FRIEDREICH ATAXIA: 150 YEARS OF BENCH AND BEDSIDE STUDIES

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Friedreich ataxia is the most frequent hereditary ataxia among Caucasians. Almost invariably, the disease is caused by homozygous GAA triplet repeat expansions in the first intron of the frataxin gene, *FXN*, whereas point mutations or deletions in conjunction with an expanded GAA tract account for the remaining cases. The expanded intronic alleles interfere with *FXN* transcription, decreasing the production of normally functioning frataxin protein to 5-20% of normal. Deficient frataxin levels result in excessive mitochondrial iron accumulation, reduced iron-sulfur clusters vital for mitochondrial energy production, and increased intracellular oxidative damage. To date, no cure has emerged and treatments remain largely supportive, despite extensive ongoing research and several rationale strategies have been attempted.

Friedreich ataxia (FRDA) is a multisystemic, degenerative disease with progressive ataxia and dysarthria, weakness, loss of position and vibration sense, scoliosis, and vision and hearing impairment (Filla et al., 1992). Most affected individuals have hypertrophic cardiomyopathy whereas glucose intolerance and diabetes mellitus occur in up to 30% of cases. The onset of symptoms is usually in the teens, but either much earlier or later onset has been less frequently observed (Table I). Initial symptoms can be purely neurological, but occasionally, cardiomyopathy can be the presenting symptom. Atypical presentations represent as much as 25% of the cases. We present a state-of-the art of FRDA in terms of clinical/genetic as well pathogenic mechanisms. A final panorama of present therapeutic options is also presented.

EPIDEMIOLOGY AND HISTORY

FRDA represents the most common hereditary ataxia in Caucasian with an estimated prevalence of 2-4 x 100,000 and a carrier frequency of approximately 1:90 to 1:60 (Filla et al.,1992; Polo et al., 1991). Nicolaus Friedreich (1825–1882) described in a series of 5 papers between 1863 and 1877 an apparently new spinal disease in nine patients from three sibships. The clinical features were well defined with onset at the time of puberty, progressive ataxia and dysarthria; sensory loss and muscle weakness were late findings; nystagmus was not consistently present. After Erb description of tendon reflexes, Friedreich reported absent tendon reflexes. He also reported scoliosis, foot deformity and cardiac symptoms. Post-mortem examination of four patients showed degeneration of dorsal columns, Clarke's columns and hypoglossal nuclei, and fatty degeneration of the heart in three cases. Friedreich believed that the four patients in whom he could secure autopsies had succumbed to 'abdominal typhus', perhaps a severe terminal febrile illness that in retrospect was unrelated to typhoid fever.

Key words: Friedreich ataxia, triplet disease, frataxin, mitochondrial iron-sulfer cluster, animal models, iPSC, antioxidant, HDACi, erythropoietin

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Table I. *Major features in Friedreich ataxia.*

Most frequent hereditary ataxia
Autosomal recessive inheritance
Onset in teen ages
Clinical hallmarks: Progressive ataxia involving spinal cord and cerebellum Peripheral neuropathy Corticospinal signs (Babinski)
Other features: Cardiomyopathy Diabetes Skeletal deformities

Friedreich's hypothesis that the disorder was an entity distinct from locomotor ataxia (tabes) or multiple sclerosis met considerable opposition. Charcot initially considered that Friedreich's patients suffered from multiple sclerosis, until he personally observed a young sufferer of hereditary ataxia, which did not conform to the diagnosis of tabes or multiple sclerosis. It was Brousse who proposed the term of "Maladie de Friedreich" in 1882 (Harding, 1981).

GENETICS

Upon position cloning on chromosome 9q21.11, the FRDA gene was identified in 1996 and a pathological expansion of a GAA/TTC repeat in the first intron of *X25*, later termed *FXN* (HGNC:3951), was detected in 98% of the disease alleles. The remaining 2% were pathological alleles harbor point mutations (missense/nonsense) (Campuzano et al., 1996) or very rare heterozygous multiexon deletions (Anheim et al., 2012). Thus, most patients carry a homozygous GAA expansion.

The GAA/TTC repeat tract is polymorphic in the human population with normal alleles containing 8 to approximately 38 repeats. The critical pathologic triplet repeat threshold in FRDA is 66 repeats, with average expansion being 630 GAA repeats on the smaller alleles and 890 GAA repeats on the larger ones. GAA expansion size affects the phenotype with an inverse correlation between number of repeats and age at onset (Filla et al., 1996; Durr et al., 1996). In addition, cardiomyopathy and diabetes have been associated with larger expansions in some series but not in others. The mitochondrial DNA haplogroup U may represent a possible modifier for age at onset and cardiomyopathy (Giacchetti et al., 2004).

CLINICAL PHENOTYPES: TYPICAL FORM AND VARIANTS

Onset age of typical FRDA is before 25 years and usually occurs between the end of the first decade and beginning of the second. Gait instability and clumsiness are typically presenting symptoms. Scoliosis is often already present when neurological symptoms appear. In rarer cases, hypertrophic cardiomyopathy becomes symptomatic before the onset of ataxia. Ataxia, that is progressive and unremitting, is a mixed cerebellar and sensory type. Truncal ataxia results in swinging, and gait becomes broad-based, with frequent loss of balance, requiring at first intermittent, then constant support. Limb ataxia causes difficulty in activities requiring dexterity and precision such as writing, dressing, and handling utensils. Dysarthria is almost universal. It usually begins within two years from the onset and progresses until speech becomes almost unintelligible. Progressive degeneration of pyramidal tracts leads to extensor plantar responses and proximal limb weakness. Tendon reflexes are usually abolished and position and vibration sense markedly impaired because of the peripheral neuropathy. Perception of light touch, pain, and temperature decreases with advanced disease. Some amyotrophy, particularly in the hands, is very common. On average, ten to fifteen years after onset, patients lose the ability to walk. However, disease progression is variable, and those with mild disease may still be ambulatory decades after onset, while those with severe disease may become wheelchair-

bound within a few years. Dysphagia, particularly for liquids, is a feature of advanced disease (Filla et al., 1990). Cognitive function is generally well preserved although subtle abnormalities, particularly of executive function, may be detected. Fixation instability, with square-wave jerks in primary position, and saccadic pursuit are the most common oculomotor abnormalities in FRDA. Nystagmus is present in a minority of patients. Ophthalmoparesis does not occur. About 30% of the patients develop optic atrophy, visual impairment, blindness and sensory hearing loss are not uncommon in the late stages of the disease. The median estimated survival from onset is 36 years. The presence of left ventricular hypertrophy or diabetes significantly reduces survival (De Michele et al., 1996).

MRI shows early cervical cord atrophy, whereas a slight cerebellar atrophy usually occurs in the late stage of the disease. Diffusion Tensor MR Imaging (DTI) shows increased diffusion in superior cerebellar peduncle (Della Nave et al., 2011). Peripheral nerve conduction study shows a mainly sensory axonal neuropathy (Caruso et al., 1983).

Atypical forms have been described, the most frequent being Friedreich ataxia with retained tendon reflexes or spasticity (FARR) and late onset Friedreich ataxia (LOFA) showing symptoms only after 25 years of age, although the disease rarely starts as late as the sixth or the seventh decade. These forms are associated with milder course and rarer occurrence of non neurological signs (Filla et al., 1996).

PATHOLOGY

Spinal involvement is more marked than that of brainstem and cerebellum. The most relevant pathological finding is atrophy of the long ascending and descending spinal tracts (dorsal columns, spinocerebellar and pyramidal tracts) with shrinkage in the cross-section of the cord. Loss of neurons in dorsal root ganglia neurons accompanied by loss of large myelinated fibers in peripheral nerves and degeneration of the posterior column in the spinal cord are the hallmark of the disease. Heart pathology mostly consists of thickening of the left ventricle and histological changes with interstitial fibrosis (Koeppen et al., 2013).

PATHOGENESIS

Expanded alleles lead to the inhibition of *FXN* expression resulting in decreased levels of the encoded protein, frataxin [Campuzano et al., 1996]. The transcriptional repression of the *FXN* gene induced by the GAA expansion is due to arrest of RNA polymerase II progression and to heterochromatin mediated gene silencing.

Frataxin is a small (210 aa) protein localized in the inner mitochondrial membrane (Campuzano et al., 1997). The structure of frataxin is conserved between species. Frataxin is a small globular acidic protein composed of a long N-terminal alpha helix and short C-terminal alpha helix that both interact with a central beta sheet structure (Musco et al., 2000; Pandolfo and Pastore, 2009). Frataxin is synthesized as a precursor protein (frataxin 1-210), then matured in two steps within the mitochondrial matrix to give an intermediate (frataxin 42-210) and a mature form (frataxin 81-210) (Condo et al., 2007, Koutnikova et al., 1998). Expression of *FXN* mRNA is decreased to 33% and the translated protein to 25% in FRDA peripheral blood monocytes in comparison to controls (Saccà et al., 2011a). Frataxin is expressed in all cells of eukaryotic organisms. However, mRNA levels and frataxin expression have tissue specificity that partially correlates with the sites of disease. In humans, the highest levels of expression are found in the heart and spinal cord, whereas lower levels are seen in the cerebellum, liver, skeletal muscle, and pancreas (Fig. 1). The differential sensitivity of tissues to frataxin deficiency remains unclear, though peculiar cellular oxidative metabolism and somatic instability of expanded GAA triplet repeats are a possible option (Bidichandani et al., 1999).

Three different pathogenetic mechanisms have been proposed as consequence of frataxin deficiency: the possibility of an abnormal structure caused by GAA expansion; a modified histone acetylation; and an abnormal DNA methylation. The first hypothesis suggests that long GAA repeats form unusual DNA structure (triplex helix) causing a "sticky" DNA that slows and inhibits the normal progression of polymerase (Sakamoto et al., 1999). The second hypothesis is based on the observation of decreased acetylation of the histones in proximity of the GAA expansion in the *FXN* gene. Hypoacetylation is

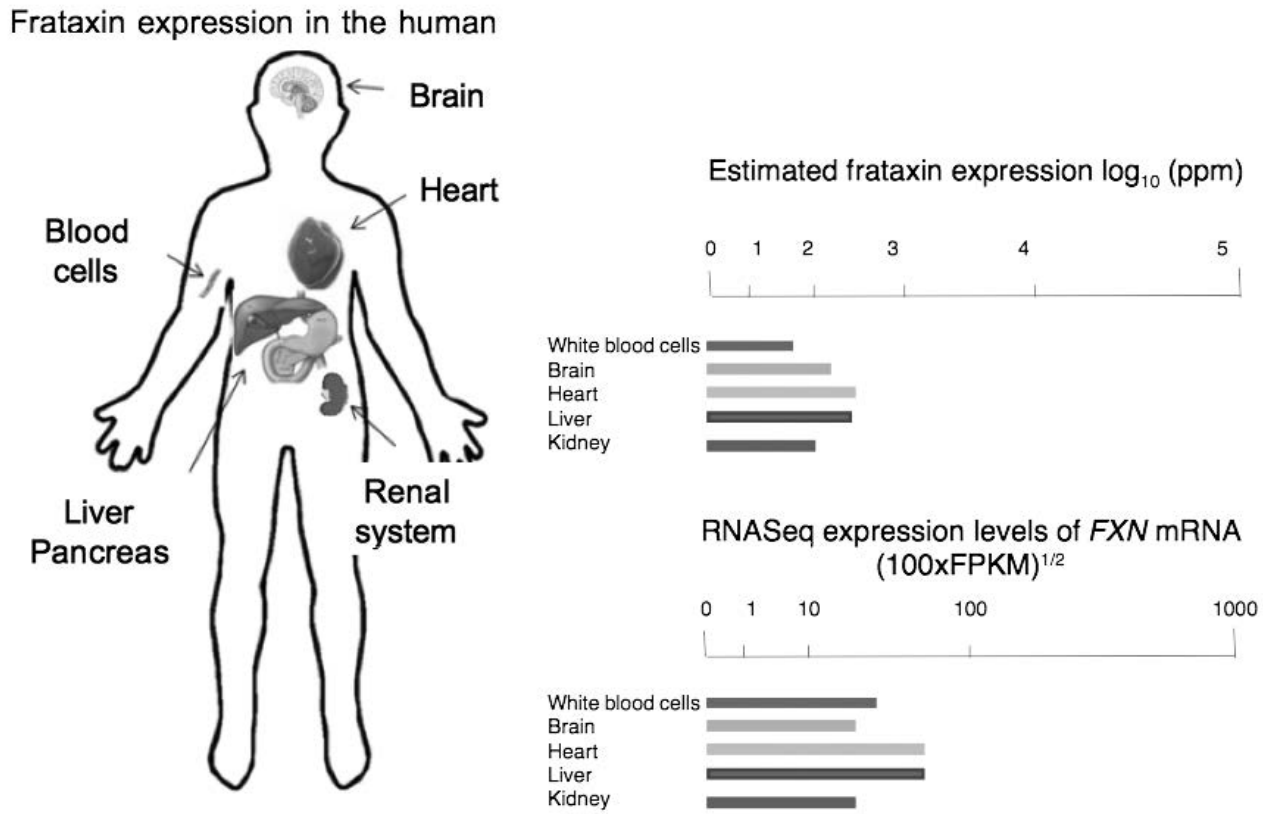

Fig. 1. *Tissues with the strongest expression of FXN (mRNA and protein) in human. Data for human tissues are retrieved from GeneCards (http://www.genecards.org) and generated using MOPED (Model Organism Protein Expression Database) and PaxDb (Protein Abundance Across Organisms) with modifications. FPKM: Fragments per kilobase of exon per million fragments mapped; ppm: parts per million.*

associated with euchromatin to heterochromatin shifts and with gene silencing. Hypoacetylation of H3 and H4 histones and trimethylation of histone 3 lysin 9 (H3K9) are seen in FRDA cells in the immediate vicinity of the expanded GAA repeat (Saveliev et al., 2003). Finally, hypermethylated CpG islands in the proximity of the abnormal *FXN* gene have been reported and the extent of methylation appears to correlate with the number of GAA repeats, frataxin/mRNA levels and disease severity (Castaldo et al., 2008). The last two mechanisms are not mutually exclusive but likely collaborate in silencing the gene.

FRATAXIN FUNCTION

Although frataxin function remains to be fully elucidated at the molecular level, a wealth of biochemical data and the determination of three-dimensional structures from several homologues of *FXN*, particularly yeast frataxin (Yfh1), have contributed to substantial progress in understanding its function. The yeast model (Babcock et al., 1997; Foury and Cazzalini, 1997) deleted of frataxin gene homolog shows mitochondrial iron accumulation, defective function of electron transport chain activities and increased sensitivity to oxidative stress. Frataxin has been supposed to be i) an iron mitochondrial storage protein; ii) a chaperone of iron in iron-sulfur cluster (ISC) biosynthesis; or iii) an allosteric regulator of ISC synthesis (Martelli and Puccio, 2014).

i) Bacterial and yeast frataxins are able to form an *in vitro* oligomeric spheroidal structure in the presence of excess iron. These oligomeric structures can capture up to 50-75 atoms of iron in a similar way as ferritin (Adamec et al., 2000). Due to its

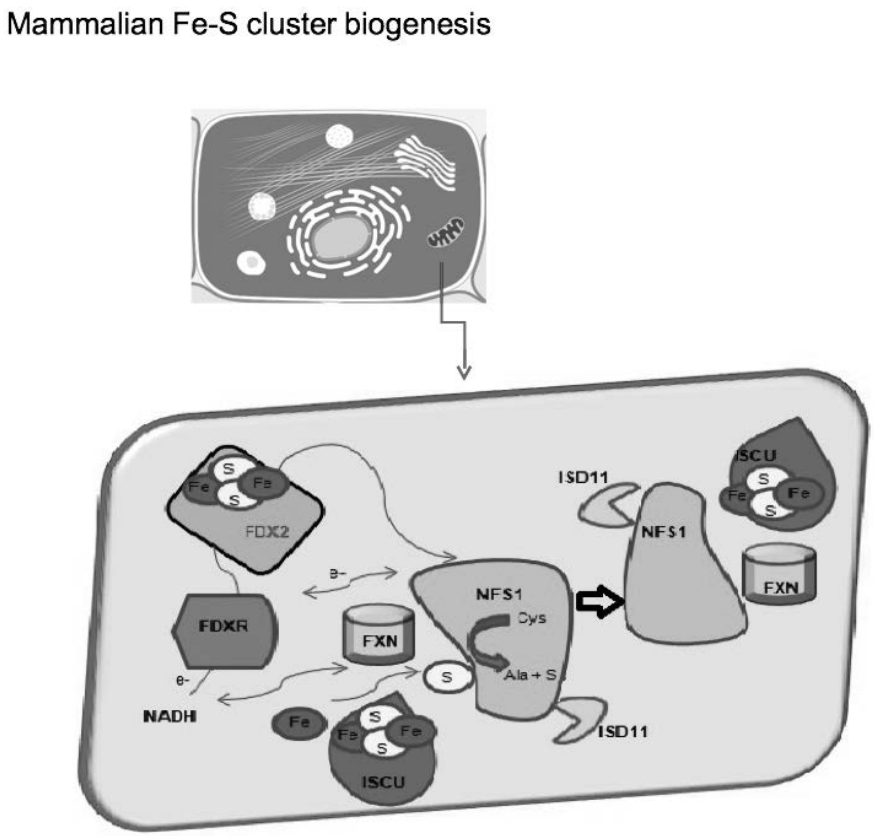

Fig. 2. *Schematic representation of the current model of mammalian Fe-S cluster biogenesis and its implication in Friedreich ataxia. The mitochondrial Fe-S assembly complex, which consists of the cysteine desulfurase NFS1 and its accessory protein (ISD11), the Fe-S cluster scaffold assembly protein (ISCU), and frataxin (FXN), converts substrates l-cysteine, ferrous iron, and electrons into Fe-S clusters. Cysteine desulfurase activity and iron entry is controlled by FXN. Electrons are provided by an electron transport chain consisting of NAD(P)H, ferredoxin reductase (FDXR) and ferredoxin (FDX2). The interaction of FDX2 with NFS1 occurs early, prior to binding of FXN/ISCU/ISD11. In particular, FXN seems to facilitate the transfer of sulfur from NFS1 to ISCU as an initial step in Fe-S cluster biosynthesis. Hence, loss of frataxin limits significantly Fe-S cluster regulatory mechanisms and biogenesis (from Beilschmidt and Puccio HM. Biochimie. 2014;100:48-60 with modifications).*

property in scavenging iron, oligomeric frataxin was initially proposed to act as ferritins by providing bio-available iron within mitochondria. The relevance of the *in vivo* function of oligomeric frataxin is questioned by *in vitro* data showing that bacterial frataxin forms iron-rich oligomeric structures only under aerobic conditions and high ionic strengths (Adinolfi et al., 2002).

