Development 114, 625-642 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

Commitment of abdominal neuroblasts in *Drosophila* to a male or female fate is dependent on genes of the sex-determining hierarchy

BARBARA J. TAYLOR* and JAMES W. TRUMAN

Department of Zoology NJ-15, University of Washington, Seattle, WA 98195, USA

*Present address for correspondence: Department of Zoology, Oregon State University, Corvallis, OR 97331, USA

Summary

Adult specific neurons in the central nervous system of holometabolous insects are generated by the postembryonic divisions of neuronal stem cells (neuroblasts). In the ventral nervous system of *Drosophila melanogaster*, sex-specific divisions by a set of abdominal neuroblasts occur during larval and early pupal stages. Animals mutant for several sex-determining genes were analyzed to determine the genetic regulation of neuroblast commitment to the male or female pattern of division and the time during development when these decisions are made. We have found that the choice of the sexual pathway taken by sex-specific neuroblasts depends on the expression of one of these genes, *doublesex* (*dsx*). In the absence of any functional dsx^+ products, the sex-specific neuroblasts fail to undergo any postembryonic divisions in male or female larval nervous systems. From the analysis of intersexes generated by dominant alleles of *dsx*, it has been concluded that the same neuroblasts provide the sex-specific neuroblasts in both male and female central nervous systems. The time when neuroblasts become committed to generate their sex-specific divisions was identified by shifting $tra\text{-}2^{ts}$ flies between the male- and female-specifying temperatures at various times during larval development. Neuroblasts become determined to adopt a male or female state at the end of the first larval instar, a time when abdominal neuroblasts enter their first postembryonic S-phase.

Key words: neural development, *Drosophila*, sex differentiation.

Introduction

In holometabolous insects, the adult central nervous system (CNS) is generated by embryonic and postembryonic divisions of neuronal stem cells, the neuroblasts (White and Kankel, 1978; Hartenstein and Campos-Ortega, 1984; Booker and Truman, 1987; Truman and Bate, 1988). During embryogenesis in all insects, neuroblasts in the ventral nervous system segregate from an undifferentiated sheet of neuroectodermal cells and subsequently divide to produce neurons for the larval nervous system (Hartenstein and Campos-Ortega, 1984; Doe and Goodman, 1985). Neuroblasts divide unequally producing a series of ganglion mother cells each of which then divides once, equally, to create two neurons (Edwards, 1969). Following their embryonic wave of mitoses, neuroblasts in holometabolous insects become quiescent, re-emerging during larval life to commence a series of postembryonic stem cell divisions which provide adult-specific neurons (Prokop and Technau, 1991). The majority of the embryonic neuroblasts in thoracic segments but only a few abdominal neuroblasts engage in postembryonic neurogenesis (Booker and Truman, 1987; Truman and Bate, 1988).

In *Drosophila*, the decision to develop as a male or female operates through the activity of a cascade of genes which lead to the appropriate differentiation of sexually dimorphic somatic tissues. These sex-determining genes, extensively characterized both genetically and molecularly, are: *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex* (*dsx*) and *intersex* (*ix*) (for reviews, Baker and Belote, 1983; Nöthiger and Steinmann-Zwicky, 1985; Cline, 1985; Baker et al., 1987; Wolfner, 1988; Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990). When homozygous for *tra* or *tra-2* loss-of-function mutations, chromosomally female flies (X/X) develop as phenotypic males in all somatic aspects, whereas, the somatic development of chromosomally male flies (X/Y) is unaffected (Watanabe, 1975; Baker and Ridge, 1980; Ota et al., 1981; Belote and Baker, 1982; Belote et al., 1985; McRobert and Tompkins, 1985; Belote and Baker, 1987). Consequently, the genes tra^+ and $tra\text{-}2^+$ are necessary for female development. In females, the primary function of the tra^+ and $tra\text{-}2^+$ gene products in somatic tissues is the preferential splicing of *dsx* pre-mRNA into the female-specific *dsx* mRNA (Nagoshi et al., 1988; Hoshijima et al., 1991; Hedley and Maniatis, 1991). In the absence of the tra^+ or $tra\text{-}2^+$ products, as

in mutant female flies, *dsx* pre-mRNA is spliced into the male-specific *dsx* mRNA (Nagoshi et al., 1988). As a result of this differential splicing, two distinct *dsx* proteins are made, one type in males and another in females (Baker and Wolfner, 1988; Burtis and Baker, 1989). Masculine or feminine sexual development depends on which wild-type *dsx* product is present. In the absence of any functional *dsx* gene product, as in flies homozygous for *dsx* loss-of-function mutations, both male and female flies develop as intersexes (Hildreth, 1965; Baker and Ridge, 1980). Dominant alleles of *dsx* express the male *dsx* mRNA in both sexes and can be used in combination with other *dsx* alleles to generate females that develop as phenotypic males or as intersexes (Denell and Jackson, 1972; Baker and Ridge, 1980; Nöthiger et al., 1980, 1987; Nagoshi and Baker, 1990).

The specification of sexually dimorphic neurons in the adult central nervous system is an attractive system to study the general processes underlying the commitment of neurons and their precursors to a particular fate. Two sources of differential neurogenesis in male and female *Drosophila* have been described: a postembryonic increase in the number of cells and Kenyon fibers in the female mushroom bodies (Technau, 1984), and divisions by terminal abdominal neuroblasts during larval and early pupal stages (Truman and Bate, 1988). We have exploited sex-specific neurogenesis in the abdominal ganglion to examine the role of the sex-determining genes in the commitment of neuroblasts to a male or female mode of division and to identify the times during development when the decision to enter and maintain a sexual fate are made.

Materials and methods

Fly stocks

Fly stocks were maintained on a standard cornmeal-diet supplemented with live yeast at 25°C. A full description of the mutants included below can be found in Lindsley and Zimm (1985; 1987; 1990).

Control larvae and pupae were derived from either Canton-S stocks or from crosses with females homozygous for the recessive mutation *cinnamon* (*cin*) (Baker, 1973). Crosses with mutant *cin* females were used to generate large numbers of single sex larvae, especially for timepoints during the first and second instar when Canton-S larvae cannot be sexed. Female flies that are *cin* homozygotes are sterile. Progeny can be recovered from these females if a cin^+ gene is introduced paternally (Baker, 1973). To produce daughters exclusively, *cin* homozygous mothers were mated to Canton-S fathers, where the wild-type gene is carried on the paternal X-chromosome (Table 1). When *cin* mothers were mated to y/y^+ cin^+ Y males, the only cin^+ gene is carried on the Y-chromosome, and therefore only sons are produced (Baker, 1973; Table 1).

The crosses used to generate mutant progeny are listed in Table 1 and the markers used to score progeny are indicated in Table 2. The tra^1 allele (Sturtevant, 1945) was shown to be a small deletion (McKeown et al., 1987) and was carried on a multiply marked *th st tra cp ri* p^p chromosome. The deficiency $Df(3L)st^{j7}$ (73A1-2; 73B1-2), *Ki roe* p^p uncovers the *tra* locus (McKeown et al., 1987). The *tra-2* alleles used were: $tra\text{-}2^1$ (Watanabe, 1975), $tra\text{-}2^B$ (Belote and Lucchesi, 1980), the deficiency *Df(2L)trix* (51A1-2;51B6; Belote et al., 1990), $tra\text{-}2^{ts1}$ (Belote and Lucchesi, 1980) and $tra\text{-}2^{ts2}$ (Belote and Baker, 1982, 1983). From sequence data, the temperature-sensitive *tra-2* alleles have been mapped to single amino acid substitutions either in, as is the case for the $tra\text{-}2^{ts1}$ allele, or near, as in the $tra\text{-}2^{ts2}$ allele, the ribonucleoprotein motif found in the *tra-2* protein (Amrein et al., 1990). All *tra-2* alleles except for $tra\text{-}2^1$ were on chromosomes that were also marked with *cn bw*. Two *dsx* recessive mutations were used: dsx^1 (Hildreth, 1965), this chromosome is also marked with p^p; and dsx^{19} (also listed as dsx^{EFH48}, Baker et al., 1991), this chromosome is also marked with the recessive mutations *red e*. The *dsx* null created as a *trans*-heterozygote of the inversion dsx^{23} ($In(3R)dsx^{D+R3}$, Baker et al., 1991) and the deficiency $Df(3R)dsx^{15}$, *bx sr e* ($Df(3R)dsx^{M+R15}$, 84D10-11; 84E8; Baker et al., 1991) has no detectable sex-specific *dsx* mRNA (R. Nagoshi, personal communication). Two dominant alleles of *dsx* were used: dsx^D, this chromosome is also marked with $Sb\ e^s$, (Denell and Jackson, 1972; Nagoshi and Baker, 1990) and dsx^M (Nöthiger et al., 1980; Nagoshi and Baker, 1990).

Table 1. *Crosses used to generate control and mutant animals*

Cross	Selected progeny
Controls	
y w cin/y w cin (X) Canton-S	*cin/cin*$^+$ female
y w cin/y w cin (X) *y w cin/y*$^+$ *cin*$^+$ Y	*cin/cin*$^+$ Y male
transformer	
y/y; tra^1/*TM6B, Tb* (X) *y/y*$^+$ Y; tra^1/*TM6B, Tb*	tra^1/tra^1
y/y; tra^1/*TM6B, Tb* (X) *y*$^+$/B^s Y; $Df(3L)st^{j7}$/*TM6B, Tb*	$tra^1/Df(3L)st^{j7}$
transformer-2	
y/y; $tra\text{-}2^1$/*In(2LR)Gla, Gla Bc* (X) *y/y*$^+$ Y; $tra\text{-}2^1$/*In(2LR)Gla, Gla Bc*	$tra\text{-}2^1/tra\text{-}2^1$
y/y; $tra\text{-}2^B$/*In(2LR)Gla, Gla Bc* (X) *y/y*$^+$ Y; *Df(2L)trix/In(2LR)Gla, Gla Bc*	$tra2^B/Df(2L)trix$
y/y: *cn* $tra\text{-}2^{ts1}$ *bw* /*In(2LR)Gla, Gla Bc bw* (X) *y/y*$^+$ Y; *cn* $tra\text{-}2^{ts2}$ *bw*/*In(2LR)Gla, Gla Bc bw*	$tra\text{-}2^{ts1}/tra\text{-}2^{ts2}$
doublesex	
y/y; dsx^1/*TM6B, Tb* (X) *y/y*$^+$ Y; dsx^1/*TM6B, Tb*	dsx^1/dsx^1
y/y; dsx^{19}/*TM6B, Tb* (X) *y*$^+$/B^s Y; $Df(3R)dsx^{15}$/*TM6B, Tb*	$dsx^{19}/Df(3R)dsx^{15}$
y/y; dsx^{23}/*TM6B, Tb* (X) *y*$^+$/B^s Y; $Df(3R)dsx^{15}$/*TM6B, Tb*	$dsx^{23}/Df(3R)dsx^{15}$
y$^+$/*y*$^+$; $Df(3R)dsx^{15}$/*TM6B, Tb* (X) *y*$^+$/B^s Y; dsx^D/*TM6B, Tb*	$dsx^D/Df(3R)dsx^{15}$
y w f/y w f (X) *y*$^+$/B^s Y; dsx^D/*TM6B, Tb*	dsx^D/dsx^+
y w f/y w f (X) *y*$^+$/B^s Y; dsx^M/*TM6B, Tb*	dsx^M/dsx^+
y/y; tra^1 dsx^1/*TM6B* (X) y/y_+ Y; tra^1 dsx^1/*TM6B*	tra^1 dsx^1/tra^1 dsx^1

The maternal genotype for each cross is given first and the designation of the mutant or wildtype progeny selected from each cross is given in the rightmost column. For each of the mutant-containing chromosomes used, the complete listing of markers is given in Materials and methods.

Staging of animals

For the developmental series, control and mutant larvae were staged independently for each instar. Larvae within an hour of their ecdysis to the next instar were collected on apple/agar

Table 2. *The markers used to distinguish male from female as well mutant from balancer larvae and pupae for each of the crosses in Table 1*

Genotype	Sexed (X/X:X/Y)*	Mutant:Balancer†
Control		
Canton-S	gonad	NA
cin/cin$^+$‡	y^+ w^+:−	NA
cin/cin$^+$ Y§	−:y^+	NA
transformer		
tra^1/*tra*^{1}	*y*:y^+	+:*Tb*
tra^1/*Df(3L)st*^{j7}	y^+:*y*	+:*Tb*
transformer-2		
tra-2^1/*tra-2*^{1}	*y*:y^+	+:*Bc*
tra-2^1/*Df(2L)trix*	*y*:y^+	+:*Bc*
tra-2ts1/*tra-2*^{ts2}	*y*:y^+	*cn bw*: *Bc bw*
doublesex		
dsx^1/*dsx*^{1}	*y*:y^+	+:*Tb*
dsx^{19}/*Df(3R)dsx*^{15}	y^+:*y*	+:*Tb*
dsx^{23}/*Df(3R)dsx*^{15}	y^+:*y*	+:*Tb*
dsx^D/*Df(3R)dsx*^{15}	gonad	+:*Tb*
dsx^D/*dsx*$^+$	y^+:*y*	+:*Tb*
dsx^M/*dsx*$^+$	y^+:*y*	+:*Tb*
tra^1 *dsx*^{1}/*tra*^{1} *dsx*^{1}	*y*:y^+	+:*Tb*

The only markers included in this Table are those important for selecting the appropriate mutant or wildtype progeny.

