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Reflections on the Diseases Linked to Mutations of the Androgen Receptor

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This review summarizes the most recent information on two pathologies linked to mutations of the androgen receptor, namely, the complete androgen insensitivity syndrome (CAIS) and the spinal and bulbar muscular atrophy (SBMA or Kennedy's disease). Data on the clinical manifestations of the two diseases are presented, together with the most relevant findings on their physiopathology and genetics.

Key Words: Complete androgen insensitivity syndrome; CAIS; spinal and bulbar muscular atrophy; SBMA; Kennedy's disease; androgen receptor; AR; brain sexual differentiation.

Introduction

Evidence accumulated over the years underlines that, among all steroid hormones, androgens probably exhibit the most complicated mode of action.

First of all, one should recall that the testes secrete two androgenic hormones, testosterone (T) and dihydrotestosterone (DHT), one of these (T) being quantitatively predominating *(1)* and the other (DHT) being more effective in the majority of the androgen-dependent structures *(2)*. The elevated concentrations of T present in the circulation represent a "reservoir" for supporting the necessity of the multiple androgen-dependent structures existing in the body. In humans, there is an additional source of production of androgens and of their precursors, namely, the adrenal gland *(3)*.

Several enzymatic systems have been shown to be able to metabolize T and other androgens. Some of these have the peculiarity of totally changing the biological properties of the substrate (e.g., the aromatase that transforms T and Δ_4-androstene-3,17-dione, Adione, respectively, into estradiol, E2, and estrone, E1) *(4)*, while others, like the 5α-reductase (5α-R), which is present in the body in two isoforms 5α-R type 1 (with a wide distribution) and 5α-R type 2 (specifically present in the androgen-sensitive structures), have the property of enhancing T activity at tissue level, by converting T into the more androgenic compound DHT *(2,5,6)* (see below). Finally, some enzymes (e.g., the 3α- and 3β-hydroxysteroid dehydrogenases, 3α-HSD and 3β-HSD) can modify the structure of DHT so as to enable the resulting metabolites (3α-diol and 3β-diol) to eventually interact with receptors other than the classical intracellular androgen receptor (AR) (e.g., 3α-diol with the $GABA_A$ receptor *(7–11)* and 3β-diol with the estrogenic receptor-β (ER-β) *(12–14)*. 3β-diol may be further hydroxylated in positions 6 and 7 to yield the so-called triols, which induce a rapid and short-lasting inhibition of LH secretion in the rat *(15)*. However, the full biological significance of these polar steroids in the brain as well as in the peripheral structures remains to be clarified. For the purpose of the present review, it is important to recall here that the 5α-reductase-3-hydroxysteroid dehydrogenase system generally coexists, in the brain, with the aromatizing enzyme, while the latter is not present in the anterior pituitary, at least in mammals.

In the brain and in the anterior pituitary of male mammals T and Δ_4-androstene-3,17-dione can also be interconverted through the action of a battery of 17β-hydroxysteroid dehydrogenase isoforms, each possessing oxidative or reductive potential *(16–19)*; Δ_4-androstene-3,17-dione can subsequently be 5α-reduced to yield 5α-androstane-3,17-dione and androsterone, or aromatized to E1. Even if E1 is a minor estrogen, it may be converted into E2 through a reversible reaction catalyzed by specific 17β-HSD isoforms *(17,18)*. The complexity of these metabolic pathways, and the reversibility of some of their reactions, are summarized in Figs. 1 and 2.

It is now clear that the main actions of T and DHT occur via their interaction with the androgen receptor (AR), located in the androgen-responding tissues *(20)*, while E2 and E1, originating from the aromatization of androgens, activate two distinct forms of the estrogenic receptor (ER-α and ER-β) *(12,21)*. At this stage, we want to emphasize once more the concept that some messages of T may be mediated by other families of receptors, as, for instance, the $GABA_A$ receptor, which comes into the picture because it binds

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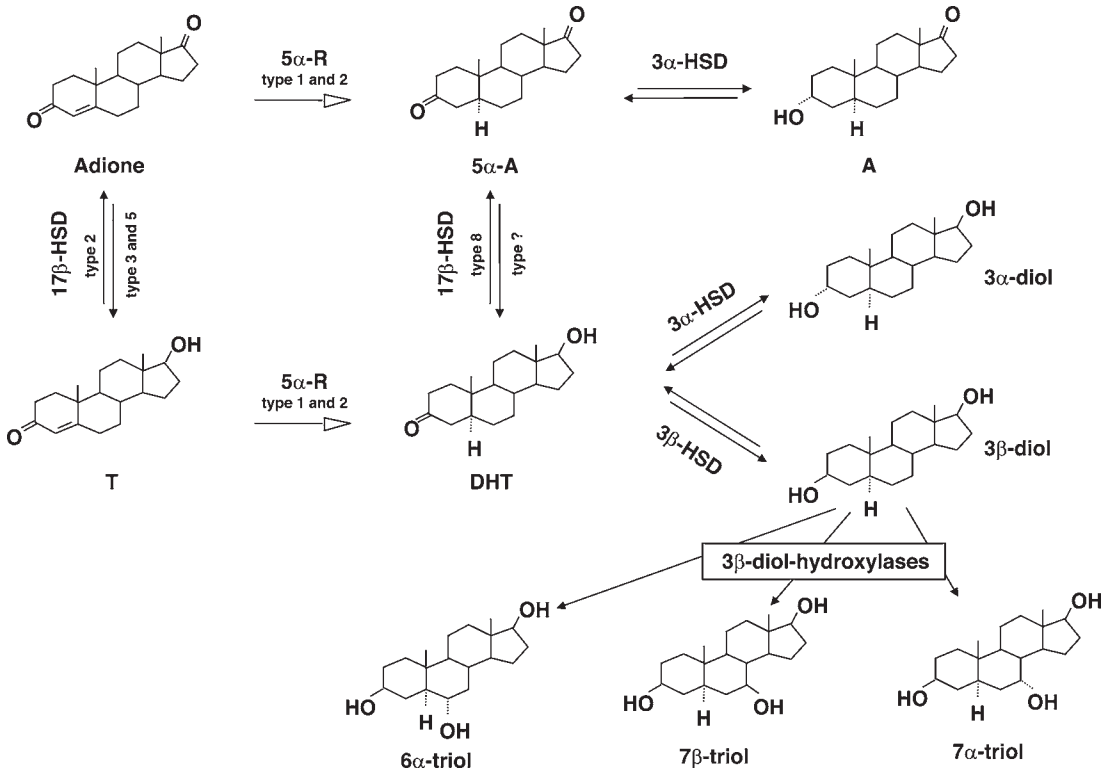

Fig. 1. Androgen metabolism: the 5α-reductase pathway. **A** = androsterone; **Adione** = Δ_4-androstene-3,17-dione; **5α-A** = 5α-androstandione; **DHT** = dihydrotestosterone; **3α-diol** = 5α-androstan-3α,17β-diol; **3β-diol** = 5α-androstan-3β,17β-diol; **T** = testosterone; **6α-3,17-triol** = 5α-androstan-3β,6α,17β-triol; **7β-triol** = 5α-androstan-3β,7β,17β-triol; **7α-triol** = 5α-androstan, 3β,7α,17β-triol; **5α-R** = 5α-reductase; **17β-HSD** = 17β-hydroxysteroid dehydrogenase; **3α-HSD** = 3α-hydroxysteroid dehydrogenase; **3β-HSD** = 3β-hydroxysteroid dehydrogenase.

3α-diol, one of the major metabolites of the process of 5α-reduction of T *(7–11)*. One should also consider the σ receptor *(22)* and even the different forms of the opioid receptors *(9)*. Absolutely peculiar appears to be the role of the ER-β receptor, which, according to recent studies of this and other laboratories *(12,14,21)*, may interact with the final products of both pathways involved in the metabolism of T (the aromatase and the 5α-R 3β-HSD system), because it binds E1 and E2 as well as 3β-diol, even if with a different affinity. Finally, the possibility cannot be overlooked that T and some of its metabolites might also act through the interaction with specific binding proteins present on the cell membrane *(23–25)*.

Bearing all this in mind, it becomes clear that, at tissue and cellular levels, the responses to androgens will depend on the types of androgenic molecules presented to the cell, on the characteristics of the metabolic events occurring in that particular district, as well as on the types of receptors (surface binding sites, AR, ER-α, ER-β, $GABA_A$, etc.) available. Actually, the final effect of androgens, in each organ, tissue, or cell considered, results from an integrated action of the signals simultaneously generated by T and by each of the metabolites formed in the cell. If one accepts this view, it becomes obvious that any event modifying one or the other of the processes just described may result in an alteration of the androgenic effects and, possibly, in the onset of pathological situations.

Scopes of the Review

The present review will be dedicated to the description of two important diseases, recognizing in their pathogenesis the presence of an aberrant AR, namely, the androgen insensitivity syndrome and the spinal and bulbar muscular atrophy, also known as Kennedy's disease. The androgen insensitivity syndrome (AIS), in its complete form, recognizes its pathogenesis in the total loss of function of all ARs present in the body, owing to various types of mutations in the coding gene. The spinal and bulbar muscular atrophy (SBMA) is a particular form of motor neuron disease, linked to a gain of function of the AR due to an abnormal expansion of a CAG (cytosine, adenine, guanine) triplet repeat in its first coding exon; this results in a polyglutamine (poly-Gln) tract of abnormal size in the mutant protein. In both cases, our discussion will be limited to the effects these receptor alterations may induce at the level of the central nervous system (CNS), because previous reviews *(26)* have exhaustively analyzed all other aspects. Before describing these two pathologies, we will provide, in summary, some information on the normal AR gene and on the normal AR.

