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Differential effects of the novel neurosteroid hypnotic (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile on electroencephalogram activity in male and female rats

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Abstract

Background: We recently showed that a neurosteroid analogue, (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH), induced hypnosis in rats. The aim of the present study was to evaluate the hypnotic and anaesthetic potential of 3β-OH further using electroencephalography.

Methods: We used behavioural assessment and cortical electroencephalogram (EEG) spectral power analysis to examine hypnotic and anaesthetic effects of 3β-OH (30 and 60 mg kg^{-1}) administered intraperitoneally or intravenously to young adult male and female rats.

Results: We found dose-dependent sex differences in 3β-OH-induced hypnosis and EEG changes. Both male and female rats responded similarly to i.p. 3β-OH 30 mg kg^{-1}. However, at the higher dose (60 mg kg^{-1}, i.p.), female rats had two-fold longer duration of spontaneous immobility than male rats (203.4 [61.6] min *vs* 101.3 [32.1] min), and their EEG was suppressed in the low-frequency range (2–6 Hz), in contrast to male rats. Although a sex-dependent hypnotic effect was not confirmed after 30 mg kg^{-1} i.v., female rats appeared more sensitive to 3β-OH with relatively small changes within delta (1–4 Hz) and alpha (8–13 Hz) bands. Finally, 3β-OH had a rapid onset of action and potent hypnotic/anaesthetic effect after 60 mg kg^{-1} i.v. in rats of both sexes; however, all female rats and only half of the male rats reached burst suppression, an EEG pattern usually associated with profound inhibition of thalamocortical networks.

Conclusions: Based on its behavioural effects and EEG signature, 3β-OH is a potent hypnotic in rats, with female rats being more sensitive than male rats.

Keywords: anaesthesia; electroencephalogram; hypnosis; neurosteroid; power spectral density; sex differences

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Editor's key points

- The novel neurosteroid analogue (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) was recently shown to induce hypnosis in rats.
- These authors examined the hypnotic and anaesthetic effects of 3β-OH administered intraperitoneally or intravenously to young adult male and female rats.
- There were dose-dependent sex differences in 3β-OH-induced hypnosis and EEG changes, with female rats generally more sensitive than male rats.
- Novel neurosteroid analogues, such as 3β-OH, that have a novel mechanism of action provide an important approach to developing safer anaesthetics.

The quest for new drugs with anaesthetic properties has slowed since the 1970s, with propofol being the last injectable hypnotic/anaesthetic drug introduced into clinical practice 30 yr ago.[1] Although relatively safe, all currently used general anaesthetics, both inhalational and injectable, are associated with many disadvantages and limitations, such as cardiovascular and respiratory depression,[2] or neurotoxic potential in the developing brain.[3] Thus, there is a growing need for a novel hypnotic/anaesthetic with an improved therapeutic profile. We recently reported that the neurosteroid analogue and T-type voltage-gated calcium channel blocker (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) displays hypnotic properties in neonatal[4] and juvenile rats[5] without associated developmental neurotoxicity.[4]

Electroencephalography is routinely used to assess depth of anaesthesia in animals[6,7] and humans,[8,9] and to record anaesthetic-induced thalamocortical oscillations.[10] The EEG is a useful tool to investigate sedative/hypnotic properties of psychotropic drugs,[11] and hypnotics/anaesthetics such as propofol.[12] However, little is known about EEG activity during 3β-OH-induced hypnosis. Given that 3β-OH blocks neuronal T-type channels and, unlike other clinically used anaesthetics, does not directly modulate gamma-aminobutyric acid type A ($GABA_A$) or N-methyl-D-aspartate receptors,[4] we hypothesised that 3β-OH also affects thalamocortical oscillations in a distinctive way. Hence, we set out to characterise the properties of cortical EEG activity in adolescent/young adult male and female rats during hypnosis induced by i.p. and i.v. 3β-OH.

Methods

Drugs

The neurosteroid analogue 3β-OH was synthesised as described,[4] and formulated in 15% or 25% w:v 2-hydroxypropyl-β-cyclodextrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Animals and drug treatment

Studies were conducted during the light cycle in adolescent and young adult Sprague-Dawley rats (P21–P57) of both sexes. The animals were housed in an accredited animal facility according to protocols approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus. A low (30 mg kg^{-1}) or high (60 mg kg^{-1}) dose of 3β-OH was given as a single bolus injection either i.p. (P29–P34 rats) over about 10 s, or i.v. (P47–P57 rats) into the tail vein of awake, un-anaesthetised, and restrained animals over 30–45 s. The animals were reused for different experiments (low/high dose, i.p./i.v, in that order) after a 'washout' period of at least 4 days. Vehicle (2-hydroxypropyl-β-cyclodextrin 15% or 25%) was devoid of any sedative/hypnotic effects or sedation-related EEG changes in rats after either i.p. or i.v. injection (Supplementary Fig. S1). Data are shown as mean (standard deviation) in the text and as mean (standard error of the mean) in the graphical presentations.

Electrode implantation and EEG recording

To record EEG signals, rats were implanted with screw electrodes under ketamine (100 mg kg^{-1}) and isoflurane (0.5–2%) anaesthesia. Lidocaine (1%) was injected locally at the surgery site to minimise incision pain. The following stereotaxic coordinates were used to place the active electrodes: anteroposterior −2.8 mm from bregma, mediolateral ±3.0 mm from midline, and dorsoventral below the skull surface and over the somatosensory cortex, as this part of the cortex receives direct input from thalamic nuclei.[13] A screw electrode placed behind the lambda on each side of the midline served as ground (right) and reference (left). Electrodes were fixed to the skull using dental acrylic. The rats were treated postoperatively with an analgesic (Banamine®, Merck Animal Health, Madison, NJ, USA) every 24 h for 48 h. At least 1 week after surgery, the synchronised, time-locked video and EEG signals were recorded from the rats using the Pinnacle system (Pinnacle Technology, Inc., Lawrence, KS, USA). The EEG signal was acquired at a sampling rate of 1000 Hz with acquisition filters set at 1 and 500 Hz, and stored for offline analysis. A notch filter with 60 Hz cut-off frequency was applied to remove line noise in some animals. Additional details are provided in the Supplementary material.

