sity of apoptotic cells identified by TUNEL. Both staining procedures produced the same pattern of degeneration in the brains of vehicle-treated rats and the same pattern of increased degeneration in the brains of (+)MK801-treated rats. To visualize degenerating cells by DeOlmos cupric silver staining, we perfused the brains with fixative containing paraformaldehyde (4%) in phosphate buffer, and serial transverse sections (70 μm thick) were cut by vibratome from the entire forebrain and stained with silver nitrate and cupric nitrate. Degenerating cells incorporate silver and appear dark against a light background.

8. Neuronal degeneration was quantified in 16 brain regions (Table 1) using the optical disector and fractionator method as described [L. L. Cruz-Orive and E. R. Weibel, *Am. J. Physiol.* **258**, L148 (1990)]. A counting frame (0.05 mm by 0.05 mm, dissector height 0.07 mm), and a high-aperture objective were used for visualizing and counting neurons. Unbiased sampling of each brain region was performed by randomly selecting 8 to 10 viewing fields over which the counting frame was positioned for counting at different focal levels by the optical dissector method. The numerical density of normal neurons in any given region was determined by counting neuronal profiles in 70-μm-thick sections stained with a Nissl stain (methylene blue, azure II). The numerical density of degenerating neurons in any given region was determined by counting argyrophilic profiles in 70-μm-thick sections stained by the DeOlmos silver method (*7*). Counting was performed in a blinded manner.
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12. Double labeling for GFAP and TUNEL was performed by first staining sections by TUNEL (*6*), washing them thoroughly at room temperature in a solution of 0.01 M phosphate-buffered saline (PBS)–Triton X-100 (0.5%), and incubating them for 1 hour in the same solution with 1% bovine serum albumin. The sections were then incubated overnight at 4°C in the presence of monoclonal antibody against GFAP (1:400 dilution; G3893, Sigma). After PBS rinses, sections were incubated with goat antibody to mouse immunoglobulin G (IgG) (1:200; BODIPY, Molecular Probes) in PBS–Triton X-100 for 1 hour at room temperature. Microscopic fields were photographed with a Kodak digital camera under fluorescence illumination to detect localization of the BODIPY fluorescence probe and under ordinary illumination to detect the TUNEL diaminobenzidine reaction product. The two images from a given field were then superimposed with PhotoShop software to determine whether the GFAP and TUNEL labels were colocalized in the same cells.
13. MK801 remains detectable in the rodent brain or cerebrospinal fluid for 3 to 4 hours after systemic administration. Elimination half-life of MK801 in rats was estimated to be 1.9 hours. The drug reaches maximal concentrations in the brain within 10 to 30 min after administration [A. Vezzani *et al.*, *J. Pharmacol. Exp. Ther.* **249**, 278 (1989)]. Phencyclidine (PCP), which freely penetrates blood-brain barriers, has a plasma elimination half-life of 3.9 hours and an even longer half-life in the rat brain [J. L. Valentine, L. W. Arnold, S. M. Owens, *J. Pharmacol. Exp. Ther.* **269**, 1079 (1994)]. A single dose of PCP (10 mg/kg ip) was used because it produces behavioral symptoms indicative of NMDA receptor blockade lasting for 8 to 10 hours in 7-day-old rats. Ketamine freely penetrates the brain but is excreted rapidly and has a short half-life in the brain [P. F. White, M. P. Marietta, C. R. Pudwill, W. L. Way, A. J. Trevor, *J. Pharmacol. Exp. Ther.* **196**, 545 (1976)]. It was administered in a series of seven injections spaced evenly over 9 hours, each injection delivering a dose of 20 mg/kg sc. CPP penetrates blood-brain barriers poorly but has a long half-life in the brain [J. D. Kristensen, P. Hartvig, R. Karlsten, T. Gordh, M. Halldin, *Br. J. Anaesth.* **74**, 193 (1995)]. To maintain a steady blockade of NMDA receptors for 8 hours, we administered CPP at a dose of 15 mg/kg ip at 0, 50, and 100 min and at 4 and 8 hours. Rat pups were killed at 24 hours after the first treatment.
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26 August 1998; accepted 17 November 1998

Role of Heteromer Formation in $GABA_B$ Receptor Function

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Recently, GBR1, a seven-transmembrane domain protein with high affinity for γ-aminobutyric acid $(GABA)_B$ receptor antagonists, was identified. Here, a GBR1-related protein, GBR2, was shown to be coexpressed with GBR1 in many brain regions and to interact with it through a short domain in the carboxyl-terminal cytoplasmic tail. Heterologously expressed GBR2 mediated inhibition of adenylyl cyclase; however, inwardly rectifying potassium channels were activated by $GABA_B$ receptor agonists only upon coexpression with GBR1 and GBR2. Thus, the interaction of these receptors appears to be crucial for important physiological effects of GABA and provides a mechanism in receptor signaling pathways that involve a heterotrimeric GTP-binding protein.

