The Journal of Neuroscience, December 1995, 15(12): 8121–8130

Uncoupling of $GABA_A$/Benzodiazepine Receptor α_1, β_2, and γ_2 Subunit mRNA Expression in Cerebellar Purkinje Cells of Staggerer Mutant Mice

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The mammalian $GABA_A$/benzodiazepine ($GABA_A$/BZ) receptor is comprised of several subunit isoforms: α_{1-6}, β_{1-3}, γ_{1-3} and δ. In the present studies, the expression of α_1, β_2, and γ_2 subunit mRNAs was examined in cerebellar Purkinje cells and deep cerebellar neurons of staggerer mutant mice during postnatal development. In control animals, the three subunit mRNAs were present at high density in Purkinje cells which, in adult animals, form a monolayer at the interface of the granule cell and molecular layers. The number of Purkinje cells in the staggerer cerebellar cortex is reduced; the majority of those that remain are retained within the granule cell layer and are unable to receive normal afferent synapses from granule cells. The three subunit mRNAs were expressed at similar levels in both staggerer and control Purkinje cells until postnatal day 9. After this time, although the α_1 subunit mRNA was maintained at control levels in staggerer Purkinje cells, the expression of β_2 and γ_2 subunit mRNAs decreased, and was largely absent by postnatal day 20. The loss of β_2 and γ_2 mRNA expression in staggerer was specific to Purkinje cells, since all three mRNAs were present throughout postnatal development in other brain regions, including the deep cerebellar nuclei. The present studies indicate that in cerebellar Purkinje cells, the $GABA_A$/BZ receptor α_1, and β_2, and γ_2, subunit mRNAs are regulated by distinct mechanisms which are differentially affected by the staggerer mutation.

[Key words: GABA, in situ hybridization, cerebellum, deep cerebellar nuclei, development, reeler, weaver]

A fully differentiated Purkinje cell phenotype is the result of interactions between genetic and epigenetic factors over a protracted period of development. The Purkinje cell is the principal integrative cell in the cerebellar cortex, relaying excitatory information from climbing fibers and mossy fibers to the deep cerebellar nuclei. Several mutations have been identified which cause abnormalities in murine Purkinje cell development (Sidman, 1968). Although some of these mutations are intrinsic to the cell, others are extrinsic and affect the Purkinje cell secondarily. Mice homozygous for the autosomal recessive mutation "staggerer" (Green and Lane, 1967) develop a pronounced cerebellar ataxia, which is detectable by postnatal days 7–8. The primary genetic defect appears to be intrinsic to Purkinje cells (Herrup and Mullen, 1979a), and leads to the failure of these neurons to acquire their mature phenotype. As a result, staggerer Purkinje cells lack several markers of terminal differentiation, including the adult form of N-CAM (Edelman and Chuong, 1982), voltage-dependent Ca^{2+} channels (Crepel et al., 1984), the IP_3 receptor (Mikoshiba et al., 1985), calmodulin (Messer et al., 1990) and the $G_{D1\alpha}$ ganglioside (Furaya et al., 1994).

An important component of the mature Purkinje cell phenotype is the presence of GABA receptors which subserve inhibitory afferents from basket and stellate cells, and from Purkinje cell collaterals. The $GABA_A$/benzodiazepine ($GABA_A$/BZ) receptor is a heteropentameric GABA-gated chloride channel (Nayeem et al., 1994) which consists of combinations of subunit polypeptides, the majority of which exist in several isoforms (α_{1-6}, β_{1-4}, γ_{1-4}, δ, and ρ_{1-2}); additional subunit diversity is generated by alternative splicing (Macdonald and Olsen, 1994; Tyndale et al., 1995). The majority of benzodiazepine and GABA binding sites are located on α and β subunits, respectively (Sigel et al., 1983; Casalotti et al., 1986; Fuchs and Sieghart, 1989). However, recombinant $GABA_A$-receptors composed entirely of α and β subunits show only a weak modification of GABA responses by benzodiazepines; the inclusion of a γ subunit, in combination with α and β subunits, confers benzodiazepine sensitivity (Pritchett et al., 1989; Knoflach et al., 1991; Horne et al., 1993; Pregenzer et al., 1993).

The α_1, $\beta_{2/3}$, and γ_2 subunits combine to form the most frequently occurring isoform of the $GABA_A$ receptor complex in the adult mammalian CNS (Benke et al., 1991; Duggan et al., 1992). In rodents, the α_1, β_2, and γ_2 subunit mRNAs are present at particularly high density in cerebellar Purkinje cells (Zdilar et al., 1991, 1992; Laurie et al., 1992; Luntz-Leybman et al., 1993); all other $GABA_A$/BZ receptor subunit genes are either absent, or expressed at much lower levels. Furthermore, Purkinje cells contain α_1, $\beta_{2/3}$, and γ_2 subunit polypeptides (De Blas et al., 1988; Fritschy et al., 1992, 1994; Gutierrez et al., 1994; Nadler et al., 1994). In this paper, we report on the effect of the staggerer mutation on the expression of $GABA_A$/BZ receptor α_1, β_2, and γ_2 subunit mRNAs in cerebellar Purkinje cells.

Materials and Methods

Riboprobe preparation. Antisense and sense ribonucleotide probes for the α_1, β_2, and γ_2 subunits of the $GABA_A$/BZ receptor were generated by polymerase chain reaction (PCR). Poly(A)$^+$ RNA was isolated from mouse brain by the method of Badley et al. (1988). Reverse transcription and PCR amplification procedures were conducted as described

Received Mar. 13, 1995; revised July 11, 1995; accepted Aug. 14, 1995.

This work was supported by grants from the National Institute of Alcohol Abuse and Alcoholism and the National Institute of Neurological Disorders and Stroke to A.R. and A.F. V.L.-L. was supported by USPHS Training Grant NS07291.

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previously (Luntz-Leybman et al., 1993). Each of the riboprobes was designed to include regions complementary to the most variable portion of the corresponding nucleotide sequence within the intracellular loop, between the putative M3 and M4 transmembrane spanning domains. The γ_2 probe recognized both long and short splice variants. The primers for the three probes, based on the published rat or mouse sequences (α_1, Wang et al., 1992; β_2, Ymer et al., 1989; γ_2, Kofuji et al., 1991), are as follows: 5′-ATA AAG CTT AAG AGA GGG TAT GCG TGG GAT-3′ (upstream α_1 primer) and 5′-ATA GGA TCC AGG CTT GAC TTC TTT CGG TTC-3′ (downstream α_1 primer); 5′-ATA AAG CTT GAG AAG ATG CGC CTG GAT GTC-3′ (upstream β_2 primer) and 5′-ATA GGA TCC GCA CGT CTC CTC AGG CGA CTT-3′ (downstream β_2 primer); 5′-ATA AAG CTT TCA GCA ACC GGA AGC CAA GCA-3′ (upstream γ_2 primer) and 5′-ATA GGA TCC ACT GGC ACA GTC CTT GCC ATC-3′ (downstream γ_2 primer).

