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ScienceDirect
Neurochemistry International 52 (2008) 541–553
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Mass spectrometric assay and physiological–pharmacological activity of androgenic neurosteroids

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Received 8 January 2007; received in revised form 15 May 2007; accepted 22 May 2007
Available online 13 June 2007

Abstract

Steroid hormones play a key role in the pathophysiology of several brain disorders. Testosterone modulates neuronal excitability, but the underlying mechanisms are obscure. There is emerging evidence that testosterone-derived ''androgenic neurosteroids'', 3α-androstanediol and 17β-estradiol, mediate the testosterone effects on neural excitability and seizure susceptibility. Testosterone undergoes metabolism to neurosteroids via two distinct pathways. Aromatization of the A-ring converts testosterone into 17β-estradiol. Reduction of testosterone by 5α-reductase generates 5α-dihydrotestosterone, which is then converted to 3α-androstanediol, a powerful $GABA_A$ receptor-modulating neurosteroid with anticonvulsant properties. Although the 3α-androstanediol is an emerging neurosteroid in the brain, there is no specific and sensitive assay for determination of 3α-androstanediol in biological samples. This article describes the development and validation of mass spectrometric assay of 3α-androstanediol, and the molecular mechanisms underlying the testosterone modulation of seizure susceptibility. A liquid chromatography–tandem mass spectrometry assay to measure 3α-androstanediol is validated with excellent linearity, specificity, sensitivity, and reproducibility. Testosterone modulation of seizure susceptibility is demonstrated to occur through its conversion to neurosteroids with ''anticonvulsant'' and ''proconvulsant'' actions and hence the net effect of testosterone on neural excitability and seizure activity depends on the levels of distinct testosterone metabolites. The proconvulsant effect of testosterone is associated with increases in plasma 17β-estradiol concentrations. The 5α-reduced metabolites of testosterone, 5α-dihydrotestosterone and 3α-androstanediol, had powerful anticonvulsant activity. Overall, the testosterone-derived neurosteroids 3α-androstanediol and 17β-estradiol could contribute to the net cellular actions of testosterone in the brain. Because 3α-androstanediol is a potent positive allosteric modulator of $GABA_A$ receptors, it could serve as an endogenous neuromodulator of neuronal excitability in men. The 3α-androstanediol assay is an important tool in this area because of the growing interest in the potential to use adjuvant aromatase inhibitor therapy to improve treatment of epilepsy.

Keywords: Neurosteroid; Testosterone; Epilepsy; 3α-Androstanediol; 17β-Estradiol; $GABA_A$ receptor; Hippocampus; Seizure susceptibility; Mass spectrometry

1. Introduction

Steroid hormones play a key role in the neuroendocrine control of neuronal excitability and brain function. There is emerging evidence that circulating steroid hormones serve as precursors for the synthesis of neurosteroids (Schumacher et al., 2003). Neurosteroids are endogenous modulators of neuronal excitability. Neurosteroids such as the progesterone metabolite allopregnanolone and the deoxycorticosterone metabolite allotetrahydrodeoxycorticosterone (THDOC) are potent positive modulators of $GABA_A$ receptors with anxiolytic and anticonvulsant properties (Harrison et al., 1987; Kokate et al., 1994; Reddy and Kulkarni, 1997; Reddy et al., 2005a). These neurosteroids have been shown to play a significant role in the pathophysiology of brain disorders such as generalized anxiety disorder, depression, epilepsy and stress (Herzog, 1995; Reddy et al., 2001; Smith et al., 1998; Monteleone et al., 2000; Purdy et al., 1991; Reddy and Rogawski, 2002; Dong et al., 2001; Reddy, 2003a, 2004a, 2005, 2006). Testosterone

Abbreviations: AED, antiepileptic drug; APCI, atmospheric pressure chemical ionization; AR, androgen receptor; CYP, cytochrome P-450; DHT, 5α-dihydrotestosterone; ECNCI, electron capture negative chemical ionization; GABA, γ-aminobutyric acid; GC, gas chromatography; 3α-HSOR, 3α-hydroxysteroid oxidoreductase; LC, liquid chromatography; MS, mass spectrometry; PTZ, pentylenetetrazol; THDOC, allotetrahydrodeoxycorticosterone; THE, tonic hindlimb extension

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doi:10.1016/j.neuint.2007.05.019

produces rapid modulation of neuronal excitability, but the underlying mechanisms are obscure. There are two potential mechanisms by which testosterone exert neuroendocrine control of brain function: binding to intracellular androgen receptors (genomic) and metabolism to neurosteroids (non-genomic). Testosterone-derived ''androgenic neurosteroids'' could be involved in mediating the testosterone effects on neural excitability. However, very little is known about the pathophysiological importance of androgenic neurosteroids in brain disorders.

This article describes the mass spectrometry (MS) assay of the androgenic neurosteroid 3α-androstanediol in biological samples and the molecular basis of testosterone modulation of seizure susceptibility via its conversion to neurosteroids with anticonvulsant and proconvulsant properties. The ultimate goal of research in this field is to explore avenues for the clinical utility of neurosteroids in treating neurological disorders such as epilepsy.

2. Biosynthesis of androgenic neurosteroids from testosterone

2.1. Androgen and estrogen pathways

Testosterone is the primary circulating androgen and a prohormone for neurosteroid synthesis. The biosynthetic pathway for the androgenic neurosteroid synthesis from testosterone is illustrated in Fig. 1. Testosterone is metabolized to neurosteroids via two distinct pathways: androgen pathway and estrogen pathway. In androgen pathway, 3α-androstanediol is synthesized from testosterone by two sequential A-ring reductions. 5α-Reductase enzyme first converts testosterone to the intermediate 5α-dihydrotestosterone (DHT), which is then further reduced by 3α-hydroxysteroid oxidoreductase (3α-HSOR) to form 3α-androstanediol (Martini, 1992; Martini et al., 1993). In estrogen pathway, testosterone is converted into 17β-estradiol by the aromatase enzyme. The 3α-androstanediol (5α-androstan-3α,17β-diol) and 17β-estradiol are synthesized in peripheral tissues and the brain (Martini, 1992; Jin and Penning, 2001). Peripherally synthesized 17β-estradiol and 3α-androstanediol could readily cross the blood-brain barrier and induce rapid effects on neuronal excitability (Reddy, 2003b).

