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# Developmental Regulation of Mossy Fiber Afferent Interactions with Target Granule Cells

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**In an *in vitro* model system based on purified target cerebellar granule neurons and explants of afferents, pontine mossy fiber afferents stop growing through contact-mediated mechanisms when they encounter granule neurons. Here we studied the developmental regulation of the stop signal posed by granule cells and the response of mossy fibers to the stop signal in two culture systems. Granule neurons presented in slices or as dissociated cells from postnatal day (P) 4 and P7 cerebellum were more potent in the arrest of P0 pontine neurites than younger (P0-P2) or older (up to P14) granule neurons. In contrast, pontine neurites at embryonic day (E) 18, during their period of normal growth toward the cerebellum, grew extensively on both cerebellar slices of all ages from P0 to P10 and dissociated P4 granule neurons. When E18 explants were maintained for 2 days before plating in medium conditioned by neonatal cerebellar cells, E18 pontine explants were rendered more responsive to the stop signal from P4 granule cells. These results indicate that the stop signal, and the response of afferents to it, are developmentally regulated. Moreover, factors within the target region may initiate these interactions.**


## INTRODUCTION

The formation of specific connections in the nervous system depends upon a series of events beginning with the establishment of cell identity, followed by axon pathfinding directed by environmental cues, and culminating with target selection and synaptogenesis (Goodman and Shatz, 1993). In target areas, the challenge facing afferents is to cease extension at the proper location and on the proper target cell. In the past few years, much progress has been made with regard to the specification of cell fate and axon guidance (Smith, 1993; Luo and Raper, 1994; Kennedy and Tessier-Lavigne, 1995; Bang and Goulding, 1996; Goodman, 1996), but the steps by which a target cell is selected by afferents are still not well understood.

In principle, several mechanisms might be used by axons to select correct target cells: (1) Target cells secrete specific chemotropic molecules that direct their afferents to grow toward them. (2) Target cells express specific cell surface-associated molecules that can be recognized by their afferents. (3) Target cells present molecules, either on the cell surface or within a short range from the cell body, that disrupt the molecular interactions required for axon extension. (4) Target cells lack the adequate substrate for axon growth, and afferents thereby stop growing when they encounter their target cells. After the appropriate target is contacted, the primary axon will cease elongating and generate synaptic boutons, even though additional growth or arborization can subsequently occur.

Our laboratory has developed an *in vitro* model system by which to test the effect of target cells on the growth of afferents, consisting of mossy fiber afferents cocultured with their target cells, granule neurons. Mossy fibers from pontine nuclei, one of several sources of mossy fibers, stop growing when they meet their target cells, granule neurons. In other words, granule cells are thought to provide a stop-growing signal for their afferents, pontine mossy fibers (Baird *et al.*, 1992b). The stop-growing signal seems to be cell surface-associated since the length of mossy fibers is not reduced if they grow at a distance from granule cells. Moreover, video microscopy studies show that mossy fibers pause after they make filopodial contact with granule cells, but continue to extend on astroglia (Baird *et al.*, 1992b). This stop-growing signal is afferent-specific in that granule neurons do not reduce the growth of climbing fibers, another cerebellar afferent which innervates Purkinje cells,

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and retinal fibers, which never grow into the cerebellum (Baird *et al.*, 1992a). Furthermore, neural activity can modulate the stop signal. In the presence of an antagonist of NMDA-type glutamate receptors, pontine neurites extend long distances over granule cell monolayers, whereas NMDA enhances the stop signal (Baird *et al.*, 1996). To date, neither the molecular nature of the stop-growing signal nor the mode of detection of the stop-growing signal by mossy fibers is clear. However, other studies from our laboratory have demonstrated that membranes from granule neurons lack growth-arresting properties, suggesting that living cells must present the stop-growing signal, and/or that components have been lost in the purification process (Ward and Mason, 1996; also see Henke-Fahle *et al.*, 1996; Ward and Mason, 1997).

Development can be considered a coordinating process that depends on progressive interactions, in strict spatiotemporal patterns. DiI labeling *in vivo* shows that in the mouse pontine fibers arrive in cerebellum at E16–E18 and then project to the internal granule layer (IGL), but by P4 are restricted to the IGL, for the most part, without ''overshooting'' their target layer in the neonatal period (Zhang and Mason, in preparation, but see Mason and Gregory, 1984). One question is just how this is accomplished - by mechanisms intrinsic to the afferents, by timing of expression of factors common to target cells, or by developmental regulation of the response of afferents to target-derived factors. We therefore wondered whether the stop signal is developmentally regulated and if so, whether the changes in stop signal are correlated with the growth of mossy fibers *in vivo*. Others have addressed this issue in heterochronic cocultures of explants of afferents with slices of target tissue in other systems, and found that the age of the target is critical to whether the afferent terminates in the proper target cell zone (Yamamoto *et al.*, 1989; Bolz *et al.*, 1990; Molnar and Blakemore, 1991; Bolz *et al.*, 1992; Yamamoto *et al.*, 1992; Erzurumlu *et al.*, 1993; Yamagata and Sanes, 1995a; Yamagata and Sanes, 1995b; Redmond *et al.*, 1997). A related question is whether the response of afferents to the stop signal is also under developmental regulation.

To address questions of developmental regulation of stop signal production and responsiveness of pontine mossy fibers, we cocultured cerebellar slices of different ages with explants of afferents of two different ages. In addition, to study granule neurons as the specific source of the stop signal, we examined afferent growth in our model *in vitro* system, consisting of coculture of pontine explants and granule cells purified from animals of different ages. We found that both the stop signal and the response of afferents are subject to developmental regulation, and that the regulation of the response of afferent to the stop signal may be under the control of factor(s) from the target.

## MATERIALS AND METHODS

### *Animals*

The C57BL/6J mice used in this study were derived from a timed-pregnancy breeding colony maintained at Columbia University College of Physicians and Surgeons. The day on which a plug was detected was considered embryonic day 0 (E0). The day when pups were born was designated P0 (postnatal day 0), equivalent to E19–19.5. Sprague–Dawley rats were purchased from Zivic Miller with the same dating system that we used for mice. In general, the dams of Sprague–Dawley strain gave birth at E21 or E22 (=P0).

### *Cocultures of Pontine Afferents and Cerebellar Slices*

In this study, interspecies cocultures were used in order to visualize pontine neurites. Thus, mouse pontine neurites cocultured with rat cerebellar slices and granule cells were immunostained with M6 monoclonal antibody, specific for mouse neurons (Lund *et al.*, 1985; Baird *et al.*, 1992b).

In the present study, rat cerebellar slices and granule cells were used with mouse pontine explants. To date, there has been no report on the growth of rat pontine mossy fibers *in vivo*. The majority of mouse pontine neurons are generated at E13–E14, 5–6 days before birth (Pierce, 1966). Similar to their mouse counterparts, rat pontine neurons are generated at E17–19, 4–6 days before birth (Altman and Bayer, 1978). Pontine afferents in the rat grow into the cerebellum just before birth, based on the appearance of the middle cerebellar peduncle (Altman and Bayer, 1978). Thus, it appears that the prenatal development of pontine neurons and their axonal projection in both rat and mouse occurs at the same pace. Comparison of development of mouse (Miale and Sidman, 1961) and rat cerebellum (Altman, 1969, 1972a,b) indicates that there is a difference of only a few days in the postnatal development of cerebellum, and in the timing of afferent ingrowth between these two species. We therefore would predict that pontine mossy fibers in the rat also stop growing within their target layer around P4.

