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Developmental Cues Modulate $GABA_A$ Receptor Subunit mRNA Expression in Cultured Cerebellar Granule Neurons

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The developmental expression of mRNAs encoding the $GABA_A$ receptor was analyzed in the rat cerebellar cortex and in cultured cerebellar granule neurons. Our studies *in vivo* reveal that the α1-, β2-, and γ2-subunit mRNA levels in the cerebellar cortex rise dramatically during the second postnatal week, a period temporally correlated with extensive cerebellar maturation. To determine if these increases were preprogrammed or dependent on extrinsic factors, we examined subunit mRNA expression in granule cell cultures prepared at embryonic day 19 (E19) and postnatal day 10 (P10), immature and mature stages of cerebellar development, respectively. In E19 cultures, the α1, β2, and γ2 $GABA_A$ receptor subunit mRNAs were present and their levels remained constant over the 21 d culture period. These results suggest that $GABA_A$ receptor gene expression is not intrinsic to the immature granule cells. A different pattern was found in P10 cultures where the three subunit mRNAs were initially present at levels approximately sixfold higher than those found at E19. The β2- and γ2-subunit mRNAs remained constant for 4 d and then increased sixfold between 4 and 7 d in culture. The magnitude and time course of these increases were similar to the developmental changes that occurred *in vivo*. Thus, our findings raise the possibility that signals encountered during development are necessary to induce $GABA_A$ receptor subunit mRNA expression. Moreover, these cues have been received by granule neurons prior to P10. In contrast to the expression of the β2- and γ2-subunit mRNAs, however, the level of the α1-subunit mRNA in P10 cultures did not change significantly. This result suggests that the $GABA_A$ receptor subunit mRNAs are differentially regulated.

[Key words: $GABA_A$ receptor, rat cerebellum, development, receptor regulation, cerebellar cultures, PCR]

Cell–cell communication in the nervous system is dependent on the correct developmental formation and regulation of neurotransmitters and their receptors. Cerebellar granule neurons, which follow an intricate and well-characterized pathway of postnatal development, offer an excellent system in which to examine the appearance of neurotransmitter phenotype. After cell birth and division in the external germinal layer, a majority of the cells that later differentiate into granule neurons migrate through the molecular layer and into the internal granule layer, a process that peaks between postnatal days 10 and 11 (P10, P11; Altman 1972a,b). Once in their adult positions in the internal granule layer, the neurons elaborate processes and form synapses with other cerebellar neurons. Thus, granule neurons migrate through different environments during maturation and presumably encounter a variety of regulatory signals.

In the mature cerebellum, granule neurons are responsive to GABA, the major inhibitory neurotransmitter in the CNS. The $GABA_A$ receptor, which also mediates the actions of the anxiolytic benzodiazepines and anticonvulsant barbiturates, comprises several subunits, most of which are encoded by families of genes. To date, numerous subunits have been cloned (Khrestchatisky et al., 1989; Lolait et al., 1989a; Pritchett et al., 1989; Shivers et al., 1989; Wisden et al., 1989; Ymer et al., 1989, 1990; Lüddens et al., 1990; Malherbe et al., 1990a; Whiting et al., 1990; Cutting et al., 1991; Kofuji et al., 1991) and designated as α, β, γ, δ, or ρ by cDNA sequence analysis. While greater than 80% sequence homology exists among subunits of a single class, the different subunit types share less than 40% identity (see Olsen and Tobin, 1990). The subunit mRNAs are expressed in developmental- and region-specific patterns in the CNS (Garrett et al., 1990; Gambarana et al., 1990, 1991; Meinecke and Rakic, 1990; Laurie et al., 1992), suggesting the presence of multiple receptors of varying composition. Moreover, many recent studies have shown that specific subunits confer unique functional properties to the receptor complex (Pritchett et al., 1989; Khrestchatisky et al., 1989; Lolait et al., 1989a,b; Puia et al., 1989; Shivers et al., 1989; Sigel et al, 1989, 1990; Ymer et al., 1989; Lüddens et al., 1990; Malherbe et al., 1990a,b; Pritchett and Seeburg, 1990; Verdoorn et al., 1990).

Although the heterogeneity of ligand-gated ion channel composition in the CNS is now well established, little is known concerning the regulation of receptor gene expression. Some clues have been provided, however, by examining $GABA_A$ receptor subunit mRNAs during postnatal ontogeny (Gambarana et al., 1990, 1991; Garrett et al., 1990; Meinecke and Rakic, 1990; Laurie et al., 1992). Such studies have demonstrated that receptor gene expression in the cerebellar cortex undergoes particularly striking changes. Whereas the mRNAs encoding the α1-, β2-, and γ2-subunits are relatively low in the first postnatal week, they rapidly increase 2.5–4.0-fold between P10 and P17 in the granule and Purkinje cell layers (Gambarana et al., 1990, 1991). This period of development temporally coincides with cerebellar maturation and synaptogenesis, raising the possibility

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that events occurring during this time modulate receptor gene expression.

Because the complexity of the intact cerebellum hinders analysis of the developmental expression of $GABA_A$ receptor genes, we have established culture systems to examine this process. Using purified cultures of granule neurons prepared at distinct developmental stages, our studies demonstrate that $GABA_A$ receptor gene expression is dependent on cerebellar maturity. In cultures prepared at embryonic day 19 (E19), a time when virtually all of the granule cell precursors are still dividing in the germinal layer and have not yet migrated, the levels of the α1-, β2-, and γ2-subunit mRNAs are constant over the entire culture period. In contrast, in cultures prepared at P10, a stage at which most cells have completed the migration process, two of the subunit mRNAs rose severalfold over a period of a few days. The magnitude and pattern of these increases in subunit mRNA levels are similar to those found *in vivo*. These findings suggest that granule neurons are not preprogrammed at E19 but receive cues, either en route to or in the internal granule cell layer, that modulate $GABA_A$ receptor gene expression.

Some of these results have been reported previously in abstract form (Beattie and Siegel, 1991).

Materials and Methods

Cell culture. Granule cell cultures were prepared from E19 and P10 rat cerebella using modifications of previously described procedures (Messer, 1977; Hockberger et al., 1989). For both ages, the cerebellar cortices were removed from many animals (two to four animals for P10 and two litters for E19), pooled, and digested in 0.05% trypsin for 12 min at 37°C. Following centrifugation for 5 min at 1000 × *g* in a Beckman clinical centrifuge, the tissue was triturated in the presence of 4000 U of DNase (type IV, Sigma). Larger pieces of tissue were discarded, and the supernatant was diluted in phosphate-buffered saline (PBS) and centrifuged at 1000 × *g* for 5 min.

