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BRIEF COMMUNICATION**Epilepsia**[®]

Augmentation of endogenous neurosteroid synthesis alters experimental status epilepticus dynamics

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Abstract

Neurosteroids can modulate γ -aminobutyric acid type A receptor-mediated inhibitory currents. Recently, we discovered that the neurosteroids progesterone, 5α -dihydroprogesterone, allopregnanolone, and pregnanolone are reduced in the cerebrospinal fluid of patients with status epilepticus (SE). However, it is undetermined whether neurosteroids influence SE. For this reason, first we evaluated whether the inhibitor of adrenocortical steroid production trilostane (50 mg/kg) could modify the levels of neurosteroids in the hippocampus and neocortex, and we found a remarkable increase in pregnenolone, progesterone, 5α -dihydroprogesterone, and allopregnanolone levels using liquid chromatography tandem mass spectrometry. Second, we characterized the dynamics of SE in the presence of the varied neurosteroidal milieu by a single intraperitoneal kainic acid (KA; 15 mg/kg) injection in trilostane-treated rats and their controls. Convulsions started in advance in the trilostane group, already appearing 90 minutes after the KA injection. In contrast to controls, convulsions prevalently developed as generalized seizures with loss of posture in the trilostane group. However, this effect was transient, and convulsions waned 2 hours before the control group. Moreover, electrocorticographic traces of convulsions were shorter in trilostane-treated rats, especially at the 180-minute ($P < .001$) and 210-minute ($P < .01$) time points. These findings indicate that endogenous neurosteroids remarkably modulate SE dynamics.

KEYWORDS

allopregnanolone, hippocampus, kainic acid, LC-MS/MS, *status epilepticus*, trilostane

1 | INTRODUCTION

Status epilepticus (SE) affects approximately 41 of every 100 000 adults and is difficult to treat. For this reason, up to 20% of cases are reported to involve mortality.¹ Various neurological disorders are associated with SE, including

cerebrovascular diseases, trauma, intoxication, and also others, but it is unclear why SE develops only in some patients affected by the previously mentioned central nervous system (CNS) disorders. In addition, SE also develops without a clear reason in some cases. Finally, patients with epilepsy may experience one or more episodes of SE, whereas others

do not. Presently, the possible factors responsible for this different propensity to develop SE are undetermined.

By characterizing neurosteroid levels in cerebrospinal fluid of patients with SE, we found that progesterone, 5 α -dihydroprogesterone, allopregnanolone, and pregnanolone were all significantly reduced in comparison to presumably healthy controls.² All the mentioned neurosteroids are known to modulate γ -aminobutyric acid (GABA) type A receptor (GABA_A) activity, and especially 3 α -reduced neurosteroids such as allopregnanolone and pregnanolone are potent positive modulators of GABA_A-mediated inhibitory currents.³ For this reason, we hypothesized that the changes observed in CNS neurosteroid levels could be relevant for SE onset or evolution.

On the other hand, the possible role of neurosteroids in SE has been questioned by a multicenter trial based on treatment with the allopregnanolone analogue brexanolone, which failed in demonstrating beneficial effects on the course of refractory SE.⁴ A possible limitation in this study was the lack of stimulation of endogenous neurosteroid production, because different types of neurosteroids can interact with the GABA_A receptor. Both allopregnanolone and pregnanolone are reduced in patients with SE; therefore, a more general approach to increase the overall neurosteroid CNS concentration should be followed to potentiate the effects of neurosteroids on GABAergic function.

To evaluate the possibility of inducing an overall increase of neurosteroid levels in the CNS, we considered the 3 β -hydroxysteroid dehydrogenase/ Δ ⁵⁻⁴ isomerase inhibitor trilostane, which is actually used for treatment of pituitary-dependent hyperadrenocorticism, primary hyperadrenocorticism, and alopecia X in dogs.⁵ A known consequence of trilostane administration is the increase in pregnenolone, allopregnanolone, and allotetrahydrocorticosterone CNS levels in castrated rodents.⁶ This occurs because trilostane blocks the conversion of pregnenolone to progesterone in the adrenal cortex, inducing an increase in the peripheral synthesis of the precursor pregnenolone that, when reaching the brain, is then used to boost overall neurosteroid production. Thus, we aimed to reproduce the same phenomenon in healthy rats and to assess whether the increased availability of neurosteroids could affect the course of SE induced by kainic acid (KA) administration.

