JCI The Journal of Clinical Investigation

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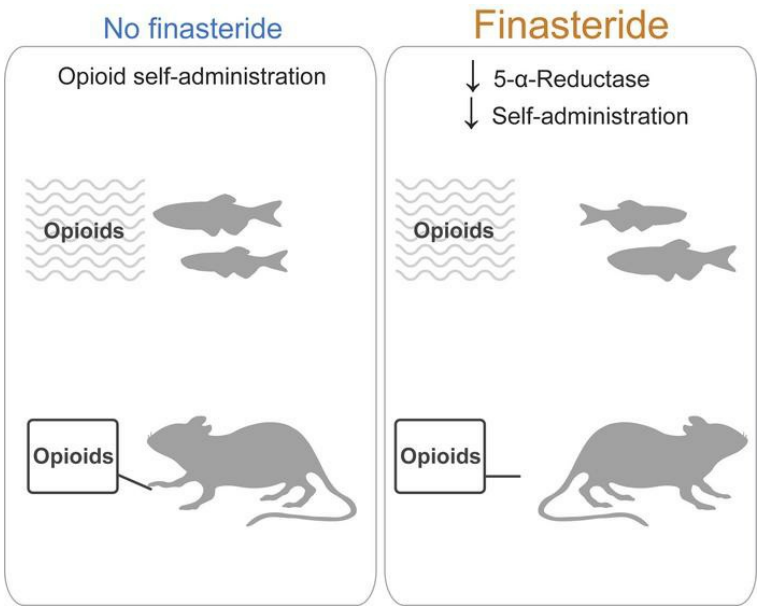
J Clin Invest. 2021. https://doi.org/10.1172/JCI143990.

Research In-Press Preview Neuroscience

Graphical abstract



Title: The 5α-reductase inhibitor finasteride reduces opioid self-administration in animal models of opioid use disorder

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Conflict of Interest Statement: The authors have declared that no conflict of interest exists.

Abstract

Opioid use disorder (OUD) has become a leading cause of death in the US, yet current therapeutic strategies remain highly inadequate. To identify potential treatments for OUD, we screened a targeted selection of over 100 drugs using a recently developed opioid self-administration assay in zebrafish. This paradigm showed that finasteride, a steroidogenesis inhibitor approved for the treatment of benign prostatic hyperplasia and androgenetic alopecia, reduced self-administration of multiple opioids without affecting locomotion or feeding behavior. These findings were confirmed in rats; furthermore, finasteride reduced the physical signs associated with opioid withdrawal. In rat models of neuropathic pain, finasteride did not alter the antinociceptive effect of opioids and reduced withdrawal-induced hyperalgesia. Steroidomic analyses of the brains of fish treated with finasteride revealed a significant increase in dehydroepiandrosterone sulfate (DHEAS). Treatment with precursors of DHEAS reduced opioid self-administration in zebrafish in a fashion akin to the effects of finasteride. These results highlight the importance of steroidogenic pathways as a rich source of therapeutic targets for OUD and point to the potential of finasteride as a new treatment option for this disorder.

Introduction

Over the last decade, the widespread abuse of prescription painkillers, such as oxycodone and hydrocodone, has led to a crisis of opioid use disorder (OUD) and a dramatic increase in opioid overdose in North America(1). The extent of this crisis is such that opioids account for more than 60% of all drug overdoses in the United States, with an estimated 47 000 to 50 000 fatalities annually. Synthetic opioids, such as fentanyl, are the major drivers of opioid overdose(1, 2). Unfortunately, current therapeutic options for OUD are highly unsatisfactory. Existing treatments rely on replacement with long-acting opioids, such as methadone or buprenorphine(3). While these options help patients cope with drug craving and manage withdrawal symptoms(3), they are not ideal due to their intrinsic liability for abuse and dependence(4). This background highlights the urgent need for improved therapeutic options to reduce the risk and severity of OUD, particularly in patients requiring opioid treatment for chronic and neuropathic pain syndromes (for which effective alternatives to these painkillers are not always available).

Rodent models have been successfully used to model substance use disorders and study the circuitry and neurobiological changes involved in drug abuse(5, 6). However, the viability of these models to screen for more effective therapeutic candidates is limited by their low throughput and high costs. In recent years, the zebrafish (*Danio rerio*) has emerged as a new alternative to study a wide range of complex behavioral and neuropsychiatric disorders, such as schizophrenia and depression(7–9). Importantly, zebrafish have been shown to develop conditioned place preference and withdrawal symptoms after exposure to opioids and other drugs of abuse, such as cocaine and alcohol(10–14). Additionally, adult zebrafish possess a complex central nervous system, including a blood-brain barrier, and considerable similarities with the mammalian homolog. Furthermore, this model is well suited for the rapid testing of candidate drugs, as compounds of interest can be

dissolved directly into the water of the tank (15–17). Therefore, zebrafish offers a unique opportunity to combine drug-discovery screening and substance abuse research.

Using a recently described paradigm to condition adult fish to self-administer the opioid hydrocodone(18), we designed a behavior-based screen to identify compounds affecting opioid self-administration in zebrafish. We screened 110 unique molecules selected for their annotated activity against processes and pathways known to be involved in substance abuse disorders. From this screen, we identified the 5α-reductase (5αR) inhibitor finasteride(19, 20) as one of the most effective compounds to reduce opioid self-administration without affecting food-seeking or overall locomotion. 5αR catalyzes the rate-limiting step of the conversion of several ketosteroids, including progesterone and testosterone, into their neuroactive metabolites dihydroprogesterone and dihydrotestosterone (DHT). In turn, these steroids are further converted into the neurosteroids allopregnanolone and 3α-androstanediol (3α-diol)(21–24), which play an essential role in behavioral regulation. Finasteride has been clinically approved for over 25 years as a treatment for benign prostatic hyperplasia and male-pattern baldness(25). These effects reflect the suppression of DHT synthesis.

The effects on self-administration were also confirmed in rats. Notably, we found that finasteride opposes the physical effects and hyperalgesia associated with opioid withdrawal but did not reduce the pain-killing properties of opioids in rat models of neuropathic pain. Finally, we identified the neurosteroid dehydroepiandrosterone sulfate (DHEAS) as a likely mediator of finasteride's effects. We thus have uncovered a role for neuroactive steroids in the control of opioid self-administration.

Results

Validation of the screening method

To screen for modulators of opioid self-administration, we utilized our newly developed assay to condition fish with the opioid hydrocodone. As previously described, small groups of adult zebrafish are conditioned to swim across an active platform to receive a dose of the drug(18). Each visit results in the delivery of hydrocodone directly at the platform. We used 15 animals per group of fish, so each n represents a unique group of 15 fish. Potential modulators of opioid self-administration are tested by training fish to self-administer hydrocodone for four days and treating with the compound of interest on the fifth day (Figure 1A). Before performing the small-molecule screen, we sought to validate the screening method by testing one of the only treatments used in the clinic for OUD, the slow-acting opioid methadone. Fish conditioned for four days to self-administer hydrocodone were treated with methadone (1 mg/L) for 60 min and then transferred to the self-administration arena. During each 30-min self-administration session, the number of triggering events for both the active and inactive platforms was recorded and used as a readout of opioid intake. Methadone significantly reduced hydrocodone self-administration (Figure 1B), suggesting that the screening assay can identify molecules that reduce opioid self-administration.

Small molecule screening identifies modulators of opioid self-administration

We conducted a small-scale screen using a targeted collection of compounds selected based on hypotheses presented in the literature regarding neuronal pathways contributing to addiction, their ability to modulate pathways identified in GWAS studies on substance abuse patients, and molecular pathways affected during substance abuse. We hypothesized that focusing on this targeted collection would increase the probability of finding effective drugs within a smaller library of compounds. To test if the compounds reduce opioid self-administration, drug-

conditioned animals were treated with 10 μM of each candidate compound 60 min before the 30-min self-administration session (Figure 1A). On average, control animals triggered the release of drug more than 1800 times per session. To reduce the number of false-positive hits, we tested each compound in duplicate, furthermore, only compounds with fewer than 1000 triggering events for both duplicates were considered hits.

Finasteride reduces opioid self-administration

After screening over 100 compounds, we identified the 5αR inhibitor finasteride as highly effective in reducing opioid self-administration. Incubation with a single dose of 10 μM of finasteride for 60 min was sufficient to reduce the number of triggering events at the active platform by 73% (Figure 1B). The complete list of tested compounds and their effects on opioid self-administration is presented in Figure S1 and Table S1).

To further validate that the inhibition of 5αR was responsible for reducing opioid self-administration, we tested a different 5αR inhibitor, dutasteride(26). Like finasteride, dutasteride reduced the number of triggering events at the active platform (Figure S2). Although research on neuroactive steroids in zebrafish has been limited, the key steroidogenic enzymes are expressed in the adult brain, and the activity of 5αR has been detected in brain extracts(27–33). Additionally, using a publicly available single-cell RNA seq library, we were able to detect 5αR transcripts in several different cell types in zebrafish brains(34) (Figure S3). Taken together, these results suggest that the inhibition of the enzyme 5αR reduces opioid self-administration in zebrafish.

To further characterize the efficacy of finasteride, we performed a dose-response experiment. We used 10-fold dilutions to test finasteride concentrations from 10 μM to 1 nM. A significant difference was detected with concentrations as low as 5 nM (Figure 1C). This dose-response

experiment supports the idea that finasteride is highly potent and has a large therapeutic window in zebrafish.

Finasteride does not affect locomotion or food self-administration

To determine if the effect of finasteride was caused by sedation, we monitored the swimming speed of finasteride-treated animals. Finasteride did not reduce locomotion in comparison with DMSO (Figure S4). Importantly, we also did not observe any significant difference in the number of triggering events at the inactive platform between the finasteride-treated fish and control animals (Figure 1). These results suggest that finasteride specifically reduces the number of triggering events at the active platform without affecting overall locomotion.

