# RESEARCH PAPER

# Cyclodextrins sequester neuroactive steroids and differentiate mechanisms that rate limit steroid actions

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**Background and purpose:** Neuroactive steroids are potent modulators of $GABA_A$ receptors and are thus of interest for their sedative, anxiolytic, anticonvulsant and anaesthetic properties. Cyclodextrins may be useful tools to manipulate neuroactive effects of steroids on $GABA_A$ receptors because cyclodextrins form inclusion complexes with at least some steroids that are active at the $GABA_A$ receptor, such as $(3\alpha,5\alpha)$-3-hydroxypregnan-20-one ($3\alpha5\alpha P$, allopregnanolone).
**Experimental approach:** To assess the versatility of cyclodextrins as steroid modulators, we investigated interactions between $\gamma$-cyclodextrin and neuroactive steroids of different structural classes.
**Key results:** Both a bioassay based on electrophysiological assessment of $GABA_A$ receptor function and optical measurements of cellular accumulation of a fluorescent steroid analogue suggest that $\gamma$-cyclodextrin sequesters steroids rather than directly influencing $GABA_A$ receptor function. Neither a 5$\beta$-reduced A/B ring fusion nor a sulphate group at carbon 3 affected the presumed inclusion complex formation between steroid and $\gamma$-cyclodextrin. Apparent dissociation constants for interactions between natural steroids and $\gamma$-cyclodexrin ranged from 10-60 $\mu$M. Although $\gamma$-cyclodextrin accommodates a range of natural and synthetic steroids, $C_{11}$ substitutions reduced inclusion complex formation. Using $\gamma$-cyclodextrin to remove steroid not directly bound to $GABA_A$ receptors, we found that cellular retention of receptor-unbound steroid rate limits potentiation by $3\alpha$- hydroxysteroids but not inhibition by sulphated steroids.
**Conclusions and implications:** We conclude that $\gamma$-cyclodextrins can be useful, albeit non-specific, tools for terminating the actions of multiple classes of naturally occurring neuroactive steroids.
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## Introduction

Neurosteroids are receiving increasing interest as potent endogenous modulators of $\gamma$-amino-*n*-butyric acid type A ($GABA_A$) receptors (Belelli and Lambert, 2005). Agents that antagonize or inhibit the effects of neurosteroids are not generally available as experimental tools. Such tools could be powerful adjuncts to the currently available inhibitors of neurosteroid function, which are largely limited to drugs that inhibit neurosteroidogenesis or neurosteroid metabolism (Concas *et al.*, 1998; Reddy *et al.*, 2001; Belelli and

Herd, 2003; Puia *et al.*, 2003). We previously demonstrated a steroid analogue, $(3\alpha,5\alpha)$-17-phenylandrost-16-en-3-ol, that inhibits the enhancing effects of neurosteroids, but the potency and water solubility of this compound are weak, and effects are relatively selective for certain structural classes of neuroactive steroids (Mennerick *et al.*, 2004). Cyclodextrins are also potential tools. We recently showed that cyclodextrins, cyclic oligosaccharides with a hydrophilic exterior and a lipophilic central cavity can be useful for manipulating the effects of the 5$\alpha$-reduced steroid, $(3\alpha,5\alpha)$-3-hydroxypregnan-20-one ($3\alpha5\alpha P$), at $GABA_A$ receptors. The effects of cyclodextrins are presumably by formation of inclusion complexes with $3\alpha5\alpha P$ (Shu *et al.*, 2004), although evidence for this conclusion is not definitive, especially given recent findings indicating that cyclodextrins may have direct effects on

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GABA$_A$ receptors (Pytel *et al.*, 2006). Although cyclodextrin inclusion complexes can be used to deliver steroids *in vivo* (Vivian *et al.*, 1997; Reddy and Rogawski, 2000; Ford *et al.*, 2005), on the time scale of brief applications to individual cells, cyclodextrins at relatively low concentrations can potentially be used to complex steroids and terminate their actions (Shu *et al.*, 2004; Akk *et al.*, 2005).

Cyclodextrins are available as $\alpha$, $\beta$ and $\gamma$ forms containing 6, 7 and 8 cyclic sugars, respectively (Wallimann *et al.*, 1997). We showed that $\alpha$-cyclodextrin does not effectively complex 3$\alpha$5$\alpha$P (Shu *et al.*, 2004). Furthermore, $\beta$-cyclodextrins may have more nonspecific effects than $\gamma$-cyclodextrins (Ohtani *et al.*, 1989). Therefore, we have focused on $\gamma$-cyclodextrin, the cyclodextrin with the largest hydrophobic cavity, to accommodate steroids of various structures.

Neurosteroids of differing structural classes have diverse effects on GABA$_A$ receptors. A hydroxyl group in the $\alpha$ configuration at C$_3$ and a hydrogen bond acceptor at C$_{17}$, on the $\beta$ face of the steroid have been shown to be important for the enhancing effects of steroids at GABA$_A$ receptors (Majewska *et al.*, 1986; Callachan *et al.*, 1987). Potentiation also tolerates a *trans* (5$\alpha$-reduced) or *cis* (5$\beta$-reduced) A/B ring fusion, despite a large difference in the three-dimensional structure of 5$\alpha$-reduced vs 5$\beta$-reduced steroids (Figure 1a1 and a2). It is not clear whether equally effective inclusion complexes can be formed between cyclodextrins and both of these structural classes of potentiating steroids. The finding that inclusion complexes form between bile steroids that are 5$\beta$-reduced, but which contain a carboxylic acid in the side chain present on the steroid, suggest that cyclodextrin/5$\beta$-reduced steroid complexes are possible (Redenti *et al.*, 2001).

Another class of neuroactive steroids inhibits GABA$_A$ receptors in a use-dependent manner (Eisenman *et al.*, 2003; Reddy, 2003). The steroids most potent at inhibiting these receptors have a sulphate group at C$_3$. This inhibitory effect appears to be tolerant of many structural modifications, including *trans* (5$\alpha$-reduced) and *cis* (5$\beta$-reduced) A/B ring fusions (Park-Chung *et al.*, 1999; Wang *et al.*, 2002). In fact, pregnenolone sulphate (PS), which contains a double bond at the A/B ring fusion (Figure 1b2), is also a potent inhibitory steroid. The blocking actions are also tolerant of positioning of the C$_3$ sulphate group in either the $\alpha$ or $\beta$ configuration. Again, whether cyclodextrins complex with sulphated steroidal GABA$_A$ receptor antagonists is unclear, although there is precedent for using $\beta$-cyclodextrins to promote PS solubility (Wang *et al.*, 1997).

We sought to test the general ability of cyclodextrins to inhibit neuroactive steroids of different structures. Our results suggest that $\gamma$-cyclodextrins are quite tolerant of a variety of structural modifications in potentiating and inhibiting steroids. However, when substitutions are made at C$_{11}$ to increase the molecular size of a potentiating steroid, potentiation was retained, but cyclodextrin effects were lost. Our data suggest that cyclodextrins can, in principle, be used to reverse the effects of any of the commonly studied endogenous neurosteroids, including the broad classes of potentiating and inhibiting neurosteroids. Using $\gamma$-cyclodextrin, we showed that different factors control the offset time
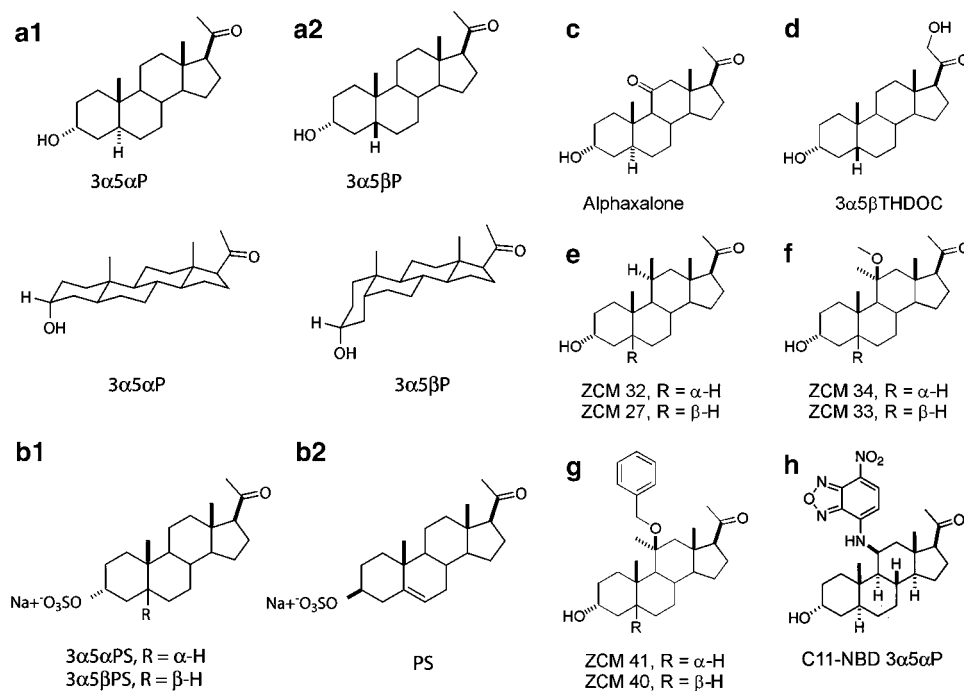