Results

Hypnotic effects and EEG signature after i.p. 3β-OH

Based on loss of righting reflex data in juvenile rats,[5] we first examined the effects of a relatively low dose of 3β-OH (30 mg kg^{-1}, i.p.) on behaviour and cortical EEG activity in male and female rats. Both the time to spontaneous immobility (male rats: 435.9 [151.9] s; female rats: 391.3 [150.0] s) and duration of spontaneous immobility (male rats: 86.8 [24.2] min; female rats: 113.8 [27.1] min) were not significantly affected by sex (Supplementary Fig. S2a). Likewise, the change in absolute spectral power after 3β-OH was similar in male and female rats (Fig. 1a): an increase in power was detected across different frequency bands (1–30 Hz) in both sexes. For example, 15 min after i.p. 3β-OH, delta power was increased >two-fold compared with baseline (Supplementary Fig. S3a; male rats: 2.1 [1.1]-fold; female rats: 2.3 [1.2]-fold). To further assess changes in power before (quiet awake) and 15 min after injection (sedation/hypnosis), we constructed power spectral density (PSD) plots. Compared with baseline, a significant increase in PSD ($P<0.05$) in frequency bands from delta to beta in both male (Fig. 1b) and female (Fig. 1c) rats was observed after 3β-OH administration. These changes in PSD from baseline were similar in both sexes (Fig. 1d).

Next, we tested a higher (60 mg kg^{-1}, i.p.) dose of 3β-OH. Although the time to spontaneous immobility was similar in both sexes (male rats: 393.8 [142.4] s; female rats: 334.1 [139.3]

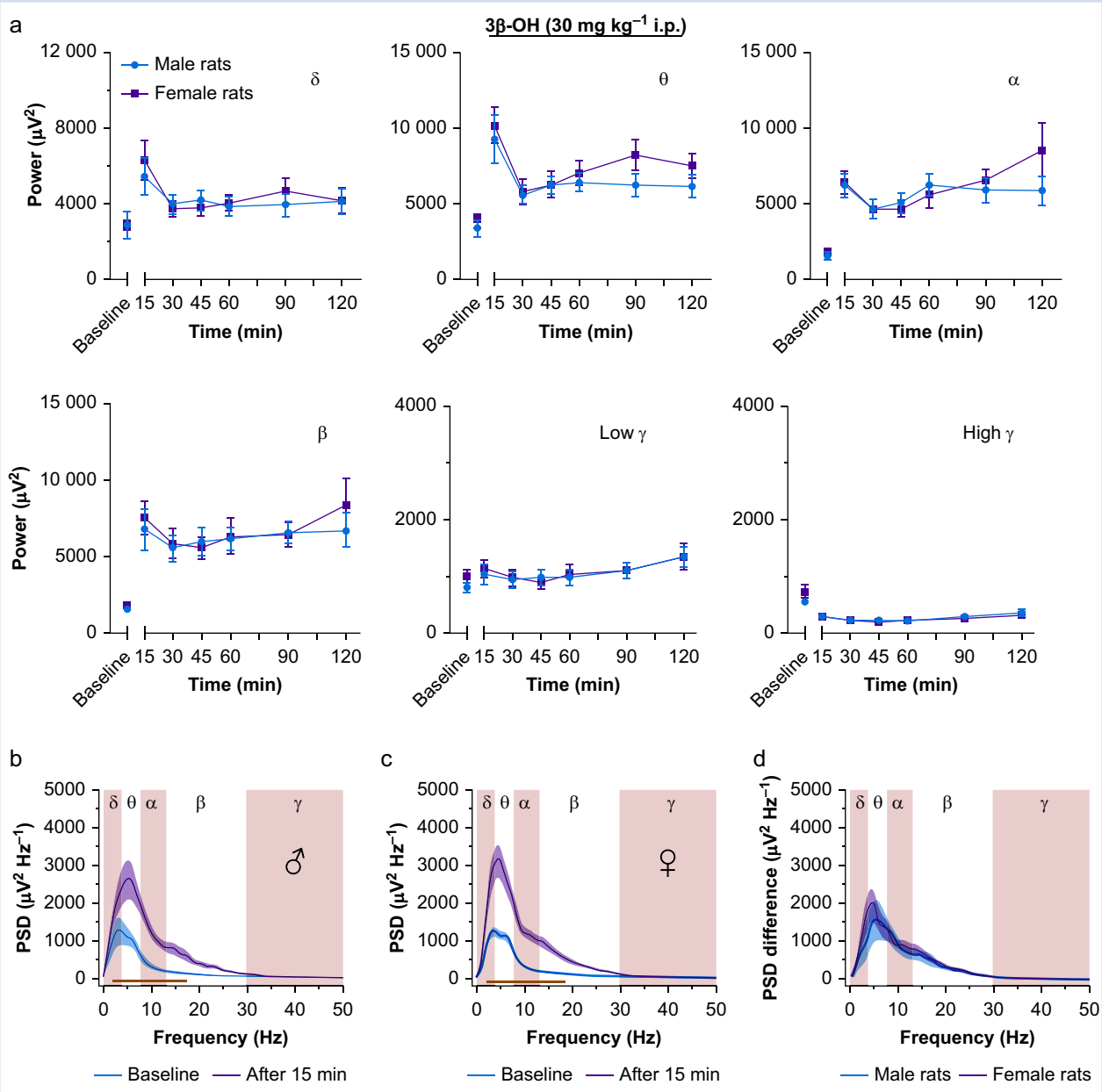

Fig 1. Effects of i.p. administration of (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) (30 mg kg^{-1}) on EEG spectral power in male and female rats. (a) Time course plots showing changes in the absolute spectral power in different frequency bands after i.p. injection of 3β-OH in male (n=8) and female (n=8) rats. (b) The power spectral density (PSD) plots during baseline EEG recording (black) and 15 min after i.p. injection of 3β-OH (red) in male rats. The dark blue line represents statistically significant change in PSD (2–18 Hz) after injection compared with baseline (P<0.05; interaction: F[103, 721]=8.12; P<0.001). (c) The PSD plots during baseline EEG recording (black) and 15 min after i.p. injection of 3β-OH (red) in female rats. The dark blue line represents statistically significant change in PSD (2–19 Hz) after injection compared with baseline (P<0.05; interaction: F[103, 721]=15.57; P<0.001). Statistical analyses for data sets presented in (b) and (c) were performed using two-way repeated measures (RM) analysis of variance (ANOVA) followed by Sidak's *post hoc* test. (d) Baseline-normalised PSD plots obtained 15 min after i.p. injection of 3β-OH showing no significant difference between male (black) and female (red) rats (interaction: F[103, 1442]=0.38, P>0.999; sex: F[1, 14]=0.15, P=0.702; frequency: F[103, 1442]=22.01, P<0.001; two-way RM ANOVA).

s), female rats had about two-fold longer duration of spontaneous immobility than male rats (male rats: 101.3 [32.1] min; female rats: 203.4 [61.6] min; Supplementary Fig. S2b). Even after this relatively high dose of 3β-OH, the toe pinch reflex was still present in both sexes. Compared with female rats, male rats had significantly higher absolute EEG power in the delta–theta and alpha ranges, but only at 120 min after injection. Except for normalised alpha power, these changes in

