Supplementary information for :

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

Supplementary material

Zebrafish

Fish handling

Adult zebrafish (*Danio rerio*) of the wild type Ekkwill (Gibsonton, FL, USA)strain were maintained in the fish facility at 28–29°C with a 14/10 hours light/dark cycle. All zebrafish experiments were approved by the University of Utah Institutional Animal Care and Use Committee. All fish experiments were performed on mixed population (50 % male/female).

Chemicals used

Finasteride (Sigma-Aldrich, USA), hydrocodone bitartrate (Spectrum Chemicals, USA and NIH), morphine (Spectrum chemicals), fentanyl (Spectrum Chemicals, USA), dutasteride (Cayman chemical company, USA), pregnenolone (Sigma-Aldrich, USA), DHEA (Sigma-Aldrich, USA), methadone (Sigma-Aldrich, USA), Allopregnanolone (Tocris, USA), 3α-diol (Steraloids, USA), androsterone (Steraloids, USA), fentanyl (Sigma-Aldrich, USA). Compounds were either re-suspended in DMSO or water according to manufacturer recommendations.

Fish locomotion

A high-speed infrared camera (Point Grey, Canada) was used to record 1-min videos of animals in the self-administration arena. Zebrafish tracking and movement quantification was performed with the software ActualTrack (United Kingdom).

Single-cell data analysis

Processed single cell data from *Raj et al. Nat Biotechnol.*(1) from 6 individual whole zebrafish brains (f1 n=6,759, f2 n=7,112, f3 n=15,156, f4 n=12,121, f5 n=9,919, f6 n=6,009) as well as manually dissected fore- (n=3,615), mid- (n=1,504) and hindbrains (n=3,894) were downloaded as an R data object from Gene Expression Omnibus (GSE105010). The R package Seurat (v3.1.1)

was used to create a new Seurat object from the raw counts data found within the downloaded InDrops object. Samples with less than 200 genes detected were already removed from these data, but we additionally removed outliers with more than 6000 genes detected and those higher than 25 % mitochondrial genes. This further processing removed an additional 371 samples (cells). We followed the SCTransform workflow as recommended in the Seurat vignette (https://satijalab.org/seurat/v3.1/sctransform_vignette.html) for scaling, normalization, and finding variable genes. Mitochondrial mapping percentage was regressed during normalization. A principal component analysis was first performed, then clustering using the Seurat FindNeighbors (using the first 30 dimensions) and FindClusters function (resolution of 2.5). The data were visualized using t-SNE dimensionality reduction using the first 30 dimensions. Similar to the published analysis in *Raj et al. Nat. Biotechnol*., we observed that the 65,718 samples/cells produced 61 clusters. t-SNE coordinates, expression values from the SCT data slot, and metadata were exported from the Seurat data object to create t-SNE figures using data.table and ggplot2 demonstrating the cluster identities, broad brain regions (fore-, mid- and hindbrain) and srd5a family expression. We plotted all family members highlighting only those cells in the foreground with relatively medium to high expression values where expression cut-offs were determined for each marker individually based on expression distribution (srd5a1 0.8-3.0; srd5a2a 1.0-3.0; srd5a2b 1.0-3.0; srd5a3 0.8-3.0)(1–7).

Rats

Hydrocodone self-administration and nociception

Animals

Adult male Sprague-Dawley and Long-Evans rats (Charles River, Roanoke, USA) were group-housed (3-4/cage) within rooms maintained at 22 ± 2 °C and 55 % humidity, on a 12/12 h light/dark

cycle (lights on at 7:00 AM). Food and water were available *ad libitum.* Following a 7-day acclimation to the housing facilities, animals were handled daily for 5 min. Behavioral measurements were carried out and analyzed by trained experimenters in a blinded fashion.

Locomotor activity

Rats were tested for locomotor activity in an open field surrounded by black Plexiglas walls (47 cm x 47 cm x 47 cm). Comparisons were drawn between self-administering animals (immediately after their performance in the operant chamber) and control animals not receiving any opioids. The locomotor analysis was performed using EthoVision XT 14 pathway tracking software (Noldus Instruments, Wageningen, The Netherlands).

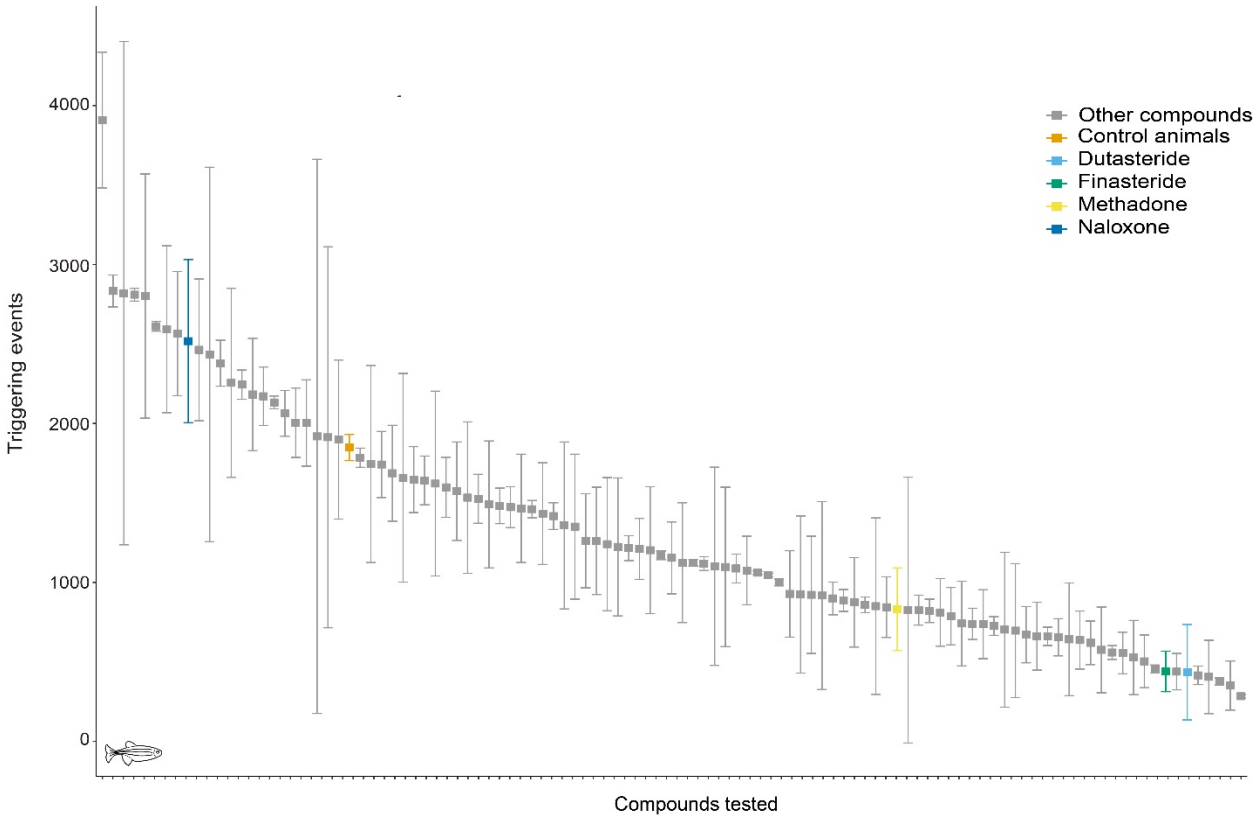

Figure S1: Results from the small molecule screen.

Overview of the effect on the number of triggering events at the active platform for each of the 110 molecules tested during the screen. The average of triggering events for untreated conditioned groups of fish (15 fish per group, 46 groups tested) compared to the average for conditioned fish treated with each candidate molecule (15 fish per group, 2 groups per compound). Each compound was tested at 10 µM. Error bars represent means +/- s.e.m. These experiments were performed using a between-subject design. Two molecules were omitted from this figure due to a drastic effect on normal fish behavior (hypolocomotion, lethality). The name of each molecule is presented in Table S1.

