Heteromeric Assembly of $GABA_BR1$ and $GABA_BR2$ Receptor Subunits Inhibits Ca^{2+} Current in Sympathetic Neurons

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Neuronal $GABA_B$ receptors regulate calcium and potassium currents via G-protein-coupled mechanisms and play a critical role in long-term inhibition of synaptic transmission in the CNS. Recent studies have demonstrated that assembly of $GABA_B$ receptor $GABA_BR1$ and $GABA_BR2$ subunits into functional heterodimers is required for coupling to potassium channels in heterologous systems. However whether heterodimerization is required for the coupling of $GABA_B$ receptors to effector systems in neurons remains to be established. To address this issue, we have studied the coupling of recombinant $GABA_B$ receptors to endogenous Ca^{2+} channels in superior cervical ganglion (SCG) neurons using nuclear microinjection to introduce both sense and antisense expression constructs. Patch-clamp recording from neurons injected with both $GABA_BR1a/1b$ and $GABA_BR2$ cDNAs or with $GABA_BR2$ alone produced marked baclofen-mediated inhibition of Ca^{2+} channel currents via a *pertussis* toxin-sensitive mechanism. The actions of baclofen were blocked by CGP62349, a specific $GABA_B$ antagonist, and were voltage dependent. Interestingly, SCGs were found to express abundantly $GABA_BR1$ but not $GABA_BR2$ at the protein level. To determine whether heterodimerization of $GABA_BR1$ and $GABA_BR2$ subunits was required for Ca^{2+} inhibition, the $GABA_BR2$ expression construct was microinjected with a $GABA_BR1$ antisense construct. This resulted in a dramatic decrease in the levels of the endogenous $GABA_BR1$ protein and a marked reduction in the inhibitory effects of baclofen on Ca^{2+} currents. Therefore our results suggest that in neurons heteromeric assemblies of $GABA_BR1$ and $GABA_BR2$ are essential to mediate GABAergic inhibition of Ca^{2+} channel currents.

Key words: $GABA_BR1$; $GABA_BR2$; Ca^{2+} channel; sympathetic neuron; antisense; G-protein

$GABA_B$ receptors play a critical role in long-term inhibition of synaptic transmission in the CNS (Bowery, 1993; Mott and Lewis, 1994). Inhibition is mainly achieved via modulation of neurotransmitter release from presynaptic terminals and hyperpolarization of postsynaptic membranes. Although activation of inwardly rectifier potassium channels (GIRKs) is thought to be responsible for a $GABA_B$-mediated membrane hyperpolarization, inhibition of presynaptic N-type Ca^{2+} channels is thought to be responsible for modulation of neurotransmitter release (Mott and Lewis, 1994; Kaupmann et al., 1998; Takahashi et al., 1998). The regulation of $GABA_B$ receptor function has been implicated in cognition enhancement and induction of long-term potentiation (Davies et al., 1991; Olpe et al., 1993). In addition, involvement of the $GABA_B$ receptor in a number of diseases of the CNS such as epilepsy, anxiety, depression, and cognitive deficits make it an attractive target for therapeutic agents (Bittiger et al., 1993; Kerr and Ong, 1995; Dichter, 1997).

The first $GABA_B$ receptors, $GABA_BR1a$ and $GABA_BR1b$, were cloned from a mouse cortical and cerebellar cDNA library (Kaupmann et al., 1997). They exhibited a low affinity for agonists and did not couple efficiently to neuronal Ca^{2+} channels when expressed heterologously in sympathetic neurons (Couve et al., 1998). Subcellular distribution of epitope-tagged $GABA_BR1$ demonstrated that recombinant receptors failed to reach the cell surface and were retained in intracellular compartments when expressed in a variety of neuronal and non-neuronal cell types. These results provided an explanation for the lack of function of recombinant $GABA_BR1$ receptors in coupling to other effector systems such as K^+ channels (Couve et al., 1998). Recently $GABA_BR2$, a second receptor ~35% identical to $GABA_BR1$, has been identified by several laboratories (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Unexpectedly for a G-protein-coupled receptor, heteromeric assembly of $GABA_BR1$ and $GABA_BR2$ has been shown to be necessary for coupling to adenylyl cyclase and activation of GIRKs in *Xenopus* oocytes and human embryonic kidney 293 (HEK293) cells (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). However, it has yet to be determined whether heterodimer formation is required for coupling to their effector systems (such as Ca^{2+} channels) in neurons.

Here we demonstrate that the heteromeric assembly of $GABA_BR1$ and $GABA_BR2$ receptor subunits is required to allow $GABA_B$ receptor-induced inhibition of Ca^{2+} currents in rat superior cervical ganglion (SCG) neurons.

MATERIALS AND METHODS

Neuron preparation and cDNA injection. Neuron isolation and injection procedures have been described previously in detail (Filippov et al., 1997, 1998; Couve et al., 1998). Briefly, single SCG neurons were dissociated

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from 15- to 19-d-old rats and plated on laminin-coated glass coverslips. Five hours after plating, neurons were microinjected into the nucleus with plasmids carrying cDNAs of the $GABA_B$ receptor subunits. Plasmids were stored at 4°C as 1 μg/μl stock solutions in 10 mM TRIS and 1 mM EDTA, pH 8, and injected at a final pipette concentration of 0.15–0.5 μg/μl. (Experimental data were indistinguishable over this range of concentrations for $GABA_B$R1a and $GABA_B$R1b.) Neurons were coinjected with cDNA for the "enhanced" S65T mutant of jellyfish green fluorescent protein (GFP) at concentrations of 0.01–0.5 μg/μl for later identification of the cells with successfully expressed cDNAs. For controls, neurons were injected with GFP cDNA alone or not injected at all. After injections, cells were incubated for 14–48 hr in a humidified incubator (5% CO_2/95% O_2) at 37°C. Injected neurons with successfully expressed cDNAs were identified as bright fluorescent cells using an inverted microscope (Diaphot 200; Nikon) equipped with an epifluorescent block N B2E (Nikon). Electrophysiological recordings were routinely made 16–20 hr after injection at room temperature (20°C). In some experiments, recordings were made 40 hr after injections. Where indicated, neurons were incubated for 12–16 hr with *pertussis* toxin (PTX; 0.5 μg/μl) added to the culture media.

