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Identification of spatial and temporal cues that regulate postembryonic expression of axon maintenance factors in the *C. elegans* ventral nerve cord

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SUMMARY

Patterns of gene expression are under precise spatial and temporal control. A particularly striking example is represented by several members of the *zig* gene family, which code for secreted immunoglobulin domain proteins required for maintaining ventral nerve cord organization in *Caenorhabditis elegans*. These genes are coordinately expressed in a single interneuron in the ventral nerve cord, known as PVT. Their expression is initiated at a precise postembryonic stage, long after PVT has been generated in mid-embryogenesis. We define spatial and temporal cues that are required for the precise regulation of *zig* gene expression. We find that two LIM homeobox genes, the *Lhx3*-class gene *ceh-14* and the Lmx-class gene *lim-6* are coordinately required for *zig* gene expression in PVT. Temporal control of *zig* gene expression is conferred by the heterochronic gene *lin-14*, a nuclear factor previously implicated in developmental timing in various contexts. Loss of the *lim-6* and *ceh-14* transcription factors and the developmental timer *lin-14* cause not only a loss of *zig* gene expression but also lead to defects in the maintenance of ventral nerve cord architecture. Overriding the normal spatiotemporal control of *zig* gene expression through expression of one of the *zig* genes under control of heterologous promoters also causes axon patterning defects in the ventral nerve cord. Our findings illustrate the importance of spatial and temporal control of gene expression in the nervous system and, furthermore, implicate heterochronic genes in postmitotic neural patterning events.

Key words: *C. elegans*, *zig*, LIM homeobox, Heterochronic, Axon maintenance

INTRODUCTION

Postmitotic neurons display specific gene expression profiles that define the differentiated state of the neuron. These gene expression profiles generally show two distinct kind of temporal dynamics. Some genes are transiently expressed until shortly after the birth of a neuron and include transcription factors, axon pathfinding and target recognition cues, which serve to wire a neuron into specific circuits (e.g. Wadsworth et al., 1996; Sharma et al., 1998; Sarafi-Reinach et al., 2001). Other genes are constitutively expressed throughout the life of a postmitotic neuron and define several functional properties of the neuron such as its neurotransmitter phenotype (e.g. Eastman et al., 1999). Transient peaks of gene expression after the birth of a neuron are usually coupled with the exit from the cell cycle, while sustained expression of genes during the life of a neuron is often controlled by transcriptional autoregulatory feedback loops (Edlund and Jessell, 1999). By contrast, little is known about temporally defined, stereotyped changes in gene expression patterns within postmitotic neurons that are induced long after neuron generation and differentiation. Although neurons are able to respond transcriptionally to signaling inputs (e.g. in the course of learning and memory) (Bailey et al., 1996), those changes in gene expression patterns usually do not occur at pre-programmed times in the life of a neuron.

Instances in which neuronal phenotypes are modified in a developmentally programmed, stereotyped temporal manner that occurs significantly past the axon outgrowth and target recognition stage include sexual differentiation in the mammalian brain at defined postnatal stages (MacLusky and Naftolin, 1981), remodeling of the insect CNS during metamorphosis (Levine et al., 1995) and synaptic rewiring of postmitotic motor neurons in the ventral nerve cord (VNC) of *C. elegans* (White et al., 1978). In all three circumstances, temporally defined alterations in gene expression are presumably mediated through hormones and their effector transcription factors to eventually cause a structural remodeling of individual groups of neurons (Levine et al., 1995; MacLusky and Naftolin, 1981; Zhou and Walthall, 1998).

We describe our analysis of another neural gene expression program that is not only under tight spatial, but also under stereotyped temporal control. Unlike the cases described above, these dynamics in gene expression do not serve to induce remodeling of the neuron but provide the neuron with a novel and specific capacity at a defined time in its life. The case studied is the PVT interneuron located in the VNC of *C.*

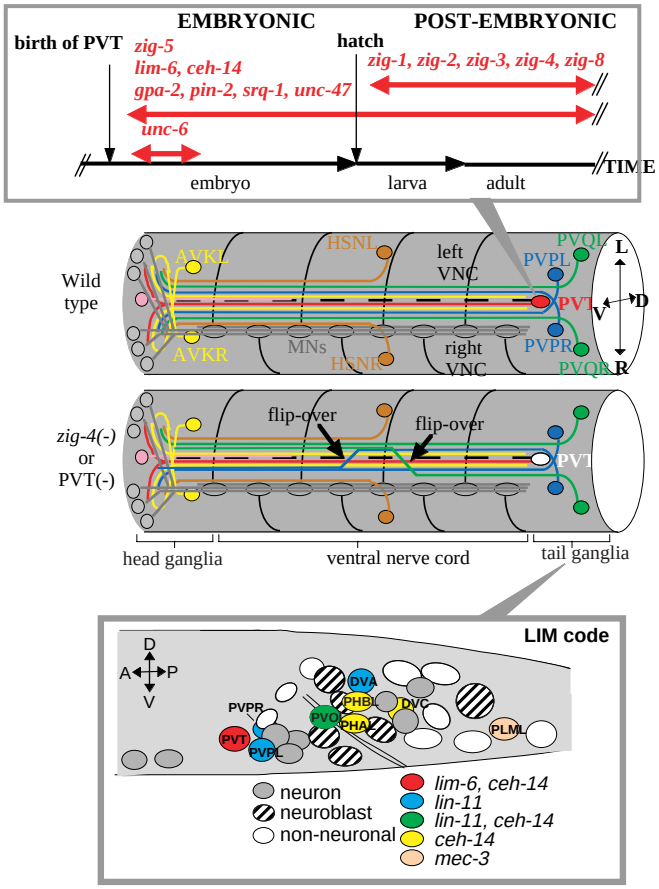


Fig. 1. Features of the *C. elegans* ventral nerve cord. The VNC in wild-type animals and consequences of microsurgical removal of PVT on VNC architecture are shown. Neurons that have terminated their axon outgrowth (PVQL/R, AVKL/R, RMEV, HSNL/R) are affected by laser removal of PVT or *zig-4* mutation, such that their axons flip over the hypodermal ridge (ventral midline) into the opposite fascicle (Aurelio et al., 2002). The upper inset shows gene expression profiles in PVT (see text for references), the lower inset the expression pattern of several LIM homeodomain transcription factors in the tail ganglia of an L1 stage animals (Cassata et al., 2000; Hobert et al., 1998; Hobert et al., 1999b; Sarafi-Reinach et al., 2001; Way and Chalfie, 1989).

elegans (Fig. 1). Using cell ablation techniques, PVT was recently found to have two temporally separable roles in axonal patterning in the VNC. In mid-embryonic stages, PVT is required for axon attraction into the VNC (Ren et al., 1999). Surprisingly, at the postembryonic larval L1 stage, PVT was found to have an additional role in non-autonomously maintaining the intact axon scaffolding in the VNC, such that loss of PVT causes axon 'flip-overs' across the ventral midline (Fig. 1) (Aurelio et al., 2002).

These two temporally separated functions of PVT correlate with the execution of a temporally controlled, biphasic gene expression program. In mid-embryogenesis, during the development of the VNC, PVT transiently expresses the UNC-6/Netrin cue to attract axons into the VNC (Wadsworth et al., 1996). Several hours later, in the larval L1 stage, PVT initiates the expression of several members of the *zig* gene family (Fig. 1) (Aurelio et al., 2002). *zig* genes encode small transmembrane (*zig-1*) or secreted (*zig-2* to *zig-8*) proteins composed exclusively of two immunoglobulin domains. Six *zig* genes are expressed in restricted domains of the nervous system, while the two remaining family members are expressed outside the nervous system. The expression of all six neuronally expressed *zig* genes overlaps in the PVT interneuron. The onset of expression of several of the *zig* genes in the PVT neuron in the L1 stage precisely correlates with the requirement for PVT in maintaining axonal organization of the VNC (Aurelio et al., 2002). Consistent with the hypothesis that *zig* genes mediate the maintenance function of PVT, we found that a deletion in the *zig-4* locus affects the maintenance of axonal position of a subset of those neurons that are affected by PVT ablation (Aurelio et al., 2002), suggesting that different *zig* family members are required to maintain the positioning of distinct subsets of VNC axons.

