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Mouse Cerebellar Granule Cell Differentiation: Electrical Activity Regulates the $GABA_A$ Receptor α6 Subunit Gene

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$GABA_A$ receptor α6 subunit gene expression marks cerebellar granule cell maturation. To study this process, we used the $\Delta\alpha$6lacZ mouse line, which has a lacZ reporter inserted into the α6 gene. At early stages of postnatal cerebellar development, α6-lacZ expression is mosaic; expression starts at postnatal day 5 in lobules 9 and 10, and α6-lacZ is switched on inside-out, appearing first in the deepest postmigratory granule cells. We looked for factors regulating this expression in cell culture. Membrane depolarization correlates inversely with α6-lacZ expression: granule cells grown in 25 mM $[K^+]_o$ for 11–15 d do not express the α6 gene, whereas cultures grown for the same period in 5 mM $[K^+]_o$ do. This is influenced by a critical early period: culturing for $\geq$3 d in 25 mM $[K^+]_o$ curtails the ability to induce the α6 gene on transfer to 5 mM $[K^+]_o$. If the cells start in 5 mM $[K^+]_o$, however, they still express the α6-lacZ gene in 25 mM $[K^+]_o$. In contrast to granule cells grown in 5 mM $[K^+]_o$, cells cultured in 25 mM $[K^+]_o$ exhibit no action potentials, mEPSCs, or mIPSCs. In chronic 5 mM $[K^+]_o$, factors may therefore be released that induce α6. Blockade of ionotropic and metabotropic GABA and glutamate receptors or L-, N-, and P/Q-type Ca^{2+} channels did not prevent α6-lacZ expression, but inhibition of action potentials with tetrodotoxin blocked expression in a subpopulation of cells.

Key words: $GABA_A$ receptor subunit; cerebellum; granule cell; β-galactosidase reporter genes; internal ribosome entry site; differentiation; cell culture; electrophysiology; transgenic mice; membrane depolarization; action potentials; tetrodotoxin; neuron-specific gene expression

At birth, rodent cerebellar granule cell precursors are found in germinative zones on the exterior surface of the cerebellum. During the first postnatal weeks, they divide, become postmitotic, and migrate across the molecular layer, finally settling in the internal granule cell layer (Altman and Bayer, 1996; Hatten et al., 1997). This maturation has distinct phases of neurotransmitter receptor expression (Farrant et al., 1994, 1995; Monyer et al., 1994; Mosbacher et al., 1994; Watanabe et al., 1994; Brickley et al., 1996; Takahashi et al., 1996; Tia et al., 1996; Wisden et al., 1996). For example, dividing precursor cells and premigratory postmitotic cells express transcripts encoding the $GABA_A$ receptor α2, α3, β3, γ1, and γ2 subunits (Laurie et al., 1992b). Later, α2, α3, and γ1 are downregulated and replaced by the adult complement (predominantly α1, α6, β2, β3, γ2, and δ) (Laurie et al., 1992a,b; Thompson and Stephenson, 1994; Caruncho et al., 1995; Gao and Fritschy, 1995; Nadler et al., 1996; Wisden et al., 1996). The α1, α6, and δ genes are expressed only when granule cells arrive in the internal granule cell layer (Zdilar et al., 1991; Laurie et al., 1992b; Korpi et al., 1993; Kuhar et al., 1993; Zheng et al., 1993; Varecka et al., 1994).

What factors determine the final stages of granule cell differentiation, e.g., $GABA_A$ receptor subunit gene induction? Granule cell entry into the internal granule cell layer coincides with their innervation by glutamatergic mossy fibers and GABAergic Golgi cell axons (Altman and Bayer, 1996). Synaptic activity, therefore, may modulate differentiation, especially because membrane potential influences neurotransmitter receptor regulation in culture (Vallano et al., 1996; Wisden et al., 1996; Gault and Siegel, 1997). Membrane potentials depend on the extracellular K^+ concentration ($[K^+]_o$). The physiological $[K^+]_o$ is ~5 mM, but rat granule cell survival *in vitro* is enhanced by depolarizing $[K^+]_o$, e.g., 25 mM (Gallo et al., 1987), so cerebellar cultures are often maintained under these conditions. The use of chronic 25 mM $[K^+]_o$ to model neuronal development, however, has been questioned. For example, in 25 mM $[K^+]_o$, rat granule cells do not correctly develop their AMPA or NMDA receptor subunit gene expression programs (Hack et al., 1995; Vallano et al., 1996), whereas they do so in lower $[K^+]_o$ (Condorelli et al., 1993; Vallano et al., 1996).

To study α6 gene regulation, we placed an *Escherichia coli* enzyme β-galactosidase (lacZ) reporter cassette into exon 8 of the mouse α6 subunit gene by homologous recombination (Jones et al., 1997). LacZ histochemistry allowed us to directly visualize the expression heterogeneity of the gene in cultured granule cells. We found that α6-lacZ gene expression fails to develop when dissociated mouse granule cells are cultured in chronic 25 mM $[K^+]_o$. By contrast, in 5 mM $[K^+]_o$, cells strongly induce the α6 gene. Induction is enhanced by action potentials but not by synaptic transmission.

MATERIALS AND METHODS

Cell culture

Homozygous $\Delta\alpha$6lacZ mice (strain C57BL/6x129S/v) (Jones et al., 1997) or C57BL/6x129S/v wild-type mice were killed at postnatal day (P) 5. The cerebellum was dissociated with trypsin, and the cells were maintained in culture (37°C, 5% CO_2) on Matrigel (Collaborative Research,

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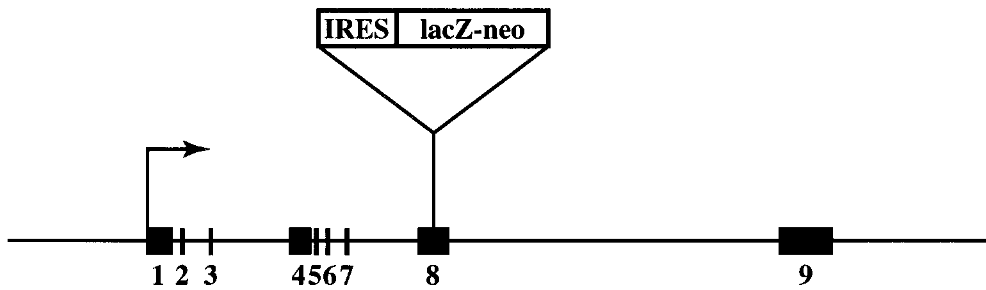

Figure 1. Structure of the Δα6lacZ mouse $GABA_A$ receptor α6 subunit gene (Jones et al., 1997). The IRES–lacZ cassette is inserted into exon 8. The *arrow* marks the transcriptional start sites; *shaded boxes* are exons; *IRES*, Internal ribosome entry site.

Bedford, MA)-coated glass coverslips (Randall and Tsien, 1995). The culture medium consisted of a minimal essential medium (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 5 mg/ml glucose, 0.3 mg/ml glutamine, 100 μg/l transferrin (Calbiochem, Nottingham, UK), and 50 mg/l insulin (Sigma, Poole, UK). As appropriate, the media was further supplemented with 4, 10, or 20 mM KCl to give a final media $[K^+]_o$ of 9, 15, or 25 mM, respectively. After 2 d in culture, all cells were fed with medium supplemented with 4 μM cytosine arabinoside (ARA-C) (Sigma) to reduce glial cell proliferation. Subsequently, cultures were fed every 5 d by a 50% replacement of ARA-C-containing medium.

Drugs and growth factors

Where appropriate, drugs and growth factors were included in the culture media from the time of cell plating: 200 ng/ml brain-derived neurotrophic factor (BDNF) (TCS Biologicals, Botolph Clayton, UK), 100 ng/ml neurotrophin-3 NT-3 (TCS Biologicals), 100 ng/ml nerve growth factor (NGF) (gift of D. Mercanti, Consiglio Nazionale delle Ricerche, Rome, Italy), and 10 ng/ml thyroid hormone (T3) (gift of P. Tirassa, Consiglio Nazionale delle Ricerche) were replenished every day; 1 μM tetrodotoxin (TTX) (RBI, Natick, MA), 10 μM CNQX (Tocris-Cookson, Bristol, UK), 10 μM (carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Tocris-Cookson), 50 μM picrotoxin (Sigma), 500 μM α-methyl-4-carboxyphenylglycine (α-MCPG) (Tocris-Cookson), 10 μM CGP 55 845A (gift of Ciba, Basel, Switzerland) (Davies et al., 1993), 300 nM ω-Aga-IVA (Peptides International, Louisville, KY), 1 μM ω-CTx-GVIA (Peninsula Laboratories, Belmont, CA), 10 μM nifedipine (RBI), and 50 nM K252a (TCS Biologicals) were replenished by the regular cell-feeding process.

