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Evidence for a G Protein-Coupled γ-Hydroxybutyric Acid Receptor

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Abstract: γ-Hydroxybutyric acid (GHB) is a naturally occurring metabolite of GABA that has been postulated to exert ubiquitous neuropharmacological effects through $GABA_B$ receptor ($GABA_BR$)-mediated mechanisms. The alternative hypothesis that GHB acts via a GHB-specific, G protein-coupled presynaptic receptor that is different from the $GABA_BR$ was tested. The effect of GHB on regional and subcellular brain adenylyl cyclase in adult and developing rats was determined and compared with that of the $GABA_BR$ agonist (−)-baclofen. Also, using guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) binding and low-K_m GTPase activity as markers the effects of GHB and (−)-baclofen on G protein activity in the brain were determined. Neither GHB nor baclofen had an effect on basal cyclic AMP (cAMP) levels. GHB significantly decreased forskolin-stimulated cAMP levels by 40–50% in cortex and hippocampus but not thalamus or cerebellum, whereas (−)-baclofen had an effect throughout the brain. The effect of GHB on adenylyl cyclase was observed in presynaptic and not postsynaptic subcellular tissue preparations, but the effect of baclofen was observed in both subcellular preparations. The GHB-induced alteration in forskolin-induced cAMP formation was blocked by a specific GHB antagonist but not a specific $GABA_BR$ antagonist. The (−)-baclofen-induced alteration in forskolin-induced cAMP formation was blocked by a specific $GABA_BR$ antagonist but not a specific GHB antagonist. The negative coupling of GHB to adenylyl cyclase appeared at postnatal day 21, a developmental time point that is concordant with the developmental appearance of [^{3}H]GHB binding in cerebral cortex, but the effects of (−)-baclofen were present by postnatal day 14. GHB and baclofen both stimulated [^{35}S]GTPγS binding and low-K_m GTPase activity by 40–50%. The GHB-induced effect was blocked by GHB antagonists but not by $GABA_BR$ antagonists and was seen only in cortex and hippocampus. The (−)-baclofen-induced effect was blocked by $GABA_BR$ antagonists but not by GHB antagonists and was observed throughout the brain. These data support the hypothesis that GHB induces a G protein-mediated decrease in adenylyl cyclase via a GHB-specific G protein-coupled presynaptic receptor that is different from the $GABA_BR$. **Key Words:** γ-Hydroxybutyric acid—$GABA_B$ receptor—Presynaptic receptor—G protein—Cyclic AMP.
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γ-Hydroxybutyric acid (GHB) is a short-chain fatty acid that occurs naturally in mammalian brain (Doherty et al., 1978). The primary precursor for GHB in the brain is GABA (Gold and Roth, 1977; Snead et al., 1989). GHB is formed in the brain from GABA-derived succinic semialdehyde via a specific succinic semialdehyde reductase (Maitre, 1997).

GHB has many properties suggesting that this compound may play a role in the brain as a neurotransmitter or neuromodulator (Cash, 1994; Maitre, 1997; Bernasconi et al., 1999; Cash et al., 1999). These characteristics include a discrete, subcellular anatomical distribution for GHB (Snead, 1987) and its synthesizing enzyme (Cash et al., 1979; Rumigny et al., 1981; Weissman-Nanopoulos et al., 1982) and the presence of specific, high-affinity [^{3}H]GHB binding sites in the brain (Benavides et al., 1982; Snead and Liu, 1984; Hechler et al., 1987, 1992), the anatomical distribution of which correlates with GHB turnover (Vayer et al., 1988). GHB is released by neuronal depolarization in a Ca^{2+}-dependent fashion (Maitre et al., 1983). Also, a Na^+-dependent GHB uptake system has been demonstrated in brain (Hechler et al., 1985), as well as a distinct ontogeny (Snead, 1994) and the ability of GHB to stimulate cyclic GMP (Vayer et al., 1987; Vayer and Maitre, 1989; Cash et al., 1999).

GHB has the ability to induce in several animal species profound EEG and behavioral changes that are dose-dependent: Lower doses of GHB result in absence-like seizures, whereas higher doses cause electrographic burst suppression associated with an anesthetic state (Snead, 1988, 1994; Snead et al., 1999). In addition, GHB has been shown to induce perturbation of several neurotransmitters (Maitre, 1997), most notably dopamine (Howard

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Abbreviations used: cAMP, cyclic AMP; $GABA_AR$, $GABA_A$ receptor; GHB, γ-hydroxybutyric acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); P, postnatal day; PTX, pertussis toxin; SN, synaptoneurosome; SY, synaptosome.

and Feigenbaum, 1997), and has found clinical use in the treatment of alcoholism (Poldrugo and Addolorato, 1999) and narcolepsy (Scrima et al., 1989) and as an anesthetic. Furthermore, GHB has emerged recently as a major recreational drug of abuse (Tunnicliff, 1997; Kam and Yoong, 1998; Bernasconi et al., 1999).

The mechanism(s) by which GHB induces these ubiquitous effects in the CNS are unknown. GHB has been postulated to act via $GABA_B$ receptor ($GABA_BR$)-mediated mechanisms in the brain (Waldmeier, 1991; Williams et al., 1995; Emri et al., 1996; Mathivet et al., 1997; Erhardt et al., 1998; Bernasconi et al., 1999; Lingenhoehl et al., 1999), but there also is evidence that GHB may act via an independent GHB-specific receptor site in the brain (Feigenbaum and Howard, 1996; Hechler et al., 1997; Maitre, 1997) that is presynaptic (Banerjee and Snead, 1995; Snead, 1996*a*,*b*) and G protein-coupled (Ratomponirina et al., 1995; Snead, 1996*a*).

The object of these experiments was to test the hypothesis that GHB binds to a GHB-specific, G protein-coupled presynaptic receptor that is different from the $GABA_BR$. We sought to test this hypothesis in four ways. First, we examined the regional effect of GHB on brain adenylyl cyclase activity in the rat. Second, we determined the effect of GHB on brain adenylyl cyclase activity in presynaptic and postsynaptic subcellular preparations prepared from rat cerebral cortex. Third, we examined the ontogeny of the GHB-induced alteration in adenylyl cyclase activity in developing rat brain. Finally, we ascertained the ability of GHB to stimulate G protein activity by studying the effect of GHB on regional guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) binding and low-K_m GTPase activity in rat brain. Furthermore, the GHB specificity of the observed GHB–adenylyl cyclase and GHB–G protein interactions was defined by the use of the GHB antagonist NCS 382 and the $GABA_BR$ antagonist CGP 35348. The results of these experiments demonstrate that GHB is negatively coupled to adenylyl cyclase and support the hypothesis that this interaction is mediated by a specific G protein-coupled, presynaptic GHB receptor.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River, Montreal, Quebec, Canada) weighing 250–300 g were used for all experiments except the ontogeny experiments. For the developmental studies, timed pregnant Sprague–Dawley females were obtained from Charles River, and rats and littermate controls were used at postnatal day (P) 14, 18, 21, 28, and 70. Adult animals were housed singly, with ad libitum access to food and water, and maintained on a 12-h light–dark cycle. Developing animals were weaned at P21 and then transferred to single cages. All animals were drug-naive.

