Cerebellar granule neurons in culture are a popular model for studying neuronal signaling and development. Depolarizing concentrations of K\(^+\) are routinely used to enhance cell survival, and kainate is sometimes added to eliminate GABAergic neurons. We have investigated the effect of these measures on expression of mRNA for \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptor \(\alpha1–6\), \(\beta1–3\), \(\gamma1–3\), and \(\delta\) subunits in cultures of mouse cerebellar granule neurons grown \(7\) or \(12\) days \(in\ vitrO\) (DIV) using semi-quantitative reverse transcription-PCR. We detected mRNA for the \(\alpha1\), \(\alpha2\), \(\alpha5\), \(\alpha6\), \(\beta2\), \(\beta3\), \(\gamma2\), and \(\delta\) subunits in all the cell cultures, but the expression levels of the \(\alpha5\), \(\alpha6\), and \(\beta2\)-subunit mRNAs were significantly dependent on the composition of the culture medium. Both an increase of the extracellular K\(^+\) concentration from 5 to 25 mM and the addition of 50 \(\mu\)M kainate immediately depolarized the neurons but prolonged exposure (7–8 DIV)-induced compensatory hyperpolarization. 25 mM K\(^+\) caused a shift from \(\alpha6\) to \(\alpha5\) expression measured at 7 and 12 DIV, which was mimicked by kainate in 12 DIV cultures. The expression of \(\beta2\) was decreased by 25 mM K\(^+\) in 7 DIV cultures and by kainate in 12 DIV cultures. The effects on \(\beta2\) expression could not be ascribed to depolarization. Alterations of \(\alpha6\) mRNA expression were reflected in altered sensitivity to GABA and furosemide of the resulting receptors. Our study has shown that a depolarizing K\(^+\) concentration as well as kainate in the culture medium significantly disturbs maturation of GABA\(_A\) receptor subunit expression.

The \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptor is a transmitter-gated Cl\(^-\) ion channel assembled from different subunits in a pentameric composition. In the mammalian central nervous system a large family of subunits exist that, based on homology, are grouped into types \(\alpha1–6\), \(\beta1–3\), \(\gamma1–3\), \(\delta\), \(\epsilon\), and \(\theta\) (1–3). The GABA\(_A\) receptor subunits are expressed in a regional- and age-specific manner (4–9). The different subunit combinations exhibit distinct properties that presumably underlie a precise physiological role for each subtype (Refs. 10–18; for review, see Ref. 19). Knowledge of the distribution of different GABA\(_A\) receptor subtypes, both anatomically and developmentally, is therefore essential for understanding the physiological actions of GABA and the pharmacological actions of drugs that act on different GABA\(_A\) receptor subtypes.

Cerebellar granule neurons are used for studying neuronal signaling and development because they have a relatively simple morphology and receive most of their inhibitory input from one cell type (for review, see Ref. 20). When cultured in serum-based medium, cerebellar granule neurons express a wide range of receptors and develop stimulus-coupled glutamate release (21). In the developing cerebellum, granule neurons express GABA\(_A\) receptor \(\alpha2\), \(\alpha3\), \(\beta3\), \(\gamma1\), and \(\gamma2\)-subunit genes (5). These subunits are replaced in the adult cerebellum where \(\alpha1\), \(\alpha6\), \(\beta2\), \(\beta3\), \(\gamma2\), and \(\delta\) predominate (4, 7, 22). The \(\alpha6\) subunit is expressed almost exclusively in cerebellar granule neurons, where it marks neuronal maturation. A similar development evidently occurs in cultures of rat cerebellar granule neurons (e.g. Refs. 23–25). It has been suggested that 45–59% of GABA\(_A\) receptors in the cerebellum of the adult rodent contain \(\alpha6\) subunits (25–28). The most predominant combinations are proposed to be \(\alpha1\beta2\gamma2\delta\), \(\alpha1\beta2\gamma2\epsilon\), \(\alpha1\beta2\gamma2\theta\), and \(\alpha6\beta2\gamma2\delta\) (25, 27, 29, 30); noteworthy is the finding that the \(\delta\) subunit is found exclusively in combination with \(\alpha6\) subunits (26, 31).

It is well known that elevated extracellular K\(^+\) concentrations or other calcium-elevating stimuli promote long term survival of rat cerebellar granule neurons in dissociated cultures (21). The physiological extracellular K\(^+\) concentration is \(5\) mM, but cerebellar granule neuronal cultures are often maintained in 25 mM K\(^+\) to enhance survival (21). This use of chronic depolarization is questionable, because it affects the subunit gene expression of neurotransmitter receptors and, hence, the receptor composition and function. More specifically, rat granule neurons do not correctly develop their AMPA or NMDA receptor subunit expression in 25 mM K\(^+\) (32–34). In addition, the K\(^+\) concentration affects GABA\(_A\) receptor subunit expression in rat (35, 36) and mouse (37) cerebellar granule neurons. Kainate is sometimes used to eliminate GABAergic neurons from cultures of cerebellar granule neurons (38). As a glutamate receptor agonist, it causes depolarization (39), but its effect on GABA\(_A\) receptor subunit expression is not as well described as that of K\(^+\).
The aim of this work was to investigate the effects of extra-
cellular K\(^+\) and kainate on cell viability and on the expression of 13 different GABA\(_A\) receptor subunit mRNAs in cultures of mouse cerebellar granule neurons. The role of membrane potential in mediating the effects of K\(^+\) and kainate was assessed by correlating the effects on membrane potential and on mRNA expression. Finally, the relative contribution of the \(\alpha\) subunit to receptor function was estimated from the sensitivity of the receptors to GABA and to the selective antagonists furosemide (11).

