The Journal of Neuroscience, February 1987, 7(2): 512–521

Immunological Probes Reveal Spatial and Developmental Diversity in Insect Neuroglia

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A set of monoclonal antibodies (MAbs) has been generated that recognizes distinct classes of neuroglia in the adult nervous system of the cricket *Acheta domesticus* corresponding to glial types distinguished by morphological criteria. These include antibodies that bind to the neuroglia of the ganglionic cortex, perineurium, neuropil, and glia associated with the glial lacunar system (interface) and fiber tracts. Another MAb specifically labels components of the neural lamella, a complex extracellular matrix secreted by underlying perineurial cells.

Selected adult glial-specific MAbs recognize particular glial antigens expressed during embryonic development of *Acheta*. Immunohistochemical staining of frozen sections of late- (90–95%) and intermediate- (50–55%) stage whole embryos reveals that the spatial distribution, degree of tissue restriction, or level of expression of some glial determinants changes as development proceeds. Labeling of certain neuroblasts in the embryonic CNS at 50–55% development by an antibody (MAb 3G6) that binds to neuropil glia in the adult CNS implies that at least 1 class of insect glia may be generated by these cells.

Glial cells regulate physiological and biochemical processes in the nervous system (Watson, 1974; Stewart and Rosenberg, 1979; Lane and Treherne, 1980; Pentreath and Kai-Kai, 1982) and are implicated in the guidance of neuron outgrowth during development and regeneration (Aguayo et al., 1981; Rakic, 1981; Silver et al., 1982; Noble et al., 1984). Recent advances in immunological methods have led to new insights about vertebrate glial cell differentiation and their interactions with neurons (Bignami and Dahl, 1974; Ranscht et al., 1982; Raff et al., 1983; Fedoroff, 1985), but the basic properties of glia in the nervous system of insects and other invertebrates remain poorly understood. An immunological approach is therefore one strategy by which highly specific probes can be generated that can serve to characterize insect glia, as has been done with vertebrates.

We report here the production of a set of monoclonal antibodies (MAbs) specifically directed against glial determinants in the adult nervous system of the cricket *Acheta domesticus*. We demonstrate that several antigenically distinct glial cell populations exist within the CNS and that some glial antigens are differentially expressed during development.

Received Mar. 18, 1986; revised June 23, 1986; accepted July 30, 1986.

We thank J. Jinneman, S. Kempf, B. Masinovsky, P. Copenhaver, and P. Brunner for their assistance and comments. This work was supported by NIH Grant NB-07778 and a Government of India Postdoctoral Fellowship (to G.R.R.).

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Materials and Methods

Immunization and production of hybridomas. To generate our initial group of MAbs, isolated mouse splenocytes were immunized *in vitro* (Reading, 1982) by incubation with a crude membrane homogenate derived from pooled adult female cricket terminal abdominal ganglia (TG) that had been fixed in buffered 4% paraformaldehyde for 2 hr at 4°C. Fixed ganglia were rinsed, homogenized on ice in a glass-glass microhomogenizer, and centrifuged at 12,000 × *g*. After 2 additional washes by centrifugation, the resulting pellet was resuspended in cell culture medium containing 0.1% gentamycin to a final concentration of ca. 2 mg/ml TG protein and preincubated for 4 hr prior to presentation of the immunogen to the isolated spleen cells. Following immunization of cultured splenocytes for 6 d, the stimulated blast cell population was harvested by separation on a sterile Ficoll-Paque (Pharmacia) gradient, and these cells were then fused with NS-1 myeloma cells by established methods (Oi and Herzenberg, 1980).

A second set of MAbs was generated directly by immunization of BALB/c mice with an immunogen enriched for adult glial cell membranes derived from pooled deafferented cercal sensory nerve stumps. Denervated stumps were harvested from animals whose cercal appendages had been removed 15 d prior to preparation, a period judged sufficient to allow the vast majority of nerve fibers in the cercal nerve to degenerate (Meyer et al., 1986). The immunization regimen consisted of 1 primary injection of 100 μg glial enriched membranes in complete adjuvant, followed by 2 successive rounds of booster immunizations (with incomplete adjuvant) at 2 week intervals.

Hybridoma cells were cultured in growth-selective medium and raised in microwells containing irradiated feeder splenocytes. Spent medium was collected from individual wells and was tested for glial-selective immunoreactivity in a series of screening procedures of increasing stringency. These included solid-phase immunoassays (Engvall and Perlmann, 1972) for antibody secretion and TG immunoreactivity and successive rounds of immunocytochemical staining of frozen sections of TG and non-neuronal tissues. Selected hybridoma lines secreting MAbs were then cloned by limiting dilution and expanded.

Immunocytochemical staining. Isolated TG, cricket tissues (e.g., skeletal muscle, gut, Malpighian tubules), and whole embryos were fixed for 2 hr at room temperature or overnight at 4°C in buffered 4% paraformaldehyde and rinsed in ice-cold cricket physiological saline (Levine, 1966). Tissues were then embedded in O.C.T. medium (Tissue-Tek) and rapidly frozen in liquid nitrogen-cooled isopentane. Cryostat sections of 6 μm thickness were collected on gelatin-coated glass slides, rinsed for 30 min in ice-cold PBS (NaH_2PO_4, 5 mM; Na_2HPO_4, 15 mM; NaCl, 150 mM, pH 7.5) and incubated for 90–120 min at room temperature in PBS containing 1% BSA, 5% heat-inactivated newborn goat serum, and 0.1% sodium azide (blocker/carrier buffer). Sections were then incubated overnight in a humidified chamber at 4°C with selected MAbs diluted 1:1 with blocker/carrier buffer or with rabbit anti-HRP (Cooper Biomedical) diluted 1:500. Following 60 min rinsing in ice-cold PBS, sections were incubated 120 min at room temperature with FITC-conjugated goat anti-mouse IgG + IgM (Cooper Biomedical) diluted 1:50 with blocker/carrier buffer or, for anti-HRP labeling, with FITC-conjugated goat anti-rabbit IgG (Cooper) diluted 1:100. Following a final 60 min rinse in PBS, slides were mounted in a 80% glycerol-based 0.05 M Tris-buffered medium containing 4% *n*-propyl gallate to

