

BRIEF COMMUNICATION

Seizure progression is slowed by enhancing neurosteroid availability in the brain of epileptic rats

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Abstract

Trilostane is a 3 β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase inhibitor able to produce a manyfold increase in brain levels of various neurosteroids, including allopregnanolone. We previously found that treatment with trilostane can slow down epileptogenesis in the kainic acid (KA) model of temporal lobe epilepsy. It is unknown whether trilostane may have a similar effect on the progression of epilepsy severity, as observed in KA-treated rats. Consequently, we investigated the effects of trilostane (50 mg/kg/day, 1 week) in epileptic rats, given 64 days after KA administration. Seizures were monitored by video-electrocorticographic recordings before and during the treatment with trilostane or vehicle (sesame oil), and neurosteroid levels were measured in serum and cerebral tissue using liquid chromatography–electrospray tandem mass spectrometry after treatment. Pregnenolone sulfate, pregnenolone, progesterone, 5 α -dihydroprogesterone, and allopregnanolone peripheral levels were massively increased by trilostane. With the only exception of hippocampal pregnenolone sulfate, the other neurosteroids augmented in both the neocortex and hippocampus. Only pregnenolone levels were not upregulated by trilostane. As expected, a significant increase in the seizure occurrence was observed in rats receiving the vehicle, but not in the trilostane group. This suggests that the increased availability of neurosteroids produced a disease-modifying effect in the brain of epileptic rats.

KEYWORDS

allopregnanolone, hippocampus, kainic acid, neocortex, neurosteroids, spontaneous recurrent seizures, trilostane

1 | INTRODUCTION

A variety of antiseizure medications are currently available to symptomatically treat epilepsy. Instead, it is challenging to develop drugs able to interfere with the

pathophysiological mechanisms of epilepsy, so as to produce healing from this major neurological disorder or, at least, to reduce the impact of the disease. Two different categories of drugs able to exert an antiepileptic action have been defined: (1) drugs with an antiepileptogenic

Mohammad Gol and Anna Maria Costa contributed equally to this work.

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activity and (2) drugs with a disease-modifying effect. In the first case, the drug should ideally preclude the onset, or at least reduce the severity of epilepsy when this outcome could be predicted on the basis of risk assessment (e.g., posttraumatic epilepsy). In the second case, a drug is expected to modify the course of the disease.¹

The kainic acid (KA) model of temporal lobe epilepsy has recently been proposed as a screening platform to disclose drugs with antiepileptogenic properties or able to produce a modification in the disease progression.² In male rats, a single KA intraperitoneal injection is followed by a self-limiting status epilepticus (SE) accompanied by widespread cerebral lesions, which pave the way to the development of epilepsy.³ We previously found that the onset of spontaneous convulsive seizures after the SE requires 18 days on average, as observed by electrocorticographic (ECoG) recordings. Moreover, long-term video-ECoG monitoring of KA-treated rats evidenced a sigmoidal increase in the seizure frequency,⁴ reaching a plateau at approximately 122 days after the KA administration.⁵ This characteristic makes the KA model attractive to test putative disease-modifying treatments.

We obtained evidence that the competitive inhibitor of 3β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase trilostane has a modulatory effect on epileptogenesis, so as to be considered a potential antiepileptogenic drug.⁶ This result was related to the notable capability of trilostane to increase the concentration of various neurosteroids in the hippocampus and neocortex of healthy rats, and also immediately after SE. Various experiments showed that different neurosteroids, especially allopregnanolone, exert antiseizure effects via γ -aminobutyric acid type A (GABA_A) receptors and can also modify the development of epilepsy (i.e., epileptogenesis) by unclear mechanisms.⁷

In view of the antiepileptogenic properties of trilostane revealed by its early administration in the KA model of temporal lobe epilepsy, which we related to a remarkable increase in allopregnanolone brain levels, we hypothesized that this drug could also increase the availability of neurosteroids in the brain of epileptic rats (i.e., in the chronic period of the model) so as to produce an antiseizure effect and, possibly, a change in the progression of epilepsy in a model in which seizures increase in frequency for many weeks.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

All experimental procedures were approved by the Italian Ministry of Health (544/2020-PR and 729/2021-PR) in agreement with European Directive 2010/63/EU.

