43. N. Jones and T. Shenk, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3665 (1979).
44. A. J. Berk *et al.*, *Cell* **17**, 935 (1979).
45. R. B. Gaynor and A. J. Berk, *ibid.* **33**, 683 (1983).
46. H. E. Ruley, *Nature (London)* **304**, 602 (1983).
47. A. Houweling, P. van den Elsen, A. van der Eb, *Virology* **105**, 537 (1980).
48. J. Banerji, L. Olson, W. Schaffner, *Cell* **33**, 729 (1983).
49. J. Banerji, personal communication.
50. A. M. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560 (1977).
51. A. J. Berk and P. A. Sharp, *Cell* **12**, 721 (1977).
52. We thank D. Golde for continued assistance and support; J. Gasson and A. Cann for helpful comments on the manuscript; and M. Ahdieh, C. Nishikubo, A. Healy, J. Fujii, H. Merriman, B. Koers, and W. Aft for technical assistance. Supported by NCI grants CA 32737, CA 38597, CA 09297, and CA 16042; by grants PF-2182 and JFRA-99 from the American Cancer Society; and by a Bank of America–Giannini Foundation Fellowship (W.W.).

12 March 1985; accepted 14 May 1985

Steroid-Dependent Survival of Identifiable Neurons in Cultured Ganglia of the Moth *Manduca sexta*

Abstract. *Adult emergence at the end of metamorphosis in the moth* Manduca sexta *is followed by the death of abdominal interneurons and motoneurons. Abdominal ganglia removed from insects before this period of naturally occurring cell death and maintained in vitro showed neuronal death confined to the same cells that normally die in vivo. Addition of physiological levels of the steroid 20-hydroxyecdysone to the culture system prevented the selective death of these motoneurons.*

KELLY L. BENNETT
JAMES W. TRUMAN*
Department of Zoology, University of Washington, Seattle, 98195

*To whom correspondence and requests for reprints should be sent.

Cell death is an important developmental force in determining the final form of the nervous system. Neuronal survival or degeneration is determined by numerous factors, including competition for target sites, the absence of trophic factors, and the presence or absence of various hormones (*1*). In the moth *Manduca sexta* the emergence of the adult at the end of metamorphosis is followed by the rapid loss of about 40 percent of the neurons in the insect's abdominal nervous system (*2*). Many of the cells that die were persistent larval neurons that maintained the behavior of the insect during metamorphosis while the adult nervous system was being formed. This period of postmetamorphic cell death occurs according to a strict temporal program, in which specific cells die in a characteristic sequence (*2*). Studies of intact insects and of isolated abdomens have suggested that the neuronal degeneration was triggered by declining levels of the ecdysteroids, the steroid molting hormones, at the end of metamorphosis (*3*). We used in vitro techniques to show that degeneration results from the direct action of ecdysteroids on the central nervous system (CNS) and also provides insight into the nature of the factors that determine the temporal sequence of cell death.

Segmental abdominal ganglia were taken early in the morning on the last day of adult development and were cultured individually for 24 or 48 hours (*4*). These times were selected because essentially all the cell death in vivo occurs within the first 48 hours after adult emergence (*2*). After the culture period, ganglia were fixed in alcoholic Bouin's fixative, sectioned at 10 μm, and stained with hematoxylin and eosin. Degenerating cells were readily distinguished from healthy cells because the cell bodies of the former were typically condensed, lacked a distinct nucleus, and stained darkly (*2*). The total number of healthy and degenerating cells in the sections from each ganglion was then counted. Interneurons and motoneurons were distinguished on the basis of cell body diameter; diameters of the former ranged from 7 to 15 μm and diameters of the latter ranged from 20 to 40 μm (*5*). Many of the large neurons had characteristic locations in the ganglion, which allowed their unambiguous identification (*6*). These included neurons that survive through adult life and others that normally die after adult emergence.

The tracheal supply was removed along with each ganglion, and an open end was floated on the surface of the medium so that oxygen had direct access to the ganglion. Of those that maintained open tracheal contact with the surface during the culture period, about half showed aberrant histology (such as highly vacuolated cells), which made cell identification and determination of fate impossible. The rest of the cultured ganglia had relatively normal histology and were used in the present analysis.