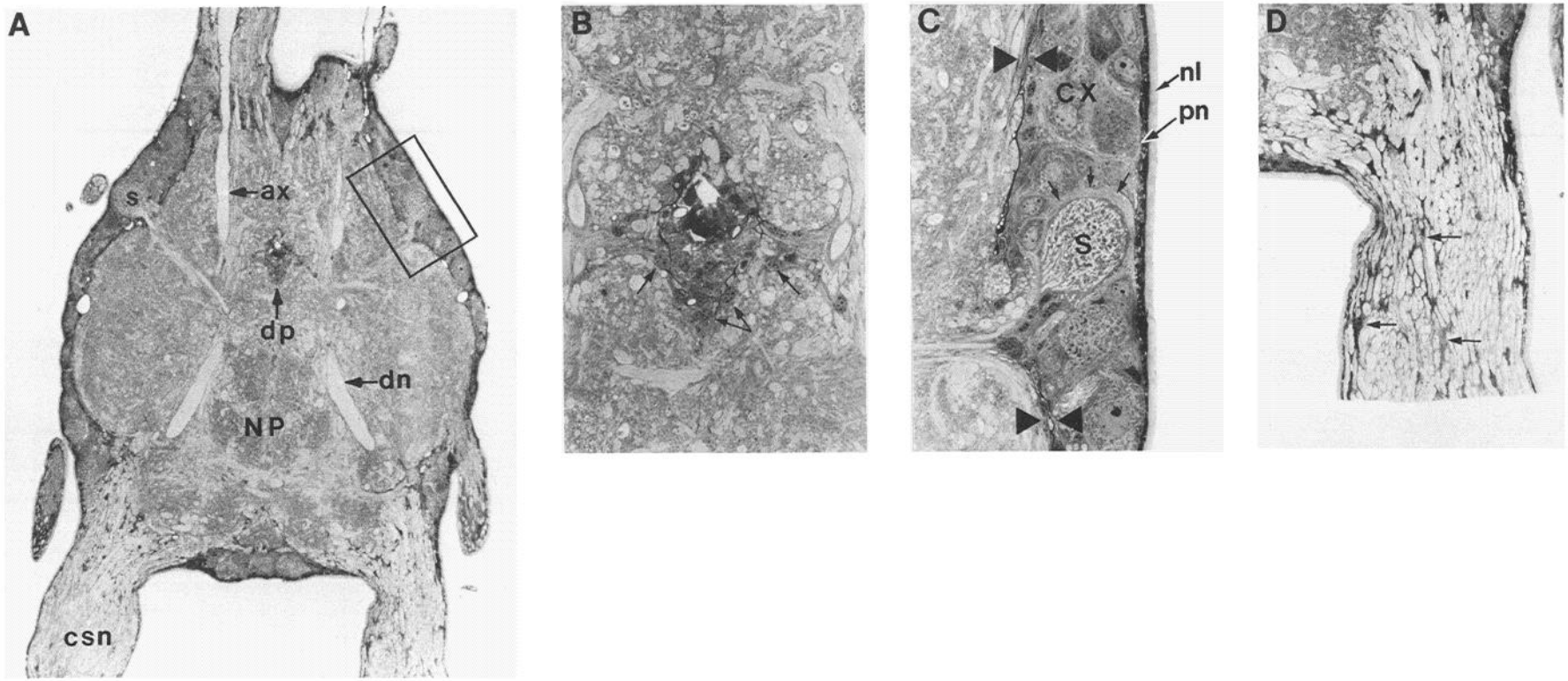

Figure 1. Anatomical landmarks within the adult terminal abdominal ganglion. Horizontal 1 μm resin-embedded section, stained conventionally with methylene blue–azure II. *A,* Cortical rind and neuropil (*NP*) are prominent domains apparent within the entire TG. Neuronal somata (*S*) are contained within the cortex. Within neuropil, large giant interneuron dendritic processes (*dn*) are prominent, as is the dorsal pit (*dp*), a structure invested with multiple wrappings of glial cells. Large cercal sensory nerves (*csn*) enter the posteriolateral regions of the TG. *B,* Enlargement of the dorsal pit region. Glial investiture stains differentially from neural tissue in the surrounding regions of neuropil. Glial cell bodies (*arrows*) lie within the dorsal pit complex. *C,* Enlargement of enclosed area shown in *A.* Cortex (*CX*) contains neuronal somata (*S*) and intimately surrounding glial wrappings (*small arrows*). Dark staining layer of the perineurium (*pn*) is apparent at outer margins of the cortex. Perineurial glial cells secrete the neural lamella (*nl*), an extracellular matrix that ensheathes the entire nervous system. Highly specialized glial lacunar system (*between arrowheads*) is located at inner margins of the cortex (i.e., the cortex–neuropil interface). *D,* Enlargement of cercal sensory nerve entering posterior of the TG. Massive parallel fiber projections are interspersed with elongated glial cell profiles (*arrows*). Magnifications: *A,* ×95; *B–D,* ×350.

Table 1.[a]

MAb designation	Glial staining selectivity	DP	PN	Glial specificity[b]	Embryonic expression[c]
1D7 (CX 1)	Cortical glia	+	±	−	+
1E7 (CX 2)	Cortical glia	+	−	+	+
2C9	General (cortex ≫ neuropil)	+	±	−	N.D.
3F6 (PN 1)	Perineurial glia	±	−	+	−
3G6 (NP 1)	Neuropil glia	−	+	+	+
5B12 (IF 1)	Interface and tract glia	+	+	+	+
5F11	General (cortex ⋙ neuropil)	+	+	−	N.D.
6D7 (CX 3)	Cortical glia	+	−	±	+
7B1 (NL 1)	Neural lamella	−	+	+	+
7C12	General (cortex > neuropil)	+	−	−	N.D.
8A4	General (cortex > neuropil)	+	+	−	N.D.
8B11 (NP 2)	Neuropil glia	−	+	+	N.D.

[a] DP, labeling of dorsal pit; PN, labeling of peripheral (i.e., cercal sensory) nerve; N.D., not done.

[b] No apparent binding of MAb to frozen sections of adult gut, flight muscle, Malpighian tubules, or to identifiable neuronal elements (e.g., somata, neurites) in frozen sections of adult terminal abdominal ganglia.

[c] Binding of MAb to nervous system or other tissues in mid-stage (50–55%) or late-stage (90–95%) embryos.

inhibit quenching. Sections were viewed and photographed with a Leitz Dialux microscope equipped with epifluorescence optics.

Histology and immunoperoxidase labeling. Selected frozen sections of whole late- and intermediate-stage embryos were stained for conventional microscopy with methylene blue–basic fuschin. Adult TG were fixed for 1 hr in buffered 2% paraformaldehyde–0.1% glutaraldehyde, dehydrated, and vacuum-embedded in LR-White resin (Polysciences, Inc.). Horizontal sections 1 μm thick were stained with methylene blue–azure II for photomicroscopy. Additional fixed, unstained 1 μm sections were prepared for immunocytochemistry essentially as described above for frozen-sectioned material but were instead labeled with affinity-purified HRP-conjugated goat anti-mouse secondary antibody (Cooper) diluted 1:50 and developed with the H_2O_2–3,3′-diaminobenzidine substrate reaction. Sections were rinsed, mounted, and photographed with a Zeiss Universal photomicroscope equipped with phase-contrast optics.

