aβ clearance, it was proposed that therapies focused on upregulation of LPR1 or down regulation of RAGE on brain endothelial cells, or aimed at restoring the Aβ peripheral "sink" action by increasing sLPR1, represent a promising approach to control Aβ levels in the brain. In line with this hypothesis, Sehgal and colleagues have recently shown that a treatment of an AD mouse model with an extract from *Withania somnifera* (WS) root reverses AD pathology *via* peripheral clearance of Aβ. In fact, treatment with WS induced LPR1 expression, but this effect was mainly mediated by hepatic and soluble LPR1 rather than brain LPR1 [126]. These results highlight the importance of the peripheral clearance of Aβ, even in the absence of changes in brain mechanism of clearance. Targeting the peripheral clearance of Aβ is a particularly appealing therapeutic approach, because it would overcome the need for BBB crossing compounds [128].

Clusterin (CLU) represents the second major apolipoprotein of the brain. It shares several features with APOE in relation not only to Aβ, but also to lipid transport. CLU is involved in the transport of cholesterol and phospholipids [129] and increased CLU levels were observed in atherosclerosis [130]. It was hypothesized that the accumulation of misfolded protein, such as Aβ fibrils, can transcriptionally activate *CLU* through the binding of a heat shock factor(s) to a heat shock element in the *CLU* promoter [131]. A genetic variation of the *CLU* gene was recently associated with LOAD risk by several GWAS studies [132, 133, 134, 135, 136], but how *CLU* SNPs influence the development of AD neuropathology is still unknown. Meanwhile, several lines of evidence, including: (i) increased levels of *CLU* transcripts and protein in AD [137, 138]; (ii) CLU presence in amyloid-beta plaques [139, 137, 140]; and (iii) CLU activity as an Aβ chaperone influencing Aβ aggregation and/or clearance by enhancing endocytosis [141] or through transport across the BBB [142] strengthen a genetic association of CLU with AD.

Several CLU transcripts are generated from a single gene located on chromosome 8. Among them, CLU1 and CLU2, differing only in the first exon and in the 5' untranslated region, are the main isoforms expressed in the human brain. The expression of *CLU1* and *CLU2* encoded proteins is differentially modulated by AD condition and AD-associated SNPs. In particular, the AD-protective allele rs11136000 was associated with increased expression of *CLU1* but not *CLU2,* even though the expression of both *CLU1* and *CLU2* is increased in AD [143].

Linking cholesterol dyshomeostasis to AD therapies

The extensive evidence indicating that cholesterol homeostasis is involved in APP processing and AD pathogenesis, suggests that a modulation of cholesterol homeostasis represents a potential therapeutic option for AD. Two different approaches have been proposed: (i) treatment with cholesterol-lowering agents; and (ii) treatment with cyclodextrins.

Even if the possible contribution of plasma cholesterol to AD development is still controversial, it has been hypothesized that lipid-lowering agents such as statins decrease the risk of AD. In fact, some, but not all, observational studies reported a beneficial effect of statins on preventing of treating AD [reviewed in 29]. However, randomized double-blind placebo-control studies also showed that statins did not have a significant beneficial effect on the progression of AD even if they were able to significantly lower plasma cholesterol level. Therefore, to date, the possible beneficial effect of statins in the treatment of AD remains still controversial.

Cyclodextrins (CD) are a family of cyclic oligosaccharide compounds that are widely used to bind cholesterol and to extract cholesterol from cultured cells [144, 145, 146]. Several *in vitro* studies have shown that CD reduce membrane cholesterol level and Aβ levels [147]. Recently, it was demonstrated in a mouse model of NPC1 that CD administration rescues the cholesterol defect, prevents neurodegenerative changes and extends life span [148, 149]. These findings have thus raised the possibility that CD treatment may be beneficial in the treatment of AD. Indeed, studies performed in cellular

models of AD have shown that the hydroxypropyl form of β-CD (HP- β-CD) reduces total cholesterol probably by direct extraction, leading to cholesterol redistribution from plasma membrane to intracellular compartments and reduces Aβ production. Most important, subcutaneous administration of HP- β-CD to the Tg19959 mice overexpressing human mutant APP, also significantly lowered β-CTF levels, Aβ production and deposition, reduced microgliosis and tau pathology and improved memory and learning abilities. In addition, HP- β-CD also increases the expression of *NPC1* and *ABCA1* mRNA [150]. Such *in vivo* effects are quite intriguing, considering that HP- β-CD can rapidly bind to the cerebral blood vessel wall, but is not further transported across the BBB [151]. Even if the data obtained with AD mouse models are quite encouraging, the mechanisms of HP- β-CD action need to be further investigated.

Concluding remarks

Cholesterol homeostasis has a strong impact on APP processing and Aβ generation in the brain. Thus, the role of several proteins involved in cholesterol metabolism in the development of AD is worthy of interest. Furthermore, in addition to *APOE*, recent studies have shown an association between LOAD and genetic variations in *NPC1,* encoding an intracellular cholesterol transporter, *ABCA1,* encoding a cholesterol membrane transporter, and *Clusterin,* encoding a cholesterol extracellular transporter. The ability of β-cyclodextrin to ameliorate Niemann-Pick C1 disease may be extended to AD.

ACKNOWLEDGMENTS

We thank F. Mangia and S. Ferraina for critical reading.

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PHARMACOLOGICAL MODULATION OF LONG-TERM POTENTIATION IN ANIMAL MODELS OF ALZHEIMER'S DISEASE

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The discovery of long-term potentiation (LTP) of hippocampal synaptic transmission, which represents a classical model for learning and memory at the cellular level, has stimulated over the past years substantial progress in the understanding of pathogenic mechanisms underlying cognitive disorders, such as Alzheimer's disease (AD). Multiple lines of evidence indicate synaptic dysfunction not only as a core feature but also a leading cause of AD. Multiple pathways may play a significant role in the execution of synaptic dysfunction and neuronal death triggered by beta-amyloid (Aβ) in AD. Following intensive investigations into LTP in AD models, a variety of compounds have been found to rescue LTP impairment via numerous molecular mechanisms. Yet very few of these findings have been successfully translated into disease-modifying compounds in humans. This review recapitulates the emerging disease-modifying strategies utilized to modulate hippocampal synaptic plasticity with particular attention to approaches targeting ligand-gated ion channels, G-protein-coupled receptors (GPCRs), Receptor Tyrosine Kinases (RTKs) and epigenetic mechanisms. It is hoped that novel multi-targeted drugs capable of regulating spine plasticity might be effective to counteract the progression of AD and related cognitive syndromes.

Alzheimer's disease (AD) is a devastating progressive neurodegenerative disease that affects more than 35 million people worldwide. AD is characterized by gradual cognitive decline associated with deterioration of daily living activities and behavioral disturbances throughout the course of the disease.

Several pathological changes have been described in post-mortem brains of AD patients, particularly in the hippocampus, including beta-amyloid (Aβ) plaques, intracellular neurofibrillary tangles (NFTs) formed by the hyperphosphorylated tau protein, inflammation and extensive cell death (1).

Growing evidence supports the idea that loss of dendritic spines, rather than Aβ plaques, NFTs or neuronal cell death, is the best pathological correlate of cognitive impairment (2). Accordingly, the early reduction in synapse number and density is higher to the damage of neuronal cell bodies (3), indicating that pruning of synaptic terminals precedes overt neuronal loss. Moreover, synapse loss is evident in patients with early AD and mild cognitive impairment (MCI) (4) and impaired synaptic function in transgenic models of AD appears long before amyloid plaque burden and neuronal cell death (2). Consequently, AD is widely recognized to be a form of synaptic plasticity failure (5). Several pathways have been implicated in the pathogenesis of AD (6,7). Central to this process is the Aβ-mediated activation of caspases, Akt and GSK-3β, which act in concert to promote spine degeneration (8-12), as well as cytokines and prostaglandins, which directly trigger neuroinflammation (13,14). More recently, several studies focused on the role of the heme oxygenase-1/biliverdin reductase (HO-1/BVR) system as a neuroprotective pathway (15,16) and drugs or natural compounds which are able to modulate this system are considered useful agents to counteract neurodegeneration (17-21). Several reasons justify the interest of the scientific community on the HO-1/BVR system and its role in AD: (i) the clearance

Key words: synaptic plasticity, long-term potentiation, hippocampus, Alzheimer's disease.

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0393-974X (2013)

of heme, which is toxic if it is in excess or in case of redox perturbation of the intracellular milieu (22), (ii) the generation of carbon monoxide, which has been shown to regulate neuropeptide release and synaptic transmission (23,24) and (iii) the reduction of biliverdin to bilirubin, the latter being an efficient neuroprotectant through the interaction with neuronal nitric oxide synthase and nitric oxide (25,26).

Also epigenetic alterations such as modifications on DNA and histone proteins, the primary elements of chromatin structure, have been associated with the development of AD (27). Recent studies have begun to elucidate the relevant mechanisms that mediate epigenetic modifications in physiological conditions and how these processes might be dysregulated in AD. Consequently, the role of specific classes of therapeutic compounds that affect epigenetic pathways has been investigated in different experimental models.

In this review, we will summarize how LTP is used to evaluate the phenotype of AD mouse models and how it represents a useful experimental tool to test the efficacy of disease-modifying approaches to the treatment of AD.

Synaptic alterations in experimental models of AD

There is no existing animal model that resembles all the cognitive, histopathological, biochemical and behavioral abnormalities observed in AD patients. However, partial reproduction of AD neuropathology and functional deficits has been achieved either through genetically-engineered mouse models of AD or with exogenous application of Aβ. As a result of the development of molecular techniques and advances in transgenic technologies, investigators have created over the past years many different transgenic lines of AD expressing human amyloid precursor protein (APP) and presenilin (PS1, PS2) mutations. These models provided excellent opportunities to examine the bases for the spatial/temporal evolution of the disease. In addition, the combination of electrophysiological and behavioral techniques together with advances in histopathological and biochemical methods has been a very powerful tool to address important questions about the pathogenic mechanisms of the disease. LTP is typically altered following manipulation of specific plasticity-related genes (28-33) and is impaired in several animal models of human neuropsychiatric disorders, such as Parkinson's disease (34), depression (35), autism spectrum disorders (36) and multiple sclerosis (37). Moreover, LTP represents a valid experimental readout to elucidate the mechanism of action of novel ligands (38-40), and to assess the efficacy of therapies for the treatment and/or prevention of AD and other neurodegenerative conditions (41,42).

Interestingly, most currently studied AD models show cognitive deficits and age-related disruption of synaptic markers and amyloid plaque deposition, but few strains show evidence of significant cell death (43). Most studies have reported, principally, either inhibition of LTP or reduction in baseline fast excitatory transmission prior to plaque deposition. However, several discrepancies emerged so far, potentially due to differences in the models and the experimental conditions used. Importantly, recent work has shown that the plasticity phenotype can be strongly influenced by the cognitive history of the animal. Thus, whilst LTP is normal at naive synapses it is severely impaired following training of a spatial task (44).

Although transgenic animals offer many advantages, it was still not possible to clearly unravel the role of APP per se or the different soluble and fibrillar Aβ species. Therefore, direct exogenous application of Aβ provided an alternative approach. Generally, LTP is impaired when synthetic Aβ are applied *in vitro* (45) and *in vivo* (46). Good evidence that disruption of LTP is caused by Aβ oligomers was also provided when naturally secreted soluble oligomers of human Aβ were injected intraventricularly into rats (47). These studies contributed to elucidate the fundamental cellular and molecular mechanisms of Aβ action on disrupted synaptic plasticity.

Targeting synaptic dysfunction in AD treatment

A disease-modifying agent should induce long-lasting functional and structural changes at the synaptic level with the aim to slow, halt or reverse disease progression. Besides providing insights into the molecular basis of learning, LTP served also as an experimental tool to test the efficacy of the different disease-modifying strategies. Among these, exogenously applied and endogenously generated anti-Aβ antibodies rapidly neutralized the synaptic plasticity disrupting effects of Aβ oligomers (48). In addition, agents that reduce nitrosative/oxidative stress or antagonize stress-activated kinases prevented Aβ inhibition of LTP in vitro (49,50). Finally, targeting putative receptors for Aβ (51) and reducing the amount of Aβ oligomers with γ-secretase inhibitors (52) or modulators (53) have all proven successful to prevent LTP impairment in AD preclinical models.

In this section we will describe emerging approaches that aim to rescue synaptic plasticity across the different AD models. We will focus on ligand-gated ion channels, G-protein-coupled receptors (GPCRs), Receptor Tyrosine Kinases (RTKs) and epigenetic mechanisms; for a fuller account the reader is referred to a recently published review (42).

Ligand-gated ion channels

Among the ligand gated ion-channels nicotinic acetylcholine receptor (nAChR) and N-methyl-D-aspartate (NMDA) receptor received the most attention.

This is also due to the fact that Aβ soluble oligomers can cause perturbation of nAChR and NMDA function, even though the mechanism remains poorly understood. On the other hand, Aβ might induce beneficial effects on synaptic plasticity when found at picomolar concentrations (as in healthy brains) via the activation of presynaptic α7 nAChRs (54). These opposing findings may be due to concentration-dependent actions of Aβ, as low levels activate and high levels desensitize α7 (55) and/or interact with other nAChRs subtypes (56).

Multiple lines of evidence suggest that α4β2 and α7 mediate the Aβ-induced suppression of LTP. In fact, antagonists at either receptor subtype have proven effective in attenuating LTP impairment following Aβ exposure (57,58).

Also the effect of nicotine has been tested in animal models of AD. Both acute and chronic nicotine administration can enhance LTP via α7 receptors (59). Accordingly, recent work showed a protective effect of chronic nicotine treatment in a rat model of AD (60,61). Similarly, the selective α7 agonist dimethoxybenzylidine (DMXB) was able to rescue LTP deficit induced by Aβ (62). Moreover, also donepezil, a widely used drug for the treatment of AD, had neuroprotective effects on synaptic plasticity following Aβ (63).

Excessive activation of NMDARs has also been implicated in AD, and the NMDAR channel blocker memantine, an uncompetitive inhibitor of NMDARs (64,65), is clinically tolerated and effective in the treatment of moderate to severe AD. Preclinical studies suggest that therapeutic concentrations of memantine reverse LTP deficiency against the rapid disruptive effects of soluble Aβ both in the CA1 (66) and DG (67) regions.

Aβ oligomers are known to interact with the GluN1, GluN2A and GluN2B subunits of the NMDA receptor (68,69) at the excitatory synapse. Specifically Aβ (70) and tau (71) are implicated in the removal of synaptic NMDARs. NR2B negative allosteric modulators ifenprodil and Ro 25–6981 can reverse Aβ-induced deficit of LTP (72-75).

GPCRs

Besides nAChR, Aβ can also directly interact with muscarinic acetylcholine receptor (mAChR). Accordingly, the selective M1 mAChR antagonist pirenzepine was able to reverse the Aβ-induced reduction of excitatory synaptic transmission on medial septum slices (76). Of note, synaptic alterations displayed by the AβPP/PS1 model were associated with a decrease in the ability of endogenous mAChR activation to reduce basal glutamatergic transmission in the CA1 area of the hippocampus (77), suggesting that muscarinic receptor dysfunction might lead to functional impairment.

Mounting evidence suggests that Aβ soluble oligomers can also cause perturbation of metabotropic glutamate (mGlu) receptors. Apart from increasing extracellular glutamate concentration Aβ forms clusters at excitatory synaptic plasma membranes, which may trigger the redistribution of mGlu5 receptor and cause an increase of synaptic mGlu5 receptors. It is believed that aberrant activation of ectopic clusters of mGlu5 receptor may increase intracellular Ca^{2+} directly or indirectly via NMDA receptors. Activation of either group I or group II mGlu receptors might also increase Aβ production although the mechanisms are not fully understood yet (78). As a consequence, MPEP, a specific negative allosteric modulator (NAM) against mGlu5 receptors (79), reversed the Aβ oligomer-induced inhibition of LTP (66).

RTKs

The growing evidence that neurotrophins are essential regulators of synaptic plasticity (80) which becomes dysfunctional before the onset of AD raise the question of whether synaptic failure could be partly ascribed to neurotrophin dysregulation. In line with this notion, a Tg mouse line expressing chronic nerve growth factor NGF deprivation displays age-related defects in dentate gyrus synaptic dysfunction (81). Moreover, a recent work suggested that alterations in the proNGF/NGF balance in the adult brain are an upstream driver of APP dysmetabolism, synaptic imbalance and learning and memory impairments (82). Both evidences support the "neurotrophic unbalance" hypothesis underlying AD-like neurodegeneration (83).

Interestingly, a similar scenario of NGF unbalance, parallel to behavioral disturbances, has been recently observed in rats chronically exposed to anabolic androgenic steroids (84).

On the other hand, exogenous supply of neurotrophins was proven effective to restore synaptic alterations in experimental AD. Accordingly, application of neurotrophin-4 (NT-4), a neurotrophic factor that signals predominantly through the TrkB receptor tyrosine kinase, prevented LTP deficits induced by Aβ both in the CA1 and DG of rat hippocampal slices (85). Similarly, also the neurotrophin brain-derived neurotrophic factor BDNF, which acts through TrkB receptors partly via the mTOR signaling pathway, has been shown to protect hippocampal synapses in a mouse model of AD (86) and to rescue plasticity defects triggered by Aβ oligomers in rat hippocampal slices and LTP-associated CaMKII activation and AMPA receptor phosphorylation at a CaMKII-dependent site (84).

Aβ can directly bind also to p75 neurotrophin receptors (p75NTR), which are best known for mediating neuronal death and have been consistently linked to the pathology

of AD (87). Therefore, blocking this receptor with the isoleucine derivative LM11A-31 rescued Aβ-induced LTP deficit (88). On the other hand, NGF was capable of restoring the LTP deficits in the APP-null mice via the p75NTR, suggesting that p75NTR may undergo a switch of function under specific conditions (89). These results highlight neurotrophins or their analogs as a new class of candidate molecule compounds for AD therapeutics. Notably, encapsulated cell biodelivery of nerve growth factor (NGF) to AD patients is currently undergoing Phase I clinical trials.

Several studies show that insulin, via the insulin receptor tyrosine kinase (IR), plays a central role in higher brain functions such as learning and memory (90) and synaptic plasticity (91) whereas deficiency of insulin signaling underlies plasticity defects and neurodegenerative disorders. Accordingly, a clinical study has showed that insulin levels were decreased in the CSF of patients with sporadic AD (92). It has also been reported that insulin can protect hippocampal neurons against Aβ-mediated toxicity (93), suggesting a potential interplay between insulin and Aβ.

In a recent work, it was demonstrated that either insulin or Insulin Growth Factor-1 (IGF-1) inhibit the formation of Aβ oligomers, thus preventing the block of LTP induced by various Aβ fragments (94). Similarly, also pre-treatment with the glucagon-like peptide-1 (GLP-1), which physiologically increases insulin release, has been proven beneficial in reversing LTP following Aβ exposure (95,96), and in aged AβPP/PS1 mice (97). Similar results were also obtained with the novel glucose-dependent insulinotropic polypeptide (GIP), a peptide hormone targeting pancreatic islets to enhance insulin secretion (98). Finally, insulin-sensitizing drugs such as the thiazolidinediones attenuated the negative effects of Aβ on LTP (99) and the PPARγ agonist rosiglitazone improved learning and memory deficits in the Tg2576 mouse model (100).

Taken together, these preclinical results raise the possibility that insulin and insulin-sensitizing drugs may serve as therapeutic agents for the treatment of AD.

Targeting epigenetics

DNA Methylation

Modifications in neuronal gene expression play an essential role in memory formation (101). Mounting evidence suggests that DNA methylation and histone modification may work in concert to dynamically regulate plasticity and memory formation in the rat hippocampus. While most studies investigated histone covalent modifications, recent literature is focusing on DNA methylation dynamics in memory and synaptic plasticity (102). Accordingly, it was demonstrated that LTP and memory formation were impaired following inhibition of DNA methyltransferase (DNMT), the enzyme responsible for DNA methylation. Pharmacologically increasing the levels of histone acetylation prior to DNMT blockade rescued both LTP and memory, suggesting that DNMT inhibition blocks the concomitant memory-associated H3 acetylation (103). That DNA methylation is important in maintaining synaptic plasticity, was also demonstrated by impairment of LTP exhibited by mice lacking methyl-CpG binding protein 1 (MBD1), a member of the methylated DNA-binding protein family (104). Moreover synaptic plasticity impairment has also been associated with DNA hypomethylation following early-life stress experiences (105), being restored by DNMT inhibitors (106).

Conversely, other evidences suggest that active DNA demethylation underlies synaptic plasticity and memory. Accordingly, mice lacking Gadd45b, a gene that modulates activity-associated DNA demethylation (107), display selective enhancements in long-term memory and synaptic plasticity (108). Altogether, these observations highlight that a complex and dynamic interplay between different epigenetic mechanisms implicated in synaptic plasticity and memory exists either in normal or pathological condition. To the best of our knowledge, whether pharmacologically targeting DNA methylation can alleviate LTP and memory impairments in AD models has not been investigated so far.

AD and histone modifications

Several studies highlighted a role for epigenetic mechanisms such as histone acetylation both in long-term potentiation (LTP) and memory formation in mice (109-112). The involvement of the epigenetic modulation of memory formation has also been investigated in disease models, although no clear-cut connection between histone modifications and the etiology of AD has yet emerged.

Studies performed on cell cultures demonstrate that β- and γ-secretase sequential cleavage of APP produces tail fragments that are able to interact with chromatin-modifying complexes. Among these, APP-CTs have been shown to form a multimeric complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60, which increases histone acetylation and stimulates transcription (113,114). Using PC12 cells and rodent primary cortical neurons, it has been demonstrated that APP-CTs induces histone H3 and H4 hyperacetylation and upregulates genes involved in cytotoxic function. These effects were potentiated in the presence of the HDAC inhibitor (HDACi) sodium butyrate (115). Thus, it is possible that downstream of histone acetylation, the TIP60 complex upregulates genes involved in the activation apoptotic pathways (116). For these reasons, the use of HAT inhibitors may hold promise for AD treatment, given the evidence for increased histone

acetylation in AD brain (117).

Several other studies suggest hypoacetylation as a potential risk factor for AD. In line with this, the therapeutic potential of different HDAC inhibitors was evaluated in several animal models of AD. Oral administration of nicotinamide, a class III HDACi, was able to reverse cognitive deficits and decrease the level of tau phosphorylation in the 3xTg-AD mouse model (118). Similarly, treatment with the HDACi sodium 4-phenylbutyrate for 5 weeks reduced tau phosphorylation and attenuated spatial learning and memory deficits in the Tg2576 mouse model of AD (119). Importantly, sodium butyrate was shown to enhance memory function even when administered at an advanced stage of disease progression (120). In addition, acute treatment with the HDACi Trichostatin A (TSA) prior to training rescued acetylated H4 levels with a parallel rescue of memory defects and hippocampal synaptic dysfunction in transgenic APP/PS1 mice (121). Likewise, in a mouse model of neurodegeneration and memory loss due to p25 overexpression and cyclin-dependent kinase 5 hyperactivation, intracerebroventricular injection of sodium butyrate elevated histone acetylation and contributed to the recovery of long-term memories and synaptic connectivity (122,123).

In line with a central role for HDACs, a recent study demonstrated that mice overexpressing HDAC2, but not HDAC1, exhibit impaired functional and structural plasticity and memory formation. These effects were attenuated by chronic treatment with vorinostat through targeting HDAC2 (124). In contrast, HDAC2 knockout mice showed facilitated memory improvement. These findings highlight that HDAC2 regulates synaptic plasticity and memory formation through epigenetic chromatin remodeling and modifications of DNA.

The role of histone-tail acetylation in these events is also sustained by several studies showing that the HAT activity of CBP is required for LTP and long-term memory in rodents (125). Importantly, intracellular Aβ and tau protein have been shown to interact with CREB/CBP signaling, downregulating CBP and in turn reducing histone acetylation in different preclinical models of neurodegeneration (126,127). Another recent study showed that increased EP300 interacting inhibitor of differentiation 1 (EID1) nuclear translocation is associated with reduced LTP and impaired spatial learning abilities through its inhibitory function on CBP/p300 mediated histone and p53 acetylation (128). Of note, the same authors find a significant enhancement of EID1 nuclear translocation also in cortical neurons of AD patient brains.

CONCLUSIONS

The finding that soluble oligomers of Aβ are capable of interfering with synaptic function and structure provides an important opening for understanding the basis of memory loss in AD. Importantly, similar findings in other disorders might indicate convergent mechanisms of synaptic plasticity failure in several neurodegenerative diseases. Emerging data suggest how different misfolded proteins that characterize neurodegenerative diseases such as AD, Parkinson's disease, Huntington's disease, Down syndrome and prion disorders share common structural features. This might indicate that assemblies produced by different disease-causing proteins might trigger similar downstream mechanisms raising the possibility of targeting their common structures for therapeutic treatment. In this context, frontal dementia exhibits neuron loss and extensive spine loss in cortex (129). Spine degeneration in neocortical neurons also occurs in other progressive neurodegenerative diseases such as Pick's disease (130) and motor neuron disease (131). Spine loss is also seen in neurons of substantia nigra, striatum, and locus coeruleus in Parkinson's disease (132-134). Similarly, striatal neurons in Huntington's disease show increased spine density in mild forms, possibly reflecting compensatory changes, but decreased spine density in severe cases (135). Notably, both the striatum and cortex express different forms of synaptic plasticity under normal and pathological conditions (41), suggesting once again that synaptic failure may contribute to memory decline also in these brain regions. It seems important to understand the molecular mechanisms that influence plasticity in the adult human brain and to determine whether regulating spine plasticity could prevent or even reverse cognitive deficits associated with neurodegenerative disease.

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BIMODAL EFFECT OF D-ASPARTATE ON BRAIN AGING PROCESSES: INSIGHTS FROM ANIMAL MODELS.

