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The *Drosophila Pox neuro* gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers

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SUMMARY

We have dissected the entire *cis*-regulatory region of the *Drosophila Pox neuro* gene with regard to its enhancers, and have analyzed their functions by the selective addition to *Pox neuro* null mutant flies of one or several functions, each regulated by a complete or partial enhancer. We have identified at least 15 enhancers with an astounding complexity in arrangement and substructure that regulate *Pox neuro* functions required for the development of the peripheral and central nervous system and of most appendages. Many of these functions are essential for normal male courtship behavior and fertility. Two enhancers regulate the development of the penis, claspers and posterior lobes of male genitalia. Three enhancers, two of which overlap, control the development of chemosensory bristles in the labellum, legs and wings, some or all of which are required for the transmission of gustatory signals elicited by female pheromones. An additional enhancer regulates in the developing brain the connectivity of two specific neuronal clusters entrusted with processing olfactory pheromone signals from the antennal nerve. Finally, functions crucial for the ability of the male to copulate depend on an enhancer that activates *Pox neuro* expression in the embryonic ventral cord. In addition to these male courtship and fertility functions of *Pox neuro*, we have identified enhancers that regulate: (1) proper segmentation of tarsal segments in the leg disc and in homologous segments of the antennal disc; and (2) proper development of the wing hinge and hence the ability of the fly to fly.

Key words: *Drosophila*, *Pox neuro*, Enhancer, Courtship behavior, Male fertility, PNS, CNS, Appendages

INTRODUCTION

The *Pox neuro* (*Poxn*) gene of *Drosophila* is a member of the Pax gene family, encoding transcription factors with a DNA-binding paired domain (Bopp et al., 1989). It plays an important role in the development of the peripheral nervous system (PNS) as determinant of poly-innervated external sensory (p-es) organs in larvae (Dambly-Chaudière et al., 1992) and adults (Awasaki and Kimura, 1997). *Poxn* is expressed in larval p-es organs and adult chemosensory organs during development, while mono-innervated external sensory (m-es) organs of larvae and adults do not express it. In the absence of *Poxn*, larval p-es organs are transformed to m-es organs (Dambly-Chaudière et al., 1992). Similarly, in *Poxn* mutants most chemosensory bristles are transformed to mechanosensory bristles (Awasaki and Kimura, 1997). *Poxn* is not only expressed in the developing PNS, but also in the CNS (Bopp et al., 1989), and plays additional roles in the development of adult appendages (Awasaki and Kimura, 2001).

In an attempt to determine all functions of *Poxn*, we have prepared a *Poxn* null mutant. Surprisingly, this mutant turned out to be male sterile, a phenotype not previously observed for *Poxn* mutants (Awasaki and Kimura, 1997; Awasaki and Kimura, 2001). Although the fertility of males depends on their initiation of courtship, which is stimulated through chemosensory bristles (Stocker, 1994), such a phenotype was not expected because male courtship is influenced by a number of additional sensory modalities such as visual, olfactory, mechanosensory and auditory signals (Hall, 1994; Greenspan and Ferveur, 2000). These sensory cues allow the male to recognize its own species, discriminate against males and to find females that have not been mated recently. The male initiates courtship by following an evading female, taps her abdomen with his foreleg, extends and vibrates the wing on the side oriented towards her (thus producing a 'love song' that reduces her locomotor activity), extends his proboscis and licks the genitalia of the female with the labellum, and finally attempts to copulate (Hall, 1994). Copulation is established by the male bending and thrusting his abdomen forward and grabbing the genitalia of the female, anchoring himself with his claspers and penis, and finally mounting the female to remain in this copulation position for about 15 minutes to transfer his sperm and accessory gland fluid.

Males are stimulated to initiate courtship by female pheromones, which are transferred over short distances to olfactory trichoid sensilla on the third antennal segment and maxillary palp or through direct contact with chemosensory bristles and sensilla on the labellum, forelegs and wing (Robertson, 1983; Gailey et al., 1986; Stocker and Gendre,

1989; Stocker, 1994; Ferveur et al., 1996; Ferveur et al., 1997; Yamamoto et al., 1997). Males not only follow chemical cues, they also react to visual stimulation by a moving object of the appropriate size. Although the visual modality seems to be a facultative input to induce courting behavior in *Drosophila melanogaster* (Spieth and Hsu, 1950; Cook, 1980), males with reduced or no eye pigment are at a distinct disadvantage in competition experiments (Conolly et al., 1969; Reed and Reed, 1950).

As *Poxn* null mutant males are sterile, we began to explore the impact of the absence of *Poxn* functions on male fertility and courtship behavior. Surprisingly, many different functions of *Poxn* are involved. To determine the contribution of each of these functions to male courtship and fertility, a complete dissection of all *Poxn* enhancers was necessary. This analysis revealed an intriguing complexity of the arrangement and substructure of *Poxn* enhancers. In addition, it demonstrated that male courtship behavior and fertility functions of *Poxn* include: (1) the regulation of the development of chemosensory bristles on the labellum, legs and wings, which receive and propagate female pheromones; (2) the regulation of the development of defined neurons in the adult brain whose proper connections are required at least for the processing of olfactory signals produced by females pheromones; (3) the regulation of proper copulation behavior, which depends on the expression of *Poxn* in specific neurons of the embryonic ventral cord; and (4) the regulation of proper development in the male genital disc to give rise to the penis, claspers and posterior lobes.

MATERIALS AND METHODS

Determination of *Poxn* transcription start by 5′ RACE

To determine the start sites of the *Poxn* transcript, polyA$^+$ RNA was isolated from 8 to 12-hour-old embryos (25°C), from which 5′ ends of *Poxn*-cDNA were synthesized by the use of a primer derived from exon 2 and the AmpliFINDER kit from Clonetech (K1800-1), amplified by PCR, subcloned and sequenced. Two *Poxn* transcription start sites, separated by 133 bp, have been mapped. The ‘upstream’ start results in a leader sequence of 844 nucleotides, the ‘downstream’ start in one of 711 nucleotides. The 5′RACE product resulting from the ‘downstream’ start site is about three times more abundant in polyA$^+$ RNA from 8 to 12-hour old embryos.

Poxn rescue and *Poxn-Gal4* driver constructs

To map the enhancers of the *Poxn* gene, rescue constructs were designed, using *Poxn* chromosomal and/or cDNA sequences (Fig. 1B), and inserted into the polylinker of the P-element vector pW6, which carries a *mini-white* marker gene (Klemenz et al., 1987). To gain additional information about the spatial and temporal expression patterns of *Poxn*, different *Poxn* enhancer fragments, the *Poxn* promoter, leader and N-terminal-coding sequence were fused at amino acid 1 or 28 of the Poxn paired domain to the coding region of the yeast transcriptional activator Gal4, joined to the 3′UTR/poly A addition site of *Poxn* (Fig. 1B), and cloned into the polylinker of pW6. For transformation, the constructs were injected into w^{1118} embryos, and the resulting lines crossed into the appropriate *w*; $Poxn^{\Delta M22\text{-}B5}$ mutant background.

Analysis of GFP expression patterns and dissection of adult brains

Poxn-Gal4 driver lines were used in combination with *P{y$^+$ UAS-GFP}* to reveal the *Poxn* expression patterns in various tissues and the axonal projection patterns of the *Poxn*-expressing neurons in the PNS and CNS. Green fluorescent protein (GFP) expression patterns were analyzed with a Zeiss Axiophot microscope under fluorescence and the filter set 450-490/FT510/LP520, or with a Leica SP1 confocal microscope and standard settings for FITC detection. The resulting Z-stacks were processed by the use of Leica ‘LCS Lite’ analysis software.

Adult brains were dissected in 0.1 M PIPES/NaOH, pH 7.5, 2 mM EGTA, 1 mM $MgCl_2$ (PEM-buffer), 4% formaldehyde, fixed for about 15 minutes, and mounted in Ringer’s solution on microscope slides bounded on two sides by cover slips to support the cover slip.

Immunohistochemistry

Tissues were fixed with 4% formaldehyde in PEM buffer, blocked with 2% fetal calf serum and incubated overnight at 4°C with purified, 1:200 diluted rabbit anti-Poxn antiserum (Bopp et al., 1989). To detect bound antibody, tissues were further incubated with biotinylated goat anti-rabbit IgG (Vector Labs), followed by incubation with avidin-peroxidase (ABC-Kit, Vector Labs) and development with 3,3′-Diaminobenzidine/H_2O_2.

Morphological analysis of the adult cuticle

Flies were anaesthetized and dissected. Body parts were incubated in 10% NaOH for 30 minutes at 80°C to remove the tissue from the cuticle structures, rinsed with PBS and mounted in glycerol. Photographs were taken with a Zeiss Axiophot microscope equipped with a Hamamatsu CCD camera.

Courting tests

Single choice courting tests were performed at room temperature in a modified 24-well microtiter plate (15×8 mm wells covered with microscope glass slides), which was placed in a tray humidified with wet filter paper. All tests were performed at room temperature either under daylight or in a darkroom 10 cm from a 15 W lamp shielded by a Kindermann 2038 red filter. Test males were collected within 12 hours of eclosion and kept isolated in a tube with fly food for 5 days at 18°C and at a regular 12-hour light/dark cycle. *w*; $Poxn^{\Delta M22\text{-}B5}$/*CyO* females were collected as virgins and kept for 5 days in groups of about 10 animals in tubes under the same conditions as the males. Before courting tests, flies were adapted to room temperature for about 4 hours. Four to 8 hours after lights had been turned on, females were aspirated without anaesthesia and placed into the courting chamber followed by the male. Observation time was limited to 15 minutes or until copulation started. Latency time for the beginning of courting (wing extension) and copulation were recorded.

The fertility of males was assessed during long-term single crosses with *w*; $Poxn^{\Delta M22\text{-}B5}$/*CyO* virgins in food tubes at 25°C and 60% humidity for 4 days at a 12-hour light/dark cycle. Males of low fertility were tested in groups of six with six virgins. In both tests the number of larvae and the genotype of the offspring were recorded.

Fly stocks

The following fly stocks were prepared:

w; Poxn$^{\Delta M22\text{-}B5}$*/CyO*,
w; Poxn$^{\Delta M22\text{-}B5}$ *Resdistal2/CyO*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; BasiK109*,
w; Poxn$^{\Delta M22\text{-}B5}$ *XK39/CyO*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; PK86*,
w; Poxn$^{\Delta M22\text{-}B5}$ *PK6/CyO*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; BsK2*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; ScK10*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; SaK64*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; BaK19*,
w; Poxn$^{\Delta M22\text{-}B5}$ *EvK12/CyO*,
w C1-1-86; Poxn$^{\Delta M22\text{-}B5}$,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; E77-19*,
w; Poxn$^{\Delta M22\text{-}B5}$*/CyO; L1-16*,

w; Poxn$^{\Delta M22\text{-}B5}$ P2-2-67/CyO,
w; Poxn$^{\Delta M22\text{-}B5}$; ΔKX2a,
w ΔKBs10; Poxn$^{\Delta M22\text{-}B5}$/CyO;
w ΔXBs6; Poxn$^{\Delta M22\text{-}B5}$,
w; Poxn$^{\Delta M22\text{-}B5}$/CyO; ΔXP1,
w; Poxn$^{\Delta M22\text{-}B5}$/CyO; ΔSH8,
w; Poxn$^{\Delta M22\text{-}B5}$ ΔBH1/CyO,
w; Poxn$^{\Delta M22\text{-}B5}$; Full1,
w; Poxn$^{\Delta M22\text{-}B5}$ SuperA-207-2/CyO,
w; Poxn$^{\Delta M22\text{-}B5}$ SuperA-243/CyO,
w; Poxn$^{\Delta M22\text{-}B5}$ EvK12; L1-16,
w C1-1-86; Poxn$^{\Delta M22\text{-}B5}$; ΔSH8,
w; Poxn-Gal4-BasiK-5/TM6B,
w; Poxn-Gal4-9-6/CyO,
w; Poxn-Gal4-6-23/CyO,
w; Poxn-Gal4-13-1 UAS-GFP/TM6B,
w; Poxn-Gal4-581-32 UAS-GFP/TM6B and
w; Poxn-Gal4-14-1-7 UAS-GFP/TM6B.