Cerebella dissected from P0, P4/P5, and P10 rats were cut parasagitally into 140 $\mu$m (for P4/P5 and P10 cerebella) or 200 $\mu$m (for P0 cerebella) slices using a McIlwain tissue chopper (Stoppini *et al.*, 1991). Slices were taken from the hemispheres, rather than the vermis or very lateral region. The medial 1/5–1/6 of the cerebellum was eliminated because of the absence of a pontine projection to the anterior vermis (Ruigrok and Cella, 1995), and the lateral region because lateral slices were difficult to maintain intact. In earlier experiments, we tried to coculture pontine explants with a whole cerebellar slice, placing the explants at the edge near ventrally positioned tracts and immature white matter or on top of the slice, but pontine neurites did not grow into the slice under such circumstances. To improve growth, we then used a trimmed-down slice configuration, cutting out individual folia that included all cellular layers and some white matter. Five to 7 slice pieces were mounted onto a laminin-coated, porous (0.4 $\mu$m) Millicell-CM membrane (Millipore). Pontine explants (300–500 $\mu$m in diameter) from P0 or E18 mouse were then placed on the membrane adjacent to the base of a folium, apposed to the white matter and IGL.

To obtain pontine explants, fresh brains were gently removed from the skull. Pontine nuclei were excised from pons, easily seen from the medial face of a parasagittally cut brain, as a bump protruding rostroventrally. After removal from the brainstem, pontine nuclei were cut into small explants with a Roboz microscalpel with a diameter ranging from 300 to 500 $\mu$m. After washing 3–6 times with medium to remove debris, explants were carefully placed adjacent to slices as described above.

After culturing for 2 days in serum-free chemically defined medium (Baird *et al.*, 1992b) at 35°C with 5% $CO_2$, the cocultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h. The filter supporting the slices with adjoining explants was

then cut from the support, and slices separated from one another by further cutting the supporting filter. All slices from the same grouping were placed in buffer in a small petri dish, and pontine explants and their neurites were visualized with a mouse-specific monoclonal antibody, M6 (gift of Dr. C. Lagenaur) by the indirect immunoperoxidase method as previously described (Baird *et al.*, 1992b). Filter pieces holding the cocultures were placed with a drop of buffer on a gelatin-coated slide, coverslipped with Aqua-mount (Lerner Laboratories, Pittsburgh, PA), and the coverslip sealed with nail polish. For each set of experiments, data were obtained from 2–3 experiments with 10–15 cocultures used in each experiment.

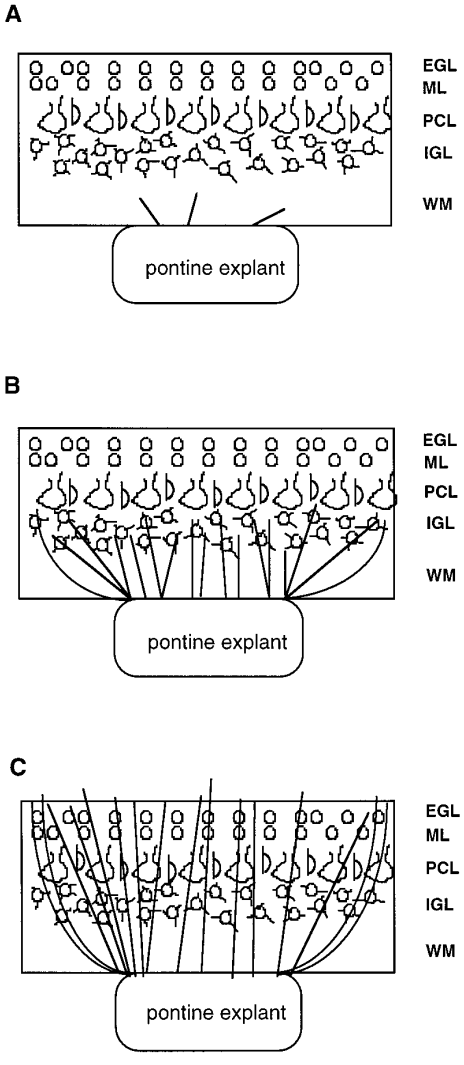



**FIG. 1.** Growth patterns of pontine neurites when cocultured with cerebellar slices. The growth of pontine neurites is divided into three categories: (A) no growth, only a few neurites extend from pontine explants into the cerebellar slice; (B) restricted growth, pontine neurites stop growing within the IGL; (C) unrestricted growth, pontine explants extend neurites throughout all the layers of the cerebellar slice. In categories B and C, in about half of the pontine explants, neurites grow around the edges of the cerebellar slice. Abbreviations: EGL, external granule layer; ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granule layer; WM, white matter.

**TABLE 1**

Neurite Growth from P0 Pontine Explants Cocultured with Cerebellar Slices

| Age of slices: / Number of cocultures: | P0 ($n$ = 36) | P4/P5 ($n$ = 30) | P10 ($n$ = 43) |
|---|---|---|---|
| No growth | 0% (0/36) | 7% (2/30) | 7% (3/43) |
| Restricted growth | 8% (3/36) | 70% (21/30) | 5% (2/43) |
| Unrestricted growth | 92% (33/36) | 23% (7/30) | 88% (38/43) |

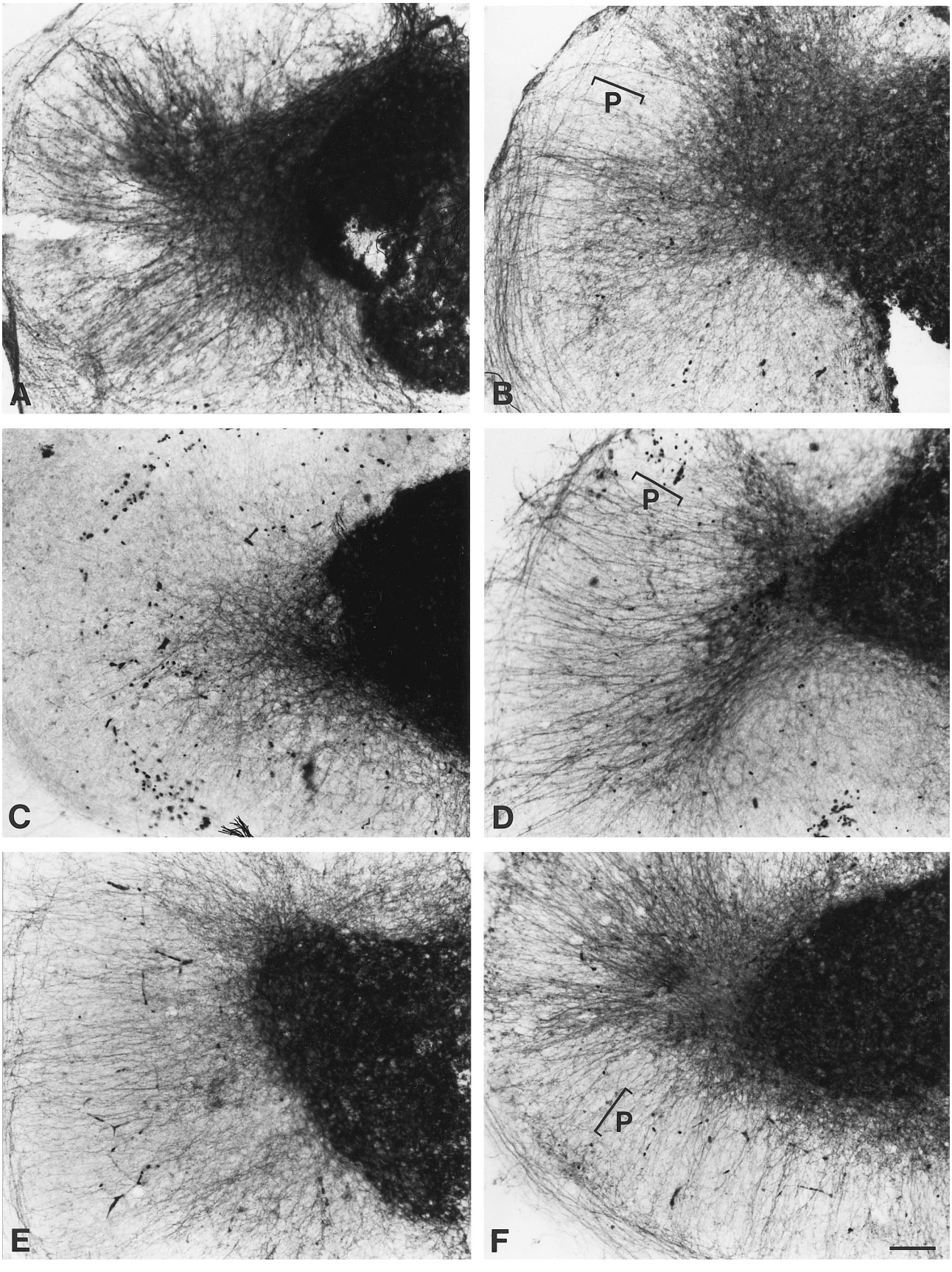
The immunostained pontine mossy fibers were traced using a camera lucida. The growth pattern of pontine fibers into the slices was divided into three categories (Fig. 1): no growth into the cerebellar slice (less than five neurites from one pontine explant); restricted growth (pontine neurite growth restricted within the IGL of the slice with fewer than 5 neurites growing through all the layers of the slice); and unrestricted growth (more than 5 pontine neurites extending into all the layers of the slice). In the latter two cases, in about half of the cocultures, neurites also grew around the edges of the cerebellar slice.

