

Segmental Determination in *Drosophila* Central Nervous System

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Summary

We have analyzed the control of two segment-specific features in the central nervous system of *Drosophila* larvae. One of them is present only in the thoracic ganglia of the larva, where it represents the anlage of the adult leg neuromeres; the other is found in the first abdominal, as well as in the thoracic, ganglia. We show that mutations within the bithorax complex have parallel but independent effects on these neural structures and on the larval epidermis. We also show that the central nervous system is very sensitive to mild perturbations of the bithorax complex, and in particular to haploinsufficiency.

Introduction

How genes control the development of an organism is a major unanswered question in biology. Some cues about this control are emerging in one case, the process of segmental determination in *Drosophila*. Segmentation is an essential step in the development of all higher organisms. In very primitive forms the segmental repeats are all alike, but in most animals there is substantial diversity in the developmental fates of the different segments. In *Drosophila*, the origin of this segmental diversity can be traced back to early embryogenesis (Capdevila and Garcia-Bellido, 1974, 1981; Simcox and Sang, 1983), probably at the time when segments are first defined (Wieschaus and Gehring, 1976), much before the first signs of diversity can be observed. This early determination is then maintained, throughout time and cell division, until the onset of differentiation. The segmental determination of all segments posterior to the mesothorax depends on a single gene cluster, the bithorax complex (BX-C; Lewis, 1978).

The combination of genetic analysis of the BX-C (Lewis, 1955, 1963, 1964, 1967, 1978, 1981) with a more recent molecular approach (Bender et al., 1983) has made the BX-C a model system for the study of how genes control development. However, most of the analysis has been of adult and larval epidermis, and we know almost nothing about whether, and how, BX-C functions are required for the segmental determination of internal tissues. This is particularly true for the central nervous system (CNS), the most highly diversified tissue of the fly. Is its diversity subject to the same control as that of the epidermis?

Various observations show that the effects of BX-C mutations on epidermis and on CNS are parallel (Jimenez

and Campos-Ortega, 1981; Green, 1981; Thomas and Wyman, 1984). In addition, at least some regions of the BX-C are expressed in the CNS, both at the RNA level, as shown by in situ hybridization (Akam, 1983; McGinnis et al., 1984), and at the protein level, as shown by use of a monoclonal antibody against the *Ubx* protein (White and Wilcox, 1984). These results may indicate that the organization of the CNS is under the direct control of BX-C functions. Alternatively, it may be that the expression of the BX-C has no direct role in the nervous system, and that segmental differences in the CNS simply result from, or are induced by, segmental differences in the epidermis and associated sensory neurons. If this were the case, the primary effect of BX-C mutations would be on the epidermis and neural transformation would be indirect.

The possibility of indirect transformation was examined in the case of a BX-C mutant, *bxd*. This mutant transforms the first abdominal segment to thoracic tissue at the level of the epidermis and produces a similar transformation in the CNS—adult *bxd* flies have an extra pair of legs, and their CNS contains an extra pair of leg neuromeres (Teugels and Ghysen, 1983). By using *bxd* alleles that give incomplete transformations, it was shown that the presence of a leg neuromere does not depend on the presence of a corresponding leg, suggesting that the development of leg neuromeres is an autonomous feature of the CNS, directly controlled by the BX-C.

These experiments, however, left the possibility that formation of leg neuromeres is initiated much before metamorphosis and is induced by some early and possibly crucial input from the periphery (Lawrence, personal communication). Two candidates for providing such an input are the larval Keilin organs and ventral pits. Both types of sensory structures are found in pairs in each of the three thoracic segments and nowhere else on the larval body, and both appear during embryogenesis. Furthermore, the Keilin organs are thought to be the sensory remnants of the larval legs (Keilin, 1915). Their sensory neurons are associated with the imaginal leg discs (Bate, submitted), where they are used as guides for the adult leg neurons, which differentiate during metamorphosis (Jan et al., submitted). These neurons, intimately associated with the leg throughout development, would seem ideally suited to trigger or induce the development of the leg neuromeres.