Additional stocks used were *Df(2R)WMG*, *Sp Bl lt/In(2LR)Gla* (Bloomington stock 1887), *w^{1118}* (Bloomington stock 5905), *w*; *sli^{F81}/CyO* (Rothberg et al., 1990), *y w*; *P{y^{+} UAS-GFP}* (P element on third chromosome; from D. Nellen) and *Oregon-R (Munich)* (from W. McGinnis).

RESULTS

Construction of a *Poxn* null allele

To generate a *Poxn* null mutation, a local hop strategy (Tower et al., 1993) was chosen by mobilizing the P element of a nearby insertion in the *slit* (*sli*) locus, *sli^{F81}* (Rothberg et al., 1990). The resulting 149 *sli^{+}* revertants, whose *w^{+}* P element had reinserted on the second chromosome, were crossed over a large deficiency *Df(2R)WMG* uncovering *sli* and *Poxn* to screen for *Poxn* lethal mutations or, if *Poxn* was not a lethal locus, for *Poxn* mutant flies with missing chemosensory bristles on the anterior wing margins and legs. Such an adult *Poxn* mutant phenotype could be expected from the expression of *Poxn* in developing adult chemosensory organs in analogy to the *Poxn* phenotype of homozygous *Df(2R)WMG* mutants, which lack all larval poly-innervated external sensory (p-es) organs, the homologs of the adult chemosensory organs (Dambly-Chaudière et al., 1992; Awasaki and Kimura, 1997) (W. B. and M. N., unpublished). As no lethals or adults of the expected phenotype were found, the DNA regions adjacent to the reinserted P elements were isolated from the 149 revertants by plasmid rescue and hybridized to a probe of a 100 kb chromosomal walk including the *Poxn* locus (Dambly-Chaudière et al., 1992) to identify P-element insertions closer to *Poxn*. In agreement with the absence of insertions into *Poxn*, four reinsertions found within the walk were all located between *sli* and *Poxn*. The closest to *Poxn*, *P{Lac-W}M22*, was mapped upstream and outside of the *Poxn* locus (Fig. 1A). This insertion mutant is homozygous viable and does not show any obvious mutant phenotype even though the insertion is located within a transcription unit.

To obtain *Poxn* null mutants, the P element in *P{Lac-W}M22* was mobilized to produce a set of imprecise excisions (Salz et al., 1987) that deleted the neighboring *Poxn* gene. One of these deficiencies, *Poxn$^{\Delta M22\text{-}B5}$*, when tested over *Df(2R)WMG*, indeed resulted in the expected phenotype of missing chemosensory bristles. This deficiency extends over 17 kb, from its proximal breakpoint in exon 2 of *Poxn* to its distal breakpoint upstream of the adjacent gene encoding a sugar transporter homolog CG8249, and includes, in addition to the *Poxn* promoter region, all upstream enhancers of *Poxn* (Fig. 1A). Because it also deletes the entire coding region of the neighboring sugar transporter gene and possibly affects the control region of the next distal transcription unit CG8253, a transgene, *Resdistal*, that completely includes both these genes (Fig. 1A) was crossed into *Poxn$^{\Delta M22\text{-}B5}$* mutants. Such transgenic *Poxn$^{\Delta M22\text{-}B5}$* flies have a phenotype indistinguishable from that of *Poxn$^{\Delta M22\text{-}B5}$* or *Poxn$^{\Delta M22\text{-}B5}$/Df(2R)WMG* flies and are viable, but all males are sterile. As no Poxn protein is detectable at any time in these mutants, *Poxn$^{\Delta M22\text{-}B5}$* is a null allele of *Poxn*.

Transformed chemosensory bristles of *Poxn* null mutants

In homozygous *Poxn$^{\Delta M22\text{-}B5}$* flies, all taste bristles on wings, legs and labellum are affected. Thus, all taste bristles on the anterior wing margin (Fig. 2A) are transformed into mechanosensory bristles, and the transformed dorsal bristles no longer constitute a second posterior row, but are interspersed with the anterior row of mechanosensory bristles, with which they form a single dorsal row (Fig. 2B). The transformed bristles are easily distinguished from wild-type mechanosensory bristles by their increased length and thickness. On the legs, taste bristles (Fig. 2C) are transformed into bracted mechanosensory bristles, most of which are indistinguishable in morphology from wild-type mechanosensory bristles (Fig. 2D), or occasionally lost. Only on the tibia, some transformed bristles are longer and thicker than the surrounding mechanosensory bristles (not shown). Finally, on the labellum, the number of taste bristles (Ray et al., 1993) (Fig. 3A) appears unchanged, but their shafts are longer, have pointed tips like those of mechanosensory bristles and are often bent or kinked (Fig. 3B).

Late enhancer active in chemosensory neurons of all taste bristles

To delimit the enhancers regulating the various *Poxn* functions, a large number of partial *Poxn* genes were constructed, in which the coding region and promoter of *Poxn* were combined with different upstream, downstream or intron regions (Fig. 1B) and which were subsequently tested as transgenes for rescue of *Poxn* functions in homozygous *Poxn$^{\Delta M22\text{-}B5}$* flies. In addition, several *Poxn-Gal4* transgenes, expressing Gal4 under the control of the *Poxn* promoter and different *Poxn* enhancers (Fig. 1B), were combined with a *UAS-GFP* reporter transgene and tested for GFP expression. The rescue experiments demonstrated that the Poxn functions required for early development of recurved taste bristles on legs and wings or on the labellum are controlled by separate ‘early’ enhancers active during late third larval instar and early pupal stages. Rescue of the taste bristles on the legs and anterior wing margins depends on an enhancer of complex structure located in the upstream region, whereas rescue of the labellar taste bristles depends on a distinct enhancer located in the downstream region of *Poxn* (Fig. 1C). In addition to these enhancers, expression of *Poxn* indicates that all chemosensory bristles share an enhancer required for their late development the functional significance of which, however, has not yet been demonstrated.

This enhancer for late *Poxn* expression in chemosensory neurons is located close to the transcriptional start site. This is evident from the observation that either one of six transgenes, *Poxn-Gal4-14*, *Poxn-Gal4-13*, *Poxn-Gal4-9*, *Poxn-Gal4-BasiK*, *Poxn-Gal4-581* or *Poxn-Gal4-6*, which share only upstream and leader sequences downstream of the *Age*I site at –0.13 kb (Fig. 1B), is sufficient to drive this late expression of a *UAS-GFP* reporter gene. In contrast to the early expression

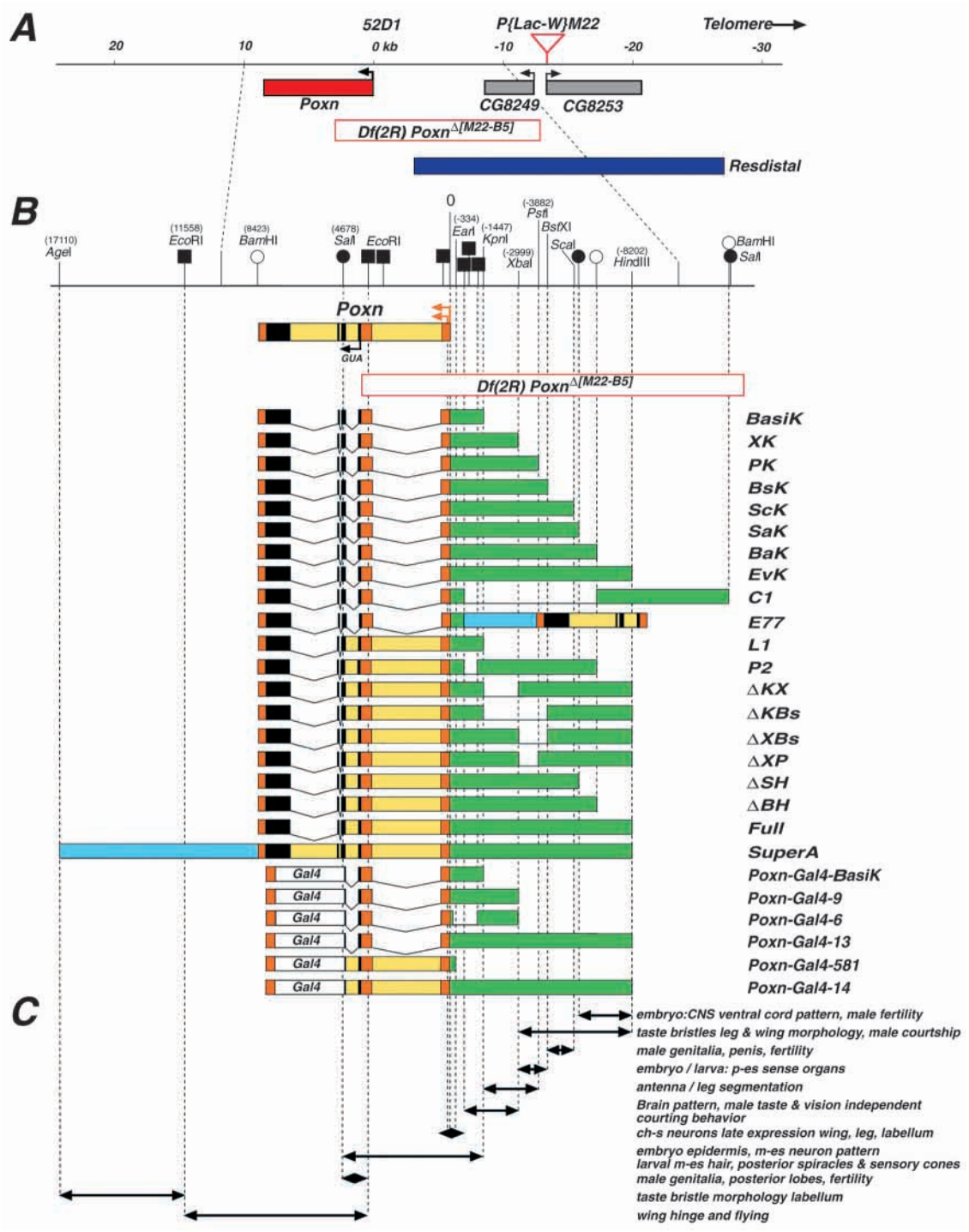


Fig. 1. Map of the *Poxn* gene and its enhancers with associated functions identified by *Poxn* rescue constructs and *Poxn-Gal4* reporter genes. (A) Map of chromosomal region at 52D1, including the *Poxn* gene. Transcribed regions of *Poxn* (red bar), and annotated genes *CG8249* and *CG8253* (gray bars) are shown with arrows indicating their directions of transcription below a scale that indicates distances in kb from the 'upstream' transcriptional start site of *Poxn*. The insertion *P{Lac-W}M22* and the *Poxn* deficiency *Df(2R)Poxn*$^{\Delta M22\text{-}B5}$ (open bar) are mapped. The deficiency deletes about 17 kb and extends from 131 bp upstream of the *Poxn* start codon in exon 2 to 730 bp proximal of the duplicated insertion site of the excised P element. In addition, the location of a genomic fragment is indicated that was used as transgene *Resdistal* (blue bar) to rescue the annotated genes affected by the *Poxn* deficiency. (B) Map of the *Poxn* gene, *Poxn* rescue constructs and *Poxn-Gal4* constructs driving the GFP reporter gene. Below a restriction map (only selected restriction sites are indicated; numbers in parentheses refer to distances in base pairs from the 'upstream' transcriptional start site marked 0) of the region including the *Poxn* gene, the composition of *Poxn* rescue constructs and of *Poxn-Gal4* driver constructs, as listed on the right, is illustrated with respect to the *Poxn* upstream region (green), 5′ leader and 3′ trailer (orange), coding region (black), introns (yellow) and downstream region (light blue). In *Poxn-Gal4* constructs, the position of the Gal4-coding region is shown as open box. (C) Map of *Poxn* enhancers and their functions. Double-headed arrows indicate location of *Poxn* enhancers, which regulate *Poxn* expression in specific spatiotemporal patterns and the associated functions listed on the right. Enhancers regulating expression during embryonic and larval stages not mapped in this study (W. B and M. N., unpublished), some of which overlap with, or even contribute to, adult functions, are also shown. In few cases, enhancers delimited by arrows have only been tested to be essential, not necessarily also sufficient, for the control of a specific *Poxn* function (see text).

of Poxn in developing adult chemosensory organs, initially observed in their SOPs of the corresponding third instar discs (Dambly-Chaudière et al., 1992), late Poxn expression in chemosensory neurons, as assayed by GFP expression, is first detectable at about 36 hours APF (after puparium formation) and is maintained in adults.

