Weaver Granule Neurons Are Rescued by Calcium Channel Antagonists and Antibodies Against a Neurite Outgrowth Domain of the B2 Chain of Laminin

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Abstract. The weaver mutation impairs migration of the cerebellar granular neurons and induces neuronal death during the first two weeks of postnatal life. To elucidate the molecular mechanisms for the impaired neuronal migration, we investigated the rescue mechanisms of the weaver (wv/wv) granule neurons in vitro. We found that Fab_2 fragments of antibodies against a neurite outgrowth domain of the B2 chain of laminin enhanced neurite outgrowth and neuronal migration of the weaver granule neurons on a laminin substratum and in the established cable culture system. The rescue of the weaver granule neurons by antibodies against the B2 chain of laminin may result from the neutralizing effect of these antibodies against the elevated B2 chain levels of the weaver brain. The L-type calcium channel blocker, verapamil (1–5 μM), also rescued the weaver granule neurons. High concentrations of MK-801 (10–20 μM), a glutamate receptor antagonist and voltage-gated calcium channel blocker, rescued the weaver granule neurons similar to verapamil, but low concentrations of MK-801 (1 μM) had no rescue effect. Simultaneous patch-clamp studies indicated that the weaver granule neurons did not express functional *N*-methyl-D-aspartate receptors further indicating that the rescue of the weaver granule neurons by MK-801 resulted from its known inhibition of voltage-gated calcium channels. The present results indicate that antibodies against the B2 chain of laminin, verapamil, and high concentrations of MK-801 protect the weaver granule neurons from the otherwise destructive action of the weaver gene. Thus, both the laminin system and calcium channel function contribute to the migration deficiency of the weaver granule neurons.

THE weaver mutant mouse provides a model system for studying mechanisms of neuronal migration and death (Sidman et al., 1965), because the granule neurons of the homozygous weaver (wv/wv) mouse cerebellum fail to migrate and die during the first two weeks of postnatal life (Rakic and Sidman, 1973 *a,b,c*; Sotelo and Changeux, 1974; Sotelo, 1975). The weaver gene defect has been genetically mapped to chromosome16 in the mouse (Reeves et al., 1989), but the affected gene(s) have not been identified. It has recently been suggested that a point mutation in a GIRK2 K^+ channel gene (Lesage et al., 1994) could be responsible for the weaver mutation (Patil et al., 1995; Slesinger et al., 1996). However, recent electrophysiological experiments contradict this view, because GIRK2 channel activity was detected neither in normal (+/+) nor weaver (wv/wv) granule neurons during the developmental stage when the neurons die (Mjaatvedt et al., 1995). Genetic studies indicate that the weaver defect is intrinsic to the granule neurons (Goldowitz and Mullen, 1982; Goldowitz, 1989), and recent in vitro and transplantation studies have further shown that the weaver granule neurons can be rescued by normal granule neurons (Gao and Hatten, 1993) or their membrane extracts (Gao et al., 1992).

The role of calcium channel function and glutamate receptors in the migratory failure and death of the weaver granule neurons has not been investigated even though recent evidence indicates that normal migratory cerebellar granule neurons express functional *N*-methyl-D-aspartate (NMDA)[1] receptors (Rossi and Slater, 1993; Farrant et al., 1994), and that both NMDA receptor blockers and voltage-gated calcium channel antagonists inhibit neuronal migration of the normal cerebellar granule neurons (Komuro and Rakic, 1992, 1993). Elevated levels of intracellular calcium are involved in neurodegenerative mechanisms of the brain tissue (Choi, 1988) and it is therefore of spe-

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1. *Abbreviations used in this paper*: GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; NMDA, *N*-methyl-D-aspartate.

cial relevance to determine the interrelationship of the calcium channel function and the weaver mouse mutation.

The B2 chain of laminin is involved in migration of the normal cerebellar granule neurons in vitro (Liesi et al., 1992) and in vivo (Liesi et al., 1995). Therefore we recently investigated the expression of laminin and its B2 chain in the migration-deficient weaver cerebellum in vivo and in cultures of the weaver granule neurons (Murtomäki et al., 1995). We found that expression of laminin, L1-antigen, and tissue plasminogen activator (tPA), all relevant for neuronal migration and survival (Sanes, 1989; Liesi, 1990), are affected by the weaver mutation (Murtomäki et al., 1995). Overexpression of laminin, its B2 chain, and tPA in neuronal and glial cells is one of the major molecular characteristics of the weaver brain (Murtomäki et al., 1995). Proteolytic activity of the weaver granule neurons is also increased in vitro, and the affected neurons deposit B2 chain–derived peptides along their surfaces (Murtomäki et al., 1995).

Survival and neurite outgrowth of the weaver granule neurons can be enhanced by a serine protease inhibitor, aprotinin, which indicates that increased tPA activity mediates the weaver gene action (Murtomäki et al., 1995). Increased proteolysis in conjunction with increased synthesis of the B2 chain of laminin may be relevant for the weaver neuronal death, since increased proteolysis and expression of the B2 chain of laminin also occur in the brain in human neurodegenerative disorders, such as Alzheimer's disease and Down's syndrome (Murtomäki et al., 1992). Proteolysis may degrade laminin and release small biologically active peptides that have been shown to accumulate in the brain in human neurodegenerative disorders (Murtomäki et al., 1992), and to bind along the surfaces of the migration-deficient weaver granule neurons (Murtomäki et al., 1995). Because high (μM) concentrations of these B2 chain–derived peptides are known to have a neurotoxic function (Liesi et al., 1989), their increased expression may participate in neuronal death, and impair neuronal migration in the affected brain tissue. In this study, we tested the hypothesis that the B2 chain–derived peptides may be neurotoxic to the (wv/wv) granule neurons. We cultured the weaver granule neurons in the presence of antibodies that specifically recognize a decapeptide (P1543) from a neurite outgrowth domain of the B2 chain of laminin (Liesi et al., 1989; Murtomäki et al., 1995) to investigate whether these antibodies can restore the neurite outgrowth potential of the weaver granule neurons.

Our results indicate that the weaver granule neurons can be rescued by antibodies specific for the B2 chain–derived peptides. This observation supports the view that overexpression of the B2 chain of laminin along surfaces of the weaver granule neurons (Murtomäki et al., 1995) may impair neuronal migration in the weaver cerebellum. We also show that the weaver granule neurons are rescued by verapamil, a specific L-type calcium channel blocker, which indicates that inhibition of calcium influx into the weaver granule neurons protects the weaver granule neurons from the action of the weaver gene. Similar rescue effect was obtained by high concentrations of MK-801 (10–20 μM), known to inhibit voltage-gated calcium channel function in hippocampal neurons (ffrench-Mullen and Rogawski, 1992). The present data demonstrate three novel ways of restoring the neurite outgrowth potential of the weaver granule neurons. Our results indicate that the weaver gene modulates the effect of laminin on migratory granule neurons and affects either the activity of voltage-gated calcium channels or neuronal sensitivity to influx of calcium to create an inhibitory environment for neurite outgrowth and neuronal migration.