Drugs of abuse are known to activate the reward pathway in the brain, which is a core contributor to motivated behaviors, such as feeding or reproduction(35). We thus decided to test if finasteride was also affecting other motivated behaviors by measuring food self-administration in zebrafish. We first trained fish as previously reported for opioids but used food instead of opioid as the reward. Food-conditioned animals were then treated with DMSO or finasteride for 60 min, and the number of triggering events at the active platform was measured. As opposed to hydrocodone self-administration, finasteride-treated animals exhibited no decrease in food-seeking (Figure 2A). These data suggest that finasteride does not affect all motivated behaviors and further validate that this drug does not impair locomotion.

Finasteride reduces self-administration of different opioids

It has been described that each class of opioids has a different abuse potential(36, 37). Therefore, we decided to test the effect of finasteride on animals conditioned with the traditional opioid most commonly used in animal models, morphine, and one of the most potent and deadly opioids, the synthetic opioid fentanyl.

In order to test the effect of finasteride, we used the same conditioning protocol to train animals to self-administer morphine and fentanyl. As morphine and hydrocodone are closely related, we used the same dose of 6 mg/L. As for fentanyl, studies suggest that it is up to 50-100x more potent than morphine(38), so a dose 50x lower (0.12mg/L) was used, to take into account its greater potency. We first confirmed that animals conditioned with either opioid had similar self-administration levels after 5 days of conditioning. There was no significant difference in the number of triggering events between fish trained with different opioids (Figure 2B). Therefore, this conditioning protocol is easily applicable to multiple classes of opioids. The conditioned animals were then treated with 10 μM finasteride for 60 min before performing a 30-min self-administration session. As observed with hydrocodone-trained animals, there was a reduction in the number of visits to the active platform without affecting the inactive platform (Figure 2B). This result suggests that finasteride reduces opioid self-administration behavior regardless of the opioid used during the conditioning phase.

Finasteride reduces opioid self-administration in rats.

The zebrafish model suggests that finasteride strongly reduces opioid consumption. To confirm this effect in mammals, we tested finasteride in a rat model of hydrocodone self-administration. Adult male Sprague-Dawley rats were first conditioned to press a lever to receive an intravenous infusion of hydrocodone (0.016-0.128 mg/kg/160μL). Operant conditioning consisted of three stages of fixed-ratio (FR) reinforcement schedules: FR1, FR2, and FR5 (i.e., FR1=each lever press resulted in a hydrocodone infusion). Animals progressed to the next stage of conditioning after reaching the criterion of >70% lever presses being on the lever on the previous schedule (Figure 3Aand S5). Importantly, to mimic the zebrafish conditions, rats had access to the drug for 60 min per day. To find the optimal concentration for hydrocodone conditioning, we tested a range of

doses from 0.016 mg/kg to 0.128 mg/kg. We found that animals conditioned with 0.032 mg/kg and 0.064 mg/kg had the highest number of lever presses after 31 days of training (Figure 3B). We then treated animals conditioned with each concentration of hydrocodone by intraperitoneal injection of finasteride (50 mg/kg, IP) or vehicle before a self-administration session. That dose of finasteride was sufficient to significantly reduce the number of active lever presses at both 0.032 and 0.064 mg/kg/infusion of hydrocodone (Figure 3B).

To determine if finasteride was effective at different doses, we treated animals conditioned with 0.064 mg/kg of hydrocodone with 25 mg/kg or 50 mg/kg of finasteride. We confirmed that injection of 50 mg/kg significantly reduced the number of active lever presses, but there was no difference in animals treated with the lower dose (Figure 3C). Importantly, locomotion or inactive lever presses were not affected by finasteride (Figure S6 and S7).

To further validate that finasteride can reduce opioid intake in mammals, we also tested the effect of finasteride on the potent synthetic opioid fentanyl. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay. Rats were trained to perform a nose-poke to trigger the release of a drop of fentanyl drinking solution (0.02 mg/kg/delivery) in a liquid magazine tray. We trained both male and female animals over 15 sessions of 60 min. As the training progressed, we observed an increase in active nose-pokes per session, and animals rapidly learned to discriminate between the active and inactive nose-poke holes (Figure 4A). Following each self-administration session, the mg/kg of fentanyl consumed was calculated for each animal by subtracting the amount of fentanyl left in the magazine tray from the total amount of drug delivered. As observed for the number of active nose-pokes, we also detected an increase in ingested fentanyl over time (Figure S8A).

After the conditioning phase, animals were separated into two groups, and the effect of finasteride was tested over 10 sessions. Each group was treated with either *an* intraperitoneal injection of finasteride (50 mg/kg) or vehicle for five out of the ten sessions. Treatments were then inversed for the remaining five sessions so that both groups received five sessions of finasteride and five of vehicle. As observed for hydrocodone self-administration, acute treatment with finasteride significantly reduced the number of active nose-pokes (Figure 4B-C), as well as the total amount of fentanyl consumed per session (Figure S8B-E), without affecting the number of inactive nose pokes (Figure S9). This acute treatment with finasteride was effective during the five days of treatment, regardless of the order of treatment (Figure 4D-E). Importantly, finasteride reduced opioid consumption in both male and female animals (Figure S10).

We also quantified the number of active nose-pokes over time during each session. In vehicle-treated animals, there was a rapid succession of active nose-pokes in the first 10 min before slowing down over the next 50 min. Finasteride treated animals followed a similar pattern, but they only acquired approximately half of the nose-pokes before slowing their intake rate (Figure 4F).

These results collectively demonstrate that the activity of finasteride on opioid self-administration is conserved in mammals, even for animals conditioned with the synthetic opioid fentanyl.

Finasteride reduces the physical effects of opioid withdrawal.

Given that previous studies have shown that neurosteroids play a role in shaping opioid withdrawal(39, 40), we tested whether finasteride may also modify the effects of opioid withdrawal, using the model of naloxone-precipitated withdrawal syndrome in adult Long Evans male rats. Finasteride significantly reduced the number of wet-dog shakes and the duration of grooming behavior induced by naloxone (Figure 5); however, it did not fully reverse the jumping

and digging responses induced by the opioid antagonist (Figure S11). These results indicate that finasteride may reduce the severity of opioid withdrawal.

Finasteride does not affect the antinociceptive properties of opioids

Despite their high abuse potential, opioids are still invaluable as analgesics. In the absence of an effective alternative, they are still an essential treatment, especially for people suffering from chronic pain. Therefore, an ideal candidate for an opioid abuse treatment would be a drug that does not affect the pain-killing properties of opioids. A previous report demonstrated that finasteride did not reduce the efficiency of morphine; however, the authors used a lower dose of finasteride (25mg/kg) and tested a single nociceptive stimulus(40). To further validate that finasteride does not interfere with the antinociceptive effects of opioids in our conditions, we used the most rigorous test available in rats by testing the impact of opioids on a rat model of neuropathy.

We performed spinal nerve ligation (SNL) surgery on adult male Sprague-Dawley rats (41), and fourteen days after surgery, animals were separated into different groups. Mechanical allodynia and nociception were measured utilizing the von Frey Hair and Randall-Selitto tests, respectively(42, 43). We also measured thermal nociception (hot plate)(44). We first validated the antinociceptive effects of opioids by testing different doses of morphine (1-3mg/kg, SC) or hydrocodone (1-10mg/kg, SC) every 30 min for 3 hours. For the Randall-Selitto test, an increase in mechanical force is applied to the paw until a withdrawal response is observed (Figure 6). Treatment with either morphine or hydrocodone significantly increased the force needed to trigger withdrawal compared with the same animals prior to treatment with the opioid (Figure 6A and Figure S12B). We then selected the most effective dose for both opioids, morphine (3 mg/kg), and hydrocodone (10 mg/kg) and tested the effect of co-injection with finasteride (50 mg/kg, IP). The antinociceptive effect of neither opioid was reduced by the co-treatment with finasteride (Figure

6B and Figure S12C). We also performed the same set of experiments to test a different mechanical stimulus, i.e., the Von Frey test. As with the Randall-Selitto test, we did not detect any reduction in the antinociceptive effects of the opioids by treatment of the rats with finasteride (Figure S13).

In order to test a different type of nociception, we used the hot plate assay to test for a thermal stimulus on a different group of neuropathic rats. Animals were placed on a hot plate analgesia meter and the latency to lick their left hind paw was measured at different temperatures (48.5°C and 51.1°C). We selected the most effective dose of opioid, based on the Randall-Selitto test, and performed the assay 30 and 60 min after opioid injection. The latency for the first lick or paw retraction of animals treated with morphine (3 mg/kg) was significantly increased at both temperatures after 30 min (Figure 6C) and 60 min (Figure S14) when compared to untreated animals. Interestingly, treatment with hydrocodone (10 mg/kg) did not reduce the latency at 51.5°C and was not very effective when given 60 min before the test. As with mechanical nociception, co-injection with finasteride (50 mg/kg, IP) did not reduce the antinociceptive efficiency of either opioid at any temperatures or pretreatment times (Figure 6C and Figure S14).

Finally, to test the effects of finasteride on the hyperalgesic effects of opioid withdrawal, we tested the effects of finasteride (50 mg/kg, IP) on the mechanical nociception induced by naloxone (1.5 mg/kg, IP) in rats subjected to SNL and treated with a 6-day escalating morphine treatment. Surprisingly, we found that finasteride significantly increased the force threshold for paw withdrawal only on the injured side (Figure 6D and Figure S15), indicating that finasteride reduced the hyperalgesia associated with opioid withdrawal.

The fact that finasteride does not inhibit the antinociceptive or antiallodynic effect of either morphine or hydrocodone in response to different painful stimuli in a rat model of neuropathic pain demonstrates that finasteride is unlikely to interfere with the principal beneficial activity of

opioids. Furthermore, the finding that finasteride reduces the hyperalgesia associated with opioid withdrawal strongly suggests it may be used to reduce the liability for opioid abuse and the untoward consequences of opioid withdrawal while retaining the clinical utility of opioids.