Furthermore, in higher eukaryotes the oligomerization process does not appear to be fully conserved. Only the precursor and intermediate forms of frataxin can form oligomers in an iron-independent way whereas mature human frataxin is not prone to oligomerization (O' Neill et al., 2005).

Although the functional relevance of an iron-rich oligomeric frataxin is questionable, there is evidence that monomeric frataxin can also bind iron *in vitro*. Several iron-binding sites have been characterized depending on the oxidative state of iron (Fe^{2+}, Fe^{3+}) and the origin of frataxin proteins (bacterial, yeast, human) (Foury et al., 2007). A primary iron-binding site appeared to be conserved in the first alpha helix of frataxin. This site binds Fe^{2+} with low-medium affinity and poor specificity as other cations were

also shown to bind.

ii) Mitochondrial ISC biogenesis in higher eukaryotes is carried out by an assembly machinery composed by many factors. The main factors involved in ISC biogenesis in humans are the scaffold protein ISCU, where the nascent ISC is assembled; the cysteine desulfurase enzyme NFS1, which acts as a sulfur donor; the small protein ISD11, which forms a complex with NFS1 and is needed for its stability and function; the ferrodoxin FDX1 and ferrodoxin reductase FDXR, which provide reducent equivalents, and NFU which may be an alternative scaffold protein (Beilschmidt and Puccio, 2014; Fig. 2).

Frataxin plays a specific role in the biosynthesis of cellular Fe/S proteins (Muhlenhoff et al., 2002). Recent work has shown that frataxin is a part of the ISC assembly complex and functions as an allosteric activator (Tsai and Barondeau, 2010). The complex without frataxin is essential in "off" state. Only when frataxin is present, ISC synthesis can activate ("on" state) and proceed at a significant rate. As a result, when frataxin levels are low, the function of multiple iron-sulfur protein is impaired. Reduced activity of aconitase and complexes I, II, and III of the respiratory chain were first detected in myocardial biopsies of children with FRDA (Rötig et al., 1997), then deficiency in iron-sulfur protein activities in mitochondria and other cellular compartments were confirmed in tissues from patients with FRDA as well as in cellular and animals models (Puccio et al., 2001).

iii) Impaired ISC synthesis alters iron metabolism. Iron is imported into mitochondria by mitoferrin; if not rapidly used in a biosynthetic process, Fe^{2+} can rapidly oxidate and form insoluble precipitates. Such precipitates have been detected in mitochondria from frataxin-depleted yeast, conditional frataxin KO mice, and also in myocardium of FRDA patients (Koeppen, 2011). Iron oxidation in mitochondria is likely to be damaging because of the toxic free radicals it generates (Tozzi et al., 2002A). Further major consequence of impaired ISC synthesis on iron metabolism is the abnormal activation on iron regulatory proteins (IRP) 1 and 2. IRP1 and 2 are cytosol proteins that control the expression of proteins involved in iron handling and distribution (Mackenzie et al., 2008). Only IRP1 contains an ISC. When this ISC is entirely assembled, the protein functions as a cytosolic aconitase. When the cytosolic iron is low, the ISC is lost and protein acts as RNA-binding protein that regulates translation of transferrin receptor (by increasing) and ferritin (by decreasing). This mechanism attempts to re-establish iron homeostasis by promoting iron uptake into the cell cytosol and then into mitochondria, to produce ISC containing proteins. When this is achieved, a negative feedback loop exists, such that the protein regains its ISC and returns to function as aconitase. In FRDA, ISC synthesis is defective so the proteins remains in "active state", promoting ongoing iron uptake into cytoplasm and subsequently into mitochondria, where it accumulates and is unable to be incorporated into ISC (Pandolfo, 2012). Reduced levels of frataxin in both human and mouse models results in defective ISC enzymes even before a pathological mitochondrial iron storage is detectable (Puccio et al., 2001; Stehling et al., 2004). However, studies on muscle creatine kinase (MCK) conditional frataxin knock-out (KO) mouse showed that an even earlier consequence of frataxin depletion is the increased fraction of labile redox-active iron inside mitochondria that prompts progressive oxidative damage (Whitnall et al., 2012), as well as a down-regulation of proteins necessary for mitochondrial iron redistribution and progressive accumulation (Huang et al., 2009). Ultimately, these pathological steps result in progressive impairment of energy metabolism and accumulation of oxidative damage (Wilson, 2006).

There are also additional changes outside of mitochondria affecting pathways involved in antioxidant, metabolic, and inflammatory responses, which are believed to contribute to disease progression (Isaya, 2014). Whilst an overwhelming body of evidence points to an important role for oxidative stress in FRDA, some controversy still exists. In patients with FRDA, some studies suggested that the oxidative stress was revealed by increased plasma levels of malondialdehyde (a lipid peroxidation product), increased urinary 8-hydroxy-2'-deoxyguanosine (a marker of oxidative DNA damage) (Emond et al., 2000; Schulz et al., 2000), but these findings have not been confirmed. Furthermore, the lack of oxidative stress is intriguing in studies of MCK conditional frataxin KO mouse

models (Seznec et al., 2005).

Decreased plasma free glutathione, and increased plasma glutathione-S-transferase activity have also been reported in FRDA patients (Tozzi et al., 2002).

Finally, an impaired antioxidant response occurs in FRDA. The pathogenesis of this impaired antioxidant response is as yet unclear, however reduced level of the transcriptional co-activator peroxisome proliferator activated receptor gamma (PPAR-γ), co-activator 1 alpha (PGC-1α), and nuclear factor erythroid to related factor 2 (Nrf2) may be in part responsible (Marmolino et al., 2010**;** Coppola et al., 2009). PGC-1α levels are decreased in fibroblasts and lymphoblasts of patients (Coppola et al., 2011).

ANIMAL AND CELLULAR MODELS

The effect of frataxin depletion has been modeled in diverse organisms, included yeast, invertebrates, Drosophila, and mouse. However, due to the complexity of the clinical phenotype of patients and the species specificity of certain fundamental pathways, mouse or mammalian cells are probably better suited to answer pathophysiological questions. It was first shown that complete deletion of frataxin in mice is not viable, resulting in high embryo lethality at day 6.5 (Cossée et al., 2000). This is in agreement with the clinical observation that there are no FRDA patients who harbor a complete loss of frataxin. To study the consequences of frataxin depletion in a tissue specific manner, several conditional KO mouse models were generated. Altogether, these different *Fxn* deletion mouse models reproduce hypertrophic cardiomyopathy, progressive spinocerebellar and sensory ataxia, and some aspects of diabetes, thus replicating most of the symptoms associated with FRDA, and have allowed better understanding of the pathophysiology of the disease (Puccio, 2009). Moreover, they are relevant for therapeutic tests, as they have been used for testing antioxidant, iron chelators and protein therapy. However, animal models with a complete deletion of frataxin from neurons presented a more severe phenotype than that observed in human disease, where residual frataxin levels are present (Perdomini et al., 2013). Furthermore, conditional deletions occur acutely at defined developmental stages, whereas the human disease is chronically progressive (Simon et al., 2004).

The main challenge for the development of better animal models derives from the technical difficulties of inserting large GAA repeats. Relatively small repeats (about 230 GAA triplets) are carried by the only current knock-in mouse models (KIKI). These are insufficient to reduce frataxin levels enough to cause a full-blown phenotype, even when in compound heterozygosity with a null allele (KIKO mice), which produces 25% to 35% of the frataxin levels of wild-type mice (Miranda et, 2002). YAC-transgenic mouse model carrying about 200 GAA triplets in two copies of the human frataxin gene on a mouse *Fxn* null background develops a motor phenotype and some FRDA-like pathology (Al-Mahdawi et al., 2006).

Cell models, including primary fibroblasts and lymphocytes from patients, showed decreased viability under stress condition, suggesting defects in cellular defense against oxidative stress (Wong et al., 1999). More recently, induced pluripotent stem cell (iPSCs) from somatic cells derived from FRDA patients have been differentiated to neurons and cardiomyocytes and showed disruption of mitochondrial homeostasis (Hick et al., 2013). The key advantage offered by human iPSC-based models over animal models is that they provide researchers for the first time with a realistic chance to work in cell culture with large numbers of human cells, which are pheno- and genocopies of those postmitotic neurons, which are affected by neurodegeneration (Eigentler et al., 2013).

THERAPEUTIC PERSPECTIVES

There is no cure for FRDA, despite the large effort in developing therapeutic strategies to intervene in the pathogenic cascade downstream of frataxin. Yet, current pharmacological attempts involve three main categories: antioxidants, implementing frataxin level, and iron chelators.

Antioxidants

Idebenone, a parabenzoquinone derivative, was initially shown to rescue *in vitro* complex II activity in heart homogenate in presence of Fe^{2+} and *in vivo* to decrease left ventricular mass index in a few

patients (Rustin et al., 1999). Afterwards, several trials, mostly open labeled, with a limited number of patients, and of short duration and sometimes retrospective were performed. Two phase III trials (IONIA and MICONOS) failed to show significant differences in primary or secondary endpoints (both neurological or cardiac) (Lagedrost et al., 2011; Lynch et al., 2010; Schulz unpublished). Other trials have been conducted with different antioxidants including alpha-tocopheryl-quinone, pioglitazone, resveratrol, Coenzime Q10 and vitamin E (Hart et al., 2005).

Implementing frataxin levels

The alternative of restoring frataxin expression in affected cells appears an appealing approach to slow down or stop disease progression. Recombinant human erythropoietin (rhuEpo) has been reported to increase frataxin protein in lymphocytes from FRDA patients (Sturm et al., 2005) and clinical trials with rhuEpo are currently in progress. Although the molecular bases of the increase of frataxin remains to be determined, it is hypothesized that it is a post translational effect since the transcript levels are unchanged. The few trials carried out reached discordant conclusions about frataxin modification in lymphocytes and clinical measure variations (Boesch et al., 2007; Saccà et al., 2011b; Mariotti et al., 2012). Carbamylated-EPO that is devoid of the hemopoyetic effect showed to be safe but failed to show significant changes in biochemical or biological outcomes (Boesch et al., 2014)

A promising therapeutic strategy that is currently the subject of intense research is to directly target the heterochromatin state of the GAA repeat expansion with histone deacetylase inhibitors (HDACi) to restore frataxin levels. HDACi prevent the deacetylation of histones making heterochromatin revert to an open active conformation that may spur gene expression. A first screening of commercially available HDACi was performed in FRDA patients and control lymphoblastoid cells, allowing the identification and the optimization of a promising compound able to increase both frataxin mRNA and protein levels in patient cells (Herman et al., 2006; Gottesfeld, 2007). After additional developments, several derivatives were tested on KIKI mice in short term studies, confirming their ability to temporarily revert frataxin gene silencing (Rai et al., 2008). A phase I trial has been recently conducted. An open-label, dose-escalation study with the HDACi nicotinamide (Vitamin B3) was recently carried out on 10 patients with FRDA, showing a partial reversal of the abnormal heterochromatinisation of the *FXN* gene and significant upregulation of frataxin protein over an 8-week period of daily dosing (Libri et al., 2014).

Iron chelators

Deferiprone, an orally active, blood-brain barrier permeable iron chelator, can potentially redistribute iron from the mitochondria to other cellular compartments and to blood transferrin. After the initial report of decreasing iron in dentate, a phase II trial was conducted with different dosages of the drug (Boddaert et al., 2007). At higher dose, deferiprone was associated with worsening of the neurological function (Pandolfo and Hausmann, 2013).

CONCLUSIONS

FRDA is a good example of a clearly defined, largely homogeneous clinical and genetic condition hiding several not trivial aspects and unclear pathogenetic steps. The functions of frataxin are still to be fully elucidated but a major role relates to impaired ISC biosynthesis. Frataxin deficiency, ISC impaired biosynthesis and mitochondrial iron accumulation might propose some drug-based approach. However, it is likely that a combination of therapies will be adopted in the future to address each significant area of cellular disorder in FRDA with the goal of maximizing benefits and reducing toxicity of any one agent. If treatment can successfully modify disease progression, there would also be room for presymptomatic diagnosis and management to prevent onset of symptoms in at-risk individuals. With the breadth of therapeutic approaches and rapid pace of development, this seems to be an achievable goal.

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NF-κB UNBALANCE AND DYSFUNCTION IN ACUTE AND AGE-RELATED NEURODEGENERATIVE DISEASE

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The mechanisms underlying the progressive loss of neurons in age-related neurodegenerative diseases remain unknown to date. NF-κB factors are cardinal transcriptional regulators of inflammation and apoptosis and have been involved in the brain programming of systemic aging as well as in the pathogenesis of brain ischemia. Studies focusing on the complexity of NF-κB transcriptional activity in neuronal cell death showed that the composition of NF-κB active dimers and epigenetic mechanisms modulating histone acetylation finely condition neuronal vulnerability to brain ischemia. The atypical activation of NF-κB RelA acetylated on lysine 310 (K310) residue can trigger the expression of apoptotic genes but also constitutes a target for a neuroprotective combination of epigenetic drugs. Conversely, activation of NF-κB/c-Rel promotes neuroprotective effects through the transcription of specific anti-apoptotic genes. In addition, the absence of c-Rel shatters the resilience of nigral dopaminergic (DA) neurons to aging and induces parkinsonian features in mice. Indeed, we found that c-Rel-deficient mice show an increased RelA activation in the basal ganglia, and develop an L-DOPA-responsive parkinsonism associated with loss of DA neurons in the substantia nigra, neuroinflammation, accumulation of alpha-synuclein and iron during aging. Here, we discuss the effect of unbalanced activation of RelA and c-Rel during aging and propose novel challenges for the development of potential therapeutic strategies for neurodegenerative diseases.

According to a recent World Health Organization report, the number of people aged 65 and over is expected to double in size within the next 30 years, thus becoming 25% of the entire population. It can be foreseen that in the next 20 years the diagnostic and therapeutic management of patients affected by age-related neurodegenerative disorders, such as brain ischemia, Alzheimer's disease (AD) and Parkinson's disease (PD), will represent a high priority health challenge for the world population. Hence, the clarification of the biological basis of these devastating disorders could facilitate the development of novel therapeutic strategies to limit, delay or halt brain damage and thus ameliorate patients' clinical cares and lifestyle.

Several studies have shown that, in the nervous system, NF-κB transcription factor acts as a pleiotropic regulator of target genes controlling physiological function (Crampton and O'Keeffe, 2013) as well as pathological processes associated with neurodegeneration (Pizzi and Spano, 2006; Camandola and Mattson, 2007).

Different members of the NF-κB family of transcription factors have been identified in mammalian cells; these include p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2).

Key words: NF-κB, c-Rel, Ac-RelA(K310), epigenetic drugs, BDNF,Parkinson's disease, stroke

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In the absence of stimuli, these factors are present as homo- and hetero-dimers bound to the inhibitor IκB family proteins. However, the transcriptional activity of NF-κB is highly complex during neuronal apoptosis with the composition of active dimers finely tuning the vulnerability of neurons to brain insults. Indeed, the RelA subunit, composing the activated p50/RelA dimer, and its post transcriptional modifications play a pivotal role in the onset of neurodegenerative processes triggered by ischemic insults as well as glutamate or beta-amyloid toxicity (Pizzi et al., 2002, 2005a,b; Inta et al., 2006; Lanzillotta et al., 2010). Conversely, the c-Rel subunit within activated NF-κB dimers counteracts the ischemic injury (Sarnico et al., 2009) and is responsible for neuroprotection. The c-Rel factor is reduced in ischemic neurons, and its overexpression can limit cell loss during ischemia. Moreover, the deficiency of c-Rel induces an age-related behavioral parkinsonism in mice, with degeneration of nigral (DA) neurons and development of a PD-like neuropathology (Baiguera et al., 2012).

Recent evidence has shown that activation of NF-κB/RelA drives the systemic and brain aging process in mice, while prevention of NF-κB/RelA activation extends lifespan (Zhang et al., 2013).

We thus postulate that while RelA activation accompanies normal brain aging, a misbalance between RelA and c-Rel might drive pathological aging by affecting the survival of substantia nigra (SN) DA neurons and turning old mice toward a parkinsonian phenotype.

Rel-A and c-Rel: two opposing regulators of neuronal resilience to brain ischemia

In the central nervous system, NF-κB factors are key players of a number of physiological processes such as neurogenesis (Koo et al., 2010), neuritogenesis (Rolls et al., 2007), synaptic plasticity, learning and memory (Levenson et al., 2004; O'Riordan et al., 2006; Ahn et al., 2008). In recent years, a body of data has shown that NF-κB dysregulation participates in neurodegenerative mechanisms that occur in brains exposed to trauma or ischaemia (Bethea et al., 1998; Schneider et al., 1999), as well as in the brains of patients affected by PD (Hunot et al., 1997; Ghosh et al., 2007) and AD (Kaltschmidt et al., 1997).

The neuronal response to external stimuli relies on a differential activation of NF-κB dimers. We found that targeting RelA or c-Rel expression by antisense oligonucleotides (Pizzi et al., 2002) or siRNAs (Pizzi et al., 2005b; Sarnico et al., 2009) produces opposite effects on neuronal survival. While over-activated p50/RelA dimers contribute to the apoptotic program, the c-Rel containing dimers increase the resilience of injured neuronal cells (Fig. 1). Neurotoxic stimuli, such as ischemia (Inta et al., 2006; Sarnico et al., 2009), glutamate (Pizzi et al., 2002), β-amyloid (Valerio et al., 2006; Pizzi et al., 2005b) or 1-methyl-4-phenylpyridinium (MPP+) (Sarnico et al. 2008; Yang et al., 2010), induce p50/RelA dimer activation and the transcription of a panel of pro-apoptotic genes (Inta et al., 2006). Conversely, c-Rel-containing dimers are responsible for anti-apoptotic gene expression by signals promoting neuroprotection in diverse neurotoxic settings, such as S100B in models of NMDA-mediated excitotoxicity (Kögel et al., 2004), agonists at mGlu5 receptors against β-amyloid (Pizzi et al., 2005b) and MPP+ toxicity (Sarnico et al., 2008) or adipocyte-derived hormone leptin in neurons exposed to oxygen glucose deprivation OGD (Valerio et al., 2009). Over-expression of c-Rel in cultured neurons promotes anti-apoptotic effects by inducing the transcription of manganese superoxide dismutase (MnSOD) and Bcl-xL (Chen et al., 2000; Bernard et al., 2001; Pizzi et al., 2005b). c-Rel overabundance also limits the generation of reactive oxygen species (ROS) by inducing transcription of the mitochondrial uncoupling proteins 4 (UCP4) (Ho et al., 2012), a brain-specific mitochondrial ion channel producing mild reduction of mitochondrial membrane potential and neuroprotection (Echtay, 2007).

