THE PHYSIOPATHOLOGICAL ROLES OF ANDROGENS IN MOTONEURONS

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SUNTO. – Il recettore degli androgeni è stato purificato negli anni '70 e clonato negli anni '80. Questa proteina fa parte della superfamiglia dei recettori steroidi e media i principali effetti degli androgeni nei tessuti dipendenti o sensibili agli androgeni. Diverse funzioni fisiologiche nel cervello sono controllate in modo differenziato nei due sessi e il recettore degli androgeni gioca un ruolo specifico nei processi di differenziazione sessuale ed è coinvolto nel mantenimento del comportamento sessuale maschile in età adulta. Se mutato, il recettore degli androgeni può avere un impatto su molte attività regolate dagli androgeni a causa di una perdita della funzione androgenica nelle cellule bersaglio. Tuttavia, nel caso di un particolare tipo di mutazione, l'allungamento del tratto di poliglutammine normalmente presente nella sua regione N-terminale, il recettore degli androgeni diventa neurotossico e può indurre la morte cellulare di motoneuroni localizzati nel midollo spinale, che esprimono livelli molto elevati di questa proteina. In questo lavoro, discuteremo brevemente le azioni più importanti delle attività androgenica mediata dal recettore degli androgeni nel cervello e i meccanismi attraverso i quali la forma mutata del recettore degli androgeni può portare alla neurodegenerazione nell'atrofia muscolare spinale e bulbare (SBMA).

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ABSTRACT. – The androgen receptor has been purified in the '70s and cloned in the '80s. It is a member of the steroid receptor superfamily and mediated the most important effects of androgen in androgen dependent or sensitive tissues. Several physiological function of the brain are differentially controlled in the two sexes and androgens play specific role in the processes of sexual differentiation and it is involved in the maintenance of male sex behaviour in adulthood. When mutated, the androgen receptor may impact on many of these androgen-regulated activities because of a loss of androgenic

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function in target cells. However, in the case of a peculiar type of mutation, the elongation of the polyglutamine tract normally present in its N-terminus, the androgen receptor becomes neurotoxic and induces cells death of a number of motoneurons in the spinal cord, which express very high level of this protein. Here, we will briefly discuss the most important actions of androgen receptor-mediated androgen activity in the brain and the mechanisms by which the mutant androgen receptor may lead to neurodegeneration in Spinal and Bulbar Muscular Atrophy (SBMA).

The androgen receptor (AR) is the factor mainly in charge in mediating the masculinizing effects of androgens in target tissue. Specific functions related to sex differences are exerted by androgens in the brain. In this area, the AR is present at high levels in the hypothalamus, particularly in neurons which are localized in the lateromamillary nucleus, in the medial mamillary nucleus, in the diagonal band (horizontal limb) of Broca, in the sexually dimorphic nucleus present in the preoptic area as well as in the paraventricular, suprachiasmatic, ventromedial and infundibular nuclei [1, 2]. AR has also been detected in other hypothalamic nuclei (e.g. nucleus basalis of Meynert, supraoptic and the periventricular nuclei, bed nucleus of the stria terminalis, medial preoptic area, etc.) [1, 2, 3]. By analysing possible differences in AR distribution in male and female brains, it emerged that many of these regions and nuclei are sexually dimorphic [4] with differential expression AR levels in the two sexes. These observation have been used to explain the existence of physiological changes of the organization of the hypothalamic-pituitary-gonadal axis in the two sexes, as well as it has contributed to explain male and female sexual behavior, or the prevalence of some psychiatric and neurological diseases in one of the two sexes [5]. Other brain regions characterized by an intense localization of AR protein are the hippocampus and the temporal cortex [6]; as opposite to the hypothalamus, these structures do not show variation of AR expression in the two sexes [7]. Finally, it has been demonstrated the presence of considerably high level of AR in the motoneurons located in the bulbar region and of the anterior horn in the spinal cord, as well as in sensory neurons present in the dorsal root ganglia, and in the sural nerve [8].

