Supplemental Information

Materials and Methods

Animals

Adult male and female C57Bl/6 wildtype (WT) mice were used to characterise the hypnotic effects and pharmacokinetic properties of 3β-OH and 3α-OH. Postnatal day (PND) 21 wildtype mice were used to compare hypnosis in adolescents versus adults. Finally, $Ca_V3.1$ global knockouts (KOs) on a C57Bl/6 background were used to examine the role of T-type calcium channels in 3β-OH or 3α-OH induced hypnosis. Wildtype mice were purchased for experiments from The Jackson Laboratory (Bar Harbor, ME, USA). $Ca_V3.1$ KO breeding pairs were obtained from Dr. Charles Adrian Handforth and maintained in house. All animals were housed in a temperature and humidity controlled facility with food and water *ad libitum*. Animals were maintained on a 14:10 light:dark cycle. All experiments were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee and are in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs and Solutions

3β-OH was synthesised as described previously and was freshly dissolved in 25% (2-hydroxypropyl)-β-cyclodextrin solution (Santa Cruz Biotechnology Inc, USA)[1]. A range of doses (20 to 120 mg kg^{-1}) of 3β-OH were used in dose response experiments. For all other experiments 100 mg kg^{-1} 3β-OH was used as a standard dose; this was the lowest dose at which 100% of males and females lost righting reflex. Sixty mg kg^{-1} 3α-OH was used for all other experiments; this dose produced relatively similar hypnotic effect as 100 mg kg^{-1} 3β-OH, and later revealed to be the lowest dose at which all males and females lost righting reflex.

Loss of Righting Reflex

Time to loss of righting reflex (LORR) and time to gain of righting reflex (GORR) was used to study the hypnotic effects of either the neuroactive steroid, 3β-OH or 3α-OH. For each

LORR experiment, animals were injected with neuroactive steroid either intraperitoneally (IP) or intravenously (IV). Shorty following injection, animals were placed on their backs and observed for righting reflex. Animals that were unable to flip themselves into a righting position within 30 seconds of being placed on their backs were said to have lost righting reflex and were considered hypnotic. Gain of righting reflex was counted as the first time animals were able to flip themselves into a righting position. Duration of hypnosis was measured from time to LORR to time to GORR.

Gonadectomy Surgeries

To determine the effects of circulating hormones on 3β-OH induced hypnosis, male and female mice underwent gonadectomy before reaching sexual maturity (postnatal day 55-58). Animals were anaesthetized with 2-3% isoflurane via inhalation. For females, a single dorsal skin incision followed by bilateral muscle incisions were performed to access each ovary. Both ovaries were ligated and removed, and the wound was closed using wound clips. Female sham surgeries consisted of skin and muscle incisions, but without ligation and removal of the ovaries. For males, a skin incision was made in the testicular sac; the testes were isolated, ligated, and remove, and the incision site was closed with wound glue. Male sham surgeries consisted of a single skin incision and wound closure without removal of testes. All animals were treated postoperatively with analgesics (banamine 2.5 mg kg^{-1} every 24 hours for 48 hours) and allowed to recover for 8 days before commencement of LORR experiments.

Pharmacokinetics

We performed pharmacokinetic experiments to measure the time-dependent changes in concentrations of 3β-OH and its metabolite 3α-OH in plasma, brain, liver, and urine. Adult mice were individually injected with 100 mg kg^{-1} 3β-OH and tissue was collected across a range of time-points (at 0, 10, 20, 30, 40, 60, 80, 120, 180, 360, 540, or 720 minutes), respectively. For studies examining sex differences in 3α-OH concentrations in brain and liver, we administered 3α-OH 60 mg kg^{-1} and collected tissue samples at 40 minutes. At 40 minutes, all animals have

lost righting reflex; this time-point also corresponded with T_{max} for 3α-OH in brain after administration of 100 mg kg^{-1} 3β-OH. For each corresponding time-point, mice were briefly anaesthetized with isoflurane and underwent necropsy for harvesting blood, urine and tissue samples. Blood plasma was transcardially collected using a heparinized needle and syringe and transferred to EDTA coated blood collection vials. Blood samples were centrifuged at 2,000 *g* for 15 minutes allowing for separation of plasma. We then collected whole brain and liver tissue samples, rinsed them in sterile saline, blotted the tissue dry, and recorded the wet weights. Finally, urine was collected directly by bladder puncture and transferred to Eppendorf tubes.

