The role of sex and sex Hormones in Neurodegenerative Diseases

Elisabetta Vegeto, Alessandro Villa, Sara Della Torre, Valeria Crippa, Paola Rusmini,

Riccardo Cristofani, Mariarita Galbiati, Adriana Maggi*, Angelo Poletti*

Department of Excellence of Pharmacological and Biomolecular Sciences

and Center of Excellence on Neurodegenerative Diseases

Università degli Studi di Milano, Italy

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*These Authors equally contributed to this manuscript

Corresponding author's contact information:
Angelo Poletti, PhD - Dipartimento di Scienze Farmacologiche e Biomolecolari, Center of Excellence on Neurodegenerative Diseases. Università degli Studi di Milano, via Balzaretti 9, 20133, Milan, Italy. Ph. +390250318215; Fax +390250318204; e-mail angelo.poletti@unimi.it

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Abstract

Neurodegenerative diseases (NDs) are a wide class of disorders of the central nervous system (CNS) with still unknown etiology. Several factors were hypothesized to be involved in the pathogenesis of these diseases including genetic and environmental factors. Many of these diseases show a sex prevalence and sex steroids where shown to have a role in the progression of specific forms of neurodegeneration. Estrogens were reported to be neuroprotective though their action on cognate nuclear and membrane receptors, while adverse effects of male hormones have been described on neuronal cells, although some data also suggest neuroprotective activities. The response of the CNS to sex steroids is a complex and integrated process that depends on: *i.*) the type and amount of the cognate steroid receptor; *ii.*) the target cell-type, either neurons, glia or microglia. Moreover, the levels of sex steroids in the CNS fluctuate due to gonadal activities and to local metabolism and synthesis. Importantly, biochemical processes involved in the pathogenesis of NDs are increasingly being recognized different among the two sexes and to be influenced by sex steroids.

The aim of this review is to present current state-of-the-art understanding on the potential role of sex steroids and their receptors on the onset and progression of major neurodegenerative disorders, namely Alzheimer's disease, Parkinson's diseases, Amyotrophic Lateral Sclerosis and the peculiar motoneuron disease Spinal and Bulbar Muscular Atrophy, in which hormonal therapy is potentially useful as disease modifier.

Essential Points

- Human neurodegenerative diseases are characterized by sex differences in term of onset, progression of disease, but current knowledge does not allow to precisely define the sex-related factors intervening in these diseases.

- Epidemiological and clinical studies linked the sex-specific synthesis of sex steroids to disease risk prevalence and incidence, but considering the hormonal pervasive effects on sexual differentiation and on brain development or functions, their sex specific influence in neurodegeneration remains obscure.

- The role played by sex steroids into functionally priming male and female brains also remains elusive thus impairing our ability to understand the extent to which brain embryonal sex differentiation may be associated with the development of a sex-specific vulnerability to neuronal death in adulthood.

- Present state-of-the-art knowledge does not allow to definitely point to sex steroids as a direct or indirect components for protective or detrimental activities in these diseases.

- The complexity of sex steroid physiological functions, the number of neural cells potentially involved, epigenetic as well as environmental factors have impaired the understanding of the role of sex on neurodegeneration so far.

- Here we provide a wide and in depth analysis of the role of sex in in four common neurodegenerative diseases: Alzheimer's, Parkinson's diseases, Amyotrophic lateral Sclerosis and Spinobulbar Muscular Atrophy because all showing a sex-specific incidence and progression.

- Our effort is aimed at facilitating the identification of all aspects that in these disorders associate sex and disease manifestation at both pre-clinical and clinical level, hoping to enable a progress in this field and underline potential ways where appropriate regulation of circulating hormones may provide benefits in these disorders where we suffer a unique lack of positive therapeutic intervention.

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1. Introduction

Neurodegenerative diseases (NDs) are devastating and largely fatal conditions that affect several million people in the world. NDs onset may occur during the entire life in human, with juvenile forms (that appear around birth, e.g.: some forms of spinal and muscular atrophy) or adult forms that may also appear very late in life. Most NDs appear after the third decade of life possibly correlating with the aging process. The causes leading to the massive neuronal death characteristic of NDs remain to be fully understood, thus developing strategies for their prevention, treatment or even delayed progression is a major challenge for modern medicine. Although it is indisputable that the main symptoms of NDs reflect neuron-specific deficits, it is also conceivable that dysfunction in other brain cells could precede and facilitate neuronal loss. Non-neuronal cells in the central nervous system (CNS) include neuroglia (oligodendrocytes and astrocytes) and microglia, a class of cells maintaining brain homeostasis through an appropriate exchange of nutrients and protection against noxious stimuli (1). Oligodendrocytes enwrap axons with myelin sheaths providing a structural and local metabolic and homeostatic protection (2); microglia ensure immune protection, the control of synaptic connections and tissue repair (3,4); astrocytes regulate the homeostasis of neurotransmission, metabolites and reactive oxygen species (5). Compelling evidence shows that these cells are impaired in NDs (1,6), as discussed in the appropriate sections of this review. Among the three major forms of ND, Alzheimer's Disease (AD) is more frequent in women than in men (ratio 2:1) (7), while Parkinson's Disease (PD) and Amyotrophic Lateral Sclerosis (ALS) affect primarily men (1.5:1, and 2:1 respectively) (8,9); peculiar is the case of Spinal and Bulbar Muscular Atrophy (SBMA), which affects men only (10). The molecular bases for this sex-related prevalence remain to be understood, although sex steroid hormones may be involved in disease pathogenesis. However, the decline of sex steroid synthesis occurring during

aging or the changes due to pharmacological supplementation may be either a risk or protective factors as a function of the neurodegenerative disease considered (11-13). Perhaps, the reason for these opposite results is that the connections between sex steroids and the manifestation of NDs are multifaceted as these hormones target all neuronal cells (neurons, glia and/or the immune cells) and control brain sexual differentiation, a process that may be relevant for the degenerative processes in adults. Moreover, another element of complexity further impairing our ability to interpret the results of investigations is that, in addition to the gonads, sex steroids may be synthesized in the brain. Epidemiological evidence indicates a sexual prevalence and incidence in most forms of ND (table 1).

Considering the reported beneficial vs. deleterious effects of sex steroids, the aim of the present review is to recapitulate current knowledge on estrogen, progesterone and androgen activities in neural cells and their potential influence on manifestation of NDs. The review will also analyze the impact of the relative contribution between circulating hormones, their local synthesis and activation (Figure 1) including their complex interplay in target cells (Figure 2). Our anticipation is that a better understanding of this field of study may lead to novel therapeutic strategies very much in need for ND.

2. The influence of sex on Neurodegenerative Diseases

a. Alzheimer's Disease: the pathogenesis

AD is the most common cause of dementia (47-49), and is a slowly progressive disease that begins well before the onset of clinical symptoms (50). With disease progression, memory loss and confusion become more severe (49), until impairments in basic physiological functions appear in the terminal stage of the disease. Hallmarks of AD include the accumulation of aggregates and

deposits of misfolded proteins, like amyloid beta (Aβ) peptides, outside neurons, and of neurofibrillary tangles (NFT), which contains abnormally phosphorylated microtubule-associated tau protein, inside neurons (50,51). Increasing evidence points to a causal role of inflammation in disease onset (52).

a.i. Pathogenic mechanisms in familiar and sporadic AD

Some AD cases (<1%) result from fully penetrant mutations of specific genes (Table 2) which associated with an onset risk before 65 years (69). Sporadic, or late onset-form of AD (LOAD), manifests later in life and is linked to genetic factors (70) acting together with environmental risk factors (71) (Table 2). The most established genetic risk factor for LOAD is the ε4 allele of the apolipoprotein E gene (*APOE*), a major cholesterol carrier responsible for lipid transport in the brain (72,73); APOEε4 accounts for differential risk to develop AD based on the gene dose (8.07 or 2.84 hazard ratio in homo- or heterozygosis, respectively) (56). Several other common genetic variants associated with LOAD highlighting the multifactorial origins of sporadic AD (Table 2). The blend of genetic, environmental and epigenetic factors (74-76) leads to a progressive decrease in synaptic density and loss of integrity in neuronal networks, resulting into neuritic atrophy and neuronal death. Several causes are involved, such as amyloidoses and tauopathy, and impairment of lipid metabolism (77-80). In the case of APOEε4 carriers hypercholesterolemia is observed (~8% higher than baseline population) together with an increased susceptibility to develop amyloid deposition (81,82). Other well-recognized etiopathogenetic elements are neuroinflammation (3,52,83,84), impairment of autophagy (85), or lysosomal degradation (86), loss of Ca^{2+} homeostasis (87,88), neuronal cycle control (89,90) and metabolic dysregulation (91-93).

a.ii Non-neuronal cells in AD.

Neuroinflammatory in AD is due to a persistent activation of microglia associated with massive release of molecules toxic to neurons and oligodendrocytes (e.g.: glutamate, free radicals, and TNF-α (52)). Indeed, in early AD stages, microglia is neuroprotective by clearing Aβ deposits and by internalize protofibrillar and fibrillar forms of Aβ peptide via endocytosis or macropinocytosis (94-97). This activity decreases during disease progression, when Aβ receptor expression (such as SRA, CD36, RAGE) is reduced and the degradation of the engulfed amyloid fibrils is impaired. In turn, the formation of amyloid deposits within microglia sustains the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) detrimental to neuron (83,98). Moreover, with aging microglia becomes dystrophic, showing morphological characteristics distinct from young, healthy cells (3,99); this process is further aggravated in pathologies involving systemic inflammation (*e.g.* obesity and diabetes mellitus) (83,100,101).

Brain aging is often associated with a loss of astrocyte functions (6), prior to AD plaque or tangle formation (102). Myelin breakdown is induced by neuroinflammation and oxidative stress and it also occurs at early stages of AD, before AD plaque or tangle formation (2), but its relevance for AD pathogenesis and the reasons why oligodendrocytes are unable to repair the initial myelin breakdown remain to be elucidated.

a.iii Sex differences in AD.

Besides age and the genetic risk factors, also sex strongly influences the risk of AD. In fact, it is estimated that almost two-thirds of Americans with AD are women (48), while among people aged 71 and older, 16% of women have AD compared with 11% of men (103,104). Also the cognitive decline is sexually dimorphic and is faster in females (22,23) with AD, but not in mild cognitive impairment (MCI) or other forms of dementia (105,106). Multimodality brain imaging indicates that in females biomarkers of the preclinical phase of AD, including failures in cerebral glucose metabolism, and a decrease in neuron mitochondrial function, appear early and overlaps the

endocrine transition of perimenopause (107). Neuropathological studies evidenced a more severe β-amyloid accumulation in women (20,21), and increased levels of tau pathology in men (24). These pathological hallmarks are reflected in most AD animal models (Tg2576, APPswe/PSEN1E9, APP23, APP_{swe}xPS1, and 3xTg-AD). Notably, a poorer behavioral performance and a greater increase of Aβ accumulation and hyperphosphorylated tau levels are observed in females than age-matched males (14-19). Several biological and social reasons were proposed to explain the AD prevalence in women (7), including the fact that women live longer than men, and older age is the greatest risk factor for AD (108). It has been suggested that men surviving to older ages may have a healthier cardiovascular risk profile, thus a lower risk for dementia than women of the same age (109); however the fact that the higher AD incidence in female is present in all age-matched groups (from 60–64 till 95 years of age) does not support this hypothesis. The role of sex hormones is still to be clarified, since mixed results were reported on the use of hormone replacement therapy (HRT) used to counteract the development and progression of AD. A strong association between APOε4 genotype with sporadic AD in women, has been reported (110,111). Indeed, women carrying both the homo- and heterozygous Apoε4 isoform have a higher rate of conversion from healthy aging to MCI and from MCI to AD. Conversely, Apoε4 variant in men has marginal effects when in homozygosity and null in heterozygosity (110,112). Memory tests done in individuals with ε3/ε3, ε3/ε4 and ε4/ε4 *APOE* genotypes showed a significant gene-dosage dependent effect of the ε4 allele on performance and male ε4 carriers at midlife showed a significant behavioral advantage in short-term memory task as compared with women (113).

b. Parkinson's Disease: the pathogenesis

PD is a progressive ND affecting 1-2% individuals over 65 years of age. It is characterized by motor symptoms, that include tremor at rest, rigidity and bradykinesia, as well as non-motor symptoms (cognitive impairment and mood, olfaction and autonomic dysfunctions). Motor symptoms are due to the selective degeneration of dopaminergic neurons located in the *substantia nigra* (SN) and innervating the *striatum*, forming the nigrostriatal dopaminergic (NSDA) system. Dopaminergic neurons have a high oxidative status at baseline, possibly because of the natural propensity of dopamine to oxidation. Antioxidant and detoxification systems are thus mainly devoted to control dopamine metabolites in dopaminergic neurons; this makes the NSDA system more susceptible and vulnerable to even mild forms of oxidative stress and energy dysmetabolism, which instead spare other neuronal cell types (114,115). PD is characterized by Lewy's bodies, which are intraneuronal aggregates mainly consisting of α-synuclein (SNCA) protein (116), in the central and enteric nervous systems. This suggested a gut-to-brain spreading of aggregated SNCA possibly due to exposure to environmental toxins (117,118).

b.i Common pathogenic mechanisms in genetic and idiopathic PD.

Familial PD (fPD) is rare and generally monogenic, while most PD are sporadic forms (85-90%) originating from both genetic causes and environmental factors (e.g.: pesticides and metals) (119,120). Some causative genes and genetic predisposition factors have been identified (Table 3) and suggested key molecular pathways as major elements in PD pathogenesis: 1) protein aggregation, since *SNCA* gene mutations render the protein (and its dopamine-induced oxidized forms) prone to oligomerize in neurons impairing protein trafficking and degradation (129-131); 2) mitochondrial defects and oxidative stress, since mutations in genes encoding leucine-rich repeat serine/threonine kinase 2 (LRRK2), DJ-1, PTEN-induced putative kinase 1 (PINK1) and Parkin produce mutated proteins causing respiratory chain dysfunctions and oxidative stress (115,132); 3) protein and mitochondria quality systems, like autophagy and the lysosomal

pathways, since mutations have been found in the glucocerebrosidase gene (*GBA*) (133-138); 4) neuroinflammation, since polymorphisms in pro-inflammatory genes potentiate the oxidative microenvironment induced by dopamine, its metabolites and oligomeric SNCA (139-145).

Animal models of PD are widely used to study disease pathogenesis, particularly those obtained by pharmacological treatments. Injection of the dopamine analogues, 6-hydroxydopamine (6-OHDA) and metamphetamine (ME), or the dopamine transporter substrates, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or the pesticide rotenone, fully reflect the biological defects of fPD (146) by inducing dopamine accumulation and production of reactive and oxidative species or by inhibiting mitochondrial activity and inducing oxidative stress and energy failure. This leads to envision that oxidative stress is a major driver of dopaminergic neuron degeneration, sustained by an interconnected network of genetic and biochemical alterations and inflammation. Oxidative stress induces chemical modification of DNA, proteins and lipids in PD brains, and promote protein aggregation engulfing the detoxification systems in dopaminergic neurons (147-151).

b.ii Non-neuronal cells involved in PD.

Besides neuron-specific defects, non-neuronal cells may be involved in PD pathogenesis. Microglia activation is present in PD patients and animal models, even before neuronal loss or the activation of other glial cells. Microglia may be detrimental to PD by secreting neurotoxic inflammatory mediators and by a limited efficiency in removing misfolded proteins by phagocytosis. Indeed, pharmacological inhibition of microglia is neuroprotective (152-163). Interestingly, microglia activation in the whole brain leads to dopaminergic neuron death only in the NSDA system, suggesting a key role for microglia and inflammation in PD motor defects (164-169). Astrogliosis is detected in specific brain regions of PD patients and animal models of the disease (170), and astrocytes contribution to PD pathogenesis is dual. On one side, activated astrocytes release inflammatory and oxidative stress mediators and sustain iron-induced

neurotoxicity in; on the other side, astrocytes display induction of autophagy and produce neuron survival factors (171-173). Importantly, many genes involved in PD are also expressed by astrocytes, suggesting that pathogenic signals may also originate from these cells (174). A sexually dimorphic reactivity of astrocytes has been reported in response to MPTP or LPS, giving rise to specific patterns of ATP/ROS and inflammatory cytokines production in astrocytes generated from male or female brains (170,175), supporting the hypothesis that they may contribute to sex differences in neuroinflammatory diseases (176).

b.iii Sex differences in PD.

Together with aging, male sex is the strongest risk factor for PD. The risk to develop PD is 1.5–fold greater in men than in women at all ages (31,177) (table 1). The phenotypic characteristics of the disease are also sexually dimorphic, since women tend to be older than men at symptom onset and more often present a tremorigenic form and a slower disease progression (31). Genomic profiling of the SN *pars compacta* (SNpc) neurons from healthy donors and PD patients further substantiated the different biological traits of men and women may have a role in disease etiology, symptoms and response to therapy (178). The difference in PD incidence among the two sexes may arise from substantial distinctions in healthy adult men and women in the composition and reactivity of the NSDA system, and this is a peculiar feature of PD among other NDs. As compared to women, the SNpc in men shows: a) a higher number of dopaminergic neurons with increased expression of SNCA and PINK1; b) more robust dopamine release induced by stimuli, such as psychomotor stimulants; and c) increased vulnerability to selective drugs in terms of addiction and toxicity. Sexual differences are also observed in regulatory systems controlling NSDA plasticity, a complex network made of interneurons, glia, microglia and input circuitries from other brain areas that is sexually distinct in terms of cell composition, function and adaptation to signals. Male-specific genetic determinants, and not organizational effects of sex steroid hormones, appear to

define this sexual dimorphism in NSDA circuitry. Importantly, the Y chromosome-specific gene *SRY* (sex determining region Y) is expressed in NSDA neurons of males and its activity is associated with a positive regulation of neuron number, dopamine synthesis and metabolism (179,180). It is thus proposed that intrinsic biological properties related with SRY gene expression predispose men to disorders associated with dopaminergic abnormalities in PD or schizophrenia.

