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Plastic neuronal changes in $GABA_A$ receptor gene expression induced by progesterone metabolites: In vitro molecular and functional studies

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Abstract

Expression of specific γ-aminobutyric acid type A ($GABA_A$) receptor subunit genes in neurons is affected by endogenous modulators of receptor function such as neuroactive steroids.

Neuroactive steroids such as the progesterone metabolite allopregnanolone might thus exert differential effects on $GABA_A$ receptor plasticity in neurons, likely accounting for some of the physiological actions of these compounds.

Here we summarise experimental data obtained in vitro that show how fluctuations in the concentration of progesterone regulate both the expression and function of $GABA_A$ receptors.

The data described in this manuscript are in agreement with the notion that fluctuations in the concentrations of progesterone and its metabolite allopregnanolone play a major role in the temporal pattern of expression of various subunits of the $GABA_A$ receptor. Thus, rapid and long-lasting increases or decreases in the concentrations of these steroid derivatives observed in physiological and patho-physiological conditions, or induced by pharmacological treatments, might elicit selective changes in $GABA_A$ receptor gene expression and function in specific neuronal populations. Given both the importance of $GABA_A$ receptors in the regulation of neuronal excitability and the large fluctuations in the plasma and brain concentrations of neuroactive steroids associated with physiological conditions and the response to environmental stimuli, these compounds are likely among the most relevant endogenous modulators that could affect emotional and affective behaviors.

Keywords: Neurosteroids; $GABA_A$ receptor; Gene expression; Cell culture; Patch-clamp; Synaptic plasticity

1. Introduction

Neurosteroids are steroid derivatives synthesized de novo from cholesterol in the central nervous system (CNS) (Hu et al., 1987) and include compounds that modulate $GABA_A$ receptor function with potencies and efficacies similar to or greater than those of benzodiazepines and barbiturates (Harrison and Simmonds, 1984; Majewska, 1992; Majewska et al., 1986). Certain neurosteroids, called neuroactive steroids, have thus been suggested to function as endogenous modulators of γ-aminobutyric acid type A ($GABA_A$) receptor-mediated neurotransmission. The progesterone metabolite 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP), called also allopregnanolone, induces opening of the $GABA_A$ receptor-associated Cl^- channel at nanomolar concentrations in vitro (Lambert et al., 1995; Majewska, 1992) as well as elicits pharmacological and behavioral effects in animals similar to those produced by other positive modulators of the $GABA_A$ receptor (Majewska et al., 1986). The anxiolytic and anticonvulsant properties of progesterone are mostly attributable to its conversion to allopregnanolone (Bitran et al., 1993, 1995; Freeman et al., 1993; Kokate et al., 1999; Picazo and Fernandez-Guasti, 1995; Reddy and Rogawski, 2000).

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1.1. $GABA_A$ receptors heterogeneity

The subunit composition of native $GABA_A$ receptors is an important determinant of the role of these receptors in the physiological and pharmacological modulation of neuronal excitability and associated behavior. For example, $GABA_A$ receptors that contain the α_1 subunit mediate the sedative-hypnotic effects of benzodiazepines (McKernan et al., 2000; Rudolph et al., 1999), whereas the anxiolytic effects of these drugs are mediated by receptors that contain the α_2 subunit (Low et al., 2000). In contrast, $GABA_A$ receptors that contain the α_4 or α_6 subunits are insensitive to benzodiazepines (Barnard et al., 1998; Wafford et al., 1996; Yang et al., 1995). Characterisation of the roles of $GABA_A$ receptors thus requires an understanding of the mechanisms by which receptor subunit composition is regulated. Many studies have established that long-term administration of sedative-hypnotic, anxiolytic or anticonvulsant drugs can affect expression of $GABA_A$ receptor subunit genes as well as the drug sensitivity and function of these receptors, suggesting that the mechanisms responsible for such changes might also underlie the physiological modulation of $GABA_A$ receptors by endogenous compounds such as neuroactive steroids.

1.2. Experimental evidences for neuroactive steroids action on δ-containing $GABA_A$ receptors

Different sensitivity to neuroactive steroids modulation of native $GABA_A$ receptors has been reported since the observation of the action of these compounds on $GABA_A$ receptors (Gee and Lan, 1991). Gene targeting in mouse producing an animal totally lacking the $GABA_A$ receptors δ subunit results in drastically reduced sensitivity to neuroactive steroids (Mihalek et al., 1999; Spigelman et al., 2003; Vicini et al., 2002). Mice lacking the δ subunit became markedly less sensitive to neuroactive steroids in vivo (Mihalek et al., 1999) and in vitro (Spigelman et al., 2003; Vicini et al., 2002). Thus, these first observations suggested that the δ subunit-containing $GABA_A$ receptors may play some specific role in the action of neuroactive steroids. Moreover, other authors have found that the $\alpha_6\beta_3\delta$ (Wohlfarth et al., 2002) and $\alpha_4\beta_3\delta$ (Adkins et al., 2001) combinations are not only sensitive to steroids, but more sensitive than the corresponding $\alpha_4\beta_3\gamma_2$ or other α subunit-containing $GABA_A$ receptors (Adkins et al., 2001; Belelli et al., 2002; Brown et al., 2002). Thus, $GABA_A$ receptors composed of the $\alpha_4\beta_2\delta$ combination show a low efficacy for GABA alone (partial agonism), resulting in a corresponding greater enhancement by steroid. These results are also consistent with a physiological role for the $\alpha_4\beta\delta$ combination in tonic inhibition involving extrasynaptic receptors that can respond to endogenous extracellular GABA and spillover (Brickley et al., 2001; Jones et al., 1997; Nusser and Mody, 2002). Accordingly, anatomical data (Laurie et al., 1992a,b; Wisden et al., 1992) show that the δ subunit containing $GABA_A$ receptors in granule neurons is likely extrasynaptic (Nusser et al., 1998).