We also verified that finasteride (50 mg/kg, IP) did not alter the antinociceptive properties of morphine (3 mg/kg, SC) in non-lesioned rats, as assessed by the Randall-Selitto test (Fig. S16A) and the hot plate test at 51.5°C (Fig. S16B). These data further suggest that finasteride does not diminish the anti-nociceptive properties of opioids.

Steroids regulate opioid self-administration

Because finasteride is a known 5αR inhibitor, we hypothesized that finasteride reduces opioid self-administration by altering the level of one or more neuroactive steroids in the brain. To investigate the landscape of changes induced by the treatment of opioid-conditioned animals with finasteride, and to identify candidate neuroactive steroids regulating opioid intake, we isolated whole brains from treatment-naive and opioid-conditioned zebrafish treated with either DMSO or finasteride (10 μM). We then performed steroid quantification using targeted ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).

In other models, finasteride induces changes in steroid levels in specific brain regions(45, 46), but because of the small size of the zebrafish brain, we used whole brains and could not achieve the same level of regional specificity. Nonetheless, we identified interesting trends, including an accumulation of several 5αR precursors and a reduction in 5αR products after treatment with finasteride. We normalized the results (min-max) for each steroid and compared the levels between DMSO- and finasteride-treated conditioned animals. The only steroid that reached significance by itself was dehydroepiandrosterone sulfate (DHEAS), which was markedly increased in finasteride-treated animals (Figure 7A). We also observed that other precursor

steroids, including testosterone and pregnenolone, also showed the same trend of accumulating in finasteride-treated animals. Surprisingly, we also detected a reduction in dehydroepiandrosterone (DHEA), which could suggest an increase in the conversion of DHEA to DHEAS in treated animals. Interestingly, we did not detect the same trend for other sulfated steroid species (Figure S17). The opposite trend of decreases in finasteride-treated animals was also observed for steroids downstream of 5αR, especially allopregnanolone (AP) and 3α-androstanediol, but these differences did not reach statistical significance (Figure 7A-B).

We tested how the accumulation of DHEAS affects opioid self-administration. Given that sulfated steroids are less effective at crossing the blood-brain barrier(47, 48), we incubated conditioned zebrafish with 10 μM DHEA for 60 min before measuring opioid-self administration. As observed with finasteride, incubation with DHEA drastically reduced the number of visits at the active platform (Figure 8A). We also tested the primary DHEA precursor, pregnenolone, and again observed a reduction in opioid self-administration (Figure 8A). Taken together, these results suggest that the accumulation of DHEAS and other precursors observed in finasteride-treated animals could play an important role in the reduction of opioid self-administration. The fact that DHEA-treated animals had a reduced number of visits at the active platform also suggests that the reduction in DHEA level observed in finasteride-treated animals is a result of an increased conversion to DHEAS.

Since we detected a reduction in some products of 5αR after treatment with finasteride, we decided to test whether their reintroduction could interfere with the activity of finasteride. However, since we did not detect any significant changes for a single product, we decided to test if a combination of steroids from the same class could act together. We chose to co-treat conditioned animals with finasteride (10μM) and allopregnanolone (0.1μM), androsterone (1μM) and 3α-diol (1μM) for 60

min. Interestingly, the presence of these products was sufficient to partially block the effect of finasteride and significantly increase the number of visits at the active platform when compared to finasteride treatment alone (Figure 8B).

Taken together, these results suggest that the accumulation of DHEA or its sulfated form might play a critical role in mediating the effect of finasteride. However, other neurosteroids such as 5αR products may also be involved in the regulation of opioid self-administration.

Discussion

We have demonstrated that finasteride modulates opioid consumption, a critical aspect of OUD, in two different animal models, and for different classes of opioids. These results suggest that finasteride or finasteride-like molecules could be a viable therapeutic strategy to treat OUD. Although finasteride is typically used for treating non-neuronal indications, there is evidence in the literature to suggest it might also have beneficial effects in the nervous system. Rodents treated with finasteride exhibit reduced reactivity towards both incentive stimuli and stress responses(49), both of which play important roles in substance abuse disorders(50, 51). Finasteride reduces risk-taking behavior, a behavioral feature typically associated with substance use disorders(49, 52, 53), and may help reduce pathological gambling(54). Since finasteride is FDA-approved and its side effects and toxicology are well-studied, it may be possible to rapidly initiate clinical trials to test the therapeutic potential of this drug for OUD. Furthermore, since finasteride does not reduce the antinociceptive effects of opioids and even opposes the hyperalgesia and physical signs associated with naloxone-precipitated opioid withdrawal, it could potentially be used as a treatment for OUD or an adjunct therapy for patients using opioids for pain relief.

We currently do not know if finasteride influences opioid self-administration by reducing motivation for drug-seeking or if treated animals are simply satisfied with a lower amount of drug. Our results in the fentanyl self-administration assay reveal that finasteride-treated rats initially perform nose-pokes to self-administer fentanyl at a rate similar to their untreated controls, but they slow down their intake sooner than controls, suggesting finasteride-treated animals may achieve satiation with a lower amount of fentanyl.

While the precise mechanism of finasteride action in opioid self-administration remains unknown, it is likely to involve changes in steroid profile in the brain. Finasteride is an inhibitor of key enzymes in steroid production, the three 5α-reductase isoenzymes SRD5A1, SRD5A2, and SRD5A3, which are expressed in different tissues, including the nervous system(55–59). Because the effects of finasteride on the steroid profile are pleiotropic(21, 22, 60), characterization of the specific steroid species involved in the regulation of opioid self-administration may eventually lead to the development of even more precise, targeted therapies for OUDs.

Our results suggest that accumulation of DHEAS plays an important role in opioid self-administration. Finasteride treatment causes a significant decrease in the non-sulfated DHEA and an accumulation of DHEAS in the brain. Importantly, treatment of opioid-conditioned zebrafish with DHEA alone reduces opioid intake, further supporting the hypothesis that DHEA/DHEAS plays an important role in opioid intake regulation. These neuroactive steroids have been shown to directly act on different neurotransmitter receptors; they can act as positive allosteric modulators of NMDA receptors, as negative modulators of the $GABA_A$ receptor, and as activators of other neuronal receptors such as σ1 and TrkA(61–63). The modulation of these pathways has been shown to be important in the regulation of opioid abuse disorders(18, 64–69) and could explain why modulation of DHEA reduces opioid intake. Furthermore, previous reports showed that repeated DHEAS treatment prevents the development of opioid tolerance without showing an effect on self-administration(70). Additionally, chronic treatment with DHEA has also been shown to reduce cocaine self-administration and reinstatement in rats(71).

Although we believe that DHEA/DHEAS plays a key role in the effect of finasteride on opioid self-administration, we also have evidence that other steroid species could be involved. Similarly, to DHEA, these other steroids have been shown to affect key neuronal pathways relevant to substance abuse. For instance, the products of 5αR, 3α-diol and allopregnanolone can act as positive allosteric modulators of $GABA_A$ receptors(72–76) and have thus been linked to neuronal stress response(77, 78). Therefore, by reducing allopregnanolone production, finasteride may reduce the negative affective state that contributes to opioid self-administration.

In this study, we performed most of the rat experiments with males since finasteride is mainly prescribed in men (although there is some history of use to treat hirsutism in women)(79) but our data suggest that both male and female rats showed reduced fentanyl self-administration upon finasteride treatment, raising the possibility that finasteride could be used as a treatment for OUD in both males and females, despite the differences in steroids levels between males and females(80).

Finasteride has been clinically used since the 1990s for the treatment of androgenic alopecia and benign prostate hyperplasia. Although it is considered a well-tolerated and relatively safe drug, there is evidence of sexual disfunction in 3.4 to 15.8 percent of men. A rare but serious side effect known as post-finasteride syndrome (PFS) has also been reported(81–83). PFS prevalence is unclear but it manifests as a range of persistent physical and neuropsychiatric disorders such as depression and anxiety that develop during or after discontinuation of finasteride use. Clinical studies will be needed to fully elucidate the treatment regime and understand the side-effects

associated with the use of finasteride for the treatment of OUDs, and careful clinical consideration must be given in weighing potential risks and benefits of finasteride use.

The optimal human dose for OUD would also have to be determined. The finasteride doses we used in rats (25-50 mg/kg, IP) are considerably higher than the dose regimens of 1-5 mg/day used in humans for alopecia and benign prostatic hyperplasia, respectively. The rat doses were selected empirically based on previous studies conducted in the Bortolato lab, showing that these doses are necessary to produce a significant reduction in 5α-reduced metabolites in rodents after acute treatment. It has also been shown empirically that the doses used in this study produce ameliorative effects in rodent models of Tourette syndrome (TS) akin to those observed within a 3-6 week period of 5 mg/day dosage in TS patients(84–86). It is important to note that the rat doses are single acute doses, whereas human dosing of finasteride is typically chronic. Finasteride is known to accumulate slowly with repeat dosing in humans, and the effects of finasteride persist for several days beyond what would be predicted based on compound half-life alone, which is attributed to persistent tight binding of finasteride to 5αR(87). Taken together, these factors suggest that the human dose necessary to treat OUD would be much lower than the acute dose used in our rat studies, but the optimal dose would need to be established empirically.

In conclusion, the present study identifies the widely-used drug finasteride as an effective agent for reducing opioid intake in both zebrafish and rat self-administration paradigms. The data further indicate that the DHEA/DHEAS pathway is a major mediator of finasteride's effect. These findings point to a promising potential therapeutic strategy in the fight against OUD and open new avenues for investigating the role of specific steroids in regulating opioid use behaviors.

Materials and Methods

Zebrafish

Adult fish treatment

Adult fish were transferred to a small treatment chamber (USplastic, USA) with 100 mL of fish water, and the compound of interest was injected directly into the water. Fish were allowed to swim in the treatment solution for one hour prior to the self-administration assay.