We describe a new monoclonal antibody that recognizes segment-specific structures in the CNS of *Drosophila* larvae. The results show that BX-C lesions have independent effects on the CNS and on the larval periphery, and strongly support the conclusion that the development of neural structures is under the direct control of the BX-C.

Results

Segment-Specific Features in the CNS of Wild-Type Larvae

The CNS of *Drosophila* larvae consists of two brain lobes and a single ventral ganglion that results from the fusion

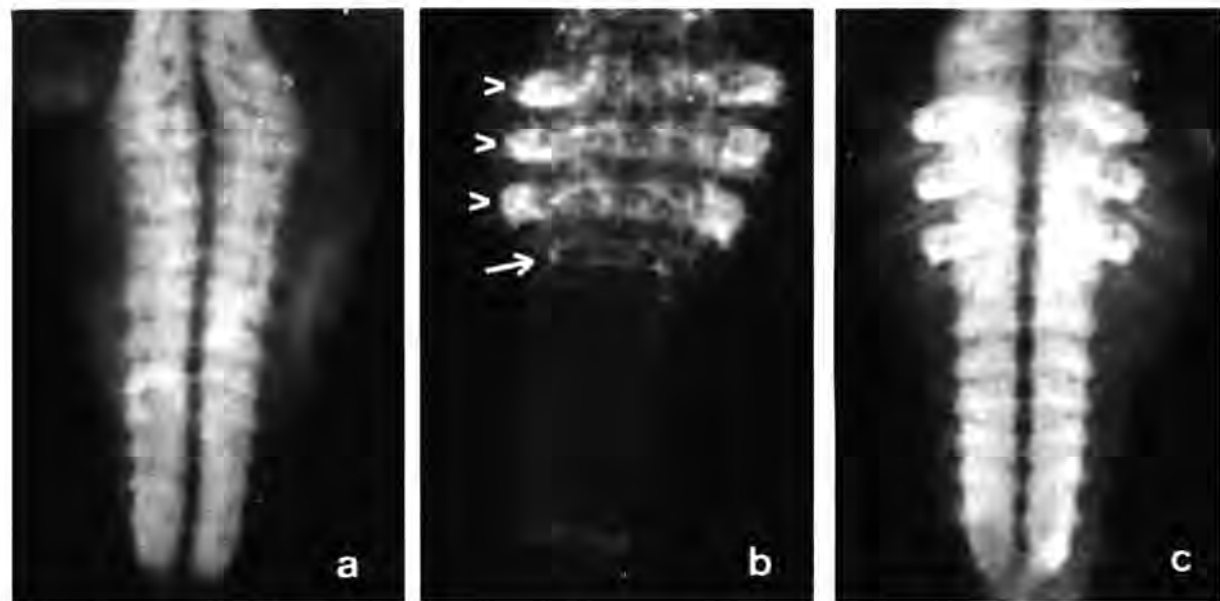


Figure 1. Ventral Ganglia from Third Instar Larvae

Ganglia are labeled with monoclonal antibody 1E8 (a), 16F12 (b), and both (c). Arrows: lateral dots. Arrowheads: presumptive leg neuromeres.

of all the segmental ganglia. As in all insects, the cell bodies of the neurons lie in the cortex of the CNS, while all the fibers and connections form the central neuropil. The neuropil of the ventral ganglion is clearly segmented in the embryo (Jan and Jan, 1982; Thomas et al., 1984). The segmental structure of the neuropil can still be detected in mature larvae (Figure 1a) when the ganglion is labeled by a monoclonal antibody, 1E8, that recognizes the association centers in the adult nervous system (Teugels and Ghysen, 1983).

