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ORIGINAL ARTICLE

GABA Receptor-Mediated Effects in the Peripheral Nervous System

A Cross-Interaction With Neuroactive Steroids

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Abstract

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the adult mammalian central nervous system (CNS), exerts its action via an interaction with specific receptors (e.g., $GABA_A$ and $GABA_B$). These receptors are expressed not only in neurons but also on glial cells of the CNS, which might represent a target for the allosteric action of neuroactive steroids. Herein, we have demonstrated first that in the peripheral nervous system (PNS), the sciatic nerve and myelin-producing Schwann cells express both $GABA_A$ and $GABA_B$ receptors. Specific ligands, muscimol and baclofen, respectively, control Schwann-cell proliferation and expression of some specific myelin proteins (i.e., glycoprotein P0 and peripheral myelin protein 22 [PMP22]). Moreover, the progesterone (P) metabolite allopregnanolone, acting via the $GABA_A$ receptor, can influence PMP22 synthesis. In addition, we demonstrate that P, dihydroprogesterone, and allopregnanolone influence the expression of $GABA_B$ subunits in Schwann cells. The results suggest, at least in the myelinating cells of the PNS, a cross-interaction within the GABAergic receptor system, via $GABA_A$ and $GABA_B$ receptors and neuroactive steroids.

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Index Entries: Peripheral nervous system; Schwann cell; myelin; $GABA_A$ receptor; $GABA_B$ receptor; progesterone; allopregnanolone.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian nervous system (Barres et al., 1990). In the central nervous system (CNS), GABA is primarily produced by inhibitory neurons and released during the firing of action potentials (Kunkel et al., 1986) in a process known as phasic inhibition (Farrant and Nusser, 2005). However, a tonic inhibition resulting from continuous activation, mainly of extrasynaptic receptors, is also present in some neurons (Semyanov et al., 2003; Belelli and Lambert, 2005; Farrant and Nusser, 2005). In common with other classic neurotransmitters in the CNS, GABA is additionally produced and

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released by glial cells (e.g., astrocytes). In the hippocampus, for example, GABA released by astrocytes regulates neuronal activity and tonic inhibition (Liu et al., 2000; Jow et al., 2004). Furthermore, in the developing CNS, different mechanisms involving the activation of GABA receptors influence various processes such as neuronal or glial precursor proliferation, differentiation, and migration (Barres et al., 1990; Al-Dahan and Thalmann, 1996; Ben Ari, 2002; McCarthy et al., 2002; Owens and Kriegstein, 2002).

Neuroactive steroids are potent modulators of some CNS functions and are therefore of interest for their sedative, anxiolytic, anticonvulsant, and anesthetic properties (Reddy, 2003; Belelli and Lambert, 2005). It is generally believed that neuroactive steroids such as dehydroepiandrosterone, pregnenolone, and their sulfates, as well as the 3α-hydroxylated-derivatives tetrahydroprogesterone (THP or allopregnanolone), tetrahydrodeoxicorticosterone (THDOC), and 5α-androstane-3α,17α-diol (3α-diol or androstenediol), might interact with different neurotransmitter receptors (Bovolin et al., 1992; reviewed in this issue by Leonelli et al. and Schlichter et al.). Arguably the best characterized nongenomic action of neuroactive steroids is the action of THP and THDOC to enhance the function of GABA type A (i.e., $GABA_A$) receptor (Majewska et al., 1986; Rupprecht and Holsboer, 1999; Lambert et al., 2003). In the nanomolar concentration range the neuroactive steroids act allosterically on $GABA_A$ receptor and enhance the action of GABA, whereas at higher concentrations (micromolar range) they directly gate the $GABA_A$ receptor channel complex (Callachan et al., 1987; Puia et al., 1990). A recent paper performed on hippocampal neurons grown in synaptic isolation demonstrated that neuroactive steroids directly gate $GABA_A$ receptors at lower concentration (about 100 n*M*) as well (Shu et al., 2004). The kinetics of this receptor activation is relatively slow, but this effect might underpin some important cellular and behavioral effects of neuroactive steroids (Shu et al., 2004). However, the GABA modulatory effects of neuroactive steroids are also evident in the spinal cord (Keller et al., 2004). In the dorsal horn neurons of the spinal cord, the strength of $GABA_A$-mediated synaptic inhibition, during development and under physiopathological conditions, can be locally modulated by controlling the synthesis of 5α-reduced neuroactive steroid metabolites (Keller et al., 2004). In the peripheral nervous system (PNS), as reported by Leonelli et al. (this issue), it has been observed that progesterone (P) and its 5α-derivatives dihydroprogesterone (DHP) and THP, are the principal neuroactive steroids able to affect many biochemical and morphological parameters in vivo (i.e., sciatic nerve of rat) and in vitro (i.e., Schwann cell cultures) (Magnaghi et al., 2001; Melcangi et al., 2003, 2005). In particular, some of these effects, described below, seem to be attributable to an involvement of the $GABA_A$ receptor (Melcangi et al., 2005).

In this review we summarize results recently obtained on the presence of GABA receptors in the PNS, specifically in Schwann cells, and then we report the subsequent effects of their modulation by specific ligands and/or neuroactive steroids. On this basis, a possible hypothesis of a cross-interaction between $GABA_A$, $GABA_B$, and neuroactive steroids is proposed.

GABA Receptors and the Nervous System

According to pharmacological and electrophysiological studies, actions of GABA in the nervous system are mediated throughout different ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors (Bowery and Enna, 2000; Bettler et al., 2004).

The $GABA_A$ receptor is a member of the ligand-gated ion channel family, composed of five subunits drawn from a repertoire of α1–6, β1–3, γ1–3, δ, ε, π, ρ1–3, and θ (Whiting et al., 1995, 1997; Lambert et al., 2003). The $GABA_A$ receptor is blocked by bicuculline and picrotoxin but is enhanced by benzodiazepines, barbiturates, a variety of general anesthetics, and neuroactive steroids (Park-Chung et al., 1999, Belelli and Lambert, 2005). In particular, THP and THDOC, as described above, do not exert a classic steroidal genomic action but act in a nongenomic manner as potent allosteric modulators of the $GABA_A$ receptor (Belelli and Lambert, 2005). Modulation of the $GABA_A$ receptor by neuroactive steroids is enantioselective and is partially dependent on receptor subunit composition (Lambert et al., 2003). The $GABA_A$ receptor is widely distributed in adult mammalian brain neurons (Sieghart and Sperk, 2002) but is also present in astrocytes (Berger et al., 1992; Hosli et al., 1997; Kang et al., 1998; Israel et al., 2003) and in certain oligodendrocyte progenitor cells (Gilbert et al., 1984; Kettenmann et al., 1984; Kirchhoff and Kettenmann, 1992).