$GABA_B$ receptors play a critical role in the fine-tuning of central nervous system synaptic transmission (*1*) and are attractive targets for the treatment of epilepsy, anxiety, depression, cognitive deficits, and nociceptive disorders (*2*). Their effects are brought about by multiple signaling cascades involving adenylyl cyclase, inwardly rectifying potassium channels (GIRKs), and voltage-dependent Ca^{2+} channels (*1*). Recently, the cDNA for a seven-transmembrane domain (7TM) protein, termed $GABA_B$ receptor 1 (GBR1), which exists in two NH_2-terminal splice forms (A and B) and has high affinity for $GABA_B$ receptor antagonists, was identified. GBR1 can account for some, but not all, of the functional properties of native $GABA_B$ receptors (*3*).

We used the yeast two-hybrid system (Y2H) (*4*) to look for intracellular proteins that mediate signaling events downstream of GBR1 activation. The COOH-terminal intracellular region of GBR1 (amino acids 857 to 960 in GBR1A) (*3*) was used as a bait (*5*) to screen a rat brain cDNA library (*6*). Our search through 2×10^6 recombinant clones yielded five positive clones, all of which encoded overlapping fragments of the COOH-terminus of a previously unidentified protein. Full-length cloning (*7*) of the identified cDNA revealed an open reading frame for a protein of 940 amino acids with an NH_2-terminal 40-residue signal sequence and seven internal hydrophobic segments characteristic for 7TM proteins (Fig. 1A) (*8*). A public database search for related sequences (*8*) revealed GBR1B with the best score (36%

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amino acid sequence identity across 804 amino acids). On the basis of this similarity, we termed the protein GBR2.

We mapped the domains mediating the interaction between GBR1 and GBR2. As identified by a series of deletion constructs and subsequent analysis in the Y2H system (*4, 5*), the interaction was mediated by two short domains centrally located in the intracellular COOH-termini of GBR1 and GBR2 (Fig. 1B). These domains span 35 amino acids in GBR1 and 32 amino acids in GBR2 and are predicted to be α-helical (*8*). Interaction was found between GBR1 and GBR2 but not between GBR1 molecules or between GBR2 molecules (*9*), suggesting a requirement for a heteromeric assembly of the two 7TM proteins.

Glutathione S-transferase (GST) pull-down assays from HEK293 cell extracts (*10*) confirmed the interaction between the COOH-termini of GBR1 and GBR2. Only the GST fusion proteins containing the GBR1–COOH-terminus or the GBR1 heteromerization domain (see GBR1Δ7 in Fig. 1B), but not GST alone, were able to bind to the GBR2–COOH-terminus. No dimerization between GBR1–COOH-termini was detected (Fig. 1C). Thus, the COOH-termini of GBR1 and GBR2 can interact in both yeast cell nuclei and the cytoplasm of mammalian cells.

The GBR2 mRNA was expressed only in the brain (Fig. 2A) (*11*), as has been described for GBR1 (*3*). To assess if GBR1 and GBR2 have the potential to interact in the brain, we compared the expression patterns of the mRNAs for the two proteins. As revealed by in situ hybridization (*12*) in serial rat brain sections, GBR1 and GBR2 are widely expressed and show considerable overlap (for example, in the cerebellum, cortex, and medial habenula, Fig. 2, B to E). Thus, GBR1 and GBR2 have the potential to interact in many neuronal populations. Indeed, the pattern as well as the strength of expression of the mRNAs is largely consistent with the distribution and density of $GABA_B$ receptor binding sites in the brain (*13*). Despite the large overlap in the expression of GBR1 and GBR2, we found spatial and temporal differences. GBR1 mRNA expression was more widespread than that of GBR2 (for example, in the striatum, olfactory bulb, and lateral habenula, Fig. 2, B, D, and E) and had an

