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Neurotoxic Properties of the Anabolic Androgenic Steroids Nandrolone and Methandrostenolone in Primary Neuronal Cultures

Filippo Caraci,1 V. Pistara,2 A. Corsaro,2 Flora Tomasello,3 Maria Laura Giuffrida,1 Maria Angela Sortino,4 Ferdinando Nicoletti,5,6 and Agata Copani1,7*

1Department of Pharmaceutical Sciences, University of Catania, Catania, Italy
2Department of Chemical Sciences, University of Catania, Catania, Italy
3PhD Program in Neuropharmacology, University of Catania, Catania, Italy
4Department of Experimental and Clinical Pharmacology, University of Catania, Catania, Italy
5Department of Human Physiology and Pharmacology, University of Rome “Sapienza,” Rome, Italy
6I.N.M. Neuromed, Pozzilli, Italy
7I.B.B., CNR-Catania, Italy

Anabolic-androgenic steroid (AAS) abuse is associated with multiple neurobehavioral disturbances. The sites of action and the neurobiological sequels of AAS abuse are unclear at present. We investigated whether two different AASs, nandrolone and methandrostenolone, could affect neuronal survival in culture. The endogenous androgenic steroid testosterone was used for comparison. Both testosterone and nandrolone were neurotoxic at micromolar concentrations, and their effects were prevented by blockade of androgen receptors (ARs) with flutamide. Neuronal toxicity developed only over a 48-hr exposure to the steroids. The cell-impermeable analogues testosterone-BSA and nandrolone-BSA, which preferentially target membrane-associated ARs, were also neurotoxic in a time-dependent and flutamide-sensitive manner. Testosterone-BSA and nandrolone-BSA were more potent than their parent compounds, suggesting that membrane-associated ARs were the relevant sites for the neurotoxic actions of the steroids. Unlike testosterone and nandrolone, toxicity by methandrostenolone and methandrostenolone-BSA was insensitive to flutamide, but it was prevented by the glucocorticoid receptor (GR) antagonist RU-486. Methandrostenolone-BSA was more potent than the parent compound, suggesting that its toxicity relied on the preferential activation of putative membrane-associated GRs. Consistently with the evidence that membrane-associated GRs can mediate rapid effects, a brief challenge with methandrostenolone-BSA was able to promote neuronal toxicity. Activation of putative membrane steroid receptors by nontoxic (nanomolar) concentrations of either nandrolone-BSA or methandrostenolone-BSA became sufficient to increase neuronal susceptibility to the apoptotic stimulus provided by β-amyloid (the main culprit of AD). We speculate that AAS abuse might facilitate the onset or progression of neurodegenerative diseases not usually linked to drug abuse. © 2011 Wiley-Liss, Inc.

Key words: testosterone; nandrolone; methandrostenolone; beta-amyloid; neuronal death

Anabolic-androgenic steroids (AASs) are synthetic derivatives of testosterone that are abused in the world of sport to build muscle and boost athletic performance (Yesalis and Bahrke, 1995). Abuse of AASs causes serious side effects that involve the cardiovascular system, the liver, and the reproductive system (van Amsterdam et al., 2010). A major concern regards their neurobehavioral actions, which are associated with stroke, mood disturbances, and psychotic symptoms (Uzych, 1992; Hall et al., 2005; Santamarina et al., 2008).

At present, neurobiological mechanisms and sites of action of AASs are unclear. In vitro, low concentrations of AASs amplify excitotoxic neuronal death (Orlando et al., 2007). In male normal volunteers, high doses of AASs induce cognitive impairment (Su et al., 1993; Daly et al., 2003). The main endogenous androgenic steroid, testosterone, has both neuroprotective effects (Hammond

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*Correspondence to: Agata Copani, MD, PhD, Department of Pharmaceutical Sciences, University of Catania, Viale Andrea Doria 6, 95125, Catania, Italy. E-mail: acopani@katamail.com

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et al., 2001; Pike, 2001; Nguyen et al., 2005; Pike et al., 2008) and neurotoxic effects (Estrada et al., 2006; Cunningham et al., 2009), depending on the experimental paradigm. Endogenous testosterone appears to exacerbate some types of neuronal injury, including ischemia-reperfusion injury (Yang et al., 2002) and methamphetamine-induced neurodegeneration of nigrostriatal dopaminergic neurons (Dluzen and McDermott, 2006). In culture paradigms, a few studies indicate that testosterone can be directly neurotoxic both at supraphysiological (Estrada et al., 2006) and at physiological (Cunningham et al., 2009) concentrations. Despite evidence that testosterone may be neurotoxic, there are conditions in which testosterone supports neuronal viability (Pike et al., 2009). In particular, it has been demonstrated that androgens selectively protect neurons against apoptosis-inducing insults (Nguyen et al., 2010), including β-amyloid (Ab) protein, the main contributor to Alzheimer’s disease (AD) pathology.