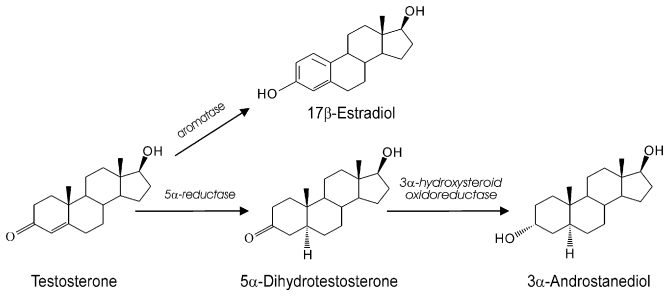

Fig. 1. Synthesis of the androgenic neurosteroid 3α-androstanediol and 17β-estradiol from testosterone. 5α-Reductase converts testosterone into 5α-dihydrotestosterone, which is then reduced further to 3α-androstanediol by 3α-hydroxysteroid oxidoreductase. The 5α-reduction is irreversible and rate limiting, while the 3α-reduction is reversible and occurs more readily. 17β-Estradiol is produced by the aromatase enzyme.

2.2. 3α-Androstanediol is a neurosteroid

The 3α-androstanediol is a neurosteroid because it is synthesized within the brain. 3α-Androstanediol is produced *de novo* by glial cells in the brain, which has 5α-reductase and 3α-HSOR enzymes (Martini et al., 1993; MacLusky et al., 1994; Zwain and Yen, 1999; Mensah-Nyagan et al., 1999; Holloway and Clayton, 2001). The 17β-estradiol is synthesized in peripheral tissues and also produced *de novo* by glial cells in the brain, which express aromatase enzyme (MacLusky et al., 1994; Mensah-Nyagan et al., 1999). In humans, activity of aromatase as well as 5α-reductase is localized in temporal and in frontal brain areas including cerebral neocortex, subcortical white matter, and hippocampus (Stoffel-Wagner et al., 2003). Similarly, *de novo* synthesis of neurosteroids in the human brain is supported by the recent reports showing the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) type 1, which catalyzes conversion of pregnenolone into progesterone (Lanthier and Patwardhan, 1986; Morfin et al., 1992; Bixo et al., 1997; Beyenburg et al., 1999; Stoffel-Wagner, 2003). Moreover, multiple isoforms of 3β-HSD are capable of exhibiting the same activity but differ by their affinity to the substrates, their optimal pH and temperature as well as by their tissue specific expression (Watzka et al., 1999; Inoue et al., 2002; Yu et al., 2002).

Testosterone mediates its cellular effects through both androgen and estrogen pathways, providing multiple possible mechanisms of action (see Fig. 1). Generally, 17β-estradiol produces excitatory effects and thereby facilitates seizures (Woolley, 2000), while 3α-androstanediol has neuroprotective and antiseizure activity (Reddy, 2004b). Therefore, a detailed study of 3α-androstanediol and related neurosteroids as mediators of the physiological effects of testosterone is required to establish the pathophysiological role of androgenic neurosteroids in the brain function.

3. Mass spectrometry assay of the androgenic neurosteroid 3α-androstanediol

3.1. Analysis of neurosteroids

Allopregnanolone and related neurosteroids have been commonly analyzed by sensitive radioimmunoassay, gas chromatography, and mass spectrometry assays (Purdy et al., 1990; Bicikova et al., 1995; Griffiths et al., 1999; Chatman et al., 1999; Kim et al., 2000). Many studies describe derivatization for the trace analysis of neurosteroids by mass spectrometry (Cheney et al., 1995; Lierre et al., 2000; Higashi et al., 2005). However, there are few validated assays for the determination of 3α-androstanediol concentrations in biological fluids. Two distinct mass spectrometry methods are described recently for measurement of 3α-androstanediol in human testicular fluid (Zhao et al., 2004) and amniotic fluid (Wudy et al., 1999), which utilized gas chromatographic

technique. Lack of a simple and specific method for 3α-androstanediol analysis is a major obstacle for further characterization of the physiological function of 3α-androstanediol and the mechanisms by which it affects brain function. Development of a radioimmunoassay is an attractive method for the analysis of 3α-androstanediol, but this assay could be associated with numerous limitations such as specificity of antisera and tedious cross-reactivity determinations and the potential risk of handling radioactive ligands. Moreover, significant cross-reactivity of antibody with chemically related steroids such as 5β-reduced metabolites (epimers) might interfere with the assay (Purdy et al., 1990; Bicikova et al., 1995). These limitations could be avoided by the development of a simple mass spectrometric assay of 3α-androstanediol. An alternative and more specific assay of 3α-androstanediol in plasma can be developed using HPLC with MS–MS detection. Moreover, liquid phase extraction followed by mass spectrometry with a short run time is the most specific and accurate method for the analysis of 3α-hydroxy neurosteroids in human and rat plasma (Cheney et al., 1995; Ramu et al., 2001). Steroids have been commonly analyzed using liquid–liquid extraction and either ECNCI–LC/MS/MS or APCI–LC/MS/MS modes (Griffiths et al., 1999; Kim et al., 2000; Kobayashi et al., 1993; Fredline et al., 1997; Vallee et al., 2000). Influence of eluent composition on ionization efficiency has been extensively studied (Volmer and Hui, 1997).

3.2. LC–MS assay of 3α-androstanediol

Recently, we have established a liquid chromatography–tandem mass spectrometry (LC–MS–MS) assay to measure 3α-androstanediol in plasma (Reddy et al., 2005b). Standard 3α-androstanediol added to plasma has been successfully analysed with excellent linearity, specificity, sensitivity, and reproducibility. In the process of optimizing conditions for 3α-androstanediol determination, we found that 0.1% of acetic acid helped improving the sensitivity. The LC–MS–MS analysis of blank plasma from five different lot numbers showed no endogenous peaks that interfered with the quantification of 3α-androstanediol. Representative chromatogram of extracted blank rat plasma with 3α-androstanediol is shown in Fig. 2A. Retention times of 3α-androstanediol and internal standard (6β-hydroxy-testosterone) are found to be 5.5 and 3.6 min, respectively, indicating that these compounds can be well separated. 3α-Androstanediol and internal standard were monitored from *m/z* 275 → *m/z* 257 and *m/z* 305 → *m/z* 269, respectively. Our prior experience indicates that 6β-hydroxy-testosterone, which is a widely used reference steroid in LC/MS identification of androgenic steroids, is an excellent internal standard because it is stable and does not interfere with the detection of 3α-androstanediol. Moreover, 6β-hydroxy-testosterone and 3α-androstanediol do not coelute. A large unidentified peak appeared at 5.8 min in blank plasma. However, this did not affect the assay as the specific peak of analyte could be readily distinguished by the different retention time. The limit of quantification for 3α-androstanediol using 50 μl of rat plasma sample is 10 ng/ml, with a signal to noise ratio of approximately

