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Intrathecal neurosteroids and a neurosteroid antagonist: Effects on inflammation-evoked thermal hyperalgesia and tactile allodynia☆

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HIGHLIGHTS

- Spinal allopregnanolone and alphaxalone increase thermal threshold in normal and inflamed paw.
- Effects of allopregnanolone were prevented by neurosteroid antagonist 17PA.
- Reversal of an equi-analgesic dose of alphaxalone occurred only at higher antagonist dosing.
- Spinal neurosteroid binding site(s) acted upon by 17PA regulates spinal pain processing

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ABSTRACT

Neurosteroids regulate neuronal excitability though binding sites associated with the ionotropic γ-aminobutyric acid ($GABA_A$) receptor. We sought to characterize the spinal analgesic actions in rats of two 5α-reduced neurosteroids, allopregnanolone and alphaxalone, on nociceptive processing and to determine whether a putative neurosteroid antagonist attenuates this effect: (3α,5α)-17-phenylandrost-16-en-3-ol (17PA). Intrathecal (IT) injection of allopregnanolone (1–30 μg/10 μL in 20% cyclodextrin) delivered through lumbar catheters produced a dose-dependent analgesia in rats as measured by thermal thresholds in the ipsilateral (inflamed by intraplantar carrageenan) and in the contralateral (un-inflamed paws). Similar observations were made with alphaxalone (30–60 μg in 20% cyclodextrin). Effective doses were not associated with suppressive effects on pinnae, blink or placing and stepping reflex. Effects of allopregnanolone (30 μg) on the normal and hyperalgesic paw were completely prevented by IT 17PA (30 μg). Reversal by IT 17PA of an equi-analgesic dose of alphaxalone occurred only at higher antagonist dosing. These results suggest that a spinal neurosteroid-binding site with which 17PA interacts may regulate spinal nociceptive processing in normal and inflamed tissue.

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1. Introduction

Neurosteroids have analgesic, anesthetic and anticonvulsant effects [14,26]. Spinal delivery of 5α-reduced neurosteroids such as allopregnanolone (3α,5α-tetrahydroprogesterone or 3α,5α-THP) reduces the facilitated nociceptive response produced by repetitive nerve stimulation [4,20,23] or visceral inflammation [31]. Antagonism of these effects by IT bicuculline [9] suggests that these agents facilitate activation by γ-amino butyric acid (GABA) of the $GABA_A$ chloride ionophore and at higher concentrations by directly activating the ionophore [1], promoting the channel open state [2]. Molecular studies have shown two neurosteroid binding sites that mediate the potentiating and direct channel activating effect of neurosteroids [12]. Ample evidence supports the ability of neurosteroids to amplify dorsal horn $GABA_A$ receptor activity regulating components relevant to dorsal horn pain processing [6,24,28,33].

Evidence for the action of neurosteroids at a specific site is further provided by the observation that neurosteroid activity is antagonized by agents such as (3α,5α)-17-phenylandrost-16-en-3-ol (17PA). 17PA has little effect on baseline GABA-mediated Cl flux but antagonizes gating and conductance augmentation produced by 5α-reduced steroids, such as allopregnanolone [15,18]. We note that two 5α-reduced steroids, allopregnanolone and alphaxalone ((3α,5α)-3-hydroxypregnane-11,20-dione), both produce sedation but only the effects of allopregnanolone are reversed by 17PA when administered systemically *in vivo*. This differential effect has been taken to support different neurosteroid binding sites [15]. Whether

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this differential effect is observed *viz.* the analgesic effects of these two neurosteroids is not known. Thus, we examined: (i) spinal actions of allopregnanolone and alphaxalone in regulating inflammation evoked hyperalgesia; (ii) dose dependency of this spinal action; and (iii) whether IT 17PA would antagonize spinal actions of IT allopregnanolone and/or alphaxalone, supporting an interaction with a spinal neurosteroid binding site.

