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GENOMIC AND NON GENOMIC EFFECTS OF ELEVATED CONCENTRATION OF ANABOLIC STEROIDS IN HUMAN NEURONAL CELLS

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Discussion

Abstract

Nandrolone and other anabolic androgenic steroids (AAS) at elevated concentration can alter the expression and function of neurotransmitter systems and contribute to neuronal cell death. This effect can explain the behavioural changes, drug dependence and neuro degeneration observed in steroid abuser.

Nandrolone treatment (10^{-8}M–10^{-5}M) caused a time- and concentration-dependent downregulation of mu opioid receptor (MOPr) transcripts in SH-SY5Y human neuroblastoma cells. This effect was prevented by the androgen receptor (AR) antagonist hydroxyflutamide. Receptor binding assays confirmed a decrease in MOPr of approximately 40% in nandrolone-treated cells. Treatment with actinomycin D (10^{-5}M), a transcription inhibitor, revealed that nandrolone may regulate MOPr mRNA stability. In SH-SY5Y cells transfected with a human MOPr luciferase promoter/reporter construct, nandrolone did not alter the rate of gene transcription. These results suggest that nandrolone may regulate MOPr expression through post-transcriptional mechanisms requiring the AR.

Cito-toxicity assays demonstrated a time- and concentration dependent decrease of cells viability in SH-SY5Y cells exposed to steroids (10^{-6}M–10^{-4}M). This toxic effects is independent of activation of AR and sigma-2 receptor. An increased of caspase-3 activity was observed in cells treated with Nandrolone 10^{-6}M for 48h.

Collectively, these data support the existence of two cellular mechanisms that might explain the neurological syndromes observed in steroids abuser.

Chapter 1 -ANABOLIC ANDROGENIC STEROIDS

The anabolic-androgenic steroids (AAS) are all synthetic derivates of testosterone, the natural male hormone produced primarily by the testes. Women also produce testosterone, but lower amount than do men. The hormone is responsible for androgenic, or masculinising, and anabolic, or tissue building, effect noted during male adolescence and adulthood.

On June 1, 1889, Charles Edouard Brown-Sequard, a French physiologist, announced that he had devised a rejuvenating therapy for body and mind. He explained that he inject himself with a liquid extract derived from the testicles of dogs and guinea pigs and observed an increased in his physical strength and intellectual energy. In the years later there were an increase of studies about the effect of organotherapy as possible therapy of a very broad range of disorder. The use of animal extract as treatment to hypogonadism and impotence continued until 1935 when testosterone was synthesize. Three research teams, subsidized by competing pharmaceutical companies, raced to isolate the hormone and publish their result in this year. Karoly Gyula David and Ernst Laqueur submitted a paper entitle "On crystalline male hormone from testicle (testosterone)". Butenandt and Hanish backed by Shering Corporation in Berlin, published a paper describing "a method for preparing testosterone from cholesterol". The Ciba scientists Ruzicka and Wettstein wrote a studies entitle "On the artificial preparation of testicular hormone testosterone (androsten-3-one-17-ol). Butenandt and Ruzicka shared the 1939 Noble Prize for Chemistry for this discovery. The possibility to obtain a high quantity of steroids lead the scientists to investigate directly in clinical trial the efficacy of this compound. This studies employing injections of testosterone propionate, a slow-release derivate, as well as oral doses of methyl testosterone. These experiment were initially as hapharzard and unregulated as the more primitive methods involving testicular extract or transplants. In its early phase synthetic testosterone therapy was reserved primarily for treating man with hipogonadism and impotence, however, the used of this hormones by elite athletes and bodybuilders show up since 1940. During 40's scientist discovered that

testosterone could facilitate the growth of muscle tissue. This findings were popularized by the writer Paul de Kruif. His book , “The Male Hormone”, published in 1945 may have helped promote testosterone use among athletes. For the past 25 year the use of testosterone and his derivates by healthy people has been officially proscribed.

AAS abuse

AAS are commonly used as sport performance enhancers in athletes (weightlifters, runners), as “muscle volume enhancer” for cosmetic purpose (bodybuilder), as performance enhancer for occupational purpose (security), and as possible “fountain of youth” in aging (Di luigi et al., 2005). The AAS used for non-therapeutic purpose are: androstenedione, DHEA; 17-βesters of testosterone (cypionate, enanthate et al.) 17-α-alkyl derivates of testosterone (methyltestosterone), 19-nortestosterone (nandrolone), tetrahydrogestrinone. More than 100 different AAS have been developed, with most of them being used illegally, synthesized in clandestine laboratories, commercialized without medical prescription or safety controls, and sometimes unknown in the scientific world. Many natural products with anabolic action are freely marketed and used to aging people. These products are weak androgenic steroids and are used as anti-obesity, anti-anging and their positive effects on sexual performance and libido.

Illicit anabolic-androgenic steroids (AAS) use is a public health problem in many country. Since 1950s and 1960s use of AAS has broadened beyond athletes and body-builder to include adolescent male seeking and idealised appearance and adolescent associated with multiple drug use. Anabolic-androgenic steroid users are often polysubstance abusers, using either traditional recreational drugs or misusing prescription drugs. Even those who avoid traditional recreational drugs are still enveloped in the drug culture to obtain their steroids (eg, suppliers or pushers), to find ways to administer them (eg, finding large-gauge needles), and to develop the means to continue to use (eg, hiding and paying for their AAS). This

immersion in the drug culture often leads to the abuse of other substances. Studies looking at AAS as a gateway drug have found that 29% of people who abuse both steroids and opioids started with steroids and were later introduced to opioids by the person who supplied them with the AAS. DuRant et al found that 25% of AAS abusers shared needles to inject drugs and that a positive correlation existed between AAS abuse and the use and abuse of cocaine, injectable drugs, and marijuana. For those who use steroids to enhance "bodily health," the use of other drugs to further enhance the AAS or decrease AAS side effects is common. Other drugs that are frequently abused as adjuncts include human growth hormone, which acts synergistically with AAS; human chorionic gonadotropin to block the testicular side effects of the anabolic steroids; diuretics to prevent water retention and improve visual muscle appearance (rippled effect); and antiestrogens such as tamoxifen to block gynecomastia. To help hide the fact that steroids are being used, some AAS abusers will take antibiotics and antiacne medications to help prevent testosterone- induced acne, which often involves the face, neck, and torso. AAS use was associated with such problem behaviour as marijuana (cannabis) involvement and overt nondestruction (e.g., aggressive-type conduct problems) and, to some extent, with involvement in power sports and disordered eating (Wichstrøm L) It is interesting to note that AAS users sometimes report a syndrome called "reverse anorexia nervosa" or "muscle dysmorphia" (Pope at al., 2005) characterized by 1) preoccupations that they look small when they are actually muscular 2) giving up social and occupational opportunities because of need to work out and 3) avoiding situations where their bodies might be seen in public. Many AAS users first developed the full syndrome of muscle dysmorphia after their first AAS use, which suggests that using AAS and gaining muscle did not necessarily resolve these men's insecurities about size (Kanyama at al., 2006).

The major differences between medically used AAS and the recreational abuse of these drugs are the dosage and schedule of administration used by illegal users. Usually, medical usage is at a physiologic replacement level (eg, hypogonadism 6 to 10 mg/d), on a continuous basis,

and with regular intervals of use. Recreational users generally develop complicated multidrug regimens (using oral and intramuscular preparations) that progressively increase in dose until 40 to 100 times physiologic levels are reached. This practice is referred to as "stacking." It is not uncommon for users to take multiple forms of AAS (five different drugs on average) from multiple classes of steroids to take advantage of the different pharmacokinetic properties of these drugs. The perceived physiologic basis for stacking is to maximize AAS receptor binding and to activate multiple steroid receptor sites. (Hall et al, 2005).

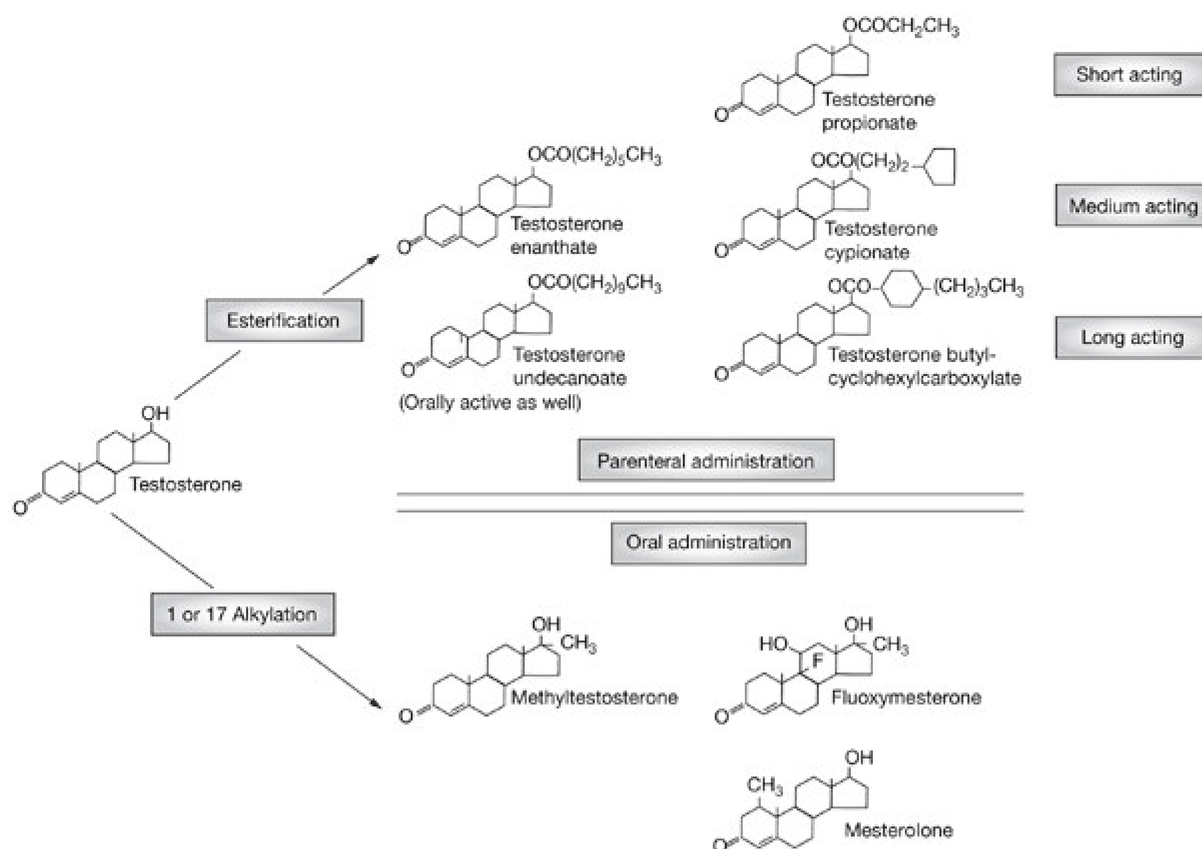
Testosterone structural modification

Testosterone molecule has been modified to obtain anabolic and androgenic effects, changing in metabolism, release and catabolism. The structural modification can be group in three principal categories

1. alkylation on 17α
2. Steroidal rings modification
3. Esterification on 17β idrossile group

17a alkylation involves the addition of an alkyl group (methyl or ethyl) to the alpha position of the 17 carbon of the steroid backbone. The alkylation at this position prevents the major route of androgen deactivaton – oxidation to a 17-keto steroid - from taking place. This allows a large part of the steroid to avoid liver first pass metabolic degradation. Examples of 17a alkylated steroids are methyltestosterone and Norethandrolone, these compounds are orally active. There is one more class of anabolic androgenic steroids that are orally active, these have unique structural modifications in the steroid A ring. This modification do is help preserving the steroid 17beta hydroxyl group, and minimize oxidation to the inactive 17-keto form. The most common A-ring modifications that shift the 17beta hydroxyl / 17-keto equilibrium to the left are methylation at the 1alpha position, and unsaturation (double bond)

in the 1(2) position. Examples of orally active steroids that contain one or more these modifications include methenolone (Primobolan), mesterolone (Proviron), and 1-testosterone.



The esterification of Testosterone is the feature of the third class of AAS . An ester is a chain composed primarily of carbon and hydrogen atoms. This chain is typically attached to the parent steroid hormone at the 17th carbon position (beta orientation), although some compounds do carry esters at position 3. Esterification of an injectable anabolic androgenic steroid basically accomplishes one thing, it slows the release of the parent steroid from the site of injection. This happens because the ester will notably lower the water solubility of the steroid, and increase its lipid (fat) solubility. This will cause the drug to form a deposit in the muscle tissue, from which it will slowly enter into circulation as it is picked up in small quantities by the blood. Generally, the longer the ester chain, the lower the water solubility of the compound, and the longer it will take to for the full dosage to reach general circulation.

Slowing the release of the parent steroid is a great benefit in steroid medicine, as free testosterone (or other steroid hormones) previously would remain active in the body for a very short period of time (typically hours). This would necessitate an unpleasant daily injection schedule if one wished to maintain a continuous elevation of testosterone (the goal of testosterone replacement therapy). By adding an ester, the patient can visit the doctor as infrequently as once per month for his injection, instead of having to constantly re-administer the drug to achieve a therapeutic effect. Clearly without the use of an ester, therapy with an injectable anabolic/androgen would be much more difficult. Esterification temporarily deactivates the steroid molecule. With a chain blocking the 17th beta position, binding to the androgen receptor is not possible (it can exert no activity in the body). In order for the compound to become active the ester must therefore first be removed. This automatically occurs once the compound has filtered into blood circulation, where esterase enzymes quickly cleave off (hydrolyze) the ester chain. This will restore the necessary hydroxyl (OH) group at the 17th beta position, enabling the drug to attach to the appropriate receptor. In summary this modification achieve a number of goals, including a) slow metabolism; b) enhanced affinity for the androgen receptor (19-nortestosterone); c) resistance to aromatization to estradiol (fluoxymesterone, 19 nortestosterone); and d) decreased binding of metabolites to androgen receptor (5α-reduced metabolites of 19-nortestosterone, 7α-19- nortestosterone). Agents such as fluoxymesterone and 19-nortestosterone (nandrolone) that resist aromatization lack the feminizing side effects of testosterone. 19-nortestosterone possesses another characteristic that increases its anabolic activity because its 5α-reduced metabolite has poor affinity for the androgen receptor. Similarly, alpha-methyl-19-nortestosterone is not a substrate for 5α reductase (Sundaram *et al.*, 1995).

Nandrolone

The drug known as nandrolone (also known commercially as Deca-Durabolin) has the IUPAC name 17b-hydroxy-19-nor-4-andro-sten-3-one, is an anabolic steroid which occurs naturally in the human body, but only in tiny quantities. Nandrolone is a 19-Nor steroid (i.e. it lacks a carbon atom at the 19 position on the steroid molecule) and is derived from the male hormone, testosterone. Nandrolone may have some legitimate medical uses, such as the treatment of major burns, malnutrition and osteoporosis. However, nandrolone has been implicated in relation to doping (especially in sports, where muscle mass and strength are deciding factors) and was banned by the International Olympic Committee Medical Commission in 1974. Athletes who resort to nandrolone do so for various reasons. The drug, which needs to be administered via intra-muscular injection to be effective, is used to increase muscle mass. However, its reputation for easing the pain and strain caused by intensive training, or hasten recovery from injury, is not scientifically based.(ASADA, 2007)

Nandrolone decanoate is the compound used for clinical and no-clinical purpose. This molecule is an ester synthesize for intra-muscle injection of the anabolic-androgenic steroid nandrolone. The ester modification make the molecule hydrophobic and extends its duration of action. The latter is dependent from slow release of the hydrophobic nandrolone ester from the muscle. A single intra-muscle injection of 50–150 mg nandrolone decanoate in healthy young men reached a peak serum levels after 2–3 days, and the maximum serum level ranged from 2.14 ng/ml in the 50-mg group to 5.16 ng/ml in the 150-mg group (Wilma et al., 2005)

Synthesis and Metabolism

Androgenic steroids are produced by the testes, adrenals and ovaries from cholesterol. In men testosterone is the principal secreted androgen. The leyding cells synthesize the majority of testosterone. In women testosterone is synthesize both in the corpus luteum and the adrenal cortex. The synthesis pathway is similar between man and women. This metabolic pathway

have a unique precursor , cholesterol, but it can have two routes. The first one (the Δ^5 pathway) involving pregnolone and dehydroepiandrosterone (DHEA) and the other (the Δ^4 pathway) involving progesterone and androstenedione. The Δ^5 pathway appears to predominate in the testis of men. Both pathways are functional in adrenals and ovaries. The precursors androstenedione and deydroepiandrosterone are weak androgens.

Testosterone can be produced by metabolism of certain steroids, such as 4-androstenedione in organs beside the testis, but this route contributes to less then 5% of plasma testosterone in men. The leyding cell produce the majority of testosterone. The production of testosterone by the leyding cells of the testis is controlled by blood levels of leuteinizing hormone (LH) release from anterior pituitary.

The adrenal synthesizes and secretes dehydroepiandrosterone and dehydroepiandrosterone sulphate, both have the potential to serve as precursors of synthesis of more potent androgens, but only 1% of the testosterone derive from this route. The secretion rate for these androgenic steroids are similar in men and women , and the rates of secretion are insufficient to produce virilisation. However , condition producing adrenal hyperplasia often lead to increase secretion of androgens and their precursor, causing virilization. About 50 percent of plasma testosterone in normal women is derived from peripheral conversion of 4-androstenedione to testosterone, and a equal amount comes from direct secretion of ovary. Secretion of androgenic steroids from by the ovary varies with the stage of the menstrual cycle and is regulated by LH and FSH, witch control steroid synthesis by various cell type in the ovary.

The metabolism of testosterone have a pivotal rule in their function. Testosterone is metabolize by the enzyme 5α-reductase to 5α-dihydrotestosterone and by aromatase to estradiol. Some effects of testosterone appear to be mediated by testosterone itself, some by dihydrotestosterone, and some by estradiol. 5α-dihydrotestosterone have a high affinity for the androgen receptor and activate gene expression more efficiently, in the tissues expressed the 5α-reductase. Two forms of 5α-reductase have been identified, type I is found

predominantly in nongenital skin and the liver, and type II is found in urogenital tissue in men and genital skin in both men and women. The conversion of testosterone to estrogens occurs predominantly in adipose tissue, with lesser amount produce in muscle, kidney, liver and hypothalamus.

Testosterone is metabolized in the liver to androsterone and etiocholanolone, which are biologically inactive. Dihydrotestosterone is metabolized to androsterone, androstanedione and androstanediol.

Mechanism of action

Testosterone and derivates can work through genomic and non-genomic mechanism. Testosterone enter a cell by passive diffusion through cell membrane. The genomic mechanism involve the activation of specific androgen receptor (AR). AR is a member of superfamily of nuclear receptors, which includes steroid hormone receptors, thyroid receptors and orphan receptors. Testosterone and dihydrotestosterone bind the hormone-binding domain of the androgen receptor allowing the ligand–receptor complex to bind, via the DNA-binding domain of receptor, to certain responsive element. The ligand-receptor complex acts as a transcription factor complex and stimulates expression of specific genes. Androgen receptor interaction with co-activators and co-repressors transcription factors lead the tissue specific gene regulation.

