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Neuronal Differentiation in Cultures of Weaver (*wv*) Mutant Mouse Cerebellum

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In the present study we report for the first time a weaver (*wv*) gene dose effect on neuron survival and neurite formation in vitro. Dissociated cerebellar cells from postnatal 7- and 8-day-old normal (+/+), heterozygous weaver (+/*wv*) and homozygous weaver (*wv*/*wv*) mice were cultured as monolayers on poly-L-lysine coated glass. Cell death occurred rapidly in *wv*/*wv* cultures. Cell counts showed that less than 20% of the total neurons and neuronal precursors (identified by "birthday" radiolabeling techniques) survived by Day 3. Cell death was less extensive in +/*wv* cultures with 65% of the total neurons and 80% of the precursors surviving by Day 3. In contrast to *wv*/*wv* cultures, younger neurons survive better than the total population in +/*wv* cultures. The impairment of neurite formation over the first week is also proportional to the number of mutant genes as shown by quantitation of (a) the percentage of cells with neurites; (b) the percentage of cells with neurites of a given length class with time; (c) the lengths of the longest processes formed per cell. The mean longest neurite lengths obtained by computer digitization at 6 days in vitro were 41.8, 26.8, and 9.0 μm for +/+, +/*wv*, and *wv*/*wv* granule cells, respectively.

Key words: neurite outgrowth, neuron survival, weaver mouse, cerebellar cultures

Among the objectives of developmental genetics are to define the primary target cell or cells affected by a mutant gene, and to elucidate the developmental processes which, when perturbed, redirect development along an altered path. These are difficult objectives to meet by in vivo descriptive analysis alone, as exemplified by study of the weaver, *wv*, genetic locus in the mouse.

The *wv* mutation causes a gene dose-dependent impairment of granule cell neuron migration and survival in the cerebellum of 4- to 14-day-old mice [1–3]. In homozygotes (*wv*/*wv*) almost all granule cells die, each within a few days of its genesis in the external granular layer, and the normal inward translocation of the cell soma to the (internal) granule layer does not occur. In heterozygotes (+/*wv*), a much smaller percentage of early postmitotic granule cells die, though a higher percentage than in normal mice, and inward translocation or migration is considerably slowed [1]. Most of the granule cell neuron somas do eventually reach the granular layer though some remain permanently ectopic in the molecular layer and others die in route. Behavior of granule cells in the heterozygote suggests that the migration defect is likely antecedent to the cell viability problem and not the

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other way around. Another major developmental defect expressed by the same population of neurons is a severe impairment of neurite formation, with virtual absence of the bipolar processes that should lie parallel to the external surface in the transverse plane of the head, and of the inward-direced leading process that normally grows along the surface of radially oriented Bergmann glial fibers. The descriptive analysis does not establish whether the migration defect, neurite formation defect, and premature cell death are related, and if so, in what causal sequences. Further, it is not settled which, if any, of these derangements of neuron differentiation is intrinsic to the granule cell or which might be a response to a primary genetic disorder in the neighboring glial cell [3, 4].

In light of these uncertainties, it appears worthwhile to examine the behavior of wild-type and mutant cells when challenged with altered local environments. One approach is to fuse preblastula embryos so as to generate chimeric mice with a variable mix of cells in each organ. Initial studies of this type with $+/wv \rightarrow +/+$ chimeras indicate that cerebellar granule cell neurons are a direct target of the *wv* locus, but offer no information about the neighboring glial cells for lack of an appropriate independent marker showing allelic differences [5]. Another approach, technically more limited in the case of the cerebellum but ultimately more powerful, is to mix cells in tissue culture. A necessary step toward this goal, not fully realized as yet, is to define conditions that allow the cell types of interest to express the relevant developmental behaviors. Messer and Smith [6] reported that monolayer cultures of cerebellar cells from 7-day-old *wv/wv*, $+/wv$, and $+/+$ mice were indistinguishable from one another. Trenkner et al [7] used a microwell reaggregation culture system and described decreased neuronal migration and neurite formation in *wv/wv* cerebellar cultures, with the defect partially reversed by alcohol–ether extraction of the horse serum prior to its addition to the culture medium. In the present study we report for the first time a *wv* gene dosage effect in vitro, with respect to both neuron survival and neurite formation.

MATERIALS AND METHODS

Mice

Mice carrying the weaver mutation are maintained on an inbred C57BL/6J background by breeding heterozygous ($+/wv$) mice. All mice used in these experiments were from the breeding colonies of the Department of Neuroscience, Children's Hospital Medical Center.

