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The Regulation of Transmitter Expression in Postembryonic Lineages in the Moth *Manduca sexta.* II. Role of Cell Lineage and Birth Order

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The expression of GABA is restricted to the progeny of only six of the 24 identified postembryonic lineages in the thoracic ganglia of the tobacco hornworm, *Manduca sexta* (Witten and Truman, 1991). It is colocalized with a peptide similar to molluscan small cardioactive peptide B (SCP_B) in some of the neurons in two of the six lineages. By combining chemical ablation of the neuroblasts at specific larval stages with birth dating of the progeny, we tested whether the expression of GABA and the SCP_B-like peptide was determined strictly by cell lineage or involved cellular interactions among the members of individual clonal groups.

Chemical ablation of the six specific neuroblasts that produced the GABA-positive neurons (E, K, M, N, T, and X) or of the two that produced the GABA+SCP_B-like–immunoreactive neurons (K, M) prior to the generation of their lineages resulted in the loss of these immunoreactivities. These results suggest that regulation between lineages did not occur. Ablation of the K and M neuroblasts after they had produced a small portion of their lineages had no effect on the expression of GABA, but did affect the pattern of the SCP_B-like immunoreactivity. Combining birth-dating techniques with transmitter immunocytochemistry revealed that it was the position in the birth order and not interactions among the clonally related neurons that influenced the peptidergic phenotype. These results suggest that cell lineage is involved in establishing the GABAergic phenotype and that both cell lineage and birth order influence the determination of the peptidergic phenotype.

The effect of lineage truncation on a morphological phenotype, axonal trajectory, also was examined for the K-lineage neurons. This lineage contains neurons equally divided into two morphologically distinct groups based on the projection of their axons (ipsilateral or contralateral). When the lineage size was reduced, the 1:1 ratio of ipsilaterally to contralaterally projecting neurons was maintained. These results suggest that the two siblings from the division of a single ganglion mother cell were making alternate growth choices. Based on our observations of the regulation of both transmitter and morphological phenotypes, we propose a model for the pattern of production of neurons in the K lineage. Our results suggest that similar developmental mechanisms used to influence transmitter and morphological phenotypes in the earliest-born members of a lineage also are used to determine the fates of the later-born members of very large clonal populations.

Evidence from studies on identified neuronal lineages in invertebrates has implicated both cell lineage and birth order within a lineage as important factors in regulating transmitter expression. Previous studies on grasshopper embryos showed that the median unpaired neuroblast (NB) generated about 80 progeny, all of which shared the properties of staining with neutral red (Goodman et al., 1979). Also, the largest of the cells, which were the first born, were shown to contain octopamine through direct chemical measurements. The later-born cells in the lineage were too small for direct measurements, but it appeared that cells that shared a common origin might be likely to share common characteristics.

In a subsequent study in the locust nervous system, Taghert and Goodman (1984) reported that only a subpopulation of the progeny of another identified NB (7-3) expressed a serotonergic phenotype. In the embryo, NB 7-3 divides three times, generating six neurons before it dies. Using antisera raised against 5-HT, they showed that this transmitter was expressed only in the progeny of the first two cell divisions of the NB, suggesting that birth order also was involved in establishing phenotypes. Furthermore, the pattern of immunoreactivity within the lineage varied according to segmental location. The heterogeneity in the expression of the serotonergic phenotype suggested that both lineage and extrinsic factors may be involved in regulating transmitter expression.

NB ablation studies have provided more evidence that cell lineage is crucial for regulating transmitter expression. Work by Sulston and Horvitz (1977) in the nematode, Stuart et al. (1987) in the leech, and Taghert and Goodman (1984) in the locust showed that, if the appropriate neuronal stem cell is removed, the nervous system does not compensate and the transmitter phenotype (dopamine or 5-HT) is lost from that region of the CNS.

In insects, transmitter determination has been studied only in the earliest-born progeny of identified lineages. It is not known whether the same mechanisms also will apply to the establishment of transmitter identity in neurons produced at a later time in very large lineages (greater than 100 cells). It is certainly possible that there may be more flexibility in transmitter determination in the later-born neurons.

In the moth *Manduca sexta,* most of the neurons in the adult nervous system are born during larval life (Booker and Truman, 1987a). They arise in discrete clusters, each of which comprises

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the progeny of a single, identifiable NB. Although the cells in each cluster are born at various times during larval life, they undergo their final maturation in synchrony at metamorphosis (Booker and Truman, 1987a,b; Witten and Truman, 1991). The advantages of studying the determination of cell fates such as transmitter expression in the postembryonic system are (1) the postembryonic NBs and associated lineages can be easily identified and manipulated; (2) many of the postembryonic clonal families are large, consisting of over 100 neurons; and (3) neurons within a given lineage tend to share features in common, but there is heterogeneity such as the presence of putative cotransmitters within the progeny of a given lineage. We report here that, in these large lineages, the NB of origin and the birth order within a lineage appear to play major roles in establishing the characteristic of transmitter phenotype.

Materials and Methods

Animals. Rearing and staging of the animals was done as described in Witten and Truman (1991).

Neuroblast ablations. Neuroblasts (NBs) generate their postembryonic lineages from the end of the second instar (II) through pupal day 2 (P+2), when they die (Booker and Truman, 1987a). To reduce the size of the lineages, the NBs were chemically ablated at specific stages by injecting larvae with the DNA synthesis blocker hydroxyurea (HU) following the protocol of Truman and Booker (1986). Caterpillars were injected on either day 1 of the third instar (III+1) or on day 0 of the fourth (IV+0) or fifth instars (V+0) with 9.5 mg HU/gm body weight via the abdominal horn. After about 1 d, the treated larvae recover from the drug, resume feeding, and eventually undergo metamorphosis, transforming into adults that appear normal externally and behave normally except they lack most of their postembryonic neurons.