The elucidation of the dual effects of NF-κB activation on neuron survival was more evident in studies of severe brain ischemia. The activation of p50/RelA rapidly occurs in neurons and glial cells and has been implicated in pathogenesis of post-ischemic injury (Crack et al., 2006; Herrmann et al., 2005; Nurmi et al., 2004). In brain ischemic tissue of mice subjected to permanent middle cerebral artery occlusion (MCAO) and in primary cortical neurons exposed to OGD, NF-κB followed a similar pattern of activation (Lanzillotta et al., 2010; Pizzi

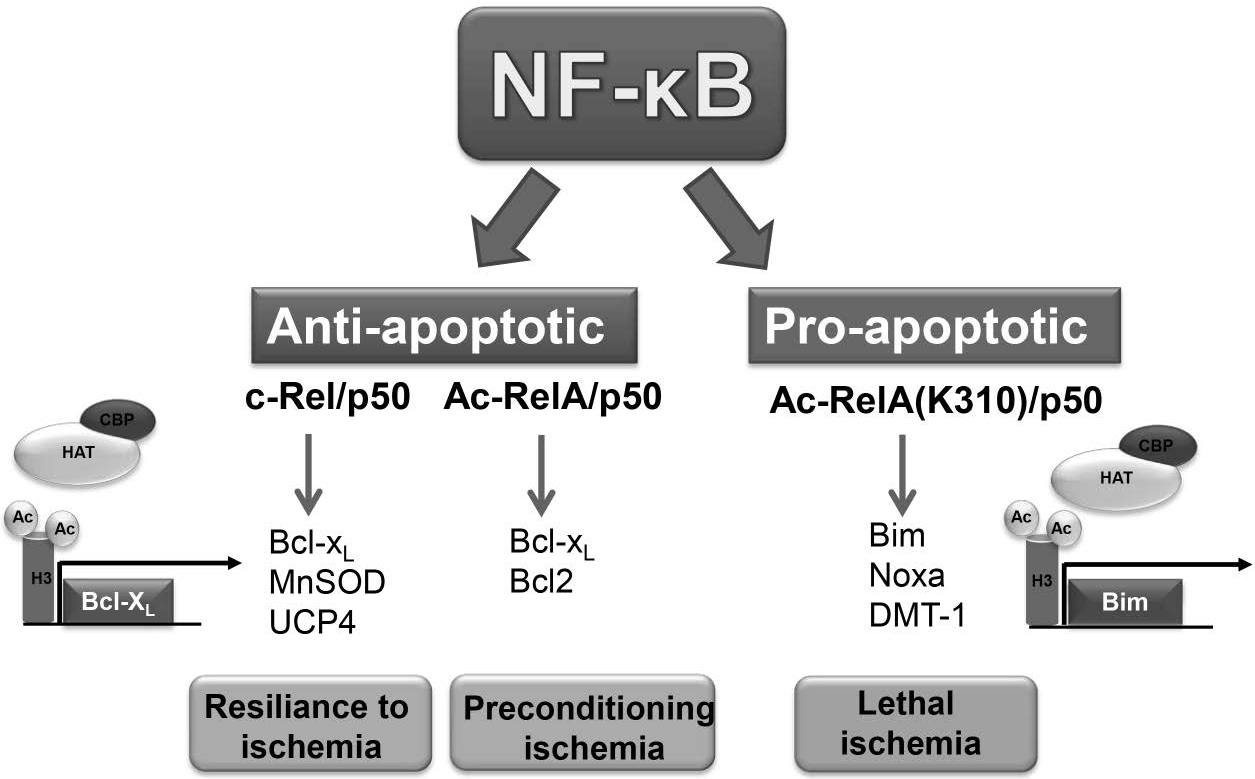

Fig. 1. *The p50/RelA and p50/c-Rel dimers regulate neuronal survival. Anti-apoptotic effects of NF-κB can be mediated by c-Rel containing dimers which enhance neuronal resilience to oxidative stress by inducing Bcl-xL, MnSOD, and UCP4 expression. NF-κB anti-apoptotic effects can also be mediated by p50/RelA during preconditioning ischemia through the transcription of Bcl-xL. The pro-apoptotic effect elicited by NF-κB p50/RelA dimer in lethal ischemia is dependent on changes in the RelA acetylation state. A lower grade of RelA general acetylation but a site-specific acetylation on K 310 residue addresses the NF-κB binding towards Bim, Noxa and DMT-1 promoters.*

et al., 2009) characterized by increased nuclear translocation of p50/RelA dimers (Crack et al., 2006; Inta et al., 2006) and decreased translocation of c-Rel-containing dimers (Sarnico et al., 2009). In these conditions, NF-κB activity was associated with an unbalanced expression of pro-apoptotic RelA target genes, i.e. an increased expression of the pro-apoptotic members of Bcl-2 family genes (Inta et al., 2006) and reduced level of the anti-apoptotic member Bcl-xL (Cao et al., 2002; Sarnico et al., 2009). During brain ischemia, NF-κB/RelA induced the expression of the 1B isoform of the divalent metal transporter-1 (1B/DMT1), the membrane carrier responsible for iron accumulation and brain damage after injury (Ingrassia et al., 2012). The RelA-induced 1B/DMT1 expression acted as an upstream mechanism responsible for iron accumulation and contributing to neuronal cell death. While the overexpression of RelA increased cell death, the overexpression of c-Rel prevented neuronal loss in cortical neurons exposed to OGD, by increasing the transcription of Bcl-xL gene (Pizzi et al., 2009; Sarnico et al., 2009). Knocking-down c-Rel expression exacerbated neuronal susceptibility to ischemic damage. Under brain ischemia, mice deficient for the c-Rel factor appeared insensitive to the neuroprotective activity of leptin, a c-Rel inducer able to limit cortical damage in wild-type mice (Valerio et al., 2009).

These data, strongly suggest that inhibition of c-Rel-containing dimers and activation of p50/RelA

are key events in the pathogenesis of post-ischemic brain injury.

In spite of these premises, p50/RelA activation per se appeared to be insufficient to drive pro-apoptotic transcription during brain ischemia. A similar pattern of p50/RelA nuclear translocation was found in mice exposed to a brief preconditioning ischemia (Lanzillotta et al., 2010), generating brain tolerance to a subsequent lethal ischemic injury (Blondeau et al., 2001). In neuronal cells, as well as in tumor cells, gene targeting by p50/RelA is finely regulated by post-transcriptional modifications of RelA subunit, such as phosphorylation and acetylation (Chen and Greene, 2004). These modifications shape the strength and specificity of the NF-κB-DNA binding and final transcriptional responses. On this line, we showed that the activation of the p50/RelA dimer, in preconditioning or in lethal ischemia, differs in the RelA acetylation state (Lanzillotta et al., 2013).

RelA acetylation is a dynamic process tuning NF-κB-mediated pro-apoptotic transcription in brain ischemia and can be modulated by epigenetic drugs

Acetylation is the key post-translational modification of histones that controls the accessibility of chromatin to the transcriptional machinery and plays an essential role in gene activation (Sweatt, 2009). Lysine acetylation is reversible and controlled by the opposing activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC).

Besides histones, diverse non-histone proteins, including NF-κB transcription factors, are modified by HAT co-activators and HDACs (Haberland et al., 2009). Acetylation of NF-κB RelA is a dynamic process, indeed the acetylation status of specific lysine residues (K122, 123, 218, 221, and 310) differently affecting the interaction with IκBα, the DNA-binding ability and transcriptional activity of the protein (Chen and Greene, 2004; Kiernan et al., 2003).

Members of class I HDACs, particularly HDAC1, HDAC2, and HDAC3, inhibited by vorinostat and entinostat (MS-275), are the most responsible for the general deacetylation of NF-κB/RelA (Ashburner et al., 2001; Chen and Green, 2004). Besides, sirtuin 1 (SIRT1), an atypical class III HDAC that requires nicotinamide adenosine dinucleotide (NAD+) rather than zinc as a cofactor (Buck et al., 2004), and is activated by resveratrol, selectively deacetylates RelA at K310 (Lanzillotta et al., 2010; Yeung et al., 2004).

Our previous studies have shown that mechanisms affecting the acetylation state of RelA might discriminate between preconditioning and neurotoxic activation of NF-κB during ischemia (Lanzillotta et al., 2010). Protective ischemic preconditioning and harmful ischemia induced similar levels of p50/RelA activation, but only the ischemic injury induced the atypical RelA acetylation. Indeed, RelA activated in mouse cortex during preconditioning ischemia appeared specifically deacetylated on K310 residue, but conserved its general acetylation. Conversely, the activated p50/RelA dimer in ischemic cortices of mice subjected to prolonged MCAO displayed a reduction of RelA general acetylation and a specific increase of RelA K310 acetylation (Lanzillotta et al., 2010).

By undergoing such aberrant acetylation, RelA detached from the anti-apoptotic Bcl-xL promoter to bind the pro-apoptotic Bim promoter (Lanzillotta et al., 2013). Lethal ischemic insult also induces a significant reduction of H3 histones acetylation, in line with previous evidence (Faraco et al., 2006; Lanzillotta et al., 2013). Prompted by these findings and in order to correct altered acetylation of RelA and histones after brain ischemia, we studied the effects of the association of the specific class I HDAC inhibitor, MS-275, (Simonini et al., 2006) with resveratrol (Baur and Sinclair, 2006).

MS-275 is a synthetic benzamide derivative that is currently under clinical evaluation for cancer therapy (Tan et al., 2010). MS-275 has been shown to inhibit HDAC 1-3 with excellent pharmacokinetic properties (Simonini et al., 2006).

Resveratrol is a polyphenol endowed with a multiple activities, including anti-oxidant, anti-tumorigenic and neuroprotective (Yu et al., 2012; Baur and Sinclair, 2006). In various models of brain ischemia, resveratrol delayed axonal degeneration after injury and mitigated the formation of free radical species as well as mitochondria-mediated apoptosis (Agrawal et al., 2011; Li et al., 2011; Morris et al., 2011; Ren et al., 2011). Widely known mechanisms of resveratrol action include the activation of the longevity factors sirtuin 1 (Howitz et al., 2003) and AMP-activated kinase (AMPK), a serine-threonine

kinase that acts as a key metabolic and stress sensor/effector (Ruderman et al., 2010).

We found that treatments with either MS-275 or resveratrol displayed a significant neuroprotective activity in cortical neurons exposed to OGD (Lanzillotta et al., 2013). Furthermore, we showed that the combination of MS-275 and resveratrol at sub-threshold doses, elicited a synergistic effect leading to maximal neuroprotection an *in-vitro* model of brain ischemia. MS-275 at the highest concentration tested of 1 μM increased acetylation of H3 histones on K9/18 residues in neurons exposed to OGD. Resveratrol, unable to modify per se the H3 acetylation, produced a synergistic increase of K9/18 H3 acetylation when used in combination with MS-275.

Notably, the synergistic effect produced by co-administration of low doses of MS-275 (0.1 μM) and resveratrol (3 μM) was sustained by AMP-activated kinase (AMPK) activation by resveratrol. This could be ascribed to the fact that AMPK can activate many catabolic pathways that produce ATP and induce acetyl-CoA (Turnley et al., 1999) generation, the fundamental co-factor for HAT activity. AMPK has also been found to indirectly support the resveratrol-dependent sirtuin 1 activation by inducing NAD+ generation (Ruderman et al., 2010). Following the AMPK-mediated enhancement of HAT and sirtuin 1 activity, the combination of MS-275 and resveratrol modulated the RelA acetylation state in neurons exposed to OGD by respectively enhancing the RelA general acetylation and reducing the acetylation at K310 residue. The neuroprotective effect and the transcription of anti-apoptotic factors observed following the treatment with the drug combination appeared to be closely related to the restoration of the optimal RelA acetylation state, a phenomenon that thus pharmacologically reproduces what occurs after a preconditioning ischemia (Lanzillotta et al., 2010; Raval et al., 2006). Treatments with MS-275 and resveratrol after OGD significantly reduced both RelA binding and H3 acetylation at the Bim promoter of neurons. The protective and transcriptional effects produced by resveratrol and MS-275 in cortical neurons were entirely reproduced in the mouse MCAO model. The combination of sub-threshold doses of the drugs, administered during the reperfusion period, elicited a synergistic effect that limited the cerebral infarct volume and the subsequent neurological deficits. MS-275 and resveratrol in combination showed a long-lasting efficacy as the beneficial effects were still evident 72 hours after the injury. Moreover, they displayed a wide therapeutic window as their efficacy was evident when administered up to 7 hours after the ischemic onset. Indeed, the treatment induced a transcriptional switch from pro- to anti-apoptotic genes, as RelA binding shifted from the Bim to the Bcl-xL promoter. Consequently, a decrease of the acetylation of histone H3 associated with the Bim promoter, and an increase in the acetylation of histone H3 at the Bcl-xL promoter was observed.

Recently, we evaluated the acetylation of histone residues at the Brain-derived neurotrophic factor (BDNF) IV promoter in primary mouse cortical neurons exposed to OGD and treated with the synergistic MS-275 and resveratrol combination. In particular, we studied the H3K9/18 and H4K12 acetylation, that are considered a part of a "backbone" of histone modifications that are associated with active promoters (Wang et al., 2008).

Transcription of BDNF is controlled by multiple promoters, driving the expression of multiple transcripts encoding for the same protein. We focused on promoter IV, that is known to be important for synaptic plasticity, both during neuronal development and in the adult brain (Hong et al., 2008). The regulation mechanisms of this promoter have been thoroughly studied and exon IV containing transcripts are highly expressed in neurons (Lyons and West, 2011; Timmusk et al., 1993). In the cortex, promoter IV-dependent BDNF transcription accounts for the majority of the neuronal activity-induced BDNF expression (Tao et al., 2012; Timmusk et al., 1993). Furthermore, several studies have posed BDNF as possible mediators of the beneficial effects of HDAC inhibitors in nervous system disorders (Chen et al., 2006; Chiu et al., 2011; Zeng et al., 2011;Yasuda et al., 2009). The ChIP analysis showed that BDNF promoter IV activity was repressed by OGD exposure, while treatment in the post-OGD period with the combination of MS-275 and resveratrol, significantly increased acetylation at H3 and H4 histones at BDNF promoter IV (Fig. 2). These histone modifications may act cooperatively and possibly in parallel to other histone modifications,

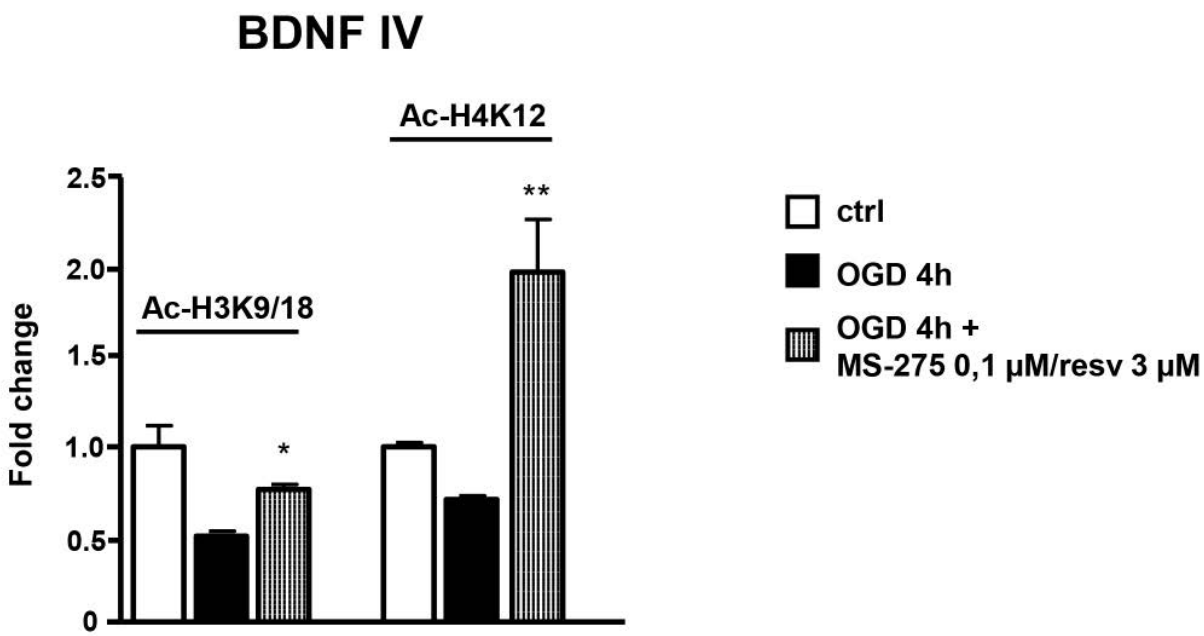

Fig. 2. *ChIP analysis of histone H3 acetylation (K9/18) and histone H4 acetylation (K12) on the BDNF IV promoter. Results are obtained by qRT-PCR analysis of BDNF IV promoter in immunoprecipitated DNA of cortical neurons exposed to OGD and treated with a combination of MS-275 (0.1 µM) and resveratrol (3 µM) during the 2 h-reoxygenation period. Treatment with drug combination significantly increases acetylation at both H3 and H4 histones associated with the BDNF IV promoter. Data are expressed as fold changes over values obtained in cells maintained in normal oxygen-glucose condition. Bars depict the mean±s.e.m. of three separate experiments run in triplicate, *p<0.05 or**p<0.01 versus the corresponding OGD value.*

to increase BDNF expression. Thus, we propose that neuroprotection elicited by MS-275 and resveratrol treatment, is also closely related to modulation of BDNF expression and may thus improve neurologic function by enhancing neuronal plasticity.

