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Research report

The neurosteroid 3α,5α-THP has antiseizure and possible neuroprotective effects in an animal model of epilepsy

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Abstract

Some anticonvulsant drugs may suppress seizures by enhancing activity of GABAergic systems. Progesterone (P)'s anti-convulsant and neuroprotective effects may be due to the steroid's actions on $GABA_A$-benzodiazepine receptor complexes (GBRs) rather than intracellular progestin receptors (PRs), as many P metabolites have a greater effect in vitro on benzodiazepine binding and Cl^- flux than P, but poor affinity for PRs. If P's actions are due to metabolism to a progestin more potent at GBRs, then systemic administration of one of those P metabolites should also prevent CNS damage. To test this hypothesis male rats were implanted with a bipolar electrode, aimed above the perforant pathway. Experimental animals received the 5α-reduced P metabolite most effective at GBRs, 5α-pregnan-3α-ol-20-one (3α,5α-THP) 2.5 mg/kg s.c., 3 h prior to perforant pathway stimulation, while control animals received sesame oil vehicle. The duration of chewing and drooling, and the incidence of wet dog shakes, partial and full seizures were reduced during perforant pathway stimulation in animals pre-treated with 3α,5α-THP compared to vehicle. Two weeks later, animals pre-treated with 3α,5α-THP had shorter latencies and distances to find a hidden platform in a Morris Water maze task. 3α,5α-THP pre-treatment also reduced damage to CA1 and CA3 layers of the hippocampus and preserved the number of neurons in the hilar region. These data indicate that the neurosteroid metabolite of P, 3α,5α-THP, can have anticonvulsant and may have neuroprotective effects in an animal model of epilepsy. Further, these data suggest that the mechanism of P's protective and anticonvulsant effects may be via GBRs rather than PRs.

Keywords: Extra-genomic; γ-Aminobutyric acid; Steroid hormone; Membrane; Hippocampus; Perforant pathway; Progesterone

1. Introduction

Findings from animal and human studies indicate gonadal steroids influence seizure processes. Estrogen (E_2) has seizure-activating effects: intraperitoneal [44] and intravenous [34] E_2 can instigate or increase firing of a seizure focus [39], lower the maximum electroshock threshold [64] and shorten the stimulation to kindling threshold in amygdala and pentylenetetrazol models [30]. Estrogen administration to women with catamenial epilepsy activates EEG abnormalities [40]. Progesterone (P) counters E_2's proconvulsant effects: P increases kindling [28] and maximum electroshock threshold [69]. In women, there is a negative correlation between seizure incidence and circulating plasma P [3,4].

In addition to anticonvulsant effects, P may also have neuroprotective properties. For example, females recover better than do males following craniotomy [55], cortical contusions [56], longitudinal incisions of the parietal cerebral cortex [21], and crush injuries to the facial nerve [33]. Pseudopregnant females with frontal aspiration lesions exhibit less behavioral impairment [1] than proestrus females with relatively suppresed P output. Pseudopregnant and P-administered females also have less cerebral edema and impairment on a Morris water maze spatial task than male rats following cortical contusions [55]. In vivo P decreases astrocytes in the vicinity of a pentrating brain injury [21] and in vitro P decreases the extent of neuronal damage produced by glutamate exposure in cultured spinal cord neurons [49].

It has been proposed that P or its metabolites' ability to rapidly alter neuronal firing [62] occurs through interactions with $GABA_A$-benzodiazepine receptor complexes (GBRs) [22,26,43]. Progestins' actions on GBRs explain the rapid analgesic [17,18], anxiolytic [7], anticonvulsant [6] and anesthetic actions of steroids. Progesterone's ef-

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fects at GBRs may also underlie P's anticonvulsant and neuroprotective effects. However, these actions are likely not direct, as P itself only has weak effects at GBRs. Many progestins, particularly 5α-pregnan-3α-ol-20-one (3α,5α-THP) are more effective than P itself at displacing TBPS binding [22,26,43], enhancing flunitrazepam binding [43] and GABA or muscimol stimulated Cl^- ion flux [5,46,47]. 3α,5α-THP has a very low affinity for PRs [13,32,53]. To test whether this naturally occurring P metabolite, more effective at GBRs than P itself, may prevent seizures and CNS damage, 3α,5α-THP was given systemically prior to stimulation of the perforant pathway.