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Nowadays it is widely recognized that D-amino acids are present in bacteria as well as in eukaryotes, including mammals. In particular, free D-serine and D-aspartate are found in the brain of mammals. Notably, D-aspartate occurs at substantial levels in the embryo brain to then consistently decrease at post-natal phases. Temporal regulation of D-aspartate content depends on the post-natal onset of D-aspartate oxidase expression, the only known enzyme able to catabolize this D-amino acid. Pharmacological evidence indicates that D-aspartate binds and activates NMDA receptors (NMDARs). To decipher the physiological function of D-aspartate in mammals, in the last years, genetic and pharmacological mouse models with abnormally higher levels of this D-amino acid have been generated. Overall, these animal models have pointed out a significant neuromodulatory role for D-aspartate in the regulation of NMDAR-dependent functions. Indeed, increased content of D-aspartate are able to increase hippocampal NMDAR-dependent long-term potentiation (LTP) and spatial memory of adult mice. However, if exposure to elevated levels of D-Asp lasts for the entire lifetime of mice, enhancement of synaptic plasticity turns into a dramatic worsening, thus triggering an acceleration of the NMDAR-dependent aging processes in the hippocampus. Nonetheless, administration of D-Asp to old mice can restore the physiological age-related decay of hippocampal NMDA-related LTP. Besides its effect on hippocampus-dependent processes in mouse models, different points of evidence are indicating, today, a potential role for D-Asp in neurologic and psychiatric disorders associated with aberrant signalling of NMDARs.

Amino acids are chiral biological molecules, as they can exist both in L- and D-form. Despite both enantiomers display similar chemical and physical properties, L-forms are the only constituents of proteins so that D-enantiomers have long been considered unnatural (1). However, after the first discovery of D-amino acids in invertebrates (2, 3), several other studies have reported their presence either in free forms or incorporated into proteins (4). In particular, the refinement of sensitive analytical techniques revealed appreciable concentrations of free D-amino acids in mammals. Among different organs, free D-amino acids, such as D-serine (D-Ser) and D-aspartate (D-Asp), were detected in the mammalian central nervous system (CNS) (5). Several studies in mammals have investigated the tissue distribution of D-Asp and D-Ser, their developmental regulation and the specific localization of these D-amino acids within different tissues and brain areas (6-9). Research on D-Ser has demonstrated that this D-amino acid is able to bind to and activate NMDA receptors (NMDARs), functioning as an endogenous co-activator at the strychnine-insensitive glycine site (10, 11). These observations, together with the existence of specific mechanisms responsible for D-Ser biosynthesis, release and degradation (10, 12-14) have been considered robust evidence for considering D-Ser as a novel neurotransmitter (13, 15). Moreover, the property to modulate the activity of NMDARs suggests an involvement of this D-amino acid in brain disorders related to altered NMDAR functionality, including schizophrenia (SCZ) (10, 14, 16, 17). In contrast to D-Ser, knowledge

Key words: D-amino acids, NMDA receptors, synaptic plasticity, spatial memory, brain aging, hippocampus, neurodegeneration.

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0393-974X (2013)

about the neurobiological role of free D-Asp in mammals has been so far less extensive, although recent evidence suggest a biological significance for this molecule (8, 11, 18). In this review, we will focus on recent reports that are aiding to the identification of a role for D-Asp in mammalian brain aging.

D-ASPARTATE IN THE MAMMALIAN BRAIN

The presence of endogenous free D-Asp has been described in mammals such as mouse, rat and human, starting from the mid-80s (19-21). Transient occurrence of D-Asp has been demonstrated in the brain, where it is selectively found at very high concentrations during embryo and perinatal phases (19-24). Surprisingly, the amount of D-Asp in the human frontal cortex at gestational week 14 even exceeds that of the corresponding L-form (20). At embryonic day 12 (E12), it has been described a faint immunostaining for D-Asp in the mid-posterior regions of rat brain (23). At this stage, D-Asp is localized in the cytoplasm of neuroblasts, which have already ceased proliferative activity, but not in mitotic cells. In migrating neuroblasts, D-Asp immunoreactivity first appears in cell bodies and then shifts to axons once neuroblasts have reached their final destination. Starting from E14, D-Asp staining becomes more intense and, between E18 and E20, extends to the whole brain (23). In another work, localization of D-Asp was analysed throughout the first post-natal month of life (24). Between post-natal day 0 (P0) and P2, D-Asp was found at considerable levels in the forebrain and midbrain, and then also in the caudal-most regions of the brain. At these perinatal stages, D-Asp is concentrated in neuronal sets of the cerebral cortex, hippocampus and cerebellum, which are actively involved in developmental processes. At P7, D-Asp immunostaining uniformly decreases in the brain, to almost disappear at P28. One important point to keep in mind is that at all phases and in all brain areas, D-Asp is exclusively restricted to neuronal population, localized both in cytoplasm and fiber tracks, without any evident staining in glia (24, 25).

METABOLISM OF D-ASPARTATE

The specific temporal and regional changes of D-Asp contents in mammalian tissues imply the existence of biochemical homeostatic mechanisms for the precise modulation of its endogenous levels. Only few years ago, a mammalian aspartate racemase (DR), which converts L-Asp to D-Asp and co-localizes with D-Asp in the adult mouse brain, has been identified and cloned (26). In line with the consistent levels of D-Asp during early neuronal ontogeny (23, 24) and its postulated role in controlling brain development, retrovirus-mediated depletion of DR elicits profound trophic defects in the dendritic arborisation and survival of newborn hippocampal neurons (26). Despite co-localization studies in the adult brain, is not yet known whether DR and D-Asp co-localize also during brain development.

While a metabolic mechanism for D-Asp synthesis has been only recently discovered, since many years has been described the existence of a catabolic enzyme, D-aspartate Oxidase (DDO), able to selectively degrade bicarboxylic D-amino acids, such as D-Asp, D-glutamate and NMDA (27). DDO is a flavin adenine dinucleotide (FAD)-containing flavoprotein (28) which oxidizes D-Asp, in presence of H_2O and O_2, producing α-oxaloacetate, H_2O_2 and NH_4^+ ions (29). DDO is inactive towards D-Ser and other D-amino acids, that are substrates of the D-amino acid oxidase (DAO) (30), another flavoenzyme belonging to the same family of DDO (31). The protein sequence possesses a functional C-terminal tripeptide for the targeting to peroxisomes (32), where DDO is supposed to oxidize D-Asp and release its catabolites (33). Localization of this enzyme into peroxisomes, which contain catalase, allows the cell to safely remove H_2O_2, a toxic product of D-amino acids metabolism (34). DDO is highly expressed in the mammalian adult kidney, liver and brain (34). In the brain, DDO is temporally expressed at post-natal phases since its activity strongly increases from birth until 6 weeks of life (28). In the adult brain, DDO is widely distributed and clearly dominant in neuronal population (35). In agreement with a physiological activity of DDO over endogenous free D-Asp, histochemical detection in the rat brain shows that DDO expression is inverse to D-Asp localization (25). The reciprocal spatial and temporal relationship between this enzyme and its substrate let hypothesize that a rigorous homeostatic control of D-Asp by DDO must likely occur in different brain regions, especially in those areas playing a major role in neural processes like the hippocampus and cortex.

D-ASPARTATE ACTS ON GLUTAMATERGIC SYSTEM

NMDA subclass of ionotropic glutamate receptors has generated for a long time an enormous interest in neuroscience due to its implication in developmental and physiological neuronal processes (36-38). In addition, alterations in NMDARs activity have also been reported in neuropathological disorders including epilepsy, Alzheimer's Disease and schizophrenia (39-41). NMDARs are heterotetramers composed of an obligatory GluN1 subunit, ubiquitously expressed in the brain, combined with GluN2A-D subunits that show different localizations and time-dependent expression, thus critically influencing

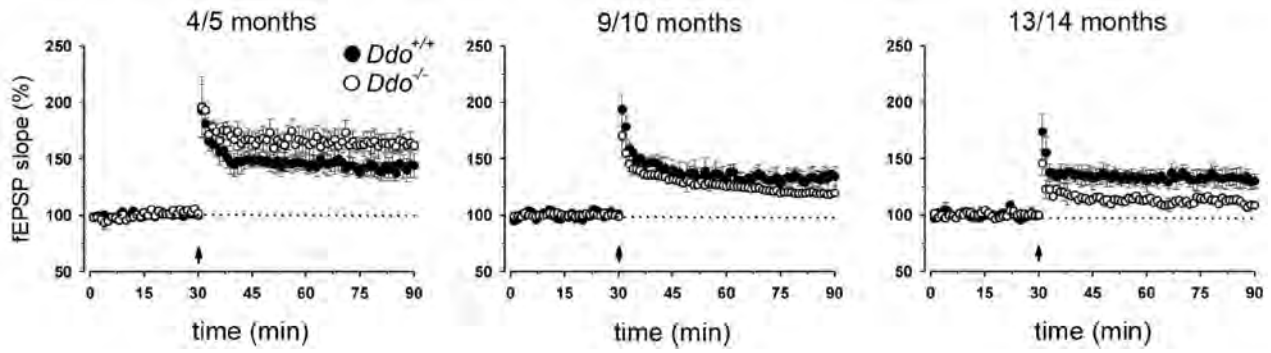

Fig. 1. *Biphasic, age-dependent effect of increased D-aspartate levels on synaptic plasticity in the hippocampus of Ddo$^{-/-}$ mice. Elevation of D-aspartate content enhances NMDAR-dependent LTP in 4-5-month-old Ddo$^{-/-}$ mice but worsens it ever more markedly at 9-10 and 13-14 months of age, respectively. Graphs show superimposed pooled data of normalized changes in field excitatory post-synaptic potential (fEPSP) slope induced by high frequency stimulation (HFS). Taken from Errico et al. (Neurobiol Aging, 2011) (46).*

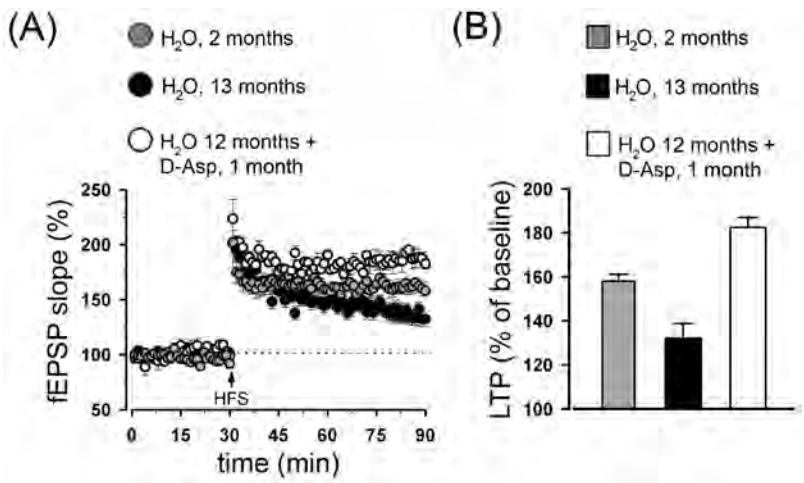

Fig. 2. *Oral administration of D-aspartate for one month to aged C57BL/6J female mice improves their hippocampal synaptic plasticity. (A) Superimposed pooled data of normalized changes in field excitatory post-synaptic potential (fEPSP) slope induced by high-frequency stimulation (HFS). (B) Summary bar graph of the fEPSP slopes quantified 50-60 min after HFS. Modified from Errico et al. (Neurobiol Aging, 2011) (43).*

the functional properties of the heteromeric assembly (42). Remarkably, the D-amino acids, D-Ser and D-Asp, are well known to bind with high affinity to the glycine and the glutamate (Glu)-binding sites of the NMDAR, respectively (6). Therefore, the transient high levels of D-Asp in the developmental brain and the high affinity of D-Asp for the Glu binding site of the NMDAR let hypothesize that this D-amino acid could play a functional role in the embryonic and early post-natal developmental regulation of glutamatergic neurotransmission at NMDAR sites, known to be implicated for neurogenesis, survival and cell migration events (36-38). On the other hand, the negligible levels of D-Asp in adult mammalian brain led, in the past, to the idea that this molecule is devoid of any physiological role. In contrast to this over-simplistic interpretation, the very abundant expression of DDO enzyme throughout the adult brain suggests that a role for this molecule could appear selectively under, yet unknown, neurological diseases and/or pharmacological treatment affecting DDO enzymatic activity. Based on this assumption, it has been shown that D-Asp, added to the medium of adult mouse brain slices, is able to

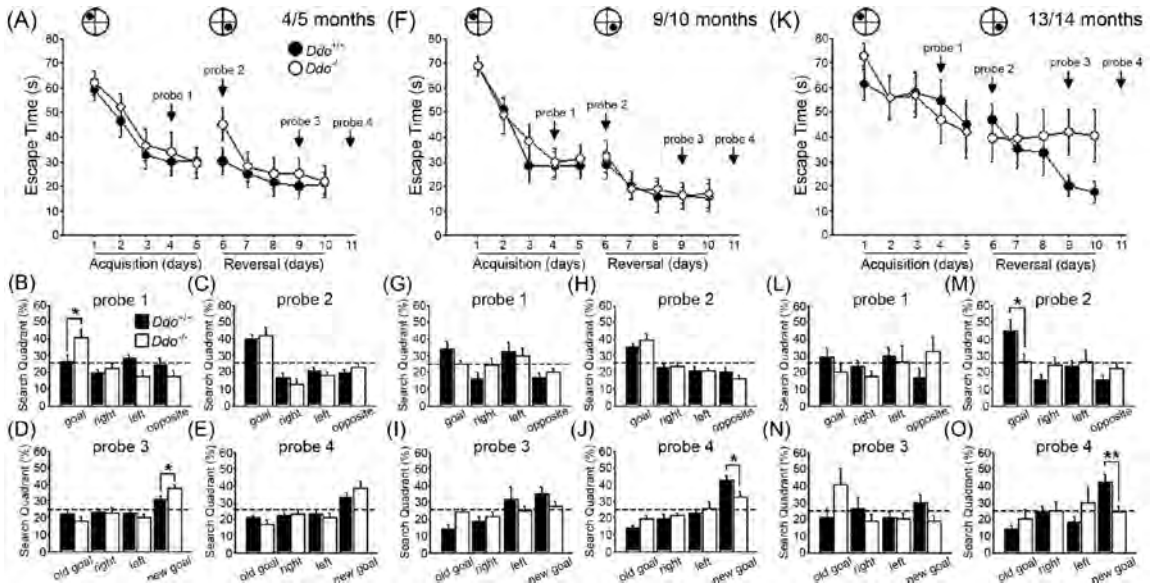

Fig. 3. *Biphasic, age-dependent effect of increased D-aspartate levels on spatial cognitive performance of Ddo$^{-/-}$ mice. Elevation of D-aspartate content enhances spatial memory of 4-5-month-old Ddo$^{-/-}$ mice but, on the other side, produces a decline of learning and memory performances in mice of 13-14 months of age. Cognitive abilities of mice were evaluated in a hidden-platform version of the Morris water maze. Graphs in A, F and K, show the times that animals employ to reach the platform (escape latencies) during acquisition and reversal phase of the training, when learning abilities of mice are evaluated. Graphs in B-E, G-J and L-O display the percentage of time spent by mice in each quadrant during the retention phases, when memory abilities of mice are evaluated. * p < 0.05, ** p < 0.01, between genotypes (Student's t test). Taken from Errico et al. (Neurobiol Aging, 2011) (46).*

trigger potent NMDAR-dependent inward currents both in CA1 pyramidal neurons of the hippocampus and in the GABAergic striatal medium spiny neurons (43, 44). Moreover pharmacological and electrophysiological studies also indicated that D-Asp specifically activates NMDARs via interaction with each of GluN2 subunits (45). Another interesting possibility supporting a further contribution of D-Asp on glutamatergic synaptic transmission at NMDAR sites is based on the knowledge that D-Asp serves as precursor of endogenous NMDA biosynthesis (46, 47). Nevertheless, it should be noted that D-Asp triggers also NMDAR-independent currents, indicating that this D-amino acid also affects other receptors or ion channels in mammalian brain (43-45, 48). Accordingly, in other reports D-Asp has also been shown to stimulate glutamatergic metabotropic mGlu receptors coupled to polyphosphoinositide hydrolysis in neonatal rat brain (49). Taken together, these electrophysiological and pharmacological investigations indicated that D-Asp strongly modulates neurotransmission in mammalian brain slices, where it seems to be mainly responsible for Glu receptors activation at NMDAR sites.

Besides its ability to bind to and stimulate NMDARs, D-Asp can also be released by neurons and recaptured in experimental conditions. In rat brain slices, intracellular radiolabeled D-Asp can be released upon chemical and electrical stimulation (50). The release of D-Asp from D-Asp-containing tissues or cells of the mammalian brain has been shown to occur in a Ca^{2+}-dependent manner (51-53). The relevant role of Ca^{2+} in triggering D-Asp release was unequivocally demonstrated in experiments using chelating agents for Ca^{2+}, able to strongly reduce D-Asp efflux after exposure to KCl (24). However, it is still unclear whether this mechanism of release can physiologically occur in the mammalian brain.

Presynaptic nerve terminals express L-Glu/L-Asp transport systems that utilize a Na^{+}-dependent mechanism to move excitatory L-amino acids against their concentration gradient. Extensive characterization of L-Glu transporters has long demonstrated that these

proteins are able to bind both L- and D-form of aspartate in a stereoblind fashion (54) and that D-Asp staining resembles that of L-Glu (55, 56). Interestingly, D-Asp uptake activity is absent in postsynaptic spines and dendrites, but appears to be concentrated in nerve terminals and/or in glial cells (55, 57), probably depending by a possible regional heterogeneity in D-Asp transport system (55).

Ddo KNOCKOUT AND D-ASPARTATE-TREATED MICE: ANIMAL MODELS WITH INCREASED LEVELS OF D-ASPARTATE

In order to disclose putative functions of D-Asp and of its metabolizing enzyme, different experimental approaches have been pursued to generate animals with deregulated high levels of D-Asp. In this respect, two knockout strains have been generated by targeted deletion of the *Ddo* gene (58, 59). Evaluation of endogenous D-Asp content in the brain and in peripheral tissues of both knockout (*Ddo*$^{-/-}$) mouse lines revealed a dramatic elevation of D-Asp, compared to wild-type littermates (43, 44, 48, 58-60). Conversely, no significant difference emerged in the brain levels of L-Asp (58, 59) and L-Glu (59). Of interest is the observation that a significant increase of endogenous NMDA content was found in the brain of *Ddo* mutant mice (58, 60). Taken together, data on deregulated high D-Asp brain levels validate *Ddo*$^{-/-}$ mice as a feasible animal model to study the *in vivo* and *in vitro* effects of increased D-Asp levels on nervous functions. In addition, besides gene-targeting approach, an alternative strategy to increase brain levels of D-Asp has also been used, through oral administration of this molecule to C57BL/6J mice (43-45). Also in this animal model a significant increase of D-Asp levels was found in each brain region analyzed, although to a lesser extent than in *Ddo*$^{-/-}$ mice.

NON PHYSIOLOGICAL, HIGH LEVELS OF D-ASPARTATE PERTURB NMDA RECEPTOR-RELATED SYNAPTIC PLASTICITY DURING AGING

The electrophysiological evidence on brain slices indicating the ability of D-Asp to act as an endogenous NMDAR agonist at glutamatergic synapses is in favour of its ability to modulate, among others, bidirectional synaptic plasticity in the brain of *Ddo*$^{-/-}$ and D-Asp-treated animals. Indeed, it is widely accepted that the activation of NMDARs can lead to long-lasting modifications in synaptic efficiency, known as long-term potentiation (LTP) and long-term depression (LTD) (61). Consistently with the feature of D-Asp to stimulate NMDARs, abnormal higher brain levels of D-Asp strongly modify striatal and hippocampal NMDAR-dependent synaptic plasticity of both *Ddo* knockout and D-Asp-treated mice (43, 44, 60).

Notably, the effect of D-Asp on NMDAR-dependent synaptic plasticity has received a special interest in the hippocampus because in this brain area under, under physiological conditions, the expression and activity of DDO are very high while D-Asp content is low (25, 35). This suggests that the hippocampal levels of this molecule should be strictly regulated. In support of this view, a previous study has demonstrated that deregulated high brain levels of this D-amino acid enhance NMDAR-dependent LTP in the CA1 area of *Ddo*$^{-/-}$ and D-Asp-treated animals (43). In particular, a two-fold increase of D-Asp levels in the hippocampus of C57BL/6J mice, orally treated with D-Asp for three months, substantially strengthens NMDAR-dependent synaptic plasticity at CA1 synapses. Interestingly, the subsequent interruption of treatment for three weeks is able to wash-out the excess of D-Asp and, in turn, to normalize LTP amplitude at physiological levels. Finally, further one-month treatment with D-Asp, after three-week withdrawal, re-establishes synaptic plasticity at previously potentiated levels (45). These results suggest a direct and plastic effect of D-Asp on hippocampal NMDAR- related synaptic processes.

The modulatory influence played by D-Asp on hippocampal circuitries is particularly intriguing if we consider the importance of this structure in neuronal processes associated with aging. Indeed, a large bulk of observations has evidenced that aged mammals are subjected to a massive loss of synapses in different hippocampal regions, among which CA1 area, that is likely to contribute to age-related impairments of synaptic plasticity and, in turn, of cognitive deficits (62). Interestingly, knockout mice for *Ddo* gene display synaptic plasticity features that dramatically change with age. In fact, while increased levels of endogenous D-Asp enhance the NMDAR-dependent LTP at 4-5 months of age, the persistent up-regulation of this D-amino acid accelerates the age-related decay of synaptic plasticity in 9/10- and, even more, in 13/14-month-old animals (Fig. 1) (48). In line with results obtained in knockout mice, long-term treatment with D-Asp for 12 months to C57BL/6J mice is able to significantly reduce LTP at CA1 synapses, compared to non-treated mice (45). The direct and reversible effect of D-Asp on hippocampus-dependent LTP is further highlighted by the fact that interruption of its administration for three weeks, after 12-month continuous treatment, can restore hippocampal synaptic plasticity at control levels (45). Changes in NMDAR-dependent LTP induced by D-Asp do not seem to depend by deregulated expression of NMDARs and AMPARs. In fact, in hippocampal homogenates from both *Ddo*$^{-/-}$ and D-Asp-treated mice, protein levels of the NMDAR subunits, GluN1, GluN2A, GluN2B, and of

AMPAR subunits, GluR1 and GluR2/3, are comparable between genotypes or treatments (45, 48).

Overall, results obtained in mice with increased levels of D-Asp show a clear biphasic modulation of persistent higher D-Asp content on hippocampal NMDAR-dependent synaptic plasticity. Both in knockout and D-Asp-treated mouse models, the increase in D-Asp levels is constant over time, since the amount of D-Asp is comparable in *Ddo*$^{-/-}$ animals of 4-5, 9-10 and 13-14 months of age (48), as well as in mice treated with D-Asp for three or twelve months (45). Therefore, the bimodal effect of D-Asp on NMDAR-dependent LTP may likely depend on a persistent abnormal stimulation of NMDARs. Accordingly, it is recognized that stimulation of NMDARs can give rise to dichotomous signaling in neurons (63): while a physiological and short-term activation of NMDARs crucially contributes to changes in synaptic strength and connectivity that are essential for learning and memory (64), on the other side, the intense and chronic stimulation of these receptors is detrimental for neurons and can contribute to the aetiology of several neurodegenerative disorders (65, 66).

The synaptic effect of D-Asp on glutamatergic neurotransmission also emerges by another study that shows a remarkable influence of this D-amino acid in old mice, when administered in a restricted short-time window at elderly phases. In particular, one-month treatment with D-Asp to twelve-month old C57BL/6J females is able to potentiate their LTP at levels even higher than those measured in two-month-old naïve controls (Fig. 2) (45).

INCREASED LEVELS OF D-ASPARTATE INFLUENCE DIFFERENT DOMAINS OF BEHAVIOUR

The two knockout lines for *Ddo* gene so far generated have been used to study both endocrine and neuronal D-Asp-related *in vivo* responses (8, 59, 67). Huang *et al*. observed that increased levels of D-Asp in the intermediate pituitary lobe of *Ddo* knockout mice elicit a reduced expression of pro-opiomelanocortin and its derivative a-melanocyte-stimulating hormone, compared to wild type littermates (59). Accordingly, also melanocortin-dependent behaviours, like penile erection, self-care activity and sexual appetite were found altered (59).

On the other side, *in vivo* studies performed in the other *Ddo* knockout line (58) have analyzed behaviours related to NMDAR-dependent functions. In these animals, D-Asp has been shown to significantly modify glutamatergic transmission in the striatum (44), a brain area primarily involved in motor and sensorimotor functions (68, 69). Of remarkable interest for its putative translational significance, D-Asp exerts protective effects against sensorimotor gating deficits produced by psychoactive drugs such as amphetamine and MK801 in *Ddo*$^{-/-}$ mice (44). A similar effect has been also found in D-Asp-treated animals (44).

Potential activation of NMDARs by D-Asp in the hippocampus of *Ddo*$^{-/-}$ and D-Asp-treated mice, as indicated by increased LTP at CA1 synapses, has been shown to trigger modifications in cognitive abilities of animals. In support for a role of D-Asp in the regulation of complex behaviours associated to NMDARs, *in vivo* studies indicate that non physiological, higher D-Asp content in the hippocampus of *Ddo*$^{-/-}$ mice is able to significantly affect learning and memory processes (44, 48). In line with electrophysiological experiments, knockout mice for *Ddo* gene, tested in a hidden-platform version of the Morris water maze, display improved reference memory at 4-5 months of age, while such cognitive ability clearly worsen at 13-14 months. In elderly phase of life, *Ddo*$^{-/-}$ mice also show an evident learning deficit (Fig. 3) (48). The biphasic cognitive response of *Ddo*$^{-/-}$ animals, that reflects exactly the age-dependent changes in NMDAR-dependent LTP, is also similarly associated with changes in ERK44/42 phosphorylation that selectively occur in the CA1 region of the hippocampus (48). Such selective localization of ERK44/42 deregulation well fits with D-Asp-related phenotypes here described since activation of CA1 area by NMDAR signalling is known to promote encoding of new spatial information in reference memory tasks (70). In the light of the biphasic responses of *Ddo*$^{-/-}$ mice to persistent increase of endogenous D-Asp levels, an unexpected physiological role emerges for DDO as a neuroprotective enzyme, able to prevent precocious deterioration processes occurring in the aging brain.

