A Stimulus Paradigm Inducing Long-term Desensitization of AMPA Receptors Evokes a Specific Increase in BDNF mRNA in Cerebellar Slices

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Abstract

Long-term desensitization of AMPA receptors (LTDA) is a core mechanism of long-term depression, a model of motor learning in the cerebellum. In this study we investigated the expression of neurotrophic factor genes after induction of LTDA in cultured cerebellar slices. LTDA was induced by application of quisqualate and monitored as a population response with a wedge recording technique. The levels of mRNA were quantified by reverse transcription followed by polymerase chain reaction. Quisqualate, at a dose and duration that reliably induced LTDA, elicited a significant and specific increase in BDNF mRNA with a peak at four hours after the application. By cell fractionation, the major source of BDNF mRNA increase was found to be in granule cells. In addition, a small but significant increase of transcripts with specific exon usage was observed in a Purkinje cell fraction. These results indicate that BDNF may be coinduced with LTDA and suggest that the slow and sustained increase of BDNF mRNA might play a role in later phases of synaptic plasticity in the cerebellum.

Introduction

Maintenance of long-term memory is reported to require protein and mRNA syntheses, whereas induction of long-term memory, in some models of synaptic plasticity (Montarolo et al. 1986; Teyler and DiScenna 1987), does not. Several genes have been identified that are coinduced in long-term sensitization, a model of Aplysia learning (Kennedy et al. 1992; Mayford et al. 1992), and in long-term potentiation (LTP), a model of memory in the cerebral cortex and hippocampus of mammals (Wisden et al. 1990; Patterson et al. 1992). This suggests that neural activity induced by learning generally initiates a cascade of gene expression, but it is not clear whether these genes are necessary and sufficient for the long-lasting synaptic modification. To answer this question clearly, ablation or suppression of specific gene expression will be of great help, but for that purpose candidate genes coinduced in long-lasting memory models must be identified first. Little is known, however, about gene expression in long-term depression (LTD), a model of motor learning in the cerebellum.
LTD is a persistent depression of transmission efficacy at synapses between parallel fibers and Purkinje cells induced by conjunctive stimulation of parallel and climbing fibers (Ito 1989). Electrophysiological studies have shown that persistently reduced sensitivity of the α-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA) type of glutamate receptor in Purkinje cells is the basic mechanism of LTD (Ito 1989; Linden and Connor 1991; Hémar et al. 1994), which we refer to as long-term desensitization of AMPA receptor (LTDA). One of the main reasons why little is yet known at the gene level about cerebellar LTD has been the difficulty in inducing, and recording LTD in large numbers of cells. LTD is difficult to detect with mass field potentials and thus induction of LTD by electrical stimulation was initially restricted to limited populations of cells (Ito 1989). Recently, however, recording from a population of Purkinje cells has become possible. Using a grease gap method in cerebellar slices trimmed to a wedge shape (wedge recording), Ito and Karachot (1989, 1990, 1992) have shown LTDA, which lasts up to 13 hr, in a population of Purkinje cells. It has also been found that joint application of the metabotropic glutamate receptor (mGluR) agonist (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD) and AMPA, or application of quisqualate alone, which activates both mGluR and AMPA receptors, can induce LTDA (Ito and Karachot 1989; Linden et al. 1991). The model of LTDA induced by pharmacological stimulus and monitored by wedge recording has a great advantage in the stability of recording and the number of neurons sampled, which are essential for the molecular biological study.

As a first step toward understanding the regulation of gene expression in cerebellar LTD, we adopted the quisqualate-induced LTDA model in cerebellar slices with monitoring by wedge recording. In this study we focused on the changes in the expressions of neurotrophic factors because they are necessary for the normal development and maintenance of neural functions (Otten et al. 1980; Diamond et al. 1987; Kalman et al. 1990; Nawa et al. 1993). Their expression in forebrain, mostly in the hippocampus, has also been shown to be increased by a variety of stimuli (Gall and Isackson 1989; Zafra et al. 1990; Ernfors et al. 1991; Isackson et al. 1991; Lu et al. 1991; Lindvall et al. 1992), including LTD (Patterson et al. 1992; Castrén et al. 1993). Quantification of neurotrophin mRNAs in the cerebellum demonstrated that brain-derived neurotrophic factor (BDNF) mRNA was specifically induced by LTDA-evoking stimulation.

