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Developmentally regulated neurosteroid synthesis enhances GABAergic neurotransmission in mouse thalamocortical neurones

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Key points

- During neuronal development synaptic events mediated by $GABA_A$ receptors are progressively reduced in their duration, allowing for rapid and precise network function.
- Here we focused on ventrobasal thalamocortical neurones, which contribute to behaviourally relevant oscillations between thalamus and cortex.
- We demonstrate that the developmental decrease in the duration of inhibitory phasic events results predominantly from a precisely timed loss of locally produced neurosteroids, which act as positive allosteric modulators of the $GABA_A$ receptor.
- The mature thalamus retains the ability to synthesise neurosteroids, thus preserving the capacity to enhance both phasic and tonic inhibition, mediated by synaptic and extrasynaptic $GABA_A$ receptors, respectively, in physiological and pathophysiological scenarios associated with perturbed neurosteroid levels.
- Our data establish a potent, endogenous mechanism to locally regulate the $GABA_A$ receptor function and thereby influence thalamocortical activity.

Abstract   During brain development the duration of miniature inhibitory postsynaptic currents (mIPSCs) mediated by $GABA_A$ receptors ($GABA_A$Rs) progressively reduces, to accommodate the temporal demands required for precise network activity. Conventionally, this synaptic plasticity results from $GABA_A$R subunit reorganisation. In particular, in certain developing neurones synaptic $\alpha2$-$GABA_A$Rs are replaced by $\alpha1$-$GABA_A$Rs. However, in thalamocortical neurones of the mouse ventrobasal (VB) thalamus, the major alteration to mIPSC kinetics occurs on postnatal (P) day 10, some days prior to the $GABA_A$R isoform change. Here, whole-cell voltage-clamp recordings from VB neurones of mouse thalamic slices revealed that early in postnatal development (P7–P8), the mIPSC duration is prolonged by local neurosteroids acting in a paracrine or autocrine manner to enhance $GABA_A$R function. However, by P10, this neurosteroid 'tone' rapidly dissipates, thereby producing brief mIPSCs. This plasticity results from a lack of steroid substrate as pre-treatment of mature thalamic slices (P20–24) with the $GABA_A$R-inactive precursor 5α-dihydroprogesterone (5α-DHP) resulted in markedly prolonged mIPSCs and a greatly enhanced tonic conductance, mediated by synaptic and extrasynaptic $GABA_A$Rs, respectively. In summary, endogenous neurosteroids profoundly influence GABAergic neurotransmission in developing VB neurones and govern a transition from slow to fast phasic synaptic events. Furthermore, the retained capacity for steroidogenesis in the mature thalamus raises the prospect that certain physiological or pathophysiological conditions may trigger neurosteroid neosynthesis, thereby providing a local mechanism for fine-tuning neuronal excitability.

Abbreviations $\alpha1^{0/0}$, GABA$_A$ receptor $\alpha1$ subunit 'knockout'; $\alpha4^{0/0}$, GABA$_A$ receptor $\alpha4$ subunit 'knockout'; $5\alpha3\alpha$, 5α-pregnan-3α-ol-20-one; α-CD, α-cyclodextrin; γ-CD, γ-cyclodextrin; 5α-DHP, 5α-dihydroprogesterone, or 5α-pregnane-3,20-dione; 3α-HSD, 3α-hydroxysteroid dehydrogenase; CNS, central nervous system; ECS, extracellular solution; GABA$_A$R, γ-aminobutyric acid type A receptor; ICS, intracellular solution; IPSC, inhibitory postsynaptic current; KS, Kolmogorov–Smirnov statistical test; mIPSC, miniature inhibitory postsynaptic current; nRT, nucleus reticularis of the thalamus; P, postnatal day; τ_w, weighted decay time constant; T90, time taken for the peak amplitude of an mIPSC to decrease by 90%; TTX, tetrodotoxin; VB, ventrobasal complex of the thalamus; WT, wild-type.

Introduction

During postnatal brain development, demand for temporal precision within neural network activity increases and in accordance, synaptic events mediated by GABA$_A$Rs become progressively reduced in their duration (Brickley *et al.* 1996; Okada *et al.* 2000; Vicini *et al.* 2001; Jüttner *et al.* 2001; Goldstein *et al.* 2002; Bosman *et al.* 2005; Takahashi *et al.* 2005; Fritschy & Panzanelli, 2014; Deidda *et al.* 2014). We reported that miniature inhibitory post-synaptic currents (mIPSCs) of thalamocortical neurones of the ventrobasal (VB) thalamus undergo considerable changes during development. In particular, their duration is greatly reduced over a critical, but relatively short, period of 24–48 h that occurs between postnatal days (P) 9–10 and P10–11 (Peden *et al.* 2008). Conventionally, such changes have been ascribed to alterations in the subunit composition of the synaptic GABA$_A$Rs. These inhibitory receptors are composed of five subunits, selected from a repertoire of 19 proteins (Olsen & Sieghart, 2008). Most neuronal GABA$_A$Rs contain two α subunits, two β subunits and either a γ or a δ subunit, permitting ~20–30 distinct naturally occurring receptor subtypes, which differ in their pharmacology, channel biophysics, anatomical location and developmental expression (Olsen & Sieghart, 2008; D'Hulst *et al.* 2009; Fritschy & Panzanelli, 2014; Rudolph & Möhler, 2014).

The developmental expression of the $\alpha1$ subunit has often been associated with a more rapid mIPSC decay time (Okada *et al.* 2000; Peden *et al.* 2008). In apparent agreement, our immunohistochemical and electrophysiological studies using both $\alpha1$ 'knock-out' ($\alpha1^{0/0}$) and $\alpha1$ 'knock-in' ($\alpha1^{H101R}$) mice revealed synaptic GABA$_A$Rs of P20 VB neurones to express the $\alpha1\beta2\gamma2$ iso-form, whereas those of younger animals were primarily composed of $\alpha2$-GABA$_A$Rs (Peden *et al.* 2008). However, the rapid transition of mIPSC kinetics between P 9 and 11 occurs at a stage when the synaptic receptors still predominantly contain the $\alpha2$ subunit and is evident prior to the increased incorporation of the $\alpha1$ subunit. These findings are complemented by the observation that identical changes in mIPSC decay kinetics occur in VB neurones derived from $\alpha1^{0/0}$ mice (Peden *et al.* 2008). Thus, mechanisms additional to GABA$_A$R

subunit composition appear to influence the development of synaptic current kinetics during this early postnatal period. In this regard, modulation of GABA$_A$R function by endogenous ligands, such as neurosteroids, represents a viable alternative mechanism.

Certain naturally occurring steroids, synthesised both in the periphery and within the central nervous system (CNS), are potent positive allosteric modulators of GABA$_A$R function (Belelli & Lambert, 2005; Carver & Reddy, 2013; Zorumski *et al.* 2013). Their brain levels are dynamically regulated in a number of physiological scenarios (e.g. development, stress, puberty, pregnancy and during the ovarian cycle; Purdy *et al.* 1991; Paul & Purdy, 1992; Grobin & Morrow, 2001; Maguire & Mody, 2009; Shen *et al.* 2010; Zorumski *et al.* 2013; Gunn *et al.* 2014). Additionally, perturbations in their levels occur in several pathophysiological conditions (e.g. panic attacks, major depression, postpartum depression, premenstrual tension and schizophrenia; Purdy *et al.* 1991; Eser *et al.* 2006; Smith *et al.* 2006; Uzunova *et al.* 2006; Longone *et al.* 2008; Zorumski *et al.* 2013, Gunn *et al.* 2014). Intriguingly, neurosteroidogenic enzymes are expressed in certain neurones (Agis-Balboa *et al.* 2006; Tsutsui, 2008; Do Rego *et al.* 2009; Castelli *et al.* 2013), suggesting that these local neuromodulators have the potential to rapidly influence neuronal excitability in a paracrine or an autocrine fashion. Here, we have recorded from VB neurones across the postnatal development period of P7–24 to investigate the influence of endogenous neuro-steroids on the profile of inhibitory neurotransmission. By exploiting the steroid-sequestering actions of the cyclic oligosaccharide γ-cyclodextrin (γ-CD; Adam *et al.* 2002; Shu *et al.* 2004), together with inhibition of neurosteroid synthesis by finasteride, we demonstrate that during a critical postnatal period, GABAergic inhibitory neuro-transmission in the thalamus is greatly influenced by local neurosteroids acting in a paracrine, or autocrine manner.

Methods

Ethical approval

All studies were conducted in accordance with Schedule 1 of the UK Government Animals (Scientific Procedures)

Act, 1986, following approval by the University of Dundee Ethical Review Committee under the auspices of a Home Office Project Licence 60/4005 to D. Belelli.

Breeding of mice

The $\alpha 4$ subunit 'knockout' ($\alpha 4^{0/0}$) mice were generated on a single C57BL6 background at the University of Pittsburgh as previously described (Chandra *et al.* 2006). Heterozygous breeding pairs were housed at the University of Dundee and brain slices were prepared from first or second generation of wild-type (WT), or $\alpha 4^{0/0}$ offspring.

Thalamic slice preparation

Thalamic slices were prepared as previously described (Belelli *et al.* 2005) from P7–24 WT, or $\alpha 4^{0/0}$ mice of either sex. Mice were killed by cervical dislocation, the brain dissected and rapidly placed in ice-cold oxygenated (95% O_2/5%CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): 234 sucrose, 2.95 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 glucose, (pH 7.4; 335–340 mosmol l^{-1}). Whilst maintained in oxygenated aCSF, the brain was sectioned in the horizontal plane using a Vibratome series 1000 PLUS Sectioning System (Intracell, Royston, Hertfordshire, UK). Tissue slices were cut at 300–350 μm thickness for P20–P24 mice and 400 μm for younger animals. The slices were immediately transferred onto a nylon mesh platform housed within a chamber containing circulating oxygenated extracellular solution (in mM: 126 NaCl, 26 $NaHCO_3$, 2.95 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose (306–309 mosmol l^{-1})) and maintained at room temperature for a minimum of 1 h prior to recording.

