MY PERSONAL MEMORIES OF RITA LEVI-MONTALCINI

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The peaceful passing of Rita Levi-Montalcini on December 30, 2012 was a great loss for the entire scientific community. Her revolutionary discovery of NGF made her the "First Lady" in Science and for all of us, her closest collaborators, she was like a "mamma" devoting time and care for her children.

I remember that I came across her work and cited her relevant papers in the '70s, when I was working at the Institute of Psychiatry at University of London. I soon realized, and was proud of it, that her milestone work on the organization of the chick brain, spinal cord and sympathetic ganglia was highly admired by English scientists and heavily quoted.

In that period we were working on the behavioral and electrocortical effects of neurotransmitters in avian species (Marley & Nisticò, 1972, *Br. J. Pharmac.* **46**: 619-636; Marley & Nisticò, 1975, *Br. J. Pharmac.* **53**: 193-205). We later produced an atlas on the distribution of monoaminergic pathways visualized by histofluorence techniques in the chick brain (Gargiulo, G. Nisticò G (1978) J Anatomy **126,** 261-274).

Some years after my return to Italy in the '80s I had the fortune to meet Rita in Rome through our common and dear friend Renato Dulbecco (Nobel Laureate, 1975). Both Rita and Renato together with Salvador Luria had worked under the direction of Giuseppe Levi in the '30-'40 at the School of Medicine, University of Turin. It is extraordinary that all three of them were awarded the Nobel Prize!

Then my relations with Rita increased with time. Thus in 1995 when I was elected Governor of Calabria Region, the first telephone call I received with enormous surprise was from Rita despite we had different political ideas. The second telephone call I received from her was when I was in the middle of a political strike. The Headquarters of Calabria Region were surrounded and occupied by hundreds of "forestal" workers who risked losing their jobs because of the drastic cuts in financial resources by the National government. I was relieved when I could leave for a while the table of negotiations with Unions and worker representatives and I could listen on the phone Rita's fresh and "excited" voice, telling me that she was happy that our joint paper on the effects of HIV gp_{120} protein on NGF receptors plasticity was accepted into PNAS (PNAS, USA, **93**: 928-933).

Since then she continued to be for me a source of inspiration throughout my political career. So thanks to her encouragement in the '90s we managed to create in Calabria (Lamezia and Piano Lago, Cosenza) an excellent research Center in Neurogenetics where still today approximately two hundred people continue to work. I still remember how happy she was when she learned that Amalia Bruni, one of the leaders of the Center in Lamezia, had discovered a new protein called "nicastrin", the catalytic site of gamma secretase, the enzyme involved in the abnormal production of beta amyloid (Abeta 42), one of the pathogenetic main factors in Alzheimer's disease.

From 1999 to 2004 I was member of the European Parliament and my prevalent interests were in scientific research. Thus I collaborated intensively with the Research Commissioner Philippe Busquin as member of an international task force that he had set up. When

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2279-5855 (2013)

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

Philippe asked me to help with the organization of a European Conference on Neuroscience, suddenly I proposed to invite for the main lecture Rita Levi-Montalcini. No one could believe that she would accept our invitation. However, despite at that time she was 94 years old, she did not hesitate to accept with pleasure our invitation and she came to Brussels. I still remember with some emotion the outstanding lecture she gave in the European Parliament on the challenges to be faced in Neuroscience. After her brilliant lecture she participated in a press Conference with approximately 100 journalists. I was sitting next to her and still remember how she fascinated the participants. The press Conference turned out to be such an extraordinary success that the European Commission and the European Parliament decided a few days later to almost triple the financial resources for the following VII Framework Program of Research.

At that time she informed me that her dream was to create in Italy an infrastructure of excellence in Neuroscience and asked me to help her in this project. Among the many proposals that arrived, we selected together to establish the laboratories in Rome and in 2005 the European Brain Research Institute (EBRI), a platform including two other Institutions (Italian Council for Research Neurobiology Institute and the Santa Lucia Foundation) was able to start its activities. According to Rita, EBRI was supposed to be an "incubator", where young Italian and foreign researchers could work together and express all their creativity under the supervision of more expert scientists until they became autonomous and independent lab leaders. She made all the efforts to select a top level international scientific Council and Management Board with some Nobel Laureates like Torsten Wiesel, Francois Jacob and Erwin Neher and other outstanding scientists like Salvador Moncada, Lamberto Maffei, Martin Raff, Tobias Bonhoeffer , Wolf Singer, Adriano Aguzzi, etc.

However, after a few years of idyllic interaction with the Santa Lucia Foundation, a legal controversy arose and the owner of the premises ordered the eviction of EBRI laboratories. But she never surrendered and she fought as a lion, with courage, stamina and intelligence. I remember that this was a sad period of her life and she had a terrible time full of bitterness and disappointments.

However, her intuition led her to elaborate new strategies to re-launch EBRI by nominating a Government Commissioner with full power. Then she proposed my name as Commissioner and despite my heavy engagements at Tor Vergata University, where I was starting a School of Pharmacy in English in collaboration with Nottingham University, I did not feel to leave Rita alone in her battle and I accepted this new challenge.

I concentrated all my energies and thanks to the collaboration of Pietro Calissano and Antonino Cattaneo and all the other young researchers and administrative personnel we recreated in the Institute a climate of trust after a long period of frustration and depression so that EBRI restarted to flourish again.

Rita was always deeply involved and determined in every decision I had to take. Sometimes she used to telephone me more that 10 times a day to solve many important problems and we used to meet at EBRI when she came to carry out some experiments with young researchers.

In addition, she was very pleased to receive in 2010 the visit of a Chinese delegation of the Bioway Company (Xiamen) and I witnessed the emotion and joy she felt when Prof. A. Pan gave her the first vial containing murine NGF, which obtained the marketing authorization in China for the treatment of some forms of peripheral neuropathies. Her dream finally became a reality. After so many years from her discovery of NGF in 1951-52 this product became an innovative instrument to alleviate suffering of human beings.

Her last original experiments led to the discovery of the role of NGF in the early stages of embryo life in chicks where it was shown to play a fundamental role in the axial rotation of the embryo (Rita Levi-Montalcini et al PNAS, 109, 2009-2014). In particular, she collaborated directly in this work with Antonino Cattaneo and many other EBRI young coworkers. This was her last scientific publication and represents an example of the exceptional neuronal plasticity in the aged brain.

Very recently we have proposed to rename EBRI as Rita Levi-Montalcini Institute in order to give tribute and the honor she deserves forever.

Now thanks to the deep relations she started with the Government and other Institutions and thanks to the selection of brilliant young researchers, EBRI is

expanding and gaining prestige on an international level.

Finally, I am pleased that we have managed to organize a meeting to give tribute to Rita Levi-Montalcini on her birthday, the 22nd of April of this year.

Many authorative personalities participated including the Economics Minister, Vittorio Grilli and the University and Research Minister, Francesco Profumo, as well as many outstanding italian and foreign scientists including the Nobel Prize Laureate Aaron Ciechanover from Tel Aviv, and the Lasker award winner Napoleone Ferrara, Moses Chao from New York University, William Mobley from University of San Diego and Philip Lazarovici from Jerusalem University.

Rita's spirit still lives in our laboratories, remains a source of inspiration and a model for young people and I am sure her heart continues to beat for us in the sky.

NEURODEGENERATION AND THE CHOLINERGIC SYSTEM

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Neurodegeneration is a feature of several chronic brain diseases which for this reason are defined neurodegenerative. Some of them are characterized by the prevalent, but not exclusive, degeneration of a type of neuron. Examples are the degeneration of the cholinergic neurons in Alzheimer's disease and dopaminergic neurons in Parkinson's disease. This review deals with neurodegenerative diseases in which a relevant loss of cholinergic neurons has been shown, including Alzheimer's disease, Parkinson's and parkinsonian diseases, and alcoholic dementia . Aims of the review are 1) to describe the alterations of the cholinergic neurons leading to their death and cholinergic denervation of some brain areas, 2) to discuss the mechanisms responsible for the loss of the cholinergic neurons, and 3) to evaluate the role of their degeneration in the clinical features of the diseases. On the basis of the existing data, it may be assumed that neurodegeneration of the cholinergic neurons in Alzheimer's disease is caused by the β-amyloid overload which exerts a direct toxic effect through p75(NTR) receptors and an indirect effect through an inflammatory reaction. The products of neuroinflammation reduce the availability of NGF, needed for the cholinergic neurons survival, and increase the level of pro-NGF which is toxic for the cholinergic neurons. In Parkinson and parkinsonian diseases, alpha-synuclein toxicity may be responsible for the degeneration of the midbrain cholinergic neurons and contribute to that of the forebrain neurons. Finally, much evidence indicate that the loss of forebrain cholinergic neurons is largely responsible for the cognitive deficits of dementias.

The term neurodegeneration refers to a progressive process of neuronal, myelin or tissue breakdown resulting in changes in the morphology and function of neurons usually leading to their death. The damage and death of the neurons is associated with an inflammatory response (Wiss-Coray and Mucke, 2002) which involves an extensive glia activation and plays a role in the neurodegenerative process (Varnum and Ikezu, 2012). Neurodegeneration characterizes several brain diseases which for this reason are defined neurodegenerative. The most important are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis, but also alcohol abuse and trauma may lead to neurodegeneration. Neurodegeneration followed by neuron death can be induced in experimental animals in discrete brain regions by the injection of neurotoxins (Olton and Wenk, 1987) and by inducing a neuroinflammatory response through intracerebroventricular infusion of bacterial lipopolysaccharides (Willard et al, 1999). Among the neurotoxins, ethylcholine mustard aziridinium (AF64A), an analog of choline which inhibits irreversibly high affinity choline uptake, has been shown to have some selectivity for the cholinergic neurons (Mantione et al, 1981). A

Key words: Acetylcholine, Alcoholic dementia, Alzheimer's Disease, Nerve Growth Factor, Parkinsons's Disease, Parkinsonian Diseases.

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2279-5855 (2013)

selective degeneration of the forebrain cholinergic neurons can be obtained by local injection of the immunotoxin 192 IgF saporin (Wiley et al, 1991; Ballmayer et al, 2001). The immunotoxin acts by coupling the ribosome inactivating toxin saporin to an antibody that recognizes low-affinity nerve growth factor (NGF) receptors, which are found in cholinergic neurons of the basal forebrain.

The neurodegeneration may be diffused throughout the brain involving neurons and glial cells. However, some neurodegenerative diseases are characterized by the prevalent, but not necessarily exclusive, degeneration of a type of neuron. Examples are the degeneration of cholinergic neurons in AD (Whitehouse et al, 1982) and dopaminergic neurons in PD (Hornykiewicz, 1971).

Aims of this review are: 1) to describe which cholinergic neurons degenerate, with the ensuing cholinergic denervation of some brain areas, in some neurodegenerative disease, namely AD, PD, Parkinsonian diseases and alcoholic dementia; 2) to discuss the mechanisms responsible for the degeneration of the cholinergic neurons, and 3) to evaluate the role of the loss of the cholinergic neurons in the clinical features of the diseases. The changes in the nicotinic and muscarinic receptors associated with the cholinergic denervation are beyond the scope of this review.

THE BRAIN CHOLINERGIC SYSTEM

A consensus exists on the anatomical organization of the central cholinergic system. It stems from investigations carried out about 25 years ago with the use of monoclonal antibodies to choline acetyltransferase (ChAT) first developed by Kimura et al (1981). From his study and the works of Fibiger (1982), Mesulam et al (1983), Levey et al (1984), Mufson et al (1988) and others, the following schematic description can be presented:

1) Forebrain cholinergic neurons, forming a series of nuclei in the medial septum, the diagonal band of Broca and the basal magnocellular nucleus of Meynert. Since there is no precise correspondence with anatomical structures, these nuclei are frequently identified, following the classification proposed by Mesulam et al (1983), in Ch1, medial septum, Ch 2 ascending limb of the Broca's band, Ch 3 horizontal part of the band of Broca and Ch 4 nucleus of Meynert. The cholinergic neurons represent from 50 to 75% of the cells present in these nuclei and their projections form the main cholinergic afference to the cerebral cortex, hippocampus, olfactory bulb and amygdala.

2) The cholinergic interneurons of the caudate nucleus and putamen

3) The cholinergic nuclei of the brain stem including Ch 5 in the tegmental pontine nucleus, Ch 6 in the dorsolateral tegmental nucleus, Ch 7 in the medial habenular nucleus and Ch 8 in the parabigeminal nucleus. Ch5 and Ch6 project to the thalamus, hypothalamus, pallidus and to the forebrain cholinergic nuclei, Ch 7 projects to the interpeduncular nucleus and Ch 8 to the superior colliculus.

4) The motor neurons of the spinal cord.

DISEASES WITH DEGENERATION OF CHOLINERGIC NEURONS.

Alzheimer's Disease

AD is the first neurodegenerative disease in which an extensive degeneration of the cholinergic neurons was observed. Davies and Maloney (1976) reported that in post-mortem brains from AD patients there was a marked reduction in ChAT which is responsible for acetylcholine (ACh) synthesis from its immediate precursors, choline and acetyl-coA. The finding was repeatedly confirmed (Perry et al, 1977, Pepeu et al, 1979, for reviews see Bartus et al, 1982, Hardy et al, 1985). A few years later, a 75% loss of cholinergic neurons in the forebrain cholinergic neurons of AD patients was detected (Whitehouse et al, 1982, Nagai et al, 1983) demonstrating that the decrease in ChAT activity in the cerebral cortex and hippocampus in AD depends on the degeneration of cholinergic nerve endings originating from cells located in the basal forebrain and septum. The degeneration of the cortical cholinergic nerve endings is confirmed by the decrease in the vesicular ACh transporter (Efange et al, 1997) and the loss of M2 muscarinic receptors in post-mortem samples taken from AD patients (Mash et al, 1985). In the brain, M2 receptors are mostly located presynaptically and regulate ACh release (Zhang et al, 2002). The cortical cholinergic denervation in AD was confirmed by

in vivo mapping using computed tomography and [^{123}I] iodobenzovesamicol as in vivo marker of the vesicular ACh transporter (Kuhl et al, 1996). Moreover, a highly significant linear relationship was found in autopsy samples between cortical gray matter volume and nucleus basalis (NB) cell number in controls and AD patients (Cullen et al, 1997).

The amyloid cascade hypothesis (Hardy and Allsop 1991; Hardy and Higgins, 1992) considers the deposition of the peptide β-amyloid (Aβ) the main pathogenetic event of AD. Much evidence demonstrate the toxicity of Aβ for the cholinergic neurons, as reported in the review by Pakaski and Kalman (2008). In our laboratory, we demonstrated that preaggregated Aβ injections in the NB of adult rats is followed by a decrease in the number of cholinergic neurons, identified by ChAT immunostaining, and an extensive glial reaction (Giovannelli et al, 1995). The number of ChAT-immunopositive neurons is significantly reduced in the forebrain of transgenic mice exhibiting extensive cerebral Aβ deposition and glial activation (Bellucci et al, 2006).

Two questions arise: 1) are all brain cholinergic neurons equally affected in AD ? 2) through which mechanism is Aβ causing the degeneration of the cholinergic neurons which appear to be more vulnerable than other types of neurons in AD ?

In contrast with the well demonstrated degeneration of the forebrain cholinergic neurons, Woolf et al (1989) found in post-mortem brains of AD patients that the neurons of the pontomesencephalo-tegmental (PMT) cholinergic nuclei, C5 – C8, (Mesulam et al, 1983, Mufson et al, 1988) do not degenerate in AD. In the striatum of AD patients a loss of ChAT activity and a decrease in the number of ChAT-positive neurons in the caudate nucleus has been reported (Perry et al, 1977, Rossor et al, 1982a), whereas in the putamen the cholinergic neurons are not affected (Rossor et al, 1982b; Nagai et al, 1983). Some decrease in ChAT activity in the anterior and posterior grey matter of the lumbar spinal cord has been described (Yates et al, 1989) and a dysfunction of the spinal motor neurons has been reported (Sica et al, 1998). However, there are no reports describing degeneration and loss of the ChAT-positive spinal motor neurons in AD.

The reasons of these remarkable regional differences in the degeneration and loss of cholinergic neurons in AD are not yet fully understood. Woolf et al (1989) observed that the number of plaques and tangles, the histopathological landmarks of AD, is smaller in the brain stem than in the cerebral cortex, and amygdala of subjects affected by AD and therefore the brain stem neurons are less affected by the disease. More important is the observation that the basal forebrain cholinergic neurons bind NGF whereas PMT cholinergic neurons do not, although phenotypically similar (Richardson et al, 1986). According to Kordover et al (1988) there is an extensive NGF binding to ChAT-positive cells of the Ch1 – Ch4 regions whereas the binding density in the putamen is much lower. Woolf et al, (1989) found that approximately 92% of all cholinergic neurons in the basal forebrain possess receptors for NGF but these receptors were not found in association with ChAT-positive somata in the pedunculopontine and laterodorsal tegmental nuclei. Pioro and Cuello (1990) reported that the degree of overlap between NGF receptor- and ChAT -containing regions in the brainstem is not as great as in the forebrain.

NGF binds to two completely different cell surface receptors—the Trk tyrosine kinase receptors, namely TrkA, and the shared p75(NTR) receptor (Roux and Barker, 2002) with high and low affinity, respectively. The expression of TrkA mRNA was found to be restricted to neurons of the basal forebrain, caudate-putamen with features of cholinergic cells and to magnocellular neurons of several brainstem nuclei (Merlio et al, 1992). The differences in NGF receptor expression of the cholinergic neurons in different brain regions offer a basis for their different dependence on NGF supply during adulthood for the maintenance of their biochemical and morphological phenotype. Basal forebrain cholinergic neurons are greatly reduced in adult mice in which phenotypic knockout of NGF was achieved by expressing transgenic anti-NGF antibodies (Ruberti et al, 2000). Unfortunately, the brain stem cholinergic neurons were not examined but, since the mice motility was not impaired, we may assume that the motor neurons are much less NGF dependent. The trophic importance of NGF for the cholinergic forebrain neurons was also confirmed by the observation that intracerebroventricular administration of NGF ameliorates their age-

associated atrophy in old rats (Fisher et al, 1987). Moreover, NGF infusions prevent the degeneration of the ChAT-positive neurons in the NB induced by local excitotoxin injection in aging rats (Casamenti et al, 1994).

Since the constant presence of NGF is necessary for the survival of the forebrain cholinergic neurons, the question arises whether their degeneration in AD depends on a reduced availability of NGF. According to Mufson et al (2003), brain NGF levels remain stable throughout the course of AD and appear to be sufficient to support the cholinergic plasticity changes occurring during the initial phases of the disease. On the basis of post-mortem studies and animal experiments, Cuello et al (2010) propose that in AD a NGF deficit results from a dysregulation of the NGF maturation cascade caused by an impaired conversion of proNGF to mature NGF and an acceleration of mature NGF degradation. According to Bruno et al (2009), the dysregulation detected in postmortem brains of AD patients can be reproduced in naïve rats by the intracerebral injection of Aβ oligomers causing microglial activation and the ensuing release of inflammatory factors. Therefore, according to the pathogenetic mechanism proposed by Cuello et al (2010), the degeneration of the forebrain cholinergic neurons in AD begins with the canonical excess in Aβ formation and deposition associated with an extensive neuroinflammatory response which leads to NGF metabolism dysregulation. This mechanism may also explain the cholinergic neuron degeneration induced in adult rats by NB injection of preaggregated Aβ . The degeneration is associated with microglia, astrocyte activation and a strong inflammatory reaction characterized by IL-1ß production and an increased inducible cyclooxygenase and nitric oxide synthase expression (Giovannini et al, 2002).

ProNGF, whose increase was observed in postmortem AD brains (Bruno et al, 2009) and in the hippocampus of subjects with mild cognitive impairment (MCI) and AD (Mufson et al, 2012), has been shown to mediate cell death in PC12 cells (Armugan et al, 2012) and oligodendrocytes (Beattie et al, 2002), through an apoptotic mechanism involving P75(NTR) receptors. Moreover, NB cholinergic neurons in subjects affected by mild to moderate AD, displayed a significant down regulation of TrkA, TrkB and TrkC expression during the progression of the disease whereas no change in p75(NTR) expression was detected (Ginsberg et al, 2006). Trk downregulation was associated with the cognitive decline. Mufson et al (2007), comparing post mortem samples of subjects with no cognitive impairment, MCI and early AD, observed that, although the number of ChAT-positive neurons in the NB was not significantly different, there was a significant reduction in the number of TrkA but not p75(NTR) receptor-containing neurons, which co-localize with ChAT, in the MCI and early AD brains. This finding indicates that in the initial AD stages there is a decrease in the response of the cholinergic neurons to neurotrophic factors. They also observed an increase in proNGF in the cortex of subjects with MCI and early AD. Since proNGF accumulates in the presence of reduced cortical TrkA and high level of p75(NTR) receptors, a shift between molecules facilitating survival and damaging molecules seems to take place in prodromal AD. The degeneration of the forebrain cholinergic neurons may therefore be caused by a decrease in NGF availability and number of Trk binding sites, resulting in a loss of trophic effect, compounded with a proNGF apoptotic effect mediated through the binding to p75(NTR) receptors.

Using rat cortical neurons and NIH-3T3 cell line engineered to stably express p75(NTR), Yaar et al (1997) demonstrated that the Aβ peptide specifically binds to p75(NTR). Furthermore, 3T3 cells expressing p75NTR , but not wild-type control cells lacking the receptor, undergo apoptosis in the presence of aggregated Aβ. By using neuroblastoma cell clones engineered to express p75(NTR), Perini et al (2002) showed that p75(NTR) is involved in the direct signaling of cell death caused by Aβ via the function of its death domain. This signaling leads to the activation of caspases-8 and -3, the production of reactive oxygen intermediates and the induction of an oxidative stress. They also showed that the direct mechanism of neuronal damage activated by Aβ acts synergistically with the inflammatory reaction induced by Aβ. Indeed, TNF-α and IL-1β, cytokines produced by Aβ -activated microglia, may potentiate the neurotoxic action of Aβ mediated by p75(NTR) signaling. These results indicate that neurons expressing p75(NTR), if expressing

also proinflammatory cytokine receptors, may be preferential targets of the cytotoxic action of Aβ in AD. The authors suggest that the high level of expression of p75(NTR) of the basal forebrain cholinergic neurons may be the reason of their vulnerability in AD, whereas the cholinergic neurons of the brainstem, which do not express p75(NTR), remain undamaged.

