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RESEARCH ARTICLE

Antiepileptogenic effects of trilostane in the kainic acid model of temporal lobe epilepsy

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Abstract

Objective: Epileptogenesis after status epilepticus (SE) has a faster onset in rats treated to reduce brain levels of the anticonvulsant neurosteroid allopregnanolone with the 5α-reductase inhibitor finasteride; however, it still has to be evaluated whether treatments aimed at increasing allopregnanolone levels could result in the opposite effect of delaying epileptogenesis. This possibility could be tested using the peripherally active inhibitor of 3β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$ isomerase trilostane, which has been shown repeatedly to increase allopregnanolone levels in the brain.

Methods: Trilostane (50 mg/kg) was administered subcutaneously once daily for up to six consecutive days, starting 10 min after intraperitoneal administration of kainic acid (15 mg/kg). Seizures were evaluated by video-electrocorticographic recordings for 70 days maximum, and endogenous neurosteroid levels were assessed by liquid chromatography–electrospray tandem mass spectrometry. Immunohistochemical staining was performed to evaluate the presence of brain lesions.

Results: Trilostane did not alter the latency of kainic acid-induced SE onset or its overall duration. When compared to the vehicle-treated group, rats receiving six daily trilostane injections presented a remarkable delay of the first spontaneous electrocorticographic seizure and subsequent tonic–clonic spontaneous recurrent seizures (SRSs). Conversely, rats treated with only the first trilostane injection during SE did not differ from vehicle-treated rats in developing the SRSs. Notably, trilostane did not modify neuronal cell densities or the overall damage in the hippocampus. In comparison to the vehicle group, repeated administration of trilostane significantly decreased the activated microglia morphology in the subiculum. As expected, allopregnanolone and other neurosteroid levels were remarkably increased in the hippocampus and neocortex of rats treated for 6 days with trilostane, but pregnanolone was barely detectable. Neurosteroids returned to basal levels after a week of trilostane washout.

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Significance: Overall, these results suggest that trilostane led to a remarkable increase in allopregnanolone brain levels, which was associated with protracted effects on epileptogenesis.

KEYWORDS
epileptogenesis, kainic acid, neurosteroids, status epilepticus, temporal lobe epilepsy, trilostane

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1 | INTRODUCTION

Epileptogenesis is usually defined as the latent period separating an initial precipitating injury from the appearance of spontaneous recurrent seizures (SRSs).[1] The key causal mechanisms of epileptogenesis are largely undetermined;[2] however, neuroinflammation and glial cell reaction have been repeatedly investigated as possibly involved factors.[3–6] Interestingly, the glial reaction that follows status epilepticus (SE) is accompanied, especially in the hippocampus, by an increased expression of CYP11A1, the cytochrome P450 cholesterol side chain cleavage enzyme that produces pregnenolone, which is known as the precursor of progesterone and other neurosteroids.[7]

Neurosteroids are so defined because they are produced in the nervous system and cooperate with peripherally born neuroactive steroids as regulators of neuronal excitability.[8–10] Their impact on different pathological conditions is well known not only for epilepsy but also for stress, anxiety, depression, psychosis, ataxia, and pain.[11–16] These different conditions share the most characterized target of neurosteroids, namely, γ-aminobutyric acid type A ($GABA_A$) receptor, which is positively modulated by allopregnanolone, pregnanolone, and tetrahydrodeoxycorticosterone to enhance inhibition.[17] Conversely, the neurosteroid pregnenolone sulfate reduces $GABA_A$ inhibitory currents, resulting in a proconvulsant effect.[18]

Epileptogenesis can be accelerated by blocking the synthesis of allopregnanolone with finasteride, which is a 5α-reductase irreversible inhibitor.[19] In view of this evidence, we hypothesized that epileptogenesis could be delayed by increasing allopregnanolone levels. This effect could be obtained by administering trilostane, a 3β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$ isomerase reversible inhibitor that targets steroid production prevalently in the adrenal cortex.[20] Interestingly, the changes in peripheral steroid production attained with trilostane effectively reduced forced swimming-induced immobility in rats and this result was prevented by the surgical removal of adrenal glands and gonads. All of this occurred because the reported behavioral changes were a consequence of the compensatory stimulation of pregnenolone production in the adrenal glands and gonads, which were promoted by the respective hypophyseal hormone.[20]

Key Points

- Trilostane did not alter the development of status epilepticus or subsequent damage in the hippocampus
- Trilostane caused a remarkable increase in allopregnanolone brain levels
- Trilostane suppressed the synthesis of pregnanolone
- Trilostane consistently delayed the development of both electrographic and motor seizures
- Trilostane modulated epileptogenesis in kainic acid-treated rats

In line with these findings, we recently reported that two injections of trilostane could be sufficient to induce a remarkable increase in pregnenolone, progesterone, 5α-dihydroprogesterone, and allopregnanolone levels in the hippocampus and neocortex of healthy rats.[21] Because trilostane is used therapeutically to control hyperadrenocorticism in dogs and is generally well tolerated,[22–25] we considered the possibility of administering this drug to rats during the latent period, to investigate whether neurosteroids, especially allopregnanolone, could be associated with a delay in epileptogenesis.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male Sprague Dawley rats ($n = 103$, Charles River), with an initial weight of 175–200 g, were housed in a specific pathogen-free facility providing a controlled environment with ad libitum access to water and food. All efforts were made to refine procedures, improve the welfare, and reduce the number of animals used for the experiments. The study protocol was authorized by the Italian Ministry of Health (323/2015-PR and 544/2020-PR). All experiments were performed in agreement with European Directive 2010/63/EU.

2.2 | Experimental design

The effects of daily[26] subcutaneous injections of trilostane (50 mg/kg in sesame oil, Cayman Chemical) were investigated in the kainic acid (KA) model.[27–29] An intraperitoneal injection of KA (15 mg/kg in saline, Sigma-Aldrich) was performed 1 week after electrode implantation. Figure 1 illustrates the experimental design, which consisted of four distinct experiments. Rats were euthanized with isoflurane 6, 7, 13, or 70 days after KA administration.

2.3 | Electrode implantation and video-electrocorticography

Electrode implantation, recording, and analysis of video-electrocorticographic (ECoG) traces were performed as previously described.[27] Briefly, 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). One electrode was implanted below lambda in the midline and used as a reference (Figure 1). Offline ECoG traces were digitally filtered (band-pass: high, 50 Hz; low, 1 Hz) and manually analyzed using LabChart 8 PRO (ADInstruments). Seizures were identified in the ECoG traces, and convulsions were analyzed by synchronized video recordings. In particular, convulsions were scored as Stage (ST.) 0 if a clear epileptiform ECoG signal was observed without behavioral changes in the video; ST. 1–2 in the presence of absence-like immobility, "wet-dog shakes," facial automatisms, and head nodding; ST. 3 when presenting with forelimb clonus and lordosis; ST. 4 corresponding to generalized seizures and rearing; and ST. 5 when seizures consisted of rearing with the loss of posture and/or wild running, followed by generalized convulsions.