**Figure 1** Structures of types of GABA potentiating and GABA antagonistic neuroactive steroids used in this study. (**a**) 3$\alpha$5$\alpha$P and 3$\alpha$5$\beta$P (allopregnanolone and pregnanolone) are GABA$_A$ receptor potentiators despite their structural differences. (**b**) Sulphated pregnane and pregnene steroids are effective non-competitive GABA$_A$ receptor antagonists. (**c–h**) Other structural modifications tolerated by potentiating steroids. (**c**) Alphaxalone is a synthetic anaesthetic steroid (Phillipps, 1975; Barker *et al.*, 1987). (**d**) 3$\alpha$5$\beta$THDOC is a naturally occurring steroid (Majewska *et al.*, 1986; Mellon *et al.*, 2001). (**e–h**) Synthetic neuroactive steroids with substitutions at C$_{11}$ are shown as ZCM 32, ZCM 34 and ZCM 41 (5$\alpha$-reduced series) and ZCM 27, ZCM 33 and ZCM 40 (5$\beta$-reduced series). C11-NBD 3$\alpha$5$\alpha$P has a fluorescent substituent at carbon 11.

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of 3α-hydroxysteroid-mediated potentiation compared to sulphated steroid-mediated inhibition of $GABA_A$ receptors.

Part of this work has been published previously in abstract form (Shu *et al*., 2005).

## Methods

*Test systems used*

Stage V–VI oocytes were harvested from sexually mature female *Xenopus laevis* (Xenopus One, Northland, MI, USA) under 0.1% 3-aminobenzoic acid ethyl ester anaesthesia, according to protocols approved by the Washington University Animal Studies Committee. Oocytes were defolliculated by shaking for 20 min at 37°C in collagenase (2 mg ml$^{-1}$) dissolved in calcium-free solution containing (in mM): NaCl 96, KCl 2, $MgCl_2$ 1 and HEPES 5 at pH 7.4. Capped mRNA, encoding rat $GABA_A$ receptor α1, β2 and γ2L subunits was transcribed *in vitro* using the mMESSAGE mMachine Kit (Ambion, Austin, TX, USA) from linearized pBluescript vectors containing receptor coding regions. Subunit transcripts were injected in equal parts (20–40 ng total RNA) 8–24 h following defolliculation. Oocytes were incubated up to 5 days at 18°C in ND96 medium containing (in mM): NaCl 96, KCl 1, $MgCl_2$ 1, $CaCl_2$ 2 and HEPES 10 at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 μl ml$^{-1}$), streptomycin (100 μg ml$^{-1}$) and gentamycin (50 μg ml$^{-1}$).

Hippocampal cultures were prepared from postnatal day 0–3 rat pups as previously described (Mennerick *et al*., 1995). Hippocampi from isoflurane-anaesthetized rats were removed and sliced 500 μm-thick. The hippocampal slices were digested with 1 mg ml$^{-1}$ papain in oxygenated Leibovitz L-15 medium, followed by mechanical trituration in modified Eagle's medium containing 5% horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 μM glutamine, 50 μl ml$^{-1}$ penicillin and 50 μg ml$^{-1}$ streptomycin. Cells were plated in modified Eagle's medium at a density of 75 cells mm$^{-2}$ onto 35 mm plastic culture dishes pre-coated with collagen microdroplets sprayed on a layer of 0.15% agarose. The anti-mitotic cytosine arabinoside (10 μM) was added on the third day after plating to halt glial proliferation. Electrophysiology was performed 6–14 days following plating.

*Measurements made*

Two-electrode voltage-clamp experiments were performed with a Warner OC725 amplifier 2–5 days following RNA injection. The extracellular recording solution was ND96 medium with no supplements. Intracellular recording pipettes were filled with 3 M KCl and had open tip resistances of ∼1 MΩ. Drugs were applied from a common tip via a gravity-driven multibarrel delivery system. Unless indicated otherwise, drugs were co-applied with no pre-application period. Cells were voltage-clamped at −70 mV for all experiments, and the peak current (for potentiated responses) or the current at the end of 20–30 s drug applications (for inhibition of responses) was measured for quantification of current amplitudes. Vertical arrows in the figures give an indication of measurements used for summary quantification.

Whole-cell, patch-clamp recordings were made from hippocampal neurons using pipettes filled with 140 mM caesium methanesulphonate (responses to exogenous GABA) or with 140 mM potassium chloride (autaptic responses). Other components of the pipette solution included (in mM): NaCl 4, EGTA 5, $CaCl_2$ 0.5, HEPES 10, pH 7.25. The bath solution for recording included (in mM) NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, D-2-amino-5-phosphonovalerate 0.05. For autaptic responses, solitary neurons devoid of other synaptic input were stimulated with a 1.5-ms depolarizing pulse to 0 mV to trigger an axonal spike and resultant recurrent transmitter release. Drug applications were made with a multibarrel pipette. $EC_{50}$ for GABA in both oocytes and hippocampal neurons is approximately 10 μM (Eisenman *et al*., 2003; Shu *et al*., 2004). Imaging of fluorescent steroid was performed using a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI, USA) mounted on a TE2000 Nikon microscope. Fluorescence was excited with a metal halide light source through a 480-nm excitation filter. Emission was collected at 535 nm. All experiments in oocytes and cultured hippocampal neurons were performed at 25°C.

To estimate solution exchange times during whole-cell recordings from hippocampal neurons, we examined the τ for perfusing a low chloride concentration (74.5 mM, 50% of normal concentration) during the steady-state response to 2 μM GABA. We found that the exchange τ was 254.7 ± 30.2 ms, well below the τ of drug offset for experiments depicted in Figure 8. Thus solution exchange time was not limiting in these experiments.

*Experimental design*

Design of individual experiments is described in the Results. Wherever possible, each cell served as its own control, and experimental conditions and drug applications were interleaved to negate any time-dependent changes in cell responsiveness.

*Data analysis and statistical procedures*

Data acquisition and analysis were performed with pCLAMP 9.0 software (Molecular Devices, Union City, CA, USA). Data plotting and curve fitting were performed with Sigma Plot 8.0 software (SPSS Science, Chicago, IL, USA). Fits to concentration response relationships were achieved using a least-squares minimization to the Hill equation:

$$\frac{ax^b}{c^b + x^b}$$

where *a* is the maximum potentiation, *b* is the Hill coefficient and *c* is the $EC_{50}$ concentration of steroid. Fits of time constants to current decays (Figure 8) were performed using a variable metric least-squares minimization algorithm (Clampfit 9.0). Data are presented in the text and figures as mean ± s.e. Statistical differences were determined using Student's two-tailed *t*-test.

Calculations of the affinity of steroid for the γ-cyclodextrin buffer were made with the equation ($K_{eq}$ = [steroid][cyclodextrin]/[complex]), where $K_{eq}$ is the dissociation constant.

The concentration of free steroid ([steroid]) was estimated from $EC_{50}$ values (or $IC_{50}$ values in the case of sulphated steroids) in the presence and absence of $\gamma$-cyclodextrin. A one-to-one binding stoichiometry was assumed for the calculations.

### Drugs, chemicals and other materials

Steroids without ZCM designations were from Sigma (St Louis, MO, USA). Steroids with ZCM designations and C11-NBD $3\alpha5\alpha$P were prepared by us using multistep synthetic procedures and had spectroscopic properties (infrared spectroscopy, and both proton and carbon nuclear magnetic resonance spectroscopy) consistent with the structures given. These compounds were determined to be pure by thin layer chromatography and elemental analysis. Full details of their synthesis will be reported elsewhere. Steroids were prepared as stock solutions in dimethyl-sulphoxide (DMSO). Final DMSO concentration was always below 0.13%, and solutions were matched for DMSO concentration.

## Results

### Steroid structures

Figure 1 shows the major structural classes of steroids used and evaluated in the present work. The most obvious structural differences between naturally occurring neuroactive steroids is the stereochemistry at the A/B ring fusion that produces $5\alpha$- and $5\beta$-reduced steroids (Figure 1a1 and a2). The different ring fusion stereochemistry produces two major diastereomer classes, both with potentiating activity at $GABA_A$ receptors.

