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Sex-specific hypnotic effects of the neuroactive steroid (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile are mediated by peripheral metabolism into an active hypnotic steroid

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Abstract

Background: The novel synthetic neuroactive steroid (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (3 β -OH) blocks T-type calcium channels but does not directly modulate neuronal γ -aminobutyric acid type A (GABA_A) currents like other anaesthetic neurosteroids. As 3 β -OH has sex-specific hypnotic effects in adult rats, we studied the mechanism contributing to sex differences in its effects.

Methods: We used a combination of behavioural loss of righting reflex, neuroendocrine, pharmacokinetic, *in vitro* patch-clamp electrophysiology, and *in vivo* electrophysiological approaches in wild-type mice and in genetic knockouts of the Ca_v3.1 T-type calcium channel isoform to study the mechanisms by which 3 β -OH and its metabolite produces sex-specific hypnotic effects.

Results: Adult male mice were less sensitive to the hypnotic effects of 3 β -OH compared with female mice, and these differences appeared during development. Adult males had higher 3 β -OH brain concentrations despite being less sensitive to its hypnotic effects. Females metabolised 3 β -OH into the active GABA_A receptor positive allosteric modulator (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (3 α -OH) to a greater extent than males. The 3 α -OH metabolite has T-channel blocking properties with sex-specific hypnotic and pharmacokinetic effects. Sex-dependent suppression of the cortical electroencephalogram is more pronounced with 3 α -OH compared with 3 β -OH.

Conclusions: The sex-specific differences in the hypnotic effect of 3 β -OH in mice are attributable to differences in its peripheral metabolism into the more potent hypnotic metabolite 3 α -OH.

Keywords: calcium channels; electroencephalogram; metabolism; neuroactive steroid; pharmacokinetics; sex-specific pharmacology

Editor's key points

- The novel synthetic neuroactive steroid 3 β -OH blocks T-type calcium channels but does not directly modulate neuronal GABA_A currents like most other anaesthetics.
- The sex-specific pharmacodynamic and pharmacokinetic effects of 3 β -OH were investigated *in vivo* and *in vitro*.
- Adult male mice were less sensitive to the hypnotic effects of 3 β -OH compared with females, and had higher brain concentrations despite being less sensitive.
- Female mice, to a greater extent than males, metabolised 3 β -OH into the active GABA_A receptor positive allosteric modulator 3 α -OH, which has sex-specific hypnotic and pharmacokinetic effects, including greater suppression of the cortical electroencephalogram.
- The greater hypnotic effect of 3 β -OH in female mice is attributable to greater metabolism into the potent hypnotic metabolite 3 α -OH, emphasising the importance of investigating sex-specific anaesthetic effects.

The novel neuroactive steroid (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (3 β -OH) has hypnotic properties, with a primary mechanism of action of blocking low voltage-activated T-type calcium channels (T channels).^{1,2} In adult rats, 3 β -OH has sex-specific hypnotic effects.⁶ Here we studied the mechanisms contributing to the sex differences in its hypnotic effect.

Addressing sex-specific differences is central to developing new therapeutics, as females may have different pharmacodynamic and pharmacokinetic responses to currently used pharmaceuticals. This includes differences in hepatic metabolism, in drug absorption and distribution, and in circulating hormones.³ The US Food and Drug Administration recommended studying sex differences in clinical trials in 1993, and until recently, there has been a disparity in the use of male and female animal models in translational research despite the importance of understanding the differential effects of new therapeutics in men and women.³

Using a combination of pharmacokinetic, behavioural, neuroendocrine, and electrophysiological approaches, we show that sex differences in the hypnotic effects of 3 β -OH are driven by differential metabolism of 3 β -OH to a γ -aminobutyric acid type A (GABA_A) receptor positive allosteric modulator (GABA-PAM) with hypnotic properties, (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile (3 α -OH).

Methods**Animals**

We used adult and adolescent male and female C57Bl/6 wild-type (WT) mice and Cav3.1 global knockouts (KOs) on a C57Bl/6J background. Animals were housed in temperature- and humidity-controlled facilities with food and water *ad libitum* and maintained on a 14:10 light–dark cycle. Experiments were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee and

comply with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments followed ARRIVE guidelines.

Behaviour and pharmacokinetics

To determine levels of hypnosis, animals were injected intraperitoneally (i.p.) or intravenously (i.v.) with either 3 β -OH (Fig 1a) or 3 α -OH and assessed for time to loss of righting reflex (LORR) and time to regaining the righting reflex as described.⁴ We performed pharmacokinetic experiments to measure the concentration of 3 β -OH and 3 α -OH over time in brain, liver, plasma, and urine. We injected adult mice with 3 β -OH 100 mg kg⁻¹ i.p. and collected tissue at 0, 10, 20, 30, 40, 60, 80, 120, 180, 360, 540, or 720 min after injection. This dose of 3 β -OH was used as a standard dose at which 100% of male and female mice lost their righting reflex. We also injected adult mice with 3 α -OH 60 mg kg⁻¹ i.p. and collected tissue at 40 min after injection. This dose of 3 α -OH produced relatively similar hypnotic effect to 100 mg kg⁻¹ 3 β -OH, and later revealed to be the lowest dose at which all male and female mice lost their righting reflex. Tissue samples were processed for high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) as described in supplemental information.

Electrophysiology

To study modulation of T channels, we cultured human embryonic kidney cells (HEK-293) stably transfected with human Cav3.1 channels and conducted *in vitro* whole-cell patch-clamp electrophysiology in voltage clamp configuration as described.⁵ We performed *in vivo* electrophysiology experiments to measure cortical EEG oscillations in adult mice after i.p. administration of 3 β -OH or 3 α -OH as described.⁴

Results**Hypnotic effects of 3 β -OH are more potent in female mice**

A dose–response curve was generated for doses of 20–120 mg kg⁻¹ i.p. to determine the pharmacodynamic properties of 3 β -OH. The Hill–Langmuir equation was used to calculate the 50% effective dose (ED₅₀) for percentage and duration of LORR. The ED₅₀ for LORR in female mice was 48 mg kg⁻¹, and the ED₅₀ in male mice was 80 mg kg⁻¹ (Fig 1b). Females also experienced longer duration of hypnosis. Males did not lose righting reflex at doses <60 mg kg⁻¹, whereas LORR was observed in females at doses as low as 30 mg kg⁻¹ (Fig 1c).

Male and female mice did not differ in the percentage of LORR at lower (20–30 mg kg⁻¹) and higher doses (80–120 mg kg⁻¹) of 3 β -OH. However, female mice were more likely to achieve LORR than male mice at 40, 50, and 60 mg kg⁻¹ (Fig 1d). For latency to hypnosis, few female and even fewer male mice lost righting reflex at doses <60 mg kg⁻¹. At doses of 80–120 mg kg⁻¹, females lost righting reflex two-fold more quickly than males (Fig 1e), and higher doses produced 3.5-fold longer duration of LORR in females than in males (Fig 1f).