Drugs

(−)-Baclofen was a gift of Dr. John Dailey (Peoria, IL, U.S.A.). The specific $GABA_B$ antagonist CGP 35348 was a gift from Novartis (Basel, Switzerland). The specific GHB receptor antagonist NSC 382 was a gift of Dr. J. J. Bourguignon (Centre de Neurochimie, Strasbourg, France). GHB sodium was obtained from Sigma (St. Louis, MO, U.S.A.). [8-^{3}H]Adenosine 3′5′-cyclic phosphate (28.0 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). [^{35}S]GTPγS (1,250 Ci/mmol) and [^{32}P]GTP (30 Ci/mmol) were obtained from DuPont NEN (Wilmington, DE, U.S.A.). 7β-Deacetyl-7β-(γ-*N*-methylpiperazino)butyrolforskolin (7β-forskolin) was purchased from Calbiochem (San Diego, CA, U.S.A.). All other analytical reagents were obtained from standard commercial sources and were of the highest available purity. All other drugs were obtained from Sigma.

In the dose–response studies of GHB and baclofen, the concentration ranged from 10^{-9} to 10^{-3} *M*. The concentration of drugs used in single-dose experiments was as follows: GHB, 250 μ*M;* baclofen, 100 μ*M;* NCS 382, 100 μ*M;* and CGP 35348, 100 μ*M*. The concentration of 250 μ*M* GHB was selected because this is the dose that has been shown to cause a robust presynaptic alteration of glutamate and GABA release (Maitre et al., 1990; Hechler et al., 1991; Banerjee and Snead, 1995; Hu et al., 2000).

Cyclic AMP (cAMP) assay: regional experiments

For dose–response studies, animals were killed, the brain was removed, and the cerebral cortex was dissected out on ice. The tissue was then homogenized in ice-cold Tris/EGTA buffer and centrifuged for 5 min at 12,000 rpm at 5°C, and a 50-μl aliquot was assayed for cAMP by the technique of Munirathinam and Yoburn (1994) in the presence and absence of varying concentrations of GHB or the $GABA_BR$ agonist (−)-baclofen, plus the GHB antagonist NCS 382 or the $GABA_BR$ antagonist CGP 35348. For forskolin and isoproterenol stimulation experiments, the tissue homogenate was incubated for 10 min at 25°C in the presence and absence of either drug at 10 μ*M* plus GHB, (−)-baclofen, NCS 382, and/or CGP 35348, the reaction was terminated by boiling the tubes for 5 min, and cAMP content was determined as described (Munirathinam and Yoburn, 1994). For the regional brain experiments animals were killed by decapitation, the brains were rapidly removed, and the cerebral cortex, thalamus, hippocampus, and cerebellum were dissected, frozen on dry ice, and subjected to forskolin stimulation experiments in the presence of GHB, baclofen, NCS 382, or CGP 35348, alone or in combination.

Pertussis toxin (PTX) experiments

Animals used for PTX experiments were implanted stereotaxically with an intraventricular cannula under halothane anesthesia as previously reported (Snead, 1992). Three days later animals were treated intracerebroventricularly with either PTX (1.5 μg) (Andrade et al., 1986; Snead, 1992) or bovine serum albumin. Three days later, the animals were killed, the brains were removed, the cortex was dissected on ice, and the effect of varying doses of GHB on forskolin-stimulated cAMP was determined as described above.

cAMP assay: subcellular experiments

Animals were killed by decapitation, the brains were rapidly removed, and the cerebral cortex was dissected on ice. Synaptosome (SY) (Snead, 1987) and synaptosoneurosome (SN) (Musgrave et al., 1994) fractions were prepared from cerebral cortex as described. The SY and SN pellets were resuspended in 1 ml of ice-cold Tris/EGTA buffer, and cAMP content was determined in the presence and absence of GHB, (−)-baclofen, NCS 382, and CGP 35348, alone and in combination, by the method of Munirathinam and Yoburn (1994). Both SY and SN preparations were used immediately for determination of ad-

enylyl cyclase activity. In the SY and SN experiments, the direct adenylyl cyclase activator 7β-forskolin was used rather than forskolin because 7β-forskolin is quite water-soluble and able to activate adenylyl cyclase activity directly in SY and SN preparations (Musgrave et al., 1994). The concentration of 7β-forskolin used in both SY and SN experiments was 1 m*M*. This has been shown to result in maximal stimulation of cAMP in these subcellular preparations (Musgrave et al., 1994).

Low-K_m GTPase assay

Animals were killed by decapitation, the brains were rapidly removed, and the cerebral cortex, thalamus, hippocampus, and cerebellum were dissected on ice and assayed for low-K_m GTPase by the method of Pacheco et al. (1994). In brief, the tissue was homogenized with 90 volumes of 50 m*M* Tris-HCl (pH 7.4) that contained 1 m*M* EGTA and 310 m*M* sucrose. The homogenate was centrifuged at 1,000 *g* for 10 min. The resulting supernatant was centrifuged at 12,000 *g* for 20 min. The P2 pellet was resuspended in 50 m*M* Tris-HCl (pH 7.4) with 1 m*M* EGTA and centrifuged at 30,000 *g* for 20 min. The final pellet was resuspended in GTPase buffer [50 m*M* Tris-HCl (pH 7.4) with 0.2 m*M* EGTA and 3 m*M* $MgCl_2$] with 1 m*M* dithiothreitol and frozen at −80°C until assayed. For the GTPase assays the frozen membranes were thawed and diluted to 5 μg of protein per tube in GTPase buffer, 1 m*M* adenylyl-5′-imidodiphosphate, 1 m*M* ATP, 1 m*M* ouabain, 5 m*M* phosphocreatine, creatine phosphokinase (2 units per tube), and GHB, (−)-baclofen, NCS 382, or CGP 35348, alone and in combination. The reaction was initiated with 0.1 μCi of [γ-^{32}P]GTP (0.5 μ*M*). Tubes were incubated for 20 min at 30°C, and the reaction was stopped in boiling water, followed by addition of 100 μl of cold 40 m*M* phosphoric acid on ice and quantitation of radioactivity. High-K_m GTPase activity was determined in the presence of 50 μ*M* GTP; low-K_m GTPase activity was determined by subtracting high-K_m GTPase activity from that determined in the presence of 0.5 μ*M* GTP.