All together, these data provide the clear cut evidence that a pharmacological intervention targeting the epigenetic machinery that regulate gene expression represents an optimal strategy to limit post-ischemic injury with an extended therapeutic window.

c-Rel deficiency causes a progressive late-onset parkinsonism in mice

After the evidence that RelA and c-Rel composing NF-κB dimers play opposing effects on neuron survival, and a misbalance of p50/RelA versus p50/c-Rel activation triggers apoptotic cell death in brain ischemia (Sarnico et al., 2009), we hypothesized that a constitutive defect in c-Rel protein expression might play a role in neurological conditions associated with age-related neurodegeneration. Hence, we studied whether c-Rel knockout mice may present symptomatological and neuropathological features of neurodegeneration. We investigated different animal groups of either c-Rel knockout or wt mice at 2, 12 and 18 months of age (Baiguera et al., 2012) and we found that c-rel-/- mice develop a PD-like pathology and substantia nigra pars compacta (SNc) revealed a loss of dopaminergic neurons that paralleled the total loss of Nissl-stained cells in c-Rel deficient animals at 18-months of age. No significant change in the estimated number of dopaminergic cells was evident in the SNc of 2- and 12-month-old c-rel-/- mice compared to age-matched controls. Notably, the loss of SNc dopaminergic neurons was associated with a decrease of TH-positive fibers as well as a reduction of dopamine transporter (DAT) and

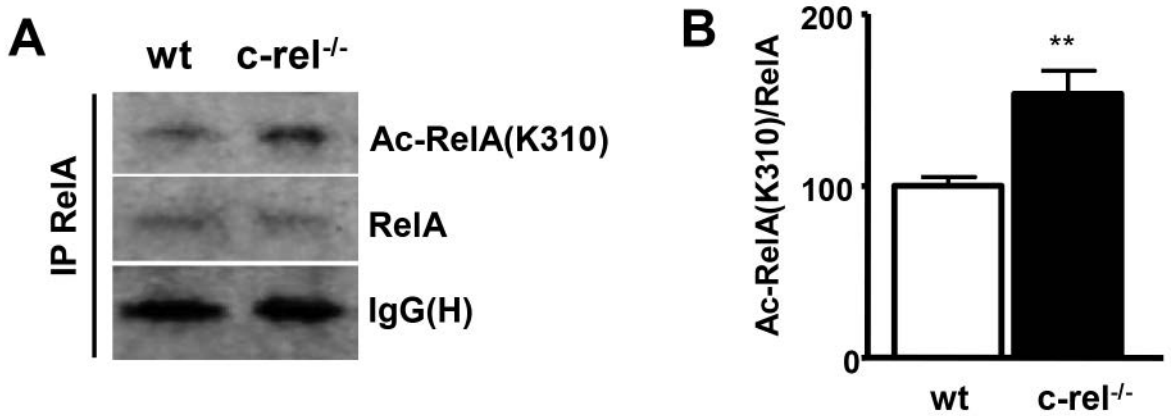

Fig. 3. *RelA acetylation at K310 residue in the striatum of c-rel-/- and wt mice.* ***A)*** *Representative picture of the immunoprecipitation analysis of RelA acetylation in total proteins of caudatus putamen. RelA acetylation at K310 residue increased in striatal total extracts of 18-month-old c-rel-/- mice. No significant change was detected in the total RelA content.* ***B)*** *Values from densitometry analysis of immunoblots are expressed as a percentage of the corresponding control value. The signal given by IgG(H) is used as a control for the quality of the immunoprecipitation. Bars depict the mean±s.e.m. of three separate experiments, **p<0.01 versus the corresponding wt value.*

dopamine content in the striatum. The 18-month-old c-rel-/- mice displayed no significant degeneration in other neuronal cell populations such as the ventral tegmental area, a region normally spared in PD, or in the nucleus basalis magnocellularis and medial septal area. Additional neuropathological examination of the aged c-rel-/- mouse brains showed the presence of a marked immunoreactivity for α-synuclein, the main protein constituent of Lewy bodies and Lewy neuritis, which represent the key pathological feature of PD (Spillantini et al., 1998), in the SNc. Of note, fibrillary aggregated α-synuclein, detected by Thiofalvin-S labelling, was present in the spared dopaminergic neurons of the SNc. Accumulation of insoluble α-synuclein in the mesencephalon was confirmed by the presence of a monomeric α-synuclein in the 8M urea/5% SDS extracts which solubilizes the insoluble fraction. In the SNc and striatum of aged c-rel-/- mice we also found increased iron levels and a significant augment of the iron transporter DMT-1 that is the above-mentioned transcriptional target of RelA acetylated on K310.

Chronic microglial activation is a main part of neuroinflammation in the post-mortem brain of PD patients (Kannarkat et al., 2013). Likewise in PD brain (Hirsch and Hunot 2009), SNc and striatum of aged c-rel-/- mice showed marked signs of microglia activation, as revealed by increased number as well as by swollen cell bodies and thickened processes of CD11b positive cells. A preliminary investigation of the state of RelA acetylation in striatal extracts of 18 month-old c-rel-/- mice showed higher levels of RelA acetylated on K310 residue, without a significant change in the total RelA (Fig. 3). Because inflammatory and neurotoxic activation of microglia also relies on K310 RelA acetylation (Chen et al., 2005), it may be plausible that microglia activation could participate in the neurodegenerative process in c-rel-/- mice. Extensive analysis of the neuroinflammatory profile of c-rel-/- mice along with disease progression will reveal the exact entity of this inflammatory process and the specific participation of innate and adaptive immunity.

The neurochemical changes observed in aged c-rel-/- mice were also accompanied by the onset of motor deficits. A significant impairment in spontaneous motor activity was evident in c-rel-/- mice at 18 months, but not in younger mice, as previously shown (O'Ryordan et al., 2006; Ahn et al., 2008). Indeed, either monitored for 1 hour or 6 consecutive days to avoid stress-related bias, 18 month-old c-rel-/- mice displayed a lower locomotor activity. Furthermore, the gaiting analysis supported the presence of a locomotor dysfunction related to

bradykinesia and rigidity. Noteworthy, the treatment with L-DOPA plus benserazide, a cocktail that is considered the gold standard for PD therapy, totally reversed the locomotor deficits and normalized most of the gaiting parameters.

Despite these findings, how the constitutive c-Rel deficiency can specifically affect DA neurons of SNc, is still an open question (Baiguera et al., 2012). The selective vulnerability of SNc neurons in PD has been attributed to the peculiar "energy-demanding" physiology of these cells (Surmeier et al., 2011) which display enormous axonal field and impressive number of synapses for each axon (Arbuthnott and Wickens, 2007). Moreover, during their pacemaking activity, SNc dopaminergic neurons, but not the VTA neurons, generate autonomous action potentials by unusual engaging of L-type Ca2+ channels which require subsequent activation of ATP-dependent Ca2+ pumps to maintain Ca2+ homeostasis (Wilson and Callaway, 2000). The energy production by mitochondria and endoplasmic reticulum in SNc dopaminergic neurons associates with the generation of large amounts of ROS that are constantly neutralized by antioxidant systems, including SODs catalases, glutathione peroxidase (Halliwell, 2006), UCP4 and UCP5. It can be inferred that in the absence of c-Rel, a reduced expression of UCP4 (Ho et al., 2012) and MnSOD (Bernard et al., 2001; Pizzi et al., 2005b) might enhance ROS accumulation during aging in SNc neurons (Cardozo-Pelaez et al., 1999) and synergize with reduced expression of anti-apoptotic Bcl-xL (Chen et al., 2000; Sarnico et al., 2009) to affect neuronal resilience. It is also feasible that this oxidative stress may contribute to elevate intracellular levels of α-synuclein (Uversky et al., 2007), DMT-1 and iron (Salazar et al., 2008) and, in turn, may lead to α-synuclein aggregation (Uversky et al., 2007), microglia activation and neuronal damage (Zhang et al., 2005). In conclusion, these findings suggest that c-Rel factor might act as a regulator of SNc susceptibility to aging.

Finally, our latest results (unpublished) suggest that, at a premotor phase (8-12 months), when there is still no loss of SNc DA neurons, c-rel-/- mice display gut dysfunctions, as shown by reduced stool frequency and stool water percentage, in addition to olfactory deficits. This disease progression mimics the pathological and clinical progression observed in PD patients, who usually show constipation and hyposmia (Simuni and Sethi, 2008), thus confirming that c-rel-/- mice represent an innovative disease model that may be useful both for studies aimed at dissecting the mechanisms of PD onset and to test novel therapeutic approaches for intervention at the premotor stages of the disorder.

CONCLUSIONS

NF-κB factors are transcriptional regulators of inflammation and apoptosis, though their relevance in aging and neurodegeneration is still underestimated.

Aberrant activation of NF-κB RelA acetylated on K310 residue triggers pro-apoptotic gene expression and can be targeted by the synergistic association of HDAC inhibitors and resveratrol. Conversely, activation of NF-κB/c-Rel promotes neuroprotective effects through the transcription of specific anti-apoptotic genes: MnSOD, BcL-XL and UCP4.

Activation of NF-κB/RelA has been found to lead the systemic aging process in mice, being negligible in the hypothalamus of young mice and progressively increasing, earlier in microglia and later in neuronal cells, as the mice become older. Aging is delayed and lifespan is extended in mice by preventing aging-related NF-κB/RelA activation in the hypothalamus and in other brain regions (Zhang et al., 2013).

If RelA activation marks physiological elderly, a deficiency of c-Rel drives mice toward a parkinsonian phenotype during aging (Baiguera et al., 2012). The aberrant acetylation of RelA on K310 in the basal ganglia occurs in c-Rel-deficient mice during aging and is associated with the development of an L-DOPA-responsive parkinsonism accompanying the degeneration of DA neurons in the SNc, activation of microglia and α-synuclein pathology.

This body of evidence supports our hypothesis that the balance between c-Rel- and RelA-mediated transcription may be at the crossroad between normal and pathological aging of the brain. In the presence of higher RelA activation, a deficit of c-Rel activity reduces SN resilience to aging, thereby leading to a late-onset form of PD.

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BENEFICIAL EFFECTS OF HYDROGEN IN THE CNS AND A NEW BRAIN-STOMACH INTERACTION

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A new medical gas, molecular hydrogen (H_2), has been reported to be effective for a variety of disorders and clinical trials have shown promising results. Though the precise mechanism is still unknown, it is obvious that H_2 has multiple effects. The first prominent effect of H_2 was as antioxidant or reactive oxygen species (ROS)-scavenger, though it blocks only hydroxyl radicals (•OH), which are the most toxic ROS, but not others. Then the possibility as a signal modulator was proposed and it became obvious that many protective effects of H_2 were not simply explained by antioxidant. Blocking reactive oxygen species (ROS) would be a rather direct anti-oxidative effect and if so, H_2 need to encounter ROS. However, long-term consumption of H_2 makes some unknown biological change which subsequently reveals resistance to ROS without any presence of H_2 itself. One of the ROS-resistant mechanisms of H_2 is due to brain-stomach interaction via ghrelin. The action of H_2 in the stomach seems to be quite specific; it activates β1 but not β2 adrenoceptor to increase the production and the release of ghrelin. Whether ghrelin secretion is a universal mechanism in all other protective effects induced by drinking H_2 water needs to be elucidated.

The history of molecular hydrogen (H_2), or hydrogen gas, according to Dixon et al, (Dixon et al. 2013), showed that the first use of H_2 was as a balloon carrier in 1783 by Frenchmen Jacques Charles. Nowadays, H_2 has become one of the most promising energy sources for future vehicles without pollution (Magdalena and Veziroglu 2005). Not only in the energy field but also in the medical field, H_2 is now getting more and more attention as a useful and unique gas.

Medical gas therapy is known for nitric oxide (NO) and helium/oxygen. It was at the end of the 1980s that NO was demonstrated to be produced by cells (Palmer et al. 1987). Seven years earlier, nobody expected that a gas as a toxic pollutant would serve as 'endothelium-derived relaxing factor'. Ever since, it has been shown that NO is involved in the regulation of the cardiovascular, immune and nervous systems. In the central nervous system (CNS), NO is shown to have an array of functions such as the regulation of synaptic plasticity, the sleep-wake cycle and hormone secretion. H_2, the lightest gas, was also hard to believe for medical therapy, though it was used in deep diving to reduce breathing resistance;

Key words: molecular hydrogen, brain-stomach interaction, ghrelin, Parkinson's disease, b1 adrenergic receptor

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

H_2-helium-oxygen mixture (Hydreliox) during an open sea dive below 130 metres (Abraini et al. 1994; Rostain et al. 1988) and H_2-oxygen mixture (Hydrox) for very deep diving (Brauer 1985).

In 2007, Ohsawa et al, first demonstrated the usefulness of H_2 as a medical gas (Ohsawa et al. 2007). They reported that H_2 is able to react with cytotoxic oxygen radicals and protect against oxidative damage, reducing the infarct volume of the brain after ischemia-reperfusion injury. Ever since, H_2 has been reported to have protective effects in a wide variety of disorders. Undoubtedly, H_2 is expected to serves as a medical gas. It is reported that H_2 is only beneficial, while NO is a Janus molecule. However, H_2 still has a narcotic potential and may cause hydrogen narcosis when it is used in breathing gas (Brauer 1985; Ornhagen 1984).

H_2 AS AN ANTIOXIDANT

The effect of H_2 as antioxidant was first reported in 2007 (Ohsawa et al, 2007), as mentioned above. It was ascribed that H_2 selectively scavenge hydroxyl radicals (•OH) which is a highly toxic dangerous radical among reactive oxygen species (ROS). Since then, an increased number of papers on the protective role of H_2 has been shown and effective disorders are well documented in a couple of reviews (Dixon et al, 2013; Ohno et al, 2012; Ohta 2011; Ohta 2014). Also, H_2 as a novel antioxidant was reported for the improvement of mitochondrial diseases (Ohta 2012) and ischemia-reperfusion injury (Eltzschig and Eckle 2011).

H_2 AS A SIGNAL MODULATOR

Though H_2 has been reported as an effective antioxidant, it is becoming clear that there is not only ROS-scavenging as an acute effect but also a chronic effect accompanying transcriptional alterations and gene expression (Hanaoka et al. 2011; Spulber et al. 2012). For example, in neurodegenerative diseases such as Parkinson's disease, assumption of H_2 in drinking water was the most effective rather than inhaling H_2 (Ito et al. 2012). Importantly the concentration of H_2 in drinking water was substantially low (Fujita et al. 2009). Consequently, increase in the concentration of H_2 in the brain was under detection, while inhaling H_2 with air showed immediate increase in H_2 concentration in striatum (supplementary data in Fujita et al,, 2009). Recent findings that H_2 in drinking water induces ghrelin release from the stomach (Matsumoto et al. 2013a) suggest a molecular mechanism underlying marked effects of H_2 without direct effect on the ghrelin receptors in the brain. In addition, in case of traumatic brain injury (TBI), not only acute change such as edema but also cytokine release in chronic phase was attenuated by drinking H_2 containing water (H_2 water). It was shown that gene expression related to oxidation/carbohydrate metabolism after TBI was reversed by drinking H_2 containing water (Dohi et al. 2014).

Another example is that H_2 inhibits Type I allergy or immediate-type hypersensitivity, which proposed "modulation of signal transduction" (Itoh et al. 2009). In a mouse model of Type I allergy, drinking H_2 water attenuated passive cutaneous anaphylaxis reaction and reduced the increase in serum histamine levels after the antigen challenge. In *in-vitro* study using leukemia cell line, pretreatment with H_2 reduced a marker of degranulation, antigen-induced phosphorylation of Lyn and its downstream signaling molecules, as well as antigen-induced intracellular calcium increase. H_2 also inhibited antigen-induced activation of NADPH oxidase (NOX) and production of ROS, which was considered to be most likely a consequence of the suppression of signal transduction. These results suggested that H_2 water could ameliorate type I allergy such as bronchial asthma, rhinitis, conjunctivitis and urticaria by modulating specific signaling pathways.

It was also demonstrated that H_2 attenuates inflammatory diseases (Kajiya et al. 2009; Xie et al. 2010). The signal transduction of H_2 was tested in lipopolysaccharide (LPS)/interferon (IFN) γ-stimulated macrophage cell line (Itoh et al. 2011). The results showed that treatment with H_2suppressed LPS/IFNγ-stimulated inducible nitric oxide synthase (iNOS) induction and NO production in macrophages, as well as in microglia (Terazawa et al. 2013). H_2 also inhibited LPS/IFNγ-stimulated phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) and its downstream signaling molecules, p38 and JNK, as well as IkBα. However, H_2 did not affect LPS/IFNγ-induced NOX activation nor ROS production.

Therefore, it was speculated that a potential target molecule of H_2 might be located at the receptor or immediately downstream of it.

Another example is LPS-induced sickness behavior. Drinking H_2 water was associated with a shift towards anti-inflammatory gene expression profile at baseline (down-regulation of tumor necrosis factor-a (TNF-α) and upregulation of interleukin (IL)-10). In addition, H_2 increases the amplitude, but shortens the duration and promotes the extinction of neuroinflammation, suggesting that the effects observed *in vivo* may involve the modulation of microglial activation (Spulber et al. 2012).

It was also demonstrated that drinking H_2 water alleviated anti-type II collagen antibody-induced arthritis in mice, which has been supported by a clinical study (Ishibashi et al. 2012). The effects of H_2 on acute liver injury (Sun et al. 2011) and amyloid-β-induced Alzheimer's disease (AD) (Wang et al. 2011) also support the hypothesis that H_2 inhibits signal transduction in these animal models.

H_2 PRODUCTION IN THE INTESTINE

There is endogenous H_2, too, though mammalian cells do not produce H_2. H_2 is produced by colonic bacteria when nondigestible and/or nonabsorbable saccharide traverses the upper gastrointestinal tract (Florent et al. 1985). About 50% of intestinal H_2 absorbed into the bloodstream by diffusion and excreted in expiratory gas throughout the lung (Calloway 1966; Muir et al, 1995). Therefore, the difference in breath H_2 excretion after the same dosage of several oligosaccharides indicates that digestibility and fermentability (Oku and Nakamura 2003). In fact, breath-H_2 after oral glucose administration was examined (Metz et al. 1976). Breath-H_2 test after oral administration of lactulose is clinically applied to examine small intestinal bacterial overgrowth, which is an underlying mechanism of irritable bowel syndrome (Ford et al. 2009). Likewise, lactulose is able to ameliorate dextran sulfate sodium (DSS)-induced intestinal inflammation in rats (Rumi et al. 2004) and cerebral infarction by producing intestinal H_2 (Chen et al. 2012). Therefore, it was speculated that lactulose may abolish development of parkinsonian symptoms in animal model. However, lactulose marginally ameliorated development of PD in rats (Ito et al. 2012).