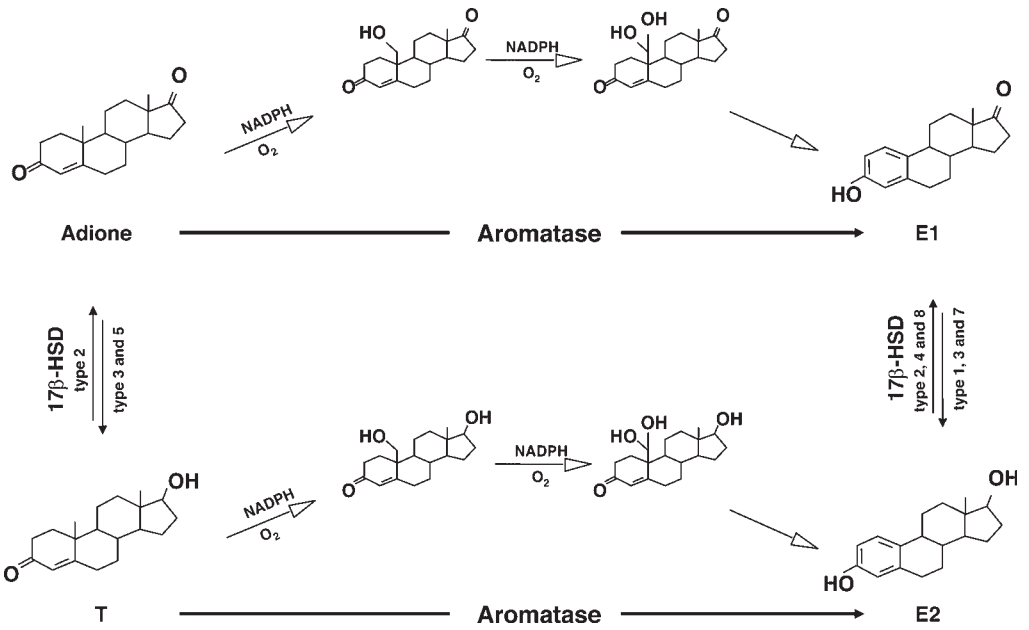

Fig. 2. Androgen metabolism: the aromatase pathway. **Adione** = Δ_4-androstene-3,17-dione; **E1** = estrone; **E2** = estradiol; **T** = testosterone; **17β-HSD** = 17β-hydroxysteroid dehydrogenase.

The Androgen Receptor Gene

The AR gene is localized in the long arm of the X chromosome, in its pericentromeric region at Xq11-q12, and spans a minimum of 54 kbp *(27–29)*. Males have a single copy of the AR gene, while females possess two copies of the gene; however, in females, one allele undergoes random X-inactivation. The open reading frame of AR, like those of the majority of the members of the steroid receptor superfamily, is encoded by eight exons, and results in a protein of approx 900 amino acids, which are organized in several well-defined regions: (1) an N-terminal transactivation region, coded by exon 1, containing stretches of glutamine (polyGln), proline (polyPro), and glycine (polyGly); this region is poorly conserved among the steroid nuclear receptor superfamily members; (2) a highly conserved DNA-binding domain, coded by exons 2 and 3, where each exon codes for one zinc (Zn) finger; and (3) a less conserved C-terminal ligand-binding domain (coded by exons 4–8). The polyamino acid sequences in the N-terminal domain are polymorphic and responsible for the variability in the size of AR; the length of the glutamine repeat (polyGln), in particular, varies between individuals from different ethnic backgrounds *(30)*.

The promoter region of the AR gene is characterized by the absence of a canonic TATA box (TATAbox-less promoter) and CAAT box, but contains GC rich elements, a SP1 binding site, homopurine stretches, cAMP responsive elements and AP1 sites (see ref. *31* for review). Two androgen responsive elements (ARE) have been identified in exons 4 and 5 of the AR gene *(32)*; these selectively control the activation of the AR promoter, being responsible for androgen-mediated upregulation of AR messenger RNA in target cells. Two principal sites of transcription initiation (TIS I and TIS II) *(33)* separated by 11 nucleotides have been identified approx 1100 bp upstream to the translation-start site *(34)*; thus, the primary transcript is characterized by an unusually long 5'-untranslated region (5'-UTR), containing a short open reading frame driven by the first AUG in the 5'-UTR and coding for a peptide of nine amino acids whose functional roles remain to be determined *(35)*. The translation into the AR protein starts at the second AUG. For additional information, see ref. *26*.

The Androgen Receptor

The AR, a member of the steroid receptor superfamily, is a ligand-activated transcription factor that, in its inactive form, is confined in a multiheteromeric inactive complex in the cell cytoplasm *(36,37)* (Fig. 3).

The major intramolecular domains of the AR (ligand-binding domain, nuclear localization signal sequence, hinge region, DNA-binding domain, and transcriptional activation domain) like those responsible for protein–protein(s) interactions, have all been well characterized. The receptor protein interacts either with molecular chaperones and steroid-receptor cofactors, both responsible for the modulation of the biological activity of the AR. The inactive complex contains several chaperones (heat shock proteins, like Hsp90 and Hsp70), that dissociate from the receptor after the binding to the ligands (T or DHT), via the C-terminal ligand-binding domain; this process allows a conformational change induced by the ligand that unmasks the nuclear localization signals of the AR. It has been proposed that this variation of the tertiary structure is also due to post-translational

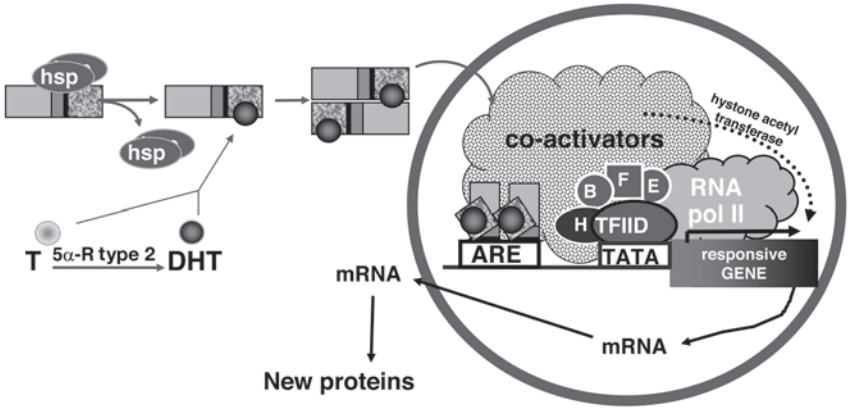

Fig. 3. Mechanism of androgen action. See text for explanation.

modifications, like the phosphorylation of specific serines *(38)*. Moreover, the role of accessory proteins is to fold the receptor to a high-affinity ligand-binding conformation and to allow constant refolding in order to protect the hydrophobic pocket in the ligand-binding domain, until the hormone becomes available *(39,40)*. In any case, the dissociation from the accessory proteins allows the translocation of AR into the cell nuclei, its dimerization, and, through the DNA-binding domain, the interaction with specific enhancer sequences, known as androgen responsive elements (ARE), located up- or downstream of the core promoter region of androgen-responsive gene.

Antiandrogenic ligands (cyproterone acetate and Casodex) also induce translocation of AR into the nucleus, but at a slower rate than DHT; this suggests that AR simply requires "a ligand" for its nuclear translocation. However, agonists or partial agonists direct AR to functional nuclear locations, which differ from those reached using antagonists (punctate vs diffuse distribution) *(37)*. As mentioned before, the nuclear translocation is also accompanied by AR dimerization, required for optimal DNA binding and essential for transcriptional activation leading to "de novo" synthesis of specific mRNAs.

At this stage, the transcriptional control by AR is modulated by complex interactions between the receptor and positive (coactivator) or negative (corepressor) factors. The dimeric receptor binds to the AREs, sequences composed of two symmetric 6 bp separated by a 3-bp spacer and oriented as direct or inverted repeat (also described as glucocorticoid responsive elements, pure GRE:5'-GGTACAnnn TGTTCT-3') *(41)*. Specificity of the AREs is offered by the context where they are located into the promoter (surrounding nucleotides), as well as from the orientation of the repeats (AREs structured as direct repeat cannot be bound by GR, several imperfect direct repeats of the 5'-TGTTCT-3' core recognition sequence are specifically bound by the AR, etc.) *(42,43)*. Moreover, steroid metabolism, the presence of tissue-specific receptors, the milieu of coactivator complexes at nuclear level, and the chromatin structure are also determinants of the specific responses to a given steroid *(44)*. When the AR dimer binds the ARE, the transcriptional activity depends on the formation of specific transcription initiation complexes and the recruitment of other nuclear factors (TFIID, H, B, F, E) *(43)* driven by transcriptional activator (AF-1, AF-2, AF-5) domains of the AR. These proteins bridge the AR dimer with some of the general transcription factors involved in the preinitiation complex on the TATAbox, and/or with other inducible transcription factors acting on a specific promoter context, stabilizing the transcription factor machinery, and allowing the initiation of transcription by RNA polymerase II (pol II) *(38)*. Some of these proteins are enzymes, like the histone acetyltransferase (HAT), that acetylates the histones bound to target genes *(45)*; histone acetyltransferases and deacetylases alter the nucleosome structure, leading to chromatin remodeling; for example, acetylation of histone tails relaxes chromatin packaging, facilitating gene transcription *(46)*. On the other hand, corepressors recruit histone deacetylases, leading to condensation of nucleosomal structures and repression of transcription.

In conclusion, the AR-coactivators complex (a) recruits the general transcription factors to the TATA box and (b) exerts histone acetyltransferase activity destructuring the nucleosomes to allow RNA pol II to transcribe the genes *(47)*; these mechanisms act jointly and activate transcription of target genes. The abnormal size of the polyGln tract in SBMA (see below) alters the transcriptional behavior of the AR, probably because of its close integration into the AR transactivation domain. The capability of AR to properly interact with coactivators and other transcriptional components may then be impaired *(48)*.

The Androgen Insensitivity Syndrome

The androgen insensitivity syndrome (AIS) is an X-linked recessive disorder, resulting in the failure of a normal mas-

culinization in chromosomally male individuals. The pathophysiology of AIS is due to the fact that cells, which normally would be androgen-sensitive, are unable to respond to androgens, because of the presence of qualitative and/or quantitative defects in the AR status. The failure of virilization can be either complete [complete androgen insensitivity syndrome (CAIS) formerly known as the "testicular feminization syndrome"] or partial [partial androgen insensitivity syndrome (PAIS)], depending on the amount of residual receptor function.