Thus, neuroactive steroids are potent modulators of $GABA_A$ receptors, and their behavioral effects are generally viewed in terms of altered inhibitory synaptic transmission. At concentrations known to occur in vivo, neuroactive steroids specifically enhance a tonic inhibitory conductance in central neurons that is mediated by extrasynaptic δ subunit-containing $GABA_A$ receptors (Stell et al., 2003). The neurosteroid-induced augmentation of this tonic conductance decreases neuronal excitability. Recognition that δ subunit-containing $GABA_A$ receptors responsible for a tonic conductance are a preferential target for neuroactive steroids may lead to novel pharmacological approaches for the treatment of those pathological conditions in which fluctuations in the circulating concentrations of endogenous neuroactive steroids have been described (Bicikova et al., 1998; Brambilla et al., 2003; Monteleone et al., 2000; Pisu and Serra, 2004; Rapkin et al., 1997; Romeo et al., 1998; Strohle et al., 2003, 2002, 1999; Uzunova et al., 1998).

1.3. $GABA_A$ receptor plasticity and neuroactive steroids

Changes in steroid sensitivity corresponding to plastic changes in the brain are induced by experiences, including chronic GABAergic drug administration, drug withdrawal and animal models of epilepsy; these are probably mediated by subunit switches (Banerjee et al., 1998; Brussaard and Herbison, 2000; Cagetti et al., 2003; Devaud et al., 1996; Mtchedlishvili et al., 2001; Reddy and Rogawski, 2000).

Some plasticity may be due to changes in neurosteroid levels in vivo, as seen in acute stress (Barbaccia et al., 1996) or stress induced by social isolation (Serra et al., 2000) and in pregnancy (Concas et al., 1998). Interestingly, chronic administration to rats of progesterone or its $GABA_A$ receptors active metabolite, allopregnanolone, and withdrawal from the steroid lead to behavioral withdrawal signs, tolerance to steroids and benzodiazepines in some assays and changes in $GABA_A$ receptor subunit composition; such changes may be a model of premenstrual dysphoria (Smith et al., 1998a).

Thus, under physiological conditions, neurons are exposed to steroids for long periods of time or to abrupt changes in steroid levels that occur in a cyclic manner. Changes in the peripheral or central production of progesterone and consequent fluctuations in the synaptic concentration of allopregnanolone might therefore contribute to regulation of $GABA_A$ receptor-mediated synaptic activity and of emotional state associated with physiological conditions such as stress, pregnancy, the menstrual cycle and menopause as well as to anxiety and mood disorders. Indeed, the concentration of allopregnanolone in plasma or cerebrospinal fluid has been shown to be altered in individuals with major depression, premenstrual syndrome, panic disorder or anxiety (Bicikova et al., 1998; Brambilla et al., 2003; Monteleone et al., 2000; Pisu and Serra, 2004; Rapkin et al., 1997; Romeo et al., 1998; Strohle et al., 2003, 2002, 1999; Uzunova et al., 1998). Fluctuations in the peripheral secretion of progesterone or allopregnanolone, together with the ability of the CNS to synthesize allopregnanolone either de novo or from peripheral progesterone, might thus play an important role in regulation of $GABA_A$ receptor gene expression and function in the CNS.

The effect of chronic progesterone and withdrawal on GABA receptor expression and function can be modeled and studied in primary cultured neurons (Follesa et al., 2000).

2. Materials and methods

2.1. Cell culture

Primary neuronal cultures enriched in granule cells were prepared from the cerebellum of 8-day-old rats as previously described (Bovolin et al., 1992; Follesa et al., 2000). All animal procedures were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Cells were cultured in basal Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mM glutamine, gentamicin (100 μg/ml, Sigma) and 25 mM KCl. For measurement of $GABA_A$ receptor subunit mRNAs, the cells were plated (12.5×10^6 cells in 10 ml/dish) in 100-mm dishes that had been coated with poly-L-lysine (10 μg/ml; Sigma, St. Louis, MO). For both immunocytochemistry and electrophysiological recording, cells were plated (3×10^5 cells in 1 ml/well) in multiwell plates containing 12-mm round coverslips also coated with poly-L-lysine. Cytosine arabinofuranoside (final concentration, 10 μM; Sigma) was added to the cultures 18 to 24 h after plating to inhibit the proliferation of nonneuronal cells. After 3 days in culture, the cells were exposed for 5 days to 1 μM progesterone, 1 μM allopregnanolone or 1 μM THDOC. In some experiments, cells were exposed to progesterone for 1 to 4 days, starting as needed, in order to always harvest the cells at the 8th day in culture. In some experiments as indicated progesterone and finasteride (10 μM, 5 days) were administered together. For steroids withdrawal, the medium containing progesterone, allopregnanolone or THDOC was then replaced with drug-free medium and the cells were incubated for an additional 3 to 24 h as indicated. Progesterone was dissolved and subsequently diluted in culture medium. The culture medium was replaced every day with fresh medium containing the appropriate drug.