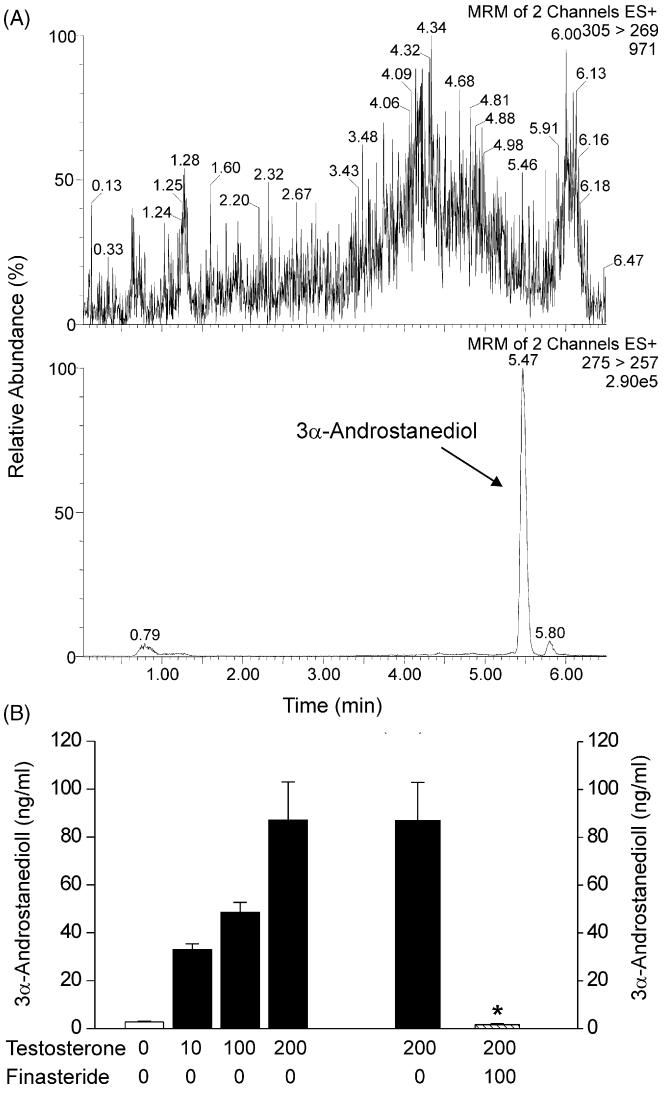

Fig. 2. Mass spectrometry assay of 3α-androstanediol. (A) Representative LC–MS–MS chromatogram of extracted blank rat plasma with 3α-androstanediol (100 ng/ml). The neurosteroid 3α-androstanediol was monitored from *m/z* 275 → *m/z* 257. (B) Plasma 5α-androstanediol concentrations following testosterone administration in rats. Pretreatment with finasteride (100 mg/kg, i.p.) completely blocked the metabolism of testosterone to 5α-androstanediol. *$p < 0.05$ vs. control ($n = 6$ per group).

2.5:1. The limit of detection for 3α-androstanediol is 2 ng/ml. The sensitivity of the method is <10 ng/ml with a detection limit of 2 ng/ml (6.8 nmol/l) and a linear range of 10–2000 ng/ml. The method has been applied for the analysis of testosterone-induced increase in plasma 3α-androstanediol levels in rats (Fig. 2B). Testosterone produced a dose-dependent elevation in plasma 3α-androstanediol, which was almost completely prevented by pretreatment with the 5α-reductase inhibitor finasteride, indicating that 3α-androstanediol is synthesized from testosterone via a 5α-reductase pathway (Reddy et al., 2005b).

3.3. Advantages and limitations of 3α-androstanediol assay

The LC–MS assay allows accurate, high-throughput analysis of 3α-androstanediol in small amounts (200 μl) of

plasma and possibly other biological samples. The advantage of the LC–MS method is that sample preparation is simple, fast and inexpensive and requires no prior derivatization for estimating 3α-androstanediol level in plasma. This assay can be utilized in pharmacological studies to measure elevated levels of free 3α-androstanediol in biological samples. The assay can be modified to estimate the total 3α-androstanediol following enzymatic hydrolysis or conjugated forms can be better analyzed by ESI without any hydrolysis. Although the precision and specificity of the assay are quite good, there are some limitations of the LC–MS assay. The major disadvantage is that the protocol appears to be not suitable for analysis of very low or physiological concentrations of 3α-androstanediol. This problem can be partly rectified by several approaches, including the use of analysis by difference approach to estimate the normal levels of 3α-androstanediol to those previously reported (Frye et al., 2004). There are several better alternatives to improve the sensitivity such as extraction of a large volume of plasma, injection of a large aliquot into the HPLC column, and reconstitution of the sample extract into a smaller volume of HPLC mobile phase to increase the analyte concentration. Moreover, the possible interference from the 3β-hydroxy isomer of 3α-androstanediol can be resolved by differences in polarity in the HPLC separation, which could be further improved using a longer column and a more gentle gradient so as to achieve better separation of the peaks. The assay sensitivity can be further increased to picomole level by additional procedures such as use of trimethyl-silyl or 2-nitro-4-trifluoromethylphenyl derivatives with negative-ion GC–MS (Kim et al., 2000; Vallee et al., 2000) or LC–MS (Higashi et al., 2005).

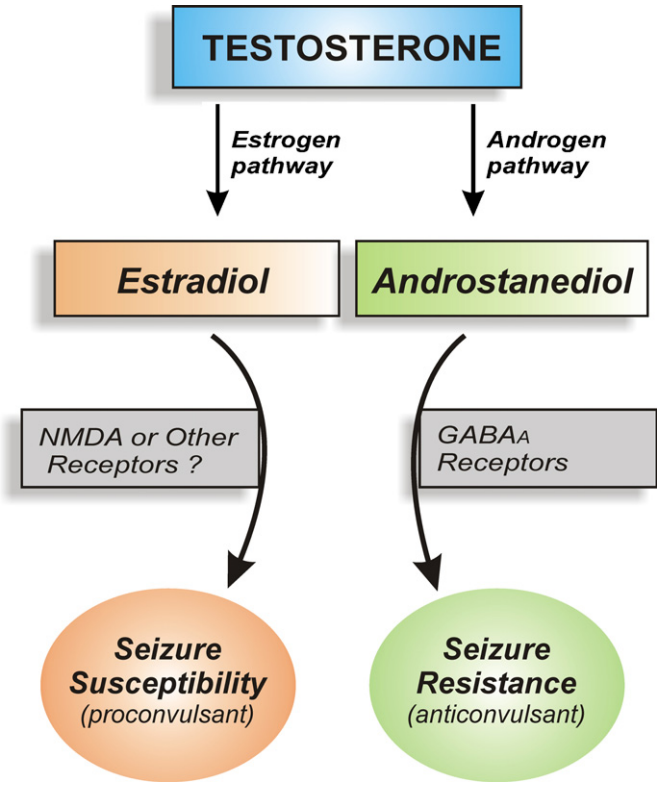

Fig. 3. Potential mechanisms of testosterone modulation of seizure activity. Androstanediol, which is synthesized through androgen pathway, produces anticonvulsant effects that are most likely due to its ability to potentiate the $GABA_A$ receptor-mediated inhibition. Estradiol, which is synthesized through estrogen pathway, facilitates seizure susceptibility by a complex mechanism, including increase in excitatory NMDA receptors because of its ability to enhance the dendritic spine density in the hippocampus.