2.4 | Immunohistochemistry

Animals were deeply anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (pH 7.4), followed by fixation in Zamboni's fixative (pH 6.9) 24 h after the last injection. Brains were kept at 4°C in the same fixative for 24 h, cryoprotected in 15% and 30% sucrose solutions, and stored at −80°C until used. A freezing stage and sliding microtome (Leica SM2000R) were used to obtain 5–6 horizontal sections (50 μm thick) from bregma level −8.04 mm to −5.04 mm.

The immunohistochemical staining was performed as previously described.[27] More specifically, we used mouse anti-neuron-specific nuclear protein (NeuN; #MAB377 clone A60, 1:200, Millipore), mouse anti-glial fibrillary acidic protein (GFAP; #G3893, 1:500, Sigma-Aldrich), and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1; #019-19741, 1:1000, Wako) antibodies. Images were acquired using an Eclipse CiL (Nikon Instruments).

2.5 | Fluoro-jade B

The brain sections were mounted on gelatin-coated slides and dried at room temperature. The following day, staining was performed as previously described,[27] using fluoro-jade B (FJB; Millipore, # AG310-30MG). Images were acquired with a Leica SP2 AOBS confocal microscope.

2.6 | Image analysis

All brain sections from −8.04 mm to −5.04 mm bregma levels were magnified (10×) and the different areas of interest (cornu ammonis 3 [CA3] stratum pyramidalis [Py], subregion B; CA3 lacunosum-moleculare [LMol]; CA1 Py; subiculum [Sub]) were analyzed.

NeuN-immunoreactive cells and FJB-positive cells were counted per square millimeter using the image analysis software NIS-Elements and ImageJ, respectively. The measured area (region of interest [ROI]) was kept unchanged for each hippocampal area regardless of the animal being analyzed. For NeuN, ROI values were .080 mm^2 in the CA3 Py, .033 mm^2 in the CA1 Py, and .113 mm^2 in the Sub. For FJB, ROI values were .119 mm^2, .047 mm^2, and .115 mm^2 in CA3 Py, CA1 Py, and Sub, respectively.

GFAP immunostaining was used to determine regions characterized by a loss of astrocytes. The image analysis software NIS-Elements was used to manually trace the unstained area (mm^2) upon GFAP detection. This unstained area was determined for each section, then the mean value of the unstained area was calculated for each animal.

Microglial activation was assessed with the same image analysis software (NIS-Elements) by calculating the binary area fraction (BinaryArea/MeasuredArea). More precisely, the *BinaryArea* corresponded to the sum of areas of all binary objects, whereas the *MeasuredArea* represented the area of the measurement frame, which was kept constant in all analyzed sections.

2.7 | Liquid chromatography–electrospray tandem mass spectrometry

The brains were carefully removed after euthanasia (isoflurane) and chilled on ice to dissect both the hippocampi and neocortices for liquid chromatography–electrospray

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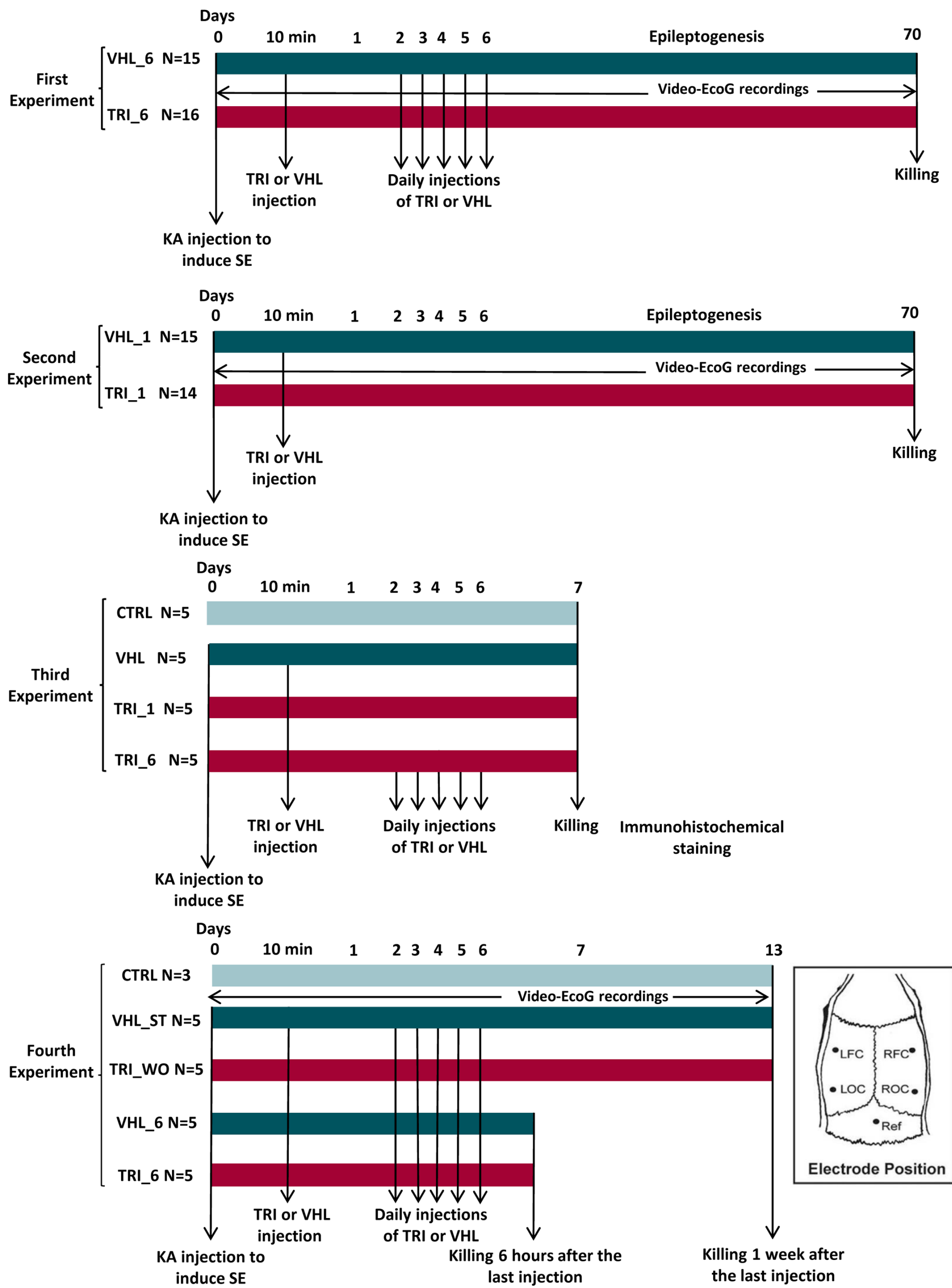

