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Differential Expression of γ-Aminobutyric Acid Type B Receptor Subunit mRNAs in the Developing Nervous System and Receptor Coupling to Adenylyl Cyclase in Embryonic Neurons

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ABSTRACT

γ-Aminobutyric acid type B receptors ($GABA_BRs$) mediate both slow inhibitory synaptic activity in the adult nervous system and motility signals for migrating embryonic cortical cells. Previous papers have described the expression of $GABA_BRs$ in the adult brain, but the expression and functional significance of these gene products in the embryo are largely unknown. Here we examine $GABA_BR$ expression from rat embryonic day 10 (E10) to E18 compared with adult and ask whether embryonic cortical neurons contain functional $GABA_BR$. $GABA_BR1$ transcript levels greatly exceed $GABA_BR2$ levels in the developing neural tube at E11, and olfactory bulb and striatum at E17 but equalize in most regions of adult nervous tissue, except for the glomerular and granule cell layers of the main olfactory bulb and the striatum. Consistent with expression differences, the binding affinity of GABA for $GABA_BRs$ is significantly lower in adult striatum compared with cerebellum. Multiple lines of evidence from in situ hybridization, RNase protection, and real-time PCR demonstrate that $GABA_BR1a$, $GABA_BR1b$, $GABA_BR1h$ (a subunit subtype, lacking a sushi domain, that we have identified in embryonic rat brain), $GABA_BR2$, and $GABA_BL$ transcript levels are not coordinately regulated. Despite the functional requirement for a heterodimer of $GABA_BR$ subunits, the expression of each subunit mRNA is under independent control during embryonic development, and, by E18, $GABA_BRs$ are negatively coupled to adenylyl cyclase in neocortical neurons. The presence of embryonic $GABA_BR$ transcripts and protein and functional receptor coupling indicates potentially important roles for $GABA_BRs$ in modulation of synaptic transmission in the developing embryonic nervous system. J. Comp. Neurol. 473: 16–29, 2004.

Indexing terms: GABA; receptor; embryo; olfactory bulb; striatum; in situ hybridization

The inhibitory neurotransmitter γ-aminobutyric acid (GABA) has been implicated in many physiological processes, including cognition, pain, anxiety, and epilepsy (Rabow et al., 1995; Couve et al., 2000; Russek, 2003). The actions of GABA are mediated through ionotropic and metabotropic receptors. At the ionotropic type A receptor ($GABA_AR$), GABA initiates fast inhibition by gating a chloride ion channel. At the metabotropic type B receptor ($GABA_BR$), GABA mediates slow pre- and postsynaptic inhibition by interaction with guanine nucleotide-binding (G) proteins that activate potassium channels, inactivate voltage-dependent calcium channels, and modulate adenylyl cyclase activity. The role of the $GABA_BR$ in these distinct processes and the molecular requirements are now being addressed after the initial cloning of two $GABA_BR$ genes (Kaupmann et al., 1997, 1998a; White et al.,

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1998; Jones et al., 1998; Kuner et al., 1999; Martin et al., 1999; Ng et al., 1999).

GABA has been proposed to act as a trophic factor during nervous system development to influence events such as proliferation, migration, differentiation, synapse maturation, and cell death (Owens and Kriegstein 2002). During embryonic development and in certain parts of the adult brain, $GABA_A$Rs may influence synaptic plasticity through an excitatory rather than an inhibitory mechanism (Stein and Nicoll, 2003; Gulledge and Stuart, 2003). There is also evidence implicating $GABA_B$Rs as mediators of motility signals for migrating embryonic cortical cells (Behar et al., 2001) and cell movement in the ventromedial nucleus of the hypothalamus (Davis et al., 2002). GABAR agonists stimulate, whereas antagonists inhibit, embryonic nervous system migration (Behar et al., 1998, 2000, 2001; Davis et al., 2002). Determining the distribution of $GABA_B$R subunit subtypes in the developing embryo and the ability of $GABA_B$R in embryonic neurons to couple to effectors will provide a basis for understanding whether one $GABA_B$R subunit subtype or both may be involved in the chemoattractant effects of GABA.

We analyzed human $GABA_B$R1 and $GABA_B$R2 gene structure and found evidence for three alternative transcripts of $GABA_B$R1 and a single transcript for $GABA_B$R2 (Martin et al., 2001). Although some initial reports indicated that expression of either $GABA_B$R1 (Kaupmann et al., 1997, 1998b) or $GABA_B$R2 (Kaupmann et al., 1998a; Kuner et al., 1999; Martin et al., 1999) could produce functional receptors in cell lines or *Xenopus* oocytes that were transiently or stably transfected, others indicated the coexpression of both $GABA_B$R1 and $GABA_B$R2. Recent studies support the hypothesis that both $GABA_B$R1 and $GABA_B$R2 subunits are necessary for $GABA_B$R function in cellular expression systems (Bowery et al., 2002; Calver et al., 2002). Mice with a null $GABA_B$R1 gene fail to respond to $GABA_B$ agonists (Prosser et al., 2001; Schuler et al., 2001), although $GABA_B$R2 protein levels are also decreased in these mice.

The requirement of two $GABA_B$R subunits for functional expression suggests that both genes should be expressed coordinately. We therefore examined, by using in situ hybridization and RNase protection analysis, whether the mRNA levels for rat $GABA_B$R1 and $GABA_B$R2 are present to the same extent in discrete regions of the embryonic and adult nervous systems. The results indicate that $GABA_B$R1 and $GABA_B$R2 transcript levels are not coordinately regulated during embryonic development but that cultured E18 neocortical neurons exhibit $GABA_B$R-activated adenylyl cyclase activity.

MATERIALS AND METHODS

In situ hybridization of rat tissue sections and probe preparation

In situ hybridization was carried out essentially as described by Martin et al. (1995) with ^{35}S-labeled rat $GABA_B$R1 and $GABA_B$R2 antisense probes. The probe templates were derived from a cDNA for $GABA_B$R1 (AF542081) and a cDNA generated by PCR for $GABA_B$R2 (AF112975; Martin et al., 1999). The region of $GABA_B$R1 used for in situ hybridization (bases 1–788 of AF542081) and RNase protection assay (RPA; bases 663–788 of AF542081, protected fragment 125 bp) probes correspond to the 3′ end of $GABA_B$R1. The $GABA_B$R2 probes used for both in situ hybridization and RPA were derived from the same cDNA (AF112975, 459 bp) and correspond to the carboxyl end of $GABA_B$R2. For RPA, the DNA was cut with BglII, and the protected fragment was 282 bp (bases 177–459 of AF112975). The $GABA_B$R2 probe is expected to hybridize to a single form of $GABA_B$R2 (Martin et al., 2001). The $GABA_B$R cDNA plasmids were linearized with restriction enzymes and used as templates to synthesize riboprobes with T7 RNA polymerase. cRNA was labeled with ^{35}S for in situ hybridization and ^{32}P for RPA. The template for cyclophilin riboprobe synthesis was purchased from Ambion (7680: pTRI-cyclophilin-rat, protected fragment 105 bp; Ambion, Austin, TX). Digital images were acquired in Adobe Photoshop 5.0 (Adobe Systems Inc., Mountain View, CA) as described by Martin et al. (1998). Adobe PageMaker was used to add text, assemble images, and print figures with a Kodak Digital Science 8650 printer.

RNA isolation

E10–E12 total RNA was purchased from Ambion. E17 rat tissue (whole embryo, head and trunk) was collected. Male Sprague-Dawley rats (225–250 g) were treated with 95% CO_2/5% O_2, and the brains were rapidly removed. The research involving rats was approved by the Boston University Medical Center animal care and use committee, which conforms to NIH guidelines. Brain regions were isolated by gross dissection on ice (whole brain, [whole brain without cerebellum], cerebellum, neocortex, hippocampus, nucleus accumbens, striatum, olfactory bulb). The retina was dissected from the lens. All samples were frozen at −80°C until RNA extraction. Total RNA was extracted (RNA isolation kit; Qiagen, Valencia, CA) and stored at −80°C.

RPA

Total RNA (10–50 μg) was hybridized overnight (12–18 hours) at 45°C to three ^{32}P-labeled cRNA probes [together: $GABA_B$R1 and $GABA_B$R2 (1.5×10^5 cpm of each probe) and the internal standard cyclophilin (5×10^3 cpm)] in hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl, 80% formamide) after a 20-min denaturation at 85°C. As a negative control, the total RNA in the hybridization reaction was replaced with either tRNA or no RNA. After hybridization, protected fragments were generated, and excess unhybridized probe was digested by 105 U of RNase T1 (Ambion) in digestion buffer (10 mM Tris-HCl, 5 mM EDTA, and 300 mM NaCl) for 60 minutes at 37°C. RNA–RNA hybrids were separated on a denaturing 6% polyacrylamide gel and visualized by autoradiography and phosphorimaging (Molecular Dynamics, Sunnyvale, CA). Levels of cyclophilin and $GABA_B$R mRNAs were evaluated by measuring the pixel intensity of the corresponding bands. The band intensity of the internal standard cyclophilin was used to normalize the $GABA_B$R subunit band intensity.