Sex hormones modulate the hypnotic effects of 3 β -OH

Our previous studies on 3 β -OH in neonatal rats found no differences in hypnotic effect between males and females,¹ whereas adult rats showed sex differences in EEG power spectra.⁶ To further explore these age differences, we gave 3 β -

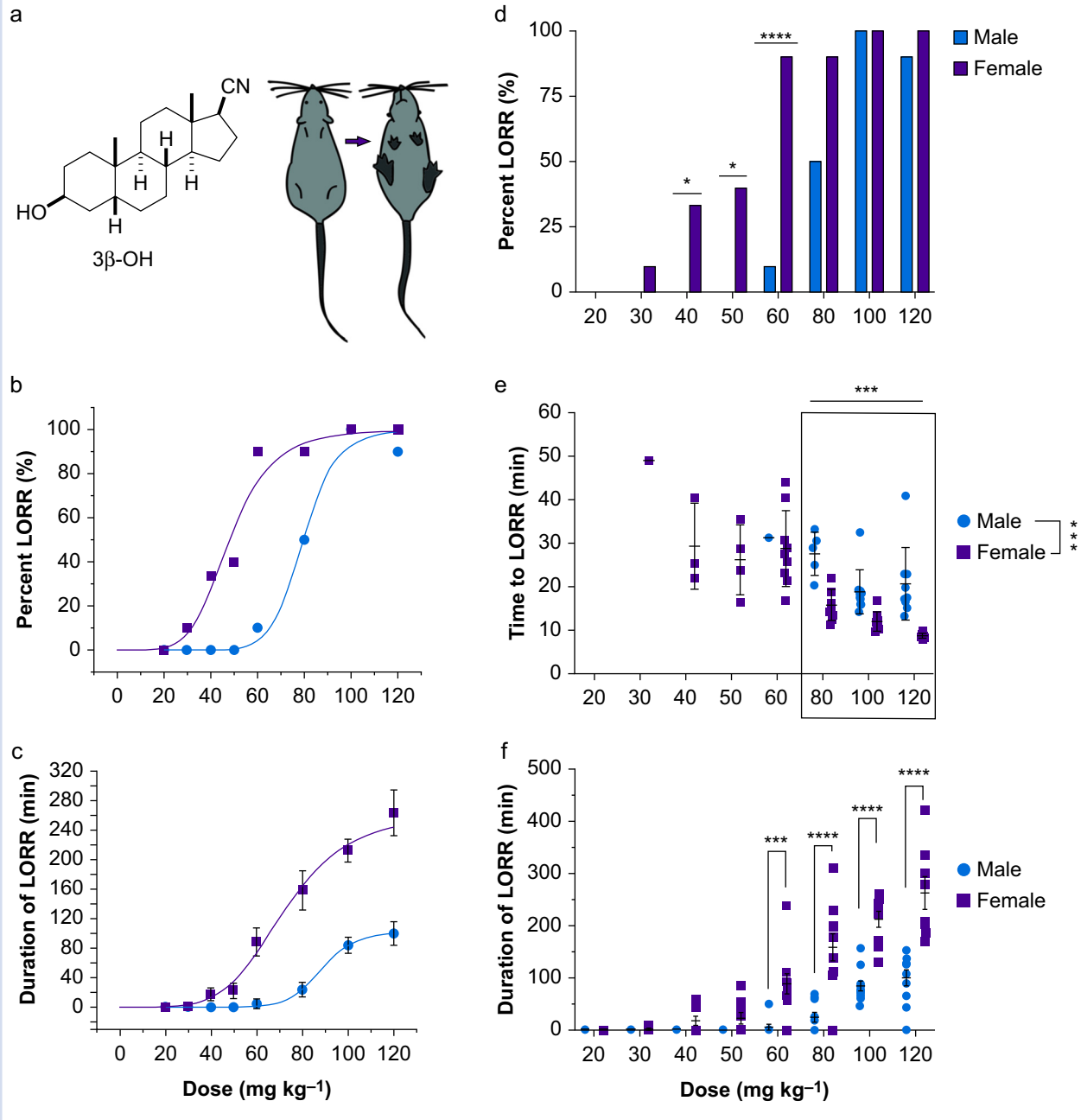


Fig 1. Hypnotic effects of 3 β -OH are more potent in female mice. (a) Male ($n=5-10$) and female ($n=8-10$) mice were injected with a range of doses of 3 β -OH and tested for LORR. (b) Dose–response results revealed that females lost righting reflex at lower doses of 3 β -OH and (c) remained unresponsive longer than males. (d) χ^2 analysis showed that males and females did not differ in percentage of LORR at lower doses of 20 mg kg⁻¹ ($\chi^2=0.0$, $P=1.0$) and 30 mg kg⁻¹ ($\chi^2=1.05$, $P=0.31$) or higher hypnotic doses of 80 mg kg⁻¹ ($\chi^2=3.81$, $P=0.051$), 100 mg kg⁻¹ ($\chi^2=0.00$, $P=1.0$), and 120 mg kg⁻¹ ($\chi^2=0.39$, $P=0.5$). At intermediate doses, females were more likely to achieve LORR than males at 40 mg kg⁻¹ ($\chi^2=3.99$, $*P=0.047$), 50 mg kg⁻¹ ($\chi^2=2.81$, $*P=0.025$), and 60 mg kg⁻¹ ($\chi^2=12.80$, $****P<0.0001$). (e) At doses between 80 and 120 mg kg⁻¹, indicated by the black box, latency to LORR decreased as dose increased and females lost righting reflex more quickly than males (two-way ANOVA sex by dose, Sidak's multiple comparisons [$F_{2,45}=8.827$, $***P=0.001$ for dose; $F_{1,45}=52.346$, $***P<0.001$ for sex]). (f) For duration of LORR, a significant interaction ($F_{7,135}=6.477$, $P<0.001$), indicated that at doses at 60 ($***P<0.001$), 80 ($****P<0.0001$), 100 ($****P<0.0001$), and 120 mg kg⁻¹ ($****P<0.0001$), females remained unresponsive longer than males. 3 β -OH, (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; LORR, loss of righting reflex; ANOVA, analysis of variance.

OH to juvenile male and female mice at P21 and tested for LORR. Similar to previous LORR experiments, adult female mice exhibited LORR sooner than adult male mice. However, P21 male mice lost righting reflex more quickly than adult males, and their LORR values were similar to those of P21 and adult female mice (Fig 2a). Furthermore, P21 male mice lost righting reflex for longer than adult males and had similar duration of LORR as P21 and adult female mice (Fig 2b). To determine the effects of circulating androgens on hypnosis, we gave 3 β -OH to gonadectomised and sham-operated female and male mice and tested for LORR. Gonadectomised female mice did not differ from sham-operated females in time to or duration of LORR (Fig 2c and d). However, gonadectomised males lost righting reflex sooner than their sham-operated counterparts (Fig 2e) and LORR lasted longer than for sham-operated males (Fig 2f).