Materials and Methods

Mice

Heterozygous (+/wv) mice carrying the weaver mutation were obtained from the Jackson Laboratories (Bar Harbor, ME), and bred by heterozygous matings at the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health. The mice were free of MHV, Sendai, and other common mouse pathogens. They were bred on a B6CBA-$A^{W\text{-}J/A}$ wv genetic background, and the homozygous weaver (wv/wv) and normal control (+/+) littermates were used for experiments 7–10 d after birth during the active migratory phase of the cerebellar granule neurons.

Cerebellar Cultures on Laminin

Cultures of granule neurons from P7 cerebella of the weaver mice (wv/wv) and their normal (+/+) littermates were initiated as previously described in detail (Murtomäki et al., 1995). 10^5 cells were plated on 25-mm glass coverslips covalently coupled with laminin (Liesi et al., 1989), and cultured in a serum-free RPMI 1640 culture medium (GIBCO BRL, Gaithersburg, MD). The coverslips were pretreated with 10 μg/ml BSA (Sigma Chemical Co., St. Louis, MO) for 1 h to saturate the free binding sites on the laminin-coated coverslips. After an initial 1–2-h period, the culture medium was changed and either the drugs or Fab_2 fragments of the laminin antibodies were added to the culture medium. The neurons were cultured for 24–48 h in 5% CO_2/95% air at +37°C and fixed in 2% paraformaldehyde in PBS for quantitation or immunocytochemistry. The drugs used were dizocilpine maleate (+MK-801; 1–100 μM), (±)-2-amino-5-phosphopentanoic acid (APV; 10–100 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), and ω-conotoxin GVIA (1–2 μM) from Research Biochemicals International (Natick, MA) and ±verapamil hydrochloride (1–5 μM) from Sigma Chemical Co. The divalent Fab_2 fragments of rabbit polyclonal antibodies against a decapeptide (P1543) from a neurite outgrowth–promoting domain of the B2 chain of laminin (Liesi et al., 1989) were used at 40 μg/ml. These B2 chain–specific antibodies recognize a free decapeptide from a neurite outgrowth domain of the B2 chain of laminin but not the native laminin molecule (Murtomäki et al., 1992). The Fab_2 fragments of the preimmune sera of the B2 chain–specific antibodies were used as controls and also applied at 40 μg/ml. Monoclonal antibodies (mAb 1533) against a 30–amino acid peptide containing a neurite outgrowth domain of the B2 chain of laminin (Murtomäki et al., 1992) were used at 40 μg/ml.

The attachment and neurite outgrowth of the weaver granule neurons on a laminin substratum were quantitated using six random fields in the fixed cultures of the normal and weaver granule neurons with or without the drug or antibody treatments. The mean numbers of neurons and of neurons with long neurites (>10× cell soma; >100 μm) were evaluated in each case. Approximately 300 neurons were evaluated per experimental group. One-way variance analysis (ANOVA) on the Instat (1.11a) program (GraphPad Software, San Diego, CA) was used for statistical analysis, if more than two groups were compared simultaneously. The statistical comparisons between individual groups of neurons were performed using a Student-Newman-Keuls multiple comparisons test or Kruskal-Wallis nonparametric ANOVA test.

Characterization of Neuronal Cultures on a Laminin Substratum

The proportions of neuronal and glial cell types in cultures of the normal and weaver cerebellum were verified using neuronal and glial marker proteins. We found that in normal cultures, ≥98% of the cells are morphologically neuronlike and express the neuronal marker L1-antigen (Rathjen and Schachner, 1984). Glial fibrillary acidic protein positive (GFAP+) astrocytes comprise ≤2% of the cells in these cultures. In the weaver cultures, ≥95% of the cells are morphologically neuronlike, and express L1

antigen. The GFAP+ astrocytes comprise ≤5% of the cells in the weaver cultures. Although L1 antigen is a neuronal marker in the CNS cultures (Lindner et al., 1986), it is not a specific granule neuronal marker (Brummendorf and Rathjen, 1995). To investigate the possibility that a proportion of the L1-positive neurons were not granule neurons but GABAergic stellate and basket cells, we applied double immunocytochemistry for L1 antigen and glutamic acid decarboxylase (GAD). GAD is the rate-limiting enzyme of the GABA synthesis, and granule neurons that are the only glutaminergic neurons in the cerebellum are negative for GAD. We found that in normal cultures, all L1-positive neurons were GAD negative, whereas in wv/wv cultures ~4% of the L1-positive neurons expressed GAD immunoreactivity indicating that they were GABAergic. The GAD+ neurons were larger than the granule neurons and had multipolar morphology. Thus, we conclude that a large majority of the cells in our cerebellar cultures are L1 antigen–positive granule neurons. The rabbit antibodies against GAD were from Chemicon International (Temecula, CA), and the mouse monoclonal antibodies to L1 antigen were from Boehringer Mannheim GmbH (Mannheim, Germany). Immunocytochemistry was performed as described (Liesi et al., 1992).

Immunocytochemistry for L1-Antigen and B2 Chain of Laminin In Vitro

Neurons were doubly immunostained using rabbit polyclonal antibodies against a granule neuronal marker, L1 antigen (Rathjen and Schachner, 1984; Lindner et al., 1986), and mouse monoclonal antibodies against the B2 chain of laminin (mAb 1533) as described (Liesi et al., 1992). The antibodies were used at 10 μg/ml and 5 μg/ml, respectively.

Cerebellar Granule Neurons in the Cable Culture System

Cerebellar neuronal migration was also studied in the cable culture system (see below), because this culture system has been widely accepted as a model system to study neuronal migration (for references see Trenkner, 1991). We have earlier demonstrated that neurons in the cable cultures migrate either along the glial fibers or along the L1-positive fibers of other neurons (Liesi et al., 1992). Thus, neuronal migration in the cable culture system appears to proceed via two modes of neuronal movement, one facilitated by the glial cells and one facilitated by the neurons. This is in line with recent work that neuronal migration of cerebellar granule neurons may occur without glial guidance (Nagata and Nagatsuji, 1990) and may proceed via nuclear movement inside the preestablished neuronal fibers (Liesi, 1992; Hager et al., 1995; Liesi et al., 1995). The cable cultures were initiated from postnatal (P7) normal and weaver granule neurons as described (Trenkner, 1991; Liesi et al., 1992). The cells were plated at 3×10^4/well on a glass surface in 16-well LabTek dishes, and allowed to reaggregate for 12 h. Either 10 μM MK-801 or 40 μg/ml of the Fab_2 fragments or preimmune sera of the B2 chain–specific antibodies were added, and the cultures were maintained for 72 h. During this time, the reaggregates of the granule neurons became connected by "cables," e.g., neuronal and glial fibers that connect the reaggregates. Neurons then started to migrate along these cables, and we investigated their migratory potential in the presence or absence of the rescuing agents. The culture medium was 5% horse serum in basal Eagle's medium (GIBCO BRL), as previously described (Trenkner, 1991). The cultures were fixed in 2% paraformaldehyde in PBS, and immunostained for L1 antigen to evaluate the numbers of migratory granule neurons along the cables with or without MK-801 or laminin antibodies. The quantitation of migratory neurons was done by counting all L1 antigen–positive neuronal cell bodies on cables in six randomly selected microwells with or without the drug treatments. All migratory neurons in the selected microwells were counted. Statistical analysis of the results was performed using Kruskal-Wallis nonparametric ANOVA test on the Instat (1.11a) program. In some experiments, double immunocytochemistry for L1 antigen and GFAP was performed to evaluate the numbers of glial cables (GFAP+ cables) as opposed to the cables formed by the migratory granule neurons themselves (GFAP− cables). We used rabbit polyclonal antibodies to L1 antigen (Rathjen and Schachner, 1994), and mouse monoclonal antibodies for GFAP (Sigma Chemical Co.). Immunocytochemistry was as described earlier (Liesi et al., 1992).