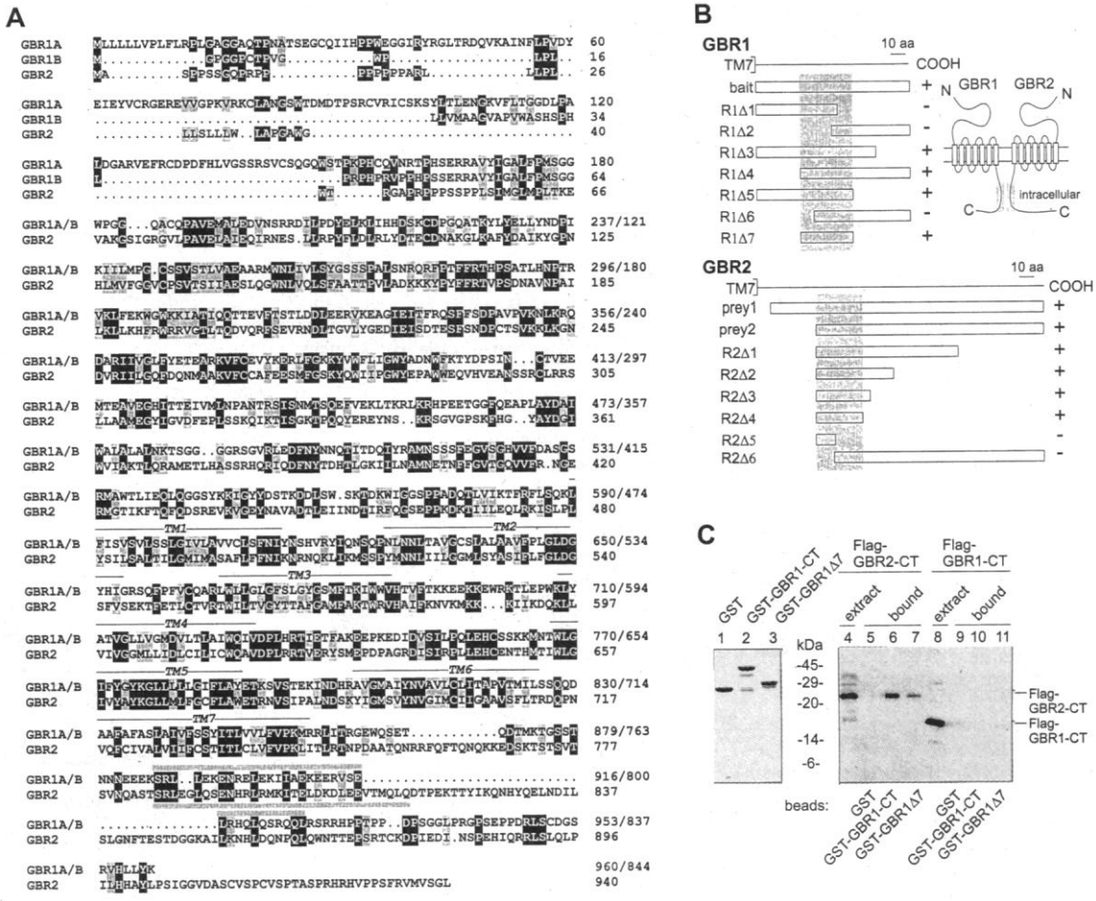

Fig. 1. Sequence comparison and interaction analysis of GBR proteins. (**A**) Amino acid sequence (*21*) alignment of GBR1A, GBR1B, and GBR2. The overall sequence similarities are 25.5% between GBR1A and GBR2, and 29.9% between GBR1B and GBR2. Identical residues are shaded in black, and conserved changes are shaded in gray. The transmembrane domains (TM1 to TM7) are indicated by thin lines. The amino acid sequences responsible for the interaction between GBR1 and GBR2 are indicated by gray bars above (GBR1) and below (GBR2) the sequences. The cDNA sequence of GBR2 was deposited in the GenBank database (accession number AF109405). A second cDNA clone for GBR2 encoded an additional proline residue at position 19. (**B**) GBR1 and GBR2 interact through amino acids in their COOH-terminal tails. The bait, two of the preys and further deletion constructs of GBR1 and GBR2 are depicted in alignment with their COOH-terminal regions. The ability of the individual GBR1 constructs to bind to the GBR2 prey 2 (upper panel) and the ability of the individual GBR2 constructs to interact with the GBR1 bait (lower panel) is indicated on the right. The regions common to all constructs scoring positive in the Y2H interaction assay are shaded in gray. They (GBR1Δ7 and GBR2Δ4) were also able to interact with each other. Their location is depicted in a membrane topology scheme of the two GBRs. The constructs were as follows. Bait, amino acids (aa) 857–960; R1Δ1, aa857–911; R1Δ2, aa908–960; R1Δ3, aa857–937; R1Δ4, aa887–960; R1Δ5, aa857–921; R1Δ6, aa898–960; and R1Δ7, aa887–921 of the immature polypeptide GBR1A. Prey1, aa754–940; prey2, aa785–940; R2Δ1, aa785–881; R2Δ2, aa785–837; R2Δ3, aa785–821; R2Δ4, aa785–816; R2Δ5, aa785–797; and R2Δ6, aa798–940 of the immature polypeptide GBR2. (**C**) The COOH-terminus of GBR2, but not of GBR1, can be pulled down by GST–GBR1-COOH-terminus fusion proteins (10). The left panel is a Coomassie blue–stained gel of the bacterially expressed GST fusion proteins GST (lane1), GST–GBR1-CT (lane 2, GBR1-CT is equivalent to GBR1 bait) and GST-GBR1Δ7 (lane 3) used for pull-down experiments. The right panel shows immunoblots of Flag-tagged COOH-termini of GBR2 (Flag–GBR2-CT, lanes 4 to 7) and GBR1 (Flag–GBR1-CT, lanes 8 to 11) recovered from cytoplasmic extracts of HEK293 cells (lanes 4 and 8) by interaction with GST (lanes 5 and 9), GST-GBR1-CT (lanes 6 and 10), and GST-GBR1Δ7 (lanes 7 and 11). The amount of extracts used for lanes 4 and 8 was 10% of the amount of extracts used for lanes 5 to 7 and lanes 9 to 11.