Samples were then processed for high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). We added 400 μl of methanol/0.2mM $ZnSO_4$ (70/30, v/v; protein precipitation solution) containing epipregnanolone (1 μg ml^{-1} final concentration) to 100 μl of plasma and urine samples, respectively. Whole brain samples and partial liver samples were homogenized in 1 ml methanol/PBS (1:1, v/v) with a Bullet Blender tissue homogenizer (Next Advance, Troy, NY, USA). All samples were vortexed, sonicated and centrifuged at 16,000 *g* at 4°C for 15 minutes.

Supernatants were collected and transferred into HPLC vials (Phenomenex, Torrance, CA, USA). For brain and liver tissue homogenates, appropriate volumes of epipregnanolone were added (0.8 μg/ml final concentration). One hundred μL of sample or calibrator were injected onto the extraction column (Eclipse XDB-C8, 4.6 x 12.5 mm, Agilent Technologies, Santa Clara, CA, USA) and after 1 minute were backflushed onto the analytical HPLC column (Eclipse XDB-C8, 4.6 x 150 mm, 3.5μm, Agilent Technologies). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of methanol. The Following gradient was used: 0 to 1 min with 60% B to 80% B; 1 to 4 min 95% B that was held at 95% for additional 5 minutes. After 9 min, the column was the re-equilibrated to the starting conditions for 1 min. We kept the column at 30°C and the flow rate at 1 ml/min.

Atmospheric pressure chemical ionization (APCI) operated in positive multiple reaction monitoring (MRM) mode was used to interface the HPLC system (Agilent 1200 components, Agilent Technologies, Santa Clara, CA, USA) with an API5500 QTRAP mass spectrometer (Sciex, Concord, ON, Canada). Peak area ratios obtained from MRM mode of the mass transition for 3β-OH and 3α-OH (m/z= 284.5→130.2 (quantifier transition; declustering potential (DP): 140V, entrance potential (EP): 8V, collision energy (CE): 55V, collision cell exit potential (CXP): 12V) and m/z= 284.5→72 (qualifier transition)) and the internal standard epipregnanolone (m/z= 301.5→269.2) were used for quantification. 3β-OH and 3α-OH peaks were baseline separated (retention times of 4.15 min and 4.45 min, respectively) and calibration and quality control samples included both compounds. The following mass spectrometer parameters were used: collision gas: medium, curtain gas: 20 L/min, ion source gases 1 and 2: 25 L/min, temperature: 500 C, ion spray voltage: 5,500 V. The method was linear from 39 ng mL^{-1} to 10 μg mL^{-1} in plasma and tissue, respectively.

Cell Culture

To study neuroactive steroid modulation of T-channels, we cultured HEK-293 cells stably transfected with $Ca_V3.1$ channels (Kerafast, Boston MA, USA). Geniticin 0.40 mg ml^{-1} was used to ensure channel specific selection. Cells were plated on Poly-D-Lysine coated coverslips 24 to 72 hours before recordings.

Patch Clamp Electrophysiology

Recordings were conducted using whole-cell patch clamp electrophysiology in voltage-clamp configuration. Electrodes were made from fire polished borosilicate glass and had a final pipette resistance between 2 and 5 MΩ. Internal solution consisted of the following: Tetramethylammonium hydroxide (TMA)-135 mM, HEPES-40 mM, EGTA-10 mM, $MgCl_2$-2 mM, and HF was used to bring pH to 7.2. Recordings were performed in Tetraethylammonium (TEA) external solution containing the following: TEA-152 mM, HEPES-10 mM, Ca^{2+}-2 mM, and TEA-OH was used to pH solution to 7.4. Membrane currents were recorded on an Axopatch 200B

amplifier (Molecular Devices, San Jose, CA, USA) and digitized using Clampx 10.7 software (Molecular Devices) running on a compatible computer. Reported capacitance values were taken from the amplifier settings and typically compensated between 50% and 80% of the series resistance. Pharmacology experiments were performed using a series of glass syringes and corresponding capillary tubes to deliver solutions. We first recorded stable baseline T-channel currents before perfusing on 3α-OH (3, 10, or 30 μM). Current recovery was measured by washing off 3α-OH with fresh external solution.