The $GABA_B$ receptor was first identified by Bowery et al. (1980) as a distinct, baclofen-sensitive, metabotropic receptor. The designation $GABA_B$ was given to distinguish it from the bicuculline-sensitive,

ionotropic $GABA_A$ receptor (Bowery et al., 1980, 2004). The $GABA_B$ receptors are members of the seven-transmembrane G protein-coupled receptor superfamily (Bowery et al., 2004), which might influence presynaptic neurotransmitter release and cause postsynaptic silencing of excitatory neurotransmission via the activation of second-messenger systems, mainly by influencing the activity of adenylate cyclase and by modulating calcium and potassium channel activity (Marshall et al., 1999; Bowery and Enna, 2000). In 1997, the first cDNAs encoding two $GABA_B$ receptor proteins, initially named $GABA_B$-1a and $GABA_B$-1b (Kaupmann et al., 1997), were identified. Subsequently, novel $GABA_B$ receptor isoforms were cloned (Isomoto et al., 1998; Pfaff et al., 1999). Of particular interest, a number of laboratories independently identified a cDNA encoding for the $GABA_B$ receptor isoform 2 ($GABA_B$-2) (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). The $GABA_B$-1 receptor is retained in the endoplasmic reticulum and is transported to the cell surface only in the presence of $GABA_B$-2; thus, the formation of a functional heterodimeric complex depends on the presence of both subunits 1 and 2 (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999; Calver et al., 2000). $GABA_B$ heterodimer component proteins (i.e., $GABA_B$-1 and $GABA_B$-2) are expressed widely throughout the neuronal compartment of the brain and spinal cord (Margeta-Mitrovic et al., 1999; Charles et al., 2001). However, recent observations suggest that certain types of glial cells (i.e., astrocytes and activated microglia) from the CNS exhibit $GABA_B$ receptor immunoreactivity (Charles et al., 2003) and might be considered a possible target for the action of $GABA_B$ receptor agonists (Kang et al., 1998; Clark et al., 2000).

$GABA_A$ Receptor in the PNS

In the CNS the $GABA_A$ receptor is expressed both in neurons and glial cells. Furthermore, studies in cat, rat, frog, and human embryo dorsal root ganglion (DRG) have shown that DRG neurons possess functionally active $GABA_A$ receptors (Deschennes et al., 1976; Gallagher et al., 1978; Inoue and Akaike, 1988; Valeyev et al., 1999). In general in the PNS, myelinated and unmyelinated fibers possess GABA receptors and GABA carriers (Brown and Marsh, 1978, Brown et al., 1979; Morris et al., 1983; Olsen et al., 1984). Although, $GABA_A$ receptors are present on normal mammalian sensory axons and are reestablished on regenerated sensory axons, however, the presence of these receptors on Schwann cells had not been investigated (Bhisitkul et al., 1987). Only the uptake of GABA by a high-affinity mechanism has been demonstrated in purified rat Schwann cells maintained in cell culture in vitro for up to 6 mo (Gavrilovic et al., 1984). Studies performed in our laboratory, using RT-PCR analysis have demonstrated that sciatic nerve and Schwann cells express mRNAs coding for some subunits for the $GABA_A$ receptor (Table 1). Primer pairs specific for rat subunits $\alpha1$, $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$, $\beta2$, $\beta3$, $\gamma1$, $\gamma2L$, and $\gamma2S$ were used, and the results were compared to those obtained with different brain regions (used as a positive control). The major bands found in the sciatic nerve of adult male rats were those of $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, and $\beta3$. Bands for $\alpha6$ and $\gamma1$ subunits were never evident, whereas faint signals for $\alpha1$, $\gamma2L$, and $\gamma2S$ subunits were observed. The $GABA_A$ subunit mRNAs in Schwann cells maintained in cell culture showed a similar pattern of expression, although with different intensities. The RT-PCR products were confirmed by sequencing, and the results indicated a 100% identity with mRNA sequences of $GABA_A$-$\alpha3$ (accession no. X51991), -$\beta1$ (accession no. X15466), -$\beta2$ (accession no. X15467), and -$\beta3$ (accession no. U14420) subunits, and a 97% homology for the $\alpha2$ subunit (accession no. L08491) (Melcangi et al., 1999; Magnaghi et al., 2001). To confirm the presence of the most representative $GABA_A$ receptor subunit proteins on rat Schwann cells (maintained in culture), an immunocytochemical analysis using a confocal microscope, was performed. Using specific anti-$\alpha2$ and anti-$\alpha3$ antibodies raised in guinea pig, we observed a clear immunoreactivity for both proteins in Schwann cells (Fig. 1). Moreover, using a pan-antibody recognizing the $\beta3$ subunit, a specific signal with a more patchy distribution in the Schwann cells was revealed (Fig. 1). The negative controls, in which the primary antibodies were substituted with a preimmune serum, revealed nondetectable signals (Fig. 1).

$GABA_B$ Receptor in the PNS

The presence of the $GABA_B$ receptor in the PNS has been demonstrated in the rat DRG, in peripheral axons, in autonomic nerve terminals, and in pig nodose ganglion cells (Bowery et al., 1981; Desarmenien et al., 1984; Liske and Morris, 1994; Sun and Chiu, 1999; Towers et al., 2000; Zagorodnyuk et al., 2002), but the expression of the receptor in Schwann

Table 1
Presence of $GABA_A$ and $GABA_B$ Receptor Subunits in the Whole Rat Sciatic Nerve and in Schwann Cell Culture of the PNS

	Subunits	Sciatic nerve	Schwann cell
$GABA_A$	α1	RNA +/–	RNA +/–
	α2	RNA +	RNA +, Protein +
	α3	RNA +	RNA +, Protein +
	α6	RNA –	RNA –
	β1	RNA +	RNA +
	β2	RNA +	RNA +
	β3	RNA +	RNA +, Protein +
	γ1	RNA –	RNA –
	γ2S	RNA +/–	RNA +/–
	γ2L	RNA +/–	RNA +/–
$GABA_B$	1a	RNA +, Protein +	RNA +, Protein +
	1b	RNA +, Protein +	RNA +, Protein +
	2	RNA +, Protein +	RNA +, Protein +

