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Target-derived influences on axon growth modes in cultures of trigeminal neurons

(vibrissa pad/barrels/tract formation/organotypic cocultures)

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ABSTRACT **Cellular and molecular signals involved in axon elongation versus collateral and arbor formation may be intrinsic to developing neurons, or they may derive from targets. To identify signals regulating axon growth modes, we have developed a culture system in which trigeminal ganglion cells are challenged by various target tissues. Embryonic day 15 (E15) rat trigeminal ganglion explants were placed between peripheral (vibrissa pad) and central nervous system targets. Normally, bipolar trigeminal ganglion cells extend one process to the vibrissa pad and another to the brainstem trigeminal complex. Under coculture conditions, the peripheral processes invade the vibrissa pad explants and form a characteristic circumfollicular pattern. Central processes of E15 ganglion cells invade many, but not all, central nervous system tissues. In isochronic (E15) central nervous system explants such as brainstem, olfactory bulb, or neocortex, these central processes elongate and form a "tract" but have virtually no arbors. However, in more mature targets (e.g., a section from postnatal brainstem or neocortex), they form arbors instead of a tract. We conclude from these observations that whether trigeminal axons elongate to form a tract, or whether they begin to arborize, is dictated by the target tissue and not by an intrinsic developmental program of the ganglion cell body. The explant coculture system is an excellent model for analysis of the molecular basis of neuron–target interactions.**

Nocturnal rodents acquire much information about their environment through a chain of neuronal assemblies that bridges the whiskers and the cerebral cortex (1). Neocortical cells receiving this information are organized into modules or "barrels" whose distribution reflects the spatial organization of the whiskers; equivalent patterns of neuronal projection are found in the trigeminal regions of brainstem and thalamus (1). Neurons of the trigeminal ganglion constitute the first level of this pathway. They have peripheral and central processes that project to the whisker pad and brainstem, respectively. Brainstem afferent terminations are clustered in relation to a corresponding arrangement of postsynaptic neurons ("barrelettes"), which reflects the arrangement of whiskers on the snout (1). Because of the point-to-point representation of the vibrissae along the trigeminal neuraxis, this system has provided a model for investigations of axon–target interactions in the formation of sensory maps in the brain (1).

During development, axons exhibit two distinct growth modes. Initially they elongate, without branching, toward their target and form a tract (2–8). Next, parent axons in the tract emit collaterals, which penetrate target zones and form arbors therein; as development proceeds, some collaterals are eliminated, whereas others are elaborated. These two major modes of axon growth, elongation and collateral/arbor formation, are also characterized by differential rates of axon extension and by changes in the levels of expression of specific proteins that are shipped to the growing axon tips (2–10).

Recently developed techniques for long-term coculturing of brain slices provide a powerful means for addressing issues of axon–target interactions and for studying the regulation of axon growth modes in a controlled environment (11–19). Investigations of axon–target relationships in organotypic cocultures have been undertaken for various parts of the mammalian brain, including the thalamocortical projection (11–15), the septohippocampal system (16), and the connections from basal forebrain to neocortex (17, 18). In such preparations, explants retain specific characteristics of their *in vivo* organization and establish connections that are morphologically and physiologically similar to those observed *in vivo*.

We have used organotypic cocultures of embryonic rat trigeminal ganglia with peripheral and central "target" explants to examine afferent–target interactions, especially in the context of the two modes of axon growth. Our results show that trigeminal ganglion explants from embryonic day 15 (E15) rats reliably innervate the vibrissa pad, surrounding each whisker follicle in a characteristic pattern. When central processes of E15 ganglion cells invade central nervous system tissue, the pattern of axon growth is different: in isochronic embryonic brainstem, the central axons form a distinct tract, whereas in slices through older brainstem or neocortex, they form arbors. Our observations demonstrate that maturity of the target tissue plays a pivotal role in triggering the shift in axonal growth mode from elongation to collateral/arbor formation. This *in vitro* system can be readily exploited to reveal mechanisms involved in the growth of peripheral and central projections of trigeminal neurons.