To measure current block, we generated current-voltage (I-V) curves by holding the membrane potential at -90 mV and recording from test potentials between -80 mV and +5 mV in 5 mV step increments. From this we generated a concentration-response curve using 3, 10, and 30 μM 3α-OH and plotted the percentage of current blocked at -30 mV. The Hill-Langumir equation was used to calculate the effective inhibitory concentration 50 (IC_{50}). To make direct comparisons between different concentrations, we normalized all data to peak baseline amplitude across all groups. Current inactivation kinetics were determined by evoking a voltage step to -30 mV after 3.5-s conditioning steps at potentials between -110 and -60 mV. Voltage-dependence of steady state activation and inactivation was described by the Boltzmann distribution $G(V) = G_{max}/1+\exp [-(V-V_{50})/k]$ and $I(V) = I_{max}/1+\exp [-(V-V_{50})/k]$ respectively.

In vivo electroencephalogram

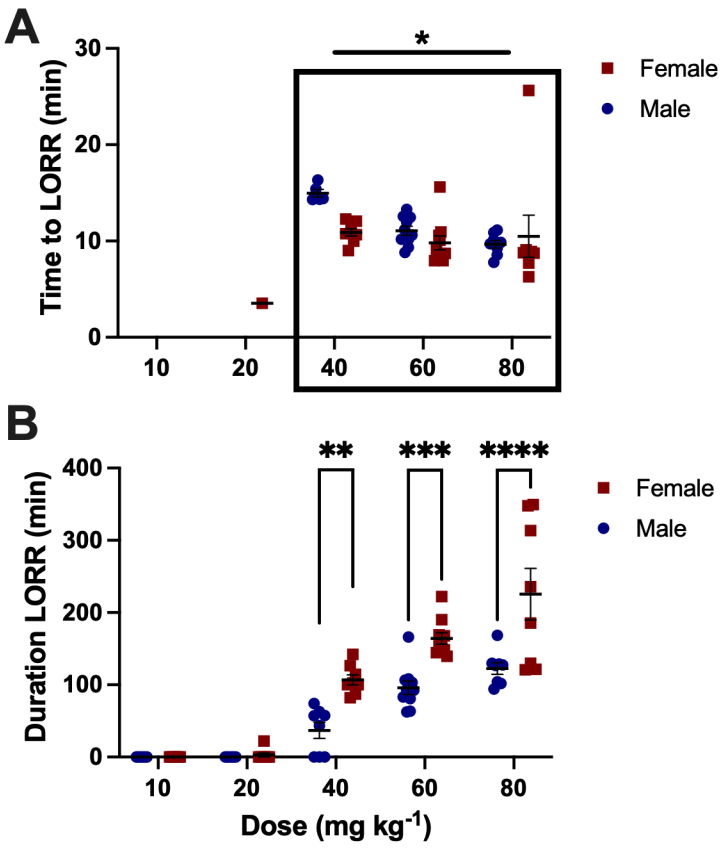
Adult male and female mice underwent stereotaxic surgeries for implantation of cortical and thalamic electrodes. During surgery, we induced anaesthesia using 3% isoflurane via inhalation and maintained between 0.5 and 2%. As a local anaesthetic, we applied lidocaine (1%) at the incision site. We implanted bilateral screw electrodes with stereotaxic coordinates in the range of sensory cortex (AP: -1 mm, MD: ± 3 mm, DV: 0). We also implanted a depth electrode with stereotaxic coordinates in the range of central medial nucleus of thalamus (CEM, anteroposterior – AP: -1.35 mm, mediolateral – MD: 0 and dorsoventral – DV: -3.6 mm) for use in another experiment.

