Serotonin Analog Selectively Ablates Identified Neurons in the Leech Embryo

Abstract. *Exposure of embryonic leeches to 5,7-dihydroxytryptamine, a cytotoxic analog of the monoamine neurotransmitter serotonin, results in the selective ablation of serotonin-containing neurons in the ventral nerve cord. Other neurons appear to be unaffected by this treatment, including those that contain another monoamine neurotransmitter, dopamine. Embryos with ablations continue to develop into juvenile leeches, but as juveniles they are unable to make normal swimming movements. However, normal swimming movements can be instated in such leeches by injecting them with serotonin.*

The ablation of embryonic cells is an important technique for revealing cell and tissue interactions that occur during development. Embryonic neurons have been ablated mechanically (*1*), enzymatically (*2*), genetically (*3*), and photolytically (*4*). We now report the use of a neurotoxin for the selective ablation of identified embryonic neurons (*5*). This toxin is 5,7-dihydroxytryptamine (5,7-DHT), an analog of the monoamine neurotransmitter serotonin. The analog has been used extensively to study the function of serotonin in adult mammalian brain (*6*), where it is taken up by and produces lesions in serotonin-containing neurons (*7*). Serotonin-containing neurons are prevalent in the nervous systems of both vertebrates and invertebrates (*8*). In particular, such neurons are present in the segmental ganglia of the ventral nerve cord of leeches, where they can be visualized by staining with the vital dye neutral red (*9*) or by making them fluorescent with the Falck-Hillarp (*10*) or glyoxylic acid (*11*) techniques. In all segmental ganglia of the leech ventral nerve cord, two lateral cell pairs and the medial, giant Retzius cell pair (*11, 12*) contain serotonin; the Retzius cells release (*13*) as well as synthesize and store (*14*) serotonin. Additional serotonin-containing neurons are present in the ganglia of more anterior segments. Furthermore, two bilateral pairs of neurons per segment, which have peripherally located cell bodies, contain another monoamine neurotransmitter, dopamine (*9, 11*). We have found that 5,7-DHT ablates the serotonin-containing neurons of embryonic specimens of the giant leech *Haementeria ghilianii* (*15*), whereas neurons containing other neurotransmitters, including the monoamine transmitter dopamine, are unaffected by exposure to the toxin.

To ablate serotonin-containing neurons during development of the nervous system, we exposed *H. ghilianii* embryos to 5,7-DHT at early stage 11 (20 days of development at 27°C), when the expanding germinal plate had just covered the entire surface of the embryos and completely enclosed the leech body (*16*). At this stage, all ganglia of the nerve cord are present and the embryonic neurons have acquired many of the morphological and electrophysiological properties of the adult. In particular, the serotonin-containing neurons have begun to accumulate serotonin. However, the embryo is still behaviorally immature and does not yet exhibit adult movements such as crawling and swimming. These movements appear late in stage 11, after another 1 to 2 weeks of development. Using a micropipette syringe, we injected 46 embryos at early stage 11 with 2 to 4 μl of 10^{-5} to $10^{-2}M$ 5,7-DHT (Sigma) in a sodium ascorbate carrier solution; 15 embryos were injected with the carrier solution only (*17*). Thirty-four of the embryos treated with 5,7-DHT and 11 control embryos survived the injection. Injections were made into the embryonic coelom, the blood-filled space surrounding the nervous system, so that the entire nervous system was