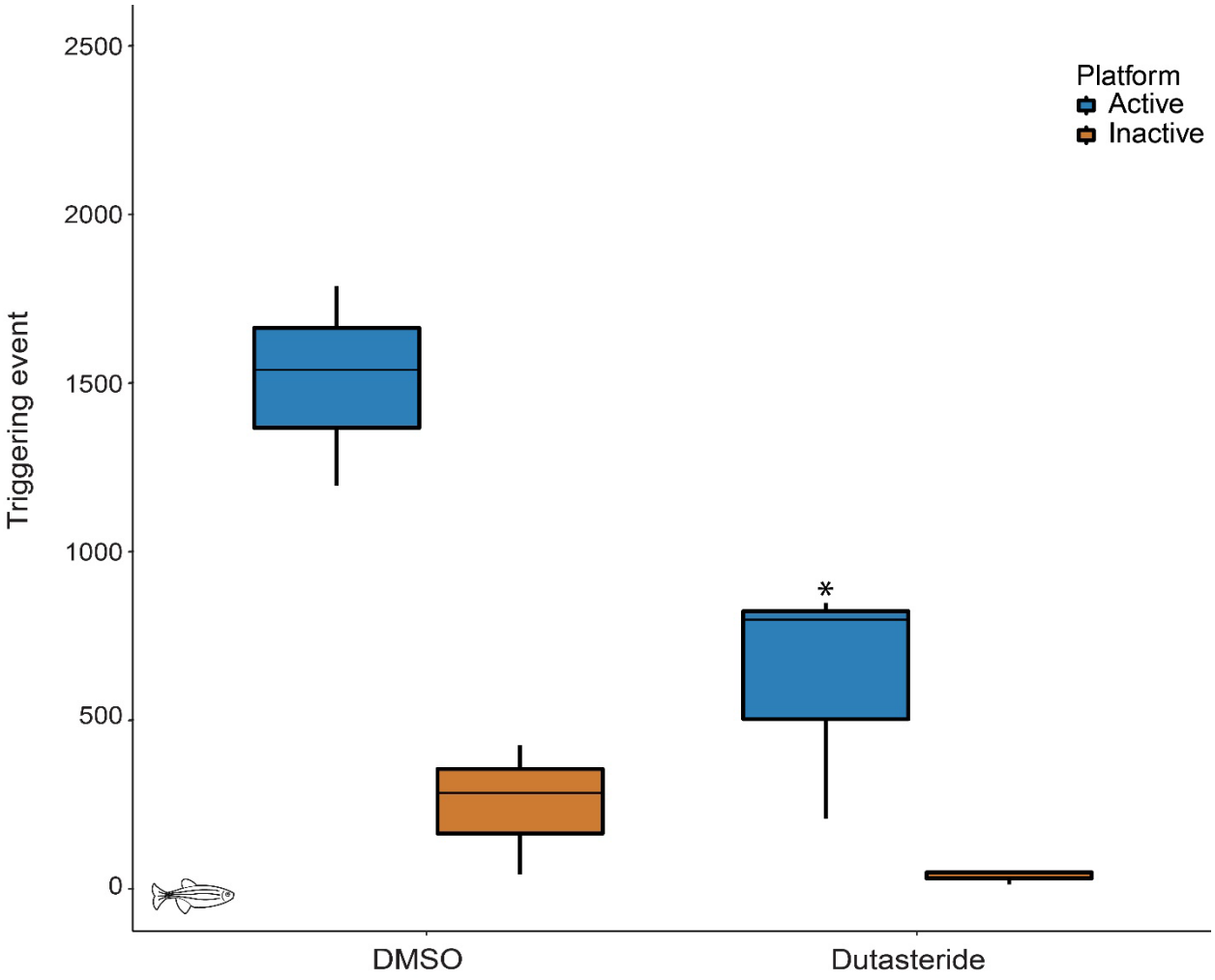

Figure S2: Dutasteride reduces opioid-self-administration.

An additional 5α-reductase inhibitor, dutasteride (10μM) also reduces the number of triggering events at the active platform. *p*-value was computed by TukeyHSD on one-way ANOVA, no significant difference was observed at the inactive platform [F(1,4)=3.65)p=0.13] and reached significance for the active platform [F(1,4),11.01, p=0.03]. DMSO: n=3, Dutasteride: n=3. **p*-value < 0.05. Each n represents a group of 15 animals. 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.

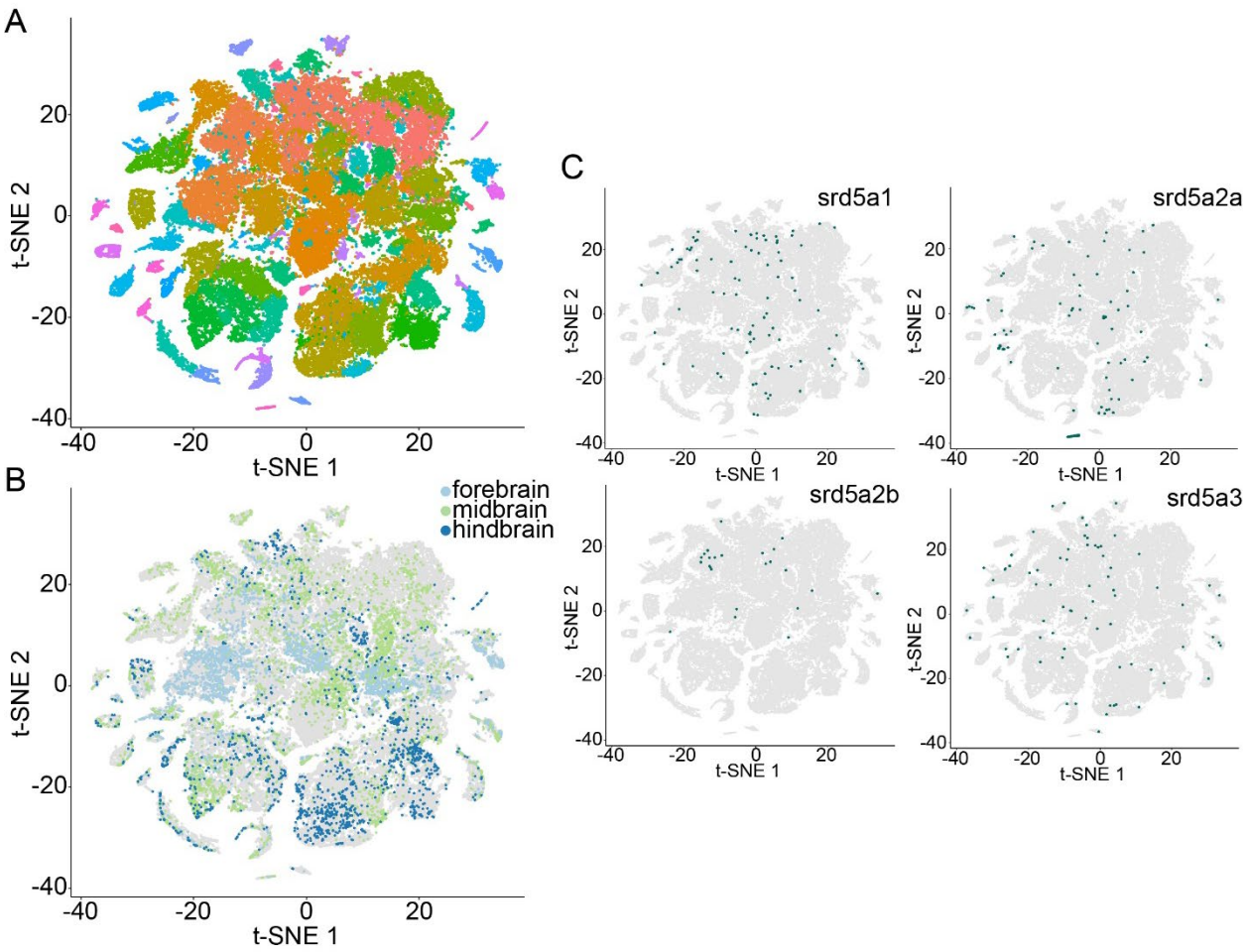

Figure S3: Srd5a family members display scattered expression in multiple brain regions. Single Cell RNA-seq data from *Raj et al. Nature Biotechnology* was reanalyzed to investigate Srd5a family member expression in specific regions of the zebrafish brain. Individual cells are visualized as unique dots on the above plots using t-SNE dimensionality reduction. **A.** t-SNE plot representing the 61 clusters produced by the 65,718 cells from the zebrafish brain. Cells cluster together based on gene expression and the various clusters are shown by color. **B**. To demonstrate the broad brain regions across the single-cell data, samples originating from manually dissected fore-, mid- and hindbrains are highlighted in light blue, green, and dark blue respectively in this t-SNE plot. The cells colored grey originate from whole-brain samples (n=6) **C**. t-SNE plots, as in A and B, are shown here with cells expressing relatively medium to high levels of srd5a family members highlighted in green showing broad expression of srd5a across brain regions and cell types. Other cells displaying little to no expression detected for srd5a are colored grey. Expression

cut-offs were determined for each marker individually based on expression distribution of scaled normalized counts (srd5a1 0.8-3.0; srd5a2a 1.0-3.0; srd5a2b 1.0-3.0; srd5a3 0.8-3.0).