Twenty-six adult male Sprague Dawley rats (Charles River, 175–200 g) were randomly assigned to the vehicle

or trilostane group. All rats first received a single dose of KA (intraperitoneal injection at a dose of 15 mg/kg)⁶ to induce SE, and then subcutaneous injections of sesame oil (the vehicle for trilostane) or trilostane (50 mg/kg) were administered for 1 week on days 64–70 after KA injection. This timing was selected because it corresponds to the rising period of seizure occurrence, as found by others.⁵

Video-ECoG recordings were performed 1 week before and during treatments for both groups ($n=13$).⁶ For this purpose, electrode implantation, recording, and analysis of video-ECoG traces were performed as previously described.³ In brief, rats were implanted with epidural electrodes in the frontal (bregma 0 mm, 3.5 mm lateral from midline) and occipital cortices (bregma -6.5 mm, 3.5 mm lateral from midline). An additional electrode was used as a reference and implanted below lambda in the midline. Offline ECoG traces were digitally filtered (band-pass: high, 50 Hz; low, 1 Hz) and manually analyzed using LabChart 8 PRO (ADInstruments). The number of convulsive spontaneous recurrent seizures (SRSs) without or with loss of posture (stage 4–5 according to the Racine scale), their total duration, and mean duration were assessed for each rat. Then, rats were euthanized by isoflurane on day 70, 6 h after the last injection, and neurosteroid levels were assessed by liquid chromatography–electrospray tandem mass spectrometry in sera, hippocampi, and neocortices.⁶ In particular, details about the performed protocol are fully available in our previously published article.⁸

2.2 | Statistical analysis

Seizure occurrence and characteristics were analyzed by repeated measures two-way analysis of variance and the post hoc Holm–Šidák test. The Mann–Whitney test was used to analyze levels of neurosteroids, which were non-normally distributed. One outlier per dataset was identified using the Grubbs test and removed from each group of treatment. Results were summarized by mean \pm SEM values, or median and interquartile range values, and $p < .05$ was considered as the threshold for statistically significant differences (SigmaPlot 13, Systat Software).

3 | RESULTS

3.1 | Trilostane increased the levels of various neurosteroids in the brain and serum of epileptic rats

The neocortical levels of pregnenolone ($p < .05$, Mann–Whitney test), pregnenolone sulfate ($p < .01$), progesterone

($p < .01$), 5α -dihydroprogesterone ($p < .01$), and allopregnanolone ($p < .01$) significantly increased in trilostane-treated rats compared to the vehicle group. No changes were found in the amount of neocortical pregnanolone (Figure 1A–F).

Trilostane significantly augmented also the hippocampal levels of pregnenolone ($p < .01$), progesterone ($p < .01$), 5α -dihydroprogesterone ($p < .01$), and allopregnanolone ($p < .05$). Consistently with the neocortex, pregnanolone was not modified by trilostane. In the hippocampus, pregnenolone sulfate was unchanged (Figure 1G–L).

By measuring the peripheral neurosteroid levels in sera, we observed a remarkable increase in pregnenolone ($p < .01$), pregnenolone sulfate ($p < .01$), progesterone ($p < .01$), 5α -dihydroprogesterone ($p < .01$), and allopregnanolone ($p < .01$) in trilostane-treated rats compared to the vehicle group of epileptic rats, whereas pregnanolone did not change (Figure S1 in the Supporting Information).

3.2 | Effect of trilostane on SRSs

There was no significant difference between the two experimental groups in the total number of stage 4–5 SRSs observed in the week preceding treatments. The weekly number of convulsive SRSs significantly increased in rats receiving the vehicle compared to the pretreatment values of the same animals ($p = .025$, Holm–Šídák test). In comparison to the vehicle-treated group, the administration of trilostane resulted in a small nonsignificant change in the occurrence of convulsive SRSs (Figure 2A). With respect to pretreatment values, trilostane did not modify convulsive SRSs.

Analysis of total duration or mean duration of stage 4–5 SRSs revealed that there was no difference between groups in the pretreatment week. Furthermore, there was no significant difference between vehicle-treated and trilostane-treated rats during the treatment. Also the intragroup comparisons between pretreatment and treatment values did not result in significant changes (Figure 2B,C).