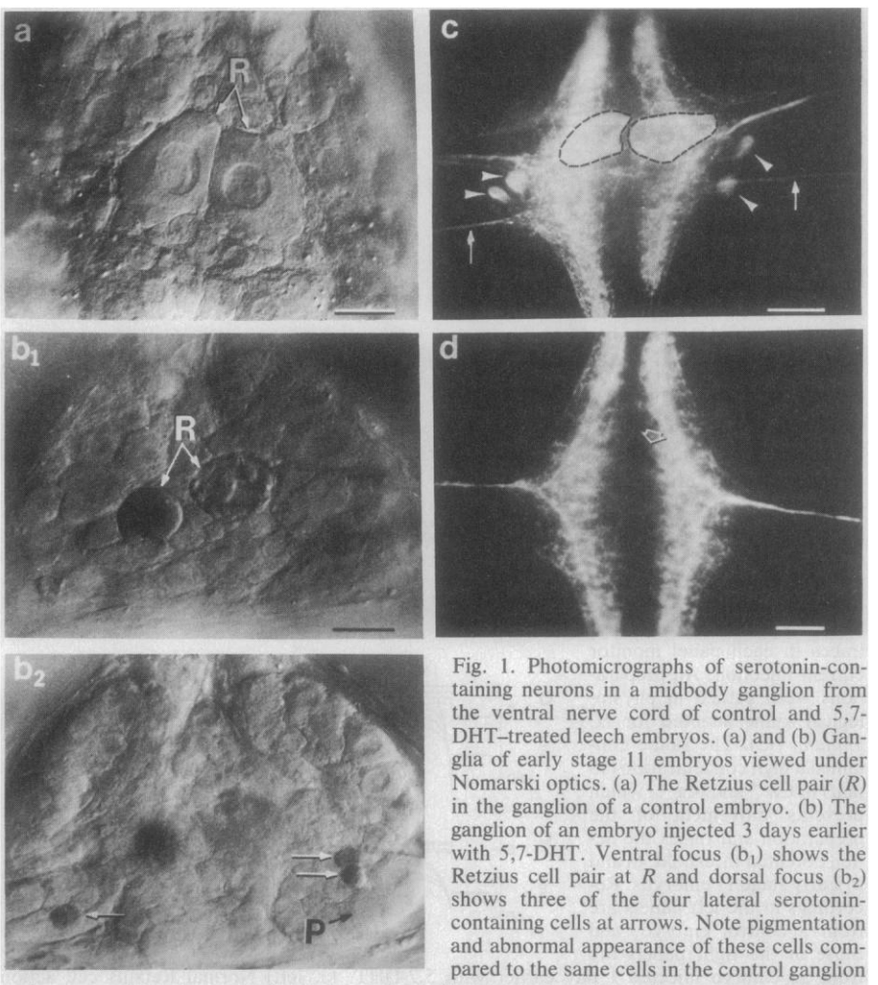

Fig. 1. Photomicrographs of serotonin-containing neurons in a midbody ganglion from the ventral nerve cord of control and 5,7-DHT–treated leech embryos. (a) and (b) Ganglia of early stage 11 embryos viewed under Nomarski optics. (a) The Retzius cell pair (*R*) in the ganglion of a control embryo. (b) The ganglion of an embryo injected 3 days earlier with 5,7-DHT. Ventral focus (b_1) shows the Retzius cell pair at *R* and dorsal focus (b_2) shows three of the four lateral serotonin-containing cells at arrows. Note pigmentation and abnormal appearance of these cells compared to the same cells in the control ganglion and compared to other cells in the treated ganglion, including a P mechanosensory cell (*P*) in (b_2). (c) and (d) Ganglia of juvenile (8-week-old) leeches treated with glyoxylic acid. (c) The distribution of monoamines in the ganglion of a control juvenile. Yellow-fluorescing cell bodies (outlined) and posterior nerve peripheral axons (arrows) of the Retzius cell pair and cell bodies (arrowheads) of the two pairs of lateral serotonin-containing cells are visible; the extensive dendritic arborizations that are visible are green-fluorescing and represent two pairs of dopamine-containing neurons whose cell bodies are located outside of the ganglion. (d) Ganglion of juvenile injected 5 weeks earlier with 5,7-DHT. There is a complete absence of serotonin-specific fluorescence from the ganglion, although a normal intensity of dopamine-specific fluorescence is present in the dendritic arborizations of the dopamine-containing neurons. The pigmented cell body remnants of the Retzius cells are indicated by an arrow. All photographs are oriented with anterior upward. Scale bars, 20 μm in (a) and (b) and 50 μm in (c) and (d).

