larized the membrane by current injection from −70 to 20 mV (n = 10) (data not shown) or by an increase of extracellular K^+ from 5 to 50 mM (n = 3) (data not shown). None of these experiments led to an increase in intracellular Ca^{2+} concentration, nor did we observe Ca^{2+} inward currents with the patch-clamp technique. We thus conclude that kainate triggers the entry of Ca^{2+} through the intrinsic pore of the kainate receptor channel.

We have demonstrated that Bergmann glial cells respond with complex behavior to the excitatory receptor ligand glutamate or to the more specific ligand kainate. This behavior includes the activation of an inward current that leads to a membrane depolarization, the entry of Ca^{2+}, and a concomitant blockade of the resting K^+ conductance. Although Ca^{2+}-dependent K^+ channels in most cell types are activated by an increase in cytosolic Ca^{2+}, adenosine 5′-triphosphate (ATP)–sensitive K^+ channels in the heart are inhibited by an increase of intracellular Ca^{2+} concentration from nominally 0 to 1 μM (9). Purkinje cells receive glutamatergic input, and the Bergmann glial cells are in intimate contact with these synaptic areas (*10*); excitation of the Purkinje cells may simultaneously trigger a cascade of events in the adjacent Bergmann glial cells. Such events could occur locally but also propagate along the cell. The functional consequences of the glial response are not known. However, the approach of assessing Bergmann glial cells in situ helps to elucidate such neuron-glia interactions.

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5. Young mice (postnatal day 17 to 22) were killed by decapitation, and 120-μm-thick slices were cut from the cerebellum. Slices were placed on a cover slip under a nylon mesh in a recording chamber [F. Edwards, A. Konnerth, B. Sakmann, T. Takahashi, *Pfluegers Arch.* **414**, 600 (1989)]. Slices were viable under these conditions for more than 3 hours. The chamber was continuously perfused with a salt solution containing 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, and 10 mM glucose and gassed with O_2. The pH was adjusted with NaOH to 7.2. Cell somata of the Bergmann glial cells were visible in normal water-immersion optics and could be approached by the patch electrode (pipette resistance ~10 megohms). The pipette contained 130 mM KCl, 0.5 mM $CaCl_2$, 5 mM EGTA, 2 mM $MgCl_2$, 10 mM Hepes, and, except for fura-2 experiments, Lucifer yellow 1 mg/ml. Membrane currents were measured with the patch-clamp technique in the whole-cell recording configuration [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *ibid.* **391**, 85 (1981)]. Current signals were amplified with conventional electronics (EPC-7 amplifier, List Electronics, Darmstadt, Germany), filtered at 3 kHz, and sampled at 5 kHz by an interface connected to an AT-compatible computer system, which also served as a stimulus generator.
6. Individual Bergmann glial cells were patch-clamped with pipettes containing 200 μM fura-2 in the tip and normal pipette solution in the rest of the pipette. In the whole-cell recording configuration, cells including their processes were filled with fura-2 within 10 to 40 min. The dye was excited at 340 and 380 nm with epifluorescence equipment (Axioplan Zeiss, Ober-kochen, Germany), and the resulting fluorescence signals were recorded with an intensified charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan). The Ca^{2+} transients are shown as the ratio of the fluorescence intensity measured during excitation at 340 nm (F_{340}) and the fluorescence intensity measured during excitation at 380 nm (F_{380}) [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985)]. Subcellular changes in Ca^{2+} concentration could be analyzed by selecting sections from the digitized image signals, and the pixels in these sections were averaged and extrapolated to continuous traces.
7. During recording, cells were filled with Lucifer yellow by dialyzing the cytoplasm with the patch pipette solution. To avoid destruction of the cell as the pipette was pulled off after recording, we destroyed the seal with a large hyperpolarizing current injection. After recording, slices were fixed for 3 to 5 hours at room temperature in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Slices were then transferred to phosphate buffer. Lucifer yellow–filled cells were examined in a microscope equipped with the appropriate filter combination (band pass, 400 to 440 nm; mirror, 460 nm; long pass, 470 nm).
8. After electrophysiology, slices were stained for glial fibrillary acidic protein (GFAP) by indirect immunofluorescence. Incubation with antibodies to GFAP (5 μg/ml diluted in 0.5% Triton X-100 in phosphate buffer, clone G-A-5) [E. Debus, U. Weber, M. Osborne, *Differentiation* **25**, 193 (1983)] was carried out overnight. Staining was visualized with goat antibody to mouse immunoglobulin. Slices were mounted with buffered glycerol.
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Calcium-Permeable AMPA-Kainate Receptors in Fusiform Cerebellar Glial Cells