2 | MATERIALS AND METHODS

2.1 | Animals and treatments

Forty-two adult male Sprague-Dawley rats (Charles River) of 175- to 200-g body weight were used in this study. All animals were housed in a specific pathogen-free facility under a controlled environment with ad libitum access to water and

food. Experiments received local approval from the Animal Welfare Body, as well from the Italian Ministry of Health (544/2020-PR). Studies were conducted in accordance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals. All efforts were made to improve welfare and to reduce the number of animals used for experiments.

2.2 | Experimental design

Two different experiments were performed, the first to determine the changes in hippocampal and neocortical levels of neurosteroids in naive rats not experiencing the SE, according to a previously established protocol of trilostane administration,⁶ and the second to characterize the dynamics of KA-induced SE in the presence of the different neurosteroid levels determined by trilostane treatment. To this aim, trilostane (Cayman Chemical) was dissolved in sesame oil and subcutaneously injected (50 mg/kg) 16 and 2 hours before euthanasia (n = 12, first experiment), or 16 and 2 hours before the induction of SE by KA (n = 9, second experiment). Control rats were also treated with sesame oil 16 and 2 hours before euthanasia (n = 12, first experiment), or 16 and 2 hours before the induction of SE (n = 9, second experiment). KA (Sigma-Aldrich) was dissolved in saline and intraperitoneally injected (15 mg/kg) as described previously.⁷

2.3 | Electrode implantation and video-electrocorticography

We implanted electrodes and obtained video-electrocorticographic (ECoG) recordings from 18 rats belonging to the previously described groups (vehicle and trilostane) and treated with KA (second experiment), starting before the induction of SE. The investigators who scored the seizures (C.L. and L.S.) were blinded to the treatment received by the animals. See the Supporting Information Data S1 for a detailed description.

2.4 | Quantitative analysis of neurosteroids by liquid chromatography–electrospray tandem mass spectrometry

See the Supporting Information Data S1.

2.5 | Statistical analysis

Data were analyzed using Sigma Plot 11 (Systat Software). Hippocampal and neocortical neurosteroid levels in treatment

groups were compared by the Mann-Whitney test. Outliers were identified using the Grubbs test and removed before analyzing video-ECoG data using a repeated measure two-way analysis of variance, considering time and treatment as the between and within factors, respectively. Then, groups were compared by post hoc Holm-Šidák test. The area under the curve of seizure duration (stage 4 + 5) was compared by the Student *t* test. Results are reported as median and interquartile range, or mean values and standard error of the mean, and considered significant when $P < .05$.

3 | RESULTS

First, we analyzed the impact of trilostane on brain neurosteroid levels. We found that the 3β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase inhibitor, injected twice before euthanasia, induced a remarkable increase in hippocampal and neocortical neurosteroid levels. Specifically, pregnenolone, progesterone, 5α -dihydroprogesterone, and allopregnanolone were respectively increased to up to 469% ($P \leq .001$ vs vehicle-treated rats, Mann-Whitney test), 592% ($P \leq .001$), 168% ($P < .05$), and 183% ($P < .01$) of control levels in the hippocampus of trilostane-treated rats; in contrast, pregnenolone sulfate and pregnanolone did not vary significantly (Table 1). Also in the neocortex, we found similar results for almost all the evaluated neurosteroids (pregnenolone sulfate, +123%, $P = .01$ vs vehicle-treated rats; pregnenolone, +606%, $P \leq .001$; progesterone, +946%, $P \leq .001$; 5α -dihydroprogesterone, +380%, $P \leq .001$; allopregnanolone, +527%, $P \leq .001$), with the only exception being pregnanolone (Table 1).

Second, we characterized the dynamics of SE in the presence of the modified neurosteroidal milieu. Although we did not find any significant difference in the latency to SE onset (trilostane-treated rats, 46.67 ± 4.9 minutes; vehicle-treated rats, 44.16 ± 7.2 minutes) and the respective SE duration (trilostane-treated rats, 9.02 ± 0.5 hours; vehicle-treated rats, 10.83 ± 0.8 hours), we observed remarkable differences in seizures recorded in the course of SE, in both occurrence and duration (Figure S1).

Specifically, 90 minutes after the induction of SE, stage 4 seizures were more frequent in the trilostane group ($P < .05$ vs vehicle-treated rats, Holm-Šidák test), reaching the peak 30 minutes later (2 hours after KA administration) and maintaining this high level up to the 150-minute time point (Figure 1A). In vehicle-treated rats, stage 4 seizures increased 60 minutes later, reaching the same level as trilostane-treated rats at the 150-minute time point. However, the reduction in stage 4 seizures was more rapid in trilostane-treated rats, as shown at the 4-hour time point, at which seizures were lower in these animals compared to controls ($P < .05$).