Cell Cultures

Cerebella were dissected from 7- and 8-day-old mice. At these ages, granule cell generation is close to its peak rate [9]. The cerebella containing the *wv* gene can be distinguished as follows: (1) *wv/wv*—The cerebellum is approximately one-third the size of that in $+/+$ mice. The vermal region is nonexistent and a foliation pattern is not recognized by gross inspection. (2) $+/wv$—The cerebellum is approximately 80% its size in $+/+$ mice. The vermal region is most affected, being similar in thickness to the lateral hemispheres, whereas the vermal region is thicker than the lateral hemispheres in $+/+$ mice. The cerebella were dissociated into single cells and cultured on polylysine-coated glass coverslips according to the methods described previously [10]. Unless stated otherwise, cells were plated at a density of 1.5×10^4 cells/mm^2.

Autoradiography

To follow the survival of granule cell precursors in vitro, mice were injected 6 hr prior to sacrifice with [^{3}H]thymidine (40–60 μCi/mole, ICN) at a dose of 10 μCi/gm body weight. Cerebellar cells were cultured, and were processed for autoradiography at 1, 2, or 3 days in vitro according to methods previously described [10].

Cell Counts

In order to count cells identified as neurons by nuclear morphology, cultures were fixed in 3% glutaraldehyde in 0.12 M phosphate, pH 7.2, for 15 min at room temperature and stained in 0.1% cresyl violet in water at pH 4.0.

Digitization of Neurite Lengths

Cultures were fixed as described above and mounted in Aquamount. Neurite lengths were digitized using phase contrast microscopy on a Zeiss Universal microscope with computer controlled stage. Neurite lengths digitized in units of 0.5 μm were stored and analyzed in a PDP 11/05 computer. A sample of 20 randomly selected cells of each genotype were digitized.

RESULTS

Dissociated cerebellar cells from 7- and 8-day -old mice were cultured as monolayers on the polylysine substrates. The predominant population (80%), uniformly distributed small round refractile cells 8–10 μm in diameter, are granule cells by ultrastructural criteria [11]. Over the first 5 days in vitro normal neurons elaborate bipolar processes and migrate to form small aggregates connected by fiber fascicles. This pattern of differentiation was not observed in cultures of weaver cerebellum, which instead showed neuronal cell death and absence of a fiber network. These parameters were analyzed in more detail.

Neuron Survival

The number of surviving neurons was quantitated at various times in vitro for cells from $+/+$, $+/wv$, and *wv/wv* cerebellum. Survival of the presumptive granule cell precursors and of the total neuron population of the three genotypes were compared to determine whether the timing of cell death in vitro is related to the postmitotic age of the neuron.

When mice are injected with a pulse of [^{3}H]thymidine, cells synthesizing DNA in the germinative zone of the cerebellar cortex become labeled. If the animals are killed after 6 hr and the cerebellar cells cultured, approximately 20% of the small neurons have grains over their nuclei in autoradiograms. These cells have properties of immature granule cell neurons at 1 day in vitro and are designated as precursor cells [10]. These cells are postmitotic and continue to mature over the next 2 days [11]. When the number of neurons with grains over their nuclei was counted at 1, 2, and 3 days in vitro, a striking loss of *wv/wv* precursors was observed within the second 24 hr. Eighty-eight percent of the *wv/wv* precursors present at 24 hr in vitro were gone by Day 3 as opposed to 10 and 20% in cultures of $+/+$ and $+/wv$ cultures, respectively (Fig. 1, upper panel). This is consistent with the timing of the death of *wv/wv* neurons in vivo, ie, soon after they become postmitotic.