The androgen receptor (AR) gene has been cloned in 1988 [9, 10, 11] and only three years later a mutated version of the AR has been associated to spinal and bulbar muscular atrophy (SBMA) or Kennedy's

disease [12] a neurodegenerative disease affecting motoneurons. The AR gene encodes a protein which acts as a ligand activated transcription factor responsible for most of the biological actions exerted by the androgenic steroids in target tissues. The AR belongs to the nuclear receptor superfamily and similarly to other member of this family control the transcription of specific target genes [13]. Structurally, the AR contains several well characterized domains, which are capable to mediate the interaction between the protein and its ligand (LBD), the protein and the DNA (DBD), and be the bases for protein-protein interactions. This protein-protein interaction is responsible for AR dimerization and AR association with co-factors and co-regulators, as well as for the interaction with molecular chaperones both responsible for the modulation of its biological activity [14, 15]. When AR is not bound to its ligand, the protein remains confined in the cytoplasm where it forms a multi-heteromeric inactive complex with Heat Shock Proteins (Hsp) [16, 17]. Upon binding to testosterone, or other androgens, the AR dissociates from the HSPs dimerizes and translocates into the nucleus. Here, the AR binds the promoter region of androgen responsive genes, typically containing androgen responsive elements (ARE), activating transcription of target genes.

In the past 30 years, several groups have described that many AR gene mutations associate to a variety of human diseases. Most of these mutations are linked to clinical conditions in which a clear loss-of-the AR-function is present. Indeed, the mutation in the gene are translated in a receptor protein with an impaired capability to activate transcription of the androgen target genes. This loss-of-AR-function could be partial or complete, causing a limited or complete androgen insensitivity in males, associated to a wide range of mixed sexual phenotypes [18]. Besides these, other AR mutations are capable to potentiate the normal AR functions, or generate a constitutively activated protein which can cause prostate cancer or other androgen-dependent diseases.

A very interesting situation occurs when the target of the mutation is the CAG (cytosine, adenine, guanine) triplet repeat stretch located in the first coding exon of the AR gene. This mutation consists in an expansion of the length of the CAG repeat, which becomes longer than 36 contiguous CAG codons. In this case, the resulting encoded AR protein contains an aberrantly long polyglutamine (polyQ) tract in its N-terminal

domain, which surprisingly confers neurotoxic properties to the mutant AR (ARpolyQ). These neurotoxic properties of the ARpolyQ are particularly manifested on motoneurons located in the anterior horns of the spinal cords, in the bulbar region of the brainstem and on sensory neurons located in the dorsal root ganglia. The death of motoneurons has the consequence to induce muscle atrophy. Individual carrying this mutant AR gene are affected by the peculiar form of motoneuron disease (MNDs), previously mentioned, the SBMA [12, 19] in which both motor and sensory functions are altered. Of note, the same type of CAG expansion encoding for the elongated polyQ tract has been reported in other genes and the coded proteins cause different types of neurodegenerative diseases [12, 20, 21, 22], which are: the Huntington's disease (HD), different types of spinal-cerebellar ataxias (SCAs), and the dentatorubral and pallidoluysian atrophy (DRPLA) [21, 23]. Singularly, these are very rare diseases, but all together constitute the most frequent class of inherited neurodegenerative diseases in human. It is likely that common mechanisms of neurotoxicity are exerted when the polyQ tract is present in a given protein [23, 24, 25].

The mutant ARpolyQ which causes SBMA is characterized by a partial loos-of-function (LOF), since its transcriptional competence is reduced when compared to wtAR [26, 27, 28, 29]. This probably accounts for the endocrine alteration typically reported in most SBMA patients, like gynecomastia and hypogonadism hypogonadic [19, 30]. Notably, we found that in immortalized moto neurons ARpolyQ has a lower transcriptional competence than wtAR when the target on promoter is a classical androgen responsive element (ARE) on which AR activates transcription mediating a positive androgenic control. Conversely, ARpolyQ and wtAR present very similar inhibitory activities if an alternative promoter region is involved, such as the AR promoter/5'-UTR activation which undergoes to a feedback mechanism exerted by the AR itself on its own promoter [26]. Indeed, we identified two opposite mechanism of the androgenic control of AR expression in moto neurons: a negative feedback on the AR promoter and a positive feed-forward activity on a region located in the AR ORF [26]. These two different androgenic modulations of AR gene could involve different regulatory elements and factors.