Sex-specific metabolism modulates the hypnotic effects of 3 β -OH

To examine differences in metabolism of 3 β -OH, we measured 3 β -OH at several times after i.p. administration. Male mice had

higher 3 β -OH concentrations in brain and liver compared with females (Fig 3a and b). However, there were no significant differences in 3 β -OH concentrations in plasma (Fig 3c). Female mice excreted approximately three-fold more 3 β -OH in urine compared with males (Fig 3d). Because males were less sensitive to the hypnotic effects of 3 β -OH despite having higher brain concentrations, we hypothesised that a metabolite of 3 β -OH contributes to hypnosis. We proposed that 3 β -OH is metabolised to (3 α ,5 β ,17 β)-3-hydroxyandrostane 17-carbonitrile (3 α -OH) by a combination of 3 β - and 3 α -hydroxysteroid dehydrogenases (3 β - and 3 α -HSD, Fig 3e). 3 α -OH is a known GABA_A receptor positive allosteric modulator and likely a hypnotic.⁷ We measured 3 α -OH concentrations in mice injected with 3 β -OH i.p., and found that female mice had higher concentrations of 3 α -OH in brain, liver, plasma, and urine compared with males (Fig. 3f–i). Moreover, females had higher C_{max} and area under the curve (AUC) concentrations of 3 α -OH compared with males (Supplementary Table S1).

We compared the effects of 3 β -OH administered i.v. with the effects of 3 β -OH administered i.p. on LORR in dose–response experiments. Both male and female mice lost righting reflex

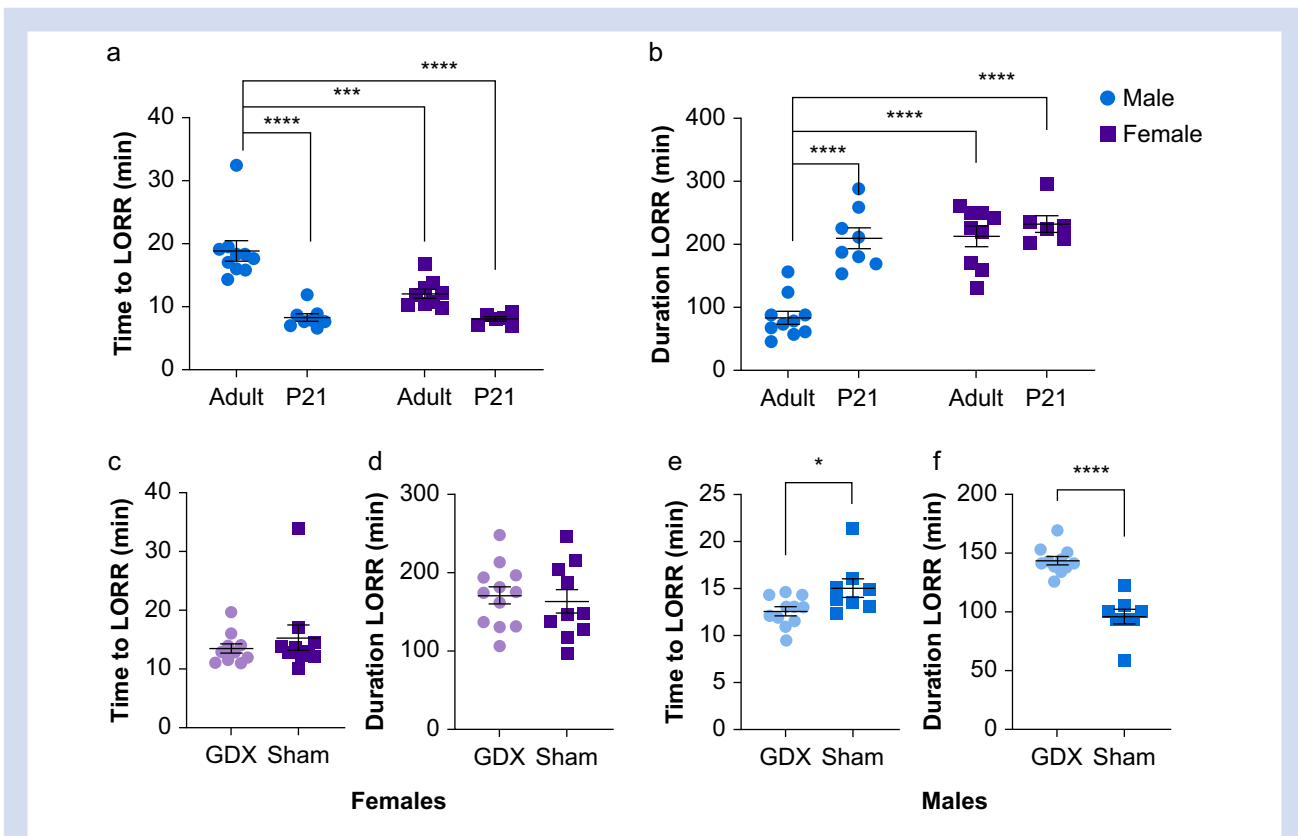


Fig 2. Sex differences in 3 β -OH hypnosis occur after hormonal maturity. (a) We administered 3 β -OH 100 mg kg⁻¹ i.p. to juvenile male ($n=8$) and female ($n=6$) mice at P21 and tested for LORR in comparison with adult mice (two-way ANOVA sex by age, Sidak's multiple comparisons; $F_{1,29}=8.657$, $P=0.006$). Adult (** $P<0.001$) and juvenile (**** $P<0.0001$) females lost righting reflex faster than adult males, and P21 males achieved LORR more quickly than adult males (**** $P<0.0001$). (b) For duration of LORR, there was a significant interaction ($F_{1,29}=13.38$, $P=0.001$) showing that adult (**** $P<0.0001$) and juvenile (**** $P<0.0001$) females were unresponsive longer than adult males. P21 males were unresponsive longer than adult males (**** $P<0.0001$) and had similar duration of LORR as P21 females and adult females. (c, d) We gave 3 β -OH 100 mg kg⁻¹ i.p. to gonadectomised (GDX) and sham-operated females ($n=10$ and $n=12$ sham and GDX, respectively) and males ($n=8$ and $n=11$ for sham and GDX, respectively) and tested for LORR. Gonadectomised females did not differ from sham females in time to or duration of LORR (unpaired t-test, $P=0.385$ and $P=0.682$). (e, f). However, GDX males lost righting reflex sooner than their sham counterparts ($t_{17}=2.416$, $*P=0.027$) and were unresponsive longer than sham males, ($t_{17}=7.071$, **** $P<0.0001$). 3 β -OH, (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; LORR, loss of righting reflex; ANOVA, analysis of variance.