4. Molecular mechanisms of testosterone modulation of seizure susceptibility

4.1. Effect of testosterone on seizure susceptibility

Testosterone has marked impact on seizure susceptibility. The potential molecular pathways for the testosterone modulation of seizure activity are illustrated in Fig. 3. Testosterone is known to produce both proconvulsant and anticonvulsant effects depending on the animal model and the seizure type (Werboff and Havlena, 1968; Thomas and McLean, 1991; Frye and Reed, 1998; Pesce et al., 2000; Mejias-Aponte et al., 2002). Both animal and clinical studies show that testosterone enhances seizure activity by metabolism to estrogens (Isojarvi et al., 1988; Thomas and Yang, 1991; Herzog et al., 1998; Edwards et al., 1999; El-Khayat et al., 2003). Epidemiological data indicate that the occurrence of focal and tonic–clonic epileptic seizures is ∼50% higher in intact than in castrated dogs (VMDB Report, 2003). On the contrary, testosterone and related androgens have protective effects against seizures induced by pentylenetetrazol and kainic acid (Schwartz-Giblin et al., 1989; Frye and Reed, 1998; Frye et al., 2001a; Reddy, 2004b). Moreover, studies in orchidectomized or castrated animals have shown that decreased testosterone is associated with higher incidence of seizures and replacement with testosterone attenuates seizures (Grigorian and Khudaverkian, 1970; Thomas and McLean, 1991; Pericic et al., 1996; Pesce et al., 2000). However, the precise mechanisms by which testosterone causes such bimodal effects on seizure susceptibility at the cellular level are unclear. Testosterone interaction with intracellular androgen receptors (ARs) is not responsible for testosterone modulation of seizure susceptibility (Cunningham et al., 1979; Roselli et al., 1987; Neri, 1989).

4.2. Resolving bimodal effects of testosterone on seizure susceptibility

To resolve the contradiction of bimodal testosterone effects, we recently studied the effects of testosterone and its neurosteroid metabolites in the pentylenetetrazol (PTZ) test, a widely used animal model of partial seizures. We demonstrated that testosterone modulation of seizure susceptibility occurs through its conversion to neurosteroids with ‘‘anticonvulsant’’ and ‘‘proconvulsant’’ actions, and hence the net effect of testosterone on neural excitability and seizure activity depends on the levels of distinct testosterone metabolites within the brain (Reddy, 2004c). Unlike 17β-estradiol, which generally facilitates seizures (Bäckström, 1976; Hom and Buterbaugh, 1986; Buterbaugh, 1989; Woolley,

2000), 3α-androstanediol has been shown to produce powerful antiseizure effects (Reddy, 2004b,c; Kaminski et al., 2005), which are not mediated by the intracellular ARs (Cunningham et al., 1979; Roselli et al., 1987). To determine the pathways of neurosteroid synthesis (see Fig. 1), the following agents were used: (i) Letrazole, an inhibitor of the aromatase enzyme (Bhatnagar et al., 1990, 2001), was used to block conversion of testosterone to 17β-estradiol; (ii) Finasteride, an irreversible inhibitor of both type 1 (brain) and type 2 (peripheral tissues) 5α-reductase isozymes in rodents (Azzolina et al., 1997), was utilized to inhibit the conversion of testosterone into DHT; (iii) Indomethacin, a powerful blocker of 3α-HSOR enzyme activity (Penning et al., 1985; Penning and Talalay, 1983), was used to inhibit reduction of DHT into 3α-androstanediol.

Consistent with our prediction, testosterone administration in intact male rats is associated with marked reduction of seizure threshold as determined by the intravenous PTZ threshold test that provides a sensitive, graded measure of seizure susceptibility (Reddy, 2004c). These effects of testosterone are dose-dependent, suggesting a proconvulsant effect. These results corroborate the reports that testosterone enhances the development of amygdala kindling seizures (Edwards et al., 1999, 2001) and lowers the threshold for electroshock seizures in rats (Woolley et al., 1961). However, these results are in contrast with two other studies that evaluated the neuroprotective actions of testosterone (Pesce et al., 2000; Frye et al., 2001a). It is likely that differences in the seizure model or the species used may have caused the discrepancies in the results. Alternatively, testosterone might have a biphasic effect on seizures: proconvulsant at higher doses, anticonvulsant at lower doses. Further, notwithstanding the modest antiseizure activity of testosterone in animals (Pesce et al., 2000; Frye et al., 2001b), testosterone itself has not been reported to improve seizures clinically (Herzog et al., 1998). Reductions of seizures were observed only when testosterone was given together with an estrogen synthesis inhibitor, suggesting the estradiol modulation of seizure activity.

5. Estrogens mediate the proconvulsant effects of testosterone

5.1. Seizure facilitating effects of testosterone are associated with elevated estradiol levels

It has previously been observed that testosterone therapy is associated with a dose-dependent increase in plasma 17β-estradiol levels (Reddy, 2004c), which is inversely correlated with the dose-response relationship for seizure susceptibility in animals. Since 17β-estradiol is derived from testosterone, these results raised the possibility that the proconvulsant-like effects of testosterone could be mediated by increased synthesis of 17β-estradiol via estrogen pathway. If the proconvulsant-like effects of testosterone are caused by its conversion to 17β-estradiol, then inhibitors of the aromatase enzymatic pathway through which 17β-estradiol is synthesized from testosterone should prevent the proconvulsant effect of testosterone. Letrozole, a selective non-steroidal aromatase inhibitor, is widely used to block conversion of testosterone to 17β-estradiol (Bhatnagar et al., 1990, 2001; Schieweck et al., 1993). Our results indicate that letrozole administration significantly decreased plasma 17β-estradiol and reversed the testosterone-induced decrease in seizure threshold (Reddy, 2004c). These results convincingly demonstrate that testosterone-induced exacerbation of seizure activity is attributable to its conversion to 17β-estradiol, which is known to have proconvulsant effects in animal models (Buterbaugh, 1989; Woolley, 2000).

5.2. Seizure facilitating effects of estradiol

Acute administration of 17β-estradiol enhances the frequency and severity of PTZ-induced seizures (Reddy, 2004c), an effect consistent with its activity in several experimental models of partial and limbic seizures (Nicoletti et al., 1985; Hom and Buterbaugh, 1986). The proconvulsant-like activity of estradiol is most consistently demonstrated after chronic treatment (Pericic et al., 1996; Saberi and Pourgholami, 2003). However, 17β-estradiol has rapid effects on increasing field potential amplitudes in hippocampus slices (Wong and Moss, 1991; Tauboll et al., 1994; Joels, 1997), and thus could produce proconvulsant effects in animal models. Thus, these reports provide strong evidence that the proconvulsant-like activity of testosterone is mediated by estrogen metabolites such as 17β-estradiol produced via aromatase pathway. Since testosterone is the common precursor for 17β-estradiol and 3α-androstanediol synthesis (Fig. 1), inhibition of aromatase enzyme could lead to enhanced testosterone availability for the 5α-reductase pathway, which generates DHT and 3α-androstanediol.