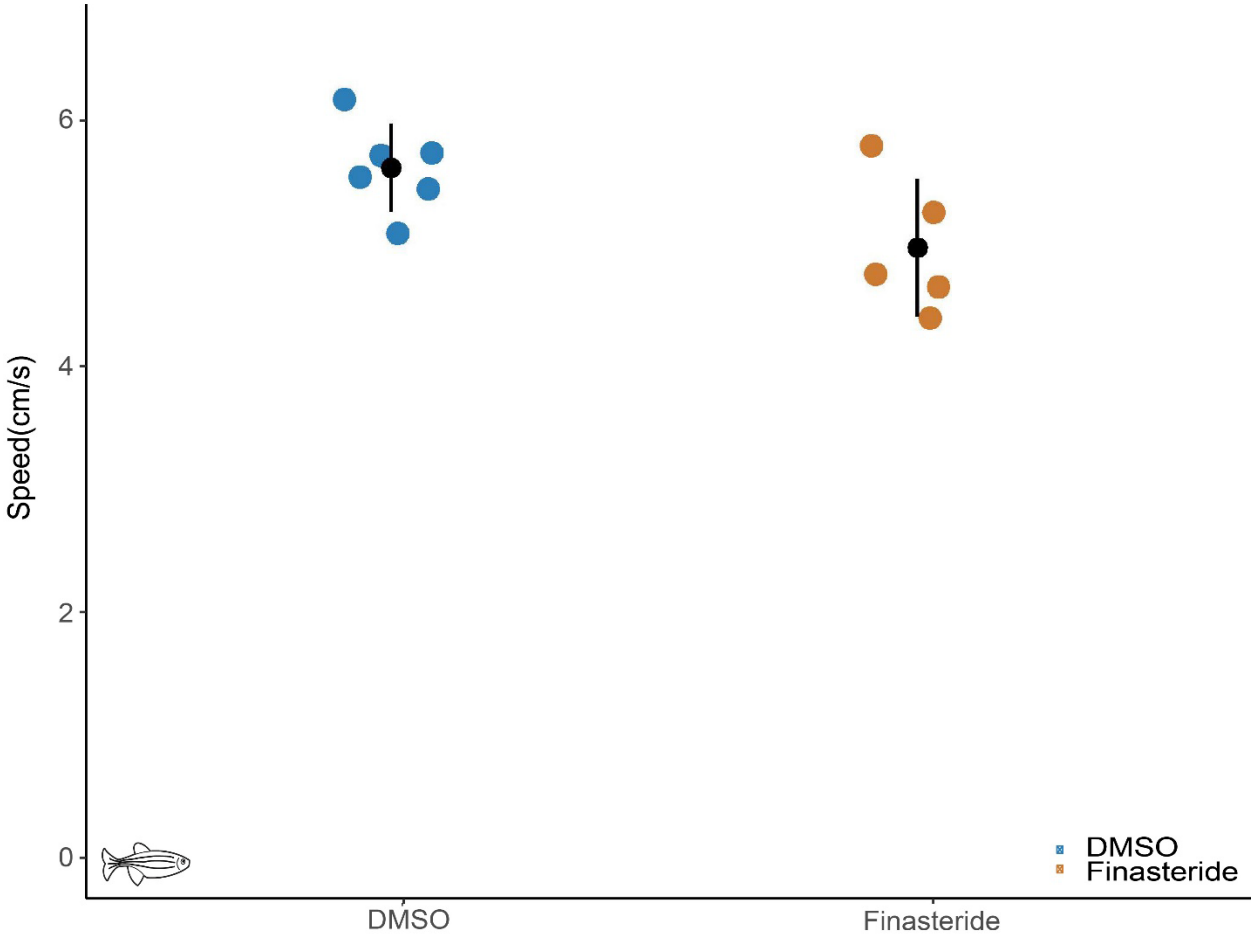

Figure S4: Locomotion is unaffected in finasteride-treated fish.

Data are the average speed of the animals in the arena (cm/s) from the different treatment conditions. DMSO: n=6, Finasteride: n=5. *p*-value was computed by TukeyHSD on one-way ANOVA [F(2,14)=2.77, p=0.1]. Each n represents a group of 15 animals. These experiments were performed using a between-subject design.

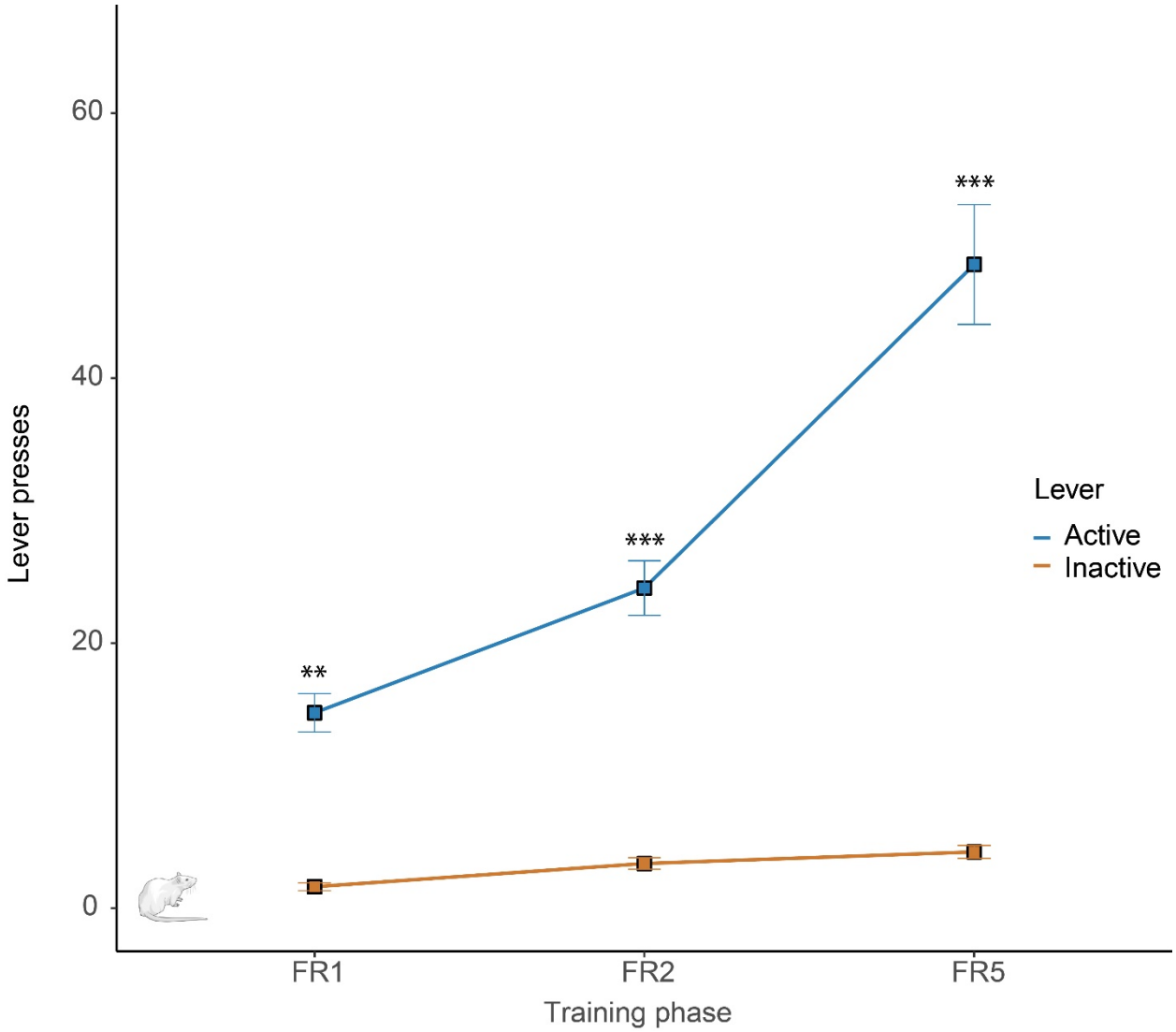

Figure S5: Number of lever presses during the hydrocodone self-administration training phase.

Rats were conditioned to self-administer hydrocodone by pressing a lever. Each rat progressed from FR1 to FR2 and from FR2 to FR5 after reaching stability, defined as >70 % of total lever presses on the active lever for three consecutive days. FR1 and FR2 stability criteria were reached form day 12 through 19 and from day 15 through 23 of training, respectively. All animals reached FR5 stability by day 31 of training and were then treated with either finasteride or its vehicle. Two-way ANOVA significant effect of interaction [$F(2,138)=29.02, p<0.0001$], as well as main effects for lever [$F(2,138)=38.29, p<0.001$] and phase [$F(1,138)=224, p<0.0001$]. ***p*-value < 0.01 ****p*-

value < 0.001. Error bars represent means +/- s.e.m. N=24 animals. Adult male Sprague-Dawley rats were used to perform the hydrocodone self-administration assay.

