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Effect of Culture Substrata and Fibroblast Inhibitors: Trigeminal Ganglion Axon Outgrowth Revisited

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Abstract.- Axon outgrowth *in vitro* can be influenced profoundly by the culture system used and may even be misleading due to certain prerequisites. Intact non-dissociated trigeminal ganglia extend neurites only when co-cultured with target tissues and not in isolation. We set out to determine if intact early age murine trigeminal ganglia extend neurites in isolation, is it the culture system involved and whether fibroblast inhibitors could actually prevent the normal outgrowth pattern. Mouse intact trigeminal ganglia before target innervation were given the choice to grow in isolation or with target tissues on three dimensional collagen gels, collagen coated platform and poly-d-lysine substrata. Cultures in this way were set up in the presence and absence of fibroblast inhibitors. Results demonstrated that; firstly, trigeminal ganglia were capable of extending neurites in isolation, Secondly, poly-d-lysine promoted extensive outgrowth of neurites, a comparable outgrowth was promoted by collagen substrata but not three-dimensional collagen gels. Thirdly, inhibitors used to prevent fibroblast outgrowth radically affected the normal outgrowth pattern of neurite fascicles and morphology. Where fibroblast inhibitors were omitted, neurites grew to several hundred micrometers distance, highly branched and established intricate neurite-non-neuronal networks. Growth cones and filopodia were well developed. Additionally, widespread outgrowth occurred only where ganglia were cultured within the first five or ten minutes after removal from embryos. Lastly, outgrowth of non-neuronal non-fibroblast cells occurred only when ganglia were cultured without fibroblast inhibitors. We conclude that evaluation of in vitro axon outgrowth and directionality should be cautious.

Key words: Trigeminal ganglion, *in vitro* axon outgrowth, mitotic inhibitors, fibroblasts, non-neuronal cells, neuronal development, poly-d-lysine, collagen gel cultures.

INTRODUCTION

A great deal of information about developing neurons as regards their outgrowth, numbers, directionality and target specificity has been gathered by growing these neurons in culture. Inhibitors are normally used in neuronal cultures to prevent fibroblast outgrowth. Furthermore, directionality of axons is best understood in three-dimensional collagen gels (Lumsden, 1988). Mouse intact trigeminal ganglia at the earliest stages of target field innervation extend neurites *in vitro* in three-dimensional collagen gels only if co-cultured with innervation targets; the maxillary and mandibular processes of the first branchial arch. The outgrowth is target directed presumably due to a target-derived chemotropic factor(s). The ganglion is unable to extend neurites in isolation state as opposed to geniculate ganglion, dorsal root ganglion and other sensory cranial ganglia (Lumsden and Davies, 1983, 1986). Conversely, when the ganglion is dissociated and cells are plated without target tissues on polyornithine substratum, they extend non-significant neurites (Davies *et al*., 1981). The earliest axon extension occurs at E9.5 from dissociated neurons and is independent of neurotrophins (Davies and Lumsden, 1984; Stainier and Gilbert, 1990). Issues that prompted the present investigation were: (i) is intact trigeminal ganglion, if cultured in isolation, capable of extending neurites well before target innervation; (ii) is collagen gel or poly-d-lysine appropriate system to evaluate neurite outgrowth and directionality; (iii) how much influence is there of the target tissues; (iv) are fibroblast inhibitors capable of affecting the magnitude of neurite outgrowth and morphology, and (v) does time in setting up cultures matter?

MATERIALS AND METHODS

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Animals

All animal handling procedures were strictly according to the guidelines laid down by The Animals Scientific Procedures Act 1986, United Kingdom. Care was taken to minimize suffering, pain and discomfort. Time mated pregnant albino mice (strain MF1) obtained from Royal Hallamshire Hospital, Sheffield were maintained at the Department of Biomedical Science, Sheffield University. They were allowed free access to food and water *ad libitum*. The day of vaginal plug was marked as day zero (E0) of gestation. Animals at the required day of gestation period were sacrificed by cervical dislocation. Uterine sacs were removed and cut opened. Embryos cleared of fetal membranes were immediately transferred to fresh sterile 0.1M cold phosphate buffered saline (PBS) and staged as described (Theiler, 1989). In all experiments, embryos older than the ages required were discarded appropriately.

Tissue culture

Trigeminal ganglia, maxillary and mandibular targets, and as well as forelimb buds (non-target tissue) were dissected using electrolytically sharpened tungsten needles. Cultures with appropriate maxillary and mandibular arches and forelimb buds served as positive and negative controls respectively. All cultures were performed using intact ganglia to mimic *in vivo* pattern and tissue dissections were performed in cold sterile PBS or DMEM F/12 medium (Sigma Chemical Co. UK).

All cultures were performed in chemically defined (serum free) medium since the presence of serum in culture medium significantly effect neurite outgrowth or morphological pattern of neurons; growth factors contained in it may in fact induce outgrowth (Luduena, 1983), and in this way could bias the normal outgrowth pattern.

Chemically defined medium was prepared from Dulbecco's Modified Essential medium (DMEM F/12) which was supplemented with 5.6% sodium bicarbonate, 200mM L-glutamine, 5μg.ml^{-1} insulin, 5μg.ml^{-1} transferrin, 2.0 x 10^{-8}M sodium selenite, trace elements, 10^{-11} M tri-idothyronine, 1μM mannitol, 10U/ml catalase and 50μg.ml^{-1} ascorbic acid. To prevent the proliferation of non-neuronal fibroblast cells, inhibitors as were added at the concentration of 1μM cytosine-β-D-arabinofuranoside, 10μM fluorodeoxyuridine and 10μM uridine as described (Davies *et al*., 1981; Lumsden, 1988), only to those cultures that had to be evaluated in the presence of fibroblast inhibitors (All reagents were obtained from Sigma Chemical Co. UK).

Explant preparation

Trigeminal ganglia were cultured on poly-d-lysine or collagen coated platform and three-dimensional collagen gels. *n* in each group represents total ganglia cultures (one ganglion per dish or well) that were conducted over several months and were later combined for analyses.

Poly-d-lysine cultures

35 mm plastic tissue culture dishes were coated with 50-100μg/ml poly-d-lysine made in sodium borate buffer, pH 8.4. Dishes were left for overnight and were thoroughly washed with cold sterile PBS 1 hour before setting up cultures.