Whole-cell voltage-clamp recordings

Patch pipettes were pulled from thick-wall borosilicate glass (Garner Glass Co., Claremont, CA, USA) using a Narashige PC-10 electrode puller (Narashige, Japan). Open tip resistances of 3–6 MΩ were obtained when pipettes were filled with intracellular solution (ICS) containing (in mM): 140 CsCl, 10 Hepes, 10 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 2 Mg-ATP and 5 QX-314 (pH 7.2–7.3, 290–300 mosmol l^{-1}). Neurones of the ventrobasal (VB) complex were visually identified using an Olympus BX51 microscope (Olympus), equipped with differential interference contrast/infrared optics and a CCD camera. Whole-cell voltage-clamp recordings were acquired at a holding potential of −60 mV using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA, USA) and filtered at 2 kHz. Isolation of mIPSCs was achieved using the solutions outlined above and by supplementing the extracellular solution (ECS) with 2 mM kynurenic acid

and 0.5 μM tetrodotoxin (TTX) to inhibit ionotropic glutamate receptors and action potential-dependent neurotransmitter release, respectively. Data acquisition and digitisation (10 kHz) were performed using a NIDAQ mx interface (National Instruments, Austin, TX, USA) and stored to a computer hard-drive using WinEDR software (Strathclyde University, UK). Series resistance was compensated for by up to 80% and recordings were considered invalid if the series resistance changed by more than 20%, or if it exceeded 15 MΩ.

Drug and solution application

Bicuculline methobromide and TTX were prepared as concentrated stock solutions in distilled water. The neurosteroids 5α-pregnan-3α-ol-20-one ($5\alpha 3\alpha$) and 5α-dihydroprogesterone (5α-DHP), the 5α-reductase inhibitor finasteride and the 3α-hydroxysteroid dehydrogenase (3α-HSD) inhibitor indomethacin were prepared as concentrated stock solutions (1000X) in DMSO. Drug stock solutions were diluted to the final required concentration in ECS (giving a final DMSO concentration of 0.1%). Kynurenic acid was dissolved directly into the ECS. Similarly, α- and γ-cyclodextrin (α-CD, γ-CD) were dissolved directly into the extracellular and intracellular solution. Two protocols were implemented for cyclodextrin studies. In the first protocol, named 'cyclodextrin pre-incubation', thalamic slices were pre-incubated in the holding chamber at room temperature with α-, or γ-CD (1 mM, >1 h). Subsequently, during the recording the tissue was continuously perfused with ECS (35°C) containing the particular cyclodextrin (1 mM), coupled with inclusion of this cyclic oligosaccharide (0.5 mM) in the recording pipette. In the second protocol, cyclodextrin was only included in the recording pipette (0.5 mM). For studies involving drug pre-incubation of thalamic brain slices prior to recording, the compound (indomethacin, finasteride or 5α-DHP) was dissolved into the holding chamber ECS (at room temperature), from a 1000X DMSO stock for the stated period. The final DMSO concentration (0.1%) had no effect on the mIPSCs, or the tonic current. Once transferred to the recording chamber, slices were maintained in ECS with the compound. The stated incubation times relate to the total time the brain slice was exposed to the compound before recording. In most cases where α- or γ-CD was applied solely to the intracellular compartment, mIPSCs were only included for analysis if they were recorded at least 6 min after acquiring the whole-cell configuration. For experiments designed to assess the temporal profile of intracellular α-, or γ-CD effects, mIPSCs were included for analysis as soon as the whole-cell capacitance transients were accurately cancelled and compensated for by the patch-clamp

amplifier circuitry. Typically, these adjustments were achieved within 1 min of attaining the 'whole-cell' recording mode. Thalamic slices were superfused using a gravity-based perfusion system set to a flow rate of 3–5 ml min^{-1} and recycled to a 50 ml oxygenated reservoir using a peristaltic pump (Minipuls 3, Gilson, Luton, UK). Drugs dissolved in ECS were applied to the slice from separate reservoirs. For all experiments, the temperature in the recording chamber was maintained at 35°C.

mIPSC analysis

Offline analysis of digitised data was performed using WinEDR/WinWCP software (Strathclyde University, UK). Identification of mIPSCs was conducted by an algorithmic detection protocol. To eliminate events affected by dendritic filtering, detected mIPSCs were subjected to a filtering protocol whereby events were discarded if their 10–90% rise time occurred outwith the limits of a Gaussian distribution, or were >1 ms. Following visual inspection of individual mIPSCs and deletion of spurious events, parameters from typically 50 or more mIPSCs were analysed with respect to their peak amplitude, 10–90% rise time, and time taken to decay from peak by 90% (T90). Subsequently, mIPSCs recorded from a single neurone were averaged by alignment of the mid-point of their rising phase and the resulting averaged mIPSC was fitted with either a mono-exponential ($y(t) = Ae^{(-t/\tau)}$), or bi-exponential ($y(t) = A_1e^{(-t/\tau 1)} + A_2e^{(-t/\tau 2)}$) decay function where $y(t)$ is the current amplitude at any given time t, A is the current amplitude and τ is the decay time constant. To assess whether the decay phase of the averaged mIPSC was best fitted by a mono- or bi-exponential, the standard deviation of the residuals was measured and an F test applied. For the vast majority of mIPSCs analysed, the decay was best described by a bi-exponential function. Accordingly, a mean weighted decay constant (τ_W) was calculated to depict the relative contribution of each decay component. Values for τ_W were derived from the following equation:

$$\tau_w = \tau_1 P_1 + \tau_2 P_2$$

where τ_1 and τ_2 are the decay time constants for the first and second exponential functions, and P_1 and P_2 are the proportions of current amplitude described by each component, i.e.

$$P_1 = \frac{A_1}{A_1 + A_2} \qquad P_2 = \frac{A_2}{A_1 + A_2}$$

Frequency analysis of mIPSCs was conducted for each recording whereby events were detected by running an offline event detection protocol (WinEDR) based on the rate of rise time threshold. For control experiments, and those involving drug pre-incubation, the number of events was determined for two bins located towards the beginning and end of a 5 min recording period. The duration of each bin varied with developmental age such that recordings from young animals (P7–8, in which mIPSCs occurred relatively infrequently) required a bin size ~1 min, whereas ~20 s bins were utilised for P20–24 animals. The accuracy of the event detection protocol was verified by visually inspecting the current recordings to ensure that all events were included and that any detected spurious noise was removed. A mean inter-event interval was then generated and converted to a frequency value.

The resident tonic current and the steroid-induced current

The resident tonic current was calculated by determining the outward current produced by bath-applied bicuculline methobromide (30 μM). Similarly, the effect of 5α-DHP pre-incubation on the tonic conductance was assessed by comparing the bicuculline methobromide-evoked outward current for control recordings (i.e. no pre-incubation) *vs.* recordings from VB neurones following drug pre-incubation. For experiments where 5α3α was applied acutely, the enhancement of the tonic current was calculated by determining the difference in holding current before and after neurosteroid application. In each case, the mean DC current of a 30–60 s current section was determined from sequential 25.6–102.4 ms epochs, depending on the mIPSC frequency of the cell. Epochs containing mIPSCs, or sections of recording which exhibited an unstable baseline, were not included in the determination of mean holding current.

Statistical analysis

All data are expressed as the arithmetic mean value ± standard error of the mean (SEM). To calculate statistical significance of mean data, Student's *t* tests (paired, or unpaired, Microsoft Excel) and ANOVA (followed *post hoc* by Tukey's HSD, SigmaStat, Systat Software Inc., San Jose, CA, USA) were used as appropriate. To compare the cumulative probability distributions of individual mIPSC properties at different developmental stages, the Kolmogorov–Smirnoff (KS) test was used (SPSS software, Chicago, IL, USA).

Results

Developmental changes to the properties of VB mIPSCs

We previously reported that the duration of VB mIPSCs is greatly reduced over a critical, but relatively short,

period of 24–48 h, between P8/9 and P10/11 (Peden *et al.* 2008). In confirmation, here, age had a significant effect on all mIPSC parameters tested ($P < 0.001$, one-way ANOVA). In particular, the mIPSCs of P7 VB neurones exhibited a relatively prolonged decay ($\tau_W = 8.5 \pm 0.4$ ms; $n = 15$), which was greatly reduced in duration by P10 ($\tau_W = 4.7 \pm 0.3$ ms; $n = 7$) and further decreased by P20–24 ($\tau_W = 3.2 \pm 0.1$ ms; $n = 28$, Fig. 1A–D, Table 1). Additionally, in comparison to P7, the mIPSCs recorded from P10 and P20–24 VB neurones occurred much more frequently (Fig. 1A and D; Table 1) and by P20–24, both

the mIPSC peak amplitude and rise time were reduced relative to earlier time points (Table 1).

Inhibitory synaptic transmission of immature VB thalamocortical neurones is influenced by locally synthesised neurosteroids

