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Mass Spectrometric Assay and Physiological-Pharmacological Activity of Androgenic Neurosteroids

Doodipala S. Reddy

North Carolina State University Department of Molecular Biomedical Sciences, Raleigh, NC 27606, USA.

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”, 3 α -androstenediol and 17 β -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 17 β -estradiol. Reduction of testosterone by 5 α -reductase generates 5 α -dihydrotestosterone, which is then converted to 3 α -androstenediol, a powerful GABA_A receptor-modulating neurosteroid with anticonvulsant properties. Although the 3 α -androstenediol is an emerging neurosteroid in the brain, there is no specific and sensitive assay for determination of 3 α -androstenediol in biological samples. This article describes the development and validation of mass spectrometric assay of 3 α -androstenediol, and the molecular mechanisms underlying the testosterone modulation of seizure susceptibility. A liquid chromatography-tandem mass spectrometry assay to measure 3 α -androstenediol 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 17 β -estradiol concentrations. The 5 α -reduced metabolites of testosterone, 5 α -dihydrotestosterone and 3 α -androstenediol, had powerful anticonvulsant activity. Overall, the testosterone-derived neurosteroids 3 α -androstenediol and 17 β -estradiol could contribute to the net cellular actions of testosterone in the brain. Because 3 α -androstenediol is a potent positive allosteric modulator of GABA_A receptors, it could serve as an endogenous neuromodulator of neuronal excitability in men. The 3 α -androstenediol 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; 3 α -androstenediol; 17 β -estradiol; GABA_A receptor; hippocampus; seizure susceptibility; mass spectrometry

Correspondence to: Doodipala S. Reddy, Ph.D., Department of Molecular Biomedical Sciences, North Carolina State University College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, NC 27606, Tel: (919) 513-6549, Fax: (919) 513-6465, E-mail: samba_reddy@ncsu.edu.

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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 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 3 α -androstenediol 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, 3 α -androstenediol is synthesized from testosterone by two sequential A-ring reductions. 5 α -Reductase enzyme first converts testosterone to the intermediate 5 α -dihydrotestosterone (DHT), which is then further reduced by 3 α -hydroxysteroid oxidoreductase (3 α -HSOR) to form 3 α -androstenediol (Martini, 1992; Martini et al., 1993). In estrogen pathway, testosterone is converted into 17 β -estradiol by the aromatase enzyme. The 3 α -androstenediol (5 α -androstane-3 α , 17 β -diol) and 17 β -estradiol are synthesized in peripheral tissues and the brain (Martini, 1992; Jin and Penning, 2001). Peripherally synthesized 17 β -estradiol and 3 α -androstenediol could readily cross the blood-brain barrier and induce rapid effects on neuronal excitability (Reddy, 2003b).

2.2. 3 α -Androstenediol is a neurosteroid

The 3 α -androstenediol is a neurosteroid because it is synthesized within the brain. 3 α -androstenediol is produced *de novo* by glial cells in the brain, which has 5 α -reductase and 3 α -HSOR enzymes (Martini et al., 1993; MacLusky et al., 1994; Zwain and Yen, 1999; Mensah-Nyagan et al., 1999; Holloway and Clayton, 2001). The 17 β -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 5 α -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 3 β -hydroxysteroid dehydrogenase (3 β -HSD) type 1, which catalyzes conversion of pregnenolone into progesterone (Lanthier and Patwardham, 1986; Morfin et al., 1992; Bixo et al., 1997; Beyenburg et al., 1999; Stoffel-Wagner, 2003). Moreover, multiple isoforms of 3 β -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, 17 β -estradiol produces excitatory effects and thereby facilitates seizures (Woolley, 2000), while 3 α -androstenediol has neuroprotective and antiseizure activity (Reddy, 2004b). Therefore, a detailed study of 3 α -androstenediol 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 3 α -Androstenediol

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 3 α -androstenediol concentrations in biological fluids. Two distinct mass spectrometry methods are described recently for measurement of 3 α -androstenediol 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 3 α -androstenediol analysis is a major obstacle for further characterization of the physiological function of 3 α -androstenediol and the mechanisms by which it affects brain function. Development of a radioimmunoassay is an attractive method for the analysis of 3 α -androstenediol, 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 5 β -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 3 α -androstenediol. An alternative and more specific assay of 3 α -androstenediol 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 3 α -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 3 α -androstenediol