The non-genomic mechanism involve the AR or a membrane androgen binding sites that are discussed to be responsible for rapid androgen signaling have been described in several tissues or

Cells. But of late, the nature of these binding sites has been controversial. The sex hormone binding globulin (SHBG) is considered to play a role in permitting certain steroids to act without entering the cell. Upon ligand binding, the SHBG interacts with a high-affinity membrane receptor. This SHBG-receptor complex has been described to cause a rapid

generation of cAMP after exposure to the respective steroid. For example, in prostate cell membranes, an increase in intracellular cAMP in response to both androgens and estradiol, via binding to a membrane SHBG receptor, has been shown. Recently, binding sites for testosterone on the surface of T cells and IC-21 macrophages have been detected with testosterone-BSA-FITC that are likely to be involved in the context of nongenomic testosterone action on [Ca2_]i (Benten et al., 1999). A study lead on pheochromocytoma rat cells PC-12 has showed a high affinity membrane testosterone binding sites. This specific androgen membrane receptor regulate cell survival. The NGF-induce neuronal differentiation of PC12 cells resulted in the suppression of the number of membrane testosterone sites (Alexaki et al., 2006).

Androgen Receptor

Androgen receptor (AR) modulate the androgen steroids action at level of gene expression directly by interacting with specific elements in the regulatory regions of target genes or indirectly by activating various growth factor signalling pathways. AR gene was cloned in 1988 (Chang et al., 1988 and Lubahn et al., 1988). Like other members of the nuclear receptor superfamily, AR has four major functional regions (Fig. 3): the N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD. Two autonomous transactivation functions, a constitutively active activation function (AF-1) originating in the N-terminal and a ligand-dependent activation function (AF-2) arising in the LBD, are responsible for the transcriptional activity of nuclear receptors.

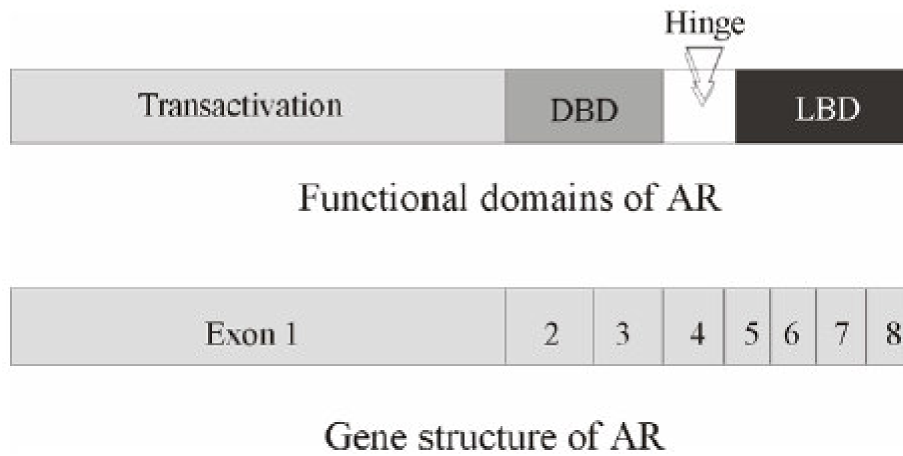


Figure 3. Androgen receptor gene organization and domain structure. The AR gene (bottom) consist of eight exons that give rise to the domain structure of receptor protein (top). The exon 1 is required for transactivation and exons 2-8 encoding a DNA-binding domain, hinge region, and hormone binding domain.

Ligand-binding initiates a series of events leading to the regulation of target genes by the receptor. The occupied receptor undergoes an allosteric change in its LBD, and was dissociated from heat shock proteins, such as hsp90, hsp70, and hsp56 (Roy *et al.* 2001), dimerized, and translocated, if it is not already present into the nucleus. Upon binding to a hormone response element (HRE), the receptor dimer recruits coactivators to form an active pre-initiation complex and interacts with basal transcription machinery to trigger the transcription of the target genes.

The DBD of AR exhibits a high degree of amino acid sequence identity to other members of the glucocorticoid receptor (GR) subfamily, the progesterone receptor (PR), and mineralocorticoid receptor (MR). Consequently, the four receptors recognize very similar, if not identical, hormone response elements (HREs). GR, PR, MR, and AR, recognize response elements that are organized as inverted repeats of 5’-TGTTCT-3’-like sequences with a three nucleotide spacer (Beato 1989). Recognition and binding of DNA is achieved by DBD. Two zinc fingers are present in the DBDs of the nuclear receptors. They differ both structurally and functionally. The first zinc finger contains so-called P-box (Gly, Ser, and Val) that dictates the sequence specificity of binding to HRE. A five amino acid-residue long D-box of the GR superfamily is located in the N-terminal side of the second zinc finger. The D-box is

important in specifying the half-site spacing requisite at the HRE. Conformation changes resulting from the binding of androgens to the LBD located at the C-terminal end of the molecule are responsible for activating the androgen response. Receptors with a deletion of their LBD are constitutively active, suggesting that the AF-1 is ligand-independent. AF-2 was demonstrated in LBDs of AR but this action seem to be weak and it is not been described to be ligand independent (McEwan, 2004). AF-2 in the LBD of AR forms a hydrophobic cleft, which core is present in helix 12, that binds the LXXLL motif of the p160 family of transcriptional coactivators , which are associated with histone acetyl transferase activity and can recruit coactivitors required for chromatin modification. The transcriptional activity of AR is affected by coregulators that influence a number of functional properties of AR, including ligand selectivity and DNA binding capacity. AR coregulators participate in DNA modification of target genes, either directly through modification of histones or indirectly by the recruitment of chromatin-modifying complexes, as well as functioning in the recruitment of the basal transcriptional machinery. Some of the better characterized coregulators are members of the p160 family, ARA70, ARA55, ARA54, ARA267-α, Smad-3, and AIB1. ARA55 and ARA70 both allow the activation of AR by 17β -estradiol (E2), with ARA70 being the most effective coactivator for conferring androgenic activity to E2. Furthermore, both ARA55 and Smad-3 have been suggested to function as bridges for cross-talk between transforming growth factor-β signalling pathway and androgen/AR action (Kang et al., 2001). Aberrant AR coregulator activity due to mutation or altered expression levels may be a contributing factor in the progression of diseases related to AR activity, such as prostate cancer (Heinlein & Chang 2002).

Nuclear receptors (NRs) may also be activated by signalling pathways that originated at the cell surface. NRs, along with other transcription factors, are regulated by reversible phosphorylation . Kinase-mediated signal transduction pathways could affect the activity of NRs. The consensus phosphorylation sites found in AR indicate that AR could be a substrate

for the DNA-dependent protein kinase, protein kinase A, protein kinase C, mitogen-activited kinase, and casein kinase II . Indeed, AR could be activated in an androgen-independent way by growth factor or cytokine signalling pathways, like those initiated by epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), keratinocyte growth factor and IL-6, which would elicit AR-mediated transcriptional activation (Wu et al., 2006). Receptor distribution and hormone metabolism can explain part of the steroid-specificity *in vivo*. Other ways of imposing selectivity have been proposed at the chromatin level (Beato *et al*. 1995) and at the level of cooperativity of receptors with other transcription factors. A recent study demonstrated that selective DNA binding by AR could be a mechanism for hormone-specific gene regulation (Schoenmakers *et al*. 2000).

Physiological effects of AAS

Testosterone and DHT play a critical roles in male sexual differentiation during embryogenesis and in the development of secondary sexual characteristics. Testosterone secreted by the fetal testis is responsible during the embryogenesis for differentiation of the Wolffian ducts into the epididymides, vas deferentia and seminal vesicles. Instead, the virilization of external genitalia is dependent from 5α-reductatse which convert testosterone into DHT. The androgen secretion lead the complete genital growth until shortly after the birth, when declining production of testosterone by testis cause a cessation of androgen-dependent development until puberty.

At puberty, synthesis and secretion of testosterone by the testis increases and blood level of testosterone gradually rise over 4-5-years period until adult levels are reached. The increase of secretion of FSH and LH from the gonadotroph cell, in turn stimulate by increased secretion of GnRH from the hypothalamus, stimulate the testis and consequently the testosterone release. Increased secretion of testosterone into the systemic circulation affect

many tissues and the changes in most of them occur gradually during the course of several years. The skin becomes coarser and oilier due to increased sebum production, with contributes to the development of acne. Sexual hair begins to grow, initially pubic and axillary hair, the air in the lower legs, and finally other body hair and facial hair. Muscle mass and strength, especially of the shoulder girdle, increased, and subcutaneous fat decreases. Epiphyseal bone growth accelerates, resulting in the pubertal growth spurt, but epiphyseal maturation leads eventually to a slowing and then cessation of growth. The androgenic effect on bone growth may involve conversion of testosterone to estradiol, which alone or in conjunction with testosterone stimulates the synthesis and secretion of growth hormone and insulin-like growth factor I. The increased in muscle mass and bone result in a pronounced increase in weight. The larynx thickens, resulting in a lower voice. In the senescence period the testosterone concentration gradually declines. The fall in serum concentration could contribute to several changes that occur with increasing age in men, including decreases in energy, libido, muscle mass and bone mineral density.

Therapeutic use of AAS

The AAS have a limited therapeutic use. Androgen replacement therapy is prescribe to patients with a androgen deficiencies. Androgens are administered to these individuals to restore a normal steroids physiological function. In male is used to restore the development of male secondary sexual characteristics, as well as to promote the effect of androgen on somatic growth. Androgen therapy also is used to normalize male sexual behaviour. In the case of hypogonadism developed prior to the normal time period for puberty the androgen replace therapy can bring about the series of changes that usually take at puberty. The exposure to a adult levels of testosterone can lead to premature closure of epiphysis, this effects is used in individuals with abnormal growth. The use of androgen therapy in aging male is submit at clinical trial evaluation. Symptoms and findings of T deficiency are similar to those

associated with aging. They include loss of energy, depressed mood, decreased libido, erectile dysfunction, decreased muscle mass and strength, increased fat mass, frailty, osteopenia, and osteoporosis. Several studies suggested that some symptoms and signs of andropause may be improved by use of androgen steroids, but the benefit/risk ratio of testosterone replacement therapy in aging men is not known, until now (Hijazi at al., 2005). The women can be affect by androgen deficiencies that cause an impaired sexual function, lessened well being, loss of energy and negative effects on bone mass. The testosterone replacement therapy result a optimal treatment for women with post-menopausal problem and premature ovarian failure. The side effects are rare when the testosterone levels are close to normal reproductive range for women (Davis et al., 1999)

Anabolic steroids have been used in a variety of catabolic states such as those involving acute and chronic illnesses, surgical trauma. The rationale for the use of androgen therapy in these case depend from two process. The first one is the ability of androgens to promote a positive nitrogen balance and overall protein synthesis, a second process is the anti-cortisol activity. The Latter decreases the catabolic effects of cortisol but not alter its protective anti-inflammatory response. These mechanisms have been shown to increase muscle and bone growth and body weight. (Demling et al., 2005). Androgen therapy receiving renewed interest in treatment of patients with chronic kidney disease. In this subjects has been observed a reduction of skeletal muscles mass that affect the physical function. A nandrolone therapy in this patients increase muscle mass, enhance quality of life and reduce mortality (McDonald et al., 2007).

Androgens enhance erythropoiesis by stimulating the production of erythropoietin. Because of this effect, androgens have been used in treatment of some haematological disorder, such as the anemia associate with bone marrow and renal failure, and with myelofibrosis. After the introduction of recombinant human erythropoietin (rHuEPO) during the late 1980s the use of these compounds has been reduced. The used of androgens as

adjuvant of rHuEPO therapy could be useful. Androgens not regulate the EPO levels but increased the erythroid progenitors to EPO. Mechanism of this action may be mediated by triggering the pluripotential stem cells from G0 phase or prolonged G1 phase into a G1 interval responsive to EPO. It can explain why a positive response to androgen therapy may depend on the severity of the anemia and that the presence of stem cells in bone marrow favourably influence the response and survivability of patients (Navarro et al., 2001).

Side effect of AAS

Androgen therapy in case of hypogonadism and androgen deficiencies aging related have been showed to be quite safety. No large-scale, long-term studies have yet been initiated to assess the risks of testosterone-replacement therapy in men. The side effects of AAS depend from the sex and health of patients and administration routes. Unwanted effects can occur in women and prepubertal boys. In these patients virilization, acne, hair growth, weight gain, gynaecomastia and male-pattern hair loss may be observed, and should be managed symptomatically. Hirutism, excessive hair growth in androgen-sensitive areas, is the major side effect of exogenous androgen administration in women (Braunstein 2007).

Several AAS-induced adverse cardiovascular effects have been reported, including hypertension, left ventricular hypertrophy (LVH), impaired diastolic filling, arrhythmia, erythrocytosis, altered lipoprotein profile, and thrombosis (Dickerman et al., 1996 and 1997).

Steroids abuser have shown psychiatric side effects. Studies compare steroids abuser with not steroid user described a higher incidence of behavioral changes as irritability, aggressiveness, euphoria, hyperactivity (Hall et al., 2005). In same case it was shown acute psychoses, exacerbation of tics and depression, and development acute confusional/delirious states. In subject that use low-dose of steroids a minimal risk of adverse psychological effects was described (Yates et al., 1999). This side effects occur, particularly, among the body-builders and athletes who are steroids abuser.

Androgens and Brain

Androgens have an outstanding part in the brain functions. It was showed a neuroprotective and neuromodulation actions of AAS during brain development but also during brain aging and the important of androgens on the systems involved in cognitive function, mood disturbances and central drive of sexuality. Androgen receptors are found in brain regions that are crucial for learning and memory including the hypothalamus, the hippocampus, prefrontal cortex, and amygdala, but are not found in other cortical regions of the brain. In these areas the androgens acting on specific nuclear receptors or throughout the metabolism into neuroactive compounds. The hormone are important during pre- and perinatal brain development for the formation of sex-specific behavioral. The brain areas involved in pre and perinatal androgens effects are the same that show functional loss with aging. Bioavailable testosterone levels decline with age in men and women and numerous studies suggest a link between testosterone and cognition in men, particularly with aging. Testosterone supplementation improves spatial cognition and working memory in healthy older men. Visual-spatial cognition improves in older men with testosterone replacement in a dose-dependent manner, but similar effects are not found in young men. Testosterone replacement has few effects in men with low or no testosterone throughout life (congenital hypogonadism). Lower testosterone levels are associated with a higher risk for Alzheimer's disease, some studies suggest that low testosterone is associated with increased beta amyloid deposition in men (Almeida et al., 2004).

The effects of hormone in brain function is observable in healthy people. It has been suggested that effects within the central nervous system (CNS) contribute to AAS effects on strength because AAS user feel more energetic and therefore train harder. The androgens have a positive (elevate mood) and negative (psychotic symptoms) effects on user behavioral.

These hormones can be consider a drug of abuse. The effect of androgens in central nervous system could cause dependence state and activation of rewarding system (Kuhn, 2002).

Neurosteroids

The brain is one of tissues where the androgens are synthesized. The steroids produce in various regions of the central and peripheral nervous system are called neurosteroids. Dehydroepiandrosterone sulfate (DHEAS), was the first steroids found at high levels in the brain long after gonadectomy and adrenalectomy, and later shown to be synthesized by the brain. Later, androstenedione, pregnenolone, their sulfates and lipid derivatives, as well as tetrahydrometabolites of progesterone (P) and deoxycorticosterone (DOC) were identified as neurosteroids. There are differences between steroid synthesis in the brain and in the adrenals. Corticosteroid synthesis involves converting deoxycorticosterone (DOC) to either aldosterone by aldosterone synthase or to corticosterone by 11β-hydroxylase. In the adrenals the enzymes are never expressed in the same cell. But in the brain, the enzymes co-express not only in the same region, but even within the same cell, therefore aldosterone synthase and 11β-hydroxylase must compete for DOC . In the brain, the pregnanes are metabolized in sequence by the enzymes 5α-reductase and 3α hydroxysteroid dehydrogenase (HSD). Progesterone (P) to tetrahydroprogesterone (THP), deoxycorticosterone (DOC) to tetrahydrodeoxycorticosterone (THDOC) and testosterone to androstanediol (Dubrovsky , 2006). The function attributed to specific neurosteroids include of $GABA_A$, NMDA and sigma receptor function , regulation of myelinization, neuroprotection and growth of axon and dendrites.

In vivo and in vitro studies

A number of excellent studies, using animal models, was performed to evaluate the effects of AAS on neural circuits that underlie the behavioral effects. The in vivo studies differentiate in treatment regiment and hormonal state, sex and age of animals.

The in vivo experiments drove to study the aggression showed a sex – species and compound specificity. The administration of testosterone propionate for a long period of time enhanced aggression in intact male rats (Breuer et al., 2001) . It was observed that aggression increase is provoked by physical stimuli (tail-pich) and in social and environmental context that do not provoke aggression in control. Other AAS failed to stimulate aggression (nandrolone) or inhibited the display of aggression (stanozolol). Testosterone and stanozolol effects on aggression appear to be dependent upon the continued presence of the AAS. In addiction, the withdrawal from testosterone propionate did not itself induce aggressive behaviour (McGinnis et al.,2002). The different effects of steroids on aggression may reflect differences in the ability of these compounds to act to androgen and estrogen receptors and resulting differences in the balance of estrogen and androgen receptor-mediated signaling. In female rats treated with AAS it was observed a striking effects on aggression (Bronson et al., 1996). It was demonstrated that decreased serotonergic tone is pivotal to the ability of the AAS to increase aggression. Treatment of Fischer rats with testosterone propionate significantly decreased both 5-HT and the 5-HT metabolite, 5-hydroxyindoleacetic acid, in the hippocampus. The serotonergic agonist, quipazine, reduced the testosterone-induced dominance in a dose-dependent fashion (Bonson et al., 1992). Moreover, the effects of quizapine were themselves antagonized by the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonist, pindolol, demonstrating that the actions of quipazine in reducing androgen-induced aggression were specific for 5-HT-mediated transmission

The Studies that investigate the effect of AAS on the sexual behaviour of intact male rodents, showed two different classes of AAS. Stanozolol, oxymetholone and 17α-methyltestosterone eliminate the display of male sexual behaviour. These compounds acting on sexual behavioral suppressing the secretion of testosterone. Methandrostenolone, nandrolone and testosterone had minimal effects on intact male animals while maintain male sexual behaviour in gonadectomised male rats (Clarck et al., 1997). Experiments led on

female rats suggest that AAS act in the brain to interfere with events necessary for the estrogen-dependent induction of female sexual behaviour and the regulation of the neuroendrocrine events required for reproductive cyclicyty.

The number of experiments that have tested the effects of AAS on anxiety behaviour is limited but the results obtained showed an anxiety-reduction a time course and dose-response dependent (Britan et al., 1993). This effects of AAS is supposed to be mediated by the $GABA_A$ receptor. AAS has been shown to be a expression and a allosteric modulator of $GABA_A$ receptor when they are given chronically or acutely.

