Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2700–2704, April 1982
Neurobiology

Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos

(neural development/segmentation mutants/differentiation antigen/pioneer neurons)

LILY YEH JAN AND YUH NUNG JAN

Department of Physiology, University of California, San Francisco, California 94143

Communicated by Seymour Benzer, January 25, 1982

ABSTRACT **Antibodies specific for horseradish peroxidase (HRPeroxase) bind to neuronal membranes in *Drosophila* and serve as a specific neuronal marker. Immunocytochemical staining with these antibodies marks sensory neurons, peripheral nerves, and fiber tracks in the central nervous system of embryos, larvae, and adult flies. Similar patterns of staining also were seen in embryos of the grasshopper. It appears that an antigen associated with the nervous system and appearing early in differentiation is recognized by antibodies to HRPeroxase. Using this staining method, we followed embryogenesis of the central nervous system in *Drosophila* and found that the organization of central fiber tracks resembled that in the previously well-characterized grasshopper. We have used the anti-HRPeroxase antibodies to show that mutations affecting segmentation in *Drosophila* affect the organization of the embryonic nervous system.**

Insects such as the grasshopper and the fruit fly have been used extensively in developmental studies. In the fruit fly, *Drosophila*, several classes of developmental mutations exist and have provided important clues to mechanisms underlying normal development. For instance, recent studies of zygotic lethal mutations, which alter segment number and polarity, indicate that segmentation involves at least three levels of spatial organization: the entire egg as one developmental unit, a repeat unit with the length of two segments, and the individual segment (1). Homeotic mutations such as *bithorax* reveal the genetic algorithm that normally operates and controls the determination of cells in different segments (2). Detailed genetic and biochemical studies of these mutations may help us to understand the early events of development. Cuticular markers are frequently used to analyze the effects of these mutations. Markers of internal tissues such as the nervous system will greatly aid the study of the developmental mutations (3–7).

Complimentary studies have been carried out in the grasshopper. For instance, pioneer neurons were found that send out processes early during embryonic life and may provide guidance for axonal growth of other neurons (8–13). In the central nervous system, processes of the central pioneer neurons may contribute to the establishment of the reiterated segmental pattern: the left and right longitudinal fiber tracts, the anterior commissure, and the posterior commissure (10, 11). In the periphery, fibers of pioneer neurons establish the first fiber pathways from the limb bud to the central nervous system, a pathway followed later in embryogenesis by axons of the peripherally derived sensory neurons (8, 9, 12, 13). A specific marker for neurons and their processes would be useful in describing the developmental sequence (13). Here we report that antibodies directed against horseradish peroxidase (HRPeroxase) may serve as a neuronal marker both in fruit flies and in grasshoppers.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Reagents. Fluorescein-coupled goat antibodies to HRPeroxase (lot 61321) and rhodamine-coupled rabbit antibodies to HRPeroxase (lot 12418) were from Cappel Laboratories (Cochranville, PA). Both gave similar results in *Drosophila* and in grasshoppers. HRPeroxase (type VI) and 3,3′-diaminobenzidine were from Sigma. A modified Robb's saline (8 mM NaCl/40 mM KCl/1 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM $MgCl_2$/10 mM glucose/4 mM Na_2HPO_4/0.4 mM KH_2PO_4, pH 7.2) (14) was used in most experiments.

Embryos. Flies were on yeast diet for 3 days before they were placed in plastic bottles covered with agar plates containing grape juice for collection of embryos. The plates were changed at 60-min intervals and incubated at 25°C for various specified times. All mutations described in this report cause lethality in homozygous embryos. The obvious abnormal development in these homozygotes (1) allowed us to collect and examine exclusively homozygotes. Grasshopper embryos were kindly provided by D. Bentley.