*Markers used to sex animals were: light cuticle color, *yellow* (*y*), versus normal cuticle color (y^+); clear malpighian tubules, *white* (*w*), versus yellow wildtype yellow colored tubules (w^+); gonads in male larvae are much larger than the gonads of female larvae, a condition that becomes easy to score by the middle of the second instar.

†Markers used to separate mutant animals from their Balancer siblings: presence of cells with a black pigment (*Bc*) versus absence of these cells (*Bc*$^+$, listed in Table 2 as +); shorter, rounder body shape (*Tubby*, *Tb*) versus wildtype body shape (*Tb*$^+$, listed in Table 2 as +); animals doubly mutant for *cinnabar* (*cn*) and *brown* (*bw*) have clear malpighian tubules versus the paler yellow tubules in animals mutant for *bw* alone. A double marker system for the selection of *tra-2*^{ts} larvae was necessary to accurately score larval genotype because the *Bc* marker is much less penetrant when larvae are raised at 29°C.

‡Only daughters were produced.

§Only sons were produced.

plates with yeast paste. The duration of each larval instar was determined for all control and mutant genotypes. For graphic representation, the number of labelled ganglia from a given timepoint during an instar was plotted as the percentage of time spent in that instar at the time the ganglia were isolated and incubated in BUdR.

Temperature shifts

Timed egg and larval collections were made at both 18°C and 29°C. Egg collections of one to two hours were then shifted at various times until the embryos hatched. Larvae were collected within an hour of their ecdysis and maintained at their rearing temperature until the time of the shift. In some cases, larvae from 2-12 hour egg collections were raised until the second or third instar before the temperature shift; the time of the shift was then computed from the length of time before they became wandering third instars and were dissected. For X/X *tra-2*^{ts} animals, the durations of embryogenesis and the first, second and third instars up to white puparium formation were 46, 53, 52 and 139 hours at 18°C and 18, 19, 20 and 41 hours at 29°C. Larval age is given with respect to the time that has elapsed within a given instar. For example, larvae that spent 16 hours as first instars at 18°C before their temperature shift to 29°C will be referred to as 30% first instars.

Labelling of ganglia with BUdR

For wandering third instar and white puparium collections, nervous systems were dissected from animals raised at 25°C. Cells undergoing S-phase were identified by their incorporation of 5-bromodeoxyuridine (BUdR; Gratzner, 1982). Ganglia were incubated in 15 μg ml^{-1} of BUdR in Schneider's *Drosophila* medium (+Glutamate; Gibco) for 2-8 hours. After fixation with 4% paraformaldehyde and treatment with 2N HCL for 0.5-1 hour, BUdR-labelled cells were detected immunohistochemically with an anti-BUdR antibody (1:200 dilution, Becton-Dickinson, Mt. View, CA) as described in Truman and Bate (1988).

For the developmental series, ganglia removed from timed collections of larvae were incubated in BUdR as described. To ensure the labelling of all of the terminal neuroblasts during their first S-phase of the postembryonic cell cycle, an additional set of collections of late first instar larvae were fed BUdR (0.5 mg ml^{-1} food; Truman and Bate, 1988) for 4-12 hours with subsequent fixation and processing for immunohistochemistry. Males and females of the following genotypes were analysed for their first postembryonic S-phase: *cin/cin*$^+$ Y, *cin/cin*$^+$, *dsx*^{M}/*dsx*$^+$ and *dsx*^{23}/*Df(3R)dsx*^{15}.

The number of ganglia examined to ascertain the pattern of terminal neuroblast divisions through embryonic and larval stages were: 78 X/X and 44 X/Y Canton-S larvae, plus 52 unsexed first and second instar larvae; 124 X/X *cin/cin*$^+$ and 95 X/Y *cin/y*$^+$Y; 75 X/X and 57 X/Y *dsx*^{23}/*Df(3R)dsx*^{15}; 44 X/X and 48 X/Y *dsx*^{1}/*dsx*^{1}; 69 X/X and 9 X/Y *dsx*^{D}/*dsx*$^+$; *145* X/X and 36 X/Y *dsx*^{M}/*dsx*$^+$; 328 X/X and 48 X/Y *tra-2*^{ts1}/*tra-2*^{ts2} shifted from 18°C to 29°C; 129 X/X and 32 X/Y *tra-2*^{ts1}/*tra-2*^{ts2} shifted from 29°C to 18°C. The number of late larval or early pupal ganglia examined is listed in Table 3.

Table 3. *Number of neuroblasts from wandering larvae or white pupae labelled during a four to six hour incubation with BUdR*

Genotype	Average number of neuroblasts ± s.e.m. (*n*) X/X	X/Y
Control		
Canton-S	0.0 (9)	4.0±0.03 (33)
cin/cin$^+$	0.0 (20)	4.0±0.0 (32)
transformer		
tra^1/*tra*^{1}	3.4±0.3 (5)	4.0±0.0 (3)
tra^1/*Df(3L)st*^{j7}	3.9±0.1 (11)	4.0±0.0 (4)
transformer-2		
tra-2^1/*tra-2*^{1}	4.1±0.1 (11)	4.0±0.0 (3)
tra-2^B/*Df(2L)trix*	4.0±0.0 (2)	4.0±0.0 (6)
doublesex		
dsx^1/*dsx*^{1}	0.0 (10)	0.0 (12)
dsx^{19}/*Df(3R)dsx*^{15}	0.0 (13)	0.0 (8)
dsx^{23}/*Df(3R)dsx*^{15}	0.0 (4)	0.0 (4)
dsx^D/*Df(3R)dsx*^{15}	3.6±0.2 (30)	3.9±0.1 (16)
dsx^D/*dsx*$^+$	0.6±0.2 (38)	3.9±0.1 (8)
dsx^M/*dsx*$^+$	0.7±0.6 (9)	4.0±0 (3)
tra^1 *dsx*^{1}/*tra*^{1} *dsx*^{1}	0.0 (9)	0.0 (6)

Results

Neurogenesis to produce adult specific neurons was monitored in larvae and pupae by the incorporation of BUdR into the DNA of dividing cells. After a brief exposure to BUdR, the ventral CNS contains small clusters of labelled cells, including the progenitor neuroblast, a few ganglion mother cells (GMCs) and, occasionally, a pair of neurons (Truman and Bate, 1988). Within these clusters, the neuroblast is distinguishable by its large nucleus and more dispersed labelling compared to the smaller, often darker, nuclei of the ganglion mother cells, or the diminutive nuclei of neurons.

Sex-specific divisions of terminal neuroblasts

The most conspicuous sexual difference in neurogenesis in *Drosophila melanogaster* is found in a set of four neuroblasts in the terminal abdominal ganglia of males that continue dividing (Fig. 1A,B; Table 3) after neuroblasts in female ganglia have stopped mitosis (Fig. 1C,D; Table 3; Truman and Bate, 1988). These neuroblasts are located near the seventh and eighth abdominal nerve roots in the larval ventral nervous system (Figs 1A,B, 2B). They are often positioned as bilaterally symmetric pairs, but in some ganglia one or more neuroblasts are displaced to the opposite side of the midline.

The developmental history and characterization of the cell cycle of the sex-specific neuroblasts was carried out using two sources of control flies: Canton-S larvae, which can be sexed beginning in the mid-second instar by the size of the larval testes, and single sex progeny from crosses with *cin/cin* mutant females and appropriate males (Table 1). Comparable results were obtained from both of these two types of control male and female larvae.

Time course of terminal neuroblast divisions during development

The time course of divisions by terminal neuroblasts was determined by incubating male and female nervous systems in BUdR at various larval and pupal stages. The developmental series of neuroblast labelling in *cin/cin*$^+$ females and *cin/cin*$^+$ Y males was established for animals raised at 25°C, and is shown in Fig. 2A. Labelled terminal neuroblasts first appeared in both sexes during the molt from the first to the second larval instar. Twelve neuroblasts incorporated BUdR in their first postembryonic S-phase but did not undergo mitosis until between 8 and 12 hours after ecdysis (33-50% second instars, Fig. 2A; Truman and Bate, 1988). Continuous divisions by all twelve neuroblasts began by 50% of second instar and persisted until 32% of third instar.

The twelve terminal neuroblasts appeared to have the same location whether they were found in males or females. These neuroblasts constituted the most posterior group of the segmentally arrayed neuroblasts in the abdominal ganglia. Within this group, they appeared to be organized into a lateral and a medial set of three pairs of neuroblasts each, although this order was not discernible in every preparation. In addition, two to four neuroblasts tended to have a more dorsal location compared to the remainder of the terminal neuroblasts. More anterior abdominal segments have between three and nine pairs of bilaterally symmetric neuroblasts that divide postembryonically (Truman and Bate, 1988; Fig. 2B,C).

The cessation of divisions by terminal neuroblasts began during the midthird instar in both sexes. By 40% of the third instar, around 10 terminal neuroblasts are labelled with BUdR in a 2 hour pulse (Fig. 2B,C). The failure of some neuroblasts to label after this time suggests that these terminal neuroblasts have stopped dividing, which has been confirmed by longer pulses (data not shown). In female ganglia, neuroblasts from both anterior and terminal abdominal segments have ceased mitosis by 56-64% of the third instar stage. Two or three terminal neuroblasts located posteriorly and medially divide slightly longer than neuroblasts from more anterior abdominal segments suggesting that there may be a very small late phase of divisions for some female terminal neuroblasts. In the male CNS, neuroblasts from anterior abdominal segments as well as eight terminal neuroblasts stopped mitoses over the same (56-64%) period in the third instar.

Four terminal neuroblasts in male ganglia can be distinguished from other abdominal neuroblasts by their exclusive incorporation of BUdR beginning at about the 75% third instar and continuing until 12 hours (12%P) after white puparium formation (Fig. 2A,B; Table 3). The duration of the cell cycle of male-specific terminal neuroblasts and GMCs was estimated by exposing nervous systems from wandering third instar larvae to pulses of BUdR of varying durations and counting the number of labelled cells. A similar analysis of cell cycle durations for thoracic and abdominal neuroblasts and GMCs was done by Truman and Bate (1988).

The data obtained for Canton-S and *cin/cin*$^+$ male ganglia is summarized in Fig. 3. In Canton-S ganglia, all four terminal neuroblasts were labelled with the shortest pulse, 2 hours. GMCs are only present transiently before they subsequently divide to generate two neurons. To determine the number of GMCs that label with successively longer pulses, we calculated the number of labelled "GMC equivalents", equalling the number of GMCs and one-half the number of labelled neurons. The number of GMC equivalents labelled with pulses of 2, 4 and 6 hours was 2.8, 3.2 and 4.2 for Canton-S larvae. The increase in GMC equivalents after longer pulses is due to the addition of neurons to the labelled clusters. The slope of the regression line fitted to these data shows that a new GMC is added about every three hours in Canton-S male ganglia. Around three GMCs are labelled for each of the three pulses, this stability in GMC numbers is due to the equilibrium reached between the addition of new GMCs and their loss through division. To account for the numbers of GMCs labelled along with the calculated rate of their addition, the interval between the