Electrophysiology of the Glutamate Receptors

Conventional whole cell patch-clamp recording techniques (Hamill et al., 1981) were applied to study the glutamate receptor function in cultures of normal and weaver granule neurons on a laminin substratum. The recordings were performed at room temperature using an EPC-7 patch-clamp amplifier. External solution for agonist trials contained (in mM): 150 NaCl, 2.4 KCl, 1 $CaCl_2$, 0.003 TTX, 10 Hepes, and 10 glucose; pH was adjusted to 7.2 with NaOH. The pipette solution contained (in mM): 100 $CsMeSO_4$, 30 CsCl, 5 BAPTA, 2 $MgCl_2$, and Hepes-K (pH 7.2). Solution was delivered by gravity flow from a multibarrel array consisting of fused silica tubing (i.d.= 200 μm) connected to independent reservoirs. Experiments were recorded on a PCM/VCR tape system (2-B; Medical Systems Corp., Greenvale, NY).

Results

Weaver Granule Neurons on a Laminin Substratum

The culture system used here is suitable to investigate the effects of various chemical compounds and neuromodulators on neuronal survival and neurite outgrowth of cerebellar granule neurons. This is because neurons in this culture system are grown at low density on a laminin substratum in a serum-free RPMI culture medium without growth factor supplementation for 24–48 h. We applied this culture system to investigate the survival and neurite outgrowth of normal (+/+) and weaver (wv/wv) granule neurons. After 24 h in vitro, the normal (+/+) cerebellar granule neurons extended long (>10× cell soma; >100 μm) neurites on a laminin substratum (Fig. 1 *A*; Fig. 2). The normal neurons had a bipolar neuronal morphology and they coexpressed L1 antigen (Fig. 1 *A*, *green*) and the B2 chain of laminin (Fig. 1 *A*, *red*) along their surfaces. In contrast, the granule neurons of the migration deficient homozygous weaver (wv/wv) mouse cerebellum attached on a laminin substratum, but failed to extend long neurites (Fig. 1 *B*; Fig. 2). Moreover, even though the weaver (wv/wv) granule neurons expressed both L1 antigen (Fig. 1 *B*, *green*) and the B2 chain of laminin (Fig. 1 *B*, *red*) along their short neurites, the weaver neurons were unable to respond to or recognize the neurite outgrowth–promoting cues of the laminin substratum.

To understand how the weaver gene impairs neuronal migration, we studied the effects of (*a*) antibodies against the B2 chain of laminin, (*b*) calcium channel blockers (verapamil and ω-conotoxin), and (*c*) NMDA receptor antagonists (MK-801 and APV) on neuronal survival and neurite outgrowth of the weaver (wv/wv)granule neurons on a laminin substratum. We found that neurite outgrowth of the weaver (wv/wv) granule neurons was significantly enhanced by 7–20 μM MK-801, added into the culture medium 1 h after the initial plating on a laminin substratum (Fig. 1 *C*; Fig. 2). In the presence of MK-801 (Fig. 1 *C*), neurites of the weaver granule neurons (see Fig. 1 *B*) extended as long as the neurites in the control cultures of the normal littermates (compare Fig. 1, *A* and *C*). While low (1 μM) concentrations of MK-801 specifically inhibit NMDA receptor function (Wong et al., 1986), high (7–20-μM) concentrations of this drug also inhibit voltage-gated calcium channels (ffrench-Mullen and Rogawski, 1992). To cover the NMDA receptor–specific functions of MK-801 (Wong et al., 1986), we used low concentrations of MK-801 (1 μM). We also applied APV (10 μM), a specific competitive antagonist of NMDA receptor function (Benveniste et al., 1990) to ensure that we did investigate the possible effect that inhibition of the NMDA receptor func-