It was further observed that the occurrence of nonconvulsive SRSs was reduced or remained stable in all animals, except for three vehicle-treated rats and one trilostane-treated rat, in which a remarkable increase ($\geq 50\%$ of the pretreatment value) of stage 0–3 SRSs occurred. For convulsive SRSs, a significant increase was observed in five rats of the vehicle-treated group and in three rats of the trilostane-treated group. However, only in the trilostane-treated group, two rats displayed a reduction $\geq 50\%$ of pretreatment values for stage 4–5 SRSs. Accordingly, a 256%

increase in the total number of SRSs in the trilostane-treated group was found, compared to the 412% increase found in the vehicle-treated group (Figure S2).

4 | DISCUSSION

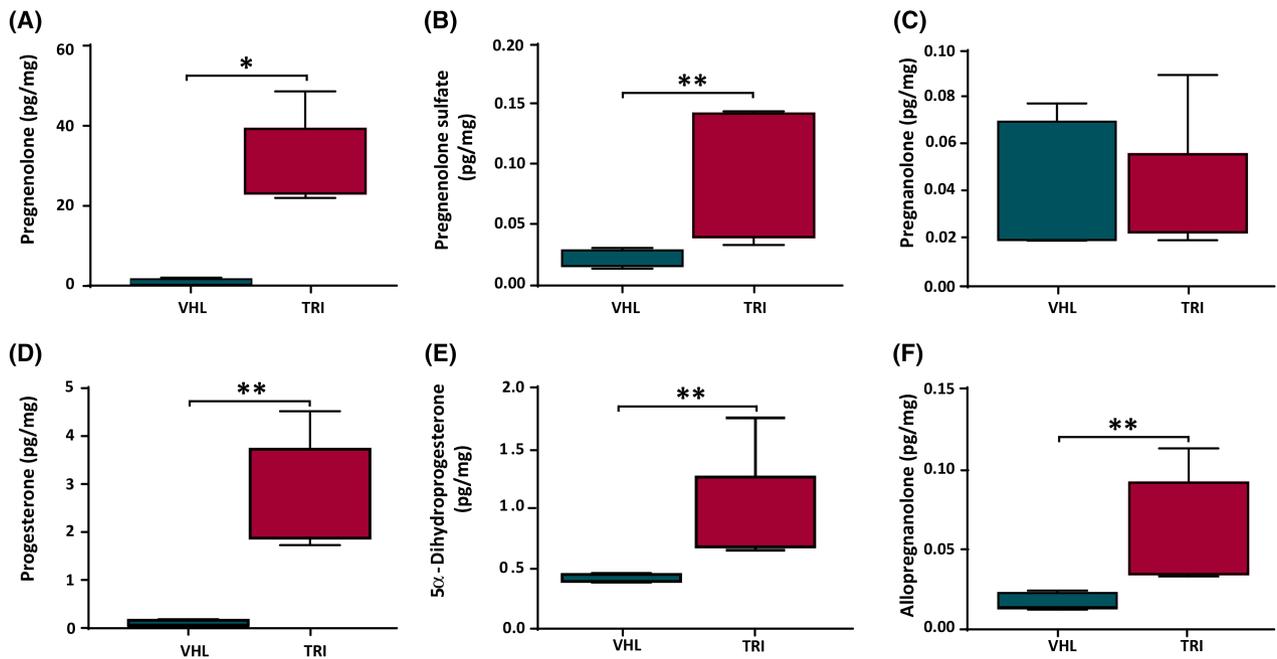
This study resulted in two major findings: (1) a consistent elevation of almost all the investigated neurosteroid levels in the injured brain of epileptic rats treated with trilostane, with the exception of pregnenolone sulfate in the hippocampus; and (2) a slowing in the occurrence of convulsive SRSs in trilostane-treated rats. We observed a statistically significant increase in the occurrence of SRSs only in the vehicle-treated group, with respect to pretreatment values.

Trilostane is a drug able to potently increase the brain levels of various neurosteroids in healthy rats⁹ as well as in those that received a KA injection to induce SE and analyzed during epileptogenesis.⁶ This same phenomenon has not been assessed in KA-treated epileptic rats, in which a reduction in allopregnanolone and pregnanolone hippocampal levels was previously found, suggesting an impaired capability to produce some neurosteroids in the sclerotic hippocampi.⁸ Interestingly, the hypothesized impairment we previously found in basal conditions in epileptic rats can be overcome by stimulating the synthesis of neurosteroids in epileptic rats with trilostane, a drug that increases the availability of neurosteroid precursors in the brain by promoting their synthesis from the periphery.