Zebrafish self-administration

The same protocol as detailed in Bosse et Peterson, 2017(18) was used to condition fish in small groups of 15 animals. For the screen, fish were conditioned for 4 days and treated with the different compounds on day 5, before being tested in the arena for 30 min. For opioid conditioning, we used the following doses: hydrocodone and morphine 6 mg/L and fentanyl 0.12 mg/L. Opioids were diluted in fish water. Between 60 to 100 animals were conditioned simultaneously and randomly assigned to different treatment conditions.

Food conditioning

The same apparatus was used as for hydrocodone conditioning. For food conditioning, fish were trained directly in the arena without performing the pre-conditioning protocol. Fish were trained for 50 min daily in a small group. Larvae food Ziegler #4 (VWR, USA) was suspended in fish water.

Steroid quantification

Brain extraction

Conditioned fish were transferred to a small treatment chamber (USplastic, USA) with 100 mL of fish water and treated with either DMSO (0.02 %) or finasteride (10 μM). Fish were allowed to swim in the treatment solution for one hour. Treated animals were then transferred to a water bath

with ice-water for euthanasia. The brain of each animal was then extracted in PBS 1X. The head was cut using a razor blade behind the gill, the skull was then carefully peeled to expose the brain using surgical forceps. The brain was then extracted by performing a cut at the base of the cerebellum. The extracted tissue was then placed in 1.5 mL self-standing microcentrifuge tube, (USAscientific, USA) on ice and the brains of 10 animals were pooled together in the same tube. Any liquid was then removed from each tube before weighing the tissues. Samples were then flash frozen in liquid nitrogen and placed at -80 °C until extraction.

Chemical

Reference standards were purchased from Steraloids (Newport, USA). All solvents were HPLC grade, and all other chemicals used were of the highest grade available. Stock neurosteroid standard mixture was prepared by mixing 5 µL of 1mg/mL solution of each steroid and adjusting the final volume to 1 mL by using methanol(88–90) All the stock solutions were stored at -80 ºC.

Sample preparation

Tissue samples were extracted as described previously(88–90). Briefly, tissue samples were extracted with 1 mL chloroform. The mixture was vortexed for 30 sec and centrifuged for 5 min; the chloroform layer was transferred to 2 mL tube and dried. The resulting residue was extracted with 1 mL methanol (MeOH). The MeOH layer mixture after 5 min centrifugation was added to the above chloroform extract. This mixture was dried and re-suspended in 125 µL MeOH and filtered using 5kD membrane filters. Filtrates were transferred to vials for UPLC-MS analysis.

UPLC-MS Analysis

Tissue sample extracts were subjected to UPLC-MS analysis for the measurement of neurosteroids, as described previously(88–90). UPLC analyses were carried out using a Waters Acquity UPLC system connected with the high-performance triple quadrupole mass spectrometer.

Analytical separations on the UPLC system were conducted using an Acquity UPLC C18 1.6 µ column (2.1 x 150 mm) at a flow rate of 0.15 mL/min and C18 1.7 µ column (2.1 x 50 mm) at flow rate 0.2mL/min. For the first column, the gradient was started with 100 % A (0.1 % formic acid in H_2O) and 0 % B (0.1 % formic acid in CH_3CN), after 0.1 min changed to 80 % A over 1 min, and then 45 % A over 5 min, followed by 20 % A in 2min. Finally, over 0.5 min, it was changed to 0 % A, then after 13 min, it was changed to the original 100 % A over 1 min, resulting in a total separation time of 13 min. For the second column, the gradient was started with 100 % A (0.1 % formic acid in H_2O) and 0 % B (0.1 % formic acid in CH_3OH), after 0.1min changed to 80% A over 2 min, and then 45 % A over 2 min, followed by 20 % A in 2min. Finally, over 1 min, it was changed to 0 % A, then after 8 min, it was changed to the original 100 % A over 2 min, resulting in a total separation time of 10 min. The elution from the UPLC column was introduced to the mass spectrometer. All MS experiments were performed by using electrospray ionization (ESI) in both positive ion (PI) and negative ion (NI) mode, with an ESI-MS capillary voltage of 3.5 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. The following MS conditions were used: desolvation gas at 400 l/h, desolvation temperature at 350 ºC and source temperature 150 ºC. Pure standards of all targeted neurosteroids were used to optimize the UPLC-MS/MS conditions prior to analysis and performing calibration curves (88–90). Reference standards were run before the first sample, in the middle of the runs and after the last sample to prevent errors due to matrix effect and day-to-day instrument variations. In addition, spiked samples were also run before the first sample and after the last sample to calibrate for the drift in the retention time of all neurosteroids due to the matrix effect. After standard and spiked sample runs several blanks were injected to wash the injector and avoid carry-over effects. Resulting data were processed by using Target Lynx 4.1 software (Waters) (88–90).

Data normalization

Steroids counts were first normalized using the initial weight of the tissue before extraction. To compare the levels of the steroids, we then used min-max normalization for steroid count in each sample.

Rats

Hydrocodone self-administration and nociception

Chemicals

Hydrocodone (Spectrum Chemical, USA), morphine (Spectrum Chemical, USA), and naloxone (Tocris, Bio-Techne) were dissolved in a solution of 2 % DMSO and 98 % saline. Finasteride (Astatech, Bristol, USA) was suspended in a solution of 5 % DMSO, 5 % Tween 80 and 90 % saline (5:5:90).

Hydrocodone self-administration.

Apparatus: The apparatus consisted of 8 operant conditioning chambers (Habitest, Coulbourn, USA), measuring 30.48 cm (W) x 25.4 cm (D) x 30.48 cm (H), and enclosed in sound-attenuating cubicles with ventilation fans. Each chamber was equipped with two retractable levers: an active lever coupled to the intravenous delivery of hydrocodone, and a control (inactive) lever. Active lever placement on the left or right side followed a counterbalanced order. Three cue lights were placed over the active lever. The apparatus was controlled by Graphic State 4 software (Coulbourn, USA).

Experimental procedure: Opioid self-administration was performed using a modified version of the protocol described by Mavrikaki et al(91). Rats weighing 225-250 g were used. Sprague-Dawley male rats (Charles River, USA) were anesthetized with ketamine and xylazine and underwent catheterization surgery. Briefly, a polyurethane catheter was inserted through the

external jugular vein, passed under the skin, and fixed in the mid scapular region. Post-operative care included buprenorphine and enrofloxacin for analgesic and antibiotic management, respectively. Catheter patency was maintained through daily flushing with a heparin (500 IU/mL) / 50 % dextrose solution.

Ten days after surgery, all rats were gently handled and kept under a food restriction regimen that maintained them at 90 % of their initial body weight and was continued throughout the whole behavioral procedure. A syringe containing a hydrocodone solution was placed in an infusion pump located outside the chamber and connected to the rat's catheter via a fluid swivel and spring-covered Tygon tube suspended through a counterbalanced swivel. The solution was administered at a dose of 0.016-0.128 mg/kg/infusion, in a volume of 160 µL/kg/infusion. Operant training began three days later and consisted of three stages of fixed-ratio reinforcement schedule: FR1, FR2, and FR5. Rats underwent daily, 1 h-long experimental sessions, between 9:00 AM and 3:00 PM and for 7 days/week, consisting of a sequence of trials (Figure 3). Each trial began with a 5-s period, during which the house light was turned off and the cue light blinked three consecutive times. Subsequently, the house light was turned on and both levers were extended. Once the rat completed the fixed ratio on either lever , both levers retracted, and a new trial began after a 15-s time-out period. Each rat progressed from FR1 to FR2 and from FR2 to FR5 after reaching stability, defined as >70 % of total lever pressed on the active lever for three consecutive days. FR1 and FR2 stability criteria were reached from day 12 through 19 and from day 15 through day 23 of training, respectively. All animals reached FR5 stability by day 31 of training and were treated with either finasteride or its vehicle.

Naloxone-precipitated opioid withdrawal.

Long Evans male rats (180-225 g) (Charles River, USA) received subcutaneous injections of morphine with the regimen previously described (cumulative doses of 5, 10, 20, 30, and 40 mg/kg per day within five days). On day 6, rats received an acute dose of morphine (40 mg/kg, SC), followed by either finasteride (50 mg/kg, IP, 100 min later) or its vehicle, and naloxone (1.5 mg/kg, IP, 120 min later), and were immediately placed inside a Plexiglass chamber with bedding. Animals were video-recorded for 30 min, and blinded observers monitored their opioid-withdrawal signs, including wet dog shakes, jumps, grooming, and digging.

Neuropathic pain assessment

Spinal nerve ligation: Sprague-Dawley rats (Charles River, USA) weighing 150-180 g were used. Following 2-3 days of handling, rats underwent mechanical nociception testing via von Frey Hair and Randall-Selitto tests. Neuropathy was then induced by SNL surgery, as previously described(41). Rats were anesthetized using xylazine and ketamine (10/75 mg/kg, IP), and their left L5 spinal nerve was exposed and tightly ligated with 4.0 silk suture (Mersilk®, Ethicon thread, Johnson). Muscle, fascia, and skin were then sutured, and the rats were treated with enrofloxacin (10 mg/kg, SC) and carprofen (5 mg/kg, SC) for post-operative care. Fourteen days after surgery, nociception was re-tested, and allodynia was confirmed in rats exhibiting a >30% reduction of their pain threshold. Rats were then assigned to different treatment groups to receive either morphine (1-3 mg/kg, SC), hydrocodone (1-10 mg/kg, SC) or saline. The antinociceptive effects of opioids were tested every 30 min for 6 consecutive observations. The analgesic effects of morphine and hydrocodone (at their most effective doses) were also tested in combination with finasteride (50 mg/kg, IP) or its vehicle, to ascertain whether finasteride altered the antiallodynic

properties of opioids. The effects of opioids and finasteride were also tested for thermal nociception in a separate group of rats with SNL using the hot plate procedure.

von Frey Hair Test: Tactile allodynia was assessed using a set of 8 von Frey monofilaments (Bioseb, Vitrolles, France) with logarithmic incremental stiffness (of 1.4, 2, 4, 6, 8, 10, 15 and 26 g). Paw-withdrawal threshold was measured, and 50 % response threshold was calculated using the Up-Down method and Dixon´s formulae, as previously described(42). Behavioral assessments were run prior to and 14 days after SNL surgery. Rats were individually placed in plexiglass compartments (17 x 11 x 13 cm) with a wire mesh bottom that allowed full access to paws. After 20-30 min of acclimation, a first 6-g hair was perpendicularly applied against the plantar surface of the left hind paw for 6 s. Paw withdrawal and/or licking reflex was considered as a positive response. Depending on the positive or negative response, the next filament with either lower or higher force was tested, respectively. Testing continued until either four consecutive negative or five consecutive positive responses were recorded after the first change of direction.