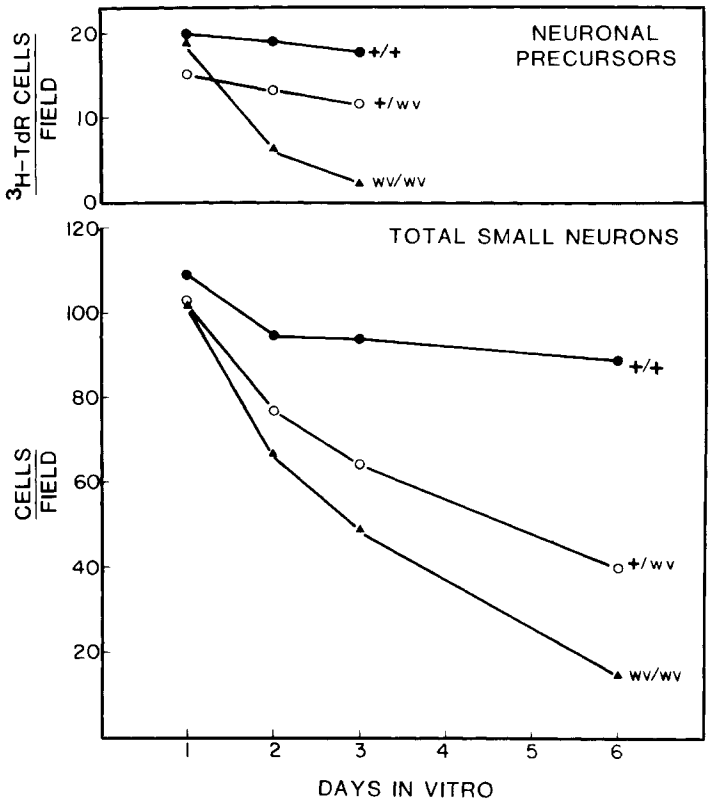

Fig. 1. Granule cell survival in cultures of weaver (*wv*) cerebellum. Upper panel: Mice were injected with [^{3}H]thymidine 6 hr prior to sacrifice on postnatal Day 8; the cerebella were dissociated and cells were plated at a density of $3 \times 10^4/mm^2$. The number of neurons with grains over their nuclei was counted in 5–10 random fields in duplicate cultures. Each point is an average of two independent experiments. Lower panel: Cells having granule cell nuclear morphology were counted in six contiguous fields in each of duplicate cultures of $+/+$, $+/wv$, and *wv/wv* postnatal Day 7 cerebellum. The points represent the mean for each genotype.

The survival of the total granule cell population in a representative experiment is depicted in Figure 1, lower panel. Cell death in $+/wv$ cultures was extensive though significantly less than in *wv/wv*. The difference between the kinetics of $+/wv$ cell death in the total population versus labeled precursor cells suggests that $+/wv$ neurons die relatively late after their last round of DNA synthesis, ie, the labeled cells survive better than the cell population as a whole in $+/wv$ cultures (Fig. 1).

Neurite Outgrowth

Neurite outgrowth was examined in cell cultures of $+/+$, $+/wv$, and *wv/wv* cerebellum to determine whether the performance of surviving mutant neurons in elaborating fibers is affected. Neurite outgrowth was quantitated in two ways: (1) by counting the number of surviving cells without neurites, with neurites less than one body length (10 μm) and with neurites longer than 10 μm, in

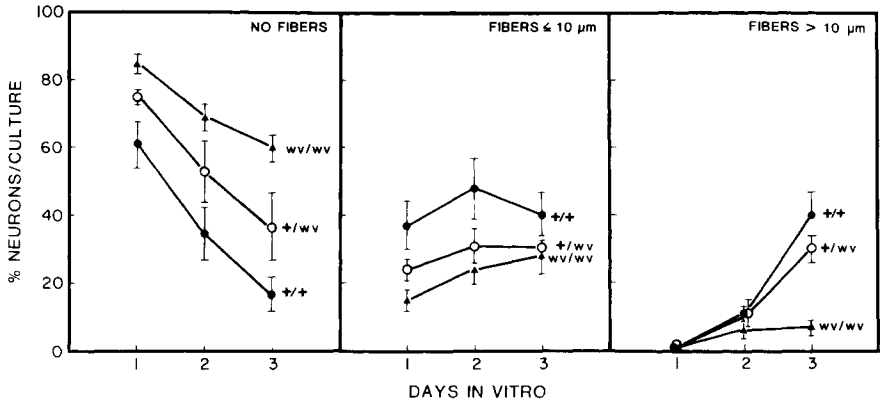

Fig. 2. Neurite outgrowth in weaver cerebellar cultures. The total number of neurons was counted on a 1-mm² field and scored as having no neurites, neurites up to one body length (≤ 10 μm) and neurites longer than one body length (> 10 μm). The data are presented as the percentage of neurons in each class of the total neurons counted per culture. At least 1800 cells were counted to obtain the data for each point. Each point is the mean and SD for three independent experiments in which three fields in triplicate cultures were counted.

a large population of cells at 1, 2, and 3 days in vitro (Fig. 2); (2) by computer-assisted digitization of a small sample at 6 days in vitro (Figs. 3, 4).