Sex-related differences are also observed in the SNpc of rodents (26,27) and in the susceptibility and progression of PD models. The loss of dopaminergic neurons in the SNpc and the reduction in dopamine levels in the striatum are more robust in males, whereas the female sex is neuroprotective when low doses of neurotoxins are used to evidence early stages of PD (27,28,30). Genetic models are only recently being studied in terms of sexual differences; neurodegeneration is increased in PARK2-null female mice, as compared with males, suggesting a higher sensibility of females to ubiquitin-proteasomal defects (26,146). Neuronal primary cultures from females survive longer and adapt to starvation through distinct metabolic programs, while autophagy is associated with cell death in male cells (181).

Oxidative stress and mitochondrial functions are also sexually dimorphic in specific brain areas; females have increased antioxidant defenses and respiratory chain activity compared to males, thus with lower mitochondrial ROS production and oxidative damage as a consequence of the higher expression of mitochondrial proteins and antioxidant enzymes (e.g.: paraoxynase-2 or thioredoxin) (182-185). Male mesencephalic neurons exposed to 6-OHDA have reduced expression of selected mitochondrial proteins, lower ATP levels and higher ROS levels compared to female neurons (186). Moreover, females show a higher stress adaptation than males mediated by the expression of stress response factors and the differential mitochondrial usage of energy sources (181,187).

Sexual differences in neuroinflammation have been scarcely analyzed in PD patients and in animal models of the disease. Using MPTP, the increase in TNFα, IL-1β and IFNγ is faster and correlates with earlier and greater decrease of TH-neurons in male than female mice striatum, (188).

c. Amyotrophic lateral sclerosis: the pathogenesis

Amyotrophic lateral sclerosis (ALS) has an incidence of 1-2:100,000 and is primarily associated with functional alterations of the upper and lower motoneurons (placed in cerebral motor cortex or brainstem, and in the ventral horns of the spinal cord or in the cranial nerves, respectively) and their target muscle cells. Neurons in the fronto-temporal regions of the brain are seldomly involved (189), and ALS may manifest as pure motor form or associated with fronto-temporal dementia (ALS-FTD). Age of onset and progression rate are highly variable (onset generally occurs around 50-60 years of age, and juvenile forms are rare (189).

c.i Sporadic versus familial forms of ALS.

ALS occurs in two, clinically indistinguishable sporadic (sALS) and familial (fALS) forms. fALS occurs only in 10-15% of patients (see table 4 for details). Several genes are associated with fALS (189); the first identified is present in 20% of fALS and encodes the antioxidant enzyme superoxide dismutase 1 (SOD1), a free radical scavenger enzyme ubiquitously expressed (232). More recently described gene mutations include the genes encoding TAR DNA-binding protein 43 (TDP-43), ALS-linked fused in sarcoma/translocated in liposarcoma (FUS/TLS), optineurin, and others (a list of gene mutations linked to ALS is reported in Table 4). Generally, the genetic alteration is associated to a gain of a toxic function due to altered protein conformation (233) (misfolding) and accumulation as protein aggregates poorly cleared by motoneurons. Alternatively, the mutation affects essential biological functions by interfering with RNA functions, axonal transport, mitochondrial and/or proteasome activities (234). Notably, the

proteins found mutated in fALS may show aberrant behavior also in their wild type (wt) form in sALS: (*e.g.*: oxydized wtSOD1, cleaved C-terminus of wtTDP-43, etc.) (235-238), suggesting the existence of common pathological mechanisms in fALS and sALS. These proteins may thus have a natural propensity to misfold, and aggregate forming insoluble inclusions.

Recent studies showed that about 50% of fALS cases are associated to an expansion of a hexanucleotide (G_4C_2) repeat located in its 5'-untranslated region of the *C9ORF72* (chromosome 9 open reading frame 72) gene. This expansion generates five different highly hydrophobic dipeptides (DPRs) via a novel mechanism named repeat-associated non-ATG (RAN) translation (239-241). The DPRs, like misfolded proteins, aggregate as insoluble inclusions, and perturb intracellular processes causing neurotoxicity (194,195,232,239,242-245).

c.ii Non-neural cells involved in ALS.

Although ALS is a motoneuron disease, it is now considered a complex multifactorial disease that may involve other cell types (i.e. skeletal muscle cells (246-248), astrocytes (249-251), oligodendrocytes (252), and Schwann cells (253,254)). Reactive microglia is generally present in the areas where the disease is manifest (255), underlining the engagement of neuroinflammation, and oxidative stress in the pathogenesis of the disease (256).

c.iii Sex differences in ALS.

ALS is characterized by high variability in the age of onset, and disease progression (table 1). Even if the overall survival is similar in the two sexes, the disease appears earlier in males than females, and with different symptomatology. In male, ALS initiates in motoneurons of the lumbar tract of the spinal cord, while in females ALS tends to begins in bulbar regions (see (38) for extensive review). The male/female ratio is between 1 and 3, depending upon the geographic area, the population considered, and the age of disease onset (254,257,258). A potential biological marker for the sex difference is mutant SOD1, whose concentration is dysregulated in the cerebrospinal

fluid (CSF) and it is higher in male than female ALS patients (41). This difference was not found in other patients, suggesting a specific alteration of SOD1 metabolism in the two sexes (41). In addition, a retrospective study, based on executive memory and language functions in ALS patients, indicated the presence of a greater executive impairment in female than in male patients. ALS females show a 2.6-relative risk for impaired executive functions (lower scores in ALS females in Phonemic Fluency, Trial Making, and Wisconsin Card Sorting test) compared with male patients, indicating an increased vulnerability in cognitive functions in female ALS patients (39). These epidemiological studies suggest that circulating estrogens may protect from some ALS alterations, while androgens might facilitate the manifestation of the pathology (259).

The molecular bases of sex-dimorphism in ALS are unknown at present. Sex steroids might directly influence specific protective or detrimental mechanisms involved in disease or determine the sex prevalence, also in relation to brain asymmetry between sexes, as further discussed. A recent study, performed on a large number of ALS women with a natural menopause and well defined oral contraceptive usage, has shown a positive association between a longer reproductive condition, and the susceptibility to ALS and the survival of ALS patients, demonstrating that the longer exposure to female hormones has neuroprotective effects on motoneurons in ALS (40). Estrogens in both sexes might directly affect spinal cord motoneurons. Indeed, in the ventral horn of the lumbar spinal cord of adult mice, cytoplasmic aromatase and nuclear estrogen receptors (ERs: ERα and ERβ) both colocalize with the motoneuron specific marker SMI-32, and with GPR30 (see below) (260); thus estradiol and phytoestrogens (which are neuroprotective in ALS mouse models (261)) may directly acts as protective agent in spinal cord motoneurons.

Apart from hormonal milieu, other genetic factors may determine gender difference in ALS onset and progression. C9ORF72 or CAG/polyglutamine (polyQ) tract expansions (e.g.: in the ataxin-2 or the androgen receptor (AR)) may play a role in ALS. Of note, AR is highly expressed in spinal

cord motoneurons and, when mutated with an expanded polyQ, causes an ALS-like form of motoneuron disease (see below) (193,262). In addition, sex is a crucial factor in the C9ORF72-linked ALS, since C9ORF72 expansion negatively impacts on survival time in men, but not in women: fALS male patients carrying the C9ORF72 expansion characterized by spinal onset have a reduced survival rate if compared to female with the same type of onset (263). Thus, this patient cluster of may be more sensitive to adverse AR action as disease modifier in males (264).

Animal studies also support the existence of a sex-dependent susceptibility to ALS. Studies in the classical animal models of ALS, tgG93A-SOD1 mice or rats (265), showed that the disease is significantly more aggressive in males than in females (32,37). In fact, male ALS mice lose weight and show motor symptoms earlier with faster symptom progression than females (32,33). Surprisingly, sexual differences are exacerbated by the strain utilized (266) and allelic variants of chromogranin B (CHGB) gene might act as ALS disease modifiers in a sex-dependent manner (34,267). Chromogranin is an important component of the secretory vesicles and binds to mutant SOD1 proteins, acting as a chaperone and promoting their secretions from neurons. Ohta and colleagues showed that, the co-expression of $CHGB^{L413}$ allelic variant in $SOD1^{G37R}$ mice results in pathological changes and earlier disease onset specifically in female mice (34). These differences may be due to a sex-determining region Y (SRY) silencer element of the CHGB promoter, which allows higher neuronal expression of CHGB in females compared to males. In patients, the sex-related effects of CHGB variants on ALS onset is still controversial: in fact, while CHGB variants are linked to an earlier disease onset in females of cohorts of Japanese and French-Canadian origins, no effect is observed in French, Swedish (34) or Italian ALS cohorts (268).

c.iv The neuroinflammatory response in ALS affected regions and in and its possible correlation with the gender differences.

Neuroinflammation, together with oxidative stress, are among the main pathogenetic mechanisms for ALS (256). Studies in autoptic tissues from ALS patients or in spinal cord of tg ALS mice (255,269-271) showed local activation of microglia (positive for the markers CD11b, Iba1, and CD68), astrocytes (GFAP and ALDH1L1 positive cells), and lymphocytes. In addition, spinal cord astrocytosis spread from the ventral horn (the site primarily affected) to the dorsal horn, and to the sites in which the cortico-spinal tract fibers enter the grey matter (272), while microgliosis was present in the corticospinal tract, and in the spinal cord ventral horn where microglia interacts with T-cell infiltrates (270). In the brain, astrocytosis was detectable in the motor cortex and in other brain regions in the cortical grey matter (273) and subcortical white matter (274), while microgliosis was present in the motor cortex, and in the motor nuclei of the brainstem (see (255) for extensive review). ALS mouse models analyzed pre or post-symptomatically showed that the presence of activated microglial cells anticipated the disease clinical manifestation and motoneuron loss (271,275), while astrocytic activation paralleled motoneuronal death (276). During disease progression, microglia and astrocytes activation increased (275,277) in parallel with an up-regulation of the expression of cell-surface markers chemokines (such as CCL2) or the colony stimulating factor 1 (CSF1) (255), that further contributed to microglia proliferation and activation. The current opinion is that microglia play a dual role in NDs exerting both neuroprotective and neurotoxic functions. In ALS, microgliosis is accompanied by the activation of myeloid cells outside of the CNS (sciatic nerve) (278); T-cells (both $CD4^+$ and $CD8^+$) increase and microglial activation occur approximately at the same time in the areas involved in ALS (279,280). So far, no studies addressed microglia and neuroinflammation as a potential component of the sex dimorphic risk of developing ALS.

d. The peculiar case of Spinal and Bulbar Muscular Atrophy.

Spinal and bulbar muscular atrophy (SBMA) is an inherited X-linked motoneuron disease characterized by early onset (30-50 years) and a very slow progression (20 to 40 years), and it occurs only in males. (44) and caused by mutations in the AR gene, and strictly depends upon the presence of testosterone (table 1). In fact, in all animal models of SBMA developed so far, physical or chemical castration counteracts disease onset and progression. SBMA is characterized by the loss of motoneurons in the anterior horn of the spinal cord and in the brain stem (motoneurons of the lower cranial nerves) (231,281-284) and of dorsal root ganglia (DRG) sensory neurons (285). SBMA symptomatology includes muscle weakness and atrophy, fasciculations, dysphagia and dysarthria with atrophy of the bulbar, facial and limb muscles, alterations in sensory function of distal extremities (283,286). The skeletal muscle cells can also be directly affected in SBMA (285,287-290)**.** Endocrine dysfunctions, such as mild androgen insensitivity, and alterations in the gonadal-hypothalamic axis, are part of the clinical manifestation of SBMA (291-293).

d.i A mutation of AR, the molecular basis of the disease.

SBMA is due to an expanded CAG (cytosine, adenine, guanine) triplet repeat sequence in the AR gene (231). The CAG sequence is translated into elongated polyQ tract in the AR N-terminus (ARpolyQ). In the healthy population, the AR CAG repeat is highly polymorphic (15-35 repetitions) (294), with variations within human races (295); in SBMA patients the AR CAG repeat becomes expanded from 37 to a maximum of 72 repetition (282,294,296). An inverse correlation exists between polyQ size and SBMA age-of-onset, progression rate and disease severity (231,297), although exceptions to this rule (evidenced in siblings) suggest that some factors may act as disease modifiers (298). The common etiopathological denominator which associates the nine CAG/polyQ diseases is the presence of neurotoxic intracellular aggregates of the mutant proteins (299). SBMA is not an exception to this rule, since ARpolyQ aggregates are present in

the nucleus of anterior horn spinal cord motoneurons, and in the cytoplasm of DRG sensory neurons of SBMA patients (300,301).

d.ii Non-neuronal cells in SBMA.

Differently from ALS, no microglia activation has been reported in SBMA. This led to hypothesize that the very slow progression of SBMA compared to ALS is due to a lack of the inflammatory process. Notably, SBMA is now classified not only as motoneuron disease, but also as neuromuscular disease. In fact, muscle biopsies from animal models and patients reveal that myopathic symptoms anticipate motoneurons degeneration and SBMA patients present myopathic symptoms not related to motoneuron degeneration (302). In a knock-in mouse model, muscle degeneration precedes the loss of motoneurons and the selective overexpression of wtAR in skeletal muscle recapitulates the disease (303). Moreover, the suppression of ARpolyQ expression exclusively in muscle ameliorates and rescues from disease (287,288). Skeletal muscle degeneration may also be responsible for alteration of retrograde axonal transport in motoneurons (304) and also defects of the neuromuscular junctions are present before the motoneuron loss (305). All these observations suggest that SBMA is not a cell-autonomous disease, and both motoneuron and skeletal muscle represent primary targets of SBMA pathogenesis, and this new perspective open new potential therapeutic approaches focused not only on the CNS, but also on the skeletal muscle.

3. Sex steroids in the mammalian nervous system

a. Morpho-functional differences between male and female brains.

Significant morphometric differences exist in the mammalian brain of the two sexes. Even after normalization for the body size, the total brain volume is significantly larger in males than females

(306-308); the global structure is different, since males, compared to females, have larger hemispheres, frontal and temporal lobes, left parietal lobe, insula, cerebellum (309), amount of CSF, volume of lateral ventricles and *sulci* (310). Compared to males, female brain has a higher proportion of gray matter (310), and higher gray/white matter *ratio* in the frontal, temporal, parietal, occipital lobes, cingulate gyrus and *insula* (reviewed in (310)). Such sexual dimorphism present at the higher organizational levels (whole brain region, or selected brain regions) reflects dimorphisms at cellular level. Indeed, neurons, neurite length and branching of specific neuronal populations differ among sexes contributing to the dimorphism of specific brain regions. Astroglia and microglia activities differ in the two sexes impacting on neuronal response to specific stimuli or insults, including those leading to neurodegeneration. Depending of the brain region analyzed, sex-specific activities may involve dopaminergic, serotonergic, and gamma-aminobutyric acid (GABA)ergic neurons, explaining some of the sex-dependent responses to physiological or pharmacological stimuli (310-313). The existence of functional differences in cognitive abilities (e.g.verbal skills or spatial abilities, reported to be better in females and males, respectively) are still object of debate (314). Studies in transsexual subjects (see (315)) showed that several sexually dimorphic brain processes change in relation to the new sexual identity. Elements of feminization can be observed in male transsexuals or masculinization in female transsexuals thus demonstrating the validity of previous observations and suggesting that specific hormonal treatments may be able to revert some of these parameters towards the characteristics of the desired sex.

b. The essential role of sex steroids in brain sexual differentiation.

During embryogenesis and early postnatal life, brain differentiation undergoes a sexually dimorphic "organization" of the brain regions controlling gender identity (316,317), sexual behavioral and endocrine functions (318,319). At this developmental stage, testosterone synthesis

by male gonads, and its estrogenic conversion by brain aromatase are responsible for brain masculinization in males that "organizes" neural circuitries and neurons persisting for the entire life to react to circulating or locally produced steroids in a male-specific manner. This process is associated to a sexually-specific expression of sex hormone receptors in numerous brain areas (3,320). The sexual differentiation involves all neural cells: in adult brains, astrocytes show a clear sexual dimorphism (321-323) in their morphology (primary process length and number distribution) (324-326), differentiation and function (327-330). Major microglia sex differences occur at the neonatal stage and in the adult brain in terms of abundance, distribution within CNS regions, response to exercise or stress, and expression of specific proteins (331,332). Male microglia has a higher density and phagocytic capacity, while female microglia is more supportive of neuronal functionality. Using transcriptomics and engrafting experiments to compare the phenotype of microglia isolated from adult male and female mice, a strict association of male microglia with inflammatory processes was observed, while female microglia was associated with inhibition of inflammatory response and promotion of repair mechanisms (333).

c. Brain expression of steroid receptors as mediators of sex hormone activities.

c.i Sex hormone receptors.

Estrogen (ER), progesterone (PR) and androgen (AR) receptors belong to the superfamily of hormone modulated-transcription factors. These receptors share structural homology in specific domains, e.g.: the central DNA-binding domain (DBD)) or the ligand binding domain (LBD), while the N-terminal domain and the very C-terminal tail greatly differ among nuclear receptors. After their synthesis, steroid receptors are bound to heat shock proteins (e.g.: Hsp90, Hsp70, etc.) that maintain the C-terminus folded in a way to expose the LBD. HSPs prevent activation and nuclear translocation of AR and PR by masking the nuclear localization signal (NLS) and the DBD

(334,335). Ligand binding induces the HSPs release and receptor conformational changes which enables post-translational modifications (PTM) (336,337), dimerization, nuclear translocation, and binding to specific HRE located in the promoter region of their target genes. This allows the recruitments of co-regulators and transcription factors to control gene transcription.

c.ii Sex hormone receptors distribution in the brain.

Brain distribution of sex steroid receptors is highly variable in function of the animal species, their developmental stage, sex, and hormonal milieu. Moreover, data have been collected both with in situ hybridization and immunocytochemistry and several differences have been found between mRNA expression and protein production. However, it must be noted that not always mRNA levels mirror protein levels and/or activity of any specific receptors because, in addition to transcriptional control, a complex regulation influences translation and posttranslational modifications. A brief overview of the major findings of the function of steroid receptor in the animal and human brain will be provided below.