An effects on brain reward has been described in rats exposed to AAS. To evaluate the rewarding properties of drugs is used, extensively, the conditioned place preference task. It has reported that testosterone induce a conditioned place preference in male rats. This effect is mediate by its metabolites 3α-androstanediol (Frye, 2007). Animal treated with androgens may have an indirect effects on brain reward. It was showed a potentiated rewarding effects of amphetamine on rats exposed to AAS (Clark et al., 1996) . In these experiments it was found that nandrolone influence morphine rewarding an the somatic expression of withdrawal when it was chronically administered on rats before the start of opioid treatment (Celelier et al., 2003). In this experiments has been describe a suppression of morphine reward and increased withdrawal. Similar results was obtain in rats treated with nandrolone and cannabinoids (Celelier et al., 2006). Nandrolone induce changes that may reflect long-term modifications in the brain reward circuits leading to a progressive decrease in the basal hedonic level, which would result in an unpleasant state facilitating the development of an addictive process (Koob and Le Moal, 2001). In hamsters was observed a self-administer effects of testosterone (Wood, 2002). This data confirm the idea about the abuse potential of AAS (Wood, 2004)

Nandrolone decanoate altered the levels of both D_1 and D_2 dopamine receptors in the mesocorticolimbic system of Sprague–Dawley rats (Kindlundh et al., 2001). Studies to date suggest that chronic exposure to high doses of AAS alters both dopamine and dopamine

receptor expression. In rats exposed to nandrolone it was showed a down-regulation of expression of D(1)-receptor and an up-regulation of D(2)-receptors (Kindlundh et al., 2003). Enhanced activity of the mesocorticolimbic dopaminergic system is critical for the acute rewarding effects of cocaine and amphetamines (Thiblin et al., 1999).

Opioids and opioid receptors are highly expressed in brain regions that mediate reward. Studies carried out by Nyberg and colleagues showed a modulation of opioid peptide Met-enkephalin-Arg-Phe, a μ and δ opioid receptor agonist, in n the hypothalamus, striatum and in the periaqueductal gray (Johansson et al., 2000). Experiments carried out in Hamster showed that self-administration of testosterone in the presence of naltrexone, an opioid antagonist, was blocked. Naltrexone inhibit, also, the onset of testosterone intoxication in hamster treated with high dose of hormone (Peter and Wood, 2004). A modulation of expression of δ opioid receptor (DOR) is described in an in-vitro study. Cell lines exposed to nandrolone showed a down-regulation of expression of DOR, mRNA and DOR binding sites. These changes in δ opioid receptor levels of mRNA and protein were not blocked by coincubation with the androgen receptor-specific antagonist, flutamide, indicating that this effect of nandrolone is independent of androgen receptor activation (Pasquariello et al., 2000).

Learning and memory are highly dependent on synaptic plasticity, which involves structural changes in neurons and synapses. The glutamate receptor *N*-methyl-d-aspartate subtype (NMDAR) plays a crucial role in synaptic plasticity. Le Greves and colleague have reported that repeated administration of AAS affect the gene regulation of NMDAR subunits in different brain areas. the drug produced a significant decrease in the mRNA expression of the NR2A and NR2B receptors subunit, while the NR1 subunit was not affect. Instead, the combination of AAS nandrolone and cocaine bring about a significant decrease in the NR1 mRNA (Le Greves et al., 1997 and 2002). A single high dose of nandrolone mediate the phosphorylation of NMDA receptor subunits and ERKs. These effects were not seen after a 2-week treatment period, indicating adaptation to high steroid levels (Rossbach et al., 2007).

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Chapter 2- Mu Opioid Receptor

Opioid systems are responsible for a variety of processes in organisms, it controls pain, reward and addictive behaviours. Opioids exert their pharmacological actions through three opioid receptors, mu, delta and kappa whose genes have been cloned (Oprm, Oprd1 and Oprk1, respectively). Neurons release a family of endogenous peptides like enkephalins, dynorphins and endorphin, which bind the opioid receptors in the brain. Opioid receptors are, also, activated exogenously by alkaloids of the opium poppy plant *Papaver somniferum,* the prototype of which is morphine. Morphine was first isolated from opium in 1805 by a German pharmacist, Wilhelm Sertürner. Sertürner described it as the *Principium Somniferum*. He named it morphium - after Morpheus, the Greek god of dreams. Alkaloids opiaces have been known to relieve pain and alter mood since the advent of recorded history. For centuries, these agents have been integrated into medical practice with varying efficacy. Morphine was first used medicinally as a painkiller. During the American Civil War it was used as a surgical anaesthetic and was sent home with many wounded soldiers for relief of pain. At the end of the war, over 400,000 people had the "army disease," morphine addiction. The Franco-Prussian War in Europe had a similar effect. In 1906 the Pure Food and Drug Act required accurate labelling of patent medicines and tonics. Various laws restricting the importation of opium were enacted, and the Harrison Narcotics Act (1914) prohibited possession of narcotics unless properly prescribed by a physician. The morphine positive (pain relieve) and negative (change behavioral and addiction) effects are carried out primarily thought the mu opioid receptor (MOPr).

Opioid receptor

The opioid receptors were discovered in the brain by binding studies using radiolabeled opioid ligands, in the early 197Os. The first evidence about being of different opioid receptors was reported by Martin and colleague in 1976. They deduced the existence of three distinct opioid receptors from the different pharmacologic effects of various opioid agonists and antagonists that selectively induce or inhibit different physiologic responses and named them μ for the morphine group, κ for the ketocyclazocine group, and σ for N-allylnormetazocine (SKF10047). In addition to these three types of receptors in the 1977 was found a high-affinity receptor for enkephalins in the mouse vas deferens and named it the δ receptor (Lord et al., 1977). All these receptors are members of the G protein-coupled family of receptors and show significant amino acid sequence homologies. Multiple receptor subtypes have been proposed based on pharmacologic criteria. However, genes encoding only one subtype from each of the μ, κ and δ receptor families have been isolated and characterized thus far.

The first opioid receptor cloning was δ. Kieffer and her colleague isolated the cDNA of δ opioid receptor (DOPr) from a expression cDNA library. The plasmid bore the cDNA encodes the 371 amino-acid residues of DOPr was cloned into COS cells and screened for it ability to binding the ligand 3H-labeled Tyr-D-Thr-Gly-Phe-Leu-Thr. Thereafter, μ and κ opioid receptor cDNAs were cloned based upon their homology to the cloned δ -opioid receptor. The human μ opioid receptor (hMOPr) cDNA has been identified from a cerebral cortical cDNA library using sequences from the rat μ opiate receptor cDNA. hMOPr shares 95% amino acid identity with the rat sequence. The human μ , δ and κ opioid receptor genes are located on chromosomes 6q24-25, 1p34.3-36.1 and 8q11.2 respectively. The three opioid receptor genes share a common genomic structure and the coding region is divided into three major exons. The promoter region of three opioid receptors genes in mouse and rat share several common feature. All three promoters lack a TATA box, are highly G/C rich and share several common transcription factors, including SP-1/2, Ikaros (IK), E-box factors and

AP1/AP2. However, each promoter has its own distinct transcription factor. The MOPr gene in both mouse and rat contain a proximal and a distal promoter (Wei and Loh, 2002). A similar structure was described for the human MOPr gene, Carr and Xu discovered a distal promoter (-827) and a proximal promoter (-252). The analysis conduct with luciferase reporter vectors bore different sequences of promoter showed a strong activity of proximal promoter in MOPr-expressing cells (SK-N-SH) and in no-expressing cells (Hela). DNA sequence analysis indicated that the hMOR proximal promoter lacked a consensus TATA box, a consensus initiator, and GC-rich sequences (Xu and Carr, 2001a). The distal promoter, instead , had a weakly activity in both cell lines. It was also identified two cis-acting elements that allow positive and negative regulation of proximal promoter activity in SK-N-SH. A fragment of 40bp (-540 to -501) containing a GCC core in MOPr gene has been described to enhance hMOPr transcription. A protein complex was described binding this fragment but remain to identified which transcription factor is. A sequence of 34-bp (-694 to 660) was discovered in the MOPr gene, this fragment have a negative effect on transcription. Comparison of the 35bp element with sequences deposited in the transcription factor databases revealed several interesting putative binding motifs, including Ikaro-2, MZF1, NFY and C/EBP$_{\beta}$. Whether these factor are functional in hMOPr gene regulation remains to be determined (Xu and Carr, 2001b). Analysis of -500 to 292 region of hMOPr promoter showed that Sp1 and Sp3 bound to the CCCTCCTCCC motif in this region. STAT1 and STAT3 transcription factors binding site was observed in -1583 to 1575 region of MOPr promoter. The interlukine-6 up-regulate the expression of mRNA of MOPr in SH-SY5Y cells via STAT1 and STAT3 activation (Börner C et al., 2004). Neuronal and immune cells express MOPr mRNA, also, under control of nuclear factor-κB. NFκB recognise three *cis*-active elements on the μ-opioid receptor gene promoter, at nt -2174, -557, and -207 (Kraus et al., 2003). Loh and colleague showed that Poly-C binding proteins (PCBPs) bound from -317 to -304 region in hMOPr proximal promoter (Kim et al., 2007). This poly-C elements is recognise

by different regulator factor whit a positive (PCBPs) or negative (alpha CP3) effects (Choi et al., 2007). In mouse was discover a sequence located at 10kb upstream the exon1 coding to a new exon called exon11. This exon have a upstream promoter that control the expression of exon11-associated variants of MOPr (Pan YX, 2002). Mouse exon11 promoter differs from mouse exon1 promoter in several aspects. First, the exon11 promoter contains a TATA box that is absent in the exon1 promoter. Second, the exon 11 promoter has one major transcription start point (tsp), while the exon 1 promoter contains multiple tsp. The exon 1 promoter contains several GC-rich *cis*-acting elements like Sp1 and AP-2 that are missing in the exon 11 promoter. The exon 11 promoter appears to be a typical eukaryote class II promoter associated with RNA polymerase II, while the exon 1 promoter favors a “housekeeping” gene mode .These preliminary results indicated a complex control tissues and spatial specific expression of hMOPr gene modulate by positive and negative regulatory element.

The opioid receptor expression are regulate by multiple promoters and alternative pre-mRNA splicing mechanism. In rat, mouse and human MOPr gene was discovered a high number of splicing variants. Early after the cloning of mouse MOR-1 (mMOR-1) was isolate the first two splice variants mMOR-1A and mMOR1-B. Using a modified 3’RACE strategy combined with a nested PCR approach, Pan and colleague have isolated 10 additional splice variants, mMOR-1B2, mMOR-1B3, mMOR-1B4, mMOR- 1B5, mMOR-1C, mMOR-1D, mMOR-1E, mMOR-1F, mMOR-1O, and mMOR-1P. These mRNA share the first three exons and differ at 3’ of the molecule utilizing different exons. These MOPr variants exhibit very similar ligand binding profile compare with the wild-type MOR-1 in transfected cells. All these variants have the same protein structure predicted from exons 1–3, which includes the N-terminus, seven trans-membrane (TM) domains, three extracellular loops, three intracellular loops, and part of the intracellular carboxyl terminus; but these variants have different carboxyl terminal tips encoded by the different downstream exons. Involvement of

these C-termini in receptor phosphorylation, internalization and desensitization in response to mu agonist has been reported. In human was identified ten MOPr splicing variants that all differ on carboxyl terminal sequence as mouse variants. It was, also, discovered a variants encoding for a single trans-membrane protein in which exon 1 was spliced to exon 4 and called hMOR-S (Du et al, 1997). Another variants of human MOPr is mu_3. This contain exon 2, 3 and 4 and it is translate in a receptor with six TM. Mu_3 expressed in CHO cells displayed a selectivity for the opioid alkaloids but is insensitive to opioids peptides (Cadet et al., 2003). In mouse was discovered MOPr variants produce by 5' splicing. These are the exon11 associate variants. They are under control of the specific promoter of exon11 and they might translate in seven TM (wild type), six TM or one TM protein. All the variants except the single one TM contain exon 2 and 3. These parts constitute the ligand binding pocket and G protein coupling, and are mainly conserved within the opioid receptor family. The generation of mRNA variants encoding similar protein products subserves a regulatory purpose, such as to control the expression level, tissue-specificity or receptor dimerization.

The fact that was discovered many MOPr splicing variants which differ in the 5' or 3' portion suggest that such regions may contain physiological-relevant element for regulation of receptor expression. The exons involved in this alternative splicing mechanism can have coding or non-coding function, these exons can act as coding sequence in one transcript and in other like a non-coding. The non-coding sequence in the 5' and 3' of mRNA molecules have a pivotal rule in modulation of mRNA stability, control of mRNA poly-A tail length, influence on mRNA localization and regulation of translation initiation and efficiency. Surratt and colleague carried out a study about the 5' and 3' noncoding regions of MOPr transcript. A plasmid containing a 2162-bp human MOPr cDNA, with 212 bp of 5' noncoding region, a 1200 bp coding region, and 750 bp of 3' noncoding region, was deleted to built different truncated construct and transfected in COS-7 cells. This experiment revealed that the non-coding region in the 5' and 3' of MOPr mRNA influence receptor expression levels.

The simultaneous deletion of 5' and 3' non coding regions up-regulate the expression in comparison to wild type. The disruption of RNA secondary structure and a cis-element with negative effects predicted in 3'non-coding sequence of MOPr mRNA might explain the increase of expression of truncate MOPr mRNA (Zöllner et al., 2000). In the 3'region of hMOR1 variant was found numerous AU-reach elements (AREs). This cis-element affecting in a negative way the stability of mRNA, suggesting low stability of hMOR-1 mRNA (Kasai et al., 2006). A study conducted on KOPr mRNA showed the existent of two transcript variants with different mRNA stability and transcription efficiency. These two mRNAs are differentially regulate by retinoic acid (Hu et al., 2002). These preliminary results about the post-transcriptional mechanisms regulating the opioids receptor expression confirm the biological and pharmacological significance of generating mRNA variants. The expression of opioids receptors in different cells and at different physiological states is regulate by complex transcriptional and post-transcriptional mechanism.

Receptor structure and function

All are members of the G protein-coupled family of receptors and show significant Amino acid sequence homologies. opioid receptors form a family of proteins that physically couple to G proteins and through this interaction affect ion channel gating, modulate intracellular Ca2+ disposition, and alter protein phosphorylation. The opioids have two well-established direct actions on neurons: (1) they close voltage-gated Ca2+ channels on presynaptic nerve terminals and thereby reduce transmitter release and (2) they hyperpolarize and thus inhibit postsynaptic neurons by opening K+ channels. Opioid receptors belong to a G protein–coupled receptor (GPCR) super family (Smith and Lee 2003) characterized by seven hydrophobic transmembrane (TM) helices (TM1–7) connected by alternating intracellular (ICL1–3) and extracellular loops (ECL1–3). The N terminus is located on the extracellular side of the membrane, whereas the C terminus is on the intracellular side.

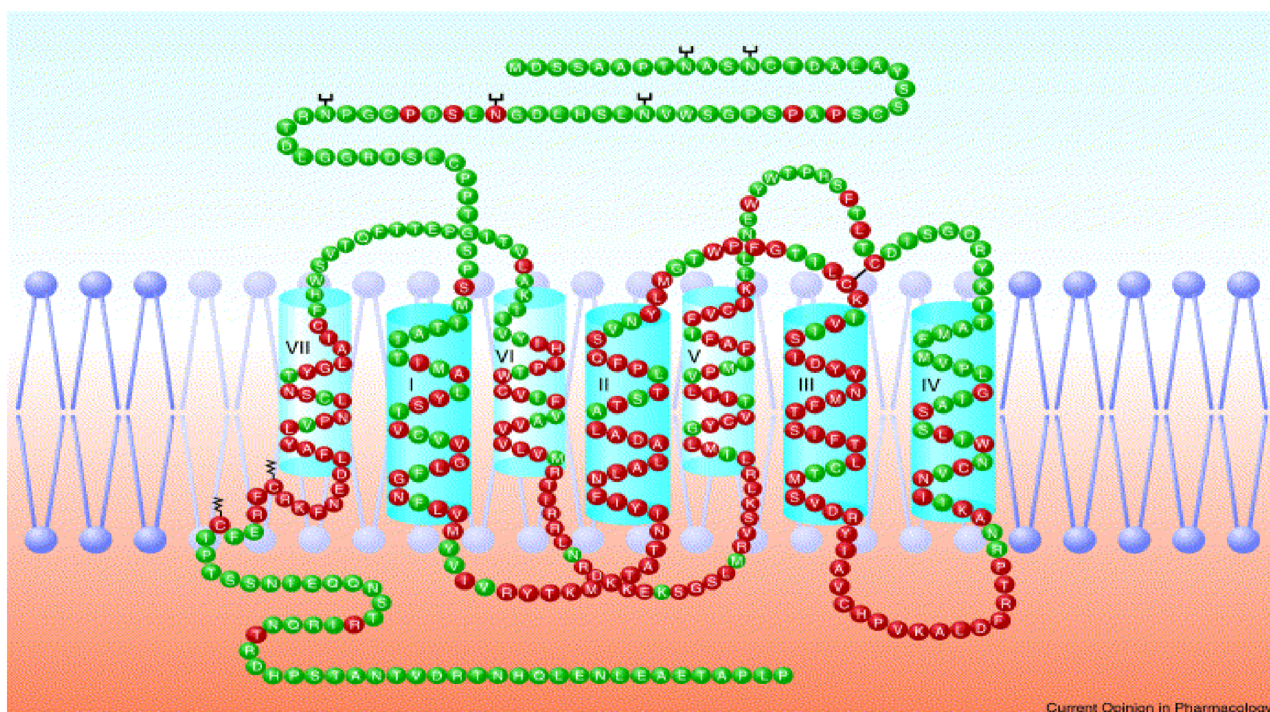

Figure 1: Mu opioid receptor (MOPr)

The GPCR acts as a link between the extracellular ligand and the intracellular G protein. Binding of an agonist to the inactive receptor leads to a structural change in the receptor primarily involving movement of helices III, VI, and VII. The active state of the receptor can then couple to a G protein via interactions with the intracellular loops (and the C-terminal in some receptors), which then initiates the subsequent intracellular signaling cascade. Opioids receptors are prototypal Gi/Go-coupled receptor. After opioid agonist bind to the receptor, dissociation of the trimeric G protein complex into Gα and Gβγ subunits can subsequently lead to inhibition of cyclin 3’5’ adenylyl cyclase (cAMP) and/or to direct interaction with K^+ and Ca^{2+}. The effects on ion channels is mediate by Gβγ subunit. The opioid suppress Ca^{2+} influx and so inhibit the excitation and neurotramitter release. The regulation of K^+ take place on the postsynaptic membrane where opioid mediate hyperpolarization that prevent excitation or propagation of action potentials. Another ion channels regulate from opioid are NMDA in the central nervous system and potential vanilloid type 1 receptor (TRPV1) mainly express in peripheral sensory neuron involved in thermosensation and nociception. (Koyama et al., 2008 and Endres-Becker et al., 2007).

Opioid activate the phospholipase Cβ(PLCβ) pathway via Gβγ-subunits or Gq proteins (Rubovithc et al, 2003). This action stimulate the open of calcium channels in cell membrane. This effect is dependent by phosphokinase C (PKC) activate by PLC. The Ca^{2+} into the cell stimulate the production of cAMP. Another , pathway involved in opioid action is the mitogen-activate protein kinase (MAPK) cascade, also known as extracellular signa-regulated kinase (ERK) (Shultz et al., 2003). The μ agonist, [D-ala^2,mephe^4,gly-ol^5]enkephalin (DAMGO), induces a transient activation of ERK that dissipates within 30 min. DAMGO induces the release of calmodulin from this receptor. Calmodulin may then activate PLC, generating diacylglycerol (DAG) that binds to PKCε, leading to its phosphorylation. PKCε can then signal to a metalloprotease, which can cleave membrane-anchored EGF-type ligands, thereby initiating EGF receptor transactivation and ultimately activation of the MAPK phosphorylation cascade.