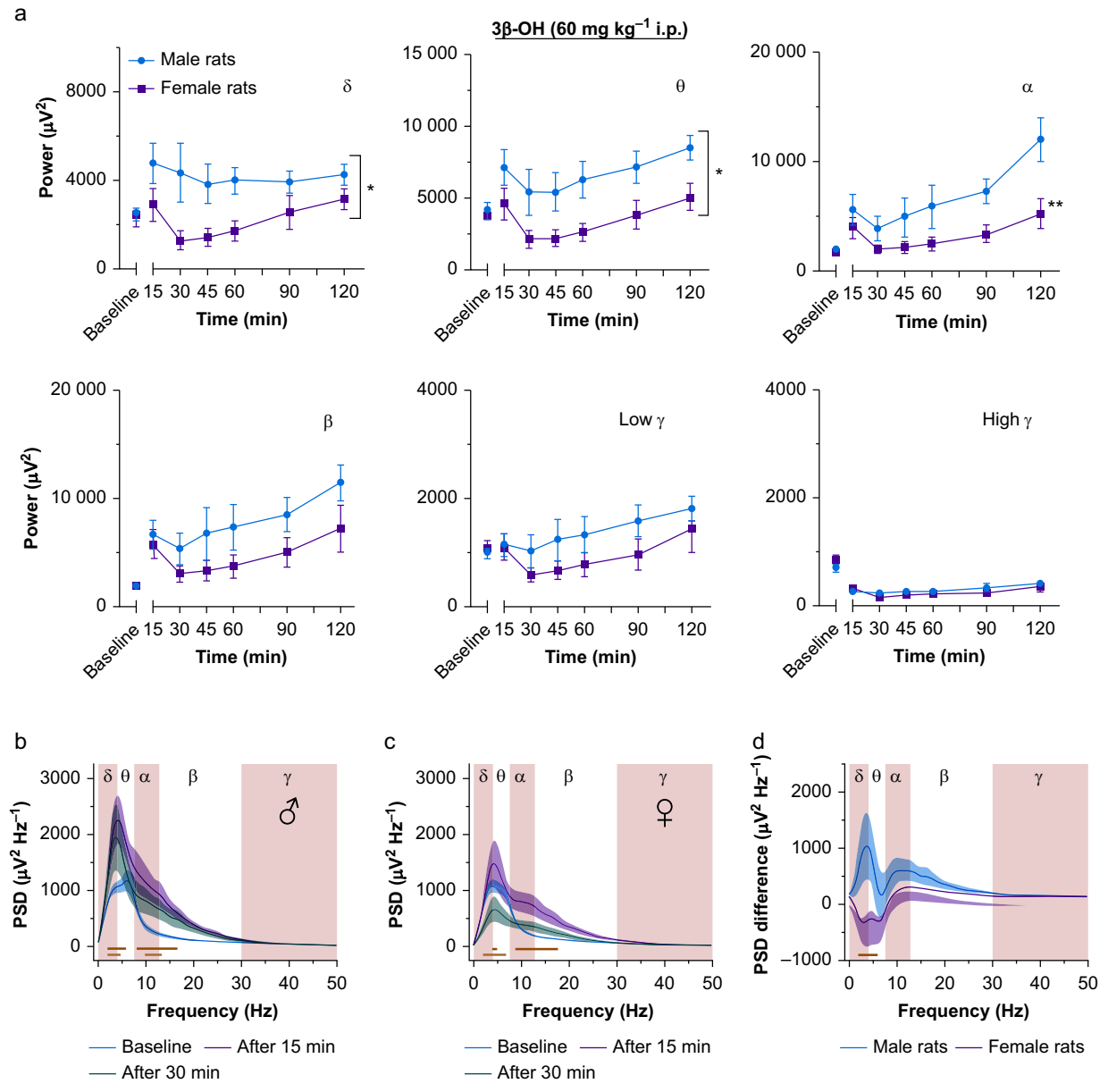

Fig 2. Effects of i.p. administration of (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) (60 mg kg^{-1}) on EEG spectral power in male and female rats. (a) Time course plots showing changes in the absolute spectral power in different frequency bands after i.p. injection of 3β-OH in male (n=8) and female (n=8) rats. The absolute spectral power in the delta and theta bands was significantly higher in male rats after 3β-OH compared with female rats. Delta: interaction: $F(5, 70)=1.06$, $P=0.389$; sex: $F(1, 14)=6.27$, $^{*}P=0.025$; time: $F(5, 70)=2.11$, $P=0.074$. Theta: interaction: $F(5, 70)=0.18$, $P=0.971$; sex: $F(1, 14)=6.72$, $^{*}P=0.021$; time: $F(5, 70)=7.03$, $P<0.001$; two-way repeated measures (RM) analysis of variance (ANOVA). A similar effect was observed in the alpha band, 120 min after i.p. injection of 3β-OH in particular ($^{**}P=0.002$; interaction: $F[5, 70]=3.80$, $P=0.004$; two-way RM ANOVA followed by Sidak's *post hoc* test). (b) The power spectral density (PSD) plots during baseline EEG recording (black), 15 (red) and 30 min after i.p. injection of 3β-OH (green) in male rats. The dark and light blue horizontal lines represent a statistically significant change in PSD after 15 min (2–6 and 8–17 Hz) and after 30 min (3–5 and 10–14 Hz), respectively, compared with baseline ($P<0.05$; interaction: $F[206, 1442]=3.36$, $P<0.001$). (c) The PSD plots during baseline EEG recording (black), 15 (red) and 30 min after i.p. injection of 3β-OH (green) in female rats. The dark and light blue horizontal lines represent a statistically significant change ($P<0.05$) in PSD after 15 min (4–5 and 9–18 Hz) and after 30 min (2–7 Hz), respectively, compared with baseline ($P<0.05$; interaction: $F[206, 1030]=2.12$, $P<0.001$). (d) Baseline-normalised PSD plots obtained 30 min after i.p. injection of 3β-OH in male (black) and female (red) rats. The dark blue line represents a statistically significant difference ($P<0.05$) in baseline-normalised PSDs (2–6 Hz) between male and female rats ($P<0.05$; interaction: $F[103, 1442]=2.73$, $P<0.001$). Statistical analyses for data sets presented in (b–d) were performed using two-way RM ANOVA followed by Sidak's *post hoc* test.

spectral power did not reach statistical significance when we normalised data from male and female rats to their respective baselines (Supplementary Fig. S3b). However, we detected a stronger decrease in high gamma (50–100 Hz) band in female than in male rats.