[^{35}S]GTPγS binding

The method of Sim et al. (1995) was used. Rats were killed by decapitation, and the brains were removed and immediately immersed in isopentane at −35°C. Coronal or horizontal sections were cut at 20 μm at −20°C and thaw-mounted onto gelatin-coated slides, which were dried and stored at −80°C until used. Tissue slices were preincubated in assay buffer (50 m*M* Tris-HCl, 3 m*M* $MgCl_2$, 0.2 m*M* EGTA, and 100 m*M* NaCl, pH 7.7) at 25°C for 10 min and preincubated further with 2 m*M* GDP for 15 min in assay buffer at 25°C. Previous studies in isolated membranes have shown that agonists of G protein-coupled receptors produce significant stimulation of [^{35}S]GTPγS binding when assays are performed in the presence of a large excess of GDP to ensure that G proteins are present in the inactivated state (Lorenzen et al., 1993; Traynor and Nahorski, 1995). Agonist-stimulated activity was determined by incubating [^{35}S]GTPγS (0.04 n*M*) with GHB, (−)-baclofen, NCS 382, or CGP 35348, alone or in combination, and 2 m*M* GDP at 25°C for 2 h. Basal activity was assessed with GDP in the absence of agonist, and nonspecific binding was assessed in the presence of 10 μ*M* unlabeled GTPγS. Following incubation, slides were rinsed twice in ice-cold Tris buffer [50 m*M* Tris-HCl (pH 7.0) at 25°C], rinsed briefly in deionized water, and air-dried.

Analysis of binding

Dried tissue sections were opposed to Hyperfilm-βmax film (Amersham) with [^{14}C]Micro-scales standards (Amersham) for 5–6 days at room temperature. The films were developed in D-19 (Kodak), fixed, and dried. Quantitative analysis of the resulting autoradiograms was performed densitometrically using a microcomputer-based densitometer system (MCID; Imaging Research, St. Catharines, Ontario, Canada). In brief, a standard curve between the optical density of ^{14}C-standards and tissue radioactivity equivalents (in picomoles per milligram of tissue) was constructed using a nonlinear regression analysis. The average optical density values of the selected brain regions were in the linear portion of this standard curve. The pmol/mg value in each brain region was calculated by interpolation using the image analyzer (Banerjee et al., 1998). Five to eight readings were determined and averaged for each anatomic area analyzed.

Protein content determination

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Data analysis

All data were expressed as arithmetic mean ± SE values. The n for each group of experiments was 6–8. Levels of significance were determined by Dunnett's two-tailed test for multiple comparisons (nonindependent samples) (Winer, 1971). Experiments that involve more than one independent variable were analyzed by ANOVA.

RESULTS

Effect of GHB on cAMP

$GABA_BR$ activation results in potentiation of isoproterenol-stimulated activation of adenylyl cyclase and inhibition of forskolin-stimulated adenylyl cyclase (Karbon and Enna, 1985; Scherer et al., 1988; Malcangio and Bowery, 1993; Knight and Bowery, 1996; Cunningham and Enna, 1996). Therefore, basal, forskolin-stimulated, and isoproterenol-stimulated cAMP levels were determined in cerebral cortex of adult animals in the presence and absence of GHB or (−)-baclofen in a concentration range of 10^{-9}–10^{-3} *M*. These experiments were repeated in the presence of NCS 382 or CGP 35348. In addition, basal and forskolin-stimulated cAMP levels were determined in cerebral cortex, hippocampus, thalamus, and cerebellum in the presence and absence of GHB (250 μ*M*) or (−)-baclofen (100 μ*M*). This concentration of GHB has been shown to produce alterations of presynaptic glutamate, GABA, and dopamine release (Maitre et al., 1990; Hechler et al., 1991; Banerjee and Snead, 1995; Hu et al., 2000). Also, basal and forskolin-stimulated cAMP levels were determined in cerebral cortex in adult animals in the presence and absence of GHB (250 μ*M*), baclofen (100 μ*M*), or a combination of the two.

GHB induced a slight but insignificant decrease in basal cAMP production with a 15% decrease observed at a GHB concentration of >100 μ*M* (data not shown). (−)-Baclofen had no effect on basal cAMP production (data not shown). GHB and (−)-baclofen both inhibited forskolin-induced cAMP formation in a dose-dependent manner with an ED_{50} of 4 and 2 μ*M*, respectively (Fig. 1A). The effect of GHB on forskolin-induced cAMP formation was abolished by PTX (Fig. 1A). Isoproterenol-induced cAMP formation was unaffected by GHB