2.2. Probe preparation

The cDNA for each subunit of the $GABA_A$ receptor studied was prepared as described previously (Follesa et al., 1998) by reverse transcription and the polymerase chain reaction (PCR). In brief, cDNA prepared from rat brain (1 to 10 ng) was amplified by PCR with *Taq* DNA polymerase (2.5 U; Perkin-Elmer/Cetus, Norwalk, CT) in 100 μl of standard buffer [100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin] containing 1 μM each of specific sense and antisense primers and 200 μM of each deoxynucleoside triphosphate. The primer pairs for the various subunits of the $GABA_A$ receptor were designed (Follesa et al., 1998, 2003, 2005) to include cDNA sequences with the lowest level of intersubunit homology. The reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) for 30 cycles of 94 °C for 45 s, 60 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 15 min. The PCR products were separated by electrophoresis, detected by staining with ethidium bromide, excised from the gel, purified and cloned into the pAMP 1 vector (Life Technologies). *Escherichia coli* DH5α was transformed with the resulting plasmids, which were subsequently purified from the bacteria and the cDNA inserts were sequenced with a Sequenase DNA sequencing kit (USB, Cleveland, OH). The determined nucleotide sequences were 100% identical to the respective previously published sequences. Plasmids containing the cDNA fragments corresponding to the various $GABA_A$ receptor subunits were linearized with restriction enzymes (Follesa et al., 1998, 2003, 2005) and then used as templates, together with the appropriate RNA polymerase (SP6 or T7), to generate [α-^{32}P]CTP-labeled cRNA probes for RNase protection assays.

2.3. RNA extraction and measurement of $GABA_A$ receptor subunit mRNAs

Total RNA was isolated from cultured cerebellar granule cells by the guanidine isothiocyanate method as previously described (Follesa et al., 1998) and was quantified by measurement of absorbance at 260 nm. RNase protection assays for the semi-quantitative detection of mRNAs encoding for the different subunits of the $GABA_A$ receptor were performed as described (Follesa et al., 1998). In brief, 25 μg of total RNA were dissolved in 20 μl of hybridization solution containing 150,000 cpm of ^{32}P-labeled cRNA probe for a specific subunit mRNA (specific activity, 6×10^7 to 7×10^7 cpm/μg) and 15,000 cpm of ^{32}P-labeled cyclophilin cRNA (1×10^6 cpm/μg) used as an internal standard. The hybridization reaction mixture was incubated overnight at 50 °C and then subjected to digestion with RNase, after which the remaining RNA–RNA hybrids were detected by electrophoresis (on a sequencing gel containing 5% polyacrylamide and urea) and autoradiography. The amounts of the different $GABA_A$ receptor subunit mRNAs and cyclophilin mRNA were determined by measurement of the optical density of the corresponding bands on the autoradiogram with a densitometer (model GS-700; Bio-Rad, Hercules, CA); this instrument is calibrated to detect saturated values, so that all our measurements were in the linear range. The data were normalized by dividing the optical density of the protected fragment for each receptor subunit mRNA by that of the respective protected fragment for cyclophilin mRNA. The amount of each receptor subunit mRNA was therefore expressed in arbitrary units.

2.4. Whole-cell patch-clamp electrophysiological recording

Immediately before recording, cerebellar granule cells on coverslips were transferred to a perfusion chamber (Warner Instruments, Hampden, CT), and neurons were visualized with a Nikon upright microscope equipped with Nomarski optics. The membrane potential was clamped at − 60 mV with an Axopatch 200-B amplifier (Axon Instruments, Foster City, CA). The resting membrane potential for the recorded neurons was approximately − 60 mV. Recording pipettes (borosilicate capillaries with a filament and outer diameter of 1.5 mm; Sutter Instruments, Novato, CA) were prepared with a two-step vertical puller (Sutter Instruments) and had resistances between 4 and 6 MΩ. Pipette

capacitance and series resistance were compensated, the latter at 60%. Currents through the patch-clamp amplifier were filtered at 2 kHz and digitized at 5.5 kHz with commercial software (pClamp 8.1, Axon Instruments).

The external solution contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES–NaOH (pH 7.3) and 11 mM glucose (all chemicals from Sigma). The internal solution contained 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES–CsOH (pH 7.3) and 2 mM ATP–disodium salt (all chemicals from Fluka, Buchs, CH, Switzerland). Drugs were applied with a fast-exchange flow-tube perfusion system driven by a motor (Warner Instruments). The partial agonist THIP (4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol, or gaboxadol; TOCRIS, Bristol, U.K.) at a concentration of 3 μM was used and the effect of allopregnanolone (1 μM; Sigma) on THIP-evoked Cl^- currents was determined.