Our discussion will be limited to CAIS, because this disorder provides the best example of a pathology in which the efficiency of a steroid hormone receptor is totally lost. For a comparison with PAIS, the reader is referred to the comprehensive review of ref. *49*.

Complete Androgen Insensitivity Syndrome (CAIS)

General Description

The best available data (Danish Patient Registry) suggest an incidence of 1 case per 20,400 live born males for the two forms of androgen insensitivity (CAIS and PAIS). However, the true incidence of AIS may be higher, since the Danish study includes only hospitalized cases. In general, CAIS appears more common than PAIS, although exact data on the respective proportions are not available. No racial differences in the incidence of AIS have been reported so far.

Individuals with CAIS have a 46XY karyotype, but exhibit a complete female phenotype in spite of the presence of a normal testicular steroidogenesis *(50,51)*, because of an abnormality of the AR gene. This abnormality deprives their bodies of functional ARs and, consequently, these individuals are entirely unable to respond to androgens. CAIS subjects exhibit external female genitalia with underdeveloped labia, a normal clitoris and vaginal introitus. The vagina, however, is blind-ending and the internal female genital organs (uterus, Fallopian tubes, upper part of the vagina) are lacking, the development of the Müllerian ducts having been suppressed, during fetal life, by a normal secretion of the Müllerian inhibiting factor [MIF, also called anti-Müllerian hormone (AMH)] by the Sertoli cells. Indeed, even during the first year of life, MIF values have been found to be elevated in patients with CAIS *(52,53)*. This is why many subjects with CAIS require surgical vaginal lengthening. The testes are undescended and are located either in the inguinal canal, in sacs of bilateral hernias, or within the abdomen. The testes of CAIS patients are small in size and their histological examination shows the presence of germ cells arrested in the early phases of their maturation. The phenotype of these patients presents essentially normal female characteristics, with a juvenile type breast development and normal female-like fat deposits *(54)*; however, male-type large hands and feet are frequently evident. Axillary, pubic, and vulvar hair is scanty or absent. Clearly, subjects with CAIS never menstruate because they do not possess a uterus. These subjects are usually raised psychologically as females and normally adopt a female gender type (see below). CAIS patients have a theoretical risk of malignant degeneration of the testes and of developing gonadoblastomas; consequently, early orchidectomy is recommended. CAIS subjects may be submitted to estrogen replacement therapy, the general belief being that these subjects do not require progesterone because they do not menstruate. Some evidence suggests, however, that progesterone therapy, combined with estrogens, may reduce the risk of breast cancer.

Gonadotropin Secretion

Of particular interest for the present review are the data regarding the secretion of the two gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] in CAIS patients. Physiologically, the secretion of these hormones is under a hypothalamic stimulatory control due to the pulsatile secretion of GnRH and under an inhibitory control due to the feedback effect of sex steroids, like androgens and estrogens (see ref. *55* for references).

In subjects with CAIS (postpubertal or adults) basal serum levels of LH are above the normal range and LH responses to exogenous GnRH are comparable to normal controls. On the contrary, the response of FSH to GnRH is greater than normal *(56)*, as it usually happens in normal adolescents who have not reached their final sexual maturity (see ref. *55*). This exaggerated response is surprising, because serum levels of inhibin are normal in CAIS patients (see ref. *55*). A differential response of LH and FSH was also found following the administration of naloxone, the opioid antagonist being able to increase LH but not FSH release *(57)*. The secretion of gonadotropins appeared to be pulsatile in six estrogen-treated orchidectomized CAIS subjects, in the only study reported in the literature *(57)*.

The increased secretion of LH found in CAIS subjects is obviously the consequence of the inability of androgens, produced by the testes, to suppress the release of this pituitary hormone. This occurs despite the fact that T levels are usually normal or slightly elevated *(50,58)*. Serum LH levels would be even more elevated if LH secretion was not tonically inhibited by the presence in the circulation of estrogens originating from the aromatization of T; the local aromatization of androgens in the brain structures controling LH secretion may also be of importance in this respect (see below). As expected, even the administration of large amounts of exogenous T produces only a partial suppression of LH levels *(56,59,60)*, possibly because of the expected aromatization of T into estrogens *(61)*. Indeed, the direct administration of exogenous estrogens to CAIS subjects inhibits gonadotropin secretion much more effectively than androgens *(60)*.

Sex Steroids

As already mentioned, in CAIS patients plasma T levels are similar to those of normal adult men or even higher *(50,*

58,62,63). The levels of plasma DHT are also within the normal male range *(64,65)*, while Δ_4-androstene-3,17-dione tends to be above the normal adult levels *(50,64)*. The values of plasma estrogens (and of estrogen metabolites in the urine) are usually intermediate between those of normal men and of normal women *(66,67)*. The estrogenic quota results from the sum of the amounts directly produced in the testes *(68)*, plus those derived from the peripheral aromatization of estrogen precursors occurring mainly in the adipose tissue *(68,69)*.

Gender Identity and Gender Role

The gender identity and the gender role of CAIS subjects are definitely female, in full agreement with their genital and pubertal development *(54)*. CAIS individuals regularly report female heterosexual activities and fantasies *(54, 70,71)* and do not experience a diminished libido or the inability to reach satisfactory orgasms *(54,70,71)*. Usually, they desire to be married and are ready to take care of adopted children *(70,71)*.

Animal Models of Androgen Insensitivity

There are at least three animal models for CAIS and PAIS: tfm mice *(72)*, tfm rats [tfm stands for "testicular feminization" *(73)*], and, the most recent, AR-knockout mice (ARKO) *(74)*.

Tfm mouse *(72)* exhibits an absolute insensitivity to androgens due to a spontaneous mutation (a single base deletion in exon 1 of the AR gene), which causes a frameshift that introduces a premature termination codon; this creates a truncated inactive form of the receptor protein. The truncated receptor of the tfm mouse still binds androgens, but lacks transcriptional activity.

The tfm rat *(73)* is unresponsive to physiological doses of androgens but responds to 10-fold higher doses *(75)*. This is linked to a single nucleotide missense mutation of the AR gene *(76)*, which results in an amino acid substitution (Arg734Gln) in the steroid-binding domain of the receptor. Reduced androgen binding explains the impaired transcriptional activity.

These two models (tfm mice and tfm rats) may be considered, respectively, the animal equivalents of CAIS and PAIS.

ARKO male mice *(74)* have a female like appearance and their body weight and genitoanal distance (an index of masculinization) are similar to those of female mice. These animals present small testes, which may reside in the inguinal canal. The external genitalia have a feminine appearance, the penis being a microphallus with hypospadia. Serum T levels are lower than in wild-type males. The few ARKO female mice obtained are fertile, with a decreased number of pups per litter *(74)*. Unfortunately, there is no information on the behavioral characteristics of either the male or the female ARKO.

Pathophysiology of the Androgen Insensitivity Syndrome

The basic etiology of CAIS is linked to a loss-of-function of the AR protein due to a mutation of the coding gene. Shortly after the cloning of the human AR *(27,77)*, Brown et al. *(78)* were the first to report that, among seven patients with CAIS, one subject and her affected sibling had a partial deletion of the AR gene, while the other five had normal restriction fragment patterns, suggesting that the molecular pathology was related to a point mutation. The number of reports has increased over the years, and, presently, a few hundred mutations have been described. A database of the mutations of the human AR has been established by Gottlieb et al. *(79, 80)* on the World Wide Web server of the McGill University Montreal, Canada (http://www.mcgill.ca/androgendb). Another database (ftp://ftp.ebi.ac.uk/pub/databases/androgenr) is available at the European Bioinformatics Institute.

Mutation analysis of the AR gene is now commercially available. It detects upward of 95% of the mutations for both CAIS and PAIS. The analysis is performed on DNA obtained from buccal swabs. However, the testing is time-consuming (about 6 wk) and expensive (not covered by insurance).

The mutations described include few cases of complete or partial gene deletions and point mutations resulting either in premature termination or in base deletions, insertion, or substitution. Even if the final result of all mutations is androgen insensitivity linked to an aberrant AR, this results, in practice, is reached via at least three different mechanisms: (1) incomplete synthesis of the AR protein; (2) failure of the physiological interaction between T or other androgenic ligands and the AR, due to alterations of the steroid-binding domain; and 3) the incapability of the complex steroid-AR to interact with the ARE.

The information that will be provided in this section is mainly derived from the article "Androgen insensitivity sindrome" written by Migeon et al. *(49)*.

Complete and Partial Deletions

Three cases of complete gene deletions have been reported in the literature. There are also reports of deletions starting from exons 2, 3, or 4 and extending to the terminus of the gene. Also intragenic deletions involving exon 2, 3, 4, or 5 have been described.

Base Deletions and Insertions

About 30 cases of this type of mutations have been reported, with half of them resulting in CAIS *(79)*. Fifty percent of these mutations involve exon 1, the others are equally distributed among exons 2–8.

Base Substitutions

By far, the largest number of mutations of the AR gene are related to base substitutions *(80,81)*. Of all the sub-

stitutions, 55% result in CAIS, 40% in PAIS, and 5% in mild androgen insensitivity. Relatively, only few mutations occur in exon 1, while the majority are found in the regions coding either for the DNA-binding domain (exons 2 and 3) or for the steroid-binding domain (exons 4–8). It is obvious that a mutation involving a cysteine in one of the two zinc fingers will result in a major disruption of the DNA-binding domain of the AR. In contrast, mutations in other parts of the zinc fingers may result only in PAIS. However, this concept cannot be generalized, as other mutations of the zinc fingers, not involving cysteine, result in CAIS. Also mutations involving an arginine residue in the second zinc finger motif may significantly modify the interaction with ARE and result in CAIS.