At all time points examined, there is a consistent effect of the weaver gene on neurite outgrowth. The percentage of cells bearing fibers of increasing length at early times in vitro (Fig. 2, left panel) is decreased proportionately to the number of weaver mutant genes. The *wv/wv* cultures are most drastically affected with 60% of the neurons lacking neurites at Day 3. In addition, fiber elongation on the part of those neurons that do form neurites is severely impaired since only 6% of the cells make fibers > 10 μm by 3 days (Fig. 2, right panel). Cells of the heterozygote are intermediate between wild-type and homozygous affected mice with respect to the percentage of neurons that fail to generate neurites, but a difference from wild type is not as clear-cut for those cells of the heterozygote that do form neurites. Forty percent of +/+ cells have fibers > 10 μm long and 32% of the +/*wv* cells have fibers of this length class (Fig. 2, right panel).

The total length of all neurites per cell originating from 20 cells in 6-day cultures was obtained from the computer-assisted digitization. The mean total lengths were 105, 49, and 14 μm for +/+, +/*wv*, and *wv/wv* neurons, respectively (Fig. 3, left panel). In general, +/+ cells tended to be bipolar; *wv/wv* cells unipolar. To illustrate further the striking gene dose-dependent difference in total neurite outgrowth observed at 6 days in vitro, the product of the mean total length by the number of cells per field is depicted in FIgure 3, right panel.

To evaluate elongation in a more quantitative manner, the length of a cell's longest neurite was digitized by computer for 20 cells in 6-day cultures of each of the three genotypes (Fig. 4). The mean longest neurite lengths also reflect a gene dosage effect ($\bar{x}$ values in Fig. 4). The distribution of the longest lengths indicates that *wv/wv* cells do not make long neurites even at 6 days: 90% have fibers < 20 μm; the longest fibers observed are 40–50 μm as opposed to 110–120 μm in +/*wv*

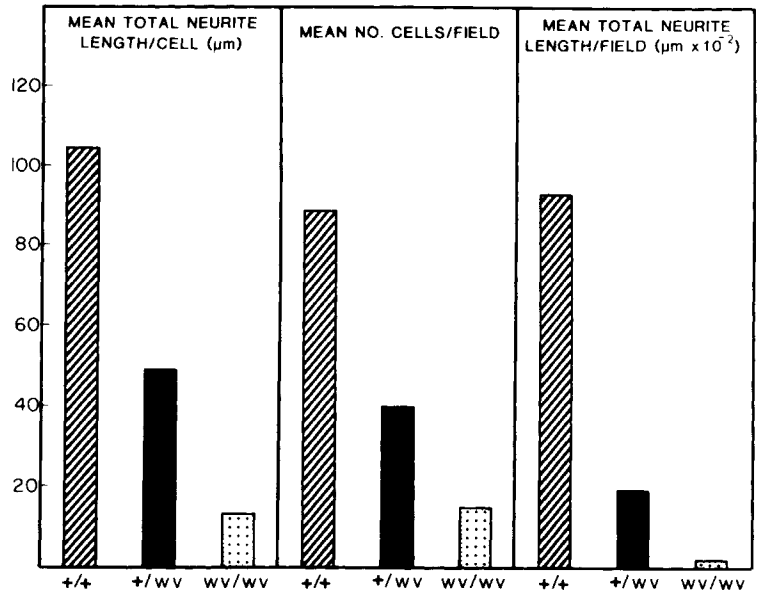

Fig. 3. Neurite growth at 6 days in vitro. The mean total neurite length/cell (left panel) was obtained by computer-assisted digitization of all fibers originating from a total of 20 neurons in triplicate cultures of $+/+$, $+/wv$, and *wv/wv* cerebellar cultures. This value was multiplied by the number of cells/field (center panel, value from Fig. 1) to obtain the mean total neurite length/field (right panel).