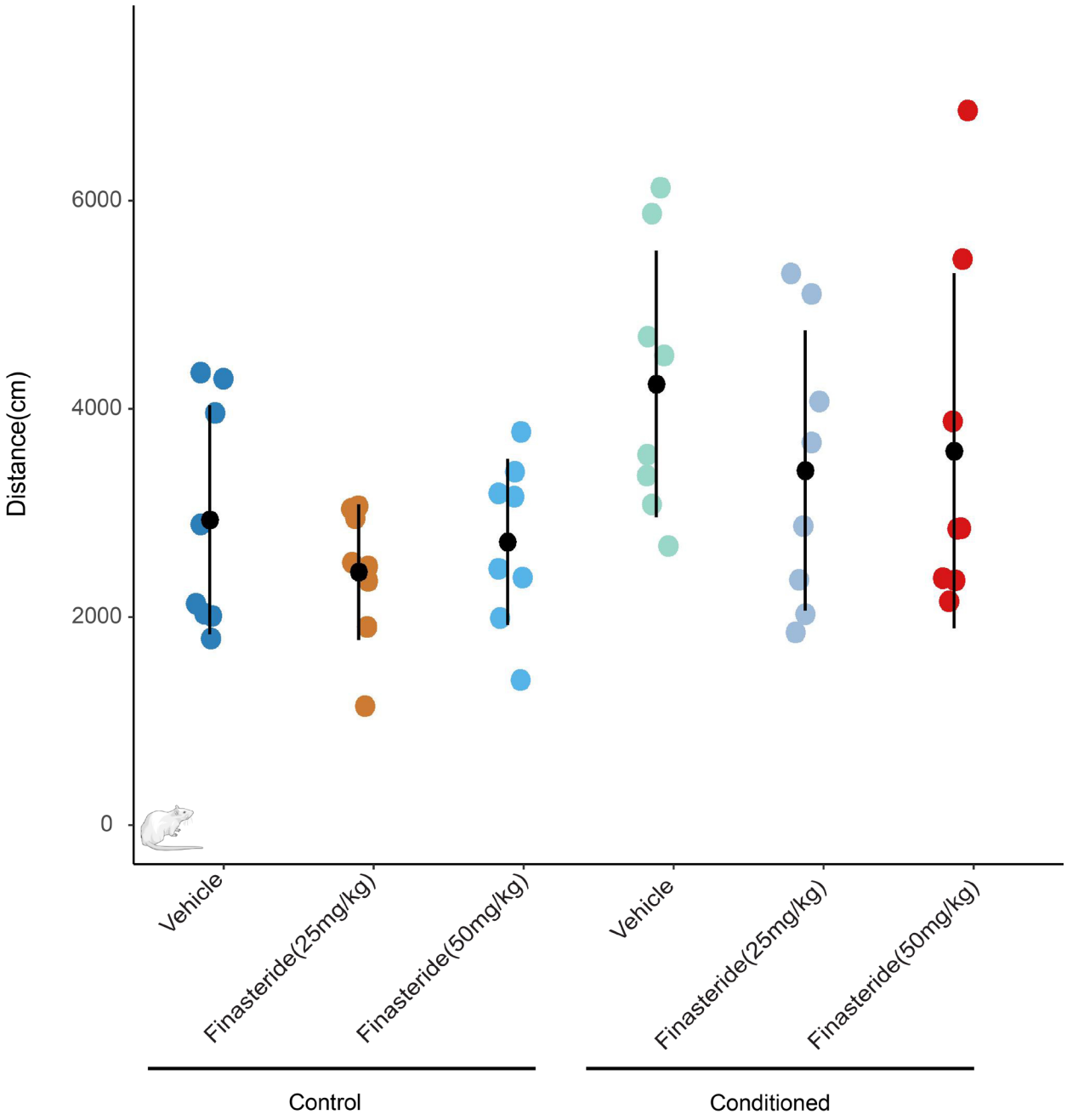

Figure S6: Locomotion is unaffected in finasteride-treated rats.

Locomotion in the open-field test of rats treated with finasteride at 25 or 50 mg/kg, IP, is not changed compared to corresponding vehicle-treated animals. N=8 per condition, two-way ANOVA. Errors bars represent means +/- s.e.m. Adult male Sprague-Dawley rats were used to measure locomotion. These experiments were performed using a within-subject design.

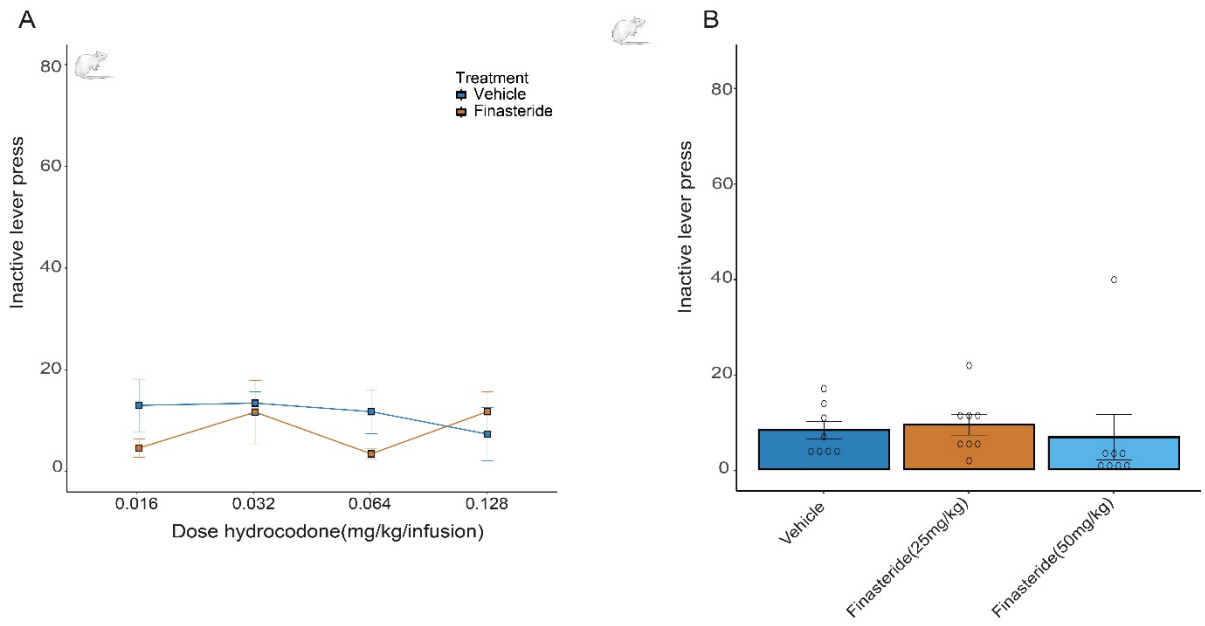

Figure S7. No difference in inactive lever press during hydrocodone self-administration in rats A. Pretreatment with 50mg/kg finasteride did not alter the number of inactive lever presses, regardless of the dose of hydrocodone. N=6 per condition. Analyses were based on two-way ANOVA [$F(3,38)=0.07$, $p=0.97$] **B.** Different doses of finasteride did not affect inactive lever presses for animals conditioned with 0.064mg/kg of hydrocodone as shown by one-way ANOVA [$F(2,21)=0.19$, $p=0.82$]. Error bars represent means +/- s.e.m. Adult male Sprague-Dawley rats were used to measure hydrocodone self-administration. Experiments were performed using a within-subject design.

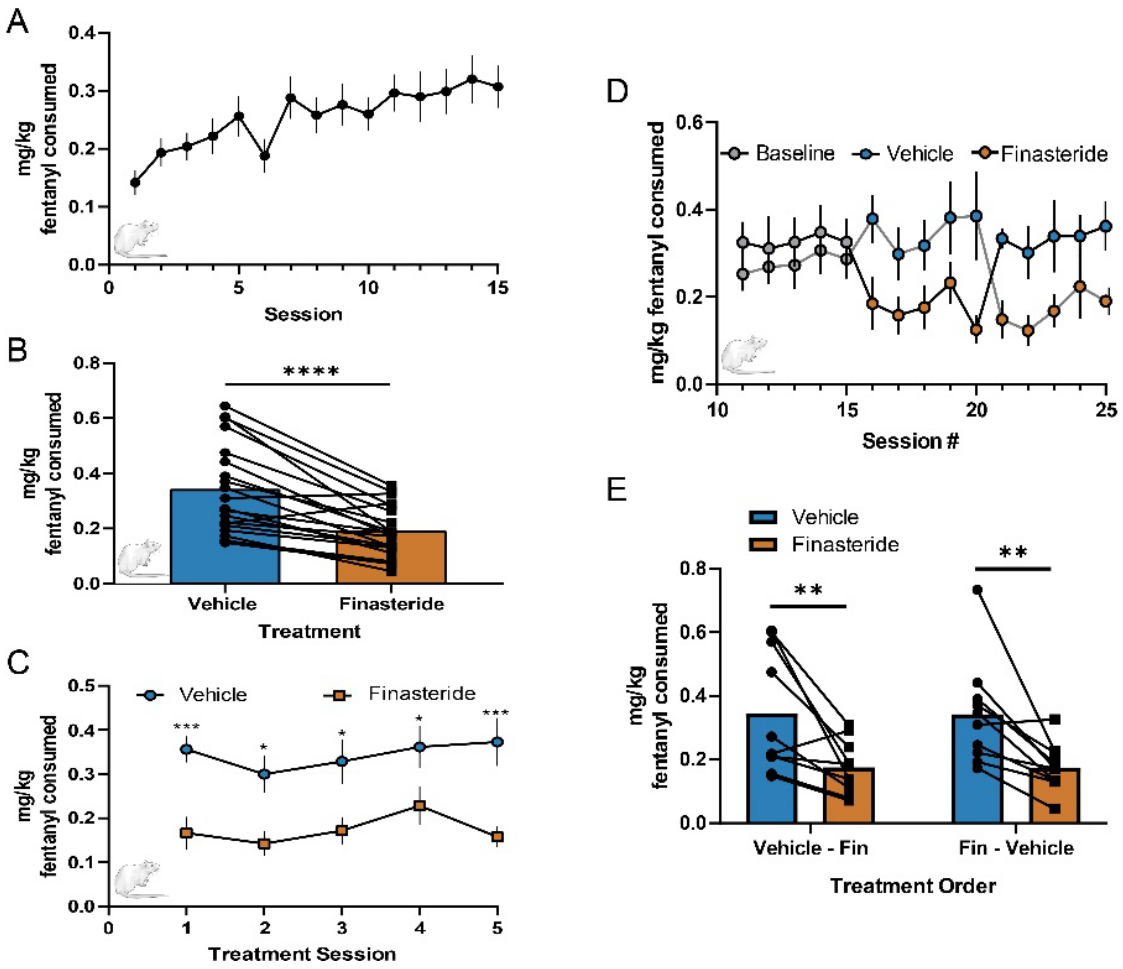

Figure S8. Finasteride decreases fentanyl consumption in rats.