What are the molecular mechanisms that underlie the biphasic gene expression program in PVT and, specifically, what are the factors that confer the precise spatial and temporal aspects of *zig* gene expression? We used postembryonically expressed *zig* gene reporter gene constructs as tools to investigate these mechanisms. We define three factors that are required for the execution of the temporal and spatial aspect of *zig* gene expression and show that their absence not only disrupts *zig* gene expression but also leads to defects in the maintenance of axon positioning in the VNC. Furthermore, we find that spatiotemporal misexpression of one of the *zig* genes, *zig-4*, causes developmental defects in the VNC, suggesting that the normal spatiotemporal control of *zig* gene expression serves to dedicate them to a role in axon maintenance and to prevent them from interfering with axon outgrowth.

MATERIALS AND METHODS

Mutant strains

OH110, *lim-6(nr2073)X* (Hobert et al., 1999b);
OH170, *ceh-14(ch3)X* (derived from TB522 (Cassata et al., 2000) through outcrossing of *dpy-20*);
OH158, *zig-4(gk34)X* (Aurelio et al., 2002);
OH636, *lim-6(nr2073) ceh-14(ch3)X*;
DR441, *lin-14(n179ts)X*;
MT1848, *lin-14(n360ts)I*;
MT1388, *lin-14(n355n679ts)I*;
MT1524, *lin-28(n719)X* (Ambros and Horvitz, 1984);
MT2257, *lin-42(n1089)X* (Z. Liu, PhD thesis, Massachusetts Institute of Technology, 1990);
NG83, *fax-1(gm83)X* (Much et al., 2000); and
CB270, *unc-42(e270)V* (Baran et al., 1999).

Reporter strains

PVT cell fate markers used in this study are *syIs7*: *Is[gpa-2::lacZ; dpy-20(+)]* (Zwaal et al., 1997), *otIs90*: *Is[pin-2::gfp; rol-6(d)]* (Hobert et al., 1999a), *otIs85*: *Is[srq-1::gfp; rol-6(d)]* and *otIs39*: *Is[unc47Δ::gfp; lin-15(+)]*. The srq-1 reporter is a translational fusion constructed from a 4.4 kb genomic fragment, encompassing 2.2 kb of sequence upstream of the predicted ATG and 2.2 kb of exonic and intronic sequences of the predicted serpentine receptor gene *F59B2.13* gene. The construct was generated using a PCR fusion protocol (Hobert, 2002). A corresponding *F59B2.13::lacZ* construct had been constructed and analyzed by Ian Hope in the course of a genome-wide expression pattern analysis (Lynch et al., 1995). The *otIs39* integrant derives from *juEx60* (Eastman et al., 1999) and contains a promoter fragment from the *unc-47* gene. The pan-neuronal marker *evIs111*

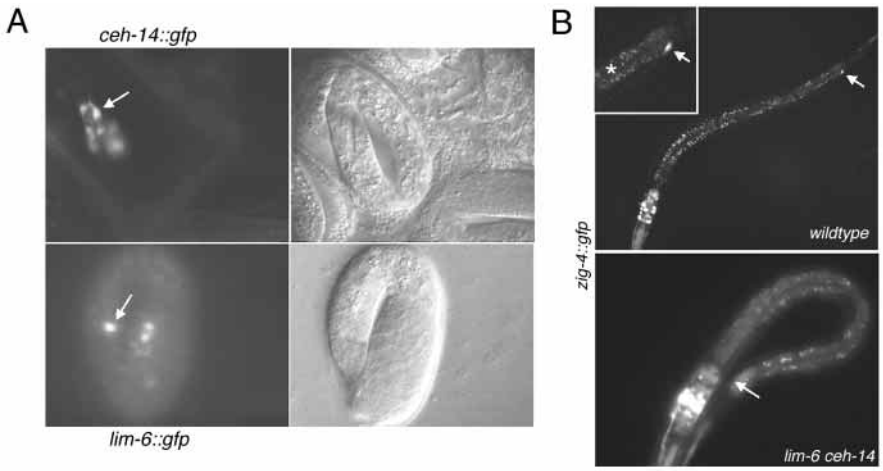


Fig. 2. *lim-6* and *ceh-14* are expressed in the PVT neuron and coordinately affect *zig-4::gfp* expression. (A) Embryonic expression of *lim-6* and *ceh-14* reporter gene constructs in PVT (see Materials and Methods for details on expression constructs). Postembryonic expression of *lim-6 ceh-14* in PVT has been previously reported (Cassata et al., 2000; Hobert et al., 1999b). (B) Effect of *lim-6 ceh-14* on *zig-4::gfp (otIs20)* expression. The inset shows an enlargement of the tail region with a *gfp*-fluorescing PVT cell. White star denotes gut autofluorescence. Note that other *zig-4::gfp* expressing cells are not affected by *lim-6 ceh-14*. See Fig. 3 for quantitative data on all PVT-expressed *zig* genes. White arrows points to PVT, which lacks a *gfp* signal in panel B.

(integrated *F25B3.3::gfp*); the PVQ marker *oyIs14* (integrated *sra-6::gfp*); and the *zig::gfp* reporter gene constructs *otIs25* (*zig-1::gfp*), *otIs7* (*zig-2::gfp*), *otIs14* (*zig-3::gfp*), *otIs20* (*zig-4::gfp*), *otIs11* (*zig-5::gfp*) and *otIs96* (*zig-8::gfp*) have been described previously (Altun-Gultekin et al., 2001; Aurelio et al., 2002). The *lim-6r::gfp* expression construct is a full length translational fusion of the *lim-6* locus to *gfp* and contains all the regulatory regions required for rescuing lim-6 mutant phenotypes (Hobert et al., 1999b); expression in PVT was scored in an extrachromosomal line, *otEx406*. The *ceh-14::gfp* construct was engineered using a PCR fusion protocol (Hobert, 2002) and encompasses 3.75 kb of promoter and parts of the first exons. A similar promoter fragment has previously been described to faithfully reflect the expression of the endogenous gene (Cassata et al., 2000). Expression of this construct was monitored in the chromosomally integrated line *otIs126*.

Scoring of neuroanatomical defects and reporter gene expression

Reporter gene expression levels and neuroanatomical defects were assayed – with the exception of the extrachromosomal *lim-6r::gfp* array – using chromosomally integrated *gfp* or *lacZ* expression constructs (see above). The integrated arrays were crossed into the respective mutant backgrounds, thus allowing a direct comparison between otherwise isogenic backgrounds. If not indicated otherwise, reporter gene expression was scored in well-fed animals. *zig-4::gfp* expression was also scored in starved animals (see text). For these starvation experiments, eggs were prepared from *otIs20 (zig-4::gfp)* animals using a standard bleaching protocol, plated onto non-seeded plates and scored for *zig-4* expression in PVT several days later.

To score neuroanatomical defects at the early L1 stage (when PVT has yet no maintenance role), eggs were prepared by bleaching and freshly hatched L1s scored 2 hours after the preparation of the eggs. With regard to neuroanatomical defects, we focused on the description of defects in the VNC. We found that in various mutant backgrounds (e.g. *lim-6 ceh-14*) neuroanatomical defects can also be observed in other axon fascicles.

Laser ablation

The nucleus of PVT was identified based on its characteristic position and morphology by Nomarski optics. Ablation was performed using a LSI VSL-337 laser as previously described (Aurelio et al., 2002; Bargmann and Avery, 1995).

Ectopic *zig-4* expression

A genomic fragment of the *zig-4* locus ranging from its start to stop codon and including all introns was amplified with restriction sites on either end of the amplicon. The amplicon was cloned behind a 1.3 kb promoter fragment from the upstream regulatory region of the *flp-1* gene (Nelson et al., 1998) and a 2.4 kb fragment from the muscle-specific *myo-3* promoter (Okkema et al., 1993). When fused to *gfp*, the 1.3 kb *flp-1* promoter (–1358 to –9 relative to ATG) is exclusively active in the AVKL and AVKR neurons. All expression constructs were injected into *oyIs14* animals at 50 ng/μl using *ceh-22::gfp* (Okkema and Fire, 1994) at 50 ng/μl as an injection marker.