Immunohistochemical Staining on Cryostat Sections. Embryos or flies were cut into 12-μm sections with a SLEE cryostat. Ribbons of cryostat sections on glass slides were fixed for 30 min in the modified Robb's saline containing 2% formaldehyde. After washing, sections were treated for 60 min with fluorescein-coupled goat antibodies to HRPeroxase (diluted 1:50 in the modified Robb's saline containing 0.3% Triton X-100). The slides were then washed, mounted, and viewed with a Zeiss fluorescence microscope with epiillumination. [Rhodamine-coupled rabbit antibodies to HRPeroxase gave a similar staining pattern when used at lower concentrations (diluted 1:200–1:500)].

Immunohistochemical Staining for Electron Microscopy. Fly brains were fixed by immersion in the modified Robb's saline containing 2% formaldehyde and 0.25% glutaraldehyde. After 30 min in the fixative, the brains were washed, cut into smaller pieces, and treated for 60 min in modified Robb's saline containing goat antibodies to HRPeroxase, 1:50 (vol/vol), 1% normal goat serum, and 0.3% Triton X-100. The pieces of brain were then washed, treated for 60 min in modified Robb's saline containing 10 μg of HRPeroxase per ml, washed again, and treated for 30 min with 0.1 M Tris-buffered saline containing 0.5 mg of 3,3′-diaminobenzidine per ml and 0.009% H_2O_2. After extensive washing, these tissues were postfixed for 30 min in 2% osmium/0.1 M cacodylate buffer, processed for electron microscopy, and examined without further staining. For control preparations, aliquots of the same goat antibodies were incubated with HRPeroxase (final concentration, 40 μg/ml) at 4°C overnight before the staining procedure.

Abbreviation: HRPeroxase, horseradish peroxidase.

RESULTS

Antibodies to HRPeroxase Bind to Neuronal Membrane in *Drosophila*. Using antibodies to HRPeroxase, we found staining of sensory neurons, motor nerves and terminals at neuromuscular junctions, and fiber tracks of the central nervous system in embryos, larvae, and adult flies (Figs. 1 and 2*A*). As far as we could tell, all neurons were stained in every developmental stage of the fly. Other structures that showed staining are pericardial cells, Garland cells, rectal papillae in adult flies, and accessory glands in the adult male's reproductive system. Whether these structures contain neural elements or otherwise have properties common to neural tissues is unknown. The pericardial cells, Garland cells, and rectal papillae apparently all show extensive pinocytotic activities (15, 16). The accessory glands, on the other hand, contain substance P-like immunoreactivity (17) and share a common antigen with a specific subset of central nerve fiber pathways, as indicated by immunohistochemical staining with a monoclonal antibody (unpublished results). Given this limited distribution of staining possibly outside the nervous system, antibodies to HRPeroxase seem to serve as a reasonably specific neuronal marker.

Antibodies to HRPeroxase appeared to bind to neuronal membranes: with immunofluorescence, the staining was most pronounced in the neuropile and appeared as a faint ring around each neuronal cell body. Using an electron microscope, we saw staining on nerve membranes of neurons (Fig. 3*A*). Preadsorption of the antibodies with HRPeroxase eliminated staining at the level of both the light and electron microscope (Figs. 2*B* and 3*B*), indicating that the staining was specific for a HRPeroxase-like immunoreactivity. Moreover, if the bivalent antibodies binding to neuronal membranes were specific for HRPeroxase, some of these antibodies should have a free, second antigen recognition site that binds HRPeroxase. Indeed, after sequential treatment with antibodies to HRPeroxase, with a dilute solution of the enzyme and with a reaction mixture containing 3,3′-diaminobenzidine and H_2O_2, HRPeroxase reaction products were found on neuronal membranes under an electron microscope (Fig. 3*A*). Similarly, under the light microscope, the same pattern of staining as revealed by immunofluorescence (Fig. 2*A*) was reproduced by the brown reaction products (Fig. 2*C*). Therefore, there must be antigenic sites on *Drosophila* neuronal membranes that crossreact with HRPeroxase. These antigenic sites do not have peroxidase activity, because treating the nervous system with 3,3′-diaminobenzidine and H_2O_2 did not generate any brown reaction products.