Figure 1b shows two GABA antagonist steroids with the characteristic sulphate substitute at $C_3$. An example of a sulphated steroid with *trans* ($5\alpha$-reduced) A/B ring fusion is shown in Figure 1b1 and the commonly used PS, with an unsaturated A/B ring fusion is shown in Figure 1b2. GABA antagonism is also tolerant of a *cis* ($5\beta$-reduced) A/B ring fusion (Park-Chung *et al.*, 1999; Wang *et al.*, 2002). Several members of all these classes exhibit similar potency for $GABA_A$ receptor antagonism (Park-Chung *et al.*, 1999; Wang *et al.*, 2002).

Figure 1c–h shows other potentiating steroids with various structural modifications at $C_{11}$ and $C_{21}$. Figure 1c and d shows the anaesthetic alphaxalone and the naturally occurring steroid $(3\alpha,5\beta)$-3,21-dihydroxypregnan-20-one ($3\alpha5\beta$THDOC). Figure 1e and f depict our synthetic steroids containing modifications at $C_{11}$. Compared to $3\alpha5\alpha$P, ZCM 32 has an additional methyl group as an axial $\beta$ substitute at $C_{11}$. ZCM 34 has a methyl group as an equatorial $\alpha$ substitute and a methoxy group as an axial $\beta$ substitute at $C_{11}$. Finally, in the $5\alpha$-series, ZCM 41 has a methyl group as an equatorial $\alpha$ substitute and a benzyloxy group as an axial $\beta$ substitute at $C_{11}$ (Figure 1g). C11-NBD $3\alpha5\alpha$P has a fluorescent 7-nitro-2,1,3-benzoxadiazol-4-amino (NBD) group as a $\beta$ sustitute at $C_{11}$ (Figure 1h), and can serve as a marker of cellular accumulation (Akk *et al.*, 2005). ZCM 27, ZCM 33 and ZCM 40 are the designations assigned to the $5\beta$-reduced

diastereomers of ZCM 32, ZCM 34 and ZCM 41, respectively. Overall, Figure 1 demonstrates a broad structural diversity of steroid analogues with $GABA_A$ receptor activity. We set out to test the breadth of cyclodextrin effects against these various classes of neuroactive steroids.

Initially, we tested some potential confounding effects of cyclodextrins on $GABA_A$ receptors. Cyclodextrins have been shown to complex DMSO, a solvent used in our steroid-containing solutions (Aree and Chaichit, 2002). If DMSO has direct effects on $GABA_A$ receptors this could confound our results. Furthermore, it was recently suggested that $\beta$-cyclodextrins may directly potentiate $GABA_A$ receptor function (Pytel *et al.*, 2006). This effect would invalidate the use of $GABA_A$ receptor-based bioassays to assess cyclodextrin effects. To determine whether cyclodextrin, DMSO, or the combination of cyclodextrin plus DMSO affected $GABA_A$ receptor function in our primary assay, we examined currents generated in voltage-clamped *Xenopus* oocytes expressing $\alpha1\beta2\gamma2L$ receptor subunit combinations. We found that $\gamma$-cyclodextrin at the highest concentrations used in our studies had no effect on GABA-gated responses (Figure 2a). Furthermore, DMSO at 0.2%, higher than the highest working concentration we used in any experiment (0.13%), had no effect on GABA responses. $\gamma$-Cyclodextrin alone (0.5 mM) had no effect on GABA-gated currents in oocytes ($0.6\pm6\%$ change, $n=6$) or in cultured hippocampal neurons ($1\pm4\%$ change in steady-state current generated by $0.5\,\mu$M GABA, $n=11$). Furthermore, in oocytes the combination of DMSO and $\gamma$-cyclodextrin had no significant effect on GABA responses (Figure 2a).

Steroids, particularly at high concentrations, can be difficult to wash out (Figure 2b1). If a primary effect of cyclodextrin is to sequester steroids, then we expect that cyclodextrin may speed washout of steroid effects. In this case, cyclodextrin is applied in the absence of GABA or DMSO solvent, so direct effects on $GABA_A$ receptor function or on solvent actions are circumvented. We tested cyclodextrin sequestration of steroid using oocytes expressing $GABA_A$ receptor subunits. We assessed baseline responses to $2\,\mu$M GABA, then responses to $2\,\mu$M GABA, co-applied with $10\,\mu$M $3\alpha5\alpha$P. As expected, $3\alpha5\alpha$P dramatically enhanced the GABA response. We then washed the ooycte with either a 60-s wash of saline alone or with 0.5 mM $\gamma$-cyclodextrin for 30 s, followed by saline alone for an additional 30 s. GABA responses were then re-assessed in the absence of steroid and $\gamma$-cyclodextrin. The cyclodextrin wash clearly accelerated recovery of the original, non-potentiated GABA response amplitude (Figure 2b and c). These results are consistent with steroid-sequestering actions of cyclodextrin.

To provide another assay, completely independent of $GABA_A$ receptor function, we examined a fluorescently tagged version of $3\alpha5\alpha$P (Figure 1h) in imaging experiments. This steroid has a NBD group attached at carbon 11 of the steroid. We previously found that this compound potentiates GABA responses, but with reduced potency compared with the parent $3\alpha5\alpha$P (Akk *et al.*, 2005). Consistent with sequestration by $\gamma$-cyclodextrin (but in contrast to the lack of effect of cyclodextrin on $GABA_A$ receptor function), we found that $\gamma$-cyclodextrin (0.5 mM) significantly reduced the potentiation generated by $2\,\mu$M C11-NBD $3\alpha5\alpha$P (Figure 3a1).
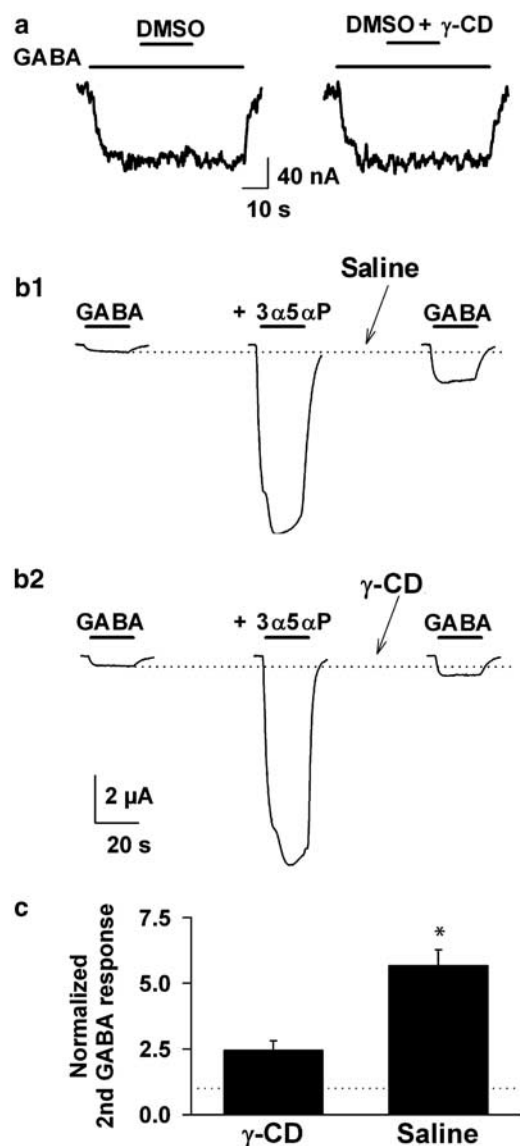
**Figure 2** The effects of $\gamma$-cyclodextrin are induced through steroid sequestration. (**a**) The formation of a complex between $\gamma$-cyclodextrin and DMSO cannot explain $\gamma$-cyclodextrin effects. Neither DMSO alone nor co-application with $\gamma$-cyclodextrin affected the GABA (2 $\mu$M) responses of *Xenopus* oocytes expressing rat $\alpha1\beta2\gamma2L$ subunit combinations. (**b**) Cyclodextrin wash in the absence of DMSO and GABA increased recovery rate for GABA responses. (**b1**) Response to 2 $\mu$M GABA, and the same concentration of GABA co-applied with 10 $\mu$M $3\alpha5\alpha$P are shown in the two left traces. The co-application trace was followed with a wash for 60 s in saline, then a re-challenge with GABA alone. Note that the response to GABA was still potentiated above the original GABA responses (dotted line). (**b2**) The same oocyte was re-challenged following the eventual return of the GABA response to baseline. In this case, the wash after drug application included 30 s of 0.5 mM $\gamma$-cyclodextrin followed by 30 s of plain saline. The response had been restored to much nearer the original GABA response. (**c**) Summary of results from six oocytes subjected to the protocol shown in (**b**). Wash with cyclodextrin significantly speeded recovery toward the original GABA response (**\*** indicates $P < 0.05$, paired *t*-test). The dotted line at a *y*-value of 1.0 indicates the original (first) GABA response, to which the other responses were normalized.