RESULTS

Two LIM homeobox genes are required for postembryonic *zig* gene expression in the PVT interneuron

LIM homeobox genes regulate various aspects of postmitotic neural differentiation (reviewed by Bach, 2000; Hobert and Westphal, 2000; Jessell, 2000; Lee and Pfaff, 2001) and have been proposed to act through combinatorial codes of overlapping expression ('LIM-code') (Sharma et al., 1998; Thor et al., 1999; Tsuchida et al., 1994). A similar LIM code may exist in the tail of an L1 stage *C. elegans* larva (Fig. 1). Specifically, two of the seven *C. elegans* LIM homeobox genes, the *Lmx*-class gene *lim-6* (Hobert et al., 1999b) and the *Lhx3*-class gene *ceh-14* (Cassata et al., 2000) are co-expressed in PVT (Fig. 1, Fig. 2A), yet their function in PVT development has not been addressed.

Six out of the eight *zig* gene family members are expressed in PVT and, in addition, in a few other cell types (Aurelio et al., 2002). We tested whether these six genes are under control of the *lim-6* and *ceh-14* LIM homeobox genes by crossing previously described, chromosomally integrated *gfp* reporter genes that monitor the PVT expression of the six genes (Aurelio et al., 2002) into *lim-6(nr2073)* and *ceh-14(ch3)*-null mutant animals, respectively. Neither LIM homeobox gene mutation had a significant effect on initiation of *zig* gene expression by itself. However, in *lim-6(nr2073) ceh-14(ch3)* double null mutants, the expression of all postembryonically expressed *zig* genes was significantly affected in PVT, if not completely abolished (Fig. 2B, Fig. 3).

Each one of the PVT-expressed *zig* genes is also expressed in a restricted subset of other cell types (Aurelio et al., 2002), yet *lim-6 ceh-14* double null mutants only show an effect on *zig* gene expression in PVT (Fig. 2B and data not shown). As

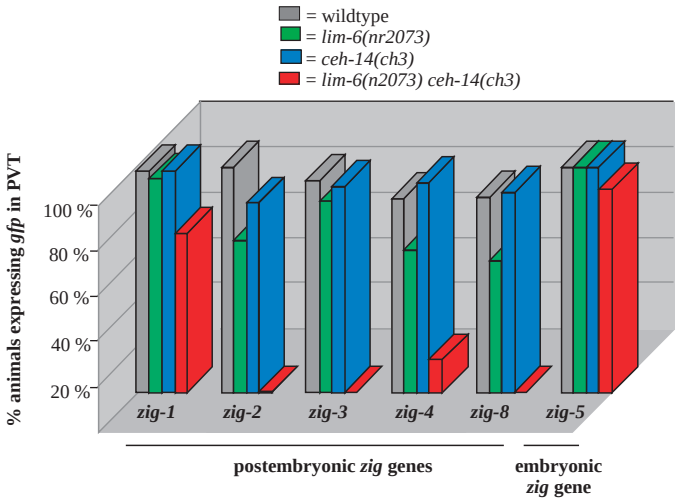
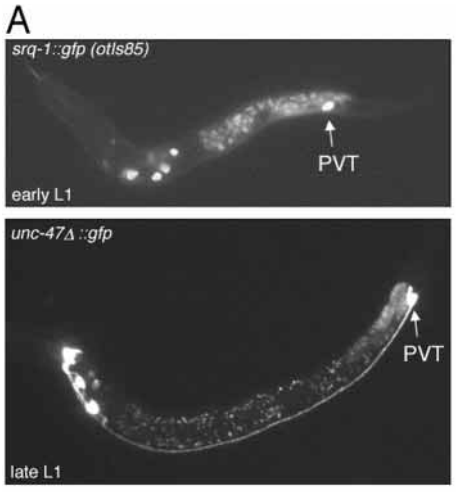


Fig. 3. *lim-6* and *ceh-14* coordinately affect expression of postembryonically expressed *zig* genes in the PVT neuron. Individuals with a chromosomally integrated *zig::gfp* reporter (see Materials and Methods) were crossed into the indicated null mutant backgrounds. Animals were scored at mixed stages. The modest effect of *lim-6* on *zig-2*, *zig-3* and *zig-4::gfp* expression (<30% off) is only manifested after the L3 stage: expression is correctly initiated but fails to be maintained. *n*=45-500, depending on genotype.



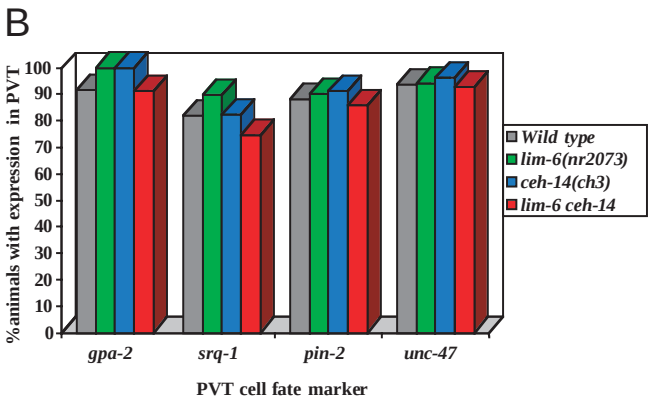


Fig. 4. Generation and fate of the PVT neuron are unaffected in *lim-6 ceh-14* double mutants. (A) *srq-1::gfp (otIs85)* and *unc-47Δ::gfp (otIs39)* are markers for PVT cell fate. In addition to PVT, *srq-1* is expressed in one more tail neuron and several head neurons. The *unc-47Δ::gfp* constructs labels not only the RMEL/R, RIS, AVL and DVB, as described previously (Eastman et al., 1999), but also the PVT neuron. Expression of the two other PVT cell fate markers used here (B), *gpa-2* and *pin-2*, has been previously documented (Hobert et al., 1999a; Zwaal et al., 1997) and is not shown. (B) Expression of PVT cell fate markers in mutant backgrounds. All alleles shown are molecular nulls. Scoring was carried out in L1 and L2 stage animals. Sample size was 28-187, depending on genotype. Expression of *unc47Δ::gfp* (*otIs39)* in PVT is seen only in 15% of post-L3 stage *lim-6 ceh-14* animals (*n*=14), while wild type shows expression in >95% at pre-L3 stages. This stage-dependent downregulation is difficult to interpret, however, because three out of four integrants of the *unc47Δ::gfp* reporter line *juEx60* show no expression in PVT beyond the L3 stage, while only one integrant, *otIs39* (tested here), shows expression after the L3 stage. We also noted that in *lim-6 ceh-14; otIs39* animals, DVB axon morphology often appears defective.

shown in Fig. 3, the impact of *lim-6 ceh-14* is strongest on those *zig* genes that are expressed strictly postembryonically (*zig-2*, *zig-3*, *zig-4*, *zig-8*), although it is weaker, but still significant, on those *zig* genes that already show some expression in embryos (*zig-5::gfp*, completely penetrant embryonic expression in wild type; *zig-1::gfp*, incompletely penetrant embryonic expression in wild type) (Aurelio et al., 2002).

Given the significant loss of *zig* gene expression in PVT in *lim-6 ceh-14* double mutants, we next examined whether the generation and overall cell fate of PVT is grossly affected in *lim-6 ceh-14* mutants. To this end, we monitored the expression of several PVT cell fate markers, namely the GTPase *gpa-2* (Zwaal et al., 1997), the LIM-only gene *pin-2* (Hobert et al., 1999a), the serpentine receptor *srq-1* (see Materials and Methods) and the *unc-47* GABA transporter gene (Eastman et al., 1999). All of these markers are expressed normally in *lim-6 ceh-14* double mutants (Fig. 4). In addition, as assessed with the *unc-47Δ::gfp* marker, PVT axon morphology appears unaffected (data not shown). Last, the embryonic, *unc-6/Netrin*-mediated role of PVT in attraction of axons from tail ganglia into the VNC (Ren et al., 1999) is unaffected in *lim-6 ceh-14* double mutants, as visualization of several of these axons in *lim-6 ceh-14* mutants reveals no defect (data not shown). Taken together, these data suggest that in the absence of the two LIM homeobox genes, *lim-6* and *ceh-14*, PVT adopts its correct cell fate, i.e. displays all known features of its terminal differentiation program, but is incapable of initiating expression of *zig* genes in the L1 stage.

