Octopod, a homeotic mutation of the moth Manduca sexta, influences the fate of identifiable pattern elements within the CNS

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Summary

Octopod (Octo) is a mutation of the moth Manduca sexta, which results in the homeotic transformation of the ventral surface of the first (A1) and less often the second (A2) abdominal segments in the anterior direction. The extent of the transformation ranges from a slight deformation of the ventral cuticle, up to the formation of miniature thoracic legs on A1. The extent of the transformation is always less within A2 as compared to A1. A genetic analysis revealed that Octo is an autosomal mutation which shows incomplete dominance. The effect of this mutation on the central nervous system (CNS) was assessed by examining the distribution and fate of the postembryonic neuroblasts in the segmental ganglia of Octo larvae. In each of the thoracic ganglia of wild-type larvae, there is a set of 45–47 neuroblasts; a reduced but homologous array of 24 and 10 neuroblasts are found in A1 and A2, respectively. Ganglion A1 of Octo larvae had 1 to 6 supernumerary neuroblasts, and 20% of the A2 ganglia showed a single ectopic neuroblast. The supernumerary neuroblasts corresponded to identifiable neuroblasts normally found in more anterior ganglia. The Octo mutation also influenced the mitotic activity of stem cells normally present in A1. In this case, the neuroblasts generated a lineage of cells that were typical of a thoracic location rather than A1. These data demonstrate that homeotic mutations can influence the fate of identifiable pattern elements within the CNS of an insect.

Key words: insect CNS, neurogenesis, homeotic mutant, Octopod, Manduca sexta.

Introduction

The central nervous system (CNS) of adult insects is characterized by dramatic regional differences in neuronal numbers (Bate, 1976; Thomas et al. 1984; Booker & Truman 1987; Truman & Bate, 1988). Typically 4–7 times as many neurons are found in the adult thoracic ganglia compared to the abdominal ganglia. Among the insects there are two strategies employed in generating these regional differences in neuronal number. In those insects that go through an incomplete metamorphosis, such as crickets and grasshoppers, all of the neurons in each segmental ganglion are generated during embryogenesis from a nearly identical array of embryonic neuroblasts (Hartenstein & Campos-Ortega, 1984; Thomas et al. 1984). However, by the end of embryogenesis, there are only small differences in the number of neurons found in the thoracic and abdominal ganglia (Booker & Truman, 1987; Truman & Bate, 1988). The dramatic regional differences in neuronal numbers found in the segmental ganglia of adult moths and flies are produced during the postembryonic period and are due to segmental differences in the number of neuroblasts found within the various regions of the larval CNS. For example, in larvae of Manduca sexta there are 45 to 47 (22 to 23 paired and 1 unpaired) neuroblasts in the thoracic ganglia, while in the typical abdominal ganglion (A3 to A8) there are only 8 neuroblasts (4 paired). The thoracic set of neuroblasts add 3000 to
4000 new neurones to each thoracic ganglion while in the abdominal segments about 100 new cells are added.

Present evidence suggests that the development of both the CNS and the epidermis of insects is influenced by segmental determination and that the genetic control of these processes at least partly overlap. Regions of the bithorax complex (BX-C) and the antennapedia complex are expressed in the CNS at both the level of RNAs, based on in situ hybridization (Akam, 1983; Hafen et al. 1983) and proteins, as shown through the use of antibodies (White & Wilcox, 1984). However, our knowledge of the role of such segmentation genes in the development of the CNS lags behind that of the epidermis. Much of the analysis has been carried out in the CNS of Drosophila melanogaster. In the fruitfly, it has been clearly shown that the branching pattern within the CNS of sensory neurones and the identifiable giant fibre can be altered in a number of homeotic mutations that affect the determined state of thoracic and abdominal segments (Palka et al. 1979; Thomas & Wyman, 1984). Moreover, staining patterns within the CNS can be transformed by certain homeotic mutations (Jimenez & Campos-Ortega, 1981; Teugels & Ghysen, 1983). The report by Green (1981) used identifiable motoneurones within affected regions to examine the role of homeotic mutations on CNS differentiation. Such studies, however, have only begun to scratch the surface. Essentially nothing is known about the influence of the homeotic genes on the fate of the neuronal precursors that generate the dramatic regional diversity in neuronal number and specificity observed within the CNS of holometabolous insects.