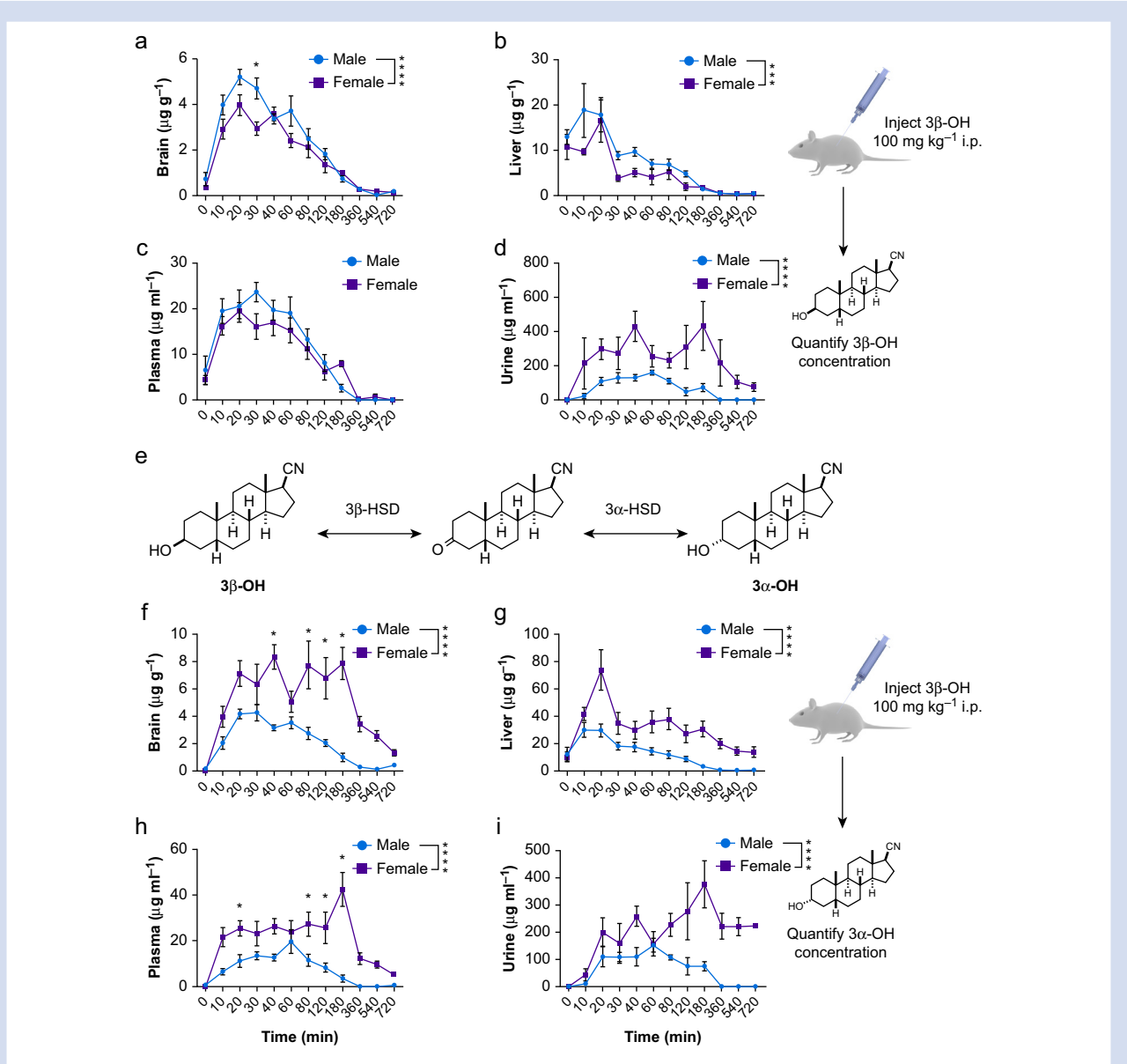


Fig 3. Sex-specific metabolism modulates hypnotic effect of 3β -OH. We harvested tissue samples after administration of 100 mg kg^{-1} of 3β -OH i.p. and measured steroid concentration using HPLC-MS/MS as indicated by cartoon inset on the right (brain and liver, $n=2-3$ at 0 min and $n=5-6$ at 10–720 min; plasma and urine, $n=1-3$ at 0 and 720 min and $n=4-6$ at 10–540 min). (a–d) In brain, liver, plasma, and urine, concentration of 3β -OH changed across different time points (two-way ANOVA sex by time-point, Sidak's multiple comparisons: $F_{11,108}=48.170$, $P<0.0001$; $F_{11,108}=16.242$, $P<0.0001$; $F_{1,104}=29.355$, $P<0.0001$; $F_{11,94}=2.114$, $P=0.026$, respectively). (a) In brain, males had higher 3β -OH concentrations than females ($F_{1,108}=12.902$, **** $P<0.0001$; inset) and the interaction showed that this was specific to 30 (* $P=0.003$) min after injection. (b) Males also had higher 3β -OH concentrations in liver ($F_{1,108}=9.103$, *** $P=0.003$; inset) but no significant interaction. (c) Plasma samples showed no sex effects. (d) In urine, females had higher 3β -OH concentrations compared with males ($F_{1,94}=24.982$, **** $P<0.0001$; inset). (e) Scheme shows that 3β -OH is metabolised into 3α -OH by 3α -HSD in a reversible reaction. (f–i) We found that after injections of 3β -OH i.p., concentrations of 3α -OH changed across different time points in brain, liver, plasma, and urine ($F_{11,108}=11.757$, $P<0.0001$; $F_{11,108}=10.623$, $P<0.0001$; $F_{11,104}=8.911$, $P<0.0001$; $F_{11,94}=2.462$, $P=0.009$, respectively). (f) For brain concentrations, females had higher 3α -OH concentrations compared with males ($F_{1,108}=85.652$, **** $P<0.0001$; inset). There was also a significant interaction ($F_{11,108}=3.161$, $P=0.001$) showing that this was specific to 40 (* $P<0.0001$), 80 (* $P<0.0001$), 120 (* $P<0.001$), and 180 (* $P<0.0001$) min after injection. (g) Females also had higher 3α -OH in liver compared with males ($F_{11,108}=64.689$, **** $P<0.0001$; inset). (h) Similarly, in plasma, females had higher 3α -OH concentrations compared with males ($F_{1,104}=83.985$, **** $P<0.0001$; inset), and there was a significant interaction ($F_{11,104}=3.703$, $P<0.0001$), indicating this was specific to 20 (* $P=0.049$), 80 (* $P=0.019$), 120 (* $P=0.012$), and 180 (* $P<0.0001$) min after injection of 3β -OH. (i) Females excreted 3α -OH in urine at higher concentrations than males ($F_{1,94}=37.160$, **** $P<0.0001$; inset), and there was no significant interaction. 3β -OH, (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; LORR, loss of righting reflex; HPLC-MS/MS, high-performance liquid chromatography coupled to tandem mass spectrometry; 3α -OH, (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; 3α -HSD, 3α -hydroxysteroid dehydrogenase; 3β -HSD, 3β -hydroxysteroid dehydrogenase; ANOVA, analysis of variance.

faster after i.v. administration than after i.p. administration. In contrast to i.p. injection, there were no sex differences in time to LORR after i.v. administration (Fig 4a). For duration of LORR, regardless of route of administration, female mice were more sensitive than males. Whereas female mice injected with 3 β -OH i.v. lost their righting reflex for a shorter duration compared with mice injected i.p., male mice given 3 β -OH i.v. lost righting for a similar duration to males given 3 β -OH i.p. (Fig 4b).