Randall-Selitto Test: Nociceptive withdrawal threshold was assessed using the Randall-Selitto algesimeter (Ugo Basile, Varese, Italy), as previously described(43). Following daily handling and acclimation to the apparatus, rats were wrapped into a cotton cloth and immobilized. The medial portion of the plantar surface of the left hind paw was carefully placed on the device's tip. An increasing mechanical force was applied until a withdrawal response was observed. Paw withdrawal threshold for Randall-Selitto experiment is set at 25 g of force applied. Rats were tested every 30 min for three consecutive hours following treatment (6 applications in total). For the assessment of morphine withdrawal-induced hyperalgesia, rats with SNL were subjected to a cumulative morphine treatment (as described above) for five days. On day 6, the effects of finasteride on naloxone-precipitated opioid withdrawal were tested using the Randall-Selitto

algesimeter immediately before naloxone treatment, as well as 30 and 60 min later. Testing was performed in both lesioned and non-lesioned rats.

Hot Plate Test. Thermal nociception was assessed using the hot plate analgesia meter (IITC Life Science, Woodland Hills, USA). The rat was placed on a plate maintained at different temperatures (48.5 and 51.5 °C), and their progressive latencies to lick the left hind paw were measured. Testing was performed in both lesioned and non-lesioned rats.

Fentanyl self-administration

Subjects

A total of 20 adult male and adult female Wistar rats (Charles River, USA) weighing 200 – 475g at the start of the experiment were individually housed and kept on a 12-h light/ 12-h dark cycle in a temperature and humidity-controlled room. Animals were provided food and water *ad libitum*.

Drugs

Fentanyl Citrate (Medisca, USA) was dissolved in deionized water at a concentration of 50 μg/mL. Finasteride (Astatech, USA) was suspended (50mg/mL) in a solution of 2.5 % ethanol, 5 % Tween80, and 92.5 % saline.

Fentanyl self-administration

Apparatus: Oral fentanyl self-administration tasks were completed in eight modular operant chambers (Med Associates, USA), equipped with a liquid magazine tray stationed between two nose-poke devices. Additionally, the operant chamber was outfitted with a solenoid controlled liquid valve (Lee Valves, USA) and a set of audiovisual cue equipment [house light, magazine light, and a tone generator].

Experimental procedure: A operant oral self-administration behavioral assay described by Shaham and colleagues(92) was utilized. Rats were trained to obtain liquid fentanyl delivered into a liquid

magazine tray following an operant response on an FR1 reinforcement schedule. During the self-administration sessions, a nose-poke in the active port (counterbalanced between animals) resulted in fentanyl delivery (0.02 mg/kg/delivery). Concurrent with drug delivery was a 10s audiovisual conditioned stimulus (CS) comprised of a 1s illumination of a light inside the nose-poke port, a 10s tone, and a 10s illumination of a light stationed above the liquid magazine tray. Any additional nose-pokes during the 10s CS were without consequence. Drug availability at the start of each session and following CS presentations was signaled by illumination of the house-light placed on the wall opposite of the nose-poke ports. All nose-pokes in the inactive port were without consequence. Animals were given two 30-min magazine training sessions, during which any active responses resulted in fentanyl and CS presentation; however, if the animal made no responses within 2-3 min of the last drug delivery (or start of the session) a non-contingent fentanyl and CS delivery occurred. Following these two training sessions, animals had 15, 1-hour sessions to self-administer fentanyl for 5 days/week. During these 15 sessions, all animals reached a response criterion of >70 % of nose-pokes occurring at the active nose-poke port. To test the effect of finasteride on fentanyl consumption animals received 10 additional self-administration sessions across 12 days, in which finasteride (50 mg/kg, 1mL/kg) or vehicle (1 mL/kg) was administered intraperitoneally 45 min prior to the session. Each treatment (vehicle or finasteride) was given for 5 consecutive days, with a 48-hour period before they received the opposite treatment. The order of treatment administration was counterbalanced across animals. Following self-administration sessions, mg/kg of fentanyl consumed was calculated for each animal by subtracting the amount of fentanyl left in the magazine tray from the total amount of drug delivered.

Statistical analyses

For zebrafish self-administration data, R graphic programming was used to generate the plots. ANOVA tests were run on plot data to test significance. ANOVA tests were performed first on inactive platforms for each dataset to validate that there was no difference between the different conditions, and then active platform values were used to test for significance. All boxplots were generated using R graphic programming and the *ggplot* module. The lower and upper hinges correspond to the first and third quartiles. The line is the median. The whiskers extend from the hinged to the maximum or minimum value at most 1.5x the inter-quartile range (IQR) from the hinge. Data points beyond that are considered outliers. No data points were excluded from the statistical analysis. For the experiment in rats, the specific statistic test has been specified in each figure legend and also what the error bar represented for each figure. A p-value of less than 0.05 was considered significant.

Study approval

All animal studies were approved by the University of Utah and the University of Washington Institutional Animal Care and Use Committees (IACUC). All zebrafish experiments were approved by the University of Utah Institutional Animal Care and Use Committee. Hydrocodone self-administration studies and nociception studies in rats were compliant with the National Institute of Health guidelines and approved by the IACUC of the University of Utah. The fentanyl self-administration studies in rats were conducted under the guidance and permission of the Institutional Animal Care and Use Committee at the University of Washington and pursuant to federal regulations regarding work with animals.

Author contributions

G.D.B designed the experiments and performed the zebrafish assays, analyzed the data and wrote the manuscript with R.T.P. and M.B. R.C. designed the nociceptive experiments in rats and performed the experiments. G.F. designed and performed the hydrocodone self-administration assay in rats assisted by E.V. T.Z. contributed to experiment design. R.T.P. designed and supervised zebrafish experiments. M.B. designed, analyzed and supervised the experiments on rat hydrocodone self-administration experiments and nociception. N.W.G. performed steroid extraction and quantification R.D.F. J.S.L designed and performed the fentanyl self-administration experiment in rats. R.D.F and P.E.M.P. designed and analyzed the fentanyl self-administration in rats. All authors contributed to data interpretation and commented on the manuscript.

Acknowledgments

We thank the NIDA Drug Supply Program for providing hydrocodone bitartrate powder. We thank Chelsea Herdman for helpful comments and bioinformatic support. We thank Karen Odeh and Stefanos Loizou for their technical support. We would like to acknowledge the Centralized Zebrafish Animal Resource (CZAR) at the University of Utah for providing Zebrafish husbandry, laboratory space, and equipment to carry out portions of this research. Expansion of the CZAR is supported in part by NIH grant # 1G20OD018369-01. This work was supported by the Charles and Ann Sanders Research Scholar Award, the L. S. Skaggs Presidential Endowed Chair, and NIH grant R21DA049530 (to M.B. and R.T.P). Oral rodent fentanyl studies were supported by National Institute of Drug Abuse (R01-DA039687. G.D.B. was supported by the Canadian Institutes of Health Research (CIHR) fellowship program. R.D.F. was supported by NIDA (T32-DA007278). T.Z. was supported by NHGRI (T32 HG008962).

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Figures and figure legends

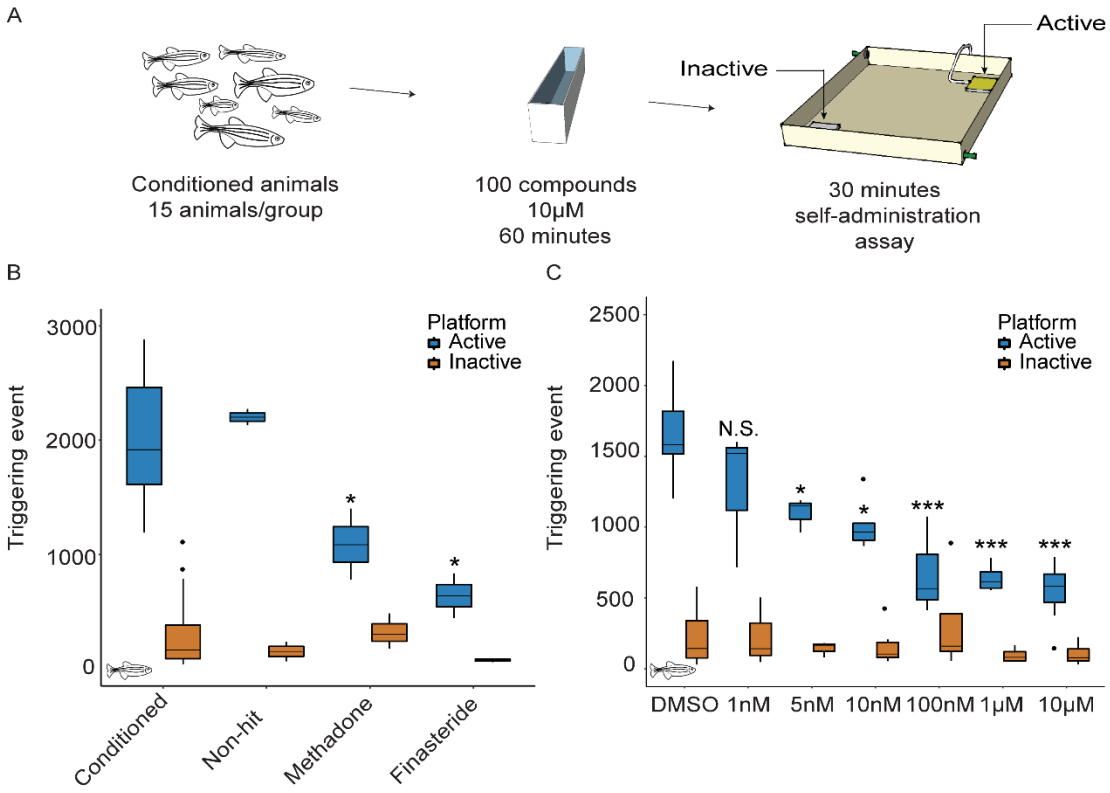

Figure 1. Small molecule screen for modulators of opioid self-administration.