(+) A reliable signal; (+/–) a faint signal; (–) lack of a signal.

cells has not yet been investigated. By utilizing RT-PCR, we have evaluated whether the mRNAs encoding for different isoforms of $GABA_B$ receptors (i.e., 1a, 1b, 1c, and 2) were present in sciatic nerve and Schwann cells. Analysis of rat whole brain was performed as a positive control. The data obtained indicated that $GABA_B$ isoforms 1a, 1b, 1c, and 2 mRNAs are not only expressed in the brain, as reported previously (Margeta-Mitrovic et al., 1999; Towers et al., 2000; Charles et al., 2001), but also in the sciatic nerve and Schwann cells (Table 1). These findings were supported by Western blot analysis, as an antibody raised against the $GABA_B$-1 protein revealed two bands of approx 130 and 100 kDa (Magnaghi et al., 2004a). As reported by others (Kaupmann et al., 1997; Ige et al., 2000), bands of this size correspond respectively to the 1a and 1b isoforms of native subunits. Furthermore, using an antibody against the $GABA_B$-2 receptor, a band of approx 110–120 kDa was evident for both sciatic nerve and Schwann cells (Magnaghi et al., 2004a). This band has a molecular weight identical to the native $GABA_B$-2 receptor protein (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ige et al., 2000).

The presence of the $GABA_B$-1 and $GABA_B$-2 protein has also been evaluated by immunocytochemistry. By utilizing epifluorescence microscopy, a longitudinal section of rat sciatic nerve reveals intense staining for the $GABA_B$-1 protein in Schwann cells, which were identified by the use of the classic marker glycoprotein P0 (Magnaghi et al., 2004a). The presence of the $GABA_B$-1 protein is also evident in rat Schwann cell cultures (Fig. 2), with staining widely distributed in perinuclear space and in cellular processes. In rat sciatic nerve the presence of the $GABA_B$-2 receptor protein was confirmed by immunocytochemistry. Intense fluorescent staining for this receptor is evident in the Schwann cells body compartment, as observed in the coronal section of rat sciatic nerve (Magnaghi et al., 2004a). Finally, the $GABA_B$-2 receptor was also identified in cultures of rat Schwann cells (Fig. 2), with an identical pattern of distribution to that established for the $GABA_B$-1 receptor. A confirmation of the presence of the $GABA_B$ receptors in the PNS comes from a study with $GABA_B$-1 knockout mice, in which it has been observed that similar to the CNS, the $GABA_B$-1 subunit is an essential requirement for $GABA_B$ function in the peripheral enteric nervous system (Sanger et al., 2002).

Effects of Specific Ligands of $GABA_A$ and $GABA_B$ Receptors in the PNS

To investigate the potential physiological significance of the expression of $GABA_A$ and $GABA_B$ receptors in Schwann cells, we determined the influence of specific receptor ligands on certain important features and properties of such cells maintained in culture. For example, peripheral myelin protein 22 (PMP22), which is one of the most important proteins required for the maintenance of the multilamellar structure of the peripheral myelin, has been considered (Quarles, 1997; Bronstein, 2000). In humans, alterations of the PMP22 gene are associated with a set of hereditary peripheral neuropathies,