We then measured 3 β -OH and 3 α -OH concentrations in brain and liver at time to LORR after i.v. administration of 3 β -OH. We observed no sex differences in 3 β -OH concentrations in brain at time of LORR. The brain had notable concentrations of 3 α -OH at time to LORR with no differences between males and females. However, 3 β -OH concentrations were approximately seven-fold greater than 3 α -OH concentrations and exceeded concentrations after i.p. administration in pharmacokinetic experiments (Fig 4c). At the time of LORR, the liver was producing large amounts of 3 α -OH, but there were no sex differences for 3 β -OH and 3 α -OH. However, 3 α -OH

concentrations were higher than those of 3 β -OH across all groups, indicating metabolism occurring early after 3 β -OH administration (Fig 4d). These results support the hypothesis that sex differences in time to LORR may be mediated by peripheral metabolism of 3 β -OH into 3 α -OH.

The metabolite 3 α -OH is a potent hypnotic agent

To test whether 3 α -OH induces LORR, we generated dose–response curves by measuring LORR between 10 and 80 mg kg⁻¹ i.p.. As the dose increased, time to LORR decreased and duration increased (Supplementary Fig. S1a and b). However, sex differences were only observed for duration of LORR (Supplementary Fig. S1b). To make direct comparisons with 3 β -OH, we compared 60 mg kg⁻¹ 3 α -OH with 100 mg kg⁻¹ 3 β -OH i.p.. Male and female mice had similar onset of LORR. Moreover, females given 3 α -OH lost righting at a similar rate as females given 3 β -OH, whereas males given 3 α -OH lost righting sooner than males given 3 β -OH (Fig 5a). For duration of LORR,

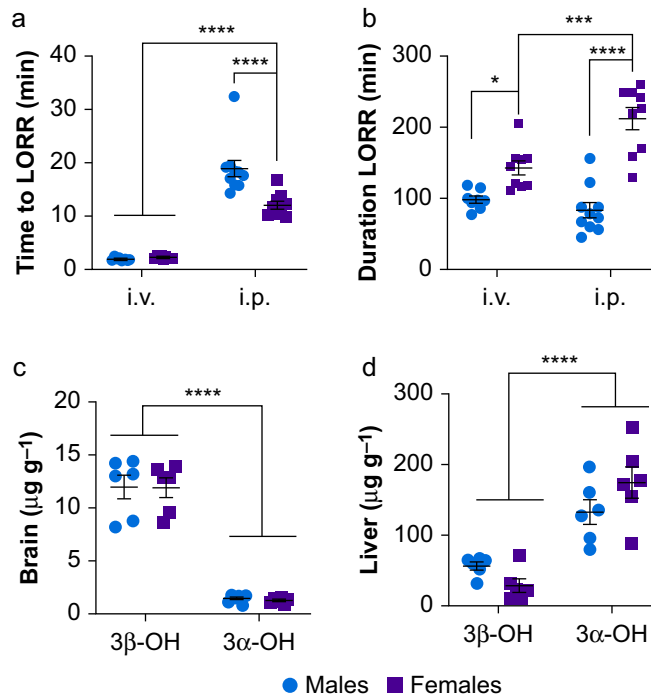


Fig 4. Sex-specific hypnotic effects are less prominent after intravenous than intraperitoneal administration of 3 β -OH. We compared the effects of administration of 100 mg kg⁻¹ 3 β -OH i.v. in adult male ($n=8$) and female ($n=9$) mice to animals that received 100 mg kg⁻¹ i.p. in dose–response experiments. (a) A significant interaction (two-way ANOVA sex by route of administration, Sidak's multiple comparisons: $F_{1,32}=13.99$, $P=0.007$) revealed that all animals lost righting reflex sooner after i.v. administration compared with i.p. administration (**** $P<0.0001$ for both), and that females lost righting reflex before males after i.p. administration (**** $P<0.0001$). However, there were no differences between males and females in time to LORR after i.v. administration ($P=0.995$). (b) For duration of LORR, a significant interaction ($F_{1,32}=13.99$, $P<0.001$) revealed that females were unresponsive longer than males regardless of route of administration (**** $P<0.0001$ and * $P=0.045$ for i.p. and i.v., respectively). Whereas females were unresponsive for a shorter duration after i.v. administration compared with i.p. administration (*** $P<0.001$), males administered 3 β -OH i.v. lost LORR for a similar duration as males given 3 β -OH i.p. ($P=0.788$). (c) We measured 3 β -OH and 3 α -OH brain and liver concentrations after i.v. administration of 3 β -OH 100 mg kg⁻¹ at time to LORR ($n=6$ males, $n=6$ females). There were no sex differences in 3 β -OH or 3 α -OH concentrations in the brain at time to LORR (two-way ANOVA sex by steroid, Sidak's multiple comparisons: $P>0.999$ and $P=0.999$, respectively). However, 3 β -OH concentrations were greater than those of 3 α -OH ($F_{1,20}=209.6$, **** $P<0.0001$). (d) At time to loss of righting reflex, a significant interaction revealed ($F_{1,20}=5.292$, $P=0.032$) that 3 α -OH concentrations were higher in liver than those of 3 β -OH across all groups ($P<0.001$ for all groups). 3 β -OH, (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; LORR, loss of righting reflex; 3 α -OH, (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; ANOVA, analysis of variance.

sex differences persisted with 3α -OH; females lost their righting reflex approximately two-fold longer than males did (Fig 5b). Whereas 3α -OH i.p. elicited similar duration of LORR in males as 3β -OH, females given 3α -OH lost righting for a shorter duration than females given 3β -OH (Fig 5b).

To determine if sex differences are attributable to sex-specific pharmacokinetic differences, we injected mice with 3α -OH i.p. and measured brain and liver concentrations of 3α -OH and 3β -OH at 40 min after LORR. Females had higher 3α -OH concentrations in both brain and liver compared with males (Fig 5c and d). Only males showed minimal conversion of 3α -OH to 3β -OH for both brain and liver, with liver showing more conversion to 3β -OH than brain (Supplementary Table S2).

Inhibition of Cav3.1 T channels contributes to the hypnotic effects of 3α -OH

We cultured HEK-293 cells expressing the human Cav3.1 subtype to investigate the effects of 3α -OH on T channels. 3α -OH blocked ~50% of T-channel currents at relevant concentrations (Supplementary Fig. S2a,b). 3α -OH also reduced normalised current after application of 30 and 10 μ M and significantly decreased current decay time (τ) compared with baseline (Supplementary Fig. S2c,d). Finally, we plotted activation kinetics (G/G_{\max}) against steady-state inactivation kinetics (I/I_{\max}) values. 3α -OH at 10 μ M produced a hyperpolarising shift in I/I_{\max} V_{50} of ~7 mV compared with baseline and shifted G/G_{\max} V_{50} to slightly more depolarised potentials ~3 mV (Supplementary Fig. S2e,f).