MATERIALS AND METHODS

Primary Cultures of Mouse Cerebellar Granule Cells—Cerebellar granule neurons were prepared from 6–8-day-old NMRI mice (Taicon M&B) according to a procedure modified from Courtney et al. (40) and Schouenbroe et al. (38). Briefly, trypsin (0.25 mg/ml, Sigma)-dissociated and DNase (50 units/ml, Sigma)-treated cells were plated at ~200,000 cells/cm\(^2\) in 35-mm Petri dishes coated with poly-d-lysine (Sigma). Cells were cultured in Dulbecco’s minimum essential medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 31 mM glucose, 0.2 mM glutamine (Sigma), 4 \(\mu\)g/\(\mu\)l insulin (Sigma), 7.3 \(\mu\)M \(p\)-aminobenzoic acid (Sigma), and 50 units/ml penicillin (LEO Pharma). As appropriate, the medium was further supplemented with 20 mM KCl (to a final K\(^+\) concentration of 25 mM) and/or 50 \(\mu\)M kainate (Sigma). Culture medium was replaced after 24 h with the inclusion of 10 \(\mu\)M cytosine arabinoside (Sigma) to reduce non-neuronal proliferation; after this treatment the medium was not changed. The cells were cultured in a humidified 5% CO\(_2\) atmosphere at 37°C. The cultures were used in experiments within 12–14 days in vitro (DIV).

Measurement of Cell Viability—The amount of viable cells in the cerebellar granule neuron cultures was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (41). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced to formazan by cells that have functional mitochondria; this process has been shown to correlate well with cell viability (42). The cultures were tested at 7 and 12 DIV.

Semiquantitative Reverse Transcription-PCR—Reverse transcription-PCR was performed as previously described (43). The cultures were tested at 7 and 12 DIV. Primers (Table I) for the GABA\(_A\) receptor subunits \(\alpha1\), \(\alpha2\), \(\alpha3\), \(\alpha4\), \(\alpha5\), \(\alpha6\), \(\beta1\), \(\beta2\), \(\beta3\), \(\gamma1\), \(\gamma2\), \(\gamma3\), and \(\delta\) were used. Briefly, RNA was isolated with RNeasy Mini Kit (Qiagen) after lysis of the cells, and the concentration and purification of RNA was measured on a spectrophotometer (Ultrospec4000, Pharmacia Biosciences). cDNA was synthesized using 0.25 \(\mu\)g of RNA, and 1 \(\mu\)l of the resulting mixture was used for PCR for each subunit. cDNA amplifications were performed in 42 cycles, and aliquots (15 of 100 \(\mu\)l in total) of the PCR products were taken from cycles 27, 30, 33, 36, and 39. Relative intensity of the ethidium bromide-stained bands on gels were measured using computer-assisted image analysis and compared with the 400-bp band of the molecular weight marker (100 Base-Pair-Ladder, Amer sham Biosciences) added in fixed amount for all gels. Because the efficiency of the PCR amplification is primer-dependent, the relative intensities could not be compared between different subunit mRNAs.

Electrophysiology—Before recordings, the culture medium was exchanged for an extracellular recording solution (artificial balanced salt solution (ABSS)), and the Petri dish with cells was transferred to an inverted phase-contrast Zeiss Axiovert 10 microscope stage. The cells were constantly perfused with ABSS (0.5 ml/min) at room temperature from a perfusion-fedy 7-barrel pipette (List) ~100 \(\mu\)m from the recorded neuron. By switching application from one barrel to another, the extracellular solution surrounding the neuron was exchanged with a time constant of ~50 ms. Individual cerebellar granule neurons were approached with micropipettes of 3–5-megohm resistance manufactured from 1.5-mm-outter-diameter glass (World Precision Instruments). Standard patch clamp technique (44) in current or voltage clamp mode was used to record from neurons in the whole-cell configuration using an EPC-9 amplifier (HEKA Elektronik). Whole-cell membrane currents and potentials were plotted on a low fidelity chart recorder during the experiment and stored on computer hard disk and video tape using a VR-100 digital data recorder (Instrutech).

For recordings of the membrane potentials with extracellular K\(^+\) concentration = 5 \(mM\) (physiological K\(^+\)), the cells were perfused with ABSS composed of 138.5 mM NaCl, 5 mM KCl, 1.25 mM Na\(_2\)HPO\(_4\), 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 10 mM glucose, and 10 mM HEPES, pH 7.35. For recordings with extracellular K\(^+\) concentration = 25 mM (depolarizing K\(^+\)) the concentration of NaCl was reduced to 118.5 mM, and the concentration of KCl was increased to 25 mM. Kainate was perfused in ABSS to a final concentration of 50 \(\mu\)M, where indicated. The intrapipette solution contained 10 mM NaCl, 130 mM potassium gluconate, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM EGTA, 2 mM MgATP, and 10 mM HEPES, pH 7.3. Initially the cells were perfused with “physiological” ABSS containing 5 mM K\(^+\) without kainate. The perfusion was then switched to ABSS with either 5 mM K\(^+\) or 50 \(\mu\)M kainate or both. After a new stable membrane potential was reached, the perfusion was switched back to physiological ABSS. Each of the depolarizing solutions was tested at least twice on each cell. Membrane potentials were corrected for liquid junction potentials.