ABNORMAL LEVELS OF D-ASPARTATE IN ALZHEIMER'S DISEASE AND SCHIZOPHRENIA

Hypofunction of NMDARs is at the basis of different neurologic disorders, among which the most representative are schizophrenia (SCZ) and a range of disorders known under the term of dementia that also include Alzheimer's, Parkinson's and Huntington's disease (71). The crucial implication of NMDARs in chronic and degenerative disorders of the CNS let hypothesize that also D-Asp, through its agonistic activity on these receptors, may have some relevance in human pathological conditions affecting NMDAR-dependent signalling. Once again, preclinical animal models with elevated levels of D-Asp hint at this hypothesis. For instance, early phenotypic deteriorations of hippocampus-dependent functions found in *Ddo*$^{-/-}$ and D-Asp-treated mice are reminiscent of the invariant synaptic and behavioral deficiencies described in senescence-accelerated mouse strains (72)

and in Alzheimer's Disease (AD)-like animal models (73-76). In particular, LTP and memory loss represent the initial manifestation of the subsequent, more complex pathophysiological framework emerging in AD-like mouse models (75-79). Precursor studies by D'Aniello and coworkers analysed regional distribution levels of D-Asp in the *post-mortem* brain of AD patients. HPLC detections revealed that levels of free D-Asp were significantly lower in AD hippocampus, frontal, temporal and parietal cortices, compared to healthy subjects (80). Such decrease is not extended to the cerebellum, a region spared from the neuropathological changes of AD (80). Regional reductions of free D-Asp levels in AD brains are reflected in increased accumulation of this D-amino acid in the ventricular cerebrospinal fluid (CSF) (81), which serves as the repository of amino acids from the brain. Interestingly, proteins and AD neurofibrillary tangles from cerebral cortex of AD patients contained significantly higher levels of D-Asp than control brains (82, 83). However, such descriptive observations do not help to clarify whether abnormal levels of free D-Asp and/or D-aspartyl residues in AD proteins from AD brains may contribute to degenerative processes occurring in AD brains.

Since the first pharmacological evidence in the 80s, a large bulk of studies has sustained a crucial effect of reduced NMDAR signaling in the pathogenesis of SCZ (84-86). To ameliorate symptoms of SCZ, today many clinical trials aim to use compounds that, like D-Ser, can enhance NMDAR-dependent transmission by targeting the Gly-binding site of NMDARs (87, 88). In support for a clinical interest in D-Ser, abnormal content and metabolism of endogenous D-Ser have been reported in the CSF and serum of SCZ patients (89-91). In line with a potential involvement of D-amino acids in SCZ, mediated by NMDARs, a very recent work has evaluated the levels of free D-Asp and NMDA in the *post-mortem* brains of SCZ patients. Relevant HPLC analyses have indicated a substantial decrease in D-Asp levels in the prefrontal cortex and striatum of patients with SCZ, compared to control individuals. In accordance to D-Asp variations, also the levels of its derivative, NMDA, are strongly reduced in both the brain regions of SCZ subjects. On the other side, the levels of the two most abundant excitatory amino acids, L-Glu and L-Asp, are overall comparable between SCZ and control samples. Interestingly, the remarkable decline of endogenous D-Asp and NMDA correlates with a selective reduction of GluN1, GluN2A and GluN2B protein levels in the prefrontal cortex of SCZ patients (92). Future studies are mandatory to understand whether alterations in the expression and/or activity of the enzymes DDO and DR are responsible for abnormal levels of D-Asp and NMDA in SCZ brains. Overall, reduction of the NMDAR agonists D-Asp and NMDA in the brain of SCZ patients is in agreement with the large body of evidence that sustain the glutamatergic hypothesis of SCZ. However, it is still unclear in which way such decrease may contribute to hypofunction of NMDARs described in SCZ.

CONCLUSIONS

Several questions related to the biological role of D-Asp in the CNS are still obscure. However, the established ability of D-Asp to function as an endogenous NMDAR agonist represents a starting point to explore new neurobiological aspects and the potential influence of this "atypical" amino acid on neurodegenerative processes. In particular, the transient abundance of D-Asp during embryonic phase, together with the role of NMDARs on survival/apoptosis, migration and differentiation, suggests that depletion of D-Asp stores, either by over-expressing *Ddo* or by ablating *Dr* gene at early embryo stages, might disclose the specific role of this molecule in modulating early brain processes. On the other hand, consistent with the beneficial effect of D-Asp administration, this molecule could be taken into account for future clinical approaches aimed at counteracting age-dependent processes related to physiological or pathological reduction of NMDAR signaling.

ACKNOWLEDGEMENTS

F.E. was supported by a Grant from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (FIRB Call - Program "Futuro in Ricerca 2010" - Project nr RBFR10XCD3). A.U. was supported by an Independent Investigator award from National Alliance for Research on Schizophrenia and Depression (NARSAD). A.U. represents the Mariano Scippacercola Foundation.

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ENDOCANNABINOID SIGNALING IN ALZHEIMER'S DISEASE: CURRENT KNOWLEDGE AND FUTURE DIRECTIONS

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The importance of the endocannabinoid system (ECS) in the modulation functions of the central nervous system has been extensively investigated during the last few years. In particular, accumulated evidence has implicated ECS in the pathophysiology of Alzheimer's disease (AD), that is a progressive, degenerative, and irreversible disorder characterized by the accumulation in the brain of β-amyloid fragments forming insoluble plaques, and of intracellular neurofibrillary tangles (NTFs) associated with synaptic and neuronal loss. In all the processes involved in the formation of both plaques and NFTs, the key-role played by the ECS has been documented. Here, we review current knowledge and future directions of ECS modulation both in animal models of AD and in human tissues, underlying the role of endocannabinoid signaling in the development of AD hallmarks. Overall, the available data suggest that next generation therapeutics might target distinct ECS elements, for instance CB_2 receptor or fatty acid amide hydrolase, as a promising approach to halt or at least to slow down disease progression.

Alzheimer's disease (AD) affects over 26 million people worldwide and it has been predicted that 1 in 85 persons will be living with the disease by 2050 (1). Thus, a better understanding of this debilitating disease and the identification of new targets to protect the brain and to slow down the AD development appear of utmost importance.

AD is a progressive, degenerative, and irreversible neurological disorder, which by impairing all the critical metabolic processes that keep the neurons healthy, causes the death of those cells responsible for the disease's features such as memory failure, personality changes and problems in carrying out daily activities. AD is characterized by the accumulation of β-amyloid (Aβ) fragments in the brain forming insoluble plaques and of intracellular neurofibrillary tangles (NFTs), both responsible for damage to synapses.

Inflammation, oxidative stress, mitochondrial dysfunction, brain cholesterol dynamics are all processes involved in the formation of plaques and NFTs (2). In all these events recent studies have pointed out the key role played by the endocannabinoid system (ECS) (3-5). This evidence, along with recent *in vitro* and *in vivo* studies, has implicated ECS in AD pathophysiology, pointing to a possible ECS-oriented intervention for halting or slowing down the disease (6,7). The present review aims at covering current knowledge and future directions of ECS involvement in AD.

THE ENDOCANNABINOID SYSTEM

The ECS includes cannabinoid receptors, their endogenous ligands and the enzymes responsible for their synthesis and degradation (8). It modulates neurotransmission at inhibitory and excitatory synapses which control different processes, such as motor behavior, nociception, appetite, cognition and reinforcement/reward (9-14). In the last decade, many efforts have been made to

Key words: Alzheimer's disease, endocannabinoid, inflammation, lipoxygenase, neuroinflammation

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0393-974X (2013)

identify distinct elements of ECS, and to better understand their involvement in human health and disease (15).

To date, two 7-transmembrane G protein-coupled cannabinoid (CB) receptor subtypes have been characterized, namely CB_1 (16) and CB_2 (17). CB_1, localized to pre-synaptic terminals, is one of the most abundant G protein-coupled receptors in brain. It is also expressed peripherally, though at lower levels (16,18). CB_2 shows only 44% overall identity to CB_1, and it was found particularly abundant in peripheral organs (17-20). More recent studies have shown that CB_2 is expressed in both normal (21-24) and diseased brain cells (25-27). The possible presence of other CB receptors has also been proposed, such as the purported "CB_3" (or GPR55) receptor and the transient receptor potential vanilloid 1 (TRPV1) channel (28). TRPV1 is expressed in several CNS nuclei (29) and the importance of endocannabinoid/endovanilloid activity in the control of brain function has been documented (30,31). Additional evidence has demonstrated the interaction of endocannabinoids also with peroxisome proliferator-activated receptors (PPARs) α and γ, although at high concentrations (32), with significant implications for gene expression regulation (33).

The two most studied and best characterized endocannabinoids, the endogenous agonists of CB receptors that mimic the effect of *Cannabis sativa* extracts, are: *N*-arachidonoylethanolamine, also called anandamide (AEA), and 2-arachidonoylglycerol (2-AG). Unlike most neurotransmitters that are mobilized from membrane-delimited storage vesicles in a bioactive form, endocannabinoids are not stored in secretory vesicles, but are released in response to different (patho)physiological stimuli through cleavage of membrane phospholipid precursors. AEA was the first endocannabinoid to be described in neurons (34), but afterwards 2-AG was found to be more abundant in the CNS (35) and to act as a full agonist of both CB_1 and CB_2 receptors (36). AEA is synthesized by *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (37,38), whereas 2-AG is formed by hydrolysis of membrane phospholipids by diacylglycerol lipase (DAGL). The latter enzyme has been found in neuronal dendritic spines (39), and has been also shown to be inducible in reactive astrocytes (40).

So far, two degradating enzymes for endocannabinoids have been described: fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). FAAH, widely expressed throughout the CNS as an integral membrane protein (34,41-43), shows complementary expression with CB_1 (44,45). It hydrolyzes both AEA and 2-AG at similar rates *in vitro* (46). MAGL acts preferentially on 2-AG (47) and is responsible for ~85 % of its hydrolysis in the brain (48). Furthermore, though endocannabinoids are mainly inactivated through hydrolysis, increasing evidence indicates that these compounds are also subject to most of the oxidative metabolic pathways that lead to eicosanoid biosynthesis. Both AEA and 2-AG, possibly under conditions in which the activity of FAAH and/or MAGL is suppressed, become substrates for cyclooxygenases (COX) and lipoxygenases (LOX), giving rise to the corresponding hydroperoxy derivatives (49). These metabolites show different activity at CB_1/CB_2 or at other ECS elements (50), or appear to act at new binding sites with distinct biological effects (51,52).

REGULATION OF ECS COMPONENTS IN AD

Cannabinoid functions in cognitive processes have been observed in the hippocampus (53), a brain region rich in CB_1 receptors (16), especially in the CA2 and CA3 subregions (54). Instead, CB_2 receptors are mainly present in the brainstem (21), cerebellum (55) and microglia (56). Alterations of ECS in AD have been recently reviewed (57), and are listed in Tab. 1. The major implications of dysregulated endocannabinoid signaling in AD are briefly discussed below.

CB receptors have been shown to be unaffected in AD (58-61), though one report documented the decrease of their levels in human brain tissues (62). In the same study, the authors claimed that discrepancies with previous findings (58) could be due to the different region under investigation, i.e. frontal *versus* parahippocampal cortex. However, no changes in CB receptors were reported in a later investigation even in the frontal cortex of human AD subjects (60).

More recently, a significant reduction in CB_1 levels was observed also in the hippocampus of double transgenic (dtg) APPswe/PS1ΔE9 mice (an animal model of AD), compared to non-transgenic animals (54). CB_1 reduction occurred mainly in CA1 region, particularly susceptible to neurodegeneration in AD (63).

CB_2 were found to be overexpressed in senile plaques, and so was FAAH (59). FAAH expression appeared to be restricted to reactive astrocytes, and CB_2 receptors were expressed only in activated microglial cells (59). Consistently with these results, the same authors found an increase of both CB_2 and FAAH expression in glial cells surrounding Aβ plaques in tissues from subjects with the Down' syndrome (64). The latter is considered a natural model of AD, because patients at an age $\geq$ 40 develop neuropathological symptoms of AD (65,66). Furthermore, in the APPswe/PS1dE9 mouse model of AD an increase in CB_2 receptors binding was observed in brain areas with Aβ amyloid plaque deposition, measured *in vivo* by positron emission tomography (PET) (67). Moreover, in peripheral blood of AD subjects, an increase in CB_2

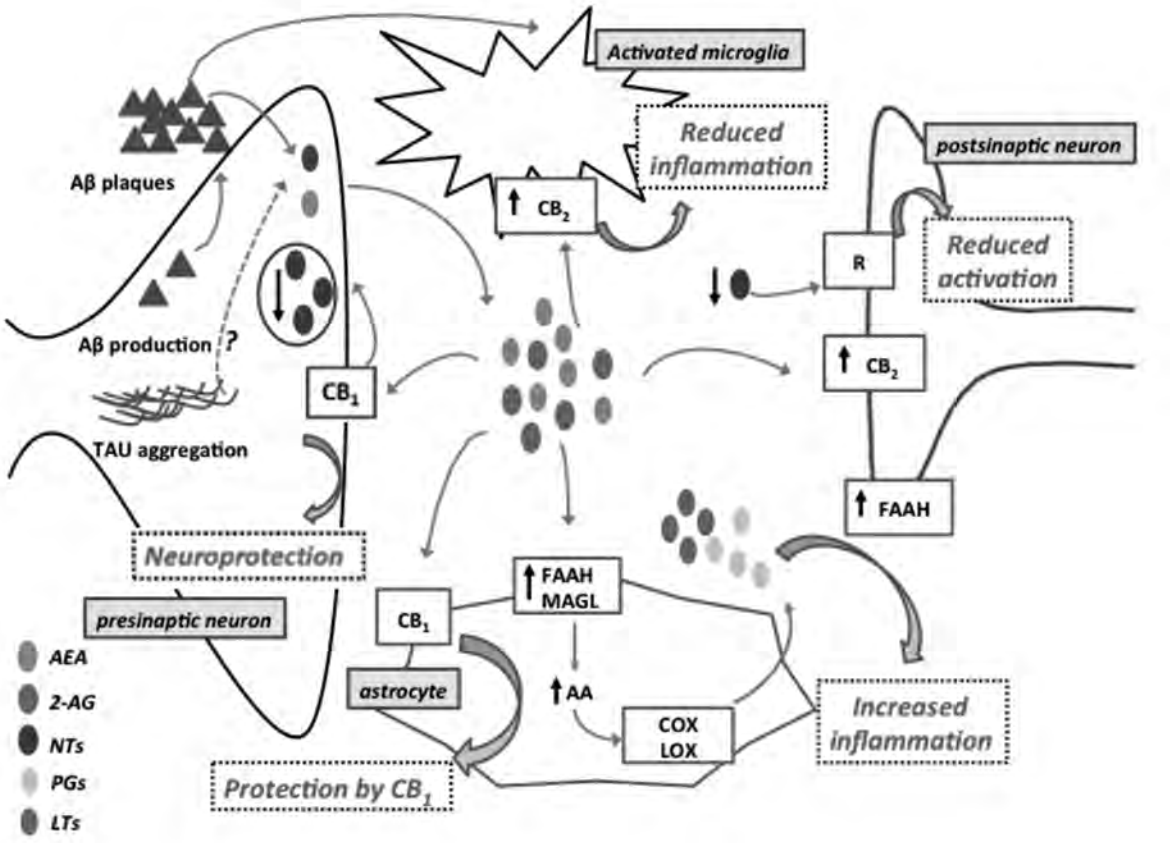

Fig. 1. *Involvement of the ECS in AD, and its possible interplay with the eicosanoid signaling (NTs = Neurotransmitter; PGs = Prostaglandins; LTs = Leukotrienes). Endocannabinoids are released in response to pathogenic events, thus representing a potential compensatory repair mechanism. The putative neuroprotective effect linked to neuronal repair and cell maintenance mainly involves: up-regulation of CB_2 in the microglia (59,69), that could reduce inflammation and presynaptic CB_1 stimulation with reduced NT release (132); up-regulation of endocannabinoid hydrolysis trough catabolic enzymes (FAAH and MAGL), that could be a significant source of AA for COX- and LOX-mediated proinflammatory eicosanoids in astrocytes surrounding neuritic plaques, with harmful effects (124,124,128,129, 133-135).*

mRNA expression was observed in patients with lower Mini Mental State Examination (MMSE) scores (68). Another report showed increased CB_2 receptor levels in severe AD, when compared with age-matched controls or subjects with moderate AD (69).

Also a reduction in the levels of AEA and of its precursor, NArPE, but not of 2-AG, has been observed in the cortex of AD patients (70). Yet, no differences in AEA or 2-AG concentrations in plasma from patients with AD and healthy controls have been detected (71). Moreover, Jung and colleagues (70) found that AEA levels correlate with the cognitive impairment of the patients, whereas Koppel and coworkers (71) did not find cognitive performance correlation with circulating endocannabinoids in subjects at risk for AD.

An early report also found an enhanced enzymatic activity of both DAGL and MAGL in the hippocampus of AD patients (72), and consistently an increase of both enzymes in human AD brains has been recently documented (61).

ENDOCANNABINOIDS AND AD HALLMARKS

AD can be distinguished from other dementias for the presence of two neuropathological hallmarks in brain regions responsible for memory: amyloid plaques and NFTs, associated with synaptic and neuronal loss.

The senile plaques are essentially composed of Aβ peptides, that are fragments of the β-amyloid precursor protein (APP), often surrounded by activated microglia and astrocytes (65). Activated microglia clusters at senile plaques seem to be responsible for the ongoing inflammatory process of the disease (73,74). Aβ accumulation can be responsible for oxidative stress,

inflammation and neurotoxicity, and hence it might trigger the pathogenic cascade that ultimately leads to apoptosis and impairment of neurotransmission networks (2).

Soluble Aβ. After several years of research on the role of Aβ in its insoluble, aggregated form, recent findings have changed the perspective of Aβ meaning. Indeed, accumulating evidence suggests that pre-fibrillar, diffusible assemblies of Aβ are also deleterious. There is now persuasive evidence that the cognitive and behavioral alterations in AD arise, at least in part, from impaired synaptic function due to soluble forms of Aβ (75). It has been shown that these forms potently alter synaptic structure and functions (76,77). In particular, soluble Aβ interferes with synaptic function that subserves higher-order neural network activity (78). Moreover, learning and memory can be improved by reducing brain soluble Aβ levels (79). In this scenario, the identification of soluble Aβ peptides as highly bioactive assemblies has furthered interest in detecting and analysing their mechanistic properties, as well as their diagnostic and therapeutic potential. Yet, we still lack insight into the *in vivo* relevance of the soluble forms of Aβ in the brain during the development of AD-related diseases.

At present, no treatment is available that stops the degenerative process of AD, and current treatments can offer only modest symptomatic benefits. Therapeutic strategies are now unable to delay disease progression by more than one year, likely because AD is diagnosed when the pathology is already permanently advanced (80).

The identification of potential new therapeutic targets is then a major need, since development of novel therapeutic approaches is inhibited by the poor knowledge of the early

Table I. *Modifications of ECS elements in AD.*

ECS element	Product analysis	Change	Tissue/Cell	Reference
CB_1	Protein	↔	Human brain	58-61
		↓	Human brain	62
		↓	Mice brain	54
	mRNA	↑	Whole blood	68
		↔	Human brain	58
CB_2	Protein	↑	Human brain	59,69
		↔	Human brain	58
	mRNA	↔	Human brain	58
2-AG	Endogenous levels	↔	Human plasma	71
		↔	Human brain	70
		↑	Human brain	61
AEA	Endogenous levels	↔	Human plasma	71
		↓	Human brain	70
FAAH	Protein	↑	Human brain, Human PBMCs	59,124
	mRNA	↑	Human PBMCs	124
DAGL	Protein	↑	Human brain	61
MAGL	Protein	↑	Human brain	61

stages of the disease, prior to Aβ plaque deposition, massive neuronal and neurotransmitter loss with irreversible neuronal damage and cognitive dysfunctions. In this context, recent studies have suggested that ECS plays a crucial role in neuroprotection, and the enhancement of the endocannabinoid tone is now considered an attractive approach for future therapeutic exploitation (81). Remarkable changes of endocannabinoid levels and receptor concentrations found in patient brains and in animal models have further strengthened the hypothesis that ECS is considerably altered in AD (82). For instance, it has been demonstrated that the enhancement of brain endocannabinoid tone, through the early endocannabinoid reuptake blockade, is able to reverse memory impairment and neurotoxic effects triggered by soluble Aβ in murine models of AD. In the same study, the authors reported that, when the treatment was done in a late phase, cognitive deficit was dramatically aggravated (83). Moreover, a novel player in brain endocannabinoid signaling has been recently identified. Indeed, it has been reported that the anti-inflammatory lipid lipoxin A_4, detected in brain tissues, enhances the affinity of AEA for CB_1 receptors, thereby potentiating the effects of this endocannabinoid. Additionally, Aβ-induced impairment of spatial memory formation was prevented by co-injection of lipoxin A_4, showing that lipoxin A_4-induced neuroprotection depends on CB_1 receptors (84). Nonetheless, it must be emphasized that the relevance of ECS for human medical care of amyloid-related diseases is still in its infancy (82). Taken together, the actual role of endocannabinoids in AD remains elusive, yet chances are that elevation of endocannabinoid levels is part of a neuroprotective mechanism that aims at counteracting Aβ-related neurotoxicity, rather than part of the pathological process.

Aβ plaques. The first study of endocannabinoids as inhibitors of Aβ toxicity was carried out on a human neuronal cell line, and showed the neuroprotective role of AEA and noladin (a putative endogenous cannabinoid with agonist activity at CB_1 receptors) through a CB_1-dependent, mitogen activated protein kinase (MAPK)-mediated mechanism (85). A previous study had already shown that the production of nitric oxide (a proinflammatory mediator) by microglial activation, is prevented by exogenous cannabinoids (86). More recently, it has also been observed that cannabinoids can counteract the Aβ-induced microglial activation via CB_2 (62), and promote microglia migration allowing clearance of the Aβ peptide (87). Consistently, the CB_2 agonist JWH-015 inhibits Aβ-induced production of proinflammatory cytokines (88). It has been observed that in microglial cells activated by interferon-γ JWH-015 suppresses the expression of CD40 (88), a glycoprotein belonging to the tumor necrosis factor receptor that is highly expressed in senile plaques in AD brain (89). Another study showed that endocannabinoids are protective in the Aβ-induced increase in DNA fragmentation and caspase-3 activation, both hallmarks of apoptosis, in primary cerebral cortical neurons (90). It was also documented that endocannabinoids can keep the cell alive by stabilizing lysosomes, that may be permeabilized by Aβ (90). Moreover, earlier studies have shown that CB_2 receptors and FAAH expression increase in immune cells surrounding senile plaques in AD subjects (59).

It was also observed that Δ^9-tetrahydrocannabinol (THC), the psychoactive principle of *Cannabis sativa* extracts, reduces Aβ aggregation through competitive inhibition of acetylcholinesterase (AChE), the enzyme responsible for the degradation of acetylcholine (91), which is generally associated with amyloid plaque deposits (92). AChE accelerates the formation of amyloid fibrils in the brain by generating stable complexes with Aβ (93) and, not surprisingly, most drugs licensed for AD treatment are AChE inhibitors. It is of noteworthy that THC appears more effective than any other drug in reducing AChE-induced Aβ deposition (91).

In animal models of Aβ-induced toxicity, many reports confirmed the beneficial effects of cannabinoids in reducing neuroinflammation; indeed, both cannabinoid agonists and phytocannabinoids like cannabidiol are able to reduce Aβ-triggered microglial activation (62,94,95). In particular, Ramírez and colleagues observed that cannabinoid administration can attenuate loss of neuronal markers and reduce cognitive deficits occurring in Aβ-treated rats, thus preventing microglial activation (62). Moreover, they showed that CB_1-positive neurons were reduced when compared to control areas of microglia activation (62). In mouse hippocampus injected with human $A\beta_{42}$ peptide, cannabidiol inhibited glial fibrillary acidic protein, as well as nitric oxide synthase and IL-1β protein expression and release (94).

Consistently, the AEA reuptake inhibitor VDM-11, which causes an elevation of the endocannabinoid tone, reversed hippocampal damage and loss of memory retention in rodents treated with $A\beta_{42}$ peptide (96). Moreover, CB_1 modulation can protect against Aβ-induced amnesia in hippocampal learning tasks (97,98). Rimonabant, a selective CB_1 antagonist/inverse agonist, improves memory deficit induced by β-amyloid fragments, probably through an increase of hippocampal acetylcholine release, or by acting directly on cannabinoid neuronal circuits involved in memory (97). Micale and coworkers focused the attention on the interaction between endocannabinoids and the dopaminergic system, in particular on dopamine D3 receptor (D3R) involvement in the neurotoxicity and amnesia induced by Aβ. They found that neurotoxin administration induced a less pronounced cognitive impairment in the passive-avoidance paradigm performance in wild-type

compared to D3R knockout mice (98). Since the latter animals exhibited higher level of endovanilloid and endocannabinoid signaling, the authors suggested a potential role for enhanced CB_1 tone in worsening memory retention (98).

NFTs. These structures result from hyperphosphorylation of microtubule-associated Tau protein, leading first to the dissociation of Tau from the microtubule, then to microtubule destabilization and Tau oligomerization within the cell, and finally to cell death (99).

Only few studies investigated the role of ECS in NFTs formation. It has been observed that cannabidiol reverses Tau hyperphosphorylation by reducing phosphorylation of glycogen synthase kinase-3β, a key kinase for both physiological and pathological tau phosphorylation in AD (100). More recently, it has been reported that MAGL expression levels are increased in neurons with hyperphosphorylated Tau (61), confirming 2-AG contribution to synapse silencing in AD. Another study showed that the reduction of AEA levels in brain tissues from AD patients correlates with patients cognitive impairment, but not with Tau hyperphosphorylation nor with amyloid plaques formation (70).

Neuronal loss of function. The third main feature of AD is the loss of neuronal ability to rapidly communicate and process signals across synapses. Synaptic function is controlled through different mechanisms, such as neurotransmitter release acting at specific pre- and post-synaptic receptors. Among the presynaptic receptors, an important role in the regulation of brain synapses is played again by those activated by endocannabinoids (101). 2-AG has been found to mediate synaptic communication in the hippocampus (102), and indeed a reduction of pre- and post-synaptic 2-AG degradation, along with an increase of its synthesis, has been demonstrated in AD, suggesting that an endocannabinoid hypertone might aggravate synapse impairment by disrupting retrograde signaling (61). However, further investigations are needed to better clarify the role of ECS in synaptic plasticity following Aβ plaque formation.