The deletion of p75(NTR) receptor in a transgenic model of AD (Thy1-hAPP(Lond/Swe) x p75(NTR-/-) mice) significantly diminished hippocampal neuritic dystrophy and completely reversed the basal forebrain cholinergic neurite degeneration in comparison with AD mice expressing wild-type p75(NTR). Aβ levels were not affected, suggesting that removal of p75(NTR) extracellular domain reduced the ability of excess Aβ to promote neuritic degeneration (Knowles et al, 2009). These findings indicate that although p75(NTR) likely does not mediate all Aβ effects, it does play a significant role in enabling Aβ-induced neurodegeneration in vitro and in vivo. Further references on the interaction between Aβ and p75(NTR) receptors can be found in a recent review by Patel and Jhamandas (2012).

Concluding this paragraph, we may assume, on the basis of the results reported above, that neurodegeneration of the cholinergic neurons in AD is caused by an Aβ overload which exerts a direct toxic effect through p75(NTR) and an indirect effect through the inflammatory reaction. The products of neuroinflammation reduce the availability of NGF needed for the cholinergic neurons survival, increase the level of proNGF which in turn exerts a toxic effect on the cholinergic neurons, and synergize the toxicity of Aβ. The difference in the expression of p75(NTR) among cholinergic neurons may explain the higher vulnerability of the forebrain cholinergic neurons in comparison to the spinal and brain stem cholinergic neurons. The pivotal role of the inflammatory reaction in the degeneration of the cholinergic neurons induced by Aβ is confirmed by the finding that chronic lipopolysaccharide infusions produced a time-dependent, but not dose-dependent, decrease in cortical ChAT activity that paralleled a decline in the number of ChAT - and p75-immunoreactive cells and a dense distribution of reactive astrocytes and microglia within the basal forebrain (Willard et al, 1999). Moreover, in the rat, the anti-inflammatory drug rofecoxib, a selective cyclooxygenase-2 inhibitor suppresses brain inflammation and protects the forebrain cholinergic neurons from the degeneration induced by Aβ injection into the NB (Giovannini et al, 2003). Finally, using tissue from subjects with no cognitive impairment, MCI, and AD and a double staining for visualizing phosphorylated tau protein and p75(NTR) expressing cells, it has been shown (Vana et al, 2011) that the increase in the number of neurons of the NB showing accumulation of tau protein is accompanied by a decrease in that of cholinergic neurons identified by p75(NTR) immunostaining. This finding suggests that also the neurofibrillary tangles within the cholinergic neurons may play a role in their degeneration during AD.

It is pertinent to remind that the degeneration of the cholinergic neurons in AD, although an important and characteristic feature of this disease, is accompanied by a diffuse neuronal damage and synaptic loss (Hardy et al, 1985; Hamos et al, 1989) involving other neurotransmitter systems (Zweig et al, 1988; Halliday et al, 1992; Lai et al, 2007), presumably caused by the Aβ peptide toxicity acting directly and indirectly through the products of the associated inflammatory reaction.

Other dementias

Although PD is considered a motor disease characterized by a degeneration of dopaminergic neurons, it may present also non motor symptoms including cognitive deficits and dementia. A moderate Aβ load was demonstrated in vivo by [^{11}C] PIB positron emission tomography (PET) in the cortex and subcortical structure in PD brains (Edison et al, 2008), in association with Lewy's bodies (see below) which are a characteristic feature of PD (Spillantini, 1999).

A loss of cells in the NB was described by Whitehouse et al (1983) in postmortem brains of 12 subjects affected by PD and this finding was repeatedly confirmed (see ref in Bigl et al, 1990, Tiraboschi et al, 2000). In vivo demonstration of the cholinergic denervation was obtained with neuroimaging techniques using ligands for vesicular ACh transport (VAChT) and acetylcholinesterase (AChE) (see ref. in Bohnen and Albin, 2011) A denervation of the limbic archicortex in PD patients was demonstrated by PET using [^{11}C]methyl-4-piperidinyl propionate

to visualize AChE and the level of denervation correlated with olfactory dysfunction (Bohnen et al, 2010). A thalamic cholinergic denervation was demonstrated (Kotagal et al, 2012) in subjects affected by PD, PD with dementia (PDD), and Lewy body dementia (LBD) but not in AD. Hilker et al (2005) reported significant reductions of cortical AChE in PD without dementia but severe reductions in PD with dementia. Dementia in PD subjects is sporadic and may begin one or more years after the onset of the motor symptoms. The development of severe cognitive deficits before or together with the motor symptoms is a characteristic feature of LBD. LBD is a type of dementia closely associated with both AD and PD. It is characterized anatomically by the presence of Lewy bodies (LB) which are clumps of α synuclein and ubiquitin proteins in neurons, detectable by post mortem brain histology (Kalra et al, 1996; Perry et al, 1997, Spillantini, 1999). The Aβ load is significantly raised in most LBD cases, as demonstrated in vivo by PET visualization (Edison et al, 2008). Two forms are described, the AD variant with LB and the diffuse Lewy Body Disease and in both forms a marked decrease, up to 75%, in ChAT activity in the midfrontal cerebral cortex and in the hippocampus was observed (Tiraboschi et al, 2000). The loss of ChAT activity is less severe and occurs later in the clinical course of AD than in LBD in which ChAT loss is already prominent in the earliest stages of the illness (Tiraboschi et al, 2002).

In post mortem brains of LBD subjects, as well as in subjects with multiple system atrophy, a significant loss of cholinergic neurons was detected by Schmeichel et al (2008) in the pedunculopontine nucleus (PPN) and the laterodorsal tegmental nuclei (Ch 5, Ch 6) which are spared in AD. The degeneration of the Ch5 and Ch6 cholinergic nuclei leads to the cholinergic denervation of the thalamus observed in LBD and PD but not in AD by Kotagal et al (2012) by measuring PPN-Thalamic AChE activity by PET imaging.

A cholinergic deficit has been also observed in atypical parkinsonian diseases. Tagliavini et al (1984) and a few other authors (see ref in Bigl et al, 1990) reported a significant loss of neurons in the NB in subjects affected by progressive supranuclear palsy (PSP).This disease, characterized by clinical features including extrapyramidal symptoms, ocular dyscontrol and cognitive impairment, is considered a tauopathy for the extensive neurofibrillary cluster of phosphorylated tau protein detected in the brains (Boewe, 2012). VAChT expression and ChAT activity in caudate nucleus and putamen were also found to be markedly decreased in postmortem brains of subject with PSP, consistent with a selective loss of striatal cholinergic interneurons (Suzuki et al, 2002).

Shinotoh et al (1999) reported a modest reduction in cortical AChE activity in patients with PSP, smaller than in PD subjects, and a 38% reduction in the thalamus. The latter result indicates a significant loss of brainstem cholinergic PPN neurons. Therefore, it appears that in this atypical parkinsonian disease there is a widespread alteration of the cholinergic neurons involving the cholinergic forebrain nuclei, the striatal cholinergic interneurons and brain stem cholinergic nuclei.

The corticobasal syndrome also belongs to the atypical parkinsonian diseases and shows a decrease in AChE activity, demonstrated by neuroimaging. The decrease was observed in the paracentral region and the frontal, parietal and occipital cortices (Shinotoh et al, 1999; Hirano et al, 2010) which are projection areas of the NB cholinergic neurons. At variance with PSP, with which the corticobasal syndrome is frequently confused (Stripp, 2011), no reduction in thalamic AChE activity was detected. Both diseases are defined tauopathies and are characterized by neurofibrillary pathology.

The frontotemporal dementias, which represent in prevalence the second group of senile dementias (Snowden et al, 2002) are also characterized by neurofibrillary pathology. However, no decrease in cortical or thalamic AChE was observed by neuroimaging (Hirano et al, 2010). They are characterized by personality, behavior disturbances, limited memory loss, and present several variants on the basis of the nature of the characteristic protein inclusions (Goedert et al, 2012). Pick's disease is included in the frontotemporal dementias (Kerstez, 2004). The studies of the cholinergic neurons of the NB report contrasting findings (Bigl et al 1990) ranging from no loss of cholinergic neurons to a 70% decrease. However, according to Hansen et al (1988), ChAT levels were normal in 5 cases of Pick's disease whereas they were reduced in AD cases studied by comparison.

Mechanisms responsible of the degeneration of the cholinergic neurons in Parkinson's disease and atypical Parkinsonian diseases.

As described above, in AD the loss of cholinergic neurons is confined to the cholinergic forebrain nuclei. Their degeneration is attributed to a dysregulation of NGF formation and metabolism presumably caused by Aβ toxicity and the associated extensive inflammatory response (Cuello et al, 2010; Mufson et al, 2007) and to direct Aβ toxicity. The degeneration of the cholinergic neurons in PD and the other neurodegenerative diseases which share the presence of tau neurofibrils, neurofibrillary tangles, and Lewy bodies, shows different patterns. In PD, PD with dementia and LBD there is a loss of forebrain cholinergic neurons (Bigl et al, 1990; Hiker et al, 2005; Tiraboschi et al, 2000) which may be caused by the presence of an Aβ load and Aβ plaques, particularly in LBD, through the mechanisms discussed above. However, in PD and LBD there is a degeneration of the midbrain cholinergic neurons located in Ch 5 and Ch 6 (Schmeikel et al, 2008) and a thalamic cholinergic denervation which do not occur in AD. The midbrain cholinergic neurons do not express NGF receptors (Woolf et al, 1989) and are less dependent on NGF supply. In PSP, besides the loss of the forebrain and midbrain cholinergic neurons, a degeneration of the striatal cholinergic interneurons was described (Suzuki et al, 2002). Therefore, different mechanisms should be responsible of the degeneration of the cholinergic neurons. Finally, the frontotemporal dementias, including Pick's disease, do not show a consistent loss of forebrain cholinergic neurons and a significant cortical and thalamic cholinergic denervation. Since the frontotemporal dementias are tauopathies characterized by neurofibrillary deposits, it appears that tau is not particularly toxic for the cholinergic neurons, even if its neurotoxicity is well documented in hippocampal organotypic slice cultures (Messing et al, 2012). Indeed, no obvious differences in the distribution and density of cholinergic and monoaminergic neurons were found comparing tau filament forming transgenic mice with wild type mice (Morcinek et al, 2012). Conversely, in A30P α-synuclein-expressing transgenic mice, a degeneration of the forebrain cholinergic neurons was observed after dopamine depletion induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration (Szego et al, 2011). This observation was confirmed and extended by Szego et al (2013) who reported that the number of neurons expressing a cholinergic marker in the medial septum-diagonal band of Broca complex decreases in A30P α-synuclein-expressing mice during aging, paralleled by a lower AChE fiber density in the dentate gyrus and in the hippocampal CA1 field. After inducing dopamine depletion by MPTP, no acute but a delayed loss of cholinergic neurons and AChE-positive fibers was observed, which was attenuated by L-3,4-dihydroxyphenylalanine (DOPA) treatment. However, P301L tau transgenic pR5 mice, overexpressing α-synuclein, develop neurofibrillary lesions but do not show the degeneration of basal forebrain cholinergic neurons observed in Alzheimer's disease (Koehler et al, 2010). It appears that α-synuclein toxicity for the cholinergic neurons is reinforced by age and dopamine depletion, a situation occurring in PD and parkinsonian diseases and therefore α synuclein may be responsible for the degeneration of the midbrain cholinergic neurons and contribute to that of the forebrain neurons.

Alcoholic dementia

Chronic ethanol abuse may lead to alcoholic dementia and the related Korsakoff's syndrome and Wernicke's encephalopathy (WE), whose cognitive deficits mimic AD. The first observation of a loss of cholinergic neurons in the NB of subjects affected by Korsakoff's syndrome was made by Arendt et al (1983). No significant decrease in the number of cholinergic neurons was found in chronic alcoholism without dementia. The loss of cholinergic neurons concurs with the large decrease in ChAT activity detected in the cortex, hippocampus and cerebellum of subjects with alcoholic dementia by Antuono et al (1980). The degeneration of the cholinergic neurons caused by ethanol abuse can be reproduced in the rat. After 6 months of ethanol intake, a loss of cholinergic neurons, affecting the forebrain nuclei, but sparing the brain stem Ch 5 and Ch6 nuclei, was observed by Arendt et al (1988) and was accompanied by a decrease in AChE activity in the cerebral cortex, hippocampus and amygdala indicating a cholinergic denervation. In rats drinking ethanol for 6 months, the decrease in ChAT activity and ACh release in the

cortex and hippocampus, together with the cognitive impairment, was observed even after four weeks withdrawal (Casamenti et al 1993). Investigating the mechanism of the neuronal degeneration, induced by ethanol abuse, Cullen and Halliday (1995a) observed that in chronic alcoholics with thiamine deficiency, neurofibrillary pathology was found in the NB, but in no other brain regions. Neurofibrillary tangles were not seen in age-matched controls and were infrequent in alcoholics without neuropathological signs of thiamine-deficiency. Neurofibrillary tangles were most numerous in the cases showing NB cell loss. The authors concluded that NB neurodegeneration in chronic alcoholics proceeds through the formation of neurofibrillary tangles. Extending their investigations (Cullen and Halliday, 1995b), they observed that tau-positive granular and fibrillary inclusions were frequently observed within the magnocellular neurons of the NB in WE subjects, occasionally in non-WE alcoholics, and never in controls. Tau immunoreactivity was not observed in cortical, brainstem, diencephalic or non-cholinergic forebrain structures. The majority of neurons in the basal forebrain showed increased peroxidase activity in all WE alcoholics and in some NB neurons of non-WE alcoholics, but was rarely seen in controls. These results suggest a link between peroxidase activity and the abnormal accumulation of phosphorylated tau. The presence of tau in the NB of alcoholics with WE suggests a thiamine-dependent mechanism in tau accumulation and cell death in the cholinergic basal forebrain. Thiamine deficiency is a common consequence of alcohol abuse and the consequences of thiamine deficiency on ACh synthesis have been demonstrated long time ago (Heinrich et al, 1973). Thiamine deficiency is therefore an important factor in the dysfunction of the cholinergic neurons in alcoholic dementia. However, it does not explain why the degeneration occurs almost exclusively in the Ch 1–Ch 4 nuclei. A neurotoxic mechanism involving neuroinflammation and possibly NGF dysfunction may be hypothesized. It must be mentioned that in organotypic brain slices of the NB exposed to ethanol, NGF, inhibition of MAPK p38 and NOS protected the cholinergic neurons against the ethanol-induced effect (Ehrlich et al, 2012) confirming the complexity of the mechanism leading to cholinergic cell degeneration in this pathology.

CONSEQUENCES OF THE DEGENERATION OF THE BRAIN CHOLINERGIC SYSTEM.

The most evident consequence of the degeneration of the forebrain cholinergic system is a cognitive deficit. More than thirty years ago in a seminal paper Drachman (1977) asked whether the cholinergic system has a specific role in memory and cognitive functions in man. Shortly later, the cholinergic hypothesis of geriatric memory dysfunction (Bartus et al, 1982) was proposed and was followed by countless papers investigating and demonstrating the role of the cholinergic system in learning and memory. An analysis of these papers is beyond the scope of this article and therefore we refer the reader to a few recent reviews (Hasselmo, 2006 Pepeu and Giovannini, 2006, Woolf and Butcher, 2010; Benarroch, 2010; Hasselmo and Sarter, 2011). ACh in the brain alters neuronal excitability, influences synaptic transmission, induces synaptic plasticity, and coordinates firing of groups of neurons. As a result, it changes the state of neuronal networks throughout the brain and modifies their response to internal and external inputs (Picciotto et al, 2012). The administration of anticholinergic drugs to humans and animals, the use of cholinergic receptor knockout mice and the lesioning of the forebrain cholinergic neurons in animals result in deficit of attention, impairment in information acquisition and amnesia. Since dementias are characterized by multiple cognitive deficits including the impairment of memory (DSM IV), it may be assumed that the loss of forebrain cholinergic neurons is largely responsible for the cognitive deficits of dementias. On this assumption, cholinesterase inhibitors are used as therapeutic agents in AD with the aim to enhance the residual cholinergic function (Giacobini, 2000).

In PD and LBD a degeneration of the midbrain cholinergic neurons located in the Ch 5 and Ch 6 nuclei was observed, with or without the concomitant loss of Ch1–Ch4 neurons. The midbrain cholinergic neurons innervate the spinal cord, brain stem, thalamus, hypothalamus, basal forebrain and medial frontal cortex and are involved in arousal and attention, the sleep-wakefulness cycle and

the regulation of muscular tone during REM sleep (Woolf and Butcher, 2011). Their loss may be a cause of the sleep behavior disorders in DLB (Schmeichel et al, 2008).

Since the first observation in transfected cultured cells (Nitsch et al,1992) that stimulation of M1 and M3 muscarinic receptor subtypes increased the basal secretion of amyloid precursor protein (APP), the possible role of the brain cholinergic system in the regulation of Aβ peptide metabolism has been the object of much investigations (see ref in Pakalski and Kalman, 2008). Experiments "in vitro" and in murine AD models showed that M1 receptors have a role in APP secretion via alpha-secretase activation and in decreasing Aβ levels, via beta-secretase inhibition (Fisher et al, 2003). Therefore it has been assumed that in AD the degeneration of the cholinergic neurons, with the ensuing cholinergic hypofunction, may aggravate the Aβ overload which is considered its main pathogenetic mechanism.

ACKNOWLEDGEMENTS

The work was supported by the University of Florence and the Foundation Cassa di Risparmio di Firenze, grants ECRF 2010.0312 and 2011.0395.

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EMERGING ROLE OF T-TYPE CHANNELS IN NEURODEGENERATION

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T-type channels are a class of voltage-gated Ca^{2+} channels that open at voltages close to resting membrane potential and show fast inactivation and slow inactivation kinetics. Because of their gating properties, T-type channels take part to burst firing in neurons and their role in pathophysiology has been traditionally linked to disorders of excitability like epilepsy. Conversely, their possible implication in neurodegeneration has been neglected so far. In the present paper we discuss a number of arguments suggesting that, instead, these channels have an important role in diverse neurodegenerative diseases. First we will report evidence showing that, because of their biophysical properties, T-type channels are expected to be implicated in neurodegeneration considering, for instance, that they can conduct steady inward Ca^{2+} currents at moderately depolarized membrane potentials. In addition, we will go through some features of T-type channels regulation that make them obvious candidates in neurodegeneration, like their redox sensitivity, modulation by Zn^{2+} or epigenetic regulation by transcription factors implicated in neurodegenerative diseases like REST or EGR1. Finally, we will review data showing that both the pharmacological modulation and the genetic ablation of T-type channels modify the progression of neuronal damage in experimental models *in vivo* and *in vitro* of diverse neurodegenerative conditions. In particular, we will show that available evidence suggests that neuroprotection can be afforded by suppressing T-type channel activity in epileptogenesis, stroke and, possibly, in brain aging, whereas T-type channel inhibition exacerbates neuronal cell damage in experimental models of motor disorders like Parkinson's disease and essential tremor.

Voltage gated calcium channels (VGCC) are a heterogeneous family of ion channels with remarkable degree of ionic selectivity that let Ca^{2+} ions enter into the cytoplasm in response to membrane depolarization. Based on their gating properties they have been traditionally subdivided into two large subfamilies, High Voltage Activated (HVA) channels that require strong membrane depolarization for opening, and low voltage activated channels (LVA) that, instead, open at voltages closer to resting membrane potential. While a huge amount of work has been performed during the last 30 years to investigate the role of HVA channels in neurodegeneration though often with controversial results (Cataldi, 2013; Yagami et al, 2012) much less attention has been paid to LVA channels. However, because of their biophysical properties and of their mechanism of regulation, these ion channels appear well suited to take part to neurodegeneration. In the present paper, we will examine the biophysical and regulatory features supporting the involvement of T-type channels in neurodegeneration and we will review the emerging evidence showing that they take part, indeed, in the pathophysiology of specific

Key words: aging, epileptogenesis, essential tremor, stroke, Parkinson's disease, spinobulbar Muscular Atrophy, T-type channels, voltage-gated Ca^{2+} channels.

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2279-5855 (2013)

neurodegenerative disorders.

BIOPHYSICAL PROPERTIES OF T-TYPE CHANNELS THAT ARE RELEVANT FOR NEURODEGENERATION

In native neuronal preparations T-type channels were identified as different from the HVA channels because of their distinctive gating properties that include channel opening at voltages close to resting membrane potential (between -65 and -50 mV), rapid deactivation and almost complete inactivation at moderately depolarized voltages (Carbone and Lux, 1984a and b; Fox et al, 1987; Fedulova et al, 1985; see also Perez-Reyes, 2003 for review). The molecular characterization of these ion channels proved much more difficult than that of HVA channels and the first T-type channel pore-forming subunit was cloned only in 1998 by E. Perez-Reyes and coworkers who used an in silico approach for gene discovery (Perez-Reyes et al, 1998). This subunit was dubbed α_{1G} and is currently designed as $Ca_V3.1$. Shortly thereafter two other members of the T-type channel family were cloned, α_{1H} and α_{1I}, now indicated as $Ca_V3.2$ and $Ca_V3.3$ (Cribbs et al, 1998; Lee et al, 1999). Electrophysiological studies performed in expression systems unraveled significant differences among these isoforms. The $Ca_V3.1$ and $Ca_V3.2$ subunits were, indeed, closer each other and to the T-type currents of native preparations than the $Ca_V3.3$ channels that diverged from them because of their slower inactivation (see Tab. 1) (Perez-Reyes, 2003). Different splicing forms have been identified for Ca_V3 channels and data are emerging suggesting a functional diversity of these isoforms thus expanding the potential complexity of T-type currents (Chemin et al, 2001; Emerick et al, 2006; Murbartián et al, 2004; Monteil et al, 2000; Zhong et al, 2006). The three cloned Ca_V3 isoforms have been all identified in the brain though their relative expression shows regional diversities (Talley et al, 1999).