Immunochemical and histochemical methods. The effect of lineage truncation on transmitter phenotype was assessed in animals just before adult emergence (pharate adults) using the immunohistochemical methods described in Witten and Truman (1991). Counts of total cells in the lineage were obtained independently from the immunochemical data. Ganglia from control and treated animals were fixed, dehydrated, embedded in paraffin, sectioned, and stained with hemotoxylin and eosin as described in Truman and Booker (1986) and Witten and Truman (1991). Raw counts from the serially sectioned material were corrected using the method of Abercrombie (1946).

Birth-dating methods. To label neurons born at specific larval stages, we used 5-bromodeoxyuridine (BUdR; Sigma, St. Louis, MO) incorporation combined with immunohistochemistry using a monoclonal antibody against BUdR (Gratzner, 1982; Becton-Dickinson, Mountain View, CA) as described in Witten and Truman (1991). Various concentrations of BUdR (0.1, 0.5, 1, 2, and 10 mg/ml) were administered to feeding III, IV, and V instar larvae by placing it in their artificial diet. A stock solution of BUdR (10 mg/ml of 40% ethanol) was prepared, and the appropriate amount was then mixed into the freshly made diet. After feeding on labeled diet for various periods, the larvae were placed on normal diet and permitted to complete growth and metamorphosis. Patterns of BUdR incorporation then were assessed in the pharate adult in combination with transmitter immunohistochemistry. The optimum concentration for survival and labeling was 0.5 mg/ml of diet (data not shown).

Depending upon the transmitter, the tissue was fixed either in 4% paraformaldehyde (peptide) or in a mixture of glutaraldehyde, picric acid, and acetic acid (GABA) as described in Witten and Truman (1991), and then postfixed in Carnoy's solution for 2 hr at room temperature. The tissue was processed sequentially, first for the BUdR labeling and then for transmitter localization. First, membranes were permeabilized, and endogenous peroxidases were blocked according to the methods of Witten and Truman (1991). Next, the tissue was incubated in 2*N* HCl in 10 mM sodium phosphate buffer with 0.9% NaCl and 0.3% Triton X-100 (pH, 7.4; PBS-X) for 60 min to denature the DNA. Following rinsing and then blocking of nonspecific staining with a 10% normal goat serum (NGS) for 2 hr, the preparations were incubated in the anti-BUdR serum at a 1:200 dilution in PBS-X containing 1% NGS for 2–3 d at 4°C. Subsequent rinsings, incubations with the secondary antiserum (biotinylated horse anti-mouse IgG; Vector Labs, Burlingame, CA) and the avidin-biotin complex conjugated to horseradish peroxidase (ABC–HRP; Vector Labs), and visualization with diaminobenzidene (DAB) followed the protocol previously described (Witten and Truman, 1991) with one modification: nickel chloride (0.04%) was added to the DAB solution to produce a black peroxidase reaction product. The black nuclear staining could be distinguished easily from the pale brown cytoplasmic reaction product formed during the subsequent transmitter immunochemical processing.

Following the DAB reaction for BUdR localization, the preparations were incubated in a 3% hydrogen peroxide PBS-X solution for 15 min. Next, they were incubated in either a 1:20 dilution of anti-SCP_B (gift of Dr. A. O. D. Willows, University of Washington) or a 1:5000 dilution of anti–GABA–keyhole limpet hemocyanin [KLH; gift of Drs. T. Kingan (USDA, Beltsville, MD) and J. Hildebrand (University of Arizona)] and processed for transmitter immunohistochemistry as detailed in Witten and Truman (1991).

Results

Relationship of transmitter expression to cell lineage

Postembryonic neurogenesis proceeds from the end of the second instar through P+2, when the NBs die. The differentiation of these new neurons is arrested shortly after their birth, and their maturation resumes when the steroid hormone 20-hydoxyecdysone, in the absence of juvenile hormone, triggers metamorphosis (Booker and Truman, 1987a,b). Transmitter expression begins after the larval–pupal transition, during the early phases of adult development (Fig. 1; Witten and Truman, 1991). In the preceding paper (Witten and Truman, 1991), we showed that the expression of two putative transmitters, GABA and an SCP_B-like peptide, is restricted to the progeny of only a subset of the 24 identified NBs in the thoracic ganglia. GABA-immunoreactive neurons are found in the E, K, M, N, T, and X lineages, and the SCP_B-like peptide immunoreactivity is colocalized with the GABA-positive neurons in the K lineage and the M lineage in the first abdominal ganglion [M(A1)].

The postembryonic lineages were truncated at various points by injection of HU at specific times during larval growth. The results of the treatment were then assessed a few weeks later, after the animals finished metamorphosis. The most extreme effects were seen after HU injection on III+1. Because most of the NBs start dividing only shortly before this time, most lineages in the resulting adults consisted of at most two to four cells rather than the approximately 100 neurons seen in control animals. Typically, a few NBs (10–15%) were not blocked by the treatment, so each ganglion showed a small number of complete lineages among the truncated ones. The NBs that escaped the HU treatment varied in an apparently random fashion from ganglion to ganglion and from animal to animal. The ability to delete randomly most of the postembryonic lineages allowed us to examine the effects of these lesions on transmitter selection in the few remaining, complete lineages. In these preparations, the expression of GABA and SCP_B-like immunoreactivities was still restricted to the same six lineages as seen in control animals (Fig. 2). GABA was found only in the three paired ventral (K, M, and N), two paired dorsal (E, T), and the one unpaired dorsal lineage (X; $n = 20$). The SCP_B-like immunoreactivity was present only in the K and M(A1) lineages ($n = 25$). In moths that were missing these particular postembryonic lineages, none of the remaining lineages began to produce GABA- or SCP_B-like–expressing cells to compensate for this lack.

