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Reciprocal interactions between olfactory receptor axons and olfactory nerve glia cultured from the developing moth *Manduca sexta*☆

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Abstract

In olfactory systems, neuron–glia interactions have been implicated in the growth and guidance of olfactory receptor axons. In the moth *Manduca sexta*, developing olfactory receptor axons encounter several types of glia as they grow into the brain. Antennal nerve glia are born in the periphery and enwrap bundles of olfactory receptor axons in the antennal nerve. Although their peripheral origin and relationship with axon bundles suggest that they share features with mammalian olfactory ensheathing cells, the developmental roles of antennal nerve glia remain elusive. When cocultured with antennal nerve glial cells, olfactory receptor growth cones readily advance along glial processes without displaying prolonged changes in morphology. In turn, olfactory receptor axons induce antennal nerve glial cells to form multicellular arrays through proliferation and process extension. In contrast to antennal nerve glia, centrally derived glial cells from the axon sorting zone and antennal lobe never form arrays in vitro, and growth-cone glial-cell encounters with these cells halt axon elongation and cause permanent elaborations in growth cone morphology. We propose that antennal nerve glia play roles similar to olfactory ensheathing cells in supporting axon elongation, yet differ in their capacity to influence axon guidance, sorting, and targeting, roles that could be played by central olfactory glia in *Manduca*.

Keywords: *Manduca sexta*; Growth cone; Glial cell; Neuron–glia interaction; Development; Olfactory system

Introduction

Reciprocal interactions between neurons and glia shape many aspects of neural development in both vertebrate and invertebrate nervous systems. Glial cells direct the differentiation, migration, pathfinding, and survival of neurons, and in turn, neurons influence the differentiation, migration, and survival of glial cells (reviewed by Auld, 1999; Lemke, 2001; Oland and Tolbert, 2002). In olfactory systems, neuron–glia interactions have been implicated in many developmental processes, including axon growth and guidance. The axons of olfactory receptor neurons (ORNs) expressing the same olfactory receptor gene converge on specific subsets of glomeruli in the brain, in mammals (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996) and insects (Vosshall et al., 2000; Gao et al., 2000) alike. In mammalian olfactory systems, a special type of glial cell, the olfactory ensheathing cell (Raisman, 1985), is thought to play multiple roles in the growth and guidance of ORN axons. Olfactory ensheathing cells arise from the olfactory placode, accompany and enwrap bundles of ORN axons from the olfactory epithelium to the bulb, and support the continuous ingrowth of ORN axons into the mature olfactory bulb (reviewed by Chuah and West, 2002). Due to their distinctive molecular profiles and their locations in the olfactory pathway, olfactory ensheathing cells have been hypothesized to play key roles in guiding ORN axons to the presumptive olfactory bulb (Liu et al., 1995; Tennent and Chuah, 1996) and in sorting and targeting ORN axons within the olfactory bulb (Puche et al., 1996; St. John and Key, 1999; Crandall et al., 2000; Schwarting et al., 2000).

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In the moth *Manduca sexta*, developing ORN axons from the antenna encounter several types of glia as they grow toward and into the antennal (olfactory) lobes of the brain. The earliest ingrowing axons cause glial cells of the CNS to proliferate and to populate the base of the developing nerve; these glial cells, which we call "sorting zone glia," in turn play a strategic role in sorting ORN axons into fascicles that are destined to terminate in particular glomeruli in the antennal lobe (Rössler et al., 1999). Their role in axon sorting suggests that sorting zone glia share certain similarities with the mammalian olfactory ensheathing cells. A second type of glial cell associated with the antennal-lobe neuropil is required to stabilize the borders of developing glomeruli (Oland and Tolbert, 1988; Oland et al., 1988; Baumann et al., 1996). In addition to having an influence on the behavior of ORN axons in vivo, we have recently shown that sorting zone and neuropil-associated glial cells have dramatic influences on ORN axons in vitro (Tucker et al., 2000, 2001). Using cocultures containing explants of olfactory receptor epithelium and glial cells, we demonstrated that contact with sorting zone and neuropil-associated glial cells leads to robust elaborations in ORN growth cone morphology and the rapid loss of ORN growth cone motility.

A third type of glial cell that ORN axons encounter, the antennal nerve (AN) glia, arises in the periphery and eventually enwraps bundles of ORN axons after they migrate down the antennal nerve to the distal margin of the sorting zone (Rössler et al., 1999). Little is known about the roles AN glia play during development, yet their peripheral origin and relationship with ORN axons suggest that they, too, may share features with olfactory ensheathing cells. The current paper presents in vitro experiments that explore cellular interactions between ORN axons and AN glial cells. Results indicate that, without undergoing long-lasting changes in morphology, ORN growth cones typically continue to advance after contacting AN glial cells. ORN axons often travel directly on the surfaces of glial processes, indicating that AN glial cells provide a permissive substrate for axon extension. Moreover, the behavior of AN glial cells is itself changed in the presence of ORN axons. Over several days in coculture, AN glial cells form multicellular arrays through proliferation and extension of processes. We propose that local cues, both contact-dependent and contact-independent, operate to produce reciprocal influences on the behaviors of ORN growth cones and AN glial cells, and that these influences are considerably different from those seen in interactions between ORN growth cones and the previously tested, centrally derived, glial cell types. Some of this work appeared previously in abstract form (Tucker et al., 2002).

Materials and methods

Animals

M. sexta (Lepidoptera: Sphingidae) were reared from eggs in environmental chambers maintained at 25°C and 50–60% relative humidity under a long day photoperiod (17 h light, 7 h dark). Under these laboratory conditions, adult metamorphic development occurs over 18 stages, each lasting 1–4 days, starting at pupation and ending with the emergence of the adult moth. Pupae were staged by examining morphological changes in external structures visible beneath the pupal cuticle after fiber-optic illumination (Tolbert et al., 1983; Oland and Tolbert, 1987; Dubuque et al., 2001). Before use, experimental animals were anesthetized on ice.

Preparation of cultures

Explants of olfactory receptor epithelium

Antennae were removed from stage 4 female pupae, placed in a petri dish containing sterile PBS, and filleted along a visible border between the olfactory receptor and nonreceptor epithelia. The olfactory receptor tissue was incubated for 2 min at 37°C in a Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (21250-014; Gibco, Grand Island, NY) containing 0.05 mg/ml collagenase (LS004196; Worthington, Freehold, NJ) and 0.2 mg/ml dispase II (165859; Boehringer Mannheim, Mannheim, Germany). After enzymatic digestion, the tissue was gently triturated with a fire-polished Pasteur pipette, layered onto 6 ml of Culture Saline, and allowed to settle by gravity. The aggregated tissue was rinsed twice more, first with Culture Saline and then with Culture Medium. Explants of olfactory receptor epithelium were evenly suspended in fresh Culture Medium and plated in 100-μl aliquots into the wells of premade culture dishes. Culture-dish wells were made by attaching coverslips beneath 8-mm diameter holes drilled into the bottoms of 35-mm Falcon dishes. Dish wells were coated with a solution containing 400 μg/ml concanavalin A (C2010; Sigma, St. Louis, MO) and 4 μg/ml laminin (40232; Collaborative Research, Bedford, MA), and rinsed with sterile water prior use. After plating, culture dishes were sealed with Parafilm to prevent evaporation and incubated in a 26°C humidified incubator with room air.

Glial cell cultures and explant–glia cocultures

For isolation of antennal nerve (AN) glia, whole brains with attached partial antennae from early stage 7 female pupae were dissected into a petri dish containing ice-cold, sterile Dissecting Medium. Antennal lobes were exposed, and the attached antennae were filleted. The antennal nerve branches were cut close to the first antennal segment, and the perineural sheath was simultaneously removed from the antennal lobes and antennal nerves. The antennal nerves were then cut distal to the sorting zone, such that the entire intracranial portion of the antennal nerve and a small amount of antennal nerve from inside the antenna were saved. Antennal nerve tissue was digested with 0.1 mg/ml papain (5125; Calbiochem, La Jolla, CA) in Simple Salt Solution for 4 min at 37°C prior to trituration. Dissociated cells were then layered onto Recovery Solution in a 15-ml Falcon tube, and 200 units of DNase (Sigma D4263) in

Simple Salt Solution were added to the top layer of suspended cells. Cells were centrifuged at 500*g* for 4 min, resuspended in fresh Culture Medium, and centrifuged again. For plating alone, cells were resuspended in Culture Medium and plated into 8-mm wells (100 μl/well) as above.

For explant–glia cocultures, glial cells were resuspended in Culture Medium and added to explant cultures previously grown for 1 day in vitro (1 DIV). For each dish, 50 μl of Culture Medium was removed from the 100-μl bubble of medium overlying the cultured explants, and gently replaced with 50 μl of the glial suspension. After plating, the culture dishes were sealed, incubated for 2 h, and flooded with at least 1 ml of Culture Medium.

For isolation of sorting zone and neuropil-associated glia, antennal lobes were dissected and desheathed as above, neuronal cell-body packets were removed, and the sorting zone and neuropil tissue were cut apart and saved separately from the antennal nerve tissue. Tissue from all three sources was processed simultaneously for tissue culture, but plated separately.

Tissue culture solutions

Culture Saline (Oland et al., 1996): 149.9 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM TES, 11 mM D-glucose, 3 g/L lactalbumin hydrosylate (11800-026; Gibco), 2.5 g/L TC yeastolate (255772; Difco, Detroit, MI), 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, pH 7.0, 360 mOsm.

Culture Medium (supplemented Leibovitz's L-15 culture medium; Lohr et al., 2002): The following ingredients were added to 500 ml L-15: 50 ml FBS, 185 mg α-ketoglutaric acid, 200 mg D-(−)-fructose, 350 mg D-glucose, 335 mg DL-malic acid, 30 mg succinic acid, 1.4 gm lactalbumin hydrosylate, 1.4 gm TC yeastolate, 0.1 mg niacin, 30 mg imidazole, 500 μg 20-hydroxyecdysone (H-5142; Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 ml stable vitamin mix (SVM). A 5-ml stock solution of SVM consisted of 15 mg aspartic acid, 15 mg cystine, 5 mg β-alanine, 0.02 mg biotin, 2 mg vitamin B_{12}, 10 mg inositol, 10 mg choline chloride, 0.5 mg lipoic acid, 5 mg p-aminobenzoic acid, 25 mg fumaric acid, 0.4 mg coenzyme A, 15 mg glutamic acid, 0.5 mg phenol red. The pH was adjusted to 7.0 and the osmolarity was raised to 390 mOsm with D-glucose prior to sterile filtration.

Simple Salt Solution: 160 mM NaCl, 6 mM KCL, 78.8 mM D-glucose, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, pH 7.0, 420 mOsm.

Recovery Solution: 50% (v/v) Culture Saline and 50% (v/v) Simple Salt Solution, pH 7.0, 380 mOsm.

Dissecting Medium: 50% Leibovitz's L-15 (Gibco BRL 41300-039), 25% (v/v) Culture Saline, 25% Simple Salt Solution with 5 mM EDTA, and 18 mM D-glucose, pH 7.0, 360 mOsm.

Live-cell microscopy

Time-lapse differential-interference-contrast imaging was performed on explant cultures grown for 1 DIV or explant–glia cocultures grown for 2 h after the plating of AN glial cells. The imaging system included an Olympus BX50WI upright microscope with water-immersion objectives, shutters, z-drive, 12V/100W halogen bulb filtered by a green optical lens (543 nm), cooled CCD camera, and computer with SimplePCI (Compix Inc., Cranberry Township, PA) acquisition and analysis software. Prior to imaging, cultures were flooded with 3 ml of Culture Medium and placed inside an insulated chamber enclosing the stage to equilibrate to 25°C in a temperature-controlled microincubator (TC202A/PDMI-2; Harvard Apparatus, Holliston, MA).

To prevent evaporation, a thin layer of canola oil was applied over the Culture Medium surface prior to imaging. Images were collected with a 60× objective at 20-min intervals for up to 24 h. To ensure that focus was maintained over these relatively long imaging periods, a series of five to six images at successive focal planes 1 μm apart were collected at each time point. Only the in-focus images were used.

Rate analysis

The distances that axons grew before and after contacting glial cells were measured in five representative recordings. Axons from explant–glia cocultures were measured provided they met the following criteria: (1) each axon could clearly be identified as an individual, (2) axon growth could be monitored before and after contact with glial cells or glial processes, and (3) at least one image from each z-series was in focus for every time point of the movie sequence. While stepping through the selected movie sequences, markers were placed at the distal tip of growing axons in each frame and the distances between markers in consecutive frames were measured in microns by using SimplePCI. The net distance grown by each individual axon at each point in time was determined by summing all distances that were recorded up to that point. Branches that clearly arose from parent axons and continued to grow for longer than 1 h after formation were also measured. All branches were measured from the time of their appearance to the end of the recording. Net distances were plotted as a function of time, and elongation rates were determined by calculating the slope of regression lines fit to the precontact and postcontact curves for the each plot.

Cytoskeletal staining, growth cone sampling, and statistical analysis

Microtubule and F-actin labeling was performed following a slightly modified version of a protocol previously developed for cultured motoneurons from *Manduca* (Matheson and Le-

vine, 1999). Cultures were rinsed with K-PIPES buffer (80 mM PIPES–KOH, pH 6.8, 5 mM EGTA, 2 mM $MgCl_2$), then fixed and extracted for 30 min in K-PIPES buffer containing 0.5% glutaraldehyde and 0.1% Triton X-100. Cultures were rinsed in phosphate-buffered saline (PBS) containing 1 mg/ml $NaBH_4$ to quench autofluorescence, then rinsed and blocked for 1 h in PBS containing 0.2% fish skin gelatin and 0.1% Triton X-100 (blocking buffer). Mouse anti-α-tubulin (T9028; Sigma) was diluted 1:800 in blocking buffer and applied for 2.0 h at room temperature. After rinsing, cultures were incubated for 1.5 h in a 1:1000 dilution of secondary antibody (goat anti-mouse Cy3; Jackson ImmunoResearch, West Grove, PA) in blocking buffer containing approximately 2.5 U/ml, or 83 nM, Alexa-488 conjugated phalloidin (A-12379; Molecular Probes, Eugene, OR). Cultures were then rinsed and mounted with coverslips in a polyvinyl alcohol (PVA)-based mounting medium that included 1,4-diazobicyclo[2,2,2]octane (DABCO) to limit photobleaching.