A. Average fentanyl consumed in mg/kg for each baseline fentanyl self-administration session (n=20 rats). **B.** Average mg/kg of fentanyl consumed during vehicle (blue) and finasteride (orange) pretreated sessions. A paired t-test revealed finasteride treatment decreased the amount of fentanyl consumed compared to vehicle ($t(19)=5.720$, $p<0.0001$. **C.** Daily injections of finasteride (50mg/kg, IP.; orange squares) significantly decreased fentanyl consumption compared to daily injections of vehicle (blue circles). A main effect of drug treatment was observed, Sidak post-hoc analysis performed for multiple comparisons on mixed-effect model [$F(1,19)=26.94$, $p<0.0001$]. **D & E.** Animals either received injections of finasteride during self-administration sessions 16-20 (n=10 rats, D: black line, E: Fin–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.** The order of treatment had no effect on fentanyl consumed [$F(1,18) = 0.002$, $p=0.97$]. No statistical interaction was observed between treatment and treatment order [$F(1,18)=0.0025$, $p=0.9604$]. A main-effect

of finasteride treatment was present [F(1,18)=22.85, p = 0.0001]. *p*-values corrected for multiple comparisons on two-way ANOVA. **p*-value < 0.05, ***p*-value < 0.001, ****p*-value < 0.0001. Error bars represent means +/- 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.

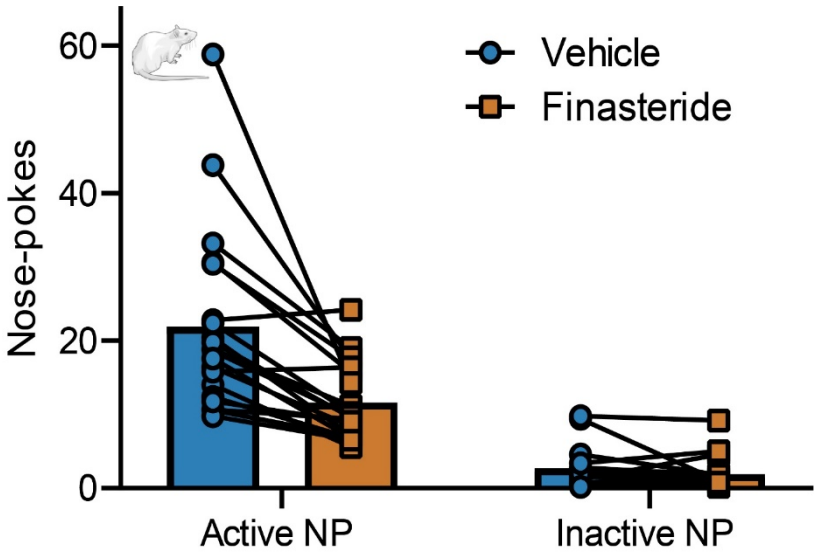

Figure S9: No difference in inactive nose pokes in fentanyl-conditioned rats.

Finasteride did not affect the number of nose pokes (NP) at the inactive port. Average operant nose-poke responses at both the active and inactive ports during finasteride (orange) and vehicle (blue) treatment sessions. N=20. 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.

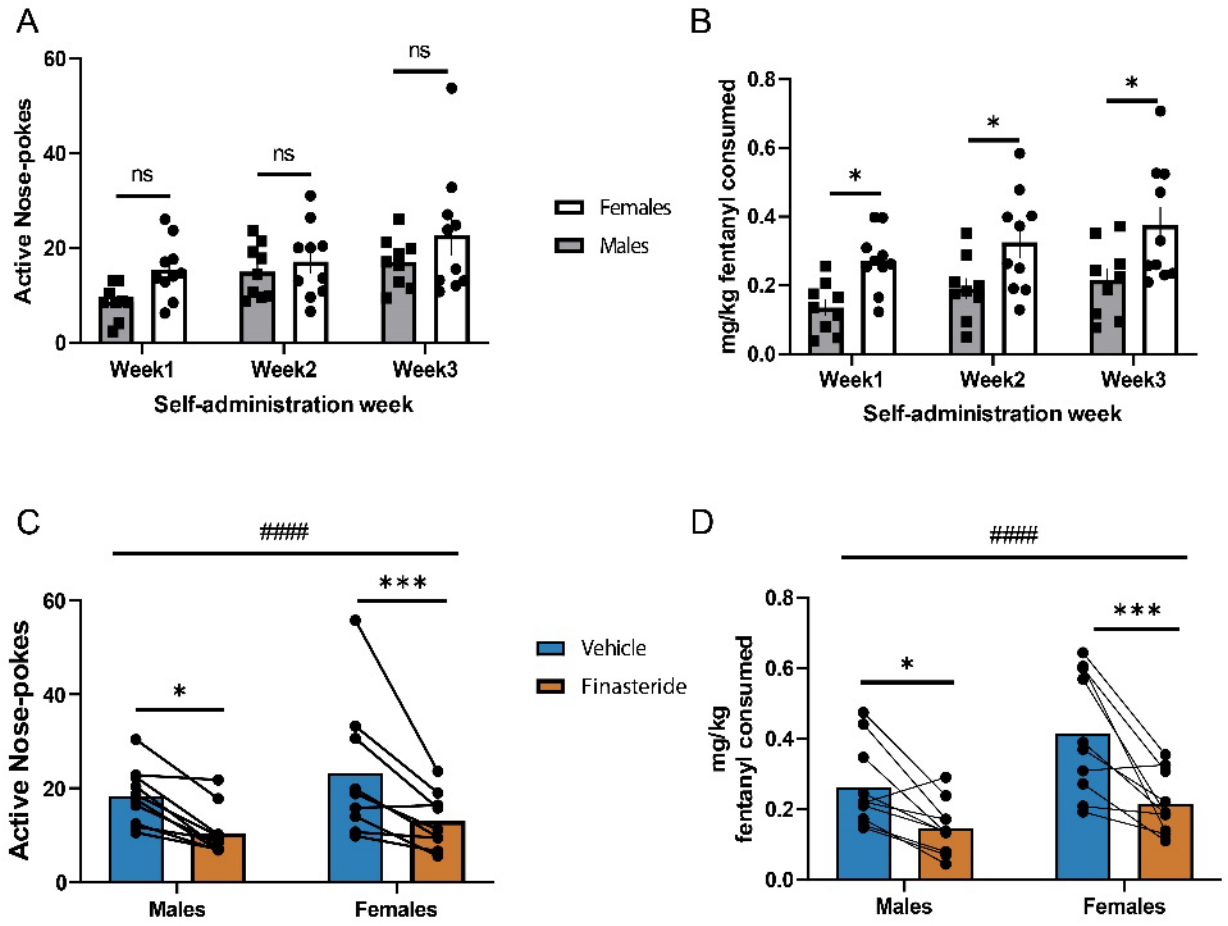

Figure S10. Finasteride decreases oral fentanyl self-administration behaviors in male and female rats.