To enrich for granule neurons, dissociated E19 cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) and applied to a 1.01/1.09 gm/ml Percoll step gradient (Hatten, 1985). The gradient was then spun for 30 min at 2300 × *g* in a Beckman clinical centrifuge. Two fractions were collected, one from the top of the gradient (fraction 1) and the other at the interface of the two Percoll layers (fraction 2). After removal from the gradient, cells were washed once with 30 ml of PBS followed by 30 ml of DMEM. The cells were resuspended in DMEM containing 10% fetal bovine serum, 6 gm/liter glucose, 100 U penicillin, and 100 μg/ml streptomycin. In addition, the growth medium contained 25 mM potassium chloride, a concentration that has been shown previously to enhance the survival of granule neurons (Lasher and Zagon, 1972; Gallo et al., 1987). While this procedure was necessary to prepare cultures enriched in granule cells at E19, cells dissociated at P10 were not subjected to a Percoll gradient. At this stage the granule cells far outnumber other cerebellar neurons, yielding cultures that are 95% enriched for this cell population.

Cells were plated in 96-well plates coated with 0.1 mg/ml poly-L-lysine and 5 μg/ml laminin at a density of 1–3 × 10^3 cells/mm^2 for the polymerase chain reaction (PCR). Alternatively, cells were plated on 12 mm glass coverslips at the same density for immunohistochemistry. After 2 d in culture, cells were treated with 60 μM 5′-fluoro-2′-deoxyuridine for 2 d to reduce the number of dividing non-neuronal cells and then returned to growth medium.

Cerebellar astrocyte cultures were generously prepared by Dr. Robert Miller (Case Western Reserve University). Briefly, cerebella from P0 rats were dissociated and plated on poly-L-lysine–coated plates for 4–6 hr as previously described (Smith et al., 1990). Nonadherent cells were removed by vigorous shaking, and the remaining astrocytes were grown in DMEM containing 10% FBS. After 7 d in culture, these cells were harvested for RNA preparation.

RNA isolation. RNA was isolated from rat tissue, cultured granule cells (E19 and P10), and cultured cerebellar astrocytes following the protocol of Chomczynski and Sacchi (1987). Briefly, tissue samples were homogenized, or cultured cells were harvested, in 5.5 M guanidinium thiocyanate, pH 7.0, containing 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The DNA was then sheared using an 18 gauge needle followed by a 21 gauge needle. RNA was isolated with 2 M sodium acetate, pH 4.0, phenol (water saturated), and 49:1 chloroform:isoamylalcohol. For each experiment, RNA was prepared from two to five cerebella for the *in vivo* studies and cells cultured from cerebella from two to four animals (P10) or two litters (E19).

Polymerase chain reaction. For analysis of tissue samples, 0.75 μg RNA was DNase treated and processed for the reverse transcriptase reaction by adding buffer (50 mM Tris-HCl, pH 8.8, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol), 200 μm of each nucleotide triphosphate (NTPs), 100 pmol of random hexamers, and 10 U of DNase. In addition, each reaction contained 125 pg of SP64 bacterial RNA transcribed from the SP64 plasmid (Promega) as an internal control. The mixtures were incubated at 37°C for 10 min followed by incubation at 95°C for 7 min to inactivate the DNase. After placing the mixture on ice, 12 U of RNasin (Promega), 100 U of SuperScript Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), and 0.1 μg/ml bovine serum albumin (BSA) were added and incubated for 1 hr at 37°C. PCR was initiated by adding buffer (15 mM Tris-HCl, pH 8.8, 60 mM KCl, 2.75 mM $MgCl_2$), 200 μM NTPs, 25 pmol of each primer, 2.5 μCi of α-^{32}P-dCTP, and 2.5 U of TAQ DNA polymerase (Perkin Elmer Cetus) to the reverse transcriptase mixture to a final volume of 50 μl. The reactions were layered with mineral oil and incubated in a Perkin Elmer Cetus thermocycler. Samples were subjected to 26 cycles consisting of 95°C for 1 min, 55°C for 30 sec, 72°C for 45 sec. For cultured cells, identical conditions were used except that 0.4 μg of RNA and 70 pg of SP64 RNA were amplified for 30 cycles.

To detect the PCR products, the samples were electrophoresed through an 8% nondenaturing polyacrylamide gel, dried, and exposed to Kodak X-AR film overnight. Radioactive incorporation was determined by excising and counting bands from the dried gel. Results for each reaction were represented as a ratio of $GABA_A$ receptor subunit PCR product to SP64 PCR product. Since the RNAs prepared from E19 and P10 granule cells in culture were processed identically for PCR, the ratios of subunit PCR product:SP64 PCR product can be compared. The *in vivo* experiments, however, were performed under slightly different conditions (0.75 μg RNA for 26 cycles vs 0.4 μg RNA for 30 cycles for cultured cells), and therefore the values obtained cannot be compared to the culture experiments. Statistics were calculated using an unpaired, two tailed Student's *t* test.

PCR primers. $GABA_A$ receptor subunit–specific PCR primers were generated from the 3′-untranslated region of the cDNAs where the sequence diversity is greatest (Khrestchatisky et al., 1989; Shivers et al., 1989; Ymer et al., 1989). The primer sequences in bases are listed below with positive (+) being complementary to the noncoding strand and negative (−) being complementary to the coding strand. All sequences are listed 5′ to 3′: α1 (+) = 1410–1429, CCCACACCCCATCAATAGGT; α1 (−) = 1501–1520, GAGGCAGTAAAGCAGACAGG; β2 (+) = 1808–1831, CTGGAAAGCTCAATGGCATGGGCA; β2 (−) = 1882–1905, CTGTCCCAACTGCGTGCCAAGCTT; γ2 (+) = 1744–1768, GGGGCATCATTAGCCTTTGATTCAC; γ2 (−) = 1807–1829, CACAACGTACCCCAAGCGAACG. In addition, SP64 PCR primers [SP64 (+) = 234–254; SP64 (−) = 344–364] were added to each reaction to generate a product of 130 base pairs. PCR products were confirmed by sequence analysis.