Androgens mediate their classical genomic effects through binding to the androgen receptor (AR), a member of the nuclear receptor superfamily that functions as a ligand-activated transcription factor (Heinlein and Chang, 2002). A CAG repeat expansion within the first exon of the AR gene is responsible for testosterone-dependent nuclear accumulation of ARs, with ensuing motor neuron degeneration in spinal and bulbar muscular atrophy (Katsuno et al., 2010). Androgens also mediate rapid nongenomic effects via the activation of signaling pathways (i.e., the MAPK pathway and the AKT pathway) triggered either by classical ARs (Heinlein and Chang, 2002; Nguyen et al., 2005; Foradori et al., 2008) or by membrane-associated ARs (Gatson et al., 2006). How the different AR activities are related to the existing discrepancies regarding whether androgens are protective or damage promoting is unclear.

Given that AAS abuse poses a significant public health problem, we investigated the potential neurotoxic effects of suprapharmacological concentrations of AASs, taking into consideration the existence of both classical and membrane-associated ARs and focusing on two largely abused steroids with different pharmacological profiles, nandrolone and methandrostenolone. Nandrolone binds to ARs to a greater degree than testosterone, whereas methandrostenolone is a weak agonist of the ARs (for review see Fragkaki et al., 2009). Hence, their neurotoxic properties were compared with those of testosterone, and the attempt to differentiate between intracellular and membrane functions of ARs was carried out by using cell-impermeable steroid analogues that preferentially bind to membrane-associated receptors.

**MATERIALS AND METHODS**

All animal experimental procedures were carried out in accordance with the directives of the Italian and EU regulations for care and use of experimental animals and were approved by the Institutional Animal Care and Use Committee of the University of Catania.

**Drugs**

Testosterone, testosterone-BSA conjugate, nandrolone, methandrostenolone, RU-486, formestane, and flutamide were purchased from Sigma-Aldrich (Milan, Italy). Nandro-

**Purified Cultures of Cortical Neurons**

Cultures of pure cortical neurons were obtained from rats at embryonic day 15 (Morini, s.p.a., Reggio Emilia, Italy). Briefly, cortices were dissected in Ca²⁺/Mg²⁺-free buffer and mechanically dissociated. Cortical cells were plated at a density of 400 × 10³/well on 24-well plates precoated with 0.1 mg/ml poly-D-lysine in DMEM/Ham’s F12 (1:1) medium supplemented with the following components: 10 mg/ml bovine serum albumin, 10 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 30 nM selenium, 2 mM glutamine, 6 mg/ml glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cytosine-1β-D-arabinofuranoside (10 μM) was added to the cultures 18 hr after plating to avoid the proliferation of non-neuronal elements and was kept for 3 days before medium replacement. This method yields >99% pure neuronal cultures (Copani et al., 1999).

**Mixed Cultures of Cortical Cells**

Cells dissected from the cortices of rat embryos and dissociated as described above were grown into MEM supplemented with horse serum (10%), fetal calf serum (FCS; 10%), glutamine (2 mM), and glucose (6 mg/ml). Cultures were kept at 37°C in a humidified 5% CO₂ atmosphere. After 3–5 days in vitro, non-neuronal cell division was halted by 3 days of exposure to cytosine-1β-D-arabinofuranoside (10 μM), and cultures were shifted to a maintenance medium identical to plating medium but lacking fetal serum. Subsequent partial medium replacement was carried out twice per week. Only mature cultures (12–14 days in vitro) were used for the experiments. Mature cultures contained about 40% neurons.