Real-time reverse transcriptase-PCR

Probes were synthesized by Applied Biosystems (Foster City, CA), and forward and reverse primers were synthesized by Oligos Etc. (Wilsonville, OR; see Table 1). Rat primers were designed to amplify the form of $GABA_B$R1 lacking the second sushi domain, described for human as $GABA_B$R1c (Martin et al., 2001). A comparable $GABA_B$R1

TABLE 1. Sequences for the Real-Time PCR Probe, Forward and Reverse Primers

Gene	Primer	Sequence 5′–3′
$GABA_BR1a$	Forward	CACACCCAGCCGCTGTG
	Reverse	GAGGTCCCCACCCGTCA
	Probe	CCGAATCTGCTCCAAGTCTTATTTGACCC
$GABA_BR1b$	Forward	GGGACCCTGTACCCCAGTG
	Reverse	GGAGTGAGAGGCCCACACC
	Probe	CCGCTGCCTCTTCTGCTGGTGATG
$GABA_BR1h$	Forward	GCTCCTGGACGGATATGGAC
	Reverse	CACGCCGTTCTGAGTGTGG
	Probe	CCCAGCCGCTGTGTGAATCGAAC
$GABA_BR2$	Forward	GCACAAACACCAGGCAGAGAG
	Reverse	CAGCCCAACGTGCAGTTCT
	Probe	ATCGTGGCCCTGGTCATCATCTTCTG
$GABA_BL$	Forward	CATCATGGTCGGAGTCAACCT
	Reverse	AAAGACCAGATTGGGCCAGG
	Probe	CCTGCTTTTTGTGGTCACCAGATACTTGCA

isoform lacking the second sushi domain has not been described for rat. We refer to this $GABA_BR1$ isoform as *$GABA_BR1h$*, the next unused letter for rat $GABA_BR1$ isoforms. The cDNA was synthesized by using random hexamers and Moloney murine leukemia virus reverse transcriptase from an Applied Biosystems kit. The real-time PCRs were set up in triplicate containing a total volume of 50 μl that was then analyzed as duplicate 20-μl aliquots in a 384-well plate in an ABI prism Applied Biosystems 7900HT Sequence Detection System. The 50-μl reaction volume contained ABI master mix, 250 μM receptor probe, 900 nM receptor forward primer, 900 nM receptor reverse primer, 1 μM rRNA probe, 250 nM rRNA forward, and 250 nM rRNA reverse primer. The rRNA probe and primers were purchased from Applied Biosystems. The reactions were incubated at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. E17 cDNA was used to generate a standard curve. The rRNA was used as an internal control and to normalize the results. The mean was taken of the three determinations, and the results are expressed in arbitrary units.

Preparation of brain membrane fractions

Membrane fractions were prepared at 4°C from male Sprague Dawley rat cerebellum, cerebral cortex, olfactory bulb, and striatum. The brain areas were removed and homogenized in 10 volumes of 0.32 M sucrose in a Teflon homogenizer. After centrifugation at 1,000*g* for 10 minutes, the pellet (P_1) was discarded and the supernatant subjected to centrifugation for 20 minutes at 20,000*g*. The pellet (P_2) was washed six times by homogenization in phosphate-buffered saline solution PBSS (123 mM NaCl, 0.4 mM KCl, 11 mM NaH_2PO_4, pH7.4, 0.9 mM $CaCl_2$, 0.4 mM $MgSO_4$, 22.2 mM glucose), followed by centrifugation (20 minutes at 20,000*g*). The final pellet was resuspended in binding buffer (50 mM Tris-HCl, pH 7.4, containing 2.5 mM $CaCl_2$).

Ligands and reagents

[S-(R*,R*)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid (CGP54626 hydrochloride) and 3-aminopropyl-methyl-phosphinic acid (CGP35024, SKF97541) were obtained from Tocris. Forskolin isolated from *Coleus forskohlii* and GABA were obtained from Sigma (St. Louis, MO). 4-Amino-3-(p-chlorophenyl)-butyric acid (baclofen hydrochloride) and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from RBI (Natick, MA). 3-Aaminopropylphosphinic acid (CGP27492) was kindly provided by Dr. Wolfgang Froestl and Dr. Johannes Mosbacher (Novartis Pharma Inc., Basel, Switzerland).

[^{3}H]CGP54626 binding assay

Competition binding experiments were performed by incubating aliquots of the P_2 membrane suspension (150 μg protein) in 500 μl of binding buffer with 1 nM [^{3}H]CGP54626 (40 Ci/mmol; Tocris) in the presence of increasing concentrations of GABA (10 nM to 1 mM) for 1 hour at 0°C. After incubation, the samples were diluted with 5 ml of buffer and immediately filtered under vacuum through glass-fiber filters (GF/B Whatman, Clifton, NJ). The filters were washed three times with 5 ml of buffer, and the radioactivity was quantitated by liquid scintillation counting in 5 ml of Ecolite scintillation fluid (ICN, Costa Mesa, CA). Nonspecific binding was determined in the presence of 100 μM GABA and was subtracted from total binding to calculate specific binding. Data were analyzed with a nonlinear regression program. Statistical comparisons were performed by one-way analysis of variance (ANOVA) and Fisher's PLSD post hoc test.

Western blot analysis

$GABA_BR1$ was immunoprecipitated by using a guinea pig polyclonal antisera raised against the synthetic peptide PSEPPDRLSCDGSRVHLLYK from rat $GABA_BR1$ (AB1531; Chemicon, Temecula, CA). $GABA_BR2$ was immunoprecipitated by using a guinea pig polyclonal antisera raised against the synthetic peptide corresponding to the c-terminal amino acids of rat and λ human $GABA_BR2$ (AB5394; Chemicon). $GABA_BR1$ was detected by Western blotting with the guinea pig polyclonal $GABA_BR1$ antisera. The polypeptides were detected with protein A horseradish peroxidase (Zymed, South San Francisco, CA) and enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Cortical neurons and cAMP assay

Primary rat E18 neocortical neurons were grown in defined media (Pike et al., 1993). The neocortical neurons were maintained in culture for 7 days in 24- or 48-well plates. The neurons were washed twice and incubated at 37°C for 20 min in Krebs-Tris buffer (20 mM Tris-HCl pH 7.3, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KPO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$) containing 1 mM IBMX, and then 20 μM forskolin and test agents were added for 20 minutes. The cells were lysed in 10% dodecyltrimethylammonium bromide in 0.05 M acetate buffer and 0.01% (w/v) sodium azide and assayed for cAMP by using the Biotrak cAMP ^{125}I scintillation proximity assay system purchased from Amersham. The results are expressed as mean percentage change in cAMP levels ± SEM. The baclofen concentration response curve shown in Figure 6 was fitted to pooled data by nonlinear regression with the logistic equation (KaleidaGraph Synergy Software, Reading, PA). The sigmoidal curve is plotted on a logarithmic scale of concentration. Emax, Hill coefficient, n_H, and EC_{50} values (as logarithms ± SEM) were averaged from five individual concentration–response curves. The antilog of the log EC_{50} is also shown as a molar concentration for comparison.