To date, there is no direct interaction between breath H_2 and various protective effects which were observed by exogenous H_2.

STOMACH-BRAIN INTERACTION VIA GHRELIN

Ghrelin as a neuroprotective peptide for dopaminergic neurons

Ghrelin is a peptide of 28 amino acids, a growth-hormone releasing acylated peptide from stomach, and an endogenous ligand specific for growth-hormone secretagogue receptor (GHS-R) (Kojima et al. 1999). Neuroprotective effects of ghrelin on dopaminergic neurons have been reported both *in vitro* (Dong et al. 2009; Liu et al. 2010) and *in vivo* (Andrews et al. 2009; Jiang et al. 2008; Moon et al. 2009). GHS-R is highly expressed by dopaminergic neurons of the substantia nigra (Zigman et al. 2006), and has been suggested that ghrelin protects nigrostriatal dopamine neurons via an uncoupling protein 2 (UCP2)-dependent mitochondrial mechanism (Andrews et al. 2009; Andrews et al. 2008).

Drinking H_2 water induces ghrelin release

Effect of drinking H_2 water was connected to that of ghrelin on dopaminergic neurons by Matsumoto et al, (2013a). Drinking H_2 water induced ghrelin gene expression in the stomach and increased plasma ghrelin levels, taking 4 days (Matsumoto et al. 2013a). In addition, the effect of drinking H_2 water observed in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease (PD) model mice was completely blocked by GHS-R inhibitor, D-Lys3 GHRP-6 (Matsumoto et al. 2013a). Therefore, induction of ghrelin production in the stomach and subsequent activation of ghrelin-initiated signal transduction via GHS-R underlies the protective effects of H_2 water in the PD model animal.

Mechanism of ghrelin release

It has been shown that secretion of ghrelin is regulated by many factors including blood glucose, estrogen, insulin and catecholamines (Kamegai et al. 2004; Kojima and Kangawa 2005; Zhao et al. 2010).

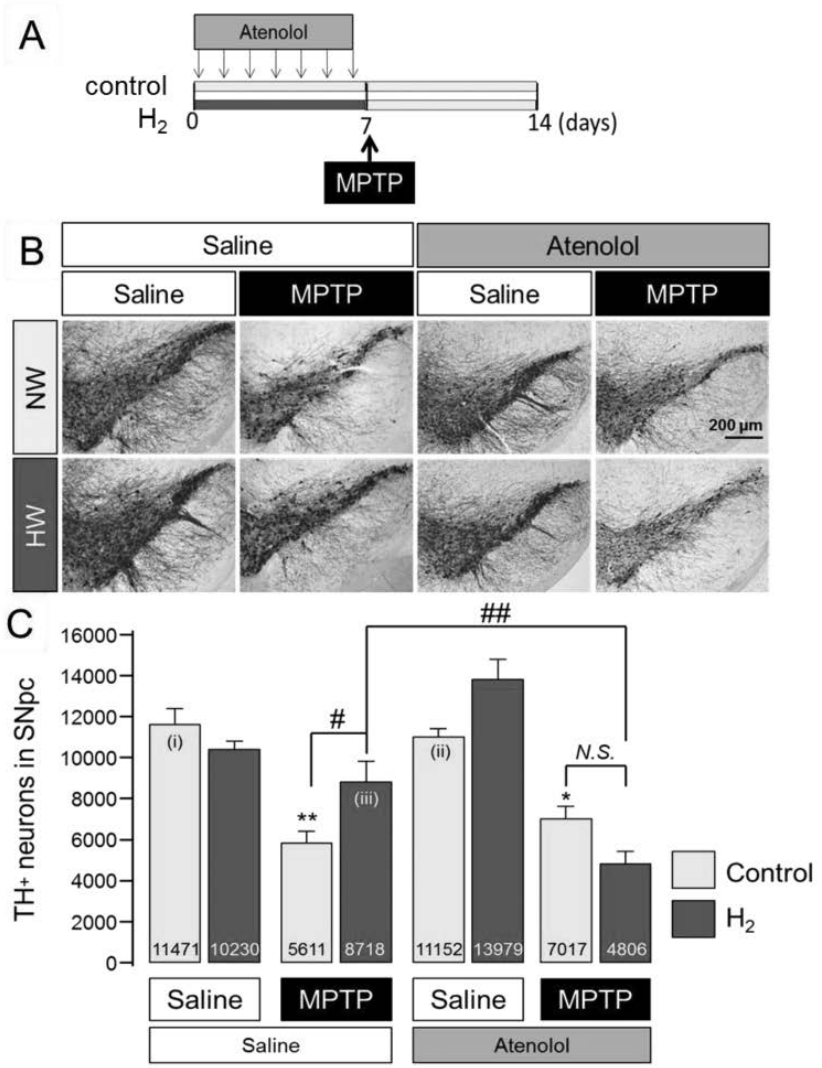

Fig. 1. *Inhibition of ghrelin secretion by β1-adrenergic receptor antagonist, atenolol, cancels the neuroprotective effect of drinking H_2 water in MPTP-induced Parkinson's disease model mice. A) Experimental protocol. Either normal tap water (control) or H_2 water were given to animals for 7 days. MPTP-HCl (15 mg/kg) was injected (i.p.) after 7 days of atenolol (10 mg/kg)- or saline-injection. B) Tyrosine hydroxylase (TH) staining was performed in substantia nigra pars compacta from saline- and atenolol-injected mice. C) Summary of the stereological analysis of nigral dopaminergic neurons. Although MPTP injections caused significant loss of TH-positive neurons, drinking H_2 water for 7 days prior to MPTP-treatment significantly attenuates the loss of TH positive cells. β1-adrenoceptor blocker, atenolol, canceled the preservation effect of drinking H_2 water. Data in figures are represented as mean ± SEM. The statistical significance of data was assessed by one-way ANOVA followed by Benferroni test. *; $p<0.05$, **; $p<0.01$ compared with control in each group; (i), (ii)#; $p<0.05$, ##; $p<0.01$ compared with (iii). (Partially from Matsumoto et al, 2013a).*

In particular, β1-adrenergic receptor stimulation increases ghrelin secretion *in vitro* and *in vivo* (Gagnon and Anini 2012; Zhao et al. 2010). Indeed, the increase in plasma ghrelin levels observed after drinking H_2 water was eliminated by administration of the β1-adrenergic receptor-specific blocker, atenolol (10 mg/kg i.p.) injected 30 min prior to H_2 water administration on each day for 4 days (Matsumoto et al. 2013a). The protective effect of drinking H_2 water was also cancelled by atenolol (Fig. 1). On the

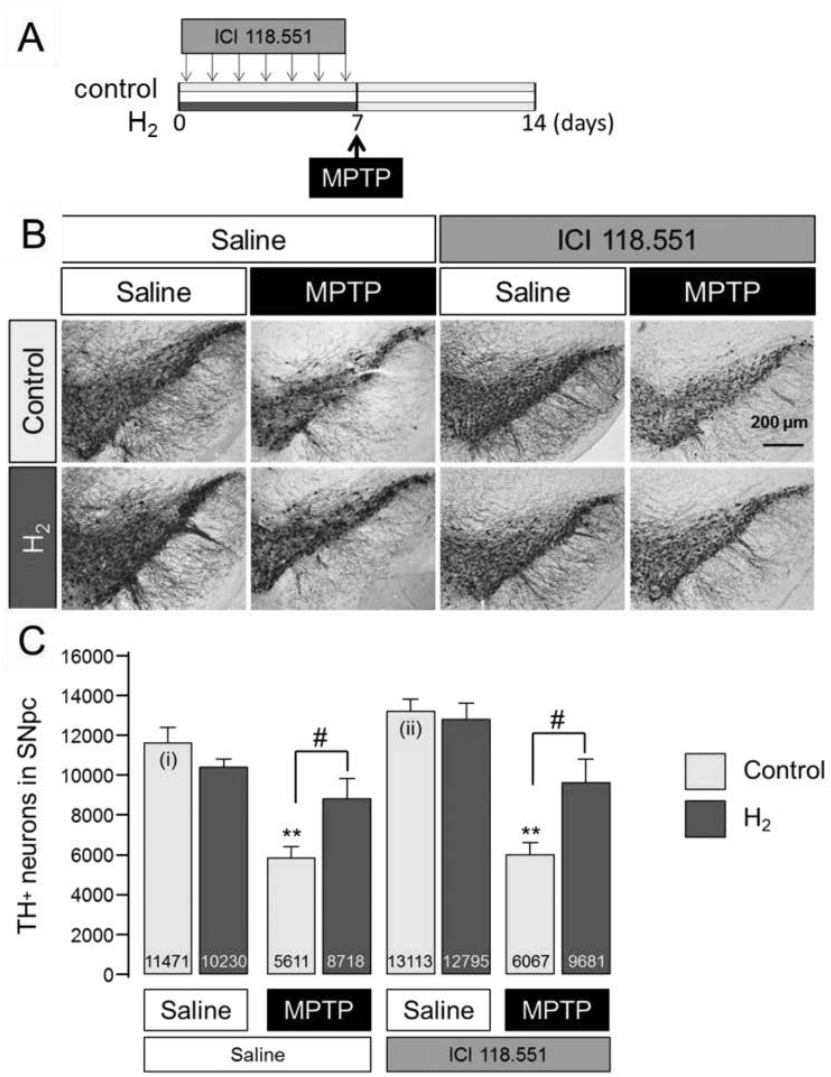

Fig. 2. *Inhibition of β2-adrenergic receptor does not affect the protective role of H_2. A) Experimental protocol. Either normal tap water (control) or H_2 water was given to animals for 7 days. MPTP-HCl (15 mg/kg) was injected (i.p.) after daily injection of ICI 118.551 (1 mg/kg)- or saline-injection for 7 days. B) Tyrosine hydroxylase (TH) staining was performed in substantia nigra pars compacta from saline- and ICI 118.551-injected mice. C) Summary of the stereological analysis of nigral dopaminergic neurons. Drinking H_2 water for 7 days prior to MPTP-treatment significantly attenuates the loss of TH positive cells. β2-adrenoceptor blocker, ICI 118.551, did not affect the effect of drinking H_2 water. Data in figures are represented as mean ± SEM. Number at the bottom of each bar represents the mean value. The statistical significance of data was assessed by one-way ANOVA followed by Benferroni test. **; p<0.01 compared with control in each group; (i), (ii) #; p<0.05. (Partially from Matsumoto et al, 2013a).*

other hand, β2-adrenergic receptor-specific blocker, ICI118.551 (1 mg/kg) did not affect the protective effect of H_2 water (Fig. 2). Thus, activation of β1-adrenergic receptors is required for H_2 water-induced enhancement of circulating ghrelin. The mechanism how H_2 stimulate β1-adrenergic receptors but not β2-adrenergic receptors needs to be investigated.

Molecular mechanism of neuroprotective effect of ghrelin

Now it is obvious that ghrelin is a key player in

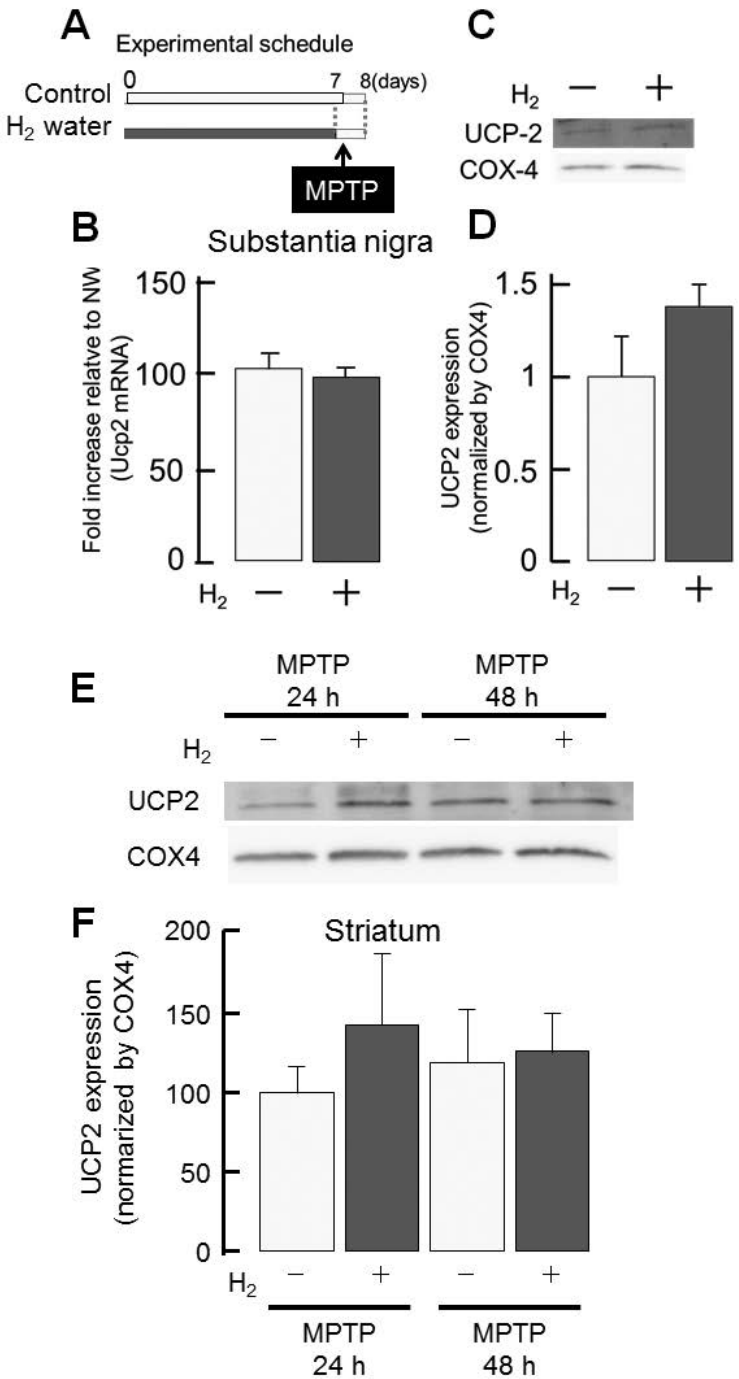

Fig. 3. *UCP2 expression in substantia nigra and striatum was not changed by drinking H_2 water. A) Experimental protocol. Either normal tap water (control) or H_2 water was given to animals for 7 days and then MPTP-HCl (15 mg/kg) was injected (i.p.). The brain was examined after 1 or 2 days of MPTP injection. B) Expression level of UCP2 mRNA in substantia nigra at 24 h after MPTP injection was almost the same with or without H_2 water. C) Western blotting of UCP2 and COX4 in substantia nigra with or without H_2 water at 24 h after MPTP injection. D) Expression level of UCP2 mRNA normalized to that of COX4. Though the mean value looks bigger in H_2 water drinking group, there was no significant difference. E, F) UCP2 expression was also tested in striatum. Western blotting of UCP2 and normalized expression level of UCP2 in striatum at 24 and 48 h after MPTP injection with or without H_2 water.*

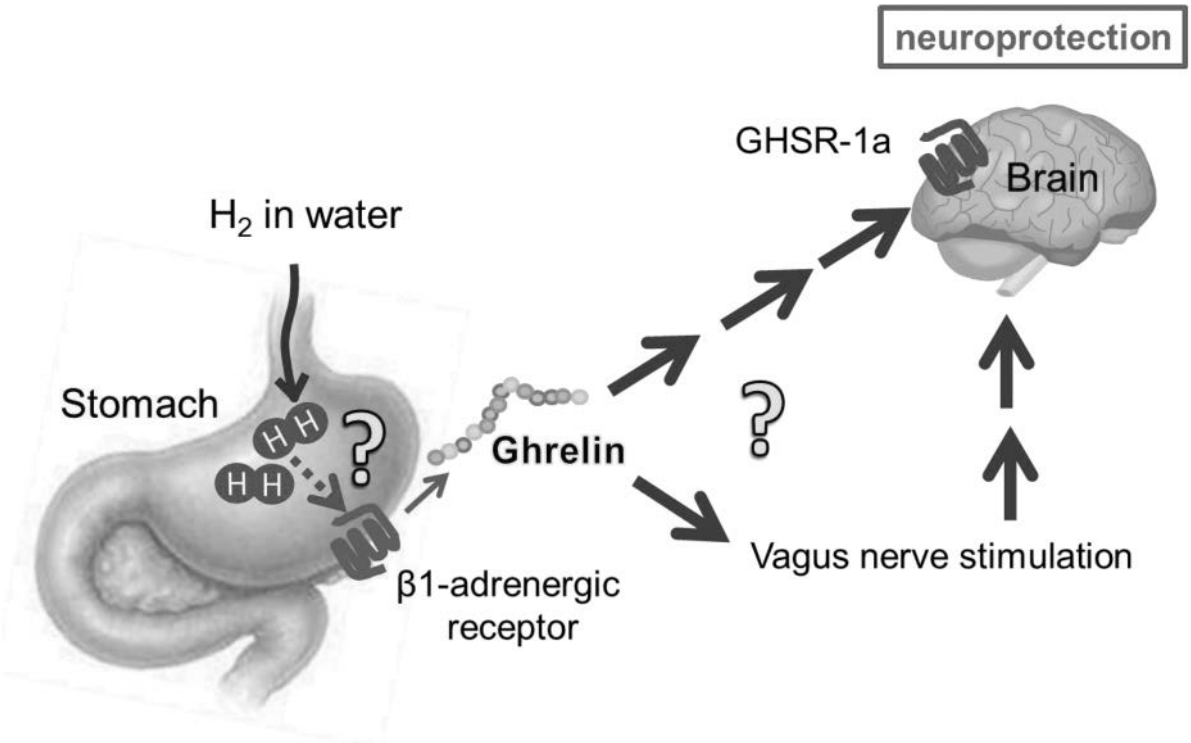

Fig. 4. *Possible mechanism of neuroprotective effect of drinking H_2-water via stomach-brain connection. H_2 in drinking water induces ghrelin production and release from the stomach via β1- but not β2-adrenergic receptor stimulation. Released ghrelin in the plasma activates its receptor, growth-hormone secretagogue receptor (GHS-R). One possibility of its cascade is that ghrelin may reach to the brain and activate GHS-R in substantia nigra directly. Another possibility is inserting neutoprotection via stimulating thevagus nerve. The precise mechanism how H_2 stimulate β1-adrenergic receptor but not β2-adrenergic receptor is also not known.*

the neuroprotective effect of drinking H_2 water in PD model. If so, the mechanism of ghrelin on PD model mice would be dependent on UCP2 as mentioned above. According to Andrews et al, (2008, 2009), ghrelin protects nigrostriatal dopamine neurons via up-regulation of UCP2. However, drinking H_2 water for 7 days did not change the expression level of UCP2 in both mRNA and protein levels (Fig. 3).