Materials and Methods

SLICE CULTURE

Cerebellar slices were cultured as described by Stoppani et al. (1991), with some modification. Cerebella were dissected from 14- to 15-day-old Wistar rats and placed on agar immersed in ice-cooled basal salt solution (BSS) containing 124 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 2.0 mM MgSO4, 2.5 mM CaCl2, 10 mM glucose, and 22.0 mM NaHCO3 equilibrated with 95% O2 + 5% CO2 gas. The vermis was cut sagittally at 400 μm thickness with a rotating blade (Rotorslicer, Dosaka-EM, Kyoto, Japan). The slices were then transferred to porous transparent membranes (Cell culture insert, pore size 0.45 μm, Becton Dickinson, Bedford, MA), placed in six-well chambers with 1.5 ml of culture medium, and maintained in a humidified atmosphere of 5% CO2 in air at 37°C. The culture medium was a mixture of 50% of Dulbecco's modified Eagle medium, 25% of Hanks' balanced salt solution, and 25% of horse serum, supplemented with HEPES (final concentration 16.7 mM, buffered to pH 7.3 with NaOH), glucose (4.5 g/l), L-glutamine (0.39 g/liter), NaHCO3 (18.9 mM), streptomycin (0.1 g/liter), and penicillin (100 U/ml) (all from GIBCO BRL, New York).

ELECTROPHYSIOLOGY

After cultivation for 2–3 days in vitro, a slice was trimmed to a wedge shape containing a few cortical folia of lobule VI with connected white matter and transferred to a recording chamber (Fig. 1A, below). The population responses of Purkinje cells in a slice to various agonists were recorded by a wedge recording technique (Garthwaite et al. 1986; Ito and Karachot 1989). Briefly, a wedge-like slice was placed in a two-compartment bath and sealed with grease in such a way that its white matter traversed a narrow slit in the septum. The direct current (d.c.) potential between the two compartments was monitored continuously with Ag/AgCl electrodes embedded in 4% agar in saline and a high-input impedance am-
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differentiator (AN-601G, Nihon-koden, Tokyo, Japan), and displayed on a chart recorder. The d.c. potential reflects the depolarization of the population of the Purkinje cells, because the only axons passing through the grease gap that give rise to the potential difference between the chambers are those of Purkinje cells. The sealing resistance was 50–120 kΩ. The chamber was perfused with BSS containing 0.5 μM tetrodotoxin (TTX) (Sankyo, Tokyo, Japan) equilibrated with 95% O₂ + 5% CO₂ at a rate of 1.5 ml/min at 35–36°C. Test drugs were added to the perfusate.

RNA ISOLATION AND QUANTITATIVE RT PCR

For RNA analysis of many slices, from which accompanying electrophysiological recordings were not made, whole slices were stimulated as a batch in the same condition, as in wedge recording. Slices on culture membranes were transferred to the chambers ("resting chamber") containing 0.5 μM TTX in culture medium and stabilized for 2 hr. They were then rapidly transferred to chambers containing test drugs and TTX in medium, stimulated for a defined period, rinsed in another chamber, and returned to the resting chamber. Total cellular RNA was extracted from slices as described by Chomczynski and Sacchi (1987). In some experiments total RNA was recovered from a wedge-like slice after electrophysiological study. The yields of total RNA from whole slices and wedge-like slices were ~10 μg and 1 μg, respectively. Reverse transcription was performed to synthesize cDNA from 0.5-μg samples of total RNA using 5 U/μl of Moloney murine leukemia virus (MMLV)–reverse transcriptase (RT) (Superscript II, GIBCO-BRL, NY) at 42°C for 1 hr in 20 μl of reaction buffer containing 50 mM Tris-HC1 (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dNTP, and 25 μg/ml of oligo(dT). A volume of 1 μl of the cDNA solution (corresponding to 25 ng of total RNA) was amplified by polymerase chain reaction (PCR) with 0.02 U/μl of Taq polymerase (AmpliTaq, Perkin-Elmer Cetus, Norwalk, CT) in 50 μl of reaction buffer containing 10 mM Tris-HC1 (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 mM dNTP, 0.21 μM of each primer, and 1.11 kbps/μl (~0.01 μM) (α-32P)dCTP. Amplified cDNA was resolved by 4% polyacrylamide gel electrophoresis (PAGE), and the dried gel was exposed to an imaging plate and analyzed with a laser-scanning imaging system (BAS2000, Fuji film, Tokyo, Japan). The imaging plate has a very wide and linear dynamic range of about four digits (i.e., 10⁴), in addition to its high sensitivity to radioactivity, and is thus best suited for quantitative measurement of the mRNA level.