In experiments addressing the GABA concentration-response relationships or furosemide sensitivities, the ABSS contained 140 mM NaCl, 3.5 mM KCl, 1.25 mM Na\(_2\)HPO\(_4\), 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 10 mM glucose, and 10 mM HEPES, pH 7.35. The intrapipette solution contained 140 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM EGTA, 2 mM MgATP, and 10 mM HEPES, pH 7.3. The neurons were voltage-clamped at ~−60 mV. The high intracellular Cl\(^−\) concentration shifted the Cl\(^−\) reversal potential to ~−90 mV. The high intracellular Cl\(^−\) concentration substantially increased the currents recorded at ~−60 mV. Series resistance was 65% compensated. GABA (Sigma) was dissolved in distilled water at a concentration at least 100× greater than that required for perfusion and diluted with ABSS. Furosemide (Sigma) was dissolved in Me\(_2\)SO and diluted with ABSS; the content of Me\(_2\)SO in the final solution was at most 0.1% and had no effect of its own on membrane current. Different concentrations of GABA were applied for 5 s at 1-min intervals. Furosemide was preapplied for 10 s immediately before the application of a premixed solution of GABA and furosemide. Between drug applications the cell was perfused with ABSS.

Membrane currents and potentials were analyzed using Pulse (HEKA Elektronik) and Igor Pro (WaveMetrics) software. Current responses were quantified by measuring the peak current during application of GABA or GABA plus furosemide. GABA concentration-response relationships were fitted to the equation:

\[
I = I_{\text{max}} \times \frac{[\text{GABA}]^n}{EC_{50} + [\text{GABA}]^n}
\]

where \(I\) is the peak membrane current induced by the GABA concentration, \([\text{GABA}]\), \(I_{\text{max}}\) is the maximum peak current that GABA can induce, \(EC_{50}\) is the GABA concentration eliciting 50% of \(I_{\text{max}}\), and \(n\) is the Hill coefficient.

Statistics—Data were described using mean and S.E. or 95% confidence intervals. Mean values were compared using either Student’s t test or analysis of variance (ANOVA); when relevant, the Tukey test was used as a means of post-hoc multiple comparisons. Two-way ANOVA was used to separate the effects of two variables and determine

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**Table I**

| Subunit | Sequence | Primer |
|---------|----------|--------|
| \(\alpha1\) | 5'-AGCTTACCCCTAAGCTTGCCAGG-3' | Up |
| \(\alpha2\) | 5'-AGCGTATCCCTCACTCGCATGGAG-3' | Low |
| \(\alpha3\) | 5'-ACACAGATTGAGGGAAAGACCATG-3' | Low |
| \(\alpha4\) | 5'-GGAGTTTGGAATGGATGACTTGC-3' | Up |
| \(\alpha5\) | 5'-GAGTGCTTCTGTTTCTCCTC-3' | Low |
| \(\alpha6\) | 5'-GAGCGAGCTTGGGAAAGATG-3' | Up |
| \(\beta1\) | 5'-CCTCTAGAGTCGAGCTGGG-3' | Up |
| \(\beta2\) | 5'-GCCAGTGCTCAGGTTATATATATAT-3' | Low |
| \(\gamma1\) | 5'-GGAGAGGTTTGGAGAGGTAGCCCCT-3' | Low |
| \(\gamma2\) | 5'-TCCTTCTACCTTGAGCAGCAGC-3' | Up |
| \(\gamma3\) | 5'-CACACCGGTCCGCTAACCCTGCCAC-3' | Up |
| \(\delta\) | 5'-TCCTATCCAGGAGCAAGAACAGC-3' | Low |

The GABA\(_A\) receptor subunits and the primer sequences for their mRNA.
their interaction. Probabilities ($p < 0.05$) were considered statistically significant. The term “occlusion” refers to situations where the effect due to simultaneous variation of two variables was smaller than the sum of the effects due to variation of each variable separately.

### RESULTS

**K⁺ and Kainate Are Acutely Depolarizing, but Prolonged Exposure Induces Compensatory Hyperpolarization**—Fig. 1 shows the influence of the culture medium as well as the extracellular recording solution (ABSS) on the membrane potentials of the neurons. The same K⁺ and kainate concentrations were used for the culture media and extracellular recording solutions: 5 or 25 mM K⁺ and 0 or 50 μM kainate.

A two-way ANOVA was used to estimate the effects of 1) culture medium and 2) extracellular recording solution on the membrane potential. At 2–3 DIV the membrane potential was independent of the culture medium but significantly dependent on the extracellular recording solution ($p = 0.002$). This dependence was further analyzed with a two-way ANOVA of the effects of 1) K⁺ concentration and 2) kainate concentration in the extracellular recording solution. Both K⁺ and kainate had highly significant depolarizing effects ($p < 0.001$ for both) and a significant interaction ($p = 0.026$), suggesting that the depolarizing effect of simultaneously increasing both K⁺ or kainate concentrations to 25 mM and 50 μM, respectively, was not as great as the sum of the depolarizations caused by increasing K⁺ or kainate separately (occlusion). Indeed, the combined effect of K⁺ and kainate did not differ significantly from the individual effects (total occlusion).