To investigate whether local neurosteroid synthesis contributes to the relatively prolonged mIPSCs of immature VB neurones, P7 thalamic slices were
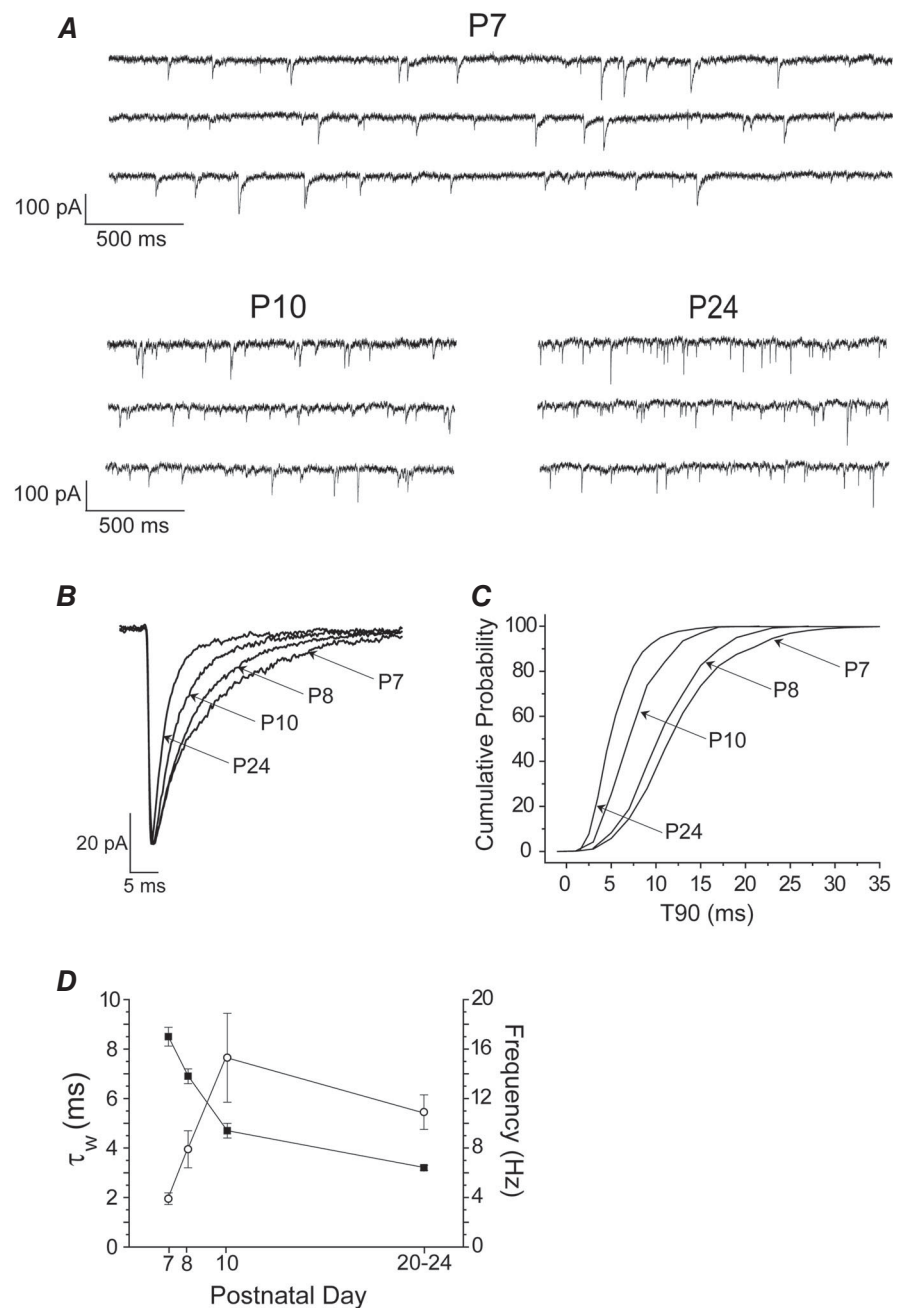

Figure 1. Postnatal development influences phasic inhibition of VB neurones

A, representative current traces recorded from VB neurones obtained from P7, P10 and P24 WT mice. *B*, representative, superimposed, averaged mIPSCs normalised to P7 peak amplitude, showing a progressive decrease in decay time with age. *C*, combined cumulative probability curves for the time taken for individual mIPSCs to decay from peak amplitude to 10% of peak (T90) for P7 (1160 events, $n = 15$ cells), P8 (559 events, $n = 9$ cells), P10 (526 events, $n = 7$ cells) and P20–24 (3642 events, $n = 28$ cells). Note the progressive leftward shift in T90 distribution indicating a developmental decrease in the mIPSC decay time ($P < 0.001$; KS test). *D*, graph illustrating the progressive decrease of the mIPSC τ_W (filled squares) and the concomitant increase of mIPSC frequency (open circles) during development ($P < 0.001$ in each case, one-way ANOVA). Each symbol represents the mean ± SEM.

Table 1. The properties of mIPSCs recorded from WT VB neurones at different stages of development

	P7 (n = 15 cells)	P8 (n = 9 cells)	P10 (n = 7 cells)	P20–24 (n = 28 cells)
Peak amplitude (pA)	−82 ± 4	−92 ± 6	−93 ± 9	−66 ± 3*
Rise time (ms)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1***
τ_W (ms)	8.5 ± 0.4	6.9 ± 0.3**	4.7 ± 0.3***	3.2 ± 0.1***
Frequency (Hz)	3.9 ± 0.5	7.9 ± 1.5	15.3 ± 3.6**	10.9 ± 1.4**

Note that all the mIPSC parameters determined here are significantly different across the developmental ages investigated ($P < 0.001$, one-way ANOVA). Asterisks indicate level of significance *vs.* those of P7 neurones as determined by Tukey's *post hoc* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2. A summary of the effects of finasteride (50 μM), γ-CD (1 mM pre-incubated/0.5 mM ICS only) and α-CD (1 mM pre-incubated/0.5 mM ICS only), upon the properties of the mIPSCs of VB neurones derived from 7-day-old mice

	Control (n = 15 cells)	50 μM finasteride (n = 8 cells)	1 mM γ-CD (n = 9 cells)	1 mM α-CD (n = 7 cells)	0.5 mM γ-CD ICS only (n = 8 cells)	0.5 mM α-CD ICS only (n = 8 cells)
Peak amplitude (pA)	−82 ± 4	−80 ± 7	−90 ± 5	−93 ± 3	−74 ± 8	−83 ± 5
Rise time (ms)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
τ_W (ms)	8.5 ± 0.4	6.0 ± 0.3***	5.8 ± 0.3***	8.8 ± 0.7	5.7 ± 0.2***	8.2 ± 0.3
Frequency (Hz)	3.9 ± 0.5	10.2 ± 3.4*	7.4 ± 1.6	5.1 ± 1.2	3.8 ± 1.2	3.8 ± 1.2

Note that τ_W and frequency were significantly ($P < 0.001$ and $P < 0.05$, respectively) affected by drug treatment whereas peak amplitude and rise time were not (one-way ANOVA). Asterisks indicate level of significance *vs.* control as determined by Tukey's *post hoc* test. *$P < 0.05$, ***$P < 0.001$, *vs.* control. Abbreviations: CD, cyclodextrin; ICS, intracellular solution.

pre-incubated with the 5α-reductase inhibitor finasteride (50 μM; for 4 h). This treatment had no effect on the mIPSC peak amplitude or rise time ($P = 0.99$ and 0.75, respectively, one-way ANOVA, Table 2), but significantly reduced the mIPSC τ_W of P7 VB neurones (control $\tau_W = 8.5 \pm 0.4$ ms; $n = 15$; finasteride $\tau_W = 6.0 \pm 0.3$ ms, $n = 8$; $P < 0.001$, one-way ANOVA, Fig. 2*A* and *D*, Table 2).

To further investigate whether early in development the duration of phasic inhibitory events is influenced by neurosteroids we utilised γ-CD, which sequesters lipophilic molecules, including steroids (Davis & Brewster, 2004; Chisari *et al.* 2010). Previous studies demonstrated the neurosteroid-scavenging properties of γ-CD (composed of 8 cyclic sugars, Shu *et al.* 2004, 2007; Akk *et al.* 2005). For P7 VB neurones, γ-CD treatment (>1 h pre-incubation, see Methods) resulted in mIPSCs with a significantly reduced τ_W (γ-CD = 5.8 ± 0.3 ms, $n = 9$; control = 8.5 ± 0.4 ms; $n = 15$, $P < 0.001$, one-way ANOVA, Fig. 2*B* and *D*, Table 2), indeed, a value similar to that of finasteride-treated neurones (6.0 ± 0.3 ms, $P = 0.99$, one-way ANOVA, $n = 8$). The averaged mIPSC decay was best described by two exponentials (τ_{fast} and τ_{slow}). Further analysis revealed that γ-CD had no effect on τ_{fast} (P7 control $\tau_{fast} = 3.6 \pm 0.3$ ms, $n = 15$, *vs.* P7 γ-CD $\tau_{fast} = 3.2 \pm 0.2$ ms, $n = 9$, $P = 0.36$, Student's unpaired *t* test), but selectively influenced τ_{slow}

(P7 control $\tau_{slow} = 13.2 \pm 0.8$ ms, $n = 15$, *vs.* P7 γ-CD $\tau_{slow} = 9.8 \pm 0.5$ ms, $n = 9$, $P < 0.05$, one-way ANOVA). Indeed, in the presence of γ-CD the value of P7 τ_{slow} is now not significantly different from that of untreated P10 neurones (P10 control $\tau_{slow} = 8.98 \pm 2.1$ ms, $n = 7$, $P = 0.65$, one-way ANOVA). Similarly, for P7 neurones, finasteride treatment selectively reduced the τ_{slow} component (P7 control $\tau_{slow} = 13.2 \pm 0.8$ ms, $n = 15$, *vs.* P7 finasteride $\tau_{slow} = 7.3 \pm 0.4$ ms, $n = 6$, $P < 0.01$, one-way ANOVA), such that it was now not significantly different from that of untreated P10 neurones ($P = 0.41$, one-way ANOVA). These observations are consistent with endogenous neurosteroid altering the kinetics of entry and exit from desensitised states of the synaptic GABA$_A$Rs (Jones & Westbrook, 1995; Zhu & Vicini, 1997).

Importantly, the sensitivity of mIPSCs to γ-CD treatment declined as development progressed, such that it had no effect on the duration of mIPSCs recorded from P10, or P20–24 VB neurones (P10 control $\tau_W = 4.7 \pm 0.3$ ms, $n = 7$, *vs.* P10 γ-CD $\tau_W = 4.7 \pm 0.1$ ms, $n = 6$, $P = 0.96$, Student's unpaired *t* test; P20–24 control $\tau_W = 3.2 \pm 0.1$ ms, $n = 28$, *vs.* P20–24 γ-CD $\tau_W = 3.2 \pm 0.2$ ms, $n = 9$, $P = 0.85$, Student's unpaired *t* test; Fig. 2*D*). As a control we utilised the structurally similar α-CD, as it is ineffective in sequestering pregnane steroids (Shu *et al.* 2004), as a consequence of

having a smaller pore diameter (6 cyclic sugars) than that of γ-CD (Davis & Brewster, 2004). The equivalent treatment with α-CD had no effect on the mIPSC τ_W of P7 neurones (Fig. 2C, Table 2). It is conceivable that the synaptic GABA$_A$Rs become less steroid sensitive during this critical developmental period. As a precedent, during parturition rat synaptic GABA$_A$Rs of oxytocin-releasing neurones become neurosteroid insensitive (Koksma et al. 2003). However, here the prolongation of mIPSC decay observed upon acute application of 5α3α (1 μM) was not significantly different between recordings obtained from P7 and P20–24 neurones (Fig. 2E, n = 3 and 5, respectively; P = 0.67, two-way mixed ANOVA). Although

interpretation of this comparison is complicated by the presence of a neurosteroid tone at P7 and that the mIPSCs are mediated by different synaptic GABA$_A$Rs (α2- and α1-GABA$_A$Rs for P7 and P20–24, respectively; Peden et al. 2008) these data confirm that synaptic GABA$_A$Rs are neurosteroid sensitive at both P7 and P20–24. Therefore, collectively our data suggest that the developmental decrease of the VB mIPSC duration is accounted for by a programmed loss of endogenous neurosteroid.