After 2 days in culture, even though the slices were trimmed down and they flattened to about 70–80 $\mu$m thickness, the typical layered structure of cerebellum was well preserved in the slice, based on the immunostaining of Purkinje cells with an antibody against a calcium binding protein, calbindin-$D_{28K}$ (SWant) (data not shown).

## *Cocultures of Pontine Afferents and Purified Granule Cells*

Pontine explants (100–300 $\mu$m in diameter) taken from P0 and E18 mice were cocultured with granule cells, purified from P2–P14 rats by using two-step Percoll gradient (Hatten, 1985). Briefly, cerebella dissected from P2–P7 rat pups were dissociated to a single cell suspension using a mixture of 1% trypsin and 0.1% DNase. Granule cells were separated from other types of cells first by means of a 60–35–0% Percoll step gradient and then further purified by two serial preplating steps on poly-D-lysine-coated (100 $\mu$g/ml, Sigma) plastic petri dish, to eliminate glia. For purifying granule cells from animals older than P7, the cerebellum was first sliced into 1-mm-thick slabs using a McIlwain tissue chopper, and then the white matter was removed using a Roboz microscalpel and forceps, before enzyme digestion. Without this step, only a small number of granule cells from older cerebellum could be separated from other cells on the Percoll gradient. Purified granule cells were plated on 16-well LabTek chamber slides or glass coverslip microculture dishes coated with poly-D-lysine (500 $\mu$g/ml, Specialty Media) and laminin (20 $\mu$g/ml, Sigma) (Baird *et al.*, 1992b), and cultured overnight. On the following day, pontine explants were added onto granule cells.

In our previous studies, the stop growing signal was detected at densities of 6000 to 8000 cells/mm$^2$. We therefore compared the growth of P0 pontine explants on granule cells plated at this density from animals of P2, P7, P10, and P14 to the growth on P4 granule



cells. However, after culturing granule cells for 3 days and pontine explants for 2 days, a greater proportion of explants on granule cells from older animals had long neurites, i.e., did not exhibit a cessation of growth. One potential factor influencing the more extensive growth was lower cell density after 3 days *in vitro*. While there was no remarkable difference in the survival rate among P2, P4, and P7 granule cells, cells from older animals plated at the same starting density showed poorer survival after 3 days in culture. As the initial plating density was the same, either plating efficiency or cell death could have been responsible for this phenomenon. Plating efficiency, checked at 2 h after plating, was good for granule cells from both young and old pups, suggesting that cell survival was poorer at older ages. Therefore, to attain the same final density of granule cells, approximately 7000 cells/$mm^2$ after 3 div, we increased the initial plating density for granule cells from older animals. About 25% more P10 and P14 granule cells were initially added into culture dish in order to obtain the same final density after 3 days *in vitro* as P4 granule cells.

Cultures were maintained for 2 days in a serum-free chemically defined medium as previously described by Baird *et al.* (1992b) at 35°C with 5% $CO_2$, and were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. Explants and their neurites were demonstrated with the M6 monoclonal antibody by the indirect immunoperoxidase method as above. After immunostaining, cell density was estimated by counting cells in an area in the center of the well and two areas in the periphery of the culture well, each area measuring 0.08 $mm^2$. The neurite lengths were measured with the aid of a camera lucida, the image of a self-made ruler calibrated with micrometer was visualized and centered on each explant. The criterion for the stop-growing response is that less than 5 neurites extend more than 200 $\mu$m from the edge of the explant. For each type of coculture experiment, data were collected from two to three experiments. Approximately 175 pontine explants were used in each experiment.

### *Preparation of Conditioned Medium*

Cerebella or cerebral cortices were dissected from P0 mice and were incubated in 0.25% trypsin and 0.025% DNase solution for 10 and 6 min, respectively. The tissue was then dissociated into a single cell suspension in 10% horse serum medium containing 0.05 mg/ml trypsin inhibitor (Type I-S, from soybean; Sigma). Cells were mixed with 30% Percoll (in a ratio of 1:1) and spun in order to remove cell debris. Cells ($10^6$) were plated with serum-free medium in a culture dish (diameter, 10.5 mm) pretreated with poly-D-lysine (Specialty Media, 500 $\mu$g/ml) and laminin (Sigma, 20 $\mu$g/ml). The next morning, cultures were washed with serum free medium. Cells ($10^6$) were used to condition 250 $\mu$l serum-free medium. Conditioned medium was collected 46–48 h later and centrifuged briefly to eliminate debris.

E18 pontine explants were treated in suspension in a 35-mm tissue culture dish (Corning) in a total volume of 2.1 ml diluted conditioned medium (in the ratio of 1 portion of serum-free medium and 2 portions of conditioned medium) or with serum-free medium, for 2 days, prior to coculture with cerebellar slices or granule cells.

## RESULTS

### *Developmental Changes in the Stop-growing Signal from Granule Neurons*

***Coculture of pontine explants with slices of cerebellar cortex.*** To test whether the stop signal in target neurons is developmentally regulated, we cocultured explants of afferent mossy fibers isolated from pontine nuclei with slices of cerebellum of different ages.

The behavior of P0 pontine neurites on cerebellar slices shows that the age of the target tissue is important in arresting the growth of pontine afferents. In slices taken from P4/5 rats, the majority of P0 pontine explants (70%) positioned below the IGL extended neurites but then stopped within the IGL (Table 1 and Fig. 2C). Pontine neurites ended in small growing tips rather than complex growth cones, as they do *in vivo* (data not shown; see Mason and Gregory, 1984). As seen *in vivo* between P2 and P4, when pontine neurites leave fiber bundles in the white matter and grow toward their target layer (Zhang and Mason, in preparation), pontine neurites in slice cocultures showed no obvious fasciculation. When P0 pontine explants were cocultured with younger (P0) or older (P10) cerebellar slices, 92 and 88% of the explants, respectively, extended neurites through all layers of the slices (Table 1 and Figs. 2A and 2E). Moreover, there was no layer selectivity: neurites grew equally into the external granule layer (EGL), Purkinje cell layer (PCL) and IGL. Taken together, the experiments on P0 pontine explants cocultured with cerebellar slices of different ages indicate that the stop signal is strongest around P4.