Immunohistochemistry. Cultured cells were processed for indirect immunofluorescence using a modification of a previously described procedure (Coons, 1958). In brief, cells were fixed in 4% formaldehyde in PBS for 15 min at room temperature. Following several rinses in PBS, cells were incubated in primary antisera (anti-GABA, 1:50, IncStar; anti-calbindin, 1:1000, Sigma) in PBS containing 1.0 mg/ml BSA, 0.5% Triton X-100, and 10% goat serum for 1 hr at room temperature in a humid chamber. After rinsing several times in PBS, coverslips were incubated for 1 hr in biotinylated secondary antibody (1:200; Vector) in the above described buffer. For peroxidase staining, the cells were processed following the manufacturer's directions in the Elite Vectastain (Vector) protocol using diaminobenzidine as a substrate. Controls were processed as above excluding incubation in primary antisera. Coverslips were mounted in PBS/glycerol (1:1) and the staining was visualized with a Nikon Microphot microscope.

To determine whether neuronal cells dissociated from E19 cerebella divided in culture, cells were processed for double-label immunofluorescence using neuron-specific enolase (NSE), a neuronal marker, and 5-bromodeoxyuridine (BrdU), a DNA analog that is incorporated only into dividing cells. E19 granule cells were pulsed for 1 hr with 10 μM BrdU (Gratzner, 1982; Houck and Loken, 1985; Hall and Landis, 1991)

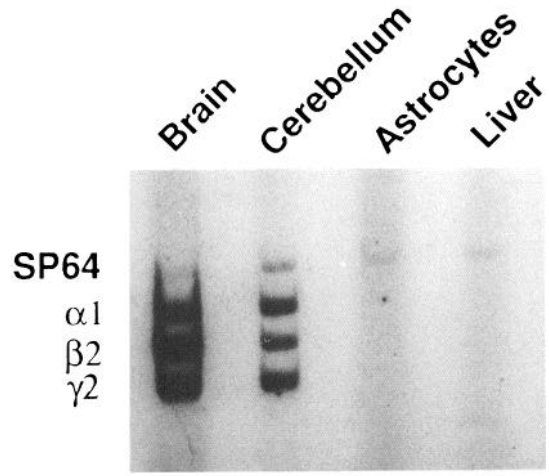

Figure 1. mRNAs encoding the α1-, β2-, and γ2-subunits of the $GABA_A$ receptor are expressed in neuronal tissues. RNA (0.75 μg) from adult rat brain, cerebellum, liver, and cerebellar astrocyte cultures and SP64 RNA (120 pg) were amplified for 26 cycles using the α1, β2, γ2, and SP64 PCR primers. All three subunit mRNAs were present in the brain and cerebellum but were undetectable in the liver and in cultured cerebellar astrocytes.

at 2, 24, or 48 hr in culture. After 4 d in culture, cells were processed sequentially for NSE and BrdU. First, cells were processed for NSE immunoreactivity (1:500; Polysciences) followed by incubation in a Texas red–conjugated secondary antiserum (1:300; Jackson ImmunoResearch). The cells were then immediately fixed again in 4% formaldehyde followed by a 30 min incubation in 2*N* HCl. To restore the pH, the cells were extensively rinsed in PBS (4 × 10 min). For BrdU staining, the cells were incubated for 30 min in primary antibody (1:100; Boehringer Mannheim) diluted in 0.5% Tween and 20% goat serum. After rinsing the cells were incubated for 1 hr in fluorescein isothiocyanate–conjugated secondary antiserum in the same buffer. Coverslips were mounted and the staining was visualized as above.

Results

Analysis of $GABA_A$ receptor subunit mRNAs in the adult rat brain

To examine the developmental expression of $GABA_A$ receptor subunit mRNAs *in vivo* and *in vitro*, a PCR protocol allowing relative quantitation of the subunit mRNAs was devised. In these assays, the levels of mRNAs encoding the α1-, β2-, and γ2-subunits were determined because previous studies found that these subunit mRNAs were abundant in cerebellar granule cells (Gambarana et al., 1991; Laurie et al., 1992) and may be colocalized within the same neurons (Gambarana et al., 1991). Moreover, these three subunits may contribute to the formation of a functional receptor complex (Verdoorn et al., 1990).

The regional distributions of mRNAs encoding the α1-, β2-, and γ2-subunits of the receptor complex were analyzed using PCR with subunit-specific primers. Our studies demonstrated that all of the subunit mRNAs were abundant in the brain and cerebellum where PCR products of 111, 97, and 84 base pairs corresponding to the α1-, β2-, and γ2-subunits, respectively, were found (Fig. 1). In contrast, none of the subunit mRNAs were detectable in the liver. Furthermore, they were undetectable in cultured cerebellar astrocytes, even though previous reports suggested that astrocytes expressed $GABA_A$ receptors (Kettenmann et al., 1988). Thus, our findings indicate that cerebellar expression of these three $GABA_A$ receptor subunit mRNAs is specific to neurons.

Conditions allowing quantitative analysis of the $GABA_A$ receptor subunit mRNAs by PCR were assessed in initial experiments. To ensure that the reaction was in the exponential phase of amplification, the PCR products were analyzed as a function of cycle number (Fig. 2; Chelly et al., 1988, 1990; Wang et al., 1989). To demonstrate that the final amount of the PCR product was dependent on the initial concentration of RNA, products were analyzed as a function of starting RNA concentration. This relationship proved to be linear (not shown). Finally, to control for experimental variation, a known amount of RNA transcribed from the bacterial plasmid SP64 and the appropriate primers were added to each reverse transcriptase reaction and carried through the PCR. All the data were then expressed as the ratio of the subunit PCR product to the SP64 PCR product, thereby allowing comparisons among experiments.

