of the interaction of NE with specific binding sites on macromolecular systems.

Apart from mechanistic considerations, the agonist specificities of the "fluoronephrines" (*20*) make them extremely useful tools in neuropharmacology. The specific α-agonist properties of 6-fluoronephrine have been utilized to investigate the α-adrenergic component of the adenosine 3′,5′-monophosphate generating system in rodent cortex (*8*). The availability of an electron microscopic technique for visualizing fluorine in low concentrations in biological specimens may make it possible to actually localize a fluoronephrine-receptor complex at an ultrastructural level (*21*).

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Embryonic Development of Identified Neurons: Temporal Pattern of Morphological and Biochemical Differentiation

Abstract. *Individually identified neurons can be recognized in grasshopper embryos, and are accessible to examination by morphological, physiological, and biochemical techniques from their birth to their maturation. Only after the axon of an identified neuron reaches its postsynaptic target does the neurotransmitter accumulate, the soma rapidly enlarge, and the central arborizations greatly expand.*

Our understanding of neuronal structure and function and of the cellular basis of behavior have profited immensely by the study of identified neurons (*1*). Identified neurons are unique cells that can be repeatedly recognized from animal to animal within the same species; such neurons have a characteristic morphology, physiology, and biochemistry. The embryonic development of these characteristic properties can potentially be examined from the birth of the neuron to its maturation. A prerequisite for such a study is the selection of a species with identified neurons that are relatively large during embryonic development and are accessible to a variety of cellular techniques.

We have been studying the development of a group of identified neurons in the embryonic central nervous system (CNS) of the grasshopper. These neurons are individually identifiable and accessible from their birth to their maturation (*2*). In this report we describe the morphological features of an identified neuron that are temporally associated with the appearance of its neurotransmitter. The cell lineage and developmental timetable of the morphological and physiological properties of these neurons are described elsewhere (*2*).

The subjects of this study are the dorsal unpaired median (DUM) neurons of the grasshopper (*3–5*). Their cell bodies (somata) form a distinct cluster on the dorsal midline of each segmental ganglion. They are termed unpaired because, unlike other neurons in the ganglion, those DUM cells thus far identified do not occur in bilaterally symmetrical pairs. Each DUM neuron soma gives rise to a median neurite which bifurcates into bilaterally symmetrical processes that in some cases exit the ganglion through peripheral nerve roots and innervate skeletal muscles. The largest individual DUM neuron in the third thoracic ganglion (soma diameter, ~ 60 μm) has axons emerging bilaterally in nerve 5 and innervates the extensor tibiae muscles of the left and right hindlegs (this neuron is therefore called Dumeti) (*4*). The neurotransmitter of Dumeti is the biogenic amine octopamine (*5*). The somata of the DUM neurons stain with neutral red (*5*), a vital dye that selectively stains monoamine-containing neurons in the leech (*6*) and lobster (*7*); evidence presented below suggests that octopamine is the transmitter for most, if not all, of the DUM neurons. Each thoracic ganglion contains about 80 DUM neurons; only six to eight of them have large-diameter somata like Dumeti, whereas most are somewhat smaller (15 to 30 μm in diameter). The only identified DUM neuron for which we know the peripheral target is Dumeti. Although the target is a muscle, this neuron is not a motoneuron but rather modulates both neuromuscular transmission and the responsiveness of the muscle (*5*).

Embryonic development in the grasshopper *Schistocerca nitens* takes 20 days at 35°C (*8*). Maintenance of a colony of the animals ensures a year-round supply of embryos. The adult CNS, consisting of a brain and a chain of ventral segmental ganglia, develops chiefly from enlarged ectodermal cells (neuroblasts) which constitute the precursor cells for most of the central neurons (*9, 10*). The three adult thoracic segmental ganglia (T_1, prothoracic; T_2, mesothoracic; and T_3, metathoracic) each contain approximately 3000 neurons which are born and largely differentiate during the 20 days of embryogenesis (*11*). By contrast to the DUM neurons, most of these neurons are bilaterally paired and have their somata on the ventral surface or lateral edge of the ganglion. These paired neurons develop from the left and right plates of ventral neuroblasts. There are about 30 neuroblasts in each ventral plate, making a total of 60 neuroblasts per segment (*10*).