Because of their activation at voltages close to membrane resting potential and of their tendency to inactivate at moderately depolarized voltages, T-type channels have been traditionally linked to burst firing in neurons (Cueni et al, 2009; Perez-Reyes, 2003). The mechanistic base of T-type dependent low-threshold calcium spikes leading to burst firing has been clearly defined (Cueni et al, 2009). Briefly, when T-type channels open during a Ca^{2+}-dependent action potential and let Ca^{2+} ions enter the neuronal cytoplasm, membrane depolarization occurs and a burst of Na^+-dependent action potential is generated by the opening of voltage gated Na^+ channels. Na^+ influx makes the membrane further depolarize and T-type channels inactivate whereas Ca^{2+}-sensitive K^+ conductances are activated. This chain of events leads to membrane hyperpolarization and the recovery of T-type channel from inactivation. The repolarization process triggers I_h channel opening causing membrane depolarization and T-type channel opening thus starting again the process that leads to burst firing generation (Cueni et al, 2009; Perez-Reyes, 2003). Burst firing is the electrophysiological signature of a high expression of T-type channels in neurons. In the next sections we will find that diverse neurodegenerative conditions are accompanied by an increase in the percentage of burst firing neurons suggesting that a higher T-type expression does occur. We will also present evidence that the switching from repetitive firing to burst firing could have a profound functional impact on neuronal network activity by affecting excitability or plasticity. While these considerations seem to suggest that they open only transiently during action potentials, in specific conditions T-type channels can also carry steady inward currents. This is due to the partial overlap of their voltage-dependence of activation and the voltage dependence of inactivation curves. When, indeed, resting membrane potential stands in this overlap region where not all the channels are inactivated and some of them have already begun to open, there will be a significant fraction of the channel population stably open and giving rise to an inward *window current* (Zhang et al, 2013; Perez-Reyes, 2003). Because the T-type window current is located at voltages only slightly more depolarized than resting membrane potential it has been speculated that a steady T-type inward Ca^{2+} current could be originated early in neurons that start to depolarize when exposed to diverse neurotoxic insults.

T-TYPE CHANNEL REGULATORY MECHANISMS POTENTIALLY RELEVANT IN NEURODEGENERATION

While a wealth of information is available on the

mechanisms regulating HVA currents in neurons, our knowledge of T-type channel regulation is still largely incomplete and data on the modulation of neuronal T-type currents by neurotransmitters are just starting to be accumulated (cf. Zhang et al, 2013 for review). Of special interest in the perspective of our review is, however, that some of the regulatory mechanisms that have been identified so far could be crucially relevant in neurodegeneration. First, it has been noticed that the amplitude of T-type currents increases parallel to $[Ca^{2+}]_i$ (Lu et al, 1994; Barret et al, 2000; Tseng and Boyden, 1991). This stands quite apart from what observed in HVA channels whose regulation is dominated by Ca^{2+}-dependent inactivation. Intriguingly, Ca^{2+}-dependent potentiation of T-type currents seems to be isoform-specific being specifically observed in $Ca_V3.2$ channels. The mechanism of $Ca_V3.2$ Ca^{2+}-dependent regulation has been elucidated and shown to be mediated by channel phosphorylation operated by Ca^{2+}-calmodulin dependent kinase II (CaMKII). Intriguingly, it has been shown that calmodulin (CaM) docks to the $Ca_V3.2$ channel subunit and that this docking contributes to amplify CaMKII activation (Lu et al, 1994; Yao et al, 2006). Considering the role of calmodulin and CaMKII in neurodegeneration (Ashpole et al, 2012; Goebel, 1999; Shirasaki et al, 2006; Vest et al, 2010), these observations could have important implications suggesting that T-type channels could be among the effectors of the transductional cascade activated by the influx of Ca^{2+} ions and their binding to CaM. An additional mechanism regulating T-type channels that could be relevant in neurodegeneration involves Zn^{2+}, a transition metal that has a major role in causing neuronal cell death in neurodegenerative diseases (Frederickson et al, 2005). Zn^{2+} is stored in and released from glutamatergic terminals both in physiological and in pathological conditions, like stroke, in which a massive release of glutamate from synaptic terminals takes place (Stork and Li, 2009). Long time ago already, it was established that this metal ion has a role as a modulator of a plethora of ion channels also including T-type channels. In particular, it was reported that Zn^{2+} blocks Ca^{2+} permeation through VGCC by interacting with the channel pore (Harrison and Gibbons, 1994). More recently we and others described a new regulatory effect of Zn^{2+} on T-type channels consisting in a profound slowing of channel deactivation (Cataldi et al, 2007; Traboulsie et al, 2007). As in the case of the modulation by Ca^{2+}/calmodulin also this effect of Zn^{2+} is isoform-specific being restricted to $Ca_V3.3$ channels. Zn^{2+}-induced slowing of channel deactivation is expected to greatly increase Ca^{2+} influx through T-type channels though this effect could be partly masked by the inhibitory effect of this ion on permeation. This is due to the fact that, even in control conditions, i.e. in the absence of Zn^{2+}, in neurons firing action potentials the majority of Ca^{2+} influx through T-type channels takes place during channel deactivation when the channel pore stays open and electrochemical gradient greatly favors Ca^{2+} permeation (Cueni et al, 2009). Moreover, channel deactivation lasts much longer in LVA than in HVA channels. Therefore, T-type channels may carry large amount of Ca^{2+} in neurons firing in bursts because they repeatedly deactivate during the down-stroke phase of each action potential. Due to the effect of this metal ion on T-type channel deactivation, it is expected that, in the presence of Zn^{2+}, much more Ca^{2+} enters through these channels in spontaneously firing neurons. In addition, computer simulation suggested that in the presence of Zn^{2+} neuronal excitability should be significantly enhanced and this hypothesis was confirmed both in current clamp experiments and in field recordings in thalamic slice preparations (Cataldi et al, 2007; Traboulsie et al, 2007). Intriguingly, Zn^{2+} modulation of T-type channels could be strictly related with another important regulatory mechanism that is also expected to be relevant in neurodegeneration: channel modulation by redox status (Todorovic et al, 2001). Oxidants cause, indeed, a marked inhibition of channel activity that is instead significantly enhanced in the presence of reducing agents (Zhang et al, 2013). The pathophysiological implications of T-type channel regulation by redox status has been extensively investigated in the case of pain perception whereas it remains unaddressed in neurodegeneration. Intriguingly, T-type channel regulation by Zn^{2+} and by redox status seems to converge to common molecular mechanism. Elegant mutation studies showed, indeed, that the reducing agent L-cysteine does increase $Ca_V3.2$ channel currents by relieving Zn^{2+} blockade because a single

His residue located in position 191 is responsible both for channel potentiation by this reducing compound and for blockade by Zn^{2+} (Nelson et al, 2007). These findings suggest that Zn^{2+} regulation of T-type channel currents could be finely modulated by changes in redox status. Both Zn^{2+} release and free radical generation take place in response to hypoxia in the brain and could, therefore, affect T-type activity in this condition. T-type channels could, therefore, act as specific sensors of anoxic conditions in the brain. This view is further supported by the evidence T-type channel activity/expression is regulated by hypoxia independently from changes in free radical generation or Zn^{2+} release. Fearon and coworkers (2000) demonstrated, indeed, that acute hypoxia causes a decrease in the amplitude of cloned $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ channels without affecting channel gating properties. Also in this case, the T-type channel isoform more intensely affected was $Ca_V3.2$. Interestingly, $Ca_V3.2$ channel modulation by hypoxia was independent from changes in the redox status (Fearon et al, 2000). Significant changes in $Ca_V3.2$ channel expression occur upon chronic exposure to hypoxia. This was demonstrated in PC12 cells and in primary cultures of rat chromaffin cells where a prolonged exposure to hypoxia induced the transcription of the $Ca_V3.2$ gene via a HIF-2α-dependent mechanism (Carabelli et al, 2007; Del Toro et al, 2003).

The hypothesis that T-type channels could be involved in cell survival and/or proliferation is supported also by data obtained in cancer cells. T-type channel hyperexpression has been observed, indeed, in a number of different cancer histotypes also including gliomas and it has been shown that the pharmacological blockade of T-type channel causes apoptotic cell death in these neoplastic cells (cf. Santoni et al, 2012). Studies aiming to identify the mechanism responsible for T-type regulation of cell survival in tumors, unraveled new unexpected transduction pathways involving these channels that could be relevant also in neurons. For instance, Choi et al (2005) showed that $Ca_V3.1$ opening causes the activation of p21 ras, a small GTPase with a marked antiapoptotic and neuroprotective activity (Shi et al, 2011). It is still controversial whether T-type channels are regulated by neurotrophic factors via tyrosine phosphorylation. Such a mechanism has been demonstrated for L-type Ca^{2+} channels that are activated by tyrosine kinases as after the exposure to the neuroprotective growth factor IGF-I (Cataldi et al, 1996; Bence-Hanulec et al, 2000). While, indeed, evidence has been reported for a tyrosine-kinase dependent modulation of T-type currents in spermatogenic cells (Arnoult et al, 1997) and for the involvement of tyrosine phosphatase in Angiotensin II-induced suppression of T-type currents in NG10815 neuronal cells (Buisson et al, 1995), other reports demonstrated that the effect of tyrosine kinase inhibitors on T-type currents are independent from tyrosine kinase inhibition and probably involve the direct interaction of these compounds with the channel (Cataldi et al, 2004; Kurejová and Lacinová, 2006).

To close this section, both the electrophysiological properties and some of the mechanisms of regulation of T-type channels suggest that these ion channels could take part to the cascade of events leading to cell demise in different neurodegenerative conditions.

T-TYPE CHANNELS IN NEURONAL CELL DEATH: THE PROTOTYPICAL EXAMPLE OF EPILEPTOGENESIS

The most convincing evidence that T-type channels could be involved in neuronal cell death comes from studies exploring their role in epileptogenesis. Epileptogenesis is the process by which a previously normal brain becomes chronically epileptic. This phenomenon is completely different from seizures, i.e. the generation and propagation of epileptic discharges, though it can be caused by epileptic activity occurring in status epilepticus (SE) (Pitkänen and Lukasiuk, 2011). Epileptogenesis also occurs after febrile seizures, encephalitis, brain trauma and brain ischemia or hemorrhage (Pitkänen and Lukasiuk, 2011). Extensive neuronal damage and network reorganization take place during epileptogenesis and, therefore, this process can be considered a "*bona fide*" neurodegenerative condition. Intense research efforts are directed to identify the molecular mechanisms of epileptogenesis, because this achievement could lead to the development of specific pharmacological approaches to prevent acquired epilepsy (Pitkänen, 2010). To this aim several epileptogenesis animal

models have been developed in which a chronic epilepsy with many similarities with human temporal lobe epilepsy (TLE) appears after the pharmacological induction of SE with either kainate or pilocarpine injection (Curia et al, 2008; Hellier et al, 1998). In both these animal models, after seizing during drug-induced SE, the rodents enter into a "*latent phase*" lasting several weeks during which epileptogenesis takes place, so that at its end the experimental animals become chronically epileptic. Using the rat pilocarpine model, evidence was recently provided that T-type channels could have a crucial role in epileptogeneis and, importantly for the issue of this review, in the related process of neuronal cell death. This was initially suggested by the observation that pilocarpine-induced epileptogenesis causes profound modifications in the intrinsic firing mode of pyramidal neurons (Sanabria et al, 2001). While, indeed, in normal rats, the vast majority (approximately 97%) of CA1 pyramidal neurons fire single spikes in response to current injection (Schwartzkroin et al, 1975), in rats made epileptic by pilocarpine 47% of these neurons respond with burst firing (Sanabria et al, 2001). As burst firing in neurons is usually dependent on T-type channel activity, it was suggested that the increase in burst firing neurons after pilocarpine could reflect an increase in T-type currents. This hypothesis was confirmed by the sensitivity of burst firing in pyramidal neurons from pilocarpine-treated rats to the T-type channel blocker Ni^{2+} (Sanabria et al, 2001) but not to nifedipine, μ-conotoxin GVIA or Agatoxin TK, that block L-, N- and P/Q channels (Su et al, 2002). Moreover, patch clamp experiments on acutely dissociated CA1 neurons showed a substantial increase in T-type current density in epileptic rats (Su et al, 2002). As these T-type currents were highly sensitive to Ni^{2+} it was supposed that they could be mainly carried by the $Ca_V3.2$ isoform that is extremely sensitive to the inhibition by this metal ion (Su et al, 2002). This hypothesis was confirmed by real time PCR and western blot experiments (Becker et al, 2008). The increase in $Ca_V3.2$ mRNA expression was only transient as it was detected only 2-3 days after SE; similarly, $Ca_V3.2$ protein expression increased during the first days and returned to basal values by 30 days after SE. Importantly, a similar time course was also observed for T-type dependent burst firing. A strong confirmation of the involvement of $Ca_V3.2$ channels came from the evidence that no increase in the percentage of burst firing neurons occurred when pilocarpine was used to induce SE in knockout mice for this T-type channel isoform (Becker et al, 2008). Therefore, the available evidence suggests that an increase in $Ca_V3.2$ is responsible for switching the firing mode of CA1 neurons to burst firing mode. The change in CA1 neuronal firing pattern is believed to contribute to spread neuronal activation and to activate the neuronal network responsible for interictal activity. More importantly, the increase in $Ca_V3.2$ expression could have a role in causing neuronal cell death and fiber sprouting that represent the neuropathological basis of epileptogenesis. While, indeed, in control animals, a marked cell loss was observable 50 days after SE both in pyramidal neurons and in granule cells of the CA1 and CA3 hippocampal subfields and in hilar cells, these neurons were virtually all spared in $Ca_V3.2$ knockout mice (Fig.1) (Becker et al, 2008). Importantly, cell loss in controls started to become evident only two days after SE suggesting that it was not due to Ca^{2+} influx through already expressed T-type channels but required the expression of new channels (Becker et al, 2008). Parallel to absence of neuronal cell death also fiber sprouting that is considered a consequence of tissue remodeling after neurodegeneration, was prevented in $Ca_V3.2$ knockout mice. In pilocarpine-treated rats, a significant increase in the density of Ni^{2+}-sensitive T-type currents, in $Ca_V3.2$ mRNA expression and in burst firing also occurs in thalamic reticular nuclei and in nucleus reuniens thalami (Graef et al, 2009). However, the time course of these changes is different from that reported in hippocampus becoming evident only 10 days after SE and being still detectable 30 days after (Graef et al, 2009). In addition, a transient increase also in $Ca_V3.3$ mRNA was observed during the latent period (Graef et al, 2009). In the cited paper a strong emphasis was given to the potential consequences of the enhanced T-type expression in thalamus considering that the reuniens nucleus is connected with both CA1 and the enthorinal cortex (Wouterlood et al, 1990; Vertes et al, 2006) and modulate the temporoammonic pathway, a privileged activation pathway for the epileptic hippocampus (Avoli et al, 2002). Conversely, the effect on neuronal cell survival was not explored

although in TLE significant degenerative changes are observed in the thalamus (Pulsipher et al, 2007).

The question of why and how $Ca_V3.2$ activation during the *latent phase* of epileptogenesis could lead to neuronal cell death and tissue remodeling still waits for a definite answer. As we will detail in the next sections there is some evidence that T-type channels could be implicated in neuronal cell death also in brain ischemia and in other neurodegenerative conditions. Therefore, this question could have more general implications than just epilepsy pathophysiology. The most obvious hypothesis is that these channels could act as a privileged Ca^{2+} influx system and, therefore, be involved in a Ca^{2+}-dependent death process. What makes this hypothesis intriguing is that, as we reported in the introduction, T-type channels may carry significant steady Ca^{2+} window currents because of the overlap of their activation and inactivation kinetics. This implies that when membrane potential attains values inside the T-type window a continuous inward Ca^{2+} current will be originated causing an overload of intracellular Ca^{2+}. Recently, Uchino et al (2013) provided some experimental evidence of this mechanism in a non-neuronal system. They demonstrated that in HEK-293 cells stably expressing the $Ca_V3.2$ T-type channel isoform, the T-type channel current window moves toward the values of the depolarized resting membrane potential of these cells when $[Ca^{2+}]_o$ is raised up to 7.2 mM (Uchino et al, 2013). Flow cytometric analysis showed that in these conditions a cytoplasmic Ca^{2+} overload occurs and the percentage of apoptotic cells significantly increases, being both these two phenomena prevented by the T-type blocker R-efonidipine (Uchino et al, 2013). A mitochondrial origin of the observed cell death was suggested based on the marked loss of mitochondrial membrane potential and of the increase in caspase 9 cleavage (Uchino et al, 2013).

The mechanism responsible for the increase in $Ca_V3.2$ expression in epileptogenesis is still unknown but several hypotheses have been proposed. First, it could be dependent on the increase in extracellular Zn^{2+} concentration that takes place in SE (Suh et al, 2001). As we mentioned above, released Zn^{2+} may affect both neuronal survival and excitability acting on multiple targets that also include T-type channels. In addition, Zn^{2+} may induce or repress gene transcription through the interaction of Zn^{2+}-binding transcription factors with Zn^{2+}-responsive sequences like the metal responsive element (MRE) or the recently identified Zn^{2+} transcriptional regulatory element (ZTRE) (Coneyworth et al, 2012; Jackson et al, 2008). The hypothesis that $Ca_V3.2$ could be one of the Zn^{2+}-regulated genes was raised by Ekstein et al (2012) on the basis of experiments that they performed by unilaterally injecting ZnCl into lateral ventricles of the rat. This treatment caused a significant cell death in the ipsilateral hippocampus and induced a transient increase in Ni^{2+}-sensitive T-type currents in surviving CA1 pyramidal neurons similar to that observed after pilocarpine-induced SE. Although, this was not directly proved at the molecular level of promoter regulation, it was proposed that in this experimental model Zn^{2+} was directly inducing $Ca_V3.2$ expression. The idea that Zn^{2+} could be a major regulator of T-type channel expression appears intriguing for its possible implications in neurodegenerative and neurovascular diseases where both T-type channels and Zn^{2+} could have a role (see below).

Another transcriptional mechanism that could account for the increase in $Ca_V3.2$ expression after SE involves the antagonistic regulation of the $Ca_V3.2$ gene by EGR and REST, two transcription factors known to have a crucial role in neurodegeneration. Recently, van Loo et al (2012) identified the $Ca_V3.2$ promoter as a region spanning 1400 bp upstream the transcription starting site. Eigtheen EGR1 putative binding sites map in this promoter region whereas a single putative binding site for REST is located in the first intron of the $Ca_V3.2$ gene. Chromatin immunoprecipitation studies confirmed that EGR1 binds to a subregion of the promoter located within the first 1188 bp upstream the transcription starting site. EGR1 hyperexpression in NG108-15 cells caused an increase in T-type current density suggesting that this transcription factor may control $Ca_V3.2$ gene transcription (vanLoo et al, 2011). Because previous studies clearly established that REST binding to the $Ca_V3.2$ gene is critical in suppressing the expression of this T-type channel isoform in adult cardiomyocites (Kuwahara et al, 2003; Kuwahara and Nakao, 2011; Kuwahara et al, 2005), the hypothesis that this transcriptional repressor could control also neuronal $Ca_V3.2$ channel expression was

proposed. However, when REST was hyperexpressed in neuronal NG108-15 cells it did not further reduce the expression of $Ca_V3.2$ channels. This finding was interpreted as an evidence that REST exerts a basal inhibitory control on the $Ca_V3.2$ gene that cannot be further enhanced (van Loo et al, 2011). Importantly, when it was cotransfected with EGR1 into NG108-15 cells, REST attenuated the stimulatory effect of this transcription factor on $Ca_V3.2$ expression (van Loo et al, 2011). Therefore, a model has been proposed assuming that $Ca_V3.2$ channel expression in neuronal cells is finely tuned by the relative balance of EGR1 and REST activity/expression (van Loo et al, 2011). This establishes a close casual link between changes in $Ca_V3.2$ channel expression and conditions leading to neuronal cell death and degeneration. EGR1 expression does increase, indeed, in response to neurovascular or neuronal injury, after brain ischemia and after seizures (Dragunow et al, 1992; Gass et al, 1992; James et al, 2005; Lu et al, 2003; Khachigian et al, 1996; Shikhanov et al, 2005). Moreover, in these conditions, EGR1 participates to the control of the inflammatory/reparative response by regulating the expression of inflammatory cytokines, chemokines, adhesion molecules and coagulation factors (Gashler et al, 1995; Cui et al, 1996; Haas et al, 1999; Khachigian et al, 1997; Liu et al, 2000; Yan et al, 1998 and 2000; Tureyen et al, 2008). EGR1 has a role in reparative gliosis (Beck et al, 2008) and cooperates with HIF-1 in regulating the expression of hypoxia-induced genes like VEGF (Sharp et al, 2004; Liao et al, 2007). Similarly, the gene repressor REST is activated in selectively vulnerable neurons after SE (Palm et al, 1998) and in neurodegenerative conditions like stroke (Calderone et al, 2003; Formisano et al, 2007; Noh et al, 2012) and Hundington disease (Zuccato et al, 2003 and 2007). By orchestrating the epigenetic response to these conditions, REST controls the expression of genes implicated in cell death or survival and/or in neurotransmission and synaptic activity like gria2, that encodes the AMPAR subunit, grin1 that encodes the NMDAR subunit GluN1 and trpv1 that encodes TRPV1 channel (Noh et al, 2012). Because some of these proteins as TRPV1 or ionotropic glutamate receptors actually promote cell death, REST-dependent decrease in their expression is currently interpreted as "defensive" response in the attempt to protect neurons from dying (Noh et al, 2012). While the data from the experiments performed in vitro in the neuronal cell line NG108-15 strongly support that idea that $Ca_V3.2$ gene transcription in epilepsy and in other neurodegenerative diseases is set by the fine balance between the stimulation by EGR1 and the repression by REST, the direct demonstration of this hypothesis is still missing.