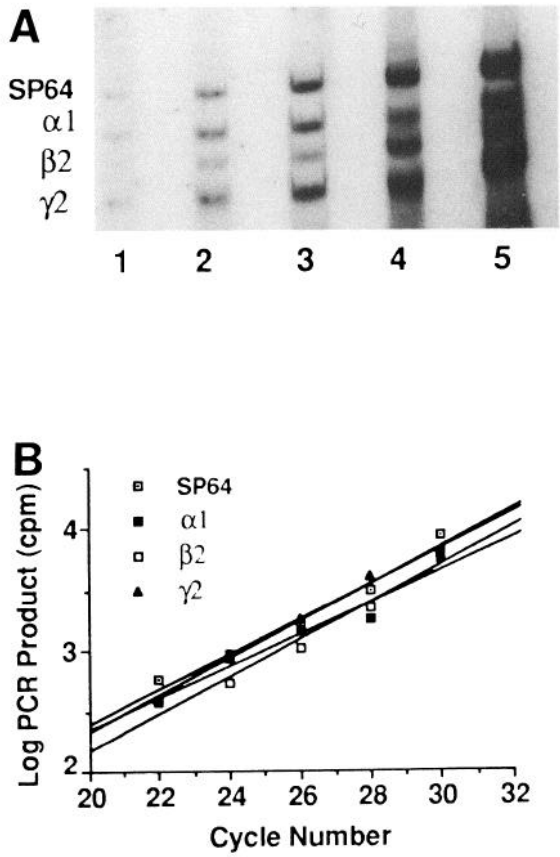

Figure 2. Kinetics of the PCR amplification on tissue. *A*, RNA (0.75 μg) isolated from adult rat brain was amplified for a variable number of cycles using the α1-, β2-, and γ2-subunit–specific primers. *Lane 1*, 20 cycles; *lane 2*, 22 cycles; *lane 3*, 24 cycles; *lane 4*, 26 cycles; *lane 5*, 28 cycles. *B*, The bands from the gel shown in *A* were cut out and counted. Log cpm was plotted versus the cycle number. These data demonstrate that the reactions were exponential up to 30 cycles for 0.75 μg of brain RNA before beginning to plateau (not shown).

$GABA_A$ receptor subunit mRNA expression in the developing cerebellum

The developmental pattern of subunit mRNAs in the cerebellum was determined using the PCR protocol. RNA was isolated from the cortical regions of cerebella from rats ranging in age from P1, an immature stage, to P23, when migration and synaptogenesis were largely complete. Relatively low signals for the α1-, β2-, and γ2-subunit mRNAs were detected from P1 through the first postnatal week (Fig. 3). However, between P10 and

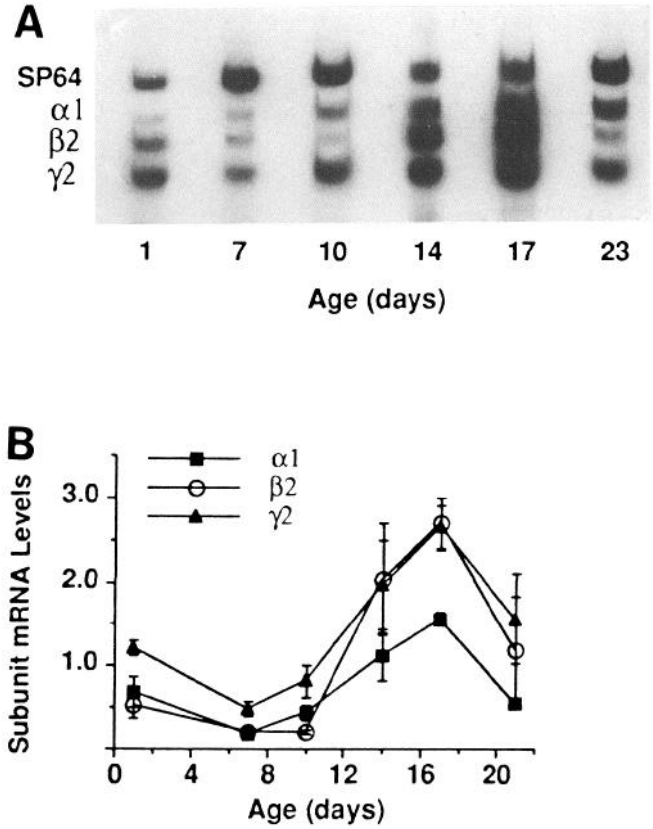

Figure 3. Expression of the α1-, β2-, and γ2-subunit mRNAs of the $GABA_A$ receptor in the developing cerebellum. *A*, The cortical region of the cerebellum was removed from rats ranging in age from P1 to P23. RNA was prepared and analyzed by PCR as described in the Materials and Methods. For each point, 0.75 μg of RNA was amplified for 26 cycles. *B*, Radioactive PCR products from four experiments were excised from the gels and counted. The data were plotted as a ratio of subunit PCR product:SP64 PCR product versus age for each subunit. Each point represents the mean ± SEM. The increases observed between P10 and P17 were significant ($p \leq 0.001$) for all three subunit mRNAs.

P17, when maximal subunit mRNA levels were obtained, the three subunit mRNAs increased three- to fourfold ($p = 0.0002$, 0.0008, and 0.0022 for α1, β2, and γ2, respectively). These increases were similar in magnitude and timing to those detected in the granule cell layer using quantitative *in situ* hybridization (Gambarana et al., 1991).

Characterization of cerebellar granule cell cultures

E19 cultures. To examine the importance of cerebellar maturation in $GABA_A$ receptor gene expression, granule cell cultures were prepared at two developmental stages, E19 and P10. At E19 granule cell progenitors comprise approximately 70% of the neurons in the cerebellum. To generate a more highly enriched population of these neurons, the dissociated cells were centrifuged over a Percoll density step gradient (Hatten, 1985). Two fractions were collected and examined over time in culture by immunohistochemical and morphological procedures.

The identity of the cells in fractions 1 and 2 was determined by staining the cells for the presence of GABA. Because granule cells are the only neurons in the cerebellar cortex that do not use GABA as a neurotransmitter, they can be identified by the absence of GABA immunoreactivity (Fig. 4). At 7 d *in vitro*,