Usually four GFP-expressing neurons innervate each taste bristle on the legs (Fig. 2E) or wings, while two to four neurons innervate each open-tipped taste bristle on the labellum (Fig. 3D). All neurons are of the chemosensory type and project into the lumen of the individual shafts. This is obvious in the labellar bristles where GFP is clearly visible in shafts (Fig. 3D), whereas the dendritic projections into shafts is not as easily detectable in legs (Fig. 2E). Two additional groups of neurons labeled with GFP innervate the labral sense organs (LSO) (Stocker, 1994; Mitchell et al., 1999). This paired organ of the internal mouth parts is composed of six mechanosensory and eight chemosensory neurons (Singh, 1997), about six of which express GFP (Fig. 3D) driven by the *Poxn-Gal4* constructs (Fig. 1B).

Complex early enhancer for development of taste bristles in legs and wings

In addition to the late enhancer active in all chemosensory bristles, an early enhancer located further upstream is necessary for the activation of *Poxn* functions in third instar larvae and pupae to restore proper morphology in taste bristles on legs and wings of *Poxn* null mutants. The complex properties of this enhancer are apparent from studies of *Poxn* transgenes under the control of incomplete enhancers (Fig. 1B) that rescue different parts of the wild-type pattern of taste bristles. Because some of these transgenes exhibit a slight dose dependence, they were always used as two homozygous copies to delimit the functions of this enhancer, which extends over a *Xba*I-*Hin*dIII fragment, located between 3.0 and 8.2 kb upstream from the transcriptional start site. Driving Poxn

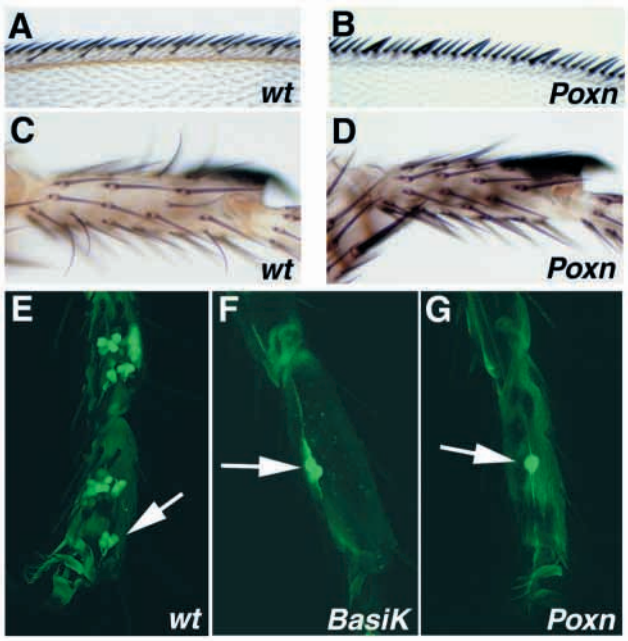


Fig. 2. Phenotypes of wing and leg bristles and of leg chemosensory neurons of wild type and *Poxn* null mutants. (A-D) Chemosensory wing and leg bristle phenotypes of wild type and *Poxn* null mutants. Bristles on the dorsal anterior wing margin (A,B) and the first male prothoracic tarsal segment (C,D) of wild type (A,C) and $Poxn^{\Delta M22\text{-}B5}$ mutants (B,D) are compared by bright field microscopy at a resolution of 20× and 25× magnification. (E-G) GFP expression in chemosensory neurons of wild-type and $Poxn^{\Delta M22\text{-}B5}$ male prothoracic legs. GFP expressing neurons are visualized in tarsal segments 4 and 5 of prothoracic legs of *w*; *Poxn-Gal4-13-1-101 UAS-GFP/+* (E), *w*; $Poxn^{\Delta M22\text{-}B5}$; *Poxn-Gal4-13-1-101 UAS-GFP/BasiK-109* (F) and *w*; $Poxn^{\Delta M22\text{-}B5}$; *Poxn-Gal4-13-1-101 UAS-GFP* (G) males by confocal fluorescence microscopy at a resolution of 40× magnification and with maximum projection of Z-stack. Note that tarsal segments 4 and 5 of $Poxn^{\Delta M22\text{-}B5}$ mutants endowed with (F) or without (G) the *BasiK* transgene are fused. Arrows indicate a cluster of four chemosensory neurons innervating a taste bristle (E) and a pair of bipolar neurons associated with a degenerated chemosensory organ (F,G). Dorsal side of legs is upwards (C,D) or towards the right (E-G).

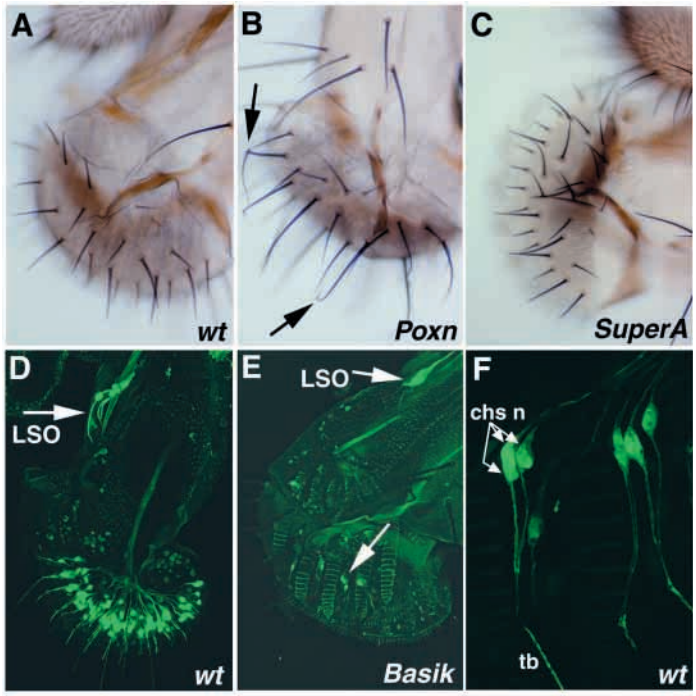
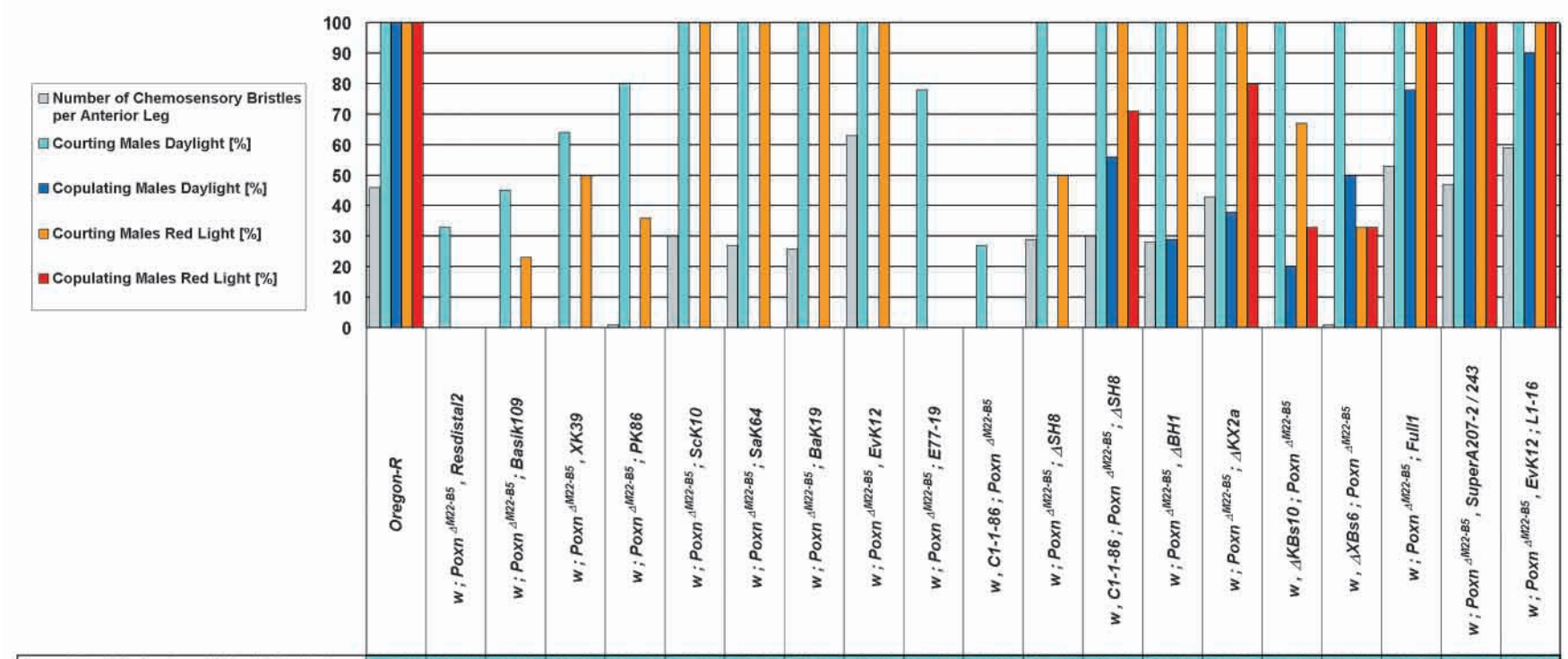


Fig. 3. Labellar taste bristle and associated chemosensory neuron phenotypes of wild-type and *Poxn* null mutant males. (A-C) Labella with chemosensory taste bristles of w^{1118} males (A), $Poxn^{\Delta M22\text{-}B5}$ males (B) and $Poxn^{\Delta M22\text{-}B5}$ males rescued with *SuperA-207* (C) are compared by bright field microscopy at a resolution of 20× magnification. Arrows in B indicate bizarre shapes of shafts of transformed taste bristles, which are completely rescued to wild-type in C. (D-F) GFP expressing labellar chemosensory neurons of *w*; *Poxn-Gal4-13-1 UAS-GFP/+* (D,F) and *w*; $Poxn^{\Delta M22\text{-}B5}$; *Poxn-Gal4-13-1 UAS-GFP/BasiK109* (E) males are visualized by confocal fluorescence microscopy at resolutions of 20× (D,E) and 100× (F) magnification and with maximum projections of Z-stacks. Arrows in D,E indicate a group of GFP-expressing neurons innervating the labral sense organ (LSO) and in E a few remaining GFP-expressing neurons in the labellum. (F) Larger magnification of the labellum shown in D, illustrating the multiple innervation of the labellar taste bristles by chemosensory neurons: shaft of taste bristle (tb) is invaded by the dendrites of three chemosensory neurons (chs n).