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Glutamate-operated ion channels (GluR channels) of the L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–kainate subtype are found in both neurons and glial cells of the central nervous system. These channels are assembled from the GluR-A, -B, -C, and -D subunits; channels containing a GluR-B subunit show an outwardly rectifying current-voltage relation and low calcium permeability, whereas channels lacking the GluR-B subunit are characterized by a doubly rectifying current-voltage relation and high calcium permeability. Most cell types in the central nervous system coexpress several subunits, including GluR-B. However, Bergmann glia in rat cerebellum do not express GluR-B subunit genes. In a subset of cultured cerebellar glial cells, likely derived from Bergmann glial cells, GluR channels exhibit doubly rectifying current-voltage relations and high calcium permeability, whereas GluR channels of cerebellar neurons have low calcium permeability. Thus, differential expression of the GluR-B subunit gene in neurons and glia is one mechanism by which functional properties of native GluR channels are regulated.

Glutamate-operated channels are present in both neurons and glial cells. In most types of neurons (*1*) and in astrocytes (*2*, *3*), the GluR channels of the AMPA-kainate type are characterized by outwardly rectifying steady-state current-voltage (*I-V*) relations and low divalent-cation permeability. In recombinant GluR channels, transiently expressed in a mammalian cell line or in amphibian oocytes, both properties have been traced to the presence of an arginine residue in the putative TM2 transmembrane segment of the GluR-B subunit. Recombinant GluR channels containing a GluR-B (GluR-2) subunit show an outwardly rectifying *I-V* relation and a low permeability ratio P_{Ca}/P_{Cs}, whereas channels lacking a GluR-B subunit are characterized by a doubly rectifying *I-V* relation and a high permeability ratio P_{Ca}/P_{Cs} (*4*). In accordance with the finding that native GluR channels in most neurons and glial cell types show outward rectification and

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low Ca^{2+} permeability, in situ hybridization experiments with brain slices from mature rats demonstrated a widespread expression of the GluR-B subunit (*5*).

In situ hybridization studies have demonstrated that there is substantial expression of all four AMPA-kainate receptor genes in the cerebellum (*5*). High-power bright-field microscopy, however, reveals differences in their cellular expression patterns (Fig. 1). As shown previously (*5*), granule cells express only GluR-B and GluR-D (GluR-4) genes. The specific probes for the GluR-B and GluR-C (GluR-3) gene mRNAs label Purkinje cells (Fig. 1, B and C), whereas the GluR-A (GluR-1) and GluR-D probes abundantly decorate cells surrounding the Purkinje cells, which most likely are Bergmann glia (Fig. 1, A and D). A more detailed study with probes for the splice variants flip and flop corroborates this finding. The GluR-A flop variant accounts for the labeling of the Purkinje cells, whereas the flip forms of GluR-A and GluR-D mRNA are heavily expressed in the putative glial cells but not in Purkinje cells (Fig. 1, E and F). These data suggest that the GluR-A flip and the GluR-D flip splice versions are coexpressed in Bergmann glia. In contrast, the GluR-B subunit is not expressed in Bergmann glia. To find out whether there is a functional correlate of the specific expression pattern in this cell type, we established primary cultures of cerebellar glial cells and measured the rectification properties and the Ca^{2+} permeability of currents activated by specific GluR agonists in cerebellar glial cells.

Primary cultures of cerebellar neurons and glia were prepared (*6*, *7*), and astrocytes were identified by monoclonal antibodies against glial fibrillary acidic protein (GFAP), which specifically label mature astrocytes, and by a fluorescein-conjugated immunoglobulin (*8*). Primary cerebellar cultures contained several cell types: neuronal granule cells, astrocytes, oligodendrocytes, microglial cells, and fibroblasts (Fig. 2, A and B). To obtain cultures enriched with astrocytes, we either subcultured cells after agitation or seeded them on uncoated culture dishes (*9*). More than 90% of the cells obtained in this way were GFAP-positive. According to morphological criteria (*3*, *10*, *11*), these astrocytes could be divided into three groups: polygonal cells (probably type 1 astrocytes), stellate cells with radially distributed fine processes (probably type 2 astrocytes), and elongated or fusiform cells. Between 5 and 50% of the GFAP-positive cells in a culture dish were fusiform (Fig. 2, C and D). Cerebellar glial cultures with a high percentage of fusiform cells showed a low expression of the GluR-B subunit (Fig. 2F), suggesting that they may be derived from Bergmann glia. In primary cultures of chick cerebellum prepared in a similar way, fusiform glial cells have been shown to represent Bergmann glia (*11*). We therefore investigated the electrophysiological properties of GluR channels in these cells.