At the time of explanation, each ganglion contained an average of about 850 neuronal profiles (*2*, *7*). In vivo neuronal counts decreased to 583 ± 20 profiles by 24 hours after ecdysis and to 542 ± 9 at 48 hours (*2*) (mean ± standard error of the mean). After 24 hours in vitro, the number of healthy cells dropped to 691 neuronal profiles and after 48 hours to 618 (Table 1). This reduction in apparent cell number was not an artifact of changes in cell size during the culture period but rather resulted from neuronal death. A moderate number of degenerating interneurons and a few dying motoneurons were evident by 24 hours in vitro (Table 1). After 48 hours in culture, both kinds of neurons showed further increases in the number of degenerating cells.

An important question was whether the neurons that died in vitro were the same cells that die in vivo. After 24 hours in culture, cell death was primarily confined to interneurons in the rostral half of the ganglion. By 48 hours, the

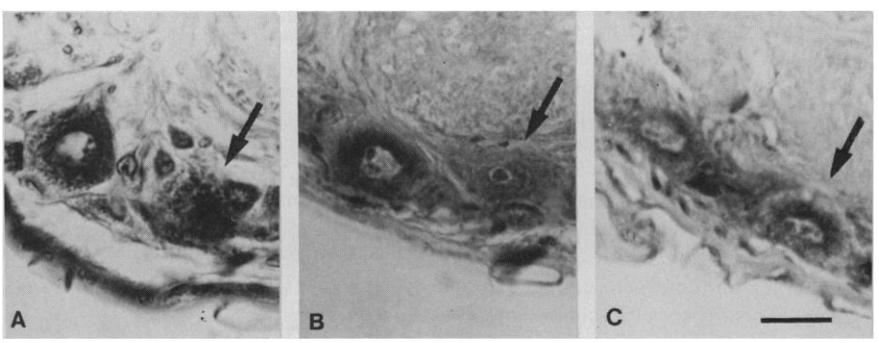

Fig. 1. Histological sections showing the appearance of motoneuron MN-11 (arrow) after exposure of the ganglia to various treatments. (A) By 20 hours after adult ecdysis in vivo, the nucleus has ruptured and condensed as a darkly staining mass in the center of the cell. A neighboring motoneuron (left) is unaffected and will survive through the remainder of adult life. (B) A dying MN-11 motoneuron in a slightly earlier stage of degeneration (the nucleolus is still visible in the middle of the collapsed nucleus) from a ganglion cultured for 24 hours with no hormone. (C) A healthy MN-11 motoneuron with an intact nucleus after 48 hours in vitro with 0.1 μg of 20-HE per milliliter. Scale bar, 20 μm.

degeneration had spread to the more caudal interneurons and also included some motoneurons. This spatial pattern of degeneration is the same as that observed for neuronal death in vivo (*2*). A more precise determination of the normal fates of the cells that died in vitro was possible in the case of the motoneurons because they could be repeatably identified from ganglion to ganglion (Fig. 1) (*2, 6*). In no case did cells that are known to survive through the life of the moth degenerate in vitro; rather, death was confined to neurons such as MN-11 and the D-IV cells, which also die in vivo (*6*). Thus in the cultured CNS, death did not occur randomly throughout the ganglion but was confined only to those cells that normally die.

Ganglia were usually placed in culture about 7 hours before the donor was scheduled to emerge. Consequently, at the end of the 24-hour culture period the ganglion should have shown neuronal loss equivalent to that from a moth 17 hours after ecdysis, which has an average of about 45 degenerating interneuron profiles and a small number of dying motoneurons (*2*). By 24 hours later in vivo, death of interneurons was finished and most of the degenerating fragments were motoneuron remnants. Thus, although the cell death in vitro occurred among the appropriate cell populations, it progressed at a slower rate than that in vivo. Whether this difference is due to a suboptimal composition of the culture medium or to problems with oxygen supply to the ganglia has not been determined.

When 0.1 μg ($2 \times 10^{-7} M$) of 20-hydroxyecdysone (20-HE) (*8*) per milliliter was present in the culture medium, the decline in cell number was retarded. After 24 or 48 hours in culture, the number of surviving neurons tended to be somewhat less than at the time of explantation, but the difference was not significant (Table 1). Few degenerating cells were seen at either time. Those that were present were presumably interneurons that normally die just before adult emergence (a few hours after the ganglia were explanted) and hence were already committed to die before the ecdysteroid exposure (*2*). These data show that the survival of neurons in the abdominal ganglion of *Manduca* is directly dependent on 20-HE or its metabolites.

