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17β-trenbolone, an anabolic–androgenic steroid as well as an environmental hormone, contributes to neurodegeneration

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ABSTRACT

Both genetic and environmental factors contribute to neurodegenerative disorders. In a large number of neurodegenerative diseases (for example, Alzheimer's disease (AD)), patients do not carry the mutant genes. Other risk factors, for example the environmental factors, should be evaluated. 17β-trenbolone is a kind of environmental hormone as well as an anabolic–androgenic steroid. 17β-trenbolone is used as a growth promoter for livestock in the USA. Also, a large portion of recreational exercisers inject 17β-trenbolone in large doses and for very long time to increase muscle and strength. 17β-trenbolone is stable in the environment after being excreted. In the present study, 17β-trenbolone was administered to adult and pregnant rats and the primary hippocampal neurons. 17β-trenbolone's distribution and its effects on serum hormone levels and Aβ42 accumulation *in vivo* and its effects on AD related parameters *in vitro* were assessed. 17β-trenbolone accumulated in adult rat brain, especially in the hippocampus, and in the fetus brain. It altered Aβ42 accumulation. 17β-trenbolone induced apoptosis of primary hippocampal neurons *in vitro* and resisted neuroprotective function of testosterone. Presenilin-1 protein expression was down-regulated while β-amyloid peptide 42 (Aβ42) production and caspase-3 activities were increased. Both androgen and estrogen receptors mediated the processes. 17β-trenbolone played critical roles in neurodegeneration. Exercisers who inject large doses of trenbolone and common people who are exposed to 17β-trenbolone by various ways are all influenced chronically and continually. Identification of such environmental risk factors will help us take early prevention measure to slow down the onset of neurodegenerative disorders.

Introduction

Alzheimer's disease (AD) is a progressive, irreversible, and so far incurable dementia. The genetic factors contributing to AD have been studied extensively. Familial Alzheimer's disease (FAD) is primarily caused by dominantly inherited mutations in the genes that encode presenilin (PS-1 and PS-2) and amyloid precursor protein (APP) (Hardy and Gwinn-Hardy, 1998). Other factors such as other genetic factors, aging, and environmental factors may lead to a chronic imbalance between β-amyloid peptide (Aβ) production and Aβ clearance in the brain (Mattson, 2000). Considering neuropathology changes may occur many years earlier than the clinical dementia, we assumed that some environmental factors which are continuously influencing the human body may play roles in the neurodegeneration of AD.

The main hallmarks of AD in the brain are extracellular β-amyloid peptide (Aβ) plaques (senile plaques) and intracellular neurofibrillary tangles (NFTs). The senile plaques consist mainly of Aβ40 and Aβ42. The initial Aβ deposition begins with Aβ42 because it is more prone to aggregate than Aβ40 (Suzuki et al., 1994). The Aβ hypothesis is one of the most prevailing hypotheses that have been proposed to explain the pathogenesis of AD. Aβ is a peptide released by proteolysis of APP. APP is a type I transmembrane protein and is ubiquitously expressed in both neuronal and nonneuronal tissues. Three secretases are involved in proteolysis of APP, α-secretase, β-secretase, and γ-secretase. Cleavage of APP by β- and γ-secretases will produce Aβ. On the contrast, Aβ production will be avoided if APP is cleaved first by α-secretase instead by β-secretase. APP is first cleaved in the extracellular domain by β-secretase, and the remnant is cleaved at least twice within the membrane by γ-secretase to produce the Aβ peptide and the intracellular domain. The produced Aβ variants contain 38–43 residues. The major Aβ variant is 40 residues in length (Wolfe and Selkoe, 2010; Wolfe, 2013). Although Aβ42 represents only 10% of total Aβ, it is the major form found in the plaques of AD. The "amyloid hypothesis"

Abbreviations: Δψm, mitochondrial membrane potential; AAS, anabolic–androgenic steroid; AD, Alzheimer's disease; APP, amyloid precursor protein; AR, androgen receptor; Aβ, β-amyloid peptide; BBB, blood brain barrier; CSF, cerebrospinal fluid; DHT, dihydrotestosterone; E_2, estradiol; ER, estrogen receptor; Flu, flutamide; Fulv, fulvestrant; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Na-DOC, sodium deoxycholate; PBST, phosphate-buffered saline containing 0.5% Tween-20; PFA, paraformaldehyde; PI, propidium iodide; PR, progestin receptor; PROG, progesterone; PS, presenilin; T, testosterone; TB, 17β-trenbolone; Tri, trilostane.