Tubulin- and F-actin-stained cultures were used to compare the morphological complexity of hundreds of growth cones across multiple dishes. At least three explants displaying radial growth and a high number of surrounding glial cells were randomly selected from each of three explant and three explant–glia dishes. The entire perimeters of the selected explants were sampled by using a 60× objective, and images (~30/dish) were saved. Unmanipulated confocal images were printed and all isolated growth cones were scored based on a qualitative scale of morphological complexity. Scoring accuracy was confirmed by comparing independent results from two separate observers. Dishes were summed only if growth cone scores were statistically similar between dishes within the same experimental group. Two-by-two contingency tables containing total numbers of simple and complex growth cones from each experimental group were constructed and tested for statistical differences by Fisher's exact test (Fischer, 1925). Qualitative variables from tested conditions were defined as statistically different at a probability value of $P < 0.05$.

Labeling of GPI-linked Fasciclin II

Cultures were fixed for 1 h in a 0.1 M phosphate buffer containing 4% paraformaldehyde (PFA), and then rinsed and blocked for 1 h in PBS containing 4% normal goat serum (NGS). Antibodies raised to specifically recognize the GPI-linked isoform of *Manduca* Fasciclin II (Wright and Copenhaver, 2000) were diluted 1:10,000 in PBS with 4% NGS, applied to cultures, and incubated overnight at 4°C. Cultures were rinsed in PBS, and incubated for 1.5 h in goat-anti-guinea pig Cy3 secondary antibody (Jackson Immunoresearch) diluted 1:500 in PBS. Cultures were then rinsed into Tris-buffered saline (TBS), incubated for 20 min at RT in a 1:1500 dilution of the nucleic acid stain Syto 13 (S-7575; Molecular Probes) in TBS, rinsed, and mounted in PVA.

CellTracker and anti-horseradish peroxidase (HRP) labeling

Prior to plating, AN glial cells were suspended in 500 μl loading buffer containing 5 μM CellTracker green (C-7025; Molecular Probes) and 0.01% Pluronic F-127 (P-6866; Molecular Probes) in standard insect saline (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes buffer, 35.5 mM D-glucose, 40 mM mannitol, pH 7.0, 390 mOsm). Cells were incubated 10–20 min with slight agitation. Labeled cells were transferred to a tube containing 6 ml of Culture Medium, pelleted by centrifugation, resuspended in Culture Medium, and plated with explant cultures grown for 1 DIV. Explant–glia cocultures were grown for 1 or 2 days in vitro, and then processed for anti-HRP immunocytochemistry. Antibodies against HRP specifically recognize cell-surface antigens on developing insect neurons (Jan and Jan, 1982). Cultures were fixed for 30 min in 0.1 M phosphate buffer containing 4% PFA, rinsed in PBS, and blocked for 1 h in PBS with 2% NGS, 2% bovine serum albumin (BSA), and 0.5% Triton X-100. Rabbit anti-HRP (323-005-021; Jackson ImmunoResearch) was diluted 1:1000 in PBS with 1% NGS and 1% BSA, added to cultures, and incubated overnight at 4°C. Cultures were then rinsed in PBS with 1% NGS, 1% BSA, and 0.25% Triton X-100, and incubated for 1 h with goat anti-rabbit Cy3 secondary diluted 1:1000 in PBS with 1% NGS and 1% BSA. Cultures were rinsed in PBS with 0.25% Triton X-100, and then rinsed again with PBS before mounting in PVA.

Laser-scanning confocal microscopy

A Nikon PCM 2000 system equipped with a Nikon E800 microscope, a 50 mW argon laser, 4 mW green and 10 mW red HeNe lasers, and a computer running SimplePCI acquisition and analysis software, was used for confocal imaging. Appropriate dichromatic filters were used for multichannel collection. Serial optical sections were taken at sequential depths, 0.3-1.0 μm apart depending on the depth of field of the required objective lens, and stored as stacks of optical images. If needed, confocal images were manipulated for brightness, contrast, and intensity with Photo Paint 9 or SimplePCI and prepared in figure format with Corel Draw 9.

Bromodeoxyuridine incorporation, immunocytochemistry, and analysis

The uridine derivative, bromodeoxyuridine, can substitute for thymidine and incorporate into DNA during the S-phase of the cell-division cycle. To monitor glial proliferation in vitro, 5-bromo-2′-deoxyuridine (BrdU; B-9285, Sigma) was added to explant–glia cocultures at a final concentration of 5 μg/ml. At the stage used to isolate glia, neuropil-associated glia (Oland and Tolbert, 1989; Kir-

schenbaum et al., 1995), some sorting zone glia (Rössler et al., 1999), and glia within the intracranial portion of the AN (unpublished observations), are dividing. Glial cells were cultured for 12 h before receiving BrdU to ensure that any cells in S-phase at the time of plating would exit this portion of the cell cycle and that all BrdU incorporation would be the result of DNA synthesis occurring in vitro. Explant–glia cocultures were grown for 13.5 h in the presence of BrdU, and then fixed with 4% PFA in 0.1 M phosphate buffer for 1 h. Following fixation, cultures were rinsed in PBS, treated with 2 N HCl in PBS for 30 min to denature DNA and facilitate antibody recognition, rinsed in PBS with 0.3% Triton X-100 (PBST), and blocked in PBST with 4% NGS for 1 h. Mouse anti-BrdU (347580; Beckton Dickinson, San Jose, CA) was diluted 1:200 in PBS with 1% NGS, applied to cultures, and incubated for 2 days at 4°C. Cultures were then rinsed in PBS with 2% NGS, incubated in a 1:800 dilution of goat-anti-mouse Cy3 secondary in PBS with 2% NGS, rinsed in PBS, incubated for 10 min in a 1-μg/ml solution of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; D9542, Sigma) to counterstain all nuclei, rinsed, and mounted in PVA. Phase contrast and two-channel fluorescence images were collected by using an inverted Olympus microscope with a Hammamatsu CCD camera and appropriate filter sets. Five nonoverlapping images were collected with a 20× planapochromat objective, covering the majority of each dish, and cell nuclei were counted in all fields provided they could be identified as glial cells in corresponding bright field images. After counting nuclei in each field and summing nuclei from similar dishes, BrdU-positive glial nuclei were expressed as a percentage of the total number of DAPI stained glial nuclei for each glial type.

Conditioned-medium experiments

Coverslips were glued beneath two physically separated 8-mm holes drilled into the bottoms of individual 35-mm Falcon dishes, and coated as described above. This technique has previously been used to prevent physical contact between cells cultured in the two adjacent wells, but allows for chemical communication between cells in the two wells through the 1 ml of culture medium that covers them after plating (Oland and Oberlander, 1994b; Luedeman and Levine, 1996). AN glial cells were grown either alone (See Fig. 10, condition 1) or physically separated from explants of olfactory epithelium (See Fig. 10, condition 2). In a third condition, AN glial cells received medium conditioned by explant–glia cocultures (See Fig. 10, condition 3). In condition 3, glial cells were plated into both wells simultaneously, such that explant-glia cultures in well “A” were established at the same time glial cells were plated in well “B.” During normal explant–glia coculture preparation, AN glial cells were bathed for 2–3 h prior to flooding in a 100-μl bubble of Culture Medium that possibly contained a concentrated supply of diffusible factors released from explants. To replicate this, AN glial cells in conditions 2 and 3 were plated into explant conditioned medium prior to flooding. Three double-well dishes were prepared per condition described in Fig. 10. For photography, slightly overlapping phase-contrast images were collected down a central strip of well B in all dishes, creating a representative montage of the AN glial cells at 24 and 72 h after plating.

Results

Isolation and culture of antennal nerve glial cells and explants of olfactory receptor epithelium

In *Manduca*, glial cells of the sorting zone and antennal lobe neuropil arise from a common pool of CNS glia (Rössler et al., 1999). AN glia have a separate origin, however, and arise in the antenna. AN glia migrate down the antennal nerve and arrive at the distal edge of the sorting zone by stage 7 (Rössler et al., 1999; Fig. 1A). AN glia were harvested for culture from pieces of early stage-7 antennal nerves that were cut distal to the sorting zone and proximal to the second segment of the antenna. This dissection excluded the sorting zone glia, which occupy the most proximal portion of the antennal nerve, and the olfactory receptor neurons (ORNs), which are located in the olfactory receptor epithelium of the antenna starting in the third segment (Fig. 1A). In vitro, AN glial cells had either oblong (Fig. 1B and C) or discoid (Fig. 1D) cell bodies, and many long, stout processes.

AN glial cells were added to cultures containing explants of stage-4 olfactory receptor epithelium grown for 24 h in vitro. Before addition of glial cells, hundreds of ORN axons extended from explants and normally displayed a radial pattern of outgrowth (Fig. 1E). ORN axon outgrowth from olfactory receptor explants was robust, particularly within the first 48 h of culture, allowing for the observation of dynamic changes in axon behavior. Individual growth cones could be identified at the peripheral margin of explants, where the extent of axon overlap was minimal (Fig. 1F). Time-lapse observations showed that ORN axons elongated by extending and retracting fine branches that extended from their growth cones. Most axons had streamlined growth cones with simple, bullet-shaped morphologies (Fig. 1F, closed arrow); however, some axons had large, flat growth cones (Fig. 1F, open arrow) at their tips.

Axon behavior after contact with AN glial cells

In cocultures, the vast majority of dissociated cells were identified as AN glial cells by size and morphology; ORNs remained clustered in explants. The general pattern of ORN axon outgrowth was not altered by the presence of AN glial cells. Out of 30 documented encounters, 93% of ORN axons continued to advance following contact with AN glial cells. The most common behavior (67%) observed after contact was the continuation of growth cone advancement directly

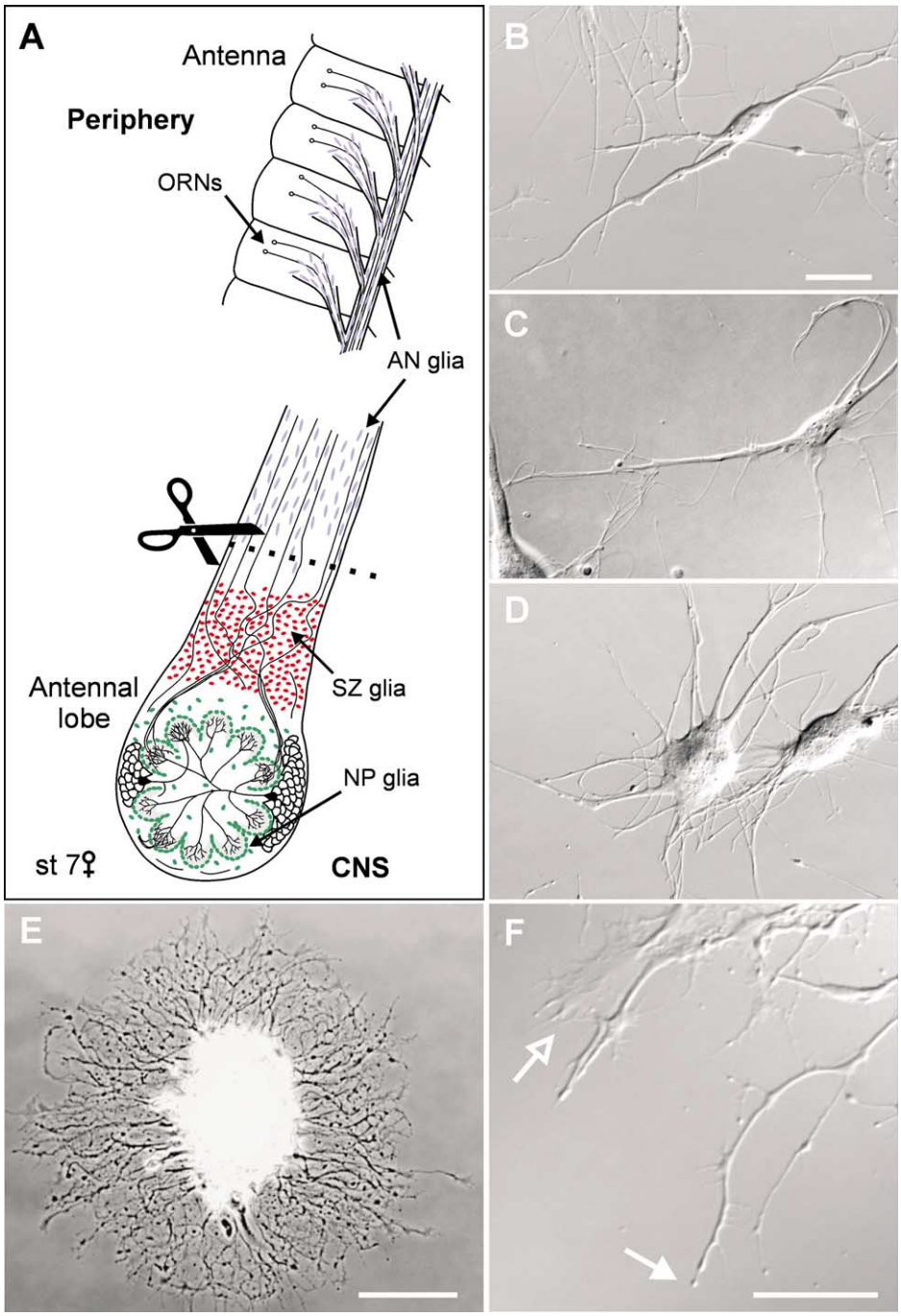


Fig. 1. Establishment of cultures containing ORN axons and antennal nerve (AN) glial cells. (A) Diagram illustrating the anatomical locations of AN, sorting zone (SZ), and neuropil-associated (NP) glial cells in the primary olfactory pathway of *Manduca sexta* at stage 7 of adult metamorphic development. (B–D) Differential interference contrast (DIC) images of freshly cultured glial cells from the antennal nerves of early stage 7 animals. (E) Low magnification phase contrast image of an explant of olfactory receptor epithelium cultured for 1 DIV. ORN axons extend in a radial pattern from explant. (F) High magnification DIC image of ORN axons at the peripheral margin of axon outgrowth form an explant. Open arrow, flattened growth cone; closed arrow, simple growth cone. Scale bars: (B) 25 μm, applies to (C, D); (F) 25 μm; (E) 100 μm.