A. Average operant nose-poke responses per session at the active port within each week of baseline fentanyl self-administration in male (filled bars) and female (open bars) Wistar rats. There was no observed statistical effect of sex on active nose-pokes [$F(1,17)=2.82$, $p=0.11$] nor was there a significant self-admin week by sex interaction [$F(2,34) = 0.96$, $p=0.39$]. A significant main effect of self-admin week was observed on a two-way ANOVA [$F(2,34) = 9.85$, $p = 0.0004$]. **B.** Average mg/kg fentanyl consumption per session during each baseline week of oral fentanyl self-administration grouped by sex. There was no significant interaction between self-admin week and sex [$F(2,34) = 0.15$]. Statistical significance was observed in both main effects of sex [$F(1,17) = 10.00$, $p = 0.0057$] and self-admin week [$F(2,34) = 6.299$, $p = 0.0047$]. A Sidak post-hoc analysis of the two-way ANOVA revealed female rats on average consumed more mg/kg of fentanyl in

each session during each week of baseline fentanyl self-administration. **C.** Average operant nose-poke responses during finasteride (orange) and vehicle (blue) treatment sessions. There was no difference in the effect of finasteride on active nose-pokes between the sexes, as evidenced by the lack of a significant sex x treatment interaction in the two-way ANOVA [$F(1,18)= 1.15$, $p= 0.30$]. An overall main effect of drug treatment was observed [$F(1,18)= 29.22$, $p < 0.0001$]. **D.** Average mg/kg fentanyl consumption during finasteride (orange) and vehicle (blue) treatment sessions. There was no observed sex difference in the effect of finasteride on mg/kg of fentanyl consumed as no significant interaction was present between sex and treatment in a two-way ANOVA [$F(1,18)=2.069$, $p=0.1674$]. There were main effects of both sex [$F(1,18)=6.110$, $p=0.0237$] and treatment [$F(1,18)=28.82$, $p<0.0001$]. **p*-value < 0.05, ****p*-value < 0.001, ##*p*-value < 0.01 and indicates a main effect of treatment. Error bars represent means +/- 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.

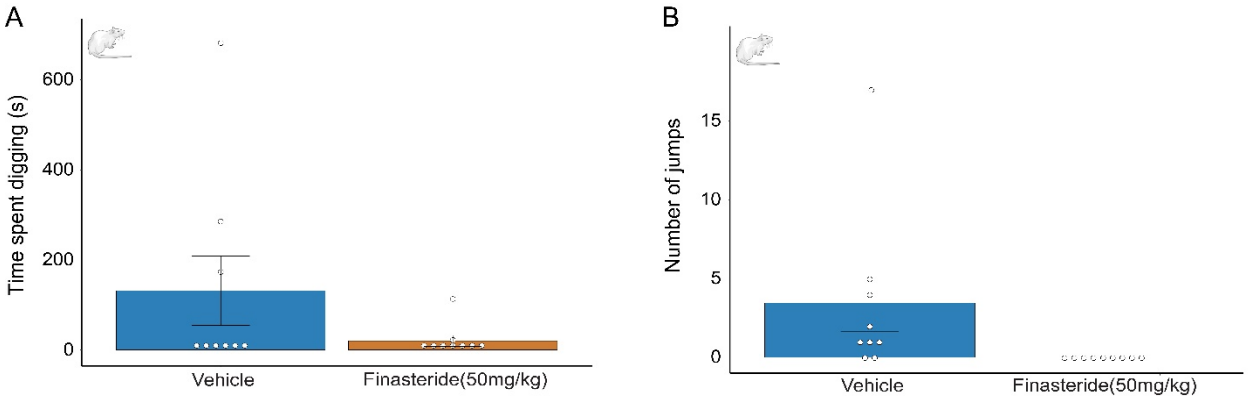

Figure S11. Finasteride did not fully reverse the jumping and digging responses induced by the opioid antagonist.

Animals were treated with an escalating dose 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 (1.5 mg/kg, IP) was administered 20 minutes later and animals were placed in a plexiglass chamber. Behavior was then recorded for 30 minutes. **A**: Although finasteride reduces the time spent digging, it did not reach significance. **B**: Acute

injection of finasteride did not fully reverse the number of jumps in animals experiencing morphine withdrawals. 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 male rats were used to test naloxone-precipitated withdrawal. This experiment was performed with a between-subject design and blind analysis.

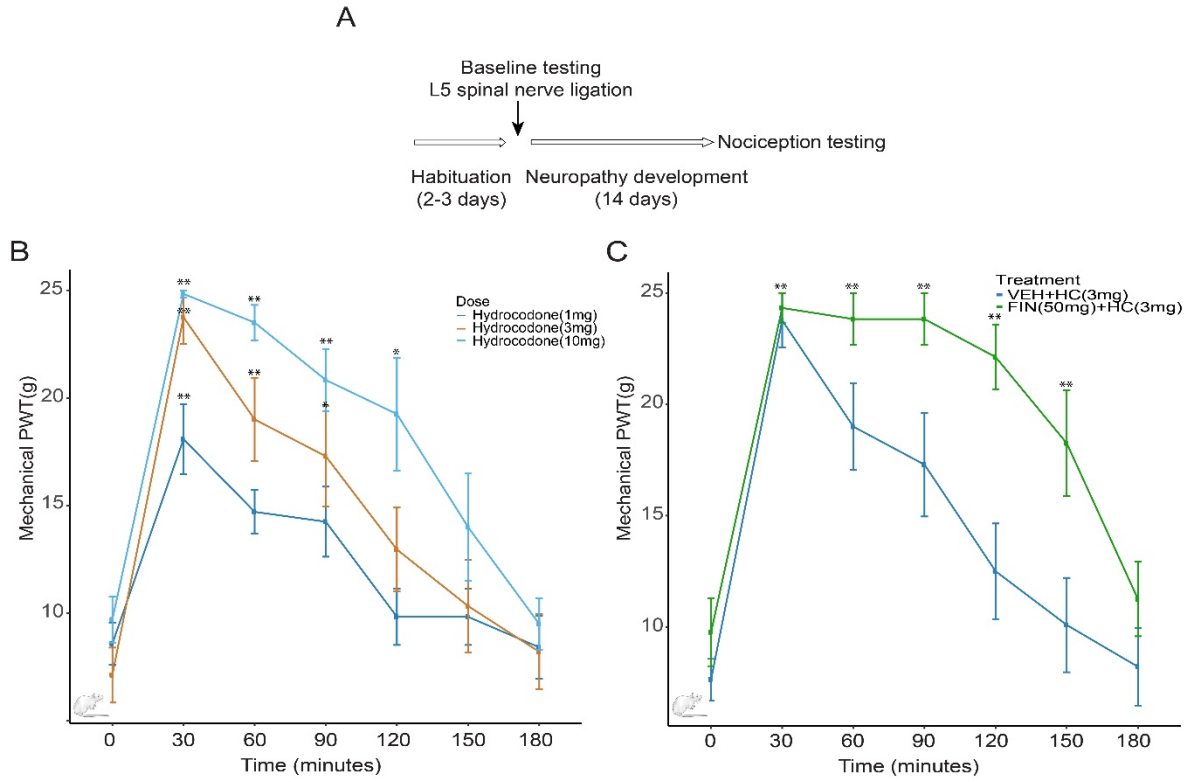

Figure S12. Randall-Sellito hydrocodone and finasteride.

A. After a 6-7 day habituation period, animals were assessed for baseline nociception tolerance followed by surgical L5 spinal nerve ligation. Neuropathy was established 13 days after surgery, and the effects of different treatments on nociception were tested the day after. **B**. Paw withdrawal thresholds (PWT) Randall-Selitto assay. The withdrawal thresholds to paw pressure were measured up to 180 minutes after treatment with different doses of hydrocodone. Different doses of hydrocodone significantly increase latency to withdrawn the paw when a pressure in g is applied, compared to animals tested immediately after injection. Two-way ANOVA significant for Time [$F(6,90)=53.90$, $p<0.0001$] and Treatment [$F(2,15)=4.50$, $p=0,030$]. N=6 per conditions

C. Co-treatment with Finasteride (50mg/kg) did not block the antinociceptive effect of hydrocodone (10mg/kg) as revealed by two-way ANOVA analysis. N=6 per condition. P compared with values measured before injection (Time 0) *p-value < 0.05, ** p-value < 0.01. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