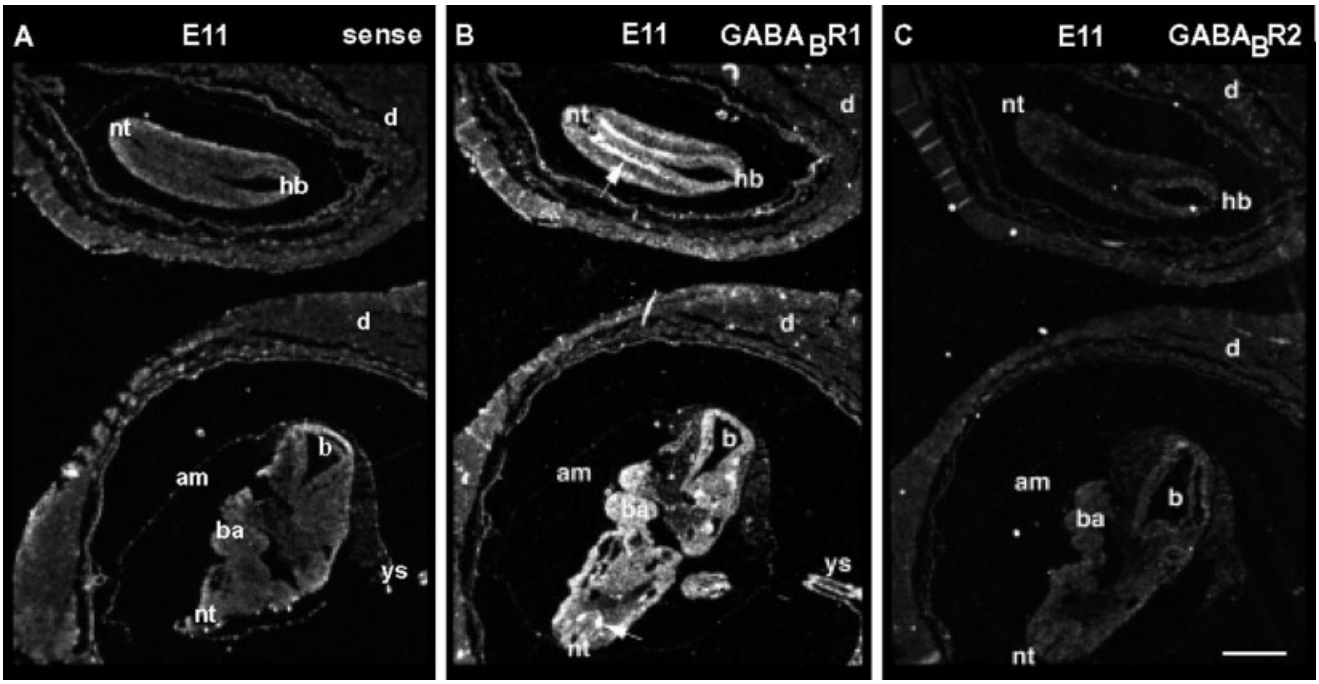


Fig. 1. GABA$_B$R1 but not GABA$_B$R2 transcripts are expressed in elements of rat E11 neural tube and brain as shown by in situ hybridization. **A–C:** In situ hybridization darkfield illuminations of GABA$_B$R1 and GABA$_B$R2 transcripts in the E11 embryo, in the decidua. Serial tissue sections were hybridized with RNA probes labeled with ^{35}S-UTP specific for sense GABA$_B$R1 (A), antisense GABA$_B$R1 (B), and antisense GABA$_B$R2 (C). GABA$_B$R1 transcripts were detected in the ventral portion of the developing neural tube (nt). The location of the amnion (am), brain (b), branchial arch (ba), decidua (d), hindbrain (hb), and yolk sac (ys) is indicated. The GABA$_B$R1 hybridization signal observed in the neural tube is indicated by an arrow. Scale bar = 50 μm.

RESULTS

Differential distribution of GABA$_B$R1 and GABA$_B$R2 transcripts in the early embryo

We used in situ hybridization to investigate whether GABA$_B$R transcripts are present during early embryonic development. At E11, GABA$_B$R1 transcripts were detected in the brain and neural tube, but GABA$_B$R2 transcripts were below the level of detection (Fig. 1). RPA was used to quantitate GABA$_B$R1 and GABA$_B$R2 transcript levels; the probes and protected fragments are shown in Figure 2 and in the early embryo (E10–E12) reveal tenfold higher levels of GABA$_B$R1 than GABA$_B$R2 mRNA (Fig. 3). The in situ hybridization and RPA data strongly suggest that GABA$_B$R1 and GABA$_B$R2 genes are not coordinately regulated during early embryonic development, even though both subunits are thought to be necessary for function.

Codistribution of GABA$_B$R1 and GABA$_B$R2 transcripts at E14 in the brain, cranial, and dorsal root ganglia

The GABA$_B$R transcript localization was examined at E14, when subdivisions of the forebrain, midbrain, and hindbrain are discernible while further structures are still developing. By E14, both GABA$_B$R1 and GABA$_B$R2 transcripts are detectable in the brain and peripheral ganglia (Fig. 4). In contrast to E11, when GABA$_B$R1 is detected and GABA$_B$R2 is virtually absent, both transcripts are detected at E14, although GABA$_B$R1 is more highly expressed. It remains to be demonstrated whether differences in transcript levels reflect alterations in the transcriptional activation or differential mRNA stability.

Abundance of GABA$_B$R1 transcripts and low levels of GABA$_B$R2 accompany the development of the basal ganglia and olfactory bulb

By E17, further brain subdivisions such as the olfactory bulb and the striatum in the basal ganglia are observed. Remarkably, the differential distribution of GABA$_B$R transcripts in the adult brain is already present by E17 (Fig. 5). GABA$_B$R1 and GABA$_B$R2 are both expressed in the cortex at E17, but, in contrast, GABA$_B$R2 transcripts are barely detectable compared with GABA$_B$R1 in the striatum and olfactory bulb. Quantitation of RNA levels from embryonic tissue by RPA demonstrates that the GABA$_B$R1 transcripts are at least twice the GABA$_B$R2 transcript levels in E17 whole embryo, E17 head and E17 trunk (Fig. 3). Results of RPA confirm the differential levels of GABA$_B$R transcripts as detected by in situ hybridization. Differences in GABA$_B$R subunit mRNA expression observed in the striatum and olfactory bulb of the embryo and adult brain are established early in development.

Detection of GABA$_B$R1 and GABA$_B$R2 proteins in embryonic tissue accompanies the presence of GABA$_B$R mRNAs

Results of in situ hybridization and RPA indicate that GABA$_B$R1 and GABA$_B$R2 mRNAs are present by E17.

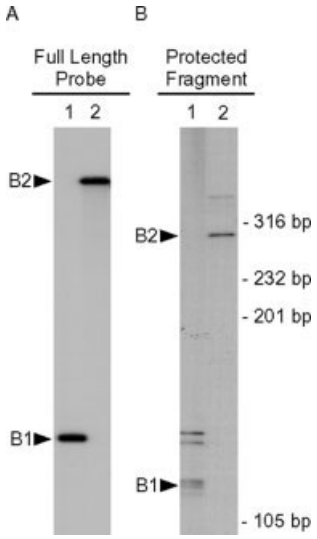


Fig. 2. $GABA_BR1$ and $GABA_BR2$ RPA probes and protected fragments. Adult rat neocortical total RNA (10 μg) was hybridized with ^{32}P-labeled cRNA probes specific to $GABA_BR1$ and $GABA_BR2$. The location of the full length (**A**) or protected fragments (**B**) corresponding to $GABA_BR1$ (B1) and $GABA_BR2$ (B2) is indicated at left. The size of protected fragments for $GABA_BR1$ is 125 bp and for $GABA_BR2$ is 282 bp. The location of radioactive RNA size markers is indicated at right.

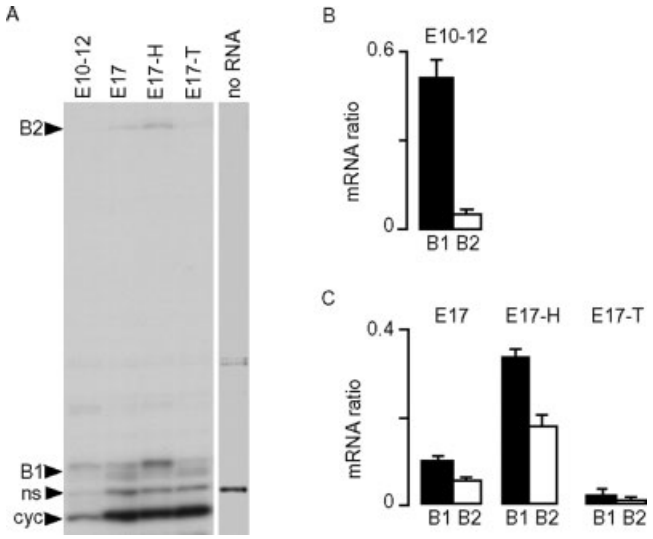


Fig. 3. $GABA_BR1$ greatly exceeds $GABA_BR2$ in transcript level in E10–E12 and E17 embryos, as shown by RPA. **A:** Rat total RNA was isolated from E10–E12 (E10–E12, 50 μg), E17 (10 μg), E17 head (E17-H, 10 μg), and E17 trunk (E17-T, 10 μg). The abundance of $GABA_BR1$, $GABA_BR2$, and cyclophilin was measured. The location of the protected fragments corresponding to $GABA_BR1$ (B1), $GABA_BR2$ (B2), and cyclophilin (cyc) is indicated at left. The size of protected fragments for $GABA_BR1$ is 125 bp, for $GABA_BR2$ is 282 bp, and for cyclophilin is 105 bp. A nonspecific (ns) fragment is present in the embryo RNA hybridizations and in the no-RNA control with all three probes. Single probe hybridizations were used to determine that the nonspecific fragment is from the cyclophilin probe (data not shown; **B,C**) The mRNA ratio (receptor/cyclophilin) was determined for $GABA_BR1$ (B1, solid bars) and $GABA_BR2$ (B2, open bars). Bars represent the mean ± SEM of between four and six determinations. Note that cyclophilin levels are different at E10–E12 and E17.

Immunoprecipitation and Western blot analysis using $GABA_BR$ antisera show that $GABA_BR1a$, $GABA_BR1b$, and $GABA_BR2$ proteins are expressed at E18 (Fig. 6), which is consistent with the presence of functional embryonic $GABA_BRs$.

$GABA_BR$-mediated adenylyl cyclase function in embryonic rat neurons

$GABA_BRs$ negatively couple to adenylyl cyclase in the adult brain (Knight and Bowery, 1996). To determine whether $GABA_BRs$ are coupled to adenylyl cyclase in embryonic neurons, we monitored cAMP levels in primary monolayer cultures of neocortical neurons. $GABA_BR$ agonists do not alter basal levels of cAMP but decrease forskolin-induced cAMP levels by 48.8% ± 3.1% for GABA (100 μM), 43.6% ± 3.6% for baclofen (100 μM), 30.2% ± 5.5% for 3-aminopropyl-methyl-phosphinic acid (SKF97541, CGP35024; 10 μM), and 41.6% ± 8.8% for 3-aminopropyl phosphinic acid (CGP27492, 100 μM; Fig. 7). Forskolin (20 μM) stimulated a 13-fold increase in cAMP levels compared with basal values (basal: 7.4 ± 0.9 pmol; stimulated: 98.4 ± 8.5 pmol; n = 24).