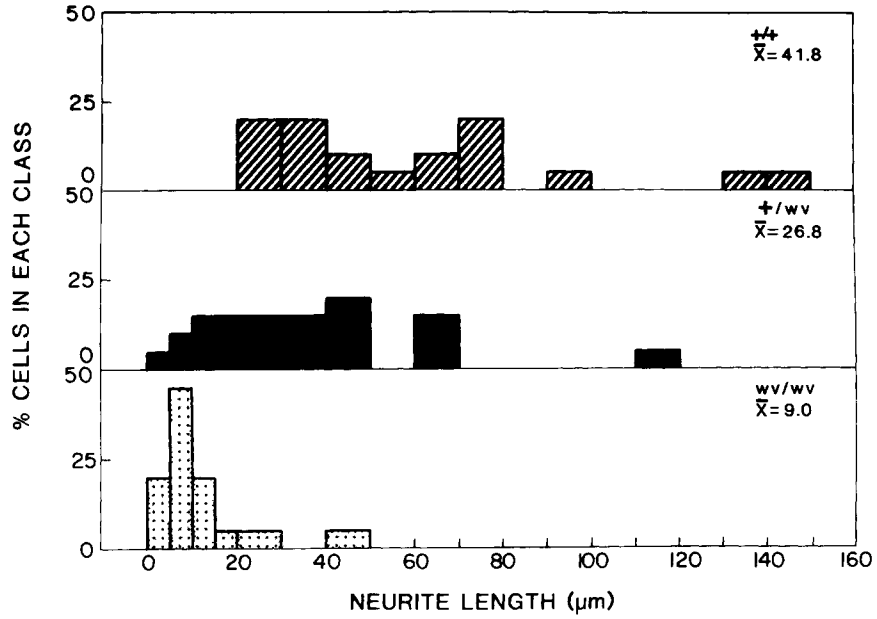

Fig. 4. Distribution of longest neurites at 6 days in vitro. The length of a neuron's longest neurite was obtained by computer-assisted digitization of 20 random cells in triplicate cultures of each genotype. The data (obtained by computer analysis) are expressed as the percentage of neurons of the total with a given longest neurite length in length intervals of 5 or 10 μm. $\bar{x}$ is the mean longest neurite length.

cultures and 140–150 μm in +/+ cultures. The distribution of longest lengths in heterozygote cultures is closer to normal than to the homozygote: 80% of +/*wv* cells have longest lengths between 10 and 70 μm, while 85% of +/+ cells have longest lengths between 20 and 90 μm.

DISCUSSION

The results presented in this report show that both survival and neurite outgrowth of granule cell neurons over the first week in vitro are affected by the weaver mutation in a gene dose-dependent manner.

The effect of the weaver gene dosage on granule cell survival in vitro resembles its action in vivo: a higher percentage of *wv/wv* neurons die and they die sooner after prior in vivo "birthday" labeling with [^{3}H]thymidine compared to +/*wv* neurons. These features appear to reflect a genetically defined neuronal developmental program rather than a nonspecific cell loss attributable to inadequate culture conditions, because the time course and extent of gene action are so faithfully adhered to in this culture system. The dose-response property makes this culture system valid for elucidating the mechanisms of action of the weaver gene. At the same time, one should emphasize that the culture system is limited by the failure of Purkinje neurons to survive and to differentiate.

The effect on neurite formation in vitro also is proportional to the number of mutant genes, as reflected by (a) the percentage of cells with neurites; (b) the probability that cells will bear fibers of increasing length class with time in vitro; and (c) the lengths of the longest neurites formed per cell. These results are consistent with a defect in neurite elongation. The in vivo analysis of granule cell neurite formation has not been quantified with respect to weaver gene dosage, and cannot be directly compared with the culture results.

Trenkner et al [7] reported that *wv/wv* reaggregate cultures do not elaborate cables to the same extent as normal cells. The present report extends this finding by quantitating fiber lengths of single cells over time and reveals a previously undetected effect in cells of the heterozygote. The absence of bipolar processes in the homozygote in vivo is most likely not due to cell death prior to neurite initiation since a proportion of *wv/wv* neurons do make neurites in culture. Three issues remain to be defined: (1) the cellular mechanism for the impaired neurite elongation; (2) the relationship between impaired cell viability and defective fiber formation; (3) the relationship between the defect in process formation and the slowed neuronal migration in vivo.

As stated in the introductory statement, the primary cellular site of action of the weaver gene is incompletely defined. The results presented in this report demonstrate an effect of the weaver gene on granule cell neurons at early times in vitro. We do not know whether the effects are intrinsic to the neuron or reflect in vitro expressions of cell interactions that may have taken place in vivo prior to culturing. These experiments will be extended with cerebellar cells from younger mice which have not yet shown overt expression of the mutant gene in vivo. In addition, we are currently investigating whether the weaver gene affects astroglial properties in vitro by analyzing differentiation in recombinant cultures of neurons and astroglia derived from normal and mutant cerebellum.

Further analyses of the expressed neuronal phenotypes at the cellular and molecular levels will provide important information concerning weaver gene function as well as more general insights into the assembly of the central nervous system.

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