All three opioid receptors (δ, κ, and μORs) have been shown to undergo homodimerization, and both δ–κ and δ–μ heterodimers have been demonstrated by coimmunoprecipitation (George et al., 2000) or BRET (Ramsay et al., 2002), whereas κ–μ heterodimers have not been observed.. That the interaction between μ- and δ-opioid receptors resulted in the creation of a unique binding site is evident from the pharmacological profile of the coexpressed receptors. This interaction is present at the cell surface, as indicated by the identical radioligand binding parameters in whole cell binding as in membranes. This would suggest that distinct conformational changes occurred, altering the original binding pockets of the μ- and δ-receptors and even altering the conformation of the G protein-interacting intracellular domains. The finding that blockade of one receptor with a selective antagonist did not restore binding of the other suggests that the binding site is indeed novel, rather than occurring as a result of altered cooperativity between ligand-binding sites on adjacent μ- and δ -receptors. Agonist treatment of the coexpressed μ- and δ-receptors revealed significant differences compared with μ- or δ-receptors expressed alone. In the combined presence of μ-

and δ-opioid receptors, there was resistance to desensitization and internalization upon exposure to DPDPE, although exposure to DAMGO resulted in an accentuated loss of receptors from the membrane. The agonist affinities for the μ · δ-receptor were not affected by the addition of guanine nucleotide or by pertussis toxin treatment of the cells, suggesting that the agonist-detected binding sites may not result from coupling to a G protein or, alternatively, may result from coupling to a pertussis toxin-insensitive G protein. he ability of the μ · δ complex to inhibit adenylyl cyclase activity following pertussis toxin treatment suggests coupling to a G protein that is pertussis toxin-insensitive, such as G_z, which has been shown to link to inhibition of adenylyl cyclase activity. The involvement of other effector pathways by the μ · δ complex remains to be established.

Physiological and Pharmacological action of opioid system

Opioid systems are responsible for a variety of processes in organisms. It control analgesia, the most well characterized effect, mood and behaviour, respiration, cardiovascular, gastrointestinal and neuroendocine function. Brain opioid peptide systems are known to play an important role in motivation, emotion, attachment behaviour, the response to stress and pain, and the control of food intake. The central nervous system in mammals is able to react to painful stimuli at many levels that are involved in transmission, modulation and sensation of pain. Endogenous opioid peptides and their receptors are located at key points in pain pathways, and response to pain can be modulated by local application of opioids at many sites.

Opioids endogenous peptides

Three families of endogenous opioid peptides have been identified: the enkephalin, endorphins and dynorphins. These endogenous opioid peptides are derived from three precursor proteins: prepro-opiomelanocortin (POMC), preproenkephalin (proenkephalin A),

and preprodynorphin (proenkephalin B) and has a characteristic anatomical distribution. Each precursor is subject to complex cleavage and post-translational modification resulting in the synthesis of multiple active peptides. The best-characterized of the opioid peptides possessing analgesic activity are the pentapeptides methionine-enkephalin (met-enkephalin) and leucine-enkephalin (leu enkephalin) both specific DOPr ligans. the Endogenous peptides endomorphin-1 and endomorphin-2, that bind MOPr, have been found to possess many of the properties of opioid peptides, notably analgesia and high affinity binding to the receptor (Fichna J et al., 2007).

Pro-enkephalin peptides are present in areas of the CNS that are presumed to be related to the perception of pain, to the modulation of effective behaviour, to the modulation of motor control and the regulation , and the regulation of the autonomic nervous system and neuro-endocrinological functions. Evidence suggests that they can be released during stressful conditions such as pain or the anticipation of pain to diminish the sensation of noxious stimuli. Endogenous opioid peptides are released from neurons and axon terminal by depolarization and can exert pre and post synaptic effects. In addition endogenous opioids are produced in many non-neuronal tissues, notably also in lymphocytes, monocytes and granulocytes in inflamed tissue (Rittner et al., 2001)

Analgesia

Opioids are the most broadly effective analgesics and are used in both acute and chronic pain. The analgesic effects of opioids arise from their ability to inhibit directly the ascending transmission of nociceptive information. Local circuits in the spinal dorsal horn play a critical role in processing nociceptive afferent input and in mediating the actions of descending pain modulating systems. Opioid receptors are present both on spinal cord pain transmission neurons and on the primary afferents that relay the pain message to them. Opioid agonists inhibit the release of excitatory transmitters from these primary afferents, and they directly inhibit the dorsal horn pain transmission neuron. Thus, opioids exert a powerful

analgesic effect directly upon the spinal cord. The depression of neuronal firing is the result of the opioid receptor activation. The inhibition Ca2+ influx and the subsequent release of glutamate and neuropeptides (substance P, calcitonin gene-related peptide) at presynaptically sites and the K+ increasing conductance that cause a hyperpolarisation of ascending neurons. Different combinations of opioid receptors are found in the supraspinal regions implicated in pain transmission and modulation. The opioid binding sites in pain-modulating descending pathways, including the rostral ventral medulla, the locus ceruleus, and the midbrain periaqueductal gray area, have a particular importance. At these sites as at others, opioids directly inhibit neurons, yet neurons that send processes to the spinal cord and inhibit pain transmission neurons are activated by the drugs. This activation has been shown to result from the inhibition of inhibitory neurons in several locations. When pain-relieving opioid drugs are given systemically, they presumably act upon brain circuits normally regulated by endogenous opioid peptides. Part of the pain-relieving action of exogenous opioids involves the release of endogenous opioid peptides. An exogenous opioid agonist (eg, morphine) may act primarily and directly at the μ receptor, but this action may evoke the release of endogenous opioids that additionally act at δ and κ receptors. Thus, even a receptor-selective ligand can initiate a complex sequence of events involving multiple synapses, transmitters, and receptor types. The analgesic effect of opioids is arisen from their binding and activation of μ,δ and κ receptor. The MOPr have a pivotal rule in this mechanism but as supported by the study of genetic knockouts of the opioid receptors and genes in mice, also DOPr and KOPr are involved. Delta receptor agonists retain analgesic properties in receptor knockout mice. The distribution of opioid receptors in descending pain control circuits indicates a overlap between MOPr and KOPr. While the MOPr agonist action are invariably analgesic, whereas those of KOPr can be either analgesic or antianalgesic. The antinalgesic actions of the KOPr agonists appear to be mediated by functional antagonism of the action of MOPr agonist. Opioids are clinical used in both acute and chronic pain. In intra-operative, post-

operative and post-traumatic pain, acute pain situations, opioids are used pre-emptively or therapeutically. The use of opioids in chronic pain, malignat (cancer-related) or non-malignant (neuropathic and imflammatory) is controversial because is not clear how well they work.

Tolerance and dependence

The major limitation to opioid long-term use is the development of physiological tolerance, a profound decrease in analgesic effect observed in during prolonged administration of these drugs. The development of opioid tolerance in humans varies depending on the route of administration and on the disease state for which the opioids are prescribed. For example, tolerance is usually not a problem with short-term postoperative epidural or intrathecal opioids but rather presents itself following chronic epidural or intrathecal opioid usage. Multiple hypotheses exist to try to explain morphine tolerance. Several manipulations of multiple mechanisms have been observed to effect the development of morphine tolerance: receptor down-regulation, receptor desensitization and cAMP superactivation. These mechanisms modulation is an adaptive response to nonphysiological doses of morphine. The downregulation of opioid receptors would lead to tolerance by reducing the number of receptors available for drug-mediated actions. A number of studies have been described a down-regulation of MOPr following chronic exposure to agonist in vitro but these data are not comparable with the in vivo time course of development of tolerance. Taken together, the available data suggest that it is unlikely that receptor downregulation is solely responsible for the development of morphine tolerance. This view has lead to the idea that rather than becoming downregulated, MOPrs may instead become desensitized or, more precisely, uncoupled from downstream signaling pathways. Several studies have examined whether there is functional desensitization of MOPrs in tolerant animals. In these reports, differences in opioid receptor G-protein coupling were measured in different brain regions and in the spinal cord before and after chronic opioid exposure in rats.

These detailed studies demonstrate that the extent of MOPr desensitization is highly dependent upon the brain region examined. The most substantial changes in desensitization/coupling were observed in brain areas that mediate nociception and where tolerance to the analgesic and respiratory depressant effects of morphine were observed. (Sim-Selley et al., 2007). Following chronic morphine treatment, cellular levels of cAMP are elevated, a phenomenon termed cAMP superactivation. Instead the elevated cAMP levels reflect cellular adaptive changes, which include increased expression of certain types of adenylyl cyclase, protein kinase A (PKA), and cAMP response element binding protein (CREB). These changes are long term and difficult to reverse.

The chronic use of opioids also causes physiological dependence. Dependence is reflected by a need for continued administration of increasing doses of drug to prevent the development of opioid withdrawal. The physiological drug-dependent state is revealed following cessation of the drug, manifested with the classic withdrawal syndrome. The signs and symptoms of withdrawal include rhinorrhea, lacrimation, yawning, chills, gooseflesh (piloerection), hyperventilation, hyperthermia, mydriasis, muscular aches, vomiting, diarrhea, anxiety, and hostility. Administration of an opioid at this time suppresses abstinence signs and symptoms almost immediately. Neuronal adaptations resulting from the long-term use of opioid drugs were first hypothesized to explain the development of drug dependence. Tolerance and dependence may share a common mechanism because the severity of withdrawal signs and the extent of the development of tolerance correlate well in vivo and in vitro. The cAMP superactivation has been suggested as a common mechanism contributing to both tolerance and dependence. However, experiment rule out in modified mice have been showed that tolerance and dependence can be dissociated. In DOPr deficient mice that not develop morphine tolerance, was observed withdrawal behaviours (Nitsche et al., 2002).

Another phenomena associated with opioids is the addiction, describe as the compulsive seeking and taking of the drug, and the persistent risk of relapse even after years of

abstinence. A specific neurocicuits mediate the transition from occasional, controlled drug use and the loss of behavioral control over drug seeking and drug taking that defines chronic addiction. Opioids are a classic drug of addiction that develop tolerance and escalation of intake, as well as profound dysphoria, physical discomfort and somatic withdrawal signs during abstinence. The neurocicuits involved in the addiction effect are the mesocorticolimbic dopamine system, which play a key role in positive aspect of opioid addiction, and the LC and the periaqueductal gray (PAG) in negative aspect. The effects of chronic morphine treatment on the activity of dopamine cells on mesolimbic system are made up of both pre- and postsynaptic adaptations. Presynaptic adaptations involve cAMP-dependent and -independent mechanisms that include the regulation of both GABA and glutamate release. Many of the postsynaptic adaptations may result from the direct activation of κ-opioid receptors or indirectly from altered afferent input. The primary result is a long-lasting decrease in dopamine cell activity. This decreased activity results in part because of an increase in GABA-mediated inhibition and possibly augmented presynaptic inhibition of glutamate release. The similar inhibitor regulation mechanism has been shown in PAG and LC, which form part of a descending (and ascending) network that plays a core role in the generation and expression of negative aspects of opioid withdrawal with diverse influences on somatic, autonomic, and aversive components of the phenomenon.

Side Effects of acute opioid application

In addiction to tolerance, dependence and addiction opioids arise other side effects dependent on distribution of their receptor. The opioids receptors MOP and DOP are located on the pre-Boetzinger complex (PBC) of the brainstem. The activation of these receptors inhibit the respiratory rhythm regulate in this brain region. Opioids produce a dose-dependent depression of ventilatory response to hypercarbia and hypoxia. It has been shown a possible involvement of serotonin 4(a) (5-HT4(a)) receptors expressed in PBC and regulate by MOPr.

Treatment of rats with a 5-HT4 receptor-specific agonist overcame opioids induced respiratory depression and re-established stable respiratory rhythm without loss of opioids analgesic effect (Manzke et al., 2003). Another central nervous system side effect is sedation, sometimes reported as fatigue and tiredness by patients. Opioids stimulate nausea and vomiting by a direct effect on the chemoreceptor trigger zone in the area postrema in the brainstem. Cough suppression effects take place on medullary cough centers (raphe nuclei). This effect is produce by dextroisomers of opioids that do not have analgesic activity. The increase of parasympatic tone in the autonomic nucleus of oculomotor nerve mediate the pupil constriction caused by opioid use. Opioid receptors are found throughout the enteric nervous system, in the nervous plexus of the bowel, in the sacral plexus, along the biliary tree, and in ureters and bladder. Opioids stimulate tonic contraction of smooth muscle at all of these sites, while reducing normal propulsive activity. Inhibition of normal intestinal secretions and peristalsis can lead to increased water absorption and constipation. This effect is mediated by the blockade of presynaptic release of acetylcholine.

Chronic morphine use and abuse has been documented to result in severe immune consequence. Acute and chronic opoids treatment affect innate and adaptive immunity. Opioids has been shown to mediate these effects by acting directly both on receptors present on the immune cells and on centrally mediated pathways. MOPr gene expression is modulate in both immune cell and neuronal cell by immune-cells derived cytokines. Kraus and colleague shown that IL-4 induce MOPr transcripts in human primary blood cells (T cells and polymorphonuclear leukocytes), immune cell lines (U-937, and HMEC-1), and dendritic cells (Kraus et al., 2001) This effect was mediated through the STAT6 family of transcription factors (Bonner et al., 2004). The indirect mechanism involving opioid receptors in the CNS, cause the release of catecholamines and steroids, which then affect the functioning of immune cells.

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Chapter 3- Neuronal cells death

Cell death does not only account for the maintenance of a constant size and cell number in proliferative tissues (skin, intestinal mucosa or the immune system), but also plays a crucial role during the development of the peripheral and central nervous system. Moreover, degeneration of one or more nerve cell populations is involved in acute and chronic neurological diseases. In neuronal cells was observed three forms of cell death : Apoptosis, necrosis and autophagy (Levine et al., 2005). This chapter is focus on apoptotic mechanism in neuronal cell death and neurodegeneration.

Apoptosis

In 1972, Kerr coined the term apoptosis, after the Greek word meaning leaves falling from a tree, to describe an intrinsic cell suicide program involved in the normal turnover of hepatocytes (Kerr et al 1972). Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear membrane breakdown and the formation of apoptotic bodies, which are small membrane-bound vesicles phagocytosed by neighboring cells. Apoptotic signals, both intracellular and extracellular, converge to activate a group of apoptotic-specific cysteine proteases termed caspases that cleave their substrates (Thornberry and Lazebnik, 1998). Two apoptotic pathways have been identified: The extrinsic pathway, which involves the activation of a death receptor upon binding of its ligand, recruitment of specific protein at death domain and downstream signaling throught a cascade of protein-protein interaction and the intrinsic pathways involves mitochondria and the release of pro-apoptotic factors into the cytosol with subsequent activation of executioner caspases.

The death receptor transmit the signal from the cell surface to intracellular pathway. The best characterized death receptor are Fas (CD95 or APO1), TNF receptor 1, TNF-related apoptosis inducing ligand receptor 1 and 2 (TRAIL-R1 and TRAIL-R2). The death ligands are constitutively homotrimeric; so binding to their receptors leads to the formation of a

minimally homotrimeric ligand–receptor complex that recruits further cytosolic factors, such as FADD and caspase-8 , forming an oligomeric death-inducing signalling complex (DISC). Caspase-8 can directly activates the direct cleavages of caspase-3. It was described, also, a crosstalk between extrinsic and intrinsic pathways. In cells where the DISC formation is weak induce an activation, via caspase-8, of pro-apoptotic protein Bid, which then triggers the release of mitochondrial proteins (Riedl and Shi et al., 2004).

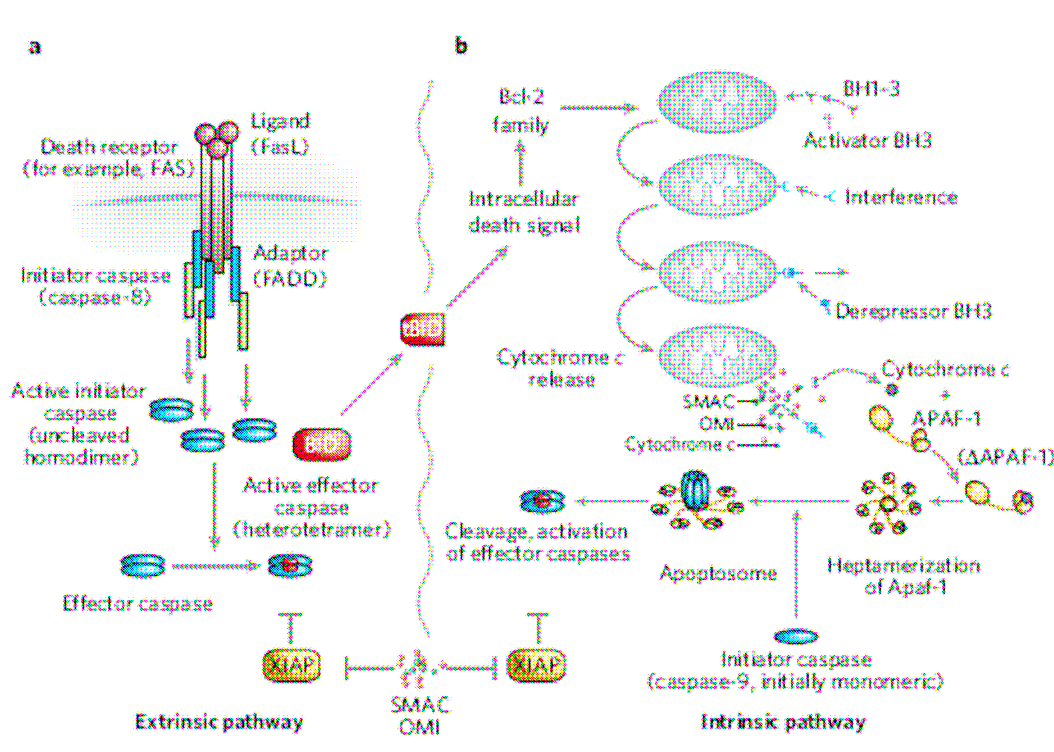

Figure 1: Apoptosis occurs through two main pathways (a) extrinsic pathway, which originates through the activation of cell-surface death receptors such as Fas, and results in the activation of caspase-8 or -10 and (b) the intrinsic pathway, which originates from mitochondrial release of cytochrome *c* and associated activation of caspase-9 (Bredesen et al., 2006).