Plasmids. A cytomegalovirus (CMV) promoter-based mammalian expression vector containing a myc-tagged version of the rat $GABA_B$R1a receptor has been described previously (Couve et al., 1998). The $GABA_B$R1a antisense cDNA was obtained by subcloning the sequence of the complete receptor in the opposite orientation with its 3′ end facing the CMV promoter. A $GABA_B$R2 receptor plasmid was obtained by subcloning an ~3.5 kb *Xba*I containing the sequence of the rat $GABA_B$R2 receptor from pC1-Neo/$GABA_B$R2 (kindly provided by Dr. Bernhard Bettler) into a CMV promoter-based mammalian expression vector.

Antibodies. A guinea pig pan antibody directed against the C-terminal domain of $GABA_B$R1a was obtained from Chemicon International. A rabbit antibody specific for $GABA_B$R1a was raised against the peptide CHPPWEGGIRYRGLTRDQVK from the N-terminal domain of $GABA_B$R1a conjugated with keyhole limpet hemocyanin and was immunopurified using the same peptide. A rabbit antibody directed against the C-terminal domain of $GABA_B$R2 was kindly provided by Dr. Bernhard Bettler. MAP2 monoclonal antibody was obtained from Sigma (St. Louis, MO). Monoclonal protein disulfide isomerase (DPI) antibody was purchased from Stress Gen. Synaptophysin antibody was kindly provided by Dr. Dan Cutler. Texas Red- and fluorescein-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Ca^{2+} channel current recording. Currents through voltage-gated Ca^{2+} channels were recorded using the conventional whole-cell patch-clamp method as described previously (Caulfield et al., 1994). Cells were superfused (20–25 ml/min) with a solution consisting of 120 mM tetraethylammonium chloride, 3 mM KCl, 1.5 mM $MgCl_2$, 5 mM $BaCl_2$, 10 mM HEPES, 11.1 mM glucose, and 0.5 μM tetrodotoxin. The pH was adjusted to 7.35 with NaOH. Patch electrodes (2–3 MΩ) were filled with a solution containing 110 mM CsCl, 3 mM $MgCl_2$, 40 mM HEPES, 3 mM EGTA, 2 mM Na_2ATP, and 0.5 mM Na_2GTP, with pH adjusted to 7.4 with CsOH. Neurons were voltage-clamped using a discontinuous ("switching") amplifier (Axoclamp 2B) sampling voltage at 6–8 kHz (50% duty cycle). Commands were generated via a Digidata 1200 interface using "Clamp 6" computer software (Axon Instruments, Foster City, CA). Ca^{2+} channel currents were routinely evoked every 20 sec with a 100 msec depolarizing rectangular test pulse to 0 mV from a holding potential of −90 mV. Currents were digitized and stored on a computer for later analysis using Clamp 6 software. Ca^{2+} channel current amplitudes were measured isochronally 10 msec from the onset of the rectangular test pulse, i.e., near the peak of the control current. To eliminate leak currents, Co^{2+} was substituted for Ba^{2+} in the external solution at the end of each experiment, blocking all Ca^{2+} channel currents, and the residual current was digitally subtracted from the corresponding currents in the Ba^{2+} solution. As reported previously (Filippov et al., 1997, 1998), currents were primarily N-type with negligible contribution by L-type channels.

Data were presented as means ± SEM as appropriate. Student's test (unpaired) was applied to determine statistical significance. The difference was considered significant if $p \leq 0.05$.

Baclofen and norepinephrine were purchased from Sigma. PTX was obtained from Porton Products (Dorset, UK).

Protein extracts, immunoblots, and immunofluorescence. Immunofluorescence and immunoblots were performed as described previously (Couve et al., 1998). For preparation of brain membranes, fresh rat brains were homogenized in 10 vol of 5 mM Tris-Cl, pH 7.4, 0.32 M sucrose, and a cocktail of protease inhibitors with 50 strokes using a glass and Teflon homogenizer. The suspension was centrifuged at 1000 × *g* for 15 min. The pellet was discarded, and the resulting supernatant was centrifuged at 17000 × *g* for 30 min. The pellet was washed twice in 50 mM Tris-Cl, pH 7.4, and resuspended at ~5 mg/ml in the same buffer. Aliquots were frozen and stored at −20°C until used. Extracts from superior cervical ganglia were prepared by homogenizing ganglia in RIPA buffer containing 50 mM Tris-Cl, 1 mM EDTA, 2 mM EGTA, 10 mM Na^+ pyrophosphate, 1 mM Na^+ orthovanadate, 50 mM NaF, 150 mM NaCl, 1% NP-40, 0.5% deoxychoic acid 0.1% SDS, and a cocktail of protease inhibitors. All extracts were stored at −20°C and mixed with SDS sample buffer before loading onto SDS-PAGE.

RESULTS

Heterologously expressed $GABA_B$R1 and $GABA_B$R2 or $GABA_B$R2 but not $GABA_B$R1 couple to N-type Ca^{2+} channels in sympathetic neurons

It has been reported previously that baclofen, a well characterized $GABA_B$ agonist, produced only a minor inhibition of Ca^{2+} channel current in neurons injected exclusively with $GABA_B$R1 cDNA, for greater than that (12.7 ± 3.2%) in control neurons injected with GFP cDNA (Couve et al., 1998). This lack of functional inhibition of Ca^{2+} currents in SCG is in agreement with the endoplasmic reticulum retention of $GABA_B$R1 after homomeric expression in neurons. In neurons injected with cDNAs for $GABA_B$R1a and $GABA_B$R2, bath application of 50 μM baclofen produced a substantial and reversible inhibition of Ca^{2+} channel current (recorded using Ba^{2+} as the charge carrier) (Fig. 1*A*). This inhibition was accompanied by a current-onset slowing similar to that reported after activation of many other endogenous or heterologously expressed G-protein-coupled receptors in SCG neurons (Hille, 1994; Ikeda et al., 1995; Filippov et al., 1998, 1999). Bath application of 1 μM CGP62349, a specific antagonist of $GABA_B$ receptors (Billinton et al., 1999), blocked the effect of baclofen (Fig. 1*C,D*). Interestingly, washing out of the agonist produced an initial over-recovery of the current followed by a slow return to the control level [as reported for some other receptors (see Meza et al., 1999)].