Recently, we have established a liquid chromatography-tandem mass spectrometry (LC-MS-MS) assay to measure 3 α -androstenediol in plasma (Reddy et al., 2005b). Standard 3 α -androstenediol added to plasma has been successfully analysed with excellent linearity, specificity, sensitivity, and reproducibility. In the process of optimizing conditions for 3 α -

androstenediol 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 3 α -androstenediol. Representative chromatogram of extracted blank rat plasma with 3 α -androstenediol is shown in Figure 2A. Retention times of 3 α -androstenediol and internal standard (6 β -hydroxy-testosterone) are found to be 5.5 and 3.6 min, respectively, indicating that these compounds can be well separated. 3 α -Androstenediol and internal standard were monitored from m/z 275 $\rightarrow$ m/z 257 and m/z 305 $\rightarrow$ m/z 269, respectively. Our prior experience indicates that 6 β -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 3 α -androstenediol. Moreover, 6 β -hydroxy-testosterone and 3 α -androstenediol 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 3 α -androstenediol using 50 μ l of rat plasma sample is 10 ng/ml, with a signal to noise ratio of approximately 2.5:1. The limit of detection for 3 α -androstenediol 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 to 2000 ng/ml. The method has been applied for the analysis of testosterone-induced increase in plasma 3 α -androstenediol levels in rats (Fig.2B). Testosterone produced a dose-dependent elevation in plasma 3 α -androstenediol, which was almost completely prevented by pretreatment with the 5 α -reductase inhibitor finasteride, indicating that 3 α -androstenediol is synthesized from testosterone via a 5 α -reductase pathway (Reddy et al., 2005b).

3.3. Advantages and limitations of 3 α -androstenediol assay

The LC-MS assay allows accurate, high-throughput analysis of 3 α -androstenediol in small amounts (200 μ l) 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 3 α -androstenediol level in plasma. This assay can be utilized in pharmacological studies to measure elevated levels of free 3 α -androstenediol in biological samples. The assay can be modified to estimate the total 3 α -androstenediol 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 3 α -androstenediol. This problem can be partly rectified by several approaches, including the use of analysis by difference approach to estimate the normal levels of 3 α -androstenediol 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 3 β -hydroxy isomer of 3 α -androstenediol 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).

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 Figure 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 (Backstrom, 1976; Hom and Buterbaugh, 1986; Buterbaugh, 1989; Woolley, 2000), 3α -androstenediol has been shown to produce powerful antiseizure effects (Reddy, 2004bc; 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) Letrozole, 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; 1983; 1985), was used to inhibit reduction of DHT into 3α -androstenediol.

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 sensitivity (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 -androstenediol synthesis (Fig.1), inhibition of aromatase enzyme could lead to enhanced testosterone availability for the 5α -reductase pathway, which generates DHT and 3α -androstenediol.

5.3. Protective effects of estradiol

The effect of estrogens on cortical excitability and seizure frequency is controversial. While estradiol have 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 3 α -androstenediol levels

Testosterone therapy has shown to be associated with marked elevation in plasma 3 α -androstenediol levels (Fig.3B) (Reddy, 2004c). Since 3 α -androstenediol 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 5 α -reductase inhibitor that blocks the conversion of testosterone to DHT and 3 α -androstenediol (Thigpen and Russell, 1992; Azzolina et al., 1997), is very helpful to investigate the role of 3 α -androstenediol in the modulation of seizure susceptibility to testosterone. Our results show that finasteride treatment completely prevented the testosterone-induced elevation of plasma 5 α -androstenediol levels (Fig.3B). Experiments involving sequential blockade of 5 α -reductase and 3 α -HSOR enzymes suggests that the testosterone modulation of seizure activity is due to its conversion to 5 α -reduced neurosteroids DHT and 3 α -androstenediol (Reddy, 2004c).