The mitochondrial release, during intrinsic apoptotic pathway, small pro-apoptotic protein normally located in internal membrane. Some of the well-characterized proteins include cytochrome *c*, SMAC (second mitochondria- derived activator of caspases)/DIABLO (direct inhibitor of apoptosis (IAP)-binding protein),AIF (apoptosis-inducing factor), EndoG (endonuclease G) and OMI/HTRA2 (high-temperature-requirement protein A2). The cytochome c, a 13 KD protein, bind to an adaptor molecule the apoptotic activator factor 1 (Apaf-1). This complex is call apoptosome. Its function is to recruit and activate caspase-9 in

the presence of ATP. Caspase-9 activate pro-caspase 3 or 7 which process substrates, like caspase activated DNase (ICAD) or PARP, and leads to DNA fragmentation. Caspase-3 is also activate by SMAC/DIABLO – IAP way. SMAC/DIABLO protein is release from mitochondria and inhibit the IAP function. IAP is an inhibitor of caspase-3 cleavage, so the effect of SMAC/DIABLO is to block the caspase-3 cleavage inhibition. OMI/HTRA2 protein is another inhibitor of IAPs that have a pro-apoptotic effect. OMI/HTRA2 can induce cell death also *via* its serine protease activity what may in turn induce caspase activation. AIF and EndoG are involved in the caspase independent apoptotic pathway. The both protein interact with DNA and or RNA to cause chromatin condensation and large scale DNA fragmentation (Lorenzo and Susin, 2007). AIF does not display intrinsic endonuclease properties and its DNA-degrading activity certainly depends on the recruitment of downstream nucleases. A cooperation between AIF and EndoG, which is a mithocondrial DNAse, was observed (Niikura et al, 2007). The mitochondrial release of these protein hinge on the membrane permeabilization regulation. Several mechanisms about membrane permeabilization have been propose. The first involve the opening of permeability transition pore (PTP), which cause swelling and rupture of membrane and the later release of the pro-apoptotic protein. A second mechanism is mediated by the Bcl-2 pro – anti apoptotic proteins. It has been hypothesized that PTP consist of the voltage-dependent anion channel (VDAC: outer membrane channel), the adenine nucleotide translocator (ANT: inner membrane channel), cyclophilin D (Cyp D). The role of these three component of the pore in the MPT mechanisms is not well know. Cyp D might be involved in the MPT Ca^{2+} -dependent events and in the cyclosporin A (CsA) sensitive MPT. The Ca^{2+} - induce MPT mechanism might activate the VDAC component of PTP.

Bcl-2 family proteins show a range of bioactivities, from inhibition to promotion of apoptosis. In mammals, there are at least 12 core BCL-2 family proteins, including BCL-2 itself and proteins that have either three-dimensional (3D) structural similarity. BCL-2 family

members have classically been grouped into three classes. One class inhibits apoptosis (BCL-2, BCL-XL, BCL-W, MCL1, BCL-B), whereas a second class promotes apoptosis (BAX, BAK and BOK). A third divergent class of BH3-only proteins (BAD, BIK, BID, HRK , BIM, BMF, NOXA and PUMA) have a conserved BH3 domain that can bind and regulate the anti-apoptotic BCL-2 proteins to promote apoptosis. BAX and BAK proteins act as pro-apoptotic factor inducing a conformation change and permeabilize the outer mitochondrial membrane. This proteins appear to be able to form membrane channels and it might be a possible mechanism of action . However, the biochemical nature of this putative BAX/BAK pore, such as the number of molecules of BAX that comprise the pore, remains unknown. BAX/BAK interact with the VDAC pore component (Antosson et al., 1997). Bcl-2 might directly inhibit a component of the PTP complex. In fact, Bcl-2 (Bcl-x_L) is capable of blocking VDAC activity and ANT activity in liposome systems and consequently inhibit the MPT (Shimizu et al., 1998). In summary, the experiments carried out until now has been shown as the formation of homo and/or hetero dimers and the equilibrium shift between pro- and anti-apoptotic members of the Bcl- 2 family may determine the sensitivity of a cell to apoptotic stimuli.

Apoptosis in neurodegeneration

Chronic neurodegenerative diseases are characterized by a selective loss of specific subsets of neuronal populations over a period of years or even decades (Okouchi et al, 2007). Recent findings indicate that components of both the extrinsic (death receptors or their ligands) and intrinsic (Bcl-2 family members) death pathways are involved during neurodegeneration or neuronal injury *in vivo.* The induction of death pathway is mediate by different mechanism. The reactive oxygen and nitrogen species are involved in the neuronal cell death activation. In neuronal cells exist an equilibrium between the reactive oxygen species (ROS) flux and the antioxidant system. A deficit of antioxidant defenses, that occurred in aging, and a ROS overload leads to oxidative stress. In Alzheimer disease (AD) has been shown that β-amyloid

production activate the generation of H_2O_2 and HNE, ROS species, which induce JNK and p38 pathways. JNK was shown to down-regulate anti-apoptotic Bcl-xL and Bcl-w expression and this effect potentiated the release of Smac and the consequent activation of caspase-9 (Yao et al., 2005). Similar mechanisms involves JNK pathways and mitochondrial released protein is been described in the Huntington's disease (HD) neuronal cells loss. This pathology cause a neuronal death in the striatum and cerebral cortex, cause motor impairment, personality change and dementia. The expansion of CAG repeats in exon 1 of the IT15(huntingtin) gene are translate into an expanded polyglutamine tract near the N-terminus of the Huntingtin protein. Huntingtin modify protein provoke the JNK activation and the release of Smac/Diablo protein form outer mitochondrial membrane. Parkinson disease (PD), the second common neurodegenerative disorder after AD, has been demonstrated to degenerate the dopaminergic neuron via a JNK/p38 pathways. The substantia nigra (SN) pars compacta, which is the primary area of the brain that is affected by PD, contain dopaminergic neuron that exhibit high levels of basal oxidative stress, rending this area vulnerably to a toxic insult. In addiction to oxidative challenge, the loss of essential growth factors (NGF and BDGF) critical for survivals of neurons also contribute to the degeneration of this cells. The familiar Parkinson disease is associated with the genetic mutation of a parkin a protein associated with outer mitochondrial membrane. Over expression of parkin wild-type has been shown protective effect on neuroblastoma cells (Jiang et al., 2004). Another PD associated gene DJ-1, even it involved in mitochondrial function, have an anti-apoptotic effect.

In the amyotrophic lateral sclerosis (ALS) has been described the activation of Fas/NOS pathaways as mediator of motoneuronal death. NO species are product by specific neuronal NO synthase (nNOS). In the Brain, nNOS, whose expression is regulated by both physiological and pathophysiological stimuli, accounts for most $NO^{\cdot}$ activity. $NO^{\cdot}$ reacts rapidly with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^{-}$) which is the most reactive species. Fas signalling activates two principal pathways that act synergistically: one involves Fas-

associated death domain protein (FADD)-mediated activation of procaspase-8 and cytochrome *c* release, and the other, which appears to be specific to motoneurons, involves the activation of p38 mitogen-activated protein kinase (MAPK) and neuronal nitric oxide synthase (nNOS). An increased sensitivity to Fas-activation and nNOS pathway has been shown in patients with a mutate superoxide dismutase-1 (SOD1). SOD1 mutation is associated with ASL disease. This mutate protein have a toxic effect for the motoneurons, a hypothesis to this effect is that SOD can directly promote the generation of active oxygen and nitrogen species. Mutate SOD1 is purpoted to down-regulate Nrf2, an inhibitor of Fas pathways (Raoul et al., 2005).

Degeneration of α-motor neurons of spinal cord is the molecular mechanism of spinal muscular atrophy (SMA). The genetic cause of this disease is the mutation of survival motor neuron (SMN1) gene. SMN1 interact with Bcl-2, attenuating Bax and Fas mediated apoptosis, and with p53. the decreases in the ability of mutate protein to bind Bcl-2 and p53 is associated with disease severity. The neuronal apoptosis inhibitory protein (NAIP) is another gene correlated with SMA. NAIP acts as an inhibitor of caspase-3 and 7.

The lost of brain function observed in diabetics patients are associated with a neuronal apoptosis. Diabetic encephalopathy is a diabetic complication which causes alteration in cognition, behaviour and neuropsychology. The neuronal death seems to be dependent of impaired insulin/IGF axis and signaling. It has been documented a possible rule of insulin/IGF axis in the regulation of β-amyloid levels (Steen et al., 2005). Another contributing pathogenic factor of diabetic encephalopathy is C-peptide. This peptide has an insulin mimetic effect on insulin receptor. Deficiency in C-peptide is associated with neuronal apoptosis (Sima and Li, 2005). The hyperglycemia associated with diabetes induce an oxidative stress and a consequent susceptibility of the hippocampus and cortical regions of brain.

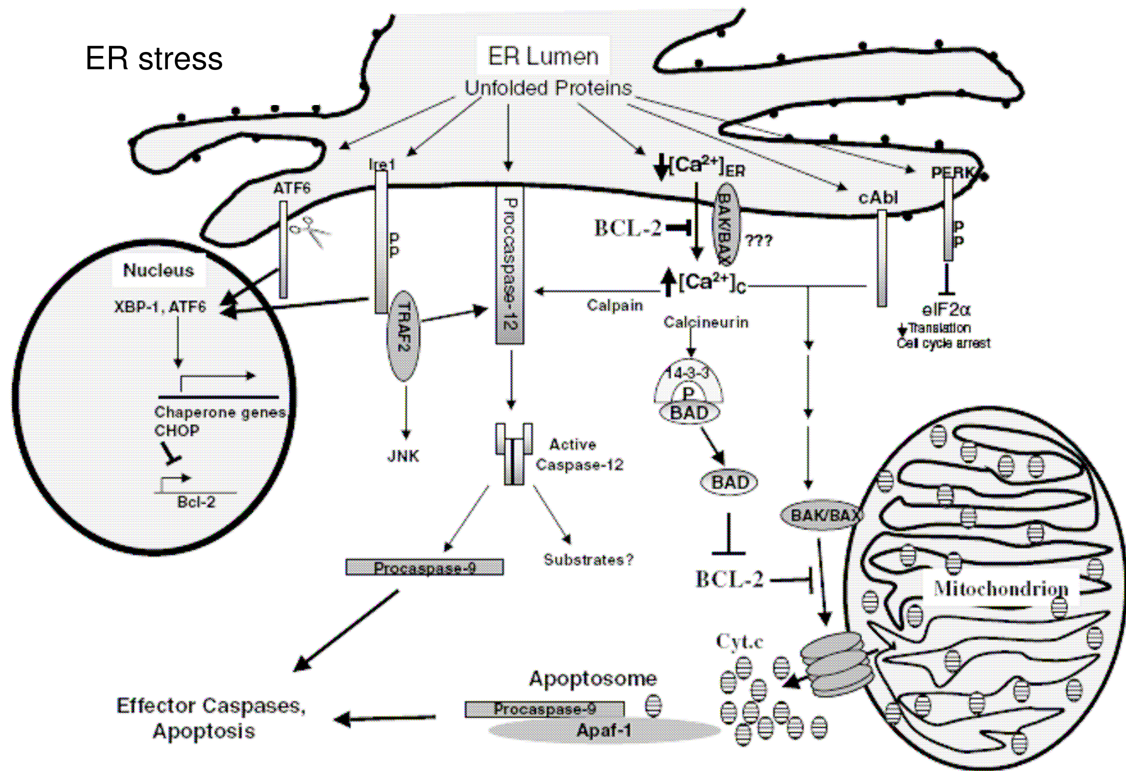

Figure 2: When ER capacity to fold properly proteins is compromised or overwhelmed, a highly conserved unfolded protein response (UPR) signal transduction pathway is activated (Breckenridge et al., 2003)

The Intrinsic and extrinsic apoptosis pathways are well studies. However it has been described a third pathways involved in neuronal death, it is the endoplasmic reticulum (ER) stress pathways. It has been showed that the activation of this pathways is caused by accumulation of unfolded or aggregate proteins and by changes in calcium homeostasis within the ER. The neurodegenerative disease are characterized by an accumulation of proteins, β-amyloid in AD, α-synuclein in PD and Huntingtin to HD. In healthy cells the ubiquitin proteasome system (UPS) and the autophagy mechanisms take under control the accumulation of aggregate of misfolding proteins in the cytosol. The control activity of UPS and autophagy is mitigated in neurodegenerative diseases either by the protein aggregates or by enhanced oxidative stress and other toxic products. Dysfunctional in these mechanism in turn cause more accumulation of proteins in the cell leading also to ER stress and consequent cell death. ER stress caused a release of Ca^{2+} from the endoplasmatic membrane and the activation of caspase pathways. The caspase proteins involved in the early stage of this pathways seem to

be caspase-12, caspase-4 and capsase-2, the later specific of neuronal cells (Dahmer , 2005). It has been recognized that exists an important crosstalk between the ER and mitochondria in the execution of cell death. It has been suggested that the Bcl-2 family proteins play a crucial role in ER–mitochondria interactions, and Bcl-2 and Bcl-xL associate with mitochondria and with the ER membrane. Mitochondrial membrane permealitation is modulate by Ca^{2+} concentration in the cytosol and it is, another, point of crosstalk between mitochondria and ER (Breckenridge DG et al., 2003). In the PD the expression of Parkin can restore proteasome function and promote survival, while loss of function of Parkin causes ER stress with accumulation of cytotoxic fibrils and protein aggregates in cells (Imai et al., 2004). ER stress is associated with the AD neuronal degeneration. ER is involved in the cleavage of β-amyloid. The PS1 and PS2 proteins, ER transmembrane proteins, are parts of the multiprotein γ-secretase complex that mediates the intramembranous cleavage of APP. Mutations in these proteins cause changes in the pattern of APP processing in the cell and increases the amount of the more toxic Aβ-42 peptide (Wilquet et al., 2004). The activation of caspase-12 and caspase-4 may contribute to neuronal death in AD as described in different studies (Nakagawa et al., 2000 and Hitomi et al., 2004).

Androgen Steroids and neuronal cell death

Androgen steroids, testosterone and its derivates, can regulate the survival and death of neuronal cells through genomic and nongenomic mechanism of actions. It has been well described the neuro-protective role of androgens in AD (Driscoll and Resnick, 2007). Androgens inhibit the accumulation of β-amyloid through two different pathways. The aromatization of AS to estradiol and activation of estrogen pathways is the first mechanism. A second pathways, estrogen independent, was observed in a series of rodent studies using the testosterone metabolite Dihydrotestosterone (DHT). It has been found that testosterone provides partial protection against Aβ toxicity , by activating a rapid, non-genomic pathway that involves MAPK signaling (Nguyen et al., 2005). This testosterone neuroprotection is

mimicked by DHT but not 3α-diol and is not attenuated by antagonists to estrogen receptors (Pike, 2001). In cerebellar granule cells treated with micromolar concentration of testosterone is observed a neuro- protective effect of steroids against the oxidative stress (Ahlbom et al., 2001).

A central nervous system toxicity is assume to be a possible factor of syndrome call steroid dementia. This is a side effect observed in some patients treated with glucocorticoid and it was assumed to be caused by a neuronal cell death (Wolkowitz et al., 2007). Similar effects of androgenic steroids are not yet described in clinical studies. Although, neurotoxic effect has been observed in vivo and in vitro studies. In a cellular model of spinal and bulbar muscular atrophy (SBMA) was demonstrated pivotal rule of androgen receptors with a repeated glutamine residues in the amino terminus region. The cells expressing this modified receptor are more susceptible to cell death when treated with androgens (Grierson et al., 2001). The cells SH-SY5Y treated with supra-physiological concentration of testosterone showed an apoptotic effects through the activation of inositol-1-4-5-trisphosphate receptor mediated intracellular Ca^{2+} release (Estrada et al., 2006). In a mouse cortical mixed cultures treated with steroids was observed an amplification of excitotoxicity induce by NMDA (Orlando et al., 2007). This effects was observed at micromolar concentration of testosterone, while nanomolar concentration is neuroprotective. Nandrolone, stanozolol and genestrione are toxic at nanomolar concentration. Testosterone effect is regulate by aromatase activity. In fact, when it was combine with anastrozole, an aromatase inihibitor, nanomolar concentration was no longer neuroprotective. Androgen receptor seems to be not involved in this pro-apoptotic effect of testosterone as show in experiment carried out with flutamide, an AR specific inhibitor. Instead, nandrolone acting via AR activation. The neurotoxic effect of steroids would be mediate by a specific membrane receptor. Studies carried out in primary cortical astrocytes and PC12 cell line shown a binding of dihydrotestosterone (DHT) or testosterone on membrane receptor. DHT conjugate with BSA enhances the toxic effect of

IAA, an inhibitor of glycolysis and a promoter of oxidative stress, and the cell death in astrocytes cells (Gatson and Singh , 2007). The PC12 cells not-differentiated express a androgen steroids membrane specific receptor. Its activation increased cytosolic Ca(2+) and induced apoptosis (Alexaki et al., 2006).

Sigma receptor and apoptosis

Sigma receptors (Walker at al., 1990) are non opiate and non phencyclidine binding sites that mediate the psychotomimetic actions of certain opioid derivatives. Sigma receptors consist of two subtypes, sigma-1 and sigma-2, that are distinguishable by biochemical and pharmacological means (Quirion et al., 1992). Sigma receptor 1 (σR1) is the best characterized. It mediates immunosuppressant, antipsychotic, and neuroprotective effects. Sigma receptor-1 ligands have demonstrated neuroprotective properties including inhibition of ischemia-induced glutamate release, attenuation of postsynaptic glutamate-evoked calcium influx, and reduced NO production. Studies anatomical distribution of σ1 subtype showed that in the central nervous system (CNS) the regions with high levels are the areas involved in motor, sensory, and endocrine functions, and memory. In peripheral tissues, they are present in placenta, liver, immune cells, and gastrointestinal tract. The high expression of σR1 receptors in steroid-producing tissues and in CNS suggests their possible role in functions of neuroendocrine and central neuroactive steroid system. Additional involvement of σR1 receptors has been postulated in psychosis with modulation of synthesis and release of neurotransmitters such as acetylcholine and dopamine.

Sigma receptor 2 (σR2) subtypes have a different pattern of anatomical distributions in CNS and in peripheral tissues. A very high concentration of σR2 receptors has been discovered in neuronal and non-neuronal tumor cell lines and this provides evidence of a possible role in cell proliferation and viability (Vilner et al., 1995). In fact, putative σR2 agonists, such as ibogaine and CB-64D, induce apoptotic death by a mechanism involving the

increase of intracellular Ca^{++} level by release from endoplasmic reticulum and subsequently from mitochondrial stores (Vilner and Bowen, 2000). These effects are inhibited by putative σR2 antagonists BD1047 and BD1063 (Bowen 2000). In a breast tumor cell line MCF-7 was observed a caspase-3 independent apoptosis pathways activate by σR2 (Crawford and Bowen, 2002). Bowen and colleagues showed in neuroblastoma cells SK-N-SH a caspase-10 dependent apoptotic pathway activated by CB-64D and associated with Bid cleavage and endonuclease G mitochondrial release (Wang and Bowen, 2006). Sigma-2 receptor activated, also, a lipid dependent apoptotic pathways. In MCF-7 was found an increase in ceramide synthesis associated with the induction of apoptosis (Crawford et al., 2002).

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Aim of the research

Epidemiological studies and in vivo experiment has been shown that administration of large doses of anabolic androgenic steroids (AAS) are correlated with neurobehavioral changes like hyperexcitability, supra-aggressive nature and suicidal tendencies. These behavioral changes could be mediated by the regulation of neurotransmission or to be the outward manifestation of neuronal damage resulting from exposure to high concentrations of AAS.

Nandrolone regulation of opioid pathway has been shown in vitro and in vivo studies. In the first part of my thesis I study the possible effects of nandrolone on MOPr expression at both the transcriptional and post-transcriptional levels in the neuroblastoma cell line SH-SY5Y, which expresses this receptor constitutively and serves as a common model for neuronal cells. AAS may modulate neural transmission via genomic and non genomic mechanisms by binding directly to the intracellular androgen receptor (AR) or acting through other signalling pathways. MOPr expression can be regulate by a direct effect on MOPr promoter or by post-transcriptional mechanism occur at mRNA and protein levels. In fact , changes in mRNA stability, differences in translation efficiency, alterations in mRNA transport, and modification of the receptor are among the possible mechanisms involved in modulation of MOPr mRNA expression. I explore the possible rule of AR activation in the MOPr regulation mediated by nandrolone with Hydroxyflutamide, a specific inhibitor of AR. A luciferase reporter vector bear the sequence of MOPr promoter and the inhibitor of transcription , Actinomycin D were used to elucidated the transcriptional and post-transcriptional mechanisms .