TREATMENTS OF AD THAT TARGET ECS

The relevance of the ECS for AD treatment has been debated for many years. The first report suggesting ECS modulation as potential therapy of AD showed that dronabinol, a synthetic form of THC, was able to improve the behavior in AD subjects and also to stimulate appetite (103). Indeed, it is known that patients with dementia start refusing food during the course of the disease (104). More recently, the same compound was found to reduce also nocturnal restlessness that frequently occurs in patients with dementia (105). In line with this, in a case report study the synthetic cannabinoid receptor agonist nabilone significantly ameliorated dementia-related restlessness (106).

CB_2 agonist JWH-015 was found to increase the ability of cultured human macrophages to remove Aβ deposits from human tissues of AD patients, as well as from synthetic Aβ fibrils *in vitro* (107). As already mentioned, by targeting the different ECS components, many studies have reported the potential neuroprotective ECS abilities in various models of AD (62,85,96-98), and highlighted disease-related alterations of the ECS occurring over time (58,59,62).

Chronic infusion of lipopolysaccharide (LPS) into the fourth ventricle of rats can induce many of the pathophysiological changes observed in neurodegenerative diseases, as well as the activation of microglia (108). The latter process is indirectly prevented by WIN-55212-2, a CB_1/CB_2 agonist (109).

The non-psychoactive component of cannabis, cannabidiol, has a number of additional characteristics that highlight the potential benefits of using cannabinoid-based therapeutics for the treatment of AD (110). Indeed, it has been observed that cannabidiol can scavenge reactive oxygen species (111), reverse Tau hyperphosphorylation (100), and reduce activation of the inflammatory transcription target nuclear factor-κB (112). Again, it has to be taken under consideration that not all animal studies agree on the possible beneficial effects of endocannabinoids for AD therapy. For example, in two animal studies using Morris water maze to measure cognitive impairment, one report documented that cannabidiol and WIN-55,212-2 could prevent memory impairment in Aβ-treated rats (87). In contrast, another report showed that HU210, a potent synthetic cannabinoid, did not improve water maze performance nor a contextual fear conditioning task in an APP23/PS45 double transgenic mouse model of AD (113). These conflicting results are likely to depend on differences in experimental sets, as well as on the drugs used to modulate ECS (e.g., phyto-, endo-, or synthocannabinoids), their administration routes, concentrations and timing of application (90,96).

INFLAMMATION IN AD: THE ENDOCANNABINOID-EICOSANOID CONNECTION

Inflammation coupled with oxidative stress plays a major role in AD, and its suppression has been shown to reduce AD pathological hallmarks as well as cognitive and behavioral deficits in AD models (114). It is well known that inflammation involves arachidonic acid (AA)-derived lipid mediators biosynthesized by pathways dependent on COX and LOX activity. Non-steroidal

anti-inflammatory drugs (NSAIDs), that exert their effects through COX inhibition, have well-documented protective effects in AD when administered at an early stage and taken over prolonged periods of time (115). It was reported that patients who suffer from inflammatory diseases (e.g., arthritis) or take anti-inflammatory drugs (e.g., anti-inflammatory COX inhibitors like aspirin and indomethacin), have a reduced risk of developing AD (116). However, an issue with NSAIDs has been their lack of efficacy in AD clinical studies (117). Also selective COX-2 inhibitors have been investigated and tested clinically as potentially better therapeutics for AD patients; yet, they failed to confirm their efficacy for actual therapy (118). Moreover, the gastrointestinal and cardiovascular toxicity of COX inhibitors limited their use for neuroinflammatory syndromes. Novel and safer anti-inflammatory strategies are thus required, not only to gain a deeper understanding of the role that inflammation plays in AD progression, but also to investigate the therapeutic potential of anti-inflammatory drugs to combat this disease. Growing evidence suggests a role for LOX, and in particular for 5-LOX, as potential target in AD (119-121). In line with this, also leukotrienes (LT), that are end-products of 5-LOX pathway, have been found to be involved in brain inflammation associated with age-related dementia, as well as with neurodegenerative diseases (122,123).

To date, the eicosanoid and endocannabinoid signaling systems have been investigated independently of each other, and indeed one is likely to operate in the absence of the other and vice versa. However, both endocannabinoids and eicosanoids are derivatives of AA, therefore a potential intersection between their signaling systems can be expected (49). In this context, it seems noteworthy that the lipases that initiate both pathways are responsive to common second messengers (e.g., elevation in intracellular Ca^{2+}), supporting the view that in cells where the enzymatic machinery for both pathways is present, endocannabinoid and eicosanoid signaling might cooperate. Such an interaction is further complicated by the ability of metabolic enzymes of eicosanoids to metabolize also (and even better) endocannabinoids (51).

We recently reported the epigenetic regulation of ECS components, and of LOX isoforms, in peripheral blood mononuclear cells (PBMCs) of subjects with late-onset AD (LOAD) and age-matched controls. Our data showed that FAAH is up-regulated in PBMCs of LOAD subjects compared to healthy individuals, without changes in the mRNA levels of any other ECS elements (124). Consistently, we also demonstrated in LOAD subjects an increase in FAAH protein levels and enzymatic activity, due to an epigenetic regulation of gene trascription. Moreover, in an independent investigation (125) we observed a significant increase of 5-LOX gene (ALOX5) expression in LOAD subjects, that was paralleled by reduced DNA methylation at gene promoter, increased 5-LOX protein and increased plasma levels of the 5-LOX end-product, LTB_4. We thus hypothesized that increased AEA hydrolysis by FAAH could contribute to the inflammatory process that occurs in AD, for instance by releasing the AA pool for neuroinflammatory LTB_4. We also provided evidence that ALOX5 and FAAH genes share common epigenetic signatures. According to this, we found a direct correlation between DNA methylation at FAAH and ALOX5 gene promoters. Moreover, LTB_4 levels were directly correlated to FAAH mRNA levels, and inversely correlated to FAAH DNA methylation, suggesting that a parallel increase of FAAH and 5-LOX expression in AD patients could evoke a sustained inflammatory condition, thus reinforcing neurodegeneration. Overall, our data highlighted the contribution of epigenetic mechanisms to the control of genes involved in AA metabolism upon AD development (126).

The possible interplay between endocannabinoid and eicosanoid signaling has been also explored in animal models of neuroinflammation. Nomura and colleagues documented the relevance of MAGL in the control of AA release for the production of proinflammatory eicosanoids in the brain of rodents (127). This observation was corroborated by the finding that mice deficient in the MAGL-encoding gene, and mice treated with the MAGL-selective inhibitor JZL184 showed elevated brain levels of 2-AG and reduced levels of AA and AA-derived prostaglandins (127). Moreover, two independent studies have documented a deregulated endocannabinoid-eicosanoid network in mouse models of AD, whereby endocannabinoid hydrolysis was recognized as the main source of AA for COX-mediated eicosanoid production in the diseased brain (128,129). In these studies, MAGL blockade was found to substantially reduce neuroinflammation and to attenuate amyloidosis, likely by suppressing pro-inflammatory eicosanoids production, thus pointing to MAGL inhibitors as an attractive therapeutic strategy for the treatment of AD.

Very recently, a functional connection between CB_2 and 5-LOX has also been documented in a zebrafish model of inflammation (130), where both a CB_2 agonist and a 5-LOX inhibitor reduced leukocyte migration in response to acute injury.

CONCLUDING REMARKS

Drugs currently used for the treatment of AD, such as AChE inhibitors (e.g., donepezil and rivastigmine) or antagonists for the N-methyl-D-aspartate glutamatergic receptors (like memantine), produce limited clinical benefit and do not correct the underlying molecular defects

(131). The prevalence of AD is expected to triple over the next 50 years, creating an urgency to develop effective disease-modifying therapies to reduce the economic burden of this devastating disorder. One of the main areas of therapeutic focus has been an anti-inflammatory strategy originated from epidemiological evidence that long-term exposure to NSAIDs protected against the development of AD. Unfortunately, subsequent large-scale double-blind placebo-controlled clinical trials failed to support the use of NSAIDs for the treatment of AD. In search for novel strategies able to halt or slow down the course of AD, and to improve the patient quality of life, here we have reviewed recent investigations that suggest a key-role for distinct ECS components in both normal and altered neuronal circuits. Therefore, ECS modulation (e.g., by targeting CB_2 and FAAH) might provide a promising arena for next generation therapeutics (132-134). In addition, evidence is mounting to support the hypothesis that endocannabinoid tone can modulate eicosanoid levels, and vice versa (see figure 1 for a schematic representation of ECS involvement in AD). This scenario is particularly likely under conditions of inflammation, that would lead to increased expression of 5-LOX. Indeed, reduction in levels of anti-inflammatory endocannabinoids may be one mechanism by which 5-LOX exerts its pro-inflammatory effects. Although the beneficial effects produced by FAAH or 5-LOX inhibition against neuropathology of AD remain to be determined, it could be anticipated that these enzymes might become a promising therapeutic target for the prevention and treatment of AD.

ACKNOWLEDGEMENTS

This investigation was partly supported by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (grant PRIN 2010–2011 to MM, and Futuro in Ricerca RBFR12DELS_001 to CDA), and by Fondazione TERCAS (grant 2009–2012) to MM. ADF was supported by a "Fondazione TERCAS-Progetto Speciale Assegni di Ricerca 2011–2013" fellowship.

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THE HEME OXYGENASE/BILIVERDIN REDUCTASE SYSTEM: A POTENTIAL DRUG TARGET IN ALZHEIMER'S DISEASE.

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive loss of cognitive function, the inability to perform the activities of daily living and psychiatric symptoms. The formation of toxic aggregates of amyloid-β-peptide (Aβ), through the activities of β - and γ- secretases, is considered as the earlier event in the pathogenesis of the disease. The deposition of both Aβ and the following hyperphosphorylation of tau protein, trigger an exaggerate immune-inflammatory response culminating with the production of excess reactive oxygen and nitrogen species responsible for damage on cellular nucleic acids, proteins and lipids. One of the mechanisms used by neural cells to counteract oxidative/nitrosative damage in AD is the enhancement of the cell stress response. Among the main components of the cell stress response is the heme oxygenase/biliverdin reductase (HO/BVR) axis, which catalyzes the degradation of heme which is toxic if produced in excess or under redox unbalanced conditions. However, the HO/BVR system and its by-products, carbon monoxide and bilirubin, have also been shown to be neuroprotective by activating pro-survival pathways and scavenging free radicals. Nevertheless, recent research demonstrated as both the inducible isoform of HO, known as HO-1, and BVR undergo oxidative/nitrosative/phosphorylative post-translational modifications in AD brain which alter the ability of HO-1 and BVR to activate the cell stress response. In this light, naturally occurring substances or drugs (e.g. statins) that prevent the post-translational modifications leading to a controlled up-regulation of the HO/BVR system have been proposed as potential new tools for the treatment of AD.

The heme oxygenase/biliverdin reductase (HO/BVR) pathway is the main metabolic system through which heme is degraded. The combined action of these enzymes converts heme into ferrous iron [Fe(II)], carbon monoxide (CO), and biliverdin-IX-alpha (BV) (Figure 1). This latter is not the final product of heme metabolism in mammals, but it is the precursor of bilirubin-IX-alpha (BR) (1,2) (Figure 2). For several years, both BR and CO were considered mere waste products, but over the past 25 years, a number of investigators have focused their attention on both HO/BVR and their products in an attempt to elucidate their true biological functions. In 1987, Roland Stocker, Tony McDonagh and colleagues published a seminal paper in which the antioxidant properties of BR were unraveled (3). In 1993, Verma *et al.* proposed a role for CO as an endogenous neuromodulator (4). These early observations were followed by many papers demonstrating CO's important role as a regulator of important brain functions such as synaptic transmission and neuropeptide release (4-6) (Table I). Carbon monoxide and BR are also involved in the regulation of several cell functions, including smooth muscle relaxation, the potentiation of the cell stress response and many others (Table I and Figure 2) (7-13). Both reactive oxygen and nitrogen species (ROS and RNS, respectively) play a main role in the pathogenesis of neurodegenerative disorders, mainly Alzheimer's disease (AD), and the activation of intracellular pathways involved in the detoxification of free radicals was claimed as an useful approach to counteract AD and other dementias (14,15). In this light,

Key words: Alzheimer's disease, biliverdin reductase, heme oxygenase, nitric oxide, oxidative stress.

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0393-974X (2013)

the up-regulation of the HO/BVR axis was considered as a promising mechanism for improving cell stress response and counteract ROS/RNS damage (2,14), and substances known to increase HO activity *in vitro* were explored as potential drugs for the treatment of free radical-induced diseases (2,15-19).

This review will examine the several lines of evidence produced over the last decades about the potential role of the HO/BVR axis in AD. In addition, novel results on the down-regulation of both HO-1 and BVR in the brain of AD subjects and the evaluation of these enzymes as peripheral biomarkers of AD will be discussed.

THE HO/BVR AXIS: FUNCTION, REGULATION AND DISTRIBUTION

Heme oxygenase

Heme oxygenase is an ubiquitous microsomal enzyme, which catalyzes the oxidative cleavage of heme moieties of hemoproteins in a 4-step, energy-dependent manner. Chemically speaking, HO itself is not a hemoprotein: it acquires this characteristic after binding to heme-Fe(III) (20). Activation of the heme catabolic pathway requires not only HO but also oxygen and NADPH-cytochrome-P-450 reductase, this latter providing the electrons necessary to catalyze the transformation of the cyclic tetrapyrrole heme into equimolar amounts of Fe(II), CO, and BV (1,2) (Figure 1).

Heme oxygenase exists in two main isoforms, HO-1 and HO-2. They are the products of 2 different genes, and their homology is limited (43%), but the active core of both enzymes is a conserved 24-amino-acid segment, which forms the hydrophobic heme-binding pocket in the folded protein (1).

Although HO-1 and HO-2 catalyze the same reaction, they play different roles in protecting tissues against injuries. Heme oxygenase-1 (HO-1), also known as heat shock protein(Hsp)-32, is induced by various stimuli, including oxidative and nitrosative stress, ischemia, heat shock, bacterial lipopolysaccharide (LPS), hemin, and the neuroprotective agent, leteprinim potassium (Neotrofin) (1,21). In addition, HO-1 activity can be increased following post-translational modifications occurring on its structure, such as the phosporylation of the aminoacidic residue Ser^{188} (22). Although constitutively expressed, HO-2 is responsive to developmental factors and adrenal glucocorticoids and it is primarily involved in maintaining cell heme homeostasis and in sensing the intracellular levels of gaseous compounds including oxygen, nitric oxide (NO), and CO (1,21,23). Currently, HO-1 induction, under condition of redox unbalance, is considered as a pivotal event in the earlier stages of cellular responses to tissue damage, since the enzyme transforms pro-oxidant intracellular heme into BV, the precursor of the antioxidant BR (24). Heme oxygenase-1 is ubiquitously distributed, but it is particularly abundant in reticuloendothelial organs, such as liver and spleen. In the central nervous system (CNS), HO-1 is present at low levels in sparse groups of neurons, including the dentate gyrus and the pyramidal neurons of CA1-CA3 areas of the hippocampus and the ventromedial and paraventricular nuclei of the hypothalamus (1,25). Heme oxygenase-1 is also found in cells of glial lineage, where its expression can be induced by oxidative stress (26). With regard to HO-2, this isoform is abundant in the brain, kidneys, and testes (1,25). In the brain, HO-2 is expressed in neuronal populations in the forebrain, hippocampus, hypothalamus, midbrain, basal ganglia, thalamus, cerebellum, and brainstem (1).

Among the three by-products of HO activity, CO received great attention as a gaseous neuromodulator. Several lines of evidence proposed HO-derived CO as a physiologic regulator of cognitive functions due to the ability of this gaseous compound to modulate hippocampal long-term potentiation (LTP) in the rat (27-29) (Table I) which represents a classical model of learning at the cellular level (30), being affected in several pathologic conditions (31-39).

Biliverdin reductase

The cytosolic BVR, is an enzyme unique in nature. This enzyme not only reduces the C10 (γ bridge) of BV thus generating BR, but it is also a serine/threonine/tyrosine kinase as well as a transcription factor involved in the regulation of various cellular functions (see below and Figure 2) (40-42). Its reductase activity is cofactor-dependent, and the cofactor itself is pH-specific (NADH at a pH of 6.8, NADPH at pH 8.7). For the activation, BVR also requires free thiols (43). Recently, it was reported that BVR's reductase activity requires the autophosphorylation of the enzyme on the specific site Ser^{149} (44). Through the reductase activity, BVR generates BR, a lipophilic molecule with strong antioxidant activity towards both ROS and RNS (2,3,8,9,11-13,45). A healthy adult produces almost 300 mg of BR each day (3). The BR formed within the cell is released and reaches the extravascular space and the bloodstream by passive diffusion or active transport (46). In the bloodstream, where concentrations normally range from 5 to 15 μM, BR is primarily bound to serum albumin, which carries it to the liver (3,10,46). Here, the BR dissociates from the albumin and enters the hepatocytes, where it is conjugated with glucuronic acid and excreted in the feces (46) .

Biliverdin reductase was initially considered a noninducible protein. Later studies showed, however, that BVR can be induced by LPS and bromobenzene at a post-

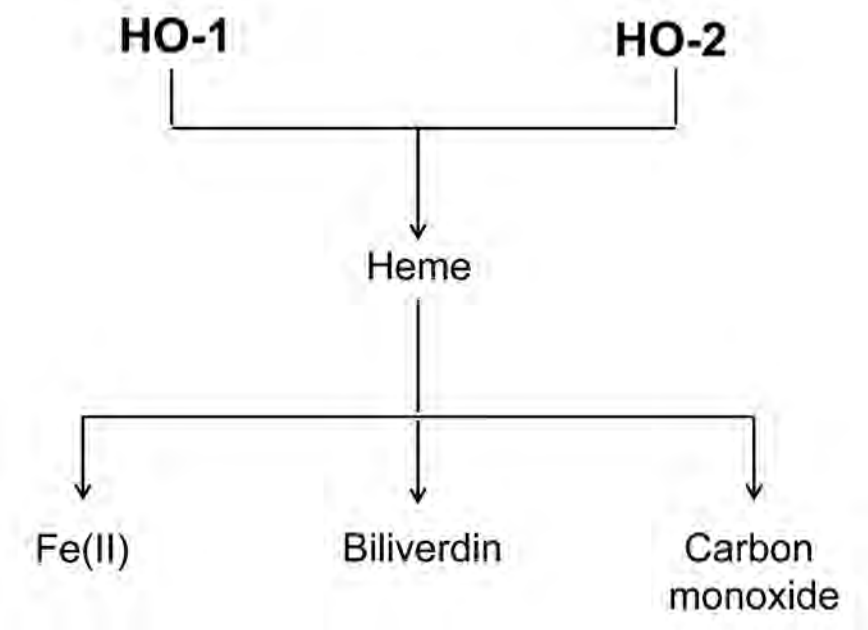

Fig. 1. *The heme oxygenase activity. Hemoprotein-derived heme moieties are transformed by either the inducible or constitutive isoforms of the microsomal enzyme heme oxygenase (HO) into equimolar amounts of ferrous iron (FeII), carbon monoxide and the linear tetrapyrrole biliverdin. HO-1, inducible isoform; HO-2, constitutive isoform.*

transcriptional level, but it is unaffected by heat shock (47,48). In rats, BVR activity increases progressively after birth and reaches adult levels by postpartum day 28 (49). Immunohistochemical studies have also revealed age-related BVR expression patterns in certain areas of the rat brain, such as the cortex, substantia nigra, hippocampus, and cerebellum (49). The enzyme is co-expressed with HO-1 and/or HO-2 in cells of the rat brain that express these enzymes under normal conditions. It is also found in regions and cell types that can express heat shock-inducible HO-1 (48). This histochemical evidence is corroborated by functional data demonstrating BVR's direct involvement in the regulation of HO-1 activity. In fact, during oxidative stress, activation of the HO-1/BVR axis causes increased heme degradation and accelerated transformation of BV to BR (42). The increasing BR levels produced by this activity eventually downregulate the reductase activity, producing a rise in BV levels, which, in turn, inhibits the oxygenase activity; this regulatory feedback loop restores heme degradation to normal levels (42).

Apart from the reductase activity, BVR plays a main role in the regulation of important cellular functions by interacting with members of the protein kinase C (PKC) family, the extracellular regulatory kinase 1/2 (ERK1/2), the PI3k/Akt pathway and the insulin receptor kinase-1 (IRK-1) (40,41). Biliverdin reductase was shown to activate the conventional PKCβII by phosphorylating the Thr^{500} and this is a prerequisite for the maturation of this latter (50). In addition, BVR activates both PKCβII and the atypical PKCζ and the novel PKCδ through protein-protein interactions (40). By interacting with PKC isoforms, BVR could have a role in breast cancer and tamoxifen resistance, in Parkinson's disease and type-2 diabetes mellitus (40). Biliverdin reductase is also a crucial component of MEK1-ERK1/2-Elk1 signaling. Biliverdin reductase functions as a scaffold protein for the activation of ERK by MEK1/2 and of Elk1 by ERK. This interaction is necessary because ERK1/2 is not able *per se* to localize the nucleus and, in order to do that, requires BVR which is endowed with both nuclear localization and nuclear export motifs (51). The first step of this process is the formation of a ternary complex constituted by BVR/MEK/ERK, which places ERK in a position that permits its activation by MEK (51). After that, the formation of this complex allows BVR to be phosphorylated by ERK (51). Once activated, the complex BVR-ERK is separated from MEK and translocates into the nucleus where it binds and activates Elk1, a transcription factor for the expression of oxidative stress-responsive genes such as *ho-1* or *inducible nitric oxide synthase (iNOS)* (41). Furthermore, BR at physiological concentrations (0.5-10 μM) and in the absence of neurotrophins, increased ERK1/2 phosphorylation with a NO/soluble guanylyl cyclase (sGC)-dependent mechanism in both PC12 and

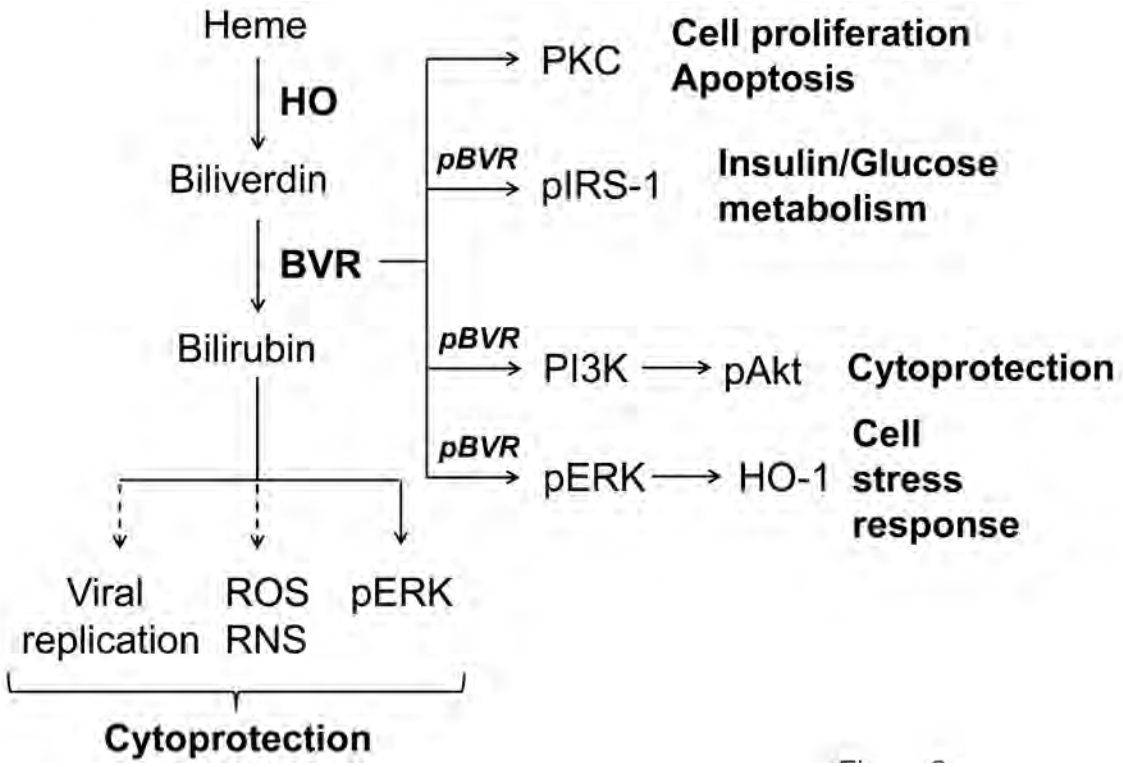

Fig. 2. *The pleiotropic activities of biliverdin reductase and its by-product bilirubin. Once formed through the heme oxygenase activity, biliverdin is then reduced by biliverdin reductase (BVR) into bilirubin. Biliverdin reductase exerts its pleiotropic effects by regulating several signalling pathways, such as those related to protein kinase-C (PKC), insulin receptor kinase 1/insulin receptor substrate 1 (IRK-1/IRS-1), phosphatidylinositide 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinases (MEK/MAPK/ERK). In addition, bilirubin was shown to be cytoprotective by inhibiting viral replication, scavenging both reactive oxygen and nitrogen species (ROS and RNS, respectively) and phosphorylating ERK. For further details see text. p, phosphorylated. Dashed arrows, inhibition.*

primary cultures of rat cerebellar granule cells. Further upstream, influx of extracellular calcium was necessary for neuronal NOS (nNOS) induction and NO release, likely through calcium-dependent phosphorylation of the transcription factor CREB. Importantly, the cascade elicited by BR through NO and ERK was cytoprotective, as revealed by exacerbated BR toxicity in cultures treated by either NOS or MEK inhibitors (52). Recently, BVR has also been shown to serve as a "shuttle" that drives heme into the nucleus, where it activates transcription of *ho-1* (53). Through the activation of ERK1/2, BVR is involved in cell proliferation, differentiation and division as well as in the stress response. The transcriptional activation of *ho-1* by BVR, together with the finding that HO-1 over-expression activates both PI3K and its downstream effector Akt in rodents (54), put forth the hypothesis of a direct role of BVR in the regulation of the PI3K/Akt system. It has been reported that (*i*) BVR co-immunoprecipitates with the p85 subunit of PI3K and (*ii*) the conversion of BV to BR *via* BVR, leads to tyrosine phosphorylation in the C-terminal domain of this enzyme which allows the binding of BVR to p85 thus activating PI3K and then Akt (40,54). By modulating the PI3K/Akt system, BVR is involved in one of the main mechanisms which regulates cell protection, and this seems to be quite important in the nervous system (55,56). Insulin signaling begins with the insulin receptor kinase(IRK)-1-mediated phosphorylation of tyrosine residues of insulin receptor substrates(IRS)-1/2 and finishes with the phosphorylation of serine/threonine residues. Specific Tyr residues of BVR, particularly Tyr^{198}, Tyr^{228} and Tyr^{291}, are substrates for IRK-1, and phosphorylated BVR serves as a Ser/Thr kinase for IRS-1, inhibiting the latter's phosphorylation by the insulin receptor. These processes represent a physiologic mechanism for increasing glucose uptake (41,57). Finally, BR blocked the replication of both type 1 herpes simplex virus and enterovirus with a mechanism related to the activation of the intracellular c-Jun N-terminal kinase