A. Conditioned animals were treated with the different molecules at 10 µM for 60 min before assessing their opioid self-administration for 30 min. **B**. Known small molecules affect opioid self-administration. Treatment with methadone (n=3), and finasteride (n=2) significantly reduces the number of triggering events at the active platform. Conditioned fish: n=56. No difference was observed at the inactive platform. *p*-value calculated with Student's T-test compared to conditioned animals. **C**. Dose response experiment for finasteride. Three doses, 100nM, 1µM, 10µM, reduce the number of triggering events below our threshold of 1000 activations. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform [F(6,36)=1.06, p=0.40] and Active platform [F(6,36)=24.60, p=2.31E-11], compared to the DMSO control. No significant difference detected for the inactive platform. DMSO: n=16, 1nM: n=3, 5nM: n=5, 10nM: n=6, 100nM: n=5, 1µM: n=4, 10µM: n=7. * *p*-value <0.05, ** *p*-value < 0.01, *** *p*-value < 1E-5. Each n represents a group of 15 animals. These experiments were performed using a between subject design. All boxplots were generated using R graphic programming and the *ggplot* module. The

lower and upper hinges correspond to the first and third quartiles. The line is the median. The whiskers extend from the hinged to the maximum or minimum value at most 1.5x the inter-quartile range (IQR) from the hinge. Data points beyond that are considered outliers.

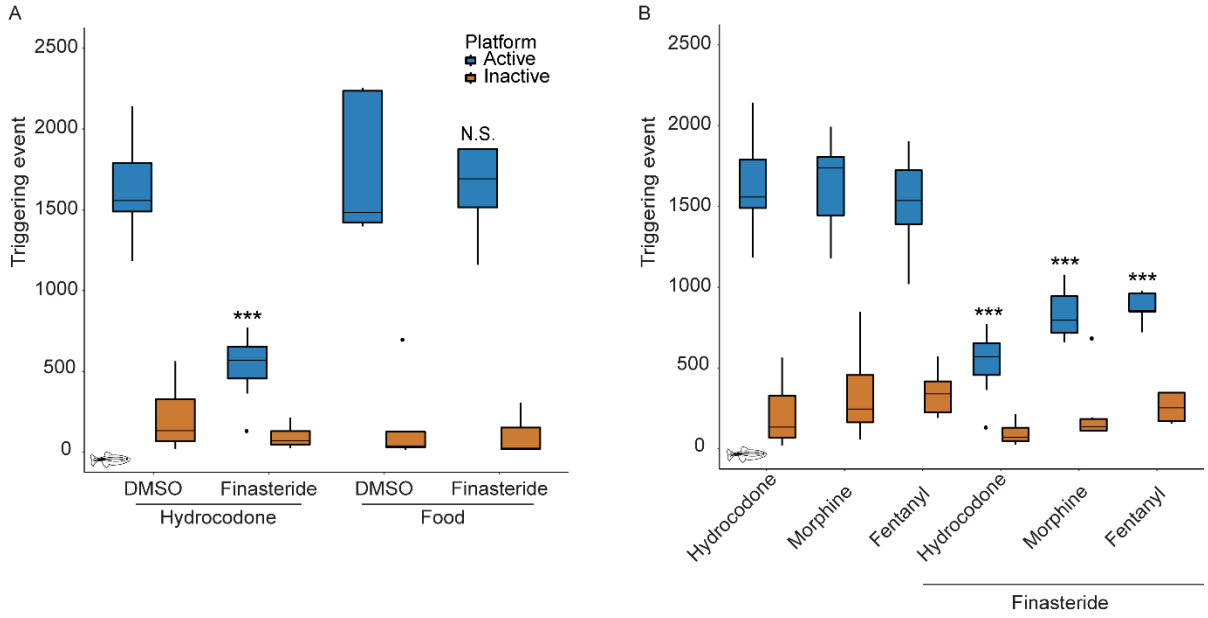

Figure 2. Finasteride effectively reduces the self-administration of different opioids, but does not affect all motivated behaviors.

A. Finasteride reduces opioid self-administration without affecting food-seeking behavior. Finasteride significantly reduces the number of triggering events at the active platform compared to DMSO control for hydrocodone. No difference was detected for fish conditioned to self-administer food. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform [F(3,29)=1.05, p=0.38] and Active platform [F(3,29)=19.37, p=4.32E-07], compared to respective DMSO control. Opioid trained animals: DMSO: n=16, finasteride: n=7. Food seeking: DMSO: n=5, Finasteride: n=6. These experiments were performed using a between subject design. **B**. Finasteride affects opioid self-administration for animals conditioned with 3 different opioids. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform [F(5,81)=1.57, p=0.18] and Active platform [F(5,81)=31.68, p=4.52E-13], compared to respective control. Hydrocodone control: n=16, Hydrocodone+Finasteride: n=7, Morphine control: n=7, Morphine+Finasteride: n=6, Fentanyl control: n=7, Fentanyl+Finasteride: n=5. No significant difference was detected for the inactive platform in any condition. * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 1E-5. Each n represents a group of 15 animals. Data for hydrocodone treatment alone reproduced from

Figure 1C for comparison. These experiments were performed using a within subject design. All boxplots were generated using R graphic programming and the *ggplot* module. The lower and upper hinges correspond to the first and third quartiles. The line is the median. The whiskers extend from the hinged to the maximum or minimum value at most 1.5x the inter-quartile range (IQR) from the hinge. Data points beyond that are considered outliers.

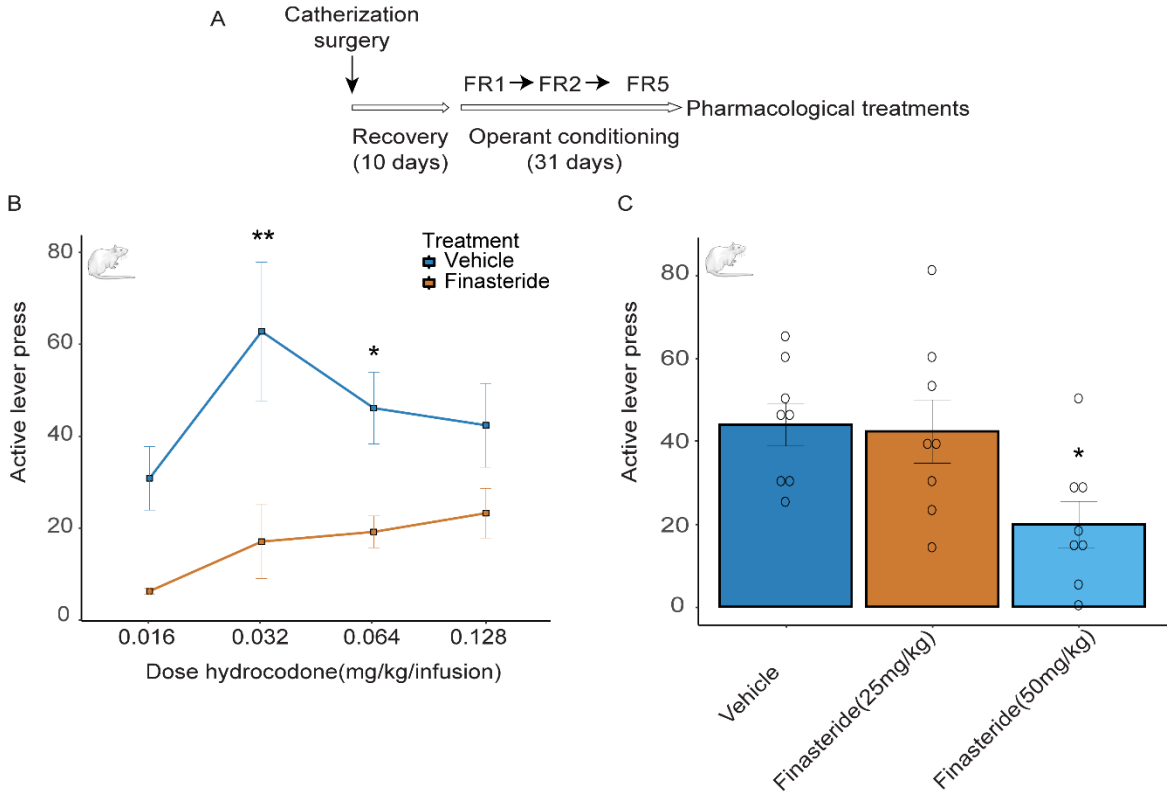

Figure 3. Finasteride treatment reduces opioid self-administration in rats.