***Coculture of pontine explants with dissociated target granule cells.*** One possible source of the stop-growing signal is the target cell itself. Alternatively, other cell types in the same cortical layer or region could provide the signal (Molnar and Blakemore, 1991; Emerling and Lander, 1994; Yamagata and Sanes, 1995a). The slice cultures cannot indicate which cell types play a role in the arrest of pontine mossy fibers within the IGL.

Our previous studies have demonstrated that purified granule neurons pose a stop-growing signal to their mossy fiber afferents (Baird *et al.*, 1992a,b). In cocultures of pontine explants on monolayers of granule neurons, we characterized explants in which neurites terminated $<200$ $\mu$m from

**FIG. 2.** Neurite outgrowth from P0 and E18 pontine explants cocultured with cerebellar slices. Mouse pontine explants were cocultured with rat cerebellar slices for 2 days and immunohistochemically stained with monoclonal antibody M6, specific for mouse neurons. Most P0 pontine neurites do not extend beyond the Purkinje cell layer (P) when cocultured with P4/5 slices (C), but grow through all the layers of P0 and P10 cerebellar slices (A, E). Irrespective of the age of cerebellar slice with which they are cocultured, E18 pontine explants show equally abundant outgrowth into the slice (B, D, F). [P], at P0 (B) and P4 (D), indicates location of Purkinje cell perikarya and immature dendrites, and at P10 (F), the monolayer of Purkinje cell perikarya and apical dendrites. Scale bar, 100 $\mu$m. Abbreviation: P, Purkinje cell layer.

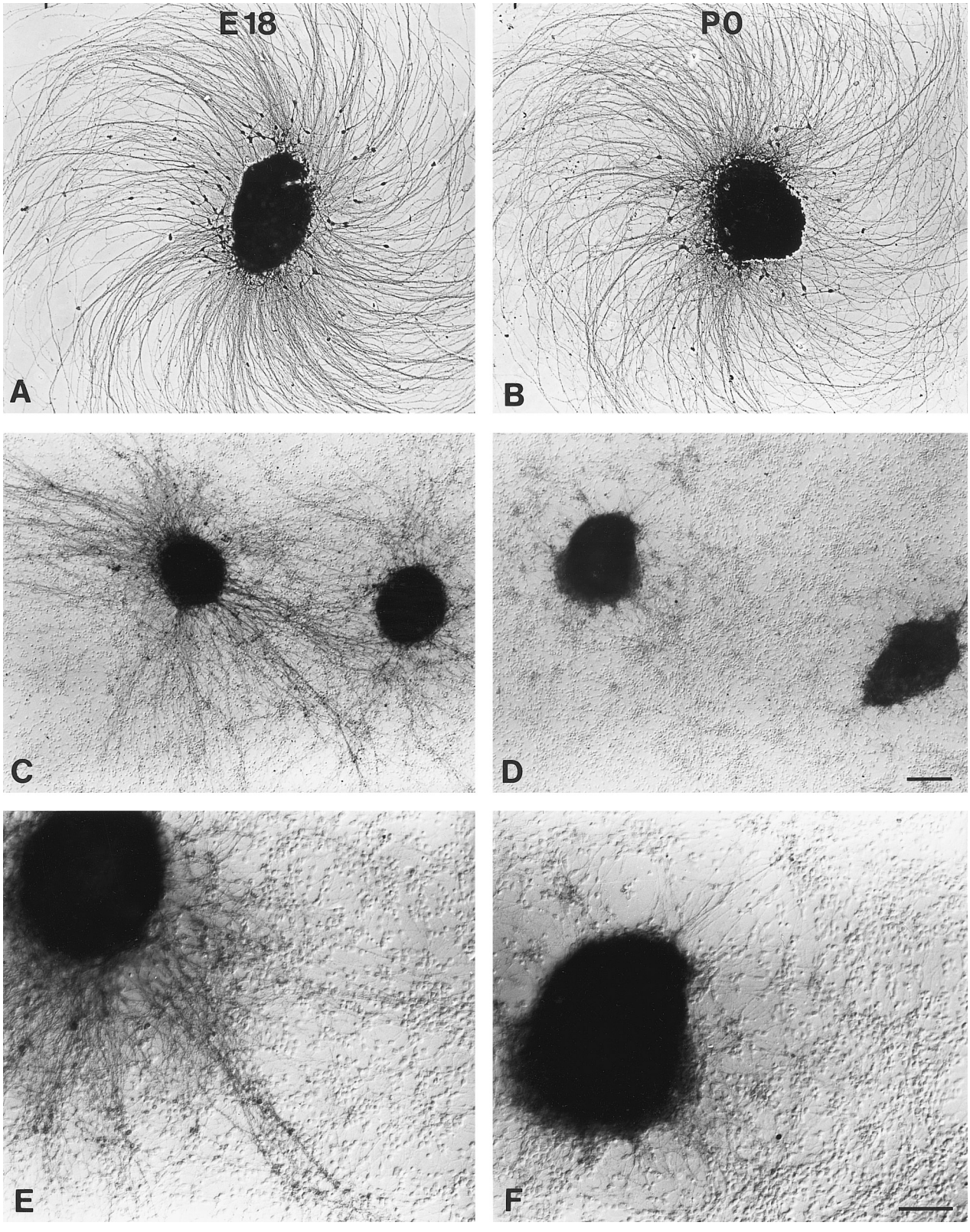



**FIG. 3.** Neurite outgrowth from E18 and P0 pontine explants on polylysine–laminin (A, B) and on P4 granule cells (C, D, E, F). There is no remarkable difference between neurite lengths from E18 (A) and P0 (B) pontine explants growing alone on poly-D-lysine and laminin substrate. Pontine explants were grown on granule cells plated at a density of 12,500 cells/mm$^2$ for 2 days, and their neurites were

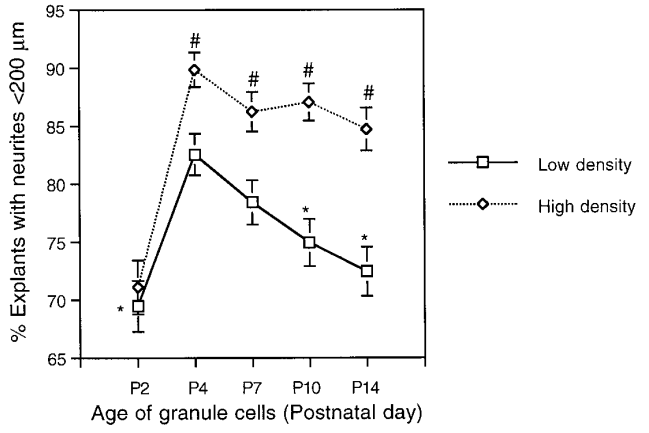



**FIG. 4.** Changes in the strength of the stop signal during development. At a low density of granule neurons (from 7380 to 8455 cells/mm$^2$) (squares), P4 and P7 granule cells provide the strongest stop signal to their afferents, reflected in the majority of neurites extending <200 $\mu$m from the edge of the explant. In contrast, granule cells from earlier and later stages present a weaker stop signal to pontine mossy fibers. Increasing the density of granule cells to 9917–12,892 cells/mm$^2$ (diamonds) compensates for the somewhat weaker stop-signal at later stages, but not for younger ages. Single asterisks (*) indicate values that are significantly different from the value at P4 ($P < 0.01$, $\chi^2$ test). The number signs (#) indicate values that are significantly different comparing low and high density in each age group ($P < 0.01$, $\chi^2$ test). Error bar represents SE.

the edge of the explants as responding to the stop-growing signal. In the following analysis, we aimed to confirm that the stop signal observed in cerebellar slices emanates from the target granule cells, and, second, asked whether it is developmentally regulated as it is in the slice cultures.