Power spectra analysis

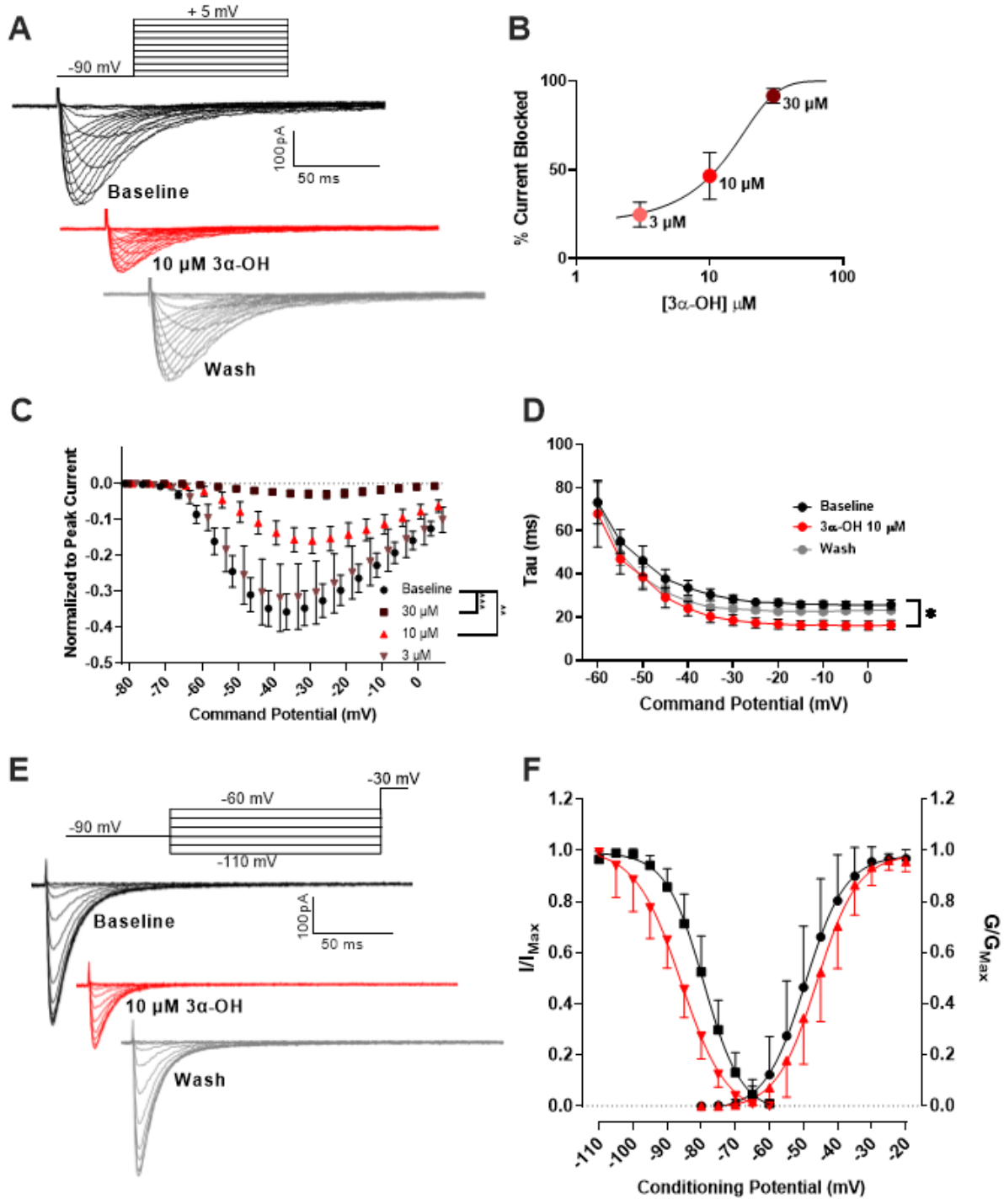
Mice were allowed to acclimate to the recording chamber for at least 30 minutes before being administered either 100 mg/kg 3β-OH or 60 mg/kg3α-OH IP. After steroid injection, animals continued to be recorded for another hour. Data for power spectra analysis was sampled from a two-minute period during the final five minutes of baseline recordings and from a two minute period 40 minutes post-steroid injection. Forty minutes corresponds to the T_{max} for 3α-OH metabolite after 3β-OH administration and represents a time-point at which both males and females are heavily hypnotic. Data were analysed using LabChart 8 software (ADInstrumetns Inc., Colorado Springs, CO). Data presented were sampled from traces from the left cortical electrode. We divided the EEG into the following frequency bands: slow (0.5-1 Hz), delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), low gamma (30-59 Hz), and high gamma (61-100 Hz). Sixty Hz was not analysed to avoid artefact from the 60 Hz electrical room noise. Parameters for analysing power density were as follows: Fast Fourier transform size of 4K, Hann window size of 4096, window overlap of 50%. Comparisons were made between baseline and 40-minute PSD values for both males and females. We also normalized data by subtracting 40-minute PSD values from baseline in order to make more direct comparisons between males and females.

Statistical Analysis

All data were considered statistically significant at $P < 0.05$. Categorical data were analysed using χ^2 analysis. Data containing only one factor and two variables were analysed using t-tests, paired for within subjects and unpaired for between subjects analysis. Data containing two or more factors and two or more variables were analysed using Factorial (ANOVA) and Sidak's multiple comparisons for post-hoc analysis. Data that did not show normality of residuals were grouped and analysed using non-parametric Mann-Whitney. Results are reported as means ± standard error of means. Statistical tests used are stated in each figure legend.