FIGURE 1 Experimental design. Rats were used in four different experiments. The first experiment consisted of six daily injections of trilostane (50 mg/kg, subcutaneously) or sesame oil. In the second experiment, rats were injected with trilostane or vehicle only one time, approximately 10 min from the kainic acid (KA; 15 mg/kg) intraperitoneal administration. A third experiment included untreated control rats and KA-treated rats receiving sesame oil or trilostane, once or repeatedly. In the fourth experiment, the hippocampal and neocortical levels of neurosteroids were measured in untreated control rats, or after six daily injections of trilostane or vehicle, and considering 6 h and 7 days as the time intervals following the last injection of trilostane or vehicle. CTRL, control; ECoG, electrocorticographic; LFC, left frontal cortex; LOC, left occipital cortex; Ref, reference electrode; RFC, right frontal cortex; ROC, right occipital cortex; SE, status epilepticus; TRI_1, trilostane single injection; TRI_6, trilostane repeated injections; TRI_WO, trilostane washout; VHL, vehicle; VHL_ST, sham-treated vehicle; VHL-1, vehicle single injection; VHL-6, vehicle repeated injections.

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tandem mass spectrometry analysis. Details about the performed protocol were published previously.[28] Only values above the limit of quantification were used for the statistical comparisons.

2.8 | Statistics

The results were analyzed by using Student *t*-test or one-way analysis of variance (ANOVA) followed by the Holm–Šídák test, depending on the number of groups and normality assessment (Shapiro–Wilk test). If required, one outlier per dataset was identified using Grubbs test and removed using SigmaPlot 13 (Systat Software). All data are presented as mean and SEM, and they were regarded as significantly different at $p < .05$.

3 | RESULTS

3.1 | Multiple injections but not a single injection of trilostane delayed the onset of SRSs

Following KA administration ($n = 95$), 3 of 45 rats in the vehicle-treated group (7%) and 5 of 50 rats in the trilostane-treated group (10%) died during or after SE. In the vehicle-treated group, two rats (4%) did not develop SE and were discarded. Moreover, two trilostane-treated rats and one vehicle-treated rat lost the ECoG implant before the first SRS and were disregarded for the analysis of SRSs. In animals monitored for SE duration, trilostane did not reduce the duration of SE at the ECoG analysis (9.121 ± .221 h, $n = 36$), as it was similar to that of the vehicle-treated rats (9.398 ± .321 h, $n = 34$; $p = .476$, Student *t*-test). After SE, the rats were used in four different experiments.

In the first experiment, we evaluated the latency for the rats to develop the first ECoG SRS and first convulsive SRS, with or without loss of posture. After repeated administration of trilostane ($n = 12$) or vehicle ($n = 13$), the latency to develop the first ECoG SRS was significantly increased ($p = .021$, Student *t*-test) in the trilostane group (Figure 2A). Moreover, the latency to develop the first convulsive SRS was also significantly increased by trilostane ($p = .038$; Figure 2B). Consistently, the latency to develop the first convulsive SRS with loss of posture (Figure 2C) was significantly longer after treatment with trilostane ($p = .018$). To explain the differences found in the time course of epileptogenesis, we hypothesized that the vehicle- and trilostane-treated rats could have developed a different SE. This hypothesis was not confirmed by analyzing the video-ECoG of vehicle- or trilostane-treated rats, which presented similar latencies to develop the seizures (Figure 2D–F) and analogue SE duration (Figure 2G–I).

Then, to evaluate whether trilostane administration during SE could modify the time course of epileptogenesis, we performed a second experiment in which we characterized the impact of a single trilostane administration on the onset of SRSs. Again, trilostane ($n = 12$) or vehicle ($n = 12$) was administered 10 min after the intraperitoneal administration of KA (Figure 3A–I). Also this protocol did not change the characteristics of SE (Figure 3D–I) but, at variance with the first experiment, the latency to develop SRSs was similar in both treatment groups (Figure 3A–C).

3.2 | Trilostane did not protect neurons in the CA3 Py, CA1 Py, and Sub

In a third experiment, we investigated the hippocampal damage in five animals per group. The animals were sacrificed on Day 7 after SE. Unfortunately, one rat in the trilostane group prematurely died. The results were analyzed by one-way ANOVA, which showed no effect of trilostane treatment on FJB positivity (cells/mm^2) in the CA3 Py ($F_{2,10} = .268$, $p = .770$), CA1 Py ($F_{2,10} = .760$, $p = .493$), and Sub ($F_{2,10} = .503$, $p = .619$). No FJB-positive cells were found in healthy rats (Figure 4A). Thus, FJB staining showed that trilostane did not reduce the number of damaged neurons in the abovementioned brain areas (Figure 4B–D).

Neuronal survival was also assessed by using NeuN immunostaining (Figure 4E). One-way ANOVA showed that KA had a significant effect on NeuN immunopositivity (cells/mm^2) in the CA3 Py ($F_{3,15} = 7.185$, $p = .003$), CA1 Py ($F_{3,15} = 21.061$, $p < .001$), and Sub ($F_{3,15} = 13.470$, $p < .001$). In comparison to healthy rats, a significant reduction in NeuN-immunopositive cells was evidenced in the CA3 Py (Figure 4F) of vehicle-treated rats ($p = .004$, Holm–Šídák test) and rats receiving a single ($p = .035$) or repeated trilostane administration ($p = .012$). Significant differences, compared to healthy rats, were also found in the CA1 Py (Figure 4G) for all the groups of rats receiving KA and, subsequently, vehicle or trilostane ($p < .001$ for all comparisons). Similarly, in the Sub (Figure 4H), the number of NeuN-immunopositive cells was significantly reduced in vehicle-treated rats ($p < .001$) and in rats treated with a single ($p = .001$) or repeated injections of trilostane ($p < .001$), in comparison to controls. Moreover, there were no beneficial effects of trilostane in any of the examined brain regions.