**Cultures of Cortical Astrocytes**

Cortical glial cells were prepared from 1–3-day-old Sprague-Dowley rats (Morini, s.p.a.). After removal of meninges and isolation of cortices, cells were dispersed by mechanical and enzymatic dissociation using a 0.25% solution of trypsin. Cells were plated onto 75-mm² flasks and maintained in DMEM culture medium, supplemented with 10% FCS, penicillin/streptomycin (100 U/ml to 100 μg/ml), and gluta-
mine (2 mM). All medium constituents were from Invitrogen (Carlsbad, CA), and all plastic materials were fromComing
Life Sciences (Acton, MA). Confluent cultures at 8–10 days in
vitro were shaken overnight at 37°C to remove microglia and
oligodendrocytes. Astrocytes were collected by trypsin diges-
tion, seeded onto 24 multiwell plates and used 6–8 days after
replating.

Handling of Aβ

Different lots of Aβ(25–35) were tested, and the same
batch was used throughout the entire study to rely on a con-
sistent profile of toxicity. Peptides were solubilized in sterile,
double-distilled water at an initial concentration of 2.5 mM
and stored frozen at –20°C. Aβ(25–35) was used at a final
concentration of 25 µM in the presence of the glutamate re-
ceptor antagonists MK-801 (1 µM) and DNQX (30 µM) to
avoid the potentiation of endogenous glutamate toxicity.

Assessment of Neuronal Injury

In mixed cortical cultures, neuronal injury was estimated
by examination of the cultures by phase-contrast microscopy
24 hr after the incubation with Aβ. Neuronal damage was
quantitatively assessed in all experiments by estimation of dead
neurons by trypan blue staining. Stained neurons were
counted from three random microscopic fields per well. In
pure neuronal cultures, neuronal injury was assessed by the 3-
[4,5-dimethylthioazol-2-yl]-2,5-diphenyl tetrazolium bromide
(MTT) assay. Cultures were incubated with MTT (0.9 mg/ml
final concentration) for 2 hr at 37°C. A solubilization solution
containing 20% sodium dodecyl sulfate was then added for an
additional 1 hr, and formazan production was evaluated in a
plate reader (absorbance = 560 nm).

Western Blot Analysis

Cells were harvested in lysis buffer containing a cocktail
of protease inhibitors (P2714; Sigma-Aldrich). After sonica-
tion, an aliquot of the sample was processed for protein concen-
trations according to the method of Bradford. Samples were
concentrated and boiled for 5 min. Proteins were sepa-
rated electrophoretically on polyacrylamide gel (30 mA/hr)
using 60–80 µg of cell proteins per lane. Proteins were trans-
ferred to nitrocellulose membranes (Hybond ECL; Amersham
Biosciences Europe GmbH, Milan, Italy) at room temperature
using a transblot semidyed transfer cell. After blocking, the
membranes were incubated with rabbit antiandrogen receptor
antibody (1:500; Ab3509; Abcam, Cambridge, United King-
dom) overnight at 4°C. Membranes were then thoroughly
washed and incubated with HRP-conjugated secondary anti-
bodies. Specific bands were visualized using the SuperSignal
chemiluminescent detection system (Pierce Biotechnology,
Rockford, IL).

Immunofluorescence Labelling and Confocal
Microscopy

Mixed cortical cultures were plated onto 35-mm dishes
with glass slides (Amnioidish Euroclone, Milan, Italy) pre-
coated with 0.1 mg/ml poly-D-lysine. Cells were fixed for 20
min in paraformaldehyde and permeabilized with 0.1% Triton
X-100 for 5 min. Non-specific binding was blocked by incu-
bation with PBS containing 3% BSA at room temperature for
1 hr. After blocking, rabbit anti-androgen receptor antibody
(1:100 dilution) was added overnight at 4°C. After washing, a
Texas red-conjugated donkey anti-rabbit antibody was used for
1 hr (1:400 dilution; Santa Cruz Biotechnology). Samples
were analyzed through a ×60 objective on a confocal laser
scanning microscope Olympus FV1000.