5.3. Protective effects of estradiol

The effect of estrogens on cortical excitability and seizure frequency is controversial. While estradiol has been shown to be proconvulsant in several studies (Buterbaugh, 1989; Reddy, 2004c), there are also studies that support an inhibiting effect of estrogens on cortical excitability (Weiland, 1992; Nakamura et al., 2004), suggesting that the effects of estrogens on seizures are contradictory. The action of estrogens on seizures depends on factors such as treatment duration, dosage, hormonal status and seizure model. For example, neuroprotective effect was observed following estradiol therapy in ovariectomized female rats (Veliskova, 2006) or aromatase inhibition in cultured hippocampal neurons (Zhou et al., 2007).

5.4. Potential mechanisms of estradiol actions on seizure susceptibility

The mechanism of estradiol action on seizure activity appears to be complex. The endocrine effects of estradiol are mediated by two distinct estrogen receptors, ER-α and ER-β, which are ligand-activated nuclear transcription factors for several genes. On a cellular level, estradiol affects neuronal excitability due to its ability to enhance glutamate receptor-mediated excitatory neurotransmission (Smith et al., 1988;

Wong and Moss, 1994) and decrease in GABAergic inhibition (Murphy et al., 1998). Estradiol acts on neurons within the limbic system, cerebral cortex, and other regions important for seizure susceptibility. Both direct effects on glutamate receptor subtypes and indirect effects through increase in dendritic spine density of hippocampal *N*-methyl-D-aspartate (NMDA) receptors have been shown to be involved in estradiol modulation of NMDA receptor function (Woolley et al., 1997; Rudick and Woolley, 2001). Chronic exposure of rats to estradiol increases the number and density of dendritic spines and excitatory synapses on hippocampal neurons that could increase the synchronization of synaptically driven neuronal firing in the hippocampus. These mechanisms could be at least partly relevant to estradiol's proconvulsant actions. In contrast, estradiol has been shown to regulate the hippocampal expression of glutamic acid decarboxylase (GAD), the principal enzyme for the synthesis of inhibitory neurotransmitter GABA (Joh et al., 2006). This conceivably could lead to decrease in seizure susceptibility. However, the exact signaling pathways of estradiol actions in the brain remain unclear.

6. Androgenic neurosteroids mediate the protective effects of testosterone

6.1. Testosterone therapy is associated with elevated 3α-androstanediol levels

Testosterone therapy has shown to be associated with marked elevation in plasma 3α-androstanediol levels (Fig. 3B) (Reddy, 2004c). Since 3α-androstanediol is derived from testosterone, these results raise the possibility that androgen pathway could be important for neuroprotective effects of testosterone or alleviation of the seizure facilitation by estrogen pathway. Finasteride, a 5α-reductase inhibitor that blocks the conversion of testosterone to DHT and 3α-androstanediol (Thigpen and Russell, 1992; Azzolina et al., 1997), is very helpful to investigate the role of 3α-androstanediol in the modulation of seizure susceptibility to testosterone. Our results show that finasteride treatment completely prevented the testosterone-induced elevation of plasma 5α-androstanediol levels (Fig. 3B). Experiments involving sequential blockade of 5α-reductase and 3α-HSOR enzymes suggests that the testosterone modulation of seizure activity is due to its conversion to 5α-reduced neurosteroids DHT and 3α-androstanediol (Reddy, 2004c).

6.2. Anticonvulsant activity of 3α-androstanediol

To further strengthen our hypothesis, we sought to demonstrate that testosterone-derived DHT and 5α-androstanediol, like progesterone-derived neurosteroid allopregnanolone (Reddy et al., 2004), has antiseizure and neuroprotective activity. Like allopregnanolone, 3α-androstanediol has powerful protective activity against seizures induced by several $GABA_A$ receptor antagonists (Reddy, 2004b,c), pilocarpine and maximal electroshock model (Kaminski et al., 2004, 2005). A dose-dependent protection by 5α-androstanediol against seizures-induced by PTZ is illustrated in Fig. 4A. The anticonvulsant ED_{50} values are listed in Table 1. In intravenous PTZ test, 3α-androstanediol causes a dose-dependent elevation of seizure threshold (Reddy, 2004c), suggesting that it acts partly by elevating seizure threshold. The seizure protecting activity of 3α-androstanediol is stereoselective and does not require activation of ARs. The 3α-androstanediol has been shown previously to reduce the behavioral seizure activity induced by kainic acid and selective hippocampal stimulation (Frye and Reed, 1998; Frye et al., 2001a). However, 3α-androstanediol at normal ED_{50} dosage does not protect seizures induced by gluatamate receptor agonists such as kainic acid, NMDA and 4-aminopyridine (Reddy, 2004b). 3α-Androstanediol is structurally similar to allopregnanolone and conferred seizure protection in the 6-Hz electroshock model of epilepsy (Kaminski et al., 2004). Overall, the anticonvulsant profile of 3α-androstanediol is highly consistent with other $GABA_A$ receptor modulating neurosteroids including allopregnanolone

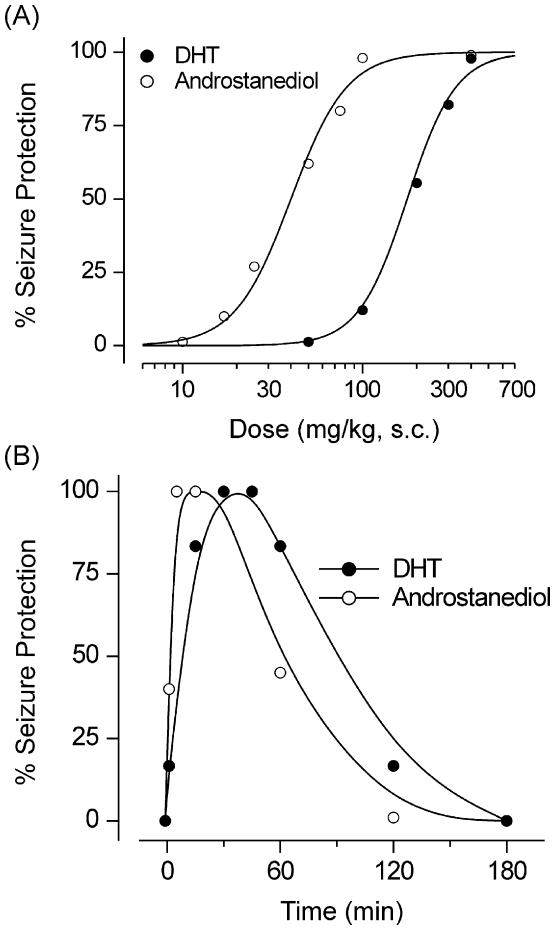

Fig. 4. Antiseizure activity of androgenic neurosteroids. (A) Dose-dependent protection by 5α-dihydrotestosterone (DHT) and 3α-androstanediol against pentylenetetrazol (PTZ)-induced seizures. DHT and 3α-androstanediol were administered, respectively, 30 and 15 min before PTZ (85 mg/kg, s.c.) injection. Mice failing to show clonic spasms lasting longer than 5 s were scored as protected (n = 6–8 mice per group). (B) Time course for protection against PTZ-induced seizures by androgenic neurosteroids DHT (442 mg/kg) and 3α-androstanediol (100 mg/kg). Steroids were given at time 0 and seizure protection was assessed at different time points (n = 6 mice per group). Adapted with permission from Reddy (2004c).