Immunohistochemical and in situ hybridisation studies suggest that certain neurones can synthesise GABA-active neurosteroids, raising the prospect that they might act in an autocrine fashion to fine-tune neuronal inhibition
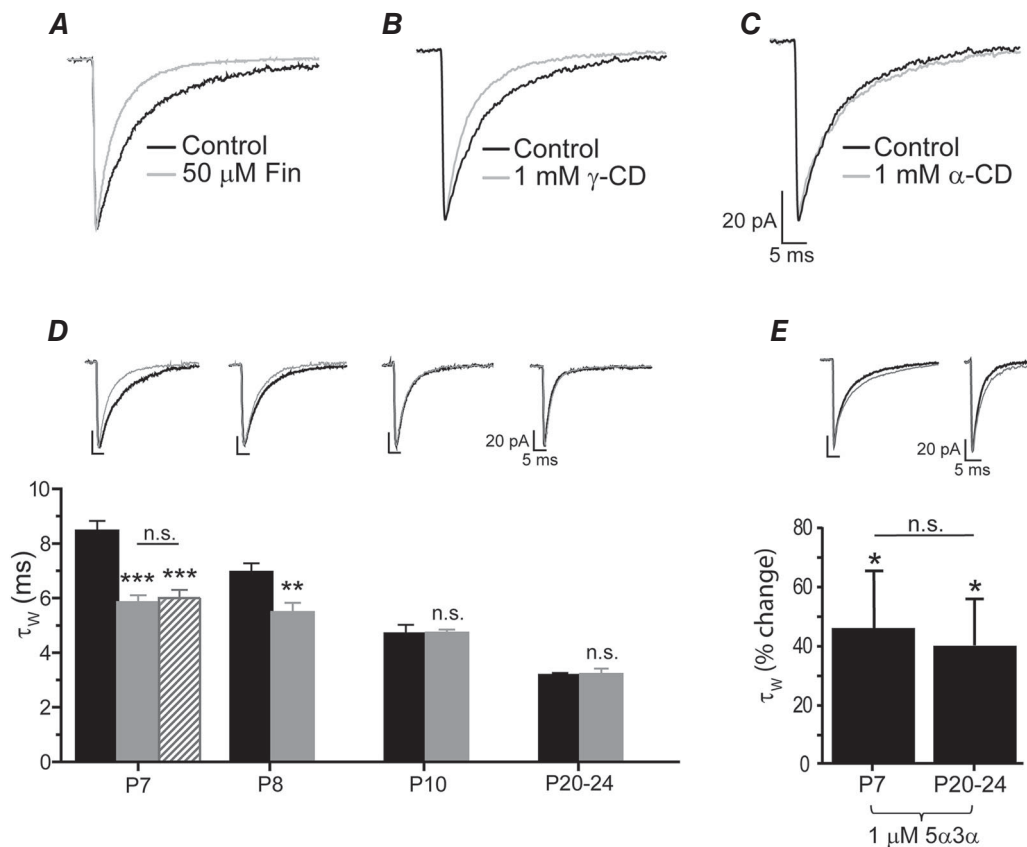

Figure 2. Early in development endogenous neurosteroids prolong GABA$_A$R-mediated mIPSCs of VB neurones

A–C, superimposed and averaged mIPSCs normalised to peak amplitude, recorded from P7 VB neurones for control and following finasteride treatment (*A*), γ-CD pre-incubation (*B*), or α-CD pre-incubation (*C*). D, bar graph comparing the effect of γ-CD pre-incubation (grey bars) on the mean mIPSC τ_W vs. control (black bars) across postnatal development. Note that for comparison, the effect of finasteride (50 μM) treatment is included for P7 VB neurones (hatched bar). The data were obtained from 7–28 neurones and are shown as the mean ± SEM. Representative corresponding averaged and superimposed mIPSCs are given above each developmental time point for control (black trace) and γ-CD (grey trace). **P < 0.01, ***P < 0.001 vs. control, one-way ANOVA (Tukey's *post hoc* test for P7 and unpaired *t* test for P8). E, bar graph illustrating the effects of acute 5α3α (1 μM) on the mIPSC τ_W recorded from P7 (n = 3) and P20–24 (n = 5) VB neurones (*P < 0.05 for each). Compared to P20–24, the percentage change in τ_W following 5α3α application at P7 was not statistically significantly different (two-way mixed ANOVA). The inset shows averaged superimposed mIPSCs before and after application of 5α3α (1 μM) for representative P7 and P20 neurones. Note for panels *A–E* the current calibration bar refers to the averaged control mIPSC. Abbreviations: n.s., not significant; CD, cyclodextrin; Fin, finasteride; 5α3α, 5α-pregnan-3α-ol-20-one.

(Agis-Balboa *et al.* 2006; Tsutsui, 2008; Castelli *et al.* 2013). In support, a study utilising an antibody raised against $GABA_A$R-active neurosteroids suggested that thalamic VB neurones contain the steroid $5\alpha3\alpha$ (Saalmann *et al.* 2007). To explore whether P7 VB neurones are a potential source of these $GABA_A$R neuromodulators, we investigated the impact of including the membrane-impermeant γ-CD (0.5 mM) solely in the recording pipette. This treatment (recordings made >6 min after achieving the whole-cell recording configuration) significantly reduced the mIPSC τ_W (5.7 ± 0.2 ms, $P < 0.001$, one-way ANOVA, $n = 8$) in comparison to control mIPSCs ($\tau_W = 8.5 \pm 0.4$ ms, $n = 15$, Fig. 3*A* and *C*, Table 2). In this respect, intracellular γ-CD was equi-effective with those recordings made with either the γ-CD pre-incubation protocol, or following finasteride pre-treatment ($P = 0.99$, one-way ANOVA, Fig. 3*C*, Table 2). In contrast, addition of α-CD (0.5 mM) solely to the ICS, had no effect on the

mIPSC τ_W ($\tau_W = 8.2 \pm 0.3$ ms, $n = 8$, $P = 0.99$ *vs.* control, one-way ANOVA, Fig. 3*B* and *C*, Table 2). When delivered from a recording electrode, relatively large molecules such as cyclodextrin require time (dependent upon molecular weight, pipette resistance, series resistance and cell geometry/volume) to equilibrate with the intracellular milieu (Marty & Neher, 1995). In agreement, upon achieving the whole-cell recording configuration, intracellular γ-CD (0.5 mM) caused a time-dependent decrease of the mIPSC τ_W, which approached equilibrium after 6–11 min (Fig. 3*D*). By contrast, both standard ICS and ICS containing α-CD (0.5 mM) were inert in this respect (Fig. 3*D*).

Neurosteroid synthesis in thalamic slices

Phasic inhibition. Our findings suggest that young (P7) thalamic tissue produces neurosteroids that influence
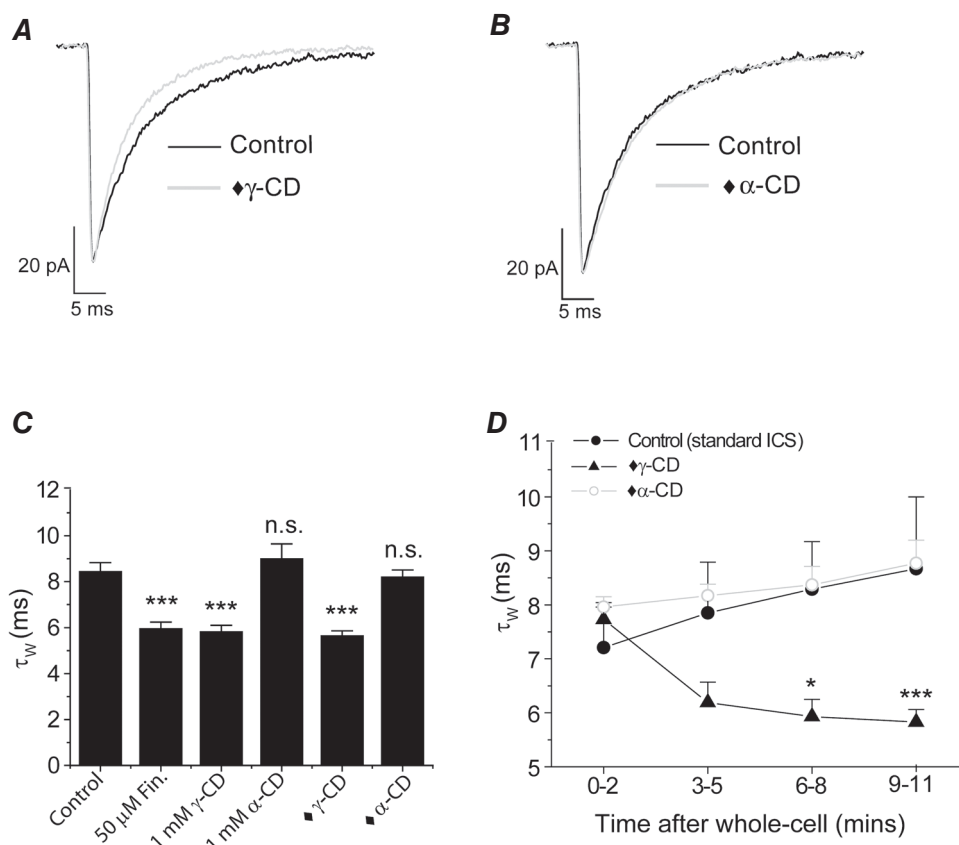

Figure 3. Intracellular γ-cyclodextrin reduces the duration of P7 VB mIPSCs
A and *B*, superimposed and averaged P7 mIPSCs, normalised to peak amplitude, for control and following intracellular γ-CD (*A*, ♦γ-CD), or intracellular α-CD (*B*, ♦α-CD) treatment. The current calibration bar refers to the averaged control mIPSC. *C*, bar graph (mean $\pm$ SEM) comparing the effects of finasteride pre-incubation, γ/α-CD pre-incubation (1 mM extracellular + 0.5 mM intracellular) and intracellular ♦γ/α-CD (0.5 mM) application alone on the mIPSC τ_W ($n = 7$–15 neurones, ***$P < 0.001$ *vs.* control, one-way ANOVA with Tukey's *post hoc* test). *D*, graph illustrating the impact of standard ICS, ICS + ♦α-CD and ICS + ♦γ-CD on the mIPSC τ_W with time after acquisition of the whole-cell recording configuration. Each data point is composed of data from 4–8 neurones. *$P < 0.05$, ***$P < 0.001$, mixed ANOVA with Tukey's *post hoc* test. Abbreviations: CD, cyclodextrin; Fin, finasteride; ICS, intracellular solution; n.s., not significant; ♦, intracellular 0.5 mM γ- or α-cyclodextrin.