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exposed to 5,7-DHT within a few seconds. Several days or weeks later, the embryos were dissected, the yolk was removed, and the nerve cord was exposed for examination under a compound microscope with differential interference contrast (Nomarski) optics. Neurons were penetrated with glass microelectrodes containing either 0.5*M* KCl or 3 percent Lucifer yellow for intracellular electrophysiological recordings or for injection of fluorescent dye to reveal their anatomy. The glyoxylic acid–induced monoamine fluorescence technique (*11*) was used to visualize serotonin and dopamine in situ.

The neurons of embryos injected with only the carrier solution appear normal throughout further development (Fig. 1, a and c, and Fig. 2, a to c, control panel). However, 2 to 3 hours after injection of $10^{-2}M$ 5,7-DHT (*18*) into embryos, their serotonin-containing neurons develop a dark brown pigmentation. Between 1 and 3 days after the injection, microscopic examination of these cells shows them to have an abnormal, granular cytoplasm, accumulations of large intracellular vesicles, and an indistinct nucleus (Fig. 1b). By contrast, cell bodies of all the other neurons in the ganglion have a normal appearance. Moreover, when injected intracellularly with Lucifer yellow, the Retzius cells are seen to have truncated axons and to have lost all dendritic processes. Nearly normal resting potentials (−45 to −50 mV) can still be recorded 1 day after injection, but only those Retzius cells that still have axon stumps respond with an action potential to injection of depolarizing current. However, this response differs from a normal action potential in both amplitude and time course (Fig. 2a, treated panel). By the third day after injection, Retzius cells have lost all axon stumps and their resting potential has fallen to less than −10 mV. During this period, the axonal and dendritic anatomy and the electrical properties of many identified non-serotonin-containing neurons are unaffected by exposure to 5,7-DHT. These include the P (pressure) mechanosensory neurons (Fig. 2b, treated column) and the AE (annulus erector) motoneuron (*12, 19*), which exhibit changes of membrane potential in response to serotonin (*20*); they also include other mechanosensory neurons, the dopamine-containing neurons, and several interneurons whose cell bodies lie adjacent to remnants of the Retzius cells. We therefore conclude that the direct cytotoxic effect of 5,7-DHT is confined to the serotonin-containing neurons.

The ablation of serotonin-containing neurons early in stage 11 does not impede further growth of the embryos; they continue to digest yolk, increase in volume, and appear morphologically indistinguishable from normal embryos of the same age. During this subsequent development, there is no replacement of the ablated serotonin-containing neurons nor accumulation of serotonin by other neurons. This is based on the following observation. Five weeks after 5,7-DHT injection, the embryo has developed into a juvenile leech. By this time, the serotonin- and dopamine-containing neurons in control preparations have taken on a nearly adult axonal and dendritic morphology (Fig. 1c); yet in treated preparations, there is a complete absence of glyoxylic acid–induced serotonin fluorescence from the nervous system (Fig. 1d) and the body wall, which normally is replete with endings of the Retzius cell axons (*21*). At this time, the cell bodies of all the original serotonin-containing neurons are visible only as tiny brown dots or areas of brown debris resulting from their disintegration (Fig. 1d). We expected the elimination of all neurons that contain serotonin to have noticeable effects on the further development of some of the other neurons, but no such effects have been found so far. The gross anatomy of the nervous system develops normally. We examined several identified sensory, motor, and interneurons (*12*), including cell P (Fig. 2c) and cell AE, in three treated juveniles and found that they had developed normal anatomical and electrophysiological properties. The dopamine-containing neurons continued to give a normal fluorescence reaction to glyoxylic acid treatment and to develop normal axonal and dendritic arborizations (Fig. 1, c and d). However, in these experiments, the serotonin-containing neurons had had accumulations of serotonin for several days before their ablation (*11*). Hence we cannot rule out that these neurons exert a more noticeable influence on neural or somatic development at an earlier stage (*22*).