The brain estrogen receptors. Estrogens modulate brain functions by binding the two intracellular steroid receptor subtypes, ERα and ERβ (338), and their several alternative splicing identified in human brain (339). Membrane receptors, like the G-protein-coupled receptor, GPR30, which binds 17β-estradiol with very high affinity inducing a fast Ca^{++} mobilization is also present in the brain (340,341). Estrogens modulate several neural functions, like mood, anxiety, fear and higher order cognitive functions by enhancing learning and memory (342). In mammalian brain, ERs distribution is generally similar throughout species (343). In humans, ERs are mainly expressed in limbic-related areas, but the two isoforms localization may differ. Indeed, ERα mRNA is highly expressed in the hypothalamus and amygdala, while the ERβ mRNA is highly expressed in the hippocampal formation, entorhinal cortex, and thalamus (344). In mice and rats, the ERα isoform is predominant in the preoptic area, in most of the hypothalamic nuclei and in the hippocampus,

while ERβ is predominant in the olfactory bulb, cerebral cortex, septum and preoptic area, bed nucleus of the stria terminalis (BST), amygdala, paraventricular hypothalamic nucleus, thalamus, ventral tegmental area, SN, raphe, *locus coeruleus* and cerebellum. Both ERs are expressed by all neural cells (345), including microglia (346). ERs are differentially expressed in neural cells of male and female rodents: ERα is higher in females than males in the hypothalamic ventromedial nucleus, the periventricular and medial preoptic area (347,348), the periaqueductal grey neurons (349) and the BST (350). Both isoforms are higher in females rat hippocampus than of male (351). In humans, ERα is higher in women in the diagonal band of Broca and in the medial mammillary nucleus, in the suprachiasmatic nucleus and ventromedial nucleus. Conversely, ERα levels are higher in men in the sexually dimorphic nucleus of the medial preoptic area, paraventricular nucleus, and lateral hypothalamic area. ERα is present in neurons, astrocytes, plexus choroideus, and other non-neuronal cells with some areas characterized by dimorphic distribution (348).

In the spinal cord, ERα and ERβ are present mainly in neurons in dorsal horn and in the area X, and at lower levels in lumbar and sacral spinal cord (see (260)). In mouse, ERα and ERβ found in SMI-32 positive anterior horn spinal cord motoneurons colocalize with aromatase, and the GPR30 (260). This localization correlates with the estrogenic induced improvement of locomotor function after spinal cord injury or in in ALS (260). Estrogens neuroprotection is also recapitulated in motoneuron cell lines and in cultured facial or spinal cord motoneurons (260).

By the availability of a transgenic model designed to induce the expression of the reporter gene luciferase under the control of specific EREs inserted into a basal promoter (the ERE-Luc reporter mice) (352), it was possible to quantify in which brain region of living animals estrogens transcriptionally activated ERs. This system enabled to demonstrate the presence of the sexually dimorphic ER activity in adult brains. In females, ER transcriptional activity is particularly elevated in the arcuate, hypothalamus septum and amygdala; very little activity is observed in the

motor areas (e.g. striatum and *substantia nigra*). When female at proestrus were compared to males, significant differences in ER activity were found. In females, ER activity was significantly higher than in males in the arcuate, hypothalamus, thalamus and septum. Interestingly the brain area with the relatively highest ER activity was the amygdala. (353). Notably, brain ER activation associates both to circulating estrogens and, particularly in males, to locally produced estrogens from circulating androgens via aromatase and 5α-reductases/3β-HSDs mediated conversion, which produces 17β-estradiol (E_2) and 3β-diol, a selective ERβ ligand (354) (see below for details). The ERs differential localization and activity explain several sex-dimorphic brain functions, e.g.: the hypothalamic GnRH release (355), the hippocampal synaptic plasticity (356), the neuroprotection against neurotoxic insults (357), the pain modulation (358), the energy metabolism regulation, the sensitivity to oxidative stress (27), the neuroinflammatory response (3), and the neuroprotection exerted in several NDs, or in brain and spinal cord injuries (260).

With regards to GPR30, in rat brain *in situ* hybridization showed its presence in the cortex (endopiriform nucleus, motor and somatosensory), hippocampus (CA1-CA3, dentate and subiculum), habenula, hypothalamus (arcuate, paraventricular, suprachiasmatic, ventromedial, central, dorsomedial and ventromedial hypothalamic nuclei), and in the SNpc (359). In these regions, GPR30 is expressed in neurons (360,361), and in astrocytes (362), while in mouse spinal cord GPR30 is found mainly in anterior horn motoneurons (260).

The brain progesterone receptors. Two forms of PR, a full-length (PR-B, 110 kDa), and a N-terminally truncated form (PR-A, 86 kDa) derived by alternative transcription of the same gene (363,364); splice PR variants have also been described (365,366). PR-A/PR-B expression *ratio* varies in the different CNS regions (367) and it is influenced by hormonal variations, age and estrous cycle after sexual maturity (364,368). In rodent brain, no PRs sex-dimorphism is observed. The PR is highly expressed in the BST, in the centromedial amygdala, in the preoptic area, in the

ventromedial and dorsomedial nucleus of the hypothalamus, and the arcuate nucleus of female rat (369), as well as in the norepinephrine neurons of the nucleus tractus solitarius of the brainstem (369). In ovariectomized (OVX) female, 17β-estradiol increases the expression of PR-B in the preoptic area, of PR-A in hippocampus and olfactory bulb, and of both PRs in the hypothalamus; no changes are present in the cortex and cerebellum (369,370). In male rats after gonadectomy PR-A mRNA highly accumulates in the cerebellum only (369,370). The lack of estrogen regulation in male rats is unexplained. However, since PR expression is regulated by estrogens, the brain area-selective regulation of PR expression could be due to different ERs and co-regulators expression in the various brain nuclei. The sexually dimorphic regulation of PR isoforms in the hypothalamus and preoptic area is functionally relevant for the control sexual behavior (371,372), anxiety (373), as well as for the production of somatostatin (374) and oxytocin receptors (375). At cellular levels, PR mRNA is present in neurons (376), in new-born rat primary cultures of CNS-derived oligodendrocytes and astrocytes (377,378), and in PNS-derived Schwann cells. No PRs expression has been found in microglia (378,379). Notably, progesterone binding activities as also been associated with the cell membrane (380-383). In female brain PRs mainly control to reproductive behavior (384-386), and is involved in the regulation of myelination and its repair after traumatic injury, neurogenesis and regeneration, inflammation, cognitive functions and mood (387-389).

The brain androgen receptor. The AR gene is located on the X chromosome (390); thus a single AR allele exists in males; also females utilize only one AR allele because of the X chromosome inactivation occurring randomly in each cell. The AR N-terminal region is encoded by exon 1 and contains the polyQ, and proline or glycine stretches (231); the DNA-binding domain and the C-terminal domain are homologous to those of ERs and PRs (391). The polyQ length is variable among individuals also in relation to ethnic backgrounds (281) and if becomes longer than 37Q

causes SBMA. AR is activated by testosterone (T), and its more potent derivative dihydrotestosterone (DHT) (392-396); both steroids positively regulate AR expression (397).

In the CNS, AR is expressed both in the CNS and PNS. In humans, AR is concentrated in specific hypothalamic nuclei, in the horizontal diagonal band of Broca, in neurons of the latero-mammillary nucleus, the medial mammillary nucleus, the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the infundibular nucleus. AR content is relatively lower in the BST, medial preoptic area, dorsal and ventral zones of the periventricular nucleus, supraoptic nucleus, and nucleus basalis of Meynert. AR is generally expressed at higher levels in males than in females, particularly in several hypothalamic regions, where androgens organize the male hypothalamic-pituitary-gonadal (HPG) axis (see (398) for details) (399), to regulate sexual dimorphic functions (400), and might be responsible for the control of sex-dependent behaviors, or for the appearance of selected psychiatric and neurological diseases, whose prevalence is sex-related (398). No major sex differences in AR expression are present in the hippocampus, and in the temporal cortex (401), which are rich of AR in both sexes. In the spinal cord, AR localizes in somatic motoneurons of the anterior horn and of the bulbar regions which directly connect to striatal skeletal muscle (284). AR is also present in the DRG sensory neurons which connect peripheral sensitive regions to posterior areas of spinal cord. Upper motoneurons in the brain motor cortex do not express AR (334). In rodents, AR localizes in somatic motoneurons of the trigeminal, facial, ambiguous and hypoglossal nuclei (391,402,403), which are androgen-target cells (10,283,284,301,334,404-407). Here, AR regulates the maturation of male motor functions, inducing neuromuscular junctions, regeneration after resection, adult dendrites and axons growth and plasticity (334,391,403,408,409).

In conclusion, the dimorphic expression of brain sex steroid receptors may explain several sex-specific behaviors. At present time, we are unable to discriminate the extent to which gonadal and brain-derived steroids play a role in the sex specific activities controlled by the brain.

d. Neurosteroids and neuroactive steroids.

In the brain, sex steroid receptors are mainly activated by the circulating sex steroids produced by the gonads, and to a less extent, by the adrenal gland, which freely enter the blood-brain barrier (BBB). In adult males, the circulating androgen levels are relatively constant with limited circadian, seasonal and annual fluctuations. In humans an androgenic peak occurs around birth and lasts for the first month of life; then androgen levels become very low until puberty when raise to high levels that gradually decline with age. However, in aged males, androgen levels are still significantly higher than in aged females. In males, circulating estrogens and progestins are low, however both steroids could be synthetized in cells expressing aromatase and even secreted into the blood in endocrine dysfunctions (e.g.: estrogens in some feminized individuals). In females, circulating levels of estrogens and progestins are very low prior puberty. After puberty, estrogens and progesterone synthesis is strictly controlled during the menstrual cycle with a great increase in case of pregnancy and lactation. In post-menopause, female sex steroid levels are very low. Androgen levels are generally very low, except for specific pathological conditions (polycystic ovary, hirsutism, etc.). In both sexes, all circulating sex steroids may reach the brain to participate in the regulation of their cognate receptors. Alternatively, sex steroids can be locally converted to more active metabolites as well as to compounds showing different biological activities. In addition, both CNS and PNS *de novo* synthetize steroid hormones locally from cholesterol (410); these steroids are indistinguishable from circulating steroids (Figure 1). CNS/PNS synthesized steroids are named "neurosteroids" to be distinguished from the gonadal sex steroids that enter the brain *via* the blood stream and called "neuroactive steroids". Several steroidogenic enzymes are present in all cell types of the CNS/PNS and neurosteroid synthesis is the result of a coordinated interaction between neurons, macroglia and microglia (see Figure 1 for a detailed view of the

different processes). CNS/PNS steroid synthesis might be an adaptive mechanism following brain damage (411) and neurodegenerative conditions which usually inversely correlate with CNS/PNS steroid levels (412). The synthesis and regulation of neurosteroids and neuroactive steroids is summarized in Figure 1. An overview of the complex distribution and activity of the various enzymes involved in sex steroids synthesis and metabolism in the brain is reported in provided below.

d.i Local activation and de novo synthesis of steroids in the brain.

Cholesterol cannot cross the BBB, thus it has to be locally produced (413). The enzyme essential for cholesterol synthesis, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA red) which converts HMG-CoA into mevalonate, is expressed by all neural cells, with highest levels in astrocytes (Figure 1). Neurosteroids biosynthesis starts with the internalization of cholesterol into mitochondria mediated by the transduceosome (414), a protein complex composed of the translocase TSPO (or "peripheral benzodiazepine receptor") and the steroid acute regulatory protein (STAR). TSPO is present both in neurons and activated glial cells (415,416) and also microglia which is activated by TSPO ligands (417-419). TSPO up-regulation in glia is a major hallmark of brain injury, inflammation and neurodegeneration (420). Because of this, TSPO is a marker widely exploited by PET imaging to investigate the dynamics of microglia activation in NDs (421).

Cholesterol conversion to pregnenolone is a rate-limiting step. Pregnenolone is substrate either of the mitochondrial enzyme P450scc (and its human counterpart Cyp11a1), that generates progesterone, or Cyp17 that produces dehydroepiandrosterone (DHEA) (Figure 1). In rodents P450scc localizes mainly in the white matter and is more expressed in females than in males (422-425). P450scc is also elevated in the cerebral cortex, hippocampus, midbrain, and amygdala (426-

428). The hortologue, CYP11A1, appears to have a similar distribution in the human brain (422,429).

3β-Hydroxysteroid Dehydrogenase (3β-HDS) is responsible for progesterone synthesis. Several 3β-HSD isoforms were identified in rodents (four in rats and six in mice) (430), while in humans only two are present: type I (mainly expressed in the placenta) (431) and type II (expressed in the gonads, adrenal gland and brain) (432,433). In humans 3β-HSD mRNA is detectable in the striatum, cortex, amygdala, midbrain, thalamus, hypothalamus, cerebellum (429). Astrocytes mainly produce the 3β-HSD derivatives. In the rodent brain, the isoform 3β-HSD-1 is the most expressed and with high concentrations in olfactory bulb, striatum, cortex, thalamus, hypothalamus, habenula, septum, hippocampus and cerebellum (434,435). 3β-HSD mRNA, is present in neurons, astrocytes and oligodendrocytes in primary culture (436,437) and Schwann cells (438).

Androgen and estrogen synthesis require P450c17 (CYP17) (439-441), generally via DHEA (442). P450c17 is highly expressed during brain development (426,443); in adult P450c17 is present in hippocampus astrocytes and neurons (437,444), but not in microglia (418).

The 17β-Hydroxysteroid dehydrogenase (17β-HSD) converts androstenedione and estrone to testosterone and 17β-estradiol, respectively (Figure 1). Different 17β-HSD isoforms exist (445), that differ in tissue and subcellular localization (446-448). Type I 17β-HSD, which mainly catalyzes the activation of estrone to estradiol (439), is present in glia of mammals and amphibians (448-450); its expression is induced by inflammatory stimuli (such as lipopolysaccharide, LPS) in primary murine microglia cultures (418). In rats, type I 17β-HSD is widely distributed in the CNS, being localized particularly in the hippocampus, cerebral cortex, thalamus, and hypothalamus. In the humans, Type I 17β-HSD is detectable in the hippocampus (451) and temporal lobe (452). Type III and Type V are the androgenic forms of 17β-HSD (439), and their expression have been

detected in the hippocampus and cerebellum of human brains. Interestingly, a Type X 17β-HSD is present in human cerebral cortex (453) and is potentially involved in AD pathogenesis as it binds to Aβ (454) and is up-regulated in AD (455).

The enzymes mentioned above are mainly responsible for the local neurosteroids production. A second group of enzymes has a catabolic role being capable to convert circulating sex steroids into products with higher (activatory enzymes) or different biological activities. An example is the enzyme 5α-Reductase (5α-R) (Figure 1), which converts testosterone to its more potent derivative 5α-dihydrotestosterone (5α-DHT) and progesterone to 5α-dihydroprogesterone (5α-DHP). DHT is the precursor of the 3β-diol which has no androgenic activity, but acquires a potent estrogenic activity (mainly mediated by ERβ binding). Instead, the 5α-DHP serves to produce allopregnenolone or 3α-hydroxy-5α-pregnan-20-one or 3α,5α-tetrahydroprogesterone (3α,5α-THP). 5α-DHP, but not allopregnenolone, binds PR; both 5α-DHP and allopregnanolone bind the GABAa receptor (456). Two isoforms of 5α-R (named type 1 and type 2) have been identified (see (457-459), with different biochemical properties, but superimposable activity. 5α-Rs reduce several androgens, progestagens and corticosteroids 3-keto-Δ4-steroids. 5α-R type 1 is expressed both in neuronal and glial cells (459), while 5α-R type 2 is confined in neuronal cells (459,460), in specific brain regions, mainly in the neuroendocrine structures (461), including in GnRH secreting neurons (293) and, at lower levels in the hippocampus. 5α-R type 2 mRNA is present in anterior horn spinal cord motoneurons (407), which also express considerably levels of AR. Instead, very low levels of type 2 mRNA are present in the amygdala, olfactory bulb and in the cerebral cortex (461). The temporal expression of the two 5α-Rs isoforms considerably differs indicating changes of their functional role from ontogeny to adulthood; 5α-R type 2 mRNA is undetectable during rat embryonic brain development at day 14, it appears at day 18 and increases

to reach a maximal level at postnatal day 2 (459). This expression pattern parallels the rate of testosterone production from fetal and neonatal testis (461). Similar changes occur for aromatase mRNA expression, but not for 5α-R type 1. Studies in cultured neurons confirmed that both 5α-R type 2 and aromatase, but not 5α-R type 1, are regulated by androgens.

Aromatase converts androgens to estrogens (Figure 1). A single gene (CYP19) encodes aromatase, under the control of multiple cell/tissue specific promoters which codes different RNAs spliced at the first exon, or 5'-untranslated region (5'-UTR) to a common splice junction immediately upstream of the AUG translation initiation site giving an identical aromatase protein. Thus, the promoter confers tissue-specific regulation to the aromatase gene (462) and those specific for the brain are 33 kb upstream of the common splicing junction.

In women, brain aromatase activity is modulated by circulating estrogens, and fluctuates with the menstrual cycle (463). In rat male brain, testosterone upregulates aromatase expression. Recent studies by PET-based imaging in the baboon brain showed that aromatase is not evenly distributed in mammalian brain as the highest uptake of the aromatase binding [11C]vorozole in the amygdala, lower level in the preoptic area and hypothalamus, basal ganglia, and cortical areas (463). Aromatase is expressed in human cerebrovascular endothelium, and has a sex dimorphic role in the protection against stroke (464,465), since aromatase expression correlate with neuroprotection. Indeed, mild neurodegenerative stimuli induce severe neurodegeneration when the enzyme is genetically or pharmacologically inhibited (466), possibly because aromatase serves to locally produce neuroprotective estrogens (463,466). In adult mice, aromatase has also been found in motoneurons of lumbar ventral horn of the spinal cord, and it is in association to nuclear ERα and ERβ expression, and with GPR30 cytoplasmic and neurite localization (260). Since ALS is more frequent and appears earlier in men than women (see below) and gender difference exists also in tg ALS mice (9) with OVX mice showing an exacerbation while estrogen treatment delays

symptoms progression in the same mice (467-469). This evidence suggests that estrogen may play a protective role in motoneuron NDs and particularly in ALS.

e. Sex steroid effects on neural cells.