To test whether the $GABA_BR$ agonist-induced change in cAMP levels could be specifically inhibited by a $GABA_BR$ antagonist, CGP54626A, we compared forskolin-stimulated cAMP levels in neocortical neuronal cultures treated with the agonist, with the agonist and antagonist together, and with the antagonist alone. Baclofen (30 μM), but not CGP54626A (1 μM), significantly reduced forskolin-induced cAMP accumulation and CGP54626A significantly antagonized the effect of the agonist (Fig. 7). Inhibition of baclofen-induced reduction of adenylyl cyclase activity by the highly selective $GABA_BR$ antagonist CGP54626A demonstrates that baclofen-induced changes in cAMP levels are mediated by $GABA_BRs$.

To compare the function of $GABA_BRs$ in different cell types and at different stages of development, we generated a dose–response curve for baclofen-mediated inhibition of adenylyl cyclase in E18 cortical neurons. The properties of the dose–response curve (Fig. 7; EC_{50}, 1.02 μM; $\log EC_{50}$, −0.044 ± 0.103; maximal inhibition, 52.18% ± 8.93%; Hill coefficient, 1.52 ± 0.38) are similar to those reported previously for adult cortical neurons (Knight and Bowery, 1996), suggesting that the two may share a similar effector system.

Differential localization of $GABA_BR1$ and $GABA_BR2$ transcripts in the adult rat brain

In situ hybridization was also used to compare the expression patterns of $GABA_BR1$ and $GABA_BR2$ transcripts in the adult rat brain. Whereas $GABA_BR1$ and $GABA_BR2$ have a similar pattern of distribution in the cortex and cerebellum, they appear to be differentially expressed in the olfactory bulb and striatum (Fig. 8). $GABA_BR1$ and $GABA_BR2$ transcripts are both detected in the accessory olfactory bulb, but $GABA_BR1$ transcripts are more highly expressed in the granule cell layer and the glomerular cell layer of the main olfactory bulb. In the striatum, $GABA_BR1$ transcripts are markedly expressed, whereas $GABA_BR2$ is barely detectable. Results of Western blot analysis demonstrate that $GABA_BR2$ protein is barely detected in the olfactory bulb and striatum, where $GABA_BR1$ is

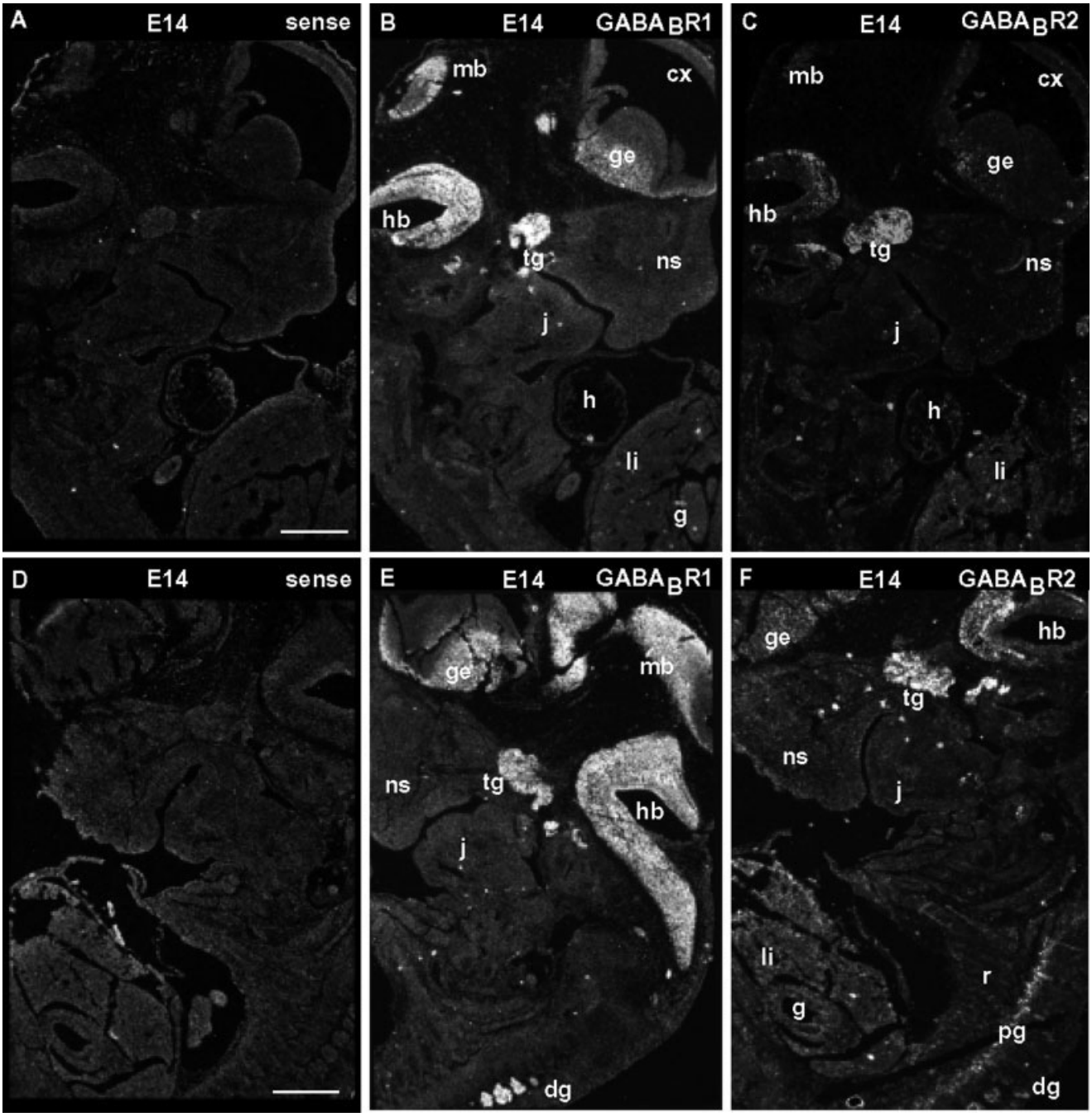


Fig. 4. GABA$_B$R1 and GABA$_B$R2 transcripts are expressed in E14 central and peripheral nervous tissue, as shown by in situ hybridization. Serial tissue sections were hybridized with ^{35}S-UTP labeled RNA probes specific for sense GABA$_B$R1 (**A,D**), antisense GABA$_B$R1 (**B,E**), and antisense GABA$_B$R2 (**C,F**). Darkfield illuminations are shown (A–F). GABA$_B$R1 and GABA$_B$R2 transcripts are observed in the brain and cranial ganglia. The location of cochlear nerves (co), cortex (cx), dorsal root ganglion (dg), ganglion eminence (ge), gut (g), heart (h), hindbrain (hb), jaw (j), liver (li), midbrain (mb), nasal sinus (ns), parasympathetic ganglion (pg), rib (r), and trigeminal ganglion (tg) is indicated. Scale bars = 500 μm.

abundantly expressed (data not shown). These results indicate that, as in the embryonic brain, GABA$_B$R1 and GABA$_B$R2 in the adult brain are not always expressed together. The differences in GABA$_B$R transcript expression found with in situ hybridization in the olfactory bulb and striatum are also evident by RPA (Fig. 9) and indicate that the GABA$_B$R1 and GABA$_B$R2 transcripts are not always expressed in an equivalent ratio.

Expression of GABA$_B$L in the adult brain

The GABA$_B$R mRNA transcript results suggest that GABA$_B$R1 may function without GABA$_B$R2 in parts of the