Here, we report the isolation of the first homeotic mutation of the moth Manduca sexta. This mutation, named Octopod (Octo), results in the transformation of the ventral surface of the first, and less often the second, abdominal segments in the anterior direction. The mutation is characterized by the appearance of ectopic thoracic legs in both larvae and adults. The array of postembryonic neuromasts within the segmental ganglia of Manduca larvae provide a nearly ideal set of pattern elements for examining the influence of Octo in the segmental determination of the adult CNS of Manduca. Reconstructions of the segmental ganglia of Octo animals reveal that the distribution and fate of specific neuromasts within the segmental ganglia are selectively influenced by the Octo mutation.

Materials and methods

Experimental animals

Larvae of the tobacco hornworm, Manduca sexta, were reared on an artificial diet under conditions of 26°C and a 17L:7D photoperiod as described by Bell & Joachim (1978). Under these rearing conditions approximately 18 days elapse from hatching to pupal ecdysis and 20 days from pupal ecdysis to the emergence of the adult. The ec dyses to the 4th and the 5th larval instars and pupal ecdysis were used as references in staging the insects (4+0, 5+0 and P+0, respectively). The Octo mutation was a spontaneous mutation first noted in our laboratory stock of Manduca sexta in 1985 by Dr Kiyoshi Hiruma. Selection of fifth instar larvae for strong expression of the Octo trait (ectopic leg on the first abdominal segment) coupled with inbreeding for approximately 12 generations has resulted in a stock that shows 100% expression.

As a wild-type stock for the genetic experiments, crosses were made to a laboratory strain carrying the recessive black (bl) mutation (Safranek & Riddiford, 1975). Larvae of this strain show melanization of the cuticle in the fourth and fifth larval stage as a result of abnormally low circulating levels of juvenile hormone. This strain has been inbred for over 17 years and shows no expression of the Octo phenotype.

For the genetic crosses, two to six pairs of freshly emerged females and males of the appropriate genotype were placed in a mating cage with a tobacco plant in a 17L:7D photoperiod at 20–21°C in a humid environment. Eggs were collected daily and transferred to 26°C.

Histological techniques

Ganglia were removed from CO2-anaesthetized animals and fixed overnight in alcoholic or aqueous Bouin’s fixative at room temperature, then dehydrated through a graded ethanol series and embedded in paraffin. Ganglia were serially sectioned at 10 μm, and the sections were stained with haematoxylin and eosin. Estimates of progeny number were determined as reported previously (Booker & Truman, 1987).

Results

The Octo phenotype

Octo larvae show a homeotic transformation of the ventral surface of A1 in the anterior direction. In the most extreme cases, the transformation was manifested in the appearance of complete but miniature thoracic legs on segment A1 (Fig. 1). The expression of the Octo mutation was typically assessed in newly moulted fifth instar larvae. A five point scoring system was devised as follows: 0 = wild-type; 1 = slight deformation of the ventral surface of the first abdominal segments often with a few bristles that are usually found on the thoracic legs; 2 = raised region of cuticle (coxa); 3 = coxa with patches of melanized cuticle; 4 = at least two well-formed distal leg segments. Often the appendages on the two sides of A1 showed different levels of expression. In such cases, the larva was assigned the higher of the two scores received. In most instances, there was no more than a one point difference in the score received by the two ectopic structures. In the homozygous condition, the penetrance of the phenotype is complete with the majority of the animals (~85%) receiving a score of 3 or 4. In a group of animals who were tracked from hatching through the moult to the fifth larval instar, there was no change in the level of expression of the Octo phenotype throughout larval life. The extent of the transformation of adult structures mirrored that seen in the larvae.

A small percentage (~10%) of the homozygous Octo individuals also showed transformation of the second abdominal segment (A2). As was the case for A1, the transformation of A2 was manifested by the formation of thoracic leg structures on the ventral surface of the segment. At the highest level of expression, the ectopic structures included only the most proximal leg segment,
the coxa. For example, among the animals that showed expression in A2, the level of expression was scored as either 1 (98%) or 2 (2%). Animals with ectopic structures on A2 were only seen among those Octo animals in which the A1 transformation received a score of 3 to 4.

The dorsal surface of A1 and A2 were unaffected by the Octo mutation. The dorsal cuticular markings of these segments were normal in mutant animals and showed no thoracic characteristics. Also, there were no signs of wing imaginal discs in segments A1 and A2.