Estrogens are extremely pleiotropic in their activities. ERs are present in all neural cells and estrogens regulate several molecular pathways, some of which involved in ND. In neurons, estrogens prevent neuronal death by increasing the endogenous synthesis of anti-apoptotic molecules (470) and the neuronal expression of growth factor receptors (471). Estrogens also improve bioenergetic activity of neurons enhancing the ATP levels, the mitochondrial membrane potential and the basal mitochondrial respiration (472), maintain the number of multisynaptic boutons (473), and enhance synapse formation (474). In astrocytes, estradiol has neurotrophic activity as facilitates the secretion of growth factors (475,476), represses the expression of glial fibrillary acidic protein (GFAP) and reduces astrogliosis (476,477), stimulates the release of the glial apolipoprotein E (apoE) necessary for neurite growth (478), and increases glutamate uptake (479). Estrogens act on microglia by attenuating the response to inflammatory stimuli, maintaining mitochondria viable, promoting the turnover of damaged proteins through the proteasome, and regulating microglial proliferation (346,480-483). These effects were extensively reviewed elsewhere (3).

f. Predicting the activity of male sex hormones in the nervous systems.

Circulating sex steroids have a major influence on brain functions, but their effects can be largely changed by selective enzymatic modification in their structure. As an example, testosterone may lead to a variety of androgen metabolites each endowed of biological specificity (Figure 2).

Aromatase changes radically the biological properties of the substrate by converting testosterone into estradiol (484); 5α-R transforms testosterone into the more potent AR ligand, DHT (293,407), but 3α-HSD and 3β-HSD convert DHT into metabolites (3α-diol and 3β-diol) which signal through membrane receptors (*e.g.* the 3α-diol with GABAa) (485) or act as estrogens activating ERs (*e.g.* 3β-diol binds ER-β specifically) (354). Therefore, the final biological function of circulating testosterone in target cells is unpredictable. This complex action becomes even more complicated when receptor dimerization is considered, since homo- and hetero-dimers may be formed providing further levels of diversification to testosterone actions. To exemplify, each monomer may associate with testosterone or DHT giving rise to three molecular species: [AR:T/AR:T]; [AR:DHT/AR:DHT]; or [AR:T/AR:DHT] and, in the case of testosterone being metabolized by CYP19 or the complex 5α-R/3β-HSDs, the active species generated may be: [ERα:E_2/ERα:E_2]; [ERα:E_2/ERβ:E_2]; [ERα:E_2/ERβ:3β-diol], etc. Considering that each dimer might be endowed of promoter selectivity, the response to the same hormone may be significantly different in each given cell. An example of the complexity of the response to the various steroids and their locally formed metabolites is reported in Figure 2.

4. The influence of Sex hormones in Neurodegenerative diseases

a. Sex hormones and AD.

The molecular bases of female prevalence of AD remain to be elucidated. The higher AD susceptibility of women may result from a combination of organizational and activational effects of sex steroids (486). In AD models, neonatal masculinization of female 3xTg-AD mice yielded to selective, region-specific decrease in Aβ accumulation, while neonatal blockage of testosterone

in male 3xTg-AD mice led to an increase in Aβ (19). Thus, the brain masculinization induced perinatally by estradiol, may certainly be a factor for AD predisposition. Conversely, epidemiological studies in aging women undergoing or not HRT showed that estradiol may be neuroprotective (487), particularly when abrupt hormonal decrease occurs as in post-menopause.

a.i Estrogen and AD.

Genetic studies suggested that an impaired ER signaling may predispose to AD, but by itself is insufficient to determine an increased risk to develop AD. Indeed, a clear association between impaired ERs signaling and neurodegenerative processes has not been found yet. Despite reports showed an association between aromatase polymorphisms and AD (488), and a significantly lower expression of ER mRNA splice variants in AD patients - particularly in women - than in healthy individuals (489,490), the human genetic studies failed in providing evidence of a clear association between AD and ER polymorphisms (491-495). Certainly, the *Apoε4* genotype, the most established genetic AD risk factor, has a stronger association in women than in men in case of homozygosity (110,111). This is possibly due to an interaction between the APOε4 variant and single nucleotide polymorphisms (SNP) of the *ESR1* (rs9340799, rs2234693; rs2228480) and *ESR2* (rs4986938) genes (496,497). The pleiotropic effects of estrogens could also intervene at different steps of AD pathogenesis, the first of which is certainly Aβ accumulation (see Figure 3).

a.ii Estrogen decreases Aβ accumulation.

Estrogens decrease the generation and secretion of β-amyloid peptides (498). In rodents, 17β-estradiol reduced Aβ accumulation in AD mouse models (482,499-502), in aromatase knockout (KO) mice (502), and after OVX (503). Several mechanisms may be involved. In neurons, the estrogen-ER complex was shown: a) to inhibit the gene expression of the β-secretase1, the enzyme required for the production of the neurotoxic Aβ peptide (504,505); b) to regulate APP processing

on cell membranes by affecting lipid composition and the lipid rafts; c) by modulating the γ-secretase and Notch signaling (506), d) by increasing the synthesis of seladin-1 (selective Alzheimer disease indicator-1, or 3-β-hydroxysteroid-Δ-24 reductase) that is involved in the generation of detergent-resistant membrane domains that influence APP cleavage by β- and γ-secretases (507,508). Finally, estrogens were shown to reduce TAU hyper-phosphorylation (509). By acting on microglia, 17β-estradiol enhances Aβ peptide removal by modulating the phagocytic activity of these cells. The proposed mechanism includes the modulation of the complement pathway, which is part of the innate immune system and includes proteins associated with the rapid recognition and internalization of pathogens, apoptotic cells, cellular debris, and misfolded proteins (510). Activated ERs were shown to bind directly to the estrogen- responsive sequences within the promoter of a key member of the complement pathway, the complement protein C3, (511) which promotes Aβ phagocytosis and clearance (510,512). In addition, estrogens may facilitate the turnover of oxidized or otherwise damaged proteins through upregulation of microglia proteasome activity via p42/44 MAPK pathway (513,514) and by preventing the induction of nitric oxide (515). It is conceivable that all these activities contribute to facilitate estrogen-dependent transit from the inflammatory to the anti-inflammatory state of microglia, perhaps contributing to slow down microglia aging. Following menopause, in the presence of Aβ deposits, the unrestrained activation of microglia may accelerate the aging process, thus resulting in the inability of these cells to acquire the anti-inflammatory phenotype involved in cell repair. This impairment in microglial function may have detrimental consequences both for microglia and surrounding cells (483). In AD models, such as the APP23 mice which overexpress the human amyloid precursor protein with the Swedish mutation, ovariectomy was shown to be associated to increased activation of the microglia at Aβ deposits; ovariectomy in these mice also facilitated the progression of microglia to their reactive state. Long-term 17β-estradiol treatment counteracted

these effects reducing microglia reactivity to that found in control animals (482), suggesting that estrogen anti-inflammatory effects on microglia protected the brain by limiting Aβ peptide overburden. In addition, microglia isolated from adult mice have distinct identities and functions determined by sex (333). In young healthy mice, male microglia expressed more inflammatory biomarkers, while female microglia showed a more neuroprotective phenotype (333). The landscape changes with aging, as aged brain females experience a higher level of microglial activation compared to age-matched males, with implications for AD development (516).

a.iii Estrogen effects on lipid metabolism.

Epidemiological studies pointed to an association between metabolic syndrome and AD in women, but not in man (517). The control of cholesterol and lipid metabolism is sexually dimorphic (319,518) and the postmenopausal reduction of circulating estrogens alters liver lipid metabolism with severe systemic consequences (319,519,520). Indeed, excessive circulating lipids causes neuroinflammation (521,522) associated cognitive impairments (523,524); thus a protective estrogenic effect could be exerted via reduced lipid biosynthesis (519). Moreover, in the hippocampus and cortex, estrogens stimulate apoE release from astrocytes (478) and affect the expression of the low-density lipoprotein receptor-related protein LRP, which triggers the formation of Aβ (525).

a.iv Progesterone and AD.

Although the positive actions of estrogens on neurodegeneration are well characterized, the role played by progesterone, also combined with estrogens, has not been fully explained yet. Progesterone exerts beneficial effects on hippocampal-dependent spatial memory (526) and on neuronal protection (526-529). These observations have not been confirmed in specific models of neuronal damage and the role of progesterone is still controversial (530-532). In general, progesterone-induced anti-oxidative (533) and anti-inflammatory actions (534) have been

described. In mice, after acute episode of global ischemia, progesterone prevented the caudate nucleus neuronal death by reducing lipid peroxidation (535). For the specific case of AD, progesterone, alone and combined with 17β-estradiol, has positive effects in murine AD models, in particular on glutamate-induced oxidative injury (536,537), glucose deprivation, and $FeSO_4$ and Aβ toxicity in primary hippocampal cultures (538). This protection mediated by progesterone could be associated both to classical genomic mechanisms (536) and to activation of membrane receptors (539) leading to the neurotrophin expression. However, it is still unclear whether progesterone or its metabolite allopregnanolone mediate these neuroprotective actions.

a.v Female hormone replacement therapy and AD.

In animal models of AD (3xTg-AD), estrogen beneficial effects on parameters like Aβ accumulation, TAU hyperphosphorylation, activity of cholinergic neurons in the hippocampus, working memory and visual attention were not modified by progesterone in acute treatments, but were decreased if the hormone was administered continuously. Most relevant, clear beneficial effects of the HRT were observed when the hormone administration mimicked the ovarian cycle (500,540) and this suggested a limited effectiveness in current HRT way of administration.

Indeed, despite these large number of data indicating that estrogens reduce AD risk, the studies on the effects of HRT in the post-menopause did not reach conclusive results, as some HRT after menopause did not provide a clear vision of the effects of the treatment. A beneficial effect of estrogen therapy with regard to the onset and prevalence of AD was reported (541-543), but these results were not reproduced in others (544-546). Studies in humans are complicated by differences among HRT, the incongruity in the time at which HRT was started (547) and the use of estrogens alone or in association with progesterone. All these elements may have a differential effect on the final AD outcome. In fact, it is known that HRT initiated few years after menopause fails to provide the beneficial effects, particularly in the nervous system. Moreover, continuous progesterone

treatment inhibits the E_2-mediated induction of neurotrophin expression and spatial memory performance (548). The inconsistency in the published results makes it impossible to produce a definitive meta-analysis able to summarize the overall effects of HRT on cognition (549). This is in contrast with pre-clinical data that are quite promising and solicit for the search of novel HRTs aimed at slowing down the progression of dementia.

a.vi Role of male sex steroids in AD.

In males, circulating testosterone decreases slowly with aging (at an estimated rate of about 2% per year) (550,551). However, with aging the Androgen Deficiency in Aging Males (ADAM) syndrome may occur and cause dysfunctions in the androgen-responsive tissues, including brain (551). In the brain of AD male rodents (552) and in AD patients (553,554) testosterone and DHT were reported to be lower than in control subjects matched for age and indeed AD is associated with a decrease in mood and libido (550,553,555,556) (see Figure 3). In orchiectomized (ORX) 3xTg-AD mice the Aβ accumulation in CSF and plasma is faster than in intact controls (557), and this correlates with an impairment of behavioral performance, while a protective effect was observed after replacement with testosterone or DHT (509). Besides the regulation of Aβ deposition, androgens naturally exert neuroprotective actions in brain that include their support to neuronal growth (558,559) and synaptic function (560-562), axonal regeneration (563,564), and protection against neuronal death (565-567). In male brain, androgens are more potent than estrogens on regulating spine density in specific regions (562,568), action that involves the AR activation (562,569). The androgen-mediated protective effects on neurons can be exerted also through the attenuation of the damages induced by serum deprivation (565,566), Aβ toxicity (570,571), and oxidative stress (567), as demonstrated by *in vitro* experiments. While *in vitro* studies were partially *in vivo* paralleled (572), a recent systematic meta-analysis of the available

randomized controlled trials did not support a beneficial effect of testosterone treatment on cognitive functioning in men (573). The inconsistency of the trials may be due to still limited knowledges of the mechanisms by which androgens regulate AD-related processes, including Aβ production, degradation and clearance. Anyway, being low testosterone level a risk factor for AD, androgen replacement is still an attractive therapeutic strategy worthy of more extensive studies.

b. Sex hormones and PD.

The NSDA neuronal circuit is highly responsive to sex steroid hormones. These signals induce specific, sometime opposite, responses in female and male brains, possibly because of the distinctive nature of the NSDA system and regulatory network in the two sexes (574) (Figure 4).

b.i Estrogens, NSDA and PD.

Under physiological conditions, the NSDA system is widely controlled by estrogens which trigger the commitment of neuroblasts towards the dopaminergic phenotype and stimulate growth and branching of neurites, metabolism and activity of DA through ERα-mediated mechanisms (575,576). Although in adulthood estrogens retain the ability to potentiate DA neurotransmission and provide neurotrophic support, differentiated TH-positive neurons do not express ERs and are thus unable to directly respond to estrogens (577,578). Yet, ovarian ablation selectively depletes DA neurons in the NSDA pathway of primates and rodents, while estradiol replacement prevents this loss when added shortly (10 days) and not long (30 days) after ovarian surgery (579,580). Estrogens action has been reconciled with the responsiveness of the microenvironment that surrounds the NSDA tract that is a complex cellular network made of interneurons, glia, input circuitries from other brain areas and microglia. Only ERα is expressed in the SN, while ERα, ERβ and GPR30 are present in the striatum (574,581-584). This immune-neural network mediates estrogens regulatory action on NSDA plasticity and, plausibly, in PD. Indeed, higher estrogens

levels inversely correlate with the severity of PD symptoms in women, while women who experienced early natural or surgical menopause show a higher risk of PD (585-588). Thus, estrogens are considered as neuroprotective agents, delaying the onset and mitigating the symptoms of PD, although confounding factors such as timing, dosage, composition and route of administration of HRT in women may limit the appreciation of hormonal effects (589,590).

b.ii Estrogens in animal models of PD.

As expected from clinical and preclinical evidence, estrogens were shown to reduce DA depletion induced by neurotoxins in the female striatum of rats and mice (29,30). This neurotrophic effect is achieved predominantly by ERα and should be activated before the toxic insult, suggesting that estrogen action does not rescue neurotransmission in damaged neurons (357,591). Estrogen responsiveness of the NSDA neural and immune network has been reconciled with organizational effects of estrogens, since early life exposure to these hormones reduces their neuroprotective effects in adult female mice following 6-OHDA injection (592). Some studies consistently showed that estrogens are ineffective in preventing DA neuronal death in the SN (27,28), although other reports recently pointed to neuroprotective effects using the 6-OHDA model of PD (593-595) and in the MPTP-induced gut neurons degeneration (596). These contrasting results could be ascribed to the specific neurotoxic mechanisms, the duration of hormone deprivation and the timing, posology and drug identity used in hormone replacement regimens. It is thus still difficult to discern whether estrogens prevent neurodegeneration or rather potentiate neurotransmission of resilient neurons. Future studies will help to understand the role of estrogens in the sexual dimorphism of neurodegeneration in PD and in addressing the role of pathogenic mechanisms, such as protein aggregation, neuroinflammation or *extra*-nigral pathology (26).

Contrary to its action in females, estrogens administration worsens striatal DA depletion in the male NSDA system, although the local production of estrogens in male brain may have protective

functions (27,30,597). Beyond effects related with the estrogen dose, the male regulatory network that controls the NSDA system has sex-specific properties and estrogen responsiveness, as further suggested by the observation that ERα and ERβ provide opposite effects in males in response to estradiol (591,598). Estrogen responses in males are different from those observed in females and are thus expected to provide specific effects in PD-like conditions.

b.iv Progesterone and PD.

Exiguous are the studies on progesterone and PD. There is no evidence for the presence of PR in striatum and SN (367,369). Clinical data related with the risk of PD and progesterone levels are not available. Only few reports have analyzed the effect of progesterone using animal models of PD, showing that low doses of progesterone are able to exert neuroprotective activities in male mice treated with MA or MPTP (599,600), while a higher dose is necessary to obtain neuroprotective effects in OVX female mice treated with MA (577). In PR-KO mice, progesterone treatment increases DA signaling in the striatum but decreases the number of TH-positive cells in the SNpc when compared to wt mice (601,602).

b.v Androgens, NSDA and PD.

AR is expressed in the SN and within TH-positive neurons, making the NSDA a direct target of androgens. Castration in adult mice causes an increase in the number of TH-positive cells in the SNpc, whereas testosterone reduced this cell population (603,604). Interestingly, an opposite effect has been reported in young mice, in which castration strongly reduces the density of TH-positive neurons in the SNpc and impairs locomotor activity, effects reverted by DHT administration (605). This suggests that organizational effects in mature, adult individuals may influence PD onset and symptomatology. However, the role of androgens in the NSDA system and PD patients is unclear, yet. Testosterone deficiency is associated with PD patients, and testosterone therapy may have beneficial effects on motor and non-motor symptoms in selected PD patients (606-609).

Testosterone AR activation potentiates NSDA toxicity in adult PD animals through the increase in intracellular DA and the production of oxidized forms and radical species; these effects are not mediated by estrogens, since dutasteride, a 5α-R inhibitor that allow testosterone conversion to estrogens, protects dopaminergic neurons in MPTP-lesioned male mice (610,611).

b.vi Sex steroid hormones and pathogenic mechanisms of PD.

Estrogens increase antioxidant responses and potentiating respiratory chain activity in the female brain, by inducing the expression of mitochondrial proteins and enzymes.; also androgens may play a role (182,183,612). Sex hormone-dependent effects on protein clearance and autophagy in the brain have been scarcely investigated. Following brain iron overload in females, estrogens were shown to reduce autophagy and severity of neuronal injury, while dopaminergic neurons from male mice are more prone to autophagy activation and more vulnerable to toxicity (182).

Although the neuroprotective effects of estrogens in brain are well established and were shown to involve inflammatory cells, little information is available on the effects of sex hormones on inflammation in PD models (483,613). Morale and colleagues showed that the amounts of toxic nitrites produced following MPTP injury in mice are inversely proportional to circulating estrogens (614). A recent study suggests that estrogens modify the reactivity of microglia to 6-OHDA in females, inducing a protective anti-inflammatory phenotype (595). Interestingly, enteric macrophages express ERα, while enteric nerve cells express both ERα and ERβ (615) and immunomodulatory and neuroprotective effects were observed for estrogens in the myenteric plexus of MPTP-treated mice (596). Although the ENS system is receiving more attention in the understanding of PD pathogenesis, the role of estrogens in this district still needs to be defined.

c. Sex hormones and ALS

c.i Sex-related molecular and biochemical alterations occurring in ALS: the role of sex steroids.