along glial processes (Fig. 2A–F; Fig. 3G–I). The majority of growth cones that advanced in contact with AN glial cells retained simple morphologies (47%), while the remainder flattened after contact (20%). Those that adopted flattened morphologies did so only temporarily, as the longest observed period of growth cone flattening lasted under 2 h. In all cases, flattened growth cones continued to advance while remaining in close contact with AN glial cells or their processes (Fig. 2A–F), contrasting previous findings that ORN growth cones remain stationary for up to 15 h after

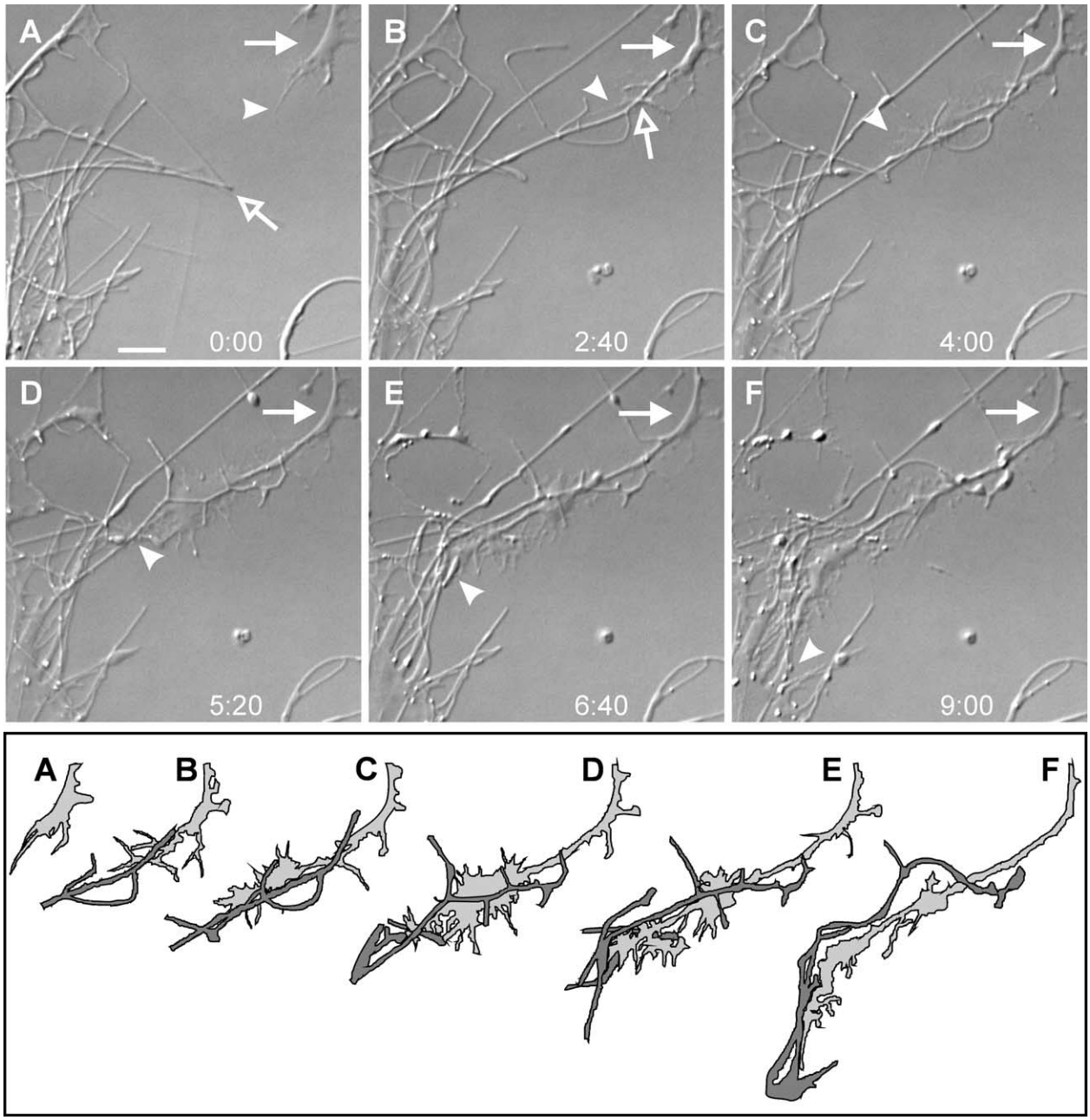


Fig. 2. After contact with AN glial cells, ORN growth cones sometimes elaborate before advancing along glial processes. (A–F) DIC images from time-lapse recording (top), and traces (bottom) of growth cone at arrow (light gray) and glial processes at open arrow (dark gray). Glial processes grow in from lower left; ORN growth cone enters from upper right. (A, B) Glial process extends toward and contacts growth cone. (C) Growth cone flattens and extends along glial process. (D) Flattened growth cone has elaborated while remaining intimately associated with glial process. (E, F) Growth cone remodels, becoming simpler, and advances along glial process. Arrows indicate axon shaft; arrowheads indicate axon tip. Time stamps are in hours and minutes. Scale bar: 10 μm, applies to all.

contacting glial cells from the sorting zone (Tucker et al., 2000, 2001). Interestingly, AN glial cell processes were motile and often contacted ORN axons before axonal growth cones reached toward them (Fig. 2A and B). After contact (Fig. 2B), the growth cone in Fig. 2 initially flattened (Fig. 2C and D), but continued to advance after remodeling to a simpler form (Fig. 2F). To help visualize the closely apposed growth cone and glial processes in this sequence, each was digitally traced, and the traced images were superimposed and arranged in sequence (Fig. 2, bottom A–F). A growth cone that retained a simple morphology after contact is shown extending along a glial process in Fig. 3. Without elaborating its form, the growth cone advanced in direct apposition to the glial process shown in the highlighted region in Fig. 3F (Fig. 3G–I).

After contact, some axons (13%) branched and continued

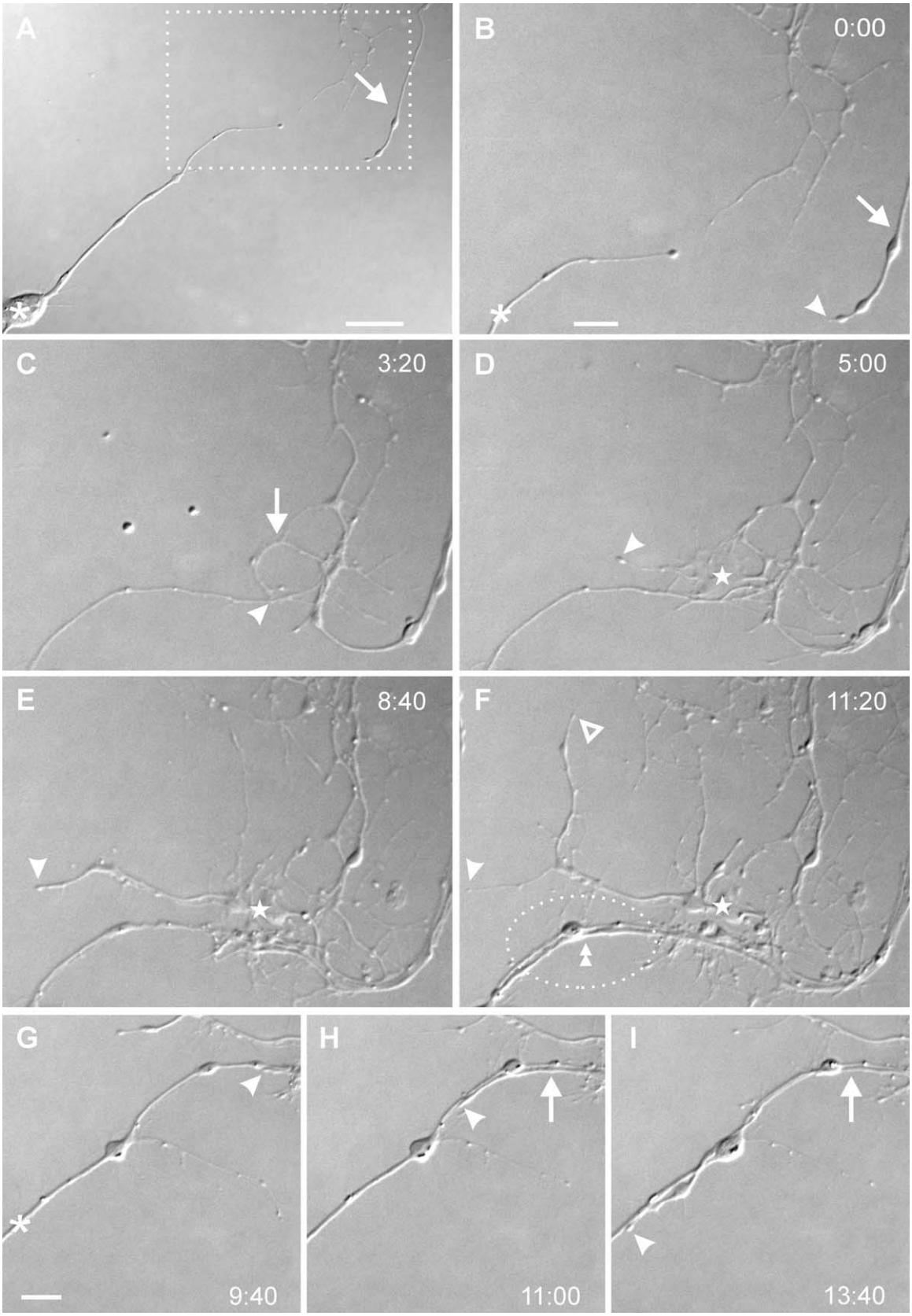
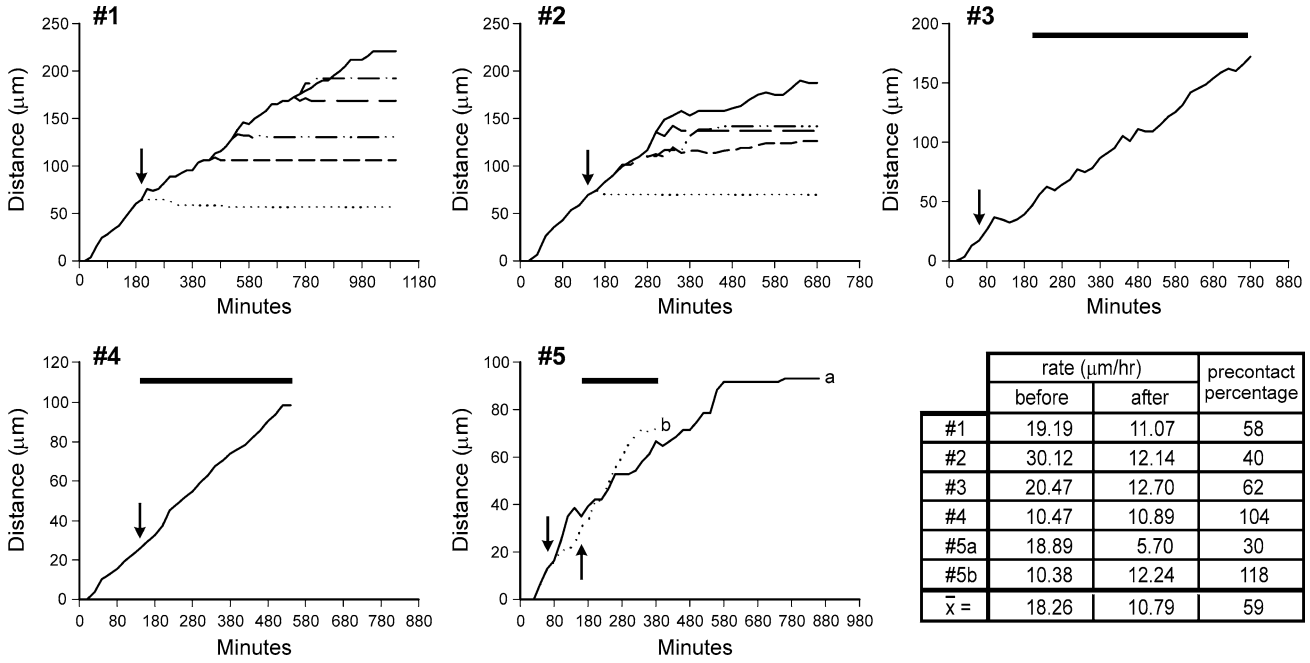


Fig. 3. Two other axon behaviors following growth cone contact with an AN glial cell. (A) Entire field of view at time zero. Cell body of AN glial cell (asterisk), bottom left; ORN axon (arrow) extends from an explant outside the field to the upper right. (B–F) Digital zoom of area outlined in (A), showing axon branching and elongation following contact with glial process (asterisk). (C) Growth cone contacts glial process at arrowhead. (D) Growth cone enlarges (star) and a small branch (arrowhead) emerges from left edge. (E) Branch continues to grow to its furthest extent. (F) New branch (open arrowhead) extends upwards as old one (arrowhead) is maintained. (G–I) Area highlighted by oval in (F), showing elongation of a different axon on a glial process. Growth cone tip (arrowhead) extends along glial process (asterisk). Double arrowhead in (F) indicates the ORN axon that elongated along glial process in (G–I). Time stamps are in hours and minutes. Scale bars: (A) 25 μm; (B–I) 10 μm.



	rate (μm/hr)		precontact percentage
	before	after	
#1	19.19	11.07	58
#2	30.12	12.14	40
#3	20.47	12.70	62
#4	10.47	10.89	104
#5a	18.89	5.70	30
#5b	10.38	12.24	118
$\bar{x}$ =	18.26	10.79	59

Fig. 4. Growth cone contact with AN glial cells leads to continued axon elongation. Plots represent the growth of five different ORN axons that encountered AN glial cells; arrows mark moment of contact. Discontinuous lines represent growth cone branches. In all five examples, ORN axons elongated after contacting AN glial cells. Axons #3, #4, and #5b elongated along glial processes, and the overhead bars correspond to the duration of contact. The table lists the individual and the average rates of axon growth before and after contact with AN glial cells.

to advance on the substrate adjacent to the contacted AN glial cells (Fig. 3A–F). At the beginning of the sequence shown in Fig. 3, axons extended from the upper right and contacted a process from the AN glial cell located at the bottom left of Fig. 3A. One growth cone (Fig. 3B, arrowhead) advanced toward and contacted the glial process (Fig. 3C), enlarged, branched, and continued to advance (Fig. 3D–F). Axons that branched but continued to elongate typically extended one branch at a time; when a growing branch stopped elongating, a new branch would grow while the first was retained but left with little cytoplasm (Fig. 3E and F).