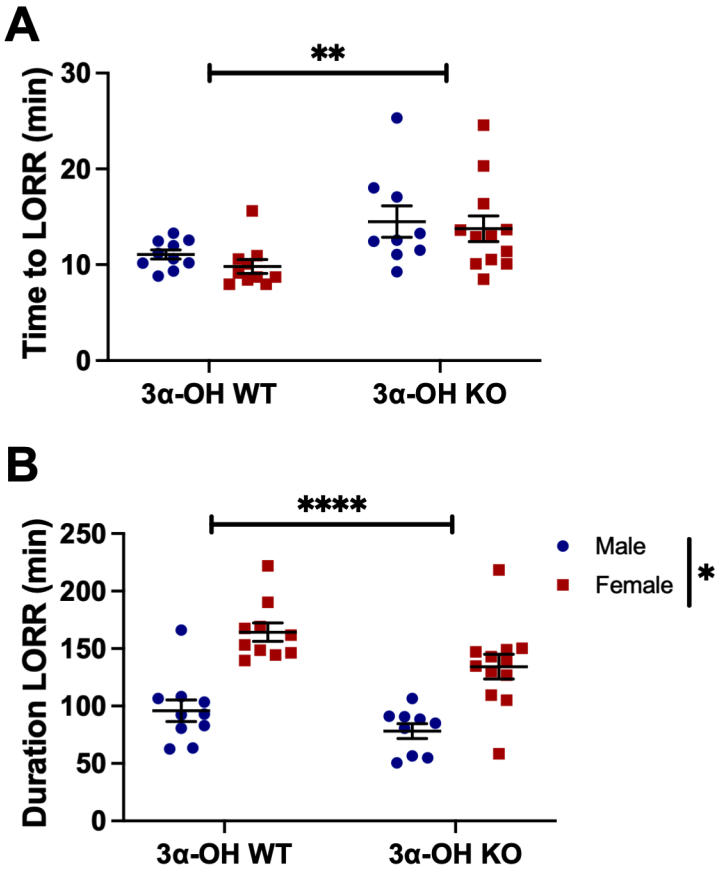


Supplemental Figure 1: Dose response effects of 3α-OH in male and female mice. a) Because no or few mice lost righting reflex at low doses, only doses with a sufficient number of data points (indicated in boxed area) were analysed for time to LORR. Males and females did not differ in time to LORR (2-way ANOVA sex by dose, P=0.084). There was a main effect of 3α-OH dose ($F_{2,43}$=3.95, $^{*}P$=0.027), and time to LORR decreased as dose increased. b) All data points were included in analysis of duration of LORR, and there was a significant dose by sex interaction (2-way ANOVA sex by dose, Sidak's multiple comparisons, $F_{4,74}$=6.28, P=0.002). Females were hypnotic longer at 40 ($^{**}P$=0.001), 60 ($^{***}P$<0.001), and 80 mg kg^{-1} 3α-OH ($^{****}P$<0.0001). The Hill Langumir equation was used for fitting the data on graph B. We obtained estimated ED_{50} of 46 ± 1 mg and slope of curve od 6 ± 1 for males with maximal effects constrained to 122 minutes, and ED_{50} of 42 ± 2 mg and slope of curve od 4 ± 1 for females with maximal effects constrained to 225 minutes.



Supplemental Figure 2: 3α-OH is a partial T-channel blocker at physiological concentrations. a) Schematic current-voltage (I-V) curves before, during, and after application of 10 μM 3α-OH by holding the resting membrane potential at -90 mV and stepping up to test potentials between -80 and +5 mV, which produces crisscrossing calcium currents typical of T-channels. b) Concentration-response curve showing that 3α-OH is a full T-channel blocker at 30