Up to date, very few data are available on the involvement of hormonal sex steroids in ALS. However, it must be recalled that sex steroids may exert organizational effects both on neuronal networks, as mentioned above, also on muscle cells (see Figure 5); these effects may be responsible for gender differences. The skeletal muscle cells differentially respond to the anabolic activity of androgens, which control muscle development in male, but also to estrogens and progestogens (616-618). In female, not only the levels of female sex hormones change during the menstrual cycle, but also the expression of their receptors (ERs and PR) is known to fluctuate in skeletal muscles, suggesting a direct role of these steroids in muscle physiology (619,620).

c.ii Male sex steroids.

An increased ratio in the risk to develop ALS is observed in correlation with age of onset. This observation apparently reinforces the hypothesis that circulating sex steroid hormones in adulthood are more relevant than their possible priming action on the brain during development. So far the contribution of the perinatal effect of sex steroids on masculinization of the nervous system is still controversial. Notably, Vivekananda and colleagues (621) pointed out the influence of prenatal factors in ALS development with the analysis of the ratio between the length of index and ring fingers (2D:4D ratio), which is known to be modulated by high prenatal testosterone levels in both sexes. In ALS patients, irrespective of sex, the 2D:4D ratio is lower than in controls, suggesting that higher prenatal circulating levels of testosterone may be an independent risk factor for ALS (621,622). Androgens might affect motoneuron development and axonal regeneration after injury, in both sexes (334,409), suggesting that prenatal exposure to testosterone or its derivatives may influence motoneurons organization, thus altering their vulnerability in later stages of life. Moreover, it has been shown that motoneuron development may be affected by the exposure of its progenitor cells to androgens (334,403). Interestingly, SOD1 mutations seem to have a significant, sex-specific effect on the proliferation and differentiation rate of these cells. As an example, studies

performed in rat neural progenitor cells (rNPCs) showed that in male, but not in female rNPCs, mutant SOD1 decreased significantly their proliferative and differentiating potential (35). This is in line with studies where the exposure to prenatal testosterone was shown to directly influence motoneuron organization (334). However, glial cells together with interneurons also play a role in the androgen priming effects on the locomotor system. Herron and colleagues (36) suggested that a specific class of synaptic input originating from a small cluster of spinal interneurons, the C-boutons, may control the altered motoneuron excitability present in ALS. The C-bouton size is increased during disease progression in tg ALS mice. Notably, C-boutons only are enlarged in male ALS mice, further suggesting a sex-specific regulation of the interplay among motoneurons and their surrounding cells (36). The role played by circulating androgens in ALS is mostly associated with the expression of AR in the motoneurons, which has an organizational role in these cells (407,408), and it is also suggested by the fact that neurons that do not express AR (cranial nerves III, IV, and VI) are spared by ALS (623). As it will be discussed later, wt AR has the propensity to form aggregates (exacerbated by the expanded polyQ tract responsible for SBMA, (624)), suggesting that wt AR might contribute to the motoneuron distress, potentiating the negative effects of other proteins capable to acquire aberrant biochemical behavior in motoneurons and involved in sALS (in their wt form) and fALS (when mutated, *e.g.* TDP-43, SOD1, etc.). However, so far, all efforts to correlate the length of the polyQ tract in wt AR with the manifestation of ALS have failed (262,625). More convincing evidence of the involvement of AR in ALS is provided by epidemiological studies on the incidence of the disease carried out in veterans of the Gulf War (626), particularly in males (627), and in soccer players (628-630). These segments of the population are thought to be inclined to the use of synthetic androgenic/anabolic sex steroids (AAS) to increase muscle strength and resistance. Synthetic selective AR modulators (SARMs) have been produced and proposed as AAS, able to circumscribe AR transcriptional

competence to the modulation of muscle anabolic activities. The effects of these AAS differs significantly from the endogenous androgens and, considering the very high levels of AR in motoneurons and muscle, it is conceivable that these AAS may have neurotoxic effects by inducing an aberrant ligand-AR conformation and/or transcriptional activity (631,632) (see Figure 5).

By analyzing whether a correlation exists between serum sex steroids and respiratory parameters in sALS, an increased level of circulating testosterone was found in female ALS patients compared to control females. In addition, while in normal individuals (female controls) the circulating levels of testosterone, dehydroepiandrosterone (and its sulfate) and progesterone, significantly declined with age, testosterone levels remained elevated with increasing age in ALS patients. Notably, in ALS patients who show higher testosterone levels and lower progesterone/free testosterone ratio a faster worsening of respiratory parameters was observed (633). Recent data from the tg SOD1 ALS mouse model have shown that castration in male prolonged survival and disease duration, while, nandrolone decanoate (an AAS) supplementation worsened motoneuron loss and reduced survival (634-636). It is of note that castration decreased AR levels in the spinal cord and muscle; nandrolone decanoate had the opposite effect and induced the formation of AR SDS-insoluble inclusions in muscle and a more pronounced astrocytic activation, supporting the negative role of androgens in ALS pathogenesis (634-636). On the contrary, in the same animal model AR blockage, obtained with flutamide, resulted in an accelerated disease onset that may contribute to muscle atrophy (637).{McLeod, 2019 #670}. These studies indicate that an impaired AR signaling, both increased or diminished, is detrimental for ALS patients. Finally, gender differences are evident in the autophagic process in muscle of tg ALS mice (248,638,639).

c.iii Female sex steroids.

The protective effects of female sex steroids may be owed to a dual action of these hormones: on one side, they may prevent cell death by acting directly on the cognate receptors expressed by

motoneuron and muscle cells; on the other side, they may quench the inflammatory component of the disease. A very recent report of the Euro-MOTOR Consortium in which over than 650 female ALS patients and 1200 controls were analyzed by correlating hormonal exposures (reproductive history, breastfeeding, contraceptive use, HRT, and gynecologic surgical history) with ALS appearance have suggested that HRT correlates with a reduced risk of ALS (263).

c.iv Neuroprotective activity of progesterone and estrogens.

The potential direct cell protection is supported by a recent study in the tg SOD1 ALS mice where progesterone slowed down the progression of the disease and extended the life span of the affected male mice, without delaying the symptom onset. IHC analysis demonstrated that progesterone reduces motoneuron death by activating the autophagy degradation of mutant SOD1, thereby decreasing the load of the toxic protein in target cells (640). The positive effects of progesterone may also be due to its action in the astrocytes that surround the diseased neurons. In fact, treatment with the autophagy inhibitor 3-MA abolished the protective effects of progesterone in cultured astrocytes derived from tg SOD1 ALS mice (640). Other data supporting the involvement of progesterone have been obtained in the Wobbler mice. These animals display a spontaneous recessive point mutation in vesicular/vacuolar protein sorting 54 ($Vps54^{wr}$) that leads to a progressive degeneration of both upper and lower motoneurons with striking similarities to ALS (641). In this model, progesterone: i) decreased oxidative stress and mitochondrial membrane disruption in motoneurons inducing the expression of BDNF; ii) restored choline-acetyltransferase (ChAT) activity and immunoreactivity in the spinal cord; iii) prevented motoneuron mitochondria vacuolization. In the same mice, progesterone treatment increased survival and muscle strength (642-644). Of note, Wobbler mice are characterized by low levels of testosterone in the testis, brain and spinal cord (645), but high levels of corticosterone and progesterone in several tissues including the brain and all regions (cervical, thoracic, lumbar) of the spinal cord. The reduced

progesterone derivatives (5α-dihydroprogesterone, allopregnanolone, and 20α-dihydroprogesterone) are also particularly elevated in the brain and in the spinal cord. It is unknown whether these changes in steroids levels correlate with motoneuron degeneration (645). Notably, a PR agonist (Nestorone) was shown to counteract several of the typical abnormalities detectable in Wobbler mice, including the presence of vacuolated motoneurons, the reduced levels of ChAT and of glutamine synthase. In the spinal cord, the Wobbler mice are characterized by increased GFAP levels and astrogliosis (646), increased levels of $Iba1^{+}$ microgliosis, of the microglial marker CD11b mRNA and of the NFκB (while IκBα is reduced), TNFα and iNOS mRNAs. All these parameters are reduced by the treatment with Nestorone, except for IκBα which is increased by the treatment (646), supporting the notion that progestogens may have beneficial effects in motoneuron diseases. These protective effects of progestagens are evident also in other animal models of neurological disorders (647). With regards to PR expression, by analyzing the spinal cord collected post-mortem from ALS patients, it has been shown that lumbar PR-A and PR-B and cervical PR-B mRNA expression are higher in ALS than controls. Even if PR-A and PR-B mainly localized in axonal processes and vessels (particularly in nerve roots and large arteries) in ALS compared with controls, they occasionally were found in motoneurons (648).

Also, estrogens may exert a protective role in ALS. The treatment with 17β-estradiol performed on pre-symptomatic or symptomatic male SOD1(G93A) mice resulted in enhanced motor performance and an increased survival of lumbar spinal cord motoneurons. This correlated with a reduced expression of the inflammasome proteins NLRP3, of the levels of activated caspase 1 and of the mature IL1beta, normally increased in the tg SOD1 ALS mice (469). Indeed, estrogens may directly target neurons and exert an antiapoptotic effect (357,470); this is also true for the motoneurons which express the classical ERs in female and it was demonstrated in tg ALS mice where treatments with 17β-estradiol delayed the disease progression and OVX had the opposite

effects (467,468,649). Further studies performed in cultured motoneurons showed that 17β-estradiol, *via* the classical ERs, increased Akt phosphorylation and, in turn, the Akt anti-apoptotic signaling pathway GSK-3β and Bcl-2 (650), effects blocked by the anti-estrogen ICI 182,780. Aromatase expression was found to be altered in the spinal cords of tg SOD1 ALS mice. Prior to ALS onset aromatase is mainly expressed in motoneurons of the anterior horn spinal cord, but when ALS is manifested, aromatase expression is mainly present in astrocytes, and the overall levels of aromatase were found to be reduced during disease progression (651).

c.v Estrogens and neuroinflammation in ALS.

Growing evidence suggests that estrogens may counteract neuroinflammation (see above, and (652)) (Figure 5). TNFα and IFNγ were indicated as essential mediators in the neuroinflammatory processes occurring in the ALS tissues (653) and both are increased in the spinal cord of tg SOD1 ALS mouse models (654) and in the blood of ALS patients (655). It has been shown that in male tg SOD1 ALS mice the treatment with 17β-Estradiol increases the survival of motoneurons and this is correlated with the downregulation of several component of the inflammatory response (e.g.: NLRP3, IL1beta, etc.), abnormally elevated in the spinal cord of these mice (469).

In cultured motoneuronal cells derived from rat embryos explants, 17β-estradiol reduced the pro-inflammatory activity of TNFα and IFNγ, an action fully reverted by the ER antagonist ICI 182,780 (650). Similar effects were also described with selective ER ligands (*e.g.*: PPT and DPN), which prevented TNFα-induced apoptosis of the cultured motoneurons (656). Notably, estrogens induced overexpression of ERα, ERβ, or both, activating a neuroprotective feedback circuit involved in the upregulation of anti-apoptotic proteins (p-AKT, p-CREB, Bcl-2, and p-Src) (656).

c.vi Metabolic disorders and ALS.

Several recent studies underlined the major involvement of estrogens, in the regulation of energy metabolism in specific cells and in most organs relevant for the metabolic control, including the

brain (319), an aspect potentially relevant also for ALS. Indeed, studies in humans and in the tg SOD1 ALS model showed a direct correlation between spinal cord and motor cortex levels of both mRNA and protein of PGC-1α [a Peroxisome Proliferator-activated Receptor γ (PPARγ) coactivator 1 α, the master regulator of cell metabolic demands] and reduced symptoms and increased survival (657); the opposite was true when the PGC-1α levels were low (658). Interestingly, this effect of PGC-1α was found in male, but not in female animals (659). In line with this observation, it was shown that the anti-type II diabetes drug metformin, which induces PGC-1α production (660), exerts negative effects on onset of neurological symptoms and on disease progression in tg SOD1 ALS female mice, but not in male (661). The extent to which sex steroid hormones are involved in these sex-dimorphic mechanisms is unknown. However, it was shown (662) that, at least in the cerebral vascular endothelium, OVX increased the expression of PGC-1α (663), thus it is likely that female mice have low PGC-1α levels in these districts.

Another interesting association was found in ALS patients with regards to cholesterol and lipid metabolism: total cholesterol, LDL, triglycerides, protein levels, and LDL/HDL ratios were significantly higher in men with ALS than in women or in the corresponding healthy controls. No alterations were found in glucose levels (664). More interesting is a study in ALS patients taking statins, drugs widely used to lower serum cholesterol. Women with ALS taking statins had a faster functional decline when compared to untreated women and men. Thus, a reduction of cholesterol levels, mediated by statins, had negative effects on ALS progression among females, but not males (665). Still unknown are the cell populations affected by the changes in cholesterol levels. It is possible that the effect of estrogens might be at the level of glia, microglia or motoneurons.

A recent study on PPARγ, which regulates genes encoding mitochondrial fatty acid β-oxidation (FAO) (666), demonstrated that the PPARγ transcriptional activity is increased in the spinal cord of symptomatic tg SOD1 ALS mice. This activity is correlated with the enhanced expression of

the PPARγ target genes (e.g.: lipoprotein lipase, glutathione S-transferase α-2) involved in scavenging lipid peroxidation by-products. In parallel, the concentration of PPARγ increases in the motoneuron indicating a role of the receptor located in motoneuron in neutralizing deleterious lipoperoxidation derivatives within these cells (667). A potential involvement of sex steroid hormones in PGC-1α and PPARγ activities may occur directly in the motoneuron through a crosstalk between ERα and PPARs known to take place in other model systems (668). As lipid metabolism is most relevant also for the state of activation of microglia cells, a contribution to the final effect of estrogens cannot be ruled out. Alternatively, the effects of estrogens may be more relevant at systemic level where estrogens regulate lipid metabolism and transport at hepatic level (319,519). Thus, estrogens may regulate neural cell metabolism by affecting the nature of the transport of lipoproteins facilitating or impairing the release of lipids to cells of the nervous system. Lipid peroxidation has also been implicated in ALS because of the high abundance of peroxidation-prone polyunsaturated fatty acids in the CNS accompanied by low antioxidant content. Diet modification designed to modify composition in favor of unsaturated fatty acid was shown to modify progression and survival of tg SOD1 ALS mice in a gender dependent manner, with high unsaturated diet correlating with an accelerating effect in female. This was related to increased levels of protein carbonyl and glycoxidative modifications and of cytoplasmic 8-oxo-dG, which correlates with oxidation of mitochondrial DNA, likely linked to an increased production of mitochondrial free radical species induced by the highly unsaturated diet (669). The reasons why these effects are more pronounced in females are still unknown. However, an evaluation of the oxygen consumption data (routine respiration is 41.7 % higher in females than in males at end stage of disease) in lumbar spinal cord slices of tg SOD1 ALS mice demonstrated the presence of early mitochondrial impairment in males due to complex I (but not in complexes II and III) dysfunction. Thus, mitochondrial function seems to be better preserved in female than

in male tg SOD1 ALS mice, in line with clinical parameters. In these animals, also the fatty acid profile evaluated using docosahexaenoic acid was significantly increased in female compared to male mice. In cultured neuronal cells (N2A9 cells) overexpressing mutant SOD1, a reduced activity of complex I function was identified, a function restored by 17β-estradiol. Thus, estradiol may delay mitochondrial dysfunction in female mice in comparison with males (670).

It is of interest that the ERα was found to regulate some mitochondrial matrix proteases and heat shock proteins involved in the classical mitochondrial unfolded protein response (UPRmt), a process which maintains the normal mitochondrial proteostasis (671). In particular, ERα upregulates the proteasome and the OMI, a mitochondrial intermembrane space (IMS) protease (671). Importantly, sex differences are detectable in the IMS-UPRmt of the spinal cords of tg SOD1 ALS mice; in fact, OMI and proteasome activity was found increased in female tg SOD1 ALS mice, while in male these parameters remained unchanged compared to age and sex matched control mice (671). This increased IMS-UPRmt response in spinal cord was not present in tg SOD1 ALS mice crossed with the male or female ERαKO, suggesting that ERα is required to mediate the activation of the OMI and proteasome activity of the IMS-UPRmt of the spinal cord (671).

c.vii Sex hormones, autophagy and proteasomal regulation.

Alternative biochemical pathways sensitive to sex-related functional alterations are those involving the intracellular degradative systems. In a recent study carried out in the mutSOD1 model of ALS, it has been demonstrated that the genes encoding proteins involved in autophagic or proteasomal regulation are differentially expressed in skeletal muscle and in spinal cord; in fact, no mutSOD1 aggregates were present in the muscle despite that the functionality of this tissue is affected by SOD1 mutation. Furthermore, autophagy or proteasomal regulatory proteins are controlled in different manners in male and female mice. Our studies showed that, at the pre-symptomatic stages, no sex related changes of heat shock protein B8 (HSPB8), BCL2-associated

athanogene (BAG) 3 and BAG1 expression, were observed in spinal cord and muscle of both tg SOD1 ALS and control mice. At symptomatic stage, HSPB8 expression was robustly increased in skeletal muscle of both female and male tg SOD1 ALS mice, and in spinal cord of male, but not female tg SOD1 ALS mice (638). HSPB8 can be induced by estrogens, and enhances the autophagic degradation of misfolded proteins (672-674), including those formed during prolonged physical exercise in muscle. Since estrogens may modulate autophagy (675) and HSPB8, this may help to explain the potential sex difference of cells to catabolize misfolded proteins (638,639,676). Indeed, HSPB8 overexpression enhances the clearance of several misfolded proteins involved in ALS (including mutant SOD1, C9ORF72 related DPRs and the disease associated TDP-43 fragments), both in flies and motoneuron cellular ALS models (673,674,676-678). HSPB8 acts in conjunction with BAG3, HSP70 and CHIP to serve as a potent facilitator of the autophagy clearance of aggregate-prone proteins, thus protecting from their neurotoxicity associated to their reduced clearance. In addition, drugs like colchicine and doxorubicin, capable to induce the *de novo* transcription of HSPB8 have been found active to reduce the accumulation of the ALS related protein TDP-43 and their disease associated fragments in a HSPB8 and autophagy dependent manner (677). In conclusion, since HSPB8 expression is strongly induced by estrogens (679), estrogens in female may exert their beneficial effects also via a stimulation of the overall autophagy processes.

d. The case of SBMA

d.i The role of testosterone.