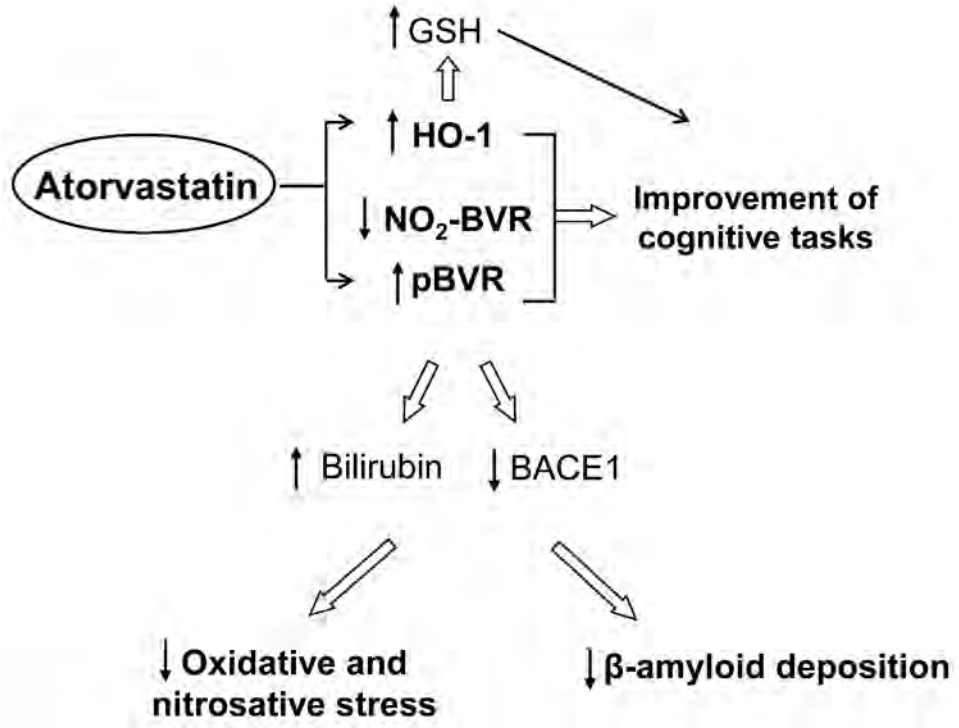

Fig. 3. *Modulation of the heme oxygenase-1/biliverdin reductase system by atorvastatin in the parietal cortex of aged canine. Atorvastatin (80 mg/day per os, 14.5 months) up-regulates heme oxygenase-1 (HO-1), decreases nitrosative and increases phosphorylative post-translational modifications of biliverdin reductase (BVR). As a consequence of these modifications on HO-1 and BVR, a significant improvement of cognitive tasks occurs. In addition, the up-regulation of HO-1 and the activation of BVR, secondary to its phosphorylation, reduce oxidative and nitrosative stress even through the increased production of the free radical scavenger bilirubin and reduced glutathione (GSH). Finally, BVR activation reduces β-secretase 1 (BACE1) protein levels thus implying a role for the reductase in lowering β-amyloid deposition.*

(JNK) and the increased production of NO (58).

THE HO/BVR SYSTEM IN ALZHEIMER'S DISEASE

Alzheimer's disease is a chronic neurodegenerative disorder characterized by progressive synaptic loss (59) cognitive dysfunction, memory impairment, inability to perform the activities of daily living, mood disorders and is considered as the leading form of dementia in the elderly. Generally speaking, about 24 million people suffered from dementia in 2001 worldwide and this figure was estimated to double in 2020 and quadruple in 2040 (60). From an epidemiologic point of view, the prevalence of AD was calculated about 1% in subjects aged 60-64 but increases up to 33% in people aged 85 or older, in the Western hemisphere (61). However, the annual incidence worldwide ranges from 1% to 7% at the ages of 70 and 85, respectively (62). Sporadic AD is the more common form of the disease, accounting for 90% of all cases, whereas only 1% accounts for the familial form (63). Most cases of sporadic AD are associated with the ε4 allele of apolipoprotein E (APOE), a plasma protein implicated in the transport of cholesterol that also binds amyloid-β-peptide (Aβ), whereas familial AD is an autosomal dominant disorder, whose early onset was associated with mutations in specific genes such as *amyloid-β precursor protein (APP), presenilin 1* and *presenilin 2* (63,64). According to the "amyloid cascade hypothesis" Aβ plays a main role in the onset and progression of AD. Aβ contains 36-43 amino acids and is produced by serial cleavage of the APP by β- and γ-secretases (65,66). Once formed, Aβ forms spontaneous aggregates in the form of oligomers or fibrils. The latter tend to form insoluble secondary structures which become the core of senile (or amyloid) plaques (65,66). β-amyloid oligomers and fibrils can be degraded by neurons through an ubiquitin-proteasome-dependent process known as the *unfolded protein response,* but when this measure is not

Table I. *Some of the main intracellular targets of CO*

Activation	**Main Outcome**
Soluble guanylyl cyclase	Smooth muscle cell relaxation Long term potentiation
Iberiotoxin-sensitive outward potassium channel	Regulation of vessel tone
Cyclooxygenase Prostacyclin	 Regulation of vessel tone
Mitochondrial biogenesis	Increase in mitochondria-derived ROS
Mitogen-activated protein kinases	Reduction in the inflammatory response
Inhibition	
NADPH oxidase	Inhibition of ROS production Antiproliferative effects
Neuropeptide release	Inhibition of the stress axis Activation of the gonadal axis

For further details about the above mentioned effects of CO, see refs. 2, 4, 5-8, 27-29.

sufficiently efficient, there is an excessive build-up of Aβ that can trigger the onset of AD (67,68). Another protein which is mainly involved in the pathogenesis of AD is tau, whose primary role is to maintain the integrity of the cytoskeleton. In AD, tau undergoes hyperphosphorylation by specific kinases such as GSK3β, cyclin-dependent kinase 5 (cdk5) and DYRK1A (69,70). Importantly, these kinases are activated by Aβ and regulated by the peptidyl prolyl cis-trans isomerase and this provides the link between Aβ formation and tau hyperphosphorylation (69,70). As a result, the hyperphosphorylated tau becomes insoluble, its affinity for the microtubules declines, and it forms aggregates with a double-helix secondary structure. As with Aβ, phosphorylated tau that is not efficiently degraded by the proteasome accumulates and exerts neurotoxic effects (71,72). As a consequence of both Aβ formation and tau hyperphosphorylation, the formation of ROS occurs. This is due to either the impairment of mitochondrial respiratory chain or the activation of enzymes such as NADPH oxidase (73-75). In addition to this, Aβ overproduction decreases also key enzymes involved in ROS detoxification, such as SOD-1 and SOD-2, thus leading to oxidative damage to the lipids and proteins of the neuron (76,77). Excess superoxide radical also reacts with NO produced by activated microglia, thereby enhancing the formation of peroxynitrite and other RNS implicated in protein nitration and neurotoxicity (78-80). The result of this excessive generation of free radicals is massive neuronal death that is particularly evident in the hippocampus, amygdala, and frontal cortex, a pattern that is consistent with the cognitive and memory deficits of AD-type dementia (81,82).

Taking into consideration the main role played by free radicals in the onset and development of neurodegeneration, the activation of intracellular pathways involved in the enhancement of cell stress response, such as the HO-1/BVR system, was proposed as an useful attempt of neural cells to counteract oxidative/nitrosative damage.

Panahian *et al*. (1999), by using transgenic (Tg) mice overexpressing HO-1 in neurons, demonstrated the neuroprotective effect of this enzyme in an experimental model of ischemic brain damage. When compared to non Tg, Tg mice exhibited significant neuroprotection with decreased dimensions of ischemic penumbra when examined at both 6 and 24 hr after induction of ischemia. The authors conclude that the neuroprotective effect of overexpressed HO-1 can be related to: (*i*) increase in both cGMP and bcl-2 levels in neurons; (*ii*) inactivation

of p53, a protein involved in promoting cell death; (*iii*) increase in antioxidant sources, as suggested by the strong reduction in the formation of lipid peroxidation products and (*iv*) increase in the iron sequestering protein, ferritin (83). In addition, in transfected neuroblastoma cells overexpressing HO-1, the activity of this enzyme was increased, and conversely, the level of tau protein was significantly decreased when compared to control (84,85). The suppression of tau protein expression was almost completely counteracted by zinc-deuteroporphyrin, a specific inhibitor of HO activity (84). Another possible mechanism through which HO activation could be useful for the AD brain is related to the ability of CO to induce LTP and improve synaptic plasticity. The inhibition of HO activity, and the following drop of CO production, significantly reduced LTP elicited by either a two-train or four-train tetanus in rat hippocampus (27). In the same experimental model, HO inhibitors blocked the *trans*-ACPD (mGlu agonist)-induced long-lasting potentiation (27). The above mentioned findings, suggest that HO-derived CO have a tonic role in the tetanus- or *trans*-ACPD- induced LTP in rat hippocampus (27). These results are consistent with previous studies which showed as CO production in the rat hippocampus is important for the early stages of memory processing of an inhibitory avoidance training (28). The cognitive effects of the HO/CO system, are restricted to the hippocampus, given that only the immediate post-training intrahippocampal infusion of zinc-protoporphyrin-IX (Zn-PP-IX, an HO inhibitor) caused amnesia for the habituation task, whereas the intra-amygdala infusion of this inhibitor did not have any effect on retention of the avoidance task in the rat (29). The sequel of HO and its by-product CO in rodent synaptic transmission seems to be age and species-specific. As shown by Vaccari et al. (86) and Mereu et al. (87), prenatal low-level exposure to CO (150 ppm from days 0-20 of pregnancy) produced a long-lasting decrease in both hippocampal HO and nNOS activities and disrupted LTP in this brain area in rat offspring. In addition, the rat seems to be the species in which CO regulates LTP because (*i*) hippocampal LTP was normal in HO-2-null mice and (*ii*) Zn-PP-IX, administered by intracerebroventricular route, did not affect either passive avoidance or spatial learnings in mice (88,89).

Although many evidence considers HO-1 an enzyme with cytoprotective function (2,21,24), other studies suggest that this enzyme can trigger neurodegeneration (90,91). Many studies reported on the excessive sequestration of redox-active iron as a characteristic feature of many neurodegenerative disorders, including AD (92), but the mechanisms responsible for this pathological iron sequestration were not extensively addressed. This last finding could be explained by keeping in mind that HO activity generates also Fe(II) which, under condition of redox unbalance, may trigger the formation of very toxic oxygen radicals which ultimately cause lipid peroxidation and cell death (93-95). Another possible mechanism to explain the neurotoxicity of HO-1 in AD brain is related to the downstream mitochondrial derangement, inflammatory cytokine release and the following cell death (91,92,96). In this frame, preclinical studies demonstrated as the administration of a blood-brain barrier-permeable HO-1 inhibitor ameliorates cognitive function in a transgenic mouse model of AD (96).

The above mentioned different roles claimed for HO-1 overexpression in AD, e.g. neuroprotective or neurotoxic, can be explained, at least in part, considering the different *in vitro* and/or *in vivo* approaches used in preclinical research. In order to try to reconcile these different views, the expression of HO-1 was evaluated in *post-mortem* samples of subjects with AD and mild cognitive impairment (MCI), this latter being the transitional stage between healthy aging and early AD. As shown by Barone et al. (97), HO-1 protein levels were up-regulated in the hippocampus of subjects with AD and MCI. At the same time, significant increases in Ser-residue phosphorylation, together with increased oxidative posttranslational modifications on HO-1, were found in the hippocampus of AD subjects (97). A similar behavior under pro-oxidant conditions was demonstrated by BVR. In specimens from AD and MCI subjects, BVR over-expression was increased in the hippocampus but underwent oxidative and nitrosative post-translational modifications (98) which were paralleled by a concomitant reduction of the phosphorylation of this enzyme on Ser/Thr/Tyr residues in this brain area (99). As a direct consequence of these post-translational modifications on HO-1 and BVR, a significant reduction in the production of BR in human hippocampus was observed (99). Furthermore, even the formation of BVR-ERK2 complex in AD and MCI hippocampi were reduced (99). These findings contributed to highlight that it is no longer correct to measure total HO-1 and BVR protein levels as indices to evaluate the involvement of these enzyme in the cell stress response since post-translational modifications, such as the phosphorylation of critical Ser/Thr/Tyr residues, play a main role in the regulation of the neuroprotective and/or metabolic activities of these enzymes.

It is noteworthy to mention that the oxidative/nitrosative post-translational modifications detected in the hippocampus were found in the plasma of AD and MCI subjects. In particular, a significant increase in nitrated BVR together with a decrease in phosphotyrosine-BVR were associated to a significant decrease in the reductase activity and cognitive function in such patients (100). This last finding proposed the HO-1/BVR system as a novel

peripheral biomarkers for the early diagnosis of AD.

CONCLUSIONS AND FUTURE DIRECTIONS.

Preclinical research generated impressive lines of evidence about the several intracellular mechanism(s) whose impairment lead to the onset and progression of AD, but, unfortunately, scientists were not able to translate these preclinical findings into clinical research (101).

The major classes of drugs currently available for the treatment of AD are acetylcholinesterase inhibitors or NMDA glutamate receptor antagonists (15,102,103). The former are used to increase synaptic levels of acetylcholine, which are reduced as a result of damage to cholinergic neurons in the amygdala, hippocampus, and frontal cortex, whereas the latter is used to prevent/reduce calcium-dependent excitotoxic neuronal cell death (15,104-106). Both acetylcholinesterase inhibitors and NMDA glutamate receptor antagonists produce some degree of improvement in the cognitive functions of patients with mild to moderate AD-like dementia, and the most marked effects are observed during the first year or so of treatment (107-109). An alternative to acetylcholinesterase inhibitors, other drugs that intervene in the pathogenesis of the disease, such as statins, are currently under the spotlight. Particularly interesting are the mechanism(s) through which statins could interfere with the development of AD which are independent of their ability to inhibit cholesterol synthesis. Among the many intracellular pathways regulated by statins (e.g. Rho-associated kinase, p21, SOD3, etc), the HO-1/BVR axis was one of those which were better investigated (110). The administration of atorvastatin (80 mg/day for 14.5 months) to aged dogs, resulted in the up-regulation of both HO-1 and BVR in the parietal cortex (111,112). Additionally, atorvastatin increased also the phosphorylation of BVR on Ser/Thr/Tyr residues (112). These atorvastatin-induced modifications on both HO-1 and BVR resulted in a significant increase in BR production and reduction in oxidative/nitrosative stress biomarkers in the parietal cortex as well as improved cognitive function of aged dogs (112) (Figure 3). Intriguingly, BVR up-regulation and post-translational modifications significantly correlated with β-secretase protein levels in the brain, suggesting a possible role for BVR in Aβ formation (112) (Figure 3). These preclinical findings contributed to unravel the HO-1/BVR system as a novel intracellular pathway involved in statins' neuroprotective function and to include HO-1/BVR as a potential target for newly developed anti-dementia drugs.

ACKNOWLEDGMENTS.

Authors are grateful to Prof. Paolo Preziosi MD FRC Path, Emeritus of Pharmacology at the Catholic University School of Medicine in Roma, for his helpful comments and suggestions. This work was supported by grants from the Catholic University (*Fondi Ateneo*) to Dr. Mancuso.

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INTRABODIES FOR PROTEIN INTERFERENCE IN ALZHEIMER'S DISEASE

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Several open questions call for new studies on pathogenic mechanisms leading to Alzheimer's Disease (AD), with the search for upstream drivers of the neurodegeneration cascade, such as neurotrophic deficits, early misfolding events of AD-related proteins (Aβ and tau) and understanding the multifactorial basis of AD pathogenesis. Since seminal immunosympathectomy experiment 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. Here, we describe a technology, pioneered by our group, based on recombinant antibody domains exploited as intracellular antibodies (intrabodies) whereby antibodies are used as genes, rather than as proteins. We discuss several applications and new promising developments of the intrabody approach for protein interference, especially in the field of AD research.

Despite intensive research, no generally accepted mechanism has yet been formulated causally linking the Alzheimer's disease (AD) triad (cholinergic deficit, amyloid-β and tau pathologies) into one unified conceptual scheme. Even though intensive research efforts, in the past two decades, there are still no effective treatments in sight to prevent, halt or reverse AD (1, 2) and the industry pipeline for drug development do not provide yet clear prospects for the future. Furthermore, the current lack of accepted biomarkers for early diagnosis represents one major problem (3, 4). These open questions call for new studies on pathogenic mechanisms leading to AD, with the search for upstream drivers of the neurodegeneration cascade, such as neurotrophic deficits (5), early misfolding events of AD-related proteins (Aβ and tau) (6) and the focus on the multifactorial basis of AD pathogenesis (1, 7, 8).

ANTIBODIES FOR TARGET VALIDATION IN 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 so for neurodegenerative diseases. Even two targets whose relevance for AD is robust and unquestionable, namely the Aβ 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 (4), 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?

One of the major obstacles in the field is the lack of techniques to reliably identify and target the assembly state of misfolded forms of Aβ and tau. In particular, soluble non-fibrillar oligomeric assemblies of Aβ are recognized as the most neurotoxic species but they are still mysterious entities in terms of size, structure and actions (9, 10).

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

Key words: Intrabodies, Alzheimer's Disease, protein interference, Amyloid-beta, tau

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0393-974X (2013)

context and subcellular location, the network of protein interaction partners, the post-translational modifications of the protein target itself etc. There is, therefore, the need for new approaches for target discovery and validation, particularly in the AD 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-based 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 post-translationally, from the same gene or mRNA, is huge. For this reason, unraveling the complexity of what really is a disease target and fully validating of 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 (11-13), 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 exploited as intracellular antibodies (intrabodies) - the so called intrabody technology - whereby antibodies are used as genes, rather than as proteins. The intrabody technology allows interfering with a protein target with a high spatio-temporal precision.

NEUROANTIBODIES: PHENOTYPIC KNOCK-OUT IN THE NERVOUS SYSTEM

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 (14)). 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 (15), 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 (16). The neuroantibody approach was instrumental to derive the AD11 mouse model, in which the postnatal expression of the anti NGF recombinant antibody aD11 in transgenic mice determines a progressive AD-related neurodegeneration, characterized by cholinergic deficit, tau and amyloid related pathology, synaptic plasticity and behavioural deficits (7, 17). 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 Aβ and tau endpoints, in the neurodegeneration cascade. More specifically, the aD11 antiNGF antibody binds mature NGF with an affinity 2000-fold higher than proNGF (18, 19). Thus, the aD11 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 (5, 19), experimentally creating a proNGF to NGF imbalance. This has allowed validating proNGF/NGF disequilibrium 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 (5). 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 phenoptype of the AD11 model (20). 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 (19), 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.

THE INTRACELLULAR ANTIBODY (INTRABODY) APPROACH

Having established that antibodies could be ectopically

expressed, as secreted proteins (15), we extended the "antibody protein silencing" concept to the intracellular targeting of antibodies to different compartments of mammalian cells (21, 22). 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 (21) (Fig.1). 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 (14). 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 (Fig.1, inset). 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 (23).

In the two decades following the first description of the use of intrabodies in mammalian cells (22), and following the initial proof of concept functional studies by us and others (24-26) several examples of intracellular antibodies effectively inhibiting the function of intracellular targets have been published (recent reviews in (27); (28); (29)), 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 ((30); (31)).

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 small 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 (32) by a labour intensive cloning of the antibody VH and VL domains into the scFv format. With the advent of phage-display technology (33, 34), 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 (35). 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 (36). Conversely ribosome display antibody libraries have been used for isolating antibody domains that are stable under reducing conditions (37). 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 (38, 39). The two hybrid method (40) 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 (38, 39, 41) (IAC or Intracellular antibody capture technology). The initial IAC method required a first round of selection of scFv from phage display antibody libraries (38, 41), but was superseded by methods allowing the direct library screening in yeast cells, expressing synthetic scFv libraries made from intracellular stable consensus scFv frameworks (38, 42), from natural immunoglobulins (43) or from immunized mice (44). These "single-pot libraries of intrabodies" (SPLINT) (43) 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.2). 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 Alzheimer's proteins microtubule associated protein tau (38), and Amyloid beta peptide (44), the proNGF precursor of NGF (45), the synaptic protein gephyrin (46), the cancer related proteins RAS (42) and transcription factor LMO2 (47). 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 (Fig.2). 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 (48). 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.

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 (49). 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 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 tanslationally 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.3). 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 3). 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 (50)). 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 (30), an important development has been the design of approaches to accelerate the specific isolation of antibodies directed against protein interaction sites (51). 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) (30). In a more direct and general approach (Fig 2), 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 (51). In the so-called 3-SPLINT approach (Fig 2), 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 transctiption 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 antibodies do not require such immune effector functions, and, also for this reason, do not carry constant immunoglobulin regions. Past work with chimaeric antibodies (52) 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 (14)). 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 (intrabody-mediated apoptosis) (53) or proteolysis of the target protein (suicide (or silencing) intrabody technology (SIT) (54) or its ER-associated degradation (55). Also a chromophore or fluorophore assisted light inactivation (CALI-FALI) of the recognized antigen could be a potential new development for intrabodies.

The "intrabody-mediated apoptosis" strategy is based on the fusion of pro-caspase to a single domain intrabody and its proximity-induced dimerization and activation (Fig.4). 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 (53). This approach might be particularly useful with fusion oncogenes, such as those occurring in cancer, or with oligomeric antigens,

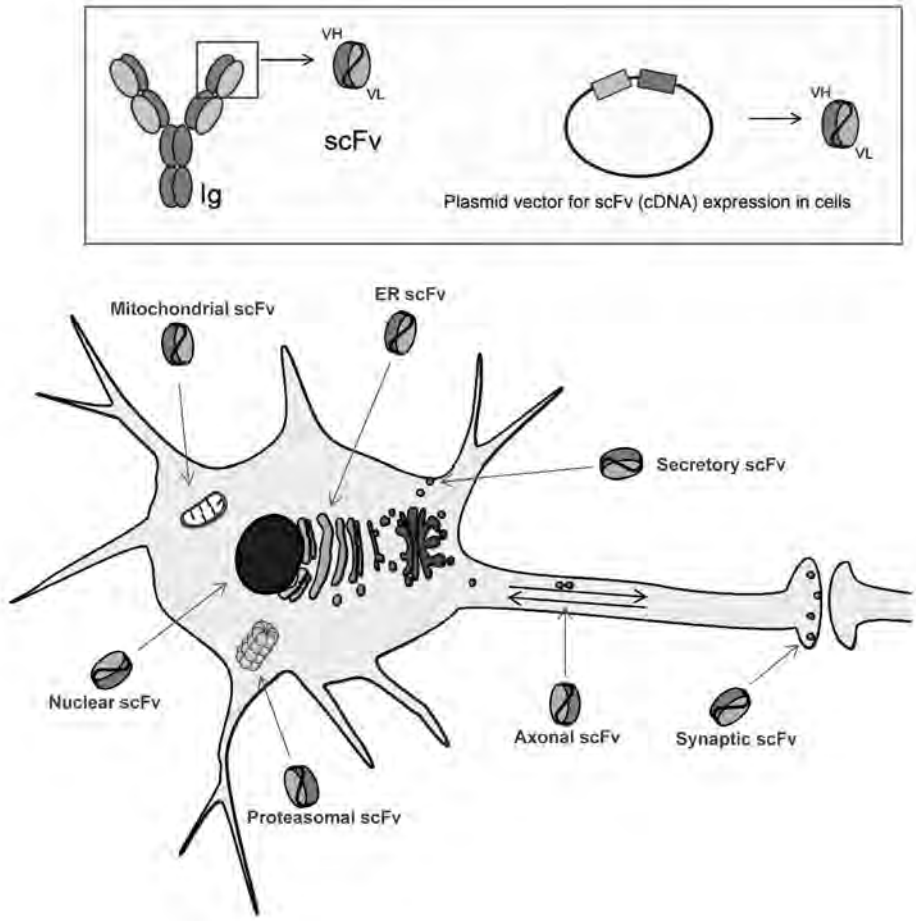

Fig. 1. *The intracellular antibody (intrabody) approach is a gene-based strategy that relies on the ectopic expression of recombinant antibodies in form of single chain variable fragment (scFv), consisting of a heavy chain (VH) and a light chain (VL) variable region linked by a flexible linker peptide (inset). By exploiting targeting sequences that normally direct the subcellular localization of proteins inside the cell, intrabodies can be expressed as genes through expression vectors (see inset) and targeted to a number of cellular compartments, including the endoplasmic reticulum (ER), nucleus, mitochondria, proteasome, axon, synaptic vesicles, secretory pathway, to locally block or modulate the function of target molecules (90, 91).*

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). Cellular proteolysis is tightly regulated process, carried out through the ubiquitin/proteasome pathway (UPP). In the approach called SIT (suicide or silencing intrabody technology), we harness the cellular machinery and signalling that regulates proteolysis to mediate degradation of cellular proteins, upon intrabody binding. An antigen-specific intracellular antibody is expressed in cells as a fusion with a known UPP substrate, IkBα, which undergoes stimulus-induced degradation. The stimulus – extracellular ligand that is used in this approach is TNFα. The intracellularly expressed scFv is non-neutralizing, and the function of the target protein is not inhibited in the absence of TNFα. Upon activation of the degradation pathway by the addition of TNFα as extracellular ligand, the complex between the intracellular antibody and its target protein will be recruited to the E2/E3 ubiquitination complex via the NF-kB signaling pathway that leads to IkBα degradation (54) (Fig 4). This protein switch for degradation provides a unique tool for rapid and reversible protein silencing on a fast time scale (15-30 min). This 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 that enter the secretory pathway, by exploiting the endoplasmic reticulum associated degradation (ERAD). ERAD is a cellular quality control mechanism that is activated in case of aberrantly folded protein in the ER.