All experiments were performed at room temperature (23 to 25 °C). Data were analyzed with pClampfit 8.01 software (Axon Instruments). Modulation of THIP-evoked Cl^- currents by allopregnanolone was calculated as percentage change, $[(I'/I) - 1] \times 100\%$, where I is the average of control responses obtained before application and after washout of drugs, and I' is the average of agonist-induced responses obtained from the same cell in the presence of drug.

2.5. Immunocytofluorescence analysis

Immunocytofluorescence analysis to detect the δ subunit of the $GABA_A$ receptor was performed as previously described (Follesa et al., 2005). Neurons cultured on coverslips were washed three times with phosphate-buffered saline, fixed for 1 h at room temperature with 4% paraformaldehyde in phosphate-buffered saline, washed three times with TN buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl] and permeabilized for 1 h at room temperature with TN-T buffer (0.1% Triton X-100 in TN buffer) containing 0.5% dried skim milk. Nonspecific binding sites for avidin and biotin were blocked by incubation of the cells for 15 min at room temperature with avidin D blocking solution and then for an additional 15 min with biotin blocking solution (Vector, Burlingame, CA). The cells were then incubated overnight at 4 °C with goat polyclonal antibodies (1:500 dilution in TN-T buffer) to the $GABA_A$ receptor δ subunit. After several washes with TN-T buffer, the cells were incubated for 1 h at room temperature with biotin-conjugated donkey antibodies (1:200 in TN-T) to goat immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) and then for 1 h with tetramethylrhodamine isothiocyanate-conjugated streptavidin (2 μg/ml in TN-T; Jackson ImmunoResearch). The cells were washed extensively with TN buffer, and each coverslip was then positioned on a glass microscope slide with a permanent aqueous mounting medium (Sigma).

For standard epifluorescence imaging, the cells were examined with an Olympus BX-41 microscope by using a UPlan FI 40× objective (numerical aperture, 0.75) and photographed with an F-View CCD camera. Semiquantitative analysis was performed with AnalySIS 3.2 software (Soft Imaging System, Münster, Germany); the acquired 8-bit gray-value images were white labeled on a black background, with a scale ranging from 0 as lower limit (black) to 255 as upper limit (white), so that the entire image histogram will be considered for calculating thresholds. Each experiment was repeated three times, 10 fields were randomly selected for each coverslip of each experimental group. In each field, four cells were randomly selected by drawing a line surrounding the cell body (region of interest; ROI), in order to measure the intensity of fluorescence, representing the abundance of the protein tested. Morphometric and statistical analysis on three-dimensional reconstructed images, were performed with the same AnalySIS 3.2 software. Fluorescence intensity, which represent δ subunit abundance, was eventually expressed in arbitrary units and compared as percentage of change between the different experimental groups.

2.6. Statistical analysis

Data are presented as means ± S.E.M. and were subjected to analysis of variance followed by Scheffe's test. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Modulation of $GABA_A$ receptor gene expression and function by progesterone metabolites: in vitro studies

The in vitro data here presented suggest that the neurosteroid allopregnanolone plays an important role in the modulation of $GABA_A$ receptor function and expression. Pharmacologically induced fluctuations in the concentration of allopregnanolone result in parallel changes in both $GABA_A$ receptor activity and the expression of specific receptor subunit genes.

3.2. Effects of chronic steroid treatment and withdrawal on $GABA_A$ receptor gene expression and function in cerebellar granule cells in culture

Long-term treatment of neurons in culture with neuroactive steroids reduces the efficacy of GABA in functional assays and induces both homologous and heterologous uncoupling between GABA, barbiturate and neurosteroid sites, and the benzodiazepine site as well as reduced efficacy of GABA in functional assays (Friedman et al., 1993, 1996; Yu and Ticku, 1995a,b). Electrophysiological measurements with cortical neurons in the whole-cell mode also revealed that chronic allopregnanolone treatment reduced both the GABA-induced current and the potentiation of this current by allopregnanolone (Yu et al., 1996b). These changes induced by long-term exposure of cultured neurons to allopregnanolone are associated with changes in the abundance of mRNAs encoding specific $GABA_A$ receptor subunits, although the subunit mRNAs affected differ among neuronal cell types (Follesa et al., 2001, 2000; Yu et al., 1996a).

Long-term exposure of cultured rat cerebellar granule cells to progesterone or allopregnanolone resulted in a marked decrease in the abundance of mRNAs for α_1, α_3, α_5 and both γ_2 subunits of the $GABA_A$ receptor (Follesa et al., 2000). Consistent with the notion that allopregnanolone, but not progesterone, exhibits a

