

# Novel neurosteroid hypnotic blocks T-type calcium channel-dependent rebound burst firing and suppresses long-term potentiation in the rat subiculum

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## Abstract

**Background:** Hypnotics and general anaesthetics impair memory by altering hippocampal synaptic plasticity. We recently reported on a neurosteroid analogue with potent hypnotic activity [(3 $\beta$ ,5 $\beta$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile; 3 $\beta$ -OH], which does not cause developmental neurotoxicity in rat pups. Here, we investigated the effects of 3 $\beta$ -OH on neuronal excitability in the subiculum, the major output structure of the hippocampal formation, and synaptic plasticity at two key hippocampal synapses in juvenile rats.

**Methods:** Biophysical properties of isolated T-type calcium currents (T-currents) in the rat subiculum were investigated using acute slice preparations. Subicular T-type calcium channel (T-channel) subtype mRNA expression was compared using qRT-PCR. Using electrophysiological recordings, we examined the effects of 3 $\beta$ -OH and an endogenous neuroactive steroid, allopregnanolone (Allo), on T-currents and burst firing properties of subicular neurones, and on the long-term potentiation (LTP) in CA3-CA1 and CA1-subiculum pathways.

**Results:** Biophysical and molecular studies confirmed that Ca<sub>v</sub>3.1 channels represent the dominant T-channel isoform in the subiculum of juvenile rats. 3 $\beta$ -OH and Allo inhibited rebound burst firing by decreasing the amplitude of T-currents in a voltage-dependent manner with similar potency, with 30–80% inhibition. Both neurosteroids suppressed LTP at the CA1-subiculum, but not at the CA3-CA1 Schaffer collateral synapse.

**Conclusions:** Neurosteroid effects on T-channels modulate hippocampal output and provide possible molecular mechanisms for the amnestic action of the novel hypnotic 3 $\beta$ -OH. Effects on T-channels in the subiculum provide a novel target for amnestic effects of hypnotics.

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**Keywords:** amnesia; general anaesthetic; hippocampus; neurosteroid; T-type calcium channels; synaptic plasticity; subiculum

### Editor's key points

- The role of T-type calcium channels as targets for the effects of general anaesthetics is unclear.
- The novel anaesthetic neurosteroid (3 $\beta$ ,5 $\beta$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile (3 $\beta$ -OH) reduced burst firing by decreasing the amplitude of T-currents and impaired long-term potentiation in the rat hippocampus.
- Modulation of hippocampal output through effects on hippocampal synaptic transmission provides a possible molecular mechanism for the amnesic action of the novel hypnotic 3 $\beta$ -OH.

Sedatives, hypnotics, and general anaesthetics cause a temporary loss of consciousness and memory loss (amnesia). While loss of consciousness may be because of effects on various subcortical and cortical targets, it is believed that temporary memory impairment originates within the hippocampal formation. These agents produce strong suppression of long-term potentiation (LTP) at the CA3-CA1 synapse,<sup>1–3</sup> a leading cellular model of synaptic plasticity and memory processing.<sup>4</sup> Both inhalation and injectable anaesthetics impair hippocampal dependent learning and memory at concentrations that are often below those needed for their hypnotic effect.<sup>5–7</sup>

The flow of information between hippocampus and numerous subcortical and cortical structures is strongly modulated by the subiculum, the major output structure of the hippocampal formation. Most subicular neurones are capable of burst firing,<sup>8,9</sup> a high-frequency (>100 Hz) barrage of action potentials, which appears to be an important means of distributing information across brain networks.<sup>10</sup> The Ca<sub>v</sub>3.1 isoform of T-type calcium channels (T-channels) contributes significantly to burst firing of subicular neurones, and T-channels are important regulators of neuronal excitability and synaptic plasticity in the subiculum.<sup>11</sup>

At a molecular level, most general anaesthetics act on N-methyl-D-aspartate (NMDA)-type glutamate, gamma-aminobutyric acid type A (GABA<sub>A</sub>), or both receptors,<sup>12</sup> but also have underappreciated effects on other targets, such as voltage-gated calcium channels.<sup>13</sup> For example, isoflurane blocks T-channels in thalamocortical neurones at clinically relevant concentrations.<sup>14</sup> We recently described a neurosteroid analogue with potent hypnotic activity in rat pups ([3 $\beta$ ,5 $\beta$ ,17 $\beta$ ]-3-hydroxyandrostane-17-carbonitrile; 3 $\beta$ -OH) that inhibits neuronal T-currents and inhibits glutamate-mediated synaptic transmission presynaptically, but unlike most other neurosteroids, it lacks effects on postsynaptic GABA<sub>A</sub> currents.<sup>15</sup> In the present study, we investigated the effects of 3 $\beta$ -OH and an endogenous neuroactive steroid, allopregnanolone (Allo), on burst firing properties of subicular neurones and synaptic plasticity at two important hippocampal synapses in

juvenile rats. We found that both neurosteroids strongly affected burst firing by decreasing T-currents and suppressed LTP at the CA1-subiculum, but not at CA3-CA1 synapses. Thus, T-channels in the subiculum are a potentially important target for the amnesic effects of anaesthetics.

## Methods

### Drugs

The neurosteroid analogue 3 $\beta$ -OH was synthesised as described,<sup>15</sup> whereas Allo was obtained from Sigma Aldrich Chemical Co. (#P-8887, Saint Louis, MO, USA). Purification of 3 $\beta$ -OH was by flash column chromatography on silica gel; preparations showed a single component on thin layer chromatography on silica gel plates. Both compounds were dissolved in 100% dimethyl sulfoxide (Sigma Aldrich Chemical Co.) as 3 or 10 mM stock solutions, and freshly diluted to the final concentrations in the external solution at the time of electrophysiology experiments. For the loss of righting reflex (LORR) study, 3 $\beta$ -OH was solubilised in 2-hydroxypropyl- $\beta$ -cyclodextrin 15% (Santa-Cruz Biotechnology, Santa Cruz, CA, USA).