To study the requirement of heterodimer formation in the ability of recombinant $GABA_B$ receptors to couple to Ca^{2+} channels, $GABA_B$R2 cDNA, in the absence of $GABA_B$R1, was injected into SCG neurons. Surprisingly, Ca^{2+} channel currents were also inhibited in neurons injected exclusively with $GABA_B$R2. This effect was indistinguishable from that obtained after coexpression of $GABA_B$R1 and $GABA_B$R2 (Fig. 1*B*). Thus, the mean inhibition of Ca^{2+} channel current (measured 10 msec after onset of the test pulse) by 50 μM baclofen was 56.1 ± 3.2% for neurons expressing recombinant $GABA_B$R1 and $GABA_B$R2 and 52.3 ± 4.8% for neurons expressing recombinant $GABA_B$R2 exclusively (Fig. 1*D*).

$GABA_B$-mediated inhibition of Ca^{2+} current is strongly voltage dependent, suggesting involvement of the G-protein βγ subunit

Slowing of the current onset by baclofen in neurons with heterologously expressed $GABA_B$R1 and $GABA_B$R2 or $GABA_B$R2 indicated that $GABA_B$ receptor-mediated inhibition of Ca^{2+} channel current was voltage dependent and relieved during depolarization (cf. Bean, 1989), as reported previously for other G-protein-coupled receptors. Indeed, a double-pulse protocol demonstrated that inhibition of Ca^{2+} current produced by baclofen was greatly reduced after a 120 mV depolarizing prepulse (Fig. 2*A*). The prepulse also abolished the slowing of the current onset produced by baclofen. Identical results were obtained with

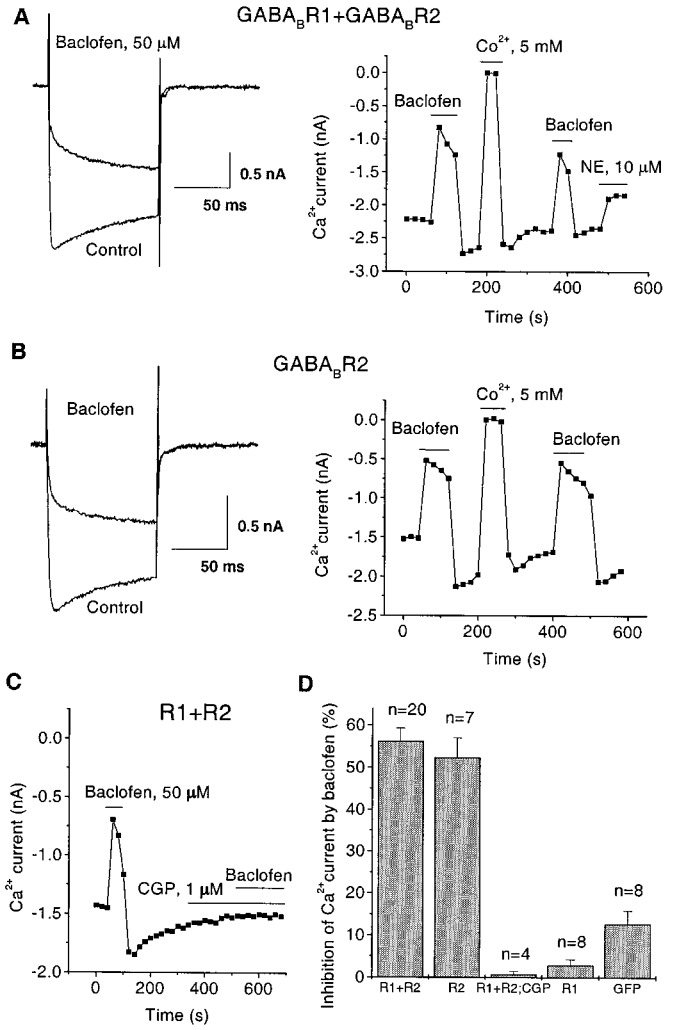

Figure 1. Coupling of heterologously expressed $GABA_BR1$ and $GABA_BR2$ receptors to N-type Ca^{2+} channels in sympathetic neurons. Currents were recorded by stepping for 100 msec every 20 sec from −90 to 0 mV and leak-corrected by subtracting currents remaining after substituting 5 mM Co^{2+} for Ba^{2+}. *A, Left, B, Left*, *Records* show superimposed leak-subtracted currents in the absence (*Control*) and presence of 50 μM baclofen. *A, Right, B, Right, C*, *Plots* show the time course of changes in current amplitude measured 10 msec from the onset of the test pulse. *Solid horizontal bars* indicate the time of exposure to baclofen, CGP62349 (*CGP*), Co^{2+}, or norepinephrine (*NE*). *D*, *Bar charts* show the mean inhibition of I_{Ba} amplitude by 50 μM baclofen in neurons injected with $GABA_B$ cDNAs + GFP cDNA or with GFP cDNA alone (*left*). Error bars show SEM (n = number of cells). Note that heterologously expressed $GABA_BR1$ + $GABA_BR2$ (*R1+R2*) or $GABA_BR2$ (*R2*) but not $GABA_BR1$ (*R1*) receptors couple to Ca^{2+} channels.