2. Materials and methods

2.1. Animals

Adult male rats (Holtzman, 200–250 g) (12/12 day–night cycle, *ad libitum* access to food/water) were used in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. To allow IT delivery, rats were prepared with chronic lumbar IT catheters placed 8.5 cm from the cisterna magna and externalized on the top of the head [17,35].

2.2. Drugs

The 5α-reduced neurosteroid agonists, allopregnanolone and alphaxalone, and the neurosteroid antagonist (3α,5α)-17-phenylandrost-16-en-3-ol (17PA) (Tocris Bioscience, Ellisville, MO, USA) were delivered in β-cyclodextrin (20%, w/v) (Sigma–Aldrich, St. Louis, MO, USA) and 0.9% NaCl with the final doses delivered in 10 μL followed by 10 μL of saline. Vehicle controls were the cyclodextrin formulation. Solutions were made and injected by two different individuals and testing was without knowledge of the solution.

2.3. Behavioral testing

IT drugs were delivered 30 min prior to unilateral intraplantar delivery of carrageenan (2% in 100 μL) at time = 0. For thermal threshold assessments, a Hargreaves system (UCSD University Anesthesia Research and Development Group, La Jolla, CA) [8] was employed. After 60 min adaptation, a radiant heat stimulus was delivered to each individual paw and the latency to withdraw noted [8]. To prevent tissue damage, a cut-off time of 20 s was used. In a limited series of experiments, we assessed tactile thresholds using von Frey hairs as previously described using the "up–down" method, with the maximum pressure being 15 g [5]. The effects of treatment on behavior and function were assessed before and during the subsequent 4 h post injection test interval during which antinociception was observed by noting the presence or absence of (i) hindpaw placing/stepping response, (ii) pinnae response, (iii) blink (corneal) reflex, (iv) righting reflex and (v) normal ambulation

2.4. Statistical analysis

Data are presented as mean and SEM of thermal escape latency (seconds) or tactile threshold (grams). Drug response data were converted to a Hyperalgesic Index (area under the curve for % Hyperalgesia: e.g. [Premeasurement − Postmeasurement/Premeasurement] × 100). In each case, hyperalgesia is indicated by a larger value whereas reversal of hyperalgesia or analgesia is indicated by a smaller or negative number. Time-dependent response data were analyzed by two-way analysis of variance-repeated measures across time. Hyperalgesic indices between treatment groups were compared using 1-way ANOVA, with a Bonferroni multiple comparison analysis for p*ost hoc* comparisons. Differences reaching a $p < 0.05$ level of significance were considered to be significant. Statistics were carried out using Prism (v4.2c) (La Jolla, CA).

3. Results

3.1. Agonist effects upon thermal hyperalgesia

Baseline thermal escape latencies prior to drug treatment was similar for all treatment groups (approximately 9–11 s) in vehicle treated animals, intraplantar carrageenan reduced the thermal escape latency of the inflamed (ipsilateral) paw, which persisted undiminished through the 4 h time point (approximately 3–5 s, see Fig. 1), with no change in the normal (contralateral) paw withdrawal latency. IT cyclodextrin (20%/10 μL) had no effect upon the thermal withdrawal latency of the ipsilateral vs. the contralateral paw, as compared to IT saline (data not shown). IT allopregnanolone (3–30 μg/10 μL) resulted in a significant dose-dependent reversal of the hyperalgesic paw latency (as measured by the latency and the hyperalgesic index) and an increase in latency above baseline for the uninjured paw (Fig. 1). Intraperitoneal delivery of the maximum IT allopregnanolone dose (30 μg) had no effect upon thermal withdrawal latencies of either the ipsilateral or contralateral paw ($N = 4$; $p > 0.05$), indicating that the IT drug effect did not represent a simple systemic redistribution (data not shown). The effects of IT alphaxalone (30 and 60 μg/10 μL) were examined on thermal escape. As shown (Fig. 2), a significant increase in the thermal escape latency was noted with both the ipsilateral (hyperalgesic) and contralateral paw.