ROLE IN BRAIN ISCHEMIA

Evidence that T-type channels could be involved in ischemic cell death in nervous system was already reported long time ago. Fern et al (1998) exposed astrocytes from neonatal rat optical nerve cultured in vitro to the combined deprivation of oxygen and glucose (OGD) and monitored the changes in $[Ca^{2+}]_i$ that occurred in response to this insult. They found that in these experimental conditions $[Ca^{2+}]_i$ showed a biphasic increase with an early rise and a later more persistent elevation (Fern et al, 1998). Importantly, the early rise of $[Ca^{2+}]_i$ was dependent on T-type channel opening being abrogated by Ni^{2+} (400 μM) whereas the late increase was prevented by the L-type channel blocker diltiazem. Cell death occurring during the 80 min of hypoxia showed a similar biphasic pattern with an early death that was entirely prevented by T-type channel blockade and a later phase significantly reduced by diltiazem (Fern et al, 1998). More recently data have been reported suggesting that T-type channels could have a role not also in astrocyte but also in neuronal ischemic cell death. In particular, Nikonenko et al (2005) evaluated the effect of a series of T-type channel blockers including mibefradil, kurtoxin, Ni^{2+}, Zn^{2+}, and pimozide on the neuronal cell damage occurring in organotypic rat hippocampal slices after OGD. Using this approach they showed that neuronal ischemic damage can be prevented by T-type channel blockade being reduced by more than 90% by 500 nM kurtoxin and than 80% by 10μM Mibefradil and 50mM Ni^{2+} (Nikonenko et al, 2005). As the neuroprotective effects of T-type channel blockers was mimicked by Ca^{2+} removal from the extracellular solution or by the chelation of Ca^{2+}_I it was proposed that these drugs protect ischemic neurons by preventing Ca^{2+} influx through T-type channels. We already mentioned above that during the early phases of ischemia membrane potential drifts

inside the T-type window. Therefore, T-type channels could represent a privileged influx pathway mediating a steady Ca^{2+} influx in this condition. Although the molecular identity of the T-type channel isoform involved was not directly assessed in this study, the evidence that low concentrations of Ni^{2+} afford a maximal neuroprotection strongly suggests that the majority of the pharmacological effect is exerted on $Ca_V3.2$ channels. More recently, Bancila et al (2011) provided evidence that T-type channel blockade could be effective in reducing neuronal cell death in the CA1 subfield of the hippocampus also in a model in vivo of brain ischemia, global ischemia in the rat. They found, indeed, that neuronal cell death in this region was reduced by more than 80% compared to controls in animals receiving the intracerebroventricular injection of mibefradil 6 hours before experimental ischemia (Bancila et al, 2011). Similar results were obtained in rats receiving pimozide by the intraperitoneal route immediately before the beginning of the neurosurgical procedure (Bancila et al, 2011). Not only neuronal cell count was higher in animals receiving these T-type blockers but also mortality was significantly reduced as only 20% of the animals receiving mibefradil died while all the controls were killed by ischemia (Bancila et al, 2011). The data *in vitro* and *in vivo* that we just reviewed have the important limitation of having been obtained with drugs that can be also provided with other pharmacological properties. Therefore, further experiments with knockout mice are eagerly awaited to confirm the involvement of T-type channels in ischemic cell death. The mechanism by which T-type channels take part to ischemic cell death is still undetermined though as described above Ca^{2+} influx seems to be required. Additional mechanisms could, however, also have a role. To be specific, we mentioned already in the previous sections that Zn^{2+} accumulates in the extracellular space after brain ischemia and

Table I. *Electrophysiological characteristics of the cloned T-type channel isoforms. The table reports the main biophysical characteristics of the three cloned T-type channels isoforms expressed in HEK-293 cells. The data reported are from Perez-Reyes (2003), Physiol Rev 83:117–161, and Klochner et al (1999) Eur J Neurosci 11:4171-4178.* V_m*: half-voltage of activation;* h_∞*: half-voltage of inactivation,*

	$Ca_V3.1$	**$Ca_V3.2$**	**$Ca_V3.3$**
Threshold, mV	-70	-70	-70
Activation kinetics, ms	1	2	7
Inactivation kinetics, ms	11	16	69
Deactivation kinetics, ms	3.0	2.2	1.1
$V_{0.5}$, mV	-45.5	-45.8	-43.8
h∞, ms	-72	-72	-72
Recovery from inactivation, ms	117	395	352
Conductance in Ba^{2+}, pS;	7.5	9	11

that this transition metal can cause cell death after entering into neuronal cytoplasm. Intriguingly, we reported evidence that T-type channels may act as an influx pathway for exogenous Zn^{2+} in heterologous expression systems (Cataldi et al, 2005) and such a mechanism could also be effective in ischemic neurons where T-type channels could act as influx route for Zn^{2+}. Before closing this section we would like to mention that studies performed in astrocytes from the retina suggest an additional unexpected mechanism by which T-type channels may affect the process of ischemic cell death. In normal conditions, neuronal activity in the retina causes neuronal cell swelling and a decrease in extracellular space volume and osmolality of extracellular milieu (Uckermann et al, 2004; Dmitriev et al, 1999); therefore, to prevent glial swelling, protective mechanisms are activated, including of purinergic P2Y1 receptors by ATP autocrinally released by the astrocytes themselves (Wurm et al, 2009; 2010). In 2011 Linnertz et al showed that exogenous VEGF mimicks this protective mechanism by inducing the release of glutamate from astrocytes through Ca^{2+}-dependent exocytosis and the following activation of group II metabotropic glutamate receptors. Importantly, VEGF-induced glutamate release from retinal astrocytes is dependent on T-type channel opening being prevented by the incubation with the selective T-type channel blocker kurtoxin or with mibefradil, whereas L-, N-, R- or P/Q-type channel blockade were all ineffective (Linnerz et al, 2011). These results suggest that VEGF promotes glutamate release from astroglia by activating T-type channels. These findings are in accordance with the well established concept that T-type channels mediate neurotransmitter release at rest, the so called *low threshold exocytosis* (Weiss and Zamponi, 2012). Considering that significant amounts of endogenous VEGF are released in brain parenchyma after stroke (Beck and Plate, 2009), it is tempting to speculate that T-type channels could control astrocyte response to this growth factor also in this condition. This hypothesis remains, however, to be directly assessed.

ROLE IN BRAIN AGING

The hypothesis that T-type channels could be implicated in neuronal aging was first proposed in 1995 by Murchison and Griffith who compared the density of whole cell VDCC currents in young (1–3 mo) and aged (24–26 mo) rats. In particular, they focused on acutely dissociated neurons from medial septum and nucleus of the diagonal band, two regions that selectively degenerate in Alzheimer disease and Lewy bodies dementia (Coyle et al, 1983; Fujishiro et al, 2006; Whitehouse et al, 1981 and 1985). The main finding was a significant increase in T-type currents in aged animals with no change in channel gating properties. Differently from what repeatedly reported in hippocampal neurons by other groups (see Cataldi, 2013 for review), no change was observed in L-type currents in these regions (Murchison and Griffith, 1995 and 1998; Murchison et al, 2009). Recently evidence has been reported that this increase in T-type channel density could affects both functional properties and cell survival of aged neurons. In particular, it was shown that the age-related increase of T-type currents in cortical pyramidal cells has a role in causing the impairment of sensory representation and temporal information processing that takes place in aging. Specifically, the electrophysiological responses elicited in deep cortical pyramidal cells of somatosensory cortex S1 by the electrical stimulation of ascending thalamocortical fibers were compared in young and aged rats (Hickmott and Dinse, 2012). What emerged from these studies is that in old animals there is a substantial increase in the strength of inhibitory neurotransmission caused by the lack of IPSP suppression during repetitive stimulation. In addition, the percentage of pyramidal neurons showing burst firing was significantly higher in aged animals. Importantly, this increase in burst firing neurons was due to a marked increase in T-type channel current density. The idea that T-type channels are responsible for the increase in burst firing was supported by the evidence that burst firing neurons were converted to regular firing neurons when resting membrane potential was raised up to -55 mV, a voltage at which T-type channels are largely inactivated. The T-type channel-dependent increase in the number of burst firing neurons could have a role in determining the age-related impairment of brain cortex integrative properties. It has been demonstrated indeed that in pyramidal cortical neurons changes in firing modes cause parallel changes in synaptic plasticity, a phenomenon

known as *firing mode-dependent synaptic plasticity* (Birtoli and Ulrich, 2004; Czarnecki et al, 2007). To be specific, the switching to burst firing mode is associated with LTD induction (Birtoli and Ulrich, 2004). Intriguingly, this burst-LTD is critically dependent on T-type current activity as shown by its abrogation in the presence of the T-type channel blocker Ni^{2+} (Birtoli and Ulrich, 2004). Interestingly, an imbalance between LTP and LTD with an increase in the threshold of the former and a lowering of that for the latter is a common finding in the aged rat cortex (Rosenzweig and Barnes, 2003) and evidence has been reported that a similar phenomenon also occurs in humans (Freitas et al, 2011). Interestingly,

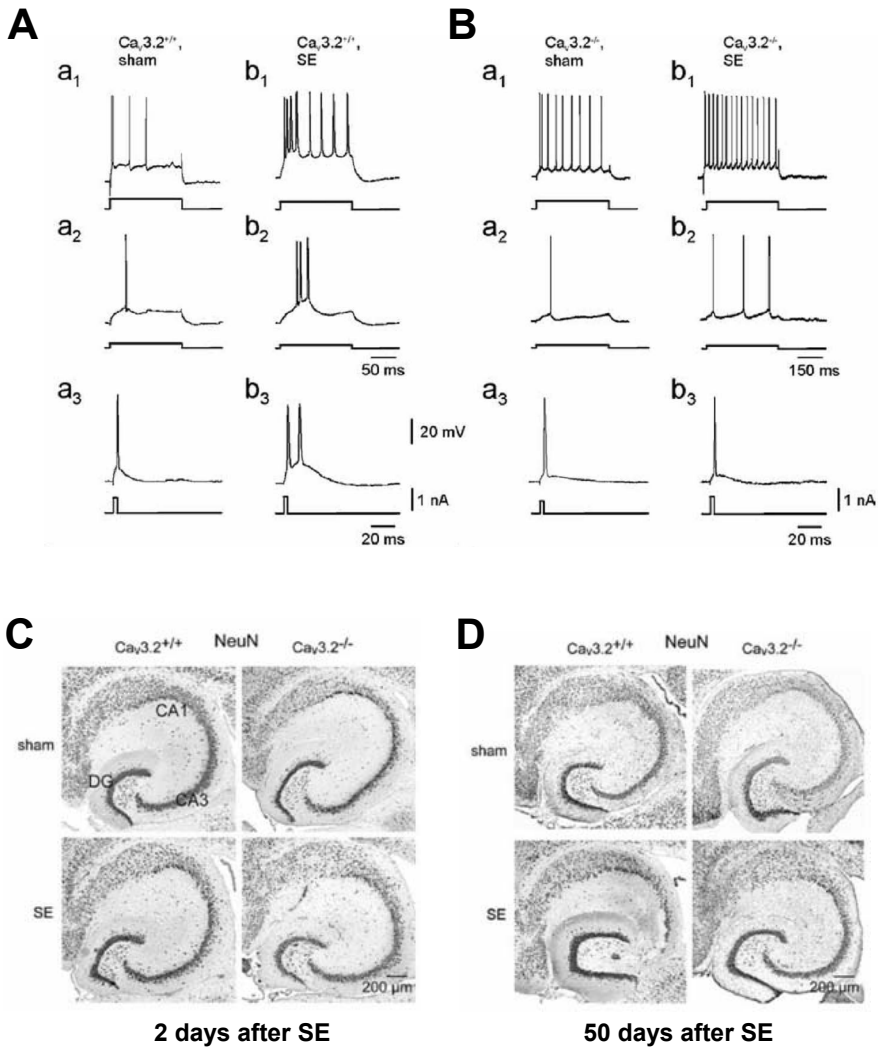
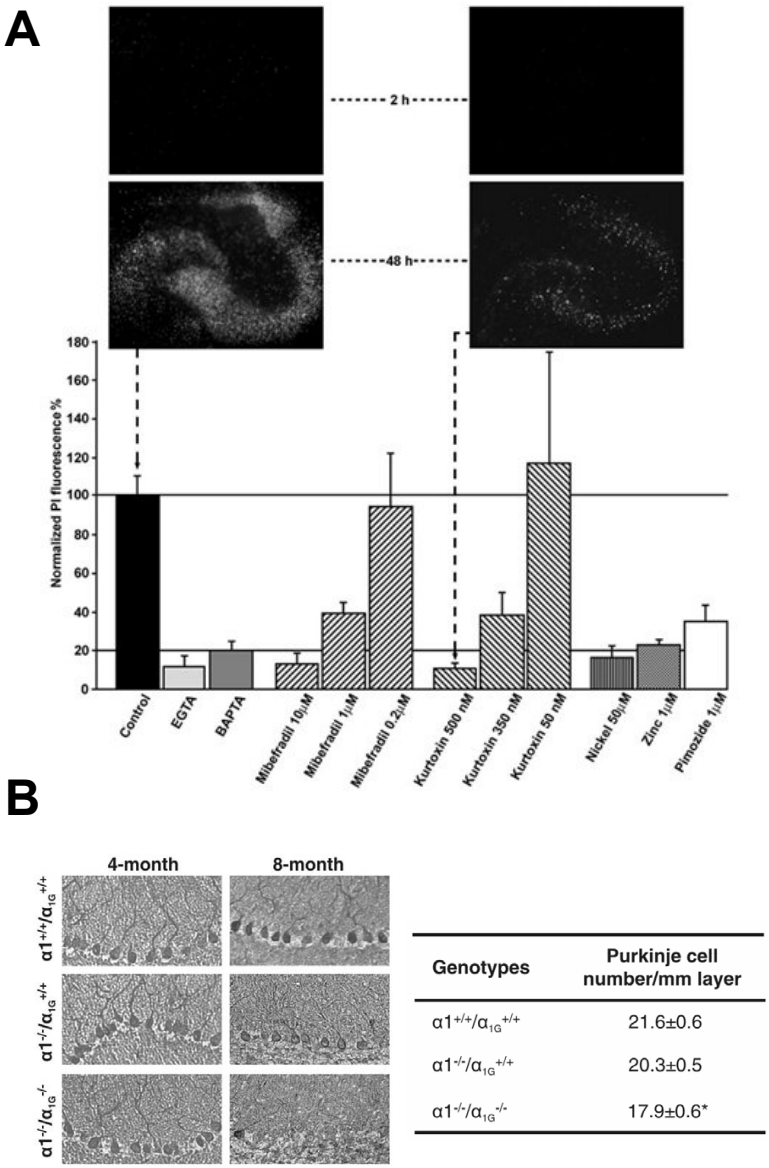


Fig. 1. *Genetic ablation of $Ca_V3.2$ prevents the emergence of burst firing and cell death in the hippocampus of rats treated with pilocarpine. Panels A and B report current clamp traces obtained in CA1 pyramidal cells from wild-type and $Ca_V3.2$ knockout mice, respectively. Current pulses of different amplitude and duration, as indicated in the insets, were injected in a1, a2 and a3. Note the presence of burst firing in the recordings obtained from pilocarpine-treated wild-type but not $Ca_V3.2$ animals. Panels C and D reports microphotographs of hippocampal sections from sham or pilocarpine treated wild-type and $Ca_V3.2$ knock-out mice, obtained 2 and 50 days after SE induction, as indicated, and stained with Neu-N to visualize neurons. Note the marked neurodegeneration occuring in the CA3 and CA1 subfields of wild type but not of knockout mice 50 days after pilocarpine administration. Reproduced with permission from Becker et al, (2008) J Neurosci 28:13341-13353.*



Genotypes	Purkinje cell number/mm layer
$\alpha1^{+/+}/\alpha_{1G}{}^{+/+}$	21.6±0.6
$\alpha1^{-/-}/\alpha_{1G}{}^{+/+}$	20.3±0.5
$\alpha1^{-/-}/\alpha_{1G}{}^{-/-}$	17.9±0.6*

Fig. 2. *A decrease in T-type channel expression or activity protects neurons from dying in brain ischemia and aging. Panel A shows the effect of different T-type blockers and of the extra- and intracellular Ca^{2+} chelation with EGTA and BAPTA, respectively, on neuronal survival in organotypic rat hippocampal slices undergoing OGD. The bar graph reports the mean+SEM of neuronal cell death evaluated as normalized propidium fluorescence measured 48h after the beginning of OGD. T-type blockers or vehicle were added to the OGD medium and the slices were exposed to these compounds for the entire duration of the experiments. The insets on the top of the graph show microphotographs of control and 500nM kurtoxin slices from a representative experiment taken 2 and 48 h after the begnning of OGD. Reproduced with permission from Nikonenko et al (2005) Mol Pharmacol 68:84-89. Panel B shows the effect of the genetic ablation of $Ca_V3.2$ channels on cerebellar Purkinje cell survival in $GABA_A$ receptor a1 subunit (a1/) knockout mice. $Ca_V3.2$ gene ablation was obtaining by crossing these mice with $Ca_V3.2$ knockout mice. In the double knockout mice so obtained both the $GABA_A$ receptor a1 subunit and $Ca_V3.2$ channels are lacking. On the left of the figure, microphotographs of the cerebellar cortex of these double knockout mice obtained at 4 and 8 month of age are shown. For comparison microphotographs of the cerebellar cortex of wild-type and of $GABA_A$ receptor a1 subunit are reported as well as indicated. The table on the rigth of the mean+SEM of neurons per mm in Purkinje cell layer. Note that in double knockout mice but not in the two other groups of animals a significant loss of Purkinje cells occurs at 8 month of age. Reproduced with permission from Chang et al (2011) Biochem Biophys Res Commun 410:19-23.*

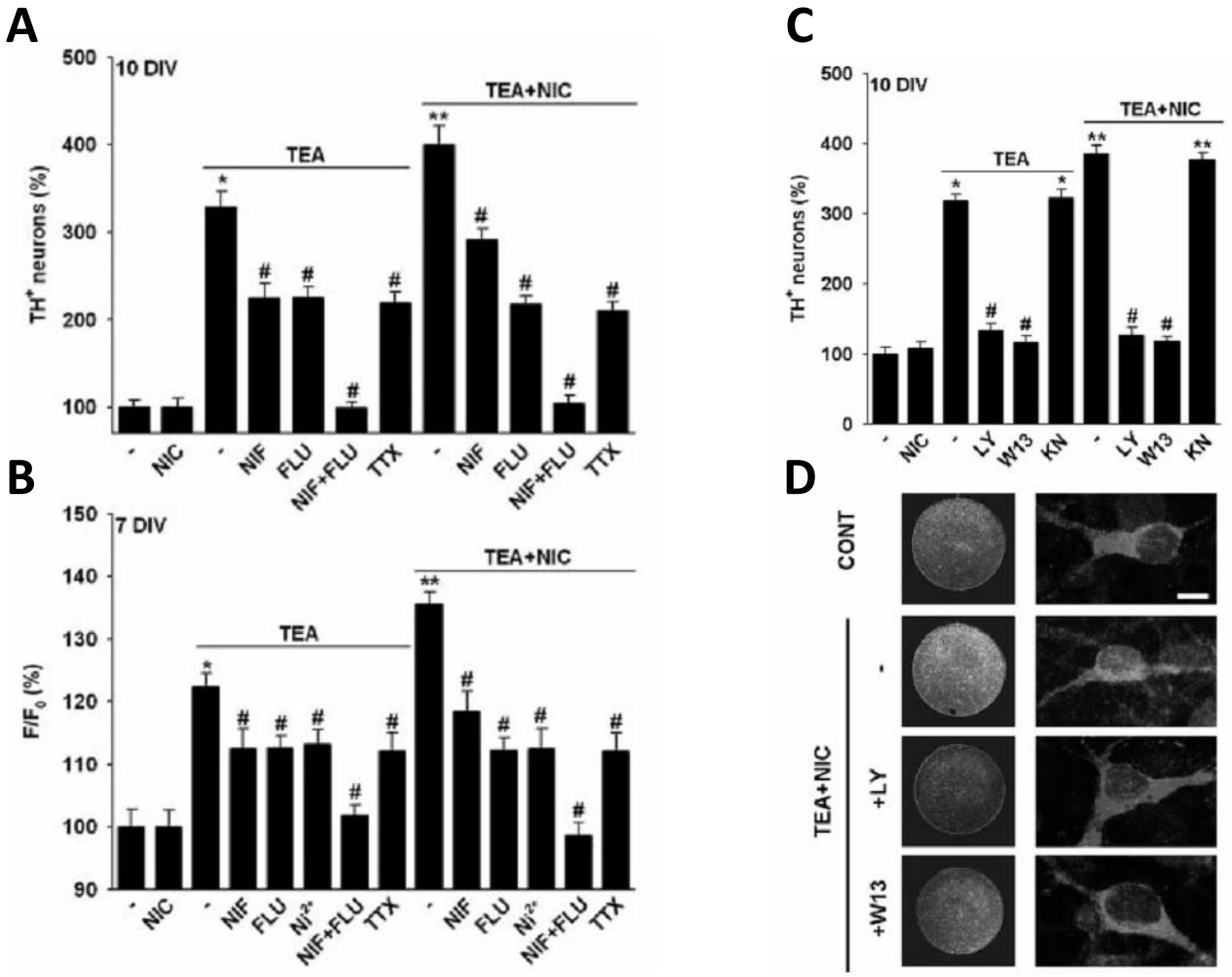


Fig. 3. *T-type channels mediate nicotine-induced neuroprotection in cultured substantia nigra dopaminergic neurons by promoting Ca^{2+} influx and and Akt activation. Panel A reports the mean+SEM of viable tyrosine hydroxylase positive neuron count measured after 10 DIV in primary cultures of mesencephalic neurons exposed to TEA, nicotine (NIC) or nicotine plus TEA in the presence of the Na^{+} channel blocher TTX, of L-type blocker nifedipine (NIF), of the T-type channel blockers flunarizine (FLU) or Ni^{2+} and of NIF+FLU. Note that nicotine is not neuroprotective by itself but potentiates TEA-induced neuroprotection and that this potentiating effect is lost when T-type channels are blocked. Panel B reports the percent change in $[Ca^{2+}]_i$, evaluated as fluo-4 fluorescence ratio, in individual neurons cultured in the same conditions as in A. Note that nicotine potentiates TEA-induced $[Ca^{2+}]_i$ and that this potentiating effect is prevented by T-type blockers. Panel C reports the mean+SEM of viable tyrosine hydroxylase positive neuron count in mesencephalic cultures treated with NIC, TEA, or NIC+TEA as in A and B in the presence of either the Akt inhibitor LY294002 (LY), or the calmodulin (CaM) inhibitor W13 (W) or the Ca^{2+}-calmodulin dependent kinase II (CaMKII) inhibitor KN93 (KN). Note that Akt or CaM inhibitions abolish TEA and TEA+NIC neuroprotection whereas CaMKII inhibition is ineffective. Panel D reports confocal images of dopaminergic neuron cultures treated for 10 days after seeding with TEA+NIC in the presence of either vehicle, W13 or LY and double immunostained with anti TH (red) and pAkt (green) antibodies. Note the strong increase in pAkt immunostaining in the presence of TEA+NIC and its abrogation in the presence of W13 or LY. *P<0.05 vs. corresponding control; **P<0.05 vs. TEA or 4-AP treatment; #P<0.05 vs. no channel blocker treatment. Reproduced with permission from Toulorge et al (2011) FASEB J 25:2563-2573.*

these functional alterations in S1 sensorymotor cortex were accompanied by relevant morphological alterations consisting in a decrease in total length of dendrites and in dendritic spine number (Hickmott and Dinse, 2012). While these data suggest that an increased density of T-type currents could be associated to "degenerative" changes in cortical neuron architecture, data from other studies suggest that it could also have a role in determining neuronal cell loss in aging. This hypothesis is supported, for instance, by the data reported by Lei et al (2011) who studied neuronal cell loss in the aging spiral ganglia of the coclea. They found that the in wild type mice the density of $Ca_V3.2$ channels in spiral ganglion neurons greatly increases with aging parallel to the progressive age-related neuronal cell loss in this ganglion. Interestingly, neuronal cell loss in spiral ganglion was significantly attenuated in knockout mice for the $Ca_V3.2$ isoform of T-type channels suggesting that the expression of this channel type is determinant for age-related neuronal cell death (Lei et al, 2011). This idea was strengthened by the evidence that in wild type mice, neuronal cell death in the spiral ganglion can be prevented by the treatment with two antiepileptic drugs with T-type channel blocking properties, ethosuximide and trimethadione (Lei et al, 2011).