When Octopod females and males were mated, relatively few eggs were laid. The mating of 4 mutant pairs resulted in a total of only 244 eggs (mean = 58 ± 39.6 s.e.m.) being laid over a six-day period, with two of the females laying no eggs. Wild-type females by comparison usually lay more than 300 eggs over the same time period. Crosses of homozygous Octo animals at 21°C resulted in 40 to 60% of the eggs producing viable larvae compared to over 95% for crosses between wild-type animals. The remaining eggs appeared to be unfertilized eggs (such as from unmated females) or they produced islands of white, flocculent material dispersed in the yolk rather than a viable embryo. When crosses between mutant animals were made at 27°C, as opposed to 21°C, fewer than 1% of the eggs were viable. It is not known if this aberrant development is indeed related to the Octo gene. There was no lethality apparent during later stages of development. Viable Octo animals were indistinguishable from wild-type animals in terms of the rate of their embryonic, larval and adult development.

The Octo genotype

The results of a series of crosses suggest that Octo is an autosomal dominant mutation (Fig. 2). When homozygous Octo males or females were crossed to bl animals approximately 60% of the progeny appeared as wild-type, while the remaining 40% expressed the Octo phenotype. Among the latter group, nearly all the

![Fig. 1. Series of photomicrographs of the ventral surface of the first abdominal segment (A1) illustrating the range of expression of the epidermal phenotype of Octo larvae. The larvae were scored using a 5-point scale (0 to 4).](image-url)
scores were in the range of 1 to 3 with no evidence of transformation of A2. Among the F1s, males and females expressed the Octo trait at an equal rate. Backcrosses to the parental strains confirmed that the Octo trait is an autosomal dominant mutation (data not shown).

Influence of Octo on the neuroblast arrays
While the influence of homeotic mutations on the fate of epidermal tissues has been extensively studied (Lewis, 1978; Akam, 1987), their role in the development of the CNS of insects is less well understood. Consequently, we examined the effect of the Octo mutation on two postembryonic processes that are important in establishing segmental differences within the CNS; namely, segment-specific differences in the number of neuroblasts and the size of specific lineages. Fig. 3A shows the complete array of neuroblasts found in ganglion T2 of wild-type larvae. Sets of neuroblasts are segmentally deleted from this array as one progresses posteriorly to A1 and A2. Reconstructions of the neuroblast arrays found in A1 of Octo larvae revealed the presence of supernumerary neuroblasts. The number of extra neuroblasts ranged from 1 to 6 (mean = 3.0 ± 0.26 s.e.m. n = 27) and they were located only in the anterior half of A1 (Figs 3B and 4). The position of these cells and the point of entry of the axons of their progeny into the neuropile indicated that these neuroblasts were members of the set of stem cells normally found only in thoracic ganglia (Figs 3 and 4).

In the 27 A1 ganglia that were examined in detail, only 3 of the thoracic specific neuroblasts were reactivated ectopically in mutant larvae; the B, K and L neuroblasts (Fig. 3B). The frequency with which these 3 neuroblasts were found in A1 of mutant larvae ranged from 26% to 72%. In many instances, only one of the neuroblast pair was found. In no instance did we find extra neuroblasts that were not homologous to those found in the more anterior ganglia.

Ganglion A2 possesses only 10 of the neuroblasts that are found in A1. Since the epidermis of Octo animals occasionally shows partial transformation of ventral A2, we examined the pattern of neuroblasts present in ganglion A2 of mutant larvae (Fig. 3). Approximately 15% of the Octo animals examined possessed at least one supernumerary neuroblast in A2. As was true of A1, the supernumerary neuroblasts were found only in the anterior half of the ganglion. In all cases, the extra neuroblasts were identified as the A neuroblast, which is normally found only in ganglia T2-A1 (Fig. 4). In all but one case, only one of the A neuroblast pair was found.

Size of neuronal lineages in Octo larvae
Segmental differences in neuronal number arise not only from differences in the number of neuroblasts, but also from variations in the number of progeny generated by homologous stem cells in different segments (Bate, 1976; Booker & Truman, 1987). Within the segmental ganglia of Manduca larvae, all the neuroblasts begin to divide at approximately the same time, the mid-second to early third instar. Yet, by the onset of metamorphosis there are dramatic differences in the number of progeny associated with each of the stem cells. Indeed, the number of progeny generated by a particular neuroblast is characteristic for that stem cell, but varies according to its segmental location (Fig. 5). The thoracic neuroblasts typically generate a significantly greater number of progeny by the end of the feeding larval stage than do their homologues located in abdominal ganglia A2 to A8. The number of progeny produced by an A1 neuroblast is intermediate between that seen for its thoracic and abdominal homologues.