After 7–8 DIV both culture medium and extracellular recording solution showed significant effects ($p < 0.001$ for both) without interaction, i.e. their effects were additive (two-way ANOVA). The effect of culture medium was further analyzed with two-way ANOVA. Increased K⁺ ($p = 0.004$) or kainate ($p = 0.034$) in the culture medium significantly hyperpolarized the membrane potentials without interaction. The effect of recording solution was also further analyzed. After 7–8 DIV (as at 2–3 DIV), both increased K⁺ ($p < 0.001$) and kainate ($p < 0.001$) concentrations were significantly depolarizing. The combined effect of increased K⁺ and kainate concentration was smaller than the sum of the individual effects ($p < 0.001$) but larger than any of the individual effects ($p < 0.001$, Tukey test), which were not significantly different. Thus, at this point the effects of K⁺ and kainate concentrations in the extracellular recording solution were only partially occluding.

In conclusion, the immediate effects of increasing either K⁺ (from 5 to 25 mM) or the kainate concentrations (from 0 to 50 μM) in the extracellular recording solution were depolarizations of similar magnitude that were occluding and independent of the culture medium. Prolonged exposure to high K⁺ or kainate concentrations in the culture medium gave rise to hyperpolarization relative to cells grown in a physiological K⁺ concentration without kainate. This suggests that the neurons develop a compensatory hyperpolarizing mechanism when exposed to depolarizing conditions for an extended period.

The membrane potentials of neurons in the different culture media may influence mRNA expression. These membrane potentials were estimated from measurements in extracellular recording solution with the same K⁺ and kainate concentrations as in the culture medium (Fig. 1, columns marked with a number sign). At 2–3 DIV elevated K⁺ concentration significantly depolarized the membrane potential (two-way ANOVA, $p = 0.005$); on the other hand, the kainate effect was not significant. After 7–8 DIV both K⁺ ($p < 0.001$) and kainate ($p < 0.001$) were significantly depolarizing and totally occluding ($p < 0.001$). The horizontal line at −42 mV shows the equilibrium potential for K⁺ with the extracellular concentration at 25 mM. No spontaneous synaptic potentials or currents were observed in any of the recorded neurons at 2–3 or 7–8 DIV. In addition, no action potentials were observed, even after depolarizations were induced with K⁺ or kainate.

**Kainate as Well as Depolarizing K⁺, Increase Cell Viability**—The survival of mouse cerebellar granule neurons in culture was strongly dependent on the concentration of K⁺ in the culture medium as indicated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 2). 25 mM K⁺ significantly ($p < 0.001$) increased the viability of mouse cerebellar granule neurons in culture regardless of the presence or absence of kainate both at 7 and 12 DIV. At 7 DIV kainate significantly ($p < 0.01$) increased cell viability in cultures grown in 5 mM but not in 25 mM K⁺, indicating that the effects of K⁺ and kainate on viability were not additive. The effect of

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**FIG. 1.** Membrane potentials of cerebellar granule neurons depend on K⁺ and kainate concentrations in the culture media as well as in the extracellular recording solution. Each column represents the mean ± S.E. from 6–11 neurons. The effects of K⁺ and kainate were analyzed using two-way ANOVA. A, at 2–3 DIV the composition of the culture medium was without effect. In the extracellular recording solution both elevation of K⁺ ($p < 0.001$) and addition of kainate ($p < 0.001$) depolarized the membrane potential with total occlusion ($p = 0.026$). B, after 7–8 DIV a significant effect of the culture medium had emerged ($p < 0.001$) in addition to, but independent of, the effect of the extracellular recording solution ($p < 0.001$). In the culture medium both elevated K⁺ ($p = 0.004$) and kainate ($p = 0.034$) hyperpolarized the membrane potential without interaction. In the extracellular recording solution both elevation of K⁺ ($p < 0.001$) and kainate ($p < 0.001$) depolarized the membrane potential with partial occlusion ($p < 0.001$). In columns marked with the symbol # membrane potentials were measured in the presence of the same extracellular K⁺ and kainate concentrations as in the culture medium. For these, at 2–3 DIV elevation of K⁺ concentration significantly depolarized the membrane potential ($p = 0.005$), whereas the kainate effect was not significant. At 7–8 DIV both K⁺ ($p < 0.001$) and kainate ($p < 0.001$) were significantly depolarizing and totally occluding ($p < 0.001$). The horizontal line at −42 mV shows the equilibrium potential for K⁺ with the extracellular concentration at 25 mM.
kainate on cell viability was not detectable after 12 DIV.

Depolarizing K⁺ Causes a Shift of mRNA Expression from α6 to α5, Which Is Mimicked by Kainate in 12 DIV Cultures—Of the six GABA_A receptor α subunit mRNAs tested for, the α1, α2, α3, and α6 mRNAs were expressed in detectable amounts, whereas α3 mRNA and α4 mRNA could not be detected.