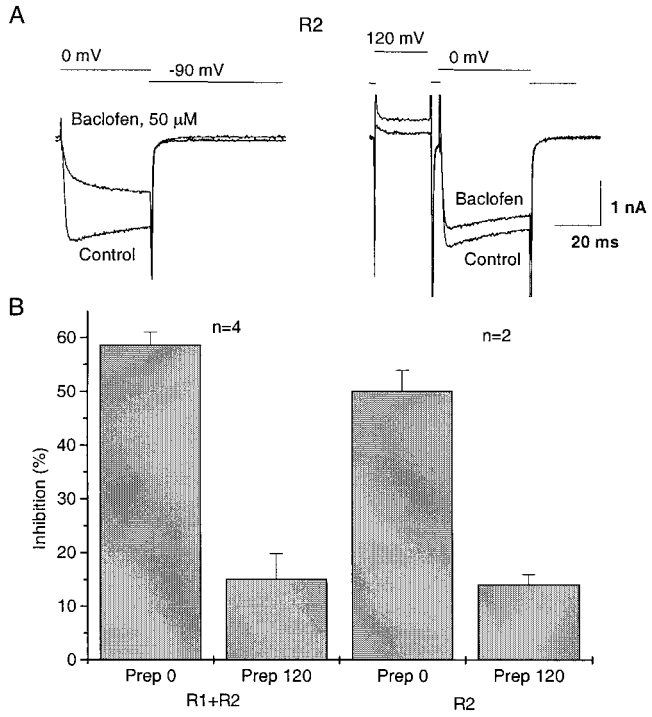

Figure 2. Voltage dependence of $GABA_B$-mediated inhibition of Ca^{2+} current. Neurons were injected with $GABA_BR1$ cDNA + $GABA_BR2$ cDNA (*R1+R2*) or with $GABA_BR2$ cDNA (*R2*). *A*, *Records* show superimposed Ca^{2+} currents obtained with a double-pulse voltage protocol (*top*) in the absence (*Control*) and presence of 50 μM baclofen. *B*, *Bar charts* show the mean percent current inhibition (measured after 10 msec at the 0 mV command potential) produced by baclofen before (*Prep 0*) and after (*Prep 120*) the +120 mV prepulse. Note that the prepulse almost abolished $GABA_B$-mediated inhibition of Ca^{2+} current and eliminated the slowing of the kinetics.

both heterologously expressed $GABA_BR1$ and $GABA_BR2$ or $GABA_BR2$ receptors. Inhibition was reduced from 58.7 ± 2.4 to 15.2 ± 4.7% in neurons with heterologously expressed $GABA_BR1$ and $GABA_BR2$ and from 50.2 ± 3.8 to 14.1 ± 1.8% in neurons expressing $GABA_BR2$ (Fig. 2*B*). A similar effect was reported for the modulation of Ca^{2+} current by endogenous $GABA_B$ receptors in sensory neurons and in calyx nerve terminals of the mammalian auditory brainstem (Grassi and Lux, 1989; Isaacson, 1998). Similar voltage-dependent effects have been generally interpreted as produced by a direct interaction of G-protein βγ subunits with an α subunit of the Ca^{2+} channel (Dolphin, 1995; Herlitze et al., 1996; Ikeda, 1996; Jones and Elmslie, 1997; Delmas et al., 1998a,b).

A PTX-sensitive G-protein mediates Ca^{2+} current inhibition after activation of recombinant $GABA_BR1$ and $GABA_BR2$ or $GABA_BR2$ receptors

A strong voltage dependency of Ca^{2+} channel current inhibition by baclofen in our experiments suggested that the recombinant $GABA_B$ receptors coupled to Ca^{2+} channels via PTX-sensitive G-proteins (for review, see Hille, 1994). Therefore, to determine the identity of the Gα-protein that mediates the Ca^{2+} current inhibition in SCG neurons, responses to baclofen were determined after pretreatment with PTX. Indeed, after PTX pretreatment (500 ng/ml), baclofen no longer inhibited Ca^{2+} channel currents in neurons expressing recombinant $GABA_BR1$ and $GABA_BR2$ receptors. PTX sensitivity was identical when cells expressing recombinant $GABA_BR2$ were studied. These observations are summarized in Figure 3*B*. Taken together they clearly indicate that PTX pretreatment prevents Ca^{2+} channel current inhibition in neurons expressing recombinant $GABA_BR1$ and $GABA_BR2$ or $GABA_BR2$ alone (Fig. 3). These results strongly suggest that only PTX-sensitive G-proteins mediate Ca^{2+} current inhibition after activation of recombinant $GABA_B$ receptors. The complete disappearance of the baclofen response in the presence of PTX was unique to $GABA_B$ receptors because the same concentration of PTX was not capable of totally abolishing the response of endogenous α-adrenergic receptors to norepinephrine (Fig. 3*A*). The small PTX-insensitive effect of norepi-

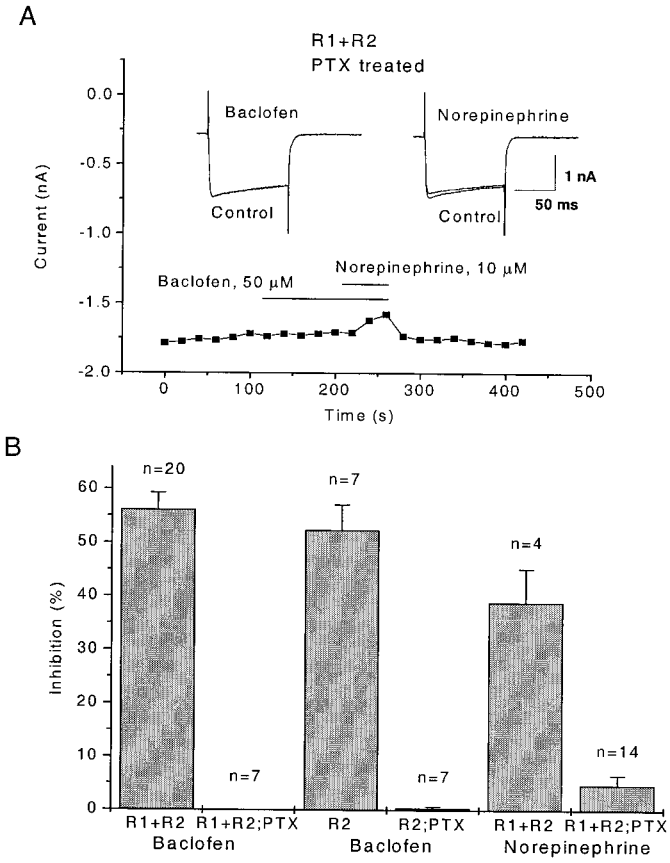

Figure 3. Recombinant $GABA_B$ receptor couples to Ca^{2+} channels only via PTX-sensitive G-proteins. *A, Top, Records* show superimposed leak-subtracted currents in the absence (*Control*) and presence of 50 μM baclofen (*left*) or 10 μM norepinephrine (*right*) in neurons pretreated with PTX (0.5 μg/ml; overnight). *Bottom*, A time *plot* of I_{Ba} amplitude is shown. *B,* Mean inhibition of I_{Ba} amplitude by 50 μM baclofen or 10 μM norepinephrine in neurons pretreated with PTX and in untreated neurons is shown. Note that PTX pretreatment completely prevented $GABA_BR1$ + $GABA_BR2$ (*R1+R2*)- or $GABA_BR2$ (*R2*)-mediated inhibition of Ca^{2+} channel current, whereas a small effect of norepinephrine remained.

nephrine is in agreement with a minor involvement of PTX-insensitive G-proteins in adrenergic receptor-mediated function (Hille, 1994; Delmas et al., 1999).