Although both *lim-6* and *ceh-14* are jointly required for initiation of *zig* gene expression, they are not jointly sufficient to induce *zig* gene expression for the following two reasons. First, both *lim-6* and *ceh-14* are already expressed embryonically (Fig. 2A) and hence significantly precede the onset of *zig* gene expression; second, pan-neuronal misexpression of *lim-6* under control of the *unc-119* promoter yields co-expression of *lim-6* and *ceh-14* in several neurons (*ceh-14* is expressed in a total of 18 neurons) (Cassata et al., 2000), yet does not cause ectopic *zig-4* expression in any of these cells (data not shown).

We also tested whether other transcription factors previously shown to be expressed in PVT, namely the nuclear hormone receptor *fax-1* (Much et al., 2000) and the paired-type

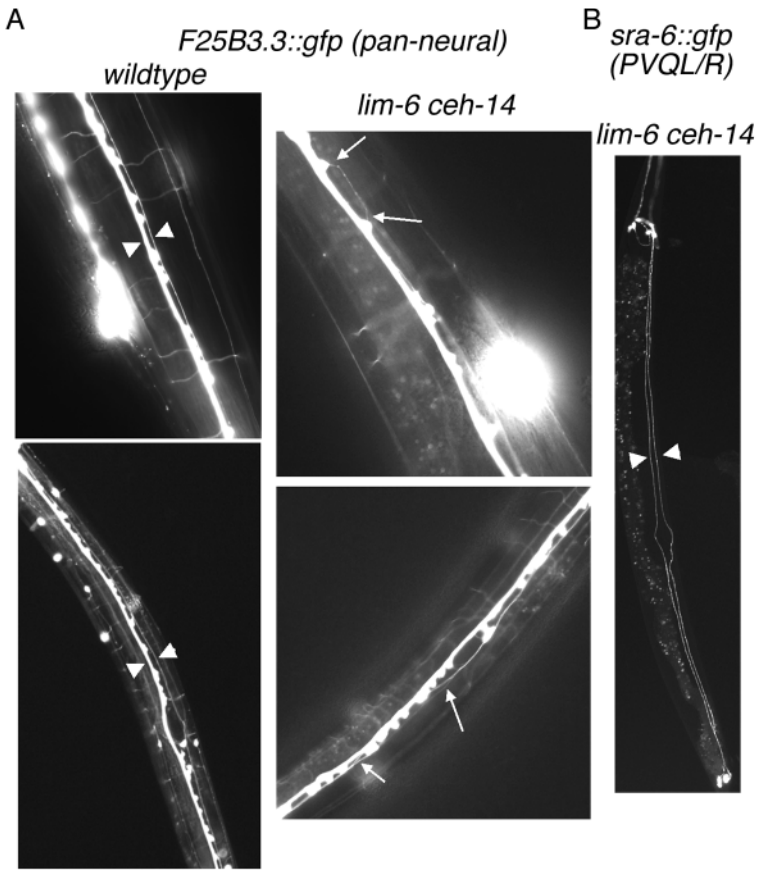


Fig. 5. Axonal defects in the ventral nerve cord of *lim-6 ceh-14* double mutants. (A) VNC defects as assayed with the *F25B3.3::gfp (evIs111)* marker. Arrows indicate the left and right VNC in wild-type animals. Arrows indicate inappropriate axon flip-overs seen in *lim-6 ceh-14* mutants. They are reminiscent of those seen in PVT(–) or *zig-4(–)* animals (Aurelio et al., 2002). Quantitative data are shown in Table 1. (B) PVQL/R neurons have a wild-type appearance in *lim-6 ceh-14* mutant animals. The marker is *oyIs14* (see Fig. 8A for *oyIs14* in a wild-type background).

homeobox gene *unc-42* (Baran et al., 1999), affect *zig* gene expression. By crossing various *zig::gfp* reporter gene integrants with *fax-1* and *unc-42* single mutants and with *lim-6; unc-42* double mutants, we found this not to be the case (data not shown).

Ventral nerve cord defects in *lim-6 ceh-14* double mutants

A loss-of-function mutation in *zig-4* or postembryonic laser removal of PVT, the cellular source of *zig-4*, causes a disorganization of the left and right axonal tracts in the VNC, characterized by a 'flip-over' of embryonically established axonal tracts into the opposite fascicle (Fig. 1) (Aurelio et al., 2002). The disruption of *zig* gene expression in PVT in *lim-6 ceh-14* double mutant animals would thus be expected to cause similar disruptions of VNC architecture. To investigate this point, we crossed *lim-6 ceh-14* double null mutants with transgenic animals in which either the whole VNC or individual neurons within the VNC are labeled with *gfp*. Using a pan-neuronal marker, we indeed observed left/right axon flip-overs from one fascicle into the opposite fascicle in the VNC of *lim-6 ceh-14* double mutant animals (Fig. 5, Table 1).

The VNC axon flip-over defect in *lim-6 ceh-14* animals manifests itself only at stages past the late L1 stage, while freshly hatched animals show no mutant phenotype (Table 1). Hence, *lim-6 ceh-14* mutants show axonal maintenance defects in the VNC that resemble those in PVT ablated and *zig-4* mutant animals not only in overall appearance but also in their temporal profile.

In summary, VNC axon flip-over defects are present in the *lim-6 ceh-14* double mutant, but not in the single mutants; the expression of *lim-6* and *ceh-14* exclusively overlaps in PVT; PVT loses the postembryonic *zig* gene expression in *lim-6 ceh-14* double mutants; and the VNC defects show a *zig-4(–)* and PVT(–)-like characteristic postembryonic profile. These points strongly suggest that *lim-6* and *ceh-14* act in PVT to affect VNC maintenance by regulating *zig* gene expression.

ceh-14 acts outside PVT to suppress the *zig-4*-induced axon flip-over phenotype

Examination of individual axons in the VNC in *lim-6 ceh-14* double mutant animals lead to a surprising observation. Although the axons of the PVQL/R neurons flip into the opposite site of the VNC in *zig-4* mutant animals, no such defect above background level can be observed in *lim-6 ceh-14* double mutant animals, in which *zig-4* expression is downregulated (Fig. 5B, Fig. 6). This observation could be explained by *zig-4* expression levels and hence *zig-4* activity not being as significantly affected in *lim-6 ceh-14* double mutants as in the *zig-4* null mutant. Alternatively, *lim-6* and/or *ceh-14* gene activity could be required for the PVQL/R flip-over event to occur in the absence of *zig-4*. To address this issue, we conducted a genetic epistasis test in which we analyzed PVQL/R neuroanatomy in *lim-6(nr2073) ceh-14(ch3) zig-4(gk34)* triple null mutant animals. We found that the *zig-4* mutant phenotype is suppressed in these triple mutant animals (Fig. 6).

We next asked whether *lim-6 ceh-14* act in PVT to suppress the *zig-4* mutant phenotype. It could, for example, be envisioned that expression of a factor X is repressed by the

Table 1. Quantification of axonal defects in the ventral nerve cord of *lim-6 ceh-14* double mutant animals

	VNC axon flip-over defects*	
Genotype	Scored past late L1 stage	Scored at the early L1 stage
Wild type	4% (*n*=50)	n.d.
lim-6(nr2073)†	10% (*n*=20)	n.d.
ceh-14(ch3)†	10% (*n*=50)	n.d.
lim-6(nr2073) ceh-14(ch3)	29.4% (*n*=51)	0% (*n*=20)
PVT(–)‡	36.4% (*n*=11)§	n.d.

*Scored with the pan-neuronal marker *evIs111 (F25B3.3::gfp)*.
†Molecular null alleles.
‡PVT was ablated in freshly hatched L1 animals.
§Aurelio et al. (Aurelio et al., 2002) showed the same penetrance of defects with a larger sample size (*n*=30).
n.d., not determined.