This inhibitory effect on potentiation was more marked against the parent natural steroid $3\alpha5\alpha$P (Figure 3a2 and b), suggesting that substitutions at $C_{11}$ may hinder inclusion

complex formation (see also Figure 7). Nevertheless, C11-NBD $3\alpha5\alpha$P was sufficiently sensitive to cyodextrin to allow us to perform imaging experiments on the fluorescent steroid in the absence and presence of $\gamma$-cyclodextrin.

We reasoned that if $\gamma$-cyclodextrin works by inclusion complex formation, then it should reduce cellular fluorescence when co-applied with C11-NBD $3\alpha5\alpha$P. We used cultured hippocampal neurons for these studies because of their amenability to imaging and to rapid solution exchange. Figure 3c shows that C11-NBD $3\alpha5\alpha$P accumulated in neurons and that simultaneously applied $\gamma$-cyclodextrin reduced this accumulation. Because $\gamma$-cyclodextrin is membrane impermeant but the neuronal fluorescence is largely intracellular, these data suggest that the intracellularly accumulated steroid is in relatively rapid equilibrium with the plasma membrane, which in turn is directly accessible to $\gamma$-cyclodextrin. Taken together, the results of Figures 2 and 3 confirm the validity of our GABA$_A$ receptor-based bioassay for cyclodextrin-steroid complex formation. The results suggest that interactions between steroid and $\gamma$-cyclodextrin arise directly from the ability of cyclodextrin to complex and sequester steroid. It is unlikely that the effect of $\gamma$-cyclodextrin is mediated by a direct action on the receptor or due to unanticipated interactions with DMSO.

*5$\beta$-Reduced neuroactive steroids and cyclodextrins*
Figures 2 and 3 suggest that $\gamma$-cyclodextrin effectively complexes $3\alpha5\alpha$P, consistent with our previous results (Shu *et al.*, 2004). To test whether the structural change induced by a $5\beta$ reduction at $C_5$ of the steroid affects the ability of cyclodextrins to complex neurosteroids, we performed experiments with $3\alpha5\beta$P and $\gamma$-cyclodextrin in *Xenopus* oocytes for comparison with our previous results. We challenged oocytes expressing the $\alpha1\beta2\gamma2L$ subunit combination with GABA (2 $\mu$M) solutions containing potentiating steroid alone (0.3–30 $\mu$M) or potentiating steroid plus 0.5 mM $\gamma$-cyclodextrin, pre-mixed in the perfusion solution. Figure 4a shows a response in which 0.3 $\mu$M $3\alpha5\beta$P increased the GABA response 3.9-fold, and 0.5 mM $\gamma$-cyclodextrin fully reversed the potentiation (to 94% of the original GABA response). By contrast, in the example of Figure 4b, 30 $\mu$M $3\alpha5\beta$P increased the GABA response 8.5-fold, which fell only slightly to 7.1-fold in the presence of $\gamma$-cyclodextrin. In some oocytes, particularly at low steroid concentration, we observed two phases (fast and slow) of potentiation elicited by co-application of steroid and GABA. We noticed no differential effect of cyclodextrin on these two phases of potentiation.

To quantify the apparent binding affinity between $3\alpha5\beta$P and $\gamma$-cyclodextrin, we created concentration–response curves for $3\alpha5\beta$P in the absence and presence of 0.5 mM $\gamma$-cyclodextrin (Figure 4c). The presence of cyclodextrin shifted the $3\alpha5\beta$P EC$_{50}$ from $0.5 \pm 0.4 \mu$M (absence of $\gamma$-cyclodextrin) to $9.5 \pm 0.7 \mu$M (presence of 0.5 mM $\gamma$-cyclodextrin, $P < 0.01$, paired *t*-test, $n = 5$) with similar predicted maximum potentiation ($16.1 \pm 3.3$, baseline vs $13.6 \pm 3.4$, cyclodextrin) and no significant effect of cyclodextrin on the Hill slope ($P > 0.3$, paired *t*-test, $n = 5$). The EC$_{50}$ shift was slightly larger than the shift in $3\alpha5\alpha$P potentiation that we
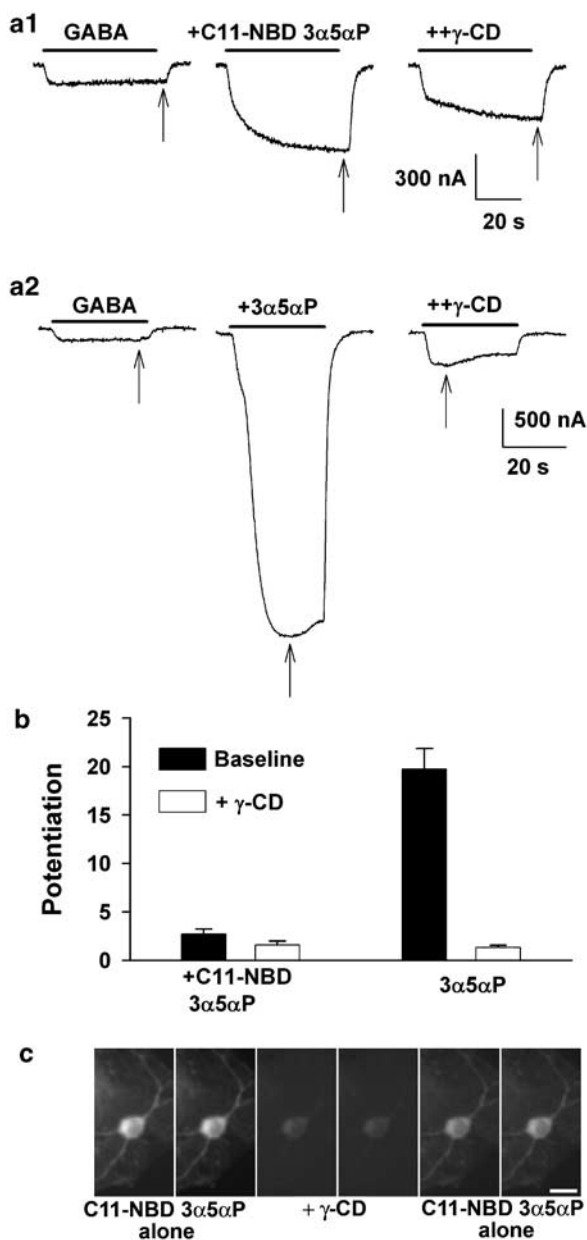
**Figure 4** Cyclodextrin binds 5$\beta$-reduced potentiating steroids. (**a**) The effect of $\gamma$-cyclodextrin co-applied with a low concentration of 3$\alpha$5$\beta$P (0.3 $\mu$M). (**b**) The effect of $\gamma$-cyclodextrin at a saturating concentration of 30 $\mu$M 3$\alpha$5$\beta$P. (**c**) The concentration–response curve of 3$\alpha$5$\beta$P was obtained in the presence and absence of 0.5 mM $\gamma$-cyclodextrin. The lines represent fits of the Hill equation to the averaged data points shown in the figure. Parameters from fits to the Hill equation were maximum response: 16.0-fold (baseline) vs 14.7 fold (cyclodextrin), EC$_{50}$: 0.5 $\mu$M (baseline) vs 10.1 $\mu$M (cyclodextrin), Hill co-efficient: 2.3 (baseline) vs 1.4 (cyclodextrin); $n = 5$ oocytes. In statistics performed on individual oocytes (see Results), the difference in Hill coefficient was not statistically significant.

obtained previously, where the EC$_{50}$ shifted from 1.1 to 9.2 $\mu$M (Shu *et al.*, 2004). The shifts in EC$_{50}$ for these two steroids, assuming a one-to-one binding stoichiometry (see Methods), predict affinities for cyclodextrin of 27.3 $\mu$M (3$\alpha$5$\beta$P) and 66.8 $\mu$M (3$\alpha$5$\alpha$P). We conclude that inclusion complexes readily form between 5$\beta$-reduced steroids and $\gamma$-cyclodextrin and that $\gamma$-cyclodextrin is a similar blocker of the effects of both 5$\alpha$- and 5$\beta$-reduced steroids (Shu *et al.*, 2004).