To correct for variation in recovery of RNA, in efficiency of reverse transcription, and in efficiency of amplification, we adopted the method of coamplification of endogenous mRNA (Chelly et al. 1988; Noonan et al. 1990). Glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA, which is expressed ubiquitously and constitutively, was thus amplified with a second set of primers in the same PCR tube and used as an internal control. The radioactivity of the cDNA of interest was normalized to that of GAPDH cDNA in each sample. To assure the linearity of our RT–PCR assay, we performed preliminary experiments for each cDNA to be analyzed, by sampling 5-μl aliquots at every third cycle of the reaction. We found, for example, that the linear range for GAPDH was between 21 and 30 cycles, and for BDNF, between 31 and 40 cycles. Thus PCR was usually carried out first with a set of primers for BDNF for seven cycles, and then a set of primers for GAPDH was added to the PCR tube for a further 27 cycles. One normalized value means that the level of BDNF mRNA is about 2⁻⁷ of that of GAPDH mRNA in this case.

One cycle of PCR consisted of denaturation at 94°C for 30 sec, primer annealing at 60°C for 45 sec, and primer extension at 72°C for 45 sec. The sequences of primer sets were as follows (from 5' to 3'): (NGF) sense TCTTCTTCAACAGGACTCA-CAG, antisense ACACACCGAGGCTGTATCTTATC; (CNTF) sense GAGAACCTCCAGGCTTACGGT, antisense TGGCTCTCAAGGTGATGATC; (FGF) sense ATCCCAAGCGGCTCTACTGCA, antisense AACAGATGCTCCCTGTGTCAG; (GAPDH) sense ATCCCAAGCGGCTCTACTGCA, antisense AAGATGCTCCCTGTGTCAG; (BDNF, exon 5 (common)) sense GCACGTGATCGAAGAGCTGCT, antisense GTCTATCCTTTGAAAGCAGCCAG; (BDNF, exon 1) sense TAAGACACTGAGTCTCCAGGAC; (BDNF, exon 2) sense AGTGTATTGCTCCAGGATCACG; (BDNF, exon 3) sense GAGAACTCTCGCTTTCTACAT; (BDNF, exon 4) sense AGCGTGATTGCTCCAGGATCAT; (BDNF, exon 1–4) antisense GGCACTGTCAAGAGCTGCT.

VIABILITY ASSAY

Lactate dehydrogenase (LDH) released from damaged cells was determined from aliquots of
culture medium. The remaining cellular LDH was determined by lysing cells with a 0.2% Triton X-100 in PBS for 10 min. LDH activity in 50-μl samples of medium or cell lysates was measured using an LDH assay kit (Sigma, St. Louis, MO) and procedures specified by the supplier. The amount of LDH activity in the medium was normalized to total activity.

ISOLATION OF PURKINJE CELLS AND GRANULE CELLS

Purkinje and granule cells were purified by reported methods (Sellinger et al. 1974; Mikoshiba et al. 1979), with minor modifications for small-scale samples. Briefly, 45 cultured slices, which were either stimulated or unstimulated (control), were cut into ~0.5-mm squares on ice and suspended in 7.5% polyvinyl pyrrolidone containing 1% bovine serum albumin (BSA) and 3.77 mM CaCl₂. The suspension was pushed gently through a series of nylon meshes with pore sizes of 750, 300, 108, and 70 μm. The sieved suspension was layered over a three-step gradient of 2.3, 1.75, and 1.0 M sucrose containing 1% BSA and centrifuged at 41,000 g for 30 min at 4°C in a Beckman SW55Ti rotor. The fine band at the 2.30--1.75 M interface, which corresponds to cerebellar neurons, was collected, filtered through a nylon mesh of 59 μm pore size, layered on a three-step gradient of 2.3, 1.9, and 1.75 M sucrose containing 1% BSA, and centrifuged at 110,000 g for 2 hr at 4°C in an SW55Ti rotor. Purkinje cells were recovered from the 1.9--1.75 M interface and granule cells from the 2.3--1.9 M interface.

The purity of preparations was determined by immunocytochemical examination of inositol trisphosphate receptor (IP₃R) protein, a specific marker of Purkinje cells. Isolated cells were plated on coverslips coated with poly-L-lysine (Sigma, St. Louis, MO), treated with anti-IP₃R monoclonal antibody (18A10), and stained by the avidin–biotin complex method with diaminobenzidine as a chromogen, as described previously (Yuzaki and Mikoshiba 1992).