We counted the number of progeny associated with each A1 neuroblast in both wild-type and mutant larvae. For the majority of neuroblasts normally found in A1, the number of progeny produced by a given stem cell by the end of the feeding period was the same in both wild-type and Octo larvae. The only exception was the M neuroblast, in which about half of the lineages showed differences in the number of progeny associated with each of the stem cells. Indeed, the number of progeny generated by a particular neuroblast is characteristic for that stem cell, but varies according to its segmental location (Fig. 5). The thoracic neuroblasts typically generate a significantly greater number of progeny by the end of the feeding larval stage than do their homologues located in abdominal ganglia A2 to A8. The number of progeny produced by an A1 neuroblast is intermediate between that seen for its thoracic and abdominal homologues.

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Of the ectopic neuroblasts found in the A1 (B, K and L) all produced the number of cells characteristic of the respective thoracic stem cell (e.g. Fig. 5C). The ectopic A neuroblast in A2 generated the number of progeny usually associated with the A neuroblast of A1, not those in the thorax (data not shown).
Influence of a homeotic mutant on the insect CNS

Fig. 3. (A) Illustrates the distribution of neuroblasts in T2, A1 and A2 of wild-type animals. The circles represent the position of the identifiable neuroblasts. The filled circles in T2 and A1 represent the neuroblasts which drop out of the array in the next ganglion. In those instances where there is uncertainty as to which neuroblasts drop out of the array (the I-J and N-T clusters) they are shaded. (B) Represents the frequency of occurrence (in per cent) of the identifiable ectopic neuroblasts found in A1 and A2 of Octo larvae (n = 27). (C) Reconstructions of A1 and A2 from two fifth instar Octo larvae showing the distribution of the postembryonic neuroblasts. Ectopic neuroblasts are identified with the letter of their thoracic counterparts.

Discussion

The Octo mutation results in the transformation of the ventral epidermis of the first, and to a lesser extent the second, abdominal segments in the anterior direction resulting in the formation of supernumerary thoracic legs. The penetrance of the Octo phenotype in homozygous animals was complete and the level of expression was relatively high. A genetic analysis of this mutation revealed that it is an autosomal mutation which exhibits incomplete dominance.

The phenotype of Octo is similar to several of the mutations of the E locus of the moth Bombby mori (Tajima, 1964). Mutations in the E locus alter segmental identity and have properties similar to mutants of the BX-C of Drosophila. The Octo phenotype is also similar to bithoraxoid (bxd), a mutation within the BX-C (Lewis, 1978). In bxd mutants, there is a transformation of the first abdominal segment to metathoracic resulting in the appearance of supernumerary thorax-like legs. Like Octopod, most bxd alleles when homozygous have little effect on dorsal structures. Unlike Octo, alleles of bxd do not result in the appearance of thoracic legs on the second abdominal segment. However, for some alleles when hemizygous there is evidence of a slight transformation of more posterior
abdominal segments (Lewis, 1978). For example, sensory structures normally associated with the ventral surface of the thoracic segments appear in all the abdominal segments in such hemizygotes. Whether the Octo mutation lies within Manduca's equivalent of the BX-C has yet to be determined. Recently, a Manduca genomic clone was isolated which, based on sequence analysis, contains a sequence similar to the homeo-domain within the abdominal-A locus of Drosophila’s BX-C (L. Nagy, R. Booker and L. M. Riddiford, unpublished data). This suggests that at least some portion of the BX-C is conserved in Manduca and that this approach may provide a handle for a more detailed analysis of the Octo mutation.