*Functional reversal of steroid effects by $\gamma$-cyclodextrin*

If cyclodextrins are to be useful for acutely reducing steroid effects *in situ*, then their effects on neuronal function are of interest. To determine whether $\gamma$-cyclodextrin sequesters steroid effects at neuronal synapses, we examined recurrent, autaptic GABA ipscs generated in microcultures of hippocampal neurons. $\gamma$-Cyclodextrin applied to naïve neurons had no effect on the peak amplitude of the ipsc (2$\pm$2% depression from a mean baseline amplitude of 7.1$\pm$2.0 nA, $n = 11$ GABAergic neurons) and a modest effect on the decay



**Figure 3** Direct imaging of a fluorescent steroid analogue suggests sequestration by $\gamma$-cyclodextrin explains reduced GABA responses. (**a**) Comparison of the effect of $\gamma$-cyclodextrin on equimolar concentrations of C11-NBD 3$\alpha$5$\alpha$P and 3$\alpha$5$\alpha$P. (**a1**) An oocyte responded to GABA (2 $\mu$M) and co-application of GABA and 2 $\mu$M C11-NBD 3$\alpha$5$\alpha$P. $\gamma$-Cyclodextrin (0.5 mM) partially reversed the effect of the tagged steroid. (**a2**) By contrast, 3$\alpha$5$\alpha$P (2 $\mu$M) more dramatically potentiated the GABA response, but $\gamma$-cyclodextrin more fully reversed the potentiation. (**b**) Summary of the protocol in (**a**) performed on four oocytes. Potentiation refers to the fold increase in GABA response, with 1.0 representing a doubling. The inhibition of 3$\alpha$5$\alpha$P potentiation was significantly larger than inhibition of C11-NBD 3$\alpha$5$\alpha$P potentiation ($P < 0.05$). (**c**) Imaging of C11-NBD 3$\alpha$5$\alpha$P applied to neurons in culture in the absence (two left panels, two right panels) and presence (two middle panels) of 0.5 mM $\gamma$-cyclodextrin. The reduction in fluorescence of the cells during co-application of steroid and cyclodextrin was consistent with sequestration of steroid by cyclodextrin. Images were taken after a 5 min pre-equilibration with steroid (left two panels), 15 s after addition of $\gamma$-cyclodextrin to the steroid-containing solution ($\gamma$-CD; middle panels) and 15 s after re-applying steroid alone. Interval between successive panels in the same experimental condition was 5 s.
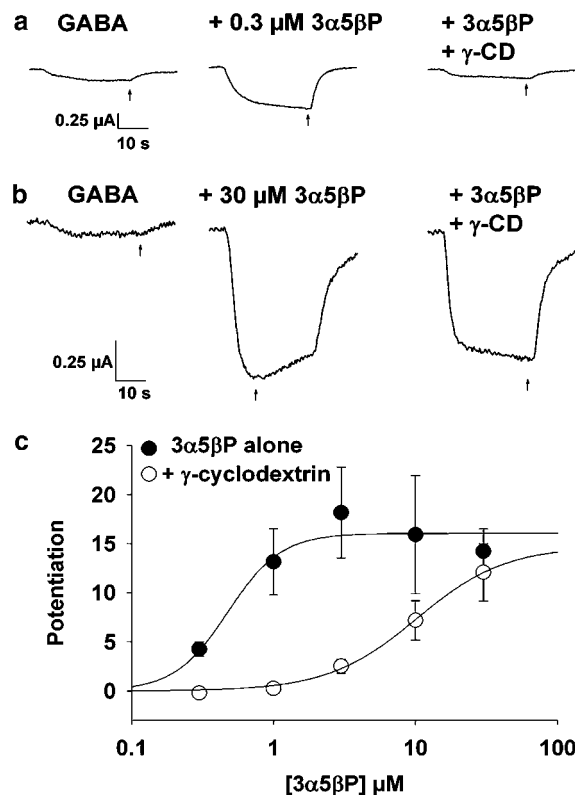
time course of ipscs ($15\pm5\%$ decrease in the 10–90% decay time: $336\pm63$ to $271\pm46$ ms, $P=0.04$, paired *t*-test, $n=11$ cells). This effect was clear and reversible in some cells (Figure 5a) but not apparent in others, and might result either from an endogenous steroid-like tone or another nonspecific effect on pre-synaptic or post-synaptic function. We found no effect of 0.5 mM $\gamma$-cyclodextrin on amplitude or decay of 2 glutamatergic neurons tested (data not shown).

We reasoned that if an endogenous steroid is responsible for the very small effect of $\gamma$-cyclodextrin on ipsc decays, we might be able to augment the effect of cyclodextrin by blocking 3$\alpha$-hydroxysteroidoxidoreductase enzymes responsible for metabolizing neurosteroids (Belelli and Herd, 2003). However, in three neurons incubated for >15 min in 1 $\mu$M of medroxyprogesterone, a potent 3$\alpha$-hydroxysteroidoxido-reductase inhibitor (Belelli and Herd, 2003), we failed to find any effect of $\gamma$-cyclodextrin on ipsc decays. These results, although somewhat inconclusive, are consistent with our previous findings that levels of endogenous neurosteroids are too low in dissociated cell culture to measure reliably by bioassay (Xu *et al*., 2000).

In contrast to modest and variable basal effects of $\gamma$-cyclodextrin on synaptic function, effects of exogenous 3$\alpha$5$\alpha$P were dramatically reduced when it was co-applied with $\gamma$-cyclodextrin (Figure 5b). In four isolated GABAergic neurons from which we elicited recurrent ipscs, 100 nM 3$\alpha$5$\alpha$P potentiated 10–90% decay time by $98\pm33\%$ ($504\pm143$ ms 10–90% decay time at baseline to $905\pm182$ ms in the presence of 100 nM 3$\alpha$5$\alpha$P). Co-application of 0.5 mM $\gamma$-cyclodextrin reversed the 3$\alpha$5$\alpha$P-mediated slowing of the ipsc decays ($450\pm109$ ms; Figure 5b). These

results demonstrate that at least acutely, $\gamma$-cyclodextrin can be used to manipulate steroid effects relatively selectively without grossly affecting basal pre-synaptic and post-synaptic function.

### $\gamma$-Cyclodextrin effects on PS

We tested the ability of sulphated neuroactive steroids to form complexes with $\gamma$-cyclodextrin to determine whether a sulphate group at $C_3$ affects this. Our goal in these experiments was to compare sulphated steroids directly with the non-sulphated potentiating steroids, using inhibition and potentiation, respectively, as bioassays for the efficiency of complex formation. Sulphated steroids typically exhibit significantly less potency for their inhibiting actions than 3$\alpha$-hydroxysteroids exhibit for potentiation, thus making direct comparisons difficult. However, because the $IC_{50}$ for sulphated steroid inhibition is dependent on the GABA concentration (Eisenman *et al*., 2003), we were able to titrate GABA concentration to yield an $IC_{50}$ for sulphated steroid inhibition similar to the $EC_{50}$'s found for potentiating steroids ($\sim$0.5–1 $\mu$M; note that the $EC_{50}$ for steroid potentiation in oocytes is higher than in native cells (Shu *et al*., 2004) for reasons that are unclear). Pilot experiments showed a GABA concentration of 30 $\mu$M to be appropriate, yielding an $IC_{50}$ concentration for inhibition of PS of 0.8 $\mu$M (Figure 6c and Eisenman *et al*., 2003). Figure 6a shows a typical example in which 1 $\mu$M PS depressed the GABA response in the absence of cyclodextrin. In this example, the degree of depression of sustained GABA response was larger (83%) than the mean depression (Figure 6c), yet co-application of PS and $\gamma$-cyclodextrin reversed the inhibition to 90% of the original sustained GABA response. By contrast, 100 $\mu$M PS abolished the sustained GABA response (99% reduction), and the reversal of PS effects by 0.5 mM $\gamma$-cyclodextrin was weak (83% depressed relative to the sustained GABA response; Figure 6b). As with potentiating steroids, the concentration–response curve of PS was shifted to the right by $\gamma$-cyclodextrin (Figure 6c). When data from individual cells were fitted with the Hill equation, the $IC_{50}$ for PS shifted from $0.9\pm0.2$ $\mu$M in the absence of $\gamma$-cyclodextrin to $27.4\pm6.9$ $\mu$M when co-applied with $\gamma$-cyclodextrin ($P<0.01$, Figure 6c). This shift in $IC_{50}$ predicts a dissociation constant for the PS/cyclodextrin interaction of 16.1 $\mu$M, similar to the range of affinities estimated for potentiating steroids (see above).