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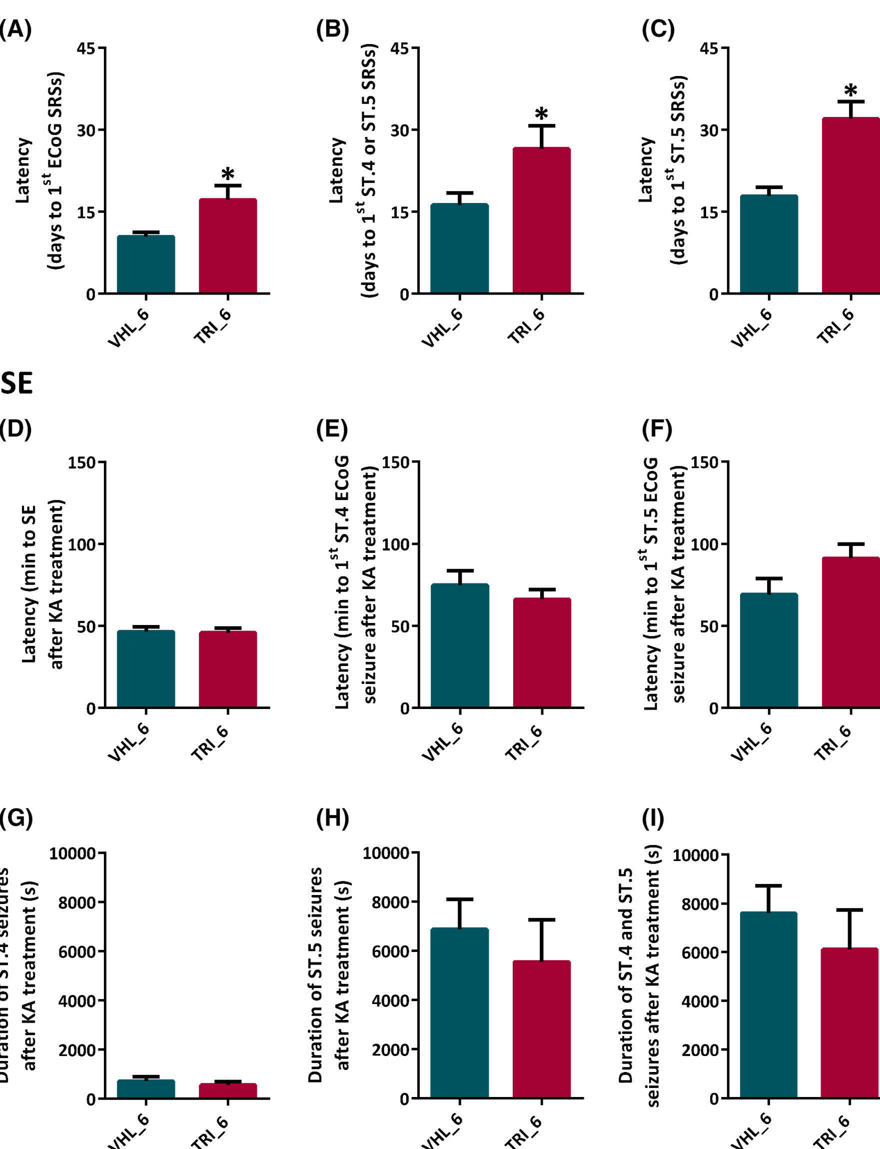

FIGURE 2 Time course of epileptogenesis and features of status epilepticus (SE) in rats undergoing a repeated administration of trilostane (50 mg/kg for 6 days) or vehicle. In comparison to the vehicle-treated group, the latency to develop the first electrocorticographic (ECoG) spontaneous seizure and then convulsive spontaneous recurrent seizures (SRSs) was significantly increased in the trilostane-treated group; trilostane was administered once daily for six consecutive days, starting 10 min after the intraperitoneal administration of kainic acid (KA; 15 mg/kg) to induce SE (A–C). No changes in the latencies to develop SE (D), or stage (ST.) 4 (E) or ST. 5 seizures (F) during SE were found. Similarly, no changes in the total duration (in seconds) of ST. 4 (G), ST. 5 (H), and a combination of ST. 4 and 5 seizures (I) were found during SE. Statistical analysis was performed using Student *t*-test. Results are shown as mean and SEM, and they are considered significant at $p < .05$. *$p < .05$. TRI_6, trilostane repeated injections; VHL_6, vehicle repeated injections.

3.3 | Trilostane did not affect the loss of astrocytes in the CA3 LMol and Sub

The impact of KA on astrocytes was characterized as a disappearance in the area of GFAP immunostaining. As expected, the GFAP immunostaining was consistent in healthy rats (Figure 5A), whereas GFAP-immunonegative areas were evident in all treatment groups where the rats were treated with KA (Figure 5B,C). The single or repeated administration of trilostane in rats did not significantly affect the damage in the examined brain regions (CA3 LMol: $F_{2,9} = .933$, $p = .428$; Sub: $F_{2,11} = .321$, $p = .732$).

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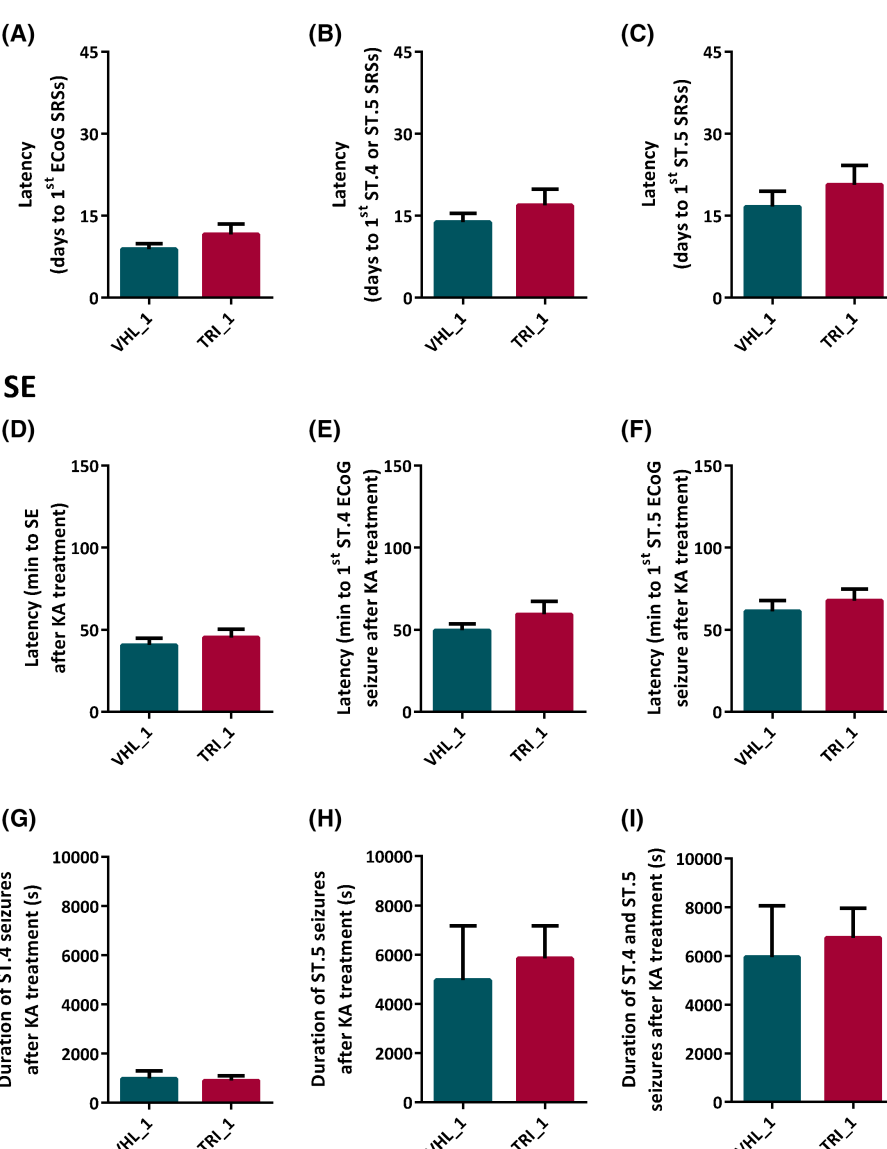