As observed previously (Baird *et al.*, 1992b), on a poly-D-lysine–laminin substrate, pontine neurites from P0 explants extended to 400–600 $\mu$m, growing symmetrically from all aspects of the explants (Fig. 3B). These neurites displayed some degree of fasciculation. In contrast, when cocultured with P4 granule cells at a final density of 7918 ± 538 cells/mm$^2$, or with P7 granule cells at a similar density, the majority of pontine explants extended short (<200 $\mu$m), unfasciculated neurites, terminating among nearby granule cells (83 ± 1.8% (mean ± SE) and 78 ± 1.9%, respectively) (Fig. 4).

However, when plated on granule neurons from periods later than P4–7, such as P10 and P14, the number of explants with neurites shorter than 200 $\mu$m was reduced (75 ± 2.1 and 72 ± 2.1%, respectively). Moreover, on granule cells from stages earlier than P4–7, such as P2, pontine explants showed effusive growth, with neurites even longer (in the range of 500–1000 $\mu$m) than either those grown on polylysine–laminin alone or those grown on granule cells from later ages, with only 69 ± 2.2% of explants extending neurites <200 $\mu$m (Fig. 4). Compared to the pontine neurites growing alone on poly-D-lysine–laminin, the long pontine neurites (>200 $\mu$m) growing on granule cells from P2, 10, or 14 cerebellum did not radiate symmetrically from the explant but, rather, joined from several sites and collected into a few thick fascicles. These differences were statistically significant, comparing values from ages P4 and P2 (pontine explants with P4 granule cells, $n = 447$ explants; with P2 granule cells, $n = 442$ explants; $\chi^2 = 20.2$, $P < 0.01$), P4 and P10 (with P10 granule cells, $n = 447$ explants; $\chi^2 = 7.28$, $P < 0.01$), and P4 and P14 (with P14 granule cells, $n = 450$ explants; $\chi^2 = 12.55$, $P < 0.01$) ($\chi^2$ test, significance level: $P < 0.01$). No statistical difference was found between P4 and P7 granule cells in their ability to elicit arrest of pontine neurite growth (with P7 granule cells, $n = 468$ explants; $\chi^2 = 2.22$, $P > 0.05$) (Fig. 4). These data indicate that the stop signal is strongest from P4 to P7, wanes somewhat after P7, and that at early stages, is weaker than at later stages. In addition, early granule cells may be more growth-supportive than granule cells at later stages.

Our previous studies have shown that the stop-signal is cell density-dependent (Baird *et al.*, 1992b). As the density of granule cells increases, pontine neurite length decreases. We then tested whether this density dependence is operative at ages earlier and later than P4–7, and, in turn, at stages when the stop signal is less strong, whether increasing the plating density would compensate for the weaker effect. P0 pontine explants were grown on granule cells of different ages at densities higher than those used in the experiments above, with some compensation for greater cell death at later ages (see Materials and Methods). When the density of P4 cells was increased, there was only a small increment in the numbers of explants with neurites <200 $\mu$m. At a final density of 7380 ± 158 cells/mm$^2$ (calculated after fixation and immunostaining), 83 ± 1.8% of pontine explants ($n = 447$) on P4 granule cells displayed evidence of arrest of growth (Fig. 4), whereas at a higher density of 10,285 ± 590 cells/mm$^2$, 90 ± 1.5% of the explants ($n = 414$) showed indication of a stop-growing response ($\chi^2 = 8.96$, $P < 0.01$). Raising the density at later ages gave a significant increase in the numbers of explants with short neurites, indicating that there is compensation for the weaker stop signal by the higher cell density. When the cell density was elevated from 7844 ± 132 to 10,096 ± 424 cells/mm$^2$, and 7448 ± 246 to 10,363 ± 340 cells/mm$^2$ for P10 and P14 granule cells, respectively, the percentage of P0 pontine explants exhibiting short neurites increased from 75 ± 2.1% ($n = 447$) to 87 ± 1.6% ($n = 441$) ($\chi^2 = 20.85$, $P < 0.01$) and from 72 ± 2.1% ($n = 450$) to 85 ± 1.9% ($n =$

visualized by immunostaining with monoclonal antibody M6. E18 pontine explants extend neurites 500–1000 $\mu$m from the explant edge (C, E). In contrast, most neurites from P0 pontine explants terminate among adjacent granule cells (D, F). Explant on left in D is shown at higher magnification in F. A, B-phase optics; C, D, E, F-Nomarski optics. Scale bars: 100 $\mu$m for A, B, C, and D; 50 $\mu$m for E and F.

**TABLE 2**

Neurite Growth from E18 Pontine Explants Cocultured with Cerebellar Slices

| Age of slices: / Number of cocultures: | P0 ($n$ = 34) | P4/P5 ($n$ = 34) | P10 ($n$ = 42) |
|---|---|---|---|
| No growth | 3% (1/34) | 3% (1/34) | 0% (0/42) |
| Restricted growth | 6% (2/34) | 24% (8/34) | 2% (1/42) |
| Unrestricted growth | 91% (31/34) | 74% (25/34) | 98% (41/42) |

373) ($\chi^2 = 17.20$, $P < 0.01$), respectively (Fig. 4). In contrast, raising the cell density of granule cells at P2 failed to make the explants respond as well as they do with P4 cells. Less than a 2% increase [from 69 ± 2.2% ($n$ = 442) to 71 ± 2.3% ($n$ = 377); $\chi^2 = 0.19$, $P > 0.05$] was observed when the cell density was raised from 8455 ± 420 to 12,892 ± 692 cells/mm$^2$ (Fig. 4).

These results further indicate that the stop signal is developmentally regulated, and that the stop signal for pontine mossy fibers does arise from granule cells. The stop signal is much weaker at neonatal stages than at any other ages tested, and wanes after P7. From slice experiments, it seems that the stop signal almost disappears by P10, but dissociated cell experiments indicate that a weaker stop signal still exists at P10 and P14. The discrepancy between the results from slice and cell cultures may reflect the possibility that other factors or cell types in the slice override the weaker stop growing signal, or that presentation of the stop signal is less effective in slices, leading to the apparent disappearance of the stop signal at P10 in slice cultures.

### *Developmental Onset of the Responsiveness of Pontine Mossy Fiber Neurites to the Stop Signal*

*In vivo*, pontine afferents are still growing to the cerebellum at E18, with some fibers in tracts, some in subcortical regions, and a few within cellular zones of the anlage below where postmigratory Purkinje cells have accumulated. In contrast, by P0, most pontine neurites have arrived in the cerebellum (Zhang and Mason, in preparation). One hypothesis is that during extension toward the cerebellum the pontine afferents are unable to respond to the stop signal, and that they must enter the target to receive target-derived signals that may prime them to respond to the stop signal. To address this, the responsiveness of younger (E18, 1–1.5 days younger than P0) pontine afferents to the stop signal was compared with that of P0 pontine neurites, in coculture with cerebellar slices at the same ages used above (P0, P4/5, and P10) or with P4 dissociated granule cells, which provide the most potent stop signal.