MATERIALS AND METHODS

Data were collected from 26 cocultures prepared from fetal and postnatal Sprague–Dawley rats. Surgical and culture procedures were performed under sterile conditions. Fetal tissue was obtained on E15 (day of sperm positivity = E0) from timed-pregnant dams (Taconic Farms) anesthetized with sodium pentobarbital. Fetuses were decapitated, and the heads were placed in ice-cold Gey's balanced salt solution (GBSS) enriched with glucose (6.5 g/liter). Under a micro-

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Abbreviations: E15, embryonic day 15; PND, postnatal day; DIC, days in culture.

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scope equipped with dark-field illumination, vibrissa pads, trigeminal ganglia, brainstem, neocortex, hypothalamus, and olfactory bulb were dissected out into cold GBSS.

Postnatal brain tissue explants were prepared from 4- and 6-day-old rat pups [day of birth = E22 = PND0 (postnatal day 0)]. At this age vibrissa-related patterns of neuron aggregation are well established in the brainstem trigeminal complex and in somatosensory cortex (20), and sensory afferents (trigeminal and thalamocortical, respectively) have formed discrete arbors within their appropriate targets (20–22). Pups were anesthetized by hypothermia, their brains were dissected out into cold GBSS, embedded in low melting point agarose, hardened on ice, and sectioned in the transverse plane on a vibratome at a thickness of 300 μm. Sections through the cerebral cortex or the brainstem were collected in GBSS. For cortical explants, 2- to 3-mm-wide pieces were cut from sections containing the barrel field (dark-field illumination allows identification of individual barrels) and collected in a culture dish. For brainstem explants, transverse sections at the level of the obex were used. Such sections invariably contain the subnucleus interpolaris of the spinal trigeminal nucleus, which exhibits the largest area of vibrissa representation of all the brainstem trigeminal nuclei (20).

Explants of trigeminal ganglia and target tissues were placed on microporous membranes (pore size, 0.4 μm) of Millicell wells and aligned in tandem, such that the peripheral target (vibrissa pad) was located on one side of the ganglion and a central target (brainstem whole-mount, hypothalamus, or olfactory bulb explant; a slice through the barrel field cortex; or a slice through the brainstem trigeminal complex) was located on the other side (Fig. 1 *A–C*). To maintain the orientation of the vibrissa pad explants, the nasal opening was preserved; the caudal edge of the explant was juxtaposed against the trigeminal ganglion. Two or three such tandem cocultures were arranged within each Millicell well. Excess GBSS was suctioned off, and the wells were placed in six-well culture plates. Serum-free medium (23) was added into each culture chamber (1 ml per well), enough to just cover the explants. The plates were maintained in a humidified CO_2 incubator at 36°C.

After 3–6 days in culture (DIC), Millicell wells were exposed to fumes of 4% paraformaldehyde for several hours and then immersed into the same fixative. A crystal of carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) (Molecular Probes) was applied to the trigeminal ganglion. Labeled cultures were left in the fixative for 3–8 weeks at room temperature to allow for dye diffusion. The extent of labeling was periodically checked through a Nikon inverted microscope equipped with epifluorescence and a rhodamine filter. Culture wells were then transferred onto glass coverslips and photographed.

In control preparations, intact whole mounts of the trigeminal pathway were prepared from E15 embryos by dissecting out the ganglia, vibrissa pads, and brainstem attached to each other. These preparations were fixed immediately after dissection and labeled as described above by placing a crystal of DiI in the ganglion to visualize the normal distribution of trigeminal fibers in the vibrissa pad and in the brainstem.

RESULTS

Peripheral Growth Patterns of Trigeminal Ganglion Cells in Organotypic Cocultures. During fetal life, the mystacial pad develops from the lateral nasal and maxillary processes (24): in rats, a follicular pattern can be distinguished on the snout after E14 (25, 26). Five rows of rostrocaudally aligned vibrissae are flanked ventrally and rostrally by an array of sinus hairs (24, 27). Trigeminal fibers penetrate the external root sheath of each vibrissa and contact Merkel cells (27–30). In intact whole mounts, a compact bundle of DiI-labeled