Thus, in *Manduca* the death of central neurons as well as some of the muscles that they innervate (*9*) occurs as a direct response to the declining titers of ecdysteroids at the end of metamorphosis. This situation is in contrast to that in the silkmoth *Antheraea polyphemus*, in which the death of certain muscle groups immediately after adult ecdysis is triggered by a two-step process involving the decline in ecdysteroid and the subsequent appearance of eclosion hormone (*10*). These results do not prove that the ecdysteroids act directly on the cells that die. It is still possible that other neurons or other cell types in the ganglion (such as glia) may be the primary target of the steroid.

Table 1. Neuronal survival and degeneration in cultured abdominal ganglia of the moth *Manduca sexta*. The number of ganglia examined is indicated in parentheses. Values are reported as means ± standard error of the mean.

Time (hours)	Conditions	Living neurons* (No.)	Dying small cells* (No.)	Dying large cells (No.)
0		848 ± 16 (5)	0	0
24	No hormone†	691 ± 23§ (6)	16 ± 4§ (6)	0.2 ± 0.2 (6)
48	No hormone†	618 ± 34§ (10)	39 ± 5§ (10)	3 ± 1 (10)
24	20-HE‡	783 ± 25‖ (8)	5 ± 1§ (9)	0
48	20-HE‡	802 ± 24‖ (3)	2 ± 1§ (3)	0

*Total number of cellular profiles in complete serial sections (10 μm) from segmental ganglia; a cell was counted when its nucleus was included in the section. †Includes both preparations in Grace medium alone and in Grace medium with isopropanol (the carrier for 20-HE). ‡Culture concentration was 0.1 μg/ml. §Significantly different from 0-hour ganglia at at least the $P < 0.05$ level. All 20-HE groups were significantly different from their respective no hormone groups at the level of at least $P < 0.05$. ‖Not significantly different from 0-hour ganglia ($P > 0.05$, *t*-test).

The data on cultured ganglia also provide some insight into the basis of the temporal sequence of neuronal deaths. Treatment of groups of *Manduca* with 20-HE at various times surrounding ecdysis showed that each neuron exhibited a discrete commitment point before which it could be saved by steroid treatment but after which such treatment was ineffective in preserving its life (*3*). The two sets of motoneurons that were examined in detail were MN-11 and the D-IV cells, which become committed to die in vivo 5 hours before emergence and 3 hours after emergence, respectively. The first signs of degeneration do not then appear until 10 to 12 hours later, at about 7 hours and 14 hours after emergence, respectively. Thus, the temporal order of the commitment points is the same as the subsequent order of deaths.

One possible interpretation for this difference in commitment times is that individual neurons may require different levels of ecdysteroid to maintain themselves. Consequently, as the ecdysteroid titer gradually declines in vivo at the end of metamorphosis, the cells that require the highest titers of steroid are the first to die and are followed by those with the next lower thresholds. In vitro, the ganglia were exposed to an abrupt step-down in ecdysteroid concentration before these neurons were committed. Therefore, all the cells should have passed through their threshold at essentially the same time, and this step-down should have initiated synchronous degeneration. However, the degeneration of motoneurons was not synchronous in vitro but rather occurred in the same temporal pattern as in vivo (for example, the death of the MN-11 cells always occurred before the death of the D-IV neurons). Thus the sequence of death is apparently not due to individual cells responding to different titers of steroid. Other classes of mechanisms, such as variable latencies or the selective withdrawal of trophic interactions within the ganglion as cells die, should be explored as alternate possibilities to explain the sequence of neuronal death in *Manduca*.

Thus, the CNS of the moth *Manduca sexta* retains the ability to show the normal progression of neuronal degeneration as well as its sensitivity to steroid regulation when maintained in vitro. It could serve as a model system to explore the trophic actions of steroids on central neurons.