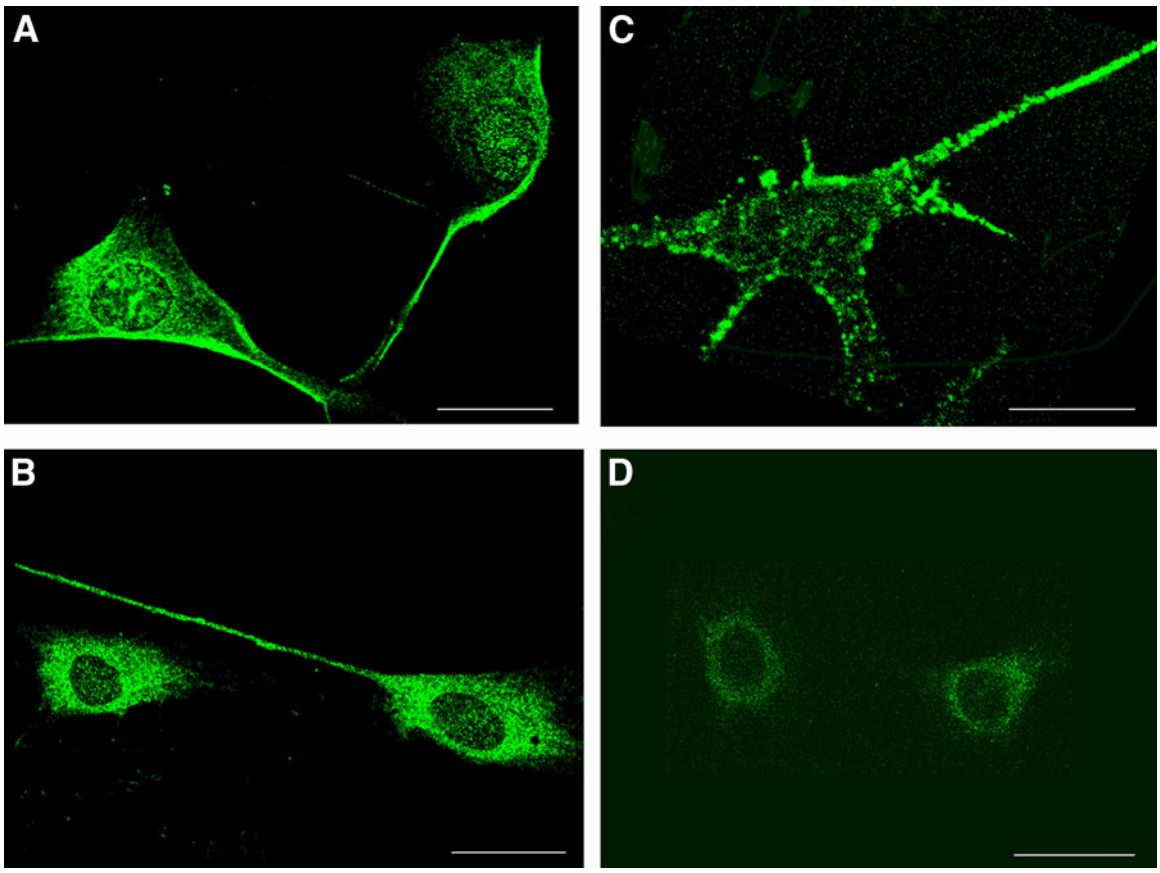

Fig. 1. Localization of $GABA_A$ receptor subunits α2, α3, and β3 in rat Schwann cell culture by confocal microscopy. Schwann cells were cultured on cover slips (Magnaghi et al., 2004a); following differentiation, they were fixed for 20 min in 4% PBS-paraformaldehyde. The cover slips were incubated overnight at 4°C with one of the primary antibodies against $GABA_A$ subunits. We have used guinea pig anti-$GABA_A$-α2 (1:500) and anti-$GABA_A$-α3 (1:300), which were a generous gift of Prof. J. M. Fritschy (University of Zurich, Switzerland), and rabbit anti-$GABA_A$-β3 (1:250). After washing, the cover slips were incubated for 2 h with Alexa-488 anti-guinea pig secondary antibody (1:200), rinsed, and mounted with PermaFluor™ mounting media. Controls for antibody specificity included a lack of a primary antibody. Confocal laser microscopy was performed using a Bio-Rad Radiance 2100 Confocal System (Bio-Rad, Milan, Italy) and a Nikon TE2000-S Eclipse microscope, utilizing the 488-nm laser. Immunoreactivity for the α2 subunit **(A)** and the α3 subunit **(B)** in the Schwann cells is evident. **(C)** A specific immunoreactivity for the β3 subunit showed a patchy distribution in Schwann cells. **(D)** Schwann cells in which primary antibodies were substituted with preimmune serum revealed no detectable signal. Scale bar, 15 μm.

e.g., Charcot-Marie-Tooth type-1A disease (Naef and Suter, 1998). We demonstrated that a 24-h exposure of Schwann cells to a relatively low concentration (1 μ*M*) of the selective $GABA_A$ receptor agonist muscimol exerted a clear stimulatory effect on the level of PMP22 mRNA, suggesting that PMP22 might be under the control of the $GABA_A$ receptor ligands (Magnaghi et al., 2001; Melcangi et al., 2005).

In the Schwann cell culture model $GABA_B$ receptors are negatively coupled to the adenylate cyclase system (Magnaghi et al., 2004a). It should be emphasized that modification of cAMP levels in Schwann cells is associated with changes in their morphological and functional parameters, including proliferation, differentiation, and their ability to synthesize certain myelin proteins (LeBlanc et al., 1992; Lee et al., 1999; Mirsky and Jessen, 1999; Mirsky et al., 2001). Consequently, we initially evaluated whether the specific $GABA_B$ agonist baclofen might influence the proliferation of Schwann cells induced by forskolin. Baclofen (100 μ*M*) counteracted the forskolin-induced proliferation of Schwann cells at 4 d in vitro, and this effect became more evident at later times of exposure (i.e., 5 and 6 d in vitro) (Magnaghi et al., 2004a). Additionally, the percentage of Schwann-BrdUrd-immunopositive cells was significantly

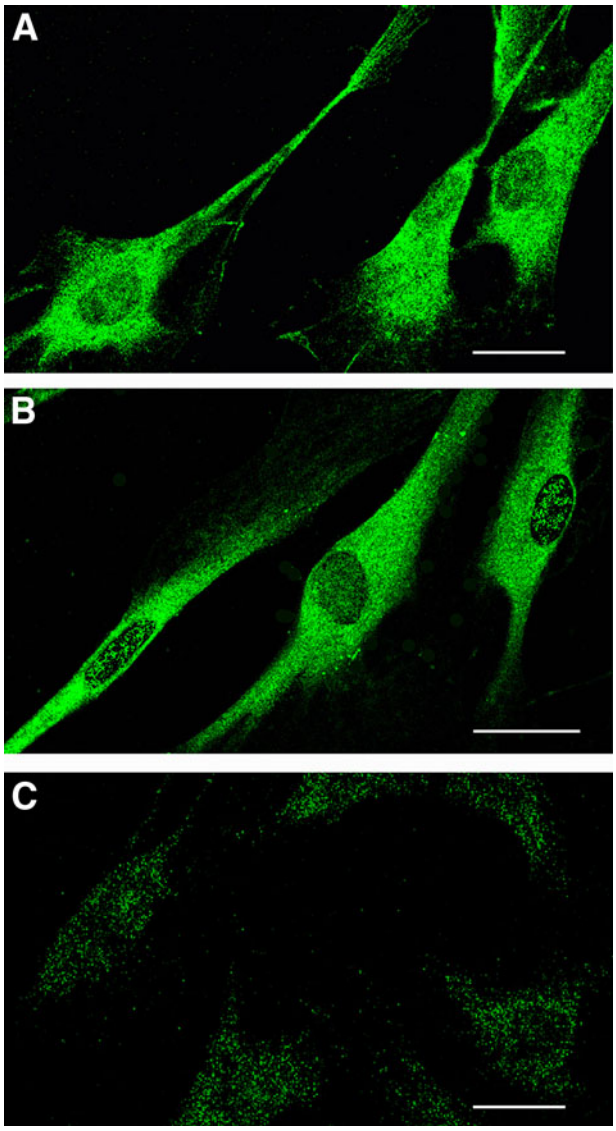

Fig. 2. Localization of $GABA_B$ receptor subunit-1 and -2 in rat Schwann cell culture by confocal microscopy. Schwann cells were obtained and treated as described in Fig. 1. The cover slips were incubated with one of the primary antibodies against $GABA_B$ subunits. We have used the guinea pig pan-anti-$GABA_B$-1 (1:300), which recognizes both 1a and 1b subunits, and anti-$GABA_B$-2 (1:250), as described in Magnaghi et al. (2004a). The immunoreactivities were revealed with Alexa-488 anti-guinea pig secondary antibody (1:250), and subsequently the samples were mounted with PermaFluor™ mounting media. Controls for antibody specificity included a lack of a primary antibody. Confocal laser microscopy was performed using a Bio-Rad Radiance 2100 Confocal System and a Nikon TE2000-S Eclipse microscope, utilizing the 488-nm laser. **(A)** Immunopositivity for the $GABA_B$-1 subunit is evident in the Schwann cells. **(B)** Schwann cells are additionally immunopositive for subunit 2. **(C)** Schwann cells with preimmune serum instead of primary antibodies revealed no detectable signal. Scale bar, 15 μm.

reduced by baclofen treatment. Furthermore, 2 h of exposure to 100 μ*M* baclofen also modified the mRNA levels of some specific peripheral myelin proteins, including the glycoprotein P0, PMP22, myelin-associated glycoprotein, and Connexin 32. The quantification of these changes revealed that mRNA levels of P0 and PMP22 are significantly decreased after exposure to baclofen. Western blot analysis indicated that protein levels of P0 and PMP22 are also similarly decreased after a 2-h exposure to baclofen (Magnaghi et al., 2004a).