The fact that ARpolyQ loose part of its activity on classical ARE-

promoters suggests that some androgenic response may be lost in motoneurons of SBMA patients. Therefore, this may have impact on the physiological regulation exerted by androgens in these cells in which, for example, androgens control the development and adult maintenance of moto neurons of the spinal nucleus of the bulbocavernosus (SNB) system at different stages [31]. Most of these androgenic activities seem to take place during development, especially around birth, or in the process of sexual differentiation when androgens exert different priming activities in the brain. Indeed, androgens modulate synapses formation at neuromuscular junctions and may regulate the growth and arborization of dendritic branches. Androgens act also in adulthood by preserving the moto neurons size [32] and the lack of androgens correlates with a reduction of moto neuron size and of the extension of dendrite [2, 33, 34]. These effects may be due to testosterone itself or to its more potent 5 alpha-reduced derivative dihydrotestosterone (DHT) that can be formed directly "in loco". In fact, spinal cord motoneurons have been found to express very high levels of the enzyme 5 alpha-Reductase type 2 responsible for this conversion and the production of DHT in most androgen dependent tissues [35]. Some of these effects can be mediated at a molecular levels by the protein neuritin (or CPG15), that is known to activate neurite outgrowth and we found to be controlled by the androgenic activation of AR in moto neurons [36, 37, 38, 39, 40, 41, 42].

Despite all these observation, so far no data seems to indicate that the LOF of the ARpolyQ may have impact on the androgenic activities in motoneuronal cells in these brain districts.

Most of the data obtained so far in SBMA strongly suggest that motoneuron death in this disease is due to neurotoxic properties which the elongated polyQ confers to the AR. This gain-of-unction (GOF) is mediated by several different mechanisms, but seems to be triggered by testosterone which is capable to induce aberrant conformations to the mutant ARpolyQ [3]. Indeed, being SBMA a disease associated to a GOF, it is surprising that only male are affected in SBMA, and female that carry the mutant gene are protected from disease manifestation. It has been initially postulated that this may involves the process of random X-chromosome inactivation; this could preserve about half of motoneurons from GOF of ARpolyQ. Surprisingly, while women heterozygous for expanded CAG repeat in exon 1 of AR gene do not

develop SBMA [43, 44], also two women homozygous for SBMA were identified and none of them showed clinical manifestation of SBMA [45]. Moreover, all mice models developed so far, present disease symptomatology only in male, even when the transgene carrying the polyQ is located randomly into the genone (and not in the X-chromosome). In these mice, castration in male ameliorates the phenotype, while the treatment with testosterone in females induces SBMA symptoms [46, 47, 48, 49, 50, 51, 52, 53, 54]. Similar data were obtained in fly models of SBMA [55, 56]. Thus, the male risk to develop SBMA is due to circulating testosterone.

At the molecular levels, we found that ARpolyQ binding to testosterone induces its aggregation [57, 58, 59]. Our data suggest that the aggregates initially sequester misfolded ARpolyQ protecting from its neurotoxicity [3], but at later stages they may become toxic by impairing important intracellular pathways (e.g.: axonal transport or mitochondria distribution [57]. In addition, ARpolyQ seems to be particularly cytotoxic at nuclear levels and cytoplasmic retention ameliorate survival of SBMA motoneurons in culture and of SBMA mouse models [60]. The neurotoxicity triggered by testosterone on ARpolyQ is likely due to the fact that this process involves the release of AR from accessory chaperones and the activation process of the AR. This requires conformational rearrangements to reach the active status of the transcription factor and the expanded polyQ might alter the correct protein folding. Of note, this process is counteracted by selected anti-androgens, like Cyproterone acetate, Flutamide or Bicalutamide [61, 62, 63, 64]. In particular, Bicalutamide has the property to act as a type I antagonist against AR and to slow down its nuclear translocation allowing ARpolyQ cytoplasmic retention and improved autophagic degradation [61, 62, 65, 66]. We combined the use of bicalutamide with that of an effective autophagic activator, the trehalose, and found a potent synergic effect of the two compounds because of the enhanced cytopasmic degradation of ARpolyQ in these conditions [65].