Other growth cones (13%) simply continued to advance across AN glial cells or processes, while a few growth cones (7%) ceased advancement after contact. Growth cones that advanced across or stopped advancing remained simple in morphology, neither flattening nor branching following contact with AN glial cells (data not shown).

Rate analysis

The net distances grown by individual ORN axons were analyzed to determine whether contact with AN glial cells influenced the overall rate of axon elongation. In all cases examined, ORN axons continued to elongate long after initial growth cone contact with AN glial cells (Fig. 4, arrows). Axon elongation rates were similar before and after contact, regardless of contact-induced changes in growth cone behavior. Whether growth cones branched and advanced away from AN glial cells (Fig. 4, axons #1 and #2), advanced near AN glial cells (Fig. 4, axon #5a), or advanced on AN glial processes (Fig. 4, axons #3, #4, and #5b), ORN axons continued to elongate for many hours after the initial contact. Axons #1 and #2 branched after contact, and then grew away from the contacted glial cells. Generally, only one axon branch advanced at a time and the growing branch always was plotted as a solid line in the graphs. The first three branches of axon #1 (Fig. 4) are shown in Fig. 3D–F. The bars above axons #3, #4, and #5b in Fig. 4 correspond to the period of time during which their growth cones extended on glial processes. Axon #4 (Fig. 4) is depicted in Fig. 2, and was measured until the tip became obscured after reaching the glial cell body shortly after Fig. 2F. The tip of axon #5b became obscured after it reached the cell body of the glial cell it had grown on, and could not be followed through the end of the recording.

After contacting glial cells, all ORN axons continued to elongate, on average, at 59% (Fig. 4, table) of their precontact rate. Axons #3, #4, and #5b, those advancing on glial processes, elongated at an average of 87% of their precontact rate. In two cases, the rate of axon elongation was actually greater on glial processes than before contact with AN glial cells (Fig. 4, axons #4 and #5b). Although they continued to grow, rates of axon elongation were considerably slower, averaging 47% of their precontact rate, for axons that did not extend directly on AN glial cells (Fig. 4, axons #1, #2, and 5b). Thus, in five separate recordings, axons always elongated after contacting AN glial cells and

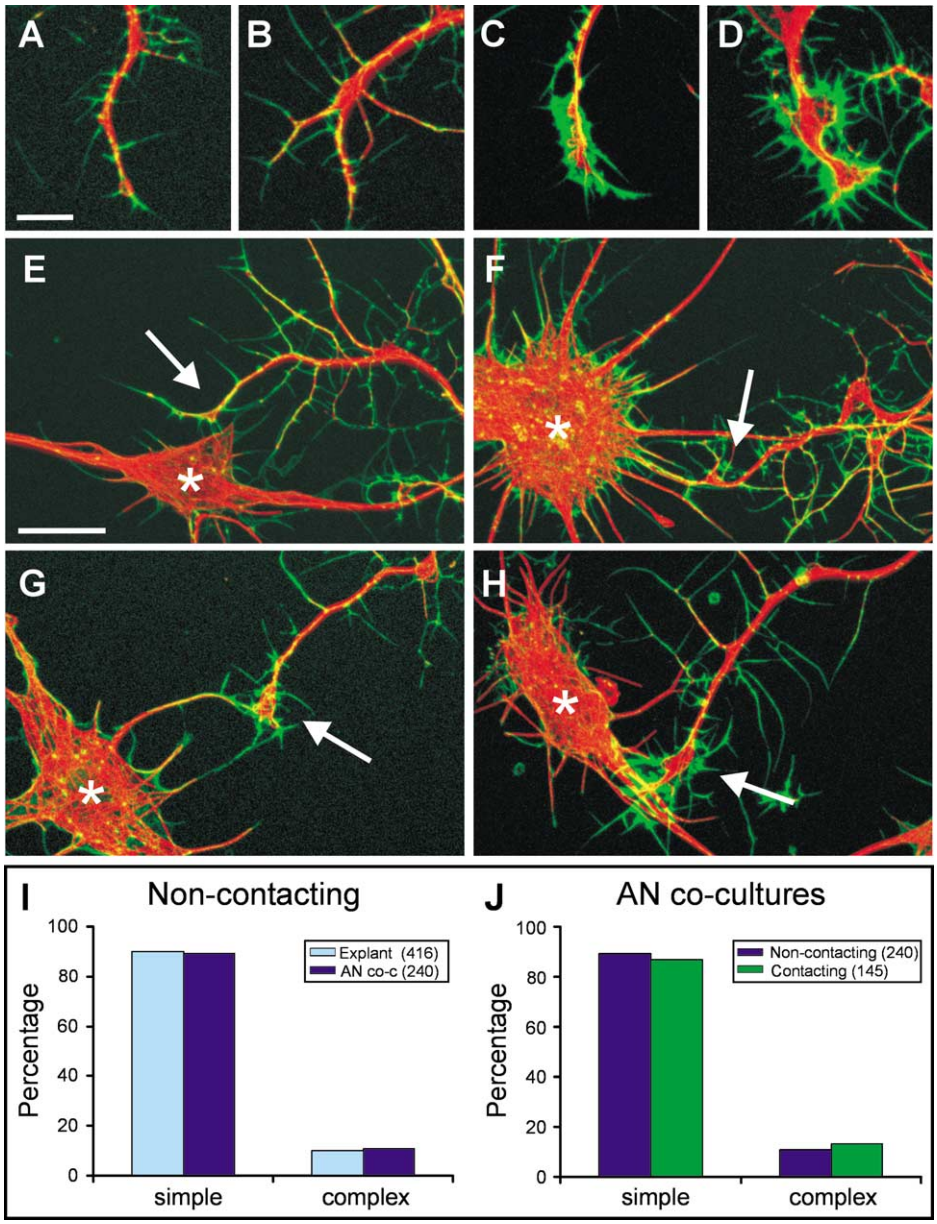


Fig. 5. Contact with AN glial cells does not elicit statistically significant changes in growth cone complexity. Cultures were fixed and stained to visualize microtubules (red) and F-actin (green). (A–D) Confocal micrographs showing morphological differences between simple (A, B) and complex (C, D) ORN growth cones. (E, F) Simple growth cones (arrows) contacting AN glial cells (asterisks). (G, H) Complex growth cones (arrows) contacting AN glial cells. Growth cones exhibit the full range of morphologies, regardless of whether they contact glial cells. (I) Noncontacting growth cones were predominantly simple, and proportions of simple and complex growth cones were not statistically different between explant only and explant–glia cocultures. (J) Proportions of simple and complex growth cones were not statistically different between growth cones that contacted glial cells (green bars) and growth cones that did not (blue bars) in explant–glia cocultures. Scale bars: (A) 10 μm; (E) 20 μm, applies to (F–H).

those that extended on glial surfaces grew faster than those that extended on the tissue culture substrate.

Statistical analysis of contact-mediated changes in growth cone morphology

To better characterize the influence that contact with AN glial cells had on ORN growth cone morphology, we used cytoskeletal labeling to analyze hundreds of growth cones from fixed cells. In confocal microscopic images of the entire perimeters of randomly selected explants, individual growth cones were scored qualitatively according to their morphological complexities. ORN growth cones had diverse shapes (Fig. 5A–D), yet they could be grouped into two broad categories: those with simple (Fig. 5A and B) and those with complex (Fig. 5C and D) morphologies. Simple

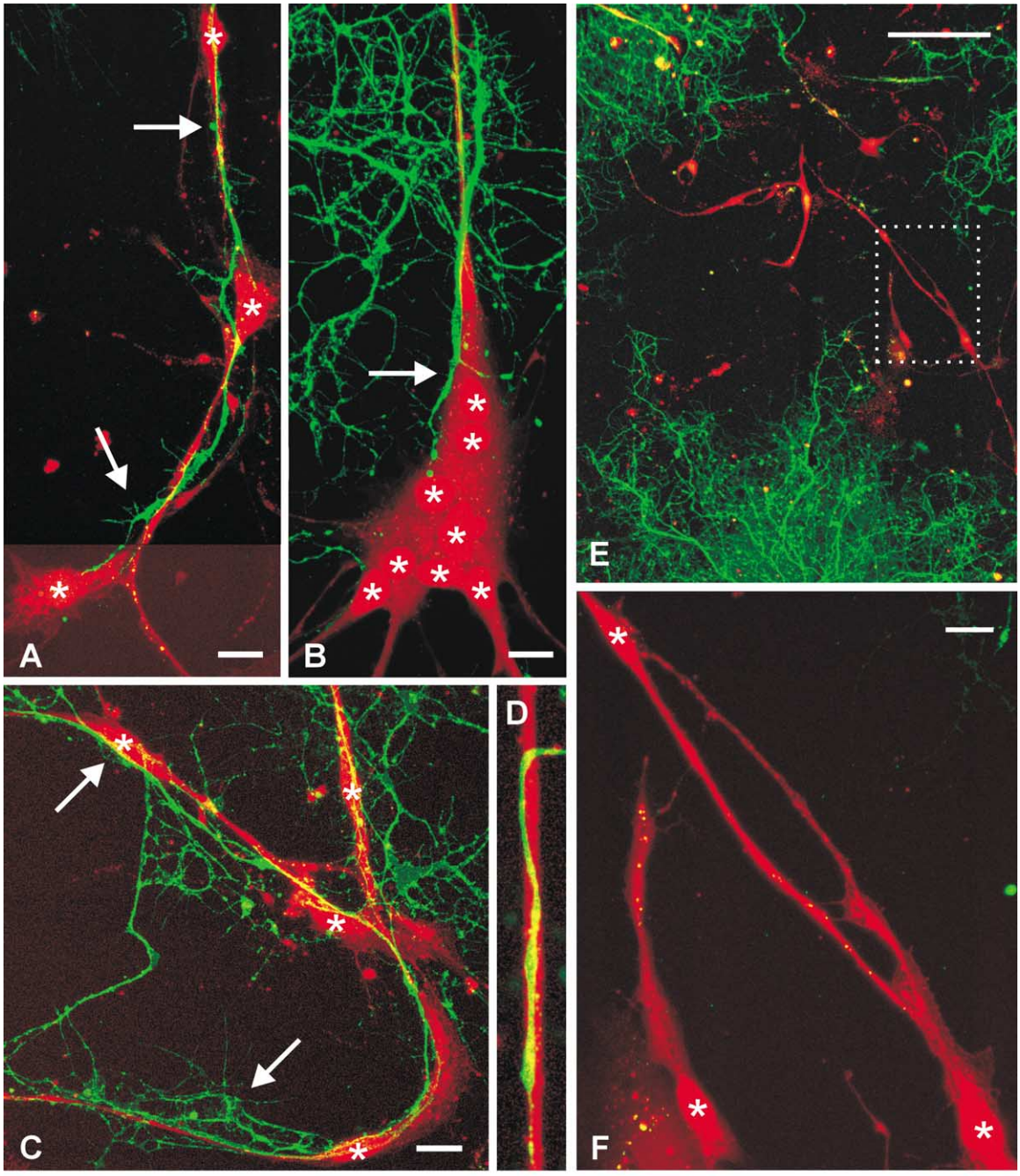


Fig. 6. ORN axons and AN glial cells form close associations. ORN axons (green), labeled with antibodies against horseradish peroxidase after 1 (A–C) and 2 days in vitro (D–F), and glial cells (red), labeled with a vital fluorescent dye prior to culturing. ORN axons, arrows; AN glial cell bodies, asterisks. (A) Single axon in close association with several AN glial cells in an array. (B) Aggregated glial cells extend process into an area of dense axon outgrowth. One axon (arrow) extended along the AN glial process, while others extended across or alongside the array. (C) ORN axons grew along the curvature of an AN glial array. (D) Individual ORN axon on the surface of a glial process, which was elevated from the substrate. (E) Low magnification view, showing a glial array between several explants. (F) High magnification view of boxed region in (E), showing that ORN axons are absent from the array. Scale bars: (A–C, F) 10 μm; (E) 100 μm; width of (D), 8 μm.

growth cones had either unbranched or branched microtubule domains, and were usually tipped by a single F-actin-based filopodium. Complex growth cones had lamellar regions containing splayed microtubules, and were surrounded by short, dense fringes of F-actin-based filopodia. Growth cones also adopted simple (Fig. 5E and F) or complex (Fig. 5G and H) morphologies when they were in contact with AN glial cells. Growth cones were predominantly simple (~85%) in morphology, regardless of glial cell contact (Fig. 5I and J). The distributions of growth cone morphologies in noncontacting conditions were not statistically different from one another ($P = 0.690$), nor were