The deletion of the postembryonic lineages had no effect on GABA expression in neurons of embryonic origin. The distribution of GABA immunoreactivity in these groups of large neurons was the same in both control and HU-treated individuals (Fig. 2*A, B*). These results also confirm that HU treatment

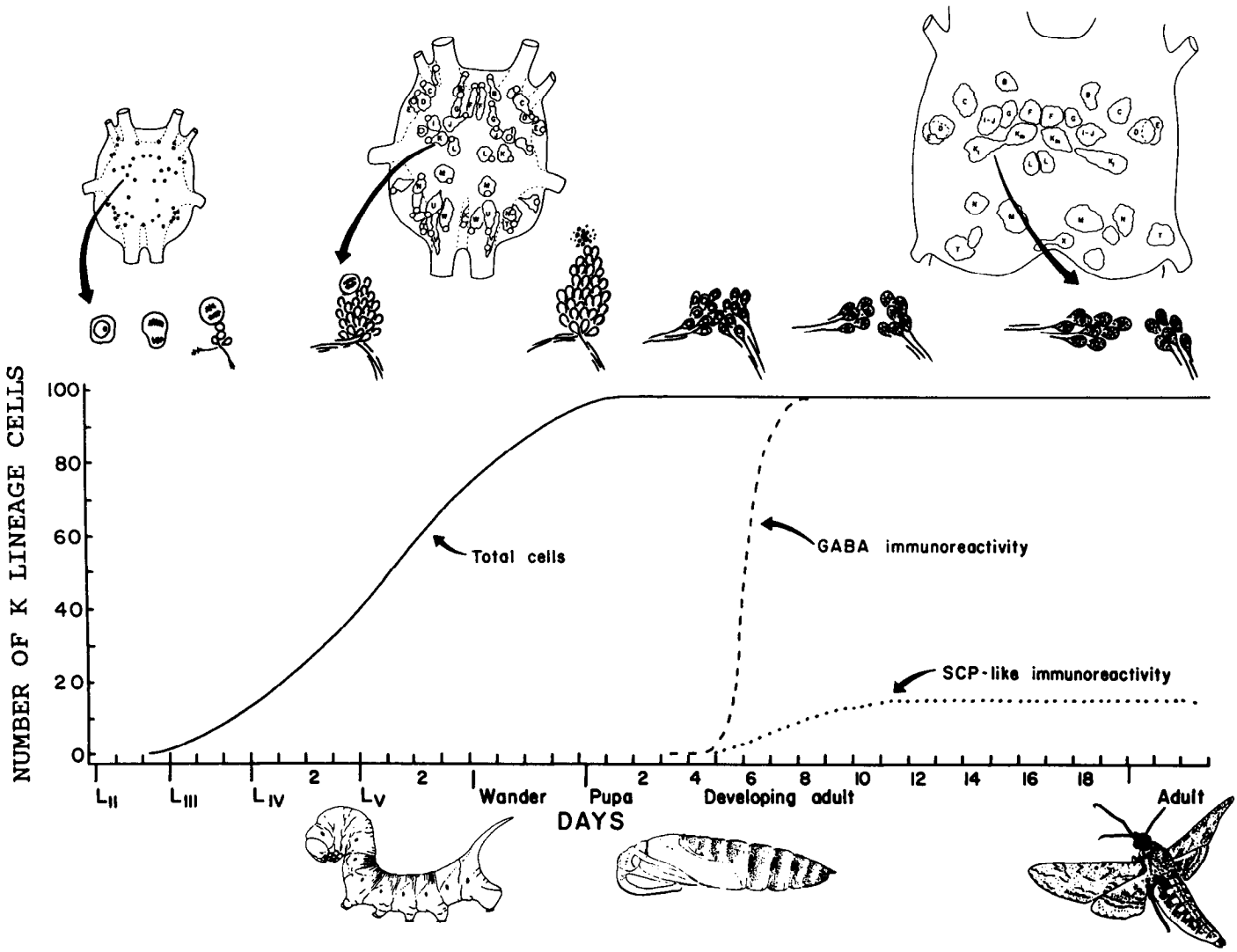

Figure 1. Developmental timetable for birth and transmitter differentiation of the postembryonic K-lineage neurons. Schematic drawings of the T2 ganglia at the *top* show the relative positions of the postembryonic NBs and their lineages during larval life [second (II) and fifth (V) instars] and in the adult. The birth and differentiation of the K-lineage neurons is illustrated (*arrow* points to the position of K NB and lineage). The histogram on the *bottom* illustrates the timetable for the birth of the K-lineage cells and when transmitter expression occurs. The K NB commences to divide at the end of L_{II} (*far left*) and continues producing neurons until its death early in adult development (P+2). After their birth, the postembryonic neurons begin to differentiate and extend short processes into the neuropil. Even at this early point in their development, the K-lineage neurons can be divided into two morphologically distinct subgroups based on the trajectories of their axons (ipsilateral or contralateral). The development of the postembryonic neurons is arrested at this partially differentiated state, and their maturation resumes at the onset of metamorphosis. Expression of GABA and the SCP_B-like peptide is not present in the developmentally arrested neurons (*open cells*). Immunoreactivity for both putative transmitters is first detected in the K-lineage neurons after metamorphosis begins, at days 5–6 of adult development (*stippled cells*).

during larval life has no adverse effects on nonmitotic cells, such as the embryonically derived neurons.

Effects of lineage size on transmitter expression

We examined the effect of lineage truncation on transmitter expression by cells in the K and M(A1) lineages. The K and M(A1) NBs begin dividing at the end of the second instar and generate lineages containing approximately 100 neurons. All the neurons express GABA immunoreactivity, but only a subset of approximately 14 (K lineage) or 15 (M lineage) cells co-express the SCP_B-like peptide (Witten and Truman, 1991). Injection of HU into III+1, IV+0, or V+0 caterpillars reduced the size of the K lineage in the pharate moth from 95 ± 7.3 cells (mean ± SEM; $n = 10$) to 3 ± 0.8 ($n = 21$), 9 ± 0.6 ($n = 21$), and 40 ± 2 ($n = 25$) cells, respectively (Fig. 3). These values represent the number of K-lineage cells that had been produced up to the time of HU treatment. In every case, all of these cells subsequently expressed GABA immunoreactivity after metamorphosis (Fig. 3). Thus, the HU treatment did not interfere with the subsequent maturation of cells that were already born. Moreover, these cells would express their GABA phenotypes despite the lack of their later siblings. Although the cell counts of the GABA-immunostaining neurons following the HU treatment (10.5 ± 3, $n = 22$; 17 ± 2, $n = 21$; 46 ± 3, $n = 25$) are slightly higher than total counts, this is attributable to the underestimation of these cells from hematoxylin- and eosin-stained sectioned material.