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identifies Aβ deposition as the primary cause of AD (Hardy, 1997; Tanzi and Bertram, 2005; Suzuki et al., 1994). The Aβ overproduction in the brain is thought to be the primary pathogenic process which causes various physiological events, such as oxidative damage, synaptic loss, formation of tau pathology, microglial and astrocytic activation, and progressive cognitive decline (Wojda and Kuznicki, 2013).

PS is a 50–55 kDa protein which contains nine transmembrane domains (Laudon et al., 2005). PS and three other proteins were discovered as essential for γ-secretase activity (Wolfe, 2013). PS-1 mRNA was expressed predominantly in the neuronal cells of the CNS, but only at low level in glial cells (Suzuki et al., 1996). Mutations in PS-1 can lead to alteration of APP processing and an increase and aggregation of Aβ42 (Haass and Strooper, 1999; Doan et al., 1996; Akbari et al., 2004). PS activity is important in learning, memory, and neuronal survival. PS-1 may control neurite outgrowth in neurons (Dowjat et al., 1999). PS is essential for synaptic contact and in regulation neurotransmitter release during synaptic transmission. Inactivation of presynaptic PS will decrease long-term potentiation (LTP) and alter short-term plasticity and synaptic facilitation (Georgakopoulos et al., 1999; Ho and Shen, 2011). PS-1 is also involved in regulation of apoptosis (Fluhrer et al., 2004).

Environmental hormones are also called endocrine disrupting compounds which are released from domestic, agricultural, and industrial sources and can interfere with the endocrine system of human beings and animal kingdom (Zeng et al., 2011). Since endogenous hormones, such as testosterone (T), dihydrotestosterone (DHT), and estradiol (E_2), exhibit protective actions in AD, the environmental hormones which may mimic or antagonize the role of endogenous hormones become our suspect for AD onset. Trenbolone acetate (TBA, 17β-hydroxyestra-4,9,11-trien-3-one 17-acetate) is a synthetic anabolic steroid that has been used extensively since the 1970s as a growth promoter for livestock in the USA. TBA is administered to livestock by subcutaneous slow-release implant (Yarrow et al., 2010). After being released, TBA is rapidly hydrolyzed to 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) in blood stream of the animals. 17β-trenbolone is a potent agonist of mammalian androgen receptor (AR) with a binding affinity to the human AR comparable to DHT (Bauer et al., 2000). Abundant studies have focused on the reproductive toxicity of 17β-trenbolone (Hemmer et al., 2001; Wilson et al., 2002; Ankley et al., 2003; Sone et al., 2005; Yarrow et al., 2010).

Besides, as an anabolic–androgenic steroid, trenbolone is used by a large portion of recreational exercisers to increase muscle size and strength (Perry et al., 2005; Parkinson and Evans, 2006; Ip et al., 2011).

Humans are at high risk of being exposed to 17β-trenbolone. There are four possible ways through which humans are exposed to 17β-trenbolone. The first one is 17β-trenbolone residue in meat. Although 17β-trenbolone is banned in livestock by some organizations, its usage is still allowed in the USA and some merchants in other areas also use 17β-trenbolone in pursuit of profit. The second way 17β-trenbolone goes to human body is through the food chains. 17β-trenbolone can be excreted by the animals and humans and it has long half-life and stable properties in the environment (Schiffer et al., 2001). It's worrying that 17β-trenbolone may be absorbed by aquatic animals (Yarrow et al., 2010) as well as plants (Schiffer et al., 2001; Blackwell et al., 2012) and can be incorporated into food chains. Thirdly, 17β-trenbolone is regarded as a promising candidate in clinical application. 17β-trenbolone can reduce incidence of androgenic and/or estrogenic side effects associated with androgen administration (Yarrow et al., 2010). The last way by which 17β-trenbolone goes into human body is direct injection. Since 17β-trenbolone can promote muscle growth and reduce fat (Yarrow et al., 2010), it is used in athletics and fit center, which is actually forbidden. The surveys indicated that trenbolone is widely used as an anabolic–androgenic steroid (AAS) mostly by recreational exercisers, in very large doses for very long times (Perry et al., 2005; Parkinson and Evans, 2006; Ip et al., 2011).