When we analysed PSD plots in male rats, 3β-OH induced a significant increase in PSD in the delta–beta range 15 and 30 min after injection (Fig. 2b). In female rats, 3β-OH first caused an increase in PSD (Fig. 2c; $P<0.05$ for the delta–beta range), followed by suppression of EEG activity 30 min after injection ($P<0.05$ for the delta–theta range). This effect was noticeable when we compared the two baseline-normalised PSD data sets, which revealed a clear difference between male and female rats in the delta–theta range (Fig. 2d).

In summary, 30 mg kg^{-1} dose of 3β-OH had a similar sedative/hypnotic effect on male and female rats, with minimal differences in EEG activity. The higher dose of 3β-OH (60 mg kg^{-1}, i.p.) had a stronger sedative/hypnotic effect in female rats, which was accompanied by suppressed EEG, especially in the low-frequency bands.

Hypnotic effects and EEG signature after i.v. 3β-OH

Although i.p. administration has some practical advantages in animals, the i.v. route provides fast onset of action and circumvents first-pass metabolism, and is thus the preferred mode of administration for injectable hypnotics/anaesthetics in clinical settings. We injected intravenously the same low (30 mg kg^{-1}) and high (60 mg kg^{-1}) doses of 3β-OH in male and female rats. The low dose had a relatively modest hypnotic effect, as assessed by time to first spontaneous immobility (male rats: 291.2 [183.7] s; female rats: 163.7 [45.0] s) and duration of spontaneous immobility (male rats: 23.8 [19.2] min; female rats: 54.2 [47.3] min; Supplementary Fig. S2c). This small sex difference in hypnotic potency was reflected in moderate EEG changes. The absolute spectral power in the delta range was significantly higher in female rats, but only 60 min after injection (Fig. 3a). This effect remained even when we normalised the delta power (Supplementary Fig. S3c). The ensuing PSD analysis confirmed these findings: 3β-OH induced an increase in PSD in a wide delta–beta range in male rats (Fig. 3b), and from theta to beta in female rats, compared with baseline (Fig. 3c). When we compared the two baseline-normalised data sets, female rats had a significantly larger increase in PSD (Fig. 3d) across the whole spectrum, but this difference was noticeable only in a narrow range within the alpha band.

Next, we analysed the EEG spectrograms from representative male and female rats before (baseline) and after i.v. 30 mg kg^{-1} of 3β-OH (Fig. 4). Baseline EEG in male rats showed a typical pattern of frequency distribution during awake periods with a prominent theta band (Fig. 4a, top). This is represented by the PSD plot in Figure 4b (grey frame). Soon after injection, power in higher-frequency bands (i.e. alpha and beta) started to rise (Fig. 4a and b, magenta frame), which was associated with a sedated behavioural state. Hypnotic effects were apparent a few minutes later, when alpha and beta frequency bands, along with delta, became even more pronounced (Fig. 4a and b, orange frame). Baseline EEG activity of a female rat was similar to that of a male rat (Fig. 4c, top; Fig. 4d, grey frame). The rise in alpha and beta bands started almost immediately after injection (Fig. 4c and d, magenta frame), signifying onset of sedation. After about a minute, a strong delta and alpha band became dominant, which correlated with the hypnotic effect of 3β-OH (Fig. 4c and d, orange frame). Taken together, the data with the low (30 mg kg^{-1}, i.v.) dose of 3β-OH revealed a relatively small difference in both EEG and hypnosis between male and female rats.

We then tested the hypnotic potency and EEG effects of a higher dose of 3β-OH (60 mg kg^{-1}, i.v.). A fast onset of action was noted in both male and female rats, as assessed by time to spontaneous immobility (67.3 [16.1] s *vs* 68.2 [8.3] s, respectively). The duration of spontaneous immobility was also similar in both sexes (87.7 [45.5] min *vs* 81.1 [60.5] min; Supplementary Fig. S2d). Analysis of EEG recordings revealed a significantly higher absolute spectral power in different frequency bands in female rats, but only 60 min after injection (Fig. 5a). This difference disappeared when we normalised the data (Supplementary Fig. S3d). We next analysed and compared the PSD plots during the initial post-injection period. Compared with baseline, PSD was significantly increased in the delta–beta range in both male (Fig. 5b) and female (Fig. 5c) rats just 1 min after injection, which corresponds to the first spontaneous immobility episode. At the second time point, slight PSD suppression was noted in male rats in the delta–theta range (Fig. 5b, green plot), followed by an increase in EEG activity in the higher alpha band ($P<0.05$). In female rats, we detected a more pronounced suppression of EEG activity (Fig. 5c, green plot; $P<0.05$ for theta), which was also noted when we compared the two data sets: female rats showed a significant decrease in PSD in the theta–alpha range compared with male rats (Fig. 5d; $P<0.05$).

To explore the sex difference in EEG activity after 3β-OH further, we examined representative EEG spectrograms (Fig. 6). Low-frequency bands dominated the male baseline EEG spectrum, as shown in the spectrogram (Fig. 6a, top) and accompanying PSD plot (Fig. 6b, grey frame). Almost immediately after injection, the male rat appeared heavily sedated with prominent alpha and beta bands (Fig. 6a and b, magenta frame). In a matter of minutes, the delta band became dominant, which indicated onset of hypnotic effect (Fig. 6a and b, orange frame). A similar pattern was observed in female rats. A typical theta-dominated EEG during baseline (Fig. 6c and d, grey frame) was replaced by a drastic increase in PSD across different frequency bands immediately after injection (Fig. 6c and d, magenta frame). This activity was followed by a decrease in alpha/beta and an increase in delta band (Fig. 6c and d, orange frame), which was directly correlated with a strong hypnotic effect. After several minutes, EEG activity started to decrease in both sexes (black frame in spectrograms), which was more pronounced in female rats, as presented by the PSD plots and original raw traces, which show a burst suppression-like pattern in female rats (Fig. 6e). All female rats, and only 50% of male rats, exhibited this pattern of suppressed EEG activity, and during these periods, the rats failed to respond to toe pinching. Importantly, none of the animals appeared hypothermic (<36°C) or hypoxic (Spo_2 <90%) (data not shown). As a burst suppression-like pattern is usually indicative of a surgical plane of anaesthesia,[14,15] we propose that 3β-OH in high doses could be used to induce general anaesthesia in rats, either as a stand-alone or as an adjuvant agent.[16]

Discussion

Administration of the novel neurosteroid 3β-OH produced potent sedative and hypnotic properties associated with significant EEG changes in young adult rats of both sexes. Both