Results

Labeling of glial tissues in the adult nervous system

The adult terminal ganglion of *Acheta* contains clearly delineated neuronal and glial domains and identifiable neuroanatomical landmarks (Figs. 1, 2*A*), which enabled us to analyze the patterns and distribution of antibody binding. Based on the results obtained from extensive immunohistochemical staining of frozen sections of TG and non-neuronal tissues, we were able to select 12 hybridoma lines (Table 1) that secreted antibodies with distinct reactivity to glial tissues located within well-defined regions of the nervous system, some of which are enriched for particular types of glia (Fig. 1, *B–D*). Of these MAbs, 5–6 appeared to be glial cell-specific in that no appreciable cross-reactivity with other adult tissues (e.g., skeletal muscle, gut, Malpighian tubules) was detected. Within this subset of MAbs are those that selectively label the neuroglia of the ganglionic cortex, perineurium, neuropil, and glial cells confined to the cortex–neuropil interface and nerve tracts.

Glial cells within the ganglionic cortex are highly immunogenic, in that at least 6 MAbs bind preferentially to glia in this domain (Table 1). Several of these antibodies (MAbs 1D7, 1E7, 6D7) bind exclusively to cortical glia. Comparison of the TG labeling patterns obtained with these MAbs with the distribution of TG anti-HRP immunoreactivity (Fig. 2*A*), which is associated almost exclusively with an antigen expressed on neuronal elements (Jan and Jan, 1982) permits neuronal and non-neuronal (e.g., glial) tissues in the highly complex cortical region to be distinguished from one another. For instance, MAb 6D7 (Fig. 2*B*) labels neither neuronal perikarya within the cortex nor centrally projected neurites; neuropil elements are most notably devoid of reactivity, with the exception of the dorsal pit, a sharply demarcated central landmark between the 7th and 8th ganglionic segments that is heavily invested with layers of cortical rind glial cells (Fig. 1*B*) and that clearly does not label with the neural antigen marker anti-HRP (Fig. 2*A*). These MAbs are thus highly selective for glia restricted to ganglion cortex and dorsal pit, generally conforming to the *type 3* glia described by Wigglesworth (1959). Detailed analysis of the localization and cellular distribution of cortical glia-associated antigens by individual MAb probes is precluded in this study by the resolution limits inherent in frozen-sectioned tissues. MAbs 1D7, 1E7, and 6D7, however, all appear to bind to the majority of non-neuronal tissues that reside within the cortical rind, including the thin layer of perineurial glia (Fig. 1*C*) located at the outer margins of the cortex. In addition, all 3 MAbs bind similarly in the extent and intensity of immunoreactivity to the layers of glial cells investing the dorsal pit. Marked labeling of the glial lacunar system, the highly specialized region at the cortex–neuropil interface (Fig. 1*C*, and see below), is not apparent. Cortical-like glia residing in the margins of the peripheral nerves, however, appear to be differentially labeled by MAbs 1D7, 1E7, and 6D7 (Table 1), which suggests that these MAbs may recognize distinct antigens which differ in their relative abundance in the PNS.

The insect CNS is enveloped by a discrete layer of specialized cells, the perineurium, which constitutes the insect blood–brain barrier (Scharrer, 1939; Lane, 1981). Although the perineurium is incorporated within the cortical rind and is in close apposition with other glial cells and extracellular components (Fig. 1*C*), MAb 3F6 recognizes a determinant that appears to be unique to perineurial cells (Fig. 2*C*). The staining pattern seen with 3F6 appears much more localized than with the MAbs that distin-