	Oregon-R	w ; Poxn $^{\Delta M22\text{-}B5}$, Resdistal2	w ; Poxn $^{\Delta M22\text{-}B5}$; Basik109	w ; Poxn $^{\Delta M22\text{-}B5}$, XK39	w ; Poxn $^{\Delta M22\text{-}B5}$; PK86	w ; Poxn $^{\Delta M22\text{-}B5}$; ScK10	w ; Poxn $^{\Delta M22\text{-}B5}$; SaK64	w ; Poxn $^{\Delta M22\text{-}B5}$; BaK19	w ; Poxn $^{\Delta M22\text{-}B5}$, EvK12	w ; Poxn $^{\Delta M22\text{-}B5}$; E77-19	w , C1-1-86 ; Poxn $^{\Delta M22\text{-}B5}$	w ; Poxn $^{\Delta M22\text{-}B5}$; ΔSH8	w , C1-1-86 ; Poxn $^{\Delta M22\text{-}B5}$; ΔSH8	w ; Poxn $^{\Delta M22\text{-}B5}$, ΔBH1	w ; Poxn $^{\Delta M22\text{-}B5}$; ΔKX2a	w , ΔKBs10 ; Poxn $^{\Delta M22\text{-}B5}$	w , ΔXBs6 ; Poxn $^{\Delta M22\text{-}B5}$	w ; Poxn $^{\Delta M22\text{-}B5}$; Full1	w ; Poxn $^{\Delta M22\text{-}B5}$, SuperA207-2 / 243	w ; Poxn $^{\Delta M22\text{-}B5}$, EvK12 ; L1-16
Latency Time Initiation Courtship Daylight (σ) [sec]	113 (50)	660 (211)	436 (192)	280 (207)	386 (151)	249 (86)	172 (25)	176 (81)	97 (40)	444 (170)	542 (234)	157 (109)	138 (60)	54 (28)	236 (177)	211 (137)	329 (242)	176 (117)	54 (42)	69 (46)
Latency Time Initiation Copulation Daylight (σ) [sec]	213 (82)	>900	>900	>900	>900	>900	>900	>900	>900	>900	>900	>900	703 (245)	357 (98)	480 (149)	720	414 (195)	319 (149)	192 (100)	276 (224)
Number of Couples Observed Daylight	9	15	11	14	10	4	4	5	5	9	15	9	9	7	8	5	8	9	8	10
Latency Time Initiation Courtship Red Light (σ) [sec]	125 (66)	>900	385 (70)	463 (208)	395 (262)	543 (171)	383 (263)	350 (117)	69 (34)	>900	>900	638 (53)	139 (58)	373 (194)	253 (118)	495 (213)	278 (83)	109 (56)	57 (19)	74 (51)
Latency Time Initiation Copulation Red Light (σ) [sec]	244 (55)	>900	>900	>900	>900	>900	>900	>900	>900	>900	>900	>900	243 (92)	>900	628 (132)	630 (210)	388 (92)	432 (189)	218 (115)	124 (44)
Number of Couples Observed Red Light	5	24	13	10	11	4	6	4	5	9	15	4	7	5	5	6	6	5	5	5
Male Genitalia: Posterior Lobe Structure	+	-	-	-	-	-	-	-	-	(+)	-	+	+	+	+	+	+	+	+	+
Male Genitalia: Penis Structure	+	-	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Brain Expression Pattern	+	-	(+)	+	+	+	+	+	+	-	-	+	+	+	(+)	(+)	+	+	+	+
Embryonic Ventral Cord Expression Pattern	+	-	-	-	-	-	-	(+)	+	-	+	-	+	(+)	+	+	+	+	+	+
Number of taste bristles per male anterior leg (σ)	46 (1)	0	0	0	1 (1)	30 (2)	27 (1)	26 (2)	63 (2)	0	0	29 (2)	30 (1)	28 (1)	43 (2)	0	1 (1)	53 (2)	47 (1)	59 (1)
Taste Bristles Labellum	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Taste Bristles Anterior Wing Margin [%]	100	0	0	0	0	76	42	84	102	0	2	42	42	89	100	0	0	98	100	104
Leg / Antenna Segmentation	+	-	-	(+)	+	+	+	+	+	-	-	+	+	+	-	-	(+)	+	+	+
Flying Capability	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
Male Fertility	+++	-	-	-	-	-	-	-	(-)	-	-	+	+++	+	++	+	++	+++	+++	+++

Fig. 4. Courting behavior of partially rescued *Poxn* males. The histogram at the top indicates the average number of recurved taste bristles per prothoracic leg (gray), the percentage of males initiating courtship under daylight (light blue) or red light (orange), and the percentage of copulating males under daylight (dark blue) and red light (red) within the observation period of 15 minutes (see Materials and Methods) for wild-type and partially or completely rescued *Poxn* males of the genotypes indicated below. The table at the bottom summarizes, as listed on the left, average latency times with standard deviations in parentheses, number of tested couples in single choice courting tests, and the rescue of phenotypic characters affected by the *Poxn*$^{\Delta M22\text{-}B5}$ mutation. Complete and partial rescue are indicated as + in dark-colored and as (+) in light-colored fields. The degree of male fertility is indicated by +++, ++ and + if more than 65%, 35-65% and 5-35% of single crosses result in offspring. In one case, male fertility is indicated as (–) because two out of 90 males produced a very low number of offspring (two and 20 larvae). The number of taste bristles on the male prothoracic leg is indicated with standard deviation in parenthesis, that of taste bristles on the anterior wing margin is given as percentage of the number observed in *Oregon-R* males.

expression under the control of increasing lengths of the upstream region rescues some taste bristles on legs and wings in homozygous $Poxn^{\Delta M22\text{-}B5}$ flies only when regions upstream of the *Pst*I site in this fragment are present as in the *BsK*, *ScK*, *SaK*, *BaK* and *EvK* transgenes (Fig. 4). Nevertheless, the enhancer crucially depends also on sequences downstream of *Pst*I, as evident from the complete absence of taste bristles in $Poxn^{\Delta M22\text{-}B5}$ flies endowed with the transgenes *C1*, Δ*KBs*, Δ*XBs* and Δ*XP* (Fig. 4), all of which lack the 0.9 kb *Xba*I-*Pst*I fragment (Fig. 1B). Omission of DNA regions proximal to the *Xba*I site, as in the *P2* or Δ*KX* transgenes, does not affect the rescue of bristles on legs and wings and thus sets a proximal limit of the early enhancer. Its distal limit is approximated by a complete rescue obtained by the *EvK* transgene that includes 8.2 kb of upstream sequences up to the *Hin*dIII site (Fig. 1B).

Thus, although the part of the enhancer included in the 0.9 kb proximal to *Pst*I of *Poxn* transgenes is not sufficient for the rescue of any taste bristles, it is necessary for the development of all taste bristles on legs and wings. The inclusion of more distal regions of the enhancer rescues increasing numbers of taste bristles, apparently with a general distal-to-proximal polarity along the leg and wing. All taste bristles on the tarsal segments 2, 4, 5 and about 40% of those on the anterior wing margin, mostly on its distal part, are rescued by the *BsK* transgene, which extends only by 0.35 kb beyond *Pst*I (Fig. 1B). By contrast, only few taste bristles are rescued on the tibia (<25%) and virtually none on tarsal segments 1 and 3 (not shown). Addition of increasing distal regions of the enhancer up to the *Bam*HI site in transgenes *ScK*, *SaK* (or Δ*SH*) and *BaK* (or Δ*BH*) does not significantly change the number of rescued bristles on the leg (Fig. 4). Only further inclusion of the most distal region of the enhancer in *EvK* restores the bristle pattern of the tibia and all tarsal segments although an excess of taste bristles appears at the expense of mechanosensory bristles in the tibia (Fig. 4). Most notably, the number of these ectopic taste bristles, which appear to arise from transformed mechanosensory bristles, are reduced by the additional presence of the first two introns in the *Full* transgene, yet completely suppressed only when also downstream control regions are present as in the *SuperA* transgene (Fig. 1B, Fig. 4). Although addition of the 2.2 kb between *Bst*XI and *Bam*HI in the absence of the most distal part of the early enhancer does not significantly alter its rescue efficiency for leg bristles, rescue of taste bristles on the anterior wing margin is enhanced from 40% to about 90% (Fig. 4). Hence, the enhancers for taste bristles on legs and wings overlap, but do not behave identically and seem to have different requirements.

Early enhancer for development of labellar taste bristles

In contrast to the taste bristles on legs and wings, the labellar bristles of $Poxn^{\Delta M22\text{-}B5}$ mutants are not simply transformed into mechanosensory sensilla, but the shafts show tips of bizarre forms (Fig. 3B, arrows). Moreover, the morphology of the labellar chemosensory bristles cannot be rescued by the presence of enhancers in the upstream region of *Poxn* transgenes. The early enhancer that, in combination with the late enhancer, restores wild-type morphology of labellar bristles is located in the downstream region of *Poxn*. This region is delimited by the *SuperA* transgene (Fig. 1B), which is sufficient for rescue of labellar bristle morphology (Fig. 3C), while the *E77* transgene including only 3.0 kb of the downstream region (Fig. 1B) is not.

Poxn males do not court receptive females in the absence of proper visual input

The observation that $Poxn^{\Delta M22\text{-}B5}$ males are sterile prompted us to examine their mating behavior towards receptive virgin females. Under daylight conditions, two thirds of the $Poxn^{\Delta M22\text{-}B5}$ males do not initiate courtship in single choice experiments within 15 minutes under standard conditions (see Materials and Methods), while the remaining third courts females very weakly, but proceeds through the entire courting sequence (for a review, see Greenspan and Ferveur, 2000). Although these males attempt to copulate by bending their abdomen, no copulation is observed. All males have well developed testes with motile sperm, accessory glands, ejaculatory duct and sperm pump. However, the cuticular structure of their genitals is aberrant, as will be shown below. Interestingly, w^{1118}; $Poxn^{\Delta M22\text{-}B5}$ double mutants do not take note of females at all under daylight (32 males tested). Similarly, $Poxn^{\Delta M22\text{-}B5}$ or w^{1118}; $Poxn^{\Delta M22\text{-}B5}$ males carrying the *Resdistal* construct, whose associated mini-white marker gene restores wild-type eye pigmentation, do not court females under red light, but only at daylight (Fig. 4). Clearly, in the absence of *Poxn* functions, male courting behavior depends entirely on proper visual input.

Influence of number of taste bristles on courting behavior of males

Surprisingly, $Poxn^{\Delta M22\text{-}B5}$ males carrying the *BasiK* transgene display a partially rescued courting behavior even though none of the recurved taste bristles on legs, wings or labellum are rescued. While under daylight they court slightly more often and with a somewhat shorter latency time before the onset of courting than their mutant counterparts, a striking difference becomes apparent when their mating behavior is compared in the absence of proper visual input. In contrast to *Poxn* males, which exhibit no courtship behavior under red light, one quarter of the transgenic *Poxn* males do court receptive females (Fig. 4). Although the *BasiK* transgene lacks the early *Poxn* function required for development of chemosensory bristles, it includes the late function expressing Poxn in chemosensory neurons. It was thus conceivable that the partial rescue resulted from the expression of Poxn in 'chemosensory' neurons able to develop even without the preceding early *Poxn* function. By introducing *Poxn-Gal4-13* (Fig. 1B) and *UAS-GFP* transgenes, we therefore investigated whether such neurons expressing Poxn, as assayed by GFP, are present in $Poxn^{\Delta M22\text{-}B5}$ adults partially rescued by the *BasiK* transgene. Indeed, five to 20 neurons, often in groups of two, are observed to express GFP in a prothoracic leg of these males (Fig. 2F), whereas about 180 chemosensory neurons (four neurons per taste bristle) express GFP (and thus Poxn) in the corresponding leg of a wild-type male (Fig. 2E). A similarly reduced fraction of GFP expressing neurons is also present in meso- and metathoracic legs (not shown) and in the labellum (Fig. 3E), as well as in legs (Fig. 2G) and labellum (not shown) of $Poxn^{\Delta M22\text{-}B5}$ mutants that have not been partially rescued by the *BasiK* transgene. Thus, the potential of expressing Poxn under control of the late enhancer in the remaining 'chemosensory' neurons associated with some of the transformed bristles of *Poxn*

mutants does not depend on the presence of the *BasiK* transgene. In legs, these neurons are most often associated with transformed bracted mechanosensory bristles, but rarely innervate structures resembling short and unpigmented bristles (less than one per leg; not shown). However, although the strongly reduced number of these neurons expressing Poxn in the partially rescued *Poxn* males would be consistent with the observed partial rescue of courting behavior, this explanation appears improbable in view of additional evidence discussed below.