2. Materials and methods

2.1. Subjects and housing

Adult, male Sprague–Dawley rats (approximately 400 g; $n = 10$) were obtained from Charles River Laboratory, Wilmington, MA and singly housed in suspended, polycarbonate cages in a temperature- (72 ± 2°F) and humidity-controlled (30–40%) room. The light cycle was 12-h light/dark, with lights on at 07.00 h. Rodent chow and water were available in animals' cages.

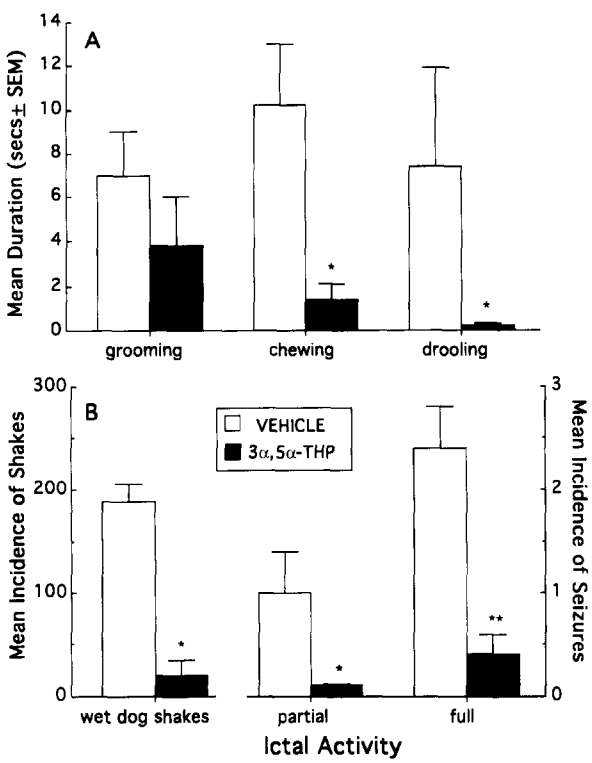

Fig. 1. Top panel A depicts mean duration (seconds ± standard error of the mean (S.E.M.)) of grooming, chewing and drooling that occurred during 60 min of perforant pathway stimulation. 2.5 mg/kg 5α-pregnan-3α-ol-20-one (3α,5α-THP), 3 h prior to stimulation (blacks bars) significantly reduced ($P < 0.05$) duration of chewing and drooling compared to sesame oil vehicle treated animals (open bars). Bottom panel B depicts mean incidence (± standard error of the mean (S.E.M.)) of wet dog shakes, partial and full seizures that occurred during 60 min of perforant pathway stimulation. Pre-treatment prior to stimulation with 3α,5α-THP (blacks bars) significantly reduced ($P < 0.05$) the number of wet dog shakes and partial seizures that occurred during perforant pathway stimulation compared to vehicle pretreated animals (open bars). * * There was also a tendancy for 3α,5α-THP pre-treatment to decrease the number of full seizures ($P < 0.08$).