E18 pontine explants cultured with cerebellar slices from P0, P4/5, or P10 rat showed predominantly unrestricted growth patterns (91, 74, and 98% of explants, respectively; Table 2, and Figs. 2B, 2D, and 2F). This pattern of neurite growth into all cellular layers of the cerebellar slices, in contrast to the restricted growth pattern of P0 pontine neurites cocultured with P4/5 cerebellar slices, suggests that E18 neurites are less able to respond to the stop-signal posed by the target cerebellum.

E18 pontine explants were also grown with and without purified granule cells. Pontine nuclei from E18 mouse were slightly smaller and the tissue somewhat more delicate during dissection than P0 pontine nuclei. When grown alone on poly-D-lysine and laminin, however, more E18 pontine explants remained attached to the substrate and grew neurites than explants from P0 pontine nuclei. Further, the number of neurites from individual E18 pontine explants was slightly higher than from P0 pontine explants, but there was no remarkable difference between the neurite lengths from E18 and P0 explants, with most neurites reaching 400 to 600 $\mu$m in both cases (Figs. 3A and 3B).

At a plating density of 12,500 cells/mm$^2$, P4 granule cells present a very strong stop signal to P0 pontine explants, inducing 90 ± 1.1% of P0 pontine explants ($n$ = 810) to stop growing at points <200 $\mu$m (Figs. 3D and 3F). However, when E18 pontine explants were cocultured with P4 granule cells at this plating density (Figs. 3C and 3E), only 46 ± 1.6% of E18 pontine explants ($n$ = 936) had neurites <200 $\mu$m (Fig. 5). The difference between these two groups of explants was statistically significant ($\chi^2 = 379.70$, $P < 0.01$). As with P0 pontine explants on P2 granule cell monolayers, most of the E18 pontine explants without evidence of growth cessation had numerous neurites extending from each explant with neurite length in the range of 500–1000 $\mu$m. These neurites showed some degree of fasciculation. These data demonstrate that E18 pontine neurites did not respond as effectively as P0 pontine neurites to the stop signal from granule cells at P4.

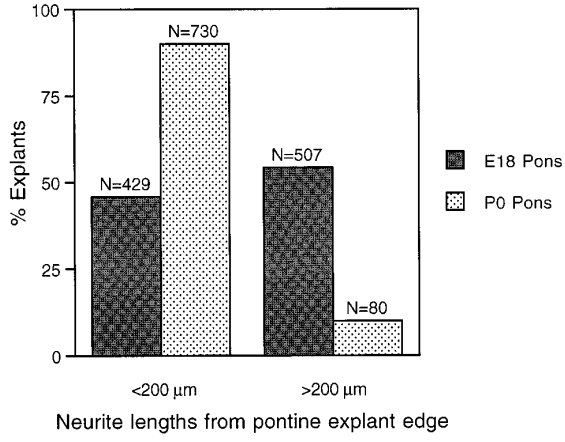



**FIG. 5.** Differential response of E18 and P0 pontine explants to P4 granule cells. When grown on a P4 granule cell monolayer at a plating density of 12,500 cells/mm$^2$, 90% of P0 pontine explants stop growing within 200 $\mu$m from the edge of pontine explants, while less than half of E18 pontine explants have neurites shorter than 200 $\mu$m. The number of pontine explants is indicated on top of the bar.

### *Enhancement of the Response of E18 Pontine Neurites to the Stop Signal by Factor(s) from the Cerebellum*

The above results demonstrate that P0 mossy fibers, most of which have arrived in the cerebellum, show arrest of growth after encountering P4 granule cells, whereas E18 mossy fibers, many of which are still projecting to the cerebellum, show a reduced ability to respond to the stop signal. One question is whether afferents must grow into the cerebellum to be primed to respond to the stop signal, or whether there is an intrinsic switch for the response that depends simply on developmental age of the afferent cells. To answer this, E18 pontine explants were cultured in suspension in a 35-mm tissue culture dish in serum-free medium (SFM) without a poly-D-lysine or laminin substrate for 1 or 2 days, during which time neurites did not emerge from the suspended explant. The isolated explants were then removed and cocultured with P4/P5 cerebellar slices. The majority of the E18 pontine explants "aged" *in vitro* extended neurites through all cellular layers of the cerebellar slice, similar to the pattern observed when E18 explants were removed and directly cocultured with cerebellar slices, as described above. Thus, aging the explants *in vitro* did not compensate for development of afferents *in vivo*.

One possible explanation for the lack of response to the stop signal is that the general differentiation program of pontine neurons is delayed in the isolated culture conditions. Alternatively, extrinsic factor(s) may be required to prime young neurites to respond to the stop signal. To distinguish between these two possibilities, medium conditioned by dissociated P0 mouse cerebellar cells for 48 h was taken as a source of cerebellar-derived factors. E18 pontine explants were held in suspension for 48 h, either in this conditioned medium, in medium similarly conditioned by P0 cerebral cortex cells, or in unconditioned SFM. The explants receiving these three treatments were then cocultured with P4 cerebellar slices. About 10% more E18 explants treated with medium conditioned by cerebellar cells had restricted growth within the IGL compared to controls. However, the difference was not statistically significant. Since other factors or cell types are also present in the slices in addition to granule cells, it is possible that the action of other factors, probably growth-promoting factors, partially masks the stop signal, as indicated by the difference between cocultures of P0 pontine explants with P10 slices and P10 granule cells.

To determine whether such a cerebellum-derived "priming" factor enhances the response of E18 pontine mossy fibers to the granule cell-derived stop signal, E18 explants treated in SFM or medium conditioned by cerebellar cells or cortex cells were cocultured with purified granule cells. After 2 days of conditioning, explants were transferred onto P4 rat granule cells plated 1 day before at a density of 12,500 cells/mm$^2$, and cultured for two additional days. Granule cells at this age and density normally provide a strong stop signal to P0 pontine explants. Treatment with medium conditioned by P0 cerebellar cells increased the proportion of E18 explants with neurites $<200$ $\mu$m over that of control explants suspended for 2 days in SFM or in medium conditioned by cerebral cortex cells. While this increase was small [64 $\pm$ 2.8% of cerebellar cell-conditioned medium-treated pontine explants ($n = 284$) display neurites shorter than 200 $\mu$m vs 48 $\pm$ 3 and 51 $\pm$ 3% treated with cortical cell-conditioned medium ($n = 280$) and SFM ($n = 282$), respectively], it was statistically significant ($\chi^2 = 15.74$, $P < 0.01$, between the cerebellar cell-conditioned medium-treated and cerebral cortical cell-conditioned medium-treated groups; $\chi^2 = 10.36$, $P < 0.01$, between the cerebellar conditioned medium-treated and SFM-treated groups) (Fig. 6A). Such a small increase may be due either to the instability of factor(s) in conditioned medium, the inefficient penetration of factor(s) into pontine explants, loss of factor(s) during dissociation and plating of cells, or dilution of factor(s). While we did not exclude the possibility that the differentiation program of pontine neurons is delayed, these experiments suggest that factor(s) from the cerebellum may render the ingrowing afferents more competent to respond to the stop signal.