Stage 5 seizures (Figure 1B) also presented accelerated development in the same animals, peaking 90 minutes after the KA injection ($P < .001$ vs vehicle-treated rats). Moreover, the peak observed in trilostane-treated rats doubled that of vehicle-treated rats, in which it appeared at the 3-hour time point. After their respective peaks, stage 5 seizures wane following a similar dynamic in both treatment groups.

By considering stage 4 and 5 seizures together (Figure 1C), it is possible to observe that convulsive seizures occurred in both treatment groups to the same extent, with early increases at 90-120 minutes in trilostane-treated rats (respectively, $P < .001$ and $P < .05$ vs vehicle-treated rats). The peak in the vehicle group instead occurred at the

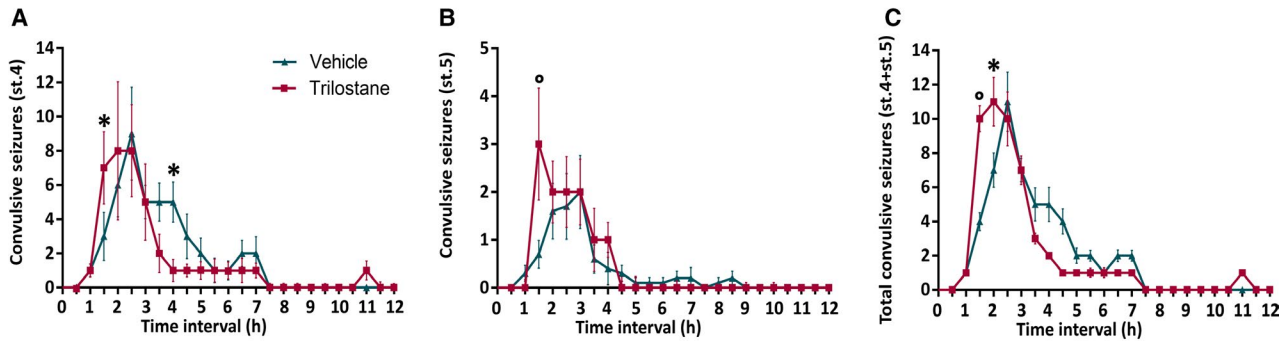
TABLE 1 Tissue levels of various neurosteroids in both hippocampus and neocortex of rats treated with trilostane and their respective controls

Analytes, ng/mg	Vehicle	Trilostane	Statistics
Pregnenolone sulfate, hippocampus	0.000529 (0.00043-0.00055)	0.000513 (0.00043-0.00058)	NS
Pregnenolone sulfate, neocortex	0.000195 (0.000190-0.000225)	0.000240 (0.000220-0.000305)	$P = .010$
Pregnenolone, hippocampus	0.0160 (0.014-0.022)	0.0751 (0.033-0.137)	$P \leq .001$
Pregnenolone, neocortex	0.017 (0.0164-0.0208)	0.103 (0.0422-0.145)	$P \leq .001$
Progesterone, hippocampus	0.00066 (0.000599-0.000798)	0.00391 (0.0019-0.0072)	$P \leq .001$
Progesterone, neocortex	0.00048 (0.00038-0.00073)	0.00454 (0.0019-0.0072)	$P \leq .001$
5α -Dihydroprogesterone, hippocampus	0.000411 (0.00037-0.00045)	0.000691 (0.00046-0.00078)	$P < .05$
5α -Dihydroprogesterone, neocortex	0.000175 (0.000155-0.000205)	0.000665 (0.000335-0.000828)	$P \leq .001$
Allopregnanolone, hippocampus	0.000253 (0.000169-0.000312)	0.000464 (0.000310-0.000576)	$P < .01$
Allopregnanolone, neocortex	0.000091 (0.00008-0.00013)	0.000480 (0.000245-0.000732)	$P \leq .001$
Pregnanolone, hippocampus	0.000216 (0.000175-0.000283)	0.000197 (0.000155-0.000251)	NS
Pregnanolone, neocortex	0.0000857 (0.0000763-0.0000912)	0.0000824 (0.000064-0.000101)	NS

Note: Results are presented as median and interquartile range. Values were compared by the Mann-Whitney test.

Abbreviation: NS, not significant.