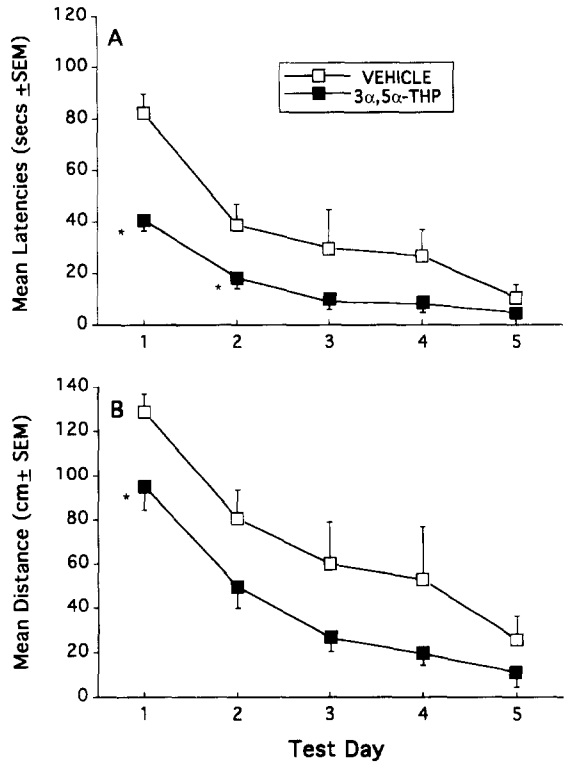

Fig. 2. Top panel A depicts mean latencies (secs ± standard error of the mean (S.E.M.)) for rats pretreated with 5α-pregnan-3α-ol-20-one (3α,5α-THP, filled squares) or vehicle (open squares) prior to perforant pathway stimulation to reach the hidden platform in the Morris water maze. Overall 3α,5α-THP pretreatment reduced latencies compared to vehicle control animals, this was particularly evident on Days 1 and 2. Bottom panel B depicts mean distances (cms ± standard error of the mean (S.E.M.)) for rats pretreated with 3α,5α-THP or vehicle. Overall 3α,5α-THP pretreatment reduced distances compared to vehicle control animals, this was particularly evident on Day 1.

2.2. Surgery

Surgical implantation of bipolar electrodes was conducted with subjects under sodium pentobarbital anesthesia (50 mg/kg, i.p. or to effect), supplemented by ethyl ether as necessary. Rats were placed in a Kopf small animal stereotaxic with the tooth bar 3.3 mm below the intraural line to stabilize the head in a horizontal position. A bipolar electrode, cut to approximately 5 mm in length, with seperated tips, was implanted unilaterally in the perforant pathway (coordinates from bregma: AP = −7.5, ML = ±4.4, DV = −4.0) [51]. The electrode was secured to the skull with small screws and dental cement.

2.3. Stimulation

One week after implantation, rats were randomly assigned to ($n = 5$) receive sesame oil vehicle (s.c., 0.2 ml) 3

h prior to stimulation or ($n = 5$) 2.5 mg/kg 3α,5α-THP (Sigma Chemical Corp; St. Louis, MO). For perforant pathway stimulation, all rats were placed in a stimulation chamber, with the implanted electrode connected to a Grass stimulator. Rats received 1 h of constant stimulation with biphasic pulses having a stimulus duration of 0.1 ms per phase, at 20 Hz with 12.5 V [59,70]. The occurrence and duration of chewing, drooling, wet dog shakes and partial or full seizures were recorded throughout the stimulation period. Partial seizures were defined as jerky tonic motor behaviors, e.g., head and ear twitches, eye blinking, isolated limb seizing and motionless staring, while full seizures were characterized by rearing on hind legs and falling over.

2.4. Behavioral testing

One week after stimulation, all rats were tested in a version of the Morris Water Tank (200 cm in diameter and 71 cm deep) to assess reference memory [11,54]. The tank was filled with 20–22°C water 36 cm deep. The water was made opaque by adding powdered milk, which enabled the white, wire mesh platform (5.3 × 5.3 × 33.5 cm) to be concealed approximately 2.5 cm below the surface of the water and 30 cm from the side of the tank. There were many constant extramaze visual cues in the room. A video camera, which was situated above the water tank, was connected to a tracking system (Multitracker, San Diego Instruments) which measured each rat's latency and distance to the platform.

Rats were tested in the water maze for 6 trials on 5 consecutive days. Each trial was initiated at one of 3 starting positions, located 43, 198 or 253° clockwise from the platform's location, as assigned randomly by the tracking program. Rats were placed in the water and the experimenter initiated the tracking system. If a rat failed to reach the platform within 120 s it was guided to the platform by the experimenter. The rat remained on the platform for 45 s to orient itself to visual cues. After completing a trial, rats were returned to their cage for a 3-min intertrial period, until six trials were completed.