We have isolated another monoclonal antibody, 16F12, which reveals two types of segment-specific structures in the ventral ganglion of mature larvae (Figure 1b). The segmental origin of these structures was ascertained by using the two antibodies simultaneously (Figure 1c). One structure (Figure 1b, arrowhead) is found laterally in each of the thoracic segments. The other structure (Figure 1b, arrows) is most clearly seen in the first abdominal segment, Ab1, where it appears as two lateral dots connected by two strands. This structure is also present in the thoracic segments and is absent in the posterior abdominal segments Ab2 to Ab7. Other structures labeled by this antibody are found at the posterior tip of the ganglion, in one or more subesophageal segments, and in the brain lobes.

Metamorphosis of the CNS

The structures labeled by 16F12 are first observed in second instar larvae and become more conspicuous at older stages. We have followed the fate of these structures during the first 36 hr after puparium formation (APF), at which time the label begins to weaken. The lateral dots in Ab1 disappear soon after the onset of metamorphosis, at about 6 hr APF. By contrast, the thoracic structures become more intensely labeled and display a progressive change in shape (Figure 2) such that, by 24 hr APF, they have assumed an aspect similar to that of the leg neuro-

meres in the adult CNS. At about 48 hr APF these structures begin to be labeled by the antibody 1E8, which recognizes the adult leg neuromeres, and can be followed during the next 3 days of metamorphosis up to the adult stage. Thus the development of the adult leg neuromeres can be traced with certainty to larval structures detected as early as the second larval instar. We call these larval structures presumptive leg neuromeres (PLN).

We have also traced the adult wing neuromere to a structure that is first detected a few hours after puparium formation and becomes progressively more distinct (Figure 2f, arrow). Interestingly, a similar structure appears in the metathoracic segment as well (Figure 2f, arrowhead). Its development parallels that of the mesothoracic presumptive wing neuromere up to about 16 hr APF, at which time the metathoracic structure begins to regress, almost disappearing by 24 hr APF (Figure 2h).

Another feature of the metamorphosing nervous system worth mentioning is that the labeled structure corresponding to subesophageal ganglia undergoes a dramatic change in shape and position; such that by 20 hr APF (Figure 2g) it has become completely separated from the other ventral ganglia and closely apposed to the brain.

The Suppression of Leg Neuromeres in Ab1

Two regions of the BX-C are known to be involved in the segmental determination of first abdominal epidermis: *Ubx*⁺ and *bxd*⁺. Mutations in either region transform the epidermal cells of Ab1 into thoracic cells. Furthermore, *Ubx* and *bxd* mutations do not complement (Lewis, 1967); hence there is a *cis* interaction between *Ubx*⁺ and *bxd*⁺. We have examined the effect of extreme *Ubx* and *bxd* mutations on the larval CNS. Larvae that are heterozygous for two extreme *Ubx*, or for one extreme *Ubx* and one extreme *bxd*, or for two extreme *bxd* mutants are shown in Figures 3a, 3b, and 3c, respectively. In all cases, the first abdomi-

