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γ-Aminobutyric Acid$_A$ Receptor Regulation: Chronic Treatment with Pregnanolone Uncouples Allosteric Interactions between Steroid and Benzodiazepine Recognition Sites

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SUMMARY

Pregnanolone [5β-pregnan-3α-ol-20-one (5β3α)] and allopregnanolone [5α-pregnan-3α-ol-20-one (5α3α)] are neuroactive steroids that are reduced metabolites of progesterone. Both 5β3α and 5α3α are potent positive modulators of the γ-aminobutyric acid response that enhance the binding of [^{3}H]flunitrazepam ([^{3}H]FNZ) to the γ-aminobutyric acid type A receptor. Chronic (48 hr) exposure of brain neurons in culture to 5β3α or 5α3α abolishes potentiation of [^{3}H]FNZ binding by these steroids. This uncoupling, or loss of allosteric interactions between steroid and benzodiazepine recognition sites, is dose dependent, stereospecific, and reversible. The number and affinity of [^{3}H]FNZ binding sites are unaffected. In contrast, the steroids 5β-pregnan-3β-ol-20-one, β-estradiol, testosterone, progesterone, deoxycorticosterone, and dexamethasone, which show little capacity to potentiate [^{3}H]FNZ binding, are also much less effective in inducing uncoupling of steroid and benzodiazepine recognition sites. These results suggest a mechanism whereby neurons could become refractory to long term modulation by neuroactive steroids. The results are discussed in terms of their possible relevance to premenstrual anxiety and enhanced frequency of seizures in certain women.

The GABA$_A$R is the target of a diverse group of anxiolytic, sedative, and anticonvulsant drugs of a variety of chemical classes, including benzodiazepines, barbiturates, and steroids, that enhance GABA$_A$R function by acting at specific allosteric modulatory sites on the GABA$_A$R. The modulator recognition sites of the GABA$_A$R are allosterically coupled both to the GABA recognition site and to one another, as evinced by the fact that the binding of [^{3}H]FNZ to the benzodiazepine recognition site is potentiated both by GABA$_A$R agonists and by positive modulators that act at the barbiturate or steroid recognition sites. The observation that prolonged administration of sedative drugs *in vivo* can evoke tolerance and dependence implies the existence of endogenous mechanisms regulating the interaction of such modulators with the GABA$_A$R.

To explore the regulation of the GABA$_A$R, we have examined the effects of chronic exposure of brain or spinal cord neurons in culture to GABA$_A$R agonists and modulators. Chronic exposure to the GABA$_A$R agonists muscimol and GABA results in a down-regulation of receptor number (1, 2), as well as a decrease in allosteric interactions among binding sites on the GABA$_A$R, which we have termed uncoupling (1). Uncoupling, but not down-regulation, is also observed after chronic exposure to benzodiazepine and barbiturate modulators (3). Interestingly, uncoupling can also be induced by chronic exposure to methylxanthines, even though methylxanthines are relatively inactive as modulators of the GABA$_A$R (3, 4).

We refer to uncoupling of the allosteric linkage between a pair of recognition sites as homologous uncoupling when it is induced by chronic treatment with a drug that binds at either of the two coupled sites and as heterologous uncoupling when it is induced by chronic treatment with a drug that acts at a different site. Thus, the decreased enhancement of [^{3}H]FNZ binding by pentobarbital that is observed after chronic exposure of neurons to pentobarbital (3) is regarded as homologous uncoupling, whereas the decreased enhancement of [^{3}H]FNZ binding by GABA after chronic exposure to barbiturates (3) or methylxanthines (4) is classified as heterologous uncoupling.

There is increasing evidence that steroids can exert direct effects on neuronal membranes by modulating neurotransmitter receptors of the CNS (5–11). Modulatory effects of steroids have been most extensively characterized at the GABA$_A$R (reviewed in Ref. 12). Progesterone and deoxycorticosterone, as well as some of their metabolites, have been shown to be positive modulators at the GABA$_A$R, in that they potentiate

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ABBREVIATIONS: GABA$_A$R, γ-aminobutyric acid type A receptor; FNZ, flunitrazepam; GABA, γ-aminobutyric acid; CNS, central nervous system; DMSO, dimethylsulfoxide; PBSS, phosphate-buffered salt solution; 5α3α, 5α-pregnan-3α-ol-20-one; 5β3α, 5β-pregnan-3α-ol-20-one.

the GABA response (13, 14), stimulate muscimol and benzodiazepine binding (15, 16), enhance $GABA_AR$-mediated chloride ion uptake (17, 18), and inhibit *t*-butylbicyclophosphorothionate binding (12, 19, 20). In particular, the reduced progesterone metabolites (21) allopregnanolone ($5\alpha3\alpha$) and pregnanolone ($5\beta3\alpha$) are exceptionally potent and efficacious positive modulators of the $GABA_AR$ (9, 16, 20).