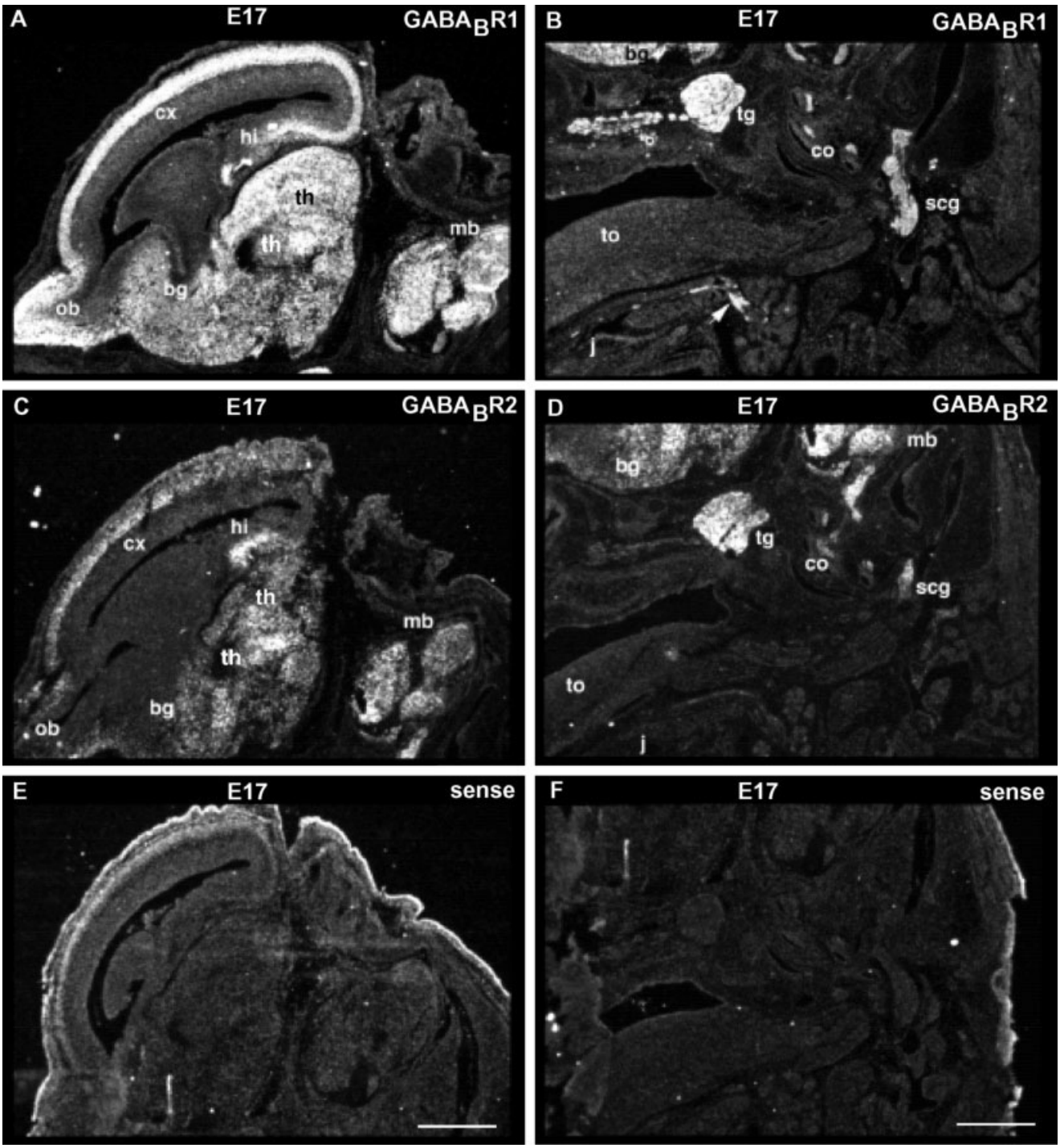


Fig. 5. GABA$_B$R1 and GABA$_B$R2 transcripts are differentially expressed in olfactory bulb and striatum by E17, as shown by in situ hybridization. Serial sagittal sections were hybridized to ^{35}S-UTP labeled RNA probes specific for sense GABA$_B$R1 (**E,F**), antisense GABA$_B$R1 (**A,B**), and antisense GABA$_B$R2 (**C,D**). Darkfield illuminations (A–F) of the E17 rat embryo include the brain (A,C,E) and cranial ganglia (B,D,F). GABA$_B$R1 and GABA$_B$R2 transcripts are detected in the brain and the cranial ganglia. GABA$_B$R1 transcripts are more highly expressed in the olfactory bulb and striatum than GABA$_B$R2 transcripts. The location of the basal ganglia (bg), cochlear nerves (co), cortex (cx), hippocampus (hi), jaw (j), midbrain (mb), olfactory bulb (ob), superior cervical ganglion (scg) thalamus (th), tongue (to), and trigeminal ganglion (tg) is indicated. The GABA$_B$R1 hybridization signal in the jaw and the hypoglossal nerve is indicated by an arrow. Scale bars = 500 μm.

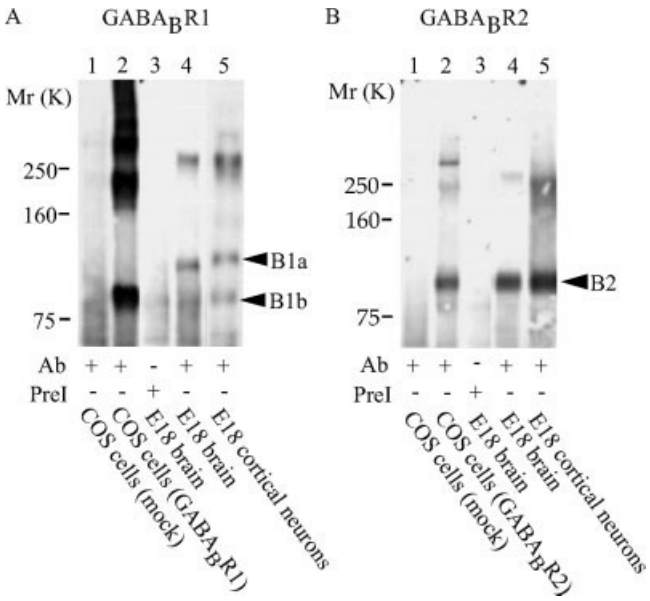


Fig. 6. E18 brain and cortical neurons in culture express GABA$_B$R1 and GABA$_B$R2 proteins, as shown by Western analysis. **A:** Extracts of COS cells expressing GABA$_B$R1 protein, of rat E18 brain, and of rat E18 cortical neurons were immunoprecipitated (IP) with GABA$_B$R1 (A, lanes 1,2,4,5) antisera, GABA$_B$R2 (B, lanes 1,2,4,5) antisera (Ab), or preimmune antisera (PreI) A, lane 3; B, lane 3. Lane 1, mock-transfected COS cells; lane 2, GABA$_B$R1 transfected COS cells; lane 3, E18 brain; lane 4, E18 brain; lane 5, E18 cortical neurons after 7 days in culture. **B:** Same as A except for GABA$_B$R2. Western analysis was performed using GABA$_B$R1 (A) or GABA$_B$R2 (B) antisera. The relative mobilities of GABA$_B$R1a (B1a), GABA$_B$R1b (B1b), and GABA$_B$R2 (B2) are indicated. These results are qualitative and can be interpreted in terms of the protein present in extracts detected by the same antisera, so the results cannot be interpreted in terms of the relative levels of GABA$_B$R1 and GABA$_B$R2 in a given tissue. The relative molecular mass, Mr (K), is indicated at left (full-range rainbow markers; Amersham).

nervous system and may have a different partner. Recently, a novel GABA$_B$ receptor sequence has been cloned and termed *GABA$_B$L* (Calver et al., 2003). We used real-time PCR to examine whether the GABA$_B$L transcript expression is consistent with GABA$_B$L being a possible partner for GABA$_B$R1 in the olfactory bulb and the striatum. Two different primers and probe sets were used to examine GABA$_B$L mRNA expression in the adult whole brain, neocortex, olfactory bulb, and striatum. Both primer sets gave a similar result (data not shown). GABA$_B$L mRNA is expressed as highly in the olfactory bulb and the striatum as the whole brain, suggesting that it could function as a partner for GABA$_B$R1 in regions of the brain where the same is not found for GABA$_B$R2 (Fig. 10). The results of real-time PCR with GABA$_B$R2 primers are consistent with our results of in situ hybridization and RPA, indicating that GABA$_B$R2 mRNA is not uniformly expressed and is present at much lower levels in the striatum and olfactory bulb. Three GABA$_B$R1 gene transcripts (GABA$_B$R1a, GABA$_B$R1b, and GABA$_B$R1h) were measured by real-time PCR. Unlike GABA$_B$R2 and more like GABA$_B$L, GABA$_B$R1a and GABA$_B$R1b transcripts are detectable at similar levels in all regions examined.

GABA$_B$R transcript levels (GABA$_B$R2, GABA$_B$L, GABA$_B$R1a, GABA$_B$R1b, and GABA$_B$R1h) were measured in the embryo at E17 (Fig. 10). GABA$_B$L is 5 times and GABA$_B$R1h 3.5 times more highly expressed in the E17 embryo than in the adult whole brain. Conversely, GABA$_B$R1a, GABA$_B$R1b, and GABA$_B$R2 are 2.4, 7, and 8 times more highly expressed in the adult than the E17 embryo, respectively.

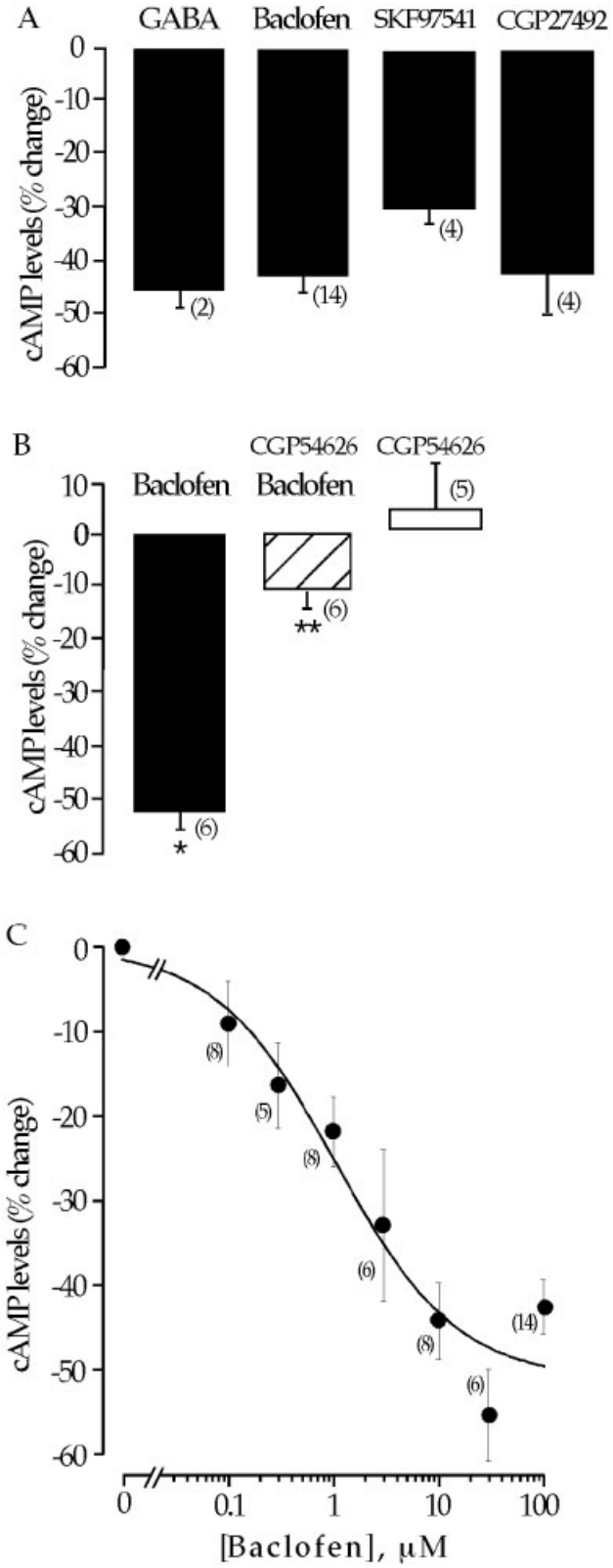
Differential pharmacology of GABA$_B$Rs in the adult cerebellum and striatum