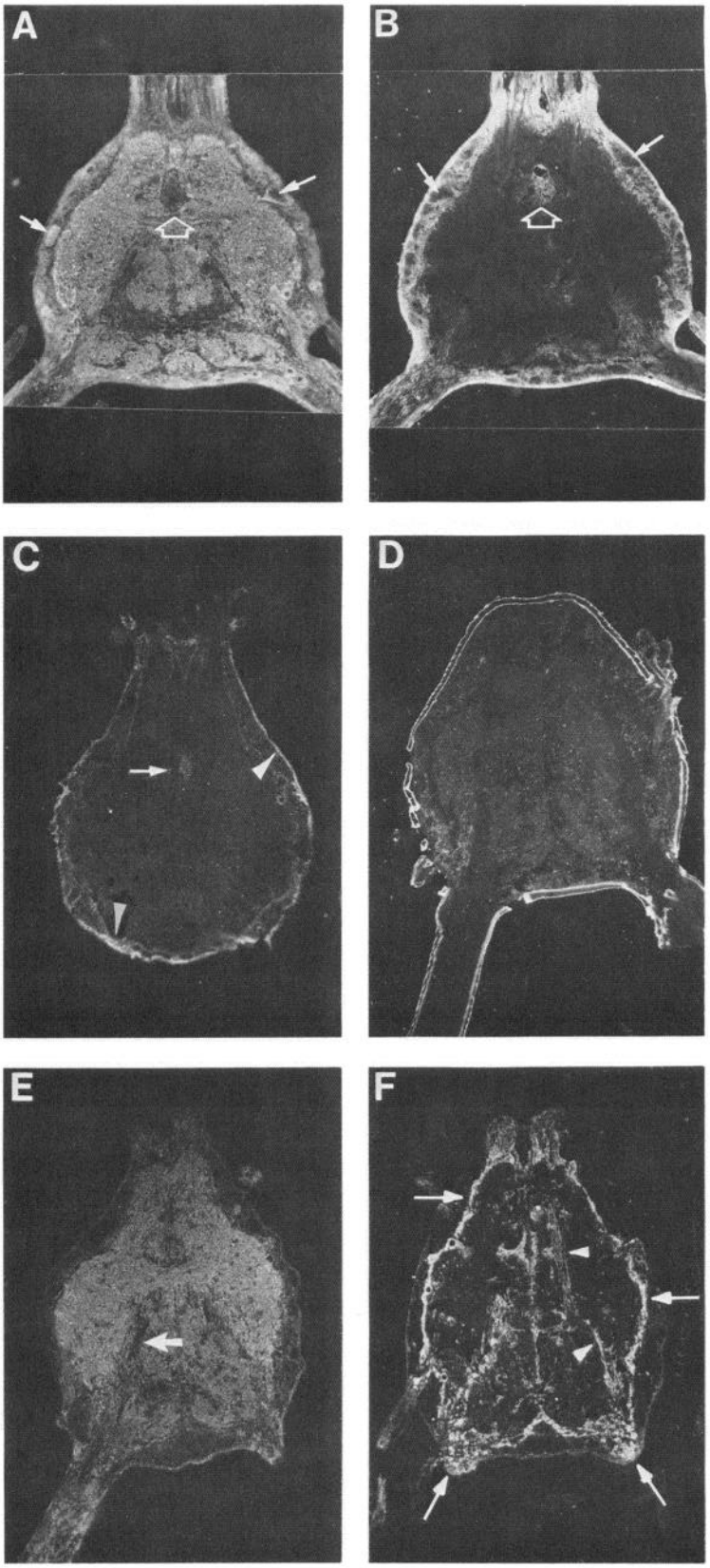

Figure 2. Immunofluorescent staining of frozen sections of adult terminal abdominal ganglia (TG) with anti-HRP antibody (*A*) and glial-selective monoclonal antibodies (MAbs) (*B–F*). *A,* Anti-HRP labels antigens present on neural elements in adult TG. Neuronal somata (*arrows*) in cortex are prominently labeled, as are neural elements within the neuropil. Note that dorsal pit (*large arrow*), a primarily glial structure located within immunoreactive synaptic neuropil, is not labeled by anti-HRP. *B,* TG labeled with cortical glia-specific MAb 6D7. Glia of cortical rind and dorsal pit (*large arrow*) are selectively labeled; neuronal somata (*small arrows*) in cortex are unlabeled, as are neuronal and glial components of the neuropil. *C,* MAb 3F6 binds specifically to glia comprising the perineurium (*arrowheads*) at outer boundary of cortex; glia of dorsal pit (*arrow*) are labeled to a lesser degree. *D,* Inner and outer surfaces of neural lamella delimiting the TG perimeter are discretely labeled by MAb 7B1. Components of the lamella are synthesized and secreted by underlying glia of the perineurium. *E,* MAb 3G6 recognizes antigen(s) expressed by presumptive neuropil glia. Neither neuronal nor glial elements within the cortical rind are labeled, nor is giant interneuron primary dendrite (*arrow*) within the neuropil. Peripheral sensory nerve entering TG at *bottom left* displays patchy pattern of immunoreactivity. *F,* Glia comprising glial lacunar system of cortex–neuropil interface and those associated with fiber tracts of large peripheral nerves entering the TG (*arrows*) are labeled by MAb 5B12. Fiber tracts within central neuropil are also labeled (*arrowheads*), as are the glia that surround circular tracheolar profiles in the cortex–neuropil interface. Identifiable neuronal elements within cortex and neuropil are unlabeled. ×125 for all.

guish cortical rind glia; labeling is confined to a thin band of cells lining the cortical perimeter, corresponding precisely with the disposition of the perineurium. The neural lamella, a complex extracellular layer that ensheathes the entire nervous system (Fig. 1*C*), is composed of a structured array of macromolecules, including collagens, synthesized and secreted by underlying perineurial cells (Scharrer, 1939; Ashhurst, 1982). The highly selective MAb 7B1 binds to and sharply delineates a distinct

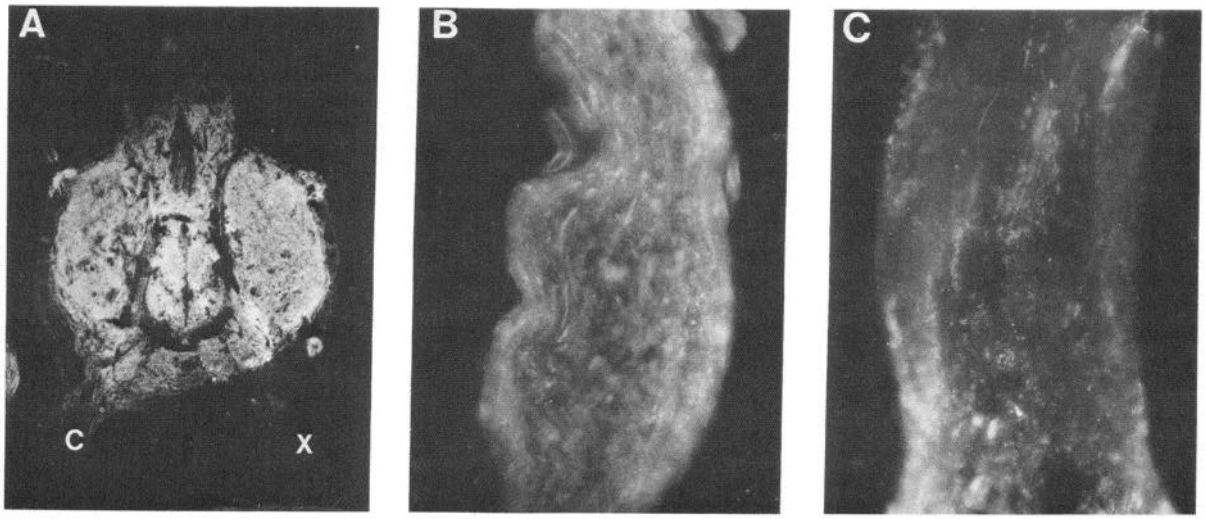

Figure 3. Immunoreactivity to MAb 3G6 is not diminished following cercal sensory deafferentation. *A,* Extent and intensity of MAb 3G6 labeling distribution in control (*C, left*) and deafferented (*X, right*) TG synaptic neuropil appears similar at 11 d following removal of right cercus. Slight displacement or loss of volume is apparent in deafferented neuropil, a consequence of the absence of substantial number of sensory axon terminals. Morphological evidence of degeneration of cercal sensory axon terminals in target neuropil has been shown to be present within less than 12 hr (Meyer et al., 1986); within 1 week, the vast majority of terminals will have degenerated. Comparison of 3G6 immunoreactivity in 15 d deafferented (*B*) and paired control (*C*) cercal sensory nerve demonstrates that 3G6 antigenicity remains in the denervated nerve stump, despite loss of majority of nerve fibers by this time. The paired nerves were photographed and printed under identical conditions, with equivalent exposure times. Magnifications: *A,* ×125; *B* and *C,* ×600.

bilayer substructure of the lamella (Fig. 2*D*). The antigen recognized by MAb 7B1 is exclusively restricted to the neural lamella in the adult animal; the 7B1 antigen is never detected within the nervous system, nor is it found in association with extracellular products that compose non-neuronal tissues in the adult cricket (e.g., connective tissues of skeletal muscle or the gut). The distribution of the 7B1 antigen on the neural lamella has no known structural correlates, based upon available ultrastructural data (Ashhurst, 1982; J. S. Edwards, unpublished observations).

