SHORT REPORT

$GABA_A$ receptor active steroids are altered in epilepsy patients with tuberous sclerosis

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Background: The neuroactive steroid 3α,5α-tetrahydroprogesterone is the most potent endogenous positive modulator of γ-amino-butyric acid $(GABA)_A$ receptors. There is evidence for a relation between neuroactive steroids and seizure susceptibility.
Objective: To evaluate the putative role of counteregulator neuroactive steroids in the occurrence of seizures in patients with tuberous sclerosis.
Methods: Plasma concentrations of the enantiomers 3α,5α- and 3α,5β-tetrahydroprogesterone ($3\alpha_s$-THP), which are positive modulators of $GABA_A$ receptors, were measured in 18 patients, along with their endogenous functional antagonists 3β,5α- and 3β,5β-THP ($3\beta_s$-THP), to assess their possible modification compared with control subjects. Neuroactive steroids were assayed using a highly sensitive and specific gas chromatographic/mass spectrometric method.
Results: In the tuberous sclerosis patients with poorly controlled seizures, there was a significantly lower $3\alpha_s/3\beta_s$-THP ratio than in seizure-free patients or control subjects.
Conclusions: The reduced $3\alpha_s/3\beta_s$-THP ratio may decrease GABAergic tone, contributing to the appearance of seizures in tuberous sclerosis patients with epilepsy.

Tuberous sclerosis is a disorder characterised by hamartomas that can affect any organ. In the brain, cortical tubers are regions of disorganised cortical lamination and are directly related to the presence of partial seizures that are often resistant to medical treatment.[1]

Surprisingly, a large increase in γ-amino-butyric acid (GABA) has been reported in brain biopsies of subjects affected by tuberous sclerosis who have intractable epilepsy, compared with intractable epilepsy from other causes.[2] Moreover, vigabatrin, a specific and irreversible inhibitor of GABA-aminotransferase, is more effective at treating infantile spasms and partial seizures caused by tuberous sclerosis than other types of epilepsy.[3 4] These findings suggest the fundamental involvement of GABAergic neurotransmission in the epileptogenesis of tuberous sclerosis. However, rather than being caused by a defect in GABA biosynthesis, the pathogenesis of epilepsy in tuberous sclerosis could involve an alteration of the $GABA_A$ receptor subunit assembly and structure,[5] or a modified expression of endogenous $GABA_A$ receptor modulatory agents. The neuroactive steroids 3α,5α- and 3α,5β-tetrahydroprogesterone ($3\alpha_s$-THP) are potent positive allosteric modulators of $GABA_A$ receptors.[6 7] Altered $3\alpha_s$-THP concentrations could be one of the reasons for the increased brain GABA levels in patients with tuberous sclerosis.[2] The rationale for this hypothesis is based on the following:

- Pharmacological studies have shown that $3\alpha_s$-THP-reduced metabolites of progesterone are positive $GABA_A$ receptor modulators, while the $3\beta_s$-THP enantiomers (3β,5α-THP and 3β,5β-THP) reduce the ability of $3\alpha_s$-THP to potentiate $GABA_A$ receptor function, acting as functional antagonists.[8–11] Moreover, antiepileptic effects of $3\alpha_s$-THP have been demonstrated in humans and in animal models with partial epilepsy and well defined epileptic foci.[12 13] In addition, $3\alpha_s$-THP may be effective only against convulsive seizures, including epilepsy caused by tuberous sclerosis, but not against non-convulsive seizures.[14]
- There is biochemical evidence from human studies showing that $3\alpha_s$-THP and its stereoisomers are synthesised in the brain and in the periphery from progesterone.[15]
- Behavioural and electrophysiological studies have shown that a decrease in brain $3\alpha_s$-THP results in a decreased $GABA_A$ receptor efficacy and in a reduction of the hypnotic/anaesthetic action of several GABAmimetic drugs.[16]

These data strongly suggest that $3\alpha_s$-THP plays an important permissive physiological role in the action of GABA at $GABA_A$ receptors. We therefore assayed $3\alpha_s$-THP, $3\beta_s$-THP, and progesterone concentrations with the appropriate gas chromatographic/mass spectrometric (GC/MS) technique,[17 18] anticipating that an alteration in their plasma levels might reflect a genetic or epigenetic defect in the enzymatic machinery that regulates THP synthesis.