The PCR product was digested with HindIII and BamHI restriction enzymes and ligated "in gel" in HindIII-BamHI treated, dephosphorylated pBluescript II SK[+] phagemid vector (Stratagene, La Jolla, CA) as described previously (Kalvakolanu and Livingston, 1991). Plasmids were linearized with HindIII and BamHI for the subsequent production of antisense and sense RNA probes, respectively. A portion of the recombinant plasmid was purified, alkali denatured and sequenced using the Amplitaq sequencing kit (#N808-0035, Perkin Elmer, Norwalk, CT). The linearized templates were purified and transcribed (Luntz-Leybman et al., 1993). The specific activity of each riboprobe was 1–1.5 × 10^9 dpm/mg.

Northern blot hybridization (data not shown), conducted under high stringency conditions, was used to determine the number and size of the mRNA species recognized by the three probes. These experiments revealed major bands at 4.0 and 4.3 kb for α_1, 8.2 kb for β_2, and 4.3 and 2.5 kb for γ_2. No cross-hybridization between these subunit mRNAs was observed.

In situ *hybridization.* Staggerer mutant mice (sg/sg) and their unaffected heterozygous or homozygous normal littermate controls (?/+) were bred from heterozygous B6C3Fe-ala-sg mice obtained from Jackson Laboratories, Bar Harbor, ME. For adult studies, five pairs of sg/sg and ?/+ control mice were examined at postnatal days (P)28, 29, 30, 60, and 80. For developmental studies, two separate groups of mice were examined at postnatal days (P)1, 6, 9, 12, 15, and 20. Mutant animals were identifiable behaviorally between approximately P7-8 when ataxia developed; at earlier data points, whole litters were sacrificed and homozygous mutant animals were identified morphologically. Animals at the P1 to P9 data points were obtained from heterozygous breeding pairs previously shown to produce large litters of which at least 25% were homozygous for the staggerer gene. In addition, adult reeler (rl/rl; $N = 3$) and weaver (wv/wv; $N = 3$) mutant mice, also bred from Jackson Laboratory stocks, were examined. Mice were decapitated after inhalation anesthesia (Metofane, Pitman-Moore, NJ); brains were rapidly removed, frozen on dry ice, and stored at −70°C. Coronal and sagittal sections, 20 μm thick, were cut in a cryostat and thaw mounted onto 3 × subbed (300 bloom gelatin and chrome alum) slides and stored at −70°C. Slide mounted sections were brought to room temperature and hybridized with ^{35}S-labeled probes as described previously (Luntz-Leybman et al., 1993). After incubation, the sections were washed in 2 × SSC (saline sodium citrate) at room temperature (RT) for 10 min, followed by incubation in RNase A solution (20 μg/ml RNase A in 10 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.0) for 30 min at 37°C. RNase treated sections were washed at increasing stringency, as follows: 2 × SSC (10 min), 1 × SSC (10 min), 0.5 × SSC (10 min), 0.25 × SSC (10 min), and 0.25 × SSC (60 min) at 70°C. After final washes in 0.25 × SSC (10 min) at RT, sections were dehydrated (1 min each) through 70%, 80%, 90%, and 100% EtOH. All washing solutions, except RNase A solution, contained 10 mM 2-mercaptoethanol to prevent nonspecific binding of riboprobe. The nonspecific hybridization signal was determined by exposing adjacent sections to sense RNA probes.

Autoradiography. Autoradiograms were generated as follows: acid-washed coverslips (No. 0, Corning Glass, Corning, NY), previously coated with a uniform layer of photographic emulsion (Kodak NTB-2, Eastman Kodak, Rochester, NY) were apposed to the slide-mounted sections and exposed for 6 d, at 4°C. Coverslips containing the autoradiographs were developed in Kodak Dektol developer (diluted 1:1 with distilled water) for 2.0 min at 17°C, fixed for 3.5 min in Kodak Rapid-Fix, washed in distilled water for 30 min, and mounted onto microscope slides. After the coverslips were developed, the slides containing the sections were dipped in Kodak NTB-2 photographic emulsion at 38°C, dried overnight, exposed at 4°C, and developed as above. Emulsion covered sections were counterstained with cresyl fast violet and used for cellular localization of grain density.

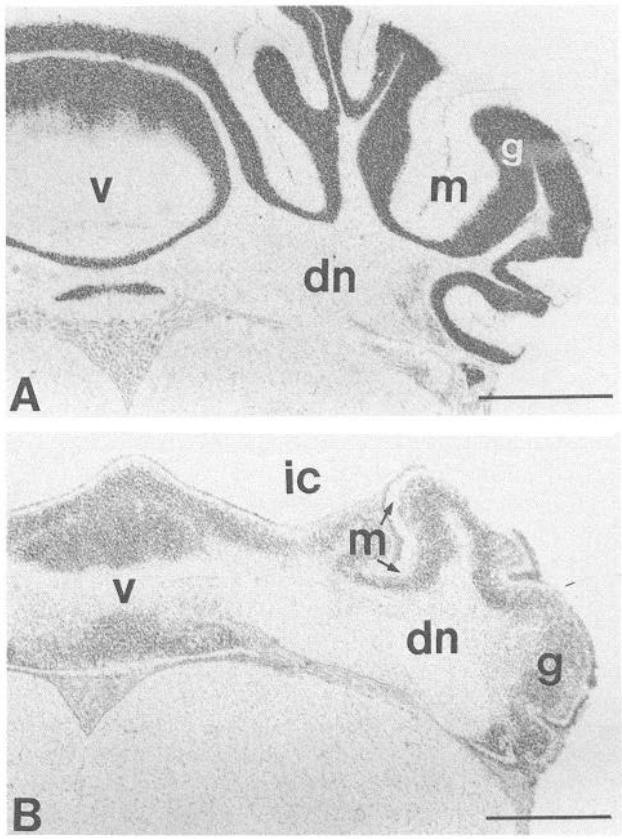

Figure 1. Cresyl fast violet stained coronal sections through the cerebellar cortex of a postnatal day 30 littermate control (*A*) and staggerer (*B*) mouse. *dn*, Deep cerebellar nuclei; *g*, granule cell layer; *ic*, inferior colliculi; *m*, molecular layer; *v*, vermis. Scale bars, 1 mm.

Optical density readings (Image-1, Universal Imaging) were used to determine the autoradiographic grain density on emulsion coated coverslips in traced regions over individual Purkinje cells and deep cerebellar neurons in coronal sections. The cortical regions selected for measurement in littermate controls were randomly selected from the vermis and hemispheres of lobules 5 through 9. Approximately the same region on the rostrocaudal axis was measured in staggerer, although the underdeveloped state of the cortex makes the precise lobular assigment difficult. Measurements of the deep cerebellar nuclei were from all three subregions (medial, intermediate, and lateral) on either side of the midline. The data are expressed as optical density units divided by the area measured: 2000 μm^2 and 1800 μm^2 for Purkinje cells and deep cerebellar neurons, respectively. Optical density measurements from equivalent regions on sense slides were deducted from the antisense measurements to obtain the specific hybridization signal.