If $Poxn^{\Delta M22\text{-}B5}$ males are rescued by *Poxn* transgenes that include increasing lengths of upstream control regions (Fig. 1B), their enhanced courting behavior appears to parallel their increasing number of rescued taste bristles on legs rather than wings. Thus, while only about one third to half of all males court under red light if no taste bristles are present, all males court if taste bristles on tarsal segments 2, 4 and 5 are rescued by *BsK* (not shown). Similarly, further increase of the upstream region in *ScK*, *SaK* and *BaK* transgenes does not change significantly the latency time before courting under daylight or red light conditions, nor the number of rescued chemosensory bristles on the legs, whereas the number of taste bristles on the wings varies by a factor of two (Fig. 4). Only when all taste bristles on the legs and wings are rescued by the *EvK* transgene, which includes 8.2 kb of the upstream region (Fig. 1B), the latency time of the rescued males is significantly reduced to that of *Oregon-R* males (Fig. 4).

Despite their normal courting behavior, $Poxn^{\Delta M22\text{-}B5}$; *EvK* males, which carry the full set of chemosensory bristles on legs and wings, but none on the labellum, have not been observed to copulate in courting tests. In fertility tests, two out of 90 $Poxn^{\Delta M22\text{-}B5}$ males carrying two copies of the *EvK* transgene produced a dramatically reduced number of offspring, while none out of 480 males carrying a single copy was able to generate any offspring.

Defective genitalia of *Poxn* males

The observation that $Poxn^{\Delta M22\text{-}B5}$ males whose taste bristles were rescued by *EvK* exhibit a wild-type courtship behavior, but are unable to copulate suggests that male fertility depends on additional functions of *Poxn*. Examining the expression of *UAS-GFP* under the control of *Poxn-Gal4-14* in pupae, we found a sexually dimorphic pattern expressed by about 30 hours APF in males (Fig. 5A), but absent in females (Fig. 5B). This male-specific pattern can be divided into two non-overlapping subpatterns regulated by separate enhancers: one located in introns 1 and 2; the other in the upstream region of *Poxn* (Fig. 5C). The absence of these enhancers can be correlated with missing *Poxn* functions resulting in defects of the male genitalia. Thus, $Poxn^{\Delta M22\text{-}B5}$ males have no penis, although the penis apodeme and protractor muscle are still present (Fig. 5D). The enhancer regulating the *Poxn* functions required for normal development of the penis is delimited by the 1.2 kb *Bst*XI-*Sca*I fragment of the upstream region (Fig. 1C), as evident from the rescue of the penis in $Poxn^{\Delta M22\text{-}B5}$ males with *Poxn* transgenes, such as *ScK*, *EvK* or Δ*KB*s, which include this fragment, but not with transgenes like *BsK* or *L1*, in which this fragment is missing (Fig. 1B, left side of Fig. 5D). A second, more subtle defect is observed on the outer genitalia of $Poxn^{\Delta M22\text{-}B5}$ males whose posterior lobes are degenerate and whose claspers display slightly aberrant bristle patterns (Fig. 5D). An essential part of the enhancer(s) regulating these functions is located in the second intron of *Poxn* (Fig. 1C). This is evident from an almost complete rescue of claspers and posterior lobes by the *E77* transgene (not shown), which includes this intron as an upstream enhancer (Fig. 1B), and from the full rescue of these structures by *L1* (Fig. 1B), whereas *Poxn* transgenes that include no intronic sequences, such as *EvK*, do not rescue the morphology of the posterior lobes and claspers at all (right side of Fig. 5D).

The copulation rate and fertility of $Poxn^{\Delta M22\text{-}B5}$ males is restored efficiently only when Poxn functions regulated by the enhancers in the introns as well as the upstream region of *Poxn* are supplied, for example by *EvK* and *L1* (Fig. 4). Hence, male fertility clearly depends on the integrity of all genital structures.

Strongly reduced male fertility in the absence of Poxn expression in the embryonic ventral nerve cord

Transgenic $Poxn^{\Delta M22\text{-}B5}$; Δ*SH* (Fig. 1B) males, which are able to sense females despite a reduced set of chemosensory bristles and whose genitals are perfectly restored, begin courting after about 3 minutes under daylight conditions (Fig. 4). Nevertheless, no copulation was observed within the 15 minutes of our tests. Long-term courting tests reveal that the males have difficulties in establishing physical contact with the females' genitalia and, after mounting, in remaining in the copulation position. In mass crosses and long-term single crosses, such males exhibit a strongly reduced fertility (<20% of single crosses have offspring), and $Poxn^{\Delta M22\text{-}B5}$; Δ*SH* flies cannot be maintained as stable lines. However, the fertility and copulation behavior of $Poxn^{\Delta M22\text{-}B5}$; Δ*SH* males can be restored by adding a copy of *C1* (Fig. 1B, Fig. 4). Although this transgene includes an enhancer region required for the rescue of the full complement of taste bristles on legs and wings, it neither enhances the incomplete number of taste bristles of $Poxn^{\Delta M22\text{-}B5}$; Δ*SH* males when provided in *trans* nor rescues any taste bristles by itself (Fig. 4). Therefore, the *C1* transgene must include a distal upstream enhancer, whose function is necessary for the full rescue of male fertility but which differs from the taste bristle enhancer of this region.

Surprisingly, this enhancer, which is included in a 2.57 kb *Hin*dIII-*Sal*I fragment, drives expression in the ventral nerve cord of the embryo (Fig. 6A-C), as evident from the expression patterns of *Poxn* transgenes and *UAS-GFP* transgenes under *Poxn-Gal4* control that comprise this upstream region (Fig. 1B). Most of the enhancer is located in the distal 1.76 kb *Hin*dIII-*Bam*HI fragment as Δ*BH* expresses Poxn only weakly in the ventral cord, while ventral cord expression of *C1*, *Full*, or Δ*XB*s is very strong. By contrast, Δ*SH* shows no Poxn expression in the ventral cord. Moreover, fertility of $Poxn^{\Delta M22\text{-}B5}$ males rescued by these transgenes and their expression in the ventral cord are in excellent agreement (Fig. 4). In accordance with our earlier conclusion, the fertility of these males does not correlate with the rescue of leg and wing taste bristles, as males rescued by Δ*XBs* have no taste bristles but a considerably higher fertility than those rescued by Δ*SH* or Δ*BH*, which possess at least half of all leg and wing taste bristles (Fig. 4).

Poxn expression under the control of the ventral cord

enhancer is first detectable during late stage 10 or early stage 11 of embryogenesis (Bopp et al., 1989) and fades only at the end of embryonic development. Many Poxn-expressing cells in the ventral cord are of neuronal morphology, as shown in a stage 17 embryo expressing GFP under the control of *Poxn-Gal4-13* (Fig. 6B,C). No Poxn expression is detectable in the ventral CNS of third instar larvae (Fig. 7B). Similarly, no expression is apparent in the adult ventral CNS (not shown). However, GFP expressed under control of the late taste bristle enhancer reveals a sexually dimorphic projection pattern of the chemosensory afferents (Fig. 6D,E), which has been observed previously for axons of some taste bristles on the prothoracic legs (Possidente and Murphey, 1989). A considerable number of chemosensory neurons from the male prothoracic legs project contralaterally into the prothoracic leg neuromere, while no arborizations of this kind are detected in the meso- or metathoracic leg neuromeres or the wing neuromere (Fig. 6D). In the female ventral CNS, all projections are exclusively ipsilateral (Fig. 6E).

Male courting behavior crucially depends on *Poxn* expression in the brain

Male fertility of *Poxn* mutants is rescued by Δ*XBs* to a much larger extent than by Δ*KBs*, even though both transgenes include the ventral cord enhancer and do not rescue any taste bristles (Fig. 4). The only difference between these two transgenes is a 1.55 kb *Xba*I-*Kpn*I upstream fragment absent from Δ*KBs* (Fig. 1B). This region includes a considerable region of an enhancer that regulates *Poxn* expression in the embryonic, larval and adult brain. *Poxn* transgenes without this upstream region, such as Δ*KBs*, Δ*KX* or *BasiK*, exhibit only a very faint Poxn expression in the brain, whereas all transgenes including this upstream fragment, such as Δ*XBs*, Δ*XP* or *XK*, rescue the complete Poxn expression pattern in the brain of