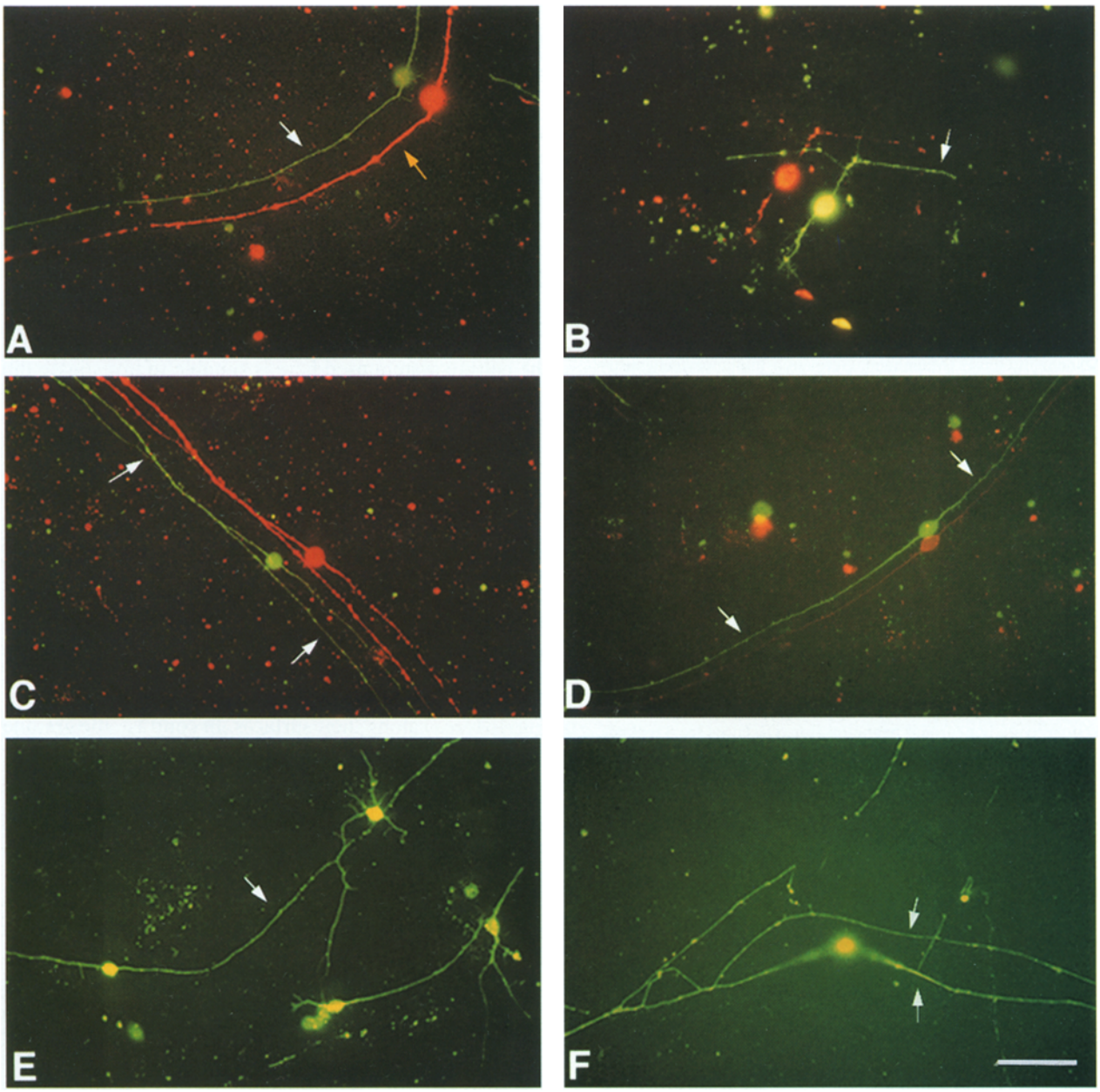


Figure 1. Demonstration of neurite outgrowth of the normal (*A*) and weaver (*B–F*) granule neurons on a laminin substratum. The neurons were identified using immunocytochemistry for the granule neuronal marker, L1 antigen (*green*), and a neurite outgrowth domain of the B2 chain of laminin (*red*). The cultures were initiated from P7 animals and maintained for 24 h. In *A*, a normal (+/+) granule neuron extends long L1 antigen–immunoreactive (*green*) neurites on a laminin substratum (*arrow*). The double immunocytochemistry shows that this neuron also expresses the B2 chain of laminin along its surfaces (*arrow*; *red*). The staining was performed on nonpermeabilized cultures to allow visualization of surface proteins. In *B*, a homozygous weaver (wv/wv) granule neuron expresses L1 antigen (*green*), but shows poor neurite outgrowth on a laminin substratum (*arrow*). The double immunocytochemistry as in *A* indicates the presence of both L1 antigen (*green*) and the B2 chain of laminin (*red*). In *C*, homozygous weaver granule neurons were cultured in the presence of 10 μM MK-801. MK-801 restores the neurite outgrowth potential of this wv/wv neuron (*arrows*) to the level of the normal granule cells (compare with *A*). L1 antigen (*green*). B2 chain of laminin (*red*). In *D*, homozygous weaver granule neurons were cultured in the presence of 100 μM APV. APV also restores the neurite outgrowth potential of this wv/wv neuron. L1 antigen (*green*). B2 chain of laminin (*red*). In *E*, homozygous weaver granule neurons were cultured in the presence of divalent Fab_2 fragments (40 μg/ml) of rabbit polyclonal antibodies against a neurite outgrowth domain of the B2 chain of laminin (anti-1543 in Murtomäki et al., 1992). These antibodies restore neurite outgrowth of the wv/wv granule neurons (*arrow*). L1 antigen (*green*). In *F*, homozygous weaver granule neurons were also rescued in the presence of a new mouse monoclonal antibody (mAb 1533; 40 μg/ml) recognizing the neurite outgrowth domain of the B2 chain of laminin (*arrows*). L1 antigen (*green*). Bar, 30 μm.

tion might have on rescue of the weaver granule neurons. We found that low concentrations of neither drug improved survival or neurite outgrowth potential of the weaver granule neurons over the untreated weaver neurons (ANOVA F = 3.1933; $P < 0.0699$, NS). 100 μM APV promoted neurite outgrowth of some of the weaver neurons (Fig. 1 *D*), but this promotion was not statistically significant as compared with the untreated wv/wv neurons (Fig. 2; $P > 0.05$, NS). Furthermore, 100 μM APV failed to promote survival of the weaver granule neurons (Fig. 2) and had no effect on survival of the normal neurons ($P > 0.05$, NS).

Our earlier results have linked overexpression of the B2 chain–derived peptides in neurotoxicity of the human neurodegenerative disorders (Murtomäki et al., 1992) and of the weaver mouse mutation (Murtomäki et al., 1995). We therefore applied divalent Fab_2 fragments of antibodies specific to the neurite outgrowth–promoting domain of the B2 chain of laminin to investigate whether these antibodies could protect the weaver granule neurons from the neurotoxicity of the B2 chain peptides and rescue their neurite outgrowth potential. When the weaver (wv/wv) granule neurons were cultured on a laminin substratum in the presence of divalent Fab_2 fragments of polyclonal antibodies (anti-1543; 40 μg/ml) against a neurite outgrowth domain of the B2 chain of laminin (Fig. 1 *E*; Liesi et al., 1989) the neurite outgrowth potential of the weaver granule neurons was rescued to the level of the normal littermates (compare Fig. 1, *E* and *A*; Fig. 2). A similar rescue effect was obtained by culturing the weaver granule neurons in the presence of the newly developed monoclonal antibodies (mAb 1533; Murtomäki et al., 1992) recognizing a neurite outgrowth domain of the B2 chain of laminin (Fig. 1 *F*; Liesi et al., 1989). Interestingly, the weaver (wv/wv) granule neurons cultured in the presence of the B2 chain antibodies adopted either bipolar or multipolar neuronal morphology and sent out neurites that extended short ramifications from the main neurites (see Fig. 1 *E*).

The result that high (7–20-μM) but not low (1-μM) concentrations of MK-801 rescued the neurite outgrowth potential of the weaver granule neurons (Figs. 1 and 2) indicated that voltage-gated calcium channels might be involved in the weaver gene action. To gain definitive evidence, we carried out a separate set of experiments to investigate the role of voltage-gated calcium channels in the migration deficiency of the weaver granule neurons. Specifically, we compared the effects of 1–5 μM verapamil (an L-type calcium channel blocker), 1–2 μM ω-conotoxin (an N-type calcium channel blocker), and 20–100 μM MK-801 on survival and neurite outgrowth of the normal (+/+) and weaver (wv/wv) granule neurons. We found that both 1 μM verapamil and 20 μM MK-801 rescued the neurite outgrowth of the weaver granule neurons to the level of the normal (+/+) neurons (Fig. 3), whereas 1 μM ω-conotoxin (Fig. 3) did not improve neurite outgrowth of the weaver granule neurons over the weaver control ($P > 0.05$). The same concentrations of these drugs had no statistically significant effect on neurite outgrowth of the normal (+/+) granule neurons (Fig. 3; $P > 0.05$). While 20 μM MK-801 rescued the weaver granule neurons (Fig. 3), 100-μM concentration of MK-801 severely impaired neurite outgrowth of both normal (+/+) and weaver (wv/wv)