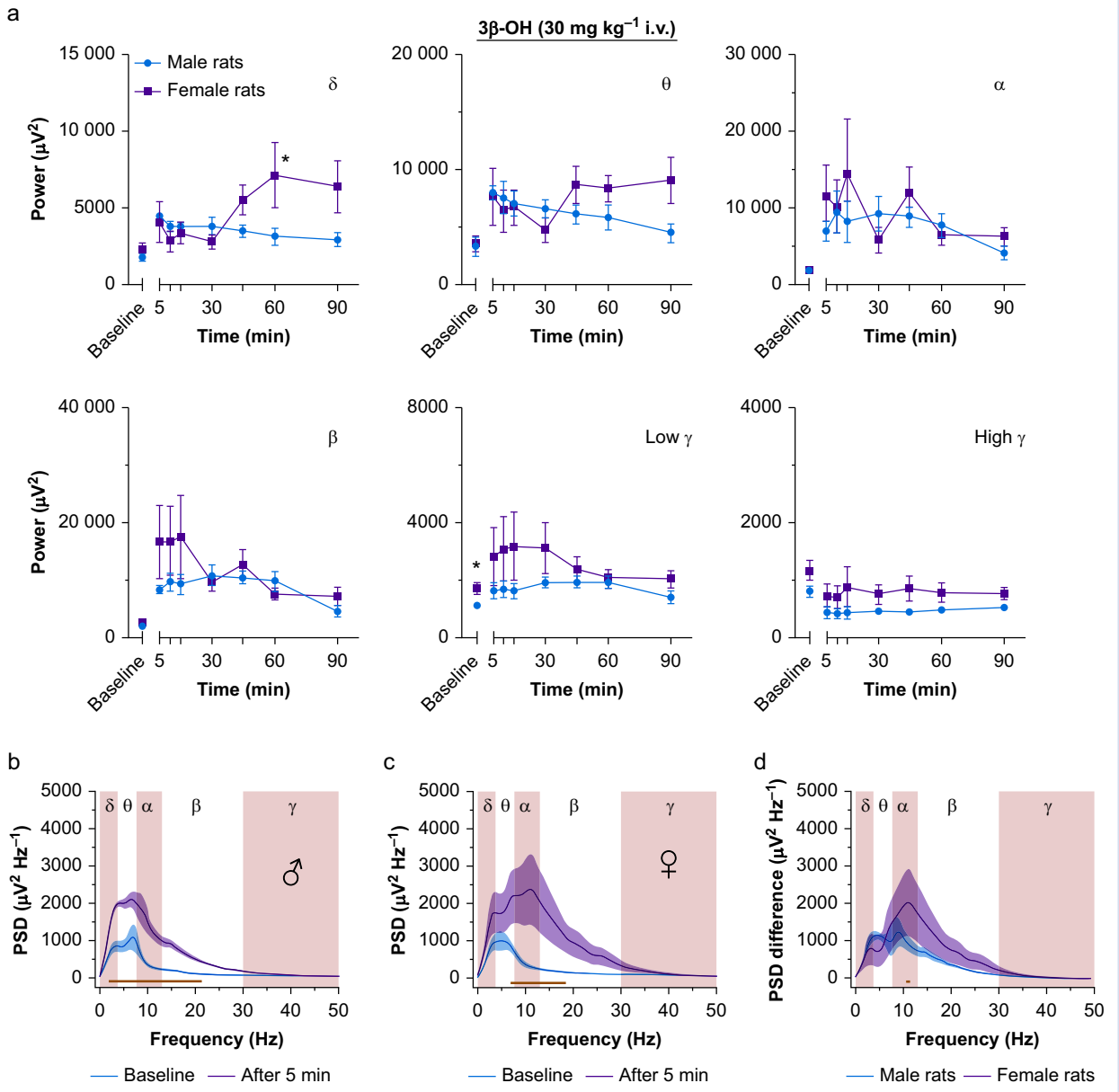

Fig 3. Effects of i.v. administration of (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) (30 mg kg^{-1}) on EEG spectral power in male and female rats. (a) Time course plots showing changes in the absolute spectral power in different frequency bands after i.v. injection of 3β-OH in male (n=6) and female (n=6) rats. The absolute spectral power in the delta band 60 min after i.v. injection of 3β-OH was significantly higher in female compared with male rats (*P=0.025; interaction: F[6, 60]=3.28, P=0.007; two-way repeated measures (RM) analysis of variance (ANOVA) followed by Sidak's *post hoc* test). The baseline value of low gamma power was higher in female than in male rats (*P<0.05; t-test). (b) The power spectral density (PSD) plots during baseline EEG recording (black) and 5 min after i.v. injection of 3β-OH (red) in male rats. The dark blue line represents a statistically significant change in PSD (2–21 Hz) compared with baseline (P<0.05; interaction: F[103, 515]=50.22, P<0.001). (c) The PSD plots during baseline EEG recording (black) and 5 min after i.v. injection of 3β-OH (red) in female rats. The dark blue line represents a statistically significant change in PSD (7–19 Hz) compared with baseline (P<0.05; interaction: F[103, 515]=9.36, P<0.001). (d) Baseline-normalised PSD plots obtained 5 min after i.v. injection of 3β-OH in male (black) and female (red) rats. The dark blue line represents a statistically significant difference in PSD (11–12 Hz) between male and female rats (P<0.05; interaction: F[103, 1040]=0.86, P=0.843; sex: F[1, 1040]=15.56, P<0.001; frequency: F[103, 1040]=8.34, P<0.001). Statistical analyses for data sets presented in (b–d) were performed using two-way RM ANOVA followed by Sidak's *post hoc* test.