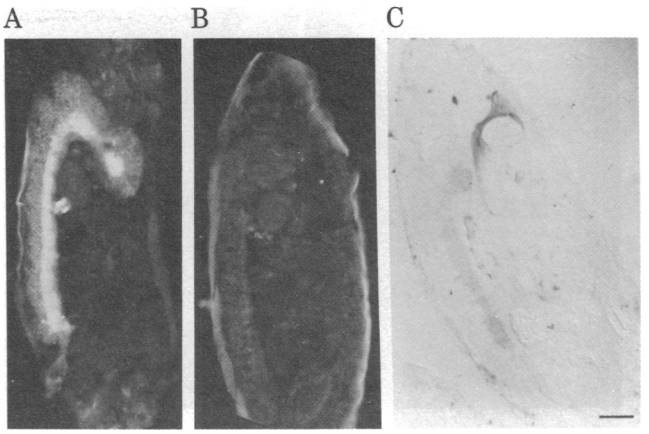

FIG. 2. Cryostat sections of 14- to 16-hr *Drosophila* embryos treated with fluorescein-coupled goat antibodies to HRPeroxase (*A*), the same antibodies preabsorbed with 40 μg of HRPeroxase per ml (*B*), and the same goat antibodies to HRPeroxase, followed by 10 μg of HRPeroxase per ml and finally by the reaction mixture containing 3,3′-diaminobenzidine and H_2O_2 (*C*). Fluorescence microscopy was used in *A* and *B*; bright field illumination was used in *C*. (Bar = 30 μm.)

Antibodies to HRPeroxase Recognize Neurons in Grasshopper Embryos. As in *Drosophila*, the neural tissues in grasshopper embryos also were marked by antibodies to HRPeroxase. A ring of staining was found around each neuron, suggesting that these antibodies were binding to the surface of neurons. In the central nervous system, antibodies to HRPeroxase marked retinae, optic ganglia, two lateral longitudinal fiber tracks in the ventral ganglia, anterior and posterior commissures of each segmental ganglion, and segmental nerves. In the periphery, pioneer neurons and sensory neurons and their processes were stained (Fig. 4), as were early efferent fibers de-

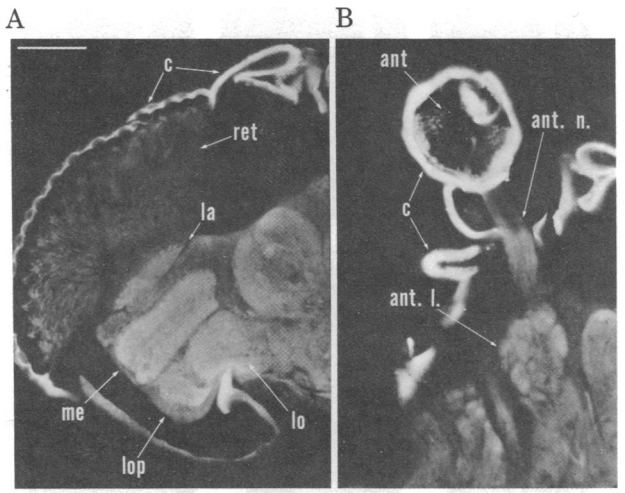

FIG. 1. (*A*) Immunofluorescent staining of the retina (ret) and optic ganglia in *Drosophila*. la, lamina; me, medula; lo, lobula; lop, lobula plate. (*B*) Immunofluorescent staining of the antenna (ant), antenal nerve (ant. n.), and antenal lobe (ant. l.) in *Drosophila*. The fly brain was cut into 12-μm cryostat sections before staining. Fluorescein-coupled goat antibodies to HRPeroxase showed green fluorescence. The cuticle (c) of the fly had yellow autofluorescence. (Bar = 50 μm.)

