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Neuronal Differentiation Rescued by Implantation of *Weaver* Granule Cell Precursors into Wild-Type Cerebellar Cortex

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The migration of postmitotic neurons away from compact, germinal zones is a critical step in neuronal differentiation in the developing brain. To study the molecular signals necessary for cerebellar granule cell migration in situ, precursor cells from the neurological mutant mouse *weaver*, an animal with phenotypic defects in migration, were implanted into the external germinal layer (EGL) of wild-type cerebellar cortex. In this region, labeled *weaver* precursor cells of the EGL progressed through all stages of granule neuron differentiation, including the extension of parallel fibers, migration through the molecular and Purkinje cell layers, positioning in the internal granule cell layer, and extension of dendrites. Thus, the *weaver* gene acts nonautonomously in vivo, and local cell interactions may induce early steps in neuronal differentiation that are required for granule cell migration.

Although a system of radial glial fibers provides an important guidance system for neuronal migrations in the central nervous system (CNS) (*1*, *2*), the molecular signals that induce postmitotic precursor cells to migrate away from germinal zones of the brain are not known. To identify the signals that induce granule neuron differentiation and migration in the developing cerebellar cortex, we used the neurological mutant mouse *weaver* as a model system. In this animal, cerebellar granule cell precursors proliferate normally in the superficial layer of the EGL (*3*, *4*) but fail to extend neurites (*5*) or to migrate away from the EGL (*1*, *6*, *7*).

To establish the dynamics of wild-type granule cell development in situ, dye-labeled, wild-type EGL precursor cells were implanted into wild-type EGL, and the differentiation of labeled cells was followed by fluorescence microscopy (*8*). Between 24 and 48 hours after implantation, labeled precursor cells moved into the deeper aspect of the EGL and initiated parallel fiber extension (Fig. 1A). These cells also generated axons 100 to 200 μm in length before the elaboration of a short, migratory process that descended perpendicular to the plane of axon outgrowth. Over the next 24 to 48 hours, labeled cells migrated through the molecular layer (ML) and Purkinje cell layer (PCL) and settled in the internal granule cell layer (IGL). Within the IGL, the labeled cells began to resemble granule cells morphologically (*1*, *3*): They extended a T-shaped axon into the ML and formed numerous short dendrites (Fig. 1B). Visualization of labeled cells provided evidence that axon extension occurs before the inward migration of the soma of granule cells. This growth matches the order of granule