positive allosteric modulatory action at $GABA_A$ receptors (Friedman et al., 1993; Majewska et al., 1986; Wu et al., 1990), these effects of chronic progesterone exposure were blocked by concomitant treatment with the 5α-reductase inhibitor finasteride (Azzolina et al., 1997; Follesa et al., 2000; Rittmaster, 1994), an effect that was observed also in vivo (Concas et al., 1998). In contrast, long-term progesterone treatment of cerebellar granule cells had no effect on the abundance of the α_2, α_4, β_1, or β_2 subunit mRNAs (Follesa et al., 2000). Differently, exposure of cultured rodent cortical neurons to neuroactive steroids had no significant effect on the abundance of mRNAs encoding the α_1, α_4 or γ_2S subunits of the $GABA_A$ receptor (Follesa et al., 2001) but down-regulated the expression of the α_2, α_3, β_2 and β_3 subunit genes (Yu et al., 1996a). It is possible that the apparent failure of long-term treatment with neuroactive steroids to affect the amounts of certain $GABA_A$ receptor subunit mRNAs in cortical neurons is attributable to the heterogeneous nature of the cortical neuronal population (compared with the homogeneity of cerebellar granule cell cultures); this heterogeneity might mask changes in subunit mRNA abundance that occur in opposite directions in different cell types. Indeed, as observed in vivo the expression of $GABA_A$ receptor subunit genes is affected by steroids in opposite directions in different subfields of neurons in the brain (Fenelon and Herbison, 1996; Weiland and Orchinik, 1995). However, it is also possible that the differences in the effects of neuroactive steroids on $GABA_A$ receptor gene expression between different cell types result from differences in the expression of enzymes that determine the abundance of progesterone and its metabolites (Hammer et al., 2004; Sanne and Krueger, 1995).

The demonstration that progesterone metabolites modulate $GABA_A$ receptor gene expression in cultured neurons is thus consistent with the results of in vivo studies in pregnant and pseudopregnant rats (Brussaard et al., 1997; Concas et al., 1998; Fenelon and Herbison, 1996; Follesa et al., 1998; Smith et al., 1998a,b). Changes in the expression of the genes for the various $GABA_A$ receptor subunits and the consequent synthesis of new receptor subtypes might thus represent a mechanism by which the sensitivity of neurons to positive and negative modulators of $GABA_A$ receptors is altered by long-term exposure to neuroactive steroids.

The changes in $GABA_A$ receptor gene expression induced in cerebellar granule cells by long-term exposure to progesterone are accompanied by changes in receptor function. Whereas the benzodiazepine diazepam markedly potentiated GABA-evoked Cl^- currents in control granule cells, this effect was greatly reduced in cells subjected to long-term treatment with progesterone (Follesa et al., 2000). Moreover, the anxiogenic and convulsant β-carboline derivative DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), a benzodiazepine receptor inverse agonist (Braestrup et al., 1983, 1982), induced a marked inhibition of GABA-evoked Cl^- currents in control granule cells but had no effect on such currents in cells chronically exposed to progesterone (Follesa et al., 2000). The reduced abilities of diazepam and DMCM to modulate GABA-evoked Cl^- currents in granule cells subjected to long-term exposure to progesterone are consistent with the down-regulation of the amounts of α_1, α_3, α_5 and γ_2 subunit mRNAs induced by such treatment (Follesa et al., 2000). Thus, both α and γ_2 subunits are required for maximal sensitivity of $GABA_A$ receptors to benzodiazepines or benzodiazepine receptor inverse agonists (Barnard et al., 1998; Pritchett et al., 1989). Although it is likely that such changes in the abundance of receptor subunit mRNAs result in corresponding changes in the synthesis of the encoded proteins, the relations between the amount of receptor subunit mRNAs and the amount of subunit proteins expressed on the cell surface remain to be determined.

The discontinuation of long-term exposure of cultured granule cells to progesterone, and the consequent sudden decrease in the production of allopregnanolone by these cells, resulted in a selective increase in the abundance of the mRNA for the α_4 subunit of the $GABA_A$ receptor (Follesa et al., 2000). The decreases in the amounts of the α_1 and γ_2 subunit mRNAs elicited by persistent exposure to progesterone also remained apparent after progesterone withdrawal (Follesa et al., 2000). These changes in $GABA_A$ receptor gene expression are identical to those induced in cultured granule cells by withdrawal of the synthetic allopregnanolone analog ganaxolone (Mascia et al., 2002).

The presence of the α_4 subunit in recombinant $GABA_A$ receptors is associated with a reduced sensitivity to classical benzodiazepine agonists and zolpidem as well as with distinct patterns of regulation by flumazenil, DMCM and other positive or negative modulators (Barnard et al., 1998). Electrophysiological recording revealed that $GABA_A$ receptors of granule cells subjected to progesterone withdrawal were both markedly less sensitive to the potentiating effect of diazepam than were those in control cells as well as positively modulated by the benzodiazepine receptor antagonist flumazenil (Follesa et al., 2000), characteristics consistent with those of $GABA_A$ receptors containing the α_4 subunit (Barnard et al., 1998). Withdrawal from chronic progesterone treatment also restored the sensitivity of cerebellar $GABA_A$ receptors to the inhibitory action of the benzodiazepine receptor inverse agonist DMCM (Follesa et al., 2000). Given that recombinant α_4 subunit-containing receptors, like α_1 subunit-containing receptors, are negatively modulated by DMCM (Whittemore et al., 1996), the increase in the sensitivity of $GABA_A$ receptors to DMCM induced by progesterone withdrawal is likely attributable to the increase in the abundance of the α_4 subunit mRNA (Follesa et al., 2000). Such an increased sensitivity to endogenous inverse agonists conferred by an increase in the expression of the α_4 subunit may contribute to the pathogenesis of progesterone withdrawal syndrome. Consistent with this notion, the increase in the abundance of the α_4 subunit mRNA apparent in the hippocampus during withdrawal from progesterone in a rat pseudopregnancy model is associated with changes in the kinetics of hippocampal $GABA_A$ receptor-mediated currents, with anxiety and with an increased susceptibility to seizures (Reddy and Rogawski, 2000; Smith et al., 1998b).