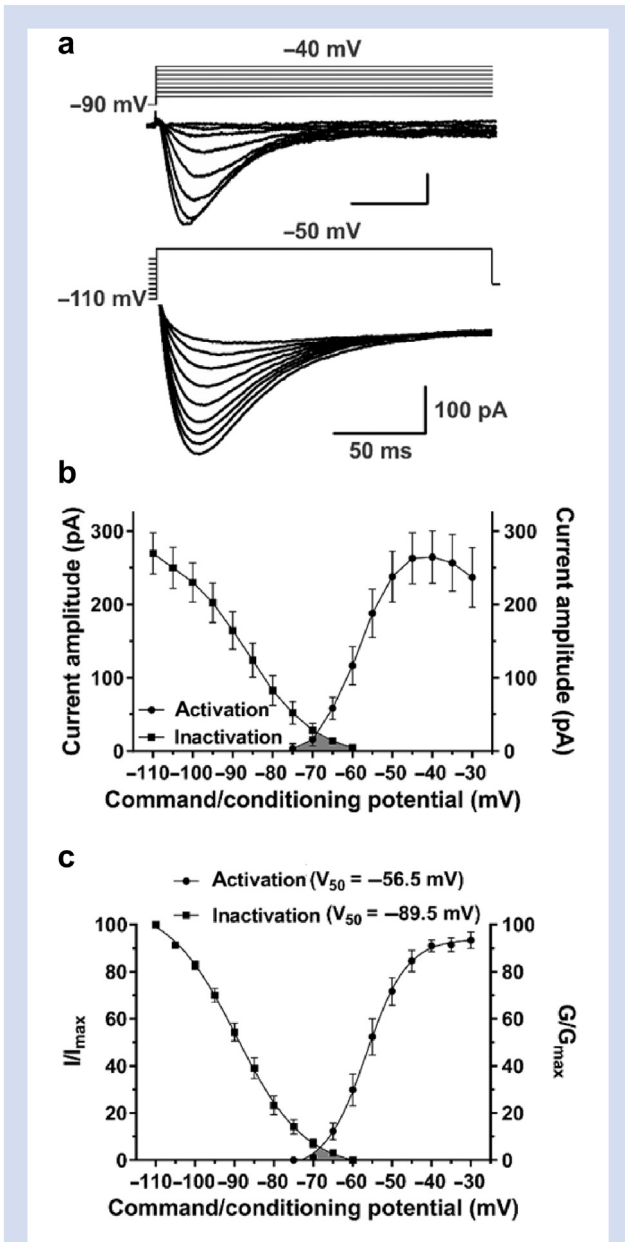
### Animals

Juvenile male and female Sprague-Dawley rats (P15–P32) were used. For patch-clamp experiments, we used P15–P28 rats, and for LTP experiments we used P28–P32 rats, in order to make a direct comparison with our previous studies of a T-channel antagonist that selectively inhibits all three subtypes without affecting other classes of voltage-gated Ca<sup>2+</sup> channels (TTA-P2; 3,5-dichloro-N-[1-{2,2-dimethyl-tetrahydro-pyran-4-ylmethyl}-4-fluoro-piperidin-4-ylmethyl]-benzamide).<sup>11</sup> Analysis of T-type calcium channel isoform mRNA expression was performed on subicular tissue of P21–P22 rats. We used P20–P29 rats to assess the LORR after i.p. administration of 3 $\beta$ -OH. Animals were housed within accredited animal facilities according to protocols approved by the University of Colorado Anschutz Medical Campus, the University of Virginia, or the Washington University Animal Studies Committee. Treatments of rats adhered to guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimise animal suffering and the number of animals necessary to produce reliable scientific data. Details of specific experimental procedures are provided in the online Supplementary material. Data are shown as mean (standard error of mean).

## Results

### Biophysical properties of T-currents in the subiculum

We previously described isolated T-currents in the subiculum of rat pups.<sup>15</sup> Here, we investigated the biophysical properties of T-currents in juvenile rats when dendritic arborisation is well underway.<sup>17</sup> The voltage dependence of T-current activation

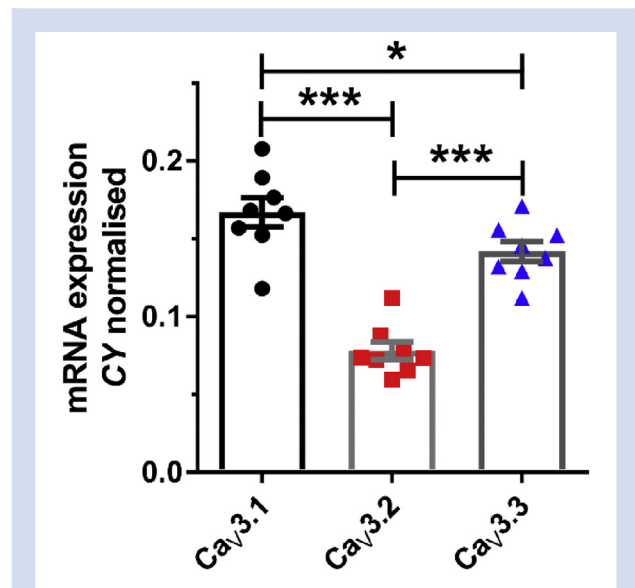


**Fig 1.** Biophysical properties of T-type calcium currents (T-currents) in the juvenile rat subiculum. (a) Average T-current I-V traces from representative subicular neurones for  $V_t$  from  $-75$  to  $-40$  mV (the peak of activation) from  $V_h$  of  $-90$  mV in  $5$  mV increments (top), and traces generated using a double-pulse protocol with  $3.6$ -s prepulses to variable voltages (from  $-110$  to  $-70$  mV in  $5$  mV increments) and test potential of  $-50$  mV (bottom); not all data points are displayed. (b) Average current amplitudes of the voltage dependence for activation and steady-state inactivation. (c) The voltage dependence of activation ( $I/I_{max}$ ) and steady-state inactivation ( $G/G_{max}$ ) is shown with  $V_{50}$  values noted in the parentheses. Note the ‘window’ current depicted by the grey area below the intersection of the curves. Results are expressed as mean (standard error of mean).