β-galactosidase (lacZ) staining

Brain slices. Timed matings of homozygous Δα6lacZ mice were set up. Four pups were used at each time point. Anesthetized animals were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed for 15 min in 4% PFA on ice, and then equilibrated overnight at 4°C in PBS/30% sucrose. Sections (40 μM) were cut on a sliding microtome and incubated free-floating in 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) at 37°C (Bonnerot and Nicolas, 1993). After X-Gal staining, sections were post-fixed in ice-cold 4% PFA for 15 min, rinsed in 100 mM phosphate buffer (PB), pH 7.4, mounted on slides, counterstained with neutral red (Sigma), coverslipped with DePeX (BDH), and photographed with a Leica Orthomat E microscope. Cerebellar anatomy was confirmed from Marani and Voogd (1979) and Altman and Bayer (1996).

Cell cultures. Coverslips were washed in PBS and fixed for 5 min in ice-cold 2% PFA/0.2% glutaraldehyde and then rinsed in PBS and incubated in X-Gal solution at 37°C overnight, although granule cells usually turned blue within 2 hr. After lacZ staining, coverslips were washed in PBS, post-fixed for 10 min in 2% PFA, rinsed in PB, counterstained with neutral red, and mounted in DePeX. Granule cells were identified by their small size and round or ovoid shape.

Controls for lacZ background staining. To check for the presence of endogenous β-galactosidase-like activity (Rosenberg et al., 1992), cultured wild-type C57BL/6x129S/v granule cells from both 5 and 25 mM $[K^+]_o$ conditions were incubated, after 14 d in culture, with X-gal. No blue staining was found in either cell culture condition or in wild-type mouse brain slices of any age (data not shown).

Immunoblotting

Membranes were prepared from cultured cerebellar cells and whole cerebella as described (Thompson and Stephenson, 1994; Jones et al., 1997). Ten micrograms of protein/lane were run in an SDS-PAGE (12% polyacrylamide) gel and immunoblotted. Immunodetection was by chemiluminescence, using a Western-Lite protein detection kit (Tropix). The α6 subunit-specific antibody α6-N (Thompson et al., 1992) was used at 2 μg/ml.

Electrophysiology

Before recording, the culture medium was exchanged for a standard Tyrode solution (in mM): NaCl 130, KCl 5 or 25, $CaCl_2$ 2, $MgCl_2$ 1, Glucose 30, HEPES-NaOH 25, pH 7.3. Coverslips were broken into pieces, and single shards were transferred to a recording chamber mounted on an inverted microscope stage (Nikon, Kingston-upon-Thames, UK). The chamber was constantly perfused with Tyrode solution at room temperature. Individual cerebellar granule cells were approached with 2–6 MΩ resistance glass pipettes, and whole-cell patch-clamp recordings were made in voltage- or current-clamp mode.

Recordings of spontaneous GABAergic miniature IPSCs (mIPSCs) were made at −70 mV in the presence of the AMPA/kainate receptor antagonist CNQX (5 μM) and the Na^+ channel blocker TTX (1 μM). The intracellular solution consisted of (in mM): CsCl 110, NaCl 10, $MgCl_2$ 5, EGTA 5, ATP 2, GTP 0.2, HEPES 35, pH 7.3. Data were filtered at 50 kHz (four-pole analog Bessel filter), recorded to digital audiotape, refiltered at 5 kHz (Brownlee Precision digital eight-pole Bessel characteristic filter; San Jose, CA), and sampled at 10 kHz to a continuous computer file. Recordings of miniature EPSCs (mEPSCs) were made at a holding potential of −70 mV in the presence of the $GABA_A$ receptor antagonist bicuculline methochloride (50 μM) and TTX (1 μM).

For analysis of membrane potentials and action potentials, the intracellular solution was (in mM): $KMeSO_4$ 110, NaCl 10, $MgCl_2$ 5, EGTA 0.6, ATP 2, GTP 0.2, HEPES 40, pH 7.3. Recordings were filtered at 2 kHz and sampled at 5–20 kHz under control of pClamp6 software (Axon Instruments, Foster City, CA). Membrane potentials were measured in the current-clamp mode within 30 sec of reaching the whole-cell configuration and corrected for liquid junction potentials (10.4 mV in 25 mM K^+, 8.8 mV in 5 mM K^+). Na^+ currents were elicited with step depolarizations to 0 mV from a holding potential of −70 mV. At this holding potential there is <0.1% Na^+ channel inactivation (data not shown). Data files were analyzed with either Axobasic- or pClamp6-based programs (Axon Instruments).

Statistical analysis

Results are presented as mean ± 1 SEM. Statistical analysis was performed using a standard two-tailed unpaired *t* test; significance was set at a confidence level of 95%. For each experimental manipulation in each individual culture, the percentage of blue granule cells was determined by manual counting of two or three randomly selected fields on two separate coverslips. The effect of each culture condition was assayed in cultures derived from at least three different litters.

RESULTS

α6-lacZ gene expression has a complex sequence of regional development correlating with cell birthday

In the Δα6lacZ mouse line, an internal ribosome entry site (IRES) lacZ cassette has been inserted into exon 8 of the $GABA_A$ receptor α6 subunit gene by homologous recombination (Fig. 1) (Jones et al., 1997). This allows cap-independent translation of β-galactosidase (β-gal) from within the α6 mRNA, and the production of β-gal in the same pattern as native α6 gene expression

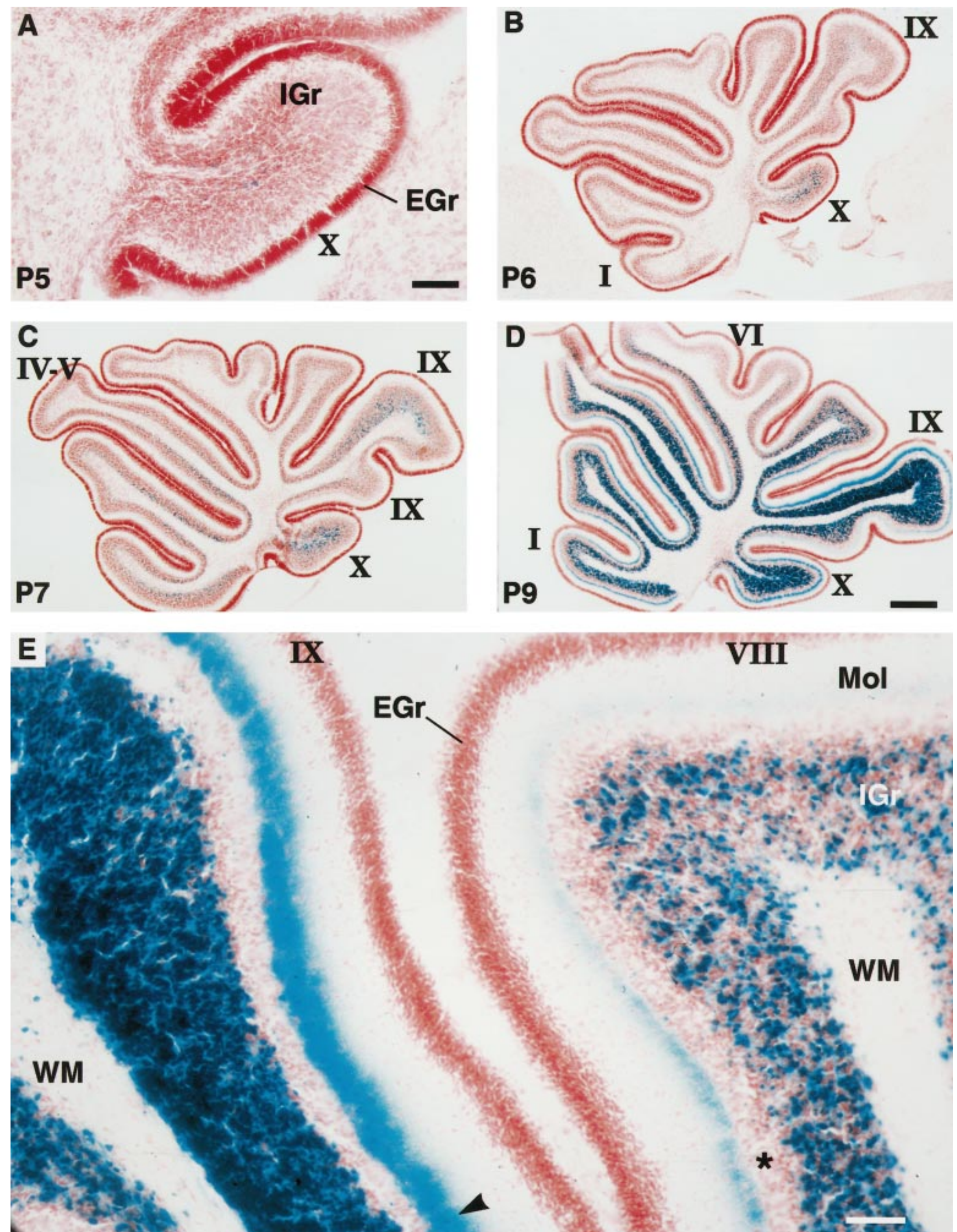

Figure 2. Sagittal sections of developing $\Delta\alpha6$lacZ mouse cerebella taken through the rostrocaudal midline, and stained for lacZ activity (*blue*). The rostral direction is on the left-hand side. *A*, Lobule X, postnatal day 5 (*P5*). *B–D*, Sections from postnatal day 6 (*P6*) to P9. *E*, P9 sagittal slice with neighboring cerebellar granule cell layers, loops of lobules VIII (*right*) and IX (*left*), at differing stages of $\alpha6$–lacZ gene expression; nonexpressing cells are *red*. *EGr*, External granule cell layer; *IGr*, internal granule cell layer; *Mol*, molecular layer; *WM*, white matter. *Roman numerals* identify the vermis lobules; *arrowhead* marks β-gal enzyme transported into granule cell axons, the parallel fibers, in the molecular layer. *Asterisk* marks postmigratory granule cells in the upper internal granule cell layer not yet expressing the $\alpha6$–lacZ gene. Scale bars: *A*, 80 μm; *D*, 0.3 mm; *E*, 40 μm.