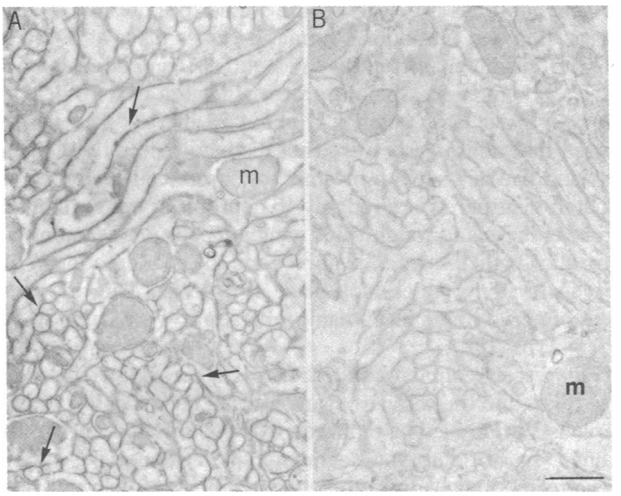

FIG. 3. (*A*) Immunohistochemical staining of the fly brain with goat antibodies to HRPeroxase. (*B*) Preadsorption control, in which the antibodies were first incubated with 40 μg of HRPeroxase per ml. Owing to difficulties of penetration, the staining due to the disposition of HRPeroxase reaction products was more intense near the cut surface (to the left of *A*). Area shown in the control (*B*) was also near the cut surface. (Bar = 1 μm.)

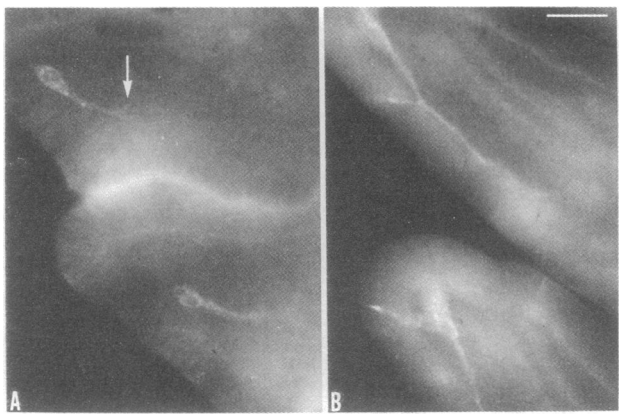

FIG. 4. Immunofluorescent staining of pioneer neurons and sensory neurons in the distal limb buds of grasshopper embryos. (*A*) At 30% development, the first pair of pioneer neurons, their axons, and growth cones are stained by antibodies to HRPeroxase. (*B*) At 45% development, other sensory neurons, their axons, and apical dendrites are stained as well. (Bar = 30 μm.)

rived from the central nervous system and nonneural accessory cells in the chordotonal organ. The HRPeroxase-like immunoreactivity appears early during neuronal differentiation: in the grasshopper nervous system (10, 11) as in *Drosophila* (18), a neuroblast divides to give rise to another neuroblast and a mother ganglion cell, which divides only once to generate two ganglion cells. These two neurons were stained shortly after their birth. Similarly, in the periphery, pioneer neurons were stained before they sent out any processes. Later in development, axons, growth cones, and the surface of cell bodies of pioneer neurons were marked by antibodies to HRPeroxase. The staining also revealed all sensory neurons that have been identified (8, 9, 12, 13) plus additional neurons that have not been identified before. A systematic survey of sensory and pioneer neurons in the grasshopper embryo and their sequence of appearance during development will be described elsewhere (H. Keshishian and D. Bentley, personal communication).

Embryogenesis of the Central Nervous System in *Drosophila*. Our knowledge of embryogenesis of the nervous system in *Drosophila* relies primarily on the studies of Poulson (18): cellularization and blastoderm formation takes place at 3–3.5 hr after fertilization. Gastrulation is essentially completed at 5.5–6 hr. Embryonic development proceeds for the next 16 hr until the first-instar larva hatches at about 22 hr (18). Poulson reported that neuroblasts were first distinguishable by their increasing size in 4– to 4.5-hr embryos. From cell counts he estimated that there were roughly 16 neuroblasts per segment in a 6-hr embryo and roughly 300 ganglion cells per segment before outgrowth of segmental nerves and the condensation of the nervous system, which took place at 9–10 hr. The first clear signs of nerve fibers in Bodian preparations were found in 10-hr embryos (18).