In contrast to the GABA data, the number of cells expressing

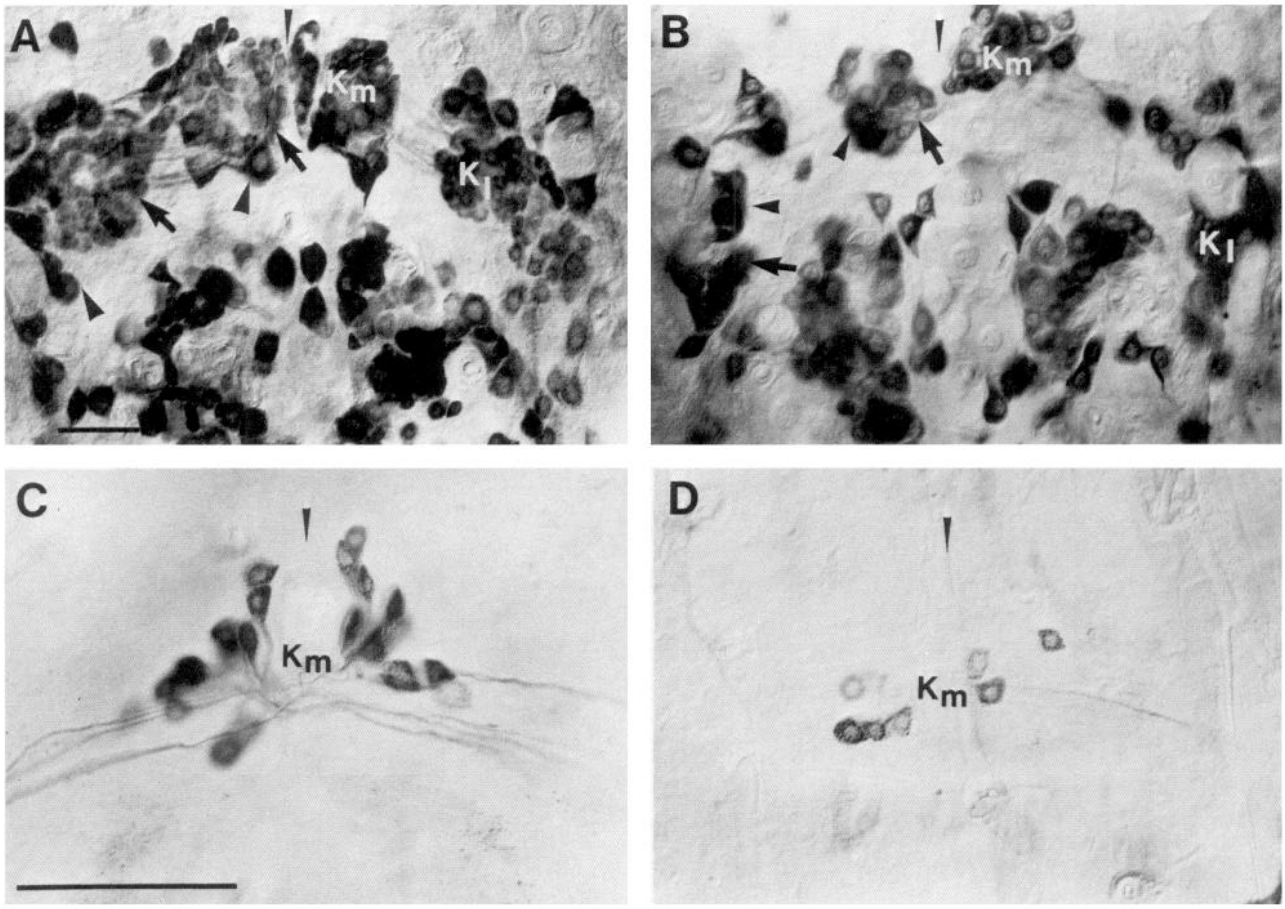

Figure 2. Whole-mount preparations of K- and M-lineage neurons in adult T2 ganglia from control (*A, C*) and HU-treated (*B, D*) animals stained with GABA (*A, B*) or SCP_B antisera (*C, D*). In the normal adult, the K lineage contains 100 cells equally divided into the medial (K_m) and lateral (K_l) subgroups. All of these neurons express GABA (*A, arrows*). After HU treatment at the beginning of the fourth instar, less than 20 postembryonic cells remain (*B, arrows*). They are surrounded by the larger embryonically derived GABAergic neurons (*wide arrowheads*). The number of SCP_B-like–immunoreactive K-lineage neurons (K_m) is also reduced from the normal number of 14 following this treatment paradigm (*C, D*). Ventral surface is shown; anterior is at the *top*. The *narrow arrowheads* indicate the midline of the ganglion. Scale bars, 100 μm.

SCP_B-like immunoreactivity in the K lineage did not increase proportionately to the total number of cells. (Fig. 3). Larvae that were injected with HU on V+0 subsequently produced the full adult complement of 14 ± 0.3 (n = 40) SCP_B-like–immunoreactive neurons despite the fact that only 40% of the lineage had been generated.