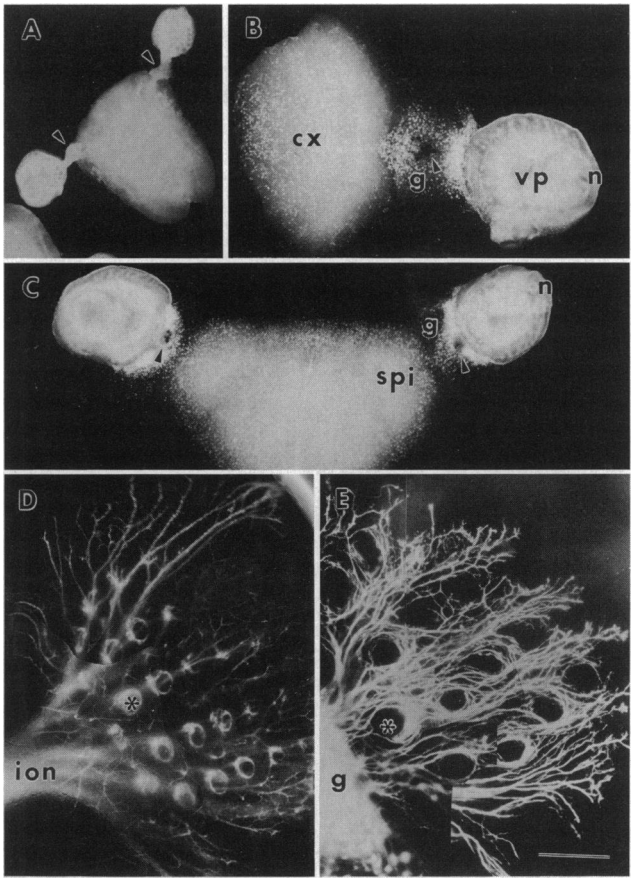

FIG. 1. Cocultures of E15 trigeminal ganglia with E15 vibrissa pads and various central targets. (*A*) Central target is a whole mount of E15 brainstem (coculture photographed while *in vitro*; arrowheads point to the ganglion explants). (*B*) Coculture of E15 ganglion (g), E15 vibrissa pad (vp), and a transverse section through PND6 barrel field cortex (cx), grown for 6 DIC. The ganglion is apposed to the pial margin of the cortical explant. (*C*) E15 vibrissa pad and ganglia grown *in vitro*, with each ganglion apposed to the trigeminal tract in a transverse section from the brainstem of a PND4 rat (6 DIC). Arrowheads in *B* and *C* point to the DiI crystals that were applied to the ganglia. (*D*) DiI-labeled trigeminal processes in the vibrissa pad from a control case in which the ganglia, brainstem, and vibrissa pads were dissected out intact from an E15 rat and fixed immediately. The infraorbital nerve (ion) breaks up into vibrissa row nerves, which fan out around the follicles. Axons from vibrissa row nerves form a "calycine" plexus around individual follicles (e.g., asterisk). (*E*) DiI-labeled peripheral processes of trigeminal ganglion cells in a coculture in which the ganglion, vibrissa pad, and brainstem whole mount were grown for 6 DIC. Fasciculated axon bundles form a circumfollicular plexus similar to the normal pattern but lacking some of the three-dimensional aspects of the normal motif. spi, Subnucleus interpolaris of the spinal trigeminal nucleus; n, nasal pole. [Bar = 1 mm (*A* and *B*), 2 mm (*C*), 400 μm (*D*), and 200 μm (*E*).]

processes approaches the caudal edge of the vibrissa field and fans out toward the nasal pole with axon fascicles coursing between vibrissa rows (Fig. 1*D*). A fiber plexus cups the base of each follicle (Fig. 1*D*; see also refs. 27, 29, and 30).

In cocultures of E15 ganglia grown with peripheral and central targets, the follicle pattern in vibrissa pads is distinct for up to 3–4 DIC (Fig. 1). With longer times, due to settling of the explants along the Millicell membrane, it becomes increasingly difficult to differentiate between the vibrissa follicles and the sinus hair follicles. As early as 3 DIC, a bundle of ganglion cell processes enters the vibrissa pad and fans out toward vibrissa follicles, as described for the intact preparation (Figs. 1*E* and 2 *B–D*). The growth of axons around the follicles is significantly more planar than normal:

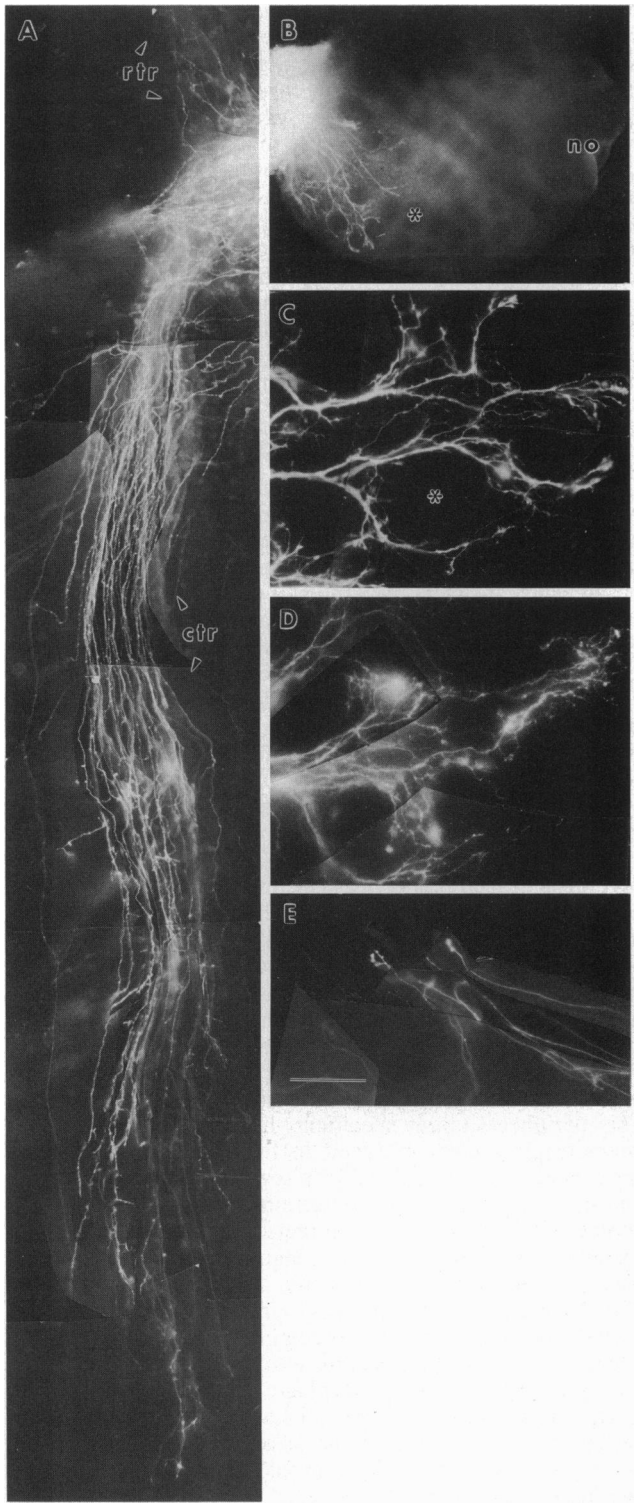

FIG. 2. (*A–C*) Growth patterns of axonal processes from E15 trigeminal ganglion cells into an E15 brainstem whole mount (*A*) and peripherally into the vibrissa pad (*B* and *C*). Axons in the brainstem form a distinct tract with rostral (rtr) and caudal (ctr) components. In contrast, ganglion cell axons that invade the vibrissa pad explants form a characteristic plexus around the whisker follicles. (*D*) A similar peripheral pattern of axon invasion is observed in cases in which E15 ganglia are cocultured with E15 vibrissa pad and brainstem slices from PND4 pups. (*E*) Ingrowth of trigeminal axons into E15 neocortex (6 DIC). Note that labeled axons have the morphology of elongating fibers with little or no arborization. no, Nasal opening. Asterisks mark individual follicles. [Bar = 500 μm for (*A*), 200 μm (*B*), 100 μm (*C* and *D*), and 500 μm (*E*).]

labeled fibers navigate toward the nasal pole between and around the follicles, but the envelope around the base of each follicle is missing. The characteristic patterning of trigeminal axons around the follicles is obtained repeatedly when E15 ganglion processes innervate isochronic vibrissa pad explants (Fig. 2 *B–D*).