FIGURE 3 Time course of epileptogenesis and features of status epilepticus (SE) in rats undergoing a single administration of trilostane (50 mg/kg) or vehicle. In comparison to the vehicle-treated group, the latencies to develop the first electrocorticographic (ECoG) spontaneous seizure and then convulsive spontaneous recurrent seizures (SRSs) were unchanged in the trilostane-treated group when the injection was only 10 min after the intraperitoneal administration of kainic acid (KA; 15 mg/kg) to induce SE (A–C). No changes in the latencies to develop SE (D), or stage (ST.) 4 (E) or ST. 5 seizures (F) during SE were found. Similarly, no changes in the total duration (in seconds) of ST. 4 (G), ST. 5 (H), and a combination of ST. 4 and 5 seizures (I) were observed during SE. Statistical analysis was performed using Student *t*-test. Results are shown as mean and SEM, and they are considered significant at $p < .05$. TRI_1, trilostane single injection; VHL_1, vehicle single injection.

3.4 | Repeated administration of trilostane reduced the microglia-activated morphology in the Sub

When analyzed by a one-way ANOVA, a significant increase of Iba1 associated with the microglia-activated morphology was found in both the CA3 LMol ($F_{3,\ 13} = 30.767$, $p < .001$) and Sub ($F_{3,\ 14} = 112.570$, $p < .001$) of KA-treated rats when compared to healthy controls ($p < .001$, Holm–Šídák test; Figure 5D–F). Nevertheless, the microglia-activated morphology in the Sub was significantly attenuated in rats treated with multiple injections of trilostane ($p = .004$), in respect to vehicle-treated rats (Figure 5F).

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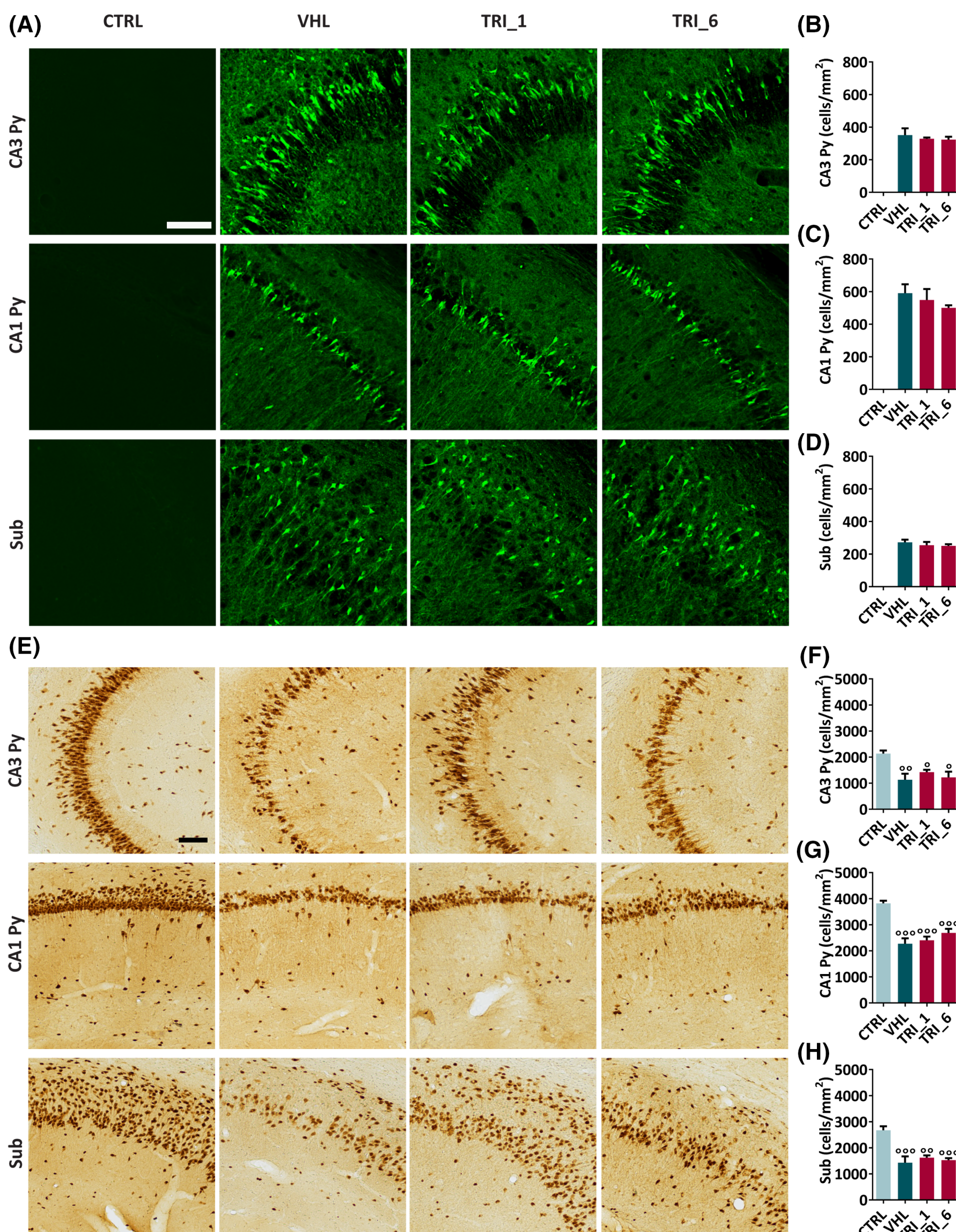

FIGURE 4 Effect of trilostane (50 mg/kg) or vehicle on neuronal cell death and survival in the cornu ammonis 3 stratum pyramidalis (CA3 Py, subregion B), cornu ammonis 1 stratum pyramidalis (CA1 Py), and subiculum (Sub) of rats after kainic acid-induced status epilepticus. Sections were stained for fluoro-jade B to evaluate neuronal cell death (A–D) or against mouse anti-neuron-specific nuclear protein to evaluate neuronal cell survival (E–H). Both a single and repeated injections of trilostane did not display neuroprotective effects, in comparison to treatment with the vehicle. Quantification was performed using ImageJ. Statistical analysis was performed using one-way analysis of variance and the Holm–Šídák test. Data are shown as mean and SEM. °$p < .05$, °°$p < .01$, °°°$p < .001$; Scale bars = 100 μm. CTRL, control; TRI_1, trilostane single injection; TRI_6, trilostane repeated injections; VHL, vehicle.