6.2. Anticonvulsant activity of 3 α -androstenediol

To further strengthen our hypothesis, we sought to demonstrate that testosterone-derived DHT and 5 α -androstenediol, like progesterone-derived neurosteroid allopregnanolone (Reddy et al., 2004), has antiseizure and neuroprotective activity. Like allopregnanolone, 3 α -androstenediol has powerful protective activity against seizures induced by several GABA_A receptor antagonists (Reddy, 2004bc), pilocarpine and maximal electroshock model (Kaminiski et al., 2004; 2005). A dose-dependent protection by 5 α -androstenediol against seizures-induced by PTZ is illustrated in Fig. 4A. The anticonvulsant ED₅₀ values are listed in Table 1. In intravenous PTZ test, 3 α -androstenediol causes a dose-dependent elevation of seizure threshold (Reddy, 2004c), suggesting that it acts partly by elevating seizure threshold. The seizure protecting activity of 3 α -androstenediol is stereoselective and does not require activation of ARs. The 3 α -androstenediol 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, 3 α -androstenediol at normal ED₅₀ dosage does not protect seizures induced by glutamate receptor agonists such as kainic acid, NMDA and 4-aminopyridine (Reddy, 2004b). 3 α -Androstenediol 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 3 α -androstenediol is highly consistent with other GABA_A receptor modulating neurosteroids including allopregnanolone 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 5 α -dihydrotestosterone as precursor of 3 α -androstenediol

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 3 α -androstenediol, which is about 5-fold more potent anticonvulsant than DHT. Unlike 3 α -androstenediol, the anticonvulsant activity of DHT was prevented by pretreatment with the 3 α -HSOR inhibitor indomethacin (Reddy, 2004c), suggesting that 3 α -androstenediol is the ultimate steroid that is responsible for the anticonvulsant effects. Indomethacin is an effective antagonist of 3 α -HSOR (Penning et al., 1985), a key enzyme for the conversion of DHT to 3 α -androstenediol (Fig.1). The time course for seizure protection following a $2.5 \times \text{ED}_{50}$ dose of 3 α -androstenediol and DHT in mice is shown in Figure 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 C₁₆-unsaturated steroid androstenediol is shown to be a strong anticonvulsant (Kaminski et al., 2006).

7. 3 α -Androstenediol Modulation of GABA_A Receptors

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

Preclinical studies in animal models of epilepsy strongly support that 3 α -androstenediol is a key androgenic neurosteroid with potent antiseizure and neuroprotective actions. However, unlike allopregnanolone, the mechanism of 3 α -androstenediol actions is not completely elucidated. Generally, 3 α -androstenediol 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, 3 α -androstenediol binds poorly to intracellular ARs (Roselli et al., 1987). Nevertheless, the extent to which ARs could contribute to the anticonvulsant activity of 3 α -androstenediol has not been fully explored.

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

The postsynaptic γ -aminobutyric acid (GABA)_A receptor appears to be a major target of 3 α -androstenediol (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 3 α -androstenediol 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 3 α -androstenediol 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 3 α -androstenediol 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, 3 α -androstenediol (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 granule cells, androstenediol caused a concentration-dependent enhancement of GABA-activated currents with an EC₅₀ of 400 nM, which is highly consistent with activity as a positive modulator of GABA_A receptors (Kaminski et al., 2006). Androstenediol is a pheromone steroid that is structurally similar to