To our knowledge, no article related to the effects of environmental hormone on AD onset has been reported in the current literature. Both the *in vivo* and *in vitro* effects of 17β-trenbolone on AD-related parameters were assessed. The experiment systems we used did not carry mutant genes that are correlated with AD.

Materials and methods

Animals and cell culture. Wistar rats were purchased from the Center of Experimental Animal of Shandong University (Shandong, China). The studies were conducted according to the regulations of the Center of Experimental Animal of Shandong University. Male and female rats were of 250 ± 10 g. Pregnant rats were shipped on the day after mating and housed individually in clean plastic cages (20 cm × 25 cm × 47 cm). The day after mating was designated as gestation day 1 (GD 1). Photoperiod was 14 h light and 10 h dark, lights on at 06:00 and off at 20:00. Rats were allowed free access to rodent chow and water. Temperature was 20–22 °C and relative humidity was 45–55%.

Hippocampal neurons from newborn rats (postnatal day 0) were cultured according to previously established procedures (Nunez, 2008). The culture medium was Neurobasal A (phenol red free, Invitrogen, USA) containing 2 mM L-Glutamine (Sigma, USA) and 2% B27 Supplement (Invitrogen, USA). All experiments were performed on 9- to 12-day-old cultures.

Drugs and treatments. 17β-trenbolone was purchased from Dr. Ehrenstorfer GmbH (Germany). T, DHT, flutamide, fulvestrant, and trilostane were purchased from Sigma (USA). In animal experiments, 17β-trenbolone was dissolved in laboratory-grade corn oil (Sigma, USA) with final concentration of 5 mg/ml, 1 mg/ml, and 0.2 mg/ml, respectively. Rats were divided into several groups with each group having six rats. Rats were injected with corn oil (control) or 17β-trenbolone solution (0.1 ml/100 g body weight) once intramuscularly on the right hind limbs. Pregnant rats were injected on GD 16. Male rats in groups Am, Bm, Cm, Dm, Em, and Fm were injected with 5 mg/ml 17β-trenbolone solution and the treating time were 0.5 h, 2 h, 6 h, 12 h, 24 h, and 48 h, respectively. Male rats in groups Gm and Hm were injected with 17β-trenbolone solution of 1 and 0.2 mg/ml, respectively. The treating time was 48 h. Male rats in group Om was control. Accordingly, female rats in groups Af, Bf, Cf, Df, Ef, and Ff were injected with 5 mg/ml 17β-trenbolone solution and the treating times were 0.5 h, 2 h, 6 h, 12 h, 24 h, and 48 h, respectively. Female rats in groups Gf and Hf were injected with 17β-trenbolone solution of 1 and 0.2 mg/ml, respectively. The treating time was 48 h. Female rats in group Om was control. Pregnant rats in group P were treated with 5 mg/ml 17β-trenbolone solution for 48 h. Group Op was control.

The largest dose used in the animal experiment was 5 mg/kg body weight. According to the dose conversion formula below (Chen, 1993) and the data in the previous paper presented, the equivalent injection dose for human should be 0.85 mg/kg body weight which is much lower than the dose injected by exercisers.

$$d_{human} = d_{rat} \times \frac{R_{human}}{R_{rat}} \times \frac{\sqrt[3]{W_{rat}}}{\sqrt[3]{W_{human}}}$$

d, dose; R, body size coefficient; W, body weight.

$d_{rat} = 5$ mg/kg, $R_{human} = 0.11$, $R_{rat} = 0.09$, $W_{rat} = 0.25$ kg, $W_{human} = 94.4$ kg (Perry et al., 2005).

The survey published in 2005 reported that 21.3% of the 207 respondents injected trenbolone at the dose of 117.1 mg every other day (*i.e.*, 1.24 mg/kg body weight) with an average cycle of 6.8 weeks (Perry et al., 2005). In 2006 it was reported that the trenbolone injection dose by exercisers was 700 mg/week with a cycle of 11–20 weeks (Parkinson and Evans, 2006). A most recent research indicated that