(Kato, 1990; Lüddens et al., 1990; Laurie et al., 1992a; Wisden et al., 1992; Varecka et al., 1994).

The developing $\alpha6$ subunit–lacZ gene expression pattern *in vivo* was traced in sagittal sections along the rostrocaudal midline of the vermis. Expression started in the most posterior part, lobule X, in agreement with the *in situ* hybridization study of Varecka et al. (1994). At P5, a few blue cells were found in the deepest part of the internal granule cell layer of lobule X (Fig. 2*A*). At this time point, other lobules were not detectably expressing β-gal. One day later (P6), more cells were expressing in the deep layers of lobule X, and scattered lacZ-positive cells were found in the dorsal aspect of lobule IX (Fig. 2*B*). By P7, expression in lobules X and IX had increased but was still confined to the deeper layers (Fig. 2*C*). At this stage, expression began to appear in the sulci (stems) of the anterior lobules I–V (Fig. 2*C*). As with lobules IX and X, these $\alpha6$ gene-expressing cells in lobules I–V were in the deeper part of the internal layer closest to the white matter tracts. Kuhar et al. (1993) obtained a similar result by *in situ* hybridization.

By P9, $\alpha6$ expression was established in all lobules except the gyri (outer folds) of VI and VII (Fig. 2*D*). In lobules X and IX, I–V, where expression first started to appear (Fig. 2*B*), β-gal activity became increasingly apparent in the internal part of the molecular layer. This presumably corresponded to transport of β-gal into the granule cell axons, the parallel fibers (Fig. 2*D,E*, *arrowhead*). By P10–11, the entire internal granule cell layer in all lobules was lacZ-positive (data not shown); over the following week, as more granule cells migrated into the internal layer and switched on the gene, the intensity of expression continued to increase (data not shown).

The contrast in $\alpha6$ subunit gene expression between developing vermis lobules is shown in detail in Figure 2*E*. At P9,

nearly all the granule cells in the internal layer of lobule IX were lacZ positive; in the internal layer of VIII, however, expression was mosaic, with many cells still not expressing lacZ (Fig. 2*E*). At this age, the heterogeneity of α6-expressing cells compares well with the results of a single-cell PCR study on juvenile rat cerebellar slices, in which α1 positive/α6 negative, α1 negative/α6 positive, and α1 positive/α6 positive cells were found (Santi et al., 1994b).

Expression of the α6 subunit gene in culture depends on $[K^+]_o$

After migration, granule cells extend dendrites that become innervated by glutamatergic mossy fibers (arriving from outside the cerebellum) and GABAergic axons of the Golgi cells (for review, see Brickley et al., 1996; Wall and Usowicz, 1997); α6 gene expression could depend on these inputs. In the following sections, we examine α6 gene expression in primary cultures of dissociated cerebellum, where the extracellular environment can be precisely controlled. Previous studies have shown that the K^+ concentration in the extracellular media influences neurotransmitter receptor expression of rat cerebellar granule cells (Cox et al., 1990; Bessho et al., 1994; Harris et al., 1994; Santi et al., 1994a; Vallano et al., 1996; Gault and Siegel, 1997); we therefore decided to look first at the effects of $[K^+]_o$ on α6 gene expression in cultured mouse granule cells.

Before we describe our experiments, it is first helpful to review the effects of extracellular K^+ concentrations in a broader context, because there seem to be species-specific differences in granule cell physiology. It is well known that elevated extracellular K^+ concentrations (e.g., 25 mM $[K^+]_o$) promote long-term survival of rat cerebellar granule cells in dissociated cultures (Gallo et al., 1987), but although chronic depolarization gives *maximal* survival, it is not essential for experiments that require long-term culture. If rodent granule cells cultured in physiological K^+ (i.e., 5 mM $[K^+]_o$) are supplemented with insulin (as in this study) or IGF-1, they also survive well (D'Mello et al., 1993; Randall and Tsien, 1995; Lin and Bulleit, 1997). Growth factors and serum also prevent apoptosis when rat cells that have been grown in 25 mM $[K^+]_o$ are switched to 5 mM $[K^+]_o$ medium (D'Mello et al., 1993). However, there could be species differences: in some conditions, mouse cells survive just as well in 5 mM $[K^+]_o$ as in 25 mM $[K^+]_o$ (Peng et al., 1991; Mogensen et al., 1994; Mogensen and Jorgensen, 1996).

We compared α6-lacZ gene expression in cultures of P5 Δα6lacZ cerebellar granule cells grown for 15 d *in vitro* (DIV) in 5, 9, 15, and 25 mM $[K^+]_o$. In 2-week-old 25 mM $[K^+]_o$ granule cell cultures, few cells stained positive for lacZ expression (Fig. 3*D*). The absence of lacZ product in 25 mM $[K^+]_o$ cultures of granule cells was independent of serum batch or type (not shown); the situation was not altered by increasing the culture period. In contrast, substantial increases in the number of lacZ-positive cells were seen if the cells were cultured in lower $[K^+]_o$ (Figs. 3*A–D*, 4*A*). Heavy blue staining was present in granule cells cultured in 5 mM $[K^+]_o$ (an average of 79 ± 3% lacZ-positive granule cells) (Figs. 3*A*, 4*A*) or 9 mM $[K^+]_o$ (82 ± 2% lacZ-positive granule cells) (Figs. 3*B*, 4*A*). The blue fibers between cell clusters indicate β-gal protein transported into the granule cell axons (Fig. 3*A,B*; compare Fig. 2*E*, *arrowhead*). Of the granule cells grown in 15 mM $[K^+]_o$ (Figs. 3*C*, 4*A*), 44 ± 5% stained positive for lacZ, a situation intermediate to that in 5 or 9 mM $[K^+]_o$ cultures and that in 25 mM $[K^+]_o$ cultures in which <1% of cells were lacZ positive (Figs. 3*D*, 4*A*). When a logistic function was fitted to the data in Figure 4*A*, the IC_{50} for $[K^+]_o$-induced suppression of α6 gene expression was 15.3 mM. This suggests that membrane potential strongly influences α6 subunit gene regulation. The $[K^+]_o$ probably influences many other aspects of gene expression in the cultured granule cells, as can be seen by the differences in clustering of the cells shown in Figure 3*A–D* (also see Peng et al., 1991).

The fidelity of α6–lacZ expression as a measure of genuine α6 expression was confirmed by immunoblotting. P5 *wild-type* cerebellar granule cells (i.e., cells with an α6 gene producing intact α6 protein) were cultured in 5 and 25 mM $[K^+]_o$ for 10 d. Cell membranes were then harvested, run in an SDS-PAGE gel, blotted, and probed with an α6-specific antibody (α6-N) (Thompson et al., 1992) (Fig. 3*I*). Only cells cultured in 5 mM $[K^+]_o$ contained detectable α6-like immunoreactivity; a doublet was present in membranes from cell cultures in contrast to the single band (57 kDa) obtained from a whole cerebellar extract (positive control) (Fig. 3*I*). The doublet could be attributable to either differential glycosylation or degradation.

The time course of α6–lacZ gene expression in a typical culture maintained in 5 mM $[K^+]_o$ is shown in Figure 3*E–H*. Data from a number of similar experiments are plotted in Figure 4*B*. In these experiments, cells prepared from P5 Δα6lacZ cerebella were plated in 5 mM $[K^+]_o$ and stained for lacZ at 4 d intervals, starting at 3 DIV. After 3 DIV there were no lacZ-positive cells (Fig. 3*E*); after 7 DIV there was significant blue staining (an average of 37 ± 3% lacZ-positive cells) (Figs. 3*F*, 4*B*). An additional increase in the culture period to 11 and 15 d produced a steady increase in the number of lacZ-positive cells (61 ± 4% and 75 ± 2%, respectively) (Figs. 3*G,H*, 4*B*). Cultures that were maintained for longer periods in 5 mM $[K^+]_o$ exhibited no significant increase in the fraction of lacZ-positive cells (data not shown). A uniform development of lacZ expression in all cells would not be expected. During preparation of cerebellar cultures, cells from all of the different regions of the cerebellum are intermixed, and these cells are not homogeneous with respect to their development of α6 gene expression (Fig. 2).