The densely packed neuronal elements within ganglionic neuropil are invested by an elaborate and heterogeneous complement of glial cells (Strausfeld, 1976). Small glial perikarya are located at the inner cortical rind margin, from which delicate cytoplasmic processes project into neuropil and ramify. Two MAbs (3G6 and 8B11) label neuropil in a pattern that suggests that they bind only to these glial elements within the CNS (Fig. 2*E*). To a lesser degree than in neuropil, these antibodies also bind to portions of peripheral nerve pathways (e.g., cercal sensory nerves). Neither of these MAbs reacts with neuronal components of the TG cortex (3G6 does, however, show slight cross-reactivity with perineurium). In addition, MAb 3G6, which is highly specific and does not bind detectably to any tissues outside the nervous system, does not label identifiable giant interneuron dendrites, axon tracts, or commissures in TG neuropil. Although analysis of frozen-sectioned TG tissue precludes certain localization of antibody binding to these complex glia, we find that the binding of 3G6 to neuropil (Fig. 3*A*) and cercal sensory nerve trunks (Fig. 3, *B, C*) is not diminished after a majority of sensory axons and terminals have degenerated following cercal deafferentation (Meyer et al., 1986). These results indicate that MAb 3G6 labels a glial, rather than a neuronal,

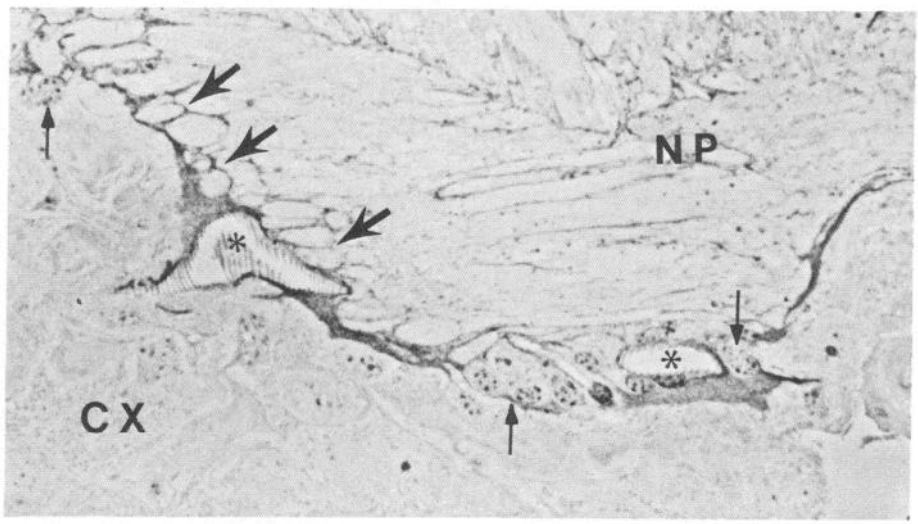

Figure 4. Immunoperoxidase labeling of cortex–neuropil interface region with MAb 5B12. Adult TG was embedded in LR-White resin and 1 μm horizontal sections were labeled with MAb 5B12 and peroxidase-conjugated secondary antibody as described in Materials and Methods. Substrate reaction product is sharply confined to the region of the cortex–neuropil interface (i.e., the glial lacunar system). Neuronal and glial elements of the cortex (*CX*) and neuropil (*NP*) are unlabeled. Labeling appears extensive in some regions where glial nuclei (*small arrows*) are abundant but also coincides with elongated projections, in association with axon profiles (*large arrows*) and tracheae (*). ×625.