DRUGS

AMPA, quisqualate, trans-ACPD, and L-aspartate (all from Tocris Neuramin, Bristol, UK) were dissolved in water as 1000× stock solutions (the pH was adjusted when necessary) and frozen at −40°C until use.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical significance was analyzed by Student’s t-test.

Results

STIMULUS CONDITIONS FOR INDUCTION OF LTDA IN CULTURED SLICES

Our initial experiments showed that the procedures for preparing acute slices sometimes significantly increased the expressions of immediate early genes (IEGs) such as c-fos, c-jun, and zif/268, which could conceivably interfere with the expression of late genes. Therefore, to investigate long-lasting changes in gene expression associated with cerebellar neuronal activity reproducibly, we used short-term cultured cerebellar slices instead of freshly isolated slices. The low levels of expression of IEGs were restored and remained at low levels after 1–2 days in culture. The slices could be maintained for >2 weeks.

The responses of Purkinje cells in cultured slices were recorded by a wedge recording technique (Garthwaite et al. 1986; Ito and Karachot 1989) (Fig. 1A). Application of 10 μM AMPA for 2 min induced responses of ~0.2–0.6 mV, which could be evoked repeatedly with little sign of desensitization (data not shown). Application of 100 μM quisqualate for 5 min as a conditioning stimulus caused the desensitization of succeeding AMPA responses (Fig. 1B, upper trace) for at least 4 hr, whereas application of 10 μM AMPA for 5 min did not (Fig. 1B lower trace). The desensitization of AMPA responses was not caused by deterioration of neurons in the slices because the responses to 3 mM L-aspartate before and after the conditioning stimuli were similar (Fig. 1B). These data are summarized in Figure 1C. In addition, lactate dehydrogenase (LDH) leakage in the medium 2 hr after stimulation, expressed as a percentage of that before stimulation, was 108±8% (n=3), 102±7% (n=3), and 111±6% (n=3) after a 5-min application of 10 μM AMPA, 100 μM quisqualate, and medium only, respectively, indicating that the desensitization of AMPA response induced by quisqualate was not attributable to damaged neurons. Thus, LTDA of Purkinje cells could be induced in cultured slices.

Next, we investigated the stimulation conditions for induction of LTDA in cultured slices in detail. As shown in Figure 3A (below), application
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Figure 1: Wedge recording and induction of long-term desensitization of AMPA responses in cultured cerebellar slices. (A) Diagram of the perfusion chamber. A cultured slice cut into a wedge shape was placed in a two-compartment bath. Gray matter containing Purkinje cell somata was perfused with test solution and separated from white matter in the adjacent compartment by grease. The potential difference between the two compartments was monitored with Ag/AgCl electrodes embedded in 4% agar in saline. (B) Representative wedge recordings. One hour after conditioning with 100 μM quisqualate (Quis) for 5 min, the response to 10 μM AMPA remained desensitized to ~35% of the response 10 min before conditioning, whereas the response to 3 mM L-aspartate (Asp) was not changed significantly (upper trace). The AMPA and Asp responses were not changed by conditioning with 10 μM AMPA for 5 min (lower trace). (C) Summary of results on conditioning stimulus. The degrees of desensitization at 1 hr after conditioning are expressed as percentages of the control response before conditioning stimulus. Conditioning with quisqualate (100 μM), but not AMPA (10 μM), for 5 min significantly desensitized subsequent AMPA responses (open columns). Responses to aspartate (3 mM) remained unchanged (shaded columns) with both conditioning stimuli. The numbers in parentheses show the number of slices. Bars with asterisks indicate significant change: (*) \( P<0.05 \); and (**) \( P<0.01 \), compared with the AMPA response after conditioning with AMPA (10 μM) for 5 min.

QUISQUALATE SPECIFICALLY INCREASED BDNF MRNA

As shown in Figure 2A, quisqualate, applied at 100 μM for 5 min increased the expression of BDNF significantly. Stimulation with 10 μM AMPA did not increase the BDNF mRNA but it caused a significant and larger increase in nerve growth factor (NGF) mRNA than quisqualate did. This suggests that quisqualate-induced NGF expression is mediated by AMPA receptor activation by quisqualate. The other neurotrophic factors studied, ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (FGF), did not show any increase on AMPA or quisqualate treatment. Thus the BDNF mRNA was increased specifically by the quisqualate stimulus.