Culture in 5 mM $[K^+]_o$ fails to induce α6 gene expression after a critical period in 25 mM $[K^+]_o$

Cultures of granule cells from P5 mouse cerebella were initiated and maintained in 25 mM $[K^+]_o$ medium for 3, 5, 7, 9, or 11 d and then switched to 5 mM $[K^+]_o$ medium until a total time in culture of 15 d had passed (i.e., 3 d in 25 mM $[K^+]_o$, then 12 d in 5 mM $[K^+]_o$; 5 d in 25 mM $[K^+]_o$, then 10 d in 5 mM $[K^+]_o$, etc.). The control culture was plated and grown in 5 mM $[K^+]_o$ for 15 DIV. Cultures were then stained for lacZ (Fig. 4*C*, *closed circles*). After 3 d in 25 mM $[K^+]_o$, a culture period that produces no lacZ-positive cells after initial plating in 5 mM $[K^+]_o$ (Fig. 4*B*), reversion to 5 mM $[K^+]_o$ media resulted in only 41 ± 4% lacZ-positive cells being present after 15 DIV (compared with 74 ± 2% in side-by-side 5 mM $[K^+]_o$ controls) (Fig. 4*B,C*). Greater depressions in the final fraction of lacZ-positive cells were seen when the cultures were switched to 5 mM $[K^+]_o$ after 5, 7, 9, or 11 d in 25 mM $[K^+]_o$ (Fig. 4*C*). Thus, the longer the cells are maintained in 25 mM $[K^+]_o$, the greater the loss in the capacity to induce the α6 gene on subsequent transfer to 5 mM $[K^+]_o$.

In reciprocal experiments, cells from P5 cerebella were maintained in 5 mM $[K^+]_o$ for the initial 3, 5, 7, 9, or 11 d before switching to 25 mM $[K^+]_o$, until a total of 15 d had

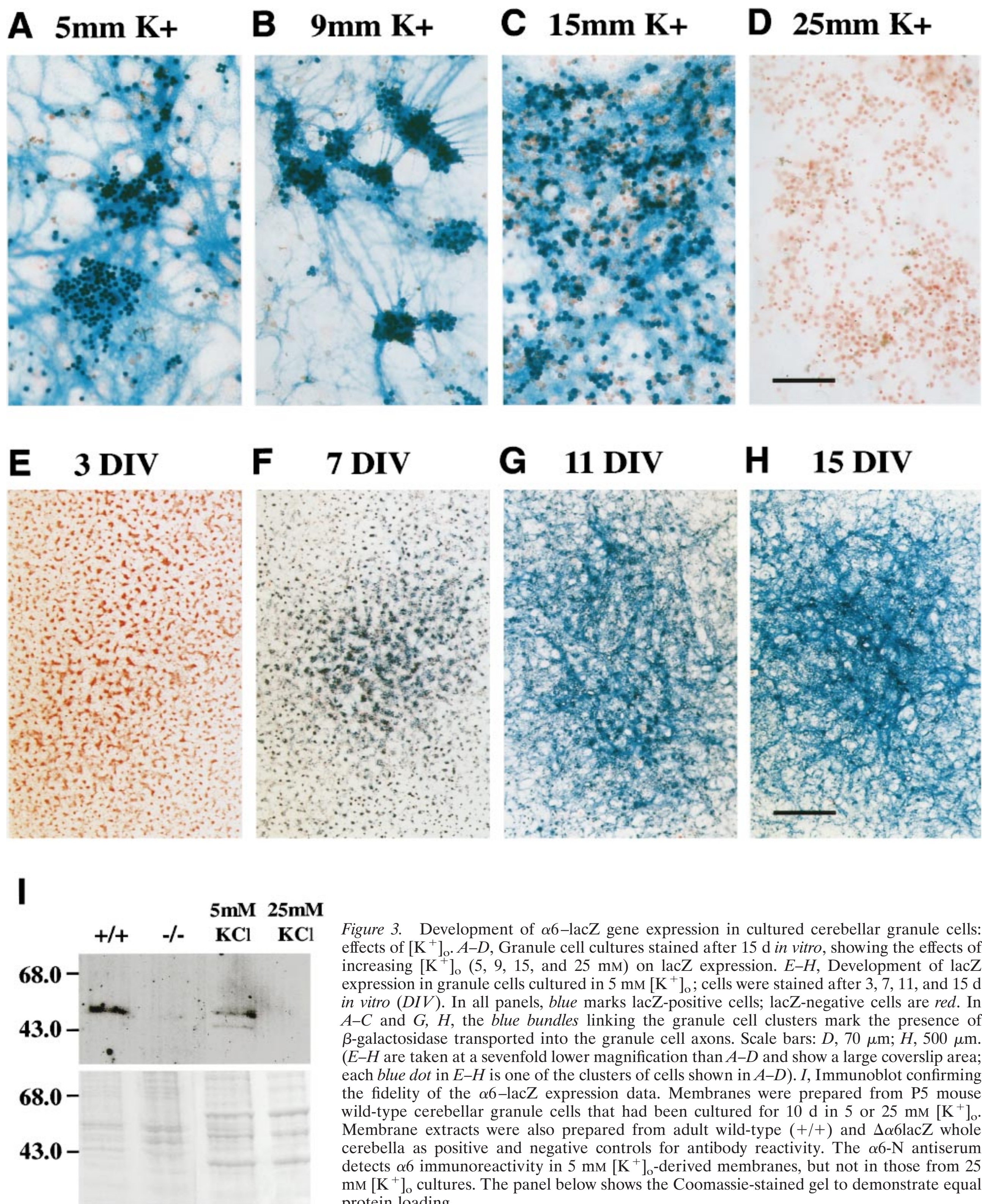

Figure 3. Development of α6–lacZ gene expression in cultured cerebellar granule cells: effects of $[K^+]_o$. *A–D*, Granule cell cultures stained after 15 d *in vitro*, showing the effects of increasing $[K^+]_o$ (5, 9, 15, and 25 mM) on lacZ expression. *E–H*, Development of lacZ expression in granule cells cultured in 5 mM $[K^+]_o$; cells were stained after 3, 7, 11, and 15 d *in vitro* (*DIV*). In all panels, *blue* marks lacZ-positive cells; lacZ-negative cells are *red*. In *A–C* and *G, H*, the *blue bundles* linking the granule cell clusters mark the presence of β-galactosidase transported into the granule cell axons. Scale bars: *D*, 70 μm; *H*, 500 μm. (*E–H* are taken at a sevenfold lower magnification than *A–D* and show a large coverslip area; each *blue dot* in *E–H* is one of the clusters of cells shown in *A–D*). *I*, Immunoblot confirming the fidelity of the α6–lacZ expression data. Membranes were prepared from P5 mouse wild-type cerebellar granule cells that had been cultured for 10 d in 5 or 25 mM $[K^+]_o$. Membrane extracts were also prepared from adult wild-type (+/+) and Δα6lacZ whole cerebella as positive and negative controls for antibody reactivity. The α6-N antiserum detects α6 immunoreactivity in 5 mM $[K^+]_o$-derived membranes, but not in those from 25 mM $[K^+]_o$ cultures. The panel below shows the Coomassie-stained gel to demonstrate equal protein loading.

passed (i.e., 3 d in 5 mM $[K^+]_o$, then 12 d in 25 mM $[K^+]_o$; 5 d in 5 mM $[K^+]_o$, then 10 d in 25 mM $[K^+]_o$, etc.). Cultures were then stained for lacZ (Fig. 4*C*, *open squares*). In three separate experiments, the longer the cells were initially cultured in 5 mM $[K^+]_o$, the greater the final number of cells that expressed lacZ after culture in 25 mM $[K^+]_o$. The time course mirrors the switching of experiments from 25 to 5 mM $[K^+]_o$: when switched after 3 d, the number of lacZ-positive cells is 30 ± 3%, after 5 d 50 ± 7%, after 7 d 70 ± 3%, after 9 d 72 ± 2%; a switch after 11 d of initial culture in 5 mM $[K^+]_o$ had no effect on the final percentage, which was 75 ± 2% (the same as cultures grown in 5 mM $[K^+]_o$ for 15 DIV) (Fig. 4*B*).