The aim of the present study was to determine the effect of chronic exposure to steroids on the $GABA_AR$. In previous studies, we found reduced allosteric interactions between GABA and benzodiazepine recognition sites after chronic exposure of neurons to benzodiazepines (3), methylxanthines (4), or barbiturates (3), with no change in receptor number or affinity. Using a similar experimental paradigm to evaluate chronic steroid effects on chick brain neurons, we now report that chronic treatment with $5\beta3\alpha$ or $5\alpha3\alpha$ results in complete homologous uncoupling of allosteric interactions between steroid and benzodiazepine recognition sites of the $GABA_AR$.

Materials and Methods

[^{3}H]FNZ (85 Ci/mmol) was purchased from Amersham. All chemicals used were obtained from commercial sources. Steroids and flurazepam were purchased from Sigma Chemical Co. (St. Louis, MO). Dialysis tubing (15,000 molecular weight cutoff; Spectrapor) was prepared by thorough rinsing with deionized water.

Cell cultures. Whole-brain cell cultures derived from 7-day chick embryos were prepared as described previously (1, 22). Briefly, brains were rapidly removed and collected in a Ca^{2+}/Mg^{2+}-free saline solution (Puck's D_1G). Tissues were finely minced, incubated with trypsin (0.025%, 5 min, 25°), and centrifuged (900 rpm, 5 min). The resulting pellet was suspended in Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, 5% chick embryo extract (prepared from 12-day chick embryos), 2.4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and was then triturated with a fire-polished Pasteur pipet. The cells were plated onto collagen-coated Falcon 60-mm culture dishes at a density of 0.4 brains/dish (approximately 2.4×10^6 cells) and were maintained at 37° in 5% CO_2 with 100% humidity. Cytosine arabinoside (1 μM) was added to the cultures after 24 h to prevent excessive proliferation of non-neuronal cells. The next day, the feeding medium was replaced with fresh medium with the same contents as described for the plating medium except that the final concentration of chick embryo extract was reduced to 2.5% and glucose was added to a final concentration of 20 mM. After 6 days in culture, the cultures were supplemented with 1 ml/dish of fresh medium.

Chronic treatment. Cultures were treated for 48 hr beginning on day 6 by addition to each dish of a small volume (6–60 μl) of a concentrated steroid solution. Concentrated solutions (10 mM) of $5\alpha3\alpha$, $5\beta3\alpha$, and 5β-pregnan-3β-ol-20-one were prepared in 100% DMSO. Concentrated solutions (1 mM) of progesterone, testosterone, deoxycorticosterone, and dexamethasone were prepared in 10% DMSO by dilution with distilled water of a 10 mM steroid/100% DMSO stock solution. Except as otherwise noted, the final steroid concentration was 5 μM for progesterone and 10 μM for other steroids. The final concentration of DMSO was 0.1%. Control cultures were treated with vehicle only. These drug concentrations produced no evident toxicity, as judged by the absence of any change in either the overall appearance of cultures under phase-contrast microscopy or the number of cells stained by trypan blue.

Cell homogenate preparation. At the end of the 48-hr treatment period, the feeding medium was removed from the dishes and the cultures were rinsed twice with ice-cold PBSS (in mM: NaCl, 123; KCl, 5.4; NaH_2PO_4, 11; $MgSO_4$, 0.4; $CaCl_2$, 0.9; glucose, 22.2; pH 7.4). The cells were scraped from the dishes with a rubber policeman and centrifuged for 5 min at 1000 rpm, to collect the cells. The cells were then homogenized by hand (35 strokes) in 1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (0.2 ml/60-mm culture dish; final protein concentration, approximately 6 mg/ml), in a glass Dounce homogenizer. To remove endogenous GABA and treatment drugs, this suspension was dialyzed against 4 liters of 25 mM potassium phosphate buffer, pH 7.0, for 24 hr at 4°, with four changes of buffer. After dialysis, the homogenate was diluted with PBSS to a final concentration of approximately 2 mg/ml protein, rehomogenized (20 strokes), and used immediately. Protein concentrations were determined by the method of Lowry *et al.* (23).