METHODS

We studied 18 patients with tuberous sclerosis and a history of epilepsy who were referred to the department of neurology of Moscow University. As shown in table 1, of these 18 patients, six had been seizure-free for at least 2.5 years (patients 1 to 6), four were experiencing monthly seizures (patients 7 to 10), four had weekly seizures (patients 11 to 14), and four had daily seizures (patients 15 to 18). Clinical information on the patients is summarised in table 1. The diagnosis of tuberous sclerosis had been made using the new revised clinical diagnostic criteria.[19]

Six healthy subjects matched for age and sex referred to the clinic for a previous infective disease formed the control group (three male, three female; mean (SD) age 10.8 (10.8) years).

Exclusion criteria were as follows: neurological disorders other than tuberous sclerosis; endocrinological and dismetabolic disorders; and other pharmacological treatments in the previous three months.

The study was approved by the local ethics committee, and informed consent was obtained from the patients or their parents before investigations were carried out.

Abbreviations: GABA; γ-amino-butyric acid; $3\alpha_s$-THP, 3α,5α- and 3α,5β-tetrahydroprogesterone; $3\beta_s$-THP, 3β,5α- and 3β,5β-THP (functional antagonists of $3\alpha_s$-THP

Table 1 Clinical profile of the patients with tuberous sclerosis

	Sex/age (years)	AED/dose (mg/kg/d)	Age at seizure onset (months)	Seizure type/age at last seizure (years)	Present seizure frequency	Behavioural and neurological findings	Tubers on MRI	Last seizure before blood sample
1	F/10	0	5	Complex partial/2	Free	Mild MH	Fro (1L/1R)	
2	M/10	0	10	Complex partial/1	Free	None	Par (1L/1R), Tem (1L), Occ (1L)	
3	F/4	0	9	Febrile/1.5	Free	Sleep problems	Cer (1)	
4	M/13	0	34	Febrile/4	Free	None	Fro (1L), Par (1L)	
5	F/35	0	18	Atypical absences/2	Free	None	Not available	
6	M/5	0	3	Complex partial, 18 months atypical absences/2.5	Free	Severe MH, autism	Fro (4R, 4L), Par (2R, 2L), Tem (3R, 2L), Occ (1R, 1L), Pas (3L)	
7	F/7	CB/20, VA/20	3	Complex partial	Daily	Severe MH, autism	Fro (1R, 1L), Par (1R, 1L)	1 h
8	M/4	CB/20, VA/20	9	Complex partial	Daily	Slight MH, autism	Fro (2R, 2L), Par (1R, 1L)	1 h
9	M/14	CB/20, VA 30	22	Atypical absences, complex partial	Daily	None	Occ (1R), Pas (2L)	3 h
10	F/2	CB/20, VA/20	6	Infantile spasms, 18 months also complex partial	Daily	Mild motor and mental handicap	Fro (2R, 1L), Pas (1R)	2.5 h
11	M/9	CB/20, VA/20	11	Simple partial	Weekly	Slight L hemiplegia	Fro (3L), Par (2R, 1L), Occ (1L), Cer (1)	26 h
12	M/7	CB/20, VA/20	28	Atypical absences up to 6 years, then complex partial	Weekly	None	Fro (2L), Par (1R, 1L), Occ (1R)	3 d
13	F/8	CB/20, VA/30	3	Complex partial	Weekly	Mild MH, autism	Fro (1L), Par (1R)	1 d
14	M/9	CB/20, VA/30	28	Atypical absences, complex partial	Weekly	None	Occ (1L)	7 d
15	M/6	CB/20, VA/40	5	Infantile spasms, 18 months also complex partial	Monthly	Mild MH	Fro (3R, 5L), Par (1R), Tem (2L), Occ (1R, 4L), Pas (3R)	18 d
16	F/29	CB/10, VA/20	18	Atypical absences, partial seizures with secondary generalisation	Monthly	None	Not available	20 d
17	F/9	CB/20, VA/20	24	Complex partial	Monthly	None	Tem (1L), Par (2R)	19 d
18	M/20	CB/20, VA/20	3	Infantile spasms up to 2 years, then atypical absences, simple partial	Monthly	Mild MH, autism	Tem (1R), Par (1R)	25 d