Ganglia were placed in isolation (one ganglion per dish) in 600-700μl of chemically defined medium and cultures were incubated for 24-48 hours at 37°C in a humidified incubator with 5% CO_2 in air. For positive and negative controls, trigeminal ganglia and target tissues were positioned approximately 200-400 μm apart, using micropipettes. Culture medium was added first and then the tissues were placed to minimize any traumatic effect such as tissue dryness that could possibly occur during transfer and placement of tissues onto coated culture dishes.

Collagen gel cultures

Rat tail collagen (gift from Prof. Marysia Placzek) and as well as vitrogen (Celltrix, CA) were used to construct three-dimensional gels. The preparation was as follows: Collagen and 10x DMEM was mixed in a ratio of 1: 9 respectively and 4.6-5μl of 0.8M sodium bicarbonate (pH 7.4) was added to the solution. Both 16mm multiwell tissue culture chambers and 35mm tissue culture dishes

were used to make a platform by spreading a 25µl drop of collagen mixture. Chambers and dishes prepared in this way were incubated for 1 hour at 37°C in a humidified incubator.

After one hour the trigeminal ganglia, and in case of controls; ganglia along with target tissues were transferred to collagen platforms. The tissues were positioned quickly approximately 200-400µm apart and overlaid with 75µl of collagen mixture. The culture set-ups were transferred to incubator at 37°C for 30-40 minutes. After the gels were set, chemically defined medium was added to each culture. They were then incubated as above. For preparation of collagen substratum, culture chambers or dishes were coated in a way similar to poly-d-lysine but for 4-6 hours, however such cultures were not overlaid with additional collagen.

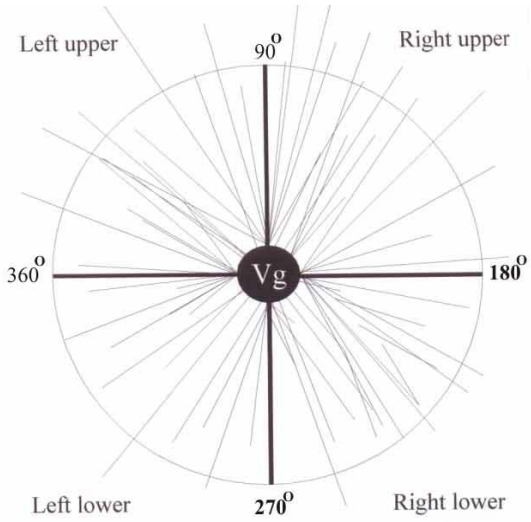
The effect of fibroblast inhibitors

In the second set of experiments; fibroblast inhibitors, cytosine-β-D-arabinofuranoside, fluorodeoxyuridine and uridine were omitted from the culture medium and E9.0-9.5, E10.0 and E10.5 mouse trigeminal ganglia were cultured in isolation or with target tissues on poly-d-lysine, three-dimensional collagen gels and collagen substrata.

To determine the effect of time, cultures were conducted as above and time taken in setting up cultures and placing them in incubator from the moment embryos were dissected out from the dams was recorded in minutes.

All cultures were observed under a phase contrast inverted microscope (Olympus IMT-2; Japan) and photographed with an attached camera. Photographs are presented as such without any manipulation such as adjusting brightness, contrast or sharpness to avoid artefacts except that they have been organized into figure plates and labelled.

Neurite fascicles were counted using a 1mm Indx grid (Tonbridge Graticules, Kent, UK) in the left and right, upper and lower quadrants of ganglia (Fig. 1). Neurite lengths were measured from the bases of neurites to their tips furthest from the ganglion using standard morphometrical methods. Single neurite fibers were not counted since major outgrowth occurred in the form of fascicles. Neurites contained in each fascicle were optically non-dissectible but careful observation demonstrated that each neurite fascicle contained 3-5 neurites. Numbers of neurite fascicle in the results section are given as mean $\pm$ standard error of mean. Comparison of neurite outgrowth was done using Mann-Whitney Rank Sum test and analysis of variance using F-test (ANOVA) using Sigma Stat (Version 2.0, Jandel Scientific Software, USA). Post-hoc Tukey's test was applied to further verify the results. Cultures in which trigeminal ganglia were dissociated or appeared dead as was indicated by cellular debris and lumps of fragmented cells were excluded from analyses. Non-neuronal cells were not counted because of enormous numbers and complexity of the outgrowth.



Schematic diagram showing counting of neurite fascicles in four quadrants of the trigeminal ganglion.

RESULTS

Isolation cultures of trigeminal ganglia with fibroblast inhibitors on Poly-d-lysine coated dishes

Of 24 E9.0-9.5 ganglia, 79.1% extended thin fine neurites that formed branches. 20.8% of ganglia did not extend neurites from any quadrant. The mean number of neurite fascicles per culture was x=9.7±1.1 (n=19). Of 41 E10.0 ganglia, outgrowth occurred in 78.0% ganglia compared with 21.9% in which no neurite outgrowth was noticeable. The

mean number of neurite fascicles per culture was x=10.9±1.2 (n=32). The difference between the number of neurite fascicles extended by E9.0-E9.5 and E10.0 ganglia was statistically non-significant (P=0.881). The mean number of neurite fascicles in total cultures (including those in which no outgrowth occurred) was x=7.7±1.2 (n=24) and x=8.5±1.2 (n=41) for E9.5 and E10.0 trigeminal ganglia, respectively.

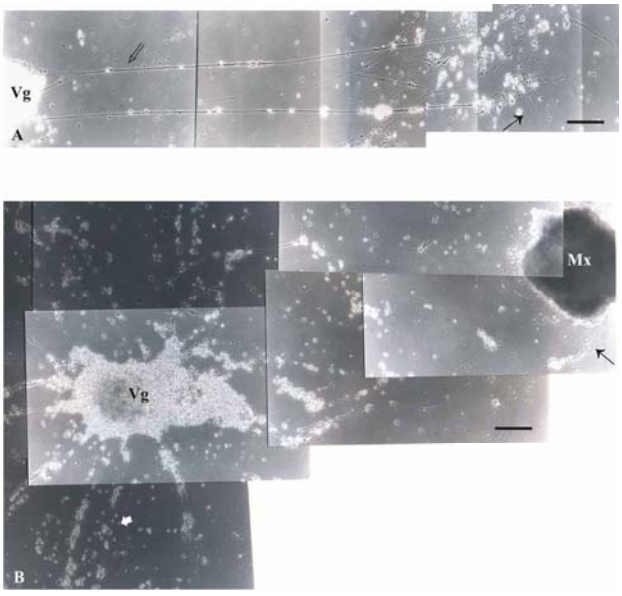


Fig. 1. Schematic diagramme showing counting of neurite fascicles in four quadrants of the trigeminal ganglion. A, Isolation culture of E9.0-9.5 trigeminal ganglion (Vg) in chemically defined medium with fibroblast inhibitors on poly-d-lysine. Note outgrowth of two long neurites (open arrow) mainly from one quadrant of the ganglion. Arrow shows growth cones. Scale bar = 100μm. B, E9.0-9.5 trigeminal ganglion (Vg) co-cultured with maxillary arch (Mx) as a control in chemically defined medium with fibroblast inhibitors on poly-d-lysine. Note neurites contacting the maxillary target (arrow). Also note radial pattern of outgrowth and neurites that were extended away from the target (arrowhead). Scale bar = 200μm.