Studies on Drosophila have given some insight into the role of homeotic mutations in determining the fate of central neural elements. In embryos deficient for the entire BX-C (Jimenez & Campos-Ortega, 1981), their CNS fails to condense and all of the segmental ganglia display a staining pattern normally observed only in the thoracic ganglia. Similarly, in adult flies carrying the bxd mutation, a supernumerary leg neuromere is often formed, suggesting transformation of A1 to a thoracic neural segment (Teugels & Ghysen, 1983; Ghysen & Lewis, 1986). Importantly, in this latter example, the presence of this transformed neuromere does not require the obvious transformation of epidermal structures. BX-C mutations also influence the segment-specific pattern of commissures within the segmented ventral nerve cord (Teugels & Ghysen, 1983). The report of Green (1981) using the triple mutant abx bx³ pbx is the only one to examine the CNS of mutant flies using an identifiable class of central neurones, the leg motorneurones. These results support a role for homeotic mutations in determining the fate of central neurones within their domain of influence. However, many of the differences observed between the motor-neurones of wild-type and mutant individuals are subtle and difficult to interpret. Taken together, the results argue that the homeotic genes of Drosophila play an
An important role in shaping the segmental differences observed within its CNS.

Analysis of postembryonic neuroblasts and their mitotic activity provides a new dimension for examining the role of homeotic genes in CNS development. One effect of the Octo mutation is to increase the number of neuroblasts found in A1 and, to a lesser extent, in A2. In all cases, the supernumerary neuroblasts were homologous to stem cells that are normally found only in more anterior segments. Interestingly, the extra neuroblasts only appeared in the anterior half of the ganglion. Whether this limited distribution of the supernumerary neuroblasts is suggestive of compartments within the CNS of Manduca cannot be determined from the analysis of this single mutation.

Only 3 out of 11 thoracic specific neuroblasts that could possibly be present were found ectopically in the ganglia of mutant larvae; the B, K and L neuroblasts. In A2 only a single ectopic neuroblast, the A neuroblast, was found. The reason for this selectivity of the Octo mutation is not known at present. One speculation is that these particular lineages may deal with sensory processing or motor activity associated with the ventral region of a thoracic segment, and might therefore be more susceptible to the influences of a gene controlling the segmental fate of ventral structures.

While the relationship between the embryonic and postembryonic neuroblasts of Manduca has not been determined, analysis of another holometabolous insect, the tsetse fly, Glossina palidipes, shows that the neuroblasts found in larval ganglia are surviving embryonic neuroblasts (Truman, unpublished results). Accordingly, in Manduca the various segments of the embryo start with essentially identical arrays of stem cells (Thomas et al. 1984). Cell death late in embryogenesis presumably establishes the segment-specific sets that one finds in the larva. Consequently, the Octo mutation presumably is involved in determining which of the embryonic neuroblasts survive the round of cell death to go on to contribute a postembryonic lineage within the affected segments.

In terms of neurogenesis, segmental differences are seen not only in the number of stems cells, but also in the number of progeny generated by a given neuroblast in different segments. Our analysis of the segments affected by the Octo mutation revealed only a single instance where a stem cell normally present in that segment had been transformed in terms of the size of the lineage it produced. In the case of the M(A1) neuroblast, about half of those examined had generated the number of progeny normally produced by their thoracic homologues. In many mutant larvae, the fate of the two M(A1) neuroblasts was mixed with one generating a thoracic-sized lineage and the other the number typical for A1.

All of the ectopic neuroblasts in A1 (B, K and L) generated the number of progeny typically associated with their respective thoracic homologues. At this level of analysis, there is no means for determining which particular thoracic segment they represent since these neuroblasts generate similar numbers of progeny in all 3 thoracic segments. Interestingly, the ectopic A neuroblasts in A2 did not produce a thoracic-sized lineage, but generated the same number of progeny that is typical for A1. Thus, the nervous system takes on a characteristic of A1, while the epidermis assumes thoracic characters with the formation of leg parts.

While care has to be taken in interpreting the results from the analysis of a single mutation, they suggest that homeotic genes can influence at least two aspects of neurogenesis. First, they can influence the number of neuroblasts present in the ganglia during the larval stage, thereby altering the array of postembryonic lineages. Second, they appear capable of altering the segment-specific number of progeny generated by a particular neuroblast. Taken together this suggests that segmental determination plays a critical role in shaping the regional differences in neuronal number observed in the segmental ganglia of adult insects.

We thank Drs David Morton, Jane Witten, Susan Fahrbach and Shirley Reis for a critical reading of the manuscript. This work was supported by a grant from NIH (ROI-NS-13079) to JWT.

References


R. Booker and J. W. Truman

Physiol. 21, 1931–1938.


(Accepted 1 December 1988)