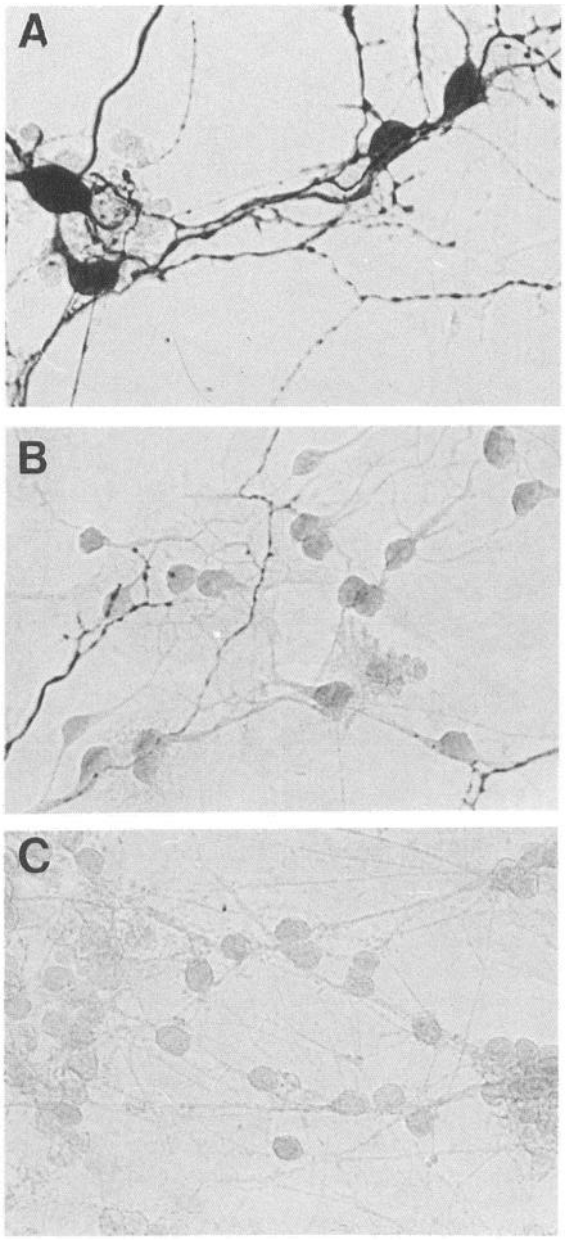

Figure 4. GABA immunohistochemistry in cerebellar cultures prepared at E19 and P10. All cells were analyzed at 7 d in culture. *A*, E19, fraction 1; *B*, E19, fraction 2; *C*, P10. Since granule cells are the only neurons in the cerebellum that do not use GABA as a neurotransmitter, the immunonegative neurons presumably represent this cell population. Magnification, 250×.

approximately 40% of the cells in fraction 1 were GABA immunoreactive. In contrast, less than 10% of the cells in fraction 2 were GABA immunoreactive, indicating that this fraction contained over 90% granule cells. This percentage of granule cells did not fluctuate over 3 weeks in culture (Table 1). In addition, while approximately 15% of the cells in fraction 1 were positive for calbindin, a calcium-binding protein whose cerebellar distribution is limited to Purkinje neurons (Jande et al., 1981), none of the cells from fraction 2 exhibited this marker (data not shown).

The cells in fraction 2 were further characterized by a number of criteria. First, the GABA-negative cells exhibited morphol-

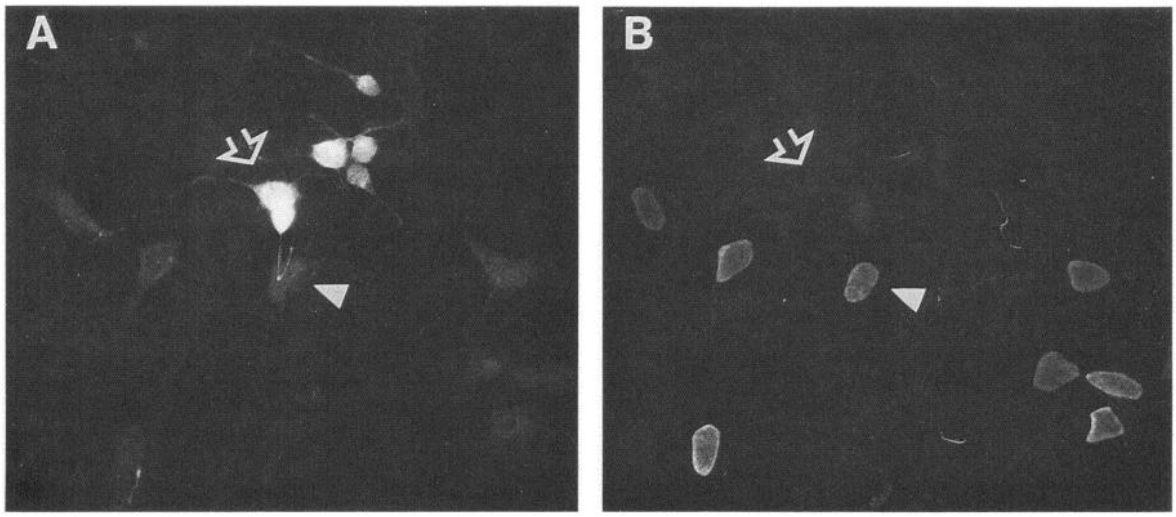

Figure 5. Granule cell precursors cease dividing when dissociated and placed in culture. Granule cells prepared from E19 cerebella were pulsed with 10 μM BrdU at 2, 24, or 48 hr. After 4 d, the cultures were double labeled for NSE and BrdU and processed for immunohistochemistry to identify neurons that had divided. *A*, NSE-positive (*arrow*) and NSE-negative (*arrowhead*) cells pulsed at 48 hr with BrdU. *B*, The same field shown in *A* following immunohistochemistry for BrdU. Less than 3% of the NSE-immunoreactive neurons were BrdU immunoreactive. Magnification, 250×.

ogies characteristic of granule cells. By 2 d in culture, these were readily identified by their small, round cell bodies (6–8 μm) and long bipolar processes. In addition, they were positive for NSE (Hockberger et al., 1987) and negative for glial fibrillary acidic protein (not shown). Since all of these findings indicate that fraction 2 is highly enriched in granule neurons, it was used for studies on $GABA_A$ receptor gene expression.

The possibility that E19 granule cell precursors divide in culture was examined by BrdU incorporation (Gratzner, 1982; Houck and Loken, 1985). Two hours after plating, 23 ± 3.4% of the NSE-immunoreactive neurons incorporated BrdU. At 24 hr this percentage decreased to 11.0 ± 2.6%, and by 48 hr only 2.7 ± 0.84% of NSE-immunoreactive neurons demonstrated BrdU staining (Fig. 5). Therefore, greater than 97% of the neuronal precursors stopped dividing within 2 d of dissociation. The majority of the cells incorporating BrdU were large and flat, suggesting that most of the dividing cells were in fact non-neuronal. These results are in agreement with those obtained in cultures of P5 mouse cerebellum (Gao et al., 1991).

P10 cultures. Since granule neurons are much more abundant at P10, cultures prepared at this stage were considerably enriched for these neurons and did not require fractionation on a Percoll gradient. As in cultures of E19 (fraction 2), greater than 90% of the neurons in the P10 cultures exhibited morphologies characteristic of granule cells. In addition, 90% of the cells were neurons, as identified by NSE immunoreactivity (not shown). Finally, greater than 90% of the neurons in P10 cultures were GABA negative (Fig. 4*C*), a percentage that remained constant over 3 weeks in culture (Table 1). Previous studies have demonstrated that non-neuronal cells are necessary for granule cell survival in culture (Hatten et al., 1985; C. E. Beattie, unpublished observations). Therefore, while an antimitotic agent was used to control proliferation, non-neuronal cells were not entirely eliminated from the cultures prepared at either P10 or E19.