Next, we analyzed the time course of expression of BDNF mRNA. As shown in Figure 2, B and C, BDNF mRNA increased significantly 4 hr after application of quisqualate and remained at a high level even 16 hr after the stimulus. NGF mRNA also followed a similar time course of expression with a peak at 4 hr after stimulation (Fig. 2C, right), but the other neurotrophic factors tested remained relatively constant during the observed period (data not shown). The level of GAPDH mRNA expression, the internal control of mRNA quantification, remained almost constant (Fig. 2B).
ACTIVITY-DEPENDENT CHANGE IN CEREBELLAR BDNF mRNA

**Figure 2:** Specific increase in BDNF mRNA by quisqualate and its time course. (A) Cerebellar slices were stimulated by 100 μM quisqualate or by 10 μM AMPA or medium alone as controls, each for 5 min. mRNAs for NGF, CNTF, FGF, and BDNF were analyzed as described in Materials and Methods. NGF mRNA was significantly elevated by AMPA stimulation (P<0.05), and BDNF mRNA specifically by quisqualate (P<0.01) (n=3). (B) Slices were stimulated in the same way as A. Total RNA was extracted 1, 4, 8, and 16 hr after conditioning, and RT–PCR was performed as described in Materials and Methods. Bands with arrows correspond to amplified cDNAs for BDNF (511 bp) and for GAPDH (228 bp). (C) Time course of increase in BDNF (left) and NGF (right) mRNA. Radioactivity of amplified cDNA was normalized to that of GAPDH cDNA and plotted against time.

**STIMULUS CONDITIONS FOR INCREASES OF BDNF mRNA AND COMPARISON WITH THAT FOR LTDA**

The BDNF mRNA level was increased by application of 100 μM quisqualate for 5 min or more, but not for 4 min or less (Fig. 3A, left). It was also increased by application of AMPA for 6 min. The expression of BDNF mRNA was thus dependent on the duration of stimulation in the same way as was the induction of LTDA (Fig. 3A, right).

The dose response of BDNF mRNA expression indicated that an increase was not observed on application of 10 μM quisqualate, but at higher concentrations of quisqualate up to 100 μM, it increased in a dose-dependent manner (Fig. 3B, left). This finding was also consistent with results on induction of LTDA (Fig. 3B, right).

Application of AMPA (10 μM) or mGluR agonist trans-ACPD (300 μM) alone failed to increase the BDNF mRNA level (Fig. 3C, left) or to induce LTDA (Fig. 3C, right). Coactivation of AMPA and mGlu receptors induced significant LTDA and increased BDNF mRNA to some extent. This suggests that both LTDA induction and BDNF mRNA expression involves the coactivation of AMPA and mGlu receptors. The difference in the ability of combined AMPA and trans-ACPD application to induce LTDA and BDNF mRNA expression may suggest that there is some mechanistic dissociation between the two phenomena, that needs further study.

It should also be noted that the BDNF mRNA expression and the LTDA induced by coapplication of AMPA and trans-ACPD (300 μM) were less than those induced by quisqualate (Fig. 3C). This was also the case when a higher concentration of trans-ACPD (1 mM) was used (data not shown). This may be because trans-ACPD is a weak and partial agonist in activating the mGluR1 subtype of metabotropic glutamate receptors, which is expressed in both granule and Purkinje cells (Aramori and Nakanishi 1992; Yuzaki and Mikoshiba 1992). In addition, trans-ACPD is a very potent agonist of mGluR2 in Golgi and granule cells in the cerebellum, and it also effectively activates mGluR3 in glial cells (Tanabe et al. 1992). Thus, the activation of mGluR1 by the more potent agonist quisqualate may be more effective for the induction of BDNF mRNA and LTDA.

In summary, quisqualate induced both LTDA and BDNF mRNA expression with a very similar dose and duration of application. Their induction involves activation of both AMPA receptor and mGluR (probably mGluR1). These results suggest that BDNF mRNA may be coinduced by LTDA-evoking stimulation and shares some common pathways of induction.