We investigated whether the different levels of GABA$_B$R mRNAs observed by in situ hybridization and RPA result in altered binding properties of GABA$_B$Rs in the brain. We measured the GABA binding affinity in four brain regions by competition experiments using [^{3}H]CGP54626, a high-affinity GABA$_B$R antagonist (Bischoff et al., 1999): cerebellum (IC_{50} = 10.22 μM; $\log IC_{50}$ = –5.06 ± 0.10), striatum (IC_{50} = 20.70 μM; $\log IC_{50}$ = −4.69 ± 0.08), cortex (IC_{50} = 13.21 μM; $\log IC_{50}$ = −4.91 ± 0.08), and olfactory bulb (IC_{50} = 15.77 μM; $\log IC_{50}$ = −4.83 ± 0.07). There is a statistically significant ($P < .05$) difference between the binding affinity of GABA for GABA$_B$Rs in the cerebellum and striatum (Fig. 11), indicating that regions of the brain with differences in GABA$_B$R transcript and protein levels also have GABA$_B$Rs with different pharmacologies.

DISCUSSION

GABA$_B$Rs mediate most slow inhibitory synaptic transmission in the nervous system (Bowery, 1993), subserving a central role in the control of neural activity. Pharmacological manipulation of nervous system function through the use of GABA$_B$R agonists has proved useful for the treatment of pain, spasticity, and drug craving, whereas antagonists suppress absence seizures and improve cognitive performance (Couve et al., 2000; Bowery et al., 2002). Understanding the expression, composition, and function of GABA$_B$Rs may provide important information allowing the development of new pharmacological agents for the treatment of neuropsychiatric disorders.

Examining gene expression during early embryonic development offers a window through which to examine coordinate gene regulation. Here we asked whether GABA$_B$R subunits are expressed coordinately during early embryonic development and compared embryonic and adult levels of expression. The results from in situ hybridization and RPA suggest that GABA$_B$R1 and GABA$_B$R2 transcript levels are not coordinately regulated in the embryo. In particular, the results of in situ hybridization indicate that GABA$_B$R1 but virtually no GABA$_B$R2 transcripts are expressed in the rat neural tube at E11. A similar difference in expression between GABA$_B$R1 and GABA$_B$R2 is also detected by RPA in E10–E12 RNA, showing that GABA$_B$R1 and GABA$_B$R2 transcripts are not coexpressed in the early rat embryo. Western analysis indicates that GABA$_B$R1 and GABA$_B$R2 proteins are present in E18 brain and E18 cultured neocortical neurons. Although RPA is quantitative, differences in antibody affinity limit the usefulness of Western analysis for quantitation of GABA$_B$R1 and GABA$_B$R2 protein when two different antisera must be used. We were initially surprised by the observation that GABA$_B$R1 mRNA can be expressed in the presence of only a small fraction of GABA$_B$R2 mRNA in both embryonic and adult striatum or olfactory bulb be-



cause coassembly of both subunits into a heterooligomeric receptor complex is believed to be required for $GABA_BR$ function (Billinton et al., 2001; Bowery et al., 2002; Calver et al., 2002).

In principle, however, largely unequal transcript levels could lead to sufficient subunit protein production to support the assembly of $GABA_BR1$ and $GABA_BR2$ heteromers. Alternatively, $GABA_BR1$ might combine with an undiscovered receptor subunit or accessory protein. Finally, the lack of mRNA colocalization in certain regions of the CNS may reflect the absence of functional $GABA_BRs$ in neurons within a particular region or stage of development.

Unequal transcript levels in a given neuron could support receptor assembly, but this is not found to be the case for $GABA_BR$ proteins in the adult striatum. Most cells in the striatum express $GABA_BR1$ but lack detectable $GABA_BR2$ protein as demonstrated by immunohistochemistry (Ng and Yung, 2001). We show here by in situ hybridization and RPA analysis that the adult olfactory bulb and striatum are unusual in having a greater expression of $GABA_BR1$ than $GABA_BR2$ transcripts. The results also demonstrate that $GABA_BR1$ expression is greater than $GABA_BR2$ expression in the embryo (E11, E14, and E17), and the most notable difference is found in embryonic olfactory bulb and striatum. The significance of these findings is emphasized by the fact that functional $GABA_BRs$ have been detected in the olfactory bulb (Olianas and Onali, 1999; Isaacson and Vitten, 2003) and striatum (Seabrook et al., 1991; Nisenbaum et al., 1992; DeBoer and Westerink, 1994) even though $GABA_BR2$ is presumably required for receptor trafficking to the cell surface and receptor function.

Our results suggest that $GABA_BR1$ is expressed without $GABA_BR2$ in certain cells within the glomerular and granule cell layers of the olfactory bulb in addition to the striatum. Cells migrate from the lateral ganglionic eminence to populate the glomerular and granule cell layer of the olfactory bulb and striatum (Wichterle et al., 2001). It is tempting to speculate that daughter cells in the olfactory bulb and striatum inherit a $GABA_BR$ gene expression profile as a result of chromatin remodeling events in the lateral ganglionic eminence.

To ask whether embryonic $GABA_BRs$ are functional, we exposed E18 cultured neocortical neurons to $GABA_BR$ agonists and measured adenylyl cyclase activity. Baclofen inhibits forskolin-stimulated adenylyl cyclase activity in embryonic rat cortical neurons with an Emax of 52.2% ± 8.9% with an EC_{50} of 1.02 μM. This is similar to the results obtained with adult rat cortical slices (Emax =

Fig. 7. E18 neocortical neurons in primary cell culture contain functional $GABA_BRs$ coupled to adenylyl cyclase. **A:** GABA (100 μM), R(–) baclofen (100 μM), SKF97541 (10 μM), and CGP27492 (100 μM) reduce cAMP levels in E18 neocortical neurons maintained in primary monolayer cell culture for 7 days (one group *t*-test, $P < .05$). **B:** CGP54626 (1 μM, hatched bar) antagonizes the effect of R(–) baclofen (30 μM, solid bar; paired *t*-test, $^{**}P < .01$). **C:** R(–) baclofen inhibits cAMP levels with EC_{50} = 1.02 μM ($\log EC_{50} = -0.044 \pm 0.103$); Emax = –51.1% ± 5.96%; $n_H = 0.74$. Cells were treated with 20 μM forskolin (fsk) to stimulate cAMP formation. The numbers of independent experiments are given in parentheses, each performed in triplicate. A single asterisk indicates significant differences from zero (one group *t*-test, $^{*}P < .05$).