was measured using typical I-V relationships ( $V_h = -90$  mV,  $V_t = -75$  to  $-30$  mV in  $5$  mV increments), whereas an independent steady-state protocol was used to measure the voltage dependence of T-current inactivation (Fig. 1a). As shown in Fig. 1b, T-current amplitudes were:  $270$  ( $28$ ) pA after the long prepulse at  $-110$  mV ( $n=19$  neurones,  $10$  rats), and  $264$  ( $36$ ) pA ( $n=15$  neurones, nine rats) at the peak of activation ( $V_t = -40$  mV).  $I/I_{max}$  and  $G/G_{max}$  curves are shown on the same graph (Fig. 1c), along with corresponding  $V_{50}$  values ( $n=20$  and  $14$  neurones, respectively). The average slope of activation curves in P15–P23 rats [ $4.5$  ( $0.5$ )] was almost identical to that reported in rat pups<sup>11</sup>; however, the average  $V_{50}$  value was significantly hyperpolarised by  $\sim 4$  mV ( $P=0.043$ , two-tailed t-test). Overlap of the activation and inactivation curves revealed a ‘window’ current ( $I_{Twindow}$ ), shown in grey shaded areas in Fig. 1b and c. These currents result from a small fraction of T-channels that are persistently available near the resting membrane potential, which in turn allows a basal inward flux of calcium ions.<sup>18</sup>

**mRNA expression of CACNA1G, CACNA1H, and CACNA1I in the rat subiculum**

The three known isoforms of T-type calcium channels ( $Ca_v3.1$ ,  $Ca_v3.2$ , and  $Ca_v3.3$ ) are encoded by CACNA1G, CACNA1H, and CACNA1I genes, respectively.<sup>19</sup> Based on our previous findings of mRNA expression levels in rat pups and insensitivity of burst firing to low concentrations of  $Ni^{2+}$ ,<sup>11</sup> and available *in situ* hybridisation data,<sup>20</sup> it appears that the  $Ca_v3.1$  isoform constitutes the largest portion of T-channels in the subiculum. To investigate whether this is also the case in juvenile rats, we used qRT-PCR to assess mRNA expression of all three genes



**Fig 2.** mRNA expression of all three T-type calcium channel isoforms in the juvenile rat subiculum. qRT-PCR analysis on excised subicular tissue revealed the dominant expression of  $Ca_v3.1$ , followed by  $Ca_v3.3$  isoform. Results are expressed as mean (standard error of mean). \* $P<0.05$  and  $^{***}P<0.001$ , one-way analysis of variance followed by Holm-Sidak’s *post hoc* test.

encoding different T-channel isoforms in the subiculum of P21–P22 rats. Indeed,  $Ca_v3.1$  was the dominant T-channel isoform at this age as well (Fig. 2;  $P < 0.001$  vs  $Ca_v3.2$  and  $P = 0.025$  vs  $Ca_v3.3$ ; one-way analysis of variance (ANOVA) followed by Holm-Sidak's test;  $n = 8$  rats). In contrast to our findings in rat pups, relatively high  $Ca_v3.3$  isoform mRNA expression was observed in juvenile rats ( $P < 0.001$  vs  $Ca_v3.2$ ), which is likely related to the presence of more extensive dendritic branching in neurones from juvenile animals.

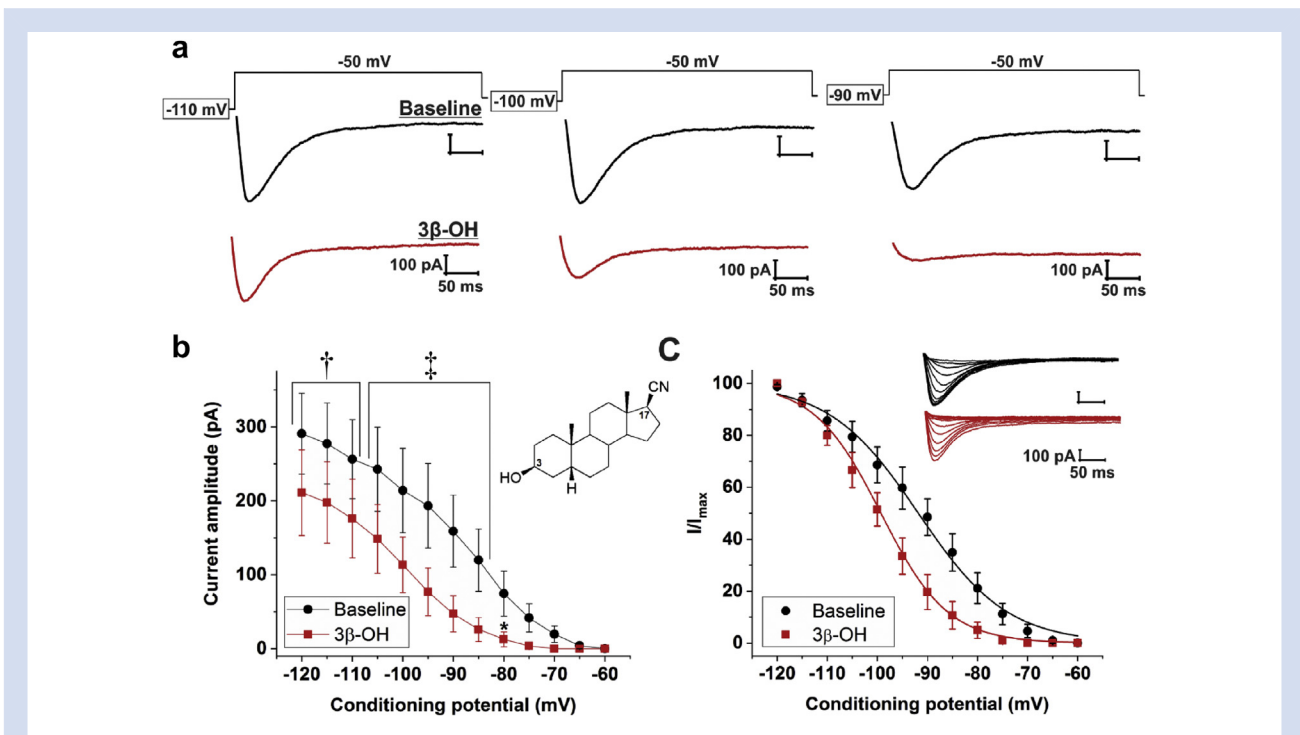
### Hypnotic effect of 3 $\beta$ -OH in juvenile rats