earlier onset (Fig. 2F). Thus, $GABA_B$ receptor isoforms lacking GBR2 may be present in the brain, and the delayed developmental expression of GBR2 may contribute to a maturation of $GABA_B$ receptors.

In keeping with the properties of native $GABA_B$ receptors (*14*), activation of heterologously expressed GBR2 elicited a decrease in forskolin-stimulated adenosine 3′,5′-monophosphate (cAMP) production (Fig. 3B) (*15*). This decrease, in our hands, was significant in contrast to the effect of GBR1 activation on forskolin-stimulated cAMP production (Fig. 3A). Coexpression of both proteins decreased forskolin-stimulated cAMP production to the same extent as GBR2 alone (Fig. 3C). The GBR2-mediated decrease in cAMP production was sensitive to pertussis toxin, suggesting the involvement of the G_i/G_o class of heterotrimeric GTP-binding proteins (G proteins). Thus, heteromeric assembly of GBR1 and GBR2 does not seem to be required for inhibition of adenylyl cyclase.

A crucial physiological effect mediated by native $GABA_B$ receptors is the activation of outward potassium currents through GIRKs (*16*). Reconstitution of GBR1 or of GBR2 with GIRK1 and GIRK2 in HEK293 cells failed to mediate GIRK activation (Fig. 3, D and E) (*17*). When coexpressed, GBR1 and GBR2 mediated a robust increase in potassium conductance through GIRK activation (Fig. 3F) in a pertussis toxin–sensitive manner. Thus, the physical interaction between GBR1 and GBR2 appears to be essential for the coupling of $GABA_B$ receptors to GIRKs. Given the importance of GIRK activation in the generation of late inhibitory postsynaptic potentials at inhibitory synapses (*16*), the interaction between GBR1 and GBR2 is likely to play a pivotal role in modulation of neurotransmission.

Thus, we have identified a COOH-terminal interaction between two $GABA_B$ receptor proteins. The resultant heteromeric assembly adds an element of complexity to G protein–mediated signaling mechanisms. Monomers of GBR1, of GBR2, and of putative additional members of this receptor family and heteromers thereof may provide a molecular basis for the different pharmacological and functional subtypes of $GABA_B$ receptors (*1*, *18*), thereby opening therapeutic avenues.