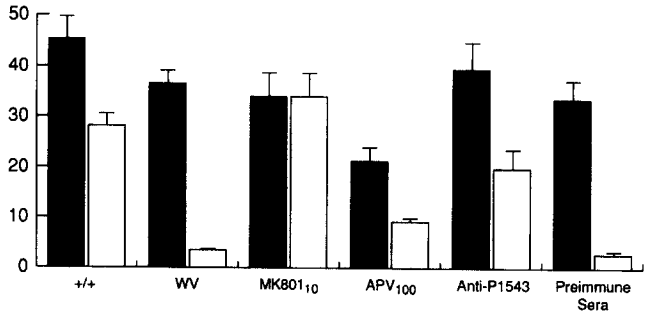


Figure 2. Quantitation of neuronal survival (*closed columns*) and outgrowth of long neurites (>10 × cell soma; >100 μm; *open columns*) in cultures of the normal (+/+) and weaver (wv/wv) granule neurons in the presence or absence of the rescuing agents. The cultures were initiated from P7 cerebella and maintained for 24 h. The error bars indicate SEM. The statistical analysis of the results was performed using one way variance analysis ANOVA. The individual groups were compared using a Student-Newman-Keuls multiple comparisons test. The results indicate that the numbers of the neuronal cell bodies (*closed columns*) are not significantly different between the normal and weaver cultures ($P > 0.05$), and that treatments with 10 μM MK-801 ($P > 0.05$) or 40 μg/ml of the anti-1543 ($P > 0.05$) or the preimmune serum for anti-1543 ($P > 0.05$) did not significantly alter the weaver neuronal attachment. However, attachment of the weaver granule neurons in the presence of 100 μM APV was impaired ($P < 0.01$) as compared with the normal neurons. Neurite outgrowth (*open columns*) of the weaver neurons was severely impaired ($P < 0.001$) as compared with the normal neurons. Both 10 μM MK-801 ($P < 0.001$) and Fab_2 fragments (40 μg/ml) of polyclonal anti-1543 ($P < 0.001$) significantly improved the neurite outgrowth of the weaver neurons as compared with the untreated weaver neurons. These treatments restored the neurite outgrowth potential of the weaver neurons to the level of the normal cultures ($P > 0.05$). The Fab_2 fragments (40 μg/ml) of the preimmune serum for the anti-1543 did not promote neurite outgrowth of the weaver neurons but the neurite outgrowth remained at the level of the untreated wv/wv neurons ($P > 0.05$). Even though APV promoted neurite outgrowth of some weaver granule neurons (see Fig. 1 *D*), 100 μM APV did not significantly improve neurite outgrowth of the weaver granule neurons ($P > 0.05$, NS) as compared with the untreated wv/wv granule neurons. ■, neurons; □, neurons with long neurites.

granule neurons ($P < 0.001$). Verapamil at concentrations up to 5 μM promoted neurite outgrowth of the weaver granule neurons ($P < 0.001$), whereas ω-conotoxin up to 2 μM had no effect on neurite outgrowth of the weaver granule neurons ($P > 0.05$). Neither 5 μM verapamil nor 2 μM ω-conotoxin affected neurite outgrowth of the normal neurons ($P > 0.05$).

Weaver Granule Neurons in the Cable Culture System

The cable culture system has been widely used for studies on neuronal migration and on neuronal–glial interactions during granule neuronal migration (Trenkner and Sidman, 1977; Trenkner et al., 1978), because neurons in this culture system have been shown to migrate along glial fibers (Trenkner et al., 1978; Hatten and Mason, 1990). In recent studies, neurons have also been shown to exhibit a second mode of neuronal migration, which occurs independent of the glial cells and proceeds along other neuronal fibers

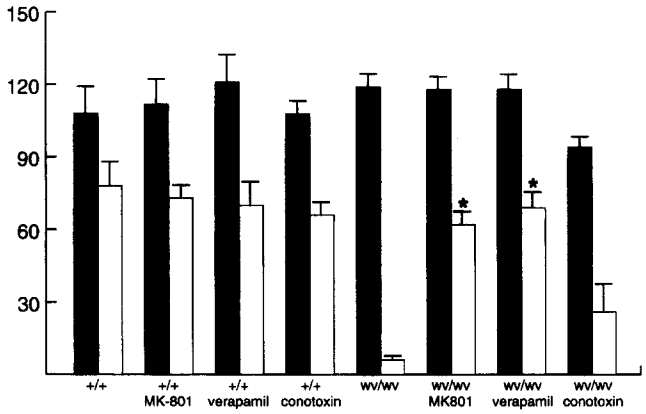


Figure 3. Quantitation of neuronal survival (*closed columns*) and neurite outgrowth of long (>100 μm) neurites (*open columns*) in primary cultures of normal (+/+) and weaver (wv/wv) cerebellum on a laminin substratum 24 h in vitro. The drugs applied were 20 μM MK-801, 1 μM verapamil, and 1 μM ω-conotoxin. All drugs were administered after the initial attachment period of neurons (1 h after plating). Neuronal survival was comparable in all cultures ($P > 0.05$, NS), whereas MK-801 and verapamil significantly improved the neurite outgrowth of the weaver granule neurons as compared with the untreated weaver neurons ($P < 0.001$). MK-801 and verapamil rescued the weaver granule neurons to the level of the normal neurons ($P > 0.05$, NS), whereas ω-conotoxin had no rescuing effect. Ω-conotoxin–treated neurons showed as poor neurite outgrowth as the untreated weaver neurons ($P > 0.05$, NS). Neurite outgrowth of the normal neurons was not affected by any of these drugs ($P > 0.05$). ■, neurons; □, neurons with long neurites.

(Liesi, 1992; Hager et al., 1995; Liesi et al., 1995). The latter mode of neuronal migration has also been demonstrated in the cable culture system (Liesi et al., 1992) which indicates that this culture system is a valuable model system for all studies concerning neuronal migration.

The weaver granule neurons have previously been shown to be deficient in their migration potential in this culture system (Hatten et al., 1986). As previously shown (Hatten et al., 1986), we found that the untreated weaver (wv/wv) granule neurons reaggregated, but developed poor cables, e.g., neuronal and glial fibers that normally connect the reaggregates (Fig. 4). A few thin L1 antigen–positive cables developed inbetween the reaggregates (Fig. 4), but few migratory neurons were seen along these immature cables (Fig. 4). L1 antigen is a neuronal marker for the cerebellar cultures (Rathjen and Schachner, 1984). Thus, L1 immunocytochemistry allowed a reliable evaluation of the migratory neurons in this culture system. However, we need to point out that even though granule neurons are thought to form the major migratory population of the neurons in the cerebellar cable cultures (Hatten and Mason, 1990), it is possible that other L1 antigen–positive neurons, such as stellate cells, basket cells, and Golgi neurons may also migrate along the cables.