Central Processes and Arbors of Trigeminal Ganglion Cells. Centrally directed processes of trigeminal ganglion cells establish a morphological pattern markedly different from that observed peripherally. Trigeminal axons stream along the lateral side of isochronic brainstem explants, forming a distinct tract with identifiable rostral and caudal components (Fig. 2*A*) reminiscent of the ascending and descending divisions of the trigeminal tract as seen *in vivo*. By 6 DIC, fibers in the caudal component have grown for a considerable distance within the brainstem, infrequently emitting short collaterals. There are virtually no arbors visible (Fig. 2*A*). Many labeled brainstem afferents are tipped with growth cones, suggesting that even after 6 DIC, trigeminal processes are still elongating.

E15 ganglion cell processes also grow into isochronic explants of neocortex and olfactory bulb (Figs. 2*E* and 3*A*). In both targets axons aggregate into bundles with sparse collateralization, similar to the growth observed in the E15 brainstem whole mounts, except without the separation of the axons into rostral and caudal components. On the other hand, when E15 ganglia are cocultured with isochronic hypothalamus, no trigeminal ingrowth is detected in the central tissue (Fig. 3*B*). However, following DiI application to the ganglion, retrogradely labeled cells are observed within the hypothalamic explants, documenting that hypothalamic neurons have projected into the ganglion. The latter experiment also serves as a control, underscoring that not all

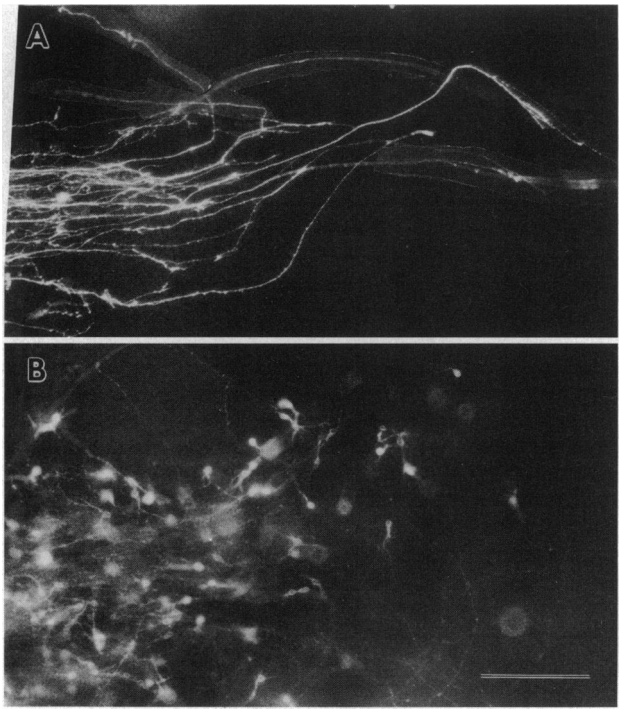

FIG. 3. (*A*) Trigeminal ganglion cells from E15 rats send axons into E15 olfactory bulb, a nontrigeminal target. Many axons grow in longitudinal arrays, which resemble tracts (4 DIC). (*B*) A strikingly different picture is seen when explants of E15 vibrissa pad, trigeminal ganglion, and hypothalamus (also an isochronic heterotypic nontrigeminal target) are cocultured. No trigeminal axonal processes enter the hypothalamus, whereas many hypothalamic cells (retrogradely labeled from DiI placement in the ganglion) project into the trigeminal ganglion explant (4 DIC). (Bar = 200 μm.)

central nervous system regions are supportive of trigeminal axon growth.