The neurosteroid analogue 3 $\beta$ -OH inhibits T-currents and produces potent hypnotic effects in rat pups.<sup>15</sup> This finding was corroborated in the present study using juvenile rats. The  $ED_{50}$  for the LORR obtained when the compound was injected i.p. was 16.0 (0.4) mg kg<sup>-1</sup>, with a slope of 3.4 (0.4). At a dose of 50 mg kg<sup>-1</sup> i.p., 3 $\beta$ -OH had an average onset of action (time to LORR) of 14.9 (1.8) min, whereas the average duration of LORR was 108 (19) min (data not shown). In juvenile rats, this  $ED_{50}$  was five-fold higher than in rat pups,<sup>15</sup> which may reflect differences in pharmacokinetics, in the maturation of thalamocortical circuitry, or both.<sup>21</sup> All animals remained euthermic during LORR and resumed normal locomotor activity after

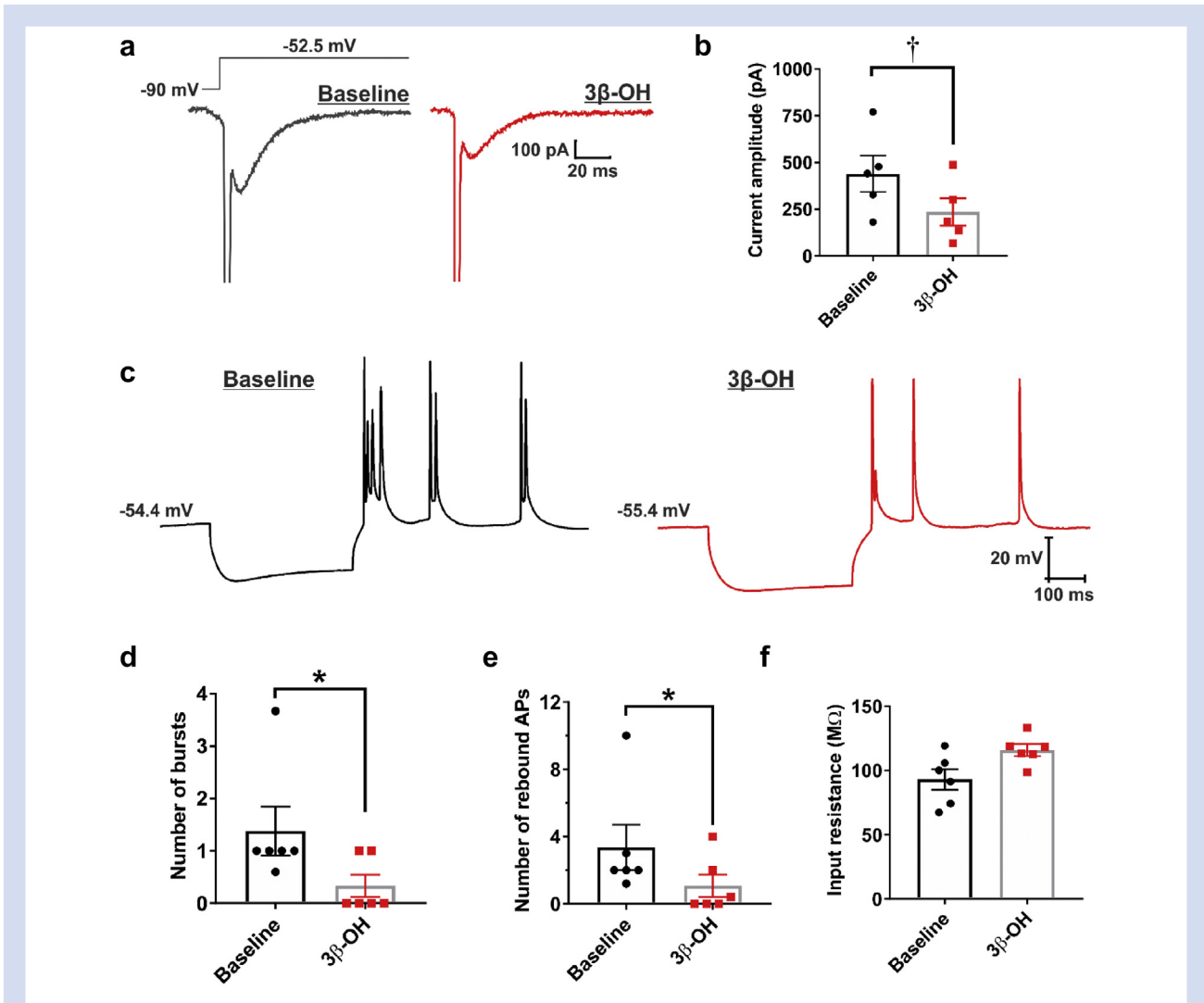
effects of 3 $\beta$ -OH dissipated (data not shown). These results clearly demonstrate that 3 $\beta$ -OH is an efficacious and apparently safe hypnotic in juvenile rats.