3.5 | Significant changes in neocortical and hippocampal neurosteroid levels were found in rats repeatedly treated with trilostane

In the fourth experiment, rats were treated daily with trilostane ($n = 10$) or vehicle ($n = 10$) for 6 days after the induction of SE, and compared to healthy controls ($n = 3$). Five animals per treatment group were sacrificed 6 h after the last injection. The remaining animals were sacrificed 1 week later. These animals were also monitored by ECoG and, consistently with the previous experiments, no significant effect of trilostane was evident for all the analyzed parameters, namely, the latency of SE development ($F_{3,\ 14} = 1.495$, $p = .259$, one-way ANOVA), the latency to the first ST. 4 seizure

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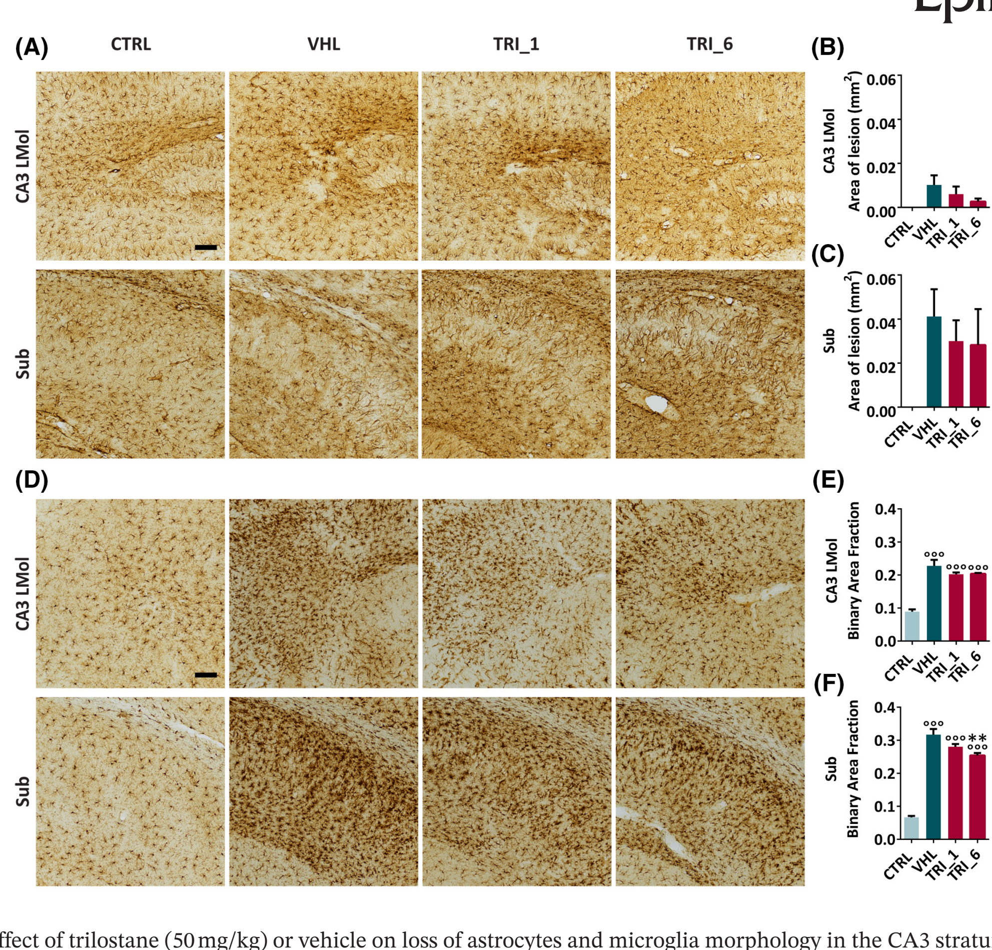

FIGURE 5 Effect of trilostane (50 mg/kg) or vehicle on loss of astrocytes and microglia morphology in the CA3 stratum lacunosum-moleculare (CA3 LMol) and subiculum (Sub) of rats after kainic acid-induced status epilepticus. Brain sections were stained against glial fibrillary acidic protein (A), a marker of astrocytes that was absent in the lesion occurring in the CA3 LMol (B) and Sub (C). The treatment with trilostane did not prevent the development of the lesion. Furthermore, brain sections were stained against rabbit anti-ionized calcium-binding adapter molecule 1 (D). In comparison to the healthy control group, the activated microglia morphology was significantly induced in the CA3 LMol and Sub after trilostane or vehicle administration. In Sub, a significant change was also observed in activated microglia morphology by comparing rats repeatedly treated with trilostane with those treated with the vehicle (E–F). Quantification was performed using the image analysis software NIS-Elements. Statistical analysis was performed using one-way analysis of variance followed by the Holm–Šídák test. Results are shown as mean and SEM, and they are considered significant at $p < .05$. Scale bars = 100 µm. $^{**}p < .01$, vehicle (VHL) versus trilostane repeated injections (TRI_6); $^{°°°}p < .001$ versus control (CTRL). TRI_1, trilostane single injection.

($F_{3,\,11} = 3.574$, $p = .050$), and the latency to the first ST. 5 seizure ($F_{3,\,12} = 2.968$, $p = .075$). Similarly, no significant effects were observed for the total duration of ST. 4 ($F_{3,\,14} = .562$, $p = .649$), ST. 5 ($F_{3,\,14} = .577$, $p = .639$), and both ST. 4 and ST. 5 seizures during SE ($F_{3,\,14} = .591$, $p = .631$; data not shown).

In these animals, we found a statistically significant increase of the neocortical (Figure 6A–F) levels of pregnenolone ($F_{4,\,17} = 132.779$, $p < .001$, one-way ANOVA), pregnenolone sulfate ($F_{4,\,16} = 17.544$, $p < .001$), progesterone ($F_{4,\,15} = 53.841$, $p < .001$), 5α-dihydroprogesterone ($F_{4,\,17} = 50.051$, $p < .001$), and allopregnanolone ($F_{4,\,17} = 24.087$, $p < .001$). In particular, this change depended on the effects of trilostane, assessed 6 h after the last injection, when compared to all the other treatment groups ($p < .001$, Holm–Šídák test; Figure 6A,B,D–F). Also, neocortical levels of pregnanolone were affected by trilostane ($F_{4,\,14} = 3.257$, $p = .044$), but in this case we found a remarkable reduction in the amount (Figure 6C).