Results

The adult cerebellar cortex is a highly organized structure consisting of five major neuronal types which are arranged in three lamina, the molecular, Purkinje cell, and granule cell layers. The deep cerebellar nuclei lie ventral to the cortex on either side of the midline (Fig. 1*A*). Unlike the normal laminar arrangement observed in unaffected littermates, the homozygous staggerer cerebellum is grossly underdeveloped (Fig. 1*B*). Although there is a normal complement of deep cerebellar cells (Roffler-Tarlov and Herrup, 1981), Purkinje cells and granule cells are reduced in number (Herrup and Mullen, 1979b). Whereas Purkinje cells in the normal adult cerebellum are aligned in a monolayer at the

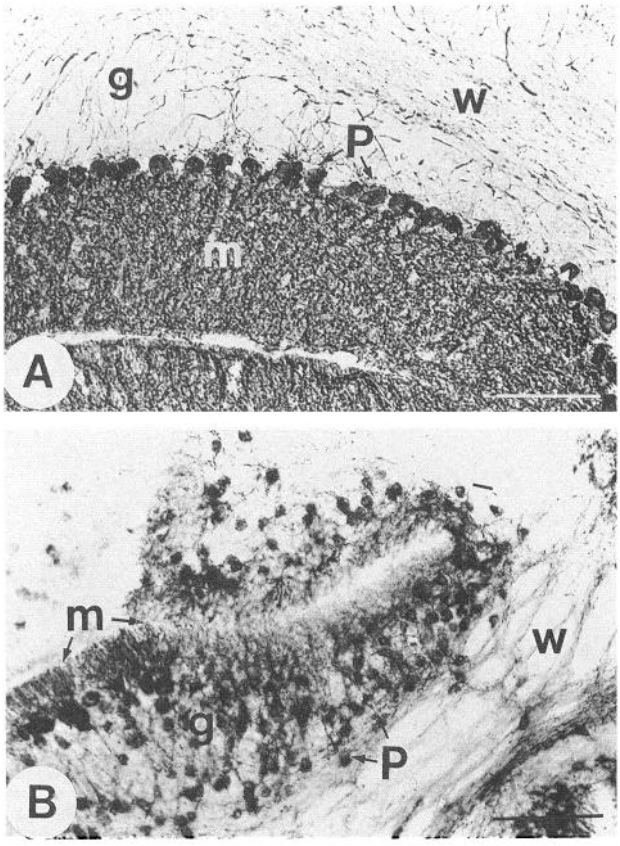

Figure 2. Immunocytochemical staining with calbindin (Sigma, St. Louis, MO) showing (*A*) Purkinje cells aligned in a monolayer in the normal cerebellar cortex, and (*B*) Purkinje cells ectopically located in the staggerer granule cell layer. *g*, granule cell layer; *m*, molecular layer; *P*, Purkinje cells; *w*, white matter. Scale bars, 100 μm.

interface of the molecular and granule cell layers (Fig. 2*A*), the majority of staggerer Purkinje cells are ectopically located within the granule cell layer and relatively few cells are found in their normal position (Fig. 2*B*).

The distribution of α_1, β_2, and γ_2 mRNAs in the adult staggerer cerebellum. $GABA_A$ receptor α_1, β_2, and γ_2 subunit mRNAs were examined in the adult staggerer and littermate control cerebellum at P28, 29, 30, 60, and 80. Each of the three subunit mRNAs were expressed at high levels in the cerebellum of unaffected littermate controls. Very low levels of autoradiographic grain density were distributed uniformly over the sense control sections. Although the three hybridization signals differed in intensity ($\alpha_1 > \gamma_2 > \beta_2$), in each case, the strongest autoradiographic signal within the normal adult cerebellum was present over Purkinje cells and deep cerebellar neurons, while granule cells, and basket and stellate cells in the molecular layer, were labeled at a lower grain density (Fig. 3*A,C,E*). In the adult staggerer cerebellum, high levels of α_1 subunit mRNA were present in Purkinje cells, while β_2 and γ_2 mRNA expression was largely absent (Fig. 3*B,D,F*; see Fig. 6). Although the overall expression of β_2 and γ_2 mRNA was reduced to background levels throughout the sg/sg cerebellar hemispheres (Figs. 4, 5; Table 1) and vermis (Fig. 6, Table 1), occasional Purkinje cells in the deeper regions of the caudal vermis retained some expression into adulthood. These cells were labeled at much lower levels than cells in the same region of the control vermis (40% and 50% below control for β2 and γ_2, respectively). By postnatal days 60–80, only infrequent Purkinje cells in the deeper regions of the caudal aspect of the staggerer vermis expressed the β_2 and γ_2 messages.

The distribution of α_1, β_2, and γ_2 mRNAs in developing staggerer mice. In order to ascertain if the relative absence of the β_2 and γ_2 subunits is correlated with known synaptogenic events within the cerebellar cortex, the expression of α_1, β_2, and γ_2 subunit mRNAs in staggerer and littermate control Purkinje cells was examined throughout the first three postnatal weeks (P1–20). In normal animals, the α_1, β_2, and γ_2 hybridization signals were diffusely distributed over Purkinje cells in the multicellular region between the external germinal layer and internal granular layer at birth. Between postnatal days (P)4–6, as cerebellar foliation increased, Purkinje cells began to form a monolayer at the interface of the internal granular layer and the molecular layer; at this time, autoradiographic grains no longer spanned the entire molecular layer, but became clustered over Purkinje cell perikarya. The intensity of the α_1 hybridization signal increased markedly between postnatal days P9–12, and maximum grain density was reached by P20. Similarly, the β_2 and γ_2 signals increased during the first two postnatal weeks. Although the neonatal staggerer cerebellar cortex was similar in size and overall structure to that of heterozygous and wild type controls at birth, staggerer animals were detectable by a slight reduction in cerebellar foliation and a thickened external germinal layer. Unlike control animals, in which Purkinje cells became aligned into a monolayer, relatively few staggerer Purkinje cells reach the normal adult position, the great majority remaining scattered within the granule cell layer. Autoradiographic grain density was measured over immature Purkinje cells in the molecular layer of the caudal vermis during postnatal week 1, and as they matured during postnatal weeks 2 and 3 (Table 2): During postnatal days 1–9, the density of the α_1, β_2, and γ_2 hybridization signals in sg/sg Purkinje cells was almost identical to that of unaffected littermate controls. By P9–12, the abnormal structure of the staggerer cerebellar cortex became more evident, and the expression of the three subunits began to diverge: although relatively normal levels of α_1 subunit mRNA were maintained in staggerer Purkinje cells, the β_2 and γ_2 mRNAs were greatly reduced by postnatal day 20.