Despite the apparently normal development of the characteristic properties of non-serotonin-containing neurons of 5,7-DHT–treated embryos, such embryos display a syndrome of behavioral abnormalities when they reach the juvenile stage, during which complex behaviors such as crawling and swimming normally appear. These abnormalities include general inactivity, hypersensitivity to mechanosensory stimulation of whole-body shortening and writhing reflexes, and delayed appearance of the normal crawling movement. Most strikingly, however, the juveniles with ablations are unable to execute the normal swimming movement, which consists of a rearward-traveling body wave of alternating dorsal and ventral contractions of segmental longitudinal muscles. The inability to swim is of particular interest because the neural circuitry underlying leech swimming has been described in

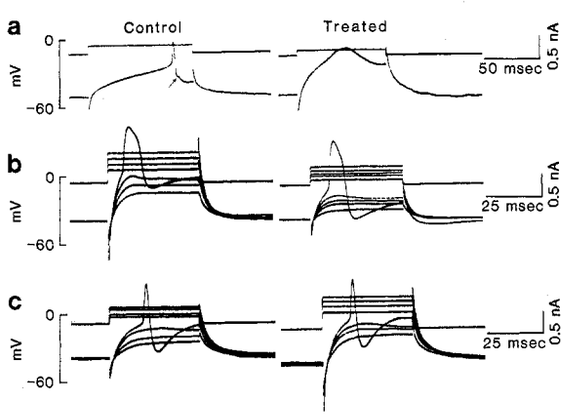

Fig. 2. Intracellular membrane potential recordings from neurons of control and 5,7-DHT–treated leech embryos. Upper traces in each panel monitor current injection. All preparations were bathed in physiological saline adapted for *H. ghilianii* (*12*). The saline contained a high concentration (15 m*M*) of Ca^{2+} and Mg^{2+}, which prevents movement of the preparation and raises the action potential threshold. (a and b) Early stage 11 embryos. Recordings in the left panels are from a control embryo, and those in the right panels are from an embryo injected 1 day earlier with 5,7-DHT. (a, left) Normal Retzius cell action potential evoked by injecting a depolarizing current pulse. Arrow indicates an action potential produced by the contralateral Retzius cell, which has passed with attenuation through an electrotonic junction coupling the Retzius cell pair. (a, right) Abnormal Retzius cell action potential evoked by injecting a depolarizing current pulse. The initial rise in membrane potential is higher in the treated than in the control embryo for about the same strength of current injection. This change reflects an increase in the input resistance of the treated Retzius cell due apparently to the loss of most of its axons and its coupling to the contralateral Retzius cell. (b, left) Normal P cell action potential, evoked by injecting depolarizing current pulses. The action potential threshold is unusually high as a result of prolonged exposure of the preparation to high Ca^{2+} and Mg^{2+} concentration. (b, right) Normal P cell action potential similar to that recorded from control embryos. (c) Juvenile (10-week-old) leeches. Normal P cell action potentials are evoked by injecting depolarizing current pulses in a control specimen (left) and in a juvenile treated 7 weeks earlier with 5,7-DHT (right).

some detail (*23*), and in the adult, the frequency and duration of spontaneous swimming episodes is directly related to the level of serotonin bathing the nervous system (*13*). When juveniles with ablations were subjected to conditions that provoke swimming in normal juveniles (*24*), they responded with uncoordinated wriggling or dorsal or ventral flexions of the whole body that alternate at the approximate frequency (1 to 3 Hz) of the swimming body wave. None of the juveniles with ablations developed the ability to swim for as long as 10 weeks after treatment. However, the ability to swim could be instated by injecting serotonin into the coelom of a treated juvenile (three animals tested) or by immersing the juvenile in water containing $10^{-5}M$ serotonin (15 animals tested). Within 10 minutes after exposure to serotonin, the juveniles with ablations exhibit normal swimming. Furthermore, their other behavioral abnormalities of general inactivity and hypersensitivity disappear. When these embryos are returned to serotonin-free water, the behavioral abnormalities return, with swimming movements gradually deteriorating over a 15- to 20-minute period. The ability to swim is not obtained by immersing juveniles with ablations in water containing other monoamines, such as dopamine, octopamine, or histamine in concentrations of up to $10^{-4}M$ (four animals tested with each monoamine).