### Effects of 3 $\beta$ -OH on T-currents in subicular neurones

The effects of 3 $\beta$ -OH on T-currents in juvenile rats were tested using a standard steady-state inactivation protocol. Original traces from a representative subicular neurone are presented in Fig. 3a. 3 $\beta$ -OH significantly decreased T-current amplitude across the range of conditioning potentials, as assessed by two-way repeated measures ANOVA followed by Holm-Sidak's multiple comparisons test (Fig. 3b; interaction:  $F [12,60] = 3.83$ ,  $P < 0.001$ ; *post hoc*:  $P = 0.002$  for potentials from  $-120$  to  $-110$  mV,  $P < 0.001$  for potentials from  $-105$  to  $-85$  mV, and  $P = 0.017$  for  $-80$  mV;  $n = 6$  neurones, three rats). This effect was strongly voltage-dependent, ranging from ~30% inhibition at  $-120$  mV to ~80% inhibition at the  $-80$  mV prepulse. Addition of 3 $\beta$ -OH 1  $\mu$ M produced a significant hyperpolarising shift in  $V_{50}$  of  $-9$  mV (Fig. 3c;  $-99.5 [2.0]$  mV vs  $-90.5 [3.0]$  mV;  $P = 0.013$ ; paired t-test). Thus, 3 $\beta$ -OH is a potent and effective T-channel antagonist, confirming our previous results in dorsal root ganglion neurones,<sup>22</sup> nucleus reticularis thalami,<sup>23</sup> and the subiculum from rat pups.<sup>15</sup>



**Fig 3.** (3 $\beta,5\beta,17\beta$ )-3-hydroxyandrostane-17-carbonitrile blocks T-type calcium currents (T-currents) in subicular neurones. (a) Original T-current traces from a representative subicular neurone at baseline (black trace) and after application of (3 $\beta,5\beta,17\beta$ )-3-hydroxyandrostane-17-carbonitrile (3 $\beta$ -OH) 1  $\mu$ M (red trace), generated using a double-pulse protocol with three different 3.6-s prepulses:  $-110$  mV (left),  $-100$  mV (middle), and  $-90$  mV (right). (b) Average current amplitudes exhibit a significant decrease after the application of 3 $\beta$ -OH 1  $\mu$ M across different conditioning potentials. The inset shows the chemical structure of 3 $\beta$ -OH, a neuroactive steroid analogue based on a progesterone scaffold with OH and CN groups in a beta-conformation at positions 3 and 17, respectively. (c) Steady-state inactivation curves ( $I/I_{max}$ ) display a leftward shift upon the application of 3 $\beta$ -OH. Inset shows original traces from a representative subicular neurone at baseline (black trace) and after 3 $\beta$ -OH (red trace), generated using our standard steady-state inactivation protocol. Results are expressed as mean (standard error of mean). \* $P < 0.05$ , † $P < 0.01$ , and ‡ $P < 0.001$ , two-way repeated measures analysis of variance followed by Holm-Sidak's *post hoc* test.



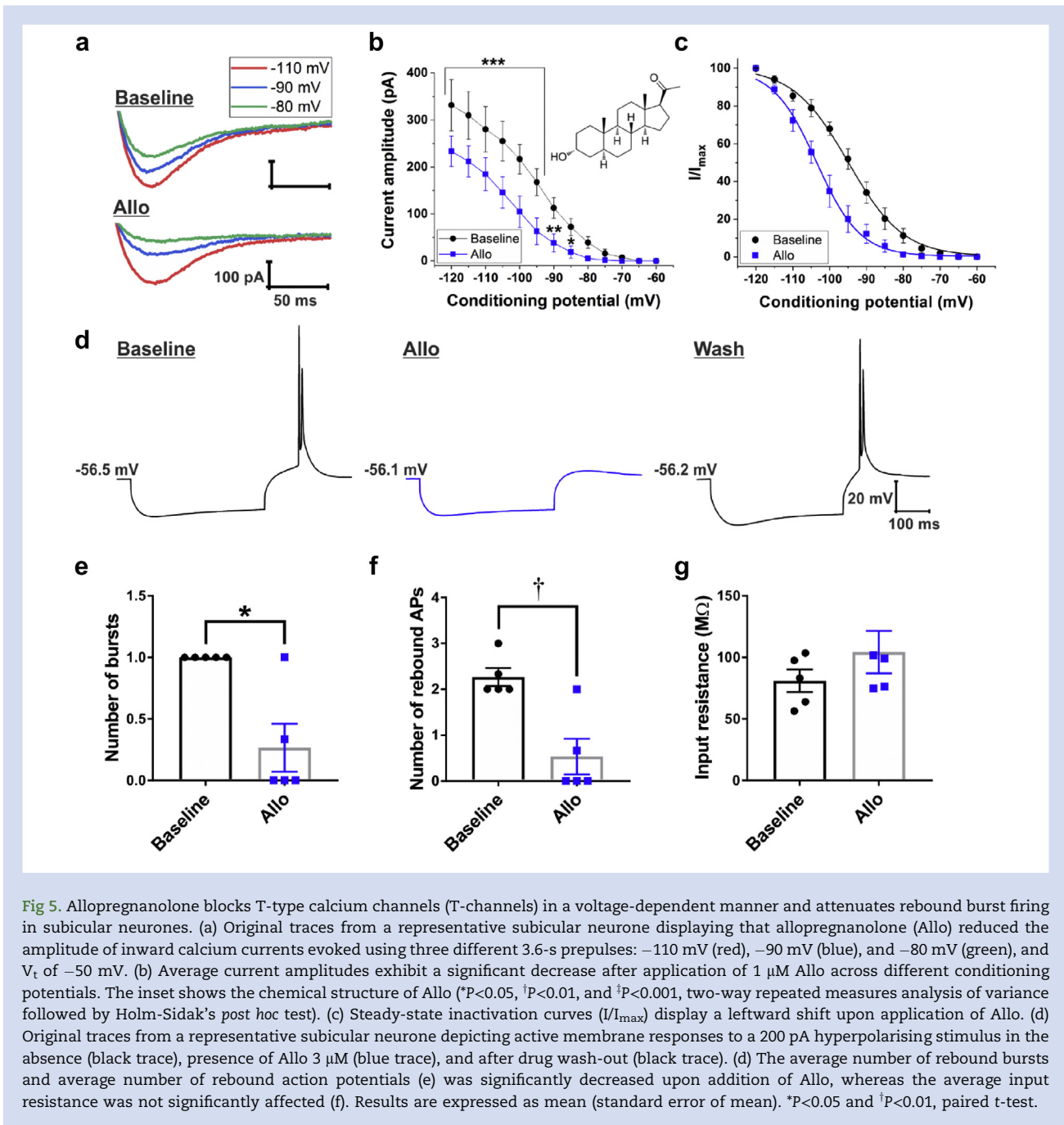
**Fig 4.** (3 $\beta$ ,5 $\beta$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile attenuates rebound burst firing in subicular neurones. (a) Original traces from a representative subicular neurone showing that (3 $\beta$ ,5 $\beta$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile (3 $\beta$ -OH) reduced the amplitude of inward calcium currents (evoked using  $V_h$  of  $-90$  mV and  $V_t$  of  $-52.5$  mV). (b) Bar graph shows a significant decrease in the average calcium current amplitude after application of  $3 \mu\text{M}$  3 $\beta$ -OH. (c) Original traces from a representative subicular neurone depicting active membrane responses to a  $200$  pA hyperpolarising stimulus in the absence (black trace) or presence of 3 $\beta$ -OH  $3 \mu\text{M}$  (red trace). (d) The average number of rebound bursts and the average number of rebound action potentials (e) was significantly decreased upon the addition of 3 $\beta$ -OH, whereas the average input resistance was not significantly affected (f). Results are expressed as mean (standard error of mean). \* $P < 0.05$  and † $P < 0.01$ , paired t-test.