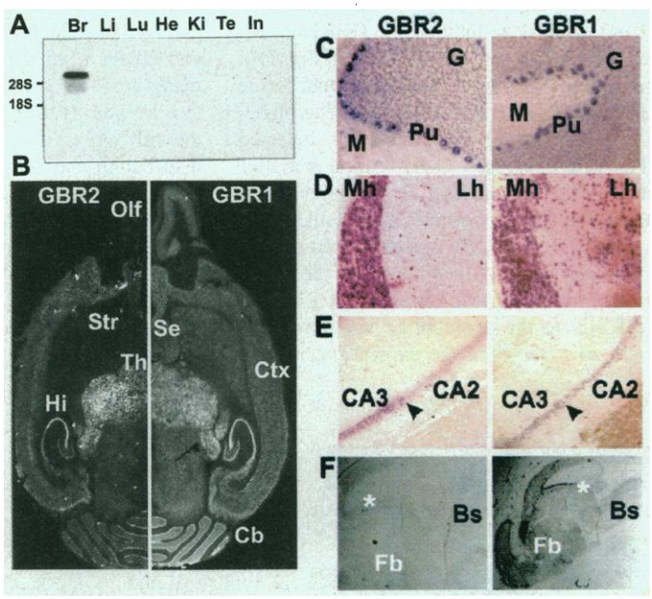

Fig. 2. Expression profile of rat GBR1 and GBR2. (**A**) A multiple tissue Northern (RNA) blot hybridized with a radiolabeled GBR2 cDNA probe revealed a strong signal for a 5.5-kb transcript in brain and a very weak signal (difficult to see on reproduction) for a shorter transcript in testis. (**B** to **F**) In situ hybridization of GBR1 and GBR2 mRNA in adult rat brain sections revealed high signal densities of both transcripts in various thalamic nuclei (B), hippocampus (B and E), cerebellar purkinje cells (C), and medial habenula (D). Both mRNA transcripts are moderately expressed in the cerebral cortex (B) and certain anterioventral thalamic nuclei (*9*) and are undetectable in white matter (*9*). Expression of GBR1 is enriched in comparison to GBR2 in the striatum (B), the olfactory bulb (B), the septal nuclei (B), the lateral habenula (D), the CA2-CA1 hippocampal subfields [(B and E); arrowheads in (E) indicate the approximate boundary between CA3 and CA2 subfields], and in the neuroepithelial cells of the ventricular zone (indicated by asterisks) of embryonic (day 17) brain (F). On the day of birth, GBR1 and GBR2 are coexpressed in the hippocampus and the thalamic nuclei and diffusely expressed in the cortex, but only GBR1 is detected in the developing cerebellum (*9*). Sense probes yielded no signal (*9*). Abbreviations: Br-brain, Bs-brainstem, Cb-cerebellum, Ctx-cortex, Fb-forebrain, G-cerebellar granule cell layer, He-heart, Hi-hippocampus, In-intestine, Li-liver, Lu-lung, M-molecular layer, Mh-medial habenula, Ki-kidney, Lh-lateral habenula, Olf-olfactory bulb, Pu-cerebellar purkinje cells, Se-septal nuclei, Str-striatum, Te-testis, and Th-thalamus.