Fluorescein-coupled antibodies to HRPeroxase stained diffuse fibers and the surface of ganglion cell bodies in the ventral nervous system of 6-hr embryos. Neuroblasts did not appear stained. In 8-hr embryos, segmentation of the nervous system was already obvious from the appearance of the left and right longitudinal fiber bundles extending along the entire length of the embryo and transverse fibers confined within each segment, as revealed by antibodies to HRPeroxase. By 10 hr after fertilization, the left and right longitudinal fiber tracts, the anterior and posterior commissures in each segment, and the segmental nerves were clearly visible in horizontal sections. This arrangement is similar to the pattern of central fiber tracks in the grasshopper embryos (10, 11). In sagittal sections, cross sections of these fiber tracks form distinct neuromeres, one in each segment. As described before (18), there are 12 segments—8 abdominal, 3 thoracic ganglia, and 1 subesophageal ganglion—in the ventral nervous system. This pattern of fiber tracts remained invariant as the ventral nervous system condensed over the next 12 hr (Fig. 5). In Bodian preparations, the longitudinal fibers and transverse commissures are discernible as areas devoid of staining. In between transverse commissures are median cells of the nervous system (18). Because there are two transverse commissures in each segment, there must be median cells between the anterior and posterior commissure of each segment and between commissures of adjacent segments.

Because segmentation of the embryonic nervous system in *Drosophila* can be followed with immunofluorescent staining, one can examine the effects of embryonic lethal mutations on the developing nervous system. Some examples are given here.

Mutations Affecting Segmentation. In a systematic search for zygotic lethal mutations, Nüsslein-Volhard and Wieschaus (1) isolated mutations that alter the segmentation of the larva. These mutations fall into three classes: (*i*) the segment polarity mutants, in which every segment has a defined region of the normal pattern deleted and the remainder is present as a mirror-image duplication; (*ii*) the pair-rule mutants, which have deletions in alternate segments; and (*iii*) the gap mutants, which have one continuous stretch of segments deleted in the embryo (1). We looked at mutants of the last two classes and found corresponding changes in the embryonic nervous system.

Of the gap mutants, *knirps* homozygotes have only two rather than eight ventral setal bands in the abdomen: the posterior terminal region including the 8th abdominal segment and part of the 7th seems normal, whereas the anterior setal band is enlarged and may be a composite of more than one segmental identity (1). Immunofluorescent staining indicated that the ventral nervous system of *knirps* homozygotes apparently consisted

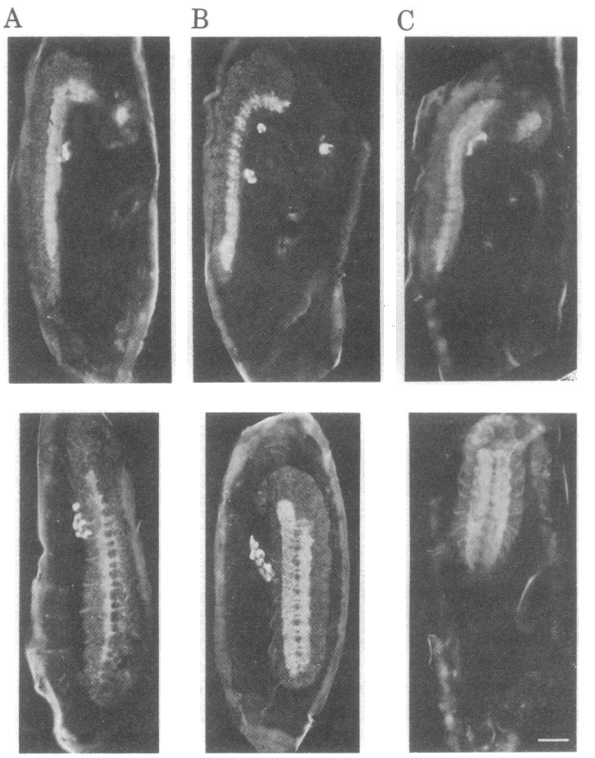

FIG. 5. Sagittal (*Upper*) and horizontal (*Lower*) cryostat sections of 12-hr (*A*), 16-hr (*B*), and 22-hr (*C*) *Drosophila* embryos stained with fluorescein-coupled goat antibodies to HRPeroxase.