Each segmental array of neuroblasts also contains a single unpaired neuroblast at the posterior end of the segment and dorsal to the paired plates of ventral neuroblasts (*9, 10*). The unpaired neuroblast gives rise to specific identified DUM neurons (*2*); it begins dividing about day 6 and degenerates about day 16. The progeny from the unpaired neuroblast extend anteriorly across the dorsal surface of the developing ganglion, with the oldest progeny furthest or most anterior from the unpaired neuroblast (*12*). This pattern of development leaves

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the progeny in an exposed dorsal cluster such that they are readily accessible to microscopic observation and microelectrode penetration.

The morphology of the DUM neurons was viewed between day 10 and day 20 of development. The embryo was removed from the egg case, the segmental ganglia were removed from the embryo (with or without attached limb buds), and their dorsal surfaces were desheathed. The desheathed ganglia were viewed with a ×40 water immersion lens of a compound microscope fitted with Nomarski interference contrast optics. Soma and nuclear diameters were measured with an ocular micrometer. Volumes were computed by assuming that the soma is a prolate spheroid and the nucleus a sphere. The development of the axons and central arborizations was revealed by microscopic visualization under ultraviolet light of cells injected with the fluorescent dye lucifer yellow (*13*).

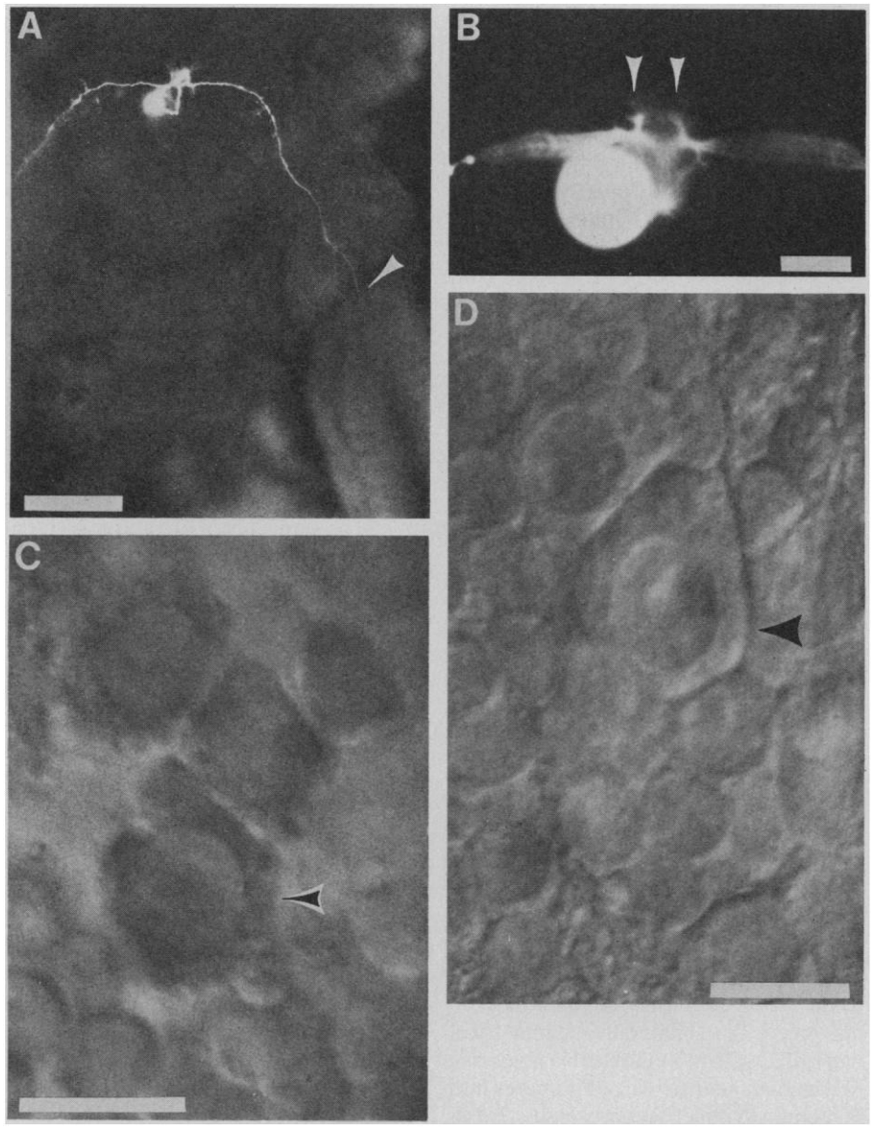


Fig. 1. Morphology of an identified DUM neuron (Dumeti) in the metathoracic ganglion (T_3) at several stages of embryonic development. (A) Peripheral morphology of Dumeti in a 13-day embryo, revealed by fluorescence of the intracellularly injected dye lucifer yellow. Arrow marks axon of Dumeti coursing over extensor tibiae muscle. The cross-striations of the extensor tibiae muscle are visible. The shallow depth of focal plane prevents visualization of the axon over the entire length of muscle, which is shown in Fig. 2A. (B) Central morphology of Dumeti in a different 13-day embryo, stained as in (A). The cell body appears unusually large because of intense fluorescence. Arrows mark initial outgrowth of central arborizations (see Fig. 2A, compare to Fig. 2B). (C) Neutral red staining of the soma of Dumeti (arrow) and two other identified DUM neurons [DUM 3,4,5 and DUM 4,5; see (*2*)] in a desheathed preparation of a 15-day embryo. A fourth and smaller soma is also stained at this stage. (D) Interference contrast view of the dorsal surface of the ganglion in an 18-day embryo; the soma of Dumeti is marked with an arrow. The volume of Dumeti (mostly cytoplasmic) is large as compared with other DUM neurons. Calibration bars: (A) 200 μm; (B) 50 μm; (C and D) 25 μm.