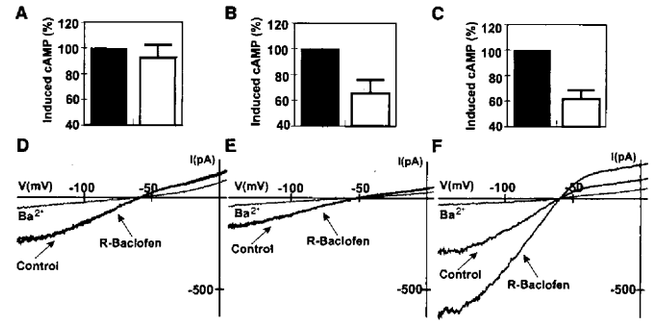

Fig. 3. Coupling of GBR1 and GBR2 expressed in HEK293 cells to effector systems. Forskolin (2 μM) treatment for 20-min stimulated intracellular cAMP concentrations by about a factor of 10 (normalized to 100%, filled bars). Treatment of parallel samples with (*R*)-baclofen (500 μM, unfilled bars) or GABA (1 mM) (*9*) attenuated forskolin-stimulated cAMP production in cells transfected with GBR2 alone (**B**) ($P = 0.001$) or with GBR1 and GBR2 (**C**) ($P = 0.019$), but not in GBR1-transfected cells (**A**) or in untransfected cells (*9*). Pretreatment of transfected cells with pertussis toxin (10 ng/ml) for 10 hours abolished the GBR2-mediated decrease in forskolin-stimulated cAMP production (114 ± 22%; $P = 0.001$) (*9*). Data are presented as mean ± SEM from at least three experiments performed in quadruplicates and were analyzed by analysis of covariance with post hoc Dunnett's test. (*R*)-baclofen (50 μM) or GABA (100 μM) (*9*) did not increase barium (Ba^{2+}, 1 mM)-sensitive GIRK currents (224 ± 46 pA, $n = 23$, measured at −140 mV) when either (**D**) GBR1 (a factor of 1.01 ± 0.01 over control, $n = 4$) or (**E**) GBR2 (a factor of 0.95 ± 0.05 over control, $n = 3$) was coexpressed with GIRK1 and GIRK2 in HEK293 cells. Thus, the current traces overlapped in the absence (control) and presence of (*R*)-baclofen. (**F**) Upon coexpression of GBR1 and GBR2, (*R*)-baclofen (50 μM) or GABA (100 μM) (*9*) reversibly increased GIRK currents by a factor of 2.2 ± 0.3 ($n = 13$) or 3.3 ± 0.8 ($n = 4$) over control values, respectively. Pretreatment of transfected cells with pertussis toxin (500 ng/ml) for 24 hours abolished the effect of GBR1 and GBR2 activation on GIRK currents ($n = 3$) (*9*). Current traces represent average currents of five voltage ramps (−150 to +5 mV over 300 ms).