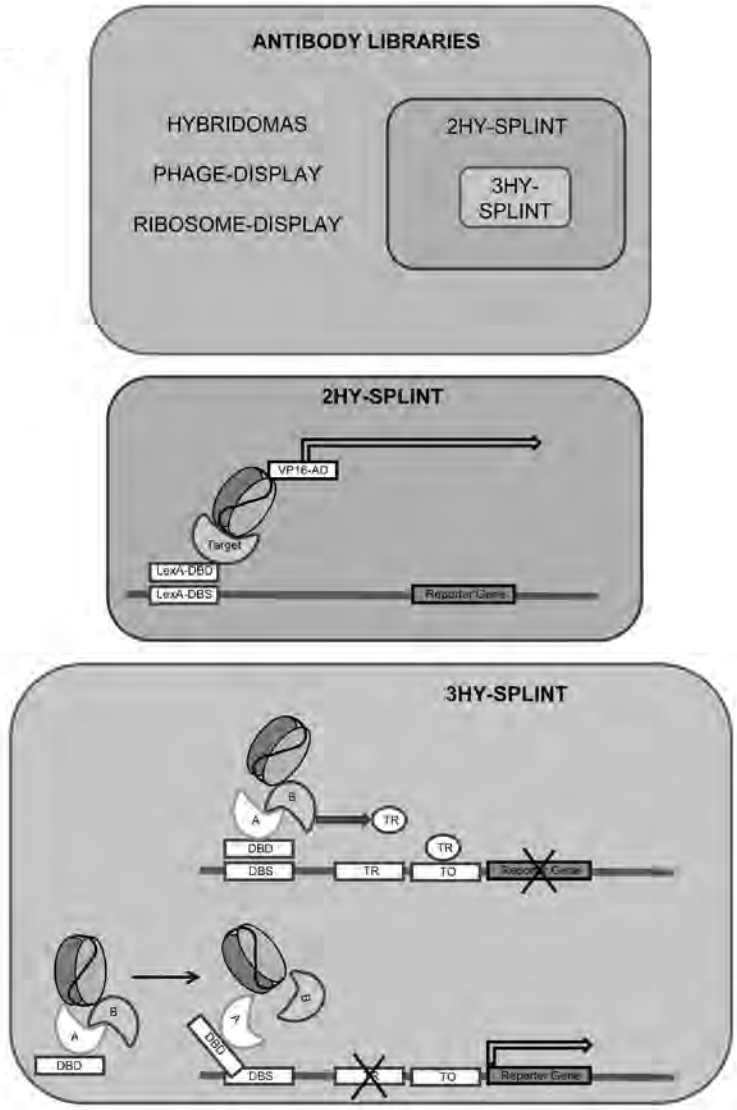

Fig. 2. *A subset of stable and functional antibodies, can be isolated and selected from the SPLINT (single-pot libraries of intrabodies). Two hybrid (2HY-SPLINT) libraries provide antibodies that bind a given target antigen bait (38), but do not ensure that the antibody will be neutralizing. In the SPLINT format, neutralization is a property that can be verified a posteriori, after the selection, or can be added by suitable effector functions. Intrinsically neutralizing, stable antibodies are a subset of 2HY-SPLINT derived antibodies. An important class of intrinsically neutralizing intrabodies is represented by those intrabodies that inhibit protein-protein interactions, that can be selected through three hybrid approach (3HY-SPLINT) (43, 92).*

When the local, ER, quality control (additional folding cycles activation) fails to correct protein misfolding, the ERAD pathway is activated. Secretory proteins that fail to reach their final folding state, become recognized as ERAD substrates, afterwhat are retro-translocated to the cytosol, ubiqutinilated and subsequently degradated by proteasome (56). HRD1 is the ubiquitin ligase involved in this mechanism. It interacts with SEL1L protein. A SEL1L is an ER resident protein, that has the adaptor role in the ERAD mechanism, by interacting with substrate recruitment proteins and HRD1. In order to force ER associated degradation of specific targets (such as misfolding proteins), fusion molecules called degradins were generated (55), by fusing a target-specific intrabody with the luminal SEL1L moiety, it is possible to promote intrabody-mediated degradation of target proteins in the

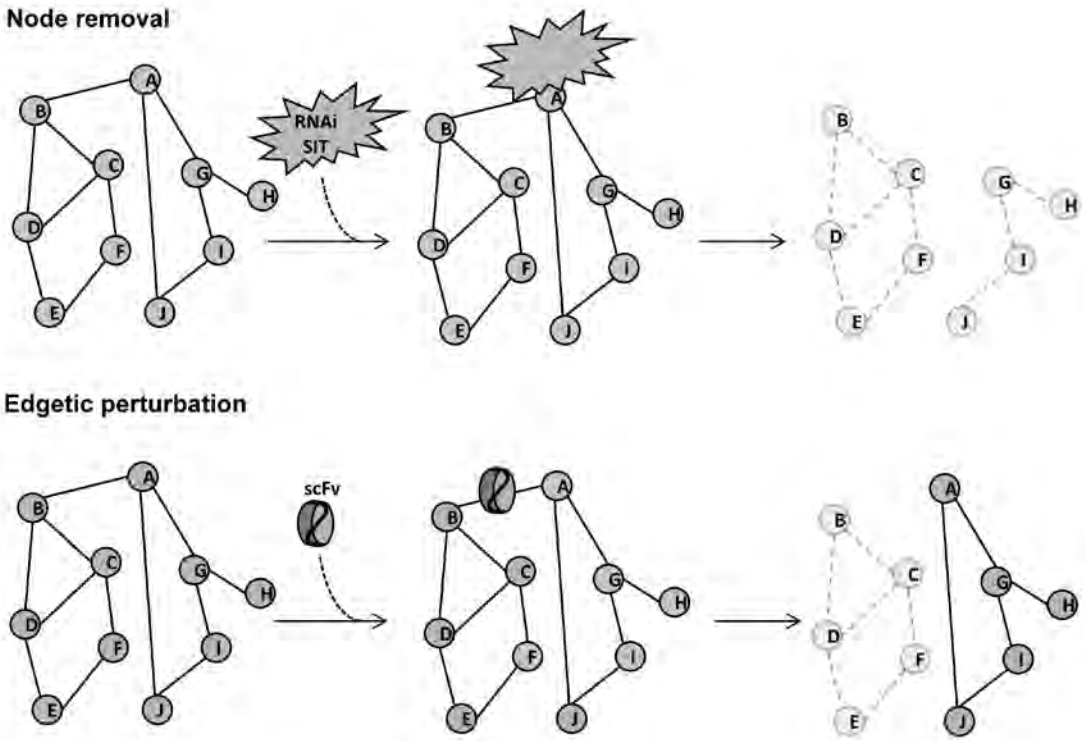

Fig. 3. *Schematic illustration of distinct outcomes in a protein network from complete loss of gene product (node removal) versus perturbation of specific molecular interactions (edgetic perturbation). Intrabodies allow edgetic perturbations, while gene- or mRNA-centered silencing approaches determine node removal.*

secretory pathway (Fig 4).

A potential development of coupled effector functions for intrabodies can exploit a chromophore or fluorophore assisted light inactivation (CALI-FALI) of the recognized antigen, in particular by combining the intrabody technology and CALI-FALI approaches, in their "genetically encoded" version (Fig.4).

Chromophore-assisted light inactivation (CALI) is a technique that allows acute, spatially and temporally controlled and localized protein inactivation. Most CALI experiments have been achieved by the laser excitation of chemically conjugated malachite-green/antibody complexes, that are targeted to a protein of interest, upon microinjection in cells (57, 58) Strong illumination of the chromophore generates short-lived reactive oxygen species, especially singlet oxygen, that can inactivate proteins in the immediate vicinity of the chromophore. More recently, it has been found that fluorescein is a more efficient photosensitizer, as a CALI reagent (59, 60). It has been estimated that the destructive effects of CALI have a half-maximal inactivation radius of 30-40 Angstrom, allowing specific target protein inactivation on the scale of protein-protein interaction. A significant improvement on the technology was introduced recently by R. Tsien and his group, who developed the tetracysteine-biarsenical dual system (61), which requires modification of the target protein by a 12-residue peptide sequence that includes four cysteines (the tetracysteine tag TC), which binds membrane-permeable biarsenical molecules, notably the green and red dyes "FlAsH" and "ReAsH", with picomolar affinity. A biarsenical fluorophore has been used to photoinactivate TC-tagged synaptotagmin I, which replaced the native synaptic protein synaptotagmin in vivo in Drosophila neurons (62). The TC motif has undergone multiple rounds of improvement to increase its affinity for the biarsenical dyes, enabling lower dye concentrations and easier wash-out of the unbound dye (63), making the CALI approach genetically encodable and non invasive. Biarsenical labelling and TC tagging has proven effective, in addition to the fluorophore-assisted laser inactivation of a number of different proteins, including that of newly and locally synthetized dendritic AMPA receptors in cultured neurons (64), for cotranslational detection of protein synthesis, for pulse chase experiments in vivo

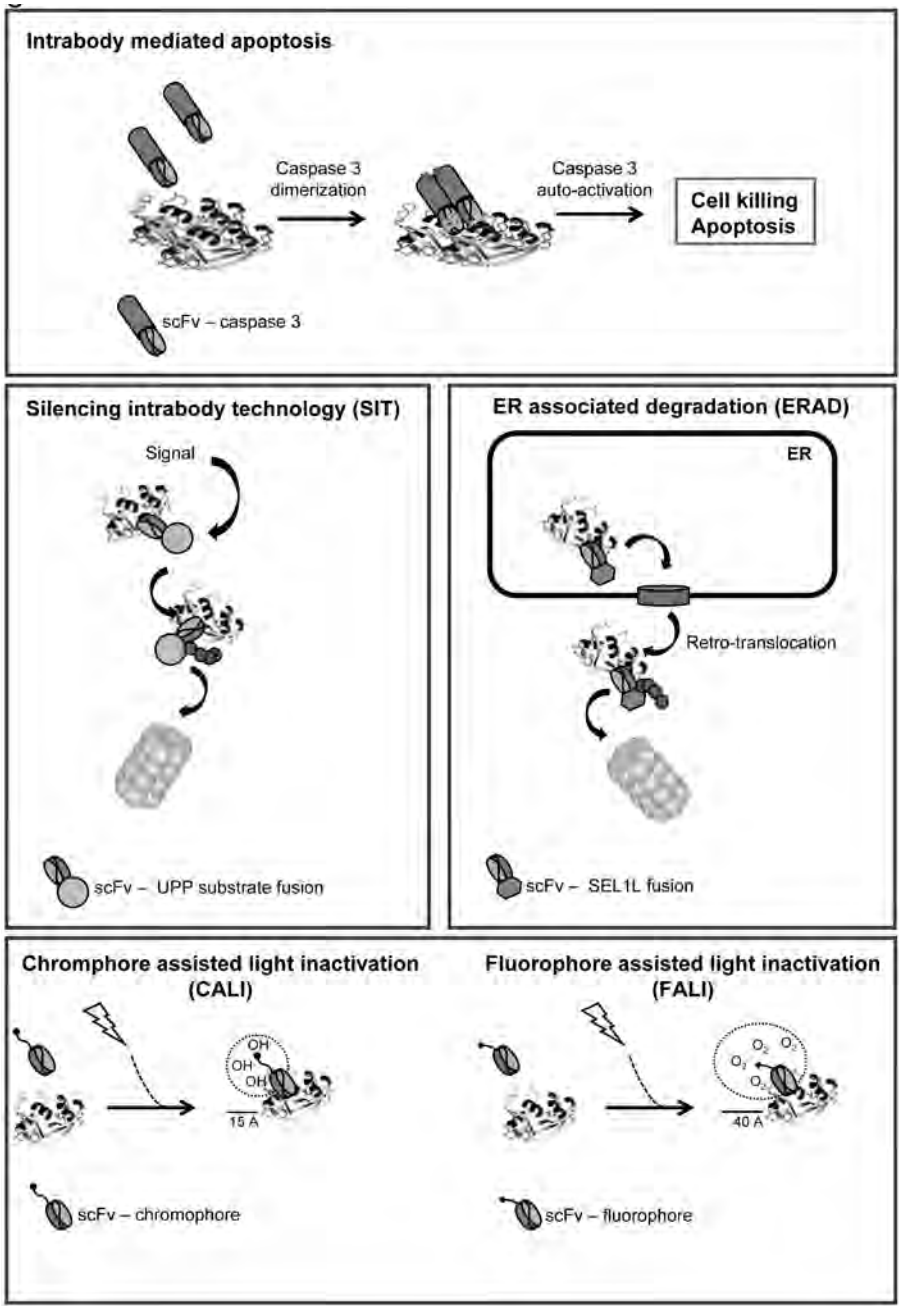

Fig. 4. *Intrabodies with effector functions interfere with cellular proteins by inducing protein knockdown or silencing. Intrabody fused to caspase 3 cause the induction of cell death upon antigen binding (antibody-antigen interaction-dependent apoptosis (53). Suicide (or silencing) intrabody technology (SIT) (54) allows the ligand-induced proteolysis of the target protein. The Endoplasmic Reticulum associated degradation (ERAD) mechanism is exploited to final proteosome-dependent degradation of proteins retro-translocated from the ER (55). Fusing intrabodies to chromophore or fluorophore could allow an antigen assisted light inactivation (CALI or FALI) of target proteins.*

and for monitoring a number of real-time processes in the cell (65). The most intrinsic limitation of CALI mediated by biarsenical staining of TC motif tags is that only exogenous tagged proteins are the direct target of inactivation. To target endogenous proteins for this local and controlled mode of inactivation, by combining

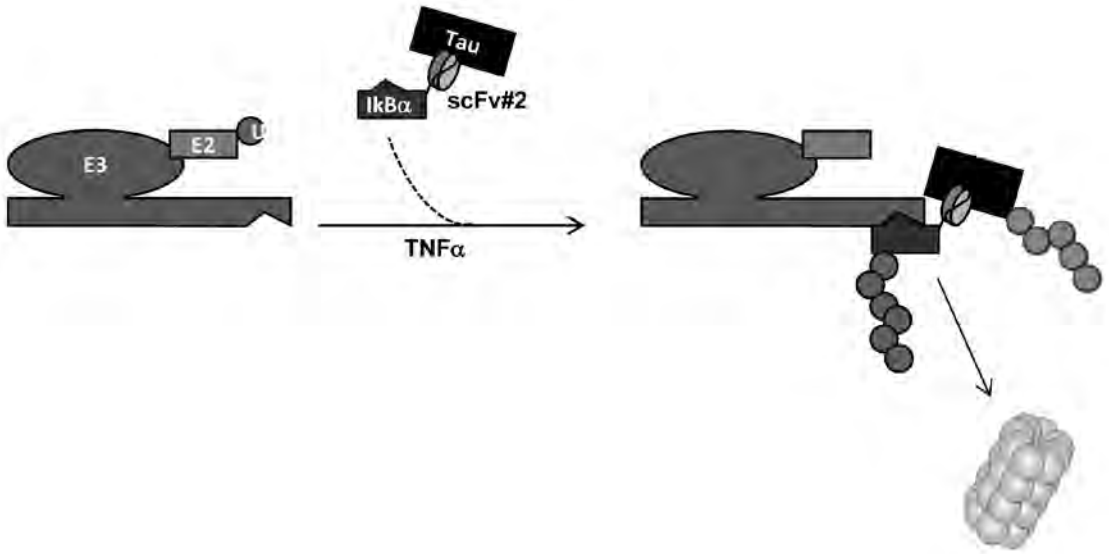

Fig. 5. I*n suicide (or silencing) intrabody technology (SIT) the protein silencing with intrabodies is targeted to degradation by engineering an antigen-specific intrabody (anti-tau scFv#2) (38) as a fusion with a known ubiquitin-proteasome pathway (UPP) substrate (IkBa), activated by an extracellular ligand (TNFa) through interaction with a membrane receptor* (54).

the intrabody technology and CALI approaches, in their "genetically encoded" version, using tetracystein-tagged intrabodies as acceptors for biarsenical dyes.

All above described intrabody targeting with effector functions could be turned into regulated neutralizing antibodies by exploiting the tetracycline-inducible expression system (66) obtaining in a such way locally and temporally regulated target neutralization.

INTRABODIES FOR ALZHEIMER'S DISEASE AND OTHER NEURODEGENERATIVE DISEASES

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 (Aβ) 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 therapeutical 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 (67); (68)).

The microtubule-associated protein Tau, together with amyloid-β, are two protein that undergoes misfolding, crucially involved in Alzheimer's Disease. Both misfolded proteins, Aβ and Tau, tend to aggregate, thus initiating the formation of major histological hallmarks of the disease, senile plaques and neurofibrillary tangles, respectively. Even if the aggregation processes are still elusive, Aβ and Tau are considered to be very valid targetas important in AD pathology. For this reasons corresponding intrabodies selection against these AD relevant targets have been done (38, 44, 54).

Anti-Tau and suicide intrabody technology (SIT)

We selected panel of 17 different anti-Tau intracellular antibodies (ICAbs) by intracellular antibody capture technology (IACT) (38). Recently, a representative anti-Tau intrabody (scFv#2) was further engineered in the form of ″switchable suicide intrabody″ by exploiting silencing intrabody technology (SIT) (54) (Fig.5). This neutralizing

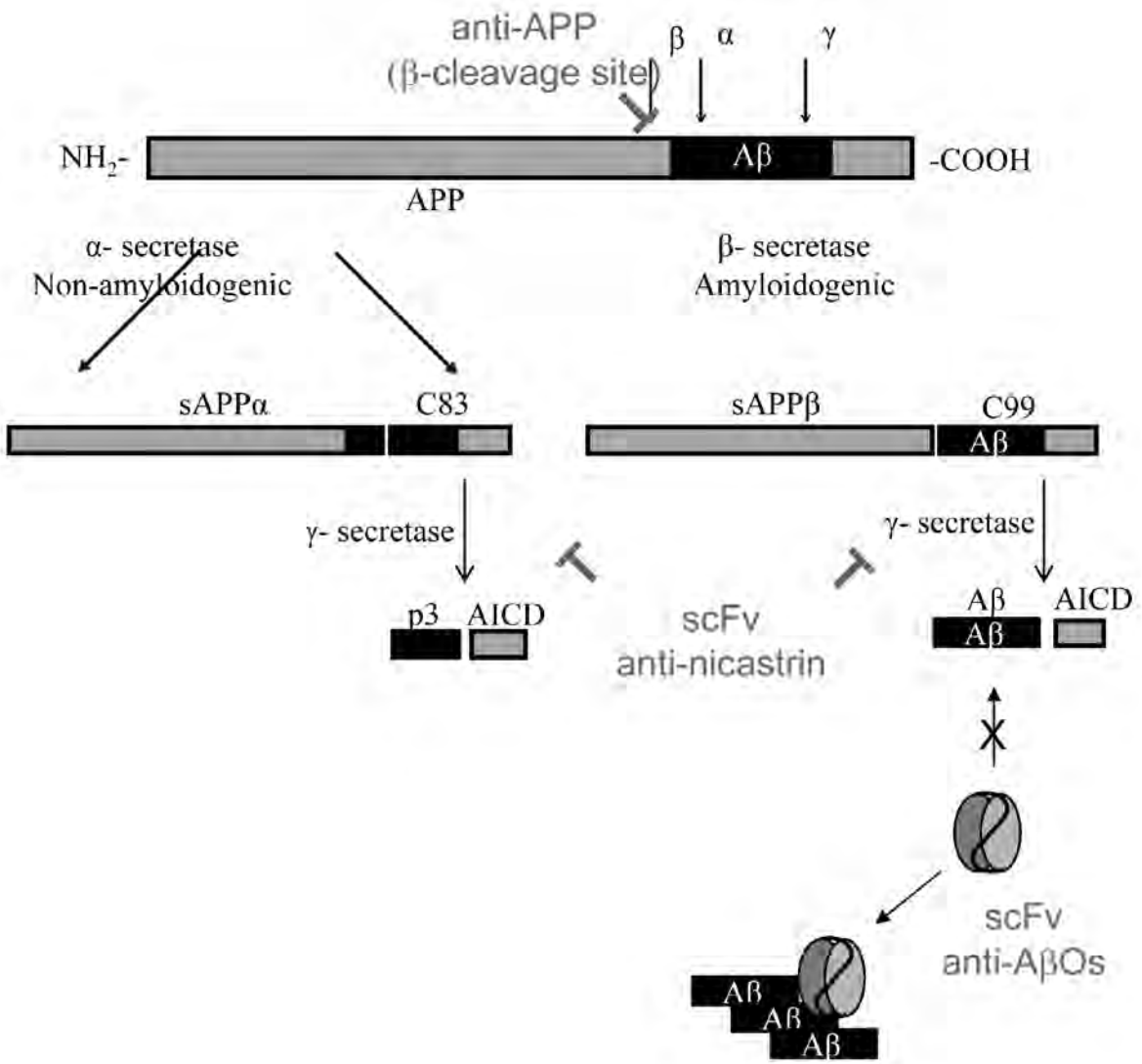

Fig. 6. *The Aβ peptide derives from proteolytic processing of its precursor APP (amyloid-β precursor protein), via sequential scission by the enzymes β- and g-secretases (amyloidogenic pathway). An alternative α-secretase pathway is considered to be non-amyloidogenic, since α-secretase cuts APP in the middle of Aβ sequence. Monomeric Aβ undergo subsequent oligomerization. Representative intrabody targeting of the Aβ/APP system raised against the APP β-cleavage site (72) or against nicastrin (component of the g-secretase complex) (73). The SPLINT-derived anti-AβOs scFvs (44) can be exploited for a selective conformational targeting of AβOs, but not of Aβ monomers.*

anti-TAU intrabody was attempted by fusing the inducible ubiquitin proteasome pathway (UPP) substrate IkBα to the C terminus of scFv#2. The chimeric complex scFv#2- IkBα was fristly co-expressed in HeLa cells with C-terminal domain of TAU protein (residues 151-422, $TAU_{151-422}$). Under cell stimulation with TNFα (ligand for IkBα degradation) (69), HeLa cotransfected cells showed reduction of $TAU_{151-422}$ protein. The maximal reduction of 70 % was obtained after 30 min of TNFα administration. The observed decrease in TAU protein level was limitted and specific to TAU only, because the protein that bind TAU (such as tubulin) remained inaffected. The TNFα-treatment of HeLa cells expressing $TAU_{151-422}$ protein alone (without scFv#2- IkBα) does not affect its level. This was the first demonstration of ligand induced neutralizing antibody, that is able to eefficiently silence TAU protein by specific intrabody-mediated degradation. This was also demonstrated to be functional in the case of endogenous TAU in human neuroblastoma cells (SHSY-5Y). The TNFα-treatment of SHSY-5Y dramatically decreased the stedy-state level of TAU in suicide anti TAU intrabody (scfv#2- IkBα) expressing cells (54). 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.

TARGETING THE AMYLOID-BETA/APP SYSTEM THROUGH INTRABODIES

There is indeed a great need to understand the cell biology and trafficking of Aβ precursor protein (APP), in relation to the cellular site(s) and timing of its processing to Aβ (70, 71) and its oligomerization. Indeed, while Aβ oligomers (AβOs) have recently been recognized as the main toxic Aβ assemblies in AD, they are still mysterious entities (9, 10) and almost nothing is known about the cellular sites and mechanisms of the oligomerization of Aβ. This is largely because no convincing AβO-specific probe has been generated yet, selectively recognizing specific, biologically relevant oligomeric forms of Aβ, with respect to Aβ monomeric or fibrillar forms. For this reason, we have decided to focus our efforts on the generation of AβO-specific recombinant antibody domains (44), to be used as intrabodies to selectively target AβOs in different subcellular compartments.

Aβ is generated by a complex proteolytic processing of APP, through sequential cleavages by β-secretase and γ-secretase (Fig.6). 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 specific cell biology methods, coupled to specific molecular probes. Thus, intrabody-based interference selective for Aβ or some of its pathological assemblies should be an extremely powerful approach.

Among the intrabody studies in AD research, both the Aβ peptide and its precursor (APP) have been targeted with intracellular antibodies (Fig.6). Paganetti et al (72) generated intrabodies directed to the the β-secretase cleavage site of human APP (Fig.6). Intracellular expression of scFv intrabody along the secretory pathway of human embryonic kidney cells shields the ß-secretase cleavage site and inhibits the formation of toxic Aß. 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 Aβ processing product.

An independent targeting of APP processing was obtained by the expression of an anti-nicastrin scFv intrabody (Fig.6); this abolished the proteolytic activity on APP, by the destabilization of the γ-secretase complex and the inappropriate glycosylation of nicastrin (73).

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 AβOs, the toxic forms of Aβ.

In vivo intrabody approaches directly targeting Aβ (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 (74-76) or ER-retained (77, 78) anti-Aβ 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-Aβ antibodies can recognize the Aβ sequence also inside APP and APP fragments, and the studies need to be interpreted taking this APP binding into account, unless specifically addressed, which is rarely the case.

Conformation-sensitive antibody domains targeting Alzheimer's Aß oligomers

Aβ oligomers (AßOs), are considered the most synaptotoxic Aβ species linked to the AD pathogenesis. Although increasing evidence supports the role of intracellular Aβ oligomerization and accumulation, as an early event in AD pathogenesis (79), little is known about the intracellular processing and trafficking events of the different forms of AβOs. Targeting the pathological assemblies of Aβ with specific probes, for mechanistic studies, for intracellular imaging or for therapeutic purposes, is therefore very important (10). Moreover, the intracellular targeting of AβOs would require the availability of antibody domains suitable for intracellular expression.