of one stretch of three (possibly thoracic) neuromeres at the rostral end, one stretch of two (possibly abdominal) neuromeres at the caudal end, and another neuromere in between (Fig. 6A). From serial sections it appeared that, despite a reduction in the number of neuromeres, there were two longitudinal fiber tracks extending for the entire length of the ventral nervous system and transverse fibers within each neuromere. However, clearly there was a gap between the isolated neuromere and neuromeres on the rostral or caudal end of the ventral nervous system.

Another mutation, *Krüppel*, deletes the entire thorax and roughly the first five abdominal segments. Thus, *Krüppel* homozygotes have a normal posterior-terminal region of the 8th, 7th, and 6th abdominal segments and, anterior to the 6th segment, a plane of mirror-image symmetry and apparently another 6th segment oriented in reverse polarity (1). Immunofluorescent staining showed that, in the ventral nervous system of *Krüppel* homozygotes, the last three neuromeres at the caudal end appeared normal. Besides these, there was only one neuromere, which was physically separated from the last three neuromeres and the brain (Fig. 6B), although these neuromeres were still connected with one another by thin bundles of longitudinal fibers. These observations indicate that the gap mutations affect segmentation of the nervous system in a manner similar to their effects on cuticular structures.

Similarly, pair-rule mutations appear to reduce the segment number in both the nervous system and cuticular structures. In pair-rule mutants such as *paired* and *even-skipped*, homologous parts of the cuticular pattern are deleted in every other segment (1). Immunofluorescent staining showed that the ventral nervous system of these mutants had a reduced number of neuromeres (Fig. 6 C and D). Whereas in normal embryos there were 12 neuromeres in the ventral nervous system—1 subesophageal, 3 thoracic, and 8 abdominal (Fig. 5)—in *paired* homozygotes, only 6 or 7 neuromeres were present (6.7 ± 0.5 average from serial sections of 14 embryos) and, in *even-skipped* homozygotes, only 7 or 8 pairs of neuromeres were present (7.7 ± 0.5 average from serial sections of 6 embryos). In both *paired* and *even-skipped* homozygotes, the ventral nervous system was condensed normally during embryogenesis. In this aspect, the effects of these two pair-rule mutations were different from those of *runt*, which is the only pair-rule mutant reported to have mirror-image duplications (1). Throughout embryogenesis, the ventral nervous system in *runt* homozygotes remained fully extended to the posterior end (Fig. 6E). Nevertheless, as in other pair-rule mutants, in *runt* homozygotes, there were also only seven to eight neuromeres.