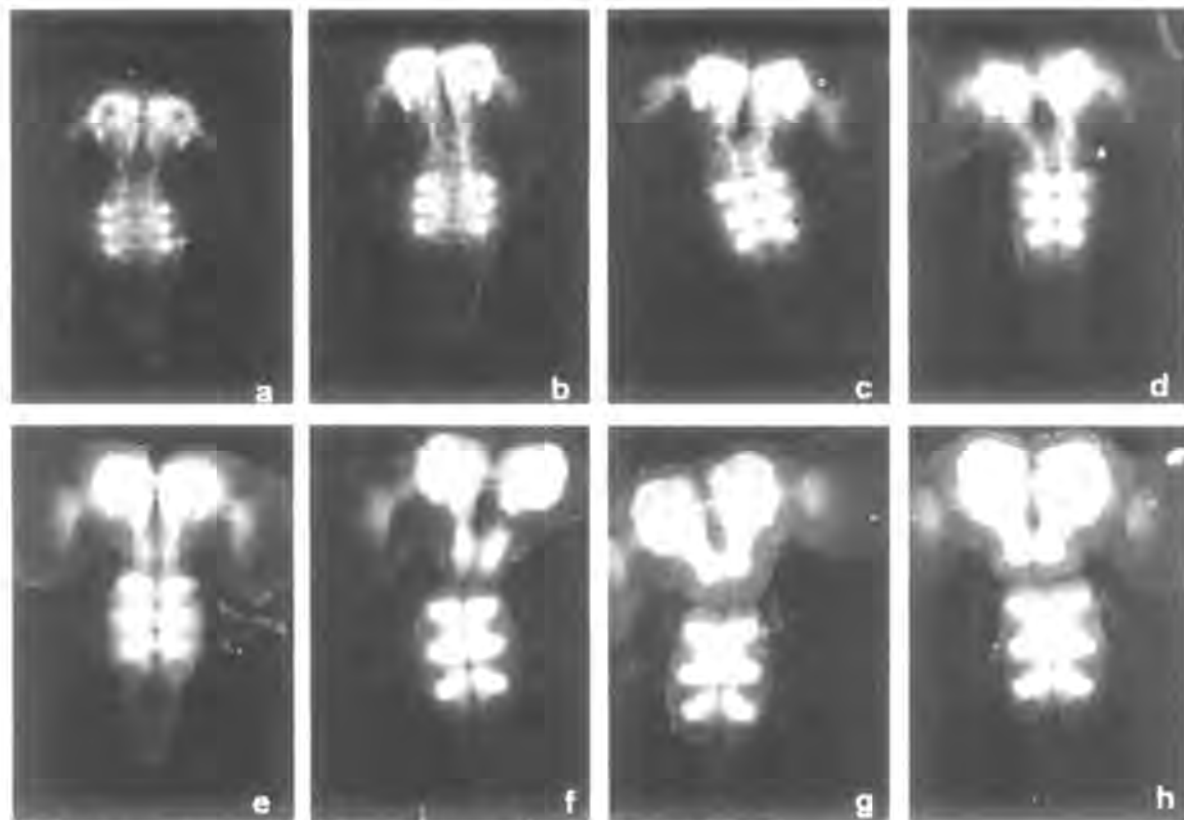


Figure 2. Metamorphosis of the Central Nervous System As Followed by the 16F12 Antibody

(a) 0 hr after puparium formation (APF); (b) 2 hr APF; (c) 4 hr APF; (d) 6 hr APF; (e) 10 hr APF; (f) 15 hr APF; (g) 20 hr APF; (h) 25 hr APF.

nal segment is completely transformed to a thoracic one as judged by the presence of additional PLN that are indistinguishable from the normal ones. This result indicates that *Ubx*⁺ and *bx*⁺ are both required for the suppression of PLN in Ab1, and that they must act in *cis*, exactly as in the case of the epidermis.

We have also examined the effect of other mutations in the *Ubx-bx* region. Extreme *abx* and *bx* mutations have no effect in Ab1. The mutations *pbx*¹ and *pbx*², on the other hand, induce a small but distinct PLN in that segment when heterozygous with extreme *Ubx* or *bx* mutations (e.g. the breakpoint *bx*¹⁰⁰, Figure 3d). Thus it may be that the region *pbx*⁺ is expressed in Ab1 and required for the complete suppression of PLN in that segment, or it may be that the mutations *pbx*¹ and *pbx*² somehow reduce the expression of the adjacent *bx*⁺ region. Whatever the case, this result suggests that *pbx* mutations have a hitherto unsuspected effect in Ab1, as has recently been found to be the case in the adult epidermis as well (Lewis, personal communication).