ROLE IN POLYGLUTAMINE EXPANSION DISORDERS

A role has been proposed for T-type channels in causing neurodegeneration in Spinobulbar Muscular Atrophy (SBMA). SBMA is a rare X-linked recessive neurodegenerative disease with a prevalence of about 1–2 cases per 100,000 (Fischbeck, 1997; Brooks et al, 1995). Also known as Kennedy's disease (Kennedy et al, 1968) this disease causes a very slowly progressive limb and bulbar muscular atrophy with fasciculations and muscle cramps. It is usually diagnosed in the third and fifth decade though the first symptoms occur in adolescence and ultimately leads to severe disability (Sperfeld et al, 2002). The disease is caused by the expansion up to more than 30 copies of the CAG triplet repeat located in the N-terminal transactivation domain of the androgen receptor (AR) gene (La Spada et al, 1991). Motor neurons in the anterior horn of the spinal cord and in cranial nerve motor nuclei show prominent neurodegenerative changes though it is currently unclear whether this neurodegeneration is the cause or the consequence of muscle atrophy (Matsumoto et al, 2013). The hypothesis that T-type channels could be involved in this disease relies on a paper published by Sculptoreanu et al in 2000. They observed that, after transfection with mutant but not with normal, ARs, androgens cause a leftward shift of the voltage dependence of activation curve of T-type currents in two different models of spinal motor neurons, the NSC-34 cell line and spinal cord motor neuron-neuroblastoma hybrid cells. This leads to a widening of the T-type window current and, presumably, to an increase in cytoplasmic Ca^{2+} load and to an increased cell vulnerability to death. Intriguingly, spontaneous cell death *in vitro* was significantly higher in cells transfected with the mutant than with the wild type AR expression construct and this difference was abrogated upon T-type blockade with Ni^{2+} (Sculptoreanu et al, 2000). However, the possible role of T-type channels in SBMA or in other polyglutamine expansion syndromes has not been explored further and it is unclear how the results reported by Sculptoreanu et al (2000) that describe the acute effect of androgen on membrane currents could fit with the current models of the disease that are based on the transcriptional dysregulation caused by the aberrant ARs (cf. Katsuno et al, 2006).

ROLE IN PARKINSON DISEASE AND OTHER MOTOR DISORDERS

While in all the neurodegenerative conditions considered so far T-type channels seem to be involved as route of excess Ca^{2+} accumulation, a decrease in their activity could contribute to neuronal cell death in specific motor disorders. Although Ca_i^{2+} overload triggers neuronal cell death, low/moderate cytosolic $[Ca^{2+}]_i$ are, indeed, essential for neuronal survival (see. Cataldi et al, 2013 for review) and neurons die when $[Ca^{2+}]_i$ decreases below physiological levels as observed, for instance, upon prolonged exposure to L-type Ca^{2+} blockers (Koh and Cotman, 1992). Recent evidence suggests that also Ca^{2+} influx through T-type channels could be important for neuronal survival. Specifically, it has been suggested that this Ca^{2+} influx route has a role in preserving the

viability of the dopaminergic neurons of substantia nigra *pars compacta*, the neuronal population that selectively degenerates in Parkinson's disease. A first indication that Ca^{2+} influx could be important for survival in these neurons came from the observation that, in this disease, the highest vulnerability to cell death is observed in a subset of calbindin-negative neurons that fire action potentials at much lower frequency than all the other neurons of this brain region (Neuhoff et al, 2002). An experimental model that is often used to evaluate *in vitro* the susceptibility to death of dopaminergic neurons of the substantia nigra is based on the evidence that when cultured in vitro many of these neurons spontaneously die during the first two weeks after seeding. Salthun-Lassalle et al (2004) explored the role of ion channels in this process of spontaneous death looking for drug treatments effective to prevent its occurrence. They found that the voltage-gated Na^+ channel (NaV) activator veratridine rescued dopaminergic neurons from dying and promotes a significant increase in $[Ca^{2+}]_I$. Importantly, both the $[Ca^{2+}]_i$ response and the neuroprotective veratridine effect were abrogated by the T-type channel blocker flunarizine but not by the L-type channel blocker nifedipine, suggesting that Ca^{2+} influx through T-type channels is responsible for neuroprotection induced by veratridine. Both the $Ca_V3.1$ and $Ca_V3.2$ isoforms of these channels are expressed in dopaminergic neurons (Salthun-Lassalle et al, 2004) where they seem to control somatodendritic dopamine release (Bergquist et al, 1998; Chen et al, 2006; Kim et al, 2008 and 2009). The hypothesis that T-type channels could be the final effector of the neuroprotection elicited by depolarizing stimuli in substantia nigra dopaminergic neurons is also supported by the evidence that they mediate the protection from spontaneous neuronal death *in vitro* induced by the activation of bungarotoxin-sensitive α7-containing receptors by nicotine (Toulorge et al, 2011). This alkaloid is not neuroprotective *per se* but significantly potentiates the neuroprotection elicited by K^+ channel blockers like TEA by leftward shifting their concentration-effect curves (Toulorge et al, 2011). The involvement of T-type channels in this phenomenon was suggested by the evidence that it was prevented when T-type channels were pharmacologically blocked with flunarizine or Ni^{2+} (Toulorge et al, 2011). T-type dependent Ca^{2+} influx leads to neuroprotection through the Ca^{2+}-CaM-dependent activation of a PI3K-Akt cascade as indicated by the increase in pAkt levels induced by nicotine plus TEA and the ability of the CaM kinase inhibitor W13 to abolish the neuroprotection elicited by this treatment. The finding that T-type channel opening could be a downstream effect following nicotinic receptor stimulation is consistent with the data reported by Tsuneki et al (2000) who demonstrated that $[Ca^{2+}]_i$ increase elicited by nicotinic receptor stimulation in substantia nigra pars compacta dopaminergic neurons depends on the opening of Na_V and T-type channels and the consequent Ca^{2+} release from intracellular stores. To close this section, current evidence suggests that T-type channel activation by different depolarizing stimuli could be neuroprotective for substantia nigra pars compacta dopaminergic neurons cultured in vitro. It remains to be investigated whether such a mechanism is also relevant in models in vivo of Parkinson's disease and, more specifically, if it could account for observation that we mentioned above that the dopaminergic neurons showing higher frequency firing are the less susceptible to die in this disease (Neuhoff et al, 2002).

The idea that T-type channel activity is required to preserve neurons from dying seems to apply also to another motor disorder, essential tremor. Essential tremor is an extrapyramidal disorder characterized by tremor in the arms and hands during voluntary movements (Hawley et al, 2010; Louis, 2009). Pathological studies showed that in patients affected by essential tremor Lewy bodies can be often found in the brain stem and neuronal cell degeneration in the cerebellum and loss of Purkinje cells occurs with aging (Louis et al, 2007 and 2010). Therefore, this disorder is now classified as neurodegenerative. Chang et al (2011) provided evidence of a role of the $Ca_V3.1$ isoform of T-type channels in determining neuronal cell loss in essential tremor. Specifically, they compared the severity of Purkinje cells neurodegeneration in the cerebellar cortex of knock-out mice for the $GABA_A$ receptor a1 subunit, which develop a spontaneous form of essential tremor, and in double knockout mice in which also the $Ca_V3.1$ gene was ablated in addition to the $GABA_A$ receptor α1 subunit (Chang et al, 2011). The main finding

of this study was that cell loss in the cerebellum was greatly enhanced by the loss of the $Ca_V3.1$ gene (Chang et al, 2011). Importantly, this effect of $Ca_V3.1$ genetic ablation was evident only if the $GABA_A$ receptor α1 subunit gene was also lacking as control homozygous $Ca_V3.1$ knockout mice did not show any cerebellar degeneration (Chang et al, 2011). The mechanism by which the loss of $Ca_V3.1$ channels causes Purkinje cell death remains undefined. However, it is important to underline that, in this experimental model, neuronal cell loss occurred well after the onset of tremor and, therefore, that the hypothesis that cell loss is the consequence of abnormal neuronal network activation cannot be *a priori* excluded. Before closing this section we would like to emphasize that, while it is certainly interesting that $Ca_V3.1$ gene ablation causes neuronal cell death in a specific experimental model of motor disorder, further studies are required to clarify whether this has any relevance in spontaneously occurring essential tremor. This consideration is also prompted by the evidence that in another experimental model of this disease, the harmaline-induce tremor, $Ca_V3.1$ channels were showed to sustain the aberrant pacemaking in the inferior olive responsible for tremor generation (Park et al, 2010).

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the evidence reviewed in the present paper suggests that T-type channels could have a role in diverse neurodegenerative diseases including motor disorders and stroke. T-type channels seem to be involved both in the chain of events leading to neuronal cell death in these conditions and/or to affect the functional properties of specific neuronal networks contributing to the genesis of the neurological symptoms of these disorders. Both these aspects of T-type channels pathophysiology are well exemplified in epileptogenesis, a condition that though not traditionally classified as "neurodegenerative" presents many of the key features of neurodegeneration. The emerging evidence that, T-type channels participate to neurodegeneration, prompts new efforts aiming to develop better pharmacological tools to target these ion channels (Rimoli et al, 2009; Cosimelli et al, 2011). It is important to underline that highly selective blockers do not represent necessarily the best way to go because simultaneously blocking other Ca^{2+} channel subtypes like L- or N-type channels could be especially advantageous as learned after decades of clinical use of L-type Ca^{2+} channel blockers for cardiovascular disorders (see Cataldi and Bruno, 2012 for review). Also promising appears the possibility to pharmacologically modulate the epigenetic mechanisms that modify the expression of these ion channels in specific neurodegenerative diseases being responsible, therefore, for the changes of T-type current amplitude observed in these conditions.

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THE HEME OXYGENASE/BILIVERDIN REDUCTASE PATHWAY IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the deposition of toxic aggregates of amyloid-β-peptide and tau protein which 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. The enhancement of the cell stress response is a common mechanism used by neural cells to counteract oxidative/nitrosative damage in AD. The main feature of the heme oxygenase/biliverdin reductase (HO/BVR) axis is to catalyze the degradation of heme, 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 exert neuroprotective effects 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) (Mancuso and Barone 2009; Maines, 1997) (Figure 1). 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 (Stocker et al, 1987). In 1993, Verma *et al* proposed a role for CO as an endogenous neuromodulator (Verma et al, 1993). These early observations were followed by many papers demonstrating CO's important role as a regulator of important functions such as synaptic transmission, neuropeptide release, cardiac function, and vessel tone (Mancuso et al, 2010; Wu and Wang, 2005). Carbon monoxide and BR contribute to maintain the cell's redox equilibrium by *(i)* activating the proto-oncogene Akt or the mitogen-activated protein kinase family (MAPK) (two important pro-survival signalling pathways) and *(ii)* scavenging free radical species including reactive oxygen and nitrogen species (ROS and RNS, respectively) (Mancuso et al, 2008, 2006a,

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

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2279-5855 (2013)

2006b, 2003; Piantadosi, 2008; Wu and Wang, 2005; Stocker et al, 2004, 1987a, 1987b; Minetti et al, 1998). Both ROS and RNS 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 a useful approach to counteract AD and other dementias (Calabrese et al, 2008a; Mancuso et al, 2007). In this light, the upregulation of the HO/BVR axis was considered as a promising mechanism for improving cell stress response and counteract ROS/RNS damage (Barone and Mancuso 2009; Mancuso et al, 2007), and substances known to increase HO activity *in vitro* were explored as potential drugs for the treatment of free radical-induced diseases (Mancuso et al, 2012; Mancuso and Barone, 2009; Calabrese et al, 2008b; Brambilla et al, 2008; Calabrese et al, 2007a,b; Mancuso et al, 2007).

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, newest 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. From a chemical point of view, HO itself is not a hemoprotein: it acquires this characteristic after binding to heme-Fe(III) (Takahashi et al, 1994). 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 (Mancuso and Barone, 2009; Maines 1997) (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 (Maines 1997).

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) (Maines 1997, 2000). 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} (Salinas et al, 2004). Although constitutively expressed, HO-2 is responsive to developmental factors and adrenal glucocorticoids (Maines 1997, 2000) 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 (Maines 2005a). 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 (Maines and Panahian, 2001). 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 ventromedial and paraventricular nuclei of the hypothalamus (Mancuso 2003; Maines 1997). Heme oxygenase-1 is also found in cells of glial lineage, where its expression can be induced by oxidative stress (Dwyer et al, 1995). With regard to HO-2, this isoform is abundant in the brain, kidneys, and testes (Mancuso 2003; Maines 1997). In the brain, HO-2 is expressed in neuronal populations in the forebrain, hippocampus, hypothalamus, midbrain, basal ganglia, thalamus, cerebellum, and brainstem (Maines 1997).

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 (Figure 1), 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) (Gibbs et al, 2012; Kapitulnik and Maines, 2009; Maines, 2005b). 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 SH groups (Maines and Trakshel, 1993). Recently, it was reported that BVR's reductase activity requires the autophosphorylation of the enzyme on the specific site Ser^{149} (Salim et al, JBC 2001). Through the reductase activity, BVR generates BR, a lipophilic molecule with strong antioxidant activity towards both ROS and RNS (Stocker et al, 2004; 1987a; 1987b; Mancuso et al, 2009; 2008; 2006a; 2006b; 2003). A healthy adult produces almost 300 mg of BR each day (Stocker et al, 1987b). The BR formed within the cell is released and reaches the extravascular space and the bloodstream by passive diffusion or active transport. 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 (Kapitulnik et al, 2004; Minetti et al, 1998; Stocker et al, 1987b). Here, the BR dissociates from the albumin and enters the hepatocytes, where it is conjugated with glucuronic acid and excreted in the feces (Kapitulnik 2004).

Biliverdin reductase was initially considered a noninducible protein. Later studies showed, however, that BVR can be induced by LPS and bromobenzene at a post-transcriptional level, but it is unaffected by heat shock (Maines et al, 2001; Ewing et al, 1993). In rats, BVR activity increases progressively after birth and reaches adult levels by postpartum day 28 (Ewing and Maines, 1995). 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 (Ewing and Maines, 1995). 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 (Ewing et al, 1993). 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 (Maines 2005b). 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 (Maines, 2005b).

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) (Gibbs et al, 2012; Kapitulnik and Maines, 2009) (Figure 2). 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 (Maines et al, 2007). In addition, BVR activates both PKCβII and the atypical PKCζ and the novel PKCδ through protein-protein interactions (Gibbs et al, 2012). 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 (Gibbs et al, 2012) (Figure 2).

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 (Lerner-Marmarosh et al, 2008). 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 (Lerner-Marmarosh et al, 2008). After that, the formation of this complex allows BVR to be phosphorylated by ERK (Lerner-Marmarosh et al, 2008). Once activated, the complex BVR-ERK is separated from MEK and translocates into the nucleus where it binds and activates Elk1, a transcription factor which plays a main role in the induction of oxidative stress-responsive genes such as *ho-1* or *inducible nitric oxide synthase*

(iNOS) (Kapitulnik and Maines, 2009). Recently, BVR has also been shown to serve as a "shuttle" that drives heme into the nucleus, where it activates transcription of *ho-1* (Tudor et al, 2008). Through the activation of ERK1/2, BVR is involved in cell proliferation, differentiation and division as well as in the stress response (Figure 2).

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 (Wegiel et al, 2009), 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 (Gibbs et al, 2012; Wegiel et al, 2009). By modulating the PI3K/Akt system, BVR is involved in one of the main mechanisms which regulates cell protection (Figure 2), and this seems to be quite important in the nervous system (Burke, 2007; Zheng et al, 2000). 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 (Lerner-Marmarosh et al, 2005; Kapitulnik and Maines, 2009).

THE HO/BVR SYSTEM IN ALZHEIMER'S DISEASE

Alzheimer's disease is a chronic neurodegenerative disorder characterized by progressive cognitive dysfunction, memory loss, 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 (Ferri et al, 2005). 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 (Mayeux 2010). However, the annual incidence worldwide ranges from 1% to 7% at the ages of 70 and 85, respectively (Seripa et al, 2009). Sporadic AD is the more common form of the disease, accounting for 90% of all cases, whereas only 1% accounts for the familial form (Bekris et al, 2010). 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* (Bekris et al, 2010; Schipper et al, 2011). 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 (Querfurth and LaFerla 2010). 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 (Butterfield et al, 2007). 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 sufficiently efficient, there is an excessive build-up of Aβ that can trigger the onset of AD (Lee et al, 2010; Calabrese et al, 2007a). 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 (Ballard et al, 2011; Keeney et al, 2012). 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 (Ballard et al, 2011; Keeney et al, 2012). As a result, the hyperphosphorylated tau becomes insoluble, its affinity for the microtubules declines, and it forms

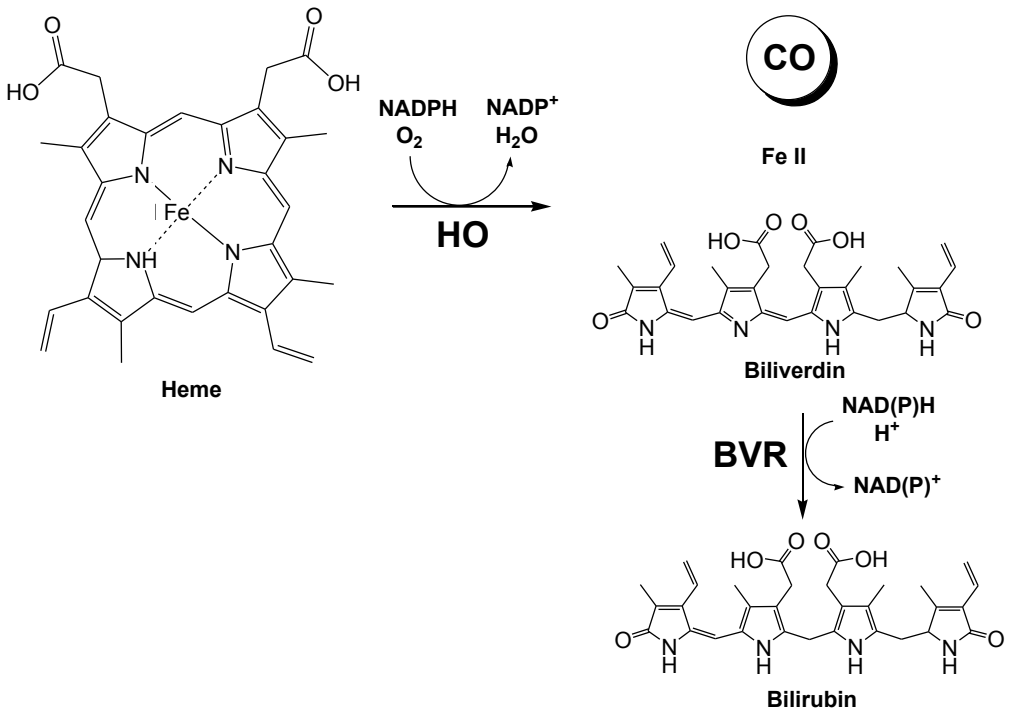

Fig. 1. *The HO/BVR system. Hemoprotein-derived heme moieties are oxidized at the α-meso-carbon bridge by heme oxygenase (HO), thus producing equimolar amounts of carbon monoxide (CO), ferrous iron (FeII) and the linear tetrapyrrol biliverdin. The latter undergoes a reductive modification at the C10 (γ bridge) by the NAD(P)H-dependent enzyme, biliverdin reductase (BVR) which yields bilirubin as the final product. For further details see text. Reprinted with permission from Mancuso et al, 2010.*

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 (Carrard et al, 2002; David et al, 2002). 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 (Sultana and Butterfield, 2009; Ferrer 2009; Rhein and Eckert, 2007). 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 (Bayer et al, 2006; Anantharaman et al, 2006). Excess superoxide radical also react with NO produced by activated microglia, thereby enhancing the formation of peroxynitrite and other RNS implicated in protein nitration (Kinouchi et al, 1998; Markesbery, 1997; Calabrese et al, 2007b). 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 (Esiri et al, 1990; Sajan et al, 2007).

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) 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. 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 with antisense HO-1 or vector transfected cells (Hui et al, 2011; Takeda et al, 2000). The suppression of tau protein expression was almost completely counteracted by zinc-deuteroporphyrin, a specific inhibitor of HO activity. The activated forms of ERK were also decreased in cells overexpressing HO-1 although no changes in the expression of total ERK were observed (Takeda et al, 2000).