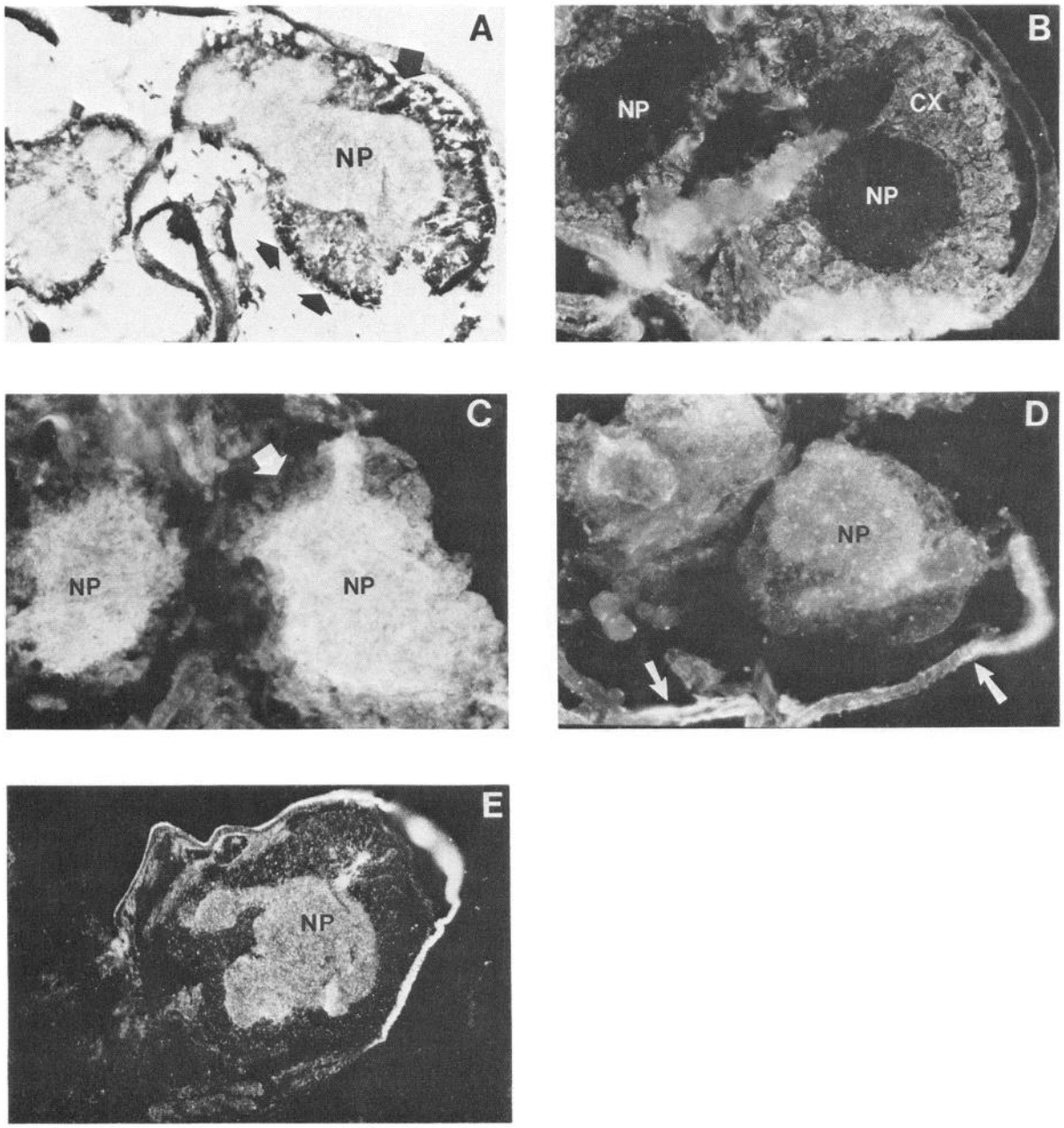

Figure 5. Immunofluorescent staining of frozen sections of late-stage (95%) *Acheta* embryos with glial-selective MAbs. *A,* Head region of late-stage (ca. 95%) embryo stained with methylene blue–basic fuschin to delineate major regions of embryonic brain. Well-developed neuropil (*NP*) is surrounded with neuronal somata and associated non-neuronal (e.g., glial) elements that compose the embryonic cortical rind (*arrows*). *B,* Embryonic brain labeled with cortical glia-selective MAb 1E7. Antibody binds strongly to cortical (*CX*) region of brain, but neuropil (*NP*) is unlabeled. *C,* MAb 3G6, which labels presumptive neuropil glia in the adult TG (Fig. 2*E*), stains corresponding regions (*NP*) in the embryonic brain at ca. 95% development. Surrounding cortical region (*arrow*) is not labeled. *D,* Late-stage embryo labeled with MAb 7B1, which, in the adult nervous system, binds only to discrete components of the neural lamella (Fig. 2*D*). Note labeling of embryonic epidermis (*arrows*), and most significantly, the recognition by 7B1 of determinants expressed in embryonic neuropil (*NP*). *E,* MAb 5B12, highly specific for interface and tract glia in the adult TG (Fig. 2*F*), binds strongly throughout neuropil (*NP*) in the 95% embryonic brain. Autofluorescence of embryonic cuticle does not represent specific immunoreactivity. Magnifications: *A–D,* ×400; *E,* ×300.

antigen which is restricted primarily to ganglionic neuropil. To this extent, this MAb appears to define the *type 4* glia described by Wigglesworth (1959).

Antibody 5B12 was generated from a fusion procedure that employed an immunogen highly enriched for peripheral glial membranes. It appears highly selective for peripheral nerve tract glial tissues (Figs. 1*D,* 2*F*), and, within the CNS, MAb 5B12 distinctly binds to and demarcates the so-called glial lacunar region (Wigglesworth, 1960; Fig. 1*C*) that receives a large complement of tracheal supply and defines the interface between ganglionic cortex and neuropil. Although neuronal and glial elements of the cortex are devoid of 5B12 immunoreactivity, peripheral nerve tracts in the neuropil and those passing through the cortex are strongly labeled; regions of the dorsal pit in the neuropil and fiber tracts in the anterior connectives are less heavily labeled. Altogether, no evidence of reactivity to neu-

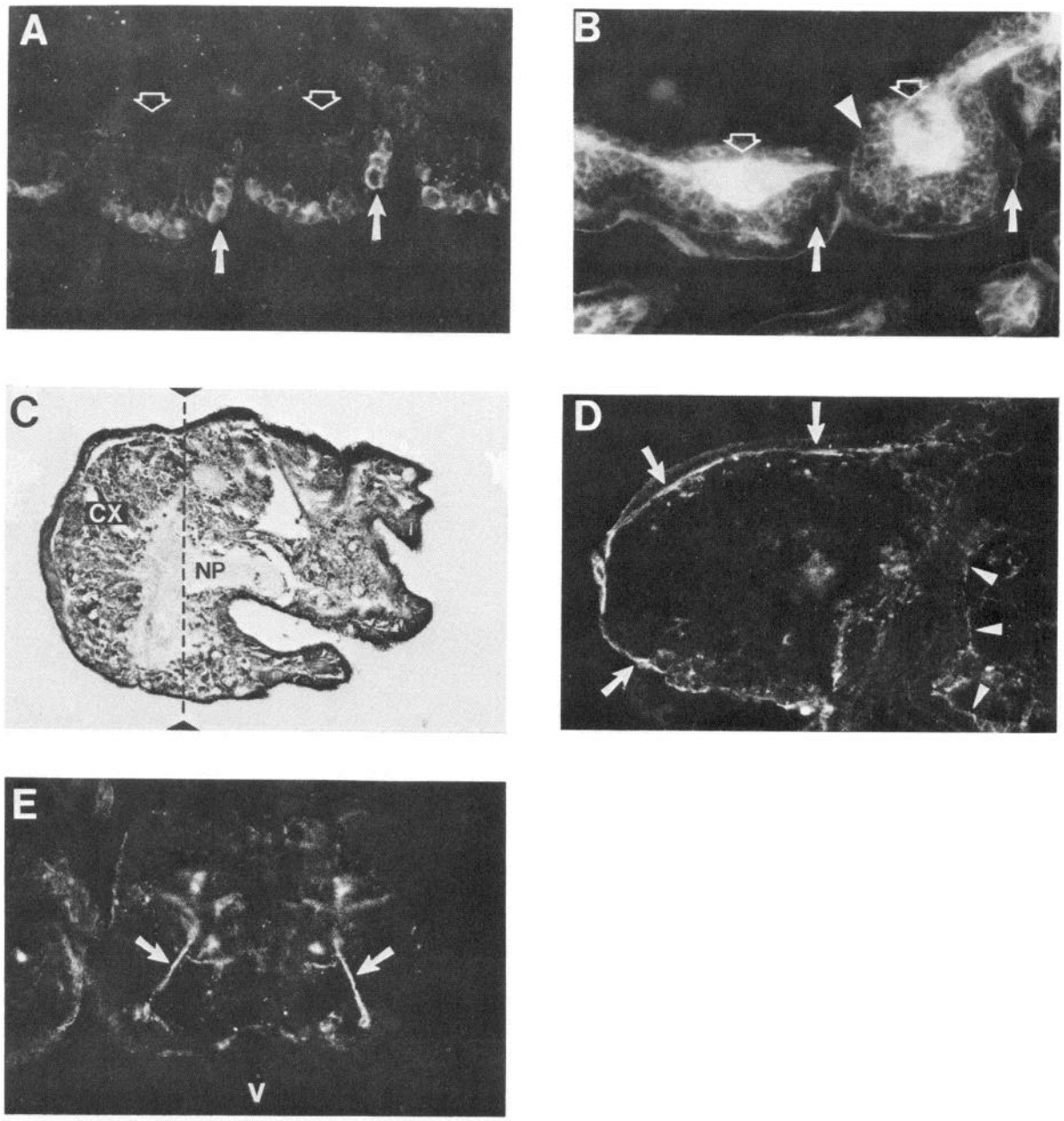

Figure 6. Immunofluorescent staining of frozen sections of intermediate-stage (50–55%) *Acheta* embryos with anti-HRP antibody (*B*) and glial-selective MAbs (*A, D, E*). *A,* Embryonic (50–55%) ventral ganglia labeled with neuropil glia-specific MAb 3G6. Discrete labeling of large neuroblasts (*solid arrows*) in neuroepithelium on ventral surface of developing ventral ganglia is apparent. Regions of underlying neuropil (*open arrows*) are not apparently labeled, nor are adjacent clusters of neuroblast progeny cells (compare with next figure). *B,* Similar region labeled with the neural antigen-specific marker anti-HRP. Note that both neuropil (*open arrows*) and most neuroblast progeny cells (*arrowhead*) strongly express anti-HRP immunoreactivity at this stage, while large neuroblasts in ventral neuroepithelium (*large arrows*) lack anti-HRP immunoreactivity. *C,* Sagittal section through head region of 50–55% embryo, stained with methylene blue–basic fuschin to reveal anatomical features. Compact neuropil (*NP*) is surrounded by extensive cortical rind (*CX*). *Dotted line* through section defines approximate transverse orientation illustrated in *E* below. *D,* Sagittal section through head region of 50–55% embryo labeled with MAb 5B12, which recognizes glia of the cortex–neuropil interface and glial lacunar system in the adult TG (see Fig. 2*F*). Antibody binds to periphery of the developing brain (*arrows*) and also delineates the surfaces of non-neuronal tissues (*arrowheads*). *E,* Transverse section of head region stained with MAb 5B12. Bilaterally organized labeled tracts or processes (*arrows*) project from periphery of ventral (*v*) regions of developing brain into central neuropil tissues. Approximate magnifications: *A* and *B,* ×400; *C–E,* ×300.