The input resistance of these fusiform cells with Na^+ as the major cation in the bath and Cs^+ as the major cation in the pipette varied between 0.3 to 0.6 gigaohm in different cells; the cell capacitance estimated from the transient current after a hyperpolarizing (50 mV) voltage step varied between 15 and 30 pF. None of the cells tested gave an action potential upon depolarization. When the selective agonists for AMPA-kainate–type GluR channels, kainate (300 μM) and AMPA (30 μM), were applied to voltage-clamped fusiform cells in normal rat Ringer solution (*12*), inward currents were evoked at negative membrane potentials (−60 mV) in 10 of the 20 cells tested. The response to 300 μM kainate was maintained during agonist application, whereas the response to 30 μM AMPA rapidly desensitized (Fig. 3A). The response to kainate was reversibly reduced by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective antagonist for GluR channels of the AMPA-kainate subtype. At 5 μM, CNQX blocked ~80% of the current evoked by 300 μM kainate (Fig. 3B). When Na^+ in the external solution was completely replaced by Ca^{2+}, large kainate-activated currents were still detectable at negative membrane potentials (Fig. 3C), indicating a high Ca^{2+} conductance of the GluR channels in fusiform glial cells. These results suggest that the native GluR channels in fusiform cells are assembled from subunits of the AMPA-kainate receptor family and do not include the GluR-B subunit.

This view is strengthened by comparison of steady-state *I-V* relations measured during kainate application in high Na^+ and high Ca^{2+} extracellular solution (Fig. 4A). In high Na^+ extracellular solution, the steady-state *I-V* relation of kainate-activated whole-cell currents, measured during a voltage-ramp stimulus, showed a characteristic doubly rectifying shape, with strong inward rectification at negative membrane potentials and a reversal potential of 3.5 ± 2.6 mV (n = 4). In extracellular solutions with high concentrations of Ca^{2+}, the *I-V* relation showed inward rectification and the reversal potential shifted to 13.3 ± 1.9 mV (n = 4) (Table 1). This result suggests an apparent permeability ratio P_{Ca}/P_{Cs} of 1.44 (*13*) and indicates that the native GluR channels in fusiform glial cells are more permeable to Ca^{2+} than to Cs^+. In accordance with our findings, a kainate-activated $^{45}Ca^{2+}$ influx into cultured fusiform glial cells was demonstrated (*11*). We also determined rectification and Ca^{2+} permeability of GluR channels in cerebellar granule cells to examine whether the prop-