GABAergic synaptic signalling. However, by P10 there is an abrupt suppression of neurosteroid synthesis, resulting in a clear reduction of the mIPSC decay time. To determine whether more mature thalamus retains the ability to synthesise neuroactive steroids we investigated the impact on GABAergic inhibition of incubating P20–24 thalamic slices with 5α-DHP, the immediate precursor of 5α3α. Limited (30–60 min) exposure to 5α-DHP (3 μM) had no effect on the mIPSC τ_W of P20–24 VB neurones ($P = 0.98$ *vs.* control, one-way ANOVA, Fig. 4*A* and *B*). However, a more extended incubation (>120 min) with this steroid caused a significant ($P < 0.001$, one-way ANOVA) prolongation of P20–24 mIPSCs (5α-DHP $\tau_W = 8.1 \pm 0.5$ ms, $n = 11$ *vs.* control $\tau_W = 3.2 \pm 0.1$ ms, $n = 28$; Fig. 4*A* and *B*). Whereas γ-CD had no significant effect on mIPSCs of P20–24 VB neurones (Fig. 2*D*, control
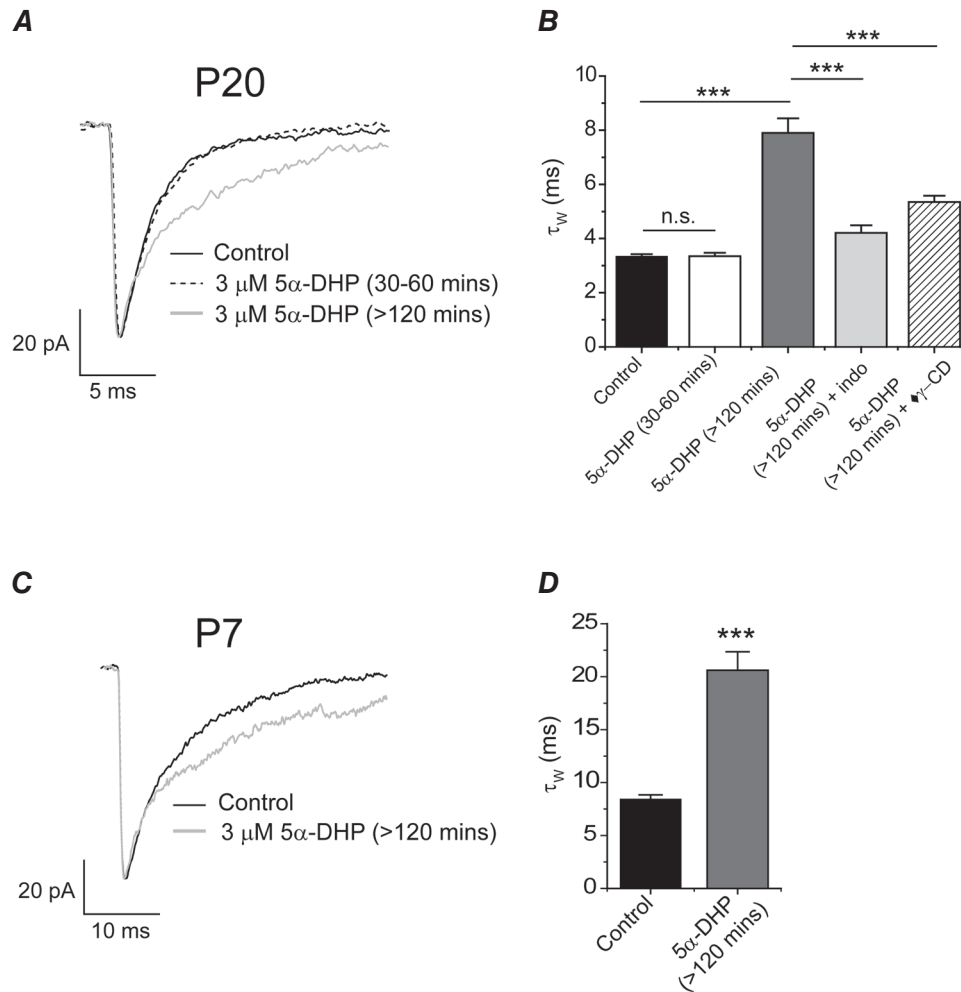

Figure 4. The neurosteroid precursor 5α-dihydroxyprogesterone prolongs the decay of mIPSCs recorded from P20 and P7 VB neurones

A, superimposed and averaged mIPSCs, normalised to peak amplitude, recorded from P20 VB neurones under control conditions (black trace), following pre-incubation of 5α-DHP (3 μM) for 30–60 min (dashed black trace) and >120 min (grey trace). *B*, bar graph (mean ± SEM) illustrating the mean P20–P24 mIPSC τ_W for control and the influence of various 5α-DHP treatments. Note that treatment with either indomethacin (30 μM; pre-incubation for 1 h and subsequent co-incubation with 3 μM 5α-DHP for >120 min) or intracellular γ-CD (0.5 mM ♦γ-CD) significantly reduced the prolongation of the mIPSC decay produced by 5α-DHP (3 μM pre-incubated >120 min) alone. Data obtained from 5–15 neurones (***$P < 0.001$, one-way ANOVA with Tukey's *post hoc* test). *C*, superimposed and averaged mIPSCs, normalised to peak amplitude, recorded from P7 VB neurones under control conditions (black trace) and following pre-incubation with 5α-DHP (3 μM) for >120 min (grey trace). *D*, bar graph illustrating the mean τ_W for control ($n = 15$ neurones) and following pre-incubation of P7 thalamic slices with 5α-DHP (>120 min, 3 μM; $n = 6$ neurones; ***$P < 0.001$ *vs.* control, Student's unpaired *t* test). Note for *A* and *C* the calibration bar for current refers to the averaged control mIPSC. Abbreviations: n.s., not significant; ICS, intracellular solution; γ-CD, γ-cyclodextrin; indo, indomethacin; 5α-DHP, 5α-dihydroprogesterone; ♦, intracellular.

$\tau_W = 3.2 \pm 0.1$ ms, $n = 28$ *vs.* γ-CD $\tau_W = 3.3 \pm 0.2$ ms, $n = 9$, $P = 0.85$, Student's unpaired *t* test), intracellular γ-CD (0.5 mM) clearly reduced the effect of 5α-DHP pre-treatment (3 μM; >120 min) on the mIPSC duration of such neurones ($\tau_W = 5.3 \pm 0.2$ ms, $n = 5$, $P < 0.001$, one-way ANOVA, Fig. 4*B*). These results suggest that 5α-DHP is converted to a GABA$_A$R-active steroid by thalamic tissue. Therefore, we used indomethacin (see Methods) to inhibit the 3α-HSD-mediated conversion of 5α-DHP to the GABA-active 5α3α (Griffin & Mellon, 2001). The effect of 5α-DHP (3 μM) to prolong P20–24 mIPSCs was substantially suppressed by indomethacin (30 μM) incubation, ($\tau_W = 4.2 \pm 0.3$ ms, $n = 8$; $P < 0.001$, one-way ANOVA; Fig. 4*B*). Therefore, in P20–24 thalamus, 3α-HSD is functional, i.e. retaining the capacity to synthesise the GABA$_A$R-active steroid 5α3α.

We next investigated whether the endogenous neurosteroid production at P7 can be further augmented. Following 5α-DHP incubation (3 μM, >120 min), mIPSCs recorded from P7 VB neurones were substantially prolonged (5α-DHP $\tau_W = 21.4 \pm 1.6$ ms, $n = 6$, *vs.* control $\tau_W = 8.5 \pm 0.4$ ms, $P < 0.001$, Student's unpaired test, $n = 15$, Fig. 4*C* and *D*), indicating that neurosteroid production and the associated GABA$_A$R modulation is not maximal at this developmental time point.

Tonic inhibition. Mature mouse VB neurones additionally possess a tonic conductance mediated by extrasynaptic GABA$_A$Rs composed primarily of α4β2δ receptors (Porcello *et al.* 2003; Belelli *et al.* 2005; Chandra *et al.* 2006; Peden *et al.* 2008). Application of bicuculline (30 μM) to P20–24 VB neurones revealed a standing tonic current (-96 ± 9 pA; $n = 27$, Fig. 5*B*). For such VB neurones, acutely applied 5α3α induced an inward current (100 nM = -32 ± 4 pA, $n = 11$; 1 μM = -55 ± 20 pA, $n = 6$, Fig. 5*A* and *B*). We have previously shown the VB tonic current to be greatly reduced by deletion of the δ, or the α4 subunit (Herd *et al.* 2009, 2013; see also Chandra *et al.* 2006; Fig. 5*B*). Furthermore, the steroid-induced inward current evident for WT neurones (5α3α, 100 nM) was absent for α4$^{0/0}$ neurones (-4 ± 3 pA, $n = 5$, Fig. 5*B*), revealing this effect of the neurosteroid to be mediated by extrasynaptic α4-GABA$_A$Rs.

Our finding that P20–24 mIPSCs are not sensitive to γ-CD treatment suggests that neurosteroid levels are relatively low at this developmental stage. Nevertheless, recombinant GABA$_A$Rs incorporating the δ-subunit are particularly sensitive to neurosteroid modulation (Belelli *et al.* 2002; Brown *et al.* 2002; Wohlfarth *et al.* 2002). With regard to untreated P20–24 VB neurones, following γ-CD pre-incubation we observed no significant change in the standing tonic current revealed by bicuculline (control = -96 ± 9 pA,

$n = 27$, *vs.* γ-CD pre-incubation = -75 ± 15 pA, $n = 4$, $P = 0.86$, one-way ANOVA, Fig. 5*D*). However, following pre-incubation with 5α-DHP (>120 min), the magnitude of the tonic current revealed by bicuculline was significantly greater (control = -96 ± 9 pA, $n = 27$, *vs.* 5α-DHP = -234 ± 27 pA, $n = 6$, $P < 0.001$, one-way ANOVA, Fig. 5*C* and *D*). By contrast, no such effect was observed following a relatively short pre-incubation period (30–60 min) with 3 μM 5α-DHP (-100 ± 28 pA, $n = 5$, $P = 0.99$ *vs.* control, one-way ANOVA, Fig. 5*D*). Therefore, although neurosteroid production is limited at P20–24, presumably due to a lack of substrate, it may be reinitiated by providing a precursor to the GABA-active steroid, which enhances both synaptic and tonic inhibition.