The distribution of α_1, β_2, and γ_2 mRNAs in the deep cerebellar nuclei. The loss of β_2 and γ_2 expression in the cerebellar cortex during postnatal week two was specific to Purkinje cells, since the staggerer deep cerebellar nuclei remained clearly labeled into adulthood (Fig. 3*D,F*). The α_1, β_2, and γ_2 hybridization signals were clustered over cell bodies in the medial, intermediate and lateral regions of the deep cerebellar nuclei (Fig. 3). Each of the messages was present at birth; quantitation of the autoradiographic grain density showed that the signal increased throughout the first three postnatal weeks (Table 3), reaching maximal levels by P20. During this time, the level of subunit expression was similar in both staggerer and control cells. Subsequently, α_1 and γ_2 mRNA expression remained stable in the deep cerebellar neurons of control mice, while the β_2 hybridization signal reached a peak at P20, after which the message became reduced. In older staggerer mice (P28, P29, P30, P60, and P80), β_2 and γ_2 mRNA expression exceeded control levels (Table 1). The degree to which the two signals increased in staggerer dcn was dependent upon the method of measurement: Optical density measurements over the whole cross-sectional area indicated that the β_2 and γ_2 signals were increased by 146% and 74%, respectively. However, although the cross-

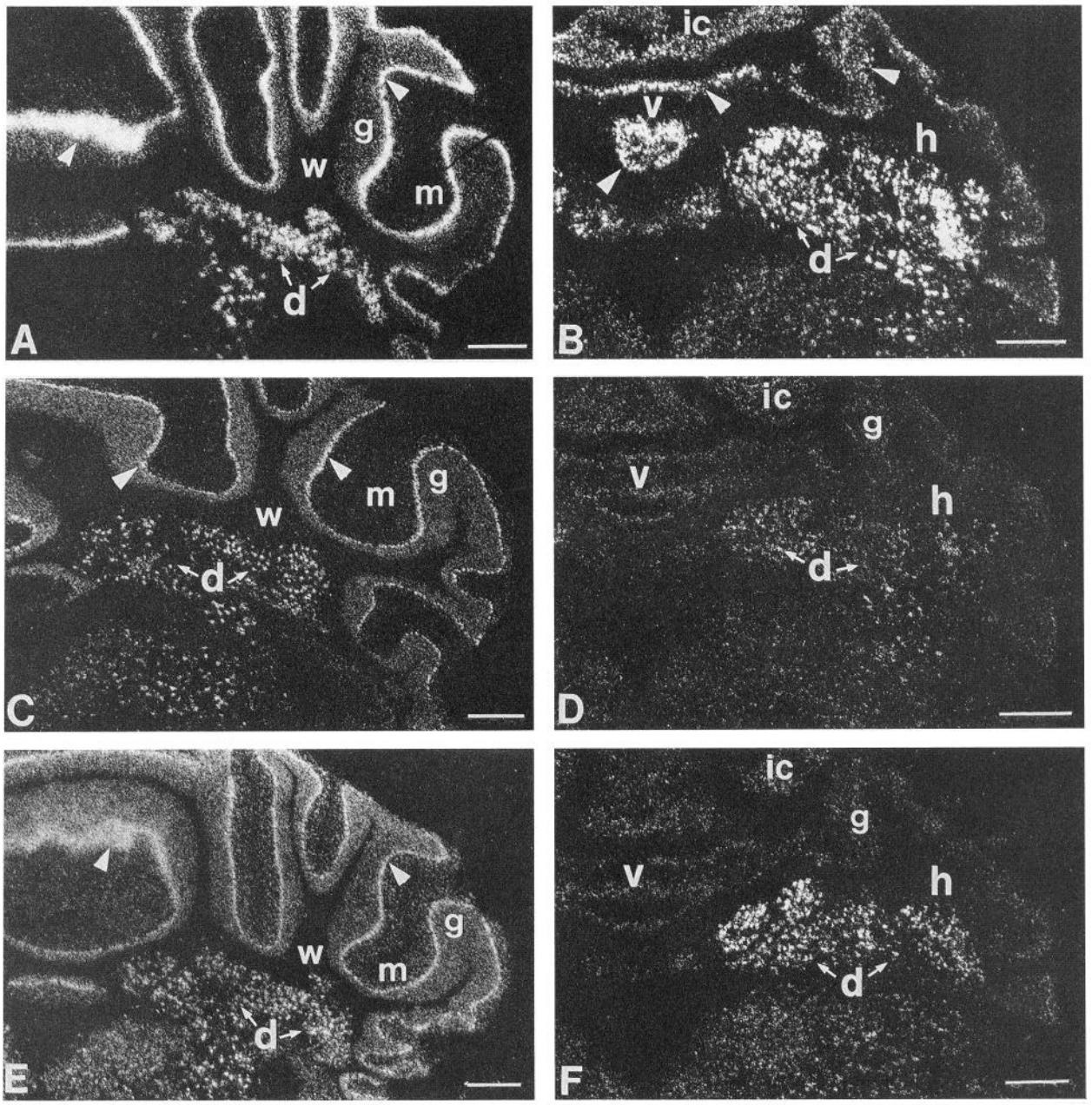

Figure 3. The expression of $GABA_A$ receptor α_1 (*A, B*), β_2 (*C, D*), and γ_2 (*E, F*) subunit mRNAs in adult littermate control and staggerer Purkinje cells. A low power photomicrograph photographed under dark-field illumination showing that each of the three subunits are expressed at high levels in Purkinje cells of normal littermate control animals (*A, C, E*). Although high levels of α_1 subunit mRNA are expressed in staggerer Purkinje cells (*B*), little or no hybridization signal is present with β_2 (*D*) or γ_2 (*F*) probes. *Arrowheads* indicate Purkinje cells; *d*, deep cerebellar nuclei; *g*, granule cell layer; *h*, hemisphere; *ic*, inferior colliculi; *m*, molecular layer; *v*, vermis; *w*, white matter. Scale bars, 500 μm.

sectional area of the sg/sg and control dcn was similar through postnatal day 9, after this time the sg/sg dcn became reduced by approximately 24%. In order to correct for the shrinkage, grain density also was measured over individual dcn cells; consequently, smaller increases of 38% (β_2) and 13% (γ_2) were obtained.

The distribution of α_1, β_2, and γ_2 mRNAs in reeler (rl/rl) and weaver (wv/wv) mice. The expression of α_1, β_2, and γ_2 subunit mRNAs also was examined in the agranular cerebellar cortex of autosomal recessive reeler and weaver mutants. As in staggerer, weaver and reeler Purkinje cells are also ectopically located. Unlike their expression in the adult staggerer, each of the three subunit messages was maintained at control levels in reeler (Fig. 7*A–C*) and weaver (Fig. 8*A–C*) Purkinje cells.