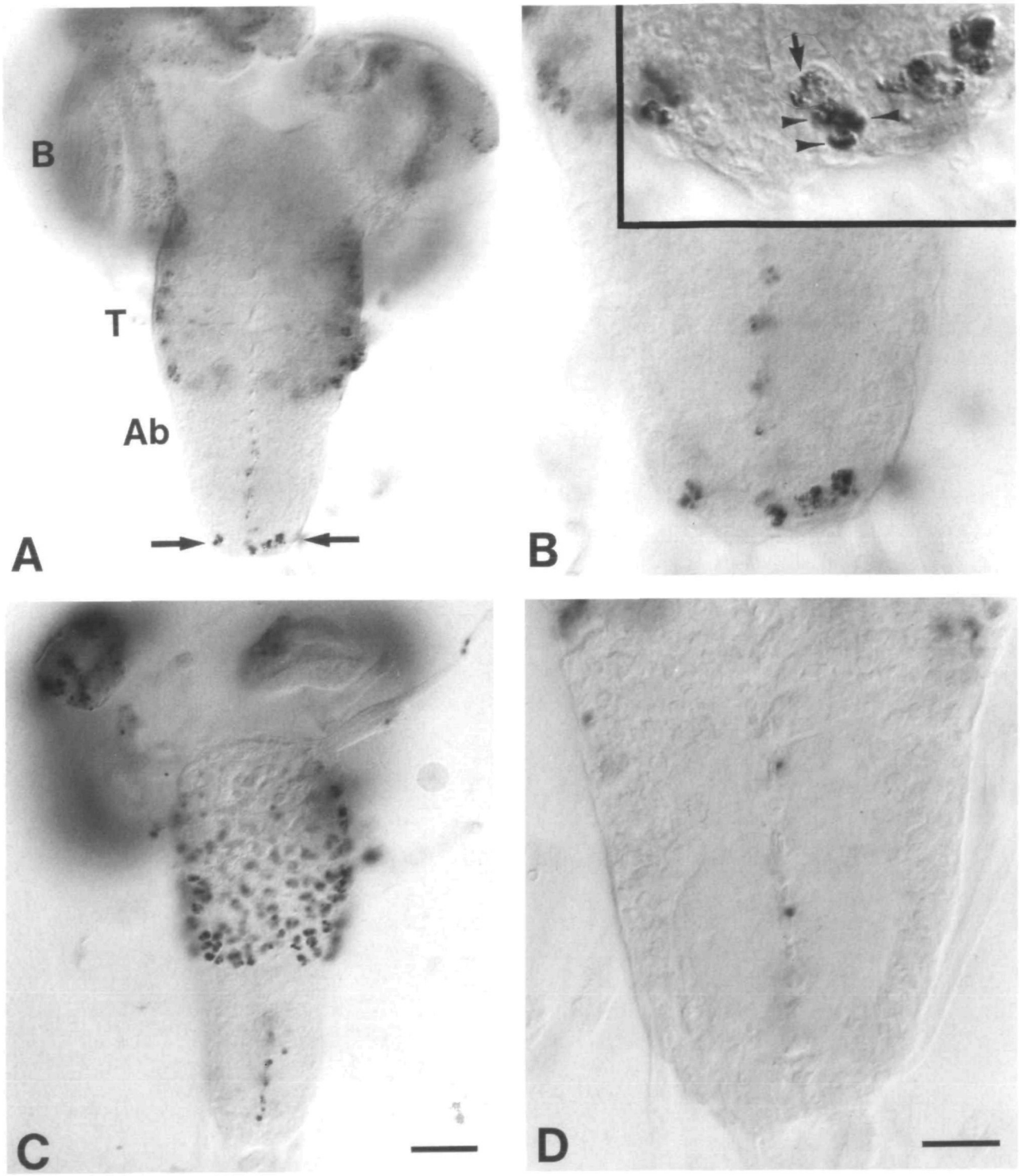
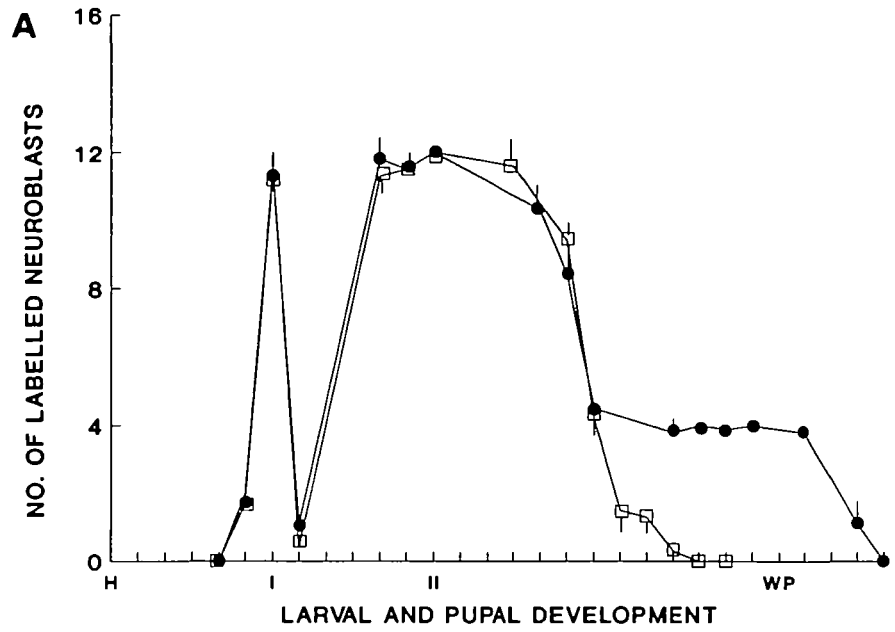
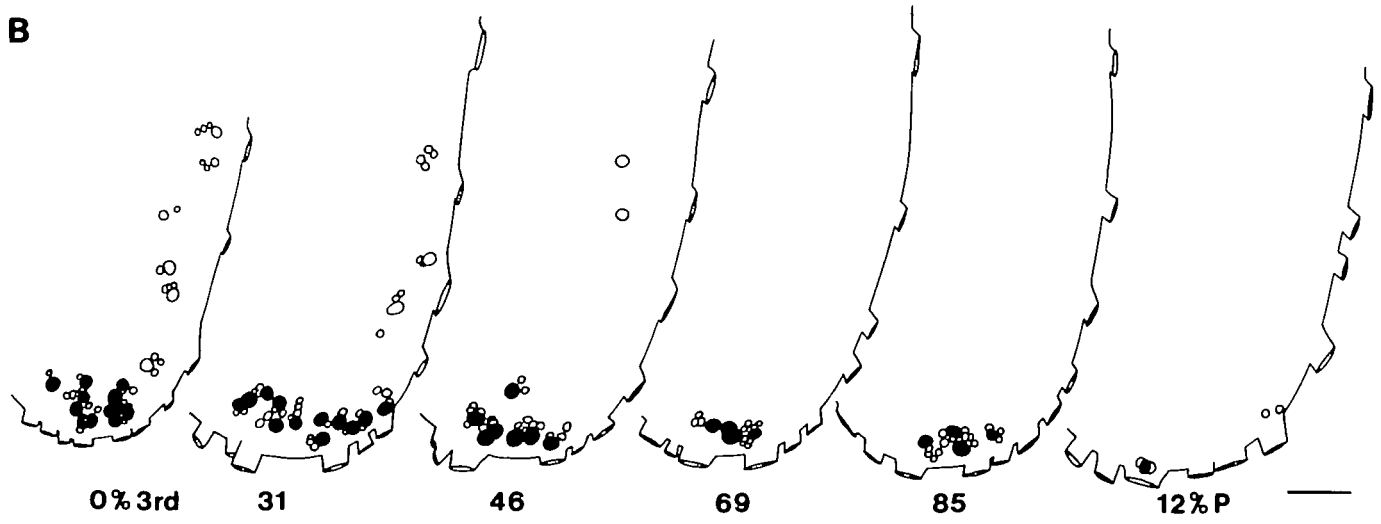
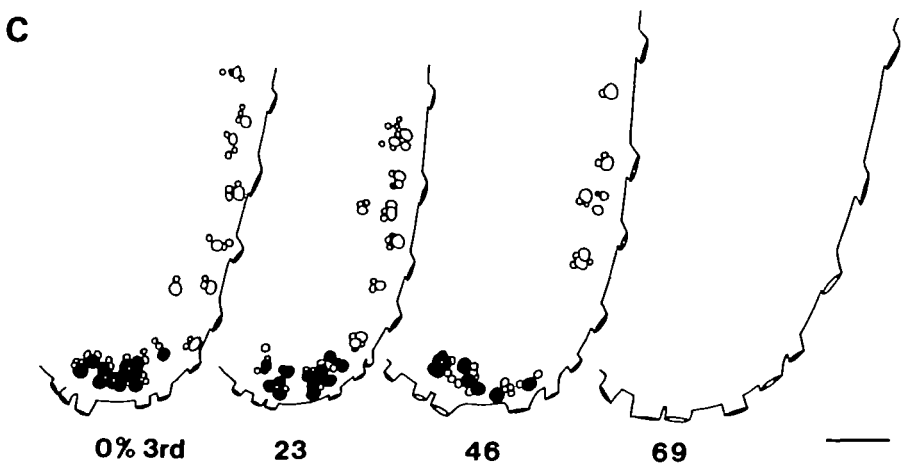


Fig. 1. BUdR-labelling of terminal neuroblasts in late third instar larvae from control male and female nervous systems. (A) CNS from a *cin/cin*$^{+}$ Y male at 88% of the third instar stage incubated in BUdR for 2 hours. Arrows mark the location of four clusters of labelled cells in the terminal abdominal region of the CNS. A number of labelled nuclei are present along the midline but are not generated by abdominal neuroblasts. B, brain; T, thoracic ganglia; Ab, abdominal ganglia. (B) Higher magnification view of the terminal region of A showing a neuroblast (inset, arrow), lighter label in a larger nucleus, and its ganglion mother cells (inset, arrowheads), darker and smaller nuclei. (C) CNS of a *cin/cin*$^{+}$ female at 88% of the third instar larval stage incubated in BUdR for 2 hours. No labelled abdominal neuroblasts are present in the terminal abdominal region. Bar, 50 μm. (D) Higher magnification of the terminal region in a *cin/cin+* female at 72% of the third instar stage incubated for 2 hours. Bar, 20 μm.

Fig. 2. Time course of BUdR incorporation into the terminal neuroblasts in cin^{+} male and female ganglia during larval and pupal stages. (A) Number of neuroblasts labelled following 2 hour *in vitro* incubations in BUdR. Each point represents the age of the animal at the beginning of the incubation period. The tick marks on the X-axis denote a four hour interval of development at 25°C. Developmental landmarks are: hatching to first instar (H), ecdysis to the second instar (I), ecdysis to the third instar (II), and white puparium formation. Male cin/cin^{+} Y (●) female cin/cin^{+} (□). (B) Camera lucida drawings of cin/cin^{+} Y abdominal ganglia during the third instar with the location and number of terminal neuroblasts (filled circles), anterior abdominal neuroblasts (large clear circles) and ganglion mother cells (small clear circles). The length of the BUdR pulse is too short to label all of the active anterior abdominal neuroblasts at the 0-31% third instar time points. (C) Camera lucida drawings of cin/cin^{+} female abdominal ganglia. Bar, 20μm.







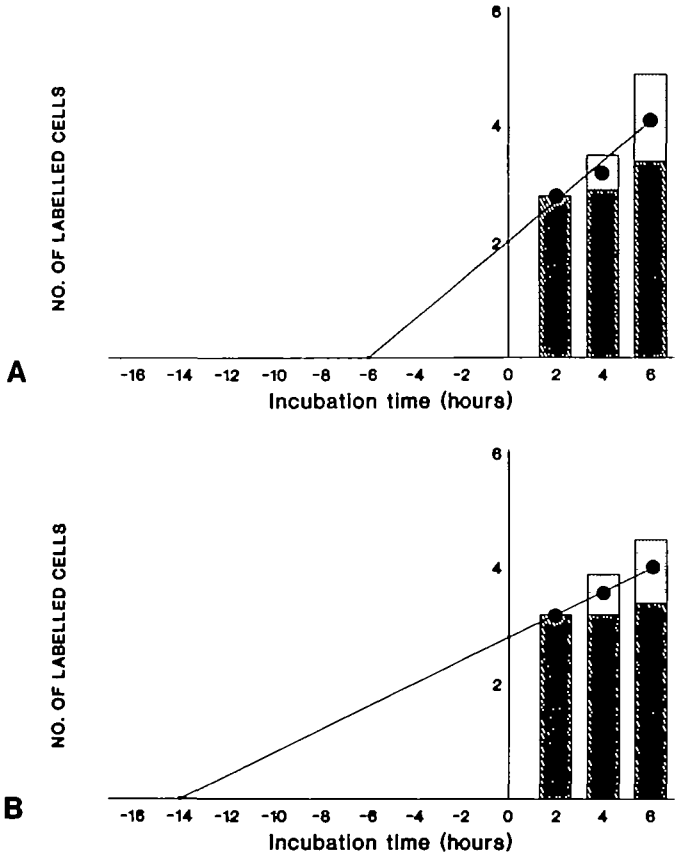


Fig. 3. Number of labelled cells associated with neuroblasts in male ganglia from wandering third instar larvae exposed to BUdR *in vitro*. The average number of ganglion mother cells (GMCs, hatched) and neurons (stippled) per neuroblast was calculated for each nervous system and then averaged for the group exposed to BUdR. The GMCs or GMC equivalents (average number of GMCs + 1/2 average number of neurons) are indicated for each group (black dot). A regression line (Cricket graphics) shows that a GMC is added to the labelled clusters in Canton-S ganglia about once every three hours. The total time to produce a neuron is about 9-10 hours, broken up into a GMC G_2M-phase of around 4 hours and a GMC S-phase of around 5.5 to 6 hours. (A) Canton-S male ganglia incubated for 2 (n=9), 4 (n=7) or 6 (n=17) hours. (B) *cin/cin*$^+$ Y male ganglia incubated for 2 (n=17), 4 (n=7), 6 (n=8).

birth and division of a GMC must be around 9-10 hours. The length of the G_2M phase of the GMC cell cycle can be determined by the time it takes for the first labelled neuron to appear, which occurred after four hours of labelling. Thus, the S-phase duration for GMCs was around 5.5 to 6 hours and is given by the X-intercept of the regression line. The Y-intercept indicates the number of GMCs (around 2) that would be labelled under very short exposures to BUdR because of the overlapping durations of the S-phases of different GMCs.

In *cin/cin*$^+$ Y ganglia, four neuroblasts were also labelled after a 2 hour pulse of BUdR (Fig. 3B). The number of GMC equivalents were 3.2, 3.6 and 4.0 for pulses of 2, 4 and 6 hours. The rate of GMC addition by the sex-specific neuroblasts at one GMC every 5 hours was slower than for neuroblasts in Canton-S ganglia. In addition, the S-phase of the GMCs was lengthened to about 14 hours with a concomitant increase in the number of GMCs that would label with instantaneous pulses (Y-intercept). The G_2M phase for the GMCs in *cin/cin*$^+$ Y ganglia, however, was around 4 hours. The differences in the cell cycle durations primarily in the S-phase reflect normal variations, since *cin/cin*$^+$ Y flies are normal and fertile, and are partly compensated by the slower development of *cin/cin*$^+$ Y larvae compared to Canton-S larvae. These cell cycle durations were obtained by averaging together the numbers of GMCs and neurons for each of the four neuroblasts per ganglion; thus, any systematic differences between the two bilaterally symmetric pairs of neuroblasts are obscured by this analysis.