Neurosteroids and GABA release

As described above, the frequency of mIPSCs recorded from P10 and P20–24 VB neurones was much greater than that of P7 neurones (Fig. 1*D*; Table 1). Whilst exploring the postsynaptic effects of finasteride, we noted that incubation with this 5α-reductase inhibitor additionally greatly increased the mIPSC frequency of P7 VB neurones (control = 3.9 $\pm$ 0.5 Hz; $n = 15$; finasteride = 10.2 $\pm$ 3.4 Hz, $n = 8$; $P < 0.05$, one-way ANOVA, Fig. 6*A* and *B*; Table 2). One interpretation of these data posits that for P7 nucleus reticularis (nRT)–VB synapses the local neurosteroid tone is sufficient to presynaptically suppress the vesicular release of GABA. Therefore, it is conceivable that the increased mIPSC frequency, evident for P10–24 neurones, reflects a loss of this neurosteroid tone. In agreement, prolonged incubation of P20–24 thalamic slices with 5α-DHP (3 μM), in addition to enhancing phasic and tonic inhibition, markedly reduced the VB mIPSC frequency (control = 10.9 $\pm$ 1.4 Hz, $n = 28$, *vs.* 3 μM 5α-DHP = 3.9 $\pm$ 0.8 Hz, $n = 11$, $P < 0.01$, one-way ANOVA, Fig. 6*A* and *B*). The effect on release appears to be mediated by 5α3α as co-incubation with 5α-DHP and indomethacin had no effect on frequency (9.9 $\pm$ 1.9 Hz, $n = 8$, $P = 0.91$, one-way ANOVA, Fig. 6*B*). Furthermore, in contrast to P20–24 neurones, for P7 VB neurones, 3 μM 5α-DHP alone (prolonged incubation) had no effect on mIPSC frequency, suggesting that for this developmental stage the effect of the native steroid to influence GABA release is maximal (5α-DHP = 2.8 $\pm$ 0.5 Hz, $n = 5$, *vs.* control = 3.9 $\pm$ 0.5 Hz, $n = 15$, $P = 0.23$, one-way ANOVA, Fig. 6*B*).

Discussion

During postnatal development, fast excitatory and inhibitory synapses are subject to considerable molecular

reorganisation resulting in brief synaptic currents, permitting increased temporal precision and fidelity. For GABAergic inhibitory neurotransmission, alterations to $GABA_AR$ subunit composition are primarily implicated in mediating these important kinetic changes (Takahashi

et al. 2005). In thalamus, the duration of GABAergic inhibition plays an important role in thalamocortical oscillations (Huguenard & McCormick, 2007; Rovó *et al.* 2014). Here, we demonstrate for thalamic relay neurones that the substantial alteration of mIPSC kinetics,
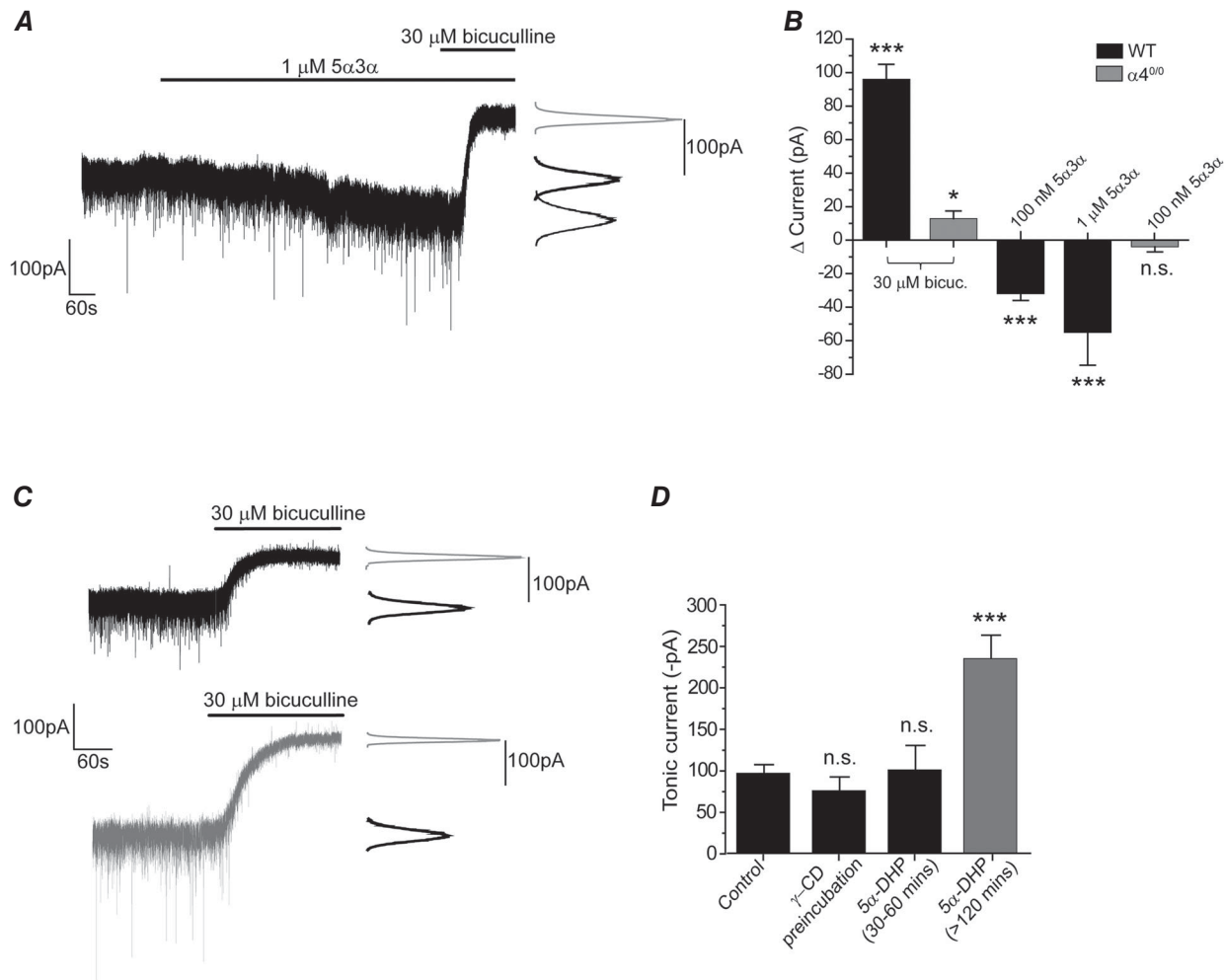
Figure 5. Neurosteroid enhancement of the tonic current mediated by extrasynaptic $\alpha4\beta\delta$ GABA$_A$Rs in P20–24 VB neurones

A, a representative current trace recorded from a P21 VB neurone. Acute application of $5\alpha3\alpha$ (1 μM) evoked an inward current. Bicuculline (30 μM), in addition to blocking the current produced by $5\alpha3\alpha$, revealed the resident tonic current. The corresponding all-points histograms are shown to the right of the trace. *B*, bar graph illustrating the impact of the $\alpha4$-subunit deletion on the mean tonic current (mean ± SEM) of P20–24 VB neurones as determined by the change in holding current following bicuculline (30 μM) treatment. Compared to WT ($n = 27$), the $\alpha4^{0/0}$ VB neurones ($n = 12$) exhibited a markedly decreased tonic conductance. For WT neurones, acutely applied $5\alpha3\alpha$ (100 nM and 1 μM) enhanced the tonic current (i.e. produced an inward current). By contrast, for VB neurones derived from $\alpha4^{0/0}$ mice 100 nM $5\alpha3\alpha$ was inert in this regard. *$^{*}P < 0.05$ vs.* control, *$^{***}P < 0.001$ vs.* control, Student's paired *t* test. *C*, representative current traces recorded from WT P20–24 VB neurones under control conditions (top trace) and following pre-incubation with 5α-DHP (3 μM; >120 min; lower trace). The corresponding all-points histograms are shown to the right of each trace. *D*, bar graph depicting the magnitude of the tonic current (mean ± SEM) recorded from P20–24 VB neurones for control and following the (i) γ-CD pre-incubation, (ii) 5α-DHP pre-incubation for 30–60 min, and (iii) 5α-DHP pre-incubation for >120 min. Data obtained from 4–27 neurones. *$^{***}P < 0.001$ vs.* control, one-way ANOVA, Tukey's *post hoc* test. Abbreviations: n.s., not significant; ICS, intracellular solution; γ-CD, γ-cyclodextrin; indo, indomethacin; 5α-DHP, 5α-dihydroprogesterone; $5\alpha3\alpha$, 5α-pregnan-3α-ol-20-one. Note the data depicted in *B* pertaining to the resident tonic current (i.e. revealed by bicuculline) in WT and $\alpha4^{0/0}$ neurones is reproduced from Herd *et al.* 2013.