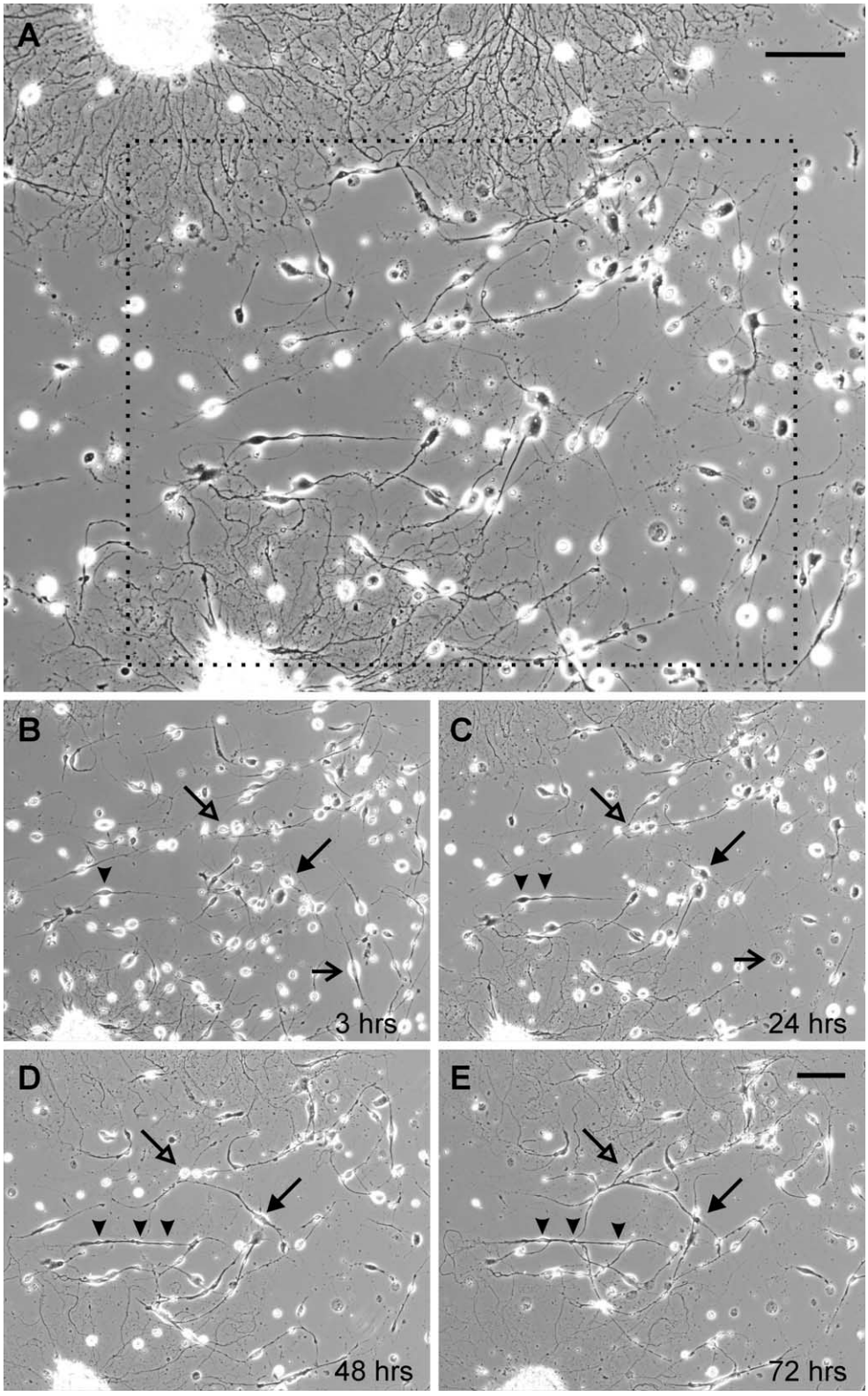


Fig. 7. AN glial cells link to form multicellular arrays near ORN axons. (A) Phase contrast image showing AN glial cells situated between explants at 24 h of coculture (B–E) Sequential photographs showing changes in glial distribution that occurred over time within the boxed region of (A). (B) Three hours after addition to explant cultures, glial cells appeared disorganized. (C) After 24 h, some glial cells died (short arrow), while others (open and closed arrows) grew processes. New phase-bright cell bodies (arrowheads) were occasionally added along glial processes, here and in (D). (D) By 48 h, many glial processes extended to join neighboring glia to form arrays. (E) Arrays continued to become more pronounced, reaching their maximal size around 72 h in vitro. Scale bars: (A) 200 μm; (B–E) 100 μm.

there statistically significant differences between contacting and noncontacting growth cones in explant–glia cocultures ($P = 0.516$), when analyzed by Fisher's exact test.

ORN axons and AN glial cells form close associations with each other

Live-cell recordings demonstrated that ORN axons and AN glial cells were often closely associated with one another. To better visualize the extent of neuron–glia interrelationships, differential staining was used to separately label ORN axons and AN glial cells. AN glial cells were labeled with a vital fluorescent dye prior to plating with explants and the explant–glia cocultures were grown for 24 or 48 h, fixed, and then stained with antibodies against horseradish peroxidase (HRP) to label ORN axons. Anti-HRP antibodies specifically detect glycoproteins on the cell-surfaces of insect neurons (Jan and Jan, 1982; Sun and Salvaterra, 1995), including cultured moth ORNs isolated from developing antennae (Lucas and Nagnan-Le Meillour, 1997).

Confocal microscopy revealed many examples of ORN axons intimately associated with AN glial cells, both at the 24-h (Fig. 6A–C) and 48-h (Fig. 6D) time points. Interestingly, AN glial cells formed multicellular arrays that were linked together by processes (Fig. 6A–C, and E). ORN axons readily grew on the AN glial cells they encountered, and their trajectories were noticeably influenced by the directional orientation of AN glial arrays (Fig. 6C). Axons grew for considerable distances along AN glial cells, sometimes shifting back and forth between glial surfaces and the adjacent substrate (Fig. 6A–D). Glial arrays often occurred between explants of olfactory epithelium (Fig. 6E; compare with Fig. 7A), in territory close to explants but devoid of ORN axons (Fig. 6F), indicating that axonal processes did not prefigure the formation of AN glial arrays.

AN glial cells form multicellular arrays on and near ORN axons

By taking phase-contrast photographs of the same fields of view on successive days in culture, we were able to characterize the behavior of individual glial cells during the formation of glial arrays. One such field of view is presented in Fig. 7, which shows many AN glial cells distributed between several explants (Fig. 7A). Three hours after plating. AN glial cells were growing in no obvious pattern on and next to ORN axons that extended from nearby explants (Fig. 7B). At 24 h, processes grew from many glial cells, and the cells had formed the rudiments of arrays (Fig. 7C). Additional cell bodies appeared to accumulate along glial processes, suggesting that glial cells proliferated during array formation (Fig. 7B–E, arrowheads). By 48 h, glial cells had assembled into clearly recognizable arrays both on and adjacent to ORN axons, with glial processes spanning between and interconnecting multiple glial cell bodies (Fig. 7D). By 72 h, the array had expanded further, with new additions of glial processes added to the array seen at 48 h (Fig. 7E). Although new additions to glial arrays were infrequent after 72 h, they were still present after 96 h of culture (Fig. 8A). Glial cells that did not incorporate into arrays apparently died, as no individual glial cells remained in isolation beyond 72 h.

When cultured in the absence of neurons, AN glial cells typically did not form arrays and died between 48–72 h in vitro. In rare cases, a small number of AN glial cells elongated and joined with several neighbors to form small glial aggregates that survived past 72 h in culture, but these arrays were always less extensive than those formed near ORN axons in cocultures.

AN glial arrays are immunoreactive for GPI-linked fasciclin II

Fasciclin II is a homophilic cell adhesion molecule in the immunoglobulin superfamily that is involved in the fasciculation and guidance of certain axons in developing insect nervous systems (Bastiani et al., 1987; Harrelson and Goodman, 1988; Grenningloh et al., 1991). In vivo, a large subset of ORN axons expresses the transmembrane isoform of *Manduca* fasciclin-II (MFas II), and a set of AN glial cells distal to the sorting zone express the GPI-linked isoform of MFas II (GPI-MFas II) (Higgins et al., 2002). Interactions between isoforms of MFas II could underlie cellular interactions between ORN axons and AN glial cells, both in vivo and in vitro. To test whether arrays of AN glial cells were GPI-MFas II-positive, explant–glia cocultures were fixed after 96 h of glial growth and labeled for GPI-MFas II. All of the AN glial cells in randomly chosen arrays were immunoreactive for GPI-MFas II (Fig. 8). As expected, ORN axons were GPI-MFas II-negative (Fig. 8B). The multicellular nature of glial arrays was apparent after counterstaining with a fluorescent nucleic acid stain. At the higher magnification shown in the boxed region in Fig. 8B, multiple nuclei were observed within the GPI-MFas II-positive array of AN glial cells (Fig. 8C–E).

AN glial cells proliferate in vitro

To determine whether AN glial cells were mitotically active in vitro, bromodeoxyuridine (BrdU) was used to label cells in the S-phase of the cell-division cycle in 12-h explant–glia cocultures. Since neuropil-associated glial cells cultured alone fail to divide (Oland, personal communication) and usually die by 48 h, we chose not to examine BrdU incorporation in cultures containing only glial cells. Before cell counts were made, glial cells were identified in phase-contrast images (Fig. 9A, E, and I). All nuclei were counterstained with DAPI, such that the total number of neuropil-associated (Fig. 9B), sorting zone (Fig. 9F), and AN (Fig. 9J) glial cells per field of view could be counted. For

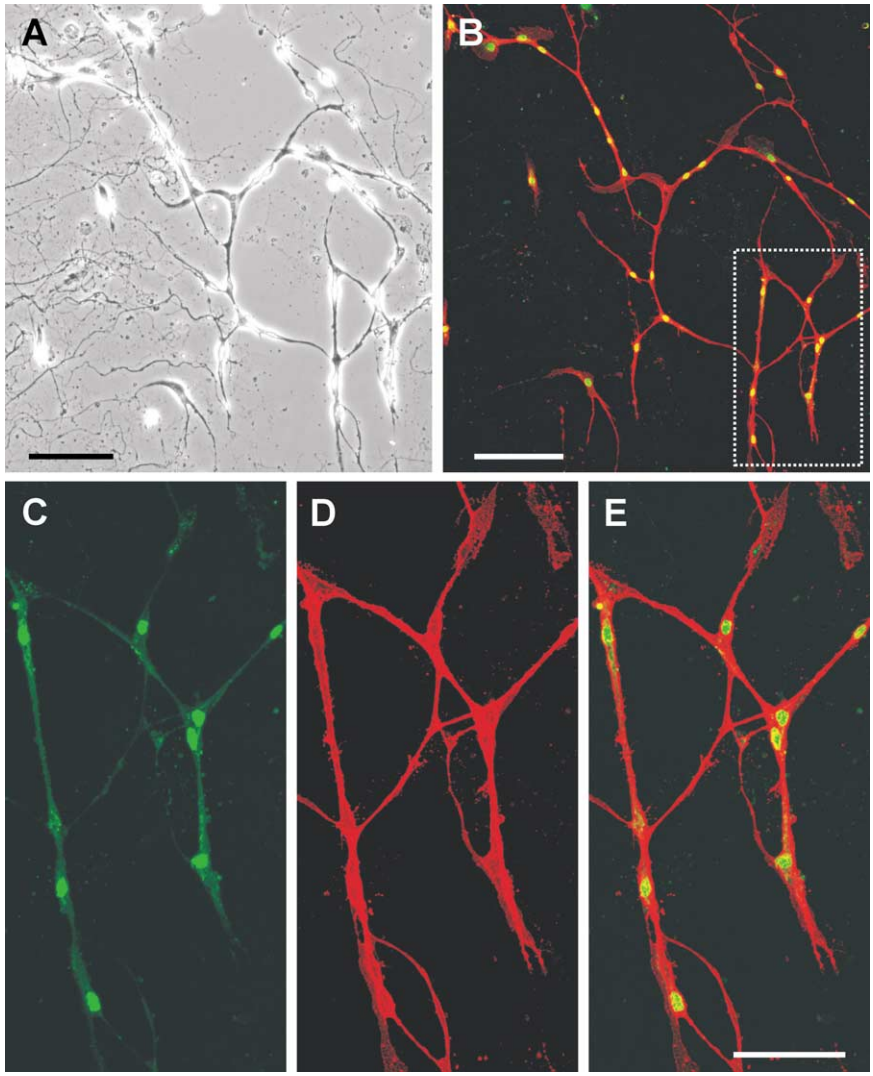


Fig. 8. AN glial arrays label with antibodies against the GPI-linked isoform of *Manduca* Fasciclin II (GPI-MFas II). (A) Phase-contrast image of an array at 96 h, prior to fixation. (B) AN glial cells in the array are GPI-MFas II-positive (red). Nuclei are counterstained (green). (C–E) High magnification view of boxed region in (B). (C) Glial nuclei are aligned. (D) GPI-MFas II-staining shows web-like appearance of arrays. (E) Merged image clearly shows that arrays consist of many glial cells. Scale bars: (A, B) 100 μm; (E) 50 μm.

the experiment presented in Fig. 9, neuropil-associated (Fig. 9A–D), sorting zone (Fig. 9E–H), and AN glia (Fig. 9I–L) were grown and examined separately, and the numbers of BrdU-positive neuropil-associated (Fig. 9C and D), sorting zone (Fig. 9G and H), and AN (Fig. 9K and L) glial nuclei were counted in each field and summed across similar dishes. Less than 2% of neuropil and sorting zone glia were BrdU-positive, while approximately 16% of glial nuclei in AN cocultures incorporated BrdU (Fig. 9M). Explants contained a few BrdU-positive nuclei (Fig. 9K and L, open arrows), presumably from nonneuronal cells, but these labeled cells were not counted. Interestingly, glial cells that normally incorporate into multicellular arrays when cultured with explants of olfactory epithelium, the AN glia, had a higher mitotic index in vitro than both sorting zone and neuropil-associated glia, which never form arrays.