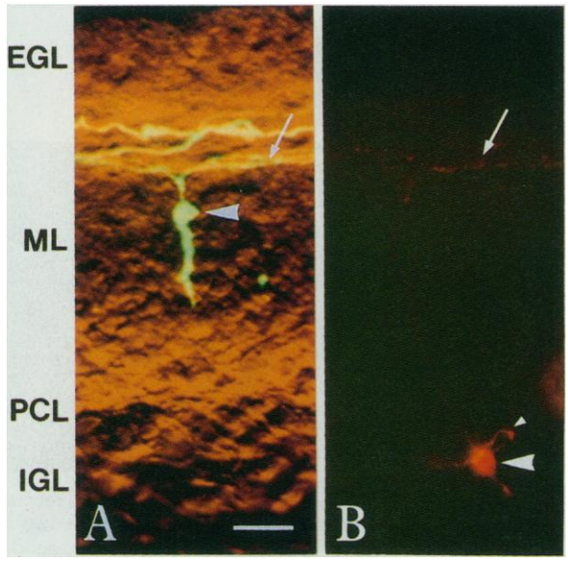

Fig. 1. Differentiation of dye-labeled wild-type EGL precursor cells after implantation into the EGL of early postnatal cerebellar cortex. (**A**) Pseudocolored confocal image. Three days after implantation, labeled cells extended long parallel fibers in the plane parallel to the pial surface (small arrow) and a migratory process (large arrowhead) perpendicular to the pial surface. Labeled cells began to move into the deeper aspect of the EGL 24 to 48 hours after implantation. Individual cells required 6 to 12 hours to transit the molecular layer (ML). (**B**) Fluorescence microscopy image. Six days after implantation, the cell soma of a labeled cell (large arrowhead) was located deep to the Purkinje cell layer (PCL), in the internal granule cell layer (IGL). The axon of the labeled neuron extended toward the molecular layer, forming a T shape characteristic of mature granule cells at the site where the axon bifurcates into the parallel fibers. Within the IGL, the cell extended short, dendritic processes (small arrowhead). Whereas the lipophilic dye PKH-26 stained both the cell body and the processes, fluorescent microbeads were confined to the cell soma (large arrowhead). Bar, 15 μm for both (A) and (B).

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cell development (Fig. 2) first proposed by Ramon y Cajal (*9*).

Implantation of *weaver* cells into the EGL of *weaver* cerebellum demonstrated the phenotypic defects in the neuronal differentiation of granule cells (*4–7, 10*). Labeled mutant cells failed to extend parallel fibers or migrate across the ML and remained at the site of injection (*11*) (Fig. 3, A and B). After implantation into wild-type EGL (*12*), however, mutant cells underwent a normal pattern of neuronal differentiation. They initiated parallel fiber extension in the deeper aspect of the EGL (Fig. 4A), migrated through the ML (Fig. 4, B and C), settled in the IGL, and extended dendritic arbors (Fig. 4D). Moreover, differentiation of implanted mutant cells occurred along the same time course that has been seen for wild-type cells (*13*). These data suggest that local interactions with wild-type cells rescued the phenotypic defects in *weaver* granule neuron differentiation.

To examine further the influence of local cell interactions between wild-type precursor cells and *weaver* cells on neuronal differentiation, we mixed labeled *weaver* cells at a 1:1 ratio with unlabeled wild-type cells before implantation of the cells into wild-type cerebellar cortex. Reaggregation of labeled *weaver* cells with unlabeled wild-type cells before implantation sharply increased the number of mutant cells that underwent axon extension and migration (*14*) (Fig. 5), which suggests that the number of rescued *weaver* cells increases with the dose of wild-type cells. This interpretation is consistent with two lines of evidence that have been obtained from in vitro experiments. (i) Rescue of the *weaver* phenotypic defect in neurite extension requires a ratio of wild-type to mutant cells >1:1 and (ii) rescue of *weaver* cells by membranes purified from wild-type cells is dose-dependent (*10*).

The rescue of *weaver* neuronal differentiation in vitro (*10*) and in the current experiments contrasts with results obtained by genetic mosaic analyses of the cell autonomy of *weaver* gene function (*15*). In homozygous chimeras, *weaver* granule cells fail to differentiate and die in ectopic positions (*15*). The discrepancy between results that were obtained with genetic mosaic analysis and those from cell mixing experiments in vitro (*10*) or cell implantation in vivo can be explained if the majority of the

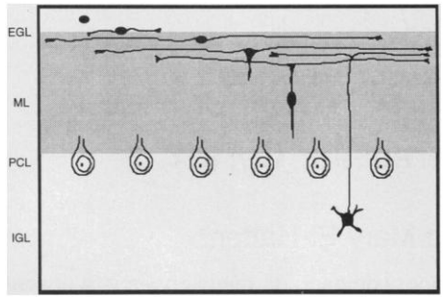

Fig. 2. Spatio-temporal pattern of granule cell differentiation and migration in vivo. Granule cell precursors proliferate in the superficial aspect of the EGL, then postmitotic cells descend into the deeper aspect of the EGL where they extend two long processes, the parallel fibers, parallel to the cerebellar laminae. Thereafter, a descending process emerges, which directs the migration of the cell body through the ML, along the radially aligned Bergmann glial fibers. Postmigratory cells settle in a dense neuronal layer, the IGL, deep to the PCL, and extend short dendrites (*9*).