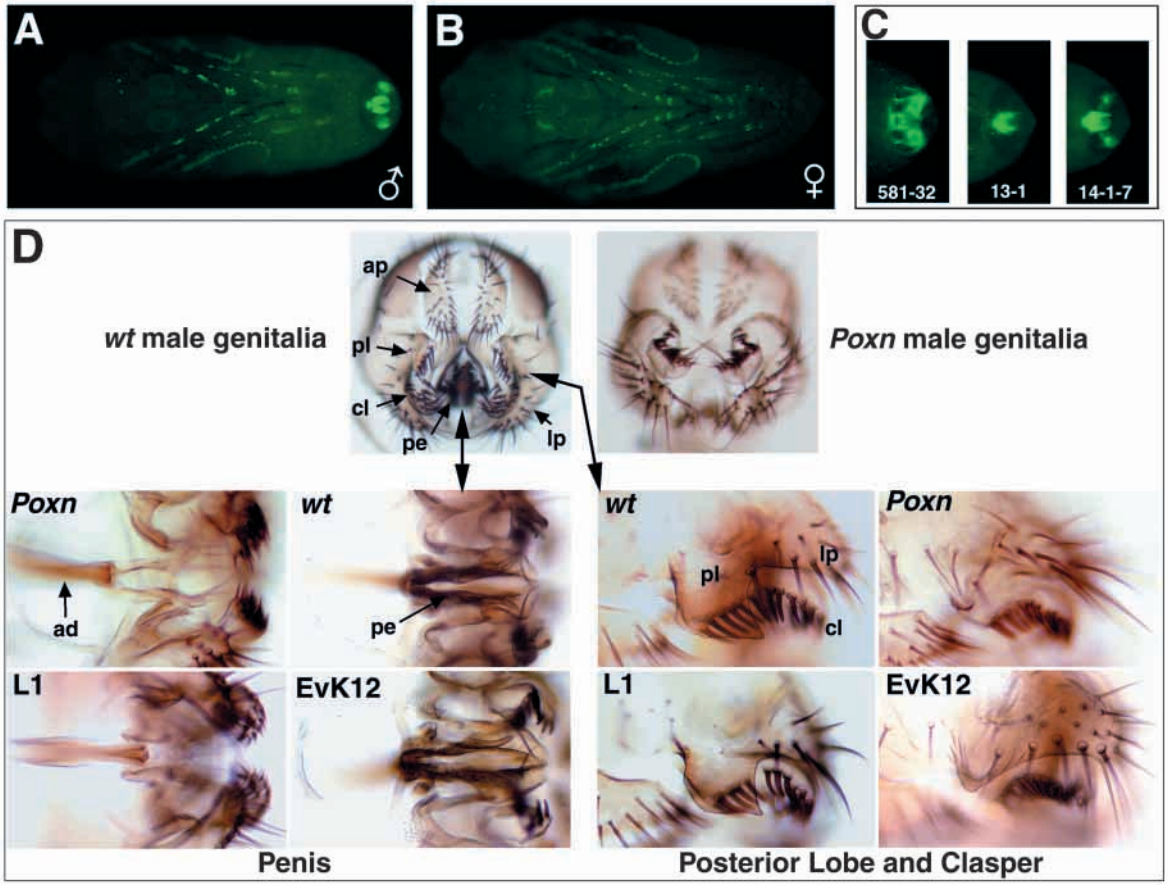


Fig. 5. Sexually dimorphic pupal expression pattern of *Poxn* in the genital region and cuticular phenotype of $Poxn^{\Delta M22\text{-}B5}$ male genitalia. (A,B) GFP expression is analyzed in ventral views (anterior towards the left) of male (A) and female (B) *w*; *Poxn-Gal4-14-1/TM6B* pupae (48 hours APF) by fluorescence microscopy at a resolution of 10× magnification. (C) Details of GFP expression in the genital region of male pupae (72 hours APF), driven by three different *Poxn-Gal4* constructs (*Poxn-Gal4-581*, *Poxn-Gal4-13-1* and *Poxn-Gal4-14-7*; compare with Fig.1B). (D) Frontal view (with dorsal side up) of cuticle preparations of male genitalia of wild type (w^{1118}; left) and *Poxn* mutant (right) under bright field microscopy at a resolution of 8× magnification. Arrows indicate anal plate (ap), clasper (cl), lateral plate (lp), penis (pe) and posterior lobe (pl). The four panels below compare details of the penis (left) and the posterior lobe, clasper and lateral plate (right) regions between wild type (*wt*) and *Poxn* mutants (*Poxn*) at a 2- to 2.5-fold greater magnification. Arrows indicate the wild-type penis (pe) and the penis apodeme (ad) in the *Poxn* mutant without penis. The four panels at the bottom illustrate, at the same magnification as the panels above, the rescue in *Poxn* mutants of the penis, but not posterior lobe and clasper, by two copies of *EvK*, which include the upstream genitalia enhancer absent from *L1*, and of the posterior lobe and clasper, but not penis, by two copies of *L1*, which include the intron genitalia enhancer not present in *EvK*. In the eight panels at the bottom, posterior is towards the right.

Poxn mutants at all stages. As *C1* exhibits no expression in the brain, it follows that the brain enhancer is completely included in a 2.38 kb *Xba*I-*Eco*RI fragment, 0.62 kb upstream of the transcriptional start site (Fig. 1C), though most of it is located in its distal two thirds.

Poxn protein in the brain is first detected during embryonic stage 12 and continues to be expressed in the embryonic, larval and adult brain throughout development. In the embryonic brain lobes, two groups of cells express Poxn with bilateral symmetry (Fig. 7A), while it is detectable only in a single group in each brain hemisphere of third instar larvae (Fig. 7B). In the adult brain, again two bilateral-symmetric groups of cells express Poxn, a ventral cluster of about 100 cells, forming a ventral arc around the region where the antennal nerve enters the brain, and a cluster of about 200 cells, located in a dorsolateral region adjacent to the antennal lobe on the surface of the ventrolateral protocerebrum (Fig. 7C). Most of these cells have the morphology of neurons, as evident from the GFP pattern driven by *Poxn-Gal4-13* (Fig. 7D). The majority of the neurons of the ventral cluster project into the antennal lobe (Fig. 7D,G) (Laissue et al., 1999), while the major target of the dorsolateral cluster is the ellipsoid body neuropil (Fig. 7D,F), a part of the central complex neuropil involved in locomotion (Strauss and Heisenberg, 1993; Martin et al., 1999). The position and architecture of the dorsolateral neurons resemble those described for large-field R neurons (Hanesch et al., 1989; Renn et al., 1999). In addition, projections from the dorsolateral cluster of cells target the lateral triangle (Hanesch et al., 1989; Renn et al., 1999), and a domain dorsal of it (Fig. 7F), while the nerve emanating from the ventral cluster arborizes on at least two additional targets in the dorsolateral part of the brain (Fig. 7G), but does not seem to connect to the dorsolateral cluster of neurons. The positions of the cell bodies and the projection pattern are symmetric in the two brain hemispheres and do not exhibit an obvious sexual dimorphism.

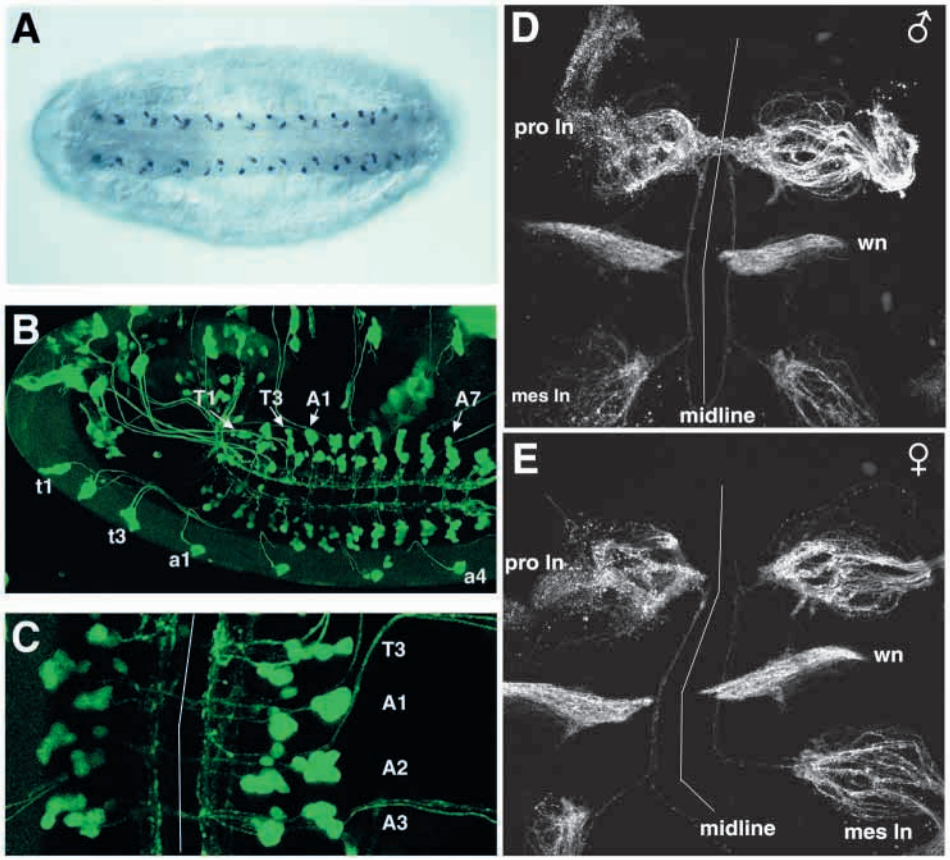


Fig. 6. Poxn expression in the embryonic and adult CNS. (A-C) Poxn expression in the developing embryonic ventral cord. (A) Segmentally repeated expression of Poxn protein in the CNS of a stage 15 *w*; *Poxn*$^{\Delta M22\text{-}B5}$ embryo rescued by *C1-1-86*, shown as ventral view under Nomarski optics at a resolution of 20× magnification. The Poxn protein is detected by a rabbit anti-Poxn antiserum. (B,C) GFP expression in the ventral CNS of a *w*; *Poxn-Gal4-13-1 UAS-GFP* embryo at stage 17, visualized in a ventrolateral overview (B) or enlarged view (C) by confocal fluorescence microscopy at a resolution of 20× magnification and with maximum projection of Z-stack. (B) Clusters of segmentally repeated GFP expressing neurons in thorax (T1-T3) and abdomen (A1-A8) of the ventral CNS and projections from the ventral and lateral p-es organs of the thoracic (t1-t3) and first four abdominal segments (a1-a4) into the ventral cord are clearly visible. (C) The morphology of the cell bodies and projections demonstrate that most, if not all, of the GFP-expressing cells in the ventral cord are neurons. The ventral midline is indicated by a white line. Note that this pattern of Poxn expression, which normally disappears by stage 17, is still clearly visible because of the high stability of Gal4 and GFP. (D,E) Poxn expression in the adult ventral ganglion. Ventral views of the anterior part of a thoracic ganglion dissected from a *w*; *Poxn-Gal4-14-1 UAS-GFP* male (D) or female (E) adult. Projections of the chemosensory neurons of the gustatory bristles on legs and wings are labeled by *Poxn*-driven GFP expression and visualized by confocal fluorescence microscopy, as in B. Note that in females, no projections from the prothoracic chemosensory neurons are crossing the midline. pro ln, prothoracic leg neuromere, wn, wing neuromere, mes ln, mesothoracic leg neuromere. Anterior is towards the left (A,B) or upwards (C-E).

Both groups of cells are also present in the adult brain of a *Poxn* mutant (Fig. 7E). The cells have a neuronal shape, as evident from GFP expression under control of *Poxn-Gal4-13*, and their number is comparable with that of the wild type. However, the projection pattern has completely changed. The ellipsoid body and other brain centers are not specifically targeted, and the arborizations are diffuse and asymmetric for the two hemispheres.

To evaluate the contribution to male fertility of *Poxn* expression in the brain, the courting behavior of *Poxn*$^{\Delta M22\text{-}B5}$; Δ*KBs* males was compared with that of *Poxn*$^{\Delta M22\text{-}B5}$; Δ*XBs* males (Fig. 4). Both transgenes do not rescue any chemosensory bristles, but restore the ventral cord expression and rescue the male genitalia as well as the late Poxn expression in the 'chemosensory' neurons associated with the transformed bristles. However, the Δ*XBs* transgene also restores Poxn expression in the brain, whereas Δ*KBs*

does not and supports it only weakly. This difference in brain expression correlates with a reduced courting intensity and fertility of the *Poxn* males rescued by Δ*KBs* when compared with those rescued by Δ*XBs* (Fig. 4). Interestingly, *Poxn* males whose Poxn expression is rescued in the brain by Δ*XBs* exhibit similar courting intensity and fertility as *Poxn* males rescued by Δ*KX*, which show only a faint expression in the brain, but whose taste bristles on legs and wings are largely rescued (Fig. 4). By contrast, *Poxn* males rescued by Δ*KBs* exhibit a considerably reduced fertility. Because these males show only faint expression of Poxn in the brain and have no taste bristles, it follows that male fertility is enhanced by the rescue of high levels of Poxn expression in the developing brain by Δ*XBs* to a similar extent as through the rescue of most taste bristles on legs and wings by Δ*KX*.