**INCREASE IN BDNF mRNA IN GRANULE AND PURKINJE CELLS**

We analyzed the cells responsible for the increase in BDNF mRNA. We used a cell fraction-
Figure 3: Stimulus conditions for increasing BDNF mRNA and comparison with that for LTDA. (A) Effect of duration of drug application. Cultured cerebellar slices were conditioned with 100 μM quisqualate (shaded columns), or with 10 μM AMPA (solid columns) for indicated durations. Radioactivity of BDNF mRNA at 4 hr after stimulation was analyzed and normalized to GAPDH mRNA (left). The degree of LTDA at 1 hr was expressed as percentage of the control AMPA response (right). Most of the LTDA results were obtained from separate experiments using wedge recording to monitor LTDA for typically 1 hr. From at least one wedge in each treatment, we recovered RNA after electrophysiological recordings. The desensitization values at 1 hr were quite similar to those at 4 hr after induction of LTDA, and increases in BDNF mRNA were also similar to the increases observed without electrophysiological recordings, giving the rationale for comparison. (B) Effect of quisqualate concentration. Slices were stimulated by indicated concentrations of quisqualate for 5 min. Expression of BDNF mRNA (left) and degree of desensitization (right) are shown as described in A. (C) Effect of AMPA and trans-ACPD. Slices were conditioned with 10 μM AMPA, 300 μM trans-ACPD, 10 μM AMPA + 300 μM trans-ACPD, or 100 μM quisqualate for 5 min. Expression of BDNF mRNA (left) and degree of desensitization (right) are shown as described in A. Numbers in parentheses indicate numbers of samples studied. Bars with asterisks indicate significant change: (*) P<0.05; (**) P<0.01, compared with the value for 5-min application of AMPA (A,C) or no drug (concentration 0) (B).

Fractionation of cerebellar neurons has the advantage that Purkinje cells and granule cells, major constituents of cerebellar neurons, differ greatly in size, and so can be separated easily by density gradient centrifugation. Contamination of the granule cell fraction with Purkinje cells was <1%, as judged by immunocytochemical staining of IP3R, a specific marker of Purkinje cells (Fig. 4A, right). Contamination of the Purkinje cell fraction with granule cells was difficult to assess because of lack of a good marker of granule cells, but estimated to be ~2% by morphological examination (Fig. 4A, left). These estimations are generally in good agreement with results of an earlier study (Sellinger et al. 1974).

In unstimulated slices, BDNF mRNA was expressed in granule cells at a level ~24 times higher than in Purkinje cells (Fig. 4B). Stimulation by quisqualate increased the expression in granule and Purkinje cells to ~2.5 and 2.8 times higher levels, respectively, than those in unstimulated slices (Fig. 4B). These values are similar to those obtained in unseparated cells (Figs. 2 and 3), indicating that the fractionation procedure did not affect the succeeding mRNA analyses. As there are ~250–900 times more granule cells than Purkinje cells (Ito 1984), the levels of BDNF mRNA observed in cerebellar slices at rest and after stimulation by quisqualate are mainly attributable to those in granule cells. It should be noted that BDNF mRNA was also expressed in the Purkinje cell fraction and was increased by stimulation.

Whereas we cannot fully rule out the possibility that some of the BDNF mRNA observed in the Purkinje cell fraction was caused by contamina-
ACTIVITY-DEPENDENT CHANGE IN CEREBELLAR BDNF mRNA

A Cell fraction
Purkinje granule

B 50 µm

Figure 4: Appearance of Purkinje and granule cells and BDNF mRNA after fractionation. (A) Appearances of the Purkinje cell fraction (left) and granule cell fraction (right) by phase-contrast microscopy. Cells were treated with monoclonal antibody against IP₃R, a specific marker of Purkinje cells, and stained by the avidin/biotin/ peroxidase method using diaminobenzidine as a chromogen. Scale bar, 50 µm. (B) Cerebellar slices were treated with 100 µM quisqualate (shaded column) for 5 min as an LTDA-evoking stimulus or medium alone as a control (open column). After 4 hr, the Purkinje cell and granule cell fractions were purified as described in Materials and Methods. BDNF mRNA normalized by GAPDH mRNA was assayed in the Purkinje cell (left) and granule cell (right) fraction as described in Materials and Methods. Data in B were obtained from two independent experiments.

Discussion

In this study we have shown delayed changes of gene expression after induction of LTDA in cer-

THE PROMOTER USED IN QUISQUALATE-INDUCED ELEVATION OF BDNF mRNA

Recently, it has been reported that the rat BDNF gene consists of four short 5' exons with different promoter regions and one 3' exon encoding the mature BDNF protein and that alternative usage of four promoters and differential splicing control tissue-specific and seizure-induced expression of BDNF mRNA (Timmusk et al. 1993; Kokaia et al. 1994). To determine which promoters are used for the increased BDNF mRNA on quisqualate stimulation, we next used exon-specific primers for RT-PCR.

We first applied RT-PCR with these primers in a kainate-induced seizure model (Timmusk et al. 1993) to evaluate the specificity of our method. As shown in Figure 5A, marked increases in BDNF mRNA containing exons 1 and 3 were found in the hippocampus 4 hr after injection of kainate, compared with the level in saline-injected control animals. Transcripts with exon 1 and exon 3 increased 18.3- and 12.1-fold, respectively, over the control levels, whereas transcripts with exons 2 and 4 increased 3.4- and 2.5-fold, respectively. These results are very similar to those obtained by Northern hybridization (Timmusk et al. 1993), confirming the specificity of our method. We found that transcripts with exons 1 and 3 also increased specifically in the cerebellum, although their total increase was less than in the hippocampus (Fig. 5A, right).