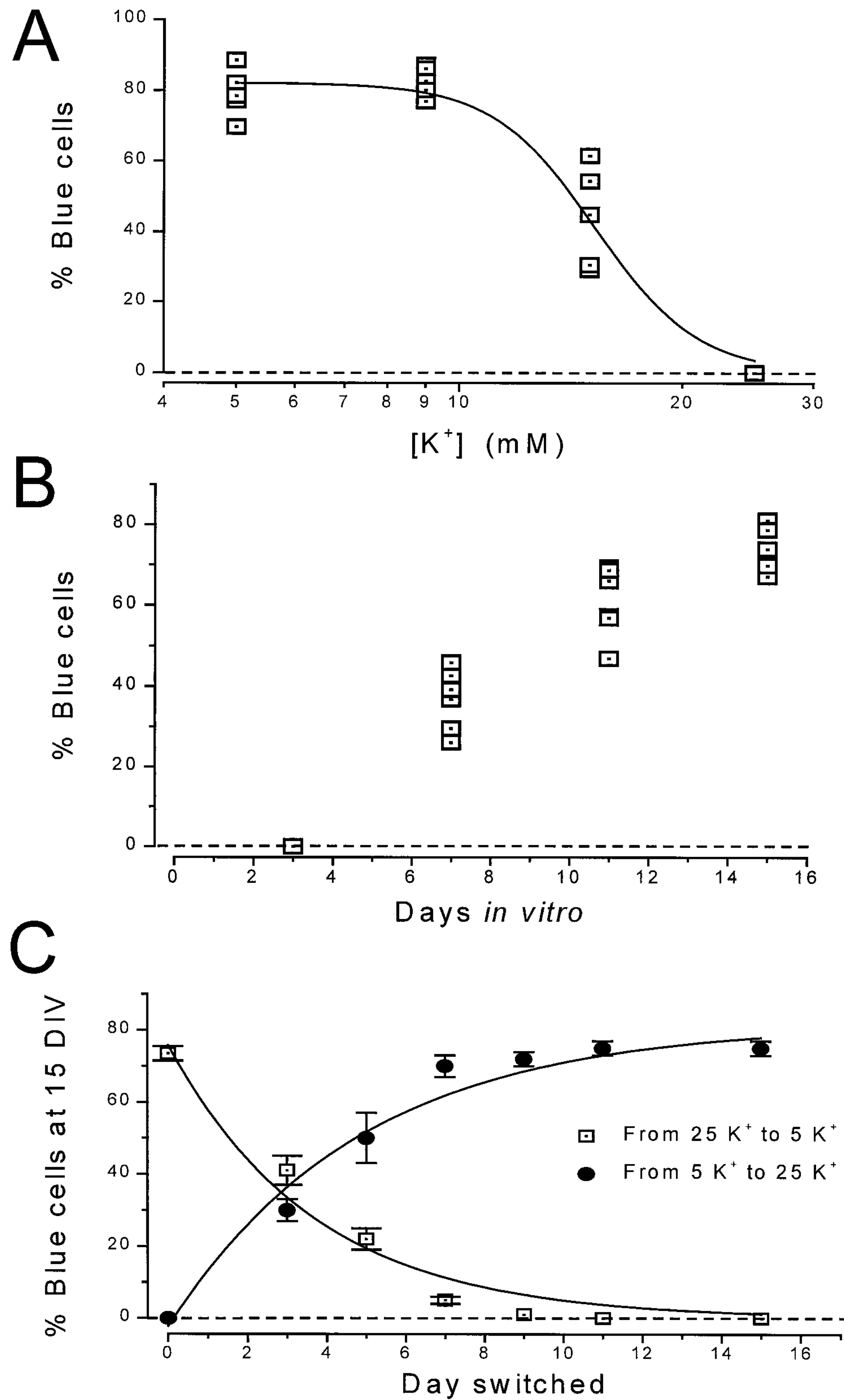

Figure 4. α6–lacZ gene expression: a summary of the effects of $[K^+]_o$ and time in culture. *Red* (negative) and *blue* (positive) cells were counted in randomly selected fields on each coverslip. *A*, The percentage of lacZ-positive cells when cultures were maintained for 15 d in media containing 5, 9, 15, or 25 mM $[K^+]_o$. Two coverslips in each of three separate cultures were analyzed. Each point corresponds to the percentage of blue cells on a single coverslip. The data are fitted with a standard logistic function (IC_{50}, 15.3 mM). *B*, Development of lacZ expression in cells cultured in 5 mM $[K^+]_o$ for up to 15 DIV. Each point represents a single coverslip. Two coverslips were examined in three separate cultures. *C*, The decline in the number of lacZ-positive cells seen as the time of switch from 25 to 5 mM $[K^+]_o$ medium was increasingly delayed (*open squares*). In all cases the number of lacZ-positive cells was assessed after 15 DIV. Each point represents the average of two coverslips from each of three cultures. Alternatively, when cells were switched from increasingly long incubations in 5 mM $[K^+]_o$ to 25 mM $[K^+]_o$ and cultured for a total of 15 DIV (*filled circles*), more lacZ-positive cells appeared the longer the initial culture period in 5 mM $[K^+]_o$.

A critical early period in culture therefore determines the ability of the cell to subsequently express the α6 subunit gene: periods of culture ≥3 d in 25 mM $[K^+]_o$ curtail the ability of cells to induce the α6 gene on transfer to 5 mM $[K^+]_o$. If the cells spend their initial culture time in 5 mM $[K^+]_o$, however, they can still express the α6-lacZ gene in 25 mM $[K^+]_o$.

Granule cells maintained in 25 mM $[K^+]_o$ are electrically silent

An increase in the $[K^+]_o$ produces a depression in α6 gene expression. Does this correlate with neural activity? We compared the electrophysiological properties of granule cells grown in 5 and 25 mM $[K^+]_o$. We analyzed (1) the resting membrane