The faint Poxn expression observed in the brain of *Poxn* males rescued by *BasiK* is important for their initiation of courtship under red light, as evident from the following observations. If late Poxn expression in 'chemosensory' neurons associated with the transformed mechanosensory bristles is rescued by *E77* or *C1*, *Poxn* males initiate courtship at daylight, but not when their vision is compromised under red light (Fig. 4). With *C1*, however, additional rescue of their embryonic ventral cord expression is observed. It follows (1) that late Poxn expression in these neurons is not sufficient to rescue any male courtship behavior under red light, even if Poxn expression in the ventral cord is rescued as well, and (2) that ventral cord expression does not affect initiation of courtship at daylight. In addition to the late Poxn expression in the remaining 'chemosensory' neurons, *BasiK* rescues only the faint expression in the brain, which is not rescued by *C1* or *E77*. Therefore, the observed initiation of courtship by $Poxn^{\Delta M22\text{-}B5}$; *BasiK* males under red light probably depends on the faint brain expression rather than the late expression in 'chemosensory' neurons, also observed in *Poxn* males rescued by *C1* or *E77*, which exhibit no courtship behavior under red light (Fig. 4). Expression of Poxn in the brain is also important for the initiation of male courtship at daylight. This is not only evident from a comparison of courtship behavior of *Poxn* males rescued by Δ*KBs* or Δ*XBs*, but also from the observation that

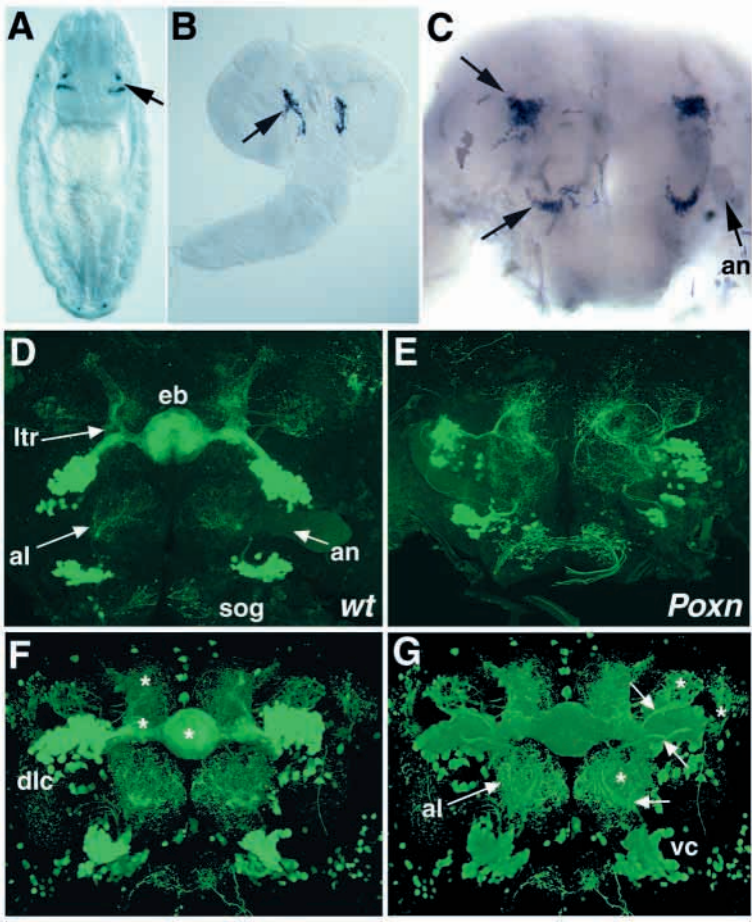


Fig. 7. Poxn expression pattern in the developing brain. (A,B) Bilateral-symmetric expression of Poxn in the two brain hemispheres (arrows) of a stage 16 wild-type embryo (A) or third instar larva (B), shown in dorsal views (anterior upwards) under Nomarski optics at a resolution of 20× magnification. (B) Note that no Poxn is detectable in the ventral ganglion. (C) Bilateral-symmetric Poxn expression in the brain of a wild-type adult male, visualized as frontal view by bright field microscopy at a resolution of 16× magnification. Arrows indicate the dorsolateral cluster of about 200 Poxn expressing cells and the ventral cluster of about 100 Poxn expressing cells, which forms an arc around the region where the antennal nerve (an) enters the brain. (D,E) Frontal views of Poxn expression in brains of 2-day-old *w*; *Poxn-Gal4-13-1 UAS-GFP* (D) and *w*; $Poxn^{\Delta M22\text{-}B5}$; *Poxn-Gal4-13-1 UAS-GFP* (E) males, visualized by GFP expression and confocal fluorescence microscopy at a resolution of 20× magnification and with maximum projection of Z-stacks. (D) The majority of cells in both GFP expressing clusters have the morphology of neurons, which project into different regions of the brain. The ventral clusters project mainly into the antennal lobes. The dorsal clusters have the ellipsoid body (eb) as major target and arborize in the lateral triangle (ltr) and in at least one additional region of the brain. The arborizations in the subesophageal ganglion (sog) of the chemosensory neurons of the labellar taste bristles and the LSO are also labeled by GFP. The arborization in the antennal lobe (al) and the antennal nerve (an) are indicated by arrows. (E) The projection pattern of the dorsal clusters has changed dramatically, the ellipsoid body is not targeted and hence not visible, and the arborizations do not reveal the striking symmetry of the wild-type pattern. Note that the disturbance of the projection pattern, as revealed by the Gal4-driven GFP expression, is completely rescued by two copies, but not by a single copy of *PK6*. This effect is presumably caused by the presence of Gal4 because the GFP pattern is not entirely wild-type even in a $Poxn^{\Delta M22\text{-}B5}$/+ background, whereas the Poxn pattern of a $Poxn^{\Delta M22\text{-}B5}$ mutant is completely rescued to wild-type by Δ*XBs* if visualized by anti-Poxn and histochemical staining. (F,G) Poxn expression in the brain of a 2-day-old *w*; *Poxn-Gal4-9/+*; *UAS-GFP/+* male, visualized as in D,E, but with average projection of the Z-stack. The *Poxn-Gal4-9* driver shows a very strong GFP expression in the Poxn expression domains of the brain with some ectopic expression, mainly in the medulla. The enhanced GFP expression combined with the average Z-stack projection produces a clearer image of the arborizations of the *Poxn* expressing neuronal clusters. (F) The major targets of the projections from the dorsal cluster (dc) of neurons are marked by asterisks. (G) Same specimen as F, but with inverted Z-stack, which offers a posterior view of the GFP expressing neuronal clusters and of the extensive arborizations of the ventral cell cluster (vc) expressing Poxn. The major neurite bundles are marked by arrows and the target areas by asterisks.

at daylight the courting activity of *Poxn*$^{\Delta M22\text{-}B5}$ males is significantly enhanced by the *BasiK* or *XK* transgenes (Fig. 4).

In summary, male courtship behavior strongly depends on an enhancer that regulates *Poxn* expression in the adult brain.

A *Poxn* wing hinge defect impairs flying but not wing extension and vibration during male courting

Homozygous *Poxn*$^{\Delta M22\text{-}B5}$ flies display morphological aberrations in the hinge region of the wing, a *Poxn* phenotype described previously (Awasaki and Kimura, 2001). Although homozygous *Poxn*$^{\Delta M22\text{-}B5}$ mutants cannot fly properly, males are able to extend and vibrate the wing during the initial courting steps. The ability to fly is controlled by an enhancer located downstream or in the last intron of *Poxn* as it is rescued by the *SuperA* or *E77*, but not by the *Full* transgene (Fig. 1B). Because the courting song of *Poxn* males was not examined, it is not clear if it is affected by the absence of this *Poxn* function. However, when *Poxn*$^{\Delta M22\text{-}B5}$; *Full* males are compared with completely rescued *Poxn*$^{\Delta M22\text{-}B5}$; *SuperA* males, no significant time difference between their onsets of courting and copulation is observed (Fig. 4). This finding suggests that the wing hinge phenotype of *Poxn* males does not interfere with courting.

DISCUSSION

Previous studies have shown that *Poxn* plays an essential early role in the specification of the larval poly-innervated external sensory organs and their adult homologs, the chemosensory taste bristles (Dambly-Chaudière et al., 1992; Awasaki and Kimura, 1997). In an exhaustive search for all enhancers, we have dissected the entire *cis*-regulatory region of *Poxn* and found a plethora of additional *Poxn* functions under the control of these enhancers. Intriguingly, many of these newly discovered functions, such as those required for the specification of taste bristles, are directly or indirectly linked to male courting behavior and fertility. Therefore, we might consider *Poxn* a male 'courtship gene' (Hall, 1994).

These male courtship and fertility functions of *Poxn* include functions required for the development of (1) taste bristles on tarsal segments and tibia, anterior wing margin and labellum, whose chemosensory neurons in part respond to female pheromone signals; (2) a ventral and dorsolateral cluster of neurons in the brain, entrusted with targeting the antennal lobe, ellipsoid body, lateral triangle and at least three additional centers in the brain and processing signals, some of which presumably originate from stimulatory olfactory signals propagated by the antennal nerve; (3) specific neurons in the larval ventral nerve cord during embryogenesis, on which the copulation behavior of the male depends; and (4) male genitalia, including penis, posterior lobes and claspers. The multitude of these courtship functions emphasizes the redundancy in the exchange of sensory information between males and females during courtship, an essential feature common to all communication systems (Shannon, 1948a; Shannon, 1948b), and obviously crucial for the survival of the species and its evolutionary success (Greenspan and Ferveur, 2000).

In addition, we have identified enhancers (W. B. and M. N., unpublished) for previously reported *Poxn* functions affecting the segmentation of legs and antennae and the structure of the wing hinge (Fig. 1C) (Awasaki and Kimura, 2001). Our analysis correlates the activity of enhancers not only with the expression patterns which they control, but also with the partial and complete rescue of mutant phenotypes of structural and behavioral nature. This approach thus reveals not merely the size, but also the complex arrangement and substructures of enhancers. The amazing complexity of the organization and substructures of the enhancers reflects their evolutionary history and thus may provide insights into the origin of their present functions.

Complex arrangement and substructures of *Poxn* enhancers

The dissection of the entire *Poxn* control region into different enhancers regulating its many functions is illustrated in Fig. 1C. The overall arrangement of the enhancers reveals an astounding density and complexity. We have delimited 11 *Poxn* enhancers in this study and estimate a total of at least 15, if we include our unpublished results that characterize enhancers active mainly in the embryo (Fig. 1C). Thus, at least nine enhancers are located in the upstream region, five in the introns and one in the downstream region of *Poxn*. Not all enhancers are separable from each other, but some overlap or interdigitate. For example, the enhancers for taste bristle development on legs and wings overlap to a large extent, but are not identical. Moreover, the region over which they extend includes completely the enhancers for (1) embryonic ventral cord expression, (2) penis development and (3) larval p-es organ development (W. B. and M. N., unpublished). Two extreme models for the arrangement of these five enhancers are conceivable. They truly overlap by sharing all or in part some of the same transcription factor binding sites, or they interdigitate without sharing any binding sites. The two models are, of course, not mutually exclusive, and in the case of *Poxn* enhancers we might indeed deal with a mixed model. Thus, the leg and wing taste bristle enhancers, located in the *Xba*I-*Hin*dIII fragment, might share binding sites at both ends (Fig. 1C), while the central region might include part of an interdigitating wing bristle enhancer and not be required for the development of leg taste bristles (Fig. 4; W. B. and M. N., unpublished). Interestingly, this region also includes the enhancers for penis development, which thus might be interdigitating or overlapping with this part of the wing taste bristle enhancer.

The leg and wing taste bristle enhancers exhibit a complex substructure. They both depend at their proximal end on binding sites in the *Xba*I-*Pst*I fragment, which cannot activate *Poxn* transcription sufficiently to support taste bristle development. Only the addition of the adjacent upstream region supports taste bristle development in distal parts of both legs and wings, while further addition of a large central region affects taste bristle development only in the wing. However, we do not know if this central region is required, together with the most distal region of the *Xba*I-*Pst*I fragment, to support the development of taste bristles in the tibia and in the first and third tarsal segments. Both proximal and distal parts of the leg or wing taste bristle enhancer are active only in *cis*, but not in *trans*, with each other or with the central region (Fig. 4; W. B. and M. N., unpublished), which implies that the leg and the wing bristle enhancer, included in the 5.2 kb *Xba*I-*Hin*dIII

fragment, are both single enhancers rather than each being composed of several independent enhancers.