The neuronal cell death caused by supra-phisiological concentration of anabolic androgenic steroids has been evaluated with three cito-toxic assays : MTT, LDH and Trypan blue exclusion assay. The neuronal cells SH-SY5Y were exposed to nandrolone, testosterone and stanozolol and a time and concentration dependent neurotoxicity has been observed. To

explore the involvement, in neurotoxicity testosterone and nandrolone dependent, of androgen receptor and sigma-2 receptor was carried out MTT experiments with Hydroxyflutamide and a competitive radioligand binding assay between testosterone and RHM-1, selective sigma-2 ligand. In vitro studies performed with steroids conjugate with bovine serum albumin (BSA) was observed a specific steroid membrane receptor, which activation lead cell death progression. To evaluate the possible activation of this pathways in my neuronal cells, I exposed the SH-SY5Y with testosterone conjugate with bovine serum albumin.

Chapter 4 - Materials and Methods

Cell Cultures

SH-SY5Y (Fig.1) is a subclone of the SK-N-SH cell line that was established from a bone marrow aspirate of a 4 year old girl with a highly malignant neuroblastoma (Biedler et al., 1973). The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites). SH-SY5Y are diploid dopaminergic neuronal cell line. Both mu and delta opioid receptors are expressed in undifferentiated human neuroblastoma SHSY5Y cells and are negatively coupled to adenylate cyclase (Zadina et al., 1994). SH-SY5Y cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown in 75-cm^2 flasks, 100 mm dishes and 96-wells plates depending on experiment, with a medium compose of 1:1 Ham's F12 and MEM supplement with 10% fetal bovine serum (FBS), L-Gluatamine (1mM), 1x of non essential Amino acid and 1x antibiotic-antimycotic solution, in a humidified environment containing 5% CO_2 and 95% air. Cells were passaged every 3–4 days detaching with a cell scraper. The cells was used until passage 20. SH-SY5Y cells was differentiate with retinoic acid (10µM- 5 days) in 100mm dishes and 96-wells.

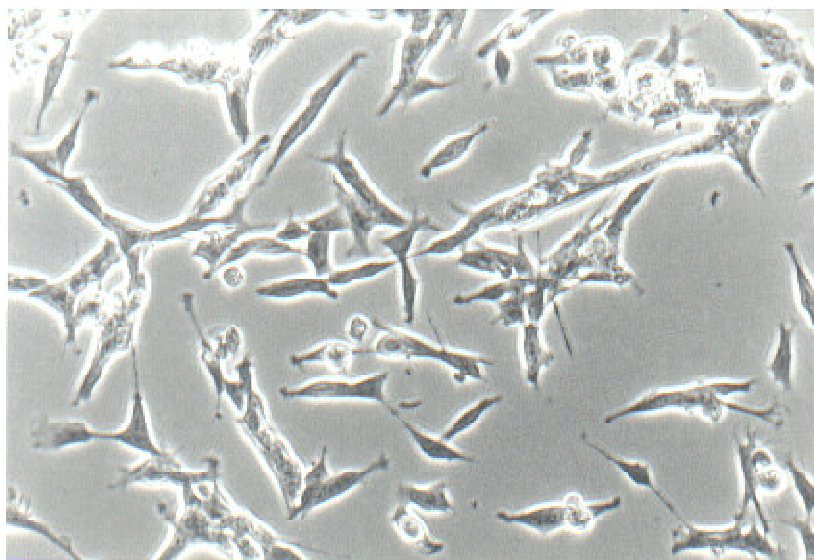

Fig.1 Picture of indifferenziate SH-SY5Y

MCF7 cells was established from the pleural effusion from a 69 year female Caucasian suffering from a breast adenocarcinoma. MCF-7 cells are a well-characterized estrogen receptor (ER) positive control cell line (cells are positive for cytoplasmic estrogen receptors) and therefore are a useful in vitro model of breast cancer to study the role of estrogen in breast cancer. This cell express high quantity of androgen receptor (Buchanan et al., 2005). MCF-7 was grown in 75-cm^2 flasks with a medium composed of RPMI medium supplemented with 10% (v/v) FCS.

Semiquantitative real-time polymerase chain reaction

For reverse transcription-polymerase chain reaction (PCR) experiments, cells were collected from tissue culture flasks, centrifuged (500 ***g*** for 5 min) and rinsed with phosphate-buffered saline (PBS). Total cellular RNA was extracted using Trizol® reagent (Invitrogen). RNA was quantify with spectrophotometere and a 2-µg aliquot was digested with RNAse-free DNAse (Invitrogen) for 15 min at 25 °C and according to the manufacturer's instructions. A 2-µg sample was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Milan, Italy) according to the manufacturer's instructions. A real-time PCR was employed for relative quantification of REST transcripts using the Light Cycler Instrument (Roche Diagnostics, Milan, Italy) and the SYBR Premix Ex Taq (Takara Bio Europe S.A., Gennevilliers, France). This 'hot start' reaction mix contains *Takara Ex TaqHS* DNA polymerase. This ready-to-use ‘hot start’ reaction mix contains *Taq* DNA polymerase, dNTP mix and the fluorescent dye SYBR Green I for real-time detection of double-stranded DNA. Reactions were set up in 20 µL including 10 ng of target DNA. To amplify the hMOPr cDNA, a sense primer (5'-CTGGGTCAACTTGTCCCACT-3') and an antisense primer (5'-TGGAGTAGAGGGCCATGATC-3') were used at 2.5 nM final concentration for amplifying a 146-bp fragment (327 to 472 bp) from GenBank™ Accession no. NM_000914. As a control, a 169-bp fragment of the human L19 ribosomal proteingene was amplified with a

sense primer (5'-CTAGTGTCCTCCGCTGTGG-3') and an antisense primer (5'-AAGGTGTTTTTCCGGCATC3') at 5 nM final concentration, producing a fragment (62 to 230 bp) from GenBank™ Accession no. BC062709. Amplification was as follows: 95°C for 10 s followed by 40 cycles of 95°C for 5 s, 62°C for 20 s and 72°C for 6 s. After that, the temperature was lowered to 40°C for 30 s and the specificity of the reaction was verified by analysis of the melting curve once the appropriate double-stranded DNA melting temperature had been reached.

Relative expression of the reverse transcription-PCR products was determined using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). This method calculates relative expression using the equation: normalized relative quantities (NRQs) = $E^{-\Delta\Delta Ct}$ (Hellemans et al., 2007). The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001), optimal and identical real-time amplification efficiencies of target and reference gene of $E = 2$ are presumed, so the final equation is $\text{NRQs} = 2^{-\Delta\Delta Ct}$. C_t is the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and $\Delta\Delta C_t$ = [C_t gene of interest (unknown sample) – C_tL19 (unknown sample)] – [C_t gene of interest (calibrator sample) – C_tL19 (calibrator sample)]. The control samples was chosen as the calibrator sample and used in each PCR. Each sample was run in duplicate and the mean C_t was used in the $\Delta\Delta C_t$ equation. L19 was chosen for normalization because this gene showed consistent expression relative to other housekeeping genes among the treatment groups in our experiments. The data was express as per cent of the $2^{-\Delta\Delta Ct}$, respect to the control assume as 100% (Fig.2)

MOR		Ct	Mean Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	%	log%$2^{-\Delta\Delta Ct}$
Sample 1	calibrator	0,00	0	0	0	1	100	2
Sample 2	calibrator	0,00						
Sample 3	Treated	0,00	0	0	0	1	100	2
Sample 4	treated	0,00						

Figure 2: An example of the Excel file created to evaluate the Real Time PCR data.

Western blotting

SH-SY5Y cells were scraped off in 10 mL of cold PBS, pelleted and resuspended in 100 µL of CER I buffer (NE-PER™ Extraction Reagent; Pierce, Rockford, IL, USA). After 10 min incubation on ice, 5,5 µL of CER II buffer (NE-PER™ Extraction Reagent; Pierce) was added and the suspension was resuspended by a vortex, incubated on ice for 1 min and then resuspended. The cytoplasmic fraction was separated by centrifugation at 16 000 ***g*** for 5 min. To obtain the nuclear extract, the cell pellet was resuspended in 50 µL of NER buffer and incubated on ice for 40 min. Soluble proteins were separated by centrifugation at 16000 g for 10 min at 4°C. The protein concentration was quantified by BCA protein assay (Pierce). Nuclear or cytoplasmic extracts (60µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cruz marker (sc-2035; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was also loaded on the gel as molecular weight standard. Proteins were then transferred to HybondTM ECLTM nitrocellulose membranes (Amersham Biotec, Milan, Italy), which were blocked in a 5% non-fat milk - TBS (10mm Tris-HCl, pH 8, containing 150 mm NaCl) - 0.1% Tween 20 solution for 1.5 h at room temperature (25°C). The blots were then probed for 1.5 h at room temperature in TBS containing 0.1% Tween 20, 5% non-fat milk and antibodies with a dilution of 1 : 800 of androgen receptor antibody (AR-441, Santa Cruz Biotechnology) Membranes were incubated with a dilution of 1:8000 peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1.5 h and the blots weredeveloped with SuperSignal

West Pico chemiluminescent substrate according to themanufacturer's protocol (Pierce). Blot images were digitally acquired by an LAS3000Imager (Fujifilm Corporation, Stamford, CT, USA). Protein expression was analyzedsemiquantitatively using AIDA (Raytest Isotopenmessgeräte GmbH, Mannheim, Germany).

Radioligand binding assay

SH-SY5Y cell membranes were prepared by homogenizing cells in 50 mM Tris- HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM benzamidine, with a Polytron homogenizer. After centrifugation (1000 x g for 10 min at 4°C), supernatants were centrifuged (18000 x g for 30 min at 4°C) and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl2. Protein concentration was determined by BCA assay (Pierce). For saturation binding experiments, cell membranes (100 µg/assay tube) were incubated in 100 mM Tris-HCl (pH 7.4) containing 0.3% BSA with increasing concentrations of [3H]-DAMGO (0.1-5 nM). Non-specific binding was determined in the presence of DAMGO (10 µM). After 90 min incubation at 25°C, bound ligand was isolated by rapid filtration on Whatman GF/B filters (Schleicher & Schuell). Filters were washed with 20 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and left in scintillation fluid for 8 h before counting. Data were fitted by nonlinear least-square regression and the LIGAND program (Munson and Rodbard 1980) was used to calculate receptor density (Bmax), Hill slopes and ligand affinity (Kd). Data are expressed as fmol of [3H]-DAMGO bound and normalized to cell protein content.

Competitive binding assay was performed incubating cell membranes (100 µg/assay tube) in 100 mM Tris-HCl (pH 7.4) containing 0.3% BSA with N-[4-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-yl)butyl]-2-methoxy-5-methyl-benzamide (RHM-1) radiolabeled with tritium (8nM) (Xu et al., 2005) alone or with increasing concentration of Testosterone (10^{-7}-10^{-5})

Cell viability Assays

The cell viability was evaluated with different methods: MTT, LDH and trypan blue. The yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color can then be quantified by spectrophotometric means. SH-SY5Y cells were seeded in 96-well plates at a density of 0.5×10^6cells/well in 100 µl of medium. The cells were treated with steroids and after 24 h, 48 h or 72 h 10 µl of MTT (5 mg/ml in phosphate buffered saline) was added at in each wells. The plates were incubated for 4h and later 100 µl of solubilization mixture (20% sodium dodecyl sulfate and 0.01 N HCl) were added and after an overnight incubation, absorbance was measured with a microplate reader at 570 nm.

To evaluate the release into the medium of lactate dehydrogenase from the cells was used the CytoTox 96 NonRadioactive Cytotoxicity Assay (Promega) 50 µl of medium from each wells were pipet in a new plate. An equal volume (50 µl) of substrate solution was added in each well. The plate is incubate 30 minute in the dark, after was added the stop buffer solution and analyzed the colorimetric changes with a microplate reader at 490 nm.

For trypan blue exclusion assay the cells were trypsinized, resuspended in 1ml PBS, and 10 µl of suspension was mixed with 10µl trypan blue dye solution (Sigma) before being counted with a hemacytometer.

Plasmid constructs

The plasmids used in this study are based on the pGL3-Basic vector system (Promega, Madison, WI) containing the luciferase reporter gene. Firstly, a fragment of the human MOPr promoter, ranging from –2447 to +8 (designated +1 at translation start codon, GenBank™ accession number AF153500) was amplified by PCR using genomic DNA obtained from SH-

SY5Y cells as template.The PCR was run using a forward primer (*5'-GTACGTCGACTCCCTCCAAAGCAACTAT-3')* and a reverse primer (5'-*GTATCTCGAGCTGTCCATGGTACTGACG-3')* bearing the *BspHI* site at its 3' terminus. This PCR product, named mu, was cloned into pCR-Blunt-II-TOPO (Invitrogen), sequenced (BMR Genomics, Padova, Italy), digested with *SacI/XhoI* and cloned into the pGL3-Basic vector or pGL3-SV40, vector containing the sequence of SV40 promoter between the multi cloning site, where the MOPr promoter is inserted, and luciferase coding sequence.

Cell Transfection and Reporter gene assays.

SH-SY5Y were plated in 24-well dishes and at 50-60% confluence were transiently transfected with pGL3-Basic, pGL3-Basic/mu or pGL3-SV40/mu reporter plasmids (1,5 mg /well) and pSV-ßGal (0,5 mg/well; Promega) by using EXGEN 500 Transfection Reagent (Fermentas, Hanover, MD). Cells were subsequently cultured in serum-free medium for 16-18 h and then exposed to nandrolone or left untreated for 48 h. Finally, cells were lysed in Reporter Lysis Buffer (Promega) and samples collected. Luciferase and beta-galactosidase activities of cell lysates were measured by Bright-Glo^{TM} Luciferase Assay System and Beta-Glo^{TM} Assay System (Promega) respectively, according to manufacturer instructions.

Immunocytochemistry of activated caspase-3

Undifferentiated SH-SY5Y cells were exposed to nandrolone and testosterone (10^{-4}M) for 48 h. After exposure the cells were washed with pre-warmed PBS and fixed in formalin (4% in PBS, pH 7.4) for 10 min, scraped into one tube per treatment, and pelleted by centrifugation (300 *g*, 5 min). The cells were washed one time with PBS and re-centrifuged. The cells were incubated with agarose (3% in PBS), 1 h, at 50°C. Subsequently, the cells were cast on ice and dehydrated in ethanol (70% overnight, 95% for 4 h, 99.5% for 3 h and xylene

for 30 min) and embedded in paraffin. The cells were sectioned (4 μm) on a microtome (Microm Heidelberg HM350, Germany).

The paraffin sections were deparaffinized in xylene and rehydrated in ethanol series (2 × 5 min in 99.5%, 95% and 70%), washed in PBS and PBS with 0.3% triton (PBST), and treated with 1% hydrogen peroxide in PBST (30 min). The sections were washed three times in PBST and PBS, and antigen retrieval was performed using microwaves. The sections were then washed three times in PBS followed by 1 h pre-incubation with 4% BSA for 1 h. The sections were then incubated with the antibody anti-cleaved caspase-3 (Cell Signaling, USA) overnight (at 5°C). After 10 min rinsing in PBS, the sections were incubated with biotinylated second antibody (30 min) and then with ABComplex/HRP (30 min) at room temperature. Immunoreactions were visualized using Vector VIP substrate kit for peroxidase. (Ostergren A et al., 2005)

Caspase-3 activity

The protein cytoplasmatic fraction of SH-SY5Y was extracted as described in previously pages in “Western blotting”. For colorimetric measurement of caspase activity, Ac-DEVD-*p*NA (BioMol, USA) was added at a final concentration of 200 μM, in a reaction buffer (40mM Hepes, 20mM NaCl, 2mM EDTA and 20% glycerol), to 100 μg of protein generated from cells under different experimental conditions. Past an incubation of 1h an 30m, caspase activity was monitored as optical absorbance at 405 nm.

Statistical analysis

In reporter gene experiments, each sample was assayed in triplicate, and each RT-PCR sample was amplified in duplicate. All data are presented as mean □SEM for the number of experiments indicated. Statistical significance was determined by Newman-Keuls test after

ANOVA using GraphPad Prism, version 3.0 (GraphPad Software, Inc., San Diego, CA, USA). P values <0.05 were considered significant.

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RESULTS

Nandrolone downregulates steady-state levels of MOPr mRNA and the density of MOPr binding sites in SH-SY5Y

SH-SY5Y cells were exposed to nandrolone (10^{-8}M-10^{-5}M) for 48 h and the endogenous MOPr transcripts were evaluated using a real time RT-PCR technique. A significant concentration-dependent (Fig. 1B) and time-dependent (Fig. 1A) downregulation of MOPr transcripts was observed. The maximum effect was at 10^{-5} M and it was present in cells exposed to this agent at least 48 h (Fig. 1B). Cells were treated, also, with testosterone (10^{-6}M) and stanozolol (10^{-6}M) for 48h and these experiments showed a downregulation of MOPr transcripts (Fig. 1C).

As show in Fig. 1D, we confirmed that in cytoplasmatic fractions of SH-SY5Y cells is present the AR showing the seem molecular weight of the AR occurring in MCF-7 cells a breast cancer cell line which express it. Therefore, it could be suggested an interaction between nandrolone and the AR expressed in SH-SY5Y.

To evaluate the potential involvement of the AR on this effect of nandrolone, the cells were exposed to hydroxyflutamide (10^{-5}M; 48 h), an androgen antagonist. This compound prevented the nandrolone-induced (10^{-6} M) reduction of MOPr transcripts (Fig. 1A) and when administered alone did not cause any change of MOPr mRNA.

A saturation binding assays was set up to evaluate MOPr density in cell cultures treated with 10^{-6} M nandrolone (48 h). The assay showed a significant decrease in the density of MOPr (B_{max}) on cell membranes (Table 1).