3.3. Effects of chronic steroid treatment and withdrawal on $GABA_A$ receptor δ subunit gene expression and related functional changes in cerebellar granule cells in culture

RNase protection assays revealed that exposure of cultures of cerebellar granule cells to 1 μM progesterone for 1 to 4 days did not

significantly modify the abundance of the mRNA encoding for the δ subunit of the $GABA_A$ receptor (Fig. 1), whereas 5 days (chronic) of treatment slightly, but not significantly, decreased (about 10%) this mRNA (Figs. 1 and 2A). On the other hand, immunocytochemical experiments performed to measure the levels of the corresponding δ subunit peptide demonstrated that 5 days of progesterone treatment induced a more robust (compared to the mRNA) and statistically significant decrease (about 21%) of the δ subunit of the $GABA_A$ receptor (Fig. 2B). The decrease in δ subunit peptide induced by chronic progesterone treatment was comparable to that induced by chronic allopregnanolone (1 μM, 5 days) or THDOC (1 μM, 5 days) (Table 1). These data further confirm, as we previously demonstrated (Follesa et al., 2000), that cerebellar granule cells in culture express the enzymatic machinery that allows the conversion of progesterone into the neuroactive steroids allopregnanolone and THDOC. Furthermore, the conversion of progesterone into allopregnanolone can be blocked by the 5α-reductase inhibitor finasteride (Follesa et al., 2000).

We next investigated the effects of progesterone withdrawal on the abundance of $GABA_A$ receptor δ subunit mRNA and peptide in cultured cerebellar granule cells. We found that even if 1 to 5 days progesterone treatment did not significantly affect the abundance of the δ subunit mRNA, progesterone withdrawal drastically reduced its abundance at any tested day of exposure in a range between 32% and 47% (Figs. 1 and 2A). Immunocytochemical experiments performed on progesterone withdrawn cells exposed for 5 days to progesterone demonstrate that also the corresponding peptide was reduced of about 46% (Fig. 2B). This last effect of progesterone withdrawal on δ peptide expression was similar to that produced by allopregnanolone withdrawal (Table 1).

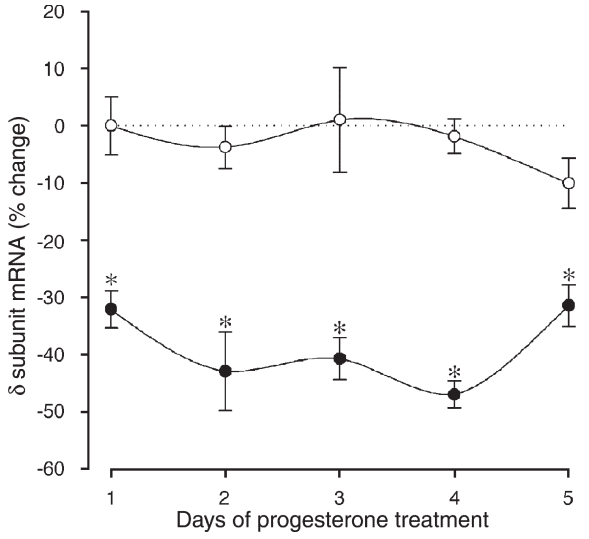

Fig. 1. Effects of progesterone exposure and progesterone withdrawal on the abundance of the $GABA_AR$ δ subunit mRNA in cerebellar granule cells. Cultured rat cerebellar granule cells were incubated for 1 to 5 days as indicated with 1 μM progesterone (white circles) and then for 6 h in progesterone-free medium (progesterone withdrawal, black circles), after which the amount of the mRNA for the $GABA_AR$ δ subunit was determined with an RNase protection assay. Data are expressed as percentage change relative to control cultures and are means±S.E.M. of values ($n=12$) from three independent experiments. *$p<0.001$ versus corresponding control.