RESULTS

To begin investigating the hypothesis that supra-
pharmacologic doses of AASs may be neurotoxic, we have
compared the effects of two synthetic AASs (nan-
drolone and methandrostenolone) and their BSA conjug-
ates (nandrolone-BSA and methandrostenolone-BSA)
with those of testosterone, using as a model rat cortical
cultures, both pure neuronal and mixed neuron–glia
types. Consistently with in vivo evidence (DonCarlos
et al., 2006; Sarkey et al., 2008), ARs were expressed by
cultured astrocytes and neurons (Fig. 1A), and AR im-
nunoreactivity was observed by confocal microscopy in
neuronal (Fig. 1B) and glial (Fig. 1C) processes. Initially,
pure neuronal cultures were exposed to different concen-
trations (0.01–10 µM) of either testosterone or nan-
drolone, and toxicity was assessed by MTT assay 48 hr
later. Testosterone and nandrolone exerted significant
toxicity only at concentrations of 10 µM (Fig. 2A).
Instead, the cell-impermeable derivatives, testoster-
olone-BSA and nandrolone-BSA, were toxic at 1 µM (Fig.
2B). Unlike testosterone and nandrolone, methandroste-
nolone had a toxic effect at 1 µM, whereas its derivative,
methandrostenolone-BSA, was toxic at concentrations as
low as 100 nM (Fig. 2C). Toxicity by testosterone, nan-
drolone, or their BSA conjugates was abrogated by the
AR antagonist flutamide (Fig. 3). Instead, toxicity by
methandrostenolone or methandrostenolone-BSA was
insensitive to flutamide (not shown), but was prevented
entirely by the glucocorticoid receptor antagonist RU-
486 (Fig. 3). Concentrations of nandrolone, methandro-
estonolone, or AAS conjugates, which were neurotoxic
following a 48-hr exposure, were also applied to the cul-
tures in a 20-min pulse and then washed out 24 hr prior
to the assessment of neuronal death. Under this experi-
mental condition, only methandrostenolone-BSA was
toxic, and its toxicity was again prevented by RU486
(Fig. 4). Finally, testosterone, nandrolone, methandroste-
nolone, or their BSA-conjugates were tested for their
ability to increase neuronal vulnerability to the apoptotic
insult provided by Aβ protein (Loo et al., 1993). Because
Aβ is able to potentiate glutamate toxicity (Koh
et al., 1990; Copani et al., 1991), and AASs might
amplify glutamate toxicity (Orlando et al., 2007), experi-
ments were carried out in the presence of a cocktail of
ionotropic glutamate receptor antagonists (1 µM MK-
801 + 30 µM DNQX) to exclude the contribution of endogenous excitotoxicity to the overall process of neu-
ronal death. Aβ(25–35) was used at concentrations
(25 µM) able to induce a 45% of neuronal death over a
24-hr period. Prior to the treatment with Aβ(25–35), pure neuronal cultures were exposed for 24 hr to the highest concentrations of the drugs devoid of intrinsic toxicity. With this particular experimental protocol, methandrostenolone-BSA amplified Aβ-induced toxicity at concentrations of 10 nM (Fig. 5).

Fig. 1. AR expression in cultured neural cells. A: Western blot analysis of AR expression in pure cultures of rat cortical neurons or in cultured astrocytes. Samples have been loaded in duplicate. A single band of about 110 kD, corresponding to the molecular weight of ARs, was observed in both cases. Confocal images with ×60 magnification of AR immunoreactivity in neurons (B) or astrocytes (C) from mixed cortical cultures. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2. Neuronal death induced in pure neuronal cultures by increasing concentrations of testosterone and nandrolone (A), their BSA-conjugated analogues (B), and either methandrostenolone or methandrostenolone-BSA (C). All drugs were applied for 48 hr. Neuronal death is represented as the percentage reduction of neuronal survival measured by MTT assay. Values are means ± SEM of six to nine determinations. *P < 0.05 vs. controls (one-way ANOVA + Fisher’s PLSD).
The set of experiments described above was repeated in mixed rat cortical cultures, which include a glial component. In mixed cultures, neuronal death was assessed by Trypan blue staining. In the presence of glial cells, testosterone and nandrolone exerted significant toxicity already at 1 μM (Fig. 6A), and nandrolone-BSA became toxic at a concentration as low as 100 nM (Fig. 6B). Instead, the presence of glia cells did not appear to affect the toxicity profile of either methandrostenolone or methandrostenolone-BSA (Fig. 6C). Similarly to what was observed in cultures of pure neurons, flutamide abrogated toxicity by testosterone, nandrolone, and their BSA conjugates (Fig. 7), whereas RU-486 prevented entirely the toxicity of methandrostenolone or methandrostenolone-BSA (Fig. 7). When tested in the brief-insult paradigm (i.e., 24 hr after a 20-min pulse), methandrostenolone-BSA was toxic once more, and its toxicity was prevented by RU486 (Fig. 8).