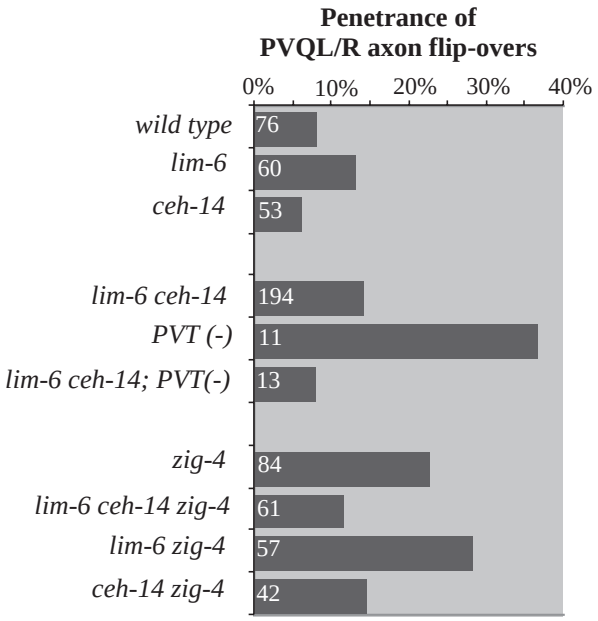


Fig. 6. Loss of *ceh-14* suppresses the flip-over phenotype of the PVQL and PVQR neurons. PVQL/R was visualized in the individual mutant backgrounds using *oyIs14 (sra-6::gfp*). All mutant alleles used [*lim-6(nr2073), ceh-14(ch3) zig-4 (gk34)*] are null alleles. PVT(–) indicates PVT ablation at the early L1 stage. All defects were scored at late larval stages. Numbers within bars indicate sample size.

LIM-6 and/or CEH-14 proteins in PVT and that its de-repression in *lim-6 ceh-14* mutants prevents an axon flip-over caused by the absence of *zig-4*. Alternatively, *lim-6* and/or *ceh-14* could act outside of PVT to suppress the *zig-4* mutant phenotype. To distinguish between these possibilities we laser ablated PVT in *lim-6 ceh-14* double mutant animals. While PVT ablation in wild-type animals causes PVQL/R axon flip-overs, the same ablation in *lim-6 ceh-14* animals causes no PVQL/R flip-overs above background levels (Fig. 6). Hence, *lim-6* and *ceh-14* must act outside of PVT to suppress the PVT(–) or *zig-4(–)*-induced axon flip-over.

Although the above mentioned experiments were all made with a *lim-6 ceh-14* double null mutant, it could be envisioned that one of the two LIM homeobox genes alone is sufficient to suppress the *zig-4* induced axonal flip-over of PVQL/R. In contrast to *lim-6*, which in the L1 stage is not expressed elsewhere in the VNC than in PVT, *ceh-14* is a good candidate as it is normally expressed in PVQL/R (Fig. 1) (Cassata et al., 2000). It was thus conceivable that *ceh-14* is required for PVQL/R to flip into the opposite cord, e.g. through regulating the expression of homophilic adhesion molecules, which we have previously implicated in playing a critical role in the axon flip-over process (Aurelio et al., 2002). We indeed found that in *ceh-14 zig-4* double null mutant animals, but not in *lim-6 zig-4* double null mutant animals, the *zig-4*-mediated PVQL/R axon flip-over phenotype is suppressed (Fig. 6).

Taken together, our data suggests that *lim-6* and *ceh-14* probably act in PVT to affect *zig* gene expression and that *ceh-14* has an additional role outside of PVT, possibly within PVQL/R, to enable axon flip-over to occur in the absence of a functional axon maintenance mechanism.

zig gene expression also depends on a *lim-6 ceh-14*-independent, intrinsic timing mechanism

Previous studies focused on postembryonic aspects of *lim-6* and *ceh-14* expression (Cassata et al., 2000; Hobert et al., 1999b). We examined the temporal dynamic of *lim-6* and *ceh-14* reporter gene expression in PVT in more detail, and found both genes to be expressed already in mid-embryonic stages (Fig. 2A) and maintained throughout the life of the animal (data not shown). Considering the postembryonic expression of most *zig* genes (*zig-1, zig-2, zig-3, zig-4, zig-8)* in PVT, the embryonic expression of *lim-6* and *ceh-14* indicates that these genes are insufficient to provide the temporal trigger for onset of *zig* gene expression at the L1 stage. Although we can not exclude the possibility that the *lim-6* and *ceh-14* reporter gene constructs do not accurately reflect endogenous gene expression, the reporter gene results provided us with sufficient motivation to search for other, temporal parameters possibly involved in timing *zig* gene expression.

An important temporal trigger for initiation and progression of larval development is the feeding state of the animal. In the absence of an external food source, animals arrest after hatching at the L1 stage. Hence, we considered the possibility that a food-dependent signal, possibly a hormonal signal, is involved in determining the timing of *zig* gene expression. By cultivating transgenic *zig-4::gfp* animals (*otIs20)* on plates that contain no food, we found that 20/20 L1 animals show *zig-4::gfp* in PVT under starvation conditions (see Materials and Methods). This results suggests that *zig-4::gfp* expression is not triggered through an extrinsic, food-dependent signal, but rather depends on an intrinsic timer.

The heterochronic timer *lin-14* regulates *zig-4* gene expression

In *C. elegans* and possibly other animals, timing of developmental events depends on well defined heterochronic genes (Ambros, 2000; Rougvie, 2001; Slack and Ruvkun, 1997). Heterochronic genes act at defined larval stages and their aberrant activity (either through loss or ectopic expression) causes either precocious or delayed execution of developmental events. The *lin-14* gene codes for a ubiquitously expressed nuclear protein, which is so far the only heterochronic factor known to be required within the L1 stage to ensure the correct execution of L1-specific blast cell divisions (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987; Ruvkun and Giusto, 1989) and neuronal rewiring events (Hallam and Jin, 1998). To investigate whether the role of this heterochronic gene also extends to the timing of a postmitotic gene expression program, we crossed a representative *zig* gene reporter, chromosomally integrated *zig-4::gfp*, with three different loss-of-function alleles of *lin-14*. We focused on the *zig-4* gene as it is the only *zig* gene for which a mutant phenotype is available so far and which would therefore allow us to compare its mutant phenotype with potential *lin-14* mutant phenotypes (see below). We found that the L1-specific onset of *zig-4::gfp* expression is abolished in all *lin-14* loss-of-function alleles tested (Fig. 7A, Table 2). Notably, onset of expression is not merely precociously executed or later initiated in the L2 stage, but absent throughout all stages. *lin-14* does not affect the generation or overall fate of PVT as the cell fate markers *unc-47Δ::gfp* and *pin-2::gfp* show normal expression in PVT in *lin-14* mutant animals; moreover, the axon anatomy of PVT is unaffected in *lin-14* mutants (Fig. 7B).

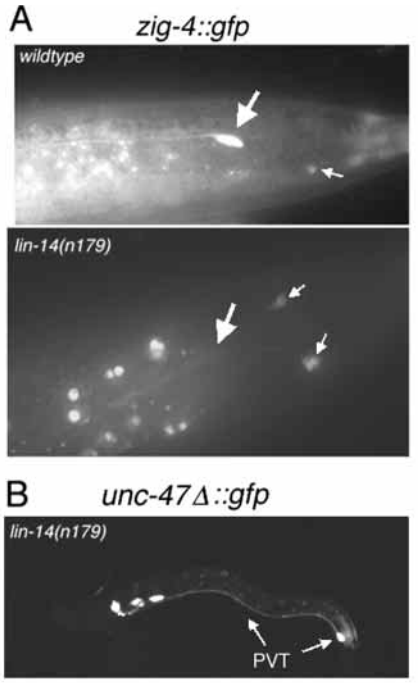


Fig. 7. The heterochronic gene *lin-14* affects *zig-4* gene expression but not PVT cell fate. (A) *zig-4::gfp (otIs20)* expression in PVT (white arrow) is absent in *lin-14* mutants. See Table 2 for quantification of defects. The small arrows indicate a pair of non-identified tail neurons that can be occasionally observed to express *zig-4::gfp* in wild-type animals, but show stronger and more consistent expression in *lin-14* mutant animals. (B) PVT adopts its normal fate in *lin-14(n179)* mutants. 21/21 late L1/L2 stage animals show normal expression of *unc-47Δ::gfp (otIs39)* in PVT and normal PVT axon morphology (white arrows indicate the cell body and axon of PVT; for wild-type control, see Fig. 4A). All strains were grown at 25°C. PVT cell fate is also unaffected as assessed with a *pin-2::gfp (otIs85)* marker (data not shown).