Strangely, drinking H_2 water neither increased appetite nor body weight in mice. Nevertheless, it might be necessary to check the involvement of vagus nerve stimulation by H_2-ghrelin cascade. Since neuroprotective effect of ghrelin via vagus nerve stimulation has been reported (Cheyuo et al. 2011), the effect of vagotomy on the effect of drinking H_2 water would be useful. Two possible cascades of H_2 in drinking water are shown in Fig. 4.

CLINICAL STUDY

In clinical study, either drinking H_2 water or infusion of H_2-rich saline is reported to be effective and the significance is noteworthy. For example, the effects of drinking H_2 water were reported in metabolic syndrome (Nakao et al. 2010; Song et al. 2013), radiation side effects (Kang et al. 2011), and PD (Yoritaka et al. 2013).

It is also noteworthy that infusion of H_2-rich saline improved MRI indices during acute stage of brainstem infarction (Ono et al. 2011) and peritoneal cavity (Terawaki et al. 2013) . A couple of studies on hemodialysis with H_2-rich saline were also reported (Nakayama et al. 2010; Nakayama et al. 2009; Terawaki et al. 2013).

To date, the effects of molecular H_2 have been tested

in more than 10 human diseases. Among the eleven human diseases or states, molecular H_2 showed mild to prominent effects in nine, and had no effect on interstitial cystitis/painful bladder syndrome (Matsumoto et al. 2013b). Double-blind randomized placebo-controlled trials have been performed in PD (Yoritaka et al. 2013), mitochondrial and inflammatory myopathies (Ito et al. 2011), diabetes mellitus type 2/glucose intolerance (Kajiyama et al. 2008), muscle fatigue in young soccer players (Aoki et al. 2012), and interstitial cystitis/painful bladder syndrome (Matsumoto et al. 2013b). As placebo effects potentially obscure the true benefit of molecular H_2, controlled studies are required for all the diseases and H_2 as a molecular gas will become a good subject in translational research for various pathologies.

CONCLUSIONS

Molecular hydrogen (H_2) is a new medical gas, shows protective effects in various diseases. The protective mechanism consists of multiple mechanisms; one is mainly anti-oxidant and a weak ROS-scavenger; another is as a signal modulator in both periphery and the central nervous system. The latter, induced by drinking H_2 water and especially in dopaminergic neurons, is due to brain-stomach interaction via stimulating β1-adrenergic receptor and release of ghrelin. Though it is still unclear how β1-adrenergic receptor is activated and how released ghrelin inserts protective effect in the central nervous system, drinking H_2 water turns out to cause distinct signaling which serves as a beneficial and simple way to protect neuronal cells. Therefore, understanding the actions of H_2 will lead to new forms of therapy or prevention for human disease. In addition, whether or not H_2 water-induced ghrelin is a key factor in all other diseases needs to be elucidated.

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NEUROIMAGING SIGNATURES OF NEURODEGENERATION IN THE AMYOTROPHIC LATERAL SCLEROSIS - FRONTOTEMPORAL LOBAR DEGENERATION CONTINUUM

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Brain imaging techniques, especially those based on magnetic resonance imaging (MRI), have been increasingly applied to study the structure and function of the human brain in health and disease. More recently, the combined investigation of genetic information and imaging data, both proposed as candidate endophenotypes or disease vulnerability markers, resulted to be considerably helpful to explore the structural and functional consequences of some genetic polymorphisms or mutations on brain connectivity, allowing to exploit the possible genotype-endophenotype associations in several neurological disorders. With regard to neurodegenerative diseases, amyotrophic lateral sclerosis (ALS), a multi-systemic neurodegenerative disease with early and prominent impairment of motor abilities, and frontotemporal lobar degeneration (FTLD), the second most common early-onset dementia, have been proven to share several clinical, neuropathological, genetic and neuroimaging features, and have been thought to belong to the same spectrum of disease. Specifically, both overlapping and diverging brain connectivity patterns, evaluated by diffusion tensor imaging (DTI), voxel- and surface-based morphometry (VBM) and resting-state functional MRI (RS-fMRI) analyses, have been described comparing several ALS and FTLD populations. In this article we review the current state of knowledge concerning the most advanced neuroimaging findings associated with clinical and genetic patterns of neurodegeneration across the ALS-FTLD continuum, underlying the usefulness of a multimodal approach to assess novel biomarkers of disease and more appropriate treatment strategies.

The substantial phenotypical heterogeneity of neurodegenerative disorders has posed a significant challenge for the discovery of biological markers (Ramanan and Saykin, 2013). Furthermore, while neuroimaging facilities such as magnetic resonance imaging (MRI) are broadly available and a vast research literature has reported on MRI methodology for *in vivo* investigating neurological disorders, conventional radiological markers of neurodegeneration, useful for both diagnosis and disease staging in clinical practice, have still not been established (Risacher and Saykin, 2013). This might be due to the fact that the current definition of neurodegenerative diseases, as delineated by clinical criteria, do not easily (and necessarily) map on the underlying neuropathology and, therefore, may provide an inadequate basis for diagnosis, therapy and outcome prediction. In this regard, the current implementation of multi-dimensional approaches, especially by combining genetic and

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MRI information, has provided interesting insights on definition of novel genetic and neuroanatomic associations that may be used to improve the diagnostic accuracy in several neurodegenerative diseases (Melville et al. 2012; McMillan et al. 2014; Jahanshad et al. 2013).

With regard to Alzheimer's disease (AD), genetic factors have been proven to influence gray and white matter (GM, WM) neuroanatomy, revealing significant associations between several single nucleotide polymorphisms (SNPs) and the risk of accumulating specific histopathologic abnormalities (Melville et al. 2012; Jahanshad et al. 2013; Shen et al. 2010). More recently, increasing evidence on the association between genetic and radiological features has also been demonstrated within the spectrum of degenerative diseases which encompasses frontotemporal lobar degeneration (FTLD), the second most common cause of early-onset dementia, and amyotrophic lateral sclerosis (ALS), a multi-systemic disease primarily but not exclusively affecting motor abilities. In fact, these two neurological disorders have been recognized as representative of a neuropathological continuum as they share several common genetic, pathogenetic and clinical features (Lomen-Hoerth et al. 2002; Murphy et al. 2007; Ling et al. 2013). Notably, the finding that mutations within chromosome 9p have been independently described in both ALS and FTLD populations, as well as in ALS/FTLD cases, has allowed to discover that exanucleotide expansions within C9ORF72 gene represent the probable genetic link between the two syndromes (DeJesus-Hernandez et al. 2011; Renton et al. 2011). Furthermore, recent genetic and pathological analyses have demonstrated that mutations of TAR DNA binding protein 43 kD (TARDBP) (Benajiba et al. 2009), fused in sarcoma/translocated in liposarcoma (FUS/TLS) (Blair et al. 2010) and ubiquilin-2 (UBQLN2) (Deng et al. 2011), apart from the above cited mutations of C9ORF72, have a key role in the pathogenesis of the ALS-FTD spectrum of disorders. In particular, all these genes have been proven to share a common link to cellular RNA dynamics (Ling et al. 2013; Thomas et al. 2012), triggering aggregations of ubiquitinated TAR DNA binding protein (TDP-43) or FUS, which may represent the major protein components of pathological inclusions detected in both neurological syndromes (Arai et al. 2006; Neumann et al. 2006).

From the phenotypical point of view, a relevant, although variable in magnitude, degree of cognitive involvement has been found in many patients with ALS. Specifically, 5-15% of ALS patients meet criteria for FTLD, while a substantial percentage of patients without dementia may show mild to moderate executive (approximately from 22 to 35%) and behavioral (up to 63%) dysfunctions (Lomen-Hoerth et al. 2002; Phukan et al. 2007; Ringholz et al. 2005). Among FTLD patients, up to 15% of them display symptoms typical of motor neuron degeneration, usually first identified by the presence of fasciculations or abnormal swallowing (Lomen-Hoerth et al. 2002; Ringholz et al. 2005; Burrell et al. 2011). Interestingly, ALS symptoms have been most commonly described in the behavioural variant subtype of FTLD (behavioral variant of frontotemporal dementia or bvFTD) (Lomen-Hoerth, 2004), while they have been detected less frequently in the language variants (i.e., progressive non-fluent aphasia or PNFA and semantic dementia or SD) (Hsiung et al. 2012; Simón-Sánchez et al. 2012). On the other hand, behavioral changes are now recognized as common symptoms in ALS and the term "behaviorally impaired (biALS)" has been recently introduced to describe ALS patients who display frontal behavioral signs but do not meet the full criteria for bvFTD (Grossman et al. 2007). Disinhibition, irritability, emotional blunting, lack of empathy, and especially apathy have been reported in several cohorts of ALS patients (Grossman et al. 2007; Gibbons et al. 2008; Lillo et al. 2011). Moreover, beyond behavioral profiles, executive performances, including verbal fluency, problem solving and execution of a dual task, have been the most commonly investigated cognitive domain in ALS research and have been found frequently impaired also in the early stages of disease (Phukan et al. 2007; Ringholz et al. 2005; Witgert et al. 2010). However, a number of neuropsychological studies identified also language deficits among non-demented ALS patients, especially in confrontation naming, conceptual semantic processing and syntactic comprehension tasks (Ringholz et al. 2005).

With regard to prognosis, ALS with or without comorbid FTLD is more disabling and progressive in

comparison to FTLD without motor neuron disease, exhibiting the shortest median survival in patients affected by ALS-FTD (Ringholz et al. 2005).

In consideration of the broadly described clinical, genetic and pathological overlap between ALS and FTLD, in the last decades structural and functional MRI correlates across this continuum have been increasingly investigated in order to discover radiological markers useful for differential diagnosis and clinical staging. Specifically, structural MRI studies, using diffusion tensor imaging (DTI) and voxel- and surface-based morphometry (VBM, SBM), and resting state functional MRI (RS-fMRI) analyses have considerably contributed to show different patterns of structural and functional abnormalities, principally involving frontal and temporal lobes, in several cohorts of ALS patients (Abrahams et al. 2005; Chang et al. 2005; Mezzapesa et al. 2007; Mohammadi et al. 2009; Agosta et al. 2010, 2013; Filippini et al. 2010; Douaud et al. 2011; Tedeschi et al. 2012; Lillo et al. 2012; Cirillo et al. 2012; Schuster et al. 2014; d'Ambrosio et al. 2014). Remarkably, in advanced stages of ALS, structural MRI alterations were found to resemble those previously described in several cohorts of FTLD patients (Seeley et al. 2008; Krueger et al. 2009; Zhou et al. 2010; Whitwell et al. 2010; Filippi et al. 2013; Farb et al. 2013). Furthermore, possible associations between genotypes and MRI patterns, considered as endophenotypes or disease vulnerability markers, have been recently explored across the ALS-FTLD continuum, allowing detection of different profiles of neurodegeneration (Whitwell et al. 2011; Mahoney et al. 2012; Dopper et al. 2013).

This paper reviews current neuroimaging knowledge within a framework of clinical and genetic changes reported across the ALS-FTLD spectrum of disease, emphasizing the crucial role which will be played in the future by multiparametric approaches for a better understanding of the neurodegenerative process.

FUNCTIONAL NEUROIMAGING

The whole-brain investigation of functional brain activity was initially carried out by applying single photon emission computed tomography (SPECT) with 99mTc-hexamethylpropylene and (18F)2-Fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) analyses, which indirectly evaluates functional brain activity by measuring respectively the regional perfusion and cerebral uptake of glucose. Several SPECT and FDG-PET studies have reported widespread frontotemporal lobe involvement in ALS patients with or without cognitive impairment (Kew et al. 1993; Abrahams et al. 1995, 1996; Vercelletto et al. 1999, 2003), showing in some cohorts of patients significant relationships between functional changes within frontotemporal areas (i.e., anterior and medial orbitofrontal cortex, anterior and medial frontal cortex and anterior temporal lobes) and neuropsychological performances (Kew et al. 1993; Abrahams et al. 1995; Vercelletto et al. 2003), with a more marked and widespread pattern of functional impairment in patients with ALS-FTLD. On the other hand, in several cohorts of FTLD patients, especially with bvFTD, FDG-PET studies, showed a symmetrical hypometabolism of the frontal lobes (sparing the motor cortex), caudate nuclei, insula and thalamus bilaterally (Jeong et al. 2005; Peters et al. 2006; Diehl-Schmid et al. 2007). Furthermore, parietal and temporal hypometabolism have been found significantly associated with disease progression in bvFTD (Diehl-Schmid et al. 2007).

Interestingly, recent evidence depicted PET signatures of neurodegeneration in some genetic variants across the ALS-FTLD continuum (Jacova et al. 2013; Lant et al. 2013; Cistaro et al. 2014). In particular, C9ORF72 mutations, (Cistaro et al. 2014) compared C9ORF72-positive ALS patients to sporadic ALS patients with or without bvFTD, showing a more widespread central nervous system involvement in C9ORF72-ALS. Moreover, C9ORF72-ALS patients exhibited a significant hypometabolism in the anterior and posterior cingulate cortex, insula, caudate and thalamus and left frontal and superior temporal cortex.

In cases of progranulin (GRN) gene mutations, it has been demonstrated that the neurodegenerative process begins many years prior to the onset of FTLD symptoms. In fact, Jacova et al. (2013) observed that GRN mutation carriers had lowered FDG uptake in the right anterior cingulate, insula and gyrus rectus compared with non-carrier subjects belonging to families with FTLD (due to GRN mutations). Finally, concerning Microtubule Associated Proteins (MAPT)

gene mutations, a PET analysis by Lant et al. (2014), using the microglial cell marker [11C](R)-PK11195, revealed significantly higher levels of microglial cell activation in several frontal subcortical WM areas in FTLD patients in comparison to healthy controls and AD patients. Moreover, a significantly higher activation in temporal subcortical regions was found in FTLD patients with MAPT mutations compared to patients with other genetic (GRN and C9ORF72 mutations) or sporadic variants of FTLD.

In the last two decades, functional activation studies by PET and fMRI while performing cognitive tasks have shown a widespread frontotemporal lobe involvement in several cohorts of ALS patients, with reduced activation in the dorsolateral prefrontal cortex (DLPFC), the anterior cingulate gyrus, the inferior frontal gyrus and insular cortex, significantly related to impairment of executive functions (Kew et al. 1993; Abrahams et al. 1995, 1996, 2004). Furthermore, similar fMRI patterns of frontal network dysfunction, especially in the right hemisphere, have been described in both ALS and FTLD patients during execution of cognitive tasks consisting of emotional attribution to visual stimuli (i.e., facial expressions or word sets, alternating blocks of unpleasant and neutral stimuli) (Palmieri et al. 2010; Virani et al. 2013). These findings suggested a widespread degeneration of neural circuits involved in social interactions and empathy, giving reason for the oppositional behaviour frequently attributed to patients with ALS or FTLD or both (Hsieh et al. 2013).

More recently, a novel focus of neuroimaging research has concerned the analysis of "functional connectivity" of spatially remote brain regions. Specifically, the whole-brain analysis of functional connectivity by RS-fMRI has allowed a better understanding of sensorimotor or cognitive functions by exploring highly reproducible networks at rest, the so-called resting-state networks (RSNs) (Damoiseaux et al. 2006; Mantini et al. 2007). From the pathophysiological point of view, RS-fMRI has been used to investigate brain functional organization in several neurodegenerative diseases, with only a few studies exploring the functional connectivity of the brain networks in ALS (Mohammadi et al. 2009; Douaud et al. 2011; Tedeschi et al. 2012; Agosta et al. 2013) and bvFTD (Zhou et al. 2010; Whitwell et al. 2011; Filippi et al. 2013; Farb et al. 2013; Dopper et al. 2013). In particular, the most consistent RS-fMRI features of the two syndromes were a suppressed connectivity within the sensorimotor network (SMN) (i.e., involving primary and supplementary motor areas) in ALS patients (Mohammadi et al. 2009; Douaud et al. 2011; Tedeschi et al. 2012) and a weakening of connectivity within the so-called "salience" network (SLN) (i.e., including the anterior cingulate and orbitofrontal-insular cortices), involved in socially-emotionally relevant information processing, in bvFTD patients (Zhou et al. 2010; Filippi et al. 2013; Farb et al. 2013).

With regard to other RSNs related to cognition, such as the default mode network (DMN), composed of the posterior cingulate, precuneus and medial prefrontal cortices, and the bilateral fronto-parietal networks (FPNs), including regions subserving attention, executive processing, planning, and working memory, some authors have described a weaker connectivity in frontal areas in both ALS (Mohammadi et al. 2009; Douaud et al. 2011; Tedeschi et al. 2012) and bvFTD (Filippi et al. 2013; Farb et al. 2013), while posterior cortical functions have been shown to survive or even thrive in some cohorts of ALS patients (Tedeschi et al. 2012; Agosta et al. 2013), similarly to what commonly reported in bvFTD patients (Seeley et al. 2008; Zhou et al. 2010). Notably, this pattern of posterior enhancement of RS-fMRI activity within the DMN, which may characterize the ALS-FTLD continuum, has not been observed in AD that, like normal aging, damages mainly the posterior part of the network (Zhou et al. 2010). Although it remains unclear whether a neurodegenerative disease can cause a network connectivity up-regulation, current hypotheses in this direction refer to the activation of neuroplasticity mechanism (i.e., compensatory reorganization of networks within less affected brain regions) (Tedeschi et al. 2012; Trojsi et al. 2012) or to a progressive imbalance between excitatory and inhibitory circuits, probably determined by degeneration of GABAergic interneurons (Turner et al. 2012).

With regard to alterations of RSNs connectivity in very early stages of FTLD, recent RS-fMRI studies, performed on pre-symptomatic carriers of GRN and MAPT mutations compared to non-

carriers, have revealed that frontoinsular RS-fMRI abnormalities may be present from the pre-symptomatic stages of disease (Whitwell et al. 2011; Dopper et al. 2013), thereby representing potential biomarkers for early diagnosis of bvFTD. However, even if divergent or overlapping features may be revealed by comparing RSNs abnormalities reported in ALS with those observed in FTLD, none of the previous RS-fMRI analyses have explored the whole ALS-FTLD spectrum of disease, investigating brain connectivity changes in large cohorts of patients belonging to this continuum and exhibiting different clinical phenotypes or mutations of genes commonly associated with ALS, FTLD or both. As a pivotal study, our research group recently evaluated RSN abnormalities in small populations of ALS and bvFTD patients in early stages of disease, showing several similarities between the two groups of patients regarding degeneration of several sensorimotor and cognitive RSNs, in favor of the existence of a functional continuum between the two syndromes (unpublished data).