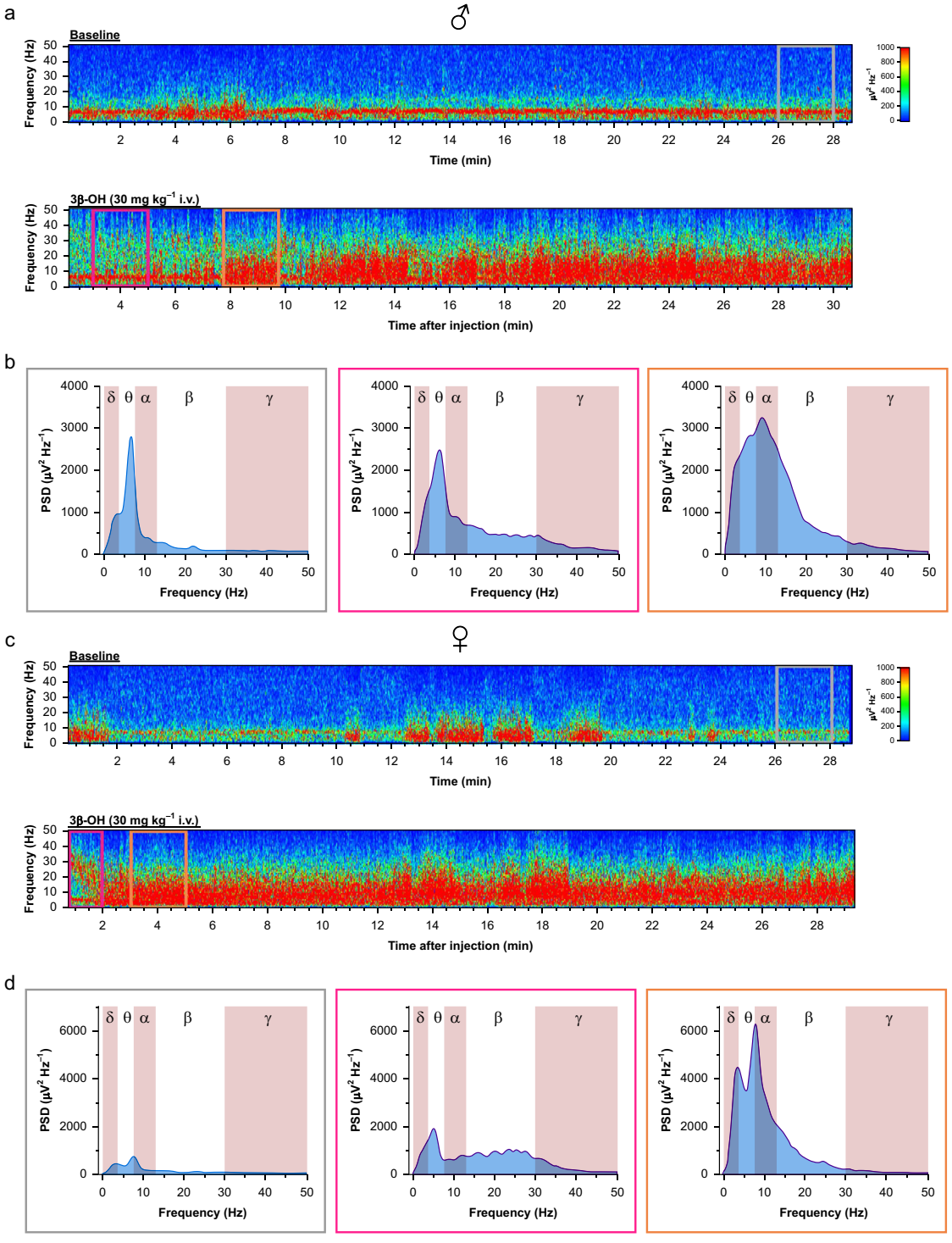

Fig 4. Spectrograms and power spectral density (PSD) plots during sedation/hypnosis induction after i.v. administration of (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) (30 mg kg^{-1}) in a male and female rat. (a) Representative spectrograms computed from the same male rat during baseline (top) and i.v. injection (bottom). Coloured rectangles indicate different behavioural states: awake (grey), sedation (magenta), and hypnosis (orange). Warm colours indicate frequency components with high power density, whereas cool colours indicate frequency components with low power density. (b) The PSD plots during different behavioural states represented in spectrograms above: awake (grey frame), sedation (magenta frame), and hypnosis (orange frame). (c) Representative spectrograms computed from the same female rat during baseline (top) and i.v. injection (bottom). Coloured rectangles indicate different behavioural states: awake (grey), sedation (magenta), and hypnosis (orange). (d) The PSD plots during different behavioural states represented in spectrograms above: awake (grey frame), sedation (magenta frame), and hypnosis (orange frame).