Heteromeric assembly of $GABA_BR1$ with $GABA_BR2$ is required to couple effectively to Ca^{2+} channels in neurons

The results presented above showing the strong inhibition of Ca^{2+} channel currents by $GABA_BR2$ cDNA alone were of interest because heterologous expression of $GABA_BR2$ is not sufficient to produce functional coupling to adenylyl cyclase or inward rectifier potassium channels in *Xenopus* oocytes or HEK293 cells (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). One possible explanation is that SCG neurons express endogenous $GABA_BR1$ subunit that is not functionally coupled to Ca^{2+} channels but that, on expression of $GABA_BR2$, coassembles with recombinant $GABA_BR2$ to constitute a functional heteromeric receptor indistinguishable from a recombinant dimer. Several experiments were designed to test this hypothesis.

First, the expression of $GABA_BR1$ and $GABA_BR2$ was analyzed in SCG neurons via immunoblotting with specific antibodies against these proteins. A specific antibody was raised against

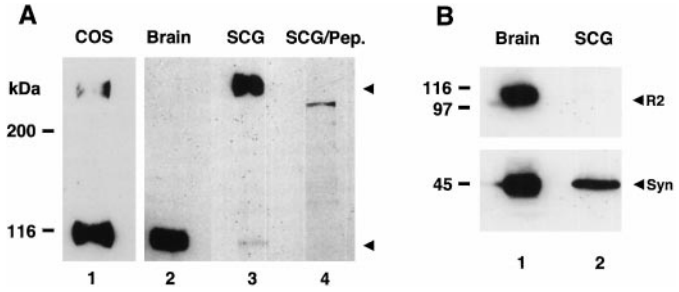

Figure 4. $GABA_BR1$ but not $GABA_BR2$ receptors are present in SCG neurons. *A,* Cell extracts derived from SCG neurons and control preparations were immunoblotted with $GABA_BR1a$ antibodies. *Lane1*, Extracts derived from COS cells transfected with $GABA_BR1a$; *lane 2*, crude brain membranes; *lane 3*, extracts derived from superior cervical ganglia; *lane 4*, same as in *lane 3* but with the antibody preincubated with 25 μg/ml $GABA_BR1a$ peptide CHPPWEGGIRYRGLTRDQVK. Relevant molecular weight markers are indicated on the *left*. *B,* Cell extracts derived from SCG neurons and control preparations were immunoblotted with $GABA_BR2$ antibodies (*R2*; *top*) or synaptophysin (*Syn*; *bottom*). *Lane 1*, Crude brain membranes; *lane 2*, extracts derived from superior cervical ganglia. Relevant molecular weight markers are indicated on the *left*. *Pep*, Peptide.

a peptide in the N-terminal domain of the $GABA_BR1a$ receptor, and expression of recombinant $GABA_BR1$ was used to test the specificity of this antisera. In agreement with previous observations, the antibody recognized $GABA_BR1a$ as two specific bands in cell lysates derived from transfected COS cells. A lower band of ~120 kDa represents the monomeric receptor, and an ~250 kDa band presumably corresponds to receptor aggregates (Fig. 4*A, lane 1*). In contrast, a single ~120 kDa band is detected in crude brain membranes in agreement with previous observations (Fig. 4*A, lane 2*) (Benke et al., 1999). Interestingly, low levels of an ~120 kDa band corresponding to the $GABA_BR1a$ protein were detected in SCG extracts. Unexpectedly, an aggregate form of the $GABA_BR1a$ protein was predominant in these cell lysates (Fig. 4*A, lane 3*). This band matches the ~250 kDa form of $GABA_BR1a$ observed in transfected COS cells. The specificity of the $GABA_BR1a$ antibody was demonstrated by competition with a $GABA_BR1$ peptide before immunoblotting (Fig. 4*A, lane 4*).

Second, to determine the expression of $GABA_BR2$ in SCG, immunoblots with antibodies against $GABA_BR2$ were performed. A single band of ~110 kDa was detected in crude brain membranes (Fig. 4*B, top*). SCG extracts showed no detectable levels of $GABA_BR2$ (Fig. 4*B*, *top*). An immunoblot with anti-synaptophysin antibodies indicates that protein levels and the integrity of cell extracts were similar between brain and SCG preparations (Fig. 4*B*, *bottom*). These observations were confirmed by detection of $GABA_BR1$ (see Fig. 6) but not $GABA_BR2$ (data not shown) by immunofluorescence in SCG neurons.

To investigate further the inhibition of Ca^{2+} current by $GABA_B$ receptor subunits in neurons, the effects of baclofen on expressed $GABA_BR1$ and $GABA_BR2$ or $GABA_BR2$ receptors were studied in detail using full dose–response curves. Baclofen inhibited Ca^{2+} currents more effectively and with higher potency in cells expressing $GABA_BR1$ and $GABA_BR2$ receptors than in cells expressing only heterologous $GABA_BR2$ receptors (Fig. 5*A*). The IC_{50} for inhibition was 0.09 ± 0.01 μM for $GABA_BR1$ and $GABA_BR2$ and approximately four times higher (0.38 ± 0.05 μM) for $GABA_BR2$. To confirm these observations using a different methodology, SCG neurons were injected either with $GABA_BR2$ cDNA or with an equal mixture of $GABA_BR2$ cDNA