STRUCTURAL NEUROIMAGING

In recent years, the development of advanced automated imaging analysis, based upon construction of statistical parametric maps, has allowed detailed anatomic studies of brain morphometry. In particular, VBM, which allows whole-brain measurement of regional brain atrophy by voxelwise comparison of GM and WM volumes between groups of subjects (Ashburner, 2007) and SBM, which allows to estimate cortical thickness (CTh) measures (Fischl and Dale, 2000), have been proven substantially contributive to investigate the extent of cortical neurodegeneration in several neurologic disorders. Specifically, the most consistent findings of VBM and CTh studies in ALS and ALS-FTD involve cortical degeneration of many regions of the frontal (i.e., the anterior cingulate, middle and inferior frontal gyrus, Broadman areas 8, 9 and 10), temporal (i.e., temporal poles, superior temporal gyrus, temporal isthmus), parietal and occipital lobes (Chang et al. 2005; Mezzapesa et al. 2007; Grossman et al. 2008; Krueger et al. 2009; Agosta et al. 2007, 2012; Lillo et al. 2012; Tedeschi et al. 2012; Mahoney et al. 2012; Verstraete et al. 2012; Mioshi et al. 2013; Schuster et al. 2014; d'Ambrosio et al. 2014). Furthermore, significant associations between cognitive dysfunctions and frontotemporal GM atrophy or cortical thinning have been reported especially in ALS patients with cognitive and behavioural symptoms (Grossman et al. 2008; Verstraete et al. 2012; Schuster et al. 2014), considerably mirroring neuropsychological and morphometric patterns identified in FTLD (Du et al. 2007; Seeley et al. 2008; Whitwell et al. 2009; Krueger et al. 2009; Agosta et al. 2012; Lillo et al. 2012; Dopper et al. 2013). In this regard, Grossman et al. (2008) reported direct correlations between measures of cognitive function and cortical atrophy in classical ALS patients. They showed that ALS patients exhibited significant difficulty on measures requiring action knowledge compared to object knowledge, and performances of these tasks were highly correlated with cortical atrophy in motor regions. Moreover, scores on tests of both action and object knowledge were correlated with decreased GM volume in inferior frontal cortex and DLPFC, known to be involved in semantic memory. Therefore, these findings indicate that deficits in semantic access, which normally mediate tasks requiring knowledge of action features, may partially influence dysfunctions of the motor system reported in patients with ALS.

More recently, Mioshi et al. (2013) characterized the patterns of brain atrophy in patients with ALS with (ALS-plus) and without cognitive and neuropsychiatric symptoms compared to healthy controls and patients with ALS-FTD. Patients with ALS-plus exhibited a significant atrophy principally confined to motor and somatosensory and adjacent frontal and parietal areas, while ALS patients without cognitive and neuropsychiatric symptoms showed only brainstem atrophy and no significant cortical atrophy. Remarkably, frontal atrophy in ALS-plus patients was less widespread than in ALS-FTLD patients, who showed more distributed frontotemporal atrophy.

Also CTh evidence, derived from the ALS-FTLD spectrum of disorders, was in favor of a major extent of cortical degeneration in case of coexistent dementia. In fact, Schuster et al. (2014) confirmed the finding that ALS-FTLD patients may exhibit a more widespread pattern of cortical thinning across the frontal and temporal lobes in comparison to CTh

changes observed in both cognitively impaired and unimpaired ALS patients, thereby revealing that the cognitive status of ALS subjects seems to be associated with different patterns of cortical atrophy. Furthermore, the CTh patterns identified in both cognitively impaired ALS and ALS-FTD patients agree with previous evidence of a widespread frontotemporal cortical thinning and atrophy, prone to extend towards contiguous parietal lobes, which have been broadly described in several cohorts of FTLD patients with many different phenotypical and genotypical variants (Du et al. 2007; Krueger et al. 2009; Whitwell et al. 2009, 2010; Rohrer et al. 2009, 2011; Agosta et al. 2012; Mahoney et al. 2012; Lillo et al. 2012; Dopper et al. 2013).

From the microsctructural point of view, DTI studies based on a whole-brain approach have demonstrated higher accuracy in detecting disease-related WM changes than region of interest (ROI)-based methods. Recent whole-brain DTI analyses have described a distributed WM damage in ALS by applying voxel-based (Grossman et al. 2008; Sach et al. 2004) or tract based spatial statistics (TBSS) (Sage et al. 2009; Agosta et al. 2010; Filippini et al. 2010; Cirillo et al. 2012; Lillo et al. 2012) approaches. Most of these studies found changes of fractional anisotropy (FA) and mean diffusivity (MD) not only in the corticospinal tracts, but also in the corpus callosum (Sach et al. 2004; Lomen-Hoerth, 2004; Abrahams et al. 2005; Chang et al. 2005; Grossman et al. 2007; Gibbons et al. 2008; Sage et al. 2009; Witgert et al. 2010; Agosta et al. 2007, 2010; Filippini et al. 2010; Burrell et al. 2011; Lillo et al. 2011; Hsiung et al. 2012; Simón-Sánchez et al. 2012; Cirillo et al. 2012) and the frontal and temporal lobes (Sage et al. 2009; Agosta et al. 2010; Cirillo et al. 2012; Lillo et al. 2012). Interestingly, in several cohorts of ALS patients the location of the diffusivity changes in the associative tracts of the frontal lobes (Agosta et al. 2010; Cirillo et al. 2012; Lillo et al. 2012) has been proven significantly consistent with similar diffusivity changes described in patients with bvFTD (Whitwell et al. 2010). In this regard, a recent TBSS DTI study by Lillo et al. (2012) investigated GM and WM changes across the whole ALS-FTLD continuum, comparing DTI and VBM patterns identified in three cohorts of patients (10 ALS, 10 ALS-FTD and 15 bvFTD) with 15 healthy controls. Lillo et al. showed that extensive prefrontal cortex changes emerged as a marker for bvFTD, while ALS-FTD was distinguished from ALS by some additional temporal GM and WM changes. Conversely, ALS seemed to be mainly distinguished from the other two disease subtypes by corticospinal tract degeneration. Firstly, Lillo et al. revealed that the morphological patterns of bvFTD and ALS exhibit a considerable overlap especially in the anterior cingulate, motor cortex and the WM tracts underneath those regions (Figs. 1, 2). However, the slight divergences in frontotemporal cortical atrophy as well as in subcortical WM damage were proven to be useful for distinguishing and classifying the different disease subtypes across the ALS-FTLD continuum (Fig. 2), inducing to prospect possible future applications of these measures as radiological markers of neurodegeneration. Notably, a greater whole-brain WM disease burden, as evaluated by DTI metrics, has been identified in FTLD cases showing Tau inclusions at autopsy when compared to FTLD cases with TDP-43 inclusions (McMillan et al. 2013), thereby emphasizing the potential role of WM neuroimaging for *in vivo* discrimination between different subtypes of FTLD pathology.

By combining DTI and "graph analytical network" approaches via a modern method that allows to examine the organization of widespread functional brain networks or "connectome", intriguing insights on knowledge of brain networks reorganization in neurodegenerative diseases have recently emerged. Specifically, concerning the ALS-FTLD spectrum of disease, Verstraete et al. (2011) have proven a significant impairment of structural networks comprising bilateral primary motor cortices, bilateral supplementary motor areas, parts of the left basal ganglia (pallidum) and right posterior cingulate and precuneus in a large cohort of ALS patients (Fig. 3). Notably, the neurodegenerative process seemed not only to affect the primary motor connections, but also the connectivity between primary motor regions and supplementary motor areas. Therefore, it has been hypothesized that ALS pathology begins in the precentral gyrus and progresses along the structural connections of the primary motor regions towards secondary motor and extra-motor regions. Furthermore, it must be taken into account that the connectivity changes of the posterior part of the

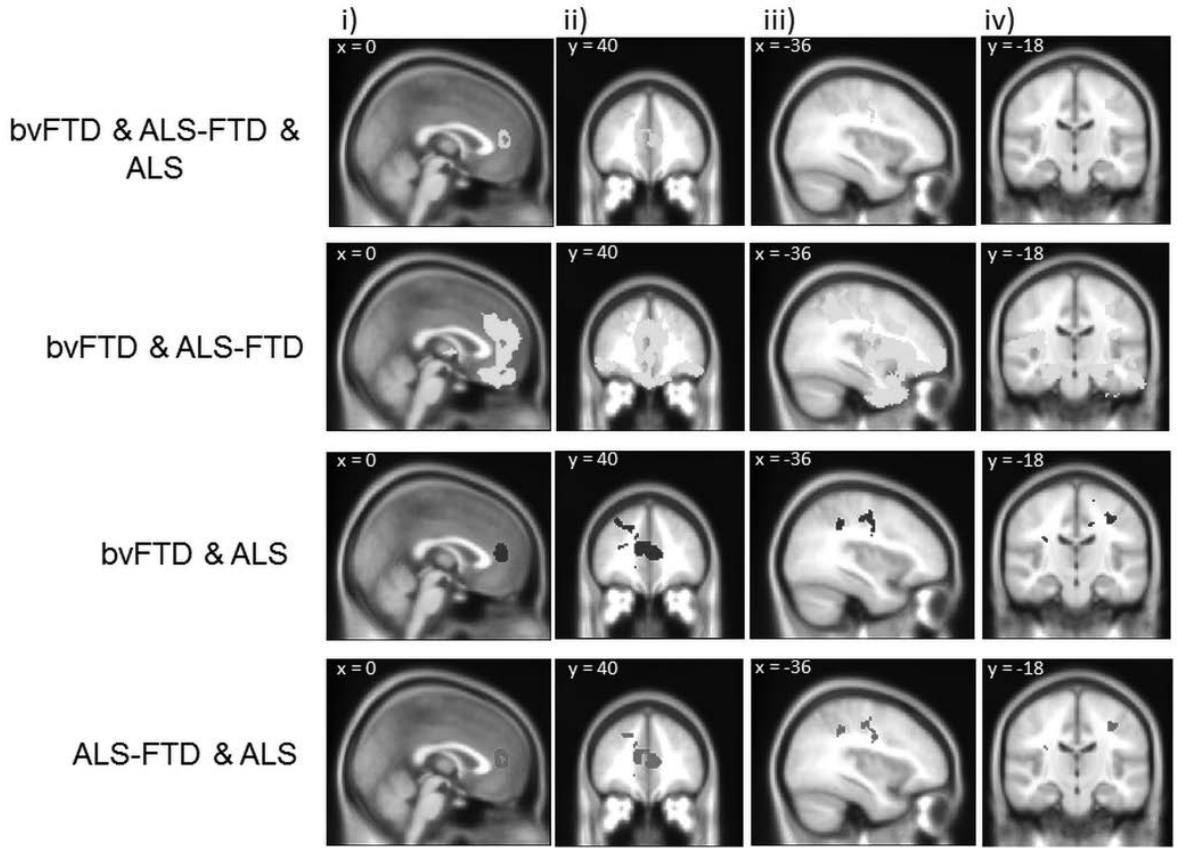

Fig. 1. *Overlap of GM atrophy clusters across patient groups belonging to the ALS-FTLD continuum: a VBM analysis by Lillo et al. 2012 showed some regions of overlap of atrophy clusters between ALS, ALS-FTD and bvFTD patients. Specifically, the first and the second columns of axial and sagittal images show overlap of atrophy clusters in anterior cingulate cortex (i, ii), while the third and the fourth columns of images show overlap of atrophy clusters in motor cortex (iii, iv) (image reproduced from Lillo et al. 2012) under the Creative Commons license (CC-BY), no permission needed).*

DMN in some cohorts of ALS patients (Tedeschi et al. 2012; Agosta et al. 2013) may be strictly related to an impaired structural connectivity of the motor network to the precuneus and posterior cingulate regions, key regions of the DMN. Conversely, with regard to FTLD, a recent "graph theoretical analysis" by Agosta et al. (2013) revealed global and local functional alterations of networks in bvFTD which exhibited some divergences in comparison to connectivity patterns described in ALS. In fact, the greatest decrease in inter-regional connectivity was shown between the frontal and occipital regions, and the insular cortices and occipital, temporal and frontal regions.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The increasing evidence of a genetic and clinicopathological continuum between ALS and FTLD has been recently reinforced by neuroimaging findings. PET and fMRI results have confirmed that connectivity changes beyond the primary motor network are a common feature in ALS patients and may exhibit relevant similarities with brain connectivity patterns described in bvFTD. At a GM and WM degeneration level, similar neural networks, comprising motor cortical, medial prefrontal cortex and temporal pole regions and their afferent and

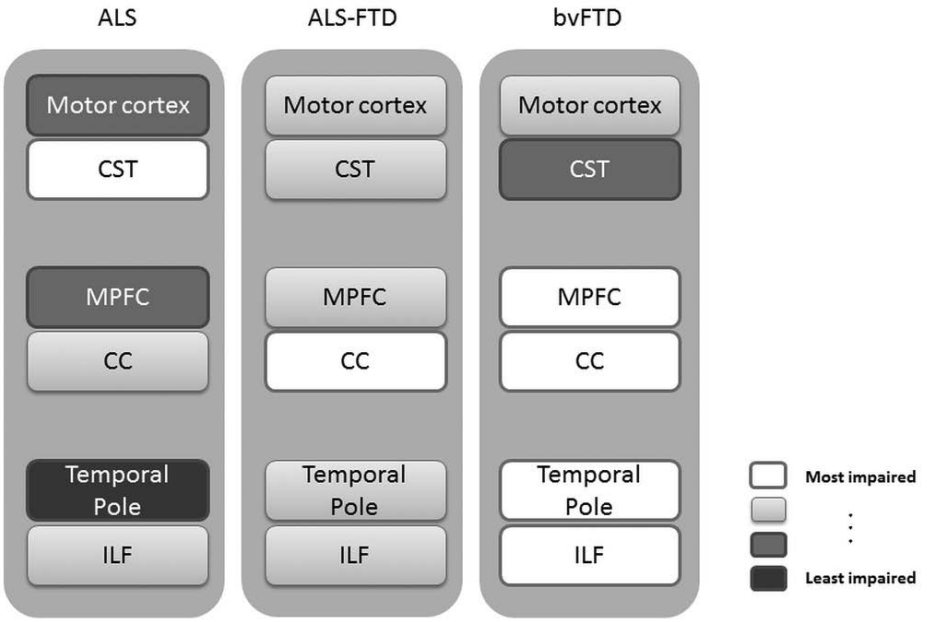

Fig. 2. *Schematic summary of VBM and DTI findings revealed by Lillo et al. 2012. The authors showed regions of GM atrophy and WM damage in ALS, ALS-FTD and bvFTD patients groups by using text boxes colored in a white-grey scale, where white indicates a more severe impairment and grey a lesser impairment (image reproduced from Lillo et al. 2012 under the Creative Commons license (CC-BY), no permission needed). CST=corticospinal tract; MPFC=medial prefrontal cortex; CC=corpus callosum; ILF=inferior longitudinal fasciculus.*

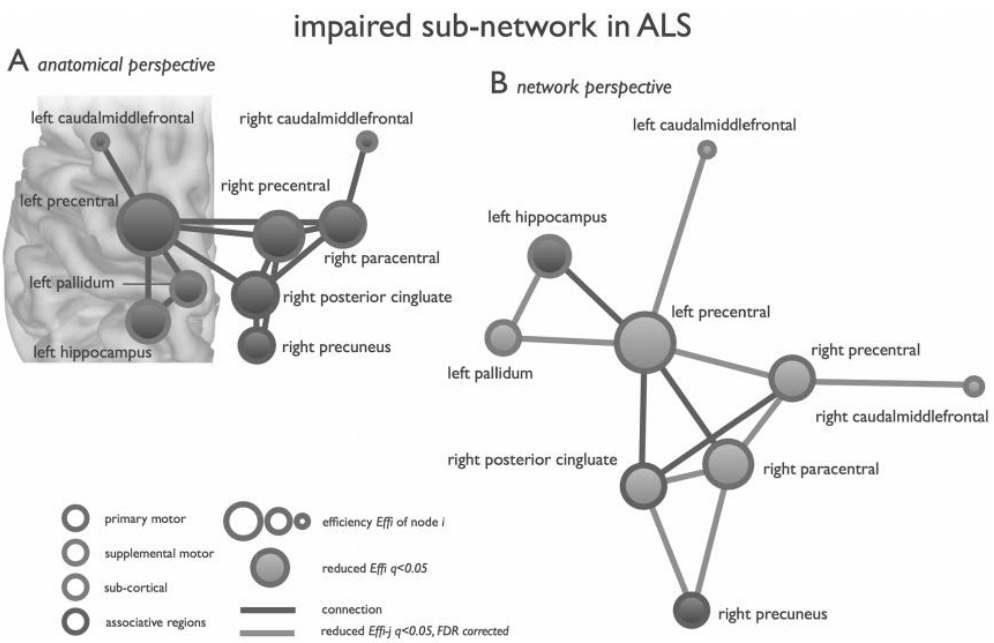

Fig. 3. *Schematic picture of impaired structural connectivity in ALS described by Verstraete et al. 2011. Nodes and connections showing significantly reduced efficiency in patients were highlighted. A significant impairment was revealed of structural networks comprising bilateral primary motor cortices, bilateral supplementary motor areas, left basal ganglia and right posterior cingulate and precuneus [image reproduced from Verstraete et al. 2011 under the Creative Commons license (CC-BY), no permission needed].*

efferents, seem to be affected across the whole ALS-FTLD continuum, showing slight but distinctive characteristics when comparing the various phenotypes of disease. Moreover, the identification of significant associations between neuroimaging profiles and genetic variants across the ALS-FTLD spectrum of disease has been proven extremely useful for investigating *in vivo* neurobiological mechanisms underlying the neurodegenerative process. Undoubtedly, future combined approaches based on neuropsychology, advanced imaging, molecular pathology and genetics will further enhance our understanding of the pathophysiology of the disease continuum existing between ALS and FTLD. We anticipate that the expected results will provide valuable information for a better classification of the clinical syndromes belonging to this spectrum of disorders and a more accurate design and management of therapeutic trials.