Trilostane also produced significant changes in the hippocampus (Figure 6G–L) by increasing pregnenolone ($F_{4,16} = 4.781$, $p = .010$), progesterone ($F_{4,\,16} = 13.408$, $p < .001$), 5α-dihydroprogesterone ($F_{4,\,16} = 30.712$, $p < .001$), and allopregnanolone ($F_{4,\,15} = 5.122$, $p = .008$) levels. Post hoc comparisons showed that pregnenolone increased after daily trilostane treatment, in respect to the vehicle-treated group with the same dosage interval ($p = .048$), or the vehicle-treated group after 1 week of washout ($p = .019$), and went back to basal levels 1 week after the last trilostane injection ($p = .034$ vs. 6 days of trilostane treatment; Figure 6G). Progesterone levels significantly increased after trilostane administration ($p < .001$ vs. all the other groups) but returned to normal after the washout ($p = .002$ vs. 6 days of trilostane treatment; Figure 6J). Six hours after

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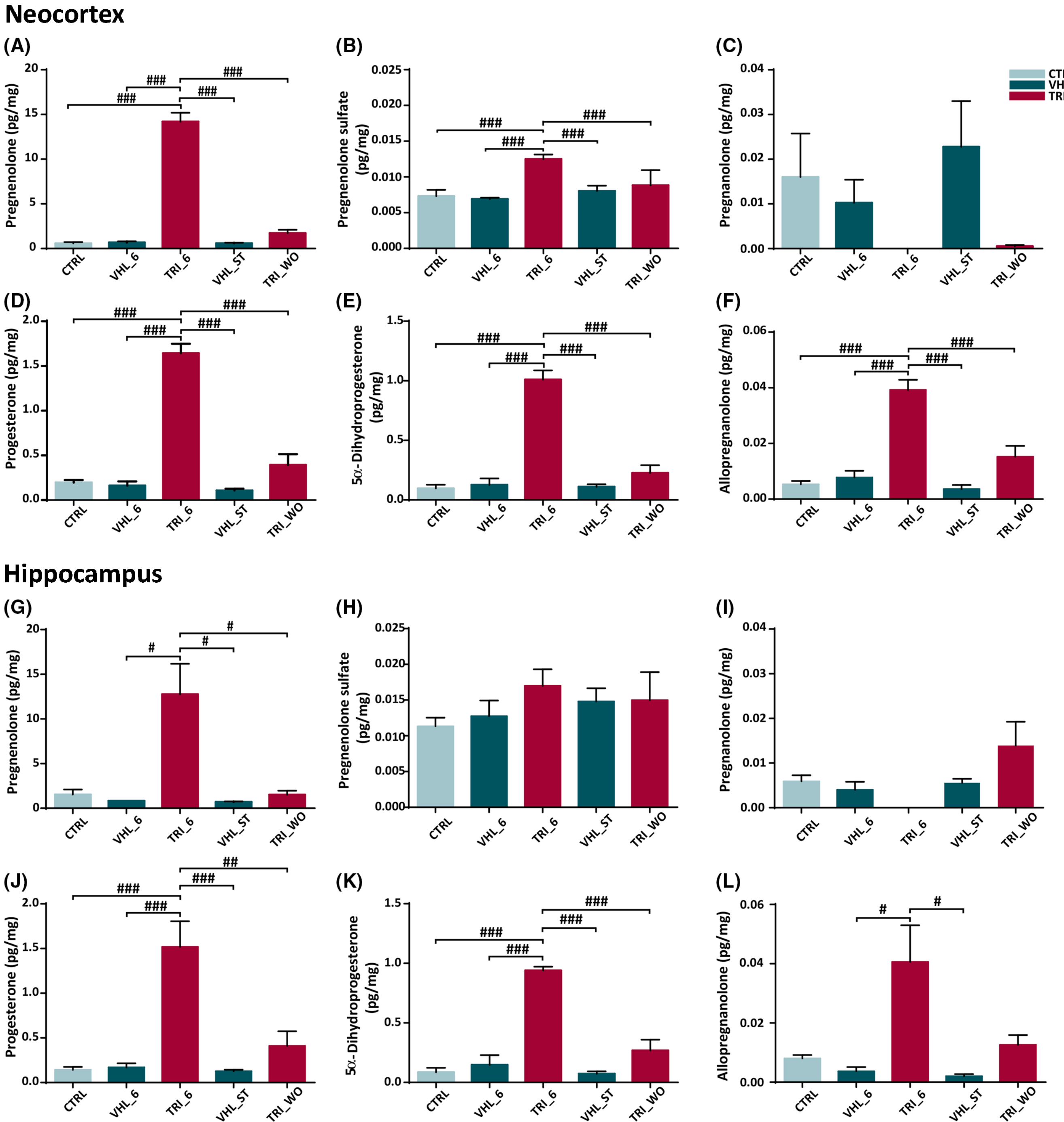

FIGURE 6 Effect of trilostane (50 mg/kg) or vehicle treatment on neurosteroid brain levels. The repeated daily injection of trilostane determined a significant augmentation of almost all of the studied neurosteroids when compared to the other treated groups (A, B, D–G, J–L). Only pregnenolone sulfate (H) did not increase its levels in the hippocampus, whereas pregnanolone was not detectable 6 h after the last injection of trilostane in both the hippocampus and neocortex (C, I). Statistical analysis was performed using one-way analysis of variance followed by the Holm–Šídák test. Data are shown as mean and SEM, and they are considered significant at $p < .05$. $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$, trilostane repeated injections (TRI_6) versus other groups. CTRL, control; TRI_WO, trilostane washout; VHL_6, vehicle repeated injections; VHL_ST, sham-treated vehicle.

the last injection of trilostane, 5α-dihydroprogesterone ($p < .001$ vs. all the other groups; Figure 6K) as well as allopregnanolone ($p = .035$ vs. the vehicle-treated group at the same time, $p = .015$ vs. the sham-treated vehicle) were also markedly increased (Figure 6L). In the hippocampus, trilostane did not affect the levels of pregnenolone sulfate ($F_{4,\ 17} = .552$, $p = .700$) or pregnanolone ($F_{4,\ 15} = 2.551$, $p = .082$; Figure 6H,I). Pregnanolone was not detectable

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6 h after the last injection of trilostane, but returned to normal after the washout (Figure 6I).

4 | DISCUSSION

This study was aimed at assessing the effects of multiple trilostane injections posttreatment on SE dynamics, brain damage, neocortical and hippocampal neurosteroid levels, and duration of epileptogenesis as evaluated by the time interval that preceded the appearance of SRSs. The main outcomes of our study were the (1) lack of trilostane effects on SE dynamics and damage to the hippocampus; (2) remarkable increase in tissue levels of various neurosteroids, with the notable exception of pregnanolone, whose synthesis was suppressed; and (3) consistent delay in the appearance of both electrographic and motor seizures in rats repeatedly treated with trilostane, but not in those receiving a single injection. Overall, these findings support a modulatory effect of trilostane on epileptogenesis.