### Effects of 3 $\beta$ -OH on rebound burst firing in subicular neurones

Hyperpolarising stimuli, such as inhibitory postsynaptic potentials, can deinactivate T-channels and cause 'rebound' burst firing of thalamocortical neurones in an oscillatory manner.<sup>24</sup> This phenomenon is also observed in a large population of pyramidal neurones of the subiculum.<sup>8,11</sup> We tested whether 3 $\beta$ -OH  $3 \mu\text{M}$  affects rebound burst firing by blocking T-channels in the subiculum of juvenile rats. Using voltage-clamp recordings, we investigated the effects of 3 $\beta$ -OH on calcium currents (mostly consisting of T-currents) evoked from  $-90$  mV (Fig. 4a). 3 $\beta$ -OH reduced the maximal

calcium current by  $\sim 50\%$  (Fig. 4b;  $P = 0.003$ , paired t-test,  $n = 5$  neurones). In current-clamp mode, we investigated burst firing properties in response to a brief hyperpolarising stimulus (Fig. 4c). 3 $\beta$ -OH significantly decreased the number of bursts (Fig. 4d;  $P = 0.034$ , paired t-test,  $n = 6$  neurones), and the number of rebound action potentials (Fig. 4e;  $P = 0.031$ ). The membrane potential was held at approximately the same value (baseline:  $-54.7$  [1.7] mV vs 3 $\beta$ -OH:  $-54.6$  [2.0] mV), whereas the average input resistance was not significantly affected upon the addition of 3 $\beta$ -OH (Fig. 4f;  $P = 0.054$ ). Our data suggest that the effects of 3 $\beta$ -OH on burst firing are preserved across different age groups in rats.





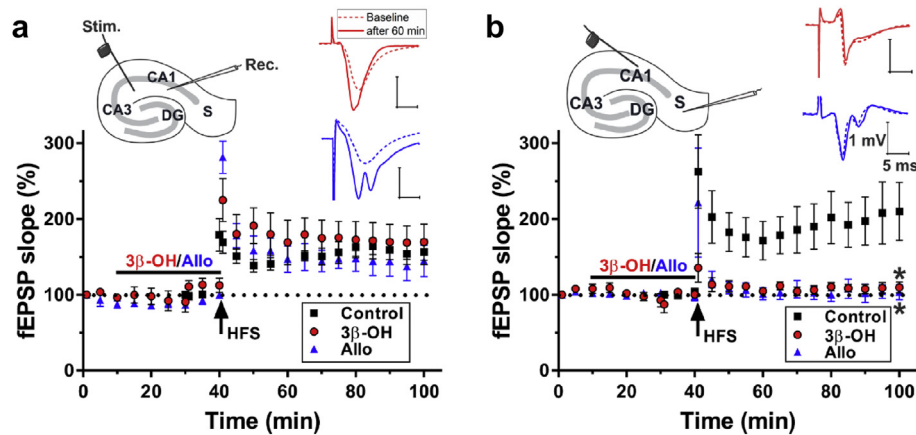
**Fig 5.** Allopregnanolone blocks T-type calcium channels (T-channels) in a voltage-dependent manner and attenuates rebound burst firing in subicular neurones. (a) Original traces from a representative subicular neurone displaying that allopregnanolone (Allo) reduced the amplitude of inward calcium currents evoked using three different 3.6-s prepulses:  $-110$  mV (red),  $-90$  mV (blue), and  $-80$  mV (green), and  $V_t$  of  $-50$  mV. (b) Average current amplitudes exhibit a significant decrease after application of  $1 \mu\text{M}$  Allo across different conditioning potentials. The inset shows the chemical structure of Allo ( $*P < 0.05$ ,  $^{\dagger}P < 0.01$ , and  $^{\ddagger}P < 0.001$ , two-way repeated measures analysis of variance followed by Holm-Sidak's post hoc test). (c) Steady-state inactivation curves ( $I/I_{\text{max}}$ ) display a leftward shift upon application of Allo. (d) Original traces from a representative subicular neurone depicting active membrane responses to a  $200$  pA hyperpolarising stimulus in the absence (black trace), presence of Allo  $3 \mu\text{M}$  (blue trace), and after drug wash-out (black trace). (d) The average number of rebound bursts and average number of rebound action potentials (e) was significantly decreased upon addition of Allo, whereas the average input resistance was not significantly affected (f). Results are expressed as mean (standard error of mean).  $*P < 0.05$  and  $^{\dagger}P < 0.01$ , paired t-test.