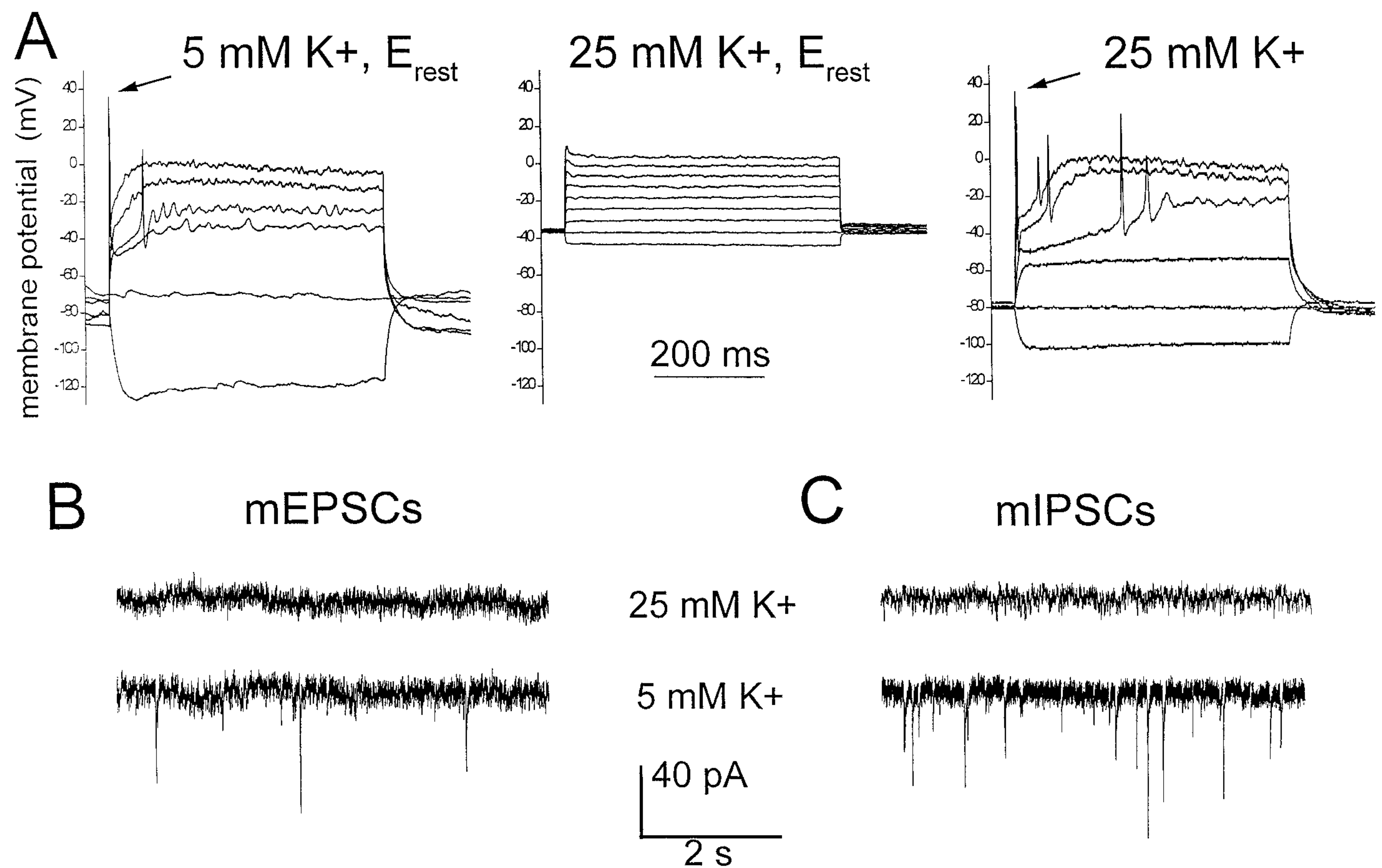

Figure 5. Effects of $[K^+]_o$ on membrane potential, action potential generation, and activity of synaptic inputs of cerebellar granule cells. *A*, Examples of granule cells recorded in current-clamp mode. The cell on the *left*, cultured in 5 mM $[K^+]_o$, is at its resting potential in an extracellular solution containing 5 mM $[K^+]_o$; the cell in the *center*, cultured in 25 mM $[K^+]_o$, is at its resting potential in a 25 mM $[K^+]_o$ extracellular solution; the cell on the *right* is similar to that in the center, but it has been hyperpolarized by current injection. The *traces* show the responses to a range of current injections (−5 to 35 pA); *arrows* mark the action potentials produced close to the start of the current injection pulse. *B*, Comparison of excitatory synaptic transmission (mEPSCs) between Δα6lacZ cultures maintained in 5 and 25 mM $[K^+]_o$. Example of current traces recorded in the whole-cell voltage-clamp at −70 mV. Δα6lacZ cells grown in 25 mM $[K^+]_o$ exhibited no spontaneous excitatory transmission. mEPSCs were isolated in TTX (1 μM) and bicuculline methochloride (50 μM). *C*, Comparison of inhibitory synaptic transmission (mIPSCs) between Δα6lacZ cultures maintained in 5 and 25 mM $[K^+]_o$. Example of current traces recorded in the whole-cell voltage-clamp at −70 mV. Δα6lacZ cells grown in 25 mM $[K^+]_o$ had no spontaneous miniature synaptic transmission in the presence of 10 μM CNQX and 1 μM TTX. In contrast, those cultured in 5 mM $[K^+]_o$ had frequent spontaneous synaptic events. These results were obtained with >10 separate culture preparations.

potential and the ability to fire action potentials in response to a depolarizing stimulus and (2) the presence of spontaneous glutamatergic and GABAergic synaptic transmission.

Resting potentials and excitability

The resting membrane potentials of Δα6lacZ cells cultured in 5 and 25 mM $[K^+]_o$ were compared (see Materials and Methods). The membrane potential of cells cultured and recorded in 25 mM $[K^+]_o$ was significantly more positive (−36 ± 1 mV; n = 11) than that in granule cells cultured and recorded in 5 mM $[K^+]_o$ (−50 ± 2 mV; n = 7). In contrast to 25 mM $[K^+]_o$ cells, a proportion of cells from 5 mM $[K^+]_o$ cultures showed spontaneous action potential firing at their resting potential (data not shown). Depolarizing stimuli (see Materials and Methods) applied to cells from 5 mM $[K^+]_o$ cultures (recorded in 5 mM $[K^+]_o$) consistently produced action potentials (Fig. 5*A, left, arrow*) within milliseconds of the start of the stimulus (n = 7). In contrast, current injection into cells from 25 mM $[K^+]_o$ cultures, at their resting potential in 25 mM $[K^+]_o$, produced no action potentials in any cell tested (n = 8) (Fig. 5*A*, *center*).

The mean amplitude of Na^+ currents recorded (under voltage-clamp) from granule cells cultured in 5 and 25 mM $[K^+]_o$ is not significantly different (250 ± 62 pA, 5 mM K^+ vs 257 ± 37 pA, 25 mM K^+), so downregulation of Na^+ channel expression is not responsible for the absence of spontaneous or stimulus-induced action potentials in cells chronically cultured in 25 mM $[K^+]_o$. In fact, action potential generation can be restored in 25 mM $[K^+]_o$ cultured cells simply by hyperpolarizing the resting membrane potential with a steady injection of negative current (Fig. 5*A, right*). Thus voltage-dependent inactivation of the Na^+ channel is responsible for the lack of action-potential firing in cells maintained in 25 mM $[K^+]_o$.

Spontaneous synaptic inputs

Reflecting spontaneous vesicular release, mEPSCs and mIPSCs arise independently of action potential firing. GABAergic interneurons are typically present in granule cell cultures, and 10 of 10 Δα6lacZ granule cells from 5 mM $[K^+]_o$ cultures had spontaneous mIPSCs (average frequency 6.2 ± 1.3 Hz) (Fig. 5*C*) (also see Martina et al., 1997). These events were reversibly blocked by

either 10 μM bicuculline methochloride or 200 μM picrotoxin (data not shown). In contrast, 10 of 10 cells from cultures grown in 25 mM $[K^+]_o$ had no detectable mIPSCs (Fig. 5*C*). A similar lack of mIPSCs was seen in wild-type cultures grown in 25 mM $[K^+]_o$ (data not shown) [note: in $\Delta\alpha$6lacZ homozygous mice, the α6 and δ subunits are eliminated and reduced, respectively (Jones et al., 1997), but the remaining α1, β2, β3, and γ2 subunits are adequate for $GABA_A$ receptor-mediated synaptic responses of granule cells].

In dissociated cerebellar cultures, rat granule cells innervate and release glutamate onto each other (Gallo et al., 1982). In cells from chronic 5 mM $[K^+]_o$ $\Delta\alpha$6lacZ mouse cultures, mEPSCs occurred at a frequency of ~0.1 Hz (Fig. 5*B*). As for mIPSCs, no spontaneous excitatory synaptic activity was detected in cells from 25 mM $[K^+]_o$ cultures (Fig. 5*B*).

The absence of spontaneous GABA and glutamate release in cells chronically cultured in strongly depolarizing media could be caused by a failure to form synaptic connections or long-term vesicle depletion from presynaptic terminals. Both mIPSCs and mEPSCs of 25 mM $[K^+]_o$ cultured cells could be reestablished by overnight culture in 5 mM $[K^+]_o$ (data not shown). This suggests that vesicle depletion is the most likely explanation for the absence of synaptic transmission in long-term 25 mM $[K^+]_o$ cultures.

Expression of the α6 subunit is depressed by tetrodotoxin but not by blockers of synaptic transmission

The electrophysiological experiments described above demonstrate that in 5 mM $[K^+]_o$ cultures, granule cells receive active excitatory and inhibitory synaptic inputs and are competent to fire action potentials; in contrast, in chronic 25 mM $[K^+]_o$, granule cells are electrically silent. Therefore, either intrinsic firing, released neurotransmitters (e.g., GABA or glutamate), or agents such as growth factors could be responsible for α6 gene induction in the 5 mM $[K^+]_o$ cultures.

To test the contribution of GABA and glutamate, 5 mM $[K^+]_o$ $\Delta\alpha$6lacZ cultures were grown in the presence of glutamate and GABA receptor antagonists for 11 d. Blockade of AMPA/kainate receptors with CNQX (10 μM), NMDA receptors with CPP (10 μM) (also see Thompson et al., 1996a), or metabotropic mGluR receptors with α-MCPG (500 μM) (Fig. 6*A*) had no influence on the number of lacZ-positive cells produced in 5 mM $[K^+]_o$. Similarly, neither the $GABA_A$/$GABA_C$ receptor antagonist picrotoxin (50 μM) nor the $GABA_B$ receptor blocker CGP 55 845A (10 μM) (Davies et al., 1993) inhibited α6-lacZ expression (Fig. 6*A*). For rat granule cells, the effects of elevated $[K^+]_o$ are mimicked by long-term culture in the presence of 5 mM $[K^+]_o$ and NMDA (Balázs et al., 1988). Chronically applied NMDA (10 μM), however, did not mimic the inhibition of lacZ expression produced by chronic 25 mM $[K^+]_o$ in our mouse cell cultures (Fig. 6*A*).

At 1 μM, the Na^+ channel blocker TTX eliminates sodium currents and action potentials in cerebellar granule cells (data not shown). TTX (1 μM) applied for the duration of the culture (15 DIV in 5 mM $[K^+]_o$) produced a significant inhibition in the number of lacZ-positive cells (Fig. 7), although many cells still expressed the gene. Many cells also died during the TTX treatment; only 45% of cells survived, compared with those grown in 5 mM $[K^+]_o$ alone (Fig. 7*C*). However, within this surviving group, only 25 ± 4% of cells express lacZ, compared with the 60 ± 8% in the parallel control groups (Fig. 7*D*). Therefore, action potential firing stimulates induction of the α6 subunit gene, either directly by regulating the gene or indirectly by promoting the health of the granule cell, because so many cells die in the presence of TTX, and the remaining nonexpressing ones may be compromised.