References and Notes

1. A. P. Arnold and R. A. Gorski, *Annu. Rev. Neurosci.* **7**, 413; M. W. Cowan, *Int. Rev. Physiol. Neurophysiol.* **17**, 149 (1978); V. Hamburger and R. W. Oppenheim, *Neurosci. Comment.* **1**, 39 (1982); R. W. Oppenheim, in *Studies in Developmental Neurobiology: Essays in Honor of Viktor Hamburger*, W. M. Cowan, Ed. (Oxford Univ. Press, New York, 1981), p. 74.
2. J. W. Truman, *J. Comp. Neurol.* **216**, 445 (1983).
3. _____ and L. M. Schwartz, *J. Neurosci.* **4**, 274 (1984).
4. Abdominal ganglia from segments A3, A4, or A5 were removed from surface-sterilized *Manduca* and cultured according to the procedure developed for epidermis [L. M. Riddiford, A. T. Curtis, K. Kiguchi, *Tissue Culture Assoc. Manual* **5**, 975 (1979)]. Ganglia were rinsed several times in sterile Grace medium (Grand Island Biological) and incubated in 1-ml culture wells at 25°C under a 95 percent O_2–5 percent CO_2 atmosphere.
5. H. M. Taylor and J. M. Truman, *J. Comp. Physiol.* **90**, 373 (1974).
6. A number of the motoneurons could be unambiguously identified on the basis of cell body location (*2*). We used MN-11 and the three D-IV motoneurons that innervate the ventral internal muscles and the MN-12 motoneuron that innervates the dorsal external 5 muscle (R. B. Levine and J. W. Truman, *J. Neurosci.*, in press). Neurons that were examined as examples of cells that do not die in vivo were MN-1, MN-4, MN-5, and cells 24 to 27 (*4*) [P. H. Taghert and J. W. Truman, *J. Exp. Biol.* **98**, 385 (1982)]. None of these cells died in vitro.
7. In making the cell counts, the number of neuro-

nal profiles that contained a portion of the nucleus was counted for each section. This technique overestimates the number of cells because a cell may be counted in more than one section. However, because the cells do not change in size during this period, this technique adequately portrays the relative changes in the number of cells.

8. Stock solutions of 20-HE (Sigma and Rhoto Pharmaceutical) were made in 10 percent isopropanol in sterile distilled water, and a measured amount was then added to the Grace medium. Ecdysteroid concentrations in the medium were similar to the levels in the blood on the last day of adult development [W. E. Bollenbacher *et al.*, *Gen. Comp. Endocrinol.* **44**, 302 (1981); J. W. Truman *et al.*, *J. Insect Physiol.* **29**, 895 (1983)].
9. L. M. Schwartz and J. W. Truman, *Dev. Biol.* **99**, 108 (1983).
10. ———, *Science* **215**, 1420 (1982); *J. Exp. Biol.* **111**, 13 (1984).
11. We thank L. M. Riddiford and D. Morton for a critical reading of the manuscript. Supported by grants from NIH, The McKnight Foundation, and NSF.

2 July 1984; accepted 4 March 1985

Reexamination of Glucose-6-Phosphatase Activity in the Brain in Vivo: No Evidence for a Futile Cycle

Abstract. *Glucose-6-phosphatase activity in the rat brain in vivo was estimated by measuring the differential loss of tritium and carbon-14 from the glucose pool labeled by a mixture of [2-^{3}H]glucose and [U-^{14}C]glucose. The results provide no evidence of significant dephosphorylation of glucose-6-phosphate and do not support the hypothesis of a futile cycle involving glucose-6-phosphatase activity in the brain.*

THOMAS NELSON
GIOVANNI LUCIGNANI
STEVEN ATLAS
ALISON M. CRANE
GERALD A. DIENEL
LOUIS SOKOLOFF
Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bethesda, Maryland 20205

It is widely believed that the mammalian brain is not a gluconeogenic organ and contains negligible glucose-6-phosphatase (G6Pase) activity (*1*). The presence of G6Pase in the brain has been demonstrated by histochemistry (*2, 3*), but quantitative assays of the enzyme activity in cerebral tissues in vitro have usually shown it to be a small fraction of that of known gluconeogenic organs, such as liver and kidney (*4, 5*), and supported the view that G6Pase activity has little, if any, role in the carbohydrate metabolism of the brain. This belief was recently challenged by Huang and Veech (*6*), who reported that there was sufficient G6Pase activity in the rat brain to maintain a steady-state hydrolysis of glucose-6-phosphate (G6P) equal to at least 35 percent of its rate of formation by hexokinase-catalyzed phosphorylation of glucose. Since the brain has a very high rate of glucose utilization (*7*), a rate of dephosphorylation of G6P equal to one-third the rate of glucose phosphorylation by hexokinase would represent a level of G6Pase activity approaching that found in the rat liver after feeding (*8*).