Divisions of terminal neuroblasts in tra *and* tra-2 *mutants*

To determine whether terminal abdominal neuroblasts had entered the male pathway in female flies sexually transformed into phenotypic males, wandering larval or white prepupal nervous systems from *tra*^{1} homozygotes and *tra*^{1}/*Df(3L)st*^{j7} *trans*-heterozygotes were incubated in BUdR. At these stages, the only abdominal neuroblasts still dividing are in the terminal portion of male ganglia (Fig. 2A). As shown in Fig. 4A, labelled terminal neuroblasts were present in ganglia derived from these chromosomally female *tra*$^-$ mutants. The posterior location of the neuroblasts and their clusters of progeny, near the 7th and 8th nerve roots, matched the position of the male-specific neuroblasts found in either X/Y *tra*$^-$ mutant (data not shown) or control male nervous systems (Figs 1B,2B). The average number of neuroblasts labelled in X/X *tra*^{1} homozygotes or *tra*^{1}/*Df(3L)st*^{j7} was similar to the number present in X/Y *tra*^{1} homozygotes or *tra*^{1}/*Df(3L)st*^{j7} or control males (Table 3). The number of GMCs and neurons produced by the late-dividing neuroblasts were similar in both male and female *tra*$^-$ ganglia. Each neuroblast from X/X *tra*^{1}/*Df(3L)st*^{j7} flies was associated with 2.5 ± 0.3 (mean ± s.e.m.) and from X/Y flies with 2.2 ± 0.2 GMCs during a 5-6 hour incubation period.

Mitotically active terminal neuroblasts were also present in abdominal ganglia from late larval and early pupal stages in X/X *tra-2*^{1} homozygotes and *tra-2*^{B}/*Df(2R)trix* flies (Table 3; Fig. 4B). To show that the sex-specific neuroblasts from X/X *tra-2*^{1} homozygotes continued to divide later into the pupal stage, ganglia from mutant pupae up to 16 hours postpupariation were incubated in BUdR. The posterior neuroblasts from X/X *tra-2*^{1} homozygotes divided up to 12 hours postpupariation (n=27, data not shown) which was similar to the temporal limits found in control male ganglia (Fig. 2A). The number of GMCs labelled after a four hour pulse in both female *tra-2*^{1}/*tra-2*^{1} ganglia was similar to the number seen in control males (3.0 ± 0.1 GMCs; Fig. 3).

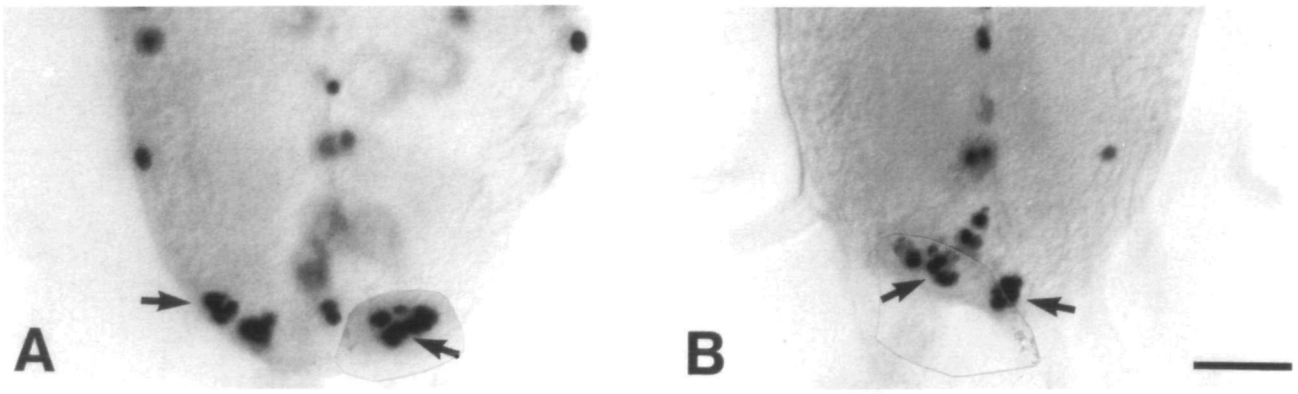


Fig. 4. Composite photomicrographs with two focal planes of wandering larval or white prepupal nervous systems from transformed female flies incubated in BUdR for 5-6 hours. (A) X/X $tra^{1}/Df(3L)st^{J7}$, arrows point to four labelled terminal neuroblasts. The other labelled nuclei laterally placed in the abdominal ganglia are from the endomitotic divisions of the nuclei of the connective tissue sheath. (B) X/X $tra\text{-}2^{B}/Df(2R)trix$, arrows point to the labelled neuroblasts. Bar, 20μm.

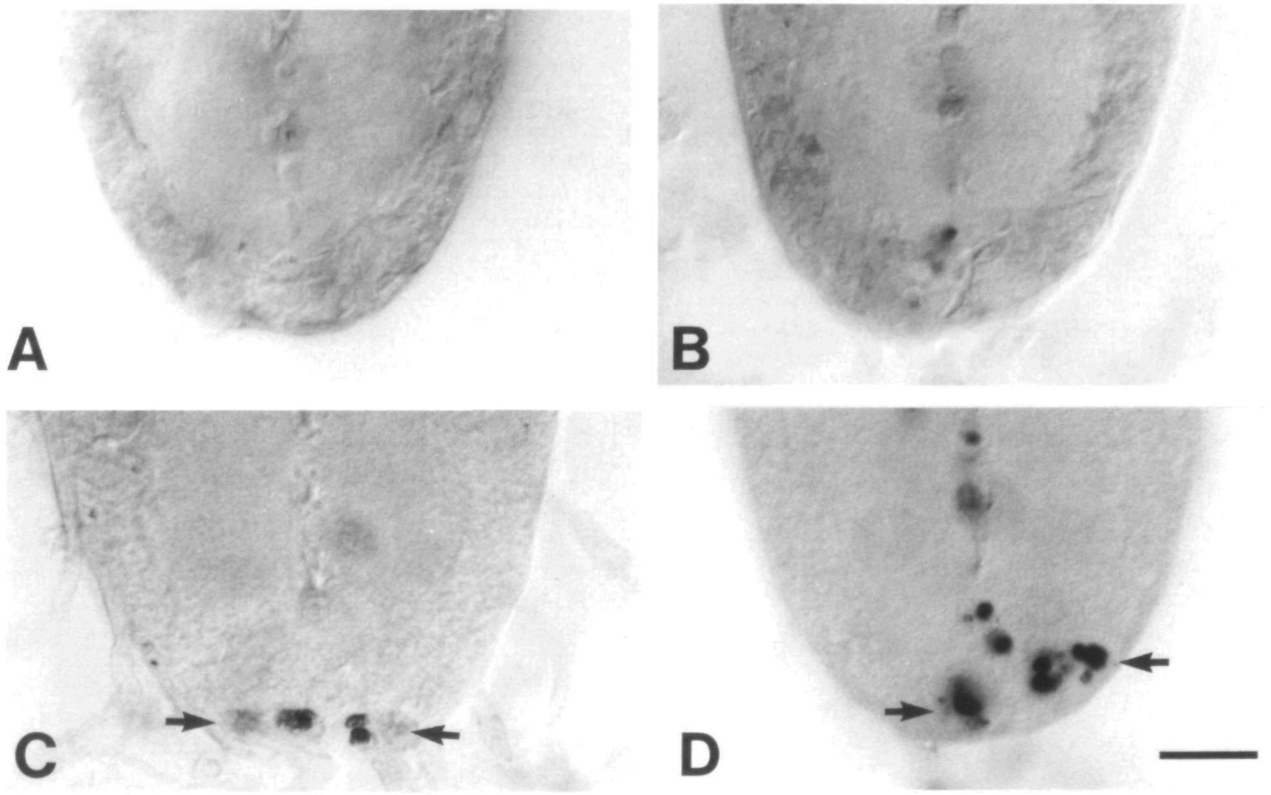


Fig. 5. Photomicrographs of wandering larval nervous systems from *dsx* mutants. (A) X/X $dsx^{23}/Df(3R)dsx^{15}$, no labelled neuroblasts. (B) X/Y $dsx^{23}/Df(3R)dsx^{15}$, no labelled neuroblasts. (C) X/X $dsx^{D}/Df(3R)dsx^{15}$, four labelled neuroblasts (arrows). (D) X/Y $dsx^{D}/Df(3R)dsx^{15}$, four labelled neuroblasts (arrows). Bar, 20μm.

Divisions of terminal neuroblasts in loss-of-function dsx *mutants*

Both male and female flies bearing loss-of-function *dsx* mutations simultaneously express masculine and feminine attributes in many of their sexually dimorphic tissues (Hildreth, 1965; Baker and Ridge, 1980; Baker and Belote, 1983; Nöthiger et al., 1987). To ascertain if any terminal neuroblasts from *dsx* null male and female ganglia had a male-specific division pattern, wandering larvae or white prepupae of three different genotypes were dissected and their nervous systems incubated in BUdR. There was no incorporation of the label into terminal neuroblasts from male or female ganglia from dsx^{1} homozygotes, $dsx^{23}/Df(3R)dsx^{15}$ (Fig. 5A,B), a *trans*-heterozygote combination that eliminates sex-specific transcripts on northern blots (R. Nagoshi, personal communication), or $dsx^{19}/Df(3R)dsx^{15}$ (Table 3). That *dsx* is epistatic to *tra* has been shown by

incubating wandering larval nervous systems from male and female *tra*^{1} *dsx*^{1} double mutants; late-dividing neuroblasts were not found in these doubly mutant abdominal ganglia, the same phenotype as expressed by *dsx* null mutants alone (Table 3).

Divisions of terminal neuroblasts in dsx^{D}/Df(3R)dsx *mutants*

The absence of late neurogenesis in loss-of-function *dsx* mutants suggests that the male *dsx*$^{+}$ product is required for terminal neuroblasts to display the male pattern of divisions. Chromosomally male or female flies mutant for *dsx dominant* alleles in *trans* with a *dsx* deficiency express only the male *dsx* mRNA (Nagoshi and Baker, 1990). Females of this genotype are phenotypically male but sterile, whereas, males are normal and fertile flies (Duncan and Kaufmann, 1975; Baker and Ridge, 1980; Nöthiger et al., 1980, 1987, 1989). To test the hypothesis that the male-specific product of *dsx* is necessary for late divisions by abdominal neuroblasts, wandering third instar nervous systems from both X/X and X/Y *dsx*^{D}/*Df(3R)dsx*^{15} animals were incubated in BUdR. Four terminal neuroblasts were labelled in X/X and X/Y *dsx*^{D}/*Df(3R)dsx*^{15} ganglia showing that the male *dsx*$^{+}$ product is required at least for the male-specific phase of divisions (Fig. 5C,D; Table 3).

The parameters of the mitotic cycles of neuroblasts and GMCs were determined for *dsx*^{D}/*Df(3R)dsx*^{15} larval ganglia. As was true for nervous systems from control males, four neuroblasts were labelled with the shortest pulse of 2 hours. The number of GMCs labelled per neuroblasts in X/X *dsx*^{D}/*Df(3R)dsx*^{15} ganglia was 2.5, 3 and 2.9 with pulses of 2, 4 and 6 hours, and for X/Y *dsx*^{D}/*Df(3R)dsx*^{15} was 2.6 and 2.8 with pulses of 2 and 6 hours, respectively. The number of GMC equivalents calculated for X/X *dsx*^{D}/*Df(3R)dsx*^{15} nervous systems was 2.5, 3.2 and 3.4 and for X/Y *dsx*^{D}/*Df(3R)dsx*^{15} nervous systems 2.6 and 3.4 for the time points listed above. From the regression line fitted to the data from X/X *dsx*^{D}/*Df(3R)dsx*^{15} ganglia, the rate of GMC addition was found to be around one GMC every four hours and the GMC S-phase (X-intercept) lasts for about 9 hours (data not shown). Labelled neurons first appear after a four hour pulse suggesting that GMCs have a G_2M-phase of about this length. Thus, the GMC cell cycle has a duration of about thirteen hours in these mutants. These values are in between those from control males (Fig. 3) showing that the male *dsx* product from the *dsx*^{D} allele is necessary and sufficient to support the normal male pattern of divisions by the terminal sex-specific neuroblasts.

Time course of terminal neuroblast divisions in dsx^{-} *mutants*

In *dsx* null flies, the failure of neuroblasts to be mitotic during the stages when male-specific divisions take place reveals an earlier block in neurogenesis. The developmental time course of sex-specific neuroblast divisions in *dsx*^{1}/*dsx*^{1} and *dsx*^{23}/*Df(3R)dsx*^{15} mutant ganglia was determined by exposure to BUdR throughout larval life (Fig. 6). Nervous systems dissected from late first instars of *dsx*^{23}/*Df(3R)dsx*^{15} *trans*-heterozygotes and incubated in BUdR over the time that they would have ecdysed to the second instar showed labelling in only eight terminal neuroblasts rather than the twelve stem cells normally seen in wild-type larvae. The absence of four neuroblasts in both male and female *dsx* mutant ganglia strongly suggests that the sex-specific neuroblasts require the presence of one or the other *dsx*$^{+}$ protein to generate any portion of their normal postembryonic lineage. These eight neuroblasts began dividing about half-way through the second instar and continued into the middle of the third instar but stop dividing at a time when the neuroblasts of other more anterior abdominal segments ceased mitosis (Fig. 6). In ganglia from mid-third instar larvae (40-48%), the last abdominal neuroblasts in mitosis were lateral and close to the 6th nerve root. Because of their more anterior location, these neuroblasts could either be lateral terminal neuroblasts or associated with neuroblasts of the sixth abdominal segment (Fig. 6B). The position of these lateral neuroblasts in *dsx* null mutants contrasts with the primarily medial and posterior location of the last dividing neuroblasts in both female and male controls (Fig. 2B,C) and may be indicative of a role for *dsx*$^{+}$ in the divisions of other abdominal neuroblasts.