The level of α5 mRNA was significantly affected by K⁺ and kainate (Fig. 3). At 7 DIV α5 expression was enhanced with 25 mM K⁺ in the culture medium relative to 5 mM K⁺ (p < 0.001). At 12 DIV both high K⁺ (p < 0.01) and kainate (p < 0.01) enhanced the expression of α5 to similar levels, but the combination of K⁺ and kainate did not further increase α5. The α6 mRNA was reciprocally affected (Fig. 3). At 7 DIV expression was significantly decreased in 25 mM K⁺ compared with 5 mM K⁺ (p < 0.001). At 12 DIV both high K⁺ (p < 0.05) and kainate (p < 0.05) inhibited α6 expression to the same extent and with additive effects. The expression of α1 and α2 mRNA was not significantly affected by the concentration of K⁺ or kainate in the culture media (results not shown).

Depolarizing K⁺ and Kainate Specifically Decrease Expression of the β2 Subunit mRNA—Of the three GABA_A receptor β subunit mRNAs studied, the β2 and β3 were expressed in significant quantities, whereas expression of β1 was not detected. Elevation of the extracellular K⁺ concentration to 25 mM significantly (p < 0.001) decreased the expression of β2 mRNA in 7 DIV but not in 12 DIV cultures (Fig. 3). Kainate had no effect on neurons cultured for 7 days, but after 12 DIV it significantly (p < 0.05) decreased the expression of β2 independently of the K⁺ concentration. Expression of β3 mRNA was not significantly affected by increased K⁺ or the addition of kainate (results not shown).

Neither Kainate nor the K⁺ Concentration Affects the Expression of γ or δ Subunit mRNA—Of the three GABA_A receptor γ subunits, only γ2 was detected. The δ subunit mRNA was also found in significant amounts. Neither 25 mM K⁺ nor kainate had a significant effect on expression of γ2 mRNA or δ mRNA (results not shown).

The GABA Concentration-Response Relationship Is Influenced by K⁺ in the Culture Medium—To investigate whether differences in mRNA expression between cultures were reflected in the functional properties of the derived membrane-bound receptors, we determined GABA concentration-response relationships resulting from the different culture conditions (Fig. 4). The EC₅₀ values and Hill coefficients are listed in Table II. Neurons cultured for 10–12 DIV in 5 mM K⁺ without kainate had a significantly lower EC₅₀ value than neurons cultured in 25 mM K⁺ without kainate (p = 0.005). The Hill coefficients were not significantly different from each other.

Furosemide Inhibition of GABA-induced Currents Is Influenced by Both K⁺ and Kainate in the Culture Medium—Furosemide has been shown to be a specific antagonist at GABA_A receptors containing α6 subunits (11). To estimate the relative contribution of α6-containing receptors we tested the sensitivity of GABA-induced currents to inhibition by 100 μM furosemide in 10–11 DIV neurons (Fig. 5). Both culture conditions (p < 0.001) and GABA concentration (p = 0.012) affected furosemide inhibition without significant interaction (two-way ANOVA). Thus, furosemide inhibition decreases with increasing GABA concentration and response levels. Because the response levels obtained with 10 and 30 μM GABA were similar for cells grown in 25 mM K⁺, with or without kainate and in 5 mM K⁺ with kainate (Fig. 4, Table II), the furosemide inhibition was directly comparable for these cells. For 5 mM K⁺ without kainate, the response levels were higher; this would have a tendency to decrease the observed inhibition. Yet, this group of cells was the most sensitive to inhibition by furosemide.

At 10 μM GABA both K⁺ (p < 0.001) and kainate (p = 0.019) had negative effects on the furosemide sensitivity (two-way ANOVA). For cells cultured in 5 mM K⁺ without kainate the control response level was higher (28%) than that of other cells (16–18%). If cells cultured in 5 mM K⁺ without kainate had been tested at the same response level the measured furosemide inhibition would have been enhanced and, thus, would strengthen the observed significance of the K⁺ and kainate effects. For 30 μM GABA, only the effect of K⁺ was found to be significant, but again, significance levels were probably underestimated because neurons cultured in 5 mM K⁺ without kainate were tested on a higher response level than the other neurons.

**DISCUSSION**

Depolarizing Effects of K⁺ and Kainate—Increased extracellular K⁺ concentration as well as the addition of kainate to the extracellular medium gives rise to depolarization. An increased extracellular K⁺ concentration inhibits and potentially reverses outward K⁺ membrane currents leading directly to depolarization. Assuming an intracellular K⁺ concentration of 130 mM as in the intrappetite solutions, increasing the extracellular K⁺ concentration from 5 to 25 mM K⁺ switches the equilibrium potential for K⁺, E_K⁺, from −84 mV to −42 mV (45). The role of kainate is more complex. Kainate activates a subset of ionotropic glutamate receptors (AMPA as well as
kainate receptors) giving rise to increased membrane permeability of Na\(^+\) and K\(^+\) (and for some AMPA receptors also Ca\(^{2+}\)), resulting in membrane depolarization (39). Whether induced by increased extracellular K\(^+\) concentration or by kainate, depolarization opens voltage-gated Na\(^+\), K\(^+\), and Ca\(^{2+}\) channels, further increasing membrane permeability of Na\(^+\) and K\(^+\) and eliciting influx of Ca\(^{2+}\). We have analyzed the effects of increased K\(^+\) and kainate on membrane potential in order to correlate these with cell viability and mRNA expression.