The peculiarity of SBMA is that only male patients are affected; even women heterozygous for the CAG expansion in the AR gene are asymptomatic (680); the hypothesis of the involvement of the random inactivation X-chromosome which would have spared at least 50% of motoneurons

from AR toxicity initially put forward was disproven by the identification of two women homozygous for SBMA. Both women did not show clinical symptom of ND (46). In addition, female carriers of the mutant AR allele have a skewed inactivation by methylation of the wild-type X-chromosome, a condition that favors the expression of mutant ARpolyQ in most cells. However, no clinical symptoms or electrophysiological signs are present in carrier females (45). Thus, the male incidence of SBMA is due to circulating testosterone. In line with this, also in tg SBMA mouse models, where the mutant AR is randomly integrated into the genome (no link with X-inactivation), only males develop motor alteration, these evidences support the involvement of testosterone in disease (42,43). It is now clear that testosterone confers to the ARpolyQ the capability to form aggregates that, at least under some circumstances, may be related to neurotoxic events (Figure 6) (334). Indeed, these aggregates may form initially in an attempt to protect neurons from mutant misfolded ARpolyQ that may alter cell homeostasis (including transcriptional dysregulation), but later may directly exert cytotoxicity (334). In male SBMA mice, castration prevents ARpolyQ accumulation and ameliorates the disease phenotype, while testosterone administration to female SBMA mice induces ARpolyQ accumulation and causes disease manifestation (see Figure 7). These observations are at the basis of the current hypothesis that testosterone is the switch transforming the ARpolyQ from a “non-toxic” to “neurotoxic” protein and for the proposal of the use of endocrine therapies in SBMA. So far, two endocrine therapies have been tested in humans: Leuprorelin, a drug that reduces testosterone production from testis (an agonist of the GnRH receptor which interrupts the gonadotropin pulsatile production from the pituitary) and Dutasteride (681), an inhibitor of the isoenzymes 5α-reductase 1 and 2 (highly expressed in spinal cord motoneurons (407)), which activates testosterone to a more potent androgen, DHT. Other studies have been based on the use of selective AR modulators (SARMs), regulators of the AF-2 region of AR, inhibitors of HSP90s, as well as with synthetic

antisense oligonucleotides (ASO) directed against AR, and provided promising results ameliorating the aberrant phenotype observed in male SBMA mice (see Figure 7 for details). However, these preclinical studies were not replicated in clinical studies performed on SBMA patients (43). A possible explanation is that SBMA patients are characterized by a large variability of the symptoms and a very slow disease progression, thus the measurement of the efficacy of these treatments is very challenging and perhaps only with very long time of treatment one will be able to provide more convincing results (10,682,683). Recently, a male-to female transgender, presented the typical SBMA symptoms, although she had been treated for 15 years with the anti-androgen spironolactone to reduce the levels of testosterone. Spironolactone is a synthetic aldosterone receptor antagonist with antiandrogen properties. It is widely used as antiandrogen for feminizing hormone therapy for its ability to compete with testosterone for AR binding, it reduces gonadal steroidogenesis and 5α-R activity. In this case, spironolactone was able to induce ARpolyQ nuclear localization and toxicity leading to SBMA pathogenesis (684). Based on our better comprehension of the mechanisms governing intracellular receptor activities, two main theories have been put forward to explain the mechanism of testosterone-induced toxicity of the mutated AR form: nuclear toxicity and conformational alterations affecting protein folding abilities. Nuclear toxicity was suggested by the finding that the survival of SBMA motoneurons in culture and of SBMA mouse models was increased with mutations preventing ARpolyQ nuclear translocation or inducing its cytoplasmic retention (685). The second is that a conformational rearrangement is required for AR activation, since the long polyQ could alter proper folding. AR folding requires post-translational modifications, including phosphorylation, sumoylation, and acetylation, that are known to contribute to ligand-dependent AR proteotoxicity (See (685,686)). Misfolding during activation results in ARpolyQ nuclear and cytoplasmic aggregation, which sequesters chaperones, UPS components, transcription factors, etc. (624). However, ARpolyQ

neurotoxicity is not directly due to aggregate formations (624,687), since an inverse correlation exists between ARpolyQ aggregation and cell viability (624). Thus, at least in the earlier stages of their formation, cytoplasmic aggregates protect from cell death and ARpolyQ aggregation could be due to the cell attempt to segregate potentially neurotoxic protein species into a physically defined intracellular compartment. At late stages, aggregates may induce cell death by generating different neuronal dysfunctions. This process occurs when the neurotoxic protein is poorly cleared from neurons by specific degradative systems (see below) (632).

Interestingly, the ARpolyQ misfolding and aggregation triggered by testosterone is prevented and/or counteracted by some anti-androgens: the steroidal Cyproterone acetate, a compound less potent than testosterone in furthering AR nuclear translocation, induces the formation of few and irregular ARpolyQ aggregates (Figure 6) (strongly sequestered into p62 bodies) (687,688). The non-steroidal Bicalutamide (Casodex®) prevents ARpolyQ nuclear translocation, permitting a more efficient cytoplasmic degradation of ARpolyQ *via* autophagy (Figure 6) (687). Interestingly, both Cyproterone acetate and Bicalutamide reduced the formation of ARpolyQ insoluble species induced by testosterone (290,631). Flutamide, another non-steroidal AR antagonist, did not induce aggregate formation in *in vitro* models and exerts protective effects in SBMA mice models (689,690). Similar data were found using selective modulators of the activation function-2 (AF2) domain of the AR, which has been proposed to be essential for disease appearance. Recently, two new molecules: the tolfenamic acid (TA) and the 1-[2-(4-methylphenoxy)ethyl]-2-[(2-phenoxyethyl)sulfanyl]-1H-benzimidazole (MEPB) rescued from the loss of body weight, and the altered rotarod activity or grip strength in SBMA mice models (691). In addition, protein misfolding and their deleterious effects triggered in cells are counteracted by different HSPs (Hsp40, Hsp70 and its C-terminus interacting protein, CHIP) (692). These observations may lead to the generation of novel treatments, *e.g.* HSPs can be pharmacologically modulated with drugs

that enhance ARpolyQ degradation *via* autophagy activation (*e.g.*: 17-AAG, trehalose, etc.) (688,693,694), preventing ARpolyQ mediated toxicity in motoneurons. Together, the data collected using the SBMA as model for NDs are a promising proof-of-principle demonstrating that by fully understanding the molecular mechanism(s) of activation of a given neurotoxic protein, there might be a possibility for pharmacological interventions aimed to modulate misfolded protein aberrant effects on neuronal survival. This clearly implies that each single mutant protein responsible for NDs must be specifically targeted with selective drugs modulating one of its functions associated to neurotoxicity (e.g., Bicalutamide, leuprorelin, dutasteride in the case of SBMA). However, more selective drugs can be combined with compounds that modulate more general pathways (e.g.: trehalose) controlling protective/aberrant functions in neurons (e.g.: autophagy stimulators, chaperones inducers, etc.) clearly suggesting that NDs might be more efficiently counteracted by an accurate formulation of drug cocktails that may greatly enhance the activity of compounds poorly active when used as single treatment (631).

5. Conclusions and future perspectives

We know since decades that the activity of sex steroid hormones in the CNS is not only restricted to the control of reproductive functions and sexual behavior, but it ranges from affective behavior to learning, cognition and motor functions. We also learned that sex steroids have a large potential for neuroprotection being able to prevent neuronal apoptosis, increase the synaptic interactions and reducing neuroinflammatory processes. Yet, our ability to fully appreciate the complexity of the action of sex steroids in the brain has been impaired by the multiplicity of their cellular and intracellular targets and by the combined ability of the CNS to metabolize peripheral steroids or to synthesize its own steroids locally. As a consequence, state-of-the-art understanding of the

physiological role of sex steroid hormones is still very basic and a number of questions are waiting for an answer: i) Which are the effects of aging and decreased gonadal function on the synthesis of sex hormones in the CNS? ii) How is receptor distribution controlled in the CNS? iii) May a change in peripheral synthesis of hormones (e.g. due to menopause) result in up-regulation of brain sex hormone receptors? iv) Which are the consequences of aging on the central activity of sex hormones in the two sexes? v) Which is the influence of central sex steroid hormones on peripheral functions not strictly associated with reproductive actions, such as energy control or behavior? vi) Are these effects sexually dimorphic? vii) How do different receptor isoforms interact with each other? viii) Which is the physio-pathological relevance of the sexual dimorphism of microglia?

The recent and major technological progress achieved in molecular imaging, genetic analysis and stem cell biology enabled to localize and measure the specific action of each steroid hormone in neural tissues along with the progression of a given neurological disease and provides the tools that may enable us to answer to all the questions above. It is our hope that this review clearly demonstrates the potential that these molecules might have in delaying and circumscribing the progression of NDs and acts as a stimulus for the progression of studies in the field. Our aim was to provide a deep perspective of our current understanding of sex steroid mode of action and by collecting current understanding on the large variety of protective functions covered by sex steroids in the brain of healthy and diseased males and females. Indeed, the identification of a correlation between estrogens deficiencies and NDs may provide a mean for the study of the efficacy of replacement therapies particularly in women after the menopause. Future studies should also include the use of modulators of androgen functions as these male hormones appear to potentiate the progress of neurodegenerative processes through different means. Even in this case, the endocrine therapy could act as a relevant complement of therapies aimed at modifying

neurodegeneration course and investigations in ALS or SBMA models should be directed at increasing our insights on endocrine therapies which decrease the toxicity of the activated AR.

To find answers, future studies should be aimed at better defining: i) the key physio-biochemical processes by which sex steroids modulate neuroinflammatory processes and promote neuronal synaptic functions and survival ii) the role played by sex steroids in neural cell interactions; iii) the role of sex-specific genetics, epigenetics and hormones in the onset and progression of NDs; iv) the relevance of brain sexual differentiation in all the previous processes.

Only a better understanding of these factors will enable the design of personalized preventive strategies and therapies aimed to reduce the continuous NDs expansion in industrialized countries.

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Figures and legends

Figure 1. Biosynthetic pathways of neurosteroid formation.
HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) is the precursor for cholesterol synthesis. ***HMG-CoA Reductase*** catalyzes the production of **mevalonic acid** from HMG-CoA, with a reaction that is rate-limiting for cholesterol synthesis. Mevalonic acid is then converted, *via* the the mevalonate pathway, to lanosterol which is in turn converted to **cholesterol** *via* either the Bloch pathway or the Kandutsch-Russell pathway. ***CYP11A1*** (P450 side-chain cleavage, named ***P450scc*** in rodents), more expressed in females than in males, catalyzes the conversion of cholesterol to **pregnenolone** through the first rate-limiting step of *de novo* steroidogenesis. Further steroid conversions are catalyzed by ***3β-HSD*** (3β-hydroxysteroid dehydrogenase), ***CYP17*** (17α-hydroxylase/17,20-lyase, or P450c17), ***5α-R*** (5α-Reductase), and ***CYP19*** (aromatase). Full line arrows indicate active steroidogenic biosynthetic pathways in the brain. Dashed line arrows represent undetermined steroidogenic biosynthetic pathways. Steroidogenic enzymes are represented by dashed line boxes. The color codes indicate their distribution in brain cells; *cyan*: neurons; *green*: microglia; *orange*: astrocytes.

Figure 2. The complexity of sex hormone-receptor interactions in the nervous system.
In neurons, 5α-R converts testosterone (T) into dihydrotestosterone (DHT), which can be converted to 3β-diol by 3β-HSD, while aromatase converts testosterone to estradiol (E_2), giving rise to a variety of androgen metabolites, particularly in males. Blood-derived or locally produced sex hormones interact with specific homo or heterodimeric receptors, giving rise to a range of ligand-receptor complexes. Each hormone-receptor complex binds to gene promoters with specific preference and affinity, resulting in a combinatorial mechanism of transcriptional regulation by sex hormones within a cell. Furthermore, sex steroid receptors are expressed in neural and microglial cells in which they activate cell-specific genetic and metabolic programs. All these molecular and cellular mechanisms make up the response to the initial hormonal signal and participate in the sexual dimorphism of neurological functions.

Figure 3. Main sexual differences and activity of sex steroid hormones on amyloid plaque deposition, neuroinflammation, and neuroprotection. The accumulation of aggregates and deposits of misfolded proteins, like amyloid beta peptides, represent the main disease-specific histopathological change in the brain of AD patients. Additional hallmarks include a diffused neuroinflammation during the advanced stages of AD. There is a strong association between APOε4 genotype, the most established genetic risk factor, with sporadic AD onset. In particular, woman carrying both the homo- and heterozygous Apoε4 isoform have a higher rate of amyloid plaque deposition, while Apoε4 variant in men has marginal effects in both homo- and heterozygous subjects. An interaction between the APOε4 variant and SNPs of the ESR1 (rs9340799, rs2234693; rs2228480) and ESR2 (rs4986938) genes is possibly involved. Estrogens, by affecting the secretase pathway in neurons and the lipid metabolism in the periphery, decrease the production of beta-amyloid peptides, thus reducing Aβ accumulation in AD. Moreover, estrogens exert anti-inflammatory effects on microglia, limiting the Aβ-induced production of ROS. The beneficial effects of estrogens are lost following menopause, or after ovariectomy (OVX). Progesterone exerts beneficial effects on neurons, but it is still unclear if this is due to an

anti-inflammatory action of progesterone per se or if the effect is mediated by the progesterone metabolite allopregnanolone. In males, AD is associated with a decrease in circulating testosterone, which – in turns – could dysregulate Aβ deposition and hinder the testosterone-mediated neuroprotective actions including the regulation of spine density. The beneficial effects of testosterone and DHT are lost in Androgen Deficiency in the Aging Male (ADAM), or after orchiectomy (ORX).

Figure 4. **Sexual differences in the activity of sex steroid hormones on dopaminergic neurons of the NSDA system.** Dopamine (DA) metabolism in the NSDA system, consisting of dopaminergic neurons that from the *substantia nigra (SN)* innervate the *striatum* (STR), triggers oxidative stress that may lead to dysfunctions in protein folding, mitochondrial activity and quality control systems, which are pathogenic mechanisms of Parkinson's disease. Astrocytes and microglia residing in the microenvironment of the SN and STR sustain DA neurotransmission, thus increasing oxidative reactions. Among sex steroid hormone receptors, only AR is expressed by DA neurons, both in males and females. In males (left side), SRY expression has been involved in DA metabolism, while high levels of testosterone sustain DA neurotransmission by targeting DA neurons and astrocytes, while only its conversion to estrogens activates microglia. Thus, male sex and hormones correlate with the potentiation DA metabolism. Higher levels of estrogens in females (right side) induce DA neurotransmission only through astrocytes and microglia. ERα-mediated microglia responses to estrogens provide anti-inflammatory and antioxidant effects.

Figure 5. Effects of sex steroid hormones on motoneurons, glial and muscle cells of ALS models.

Estrogens, progestagens, and androgens through their receptors (respectively, ERα, ERβ, PR, and AR) modulate different pathways in motoneurons and neighbor cells. Estrogens and progestagens extend the life span of ALS animal models through a reduced production of inflammasome proteins, directly on motoneuron or through microglial cells. Estrogens also act on motoneuron lipid metabolism, and mitochondrial functions.

Furthermore, progestagens block oxidative reactions allowing a better mitochondrial activity. Androgens and anabolic-androgenic steroids (AAS) may lead to dysfunctions in protein folding.

Skeletal muscle cells express sex steroid receptors, but while ER and PR activation in female triggers a better motor performance, in male an excessive activation of AR leads to muscle fiber hypertrophy and diminished survival of the ALS animal model.

Figure 6. Effects of testosterone and anti-androgens on ARpolyQ aggregation in SBMA cell model.

Confocal fluorescence microscopy analysis on immortalized motoneuronal (NSC34) cells transfected with plasmid coding for a chimera of green fluorescent protein (GFP) and the AR containing an elongated polyglutamine tract (GFP-AR.Q48). Cells have been treated with ethanol (as vehicle control), 10 nM testosterone, 100 nM Casodex (Cas), or 100 nM Cyproterone acetate (Cypr) for 48h. Nuclei were stained with Hoechst (blue) (63X magnification). Scale bar = 10 μm. Aggregation of GFP-AR.Q48 is induced by testosterone and cyproterone acetate, but not by Casodex. Casodex, but not cyproterone acetate reverts the testosterone induced aggregation of GFP-AR.Q48.

Figure 7. Ligand-dependent toxicity of mutant ARpolyQ in SBMA.
Neurotoxicity of the mutant ARpolyQ associated with SBMA is triggered by the endogenous AR ligand testosterone. Pharmacological treatments (GnRH analogs) or surgical castration, which abolish testosterone production from the male gonads, completely rescue from the aberrant motor behavior phenotype and extend survival of all tg SBMA mouse models tested so far. Antiandrogens (e.g.: flutamide), SARMs inhibiting the AF-2 of AR, inhibitors of HSP90 also ameliorate motor behavior in the same tg SBMA mice. Similar results have been obtained using a genetic approach based on the administration of antisense oligonucleotides (ASO) against the AR mRNA (particularly active in muscle tissue). Conversely, female tg SBMA mice normally do not develop SBMA; testosterone treatment in female tg SBMA mice induces similar motor alteration to those described in male tg SBMA mice.