Outgrowth pattern and morphology

The outgrowth in 66.1% of growing cultures of both E9.0-9.5 and E10.0 ganglia was confined to either one or two same or opposite quadrants. Occasionally, neurites grew from whole circumference of the ganglia (radial pattern of outgrowth) but the major neurite outgrowth was still confined to one or two adjacent or opposite quadrants. Neurites grew out as straight and parallel bundled fibers but showed curved trajectories toward distal ends. Most grew to 150-200μm and occasionally to more than 400μm distance (Fig. 1A). Neurites were neither strongly adhered to the tissue culture substratum nor adjacent neurites. They were branched and formed collaterals.

When co-cultured with maxilllary or mandibular targets or forelimb buds, an outgrowth (x=17.7±2.1; n=20) was observed from 90.9% trigeminal ganglia which was significantly greater than cultures maintained without targets (P<0.001). The outgrowth appeared non-extensive and non-directional as neurites not only grew towards appropriate targets (Fig. 1B), but they also grew in other directions and toward inappropriate forelimb bud target (not shown).

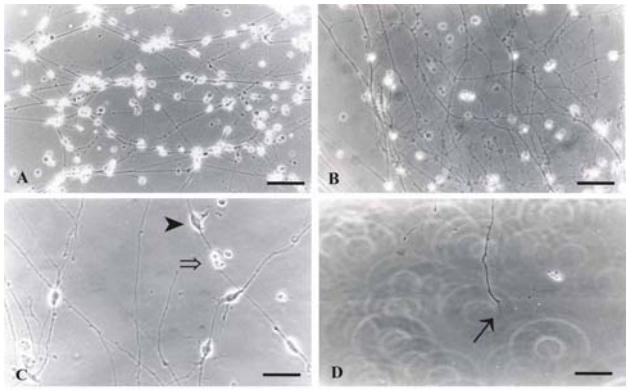


Fig. 2. A-B. Neurite networks from isolation cultures of trigeminal ganglia with fibroblast inhibitors on poly-d-lysine. Scale bar = 200μm. C, Phase bright spindle shape (arrowhead) and spherical structures (open arrow) along neurites. Scale bar = 100μm. D, Neurite growth cones (arrow). Scale bar = 25μm.

In both E9.0-9.5 and E10.0 cultures, neurites formed fine neurite networks as a result of crossing each other or branching and were accompanied by a large number of phase bright spherical structures that were present at locations where neurites

emerged from the ganglia and as well as along neurite surfaces after they had extended some distance (Fig. 2A-B). Other structures, which appeared as phase bright spindle shape swellings were also evident along neurite surfaces. At least three or more of these structures were found along a single neurite (Fig. 2C). Growth cones were fine, appeared delicate structures and had fine filopodial-like processes (Fig. 2D). Other non-neuronal structures such as fibroblasts did not grow out in these cultures.

Because sparse neurite outgrowth was observed on two-dimensional poly-d-lysine substratum even in the presence of target tissues and the outgrowth was mostly random and non-directional, the next question addressed was: Is it due to a difference of substratum used? Should cultures conducted on collagen coated surfaces or three-dimensional collagen gels make any difference?

Isolation cultures of E9.5-E10.0 trigeminal ganglia on collagen platform and collagen gels

E9.5-E10.0 trigeminal ganglia were cultured in isolation on three-dimensional collagen gels (n=33) and collagen coated dishes (n=30). Control cultures (n=15) with maxillary or mandibular processes were set in parallel. Trigeminal ganglia cultured in isolation in three-dimensional collagen gels (x =4.0$\pm$0.2) or collagen substrata (x =7.6$\pm$0.1) did not extend appreciable neurites. Neurites were confined to one quadrant but an outgrowth from whole surfaces of ganglia was also evident. Neurites were, thin and fine and did not show network formation. Spindle-shape structures on neurite surfaces were evident. Fibroblasts or non-neuronal cells did not grow out in these cultures (Fig. 3A-C). Ganglia co-cultured with maxillary or mandibular targets extended comparatively greater number of neurites (x=13.4$\pm$0.1).

Isolation cultures of trigeminal ganglia in chemically defined medium exclusive of fibroblast inhibitors on poly-d-lysine

Of E9.0-9.5 (n=31) and E10.0 (n=97) trigeminal ganglia, 90.3% produced extensive neurite outgrowth with a mean number of x=32.6±3.6 and x=100.0±8.7 neurite fascicles respectively, compared with similar age ganglia cultured in the presence of inhibitors (P<0.001).

In 83.2% cultures, the outgrowth was radial (Fig. 4A-B) while it was confined to only one or two quadrants in remaining cultures. Ganglia that extended more than 50 neurite fascicles were 68.0% and the maximum number of fascicles observed from a single ganglion was 442 compared to controls in which the maximum number observed was 30 with a mean number of x=10.9$\pm$1.2 neurite fascicles. Outgrowth from E10.0 ganglia was significantly greater than E9.5 (P<0.001).

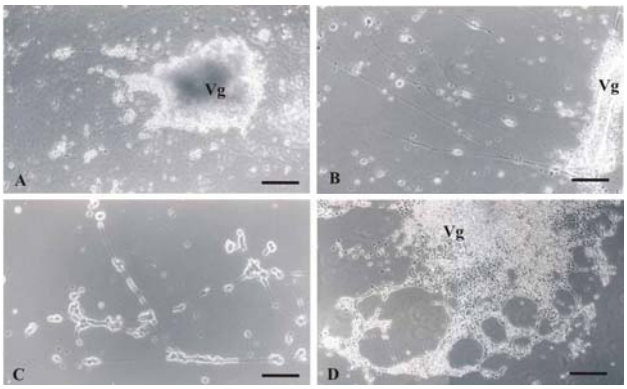


Fig. 3. A-B, E9.5 trigeminal ganglion (Vg) cultured in chemically defined medium with fibroblast inhibitors on three dimensional collagen gels. Note sparse neurite outgrowth from the ganglion; C, Shows neurites and neurite networks elaborated on collagen gels; D, E9.5 trigeminal ganglion cultured in chemically defined medium without fibroblast inhibitors on three dimensional collagen gels. Note a greater neurite outgrowth from the ganglion. Neurite-non-neuronal networks are also evident. Scale bars A-D = 100μm.