Number of convulsive seizures over time



Convulsive seizures duration over time

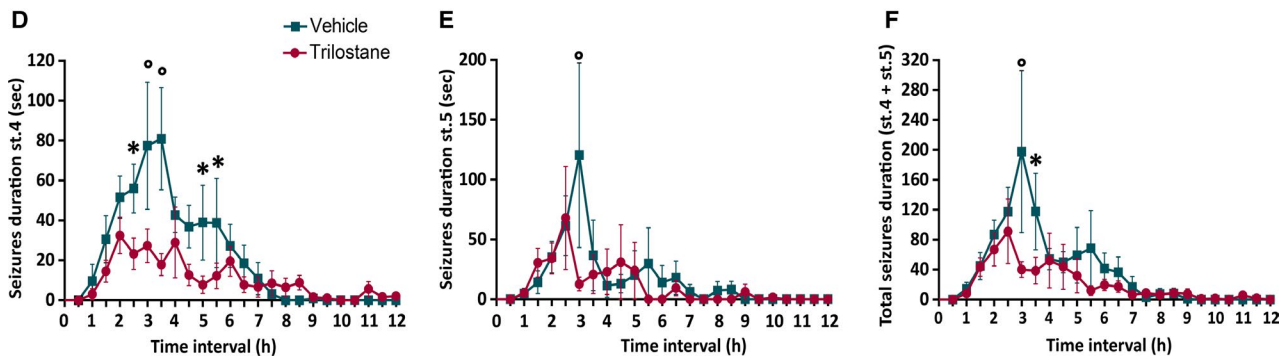


FIGURE 1 Occurrence and duration of video-recorded seizures during kainic acid (KA)-induced status epilepticus (SE). A-C, Seizures induced by KA were counted every 30 minutes for 12 hours. A, Note that stage (st.) 4 seizures increased early ($*P < .05$ vs vehicle-treated rats, Holm-Šídák test) in the trilostane group 90 minutes after induction of SE. Conversely, 150 minutes later these seizures were remarkably reduced in the same group ($*P < .05$ vs vehicle-treated rats). B, Stage 5 seizures also developed precociously in trilostane-treated rats, peaking at the 90-minute time point ($^{\circ}P < .001$ vs vehicle-treated rats, Holm-Šídák test). C, The average occurrence of stage 4 and 5 seizures was respectively reached at 90 minutes ($^{\circ}P < .001$ vs vehicle-treated rats) and 120 minutes ($*P < .05$) in trilostane-treated rats. D-F, Duration of convulsive seizures over time. D, Interestingly, stage 4 seizures presented longer duration in vehicle-treated rats (150 minutes, $*P < .05$; 180 minutes, $^{\circ}P < .001$; 210 minutes, $^{\circ}P < .001$; 300 minutes, $*P < .05$; 330 minutes, $*P < .05$ vs trilostane-treated rats, Holm-Šídák test). E, In contrast, stage 5 seizures were longer in the vehicle-treated group only at the 180-minute time point ($^{\circ}P < .001$). F, Considering stage 4 and stage 5 convulsive seizures together, rats treated with trilostane presented a significant reduction in seizure duration at the 180-minute ($^{\circ}P < .001$) and 210-minute ($*P < .05$) time points

150-minute time point. Subsequently, convulsive seizures similarly decreased in both groups, almost disappearing 7 hours after KA administration.

Then, we evaluated the duration of convulsive seizures over time. Interestingly, stage 4 seizures had a longer duration in vehicle-treated rats (Figure 1D), starting from the 150-minute time point ($P < .05$ vs trilostane-treated rats) up to the 330-minute time point ($P < .001$ vs trilostane-treated rats at 180-210 minutes; $P < .05$ at 300-330 minutes) with the exception of the 240- to 270-minute time point. The same peak of stage 4 seizure duration observed in vehicle-treated rats was never reached in trilostane-treated animals.

Consistently, stage 5 seizures also appeared to be more durable in vehicle-treated rats compared to trilostane-treated

rats 3 hours after KA administration ($P < .001$, Figure 1E). In the vehicle group, duration of stage 5 seizures evaluated at its peak doubled that of trilostane-treated rats. However, in the following time point duration of stage 5 seizures was comparable in both treatment groups.

When stage 4 and stage 5 convulsive seizures were averaged, duration of convulsive seizures was significantly lower in rats treated with trilostane at the 180-minute ($P < .001$ vs vehicle-treated rats) and 210-minute ($P < .05$) time points (Figure 1F). Additionally, by considering the area under the curve for each rat of both groups, we found a remarkable 2.5-fold difference in the overall seizure duration, which was $1\,666\,171.11 \pm 476\,331.82$ seconds² in vehicle-treated rats versus $657\,987.75 \pm 155\,321.00$ seconds² in trilostane-treated rats ($P = .038$, Student *t* test).