Table 1
Antiseizure profile of testosterone-derived 3α-androstanediol and progesterone-derived allopregnanolone in mouse models of epilepsy

Seizure model	Antiseizure potency (ED_{50})[a]		Reference
	3α-androstanediol	Allopregnanolone	
$GABA_A$ receptor antagonists			
Pentylenetetrazol	40 (27–60)	12 (10–15)	Reddy (2004c); Reddy et al. (2004)
Bicuculline	ND	12 (10–15)	Reddy (2004b, 2006)
Picrotoxin	44 (24–81)	10 (5–19)	Reddy (2004b)
DMCM	39 (21–74)	ND	Reddy (2004b)
Glutamate receptor agonists			
Kainic acid	>200	Inactive	Reddy (2004b)
NMDA	>200	Inactive	Reddy (2004b)
4-Aminopyridine	>200	Inactive	Reddy (2004b)
Status epilepticus models			
Pilocarpine	105 (48–232)	7 (4–13)	Kaminski et al. (2005)
Electroshock models			
Maximal electroshok	224 (182–274)	>100	Kaminski et al. (2005)
6-Hz model	29 (16–52)	14 (10–19)	Kaminski et al. (2004,2005)

[a] ED_{50} is the dose in mg/kg producing seizure protection in 50% of animals. Values in parentheses are 95% confidence limits. DMCM, methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate; NMDA, *N*-Methyl-D-aspartate; ND, not determined.

and THDOC, which have similar spectrum of anticonvulsant activity in animal seizure models (Belelli et al., 1989; Kokate et al., 1994; Reddy and Rogawski, 2002; Reddy et al., 2004; Reddy, 2006).

6.3. The anticonvulsant 5α-dihydrotestosterone as precursor of 3α-androstanediol

Our recent study demonstrated that DHT itself is an anticonvulsant (Fig. 4A). However, the seizure protection has been observed at supraphysiological doses. This raises the possibility that DHT may serve as an intermediate precursor for the synthesis of 3α-androstanediol, which is about 5-fold more potent anticonvulsant than DHT. Unlike 3α-androstanediol, the anticonvulsant activity of DHT was prevented by pretreatment with the 3α-HSOR inhibitor indomethacin (Reddy, 2004c), suggesting that 3α-androstanediol is the ultimate steroid that is responsible for the anticonvulsant effects. Indomethacin is an effective antagonist of 3α-HSOR (Penning et al., 1985), a key enzyme for the conversion of DHT to 3α-androstanediol (Fig. 1). The time course for seizure protection following a $2.5 \times ED_{50}$ dose of 3α-androstanediol and DHT in mice is shown in Fig. 4B. Both androgenic steroids exhibited a rapid onset to peak effect (20 min) and protection diminished during the 120-min period after the injection. Moreover, the androgenic C_{16}-unsaturated steroid androstenol is shown to be a strong anticonvulsant (Kaminski et al., 2006).

7. 3α-Androstanediol modulation of $GABA_A$ receptors

7.1. Mechanism of 3α-androstanediol actions in the brain

Preclinical studies in animal models of epilepsy strongly support that 3α-androstanediol is a key androgenic neurosteroid with potent antiseizure and neuroprotective actions. However, unlike allopregnanolone, the mechanism of 3α-androstanediol actions is not completely elucidated. Generally, 3α-androstanediol lacks classical hormonal properties since its actions occur rapidly (within minutes), even in the presence of the AR antagonist flutamide (Reddy, 2004b), suggesting that ARs are not involved in its anticonvulsant actions. Moreover, 3α-androstanediol binds poorly to intracellular ARs (Roselli et al., 1987). Nevertheless, the extent to which ARs could contribute to the anticonvulsant activity of 3α-androstanediol has not been fully explored.

7.2. 3α-Androstanediol is a positive modulator of $GABA_A$ receptors

The postsynaptic γ-aminobutyric acid $(GABA)_A$ receptor appears to be a major target of 3α-androstanediol (Fig. 5). The $GABA_A$ receptor, a subtype of receptor for the neurotransmitter GABA, mediates the bulk of synaptic inhibition in the brain (Mehta and Ticku, 1999). Because 3α-androstanediol is structurally very similar to allopregnanolone (Gee et al., 1988; Rogawski and Reddy, 2004), it is thought that its anticonvulsant actions are conferred by its selective interaction with $GABA_A$ receptors. Although 3α-androstanediol meets the structural requirements for steroid allosteric modulator of $GABA_A$ receptors (Lambert et al., 2001), its effects on $GABA_A$ receptor function have not been widely investigated in electrophysiological studies. There are, however, studies showing that 3α-androstanediol can alter GABA-stimulated chloride flux and muscimol binding, supporting the view that it could have positive allosteric activity at $GABA_A$ receptors (Frye et al., 1996, 2001b; Rogawski and Reddy, 2004). In an electrophysiology study, 3α-androstanediol (100 μM) produced a significant potentiation ($341 \pm 73\%$) of GABA-evoked Cl^- currents in the voltage clamped primary spinal cord neurons (Park-Chung et al., 1999). In patch-clamp recordings from cerebellar