Table 1. Granule neurons in cerebellar cell cultures (% of total neurons)

	Days in culture		
	1	7	17
E19	93.8 ± 1.30 (543)	94.1 ± 1.09 (761)	94.6 ± 1.60 (605)
P10	93.6 ± 0.78 (406)	91.8 ± 1.70 (697)	97.5 ± 0.67 (793)

Granule cells, isolated from E19 (fraction 2) and P10 cerebella, were processed after 1, 7, and 17 d in culture for GABA immunoreactivity. The values reported represent the mean ± SEM with the number of neurons counted from three experiments in parentheses. The percentage of granule neurons in both types of cultures remained constant throughout the experimental time course.

Subunit mRNA patterns of expression differ in granule cells isolated from E19 and P10 cerebella

The expression of $GABA_A$ receptor subunit mRNAs was examined over time in cultures prepared from E19 and P10 cerebella. Initial experiments were performed to establish a quantitative PCR procedure as described above for the *in vivo* studies (Fig. 6). When these conditions were used for granule cells isolated at E19, the α1-, β2-, and γ2-subunit mRNAs were expressed at constant levels from 2 to 21 d in culture (Fig. 7). Although a slight increase in all three subunit mRNAs occurred between 2 and 7 d in culture, this change was not significant ($p = 0.20$–0.30).

A very different pattern of mRNA expression was exhibited in granule cells isolated from P10 cerebella. Two hours after plating, the α1-, β2-, and γ2-subunit mRNAs were present and remained at constant levels for 4 d in culture (Fig. 8). During this time, the levels of the subunit mRNAs were five- to sevenfold higher than those found in the E19 cultures. Between 4 and 7 d, the expression of the β2- and γ2-subunit mRNAs rose 6.1-fold ($p = 0.05$) and 6.5-fold ($p = 0.003$), respectively (Fig. 8). This time in culture corresponds to P14–P17, a period during which increases in the subunit mRNAs are observed *in vivo*. Surprisingly, the increase in mRNA levels was found for only two of the three subunits. While a slight increase in the α1-subunit mRNA was seen in all experiments between 4 and 7 d in culture, this change was not statistically significant ($p = 0.27$).

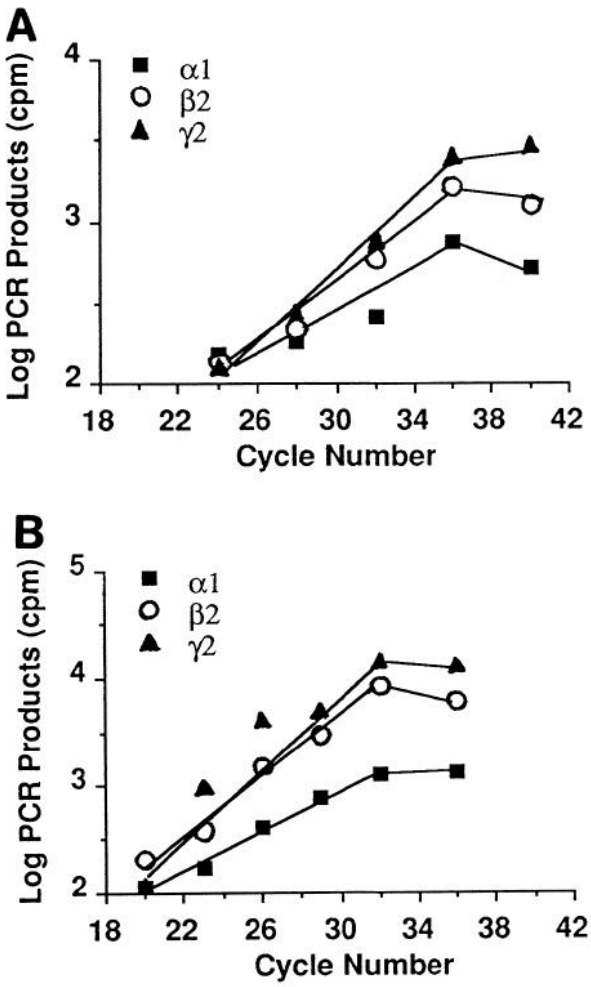

Figure 6. Kinetics of PCR amplification: E19 and P10 cultured granule cells. RNA (0.4 μg) was amplified for varying number of cycles and represented as described in Figure 2. *A*, Amplification of RNA isolated from E19 granule cells cultured for 7 d was exponential for 36 cycles and then began to plateau. *B*, Amplification of RNA from P10 granule cells cultured for 7 d was exponential for 32 cycles and then began to plateau.