The following is the timetable from day 10 to day 20 of the morphological differentiation of Dumeti, one of the oldest DUM neurons (*14*).

Day 10. The axons of Dumeti leave the CNS and begin growing out the peripheral nerves. The soma is small; the nucleus occupies most of the volume.

Day 11. The axons continue to grow out the peripheral nerves. The soma remains relatively unchanged. The central arborizations are beginning to develop but are very small.

Day 12. The axon of Dumeti reaches the proximal portion of the extensor tibiae muscle in the femur of the limb bud. The soma and central arborizations remain relatively unchanged.

Day 13. The axon of Dumeti extends over the entire extensor tibiae muscle (Figs. 1A and 2A). The soma and central arborizations remain relatively unchanged (Figs. 1B and 2A).

Day 14. The soma of Dumeti begins rapidly to enlarge in diameter (Figs. 1D and 2B), with most of the increase in soma volume being cytoplasmic (Fig. 3, D and E). The central arborizations begin to expand greatly in size and in number of fine branches (Fig. 2B). Some of the other DUM neurons are still being born from the dividing unpaired neuroblast.

Day 16. The unpaired neuroblast stops dividing and degenerates.

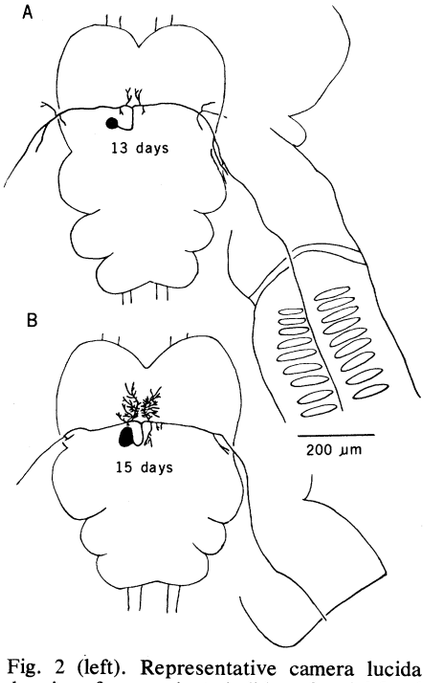
Day 20. By the time the animal hatches, some of the somata (including Dumeti) have greatly enlarged in diameter, while most remain small.

The onset of neutral red staining may be indicative of the temporal pattern of the chemical differentiation of the DUM neurons. Before day 14 none of the DUM neuron somata stain with neutral red. As early as day 14, the three oldest DUM somata, including Dumeti, stain with neutral red (Fig. 1C); few other somata stain on this day. At hatching (day 20), most of the DUM neurons' somata stain with neutral red.

The appearance of the DUM cell neurotransmitter, octopamine, was studied by performing radioenzymatic assays for octopamine (*15*) on extracts of embryonic ganglia; extracts of individual neuron somata from the adult ganglia were also assayed. The detection of octopamine in the embryonic ganglia required pooling of chains of meso- and metathoracic ganglia dissected from embryos of known ages; we included the first three abdominal ganglia (A_1 to A_3) at all ages, since by

day 14 they fuse with the metathoracic ganglion (Fig. 3C). The assay was performed three times for each day of development, using at least four separate chains of embryonic ganglia for each day for each assay (Fig. 3B). The chains of ganglia were combined in a single microtube, the amine extracted (repeated freeze-thaw cycles in 30 μl of 0.1*N* HCl), and replicate portions were removed from the amine assay (*15*). Known amounts (0.2 pmole) of octopamine were added to additional samples to serve as recovery controls and to monitor for tissue interference. The acid-insoluble tissue residues were used for protein measurements (*16*).