Control ganglia co-cultured with appropriate maxillary, mandibular or inappropriate forelimb bud targets also extended extensive and radial outgrowth of neurites (Fig. 5A). Neurites contacted the target tissues but also grew in large numbers from opposite or lateral quadrants. The outgrowth was significantly greater (P<0.001) with a mean number of x=95.9$\pm$10.3 (n=13) neurite fascicles than control co-cultures performed in the presence of inhibitors (x=17.7$\pm$2.1, n=20). However, when this outgrowth was compared with similar age

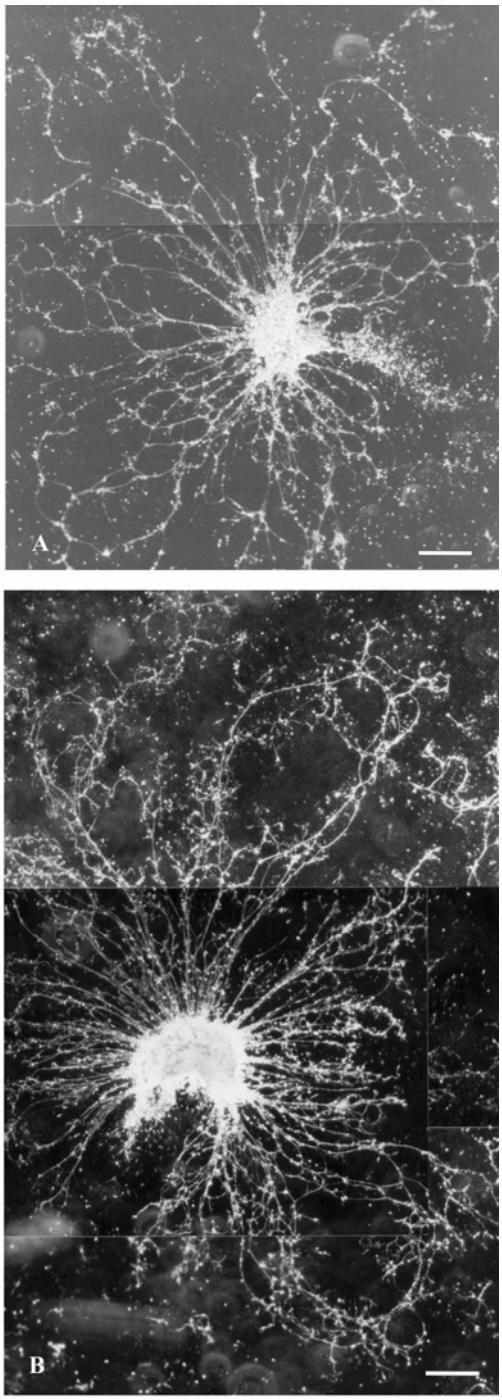


Fig. 4. Dark-field photomontages of isolation cultures of E9.5 (A) and E10.0 (B) trigeminal ganglia cultured without fibroblast inhibitors on poly-d-lysine showing extensive and radial pattern of neurite outgrowth and non-fibroblast non-neuronal cells.

Scale bar = 500μm.

ganglia that were cultured in isolation but exclusive of inhibitors, the difference was non-significant (P=0.476), indicating that the outgrowth that occurred in control cultures was due to exclusion of inhibitors rather than the influence of target tissues.

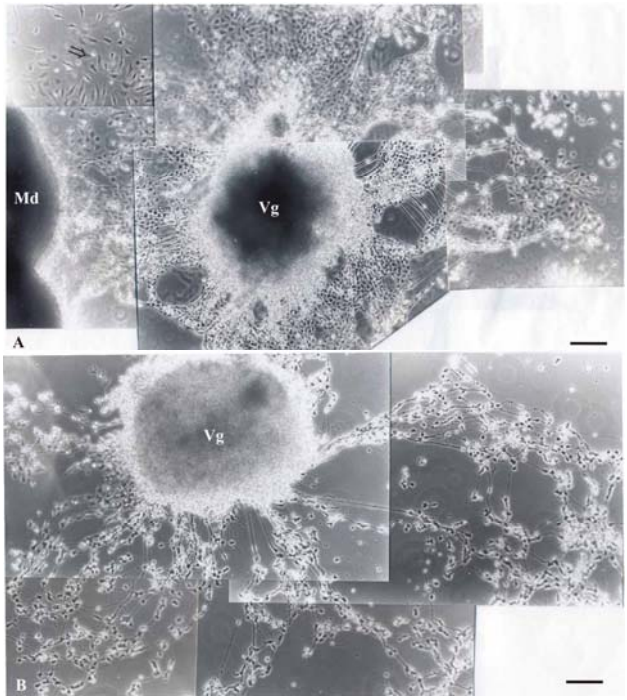


Fig. 5. A, E10.0 trigeminal ganglion (Vg) co-cultured with mandibular arch (Md) as control in chemically defined medium without fibroblast inhibitors on poly-d-lysine. Note extension of neurites and non-fibroblast non-neuronal cells. Neurite outgrowth is radial. Neurites grew and contacted mandibular target but also grew out from other quadrants and away from the ganglion (arrow). Open arrow shows fibroblasts; B, Isolation culture of E10.0 trigeminal ganglion (Vg) cultured in chemically medium without fibroblast inhibitors on poly-d-lysine. Note extension of neurites and non-neuronal non-fibroblast structures from two quadrants of the ganglion. Scale bar = 100μm.

Outgrowth pattern and morphology

The neurites were several hundred micrometers long (max. length noted: 2.5±2.1 mm), fasciculated, optically non-dissectible, branched, formed networks and were accompanied with a large number of non-fibroblast non-neuronal cells (Fig. 5B). Both neurite numbers and lengths were

however inconsistent between ganglia, whereby some neurites were only 20-50μm long, others grew to a more than 1800±4.5μm distant. Likewise, similar age ganglia sometimes extended more than 300-500 neurite fascicles in all four quadrants while at other times the same age ganglia extended only 20-30 neurites. Neurites made complicated networks whereby each fiber had developed contacts with several others at numerous locations (Fig. 6A-B). The neurites were both straight and curved. They extended side branches or collaterals which were of two kinds; one, which were short, thick and were mostly found near neurite origins at surfaces of ganglia while the others, were long and thin and were found at distal neuritic ends.