Divisions of terminal neuroblasts in dsx *dominant*/dsx^{+} *mutants*

In both male and female CNSs, four terminal neuroblasts require *dsx*$^{+}$ function for their postembryonic divisions (Fig. 6). Whether the neuroblasts in female ganglia correspond to those in male ganglia was elucidated by examining ganglia from female *dsx*^{D}/*dsx*$^{+}$ and *dsx*^{M}/*dsx*$^{+}$ larvae. These animals exhibit intersexual differentiation resulting from the presence of both male and female *dsx*$^{+}$ products (Denell and Jackson, 1972; Duncan and Kaufmann, 1975; Baker and Ridge, 1980; Nöthiger et al., 1980, 1987, 1989). Twelve terminal neuroblasts incorporated BUdR when ganglia of *dsx*^{M}/*dsx*$^{+}$ females were exposed throughout the period when first instar larvae molt into second instars (Fig. 7A). The same number of terminal neuroblasts were found in both male or female wild-type larvae at these times (Fig. 2A). If the sex-specific neuroblasts in female ganglia were distinct from those in male ganglia then sixteen neuroblasts would have been expected in *dsx*^{M}/*dsx*$^{+}$ female ganglia. The twelve neuroblasts found in *dsx*^{M}/*dsx*$^{+}$ ganglia began their postembryonic divisions in mid-second instar larvae (50%) and continued dividing until the middle of the third instar (Fig. 7A).

During the third instar when female neuroblasts normally become quiescent, some neuroblasts persisted in both X/X *dsx*^{D}/*dsx*$^{+}$ and *dsx*^{M}/*dsx*$^{+}$ ganglia (Table 3; Figs 7, 8). In these mutants, neuroblasts and their progeny could be labelled until the time when divisions would normally stop in control male ganglia. The number of neuroblasts labelled in pulses during the late third instar and through the early pupal period was

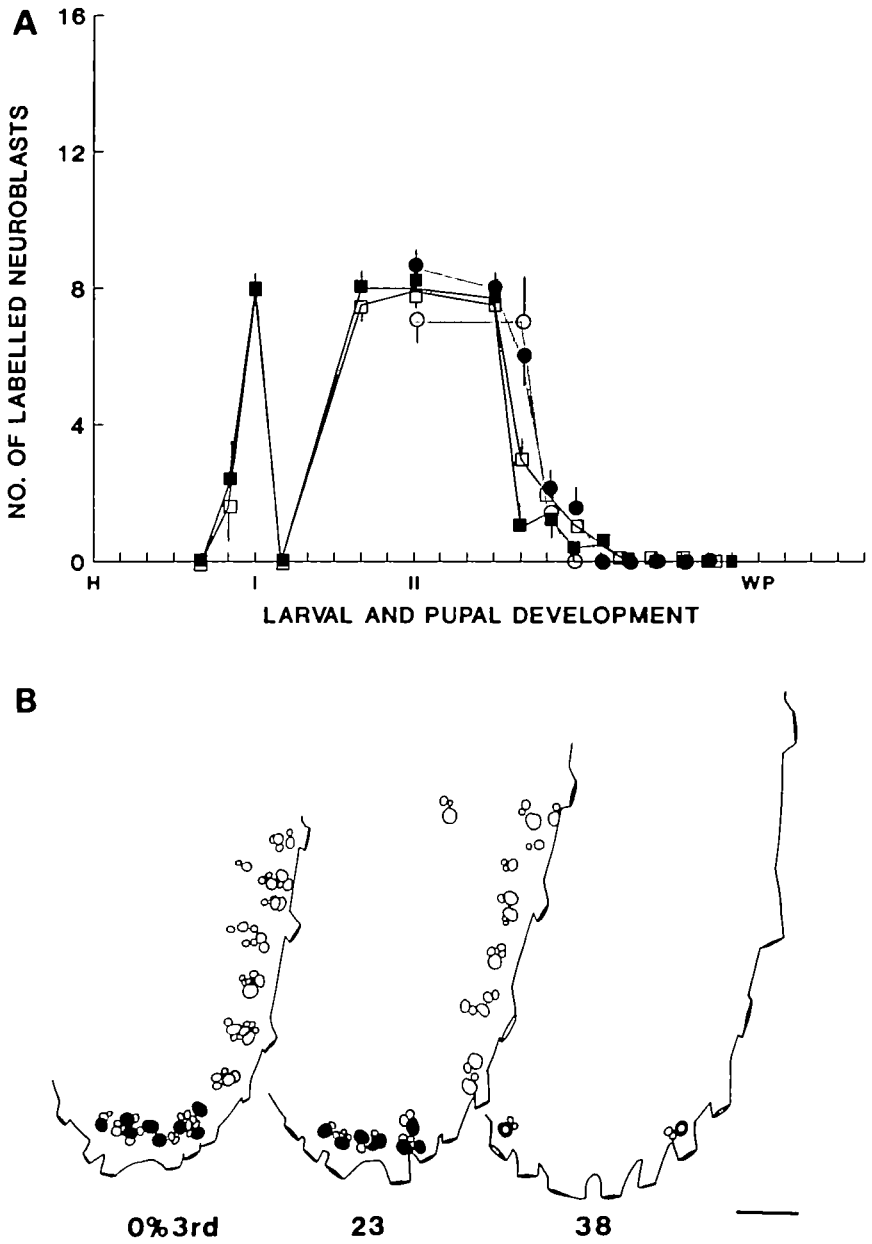


Fig. 6. Time course of BUdR incorporation into terminal neuroblasts in loss-of-function *dsx* mutants. (A) Number of terminal neuroblasts that can be labelled at different times during larval life in dsx^1/dsx^1 (X/X ●; X/Y ○) and $dsx^{23}/Df(3R)dsx^{15}$ (X/X ■; X/Y □). (B) Camera lucida drawings of X/X $dsx^{23}/Df(3R)dsx^{15}$ nervous systems at different stages of the third instar. The lateral neuroblasts in the ganglion from the 38% third instar are marked as annuli to indicate the uncertainty of their inclusion within the terminal neuroblast group. Tick marks as in Fig. 2. Bar, 20μm.

quite variable. On average, less than one neuroblast per ganglia was found to be dividing (Table 3); in a number of ganglia no neuroblasts were labelled with BUdR while in others two or three neuroblasts were present.

For the neuroblasts in dsx^M/dsx^+ females displaying the male pattern of divisions, their cell cycle properties were similar to those of control cin/cin^+ Y male neuroblasts and GMCs. The number of labelled GMCs per neuroblast were 3.1, 3.6 and 3.3 for pulses of 2, 4 and 5.5-6 hours. The GMCs equivalents were calculated to be 3.1, 3.9 and 3.8 for the three pulses tested. From the plotted regression line, the addition of new GMCs occurred at a rate of one for every five hours and the S-phase (X-intercept) duration was around fourteen hours (data not shown). Labelled neurons first appeared after a four hour pulse indicative of a G_2M duration of this length.

We found no evidence that neuroblasts in these mutants once they had entered the male pathway were capable of premature cessation of mitosis rather than being stably committed for the entire period of male-specific divisions. GMCs were labelled only in association with a neuroblast; and neuroblasts, when labelled, had GMCs and neurons. Once neuroblasts from mutant ganglia have entered the male pathway, they seem to perform as efficiently as those in ganglia from male siblings or control males irrespective of the presence of a normal dsx^+ allele.

An initial commitment step for the male pathway occurs at the first larval molt

The time when neuroblasts adopt a particular sex-specific developmental program can be discerned by using conditional *tra-2* mutations. When X/X *tra-*

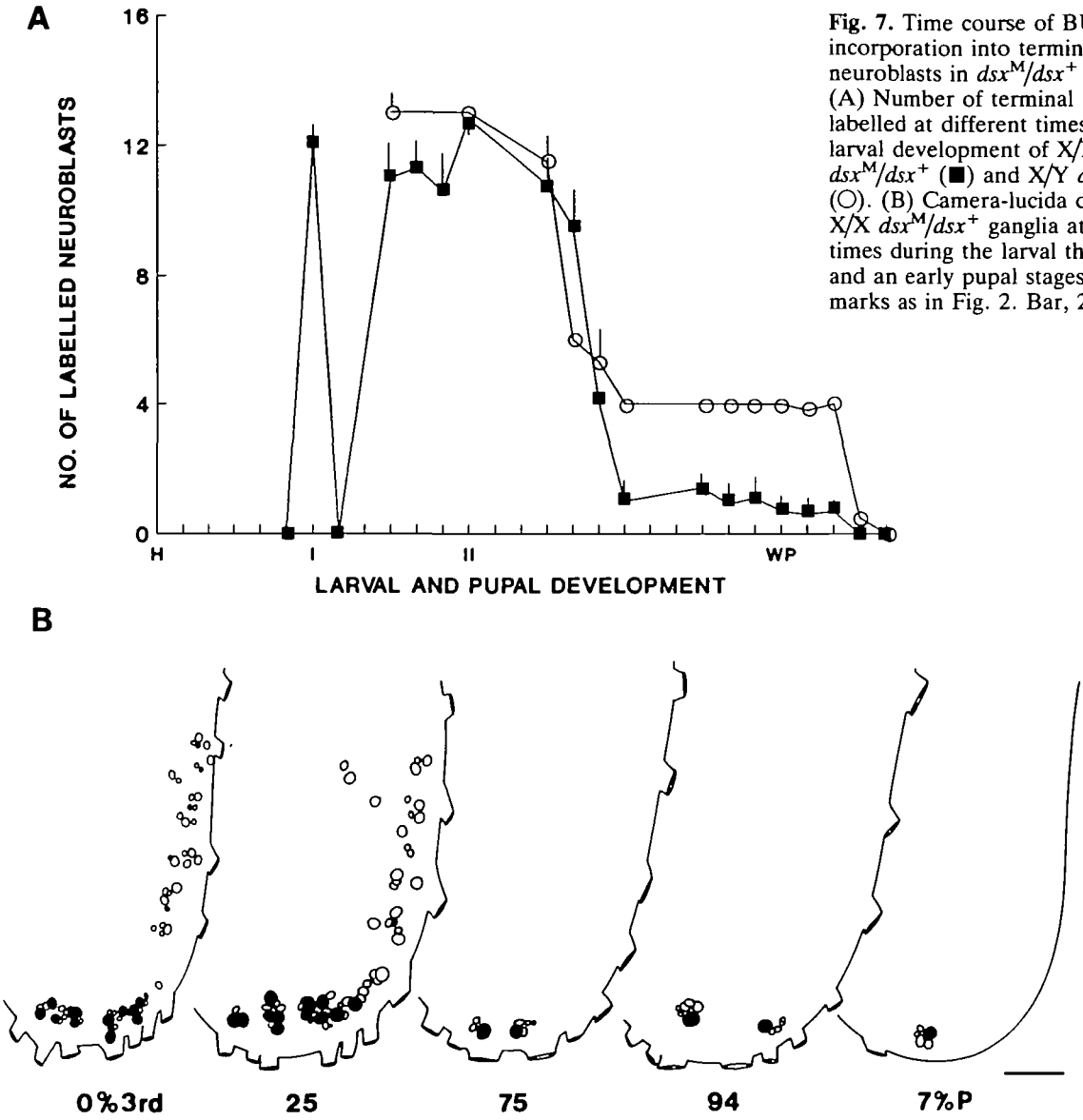


Fig. 7. Time course of BUdR incorporation into terminal neuroblasts in dsx^{M}/dsx^{+} mutants. (A) Number of terminal neuroblasts labelled at different times during larval development of X/X dsx^{M}/dsx^{+} (■) and X/Y dsx^{M}/dsx^{+} (○). (B) Camera-lucida drawings of X/X dsx^{M}/dsx^{+} ganglia at different times during the larval third instar and an early pupal stages. Tick marks as in Fig. 2. Bar, 20μm.

$2^{ts1}/tra\text{-}2^{ts2}$ ($tra\text{-}2^{ts}$) flies were raised at the female-determining temperature (18°C), no neuroblasts in the terminal abdominal region were labelled by BUdR incorporation in ganglia from wandering larvae or white prepupae. In contrast, in ganglia from flies of the same genotype raised at the male-determining temperature, (29°C) four neuroblasts were labelled (Table 3; Figs 9,10A). Mutant animals were shifted between the female-determining temperature and the male-determining temperature during embryogenesis and larval life. The presence or absence of late-dividing neuroblasts was then monitored by *in vitro* BUdR incorporation in ganglia removed when animals became wandering third instars or white puparia (Fig. 9). In these experiments, only labelled neuroblasts can unambiguously be assigned a fate, which is that of male-specific neuroblasts; unlabelled ganglia could represent cases where neuroblasts were unable to incorporate BUdR because they were in the female-specific pathway or simply absent. Nervous systems of male $tra\text{-}2^{ts}$ larvae raised under the temperature regimes employed in these experiments and incubated as wandering third instar larvae had four labelled neuroblasts (data not shown).