The observed magnitude of trilostane stimulatory activity was roughly the same observed in our previous experiments,^{6,8} thus suggesting a major role of the peripheral source of neurosteroids detected in the brain, namely, adrenal glands.^{10,11} However, this was not the case for pregnanolone, levels of which were not modified by trilostane both peripherally and centrally. Intriguingly, we found that pregnenolone sulfate was not increased in the hippocampus despite the massive increase observed in the sera of trilostane-treated epileptic rats, whereas in the neocortex a significant elevation in levels of this neurosteroid was evident. This finding might suggest a reduced transport of pregnenolone sulfate in the hippocampus of KA-treated rats, or its enhanced local metabolism.

The KA model represents a very useful tool to investigate epileptogenesis and the progression of epilepsy after brain damage.^{12,13} Using this model, myo-inositol administered 4 h after KA injection and continued for 4 weeks reduced the occurrence and duration of SRSs without affecting the onset of epilepsy, thus documenting a disease-modifying effect independent of any activity on epileptogenesis.¹⁴ Interestingly, this result was ascribed to

Neocortex

Hippocampus

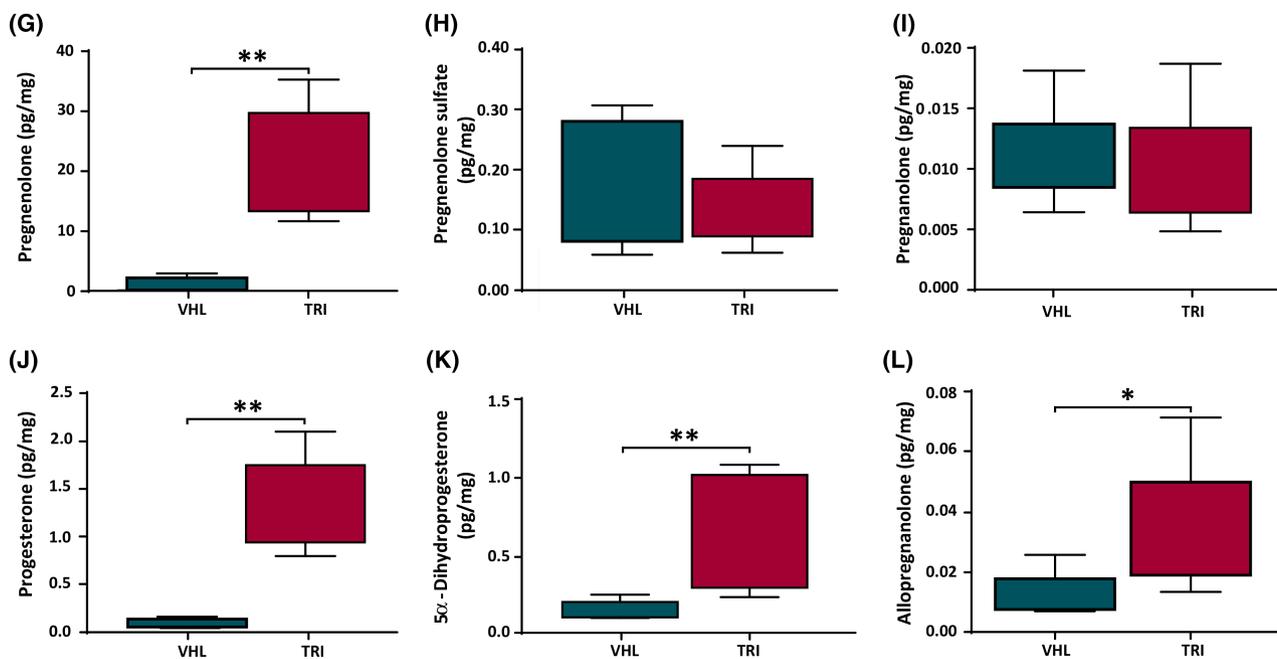


FIGURE 1 Effects of trilostane on neurosteroid levels in neocortex and hippocampus. The repeated daily administration of trilostane (TRI) resulted in a significant increase (A, B, D–F, G, J–L) of almost all of the examined neurosteroids when compared to the vehicle group (VHL). Only pregnenolone sulfate (H) levels in the hippocampus, and pregnanolone in both the hippocampus and neocortex (C, I) were not increased in the trilostane group compared to the vehicle group. Statistical analysis was performed by the Mann–Whitney test. Results are shown as median \pm interquartile range values; * p < .05, ** p < .01 versus the vehicle.