### Effects of Allo on T-current properties and rebound burst firing in subicular neurones

Allo partially blocks T-currents in rat sensory neurones with an  $\text{IC}_{50}$  of  $0.9 \mu\text{M}$ .<sup>25</sup> We assessed whether Allo exhibits similar effects on T-currents in subicular neurones, and compared these effects with those of  $3\beta\text{-OH}$  using the same voltage- and current-clamp protocols (Fig. 5a). Allo ( $1 \mu\text{M}$ ) showed a strikingly similar T-current blocking effect as the same concentration of  $3\beta\text{-OH}$  (Fig. 5a–c). Allo significantly decreased T-current amplitudes starting from the prepulse of  $-120$  mV (30% inhibition) to a prepulse of  $-85$  mV (75% inhibition; Fig. 5b; interaction:  $F[12,72]=5.34$ ,  $P < 0.001$ ; post hoc:  $P < 0.001$  for potentials from  $-120$  to  $-95$  mV,  $P=0.002$  for  $-90$  mV, and

$P=0.046$  for  $-85$  mV;  $n=7$  neurones, three rats), which was accompanied by a hyperpolarising shift in  $V_{50}$  of  $10$  mV (Fig. 5c).

The effects of Allo on T-current-dependent rebound burst firing was investigated in subicular neurones (Fig. 5d). The effects of Allo mirrored those of  $3\beta\text{-OH}$   $3 \mu\text{M}$ : the number of bursts (Fig. 5e;  $P=0.020$ , paired t-test,  $n=5$  neurones, three rats), and the number of rebound action potentials (Fig. 5f;  $P=0.002$ ), were significantly decreased, whereas the average input resistance was slightly higher upon the addition of Allo (Fig. 5g;  $P=0.103$ ). We conclude that both the endogenous (Allo) and synthetic ( $3\beta\text{-OH}$ ) neuroactive steroid analogues block isolated T-type calcium currents and rebound bursting in the subiculum, with similar potency.



**Fig 6.** Long-term potentiation is impaired by (3 $\beta$ ,5 $\beta$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile or allopregnanolone at the CA1-subiculum, but not at the CA3-CA1 synapse. (a) Schematic diagram of hippocampal formation with stimulation probe for CA1 recordings placed along the Schaffer collateral pathway. Extracellular recordings were obtained in the apical dendritic layer of the CA1 region. Inset shows original excitatory postsynaptic potential (EPSP) traces in baseline (dashed line) conditions and 60 min after high frequency stimulation (HFS) in the presence of (3 $\beta$ ,5 $\beta$ ,17 $\beta$ )-3-hydroxyandrostane-17-carbonitrile (3 $\beta$ -OH) 1  $\mu$ M or Allo 1  $\mu$ M (solid line). Constant current pulses (0.1 ms, 100 Hz  $\times$  1 s) HFS successfully induced long-term potentiation (LTP) in CA1 in the presence of either 3 $\beta$ -OH or Allo measured 60 min after HFS. (b) Schematic diagram of hippocampal formation with stimulation probe for LTP induction in the subiculum located towards the end of CA1 in the stratum oriens/alveus region, and with the recording pipette in the molecular layer of the subiculum. Original EPSP traces shown in inset depict the absence of LTP at the CA1-subiculum synapse after the addition of 3 $\beta$ -OH 1  $\mu$ M or Allo 1  $\mu$ M (solid line), as compared with baseline conditions (dashed line). The normalised EPSP slopes show that HFS (200 Hz  $\times$  1 s  $\times$  2) did not induce subicular LTP in the presence of either 3 $\beta$ -OH or Allo. Results are expressed as mean (standard error of mean). \* $P$  < 0.05 vs control, one-way analysis of variance followed by Holm-Sidak's *post hoc* test.

### Effects of 3 $\beta$ -OH and Allo on synaptic plasticity at the CA3-CA1 and CA1-subiculum synapses

Most hypnotics and general anaesthetics suppress LTP at the CA3-CA1 synapse.<sup>26–28</sup> TTA-P2, a structurally unrelated T-channel antagonist, alters LTP at the CA1-subiculum, but not at the CA3-CA1 synapse.<sup>11</sup> As our results revealed T-channel-blocking properties of 3 $\beta$ -OH and Allo on subicular T-currents, we speculated that these neuroactive steroids also have significant effects on synaptic plasticity in hippocampal slices. 3 $\beta$ -OH or Allo at 1  $\mu$ M did not significantly alter basal transmission (control: 100 [2]% of baseline; 3 $\beta$ -OH: 112 [10]%; Allo: 99 [2]%,  $n=5$  in each group;  $F(2,12)=1.54$ ,  $P=0.255$ , one-way ANOVA) or LTP at the CA3-CA1 synapse (Fig. 6a; control: 156 [16]% of baseline 60 min after high frequency stimulation [HFS]; 3 $\beta$ -OH: 169 [24]%, red trace; Allo: 144 [21]%, blue trace). This finding was confirmed using 3 $\beta$ -OH 10  $\mu$ M (138 [9]%,  $n=5$ ; data not shown). These data are consistent with our previous study that showed the lack of effect of Allo on LTP induction at this synapse.<sup>29</sup> Conversely, 3 $\beta$ -OH and Allo, both at 1  $\mu$ M, significantly suppressed LTP at the CA1-subiculum synapse (Fig. 6b; control: 210 [38]% of baseline 60 min after HFS; 3 $\beta$ -OH: 110 [7]%; Allo: 103 [10]%;  $n=5, 7$ , and  $5$ , respectively), as assessed by one-way ANOVA followed by Holm-Sidak's *post hoc* test ( $F(2,14)=7.86$ ,  $P=0.005$ ; 3 $\beta$ -OH:  $P=0.010$  and Allo:  $P=0.010$ , both vs control). Taken together, these results confirm our findings that modulating T-channels can have a crucial influence on synaptic plasticity in the CA1-subiculum, but not in CA3-CA1 pathway.