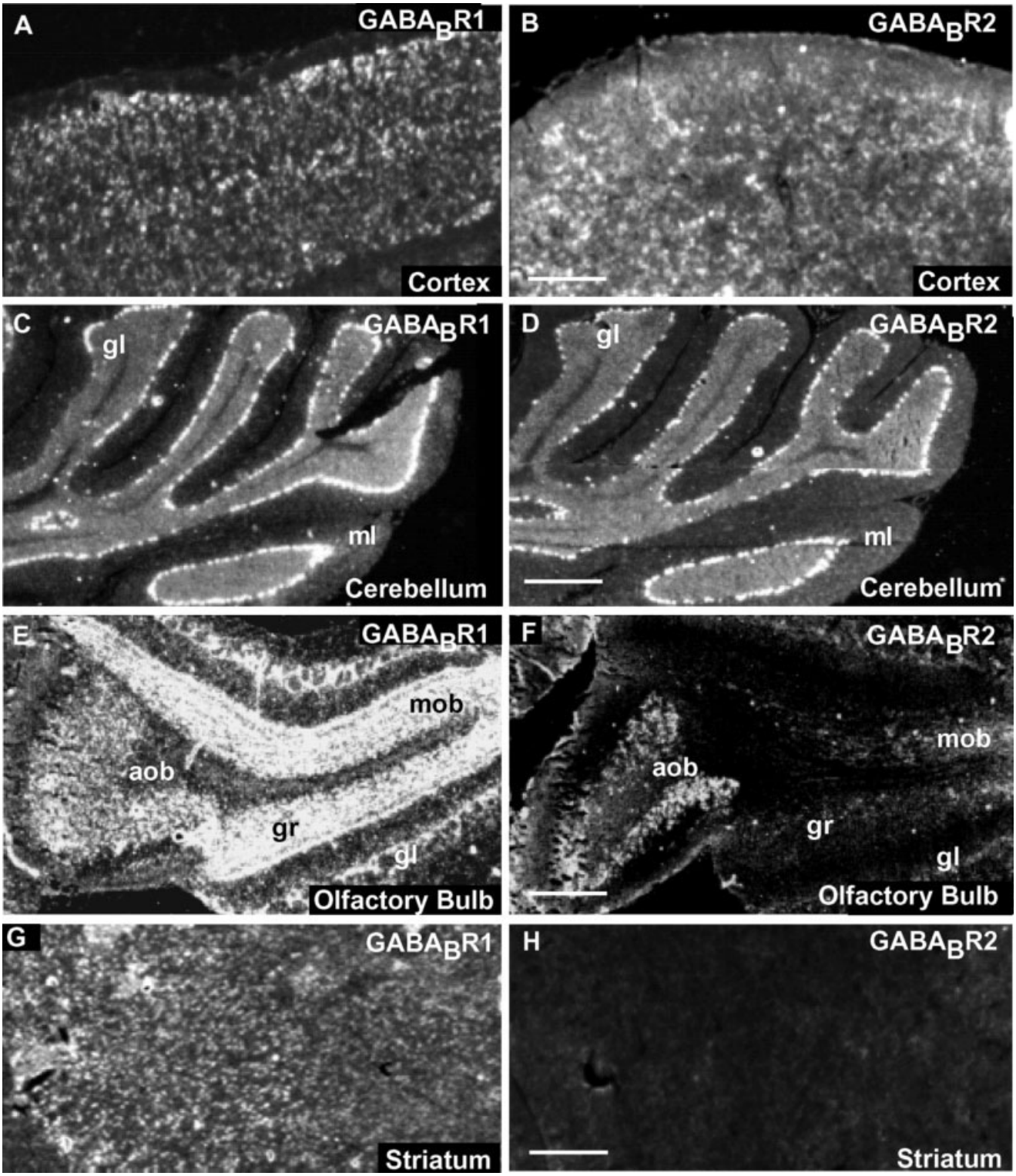


Fig. 8. $GABA_BR1$ exceeds $GABA_BR2$ in transcript level in the adult olfactory bulb and striatum, as shown by in situ hybridization. ^{35}S-UTP-labeled RNA probes specific for $GABA_BR1$ (**A,C,E,G**) and $GABA_BR2$ (**B,D,F,H**) were hybridized to adult rat brain sections. Darkfield illuminations are shown of the cortex (A,B), cerebellum (C,D) olfactory bulb (E,F), and striatum (G,H). The location of the cerebellum molecular layer (ml), granular layer (gl), main olfactory bulb granule cell layer (gr), glomerular cell layer (gl), accessory olfactory bulb (aob), and main olfactory bulb (mob) is indicated. These results are qualitative and can be interpreted in terms of differences in the expression of specific mRNAs between subregions of the cortex, cerebellum, olfactory bulb, and striatum. Scale bars = 50 μm.

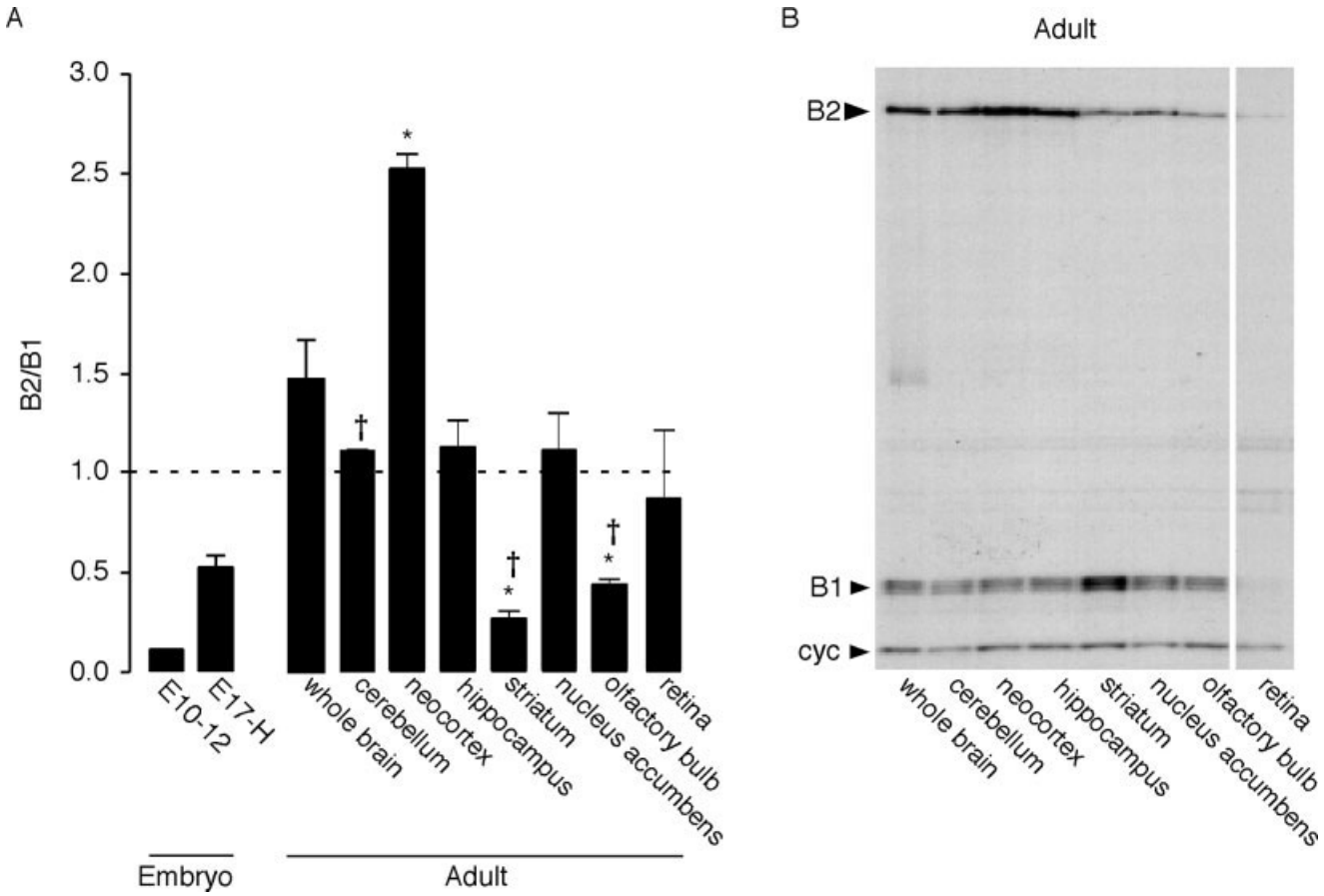


Fig. 9. $GABA_BR1$ and $GABA_BR2$ transcripts are differentially expressed in neocortex, striatum, and olfactory bulb, as shown by RPA. **A:** The ratio between $GABA_BR2$ and $GABA_BR1$ transcripts was determined for the embryonic and adult rat brain. Total RNA was isolated from E10–E12 (50 μg) and E17 head (E17-H, 10 μg). Total RNA (10 μg) was isolated from the adult brain: whole brain, cerebellum, neocortex, hippocampus, striatum, nucleus accumbens, and olfactory bulb. Bars represent the mean ± SEM of three or four determinations. Statistical analysis was performed by using ANOVA and the Fisher's post hoc test. $^*P = .001$, neocortex compared with striatum and olfactory bulb; $^\dagger P = .05$, cerebellum compared with striatum and olfactory bulb. **B:** The location of the protected fragments corresponding to $GABA_BR1$, $GABA_BR2$, and cyclophilin is indicated at left.

42.1% ± 5.6% inhibition, EC_{50} = 0.85 μM; Knight and Bowery, 1996). $GABA_BR$ mediates inhibition of calcium elevations in developing hypothalamic neurons (Obrietan and van den Pol, 1998) and couples negatively to adenylyl cyclase in cultured cerebellar granular cells derived from postnatal day 8 rats (Xu and Wojcik, 1986). The adenylyl cyclase response in embryonic neurons and adult rat cortical tissue indicates that functional $GABA_BRs$ are expressed at both stages of development with a similar pharmacology.

GABA action at embryonic $GABA_ARs$ is excitatory in the hippocampus (Ben-Ari, 2002), but it is less clear which transmitter system causes inhibition in the embryo (Obrietan and van den Pol, 1998). $GABA_BRs$ are reasonable candidates for mediators of inhibition in the embryonic CNS. However, a delay in the onset of postsynaptic $GABA_BR$ function postnatally has been described, suggesting that at least some components of $GABA_BR$ coupling are not present in early development (Owens and Kriegstein, 2002). $GABA_BR$-stimulated migration has been detected in the embryonic nervous system (Behar et al., 2001), but the type of coupling that could be potassium channels, voltage-dependent calcium channels, or adenylyl cyclase has not been described. Our results demonstrate that $GABA_BR$ is coupled to adenylyl cyclase in embryonic cortical neurons, indicating that embryonic cortical neurons contain all of the components required for this type of G-protein-coupled receptor function. The results presented in this report suggest that $GABA_BRs$ may play an inhibitory role in the embryonic nervous system by coupling to adenylyl cyclase.