the modulation of GABA_A receptors, which notoriously are potentiated by neurosteroids such as allopregnanolone and pregnanolone, whereas pregnenolone sulfate displays opposite properties.⁷

Other, different mechanisms could also be involved in the modification of epilepsy in KA-treated rats, for instance, neuroinflammation. This was suggested by another investigation showing that simvastatin,

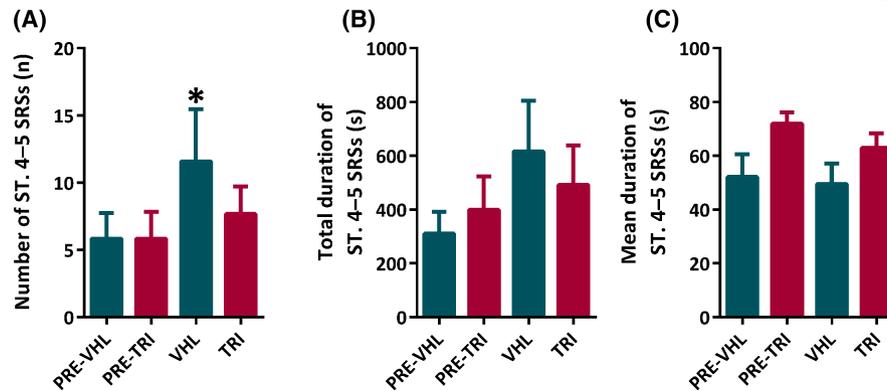


FIGURE 2 The effect of trilostane (TRI) on (A) the number of stage (ST.) 4–5 spontaneous recurrent seizures (SRSs), (B) total duration, and (C) mean duration of ST. 4–5 SRSs. (A) The number of convulsive seizures was significantly increased in rats receiving the vehicle (VHL) compared to pretreatment (PRE-VHL). (B, C) No changes in total duration (B) or mean duration of ST. 4–5 SRSs (C) were found in both groups. * $p < .05$ compared to the PRE-VHL. Statistical analysis was performed by two-way repeated measures analysis of variance and the Holm–Šidák test. Results are shown as mean \pm SEM; $p < .05$ was considered statistically significant.

administered 30 min after KA and then for 2 additional weeks, reduced the epileptic activity in video-ECoG recordings of epileptic rats, which also presented a reduction in interleukin-1 β and tumor necrosis factor- α hippocampal levels.¹⁵ Interestingly, also neurosteroids are antiinflammatory agents, especially progesterone and allopregnanolone.¹⁶

Finally, neuroinflammation is linked to oxidative damage, and the increase of endogenous antioxidant activity effectively modified the occurrence of weekly seizures in KA-treated rats, suggesting that multiple factors may participate in the modulation of epileptogenesis to determine a different epilepsy phenotype in the KA model. In this regard, neurosteroids such as allopregnanolone are involved in the microglial response to oxidation and may counteract the consequences of the oxidative stress.^{17,18}

To conclude, the main outcome of our study is that the increase in brain neurosteroid availability can slow down the progression of seizure occurrence in the KA model, which was further affected in our experiment by the possible appearance of reflex seizures induced by the handling of the animals during the repeated injections. Thus, the administration of trilostane could have a disease-modifying effect. To be definitely demonstrated, this possibility requires confirmation of the slowdown for a longer period of observation and possibly after the interruption of trilostane administration. The present findings and the previous ones suggest that trilostane could be eligible as a drug able to deeply influence the course of temporal lobe epilepsy, as modeled in KA-treated rats.

AUTHOR CONTRIBUTIONS

Concept and design of the study: all authors. *Experiments, data acquisition, and analysis:* Anna Maria Costa,

Mohammad Gol, Chiara Lucchi. *Drafting the manuscript and figures:* all authors. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

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

ETHICAL PUBLICATION STATEMENT

The Ethics Committee of the University of Modena and Reggio Emilia, Italy, approved this study. 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 can be found online in the Supporting Information section at the end of this article.

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