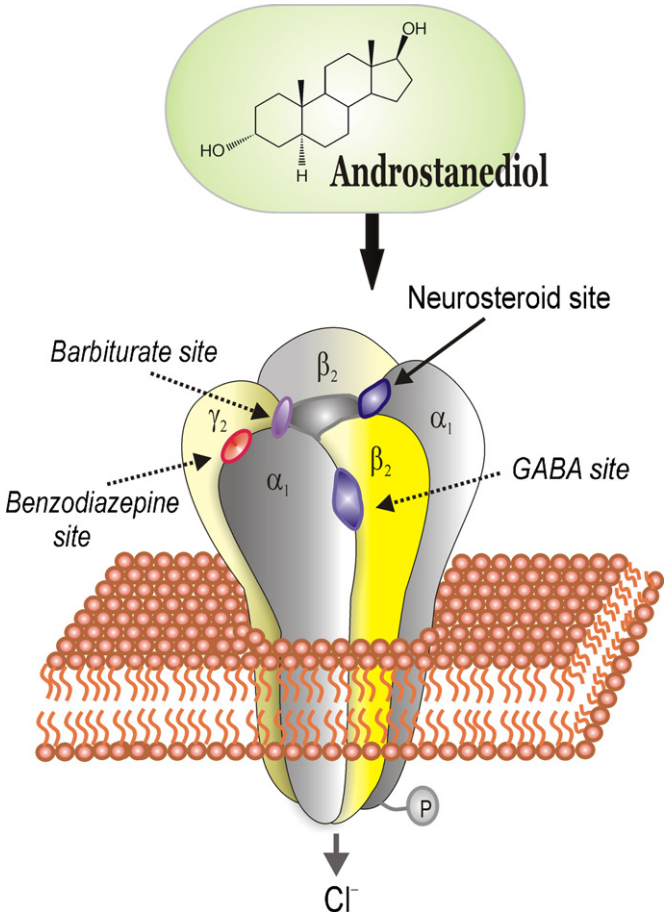

Fig. 5. Androstanediol potentiation of $GABA_A$ receptor function. Like allopregnanolone, 3α-androstanediol is believed to bind at $GABA_A$ receptors and enhance GABA-mediated inhibitory neurotransmission in the brain. The $GABA_A$ receptor is built from several subunits and composed of pentameric channel made of two α subunits, two β subunits and a γ subunit. $GABA_A$ receptor are pluripotent drug targets mediating anxiolytic, sedative, anticonvulsant, and amnesic activities. Neurosteroids have a specific binding site at the $GABA_A$ receptor and the subunit composition appears to have a great impact on neurosteroid modulation of receptor function. The binding site(s) for 3α-androstanediol is proposed to be distinct from that of the GABA, benzodiazepine and barbiturate sites. However, the exact location of 3α-androstanediol binding site is currently unknown. Thus, 3α-androstanediol, by allosteric potentiation of $GABA_A$ receptor-mediated inhibitory synaptic currents, could promote enhanced inhibition and seizure protection.

granule cells, androstenol caused a concentration-dependent enhancement of GABA-activated currents with an EC_{50} of 400 nM, which is highly consistent with activity as a positive modulator of $GABA_A$ receptors (Kaminski et al., 2006). Androstenol is a pheromone steroid that is structurally similar to endogenous neurosteroid 3α-androstanediol. Similarly, 3α-androstanediol inhibits spontaneous epileptiform bursting in hippocampus slices in a stereoselective fashion (Reddy, 2004c; Kaminski et al., 2005). This is extremely consistent with the stereoselective effects of neurosteroids such as allopregnanolone at $GABA_A$ receptors (Gee et al., 1988; Kokate et al., 1994). Overall, these studies strongly support that 3α-androstanediol act as a positive modulator of $GABA_A$ receptors.

8. Pathophysiological role of androgenic neurosteroids

8.1. Androgenic neurosteroids in aging and brain disorders

Changes in brain androgenic neurosteroid biosynthesis could affect neuroendocrine conditions such as anxiety, aggressive behavior, cognitive function and seizure susceptibility (Rogawski and Reddy, 2004; Pinna et al., 2005). Aging is associated with low levels of testosterone that might be linked to several conditions, including muscle weakness, sexual dysfunction and cognitive dysfunction (Schumacher et al., 2003). It is believed that changes in circulating testosterone can affect brain levels of androgenic neurosteroids. Testosterone therapy is used to alleviate some of these conditions, but the pathophysiological role of androgenic neurosteroids is not completely understood.

8.2. Androgenic neurosteroids in epilepsy

In many men with epilepsy, testosterone deficiency is an unusually common clinical observation (Macphee et al., 1988; Herzog, 1991; El-Khayat et al., 2003). Temporal lobe epilepsy surgery has been shown to reduce seizure occurrence and normalize serum androgen concentrations in men with epilepsy (Bauer et al., 2000). Alterations in testosterone levels, therefore, may possibly contribute to exacerbation of seizures. Despite the testosterone-derived 3α-androstanediol's antiseizure effects in animals (Table 1) (Frye and Reed, 1998), however, testosterone itself has not been reported to improve seizures clinically (Herzog et al., 1998). One possible explanation is that antiepileptic drugs that induce enzyme synthesis may enhance the conversion of testosterone to 17β-estradiol, and presumably reduce the net availability of testosterone for the synthesis of 3α-androstanediol. This conjecture is supported by the improved seizure control achieved with testosterone therapy when testosterone was used along with an aromatase inhibitor testolactone that inhibits 17β-estradiol synthesis (Herzog et al., 1998). The introduction of finasteride (*Propecia*®), which inhibits DHT and 3α-androstanediol synthesis, for the treatment of male pattern baldness led to recurrent seizures, which then subsided once the drug was discontinued. Finasteride induced seizure exacerbation has also been reported recently (Herzog and Frye, 2003).

8.3. Androgenic neurosteroids in antiepileptic drug actions

It is well known that chronic therapy of antiepileptic drugs (AEDs) such as phenytoin leads to profound changes in steroid hormones, including enhanced metabolism of testosterone mediated by cytochrome P450 isoforms (Duncan et al., 1999). Recently, Herzog et al. (2006) compared serum levels of neurosteroids among men with epilepsy who take various AEDs. Enzyme inducing AEDs (carbamazepine and phenytoin) are associated with a more favorable neurosteroid balance (lower DHEAS and higher androstanediol/estradiol ratio) for seizure management. Moreover, a markedly reduced serum bioavailable testosterone levels and sexual function was

reported (Herzog et al., 2006). Two-week phenytoin treatment has been shown to affect the hippocampal levels of testosterone, CYP isoforms, and AR expression in a mouse model (Meyer et al., 2006). The increased metabolism of testosterone leading to augmented androgen metabolite formation most likely led to enhanced expression of CYP19 and AR in hippocampus. Thus, AEDs could modulate the androgen signaling in the hippocampus, which is a critical area for epileptogenesis.

8.4. Androgenic neurosteroids in developing brain

There is experimental evidence to suggest that estrogens could dampen the 3α-androstanediol protective actions. For example, 3α-androstanediol has been shown to play a crucial role in guarding against estrogen toxicity in mice lacking 5α-reductase (Mahendroo et al., 1996, 1997). The 5α-reductase knockout mice have normal estrogen levels but are deficient in 3α-androstanediol synthesis. Similarly, testosterone has been shown to reduce the anticonvulsant effect of the $GABA_A$ receptor-modulating benzodiazepine flurazepam in adult male mice (Rosse et al., 1990). Overall, these results suggest that 3α-androstanediol plays a physiological role in mediating the effects of testosterone on seizure susceptibility. Therefore, pharmacological blockade of the estrogen pathway or stimulation of the 3α-androstanediol pathway may represent novel therapeutic strategy for certain neurosteroid-sensitive brain conditions.