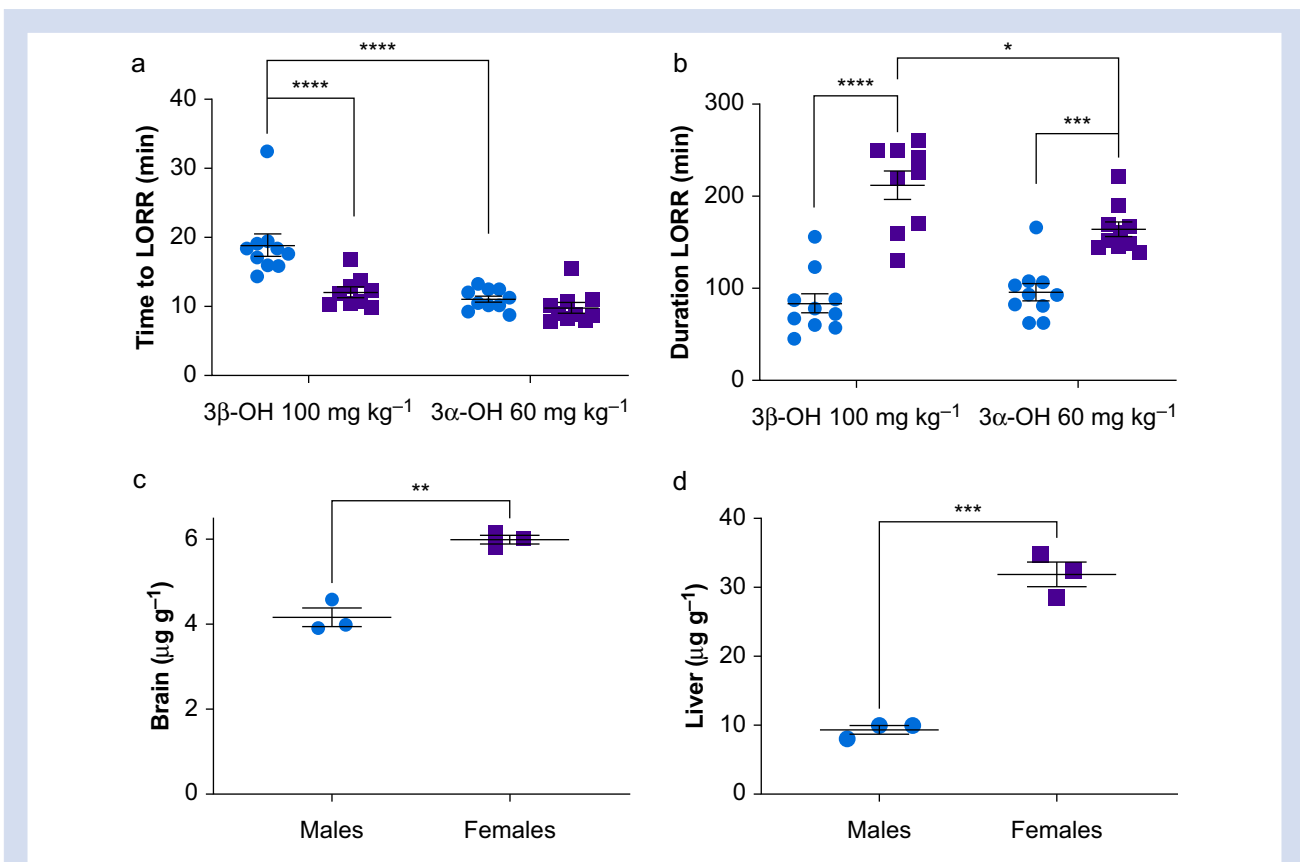


Fig 5. 3α -OH is a potent hypnotic. We administered 3α -OH 60 mg kg⁻¹ i.p. to adult male ($n=10$) and female ($n=10$) mice and measured loss of righting reflex. (a) A significant interaction (two-way ANOVA sex by steroid, Sidak's multiple comparisons: $F_{1,35}=7.872$, $P=0.008$) revealed that males and females lost righting reflex at similar times after i.p. injection of 60 mg kg⁻¹ 3α -OH ($P=0.806$). Females given 60 mg kg⁻¹ 3α -OH lost righting reflex at a similar time as females given 100 mg kg⁻¹ 3β -OH ($P=0.391$), and more quickly than males given 100 mg kg⁻¹ 3β -OH (**** $P<0.0001$). Males given 3α -OH lost righting reflex sooner than males given 3β -OH (**** $P<0.0001$). (b) For duration of LORR, a significant interaction ($F_{1,35}=7.482$, $P=0.010$) revealed that females lost righting longer than males after injection of 100 mg kg⁻¹ 3β -OH (**** $P<0.0001$) and 60 mg kg⁻¹ 3α -OH (*** $P=0.0004$). Also, females given 60 mg kg⁻¹ 3α -OH lost righting for a shorter duration than females given 100 mg kg⁻¹ 3β -OH (* $P=0.022$). To determine if sex differences were also attributable to the sex-specific pharmacokinetics of 3α -OH, we injected male ($n=3$) and female ($n=3$) mice with 60 mg kg⁻¹ 3α -OH and measured brain and liver concentrations of 3α -OH and 3β -OH at 40 min after LORR. (c) Females had higher 3α -OH brain concentrations compared with males (unpaired t -test $t_4=7.907$, ** $P=0.0014$). (d) Similarly, females had higher liver 3α -OH concentrations than males ($t_4=11.167$, *** $P=0.0003$). 3β -OH, (3 β ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; LORR, loss of righting reflex; 3α -OH, (3 α ,5 β ,17 β)-3-hydroxyandrostane-17-carbonitrile; ANOVA, analysis of variance.