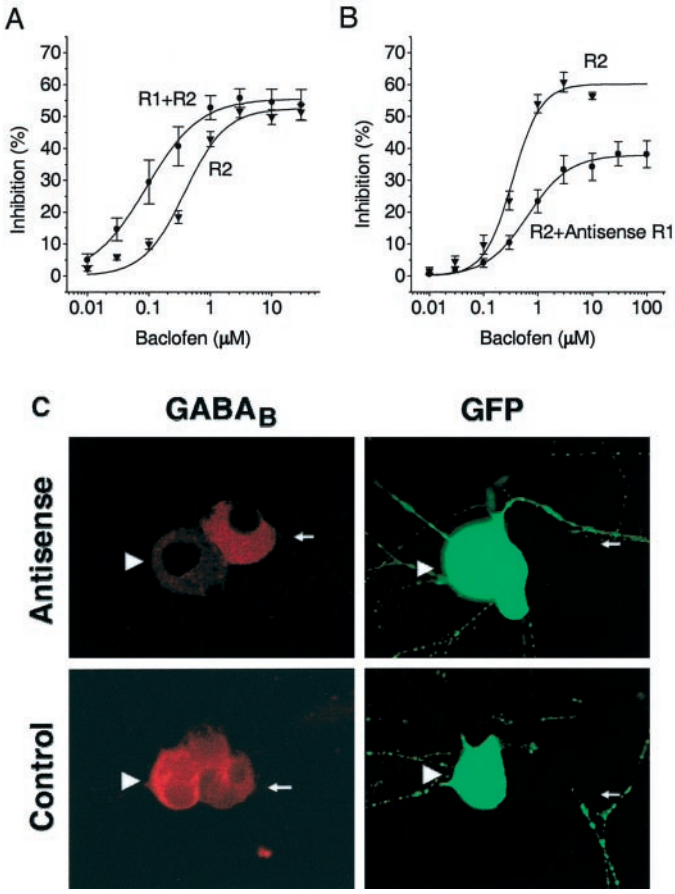

Figure 5. Heteromeric assembly of $GABA_BR1$ with $GABA_BR2$ is required to couple effectively to Ca^{2+} channels in neurons. *A, B*, *Plots* show the concentration dependence of Ca^{2+} current inhibition by baclofen in SCG neurons injected with $GABA_BR1$ cDNA + $GABA_BR2$ cDNA (*R1+R2*) or $GABA_BR2$ cDNA (*R2*) ~16–20 hr before recordings (*A*) or with $GABA_BR2$ cDNA (*R2*) or $GABA_BR2$ cDNA + $GABA_BR1$ antisense cDNA (*R2+antisense R1*) ~40 hr before recordings (*B*). *Curves* were fitted to pooled data points (mean ± SEM) using Origin 4.1 software to the Hill equation: $y = y_{max}x^{nH}/(x^{nH} + K^{nH})$, where y is the observed percent inhibition, y_{max} is the extrapolated maximal percent inhibition, x is the baclofen concentration (micromolar), K is the IC_{50} (micromolar), and n_H is the Hill coefficient. *A*, For *R1+R2*, IC_{50} = 0.09 ± 0.01 μM; Hill coefficient = 1.00 ± 0.13; and maximal inhibition = 55.55 ± 1.68%. For *R2*, IC_{50} = 0.38 ± 0.05 μM; Hill coefficient = 1.30 ± 0.20; and maximal inhibition = 52.55 ± 1.90%. *B*, For *R2*, IC_{50} = 0.34 ± 0.04 μM; Hill coefficient = 1.66 ± 0.28; and maximal inhibition = 60.14 ± 2.30%. For *R2+antisense R1*, IC_{50} = 0.66 ± 0.15 μM; Hill coefficient = 1.14 ± 0.24; and maximal inhibition = 37.93 ± 1.92%. Note that heterologously coexpressed $GABA_BR1$ and $GABA_BR2$ inhibit Ca^{2+} current more effectively than does heterologously expressed $GABA_BR2$ and that reduction of endogenous $GABA_BR1$ expression by antisense cDNA significantly reduces inhibition of Ca^{2+} current. *C,* Immunofluorescence with $GABA_BR1$ antibodies in cells injected with $GABA_BR1$ cDNA antisense is shown. Cells were injected with either GFP (*bottom panels)* or GFP plus $GABA_BR1a$ antisense (*top panels*) cDNA. *Right panels* show GFP fluorescence of injected neurons. *Left panels* show the same neurons stained for $GABA_BR1$. In both cases the *leftmost* cell has been injected (*arrowheads*), whereas the *rightmost* cell has not (*arrows*). Only cells injected with $GABA_BR1$ antisense cDNA show a decrease in $GABA_BR1$ expression when compared with noninjected cells.

and $GABA_BR1$ antisense cDNA to reduce the expression of endogenous $GABA_BR1$ protein. As expected, immunofluorescence with $GABA_BR1$ antibodies revealed detectable levels of $GABA_BR1$ in all sympathetic neurons, and levels were reduced in cells injected with GFP and $GABA_BR1$ antisense cDNA (Fig. 5*C*, *top left*, *arrowhead*). Furthermore, coexpression of the sense and antisense cDNAs of $GABA_BR1$ resulted in a significant decrease in expression of recombinant $GABA_BR1$ protein (data not shown). Control cells injected with GFP alone showed no difference in expression of $GABA_BR1$ (Fig. 5*C*, *bottom left*, *arrowhead*). In accordance with these observations, inhibition of Ca^{2+} channel currents in neurons injected with $GABA_BR1$ antisense cDNA was significantly lower than inhibition in control neurons for all effective doses of baclofen (Fig. 5*B*).

Taken together, the results presented above indicate that SCG neurons contain detectable levels of $GABA_BR1a$ and suggest that responses to baclofen in neurons injected with $GABA_BR2$ result from activation of a heterodimer formed between endogenous $GABA_BR1a$ and recombinant $GABA_BR2$ receptors. Interestingly, trace levels of $GABA_BR2$ were observed occasionally in SCG extracts. This might generate trace levels of endogenous $GABA_BR1/R2$ dimer and hence provide an explanation for the low response of control SCG neurons to baclofen (Fig. 1*D*).

Endogenous $GABA_BR1$ is predominantly localized to the endoplasmic reticulum in SCG neurons

As reported previously, recombinant $GABA_BR1$ receptors are retained in an intracellular compartment when expressed in neuronal and non-neuronal cell types (Couve et al., 1998). To determine whether endogenous $GABA_BR1$ receptors failed to reach the cell surface in SCG neurons, immunofluorescence with $GABA_BR1$ antibodies was performed. The $GABA_BR1$ receptor was found mainly restricted to the cell body of SCG neurons (Fig. 6*A*, *left*). Detection of MAP2, a somatodendritic marker, shows a different distribution pattern with predominant staining in cell projections (Fig. 6*A*). The pattern difference is still apparent after the levels of fluorescence have been equalized.