### Cyclodextrin effects on other commonly used neuroactive steroids

Figure 7a summarizes the effect of 0.5 $\mu$M of various commonly used neuroactive steroids applied alone or with 0.5 mM $\gamma$-cyclodextrin. It is clear that several chemical substitutions are unimportant for the ability of inclusion complexes to form between $\gamma$-cyclodextrin and steroid. Notably, substitutions at $C_{11}$ in alphaxalone or $C_{21}$ in 3$\alpha$5$\beta$THDOC (see Figure 1) were unimportant for the ability of cyclodextrin to sequester steroid (Figure 7a). For steroid-mediated inhibition of GABA currents, substitution of a pregnane backbone for the pregnene backbone of PS (Figure 1b) did not appreciably affect the ability of
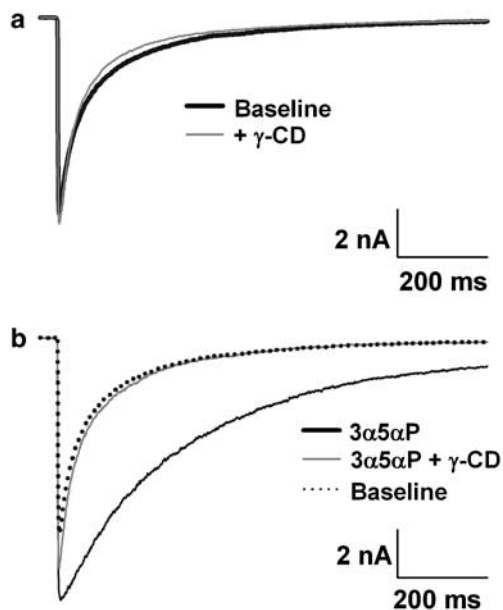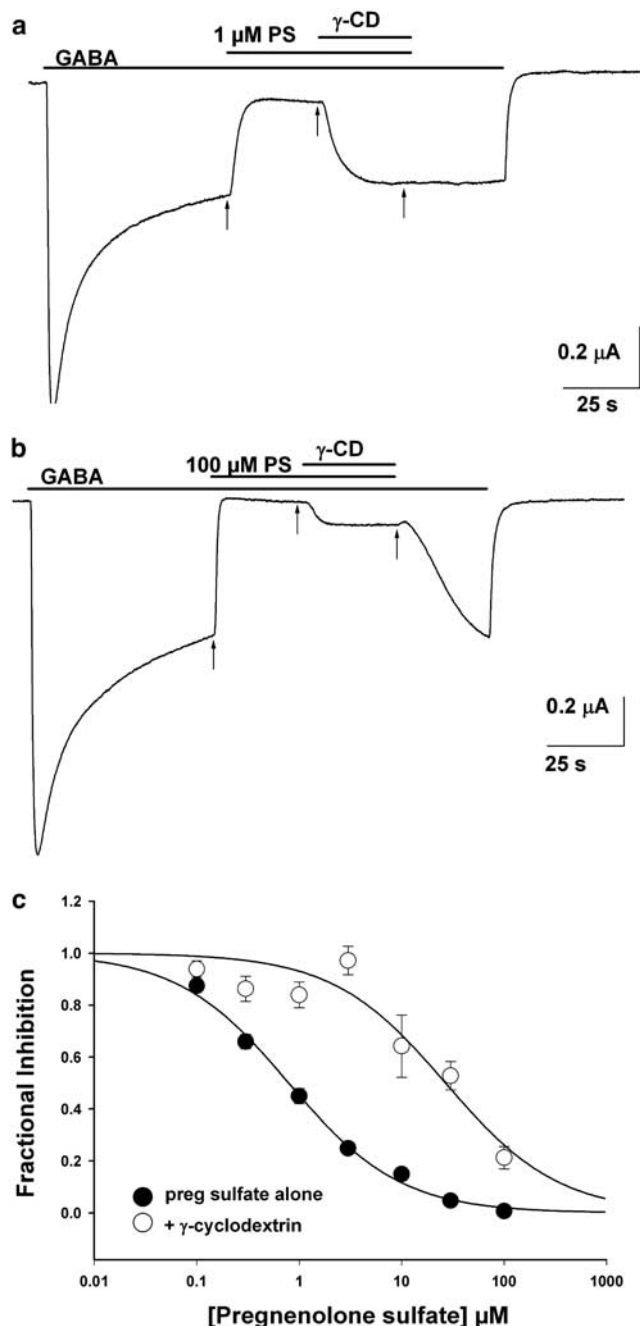


**Figure 5** Functional use of $\gamma$-cyclodextrin to reverse exogenous steroid effects in neurons. (**a**) The ipscs generated from autaptic hippocampal neurons cultured in synaptic isolation. $\gamma$-Cyclodextrin (0.5 mM) has little effect on ipscs under basal conditions. (**b**) In the same cell as in (**a**), the ipsc is prolonged by 100 nM 3$\alpha$5$\alpha$P in the absence, but not in the presence of 0.5 mM $\gamma$-cyclodextrin. The dotted trace is a re-plot of the baseline ipsc from (**a**).

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**Figure 6** PS and cyclodextrins. (**a**) Effect of $\gamma$-cyclodextrin on a low (1 $\mu$M) concentration of PS during the steady-state response of an oocyte to 30 $\mu$M GABA. Co-application with 0.5 mM $\gamma$-cyclodextrin ($\gamma$-CD) completely reversed PS inhibition. (**b**) The effect of $\gamma$-cyclodextrin at a saturating concentration of 100 $\mu$M PS. Arrows in (**a**) and (**b**) indicate the time points from which measurements were taken. (**c**) The concentration–response curve of PS was shifted to the right in the presence of $\gamma$-cyclodextrin compared with the curve in the absence of 0.5 mM $\gamma$-cyclodextrin. The solid line indicates a fit of the Hill equation to the average data points shown in the Figure. IC$_{50}$ of PS in the presence of $\gamma$-cyclodextrin shifted from 0.8 $\mu$M (baseline) to 27 $\mu$M (in the presence of 0.5 mM $\gamma$-cyclodextrin) ($n = 15$).

$\gamma$-cyclodextrin to interact with sulphated steroids (Figure 7b). As with 3$\alpha$-hydroxysteroids, 5$\beta$ reduction of sulphated steroids (3$\alpha$5$\beta$PS), a 5$\beta$ A/B ring fusion, did not affect complex formation (Figure 7b). We conclude that commonly

used neuroactive steroids active at GABA$_A$ receptors are effectively sequestered by $\gamma$-cyclodextrin.

### Interaction of synthetic 5$\alpha$-reduced and 5$\beta$-reduced neuroactive steroids with $\gamma$-cyclodextrin

Based on previous structural information about steroid–cyclodextrin inclusion complexes (Adam *et al.*, 2002) and our own data suggesting that an NBD substitution at C$_{11}$ reduced inclusion complex formation (Figure 3a and b), we hypothesized that the ability of steroids to form inclusion complexes with cyclodextrin would be particularly sensitive to substituents located on the steroid C-ring. We tested several synthetic steroid analogues with substitutions at C$_{11}$ (Figure 1e–g; ZCM compound series). All of these compounds retained at least some potentiation at GABA$_A$ receptors, allowing us to assay presumed inclusion complex formation. In the 5$\alpha$-reduced series, we found that all but the largest substitutions at C$_{11}$ still permitted $\gamma$-cyclodextrin modulation (Figure 7c). For 5$\beta$-reduced steroids, compounds containing the same structural modification at C$_{11}$ (Figure 7d; ZCM 27, ZCM 33, ZCM 40) were also studied. We found that $\gamma$-cyclodextrin effects for both the 5$\alpha$- and 5$\beta$-reduced series of C$_{11}$-modified steroids were similar (Figure 7c vs d), confirming that 5$\beta$ reduction does not affect the ability of inclusion complexes to form, nor does 5$\beta$ reduction appear to interact with C$_{11}$ modifications to influence inclusion complex formation.

### Differences in rate-limiting factors for potentiating and inhibitory steroids

We have recently suggested that cellular retention of steroid unbound to GABA$_A$ receptors is responsible for long-lived currents directly gated by GABA-active steroids in the absence of GABA (Shu *et al.*, 2004). The results presented here (Figure 2b) suggest that removal of potentiation is also cyclodextrin-sensitive, implying that non-receptor-based cellular retention rate limits the potentiating effects of steroids. Does cellular retention also rate limit the effects of steroidal GABA$_A$ receptor antagonists? To determine directly and quantitatively whether cellular retention is responsible for rate-limiting steroid potentiation and inhibition of GABA responses by 3$\alpha$-hydroxysteroids and sulphated steroids, respectively, we examined whether $\gamma$-cyclodextrin could speed recovery from the effects of the two classes of steroids at GABA$_A$ receptors. We used hippocampal neurons for these studies to optimize solution exchange times.