ronal elements in the PNS or CNS is observed. This MAb, then, appears highly specific for peripheral and tract glia, and the glial cells or products that reside in the glial lacunar system. Preliminary results obtained from immunoperoxidase staining of resin-embedded sections of TG demonstrate that 5B12 labels the thin cytoplasmic processes of glial cells that surround tracheolar roots and nerve fibers in the cortex–neuropil interface (Fig. 4) and elongate glial cells that are incorporated within peripheral fiber tracts. In this regard, MAb 5B12 labels a class of glial cells that resembles to some degree the *type 2* nerve tract glia categorized on morphological and topographical criteria by Wigglesworth (1959).

In addition to the more specific MAbs described above, others (Table 1) show a more general labeling distribution and bind to the glial tissues of both cortex and neuropil but do not label neuronal elements within these domains. Some of these antibodies also display notable cross-reactivity with tissues outside the nervous system. Despite their lower specificities, these antibodies nonetheless readily discriminate between neuronal and glial tissues in the adult nervous system and thereby emphasize the immunological identity of insect neuroglia.

Immunostaining of embryonic tissues

Little is known about the properties and functions of insect neuroglia during development. It was of interest, therefore, to determine whether any of the MAbs, raised against determinants expressed in the adult nervous system, would recognize glial elements in the embryonic nervous system. We incubated frozen sections derived from embryos fixed at intermediate (50–55%) and late (90–95%) stages of development (Lauga, 1969; Edwards and Chen, 1979) with selected glial-specific MAbs. We found that many of the antigens detected in the adult nervous system are also expressed in embryonic tissues, but often with marked differences in their distribution (Table 1; Figs. 5, 6).

Antibody 3F6, which specifically labels perineurial cells in the adult TG, did not bind detectably to any embryonic tissues within or without the nervous system, even though many components of the perineurium are present in late-stage embryos of orthopteroid insects (Swales and Lane, 1985). The developmentally regulated expression of the 3F6 antigen could reflect the postembryonic synthesis or modification of a macromolecule important in the formation of the mature insect blood–brain barrier. In this regard, a number of studies on the morphological, physiological, and biochemical properties of the insect blood–brain barrier (see Lane, 1985, and Treherne, 1985, for reviews) report significant changes that occur in a developmental stage-dependent manner.

In contrast to probe 3F6, many other MAbs were highly reactive in embryos, the intensity of labeling in some cases surpassing that observed in the adult nervous system. Cortical glial antigens, in particular, were clearly detectable in late-stage embryos (Fig. 5*B*). Unlike the apparent glial specificity displayed in the adult animal, most of the cortical glia-selective MAbs (e.g., 1E7 and 6D7) showed extensive cross-reactivity with embryonic ectodermal tissues (e.g., epidermis, gut) or extracellular matrix outside the nervous system. Central neuropil, however, remained unlabeled in embryos. Thus, as development proceeds, the distribution of certain antigens common to both cortical glia and other embryonic ectoderm-derived tissues becomes restricted to glial cells within the nervous system.

MAb 3G6, which labels presumptive neuropil glia in the adult TG (Fig. 2*E*), reacts similarly in late-stage (ca. 95%) embryos (Fig. 5*C*). At intermediate (50–55%) stages of development, however, labeling is not observed in the neuropil but, rather, is associated with the cytoplasm of large cells in the ventral neuroepithelium of developing segmental ganglia, which have the characteristic appearance of neuroblasts (Fig. 6*A*). Neither the underlying progeny of the neuroblasts nor epidermal cells surrounding the neuroblasts are recognized by MAb 3G6. In striking contrast to the apparent neuroblast-selective labeling observed with MAb 3G6, staining of the 50–55% embryonic nervous system with the neural antigen-selective marker anti-HRP (Fig. 6*B*) discloses an abundance of small immunoreactive neural progeny cells clustered above the neuroblasts, which are prominent by their large size, position, and, notably, the absence of appreciable anti-HRP immunoreactivity (in other studies, neuroblasts do not express the antigen recognized by anti-HRP; Jan and Jan, 1982). The 50–55% embryonic neuropil also stains intensely with anti-HRP, at a time when it is essentially devoid of both MAb 3G6 immunoreactivity and a well-developed complement of glial cells. In the developing embryonic brain at this time, 3G6 labels similar large neuroblast-like cells that reside in the germinal neuroepithelium on the periphery. In both the developing brain and segmental ganglia, we have noted that the neuroblast labeling is selective; that is, only a rather limited ($\geq$50–60%) proportion of the total number of neuroblasts observable in a given section through the embryonic CNS binds MAb 3G6. Furthermore, there is some evidence that the neuroblast labeling pattern is also position-specific. In repeated preparations of the developing 50–55% ganglionic chain, 2 adjacent neuroblasts, located in the anteriolateral regions of each segmental ganglion, were consistently labeled with MAb 3G6.

Differences observed in the labeling of the adult and embryonic nervous system by another MAb, 7B1, demonstrate that some glia-associated antigens may undergo major changes in distribution during development. MAb 7B1, which selectively labels only discrete components of the neural lamella surrounding the nervous system in the adult (Fig. 2*D*), binds in the late-stage embryo to determinants expressed throughout central neuropil (Fig. 5*D*). Although no characteristic labeling of embryonic neural lamella is present, MAb 7B1 does nevertheless distinctively label the inner and outer surfaces of the embryonic epidermis. The finding of such transient expression within the embryonic CNS of an antigen that is associated only with an extracellular matrix secretion product surrounding the adult nervous system suggests that this macromolecule may be involved in structural organization of the CNS during development.