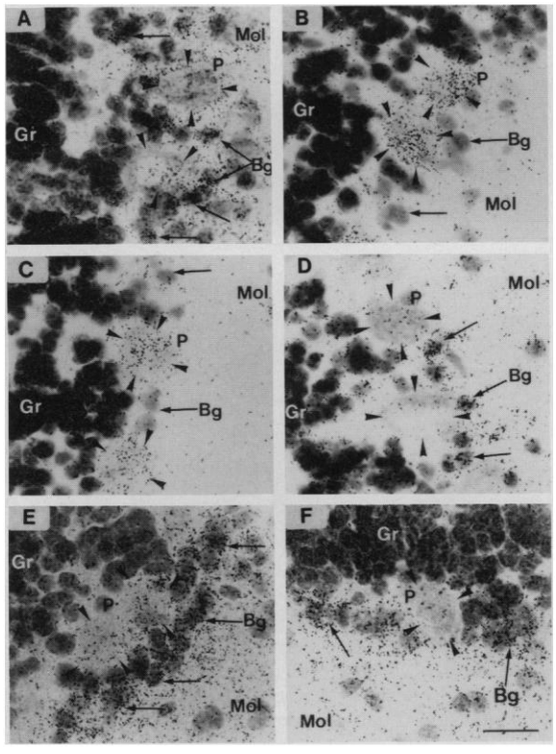

Fig. 1. In situ hybridization of AMPA-kainate–type GluR mRNAs in rat cerebellum. (**A**) GluR-A mRNA distribution (pan probe), (**B**) GluR-B pan probe, (**C**) GluR-C pan probe, (**D**) GluR-D pan probe, (**E**) GluR-A flip (probe specific for the flip splice version), and (**F**) GluR-D flip probe. High-power bright-field microscopy was used; oligonucleotides and procedures were as described (*5*). Purkinje cells are marked by arrowheads, and somata of putative Bergmann glial cells close to the Purkinje cell layer are indicated by arrows. Bg, putative Bergmann glia; Gr, granule cell layer; P, Purkinje cells; and Mol, molecular layer. Bar in (F), 22 μm.

erties of GluR channels in culture conditions remain cell-specific. Cerebellar granule cells were identified by their morphology and their ability to produce action potentials. The whole-cell *I-V* relation of the current activated by 300 μM kainate was almost linear in extracellular solutions with high Na^+ concentrations and indicated a reversal potential of -1.1 ± 3.0 mV (n = 4) but showed strong outward rectification in extracellular solutions with high concentrations of Ca^{2+} (Fig. 4B). The reversal potential shifted to -52.4 ± 4.7 mV (n = 3) (Table 1). This shift indicates a low permeability ratio P_{Ca}/P_{Cs} of 0.05 in cerebellar granule neurons, in agreement with published results (*3*).

The rectification and divalent permeability of GluR channels in cultured fusiform glial cells are very similar to those of recombinant GluR channels transiently expressed in mammalian host cells from GluR-A and GluR-D subunits of the AMPA-kainate receptor family (Table 1). The doubly rectifying shape of the *I-V* relation and the high Ca^{2+} permeability of GluR channels in fusiform glial cells of the cerebellum might therefore be a key to identifying the subunit composition of native GluR channels in Bergmann glia. Native channels in Bergmann glial cells probably consist of homomeric GluR-A and GluR-D channels or of heteromeric GluR-A–GluR-D channels. Although we cannot completely exclude that the expression pattern of GluR channels in glial cells changes under culture conditions, one feature of GluR channels lacking the GluR-B subunit, the doubly rectifying shape of the *I-V* relation, was recently confirmed in brain slices (*14*). A somewhat comparable behavior of native GluR channels has been reported in a subpopulation of cultured rat