3.2. Agonist effects upon tactile allodynia

After carrageenan, rats displayed a tactile allodynia in the inflamed (ipsilateral) paw with no change in the contralateral paw. Rats pre-treated with IT allopregnanolone or alphaxalone (but not 20% cyclodextrin) displayed a reduction in the tactile allodynia otherwise observed after intraplantar carrageenan as compared to vehicle: Post IT Vehicle threshold at 3 h = 3.1 ± 0.6 g vs. IT allopregnanolone, 30 μg/10 μL: 11.6 ± 1.9 g; ($p < 0.05$ vs. vehicle) or alphaxalone (60 μg/10 μL): 13.5 ± 2.1 g ($p < 0.05$ vs. vehicle) (data not shown).

3.3. Agonist effects upon behavior

Over the intrathecal doses examined for alphaxalone (30–60 μg) and allopregnanolone (3–30 μg), there was no change in the ability to evoke a pinnae or blink response. There was no loss of hind paw placing/stepping, or the righting response secondary to being placed recumbent. In the uninflamed (contralateral) paw, there was typically a modest increase in the thermal escape latency, but most animals even at the highest doses continued to display withdrawal. Though not systematically examined, in the absence of stimulation, high dose alphaxalone animals appeared to show less spontaneous activity in the test cage, than adjacent animals run in parallel receiving vehicle only, though they were not immobile and showed the previously noted evoked responses.

3.4. Intrathecal antagonist

IT 17PA (30 μg/10 μL) alone had no effect upon either the hyperalgesic thermal escape latency (ipsilateral paw) or the normal escape latency (contralateral paw). To examine antagonism by 17PA of the agonist-evoked antihyperalgesia, IT 17PA (30 μg) was given 15 min prior to the agonist. At −20 min, the just maximally effective IT doses of allopregnanolone (30 μg/10 μL) or alphaxalone (60 μg/10 μL) were given, followed at $T = 0$ by intraplantar