AED, antiepileptic drug; CB carbamazepine; Cer, cerebellar lobe; d, day; F, female; Fro, frontal lobe; h, hour; L, left; M, male; MH, mental handicap; Occ, occipital lobe; Par, parietal lobe; Pas, parasagittal lobe; R, right; Tem, temporal lobe; VA, valproic acid.

Heparinised blood samples were taken between 9 am and 10 am during the interictal period for plasma determination of $3\alpha_s$-THP, $3\beta_s$-THP, and progesterone. In patients 7 to 18, carbamazepine and valproic acid were given 12 to 13 hours before blood sampling. In female subjects with menstrual cycles, the blood samples were taken during the follicular phase.

We used a GC-MS method for the determination of neuroactive steroids.[17 18] This profiles and quantifies very small amounts (fmol) of steroidal compounds with high accuracy and allowed us to identify the stereoisomers. Briefly, approximately 5000 dpm of [H^3]-progesterone were added to the plasma to monitor recovery. Ethyl acetate was used for extraction (3×2 ml) and thin layer chromatography for separation (2 mm silica gel 60; solvent systems: carbon tetrachloride/methanol 99:1 (vol/vol), followed by a consecutive run in one direction using cyclohexane/ethyl acetate (3:2, vol/vol)). We then added 7 pmol of progesterone to the eluate containing $3\alpha_s$-THP or $3\beta_s$-THP as an internal standard, and 10 pmol of 3α,α-THP to the eluate containing progesterone. These eluates were freeze dried and derivatised with heptafluorobutyric acid anhydride. Derivatised steroids were analysed using a Finnigan Trace GC/MS instrument equipped with a capillary column (HP-35MS: length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm). The derivatised steroids were assayed in the negative ion chemical ionisation mode. The recovery of tritiated steroids ranged from 80% to 90%. The detection limit for the steroids studied was approximately 10 fmol.

Statistical analysis

Differences in steroid concentrations and in the ratio between $3\alpha_s$-THP and $3\beta_s$-THP within the tuberous sclerosis groups of patients and the control subjects were tested for significance using a one factor analysis of variance (ANOVA). The Tukey–Kramer test was subsequently used for any steroid or $3\alpha_s/3\beta_s$-THP ratio that showed a significant effect at $p < 0.05$ on ANOVA. Statistical analysis was done using SPSS for Windows 10.8.

RESULTS

No significant age effect was detected by ANOVA between the groups of tuberous sclerosis patients and controls. No significant differences were found between the tuberous sclerosis groups for age of seizure onset and the number or localisation of cortical tubers.

In comparisons of steroid concentrations, ANOVA showed significant differences in the concentrations of $3\alpha_s$-THP and $3\beta_s$-THP between seizure-free tuberous sclerosis patients, tuberous sclerosis patients with epilepsy, and the control group (fig 1), but not in the concentrations of progesterone ($F = 2.36$; $df = 159.54$; $p = 0.08$).

As shown in fig 1, plasma levels of $3\beta_s$-THP were increased in all tuberous sclerosis patients with epilepsy, independent of seizure frequency, when compared with seizure-free patients and control subjects. The concentrations of $3\alpha_s$-THP were unchanged in patients with monthly seizures, were decreased (although not significantly) in patients with weekly seizures, and were significantly reduced in patients with daily seizures compared with controls and with seizure-free tuberous patients.

There was a highly significant decrease in the ratio $3\alpha_s/3\beta_s$-THP in all tuberous sclerosis patients with epilepsy compared with seizure-free patients and controls (fig 1).