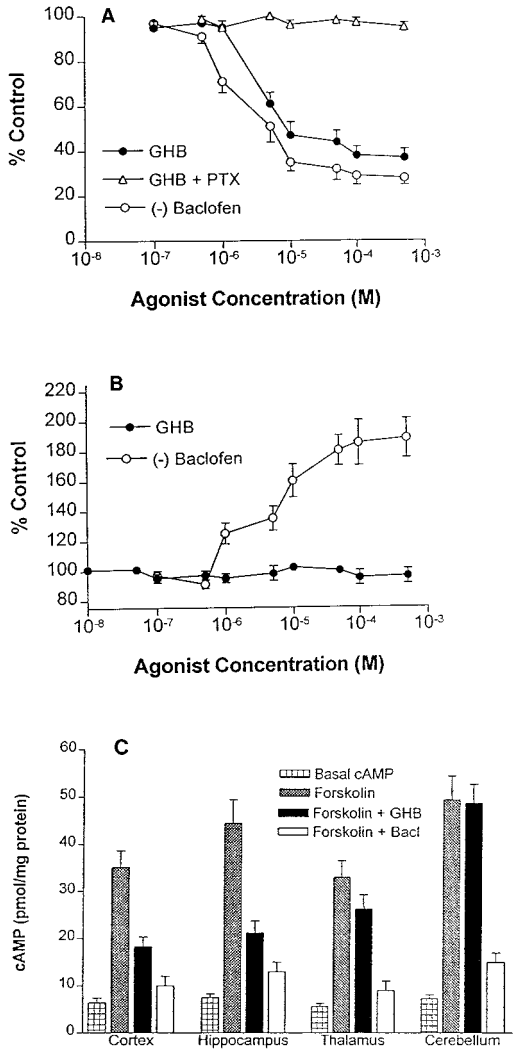

FIG. 1. A: Effect of GHB or (−)-baclofen on forskolin-induced formation of cAMP in rat cerebral cortex. Data are mean ± SEM (bars) values of eight determinations. There was no significant effect of GHB on basal cAMP levels. Forskolin (10 μM) resulted in a significant ($p < 0.0001$; ANOVA) elevation of cAMP level compared with basal cAMP levels. (−)-Baclofen and GHB both resulted in a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls with an ED_{50} of 2 and 4 μM, respectively. The suppressive effect of GHB on forskolin-induced formation of cAMP was abolished by PTX treatment. **B:** Effect of GHB or (−)-baclofen on isoproterenol-induced formation of cAMP in rat cerebral cortex. Data are mean ± SEM (bars) values of eight determinations. There was no significant effect of GHB on basal cAMP levels. Isoproterenol (10 μM) resulted in a significant ($p < 0.0001$; ANOVA) elevation of cAMP level compared with basal cAMP levels. There was no significant alteration from control in isoproterenol-induced cAMP formation observed at any concentration of GHB used in these experiments. (−)-Baclofen resulted in a significant ($p < 0.005$; ANOVA) enhancement of isoproterenol-induced elevation of cAMP levels in concentrations of $>5 \times 10^{-7}$ M with an ED_{50} of 4 μM. **C:** Regional effect of GHB or (−)-baclofen on forskolin-induced formation of cAMP in rat brain. Data are mean ± SEM (bars) values of eight determinations. Forskolin (10 μM) resulted in a significant ($p < 0.0001$; ANOVA) elevation of cAMP level compared with basal cAMP levels in all regions tested. GHB (250 μM) produced a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls in cortex and hippocampus but not thalamus or cerebellum. (−)-Baclofen (100 μM) produced a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls in all brain regions examined, including the cerebellum.

but was significantly enhanced by (−)-baclofen with an ED_{50} of 4 μM (Fig. 1B). There was a regional specificity to the observed inhibition of forskolin-stimulated cAMP activity by GHB, with significant decreases being observed in cortex and hippocampus but not in thalamus or cerebellum. (−)-Baclofen-inhibited forskolin-stimulated cAMP activity was seen in all regions of brain examined, including the cerebellum (Fig. 1C).

GHB inhibition of forskolin-stimulated cAMP activity was blocked by NCS 382 but not by CGP 35348, whereas baclofen inhibition of forskolin-stimulated cAMP activity was blocked by CGP 35348 but not by NCS 382 (Fig. 2A). GHB and (−)-baclofen were partially additive in their ability to inhibit forskolin-stimulated cAMP activity (Fig. 2B). Neither NCS 382 nor CGP 35348 alone had any effect on basal, forskolin-stimulated, or isoproterenol-stimulated cAMP levels in cerebral cortex (data not shown).

Effect of GHB on cAMP in developing brain

Basal and forskolin-stimulated cAMP levels were determined in cerebral cortex of developing animals in the presence and absence of GHB (250 μM) or (−)-baclofen (100 μM). GHB inhibition of forskolin-stimulated cAMP activity was not observed in cerebral cortex before P21, but (−)-baclofen inhibition of forskolin-stimulated cAMP activity was demonstrated at the earliest age tested, i.e., P14 (Fig. 3).

Effect of GHB on pre- and postsynaptic cAMP levels

Basal and 7β-forskolin-stimulated cAMP levels were determined in SY and SN fractions prepared from cerebral cortex in the presence and absence of GHB (250 μM) or baclofen (100 μM). In both SY and SN preparations 7β-forskolin produced a statistically significant increase in adenylyl cyclase activity. Incubation of the SY and SN preparations with GHB resulted in a significant decrease in 7β-forskolin-stimulated cAMP activity only in the SY fraction. However, incubation of the SY and SN preparations with (−)-baclofen resulted in a significant decrease in 7β-forskolin-stimulated cAMP activity in both SY and SN fractions (Fig. 4).

Effect of GHB on [^{35}S]GTPγS binding

Basal [^{35}S]GTPγS binding was determined in cerebral cortex in adult animals in the presence and absence of GHB in a concentration range of 10^{-9}–10^{-3} M. These experiments were repeated in the presence of the GHB antagonist NCS 382 (100 μM) and/or the $GABA_B$ antagonist CGP 35348 (100 μM). In addition, [^{35}S]GTPγS binding was determined in the presence and absence of

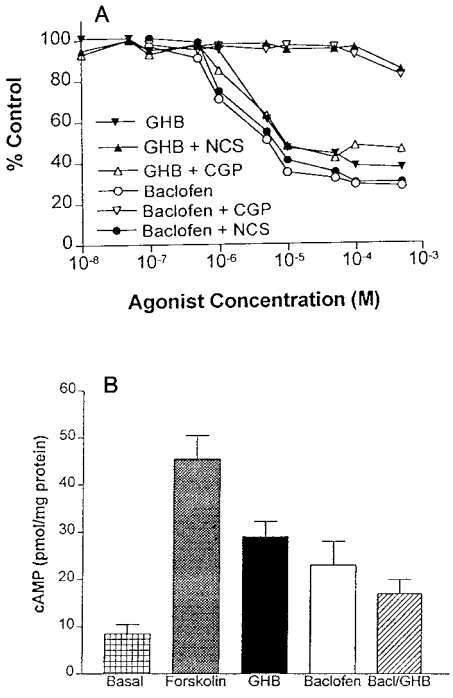

FIG. 2. A: Effect of GHB or (−)-baclofen on forskolin-induced formation of cAMP in rat cerebral cortex in the presence and absence of the GHB antagonist NCS 382 (NCS) or the $GABA_BR$ antagonist CGP 35348 (CGP). Data are mean ± SEM (bars) values of eight determinations. Concentrations of GHB $>10^{-6}$ *M* resulted in a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls in both the presence and absence of CGP (100 μM). However, in the presence of NCS (100 μM), there was no significant alteration from control in forskolin-induced cAMP formation observed at any concentration of GHB. (−)-Baclofen concentrations of $>5 \times 10^{-7}$ *M* resulted in a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls in both the presence and absence of NCS (100 μM). However, in the presence of CGP (100 μM), there was no significant alteration from control in forskolin-induced cAMP formation observed at any concentration of (−)-baclofen. **B:** Effect of (−)-baclofen on forskolin-induced formation of cAMP in rat cerebral cortex in the presence or absence of GHB. Data are mean ± SEM (bars) values of eight determinations. Forskolin (10 μM) resulted in a significant ($p < 0.0001$; ANOVA) elevation of cAMP level compared with basal cAMP levels. GHB (250 μM) produced a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls. Similarly, 100 μM (−)-baclofen resulted in a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation compared with forskolin controls. Baclofen (100 μM) in combination with GHB (250 μM) (Bacl/GHB) produced a decrease in forskolin-induced cAMP formation that was significantly different from control ($p < 0.005$; Dunnett's two-tailed test for multiple comparisons), baclofen alone ($p < 0.01$; Dunnett's two-tailed test for multiple comparisons), or GHB alone ($p < 0.01$; Dunnett's two-tailed test for multiple comparisons), indicating additivity.

baclofen (100 μM) or GHB (250 μM), alone, together, and in combination with their respective antagonists each in a concentration of 100 μM. Regional analysis of the effect of GHB (250 μM) or (−)-baclofen (100 μM) on [^{35}S]GTPγS binding also was determined. The regions analyzed in these experiments were laminae I–III of cerebral cortex, CA1 of the hippocampus, ventrolateral nucleus of the thalamus, and the granular cell layer of the cerebellum. These areas were chosen because the cortical, hippocampal, and thalamic regions show [^{3}H]GHB binding, but there is no [^{3}H]GHB binding in cerebellum (Hechler et al., 1992); hence, any observed effect of GHB on [^{35}S]GTPγS binding in cerebellum would not be caused by the GHB receptor.