Discussion

We have shown that the expression of $GABA_A$ receptor α_1, β_2, and γ_2 subunit genes in staggerer Purkinje cells diverges during the second postnatal week, and that although α_1 subunit mRNA expression is maintained near control levels into adulthood, the β_2 and γ_2 subunit mRNAs become developmentally downregulated and are virtually absent in adult staggerer Purkinje cells. In contrast, β_2 and γ_2 subunit mRNAs are expressed at, or slighty above, normal levels in staggerer deep cerebellar neurons. The

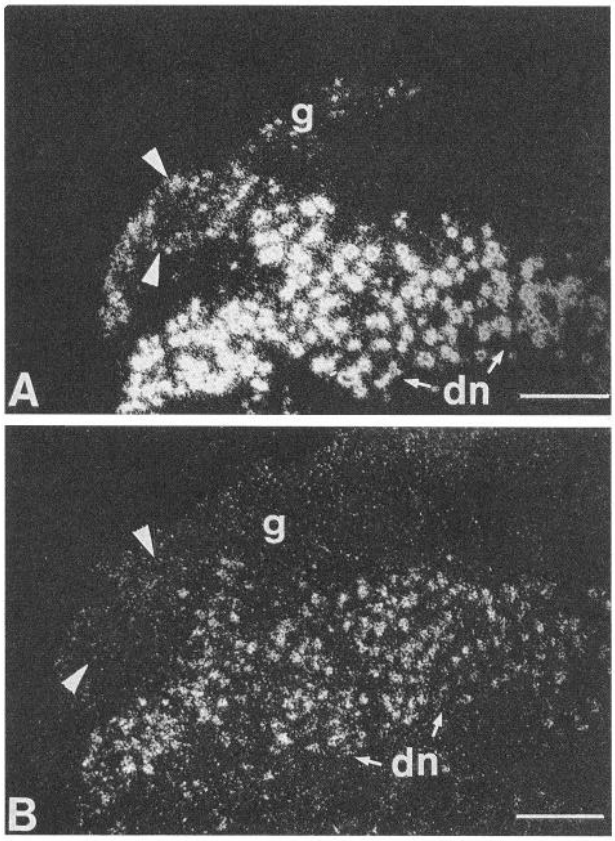

Figure 4. The expression of α_1 (*A*) and β_2 (*B*) subunit mRNAs in Purkinje cells in adjacent sections through the staggerer cerebellar hemisphere at P30. *Arrowheads* indicate labeled (*A*) or unlabeled (*B*) Purkinje cells; *g*, granule cell layer; *dn*, deep nuclei. Scale bars, 250 μm.

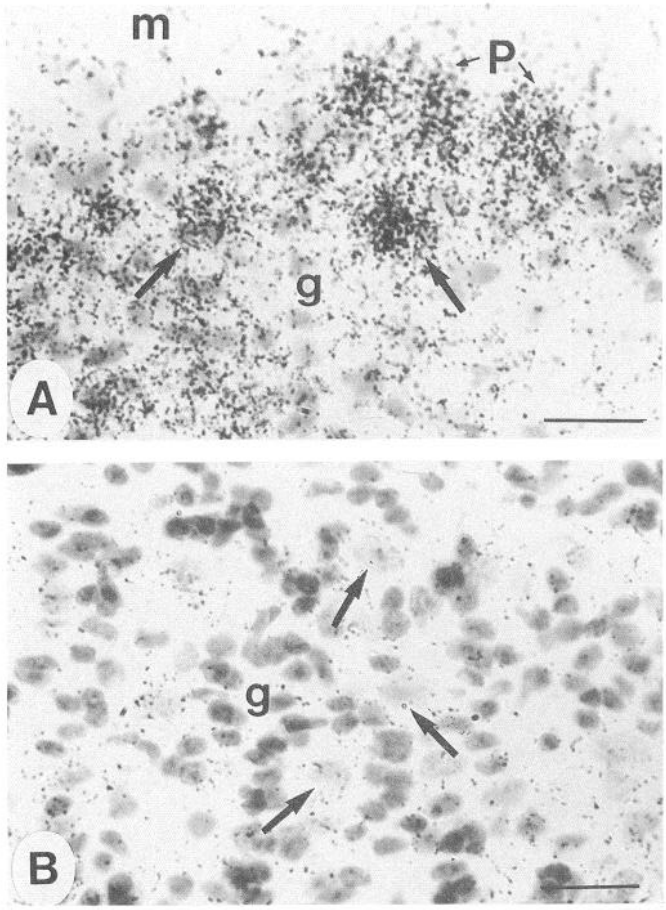

Figure 5. High power photomicrographs showing high levels of α_1 subunit mRNA (*A*) and little or no γ2 hybridization signal (*B*) in the staggerer cerebellar hemisphere. *P* indicates three Purkinje cells in the normal position at the interface of the granule cell and molecular layers. *Arrows* indicate Purkinje cells ectopically located in the granule cell layer. *g*, Granule cell layer; *m*, molecular layer. Scale bars, 50 μm.

majority of staggerer Purkinje cells are ectopically located in the granule cell layer and are fewer in number and smaller in size than those of unaffected littermates, making it difficult to differentiate them from Golgi II neurons which are also scattered throughout this region. Therefore, both cell types have been grouped together as "medium to large neurons" (Herrup and Mullen, 1979b). Although there is mediolateral variation in the degree of the cellular disorder, all medium to large cells, regardless of their mediolateral and rostrocaudal position in the staggerer cerebellar cortex, showed a reduction or absence, of β_2 and γ_2 mRNA while retaining the α_1 signal. While the presence and identity of specific mRNAs in normal Golgi II cells has not been ascertained, the lack of punctate α_1, β_2, and γ_2 signals in the granule cell layer of normal animals argues strongly that Golgi II cells do not express these particular subunit mRNAs at high levels. It seems most likely, therefore, that the α_1-labeled medium to large neurons which remain in the staggerer cerebellar cortex represent Purkinje cells, rather than Golgi II cells.

Whether the α_1 subunit mRNA retained in staggerer Purkinje cells is transcribed into the corresponding subunit polypeptide

Table 1. Optical density (OD) measurements of autoradiographic grain density over Purkinje cells in the hemispheres and vermis, and over deep nuclear cells, in the adult (P28–P80) staggerer (sg/sg) and control (?/+) cerebellum

	α_1 mRNA		β_2 mRNA		γ_2 mRNA	
Region	?/+	sg/sg	?/+	sg/sg	?/+	sg/sg
P Cells (vermis)	820 ± 160	807 ± 42 (2%↓)	89 ± 15	11 ± 3 (88%↓)	421 ± 43	28 ± 5 (93%↓)
P Cells (hemis)	880 ± 140	830 ± 45 (6%↓)	98 ± 17	9 ± 2 (91%↓)	480 ± 39	38 ± 6 (92%↓)
dcn (area)	1300 ± 214	1380 ± 231 (6%↑)	130 ± 23	320 ± 29 (146%↑)	420 ± 52	730 ± 64 (74%↑)
dcn (cells)	719 ± 137	713 ± 71 (1%↓)	117 ± 7	161 ± 14 (38%↑)	274 ± 27	309 ± 36 (13%↑)

Data are expressed as optical density units (mean ± SEM; % change from control) per 2000 μm² (Purkinje cells) and 1800 μm² (deep nuclear cells) areas. Values for "dcn (area)" were obtained by measuring the average OD within the cross-sectional area of the dcn throughout the rostrocaudal extent of the nucleus. N = 5; 5–6 sections for each animal; approximately 50–150 measurements per section (Purkinje cells), and 200 measurements per section (deep cerebellar cells; left and right nuclei).