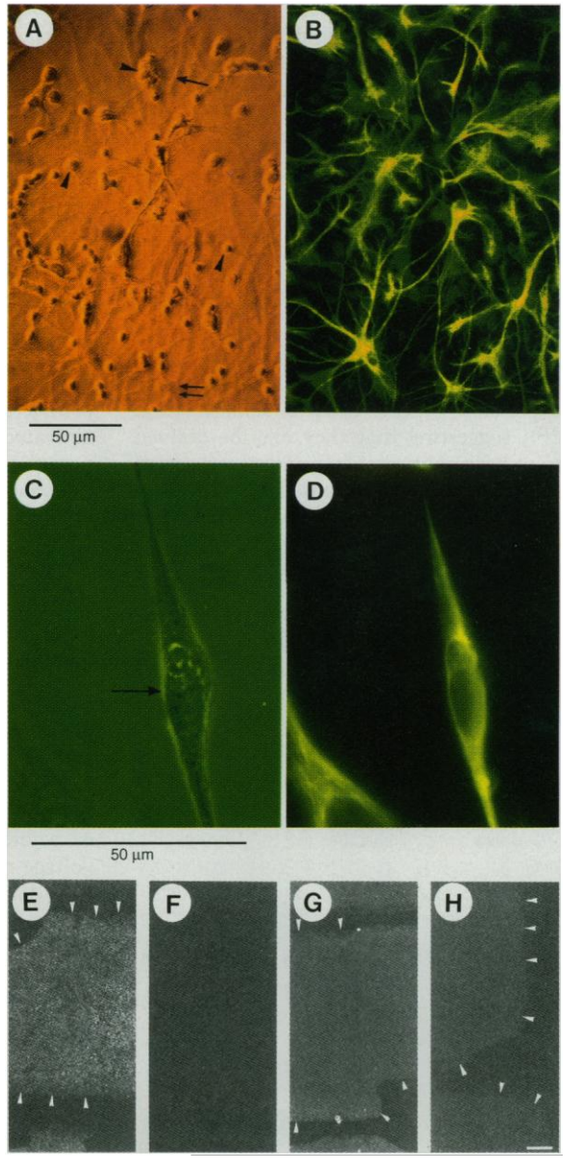

Fig. 2. GFAP immunofluorescence staining and expression pattern of GluR mRNAs of glial cells in primary cultures of rat cerebellum. (**A** and **B**) Mixed cultures of granule neurons and glial cells (13 DIV); same field of view, with (A) differential interference-contrast optics and (B) fluorescein isothiocyanate (FITC) fluorescence optics. The bright, single spherical granule neurons and aggregates of them (arrowheads) show no GFAP staining. The flat cells, which are less visible with differential interference-contrast optics, are GFAP-positive. Most of these GFAP-positive cells show stellate morphology (double arrow), some are polygonal, and some are elongated (arrow). (**C** and **D**) Glial cells of fusiform shape (13 DIV) 1 day after the agitation procedure and subculturing. Same field of view, with (C) phase-contrast optics and (D) FITC fluorescence optics. Cells were immunostained for GFAP with mouse anti-GFAP antibody as the primary antibody, followed by goat antibody to mouse immunoglobin G that was labeled with FITC (*8*). The fusiform cell (arrow) shown in (C) and (D) is characteristic of the cells used in the electrophysiological experiments. (**E** through **H**) Detection of AMPA-kainate–type GluR mRNAs in cultured glial cells (10 DIV) 2 days after the agitation procedure. The cells were seeded on poly-L-lysine–coated microscope slides (*9*), and low-power dark-field images using ^{35}S-labeled oligonucleotides were taken (exposure time, 4 weeks). The slides contained a high density of fusiform cells (about 50%). (E) GluR-A pan probe, (F) GluR-B pan probe, (G) GluR-C pan probe, and (H) GluR-D pan probe. Arrowheads delineate the signal boundary from the background. No signal could be detected with the GluR-B probe in glial cultures (F), even though the same probe hybridized strongly to cultured cerebellar granule cells and brain sections (data not shown). Similar results were obtained in five other cultures. Because no GluR-C mRNA is present in Bergmann glia in hybridized cerebellar sections (Fig. 1), the specific signal obtained with the GluR-C probe to the glial cultures (G) probably represents GluR-C subunit gene expression in another type of astrocyte. Bar in (H), 3 mm.

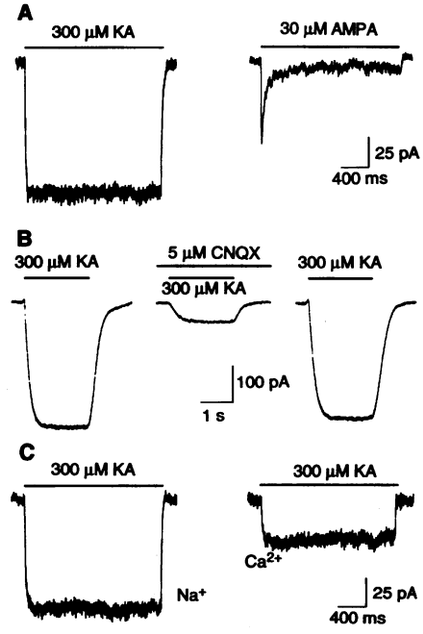

Fig. 3. Properties of AMPA-kainate–type GluR channels in fusiform glial cells determined from whole-cell current responses to different agonists. (**A**) Response of a fusiform cell to fast application of 300 μM kainate (KA, left trace) and 30 μM AMPA (right trace). The duration of agonist application is indicated by the bar above each trace. The cell was lifted from the bottom of the dish to obtain a more rapid solution change. (**B**) Block of responses to kainate by the AMPA-kainate receptor antagonist CNQX. Each trace represents the whole-cell current response to 300 μM kainate in control solution, in the presence of 5 μM CNQX, and after a 20-s washing period. The fusiform cell (10 DIV) underwent the agitation procedure and subculturing 1 day before. (**C**) Whole-cell current responses to 300 μM kainate in high Na^+ (140 mM) extracellular solution or high Ca^{2+} (110 mM) extracellular solution. The recording pipette contained high Cs^+ (140 mM) solution. The fusiform cell (8 DIV) in primary culture was seeded on a noncoated petri dish. The membrane potential was −60 mV for all recordings.