To determine if enhanced neurite outgrowth and migration of the weaver granule neurons on a laminin substratum by the B2 chain–specific laminin antibodies and by MK-801 was a general phenomenon, independent of the culture conditions, we examined the effects of these compounds on neurite outgrowth of the weaver granule neurons in the established cable culture system. When MK-801 (10–20 μM) was included into the culture medium of the weaver (wv/wv) granule neurons, the neurons reaggregated as usual, but cable formation between the L1 antigen–immunoreactive reaggregates was clearly enhanced (Fig. 4). Furthermore, MK-801 significantly promoted migration of the weaver granule neurons along the cables (Fig. 4; $P < 0.0001$). Polyclonal antibodies against a neurite outgrowth domain of the B2 chain of laminin (anti-1543; 40 μg/ml) also enhanced both cable formation and neuronal migration of the weaver granule neurons (Fig. 4). The preimmune sera of the B2 chain–specific antibodies (40 μg/ml) did not promote neuronal migration of the weaver granule neurons as compared with the untreated weaver cable cultures ($P > 0.05$, NS).

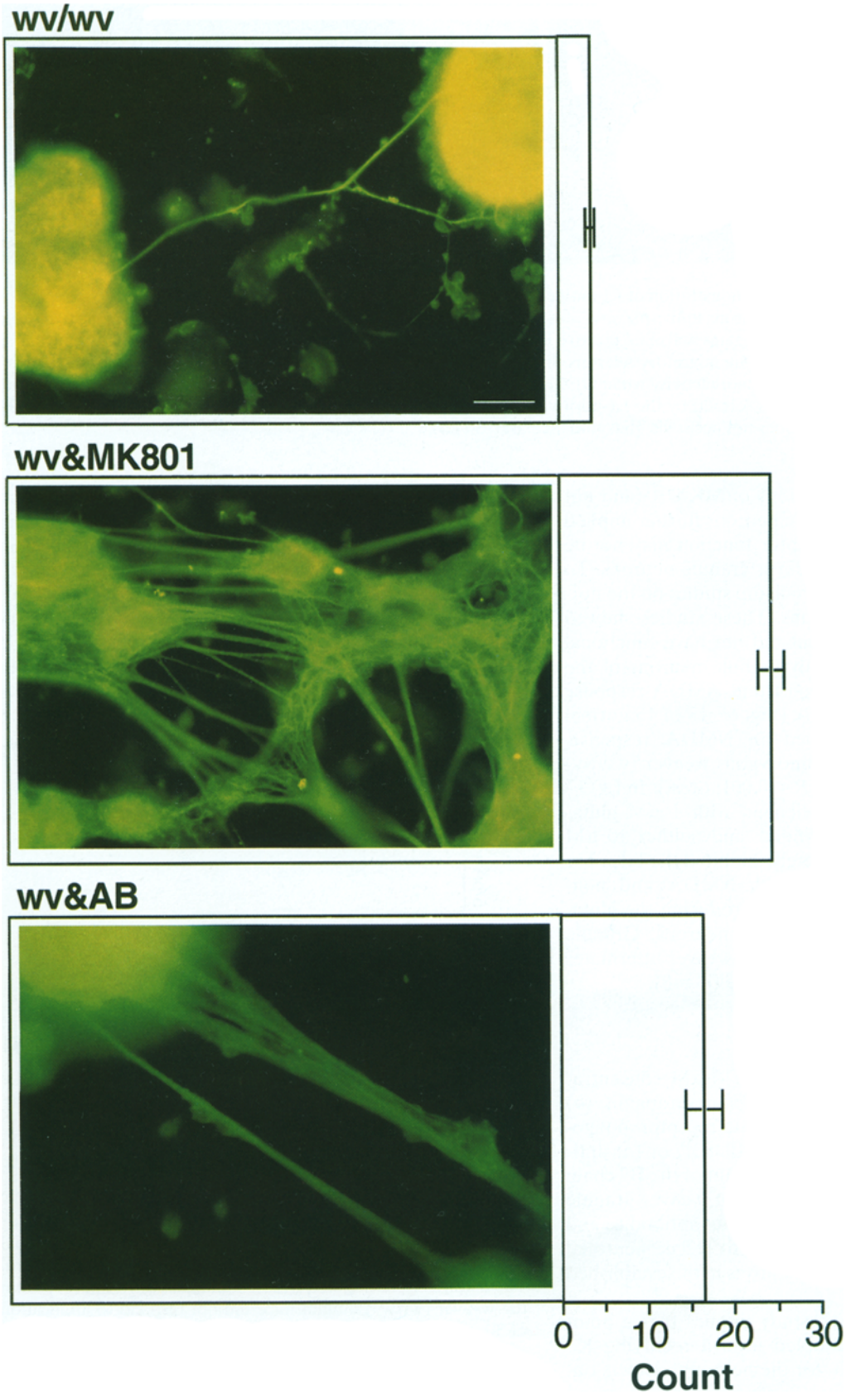
As shown in Fig. 4, all reaggregates and cables formed by the weaver granule neurons were strongly L1 antigen positive as if the cables were largely formed by L1 antigen–positive neuronal fibers. We studied this possibility further by using double immunocytochemistry for L1 antigen and GFAP in normal (+/+) cerebellar cable cultures. We found that a large number of cables that connected the reaggregates were formed by L1+/GFAP− fibers (Fig. 5, *A–B*) indicating that they were formed by L1-positive fibers of the migratory neurons themselves. We further found that ~22% of the L1-positive cables included GFAP positive glial fibers, whereas most of the cables were L1+/GFAP− even though the GFAP-positive astrocytes were present inside the reaggregates (Fig. 5 *B*). This result indicates that neuronal migration in the cable culture system occurs along both neuronal and glial fibers, but that the neuronal migration mode prevails.

Glutamate Receptor Function of the Weaver Granule Neurons

Our pharmacological results indicated that inhibition of the L-type calcium channel function of the weaver granule neurons rescued their neurite outgrowth potential in primary cultures on a laminin substratum (Fig. 3). The fact that

Figure 4. Neuronal migration of the weaver granule neurons in the established cable culture system was evaluated using L1 antigen immunocytochemistry in the presence or absence of MK-801 (10 μM) or anti-1543 (40 μg/ml). Since L1 antigen is a cerebellar neuronal marker, it was possible to identify and quantitate the individual granule neurons along their migratory pathways. Note that the shown cables are formed by L1 antigen–immunoreactive neuronal fibers of the L1 antigen–positive neurons that are present in the reaggregates and extend their processes to connect the reaggregates. Neuronal migration here occurs along the neurites as shown previously for other systems (Liesi et al., 1992; Hager et al., 1995). The results indicate that both MK-801 and Fab_2 fragments of antibodies against the B2 chain of laminin significantly enhanced migration of the L1 antigen–immunoreactive granule neurons along the L1 antigen–positive neuronal processes, e.g., cables, as compared with the untreated weaver cultures. ANOVA $F = 39.941$ ($P < 0.0001$). Numbers of reaggregates of the granule neurons were not affected by the drug or antibody treatments (data not shown). Bar, 30 μm.