Reversible binding of [^{3}H]FNZ. Enhancement of [^{3}H]FNZ binding by $5\beta3\alpha$ and other steroids was measured by addition of 100-μl aliquots of homogenate (approximately 200–250 μg of protein) to assay tubes containing [^{3}H]FNZ (final concentration, 1 nM) in a total volume of 0.5 ml of PBSS, for 60 min on ice, in the presence and absence of steroid. Saturation analysis was conducted in the presence of 0.5–30 nM [^{3}H]FNZ. The reaction was quenched by the addition of 5 ml of ice-cold PBSS, rapidly followed by vacuum filtration over Whatman GF/B glass fiber filters, which were then washed three times with a total of 15 ml of PBSS. Nonspecific binding (approximately 10–20% of total) was determined in the presence of 10 μM flurazepam and was subtracted from total binding to yield specific binding. For saturation binding experiments involving high concentrations of [^{3}H]FNZ, total binding and nonspecific binding were determined in the presence of 2.5 μM Ro5–4864 to block binding to low affinity, "peripheral" sites, which are abundant in embryonic chick brain (24, 25). All binding determinations were performed in quadruplicate. Radioactivity retained on the filters was determined by liquid scintillation counting in 5 ml of Liquiscint (National Diagnostics). Potentiation of [^{3}H]FNZ binding by steroids was calculated as follows:

$$\%\ \text{Potentiation} = \left(\frac{\text{specific binding in the presence of steroid}}{\text{specific binding in the absence of steroid}}\right) - 1 \times 100$$

In some cases, changes in enhancement of [^{3}H]FNZ binding after chronic treatment are expressed as percent uncoupling, defined as follows:

$$\%\ \text{Uncoupling} = \frac{\%\ \text{potentiation of control} - \%\ \text{potentiation of treated}}{\%\ \text{potentiation of control}} \times 100$$

Data analysis. Dose-effect data were analyzed by computer-aided nonlinear regression. Data were weighted according to the standard error of replicate samples. Results are presented as the mean ± standard error. Significance was determined using Student's paired or unpaired *t* test.

Results

As shown in Fig. 1, $5\beta3\alpha$ dose-dependently enhanced the binding of [^{3}H]FNZ to cell homogenates derived from embryonic chick brain, with an EC_{50} of 204 nM and maximum enhancement of about 40%. Enhancement of [^{3}H]FNZ binding by 5 μM $5\beta3\alpha$ was completely abolished in the presence of 200 μM picrotoxin (data not shown). $5\alpha3\alpha$ (5 μM), the 5α-OH enantiomer of $5\beta3\alpha$, was even more effective in stimulating [^{3}H]FNZ binding (Fig. 2), but $5\beta3\alpha$ was used for most chronic treatment experiments because of its lower cost. In contrast, 17β-estradiol did not stimulate [^{3}H]FNZ binding at any of the concentrations (0.01–10 μM) tested. Other steroids that failed to increase [^{3}H]FNZ binding were dexamethasone, testosterone, progesterone, deoxycorticosterone, and 5β-pregnan-3β-ol-20-one, the 3β-OH enantiomer of $5\beta3\alpha$ (Fig. 2).

When cultures were chronically exposed to 10 μM $5\beta3\alpha$ for

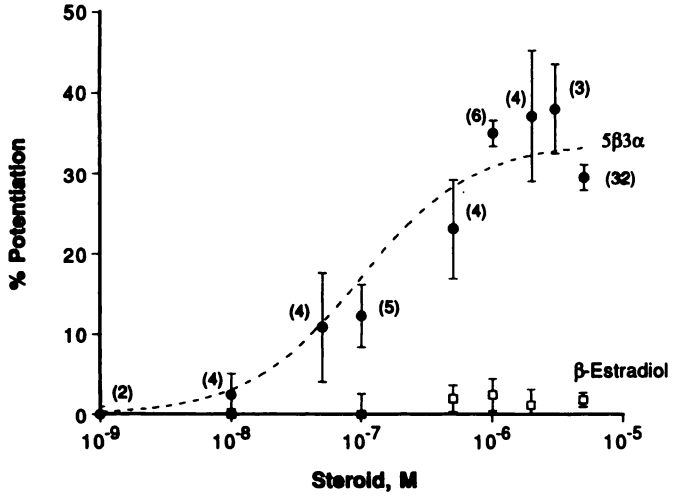

Fig. 1. [^{3}H]FNZ binding is enhanced by 5β3α but not by 17β-estradiol. Cell homogenates derived from embryonic chick brain cultures were incubated with 1 nM [^{3}H]FNZ in the presence of 5β3α (●) or β-estradiol (□). Values are percent enhancement of [^{3}H]FNZ binding over binding in the absence of steroids. *Smooth curve*, best fit to the Hill equation, % enhancement = E_{max}[steroid]^n/[[steroid]^n + $(EC_{50})^n$], with EC_{50} = 204 nM, E_{max} = 40% enhancement, and n = 1.15. Values are the means ± standard errors (five experiments for β-estradiol; the number of experiments is given in parentheses for 5β3α.