The sensitivity of our assay (0.08 pmole) (*15*) required a minimum sample of two ganglionic chains from a 13-day embryo, and less thereafter. We detected no octopamine in the ganglia before day 13, even when we pooled six ganglionic chains for a single assay. If octopamine is present, there is less than 0.015 pmole per chain of ganglia at this time. From day 13 until day 20, there is a continuous increase in the amount of octopamine, whether expressed per ganglionic chain (from 0.024 to 0.975 pmole) or per microgram of protein (from 0.02 to 0.35 pmole). A single adult male metathoracic ganglion contains 34 pmole of octopamine or 0.32 pmole per microgram of protein. This appearance of octopamine on day 13 correlates well with the



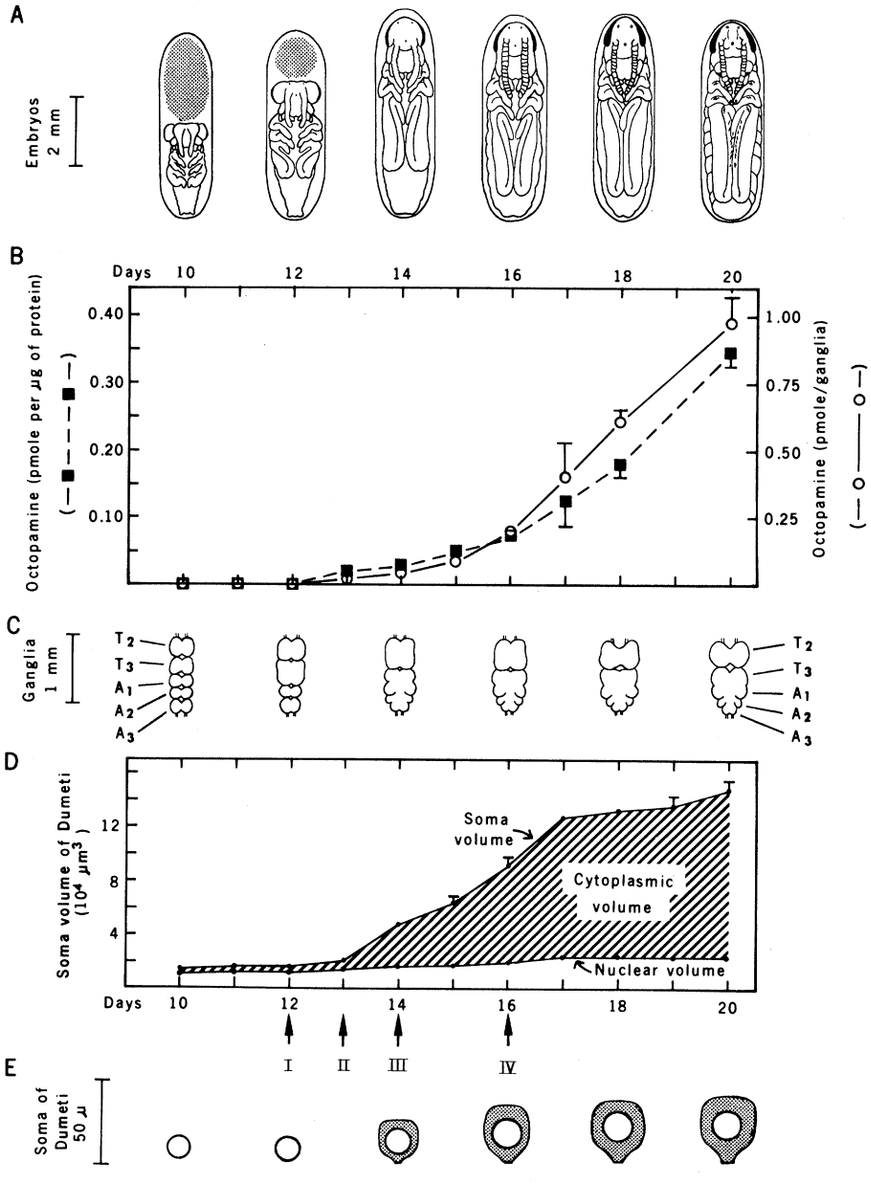


Fig. 2 (left). Representative camera lucida drawings from projected slides of serial focal planes of a dye-injected identified neuron (Dumeti) in fixed preparations, at two stages of development. (A) At 13 days, the axon of Dumeti extends over the entire extensor tibiae muscle. The soma and central arborizations remain relatively small. (B) At 15 days, the soma has enlarged markedly and the central arborizations are vastly expanded. The axon extends over the entire muscle beyond the boundary of the figure. Fig. 3 (right). Temporal pattern of accumulation of octopamine and morphological development of octopaminergic DUM neurons during grasshopper embryogenesis. (A) Development of embryo within the egg case; major features traced from camera lucida projections. Stippling indicates yolk not yet incorporated within the embryo. Hatching occurs at day 20. (B) Time course of accumulation of octopamine in embryonic ganglia, given as picomoles of octopamine per microgram of protein (■), and as picomoles of octopamine per set of T_2 and T_3 and A_1 and A_3 ganglia (●). Each point represents the mean of three separate determinations, each determination from four pooled chains of ganglia (T_2 and T_3 and A_1 to A_3) from animals of a given age. The standard error of the mean (S.E.M.) is indicated by error bars; where not shown, S.E.M. ≤ diameter of the point. (C) Development and fusion of last two thoracic (T_2 and T_3) and first three abdominal (A_1 to A_3) ganglia. Outlines from camera lucida drawings of dissected ganglia are shown. (D) Developmental changes in nuclear and cytoplasmic volumes of a single identified DUM neuron (Dumeti). Preparations were visualized with Nomarski optics and diameters measured with an ocular micrometer; volumes were computed by assuming the soma to be a prolate spheroid and the nucleus a sphere. Each point represents the mean of three separate determinations; the S.E.M. is indicated by error bars, and when not shown is very small. Roman numerals: *I*, axon of Dumeti reaches extensor tibiae muscle; *II*, axon of Dumeti extends over entire extensor tibiae muscle; *III*, soma of Dumeti stains with neutral red; and *IV*, DUM neuroblast stops dividing and degenerates. (E) Representative camera lucida drawings of the soma of Dumeti at different stages of development. The stippled area indicates cytoplasm. Most of the increase in volume is cytoplasmic.