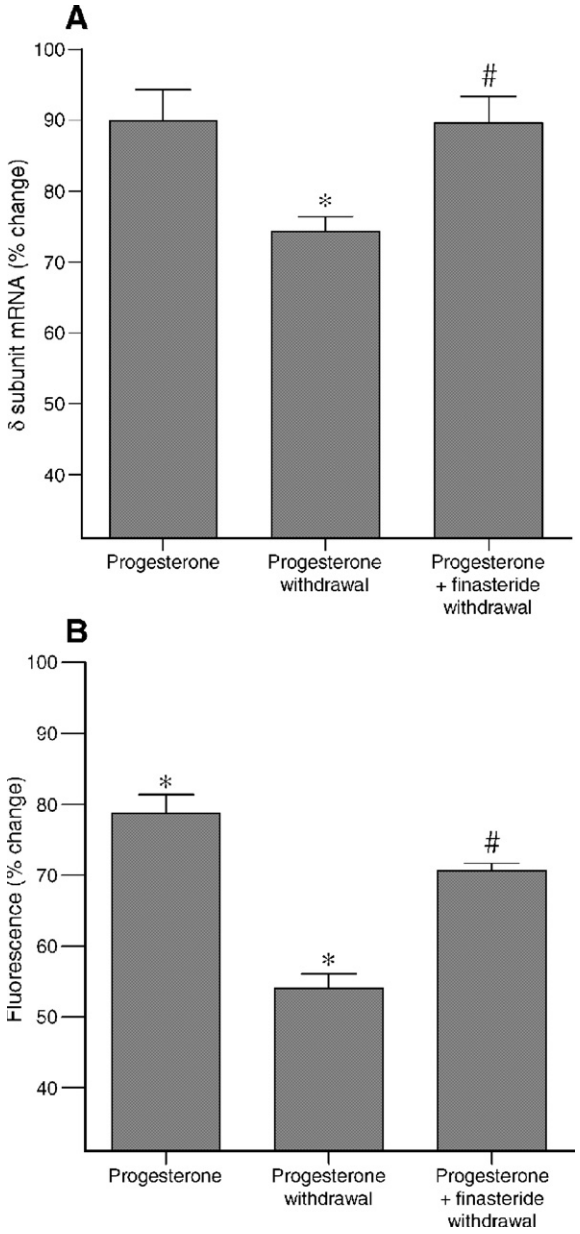

Fig. 2. Effects of finasteride on the abundance of the $GABA_AR$ δ subunit mRNA and corresponding peptide, in cerebellar granule cells subjected to progesterone withdrawal. Cultured rat cerebellar granule cells were incubated for 5 days with 1 μM progesterone with or without 10 μM finasteride and then subjected to withdrawal for 6 h, after which the amount of the mRNA for the $GABA_AR$ δ subunit (A) and corresponding peptide (B) were determined with an RNase protection assay or by immunocytofluorescence respectively. Data are expressed as percentage change relative to control cultures and are means±S.E.M. of values ($n=12$) from three independent experiments. *$p<0.001$ versus corresponding control; #$p<0.001$ versus progesterone withdrawal.

The effects of progesterone withdrawal after 5 days exposure was abolished, both at mRNA and peptide levels, by concomitant treatment of cerebellar granule cells with finasteride (Fig. 2A and B). Furthermore, time course studies demonstrate that the effect of progesterone withdrawal on the down-regulation of the δ subunit mRNA was time dependent and reversible, with the levels returning to control 24 h after progesterone removal (Fig. 3).

Table 1
Effects of neuroactive steroids treatment and withdrawal on $GABA_A$ receptor δ subunit peptide levels

Steroid treatment	$GABA_A$ receptor δ subunit peptide levels immunofluorescence (% change)
THDOC (1 μM, 5 days)	−27.33±1.86
Allopregnanolone (1 μM, 5 days)	−32.66±7.93
Allopregnanolone withdrawal (6 h)	−57.00±1.20

Cells were cultured for 5 days in the absence (control) or presence of the indicated steroid, after which the allopregnanolone-treated cells were incubated for an additional 6 h (withdrawal), in allopregnanolone-free medium. The cells were subjected to immunofluorescence analysis with antibodies to the $GABA_AR$ δ subunit and an epifluorescence microscope. The obtained images were subjected to semiquantitative measurement of the level of δ subunit immunoreactivity. Data are expressed as percentage change in fluorescence intensity relative to cells incubated in the absence of steroids (control) and are means±S.E.M. of values from 30 randomly selected cells in three independent experiments. *$p<0.001$ versus control neurons.

We next examined whether the changes in expression of the $GABA_A$ receptor δ subunit gene induced by chronic exposure to and withdrawal of progesterone in cerebellar granule cells were accompanied by parallel changes in $GABA_A$ receptor function.

The modulatory effect of several neuroactive steroids on $GABA_A$ receptor function is markedly enhanced by the presence of the δ subunit (Adkins et al., 2001; Brown et al., 2002; Wohlfarth et al., 2002). Consistent with such observations, this modulatory action is impaired in mice that lack the δ subunit (Mihalek et al., 1999; Spigelman et al., 2003). We therefore examined whether the changes in δ subunit expression elicited by chronic progesterone exposure and progesterone withdrawal in cerebellar granule cells were associated with parallel changes in the effect of allopregnanolone.

Given that receptors containing the δ subunit manifest a greater sensitivity to the partial agonist THIP (4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol, or gaboxadol) than do those containing the γ_2 subunit (Adkins et al., 2001; Brown et al., 2002), we used this compound to evoke $GABA_A$ receptor-mediated Cl^- currents in cerebellar granule cells in culture. We utilized a THIP concentration previously used (Follesa et al., 2005) extrapolated by the concentration–response relations for THIP-evoked Cl^- currents in cerebellar granule cells in culture in the whole-cell patch-clamp configuration (Follesa et al., 2005).

Chronic progesterone exposure slightly reduce the modulatory effect of 1 μM allopregnanolone (144±48% potentiation) on THIP-evoked Cl^- current compared with that apparent in control cells (187±59%) (Fig. 4). However, withdrawal of progesterone for 6 h was associated with a significant decrease (51±7% potentiation, $p<0.05$) in allopregnanolone efficacy (Fig. 4). These last observations well correlate with the progesterone withdrawal-induced down-regulation of the $GABA_A$ receptor δ subunit.