DISCUSSION

The $3\alpha_s$-THP metabolites are potent positive allosteric modulators of GABA action at $GABA_A$ receptors in the central nervous system, and may serve as endogenous anxiolytic and analgesic agents. In contrast, the $3\beta_s$-THP are devoid of intrinsic activity at $GABA_A$ receptors and behave as functional antagonists of the actions of $3\alpha_s$-THP in potentiating $GABA_A$ receptor function.[8–11]

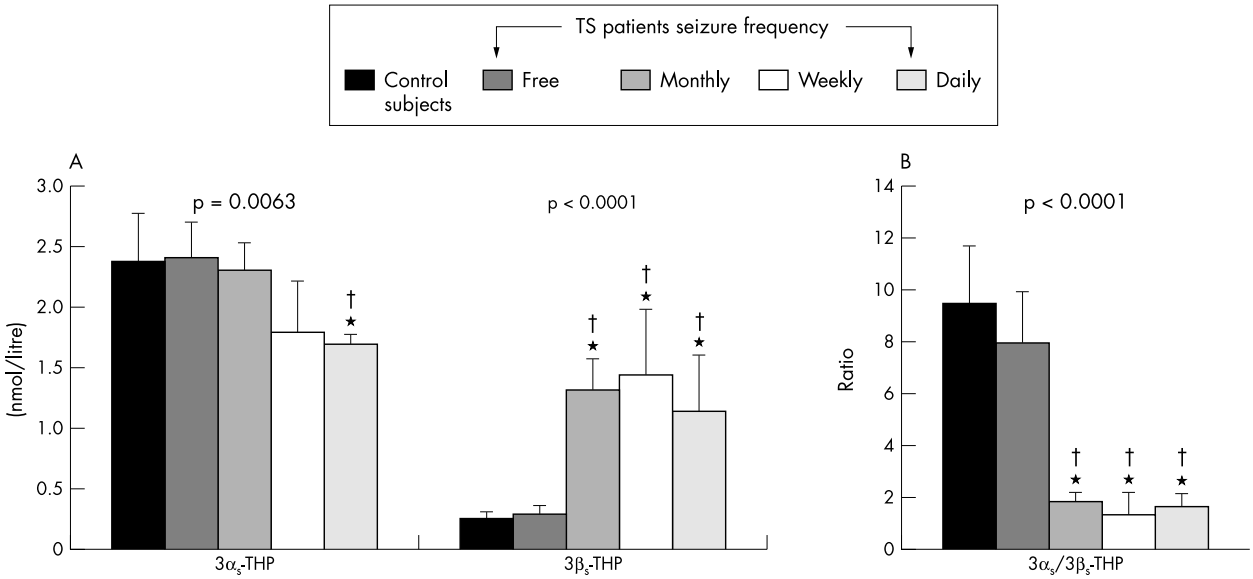

Figure 1 Histograms showing: (A) mean and SD (error bars) of the neuroactive steroids $3\alpha_s$-THP and $3\beta_s$-THP; (B) Mean and SD of the ratio between $3\alpha_s$-THP and $3\beta_s$-THP values, in control subjects and patients with tuberous sclerosis (TS) seizure-free, or with monthly, weekly, and daily seizure frequencies. Analysis of variance probability (p) values are shown above each group of neuroactive steroids and their ratio; asterisks and daggers indicate significant post hoc results. (A) Post hoc results showed that $3\alpha_s$-THP levels were decreased in TS patients with daily seizures compared with control subjects ($p < 0.05$) and seizure-free TS patients ($p < 0.05$). The $3\beta_s$-THP concentrations were increased in TS patients with monthly, weekly, and daily seizures compared with control subjects ($p < 0.0001$, $p < 0.0001$, and $p < 0.005$, respectively) and seizure-free TS patients ($p < 0.0001$, $p < 0.0001$, and $p < 0.005$, respectively). (B) Post hoc results showed that the ratio between $3\alpha_s$-THP and $3\beta_s$-THP values was decreased in TS patients with monthly, weekly, and daily seizures compared with control subjects ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively) and seizure-free TS patients ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively). *Post hoc significance compared with control subjects; †Post hoc significance compared with seizure-free TSC patients. $3\alpha_s$-THP, 3α,5α-tetrahydroprogesterone and 3α,5β-tetrahydroprogesterone; $3\beta_s$-THP, 3β,5α-tetrahydroprogesterone and 3β,5β-tetrahydroprogesterone.