A *lin-14* gain-of-function allele that causes LIN-14 protein to be present at all larval stages (Ambros and Horvitz, 1984; Arasu et al., 1991) has no effect on *zig-4* expression (Table 1). A heterochronic gene that is required for L2 specific developmental events, *lin-28* (Ambros and Horvitz, 1984), also has no effect on *zig-4* expression (Table 2). The *period* homolog *lin-42*, which affects timing of L4 stages (Z. Liu, PhD thesis, Massachusetts Institute of Technology, 1990), but whose expression has been shown to peak in each individual larval stage (Jeon et al., 1999) leaves *zig-4* expression unaffected as well (Table 2). Taken together, while *zig-4* is expressed throughout all larval stages well into adulthood, it is specifically the passing through the L1 stage but not any other larval stage that is required for initiation of *zig* gene expression.

Expression of *zig-4::gfp* is not affected by *lin-14* in cells other than PVT. Consistent with this observation, none of the other *zig-4::gfp* expressing cells (ASK, ASI, BAG, M2) show stage-specific regulation of *zig-4* expression in wild-type animals.

lin-14 affects ventral nerve cord architecture

Loss of *zig-4* expression in *lin-14* mutant animals would be expected to cause axon maintenance defects in the VNC of *lin-14* mutants. We analyzed VNC architecture by crossing three different loss-of-function alleles of *lin-14* with transgenic animals that express a *gfp* reporter either in all VNC neurons or in a selected subset. As expected from the loss of *zig-4* gene expression, we found that *lin-14* animals display axon flip-overs in the VNC (Fig. 8; Table 3). The defects can be observed with a pan-neuronal *gfp* marker as well as with the PVQL/R specific marker and are thus very similar to defects observed in *zig-4* mutant animals. Heterochronic genes that affect later stages of larval development do not affect VNC architecture (data not shown).

The VNC defects in *lin-14* mutant animals display temporally dynamic axonal defects. Freshly hatched *lin-14* animals show no defects above background level, while animals at later larval and adult stages display significant defects (Table 3). These temporal dynamics precisely reflect the temporal dynamics observed in *zig-4* mutants animals and are also consistent with the observation that PVT is specifically required in the L1 stage to affect VNC architecture (Aurelio et al., 2002).

lin-14 is ubiquitously expressed in neuronal and non-neuronal cells (Ruvkun and Giusto, 1989). Its expression is initiated in embryogenesis, peaks right after hatching, then rapidly fades and is absent by the L2 stage (Ruvkun and Giusto, 1989). To affect VNC architecture, *lin-14* could conceivably act in neurons or, alternatively, in the underlying hypodermis. The later possibility seems unlikely, because a null mutation in the *lin-28* gene, which diminishes LIN-14 protein levels in the hypodermis, but not in neurons (Arasu et al., 1991), has no effect on VNC architecture (Table 2). As we

Table 2. Expression of *zig-4::gfp* in various heterochronic mutant backgrounds

Genotype	LIN-14 levels/activity* L1	L2	L3	L4	Loss of *zig-4* expression†
Wild type	+	–	–	–	3.1% (*n*=33)
lin-14(n179)‡	–	–	–	–	75% (*n*=52)
lin-14(n360)§	–	–	–	–	62.5% (*n*=32)
lin-14(n355n679) (25°C)¶	–	–	–	–	76.9% (*n*=52)
lin-14(n355n679) (15°C)¶	+	+	+	+	0% (*n*=44)
*lin-28(n719)***	+††	–	–	–	9.4% (*n*=53)
lin-42(n1089)	n.a.				0% (*n*=70)

*Activity refers to blast cell divisions (Ambros and Horvitz, 1987). Protein levels were determined by antibody staining (Arasu et al., 1991; Ruvkun and Giusto, 1989).

†Complete absence of *gfp* in the PVT cell. The *zig-4::gfp* marker *otIs20* was crossed into the respective mutants alleles. All animals were scored as late larvae. If not explicitly indicated otherwise all animals referred to in this table were raised and scored at 25°C.

‡*n179* is a strong loss-of-function *ts* allele that affects all *lin-14* isoforms (Ambros and Horvitz, 1987; Reinhart and Ruvkun, 2001).

§*n360* is a hypomorph that only affect the *lin-14b* isoform, but not the *lin-14a* isoform (Ambros and Horvitz, 1987; Reinhart and Ruvkun, 2001).

¶The *n355* lesion, a rearrangement in the 3′UTR (Reinhart and Ruvkun, 2001), leads to constitutive expression of LIN-14 protein throughout all larval stages (Arasu et al., 1991) and confers a temperature-independent gain-of-function phenotype (Ambros and Horvitz, 1984). *n679* is a point mutation in the LIN-14 protein that leads to a temperature-sensitive loss of LIN-14 protein function (Ambros and Horvitz, 1987; Reinhart and Ruvkun, 2001).

**A probable molecular null allele (Ambros and Horvitz, 1984).

††*lin-28* affects LIN-14 protein levels in hypodermal and intestinal cells, but not in neuronal cells (Arasu et al., 1991).

n.a.: not applicable.

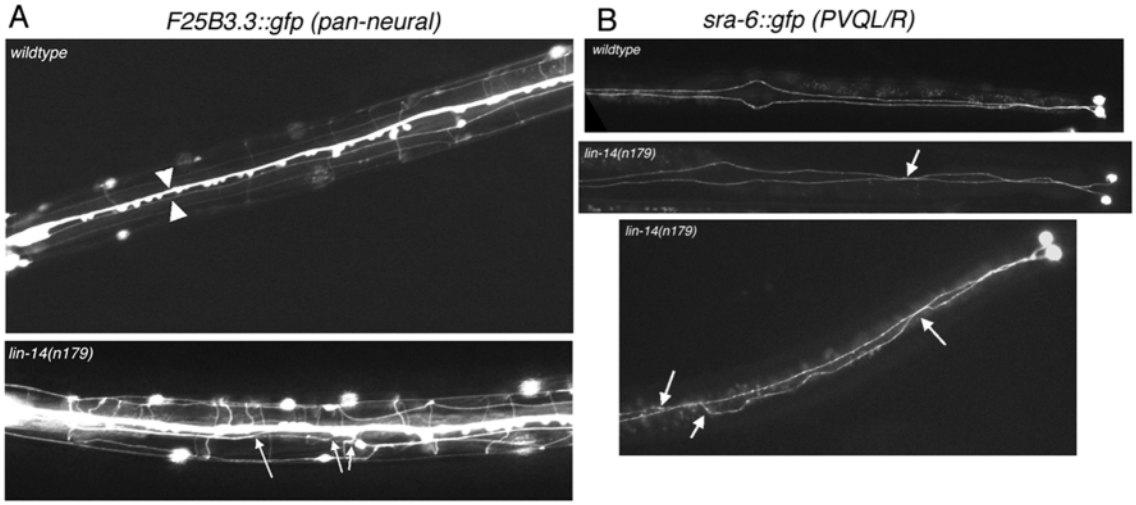


Fig. 8. The heterochronic gene *lin-14* affects ventral nerve cord structure. (A) Axonal defects in *lin-14(n179)* mutants visualized with the pan-neuronal reporter *erIs111*. (B) Two examples of axonal defects in *lin-14(n179)* mutants visualized with the PVQL/R reporter *oyIs14 (F25B3.3::gfp)*. See Table 3 for quantification of defects. All strains were grown at 25°C. Arrows indicate inappropriate axon flip-overs; arrowheads indicate correctly positioned left and right VNC axons.

have so far been unable to express *lin-14* under control of a PVT-specific promoter, we sought to further narrow the focus of action of *lin-14* action by conducting a laser ablation experiment. We reasoned that if *lin-14* acts in a PVT-dependent manner, laser ablation of PVT in a *lin-14* mutant background would not enhance the *lin-14* mutant phenotype. By contrast, if *lin-14* were to act independently of PVT in some other cell, laser ablation of PVT would enhance the *lin-14* mutant phenotype. Laser ablation of PVT causes a 36.4% penetrant PVQL/R phenotype (Fig. 6). We found that 38.1% of *lin-14(n179)* animals in which PVT was ablated showed defects in PVQL/R axon positioning (Table 3). This number is virtually the same as the 40.8% defects observed in unoperated *lin-14(n179)* animals (Table 3). Although this result does not conclusively prove that *lin-14* acts within PVT, it provides strong suggestive evidence that *lin-14* activity is mediated through PVT to affect VNC structure. Together with the effect of *lin-14* on *zig-4* expression, we conclude that the *lin-14* defects are probably due to absent *zig* gene expression in PVT.