Independence of Epidermal and Neuronal Transformations

We have used different combinations of *bx* and *Ubx* to try to assess whether there is a correlation between the presence and size of the PLN and the presence and size of the Keilin organs and ventral pits in Ab1. More specifically we combined an extreme and a moderate *bx* mutation,

respectively *bx*¹⁰⁰ and *bx*¹, to a deficiency, a *Ubx* breakpoint (*Ubx*⁸⁰) to a *Ubx* point mutation (*Ubx*¹). We observed that *bx*¹⁰⁰ gives additional PLN indistinguishable from the normal PLN (Figures 4a, 4b), while *bx*¹ gives additional PLN of smaller than normal size (Figures 4c, 4d) when hemizygous or combined to either *Ubx* mutation. As for the epidermal transformations, we found that all combinations generally display incomplete additional Keilin organs containing two, instead of the normal three, hairs (Lewis, 1978; Struhl, 1984). However, in exceptional cases the Keilin organ on one side of Ab1 may have three hairs or only one, or be absent. We observed two *bx*¹⁰⁰/*Ubx*¹ larvae with no Keilin organ on one side of their first abdominal segment; nevertheless both had a fully developed PLN on that side of the ganglion. Conversely, exceptional cases were found where *bx*¹/*Df* larvae had an additional Keilin organ containing three hairs; the additional PLN on that side remained as small as usual. Ventral pits are almost always observed in the first abdominal segment in all the *bx* combinations used here, irrespective of the size of the additional PLN. Furthermore, additional ventral pits are often observed in posterior abdominal segments as well (Lewis, 1978), although no trace of PLN was ever observed posterior to Ab1.

We have examined other epidermal features such as the extent of the transformation of the ventral setal belt and the size of the leg discs in Ab1. None of the variations observed between different mutant combinations was strictly correlated to the extent of the neuronal transforma-

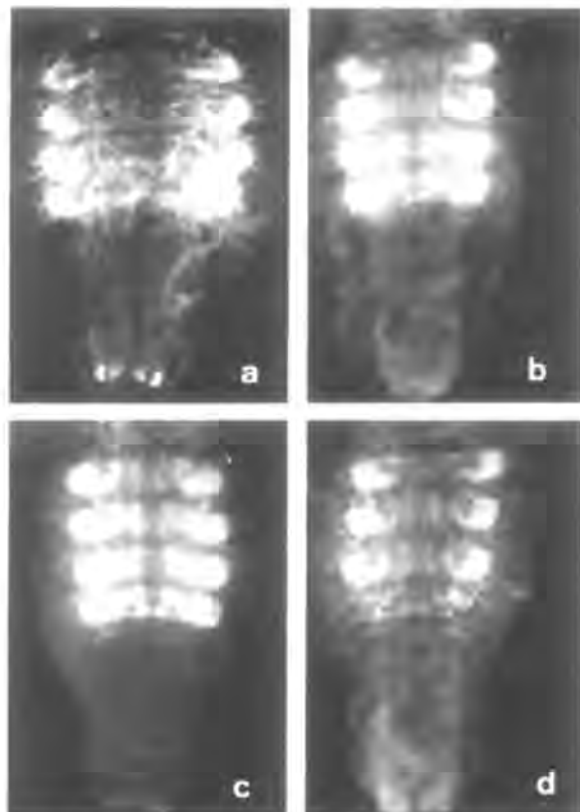


Figure 3. Suppression of Presumptive Leg Neuromeres in the First Abdominal Segment

(a) *Ubx*¹/*Ubx*⁸⁰; (b) *Ubx*⁸⁰/*bxd*¹⁰⁰; (c) *bxd*¹⁰⁰/*bxd*¹²⁵; (d) *pbx*²/*bxd*¹⁰⁰.

tion. We observed that the extent of epidermal transformation is in general more extreme in *bxd/Df* and *bxd/Ubx*⁸⁰ than in *bxd/Ubx*¹ for both *bxd*¹ and *bxd*¹⁰⁰; this is not true for the extent of the neuronal transformation, which is moderate for all three *bxd*¹ combinations and complete for all three *bxd*¹⁰⁰ combinations (Figure 4). Thus the behavior of *Ubx*¹ is completely defective in neurons and partly defective in epidermis.