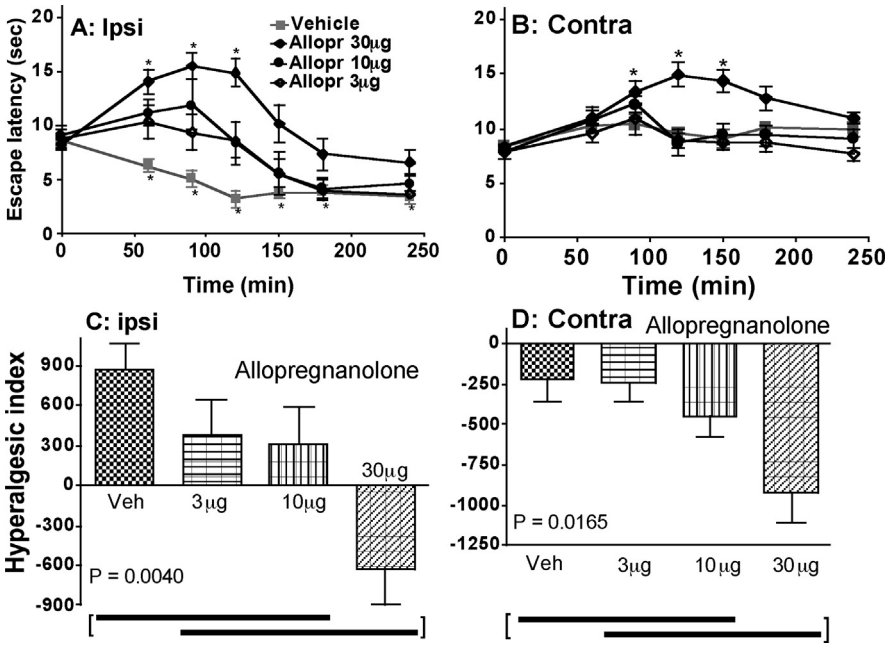

Fig. 1. TOP: Effect of IT allopregnanolone or cyclodextrin (20%) vehicle on escape latency of ispilateral (A) and contralateral (B) hindpaw as a function of time after ipsilateral intraplantar carrageenan given at $T = 0$. BOTTOM: Hyperalgesic index for ipsilateral (C) and contralateral (D) hindpaw shows that IT allopregnanolone resulted in dose dependent reversal of thermal hyperalgesia of inflamed paw and a significant increase above baseline (analgesia) in contralateral, un-inflamed paw. Positive scores indicate hyperalgesia; negative scores indicate analgesia. Each plot presents means ± SEM, 5–9 animals. Comparisons: A and B: Two Way repeated measures ANOVA with Bonferroni post hoc comparison: $^{*}p < 0.05$ vs baseline; C and D: Bars under each AUC graph link treatments that do not differ from each other (Bonferroni multiple comparisons, $p < 0.05$).

carrageenan. IT 17PA pretreatment (Fig. 3) resulted in a near complete reversal of the antinociceptive effects of IT allopregnanolone as measured by the latency and the hyperalgesic index on both the ipsilateral and contralateral paw (e.g. no statistical difference between allopregnanolone + 17PA vs. vehicle).

In contrast, as shown in Fig. 4, IT 17PA (30 μg) produced only a modest none significant reversal of the antihyperalgesic actions of IT alphaxalone in the ipsilateral and contralateral paws. Increasing the 17PA dose to 60 μg resulted in a reduction in the antihyperalgesic effects in the ipsilateral paw and contralateral paw.

In separate experiments, we sought to determine if the post-treatment of 17PA at 120 min post-carrageenan would reverse the effects of IT allopregnanolone (30 μg/10 μL). In these studies (data not shown), there was a modest but statistically significant reversal after the IT 17PA injection: Post-Vehicle latency = 3.4 ± 0.8 s vs. Post 17PA 6.9 ± 1.8 s ($p < 0.05$).

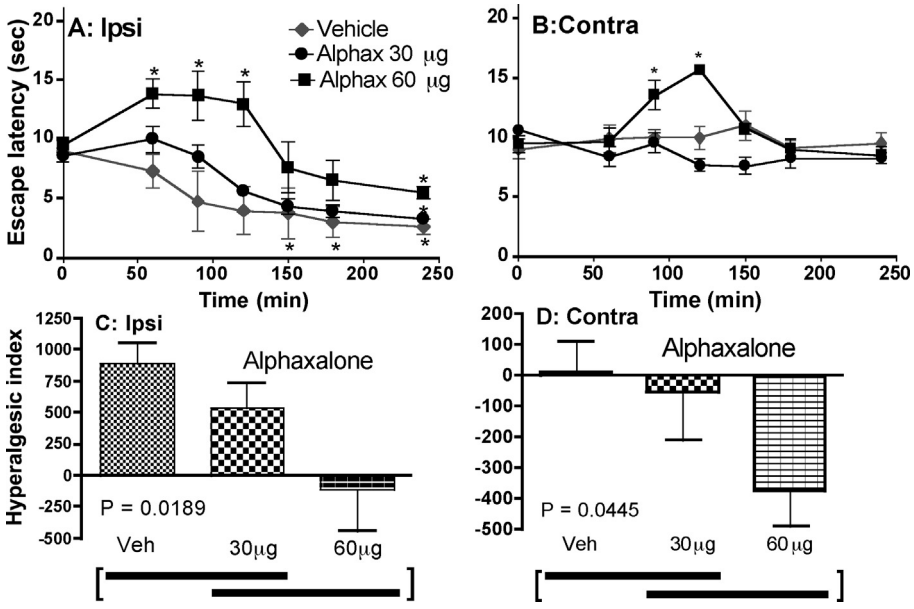

Fig. 2. TOP: Effect of IT alphaxalone or cyclodextrin (20%) vehicle on escape latency of ipsilateral (A) and contralateral (B) hindpaw as a function of time after ipsilateral intraplantar carrageenan given at $T = 0$. BOTTOM: Hyperalgesic index for ipsilateral (C) and contralateral (D) hindpaw shows that IT allopregnanolone resulted in dose dependent reversal of thermal hyperalgesia of the inflamed paw and a significant increase above baseline (analgesia) in contralateral, un-inflamed paw. Positive scores indicate hyperalgesia; negative scores indicate analgesia. Each plot presents means ± SEM, 5–9 animals. Comparisons: A and B: Two Way repeated measures ANOVA with Bonferroni post hoc comparison: $^{*}p < 0.05$ vs baseline; C and D: Bars under each AUC graph link treatments that do not differ from each other (Bonferroni multiple comparisons, $p < 0.05$).