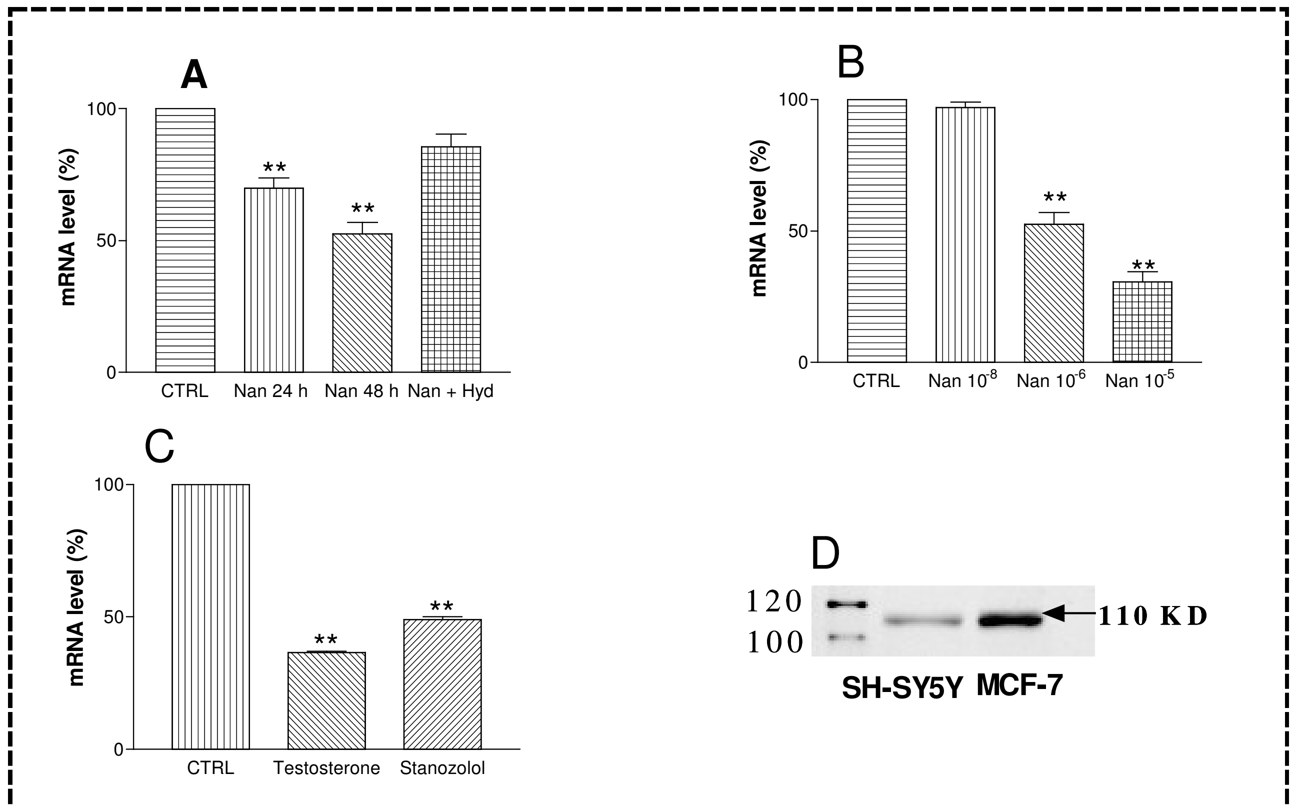


Figure 1. Nandrolone downregulates MOPr mRNA levels. (A) Time-dependent effect of nandrolone on steady state levels of MOPr mRNA and the effect of hydroxyflutamide. Cells were exposed to nandrolone for 24 or 48 h and the effect of selective androgen receptor inhibitor hydroxyflutamide (10^{-5} M) on nandrolone (10^{-6} M; 48 h)-induced downregulation of MOPr mRNA was evaluated. Nandrolone was added 1h after the antagonist. Steady-state levels of MOPr and L19 mRNA were calculated using the $\Delta\Delta Ct$ method. MOR mRNA levels were normalized to those of L19 and presented as % relative to those of control cells, which were arbitrarily set as 100%. Values are the mean ± S.E.M. of six experiments done in duplicate. **p<0.01 *vs.* CTRL. (B) Concentration-dependent effect of nandrolone. The Cells were growth for 48 h in culture medium (CTRL) or in medium supplemented with nandrolone (10^{-8}-10^{-5}M). (C) Testosterone and stanozolol at 10^{-6}M for 48h cause down-regulation of MOPr mRNA expression in SH-SY5Y (D) Representative western blot analysis of androgen receptor (AR) in cytoplasmatic extracts from SH-SY5Y and MCF-7 cells. Approximate molecular mass of AR (only a single band of approx. 110 kDa was observed) was determined by comparison with molecular mass standards (shown in the first line). A representative experiment, repeated three times with similar results, is shown **Table 1.** Binding Characteristics (Bmax and Kd values) of MOPr agonist [^{3}H]DAMGO in SH-SY5Y cells exposed to culture medium (CTRL) or to nandrolone (10^{-6}M) for 48 h.

Treatment	Bmax (fmol/mg protein)	KD (nM)
CTRL	**84±3**	**1,5±0.12**
Nandrolone	**56±7***	**0,92±0.34**

Mean ± S.E.M. of three experiments performed in triplicate. *p<0.05 *vs.* CTRL.

Nandrolone reduces MOPr mRNA levels through a post-transcriptional effect

To evaluate the effects of nandrolone on MOPr mRNA stability, cells were treated with the transcription inhibitor actinomycin D. By blocking transcription, we were able to determine whether nandrolone affects mRNA stability of MOPr transcripts. The cells were pre-treated with actinomycin D (1µg/ml) for 30 minutes and then exposed to nandrolone (10^{-6} M) for 48 hours. Real-time RT-PCR showed that the rate of MOPr mRNA decay was significantly increased. Therefore, this steroid seems to reduce the stability of MOPr mRNA (Fig. 2).

To investigate the effects of nandrolone on MOPr promoter transcriptional activity, a DNA construct containing a portion of the human MOPr promoter (from -2447 to +8 bp) including a GRE responsive element was generated. This cDNA was cloned into a pGL3-Basic plasmid, which contains a luciferase reporter, or into a pGL3-SV40 plasmid, containing the luciferase gene under the positive control of the SV40 promoter. The transcriptional activity of these plasmids was evaluated in transient transfection assays. Luciferase is express in response to promoter activation and will catalyzes the oxidation of substrate luciferin (added during the assay), resulting in photon production. Light output is directly proportional to amount of luciferase present in each sample. As show in Fig. 2B, nandrolone treatment (10^{-6} M; 48h) did not influence MOPr transcription in SH-SY5Y cells transfected with the pGL3-Basic/mu plasmid or with the pGL3-SV40/mu plasmid. As consequence of SV40-positive promoter activity, a more pronounced elevation of MOPr transcripts was observed in cells transfected with the pGL3-SV40/mu plasmid; however, nandrolone treatment did not modify this transcriptional activity.

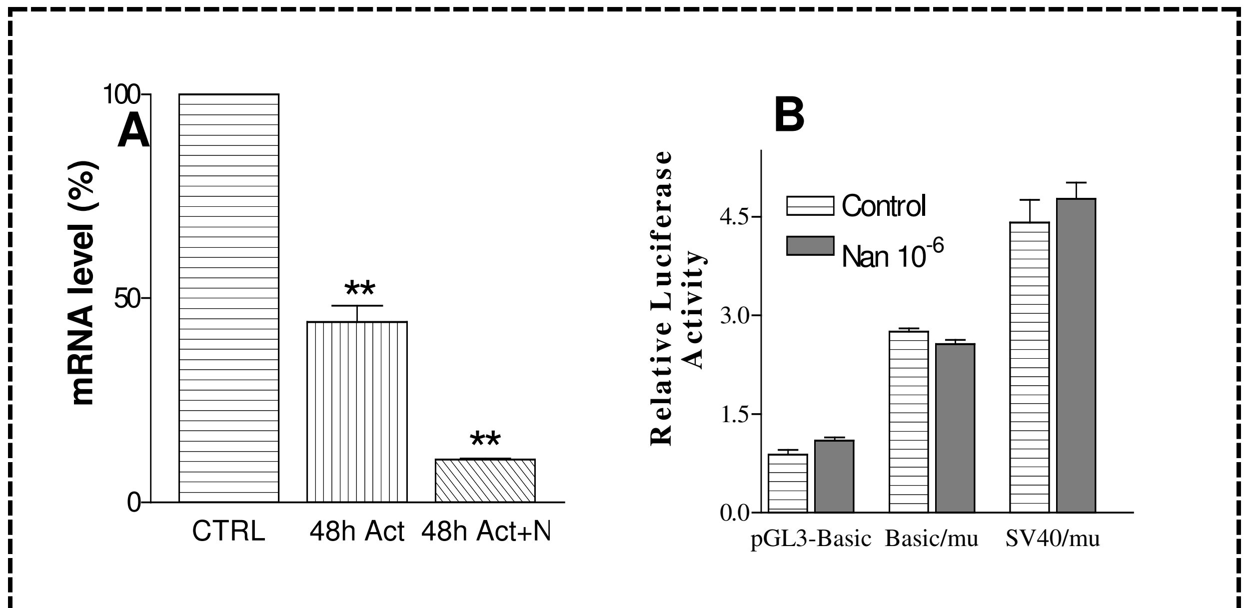


Figure 2. Nandrolone reduces MOPr mRNA through a pos-transcriptional effect.(A) SH-SY5Y were exposed to actinomycin D (1µg/ml)for 30 minutes and growth for 48 h in culture medium containing actinomycin D alone (Act) or in medium supplement with actinomycin D and Nandrolone 10^{-6} M (Act+Nan). For further details see the legend to Fig. 1. Values are the mean ± S.E.M. of six experiments done in duplicate. ** $p<0.01$ *vs.* CTRL. (B) Cells were transiently transfected with pGL3-Basic, pGL3-Basic/mu or pGL3-SV40/mu reporter plasmids and grown for 48 h in culture medium (CTRL) or in medium supplement with Nandrolone (10^{-6} M); thereafter, luciferase activity was measured on cell lysates. For detailed experimental conditions see " Materials and Methods". The transcriptional activity of each construct is expressed as relative luciferase activity, and transfection efficiencies are normalized to β-galactosidase activity by co-transfection of the internal control plasmid, pSV-βGal. The transcriptional activity of each construct is expressed as luciferase activity relative to the activity of cells transfected with pGL3-Basic, which had assigned a value of 1.0. The data are the mean ± S.E.M. of six independent experiments done in triplicate with at least three different plasmid constructs.

Supra-physiological concentration of androgen steroids induce toxicity in SH-SY5Y cells

To examine the effect of androgen steroids supra-physiological concentration, we set up a MTT assay. Reduction of yellow methyltiazoletetazolium (MTT) into blue insoluble formazan crystals is drived by mitochondrial of living cells. The viability of SH-SY5Y cells seeded in 96-wells plates were evaluated after to be exposed to nandrolone, testosterone and stanozolol (10^{-4}M-10^{-6}M) for 48h and 72h (Figure 3). The data collected show a toxic effect concentration and time dependent for the three androgen steroids. Nandrolone have a weak toxic effect at 10^{-5}M and more high effects at 10^{-4}M but no effects at 10^{-6}M. Testosterone shown a similar result but a highest potency as demonstrated by EC50 value while stanozolol has the comparable dose respond curves and EC50 values with nandrolone. (Figure 4)

Toxic effects of AS nandrolone and testosterone are evaluated , also, with the LDH test. The measurement of lactate dehydrogenase release from damaged cells is correlated with cell death. The medium of each samples was collected and analysed to evaluate the enzymatic activity of LDH release from the cells This assay performed on SH-SY5Y cells exposed to nandrolone and testosterone (10^{-4}M-10^{-5}M) for 72h, confirm the toxic effect time- and concentration dependent of AAS (Figure 5)

The trypan blue exclusion test is another experiment carried out to confirm the toxicity of supra-physiological concentration of AS. A decreased in the living cells number was observed in SH-SY5Y cells treated with nandrolone and testosterone (10^{-4}M and 10^{-5}M) for 48h in comparison with control cells not treated (Figure 5).

SH-SY5Y cells are differentiated with retinoic acid to evaluated a possible changes in cell death hormones dependent. Cells exposed with retinoic acid for 5 days were after treated with the three steroids (10^{-4}M-10^{-5}M) for 48h. Data collected did not show any remarkable different in neurotoxicity compare with undifferentiated cells (Figure 3).

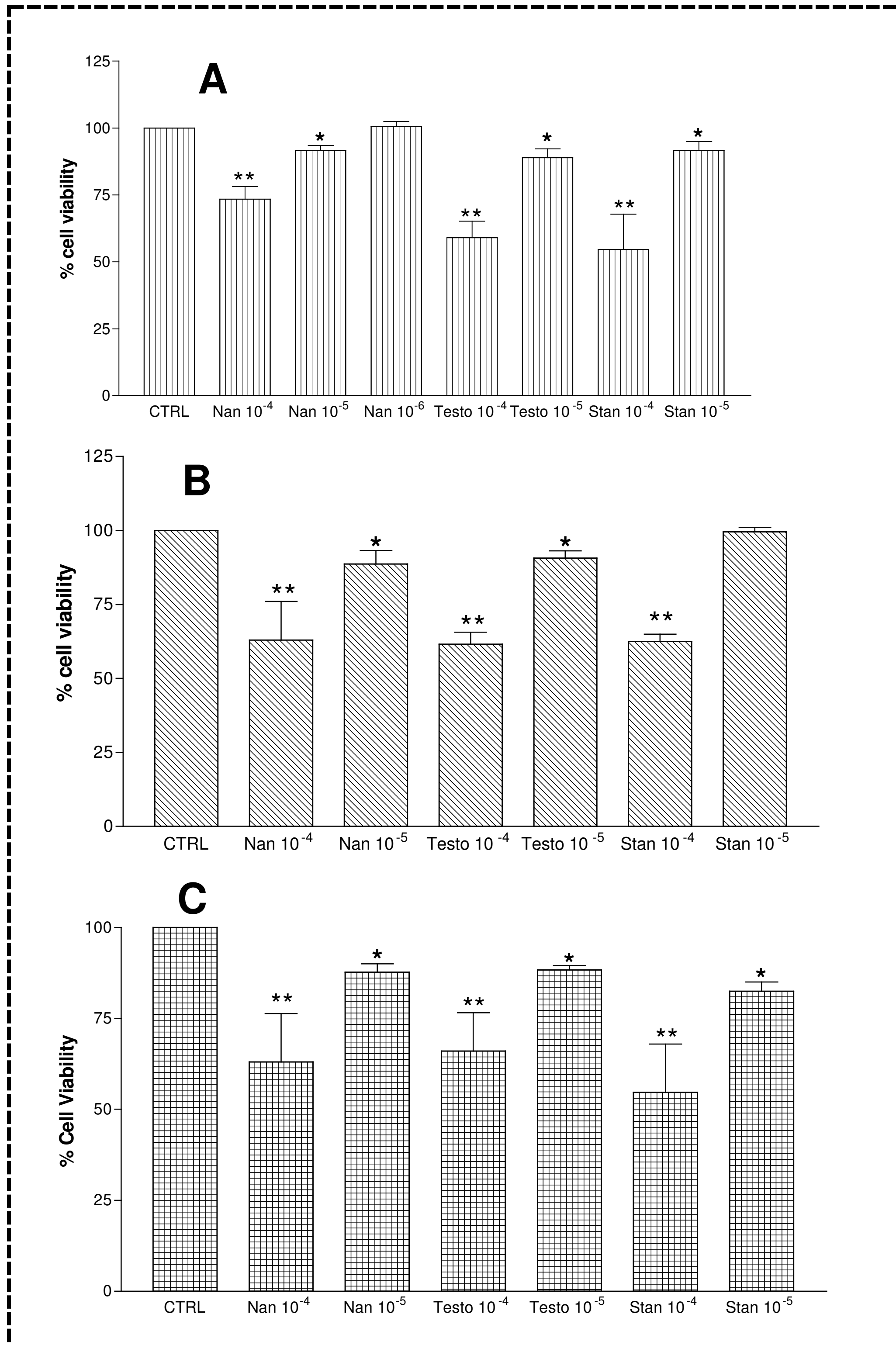
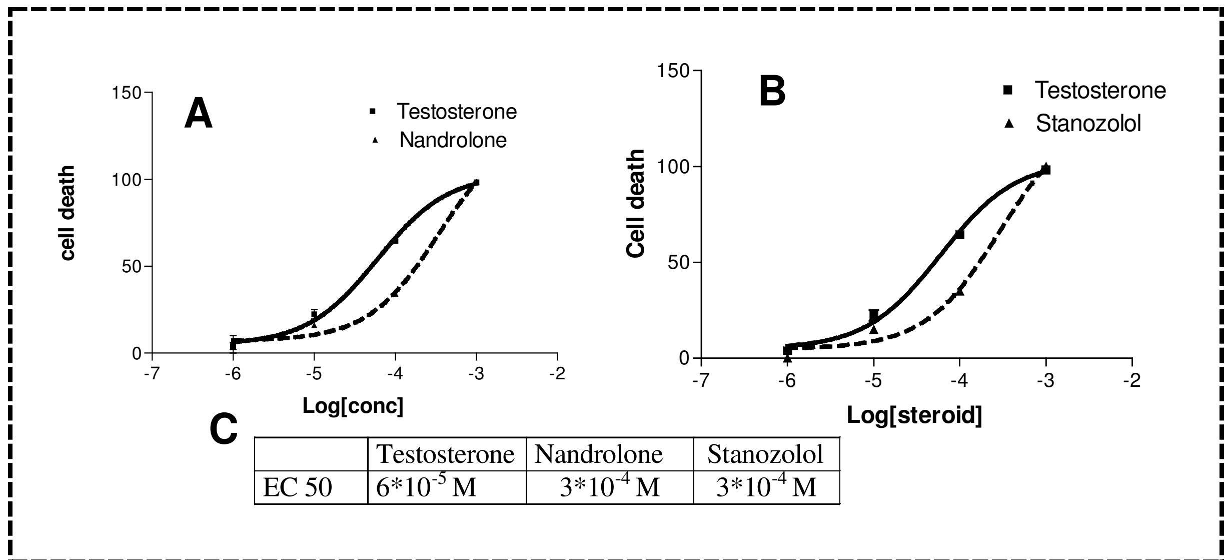


Figure 3 : MTT assays (A) SH-SY5Y cell seed on 96 wells plates were treated with nandrolone (Nan), testosterone (Testo) and stanozolol (Stan) with various concentration and were scored for cell viability with MTT assays 48h after (B) 72h of hormones exposure (C) SH-SY5Y cells differentiated with retinoic acid (10µM- 5 days) and after exposed with hormones for 48h. The values represent the mean ± S.E. of four independent experiment done in quintuple (* $p<0.05$; ** $p<0.001$)



	Testosterone	Nandrolone	Stanozolol
EC 50	$6*10^{-5}$ M	$3*10^{-4}$ M	$3*10^{-4}$ M

Figure 4: Dose response curve set up to evaluated the EC50 values of the three steroids. SH-SY5Y cells were treated for 48h with various concentration (10^{-3}M-10^{-6}M). (A) nandrolone (thickness line) testosterone (Dash line) (B) stanozolol (thickness line) testosterone (Dash line) (C) EC50 values table

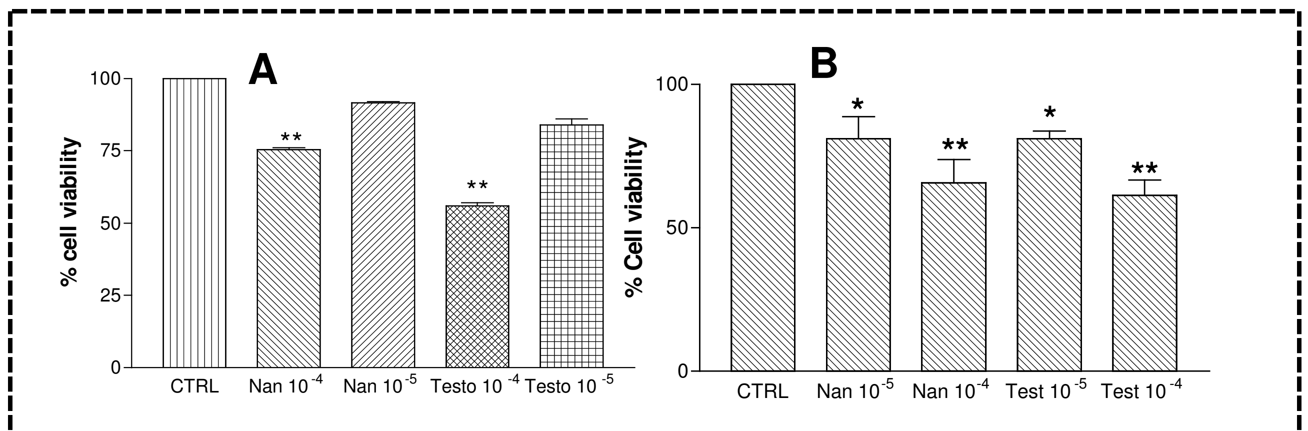


Figure 5: (A) Cell death was assayed using LDH release assay. SH-SY5Y cells were exposed to nandrolone and testosterone for 72h. The values represent the mean ± S.E. of three independent experiment done in quintuple (* $p<0.05$; ** $p<0.001$) (B) Tryplan blue exclusion assay. SH-SY5Y cells are counted with Neubauer camera after to be exposed with steroids for 48h. Results are expressed as percentage viability (number of viable cells/number of total cell) vs control set as 100%. The values represent the mean ± S.E. of four independent experiment (* $p<0.05$; ** $p<0.001$)