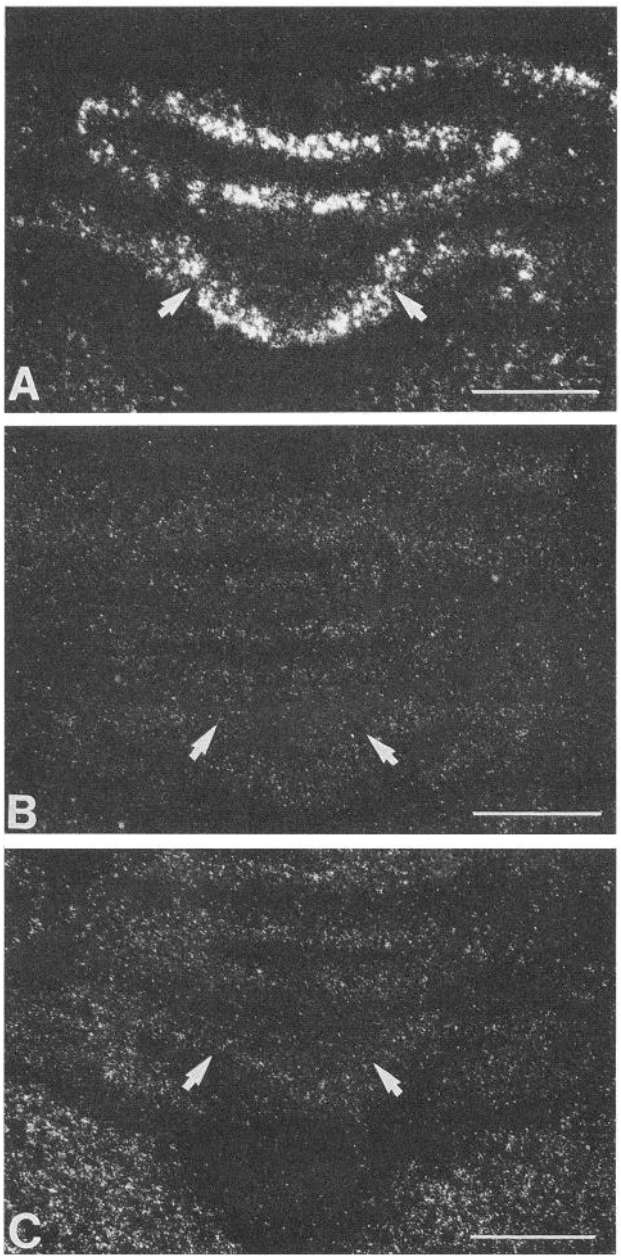

Figure 6. The expression of α_1 (*A*), β_2 (*B*), and γ_2 (*C*) subunit mRNAs in Purkinje cells in adjacent sections through the staggerer caudal vermis (a region approximately equivalent to lobule 9) at P60. *Arrows* indicate Purkinje cells. Scale bars, 200 μm.

is, as yet, unknown. In earlier studies, a considerable reduction in ^{3}H-flunitrazepam (Rotter and Frostholm, 1988) and ^{3}H-muscimol (Rotter et al., 1988) binding sites was observed in the staggerer cerebellar cortex, indicating the absence of a functional $GABA_A$/BZ receptor complex. Although the lack of β_2 subunit mRNA, and presumably the corresponding polypeptide, accounts for the loss of GABA agonist binding, the presence of normal α_1 mRNA levels together with a large decrease in ^{3}H-flunitrazepam binding are more difficult to reconcile. At least two mechanisms may account for the latter findings: it has been suggested that unassembled subunits may be degraded rapidly within the cell, whereas assembled subunits may be more stable (Nadler et al., 1994). If this were the case, α_1 subunit polypeptides in staggerer Purkinje cells may fail to form a receptor complex and subsequently become degraded. Alternatively, in the event that α_1 subunit polypeptides carrying the flunitrazepam binding site are capable of forming a homo-oligomeric receptor complex, the absence of the γ_2 subunit would prohibit such a receptor from binding benzodiazepine ligands with high affinity.

A variety of studies support the linkage of the α_1, β_2, and γ_2 subunits in the native Purkinje cell $GABA_A$ receptor. The three subunit genes map closely together on human chromosome 5 (Buckle et al., 1989; Wilcox et al., 1992; Russek and Farb, 1994); other $GABA_A$ receptor subunit genes also colocalize in clusters which are widely distributed throughout the human and murine genomes (Cutting et al., 1992; Wilcox et al., 1992; Danciger et al., 1993; Nakatsu et al., 1993; Hicks et al., 1994). Thus, the α_1, β_2, and γ_2 syntenic region is likely to exist in the mouse, in which the α_1 (Keir et al., 1991; Buckwalter et al., 1992) and γ_2 (Buckwalter et al., 1992) subunits have been localized on chromosome 11. Although the precise subunit composition is unknown, it has been proposed that the native $GABA_A$ receptor has a pentameric structure (Nayeem et al., 1994). Bachus et al. (1993) have suggested that a recombinant receptor consisting of α, β, and γ subunits has the stoichiometry of 2α/1β/2γ. Furthermore, Khan et al. (1994) have demonstrated that, in the cerebellum, both long and short forms of the γ_2 subunit are coimmunoprecipitated within single receptor complexes. Each of the α_1, $\beta_{2/3}$, γ_{2L}, and γ_{2S} variants is present in adult Purkinje cells (Fritschy et al., 1992, 1994; Gutierrez et al., 1994). Together, these studies make it highly plausible that in the adult Purkinje cell $GABA_A$/BZ receptor has the composition of $\alpha_1/\alpha_1/\beta_2/\gamma_{2L}/\gamma_{2S}$. The close proximity of the α_1, β_2, and γ_2 subunit genes on a particular chromosome, and the similar spatial and temporal developmental profiles of their mRNAs (Luntz-Leybman et al., 1993; Zdilar et al., 1991, 1992) suggests that there may be a common gene cassette involving the α_1, β_2, and γ_2 genes, similar in concept to that proposed by Jan and Jan (1993) for *Drosophila* homeotic genes. The concept of a functional gene cassette is used to predict that if one member of the cassette is expressed in a group of cells at a particular developmental stage, it is highly likely that other members also may be present in those cells at the same developmental period. The existence of such a functional gene cassette would imply that the expression of the α_1, β_2, and γ_2 genes are linked both temporally and spatially. However, although the three gene products are coordinately expressed within the cerebellum, acute and chronic ethanol exposure has been shown to result in differential changes in levels of $GABA_A$ receptor α_1, β_2, and γ_2 subunit mRNAs, polypeptides and agonist binding sites in cerebellar neurons (Mhatre and Ticku, 1992; Morrow et al., 1992; Mhatre et al., 1993; Wu et al., 1995). There is, in addition, evidence which indicates that GABA itself can influence the expression of individual subunit genes (Hansen et al., 1991; Montpied et al., 1991; Kim et al., 1993; Mhatre and Ticku, 1994). Thus, although the three subunit mRNAs are coordinately expressed in normal Purkinje cells, the level of expression of each message may be independently regulated by extracellular signals.

The question arises whether the defect in β_2 and γ_2 expression is a direct result of the staggerer mutation acting intrinsically on the Purkinje cell's developmental program, or whether it is secondary to altered Purkinje cell interactions with other neurons. This intrinsic defect in staggerer Purkinje cells is unlikely to be due to β_2 and γ_2 gene deletions, since the two mRNAs are expressed together with the α_1 mRNA in other cerebellar regions.