In conclusion, the analysis of individual mutant larvae has shown that the presence of additional PLN does not depend on the presence of Keilin organs or ventral pits, and more generally that there is no clear correlation between the extent of neuronal transformation and the extent of epidermal transformation.

Presence of Additional Lateral Dots in Hemizygous Larvae

In the course of the experiments described above, we noticed that all hemizygous *bxd* combinations have additional lateral dots in all abdominal segments, while in wild-type larvae no lateral dots are ever found in segmental ganglia posterior in Ab1 (Figure 5a, where *Df(3R)P115* deletes all BX-C functions). This phenotype is entirely due to the deficiency, since in larvae where one copy of BX-C is normal and the other is deleted, lateral dots that resemble those of Ab1 are also found in all abdominal segments (Figure 5b). This indicates that two doses of BX-C are required for the suppression of lateral dots in segments Ab2

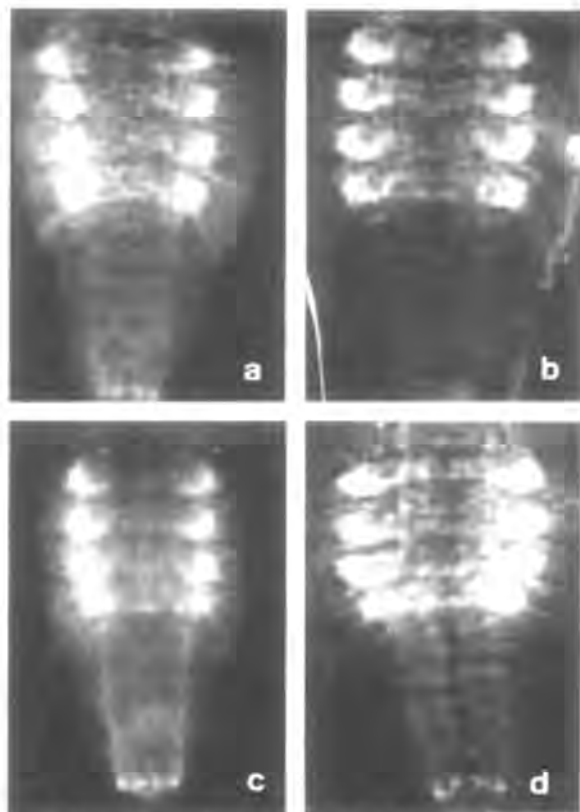


Figure 4. *bxd/Ubx* Combinations Used in the Analysis of the Correlation between Neural and Epidermal Phenotypes

(a) *bxd*¹⁰⁰/*Ubx*¹; (b) *bxd*¹⁰⁰/*Ubx*⁸⁰; (c) *bxd*¹/*Ubx*¹; (d) *bxd*¹/*Ubx*⁸⁰.

to Ab7. By contrast, the hemizygosity for the BX-C has no reproducible effect on any cuticular or sensory structure of the larva.

Effect of a Regulator of BX-C, *trx*, on the Nervous System

The expression of the BX-C is controlled by various regulatory genes (Lewis, 1968, 1978; Ingham and Whittle, 1980; Garcia-Bellido, 1981; Struhl, 1981; Duncan and Lewis, 1982). One of them, *Rg-bx*, is required for the normal expression of the complex (Lewis, 1968; Capdevila and Garcia-Bellido, 1981). A leaky allele of *Rg-bx*, named *trx*, is homozygous viable (Ingham, 1981). Mutant adults show patchy transformations of the metathorax and all abdominal segments toward more anterior segments, while mutant larvae show only a slight epidermal transformation that appears limited to the posterior abdominal segments, most notably Ab8 (Ingham, 1983). Furthermore, there is no trace of transformation to a thoracic segment, nor even to Ab1. By contrast, the CNS of all 23 *trx* larvae examined showed patchy transformations in one or more abdominal segments. Some of them are clearly lateral dots (Figure 6a), suggesting a transformation to Ab1, while others have the appearance of small PLN (Figure 6b), suggesting a transformation to thoracic segment.