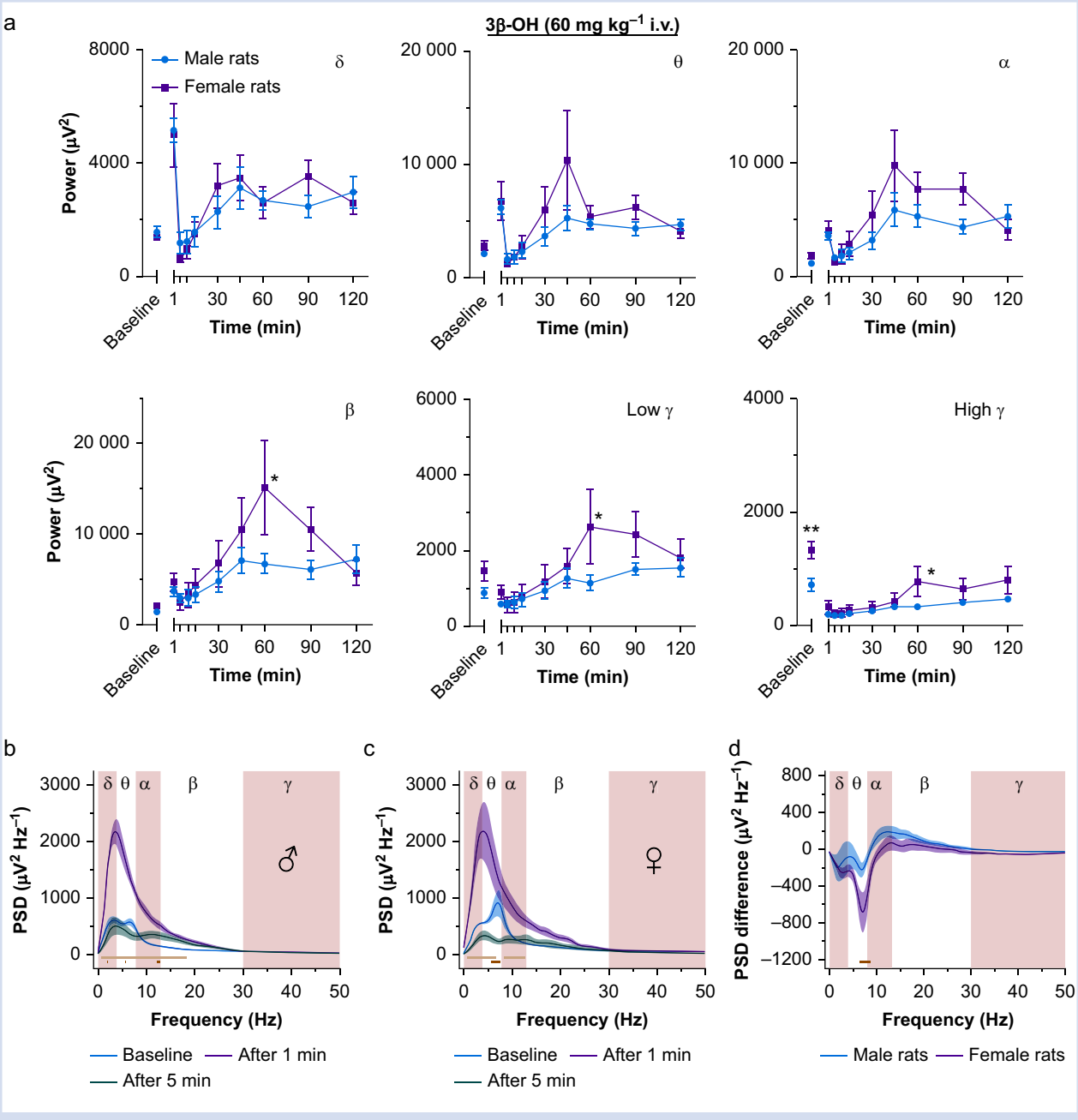

Fig 5. Effects of i.v. administration of (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) (60 mg kg^{-1}) on EEG spectral power in male and female rats. (a) Time course plots showing changes in the absolute spectral power in different frequency bands after i.v. injection of 3β-OH in male (n=6) and female (n=5) rats. A significantly higher absolute spectral power in beta, low, and high gamma frequency bands was detected in female rats 60 min after injection, as compared with male rats. Beta: *P=0.017; interaction: $F(8, 72)=2.77$, $P=0.010$. Low gamma: *P=0.031; interaction: $F(8, 72)=2.62$, $P=0.014$. High gamma: *P=0.040; $F(8, 72)=2.75$, $P=0.011$; two-way repeated measures (RM) analysis of variance (ANOVA) followed by Sidak's *post hoc* test. The baseline value of high gamma power was higher in female than in male rats (**P<0.01; *t*-test). (b) Power spectral density (PSD) plots during baseline EEG recording (black), and 1 (red) and 5 min after i.v. injection of 3β-OH (green) in male rats. The light and dark blue horizontal lines represent a statistically significant change in PSD after 1 min (1–18 Hz) and after 5 min (2, 7, and 12–13 Hz), respectively, compared with baseline ($P<0.05$; interaction: $F[206, 1030]=28.85$, $P<0.001$). (c) The PSD plots during baseline EEG recording (black), 1 (red) and 5 min (green) after i.v. injection of 3β-OH in female rats. The light and dark blue horizontal lines represent a statistically significant change in PSD after 1 min (1–7 and 9–13 Hz) and after 5 min (6–8 Hz), respectively, compared with baseline ($P<0.05$; interaction: $F[206, 824]=9.41$, $P<0.001$). (d) Baseline-normalised PSD plots obtained 5 min after i.v. injection of 3β-OH in male (black) and female (red) rats. The dark blue line represents a statistically significant change in PSD (6–9 Hz) between male and female rats ($P<0.05$; interaction: $F[103, 927]=1.95$, $P<0.001$). Statistical analyses for data sets presented in (b–d) were performed using two-way RM ANOVA followed by Sidak's *post hoc* test.