Following 5 days of behavioral testing, animals were

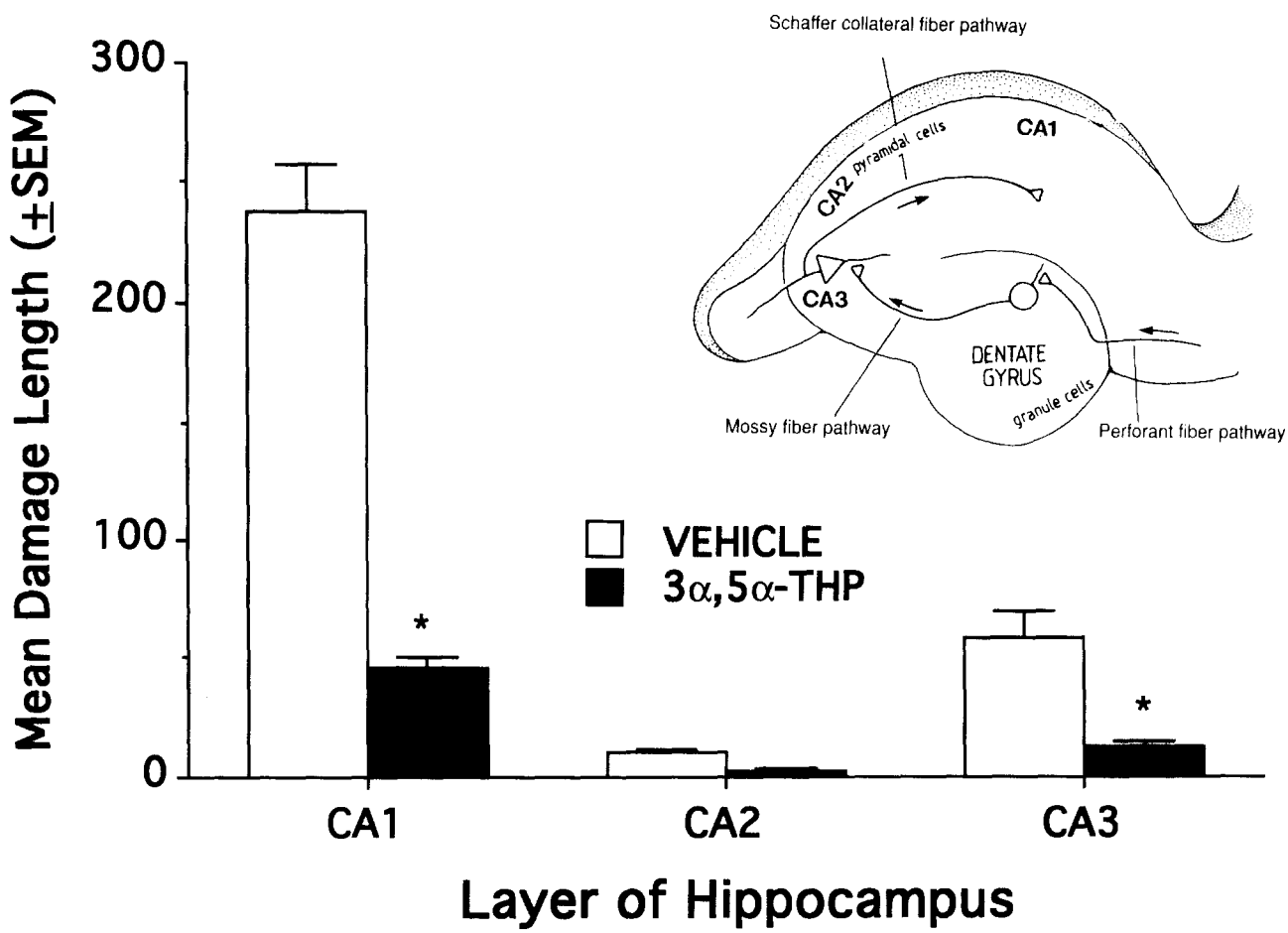

Fig. 3. Mean damage length (±standard error of the mean (S.E.M.)) of CA1, CA2 and CA3 for rats pretreated with 5α-pregnan-3α-ol-20-one (3α,5α-THP, black bars) or vehicle (open bars) prior to perforant pathway stimulation. 3α,5α-THP pretreatment reduced damage length of CA1 and CA3. The inset schematic indicates the areas considered CA1, CA2, CA3 for quantification of damage length. Cytoarchitechtonically, CA1 was identified as the area of smaller-sized pyramidal cells and greater pyramidal cell density. CA3 was considered the layer which began parallel to the dentate gyrus. CA2 was defined as the area between CA1 and CA3.