μM and a partial T-channel blocker at more physiological concentration of 10 μM. c) Data across concentrations were normalised to the peak baseline amplitude across all groups. A significant interaction (2-way ANOVA steroid concentration by command potential, Sidak's multiple comparisons, $F_{51,748}$=4.930, P< 0.0001) revealed a decrease in current after application of 30 μM ($^{****}P$<0.0001 at 30 mV) and 10 μM ($^{*}P$=0.019 at 30 mV) 3α-OH compared to normalised baseline current. d) At 10 μM 3α-OH also significantly decreased current decay time, a measure of inactivation kinetics, compared to baseline ($F_{2,16}$=5.614, $^{*}P$=0.0142). e) Schematic of steady-state inactivation kinetics (I/I_{max}) and corresponding traces. We held the cell at conditioning potentials between -110 and -60 mV and recorded currents at a test potential of -30 mV. f) Average steady-state inactivation curves and activation curves (baseline black and 3α-OH red). Ten μM 3α-OH creates a hyperpolarizing shift in the V_{50} (Paired t-test, t_4=3.20, P=0.033, V_{50} baseline= -79.42 mV, V_{50} 3α-OH= -87.03mV). We also noted a small but statistically significant depolarizing shift in activation kinetics (t_8=2.314, P=0.049, V_{50} baseline= -50.19 mV, V_{50} 3α-OH= -47.22 mV)



Supplemental Figure 3: $Ca_V3.1$ T-channels partially contributes to the hypnotic effects of 3α-OH. We administered 60 mg kg^{-1} 3α-OH IP to male (n=9) and female (n=12) $Ca_V3.1$ KOs and compared hypnosis to data from WT animals. a) For time to LORR there was a significant effect of genotype in which WTs became hypnotic sooner than KOs (2-way ANOVA sex by genotype, Sidak's multiple comparisons ($F_{1,37}$=10.19, $^{**}P$=0.003). b) For duration of LORR, there was also a significant main effect of genotype ($F_{1,37}$=45.72, $^{****}P$< 0.0001). KO animals were hypnotic for a

significantly shorter duration compared to WT animals. There was also a significant effect of sex in which females were hypnotic for a longer duration than males ($F_{1,37}$=6.716, $^{*}P$=0.014, inset).

Matrix	Compound	Sex	$T_{1/2}$	T_{max}	C_{max}	T_{last}	C_{last}	AUC_{last}	AUC_{inf}
			min	min	μg g^{-1}	min	μg ml^{-1}	μg ml^{-1}·min	μg ml^{-1}·min
Brain	3β-OH	F	147.4	20	4.1	720	0.14	573	602
	3β-OH	M	266.9	20	5.01	720	0.14	530	583
	3α-OH	F	215.7	40	8.6	720	1.28	3215	3614
	3α-OH	M	153.1	20	4.2	720	0.28	589	651
Liver	3β-OH	F	191.4	20	14.6	720	0.52	1029	1173
	3β-OH	M	129.5	20	14.66	720	0.37	1421	1490
	3α-OH	F	357.5	20	64.0	720	11.83	15598	21700
	3α-OH	M	132.7	20	30.7	720	0.76	2579	2725
Plasma	3β-OH	F	102.4	20	19.0	180	7.56	1961	3078
	3β-OH	M	49.7	30	24.8	180	2.91	2193	2402
	3α-OH	F	248.6	180	34.9	720	5.38	12383	14312
	3α-OH	M	71.3	60	16.7	180	3.44	1559	1913
Urine	3β-OH	F	308.1	40	418.9	720	75.29	121178	154648
	3β-OH	M	63.1	60	145.0	720	0.24	23537	23559
	3α-OH	F	61.4	60	134.8	720	0.31	26370	26397
	3α-OH	M	63.1	60	145.0	720	0.24	23537	23559

Supplemental Table 1: Pharmacokinetic parameters showing sex-specific changes in steroid metabolism. After injection of 100 mg kg 3β-OH, females produce high concentrations of the active metabolite 3α-OH in brain, liver, plasma, and urine. Although steroid concentration is maximal in the first few hours to minutes, even at the last time-point of 12 hours, steroid compound is still detected in the samples.

Matrix	Sample	Compound	Sex	[Steroid]
				μg g^{-1}
Brain	1	3β-OH	F	0
	2	3β-OH	F	0
	3	3β-OH	F	0
	4	3β-OH	M	0.99
	5	3β-OH	M	0
	6	3β-OH	M	0.67
Liver	1	3β-OH	F	0
	2	3β-OH	F	0
	3	3β-OH	F	0
	4	3β-OH	M	3.01
	5	3β-OH	M	1.9
	6	3β-OH	M	3.36

Supplemental Table 2: Brain and liver concentrations of 3β-OH at the time point of 40 minutes after injection of 60 mg kg^{-1} 3α-OH. Note that there is no conversion into 3β-OH in females in the brain, and only minimal conversion in male brains. However, males do show conversion of 3α-OH into 3β-OH in the liver.