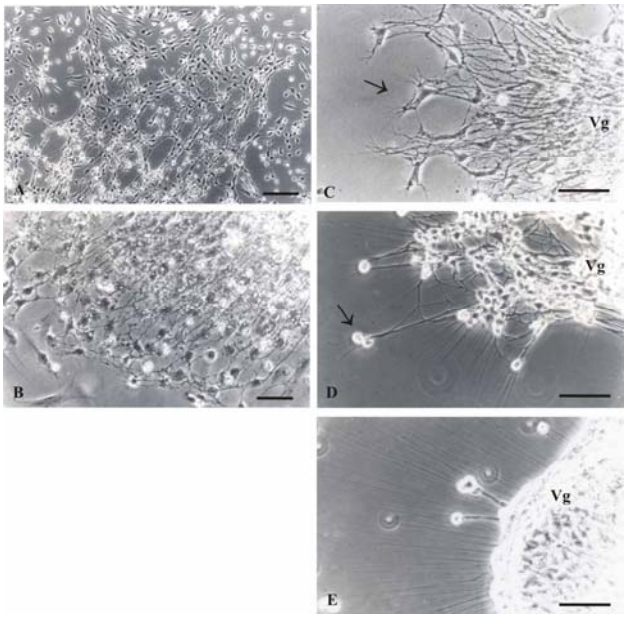


Fig. 6. A-B, Neurite networks, growth cones and filopodia from isolation cultures of E9.5-E10.0 trigeminal ganglia (Vg) cultured without fibroblast inhibitors on poly-d-lysine. Scale bar = 100μm. C-E, Arrows show growth cones and phase bright spherical structures. Filopodia are also visible. Scale bar = 50μm.

Extremely complex and highly developed growth cones were observed from both E9.0-9.5 and E10.0 ganglia. They were found at leading edges of neurites and were equipped with 80-100μm long filopodial-like processes while veil-like lamelli-podia among them were also evident (Fig. 6C-E). The processes which appeared filopodial-like were very fine spine-like and easily visible as clusters of 8-10 at advancing ends of growth cones. Growth cones showed adhesion with the tissue culture substratum but filopodial structures appeared floating in the culture medium. In such cultures, the growth cones were not attached to the substratum and were only found at neurite ends. In addition, both E9.0-9.5 and E10.0 trigeminal neurites were accompanied by a large number of sphere shape structures which were located on neurite surfaces. They formed clusters among neurites near ganglia but showed a more dispersed pattern toward distal neuritic ends. They were nucleated and a variation in their size was also noticeable (Fig. 6C-E).

The second type of structures was spindle shaped swellings along neurite membranes. Several such structures were found along each neurite and were also present few micrometers behind growth cones and distal tips of neurites. These were phase bright and were accompanied by several tiny vesicles that had trailed behind them. They were strictly associated with neurite surfaces and were also present at the branching points of neurites. Both structures were similar to the ones observed in ganglia cultured in the presence of mitotic inhibitors. Such an outgrowth was not observed from trigeminal ganglia cultured in the presence of mitotic inhibitors.

The neurite outgrowth from slightly older age E10.5 ganglia was not extensive. It was although radial but neurites were short, thick and highly fasciculated, did not grow to a long distance and most of them were only 100-150μm long. It was noticeable that neurites grew in separate groups of 5-6 neurite fascicles with gaps between adjacent fascicles; a pattern not observed in younger age ganglia.

For a quick overview, pattern of neurite outgrowth that occurred from mouse trigeminal ganglia in the presence of fibroblast inhibitors and when these inhibitors were omitted from the culture medium is demonstrated as line drawings (Fig. 7).

Outgrowth pattern of non-neuronal cells

The striking feature of ganglionic cultures grown without mitotic or fibroblast inhibitors was