morphological differentiation of the DUM neurons and with the onset of neutral red staining. Not all neurotransmitters appear this late in embryogenesis; for example, acetylcholine is present within the thoracic ganglia by day 10 and within the embryo as a whole as early as day 5 (*17*). Thus, octopamine begins to appear in the DUM neurons only after the axons of Dumeti, one of the oldest DUM neurons, have reached and extended over their peripheral target.

Before we can specifically associate the appearance of octopamine in the embryonic ganglia with the morphological development of the DUM neurons, we have to answer the following two questions. First, do all of the DUM neurons, and in particular those with large somata, contain octopamine? Second, is octopamine associated exclusively with DUM neurons or is it found in other neurons within the thoracic ganglia?

To answer the first question, we removed large numbers of somata of individual DUM neurons from the adult metathoracic ganglion and assayed for octopamine and another biogenic amine, serotonin (*18*). Octopamine was detected in all assays in the amount of 0.14 ± 0.02 pmole per soma. This amount agrees well with the level of octopamine detected in the soma of Dumeti (*5*), and strongly suggests that most if not all of the large DUM neurons contain octopamine. We have not detected significant levels of serotonin, even in samples of 10 to 20 pooled DUM somata; if present, we estimate its level to be less than 3 percent that of octopamine. Furthermore, none of the large DUM somata show a formaldehyde-induced fluorescence when treated by the Falck-Hillarp method, which suggests that they must contain an amine other than serotonin, dopamine, adrenaline, or noradrenaline.

To answer the second question we removed neuronal somata along the lateral edge of the ganglion which also stain with neutral red. Octopamine is not present in significant amounts (less than 0.007 pmole per soma) in these neurons. Octopamine, therefore, appears to be exclusively associated with the DUM neurons in the segmental ganglia, and is probably in all of the large DUM cells.

We interpret the increase in octopamine levels (Fig. 3B) during embryogenesis in the following way. (i) The amount of octopamine per neuron is increasing during embryogenesis, since the somata of Dumeti and other large DUM neurons each contain approximately 0.1 pmole in the adult whereas this level is not reached for the combined meso- and metathoracic ganglia until day 15. (ii) Just as there is a sequential onset of neutral red staining, there may be a sequential appearance of octopamine in different DUM neurons, first in the oldest and then in the younger DUM neurons as they reach a similar stage in their differentiation. (iii) Octopamine begins to appear only after the axons of one of the oldest DUM neurons, Dumeti, reach their peripheral target (*14*).