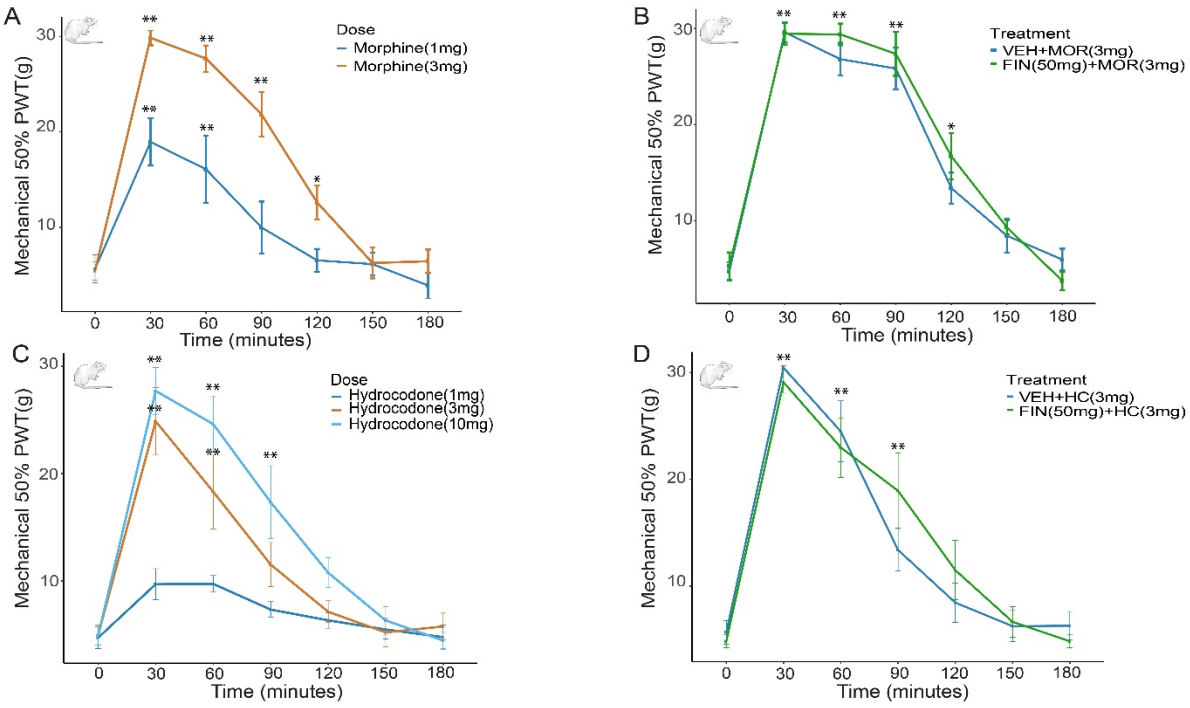

Figure S13. Von-Frey morphine and hydrocodone and finasteride

Finasteride does not affect the antinociceptive effect of opioids in a neuropathic pain model; paw withdrawal thresholds (PWT) to Von Frey filament. **A**. Different doses of morphine increase the PWT threshold. Two-way ANOVA revel a significant effect of time [$F(6,72)=56.41$, $p<0.0001$] and treatment [$F(1,12)=14.40$, $p=0.0026$]. 1mg/kg: n=6, 3mg/kg: n=8. **B**. Co-treatment with finasteride (50mg/kg, IP) did not block the antinociceptive effect of morphine(3mg/kg). Two-way ANOVA significant effect of time [$F(6,72)=125.4$, $p<0.0001$], but no effect of treatment [$F(1.12)=0.37$, $p=0.56$] and no significant interaction [$F(6,72)=0.41$, $p=0.87$]. Vehicle: n=6, Finasteride: n=8 **C**. Hydrocodone at doses of 3 and 10 mg/kg increased the PWT to Von Frey filament. N=6 per condition Two-way ANOVA identified significant main effects of time ($F(6.126)=56.67$)$p<0.0001$ and treatment [$F(2,21)=9.50$, $p=0.0012$]. **D**. Co-injection with

finasteride (50mg/kg) did not affect the antinociceptive activity of hydrocodone (10mg/kg), as the two-way ANOVA revealed a significant effect of time [F(6.72)=60.06, p<0.0001], but no main effect of treatment [F(1,12)=0.046, p=0.83] and no significant treatment x time interaction [F(6,72)=1.37, p=0.24]. Vehicle: n=6, Finasteride: n=8. *p*-value compared with values measured before injection (Time 0), **p*-value < 0.05, ** *p*-value < 0.01. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

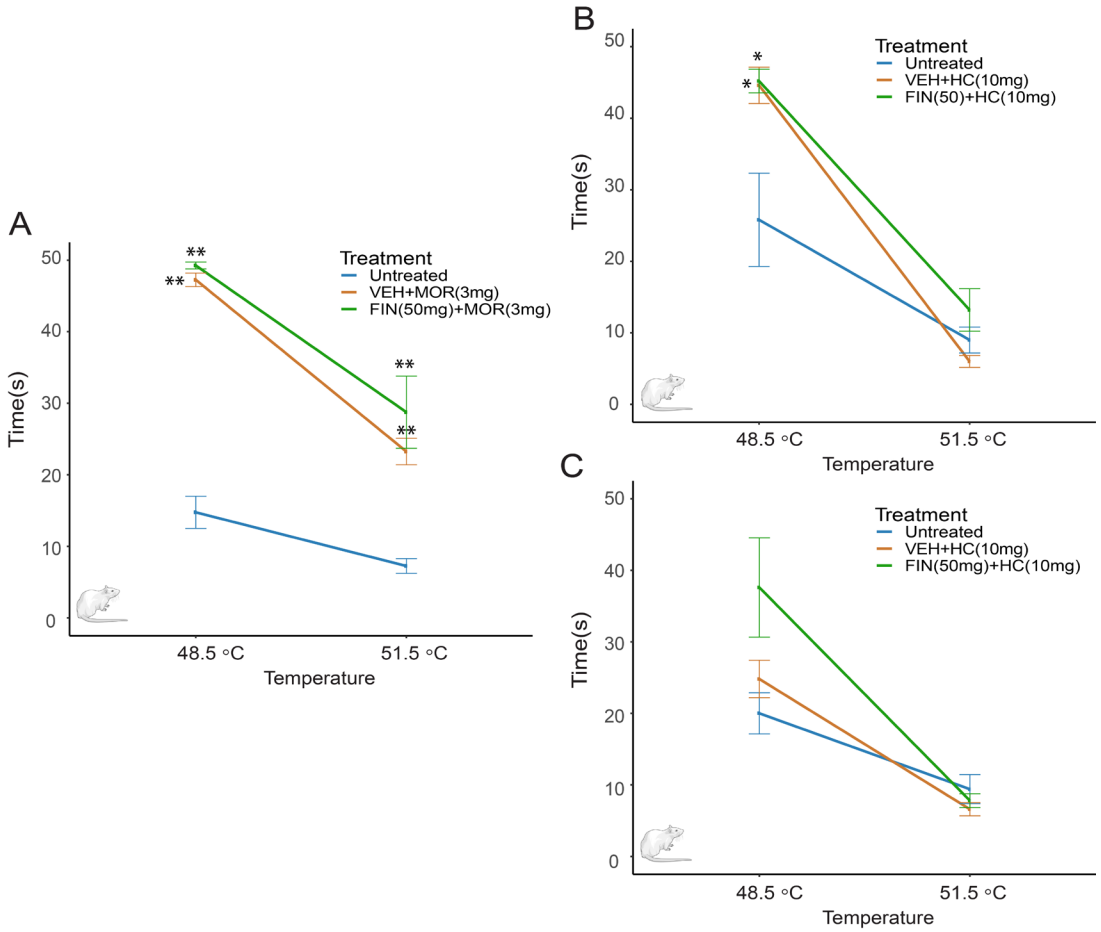

Figure S14. Hot-plate morphine and hydrocodone and finasteride.

Finasteride did not affect the thermal antinociceptive effect of hydrocodone, as measured by the time before paw lick/retraction at different temperatures. *p* value compared with untreated animals. **A**. Finasteride did not affect the thermal antinociceptive effect of morphine administered 60 min prior to the test, which measured the time before paw lick at different temperatures. 48.5°C :Two-

Way ANOVA significant effect of treatment [F(2,9)=770.3, p<0,0001]. 51.5°C two way-ANOVA significant effect of treatment [F(2,12)=7.66, p=0.0072]. Both vehicle+morphine and finasteride+morphine are significantly different from untreated animals. No difference observed between vehicle+morphine and Finasteride-Morphine. **B**. Finasteride did not affect the thermal antinociceptive effect of hydrocodone administered 30 min prior to the test, which measured the time before paw lick at different temperatures. Two-Way ANOVA significance effect of treatment [F(2,12)=6.1119, p=0.015]. N=5 per condition. Both vehicle+hydrocodone and finasteride+hydrocodone are significantly different from untreated animals at 48.5°C. No difference observed between vehicle+hydrocodonde and finasteride+hydrocodone. **C**. Hydrocodone given 60 min prior to the test did not have a thermal antinociceptive. N=5 per condition. *p-value < 0.05, ** p-value < 0.01. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