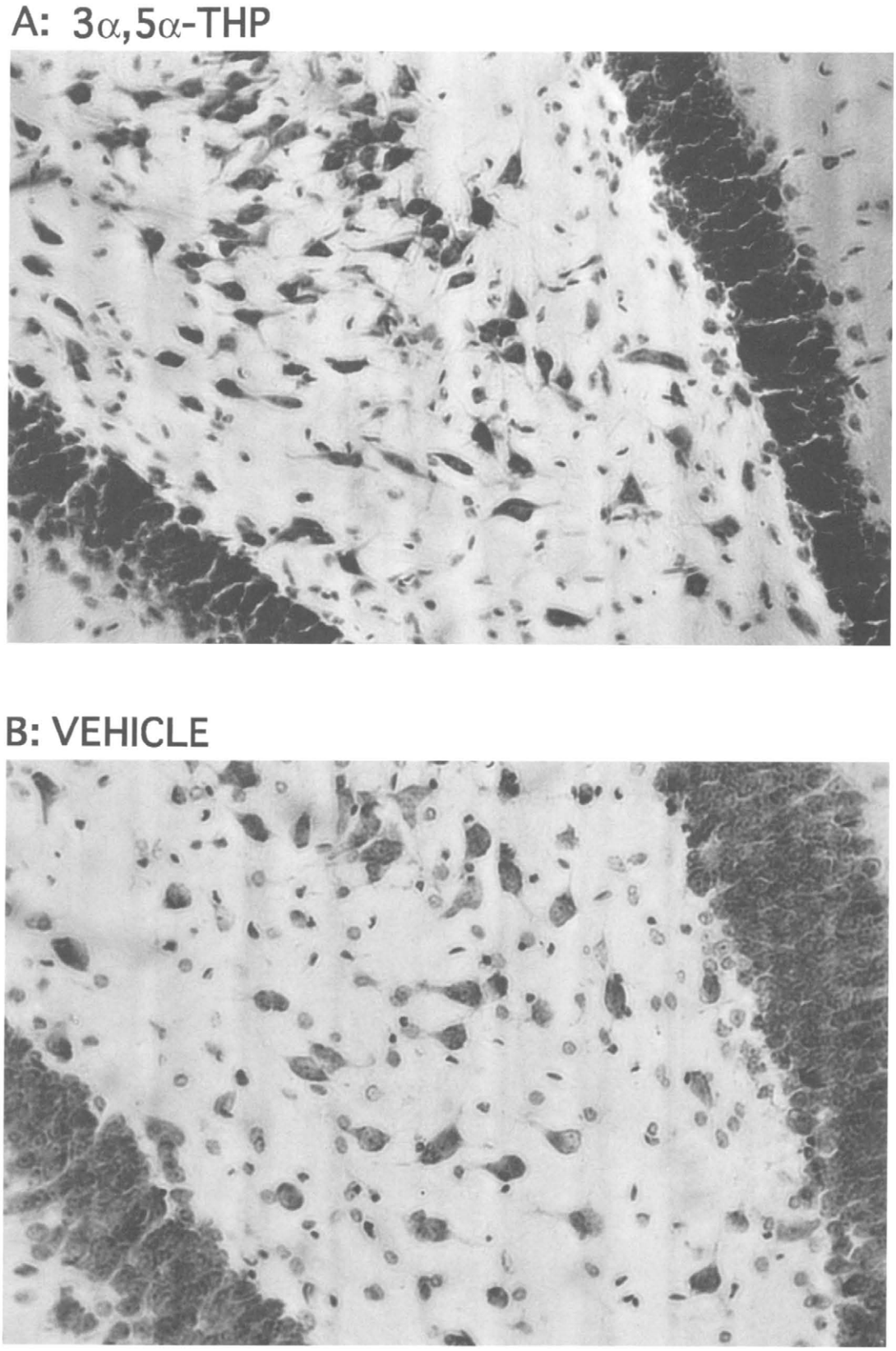

Fig. 4. Representative photomicrographs of a section of the middle most portion of the hilar region of an animal pretreated with 5α-pregnan-3α-ol-20-one (3α,5α-THP, top panel, A) or vehicle (bottom panel, B) prior to perforant pathway stimulation. 3α,5α-THP pretreatment prevented neuronal loss in the hilar region.

administered an overdose of sodium pentobarbital (150 mg/kg i.p. or to effect), and perfused intracardially with 0.9% saline followed by 10% formalin. The frozen brains were sliced in 20 μm coronal sections and stained with Cresyl violet.

2.5. Histological analysis

Histological analyses were performed by an observer, uninformed of the experimental condition of each animal, using Jandel JAVA software to measure damage length of CA1, CA2 and CA3 pyramidal layers under 4 × magnification [54,59]. CA1 was identified as the area of smaller-sized pyramidal cells and greater pyramidal cell density [41]. CA3 was considered the layer which began parallel to the dentate gyrus. CA2 was defined as the area between CA1 and CA3. To assess the hilar region, neurons were counted from a single photomicrograph for each subject, taken at 10 ×, from the middle most extent of the hilus.