which occurs during a developmentally critical (P10) window, results not from changes in GABA$_A$R expression, but from decreased local neurosteroid production. Although this GABA-modulatory tone dissipates with maturation, the neurosteroid synthesising enzymes remain functional, permitting the adult thalamus to potentially respond to physiological/pathophysiological stimuli with renewed steroidogenesis and thereby 'fine-tune' neuronal inhibition.
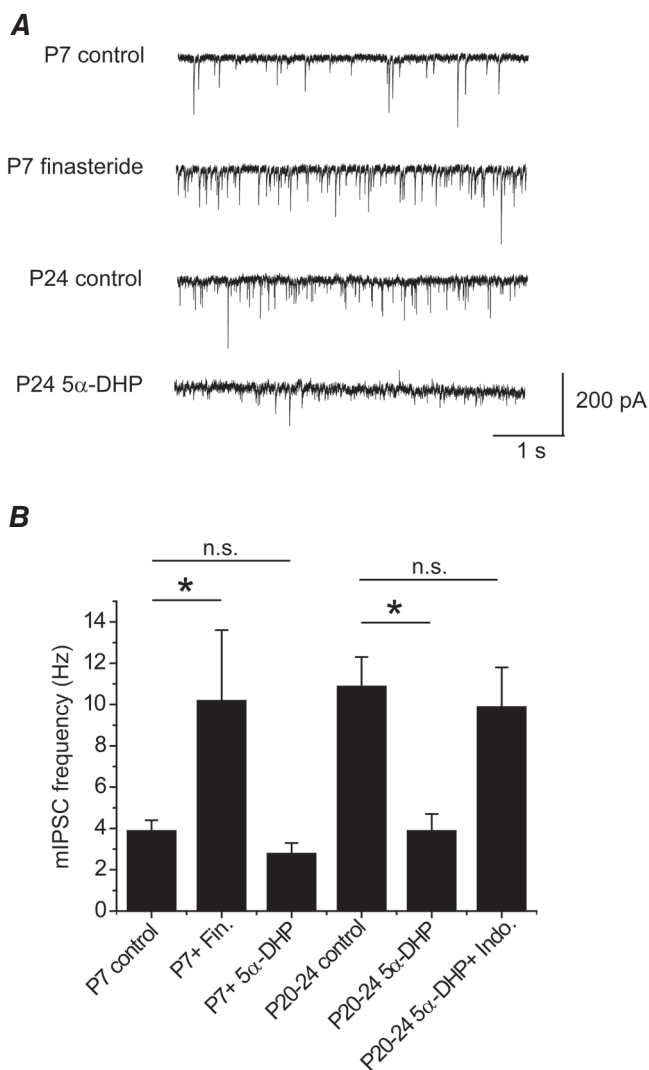

Figure 6. Neurosteroids influence the mIPSC frequency of VB neurones

A, representative traces depicting the frequency of mIPSCs for an untreated P7 neurone, a P7 neurone pre-treated with 50 μM finasteride, an untreated P24 neurone and a P24 neurone pre-treated with 3 μM 5α-DHP. *B*, summary bar graph of mIPSC τ_W for various manipulations of neurosteroid synthesis at P7 and P20–24. Data from 5–28 neurones. *$P < 0.05$, one-way ANOVA with Tukey's *post hoc* test. Abbreviations: 5α-DHP, 5α-dihydroprogesterone; Indo, indomethacin; Fin, finasteride; n.s., not significant.

In thalamus a developmentally regulated endogenous neurosteroid tone influences phasic inhibition

Studies of a variety of CNS neurones report that an α2/3- to α1-GABA$_A$R subunit switch probably underlies the developmental decrease in the duration of IPSCs (Takahashi *et al.* 2005; Fritschy & Panzanelli, 2014). In support, in thalamic neurones α1-subunit over-expression reduced the mIPSC decay time (Okada *et al.* 2000). Furthermore, for developing thalamic VB neurones synaptic α2-GABA$_A$Rs are replaced by α1-GABA$_A$Rs (Peden *et al.* 2008). However, in these neurones the major change to mIPSC kinetics occurs during a critical, brief (P9–10) time window, which precedes the GABA$_A$R subunit switch (Peden *et al.* 2008). Therefore, what underpins this rapid change of synaptic α2-GABA$_A$R function? Using finasteride to inhibit synthesis, or γ-CD to sequester neurosteroids, we demonstrate that during postnatal development, thalamic synaptic inhibition is influenced by an endogenous neurosteroid tone that rapidly dissipates by P10, consequently reducing mIPSC duration. Previous studies demonstrated γ-CD, but not α-CD, to complex with 5α3α (Shu *et al.* 2004). In agreement, only γ-CD reduced the mIPSC decay of P7 VB neurones. Furthermore, γ-CD had no effect on >P10 mIPSCs, consistent with previous observations revealing it to be inert on recombinant GABA$_A$Rs (Shu *et al.* 2004).

The effect of γ-CD may reflect sequestration of alternative endogenous GABA$_A$R modulators. A study of mouse nRT neurones reported the presence of an endogenous benzodiazepine-site 'agonist', sufficient to prolong spontaneous IPSCs (Christian *et al.* 2013). Irrespective of whether γ-CD complexes such 'endozepines', VB inhibitory synapses are not subject to this form of modulation (Christian *et al.* 2013). In neocortex, endocannabinoids influence synaptic inhibition by a direct interaction with GABA$_A$Rs (Golovko *et al.* 2014). However, crucially, the impact of γ-CD treatment on P7 VB neurones was mimicked by inhibiting neurosteroid synthesis with finasteride. Furthermore, although γ-CD had no effect on the mIPSCs of P20–24 VB neurones, it reduced their duration when prolonged by prior 5α-DHP treatment (i.e. by sequestering newly synthesised 5α3α; see below). Collectively, these findings implicate local GABA$_A$R-active neurosteroids in the developmental change to mIPSC duration. Note that after P10, the mIPSC duration is further reduced in a γ-CD-insensitive manner. This subsequent change exhibits a temporal profile consistent with the replacement of synaptic α2-GABA$_A$Rs with α1-GABA$_A$Rs (Peden *et al.* 2008). The neurosteroid-mediated change in mIPSCs could result from an alteration of the sensitivity of synaptic GABA$_A$Rs to this endogenous modulator, governed by various factors including post-translational receptor modification (Koksma *et al.* 2003; Belelli & Lambert, 2005). However,

P7 and P20–24 VB mIPSCs were similarly sensitive to exogenous $5\alpha3\alpha$. Given the presence of an endogenous neurosteroid tone at P7, and that the different subtypes of synaptic $GABA_ARs$ mediate these mIPSCs at P7 cf. P20–24, caution is warranted in making a meaningful quantitative comparison of the effects of exogenous neurosteroid at the two developmental stages. Nevertheless these data, together with those obtained with the $5\alpha3\alpha$ precursor DHP, establish that the synaptic $GABA_ARs$ at both developmental stages are neurosteroid sensitive, therefore suggesting that this perturbation of mIPSC kinetics primarily results from a dynamic change in the level of local neurosteroid.

Our recent findings suggest that the developmental changes to IPSC kinetics will be complex. VB neurones express extrasynaptic $GABA_ARs$ that mediate an inhibitory tonic conductance (Belelli *et al.* 2005; Cope *et al.* 2005; Jia *et al.* 2005). Utilising paired recordings of nRT and VB (P15–23) neurones, we demonstrated that GABA, released in response to physiological frequencies of nRT excitation, not only activates synaptic ($\alpha1\beta2\gamma2$) receptors, but additionally engages extrasynaptic ($\alpha4\beta2\delta$) $GABA_ARs$, by GABA 'spill-over', to produce a slow IPSC component (Herd *et al.* 2013; see also Rovó *et al.* 2014). Intriguingly, at P8–9, when the IPSC duration is prolonged by resident neurosteroid, the expression of extrasynaptic $\alpha4$-$GABA_ARs$ is modest (Peden *et al.* 2008) and therefore these receptors presumably have only a limited impact on IPSC duration. However, with further development ($>$P10) the loss of the neurosteroid influence on the IPSC duration is temporally coincident with increased $\alpha4$-$GABA_AR$ expression (Peden *et al.* 2008), presumably leading to an increased slow component of the IPSC mediated by GABA 'spill-over'.

P20–24 VB neurones synthesise $GABA_AR$-active neurosteroids that enhance phasic and tonic inhibition

The abrupt cessation during development of a neurosteroid influence on synaptic $GABA_ARs$ may result from changes to enzyme expression, or loss of steroid substrate(s). To explore whether more mature thalamus can synthesise $GABA_AR$-active neurosteroids we investigated the $5\alpha3\alpha$ precursor, 5α-DHP. Acute or short application of 5α-DHP had no effect on the mIPSCs of P20–24 VB neurones, confirming that this steroid does not directly interact with synaptic $GABA_ARs$. However, a >2 h application produced a clear prolongation of the mIPSC decay and significantly increased the tonic conductance. The requirement for a prolonged incubation with DHP is not known, but may reflect time required for penetration of the steroid into the slice preparation (Gedell *et al.* 2004), equilibration of the steroid with intracellular membrane

compartments (Li *et al.* 2007) and conversion to the $GABA_AR$-active $5\alpha3\alpha$. Given the coordinated effects of synaptic and extrasynaptic $GABA_AR$ activation on IPSC duration (Herd *et al.* 2013), enhancement of neurosteroidogenesis during physiological/pathophysiological scenarios, or in response to psychoactive drugs (see below), should greatly influence thalamic inhibition and consequently behaviourally relevant thalamocortical network oscillations (Huguenard & McCormick, 2007). However, note the neurosteroid effects on GABA release also requires consideration in this regard (see below).

Although 5α-DHP may engage genomic mechanisms to alter gene expression (Schumacher *et al.* 2014), both the prolongation of GABA-mediated synaptic currents and the increased tonic current were substantially prevented by the 3α-HSD inhibitor indomethacin (30 μM), or by intracellular γ-CD. Therefore, the effects of 5α-DHP on phasic and tonic inhibition result from the local conversion of this precursor into $5\alpha3\alpha$ by 3α-HSD. Consequently, the decreased neurosteroid tone observed later in development probably results from a lack of substrate, suggesting that one, or more steps 'upstream' from 5α-DHP synthesis are inactive. Similarly, in neonatal spinal dorsal horn neurones, a developmentally regulated neurosteroid presence prolongs the mIPSC decay, an effect that similarly dissipates upon maturity (Inquimbert *et al.* 2008).

Developmental changes in quantal GABA release are influenced by neurosteroids

Temporally coincident with the postsynaptic changes to phasic inhibition, there is a large increase by P10 of mIPSC frequency, which remains elevated for P20–24 neurones. For P7 neurones, finasteride greatly increased mIPSC frequency, such that it approached that of older neurones. Furthermore, increasing $5\alpha3\alpha$ synthesis with 5α-DHP clearly reduced the elevated mIPSC frequency of P20–24, but not of P7 neurones. This 5α-DHP effect on P20–24 neurones was prevented by co-incubation with indomethacin, implicating the involvement of $5\alpha3\alpha$. These results suggest that early in development (P7), the neurosteroid tone is sufficient to suppress spontaneous vesicular GABA release from nRT terminals, an effect mimicked by exogenous 5α-DHP when added to more mature neurones. These findings are difficult to reconcile with the relatively depolarised chloride reversal potential reported for nRT neurones, where $GABA_AR$ activation results in a net Cl^- efflux and consequently depolarisation (Ulrich & Huguenard, 1997; Sun *et al.* 2012), i.e. neurosteroid enhancement of presynaptic $GABA_ARs$ should increase, not decrease, vesicular release. However, other $GABA_AR$ modulators including zolpidem (1 μM), pentobarbital (100 μM) and etomidate (3 μM), at concentrations

that produced a clear prolongation of P18–24 VB mIPSCs, had no effect on their frequency (Belelli *et al.* 2005; authors' unpublished observations), suggesting that this neurosteroid effect may not be mediated by presynaptic GABA$_A$Rs, but involve other targets, e.g. voltage-gated T-type calcium channels (Jevtovic-Todorovic *et al.* 2009).