GHB induced a dose-dependent increase in [^{35}S]GTPγS binding. The effects of GHB on [^{35}S]GTPγS binding were blocked by NCS 382 but not by CGP 35348 (Fig. 5A). (−)-Baclofen also stimulated [^{35}S]GTPγS binding (Fig. 5B). The baclofen-stimulated [^{35}S]GTPγS binding was blocked by CGP 35348 but not by NCS 382. The effects of GHB and (−)-baclofen on [^{35}S]GTPγS binding were additive (Fig. 5B). GHB-induced stimulation of [^{35}S]GTPγS binding was seen in cortex and hippocampus but not in thalamus or cerebellum; however, (−)-baclofen-induced stimulation of [^{35}S]GTPγS binding was seen in all regions of brain examined, including the cerebellum (Fig. 5C). Neither GHB antagonists nor $GABA_B$ antagonists alone had any effect on [^{35}S]GTPγS binding (data not shown).

Effect of GHB on low-K_m GTPase

Basal low-K_m GTPase activity was determined in cerebral cortex in the presence and absence of baclofen (100 μM) and GHB (250 μM), alone, together, and in combination with their respective antagonists, each in a concentration of 100 μM. The effect of GHB (250 μM) or (−)-baclofen (100 μM) on low-K_m GTPase in cortex, thalamus, hippocampus, and cerebellum was also analyzed. High-K_m GTPase represented 60–65% of total GTPase activity in brain membranes. GHB induced an increase in low-K_m GTPase activity that was blocked by NCS 382, unaffected by CGP 35348, and potentiated by (−)-baclofen. (−)-Baclofen induced an increase in low-K_m GTPase that was blocked by CGP 35348, unaffected by NCS 382, and potentiated by GHB (Fig. 6A). Neither CGP 35348 nor NCS 382 alone had any effect on low-K_m GTPase (data not shown). GHB induced low-K_m GTPase activity in cortex and hippocampus but not thalamus or cerebellum, but (−)-baclofen induced low-K_m GTPase activity in all brain regions examined, including the cerebellum (Fig. 6B).

DISCUSSION

These experiments were designed to test the hypothesis that GHB binds to a GHB-specific, G protein-coupled presynaptic receptor that is different from the $GABA_BR$. The data show that GHB has no effect on basal or isoproterenol-stimulated cAMP levels but that GHB decreases forskolin-stimulated cAMP levels in cortex and hippocampus and that the effect of GHB on forskolin-stimulated cAMP is observed in presynaptic and not postsynaptic subcellular tissue preparations. The GHB-induced alteration in forskolin-induced cAMP for-

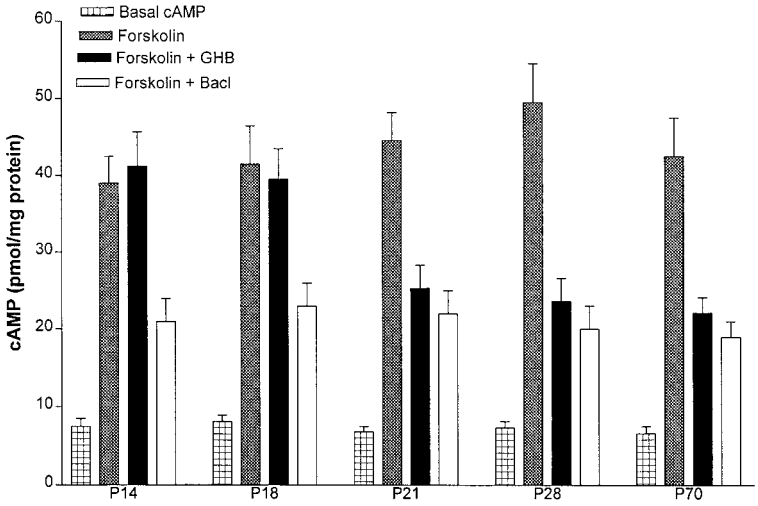

FIG. 3. Effect of GHB or (−)-baclofen (Bacl) on forskolin-induced formation of cAMP in rat cerebral cortex in developing animals from P14 to P70. Data are mean ± SEM (bars) values of eight determinations. There was no significant change in basal cAMP level at any age group. Forskolin (10 μM) resulted in a significant ($p < 0.0001$; ANOVA) elevation of cAMP level compared with basal cAMP levels in all age groups. GHB (250 μM) produced no significant alteration of forskolin-induced cAMP formation in the P14 or P18 animals, but beginning at P21, a significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation was observed with GHB. There was no age difference in GHB-induced alteration of forskolin-stimulated cAMP formation after P21. A significant ($p < 0.005$; ANOVA) decrease in forskolin-induced cAMP formation was observed with Bacl in all age groups tested.

mation is blocked by a specific GHB antagonist but not a specific $GABA_BR$ antagonist and is sensitive to PTX. Furthermore, we have shown that the negative coupling of GHB to adenylyl cyclase emerges at a developmental time point that is concordant with the developmental appearance of [^{3}H]GHB binding in cerebral cortex, i.e., P21 (Snead, 1994).

These data also demonstrate that there is a distinct and differential regional distribution of both [^{35}S]GTPγS binding and low-K_m GTPase activity following GHB-mediated G protein activation in the brain. The regional specificity of GHB-stimulated [^{35}S]GTPγS binding and low-K_m GTPase activity was identical to that observed for GHB-induced inhibition of forskolin-induced second messenger accumulation, i.e., GHB-stimulated [^{35}S]GTPγS binding and low-K_m GTPase activity were observed in cortex and hippocampus but not thalamus or cerebellum. As with the cAMP studies, the effect of GHB on [^{35}S]GTPγS binding and low-K_m GTPase activity was blocked by a specific GHB antagonist but not a specific $GABA_BR$ antagonist, and GHB and (−)-baclofen were partially additive.