Consistent with our previous results from studying interactions between 3$\alpha$-hydroxysteroids and GABA$_A$ receptors in the absence of GABA, we found that the recovery from the potentiating actions of 3$\alpha$-hydroxysteroids was sensitive to wash with $\gamma$-cyclodextrin (Figure 8a). Responses to GABA (0.5–2 $\mu$M) were potentiated by 300 nM 3$\alpha$5$\alpha$P by 9.2$\pm$2.3-fold. Upon removal of steroid, the relaxation from the potentiated response back towards the original GABA response was described by a time constant of 30.6$\pm$12.6 s ($n = 11$). This was decreased to 0.9$\pm$0.1 s in the same cells washed with GABA plus $\gamma$-cyclodextrin (Figure 8a, $P < 0.05$).
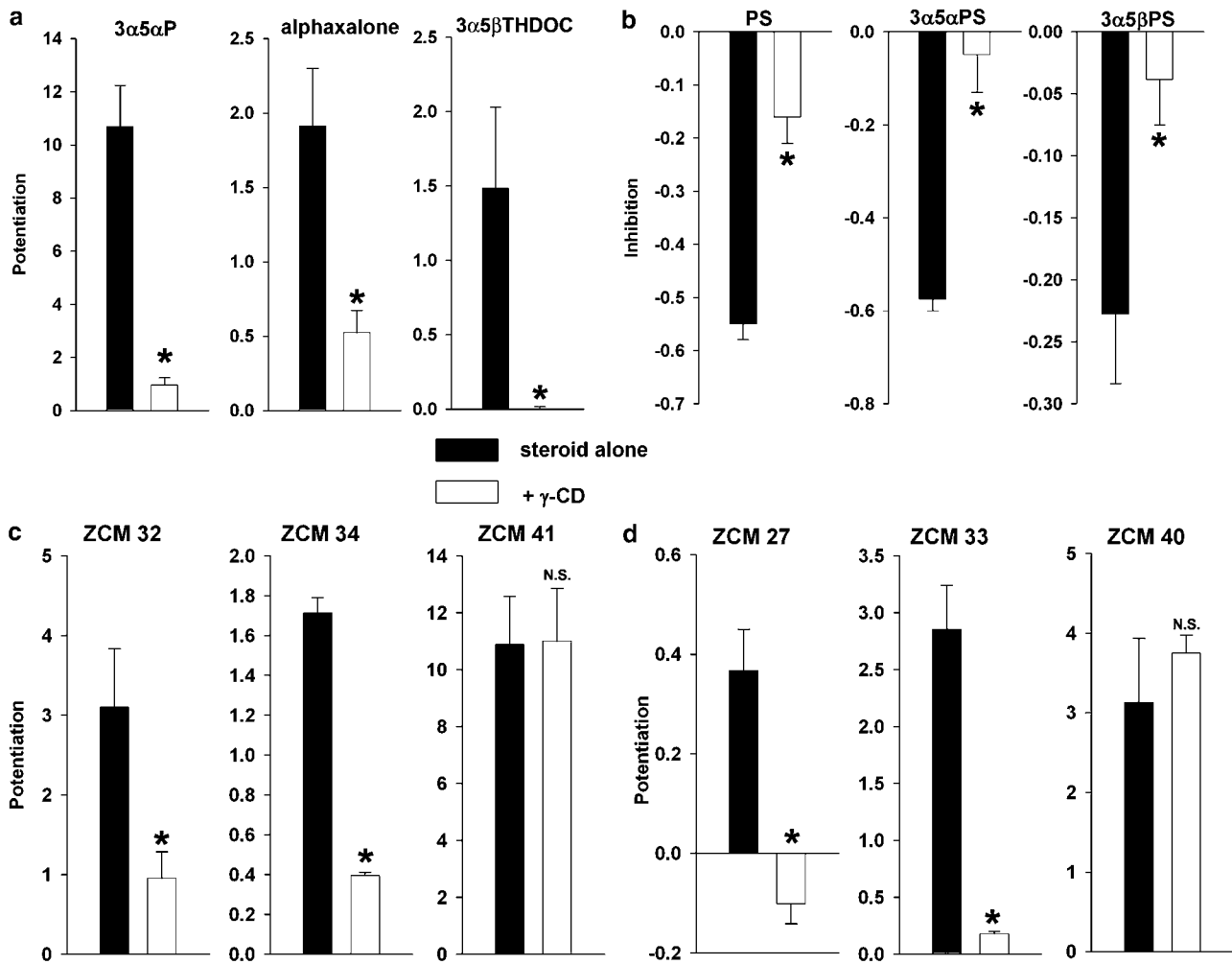
**Figure 7** Cyclodextrin effects on neuroactive steroid potentiation and inhibition. (**a**) Neuroactive steroid potentiators were used to modulate responses to $2\,\mu$M GABA, $3\alpha5\alpha$P at $0.5\,\mu$M, alphaxalone at $0.5\,\mu$M and $3\alpha5\beta$THDOC at $0.5\,\mu$M. Co-application with $0.5\,$mM $\gamma$-cyclodextrin ($\gamma$-CD) significantly blocked potentiation ($n = 4$–13 for each experiment, asterisks indicate $P < 0.05$ compared to steroid alone). (**b**) Neuroactive steroid inhibitors were used to antagonize responses to $30\,\mu$M GABA, PS at $1\,\mu$M, ($3\alpha,5\alpha$)-3-hydroxypregnan-20-one sulphate ($3\alpha5\alpha$PS) at $1\,\mu$M, and $1\,\mu$M ($3\alpha,5\beta$)-3-hydroxypregnan-20-one sulphate ($3\alpha5\beta$PS). Co-application with $0.5\,$mM $\gamma$-cyclodextrin significantly blocked inhibition. (**c** and **d**) The interaction of synthetic $5\alpha$- and $5\beta$-reduced neuroactive steroids and cyclodextrins. (**c**) Synthetic $5\alpha$-reduced neuroactive steroids with small to large substitutions at $C_{11}$ (ZCM 32 < ZCM 34 < ZCM 41) were applied at $0.25\,\mu$M with $2\,\mu$M GABA. Co-application with $0.5\,$mM $\gamma$-cyclodextrin blocked potentiation in steroids with the smaller substitution at $C_{11}$ but failed to block potentiation of ZCM 41, which contains a large aromatic substitution at $C_{11}$. (**d**) Synthetic $5\beta$-reduced neuroactive steroids in the same series (ZCM 27 < ZCM 33 < ZCM 40). Cyclodextrin blocked potentiation of the smaller steroids but not that of the larger steroids. The mean potentiation was apparently reduced below zero in the case of ZCM 27, but this appeared to be due to nonspecific small rundown of the GABA response in several cells, and the depression below zero was not statistically significant ($P > 0.05$). In (**b**, **c** and **d**) $n = 8$, asterisks indicate $P < 0.05$ compared with steroid alone, NS indicates not significant.

A similar protocol employing $2\,\mu$M GABA and $5\,\mu$M PS was used to study the removal of steroid inhibition. PS inhibited GABA responses by $75 \pm 3\%$. Recovery from inhibition was much faster than recovery from potentiation ($\tau = 3.7 \pm 0.6\,$s, $n = 5$), and this recovery was not affected by the presence of $\gamma$-cyclodextrin ($\tau = 4.6 \pm 0.6$, $P > 0.05$, Figure 8b). Taken together, these results suggest that similar factors (i.e. cellular retention of steroid) are responsible for rate limiting both major actions of $3\alpha$-hydroxysteroids: potentiation of GABA-gated currents and direct activation of receptors in the absence of GABA. A different factor, probably the dissociation rate of steroid from receptors, is responsible for rate limiting the effect of the sulphated inhibitory steroids.