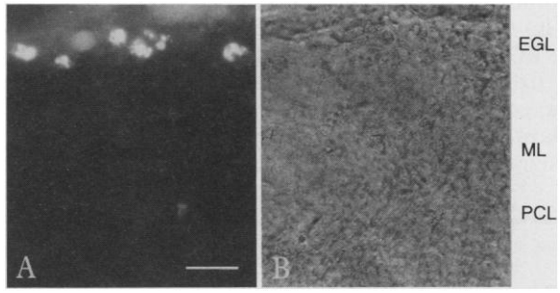

Fig. 3. Precursor cells of the *weaver* EGL fail to differentiate after implantation into the EGL of early postnatal *weaver* cerebellar cortex. (**A**) Five days after implantation into the midline portion of *weaver* cerebellar EGL, labeled mutant cells remained at the site of injection. (**B**) A bright-field view of the region shown in (A) reveals the layers of the *weaver* cerebellar cortex. In nine implantations of ~25,000 labeled cells, no labeled *weaver* cells extended neurites or migrated away from the site of injection. Bar, 20 μm for both (A) and (B).

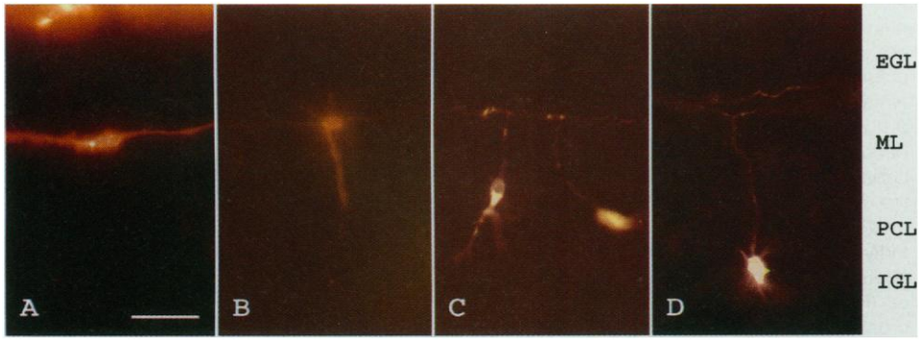

Fig. 4. Differentiation of *weaver* EGL precursor cells is rescued by implantation of the cells into the EGL of early postnatal wild-type cerebellar cortex. (**A**) Two days after implantation into the EGL of wild-type cerebellar cortex, a labeled *weaver* cell extended parallel fibers parallel to the pial surface. (**B**) Three and (**C**) 4 days after implantation, labeled mutant cells had migrated into the molecular layer, where they displayed the cytological features characteristic of migrating granule neurons (*21*), including caudal positioning of the nucleus, extension of a leading process, and a trailing axon. (**D**) Five days after implantation into wild-type EGL, a labeled *weaver* cell had successfully migrated into the internal granule cell layer and had formed a T-shaped axon and short dendrites. As in Fig. 1B, whereas the lipophilic dye PKH-26 labels both the cell body and the processes, the fluorescent microbeads are confined to the cell soma. Bar, 20 μm for (A) to (D).

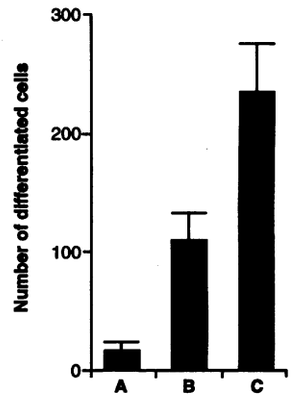

Fig. 5. Rescue of *weaver* EGL precursor cell differentiation is enhanced when mutant cells are preincubated with wild-type cells before implantation. The number of rescued mutant cells varies with the ratio of mutant cells to wild-type cells injected. To identify mutant cells, we double-labeled them with the dye PKH-26, which is incorporated into cellular membranes, and with microbeads (*22*). (**A**) Double-labeled *weaver* cells were injected into wild-type cerebellar EGL. (**B**) Double-labeled *weaver* cells mixed with unlabeled wild-type cells at a ratio of 1:1 before implantation. (**C**) Double-labeled wild-type cells were injected into the P5 wild-type cerebellum. For each of the three experiments, data were collected from 14 cases and are expressed as mean ± SEM (bar). Approximately 2500 differentiated granule neurons were scored.