The expression of *trx* is increased in zygotes derived from *trx/Df-red* mothers, where *Df-red* deletes the *trx* gene (Ingham and Whittle, 1980). This maternal effect is also

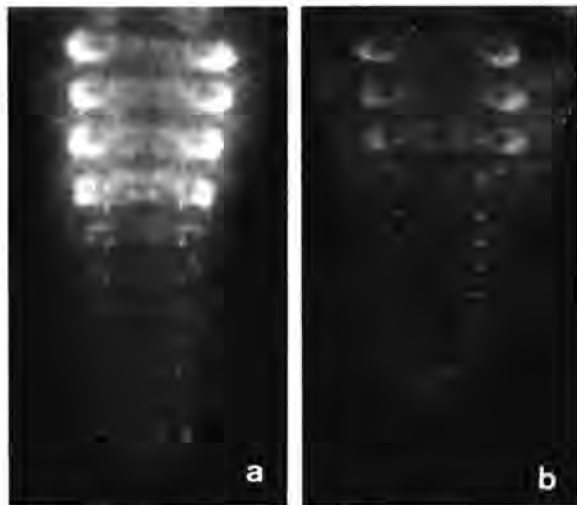


Figure 5. Effect of the Haploinsufficiency for BX-C
(a) *bxd¹⁰⁰/Df(3R)P115*; (b) *+Df(3R)P115*.

observed at the level of the CNS (Figures 6c, 6d), without any parallel effect on the epidermis.

Discussion

The question we have addressed is whether BX-C functions act directly in the CNS or whether the segmental diversity of this tissue is induced by the diversity of the epidermis and associated sensory structures. As far as we know, all the sensory structures that develop in the embryo remain present in the larva. Therefore, isolation of a monoclonal antibody that labels segment-specific features in the larval nervous system allowed us to analyze directly the relation between these CNS structures and the early sensory inputs, and to assess whether one depends on the other. We present three lines of evidence that there is no such dependence.

The first line of evidence relies on the fact that the adult leg neuromeres can be traced back to structures that we first detect during the second larval instar, much before the adult legs begin to differentiate. These larval structures, which we call presumptive leg neuromeres (PLN), are normally present only in the three thoracic segments. However, in *Ubx* or *bxd* mutants, they are also found in the first abdominal segment, Ab1. A detailed analysis of mutant larvae demonstrated that the presence of an additional PLN in Ab1 does not correlate with the presence of any known sensory or epidermal thoracic feature in that segment, such as Keilin organs, ventral pits, or metathoracic ventral setae. Furthermore, we never detected any epidermal transformation of the larval Ab1 in *pbx* mutants, indicating that the small PLN that develop in that mutant are unlikely to be induced by the periphery.

A second line of evidence depends on the discovery that the haploinsufficiency for the complex leads to the development of lateral dots in Ab2 to Ab7, suggesting that these segments are partly transformed to Ab1. No known