A. Rats were conditioned to self-administered hydrocodone and after establishing robust FR5, they were treated with either vehicle or finasteride. **B**. Active lever presses for animals trained with different concentrations of hydrocodone, a total of 6 animals per conditions were tested. Treatment with finasteride (50mg/kg) reduces opioid self-administration of hydrocodone for animals conditioned with 0.032 and 0.064 mg/kg. *p*-value corrected for multiple comparison two-way ANOVA [F(1,38)=30.5, p=0.0001]. *p*-value compared vs vehicle-treated animals **p*-value < 0.05, ** *p*-value < 0.01. **C**. IP injection with 50 mg/kg, but not 25 mg/kg finasteride reduces the number of active lever presses for *0.064 mg/kg hydrocodone*, 8 animals were treated for each dose of finasteride. *p*-value corrected for multiple comparisons on one-way ANOVA [F(2,21)=4.69, p=0.02]. Error bars represent means +/- s.e.m. Adult male Sprague-Dawley rats were used to

perform the hydrocodone self-administration assay. These experiments were performed using a within subject design.

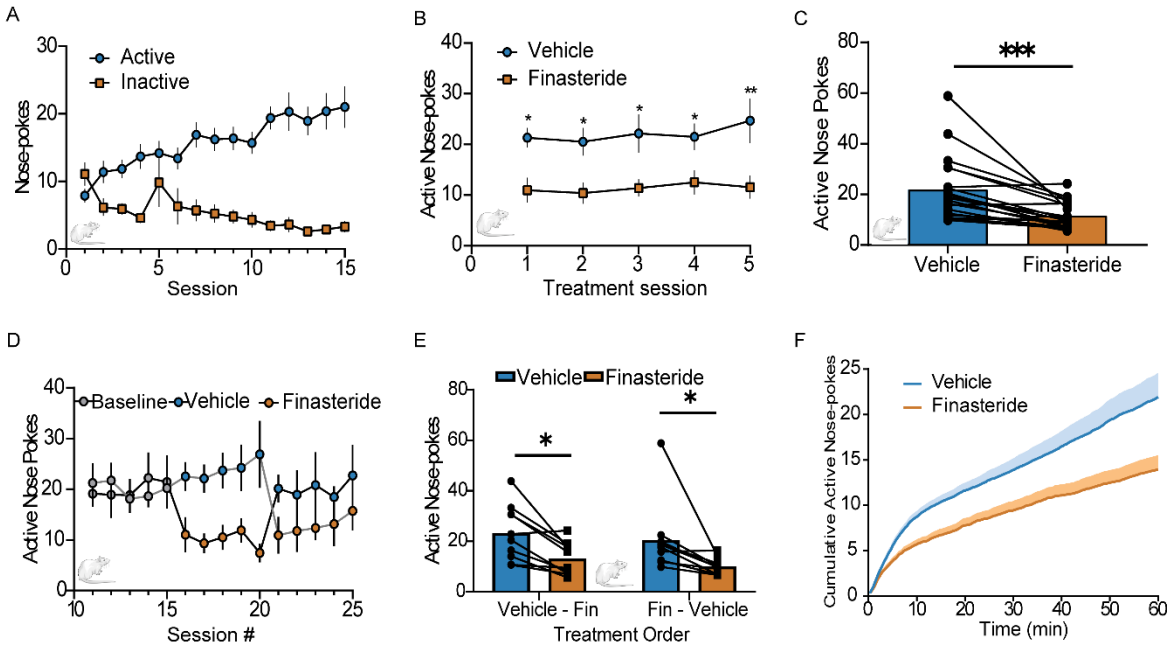

Figure 4. Finasteride decreases operant response for oral fentanyl self-administration.

A.Operant nose-poke responses at the active (blue circles) and inactive (orange squares) nose-poke ports during baseline fentanyl self-administration sessions (n=20 rats). **B.** Daily finasteride injections (IP, 50mg/kg) (orange squares) decreased active nose-pokes compared to vehicle injections (blue circles). A main effect of drug treatment was observed, Sidak post-hoc analysis was performed for multiple comparisons on mixed-effect model [$F(1,19)=20.77$, $p=0.0002$]. **C.** Average operant responses during vehicle (blue) and finasteride (orange) treatment for each individual animal. A paired t-test revealed finasteride significantly decreased animals' active responding for fentanyl delivery [$t(19)=4.481$, $p=0.0003$]. **D & E.** Animals received daily injections of finasteride during sessions 16-20 (n=10 rats, D: black line, E: Finasteride-Vehicle) or during sessions 21-25 (n=10 rats, D: gray line, E: Vehicle - Fin). **D.** Baseline refers to animals responding during sessions 10-15. **E.** Order of treatment had no interaction with the effect of the treatment[$F(1,18)=0.007$, $p=0.93$]. A main-effect of treatment was observed [$F(1,18)=19.03$, $p=0.0004$]. Sidak analysis was performed for multiple comparisons on two-way ANOVA. **F.** An average cumulative plot of active nose-poke responses during the vehicle- (blue) or finasteride-(orange) treated sessions (n=20). *p-value < 0.05, ** p-value < 0.01, ***p-value < 0.001. Error

bars represent the mean +/- s.e.m. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay. These experiments were performed using a within subject design.

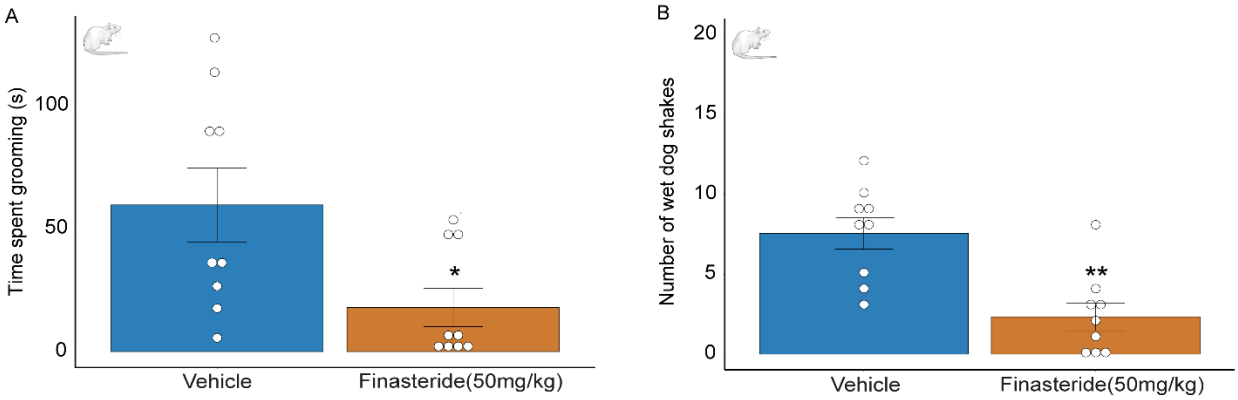

Figure 5. Finasteride reduces the physical effects of opioid withdrawal.

Animals were treated with escalating doses of morphine for five days. On day 6, rats received an acute dose of morphine (40 mg/kg, SC) followed by either finasteride (50mg/kg, IP) or vehicle. The opioid receptor antagonist naloxone (1.5mg/kg, IP) was administered 20 minutes later and animals were placed in a plexiglass chamber and their behavior was then recorded for 30 minutes. **A**: Animals in withdrawal spent less time grooming after finasteride treatment. **B**: Acute injection of finasteride reduces the number of wet-dog shakes in animals in morphine withdrawal. **p*-value < 0.05, ** *p*-value<0.01. Error bars represent means +/- s.e.m. n=9 for both conditions. *p*-value calculated using unpaired t test with Welch's correction between finasteride- and vehicle-treated animals. Adult male Long Evans rats were used to test naloxone-induced withdrawal. This experiment was performed with a between-subject design and blind analysis.

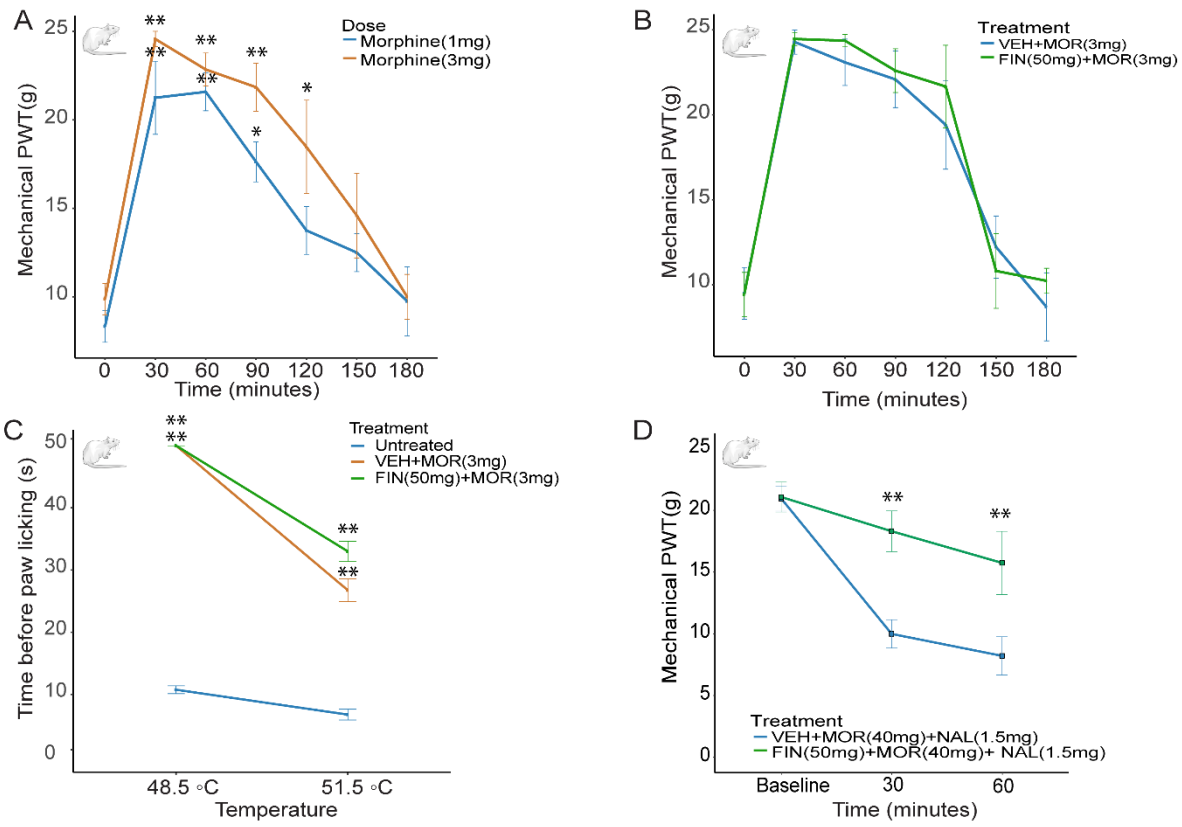

Figure 6. Finasteride does not affect the antinociceptive effect of opioids in a neuropathic pain model.