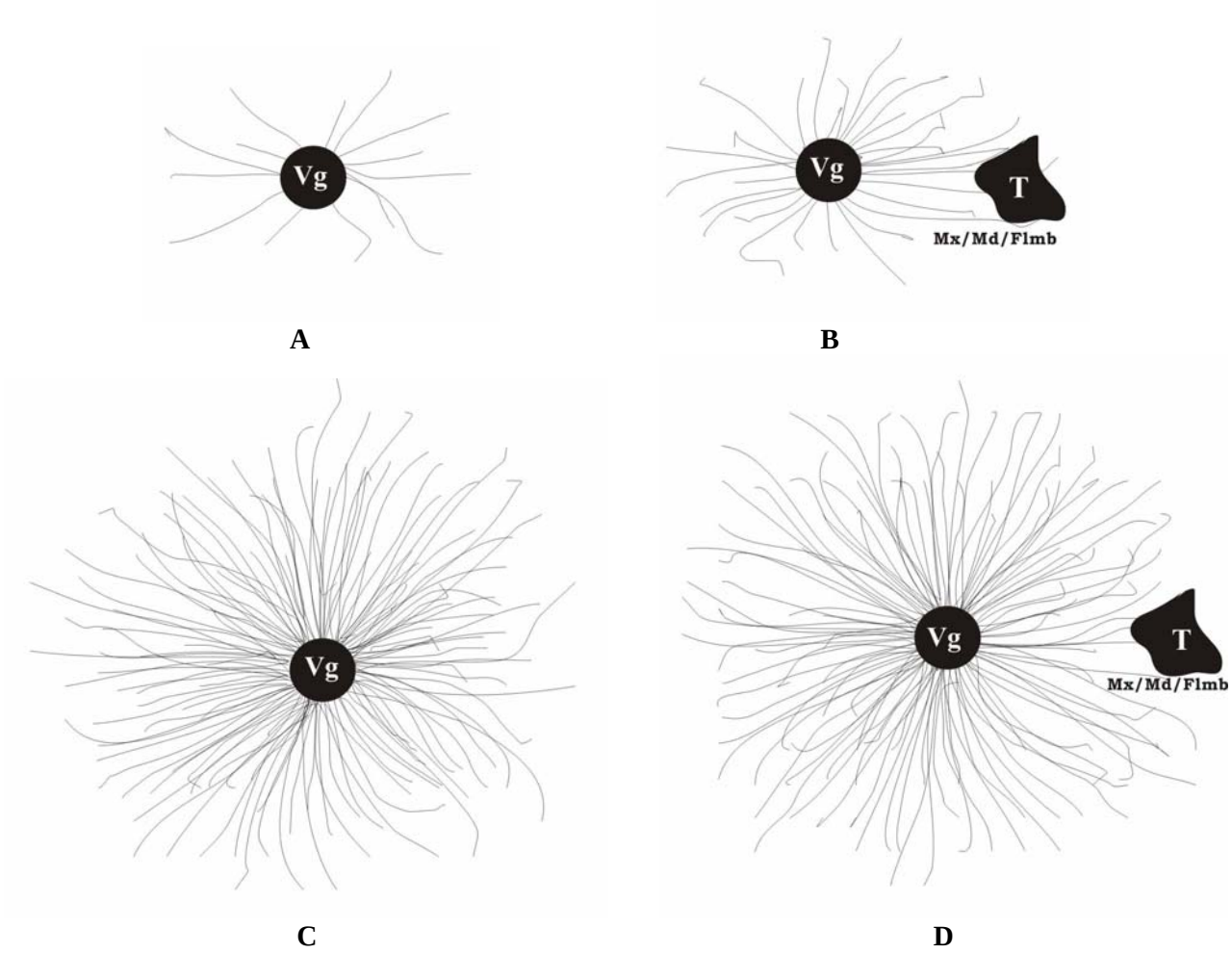


Fig. 7. Schematic drawings of E9.5-E10.0 trigeminal ganglion summarizing the extent of neurite outgrowth with or without fibroblast inhibitors. Cultures without fibroblast inhibitors show extensive outgrowth of neurites irrespective of target tissues. For simplicity, non-neuronal structures are not shown. Mostly, neurites ended as straight growing fibers. (Curved ends at line tips are limitation of the software used). A, isolation cultures of trigeminal ganglion (Vg) with fibroblast inhibition; B, co-cultures of trigeminal ganglia (Vg) with appropriate (Flmb) targets with fibroblast inhibition; C, isolation cultures of trigeminal ganglion (Vg) without fibroblast inhibitors; D, co-cultures of trigeminal ganglia (Vg) with appropriate maxillary (mx) or mandibular (md) or inappropriate (Flmb) targets without fibroblast inhibitors.

the enormous outgrowth of non-neuronal structures that grew out from E9.0-10.5 trigeminal ganglia. Such structures were never observed in the presence of fibroblast inhibitors. They were mostly triangular, polygonal or irregular shape and were phase dark. They showed fibroblast-like morphologies but were smaller than fibroblasts and were readily distinguishable from them (Fig. 5B). Some of these structures formed flattened and broadened sheet-like aggregates. They had several short processes with which they were attached to other similar structures and as well as neurites. They were of two kinds: one, typified by more irregular shaped and flattened morphologies with several processes, were mostly found at the emergence points of neurites, had tiny phase-bright vesicles located in their centers due to which they portrayed granular appearance; the second were triangular or tripolar and were present

in the middle and distal ends of neurites. They had large disc shaped cell bodies and well-formed processes as well as growth cones. Both kinds of structures were found in tight contact with neurites. They were present in large numbers between parallel growing neurites and appeared to have grown one after the other along neurite surfaces.

Isolation cultures of E9.5-10.0 trigeminal ganglia in chemically defined medium exclusive of fibroblast inhibitors in collagen gels and collagen substrata

Trigeminal ganglia cultured in isolation in three-dimensional collagen gels in the absence of fibroblast inhibitors could extend only few neurite fascicles (x=10$\pm$1.6, n=21) but extended a large number of neurite fascicles (x=70.8$\pm$12.2, n=28) and non-neuronal cells on collagen substrata in a pattern similar to that observed on poly-d-lysine. The difference between the two groups was statistically significant (P<0.001). Outgrowth pattern and neurite morphology were similar as observed on poly-d-lysine (Fig. 3D).

The effect of time taken in setting cultures

While meager outgrowth of neurites in the presence of fibroblast inhibitors can be accounted for the inhibitors themselves; some cultures still showed poor outgrowth in the absence of inhibitors compared to outgrowth observed from E9.5 or E10.0 ganglia. There might have been two reasons; 1) the time taken during dissection of tissues and setting cultures especially where the cultures were conducted on collagen gels which normally take at least 30 minutes to set, may affect the living status of tissues and consequently the normal outgrowth pattern or 2) ganglia were of slightly older age (E10.5).

To address this, E10.5 trigeminal ganglia (n=36) were cultured in isolation without inhibitors on poly-d-lysine (because an excess outgrowth was observed on this substratum) and the time required to dissect tissues from one litter and setting up each culture was counted in minutes using a timer clock. Trigeminal ganglia (n=7) which were dissected and put into culture quickly within the first 5-10 minutes produced extensive outgrowth of neurites and non-neuronal cells after a period of 24 hours in culture. In contrast, ganglia (n=18; n=9) that were put into culture within the next 10-15 and 15-30 minutes showed a stunted pattern of outgrowth. Although ganglia appeared alive they could extend only a few long neurites. In addition, non-neuronal cells formed lamellar structures at distal ends of neurites beyond which very few neurites managed to grow and instead showed fasciculations amongst them. Neurites only grew to 100μm or less and appeared short fasciculated fibers. Non-neuronal cells were present amongst them. Trigeminal ganglia (n=9) which were kept in DMEM but not placed in incubator until 30-40 minutes after dissection appeared dead and did not show any appreciable outgrowth of either neurites or non-neuronal structures.

Statistical analysis among the three groups compared with controls demonstrated a significant reduction (P<0.05) in the amount of neurite outgrowth with the passage of time. Thus those ganglia that were not put into culture until 30-40 minutes extended negligible number of neurites (Fig. 8). Non-neuronal cell density also decreased.

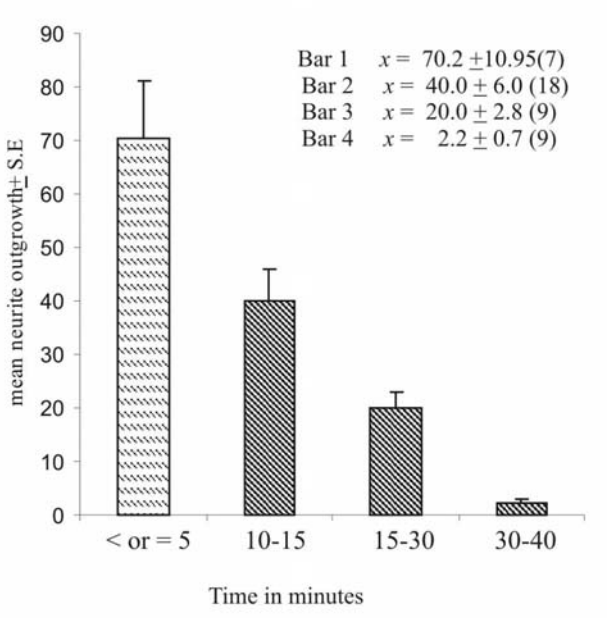


Fig. 8. Magnitude of neurite outgrowth is shown as mean ± S.E.M (n).