Figure 1

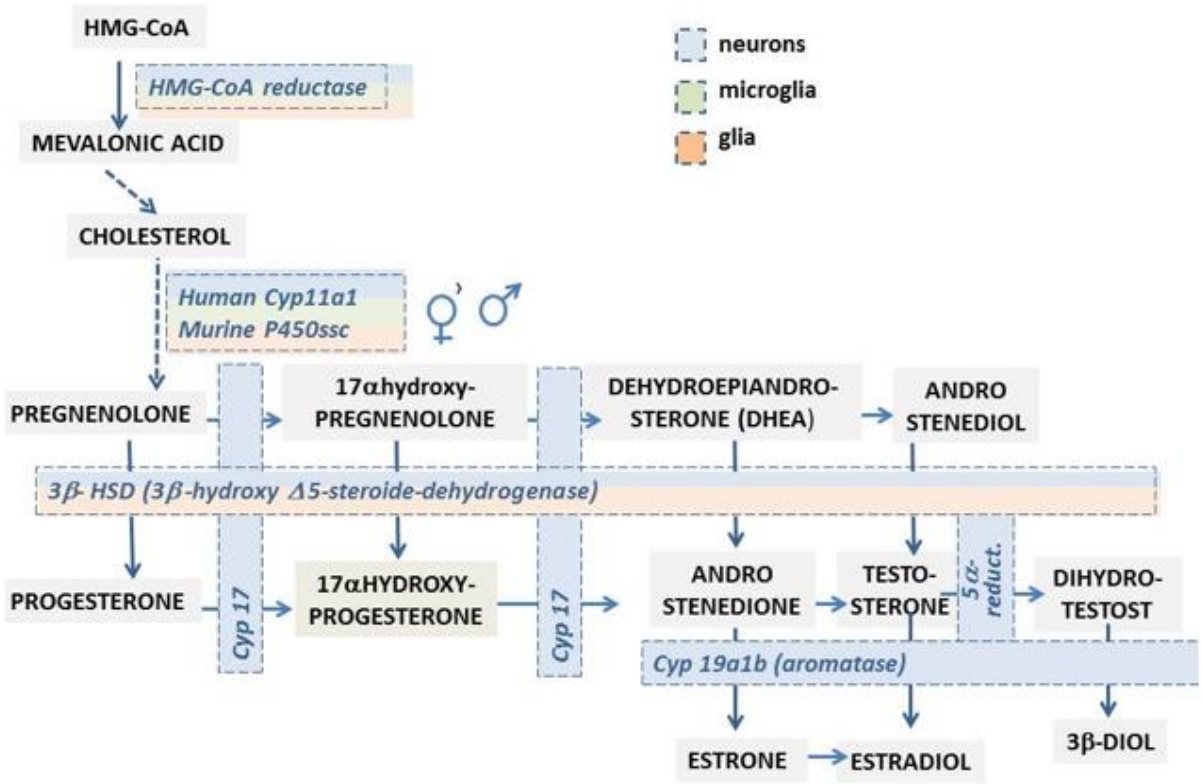


Figure 2

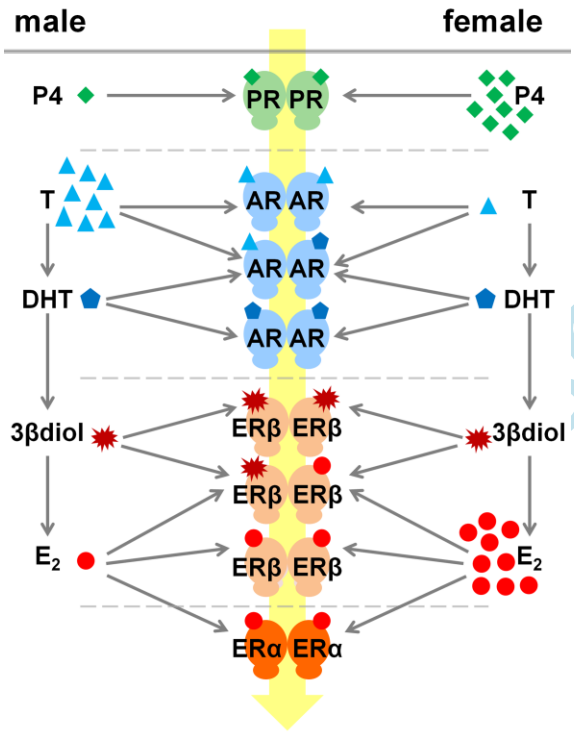


Figure 3

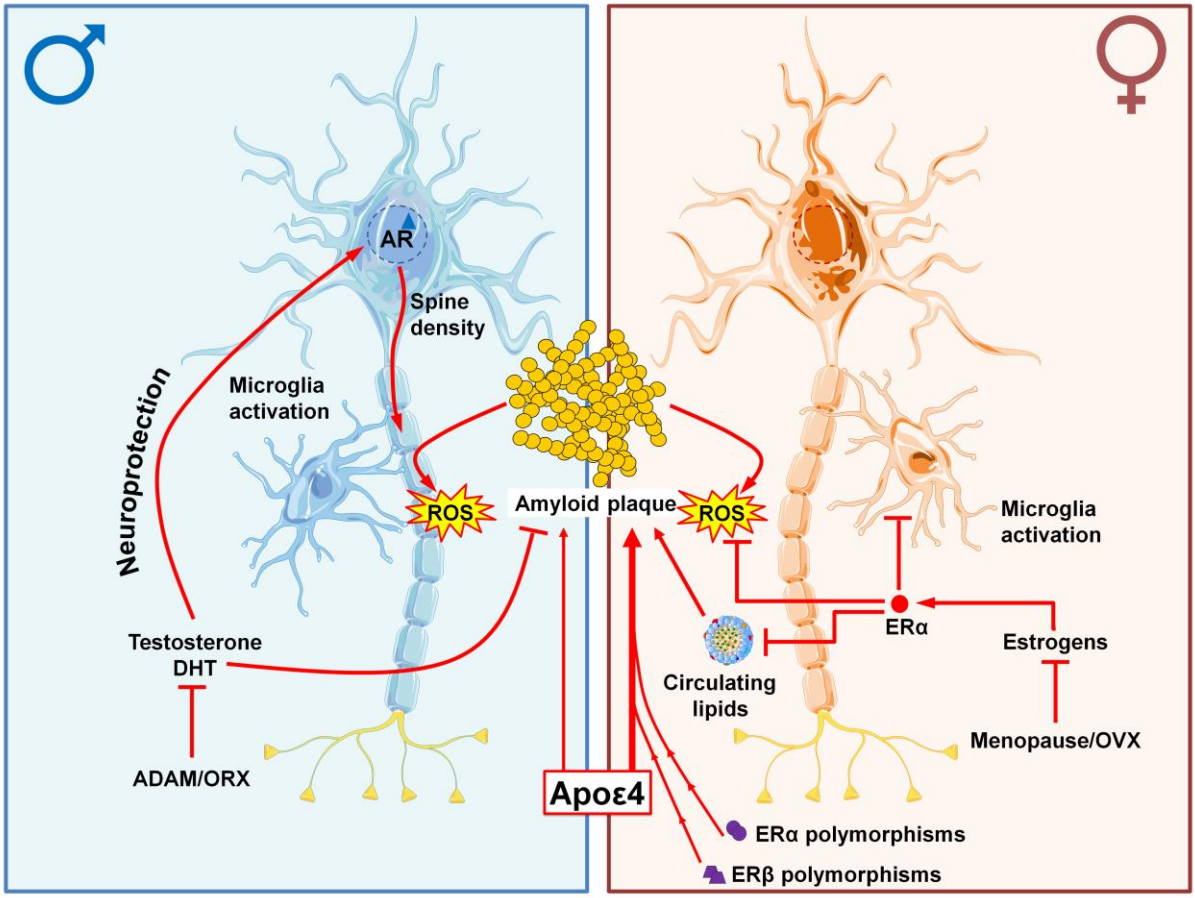


Figure 4

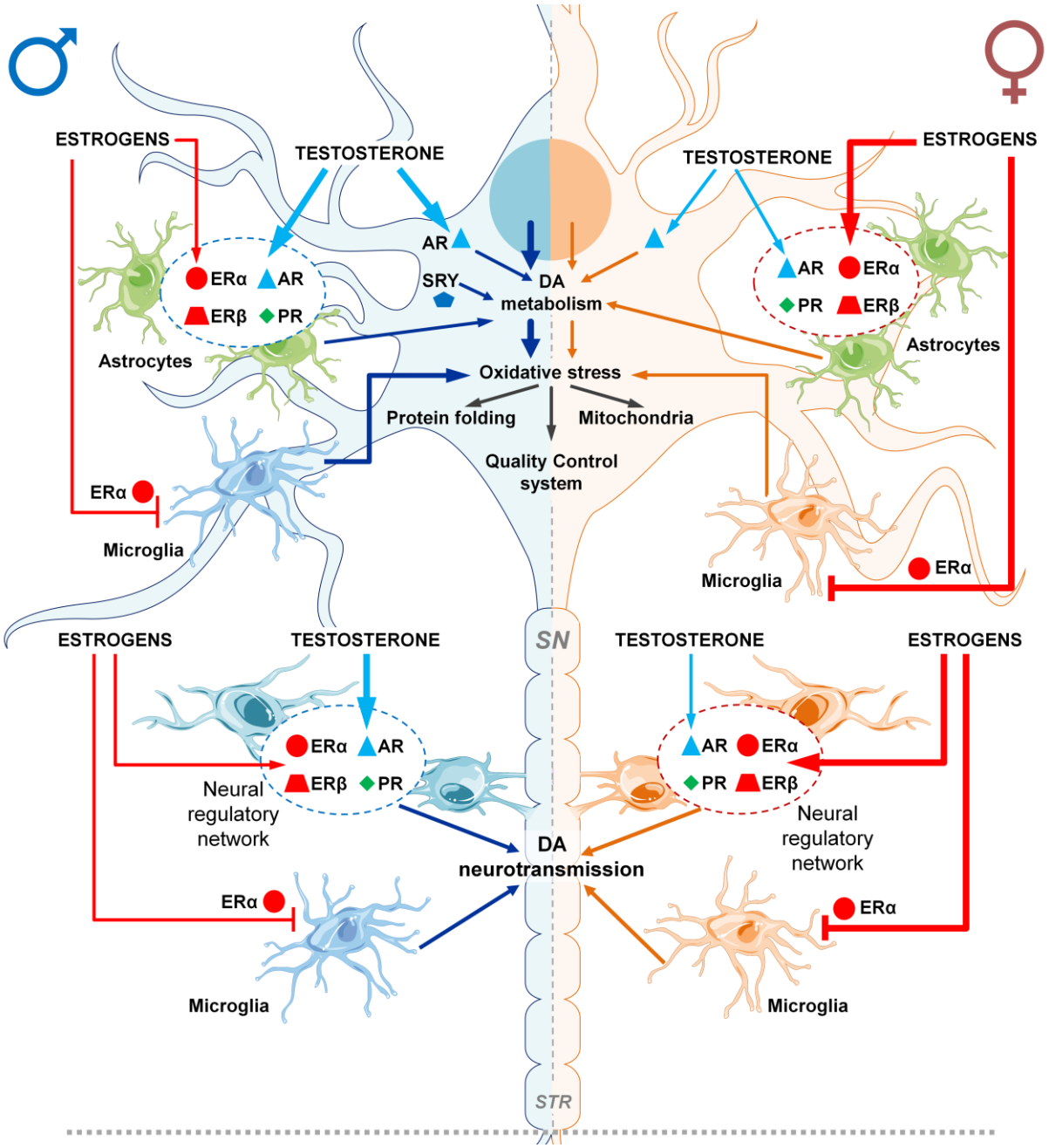


Figure 5

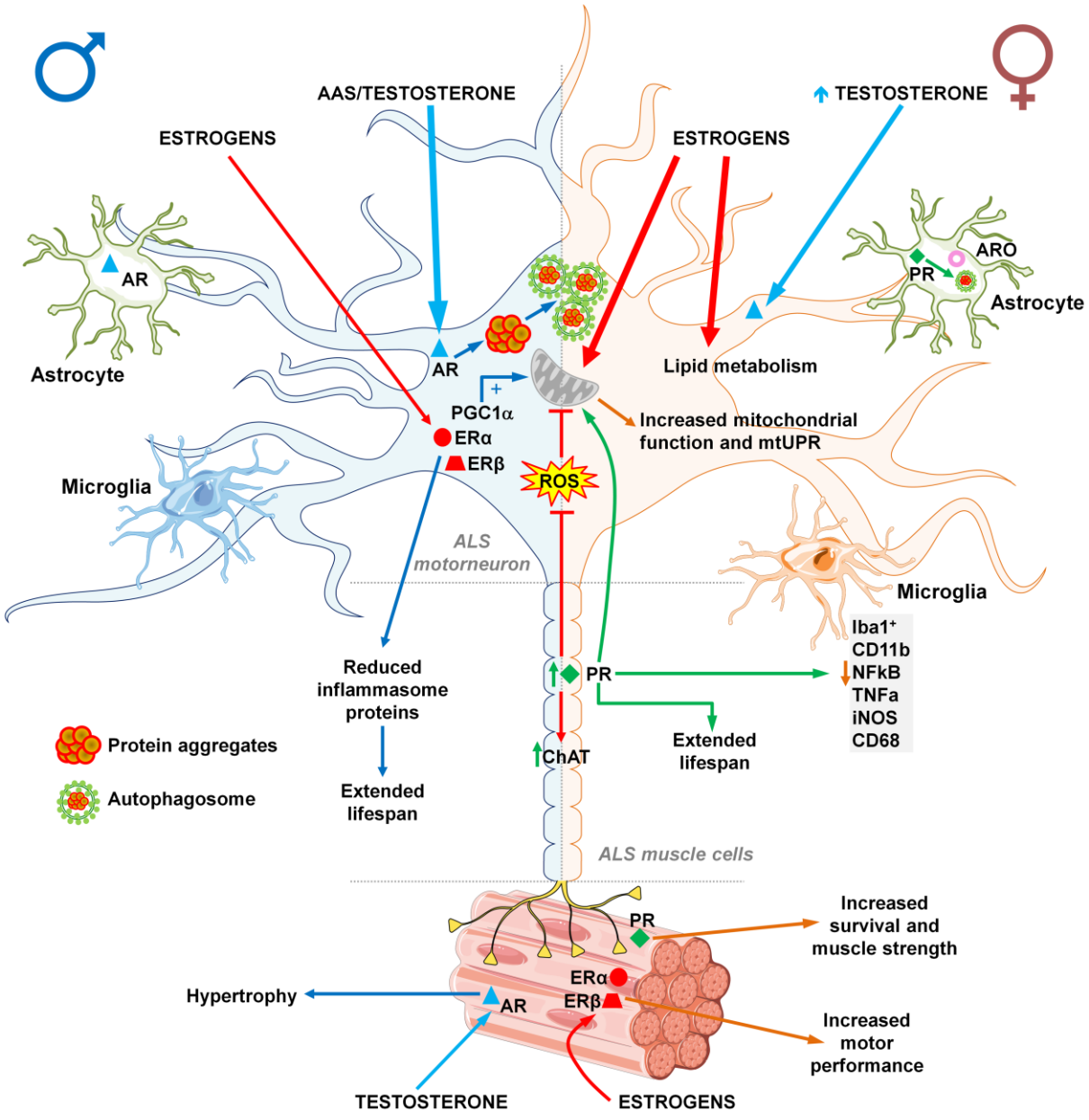


Figure 6

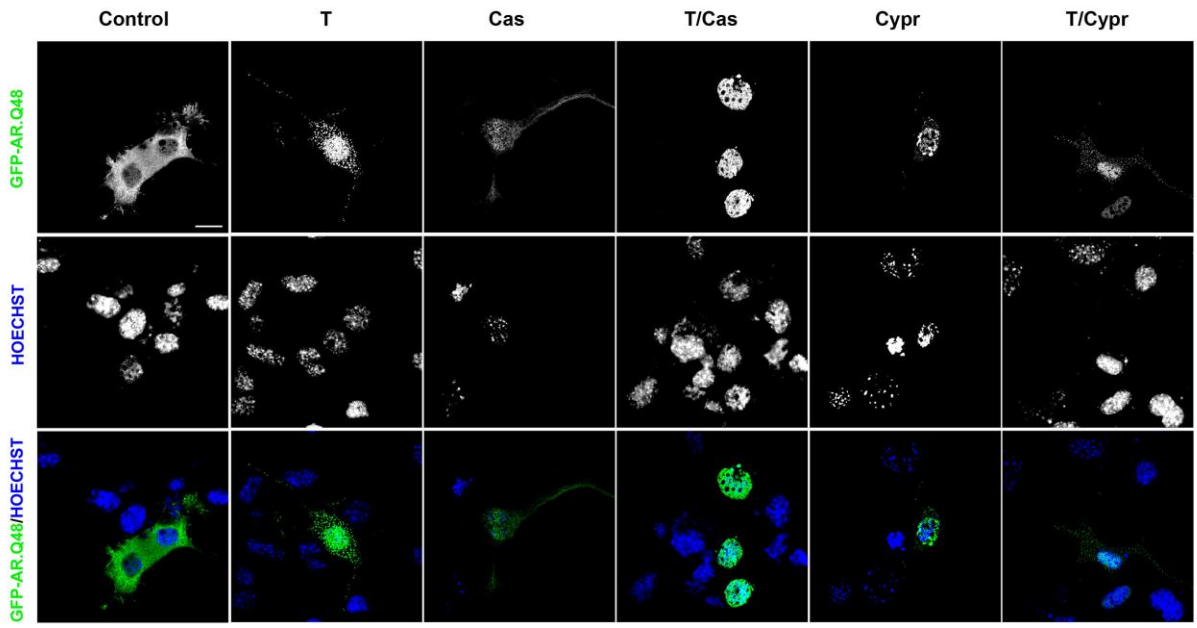
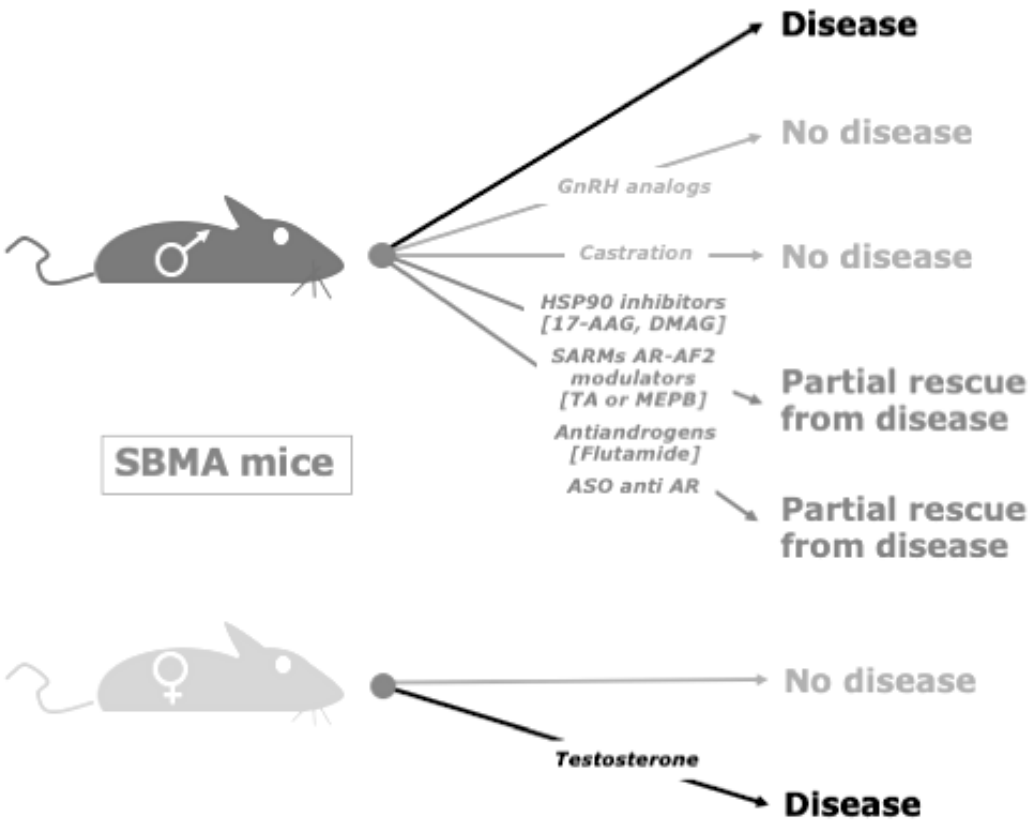
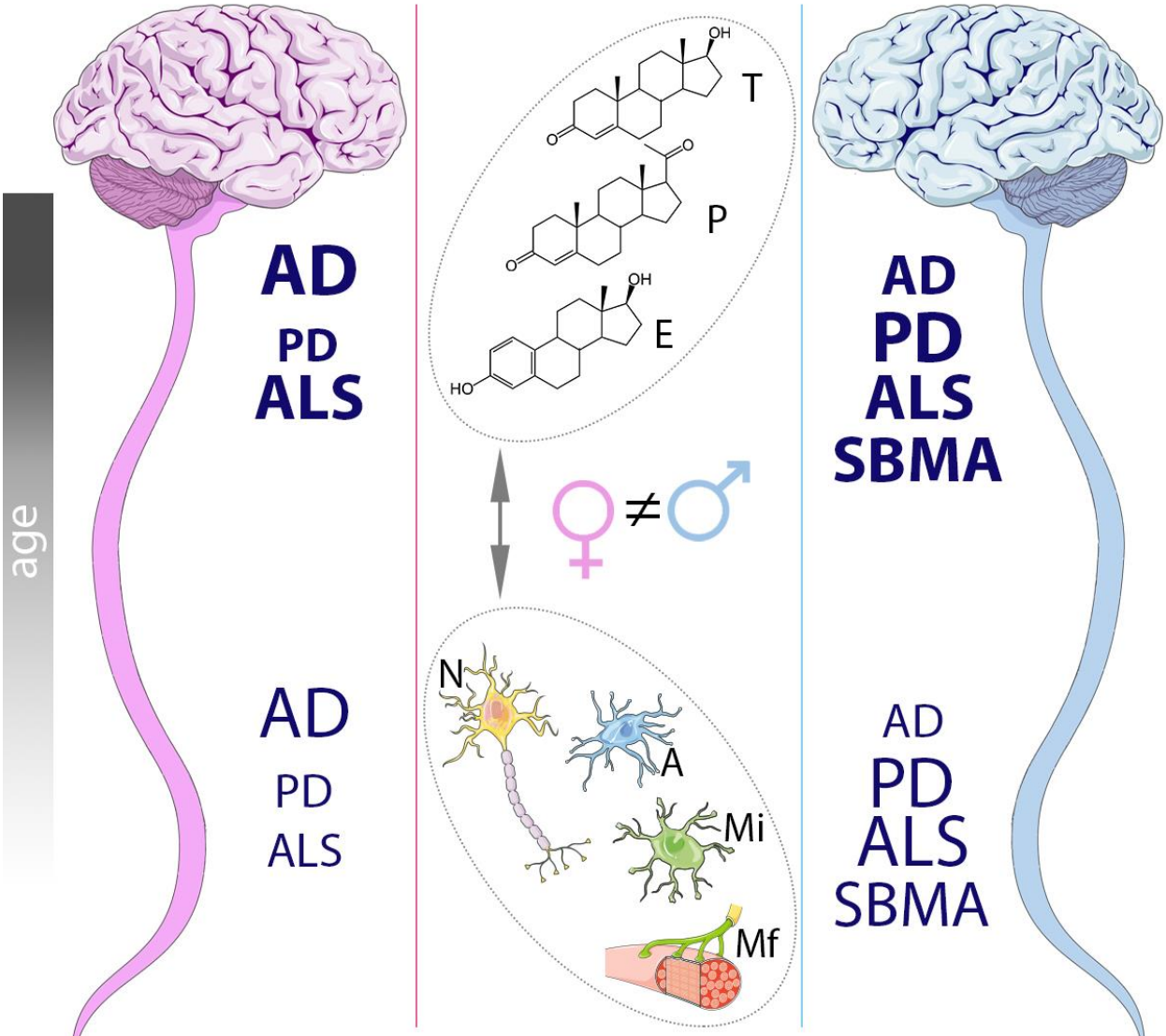


Figure 7



Graphical Abstract



AD: Alzheimer's disease, PD: Parkinson's disease, ALS: Amyotrophic Lateral Sclerosis
SBMA: Spinobulbar Muscular Atrophy, T: Testosterone, P: Progesterone, E: 17β–Estradiol
N: Neuron, A: Astrocyte, Mi: Microglia, Mf: Muscle fiber

Table 1. The gender related risk to develop neurodegenerative diseases		
AD incidence		
ANIMAL MODELS	- higher Ab accumulation (14); - higher levels of hyperphosphorylated tau (14); - poorer behavioral performance (15-19).	
PATIENTS	- more severe b-amyloid accumulation (20,21); - faster cognitive decline (22,23).	- increased tau pathology (24).
PD incidence		
ANIMAL MODELS	- favorable performance using low neurotoxin doses, that mimic the early stages of the disease (25); - higher sensibility to ubiquitin-proteasomal defects in PARK2 null mice (26).	- significantly more robust reduction in DA levels in the striatum and loss of dopaminergic neurons in the SNpc (27-30).
PATIENTS	- older than men at symptom onset (31); - present more often with a tremorigenic form and a slower progression of the disease (31).	- Increased risk due to the sexual dimorphism of the NSDA system (29).
ALS incidence		
ANIMAL MODELS	- outperform on batteries of behaviour tests measuring tremor, motor impairment, motor strength and coordination with rotarod and grip strength, as well as measuring tail elevation, footprint analysis, or motor activities (32,33);	- SOD1 mutation decreased proliferative and differentiating potential of rat neural progenitor cells (35); - C-boutons enlargement (36); - the disease is significantly more aggressive; in fact,

	- earlier disease onset in mice co-expressing mutant chromogranin and SOD1 (34).	males ALS mice lose weight and show motor symptoms earlier than females (32,37); - the disease progression is much shorter in males (32,37).
PATIENTS	- older than males at the age of onset (38); - prevalence of the bulbar form (38); - significant vulnerability to develop cognitive dysfunctions related to ALS (39); - positive association between longer reproductive time-span and susceptibility and survival of ALS (40).	- prevalence of the forms with limb onset (38); - higher CSF levels of SOD1 (41).
SBMA incidence		
ANIMAL MODELS	- in tg mouse models females are not affected (42,43); - testosterone induces SBMA symptoms	
PATIENTS	- female carriers are asymptomatic or present mild clinical abnormalities (44,45); - women homozygous did not show any clinical symptom of ND (46).	

Table 2. Genes/proteins involved in AD					
Gene symbol	**Gene/Protein name**	**Protein function**	**Reference**	**sporadic (s) /familial (f)**	**notes**
APP	amyloid beta precursor protein	Precursor of beta amyloid (Aβ)	53	f	
ABCA7	ATP binding cassette subfamily A member 7	Regulates the homeostasis of phospholipids and cholesterol in the CNS	54	s	
ADAM10	ADAM metallopeptidase domain 10	The main α-secretase that cleaves APP in the non-amyloidogenic pathway	55	s	
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif 4	Facilitates Aβ4-x peptide generation	55	s	
ALPK2	alpha kinase 2	Protein serine/threonine kinase activity	55	s	
APH1B	aph-1 homolog B, gamma-secretase subunit	A functional component of the gamma-secretase complex	55	s	
APOE	apolipoprotein E	Lipid metabolism	56	s	The ε4 allele of *APOE* is the major genetic risk factor for LOAD
ATP5H	ATP synthase peripheral stalk subunit d	ATP biosynthetic process	57	s	
BIN1	bridging integrator 1	Involved in synaptic vesicle endocytosis	58	s	
CASS4	Cas scaffold protein family member 4	It may have a role in axonal transport	59	s	
CD2AP	CD2 associated protein	Regulates the actin cytoskeleton	54	s	
CD33	CD33 molecule	It may have a *role* in cell-to-cell adhesion	54	s	Expressed on cells of myeloid lineage
CELF1	CUGBP Elav-like family member 1	Involved in pre-mRNA alternative splicing, mRNA translation and stability.	59	s	
CLNK	cytokine dependent hematopoietic cell linker	Plays a role in the regulation of immunoreceptor signaling	55	s	Expressed on cells of myeloid lineage
CLU	clusterin	A Golgi chaperone that facilitates the folding of secreted proteins	60	s	
CNTNAP2	contactin associated protein like 2	It may play a role in the local differentiation of the axon	55	s	
CR1	complement C3b/C4b receptor 1 (Knops blood group)	Act as a negative regulator of complement cascade, mediating phagocytosis	61	s	
DRB1	RNA binding motif protein 45	Binding to poly(C) RNA	59	s	
EPHA1	EPH receptor A1	Regulates cell proliferation, may play a role in apoptosis	54	s	
FERMT2	fermitin family member 2	Regulates the activation of integrins	59	s	
HESX1	HESX homeobox 1	Involved in forebrain development	55	s	
INPP5D	inositol polyphosphate-5-phosphatase D	Regulation of cellular activation	59	s	
KAT8	lysine acetyltransferase 8	Selectively inhibits antiviral immunity	55	s	
MAPT	microtubule associated protein tau	Encodes for tau, the predominant component of neurofibrillary tangles	62	f	Unclear Pathogenicity for AD
MEF2C	myocyte enhancer factor 2C	Involved in neurogenesis and in the development of cortical architecture	59	s	
MS4A	membrane-spanning 4A	Participates in the regulation of calcium signaling	54	s	
NME8	NME/NM23 family member 8	Nucleoside diphosphate kinase activity	59	s	

PICALM	phosphatidylinositol binding clathrin assembly protein	Involved in cellular trafficking and regulation of endocytosis	60	s	
PLD3	phospholipase D family member 3	Involved in APP processing	63	s	
PSEN1	presenilin 1	Part of the γ-secretase complex, regulating APP processing	64	f	
PSEN2	presenilin 2	Part of the γ-secretase complex, regulating APP processing	65	f	
PTK2B	protein tyrosine kinase 2 beta	Enhances signals that regulate neuronal activity	59	s	
RIN3	Ras and Rab interactor 3	Involved in the early endocytic pathway	59	s	