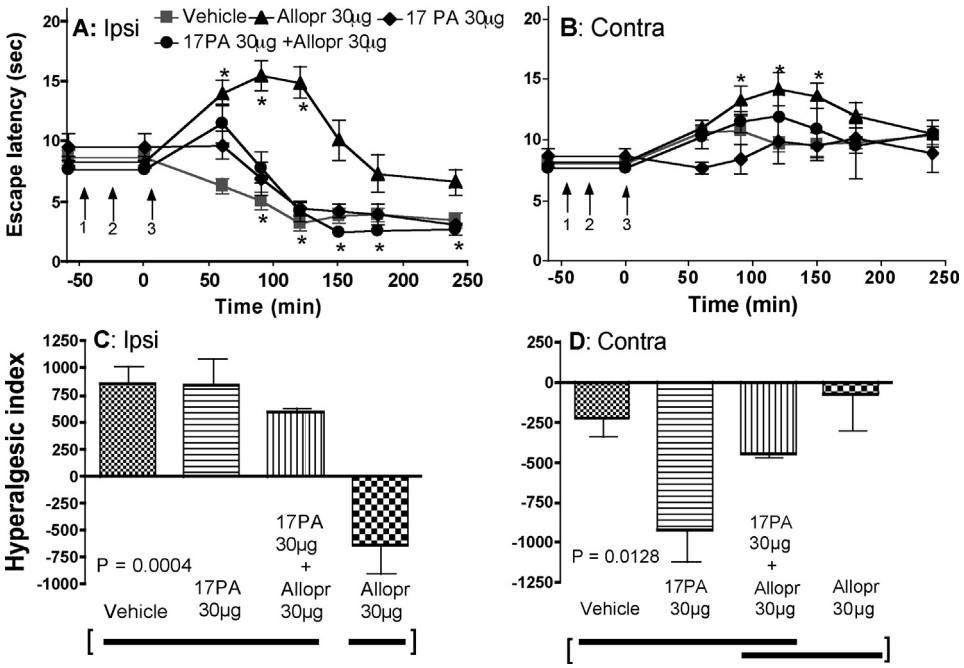

Fig. 3. TOP: Effect on escape latency of IT neurosteroid antagonist, (17PA) on escape latency in the ipsilateral-carrageenan injected (A) and the contralateral (B) hindpaw when given alone or in the presence of allopregnanolone (30 μg/10 μL). IT 17PA (30 μg/10 μL) was given 15 min (arrow 1) prior to IT allopregnanolone or vehicle (arrow 2). Carrageenan was injected into the ipsilateral hind paw at $T = 0$ (arrow 3). BOTTOM: Hyperalgesic index for ipsilateral (C) and contralateral (D) hindpaw shows that IT 17PA had no effect upon the carrageenan evoked thermal hyperalgesia, but reversed effects of IT allopregnanolone. Each plot presents means ± SEM, 5–9 animals. Comparisons: A and B: Two Way repeated measures ANOVA with Bonferroni post hoc comparison: $^*p < 0.05$ vs baseline; C and D: Bars under each AUC graph link treatments that do not differ from each other (Bonferroni multiple comparisons, $p < 0.05$).