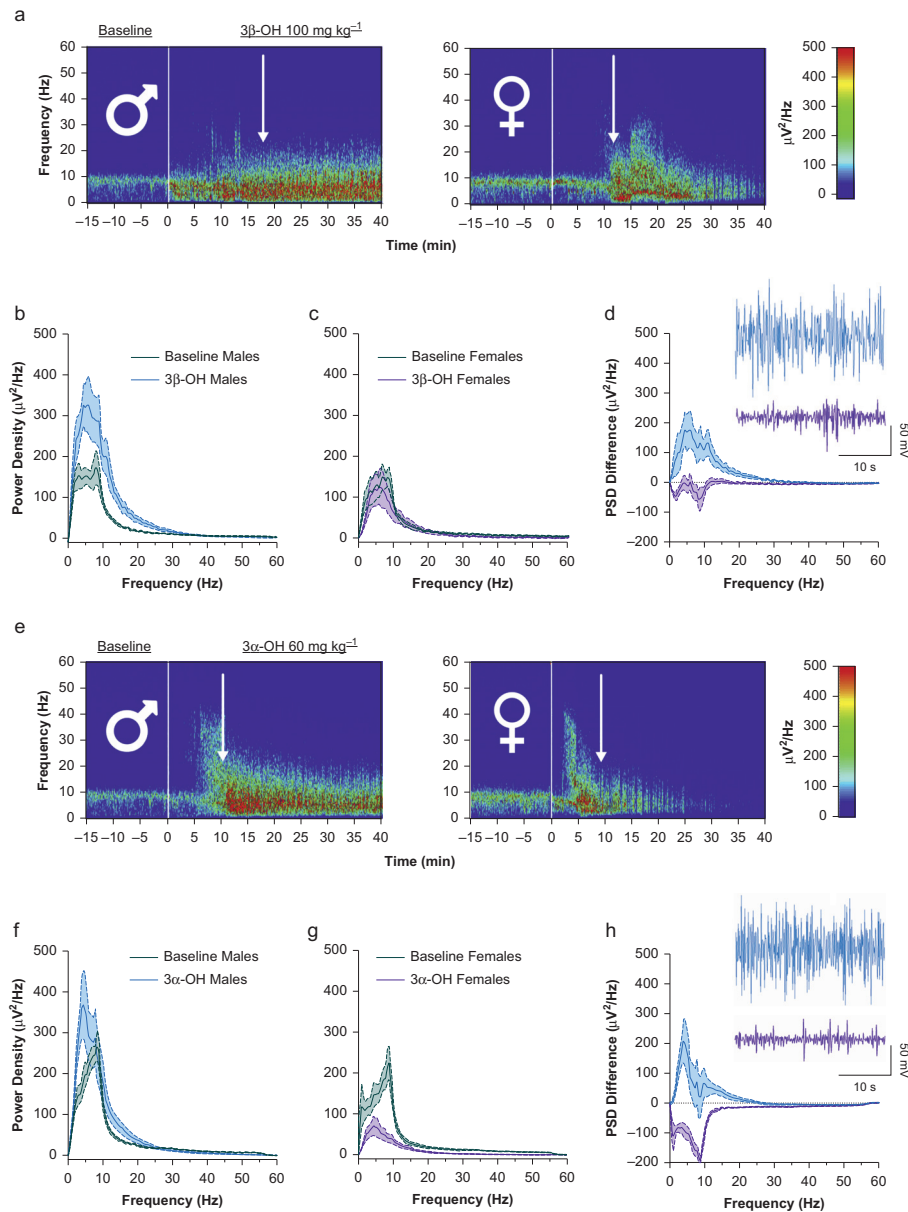


Fig 6. $3\beta\text{-OH}$ and $3\alpha\text{-OH}$ show sex-specific changes in cortical EEG recordings. We analysed power spectral density (PSD) from male ($n=7-9$) and female mice ($n=7-9$) before (baseline) and after receiving either 100 mg kg⁻¹ $3\beta\text{-OH}$ or 60 mg kg⁻¹ $3\alpha\text{-OH}$ i.p. (a) Spectral plots showing sustained increase in PSD in males (left panel) and progressive decrease in PSD in females (right panel) after administration of $3\beta\text{-OH}$. Arrows indicate average time of LORR from dose-response experiments. Note that females show segments of near-complete suppression over time. (b) At 40 min after injection, males showed increase in PSD compared with baseline, peaking in the delta to theta range (Mann-Whitney $U=17\,119$, $P=0.0007$). (c) Female PSD is instead suppressed, and does not differ from baseline after 40 min (Mann-Whitney $U=12\,212$, $P<0.0001$). (d) After subtracting 40 min PSD values from baseline, we found clear sex differences showing that females had suppressed power (Mann-Whitney $U=7961$, $P<0.0001$). Representative traces from males in blue and females in pink visually show stark contrast in the raw EEG waveforms. (e) Spectral plots showing sustained increase in PSD in males (left panel) and profound progressive decrease in female (right panel) PSD with consistent patterns of burst-like suppression. Arrows represent average time to LORR from dose-response curves. (f) 40 min after injection, males showed an increase in PSD values compared with baseline (Mann-Whitney $U=16\,020$, $P<0.0001$), and the peak appeared to shift from around 10 Hz to slower delta oscillations around 4 Hz. (g) Female PSD also showed a leftward shift toward lower frequency oscillations from baseline. In addition, female EEG showed suppression from baseline at 40 min after injection (Mann-Whitney $U=9227$, $P<0.0001$). (h) We subtracted PSD values after 40 min from baseline PSD values and found that females had suppressed power compared with males ($t_{410}=8.46$, $P<0.0001$). Representative traces from males in blue and females in pink again highlight the profound suppression in the female EEG waveforms. $3\beta\text{-OH}$, ($3\beta,5\beta,17\beta$)-3-hydroxyandrostane-17-carbonitrile; LORR, loss of righting reflex; $3\alpha\text{-OH}$, ($3\alpha,5\beta,17\beta$)-3-hydroxyandrostane-17-carbonitrile; ANOVA, analysis of variance.

Because knockout of T-channel isoform $\text{Ca}_v3.1$ reduces the hypnotic effect of $3\beta\text{-OH}$ in male mice,⁴ we determined the possible role of $\text{Ca}_v3.1$ channels in the hypnotic effects of $3\alpha\text{-OH}$. Adult WT and $\text{Ca}_v3.1$ KO mice were injected with $3\alpha\text{-OH}$ i.p. and tested for LORR. KO mice took ~40% longer to LORR compared with WT mice, with no sex differences (Supplementary Fig. S3a). KO mice lost righting reflex for a shorter duration compared with WT mice, and females lost righting reflex longer than males (Supplementary Fig. S3b). These results confirm that $3\alpha\text{-OH}$ can partially block T channels at relevant brain concentrations, which contributes to its hypnotic effects.

$3\beta\text{-OH}$ and $3\alpha\text{-OH}$ show sex-specific effects on cortical EEG

We recently showed that $3\beta\text{-OH}$ causes sex-specific changes in cortical EEG associated with hypnosis⁶; however, the mechanism driving these differences is unknown. We analysed power spectral density (PSD) of the EEG in male and female mice before and after receiving either $3\beta\text{-OH}$ or $3\alpha\text{-OH}$ i.p.. Male mice given $3\beta\text{-OH}$ showed a sustained increase in PSD in the delta and theta range (1–8 Hz), whereas after an initial increase, PSD in females decreased over time (Fig 6a). To examine this further, we plotted PSD for males and females at baseline and at 40 min after injection (when females achieve C_{max} for $3\alpha\text{-OH}$ metabolite). We found that PSD increased in males, but not in females (Fig 6b and c). When we subtracted $3\beta\text{-OH}$ values from baseline, females showed greater suppression in the EEG compared with males (Fig 6d).

We repeated these experiments with $3\alpha\text{-OH}$ and found even more pronounced effects. After administering $3\alpha\text{-OH}$ i.p., male mice showed a sustained increase in PSD in the delta to theta range, whereas PSD in females decreased over time (Fig 6e). When we plotted the PSD values, PSD in males after 40 min increased from baseline, but PSD in females significantly decreased from baseline (Fig 6f and g). Subtracting $3\alpha\text{-OH}$ values from baselines revealed that females had greater suppression of the EEG compared with males (Fig 6h). Because at 40 min after injection females showed no conversion of $3\alpha\text{-OH}$ to $3\beta\text{-OH}$ (Supplementary Table S2), we conclude that suppression of EEG in females receiving $3\beta\text{-OH}$ or $3\alpha\text{-OH}$ may be attributable to the effects of $3\alpha\text{-OH}$.