## Discussion and conclusions

We have tested the ability of cyclodextrins functionally to sequester neuroactive steroids. We found that all commonly used neuroactive steroids that we tested exhibited approximately equivalent binding to $\gamma$-cyclodextrin, assayed using GABA currents in oocytes expressing recombinant GABA$_A$ receptor subunits. Neither the configuration of the A/B ring fusion nor addition of sulphate groups to pregnene or pregnane steroid backbones affected cyclodextrin binding. Large, hydrophobic substituents at $C_{11}$, however, affected the ability of $\gamma$-cyclodextrin to bind steroid. We conclude that $\gamma$-cyclodextrin is a useful, albeit nonspecific, tool for

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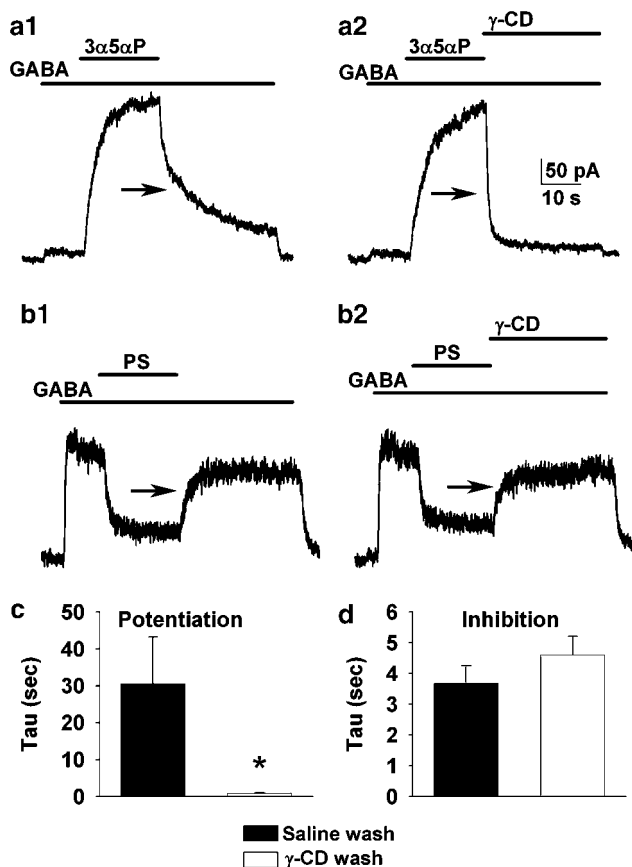H-J Shu *et al*
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**Figure 8** Cyclodextrins demonstrate different factors governing time course of potentiation vs inhibition by steroids. (**a1**) $3\alpha5\alpha P$ (0.3 $\mu$M) potentiated the response to 0.5 $\mu$M GABA. Removal of the steroid produced a slow relaxation (arrow) back towards the original GABA response when GABA alone was applied. (**a2**) When $\gamma$-cyclodextrin (0.5 mM) was included in the GABA solution upon steroid removal, the relaxation (arrow) was accelerated. (**b1** and **2**) When PS (5 $\mu$M) was used to inhibit the response to 2 $\mu$M GABA, $\gamma$-cyclodextrin (0.5 mM) had no effect on the recovery time course (arrows) of PS offset. (**c** and **d**) Summary of the effect of $\gamma$-cyclodextrin on the offset $\tau$ for potentiation (**c**, $n=11$) and inhibition (**d**, $n=5$) from experiments like those shown in (**a**) and (**b**), respectively. Asterisk indicates $P<0.05$. In (**d**), there was no significant difference between the offset $\tau$ for inhibition (paired *t*-test).

terminating the actions of multiple classes of neuroactive steroids.

In the course of these studies, we determined several novel characteristics of the interaction of steroids with GABA$_A$ receptors, and several features of $\gamma$-cyclodextrin-mediated sequestration of neuroactive steroids. Firstly, we directly showed that $\gamma$-cyclodextrin, acutely applied, functionally reversed effects of exogenous steroid at synapses, with little influence on presynaptic or postsynaptic function at baseline. Secondly, we showed that different factors govern the time course of the two major classes of neuroactive steroids at GABA$_A$ receptors. GABA$_A$ receptor potentiation (present work), like direct gating (Shu *et al.*, 2004; Akk *et al.*, 2005), is rate limited by accumulation in cyclodextrin-accessible reservoirs rather than by inherent drug dissociation rates from receptors, which are not influenced by $\gamma$-cyclodextrin. In contrast, the offset rate of GABA inhibition by sulphated

steroids is faster than potentiation offset, and is governed by a cyclodextrin-inaccessible mechanism, probably the drug–receptor dissociation rate. This conclusion is consistent with the negative charge on PS, which probably prevents this steroid from accumulating in the same reservoirs, including intracellular compartments, that $3\alpha5\alpha P$ accesses. This finding is also consistent with our previous suggestion that rates of block by sulphated steroids are influenced by receptor conformation (Eisenman *et al.*, 2003) rather than by cellular retention.

Nonspecific effects need to be carefully considered when working with cyclodextrins. Cyclodextrins have been used for other purposes, such as extracting cholesterol from membranes (Yancey *et al.*, 1996; Haynes *et al.*, 2000; Westover *et al.*, 2003), for sponging lipophilic fluorescent markers from plasma membranes (Kay *et al.*, 1999), for terminating the actions of peripherally active steroids (Adam *et al.*, 2002) and for complexing solvents like DMSO (Aree and Chaichit, 2002). We focused our analysis on $\gamma$-cyclodextrin, because it appears to be less effective than $\beta$-cyclodextrins at removing cholesterol from membranes (Ohtani *et al.*, 1989), thus reducing the possibility that effects on membrane cholesterol might contribute to our results.

$\beta$-Cyclodextrins have recently been suggested to have direct potentiating effects on GABA$_A$ receptors (Pytel *et al.*, 2006), which would confound our results. However, we have found that $\gamma$-cyclodextrin alone does not affect responses to exogenous GABA in either hippocampal neurons or oocytes (Shu *et al.*, 2004) and does not potentiate non-equilibrium synaptic GABA responses. Also, our results have eliminated an important potentiating effect of $\gamma$-cyclodextrin on synaptic, non-synaptic and recombinant $\alpha1\beta2\gamma2L$ receptors. It is possible that subunit combinations not identified in our hippocampal cultures or oocytes are potentiated by the $\beta$-cyclodextrin used by Pytel *et al.* (2006). It is also possible that patch excision in the fast application studies of Pytel *et al.* (2006) renders receptors sensitive to $\beta$-cyclodextrin. However, preliminary results suggest that up to 5 mM methyl-$\beta$ cyclodextrin has no effect on intracluster channel kinetics of $\alpha1\beta2\gamma2L$ receptors in cell attached patches (G Akk, personal communication). Although only structural methods such as nuclear magnetic resonance or crystallography can definitively determine inclusion complex formation, our results, including control experiments (data shown in Figures 2 and 3), strongly support our conclusion that the attenuation of the steroid effects results directly from cyclodextrin–steroid interactions rather than from direct effects of cyclodextrin on GABA$_A$ receptors or other targets.

Cyclodextrins are also used to dissolve and deliver steroids systemically (Wallimann *et al.*, 1997). At first glance, this use of cyclodextrins seems paradoxical in the light of our demonstration that cyclodextrins inhibit the actions of steroids. How can cyclodextrins be effective delivery agents if they inhibit the ability of the drug to act, as shown in the present work? It is important to consider the differences in paradigms when considering the different uses of cyclodextrins. Cyclodextrins are often used to deliver steroids by dissolving a high ratio of steroid to cyclodextrin. The low affinity of the steroid for cyclodextrin (15–60 $\mu$M estimated in the Results) thus favours delivery of high steroid concentra-

tions to tissues. *In vivo* cyclodextrin complexes may also be protected from enzymatic degradation, imparting some of their usefulness as drug delivery vehicles (Wallimann *et al.*, 1997). In our *in vitro* assay, excess free cyclodextrin will out-compete receptors and lipophilic reservoirs for steroid retention. It should be noted that delivery of free cyclo-dextrins *in vivo* has been used to speed recovery from the steroid neuromuscular blocking agent rocuronim (de Boer *et al.*, 2006; Sorgenfrei *et al.*, 2006).

Assayed by phase solubility, dissociation constants of steroids for cyclodextrins range considerably (Wallimann *et al.*, 1997). Our bioassay of neuroactive steroids showed that over a relatively wide range of structures, neuroactive steroids have a similar affinity for $\gamma$-cylcodextrin, but this affinity is reflected in estimated dissociation constants of tens of micromolar. Thus, sequestration of steroids by $\gamma$-cyclodextrin under the conditions of our experiments is a relatively low-affinity interaction, and this limitation needs to be considered in potential uses of cyclodextrins.

In summary, we have found that cyclodextrins are useful tools for reversing the effects of a wide range of neuroactive steroids, including both potentiators and inhibitors of GABA$_A$ receptor function. The utility of cyclodextrins is limited by their effects on other cellular processes, notably the cholesterol content of membranes. However, in the short term, we have directly demonstrated that cyclodextrins are useful for functionally sequestering steroids, as they have few effects during brief exposures on other cellular processes such as those governing acute signalling. Further, we have used cyclodextrin to elucidate differences in the mechanisms governing the time course of steroid potentiation vs inhibition of GABA actions.

## Acknowledgements

## Conflict of interest

The authors state no conflict of interest.

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