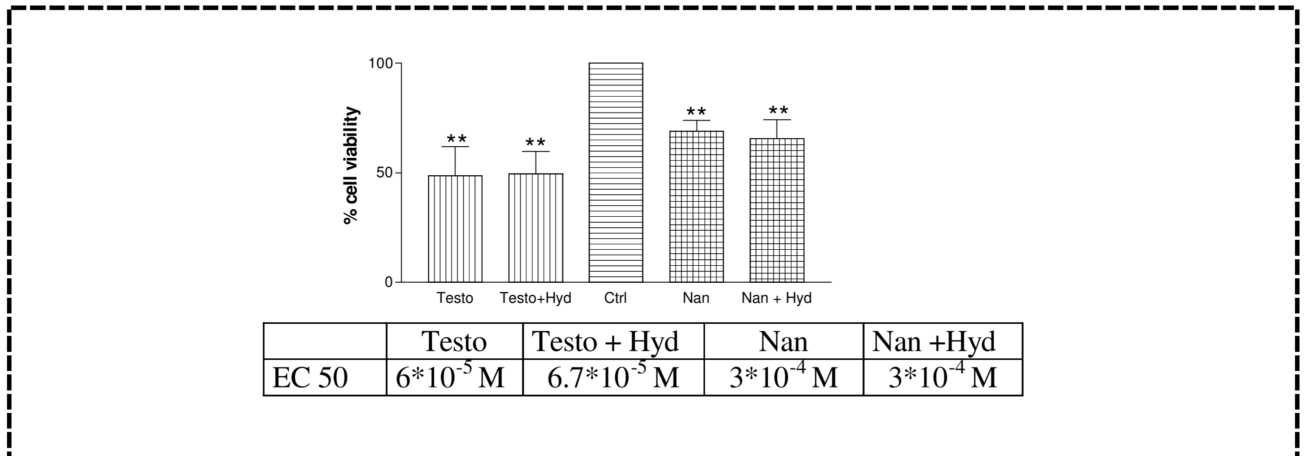
Mechanisms mediated toxic effect of supra-physiological concentration of AAS

The MTT assay set up previously was used to investigate the possible molecular mechanisms activated by androgen steroids. To explore the possible rule of androgen receptor (AR) in toxicity caused by nandrolone and testosterone treatment in SH-SY5Y cells, I used Hydroxyflutamide, a specific inhibitor of AR. Hydroxyflutamide at 10^{-5}M administered alone for 48h not cause relevant cell death in our cellular model. The cells are pre-treated for 1h with Hydroxyflutamide (10^{-5}M) before to be exposed with nandrolone and testosterone (10^{-3}M and 10^{-6}M) for 48h. The toxic effect of AS in present of Hydroxyflutamide not change as show in figure 4 and in the EC50 values table. This data describe as AR is not involved in neurotoxic mechanism of elevated dose of AAS (Figure 6)

A competitive radioligand binding assay was carried out to studies whether sigma-2 receptor would be activated by testosterone. Sigma-2 specific radioligand [^{3}H]RHM-1 at concentration of 8nM was used in competition with testosterone (100nM - 100µM). Only the high concentration of testosterone (100µM) showed a weak competition with RHM-1 (Figure 7). To explore the possible activation of specific membrane receptor I treated the SH-SY5Y with testosterone conjugate with BSA (T-BSA). MTT test performed with T-BSA at (10^{-4}M and 10^{-5}M) for 48h not showed remarkable toxic effect if compare to control (Figure 7)

To evaluated whether the death of neuroblastoma cells is indeed due to a apoptotic pathway, it was set up an immunocitochemistry experiments and caspase-3 activity assay. The cells treated with nandrolone and testosterone (10^{-4}M) for 48h are analyzed with a specific antibody that recognize the active form of caspase-3. The cells exposed to steroids showed a per cent number of cells positive to the antibody greater then the control. The caspase-3 activity was evaluated with a colorimetric substrate Ac-DEVD-*p*NA, cleaved by active caspase-3. The cytosolic protein lysates of treated SH-SY5Y cells display an caspase activity

three folds greater than cells not treated. These data confirm the activation of apoptotic pathways caspase-3 dependent in cells SH-SY5Y exposed to AAS (Figure 8)



	Testo	Testo + Hyd	Nan	Nan +Hyd
EC 50	$6*10^{-5}$ M	$6.7*10^{-5}$ M	$3*10^{-4}$ M	$3*10^{-4}$ M

Figure 6 : Cell viability bar graph of SH-SY5Y pre-treated for 1h with hydroxyflutamide (Hyd) and after exposed for 48h with nandrolone and testosterone. The values represent the mean ± S.E. of three independent experiment done in quintuple (* $p<0.05$; ** $p<0.001$). Table containing EC50 values.

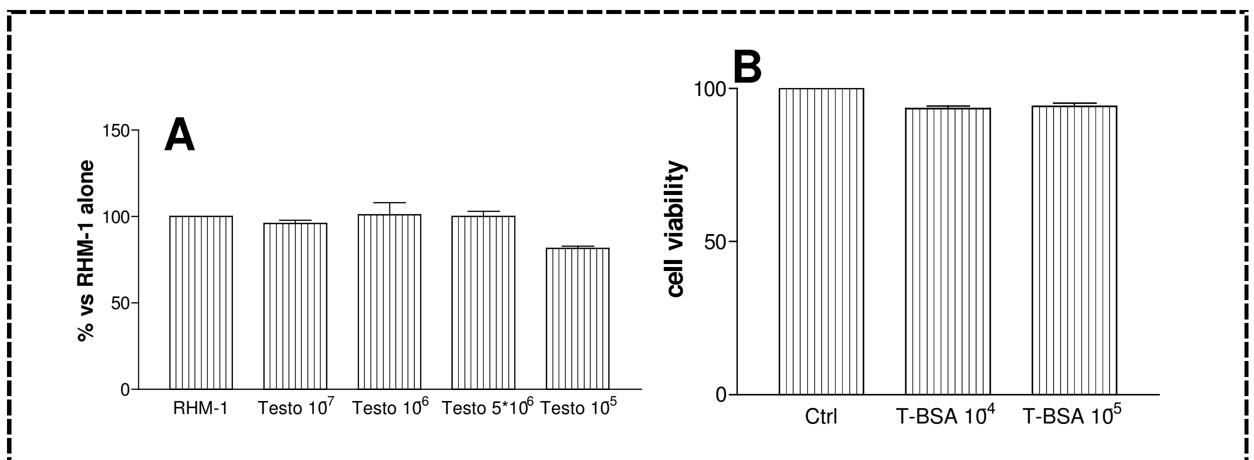


Figure 7 : (A) Competitive radioligand binding assay on sigma-2 receptor. [^{3}H]RHM-1, selective sigma-2 ligand, were used in competition with growing dose of testosterone The values represent the mean ± S.E. of two independent experiment done in triplicate (* $p<0.05$; ** $p<0.001$)

(B) Cell viability bar graph of SH-SY5Y cells treated with testosterone conjugate with bovine serum albumine (T-BSA) for 48h. The values represent the mean ± S.E. of three independent experiment done in quintuple (* $p<0.05$; ** $p<0.001$)

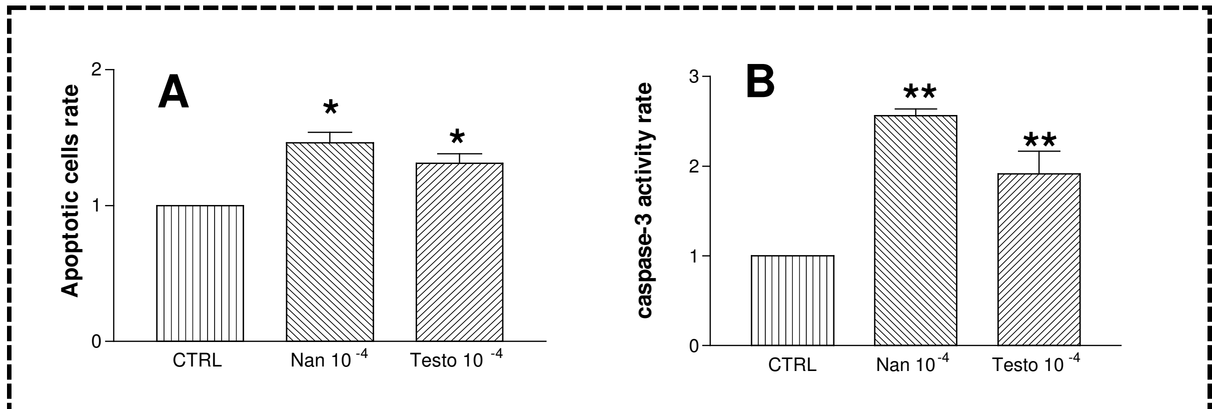


Figure 8 : (A) Immunocitochemistry experiment carried out on SH-SY5Y exposed to nandrolone and testosterone for 48h. caspase-3 cleavage active form was detected with specific antibody (B) Caspase-3 enzymatic assays. 100µg of protein, extracted from SH-SY5Y treated with steroids for 48h , were assayed with a specific caspase-3 colorimetric substrate Ac-DEVD-pNA (200 µM).

Discussion

Anabolic androgenic steroids (AAS) cause numerous side effects at both the central and peripheral nervous system, including increased aggression, irritability, mania, depression, and suicidal tendencies. Changes elicited by AAS have been observed for the neurotransmitters $GABA_A$ (γ-aminobutyric acid) (Britan et al., 1993) and 5-HT (serotonin) (Kindlundh et al., 2004) as well as for the neurotransmitter receptors dopamine D1 and D2 (Kindlundh et al., 2003), NK-1 (neurokinin-1) (Hallberg et al, 2005), and NMDA (N-methyl-D-aspartic acid) (Rossbach et al., 2007). AAS exposure has been proposed as a risk factor for other drugs of abuse. Animal studies, in fact, have confirmed that AAS evoke neurobiochemical alterations related to behavioral responses and affect the endogenous opioid and dopamine systems (Clark et al, 1993). A possible explanation for this effect is that chronic nandrolone treatment results in long-term changes in reward brain circuits and leads to a progressive decrease in the basal hedonic level, which results in an unpleasant state that renders the organism susceptible to the development of an addictive process (Celierer et al., 2003). Based on these data, AAS have been proposed to serve as ''gateway'' drugs to opioid dependence (Arvary et al., 2000). Nyberg and colleagues have determined the molecular mechanisms of the rewarding effects of nandrolone on different opioid peptides (Magnusson et al, 2007). Moreover, the effects of nandrolone on delta opioid receptor (DOPr) expression have been evaluated in neuronal cell lines (Pasquariello et al., 2000) . In this study, downregulation of DOPr in cell exposed to nandrolone was shown to be independent of AR.

I found, in this thesis, that treatment of SH-SY5Y neuronal cells with nandrolone at a supra-physiological concentration (10^{-6} M), which may mimic abuse regimes (Clark et al., 1993), decreased the expression of MOPr mRNA. Exposure to nandrolone also significantly decreased the number of MOPr binding sites. This finding suggests that a prolonged exposure to nandrolone affects MOPr function.

Opioid receptor expression is regulated by multiple mechanisms, including transcriptional and post-transcriptional events (Law et al., 2004). MOPr promoter activity is

under the control of various transcription factors (Kim et al., 2006) that interact with DNA responsive elements mainly located in the 5' upstream region of this promoter. Interestingly the human MOPr promoter contains a glucocorticoid responsive element GRE (from -2215 to -2195 bp), which can bind the glucocorticoid receptor or the AR (Takai et al., 2007). Post-transcriptional regulation occurs at the mRNA or protein level via control of mRNA stability, alterations in translation efficiency, alterations of mRNA transport, and covalent modification of receptor molecules. Recent studies have indicated that the 5' and 3' untranslated regions (UTR) of MOPr mRNA affect the overall activity of the transcripts (Law et al., 2004). Thus, post-transcriptional regulation of the MOPr gene may play an important role in the spatial and temporal expression of the receptor protein. A correlation between *cis* elements in the 5' and 3' UTR regions and the alteration in MOPr expression has been described (Xu and Carr et al., 2001). In addition, the 5' UTR in the MOPr mRNA is a key site of post-transcriptional regulation. This mechanism is mediated by uAUG/uORF (open reading frame) sequences that control translation initiation. Recent discoveries in hormone action emphasize that regulation of gene expression is not restricted to alterations in the rate of gene transcription. On the contrary, hormonal effects on the stability of a specific mRNA can profoundly alter its steady-state concentration (Gies et al., 1997). The down-regulation of MOPr mRNA nandrolone dependent may be due to a decrease in the stability of mRNA since no change was observed in the rate of MOPr promoter/luciferase transcription in a gene reporter system containing a sequence of 2455 bp of the 5'upstream region of the human MOPr promoter. Until now, the identities of the cellular proteins that are involved in the post-transcriptional mechanisms that regulate MOPr and are modulated by nandrolone are unknown.

Nandrolone may regulate MOPr mRNA levels through mechanisms involving the classic intracellular AR. This notion is supported by the finding that nandrolone-induced downregulation of MOPr mRNA levels in SH-SY5Y cells was blocked by hydroxyflutamide, an androgen antagonist that binds to the AR and is expressed in the SH-SY5Y cell line. In

previous studies, was showed that nandrolone modulates DOPr expression in neuronal cells but that this action is independent from AR involvement (Pasquariello et al., 2001). Therefore, the present data demonstrate that nandrolone activates different pathways to modulate the expression of MOPr and DOPr in neuronal cells. A down-regulation of MOPr expression is observed also in cells treated with testosterone and stanozolol (10^{-6} M) for 48h. This in vitro observation need to be investigate deeply and correlate with the stacking administration regime common in AS abusers.

The second part of my studies was focus on the explanation of androgen steroids toxic effect at supra-physiological concentrations. The neurotoxicity of steroids on neuronal cells can negatively affect the behaviour of user and might induce a degenerative process. The "steroid dementia" syndrome found in some patients using high concentration of steroids could be caused by neuro-toxic consequence (Wolkowitz et al., 2007). This thesis show , with different cito-toxic assays and condition, the toxic effect of supra-physiological concentration of testosterone, nandrolone and stanozolol in SH-SY5Y neuroblastoma cells. These steroids show this effect at micromolar concentration after 48h and 72h of exposure. A study lead in PC12 cell line exposing to testosterone showed opposite result between differentiate and undifferentiated cells. Instead, SH-SY5Y cells differentiated with retinoic acid show similar susceptibly cell death when treated with steroids in confront with undifferentiated cells. Studies carried out in different cellular model, PC12 (Alexaki et al., 2006), cortical astrocytes (Gatson and Singh, 2007), and mouse cortical cells (Orlando et al., 2007), described that AS can promote cell death. This effect is time- and concentration-dependent and various mechanisms might be involved. In mouse cortical cells was described an AR dependent action of nandrolone that promotes the NMDA toxic effect, in the same models testosterone effects are not mediated by AR but it is regulated by aromatase conversion of testosterone to estradiol. At nanomolar concentration testosterone is completely converted in 17β-Estradiol, a molecule with a protective effect on neuronal cells. Nanomolar concentration of testosterone

became neurotoxic in the present of aromatase inhibitor. This indicates that testosterone is intrinsically toxic to cortical neurons and high dose of steroid overwhelmed the neuroprotection effect aromatase dependent and showed a toxic effect. I investigated the possible rule of AR in testosterone and nandrolone toxic effect in SH-SY5Y and I found that AR seems not mediated this mechanism. In neuroblastoma cells model, used in this thesis, is not yet know whether the metabolism of steroids , aromatization of testosterone or 5α-reductase conversion of nandrolone, regulate the cito-toxicity of this two steroids.

Another possibility, described in various studies, associated with neuronal cell death dependent from steroids, is the activation of membrane specific androgen receptor. Recently, it was demonstrated that testosterone induces intracellular Ca^{2+} oscillation in the cytosol and nucleus, wich are an important mediator of downstream events as apoptosis (Estrada et al., 2006). Sigma-2 receptor, which activate an apoptotic pathways, has been described to activate Ca^{2+} oscillation in SK-N-SY cells, progenitor of SH-SY5Y cells (Vilner and Bowen, 2000). The competitive radio binding assay set up to evaluated a possible activation of this apoptosis pathways by testosterone not show a positive result. Testosterone not seems to compete with specific sigma-2 ligand RHM-1 to binding the receptor. To elucidate the hypothesis of involvement of a membrane receptor in the toxic effects of steroids on SH-SY5Y cells. I made an experiment with testosterone-BSA. The results obtained did not show any direct toxic effect at micromolar concentration after 48h of exposure. In PC12 and astrocytes was hypothesized to exist a receptor on the cytoplamatic membrane of this cells recognize specifically by steroids and that steroids binding on this receptor activate a pro-apoptotic pathways. This receptor seems to have a low affinity to AS and it could explain the high-concentration need to observe the toxic effect. Until now it is not clear which is this receptor. In astrocytes exposed to DHT-BSA and iodoacetic acid (IAA) was observed an increase of the toxic effect provoke by IAA alone. DHT-BSA alone not show any toxic effect but it makes the cells more susceptible to oxidative stress. My data seems not to be comparable to

PC12 and astrocytes results. Therefore, it might be hypothesized a mechanism that involved membrane fluidity. It was observe the intercalation of the lipophilic steroids into membrane of target tissues, resulting in perturbation of lipid-lipid interactions that may, in turn, alter the function of membrane proteins (Withing et al., 2000).

In a in vitro cellular model of neurodegenerative disease, spinal bulbar muscular atrophy (SBMA), was detected cleavage of caspase-3 upon androgen stimulation, suggesting that this caspase is involved in the cell death induced by androgen treatment (Grierson et al., 2001). In SH-SY5Y cells exposed to testosterone was obersved caspase-3 activation (Estrada et al., 2006). I found that the androgen steroids nandrolone induce toxic effect throught activation of caspase-3 pathway in my time and concentration experimental condition (10^{-4}M;48h). The immunocitochemistry and caspase-3 activity assays results showed the activation of this pathway.

In summary, in this thesis I observed genomic and non-genomic effects of high dose of anabolic androgenic steroids in a neuronal cellular model. Development of neurological syndromes in steroids abuser might be explain by a complex molecular mechanisms. Together, my results described two mechanisms which could be correlate with behavioural changes and neuro degeneration observed in steroids abuser. I showed as steroids regulated in SH-SY5Y the opioid neuronal circuit through the control of MOPr expression via androgen receptor (AR) activation and mRNA stability inhibition and a neurotoxic effect, AR independent, associated with elevate dose of steroids. The beneficial effects of AAS are carefully regulated over a narrow (nanomolar) range of concentration, which is crucial for normal neuronal functions. Elucidation of androgens effects in neuronal cells is, also, important to define the safety of androgen supplementation therapy.

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