Long-range diffusible factors fail to promote the formation of AN glial arrays

Formation of AN glial arrays depended on the presence of explants of olfactory epithelium. Axon contact was unlikely required for array formation, however, since AN glial cells formed arrays at locations devoid of ORN axons (Fig. 6E and F; Fig. 7B; unpublished observations). To test whether a soluble signal released from explants could influence the development of glial arrays, we used two-well culture dishes that allowed cells in the two wells to be in chemical communication with one another. This paradigm allowed us to test the effect of culturing AN glial cells alone (condition 1), culturing AN glial cells with medium conditioned by explants (condition 2), and culturing AN glial cells with medium conditioned by explants and AN glial

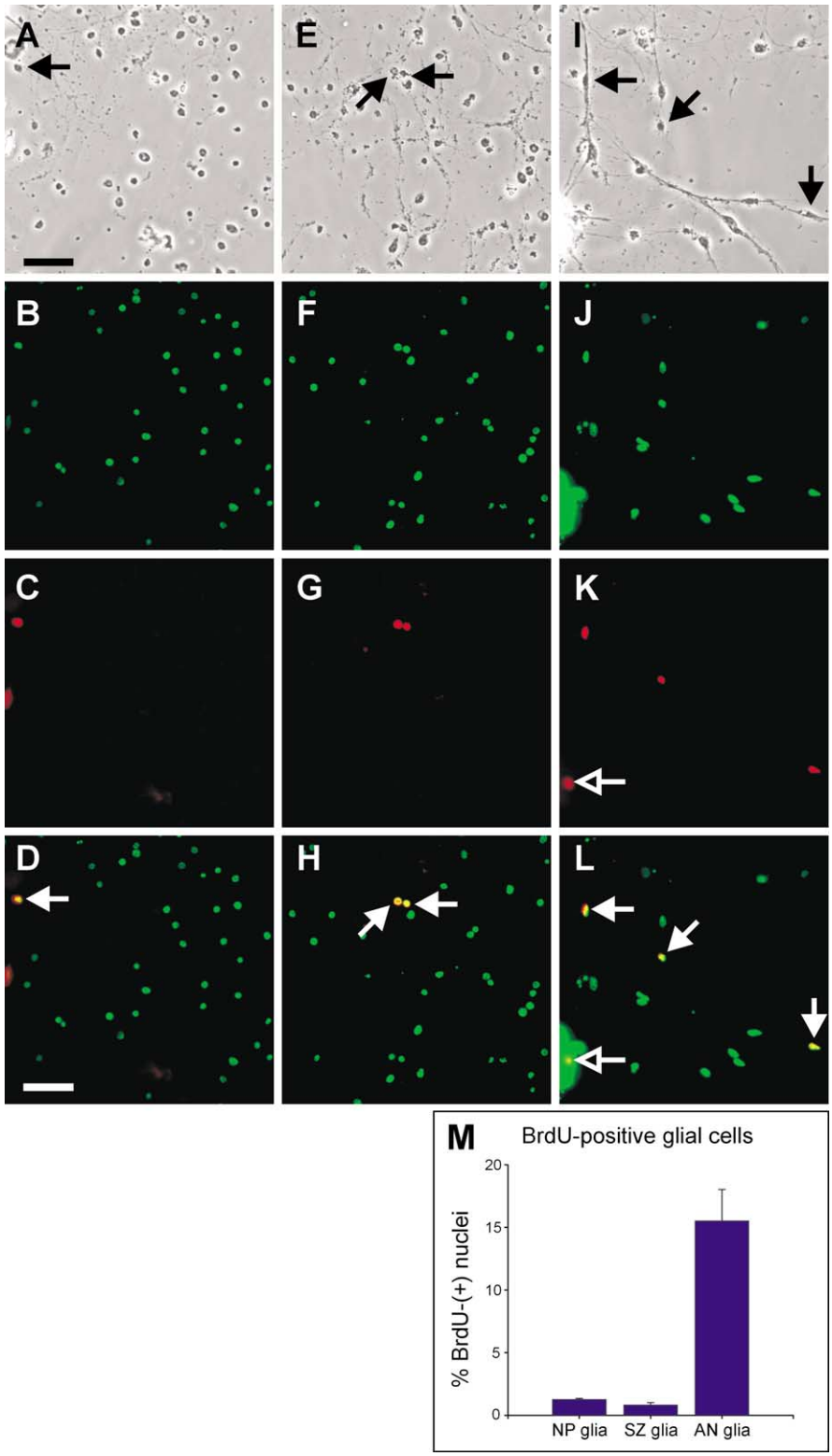


Fig. 9. AN glial cells are mitotically active in vitro. Three fields of view containing neuropil-associated (A–D), sorting zone (E–H), and AN (I–L) glia, after bromodeoxyuridine (BrdU) incubation and anti-BrdU immunocytochemistry. (A, E, I) Phase contrast images. (B, F, J) DAPI counterstained nuclei. (C, G, K) BrdU-positive nuclei. (D, H, L) Merged images; DAPI (green), BrdU (red). (M) The percentage of glial cells synthesizing DNA is highest in explant–glia cocultures containing AN glial cells. Arrows correspond to BrdU-positive glia; open arrows correspond to a BrdU-positive explant cell. Scale bars: (A) 50 μm, applies to (E and I); (D) 50 μm, applies to (B–L).

cells (condition 3). Condition three was included to test whether a diffusible signal that could influence the formation of AN glial arrays was generated in explant–glia cocultures. Photographs were taken at 24 and 72 h after glial addition. Arrays of AN glial cells did not form in well B of condition 1 (Fig. 10B), in well B of condition 2 (Fig. 10C), or in well B of condition 3 (Fig. 10D), suggesting that a long-range diffusible signal did not stimulate array construction. The small glial aggregates that were occasionally seen in the absence of explants were not present in any of the three conditions. In well A of condition 3, however, AN glial cells that were grown with explants of olfactory epithelium did form arrays (Fig. 10E), suggesting that AN glia were competent to form arrays, but required the local presence of explants to do so.

Since AN glial cells very rarely formed multicellular arrays when cultured alone, even at higher plating densities (approximately 2-fold greater), array formation presumably depended on their combined culture with explants of olfactory epithelium. Furthermore, unlike their vertebrate counterparts, insect neuroglia in general and moth olfactory glia in particular are not robust in primary culture and often require the presence of neurons to enhance their survival (Oland and Oberlander, 1994a; Oland et al., 1996). Over many years of studying moth glial cells in vitro, we have never observed array formation by other types of olfactory glia (Oland, personal communication). In fact, sorting zone and neuropil-associated glial cells did not incorporate into multicellular arrays, even when they were grown with explants of olfactory epithelium (Fig. 9; unpublished observations). Therefore, only AN glial cells formed arrays and although direct contact with ORN axons was not required, long-range soluble factors alone were insufficient to promote array formation.

Discussion

Axon–glia interactions underlie critical developmental events in the formation of the adult olfactory system in the moth *Manduca sexta.* In the present in vitro study, we characterized reciprocal interactions between ORN axons and glial cells isolated from the developing antennal nerve of *Manduca.* Contact with AN glia overwhelmingly led to continued axon elongation, without causing dramatic or long-lasting changes in growth cone complexity. In addition, growth cones preferentially extended along glial surfaces, indicating that AN glial cells provided a permissive substrate for axon elongation. AN glial cells were active during interactions with ORN axons; they often initiated encounters by extending processes to meet advancing growth cones. Furthermore, glial cells proliferated and actively organized into multicellular arrays on and near ORN axons. Arrays rarely developed when AN glia were grown alone, and those that did form were always considerably smaller than those that formed in explant–glia cocultures. Exposing AN glial cells to conditioned medium from explants or explant–glia cocultures failed to induce the formation of glial arrays, indicating that this glial behavior was dependent on short-range interactions with ORN axons. We hypothesize that AN glial cells provide permissive but not instructive cues for growing ORN axons in vivo, and in turn, that ORN axons stimulate changes in the behavior of AN glia that allow them to migrate and enwrap small bundles of ORN axons in the nerve.

ORN axons respond differently to sorting zone and neuropil-associated glial cells

In marked contrast to the findings reported here for AN glial cells, the glial cells that populate the sorting zone and antennal-lobe neuropil induce extensive contact-dependent elaborations in growth cone morphology and cause the cessation of ORN axon elongation (Tucker et al., 2000, 2001). Unlike AN glia, which arise in the periphery, sorting zone and neuropil-associated glia arise from precursors in the CNS and are present at key decision regions of the olfactory pathway during early stages of ORN axon ingrowth. We have shown that both have decisive influences on the behavior of ORN axons in vivo (Oland and Tolbert, 1988; Oland et al., 1988; Baumann et al., 1996; Rössler et al., 1999). In the glia-rich sorting zone, ORN axons lose associations with neighboring axons, abruptly change trajectories, and join with axons of like identity to form new fascicles that terminate in particular glomeruli (Oland et al., 1998; Rössler et al., 1999; Higgins et al., 2002). In the olfactory neuropil, axons make terminal branches that coalesce into protoglomeruli after passing through the shell of neuropil-associated glia (Oland et al., 1990, 1998). The dramatic differences seen in vitro in growth cone responses to AN glial cells versus sorting zone and neuropil-associated glial cells likely have functional consequences in vivo; AN glia may provide a permissive substrate for axon elongation, whereas sorting zone and neuropil-associated glia may provide cues necessary for axon sorting, targeting, and branching.

Potential roles for AN glia in vivo: glial influences on axon behavior

AN glia are born peripherally in the antennal epithelium, and migrate to populate the entire length of the antennal nerve distal to the sorting zone (Rössler et al., 1999). The AN glia first appear in the intracranial portion of the antennal nerve at stage 6, label with antibodies against the GPI-linked isoform of *Manduca* fasciclin II (GPI-MFas II), and have longitudinally oriented processes that extend to the distal margin of the sorting zone (Higgins et al., 2002). AN glia completely invest the intracranial portion of the antennal nerve by mid-stage 7, after most ORN axons have

reached the brain (Rössler et al., 1999). Since AN glia do not accompany early growing ORN axons to the brain, AN glia are unlikely to influence axon outgrowth or guidance during the initial establishment of the antennal pathway. The AN glia could, however, provide a permissive substrate for later growing ORN axons, which continue to arrive through stage 9 of adult development (Sanes and Hildebrand, 1976).

Our current in vitro findings suggest that growth cones readily traverse AN glial surfaces, without prolonged changes in growth cone morphology or dramatic alterations in the rates of axon elongation. Time-lapse movie sequences and differential labeling of fixed preparations demonstrated that ORN axons formed intimate associations with AN glial cells, an observation corroborated by electron microscopy (L.A. Oland, unpublished observations). We hypothesize that cell–cell and/or cell–matrix adhesion molecules mediate axon-glia interactions and allow ORN axons to elongate on glial processes in vitro.

Potential roles for AN glia in vivo: axon influences on glial behavior

AN glia are intimately associated with ORN axons in the mature antennal nerve, where glial processes enwrap fascicles that include 10–70 ORN axons (Sanes and Hildebrand, 1976). Results from the current and previous studies suggest that the development of this arrangement may require both physical and chemical communication between AN glial cells and ORN axons.

First, ORN axons likely provide a substrate for glial migration in vivo, as AN glia course down the antennal nerve along axons. We report in the current study that, before forming arrays, AN glial processes were often seen extending toward, adhering to, and growing along ORN axons. Intimate associations between ORN axons and AN glial cells were therefore formed mutually, with both axons and glial cells contributing to the response. We hypothesize that contact-dependent processes similar, if not identical, to those predicted to regulate axon extension on glial surfaces, underlie the association of AN glial processes with ORN axons.

Second, nitric oxide (NO) signaling appears to regulate glial migration in the developing primary olfactory system of *Manduca.* A calcium-dependent NO synthase (NOS) gene has been cloned in *Manduca*, and is strongly expressed in the antenna (Nighorn et al., 1998). The spatial location of NOS immunoreactivity in ORN axons changes during development, such that at stage 5, NOS-positive axons are restricted to the region of nerve adjacent to the rootlets, and at stage 7, NOS-positive axons are distributed throughout the entire width of the antennal nerve (Gibson and Nighorn, 2000). The distribution of glial cells in the antennal nerve also changes developmentally, and coincides spatially and temporally with the location of NOS-positive ORN axons (Gibson and Nighorn, 2000). Treatment of developing animals with agents that block NO signaling disrupts the migration of AN glia in the antennal nerve, suggesting that NO released from ORN axons could either directly or indirectly stimulate glial migration (Gibson et al., 2001).

In the current study, AN glial cells preferentially formed arrays in cultures containing explants of olfactory epithelium, but they often did so adjacent to, not directly on, ORN axons. This suggests that direct physical contact with axons is not necessary for array formation. Since potential long-range soluble factors presented through conditioned medium did not substitute for the absence of explants, the simplest hypothesis is that short-range secreted signals, either diffusible or substrate-bound, act to stimulate the formation of glial arrays in vitro. An intriguing possibility is that NO, which has a short half-life and thus a spatially discrete sphere of influence (Philippides et al., 2000), induces the formation of AN glial arrays after being released from NOS-positive ORN axons.

The axon-induced formation of multicellular glial arrays in vitro likely represents a 2-D reflection of more complex changes in glial behavior that normally occur in vivo. Array formation could represent an in vitro response to a signal that normally leads to glial elongation and migration along axons in vivo. In addition, AN glial association with and alignment near ORN axons could represent an in vitro behavior that reflects the process of glial enwrapment of axon fascicles in vivo.