8.5. Androgenic neurosteroids in the hippocampus functions

Although the brain site at which androgenic neurosteroids exerts their protective effect is not known, several lines of evidence suggest that the hippocampus could be a key target. First, the hippocampus is a critical region for the control of epileptic seizures. Second, the hippocampus is known to contain enzymes that convert testosterone into 3α-androstanediol (Mensah-Nyagan et al., 1999). Third, $GABA_A$ receptors, which are the major target for neurosteroids, are abundant in the hippocampus subfields. Finally, 3α-androstanediol suppresses epileptiform activity in hippocampus slices (Reddy, 2004c; Kaminski et al., 2005). Thus, 3α-androstanediol modulation of hippocampal $GABA_A$ receptors may be an interesting area for further research.

8.6. Gender-related seizure susceptibility

Steroid hormones play a key role in the gender-related differences in susceptibility to several brain disorders such as sensitivity to seizures and chronic stress-related conditions. However, the precise mechanism underlying such sexual dimorphism is obscure. It is suggested that the sex differences could be due to steroid hormones or sexually dimorphic characteristics in specific brain areas relevant to epilepsy (Cooke et al., 1999; Reddy, 2003b; Ravizza et al., 2003). Current experimental evidence indicates that progesterone- and testosterone-derived neurosteroids could be involved in sexual dimorphism in neural excitability and seizure susceptibility (Cooke et al., 1999; Reddy et al., 2004; Reddy, 2006). The progesterone-derived neurosteroid allopregnanolone is a powerful $GABA_A$ receptor-modulating neurosteroid with anticonvulsant properties (Reddy et al., 2001, 2004). This neurosteroid has a dose-dependent protection against pentylenetetrazol seizures in both male and female mice lacking progesterone receptors (Reddy et al., 2004). However, female mice exhibited significantly enhanced sensitivity to the protective activity of allopregnanolone as compared to males. In the pilocarpine seizure test, 3α-androstanediol has similar increased potency in female mice. These results underscore the possible role of GABAergic neurosteroids such as allopregnanolone and 3α-androstanediol in the gender-related differences in seizure susceptibility and protection.

9. Clinical application of aromatase inhibitors in epilepsy

9.1. Aromatase enzyme as a new target of epilepsy therapy

Aromatase is the key enzyme for the conversion of testosterone to 17β-estradiol, a neuroactive steroid that promotes seizures (Fig. 3). Aromatase enzyme is expressed in discrete areas in the brain such as hippocampus and neocortex that are involved in epileptogenesis. Aromatase inhibitors could decrease brain excitability by decreasing local estradiol levels and therefore, could be beneficial for the treatment of epilepsy (MacLusky et al., 1994). Consequently, aromatase inhibitors have been proposed as a suitable approach to seizure therapy in some men with epilepsy.

9.2. Efficacy of aromatase inhibitors in epilepsy therapy

Three different aromatase inhibitors have been tested in men with epilepsy: testolactone, letrozole and anastrazole. Herzog et al. (1998) tested the efficacy of testosterone and testolactone in men with intractable complex partial seizures. Improvement in seizure control was reportedly achieved with testosterone therapy when testosterone was used along with testolactone. In a case report, letrozole has been shown to improve seizure control in a 61-year-old man with epilepsy (Harden and MacLusky, 2004). In a pilot study, the safety and efficacy of add-on anastrazole therapy was tested in men with intractable epilepsy (Harden et al., 2004). Men with the greatest seizure reduction showed unexpectedly elevated levels in FSH, a pituitary-derived gonadotropin. Hence, the outcome of trials with three distinct aromatase inhibitors – testolactone, letrozole, and anastrazole – suggests a beneficial treatment modality for men with epilepsy (Harden and MacLusky, 2005).

9.3. Beneficial effects of aromatase inhibitors

Aromatase inhibition affects testosterone metabolism with variable effect on estradiol levels (Harden and MacLusky, 2005). Testosterone levels did increase, but not to above the normal range. Whether aromatase inhibition leads to

normalization or elevation of androgen levels remain unclear. There is little information on whether letrozole or anastrazole therapy increase serum levels of androgenic neurosteroids DHT and androstanediol. This information would help confirming the mechanism(s) by which aromatase inhibitors improves seizure control in men with epilepsy. If aromatase inhibition is associated with elevation in 3α-androstanediol levels, aromatase inhibitors may represent a rationale approach for epilepsy therapy that would not produce sedative side effects, which is often a limiting factor with standard AEDs. Testolactone is a steroid-based competitive inhibitor of the aromatase enzyme, and therefore the clinical use of testolactone may result in androgenic side effects. Because of FDA-approved safety and ready availability, non-steroidal aromatase inhibitors such as letrozole (*Femara*®) and anastrazole (*Arimidex*®) may provide additional seizure control in some men with epilepsy. Many men with epilepsy have low testosterone, and aromatase inhibition may be helpful in maintaining normal testosterone levels and thereby improving sexual dysfunction. However, further trials are clearly warranted to determine the efficacy of aromatase inhibitors in epilepsy.

10. Conclusions

Testosterone is metabolized in the brain to the androgenic neurosteroid 3α-androstanediol and 17β-estradiol. Although 17β-estradiol has long been known to facilitate seizure activity, the physiological role of androgenic neurosteroids is still uncertain. The 3α-androstanediol is synthesized from testosterone by two sequential A-ring reductions via the intermediate DHT. 3α-Androstanediol is a neurosteroid because it is produced de novo by glial cells in the brain, which has 5α-reductase and 3α-hydroxysteroid oxidoreductase enzymes. 3α-Androstanediol has been shown to be a positive modulator of $GABA_A$ receptors with powerful anticonvulsant and protective effects. Thus, 3α-androstanediol could play a key physiological role in mediating the effects of testosterone on cortical excitability, seizure activity, and neuroprotection.

Recent evidence suggest that testosterone modulation of seizure susceptibility occurs through its conversion to neurosteroids with anticonvulsant and proconvulsant actions, and hence the net effect of testosterone on neural excitability and seizure activity depends on the levels of distinct testosterone metabolites within the brain. The 3α-androstanediol assay is an important tool in this area because of the growing interest in the potential to use adjuvant hormonal therapy to improve treatment of epilepsy. Men with epilepsy exhibit unusual testosterone deficiency. Aromatase inhibition and consequent reduction in estradiol or elevation of androgenic neurosteroid levels may be a suitable adjunct approach to the treatment of epilepsy. While recent studies provide a better understanding of the role of 3α-androstanediol, further studies are clearly warranted to ascertain the specific role of androgenic neurosteroids in the pathophysiology of epilepsy and other neurological conditions.

Acknowledgements

The author thanks Drs. Roberto C. Melcangi and AG Mensah-Nyagan for an expert advice with the manuscript. This work was supported partly by NIH R21NS052158 and NC State CVM grants.

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