Putative autocrine and paracrine actions of neurosteroids

Studies of steroidogenic enzyme expression indicate that neurosteroid synthesis occurs in both glia and neurones (Do Rego *et al.* 2009), suggesting neurosteroids act as either paracrine or autocrine messengers to influence neuronal GABA$_A$Rs (Belelli & Lambert, 2005; Lambert *et al.* 2009). However, a subsequent investigation revealed that expression of 5α-reductase and 3α-HSD enzymes is restricted primarily to particular neuronal populations in mouse brain (Agis-Balboa *et al.* 2006). Studies using an antibody raised against the neurosteroid suggest that the distribution of the 5α3α steroid in adult rat brain exhibits a similar neuronal localisation, with staining restricted predominantly to intracellular and membrane compartments of principal neurones (Saalmann *et al.* 2007; Tokuda *et al.* 2011). Adult rodent nRT neurones exhibited staining for the 5α-reductase enzyme and for the neurosteroid in nRT and VB neurones (Agis-Balboa *et al.* 2006; Saalmann *et al.* 2007; Castelli *et al.* 2013). Clearly, it would be of interest to perform such immunohistochemical investigations earlier in development.

Therefore, in certain neurones the neurosteroids may act in an autocrine fashion to fine-tune neuronal inhibition. In this scenario, the neurosteroid accesses the GABA$_A$R by lateral diffusion in the plasma membrane, without the necessity for being secreted in a paracrine manner into the extracellular milieu from surrounding neurones or glial cells (Agis-Balboa *et al.* 2006; Chisari *et al.* 2010), a scenario consistent with the demonstration of a neurosteroid transmembrane binding site on the GABA$_A$R and the lipophilic nature of the neurosteroid (Chisari *et al.* 2010). Although low nanomolar aqueous concentrations of 5α3α enhance GABA$_A$R function *in vitro*, given their lipophilicity, much greater concentrations (μM) may accumulate in the membrane, raising the prospect of a relatively low affinity steroid binding site on the GABA$_A$R (Chisari *et al.* 2010), i.e. consistent with the observation that γ-CD efficiently sequesters the neurosteroid of VB neurones. Note that our studies with finasteride and γ-CD do not address the neurosteroid source. Indeed, our observation that the developmental changes to GABA release also appear to be governed by local neurosteroids suggests that their ambient concentrations are sufficient to be biologically active, and/or that nRT neurones may synthesise neurosteroids (Agis-Balboa *et al.* 2006:

Saalmann *et al.* 2007). Further studies are required to clarify the relative contribution of autocrine and paracrine neurosteroids to inhibition in developing thalamus.

Do neurosteroids influence GABA$_A$R-mediated inhibition in the adult brain?

The neurosteroid tone dissipates by P10, prompting the question as to whether they play a role in the adult. Indeed, it will be of interest to establish whether a thalamic neurosteroid tone re-emerges at >P24. Certainly, as described above, the synthetic enzymes and the neurosteroid are present in particular neurones of adult rodents (Agis-Balboa *et al.* 2006; Saalmann *et al.* 2007; Castelli *et al.* 2013; Gunn *et al.* 2014). Furthermore, when provided with substrate, the mature thalamus can synthesise GABA$_A$R-active neurosteroids. Irrespective of the source, in adults neurosteroid levels are not static, but change in a variety of physiological and pathophysiological scenarios, including ageing, stress, pregnancy, the oestrous cycle, postpartum depression, premenstrual tension, inflammatory pain and certain forms of epilepsy (Belelli & Lambert, 2005; Schumacher *et al.* 2014). Furthermore, certain antidepressants and various psychoactive drugs also perturb their levels (Belelli & Lambert, 2005; Lambert *et al.* 2009). Note that peripheral endocrine glands such as the adrenal cortex and ovaries provide an important additional source of brain GABA$_A$R-active neurosteroids as they can readily cross the blood–brain barrier (Belelli & Lambert, 2005; Schumacher *et al.* 2014). How these endocrine messengers synergise with local autocrine and paracrine steroids to influence neuronal inhibition remains to be clarified. In adults, what initiates local neurosteroid production in the brain? A recent report demonstrated activation of NMDA receptors to increase the staining for 5α-reduced neurosteroids in CA1 hippocampal neurones (Tokuda *et al.* 2011). By enhancing inhibition, such a mechanism may provide a balance in situations associated with excessive glutamate-induced excitation (Chisari *et al.* 2010; Tokuda *et al.* 2011).

Neurosteroid influence on thalamic neuronal excitability during development

What are the likely effects of neurosteroids on the excitability of mouse thalamic relay neurones? Clearly this will depend on the developmental age studied. Developed (P18–27) VB neurones express both synaptic $\alpha 1\beta 2\gamma 2$ GABA$_A$Rs and extrasynaptic $\alpha 4\beta 2\delta$ GABA$_A$Rs, and their activation produces a hyperpolarisation of the resting membrane potential (Belelli *et al.* 2005; Cope *et al.* 2005; Peden *et al.* 2008; Herd *et al.* 2009; authors' unpublished observations). Although here we have not directly investigated the effects of the neurosteroid

upon relay neurone excitability, our previous studies on etomidate may be instructive. Low concentrations of this general anaesthetic, in common with $5\alpha3\alpha$, enhance both synaptic and extrasynaptic $GABA_A R$ function, consequently increasing the tonic conductance, thereby causing membrane hyperpolarisation and prolonging the neurally evoked IPSP (Herd *et al.* 2014). Functionally, the increased tonic conductance suppresses tonic spiking and the associated hyperpolarisation may favour burst firing due to relief of T-type calcium channel inactivation (Cope *et al.* 2005; Herd *et al.* 2014). Additionally, prolongation of the IPSP caused a delay in the timing of post-inhibitory rebound burst firing. As neurosteroid synthesis also resulted in an enhanced tonic conductance and prolonged synaptic inhibition, presumably it would exert a similar influence to etomidate upon thalamocortical excitability. However, caution is warranted in making this assumption, as in contrast to etomidate (Belelli *et al.* 2005), the neurosteroid additionally acted upon nRT neurones to substantially reduce the frequency of mIPSCs. Nevertheless, given that the architecture of thalamocortical network oscillations is considered to be partly dependent on the duration of synaptic inhibition (Kim *et al.* 1997; Huguenard & McCormick, 2007), a neurosteroid-induced IPSP prolongation may simplistically be predicted to lengthen the period of rebound-dependent oscillations (e.g. sleep spindles). However, in contrast, more modest increases in IPSC decay time have been shown to paradoxically reduce rebound latency and network synchrony (Sohal *et al.* 2006), suggesting that any spatio-temporal variation of endogenous neurosteroidogenesis may differentially impact upon network operations. Adding further complexity, an enhanced tonic conductance increases temporal jitter in rebound burst timing and may reduce network synchrony (Sohal *et al.* 2006; Bright *et al.* 2007). Future computational modelling studies are thus required to dissect the relative impact of a dynamic steroid-induced modulation of tonic and phasic inhibition on thalamic network functions.

Even less predictable are the effects of the neurosteroid tone upon developing thalamocortical activity. Immature (P8–9) VB neurones express synaptic $\alpha2\beta\gamma2$ not $\alpha1\beta2\gamma2$ $GABA_A Rs$ and the tonic current is only $\sim25\%$ of that exhibited by more mature (P23–27) neurones (Peden *et al.* 2008). Adding complexity, at P7 we illustrate here that the endogenous neurosteroid tone is sufficient to act presynaptically to substantially reduce the quantal release of GABA onto VB neurones. Hence, at this developmental stage the neurosteroid appears to act both pre- and postsynaptically to reduce and facilitate the actions of GABA, respectively. Furthermore, determining whether GABA is excitatory or inhibitory will be fundamental to elucidating the action of neurosteroids on developing thalamus. GABA in (P9–12) rat VB neurones, in common with older mouse VB neurones, is reported to

be hyperpolarising (Ulrich & Huguenard, 1997; Herd *et al.* 2009; authors' unpublished observations). However, critically, whether GABA causes a depolarisation or hyperpolarisation in P7 VB neurones to date has not been investigated. An additional consideration concerns the intracellular concentrations of Cl^- within different neuronal compartments (cell body, dendrites, axon terminals), which may not be uniform, reflecting differential expression of cation–chloride co-transporters, e.g. KCC2 and NKCC1 (Trigo *et al.* 2008). Clearly a specific study on young thalamocortical neuronal activity is now warranted to elucidate the physiological relevance of the changing neurosteroid tone during development.

Conclusion

We reveal an important developmental role for neurosteroids, underpinning the dramatic changes to synaptic inhibition that occur early in thalamocortical neurone development. This precisely timed neurosteroid loss appears to be temporally harmonised with the emergence of an increased contribution from extrasynaptic $GABA_A Rs$, allowing a transition from immature to mature inhibition. Interestingly, this attenuation of neurosteroid tone at P9–10 coincides precisely with the onset of coordinated, targeted movements and mobility in mice (Dehorter *et al.* 2011) and in many neurones with the dramatic change in the effects of GABA from depolarising to hyperpolarising (e.g. Tyzio *et al.* 2007). Although the neurosteroid influence dissipates by P10, we demonstrate that upon provision of substrate, these thalamic neurones retain the ability to synthesise $GABA_A R$-active neurosteroids. A future challenge will be elucidating whether under certain physiological or pathophysiological circumstances local neurosteroids play a role in thalamocortical inhibition of mature circuits and, if so, determining the molecular mechanism(s) that triggers the synthesis of these potent neuromodulators.

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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

All experiments were performed and analysed by A.R.B. and M.B.H. A.R.B., D.B., M.B.H. and J.J.L. designed the experiments. All authors contributed to the interpretation of data, the drafting of the article or revising it critically for important intellectual content. We hereby confirm that all authors approved the final version of the manuscript and that all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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