The effect of HU treatment on SCP_B-like expression in the M(A1) lineage differed from that seen in the K lineage. Normally, 15 SCP_B-like–immunoreactive cells are found in this lineage, but significant numbers were not detected in any of the three treatment groups (III: 0.75 ± 0.75, n = 26; IV: 0.2 ± 0.1, n = 32; V: 0.1 ± 0.1, n = 26; Fig. 4). These very low values with high SEMs are due to the rare occurrence (two preparations) of a few peptide-immunoreactive M-lineage neurons in the approximately 30 HU-treated ganglia examined. The presence of these few immunostaining neurons is most likely due to a few NBs initially escaping the HU treatment and producing truncated lineages prior to their death, as was reported by Truman and Booker (1986). There are two possible mechanisms to account for the patterns of SCP_B-like expression in these two lineages: (1) transmitter expression may be a function of birth order with the SCP_B-like cells being among the first to be born in the K lineage (during the third and fourth instars) or late in the M lineage (during the fifth instar), or (2) interactions among lineage cells at metamorphosis may determine expression with the SCP_B-like phenotype being preferred in the K lineage and a less preferred phenotype in the M-lineage cells. To distinguish between these possibilities, we birth-dated the progeny of the K and M NBs using BUdR to label dividing cells. The results of the birth-dating experiments are detailed below.

Relationship of transmitter expression to birth order

To determine the time during larval life when the GABA and GABA + SCP_B-like neurons are born, transmitter immunocytochemistry was used in conjunction with BUdR birth-dating. As expected, the GABAergic cells are born throughout postembryonic life, commencing at the beginning of the third instar and ending early in the pupal stage (P+2), when the NB dies. The birth dates of the subset of K- and M(A1)-lineage neurons that also expressed the SCP_B-like peptide were not distributed randomly during the different larval stages, but were found clustered together. K-lineage neurons that showed both BUdR incorporation and anti–SCP_B-like immunoreactivity were seen

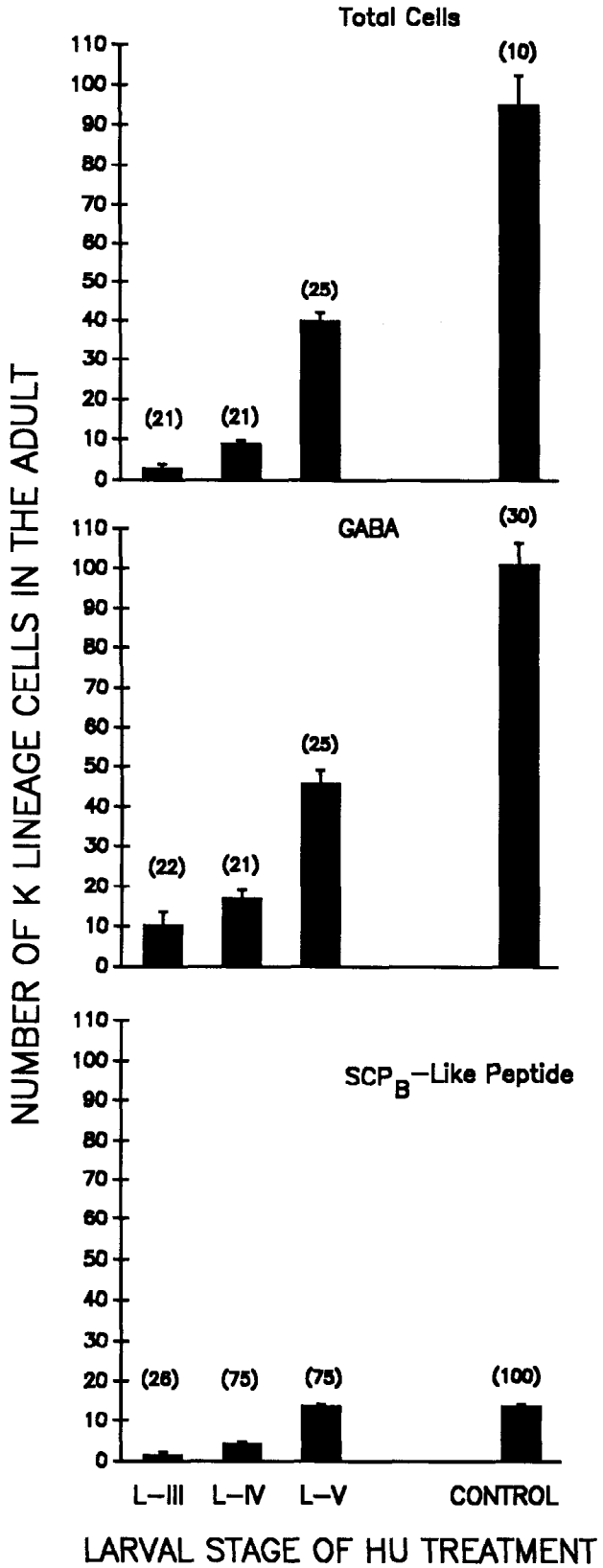

Figure 3. Cell counts of K-lineage neurons in adults following NB ablations at specific larval stages. NBs were ablated by injection of HU at the beginning of III, IV, and V larval stages (L). Cell counts were made in pharate adults after the postembryonic neurons had matured and were expressing transmitters. In the untreated adult, the K lineage contains approximately 100 neurons, all of which express GABA immunoreactivity, and 14 of which co-express GABA and SCP_B-like immunoreactivities (*control* values). NB ablations reduced the size of the K lineage. Total cell counts were obtained from sectioned material (*top*). Lineage truncation did not alter the expression of the GABAergic phenotype because all neurons regardless of the size of the lineage were GABA immunopositive (*center*). The number of SCP_B-like–immunoreactive neurons, however, did not continue to increase proportionally as the total number of neurons increased (*bottom*). Rather, the full complement of peptide immunoreactive cells were produced following HU injection at L-V. Values represent mean and SEM; the numbers of preparations examined are indicated in *parentheses*.