Collectively, the observations presented here indicate that Schwann cells are a potential target for GABA action. The activation of $GABA_A$ receptors exerts a stimulatory effect on PMP22 (Magnaghi et al., 2001), whereas the activation of $GABA_B$ receptors influences important cellular processes in these peripheral glial cells (i.e., proliferation and myelin protein expression). Consequently, at least in the case of PMP22, these findings reveal that depending on the receptor involved ($GABA_A$ or $GABA_B$), GABA might increase or decrease the synthesis of this myelin protein.

Neuroactive Steroids and GABA Receptors in the PNS

Neuroactive steroids such as THP, THDOC, and 3α-diol exert their actions primarily by binding to the $GABA_A$ receptor to enhance the function of this transmitter-gated ion channel (Park-Chung et al., 1999; Rupprecht and Holsboer, 1999; Lambert et al., 2003; Belelli and Lambert, 2005). Although the effects of these endogenously neuroactive steroids on neuronal function in the CNS have been studied extensively, their actions in the PNS have received little consideration (Melcangi et al., 1999, 2000b, 2003).

Similarly, investigations on the effects of neuroactive steroids, such as P or estradiol, on $GABA_B$ receptors, have been restricted to the CNS (Al-Dahan and Thalmann, 1996; Kelly et al., 2003). However, it could be suggested that in comparison to what happens on the $GABA_A$ receptor, the neuroactive steroids seem to modulate $GABA_B$ with different mechanisms. Namely, the neuroactive steroid action on $GABA_B$ does not seem to be related to a direct interaction with the receptor sites. For example, in neocortex of ovariectomized rats, physiological levels of P increased the apparent $GABA_B$ receptor density (defined by B_{max}), whereas the antiprogestin RU38486 produced the opposite effect (Thalmann and Tehrani, 2000). Moreover, during the estrous cycle, variations

of P concentration in the cerebral cortex influence the binding of GABA to $GABA_B$ receptors (Al-Dahan and Thalmann, 1996).

Further examples of an interaction between neuroactive steroids and GABA receptors in the CNS are represented by GABA-regulated action on endogenous neuroactive steroid synthesis. In the frog hypothalamus, GABA inhibits, in a dose-dependent manner, the activity of several key steroidogenic enzymes, including 3α-hydroxysteroid-dehydrogenase and cytochrome P450-C17 (Do-Rego et al., 2000; Mensah-Nyagan et al., 2001). This effect is mimicked by muscimol and blocked by the specific $GABA_A$ antagonist bicuculline (Do-Rego et al., 2000; Mensah-Nyagan et al., 2001). However, in rat retinal ganglion cells, the synthetic $GABA_A$ agonist muscimol stimulates the biosynthesis of pregnenolone, an effect reversed by the antagonists bicuculline and picrotoxin (Guarneri et al., 1995). The discrepancies in these studies could be attributable to the different species utilized and/or to the different pharmacological properties of GABA with respect to those of synthetic ligands utilized. Moreover, in rat cortex a $GABA_B$-mediated mechanism underpins the increase in THP and THDOC synthesis induced by γ-hydroxybutyric acid (GHB, a metabolite of GABA) (Sanna et al., 2004; Belelli and Lambert, 2005). Successively, neuroactive steroids, acting as amplifiers of the GABA neurotransmission, play a role in the $GABA_A$-mediated actions of GHB (Barbaccia et al., 2002).

The literature described above for the CNS suggests a mutual interplay between the GABAergic system (via $GABA_A$ and $GABA_B$ receptors) and neuroactive steroids, such as P and its derivatives. We therefore investigated whether there is similar crosstalk between these neuroactive steroids and $GABA_A$/$GABA_B$ receptors in the PNS.

Effects of Neuroactive Steroids on $GABA_A$ Receptors

As reported above, P and its 5α-derivatives DHP and THP are unusual modulators of several biochemical and morphological parameters in the PNS. In particular, as reviewed by Leonelli et al. (this issue), these neuroactive steroids stimulate the expression of specific peripheral myelin proteins, such as P0 and PMP22 (Magnaghi et al., 2001; Melcangi et al., 2005). However, whereas the $GABA_A$ receptor-enhancing steroid THP also increases the levels of P0 mRNA, this steroid is the only derivative of P identified to date that can stimulate the levels of PMP22 mRNA and protein (Melcangi et al., 1999, 2000a, 2000b, 2003). The testosterone derivative 3α-diol, another positive allosteric modulator of the $GABA_A$ receptor (Frye et al., 1996a, 1996b), is able to significantly increase gene expression and protein levels of PMP22 (Magnaghi et al., 2000a, 2004b).

In conclusion, these observations with $GABA_A$ receptor modulatory steroids complement those made with muscimol, suggesting that the expression of PMP22 seems to be under the control of the $GABA_A$ receptor in Schwann cells.

Effects of P and its Derivatives on $GABA_B$ Receptors

Certain steroids that enhance $GABA_A$ receptor function, in common with the $GABA_A$ receptor agonist muscimol, influence the expression of the important Schwann cell protein PMP22. Therefore, the nongenomic actions of these steroids somehow produce changes in Schwann cell gene/protein expression. As described above, activation of the $GABA_B$ receptor influences Schwann cell proliferation and expression of some important myelin proteins (Magnaghi et al., 2004a). We therefore investigated in Schwann cells the effects of neuroactive steroids on $GABA_B$ receptor subunit expression. Hence, we initially analyzed the possible effects exerted by P and its 5α-reduced derivatives, DHP and THP, respectively (10 n*M*). Schwann cells in culture were exposed to the steroids for 1, 2, 4, and 24 h, and $GABA_B$-1a, -1b, and -2 subunit expression was analyzed by real-time PCR. We examined all three subunits as $GABA_B$-1a, $GABA_B$-1b, and $GABA_B$-2 because they are essential for the expression of a functional $GABA_B$ receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999; Calver et al., 2000). The experiments revealed that 4 h of exposure to THP produces a robust stimulation of the mRNA of all three subunits, $GABA_B$-1a (Fig. 3a), $GABA_B$-1b (Fig. 3b), and $GABA_B$-2 (Fig. 3c), whereas 24 h of exposure to this neuroactive steroid decreased the expression of the 1b (Fig. 3b) and 2 (Fig. 3c) subunits.