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NERVE GROWTH FACTOR, SYNAPTIC PLASTICITY AND ALZHEIMER'S DISEASE

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Nerve growth factor (NGF) modulates multiple signaling pathways to regulate neuronal survival, synaptic plasticity and cognitive processes. On the other hand, NGF has been implicated in the pathophysiology of a wide variety of neurological and psychiatric diseases and may serve as a potential therapeutic strategy for the treatment of neurodegenerative disorders. Here we explore the interactions between NGF, synaptic plasticity and Alzheimer's disease (AD), and highlight a novel NGF variant which might provide an innovative therapeutic option to restore neuroplasticity deficits in AD.

Release of neurotrophins during synaptic activity plays a key role in processes that lead to normal synaptic plasticity and memory (Arancio and Chao, 2007). As a result, alteration in neurotrophin signaling has been implicated in the pathophysiology of a wide variety of neurodegenerative and cognitive disorders (Lu et al, 2013). The finding that neurotrophic factors, such as Nerve Growth Factor (NGF), modulate neuronal survival and axonal growth has been used as the rationale in developing therapies for Alzheimer's disease (Cattaneo and Calissano, 2012), Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis, and also for spinal cord injury (Arancio and Chao, 2007). However, the development of an NGF-based therapy for neurodegenerative diseases remains a difficult task (Thoenen and Sendtner, 2002) due to incomplete access of NGF to the brain (Poduslo et al, 1994) and to its potent nociceptive effects in animals and humans (Pezet and McMahon, 2006).

Our group has recently demonstrated that mouse NGF is able to reverse synaptic dysfunction in a mouse model of cognitive impairment (La Rosa et al, 2013). Moreover, a ''painless'' human NGF was recently designed by mutation at residue R100 (Covaceuszach et al, 2010), displaying full neurotrophic and anti-amyloidogenic activities in neuronal cultures, and in Alzheimer's models, while keeping a reduced nociceptive activity in vivo (Capsoni et al, 2011).

In this review we will focus on the interaction between NGF, synaptic plasticity and AD, and we will present data concerning the efficacy of the "painless" NGF mutant hNGFP61S/R100E in rescuing long-term potentiation (LTP) in the Tg2576 mouse model of AD. Our understanding of the role of NGF in synaptic plasticity and memory will hopefully lead to the generation of novel disease-modifying therapies for neurodegenerative disorders.

NGF AND SYNAPTIC PLASTICITY

There is considerable evidence that NGF and brain derived neurotrophic factor (BDNF) signaling are crucially involved in learning and memory. While BDNF signaling is established in hippocampus-dependent learning and plasticity, NGF effects have been mainly associated with retention and consolidation of memory tasks in the hippocampus and cortex (Dudai, 2004). Moreover, BDNF seems

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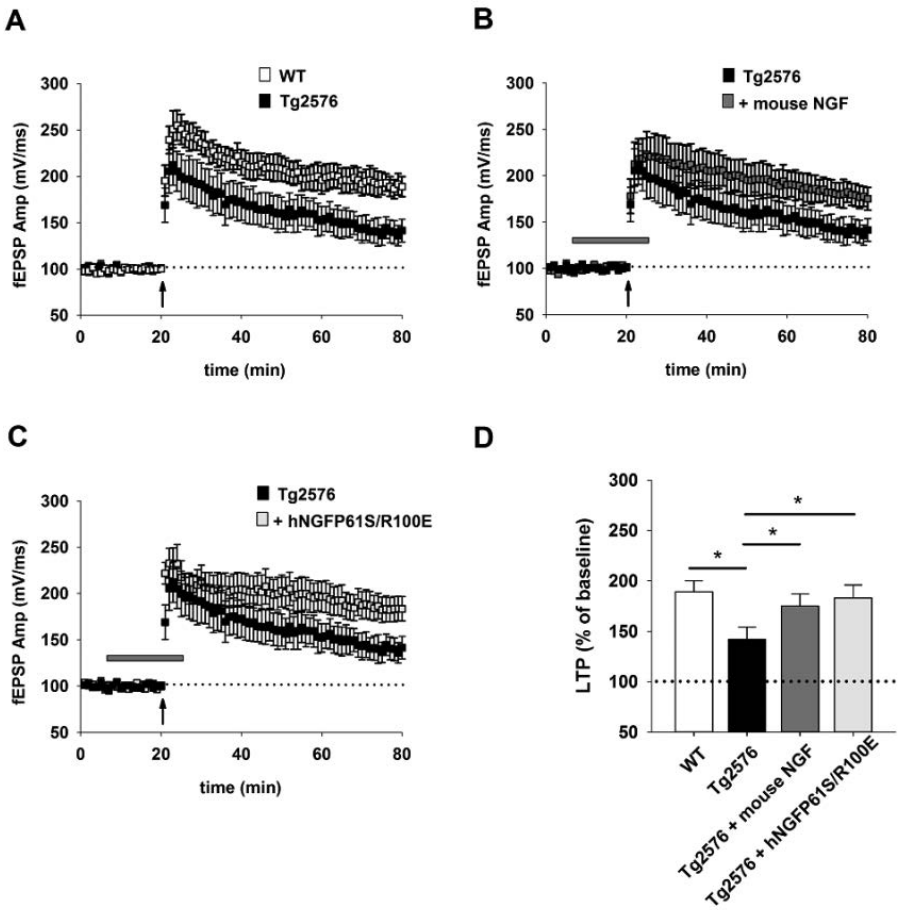

Fig. 1. *LTP impairment in 6 month-old Tg2576 mice is restored by NGF variants. A) Each point represents the mean ± SEM of the field excitatory postsynaptic potential (fepsp) amplitude expressed as a percentage of the mean control from 6 month-old WT and Tg2576 mice. In these, and subsequent experiments, picrotoxin (100 μm) was always present throughout recordings. Arrow indicates application time point of conditioning stimulus train (HFS: eight trains, each of eight stimuli at 200 Hz, and an intertrain interval of 2 s). B-C) Either mouse NGF (50 ng/ml) (B) or hngfp61s/R100E (50 ng/ml) (C) rescued the magnitude of DG-LTP in 6 month-old Tg2576 slices. Bar indicates the exposure time to NGF variants. D) Bar graph summarizes LTP (mean ± SEM) measured at 60 min post-HFS under the different experimental conditions described above. A minimum of 6 experiments are shown for each condition.*

to have a direct, and rapid role in the induction of LTP (Kang and Schuman, 1995), or at least in the transition from early phase LTP (E-LTP) to late phase LTP (L-LTP) in CA1 synapses (Lu et al, 2008), whereas NGF displays a more indirect role. Indeed it has been suggested that NGF, via enhancement of cholinergic signaling, regulates intrinsic plasticity of pyramidal cells in the hippocampus by preserving the expression of the cation-chloride cotransporter KCC2 (Lagostena, 2010). Of note, following NGF withdrawal, the action of GABA becomes depolarizing therefore disrupting normal plasticity at CA3-CA1 synapses (Rosato-Siri et al, 2006).

To date, only few reports documented an involvement of NGF in the regulation of synaptic plasticity in healthy animals. NGF was shown to enhance the amplitude of hippocampal LTP (Wang et al, 2012) by increasing the activity of PARP-1, a polymerase mediating the PolyADP-ribosylation and important for memory formation. It is believed that NGF exerts a direct effect in the hippocampal dentate gyrus where expression of NGF and its receptors

are relatively high (Mrzljak and Goldman-Rakic, 1993; Dougherty and Milner, 1999). Accordingly, it was demonstrated that intracerebroventricular injection of NGF, administered 30 min before the in vivo recording, could restore synaptic plasticity in the dentate gyrus of rats deficient in NGF (Kelly et al, 1998). Moreover, a recent finding provides the first direct demonstration that NGF availability can influence hippocampal physiology and behavior in the intact, adult brain by modulating brain cholinergic systems. Selective augmentation of septo-hippocampal function with NGF significantly facilitates hippocampal plasticity, whereas NGF blockade impairs hippocampal plasticity. Furthermore, NGF blockade significantly impairs memory retention (Conner et al, 2009).

NGF AND ALZHEIMER'S DISEASE

The "neurotrophic factor hypothesis of AD" emerged in the 1980s, which postulates that a loss of NGF in AD brain may account for the loss of cholinergic function. However, no change was found in NGF mRNA levels in AD cerebral cortex and hippocampus compared with normal brain (Goedertet al, 1986). Furthermore, measurements of NGF protein in AD brain unexpectedly showed either significant or non-significant increases in the cerebral cortex and hippocampus (Allen et al, 1991), although NGF levels in the cholinergic basal forebrain (ChBF) were decreased (Mufson et al, 1996). One explanation for this decrease in ChBF NGF levels might be a failure in retrograde transport of NGF from target tissues to the cell bodies (Cooper et al, 2001).

Nerve growth factor (NGF) is highly expressed in major targets of the ascending projection from cholinergic basal forebrain neurons (Bigl et al, 1982; Mesulam et al, 1983) that retrogradely transport NGF from hippocampal and cortical regions to the cholinergic cell bodies of the basal forebrain (Seiler and Schwab, 1984). After cortical/hippocampal release, NGF mediates its actions on cholinergic basal forebrain neurons via the p75NTR and TRKA receptor (Bickel and Kewitz, 1990; Lauterborn et al, 1991; Holtzman et al, 1992; Gibbs and Pfaff, 1992).

Exogenous NGF treatment increases choline acetyltransferase (ChAT) activity and choline uptake activities and enhances acetylcholine synthesis as well as basal and depolarization-induced acetylcholine release in adult rats (Rylett et al, 1993).

Moreover, NGF modulates AβPP expression (Rossner et al, 1998) and signaling (Matrone et al, 2011) by inducing Tyr(s) phosphorylation on AβPP C-terminal domain (Matrone et al, 2011). In turn and relevantly, AβPP plays a role in the activation and localization of NGF/TRKA signaling pathway in vivo, thereby regulating the sensitivity of neurons to the trophic action of NGF (Matrone et al, 2011). These data support the hypothesis of a functional interconnection between AβPP and TRKA pathway(s) (Calissano et al, 2010). The neuroprotective effects of NGF on the cholinergic system could explain the disease-modifying proprieties exerted by this molecule in preclinical models. Indeed, recent data suggests that NGF and its receptors could be the upstream drivers of both altered AβPP processing and cholinergic deficit.

The anti-NGF AD11 mouse model (Capsoni et al, 2000) expresses a recombinant, highly specific anti-NGF antibody in the adult brain that neutralizes NGF in the adult brain, after normal development. This anti NGF antibody has the remarkable property that it binds mature NGF with a 2000 fold higher affinity than unprocessed proNGF, thus leading effectively to an imbalance of proNGF to NGF in the adult brain (Capsoni and Cattaneo, 2006).

The anti-NGF AD11 mouse model (Capsoni et al, 2000) expresses a recombinant, highly specific anti-NGF antibody in the adult brain that neutralizes NGF after normal development.

These mice develop an AD-like phenotype, with progressive impairment of working memory (Berardi et al, 2007) accompanied by synaptic plasticity deficits in the cortex (Origlia et al, 2006) and hippocampus (Sola et al, 2006). The lack of NGF per se is sufficient to produce accumulation of β-amyloid in the hippocampus and a progressive neuronal expression of hyperphosphorylated tau (Capsoni et al, 2002). Moreover, the neurodegeneration can be fully reverted with NGF administration, but not with cholinergic drugs (De Rosa et al, 2005).

The NGF deficit responsible for this progressive neurodegeneraiton is, most likely, a proNGF/NGF imbalance, that could represent a driver of neurodegeneration and affect excitatory/inhibitory

homeostasis (Capsoni and Cattaneo, 2006; Capsoni et al, 2010). Recently, we generated transgenic proNGF mice (TgproNGF#3 mice), expressing postnatally furin-cleavage resistant proNGF as a transgene in the neuronal compartment (starting from p6-p10) (Tiveron et al, 2013), and directly proofed that proNGF/NGF imbalance induce cognitive deficit and a neurodegenerative-like phenotype. These changes are mirrored in humans where MCI and early AD show an increased level of pro-NGF and co-receptor sortilin with parallel decrease of TRKA receptors (Counts et al, 2004; Fahnestock et al, 2001).

Moreover, a diminished cleavage of proNGF to NGF and an increased NGF degradation in AD human brains has been shown (Bruno et al, 2009).

NGF RESCUES SYNAPTIC DYSFUNCTION IN ALZHEIMER'S DISEASE

The growing evidence that neurotrophins are essential modulators of synaptic plasticity and that synaptic transmission becomes dysfunctional before the onset of AD raise the question of whether synaptic failure could be ascribed to neurotrophin dysregulation. In line with this notion, a Tg mouse line expressing chronic nerve growth factor (NGF) deprivation displays age-related defects in synaptic plasticity, supporting a "neurotrophic unbalance" hypothesis underlying AD-like neurodegeneration (Houeland et al, 2010). Accordingly, exogenous supply of neurotrophins was proven effective to restore synaptic alterations in experimental AD. Indeed, it was recently demonstrated that application of neurotrophin 4 (NT4), a neurotrophic factor that signals predominantly through the TrkB receptor tyrosine kinase, prevented LTP deficits induced by Aβ both in the CA1 and DG of rat hippocampal slices (Zeng et al, 2010). This rescuing effect was associated with enhanced CaMKII autophosphorylation, a signaling event normally stimulated by LTP but suppressed by Aβ42. Aβ can interact directly with p75NTRs, which are known to mediate neuronal death and have been consistently linked to the pathology of AD (Costantini et al, 2005). Blocking p75NTRs with the isoleucine derivative LM11A-31 rescued Aβ-induced LTP impairment with nanomolar concentration, without affecting baseline transmission (Yang et al, 2008).

Previous studies examined the expression of LTP within the DG of the hippocampus from the Tg2576 AD mice model (Jacobsen et al, 2006). Indeed, it was shown that the DG is synaptically impaired in 4- but not 2-month-old Tg2576 mouse hippocampal slices. In the study here presented, we examined synaptic plasticity at medial perforant pathway to granule cell synapses (PP-DG) in 6 month-old Tg2576 mice compared to age-matched controls. The response of 6 month-old Tg slices to the conditioning stimulus train was significantly lower from that of their WT controls ($P < 0.05$). In fact, LTP at 60 min was 189 ± 11% for WT (n=7) and 142 ± 12% for Tg (n=8) mice (Fig. 1A). Notably, pre-treatment with bath-applied mouse NGF, at a concentration not affecting baseline transmission per se, was able to fully rescue LTP magnitude in Tg2576 mice (175 ± 12%, n=6, $P < 0.05$) (Fig. 1B). Similarly, also the "painless" hNGFP61S/R100E (50 ng/ml) (Capsoni et al, 2011) was able to significantly recover LTP deficit in Tg2576 mice (183 ± 13%, n=4, $P < 0.05$) (Fig. 1C). Data obtained are summarized in Fig. 1D. Overall, these results highlight that either mouse NGF or their analogs such as the "painless" human NGF might represent candidate molecule compounds for AD therapeutics. Intriguingly, encapsulated cell biodelivery of NGF to AD patients is currently undergoing clinical trials (Tuszynski et al, 2005; Ferreira et al, 2015).

NGF-BASED THERAPIES FOR AD: PERSPECTIVES

While many clinical trials in the 1990s failed to reverse ALS phenotype and several neuropathies with administration of BDNF and NGF (Thoenen and Sendtner, 2002), there is still considerable promise for the use of neurotrophins in neurodegenerative diseases, such as AD.

The main hypothesis postulates that lack or lowering of specific neurotrophic factors contributes to age-related neurodegeneration (Helen et al, 2013). Therefore, defining the mechanisms that drive age-related neurodegeneration could lead to proper use of neurotrophins in neurodegenerative disorders.

The actions of these growth factors have important effects upon LTP, LTD and other forms of synaptic

plasticity; hence it is likely that small molecule agonists that promote these enzymatic activities may prove to be clinically relevant. It is also likely that other growth factors may also influence these complex disorders.

However, several problems emerged in relation to the therapeutic use of NGF. One major problem concerns the brain penetrability, since NGF does not readily cross the blood brain barrier (Pardridge, 2002), and also when released intracranially it shows a restricted diffusion in brain (Saltzaman et al, 1999).

Different highly invasive approaches have been then tested, such as neurosurgery for the transplant of autologous fibroblasts engineered to secrete NGF directly in the brain (Tuszynski et al, 2005). An alternative way to deliver macromolecules into the brain is the nose-to-brain route that is a rapid and non-invasive administration delivery to the brain, with negligible systemic exposure (Cattaneo et al, 2008). This approach is suitable for repeated administrations (Cattaneo et al, 2008).

Another major setback for the therapeutic use of NGF is represented by the pro-nociceptive actions of NGF. Indeed, NGF has a well-known role in neural and inflammatory pain (Pezet and McMahon, 2006), which has been shown in the course of pilot clinical trials in AD patients (Eriksdotter et al, 1998). However, a rare human syndrome, the hereditary sensory and autonomic neuropathy type V (HSAN V) provided the rationale to bypass this problem. Indeed, this rare syndrome shows congenital insensitivity to pain, without any major neurological symptoms due to a mutation (R100W) in the NGF gene (Einarsdottir et al, 2004). This formed the basis for the development of a painless form of NGF, with equal neurotrophic potency (Capsoni et al, 2011).

NGFR100 has a normal affinity for TRKA, but the binding to p75NTR is lower than its non-mutant counterpart (Covaceuszach et al, 2010). Moreover, hNGFR100 fails to activate PLC-1γ signaling when it binds to TRKA, an effect that is implicated in TRKA-mediated sensitization of sensory nociceptors (Capsoni et al, 2011). Most importantly, the ability of this painless variant to rescue synaptic impairment (see Fig. 1) in a mouse model of AD highlights its therapeutic potential for the treatment of AD.

Thus, besides NGF itself as potential drug, some different approaches could be used to manipulate NGF–TRKA signaling, including small-molecule TRKA agonists (Jang et al, 2007), or p75NTR antagonists, such as LM11A-31 that blocks the action of pro-NGF (Tep et al, 2013). Moreover, LM11A-31 could take the advantage of oral administration due its stability and capacity to cross the bloodbrain barrier (Tep et al, 2013). Of note, also amitriptyline, a classical antidepressant drug, has been recently recognized as ligand both for TRKA and TRKB receptors, possibly promoting TRKA–TRKB heterodimerization which does not occur with NGF or BDNF (Jang et al, 2009).

By departing from conventional neurotrophin targeting, we believe that novel NGF-based ligands might provide novel therapeutic options for a broad range of neurodegenerative disorders.

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