A high-affinity binding site for GHB has been identified in the brain (Benavides et al., 1982; Snead and Liu, 1982), and it has been proposed that this binding site represents a specific receptor belonging to the G protein superfamily (Ratomponirina et al., 1995; Kemmel et al., 1998). There appear to be high- and low-affinity components to the GHB binding site with a K_D of 30–580 nM and 2.3–16 μM, respectively (Maitre, 1997), and GHB binding is sensitive to PTX (Ratomponirina et al., 1995; Snead, 1996*b*). There is no GHB binding in cerebellum, so it is not surprising that GHB had no effect on forskolin-stimulated cAMP levels or G protein activation in that brain region; however, it is not clear why GHB had no effect on either forskolin-stimulated cAMP levels or G protein activation in the thalamus. Although the density of GHB binding in thalamus is less than in cortex,

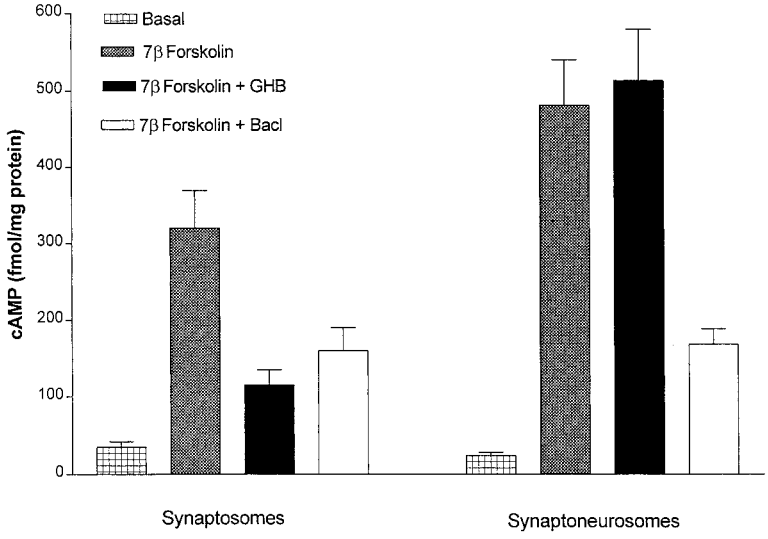

FIG. 4. Effect of GHB (250 μM) or (−)-baclofen (100 μM; Bacl) on 7β-forskolin-induced formation of cAMP in SY and SN fractions prepared from adult rat cerebral cortex. Data are mean ± SEM (bars) values of eight determinations. The concentration of 7β-forskolin in all experiments was 1 mM. There was no significant difference between SYs and SNs in basal cAMP levels. The 7β-forskolin (1 mM)-stimulated cAMP level in SYs was significantly ($p < 0.01$; ANOVA) lower than in SNs. 7β-Forskolin-induced formation of cAMP in SNs was not significantly altered in the presence of 250 μM GHB. However, 7β-forskolin-induced formation of cAMP in SYs was significantly ($p < 0.005$; ANOVA) reduced in the presence of GHB. 7β-Forskolin-induced formation of cAMP was significantly ($p < 0.005$; ANOVA) reduced in both SYs and SNs in the presence of Bacl.

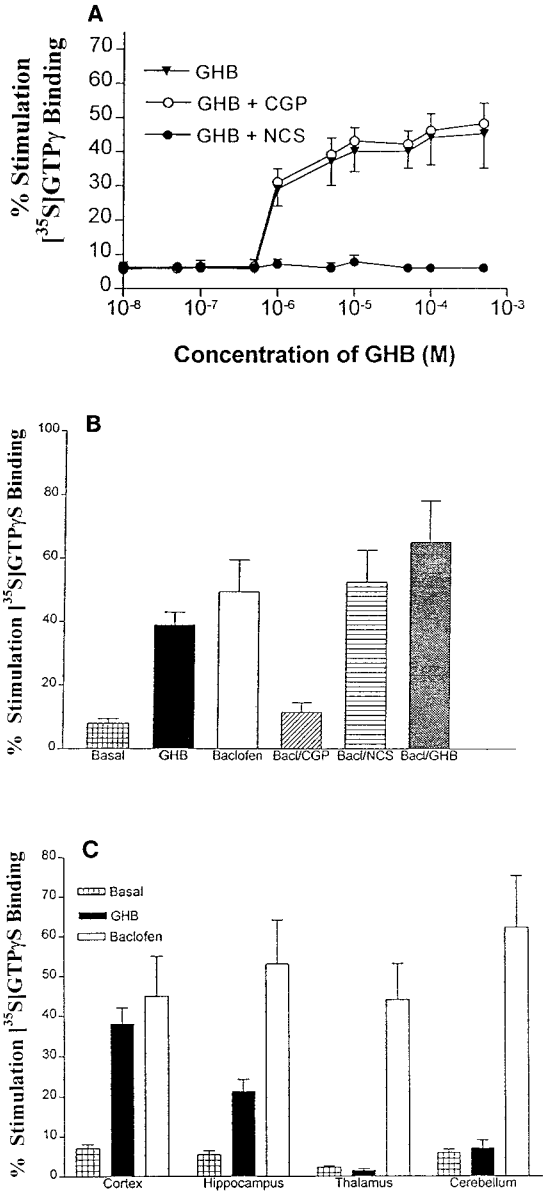

FIG. 5. A: Effect of GHB on [^{35}S]GTPγS binding in rat cerebral cortex in the presence or absence of the GHB antagonist NCS 382 (NCS) or the $GABA_BR$ antagonist CGP 35348 (CGP). Data are mean ± SEM (bars) values of eight determinations. Concentrations of GHB of $>10^{-6}$ *M* resulted in a significant ($p < 0.005$; ANOVA) increase in [^{35}S]GTPγS binding that was unchanged in the presence of CGP (100 μ*M*). However, in the presence of NCS (100 μ*M*), GHB had no effect on [^{35}S]GTPγS binding at any concentration tested. **B:** Effect of (−)-baclofen on [^{35}S]GTPγS binding in rat cerebral cortex in the presence or absence of GHB, the GHB antagonist NCS, or the $GABA_BR$ antagonist CGP. Data are mean ± SEM (bars) values of eight determinations. Both baclofen (100 μ*M*) and GHB (250 μ*M*) produced a significant ($p < 0.005$; ANOVA) increase in [^{35}S]GTPγS binding compared with controls. (−)-Baclofen (100 μ*M*) in combination with CGP (100 μ*M*) (Bacl/CGP) had no significant effect on [^{35}S]GTPγS binding, but the effect of (−)-baclofen on [^{35}S]GTPγS binding was unaffected by NCS (100 μ*M*) (Bacl/NCS). (−)-Baclofen (100 μ*M*) in combination with GHB (250 μ*M*) (Bacl/GHB) produced an increase in [^{35}S]GTPγS binding that was significantly different from control ($p < 0.005$; Dunnett's two-tailed test for multiple comparisons), baclofen alone ($p < 0.01$; Dunnett's two-tailed test for multiple comparisons), or GHB alone ($p < 0.01$; Dunnett's two-tailed test for multiple comparisons), indicating additivity. **C:** Regional effect of GHB or (−)-baclofen on [^{35}S]GTPγS binding in rat brain. Data are mean ± SEM (bars) values of eight determinations. GHB (250 μ*M*) produced a significant ($p < 0.005$; ANOVA) increase in [^{35}S]GTPγS binding compared with controls in cortex and hippocampus but not thalamus or cerebellum. (−)-Baclofen (100 μ*M*) stimulated [^{35}S]GTPγS binding in all brain regions examined, including cerebellum.