The observed effects of increasing the extracellular concentration of K\(^+\) from 5 to 25 mM or adding 50 \(\mu\)M kainate to the extracellular medium are 2-fold. That is, the acute effects of both are depolarizations of similar magnitude that are mutually occluding and independent of the preceding culture conditions. Prolonged exposure to elevated K\(^+\) or kainate in the culture medium induces compensatory hyperpolarizing mechanisms; in this respect the effects of K\(^+\) and kainate are additive. Depolarization and compensatory hyperpolarization in concert determine the membrane potentials in the culture media.

The mutual occlusion of the depolarizations induced by in-
K+ concentration and kainate in the extracellular recording solution is predicted by the Goldman equation (45). This equation also states that the greater the concentration and membrane permeability of a particular ionic species, the greater will be its role in determining the membrane potential (45). From Fig. 1 it is apparent that, with 25 mM K+ and no kainate in the extracellular recording solution, the membrane potential is, indeed, close to the E_K. An effect of depolarizing K+ concentrations on expression of AMPA receptor subunits has been reported (46) that might influence the depolarizing ability of kainate. Because we did not observe any interaction between the composition of the culture medium and the depolarizations induced when adding kainate to the extracellular recording solution, such an effect was not important in our experiments.

How does this correlate with cell viability and mRNA expression? This question is addressed in the following paragraphs. The interpretation is complicated by the fact that the measurements of membrane potentials are made at two time points, but the derived effects (viability and mRNA expression) are influenced by the membrane potential experienced by the cell throughout the culture period and possibly with some delay.

Effect of Depolarizing K+ and Kainate on Cell Viability—
During cerebellar development, excess granule neurons are produced and subsequently lost via apoptosis (47). Also in culture, a proportion of the rat cerebellar granule neurons die relatively soon. This apoptotic cell death is prevented or reduced if the extracellular K+ concentration is elevated to depolarizing concentrations (21). Our results show, in conflict with an earlier study (48), that also mouse cerebellar granule neurons survive significantly better when grown in a depolarizing than in a physiological K+ concentration. Depolarizing concentrations of K+ have been suggested to promote survival of cerebellar granule neurons in dissociated cultures by increasing the intracellular Ca2+ concentration via L-type voltage-gated Ca2+ channels (21).

In the developing brain, excitatory amino acids appear to exert trophic effects. NMDA has been shown to reduce apoptotic cell death in rat cerebellar granule neuron cultures by stimulating brain-derived neurotrophic factor (49). In cultures of rat cortical neurons kainate has been reported to elevate intracellular calcium, presumably in part via G protein activation, leading to enhanced nerve growth factor-mediated increase in cell viability (50). Significant neurotoxicity of kainate was not seen before 14 DIV (50). In the present study, kainate increased the viability of mouse cerebellar granule neurons cultured in physiological but not depolarizing K+ at 7 DIV. In 12 DIV cultures no effect of kainate on viability was detected. This profile seems not to correlate with kainate depolarization, which increased from 2–3 DIV to 7–8 DIV. It is possible that depolarization decreased again between 8 and 12 DIV or that a neurotoxic effect of kainate developed and counteracted the positive effect on cell viability. Alternatively, depolarization may not be a main mediator of the effect of kainate on cell viability.

Effect of Depolarizing K+ and Kainate on the Expression of GABAA Receptor Subunit mRNA—The present study provides, for the first time, a simultaneous semi-quantitative analysis of the expression of 13 of the main mammalian GABAA receptor subunit mRNAs (α1–6, β1–3, γ1–3, and δ) in cultured mouse cerebellar granule neurons. The GABAA receptor subunit mRNA species that were detected in this study (α1, α2, α5, α6, β2, β3, γ2, δ) are generally in good agreement with earlier studies of rat cerebellar granule neuron mRNAs in vivo (7, 20) and in vitro (20, 36). Most importantly, our results show that the expression of some receptor subunit mRNAs (α5, α6, β2) is strongly dependent on culture conditions.

Depolarizing K+ and Kainate Hinders Maturation of a Subunit Expression—Our results demonstrate that mouse cerebellar granule neuron cultures maintained in depolarizing K+ express the α5 subunit as a substitute for α6, the latter prominent in cultures grown in physiological K+. At 12 DIV the effect of high K+ is mimicked by kainate in the culture medium, occluding the effect of K+ on α5 mRNA. Although kainate is significantly depolarizing already at 7–8 DIV (and maybe earlier), the changes of mRNA expression are likely to be delayed relative to changes in membrane potential. Thus, on this basis, it is feasible that the reciprocal changes in α5 and α6 mRNA

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**Fig. 5.** Furosemide inhibition of GABA-induced peak currents in mouse cerebellar granule neurons cultured in different media for 10–11 DIV. A, representative current traces of the inhibitory effect of furosemide (100 μM) on peak currents induced by 10 μM GABA in neurons cultured with 25 mM K+ with 50 μM kainate, 25 mM K+ without kainate, 5 mM K+ with 50 μM kainate, and 5 mM K+ without kainate, respectively. The increased current variation in the beginning of the furosemide-modulated traces was caused by transient voltage pulses used to monitor cell membrane conductance and capacitance. These pulses were suspended ~5 s before GABA application. B, each column represents the means ± S.E. (n = 4–14 neurons) for the inhibition of GABA-gated peak currents by 100 μM furosemide. The respective culture media are indicated below the columns. The asterisks denote the significance levels of the inhibition (*, p < 0.05; ***, p < 0.001). The two GABA concentrations employed, 10 and 30 μM, corresponds to the following response levels for each culture medium: 25 mM K+ with 50 μM kainate, 16 and 36%; 25 mM K+ without kainate, 17 and 34%; 5 mM K+ with 50 μM kainate, 18 and 37%; 5 mM K+ without kainate, 28 and 47% (calculated from the parameters in Table I).
expression are mediated more or less by differences in membrane potential.