Table 2. Optical density measurements of autoradiographic grains over cerebellar Purkinje cells in the vermis of developing staggerer (sg/sg) and control (?/+) mice

	α_1 mRNA		β_2 mRNA		γ_2 mRNA	
Age	?/+	sg/sg	?/+	sg/sg	?/+	sg/sg
P1	153 ± 87	168 ± 84 (10%↑)	40 ± 10	35 ± 20 (13%↓)	227 ± 57	234 ± 75 (3%↑)
P6	142 ± 74	165 ± 83 (16%↑)	63 ± 15	70 ± 18 (11%↑)	241 ± 69	235 ± 79 (2%↓)
P9	253 ± 93	255 ± 92 (1%↑)	72 ± 24	76 ± 26 (6%)↑)	250 ± 80	232 ± 70 (7%↓)
P12	943 ± 239	736 ± 282 (22%↓)	136 ± 28	106 ± 25 (22%↓)	405 ± 127	274 ± 97 (32%↓)
P15	896 ± 236	789 ± 246 (12%↓)	117 ± 24	34 ± 12 (71%↓)	456 ± 109	210 ± 68 (54%↓)
P20	1302 ± 310	1120 ± 306 (14%↓)	116 ± 26	28 ± 11 (76%↓)	466 ± 91	77 ± 25 (83%↓)

Data from two sg/sg and two ?/+ controls are pooled at each age; 4–5 sections for each animal; approximately 35–100 measurements per section. Data are expressed as optical density units per 2000 μm² area (mean ± SD; % change from control).

Deep cerebellar neurons have a developmental timecourse and migratory pathway similar to that of Purkinje cells (Yuasa et al., 1991). Although these cells have been shown to be similar in number in staggerer and control animals (Roffler-Tarlov and Herrup, 1981), measurement of grain density over the entire cross-sectional area of the nucleus indicated that β_2 and γ_2 expression was much higher in staggerer than in controls. Since measurements over individual cells gave much lower estimates of these differences, these apparently higher levels of β_2 and γ_2 mRNA expression may be explained by closer cell proximity resulting from deafferentation-induced dendritic shrinkage. The smaller residual increases in β_2 and γ_2 mRNA expression in adult staggerer deep cerebellar cells are unlikely to be intrinsic to the cell, but rather, may indicate a slightly increased synthesis of the two subunits in response to the partial loss of Purkinje cell input. It is possible that exposure to GABAergic afferents during an early developmental period may be crucial for normal $GABA_A$ receptor subunit expression in the target cell, since β_2 and γ_2 mRNAs were not observed to be increased over control levels in the deep cerebellar neurons of Purkinje cell degeneration mice (Gambarana et al., 1993), in which Purkinje cell loss occurs at a later time.

The loss of β_2 and γ_2 mRNAs in Purkinje cells of staggerer mutant mice during postnatal week two occurs at a time of rapid changes in their afferent and efferent connections. During the first two postnatal weeks, normal Purkinje cells undergo at least three major developmental events: they become aligned into a monolayer (Hendelman and Aggerwal, 1980) and receive excitatory synapses from granule cell parallel fibers (Larramendi, 1969; Landis and Sidman, 1978); in addition, the multiple climbing fiber innervation of Purkinje cells observed in neonates regresses to the one to one relationship observed in adult mice (Crepel et al., 1980). Each of these steps is abnormal in staggerer. It is tempting to speculate, therefore, that loss of synaptic input to the ectopically located staggerer Purkinje cell results in the downregulation of the two mRNAs. However, since the timing and number of basket cell, stellate cell and Purkinje cell collateral contacts with Purkinje cells appears to be relatively normal (Landis and Sidman, 1978), there is no loss of GABAergic innervation. Neither does the absence of excitatory parallel fibers appear to be involved in the downregulation of β_2 and γ_2 expression: mice homozygous for the "reeler" and "weaver" mutations are similar to staggerer in that the majority of Purkinje cells do not form a monolayer, do not receive parallel fiber input, and remain multiply innervated by climbing fibers into adulthood. The present results, and those of earlier studies (Frostholm et al., 1991; Luntz-Leybman et al., 1993), show that in each of these mutants, β_2 and γ_2 subunit mRNAs, in addition to the α_1 mRNA, continue to be expressed throughout development. Furthermore, the density of ^{3}H-flunitrazepam and ^{3}H-muscimol binding sites over dendritic arborizations in the two mutants is comparable to that of normal animals (Rotter and Frostholm, 1988; Rotter et al., 1988). Taken together, these observations indicate that the disruption in staggerer subunit gene expression is due to a defect intrinsic to the Purkinje cell, rather than to altered cell–cell interactions.

The lack of β_2 and γ_2 gene expression in adult staggerer Purkinje cells is by no means a unique defect. Although Purkinje cell GAD and calbindin mRNAs do not appear to be disrupted by the staggerer mutation (Frantz and Tobin, 1990), previous studies have revealed that the adult form of N-CAM is largely absent from staggerer Purkinje cells (Edelman and Chuong, 1982), as are the ganglioside, $G_{D1\alpha}$ (Furaya et al., 1994), voltage dependent calcium channels (Crepel et al., 1984), zebrin 1 (So-

Table 3. Optical density measurements of autoradiographic grains over individual cells in the medial, intermediate, and lateral regions of the deep cerebellar nuclei of developing staggerer (sg/sg) and control (?/+) mice

	α_1 mRNA		β_2 mRNA		γ_2 mRNA	
Age	?/+	sg/sg	?/+	sg/sg	?/+	sg/sg
P1	68 ± 23	59 ± 18 (13%↓)	96 ± 27	97 ± 30 (1%↑)	140 ± 37	131 ± 44 (6%↓)
P6	87 ± 27	95 ± 30 (9%↑)	110 ± 35	114 ± 31 (4%↑)	169 ± 46	157 ± 49 (7%↓)
P9	143 ± 51	138 ± 46 (4%↓)	127 ± 45	118 ± 46 (7%↓)	187 ± 53	181 ± 57 (3%↓)
P12	275 ± 87	284 ± 118 (3%↑)	135 ± 47	149 ± 53 (10%↑)	190 ± 61	197 ± 61 (4%↑)
P15	440 ± 145	436 ± 108 (1%↓)	178 ± 59	156 ± 51 (12%↓)	245 ± 79	227 ± 82 (7%↓)
P20	513 ± 143	445 ± 126 (13%↓)	213 ± 65	235 ± 64 (10%↑)	268 ± 96	270 ± 86 (1%↑)

Data from two sg/sg and two ?/+ controls are pooled at each age; 5–6 sections for each animal; approximately 100 measurements from each nucleus (left and right). Data are expressed as optical density units per 1800 μm² area (mean ± SD; % change from control).