Table 3. Quantification of axonal defects in the ventral nerve cord of *lin-14* mutant animals

	VNC axon flip-over defects*	
Genotype	Pan-neuronal†	PVQL/R‡
Wild type adult	6.7% (*n*=30)	10.5% (*n*=85)
lin-14(n179) adult	41.4% (*n*=58)	40.8% (*n*=147)
lin-14(n179) adult; PVT(–)§	n.d.	38.1% (*n*=21)¶
lin-14(n360) adult	25% (*n*=60)	25% (*n*=36)
lin-14(n355n679) adult	44.4% (*n*=27)	27% (*n*=100)
lin-14(n355n679) early L1	4.3% (*n*=47)	2.7% (*n*=36)

*All animals were raised and scored at 25°C.
†Marker: *evIs111 (F25B3.3::gfp)*.
‡Marker: *oyIs14 (sra-6::gfp)*.
§PVT was ablated in freshly hatched *lin-14(n179)* animals.
¶As shown in Fig. 6, PVT laser ablation in a wild type background results in a 36.4% penetrant mutant phenotype.
n.d., not determined.

Ectopic expression of *zig-4* causes VNC defects

We have defined three factors, *lim-6*, *ceh-14* and *lin-14* that are required for the correct spatiotemporal expression of *zig-4*. We investigated the consequences of overriding the spatiotemporal control of *zig-4* mediated through these factors by ectopically expressing *zig-4* under the control of heterologous promoters at earlier time points and at different locations in the VNC. To this end, we made use of the promoter fragments from the *flp-1* and *myo-3* genes (Nelson et al., 1998; Okkema et al., 1993). The *flp-1* promoter fragment is exclusively active in the AVKL and AVKR neurons (see Materials and Methods), which send axons along both sides of the VNC, while the *myo-3* promoter is active in body wall muscle cells which abut the left and right VNC and send muscle arms into the VNC. As assessed through analysis of the expression of corresponding *gfp* fusion constructs, both promoters are already embryonically active (data not shown). We find that adult animals that misexpress *zig-4* under control of either of the two promoters show mispositioned PVQL/R axons in the VNC (Fig. 9). The observed defects could be caused through two distinct mechanisms. In one scenario, precise levels and/or a defined localization of the endogenous ZIG-4 protein is required in the L1 stage to ensure axon maintenance and ectopic ZIG-4 expression obscures this finely tuned distribution. In an alternative scenario, ectopically expressed ZIG-4 may already act in embryonic stages to affect PVQL/R axon outgrowth. In an attempt to distinguish between these two possibilities, we assessed PVQL/R axon anatomy right after hatching, that is, before PVT and *zig-4* are required to maintain axon anatomy in the VNC. We find that animals that ectopically express *zig-4* already display defects at this early stage (Fig. 9). We thus conclude that ectopically expressed *zig-4* affects development of the VNC, probably during the axon outgrowth stages.

Several of the transgenic lines revealed another intriguing aspect of *zig-4* function. A subset of the transgenic lines (*flp-1* promoter: line #2 and #3, *myo-3* promoter: line #2) show no developmental defects, yet showed maintenance defects in the

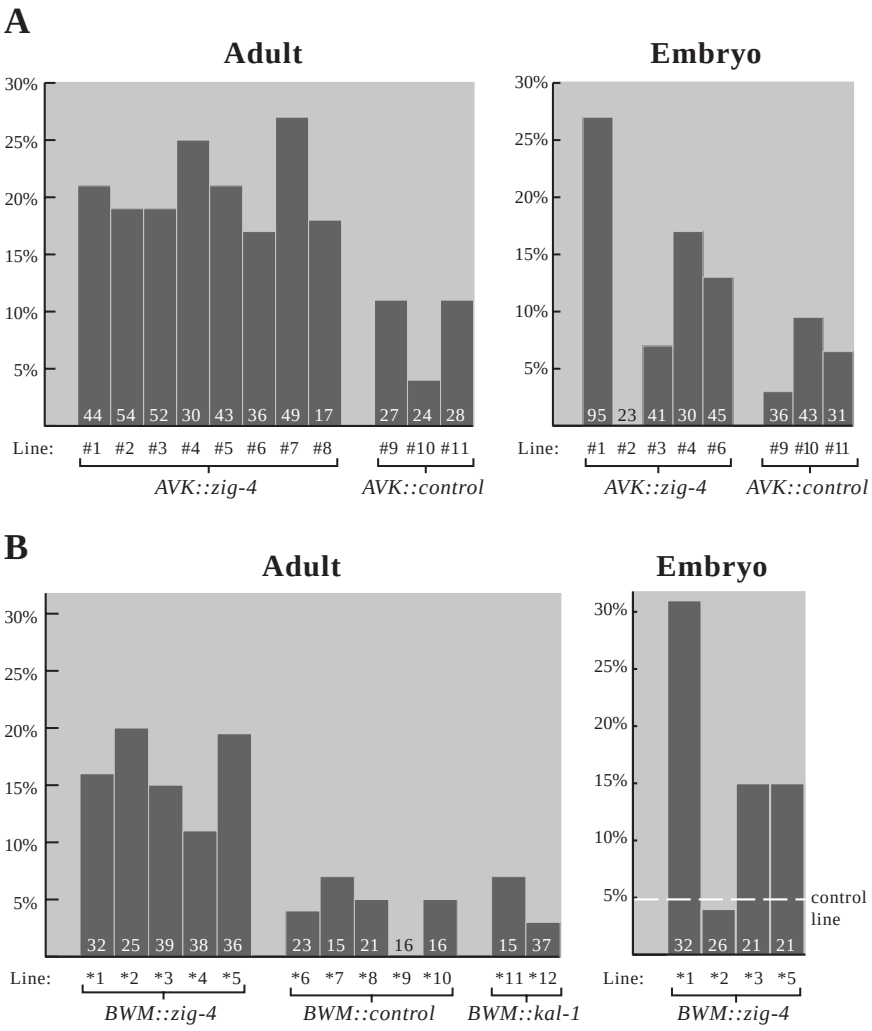


Fig. 9. Misexpression of *zig-4* causes PVQL/R axon patterning defects. PVQL/R defects can be observed in animals that misexpress *zig-4* under the control of the *flp-1* promoter, expressed in the AVKL/R neurons (A), or the *myo-3* promoter, expressed in body wall muscles (BWM; B). Animals were scored as adults to reveal maintenance defects (left panels, 'Adult') and a subset of those lines were scored as freshly hatched L1 animals to allow scoring for embryonic defects (right panel, 'Embryo'). Numbers within bars represent sample size. The broken 'control line' in B refers to the average value of the five BWM::control lines that were scored as adults (left). We could not score control lines as freshly hatched larvae (as in A) because of a strong *myo-3::gfp* background signal. 'Control' refers to the respective promoter driving expression of *gfp*. The *kal-1* gene, which codes for a secreted protein (Bülow et al., 2002) was used as an additional control. Control and transgenic lines all contain *oyIs14* in the background to allow scoring PVQL/R anatomy.

adult (Fig. 9). As levels of expression from independent extrachromosomal arrays can be highly variable, we consider it possible that these lines do not express enough *zig-4* to cause embryonic defects, but enough *zig-4* to interfere with the maintenance role of endogenous *zig-4* at the L1 stage. In other words, ectopic *zig-4* has two separable effects: it can interfere with axon outgrowth but it can also disrupt the normal function of *zig-4* at postembryonic stages to affect VNC axon positioning. Taken together, the precise spatiotemporal control of *zig-4* gene expression is necessary to prevent it from inappropriately interfering with VNC patterning at distinct stages.