To determine whether Purkinje and granule cells use different promoters, we studied exon usage in isolated cells (Fig. 5B, right). Exon 1 was mainly used in both cell types after stimulation with quisqualate, its usage increasing about 2.6- to 2.7-fold. In granule cells, transcripts with exons 3 and 4 increased 1.9- and 2.1-fold, respectively. These increases were similar to those in unfractio-
A Kainate-induced seizure in vivo

Hippocampus Cerebellum

Quisqualate-induced Total cell fraction

BDNF mRNA relative to control

BDNF mRNA relative to control

Figure 5: Expression of BDNF mRNA containing different 5′ exons after seizure induced by kainate and after stimulation by quisqualate. (A) Adult rats (weight 210–220 grams) were injected intraperitoneally with kainate (12 mg/kg body weight) or saline as a control. Total RNA was prepared from the hippocampus (left) and cerebellum (right) 4 hr later, and BDNF transcripts were analyzed with different exon-specific primers as described in Materials and Methods. Data are expressed relative to control levels. (B) Cultured cerebellar slices were treated with 100 μM quisqualate for 5 min as an LTDA-evoking stimulus or medium alone as a control (45 slices for each treatment). After 4 hr, slices were cut into pieces as described in Materials and Methods, and ~1 out of 45 chopped slices were reserved for the analysis of unfractionated slices, and the remainder were fractionated. BDNF transcripts with different 5′ exons were analyzed in unfractionated slices (left) or in fractionated granule and Purkinje cells (right). Data were obtained from one experiment and expressed relative to control levels. Similar results were obtained in another set of experiments.

SPECIFIC INDUCTION OF BDNF mRNA BY QUISQUALATE IN THE CEREBELLUM

Quisqualate, applied under conditions similar to those for inducing LTDA, increased BDNF mRNA after 4 hr. Neurotrophin expression in neurons has been shown to be increased by neuronal activity in the forebrain, especially in the hippocampus (Gall and Isackson 1989; Zafra et al. 1990; Ernfors et al. 1991; Isackson et al. 1991; Lu et al. 1991; Lindvall et al. 1992). The stimulation used in most studies involved a variety of strong pathological stimuli, such as experimentally induced seizure (Gall and Isackson 1989; Zafra et al. 1990; Ernfors et al. 1991; Isackson et al. 1991), insulin-induced hypoglycemic coma and cerebral ischemia (Lindvall et al. 1992), and prolonged exposure to high potassium solution in vitro (Lu et al. 1991). Recently, Bessho et al. (1993) showed that BDNF mRNA was increased by quisqualate in cerebellar granule cells, although the condition for stimulation was not physiological, as it involved 0.5–4 hr in dispersed culture. We found that the expression of this neurotrophin is increased in the cerebellar slice by more moderate stimulation, suggesting that BDNF mRNA may be controlled under physiological neuronal activity in the cerebellum.

The level of increase in BDNF mRNA in the cerebellum following mild quisqualate stimulation was about threefold above that of the control value and less than one-fifth of that in the hippocampus after kainate-induced seizure (Fig. 5A), and that of other earlier studies using strong stimuli. The level of increase was, however, comparable to the two- to threefold increase in the hippocampus after LTP-evoking stimulation (Patterson et al. 1992; Castrén et al. 1993), suggesting that the physiological range of increase in BDNF mRNA may be similar in different brain regions.

It is interesting that of the neurotrophins studied, BDNF mRNA was specifically increased, and using cell fractionation, the observed changes could be shown to occur in two main types of neurons, Purkinje and granule cells. In addition, quisqualate-induced expression of BDNF mRNA in the cerebellum and in fractionated cells was found to involve a unique pattern of exon usage.
et al. 1991; Lindvall et al. 1992; Zafra et al. 1992). One explanation for the specific induction of BDNF mRNA that was observed in the cerebellum is that it results from the milder stimulus used, consistent with specific increases in BDNF mRNA in the visual cortex following illumination (Castrén et al. 1992) and in the hippocampal CA1 area following LTP (Patterson et al. 1992). If this were the case, then NGF mRNA would be expected to be coinduced with BDNF mRNA by a stimulus of higher intensity. However, AMPA, which caused more depolarization than quisqualate (Fig. 1B) and increased NGF mRNA, failed to increase BDNF mRNA (Fig. 2A). This suggests the alternative explanation that the specific increase of BDNF mRNA may mainly be attributable to the activation of mGluR by quisqualate.