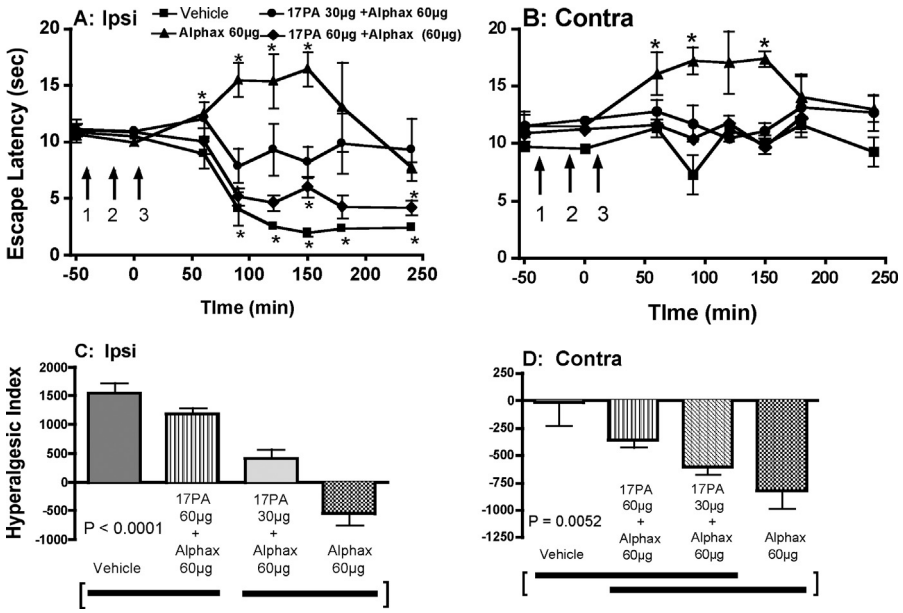

Fig. 4. TOP: Effect on escape latency of IT neurosteroid antagonist, (17PA) on escape latency in the ipsilateral-carrageenan injected (A) and contralateral (B) hindpaw when given in the presence of alphaxalone (60 μg/10 μL). IT 17PA (30 or 60 μg/10 μL) was given 15 min (arrow 1) prior to IT alphaxalone or vehicle (arrow 2). Carrageenan was injected into ipsilateral hind paw at $T = 0$ (arrow 3). BOTTOM: Hyperalgesic index for ipsilateral (C) and contralateral (D) hindpaw shows that IT 17PA, 60, but not 30 μg/10 μL resulted in reversal of the antinociceptive effects of IT alphaxalone. Each plot presents means ± SEM, 5–9 animals. Comparisons: A and B: Two Way repeated measures ANOVA with Bonferroni post hoc comparison: $^*p < 0.05$ vs baseline; C and D: Bars under each AUC graph link treatments that do not differ from each other (Bonferroni multiple comparisons, $p < 0.05$).

4. Discussion

Intrathecal delivery of 5α-reduced neurosteroid agonists allopregnanolone and alphaxalone in a cyclodextrin-water-based vehicle reverse, in a dose-dependent fashion, thermal and mechanical hyperalgesia. These effects occurred at doses that are not systemically active and which have no effect upon general function or motor function. These results are in accord with previous work showing that IT delivery of several neurosteroid-like molecules produces a significant effect upon facilitated pain states [9,20,31]. Aside from their antihyperalgesic action, these agents also modestly increased the response latency of the un-inflamed paw,

suggesting that their action was not limited to a facilitated state. This observation is comparable to work showing the effects of IT neurosteroids on tail flick response latency [9].

4.1. Spinal pharmacology of neurosteroid agonist action

Neurosteroids interact with an allosterically coupled binding complex of the $GABA_A$ chloride ionophore. This coupling augments the GABA initiated opening of the channel. At higher concentrations, neurosteroids can activate the channel. The likelihood that the neurosteroids are exerting their spinal actions through a $GABA_A$ channel is supported by reports that the antihyperalgesic actions are prevented by $GABA_A$ block with bicuculline [9].