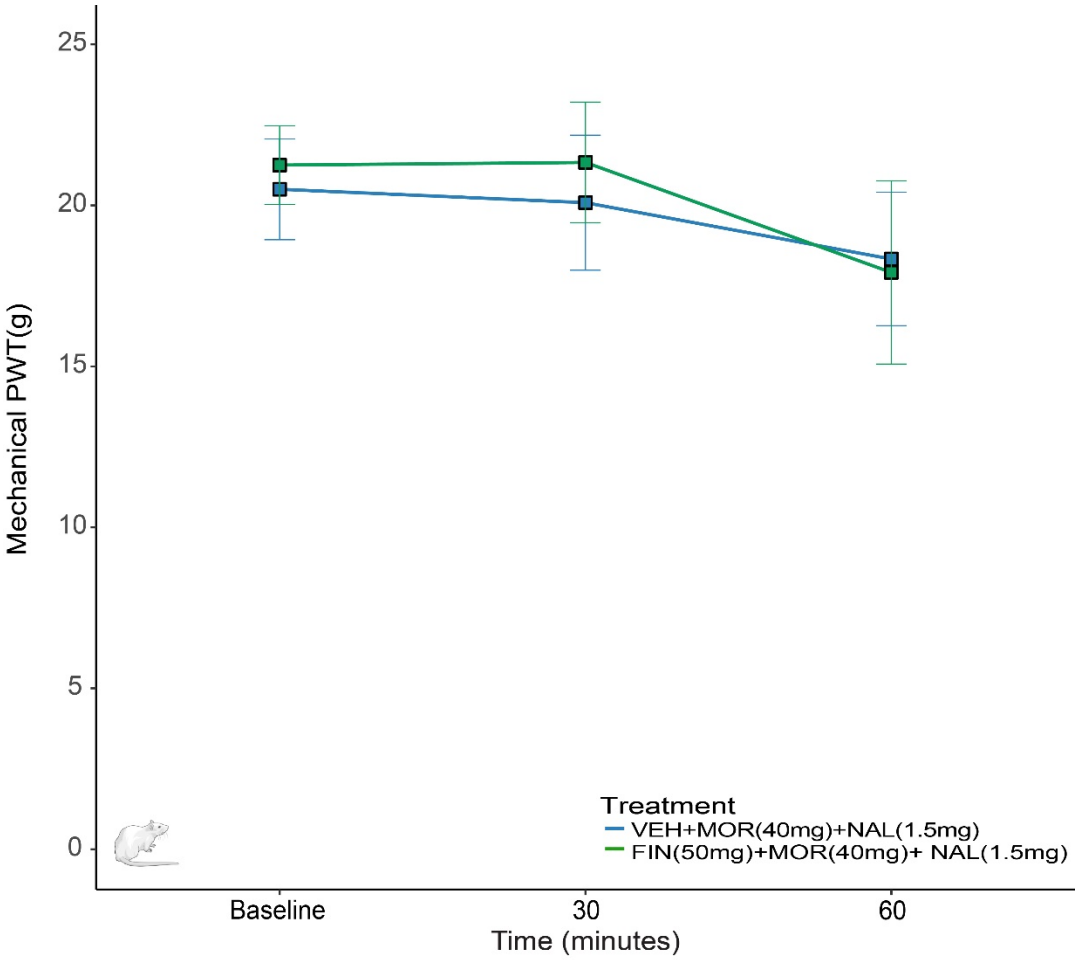

Figure S15. Finasteride did not affect pain tolerance on the un-injured paw.

Rats subjected to spinal nerve ligation and treated with a 6-day escalating morphine treatment were injected with finasteride (50mg/kg, i.p.) or vehicle. Mechanical nociception was measured immediately after naloxone treatment and repeated 30 and 60 min later. Two-way ANOVA no significant effect of Interaction [$F(2,20)=0.093$, $p=0.91$], Time [$F(2,20)=1.21$, $p=0.32$], Treatment [$F(1,10)=0.1$, $p=0.10$]. Error bars represent means +/- s.e.m.. N= 6 per condition. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

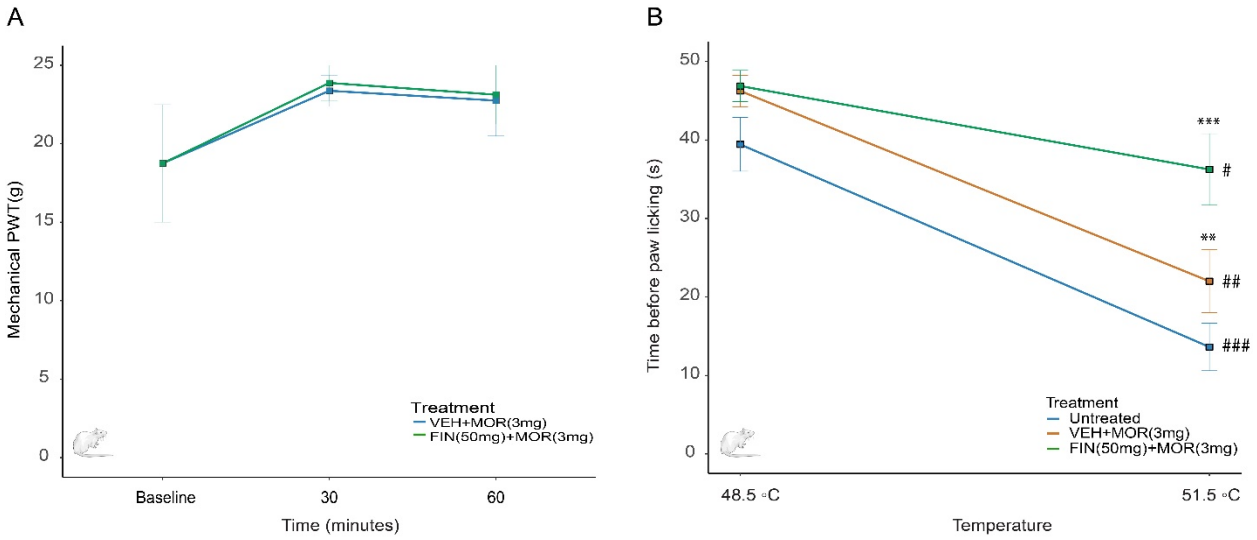

Figure S16 Finasteride does not significantly affect the antinociceptive properties of morphine in non-lesioned rats.

A. Paw withdrawal thresholds (PWT) to the Randall-Selitto test. Pain tolerance was measured over 60 min after treatment with morphine and either finasteride (50mg/kg) or vehicle. Treatment with either morphine+vehicle or morphine+finasteride does not affect mechanical pain tolerance in healthy rats. Two-way ANOVA no significant effect of Time [$F(2,12)=2.60$, $p=0.12$], treatment [$F(1,6)=0.015$, $p=0.91$] and no effect of interaction [$F(2,12)=0.0063$, $p=0.1$]. N=4 per condition. **B**. 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 significant effect of Treatment [$F(2,21)=8.81$, $p=0.011$]. Both vehicle+morphine and finasteride+morphine are significantly different from untreated animals; -** p-value < 0.01, *** p-value < 0.001. No difference was observed between vehicle+morphine and finasteride+morphine. The time before paw lick was also significantly different between 48.5 °C and 51.5 °C for each condition; #p-value < 0.01, ## p-value < 0.001, ### p-value < 0.0001. N= 8 per condition. 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. These experiments were performed using a between-subject design.

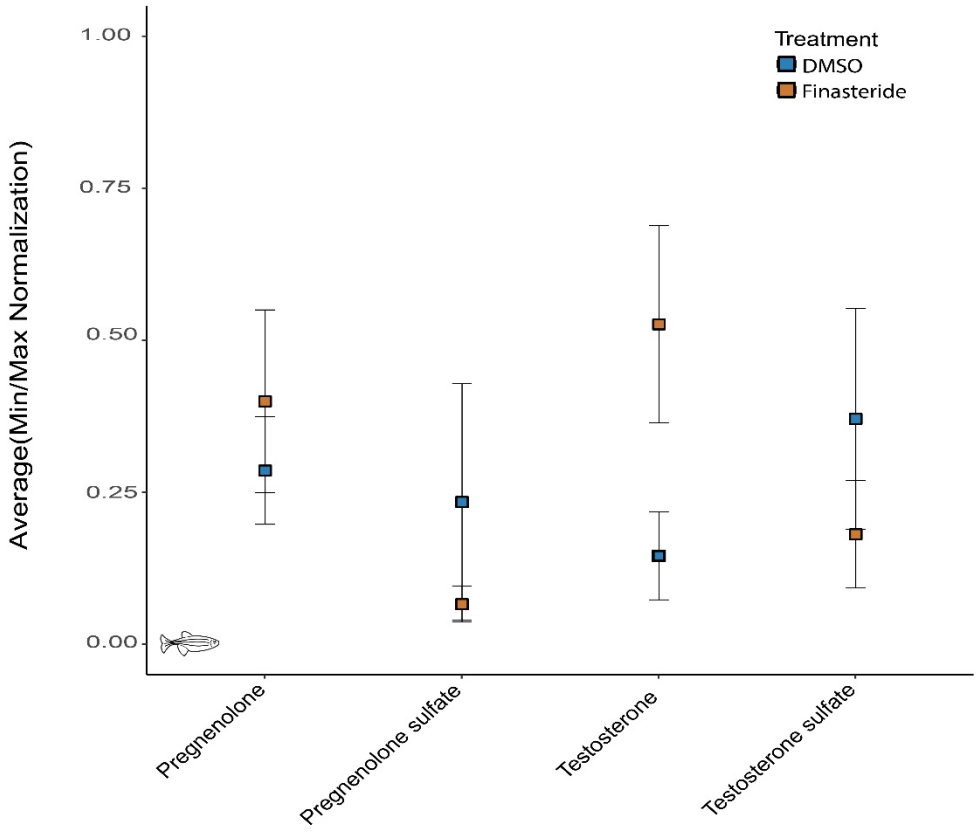

Figure S17. Other sulfated steroids are not accumulating in finasteride-treated fish.

Other sulfated steroids were not affected by the treatment with finasteride. Data are the normalization scores for the quantification of steroids in conditioned brains treated with DMSO or finasteride (10 µM). N=5 per condition. Each n represents a set of 10 brains.

Supplementary references

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