An additional complication of the leg taste bristle enhancer is the fact that, if intron and downstream control regions are absent, it produces in the tibia a large excess of ectopic taste bristles at the expense of mechanosensory bristles, an effect that is more pronounced in the male than in the female (Fig. 4; W. B. and M. N., unpublished). Thus, the balance between taste and mechanosensory bristles in the tibia, yet not in other leg segments, clearly depends on the presence of additional intron and downstream elements. Interestingly, one to three ectopic taste bristles, located in the proximal region of the wing, are similarly suppressed by the additional presence of introns (Fig. 4; W. B. and M. N., unpublished). This situation is further complicated by preliminary results with $Poxn^{\Delta M22\text{-}B5}$ flies rescued by a *Poxn* transgene that completely lacks the upstream enhancers for wing and leg taste bristles, but includes all downstream and intron enhancers. As expected, all labellar taste bristles of these flies are rescued. Surprisingly, however, some of the leg taste bristles are rescued only in male forelegs, but none in female legs, while all ventral and about a third of the dorsal wing taste bristles are rescued in both males and females. It appears, therefore, that the downstream labellar taste bristle enhancer shows considerable redundancy with the upstream wing and leg taste bristle enhancers, yet not vice versa (Fig. 1C). Future detailed analysis of which binding sites are part of these enhancers is expected to shed light on their intricate structure and function relationships and to reveal insights into their evolutionary origin.

Role of *Poxn* in the development of leg, antennal, wing and male genital discs

In addition to the enhancers that control male courtship functions discussed below, we have identified two enhancers (Fig. 1C) whose function is required in the male genital disc for the development of the penis, claspers and posterior lobe (Fig. 5). Moreover, in leg and antennal discs, *Poxn* is expressed in, and required for the development of, two segment primordia (W. B. and M. N., unpublished) that give rise to homologous segments (Fig. 4) (Postlethwait and Schneiderman, 1971). Their homology is reflected at the molecular level by the fact that their expression in leg and antennal discs is regulated by the same enhancer (Fig. 1C). It has been proposed that the genital disc is a ventral disc which behaves in a manner similar to the leg and antennal discs (e.g. Gorfinkiel et al., 1999). However, our identification of two enhancers required for the development of penis, claspers and posterior lobes that are different from the leg/antennal enhancer argues that these structures are not homologous to the leg/antennal segments and that the genital and leg/antennal discs may exhibit only a distant evolutionary relationship (Hadorn, 1978). A similar argument can be made for the *Poxn* enhancer, the function of which is required in the wing disc (Fig. 1C) for proper development of the wing hinge (Awasaki and Kimura, 2001) (W. B. and M. N., unpublished). It therefore appears that the wing is not homologous to the leg or antenna, but only distantly related to it, a notion in agreement with the current model (Wigglesworth, 1973; Kukalová-Peck, 1983; Averof and Cohen, 1997). Nevertheless, it is intriguing that *Poxn* has acquired during evolution enhancers that regulate functions in leg/antennal, wing and genital discs. Rather than homology of the structures derived from the different parts of the discs expressing *Poxn*, *Poxn* activity may reflect the close relationship of the gene networks in which *Poxn* participates, in agreement with the gene network hypothesis (Noll, 1993).

Role of *Poxn* in the reception of pheromone signals through taste bristles

Our analysis of enhancer functions was not limited to their more direct effects such as, for example, the regulation of Poxn expression in developing taste bristles and specification of their chemosensory fate. Our main interest in this study was rather to assess and measure the indirect effects of separate *Poxn* enhancers and functions on male fertility and courting behavior. We were able to test primarily the first step in this communication system, by which females arouse the interest of males, which react by extending and vibrating a wing and thus initiate courting the female (Greenspan and Ferveur, 2000). The male receives through its sensory organs, and reacts to, many types of signals, which are additive and, at least under laboratory conditions, redundant, i.e. as long as the combined input signal exceeds a threshold, the male begins courtship (Hall, 1994). Our approach to analyze the various *Poxn* functions by a dissection of its enhancers allows us to answer which *Poxn* functions are involved in the reception and processing of these signals. These partially characterized courtship functions of *Poxn* can be divided into primary functions of the peripheral nervous system, which receives and propagates the different signals, and secondary functions of the CNS and brain, on which processing and integration of the signals depends.

The following sensory modalities play a role in courtship (Greenspan and Ferveur, 2000): (1) visual input received by the photoreceptors of the eye; pheromone signals received (2) by gustatory receptors in the neurons of the taste bristles on legs, wing and labellum, and (3) by olfactory receptors in the neurons of the olfactory sensilla in the third antennal segment and maxillary palp; (4) auditory signals innervating neurons of the chordotonal organs of Johnston's organ in the second antennal segment; and (5) mechanosensory input innervating neurons of mechanosensory bristles. Courting tests with *Poxn* mutant males some of whose *Poxn* functions have been rescued showed that these are important only for the reception of signals by neurons of taste bristles. As *Poxn* is never expressed in developing and adult olfactory or chordotonal organs and mechanosensory bristles, it might have functions in the reception of only the first two types of signals. However, an essential function of *Poxn* in the reception of light input is ruled out by two observations: $Poxn^{\Delta M22\text{-}B5}$ males are able to initiate courtship at daylight, but not under red light (Fig. 4), and *w*; $Poxn^{\Delta M22\text{-}B5}$ double mutants also fail to initiate courtship at daylight. This result further supports the notion that visual and chemosensory taste and olfactory inputs play the major role in the initiation of male courtship behavior, while mechanosensory and auditory inputs play a subordinate role (Cook, 1980; Tompkins et al., 1980; Gailey et al., 1986; Markov, 1987; Heimbeck et al., 2001). Moreover, it demonstrates that the visual input alone is sufficient to trigger male courtship, though at a much reduced efficiency when compared with the use of all sensory modalities affecting courtship initiation. Finally, it follows that *Poxn* plays an important role in the reception of pheromones by gustatory receptors as evident from the observation that the latency times of courtship initiation at daylight and under red light are

considerably prolonged by the selective removal of taste bristle functions (compare with rescue of *Poxn*$^{\Delta M22\text{-}B5}$ by Δ*XBs* and *Full* in Fig. 4). The fact that the selective removal of all taste bristles does not eliminate courtship in the dark strongly suggests that *Poxn* has functions crucial for the processing of signals elicited by female pheromones in the olfactory receptors (see below). It further follows that not only visual, but also olfactory input alone is sufficient to trigger male courtship, though also at reduced efficiency, illustrating the redundancy of the system.

At present, we cannot answer what the contributions of leg, wing and labellar taste bristles are in the reception of the female pheromone signals. We do not know, for example, if wing taste bristles recognize pheromones or are redundant for this function because no significant change in courtship behavior is noticed between males rescued by *SaK* or *BaK* despite a considerable difference in the number of wing, but not leg or labellar, taste bristles (Fig. 4). These and related questions are now amenable to an experimental approach if future analysis of the taste bristle enhancers permits a selective removal of the different taste bristle functions.

It may be important that Poxn is expressed in chemosensory neurons of prothoracic legs that connect contralaterally in the male, but not in the female (Possidente and Murphey, 1989). This and the additional sexual dimorphism that males have about 50% more chemosensory bristles on their forelegs than females (Nayak and Singh, 1983) suggest that pheromone receptors on male leg taste bristles are restricted to the forelegs.

Role of *Poxn* in processing olfactory signals in defined neurons of the brain

We have identified two *Poxn* enhancers that regulate secondary courtship functions of *Poxn*, one active in the developing brain (brain enhancer), the other in the embryonic ventral CNS (ventral cord enhancer). Our results suggest that Poxn expression under control of the brain enhancer in the developing and adult brain is crucial for the proper processing of courtship signals elicited by female pheromones in the olfactory receptors (see above). This conclusion is further supported by comparing *Poxn* males without taste bristle and ventral cord functions in the presence (*Poxn*$^{\Delta M22\text{-}B5}$; *XK*) and absence of the brain function (*Poxn*$^{\Delta M22\text{-}B5}$; *E77*). In the absence of the brain function, these males do not initiate courtship under red light, while no difference in courting between the two types of males is apparent in daylight (Fig. 4). Similarly, if we compare *Poxn* males without taste bristle functions, but with the ventral cord function, in the presence (*Poxn*$^{\Delta M22\text{-}B5}$; *XK*) and absence of the brain function (*Poxn*$^{\Delta M22\text{-}B5}$; *C1*), we find that the brain function is crucial for courting under red light, but not in daylight (Fig. 4). As these males have no taste bristle input, they are able to court only in the absence of proper visual input if the olfactory input is processed by the brain function of *Poxn*. Our results, therefore, demonstrate that the *Poxn* brain function is necessary for the processing of olfactory input, to which the ventral cord function does not contribute. This conclusion is also consistent with the observation that the antennal lobe, which receives the olfactory signal through the antennal nerve, is targeted by the Poxn-expressing ventral and ventrolateral neuronal clusters in the brain (Fig. 7D,F,G). In the *Poxn* mutant, however, the Poxn-expressing neurons in the brain fail to make their proper connections (Fig. 7E).

In summary, *Poxn* includes two courtship functions involved in the reception and processing of sensory input: (1) the reception and propagation of female pheromone signals through taste bristles, and (2) the processing in the brain of olfactory signals elicited by female pheromones. A third function of *Poxn* during copulation is discussed below. Because the brain enhancer has not been removed completely in any of the *Poxn* rescue constructs that include taste bristle enhancers, it is possible that the brain enhancer is also required for the processing of signals received from taste bristles. Similarly, we cannot exclude a role of the brain enhancer in the processing of mechanosensory and auditory input.

Role of *Poxn* in the processing of courtship signals during copulation

Also the function of the ventral cord enhancer appears to be required for the processing of sensory signals that affect male courting behavior. However, in contrast to the brain enhancer, the ventral cord enhancer controls a *Poxn* function that does not influence the initiation of courtship, but somehow affects the success of copulation. In the absence of the ventral cord function, males attempt to copulate, but are unable to attach themselves to the female genitalia or soon fall off after copulation and remain on their back shivering for several minutes before they recover. By contrast, the ventral cord function is dispensable for the processing of input received through taste bristles. This is evident from the courtship behavior of males that lack the ventral cord but not the brain function: *Poxn*$^{\Delta M22\text{-}B5}$; *SaK* males, many of whose leg and wing taste bristles are rescued, initiate courtship at a considerably enhanced frequency both at daylight and under red light when compared with *Poxn*$^{\Delta M22\text{-}B5}$; *XK* males, which have no taste bristles (Fig. 4). It follows that the ventral cord function is not primarily required for the reception of signal(s) received from sensory organs, but rather for their processing and input into the efferent nervous system, such as for the activation of certain motorneurons required to initiate and maintain copulation.

To evaluate how the ventral cord function influences copulation, we compared *Poxn*$^{\Delta M22\text{-}B5}$; Δ*SH* males, whose genitalia are rescued and thus are able to copulate, in the presence and absence of the ventral cord function carried by *C1*, which cannot contribute to taste bristle development in *trans* (Fig. 1B,C). As evident from Fig. 4, the ventral cord function dramatically enhances successful copulation and hence male fertility. Thus, the ventral cord function of *Poxn* is not responsible for the processing of courtship signals, except during its last phase to initiate and maintain copulation.

What could the ventral cord function of *Poxn* be? Most probably it is required to orchestrate the complex movements of copulation. Thus, the male initiates copulation by bending the abdomen forward, attaching its end to the female genitalia through its claspers, mounting the female and inserting the penis into the vagina. It maintains this position by anchoring the penis within the vagina. Apparently, this complex coordinated movement of male abdomen and genitalia, which is regulated by efferents of motorneurons, is disturbed by the absence of the ventral cord function of *Poxn*. Specifically, mechanosensory signals in the male genital region may not be properly processed and thus impair the coordinated movement regulated by motorneurons.

An intriguing feature of the ventral cord function of *Poxn* is

its early expression during embryogenesis, whereas its mutant phenotype becomes apparent only in the adult. A probable explanation is that many of the larval ventral cord neurons specified during embryogenesis persist to the adult stage (Truman, 1990). Although they constitute only a small fraction of about 5-10% of the adult CNS, they contribute disproportionately to certain neuronal classes and thus may provide clues that are important for the proper organization of the adult CNS (Truman et al., 1993). Future studies investigating the function of *Poxn* in these neurons might not only shed light on their role in adult courtship behavior, but also on the complex developmental changes in the neurons of the larval CNS that are conserved during metamorphosis.

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