Weaver Rescue in Cable Cultures



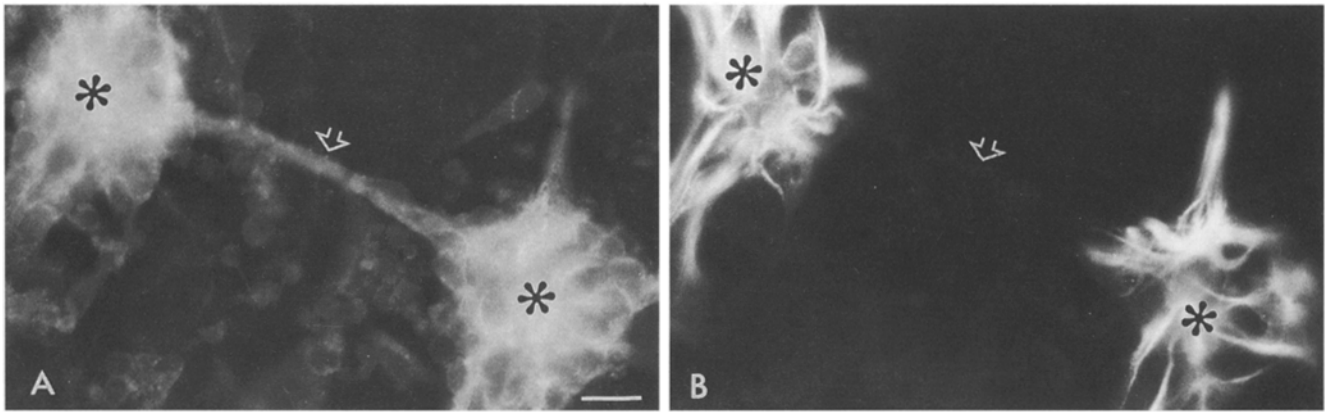


Figure 5. Double immunocytochemical demonstration of L1 antigen (*A*) and GFAP (*B*) in cable cultures of the normal mouse cerebellar cells after 72 h in vitro. In *A*, all the neurons in the reaggregates (*asterisk*) are immunoreactive for L1 antigen. The L1-positive cable (*open arrow*) that connects the two reaggregates is also L1 positive indicating that it contains L1-positive fibers of the granule neurons as well as L1 antigen–positive cell bodies of the migratory neurons. In *B*, double immunocytochemistry reveals that the L1 antigen–positive cable (in *A*) is devoid of GFAP immunoreactivity while GFAP immunoreactivity is abundant inside the reaggregates. This double labeling result indicates that this cable is formed by the L1-positive neuronal fibers and not by GFAP-positive astrocytes, which indicates that migration along this cable does not occur via glial guidance. Bar, 30 μM.

only high (7–20 μM) concentrations of MK-801 (and not APV) rescued the weaver granule neurons further implied that the glutamate/NMDA receptor function may not be involved in the rescue of the weaver granule neurons. To confirm this we performed patch-clamp studies on the normal and weaver granule neurons. These studies showed that the weaver granule neurons did not have functional NMDA receptors (Fig. 6). Only granule neurons of the normal littermates showed a consistent NMDA response when exposed to 30 μM NMDA (Fig. 6). 13 of 15 normal (+/+) granule neurons showed an NMDA response, whereas only 2 of 17 of the homozygous weaver (wv/wv) littermate neurons responded ($P < 0.001$; *t*-test). In (+/+) granule neuronal cultures challenged with 1 μM glutamate, four of eight cells responded, and neither 10 μM APV nor 10 μM CNQX completely inhibited the response indicating the involvement of both NMDA and non-NMDA type glutamate receptors in producing the glutamate currents of the normal granule neurons. Granule neurons from the homozygous (wv/wv) weaver littermates did not respond to 1 or 10 μM glutamate ($n = 8$).

Discussion

The present results indicate that (*a*) 1–5-μM concentrations of a L-type calcium channel blocker verapamil, (*b*) 10–20 μM concentrations of a glutamate receptor antagonist and a calcium channel blocker MK-801, or (*c*) antibodies against a neurite outgrowth domain of the B2 chain of laminin rescue the migration deficient weaver granule neurons on a laminin substratum. Laminin antibodies and MK-801 were also applied and found to rescue neuronal migration of the weaver granule neurons in the established cable culture system.

A recent molecular genetic study implies that a point mutation in a G protein coupled inward rectifying K^+ channel could be responsible for the neuronal defects observed in the weaver mutation (Patil et al., 1995). Expression of the mutated wv/wv GIRK2 channel in Xenopus oocytes showed that this mutated channel also gates sodium (Slesinger et al., 1996), which may explain the poor resting membrane potentials reported for the weaver neurons in vitro (Murtomäki et al., 1995). However, a recent electrophysiological study indicates that neither normal nor weaver granule neurons express functional GIRK2 channels during the developmental stage when the neurons die (Mjaatvedt et al., 1995), which implies that the the mu-

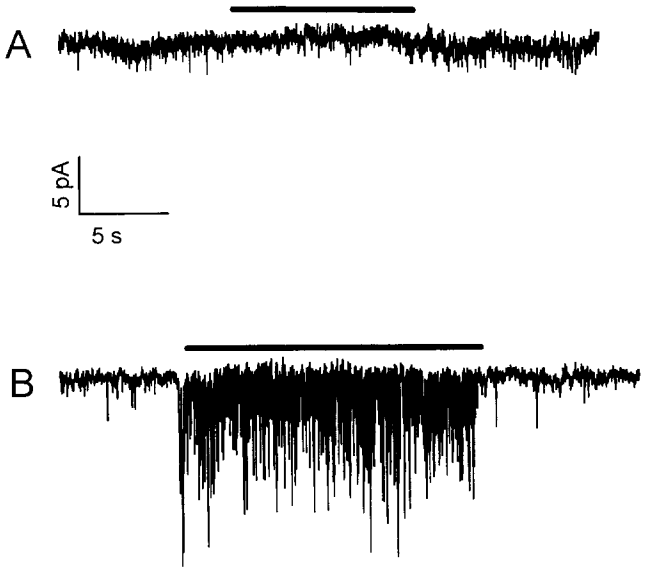


Figure 6. Whole cell recordings from the weaver (*A*) and normal (*B*) granule neurons on a laminin substratum after 24 h in vitro. The cultures were initiated from P7 animals. In *A*, neurons from the weaver (wv/wv) mutant mice did not respond to application of 30 μM NMDA. In *B*, neurons from the normal (+/+) mouse cerebellum responded to application of 30 μM NMDA. Saline bath and drug solutions contained 1 μM glycine. Whole cell recording, voltage clamp at −50 mV, data filtered at 100 Hz. Bars indicate NMDA application.

tated GIRK2 gene may not be the weaver gene. Thus, developmental expression of the GIRK2 channel gene and its role in the weaver mutation needs to be further explored. However, the present rescue mechanisms of the weaver granule neurons and the rescue of the weaver granule neurons by a protease inhibitor aprotinin, which also restored the resting membrane potentials of the weaver granule neurons (Murtomäki et al., 1995), suggest that a point mutation in a K^+ channel gene may not be entirely responsible for the weaver phenotype.