The effect of THP on $GABA_B$ receptor expression is comparable to the influence of this steroid on $GABA_A$ receptor subunit expression in the CNS. Long-term exposure (i.e., 5 d) of cortical neurons in vitro to THP decreases the levels of α and β $GABA_A$ subunit mRNAs (Yu et al., 1996). Similarly, changes in $GABA_A$ receptor subunit composition in rat cerebral cortex

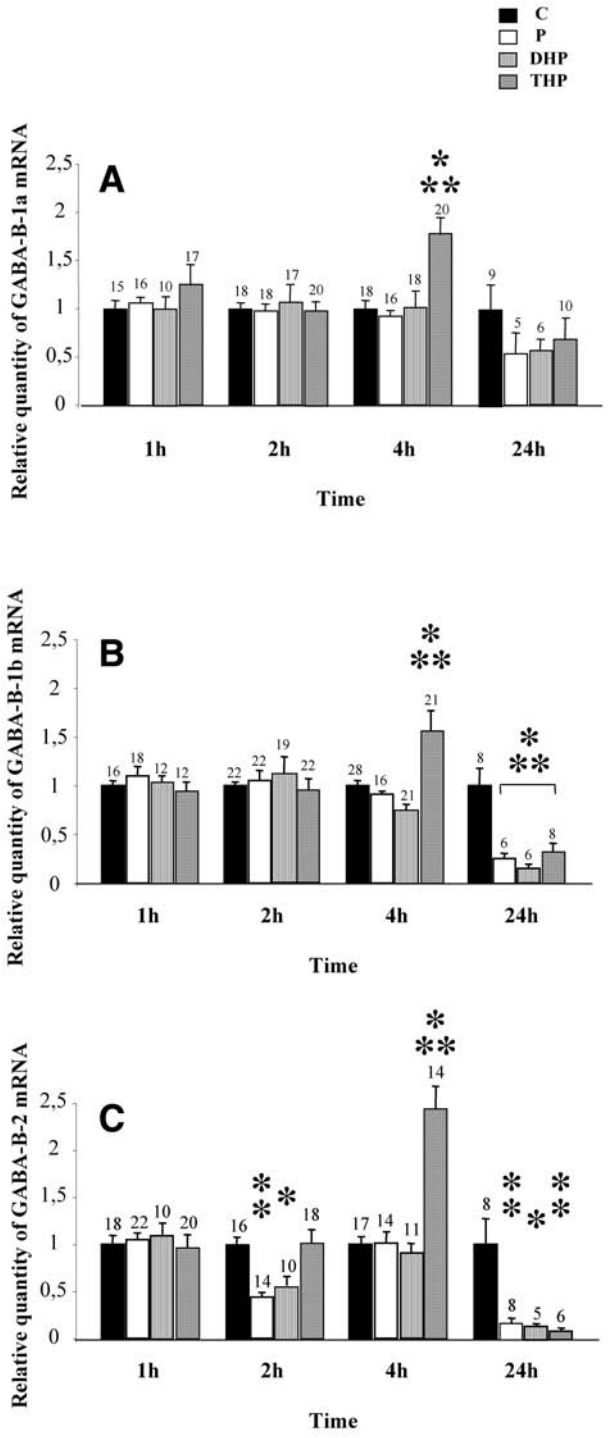

Fig. 3. The effect of progesterone (P), dihydroprogesterone (DHP), and tetrahydroprogesterone (THP) on $GABA_B$-1a **(A)**, -1b **(B)**, and -2 **(C)** subunit expression in rat Schwann cell culture. Schwann cells were exposed for 1, 2, 4, and 24 h to P, DHP, or THP at 10 n*M*; following total RNA phenol-chloroform extraction, the samples were processed for Multiplex PCR assays with specific primers set for 1a, 1b, and 2 subunits. A 1-µg aliquot of each sample was treated with DNAsi, to avoid DNA contamination, then reverse transcribed, according to the High-Capacity cDNA Archive commercial kit (Applera). The PCR reaction was performed using the TaqMan Universal PCR Master Mix (Applera), with the specific TaqMan MGB probe (i.e., for 1a, 1b, and 2 subunits), labeled with fluorochrome 6-FAM (Applera), and the primer pairs for the specific 1a, 1b, and 2 subunit mRNAs. The PCR reactions were performed in multiplex with a housekeeping internal control for the 18S rRNA, labeled with VIC (Applera). We have utilized the ABI Prism 7000 Sequence Detection System (Applera). PCR parameters were 50°C for 2 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. Data analysis has been taken as the cycle at which present threshold value of amplification was reached. Reactions containing a serial dilution of control samples (also called calibrator) have been included on each plate to quantify the relative level of the specific mRNA of interest by the relative standard curve method, as suggested in the manufacturer's user bulletin, no. 2 (Applera). Finally, after normalization for the 18S rRNA, data were expressed as relative quantity to control (C, vehicle treatment). The columns represent mean ± S.E.M. of the number of determinations performed (numbers at top of columns). (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$.

and hippocampus, during pregnancy and after delivery, are influenced by fluctuations in endogenous brain concentration of THP (Concas et al., 1998; Maguire et al., 2005). Therefore, we hypothesize that in the PNS, the neuroactive steroid THP exerts a $GABA_A$-mediated regulation of the $GABA_B$ receptor expression. The intracellular mechanism leading this control is presumably complex and to date has not been identified. However, possible mechanisms by which GABA, after $GABA_A$ receptor activation, might control transcriptional activity have been hypothesized (Obrietan et al., 2002; Galanopoulou et al., 2003; Mantelas et al. 2003). In the developing rat cortex, for instance, the neuronal nitric oxide synthetase and brain-derived neurotrophic factor are controlled via the $GABA_A$ receptor (Mantelas et al., 2003); it has been proposed that the depolarization following $GABA_A$ receptor activation leads to the opening of L-type voltage-gated calcium channels, resulting in an increase of calcium influx, which in turn leads to phosphorylation and activation of the transcription factor cAMP response element-binding (CREB) protein (Mantelas et al., 2003). Interestingly, in rat hippocampal neurons it has been demonstrated that $GABA_B$-1a and $GABA_B$-1b subunit expressions are mediated by CREB protein (Steiger et al., 2004).