One link between changes in membrane potential (e.g., action potential firing) and gene expression is through activation of voltage-dependent dihydropyridine-sensitive L-type Ca^{2+} channels (Bading et al., 1993). Although these channels are present on rodent granule cells grown in 5 mM $[K^+]_o$ (Randall and Tsien, 1995), their chronic inhibition with the antagonist nifedipine (10 μM) had no effect on the number of lacZ-positive cells in cultures grown in 5 mM $[K^+]_o$ (Fig. 6*B*). Rat granule cells cultured in 5 mM $[K^+]_o$ also express considerable current components that are sensitive to antagonists of N- and P/Q-type Ca^{2+} channels (Randall and Tsien, 1995). Chronic applications of the N-type channel antagonist ω-CTx-GVIA (1 μM) and the P/Q-type antagonist ω-Aga-IVA (300 nM), however, produced no change in the number of lacZ-positive granule cells in 5 mM $[K^+]_o$ cultures (Fig. 6*B*). Therefore, *in vitro* expression of the α6 subunit gene is not specifically coupled to the opening of L-, N-, P-, or Q-type Ca^{2+} channels.

Growth factors

Growth factors, such as brain-derived neurotrophic factor (BDNF) or thyroid hormone (T3), promote granule cell function and differentiation (Leingärtner et al., 1994; Gao et al., 1995; Neveu and Arenas, 1996; Nonomura et al., 1996). We examined whether α6 gene expression in cultures grown in 25 mM $[K^+]_o$ could be rescued by these or other growth factors. P5 granule cells were cultured in 25 mM $[K^+]_o$ for 11 d, and the following growth factors were included individually in the media throughout the culture period: BDNF (200 ng/ml), NT-3 (100 ng/ml), NGF (100 ng/ml) or T3 (10 ng/ml). None of these factors were able to induce lacZ expression in 25 mM $[K^+]_o$ media (data not shown). The trk antagonist K252a (used at 50 nM) (Leingärtner et al., 1994) produced no change in the number of lacZ-positive cells in a 5 mM $[K^+]_o$ culture of $\Delta\alpha$6lacZ cerebellum: 68% blue cells in K252a versus 71% blue cells in control [also reported with mouse cells (Lin and Bulleit, 1997)]. Therefore, various growth factors implicated in cerebellar development are not required for the induction of the mouse α6 subunit gene.

DISCUSSION

We have used a lacZ reporter inserted into the $GABA_A$ receptor subunit α6 gene to follow α6 expression in developing mouse cerebellum and to assay factors regulating this expression in differentiating mouse granule cells in culture. At early stages (the first 3 postnatal weeks), α6 expression is strongly mosaic within and between developing cerebellar lobules (Fig. 2). This may account for differences in $GABA_A$ receptor subunit expression assayed by single-cell PCR on juvenile slices, in which 50% of rat granule cells are α6-negative (Santi et al., 1994b). In culture, cells express the α6–lacZ gene only in low (5–15 mM) $[K^+]_o$, whereas under chronic depolarizing conditions (25 mM $[K^+]_o$) α6 is induced in few cells. The use of lacZ as a reporter allowed the heterogeneity in the cultures to be seen directly. In most previous studies of $GABA_A$ receptor subunit development in culture, mRNA or membranes have been pooled from populations of cells. In agreement with the lacZ gene expression results, benzodiazepine enhancements of $GABA_A$ receptor responses recorded from granule cells cultured in 25 mM $[K^+]_o$ did not differ between

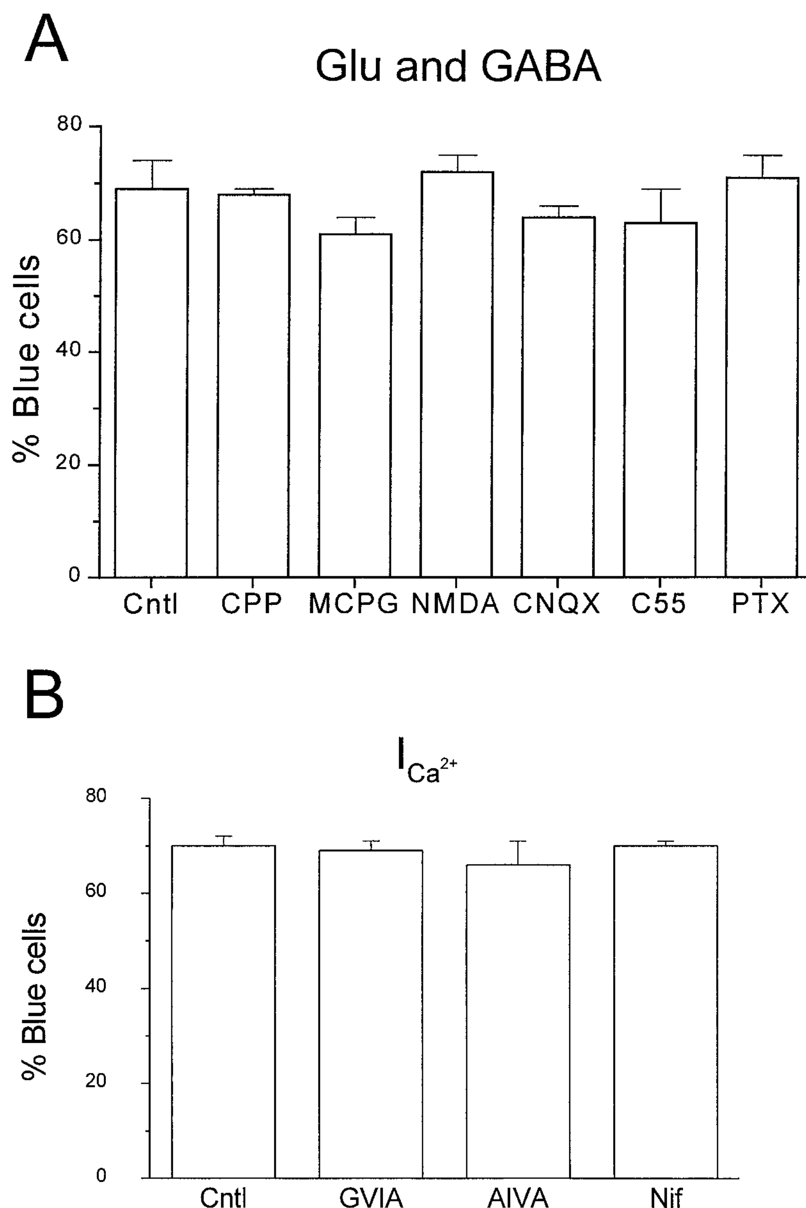

Figure 6. The effects of ion channel activity on the development of α6-lacZ granule cell expression in 5 mM $[K^+]_o$. *A*, The effects of 10 μM CPP, 500 μM MCPG, 50 μM picrotoxin (*PTX*), 10 μM CNQX, 10 μM CGP55845A (C55), and 10 μM NMDA on lacZ expression in cultures maintained in 5 mM $[K^+]_o$ for 11 d *in vitro*. Each bar represents data pooled from two coverslips in each of three separate Δα6lacZ cultures. *B*, The fraction of lacZ-positive cells present in 5 mM $[K^+]_o$ Δα6lacZ cultures grown under control conditions (*Cntl*) or in 1 μM ω-CTx-GVIA (*GVIA*), 300 nM ω-Aga-IVA (*AIVA*), or 10 μM nifedipine (*Nif*). Each bar represents the average of two coverslips in each of three cultures.

Δα6lacZ +/+ and −/− cells (J. R. Mellor and A. D. Randall, unpublished observations), and no α6 immunoreactivity could be detected in membrane extracts prepared from +/+ mouse cells cultured in 25 mM $[K^+]_o$. (Fig. 3*I*). By contrast, when cells were grown in 5 mM $[K^+]_o$ for 2 weeks, +/+ and −/− granule cells differed in benzodiazepine sensitivities, as expected from the loss of the α6 subunit in −/− cells (Jones et al., 1997), and α6 immunoreactivity could be detected from +/+ membranes (Fig. 3*I*).

Factors regulating $GABA_A$ receptor α6 subunit gene expression: subtle species differences?

There are many descriptions of the development of $GABA_A$ receptor α6 subunit expression in dissociated cultures of rat cerebellum (for review, see Wisden et al., 1996), but few using mice [only Lin and Bulleit (1996, 1997), Lin et al. (1998), and this report]. The majority finding is that in rat cultures using 20–25 mM $[K^+]_o$, α6 subunit gene expression, as assayed by RNA and protein levels or with electrophysiology and drug-binding profiles, either increases with time in culture or is at least abundantly present (Malminiemi and Korpi, 1989; Bovolin et al., 1992; Mathews et al., 1994; Thompson and Stephenson, 1994; Zheng et al., 1994; Caruncho et al., 1995; Gao and Fritschy, 1995; Thompson et al., 1996a,b; Zhu et al., 1996; Ghose et al., 1997). There is no evidence for the effect of $[K^+]_o$, and therefore membrane depolarization, on rat α6 gene expression: the rate of α6 gene transcription does not vary between rat cells cultured in 12.5 versus 25 mM $[K^+]_o$ (Harris et al., 1995). α6 mRNA steady-state levels are not significantly different in rat granule cells (prepared from P8 cerebella) after 5 DIV in either 12.5 or 25 mM $[K^+]_o$ cultures (Harris et al., 1994).