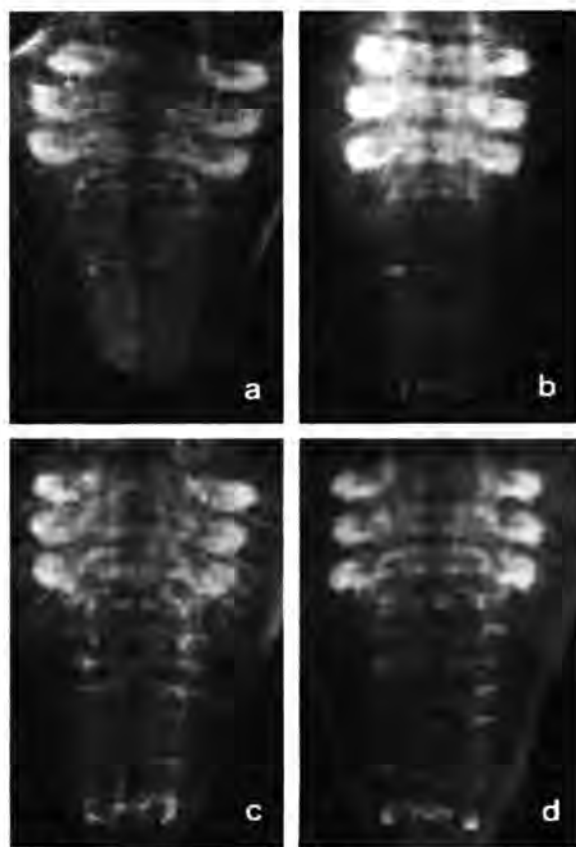


Figure 6. Effect of a Regulator Gene, *trx*, on the Larval Central Nervous System
(a, b) *trx/trx*; (c, d) *trx/trx* from *trx/Df-red* mothers.

peripheral structure is similarly affected in flies hemizygous for the BX-C.

The third indication that the BX-C acts directly in the CNS comes from the analysis of *trx* larvae. Here again clear segmental transformations were observed in the CNS, in the absence of any detectable effect on the periphery.

These results leave little doubt that BX-C functions are directly responsible for the segmental diversity of the CNS. Furthermore, this tissue appears very sensitive to BX-C defects, as indicated by the mutant phenotype associated with hemizygosity. These conclusions are entirely consistent with the finding that the BX-C is heavily expressed in the embryonic CNS (Akam, 1983; McGinnis et al., 1984).

The observation that BX-C mutations result in the formation of additional PLN and lateral dots suggests that BX-C functions are required for the suppression of PLN and lateral dots in the segments where they are normally absent. This extends to the CNS the concept of an archetype, or primitive type, that would be progressively modified in consecutive segments (Garcia-Bellido, 1977; Lewis, 1978). Thus the ability to develop PLN and lateral dots would be an autonomous feature of the primitive segmental ganglion; this ability is then suppressed in abdominal ganglia by some BX-C functions, irrespective of the peripheral input.

Experimental Procedures

Fly Strains

All mutants were from E. Lewis except *trx* (Ingham and Whittle, 1980). All crosses were performed at 23°–25°C. In all cases where the mutant larvae did not display some obvious external phenotype, the mutant chromosomes carried the mutation *red*, so that mutant could be distinguished from normal progeny.

Antibody

The monoclonal antibody 16F12 was obtained in the laboratory of two of us (Y. N. J. and L. Y. J.) after immunization of mice with *Drosophila* embryo extracts, as will be described elsewhere.

Immunofluorescence

Larval ganglia were dissected in phosphate buffer (pH 7.6), fixed in Carnoy (alcohol:chloroform:acetic acid, 6:3:1), and rinsed in phosphate buffer. All the following steps were performed at room temperature in phosphate buffer containing 0.3% deoxycholate and 0.3% triton (PDT buffer). The ganglia were incubated for 3 hr with the monoclonal antibody (1:8) in the presence of normal goat serum (1:10), rinsed for 3 hr, incubated for 3 hr with the second antibody (FITC conjugated anti-mouse goat antiserum, Nordic, 1:40), rinsed for 3 hr, and mounted in glycerin. Some batches of second antibody gave a relatively high background; in those cases the antibody was preincubated with a larval extract made by grinding ten Carnoy-fixed larvae in 500 µl of PDT. This extract was centrifuged for 2 min, the antibody was added (1:40), the mixture was centrifuged again after 10–20 min, and the supernatant was used as such for the incubation. The ganglia were viewed with a Zeiss microscope equipped with epifluorescence and a 16× immersion lens. From ten to 30 larvae were examined for each genotype. All photographs were taken on Ilford HP5 film pushed to 1500 ASA by using the Acufine developer.

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