Temperature shifts from the female- to the male-determining temperature indicate the latest time in development when neuroblasts could enter the male-specific pathway. Late-dividing neuroblasts were found when embryos or first instar larvae were shifted to the male-determining temperature (Fig. 9). Flies shifted as embryos have from three to four neuroblasts expressing the male pattern whereas flies shifted as first instars generally have two labelled neuroblasts (Figs 9,10B). Because of the normal variability in the location of the four male-specific neuroblasts, we could not establish whether these two labelled neuroblasts represented one of the two bilaterally symmetric pairs or a random sample of the possible pairs. The latest time when larval shifts could lead to the activation of the male-specific pathway was at the end of the first instar. Ganglia from

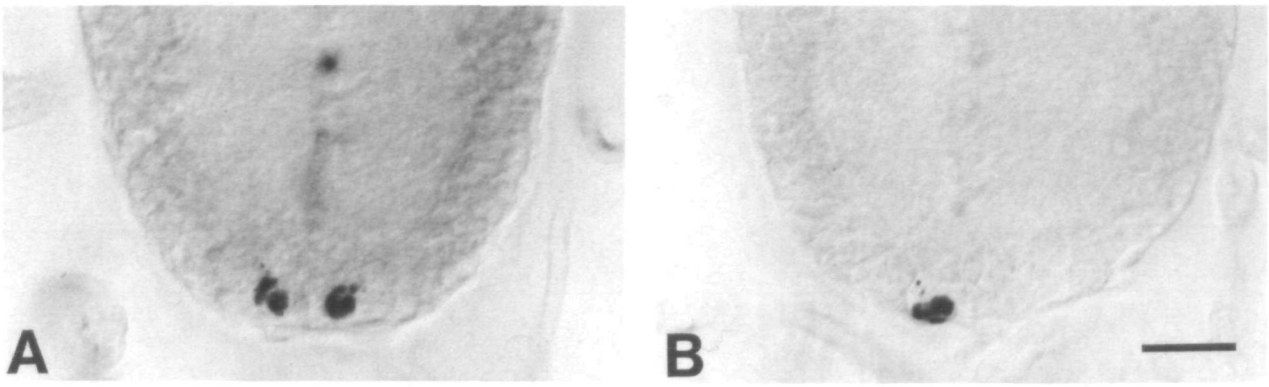


Fig. 8. Photomicrographs of X/X dsx^{M}/dsx^{+} abdominal ganglia. (A) 75% third instar larva, 5 hour incubation in BUdR. (B) 83% third instar larva, 4 hour incubation in BUdR. Bar, 20μm.

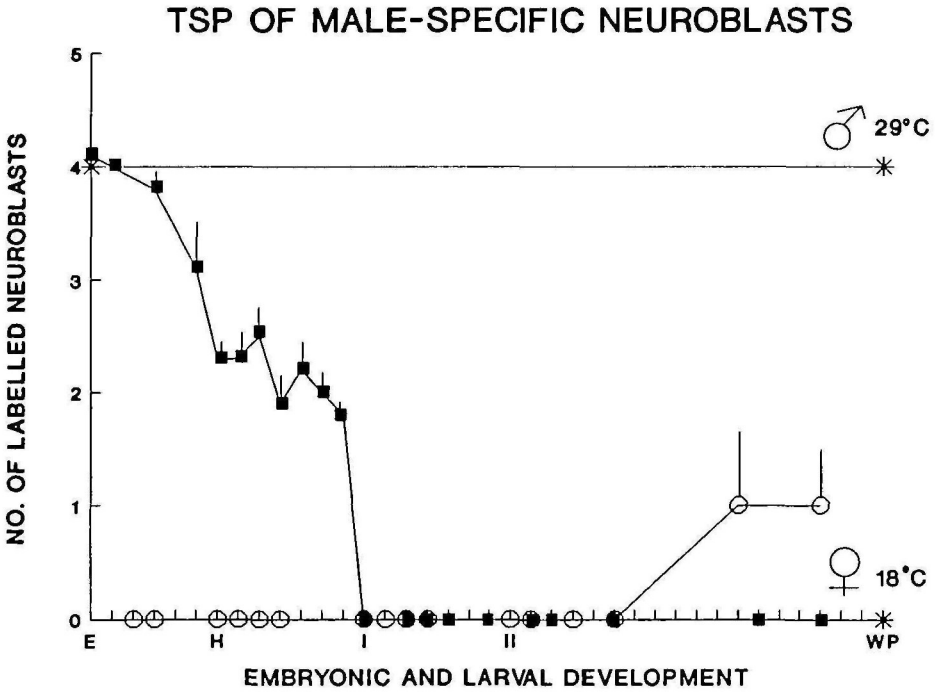


Fig. 9. Temperature-sensitive period of the male pattern of divisions in X/X $tra\text{-}2^{ts1}/tra\text{-}2^{ts2}$ ganglia. Nervous systems were assayed as late third instar larvae or white prepupae, a time when only the male-specific neuroblasts would still be dividing. Each point represents the number of labelled neuroblasts in CNSs that had been shifted at the time shown on the X-axis. The tick marks represent about eight hours of development at 18°C. Temperature shifts from the male- (29°C) to the female-specifying temperature (18°C) (○). Temperature shifts from the female- to the male-specifying temperature (■).

animals shifted to the male-determining temperature as late first instar larvae still had some late-dividing neuroblasts, whereas, ganglia from larvae transferred five hours later as freshly ecdysed second instars, had neuroblasts that were not labelled (Fig. 10C). Similarly, when $tra\text{-}2^{ts}$ larvae were shifted as second or third instars, there was no incorporation of BUdR in neuroblasts when tested as wandering larvae (Figs 9,10D).

The opposite set of temperature shifts, from the male- to the female-determining temperature, identify the earliest time when neuroblasts could be irreversibly committed to the male-specific pathway. When larvae were shifted to the female-specifying temperature as first, second or early third instar larvae and the ganglia assayed from wandering third instar larvae, terminal abdominal neuroblasts did not incorporate BUdR, indicating that no neuroblasts were in the male-specific mode of divisions (Fig. 9). To examine more closely the fate of neuroblasts that had entered the male-specific pathway, X/X $tra\text{-}2^{ts}$ animals were shifted to the female-determining temperature during the mid-third instar and examined at two different time points. Twenty-four hours after the shift (about 17% of the duration of the third instar), four sex-specific neuroblasts could still be labelled (n=9; Fig. 11A), but by 72 hours at 18°C, at most only one terminal neuroblast still labelled (n=4; Fig. 11B).

To determine if the commitment of sex-specific neuroblasts to a sex-specific pathway could be affected at any time during their postembryonic divisions, mutant larvae raised at one temperature were shifted after ecdysis to the second instar and their nervous systems were incubated in BUdR as soon as they became third instars. In control ganglia, both male and female flies have twelve terminal neuroblasts that are mitotically active at the beginning of the third instar (Fig. 2). In ganglia from larvae raised at the male-

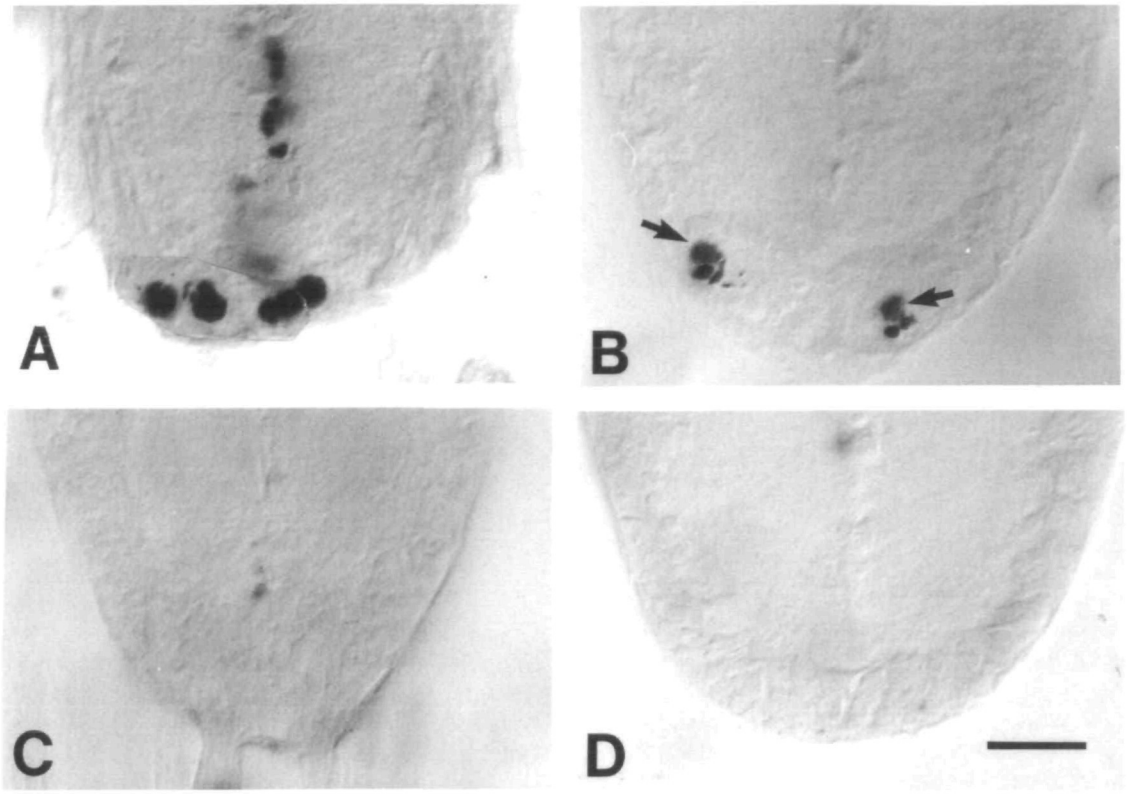


Fig. 10. Photomicrographs of ganglia from X/X *tra-2*^{ts} wandering third instar larvae that had been shifted earlier in development from the female- to the male-specifying temperature. (A) Composite photograph of ganglia from a larva raised at the male-specifying temperature continuously. (B) Shifted as a 30% first instar, arrows indicate two labelled neuroblasts and their progeny. (C) Shifted as a newly ecdysed 2nd instar. (D) Shifted as a 25% third instar. Bar, 20μm.

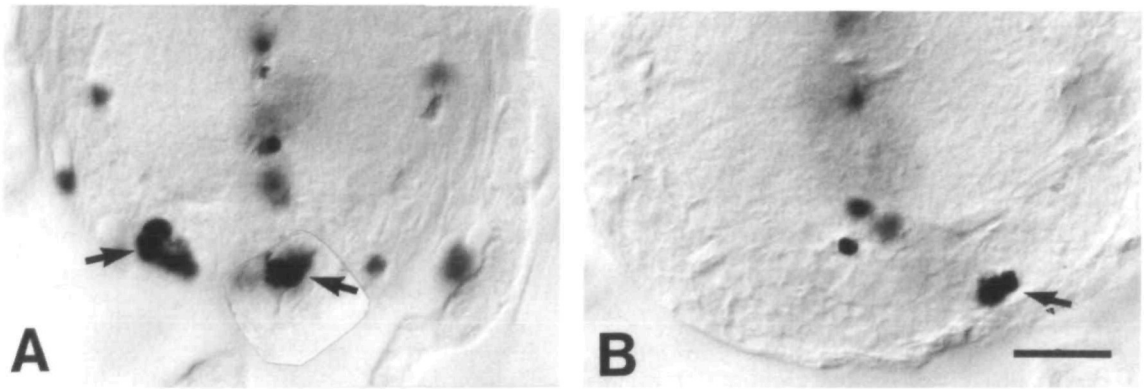


Fig. 11. Cessation of divisions by male-specific neuroblasts in flies raised to 34% of the third instar at the male-specifying temperature then shifted to the female-specifying temperature. (A) After 24 hours at 18°C, a period equalling 17% of the third instar at this temperature. Arrows indicate the presence of four labelled neuroblasts and their progeny. (B) After 72 hours at 18°C, a period equalling 52% of the third instar. An arrow indicates the single labelled neuroblast and progeny. Bar, 20μm.

specifying temperature then shifted to the female-determining temperature, only eight neuroblasts were dividing in ganglia from recently ecdysed third instar larvae (Fig. 12A). Likewise, larvae raised at the female-specifying temperature and shifted to the male-determining temperature showed that only eight terminal neuroblasts were still dividing in newly ecdysed third instars (Fig. 12B). These experiments show that the commitment of neuroblasts to either a male or female fate must be maintained throughout the period when their sex-specific lineages are being produced.