mutant cells within the genetic mosaics had an insufficient number of contacts with wild-type cells for rescue to occur. Other studies support the conclusion that the *weaver* gene acts nonautonomously (*10*) and demonstrate that rescued mutant cells undergo granule cell maturation normally. The differentiation of ~2500 labeled *weaver* and wild-type cells was assayed by microscopy. In this population, >99% of the cells displayed the morphological features of granule neurons. None resembled the morphology of other classes of cerebellar neurons, including those of Purkinje cells. The rescued *weaver* cells produced proper patterns of axon outgrowth, underwent cell migration, were positioned in the correct neuronal layer, and extended dendrites on the same timetable as wild-type granule cells (*12*).

Several general models can explain the rescue of *weaver* cells by implantation into the EGL of early postnatal wild-type cerebellar cortex. In one model, supported by previous in vitro experiments, the *weaver* gene could function in a single, early event that is required to initiate the program of granule cell development in the EGL. Alternatively, the *weaver* gene could act at each step of granule cell development, other cells could provide signals that regulate the subsequent steps of granule cell differentiation in situ, or both of these processes could occur. Thus, the rescue of axonal patterning in *weaver* granule cells, directed migration, final cell positioning, and dendritic maturation, which were seen in this set of implantation experiments, could involve signals contributed by Bergmann glia (*1*), Purkinje cells, mature granule cells, and afferent axons (*2*).

Experiments in vitro demonstrated that membrane-bound signals expressed by wild-type but not *weaver* EGL cells are required for the extension and migration of granule cell axons (*10*). A general role for cell contact in CNS neuronal differentiation is supported by studies on the developing vertebrate spinal cord that show that induction of the floor plate by the notocord requires close contact of the two tissues (*16*). Further support for contact-mediated mechanisms of neural specification has been obtained in studies on *Drosophila* and mouse mutations, in which a number of genes that control neuronal differentiation, including *Notch* and *Delta* (*17*), *sevenless* and *Boss* (*18*), and *Steel* and *c-kit* (*19*), encode ligand-receptor systems that are components of the cell membrane. Alternatively, the *weaver* gene could function in each step of granule cell development, including axon extension, acquisition of laminar position, and dendritic arborization. The complex features of granule cell development analyzed here in vivo, including guidance of the granule cell axons within the ML, acquisition of correct laminar position in the IGL, and formation of synaptic contacts with ingrowing afferents, could require diffusible signals in addition to membrane-bound signals.

The implantation of purified populations of cells with specific phenotypic defects in neuronal development into germinal zones of developing mammalian brain provides a general approach to the analysis of the autonomy of gene expression required for mammalian CNS differentiation. Together with our in vitro experiments (*10*), the findings here on the rescue of *weaver* granule cell differentiation suggest that local interactions among progenitor cells induce neuronal differentiation in germinal zones of brain (*20*). The identification of the *weaver* gene should provide important insights on the signals for CNS neural specification.

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22. To identify *weaver* cells, we double-labeled EGL cells that were purified from the midline portion of *weaver* cerebella with the dye PKH-26 and with microbeads as described (*8*). Unlabeled EGL cells purified from wild-type cerebella were mixed with labeled *weaver* cells at a ratio of 1:1 (*10*) and at a final cell density of ~2.5 × 10^{6} cells per milliliter for 10 to 30 min. Previous experiments confirmed that PKH-26 does not transfer to unlabeled granule cells (*10*). The double-labeling precluded transfer of either the membrane-bound PKH-26 or microbeads to wild-type cells in situ.

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