4 | DISCUSSION

Our purpose was to describe the development of SE in the presence of altered neurosteroid levels in the brain. For this reason, first we evaluated the effects of trilostane administration on neocortical and hippocampal neurosteroid levels. Trilostane is an inhibitor of 3β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase that was developed as a cancer drug for humans, then used to reduce hyperadrenocorticism in dogs. Specifically, trilostane blocks corticosterone synthesis and removes the inhibitory control of adrenocorticotrophic hormone (ACTH), leading to enhanced stimulatory drive on adrenocortical activity. Thus, ACTH stimulates the conversion of cholesterol to pregnenolone and overcomes the block of 3β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase to restore corticosterone production and to increase the availability of the neurosteroid precursor pregnenolone.⁸ A previous investigation in castrated mice showed that trilostane increases levels of pregnenolone and allopregnanolone in the CNS,⁹ similarly to our results. In the same work, trilostane also induced a remarkable increase in allotetrahydrocorticosterone, which was not measured in our animals.

The changes in CNS neurosteroid levels we found in trilostane-treated rats resulted in remarkable effects on the dynamics of SE induced by KA. Although the latency to SE onset was not affected, trilostane-treated rats anticipated the occurrence of convulsive seizures in response to KA. The most severe stage 5 seizures were also more frequent in trilostane-treated rats, suggesting a proconvulsant effect of the increase in neurosteroid levels. However, this initial effect was not durable. Moreover, considering the seizure duration, the overall response to trilostane appeared to be more anticonvulsant than proconvulsant, because it was mainly characterized by reduction in duration of convulsive seizures. In particular, trilostane-treated rats presented with shorter stage 4 seizures for hours.

We have no simple explanation for the changes described in trilostane-treated rats. GABAergic neurons play a complex role in establishing seizure onset and duration. By optogenetically stimulating parvalbumin interneurons in epileptic mice, Lévesque and collaborators found that the seizure rate was reduced, but the seizure probability instead increased, indicating that the enhanced GABA release from parvalbumin interneurons can paradoxically promote seizures.¹⁰ Although we have not evaluated these phenomena in our animals, the GABAergic tone could be expected to increase in trilostane-treated animals, possibly reproducing a situation similar to that characterized by Lévesque and collaborators in their epileptic mice.

Neurosteroids are currently under investigation for their possible therapeutic effects in epilepsy and SE. Encouraging preclinical evidence for allopregnanolone and its 3β -methyl

derivate ganaxolone, administered 30 minutes after SE onset, has been obtained in a mouse model based on tetramethylenedisulfotetramine injection.¹¹ For the same molecules, the positive findings were confirmed in the lithium-pilocarpine model of SE.¹² Interestingly, additive properties were also described by combining ganaxolone with tiagabine or midazolam.¹³ This further evidence suggests that neurosteroids could be used in polytherapy to address SE. Not only allopregnanolone but also other neurosteroids could be candidates for this purpose, because both androstanediol and 5α -dihydroprogesterone have displayed anticonvulsant effects.^{14,15} However, the use of neurosteroids could be limited in male subjects, who have appeared to be less sensitive than female subjects to the anticonvulsant properties of at least some of them.¹⁴

The use of trilostane to increase the availability of a variety of neurosteroids in the brain could be an alternative to the administration of a single steroid. Steroids are generally metabolized slowly and could afford longer protection than other endogenous anticonvulsant molecules, such as the peptides. For instance, in ovariectomized rats, the half-life of exogenously administered progesterone was 1.21 ± 0.21 hours.¹⁶ Because we found a sixfold increase in progesterone levels in the brain, we hypothesized that this neurosteroid was more available during SE induction and possibly also in the period of convulsive seizures. Trilostane might have contributed to maintaining elevated brain neurosteroid levels, because its effects on the suppression of cortisol production in dogs was shown to last up to 12 hours.⁵ This is because the reported half-life of trilostane is 8 hours. Although trilostane is reported to be specific for the adrenal cortex, we are not aware of any possible direct effect of this drug on the brain or, especially, whether this effect may appear because of dysfunction of the blood-brain barrier in the course of SE. This question must be addressed in future experiments aimed at establishing the time course of the changes in neurosteroid levels in cerebral tissue during SE. However, our findings suggest that the neurosteroids are able to modulate SE, providing that most of them are increased. It remains to be established whether similar effects could be observed if trilostane is administered after the onset of SE, so as to assess the possible clinical use of this drug in the course of SE.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

Concept and design of the study: C.L., C.R., G.B. Experiments, data acquisition, and analysis: A.M.C., C.L., C.R., L.S., S.M. Drafting the manuscript and figures: C.L., G.B. All authors read and approved the final version of the manuscript.

ETHICAL APPROVAL

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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