Trigeminal Ganglion Ingrowth into More Mature Targets. Heterochronic trigeminal targets (brainstem slices at the level of subnucleus interpolaris from PND4 rats and slices through barrel field cortex from PND4 or PND6 rats) were used to assay the growth of E15 trigeminal ganglion cells into more mature central tissue. During normal development in the rat, vibrissa-specific axonal and neuronal modules are clearly defined in the brainstem as well as in barrel field cortex by PND4 (20–22). The pattern of ganglion cell axons in these more-mature central targets is strikingly different from that observed in isochronic tissues: central trigeminal axons enter subnucleus interpolaris along a lateral to medial trajectory and form restricted arbors (Fig. 4 *A–C*). In tandem cocultures of E15 vibrissa pads, E15 trigeminal ganglia, and postnatal barrel field cortex, central processes of trigeminal ganglion cells invade the cortical tissue in much the same way as seen in cases with older brainstem slices. To test whether traversing across existing axonal pathways in the white matter is prerequisite to the formation of arbors, in some cocultures the ganglion was apposed to the ventricular surface of the neocortical slice and in others it was apposed to the pial surface (Fig. 1). Irrespective of the site of ganglion placement, parallel arrays of trigeminal axons enter the cortical slice radially and form restricted arbors within a short distance (Fig. 4 *D* and *E*). In most cases, the arbors are located 200–300 μm from the pial or white matter edge of the neocortical explants, suggesting that they are confined to layers IV–VI. However, due to a flattening of the neocortical explant after several DIC, we could not precisely localize these arbors to barrels or to specific cortical laminae. Previous studies have shown that in heterochronic cocultures of thalamus and neocortex, thalamic axons invade the cortical explant and arborize in layer IV while receiving a reciprocal projection primarily from neurons in layer VI, also pointing to an important role for target-specific cues in the establishment of neural connections (13–15).

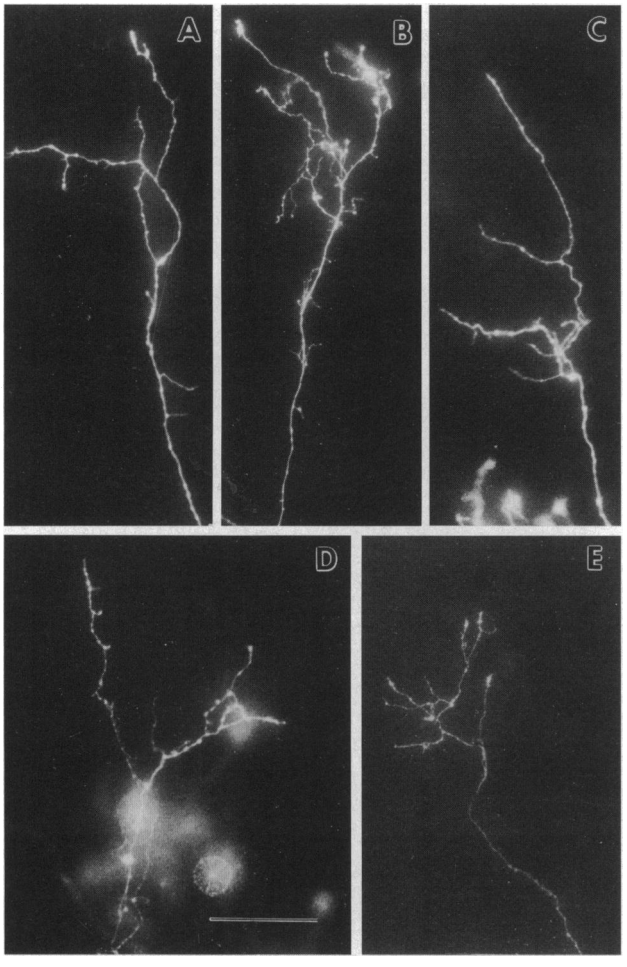

FIG. 4. (*A–C*) In cocultures of E15 vibrissa pads with E15 trigeminal ganglia and a transverse slice through PND4 brainstems, sensory axons invade the subnucleus interpolaris and form restricted arbors (6 DIC). (*D* and *E*) Similar small, circumscribed arbors are also seen within PND4 (*D*) or PND6 (*E*) barrel field cortex. Regardless of the relative positions of the cortical explant and the ganglion (i.e., ventricular versus pial surface of cortex apposed to the ganglion), trigeminal axons succeed in entering the cortex and forming focalized arbors. There is no indication of tract formation within these more mature cortical targets (6 DIC). (Bar = 100 μm.)

DISCUSSION

Organotypic cocultures of trigeminal ganglia with explants of peripheral and central target tissues reveal that primary sensory neurons are able to invade the vibrissa pad and form a characteristic circumfollicular pattern within this peripheral target. The consistent patterning of the peripheral trigeminal projection in all the coculture variations used in this study suggests that specific feedback from central targets is not crucial for directing sensory processes to develop their characteristic distribution around the follicles. Whether the patterning is fully dictated by the periphery, or whether there is a specific interaction of trigeminal processes with the whisker pads, can now be tested in this culture system by examining the ingrowth of other sensory afferents into the vibrissa pads.