SORL1	sortilin related receptor 1	A neuronal apolipoprotein E receptor	59	s	
TREM2	triggering receptor expressed on myeloid cells 2	Phagocytosis, migration, activation	66	s	Exclusively expressed on cells within the myeloid lineage. SNPs increase disease risk by 2- to 4-fold.
TRIP4	thyroid hormone receptor interactor 4	Paly a role in the estrogen receptor and NF-κB transactivation	67	s	
UNC5C	unc-5 netrin receptor C	Direct axon extension and cell migration during neural development	68	s	
ZCWPW 1	zinc finger CW-type and PWWP domain containing 1	A histone modification reader involved in epigenetic regulation	59	s	

Table 3. Genes/proteins involved in PD					
Gene symbol	**Gene/protein name**	**Protein function**	**Reference**	**sporadic (s) /familial (f)**	**notes**
ATP13A2	ATPase cation transporter 13A2	lysosomal transmembrane protein associated with inorganic cations transport	121	f	Mutations produce truncated inactive proteins and cause an atypical form of PD (Kufor-Rakeb syndrome), with juvenile onset and rapid progression. Recessive inhheritance.
DJ-1	DJ-1	Molecular chaperone which acts as cellular sensor of oxidative stress and	122	f	Earky onset and slow disease progression. Recessive inhheritance.
GBA1	Beta-glucosylceramidase - glucocerebrosidase	lysosomal enzyme involved in glycolipid metabolism	123,124	s	Heterozygous mutations cause accumulation of glucocerebroside in different organs and are considered the greatest genetic risk factor for PD also associated with reduced age of onset.
LRRK2	-rich repeat kinase 2		125	s/f	Missense mutations result in amino acid substitutions that increase protein activity. Mutations in the LRKK2 gene are the most common cause of inheritable and sporadic PD, with mid-to-late onset and slow progression. Dominant inheritance.
PINK1 (PARK6)	PTEN-induced putative kinase	serine-threonine mitochondrial kinase involved in mitochondrial function together with parkin	126	f	Truncating, missense and nonsense point and frameshift mutations result in dysfunctional protein; early onset. Recessive inhheritance.
PRKN	Parkin	E3 ubiquitin ligase regulating mitochondrial function and protein quality control system	127	s/f	It is the most common cause of autosomal recessive PD, with early onset (less than 40 years old) and slow progression. Recessive inheritance
SNCA	α-synuclein	synaptic function and neurotransmission	128	s (rarely)/f	Amino acid substitutions, due to missense mutations ,or increased protein expression, due to gene locus multiplications, render α-syn prone to aggregation. Dominant inheritance.

Table 4. Genes/proteins involved in motoneuron diseases (ALS and SBMA)

ALS

Gene symbol	Gene | Protein name	Protein function	Reference	sporadic (s) /familial (f)	notes
ALS2	Alsin Rho guanine nucleotide exchange factor ALS2 | Alsin	Rho Guanine Nucleotide Exchange Factor	190	f	
ANG	Angiogenin	Actin binding; ribonuclease	191	s/f	
ANXA11	Annexin A11	Vesicle trafficking, apoptosis, exocytosis, and cytokinesis	192	s/f	
ATXN2	Ataxin 2	Endocytosis/RNA metabolism	193	s/f	CAG repeat sequence longer than 35 CAG causes Spino Cerebellar Ataxia (SCA)-2; shorter repeats are linked to ALS.
C9orf72	C9orf72-SMCR8 complex subunit | Guanine nucleotide exchange C9orf72	–	194,195	s/f	abnormally translated (sense and anti-sense mRNAs)by RAN-translation producting five dipeptide-repeat (DPR) proteins
CCNF	Cyclin F	Catalyzes ubiquitin transfer to substrates for UPS degradation	196	s/f	
CFAP410			197	s/f	also: chromosome 21 open reading frame 2 (C21orf2)
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain-10		198	s/f	
CHMP2B		(ESCRT-III); involved in sorting of endosomal cargo proteins.	199	f	May also be involved in FTD
DAO	D-amino acid oxidase	-	200	f	May also be involved in FTD
DCTN1			201	s/f	
ELP3	Elongator complex protein 3		202	s	
ERBB4	-b2 receptor tyrosine kinase 4	Member epidermal growth factor (EGF) receptor tyrosine kinases	203	s/f	
EWSR1	-binding protein EWS	RNA/DNA binding	204	s	1
FIG4	FIG4 phosphoinositide 5-phosphatase | Polyphosphoinositide phosphatase	- bisphosphate	205	s/f	
FUS	FUS RNA binding protein	-	206,207	s/f	
GLE1			208	s/f	
HNRNPA1		-binding protein	209	s/f	
HNRNPA2/B1		-binding protein	209	s	H NRNPA2B1 associated with ALS are extremely rare
KIF5A		-based motor protein	210	s	
MATR3		RNA-binding protein	211	s/f	
NEFH			212	s/f	
NEK1	NIMA Related Kinase 1 | Serine/threonine-protein kinase Nek1		213	s/f	
OPTN	Optineurin		214	s/f	
PFN1		Actin-binding protein	215	s/f	
SETX			216	s	

SIGMAR1	Sigma non-opioid intracellular receptor 1	Lipid transport from the endoplasmic reticulum	217	f	May also be involved in FTD
SOD1	Superoxide dismutase 1	Superoxide dismutase	218	s/f	First ALS gene identified
SPG11	SPG11 vesicle trafficking associated, spatacsin | Spatacsin	Maintenance of cytoskeleton stability/regulation of synaptic vesicle transport	219	f	
SQSTM1	Sequestosome 1	Autophagy adaptor	220	s/f	May also be involved in FTD
TAF15	TATA-box binding protein associated factor 15	RNA-binding protein	221	s/f	
TARDBP	TAR DNA Binding Protein | TAR DNA-binding protein 43	RNA-binding protein	222,223	s/f	TDP-43 and its C-terminal fragments (TDP-35 and TDP-25) are component of pathological inclusions found in almost all ALS patients (not in mutant SOD1s). May also be involved in FTD
TBK1	TANK binding kinase 1 | Serine/threonine-protein kinase TBK1	Innate immune response, autophagy, inflammation and cell proliferation	224	s/f	
TIA1	TIA1 cytotoxic granule associated RNA binding protein | Nucleolysin TIA-1 isoform p40	RNA-binding protein	225	s/f	
TUBA4A	Tubulin alpha 4a	Microtubule subunit	226,227	s/f	
UBQLN2	Ubiquilin 2	Protein degradation	228	s/f	May also be involved in FTD
VAPB	VAMP associated protein B and C	ER-membrane protein	229	f	
VCP	Valosin containing protein | Transitional endoplasmic reticulum ATPase	Ubiquitin segregase	230	s/f	May also be involved in FTD
SBMA					
Gene symbol	**Gene/Protein name**	**Protein function**	**Rreference**	**sporadic (s) /familial (f)**	**notes**
AR	Androgen receptor	Steroid hormone receptor	231	f	CAG repeat loger than 36-37 repeats causes SBMA