We recently generated a large panel of anti-AßOs recombinant scFv antibodies (44), exploiting the "Intracellular Antibody Capture Technology" (IACT). To this attempt, a human Aβ1-42 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 (43), and an Aβ1-42 immune SPLINT library, derived from V regions isolated from Aβ-immunized mice.

The selected anti-AβOs scFvs show unique properties

in terms of sequence, epitope recognition, conformational selectivity for Aβ oligomers in vitro, immunoreactivity towards naturally-produced Aß deposits in AD brains, inhibition of synaptic binding of Aß oligomers and neutralization of their-induced cyto-toxicity (44).

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

The panel of anti-AβOs 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 (44, 80, 81) and neutralization assays in cells (44, 82).

The conformation specificity of anti-Aβ antibodies is most often not associated with sequence specificity for the epitope recognized on Aβ (83-85) and the coexistence of conformation sensitivity, together with sequence specificity is a relatively rare property of anti-Aβ antibodies. For immunotherapy applications, the sequence specificity of anti-Aβ antibodies, is an essential property to be considered, besides their conformation specificity, due to in vivo mechanistic and safety reasons. Indeed, the AβOs scFvs were mentioned as new potential tools of study (10) and for new generation Aß immunotherapies (86). Moreover, as intrabody domains the anti-AβOs 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 AβOs formation and actions in cellular models, by using conformational anti-AβOs scFvs as intracellular antibodies (intrabodies) (Fig.6). Remarkably, the anti-AβOs scFvs show the peculiar conformation selectivity for AβOs even when expressed as intrabodies (44), essential prerequisite for in vivo applications. This provides the unique opportunity to study the detailed genesis and traffic of AβOs in living cells.

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 aproaches 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 (87-89), the availability of recombinant antibodies against TAU (38, 54) and AßOs (44) will allow their expression in the nervous system of transgenic animals (16) or with viral vectors, the best approach to test the feasibility and efficacy of therapeutic approaches based on Tau or AβO vaccination.

Following the initial proof of concept studies in the early (14, 22), 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 s, prions 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.

ACKNOWLEDGEMENTS

This work was supported by grants from FIRB MIUR

(RBAP10L8TY), Fondazione Roma to A.C. and from Alzheimer's Association (NIRG-12-237751) to G.M.

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Cell Death Differ 2013; doi: 10.1038/cdd.2013.22

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POTENTIAL NEURODEGENERATIVE EFFECT OF ANABOLIC ANDROGENIC STEROID ABUSE

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Anabolic androgenic steroids (AASs) are synthetic androgen-like compounds which are abused in sport communities despite their side effects. AAS abuse has been coupled with several medical complications, such as sterility, gynecomastia, and increased risk of cardiovascular and hepatic diseases. More recently, it has been observed that non-medical use of these steroids is frequently associated with changes in mood as well as cognitive deficits. Although the nature of this association is still largely unexplored, recent animal studies have shown the neurodegenerative potential of these compounds ranging from neurotrophin unbalance to increased neuronal susceptibility to apoptotic stimuli. Hence, exposure to AASs may result in a compromised brain, more susceptible, later in life, to the onset or progression of diseases not usually linked to drug abuse, especially neurodegenerative diseases.

The term anabolic androgenic steroids (AASs) indicates a group of synthetic compounds derived by selective chemical manipulations of the 19-carbon testosterone molecule. These modifications affect the pharmacokinetics of the resulting molecule (e.g., orally active compounds), as well as the ratio of the anabolic/androgenic effect. Clinically, AASs have been used to treat a variety of conditions characterized by profound body wasting, such as in protein-calorie malnutrition with associated weight loss or in the HIV-wasting syndrome (1), and to counteract pathological conditions characterized by low amount of testosterone (e.g., delayed puberty or some type of impotence) (2).

Although body builders and athletes of both sexes seeking to enhance their performance have often abused AASs, now individuals use these steroids without any athletic ambition with the only purpose of "body image drugs" (3). Moreover, this public-health issue, which nowadays includes children (4), is influenced by the relatively easy supply of these drugs through the web (5). As reviewed by van Amsterdam and colleagues, AASs abuse induces several side effects, such as sterility, gynecomastia, and increased risk of cardiovascular and hepatic disease (2). Although the severity of these negative effects depends on the specific steroid, the dose and the duration of exposure (6), it is notable that therapeutic doses of AASs as those used to treat hypogonadism have been linked to a higher cardiovascular event rate (7).

The earliest reports on the toxic effects of AASs on hepatic and endocrine functions have been published in the late '70s, while one decade later the first review covering the potential neuropsychiatry deleterious impact of AASs abuse appeared (8). In 1993 Su and colleagues demonstrated in male volunteers that exposure to methyltestosterone induced negative mood and cognitive impairment (9). On the one hand, investigations of the neuropsychiatric effects of AASs have been hampered by several methodological issues (e.g., lack of placebo control, concomitant drug coadministration, etc.), on the other hand, these initial reports have stimulated experimental researches aimed at elucidating the biochemical effects induced by AASs. Although several evidences demonstrate that behavioral disturbances of AASs abuse are recapitulated in animal models, only recent experimental studies have focused on the neurodegenerative potential of these compounds. After a brief examination of the mechanism of action of AASs, here, we review these recent studies and highlight the possible neuronal mechanism behind AASs-induced

Key words: Anabolic androgenic steroids, neurodegenerative diseases, Drug of abuse, Nerve growth factor.

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0393-974X (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

neuropsychiatric symptoms in humans.

Mechanism of action

Anabolic steroids exert their biological effects on several different cellular mechanisms including proliferation, differentiation and homeostasis. The classical genomic effect of androgens presumes that steroid hormones can freely cross the plasma membrane and reach the cytoplasm where they can interact with specific intracellular steroid receptor proteins, the androgen receptor (AR). The bound steroid receptors act as transcription factors and bind as homodimers or heterodimers to specific DNA response elements in target gene promoters, inducing activation or repression of transcription and subsequently protein synthesis (10-14). The anabolic effect of AASs is mediated primarily by ARs in skeletal muscle (15) where they regulate the transcription of target genes that control the accumulation of DNA required for muscle growth. Moreover, this ligand-dependent transcriptional factor modulates gene expression through the recruitment of various coregulator complexes, the induction of chromatin reorganization, and epigenetic histone modifications at target genomic loci. Dysregulation of androgen/AR signaling perturbs normal reproductive development and accounts for a wide range of pathological conditions such as androgen-insensitive syndrome, prostate cancer, and spinal bulbar muscular atrophy (16). The AR is a member of the nuclear receptor superfamily, members of which function as ligand-inducible transcription factors that mediate the expression of target genes in response to ligands specific to each receptor (17). The classical steroid receptors such as AR belong to the type 1 of nuclear receptors that typically form ligand-induced homodimers, binding to inverted repeat DNA response elements. The type 2 nuclear receptors dimerize with the 9-*cis* retinoic acid receptor and include the receptors for vitamin D3, thyroid hormone, all-*trans* retinoic acid, and the peroxisome proliferator-activated receptors. The DNA response elements of this group of nuclear receptors are characteristically direct repeats. Finally the third types of nuclear receptors are the orphan receptors, such as TR2, TR4, and chicken ovalbumin upstream promoter transcription factor (18) the ligands for which remain unclear. As mentioned above, the transcriptional activity of AR, as well as other members of the nuclear receptor superfamily, is modulated by coregulatory proteins. Coregulators are generally defined as proteins that interact with nuclear receptors to increase transactivation (coactivators) or reduce transactivation (corepressors) of target genes but do not significantly alter the basal transcription rate (19). Steroid receptors have been shown to interact with other DNA-binding proteins, resulting in modulation of steroid receptor transcriptional activity. AR has been found to interact with a number of transcription factors including AP-1 (20), Smad3 (21), nuclear factor kB (22), sex-determining region Y (23), and the Ets family of transcription factors (24). Although AR is normally thought to function as a homodimer, it has been found to heterodimerize with other nuclear receptors including the estrogen receptor (ER) (25), glucocorticoid receptor (26), and testicular orphan receptor 4 (27). Over the past two decades numerous experiments support the hypothesis that many steroid responses involve non-genomic mechanism. Such as, hormone-bound/activated nuclear receptors are able to interact with other transcription factors on target gene promoters without direct binding to DNA (28, 29). Steroid receptors are able to activate intracellular signaling molecules, such as the PI-3K/Akt signaling pathways, the mitogen-activated protein kinase (MAPK) family, and the ERK1/2, by transcription-independent mechanisms (30, 31). Steroids have also been shown to elicit cellular responses in a rapid way even when prevented from entering the cell. Perhaps the most conserved cellular response to steroid hormones indicating a non-genomic action is the rapid rise of intracellular calcium concentration observed in a variety of cell types (32-35). The rapid non-genomic effect of androgens is mediated either by the classical intracellular androgen receptor (iARs) (36) or by membrane-associated ARs (mARs) (37). Although the exact molecular identity of mAR still remains unknown, it is believed that mAR may represent either a pool of iAR targeted to the plasma membrane and/or associated membrane structures (e.g., lipid rafts or caveolae), mediating rapid androgen effects in the absence of transcriptional activity (38) or an unknown G-protein coupled receptor (GPCR) (or a receptor associated with a GPCR) triggering a variety of iAR-independent signaling cascades. How the different AR activities are related to the existing discrepancies regarding whether androgens are protective or damage promoting is unclear.

Animal studies

The potential neurotoxic effects of supra-pharmacological doses of AASs, have been evaluated through several experimental paradigms based on *in vivo* and *in vitro* approaches. Among rodents, the rat is the most used species. According to the National Institute on Drug Abuse (NIDA), nandrolone and stanozolol are two of the most frequently AASs abused. Therefore, experimental studies mainly focused on these compounds with a dosage schedule aimed to mimic those usually taken by AASs abusers (39, 40). Our group demonstrated in the rat that repeated exposure to AASs caused a derangement in the brain-derived neurotrophic factor (BDNF) status with a concomitant induction of a depressive phenotype (41).

This finding prompted us to investigate the effects exerted by these steroids on another neurotrophic factor, namely, nerve growth factor (NGF). It is currently hypothesized that Alzheimer's disease-related loss of cholinergic signaling and altered amyloid precursor protein (APP) processing are due to alterations in nerve NGF trophic support, an hypothesis known as the "neurotrophic unbalance hypothesis" (42-44). We have found that AASs treatment caused region-specific changes in the expression of NGF and its receptors. Both nandrolone and stanozolol increased NGF levels in the hippocampus and reduced NGF levels in the basal forebrain while reduced p75NTR expression in the hippocampus. Finally, AASs treatment reduced the expression of choline acetyltransferase in the basal forebrain and impaired the behavioral performance in the Morris water maze (45). These data suggests that AASs caused an impairment of the retrograde transport of NGF from the hippocampus to the basal forebrain possibly due to a defect in p75NTR expression in hippocampal cholinergic nerve terminals. Interestingly, a similar scenario has been observed in mutant mice modeling Down's syndrome (46), which show an impairment of spatial memory in the Morris water maze (47). In these mice, NGF levels are increased in the hippocampus and reduced in the septum, again suggesting a disconnection between NGF production and its retrograde transport (48). It has been demonstrated that testosterone exerts both neuroprotective (49-52) and neurotoxic effects (53, 54). These apparently diverging findings may be related to the different experimental paradigms and/or the doses of hormone employed. Nevertheless, the study by Estrada and coworkers (53), which demonstrated that testosterone at 1 μM concentration decreased human neuroblastoma cell viability and activate apoptotic program, prompted the investigator to speculate that the observations obtained at a single cell level might have long term effects at the system level. A recent study has deeply addressed the issue of AASs neurotoxicity in primary neuronal cultures (55). Results have revealed that micromolar concentrations of nandrolone are detrimental to cortical neurons. This action, which requires a 48-h exposure, was prevented by pharmacological blockade of ARs with flutamide suggesting an AR-mediated genomic mechanism. However, the cell-impermeable analogue nandrolone-BSA, which preferentially targets membrane-associated ARs, was also neurotoxic in a time-dependent and flutamide-sensitive manner. This latter finding reinforces the notion that membrane and intracellular ARs might share similarities in their pharmacological profile (56). Finally, activation of androgen membrane receptors by nontoxic concentrations of cell-impermeable AASs analogues potentiates the apoptotic stimulus induced by β-amyloid. AASs not only synergize the neurotoxic effect of β-amyloid but also potentiate the neuronal death triggered by *N*-Methyl-D-aspartate (NMDA). In fact, Orlando and colleagues (57) have demonstrated that several AASs (e.g., nandrolone, stanozolol and gestrinone) amplified, at nanomolar concentrations, NMDA toxicity in mixed mouse cortical cultures. Interestingly, aromatase inhibitors did not abolish this action, while flutamide completely prevented any synergic effect of the hormones. This latter finding strongly suggests an ARs-dependent action. Hippocampal synaptoneurosomes prepared from rats injected with nandrolone, revealed that a single injection of the hormone increased phosphorylation of the NMDA receptor subunits NR2A and NR2B and ERK1/2, while the levels of phosphorylated CaMKIIα were unaltered (58). Intriguingly, daily injection of nandrolone for 2 weeks did not affect the content of any of the proteins tested, suggesting some form of adaptation to high steroid levels. After a single AAS injection, the NR2A subunit was phosphorylated at Ser^{1232}, whereas the NR2B at Tyr^{1472}. While these data are closely related to changes in synaptic plasticity and relevant for the formation of LTP and LTD (59) it is noteworthy to recall that cyclin-dependent kinase 5 (Cdk5) phosphorylation of NR2A (Ser^{1232}) induces hippocampal CA1 cell death (60). It has been proposed the use of RNA interference for Cdk5 silencing in Alzheimer's disease and other tauopathies (61), further emphasizing the role of Cdk5 in neurodegenerative disease.

Several studies have investigated the relation of apoptosis and AASs treatment in different tissue and organs. Using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL), caspase-3 assay and transmission electron microscopy, Shokri and colleagues (62) demonstrated in the male Wistar rat that nandrolone exposure increased apoptosis in spermatogenic cells, an action which may related to infertility often observed in AASs abusers. In adult rat ventricular myocytes, AASs induce apoptotic cell death in a dose-dependent manner and markedly increased the expression of the pro-apoptotic oncogene Bax-alpha (63). These results might shed some light in the understanding of ventricular remodeling, cardiomyopathy, and sudden cardiac death associated with AAS abuse. In agreement with these latter findings, Fanton and colleagues (64) have observed elevated caspase-3 activity in the heart of rabbits subjected to long-term norethandrolone treatment suggesting that apoptosis is involved in the induction of cardiac lesion. Apoptosis has been also observed in differentiated skeletal muscle fibers. In fact, in differentiated murine C2 skeletal muscle cells, short-term exposure to supraphysiologic doses (>10 μM) of stanozolol showed pathologic features which might be related to programmed cell death such as cytoplasmic shrinkage and chromatin condensation. Moreover, cells

also showed positive in situ nick-end labeling of nuclear chromatin, indicating DNA strand breakage (65). Among peripheral tissues, flow cytometry demonstrated in human umbilical vein endothelial cells (HUVECs) an apoptotic effect exerted by several AASs (66). Although most of the steroids used in this study were toxic at high micromolar range, it is to note that nandrolone at concentrations as low as 9 μM, significantly reduced the proliferation rate of HUVECs, as well as induced apoptosis. Recently, Tugyan and colleagues (67) have evaluated the effects of long-term (8 weeks) exposure to nandrolone decanoate on brain tissue. By combining TUNEL staining and caspase-3 assay, these Authors have demonstrated a significant decrease in neuronal count concomitant to an increase in apoptotic cells in the parietal cortex, prefrontal cortex and hippocampal regions (i.e., CA1, CA2, CA3 and dentate gyrus) as well as an increase in oxidative stress in the brain as reflected by a decreased glutathione peroxidase activity and increased malondialdehyde levels. Moreover, in those experimental conditions, they found that the hematopoietic cytokine erythropoietin (EPO) dose-dependently preserved the number of neurons in the hippocampus. Although the design of the study does not allow definite disambiguation of neuroprotective or neuroreparative EPO effects, it may constitute a rational for EPO use in AASs-induced neurodegeneration. In neuron-like differentiated pheochromocytoma cell line PC12, methandienone and 17-α-methyltestosterone have been shown to modulate survival and apoptosis-related protein (i.e., ERK, caspase-3, poly (ADP-ribose) polymerase and heat-shock protein 90) causing an increase in the activity of the intrinsic apoptotic pathway as well as abnormalities in neurite network (68). Interestingly, a short-term increase in neuritin expression was also observed indicating a possible reparative reaction. Nevertheless, these data reinforce the hypothesis of a potential neurodegenerative effects produced by AASs. Moreover, as reported above, AASs may enhance excitotoxic death at low (nanomolar) concentrations which are below those observed in AASs abusers (69, 70). This raises a serious concern since excitotoxic mechanism can be triggered by a series of vascular, metabolic and toxic insults as well as concomitant psychoactive drug abuse. Under these conditions, the abuse of AASs might accelerate the rate of neuronal death.

Human studies

Along with peripheral toxic effects observed after prolonged use of supra-pharmacological doses of AASs (see above), a large body of the literature describes psychiatric side effects induced by anabolic steroids (71-74). As elegantly and detailed reviewed by Oberlander and Henderson (75) abuse of AASs leads to hypomanic or manic symptoms, sometimes accompanied by aggression or violence (so termed "roid-rage"), irritability and anxiety. Opposite to many findings related to AAS-induced anxiety or aggression, only few studies have been addressed on cognitive function in AASs abusers. In this section we will consider these studies in which detrimental cognitive functions might arise from a potential neurodegenerative insult. Assessing the precise mechanism responsible for AASs-induced behavioral disturbances is hindered by several factors. Abusers frequently self-administer multiple AASs, a procedure known as "stacking", in which doses may be increased and then decreased. To make the picture even more complicated, steroids users often employ non-AAS compounds in order to mask hormonal abuse, for energy replacement (e.g., insulin) or for fat loss (e.g., triiodothyronine). Moreover, as stigmatized by Kanayama and colleagues (76) although AASs have been used since the 1950s, the spread of illicit AAS did not begin until the 1980s. Thus, knowledge of the toxic effects of AASs abuse is still evolving and it might be possible that frank, diagnosable AAS-induced neurotoxicity will emerge in the coming years. The first study that examined the cognitive effects of AASs has been published in 1993 by Su and colleagues (77). In healthy male volunteers, these authors demonstrated in a placebo-controlled prospective study that methyltestosterone induced cognitive impairment (e.g., distractibility, forgetfulness and confusion) as determined by a visual analogue self-rating scale. In a following study, the same research group correlated neuroendocrine and behavioral effects of AASs in male normal volunteers (78). While increased plasma levels of free thyroxine significantly correlated with changes in aggressiveness (i.e., anger, violent feelings, irritability), decreased concentrations of total testosterone correlated with an increase in the cognitive cluster symptoms (i.e., distractibility, forgetfulness). These data suggest a possible causative relationship between AASs-induced hormonal changes and adverse mood and behavioral symptoms observed in steroids abusers. A question remains to be addressed: could be the cognitive findings obtained in normal volunteers generalized to "real" AASs abusers? The recent study by Kanayama and coworkers (79) might help in resolving this issue. British male weightlifters (age 29-55), with a reported life-time duration of AASs use ranging from 8 to 640 weeks were recruited and administered five cognitive tests; age-matched non-AASs-using weightlifters served as control. In agreement with studies conducted in normal volunteers, this work confirmed that long-term AASs users are characterized by cognitive deficits. In fact, although no significant differences were observed in response speed, sustained attention and verbal memory, steroids abusers performed more poorly than non-users on visuospatial

memory. Moreover, a significant negative correlation was noted between visuospatial memory and total lifetime exposure to AASs. Although was not the main purpose of this observational study to identify the neurobiochemical events underlining AAS-induced cognitive deterioration, it is interesting to note that spatial memory impairment has been observed in rats after prolonged AAS treatment (45,80). Indeed, more studies are needed to firmly establish the relevance (if any) of neurodegenerative events as determinants of cognitive deficits in AASs abusers. Nevertheless, the use of animal studies might help in elucidating this issue since such experimental approach can offer the opportunity to explore additional signal pathways that have been recently causatively linked to neurodegenerative processes (e.g., the Wnt signaling pathway).

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NEUROAIDS: VIROLOGICAL ASPECTS OF HIV INFECTION

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NeuroAIDS is one of the main complications of chronic HIV-infection. The Central Nervous System is an immunologic sanctuary for HIV and allows the persistence of the virus despite an efficient antiretroviral therapy. HIV-1 could promote the neurodegeneration through the induction of inflammation by the release of neurotoxins from infected cells. In addition, several viral proteins can directly contribute to the neuronal damages, activate cell-signaling involved in the control of cellular survival and apoptosis, favoring functional alterations in the target cells. Macrophages play a key role in the pathogenesis of NeuroAIDS, they are the main reservoirs of the infection in brain, promoting the inflammatory escalation, astrogliosis and degeneration process. This review aims to highlight the virological aspects associated with NeuroAIDS including pathogenesis, and treatment of HIV-1 in the CNS sanctuaries.

More than 34 millions of people are still living with Acquired Immune Deficiency Syndrome (AIDS) worldwide, however last data from the Joint United Nations Programme on HIV/AIDS (UNAIDS) relative to the 2011, report a decline of 24% of AIDS-associated mortality compared to the 2005 [NAIDS]. AIDS-related neurocognitive disorders are one of the major complications of chronic HIV-infected patients. In general, it is represented by a combination of neuronal-tissue inflammation and virus-related neurological disorders (1). Despite the high efficacy of Antiretroviral Therapy (ART) against AIDS-associated syndromes (2,3), HIV cannot be completely eradicated. In particular, the compartment of the Central Nervous System (CNS), isolated from the rest of the body, represents a "sanctuary" for the infection (4). Several risk factors are associated with the incidence of HIV-neurocognitive impairment such as low CD4+ cell count, high viral load at baseline, low CD4+ nadir, HCV-coifection, drug abuse and metabolic comorbidities (1,5–13).

Clinical aspects and classification of neurological disorders

HIV-nervous disorders (NeuroAIDS) is one of main issues in patients with AIDS despite the antiretroviral therapy, and it is characterized by a rich set of dysfunctions such as decrease of attention, mood alterations, depression, psychomotor disturbs, alteration in the extrapyramidal movements and spasticity, associated with morphological profiles characterized by atrophy, neuro-degeneration, persistent inflammation with microglial nodules, perivascular lymphocytes cuffing, accumulation of multinucleated cells expressing HIV antigens (probably derived from the fusion of the uninfected and infected perivascular macrophages), demyelinization and white matter gliosis (1,14–16). According to the American Academy of Neurology criteria, HIV-associated neurocognitive disorders (HAND) can be divided in HIV-associated dementia (HAD) and minor cognitive motor disorders (MCMD) (17,18). Conversely, the new criteria developed by the HIV Neurobehavioral Research Center (HNRC) define three conditions with a progressive evolution. The ANI (HIV-associated asymptomatic neurocognitive impairments) are characterized by the presence of cognitive function impairment in at least two domains without interfering with everyday function, with no signs of delirium or dementia. The MND (HIV-1 associated mild neurocognitive disorders) are characterized by cognitive function impairment in mild matter interfering with normal daily activation. This status

Key words: HIV-1, CNS, Macrophages, HAND

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0393-974X (2013)

could evolve in a more serious condition called HAD (HIV-1-associated dementia), markedly incompatible with normal day-by-day life (1,13,17).

Cells involved in the pathogenesis of NeuroAIDS.

HIV can infect different cellular target within the CNS such as parenchymal microglia, neurons and astrocytes (19,20). CCR5 and CCR3 are the main co-receptors used by HIV in brain (21). CCR5-using viruses, in fact, are present with an high prevalence but also X4- and dual-tropic variants were isolated (4,21–23). Overall, macrophages are the main cellular targets of HIV in the brain tissue playing a key role in the neurodegeneration process (24). Four major types of macrophages were identified within the CNS: meningeal macrophages, macrophages of the choroid-plexus, perivascular macrophages and microglial macrophages (25,26). In particular, perivascular macrophages and microglial macrophages seem to act a fundamental role in the HAND- pathogenesis (25).

Macrophages release viral proteins, inflammatory cytokines and neurotoxins, inducing inflammation, astrocytes differentiation, apoptosis and alteration of the normal neurogenesis (27,28). They are the main cellular reservoir of HIV infection (29) and their role, in CNS infection, will be further discussed.

Microglial resident cells are an hybrid between white and glial cells, derived from hematopoietic precursors and monocytes (CD11+, CD14+ and CD16+) during the postnatal period of microglia infiltration (30–32). These cells are responsible of the immunological surveillance within the central nervous system, and in vivo studies demonstrated their crucial role in neuronal function and regeneration (31). They play also an important role in the HAND pathogenesis, contributing to the neurodegenerative events through various mechanisms. Glial cells infected by HIV are able to release factors and toxins which cause bystander damage in neurons and astrocytes (33,34). Microglia are the main source of cytokines in brain (34). They could initiate and support the astrogliosis with a feedback-loop of cytokines between glial cells and astrocytes (34). Astrogliosis is a cellular reaction, characterized by a pattern of functional and structural changes involving astrocytes (34). It plays several roles: protects neurons and their function, participates to the remodeling of the neurovascular unit, but at the same time it could contribute to the neuronal injury (34).

Astrocytes are neuroectodermal-derived cells, important components of the BBB, which support the function and metabolism of neurons, control the state of the neuronal synapses by the uptaking of neurotransmitters, the ionic homeostasis into the CNS, scar formation, tissue repair and they also regulate the immune response in the brain (30,35,36), playing a leading role in the HAND-pathogenesis (24). Despite the lack of the CD4 receptor on their membrane, HIV DNA has been found in vivo in astrocytes, but the number of p24+ cells is very low, indicating that HIV can enter into these cells maintaining a limited replication capacity and becoming a driving force for the residual replication in the CNS (37). In vitro studies have shown that HIV-1 initially replicates without cytopathic effect in human astrocytes and then evolves in a latent infection, including reduction of viral proteins expression. Several viral genetic factors could modify the state of activation of the host cells, inducing the chemoattractive factors' release and promoting the recruitment of monocyte and microglia. This mechanism could amplify the neuronal tissue damage, the production and secretion of Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS), the deregulation of the glutamatergic transmission, contributing to the excitotoxic injuries (24,38,39). Moreover, several cellular factors such as IL-1β, TNF-α or IFN-γ are able to stimulate and reactivate the latent phase of these cells (4,37,40,41).