Finally, all drugs were tested for their ability to affect neuronal susceptibility to Aβ-induced toxicity in mixed cortical cultures. For hippocampal cultures, testosterone has been reported to be neuroprotective against Aβ(25–35) at 10 nM concentrations (Pike, 2001). At these concentrations, testosterone protected mixed cortical cultures against Aβ toxicity (Fig. 9), and protection was abolished by the combined addition of flutamide and the aromatase inhibitor formestane (Fig. 9). However, at higher concentrations (100 nM), which were not toxic per se, testosterone did not influence Aβ toxicity (Fig. 10). At the highest concentrations devoid of intrinsic toxicity, nandrolone-BSA, methandrostenolone, and methandrostenolone-BSA all amplified Aβ-induced toxicity in mixed cortical cultures (Fig. 10).

DISCUSSION

We have demonstrated that suprapharmacologic doses of two different AASs, namely, nandrolone and methandrostenolone, are toxic for cultured cortical neu-
rons. However, the neurotoxic properties of nandrolone and methandrostenolone diverge.

Similarly to the case with testosterone, micromolar concentrations of nandrolone were detrimental to cortical neurons. The actions of testosterone and nandrolone were prevented by pharmacological blockade of ARs with flutamide, suggesting that toxicity was dependent on ARs. The evidence that the neurotoxic effects of testosterone and nandrolone required a 48-hr exposure suggests that high concentrations of androgens may affect neuronal viability by acting through AR-mediated genomic mechanisms. Both testosterone and nandrolone were more potent in cortical cultures containing glia cells, suggesting that AR-expressing glia cells could be implicated in the regulation of neuronal survival. The role of AR activation in glia cells is currently unclear. Based on the evidence that androgens can promote the induction of nuclear factor-κB-dependent proinflammatory genes, which lead to brain inflammation (Gonzales et al., 2009), it cannot be excluded that glial response factors synergize with androgens in reducing neuronal viability.

Confocal studies using an antibody against the classical nuclear AR revealed the localization of ARs to extranuclear sites, including neuronal axons and glia processes. AR immunoreactivity also profiled plasma membranes, indicating the presence of putative membrane receptors both in neurons and in astrocytes. Evidence exists that membrane-associated ARs may be either related or unrelated to the classical nuclear receptors (for review see DonCarlos et al., 2006). We found that testosterone-BSA and nandrolone-BSA, which preferentially target membrane ARs, exerted neurotoxic effects both in pure neuronal and in mixed cultures. These effects were prevented by flutamide, thus support-
ing the notion that membrane and intracellular ARs might share a similar pharmacological profile.

It has been proposed that activation of ARs may elicit opposite effects on cell survival (i.e., detrimental or beneficial) depending on whether membrane ARs or intracellular ARs are activated (Gatson et al., 2006; Gatson and Singh, 2007). In pure neuronal cultures, both testosterone-BSA and nandrolone-BSA were toxic, with a greater potency than their parent compounds. A similar effect was also observed following the exposure of mixed cultures to nandrolone-BSA, indicating that AAS-related toxicity depends on the preferential activation of putative membrane ARs over intracellular ARs. Interestingly, activation of putative membrane ARs by low nanomolar concentrations (10 nM) of nandrolone-BSA did not itself lead to neuronal death, but was sufficient to increase neuronal susceptibility to the apoptotic stimulus provided by Aβ(25–35).

Consistently with the hypothesis that androgens may activate two competing pathways for the regulation of neuronal survival, physiological concentrations (10 nM) of testosterone were instead protective against Aβ(25–35)-induced apoptosis in mixed cultures. The protective effect of 10 nM testosterone was fully blocked by combining flutamide with the aromatase inhibitor formestane. We speculate that, under physiological conditions (i.e., low concentrations of the hormone in a neuron–glia interplay), testosterone might undergo aromatization to the neuroprotective molecule 17β-estradiol (García-Segura et al., 2003). After that, the residual concentrations of testosterone might promote neuronal survival if intracellular ARs are abundant with respect to membrane-associated ARs.