The explanted ganglion cells are competent to grow into several central targets such as isochronic brainstem explants, neocortex, and olfactory bulb, as well as more mature central nervous system trigeminal areas like the spinal trigeminal nucleus and barrel field cortex. The relative lack of target-related specificity in the central trigeminal axons contrasts with that reported for their peripheral counterparts: culture studies with embryonic mice have documented that given a choice between maxillary pad and other cutaneous fields, trigeminal axons are selectively attracted to the former (31). This peripheral target specificity has been attributed to a chemotropic factor produced in the newly differentiating snout epidermis (see ref. 31 for a review). Although we have not specifically challenged trigeminal ganglion cells with a choice of two or more central targets, our results indicate that a similar, regionally restricted chemotropic influence is not in place for central trigeminal axons. Nevertheless, the ability of trigeminal ganglion processes to innervate central nervous tissue is not universal: E15 trigeminal ganglion cells do not invade isochronic hypothalamic explants but instead receive a projection from this diencephalic region. One possibility worth considering is that hypothalamic tissue may be nonpermissive for trigeminal axons. In our cultures we could not directly assess whether trigeminal axons were repelled by the hypothalamic explants. However, it is important to note that during development, central processes of trigeminal ganglion cells do not normally have access to brain regions such as the olfactory bulb or neocortex, whereas they do traverse close to hypothalamus; further studies are needed to address whether hypothalamic tissue is repulsive or nonpermissive for trigeminal axon growth.

The different modes of axon growth obtained in immature versus older tissue provide evidence that maturational changes in the target can have a dramatic effect on the morphological characteristics of developing axons. In the normal brainstem, trigeminal nuclei have not fully differentiated by E15, but a distinct trigeminal tract has already formed (25, 32). The existence of this tract may present a

template for ganglion cell axons to follow when extending in culture. On the other hand, in slices through PND4 brainstem and PND4 or -6 barrel field cortex, the target neuropil is more mature and ingrowing afferents readily arborize within this tissue. Whether trigeminal axons can form discrete arbors in explants or slices of more mature nontrigeminal regions (e.g., older olfactory bulb) or whether such axonal ramifications are induced only by trigeminal regions that contain vibrissa-related modules (e.g., brainstem trigeminal nuclei, barrel cortex) remains to be determined.

Several studies show that the elongation of afferents to their targets precedes the completion of neurogenesis, migration, and differentiation of postsynaptic neurons (2–8, 33, 34). In neuronal systems where there is considerable discrepancy between the time of arrival of afferents and the differentiation of their target cells, collateral innervation begins only after a significant waiting period (2–8, 35–39). Perhaps the length of this period reflects the process of target maturation, which is prerequisite for triggering the second growth phase of afferent axons. The fact that E15 ganglion cell processes elongate and form a distinct tract in E15 brainstem whole mounts, whereas in PND4 brainstem and neocortex slices they form discrete arbors but no tract, supports the hypothesis that extrinsic signals deriving from the target have a pivotal role in triggering the shift from elongation to collateralization and arborization of afferent axons. The molecular makeup of such target-derived signals, which may initiate a new growth mode, remains elusive. Differential expression of cell and substrate adhesion molecules within the target, or a temporally restricted release of trophic substances by postsynaptic cells, may contribute to this phenomenon (31, 36–42). If sensory afferents are capable of switching from one growth mode to another as a function of target maturation, then in cocultures of "older" ganglion explants and "younger" target explants these axons may revert back to their elongation mode.

In conclusion, we have shown that organotypic cocultures of trigeminal ganglia with peripheral and central targets present a simple and highly advantageous model system for delineating cellular mechanisms underlying specific interactions between growing axons and their targets. In recent years, considerable effort has been directed toward understanding the molecular basis of neural circuit formation, but progress has been limited by the lack of simple models to establish a relationship between molecular function and axon organization in the brain. Organotypic culture systems bridge the gap between the molecular and systems levels and allow easier access to many aspects of the study of axon guidance and neuron–target interaction.

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