The pool of permanently infected cells established during the earliest stages of acute HIV infection and persisting with a long half-life (42–46), represent the major barrier to eradication. These kind of cells were mainly represented by T CD4+ memory cells and macrophages. (47–50). Indeed, macrophages can sustain viral infection for long periods of time, from weeks to months, both in vitro and in vivo (51–56) and they can efficiently transfer the virus to CD4+ T-lymphocytes contributing to their depletion in human cellular compartments (57).

Macrophages and dendritic cells represent the main antigen presenting cells involved in the first line response to infections in the body, including the CNS (26). HIV induces a persistent infection in macrophages promoting long-term cell survival (53) and determining resistance to the apoptotic signaling through the pathway of both NF-kB and Bcl-2 (26). Furthermore, other in vitro studies showed an up-regulation of the telomerase enzyme that can increased the resistance of the DNA to the oxidative infection-induced damages (58,59). The replication kinetic between lymphocytes and macrophages may be different due to their peculiar characteristics. HIV has an exponential rate of replication in lymphocyets, inducing rapid cells death. Differently, in vitro studies showed that macrophages are less sensitive to the virus-associated cytopathic effects with a replication rate that tends to increase after 14 days post infection reaching a plateau until 45 days (29,53,60,61). Macrophages can transmit infection to the uninfected lymphocytes through cell-cell synapse involving the VCCs (Virus- Containing Compartments), which are intracellular compartments acting as site for the virus assembly and as a vehicle for

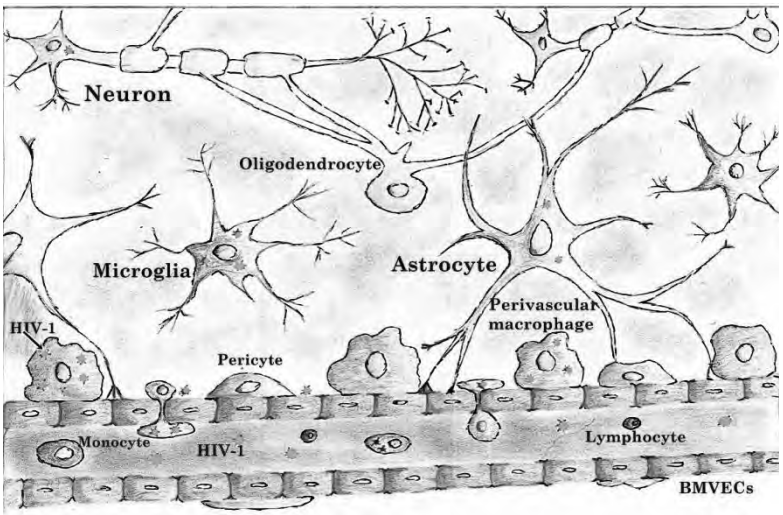

Fig. 1. *HIV infection into CNS. HIV could enter in the CNS both directly from blood circulation but also infected monocytes, crossing the BBB, could transfer the infection to neighboring cells (astrocytes, perivascular macrophages, microglia).*

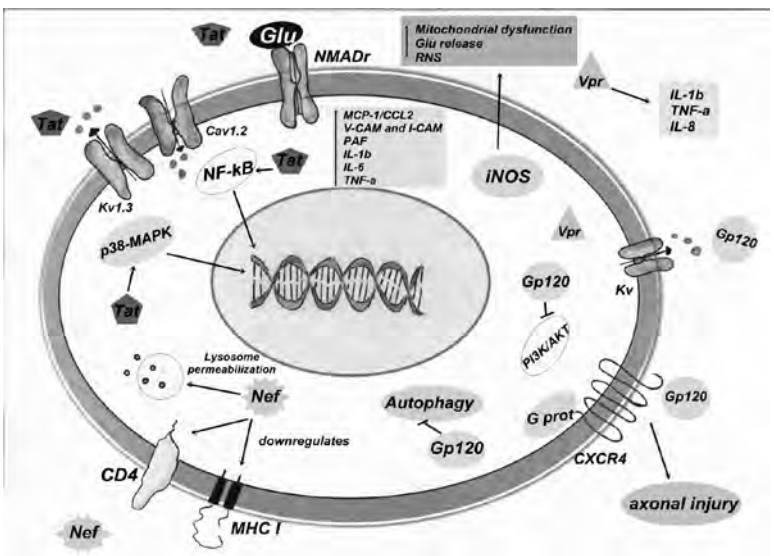

Fig. 2. *Effects of viral proteins. Tat increases the expression and activity of ionic channels, like Kv1.3 and Cav1.2. It is a "promiscuous agonist" of the NMDA receptor, whose activation induces the inducible form of the Nitric Oxide Synthase, contributing to the excitotoxic damage glutamate-dependent. Furthermore, Tat actives cellular pathway involved in the genetic expression, contributing to the release of inflammatory cytokines. The delivery of pro-inflammatory proteins is also attributed to other viral proteins, like Vpr. Gp120 enhances the outward K+ current, inhibits the autophagic pathway, inducing axonal injury. Nef downregulates the expression of CD4 and MHC I, participating to the cellular damage and allowing the lysosome permeabilization.*

the cell-to-cell spread (62,63). In this way macrophages could contribute to the progression of the infection, promoting T-CD4+ cells depletion and inducing also apoptosis of different cells (CD8+, CD4+, neurons and astrocytes) caused by the release of cytotoxic factors (60).

Thanks to their biological properties, HIV replication into macrophages presents a different sensibility to the drugs compared to lymphocytes. P-glycoprotein and Multidrug Resistance Transporter (MRP 1, MRP4 and MRP 5), expressed upon these cells (29), protect them against external toxic substances, but at the same time, they limit the intracellular bioavailability of drugs. Several studies analyzed antiretroviral drug's activity in both macrophages and lymphocytes. In particular, nucleoside analogues showed a remarkable EC50 (Effective Concentration 50) value in macrophages

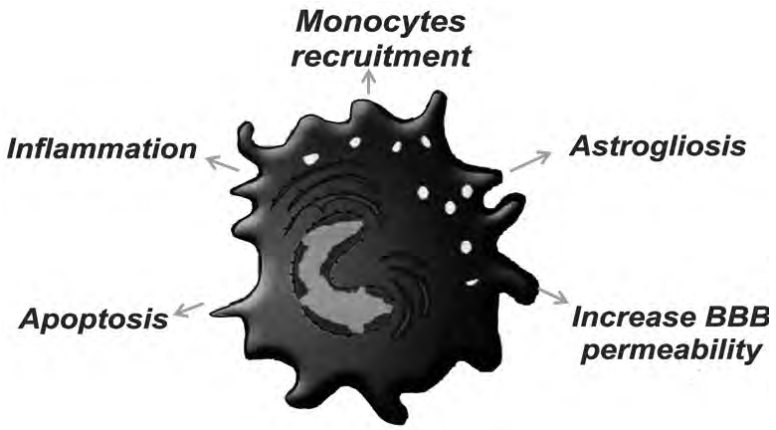

Fig. 3. *Macrophages play a key role in the pathogenesis of NeuroAIDS. Macrophages are the main reservoirs of the infection, allowing its persistence despite the ART. They release neurotoxins inducing apoptosis of the neighboring infected and uninfected cells, initiate and support the inflammatory process and increase the BBB permeability, promoting the recruitment of other cells in the site of infection. Furthermore, they could promote astrogliosis thus amplifying the damage.*

(60,61,64). A good efficacy was evidenced also for protease inhibitors and integrase inhibitors, with similar EC50 values between the two cell types (65).

The suboptimal drug concentration in the cellular compartment favors the emergence of resistant strain in reservoirs thus promoting the replenishment of circulating viral population with resistant variants, determining a therapy failure (29).

All the features reported above, which characterized HIV infection in macrophages, account for the peculiar role of these cells in the pathogenesis of HAND. Activated macrophages promote the induction and support the maintenance of the neuroinflammation in CNS (25,66). They can deliver reactive species and metalloproteinases initiating and supporting the astrogliosis, thus contributing to the neuronal damage (25,34,67,68).

Virological aspects of HIV-associated neurodegeneration

HIV could cross the BBB during the early stage of infection (69) through three hypothetical, not mutually, mechanisms:

a) In the "Trojan horse" hypothesis, infected monocytes, leukocytes and perivascular macrophages crossing the BBB could release viral particles able to infect resident cells like microglia establishing a persistent infection. This mechanism has been observed also with other retroviruses and lentiviruses and it is probably the main gate for the penetration into the brain (70). Several observations suggest that monocytes may result infected before leaving the bone marrow (58). In particular, an amount of proviral DNA was found in these cells also without the expression of viral protein, thus allowing the dissemination of the infection (58,71). A relevant role is covered by a little subset of monocytes which tend to increase during HIV infection (72), CD14lowCD16high (26,29,73–75). These cells show intermediate characteristics between monocytes and differentiated cells (macrophage and dendritic cells) (29,74). They are more permissive to HIV replication, probably for the lower activity of the host restriction factors than the CD14highCD16low cells (71,75), and they can better cross the BBB (29,72,73).

b) Another viral access is represented by the direct infection of endothelial cells located in the CNS's edge which express on their surface chemokine receptors involved in the HIV-1 entry, like CXCR4, CCR3, DC-SIGN (70,76).

c) Viral particles may cross the barrier in case of altered tissue and/or increased permeability due to other dysfunctions (4,77).

HIV replication and the release of different viral proteins into the central nervous system could amplify the level of alteration and permeability of the BBB (24,39).

The BBB is a critical protective structure that physically separates the CNS from the systemic circulation, regulating the transition of cells, proteins and molecules into the nervous tissue and maintaining its homeostatic equilibrium. It is composed by microvascular

Table 1. *Drugs that can cross the BBB with relative CNS Penetration-Effectiveness rank (CPE) [(121)Letendre et al. 2010]. Drugs with high CPE rank could efficiently cross the BBB allowing a better effectiveness of the therapy.*

Class	Name	CPE rank
NRTI	Zidovudine (AZT)	4
	Abacavir	3
	Emitricitabine (FITC)	3
	Stavudine (d4T)	2
	Lamivudina (3TC)	2
NNRTI	Nevirapine	4
	Delavirdine	3
	Efavirenz	3
	Etravirine	2
PI	Indinavir/r	4
	Lopinavir/r	3
	Darunavir/r	3
	Fosamprenavir/r	3
	Indinavir	3
	Fosamprenavir	2
	Atazanavir	2
	Atazanavir/r	2
INI	Raltegravir	3
Entry/Fusion	Maraviroc	3
Inhibitors	Enfuvirtide	1

endothelial cells over a basal lamina followed by other cells type like astrocytes, pericytes, perivascular macrophages and parenchymal microglia. Astrocytes, with their extroversions provide to the maintenance of the barrier (72,78), avoiding the passage through gap-junctions of metabolites including calcium, cyclic nucleotides and neurotransmitters. Moreover apoptotic signals could be release from HIV-infected to uninfected astrocytes and neurons (77). Few number of infected astrocytes are sufficient to alterate the BBB integrity by inducing endothelial cells apoptosis (78). In addition, several viral proteins could also alter the BBB permeability by inducing apoptosis (79,80) and increasing the neuroinvasion of HIV and other viruses (4).

Viral populations found in CNS could be different from viruses of the systemic circulation (13,14), underlining the importance to prevent the transition of the virus into the CNS using specific antiretroviral therapy. Moreover, recent data, obtained from patients in a late state of disease, showed an independent evolution of viral

tropism between brain and immune system, evidencing an evolution toward macrophages tropism overtime (69).

This manuscript will mainly focus on the role of macrophages in the pathogenesis of NeuroAIDS.

HIV: direct and indirect mechanism of neuronal damage

Despite the limited number of infected cells in the brain, HIV can directly or indirectly affect the neuronal tissue (81). In particular, an extent virus-associated damage is observed during encephalopathy due to the release of viral proteins and cellular neurotoxic molecules (4,82). Apoptosis is the main pathogenic mechanism observed in HAND. Viral proteins released by infected cells, mainly by macrophages, could induce apoptosis with both direct and indirect mechanism. HIV can induce neuronal apoptosis by interfering with lysosomal enzyme acting at both mRNA and protein levels. This induces its release from the organelle like cathepsin B interfering with its natural inhibitors. Ex vivo analysis showed an increased expression of the cysteine protease and the intracellular inhibitor cystatin B within hippocampus and basal ganglia of HIV+ patients with MCMD and HAD. This unbalance in the control of cathepsin B activity is associated with high neuronal apoptosis (83). HIV infection induces oxidative stress with an increase of the oxidized glutathione form. The oxidize stress infection-derived can promote in vitro shortening telomere length, becoming a key sensor of cellular apoptosis (67,84). The inflammatory cascade plays a key role in these processes. The rate of infected cells can't justify the extent damage observed in HIV Encephalopathy (HIVE) (4).

HIV-associated neuroinflammation could depend on three independent events: infiltration of infected monocyte and lymphocytes in CNS, release of viral and cellular factors from these infected cells, and infection of resident cells by viral particles infiltrating into CNS or released from infected cells (85). Some cellular cytokines, released during HIV infection, are neurotoxic like TNF- α, platelet derived growth factor (PDGF), nitric oxide and quinolinic acid (QUIN), while others factors can promote the recruitment of immunological cells in the CNS through the BBB like CCL2 (4). The presence of these proteins is maintained also during a suppressive ART. In CSF, cytokines like CCL2, IL-8, CCL3, CXCL10, IFN-γ and IL-6, are expressed even in presence of an adequate therapy, indicating the continuous neuronal inflammation promoting the HAND-associated encephalopathy (4). Up-regulation of COX-2 enzyme is observed in infected astrocytes, macrophages and endothelial cells (32). But some of these products can negatively modulate HIV infection. LTB4 and LTC4, produced by monocytes and microglia, could modulate HIV infection in macrophages reducing the expression of CCR5 in PKC-dependent way (32). Several viral proteins could be released mainly during the uncoating and the budding of the virus. In particular, in the case of non-productive infection, as occurs in astrocytes, certain viral regulatory proteins are delivered outside the cell (81). Moreover, some cellular factor can increase HIV replication. Nerve Growth Factor (NGF) is an neurotrophin factor that can promote the survival of infected macrophage through NGF-trkA and p75NTR receptors, and consequently allows the long-term production of viral particles (28,86). Some in vitro studies showed an increased level of expression during HIV infection (86). This factor is also associated with restoring long-term potentiation (LTP) in mice with cognitive impairment, and it could be crucial for the regeneration of functional plasticity (87).

Tat

HIV-1 Tat is a regulatory protein that plays a pivotal role in HIV pathogenesis (88). It forms a ternary complex with the cyclin T1 and CDK9 that binds TAR, phosphorylates cellular RNA polymerase II thus enhancing its HIV DNA transcriptional activity (89). Tat is released by infected cells and can modulate the cellular protein expression profile (89,90) inducing also cellular apoptosis (91). Exposition of human astrocytoma cells to HIV-1 Tat recombinant protein demonstrated a modest level of apoptosis (< 8%) compared with untreated cells (28). However, Tat can contribute to the neuronal damage though various mechanisms.

Some studies reported an association of Tat with a potassium voltage-gated channel activity, Kv1.3. The microglial cells express Kv1.2, Kv1.3 and Kv1.5 transcripts and proteins, but only Kv1.3 activity has been correlated with Tat in the rat's brain (92). Exposure to this protein is correlated with an increased outward K+ current and expression of the channel protein (93) . Some molecules such as LPS, are able to activate microglial cells guiding the release of inflammatory cytokines and neurotoxic substances like RNS and ROS, inducing also an increase of the K+ current (92). Recent in vitro studies demonstrated the neuroprotective effect of minocycline, a semi-synthetic tetracycline derivate, is able to block of the Kv1.3 (94). Transient exposition to HIV-Tat determines in rats an increase in the number of Ca2+v1.2 channels inducing astrogliosis in the cortical region. In particular, this increased Ca2+ current is correlated with death of cortical neurons, microglia and monocytes (95). In addition, in vitro studies showed that 24h exposure of rat microglial cells to HIV-Tat determines an upregulation of the Ca2+ channel, correlated with high secretion of pro-inflammatory and neuro-toxic molecules and determines death of the neuronal cells with a mechanism involving p38 MAP Kinase (93). Furthermore, HIV Tat protein

is responsible of leukocytes infiltration and invasion with inflammatory phenotype and an engagement of microglia in cell-to-cell contact between synapse in rat CNS. In addition, an high release of chemokines and cytokines, like MCP-1/CCL2 (Monocyte chemoattractant protein type 1), protein of the CAM family (V-CAM 1 and I-CAM1), and production of platelet-activator factor PAF (96) is observed. The Tat-induced expression of these cytokines (via NF-kB) could be different between monocytes and astrocytes. Monocytes produces three types of cytokines (IL-1β, IL-6 and TNF-α), while astrocytes produces only IL-6, even though the mRNA of IL-1β is increased. Tat induces IL-1β mRNA expression in a dose-dependent manner (97). The expression of these cytokines is maintained for long period even though Tat is not detectable anymore. So a transient exposure of Tat results in a cascade of events of glial activation like the "hit and run" phenomenon (97). Tat promotes the excitotoxic Glutamate-mediated damage. Chronic and low presence of this viral protein in transgenic mice induces alteration of the equilibrium between Glutamate and GABA (98) evoking an high Glutamate-overflow in the cortex and hippocampus compared to the control, and a reduced GABA overflow in cortex but not in the hippocampus. The release of Glu under resting conditions is the same of the control, indicating that Tat enhances the glutamatergic transmission secondary to a positive stimulus. Tat is known to be a "promiscuous agonist" for its capability to bind receptors as NMDA, CCr2 and mGluR1, but it can also modulates cellular gene expression. The expression of GLUT1, a marker of glutamate synaptic vesicles, is increased in cortex and hippocampus of TT mice in Tat expression-dependent manner (98). In vitro studies demonstrated that Tat could also increase the expression of the isoform GSA of the Glutaminase enzyme probably in a STAT-1 dependent manner, which is responsible of the conversion of the glutamine in its corresponding acid (99). Indeed, a high expression profile of Glutaminase in HIV-infected patients with dementia is observed (99). HIV Tat also interacts directly with NMDA and LRP (Lipoprotein receptor related-protein) receptors of the hippocampal cells, inducing a decrease in the synaptophysin (SYN) expression (a glycoprotein presents on the presynaptic vescicles), and a progressive, but reversible, loss of the presynaptic contact (100) . Treatment with antagonist like RAP and MK801, inhibitors of LRP and NMDA respectively, could prevent the presynaptic loss and the reduction of the SYN expression indicating that this phenomenon is post-synaptic (100). Moreover, LRP receptor and its ligand ApoE4 are associated to Alzheimer's disease, representing a possible correlation with HAND (77).

Calcium influx through NMDA receptors activates pathways leading neuronal damage (101) and induces the Nitric Oxyde Synthetase (NOS) determining the production of NO, that can amplify the excitotoxic damages, inhibiting the mitochondrial respiration and favoring the accumulation of reactive derivates such peroxynitrite, which is associated with apoptosis in astrocytes (68,102).

Finally HIV Tat could determine an engulfment in the microglial processes and dendritic spines by increasing the leukocytes infiltration and the microglial activity against the invading leukocytes (96). The loss of postdendritic spines probably due to the ubiquitin-proteosome pathway independent from NMDA and the activation of the inducible Nitric Oxyde Synthetase (iNOS) is also observed. Treatment with Ca+2 chelators or NMDA antagonists like MK801, which is able to prevent cellular death, doesn't influence the synapses loss while retards synapse formation (103). However, the glutamatergic signaling is not the only neurotransmission influenced by HIV proteins. Several studies have shown an interference with the dopamine transporter in both the striatum and midbrain and the vescicular monoamine transporter-2, but in the last case only in the striatum (104,105).

Gp120

The envelope glycoprotein gp120 induces the release of inflammatory cytokines and toxic substances like glutamate, leading to neuronal damage by indirect mechanism (101). In vitro studies showed that gp120 didn't present pro-apoptotic capacities on human astrocytoma cells (28), because of the lack of the CD4 receptor on the surface of these cells.

It can induce alteration of the Toll Like Receptors (TLR) expression on the astrocytes surface thus increasing HIV pathogenicity (106). The envelope glycoprotein secreted by infected cells can also alter the autophagy process normally induced by stress conditions, like nutrients deficiency and infections (107). In addition, it is involved in the pathogenesis of Alzheimer and Parkinson and other aging-diseases (108). Analysis of autophagy's markers in the brain of gp120-expressing mice showed a reduced expression of beclin-1, LC3 and the neuronal marker MAP2 (108). In the II, III and V layers of pyramidal neurons of the midfrontal cortex this catabolic process is reduced in aged patients compared to young HIV+ and HIVE patients. This leads to the accumulation of altered proteins that can damage neuronal tissue (108). Ex vivo studies on the Corpus Callosum (CC) of HIV infected rats, showed that gp120 induces axonal injury though the interaction with CXCR4 receptors. This is demonstrated also by the accumulation of the β-APP (a β-amyloid precursor) in the axons, representing an axonal impairment. The treatment with a CXCR4-antagonist

(T140), interrupts this phenomenon (91). Moreover, rat cortical neuronal cultures chronically treated with gp120 showed an increased outward K+ current in dose-dependent manner (109). Decreased K+ current is associated with LTP and memory processes, while enhanced current determines learning and memory deficiencies. The increased current of K+ ions in neuronal rat cells also induces apoptosis. These evidence is corroborated by the reduced percentage of apoptotic neurons after the treatment with 4-Aminopyridine that blocks Kv channel (109). The phosphotidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, involved in cellular growth and survival (110), is reduced in both gp120- expressing mice brains and human forebrain from HAND patients, and it is correlated with HIV-1 neuropathogenesis (111).

Vpr

HIV Vpr (Viral Protein R) is an accessory protein with various, sometimes controversial, functions during the viral life cycle. In particular, it is involved in the pre-integration complex (PIC) nuclear import, induction of G2 cell cycle arrest, transcriptional co-activation of viral and host genes and inhibition of nuclear kappa B factor (NF-κB) (112). This viral protein is released by infected cells, but it can be incorporated in both defective and complete viral particles (85). Vpr is able to increase the release of pro-inflammatory cytokines like TNF-α, IL-1β and IL-8 in macrophages, probably acting on MAPK pathway. Furthermore it induces apoptosis, probably with the brokerage of IL1-β and IL-8. In particular, it is known that IL-8 induces the release of neurotoxins like metalloproteinase of the matrix, and promotes cell cycle and pro-apoptotic proteins (85).

Nef

HIV Nef is an important viral protein that enhances viral replication and infectivity, down regulating CD4 and MHC I receptors (113). It increases the sensibility of astrocytes to hydrogen peroxide (114), promotes astroglial activation and astrogliosis (38), induces the permeabilization of lysosomes with resulting enzyme release (115), and it causes apoptosis of Micro Vascular Endothelial Cells (MVEC) (80).

Antiretroviral Therapy and HAND

In order to prevent and reduce the incidence of neurological HIV-associated dysfunctions (22), international guidelines (last update on February 2013) (116) recommend four possible first line treatment regimens for HIV naïve infected patients. Each regimen includes at least two NRTI plus an NNRTI, a PI or an INI. For experienced patients, the antiretroviral regimen depends from the treatment history and the data obtained from the resistance test, identifying at least two drugs fully active to add to the background therapy.

The main limitation for the treatment of HIV in the CNS is represented by the capability of the drugs to cross the blood brain barrier. Neurons are extremely sensible to every minimal environmental change (117), for this reason and as explained before, the critical importance of the BBB is in the physical separation of the CNS from the rest of the body regulating the passage of substances and cells from blood and guaranteeing the neurovascular equilibrium (117). Endothelial cells (BMVECs) are linked each other through tight junctions and cover an area of approximately 20m2, representing more than 100 billion of capillaries. Despite this wide surface of absorption, very few molecules can overcome this barrier, especially in case of drugs (117). Molecular weight, lipophilicity and blood's protein binding are the main pharmacological factors influencing the distribution of the drug into the brain tissue (Table 1) (118–121).

The CNS Penetration-Effectiveness is a parameter (CPE) that correlates drug's penetration into CNS with the effectiveness of the therapy, and it is related to plasma, CSF viral load and blood CD4+ counts. Regimens with lower CPE are more prone to show evidence of residual viral replication in CSF (122–124), associated with cognitive impairment (125).

New drugs delivery technologies try to overcome the limit of BBB permeability to antiretroviral drugs. Some method aim to modulate the BBB permeability, for example applying electromagnetic interference, hypertonic solution of urea or mannitol (122,126), inhibiting drugs efflux transports, targeting nanoparticles and using cell-mediated nanoART (122). For example magnetic azidothymidine 5'-triphosphate (AZTTP) liposomal nanoformulation can cross the BBB and taken up from monocytes (127). Several in vitro studies showed that Tat-conjugated Ritonavir- loaded nanoparticles effectively inhibits viral replication in macrophages without inducing neuronal-toxicity (128).

Moreover, long-term ART treatment could favor the emergence of resistant HIV-1 in target cells. In macrophages, representing the main sanctuary of the infection in the CNS, the generation of a resistant HIV-1 viral reservoir could be promoted. In particular, several cellular transporters (P-gp, MRP4 and MRP5), reducing the optimal intracellular concentration of the drugs, could favor both the emergence in later stage of disease of resistant viruses and their productive infection to other target cells. (29,60,61,64).

Furthermore, given the importance of these cells in the pathogenesis of NeuroAIDS (129), new in vitro experiments on macrophages, based on the use of promising antiretroviral drugs, could help the design of

advanced therapeutic regimens aimed to block or interfere with the CNS infection (60).

ACKNOWLEDGEMENTS:

We thank Francesca Stafoggia for her important contribution to this work.

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