Reciprocity in vertebrate neuron–glia interactions

The current study describes reciprocal interactions between cultured ORN axons and AN glial cells from *Manduca.* Such two-way interactions between cultured neurons and glia have been studied in considerable detail in vertebrates. In vitro, cellular behaviors including neurite elongation on glial cells and glial enwrapment of axons have been well characterized, and are dependent on the proper function of cell–cell and cell–matrix adhesion molecules. Across vertebrate species, function-blocking experiments demonstrate that the outgrowth of motor and sensory neurites on cultured Schwann cells is regulated by a variety of glycoproteins, including integrins and the homophilic adhesion molecules L1 and N-cadherin (Bixby et al., 1988; Kleitman et al., 1988; Seilheimer and Schachner, 1988; Letourneau et al., 1990). Adhesion molecules and integrins also regulate neurite elongation on CNS-derived astrocytes and Müller cells (Tomaselli et al., 1988; Neubauer et al., 1988; Drazba and Lemmon, 1990; Yazaki et al., 1996). Furthermore, L1 and N-cadherin act reciprocally to mediate axon-induced changes in Schwann cell behavior, including the linear alignment along, adhesion to, and enwrapment of individual axons (Seilheimer et al., 1989; Wood et al., 1990; Letourneau et al., 1991; Wanner and Wood, 2002). Hetero-

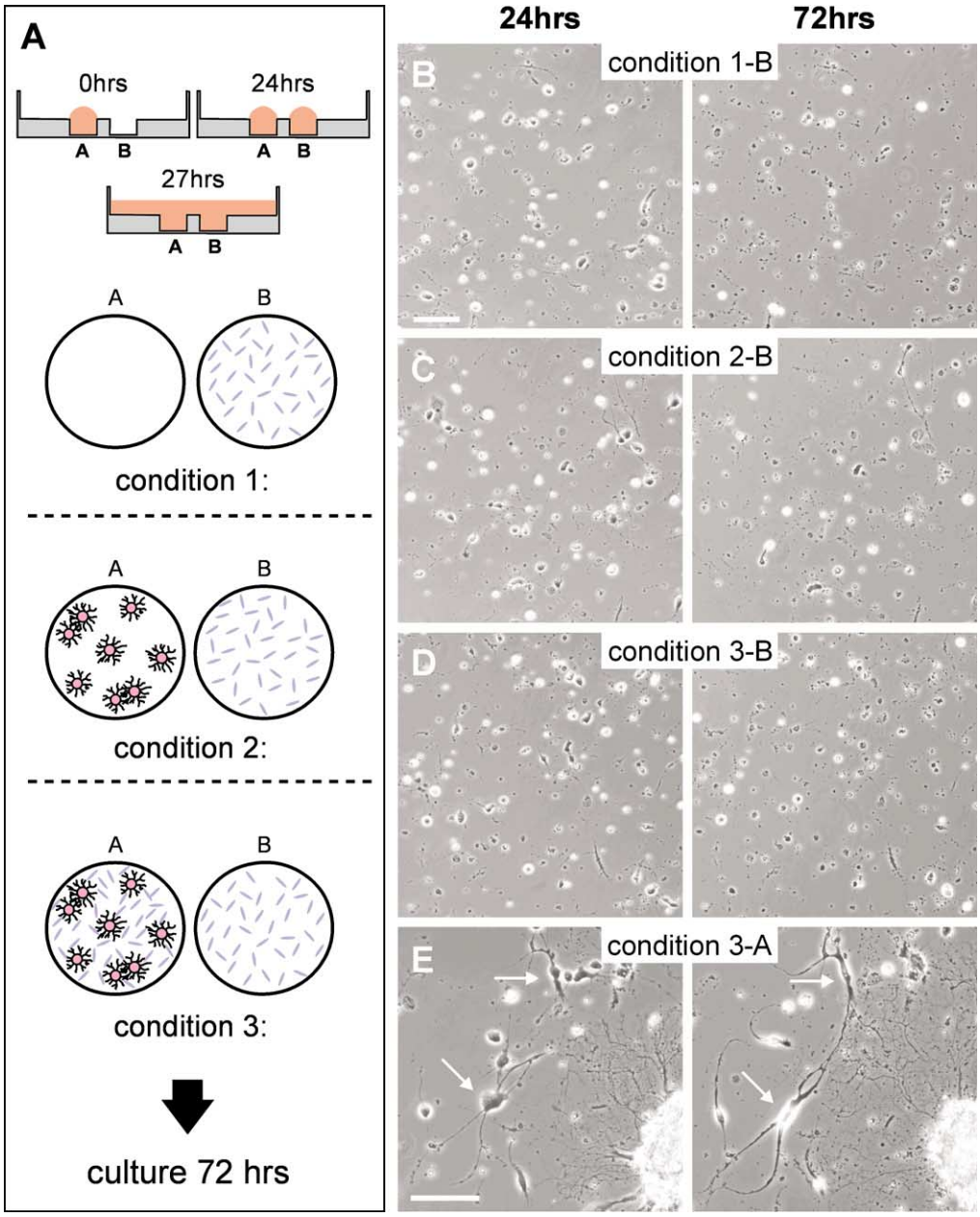


Fig. 10. Explant-conditioned medium does not stimulate array formation. (A) Diagram illustrating the arrangement of explants and glial cells within double-well dishes in each of three experimental conditions (see Materials and methods). (B) Phase contrast images of AN glial cells at 24 h (left) and 48 h (right) after flooding. Glial cells did not form arrays when the opposite well was left blank. (C) Phase-contrast images of AN glial cells plated opposite to wells containing explants. Glial cells did not form arrays after 24 or 48 h in chemical communication with explants. (D) Phase-contrast images of AN glial cells plated opposite to an explant–glia coculture. Explant–glia cocultures failed to induce physically separated AN glial cells to form arrays. (E) Glial cells formed arrays when plated in the same wells as explants. Arrows point to two glial cells linking to form an array. Scale bars: (B) 100 μm, applies to (C, D); (E) 100 μm.

philic adhesion molecules also mediate neuron–glia interactions and allow for the formation of contacts between DRG neurites and Schwann cells during the early stages of glial enwrapment (Suter et al., 1995). Like vertebrate neuron–glia interactions, adhesive interactions between moth ORN axons and AN glial cells likely promote bidirectional changes in cellular behavior that lead to axon extension on glial surfaces and glial process association with ORN axons.

Comparison of AN glia and ensheathing cells of the mammalian olfactory system

The arrangement of glia within the primary olfactory pathway of mammals has been studied in considerable detail, in part because the adult olfactory bulb retains the ability to support the continuous ingrowth of axons from the periphery. This ability has been attributed to a specialized

population of glia, the olfactory ensheathing cells, that display a blend of Schwann cell and astrocyte characteristics (Barber and Lindsay, 1982; Raisman, 1985; Doucette, 1990; Ramón-Cueto and Avila, 1998). AN glia and olfactory ensheathing cells have certain similarities and some key differences, highlighted by: (1) their anatomical locations within their respective olfactory pathways, (2) their roles in promoting neurite growth, and (3) their roles in axon guidance.

During development, olfactory ensheathing cells arise from the olfactory placode (Chuah and Au, 1991) and accompany the first ORN axons to the presumptive olfactory bulb (Marin-Padilla and Amieva, 1989; Doucette, 1989; Valverde et al., 1992). Olfactory ensheathing cells have been hypothesized to guide ORN axons to the brain since their migration from the olfactory epithelium is directed by a diffusible signal released from the olfactory bulb (Liu et al., 1995), and since ensheathing cell processes extend ahead of ORN growth cones during the pioneering of the olfactory pathway (Tennent and Chuah, 1996). AN glia also have a peripheral origin, but unlike olfactory ensheathing cells, AN glia migrate toward the CNS only after the first ORN axons have reached the brain and are therefore unlikely to influence the initial growth or guidance of ORN axons (Rössler et al., 1999). AN glia migrate to the base of the antennal nerve and stop short of CNS tissue at the sorting zone, whereas olfactory ensheathing cells migrate into the CNS, populate the olfactory nerve layer of the olfactory bulb, and intermingle with centrally derived astrocytes (Doucette, 1989). In the antennal and olfactory nerves, respectively, AN glia and olfactory ensheathing cells do not enwrap individual ORN axons, but in a similar fashion, they ensheath small axon bundles (De Lorenzo 1957; Sanes and Hildebrand, 1976; Barber and Lindsay, 1982).

Olfactory ensheathing cells express extracellular matrix components and adhesion molecules in vivo that likely promote the outgrowth of ORN axons (Doucette, 1990; Gong and Shipley, 1996; Treloar et al., 1996). In vitro, olfactory ensheathing cells enhance olfactory neurite outgrowth and provide a substrate that is conducive for the elongation of ORN neurites (Chuah and Au, 1991, 1994; Kafitz and Greer, 1998, 1999; Tisay and Key, 1999). In the present study of short-term interactions, ORN axons preferentially extended on and adjacent to the surfaces of AN glial cells. The overall outgrowth of ORN axons was not noticeably different in explant–glia cocultures, but we did not compare the lengths of axons with and without AN glia. In vivo and in vitro, olfactory ensheathing cells express growth factors that may enhance neurite elongation and provide trophic support for ORN axons during development and in normal adult-turnover (Key et al., 1996; Woodhall et al., 2001; Martin et al., 2002). Although it remains possible that AN glia display similar trophic interactions with ORN axons during development, ORN turnover is not likely to occur in *Manduca* due to its brief adult lifespan.

To date, the ability of ORN axons to influence the behavior of mammalian olfactory ensheathing cells has not been explored in detail. Although ensheathing cells retain the ability to enfold olfactory neurites in vitro (Ramón-Cueto et al., 1993), no reports have suggested that ORN axons induce ensheathing cells to proliferate and reorganize into large multicellular arrays. In fact, mammalian olfactory ensheathing cells divide slowly in vitro unless stimulated by exogenously applied mitogens (Chuah and Teague, 1999; Yan et al., 2001; Alexander et al., 2002). Recently, however, time-lapse observation of individual ensheathing cells revealed that they rapidly change shape and size to divide, show morphological plasticity by switching between flattened and fusiform morphologies, and either actively or passively aid in neuronal migration and axon elongation (Van Den Pol and Santarelli, 2003). Further studies examining interactions between ORN axons and olfactory ensheathing cells are needed to determine whether similarities exist between the in vitro behavior of mammalian ensheathing cells and AN glia.

In mice, olfactory ensheathing cell processes envelop fascicles of ORN axons in the olfactory nerve and outer olfactory nerve layer of the adult olfactory bulb (Au et al., 2002). Olfactory ensheathing cells express molecules known to influence the growth, sorting, and targeting of ORN axons as they extend from the olfactory nerve layer to the underlying glomerular layer of the olfactory bulb (Puche et al., 1996; St. John and Key, 1999; Tisay et al., 2000; Crandall et al., 2000; Schwarting et al., 2000; Gilbert et al., 2001). Ensheathing cells of a different molecular profile, coined olfactory ensheathing cell-like cells, loosely associate with ORN axons in the inner olfactory nerve layer, and may play a role in axon reorganization and targeting (Au et al., 2002). Instead of sorting in the nerve layer that circumscribes the olfactory neuropil, ORN axons in *Manduca* reorganize extensively within the sorting zone before reaching target glomeruli within the antennal lobe (Oland et al., 1998; Rössler et al., 1999). Since AN glia arrive late and do not enter the sorting zone, they are unlikely to mediate axon sorting or targeting. Instead, the centrally derived glia residing in the sorting zone could play functionally equivalent roles to the ensheathing cells that reside in the nerve layer of the olfactory bulb (Valverde, 1999; Key and St. John, 2002).

Summary

Glial cells play critical roles during the development of the adult moth olfactory system. The results of the current in vitro study suggest that reciprocal interactions between ORN axons and AN glial cells could influence axon–glia interrelationships in vivo. AN glia support the elongation of ORN axons in vitro, and, although they are not required for the initial growth or guidance of axons in vivo, AN glia may promote the growth of late-arriving ORN axons. In addition, ORN axons have the capacity to influence the behavior of

AN glial cells, by triggering the formation of multicellular arrays in vitro, and by triggering the migration of AN glia in vivo. The intimate relationships seen between ORN axons and AN glial cells in vitro likely reflect the mutual associations that are required to support glial migration, glial enwrapment of axon bundles, and axon elongation on glial processes in vivo. Future studies will more closely examine the interdependence of ORN axon and AN glial cell behavior in vivo, and probe the molecular bases of neuron-glia interactions in vitro.

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References

Alexander, C.L., Fitzgerald, U.F., Barnett, S.C., 2002. Identification of growth factors that promote long-term proliferation of olfactory ensheathing cells and modulate their antigenic phenotype. Glia 37, 349–364.

Au, W.W., Treloar, H.B., Greer, C.A., 2002. Sublaminar organization of the mouse olfactory bulb nerve layer. J. Comp. Neurol. 446, 68–80.

Auld, V., 1999. Glia as mediators of growth cone guidance: studies from insect nervous systems. Cell. Mol. Life Sci. 55, 1377–1385.

Barber, P.C., Lindsay, R.M., 1982. Schwann cells of the olfactory nerves contain glial fibrillary acidic protein and resemble astrocytes. Neuroscience 7, 3077–3090.

Bastiani, M.J., Harrelson, A.L., Snow, P.M., Goodman, C.S., 1987. Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. Cell 48, 745–755.

Baumann, P.M., Oland, L.A., Tolbert, L.P., 1996. Glial cells stabilize axonal protoglomeruli in the developing olfactory lobe of the moth *Manduca sexta*. J. Comp. Neurol. 373, 118–128.

Bixby, J.L., Lilien, J., Reichardt, L.F., 1988. Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro. J. Cell Biol. 107, 353–361.

Chuah, M.I., Au, C., 1991. Olfactory Schwann cells are derived from precursor cells in the olfactory epithelium. J. Neurosci. Res. 29, 172–180.

Chuah, M.I., Au, C., 1994. Olfactory cell cultures on ensheathing cell monolayers. Chem. Senses 19, 25–34.

Chuah, M.I., Teague, R., 1999. Basic fibroblast growth factor in the primary olfactory pathway: mitogenic effect on ensheathing cells. Neuroscience 88, 1043–1050.

Chuah, M.I., West, A.K., 2002. Cellular and molecular biology of ensheathing cells. Microsc. Res. Tech. 58, 216–227.

Crandall, J.E., Dibble, C., Butler, D., Pays, L., Ahmad, N., Kostek, C., Puschel, A.W., Schwarting, G.A., 2000. Patterning of olfactory sensory connections is mediated by extracellular matrix proteins in the nerve layer of the olfactory bulb. J. Neurobiol. 45, 195–206.

De Lorenzo, A.J., 1957. Electron-microscopic observations of the olfactory mucosa and the olfactory nerve. J. Biophys. Biochem. Cytol. 3, 839–863.

Doucette, R., 1989. Development of the nerve fiber layer in the olfactory bulb of mouse embryos. J. Comp. Neurol. 285, 514–527.

Doucette, R., 1990. Glial influences on axonal growth in the primary olfactory system. Glia 3, 433–449.

Drazba, J., Lemmon, V., 1990. The role of cell adhesion molecules in neurite outgrowth on Muller cells. Dev. Biol. 138, 82–93.

Dubuque, S.H., Schachtner, J., Nighorn, A.J., Menon, K.P., Zinn, K., Tolbert, L.P., 2001. Immunolocalization of synaptotagmin for the study of synapses in the developing antennal lobe of *Manduca sexta*. J. Comp. Neurol. 441, 277–287.