Mutations in the steroid-binding domain may affect the levels of androgen binding, the specificity of the binding, and the association/dissociation kinetics of the ligand–receptor interactions. For example point mutations in exon 4 may lead to CAIS, due to transcription of a mutant receptor with decreased stability and an accelerated rate of steroid dissociation. Over 30% of point mutations of the AR gene are located in exon 5, many of which result in CAIS. This is suggestive of an important role of this area of the protein for the binding of the ligand. In this exon, a mutation has been found to be identical to that which occurs in the tfm rat at the homologous residue (arginine 734).

In exons 6 and 7, several mutations of arginine into other amino acids have resulted in CAIS, suggesting an important role for this amino acid. It is of interest that mutations in exon 8, resulting in amino acid substitutions or in a stop codon, also result in CAIS, indicating that this terminal area of the receptor is very important.

Conclusions

This short analysis of the genetics of CAIS suggests some important considerations. The first and more relevant one is that, at the molecular level, CAIS is a disease that recognizes the same pathogenesis, but whose etiology is highly variable. One might go so far as to say that CAIS, at the molecular level, is not a single disease. This conclusion, probably, does not have a great clinical relevance as per today, but must be kept in mind for future attempts of genetic therapy.

Sexual Differentiation of the Brain

The typical female psychosexual orientation present in CAIS subjects undoubtedly stimulates a discussion on the hormonal factors possibly involved in controlling the sexual differentiation of the human brain and in inducing the pre- and/or post-natal "organization" of the nervous centers controlling sex orientation and gender identity. In humans, like in several animal species *(82)*, a sexual dimorphism is definitely present in the morphology of some CNS structures (e.g., the amygdala, especially in its medial portion) *(83,84)*, the frontomedial cortex *(84)*, the hypothalamus *(84)*, the angular gyrus *(84)*, the bed nucleus of the stria terminalis *(85,86)*, as well as in the mechanisms controlling crucial endocrine and behavioral phenomena. For instance, the pulsatile regulation of the secretion of the gonadotropins *(87)*, of prolactin, and of growth hormone *(88)* are typically dimorphic. A sexual dimorphism has also been reported in the expression of different types of behaviors like spatial ability and verbal fluency: this dimorphism is specially interesting, because these particular behaviors are not sexually oriented *(89)*. Data have also appeared indicating that the brain concentrations of a series of central neurotransmitters (e. g., epinephrine, norepinephrine, dopamine, serotonin) and of some neuropeptides (like substance P, cholecystokinin, vasopressin, vasoactive intestinal peptide, the opioids and their receptors) may be different in men and in women *(86,90–97)*. Moreover, some CNS pathologies seem to occur more frequently in one of the two sexes. For instance, Parkinson's disease is a characteristic of men, while depression, insomnia, and other psychological disturbances (e.g., anorexia nervosa) are the prerogative of women.

Because one of the major interests of this article is related to the pathophysiology of CAIS, a short description of the sex differences encountered in the distribution of the AR in the normal human brain seems in order. AR is present in all brain structures, but shows higher concentrations in some specific areas. For instance, in several hypothalamic nuclei, which stain positively for the AR, the staining is generally more intense in men than in women. A sex-linked difference in AR concentrations has been found also in the horizontal diagonal band of Broca, the sexually dimorphic nucleus of the preoptic area (SDN-POA), the medial preoptic area, the dorsal and ventral zones of the periventricular nucleus, the paraventricular nucleus, the supraoptic nucleus, and the infundibular nucleus. The major sex difference in the amounts of AR has been found, however, in the lateral and medial mammillary nucleus, where, again, the concentrations in men are clearly more elevated than in women *(98)*. Because castration is followed by a decrease of the expression of AR in this particular region, it is also possible that the endocrine milieu present at time of observation might partially influence the expression of AR in these areas *(99)*.

In CAIS subjects, the pattern (male or female) of distribution of the anatomical and functional dimorphic parameters described above is totally unknown, one of the possible reasons being that, when the syndrome was first described, there was only scanty information on how CNS parameters might be influenced by the processes leading to the sexual differentiation of the various centers. We believe that, on this particular point, an extrapolation from data obtained in animal models of CAIS is totally acceptable. In adult normal rats, the posterodorsal medial amygdala (MePD), the SDN-POA, and the suprachiasmatic nucleus (SCN) are

sexually dimorphic in volume and neuronal size, both parameters being larger in males than in females. In tfm rats that represent, as we have seen, a typical animal model for CAIS, the volume of all these nuclei and the size of their neuronal bodies have been found to be intermediate between those of normal males and females.

The analysis of the contribution of sex hormones in creating these sex-related differences in the morphology, in the biochemistry, and in the physiology of the human brain deserves a lot of attention, especially if one wants to formulate a clear hypothesis on the situations encountered in CAIS subject. Before entering a more clinically oriented discussion, we will briefly describe the major hypotheses available to explain the sexual "organization" of the mammalian brain.

The first hypothesis assigns the major "organizing" role to the estrogenic molecules deriving from the aromatization of fetal androgens; the second one points to a direct participation of androgenic molecules in this process; while the third underlines the role of sociocultural experiences and of the methodologies of rearing. Obviously, these three hypotheses are not mutually exclusive.

Before entering a detailed discussion of these hypotheses, we feel important to familiarize the reader with the major metabolic pathways metabolizing T and other androgens in the brain and especially in its neuroendocrine components.

The Aromatase

Aromatase is a member of the P450 cytochrome superfamily and is coded by a single copy gene (*CYP19*) located on chromosome 15q21.1. It is a highly conserved enzymatic complex able to bind with high affinity (n*M* range) 3-keto-Δ_4-androgens (T and Δ_4-androstene-3,17-dione) and to transform them into estrogens (E2 and E1, respectively). The process of androgen aromatization (Fig. 2) consists in a series of reactions, occurring in a single catalytic site, involving two consecutive hydroxylations at the C19 methyl group (each requires one oxygen mole and the participation of NADPH), the cleavage of the 19 methyl group, and the rearrangement of the A ring to form the phenolic ring characteristic of estrogens *(100)*. Aromatase expression is restricted to the gonads and to the brain in many vertebrates (from aquatic and avian species to mammals); by contrast, in humans the enzyme is also present in a wide variety of other tissues, including the placenta, the adipose tissue, the breast, the bone, the skin, and the prostate *(101)*. The *CYP19* gene is a very large one (about 120 kbp in length) and is composed of 10 exons. The coding region of the gene spans from exon 2 to exon 10. Upstream to exon 2, there are a number of alternative exons 1, that are spliced into the 5'-untranslated region of the transcript in a tissue-specific fashion *(102)*. As a consequence, the translated protein (503 aa, 58 kDa) has the same amino acid sequence in all tissues, but the factors affecting its expression may vary depending on their interaction with specific promoters upstream of the different exon 1 subtypes [see refs. *103* and *104* for references]. To underline the importance of aromatase in brain differentiation and function, the major transcript present in the brain of rodents *(105)* and humans *(106)* possesses a specific exon 1 (exon 1-f), even if small amounts of transcripts carrying other exon 1 subtypes may also be present *(105,107)*.

Within the normal brain of mammals, aromatase is expressed only in neurons *(108)* and is mainly localized in structures involved in the control of the reproductive axis, such as the hypothalamus, the preoptic area, and the limbic system *(103)*. A recent study performed in our laboratory in the developing rat has demonstrated that, during embryogenesis, there is a clear-cut sex-related dimorphism in aromatase expression, the enzyme being significantly higher in the male than in the female hypothalamus *(109)*. Moreover, the analysis of the relative expression of "brain-specific" and "total" aromatase transcripts reveals that, in fetal life, all the aromatase mRNAs carry the exon 1-f, both in males and in females. After birth there are no changes in males, while in females other transcripts might also be present *(109)*.

The 5α-Reductase

Two isoforms of the 5α-reductase (5α-R type 1 and 5α-R type 2) have been cloned (see ref. *5* for review). The protein sequences show a modest homology (about 47%), a molecular weight of 28–29 kDa, and a high number of hydrophobic amino acid residues, which accounts for the intrinsic membrane localization of both enzymes. Both forms of the enzyme 5α reduce all 3-keto-Δ_4-steroids, like T and other androgens, progestagens, and corticosteroids. However, they possess different kinetics and specificity. The affinity for the type 1 isoform is in the m*M* range for all the substrates, and is much lower than that for the type 2 isoform, which is in the n*M* range *(110)*. The two isoforms exhibit different pH optima: 5α-R type 1 works well over a wide pH range (from 5 to 8), while the 5α-R type 2 shows a narrow pH optimum around 5.5 and a very low activity at pH 7.5. "Selective" blockers of the 5α-R type 2, like finasteride *(5)*, "preferential" type 2 blockers like Suramine *(111,112)*, and "specific" inhibitors for the type 1 isoform *(113)* have already been obtained. Generally, the type 1 gene is widely expressed in various tissues, with the highest levels in the liver. On the other hand, the type 2 isoform is mainly concentrated in the androgen-dependent structures, such as the stromal component of the prostate, the epididymis, and the seminal vesicles; low levels, if any, have been detected in other tissues. Subcellular localization studies performed on the two 5α-Rs indicate that they are strictly associated with cellular membranes *(6)*, but in different cellular compartments: the 5α-R type 1 is associated with cell nuclei and the 5α-R type 2 mainly with the microsomal fraction *(6)*.

A considerable 5α-R activity is present in the CNS, especially in its neuroendocrine components *(114,115)*. The

5α-R type 1 is expressed both in the neuronal and in glial cells *(116)*, while the 5α-R type 2 is present exclusively in neurons *(109,117)*, indicating its particular relevance.

The Aromatase Hypothesis

According to this hypothesis, the sexual differentiation of the brain toward male patterns is thought to be "imprinted" or "organized" by hormonal signals originating from the developing male gonads. Mainly on the basis of animal experiments (conducted principally in rodents), it is assumed that this process is initiated by the androgens secreted by the fetal and neonatal testis *(82,97,104)*. Two subsequent peaks of androgen secretion (one during the fetal life and the second during the first days following birth) occur in the majority of mammalian species as well as in humans *(1,118,119)*. Androgens are then converted intracellularly, in specific brain regions (e.g., hypothalamus, limbic system) into estrogens by the enzyme aromatase *(103,104)*. Locally formed estrogens become then, paradoxically, the major effectors of the permanent masculinization of the brain.