2.6. Statistical analysis

Multiple one-way analysis of variance (ANOVA) examined behavioral differences between vehicle control and 3α,5α-THP pre-treated rats during stimulation. Separate two-way ANOVAs with repeated measures were used to determine if latency, distance, or hippocampal damage varied with 3α,5α-THP or vehicle pre-treatment. One-way ANOVA examined differences in number of neurons in the hilar region. ANOVAs were followed by Newman–Keuls post-hoc tests; significant differences at the $P \leq 0.05$ level are reported.

3. Results

3.1. Ictal activity

Pretreatment with 3α,5α-THP significantly decreased ictal activity during perforant pathway stimulation. As Fig. 1A illustrates, 3α,5α-THP animals chewed ($F_{1,8} = 8.80$, $P < 0.01$) and drooled ($F_{1,8} = 3.55$, $P < 0.05$) significantly less than vehicle animals. Animals that received 3α,5α-THP also had fewer wet dog shakes ($F_{1,8} = 56.34$, $P < 0.001$), fewer partial seizures ($F_{1,8} = 5.00$, $P < 0.05$) and a tendancy towards fewer full seizure ($F_{1,8} = 4.34$, $P < 0.07$) (see Fig. 1B).

3.2. Water maze performance

Pretreatment with 3α,5α-THP attenuated deficits in reference memory. There were main effects of 3α,5α-THP pre-treatment ($F_{1,32} = 6.76$, $P < 0.05$) and day of testing ($F_{4,32} = 31.26$, $P < 0.05$), as well as an interaction between these variables ($F_{4,32} = 3.03$, $P < 0.05$) on latency to the hidden platform. As Fig. 2A illustrates, 3α,5α-THP animals had shorter latencies overall, particularly on Days 1 and 2. There was also a tendancy for a main effect of 3α,5α-THP pre-treatment ($F_{1,32} = 3.79$, $P < 0.08$) and a main effect of day of testing ($F_{4,32} = 35.46$, $P < 0.05$) on distance to the platform. 3α,5α-THP animals had shorter distances overall, particularly on Day 1 (Fig. 2B).

3.3. Hippocampal morphology

3α,5α-THP also attenuated cell loss in the hippocampus. There were main effects of 3α,5α-THP pre-treatment ($F_{1,24} = 57.86$, $P < 0.001$) and pyramidal cell layer ($F_{3,24} = 86.91$, $P < 0.001$), and an interaction between these variables ($F_{3,24} = 49.01$, $P < 0.001$) on damage length. As Fig. 3 illustrates, 3α,5α-THP animals had significantly less damage to CA1 and CA3 than vehicle animals.

3α,5α-THP pre-treatment also preserved the number of neurons in the hilar region ($F_{1,8} = 10.95$, $P < 0.01$). There was an average of 21 ± 1.4 neurons identified for 3α,5α-THP pretreated animals compared to 9.6 ± 3.1 neurons in the hilar region for vehicle-treated animals (see Fig. 4).

4. Discussion

Pretreatment with the P metabolite, 3α,5α-THP effectively prevented ictal activity during perforant pathway stimulation, as well as subsequent deficits in reference memory and hippocampal morphology. It has been shown previously that P, as well as other GABAergic agents, has anti-convulsant properties and can prevent neuronal loss. That 3α,5α-THP also has these effects suggests a GBR-mediated mechanism of action, because 3α,5α-THP is a potent modulator of GBRs but lacks a high affinity for intracellular PRs. Consistent with this, P's previously reported anti-convulsant and neuroprotective effects may be due, in part, to P's metabolism and subsequent effects at GBRs.