To exclude the possibility that conditioned medium from cerebellar mixed cultures might have toxic effects on the growth of pontine neurites, E18 pontine explants held in suspension in conditioned or serum-free media were plated alone on a poly-D-lysine and laminin substrate. Two days later, they were fixed and immunostained with M6 monoclonal antibody, and lengths of explant neurites were measured. Because pontine neurites tend to grow clockwise when on a poly-D-lysine and laminin substrate alone compared to radial growth on granule cells, the outgrowth was divided into 4 quadrants and the neurite lengths were measured from the edge of explant to the farthest point on each of 4 axes. All three groups of pontine explants grew well on poly-D-lysine and laminin, but the neurites of pontine explants treated with conditioned media were longer than neurites from pontine explants without conditioned medium pretreatment: 601 $\pm$ 12 $\mu$m ($n = 70$) for cerebellar cell-conditioned medium-treated explants and 578 $\pm$ 11 $\mu$m ($n = 66$) for cerebral cell-conditioned medium-treated explants, compared to 514 $\pm$ 9 $\mu$m ($n = 72$) for explants in SFM alone (Fig. 6B). These differences were significant between the group treated with cerebellar cell-conditioned medium vs. that in SFM ($P < 0.01$, Bonferroni's $t$ test), and the groups treated with cortical cell-conditioned medium and SFM ($P < 0.01$, Bonferroni's $t$ test). These results not only exclude the possibility of toxic effects of conditioned medium from cerebellar cell cultures on the growth of pontine neurites, but indicate that factors in conditioned medium from the cerebellar target can enhance the ability of pontine neurites to grow as well as prime them to respond to the stop signal.

## DISCUSSION

The present analysis by both slice culture and cell culture reveals that granule neurons, as target cells, are most potent over a period of P4–P7 in arresting the extension of their afferents, pontine mossy fibers. Subsequently, the stop signal begins to wane. In addition, younger afferents which *in*

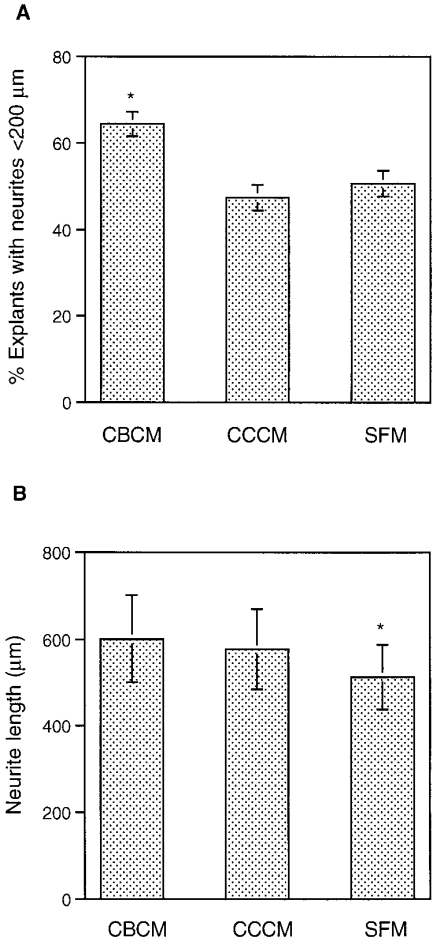



**FIG. 6.** Effects of conditioned medium on the response to the stop signal and neurite outgrowth of E18 pontine explants. (A) Medium conditioned by cerebellar mixed cultures (CBCM) enhances the response of E18 pontine explants to the stop signal. Pretreatment of E18 pontine explants for 2 days with conditioned medium from P0 cerebellar mixed cultures results in an increase in the number of pontine explants displaying signs of growing arrest on P4 granule cells (pontine neurites $<200$ $\mu$m from the edge of the explant). In contrast, factors in cerebral cortex cell-conditioned medium (CCCM) or serum-free medium (SFM) do not increase the response of pontine explants to the stop-growing signal. Asterisk indicates significant difference from controls ($P < 0.01$, $\chi^2$ test). Error bar represents SE. (B) Conditioned medium increases neurite outgrowth from E18 pontine explants. E18 pontine explants grown on poly-D-lysine and laminin alone for 2 days after 48 h in suspension in CBCM or CCCM show enhanced neurite outgrowth, compared to explants maintained in SFM. Asterisk indicates that pontine explants previously cultured in serum-free medium grow shorter neurites on laminin than pontine explants cultured in conditioned media ($P < 0.01$, Bonferroni's $t$ test). Error bar represents SD.

*vivo* are growing to or have just arrived in the cerebellum are less capable than more mature afferents of responding to the stop signal. Moreover, factors from the cerebellum may render the pontine fibers able to respond to the stop signal. These results indicate that there may be multiple interactions between granule cells or other cerebellar cells and pontine mossy fibers during development.

### *Timing of Target Cell Regulation of the Stop Signal*

The stop signal is strongest between P4 and P7. This corresponds to the cessation of the growth of pontine mossy fibers in the IGL *in vivo*, and the commencement of granule cell migration inward from the EGL to the IGL. DiI labeling *in vivo* (Zhang and Mason, in preparation) shows that in the mouse, pontine fibers begin to enter the cerebellum at E16–E18 in a lateral tract and project into the cerebellar anlage as thick fasciculated bundles. By P2, pontine mossy fibers have already left the tract projecting toward the IGL. At P4, pontine mossy fibers are restricted to the IGL as fine unfasciculated fibers. By P7, they still remain below the PCL without "overshooting" their target layer, except for some fine filopodial extensions onto Purkinje cells (Mason and Gregory, 1984).

The maximum strength of the stop signal matches the beginning of protracted granule cell migration, with a lower efficacy at P2, when most granule cells are in the EGL. Two caveats apply to the interpretation of our data that this peak reflects the waxing and waning of the stop signal. First, granule neurons with their protracted development may be proliferating, migrating and maturing in different relative numbers at the different ages tested. The apparent peak of the stop signal at P4–7 could thus reflect the average developmental state of the fraction as a whole. Second, the EGL cells, which are a major component of the granule cell fraction in our assay, may, like neural crest cells (Bronner-Fraser, 1993), generate precursors with different intrinsic properties, or ones that will be targets of different mossy afferent systems and thus modalities. Each mossy population has their own arrival time (Morris *et al.*, 1988; Ashwell and Zhang, 1992; Grishkat and Eisenman, 1995). Because the pontine afferents are the latest to arrive, we have assumed that their targets are produced in time for their arrival, and are among the total cell population. Future analyses will determine whether granule cells at a certain developmental stage or a particular subpopulation of targets has a more or less potent ability to arrest mossy afferent growth.

### *Mechanisms of the Target-Derived Stop Signal*

Two possible mechanisms may explain the cessation of pontine afferents by their target cells. First, granule cells could express specific cell surface-associated molecule(s) that can be recognized by receptors on the pontine afferents. Second, granule cells could lack adequate substrate molecule(s) required for the extension of pontine mossy fibers. The developmental timetable of regulation of the stop signal suggests that signals are upregulated or downregulated during the period between P4 and P7, leading to the cessation of pontine mossy fiber extension. Our results point to factors produced by the granule cell that are under develop-

mental regulation. Studies have shown that a number of molecules in granule cells show this pattern of developmental regulation. For example, the mRNAs encoding the $\alpha$1-, $\beta$2-, and $\gamma$2-subunits of $GABA_A$ receptors are relatively low in the first postnatal week, then rise dramatically between P10 and P17 in the granule cell layer (Gambarana *et al.*, 1990, 1991). In addition, whereas the mRNAs of NR2A and 2C subunits of NMDA receptors appear in granule cells during the second postnatal week and are predominant in the adult cerebellum (Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992; Watanabe *et al.*, 1992), NR2B mRNA is only transiently expressed in granule cells during the first 2 postnatal weeks (Akazawa *et al.*, 1994). In an extensive analysis of a cDNA expression library from granule cells, Kuhar *et al.*, (1993) found that 22 cDNAs have high levels of expression at P0 and P10; by P20 and P40, the expression of these cDNAs decreases to low or undetectable levels. Future investigations will determine whether these molecular components participate in the stop-growing signal(s) or reflect other aspects of the maturation of granule neurons.