A. Paw withdrawal thresholds (PWT) to the Randall-Selitto test. Pain tolerance was measured over 180 min after treatment with different doses of morphine. Both doses of morphine significantly increased PWT compared to animals tested immediately before injection. Two-way ANOVA significant effect of Time [$F(6,72)=31.09$, $p<0.0001$]. Morphine 1mg/kg: n=6, Morphine 3mg/kg: n=8 per condition. **B**. Co-treatment with finasteride (50mg/kg) did not block the antinociceptive effect of morphine (3mg/kg). Two-way ANOVA significant effect of Time [$F(6,48)=45.31$, $p<0.0001$], but no significant effect of treatment [$F(1,8)=0.19$, $p=0.68$] and no effect of interaction [$F(6,48)=0.37$, $p=0.89$]. N=5 per condition. **C**. Finasteride did not affect the thermal antinociceptive effect of morphine as measured by the time before paw lick in response to different temperatures 30 min after treatment with morphine. Two-way ANOVA 48.5°C significant effect of Treatment [$F(2,9)=770$, $p<0.0001$], 51.5°C significant effect of Treatment $F(2,9)=33.12)p<0.0001$. Both vehicle+morphine and finasteride+morphine are significantly different from untreated animals. No difference observed between vehicle+morphine and

finasteride+morphine. N=4 per condition. **D:** Finasteride reduces hyperalgesia associated with opioid withdrawal. Rats subjected to SNL and treated with a 6-day escalating morphine treatment were injected with finasteride (50mg/kg, IP) or vehicle. Mechanical nociception was measured immediately, on the injured paw, after naloxone treatment, and repeated 30 and 60 min later. Two-way ANOVA significant effect of Interaction [F(2,20)=4.53, p=0.024], Time [F(2,20)=19.4, p<0.0001], Treatment [F(1,10)=13.1, p=0.0046].*p-value < 0.05, ** p-value < 0.01. Error bars represent means+/- s.e.m. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect and adult male Long Evans rats were used for the naloxone-precipitated withdrawal experiment . These experiments were performed using a between subject design.

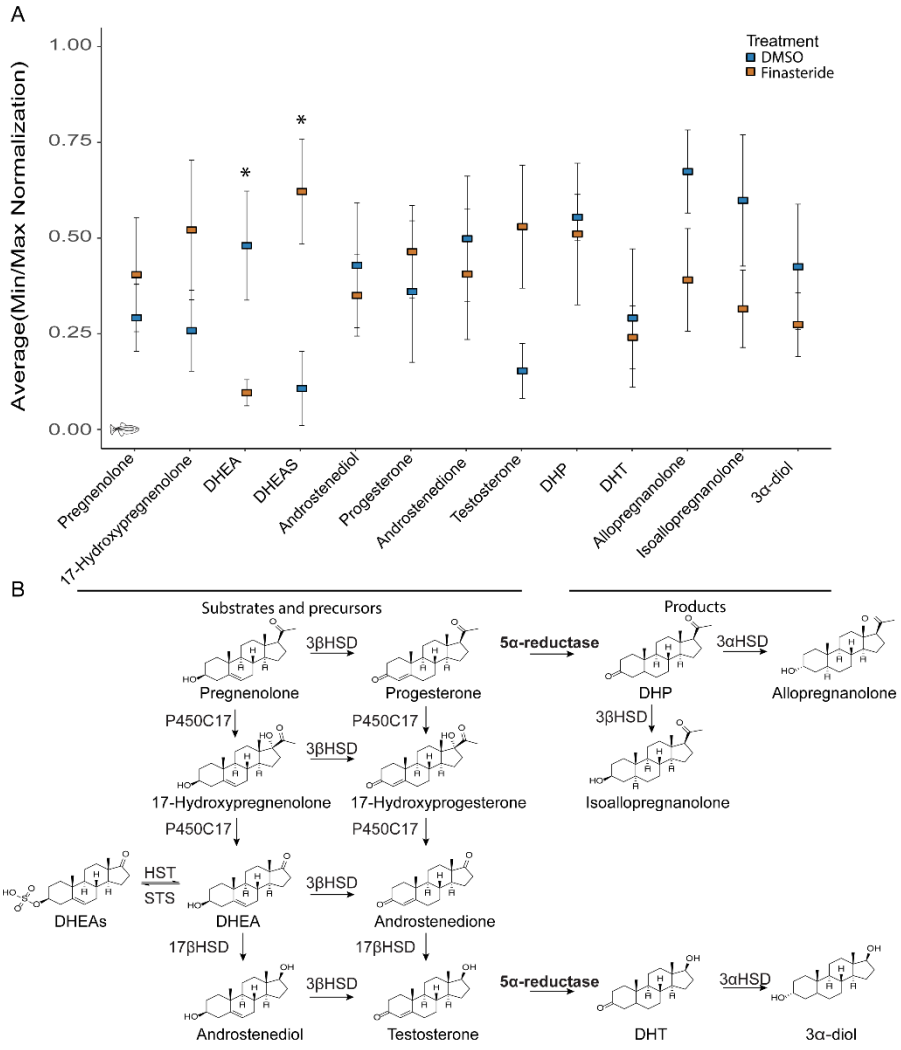

Figure 7. Finasteride treatment changes neurosteroid levels in the conditioned animal brain. **A**. Normalization score for the quantification of steroids in conditioned brains treated with DMSO or finasteride (10 µM). *p*-value calculated with Student's T-test. N=5 set of 10 brains per condition. **p*-value < 0.05 **B**. Partial neurosteroidogenesis pathways. Finasteride blocks the rate limiting enzyme, 5α-reductase, causing accumulation of upstream neurosteroid species.

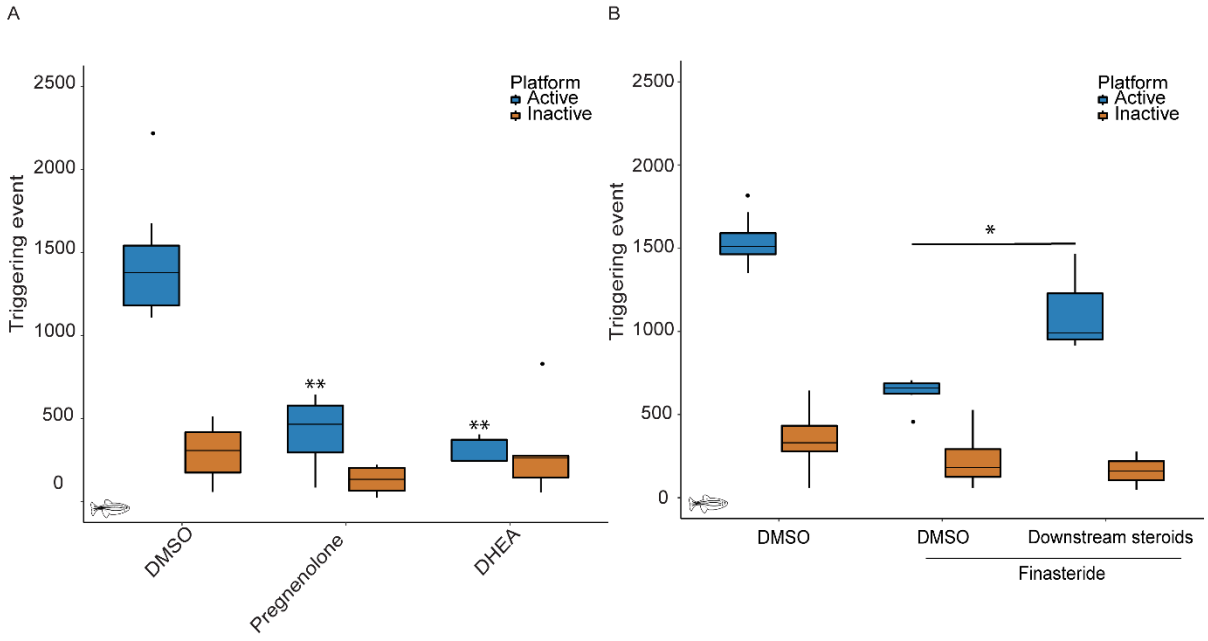

Figure 8. Specific neurosteroids also affect opioid self-administration.

A. Incubation with steroids upstream of 5αR, DHEA (10 µM) or pregnenolone (10 µM) reduces the number of triggering events at the active platform. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform [F(2,21)=2.18, p=0.14] and Active platform [F(2,21)=51.09, p=8.57E-09]. No significant difference was detected for the inactive platform compared to the DMSO control. DMSO: n=12, Pregnenolone: n=8, and DHEA: n=5. **B**. Co-treatment with finasteride (10 µM) and a selection of steroids downstream of 5αR partially blocks the reduction in opioid self-administration induced by finasteride. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform [F(2,16)=1.58, p=0.24] and Active platform [F(2,16)=68.93, p=1.37E-08]. No significant difference was detected for the inactive platform. DMSO: n=8, Finasteride: n=8, Finasteride+downstream steroids: n=3. **p*-value < 0.05, ** *p*-value < 0.01. Each n represents a group of 15 animals. These experiments were performed using a between subject design. All boxplots were generated using R graphic programming and the *ggplot* module. The lower and upper hinges correspond to the first and third quartiles. The line is the median. The whiskers

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