The δ subunit has not been found (4, 26, 27) or found in very low levels (single cell bodies) (7) in rat cerebellum in vivo. In rat cerebellar granule neurons cultured in 12.5 or 25 mM K⁺, however, δ subunit mRNA has been found (24, 35). During development of the rat central nervous system, the δ gene expression in particular seems to undergo a prominent peak in the early brain (5, 6), i.e., the expression of the δ gene might, in some brain areas, indicate undifferentiated neurons. This is opposite to the α6 subunit, which is reported to mark maturation of cerebellar granule neurons in vivo (5, 7, 22).

Several studies show that the level of the α6 subunit mRNA and protein increases during the development of rat cerebellar granule neurons cultured in 25 mM K⁺ (e.g., Refs. 23, 24, and 51). Depolarizing K⁺ concentrations do not diminish the α6 level (35). Actually, one study shows that α6 expression is maintained by depolarizing K⁺ (36).

In contrast to this but in agreement with the present study, cultures of mouse cerebellar granule neurons grown in 25 mM K⁺ for 11–15 DIV have been shown not to express the α6 gene, whereas cultures grown for the same period in 5 mM K⁺ did (37). These results suggest a species-specific difference regarding regulation of α6 expression, which, however, may not apply to α6 subunit expression. In accordance with our results for mouse cerebellar granule neurons, Harris et al. (35) find that in rat granule neurons the level of α6 mRNA was higher after 5 DIV in 25 mM K⁺ than in 12.5 mM K⁺.

In the present study, the effect of K⁺ on α5 and α6 expression was mimicked by kainate at 12 DIV but not 7 DIV. To our knowledge, no AMPA or kainate receptor-mediated effects on α5 expression in mouse or rat cerebellar granule neurons have been reported. The α6 gene expression in rat cerebellar granule neurons has been found to be increased by glutamate. This effect was not sensitive to the NMDA receptor antagonist MKS01 (5SR,10S)-(−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine), suggesting involvement of AMPA/kainate and/or metabotropic glutamate receptors (35). In light of the proposed species-specific difference it may not be surprising that we found the opposite effect on α6 expression when using kainate, but this could also be due to activation of metabotropic glutamate receptors by glutamate in the rat experiments.

The Effect on β2 Subunit Expression Does Not Correlate with Membrane Potential—Compared with the α5 and α6 subunits the effects of K⁺ and kainate on β2 mRNA expression showed a very different pattern; at 7 DIV only change of K⁺ concentration was effective, whereas at 12 DIV only kainate had a weak effect. As for α5 and α6 mRNA, the effect of increased K⁺ at 7 DIV could potentially be due to depolarization, but because the K⁺ effect had vanished after 12 DIV we can postulate that either the mechanism had changed or it was never mediated through membrane potential. The lack of K⁺ effect after 12 DIV excludes the possibility that the kainate effect was mediated by depolarization. If expression of β2 mRNA is influenced by membrane potential at all, the effect seems to be transient. To our knowledge, there are no reports on the effect of kainate on the expression of GABA₆ receptor β subunits.

δ mRNA Expression—Interestingly, it has been reported that rat cerebellar granule neurons show very low expression of δ mRNA when cultured in 5 mM K⁺. However, δ mRNA expression increases significantly in culture medium with 25 mM K⁺− or in Mg²⁺−free medium containing NMDA (52). As for α6 mRNA, depolarizing medium is important for maintenance of δ mRNA expression (36). The lack of effect of depolarization on δ mRNA expression in mouse cerebellar granule neurons found in the present study could indicate a species difference in regulation of expression of the δ subunit, although it could also be due to differences in culture conditions.

GABA Sensitivity and Inhibition by Furosemide Reflect the α6 mRNA Level—The presence of α6 subunit in the GABA₆ receptor complex has functional implications with regard to GABA and furosemide sensitivity. Provided that a proportion of the α6 mRNA is normally translated into protein and incorporated into receptor complexes in the cell membrane, a correlation between mRNA expression and these functional properties would be expected.

Recombinant expression of α1β2γ2, α6β2γ2, and α1α6β2γ2 combinations in human embryonic kidney 293 cells (24) and oocytes (30) have demonstrated a higher EC₅₀ value for GABA in the α1α6β2γ2 combination (34 and 107 μM, respectively) than in the α1β2γ2 (14 and 41 μM, respectively) and α6β2γ2 (2 and 6.7 μM, respectively) combinations. A relatively modest expression of α6 subunit would probably result primarly in α1α6β2γ2 receptors, whereas a relatively high level of α6 would favor α6β2γ2 receptors with high sensitivity to GABA. In agreement with this we found that only the neurons with the highest level of α6 mRNA expression, namely those cultured in physiological K⁺ concentration without kainate, differed with regard to GABA sensitivity. These neurons had the lowest EC₅₀, e.g., significantly lower than neurons grown in 25 mM K⁺ (Table II, Fig. 4).