To determine the subcellular distribution of $GABA_BR1$, SCG neurons were stained for $GABA_BR1$ and DPI, an endoplasmic reticulum marker. DPI is present in neuronal and non-neuronal cell types from the SCG preparation (Fig. 6*B*). In contrast, $GABA_BR1$ is present exclusively in neurons, and it overlaps almost entirely with DPI in these cells. These observations strongly suggest that both proteins colocalize in the endoplasmic reticulum.

$GABA_BR1$ is localized to the plasma membrane after coexpression of $GABA_BR2$

To provide additional evidence of the formation of functional, cell surface heterodimers after expression of $GABA_BR2$, SCG neurons were microinjected with $GABA_BR1$ or $GABA_BR2$ independently or with $GABA_BR1$ and $GABA_BR2$ simultaneously. As reported previously (Couve et al., 1998), a myc-tagged version of $GABA_BR1$ is retained intracellularly in SCG neurons (Fig. 7, *top*). In contrast, a FLAG-tagged version of $GABA_BR2$ is targeted efficiently to the cell surface (Fig. 7, *middle*). Consistent with our electrophysiological findings, expression of $GABA_BR2$ changes the subcellular distribution of $GABA_BR1$ from the endoplasmic reticulum to the plasma membrane (Fig. 7, *bottom*). These observations support the idea that $GABA_BR2$ rescues $GABA_BR1$ from intracellular compartments and strongly suggest that heterodimer formation is a requirement for $GABA_B$ receptor function.

DISCUSSION

In this report we demonstrate that coexpressed $GABA_BR1$ and $GABA_BR2$ subunits produce a functional receptor in sympa-

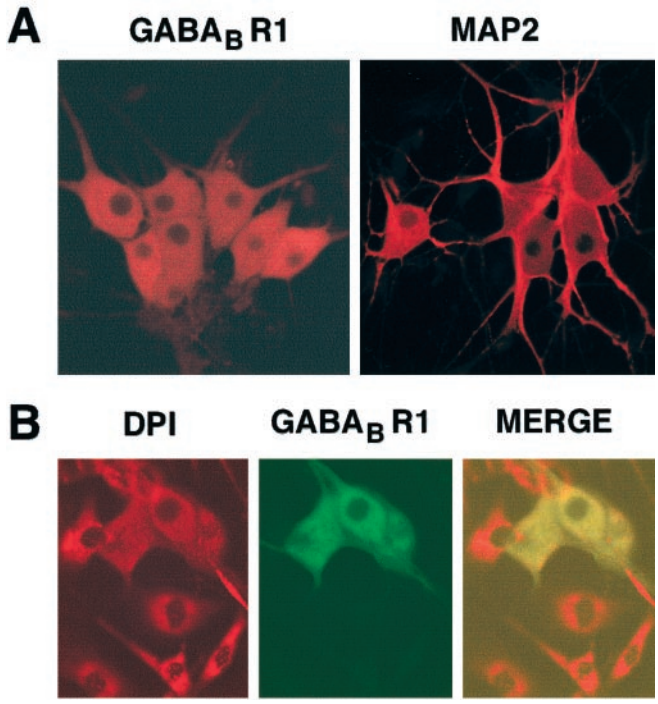

Figure 6. $GABA_B$R1 is predominantly localized to the endoplasmic reticulum in SCG neurons. *A,* Expression and distribution of $GABA_B$R1 in SCG neurons were examined after staining with a $GABA_B$R1 antibody directed against the C-terminal domain of $GABA_B$R1 (*left*) or with antibodies directed against MAP2 (*right*). The levels of fluorescence were equalized to compare distribution of the two proteins. *B,* Subcellular distribution of $GABA_B$R1 in SCG neurons was analyzed by coimmunofluorescence with DPI (*left*) and $GABA_B$R1 (*middle*) antibodies. DPI staining is not restricted to neurons and overlaps almost completely with $GABA_B$R1 staining in SCGs (merged image; *right*).

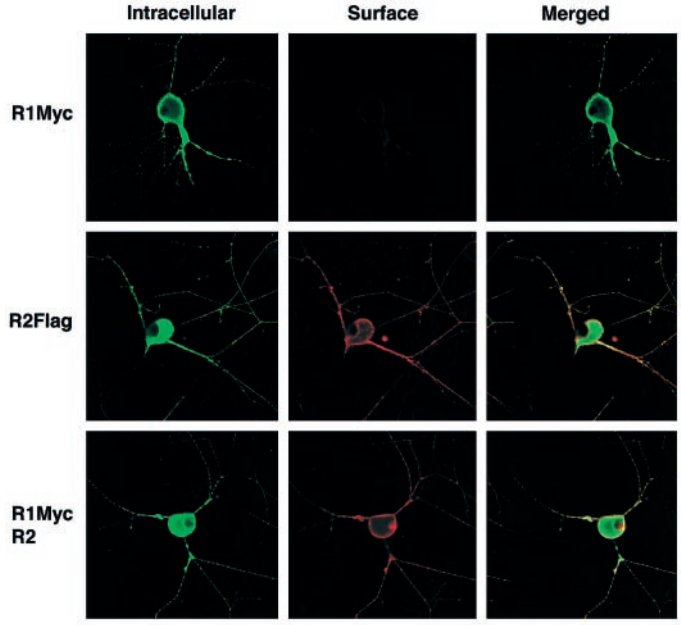

Figure 7. Recombinant $GABA_B$R1 is expressed at the cell surface after coexpression of $GABA_B$R2 in SCG neurons. Expression and distribution of $GABA_B$R1 tagged at the N-terminal domain with the myc epitope (*R1Myc; top*) and $GABA_B$R2 tagged at the N-terminal domain with the FLAG epitope (*R2Flag; middle*) were examined after staining with 9E10 and FLAG antibodies, respectively. The distribution of *R1Myc* after coexpression of $GABA_B$R2 (*R2*) was analyzed by immunofluorescence with 9E10 antibodies (*bottom*). On the *right* are merged images of intracellular and cell surface staining. The *R1Myc*-staining pattern changes dramatically after injection of $GABA_B$R2.

thetic neurons that couples effectively to N-type Ca^{2+} channels. Thus, heteromeric assembly of $GABA_B$R1 and $GABA_B$R2 receptor subunits appears to be necessary for coupling to all three known effectors: Ca^{2+} channels, adenylyl cyclase, and inward rectifier K^+ channels (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). It might be noted, however, that the IC_{50} for inhibition of the Ca^{2+} current in sympathetic neurons (~90 nM) was appreciably lower than that required to inhibit GIRK channels in non-neuronal cells, suggesting that expression of $GABA_B$ receptor dimers in their natural (neuronal) environment allows more effective ion channel coupling.