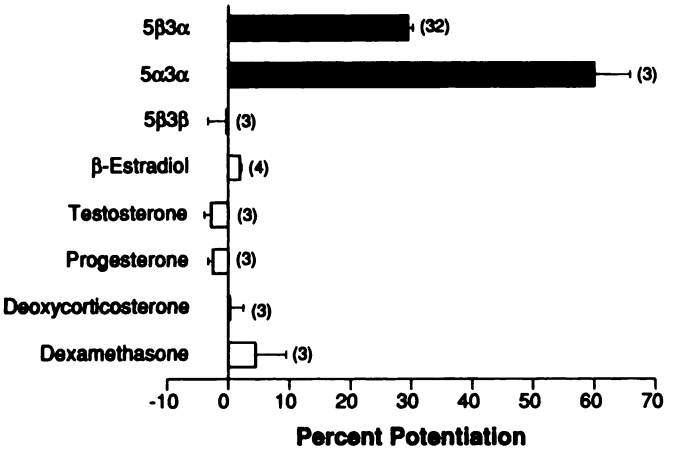

Fig. 2. Enhancement of [^{3}H]FNZ binding is specific for 5β3α and 5α3α. Binding of 1 nM [^{3}H]FNZ to culture homogenate was determined in the presence and absence of the indicated steroid (10 μM). Potentiation of [^{3}H]FNZ binding was significant only for 5β3α ($p < 0.0001$, unpaired t test) and 5α3α ($p < 0.015$). *Error bars*, standard error. The number of experiments is shown in parentheses. *5β3β*, 5β-pregnan-3β-ol-20-one.

48 hr before assay, enhancement of [^{3}H]FNZ binding by 5β3α was reduced from 26 ± 2% to −1 ± 1% (23 experiments), indicating complete (105 ± 5%) uncoupling of allosteric interactions between the steroid and benzodiazepine recognition sites on the $GABA_AR$. A briefer (24-hr) period of exposure to 5β3α resulted in partial (63 ± 6%, three experiments) uncoupling. To investigate the reversibility of 5β3α-induced uncoupling, cultures were treated with 10 μM 5β3α for 48 hr, washed three times at 37° to remove the drug, and returned to the incubator for an additional 24 hr. Washed cultures exhibited nearly complete recovery of 5β3α potentiation of [^{3}H]FNZ binding, whereas no recovery was observed with sister cultures maintained in the continued presence of 5β3α (Fig. 3). Induction of uncoupling by 5β3α was dose dependent, with an apparent EC_{50} of about 1 μM (Fig. 4).

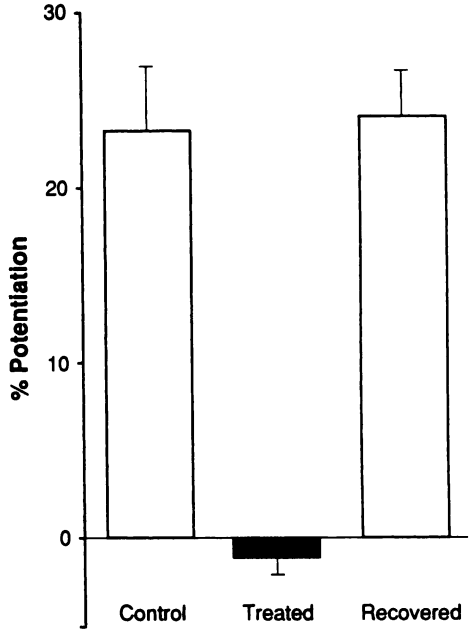

Fig. 3. Reversible elimination of enhancement of [^{3}H]FNZ binding after chronic treatment with 5β3α. Cultures were incubated in the absence or presence of 5β3α (10 μM) for 48 hr. One group of treated cultures was then washed three times at 37° with warmed sterile PBSS supplemented with 3% horse serum and was allowed to recover for an additional 24 hr in conditioned medium in the absence of 5β3α. A second group of treated cultures was washed three times at 37° with warmed sterile PBSS supplemented with 3% horse serum and containing 10 μM 5β3α and was then incubated for an additional 24 hr in conditioned medium containing 10 μM 5β3α. Cultures (treated and control dishes) were then scraped, dialyzed, and assayed for enhancement of [^{3}H]FNZ binding by 5β3α (5 μM), as described in Materials and Methods. *Bars*, means ± standard errors of three independent experiments.