endogenous neurosteroid 3 α -androstenediol. Similarly, 3 α -androstenediol 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 3 α -androstenediol 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 3 α -androstenediol'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 17 β -estradiol, and presumably reduce the net availability of testosterone for the synthesis of 3 α -androstenediol. 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 17 β -estradiol synthesis (Herzog et al., 1998). The introduction of finasteride (*Propecia*®), which inhibits DHT and 3 α -androstenediol 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 androstenediol/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 3 α -androstenediol protective actions. For example, 3 α -androstenediol has been shown to play a crucial role in guarding against estrogen toxicity in mice lacking 5 α -reductase (Mahendroo et al., 1996; 1997). The 5 α -reductase knockout mice have normal estrogen levels but are deficient in 3 α -androstenediol 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 3 α -androstenediol plays a physiological role in mediating the effects of testosterone on seizure susceptibility. Therefore, pharmacological blockade of the estrogen pathway or stimulation of the 3 α -androstenediol 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 3 α -androstenediol (Mensah-Nyagan et al., 1999). Third, GABA_A receptors, which are the major target for neurosteroids, are abundant in the hippocampus subfields. Finally, 3 α -androstenediol suppresses epileptiform activity in hippocampus slices (Reddy, 2004c; Kaminski et al., 2005). Thus, 3 α -androstenediol 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, 3 α -androstenediol has similar increased potency in female mice. These results underscore the possible role of GABAergic neurosteroids such as allopregnanolone and 3 α -androstenediol 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 17 β -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 anastrozole. Herzog and colleagues tested the efficacy of testosterone and testolactone in men with intractable complex partial seizures (Herzog et al., 1998). 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 anastrozole 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 anastrozole - 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 anastrozole therapy increase serum levels of androgenic neurosteroids DHT and androstenediol. 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 3 α -androstenediol 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 anastrozole (*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 3 α -androstenediol and 17 β -estradiol. Although 17 β -estradiol has long been known to facilitate seizure activity, the physiological role of androgenic neurosteroids is still uncertain. The 3 α -androstenediol is synthesized from testosterone by two sequential A-ring reductions via the intermediate DHT. 3 α -Androstenediol is a neurosteroid because it is produced de novo by glial cells in the brain, which has 5 α -reductase and 3 α -hydroxysteroid oxidoreductase enzymes. 3 α -Androstenediol has been shown to be a positive modulator of GABA_A receptors with powerful anticonvulsant and protective effects. Thus, 3 α -androstenediol 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 3 α -androstenediol 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 3 α -androstanediol, further studies are clearly warranted to ascertain the specific role of androgenic neurosteroids in the pathophysiology of epilepsy and other neurological conditions.

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Glossary

ABBREVIATIONS

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

Fig. 1. Synthesis of the androgenic neurosteroid 3 α -androstenediol and 17 β -estradiol from testosterone

5 α -Reductase converts testosterone into 5 α -dihydrotestosterone, which is then reduced further to 3 α -androstenediol by 3 α -hydroxysteroid oxidoreductase. The 5 α -reduction is irreversible and rate-limiting, while the 3 α -reduction is reversible and occurs more readily. 17 β -Estradiol is produced by the aromatase enzyme.

Fig. 2. Mass spectrometry assay of 3α-androstenediol

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

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.

Fig. 4. Antiseizure activity of androgenic neurosteroids

(A) Dose-dependent protection by 5 α -dihydrotestosterone (DHT) and 3 α -androstanediol against pentylenetetrazol (PTZ)-induced seizures. DHT and 3 α -androstanediol were administered, respectively, 30 and 15 min before PTZ (85 mg/kg, sc) injection. Mice failing to show clonic spasms lasting longer than 5 seconds 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 3 α -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.

Fig. 5. Androstenediol potentiation of GABA_A receptor function

Like allopregnanolone, 3 α -androstenediol 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 receptors 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 3 α -androstenediol is proposed to be distinct from that of the GABA, benzodiazepine and barbiturate sites. However, the exact location of 3 α -androstenediol binding site is currently unknown. Thus, 3 α -androstenediol, by

allosteric potentiation of GABA_A receptor-mediated inhibitory synaptic currents, could promote enhanced inhibition and seizure protection.

Table 1

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

Seizure Model	Antiseizure Potency (ED ₅₀)*		(Reference)
	3 α -androstanediol	Allopregnanolone	
<i>GABA_A Receptor Antagonists</i>			
Pentylenetetrazol	40 (27–60)	12 (10–15)	(Reddy, 2004c; Reddy et al. 2004)
Bicuculline	ND	12 (10–15)	(Reddy, 2004b; Reddy, 2006)
Picrotoxin	44 (24–81)	10 (5–19)	(Reddy, 2004b)
DMCM	39 (21–74)	ND	(Reddy, 2004b)
<i>Glutamate Receptor Agonists</i>			
Kainic acid	>200	Inactive	(Reddy, 2004b)
NMDA	>200	Inactive	(Reddy, 2004b)
4-Aminopyridine	>200	Inactive	(Reddy, 2004b)
<i>Status Epilepticus Models</i>			
Pilocarpine	105 (48–232)	7(4–13)	(Kaminski et al., 2005)
<i>Electroshock Models</i>			
Maximal electroshock	224 (182–274)	>100	(Kaminski et al., 2005)
6-Hz model	29 (16–52)	14 (10–19)	(Kaminski et al., 2004; 2005)

* ED₅₀ 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.