Because the swimming incapacity of juveniles lacking serotonin-containing neurons is eliminated by exogenous serotonin, that incapacity must be a consequence of depletion of serotonin in the nervous system or body wall, rather than being attributable to deterioration or altered development of swim generator neurons or muscles. Moreover, although at least some serotonin-containing neurons are essential for the expression of the normal swimming movement, this serotonin-mediated expression of swimming is of minor importance during genesis of the swim generator circuit because a functional circuit develops without it. These results resemble findings made with amphibians (*25*) and arthropods (*26*), in which execution of a locomotory behavior during embryogenesis is unnecessary for development of the pattern generator of that behavior.

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References and Notes

1. C. Lance-Jones and L. Landmesser, *J. Physiol. (London)* **302**, 559 (1980); J. R. Sanes, J. G. Hildebrand and D. J. Prescott, *Dev. Biol.* **52**, 121 (1976).
2. D. A. Weisblat, S. L. Zackson, S. S. Blair, J. D. Young, *Science* **209**, 1538 (1980).
3. M. Chalfie and J. E. Sulston, *Dev. Biol.* **82**, 358 (1981); see also R. J. Mullen, E. M. Eicher, R. L. Sidman, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 208 (1976).
4. E. R. Macagno, *Nature (London)* **275**, 318 (1978); see also J. E. Sulston and J. G. White, *Dev. Biol.* **78**, 577 (1980).
5. C. Sachs and G. Jonsson [*Med. Biol.* **53**, 156 (1975)] used 5,7-DHT in neonatal rats where it only caused partial damage to serotonin-containing neurons rather than the complete ablations reported here. T. Kasamatsu and J. D. Pettigrew used 6-hydroxydopamine in kittens to deplete the immature visual cortex of norepinephrine [*J. Comp. Neurol.* **185**, 139 and 163 (1979)].
6. J. H. Jacoby and L. D. Lytle, Eds., *Serotonin Neurotoxins* (New York Academy of Sciences, New York, 1978), vol. 305.
7. H. G. Baumgarten and L. Lachenmayer, *Z. Zellforsch.* **135**, 399 (1972); A. Bjorklund, A. S. Horn, H. G. Baumgarten, A. Nobin, H. G. Schlossberger, *Acta Physiol. Scand. Suppl.* **429**, 29 (1975); H. G. Baumgarten, A. Bjorklund, L. Lachenmayer, A. Rensch, E. Rosengren, *Cell Tissue Res.* **152**, 271 (1974).
8. A. Parent, in *Serotonin Neurotransmission and Behavior*, B. L. Jacobs and A. Gelperin, Eds. (MIT Press, Cambridge, Mass., 1981), p. 3.
9. A. E. Stuart, A. J. Hudspeth, Z. W. Hall, *Cell Tissue Res.* **153**, 55 (1974).
10. B. Ehinger, B. Falck, H. E. Mybrberg, *Histochemie* **15**, 140 (1968).
11. D. K. Stuart, *Soc. Neursci. Abstr.* **7**, 2 (1981).
12. A. Kramer and J. Goldman, *J. Comp. Physiol.* **144**, 435 (1981).
13. A. Willard, *J. Neurosci.* **1**, 936 (1981).
14. S. Rude, *J. Comp. Neurol.* **136**, 349 (1969); R. E. Coggeshall, S. A. Dewhurst, D. Weinreich, R. E. McCaman, *J. Neurobiol.* **3**, 259 (1972); C. M. Lent, J. Ono, K. T. Keyser, H. J. Karten, *J. Neurochem.* **32**, 1559 (1979).
15. Our leeches and embryos were obtained from a colony maintained at the University of California, Berkeley; see R. T. Sawyer, F. LePont, D. K. Stuart, and A. P. Kramer [*Biol. Bull.* **160**, 322 (1981)].
16. J. Fernandez, *Dev. Biol.* **76**, 245 (1980); D. A. Weisblat, G. Harper, G. S. Stent, R. T. Sawyer, *ibid.*, p. 196.
17. Sodium ascorbate is included in the carrier solution at a concentration of 1 percent (weight to volume) to prevent the oxidation of 5,7-DHT. The carrier solution was designed to be isotonic, with physiological saline adapted for *Haementeria ghilianii* by substituting sodium ascorbate for NaCl. Its composition was 65 m*M* NaCl, 65 m*M* sodium ascorbate, 4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.8 m*M* $MgSO_4$, and 10 m*M* Hepes buffered to *p*H 7.6. It is estimated that upon injection a two- to five-fold dilution of 5,7-DHT occurred.