DISCUSSION

The PVT neuron displays unusual gene expression profiles. Shortly after its birth, the expression of several genes that define the differentiated state of PVT is initiated. One of these genes, *unc-6/Netrin* is only transiently expressed (Wadsworth et al., 1996), while other genes, such as *gpa-2*, *pin-2* and *srq-1* are expressed throughout the life of the animals (Fig. 1). Yet another set of genes, namely several *zig* genes, is expressed in a strictly postembryonic manner. The functional relevance of these genes has been demonstrated through laser ablation and mutational analysis (Aurelio et al., 2002) (T. B. and O. H., unpublished). We extend the previous finding of the requirement for *zig* genes in VNC patterning, by showing that ectopic expression of *zig-4* causes VNC patterning defects, thus underscoring the necessity of precise temporal and spatial control of *zig* gene expression. We have described separate sets of factors that define the spatial and temporal expression domains of *zig* genes in the PVT neuron.

The *lim-6* and *ceh-14* LIM homeobox genes define the spatial domain of *zig* gene expression. The well defined cooperative action of several LIM homeobox genes in cell specification in the vertebrate spinal cord (Sharma et al., 1998; Tsuchida et al., 1994; Jessell, 2000; Lee and Pfaff, 2001) and in *Drosophila* (Thor et al., 1999), collectively referred to as a LIM-code, and the biochemical evidence for hetero- and homodimerization of individual LIM homeodomain proteins, including the vertebrate LIM-6 ortholog LMX-1 and the vertebrate CEH-14 ortholog LHX3 (Jurata et al., 1998; Thaler et al., 2002), suggest a model in which LIM-6 and CEH-14 bind as heterodimers either directly to *zig* gene promoters or regulate the expression of intermediary factors that directly control *zig* gene expression. In the absence of either LIM homeodomain protein alone, the other LIM homeodomain protein may still be capable of activating its target gene(s) as a homodimer, thus explaining why each single mutant has little observable effect on *zig* gene expression.

Although *lim-6* and *ceh-14* are both jointly required for *zig* gene expression, they are not sufficient to induce *zig* gene expression, as inferred from the observation that the embryonic expression of both genes is insufficient to yield embryonic *zig* gene expression and from the observation that supplying *lim-6* in other *ceh-14*-expressing cells does not cause ectopic *zig* gene expression. The effects of *lim-6* and *ceh-14* are thus strictly dependent on the cellular context and strongly suggests

the existence either of other activators required in PVT to induce *zig* gene expression or of repressors that prevent *lim-6* and *ceh-14* from activating *zig* genes in embryonic stages and/or other cell types. The strict cellular context-dependence of target gene regulation by *lim-6* and *ceh-14* is reminiscent of several other *C. elegans* LIM homeobox genes, such as *mec-3* (Duggan et al., 1998) or *ttx-3* (Altun-Gultekin et al., 2001). Each of these genes is required for expression of all subtype characteristics of specific neuron classes, but incapable of inducing these characteristics in many other cell types when ubiquitously expressed. A similar context-dependent activity of the *ceh-14* ortholog *Lhx3* has also recently been described in vertebrates (Thaler et al., 2002).

Although the expression of all neuronally expressed *zig* genes overlaps uniquely in PVT, individual *zig* genes are expressed in cells other than PVT. In those cells, *zig* gene expression does not appear to be temporally regulated in a similar manner as it is in PVT. Moreover, there is hardly any overlap of expression of *zig* genes, *lim-6* and *ceh-14* in cells other than PVT. This is again consistent with the previously discussed case of the *ttx-3* homeobox gene. All genes that are under control of *ttx-3* in the AIY interneuron class, are also expressed in cells other than AIY and are under control of different transcription factors in those other cell types (Altun-Gultekin et al., 2001). This point again illustrates that *zig* gene regulation by LIM homeobox genes in PVT falls into the general paradigm of context dependent regulation of gene expression.

An interesting feature of *lim-6* and *ceh-14* activity is the specificity of their impact on mostly those genes that show a temporally regulated profile of expression: *lim-6* and *ceh-14* affect all postembryonically expressed *zig* genes, but exert a lesser effect on *zig* genes that show a certain component of embryonic expression and no effect on any other of the embryonically expressed PVT cell fate markers available. In classical developmental terms, *lim-6* and *ceh-14* thus appear to define the competence of the PVT cell to respond to a temporal cue.

One co-factor that either directly or indirectly contributes to *zig* gene expression is the heterochronic factor LIN-14. This protein defines the temporal domain of *zig* gene expression. Recent experiments show that LIN-14 acts as a DNA-binding transcription factor (V. Ambros, personal communication), which prompts the speculation that LIN-14 directly binds to *zig* gene promoters, possibly in conjunction with LIM homeodomain proteins. Initially, *C. elegans* heterochronic genes were defined through their effect on developmental stage-specific cell division events (Ambros and Horvitz, 1984). The first evidence that they may also act in postmitotic processes in the nervous system was provided by the observation that loss of *lin-14* leads to a precocious re-wiring of D-type motor neurons, an event that is normally only observed in late L1-stage animals (Hallam and Jin, 1998). Our observation of a function of *lin-14* in temporally controlled induction of *zig* gene expression is qualitatively different from the D-type motoneuron case. D-type motoneuron rewiring is executed in *lin-14* mutants, though at an inappropriate time; hence, LIN-14 acts as a repressor of the rewiring process. By contrast, *zig* gene expression in *lin-14* mutants is not merely observed at an inappropriate time, but largely absent; hence, LIN-14 acts as an activator in PVT. In addition, while the molecular targets of LIN-14 that cause the D-type rewiring defect are as yet elusive, the VNC axonal patterning that we observe in *lin-14* mutants is consistent with the notion that the *zig* genes are (direct or indirect) effectors of *lin-14* in axonal patterning.

If LIN-14 is the temporal trigger for activation for *zig* gene expression, what is the temporal mechanism that restricts LIN-14 activity to the L1 stage? This question is particularly relevant as LIN-14 antibody staining can already be observed during late embryogenesis (Ruvkun and Giusto, 1989). Two models could be envisioned: LIN-14 protein levels may have to accumulate to a critical threshold level that does not occur until the L1 stage. Observable embryonic expression may still be at subthreshold levels. Alternatively, restriction of *lin-14* activity to the L1 stage may be dictated through the presence of a co-factor that is temporally regulated, such as a nuclear hormone receptor; the secretion of its ligand may be coupled to hatching of the embryo. The feeding state of the animal cannot be a determinant as we found *zig-4* expression to be normal in starved animals.

We have previously suggested that *zig* genes may be required specifically during the L1 stage as a stabilizing factor to ensure that already established axonal tracts can cope with mechanical stress and changes in the local molecular environment occurring in the VNC in the L1 stage (Aurelio et al., 2002). But why is it that *zig* genes are under control of an L1-specific timer and not just simply expressed throughout embryonic, larval and adult stages, like other PVT cell fate markers? We show that inappropriate expression of the *zig* maintenance factors during embryonic development of the VNC interferes with axonal patterning possibly through interfering with the axonal outgrowth machinery. For example, during embryonic axon outgrowth, the SAX-3/Robo protein is required to prevent PVQL/R axons from crossing the midline inappropriately (Aurelio et al., 2002; Zallen et al., 1998). It is conceivable that precocious expression of the Ig-domain containing ZIG-4 protein may interfere with the activity of SAX-3, an Ig domain-containing transmembrane protein, thus causing the midline crossover defects that we observe. The tight postembryonic temporal control of *zig* gene expression in wild-type animals thus may serve to prevent *zig-4* from inappropriately acting to affect VNC development.

The observation that loss of a developmental timer, i.e. the LIN-14 protein, causes severe disruption of axonal organization in the VNC, presents a striking example for the importance of precise temporal orchestration of gene expression events in the nervous system. Does the concept of temporal control of neural gene expression programs by heterochronic genes, as described by Hallam and Jin for motoneuron rewiring and as described here in this paper for *zig* genes, apply to species other than *C. elegans?* The recent identification of temporally regulated microRNAs in vertebrates (Banerjee and Slack, 2002; Pasquinelli et al., 2000), some of which are orthologous to *C. elegans* heterochronic microRNAs (Lagos-Quintana et al., 2002; Pasquinelli et al., 2000) and the sequence similarity between heterochronic genes and circadian clock genes (Jeon et al., 1999) (F. Slack, personal communication), suggests not just a conservation of general concepts of heterochronic regulation of gene expression but also a conservation on the mechanistic levels.

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