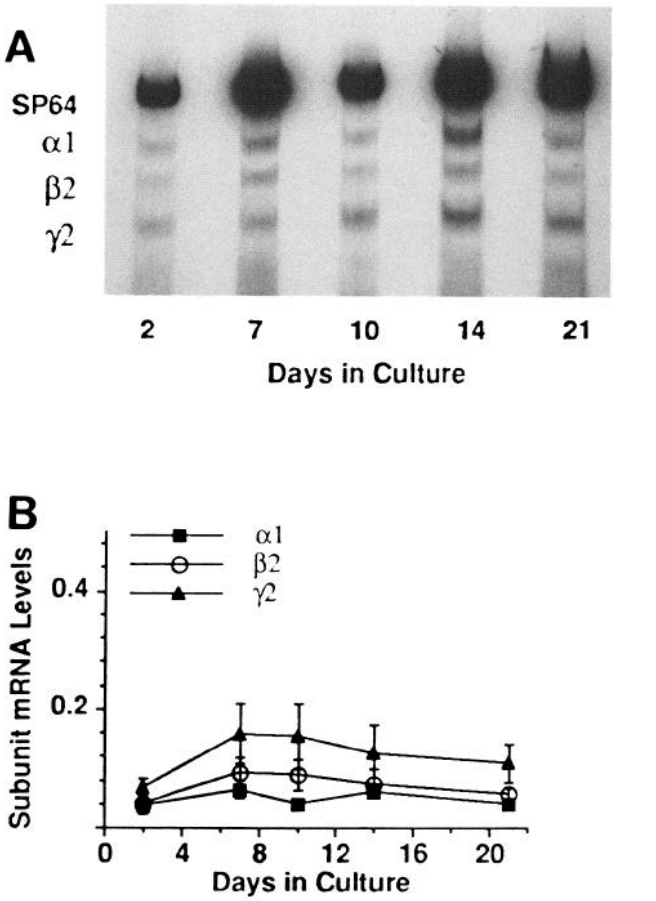

Figure 7. Expression of the α1-, β2-, and γ2-subunit mRNAs in granule cell cultures prepared at E19. *A*, Cells were harvested and analyzed for the presence of the α1-, β2-, and γ2-subunit mRNAs by PCR. For each point, 0.4 μg of RNA was amplified for 30 cycles, which falls into the exponential range of amplification for both sets of cultured neurons. *B*, Radioactive bands from the gels were counted. Subunit PCR products were expressed as a ratio of the SP64 PCR product and plotted versus the age of the cultures. Each point represents the mean ± SEM for four to six determinations. No significant changes in subunit mRNA levels were found.

Discussion

Our studies demonstrate that cultured cerebellar neurons provide a unique system for examining the developmental regulation of $GABA_A$ receptor gene expression in the CNS. Cultures prepared at E19, an age at which cells are still dividing in the germinal cell layer, and at P10, when the cerebellum is undergoing extensive maturation, are both highly enriched in granule neurons. Previous studies have demonstrated that cultured granule cells differentiate morphologically and physiologically to exhibit many properties characteristic of this cell population *in vivo* (Gallo et al., 1987; Hockberger et al., 1987; Gruol and Crimi, 1988). Our studies now show that the cultured granule neurons express other characteristic phenotypes including the mRNAs encoding the α1-, β2-, and γ2-subunits of the $GABA_A$ receptor.

The developmental expression of the $GABA_A$ receptor subunit mRNAs in P10 granule cells in culture in many ways parallels that seen *in vivo*. The mRNAs encoding the α1-, β2-, and γ2-subunits were expressed at relatively low levels in granule neurons *in vivo* through the first postnatal week. Striking increases were observed, however, between P10 and P17, when the levels of all three subunit mRNAs rose three- to fourfold. In addition, others have demonstrated that the α1-subunit mRNA and its encoded polypeptide become abundant in granule neurons only after migration to their adult positions (Meinecke and Rakic, 1990). Consistent with these findings *in vivo*, all of the subunit mRNAs were present in granule cell cultures prepared at P10. Moreover, the mRNAs were expressed at levels approximately sixfold higher than those found at E19. In addition, the β2- and γ2-subunit mRNAs increased sixfold between 4 and 7 d in culture. Although these increases occurred approximately 2 d later than those seen *in vivo*, our results indicate that cultured P10 granule cells mimic many aspects of their normal developmental program. The delay in the observed increases presumably reflects the time required for recovery following tissue dissociation and cell culture. In contrast to the β2- and γ2-subunit mRNAs, the level of the α1-subunit mRNA did not change significantly over the entire culture period. This result suggests that signals necessary to induce α1-subunit mRNA levels are absent from the P10 cultures. Moreover, it indicates that although the α1-, β2-, and γ2-subunit mRNAs increase

coordinately *in vivo*, their expression may be regulated by different mechanisms.

Our findings concerning $GABA_A$ receptor subunit mRNA expression *in vivo* and in P10 culture agree in part with the results of a recent report by Bovolin et al. (1992). As found in our studies in the developing cerebellum, these investigators demonstrated that the levels of the mRNAs encoding the α1- and γ2-subunits rose during the second postnatal week. However, in contrast to our *in vivo* studies reported here, as well as to our previous studies using *in situ* hybridization histochemistry, which revealed that the mRNAs rose 2.5–4-fold (Gambarana et al., 1990, 1991), increases of approximately 16-fold for the α1-subunit mRNA and 2-fold for the mRNAs encoding the two γ2-subunit isoforms together (γ2L and γ2S) were observed. In addition, the results obtained in cultured granule neurons also differ from ours in some respects. Most importantly, Bovolin and coworkers reported that the α1-subunit increased over time in culture whereas no change was found in our system. The reasons for this disparity may reflect differences in experimental time course. While our studies followed subunit mRNA expression for 10 d in cultures prepared at P10, these investigators examined changes over a 4 week period in cultures prepared at P8. Relatively low levels of the α1-subunit were found in the first 2 weeks of culture and rose approximately twofold between days 14 and 18. Thus, although α1-subunit levels may increase in culture, the time course of this change is different from that found for the β2- and γ2-subunit mRNAs, suggesting that the mRNAs are subject to different regulatory signals.

Insight into the regulation of the $GABA_A$ receptor genes in cerebellar cells is provided by our studies on E19 cultures. Although all three subunit mRNAs were detected, the levels of expression did not change over 3 weeks, a pattern distinctly different from that seen in P10 cultures. Had the E19 cells been programmed to follow the pattern observed *in vivo*, increases in subunit mRNA levels would have been anticipated at approximately 13–17 d in culture. These findings suggest that environmental signals present in the maturing cerebellum between E19 and P10 modulate $GABA_A$ receptor gene expression. The maturity of the neurons at the time of dissociation is presumably a critical determinant in their ability to express $GABA_A$ receptor subunit mRNAs. In addition, the maturity of non-neuronal cells in our cultures could also affect gene expression by granule neurons. Non-neuronal cells comprised 5–7% of the cells in both the E19 and P10 cultures, and these cells are required to maintain neuronal viability (Hatten, 1985; Hatten et al., 1988). In previous studies on cultured neurons it has been demonstrated that glia can alter neuronal morphology (Denis-Donini et al., 1984; Denis-Donini and Estenoz, 1988) and that factors released from non-neuronal cells can affect neurotransmitter and neuropeptide phenotype (Yamamori et al., 1989; Nawa and Patterson, 1990). Differences in the non-neuronal cells in our E19 and P10 cultures could influence the ability of the granule neurons to follow the normal developmental program *in vitro*.