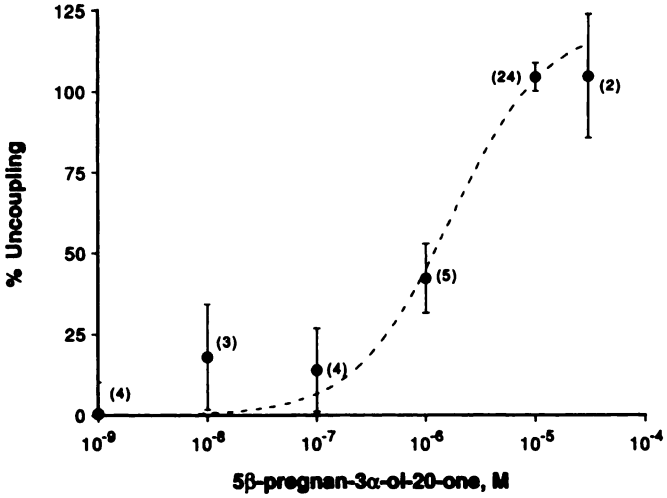

Fig. 4. Uncoupling by 5β3α is dose dependent. Cultures were incubated with the indicated concentration of 5β3α for 48 hr. Cell homogenates were dialyzed and percent uncoupling was calculated from the change in potentiation of 1 nM [^{3}H]FNZ binding by 5 μM 5β3α in treated cultures, compared with untreated sister cultures. *Error bars*, standard errors. The number of experiments is given in parentheses. *Smooth curve*, best fit to the Michaelis-Menten equation, with EC_{50} = 1.7 μM and maximum uncoupling = 121%.

Because steroids are lipophilic and partition readily into membranes, membrane homogenates were dialyzed extensively before assay to remove the drugs used for chronic treatment. Nevertheless, it seemed possible that the loss of enhancement of [^{3}H]FNZ binding could be due to a small amount of residual 5β3α remaining dissolved in the lipid bilayer. To test this hypothesis, cultures were homogenized and the homogenate was incubated for 48 hr at 37° in the presence of 10 μM 5β3α, dialyzed, and then assayed for enhancement of [^{3}H]FNZ binding by 5β3α. No uncoupling was observed in treated homogenates, although intact sister cultures homogenized after exposure to 5β3α over the same period of time exhibited a complete loss of 5β3α potentiation of [^{3}H]FNZ binding (Fig. 5). This indicates that uncoupling cannot be due to a passive mechanism, such as partitioning of steroids into membranes, but is an active process mediated by intact cells.

Chronic treatment with 5β3α did not induce down-regulation of benzodiazepine binding sites. In saturation binding experiments, there was no difference between treated and untreated cells in either the number or affinity of [^{3}H]FNZ binding sites (Fig. 6). This result also provides further assurance that uncoupling is not an artifact due to residual 5β3α, which would be expected to decrease the apparent K_d of [^{3}H]FNZ binding.

To examine the specificity of uncoupling, we measured potentiation of [^{3}H]FNZ binding by 5β3α after chronic treatment of cultures with a series of other steroids (Fig. 7). Except for 5α3α, all were clearly less effective than 5β3α in inducing uncoupling, although perhaps not entirely without activity. Dexamethasone, in particular, elicited a smaller but statistically significant degree of uncoupling, and uncoupling by progesterone fell just short of statistical significance ($p = 0.056$).

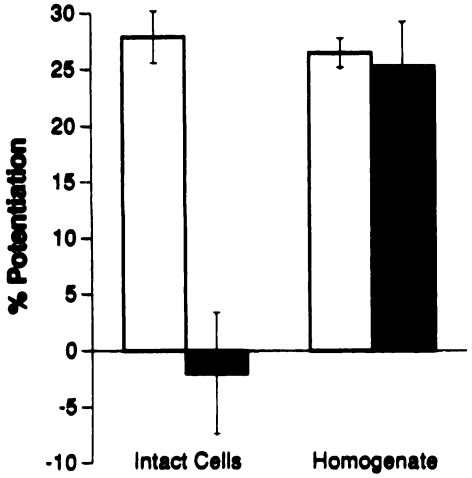

Fig. 5. 5β3α-induced uncoupling requires intact cells. Homogenates were prepared from intact cell cultures under sterile conditions and were incubated for 48 hr at 37° in the presence (□) or absence (□) of 10 μM 5β3α, in loosely capped vials. Intact sister cultures were maintained with (□) or without (□) 5β3α for the same time period, scraped, and homogenized. Both tissue preparations were dialyzed before the binding assay to remove the treatment drug, as described in Materials and Methods. Potentiation of [^{3}H]FNZ binding by 5β3α (5 μM) was measured as described in Materials and Methods. Data shown are the mean ± standard error of quadruplicate determinations, expressed as percentage potentiation of [^{3}H]FNZ binding. Binding of [^{3}H]FNZ in the absence of 5β3α was similar in all groups (untreated intact cells, 45 fmol/mg of protein; 5β3α-treated intact cells, 56 fmol/mg of protein; untreated homogenate, 50 fmol/mg of protein; 5β3α-treated homogenate, 46 fmol/mg of protein). This experiment was repeated three times with similar results.