Once activated by testosterone, the mutant ARpolyQ can thus generate misfolded species that must be removed from cell in order to prevent aggregate accumulation and possible damages of the degradative pathways. This process is generally regulated by specific chaperones, including the heat shock proteins (HSPs), which are overex-

pressed upon different cell stresses, including proteotoxic stresses [67]. Chaperones are a large family with more than 150 members (subgrouped in: small HSPs, HSP40s, HSP60s, HSP70s, HSP90s and HSP100) [68]) and work often in conjunction with co-chaperones (e.g.: nucleotide exchange factors (NEFs), such as BCL2-associated athanogene (BAG) family of proteins [69]).

It has been reported that several of these chaperones/co-chaperones are linked, when mutated, to neurodegenerative diseases of other disorders in which neurons are affected [70]. The function of chaperones is to promote protein folding, counteracting misfolding and aggregation. If this process fails, they are able to direct misfolded proteins to the degradative systems. Among these, the most important are: the ubiquitin-proteasome system (UPS) and the autophagic pathway, which are finely tuned by specific chaperones and co-chaperones [71, 72, 73, 74, 75]. We found that some smallHSP (also named HSPBs), like HSPB8, are able to fully counteract ARpolyQ aggregation under several different circumstances [76]. This occurs by facilitating a peculiar form of autophagy, named chaperone-assisted selective autophagy (CASA), which is based on the activity of the CASA complex. Indeed, this complex includes two molecules of HSPB8 interacting with BAG3, HSP70 and CHIP/STUB1. Once the CASA complex has recognized mutant ARpolyQ, it interacts with SQSTM1/p62, an autophagy receptor which recognizes both the ubiquitinated proteins and the lipidated form of LC3 (LC3-II) associated to the autophagosome membranes to target the misfolded proteins of the CASA complex to degradation [77]. Alternatively, the complex HSP70-CHIP/STUB1 and SQSTM1/p62 can works in conjunction with BAG1 to target ARpolyQ to UPS [75]. When this equilibrium is imbalanced, ARpolyQ accumulated into intracellular aggregates into motoneurons.

HSPB8 is widely distributed in most human tissues, and it is upregulated in SBMA [61, 78, 79, 80]. When mutated it causes Charcot-Marie-Tooth type 2L disease, hereditary distal motor neuropathy type II (dHMN-II) or distal myopathy [81, 82, 83]. HSPB8 is highly expressed in anterior horn motoneurons [80] and in skeletal muscle, two tissues potentially affected in SBMA [66]. In addition HSPB8 becomes overexpressed during disease progression, thus contributing to the removal of misfolded ARpolyQ. When expressed in cells, HSPB8 facilitates ARpolyQ clearance via autophagy removing the autophagy flux blockage which characterize this disease [61, 65, 75,

84]. HSPB8 also removes other misfolded proteins responsible for neuronal death in other neurodegenerative diseases [61, 75, 80, 85, 86, 87, 88, 89, 90, 91], suggesting that this factor can be a potential target to counteract proteotoxicity in a wide variety of disorders affecting the brain.

In conclusion, androgens have several role in the brain and most of the effects on neuronal cells are mediated by the AR. Reduced function of AR may impact on the sex and aggressive behavior in male and may be linked to depression, while aberrant functions associated to the presence of the elongated polyQ tract may cause death of motoneurons and their target muscle cells. There are several strategies potentially useful to be use to counteract the toxicity of the mutant ARpolyQ, including the approaches aimed to prevent its activation, but that unfortunately cause heavy side effects at endocrine levels. We have identified a factor, HSPB8 which when activated protect against mutant ARpolyQ toxicity and may be an important target for future therapeutic approaches in SBMA.

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The Author wish to dedicate this manuscript to the memory of his

Master of Science and Mentor professor Luciano Martini who founded the Institute of Endocrinology, and thanks to his endless support, greatly contributed to the development of the scientific vocations of many of his fellows, allowing them to become independent investigators.

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Both will always be remembered by all their fellows.

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