Data suggesting that estrogens might play a role as differentiating agents also in primates and in humans are available *(120)*. In the first place, one must consider that all the components of the system (androgenic precursors, the enzyme aromatase, the estrogenic receptors, and so on) are present in the normal human brain. Aromatase has been shown to be expressed throughout the brain, including the hypothalamus, both in men and in women *(98,107)*. The occurrence, in the human brain, of the two estrogenic receptors ER-α and ER-β *(13,99,121,122)*, and particularly the presence of high levels of ER-β in the hypothalamic–preoptic area *(12,13,122)*, imply the existence of an operational estrogenic mechanism and underline, at the same time, the fact that ER-β may play a major role in the control of the sexual differentiation of the human CNS. Because ER-α and ER-β can act on the estrogen responding element (ERE) of the responding genes not only as homodimers, but also as heterodimers *(123)*, it is even possible that some aspects of brain differentiation could be triggered only if the ER-β is present.

Also observations made in clinical cases tend to underline the validity of the aromatase hypothesis for the fine tuning of the human CNS. For instance, male subjects with cloacal exstrophy, who were assigned to the female sex at time of birth and who later declared themselves males, seem to prove that the prenatal T surge may also act as a differentiating signal in the human brain *(124)*. Additional support to the hypothesis that estrogens may also exert an "imprinting" role in humans comes from the observations that girls whose mothers were exposed during pregnancy to the synthetic estrogen diethylstilbestrol (DES) showed a high risk of developing at least a bisexual behavior *(125,126)*. For additional comments see the section "The lesson from other pathologies."

Finally, it is interesting to note that some xeno- and phytoestrogens can act as specific agonists at the level either of the ER-α or of the ER-β *(21)*, an observation that suggests also including nutritional estrogens and estrogen-like environmental chemicals in the list of the possible "organizers" of some specific CNS mechanisms, including those leading to the masculinization of the brain.

The Androgenic Hypothesis

Although it is generally accepted that the aromatization of T into estrogens is the major mechanism responsible for the sexual differentiation of the brain toward male patterns, the participation in this process of androgens "per se" cannot be excluded. The ARs are widely distributed in the fetal/neonatal brain and show peak concentrations in the areas controlling the reproductive system *(127,128)*. Moreover, the fact that AR concentrations are particularly elevated in the brain around birth, and are higher in male than in female neonatal animals *(127)*, also support the concept of a direct effect of androgens on some developmental phenomena. A role for androgens is also underlined by the results which indicate the existence of a selective ontogenetic pattern of expression and regulation of the two 5α-R genes (particularly 5α-R type 2) in the brain of the fetal and neonatal rat. It has been reported that the messenger of 5α-R type 2 is undetectable on pregnancy d 14, increases after pregnancy d 18 and peaks on postnatal d 2 *(109,117)*. This pattern of expression appears to be correlated with the rate of production of T from the fetal and neonatal testis, as shown by the fact that it can be suppressed by the antiandrogen flutamide *(117)*.

In this context, it is relevant to quote that DHT seems to be essential for the control of the growth and of the organization of selected neuronal populations like the sexually dimorphic nucleus of the accessory olfactory tract, which is smaller in males than in females, and which further decreases, in males, when DHT is administered *(129)*. Moreover, in male rats, DHT is needed, together with estrogens, to allow a full masculine development of the sexually dimorphic spinal nucleus of the bulbocavernosus *(130)*. For additional information see the section on "The spinal and bulbar muscular atrophy."

Finally, it is interesting to note that both T and DHT are morphogenetic signals for the development of hypothalamic neurons expressing the aromatase *(130,131)*, and may influence the plasticity and the synaptic connectivity of hypothalamic aromatase-positive neurons *(132)*. These effects are AR-mediated, because they are suppressed by the antiandrogen flutamide, but not by the antiestrogen tamoxifen *(132)*. These interesting observations underline the existence of a very strict link and of a mutual interplay between the direct effects androgens may exert in the process of sexual differentiation of the brain and the mechanisms depending, on the contrary, on the aromatization of androgenic precursors.

One additional consideration may reinforce the argument that androgens may also directly masculinize the brain in humans: during fetal life, both sexes are exposed to high levels of estrogens, while only males are also confronted with elevated amounts of androgens *(133)*. Indeed, in human fetuses, at 34–41 wk of gestation, and in neonates, serum T levels are 10-fold higher in males than in females *(134)*.

Before concluding this section dedicated to the role of sex steroids in the control of the sexual differentiation of the brain, the authors want to underline that specific receptors for practically all hormonal steroids are present not only in neurons, but also in glial elements (e.g., astrocytes, oligodendrocytes, Schwann cells) *(135–138)*. The 5α-R type 1 is also regularly found in glial elements, while the aromatase appears only when glial cells are induced to react to some stimuli *(139)*. These observations may have important physiological implications, because of the close cross-talk existing between neurons and glial elements. However, the studies on the possible participation of the glia in the process of the sexual differentiation of the brain are still in their infancy *(140)*. The problem remains then elusive at the moment, but certainly deserves to be deeply investigated in the future.

The Sociocultural Experience Hypothesis

There is no doubt that sociocultural experiences play a major influence in facilitating the expression of the differentiation of the brain. These influences become more relevant in the higher species and certainly are absolutely essential in humans. Of particular importance are those pertaining to the world of rearing and of infant education.

Interpretations of the Complete Androgen Insensitivity Syndrome

As we have previously discussed in detail, individuals with CAIS behave psychologically like females, and desire to be assigned to the feminine sex. This occurs even if their brains possess a full aromatization capability and are exposed to estrogen precursors of testicular and adrenal origin from the beginning of fetal and placental steroidogenesis. Moreover, because ARs are absent or nonfunctioning, the only sex hormone receptors capable of eliciting hormonal responses are the ERs in their two forms, ER-α and ER-β. Unfortunately there is no direct evidence available, but there is no reason to believe that their presence and distribution in the brain of CAIS subjects should be different from normal. Even if all conditions for an effect of estrogens as "masculinizing" agents are probably present, the brains of these patients are not masculinized. Several hypotheses may be put forward to explain this anomaly. First of all, studies performed in rodents, in primates, and even in humans *(120)* have clearly indicated that, for this type of "imprinting," the time and the amplitude of the estrogenic signals are of crucial importance *(141)*. Obviously, these two parameters (time and amplitude of the signals) are not respected in CAIS subjects. It is possible that the excess of estrogen formation might have disrupting rather than organizing effects on the brain. Brawer et al. *(142)* have reported that the administration of elevated doses of estrogens induces progressive multifocal lesions of the arcuate nucleus, one of the hypothalamic nuclei crucial for the control of the sexual differentiation of the brain in rodents. Moreover, Davis et al. *(143)* and Nordeen et al. *(144)* have reported that the incidence of apoptosis is higher, in several cerebral districts, in females than in males. A second possible explanation is provided by the fact that, in the affected individuals, estrogens are working alone, in the absence of any action of T and DHT, in a situation in which a cooperation of estrogens with androgens might be normally of importance. As previously mentioned, observations made in rodents, in non-human primates, and in humans have underlined possible direct effects of androgens in the process of the masculinization of some brain structures *(124,145)*. For instance, as already seen, DHT seems to be essential, alone or together with estrogens, for the development and the organization of selected neuronal populations like the sexually dimorphic nucleus of the accessory olfactory tract *(129)* and the sexually dimorphic spinal nucleus of the bulbocavernosus *(130)*.

A third hypothesis may sound like a paradox. We have previously stressed the fact that androgens, via an action mediated by AR and exerted on nervous cells that are positive for the aromatase, may not only increase the number of these cells, but also facilitate the biosynthesis of their specific enzyme. This increase of the amounts, and, consequently, of the efficiency, of the aromatase would obviously be absent in patients with CAIS, owing to the total inactivity of T and other androgens.

It is also evident that, in this disease, the sociocultural experience is essential in determining sex-related behaviors, since these subjects are usually assigned to the female sex at time of birth and are consequently educated and reared as females from the very beginning of their lives.

The Lesson From Other Pathologies

For those who will reject the hypotheses we have just proposed, the female sexual differentiation of the brain of CAIS subjects would provide one argument against the theory that the process of aromatization might play a pivotal role in inducing the sexual "organization" of the human brain toward male patterns. We will consider, in the next few paragraphs, whether this conclusion will find support in other human pathologies.

Deficiency of the 5α-Reductase Type 2

The genetic deficiency of the enzyme 5α-R type 2, or Imperato-McGinley syndrome *(55,146)*, is a disease that should be critically analyzed in order to better understand

the mechanisms supervising the sexual organization of the human brain. The syndrome, which is inherited as an autosomal recessive trait, is due to a mutation of the 5α-R type 2 gene, which results in the synthesis of a mutated enzyme unable to convert T into DHT in the androgen-dependent tissues, where this isoenzyme is the predominant form. Over 30 different mutations scattered over the gene have been described so far. The majority of these mutations are homozygous (see ref. *147* for references). Males affected by this pathology present ambiguous external genitalia at birth and, before this syndrome was discovered, were brought up as females. However, at time of puberty, the increased secretion of T from the testes, and the presence of a functional 5α-R type 1, could overcome the deficiency of the type 2 isoenzyme, leading to a normal male development of the secondary sex characteristics *(148)*. After puberty, these subjects claimed then to be assigned to the male gender *(146)*. Because the aromatase was working in their prenatal brains, these subjects may be quoted as an example proving the validity of the aromatization theory also for the human brain. It emerges that, following a precocious screening, the subjects affected by this disease should be brought up as males and that their genitalia, when necessary, should undergo plastic surgery in a male direction. It is also evident that subjects with the Imperato-McGinley syndrome demonstrate that exposure of the brain to T and to other aromatizable androgens during development appears to have a greater impact in determining male gender identity than do the sex of rearing and other sociocultural influences *(149)*. Similar general conclusions may be reached following a correct analysis of patients with the deficiency of the enzyme 17β-hydroxysteroid dehydrogenase type 3 *(150,151)*, the 17β-HSD isoform involved in the formation of T and E2, respectively, from Δ_4-androstene-3,17-dione and E1.