There are several other possibilities for the differences in $GABA_A$ receptor subunit mRNA expression between E19 and P10 cultures that we believe are unlikely. First, the E19 cells were subjected to Percoll gradient fractionation before plating whereas the P10 cells were not. While it is possible that the gradient fractionation affects the properties of the cells, our preliminary data suggest that this is not the case. When granule neurons were dissociated from P2 animals, plated without fractionation, and maintained for 16 d in culture, they expressed constant

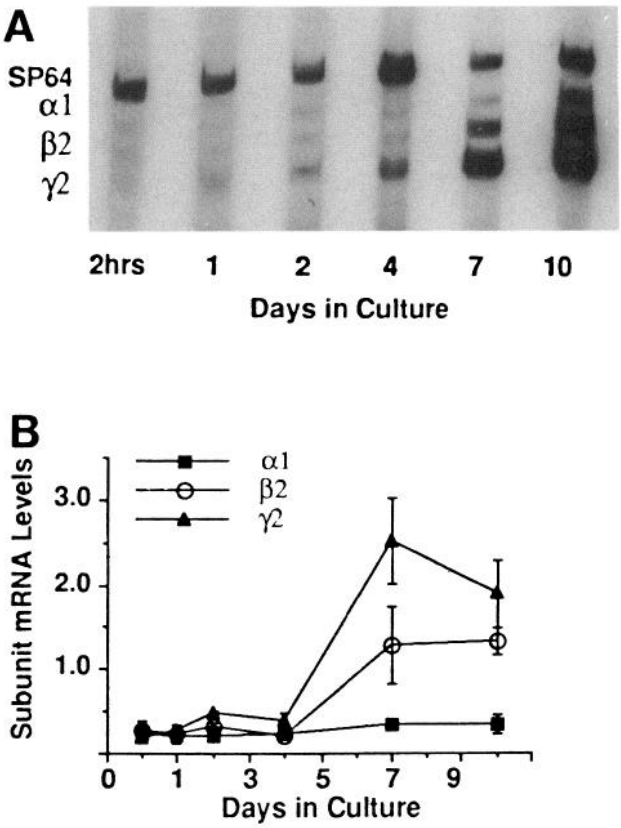

Figure 8. Expression of the α1-, β2-, and γ2-subunit mRNAs in granule cell cultures prepared at P10. *A*, Cells were harvested and analyzed for the presence of the α1-, β2-, and γ2-subunit mRNAs by PCR. For each point, 0.4 μg of RNA was amplified for 30 cycles. *B*, Radioactive bands from the gels were counted and plotted as described in Figure 7. Each point represents the mean ± SEM for four to six determinations. The increases in the β2- and γ2-subunit mRNAs between 4 and 7 d in culture were statistically significant ($p \leq 0.002$), while changes in the α1-subunit mRNA levels were not.

levels of subunit mRNAs. Because this result is identical to that obtained with E19 cells plated after fractionation, the Percoll gradient does not appear to affect receptor gene expression (K. Behringer and R. E. Siegel, unpublished observation). Second, the culture conditions used in our experiments could favor P10 granule cells. However, E19 granule cells cultured for 20 d were morphologically identical to P10 granule cells cultured for 7 d; this suggests that E19 and P10 cells grow equally well under our culture conditions.

The cues modulating $GABA_A$ receptor gene expression in the cerebellum *in vivo* are unknown. The fact that extensive migration, maturation, and synaptogenesis temporally coincide with the observed increases in subunit mRNA levels raises the possibility that an event(s) during this period alters gene expression. By P10–P14, many neurons from the external germinal cell layer have already migrated to their adult positions in the granule cell layer. Once in their mature position, the neurons begin extending dendritic processes and forming synapses with GABAergic Golgi neurons as well as with excitatory mossy fibers (Altman, 1972a; Shimono et al., 1976). In addition, granule cell axons of the parallel fiber network are forming synaptic interactions with Purkinje, basket, and stellate cell dendrites. Thus, possible modulatory signals include contact with neighboring neuronal and non-neuronal cells, interactions with neurotransmitters and growth factors, or the migration process itself. In fact, our pre-

liminary results suggest that granule neurons become responsive to these regulatory signals only after entering the migratory pathway.

A number of recent studies have raised the possibility that neurotransmitters play a role in regulating $GABA_A$ receptor gene expression. For example, the transmitter GABA appears to modulate the expression of its own receptor. When granule neurons isolated from P7 cerebella were grown in the presence of GABA, low-affinity GABA receptors were found in addition to the high-affinity sites that are present in the absence of the transmitter (Meier et al., 1984). The relationship between these changes in receptor properties and subunit mRNA expression is currently unknown. Additional studies have suggested a role for another neurotransmitter, glutamate, in $GABA_A$ receptor mRNA expression. When granule cells from P6 rats were grown under depolarizing conditions in the presence of either competitive or noncompetitive antagonists of the NMDA-selective glutamate receptor, decreased levels of some $GABA_A$ receptor subunit mRNAs were found (Memo et al., 1991). These results suggest that glutamate regulates the expression of $GABA_A$ receptors via action at the glutamate receptor.

While the complexity of the CNS has inhibited progress in identifying cues regulating the $GABA_A$ receptor and other neuronal receptors, similar studies have begun to elucidate events and signals modulating the expression of the ACh receptor (AChR) gene family in the PNS. In the ciliary ganglion, for example, it has been demonstrated that the levels of the mRNA encoding the α3-subunit of the neuronal nicotinic AChR decrease in response to axotomy and denervation (Boyd et al., 1988). Similarly, studies on the peripheral nicotinic AChR at the neuromuscular junction have demonstrated that denervation or treatment with TTX induces rises in the level of the α-subunit mRNA in skeletal muscle cells without altering the levels of those encoding the δ- and γ-subunits (Goldman et al., 1985; Klarsfield and Changeux, 1985). In addition, the AChR-inducing factor ARIA produces increases in the α-subunit mRNA almost exclusively (Harris et al., 1989). These studies demonstrate that distinct signals selectively modulate individual subunit levels of the AChR, and it is likely that the expression of the $GABA_A$ receptor, another ligand-gated ion channel, may be similarly regulated.

In summary, our studies have demonstrated that purified cultures of cerebellar granule neurons express $GABA_A$ receptor subunit mRNAs. Differences in the patterns of subunit mRNAs in cultures prepared at E19 and P10 suggest that $GABA_A$ receptor gene expression is developmentally regulated. Moreover, the finding that changes in specific subunit mRNA levels in cultured P10 neurons mimic those seen *in vivo* suggests that these cultures can provide a model system to examine regulation of $GABA_A$ receptor expression in the CNS. Studies are currently underway to evaluate the exact timing and importance of environmental cues in the expression of receptor mRNAs and the encoded polypeptides in cultured granule neurons.

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