We did not observe any effect of trilostane administration on the investigated features of SE induced by KA. This finding was at odds with our previously reported result, in which KA-treated rats that received two trilostane injections before the SE induction presented an anticipated disappearance of convulsive seizures.[21] The difference between the present and previous studies suggests that trilostane could not be effective in counteracting the seizures when administered after SE onset. Other investigators reported that allopregnanolone or its analogue ganaxolone were effective in terminating SE induced by tetramethylenedisulfotetramine[30] or lithium–pilocarpine,[31] even when administered after SE induction. This suggests that trilostane is probably unsuitable for the acute treatment of seizures and SE. A single trilostane injection during SE, not followed by other administrations, was also unable to modify the time course of epileptogenesis, thus suggesting that trilostane should be repeatedly administered to be effective.

Another important point was the lack of trilostane effects on brain damage, with a limited but significant reduction in microglia reactivity in the subiculum. This was surprising in view of the multiple mechanisms involved in neuroprotection, which could be activated by progesterone, 5α-dihydroprogesterone, and allopregnanolone, which involve both membrane and intracellular progesterone receptors, and also $GABA_A$ receptors.[32] It is well known that the potentiation of the $GABA_A$ receptor by diazepam[33] can produce protective effects in the pilocarpine model of SE.[34,35] Conversely, diazepam administered 3 h after the onset of SE in KA-treated rats did not afford neuroprotection in the hippocampus, but only in some extrahippocampal regions.[36] This result was still associated with the successful termination of SE by diazepam, whereas trilostane was not effective in modifying the course of SE in our animals. For this reason, we believe that the absence of neuroprotective effects exerted by trilostane could be due to its inefficacy in modulating SE dynamics.

Despite the lack of effects in controlling seizures during SE, and in producing neuroprotective effects, the repeated administration of trilostane consistently modified the time course of epileptogenesis. This apparently surprising result is consistent with the observation that finasteride could anticipate the appearance of SRSs in pilocarpine-treated rats, as reported by two independent groups.[7,37] Finasteride is known to reduce the availability of allopregnanolone in the brain,[38] so as to exert an effect opposed to that produced by trilostane. Thus, we interpreted the delayed epileptogenesis observed in rats treated with trilostane as the consequence of increased allopregnanolone availability; however, only indirect evidence supporting this hypothesis is found in the literature. Specifically, progesterone administration in mice was followed by a remarkable delay in the progression of epileptogenesis induced by kindling, an effect completely prevented by administering finasteride.[39] Interestingly, other investigators reported that allopregnanolone administered for 12 days to pilocarpine-treated rats after SE was able to reduce the occurrence of ripples and interictal spikes,[40] but these authors did not systematically investigate the allopregnanolone effects on epileptogenesis. For these reasons, the leading role of allopregnanolone in the modulation of epileptogenesis remains to be fully demonstrated.

We tried to clarify the role of allopregnanolone by evaluating its hippocampal and neocortical levels, along with the levels of other allopregnanolone-related neurosteroids, in rats at the end of the period of trilostane administration and after a week of drug washout. The remarkable 12-fold increase in allopregnanolone hippocampal levels found in the trilostane group compared to vehicle-treated rats supports a major role for this neurosteroid in the effects observed for epileptogenesis; however, changes similar to those observed for allopregnanolone were also found for pregnenolone, progesterone, and 5α-dihydroprogesterone, all neurosteroids whose levels were probably not affected by the drug finasteride in the mentioned previous experiments. Unfortunately, we could not include a treatment group with finasteride to block the effects of trilostane, because finasteride also modifies testosterone metabolism, with obvious consequences on levels of the other neurosteroids[41] such as dihydrotestosterone, and the respectively targeted receptors. No effects of pregnenolone and 5α-dihydroprogesterone on $GABA_A$ receptors have been reported until now, and progesterone effects are known to be mediated by its conversion to allopregnanolone.[42]

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Trilostane could produce additional metabolic effects, because this drug was also reported to inhibit 11β-hydroxysteroid dehydrogenase in dogs, so as to impair the inactivation of cortisol, a hormone that is not produced in rats.[22] Unexpectedly, we found a complete inhibition of pregnanolone production, which was recovered after the trilostane washout, suggesting an inhibitory effect of trilostane on 5β-reductase. This activity is indirectly suggested by the finding that allopregnanolone, which shares the metabolic activity of 3β-hydroxysteroid dehydrogenase with pregnanolone,[43] increased greatly. In view of this finding, a question could be raised on the overall effect of trilostane on the balance of anticonvulsive neurosteroids produced in the brain of rats with epilepsy, which include pregnanolone and tetrahydrodeoxycorticosterone; however, the reduction in pregnanolone levels was more than compensated for by the massive increase in allopregnanolone production observed in the hippocampus. The main role of allopregnanolone was also indicated by the absence of changes in the levels of the proconvulsant agent pregnenolone sulfate in the hippocampus of trilostane-treated rats, as well as by the limited increase of this neurosteroid observed in the neocortex. The change in pregnenolone sulfate neocortical levels was not so large as to possibly overcome the effects of allopregnanolone, whose levels were 52-fold higher than those found in the vehicle group.

We could not exclude a possible contribution of other hormones involved in the adrenal cortex and/or gonadal regulation to the findings of our experiments. By blocking 3β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$ isomerase to reduce steroidal production in the adrenal cortex, adrenocorticotropic hormone (ACTH) would be expected to increase.[44] ACTH notoriously affects seizures, but its activity is thought to be dependent on the synthesis of deoxycorticosterone by the adrenal gland,[45] which was impaired by trilostane in our rats. Similarly, a role for corticotropin-releasing hormone could also be considered, but this neuropeptide is a proconvulsant[46] and, to the best of our knowledge, could have opposed the delay in epileptogenesis found in trilostane-treated rats. Also, the gonadal axis[47] would need to be considered to interpret our findings. Testosterone levels were reported to be reduced by trilostane in rats.[48] The effects of testosterone on seizures were investigated in both the pilocarpine and KA models of SE, with the authors reporting[49] increased seizure occurrences in animals that received testosterone replacement after castration; however, these changes were observed only in the course of KA-induced SE, and no study has yet to establish a role for testosterone in epileptogenesis.

The KA model of temporal lobe epilepsy has been suggested to be suitable for the screening of putative disease-modifying/antiepileptogenic agents. In this regard, it was shown that oral administration of everolimus (2–3 mg/kg) or an intraperitoneal injection of phenobarbital (60 mg/kg), at several time points after SE onset, were not able to prevent the development of SRS.[50] On the other hand, our results suggest that trilostane displays potential as an antiepileptogenic drug, possibly by increasing allopregnanolone levels in the brain. Our study was based on a relatively short-term period of administration, so we cannot exclude that a longer period of treatment may result in more beneficial effects. It is nonetheless interesting to note that a delay in the onset of convulsive seizures occurred following the drug washout and the return of neurosteroid levels to the baseline, suggesting that these agents produced a change in the development of epileptogenesis rather than having exerted a transient antiseizure effect.

AUTHOR CONTRIBUTIONS

Concept and design of the study: Anna Maria Costa, Chiara Lucchi, Giuseppe Biagini. 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.

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