Several lines of evidence suggest that pregnane steroids produce their anticonvulsant effects through specific and stereoselective actions at GBRs [6,48,61]. That 3α,5α-THP is responsible for the anti-convulsant and neuroprotective effects of P is strongly supported by the finding that local application of 3α,5α-THP, but not P, produced immediate potentiating effects on GABA-mediated inhibition [62,63]. Within 40–80 s after local continuous application of 3α,5α-THP to Purkinjie cells, 121% enhancement of GABA inhibition is noted. In contrast, P enhances GABA inhibition less (83%) only after a 9 minute latency, which is sufficient time for P's conversion to 3α,5α-THP. Administration of 4MA, a 5α-reductase inhibitor, prevents the potentiating effect of P on GABA inhibition [60]. Similarly, IV 3α,5α-THP immediately and more potently (20 fold) increases threshold to focal eplileptic seizures in a cerveaux-isole preparation than P, effects that are delayed about 20 min, again sufficient time for conversion to

3α,5α-THP [65]. Within seconds of intraarterial 3α,5α-THP, there is a marked depression in interictal epileptiform activity [37]; such rapid effects preclude 3α,5α-THP's action on intracellular PRs and suggest direct effects on neuronal membranes. Longer-term treatment with P's increase in [3H]muscimol binding in CNS areas without PRs [9,10,57] also suggests that P's effects may be due to metabolism and subsequent actions at GBRs.

The present data support the notion that 3α,5α-THP has anti-convulsant effects in the perforant pathway stimulation model. Whether the decreased hippocampal damage presently seen were solely due to the preventive effects of 3α,5α-THP on seizures or additional neuroprotective effects of 3α,5α-THP remains to be seen. Neuroprotective effects of in vivo 3α,5α-THP have not been reported previously, but there are separate accounts of activational effects of gonadal and neurosteroids on spatial memory [16,19], hippocampal morphology [8,67,68] and physiology [15,35,66]. Peaks in the density of dendritic spines in CA1 hippocampal pyramidal cells [67,68] positively correlate with independently measured deficits in acquisition of spatial task during behavioral estrus [16], when P and 3α,5α-THP levels are low [29,31,50]. Although the direction of 3α,5α-THP's effects on memory are task- and dose-dependent, 3α,5α-THP can enhance acquisition of memory task [19] and decrease the area and length of neurites [8]. Together with the present findings this suggests 3α,5α-THP may protect neurons in the hippocampal complex, particulary cells in pyramidal cell layers CA1 and CA3, where reductions in neurons are found in human eplileptic patients [12].

Although these data are consistent with 3α,5α-THP's effects in this animal model of epilepsy being a consequence of the neurosteroids' acting directly at GBRs or indirectly via glycine or glutamate mediated actions [14] but not at PRs, there are other, as of yet uninvestigated, possible modes of action. Like P, 3α,5α-THP may have its antiseizure or putative neuroprotective effects by any of the following mechanisms. (1) In vitro P decreases oxygen utilization by neurons [45]. As seizure activity depends on increases in oxygen and glucose [23,36,42], P's or 3α,5α-THP's anticonvulsant activity may occur by depressing CNS oxygen and glucose. (2) Local cortical application of P inhibits electrical discharge from a penicillin focus [38], suggesting P or 3α,5α-THP may act directly on GBR neurons in the cortex to suppress epileptiform discharges [2]. (3) Progesterone, and possibly 3α,5α-THP, may potentiate the effects of endogenous anticonvulsant such as adenosine [52]. (4) Steroid hormones are degraded by the same hepatic microsomal enzyme system as are many antiseizure medications [58]; thus, in women, P or 3α,5α-THP may compete for sites of hepatic inactivation. (5) P, and possibly 3α,5α-THP, can alter membrane stabilization [25], which may limit tissue destruction by reducing free radical related membrane peroxidation [24].

Despite the numerous possible mechanisms of P and 3α,5α-THP, the present findings indicate 3α,5α-THP has anticonvulsant and may have neuroprotective effects in this animal model of epilepsy. Thus, further investigation of 3α,5α-THP's mechanism is warranted. These findings support clinical and animal studies that reveal P's anticonvulsant and neuroprotective properties and extend them by demonstrating that 3α,5α-THP can have similar effects, which suggests P's effects may be due to conversion to 3α,5α-THP. Interestingly, epileptic women who abruptly discontinue P therapy experience their usual premenstrual exacerbation of seizures, but this effect can be eliminated or reduced by gradually tapering P over 3 or 4 days [27]. Withdrawal from chronically administered P is blocked by prior exposure to indomethacin [20], which prevents P's conversion to 3α,5α-THP; which further this suggests eplileptiform activity and sequelae may be a consequence of withdrawal from 3α,5α-THP, rather than P itself.

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