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11. Total RNA was isolated from the respective rat organs, separated, and blotted according to standard procedures (*7*). A 702–base pair (bp) cDNA probe of GBR2 was labeled with [α-^{32}P]deoxycytidine triphosphate with a random primed labeling kit (Boehringer Mannheim). The blot was hybridized with QuikHyb solution (Stratagene), washed [0.1× sodium chloride/sodium citrate (SSC), 60°C], and exposed to x-ray film.
12. Digoxigenin (Dig)–uridine triphosphate–labeled complementary RNA (cRNA) probes were synthesized from linearized template DNA (Boehringer Mannheim). The open reading frame of GBR1A (*20*) was used for generating sense (1948 bp) and antisense (1751 bp) cRNA probes codetecting transcripts of GBR1A and GBR1B. Sense (1815 bp) and antisense (1763 bp) cRNA probes detecting the GBR2 message were synthesized from the 3′ untranslated region of GBR2. In situ hybridization was performed on frozen brain sections (15 μm) from Wistar rats as described [M. J. Rossner, J. Dorr, P. Gass, M. H. Schwab, K. A. Nave, *Mol. Cell. Neurosci.* **10**, 460 (1997)] with 50 to 100 ng of cRNA probes overnight at 65°C (50% formamide, 5× SSC). Nonspecific binding of probes was eliminated by sequential washes in 2× SSC and in 0.2× SSC at room temperature and at 65°C, and the single-stranded probe was removed by treatment with ribonuclease A (2 μg/ml, 37°C, 30 min). Bound Dig-labeled cRNA probes were detected with alkaline phosphatase–conjugated antibody to Dig. (1:1500 to 1:2000, 1 hour) and standard reagents (Boehringer Mannheim).
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15. Transiently transfected HEK293 cells or untransfected controls were incubated in serum-free medium containing 1 mM 3-isobutyl-1-methyl-xanthine for 30 min at 37°C. Cells were harvested, washed, and resuspended in Krebs-tris buffer (*3*). We treated 500,000 cells per sample in quadruplicates with either GABA (1 mM) or (*R*)-baclofen (500 μM) or respective vehicles for 5 min followed by incubation with forskolin (2 μM) or dimethyl sulfoxide (vehicle) for 20 min at 37°C. Cells were pelleted and boiled for 8 min in tris-EDTA buffer (Amersham Pharmacia Biotech), and cell extracts were assayed for cAMP concentrations with a standard cAMP assay kit (Amersham Pharmacia Biotech).
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17. Transfected cells (*19*) on cover slips were transferred to the stage of an inverted microscope and were continuously perfused with a solution containing 115 mM NaCl, 25 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, and 10 mM glucose (pH 7.25, adjusted with NaOH). The whole-cell configuration of the patch-clamp technique was applied at room temperature with pipette solutions containing 115 mM K-gluconate, 20 mM KCl, 10 mM Hepes, 5 mM EGTA, 4 mM adenosine triphosphate (Mg^{2+} salt), and 0.3 mM guanosine triphosphate (Na^{2+} salt) (pH 7.25, adjusted with KOH). GABA (100 μM) or (*R*)-baclofen (50 μM) was dissolved in the extracellular solution and was applied steadily to single, lifted cells by a double-barreled application pipette. The inward rectifier currents were activated with voltage ramps. Membrane potentials were corrected for the liquid junction potential (−11 mV). Data collection and analysis were performed as described [G. Köhr and P. H. Seeburg, *J. Physiol.* **492.2**, 445 (1996)].
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19. HEK293 cells (American Type Culture Collection) were cultured in minimal essential medium with Earles's salts supplemented with L-glutamine, 10% fetal calf serum, and 1% Pen/Strep (Gibco-BRL), transiently transfected with standard calcium phosphate–transfection procedures (*7*) with 10 μg of combined cytomegalovirus (CMV) expression plasmids (*20*) per 2 million cells, and used for analysis at 48 hours after transfection.
20. The coding regions for GBR1A (*3*), GIRK1 [Y. Kubo, E. Reuveny, P. A. Slesinger, Y. N. Jan, L. Y. Jan, *Nature* **364**, 802 (1993)], and GIRK2 [M. Stoffel *et al.*, *Biochem. Biophys. Res. Commun.* **212**, 894 (1995)] were amplified from rat brain cDNA with gene-specific primers. The amplicons and the cDNA for GBR2 were subcloned into pBluescript (Stratagene) and also into a CMV expression vector [T. J. Schall *et al.*, *Cell* **61**, 361 (1990)] for transfection in HEK293 cells (*19*).
21. The amino acid sequences were aligned by the clustal method (Lasergene, DNAStar, Madison, WI). Amino acid abbreviations are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
22. We thank K. Hirschfeld and A. Hesselschwerdt for expert technical assistance, our colleagues at BASF-LYNX Bioscience AG for discussions and help, and P. H. Seeburg and colleagues for suggestions, discussions, and support. Supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (grant 0311633).

18 November 1998; accepted 7 December 1998

Rule Learning by Seven-Month-Old Infants

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A fundamental task of language acquisition is to extract abstract algebraic rules. Three experiments show that 7-month-old infants attend longer to sentences with unfamiliar structures than to sentences with familiar structures. The design of the artificial language task used in these experiments ensured that this discrimination could not be performed by counting, by a system that is sensitive only to transitional probabilities, or by a popular class of simple neural network models. Instead, these results suggest that infants can represent, extract, and generalize abstract algebraic rules.

What learning mechanisms are available to infants on the cusp of language learning? One learning mechanism that young infants can exploit is statistical in nature. For example, Saffran *et al.* (*1*) found that the looking behaviors of 8-month-old infants indicated a sensitivity to statistical information inherent in sequences of speech sounds produced in an artificial language—for example, transitional probabilities, which are estimates of how likely one item is to follow another. In the corpus of sentences "The boy loves apples. The boy loves oranges." the transitional probability between the words "the" and "boy" is 1.0 but the transitional probability between the words "loves" and "apples" is 1/2 = 0.5.

It has been suggested that mechanisms that track statistical information, or connectionist models that rely on similar sorts of information [for example, the simple recurrent network (SRN) (*2*)], may suffice for language learning (*3*). The alternative possibility considered here is that children might possess at least two learning mechanisms, one for learning statistical information and another for learning "algebraic" rules (*4*)—open-ended abstract relationships for which we can substitute arbitrary items. For instance, we can substitute any value of x into the equation $y = x + 2$. Similarly, if we know that in English a sentence can be formed by concatenating any plural noun phrase with any verb phrase with plural agreement, then as soon as we discover that "the three blickets" is a well-formed plural noun phrase and that "reminded Sam of Tibetan art" is a well-

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