18. Maximal effects were observed at this concentration, and almost no effects were observed at a concentration of $10^{-5}M$.
19. J. G. Nicholls and D. A. Baylor, *J. Neurophysiol.* **31**, 740 (1968); A. E. Stuart, *J. Physiol. (London)* **209**, 627 (1970).
20. P. B. Sargent, thesis, Harvard University, Cambridge, Mass., (1975); P. Fuchs, L. Henderson, J. G. Nicholls, *J. Physiol. (London)*, in press.
21. Because of the reaction of known concentrations of serotonin with glyoxylic acid, we believe that as little as 0.1 m*M* serotonin would be detected by this fluorescence method. This amount is 50 to 1500 times less than the concentration found in serotonin-containing neurons of the leech (unpublished observation).
22. Evidence that serotonin may have an important role during very early development has been presented by, for instance, G. A. Buznikov, A. V. Sakharova, B. N. Manukhin, and L. N. Markova [*J. Embryol. Exp. Morphol.* **27**, 339 (1972)]; S. S. Deeb [*J. Exp. Zool.* **181**, 79 (1972)]; and J. M. Lauder and H. Krebs [*Dev. Neurosci.* **1**, 15 (1978)].
23. G. S. Stent, W. B. Kristan, Jr., W. O. Friesen, C. A. Ort, M. Poon, R. L. Calabrese, *Science* **200**, 1348 (1978); J. C. Weeks, *J. Neurophysiol.* **45**, 698 (1981).
24. Release of specimens at the surface of a large volume of water stimulated swimming in control juveniles. A few specimens were released into seawater, which is noxious and stimulated vigorous swimming in control juveniles; yet the juveniles with ablations responded only with vigorous flexions of the whole body and could not generate swimming movements.
25. L. Carmichael, *Psychol. Rev.* **33**, 51 (1926); S. A. Matthews and S. R. Detwiler, *J. Exp. Zool.* **45**, 279 (1926).
26. W. J. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 954 (1973); W. J. Davis and K. B. Davis, *Am. Zool.* **13**, 409 (1973); D. R. Bentley and R. R. Hoy, *Science* **170**, 1409 (1970); A. E. Kammer and S. C. Kinnaman, *J. Comp. Physiol.* **130**, 29 (1979).
27. We thank W. B. Kristan, Jr., and G. S. Stent for helpful discussions and advice. This work was supported by NIH training grant GM 07048, NIH research grants NS 06456 and NS 12818, and NSF research grants BNS77-19181 and BNS79-23459.

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Dispersal by Swarming in a Social Spider

Abstract. *Groups of* Achaearanea wau *(Theridiidae) disperse and found new colonies by means of synchronized emigrations of adult and subadult females. Emigrations involve the construction of silk highways from parent colonies to new web sites, synchronized migrations along the highways, and the establishment of daughter colonies. Emigrations of* Achaearanea wau *are similar in timing, group composition, and in some behavior components to swarming of social bees and wasps.*

Some social bees and wasps disperse and found new colonies by swarming, a process which has been described in wasps as "movements between old and new nest sites effected by coordinated, synchronous migration of large numbers of individuals including both workers and queens" (*1*). Swarming of this sort was considered unique to apid bees and some polybiine wasps. As a means of dispersal, swarming may have profound effects on the structure and dynamics of populations (*2*). We found a similar phenomenon, involving the formation of new colonies by synchronized, group emigration, in a social theridiid spider, *Achaearanea wau* Levi (*3*), in Papua New Guinea.

Achaearanea wau was studied near Wau, Morobe Province, Papua New Guinea (7°19′S, 146°44′E). Colonies of these spiders, often containing several hundred individuals, occurred in discrete populations in treefall gaps and along edges of montane forest. One population of 13 to 35 colonies on Mount Kaindi (at

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