Various investigators have shown that naturally occurring 3α reduced neuroactive steroids (such as $3\alpha_s$-THP) exert anticonvulsant effects.[12 13] To our knowledge, no similar investigations have examined the 3β reduced compounds, owing to the difficulty in detecting stereoisomers by simple radioimmunoassay methods. Thus this is the first clinical study on epilepsy that has evaluated the entire spectrum of THP enantiomers by means of a GC-MS technique, which is highly sensitive and specific.[18 19]

The finding of unchanged THP enantiomer plasma levels in seizure-free tuberous sclerosis patients compared with controls may suggest that alterations in neuroactive steroids are not primary in tuberous sclerosis but represent an epiphenomenon related to seizures or their pharmacological treatment.

The reduced $3\alpha_s$-THP concentrations found only in patients with daily seizures, in which the interictal period is very short, may be caused by seizure induced stress. However, in a previous study by Galli *et al* it was reported that there was an increase in allopregnanolone (3α,5α-THP) in the postictal phase.[13] A possible explanation for this discrepancy is that the radioimmunoassay method used in that study did not discriminate between the different enantiomers and thus considered the whole pool of THP stereoisomers. Furthermore, they reported increased allopregnanolone levels up to 15 minutes after seizure occurrence, which is a very short time span compared with our paradigm, where the minimum interval was never less than one hour.

However, the major finding of our study was the decreased ratio between $3\alpha_s$ and $3\beta_s$-THP in all tuberous sclerosis patients with epilepsy (fig 1B). This ratio should be considered the most relevant index of $GABA_A$ receptor function. A decrease in the modulatory effect of $3\alpha_s$-THP, caused mainly by the augmented $3\beta_s$-THP levels in the brain, could result in a reduced sensitivity of $GABA_A$ receptors to GABA, and in a great reduction in the sedative anticonvulsant action of several GABAmimetic drugs.[16] Thus the high GABA levels found in brain biopsies of drug resistant tuberous sclerosis patients[2] could represent a compensatory mechanism for the down-regulation of $GABA_A$ receptor function resulting from a decreased $3\alpha_s/3\beta_s$-THP ratio.

These findings are consistent with the evidence of a relation between neuroactive steroids and seizure susceptibility.[12] A possible hypothesis to explain our data is that there might be an inadequate induction of the enzymes 5α/5β-reductases or the 3α/3β-hydroxysteroid oxidoreductases responsible for the biosynthesis of the $3\alpha_s$ or $3\beta_s$ reduced derivatives of progesterone in tuberous sclerosis patients with seizures.[15 20]

On the other hand, treatment with antiepileptic drugs could cause changes in the enzymes that metabolise $3\alpha_s$- or $3\beta_s$-THP. It has been shown, for instance, that carbamazepine induces steroidogenesis in rat brain, increasing 3α,5α-THP concentrations.[21] However, we did not found significant changes in the $3\alpha_s$-THP levels except for a decrement in patients with daily seizures.

Although we need further analyses of samples from a larger population of patients with tuberous sclerosis before and after antiepileptic treatment, and also appropriate pharmacological studies in animal models, the decreased $3\alpha_s/3\beta_s$-THP ratio in the patients with epilepsy may reflect an altered $GABA_A$ receptor mediated excitability which could facilitate the occurrence of seizures.

Conclusions

This preliminary evidence is significant not only for a better understanding of the pathophysiology of seizures but also in the clinical perspective in tuberous sclerosis, as it opens the field to the development of possible therapeutic agents. Recently, a $3\alpha_s$-THP analogue, ganaxolone, with anticonvulsant properties in various seizure models such as partial epilepsy, has been synthesised.[22] Ganaxolone might also be considered as a potential therapeutic agent in patients with tuberous sclerosis who have epilepsy.

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