The axon of Dumeti reaches the extensor tibiae muscle by day 12 and extends over the whole muscle by day 13. Octopamine first appears in the ganglion on day 13, and after this time the soma of Dumeti begins to enlarge rapidly in diameter, the soma begins to stain with neutral red, and the central arborizations begin to expand quickly. Are any of these events causally related? At present we can not say, but in the future we hope to perturb this sequence, for example, by prior removal of the target muscle, to elucidate further the rules and mechanisms of neuronal development.

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8. The colony is maintained at 35°C, 60 percent humidity, and with a 16:8 light-dark cycle. The generation time is 3 months. Individual clutches of 80 to 100 eggs are collected daily and incubated at 35.0° ± 0.5°C. Hatching is synchronous to within a few hours and occurs on day 20; embryonic development proceeds through a well-defined pattern of stages (D. Bentley, H. Keshishian, M. Shankland, A. Raymond, *J. Embryol. Exp. Morphol.*, in press).
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11. Grasshoppers (order Orthoptera) are hemimetabolous insects which hatch from their egg case as larvae that resemble miniature adults. Many new sensory neurons, which differentiate from peripheral epithelial somata, are added postembryonically. But in the segmental ganglia, the neuroblasts degenerate before the end of embryogenesis and thus no new central neuronal proliferation occurs after hatching (*9*, *10*) [A. Gymer and J. S. Edwards, *J. Morphol.* **123**, 191 (1967); A. A. Panov, *Entomol. News* **45**, 179 (1966); G. Sbrenna, *Boll. Zool.* **38**, 49 (1971)].
12. By contrast, the columns of progeny from the numerous paired ventral neuroblasts extend dorsally into the developing ganglion.
13. Glass microelectrodes are filled with 5 percent lucifer yellow (the generous gift of Walter Stewart), and have resistances of 150 to 250 megohms. Tissue is either viewed living or viewed after being fixed in 4 percent formaldehyde, dehydrated in an ethanol series, cleared in methyl benzoate, and mounted in Fluormount.
14. Of the three oldest DUM neurons which develop in relative synchrony (*2*), Dumeti is the only one with a known peripheral target and is, therefore, the major subject of this study. The oldest DUM neurons in the mesothoracic ganglion appear to follow a developmental timetable similar to that followed by the oldest DUM neurons in the metathoracic ganglion.
15. Octopamine was assayed by a modification of a radioenzymatic procedure originally described by P. B. Molinoff, L. Landsberg, and J. Axelrod [*J. Pharmacol. Exp. Ther.* **170**, 253 (1969)]. Our modifications have been described [M. W. McCaman and R. E. McCaman, *Brain Res.* **141**, 347 (1978)] and the sensitivity of the assay was such that 0.08 pmole of octopamine yielded a net radioactivity equal to that of the blank, 150 count/min.
16. Protein was measured by the procedure of O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall [*J. Biol. Chem.* **193**, 265 (1951)].
17. Acetylcholine (ACh) was measured with a radiochemical assay described by R. E. McCaman and J. Stetzler [*J. Neurochem.* **28**, 669 (1977)], which is sufficiently sensitive to measure 0.05 pmole. Before day 10, it is difficult to separate the nervous system from the rest of the embryo. At day 6, the embryo without the head contains 0.22 pmole of ACh (0.07 pmole per microgram of protein) and additionally without the limb buds contains 0.09 pmole of ACh (0.03 pmole/μg). The meso- and metathoracic and first three abdominal ganglia from an 11-day embryo contain 1.4 pmole of ACh (1.0 pmole/μg) and from a 20-day embryo contain 68.5 pmole of ACh (19.0 pmole/μg).
18. Serotonin was assayed by a modification of a radioenzymatic procedure described by J. M. Saavedra, M. Brownstein, and J. Axelrod [*J. Pharmacol. Exp. Ther.* **186**, 508 (1973)]. The sensitivity of the assay was sufficient to measure 0.05 pmole.
19. We thank J. Ono and J. Stetzler for technical assistance in performing some of the transmitter assays, J. Raper for critical reading of the manuscript, W. Kristan and A. Selverston for generous use of facilities, J. Coulombe for technical assistance, and N. Cooley for preparation of Fig. 3. This work was supported by a Helen Hay Whitney Fellowship to C.S.G., NIH grants to M.O., R.M., and N.C.S., and an NSF grant to N.C.S.

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