Discussion

We found that $3\beta\text{-OH}$ has sex-specific hypnotic effects in mice. Males become less sensitive to $3\beta\text{-OH}$ as they age and more sensitive after gonadectomy. Furthermore, sex differences are directly related to sex-specific pharmacokinetic effects, as males had more $3\beta\text{-OH}$ than females in brain, but females produced higher concentrations of the metabolite $3\alpha\text{-OH}$, a GABA_A receptor positive allosteric modulator. Both pharmacokinetic and EEG data suggest that $3\alpha\text{-OH}$ contributes to the hypnotic and sex-specific effects of $3\beta\text{-OH}$. Similar to its pro-compound $3\beta\text{-OH}$, $3\alpha\text{-OH}$ blocks the $\text{Ca}_v3.1$ T-channel subtype in a voltage-dependent manner, which partially contributes to its hypnotic effects.

Sex differences in therapeutic responses is common. For example, women emerge from propofol anaesthesia more quickly than men.^{8,9} These differences can be attributable to hepatic metabolism, drug absorption, or effects of circulating hormones.³ Previous research on endogenous and synthetic neuroactive steroids have reported that female rodents exhibit longer LORR than their male counterparts, which can be

influenced by sex hormones.^{10–12} Similar to work by others, we found that female gonadectomy did not diminish neuroactive steroid induced hypnosis, but that male gonadectomy made males more sensitive to hypnosis.¹² Our results suggest circulating androgens may contribute to duration of hypnosis induced by $3\beta\text{-OH}$ in sexually mature mice, but more experiments manipulating oestrogens and androgens are needed.

We found that the observed sex differences are directly related to sex-specific pharmacokinetic effects, specifically drug metabolism, as females produced more of the metabolite $3\alpha\text{-OH}$ compared with males, indicating that sex differences in $3\beta\text{-OH}$ -induced hypnosis may be attributable to increased concentrations of $3\alpha\text{-OH}$. This is supported by studies showing that there were no sex differences in hypnotic effects or brain concentrations of $3\beta\text{-OH}$ or $3\alpha\text{-OH}$ administered i.v. in time to LORR. Sex differences appear when measuring duration of LORR after sufficient time for drug metabolism has passed.

$3\alpha\text{-OH}$ is a potent hypnotic, and lower doses of $3\alpha\text{-OH}$ are required to achieve similar hypnotic effects as $3\beta\text{-OH}$. The potent hypnotic effects of $3\alpha\text{-OH}$ are likely attributable to its effect as a potent GABA_A receptor modulator.⁷ Female mice do not convert $3\alpha\text{-OH}$ to $3\beta\text{-OH}$ after administration of $3\alpha\text{-OH}$, supporting the conclusion that the hypnotic effects, at least in females, are solely attributable to $3\alpha\text{-OH}$. These results indicate that sex differences in $3\beta\text{-OH}$ -induced hypnosis may be caused by increased concentrations of its active metabolite $3\alpha\text{-OH}$.

Our data suggest that $3\beta\text{-OH}$ is metabolised by 3β -hydroxysteroid dehydrogenase into a 3-keto steroid that is further reduced by 3α -hydroxysteroid dehydrogenase into $3\alpha\text{-OH}$. We speculate that circulating androgens in adult animals inhibit conversion of $3\beta\text{-OH}$ to $3\alpha\text{-OH}$. Mouse 3β -hydroxysteroid dehydrogenase groups I, II, III, and VI convert 3β -reduced steroids into active 3-keto compounds, such as the conversion of pregnenolone to progesterone. Mouse 3β -hydroxysteroid dehydrogenase groups IV and V act as keto-reductases.¹³ Unlike adult male mice, adult females do not express liver 3β -hydroxysteroid dehydrogenase V, the expression of which increases during sexual maturity.¹³ Liver 3β -hydroxysteroid dehydrogenase V may be necessary for back conversion of $3\beta\text{-OH}$. In humans, 3β -hydroxysteroid dehydrogenase isoforms 1 and 2 are primarily expressed in reproductive organs and adrenal glands as opposed to liver. Various 3α -hydroxysteroid dehydrogenase isoforms are expressed in liver in both humans and mice.^{14–17} Although there is sex-specific distribution in some tissues, there are no reported sex differences in liver expression between mouse and humans.^{17–19} Furthermore, 3α and 3β reduction are often initial steps in steroid metabolism with sulphation and glucuronidation likely second steps.¹⁴ Further studies are warranted to identify the isoforms of 3β -hydroxysteroid dehydrogenase, 3α -hydroxysteroid dehydrogenase, and other liver enzymes that might mediate sex differences in neurosteroid-induced hypnosis.

Our studies are in line with previous work showing that GABA_A positive allosteric modulator anaesthetics are T-channel blockers.^{20,21} $3\alpha\text{-OH}$ is a partial T-channel blocker at relevant brain concentrations, and knockout of $\text{Ca}_v3.1$ reduces its hypnotic effects. After intravenous administration of $3\beta\text{-OH}$, its concentrations are eight times higher than those of $3\alpha\text{-OH}$, and twice as high after intraperitoneal administration at the time to LORR. We posit that T channels play a role in induction of hypnosis by decreasing thalamocortical excitability.^{4,22–24} We have shown previously that after intravenous administration of $3\beta\text{-OH}$, male and female rats had similar PSD patterns soon after they became sedated.⁶

Our current findings confirm that 3β -OH increases PSD in the delta and theta frequency range and that EEG power is suppressed in females.^{4,6} We hypothesise that suppression of the EEG in females is driven by the active metabolite 3α -OH. Burst suppression activity may be caused by changes in cortical metabolism resulting in inhibition of the EEG with periods of excitatory bursts.^{25,26} This suppression is similar to that of other GABA_A receptor positive allosteric modulator anaesthetics, including propofol, isoflurane, and alfaxalone,^{27,28} but not of the N-methyl-D-aspartate (NMDA) antagonist ketamine.²⁹ Because females do not convert 3α -OH to 3β -OH, we conclude that suppression in the EEG is not attributable to 3β -OH. Alternatively, 3α -OH metabolism may be faster in females than in males, causing the increased power of delta and theta to return to baseline faster in females. However, this is less likely given that female mice exhibited longer LORR and greater suppression of EEG power after injection of 3α -OH compared with males.

Conclusions

Current GABA_A receptor modulating general anaesthetics are associated with neurotoxicity in the developing brain and have low therapeutic indexes, highlighting the need for novel, safer anaesthetics.^{30,31} Our results show that neuroactive steroids act as potent hypnotics that should be investigated for clinical use. We also demonstrate the importance of studying sex-specific effects of anaesthetics, especially pharmacokinetic differences. Because females can have different pharmacodynamic and pharmacokinetic responses to pharmaceuticals, addressing sex-specific differences is central to developing new therapeutics.

Authors' contributions

Conceptualisation of experiments: FMM, DFC, VJT, SMT

Conduct of majority of the experiments: FMM

Data analysis: FMM

Writing of the manuscript: FMM, JK

Pharmacokinetic experiments: OHC, JK

Editing of the manuscript: OHC, DFC, VJT, SMT

Perform loss of righting reflex experiments: DW, BFR

Provided steroid compounds: DFC, KK

Supervision of experiments: SMT

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Declaration of interest

The authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bja.2022.09.025>.

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