hippocampus, and striatum, binding is still significant in that brain region (Hechler et al., 1987, 1992; Snead et al., 1990). One possible explanation might be that an effect was not detected in thalamus because of a lack of sensitivity of assay measures for the thalamus. Although this may be plausible for the cAMP experiments, it seems unlikely that an effect of GHB on [^{35}S]GTPγS binding would have been missed by the autoradiographic techniques used. The ED_{50} observed for GHB in the forskolin experiments is consistent with activation of the low-affinity GHB binding site. The density of the low-affinity GHB binding site in the thalamus could be too low to pick up an effect in that region. Alternatively, GHB might couple to a second messenger system in the thalamus that differs from the GHB-coupled second messenger system in the cortex and hippocampus.

The question whether this GHB binding site is a $GABA_BR$ or a specific GHB receptor remains unresolved to date. GHB has been hypothesized to be a weak agonist at the $GABA_BR$ based on binding (Mathivet et al., 1997; Bernasconi et al., 1999) and electrophysiological (Erhardt et al., 1998; Madden and Johnson, 1998) studies. However, those electrophysiological experiments indicating that GHB induces long-lasting inhibitory postsynaptic potentials and rebound Ca^{2+} spikes in a manner similar to (−)-baclofen (Xie and Smart, 1992; Williams et al., 1995) indicate that the concentration of GHB required to mimic the postsynaptic effects of baclofen is in the millimolar range. This inordinately high concentration of GHB is commensurate with that achieved in brain with systemic administration to rats of high doses (>500 mg/kg) of GHB that are associated with burst suppression on the EEG, loss of righting reflex, and "anesthesia" (Snead, 1991); however, normal brain concentrations of GHB in rat brain are on the order of 2 μ*M* (Snead and Morley, 1981). Moreover, the electrophysiologic effects of GHB on the postsynaptic $GABA_BR$ are not blocked by a specific GHB antagonist (Williams et al., 1995; Emri et al., 1996). Additional lines of evidence that mitigate against the hypothesis that GHB is a physiologically significant $GABA_BR$ agonist are the inability of baclofen to displace [^{3}H]GHB binding and the differences between [^{3}H]GHB and [^{3}H]$GABA_B$ binding in rat brain in their ontogeny and regional distribution (Snead, 1994).

There are few data that address the molecular aspects of the $GABA_BR$ in relation to GHB. $GABA_BR1a$ and $GABA_BR1b$ were cloned in 1997 (Kaupmann et al., 1997). Recently, it has been shown by several laborato-

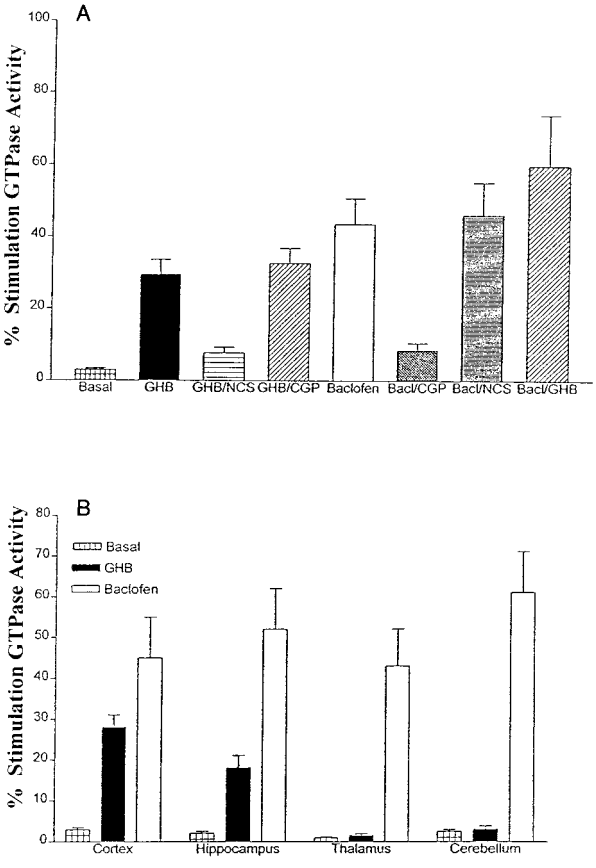

FIG. 6. A: Effect of GHB or (−)-baclofen on low-K_m GTPase activity in rat cerebral cortex in the presence or absence of GHB, the GHB antagonist NCS 382 (NCS), or the $GABA_BR$ antagonist CGP 35348 (CGP). Data are mean ± SEM (bars) values of eight determinations. Both baclofen (100 μM) and GHB (250 μM) produced a significant ($p < 0.005$; ANOVA) increase in low-K_m GTPase activity compared with controls. (−)-Baclofen (100 μM) in combination with CGP (100 μM) (Bacl/CGP) had no significant effect on low-K_m GTPase activity, but the effect of (−)-baclofen on low-K_m GTPase activity was unaffected by NCS (100 μM) (Bacl/NCS). (−)-Baclofen (100 μM) in combination with GHB (250 μM) (Bacl/GHB) produced an increase in low-K_m GTPase activity that was significantly different from control ($p < 0.005$; Dunnett's two-tailed test for multiple comparisons), baclofen alone ($p < 0.01$; Dunnett's two-tailed test for multiple comparisons), or GHB alone ($p < 0.01$; Dunnett's two-tailed test for multiple comparisons), indicating additivity. **B:** Regional effect of GHB or (−)-baclofen on low-K_m GTPase activity in rat brain. Data are mean ± SEM (bars) values of eight determinations. GHB (250 μM) produced a significant ($p < 0.005$; ANOVA) increase in low-K_m GTPase activity compared with controls in cortex and hippocampus but not thalamus or cerebellum. (−)-Baclofen (100 μM) stimulated low-K_m GTPase activity in all brain regions examined, including cerebellum.

ries that $GABA_BR2$ is coexpressed with $GABA_BR1$ in many brain regions and that inwardly rectifying potassium channels are activated by $GABA_BR$ agonists only on coexpression of $GABA_BR1$ with $GABA_BR2$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Lingenhoehl et al. (1999) have shown that GHB can activate $GABA_BR1/R2$ coexpressed with Kir3 channels in *Xenopus* oocytes with an EC_{50} of ~5 m*M*. This affinity is too low to explain a high-affinity GHB binding site on the $GABA_BR$. Furthermore, an EC_{50} of 5 m*M* in these experiments makes it unlikely that the $GABA_BR$ is the receptor that mediates the depolarizing effects of low doses of GHB (Diana et al., 1991; Godbout et al., 1995) or the inhibition of Ca^{2+} conductances in neurohybridoma cells in culture (Kemmel et al., 1998) because the concentration of GHB used in those studies is too low to activate recombinant $GABA_BR1/R2$.