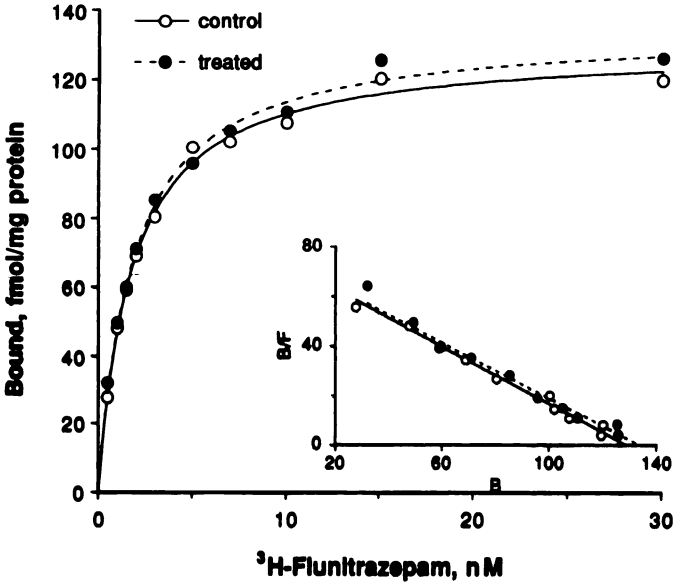

Fig. 6. Chronic exposure to 5β3α does not alter [^{3}H]FNZ binding affinity or number of sites. Brain cultures were incubated for 48 hr in the presence (●) or absence (○) of 5β3α (10 μM). Binding of 0.5–30 nM [^{3}H]FNZ to dialyzed culture homogenates was determined by filtration. Nonspecific binding determined in the presence of 10 μM flurazepam has been subtracted. To prevent binding to low affinity, peripheral sites, both total binding and nonspecific binding were determined in the presence of 2.5 μM Ro5–4864 (24, 25). Each *point* is the mean of quadruplicate determinations. *Inset*, Scatchard plot of the same data. The *lines* were calculated from the Michaelis-Menten equation, using K_d and B_{max} values determined by nonlinear regression (control, K_d = 1.78 nM, B_{max} = 134 fmol/mg of protein; 5β3α-treated, K_d = 1.73 nM, B_{max} = 129 fmol/mg of protein). This experiment was repeated two more times with similar results; the average B_{max} ratio (treated/control) was 1.01 ± 0.04 and the K_d ratio was 0.87 ± 0.08 (mean ± standard error, three experiments).

To investigate the pharmacology of uncoupling, cultures were chronically treated with 5β3α in combination with either picrotoxin or SR-95531. Interestingly, picrotoxin significantly reduced the extent of uncoupling, whereas SR-95531 had no effect. Chronic treatment with picrotoxin or SR-95531 alone did not affect enhancement of [^{3}H]FNZ binding by 5β3α (data not shown).

Discussion

In spite of the widespread medical and nonmedical use of steroids, there is surprisingly little information on the long term effects of steroids on the CNS. In particular, the effects of chronic treatment with reduced progesterone metabolites such as 5β3α and 5α3α have not been previously examined either *in vivo* or *in vitro*. However, there have been several reports indicating that chronic progesterone or β-estradiol treatment *in vivo* can affect the $GABA_AR$. For example, progesterone has been reported to produce a regionally specific up-regulation of [^{3}H]FNZ binding to central cerebrocortical membranes, with no change in the number of hippocampal binding sites (26). A similar up-regulation of benzodiazepine binding sites has been observed in spinal cord and some peripheral organs after progesterone treatment (26, 27). Effects of chronic treatment with β-estradiol on the CNS have been more variable, possibly due to differences in surgical procedures. An up-regulation of [^{3}H]muscimol binding sites in hypothalamus (28) and other brain regions (29) after chronic administration of 17β-estradiol to ovariectomized rats has been reported. In contrast,