A difference between methandrostestosterone and either nandrolone or testosterone could be observed when the drug was first tested for its intrinsic toxicity. The action of methandrostestosterone was not blocked by flutamide, suggesting that toxicity was independent of AR activation. Consistently with the notions that methandrostestosterone is only a weak agonist of the ARs and that most of its anabolic activity likely comes from non-AR-mediated effects (Fragkaki et al., 2009), the neurotoxicity of the drug was fully prevented by the glucocorticoid receptor (GR) antagonist RU486. RU486 also has a remarkable antiprogesterone activity (Baulieu, 1989); however, unlike other AASs (McRobb et al.,

![Fig. 7. Neuronal death induced in mixed cortical cultures by a 48-hr exposure to testosterone, nandrolone, methandrostenolone, or their BSA-conjugated analogues alone or combined with either 10 μM flutamide or RU486. After trypan blue staining, dead neurons were counted in three random microscopic fields/well. Values are means ± SEM of six determinations. *P < 0.05 vs. controls and #P < 0.05 vs. the values obtained in the absence of flutamide or RU486 (one-way ANOVA + Fisher’s PLSD).](image)

![Fig. 8. Neuronal death by nandrolone and methandrostenolone (A) and their BSA-conjugated analogues (B) applied for 20 min to mixed cortical cultures. In B, RU486 (10 μM) was coadded with methandrostenolone-BSA during the 20-min pulse. Neuronal death was assessed by trypan blue staining. Values are means ± SEM of four determinations. *P < 0.05 vs. control and #P < 0.05 vs. methandrostenolone-BSA alone (one-way ANOVA + Fisher’s PLSD).](image)
methandrostenolone lacks significant progestational properties (Wynn and Landon, 1961; Fragkaki et al., 2009), suggesting that progesterone receptors (PRs) were not involved in the neurotoxic action of the drug. Notably, nandrolone is among the AASs endowed with progesterone-like actions (McRobb et al., 2008; Fragkaki et al., 2009); the evidence that flutamide prevented entirely the toxicity of nandrolone provides hints that PRs might not be relevant to the neurotoxic properties of AASs.

Activation of GRs has been shown to exacerbate a variety of neuronal insults, including excitotoxicity and Aβ toxicity (Goodman et al., 1996). In mixed cultures, methandrostenolone was able to exacerbate Aβ(25–35)-induced toxicity at concentrations that were not toxic per se (100 nM), and exhibited intrinsic toxicity at 1 μM concentrations independently of the presence of glia cells. Methandrostenolone-BSA, which likely binds membrane-associated GRs, was always more potent than the parent compound, suggesting that its toxicity relied on the preferential activation of putative membrane GRs over intracellular GRs. Recently, the activation of putative membrane-associated GRs has been shown to mediate rapid, nongenomic effects able to potentiate NMDA-evoked toxicity in hippocampal neurons (Xiao et al., 2010). Consistently with this evidence, a brief challenge with methandrostenolone-BSA, but not with nandrolone-BSA, was sufficient to promote neuronal toxicity both in pure and in mixed neuronal cultures. The identity of membrane-bound GRs has not been elucidated yet, and both an unknown receptor type and classical intracellular GRs that associate with the membrane have been proposed (for review see Tasker et al., 2006). The “fast” toxicity induced by methandrostenolone-BSA was prevented by RU486, suggesting that the drug was acting at classical intracellular receptors associated with the plasma membrane.

Overall, we have provided evidence that two AASs with a different pharmacological profile, namely, nandrolone and methandrostenolone, can affect neuronal survival at suprapharmacologic doses, raising a serious concern for steroid abusers, who have micromolar concentrations of AASs in their brain (Lukas, 1996; Wu, 1997; Daly et al., 2001). The relevant sites for the neurotoxic action of nandrolone and methandrostenolone appear to be membrane-associated ARs and membrane-associated-GRs, respectively. Noteworthy, concentrations of the drugs that were not directly neurotoxic were, however, able to increase neuronal susceptibility to the apoptotic stimulus provided by Aβ(25–35). Hence, in vivo, exposure to AASs may result in a compromised brain, more susceptible, later in life, to the onset or progression of diseases not usually linked to drug abuse, especially neurodegenerative diseases (e.g., Alzheimer’s disease).

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