Conversely, in our experiments we have also observed that at early times of exposure (i.e., 2 h), P

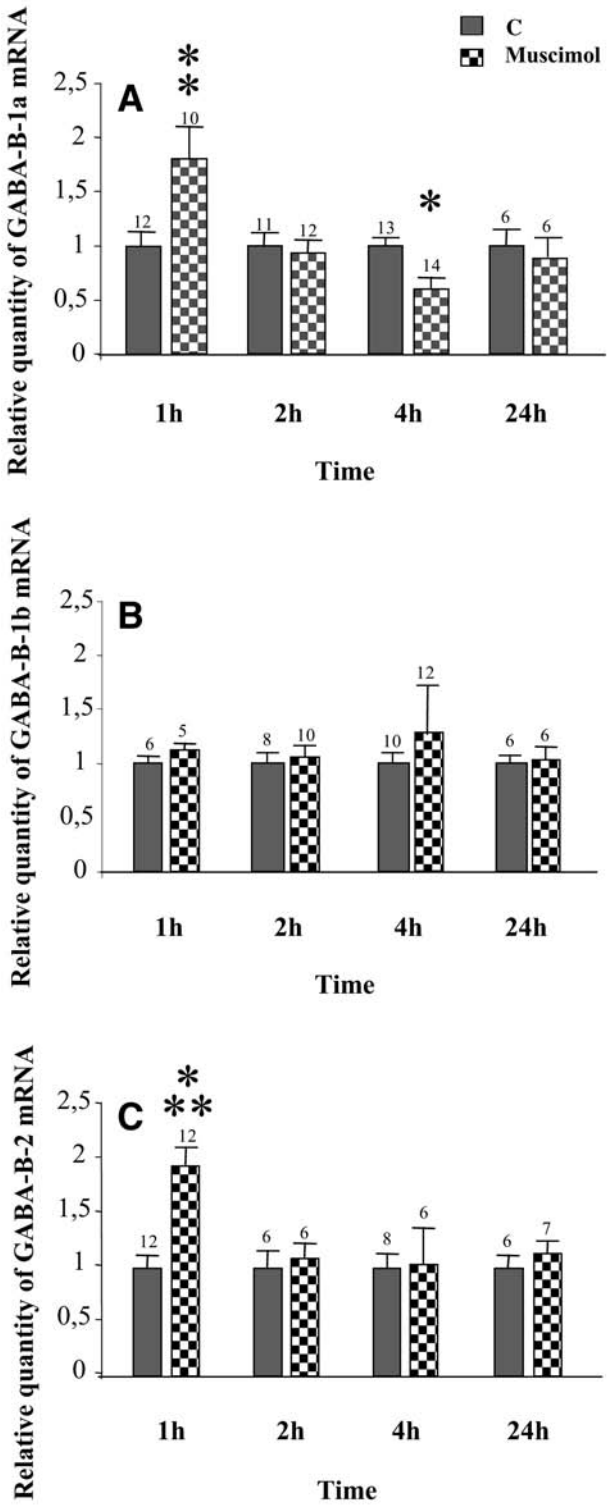

Fig. 4. The effect of muscimol on $GABA_B$-1a **(A)**, -1b **(B)**, and -2 **(C)** subunit mRNA expression in rat Schwann cell culture. Schwann cells were exposed to 100 μ*M* muscimol for 1, 2, 4, and 24 h. After total RNA extraction the samples were then processed for the Multiplex PCR assays with specific primers set for 1a, 1b, and 2 subunits, as described in Fig. 3. After normalization for 18S rRNA, data were expressed as relative quantity to control (C, vehicle treatment). The columns represent mean ± S.E.M. of determinations performed (numbers at top of columns). (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$.

and DHP are able to decrease the expression of the $GABA_B$-2 subunit (Fig. 3c), whereas the mRNA levels of other subunits are not affected (Fig. 3a,b). This effect is evident only with P and DHP, as THP is ineffective at shorter exposure times (i.e., 1 and 2 h), suggesting a different mechanism of action in such a decrease of $GABA_B$-2 subunit expression. The $GABA_B$-2 subunit is important for $GABA_B$ receptor trafficking to the cell surface (Calver et al., 2001). Therefore, in parallel to the effect of P in the CNS (Thalmann and Tehrani, 2000), it is possible to hypothesize that P and DHP rapidly control cell-surface $GABA_B$ receptor availability also in Schwann cells. These effects do not seem to be genomic in nature, because they are not blocked by the antiprogestin RU38486 (1 μ*M*) (data not shown).

Interestingly, at later times of exposure (i.e., 24 h), P and DHP significantly decrease the expression of either subunit 1b or 2 (Fig. 3b,c). This effect is similar to that evoked by THP treatment (Fig. 3b,c). Consequently, a possible hypothesis might be that the effects of P and DHP are attributable to their conversion into THP by the 5α-reductase-3β-hydroxysteroid-dehydrogenase enzymatic complex, which is present in Schwann cells (*see* Leonelli et al., this issue).

Collectively, these observations suggest that the mRNA levels of $GABA_B$ subunits might be influenced differently, mainly by THP, via a $GABA_A$-mediated mechanism, but also partially by its precursors P and DHP.

Effects of Specific GABA Receptor Ligands on $GABA_B$ Receptors

Given that the $GABA_A$ receptor modulator THP influences $GABA_B$ receptor expression, we have evaluated whether this effect is mimicked by activation of the $GABA_A$ receptor. To this purpose we have analyzed, by real-time PCR, $GABA_B$-1a , $GABA_B$-1b, and $GABA_B$-2 subunits in Schwann cell cultures exposed to 100 μ*M* muscimol for 1, 2, 4, and 24 h. The results demonstrate that after 1 h of treatment the $GABA_A$ agonist muscimol exerts a significant increase of the expressions of subunits 1a and 2 (Fig. 4a,c), with a similar pattern of action to that observed with THP