Congenital Adrenal Hyperplasia

The congenital adrenal hyperplasia is a rather common form of hyperandrogenism in women, which occurs in two forms, one precocious and the other called "late-onset" (see ref. *55*). Both forms are due to an inherited defect of the P450c21 hydroxylase, one of the key enzymes involved in the biosynthesis of cortisol. The deficiency of this enzyme results in a decreased capability of the adrenal gland to form glucocorticoids and, consequently, in an accumulation of cortisol precursors that may be converted into androgens *(152,153)*. Because the feedback regulation of ACTH secretion is lost due to the deficiency of cortisol, in these subjects an increased adrenal size and an overproduction of androgens are observed. The excess of adrenal androgens during pregnancy induces the virilization of the external genitalia of female fetuses. Females born to mothers with this disease (or having the same mutation) and not treated during pregnancy (see below) present signs of tomboyism, as well as other aspects of male-oriented behaviors *(154)*. This fact would suggest that, in these subjects, the masculinizing "imprinting" linked to the aromatization of the excess of circulating testosterone is prevailing over the spontaneous differentiation toward a female brain that otherwise would have occurred. However, the role of the sociocultural influences cannot be disregarded in these patients. Actually, because of the ambiguous genitalia noted at birth, they were usually assigned to the male sex and raised as males. Obviously, this should be a story of the past, because it is now imperative to treat the pregnant mothers bearing these fetuses *in utero* with dexametasone. This synthetic corticoid would reestablish normal ACTH secretion and eliminate the adrenal hypertrophy together with the ensuing hyperandrogenism.

Aromatase Deficiency

A final answer to the problem of the role eventually played by the aromatization hypothesis in humans was expected from the description of clinical cases of aromatase deficiency.

Aromatase deficiency is a very rare disease, only 11 cases having been described so far (six females and five males). In this pathology, a mutation in the aromatase gene results in an inactive enzyme, which is unable to convert androgens into estrogens. We are confronted with an autosomal recessive condition (the point mutations identified so far are mainly located in exons 9 and 10 of the aromatase gene) in which estrogen biosynthesis is virtually absent. During pregnancy, fetal androgens are not converted into estrogens owing to the absence of the aromatase in the placenta, and this results, in the mother, in increased levels of plasma T and in low estrogen levels. Thus, pregnant women bearing a child with aromatase deficiency exhibit hirsutism, that spontaneously resolves post-partum. Serum levels of E2 and particularly of estriol are extremely low in the third trimester of pregnancy in the mothers bearing infants with aromatase deficiency.

The disease manifests itself both in female and in male subjects. In females, it may be suspected at birth, because the female baby presents pseudohermaphroditism due to virilization of the external genitalia. In adult females, manifestations include delay of puberty, breast hypoplasia, and primary amenorrhea with multicystic ovaries.

In males, the diagnosis occurs later (frequently in adulthood) mainly on the basis of the tall stature of the subjects, who also exhibit eunuchoid proportions of the skeleton, due to incomplete epiphyseal closure. Osteoporosis and obesity are usually present. Administration of low doses of estrogen leads to bone maturation, to complete epiphyseal closure, and to an increase in bone mineral density. Plasma levels of estrogens are very low, while FSH, LH, and T are increased. The testes are usually small or atrophic, two patients (out of five) presenting uni- or bilateral cryptorchidism. Spermogram shows an oligoasthenospermia *(155–157)*.

The disease may occur in the same family. A mutation in the *CYP19* gene has indeed been described in a sister and a

brother. The 28-yr-old XX proband, followed since infancy, exhibited the cardinal features of the aromatase deficiency syndrome as previously defined. She had non-adrenal female pseudohermaphrodism at birth and underwent repair of the external genitalia, including a clitorectomy. At the age of puberty, she developed progressive signs of virilization, with no signs of estrogen action, hypergonadotropic hypogonadism, polycystic ovaries, and tall stature. The basal values of T, Δ_4-androstene-3,17-dione, and 17-hydroxyprogesterone were elevated, whereas plasma estradiol was low. Cyst fluid from the polycystic ovaries had a strikingly abnormal ratio of Δ_4-androstene-3,17-dione and T to estradiol and estrone. Hormone replacement therapy led to breast development, resolution of ovarian cysts, and suppression of the elevated FSH and LH values. Her adult height was 177 cm. Her only sibling, an XY male, was studied at 24 yr of age. The height of the brother was 204 cm with eunuchoid skeletal proportions; the weight was 135 kg. He was sexually fully mature but had macroorchidism. The plasma concentrations of T, DHT, and Δ_4-androstene-3,17-dione were elevated, while E2 and E1 were less than 7 pg/mL. In this case, plasma FSH and LH concentrations were more than three times the mean normal values. The bone age was 14 yr at a chronological age of 24. Bone mineral densitometric indexes were consistent with osteoporosis. Hyperinsulinemia, increased serum triglycerides, total cholesterol, and low-density lipoprotein cholesterol and decreased high-density lipoprotein cholesterol were detected. For the reasons mentioned above, during both pregnancies, the mother exhibited signs of progressive virilization that regressed post-partum.

The possible impact of lack of estrogens on the psychosexual development of both women and men with aromatase deficiency was obviously considered *(155,158,159)*. Thus far, no evidence is available to suggest gender identity problems in these patients. Even in the absence of the aromatase, men affected by this pathology display a masculine identification and a psychosexual orientation consistent with their genotypic sex *(160)*. Also, sexual activity was reported to be normal. However, the libido of at least one of the male patients was severely depressed and could be restored with estrogen treatment (see ref. *160*).

This disease, at a first glance, seems to provide evidence showing that the conversion of androgens into estrogens does not exert, in the developing human brain, the same crucial role played in the animal species studied so far. There are, however, a few points that deserve discussion. First of all, in the majority of the studies so far reported, estrogens have been found to be low but not totally absent; moreover, the deficit of estrogens during pregnancy appears particularly in the third trimester, when, probably, the process of the "imprinting" of the brain is already concluded. Finally, the possible role of exogenous estrogens has never been taken into consideration in analyzing this pathology. From these considerations it appears that the negative conclusion reached by some authors is at least premature and that additional studies on new cases are absolutely necessary.

Estrogen Insensitivity

Usually, the only case of a male subject with insensitivity to estrogen linked to a deficit of ER-α is discussed together with the cases presenting aromatase deficiency, because his phenotype showed anomalies similar to those we have just described. This 28-yr-old man presented with a tall stature (204 cm), incomplete closure of the epiphyses (bone age 15 yr), with a history of continued linear growth into adulthood despite normal pubertal development and a normal gender identification. Serum FSH, LH, E2, and E1 concentrations were elevated, but T concentrations were normal. There were no physical or biochemical responses to exogenous estrogens administered chronically in high doses. DNA analysis revealed a homozygous status with a point mutation of both ER-α alleles; specifically, a cytosine to thymine transition at codon 157, predicting a truncated receptor protein lacking the DNA- and ligand-binding domains. The parents, in good health, were heterozygous carriers *(161)*.

In our opinion this subject should be kept totally separated from the cases presenting aromatase deficiency, because, even if not proved clinically, some efficacy of estrogens should have been expected in this subject: especially at the level of the brain and of the other structures where ER-β is the prevailing form of ER. At time of the discovery of this patient, ER-β was not known and it is at least surprising that the subject was not evaluated later for its presence or for its responses to ER-β specific ligands. In our opinion this case provide two types of information: (a) the aromatase hypothesis keeps its validity also in humans; and (b) as discussed in another section of this article, ER-β may participate in the masculine sexual differentiation of the brain in humans. In this line of thinking, one may quote the evidence originating from three animal models. In male mice with a knockout of the ER-α gene (αERKO) mounting behavior was normal *(162)*; all components of sexual behavior were intact also in male mice lacking the ER-β gene (βERKO) *(163)*; by contrast, double knockout (α-βERKO) male mice did not exhibit any component of sexual behavior *(164)*, indicating that some sort of redundancy is present in this important system.

Conclusions

In conclusion, the human data contradicting the aromatization hypothesis appear to have the same weight of those favoring it. Moreover, it is conceivable that, in higher vertebrates and particularly in primates and in men, the process of sexual differentiation of the brain, which plays such a crucial role for the propagation of the species, might be controlled by multiple mechanisms including the sociocultural environment and hormonal factors, like estrogens and

androgens. In this context, it would be extremely useful to obtain also data on the effects exerted on this process by other hormones, which, like progesterone and cortisol, are highly produced during pregnancy. Classical intracellular receptors for these hormonal steroids have been shown to be present not only in the adult CNS, but also in many structures of the developing brain *(165,166)*.

The Spinal and Bulbar Muscular Atrophy

The spinal and bulbar muscular atrophy (SBMA) is the first identified member of a large class of neurodegenerative diseases, which includes Huntington's disease (HD), the spinal–cerebellar ataxias (SCAs) 1, 2, 3 (or Machado–Joseph disease), 6, 7, and 17; as well as dentatorubral and pallidoluysian atrophy (DRPLA) *(167,168)*. These disorders, although rare, represent the most frequent type of human hereditary neurodegenerative diseases, and are possibly due to similar neurotoxic mechanisms *(168,169)*. These pathologies are presently known as "CAG/polyGln-related inherited neurological disorders" *(167,170,171)*, because a common feature of all these diseases is the abnormal presence of disease-specific but unrelated proteins containing an elongated polyGln tract (e. g., huntingtin in Huntington's disease, ataxins in SCAs, atrophin in DRPLA). The presence of these pathologically mutated proteins is responsible for the appearance of intracellular aggregates, which may participate in the process of neurodegeneration. Surprisingly, the mutated protein present in SBMA is the receptor for androgenic hormones (AR), which is known in its structure since 1988 *(27,77,172)*. This has obviously facilitated the identification of the mutated protein. In SBMA patients, the majority of the aggregates are localized in the cell nuclei or in the cell cytoplasm *(173)*. The aggregates display a pale granular and fibrillary morphology, with no membranes *(174)*, and resemble those detected in several other neurodegenerative pathologies with a different etiology (e.g., Alzheimer and Parkinson disease, prion disorders), suggesting the existence of common deleterious effects on neuronal functions and survival.