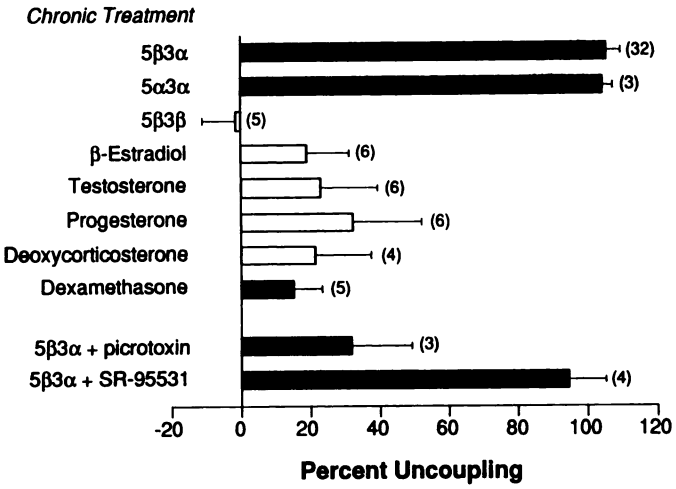

Fig. 7. Pharmacological specificity of steroid-induced uncoupling. Cultures were treated for 48 hr with the indicated steroid (progesterone, 5 μM; others, 10 μM), homogenized, and dialyzed. Uncoupling of 5β3α (5 μM) enhancement of [^{3}H]FNZ binding by steroids was determined as described in Materials and Methods, except for chronic treatment experiments with 5α3α, in which 5 μM 5α3α, rather than 5β3α, was used to potentiate [^{3}H]FNZ binding. Picrotoxin and SR-95531 (100 μM) were included during the chronic treatment period where indicated. ■, Statistical significance at the $p < 0.01$ level; □, statistical significance at the $p < 0.05$ level (unpaired t test). Uncoupling was of borderline significance for β-estradiol ($p \leq 0.066$), testosterone ($p \leq 0.097$), progesterone ($p \leq 0.066$), and deoxycorticosterone ($p \leq 0.089$). The decrease in uncoupling by 5β3α in the presence of picrotoxin was also statistically significant ($p < 0.0001$).

regionally specific decreases in [^{3}H]muscimol binding *in vivo* in response to chronic 17β-estradiol treatment of nonovariectomized animals have been reported (30, 31). Moreover, a decrease in high affinity [^{3}H]muscimol binding, with little change in [^{3}H]FNZ binding, and a decreased ability of GABA to enhance [^{3}H]FNZ binding after chronic β-estradiol treatment have also been observed *in vivo* (30).

A difficulty in interpreting experiments performed *in vivo* is that it is often unclear whether the observed changes are due to the administered steroid or to a metabolite. Moreover, steroids may act indirectly by stimulating the release of other factors, such as prolactin (32). To minimize these concerns, we examined the effects of chronic steroid exposure on neurons in tissue culture.

After exposure of cultures to 10 μM 5β3α for 48 hr, enhancement of [^{3}H]FNZ binding by 5β3α was completely eliminated, consistent with allosteric uncoupling of steroid and benzodiazepine recognition sites of the $GABA_AR$. This is a more pronounced effect than the partial uncoupling of GABA and benzodiazepine sites induced by chronic treatment with GABA or benzodiazepines, in which some enhancement of [^{3}H]FNZ binding by GABA remains after chronic treatment. A similarly profound homologous uncoupling of the benzodiazepine and barbiturate recognition sites is observed after chronic treatment of cultures with pentobarbital or barbital (3).

This loss of the allosteric modulation of [^{3}H]FNZ binding by 5β3α is referred to as homologous uncoupling, because it is observed after chronic treatment with 5β3α. Uncoupling is not necessarily entirely specific for compounds related to that to which the cell has been chronically exposed, because we have also observed instances of heterologous uncoupling, in which decreased allosteric coupling between two recognition sites is induced by chronic treatment with a drug believed to bind to a separate site. For example, chronic treatment with pentobarbital produces both homologous uncoupling of barbiturate and benzodiazepine recognition sites and heterologous uncoupling of GABA and benzodiazepine recognition sites (3). Similarly, chronic treatment with methylxanthines induces heterologous uncoupling of GABA and benzodiazepine recognition sites. As noted above, the two most extreme instances of uncoupling that we have observed are examples of homologous uncoupling; chronic treatment with barbiturates virtually eliminates enhancement of [^{3}H]FNZ binding by barbiturates, and chronic treatment with 5β3α likewise nearly eliminates enhancement by 5β3α. On the other hand, chronic treatment with GABA or muscimol produces only a comparatively modest 40% homologous uncoupling of enhancement of [^{3}H]FNZ binding by GABA, indicating that homologous uncoupling is not necessarily complete.

The observation that 5β3α and 5α3α were the only steroids tested that produced complete uncoupling, and also the only ones that enhanced [^{3}H]FNZ binding, suggests that these two effects may be linked. On the other hand, there was some evidence of uncoupling in cultures treated with β-estradiol, testosterone, progesterone, deoxycorticosterone, or dexamethasone (although uncoupling was statistically significant only for dexamethasone), so the absence of measurable enhancement of [^{3}H]FNZ binding is probably not a reliable indicator of inability to induce uncoupling.