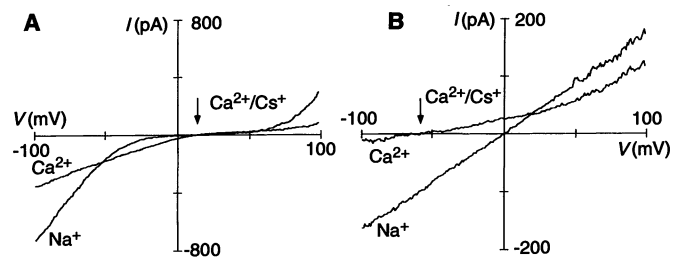

Fig. 4. High Ca^{2+} permeability of AMPA-kainate–type GluR channels in fusiform glial cells and low Ca^{2+} permeability in granule neurons. (**A**) Fusiform glial cell (8 DIV) in primary culture, seeded on a noncoated petri dish. (**B**) Granule neuron from a mixed primary cerebellar culture (10 DIV). Steady-state *I-V* relations of kainate-activated whole-cell currents were obtained in high Na^+ or high Ca^{2+} extracellular solution with voltage ramps. The curves represent the difference between membrane currents measured in the presence of and before application of 300 μM kainate. The reversal potential shifts in the positive direction from 4 mV in high Na^+ extracellular solution to 13.5 mV in high Ca^{2+} extracellular solution for the fusiform glial cell and in the negative direction from −1.6 to −61 mV for the granule neuron. The recording pipette contained high Cs^+ (140 mM) solution. Reversal potentials in high Ca^{2+} extracellular solution are indicated by arrows.

Table 1. Reversal potentials and rectification ratios of AMPA-kainate–type GluR channels in cerebellar cells compared to recombinant GluR channels expressed in human embryonic kidney cells (*4*). Reversal potentials (V_{rev}) were measured in the whole-cell recording configuration in high Ca^{2+} extracellular solution (110 mM Ca^{2+}). Rectification ratios G_{+80}/G_{-80} were measured as ratios of chord conductances at +80- and −80-mV membrane potential, respectively, in high Na^+ solution (140 mM Na^+). The recording pipette contained high Cs^+ (140 mM) solution. All data were obtained with 300 μM kainate. The means ± SEM for *n* cells are shown. Note that the experimental conditions for the two sets of experiments are similar. A *t* test was used for significance tests.

Channel	V_{rev} (mV) Ca^{2+}/Cs^+	*n*	G_{+80}/G_{-80}	*n*
Fusiform glia	13.3 ± 1.9	4	0.30 ± 0.06	4
Granule cells	−52.4 ± 4.7*	3	1.00 ± 0.10*	4
GluR-A	13.0 ± 5.0†	9	0.21 ± 0.13†	7
GluR-D	12.7 ± 2.3†	8	0.12 ± 0.05	5
GluR-A/D	3.5 ± 1.5	6	0.12 ± 0.04	6

*The values are significantly different from the corresponding values in fusiform glia ($\alpha = 0.01$). †The values are not significantly different from the corresponding values in fusiform glia ($\alpha = 0.2$).

hippocampal neurons (*15*), but nothing is known about the expression pattern of GluR-subunit genes in these cells, nor is the identity of these cells known. Correlations between the subunit expression pattern and the functional properties of native GluR channels have been noted in hippocampal neurons of the brain slice (*16*), but our results demonstrate the most striking correlation between functional properties of native GluR channels and recombinant channels constructed from those GluR subunits used as probes in in situ localization experiments.

The functional significance of the high Ca^{2+} permeability of GluR channels caused by the lack of expression of the GluR-B subunit in Bergmann glial cells remains unclear. The Ca^{2+}-permeable GluR channels might be involved in the control of glial-guided migration of cerebellar granule cells during development (*17*) but could also be related to the role of Bergmann glia in cellular signaling (*18*). Glutamate released from excitatory synapses made by the parallel-fiber nerve endings on Purkinje cells could spill over from the synaptic cleft and activate GluR channels of Bergmann glial cells, which ensheathe these synapses (*19*). The Ca^{2+} influx through glial GluR channels might trigger second messenger cascades that depend on excitation of parallel-fiber synapses. Alternatively, the Ca^{2+} influx could trigger the secretion of homocysteate, a transmitter-like substance present in Bergmann glia and released by Ca^{2+}-dependent mechanisms (*20*).