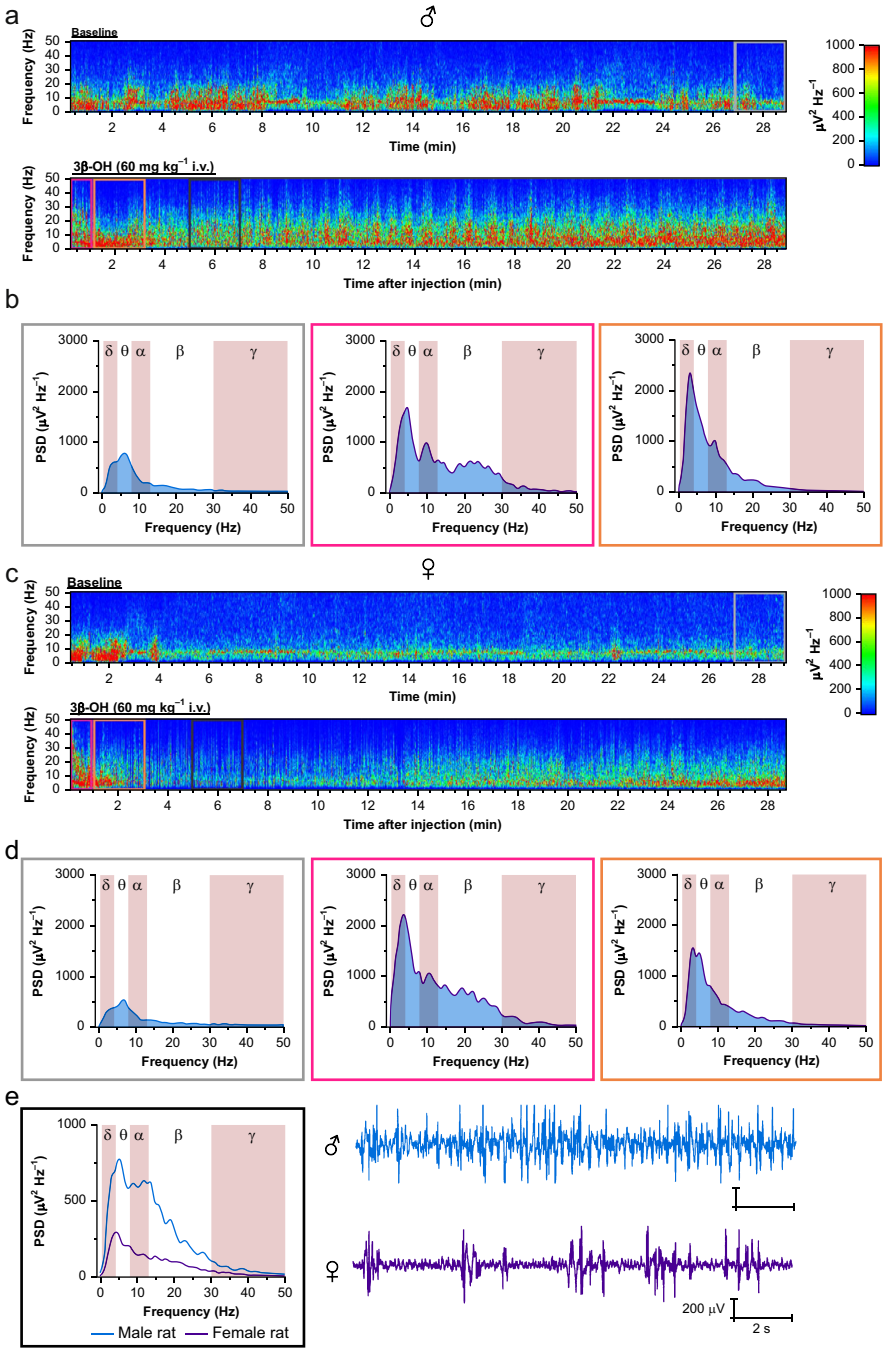

Fig 6. Spectrograms and power spectral density (PSD) plots during sedation/hypnosis/anaesthesia induction after i.v. administration of (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) (60 mg kg^{-1}) in a male and female rat. (a) Representative spectrograms computed from the same male rat during the baseline (top) and i.v. bolus injection (bottom). Coloured rectangles indicate different behavioural states: awake (grey), sedation (magenta), and hypnosis (orange). The black rectangle denotes 5–7 min after injection. (b) The PSD plots during different behavioural states represented in spectrograms above: awake (grey frame), sedation (magenta frame), and hypnosis (orange frame). (c) Representative spectrograms computed from the same female rat during baseline (top) and i.v. bolus injection (bottom). Coloured rectangles indicate different behavioural states: awake (grey), sedation (magenta), and hypnosis (orange). The black rectangle denotes 5–7 min after injection. (d) The PSD plots during different behavioural states represented in spectrograms above: awake (grey frame), sedation (magenta frame), and hypnosis (orange frame). (e) The PSD plots 5–7 min after i.v. injection (black frame) obtained from spectrograms above (a and c). Original EEG traces extracted from the same time period after the injection. Note a typical burst suppression-like pattern in the female, but not in the male, rat.

male and female rats responded similarly to a 30 mg kg^{-1} i.p. dose, whilst female rats remained immobile longer and their EEG activity was suppressed in the low-frequency range as compared with male rats after 60 mg kg^{-1} i.p. Female rats also appeared more sensitive to 30 mg kg^{-1} i.v. than male rats, with a small difference in PSD noted within the alpha band, and after 60 mg kg^{-1} i.v., all female rats tested, but only half of the male rats, reached an EEG burst suppression-like pattern, which represents a profound inhibition of thalamocortical circuitry.

Little is known about sex differences in response to anaesthesia exposure. It has been reported that women emerge faster from propofol anaesthesia than men,[17,18] presumably because of sex differences in its pharmacokinetics.[19] Whilst the pharmacokinetic properties of 3β-OH in the adult rat brain are not currently known, it is possible that this may have contributed to the observed sex-dependent EEG and behavioural differences in our study that suggest that female rats are more sensitive to neurosteroid-induced hypnosis. Interestingly, another neurosteroid analogue, alphaxalone, also caused a two- to three-fold longer time of spontaneous immobility in female rats after i.p. administration, although a surgical plane of anaesthesia was not achieved in either sex.[20] Similar sex differences were observed in an EEG study of alphaxalone,[21] in which the hypnotic effect and the burst suppression EEG pattern after i.p. alphaxalone were more prominent in female than male rats. We found that sex difference in the duration of spontaneous immobility largely disappeared after i.v. administration. However, changes in EEG persisted and were more prominent in female rats up to 1 h after 60 mg kg^{-1} i.v. injection. Bearing in mind its rapid onset of action, this finding suggests that peripheral metabolism may also play a role in the sex-dependent hypnotic effects of 3β-OH after i.v. administration, particularly at later time points that are more relevant for the maintenance or emergence from hypnosis and anaesthesia.

Increased alpha and beta EEG power is associated with sedation, whereas increased delta power (often with alpha) denotes onset of a hypnotic state, at least after administration of typical GABAergic anaesthetics and sedatives.[10,22] Besides these typical changes in EEG, we also detected a large increase in theta band activity during 3β-OH-induced hypnosis, similarly to sub-anaesthetic doses of ketamine in rats[23,24] and ketamine anaesthesia in humans.[25,26] We propose that inhibitory presynaptic actions of 3β-OH on glutamatergic activity[4] may account for this similarity with ketamine. Thus, it is possible that 3β-OH, especially at lower doses, may produce a kind of 'dissociative state' similar to that of ketamine.[27]

Appearance of burst suppression episodes in cortical EEG recordings is generally accepted to be attributable to $GABA_A$-mediated hyperpolarisation of thalamocortical neurones.[28] This indicates a nearly complete disconnect of thalamocortical information transfer, which is usually accompanied with a surgical level of anaesthesia. An inhibitory effect of commonly used anaesthetics, and 3β-OH, on glutamate-mediated synaptic transmission during burst suppression may also contribute to this EEG effect.[29]

Alphaxalone, a neurosteroid analogue with prominent GABAergic properties[30] that also inhibits the $Ca_V3.2$ Ca^{2+} channel isoform of T-currents in sensory neurones,[31] is another hypnotic with a typical pattern of EEG changes.[32,33] Although 3β-OH is devoid of direct GABAergic activity in both thalamic[34] and hippocampal brain slices,[4] it produces an apparently similar EEG signature in rats. We have shown previously that 3β-OH may suppress neuronal excitability by inhibiting T-channel-dependent rebound bursting[5] and by decreasing presynaptic glutamatergic transmission.[4] Furthermore, 3β-OH may hyperpolarise thalamocortical neurones by inhibiting the baseline influx of Ca^{2+} *via* T-type 'window' currents.[34] Although precise molecular mechanisms underlying sex-dependent differences of neurosteroid-induced hypnosis are not known, it is possible that 3β-OH, by decreasing neuronal excitability and hyperpolarising the neuronal membrane, may produce an EEG pattern resembling a typical GABAergic neurosteroid hypnotic, such as alphaxalone. Consistent with this hypothesis, a structurally unrelated T-channel selective antagonist (TTA-P2) also produces hyperpolarisation of thalamocortical neurones by inhibiting T-type window current and promotes generation of delta oscillations both *in vitro*[35] and *in* vivo.[36] These effects of 3β-OH and TTA-P2 are either absent or severely diminished in mice lacking the $Ca_V3.1$ Ca^{2+} channel isoform that mediates T-currents.[34,36]

In conclusion, we found that 3β-OH is a potent hypnotic in rats with a rapid onset of action and an EEG signature comparable with other neurosteroid hypnotics/anaesthetics (e.g. alphaxalone). Furthermore, we found that 3β-OH exhibits sex-dependent hypnotic effects in rats, particularly after i.p. administration, which was confirmed by EEG power spectral analysis. Finally, we propose that neurosteroid analogues with a novel mechanism of action, such as 3β-OH, may provide a new avenue for development of efficacious and safe anaesthetics that can be tailored to individual patient needs.

Authors' contributions

Study design: SMJ, VJ-T, DFC, SMT
Experimentation: SMJ, DS, YHR, KK, VJ-T
Data analysis: SMJ, DS, YHR, KK, VJ-T
Overall project supervision: SMJ, VJ-T, DFC, SMT
Writing of paper: SMJ, VJ-T, DFC, SMT

Declarations of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

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