Temperature-pulse experiments were used to assay whether the male-specific neuroblasts could recover from treatment at the female-specific temperature. When larvae were shifted as early to mid-second instars from the male- to the female-specifying temperature

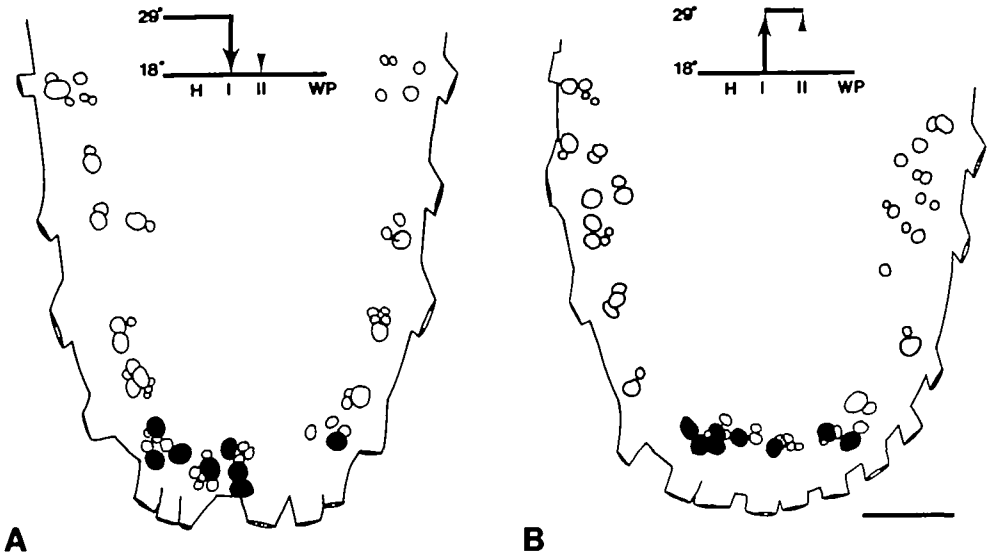


Fig. 12. The effect of temperature shifts on sex-specific neuroblast divisions after commitment to a sexual pathway. X/X *tra-2*[15] larvae raised at one temperature until ecdysis to the second instar were shifted to the opposite temperature (arrow in inset) and examined in recently ecdysed third instars (arrowhead in inset). (A) Shift from the male- to female-specifying temperature. (B). Shift from the female- to male-specifying temperature. Bar, 20μm.

and then returned to the male-specifying temperature in the mid-third instar, the male-specific neuroblasts did not resume divisions when examined by BUdR incorporation as white puparia (data not shown). The exposure of neuroblasts that had been committed to the male division pattern to the female-specifying temperature, under the conditions employed here, appears to irreversibly block the expression of the male mode of late divisions by these neuroblasts.

Discussion

Characteristics of terminal sex-specific neuroblasts

In the terminal region of the abdominal CNS of larvae, twelve neuroblasts are found in both males and females. Terminal neuroblasts exhibit the same behavior in both sexes as gauged by BUdR incorporation until the middle of the third instar. They enter their first "S" phase during the late first instar and begin divisions during the mid-second instar. Eight of these twelve neuroblasts in males and all twelve neuroblasts in females stop dividing in the middle of the third instar.

In male larvae, four neuroblasts continue to divide until around 12 hours after pupariation (12%P). With BUdR labelling alone, it is not possible to identify which of the twelve terminal neuroblasts enters the male pathway until the other eight neuroblasts cease divisions. During the male-specific period of divisions in Canton-S ganglia, each neuroblast divides approximately once every three hours over this roughly 30 hour period, adding about twenty extra neurons to the adult nervous system of male compared to female flies (schematized in Fig. 13A). From preliminary experiments, when BUdR is fed to males as late third instar larvae or injected into white prepupae, labelled neurons are present in the terminal part of the adult abdominal ganglia suggesting that the male-specific cells persist into the adult (data not shown).

Role of the sex-determining genes in establishing and maintaining the sex-specific divisions of terminal neuroblasts

The genes of the sex-determining hierarchy are essential for the appropriate development and divisions of sex-specific neuroblasts. The male-specific product of dsx^+ is needed for four neuroblasts to enter the male pathway, which has been monitored by their division pattern during the later part of the third instar through the early pupal stages. In females sexually transformed into phenotypic males as in tra^-, $tra\text{-}2^-$ or $dsx^D/Df(3R)dsx^{15}$ mutants, four terminal neuroblasts have the male pattern of persistent divisions with roughly the same mitotic rate as control males (Fig. 13B). In $dsx^D/Df(3R)dsx^{15}$ males, the male dsx^+ product, provided by the dsx^D allele, is necessary and sufficient for four terminal neuroblasts to enter the male pathway.

In both sexes, wild-type dsx^+ gene products are necessary for the generation of a postembryonic lineage by the sex-specific neuroblasts. In dsx^- homozygotes, four terminal neuroblasts never incorporate BUdR at any time during the second or third instar stages when all other terminal neuroblasts are mitotically active. The failure to specify the sexual identity of these neuroblasts results in the complete inability of these neuroblasts to perform their normal divisions, not just to engage in a series of sexually indifferent mitoses. From these experiments, it cannot be determined whether the neuroblasts are present and unable to divide, or whether they fail to initiate any postembryonic development such as re-enlarging during the first instar (White and Kankel, 1978; Truman and Bate, 1988).

The absence of sex-specific neuroblasts in *dsx* null ganglia is unusual compared to that of other sexually dimorphic tissues that require the activity of the dsx^+ gene. For example, in the genital disc, both genital primordia differentiate in *dsx* null mutants (Hildreth, 1965). Other structures, like the sexcombs bristles on

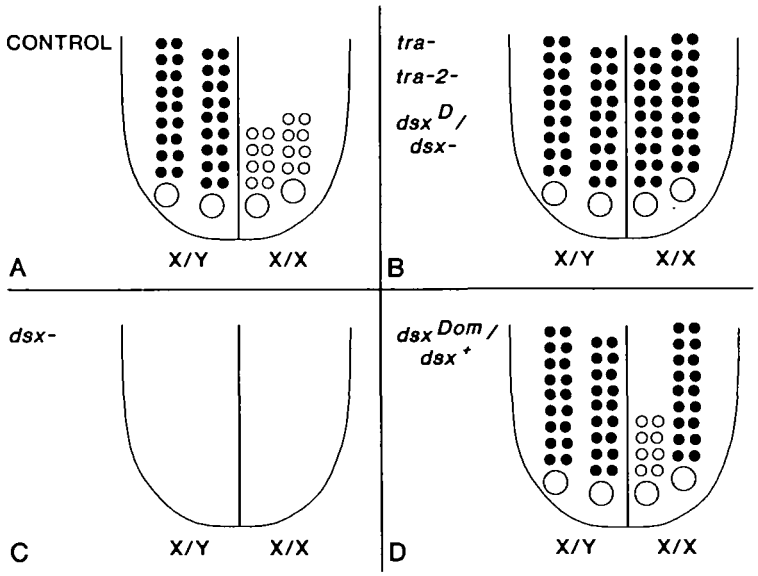


Fig. 13. Summary of the divisions of sex-specific terminal neuroblasts. (A) Control male (X/Y) and female (X/X) hemiganglia of the terminal abdominal region. Neuroblasts (stippled) in the female pathway generate a few neurons (clear circles) while those with male pattern make many more neurons (black circles). (B) Mutant conditions that transform females into phenotypic males cause terminal neuroblasts to express only the male type of divisions. (C) Loss-of-function *dsx* mutants have no sex-specific terminal neuroblasts. (D) X/X dsx^{Dom}/dsx^{+} ganglia have some neuroblasts that adopt the male and others that take the female pathway and produce appropriately sized lineages in accordance with that choice.

the foreleg, differentiate with an intermediate number and intersexual morphology in dsx^{-} mutants (Hildreth, 1965; Baker and Ridge, 1980). Biochemical sexual dimorphisms show similar effects; for example, yolk proteins, normally a female-specific product of fat body cells, are synthesized by both male and female fat body cells in dsx^{-} flies (Postlethwait et al., 1980; Ota et al., 1981; Bownes and Nöthiger, 1981). Consideration of these findings led to the hypothesis that the products of the dsx^{+} gene suppress the differentiation of the opposite sexual phenotype in these tissues (Baker and Ridge, 1980; Baker and Belote, 1983). Although the molecular mechanisms by which dsx^{+} controls the specification of sex-specific neuroblasts is unknown, the dsx^{+} gene products appear to act by stimulating neuroblast activity, not solely by suppressing inappropriate sexual differentiation.

For many tissues the intersexual development that results from loss-of-function *dsx* mutations is very similar to that obtained when both male and female *dsx* products are active, as in X/X dsx^{D}/dsx^{+} or dsx^{M}/dsx^{+} flies (Nöthiger et al., 1980, 1987; Baker and Ridge, 1980). For the sex-specific neuroblasts, their development and differentiation is very distinct in these two type of mutants. Whereas the sex-specific neuroblasts in *dsx* null mutants completely fail to divide, those in dsx^{D}/dsx^{+} or dsx^{M}/dsx^{+} appear to adopt either the female or male pathway and these decisions seem to be stable (Fig. 13C,D). Less than one-quarter of the sex-specific neuroblasts in X/X dsx^{M}/dsx^{+} ganglia entered the male-specific pathway and late-dividing neuroblasts were found in these mutant ganglia up until the time when the male-specific divisions normally cease.

The relationship between male- and female-specific terminal neuroblasts

At this time, there is no separate marker for the male- and female-specific neuroblasts. They can be distinguished only on the basis of the temporal limits of their incorporation of BUdR. Nevertheless, the analysis of the different mutants in the sex-determining genes has provided some insight into the relationship between the sex-specific neuroblasts found in both sexes. Two general models can be proposed to account for these sexually dimorphic neuroblasts. On one hand, the same four neuroblasts in the late first instar larva could opt for either a male or a female pathway depending on the activity of the dsx^{+} gene. An alternative hypothesis would be that first instar larvae possess two distinct sets of four neuroblasts with one set becoming active and the other dormant depending on the activity of the sex-determining genes.

The results from a phenotypic analysis of *dsx* mutants supports the first hypothesis. In the absence of dsx^{+} function, four neuroblasts in both male and female ganglia fail to make any postembryonic divisions. When both male and female dsx^{+} products can be produced in the same individual only twelve neuroblasts are found at times, from the mid-second to mid-third instar, when all terminal neuroblasts are normally dividing. This finding is consistent with the hypothesis that a single set of four neuroblasts exist that can choose either the male- or female-specific pathway depending on which dsx^{+} gene product is present. Because additional terminal neuroblasts are not labelled in X/X dsx^{M}/dsx^{+} ganglia, the other possibility that there are two sets of terminal neuroblasts is less tenable. However, a condition of imperfect cross-inhibition between the male- and female-specific dsx^{+} products acting in two distinct sets of sex-specific neuroblasts, which results in four active sex-specific neuroblasts per ganglia, cannot be entirely resolved until individual markers for the male- and female-specific neuroblasts are discovered.

An additional complication in the interpretation of the dsx^{M}/dsx^{+} results would occur if the male-specific

neuroblasts were a separate group from the twelve terminal neuroblasts and only became mitotically active late in the third instar. The assignment of the four late-dividing neuroblasts in male ganglia as members of the twelve terminal neuroblasts is supported by the findings that only eight terminal neuroblasts divide in male *dsx*$^-$ nervous systems, and that temperature-shifts from the male to the female-specifying temperature after neuroblast commitment to the male fate resulted in only eight dividing neuroblasts when assayed by BUdR incorporation at the beginning of the third instar (see Fig. 12), a time when twelve neuroblasts are dividing in control male central nervous systems.

Time of commitment to the male or female division patterns

During development, there are three likely decision points for control of sex-specific neurogenesis. The first is the initial emergence of neuroblasts during embryogenesis when the divisions necessary to create neurons for the larval nervous system occur. The second point is at the end of the embryonic neurogenic period when the neuroblasts decide between becoming dormant or permanently postmitotic. The third point is late in the first instar when the abdominal neuroblasts enter their first postembryonic S-phase in preparation for mitosis.

By performing temperature-shift experiments using *tra-2*^{ts} larvae, we determined that the time when sex-specific neuroblasts become committed to a male or female pathway was late in the first instar (Fig. 14). The commitment event then immediately precedes or is coincident with the first S-phase associated with the postembryonic divisions of these neuroblasts. Temperature shifts of X/X *tra-2*^{ts} flies cause the type of *dsx* mRNA produced to change; a temperature shift from the female- to the male-specifying temperature results in the expression of the male-specific form of *dsx* mRNA at the expense of the female-specific form of *dsx* mRNA (Nagoshi et al., 1988). We conclude that the presence of either the male- or female-specific dsx proteins at the end of the first instar determines whether sex-specific neuroblasts enter the male or female pathway (Fig. 14).

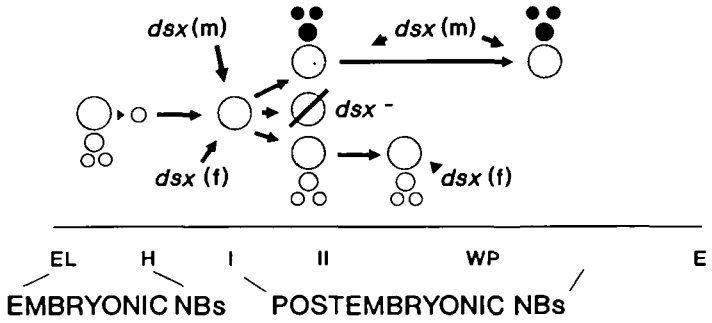


Fig. 14. Summary of the commitment of the neuroblasts to a sex-specific pathway and the timing of this decision. This schematic figure indicates the presumed embryonic divisions by the neuroblasts that will become the sex-specific neuroblasts and their subsequent postmitotic regression. The entry of the sex-specific neuroblasts during the late first instar into their first postembryonic S-phase is coincident with their commitment to a sexual pathway established by the temperature-shift experiments with *tra-2*^{ts} larvae. In X/X *tra-2*^{ts} larvae, these temperature shifts determine which of the sex-specific products of *dsx* are made and this then leads to the expression of a male or female phenotype of the neuroblasts. In the absence of any functional *dsx* products, the sex-specific neuroblasts in both sexes exhibit a third possible fate, that of failure to engage in postembryonic divisions. The ability of neuroblasts to divide throughout the larval period and produce its sex-specific lineage requires the continued presence of the *dsx*$^+$ gene products.