Our recent studies have established that the weaver granule neurons are proteolytically overactive and produce excessive amounts of the B2 chain of laminin (Murtomäki et al., 1995). The weaver neurons also show accumulation of the B2 chain–derived peptides along their surfaces (Murtomäki et al., 1995), which may be due to the proteolytic cleavage of the B2 chain into biologically active peptides that have a dual neurotrophic/neurotoxic function (Liesi et al., 1989). These B2 chain–derived peptides may be involved in weaver granule neuronal death and migration deficiency (Murtomäki et al., 1995), because accumulation of antigenetically related peptides has also been demonstrated in human neurodegenerative disorders, such as Alzheimer's disease and Down's syndrome (Murtomäki et al., 1992). The B2 chain of laminin is involved in neuronal migration in the normal cerebellum in vivo and in vitro (Liesi et al., 1992; Liesi et al., 1995). Thus, overexpression of the B2 chain of laminin in the weaver brain may interfere with the granule neuronal migration. In line with this hypothesis, we show that antibodies against a decapeptide (P1543) from a neurite outgrowth domain of the B2 chain of laminin (Liesi et al., 1989) enhance neurite outgrowth and migration of the weaver granule neurons in two separate culture systems (Figs. 2 and 4). The rescue of the weaver granule neurons by the B2 chain–specific antibodies may result from the neutralizing function of these antibodies against the molecular action of the B2 chain of laminin and its neuroactive peptides on the weaver granule neurons. This hypothesis could also explain the rescue of the weaver granule neurons by normal cerebellar neurons or their membrane extracts (Gao et al., 1992, 1993). The normal neurons or their extracts could act similarly to the B2 chain antibodies in neutralizing the neuroactive B2 chain peptides by binding these peptides and preventing their binding to the vulnerable weaver neuronal surfaces.

Earlier studies indicate that normal migratory cerebellar granule neurons express functional NMDA receptors (Rossi and Slater, 1993; Farrant et al., 1994), and that calcium channel and NMDA receptor antagonists inhibit neuronal migration in cerebellar slice cultures (Komuro and Rakic, 1992; Komuro and Rakic, 1993). Our results concur with these findings by showing that while normal granule neurons express both NMDA- and non-NMDA–type glutamate receptors, the migration deficient weaver granule neurons fail to express functional NMDA (Fig. 6) or other types of glutamate receptors (not shown). Thus, the weaver gene may inhibit neuronal glutamate receptor function, which may in part result in migration deficiency of the weaver granule neurons.

Our present results indicate that calcium channel function is essential in mediating the weaver gene defect. Inhibition of the calcium channel function of the weaver granule neurons by verapamil and high concentrations of MK-801 resulted in rescue of the weaver granule neurons. The result that verapamil, an L-type calcium channel blocker, was effective at 1–5-μM concentrations, whereas ω-conotoxin, an N-type calcium channel blocker, failed to rescue the weaver granule neurons at 1–2-μM concentrations implies that inhibition of the L-type calcium channels is primarily involved in rescue mechanisms of the weaver granule neurons. The involvement of L-type calcium channel function in rescue of the weaver granule neurons is consistent with our earlier data showing that the weaver granule neurons in our culture system are in a depolarized state (Murtomäki et al., 1995). In depolarized state both N- and T-type calcium channels are only transiently active and quickly desensitize, whereas the L-type channels are known to be constantly active at such potentials (Nowycky et al., 1985). Thus, if influx of calcium were responsible for the death and migration deficiency of the weaver neurons, drugs that inhibit this influx, such as verapamil, are likely to rescue the weaver neurons. The rescue effect of high but not of low concentrations of MK-801 is consistent with the reported inhibitory effect of high concentrations of MK-801 on the voltage-gated calcium channels (ffrench-Mullen and Rogawski, 1992). Neither viability nor neurite outgrowth of the normal cerebellar neurons was affected by 1–5 μM verapamil or 10–20 μM MK-801 as compared with the normal untreated neurons ($P > 0.05$, NS). MK-801 at 100 μM caused a significant reduction in both viability and neurite outgrowth of the normal neurons ($P < 0.001$). These results indicate that neuronal migration of the weaver neurons, but not that of the normal neurons, is affected by drugs that may inhibit voltage-gated L-type calcium channels. Thus, the effects of verapamil and MK-801 on the rescue of the weaver neurons are highly specific and may relate to the fact that the weaver granule neurons are in a depolarized state whereas the normal granule neurons have normal resting membrane potentials (Murtomäki et al., 1995).

The pharmacological studies by Komuro and Rakic (1992, 1993) report that both ω-conotoxin (30–3,000 nM) and MK-801 (10 μM) inhibit neuronal migration in slice cultures of the P10 mouse cerebellum, whereas we detected no inhibition of neuronal migration in purified neuronal cultures of the normal mouse cerebellum using similar concentrations of these drugs (Fig. 3). It is possible that an age difference between the neurons used in our study (P7) and those (P10) used by Komuro and Rakic (1992) could explain the differing results in the effects of these drugs. Another possibility is that purified normal neurons would not be affected by MK-801 and ω-conotoxin or would require higher concentrations than neurons in slice preparations. This would imply that a glial influence could modulate the inhibition observed in slice preparations. However, the most logical explanation for this contradiction is that there is no contradiction, because we observed a different part of the neuronal migration process than Komuro and Rakic (1992, 1993). Komuro and Rakic (1992, 1993) observed nuclear movement of the granule neurons from point A to point B with or without the drug treatments using DiI-labeled neurons. Thus, they observed the nuclear movement phase of neuronal migration

(Liesi, 1992; Hager et al., 1995; Liesi et al., 1995) which may be inhibited by MK-801 and ω-conotoxin. However, we performed neither video nor labeling studies to observe nuclear movement, but we investigated the ability of the granule neurons to initiate their migration by extending neurites on a laminin substratum. Thus, we observed the neurite extension phase of neuronal migration that always precedes the second phase of neuronal migration that involves nuclear movement (Liesi, 1992; Liesi et al., 1995). This hypothesis that different drugs may specifically affect different parts of neuronal migration needs to be tested in future experiments.

In summary, our results show that weaver granule neurons can be rescued by antibodies against the B2 chain of laminin, and by inhibition of calcium channel function using a L-type calcium channel blocker verapamil and high concentrations of MK-801. Since our previous studies have shown that inhibition of excessive proteolytic activity of the weaver neurons by aprotinin also rescues the weaver neurons (Murtomäki et al., 1995), an experimental modulation of proteolytic enzymes, extracellular matrix molecules, and intracellular levels of calcium in the weaver neurons protects these neurons from the destructive effect of the weaver gene.

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