Although many evidence considers HO-1 an enzyme with cytoprotective function (Mancuso and Barone, 2009; Maines and Panahian, 2001; Maines 2000), other studies suggest that this enzyme can trigger neurodegeneration (Koeppen and Dickson, 1999; Schipper et al, 1999). Many studies reported on the excessive sequestration of redox-active iron as a characteristic feature of many neurodegenerative disorders, including AD (Schipper 2000), 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

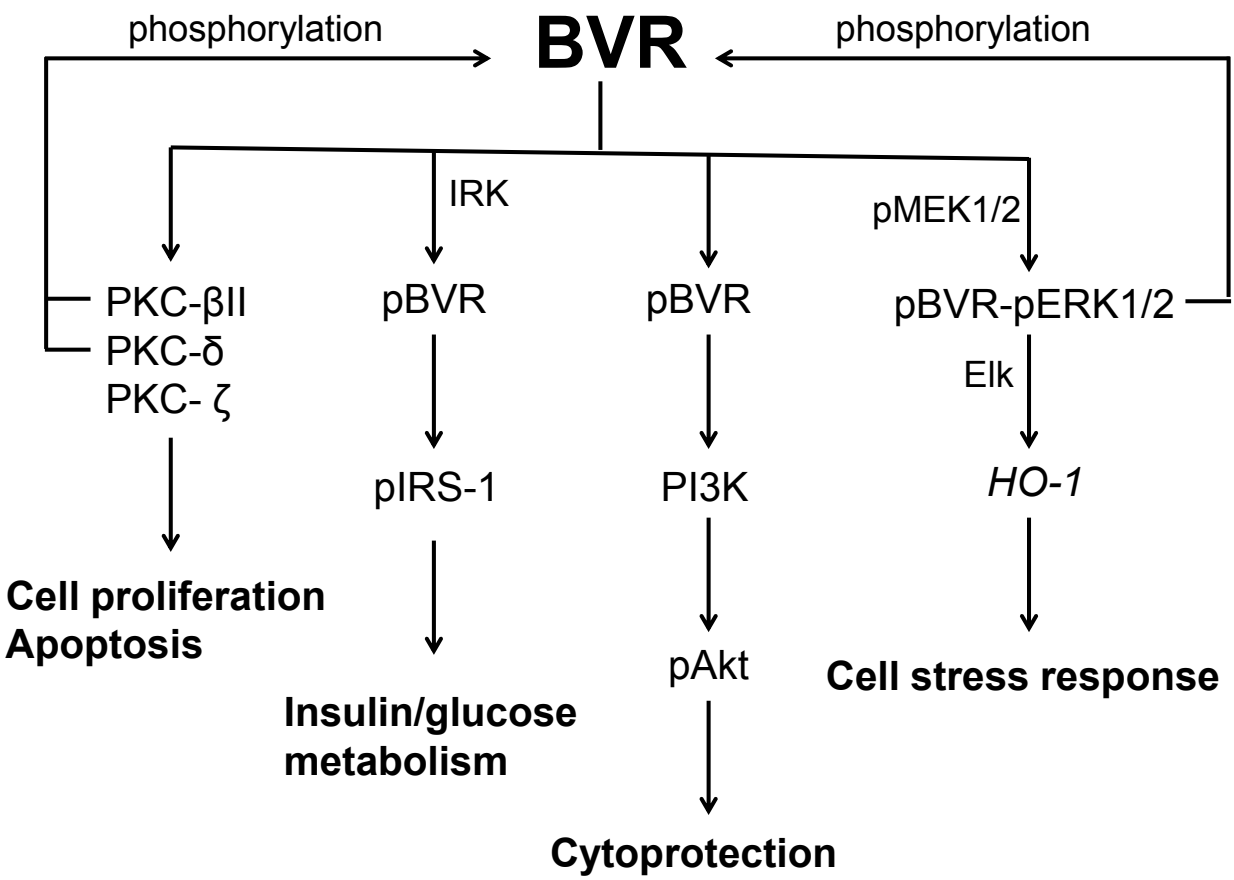

Fig. 2. *The main intracellular pathways involved in the pleiotropic actions of biliverdin reductase. Biliverdin reductase (BVR) exerts its pleiotropic effects by regulating several signalling pathways, such as those related to protein kinase-C (PKC), insulin receptor kinase 1/insulin receptor substrate 1 (IRK-1/IRS-1), phosphatidylinositide -3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinases (MEK/MAPK/ERK). For further details see text. p, phosphorylated*

(Goldstein et al, 2003; Chiueh et al, 2001; Van Bergen et al, 1999). 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 (Schipper et al, 2009; Schipper 2000). 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 (Schipper et al, 2009).

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 (2012a), 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 (Barone et al, 2012a). 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 (Barone et al, 2011b) which were paralleled by a concomitant reduction of the phosphorylation of this enzyme on Ser/Thr/Tyr residues in this brain area (Barone et al, 2011a). 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 (Barone et al, 2011a). Furthermore, even the formation of BVR-ERK2 complex in AD and MCI hippocampi were reduced (Barone et al, 2011a). 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 (Di Domenico et al, 2012). This last finding proposed the HO-1/BVR system as a novel peripheral biomarkers for the early diagnosis of AD.

CONCLUSIONS AND FUTURE DIRECTIONS

Although preclinical research generated impressive lines of evidence about the several intracellular mechanism(s) whose impairment lead to the onset and progression of AD, clinical research aimed at the development of new drugs capable of preventing or delaying the onset of neuronal damage in AD patients has produced limited results. The major classes of drugs currently available for the treatment of AD are acetylcholinesterase inhibitors or NMDA glutamate receptor antagonists (Mancuso et al, 2011; Massoud and Leger, 2011; Siciliano et al, 2011). 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 (Mancuso et al, 2011; Cosman et al, 2007; Geerts and Grossberg, 2006; Lipton, 2005). Both acetylcholinesterase inhibitors and NMDA glutamate receptor antagonists can be considered as "symptomatic drugs" because they 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 (Martorana et al, 2010; Birks, 2006; Birks et al, 2009). An alternative to symptomatic treatment with acetylcholinesterase inhibitors involves the use of drugs that intervene in the pathogenesis of the disease, such as statins. 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 (Butterfield et al, 2011). 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 (Butterfield et al, 2012; Barone et al, 2012b). Additionally, atorvastatin increased also the phosphorylation of BVR on Ser/Thr/Tyr residues (Barone et al, 2012b). 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 (Barone et al, 2012b). 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 (Mancuso et al, 2011; Mancuso and Barone, 2009).

ACKNOWLEDGMENTS

The author is 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 and for reading the manuscript. This work was supported by grants from the Catholic University (*Fondi Ateneo*) to Dr. Mancuso.

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STEM CELLS: THE LONG ROAD MAP TO NEURODEGENERATIVE DISEASES, A PHARMACOLOGIST'S STANDPOINT

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The perspective of so-called regenerative medicine, greatly emphasized by the media and the stagnation of valid therapy, has led to the claimed therapy of the use of stem cells, among which only embryonic or adult stem cells are foreseen for possible applications in humans to date. . However, until now, clinical trials on humans have not been performed with so-called induced pluripotent stem cells. Since all the above-mentioned types of cells are considered "drugs" and biological products" by the FDA, it is clear interest of pharmacologists to follow clinical trials performed with stem cells in neurodegenerative diseases, their risks and adverse reactions. This detailed analysis considers authorized or non-authorized clinical trials on stem cells, by the regulatory agencies, which have been carried out with stem cells mainly provided by the U.S. and European authorized factories or biotechnological centers. However, a wide use of stem cells takes place in unauthorized Centers and triggers a tourism of stem cells and some severe adverse reactions have occurred, equally treated in the aforementioned Centers.

The identification of embryonic stem cells (eSC; *human embryonic stem cells*, hESCs) (Thomson et al,1998), which took place about 13 years ago, has entailed an unprecedented and growing hope for the treatment of many severe pathologies, in particular, of degenerative diseases affecting the central nervous system [amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease] or immuno-inflammatory diseases (multiple sclerosis, MS), cardiovascular (myocardial infarction), ocular and severe metabolic diseases (diabetes), although, the list continues (Kuehn, 2012). Stem cells are the only realistic method to generate sufficient human material that is not transformed or genetically modified, for laboratory study, drug research and disease modelling (Chandran, 2008) (fig.1).

The *Food and Drug Administration* (FDA) has considered the products of the so-called regenerative medicine (*e.g.* stem cells) as drugs according to the 1938 *Federal Food Drug and Cosmetic Act* with further amendments, and as organic products by the *Public Health Service Act* (Cyranoski, 2010).

After a general overview of information, this paper will take into detailed review all the reliable clinical trials hereto carried out or in progress on stem cells for neurodegenerative diseases authorized by regulatory Authorities and we hereinafter report the unauthorized therapeutic uses and hazards of employing stem cells in inappropriate situations. Regrettably, reported data show that research is still a long way from a present application with an acknowledged therapeutic use in humans.

GENERAL INFORMATION

Currently available stem cells are classified into the following categories:

a) embryonic stem cells
b) amniotic, fetal, cord, placental stem cells
c) adult stem cells

Key words: clinical trials; regenerative medicine; regulatory issues; stem cells

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2279-5855 (2013)

d) induced pluripotent stem cells

Embryonic Stem Cells

Embryonic stem cells (ESCs), for human stem cells (hESCs), derive from the inner cell mass of human blastocysts, on the 5th -6th day of development, originally created with the aim of IVF. They differ from adult cell tissues because of their pluripotency (i.e. the ability to produce all the different types of body cells *in vitro* and *in vivo* and give rise to functional specialized progeny), because of their clonogenic properties and renovating abilities (*self renewing*), to grow, and expand in culture (Gonzales and Bernard, 2012; Mummery et al, 2011; Perez López and Otero, 2012). Their pluripotent property is shown by the fact that, once injected subcutaneously, they determine teratomas composed of cells with the primary three germ layers, ectoderm, endoderm and mesoderm (fig.2). The possible formation of teratomas is one of the potential adverse actions of hESCs in clinical practice (see paragraph "Dangers on the use of stem cells in inappropriate situations") and specific guidelines have recently been developed in the U.S. for the use of embryonic stem cells (No author listed, 2009; Lo et al, 2010).

Amniotic, fetal, cord and placental stem cells

Cord blood cells are an acknowledged and non-controversial source of hematopoietic and non-hematopoietic progenitors and the second most common source of stem cells in cell substitution therapies in autologous and allogenic transplants (Ilic et al, 2012). Cord blood cells seem to be very useful for regenerative medicine (Brown, 2012; Ierc et al, 2012; Roura et al, 2012), but they are relatively limited in number and, thus, need to be expanded *in vitro* in order to provide a sufficient number of cells for autologous and allogenic implants. The controversy on the manipulation of cells, before their (re)implantation, will hereinafter be taken into consideration. A product made from cord stem cells, Hemacord, is available for some blood diseases in the USA (Kuehn, 2012). A real market of umbilical cords, prematurely clamped and paid up to 15.000-30.000 euro, has been reported in the UK (Brown, 2012).

Different types of stem cells may be derived from amniotic membranes (Ilancheran et al, 2009; Moodlsey et al, 2010), placenta (Yuan et al, 2012; Wang et al, 2012) and fetuses (Carpino et al, 2012).

Autologous Stem Cells

Li and Clevers (2010) have distinguished, quiescent cells (beyond the cell cycle and in a lower metabolic state) and active cells within the cell cycle in adult tissues. These cells may be found in: the ovary (Bhartiya, 2012; Hosni and Bartu, 2012; Powell, 2012; Telfer and Albertini, 2012; Virant Klun et al, 2012; Vogel, 2012; White et al, 2012), breast (Bandyopadhyay et al, 2012; Choudhary and Capuco, 2012; Kaimala et al, 2012; Spaars et al, 2012), via expansion with xantosine (Choudhary and Capuco, 2012), and from breast milk (Beltran, 2012), dental pulp (Salai et al, 2012), hair follicles and sweat glands (Lu et al, 2012), intestine (Yilmaz et al, 2012), eyes (Salero et al, 2012), lungs (Kotton, 2012) and hematopoietic marrow after chemoablation.

Mesenchymal autologous stem cells present in postnatal tissues are defined adult stem cells (*adult stem cells, ASCs*) (Boregowda and Finney, 2012). They differ greatly, *e.g.* some tissues, such as the bone marrow, contain more than one type of ASCs (Gonzales and Bernard, 2012).

The way in which stem cells, recently derived from dental pulp (Sakai et al, 2012), sweat glands (Lu et al, 2012), retinal pigmented epithelium (Solero et al, 2012) and lungs (Kotton, 2012), may possibly develop remains largely unclear (Boregowoda and Finney, 2012).

Specific cellular elements present in some areas of the CNS have features of expandable neural stem cells (eNSCs). In this respect, it is worth recalling that neurogenesis implies the generation of a neuron from a cell division. This cell can be a limited precursor (a cell which can only rarely divide and give rise merely to neurons) or a stem cell (a cell which can divide many times, which is capable of generating all the major cell types of the organ it is located in, as in the case of the CNS: neurons, astrocytes and oligodendrocytes) (fig. 1). Indeed, the discovery of neurogenesis in adults has sparked the search and the discovery of neural stem cells (NSCs), firstly, in the developing CNS and, consequently, in the adult CNS (Conti and Cattaneo, 2010).

The neurogenesis in the adult brain can be easily detected in two areas of mammal brains : the sub-

ventricular zone (SVZ), lining the lateral ventricles of the brain, provided with nervous stem cells and neural progenitor cells, and the sub-granular zone (SGZ) of the dentate gyrus (which is part of the hippocampus). The SVZ concurs to the response of cells as a result of injuries. It has been found that the SVZ has nerve stem cells (*neural stem cells*) and nerve progenitor cells (*neural progenitor cells*) and the balance between the two is vital in normal brain development. It is known that the signaling pathway, *Notch*, regulates the self renewal of nerve stem cells, whereas the signaling pathway, referring to the receptor of the *epidermal growth factor* (EGF), is linked to nerve progenitor cells (Aguirre et al, 2010). These pathways interact to maintain a balance between the populations of nerve stem cells and nerve progenitor cells. The presence of stem cells in the adult *neocortex*, the area that regulates language and thought, with partially positive findings by Ohira et al (2010), and for temporal lobe by Vaysse et al (2012), remains controversial. Interneurons capable of modulating and synchronizing the activity of the principle neurons which subsequently disperse throughout the neo-cortex may be generated. This process is rare under normal conditions, yet strongly increased by ischemia (Ohira et al, 2011).

Unfortunately, an increase of neurogenesis due to injury is difficult to test.

Past and present efforts to transplant eNSCs or young neurons in patients with neurodegenerative diseases have only accounted for disillusioned hopes, although, more than restoring lost neuronal circuitry (which neurons normally spread among very precise locations from a distance of several centimeters), it has been found that the cells seem to merge locally in the tissue, providing neurotransmitters to the other neurons that have not yet been destroyed by the disease. Moreover, transplanted cells may be less able than stimulated cells to merge into distinct neuronal circuits and, in this case as well, exert paracrine effects (Marin, 2010).

In rodent and monkey models of neurodegenerative diseases, the increase of eNSC may have beneficial results (Androutsellis-Theotokis et al, 2010; Kittappa et al, 2012).

Induced pluripotent stem cells

Ethical and safety concerns related to hESCs, the capacity to address specific structures and safety, particularly concerning the possibility of therapeutic use, have led to implementing stem cells that would not pose such issues, whilst maintaining the pluripotency features of hESCs, being able to develop any type of organ tissue. Six years after hESCs, Shinya Yamanaka managed to obtain so-called induced pluripotent or reprogrammed stem cells (iPSCs) from somatic cells ofice (Takahashi and Yamanaka, 2006) or man (Takahashi et al, 2007 and the Japanese scientist won the Nobel Prize in 2012 for this discovery). The reprogramming was achieved by using a transcription-factor transduction, a mixture of definite transcription factors (Yamanaka and Blau, 2010) (see hereinafter). iPSCs cells share many phenotypic (morphology, growth and ability to differentiate) and molecular properties (epigenetic changes and gene expression profiles) that are characteristic of stem cells, from naturally fertilized embryos or generated by somatic cell nuclear transfer (SCNT) through a micromanipulation technique (Yamanaka and Blau, 2010). However, the two types of cells (hESC and iPSCs) are not identical (Halzer and Moss, 2007; Hackett and Fortier, 2011; Dolgin, 2010) as the iPSCs possess an epigenetic memory of the tissue they derive from, that influences the differentiation in the type of required cells, while SCNT are similar to eSC-derived cells (Zwaka, 2010). ESCs from mice that waste their potency after 7.5 days may be reprogrammed through exposure to LIF/ STAT signals with associated changes of the transcriptome of the phenotype memory (total RNA in a cell), loss of phenotypic memory and of the memory of epiblastic cells (Bao et al, 2009; Dolgin, 2010). The most important transcription factors for reprogramming somatic cells are *c-Myc* (also known as *OSKM*), *OCT3 / 4* (also known as *Pou5f1*), *Sox2, Klf4. NANOG, Lin 28* (Halzer and Moss, 2007; Kaimala et al, 2012; Takahashi and Yamanaka, 2006; Takahashi et al, 2007). These transcription factors break up the chromatin (*c Myc*), allow *OCT3 / 4* and *Sox2* to identify the regulatory areas of the gene and restore pluripotency. In addition to being a cofactor of *OCT3 / 4* and *Sox2*, *Klf4* is also capable of inhibiting the programmed cell death that may otherwise be induced (Zwaka, 2010).

In contrast to adult myoblasts (Darabi et al, 2012; Meregalli et al, 2012), myogenic muscular and fetal

cells, changed into iPSCs through *PAX7* conditional application, have developed great quantities of myogenic precursors that, once transplanted in mice dystrophic muscles, appeared to be well distributed and were detected 11 months after being transplanted, producing abundant positive myofibers dystrophin positive (Darabi et al, 2012). These results are encouraging for the treatment of muscular dystrophy, hereditary heterogeneous neuromuscular disorders that include (Meregalli et al, 2012) Duchenne's b (DMD) and Becker's Muscular Dystrophy (BMD).

Despite the expected therapeutic concern on iPSCs and their possible use other than ethical considerations, increasing worries are being raised on the non-compliance between these cells and ESCs (Dolgin, 2011), including the ability to relate hereinafter with structures of the body they are designed for (Balzer and Moss, 2007). Furthermore, antigenicity studies (Yamanaka and Blau, 2010), DNA damage mediated by p53 (Krizhanovski and Lowe, 2009), the possible formation of teratomas (Atala, 2012), neurocytomas (Sim et al, 2006) and teratocarcinomas (Germain et al, 2012) and, even so, concern on carcinogenicity (Schlűter and Kaur, 2009; Dolgin, 2011; Krizhanovski and Lowe, 2009; Germain et al, 2012; Wadman, 2009; No author listed, 2012a) have been envisaged. Indeed, *Soof iPSAx2* is a cancer driving factor (Bass et al, 2009); *fl4* and *c-Myc* are also involved in neoplastic growth. Issues have also been raised regarding p53 (Krizhanovski and Lowe, 2009). The question of immunogenicity of iPSCs is also relevant, moreover with regard to the formation of teratomas and teratocarcinomas (Zhao et al, 2011; Apostolou and Hochedlinger, 2011) (fig.2). Recent findings from Arachi et al (2013) suggest limited immunogenicity of transplanted cells differentiated from iPSCs and ESCs cells.

No clinical experiments with iPSCs have hitherto been carried out. It has been reported that the first study in the world on iPSCs has been authorised in Japan on February 13 of this year for the treatment of macular degeneration of the retina by a review board of the National Institute of Biomedical Innovation in Osaka, however this study still requires approval by the health minister that does not seem imminent (go. nature.com./bkvonk)

Furthermore, it is worth mentioning that the Nobel Prize winner Japanese scientist, who discovered iPSCs has quite recently founded a big center in Japan, which is able to provide a large number of iPSCs that are also antigenically compatible. American researchers express their appreciation, but also object to the project (Cyranoski, 2012a), as it seems that clinical trials cannot be started before 3 years' time.

USE OF STEM CELLS IN REGENERATIVE MEDICINE

General Principles

The so-called regenerative medicine has represented a great breakthrough after the discovery of stem cells and particular interest has hereto been addressed to cell replacement therapy in neurodegenerative diseases. The general approach has been grafting specific cell types in the affected areas in order for these cells to be embedded in the tissue and, thus, replace the lost cell components. The grafts have been represented by hESCs and specific progeny of stem cells resulting from the direct differentiation of stem cells (eNSCs). As regards the use of human stem cells in neurodegenerative diseases, it is important to consider the strategy for a possible implementation of antibodies that block *lingo 1*, a cell surface molecule that prevents the inoculated stem cells of the CNS from becoming oligodendrocytes (Mosyak et al, 2006), an essential element to obtain a remyelination. The hypothesis whether drugs that inhibit *lingo 1* can accelerate myelination is being viewed.

The source of stem cells

The purity of stem cells and the storage until the use are important issues to be addressed. Only a few biotechnology facilities (*Advanced Cell Technology, Stem Cell Inc., Neuralstem, ReNeuron)* were authorized by the regulatory authorities in the U.S. and are able to supply stem cells for reparative medicine.

Geron had started conducting a Phase I in which oligodendrocyte progenitor cells, derived from hESCs, were injected in 8 patients with total thoracic spinal cord injury on which further details will hereinafter be provided. However, Geron suspended his work due to a lack of state funding of the project (Hayden,2012) (see hereinafter, paragr. 3 a) 1).

The *California Institute for Regenerative Medicine*, a public institution in San Francisco, one of the world's largest supporters of stem cell research, has an ambitious research program and clinical use of stem cells, although it was severely restrained by severe California budget shortfalls and a shortage of state support following the economic crunch (Haiden, 2012), thus leading to a suspension of Geron's clinical trials because of the failing of state subsidies.

Celltex Therapeutics, is a controversial biotechnology company based in Houston, Texas, USA, for which there is extensive evidence showing that it performs stem cell treatments on fee-paying patients. This is in contrast with the decisions of the FDA which considers a crime injecting unauthorized adult cells in patients (Kuehn et al, 2012; No Author listed, 2012a). Paragraph "Regulatory issues" deals with this topic.

ESC for clinical trials are studied at the *King's College* in the UK (Hawkes, 2011) and additional information can be found at www.clinicaltrials.gov

In Italy *Cell Factory*, a GMP certified laboratory, in association with the Center for Transfusion Medicine of the IRCSS of the Policlinico in Milan and also with the unit of the placental blood bank with 9000 units ready for transplantation, provides bone marrow mesenchymal cells for the treatment of neurological patients (Lazzari and Giordano, 2012).

Stamina Foundation of Turin provided stem cells to the Ospedali Riuniti of Brescia although it was blocked by the NAS (Nucleo Antisofisticazioni Sanità dei Carabinieri), a specialized Healthcare Control Corps of Italian gendarmes, and deemed invalid by the Italian Drug Agency (AIFA) on the grounds of serious flaws in its cell processing, non-compliances and irregularities (Toresini, 2012) (see below).

In May 2012, a biopharmaceutical company, Stemedica, based in San Diego, was authorized by the FDA to produce stem cells, which will be used by a Mexican healthcare team, Grupo Angeles, for the treatment of ischemic heart disease, in a specific private clinic. In this treatment, mesenchymal stem cells will be administered intravenous for an allogeneic transplant. Grupo Angeles was authorized to carry out such trials by COFEPRIS, the Mexican agency for drugs, similarly to the FDA (No Author listed, 2012b).