DISCUSSION

The results obtained from cultures of early developing mouse trigeminal ganglia provided

evidence that intact trigeminal ganglion can extend neurites *in vitro* in the absence of target tissues; the ganglion extends significantly greater neurite fascicles on poly-d-lysine than collagen, collagen in three-dimensional state does not allow free outgrowth of neurites. Time required in setting cultures may significantly affect the neurite outgrowth. If ganglia are not put into culture immediately after taking out from the animals they show least neurite outgrowth. Most importantly, inhibitors used *in vitro* to prevent fibroblast outgrowth radically affect the outgrowth of neurites as well as non-neuronal non-fibroblast cells. We suggest that data obtained form any neuronal cultures performed in the presence of fibroblast inhibitors must be assessed extremely cautiously.

Target independence and choice of substratum

Contrary to the previous demonstrations that intact trigeminal ganglion is a special case and does not extend neurites in isolation compared with other sensory cranial ganglia that produce neurites without target tissues (Lumsden and Davies, 1983, 1986), the present *in vitro* cultures indicate that the trigeminal ganglion may not be a special case if a different culture system is used and fibroblast inhibitors are excluded.

A significant number of trigeminal ganglia could extend neurites if cultured in isolation on poly-d-lysine substratum. However it was noticeable from the mean number of neurites that the magnitude of outgrowth was still not extensive. The outgrowth was significantly less on collagen coated platform or three-dimensional gels than which occurred on poly-d-lysine. Although, collagen is an extracellular matrix molecule and has been implicated in neurite outgrowth (Adams and Watt, 1993), collagen is a less permissive substratum and neurites prefer to grow on poly-d-lysine or poly-ornithine or glial cell surfaces (Letourneau, 1975ab). Thus meager outgrowth from trigeminal ganglia cultured on collagen gels was possibly due to the non-permissive nature of collagen or its three-dimensional state because trigeminal ganglia that were allowed to grow on collagen coated dishes rather than three-dimensional gels; extended neurites consistent to that reported in earlier studies (Lumsden, 1988). This indicates that trigeminal ganglia in intact state extend neurites only if poly-d-lysine or collagen is presented as an even spread substratum. This might have been due because collagen gels take longer time to set and furthermore, it is necessary to overlay the explanted tissues with more collagen to create a three-dimensional state. This set up is incubated again for a further 20-30 minutes before more culture medium could be added. During this time period while the collagen transforms into gel form, the ganglia may suffocate since neuronal cells need excess oxygen to survive. This points out that most of the cells die before they could send out neurites. Thus when ganglia are cultured on even spread substratum and are not overlaid with collagen they extend more neurites. A comparison of the number of neurites that grew out from trigeminal ganglia cultured on poly-d-lysine as opposed to collagen is indicative of this effect. Previous work provides evidence that both poly-d-lysine and collagen promote extensive branching but poly-d-lysine promotes maximal elongation rates of rat superior cervical ganglion neurons (Camella and Ross, 1987). Although presently, we did not measure neurite growth rates we did observe extensive branch formation in poly-d-lysine cultures. Also, poly-d-lysine yields highly enriched neuronal cultures (Lucius and Mentlein, 1995). We suggest that poly-d-lysine is a permissible substratum to evaluate axon outgrowth but one should also take into consideration that poly-d-lysine alters neuronal morphology, ultrastructure and metabolism (Harry *et al*., 1998).

Effect of fibroblast inhibitors

Because fibroblast inhibitors are routinely used in ganglionic cultures to prevent non-neuronal fibroblast cell outgrowth for assessment of neurite directionality, these inhibitors were used presently in the first set of cultures at the same concentration as has been previously described (Lumsden, 1988). Trigeminal ganglia cultured in isolation before target innervation extended neurites but the outgrowth was minimal and neurites grew only a short distance. It was hypothesized that mitotic inhibitors were possibly inhibiting the normal outgrowth of neurites. To our surprise, exclusion of inhibitors from the culture medium resulted in a several fold increase in the number of neurites along

with non-fibroblast non-neuronal cells that never grew out in the presence of inhibitors. In addition, neurites grew to a long distance, appeared highly fasciculated and formed complex networks together with non-neuronal cells. This suggested that the trigeminal ganglion outgrowth was in fact arrested by fibroblast inhibitors.

Elimination of fibroblast inhibitors therefore provided evidence that inhibitors used to prevent fibroblast outgrowth not only drastically reduce the outgrowth of neurites but have severe inhibitory effects on neurite pattern and morphology.

The extent of outgrowth and morphological pattern of neurites and non-neuronal cells showed by ganglia cultured in isolation exclusive of inhibitors was similar to ganglia that were co-cultured with maxillary or mandibular targets under these conditions indicating further that the initial outgrowth from early developing intact trigeminal ganglia may not occur under the influence of target tissues. The drawback however is that it was difficult to assess the role of target tissues in the absence of inhibitors because neurites grew in all quadrants and showed subtle directionality. Similarly when trigeminal ganglia were co-cultured with two or three targets or with inappropriate forelimb buds in the absence of fibroblast inhibitors (unpublished observations), assessment of directionality became extremely difficult. However where the ganglia are cultured in isolation at the earliest stages of target field innervation to investigate if an outgrowth occurs, the directionality of neurites can be ruled out because of absence of target tissues.

All fibroblast inhibitors including arabinose nucleosides (adenine, guanine, cytosine and thymine) induce apoptosis at micromolar (μM) concentrations. Cytosine arabinoside when combined with 5-fluorodeoxyuridine induces apoptotic cell death of postnatal rat sympathetic neurons even in the presence of NGF and despite the fact that neurons are post-mitotic. Fluorodeoxyuridine-dependent apoptosis induced by arabinosides is mediated by topoisomerase II inhibitors and they cause double-strand breaks in the DNA thus inducing cell death. In addition, chromosomal aberrations and damage to repair systems occurs because fluorodeoxyuridine treatment results in dUTP incorporation in neurons (Tomkins *et al*., 1994).