Both allopregnanolone and alphaxalone display potent anesthetic effects. Although previous systemic work with alphaxalone reported no specific antinociceptive effects [20], both agents comparably activate the $GABA_A$ ionophore [7] and prevent thermal hyperalgesia. To address the likelihood that these agents were interacting with a neurosteroid binding site after IT delivery, we examined the ability of the putative neurosteroid antagonist 17PA to reduce the observed antihyperalgesic action. This agent behaves as a competitive antagonist at a $GABA_A$-associated binding site [18]. IT-17PA abolished the antihyperalgesic and analgesic actions of IT-allopregnanolone. The antihyperalgesic effects of an equianalgesic IT dose of alphaxalone were also reversed, albeit only at a higher 17PA dose. Given the assertion that 17PA is a competitive antagonist, then the apparent difference in antagonist potency of 17PA against equianalgesic doses of the two agonists suggests distinguishable binding sites for these two agonists, e.g. a binding site interaction with an alphaxalone site at which 17PA had a lower affinity than the binding site for allopregnanolone. Whether or not this hypothesis is correct, the observations showing a different antagonist potency are comparable to previous work in which it was shown that systemic 17PA reversed the sedative effects of allopregnanolone, but had little effect upon sedation after alphaxalone [15].

4.2. Cyclodextrin as a vehicle

Cyclodextrin was employed as a vehicle compatible with IT delivery. It had no effect when given alone as compared to IT saline. IT cyclodextrin is a vehicle for solubilizing some lipophillic compounds, such as capsaicin, small peptides and neurosteroids [34]. Cyclodextrin the permitted use of a water-based vehicle that is generally preferable to other lipid solubilizing or detergent-like vehicles for neuraxial delivery. There are several *caveats* to the use of IT cyclodextrin. First, previous work with gamma-cyclodextrin suggested that the complex may result in a reduction in the apparent potency of steroids. Nevertheless, the use of IT β-cyclodextrin revealed a monotonic dose-effect relationship with drug effects of several hours duration. Accordingly, to the degree that cyclodextrin sequesters neurosteroids, the observed actions are compatible with a dose-proportional delivery of drug to the active site after IT delivery. Second, β-cyclodextrins can exert direct potentiating effects on $GABA_A$ receptors [25,28]. We did not see evidence of this action with the present study, as the IT cyclodextrin alone had no effect upon either normal or hyperalgesic paw latencies. Whether other response systems might be affected by this action remains to be determined. Third, cyclodextrin binds cholesterol and extracts cholesterol from cell membranes and disrupts caveolae formation [10,21,36].

4.3. Mechanisms of neurosteroid associated antinociception

The interaction of neurosteroids with the GABA ionophore involves specific subunits, notably those of the alpha and beta forming binding pockets with which the neurosteroids may interact [11,12]. In the spinal cord, alpha subunits are expressed in the superficial dorsal horn [3] on primary afferents, which, because of the superficial laminar distribution, likely represent nociceptive afferents, and on second order neurons. The presynaptic location is believed to represent mechanisms underlying mediation by GABA of preterminal inhibition (e.g. primary afferent depolarization) [27] while the postsynaptic effect is considered to be associated with a hyperpolarization resulting from the increased chloride conductance [31]. Knockout of the α2-$GABA_A$ subunit revealed that the antihyperalgesic effects of spinal benzodiazepines were prevented [32]. We note that the reduced effect of IT-17PA on the antihyperalgesic effects of alphaxalone in contrast to the allopregnanolone may also be interpreted as suggesting multiple potential targets other than the $GABA_A$ receptor. Interactions of neurosteroids with NMDA ionophores [13,16,29] and T type calcium channels [22] have been described. Neurosteroids have also been shown to interact with glycine receptor mediated chloride currents [19,30]. The role of these interactions at the spinal level is not excluded in the present study and requires further investigation. We are however aware of no data considering an interaction of 17PA with such binding.

5. Conclusions

We demonstrated that two structurally similar 5α-reduced neurosteroids produce a potent antinociception and antihyperalgesic action. A putative neurosteroid receptor antagonist had distinguishable potency in reversing the effects of the two agents, consistent with the hypothesis of possible multiple targets for spinal neurosteroids.

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