To the degree that the present experiments allow, the mechanism by which these exogenously expressed receptors inhibit neuronal Ca^{2+} channels appears identical to that used by native $GABA_B$ receptors present in other neurons. Thus, inhibition was strongly reduced by *pertussis* toxin, implying mediation by members of the G_i/G_o family of G-proteins [probably G_o (see Campbell et al., 1993; Menon-Johansson et al., 1993)]. Also, the strong voltage dependence of inhibition in sympathetic neurons accords with previous observations on sensory neurons (Grassi and Lux, 1989), suggesting that it was mediated by $\beta\gamma$ subunits of the G-protein (Dolphin, 1995; Jones and Elmslie, 1997). Thus, our observations on the expression and functional coupling of recombinant $GABA_B$ receptors in sympathetic neurons are likely to be relevant to the properties of natively expressed receptors.

Immunoblot analysis demonstrated that the predominant form of $GABA_B$R1 in SCG corresponds to a large ~250 kDa protein band. The nature of this high-molecular weight form of $GABA_B$R1 is not known, but it is unlikely to correspond to receptor dimers because homomeric interactions have not been shown to occur (Fritschy et al., 1999). A similar protein band is normally observed in other heterologous expression systems in which the $GABA_B$R1 receptor is not functional (Couve et al., 1998). We hypothesize that this structure corresponds to nonfunctional $GABA_B$R1 receptor aggregates retained in the endoplasmic reticulum.

In this context, our experiments strongly suggest that $GABA_B$R2 forms an essential subunit of the functional receptor and that it is required both for assembly and membrane insertion of the receptor. Thus, functional membrane receptors were generated not only by coexpression of recombinant $GABA_B$R1 and $GABA_B$R2 plasmids but also on expression of exogenous $GABA_B$R2 subunits alone. We attribute this to coassembly with the native $GABA_B$R1 subunits normally present in these sympathetic neurons, because reduction of $GABA_B$R1 protein by antisense expression also reduced the response of $GABA_B$R2-expressing cells to baclofen (Fig. 5). Because $GABA_B$R1 protein was predominantly confined to the endoplasmic reticulum, this also suggests that $GABA_B$R2 plays a crucial role in trafficking and membrane insertion of the dimeric receptor. The fact that $GABA_B$R2 is localized to the plasma membrane and traffics $GABA_B$R1 in heterologous cells (White et al., 1998; Martin et al., 1999) and in SCG neurons supports these observations.

Cultured SCG neurons respond poorly to $GABA_B$ agonists and are generally thought to be devoid of $GABA_B$ receptors. However, $GABA_B$ responses may appear as a result of $GABA_B$R2 expression during pathology, in certain stages of development, or

under specific and local stimuli. Our findings together with the fact that $GABA_B$ agonists have been shown to modify superior cervical ganglia after prolonged applications (Parducz et al., 1990), that SCG neurons contain $GABA_A$ receptors (Brown et al., 1979), and that SCG neurons are innervated by GABAergic terminals (Kasa et al., 1988) certainly suggest that $GABA_B$ receptors have the potential to function in sympathetic neurons. Nevertheless, the conditions under which $GABA_B$ receptors may be physiologically expressed remain to be determined. If this hypothesis is correct, other neuronal types might use a similar regulatory mechanism, in which expression of the $GABA_BR2$ subunit or other accessory protein determines the formation of functional receptors by trafficking existent $GABA_BR1$ from the endoplasmic reticulum to the plasma membrane.

In situ hybridization studies have revealed certain areas in the CNS where expression of $GABA_BR1$ is much greater than $GABA_BR2$ and others where $GABA_BR1$ is present and $GABA_BR2$ is not (Jones et al., 1998; Kaupmann et al., 1998; Martin et al., 1999). These observations have led to the suggestion that $GABA_BR1$ can function as a monomer or with accessory proteins other than $GABA_BR2$ in some areas of the CNS (Marshall et al., 1999). The fact that baclofen produced a small inhibition of the Ca^{2+} current in sympathetic neurons in the absence of exogenous $GABA_BR2$ subunits might appear to offer some support for this view. However, because trace levels of endogenous $GABA_BR2$ protein were occasionally detected in SCG extracts, we cannot exclude the possibility that the small effects of baclofen on uninjected cells might have resulted from activation of a small number of endogenous $GABA_BR1/R2$ dimers.

The question also arises whether $GABA_BR2$ subunits might form functional receptors independent of $GABA_BR1$ subunits. $GABA_BR2$ has been reported to couple negatively to adenylyl cyclase in transfected COS cells (Martin et al., 1999) and HEK293 cells (Kuner et al., 1999). However, the effectiveness of this coupling is under question because the baclofen concentrations used in these experiments were very high (300–500 μM; compared, for example, with an IC_{50} of ~90 nM for Ca^{2+} current inhibition mediated by $GABA_BR1$ and $GABA_BR2$ in the present experiments), and $GABA_BR2$ does not couple to the inward rectifier GIRK channels coexpressed in *Xenopus* oocytes or HEK293 cells (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Our experiments with $GABA_BR1$ antisense cDNA injections in SCG neurons confirm that responses apparently mediated by exogenous $GABA_BR2$ receptors are primarily (and possibly entirely) caused by formation of $GABA_BR1/GABA_BR2$ dimers with endogenous $GABA_BR1$ subunits. Nevertheless, more conclusive evidence is necessary to exclude the possibility that either $GABA_BR1$ or $GABA_BR2$ might be able to function independently. New subunit-specific agonists and definitive binding studies are necessary to resolve this issue.

Notwithstanding such considerations, the principal point emerging from the present experiments is that efficient coupling of $GABA_B$ receptors to Ca^{2+} channels in sympathetic neurons requires the heteromeric assembly of both $GABA_BR1$ and $GABA_BR2$ subunits and therefore that this is likely to be true for those endogenous $GABA_B$ receptors responsible for Ca^{2+} current inhibition (and resultant presynaptic inhibition) in other parts of the mammalian nervous system.

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