EXON USAGE OF BDNF mRNA IN THE CEREBELLUM AND CELLULAR LOCALIZATION

Recently, alternative usage of four exons of the rat BDNF gene has been reported to control tissue-specific and stimulus-specific regulation of BDNF mRNA expression (Timmusk et al. 1993; Kokaia et al. 1994). However, these reports concern the forebrain, and not the cerebellum. We have showed here that kainate also increases the expression of BDNF mRNA in the cerebellum, with very similar usage of exons as in the hippocampus (Fig. 5A).

Basal and activity-dependent expression of BDNF has been found mainly in granule cells. We consider that at least part of the BDNF mRNA observed in the Purkinje cell fraction reflected the low level of expression of BDNF mRNA in those cells, because the exon usage was different from that in the granule cell fraction. Studies using ISH have shown that BDNF mRNA was not localized to Purkinje cells, but to granule cells in the cerebellum (Hofer et al. 1990; Rocamora et al. 1993). The discrepancy between our results and those of earlier studies can be explained by the higher sensitivity of RT–PCR over ISH. It should also be noted that granule cells are packed more densely than Purkinje cells, as there are ~250–900 times more granule cells than Purkinje cells (Ito 1984), and thus grain density in granule cell layers is sometimes exaggerated in ISH. Further studies using RT–PCR with single Purkinje cells will be needed to clarify the discrepancy.

FUNCTIONAL SIGNIFICANCE OF INCREASED BDNF mRNA AND RELATIONSHIP WITH LTD

BDNF was initially identified as a trophic factor for peripheral sensory neurons (Barde et al. 1982) and recently has been shown to promote the survival and/or differentiation of mesencephalic dopaminergic neurons and basal forebrain cholinergic neurons (Alderson et al. 1990; Hyman et al. 1991; Kniisel et al. 1991). BDNF has also been shown to regulate the expression of neuropeptides in GABA-ergic neurons (Nawa et al. 1993). During development of the cerebellum, expression of BDNF mRNA is mainly localized in the internal granule cell layer and increases dynamically with a peak around postnatal day 20 (Rocamora et al. 1993), at which time the synapse formation between parallel fibers of granule cells and Purkinje cells is prominent (Altman and Winfree 1977). In contrast, transcripts of the functional BDNF receptor gene, trkB, are expressed predominantly in Purkinje cells (Klein et al. 1990). Although the effect of BDNF on Purkinje cells remains to be determined, these observations suggest that BDNF expressed in granule cells may have a trophic action on Purkinje cells such as maintenance or regulation of synapses between granule cells and Purkinje cells. The BDNF mRNA observed in the Purkinje cell fraction also raises the possibility of autocrine actions of BDNF in these neurons (see also Kokaia et al. 1993; Miranda et al. 1993).

It is not clear whether BDNF mRNA elevation and LTD are causally related or whether BDNF is involved in the process of LTD at all. LTD and LTDA are attributable to the reduced AMPA receptor sensitivity in Purkinje cells (Ito and Karachot 1989; Linden et al. 1991). The desensitization of AMPA receptors in Purkinje cells, probably by phosphorylation, is a phenomenon observed within a few minutes of conditioning stimulus that persists for many hours. But information storage, based solely on post-translational modification of proteins such as phosphorylation, is generally considered to deteriorate rapidly (Dudai 1989). Thus, although the time course of BDNF mRNA expression is too slow to account for initial induction, it is possible that BDNF may have some role in later phases of synaptic plasticity, because if LTD is a basic mechanism of motor learning, it should eventually be transformed into a permanent memory. The efficacy of synaptic transmission can be affected by the morphological changes in the syn-
aptic spine or the formation of new synapses, as has been suggested in the hippocampal LTP (Dudai 1989). For example, if the electrical resistance of the synaptic spine neck is decreased or the new synapses are formed on more distal dendrites, the voltage change at the soma induced by the synaptic input may be depressed. The number of synapses per Purkinje cell is reported to increase after motor learning but not after simple exercise (Black et al. 1990; Isaacs et al. 1992). Consequently, BDNF mRNA expression in granule and Purkinje cells might play a role in such morphological manifestations. Further studies, for example, involving functional ablation of BDNF, will be useful in clarifying the role of this neurotrophin in cerebellar learning.

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