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9. Cerebellar cultures were prepared from Wistar rat pups 8 days after birth by methods described for cerebellar granule cells (*6*) and astrocytes (*7*), with some modifications. The glial cells and granule neurons were dissociated with 0.025% trypsin (type III from bovine pancreas, Sigma T8253) as described (*6*); trypsinization was terminated by the addition of soybean trypsin inhibitor (Sigma T9003) and deoxyribonuclease (DNase) (type IV from bovine pancreas, Sigma D5025). After the last centrifugation step (160*g*, 5 min), the cell pellet was resuspended in culture medium containing Dulbecco's modified Eagle's medium without sodium pyruvate, with glucose (4500 mg/liter) (Gibco 041-01965), 10 to 15% heat-inactivated fetal calf serum (Boehringer 210471), 2 mM L-glutamine (Sigma G3126), penicillin (100 IU/ml), and streptomycin (100 μg/ml). When the cells were plated on poly-L-lysine–coated petri dishes, the mixed primary culture contained several neuronal and nonneuronal cell types. For enrichment of cerebellar granule cells, cells were seeded on poly-L-lysine–coated (5 μg/ml, Sigma P1524) 3.5-cm petri dishes, the concentration of KCl in the medium was increased to 25 mM, and 10 μM cytosine β-D-arabinofuranoside (Sigma C1768) was added to the medium after 1 day in vitro (DIV). For enrichment of astrocytes and putative Bergmann glial cells, two different procedures were used, both exploiting the fact that astroglia-related cells adhere more readily to surfaces than neurons and oligodendrocytes (*7*). In the first procedure, the cells were seeded into 3.5-cm petri dishes that were not covered with poly-L-lysine (2 ml of suspension per dish). Floating cells were removed by exchanging the medium every 3 to 4 days. The adhering cells were kept in primary culture and were used after 8 to 12 DIV for electrophysiological experiments. In the second procedure, cells were grown in 250-ml Falcon flasks covered with poly-L-lysine (8 ml of suspension per flask), submitted to an agitation procedure after 8 to 12 DIV (orbital shaker, 280 to 300 rpm, 37°C for 16 hours), and then subcultured. The flasks were rinsed three times each with culture medium and with phosphate-buffered saline (PBS) containing 8 g/liter NaCl, 2.16 g/liter $Na_2HPO_4 \cdot 7H_2O$, 0.2 g/liter KCl, and 0.2 g/liter KH_2PO_4. Then PBS with 0.05% trypsin and 10 μM EDTA was added for 3 min (room temperature, 5 ml per flask). Trypsinization was stopped with culture medium containing 0.1% DNase (10 ml per flask). Cells were resuspended with the fluid stream from a pipette, centrifuged at 160*g* for 5 min, resuspended in culture medium, and plated onto uncovered or covered 3.5-cm dishes (25,000 to 100,000 cells per dish) or on covered microscope slides. The plated cells were used for electrophysiological experiments 1 to 2 days after subculturing. Fusiform GFAP-positive cells were obtained after both culture procedures; the yield was higher with the method of agitation and subculturing. No difference in the electrophysiological properties was noted.
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12. Whole-cell current-recording experiments were performed on cells beginning from 8 DIV and up to 13 DIV in primary culture and 1 to 2 days after subculturing and after the agitation procedure to ensure a sufficiently low density of cells and to avoid electrical coupling [H. Kettenmann and B.

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Apparent permeability ratios P_{Ca}/P_{Cs} were calculated as described (*15*) from average values of reversal potentials; we assumed biionic conditions and used the following ion concentrations: 140 mM Cs^+ and 110 mM Ca^{2+}.

14. The doubly rectifying *I-V* relation of a kainate-type glutamate receptor and the Ca^{2+} influx in Bergmann glial cells of mouse cerebellar slices have recently been demonstrated by T. Müller, T. Möller, T. Berger, J. Schnitzer, and H. Kettenmann [*Science* **256**, 1563 (1992)].
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Hebbian Depression of Isolated Neuromuscular Synapses in Vitro

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Modulation of synaptic efficacy may depend on the temporal correlation between pre- and postsynaptic activities. At isolated neuromuscular synapses in culture, repetitive postsynaptic application of acetylcholine pulses alone or in the presence of asynchronous presynaptic activity resulted in immediate and persistent synaptic depression, whereas synchronous pre- and postsynaptic coactivation had no effect. This synaptic depression was a result of a reduction of evoked transmitter release, but induction of the depression requires a rise in postsynaptic cytosolic calcium concentration. Thus, Hebbian modulation operates at isolated peripheral synapses in vitro, and transsynaptic retrograde interaction appears to be an underlying mechanism.