### *Response of Afferents to the Stop Signal on Targets*

As in the present study, other work has shown that the influence of target tissue on afferents is stage-specific (Molnar and Blakemore, 1991; Bolz *et al.*, 1992; Erzurumlu *et al.*, 1993; Redmond *et al.*, 1997). Recent studies in other regions of the nervous system have demonstrated that the target cells or layers can affect growth of afferents differentially depending on age of the target. When explants of lateral geniculate nucleus (LGN) are cocultured with cerebral cortex, the cortex promotes outgrowth of thalamic neurites at earliest stages, then at P4, provides a stop signal in layer 4 (the target layer) (Molnar and Blakemore, 1991; Bolz *et al.*, 1992; Molnar and Blakemore, 1995). Using a coculture system in which a tectal slice is overlaid with a retinal explant, Yamagata and Sanes (1995a) showed that intrinsic, localized, membrane or matrix associated cues are responsible for the lamina-specific outgrowth and arborization of retinal axons in the optic tectum. Likewise, when trigeminal ganglia at early stages are cocultured with isochronic target tissue, they extend loosely bundled neurites in distinct fiber tracts similar to those *in vivo*. With more mature target tissue, the trigeminal neurites from early stage animal form discrete arbors (Erzurumlu *et al.*, 1993).

In contrast to the change in the response of pontine neurites to the granule cell-derived stop signal observed in the present study, in other systems the response of afferents at different stages to targets does not appear to be under developmental regulation. Explants of lateral geniculate nucleus from E16 animals show the same response to the cerebral cortex as P0 explants (Molnar and Blakemore, 1991; Bolz *et al.*, 1992). When trigeminal ganglia from late stages that have already formed arbors *in vivo* are cocultured with young target tissue, their growth pattern reverts to early elongation stages in response to the younger target (Erzurumlu and Jhaveri, 1995). Likewise, heterochronic cultures of spinal cord and dorsal root ganglia (DRG) show that DRG axons from E14–P0 animals grow into and arborize in the spinal cord of E14–E18 but not of E20–P0 animals. This indicates that the ability of DRG axons to enter and arborize in the spinal cord is determined by the age of target tissue and not by the age of the afferents (Redmond *et al.*, 1997).

Our observations show that the ages of afferents and target cells are *both* critical factors in determining whether afferent growth is arrested by target cells. Since the neurite lengths of E18 and P0 pontine explants after 2 days alone on poly-D-lysine and laminin substrate show no remarkable difference, it seems unlikely the differential response of E18 and P0 pontine neurites to the stop signal is due to a difference in the inherent rate of neurite extension between the two ages. An alteration in the signaling systems of pontine neurites may better explain the differential response of E18 and P0 pontine explants to P4 granule cells. Receptor(s) on the pontine afferents for detecting the stop signal may be upregulated as the neurites mature. In addition, intracellular components of signal transduction systems that link the detection of the stop signal with the change in the motility of pontine growth cones may be upregulated from E18 to P0.

An additional interpretation of these data is that granule neurons and their precursors from the EGL are subdivided with respect to their mossy inputs and modalities. Thus, it may be that only the stop-signal for pontine afferents peaks at P4–7. It would therefore be of interest to test whether the spinocerebellar mossy fibers (Grishkat and Eisenman, 1995), which arrive earlier than the pontine mossy fibers, have a different peak of responsiveness to the granule neurons.

### *Arrest of Afferent Growth May Be Dependent on Reciprocal Interactions between Afferent and Target Region*

The present analysis raises the possibility of reciprocal interaction(s) between pontine afferents and granule cells or cerebellum during development. A dramatic change in the responsiveness of pontine mossy fibers to the stop signal occurs within 1–1.5 days between E18 at the time the pontine mossy fibers have just entered the cerebellum, and birth. Aside from any intrinsic maturational changes not excluded by our analysis, results from conditioned medium experiments show that factor(s) from the cerebellum may render the pontine afferents more competent to respond to the stop signal. Further studies will investigate the source of the factor(s) (granule cells or other cell types in the cerebellum); the molecular nature of the factor(s) (protein(s) or small molecule(s)); the mechanism through which the factor(s) functions (transcriptional or translational regulation).

The developmental regulation of the stop signal in granule cells could be key to the onset of the stop signal. Even though the period during which the stop signal is very strong roughly corresponds to the commencement of massive migration of granule cells from the EGL into the IGL, an argument against intrinsic control of the stop signal by granule cells is that after P7, granule cells in the EGL still divide and migrate into the IGL, and the migration of gran-

ule cells is completed around P20 (Altman, 1969, 1972a). Preliminary results showed that whereas the P4 granule cells aged 3 days *in vitro* were as potent as fresh P4 granule cells to provide a stop signal to P0 pontine mossy fibers, P1 granule cells aged *in vitro* for 3 days still could not effectively arrest the growth of P0 pontine neurites, as is the case for newly purified P1 granule cells (Zhang and Mason, unpublished results). It appears that the change in the strength of the stop-growing signal is regulated by environmental factors rather than by intrinsic properties of granule cells. One possible source of these factors is the afferents themselves, since during this period pontine mossy fibers are approaching their target layer. It is possible that the differentiation and/or maturation of granule cells in culture could be delayed in the absence of afferents. Our *in vitro* system will provide a convenient way to test whether the changes in the stop signal are affected by extrinsic factor(s).

In support of the notion that afferents stimulate targets to upregulate the stop signal and in turn targets prime afferents to respond to the stop signal, a number of studies have demonstrated that there are indeed complex reciprocal interactions between afferents and their targets or intermediate targets during specific growth phases. In the spinal cord, commissural axons are guided by their intermediate target, floor plate, to the midline through the action of netrins (Kennedy *et al.*, 1994). E11 commissural axons are not as sensitive as E13 commissural axons to netrins, but addition of netrin-synergizing activity purified from the brain dramatically potentiates the response of E11 commissural axons to netrins (Serafini *et al.*, 1994). In addition, substance P released from a subset of commissural neurons can modulate the chemotropic effect of the floor plate on commissural fibers (De Felipe *et al.*, 1995).

An additional example of the reciprocal interactions between afferents and targets is found in sweat glands and the sympathetic neurons which innervate them. One subpopulation of sympathetic neurons undergoes a striking alteration in neurotransmitter phenotype during development, from noradrenergic to cholinergic (Schotzinger *et al.*, 1994). The target tissue, the sweat gland, plays a critical role in the respecification of neurotransmitter phenotype. Recent studies reveal that only previously innervated sweat glands have the ability to induce expression of cholinergic traits by this subset of sympathetic neurons (Rohrer, 1992; Tresser *et al.*, 1992), and the noradrenergic innervation of sweat glands is essential for production of the factor that promotes cholinergic differentiation. In addition, the development of secretory function of sweat gland cells is dependent upon the neurotransmitter change in the afferents (Stevens and Landis, 1987, 1988).

In conclusion, both the response of pontine afferents to the stop signal posed by their target granule cells and the stop signal itself undergo developmental regulation. Both properties are well timed for the ingrowth into cerebellum and termination within the IGL of pontine neurites *in vivo*. These results also indicate there may be multistep interactions between pontine mossy fibers and their target cells or target region.

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