Huang and Veech injected a mixture of [2-^{3}H]glucose and [U-^{14}C]glucose into one carotid artery of rats, removed the brains by freeze-blowing (*9*) at various times up to 5 minutes later, separated free glucose from the tissue, and determined its ^{3}H/^{14}C ratio by liquid scintillation counting. The principle behind these procedures is as follows. Any [2-^{3}H]glucose metabolized as far as fructose-6-phosphate in the glycolytic pathway loses most of the ^{3}H label, but [^{14}C]fructose-6-phosphate retains the ^{14}C (*8*). Some fructose-6-phosphate is converted back to G6P by reversal of the reaction catalyzed by hexosephosphate isomerase, and, if there is G6Pase activity, the glucose moiety is returned to the free glucose pool with its ^{14}C but without ^{3}H. The ^{3}H/^{14}C ratio in the free glucose pool should then decline progressively with time if there is G6Pase activity. Huang and Veech reported such a decline, suggesting previously unsuspected levels of G6Pase activity in the brain.

A key issue is the purity of the free glucose pool in which they measured the ^{3}H/^{14}C ratio. Most products of glucose metabolism beyond the G6P step lose the ^{3}H but not the ^{14}C label; if any of these products were to contaminate the glucose fraction, they would lead to low ^{3}H/^{14}C ratios. In their experiments Huang and Veech relied on Dowex 1-formate and Dowex 1-borate column chromatography of perchloric acid extracts of the brain tissue for purification of the glucose. In a subsequent study (*10*) they added derivatization of the glucose fraction by hexokinase-catalyzed phosphorylation.

We repeated the experiments of Huang and Veech (*6*) but with special efforts to ensure the purity of the glucose fraction in which the ^{3}H/^{14}C ratio was measured. Normal male Sprague-Dawley rats (330 ± 9 g) were anesthetized with pentobarbital (30 mg/kg, intraperitoneally), and one femoral artery and one external carotid artery were catheterized in each animal. The carotid catheter was inserted 5 mm past the carotid bifurcation into the common carotid artery. Both catheters were secured by ligatures. In one group of seven rats the internal carotid artery contralateral to the side of catheterization was ligated as in the studies of Huang and Veech (*6*). Because these animals occasionally exhibited neurological and behavioral defects at the time of the experiment, a second group of eight animals was similarly prepared except that the contralateral internal carotid artery was not permanently ligated but was encircled with a loose ligature that was temporarily pulled taut during injection of the labeled glucose through the carotid catheter to ensure bilateral distribution of tracer.

Approximately 24 hours after surgery a mixture of 50 μCi of [2-^{3}H]glucose, 5 μCi of [U-^{14}C]glucose (*11*), and 5.1 m*M* glucose in 0.05 ml of 0.9 percent saline was injected through the carotid catheter. At various times between 2 and 7 minutes after the injection the brain was removed by freeze-blowing (*9*). Several blood samples were drawn from the femoral artery at various times during the interval between the injection of labeled glucose and freeze-blowing.

The frozen brains were powdered under liquid nitrogen in a cryostat at −35°C and perchloric acid (0.6*M*) extracts of the brain tissue and plasma were prepared. Acidic metabolites were removed from the neutralized supernatant fractions by passage through columns containing 2 ml of Dowex AG 1-X8 formate (200 to 400 mesh). After adjustment of the *p*H of the effluent to 4.0, basic metabolites were removed by cation-exchange chromatography on columns with 2 ml of Dowex AG 50-X8 H^+ (200 to 400 mesh). The final effluent contained approximately 96 percent of the glucose originally present in the perchloric acid extracts. Tritiated water released by the metabolism of [2-^{3}H]glucose was also present.

The extracts, now depleted of all anionic and cationic metabolic products of glucose metabolism, were evaporated to dryness to eliminate the [^{3}H]H_2O and chromatographed as 4-cm bands on Whatman 3MMChr paper in the ascending direction with a solvent system consisting of isobutyric acid, water, and concentrated ammonium hydroxide (66:33:1 by volume). [^{14}C]Glucose standards were chromatographed in parallel lanes on the same chromatographic papers. Bands migrating to the same position relative to the solvent front (R_F) as authentic [^{14}C]glucose were eluted with water; this eluate contained 25 to 40