3.4. Mechanism of the effect of long-term progesterone exposure on $GABA_A$ receptor plasticity

It remains to be determined whether changes in $GABA_A$ receptor plasticity induced by long-term exposure to progesterone or its metabolites are mediated by allosteric modulation of $GABA_A$ receptors or by an indirect genomic action. The progesterone metabolite allopregnanolone do not influence gene expression by direct interaction with the intracellular progesterone receptor but do modulate neuronal excitability by acting at membrane-bound $GABA_A$ receptors. However, it remains possible that neuroactive steroids influence gene expression indirectly by their conversion to other metabolites (5α- or 5β-dihydroprogesterone, DHP) that could bind to and activate the intracellular progesterone receptor (Rupprecht et al., 1996, 1993). However, the changes in $GABA_A$

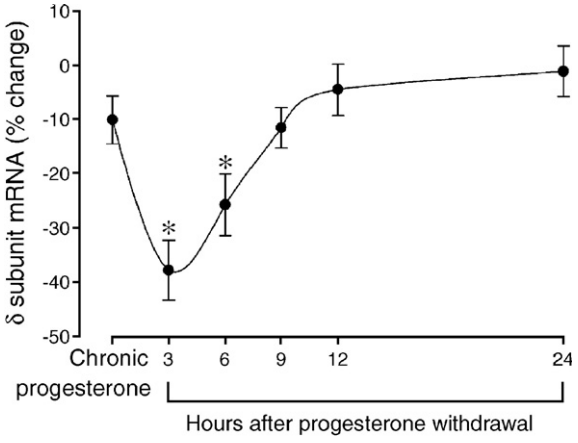

Fig. 3. Time dependent effect of progesterone withdrawal on the abundance of the $GABA_AR$ δ subunit mRNA in cerebellar granule cells. Cultured rat cerebellar granule cells were incubated first for 5 days with 1 μM progesterone (chronic progesterone) and then for the indicated times in progesterone-free medium (progesterone withdrawal), after which the amount of the mRNA for the $GABA_AR$ δ subunit was determined with an RNase protection assay. Data are expressed as percentage change relative to control cultures incubated in the absence of progesterone and are means±S.E.M. of values ($n=9$) from three independent experiments. *$p<0.01$ versus corresponding control.

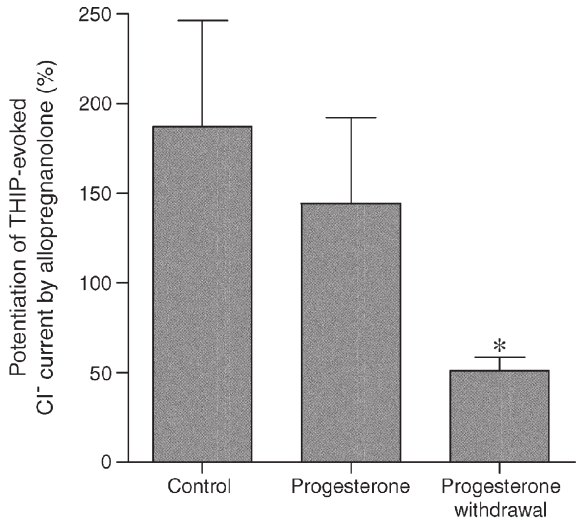

Fig. 4. Effects of chronic exposure to and subsequent withdrawal of progesterone on $GABA_A$ receptor THIP-evoked Cl^- currents modulated by allopregnanolone in cerebellar granule cells. Cerebellar granule cells were incubated for 5 days in the absence (control) or presence of 1 μM progesterone (progesterone) or subjected to 6 h withdrawal (progesterone withdrawal) and then subjected to recordings of Cl^- currents evoked by 3 μM THIP in the presence of 1 μM allopregnanolone. Data are expressed as percentage potentiation of the THIP response and are means±S.E.M. of values from nine neurons for each experimental group. *$p<0.05$ versus control cells.

receptor gene expression induced by progesterone treatment or pregnancy are unlikely to be mediated by the interaction of other metabolites with intracellular progesterone receptors. The high concentrations of progesterone present both in pharmacologically treated cells (Follesa et al., 2000) and in the brain of pregnant rats (Concas et al., 1998) would be expected to preclude an action at the intracellular progesterone receptor of DHP, given that the concentrations of this metabolite and its affinity for this receptor is markedly smaller than that of progesterone. Although a genomic action of progesterone mediated by other transcriptional regulatory proteins that contribute to the expression of $GABA_A$ receptor subunit genes cannot be excluded, the observations that finasteride prevents the increase in allopregnanolone concentrations both in cultured cerebellar granule cells (Follesa et al., 2000) and in the brain of pregnant rats (Concas et al., 1998), as well as inhibits the associated changes in $GABA_A$ receptor function (Concas et al., 1998) and gene expression (Concas et al., 1998; Follesa et al., 2000) suggest that these latter changes are the consequence of allopregnanolone action at the steroid recognition site of the $GABA_A$ receptor.

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