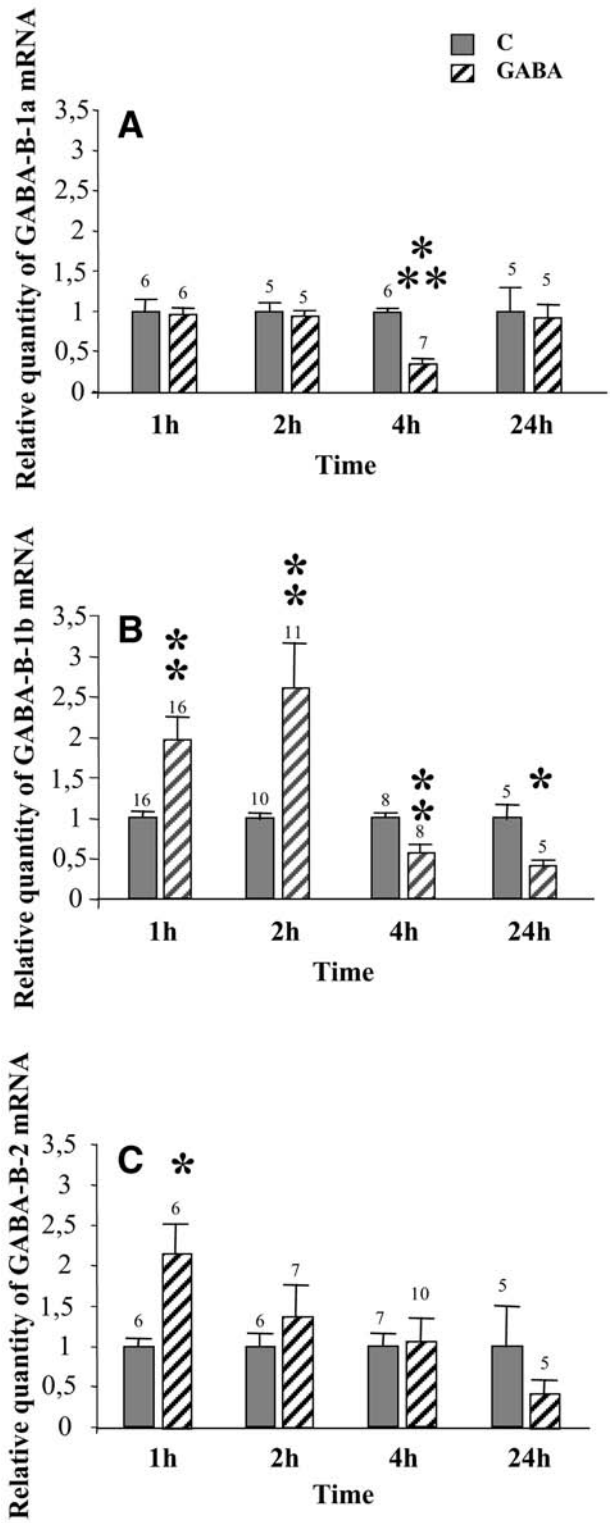

Fig. 5. The effect of GABA on $GABA_B$-1a **(A)**, -1b **(B)**, and -2 **(C)** subunit mRNA expression in rat Schwann cell culture. Schwann cells were exposed to 1 m*M* GABA for 1, 2, 4, and 24 h. After total RNA extraction the samples were then processed for Multiplex PCR assays with specific primers set for 1a, 1b and 2 subunits, as described in Fig. 3. After normalization for 18S rRNA, data were expressed as a relative quantity to control (C, vehicle treatment). The columns represent mean ± S.E.M. of determinations performed (numbers at top of columns). (***) $p < 0.001$; (**) $p < 0.01$; (*) $p < 0.05$.

(Fig. 3a,c). After 4 h of exposure to muscimol, moreover, the expression of the $GABA_B$-1a subunit decreases significantly (Fig. 4a). Thus, the $GABA_B$-1a and -2 subunits are similarly modulated by THP and muscimol. It might be speculated that in Schwann cells of the PNS, the $GABA_B$-1a subunit is under the control of the neuroactive steroid THP via a $GABA_A$-mediated mechanism. Otherwise, at all times of exposure the 1b subunit is never affected by muscimol treatment (Fig. 4b), excluding a mechanism through $GABA_A$ in the control of such a subunit; however, a rapid $GABA_A$ desensitization should be considered.

Finally, regulation of Schwann cell $GABA_B$ subunit expression by GABA (1 m*M*) was investigated. In this regard, the main effect of GABA was on the level of the $GABA_B$-1b subunit mRNA, with 1 and 2 h exposure to this agonist producing a significant stimulation (Fig. 5b). In contrast, 4 and 24 h of treatment significantly decreased 1b expression (Fig. 5b). Furthermore, treatment with GABA decreased 1a subunit expression (evident after 4 h of treatment [*see* Fig. 5a]), which is a similar effect to that observed with muscimol (Fig. 4a). In addition, the stimulation of $GABA_B$-2 subunit after 1 h of exposure (Fig. 5c) mirrored that produced by muscimol (Fig. 4c).

Collectively, our data indicate that in Schwann cells there is a complex interplay between neuroactive steroids, $GABA_A$ and $GABA_B$ receptors, which can influence important and fundamental properties of Schwann cells.

Conclusions

Here, we have demonstrated that the sciatic nerve and myelin-producing Schwann cells express both the $GABA_A$ and $GABA_B$ receptors. Furthermore, the specific $GABA_B$ and $GABA_A$ receptor ligands baclofen and muscimol, respectively, influence the proliferation of the Schwann cell and the expression of specific myelin proteins, suggesting a role of these receptors in Schwann cell biology. The P metabolite THP, probably by enhancing the function of the $GABA_A$ receptor, influences the synthesis of the peripheral myelin protein PMP22. Adding further

complexity, THP and the progestagens P and DHP can influence the expression of $GABA_B$ subunits in Schwann cells. Furthermore, some of these effects are mimicked by muscimol and GABA, suggesting $GABA_A$ involvement in the control of $GABA_B$ receptor expression by neuroactive steroids.

In conclusion, the results obtained to date suggest, at least in the myelinating cells of the PNS, a cross-interaction between $GABA_A$ and $GABA_B$ receptors and certain neuroactive steroids. Recent observations on polysialylated form of neural cell-adhesion molecule (PSA-NCAM) progenitor cells in the CNS, which in vivo differentiate into glial cells, revealed that neuroactive steroids and GABA signaling are involved in autocrine/paracrine loops in the control of PSA-NCAM progenitor proliferation and differentiation (Gago et al., 2004). This suggests, as reported above, a key role for THP in the development of the nervous system, specifically of its glial components (Ben Ari, 2002; Gago et al., 2004).

Our results therefore suggest that neuroactive steroids and GABA might similarly play an essential role in the development and function of certain components of the PNS. For instance, by changing the expression of the $GABA_B$ receptor, it is possible that neuroactive steroids modify the sensitivity of $GABA_B$ to GABA influencing Schwann cell proliferation and differentiation. Our future research aims to better understand this intriguing interaction of steroids with G protein-coupled and Cys loop receptors, both in health and in disease, in the hope of identifying novel therapeutic strategies for the treatment of peripheral neuropathies.

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