Because similar fibroblast inhibitors were used, it is conceivable that a significantly less or none neurite outgrowth was due to the inhibitory action of these inhibitors. This suggests that such inhibitors may arrest neurons while they are completing mitosis. Although pioneer fibers emerge from E8.5-9.0 trigeminal ganglion (Stainier and Gilbert, 1990); at this stage the ganglion is difficult to dissect as it is an aggregate of cells and readily dissociates *in vitro* within a few hours. Very few neurons which emerge at E9.0 possibly belong to placode-derived cells from the first wave of neurogenesis and reach the maxillary and mandibular epithelium by E10.0 (Stainier and Gilbert, 1990). Neurons from the second wave are generated between E9.5 and E10.0 and reach the maxillary process at E11.0 and mandibular process at E10.5 (Lumsden and Davies, 1983, 1986). It is because of this reason Stainier and Gilbert (1990) argued that target-directed outgrowth observed by Lumsden and Davies (1983, 1986) was re-growth. To a certain extent this seems true because E10.5 and later age trigeminal ganglia become dependent on neurotrophic factors (Davies and Lumsden, 1984; Buchman and Davies, 1993). Moreover, for lingual afferents that first enter the tongue *in vivo*, Rochlin and Farbman (1998) have shown *in vitro* that rat trigeminal neurons are repelled by their presumptive targets, the maxillary and mandibular arches during the initial stages of advance from the trigeminal ganglion and do not have a net attractive influence until after afferents have arrived near the target suggesting that initial guidance is possibly provided by midline intermediate targets. This observation contrasts with Lumsden and Davies (1986) work in mouse that maxillary or mandibular arch targets attract pioneer neurons from the time their growth cones exit the ganglion.

Presently therefore, the outgrowth which was observed from E9.0-E10.0 trigeminal ganglia was primary outgrowth and not re-growth. Because developing mouse trigeminal neurons are generated in waves of neurogenesis, it is likely that sparse neurite outgrowth observed in the presence of inhibitors from trigeminal ganglia occurred only from those neurons which had already ended their

terminal mitosis and were post-mitotic by the time ganglia were put into culture. Since the major outgrowth occurs from the second wave of neurogenesis that begins at E10.0 (Stainier and Gilbert, 1990), a significant increase in neurite number observed from E10.0 ganglia compared to E9.0-9.5 might have occurred from the second wave of neurogenesis. This is consistent with previous observations on mouse trigeminal ganglia that maximum neurite outgrowth occurs during a period when axons are actively growing towards their final targets (Davies and Lumsden, 1984). This shows an age-related effect demonstrating that E10.0 ganglia extend maximum neurites. Thus the results at one end demonstrate that fibroblast inhibitors significantly affect the normal pattern of neurite outgrowth and on the other that intact trigeminal ganglion at earliest stages of target field innervation extends neurites even when its target tissues are not there. As to which outgrowth, with or without inhibitors, should be considered normal compared with *in vivo* outgrowth remains an open possibility to be examined.

The effect of time

Most imperative is the time factor; ganglia which were put into culture within 10 minutes of dissection extended greater number of neurites but showed a restricted (stunted) pattern of outgrowth when this time period was increased to 20 minutes, while those ganglia that were not put into culture until 30-40 minutes extended very few neurites. This indicates that tissues kept longer in PBS or DMEM eventually die after 20-30 minutes. This to some extent also explains why substantial outgrowth could not occur in three-dimensional collagen gels, the reason being collagen gels take more than 30 minutes to set before culture medium could be added. Because cultures can be set up quickly within 5-10 minutes on poly-d-lysine coated substratum, a greater neurite outgrowth can occur easily because tissues remain alive within this time period. In addition, trigeminal ganglia at later stages become dependent on target-derived neurotrophic factors for survival (Davies, 1997).

Trigeminal ganglion axon outgrowth and migration is intrinsically determined

Extension of neurites without target tissues suggests that trigeminal ganglia were determined to extend neurites at earliest stages of target field innervation according to intrinsically determined programs. This raises the possibility that neurite outgrowth promoting factors are available to growing neurons via an autocrine source. Intrinsically produced neurotrophins (Arumae *et al.*, 1993; Davies, 1997) and neuregulins (Gassman and Lemke, 1997) may be the factors behind initiating neuron outgrowth and guiding them toward target tissues. Although neurotrophic factors do not act as neurotropic factors (Davies, 1997), in vitro cultures on dorsal root ganglia from p75-/- mice show that p75 is important in the developing embryo for regulating axon outgrowth and arborization and for Schwann cell migration during E11.5 to E14.5 (Bentley and Lee, 2000).

In addition to growth factors, basic helix-loop-helix (bHLH) transcription factors, the neurogenins may determine formation of neuronal precursors. Neurogenin-1 is expressed by placodal ectodermal cells, and acts prior to neuroblast delamination. Its involvement in the formation of sensory neurons is evident from the fact that mouse embryos lacking neurogenin-1 fail to generate the proximal (neural crest-derived) subset of cranial sensory neurons (Fode *et al*., 1998). Similarly, neurogenin-2 is an essential determination factor for the development of epibranchial placode-derived cranial sensory ganglia. A neurogenin-2 null mutation blocks the delamination of neuronal precursors from the placodes (Ma *et al*., 1998). Besides these, the role of extracellular matrix molecules laminins in trigeminal ganglion axon outgrowth still appears alluring because tooth pulp fibroblasts synthesize laminin-2 and laminin-8 that promote neurite outgrowth from trigeminal neurons (Fried *et al*., 2005).

Non-neuronal cells

The striking finding of present *in vitro* cultures of trigeminal ganglia in the absence of fibroblast inhibitors was a simultaneous outgrowth and proliferation of a large number of non-neuronal cells morphologically different from fibroblasts that never grew out in the presence of inhibitors. It should be noted that Lumsden and Davies (1983,

1986) made no mentioning about non-neuronal non-fibroblast structures or Schwann cells. It is presumed that in their trigeminal ganglionic cultures such structures did not grow out because they used fibroblast inhibitors. This is similar to ours because we also observed that in the presence of fibroblast inhibitors such structures do not grow out at all and appear in cultures only when fibroblast inhibitors are not added to cultures. Accordingly, it is suggested here that inhibitors used to block fibroblast outgrowth were equally effective in preventing their outgrowth and proliferation. Their identity in trigeminal ganglion cultures is uncertain but it is likely that they were Schwann cells and if they in fact were Schwann cells, their outgrowth from E9.0 mouse trigeminal ganglia is astounding.

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