A somewhat similar redistribution of a glia-associated antigen during embryonic CNS development is shown with MAb 5B12, which, in the adult nervous system, is specific for glial cells or products associated with peripheral fiber tracts and the glial lacunar system (Fig. 2*F*) but which, in the late-stage embryo, labels an antigen that is predominantly, if not exclusively, localized within the neuropil and central connectives (Fig. 5*E*). At the 50–55% stage of development, however, the 5B12 antigen is less abundantly distributed and more discretely organized within the nervous system. Labeling is most often observed as a thin layer uniformly surrounding the outer regions of the neuroepithelial surface (Fig. 6*D*). In transverse sections through the developing CNS (Fig. 6*E*), however, 5B12 immunoreactivity is associated with what appear to be well-defined, symmetrically organized tracts or pathways that project from the outer neuroepithelial layers into the interior core of developing neuropil. In addition, the 5B12 antigen is detectable at the peripheral margins and on the luminal surfaces of many non-neuronal tissues (e.g., limb buds, antennae, cercal appendages) throughout the entire 50–55% embryo, where it displays a basal lamina-like distribution (M. R. Meyer and J. S. Edwards, unpublished observations).

Discussion

We have shown here that highly specific immunological probes can be produced with which we can begin to address questions regarding the diversity and development of glial cells in the insect nervous system. Our hydridoma fusion procedures using both heterogeneous and glial-enriched immunogens resulted in

the generation of a relatively large complement of glial-reactive MAbs. Of these, a subset appears to be glia-specific MAbs that bind to major classes of glial cells in the CNS and PNS of *Acheta*. Thus, like their vertebrate counterparts, insect glia appear to be highly immunogenic and possess unique determinants that can clearly distinguish them from neurons and other cells.

Insect glial cells have been classified into 3–5 types based entirely upon morphological criteria (Wigglesworth, 1959; Pipa, 1961; Nordlander and Edwards, 1968; Sohal et al., 1972; Strausfeld, 1976) and generally comprise those cells associated with the perineurium, cortex, neuropil, and nerve tracts of the CNS and PNS. As judged by analysis of the immunohistochemical staining patterns observed in sections of adult cricket TG, our MAbs appear to label selectively 4 major postulated glial cell types or classes that can be discriminated on the basis of antigenic (i.e., molecular) identity. These findings thus support the separation of insect glia into separate classes and are in general accord with the classification scheme put forth by Wigglesworth (1959), which is widely employed for characterization of glia throughout the insect taxa. Although the correlations between morphological and immunohistochemical observations are by no means absolute, an immunological approach to glial cell diversity, in concert with the appropriate techniques to resolve cellular detail, may help to clarify some of the ambiguities that have arisen from classifications based solely upon anatomical descriptions of highly complex, pleiomorphic cells.

In vertebrates, the distinct molecular heterogeneity of neuroglia characterized by the use of highly specific immunological probes has allowed glial cell lineage and differentiation to be explored both *in vivo* and *in vitro* (Raff et al., 1983; Federoff, 1985). For instance, it has now been shown rather convincingly that unique sets of glial precursor cells coexist in the developing and mature CNS that give rise to different populations of class-specific macroglial cells (Raff et al., 1984; ffrench-Constant and Raff, 1986). Furthermore, the time and rate at which certain glial cell precursors divide may be influenced by environmental cues or local factors (Temple and Raff, 1986). Despite these and other recent advances in vertebrate glial cell biology, basic questions regarding the origin and lineage of stem cells or precursors destined to become mature neurons and glia remain yet to be answered (Fujita, 1963; Levitt et al., 1981, 1983).

Glial cell lineage and differentiation have not been explored in depth in insects, but it is widely assumed that during early embryogenesis both neurons and glial cells differentiate from neuroectoderm (i.e., germinal neuroepithelium). The possibility that both neurons and glia may be generated by common precursor cells or neuroblasts has been put forth (Panov, 1963; Edwards, 1969; Vanhems, 1985), but this view remains speculative pending further analyses focused more sharply on the issues of cellular lineage and differentiation (Doe and Goodman, 1985). The use of immunological probes to detect the early expression of glial cell-specific antigens in insects during embryonic and postembryonic development may thus be useful in investigating glial lineage, in a manner analogous to the use of neuron-specific antibodies to track key events in neurogenesis (Kotrla and Goodman, 1981; McKay et al., 1983).

It is intriguing that a MAb (3G6) that is highly selective for presumptive neuropil glia in the adult CNS binds to a discrete population of neuroblasts in the developing segmental ganglia and brain of embryos at the 50–55% stage of development. These results favor the possibility that determinants are shared between neural precursors and glial cells. The selective labeling of certain neuroblasts rather late in the course of neurogenesis suggests that they give rise to a class of glial cells after having generated their quota of neuron precursors. Further, the total absence of MAb 3G6 binding to the progeny of neuroblasts, most if not all of which are destined to become neurons, further supports our belief that this antibody recognizes glial, and not neuronal, determinants in the neuropil. This hypothesis is reinforced by results obtained from comparison of the relative labeling distributions in the 50–55% embryonic nervous system of the neuron-specific marker anti-HRP and the glia-selective MAb 3G6; only neuroblasts express the 3G6 antigen, while only their progeny and adjacent neuropil strongly exhibit anti-HRP reactivity. These findings, based on differential antibody labeling of cells in the developing nervous system, are therefore in close agreement with observations from previous morphological studies describing the possible origin of glial cells from neuroblasts (Panov, 1963; Edwards, 1969; Vanhems, 1985). Nonetheless, more detailed analysis of the time course and distribution of antigenic expression will be required to successfully resolve the events that occur between gliogenesis and the arrival and maturation of glia in the neuropil.

Many of our glial cell-specific MAbs that bind to tissues in the adult cricket nervous system label antigens expressed in the embryo (Table 1), and several lines of evidence demonstrate that the distribution of glial determinants is often dramatically altered as development proceeds. For instance, MAbs that specifically label cortical glia in the adult CNS (e.g., 1E7 and 6D7) show extensive cross-reactivity with many ectoderm-derived tissues in the intermediate-stage embryo, but these antigens become progressively more restricted to the glial components of the nervous system later in development. Similar redistribution of glia-associated macromolecules with ensuing development can be traced with the MAbs specific for neural lamella (a glial secretion product) and interface-tract glia (i.e., 7B1 and 5B12). Apparently, at rather late stages of embryonic development, and even during postembryonic development, the expression of glia-related determinants undergoes significant regulation, as evidenced by progressive restriction of antigen localization. It is intriguing to ask whether any of these observed alterations in expression may be correlated with recognizable ongoing events in neuronal growth and function.

We have demonstrated here, using a panel of glia-selective MAb probes, that insect glia are immunologically distinct from neurons and other cells and, more importantly, that such glia may be classified into 4 major types based upon their antigenic expression in the adult nervous system. Furthermore, because of their molecular identity, we have found it possible to label and observe glial cells at various stages of development. Future efforts may now be directed towards closer examination of insect glial cell lineage, development, and differentiation, and the molecular analysis of the glia-associated macromolecules labeled by specific MAbs.

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