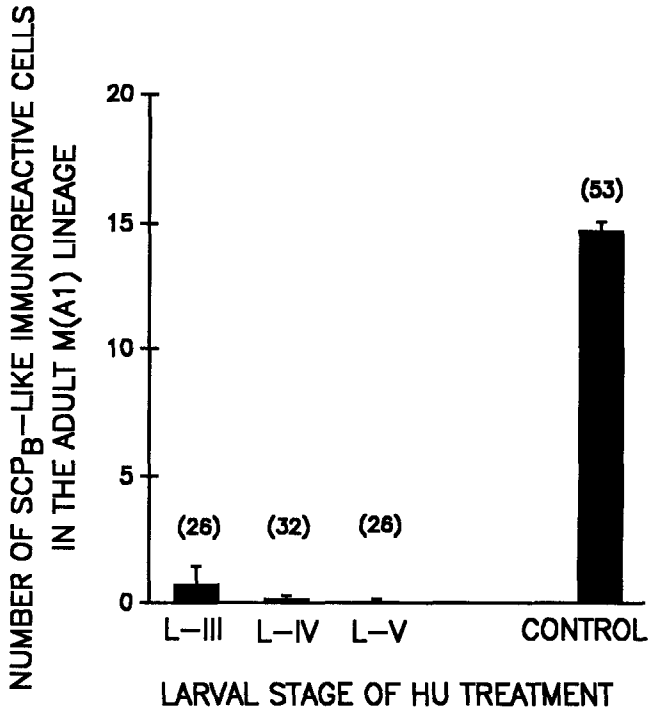

Figure 4. Effect of HU treatment during larval life on the total number of SCP_B-like–immunoreactive neurons in the adult M(A_1) lineage. Truncation of the M(A_1) lineage at the onset of the L-III, L-IV, or L-V larval instars significantly reduced or eliminated all SCP_B-like immunoreactivity. Values represent mean and SEM; numbers of preparations examined are in *parentheses*.

primarily when BUdR was administered during the fourth larval stage (Figs. 5, 6). Only occasionally did feeding BUdR in the third stage result in double-labeled cells, while SCP_B-like–immunoreactive neurons were never labeled by feeding during the fifth instar. In contrast to the K lineage, the SCP_B-like–immunoreactive cells in the M(A1) lineage showed BUdR incorporation only during the fifth larval instar (Fig. 5). Thus, the birth dates for the SCP_B-like cells in K and M(A1) are at different times, during the fourth and fifth larval instars, respectively.

Effect of lineage size on axonal trajectory

In addition to the studies of the influence of cell lineage and cellular interactions on the establishment of transmitter determination described above, we also examined the role of cellular interactions on determination of a morphological phenotype, axonal trajectory. The K NB generates approximately 100 neurons that are divided equally into two subgroups based on the tracts in which their primary neurites insert and the positions of the somata in the adult ganglion (Witten and Truman, 1991). These initial neurite trajectories are established in the larval

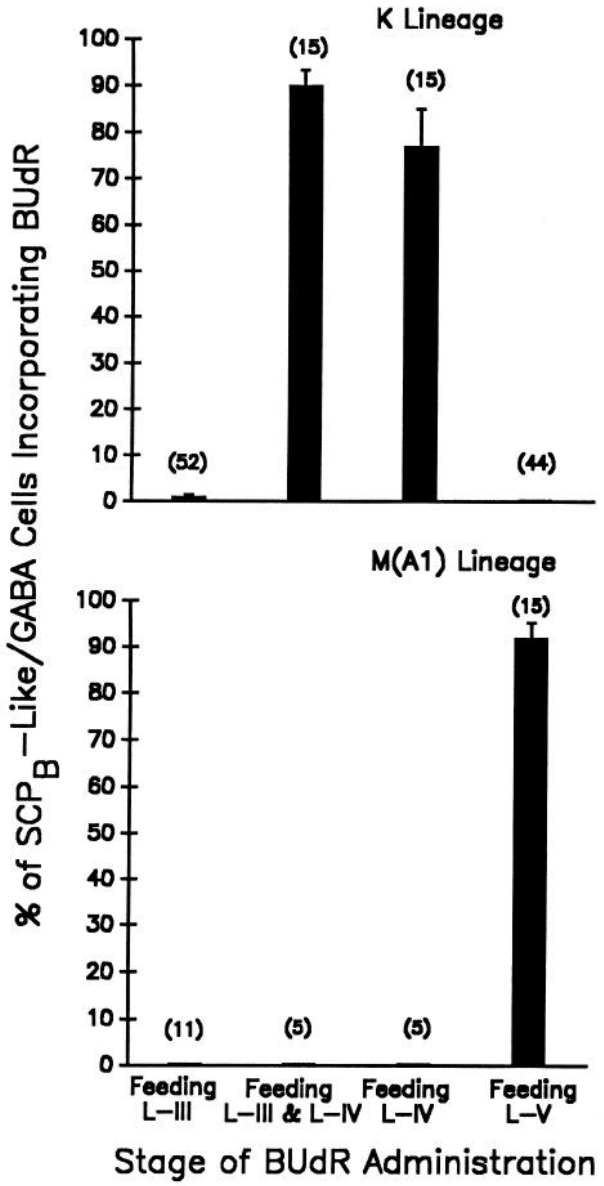

Figure 5. Birth-dating of the SCP_B-like neurons in the adult K and M(A1) lineages. Larvae were fed a diet containing 0.5 mg BUdR/ml during L-III, L-IV, and L-V. SCP_B-like immunoreactivity was found in adult K-lineage neurons only when larvae were fed BUdR during L-III and L-IV (*top*). The M-lineage peptide-immunopositive cells, however, are born later, during the feeding stage of L-V (*bottom*). Thus, the SCP_B-like–immunoreactive neurons in these two lineages are born at different times during larval life. Values represent mean and SEM; number of preparations examined are in *parenthesis*.

stage soon after the neurons are born: half of the cells project contralaterally into the ventral medial tract, and the others project ipsilaterally into a lateral tract. As the neuropil enlarges during metamorphosis, the two groups are separated physically so that somata of the contralaterally projecting neurons are in a medial cluster of the K lineage (K_m) and the ipsilaterally projecting cells are in a lateral group (K_l). All the K-lineage neurons express GABA. We examined whether lineage truncation influenced the determination of axonal trajectory by counting the number of GABA-immunoreactive neurons present in the two K sublineages. Regardless of the stage at which the NBs were