Selig and Baur (30) show that oocytes expressing the α1α6β2γ2 combination with regard to furosemide inhibition were more like the α6β2γ2 combination. Furthermore, it was possible to gradually vary the IC₅₀ for furosemide between the values for the α1β2γ2 and the α6β2γ2 combination by adjusting the α1/α6 cDNA ratio. These observations suggest that furosemide inhibition is a more sensitive indicator of α6 protein in the receptors than GABA EC₅₀. In agreement with this notion we found that furosemide inhibition at 10–11 DIV was significantly dependent on both K⁺ and kainate; this was also the case for α6 mRNA expression at 12 DIV. In addition, the rank order of α6 mRNA expression at 12 DIV and furosemide inhibition is in good agreement; that is, 5 mM K⁺ without kainate > 5 mM K⁺ with kainate > 25 mM K⁺ without kainate > 25 mM K⁺ with kainate. The kainate effect on α6 expression was not significant at 7 DIV but increased to a significant level between 7 and 12 DIV. Thus, the functional properties of the GABA₆ receptors at 10–11 DIV seem to be in accordance with development of mRNA levels from 7 to 12 DIV.

Our Cerebellar Granule Neurons Were Electrically Silent—Mellor et al. (37) report spontaneous miniature synaptic currents in mouse cerebellar granule neurons cultured and recorded in 5 mM K⁺, but not in 25 mM K⁺, at 15 DIV. Action potentials were also reported subsequent to adequate depolarization, but for neurons cultured and recorded in 25 mM K⁺, an initial hyperpolarizing current injection was required to reactivate the inactivated Na⁺ channels. In contrast, we did not observe spontaneous synaptic potentials or action potentials in any of the recorded neurons at 2–3 or 7–8 DIV. Because the membrane potentials in our neurons cultured and recorded in 25 mM K⁺, an initial hyperpolarizing current injection was required to reactivate the inactivated Na⁺ channels. In contrast, we did not observe spontaneous synaptic potentials or action potentials in any of the recorded neurons at 2–3 or 7–8 DIV. Because the membrane potentials in our neurons cultured and recorded in 5 mM K⁺ without kainate were more negative (−56 ± 3 mV at 2–3 DIV and −56 ± 1 mV at 7–8 DIV) than those of Mellor et al. (37) (−50 ± 2 mV at 15 DIV), inactivation of voltage-gated Na⁺ channels is not likely. The most probable reason for the lack of electrical activity is that necessary features such as synapses and high density of voltage-gated Na⁺ channels might be less developed at 7–8 DIV.

Concluding Remarks—Both a depolarizing K⁺ concentration and kainate in the culture medium have the potential to enhance survival of mouse cerebellar granule neurons, but this happens at the expense of maturation of the GABA₆ receptor
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This is normally not desirable, but with sufficient knowledge of the effects of K<sup>+</sup> and kainate these parameters may perhaps be exploited to adjust the subunit expression in a preferred direction. Species-specific effects on expression of some subunits make extrapolation from one species to another difficult. The actions of extracellular K<sup>+</sup> and kainate described here can probably be attributed to alterations of the membrane potential. To clarify the exact mechanism of action, more detailed experiments employing subtype-specific glutamate receptor antagonists and Ca<sup>2+</sup> channel blockers are required.

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Note Added in Proof—After acceptance of the present manuscript, we learned that J. H. Ives et al. found a decreased GABA<sub>A</sub> receptor α1, β6, β2 and an increased β3 subunit expression when mouse cerebellar granule neurons were cultured in 25 mM K<sup>+</sup> (Ives, J. H., Drewery, D. L., and Thompson, C. L. (2002) Neuropharmacol. 43, 715–725).

REFERENCES

1. Barnard, E. A., Skolnick, P., Olsen, R. W., Mohler, H., Sieghart, W., Biggin, G., Braestrup, C., Bateson, A. N., and Langer, S. Z. (1998) Pharmacol. Rev. 50, 291–313
2. Bonnert, T. P., McKernan, R. M., Farrar, S., Le Bourdelles, B., Heavens, R. P., Smith, D. W., Hewson, L., Rigby, M. R., Sirinathsinghji, D. J. S., Brown, N., Wafford, K. A., and Whiting, P. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9891–9896
3. Korpi, E. R., Grunder, G., and Liddens, H. (2002) Prog. Neurobiol. 67, 113–159
4. Laurie, D. J., Seeburg, P. H., and Wisden, W. (1992) J. Neurosci. 12, 1063–1076
5. Sigel, E., and Baur, R. (2000) J. Neurochem. 74, 2590–2596
6. Sinkkonen, S. T., Hanna, M. C., Kirkness, E. F., and Korpi, E. R. (2000) J. Neurosci. 20, 3588–3595
7. Brooks-Kayal, A. R., Shumate, M. D., Jin, H., Bickler, T. Y., Kelly, M. E., and Coulter, D. A. (2001) J. Neurosci. 21, 12039–12049
8. Verdoorn, T. A. (1994) Mol. Pharmacol. 45, 475–480
9. Korpi, E. R., Kuner, T., Seeburg, P. H., and Liddens, H. (1995) Mol. Pharmacol. 47, 283–289