Ongoing experiments with stem cells approved by the U.S. (FDA) and European regulatory authorities and trials conducted without authorization will be dealt with in the following paragraphs.

Several ongoing attempts show that regulatory authorities are starting to authorize invasive generative medicine interventions with stem cells or cells derived from them.

PHASE I AND IIA TRIALS WITH STEM CELLS FOR NEURODEGENERATIVE DISEASES AUTHORIZED BY REGULATORY AGENCIES

Significant issues are related to transfer the therapeutic use of stem cells in humans**,** notwithstanding the very extensive research performed on animals. It is worth mentioning the obvious differences among species, the need for immunosuppression to prevent rejection, with the exception of ASC, the duration of therapy and the potential carcinogenicity.

An important factor is the purity of the cells to be injected, the safety and certification that they are produced by specifically acknowledged biotechnological industries authorized by the regulatory authorities, *e.g.* some mentioned in previous paragraph.

The issues regarding the use of hESCs in degenerative pathologies will be dealt with by various Authors (Chandran, 2008; Daley, 2012; De Feo, 2012).

Recently, two US independent groups demonstrated that human neural stem cells transplanted into the brain can mature into myelinated brain cells such as oligodendrocytes. The cells produced a myeline sheath that boosts the speed of neural signaling. Magnetic Resonance Imaging (MRI) may indicate transplanted cell engraftment and successfully myelinated brain cells (Uchida et al.2012).

Twenty three clinical trials to date performed on human subjects have been approved by regulatory authorities. They concern several neurodegenerative diseases as hereinafter referred to, and all the trials are substantially in phase 1 and I/IIa.(Table 1).

I. *Spinal cord injury.* In March 2008 an experimentation request, based on more than 24

studies on 2000 animals and i in 22.000 pages, was presented to study the result of hESCs in patients with spinal cord injury ranked "A" according to the scale of bone marrow damage by the *American Spinal Injury Association* (Mayor, 2010). The research, approved by the FDA (Wadman, 2009) was being performed in 7 U.S. medical centers. Preclinical studies with oligodendrocyte progenitor cells, in spinal cord injuries in animals, had shown that these cells move through the site of the injury. Subsequently, they mature in functional oligodendrocytes that remyelinize axons and produce neurotrophic factors, implying an improved locomotion of the treated animals (Pang et al, 2011). Patient selection was carried out on the grounds of a complete loss of sensory and motor functions, with the last spinal segments, T3 to T10, which were intact and 2.000.000 stem cells developed by Geron (GRNOPC1) were injected into the spinal injury site of each patient, which had occurred 7-14 days before. They are oligodendrocyte precursors which perform different functions in the CNS, including the production of myelin that allows an efficient conduction of nerve impulses. Oligodendrocytes are lost in spinal cord injury with a consequent loss of myelin and neurons, which disarranges the conduction of nerve impulses, resulting in paralysis.

Let us to remember that human neural stem cells transplanted into the brain can mature in muyelinated brain cells such as oligodendrocytes (Uchida et al, 2012).

The primary end point of the study was the safety of the injected cells, the injection procedure, the necessary concurrent immunosuppression for a year, whereas the secondary end point was the evolution of the neurological function. The purpose of the entire study was to determine whether, under the best safety conditions, injecting stem cells into the injury site could foster a repair mechanism. Unfortunately, as previously mentioned, the reliable and well-designed study has been suspended due to a lack of funds and support (Baker, 2011; Gornall, 2010). The issues related to the treatment of spinal injuries have been tackled in a recent paper (Snyder and Teng, 2012) and Lucovic et al (2012) have discussed the issue of hESCs in spinal cord injuries.

II. *Macular degeneration*. Schwartz et al (2012) have performed a clinical phase I / IIa trial on a patient with a rare form of blindness, Stargadt disease, which usually affects children, and a form of age-related atrophic macular degeneration. Cells provided by *Advanced Cell Technology* were employed (retinal injections) (approximately 5000 per injection) and approved by the FDA for the survey and immunosuppressants were administered. In addition to an excellent tolerance, remarkable clinical results, although downsized and criticized by other researchers, were achieved considering the severity and incurability of the disease (Falsini and Bisti, 2012; Huang et al, 2012).

III-V *Stroke*. Approved by the UK regulatory authorities (*Medicines and Healthcare Products Regulatory Agency*), a clinical trial with stem cells in stroke was initiated in 2010 (PISCES: *Pilot Investigation of Stem Cells in Stroke*) and had been scheduled to be carried out within a two-year span, as a treatment option for *stroke* survivors, with the possibility of restoring lost or impaired functions. The research is monitored by prof. K. Muir, *Synapse Chair of Clinical Imaging*, University of Glasgow, in cooperation with the group, ReNeuron, that develops and uses fetal brain stem cells and has provided ReN001 cells for the therapy.

The cells will be injected into the involved area of the brain by means of a neurosurgical procedure, and an observation period of 2 years, which has not been concluded yet, is envisaged. Patients aged 60 years and above, with scarce or little improvement 6-24 months after *stroke* are taken into account. The type of procedures and the characteristics of ReN001 cells guarantee that patients enrolled in the trial will not receive immunosuppression after the injection of stem cells (Wise, 2010; Smith and Gavins, 2012). The evaluation of results of the treatment with stem cells in stroke has been tackled by various Authors (Kim et al, 2012; Linvall and Kokaya, 2011; Oki et al, 2012). Oki et al (2012) have discussed the improvement in the recovery phase; Kim et al (2012), in a retrospective study on different parameters on stroke patients treated with stem cells, have criticized the publicized effectiveness of the results, essentially based on the individuals' impressions.

VI-VIII *ALS*. Two phase I trials (mentioned in Gornall et al, 2010) are conducted on ALS in the USA. In September 2009 a trial, sponsored by a private industry, Neuralstem, was authorized, under

the aegis of Professor Eve Feldmann, Professor of Neurology at the University of Michigan (Gornall, 2010). Another phase I trial was initiated in January 2010 using the patient's stem cells and followed by another biotechnology company, TCA Cellular Therapy (Gornall, 2010). A further phase I trial on patients with ALS started in Italy using brain stem cells of spontaneously aborted fetuses in the 3rd month, authorized by the Istituto Superiore di Sanità (ISS) has just begun at the IRCSS, Casa Sollievo della Sofferenza S. Pio (San Giovanni Rotondo), organized by Professor Angelo Vescovi and Prof. Letizia Messina at the ALS Center of the Ospedale Maggiore di Novara. The first patient was treated with 2500000 brain stem cells (Arcovio et al, 2012).

IX-XX *Multiple sclerosis*. There are several reliable studies (Ben Hur, 2011; Burt et al, 2009; 2011; 2012a and 2012b; Burt and Milanetti, 2011; Farge and Gluckman, 2011; Franklin and French Constant, 2010; Mancardi and Saccardi, 2008; Saccardi et al, 2012; Sullivan et al, 2012) on the use of ASC in multiple sclerosis (MS), a multifocal autoimmune disease, which was treated with hematopoietic ASC transplantations (HSCT) (146-151). A trial has been funded and monitored in the UK by the *Medical Research Council* and involves the use of mesenchymal ASC in the early stages of MS, on which clinical trials are being conducted between Cambridge and London. The injections are not performed systemically, yet restricted to the affected areas (Franklin and French Constant, 2010)146).

In the treatment of MS, in which inflammatory stages precede degenerative axonal stages, the use of these stem cells must be assessed in the context of greater or less intense immunosuppressive treatments, based on one (*e.g.*, cyclophosphamide) or more cytotoxic anticancer drugs or antilymphocyte antibodies (ALG), according to the disease stage.

It seems that the use of stem cells in the first phase of the disease plays a rather immunosuppressant role and a neuro-repairing action may be performed solely in the degenerative phase. Hence, the great difficulty of the appropriate use of stem cells according to the pathologic and developmental stages of the disease are at times difficult to identify. With regard to this issue, Burt (2012a) has recently stated that "if confused or hesitant, remember" treat with standard immune suppressive drugs and, if no inflammation, no response".

In MS, stem cells could be useful to promote tissue protection and limit the loss of motor neurons, thereby delaying the period of assisted ventilation and artificial feeding for patients (Chandran, 2008). However, the information resulting from conducted or ongoing clinical trials are essential (Burt et al, 2009; 2012; Franklin and Ffrench Constant, 2010; Mancardi and Saccardi, 2008; Saccardi et al, 2012).

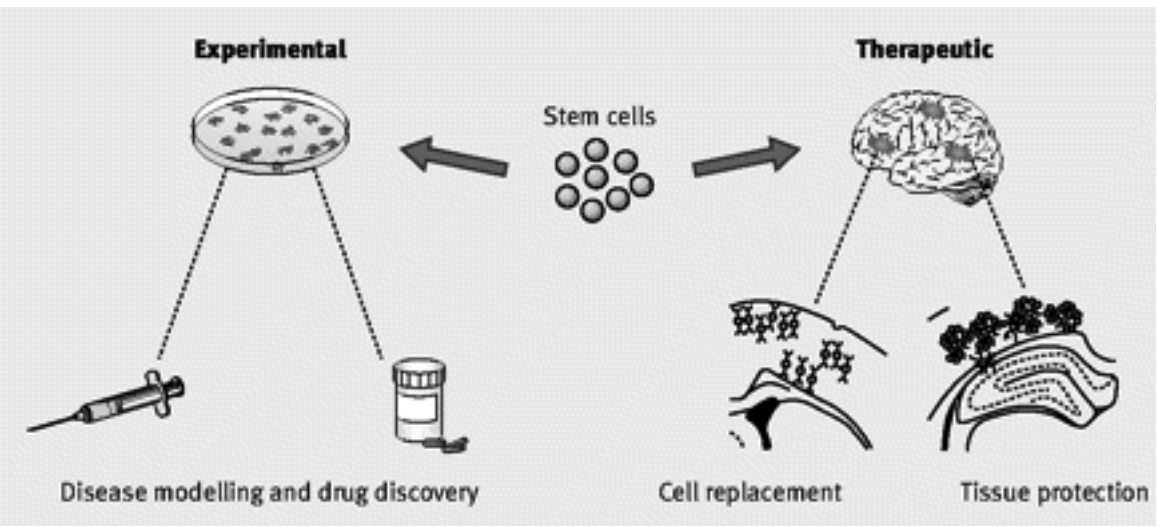

Fig. 1. *Stem cells allow the generation of near unlimited numbers of non-transformed human neural cells for experimental and therapeutic purposes. (From Chandran S, 2008. Reproduced with permission from BMJ)*

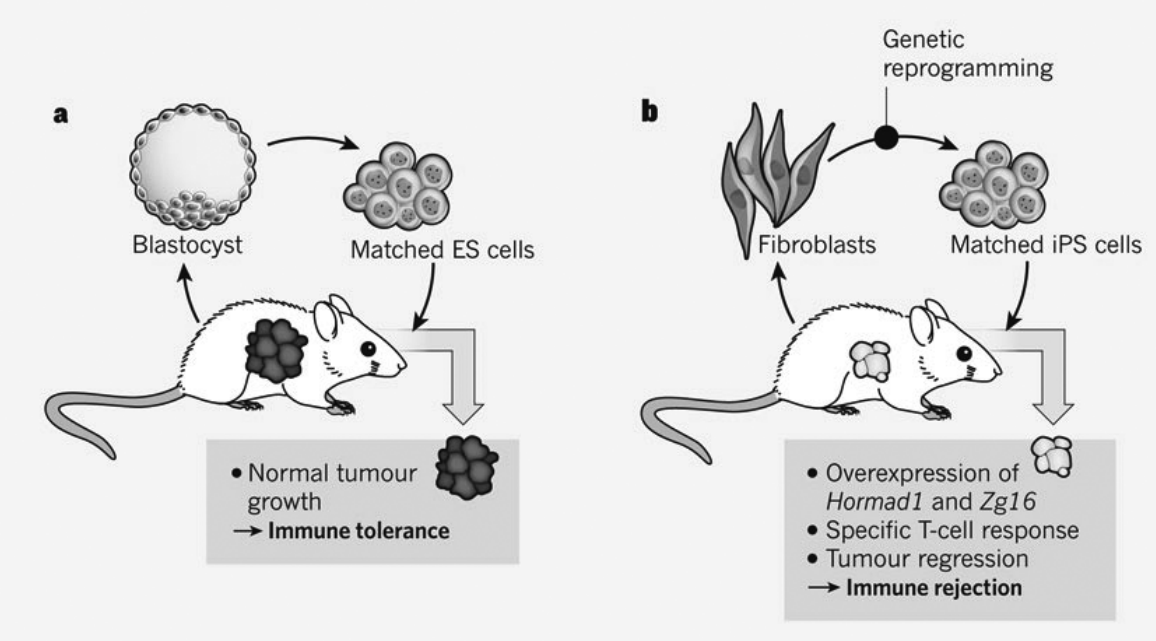

Fig. 2. *Panel a: The immune system tolerates autologous ESCs derived from blastocyst embryos grow teratomas on transplantation into mice of the same genetic background. Panel b) iPSCs reprogrammed from fetal fibroblasts by different approaches elicit an unspected immune reaction in genetically identical mice, resulting in their rejection (from Apostolou E and Hochedlinger K, 2011. Reproduced with permission from Nature)*

XXI *Parkinson's Disease* At present, a trial of mesenchymal stem cells from bone marrow in patients with a particular and debilitating form of Parkinson's Disease, progressive supranuclear palsy, is being performed in Italy at the Ospedale Policlinico in Milan. However, few patients have hereto been treated. The cells, provided by the *Cell Factory* of the hospital, are introduced by means of a catheter inserted in the femoral artery up to the CNS basal nuclei (Lazzari and Giordano, 2012).

The problems for efficient stem cell treatment in nigrostriatal degenerations are broadly considered in a work by Lindvall and Kokaia (2009). These Authors report experiences with human fetal mesencephalic tissue from 300-400 patients and research has shown that grafted dopaminergic neurons reinnervate the striatum and, in some patients, yield considerable benefits for years. Despite the achieved improvements, compared to placebo, the occurrence of side effects, including dyskinesias, have been debated. Dopaminergic neurons can be developed from different sources, including reprogrammed somatic cells. Concerns still persist on the ability of these cells to provide a functional reinnervation and motor recovery of animals with experimental models of Parkinson's Disease. The graft should be designed according to the patient's conditions and taking into account the possible formation, of a number of injected cells, of dopaminergic foci responsible for dyskinesias or the release of dopamine in an inappropriate area. It is worthy taking into consideration the presence of serotonergic neurons in the inoculation. The injection of 100,000 cellular components in the putamen is suggested, with the least possible number of serotonergic neurons and considering immunosuppression for 12 months. Significant contributions have been provided by other Authors (Loewenbrũck and Storch, 2011; Gibson et al, 2012).

XXII. A survey, initiated in Italy at the Ospedali Civili of Brescia, on the use of stem cells on 19 patients affected by neurodegenerative diseases, was blocked by the Italian Agency for Drugs (Agenzia Italiana per il Farmaco, AIFA) due to the inadequacy

Table I. *Neurodegenerative disease clinical trials*

Neurodegenerative disease (studies)	Stem cell type	Phase of clinical trials	Event date	The state of trials and possible outcome
Spinal cord injury (1)	hESCs	I	2009	Interrupted (2010)
Stargadt disease; age-related atrophic macular degeneration (1)	hESCs	I/IIa	2010	Remarkable clinical results, downsized and criticized (2012)
Stroke [3; 1 ongoing (PISCES)]	ASCs or*	I/II	2008-2010	Debated results
Amyotrophic lateral sclerosis (3; 1*)	ASCs or *	I	2009-2012	Unknown
Multiple sclerosis (11)	ASCs	I/II	2008	Partial or uncertain results
Sovranuclear cerebral palsy (1)	ASCs	I/II	2012	Ongoing
Autism (1)	Umbilical cord cells		2012	Ongoing

**Fetal brain stem cells*

of the laboratory supplier of cells (Stamina Foundation) and a number of other problems, such as the lack of required authorization by the regulatory authorities and only one favorable opinion of the hospital Ethics Committee (Toresini, 2012).

After the intervention requested by the parents of a child with muscular dystrophy treated in the aforesaid hospital, the magistracy has also decided to continue the treatment for a "compassionate use". On 31 August 2012, the Ministry of Health issued a position paper stating that the treatment was performed on the grounds of no certified scientific method and there is no scientific data proving its effectiveness. Subsequently, on March 7th present year, the Minister authorized similar treatment, which had been denied by a previous judge, to another young patient affected by methacromatic dystrophy. This decision caused a strong reaction by Italian researchers (go.nature.com/pbwdl; in Italian) and European Medicines Agency. In this regard, it is worth noting that allowing a compassionate use would require convincing bibliographical evidence and reliable clinical outcomes, both non-existing in the examined cases. Significantly different is the position of the FDA in the U.S. which considers the unauthorized use of stem cells a crime and the US magistracy, which entitles the FDA solely to grant the authorization. Finally the Italian Parliament has considered stem cells as drugs entailing subsequent regulatory and legislative policies.

XXIII. In August 2012, the FDA gave the green light for a clinical trial on autism to be held in the Setter Neuroscience Institute in Sacramento, with CBR (cord blood registry). The treatment will be performed with umbilical cord cells injected intravenously (No Author listed 2012c). No clinical results were found in subjects with severe infantile muscular atrophy (SMAI) treated with intrathecal injections of mesenchimal stem cells supplied by an hospital stem cells facility (Carrozzi et al, Neuromuscul. Disord. 2012; 22:1032-1034)].

THERAPEUTIC USES OF NON-AUTHORIZED STEM CELLS BY REGULATORY AUTHORITIES

Patients with more or less advanced severe diseases and who cannot wait a decade to start following clinical guidelines for stem-cell treatment, validated by authorized experiments, consult centers worldwide (about 300, including more 100 in China), often inadequately advertised (on dozens of websites!), where stem cell treatments are performed without specific controls leading to a sore tourism of stem cells (Basrclay, 2009).

Regarding ALS, important results have been

reported from a center in Dubai, run by the Turkish surgeon, Deda (2008; 2009) and by Martinez et al (2012) of the San José Tec Hospital of Monterrey in Mexico. Dr. Deda extracted bone marrow from the outer edge of the patient's external pelvic girdle, subsequently processed in a specialist laboratory in Jordan to obtain "purified" mononuclear cells. He then performed a laminectomy at the C1-C2 level under general anaesthesia in order to locate the spinal cord and he injected the cells in different areas (Deda et al, 2009). Results have been published (Deda et al, 2008; 2009), but skilled British neurologists have stated that there are no scientific grounds to approve them because of a lack of randomization and blindness and basic science does not provide any indication that the grafted cells may be effective in ALS (Gornall, 2010). The same observations can be stated concerning the results obtained by Martinez et a (2012), according to which the transplantation of stem cells in the frontal motor cortex delays the progression of ALS and improves the quality of the life.

Many objections have been raised with regard to stem-cell treatments and their effects in Parkinson's disease.

Since 2007, XC-Center (www.xcell-center.com), based in Cologne and Dusseldorf has drawn more than 3500 patients worldwide affected by 17 different types of chronic degenerative diseases including: cerebral palsy, multiple sclerosis, autism, Parkinson's disease, Alzheimer's disease, heart disease and diabetes (Tuffs, 2010).

Patients with MS were treated, as mentioned, in Monterrey (Martinez, 2012) and Costa Rica (50-70 patients) (158), reporting good results. A case of type I diabetes was treated positively in Peru. A Dutch doctor, working in England, was prosecuted by the English medical Association for carrying out stem cell treatments, requesting remuneration (Dyer, 2010) .

In China, the most diverse stem cells are injected in hundreds of centers (over 100 known in 2009) (Cyranoski, 2012b; Dolgin, 2010, 2012d), although three are particularly well known:

In May 2009 the Minister of Health in China classified stem cell treatments as a "high risk" and required authorization by a special Committee for any use (No Author listed, 2011; 2012f and 2012g). The provision was strongly reinforced on January 10 this current year, each trial was discontinued without prior and specific authorization and a program for new trials was submitted in July (No Author listed 2012d, 2012e).

This provision seems to recall a proclamation by Alessandro Manzoni, in "The Betrothed" ("I Promessi Sposi"). To our knowledge, only *Beijng Puhua International* followed the Government regulation on stem cells (Cyranoski, 2012b).

DANGERS ON THE USE OF STEM CELLS IN INAPPROPRIATE SITUATIONS

Bruce Dabkin from the University of California, Los Angeles, reports that patients treated in centers in China have had spinal cord injury, meningitis, nerve complications and infections after stem cell treatments for blood diseases (Barclay, 2009). An Israeli boy affected by ataxia teleangectasia and treated with stem cells in Russia in 2001, developed brain and spinal cord tumor in 2005, probably due to the injected cells (Barclay, 2009; Dolgin, 2010). Gornall (2010) reports further cases of stem cell therapies in unauthorized centers. Strange lumps of cells were detected in the kidneys of a woman who had undergone a stem cell treatment in Thailand (Dolgin, 2010). An 18-month-old baby with cerebral palsy had a severe disorder after the injection of stem cells into the brain in a private hospital in Dusseldorf (2010).

A so-called colitis cord syndrome has developed by 10% in subjects who received cord blood 88-114 days (median 131) after transplantation (Herrera et al, 2011).

Even under the best conditions, stem cell treatments imply risks and it has been pointed out that the cells obtained may be contaminated, in particular in the expansion phase, by bacteria and viruses and the procedures used for administration can cause tissue damage (Kuehn, 2012).

Tumors (Atala, 2012; Cyranosky, 2012a; Germain et al, 2012; Krizhanovski and Lowe, 2009; No Author listed, 2012a) and autoimmune diseases (Cyranoski, 2012b) must always be considered (fig. 2).

REGULATORY ISSUES

The current approach of the FDA has had to deal

with a large number of supposed centers in the U.S. as well (about one hundred) (Cyranoski, 2012c) where stem cell therapies were carried out for spinal cord injury, ALS, multiple sclerosis, autism, etc., without the FDA approval of the treatments performed (Cyranoski, 2012d). In one of these advanced centers, the FDA has blocked the use of bone marrow and synovial fluid ASCs and the potential conversion of mesenchymal cells into bones, cartilage and fat (Cyranoski, 2012c).Very recent decisions of the U.S. courts have clearly established the role of the FDA on trials carried out with stem cells (Cyranoski, 2012c; No Author listed, 2012g). A consumer warning was broadcast by the FDA in January 2012 (htpp:// tinyurl.com/7f4dlchc). On 27 December 2011 a university researcher was arrested, charged with providing stem cells in unapproved therapies (No Author listed, 2011).