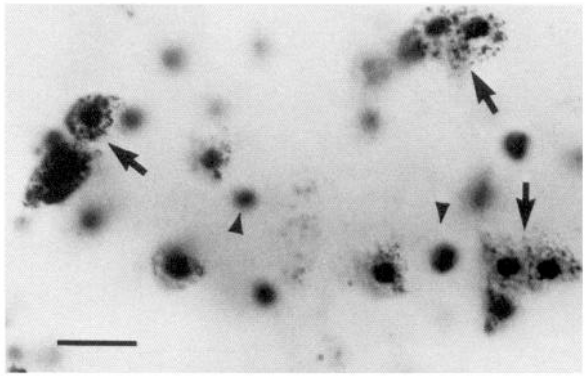

Figure 6. Photograph of SCP_B-like and BUdR double-labeled K-lineage neurons from an adult T2 ganglion. This ganglion is from an adult that was fed BUdR during L-III and L-IV. All K-lineage SCP_B-like–immunopositive neurons also have labeled nuclei showing that they were born during these two larval stages (*arrows*). The other labeled nuclei (*arrowheads*) belong to K-lineage neurons born at this time that do not express immunoreactivity to this peptide. Scale bar, 20 μm.

ablated, there tended to be approximately equal numbers of neurons in the K_m and K_l subgroups (Fig. 7).

Discussion

In the adult moth, only six of the 24 postembryonic lineages (E, K, M, N, T, and X) contain neurons immunoreactive to GABA, and two of these lineages [K, M(A1)] also produce SCP_B-like–immunoreactive cells. The SCP_B-like immunoreactivity is colocalized with GABA in a small percentage of K and M cells. By ablating the NBs at various times in postembryonic life, the lineages were truncated or eliminated. Thus, we were able to test whether expression of GABA or the SCP_B-like peptide was dependent on the presence of these specific lineages, and also, if it was influenced by birth order or cellular interactions within a lineage group.

When the six NBs (E, K, M, N, T, and X) that normally produced the GABA- or SCP_B-like–immunoreactive neurons were ablated, no neurons were found in the remaining postembryonic lineages that expressed these immunoreactivities. This suggests that there is no regulation between lineages, at least in the expression of these transmitters. Thus, cell lineage is important for transmitter selection for the postembryonic progeny of the NBs. It seems, therefore, that developmental mechanisms similar to those at work embryonically to establish the characteristics of the early-born progeny (Goodman et al., 1979; Taghert and Goodman, 1984) are also used to determine the transmitter fates of the later-born, postembryonic progeny.

The mechanisms that are involved in establishing transmitter phenotypes within a clonal family are more difficult to assess. A number of mechanisms might be employed alone or in combination: (1) transmitter type could be a rigid function of birth order; (2) all cells could initially be equivalent, and interactions between neurons at the start of metamorphosis would determine which transmitters a cell produces; or (3) the targets of the cells or their afferent inputs could influence the transmitter. The tools that we have used in this study have allowed us to divide the cells into three categories: GABA-negative, GABA-positive, and both GABA- and SCP_B-like–positive. The cells in the K and M(A1) lineages fall into the two latter classes. One interesting finding is that the cells expressing GABA+SCP_B-like immu-

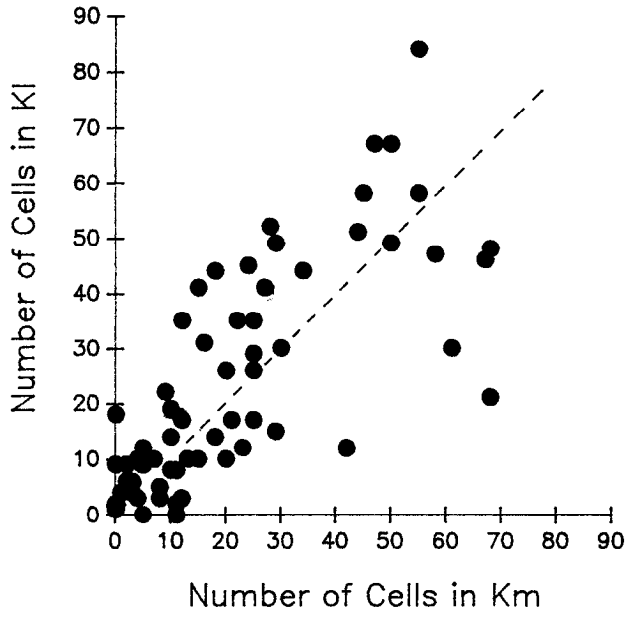

Figure 7. Effect of lineage truncation on the distribution of cells in the adult K lineage subgroups, K_m and K_l. In the adult, the K lineage contains 100 neurons, equally divided into two positionally and morphologically distinct groups. Counts of the number of cells found in the K_m and K_l subgroups in adults that were injected with HU during larval life (L-III, L-IV, or L-V) are plotted. The data closely approximate a 1:1 correspondence as indicated by the dotted line.