General Description

This disease is characterized by the selective loss of motoneurons in the anterior horn of the spinal cord; this process is accompanied by the depletion of sensory neurons in the dorsal root ganglia (DRG), as well as by the selective degeneration of motoneurons also in the brainstem (motoneurons of the lower cranial nerves). The typical age of onset occurs around 30–50 yr and the progression rate may vary between 20 to 40 yr; therefore, the neurodegeneration progresses very slowly; however, both parameters (age-of-onset and progression rate) are highly variable. In general, the elevated loss of motoneurons occurring in the spinal cord and in the bulbar region results in muscle weakness and atrophy, fasciculations, dysphagia, and dysarthria. The loss of DRG neurons is responsible for abnormalities in sensory function in the distal extremities occasionally seen in some patients *(175)*. The neurological symptoms are often associated with mild androgen insensitivity, i.e., gynecomastia, feminized skin changes, testicular atrophy with involution of Leydig cells, and occasionally impotence, oligospermia or azoospermia.

Pathophysiology of the Spinal and Bulbar Muscular Atrophy

As stated before, the AR gene has been found mutated in this particular type of motoneuron disease. The AR mutation consists of an abnormal expansion of a CAG (cytosine, adenine, guanine) triplet repeat sequence located in the first coding exon of the AR gene; the expanded CAG repeat encodes a polyglutamine (polyGln) tract of abnormal size in the mutant protein.

This PolyGln tract also presents several peculiar features in a normal population; for example, the tract is highly polymorphic and ranges from 15 to 37 polyGln *(176)*; notably, the CAG repeat length varies in different racial populations, African-Americans having the shortest and Asians the longest; Caucasians have CAG repeats intermediate between these two populations *(30)*. In SBMA patients, the CAG repeat is expanded over 38 *(170,176)*, at least twice as long as in the normal population average. Several studies have demonstrated that there is a strong inverse correlation between the size of the polyGln expansion and the age-of-onset, severity of the disease, and progression rate *(171,177)*; however, some exceptions are known; for example, in two siblings with different pathologic elongations of the AR polyGln tract, neurodegeneration preferentially occurs earlier in the sibling with the shorter repeat length *(178)*. Therefore, in addition to CAG repeat length, other factors may modify the onset and the progression of the disease.

As mentioned before, mild androgen insensitivity (i.e., gynecomastia, feminized skin changes, testicular atrophy with involution of Leydig cells, and occasionally impotence, oligospermia or azoospermia) may be observed in several cases of this disease. These endocrine signs in SBMA are usually ascribed to a reduction of the transcriptional activity of the mutant AR in the structures involved *(179)*. However, another explanation may be suggested. We have been able to show that normally the hypothalamic neurons that secrete GnRH (i.e., the major controller of all reproductive phenomena) express the AR and the 5α-R type 2 *(180–182)* and are sensitive to the feedback effects of T and DHT through their interaction with the AR *(180)*. In our opinion, the decreased fertility and the endocrine signs present in Kennedy's patients might be linked also to the fact that the AR present in GnRH neurons is mutated, and, consequently, may alter the functionality of the GnRH system (see below). Unfortunately, the low number of GnRH secreting cells in the human brain (about 200–1000 scat-

tered in the hypothalamus) does not allow a direct evaluation of this hypothesis.

To design future approaches to treat SBMA patients, it is extremely important to understand the physiological role of androgens in spinal cord motoneurons. This has been studied extensively, but several aspects are still obscure. One of the major target of androgen action in the CNS are the motoneurons of the spinal nucleus of the bulbocavernosus (SNB) system *(183)*, known as Onuf's nucleus in humans. In this structure, androgens control the development and adult maintenance of SNB at different stages *(184)*, by acting in two steps at time of birth (during sexual differentiation) and at the beginning of puberty, when androgens modulate the formation of synapses at neuromuscular junctions controlling the growth and maturation of SNB dendritic branches. In adulthood, androgens are involved in the maintenance of the size of motoneurons *(185)* and of their dendrites in SNB; moreover, androgens act also on the muscle fibers, the target of motoneuron *(186)*. It is known that androgen deprivation reduces the somatic size and the dendritic length of motoneurons, affecting also the number of chemical and electrical (gap junction) synapses; by contrast, androgen replacement therapy reverses these phenomena *(187–189)*. In addition, androgens promote the re-growth of several peripheral nerves after resection, like the hypoglossal *(190)*, facial, and sciatic *(191)* nerves; all these nerves derive from motoneurons that express high levels of AR *(192–195)*. The molecular mediators of the androgenic effects on motoneurons are generally structural proteins like actin, tubulin, and gap junction channels *(183,189)*, modulated by the native androgen T or by its 5α-reduced derivative DHT formed *in loco* by the 5α-R type 2; in fact this isozyme is highly expressed in these cells *(196)*. Using the differential display–polymerase chain reaction technique (DDPCR) and subsequently real time RT-PCR analysis on RNA derived from immortalized motoneuronal cells, we have recently demonstrated that activated AR controls the expression of neuritin *(197)* a protein responsible for neurite elongation *(198–201)*. Neuritin gene is already expressed in basal condition in immortalized motoneurons, but is selectively up-regulated by androgens in a specific manner, since the DHT effect is counteracted by the anti-androgen Casodex. DHT also induces neurite outgrowth in immortalized motoneurons expressing AR, while testosterone is less effective and its action is counteracted by the 5α-R2 inhibitor finasteride, suggesting that the only active androgen in motoneuron is the 5α-reduced compound DHT. Moreover, the androgenic effect on neurite outgrowth was completely abolished by silencing neuritin with siRNA, suggesting that neuritin is required for the effects of DHT in motoneuron *(197)*.

Because the transcriptional activity of the mutant SBMA AR is reduced *(167,202)*, it may be proposed that a loss-of-function of the receptor may be involved in the pathogenesis of the disease, possibly through a reduced activation of the neuritin-mediated dendrite or axonal outgrowth in target motoneuronal cells. This mechanism is almost certainly involved, even if it is not the only one. In fact, a large body of experimental and clinical observations collected in all CAG/PolyGln-related diseases strongly suggest that the elongation of the polyGln tract confers a neurotoxic gain-of-function to the mutant proteins. First of all, as extensively discussed in a previous section of this article, a large number of AR mutations may partially or completely inactivate AR functions, or even prevent the synthesis of the AR protein: these mutations, when are responsible for CAIS, do not induce neurological signs of motoneuronal degeneration in patients with this disease. Second, the mice carrying an inactivated AR gene, the tfm mice, also have no signs of motoneuronal loss *(203)*, and similar negative observations have been also made in an ARKO mouse *(74)*. Finally, the transgenic mouse models for SBMA obtained so far *(204–208)* showed motoneuronal dysfunction or death even in the presence of endogenous wild-type AR, suggesting that the mutant AR is responsible for neurotoxicity.

Interestingly, females carrying the heterozygous CAG expansion in the AR gene are asymptomatic *(209)*. Initially, this has been explained by the fact that the X-chromosome in female is randomly inactivated, preserving at least 50% of total motoneurons from AR toxicity. However, recently, two women homozygous for SBMA have been found and they do not show clinical sign of neurodegeneration *(210)*. In these two cases, the gain of neurotoxic function in SBMA AR could be balanced by a lower level of expression of AR in female motoneurons, or linked to lower amounts of circulating T. Katsuno et al. *(206,211)* developed a SBMA mice model and found that clinical signs of the diseases only appear in male animals. Female transgenic mice showed only minor manifestations, that markedly deteriorated with T administration. In this case, the mutant AR is randomly distributed into the genome, and the X-inactivation effect cannot be involved; therefore, T seems to be responsible for the appearance of the disease *(206)*. The same authors have then examined the effect of androgen-blocking drugs on SBMA mouse model, and demonstrated that leuprorelin, a GnRH agonistic analog that reduces T release from the testis, rescued motor dysfunction in male transgenic mice; therefore, leuprorelin may be a good candidate for the treatment of SBMA *(211)*.

Role of Aggregates

It is still unknown why aggregates appear only when the polyGln length reaches a critical threshold size. It has been proposed that the polyGln tract of abnormal size may intrinsically self-associate, and then recruit other molecules of mutated proteins. Synthetic peptides containing poly-L-Gln are insoluble in vitro and tend to acquire a beta-pleated sheets structure, stuck together by hydrogen bonds *(212–214)*.