Fischer, R.A., 1925. Statistical Methods for Research Workers. Oliver and Boyd, Edinburgh.

Gao, Q., Yuan, B., Chess, A., 2000. Convergent projections of Drosophila olfactory neurons to specific glomeruli in the antennal lobe. Nat. Neurosci. 3, 780–785.

Gibson, N.J., Nighorn, A., 2000. Expression of nitric oxide synthase and soluble guanylyl cyclase in the developing olfactory system of *Manduca sexta*. J. Comp. Neurol. 422, 191–205.

Gibson, N.J., Rossler, W., Nighorn, A.J., Oland, L.A., Hildebrand, J.G., Tolbert, L.P., 2001. Neuron-glia communication via nitric oxide is essential in establishing antennal-lobe structure in *Manduca sexta*. Dev. Biol. 240, 326–339.

Gilbert, M., Smith, J., Roskams, A.J., Auld, V.J., 2001. Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. Glia 34, 151–164.

Gong, Q., Shipley, M.T., 1996. Expression of extracellular matrix molecules and cell surface molecules in the olfactory nerve pathway during early development. J. Comp. Neurol. 366, 1–14.

Grenningloh, G., Rehm, E.J., Goodman, C.S., 1991. Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. Cell 67, 45–57.

Harrelson, A.L., Goodman, C.S., 1988. Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. Science 242, 700–708.

Higgins, M.R., Gibson, N.J., Eckholdt, P.A., Nighorn, A., Copenhaver, P.F., Nardi, J., Tolbert, L.P., 2002. Different isoforms of fasciclin II are expressed by a subset of developing olfactory receptor neurons and by olfactory-nerve glial cells during formation of glomeruli in the moth *Manduca sexta*. Dev. Biol. 244, 134–154.

Jan, L.Y., Jan, Y.N., 1982. Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. Proc. Natl. Acad. Sci. USA 79, 2700–2704.

Kafitz, K.W., Greer, C.A., 1998. The influence of ensheathing cells on olfactory receptor cell neurite outgrowth in vitro. Ann. N. Y. Acad. Sci. 855, 266–269.

Kafitz, K.W., Greer, C.A., 1999. Olfactory ensheathing cells promote neurite extension from embryonic olfactory receptor cells in vitro. Glia 25, 99–110.

Key, B., Treloar, H.B., Wangerek, L., Ford, M.D., Nurcombe, V., 1996. Expression and localization of FGF-1 in the developing rat olfactory system. J. Comp. Neurol. 366, 197–206.

Key, B., St. John, J., 2002. Axon navigation in the mammalian primary olfactory pathway: where to next? Chem. Senses 27, 245–260.

Kirschenbaum, S.R., Higgins, M.R., Tveten, M., Tolbert, L.P., 1995. 20-Hydroxyecdysone stimulates proliferation of glial cells in the developing brain of the moth *Manduca sexta*. J. Neurobiol. 28, 234–247.

Kleitman, N., Simon, D.K., Schachner, M., Bunge, R.P., 1988. Growth of embryonic retinal neurites elicited by contact with Schwann cell surfaces is blocked by antibodies to L1. Exp. Neurol. 102, 298–306.

Lemke, G., 2001. Glial control of neuronal development. Annu. Rev. Neurosci. 24, 87–105.

Letourneau, P.C., Roche, F.K., Shattuck, T.A., Lemmon, V., Takeichi, M., 1991. Interactions of Schwann cells with neurites and with other Schwann cells involve the calcium-dependent adhesion molecule, N-cadherin. J. Neurobiol. 22, 707–720.

Letourneau, P.C., Shattuck, T.A., Roche, F.K., Takeichi, M., Lemmon, V., 1990. Nerve growth cone migration onto Schwann cells involves the calcium-dependent adhesion molecule, N-cadherin. Dev. Biol. 138, 430–442.

Liu, K.L., Chuah, M.I., Lee, K.K., 1995. Soluble factors from the olfactory bulb attract olfactory Schwann cells. J. Neurosci. 15, 990–1000.

Lohr, C., Tucker, E., Oland, L.A., Tolbert, L.P., 2002. Development of depolarization-induced calcium transients in insect glial cells is dependent on the presence of afferent axons. J. Neurobiol. 52, 85–98.

Lucas, P., Nagnan-Le Meillour, P., 1997. Primary culture of antennal cells of *Mamestra brassicae*: morphology of cell types and evidence for biosynthesis of pheromone-binding proteins in vitro. Cell Tissue Res. 289, 375–382.

Luedeman, R., Levine, R.B., 1996. Neurons and ecdysteroids promote the proliferation of myogenic cells cultured from the developing adult legs of *Manduca sexta*. Dev. Biol. 173, 51–68.

Marin-Padilla, M., Amieva, M.R., 1989. Early neurogenesis of the mouse olfactory nerve: Golgi and electron microscopic studies. J. Comp. Neurol. 288, 339–352.

Martin, L.V., Weston, S., West, A.K., Chuah, M.I., 2002. Nerve growth factor promotes olfactory axonal elongation. Neuroreport 13, 621–625.

Matheson, S.F., Levine, R.B., 1999. Steroid hormone enhancement of neurite outgrowth in identified insect motor neurons involves specific effects on growth cone form and function. J. Neurobiol. 38, 27–45.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., Axel, R., 1996. Visualizing an olfactory sensory map. Cell 87, 675–686.

Neugebauer, K.M., Tomaselli, K.J., Lilien, J., Reichardt, L.F., 1988. N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes in vitro. J. Cell Biol. 107, 1177–1187.

Nighorn, A., Gibson, N.J., Rivers, D.M., Hildebrand, J.G., Morton, D.B., 1998. The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*. J. Neurosci. 18, 7244–7255.

Oland, L.A., Muller, T., Kettenmann, H., Hayashi, J., 1996. Preparation of primary cultures and acute slices of the nervous system of the moth *Manduca sexta*. J. Neurosci. Methods 69, 103–112.

Oland, L.A., Oberlander, H., 1994a. Growth and interactions of cells from the insect nervous system in vitro, in: Maramorosch, K., McIntosh, A.H. (Eds.), Insect Cell Biotechnology, CRC Press, Boca Rotan, FL, pp. 107–125.

Oland, L.A., Oberlander, H., 1994b. Factors that influence the development of cultured neurons from the brain of the moth *Manduca sexta*. In Vitro Cell Dev. Biol. Anim. 30A, 709–716.

Oland, L.A., Orr, G., Tolbert, L.P., 1990. Construction of a protoglomerular template by olfactory axons initiates the formation of olfactory glomeruli in the insect brain. J. Neurosci. 10, 2096–2112.

Oland, L.A., Pott, W.M., Higgins, M.R., Tolbert, L.P., 1998. Targeted ingrowth and glial relationships of olfactory receptor axons in the primary olfactory pathway of an insect. J. Comp. Neurol. 398, 119–138.

Oland, L.A., Tolbert, L.P., 1988. Effects of hydroxyurea parallel the effects of radiation in developing olfactory glomeruli in insects. J. Comp. Neurol. 278, 377–387.

Oland, L.A., Tolbert, L.P., 1987. Glial patterns during early development of antennal lobes of *Manduca sexta*: a comparison between normal lobes and lobes deprived of antennal axons. J. Comp. Neurol. 255, 196–207.

Oland, L.A., Tolbert, L.P., 2003. Key interactions between neurons and glial cells during neural development in insects. Annu. Rev. Entomol. 48, 89–110.

Oland, L.A., Tolbert, L.P., 1989. Patterns of glial proliferation during formation of olfactory glomeruli in an insect. Glia 2, 10–24.

Oland, L.A., Tolbert, L.P., Mossman, K.L., 1988. Radiation-induced reduction of the glial population during development disrupts the formation of olfactory glomeruli in an insect. J. Neurosci. 8, 353–367.

Philippides, A., Husbands, P., O'Shea, M., 2000. Four-dimensional neuronal signaling by nitric oxide: a computational analysis. J. Neurosci. 20, 1199–1207.

Puche, A.C., Poirier, F., Hair, M., Bartlett, P.F., Key, B., 1996. Role of galectin-1 in the developing mouse olfactory system. Dev. Biol. 179, 274–287.

Raisman, G., 1985. Specialized neuroglial arrangement may explain the capacity of vomeronasal axons to reinnervate central neurons. Neuroscience 14, 237–254.

Ramón-Cueto, A., Perez, J., Nieto-Sampedro, M., 1993. In vitro enfolding of olfactory neurites by p75 positive ensheathing cells from adult rat olfactory bulbs. Eur. J. Neurosci. 5, 1172–1180.

Ramón-Cueto, A., Avila, J., 1998. Olfactory ensheathing glia: properties and function. Brain Res. Bull. 46, 175–187.

Ressler, K.J., Sullivan, S.L., Buck, L.B., 1994. Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell 79, 1245–1255.

Rössler, W.R., Oland, L.A., Higgins, M.R., Hildebrand, J.G., Tolbert, L.P., 1999. Development of a glia-rich axon-sorting zone in the olfactory pathway of the moth *Manduca sexta*. J. Neurosci. 19, 9865–9877.

Sanes, J.R., Hildebrand, J.G., 1976. Origin and morphogenesis of sensory neurons in an insect antenna. Dev. Biol. 51, 300–319.

Schwarting, G.A., Kostek, C., Ahmad, N., Dibble, C., Pays, L., Puschel, A.W., 2000. Semaphorin 3A is required for guidance of olfactory axons in mice. J. Neurosci. 20, 7691–7697.

Seilheimer, B., Persohn, E., Schachner, M., 1989. Antibodies to the L1 adhesion molecule inhibit Schwann cell ensheathment of neurons in vitro. J. Cell Biol. 109, 3095–3103.

Seilheimer, B., Schachner, M., 1988. Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neuron growth on Schwann cells in culture. J. Cell Biol. 107, 341–351.

St. John, J.A., Key, B., 1999. Expression of galectin-1 in the olfactory nerve pathway of rat. Brain Res. Dev. Brain Res. 117, 171–178.

Sun, B., Salvaterra, P.M., 1995. Characterization of nervana, a Drosophila melanogaster neuron-specific glycoprotein antigen recognized by anti-horseradish peroxidase antibodies. J. Neurochem. 65, 434–443.

Suter, D.M., Pollerberg, G.E., Buchstaller, A., Giger, R.J., Dreyer, W.J., Sonderegger, P., 1995. Binding between the neural cell adhesion molecules axonin-1 and Nr-CAM/Bravo is involved in neuron–glia interaction. J. Cell Biol. 131, 1067–1081.

Tennent, R., Chuah, M.I., 1996. Ultrastructural study of ensheathing cells in early development of olfactory axons. Brain Res. Dev. Brain Res. 95, 135–139.

Tisay, K.T., Bartlett, P.F., Key, B., 2000. Primary olfactory axons form ectopic glomeruli in mice lacking p75NTR. J. Comp. Neurol. 428, 656–670.

Tisay, K.T., Key, B., 1999. The extracellular matrix modulates olfactory neurite outgrowth on ensheathing cells. J. Neurosci. 19, 9890–9899.

Tolbert, L.P., Matsumoto, S.G., Hildebrand, J.G., 1983. Development of synapses in the antennal lobes of the moth *Manduca sexta* during metamorphosis. J. Neurosci. 3, 1158–1175.

Tomaselli, K.J., Neugebauer, K.M., Bixby, J.L., Lilien, J., Reichardt, L.F., 1988. N-cadherin and integrins: two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces. Neuron 1, 33–43.

Treloar, H.B., Nurcombe, V., Key, B., 1996. Expression of extracellular matrix molecules in the embryonic rat olfactory pathway. J. Neurobiol. 31, 41–55.

Tucker, E.S., Oland, L.A., Tolbert, L.P., 2000. In vitro study of interactions between olfactory receptor growth cones and glial cells of the axonal sorting zone. Soc. Neurosci. Abstr. 26, 1611.

Tucker, E.S., Oland, L.A., Tolbert, L.P., 2001. In vitro analysis of interactions between olfactory receptor growth cones and centrally derived glia. Assoc. Chemoreception Sci. Abstr. 23, 72.

Tucker, E.S., Oland, L.A., Tolbert, L.P., 2002. Growing olfactory receptor axons from *Manduca sexta* display different interactions with central and peripheral glia in vitro. Assoc. Chemoreception Sci. Abstr. 24, 7.

Valverde, F., 1999. Building an olfactory glomerulus. J. Comp. Neurol. 415, 419–422.

Valverde, F., Santacana, M., Heredia, M., 1992. Formation of an olfactory glomerulus: morphological aspects of development and organization. Neuroscience 49, 255–275.

Vassar, R., Chao, S.K., Sitcheran, R., Nunez, J.M., Vosshall, J.B., Axel, R., 1994. Topographic organization of sensory projections to the olfactory bulb. Cell 79, 981–991.

Vosshall, L.B., Wong, A.M., Axel, R., 2000. An olfactory sensory map in the fly brain. Cell 102, 147–159.

Wanner, I.B., Wood, P.M., 2002. N-cadherin mediates axon-aligned process growth and cell-cell interaction in rat Schwann cells. J. Neurosci. 22, 4066–4079.

Wood, P.M., Schachner, M., Bunge, R.P., 1990. Inhibition of Schwann cell myelination in vitro by antibody to the L1 adhesion molecule. J. Neurosci. 10, 3635–3645.

Woodhall, E., West, A.K., Chuah, M.I., 2001. Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. Brain Res. Mol. Brain Res. 88, 203–213.

Wright, J.W., Copenhaver, P.F., 2000. Different isoforms of fasciclin II play distinct roles in the guidance of neuronal migration during insect embryogenesis. Dev. Biol. 225, 59–78.

Yan, H., Bunge, M.B., Wood, P.M., Plant, G.W., 2001. Mitogenic response of adult rat olfactory ensheathing glia to four growth factors. Glia 33, 334–342.

Yazaki, T., Martuza, R.L., Rabkin, S.D., 1996. Expression of L1 in primary astrocytes via a defective herpes simplex virus vector promotes neurite outgrowth and neural cell migration. Brain Res. Mol. Brain Res. 43, 311–320.