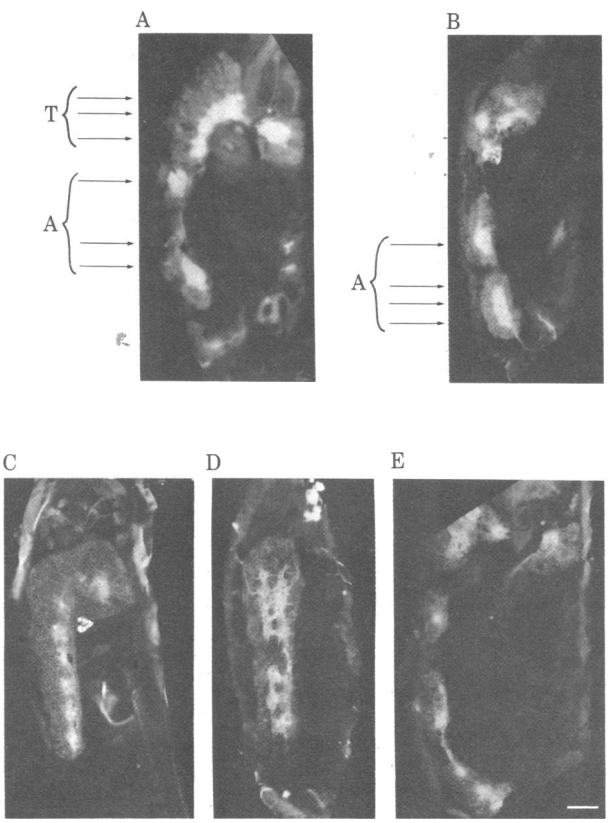

FIG. 6: Cryostat sections of *knirps* (A), *Krüppel* (B), *paired* (C and D), and *runt* (E) mutant *Drosophila* embryos stained with fluorescein-coupled goat antibodies to HRPeroxase. A horizontal section is shown in D. All others are sagittal sections. All embryos were obtained about 18 hr after fertilization. (Bar = 30 μm.)

DISCUSSION

Our results show that antibodies to HRPeroxase recognize neuronal membranes in *Drosophila*. This provides a simple staining method for nerve fibers at the level of both light and electron microscopes. The antigen recognized by antibodies to HRPeroxase appears early during neuronal differentiation; therefore, this staining method can be used throughout the development of the *Drosophila* nervous system.

Using antibodies to HRPeroxase, we have examined embryogenesis of the nervous system in normal flies and in segmentation mutants. We found that certain genes controlling segment number of cuticular structures affect the organization of the central nervous system in a parallel manner. This is perhaps best demonstrated in the case of *knirps* and *Krüppel*, where mutations causing reduction of segment number as evident from cuticular structures (1) caused similar reductions of segment number in the embryonic nervous system (Fig. 6). Similar findings have been made in surgical studies (19), where ligation of embryos caused a reduction in the number of segments in both the central nervous system and cuticular structures. Whether the primary effect of the segmentation mutations is on nervous tissues or cuticular tissues is not known. An answer to this important question should be obtainable by mosaic studies.

Antibodies to HRPeroxase also marked specifically neurons in grasshopper embryos. A striking example is the staining of the limb bud: at about 30% development (i.e., in about 6-day-old embryos), the only neurons present in the distal limb are a pair of pioneer neurons (9). This pair of neurons alone were stained by antibodies to HRPeroxase before they sent out processes. This is also true for the base pioneers (13) in the proximal limb. Later in development, the axons and growth cones of pioneer neurons were stained as well (Fig. 4). In addition, this staining method revealed perhaps all sensory neurons in the limb bud, some of which had not been identified previously (H. Keshishian and D. Bentley, personal communication). Thus, use of antibodies to HRPeroxase, similar to monoclonal antibodies raised against grasshopper embryo (13), offers a fast and easy way to monitor neural development. For instance, at various stages during and after the growth of pioneer fibers, one could monitor the growth of other sensory and central fibers and possibly obtain a better assessment of the guiding function of the pioneer fibers (ref. 13; H. Keshishian and D. Bentley, personal communication).

A similar reiterated segmental pattern of central fiber tracks was seen in the fruit fly and in the grasshopper: a left and a right longitudinal fiber track running the entire length of the ventral nervous system and an anterior commissure and a posterior commissure within each segment. Further, in both *Drosophila* embryos and grasshopper embryos, this segmental pattern of fiber tracks was established at about 40% development. In the

grasshopper, these fiber tracks are apparently established by central pioneer neurons, which are the daughter cells of seven median precursor cells (10, 11). In *Drosophila*, median cells are present dorsal to the nervous system and in between transverse commissures (18). Some of the smaller median cells between transverse commissures may supply sheath cells to developing nerve fibers between ganglia and to outgrowing segmental nerves (18). Poulson suggested that some dorsal median cells also might serve to guide the outgoing segmental nerves to the lateral mesoderm (18). It might be interesting to ask whether cells similar to the central pioneer neurons in grasshopper embryos exist in *Drosophila* and, if so, whether mutations could be found that alter the differentiation of these cells.