In formulating a cellular mechanism underlying the temporal specificity in associative learning, D. O. Hebb (*1*) postulated that the coincidence of electrical activities in the pre- and postsynaptic cells helps modulate the strength of the synaptic connection. Extensions (*2*) of Hebb's postulate suggest that synaptic efficacy may be potentiated or stabilized by synchronous pre- and postsynaptic activities but weakened by asynchronous activities. This Hebbian mechanism has been used to account for several forms of synaptic plasticity in mature and developing central nervous systems (*3*), but whether it operates at peripheral synapses is unknown. We investigated whether Hebbian modulation occurs at isolated neuromuscular synapses in culture, where the simplicity of the cellular environment facilitates the study of its mechanisms.

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Isolated myocytes innervated by single cocultured spinal neurons in 1-day-old *Xenopus laevis* nerve-muscle cultures (*4*) were used (Fig. 1A). In the first set of experiments, the postsynaptic myocyte was repetitively activated by iontophoretic application of acetylcholine (ACh) pulses to the myocyte surface near the synapse either synchronously or asynchronously with suprathreshold stimulation of the presynaptic neuron. The membrane current of the myocyte was monitored by whole-cell voltage-clamp recording (*5*). The amplitude and duration of the ACh pulses were adjusted to produce a membrane current equal to that of a usual impulse-evoked postsynaptic current (EPC). We assayed the synaptic efficacy by measuring the EPC amplitude before and after repetitive coactivation (Fig. 1, B and C). During the first 10-min control period, the mean amplitude of EPCs elicited by low-frequency test stimuli remained relatively constant. The mean EPC amplitude was unaffected by synchronous coactivation (100 stimuli at 2 Hz). In contrast, coactivation with the same number of stimuli but applied asynchronously to the same synapse with a delay of 100 ms between the post- and presynaptic activation, reduced the EPC amplitude. The result was the same regardless of whether the synchronous coactivation was applied before or after the asynchronous one.

The temporal specificity of coactivation in producing synaptic depression was revealed by a series of experiments in which the presynaptic stimulation was applied either at or ± 10, ± 63, ± 125, and ± 250 ms after the onset of each ACh pulse applied to the myocyte (Fig. 1D). With no delay (synchronous coactivation), depression was not observed. Small but significant depression was found when a ± 10-ms interval was imposed between the pre- and postsynaptic stimuli. The effect increased to a maximum of about 50% at intervals above 63 ms. This critical dependence of synaptic depression on the asynchrony between pre- and postsynaptic activation agrees with the expected temporal specificity in Hebbian modulation. In these experiments, the myocyte was voltage-clamped at the resting potential during repetitive coactivation to allow assessment of the induced membrane current at the myocyte. Similar results were obtained when the myocyte was held in current-clamp condition, allowing depolarization of the myocyte membrane potential (*6*).

We next tested whether synaptic depression required postsynaptic ACh receptor activation, presynaptic stimulation, or both. Repetitive postsynaptic iontophoresis of ACh in the absence of presynaptic stimuli induced synaptic depression similar to that induced by the asynchronous coactivation. Significant depression was observed when the myocyte was held in either voltage-clamp or current-clamp condition during ACh application, which suggests that postsynaptic receptor activation in the absence of synchronous presynaptic activity was sufficient to induce synaptic depression (Fig. 2A). Thus, presynaptic activation is not required. Furthermore, presynaptic suprathreshold stimulation (100 pulses at 2 Hz) alone did not result in significant depression ($n = 4$), which indicates that asynchronous postsynaptic activation is not only sufficient but also necessary for the induction of depression.

The depression induced by asynchronous postsynaptic activation was long lasting (Fig. 2B). Pulses of ACh were applied in current-clamp condition, and synaptic responses were monitored for 20 min after ACh application. No significant recovery of synaptic responses was observed. Persistent depression was observed in all four cases for which synaptic responses were monitored for 1 hour after ACh applica-