It may be that mouse and rat α6 gene regulation differ subtly. For example, mouse and rat granule cells have differing survival requirements, possibly reflecting physiological differences. It is well known that elevated extracellular K^+ concentrations (e.g., 25 mM $[K^+]_o$) promote long-term survival of rat cerebellar granule cells in dissociated cultures (Gallo et al., 1987), but they are

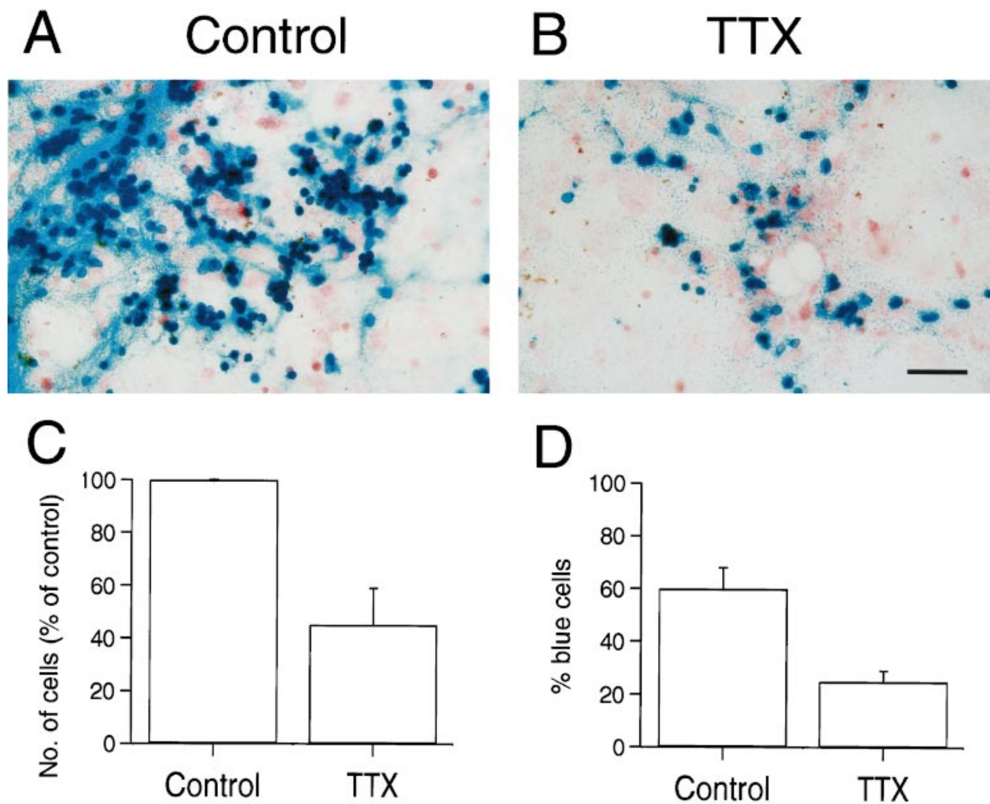

Figure 7. Development of α6–lacZ gene expression in cultured cerebellar granule cells: effects of TTX. *A, B*, Cultures maintained in 5 mM $[K^+]_o$ for 15 DIV in the absence (*A*) and presence (*B*) of 1 μM TTX. *C*, The percentage of surviving cells in 5 mM $[K^+]_o$ and 1 μM TTX compared with cells in the same media but with no added TTX. *D*, The percentage of α6–lacZ-expressing cells present in 1 μM TTX-containing and control media for the experiments described in *C*. The cells were counted at the cell-dense area of the coverslips; the average of four separate experiments was calculated. In all panels, *blue staining* indicates lacZ-positive cells; lacZ-negative cells are *red*. Scale bar, 30 μm.

not essential for experiments requiring long-term culture of mouse cells. In some conditions, mouse cells survive as well in 5 as in 25 mM $[K^+]_o$ (Peng et al., 1991; Mogensen et al., 1994; Mogensen and Jorgensen, 1996).

Our results for mouse cells are partially supported, however, by observations that in some cases α6 expression in rat cells is not necessarily constitutive. One group has reported that regardless of culture conditions, α6 mRNA fails to increase from low basal levels in 25 mM $[K^+]_o$, although the RNA levels of some other subunits increase over time in the same cultures (Behringer et al., 1996; Gault and Siegel, 1997). *In vivo*, the α6 and δ subunit genes have similar developmental profiles: both genes switch on as the cells reach the internal granule cell layer (Laurie et al., 1992b), and the two proteins specifically associate in a $GABA_A$ receptor subtype (Jones et al., 1997; for review, see Wisden and Moss, 1997). Interestingly, they are not regulated in the same way: δ subunit mRNA increases in rat granule cells cultured in chronic 25 mM $[K^+]_o$, but not in 5 mM $[K^+]_o$, and is also regulated by cell density (Behringer et al., 1996; Gault and Siegel, 1997). Chronically depolarized cells have higher calcium loads than those maintained in 5 mM $[K^+]_o$, and Ca^{2+}/calmodulin-dependent protein kinases are implicated in δ gene regulation (Gault and Siegel, 1997). We have found that cells maintained in chronic 25 mM $[K^+]_o$ are electrically silent (see below), suggesting that δ subunit gene expression is inversely related to the amount of synaptic transmission and action potential firing.

Action potential firing, but not synaptic transmission, stimulates α6 gene induction

There is a correlation between neuronal activity and α6 gene induction. In 5 mM $[K^+]_o$, a proportion of granule cells fire spontaneous action potentials and have spontaneous excitatory and inhibitory synaptic transmission. In chronic depolarizing conditions (25 mM $[K^+]_o$), voltage-gated Na^+ channels are inactivated (no action potentials), and transmitter vesicle pools are probably depleted (no mIPSCs or mEPSCs). Despite the correlation with activity, GABA receptor, glutamate receptor [see also Thompson et al. (1996a) for ionotropic glutamate antagonists on rat cells in 25 mM $[K^+]_o$], and voltage-gated Ca^{2+} channel activation are not necessary for α6 gene induction in cultures grown in 5 mM $[K^+]_o$ (Fig. 6).

Nevertheless, elimination of Na^+ channel function with chronic TTX treatment blocks α6–lacZ induction in some cells, as well as killing many of them, although a proportion of surviving cells remain intensely stained for lacZ (Fig. 7). This mixed result could be because dissociated granule cell cultures are made by pooling granule cells at different stages of their development (i.e., at P5, vermis lobule IX and X are already beginning to express the α6 gene, whereas other lobules and the hemispheres are several days behind) (Fig. 2). At the time of plating, some cells may already be committed to expressing α6 and may be unresponsive to the inhibition of Na^+ channels; other cells could still be at an earlier and more malleable stage.

There could be factors (other than GABA or glutamate) endogenously released in 5 mM $[K^+]_o$ that promote α6 expression. For instance, in the granule cell layer of the mutant mouse *stargazer*, BDNF mRNA levels are attenuated (Qiao et al., 1996), and α6 protein levels are reduced to 20% of wild-type (Barnes et al., 1997). However, K252a, a selective blocker for trk tyrosine kinases (receptors for BDNF, NT-3, and NGF) (Leingärtner et al., 1994), did not stop an increase of α6 mRNA in cultured mouse cells over a 4 d period (Lin and Bulleit, 1997; and our results). Although BDNF is not essential for α6 expression, it does enhance the rate of appearance of α6 mRNA in cultured mouse granule cells, possibly by promoting the general maturation of the cell (Lin et al., 1998).

Conclusions

The best predictor of α6 gene expression is simply cell age: α6 expression may be "hard-wired" into the terminal differentiation program of granule cells (Lin and Bulleit, 1996), with the commitment to express α6 starting at an earlier point in granule cell development, as suggested for other $GABA_A$ receptor subunit genes (Beattie and Siegel, 1993). For example, this could be regulated by a cellular clock initiating from the last mitotic division in the external granule cell layer. The development of α6 expression is resistant to most experimental modulations. During the first days in culture, however, depolarizing $[K^+]_o$ blocks subsequent α6 gene expression, whereas normal $[K^+]_o$ permits future induction. As the period of initial plating and growing in 25 mM $[K^+]_o$ is increased, fewer cells are capable of switching on the α6–lacZ gene after they are transferred to 5 mM $[K^+]_o$. Conversely, prolongation of the initial culture period in 5 mM $[K^+]_o$ enables more cells to express the α6-lacZ gene after transfer to 25 mM $[K^+]_o$. Although the effect of an initial plating in 25 mM $[K^+]_o$ for 11 DIV is absolute (no expression), voltage-gated Na^+ channel activity acts on subpopulations of developing cells; cAMP elevation also reduces α6 levels (Thompson et al., 1996b; rat cells, Ghose et al., 1997). Stimuli that pattern action potential firing may therefore influence the timing of α6 induction. Identification of the proteins that bind to the regulatory regions of the α6 gene will explain how the final stages of granule cell maturation take place (Jones et al., 1996; Bahn et al., 1997).

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