The rationale for choosing adenylyl cyclase as a putative presynaptic effector that might be negatively coupled to GHB is based on data showing that $GABA_BR$ activation results in potentiation of isoproterenol-stimulated activation of adenylyl cyclase and inhibition of forskolin-stimulated adenylyl cyclase (Karbon and Enna, 1985; Scherer et al., 1988; Malcangio and Bowery, 1993; Cunningham and Enna, 1996; Knight and Bowery, 1996). In addition, presynaptic adenylyl cyclase has been shown to be functionally coupled to presynaptic neurotransmitter release (Chavez-Noriega and Stevens, 1994; Kemp et al., 1994; Fedorovich et al., 1999), and there is evidence from in vivo microdialysis experiments that GHB induces a robust decrease in the basal presynaptic release of GABA that is specifically blocked by a GHB antagonist (Maitre et al., 1990; Hechler et al., 1991; Banerjee and Snead, 1995; Gobaille et al., 1999; Hu et al., 2000). The GHB-induced presynaptic inhibition of GABA release in thalamus occurred at 250 μM GHB, a concentration that was shown in the current experiments both to inhibit forskolin-induced cAMP formation and to stimulate [^{35}S]GTPγS binding and low-K_m GTPase activity, all indicative of GHB-mediated G protein activation in the brain.

SYs represent functionally intact neuronal terminals (Musgrave et al., 1994) and have been used as an investigative tool to study the basic mechanisms of presynaptic neurotransmitter release (Goldbach et al., 1998; Cattabeni et al., 1999; Dam et al., 1999; Sunderland et al., 2000), presynaptic ion fluxes (Perkinton and Sihra, 1998; Vinje et al., 1999), and presynaptic phosphorylation (Dekker et al., 1990). GHB has been shown to be present in SY-enriched fractions of brain with no evidence of leakage or metabolism, and the adequacy of the SY preparation used in these experiments in representing presynaptic nerve endings has been confirmed by electron microscopy and by the relative increase in activity of choline acetyltransferase and succinic semialdehyde dehydrogenase in the SY and mitochondrial fractions, respectively (Snead, 1987). SNs contain both post- and presynaptic elements (Johnson et al., 1997), but there is evidence that SNs represent a primarily postsynaptic neuronal fraction (Daly et al., 1980, 1982; Musgrave et al., 1994). SNs have been used to investigate postsynaptic phenomena such as GABA-stimulated Cl^- flux (Buck and Harris, 1990; DeLorey and Brown, 1992) and postsynaptic cAMP accumulation in response to catecholamines (Chasin et al., 1974), serotonin (Psy-

cholyos, 1978), and adenosine (Daly et al., 1980). The finding that GHB negatively coupled to cAMP in SY preparations and not SN preparations, taken together with the observation that the effects of a $GABA_BR$ agonist known to stimulate both pre- and postsynaptic $GABA_BRs$ (Mott and Lewis, 1994) were seen in both SYs and SNs, gives additional credence to the idea that the GHB receptor is presynaptic. These findings agree with published pharmacological (Maitre et al., 1990; Hechler et al., 1991; Banerjee and Snead, 1995; Snead, 1996*a*; Gobaille et al., 1999; Hu et al., 2000) and physiologic (Berton et al., 1999) data suggesting that GHB acts primarily at a presynaptic site. Alternatively, there may be postsynaptic GHB receptors that couple to a different second messenger system from that examined in the current experiments. If this hypothesis is correct, any postsynaptic GHB-mediated effects would have been missed.

The ontogeny data in the current experiments suggest that the effects of GHB on cAMP do not appear until the third week of postnatal life, whereas the effects of the $GABA_BR$ agonist emerge earlier in development. These findings are in agreement with data showing that the developmental appearance of the $GABA_BR$ is early in life (Fukada et al., 1993; Turgeon and Albin, 1993; Snead, 1994; Fritschy et al., 1999) and antedates that of the GHB binding site, which does not appear until P17 (Snead, 1994). Therefore, the ontogeny data suggest that the effects of GHB on forskolin-stimulated cAMP are GHB-specific.

In summary, the present studies do not support the hypothesis that the GHB binding site is part of the $GABA_BR$ or that GHB exerts its physiologic effects via $GABA_BR$-mediated mechanisms. The absence of a significant effect of GHB on basal cAMP levels in brain and the ability of GHB to decrease forskolin-stimulated activity in brain are similar to the effects of $GABA_BR$ agonists on adenylyl cyclase; however, the lack of effect of GHB on isoproterenol-stimulated adenylyl cyclase activity is different from the predicted increase that would be seen with $GABA_BR$ agonists (Knight and Bowery, 1996). Also, the fact that the effect of GHB on forskolin-stimulated cAMP did not appear until P21 makes it unlikely that this was $GABA_BR$-mediated because the $GABA_BR$ is present in the brain at birth and is functional well before P21 (Al-Dahan and Thalmann, 1989; Fukada et al., 1993; Zhang et al., 1999). Similarly, the absence of an effect of GHB on forskolin-stimulated cAMP, [^{35}S]GTPγS binding, or low-K_m GTPase activity in cerebellum makes it unlikely that this is a $GABA_BR$-mediated effect because the cerebellum is replete with $GABA_BRs$ but devoid of GHB binding sites (Hechler et al., 1992; Maitre, 1997). Conversely, the regions in which GHB exerted significant effects on cAMP and G protein were those where the density of [^{3}H]GHB binding sites is the highest, i.e., cortex and hippocampus (Hechler et al., 1987, 1992). Finally, the pharmacology data do not support the premise that GHB decreased forskolin-induced cAMP formation or stimulated G protein via a $GABA_BR$-mediated mechanism of action because these effects were blocked by a specific GHB antagonist but not a specific $GABA_BR$ antagonist. Therefore, the data from these experiments support the hypothesis that GHB induces a G protein-mediated decrease in adenylyl cyclase activity via a GHB-specific, G protein-coupled presynaptic receptor that is different from the $GABA_BR$.

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