The situation was made even more confusing by the positions taken by scientific associations, such as the *International Cellular Medicine Society* (ICMS) and the *International Society for Stem Cell Research* (ISSCR). The ISSCR, a team of biologists and clinicians in the stem cell field, based in Deerfield, Illinois, yet diffused worldwide, has developed specific guidelines (*Guidelines for the conduct on Human Embrionic Stem Cell Research*) and launched a website (go. nature.com/zK3L4c) (Dolgin, 2010; Dickens BM, 2006). The ISSCR has also decided to expel its affiliated members from clinics offering unauthorized stem cell treatments and not strictly in compliance with the *Guidelines*.

12% of the funds the ISSCR receives are financed by the industries, a detail that was noted by the ICMS, despite, its subsequent withdrawal , by stating that the ICSSR funds are aimed to "financing basic research" and not supporting industrial interests (Cyranoski, 2012).

The FDA position on the initiatives and proposals of the two above-mentioned associations and their credibility ranges from critical to cautious (Dolgin, 2010), even on the grounds of the undisputed and legally acknowledged role of the Agency in terms of authorizations of clinical trials on embryonic stem cells conducted in the USA. (Cyranoski, 2010; Cyranoski, 2012c; No Author listed 2012h) The type of IRB developed by the ICMS, the lack of company alien members, the need for an adequate presence and the voting system have been strongly criticized (Kuehn, 2012).

There are specific provisions on cell-based therapies in Europe (EMEA, 2006-2008; 2008-2010)187-188).

As previously stated, serious measures were taken in the UK against a doctor who performed treatments with stem cells (Dyer, 2010).

CONCLUSIONS

Stem cells and actions taken on them are still considered an important source of experimental and clinical research (Aznar and Sanchez, 2011; Chandran, 2008; Daley, 2012; De Feo, 2012). These cells are indeed considered useful not only in the so-called regenerative therapies, but also in cell-based studies of the pathogenesis of diseases, in the field of medicine discovery and in the screening for drug toxicity. The pharmaceutical industry has shown a lack of interest in stem cells as the development of innovative drugs does not apply to stem cells, in that the use of stem cells is unrealistic in any pharmaceutical preparation.

The unfortunately widespread and indiscriminate journalism zeal for reparative therapies with stem cells is based on ongoing reports of the results obtained in experiments, whereas current clinical trials hitherto carried out, approved by regulatory authorities, and not those independently conducted in unauthorized locations and manners are an uncertain outset and an undefined perspective. (Daley, 2012). It was pointed out that the only safe and controlled stem cell therapy in current use is probably only represented by the transplantation of autologous cells from bone marrow, umbilical cord blood or from peripheral blood for the treatment of serious haematological diseases (Ilic, 2012, Kuehn, 2012). The stem cells involved in such treatments simply continue to perform their natural functions (Dolgin, 2010).

The recent Nobel Prize awarded to Yamanaka has led the media to an overvaluation of the discovery of iPS cells for therapeutic purposes. Yamanaka himself has recommended a period of about three years for the first possible applications and, more recently, Masayo Takahashi, from theJapanese RIKEN Center for Develpmental Biology, has reported a cautious use of such cells to treat retinal diseases (Cyranoski,

2012d).

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BIOMARKERS TO PREDICT DRUG EFFICACY AND SAFETY IN NEURODEGENERATIVE DISEASES

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Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Multiple sclerosis (MS) (at later stages) require expensive long-term care, that rarely reduce disease progression. For this reason, the treatment of these disorders represents a major challenge for the pharmaceutical industry. The identification of genetic factors implicated in the drug response variability has become a crucial step for the future of patients care in the prospective of identification of genomic biomarkers of drug efficacy and safety with the ultimate goal of facilitating a more personalized therapy. Pharmacogenomics studies the relationship between inter-individual genetic variability and differential drug response, both in term of safety and efficacy, with the aim to identify patients who are most likely to benefit from a particular treatment or are at high risk for drug adverse reactions. In this review we focused on current knowledge of genetic factors contributing to different response to therapy of the principal neurodegenerative diseases such as AD, PD, MS. Although in recent years pharmacogenomics of these disorders have had important progress towards identification of safety and efficacy genomic biomarkers, the translation to clinical practice is still a long process.

Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) and multiple sclerosis (MS) at the later stages of the disease, have common and unique molecular pathological characteristics that result in serious reductions in nervous-system functionality. Some of these disorders only marginally affect the lifespan of the patient, thereby requiring expensive long-term care; however, current treatments rarely reduce disease progression. For this reason, the treatment of these disorders represents a major challenge for the pharmaceutical industry.

Although it is demonstrated a clear multifactorial origin of such neurodegenerative disorders, genetic

Key words: Pharmacogenomics, Genomic Biomarkers, Single Nucleotide Polymorphism (SNP), neurodegenerative diseases, Alzheimer's disease (AD), Parkinson's disease (PD), Multiple sclerosis (MS).

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2279-5855 (2013)

factors play an important role in establishing the pathological events, and even dramatically changes the disease phenotype from patient to patient. Many genes have been identified as susceptibility factors, increasing the risk of disease development, operating as regulatory factors, modulating the magnitude and severity of pathogenic processes and also the response to drug treatment (Peden and Ironside, 2012; Schellenberg and Montine, 2012; Rocket et al., 2012; Gorraud et al., 2012; Kumar et al., 2012). Genetic variability in genes encoding drug-metabolizing enzymes, drug receptors and proteins involved in pathway signalling may be implicated in the drug response variability. The identification of such variability has become a crucial step for the future of patients care in the perspective of identification of genomic biomarkers of drug efficacy and safety with the ultimate goal of facilitating the individualization of therapy (Currow and Abernethy, 2012; Doerr and Eng, 2012; Holmes et al., 2009).

In last years, pharmacogenomics, has obtained a growing interest in all fields of medicine, including neurology (Wilkinson, 2006; Davis and Khoury, 2006; Shabo, 2006; Crews et al., 2012; Lesko and Schmidt, 2012). However, even though in recent years the neurodegenerative disorders care have experienced considerable progresses, there is still an increasing need to tailor therapeutic options to optimize the benefit- risk relationship. Identification of predictive biomarkers would identify those individuals who are more likely to respond to a specific treatment, while directing non-responders toward alternative treatment. Different categories of genes could be mainly involved in pharmacogenomics of neurodegenerative disorders, these include: genes associated with disease pathogenesis, genes associated with the mechanism of action of a particular drug, genes associated with phase I and phase II metabolic reactions, genes associated with transporters, and pleiotropic genes and/or genes associated with concomitant pathologies. Considering the important contribution given by the inter-individual genetic variability to the drug response, also the International Regulatory Agencies (European Medicine Agency –EMA– and Food and Drug Administration -FDA) in the last years began to face these new issues and together initiated a series of international conferences with the objective of obtaining input from the pharmaceutical industry, academia and other stakeholders (Novelli et al., 2008; Novelli et al., 2010; Goodsaid and Papaluca, 2010). Anyway, it must be highlighted that also non-genetic factors may influence drug disposition; for example, adverse drug reactions are frequently caused by the presence of non-genetic factors that alter the pharmacokinetics or pharmacodynamics of a drug, such as diet, social habits and renal or hepatic dysfunction (Shah and Shah, 2012).

In this review we focused on current knowledge of genetic factors contributing to different response to therapy of the principal neurodegenerative diseases such as AD, PD, MS, both in terms of safety and efficacy.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a complex neurodegenerative disease that leads to a chronicle progressive decline and loss of cognitive function. The clinical spectrum of AD is various and includes both behavioural and psychological symptoms. It is the most frequent cause of dementia in the elderly (Cacabelos et al., 2012) and represents a major problem of health in developed countries. AD clinical picture usually requires the utilization of different drugs administered simultaneously, including memory enhancers such as the conventional antidementia drugs, psychotropics, anticonvulsants, antiparkinsonians, and others.

The mainstay of treatment for the cognitive symptoms of AD is aimed at addressing the limbic and cortical cholinergic deficit. At present, there are different cholinesterase inhibitors commonly used: donepezil, rivastigmine, galantamine, tacrine and memantine. Clinically, there is significant variability in the response and side effects to cholinesterase inhibitors (ChEIs) in AD, ranging from no response or gradual worsening, to a slowed rate of symptomatic decline, to improvement in cognition and behaviour, although this is time limited. Several pharmacogenomics studies have demonstrated that the therapeutic response in AD is genotype-specific, and that APOE genotype status affects the quality and size of drug responsiveness in AD patients treated with ChEIs (Cacabelos et al., 2012). Early studies found that drug efficacy was reduced in patients

treated with tacrine, carrying the ApoE-ε4 allele (Poirieret al., 1995, Farlow et al., 1998). Anyway, later studies have found discrepant results (Rigaud et al., 2002). Different treatment responses in APOE-ε4 carriers have been observed also for donepezil (Bizzarro et al., 2005; Choi et al., 2008). Prospective studies with galantamine in large samples of patients in Europe (Aerssens et al., 2001) and in the United States (Rasking et al., 2000) showed no effect of APOE genotypes on drug efficacy.

Recent studies indicated Tomm40 gene as a potential biomarker to identify people at high risk of developing sporadic AD at relatively young ages (Roses 2010; Johnson et al., 2011). This gene is close to ApoE and its variants are co-inherited with particular ApoE alleles; if Tomm40 will be validated as a biomarker for individual AD risk, it should be interesting to test if it can also influence the drug efficacy.

Tacrine treatment is also associated with adverse drug reaction, such as liver damage. A study by Alfiveric et al, reported that genetic variants in ABCB4 transporter may influence tacrine-induced elevation of liver transaminases (Alfiveric 2007).

Harold et al (2006), found one SNP, rs733722, in a promoter region of Choline acetyltranferase gene (ChAT), that was associated with AD patients response to cholinesterase inhibitors (P = 0.03) and accounts for 6% of the variance in response to AChE inhibitors.

Different studies have found a positive association between polymorphisms in CYP2D6 gene and ChEIs response. Pilotto et al. (2009), have evaluated the influence of the CYP2D6 gene in the clinical efficacy of donepezil in patients with mild to moderate AD: a significantly higher frequency of patients with the G allele of rs1080985 was found in non-responders than in responders (58.7% vs 34.8%, p = 0.013) (Pilotto et al., 2009). Recently, another study has confirmed that functional polymorphisms in the CYP2D6 gene can influence the clinical efficacy of donepezil (Seripa et al., 2011). The analysis of CYP2D6 genotypes may be useful in identifying subgroups of AD patients with different clinical response to donepezil treatment.

However, it is crucial to consider the ethnicity of a population when a *CYP2D6* genotypes analysis is performed. Indeed, *CYP2D6* gene is highly polymorphic and heterogeneous (Bertilsson, 1995; Bradford, 2002; Teh and Bertilsson, 2012) and the type and frequencies of alleles show ethnic specifities. For instance, the type and frequencies of *CYP2D6* among Caucasian are different from those in Asians and Africans (Ismail and Teh, 2001).

Other studies have described positive association between polymorphisms in acetylcolinesterase gene and response to rivastigmine, choline acetyltranferase gene and response to donepezil, galantamine and rivastigmine (Harold et al., 2006; Santoro et al., 2010).

Polymorphisms in *CYP2D6* gene, as well as polymorphisms in acetylcholinesterase (*AChE*), butyrylcholinesterase (*BChE*), choline acetyltransferase (*ChAT*) were found to be associated with better clinical response to donepezil and galantamine (Noetzli and Eap, 2013). In conclusion, although pharmacogenomics findings could be useful in AD to avoid medication changes due to non-response or/and adverse events, confirmation studies in larger populations are necessary to establish evidence of which subgroups will most likely benefit from treatment.

PARKINSON'S DISEASE

Parkinson's disease (PD) is a common neurodegenerative disease affecting about 1% of the population over the age of 60 years. Current therapy for PD is limited to the symptomatic relief of patients, having so far failed to prevent or inhibit the neuro-degeneration process. L-dopamine (l-dopa) is considered the standard treatment for PD. However, it is known a marked inter-individual variability in drug response and the occurrence of adverse events (such as motor complications, motor fluctuations, dyskinesias, confusion, hallucinations, psychosis and impulse control disorders). Different studies have evaluated the role of polymorphisms in monoamine degradation enzymes (COMT and MAOB) in relation to L-dopa response. No differences were found between COMT genotypes and response to L-dopa (Lee et al 2002; Watanabe et al., 2003; Contin et al., 2005). However, Bialecka et al. (2004), reported an higher frequency of COMT L/L (low activity variant) in patients treated with low doses of L-dopa. Moreover, the COMT L/L genotype was also associated with less dyskinetic symptoms in patients

treated with L-dopa and entacapone (a COMT inhibitor that increases the L-dopa elimination half-life) (Bialecka et al., 2004). On the other hand, other studies demonstrated that the COMT H/H (High activity variant homozygotes) genotype increases the positive effect of entacapone on the response of PD patients to L-dopa (Corvol et al., 2011). In addition, COMT inhibition was correlated with the plasma concentration of entacapone, suggesting that higher doses of the drug might be more effective in COMT L/L patients.

Other genes investigated as potential modulators of PD therapy were MAO-B and MAO-A. Anyway the study of Bialecka et al. (2004), reported a difference in the daily dose of dopa in patients with different alleles in MAO-B, without reaching statistical significance (Bialecka et al, 2004).

Pramipexole is a dopamine agonist of the non-ergoline class indicated for treating early-stage PD. Liu et al. (2009), investigated the role of dopamine receptor polymorphisms (DRD2 and DR23 genes) in the response to pramipexole treatment (Liu et al., 2009). They found a significant association between the DRD3 Ser9Gly (rs6280) polymorphism and response rate to pramipexole in PD patients ($P = 0.024$): in particular an higher response rates in patients Ser/ser homozygotes (Liu et al., 2009). SNPs and genotypes at this locus have been also associated with developing hallucinations, a side effect in long term PD therapy (Makoff et al., 2000).

Another DRD2 polymorphism in the promoter region, -141C ins/del (rs1799732), has been associated with the developing of hallucinations; in particular patients with C/C genotype show a greater risk to develop hallucinations (Makoff et al., 2000). Moreover, a dinucleotide polymorphism in intron 2 (CA repeat) has been associated with an increase risk of diskynesia problems (Zappia et al., 2005; Strong

Table. 1. *Principal genes associated with treatment efficacy and/or adverse drug reactions in neurodegenerative diseases*

Disease	Drug	Gene	Associated with	References
Alzhaimer's disease	Donepezil	APOE (e4 carriers) CHAT CY2D6	Response	Bizzarro et al. 2005, Harold et al. 2006, Pilotto et al.2009
	Galantamine	CHAT	Response	Harold et al. 2006
	Rivastigmine	ACHE CHAT	Response	Harold et al. 2006
	Tacrine	ABCB4	Liver toxicity	Alfirevic et al. 2007
Parkinson's disease	Levodopa	COMT (LL or HH alleles)	Response	Bialecka et al. 2004
	Levodopa	DAT (VNTR)	Dyskinesia and psychosis	Kaiser et al. 2003
	Levodopa + entacapone	COMT (HH alleles)	Response	Corvol et al. 2011
	Levodopa + entacapone	COMT (LL alleles)	Dyskinesia	Bialecka et al. 2004
	Pramipexole	DRD2 and DRD3	Hallucinations	Makoff et al. 2000
Multiple sclerosis	IFN-β	IFNAR1, IRF5, GPC5	Response	Cunningham et al. 2005, Comabella et al. 2009, Vandenbroeck et al. 2011, Graham et al. 2007, Cenit et al. 2009
	Glatiramer acetate	HLA-DR1*1501	Response	Fusco et al.2001
	Mitoxantrone	ABCB1 and ABCG2	Response	Cotte et al. 2009
	Mitoxantrone	ABCB1 and ABCG2	Cardiotoxicity	Dorr et al. 2009

et al., 2006).

The rs28363170 polymorphism (40 bp VNTR) in the 3'UTR of dopamine transporter gene DAT (SLC6A3) has been shown to be a risk factor for developing dyskinesia and psychosis, in patients carrying multiple copies of this repeat polymorphism (Keiser et al., 2003). Anyway, another study by Contin et al, found no significant difference in levodopa main outcome variables and dyskinesia incidence between two groups of patients stratified by DAT VNTR polymorphism. (Contin et al., 2004). Further investigation should be carried out to reach more confirmed results.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a complex genetic diseases, characterized by modest disease risk heritability, polygenic inheritance, multifaceted gene-gene and gene-environment interactions, and clinically variable phenotypic presentation. Although many clinical trials are in progress, only few drugs have been approved for the MS treatment. However, none of these can halt disease progression indefinitely or reverse previous neuronal damage; the two most frequently used first-line disease treatments, glatiramer acetate (GA) and interferon beta (IFN-β), have been clearly shown to reduce the relapse rate, slow the appearance of new and enhancing lesions, and delay progression of disability. However, MS is a heterogeneous disease, and patients vary in their response to treatment with either drugs.

IFN-β was one of the first drug to demonstrate a clinical efficacy in MS treatment. Anyway, the efficacy is restricted to a subgroup of patients with relapsing remitting MS that represents about 30% of all MS patients. Prior attempts to identify 'predictors of response' were based on pre-selection of candidate genes associated with Type I interferon pathways. IFN-β, a pleiotropic cytokine, binds to a heterodimeric receptor (IFNAR1/IFNAR2) of the Jak/STAT signaling pathway. Cunningham et al, have studied polymorphisms in genes containing interferon-stimulated response elements and they identified 4 genes associated with the INF-β response (IFNAR1, LMP7, CTSS and MxA) (Cunningham et al., 2005). Some studies have confirmed an association between a GT repeat in the IFNAR1 promotor and the effective response (Cunningham et al., 2005; Matsuyama et al., 2003).

Two recent studies have assessed the association of IRF5, a transcription factor that regulates expression of various genes, with clinical response to IFN-β (Vandenbroeck et al., 2011; Graham et al., 2007): a poor pharmacologic response was found to be associated with rs200460 T/T genotype (P=0.0006) and rs478142 A/A genotype (P=0.002) (Vandenbroeck et al, 2011).

Also the APOE gene has been linked with clinical response, even if two different and recent studies reported conflicting results (Guerrero et al., 2011, Carmona et al., 2011). In the first study, authors did not find any association with clinical variables after two year of INF-β treatment (Guerrero et al., 2011); in the second study authors found an increased time to moderate disability in patients carrying the APOE-ε2 allele (Carmona et al., 2011).

Two recent whole genome association studies revealed associations of genes involved in neuroprotection and neurogenesis, such as GPC5 and NPAS3, and neurotransmission such as GRIA3 (Byun et al., 2008; Comabella et al., 2009). A subsequent replication study has confirmed the association of GPC5 with IFN-β response (Cenit et al., 2009): in particular for the rs10492503, the authors observed a significant differences between responder and non-responder patients (P = 0.0005).

Glatirate acetate (GA) is a immune-modulator drug, currently used to treat multiple sclerosis for reducing the frequency of relapses, but not for reducing the progression of disability. In 2001, an association of HLA DRB1*1501 (rs3135388) with better response to GA but not IFN β was described (Fusco et al., 2001). A recent study by Gros et al. (2011), validated this polymorphism as associated with clinical response: authors reported that homozygotes for the A allele of rs3135388 experienced a significantly longer event-free survival than A/G heterozygotes or G/G homozygotes (Gros et al., 2011). Anyway, the authors acknowledge that the effect of HLA DRB1*1501 by itself is not sufficient to explain the decreasing hazard rate among GA treated subjects, and they suggest that other alleles of modest effect and non-genetic factors affecting response to GA remain to be discovered.

Mitoxantrone is an immunosuppressor, restricted

to escalation therapy where other therapies have failed or as first line therapy for malignant MS forms. Genetic variants in ABCB1(rs1045642 and rs2031137) and ABCG2 (rs2231137 and rs2231142) genes were identified as potential predictor of drug response (Cotte et al., 2009). Morevover, rare genotypes in these genes are associated with early cardiotoxicity (Dorr et al., 2009).

Finally, it deserves to be quoted the purine analogue azathioprine (AZA) that is a good example of potential impact of pharmacogenomics in clinical practice. Although AZA is used in a variety of neuroimmumologic disorders, in MS it is used for maintenance treatment only for patients who frequently relapse. The drug presents hematological toxicity as severe adverse event that can be fatal in about 0.3% of cases. AZA conversion to inactive metabolites is mediated by TPMT (thiopurine-methyl transferase) and it is well described a correlation between TMPT genotypes and thiopurine toxicity (Ford and Berg, 2010; Sahasranaman et al., 2008). For this reason, AZA treatment is contro-indicated in patients with low/absent TPMP activity (about 0.3%).

CONCLUSIONS

In the last years pharmacogenomics of neurodegenerative disorders had important progresses towards identification of safety and efficacy genomic biomarkers. However, since replication studies of genetic factors associated with drug response often have reported conflicting results, the translation to clinical practice is still a long process.

The difficulty in finding clear genetic biomarkers linked to efficacy and safety of drugs is due to the fact that neurodegenerative diseases are complex, multifactorial diseases, in which different genes could be involved. Moreover, the identification of efficacy and/or safety biomarkers is further complicated by the presence of complex phenotypes affecting mood, personality, behavior, cognition and functioning. The heterogeneous clinical picture requires the utilization of different drug, used simultaneously: this further complicates the picture, since drug-drug interactions could alter the response in term of efficacy and safety. With increasing advances in science and technology and better understanding of the complex mechanisms that underpin drug response, personalized medicine may become a reality one day but these are very early days. For some drugs, the role of non-genetic factors may be so important that it may be difficult to personalize therapy (Shah and Shah, 2012).

Research in the neurodegenerative disorders is currently producing new potential genomic biomarkers of drug response. However, the confirmation of the association of identified polymorphisms described in this review, the identification of new polymorphisms linked to drug efficacy and/or drug safety, and the understanding of functional role of these variants are necessary to clarify the potential impact of genetic variants on drug response. Moreover, prospective studies should be conducted to demonstrate the clinical utility of these genomic biomarkers. Ongoing progress in the pharmacogenomics of neurodegenerative disease will contribute in a near future to the development of a more personalized and/or a population-specific treatment providing an important tool to make the dream of personalized medicine a reality.

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