The low level of $GABA_BR2$ transcripts in adult rat (Durkin et al., 1999; Clark et al., 2000) and human (Martin et al., 1999; Clark et al., 2000; Berthele et al., 2001) striatum suggests that different functional $GABA_BR$ isoforms might form in the adult striatum and olfactory bulb, where $GABA_BR1$ is in great excess over $GABA_BR2$. We asked whether the differences in $GABA_BR$ transcript expression between the cerebellum and the striatum result in a difference in GABA binding and, by inference, receptor subunit composition. A significant difference in binding was detected when the striatum and cerebellum were compared. Striatum (which contains a predominance of $GABA_BR1$ mRNA) exhibits a lower affinity of GABA bind-

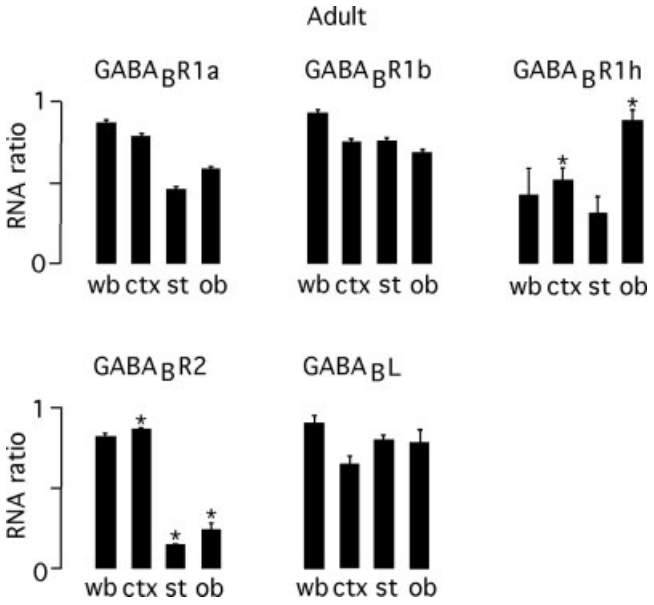


Fig. 10. GABA$_B$R1a, GABA$_B$R1b, and GABA$_B$L share a similar transcript profile that is distinct from that of GABA$_B$R2 and GABA$_B$R1h in adult brain as shown by real-time PCR. The RNA ratio (receptor/rRNA) in the whole brain (wb), neocortex (ctx), striatum (st), and olfactory bulb (ob) for GABA$_B$R1a, GABA$_B$R1b, GABA$_B$R1h, GABA$_B$R2, and GABA$_B$L was measured relative to the E17 total RNA. The bars represent the mean ± SEM of three determinations. Statistical analysis was performed by using ANOVA and the Fisher's post hoc test. GABA$_B$R2: *P = 0.001, neocortex compared with striatum and olfactory bulb; GABA$_B$R1h: *P = 0.05, neocortex compared with olfactory bulb.

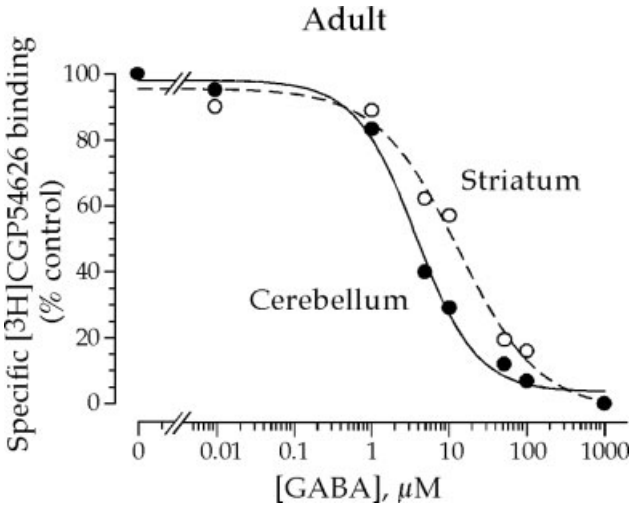


Fig. 11. GABA exhibits somewhat higher affinity for GABA$_B$R in adult cerebellum compared with striatal membranes. Competition for [^{3}H]CGP54626 binding by GABA in the cerebellum (solid circles) and striatum (open circles) is shown for a typical experiment performed in triplicate. Five independent determinations were used to calculate the IC$_{50}$ values: IC$_{50}$ (cerebellum) is 10.22 μM (logIC$_{50}$ = –5.06 ± 0.10) and IC$_{50}$ (striatum) is 20.70 μM (logIC$_{50}$ = −4.69 ± 0.08).

ing than cerebellum (which contains both GABA$_B$R1 and GABA$_B$R2 transcripts at similar levels). This result is consistent with the lower affinity of binding measured when GABA$_B$R1 is expressed alone in comparison with the affinity measured when GABA$_B$R1 is expressed together with GABA$_B$R2 (Kaupmann et al., 1998a; White et al., 1998).

We previously demonstrated the presence of human GABA$_B$R1c, a subunit isoform lacking one of two sushi domains (Martin et al., 2001). The equivalent rat GABA$_B$R1 isoform has not been described. To determine whether GABA$_B$R1c is present in rat brain, we designed primers to amplify a GABA$_B$R1 sequence lacking the second sushi domain. GABA$_B$R1c was found in rat embryonic and adult brain mRNA. The term *GABA$_B$R1c* has already been used for rat to describe an isoform that contains an insertion at the start of the fifth transmembrane domain (Isomoto et al., 1998). We propose that the novel rat splice form without the second sushi domain be termed *GABA$_B$R1h*; this is the next unused letter for GABA$_B$R1 subunit isoforms in rat. Our results indicate that GABA$_B$R1h is three times more highly expressed in the embryo than in the adult and that expression in olfactory bulb is significantly greater than in whole brain, cortex, or striatum. The sushi domain of neurocan has been implicated in cell adhesion and axonal outgrowth and pathfinding (Oleszewski et al., 2000). The importance of deleting one sushi domain in GABA$_B$R1h may relate to its location and cell surface expression in neurons.

Extensive pharmacological investigation of GABA$_B$Rs in vivo reveals evidence for GABA$_B$R subtypes (Calver et al., 2002), suggesting the existence of a corresponding heterogeneity in GABA$_B$R subunits and isoforms. However, a single heterodimeric receptor composed of GABA$_B$R1 and GABA$_B$R2 subunits has not been able to reproduce the varied pharmacology observed in the brain (Calver et al., 2002). GABA$_B$R1 requires a partner to reach the cell surface and signal to effectors (White et al., 1998). Additional complexity may be provided by a third GABA$_B$R subunit, which could function as an alternative partner for GABA$_B$R1. In *Drosophila*, an additional GABA$_B$R, termed *GABA$_B$R3*, has been discovered, but the function of this receptor remains unknown (Mezler et al., 2001). Recently, a novel GABA$_B$R subunit has been described for mammals, termed *GABA$_B$L* (Calver et al., 2003). The reported GABA$_B$L sequence is unusual for a G-protein-coupled receptor in that it does not contain a signal sequence. In addition, unlike the related GABA$_B$Rs and metabotropic glutamate receptors, it does not contain a large extracellular N-terminal ligand binding region. The C-terminal intracellular sequence of GABA$_B$L contains a putative coiled-coil domain, which has been shown to be critical in GABA$_B$R subunit function. As with GABA$_B$R1 but not GABA$_B$R2, the expression of GABA$_B$L transcripts was not significantly different in the cortex, olfactory bulb, and striatum. Recently, moderate GABA$_B$L protein expression was detected by immunohistochemistry in the caudate-putamen (Charles et al., 2003). Based on expression patterns, GABA$_B$L may be a possible partner for GABA$_B$R1. Although no functional interaction has been demonstrated between GABA$_B$R1 and GABA$_B$L to validate this possible subtype, the lack of function may be a consequence of missing N-terminal GABA$_B$L sequences or the assays used.

The differential distribution of GABA$_B$R subunit mRNAs in the embryo, striatum, olfactory bulb, and neocor-

tex leads to the conclusion that $GABA_BR1$ and $GABA_BR2$ transcripts are not necessarily expressed coordinately. This is consistent with the observation that $GABA_BR1$ and $GABA_BR2$ are located on different chromosomes and indicates that their promoter regions are likely to be under the control of distinct transcription factor(s). The current model of $GABA_BR$ function is that $GABA_BR1$ is not able to function without a partner, such as $GABA_BR2$, that transports it to the cell surface, where coupling to G proteins can occur. Thus, the results described here suggest that the early embryonic CNS (day <E14) lacks functional $GABA_BRs$. Alternatively, another partner, such as $GABA_BL$, could function with $GABA_BR1$ in the early embryo. $GABA_BL$ mRNA is present in regions where $GABA_BR2$ is low, and it remains to be determined whether $GABA_BL$ could be a possible partner for $GABA_BR1$ as well.

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