Another hypothesis is that aggregates are formed as abnormal cell response to the elongated polyGln. In fact,

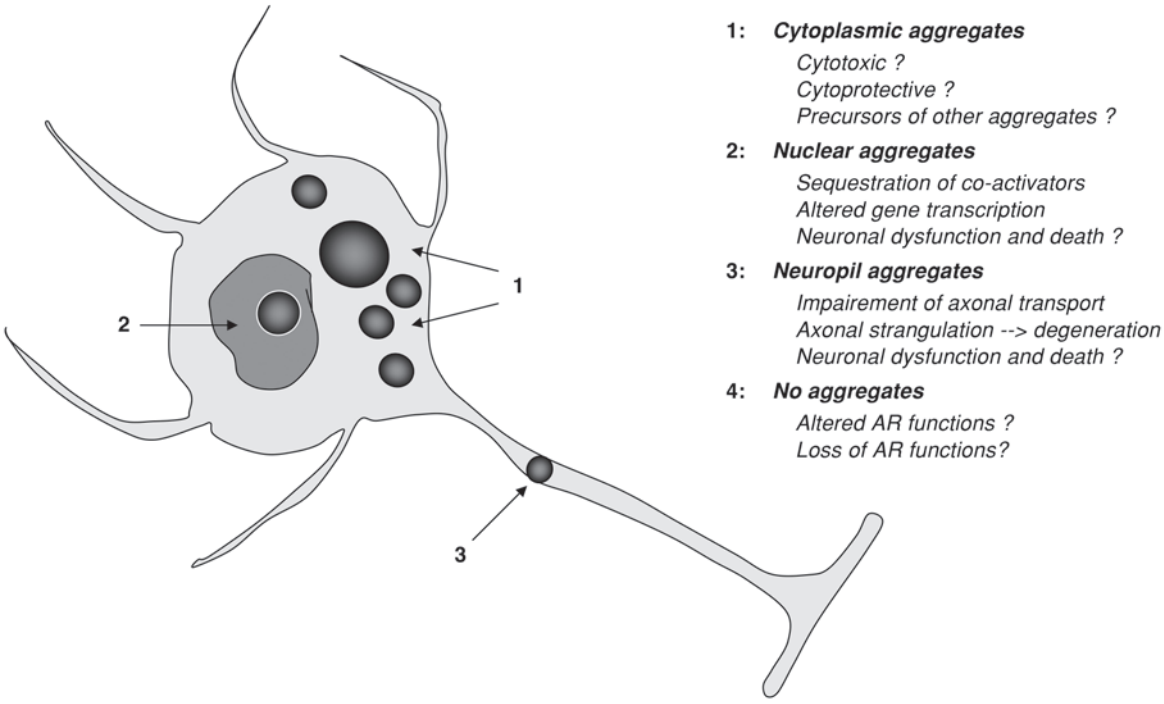

Fig. 4. Role of the different types of AR aggregates in SMBA.

aggregates entrap ubiquitin, suggesting that the proteolytic pathway is impaired *(215)*. It has been shown that soluble polyGln proteins can be normally degraded, but the elongated polyGln seems to be resistant to proteasomal degradation *(216,217)*. Whether the formation of inclusions is the cause or the effect of the toxicity of expanded polyGln tracts is unclear. In fact, in cellular models, not always the formation of inclusions has been associated with cell death *(218–222)*; moreover, in mouse models of SBMA, neurological dysfunctions are detectable even in the absence of neuronal loss *(205)*, suggesting that cell death may be a late event in the progression of the disease.

We have initially shown that SBMA AR aggregation is dependent on the degree of AR activation by ligands. Aggregation does not occur in living neuronal cells expressing the unactivated mutated SBMA AR; in contrast, cytoplasmic (perinuclear and neuropil) inclusions are formed after activation of SBMA AR by T *(174,221)*. Therefore, it is possible that the unligated AR, entrapped with accessory chaperone proteins (Hsp90, Hsp70), is protected from aggregation; this may occur either because the chaperones may mask the polyGln tract or because the chaperones may repair the misfolding of the mutant AR. Because T sheds the chaperones, the polyGln may start intermolecular interactions with other polyGlns and/or the AR may not gain the correct active conformation and accumulate within the cell *(223)*. It is also possible that, when AR translocates to the nuclei (as a consequence of androgen binding), some unknown nuclear factors will initiate the aggregation processes. Therefore, androgen levels are critical for the appearance of aggregates in SBMA; thus, the high variability of clinical progression and in the age-of-onset in males as well as the lack of symptoms in female, may be due to the individual variation of circulating androgens.

Once activated, the SBMA AR forms aggregates that may have different subcellular localization (see Fig. 4): in the cytoplasm, in the nucleus, and in the neuropil; because of this, they may have different effects on neuronal cell functions and play different role in neurotoxicity. By studying AR aggregation in immortalized motoneurons, we have shown that the toxicity of the mutant SBMA AR is distinct from the appearance of cytoplasmic aggregates *(221)*; in the short-term period, cytoplasmic aggregates may even be protective, by removing the toxic proteins from the cell soluble fraction, and confining SBMA AR into an insoluble subcellular compartment. Preliminary data obtained in our laboratory, using a reporter plasmid for proteasome activity (YFPu), have shown that alterations of this degradative pathway are present when the mutant AR is in its soluble unactivated form; on the other hand, when the cells are treated with T to induce cytoplasmic aggregate formation, the degree of proteasome impairment is markedly reduced. This strongly suggests that the accumulation of the neurotoxic protein into a defined subcellular compartment, in an inaccessible insoluble form, may help to protect motoneuron from mutant AR toxicity; in this view, the cytoplasmic aggregates, at least in the earlier stages of formation, may play a beneficial role in the SBMA. The aggregates may also contribute to de-saturate the proteasome to allow the activation of different degradative pathways (i.e., the autophagocytosis, and the lysosomial pathway); this hypothesis, however, remains to be experimentally tested. It is very

interesting to note that different antiandrogens, like Casodex or cyproterone acetate, may either induce or protect against cytoplasmic aggregates, suggesting that their use may have different impacts as potential new approaches to treat SBMA.

T-induced toxicity of mutant AR can be explained in other ways; in fact, a second type of aggregates found in cells are the neuropil aggregates. It is unclear whether they derive from cytoplasmic aggregates migrating into the neuronal processes, or whether they are formed *in loco (224,225)*; since motoneuronal cells are characterized by very long cell processes, their potential toxicity is extremely elevated. For example, neuropil aggregates could mechanically alter neurite functions by blocking the transport of organelles, vesicles, and polyribosomes along the axons, as well as by modifying the accessibility of factors required by the neurofilament network *(226,227)*. We have clearly proved, in living cells expressing the mutated SBMA-AR, that neuropil aggregates induced by T alter the axonal and/or the dendritic distribution of mitochondria (labeled with a blue fluorescent protein containing a mitochondrial localization signal). The mitochondria tend to accumulate in the cell processes in close association to neuropil aggregates, but are not physically sequestered. Occasionally, in correspondence with the mitochondrial accumulation and the AR inclusions, neurite morphology shows a high degree of caliber variation; this could deprive the downstream compartment of nutrients coming from the soma *(224)*. This axonal swelling has also been described in other neurodegenerative disorders [like amyotrophic lateral sclerosis (ALS)], and may be secondary to the formation of inclusions in neurites. Because in neurites, mitochondria are actively transported via the microtubule-based system using the specific motor proteins kinesin and dynein, we have further analyzed the axonal transport and found that neuropil aggregates also alter kinesin distribution *(224)*. Microtubules, which apparently are normal, seem to be forced at the surface of the inclusions *(26)*. Therefore, alteration of fast axonal transport mediated by kinesin can be modified by the neuropil aggregates by decreasing the bioavailability of components essential for synaptic functions and/or by inducing axonal strangulation; both processes may cause neuronal dysfunctions *(26)*. Therefore, in SBMA and possibly in other motoneuron diseases, motoneurons, or neurons of the DRG, may selectively degenerate as a consequence of alteration of axonal transport along the very long axons (up to 1 m), which maximizes the requirement for essential components of the synapses located far away from the cell body *(228)*.

With regard to nuclear aggregates, their formation has been associated with the production of a specific AR N-terminal fragment containing the polyGln tract; these aggregates seem to depend on the polyGln length *(229,230)*. These aggregates may also be induced by ligand treatment *(231)*; therefore, it is conceivable that the proteolytic cleavage of the mutated AR alters the release of the chaperons *(205)*. It has been shown that the AR fragment starts nuclear aggregation and that nuclear inclusions found in SBMA patients are formed by the AR N-terminal fragments *(231–233)*. Interestingly, the mutation of a caspase-3 cleavage site, located at Asp146 of AR, inhibits aggregate formation, suggesting that fragmentation is due to the activation of the apoptotic signals and may be a late event in neuronal cell death *(228)*. It has also been demonstrated that specific intracellular signaling pathways, like the mitogen-activated protein kinase (MAP kinase) pathway, may play a role in neuronal death; for example, the mutation of the MAP kinase consensus phosphorylation site of AR (ser 514) counteracts the formation of the AR N-terminal fragment and neuronal death, suggesting that phosphorylation allows caspase-3 cleavage of AR generating cytotoxic polyGln fragments *(234)*.

Also, nuclear aggregates are ubiquitinated and sequester the 26S proteasome components; moreover, Hsp70 and Hsp40 reduce the number of nuclear aggregates *(233,235, 236)*, ameliorating the clinical signs of SBMA transgenic mice *(237)*, by increasing the AR solubility of AR nuclear aggregates, and also by enhancing the degradation of SBMA AR through the proteasome *(235)*. The mechanism proposed to explain the neurotoxicity of nuclear aggregates suggests that, once the AR migrates into the nuclei and aggregates, it sequesters the steroid receptor coactivator (SRC1) *(174)* and the CREB-binding protein (CBP) *(238)*, two important transcriptional regulators. The deprivation of CBP alters AR-mediated and AR-independent gene transcription, because CBP is a histone deacetylase enzyme required to unpack the nucleosome; this process allows chromatin decondensation at the transcription start site, permitting the access of the RNA polymerase II to initiate gene transcription *(238)*. Upregulation of endogenous CBP (dCBP), in a *Drosophila* model of SBMA, reversed the aberrant functional and morphological alterations observed in basal conditions *(168,239)*. Histone deacetylase inhibitors, like suberoylanilide hydroxamic acid (SAHA), may thus become useful to try to counteract polyGln toxicity *(168,239–241)*.

The mutant AR protein may also aberrantly interact with other proteins, inducing a transcriptional dysregulation *(242)*; for example, the elongated polyGln reduces the interaction of AR with its coactivator p160 *(243)*; it may modify the mechanism of activation of the AR/c-Jun-mediated transcription *(244)* and it may decrease the interaction of AR with a Ras-related nuclear G protein (ARA24) that directly binds the N-terminal of AR *(245)*. It has also been shown that a small set of genes respond to mutant AR, but not to activated wild-type receptor *(242)*.

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