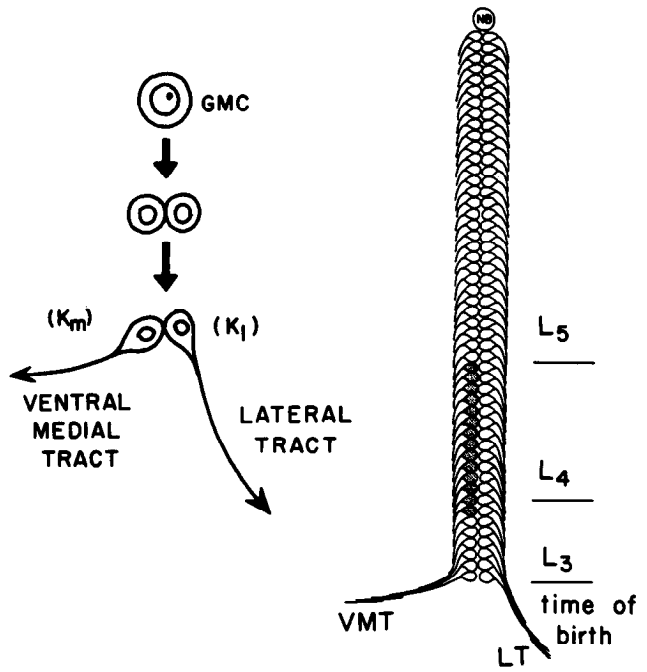

Figure 8. Model for pattern of production of the 100 neurons in the K lineage. During larval life, the K NB divides asymmetrically 50 times, producing 50 GMCs, which then divide once symmetrically, each producing two sibling neurons. *Left,* We propose that the siblings from each GMC division make different growth choices: 50 send axons contralaterally into the ventral medial tract, and the other 50 project ipsilaterally into the lateral tract, thus forming the two K lineage subgroups, K_m and K_l, respectively. *Right,* The K NB and its lineage are illustrated. All K-lineage neurons express GABA (*open cells*). The cells that co-express GABA and the SCP_B-like peptide (*stippled cells*) are clustered within the lineage. They are a subset of the contralaterally projecting neurons (K_m), which are born during the III (L_3) and IV (L_4) instars. Transmitter phenotype, therefore, is a function of both lineage and birth order within the lineage.

noreactivities are not distributed randomly throughout the lineages in which they are found. The BUdR labeling experiments show that they are born during the fourth larval stage in the K lineage and during the fifth larval stage in the M(A1) lineage. Thus, neurons that share particular phenotypes appear to be clustered together.

Chemical ablations of the NBs at various stages reduced the size of the lineages. The few neurons that were produced expressed the transmitters that were expected in cells normally born up to the time of the ablations. For example, when the K NB was arrested at IV+0, only an occasional SCP_B-like–immunoreactive cell appeared, whereas after arrest at V+0, all were present. This response to lineage truncation is consistent with the fourth instar birth dates for the SCP_B-like–positive cells in this lineage. Similarly, in the M(A1) lineage, termination of neuron production at V+0 resulted in almost no SCP_B-like–positive cells in this lineage, though the K lineage showed its full complement under the same treatment. Again, this result is consistent with the fifth instar birth date for the M(A1) peptide-immunoreactive cells. These data show that, once a set of cells have been born, their transmitter expression appears to be independent of whether or not later-born cells are present. It should be noted, however, that our experiments have not ruled out the possibility that early-born cells may effect the phenotype of the later-born progeny.

Within the K lineage, we also documented the effect of lineage truncation on a morphological phenotype, axon trajectory. In the adult, the K lineage consists of 100 cells equally divided into two subgroups. The K_m group, which is in a medial position in the ganglion, has neurons that project contralaterally inserting into the ventral commissure II (VCII) tract. The 50 laterally positioned K_l neurons send their axons ipsilaterally into a lateral tract. When the lineages were truncated, approximately equal numbers of K_m and K_l neurons were still produced. One mechanism for generating the pattern described above is for the two daughters of each ganglion mother cell (GMC) to choose divergent growth responses: one projects ipsilaterally; the other, contralaterally. Such divergent choices among siblings from a given GMC have been described in studies of grasshopper embryos, where interactions between the two sibling neurons (Doe and Goodman, 1985; Doe et al., 1985; Kuwada and Goodman, 1985) are important. An alternate explanation for our findings is that the two daughters of one GMC may grow ipsilaterally, while those from the next GMC project contralaterally. At this time, we cannot exclude either option, but the precedence of the grasshopper data causes us to favor the first hypothesis.

Based on the results from these studies, we propose the following simple model for the pattern of production of neurons in the K lineage (Fig. 8). The K NB divides 50 times during the third through fifth larval instars, generating approximately 100 neurons. Each division is asymmetric, producing an NB and a smaller cell, the GMC, which divides only once to produce two sibling neurons (Fig. 8, left). Following their birth, the two daughters of each GMC make different axonal growth choices: one grows contralaterally into VCII, while the other extends ipsilaterally. Data from the grasshopper embryo suggest that the

decision as to which cell makes which growth response would come about through interactions between the two daughters (Doe and Goodman, 1985; Doe et al., 1985; Kuwada and Goodman, 1985). These sets of interactions are probably repeated about 50 times, once for the daughters of each GMC that is produced. This would result in the two equal subgroups K_m and K_l. Later, during development of the adult, the cells within the K lineage would become further distinguishable as they express their putative transmitters. All express GABA, but only a subset co-express the SCP_B-like peptide. Given the above scheme and the clustering of the birth of these cells, we expect that the GABA+SCP_B-like phenotype is assumed by the contralaterally projecting daughters from 14 successive GMCs that are born during the fourth instar.

Early in embryonic lineages, the progeny from one GMC may be radically different from those of the next. Laser ablation experiments suggest that birth order of the GMCs is of paramount importance in establishing these differences (Doe and Goodman, 1985; Doe et al., 1985; Kuwada and Goodman, 1985). The clustering of phenotypes that we find for the later parts of large lineages does not negate the issue of birth order. Rather, early in lineages the counting "unit" may be a single GMC, whereas later in a lineage it may extend over a block of sequentially born GMCs.

The regulation of transmitter expression in the K lineage appears to be hierarchical, with lineage being the first step followed by birth order within the lineage. The regulation of the expression of GABA and the SCP_B-like peptide in the M lineage, however, adds an additional complication. Only the M-lineage neurons in A1 of the adult express both GABA and the peptide, even though the lineage is represented in all segments (Witten and Truman, 1991). The thoracic M lineages [M(T)], however, do transiently express the SCP_B-like peptide during the early stages of adult development. This immunoreactivity disappears prior to its emergence, and thus adult M(T) neurons are immunopositive for GABA only. This segmental specificity may be conveyed by positional information to the M NB, or extrinsic factors such as hormones, afferent input, and targets may be interacting with cell lineage to produce this segmental specificity of transmitter expression.

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