### Discussion

We have shown that burst firing of subicular neurones relies on T-channel activity,<sup>11</sup> and that 3 $\beta$ -OH, a novel neuroactive steroid with hypnotic effects, is a potent blocker of subicular and thalamic T-currents in rat pups.<sup>15</sup> Here, we confirm the hypnotic and T-channel-blocking properties of 3 $\beta$ -OH in juvenile rats and, furthermore, report on its differential effects at two hippocampal synapses important for memory processing: CA3-CA1<sup>4</sup> and CA1-subiculum.<sup>30</sup>

First, we detected isolated T-currents in subicular neurones with minimal differences in biophysical properties compared with rat pups,<sup>11</sup> which clearly showed a dominant expression of the Ca $_v$ 3.1 T-channel isoform. However, here we report an increase in mRNA expression of Ca $_v$ 3.3 isoform compared with rat pups. This increase of Ca $_v$ 3.3 expression coincides with distal dendritic localisation of this T-channel isoform in the subiculum,<sup>31</sup> which may explain why we did not detect substantial T-currents with Ca $_v$ 3.3-like properties (e.g. slow activation and inactivation kinetics) using somatic recordings in rat pups. The development of the dendritic arbor and change in the expression of Ca $_v$ 3.3 may also account for the slight but significant hyperpolarising shift in  $V_{50}$  for T-current activation, which yielded a 'window current'.

The neurosteroid analogue 3 $\beta$ -OH and the endogenous neurosteroid Allo blocked T-currents in subicular neurones with similar potency as described.<sup>15,25</sup> Inhibition of T-currents was accompanied by the attenuation of rebound burst firing without significant changes in passive membrane properties

of subicular neurones. These effects largely mirror our findings with the structurally unrelated selective T-channel antagonist TTA-P2,<sup>11</sup> thus indirectly confirming the crucial role of T-channels in mediating burst firing in the subiculum. 3 $\beta$ -OH also blocks Cav3.2 T-currents in dissociated neurones of the dorsal root ganglion<sup>25</sup> and in the nucleus reticularis thalami,<sup>23</sup> an inhibitory thalamic nucleus rich in Cav3.2 and Cav3.3 T-channel isoforms.<sup>20</sup> Taken together, our results show that 3 $\beta$ -OH modulates neuronal excitability acting through T-channels, regardless of which T-channel isoform is dominant in neuronal tissue.

LTP represents a persistent activity-dependent change in synaptic efficacy usually studied by stimulating the Schaffer collateral pathway (CA3-CA1 synapses). Amnestic actions of hypnotics and anaesthetics are usually studied using this particular synapse as a cellular model of learning and memory, with limited data on their effects on other hippocampal synapses. Most studies have focused on either GABA<sub>A</sub> or NMDA receptors as the major molecular targets for hypnotic/anaesthetic-induced impairment of synaptic plasticity,<sup>3,32,33</sup> with very little, if any, data on the contribution of voltage-gated calcium channels to these effects. The results presented here show that 3 $\beta$ -OH and Allo are devoid of effects on LTP induction at the CA3-CA1 synapse, thus corroborating our previous findings that T-channels do not play an important role in regulating synaptic plasticity in this hippocampal subregion.<sup>11</sup> This is also consistent with a lack of effect of 3 $\beta$ -OH on NMDA receptors,<sup>15</sup> as LTP in the CA3-CA1 pathway relies heavily on activation of these glutamate receptors.<sup>34</sup> In contrast, both 3 $\beta$ -OH and Allo strongly suppressed the induction and maintenance of LTP at the CA1-subiculum synapse, very similar to TTA-P2.<sup>11</sup> This effect should likely be accompanied with specific alterations in memory processing, and this hypothesis needs to be tested in future behavioural experiments aimed at assessing learning and memory processes that require intact function of the hippocampal formation. Towards this end, we recently reported that Cav3.1<sup>-/-</sup> mice exhibit specific deficits in spatial navigation and recognition memory.<sup>35</sup>

3 $\beta$ -OH is a potent hypnotic in both rats and mice<sup>15</sup> (unpublished data). This hypnotic effect is apparently preserved across different age groups. The concentration range used in this study (1–3  $\mu$ M) probably reflects sub-hypnotic doses in juvenile rats, but is nonetheless high enough to modulate both neuronal excitability and synaptic plasticity in the subiculum. We propose that the predominant action of 3 $\beta$ -OH on subicular T-channels and LTP in the CA1-subiculum synapse may result in amnestic effects different from those seen with other commonly used injectable anaesthetics, such as propofol or ketamine, that have very little effect on neuronal T-type currents at clinically relevant concentrations.<sup>16,36,37</sup>

### Authors' contributions

Performed experiments and analysed the data: SMJ, YI, SlJJ, VT, KK, BA.

Designed the studies, supervised the overall project, and performed final manuscript preparation: SMJ, VJT, DFC, CFZ, SMT.

### Declaration of interest

CFZ serves on the Scientific Advisory Board of Sage Therapeutics and holds stock in Sage Therapeutics. Sage

Therapeutics was not involved in the funding or design of the present study.

Other authors have no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bja.2019.01.029>.

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