For all of the neuroblasts that have been studied to date, all postembryonically active neuroblasts also have embryonic divisions (Prokop and Technau, 1991). If neuroblasts that will become the sex-specific neuroblasts segregate during embryogenesis and produce an embryonic lineage, this initial emergence is independent of the functioning of the sex-determining genes (Fig. 14). These neuroblasts, then, may be unique in having two distinct determination steps, one necessary for their embryonic and another required for their postembryonic divisions, controlling their fate and that of their progeny. The genetic and molecular mechanisms that control the embryonic phase of neuroblast segregation and identity are beginning to be established. For embryonic neurogenesis, several genes are known to contribute to these processes (for review, Campos-Ortega and Jan, 1991). Expression of three genes within the *achaete-scute* complex correlates with neuroblast segregation and when absent, as in embryos with a deficiency for the locus, at least the posterior median neuroblast does not appear to segregate (Cabrera et al., 1987). Another gene, *prospero*, has been found to be important in designating neuroblast identity, resulting in the proper differentiation and axonal pathfinding of its neurons, but not necessary for the segregation of these neuroblasts, their divisions or that of their GMCs (Doe et al., 1991). The requirements for the activity of the *dsx* gene in sex-specific neuroblasts may encompass both reactivation as well as identity functions for neuroblasts and their progeny.

Stability of the commitment to the sex-specific pathway

The commitment of sex-specific neuroblasts to a male or female mode of division must be maintained throughout the period when these neuroblasts are engaged in generating their postembryonic lineages. Following an initial determinative event during the late first instar, there appears to be no other single time point after which neuroblasts become independent of the activity of *tra-2* and other downstream sex-determining genes to express a sex-specific pattern of divisions. In X/X *tra-2*^{ts} larvae, when sex-specific neuroblasts are exposed to the temperature conducive to the differentiation of the opposite sex, they cease dividing, even at times when these neuroblasts would be expected to be mitotic by comparison to control male and female CNSs (see Figs 11,12). In addition to their inability to execute a particular developmental program, this finding suggests that neuroblasts, once committed to a sex-specific neuroblast pathway, cannot

switch to the neuroblast pathway of the opposite sex. For male-specific neuroblasts, the instability of the commitment to the male-specific mode of divisions occurs under conditions where the female-specific form of *dsx* mRNA comes to predominate, as happens when X/X *tra-2*^{ts} animals are shifted from the male- to the female-specifying temperature (Nagoshi et al., 1988). Indeed, under different conditions where both male and female *dsx* products can be made, as in X/X *dsx*^{M}/*dsx*$^{+}$ animals, the commitment to the male-specific pathway appears to be stable. The decisions as to the differentiated state of sex-specific neuroblasts differs from the irreversible commitment that exists for a set of male-specific transcripts made by the male accessory glands (Chapman and Wolfner, 1988) or the reversible expression of the yolk protein genes in fat body cells in X/X *tra-2*^{ts} flies (Belote et al., 1985).

We wish to thank Drs John Ewer, Steven Robinow, Angela Giangrande, Michael McKeown and Kenneth Burtis for their careful reading of the manuscript and helpful suggestions. The *Drosophila* stocks were generously provided by Drs Bruce Baker, Rodney Nagoshi, Bruce Chase, John Belote and Michael Mckeown. The anonymous reviewers contributed many thoughtful comments that improved the manuscript. B.J.T. was supported by an NIH postdoctoral fellowship, 5F32NS08618.

References

Amrein, H., Maniatis, T. and Nöthiger, R. (1990). Alternatively spliced transcripts of the sex-determining gene *tra-2* of *Drosophila* encode functional proteins of different size. *Embo J.* **9**, 3619-3629.

Baker, B. S. (1973). The maternal and zygotic control of development by *cinnamon*, a new mutant in *Drosophila melanogaster*. *Dev. Biol.* **33**, 429-440.

Baker, B. S. (1989). Sex in flies: the splice of life. *Nature* **340**, 521-524.

Baker, B. S. and Belote, J. M. (1983). Sex determination and dosage compensation in *Drosophila melanogaster*. *Ann. Rev. Genet.* **17**, 345-393.

Baker, B. S., Hoff, G., Kaufman, T. C., Wolfner, M. F. and Hazelrigg, T. (1991). The *doublesex* locus of *Drosophila melanogaster* and its flanking regions: A cytogenetic analysis. *Genetics* **127**, 125-138.

Baker, B. S., Nagoshi, R. N. and Burtis, K. C. (1987). Molecular genetic aspects of sex determination in *Drosophila*. *BioEssays* **6**, 66-70.

Baker, B. S. and Ridge, K. A. (1980). Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* **94**, 383-423.

Baker, B. S. and Wolfner, M. F. (1988). A molecular analysis of *doublesex*, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster*. *Genes Dev.* **2**, 477-489.

Belote, J. M. and Baker, B. S. (1982). Sex determination in *Drosophila melanogaster*: Analysis of *transformer-2*, a sex-transforming locus. *Proc. Natl. Acad. Sci. USA* **79**, 1568-1572.

Belote, J. M. and Baker, B. S. (1983). The dual functions of a sex determination gene in *Drosophila melanogaster*. *Dev. Biol.* **95**, 512-517.

Belote, J. M. and Baker, B. S. (1987). Sexual behavior: Its genetic control during development and adulthood in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* **84**, 8026-8030.

Belote, J. M., Handler, A. M., Wolfner, M. F., Livak, K. J. and Baker, B. S. (1985). Sex-specific regulation of yolk protein gene expression in *Drosophila*. *Cell* **40**, 339-348.

Belote, J. M., Hoffman, F. M., McKeown, M., Chorsky, R. L. and Baker, B. S. (1990). Cytogenetic analysis of chromosome region 73AD of *Drosophila melanogaster*. *Genetics* **125**, 783-793.

Belote, J. M. and Lucchesi, J. C. (1980). Male-specific lethal mutations of *Drosophila melanogaster*. *Genetics* **96**, 165-186.

Booker, R. and Truman, J. W. (1987). Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca Sexta*. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* **255**, 548-559.

Bownes, M. and Nothiger, R. (1981). Sex determining genes and vitellogenin synthesis in *Drosophila melanogaster*. *Mol. Gen. Genet.* **182**, 222-228.

Burtis, K. C. and Baker, B. S. (1989). *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell* **56**, 997-1010.

Cabrera, C., Martinez-Arias, A. and Bate, M. (1987). The expression of three members of the *achaete-scute* gene complex correlates with neuroblast segregation in *Drosophila*. *Cell* **50**, 425-433.

Campos-Ortega, J. A. and Jan, Y. N. (1991). Genetic and molecular bases of neurogenesis in *Drosophila melanogaster*. *Ann. Rev Neurosci.* **14**, 399-420.

Chapman, K. and Wolfner, M. F. (1988). Determination of male-specific gene expression in *Drosophila* accessory glands. *Dev. Biol.* **126**, 195-202.

Cline, T. W. (1985). Primary events in the determination of sex in *Drosophila melanogaster*. In *The Origin and Evolution of Sex*, (ed. H. O. Halvorson and A. Monroy), pp. 301-327. New York: Alan Riss,Inc.

Denell, R. E. and Jackson, R. (1972). A genetic analysis of *transformer-Dominant*. *Dros. Inf. Serv.* **48**, 44-45.

Doe, C. Q., Chu-Lagraff, Q., Wright, D. M. and Scott, M. P. (1991). The *prospero* gene specifies cell fates in the *Drosophila* central nervous system. *Cell* **65**, 451-464.

Doe, C. Q. and Goodman, C. S. (1985). Early events in insect neurogenesis I. Development and segmental differences in the pattern of neuronal precursor cells. *Dev. Biol.* **111**, 193-205.

Duncan, I. W. and Kaufman, T. C. (1975). Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: mapping of the proximal portion of the right arm. *Genetics* **80**, 733-752.

Edwards, J. S. (1969). Postembryonic development and regeneration of the insect nervous system. *Adv. Insect Physiol.* **6**, 98-137.

Gratzner, H. G. (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* **218**, 474-475.

Hartenstein, V. and Campos-Ortega, J. A. (1984). Early neurogenesis in wildtype *Drosophila melanogaster*. *Wilhelm Roux's Archiv. Dev Biol.* **193**, 302-325.

Hedley, M. L. and Maniatis, T. (1991). Sex-specific splicing and polyadenylation of *dsx* pre-mRNA requires a sequence that binds specifically to *tra-2* protein in vitro. *Cell* **65**, 579-586.

Hildreth, P. E. (1965). *Doublesex*, a recessive gene that transforms both males and females of *Drosophila* into intersexes. *Genetics* **51**, 659-678.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H. and Shimura, Y. (1991). Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in *Drosophila*. *Science* **252**, 833-836.

Lindsley, D. L. and Zimm, G. (1985). The genome of *Drosophila melanogaster*. Part 1:Genes A-K. *Dros. Inf. Serv.* **62**.

Lindsley, D. L. and Zimm, G. (1987). The genome of *Drosophila melanogaster*. Part 3: Rearrangements. *Dros. Inf. Serv.* **65**.

Lindsley, D. L. and Zimm, G. (1990). The genome of *Drosophila melanogaster*. Part 4: Genes L-Z. *Dros. Inf. Serv.* **68**.

McKeown, M., Belote, J. M. and Baker, B. S. (1987). A molecular analysis of *transformer*, a gene in *Drosophila melanogaster* that controls female sexual differentiation. *Cell* **48**, 489-499.

McRobert, S. P. and Tompkins, L. (1985). The effect of *transformer*, *doublesex*, and *intersex* mutations on the sexual behavior of *Drosophila melanogaster*. *Genetics* **111**, 89-96.

Nagoshi, R. N. and Baker, B. S. (1990). The regulation of sex-specific RNA splicing at the *Drosophila doublesex* gene: Cis-acting mutations in exon sequences alter sex-specific RNA splicing patterns. *Genes Dev.* **4**, 89-97.

Nagoshi, R. N., McKeown, M., Burtis, K. C., Belote, J. M. and Baker, B. S. (1988). The control of alternative splicing at genes

regulating sexual differentiation in *D. melanogaster*. *Cell* **53**, 229-236.

Nöthiger, R., Jonglez, M., Leuthold, M., Meier-Gerschwiler, P. and Weber, T. (1989). Sex determination in the germ line of *Drosophila* depends on genetic signals and inductive somatic factors. *Development* **107**, 505-518.

Nöthiger, R., Leuthold, M., Andersen, N., Gerschwiler, P., Gruter, A., Keller, W., Leist, C., Roost, M. and Schmid, H. (1987). Genetic and developmental analysis of the sex-determining gene *doublesex* (*dsx*) of *Drosophila melanogaster*. *Genet. Res. Camb.* **50**, 113-123.

Nöthiger, R., Roost, M. and Schupbach, T. (1980). *Masculinizer* is an allele of *doublesex*. *Dros. Inf. Serv.* **55**, 118.

Nöthiger, R. and Steinmann-Zwicky, M. (1985). Sex determination in *Drosophila*. *Trends Genet.* **3**, 209-215.

Ota, T., Fukunaga, A., Kawabe, M. and Oishi, K. (1981). Interactions between sex-transformation mutants of *Drosophila melanogaster*. I. Hemolymph vitellogenins and gonad morphology. *Genetics* **99**, 429-441.

Postlethwait, J. H., Bownes, M. and Jowett, T. (1980). Sexual phenotype and vitellogenin synthesis in *Drosophila melanogaster*. *Dev. Biol.* **79**, 379-387.

Prokop, A. and Technau, G. M. (1991). The origin of postembryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster*. *Development* **111**, 79-88.

Slee, R. and Bownes, M. (1990). Sex determination in *Drosophila melanogaster*. *Quart. Rev. Biol.* **65**, 175-204.

Steinmann-Zwicky, M., Amrein, H. and Nöthiger, R. (1990). Genetic control of sex determination in *Drosophila*. *Adv. Genet.* **27**, 189-237.

Sturtevant, A. H. (1945). A gene in *Drosophila melanogaster* that transforms females into males. *Genetics* **30**, 297-299.

Technau, G. M. (1984). Fiber growth and decay in the mushroom bodies of adult *Drosophila melanogaster* as a function of age and environmental stimulation. *J. Neurogenet.* **1**, 113-126.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* **125**, 145-157.

Watanabe, T. K. (1975). A new sex-transforming gene on the second chromosome of *Drosophila melanogaster*. *Jap. J. Genet.* **50**, 269-271.

White, K. and Kankel, D. (1978). Patterns of cell divisions and movement in the formation of the imaginal nervous system in *Drosophila melanogaster*. *Dev. Biol.* **65**, 296-321.

Wolfner, M. F. (1988). Sex-specific gene expression in somatic tissues of *Drosophila melanogaster*. *Trends Genet.* **4**, 333-337.

(Accepted 25 November 1991)