The relationship between enhancement of [^{3}H]FNZ binding and potentiation of the GABA response is somewhat unclear. 5β3α and 5α3α both strongly potentiate the GABA response and enhance [^{3}H]FNZ binding, whereas their corresponding 3β-enantiomers neither potentiate the GABA response nor increase [^{3}H]FNZ binding (9, 16, 20, 33). We were initially somewhat surprised that progesterone and deoxycorticosterone did not enhance [^{3}H]FNZ binding, because we previously found that these steroids are positive modulators of the GABA response in chick spinal cord cultures (9). This could indicate that enhancement of the GABA response and enhancement of [^{3}H]FNZ binding are unrelated. On the other hand, the maximum enhancement of [^{3}H]FNZ binding by 5β3α is only about 40%, and the efficacy of 5β3α as a modulator of the GABA response is >4-fold greater than that of progesterone or deoxycorticosterone.[2] Assuming that progesterone and deoxycorticosterone are similarly less efficacious than 5β3α in enhancing [^{3}H]FNZ binding, the expected degree of potentiation would be <10%, which is about the limit of reliable detection for this assay.

Chronic exposure to $GABA_AR$ agonists can induce uncoupling of GABA and benzodiazepine recognition sites (1). In addition to potentiating the action of GABA, 5β3α and 5α3α are able to open the $GABA_AR$ chloride channel in the absence of GABA, so it is possible that direct activation of the $GABA_AR$ by these steroids may play a role in uncoupling. However, there was no evidence of down-regulation of [^{3}H]FNZ binding sites, such as that seen after chronic exposure of cultures to GABA or muscimol (1, 2). For the same reason, it seems unlikely that uncoupling by 5β3α is an indirect consequence of enhancement of the action of endogenous GABA in the cultures. As an additional test, we chronically exposed cultures to 5β3α in

[2] J. Celentano, unpublished observations.

combination with SR-95531, a competitive GABA antagonist that inhibits GABA-induced uncoupling and down-regulation (1). SR-95531 did not block 5β3α-induced uncoupling of steroid/benzodiazepine interactions, excluding endogenous GABA as a causative factor. In contrast, the noncompetitive GABA antagonist picrotoxin significantly decreased 5β3α-induced uncoupling, consistent with the ability of picrotoxin to block enhancement of [^{3}H]FNZ binding by steroids (20).

Although a detailed time course of 5β3α-induced uncoupling was not determined, partial uncoupling was observed after 24 hr, suggesting that the $t_{1/2}$ for 5β3α-induced uncoupling of steroid/benzodiazepine site interactions is not greatly different from that for flurazepam-induced uncoupling of GABA/benzodiazepine site interactions, which is approximately 18 hr (3). This is similar to the average $t_{1/2}$ for turnover of $GABA_AR$s photoaffinity labeled with [^{3}H]FNZ (22, 34). In view of the existence of multiple $GABA_AR$ subunit variants (35–39), it is tempting to speculate that uncoupling could be due to changes in receptor subunit composition (40, 41). Chronic exposure to GABA, which results in both GABA/benzodiazepine recognition site uncoupling and $GABA_AR$ down-regulation (1), is associated with a decrease in $GABA_AR$ α subunit mRNA levels (2). The effect of chronic exposure to 5β3α differs, in that there is no decrease in maximal [^{3}H]FNZ binding, suggesting that if uncoupling does reflect a change in receptor subunit synthesis there is likely to be some mechanism for maintaining total receptor levels constant. Alternatively, uncoupling could be due to a post-translational modification of the $GABA_AR$, such as phosphorylation. A number of the identified $GABA_AR$ subunits contain consensus phosphorylation sequences, and there is evidence that phosphorylation can regulate $GABA_AR$ function (42–46).

If uncoupling of steroid/benzodiazepine site interactions reflects an overall decrease in the sensitivity of the $GABA_AR$ to steroids, it could be clinically relevant. Progesterone is anxiolytic in humans and increases seizure thresholds in animals, effects that may be due either to the direct action of progesterone on the $GABA_AR$ (9) or, more probably, to the effects of more potent and efficacious progesterone metabolites, such as 5α3α (16). Direct modulation of the $GABA_AR$ by steroids requires relatively high steroid concentrations, but there is evidence that such high concentrations may be attained *in vivo*. For example, 5β3α and 5α3α levels in human plasma average about 100 nM in the third trimester of pregnancy (47), and 5α3α levels in excess of 1 μM have been measured in rat ovarian venous plasma during estrus (48). Moreover, there is evidence for *de novo* synthesis of steroids in brain, raising the possibility that local brain concentrations could be even greater (6, 47, 49–51).

Progesterone levels in women peak in the latter part of the menstrual cycle and then fall sharply immediately before menstruation, and this drop is correlated with premenstrual anxiety and with increased seizure frequency in certain women with epilepsy (52, 53). The increase in seizure frequency has been attributed to low levels of progesterone at this time, but an additional contributing factor could be a rebound effect due to $GABA_AR$ steroid subsensitivity resulting from high levels of progesterone and its metabolites earlier in the cycle.

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