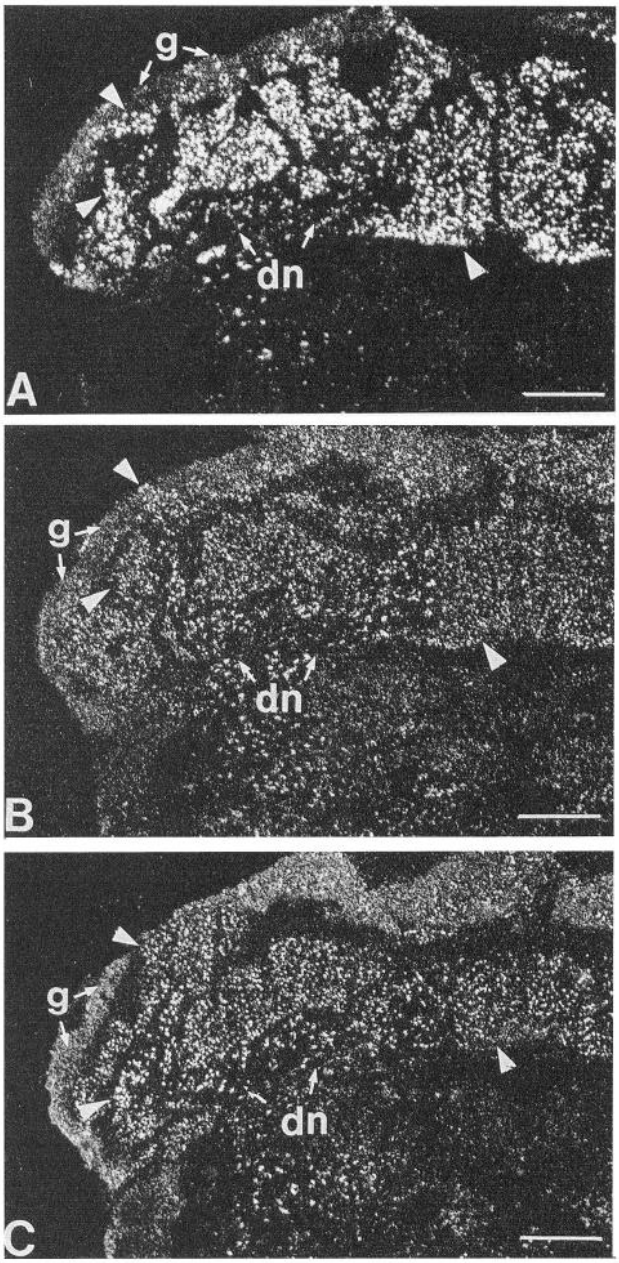

Figure 7. The expression of $GABA_A$ receptor α_1 (*A*), β_2 (*B*), and γ_2 (*C*) subunit mRNAs in Purkinje cells of the adult reeler mutant. Each of the three subunits are expressed at high levels throughout the cerebellar cortex. *Arrowheads* indicate Purkinje cells in the vermis and hemispheres; *dn*, deep cerebellar nuclei; *g*, granule cell layer. Scale bars, 500 μm.

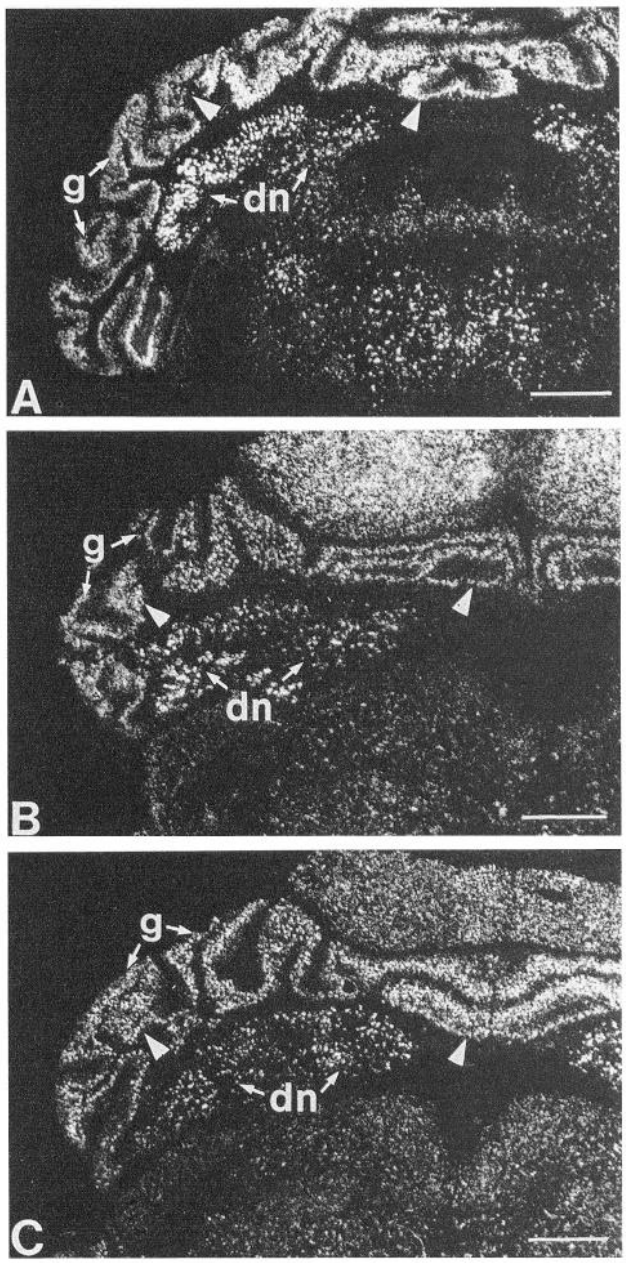

Figure 8. The expression of $GABA_A$ receptor α_1 (*A*), β_2 (*B*), and γ_2 (*C*) subunit mRNAs in Purkinje cells of the adult weaver mutant. Each of the three subunits are expressed at high levels throughout the cerebellar cortex. *Arrowheads* indicate Purkinje cells in the vermis and hemispheres; *dn*, deep cerebellar nuclei; *g*, granule cell layer. Scale bars, 500 μm.

telo and Wassef, 1991) calmodulin mRNA (Messer et al., 1990), thymidine kinase activity (Messer et al., 1981; Messer, 1988) and the P400 protein (Mikoshiba et al., 1985). Several of these earlier studies suggest that the intrinsic developmental program of sg/sg Purkinje cells is arrested prior to postnatal day 5 (Messer, 1988; Messer et al., 1988, 1990, 1991). In the present study, however, the expression of the three receptor subunit mRNAs diverges slightly later, during postnatal week 2. This indicates that certain aspects of the sg/sg Purkinje cell phenotype continue to change beyond postnatal day 5, and raises the possibility that the β_2 and $\gamma 2$ receptor subunit genes may be controlled by a mechanism different from that responsible for the developmental block which occurs at, or before, this time. Taken together, these studies suggest that the primary defect in the staggerer Purkinje cell involves a regulatory "cascade" of events which affect the expression of a number of molecules, including the $GABA_A$ receptor β_2 and γ_2 subunit gene products. One plausible explanation is that the product of the staggerer gene is a "master" transcription factor which controls the expression of other downstream transcriptional regulators which act at different developmental stages. Some of these regulators may be crucial for normal gene expression at, or before P5, while others may regulate gene activity at later stages. The pentameric composition

of the Purkinje cell $GABA_A$/BZ receptor adds a further degree of complexity, in that each subunit may be differentially regulated, rather than being controlled as a whole receptor complex. In the case of staggerer Purkinje cells, only the β_2 and γ_2 subunit genes are affected by the mutation, while the α_1 subunit gene is expressed normally.

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