This study using antibodies to HRPeroxase suggests a general strategy in developmental studies. By obtaining antibodies that recognize antigens common to *Drosophila* and larger insects such as grasshoppers, one may use antibodies to HRPeroxase and other molecules as a link to take advantage of both systems. Much is known about the lineage and differentiation of identifiable neurons in the grasshopper because they are large enough for intracellular recording and injection of tracers (8–13). Hence, from studies of the grasshopper, one may obtain information concerning the nature of the neuronal antigens. On the other hand, in *Drosophila*, one can systematically identify small regions of a chromosome as regions containing genes important for neural development (20, 21). Conceivably, one can localize structural genes coding for the neuronal antigens, induce mutations of these genes, and learn from the effects of those mutations normal functions of the gene products.

We thank Drs. C. Nüsslein-Volhard and E. Wieschaus for mutant stocks and Ms. Sandra Barbel and Louise Evans for technical assistance. This work is supported by National Institutes of Health Grant 1R01NS15963 (to L.Y.J.) and a Muscular Dystrophy Association grant (to Y.N.J.). L.Y.J. is an established investigator of the American Heart Association. Y.N.J. is a McKnight Scholar.

1. Nüsslein-Volhard, C. & Wieschaus, E. (1980) *Nature (London)* **287**, 795–801.
2. Lewis, E. B. (1978) *Nature (London)* **276**, 565–570.
3. Pearse, A. G. E. (1972) *Histochemistry* (Churchill, London), Vol. 11, 3rd Ed., pp. 1342–1343.
4. Janning, W. (1972) *Naturwissenschaften* **11**, 516–517.
5. Kankel, D. R. & Hall, J. C. (1976) *Dev. Biol.* **48**, 1–24.
6. Lawrence, P. A. (1981) *J. Embryol. Exp. Morphol.* **64**, 321–332.
7. Fujita, S. C., Ferrus, A., Shotwell, S. L. & Benzer, S. (1981) *Neurosci. Abstr.* **7**, 120.
8. Bate, C. M. (1976) *Nature (London)* **260**, 54–55.
9. Keshishian, H. (1980) *Dev. Biol.* **80**, 388–397.
10. Goodman, C. S., Bate, M. & Spitzer, N. C. (1981) *J. Neurosci.* **1**, 94–102.
11. Bate, C. M. & Grunewald, E. B. (1981) *J. Embryol. Exp. Morphol.* **61**, 317–330.
12. Keshishian, H. & Bentley, D. (1981) *Neurosci. Abstr.* **7**, 347.
13. Ho, R., Chang, S. & Goodman, C. S. (1981) *Neurosci. Abstr.* **7**, 348.
14. Robb, J. A. (1969) *J. Cell Biol.* **41**, 876–884.
15. Rizki, T. M. (1978) in *The Genetics and Biology of Drosophila*, eds. Ashburner, M. & Wright, T. R. F. (Academic, London), Vol. 2, pp. 307–452.
16. Wessing, A. & Eichelberg, D. (1978) in *The Genetics and Biology of Drosophila*, eds. Ashburner, M. & Wright, T. R. F. (Academic, London), Vol. 2, pp. 1–42.
17. Jan, Y. N. & Jan, L. Y. (1982) in *Neuropharmacology of Insects*, Ciba Foundation Symposium 88 (Pitman Medical, London), pp. 221–239.
18. Poulson, D. F. (1950) in *Biology of Drosophila*, ed. Demerec, M. (Hafner, New York), pp. 168–274.
19. Brown, E. E. & Schubiger, G. (1981) *Wilhelm Roux's Arch. Dev. Biol.* **190**, 62–64.
20. Campos-Ortega, J. A. & Jimenez, F. (1980) in *Development and Neurobiology of Drosophila*, eds. Siddiqi, O., Babu, P., Hall, L. M. & Hall, J. C. (Plenum, New York), pp. 201–222.
21. White, K. (1980) *Dev. Biol.* **80**, 332–344.