The mesoderm determinant Snail collaborates with related zinc-finger proteins to control *Drosophila* neurogenesis

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The Snail protein functions as a transcriptional regulator to establish early mesodermal cell fate. Later, in germ band-extended embryos, Snail is also expressed in most neuroblasts. Here we present evidence that this expression of Snail is required for central nervous system (CNS) development. The neural function of *snail* is masked by two closely linked genes, *escargot* and *worniu*. Both *Escargot* and *Worniu* contain zinc-finger domains that are highly homologous to that of Snail. Although not affecting expression of early neuroblast markers, the deletion of the region containing all three genes correlates with loss of expression of CNS determinants including *fushi tarazu*, *pdm-2* and *even-skipped*. Transgenic expression of each of the three Snail family proteins can rescue efficiently the *fushi tarazu* defects, and partially the *pdm-2* and *even-skipped* CNS patterns. These results demonstrate that the Snail family proteins have essential functions during embryonic CNS development, around the time of ganglion mother cell formation.

Keywords: *Drosophila*/neuroblast/neurogenesis/Snail/zinc finger

Introduction

The *snail* (*sna*) mutant was first identified in a large-scale screening for genes involved in embryonic patterning (Nusslein-Volhard *et al.*, 1984). Embryos that are homozygous for loss-of-function mutations of *sna* exhibit defects in gastrulation, mesoderm formation and germ band retraction (Simpson, 1983; Grau *et al.*, 1984; Nusslein-Volhard *et al.*, 1984). *sna* expression is detected first in the ventral cells of the blastoderm stage embryo (Alberga *et al.*, 1991; Kosman *et al.*, 1991; Leptin, 1991). This ventral expression has been proposed to define the limit and to direct the invagination of the ventral presumptive mesoderm. The Sna protein contains five zinc fingers and functions as a DNA-binding transcriptional regulator (Boulay *et al.*, 1987; Ip *et al.*, 1992a; Kasai *et al.*, 1992; Mauhin *et al.*, 1993). In the blastoderm stage embryo, Sna prevents the mixing of cell fates by directly repressing neuroectodermal genes such as *rhomboid* and *single-minded* in the mesodermal territory (Kosman *et al.*, 1991;

Leptin, 1991; Ip *et al.*, 1992a). Sna may also regulate other target genes that are important for ventral cell invagination (Ip *et al.*, 1994a; Casal and Leptin, 1996; Hemavathy *et al.*, 1997).

The expression of Snail disappears from the mesoderm ~30 min after invagination, then reappears in the neuroectoderm (Alberga *et al.*, 1991; Kosman *et al.*, 1991; Leptin, 1991; Ip *et al.*, 1994b). *Drosophila* embryonic nervous system development starts with groups of cells, proneural clusters, in the neuroectoderm that acquire the competence to become neural precursors (Campos-Ortega, 1993; Goodman and Doe, 1993). This initial determination depends on genes such as *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*), *muscle segment homeobox* (*msh*) and those in the *achaete-scute* complex (Isshiki *et al.*, 1997; Campos-Ortega, 1998; Chu *et al.*, 1998; McDonald *et al.*, 1998; Weiss *et al.*, 1998). Through the process of lateral inhibition (Bhat, 1998a; Rooke and Xu, 1998), a cell within each proneural cluster is selected to become the neuroblast, which then delaminates into the interior of the embryo. Neuroblasts function as stem cells of the nervous system. They divide repeatedly and asymmetrically to produce another neuroblast and a ganglion mother cell (GMC). The GMC divides once more to produce a pair of post-mitotic neurons. The fate of GMCs and postmitotic neurons follows a well-defined pattern depending on the input of many genes (Campos-Ortega, 1993; Goodman and Doe, 1993; Doe and Skeath, 1996).

By the time of neuroblast delamination, Sna is present in most of the neuroblasts that have segregated from the ectoderm. Despite the extensive expression in the neuroblasts, Sna had no known function in the developing nervous system (Alberga *et al.*, 1991; Kosman *et al.*, 1991; Leptin, 1991; Roark *et al.*, 1995).

The neuroblast pattern of *sna* resembles that of a group of genes called pan-neural genes (Bier *et al.*, 1992; Roark *et al.*, 1995). One of these genes, *scratch* (*scrt*), encodes a protein that has sequence similarity to Sna in the zinc-finger domain (Roark *et al.*, 1995). Mutations of *scrt* have no obvious phenotype except that viable escapers have morphological defects in the eyes. Furthermore, no nervous system defect can be seen in *sna scrt* double mutants (Roark *et al.*, 1995). On the other hand, the *scrt dpn* double mutants [*deadpan* (*dpn*) is another pan-neural gene that encodes a basic helix-loop-helix protein (Bier *et al.*, 1992)] exhibit some defects in nervous system development (Roark *et al.*, 1995). Therefore, *scrt* does have a function in the central nervous system (CNS), but the function of *sna*, if any, in the nervous system does not overlap with that of *scrt*.

Escargot (*Esg*) is another protein that contains five zinc fingers with sequences highly homologous to those of Sna (Whiteley *et al.*, 1992). The expression of *esg* is rather

1	MAANYKSC	PLKKRP	IVFV	EERLPQTEAL	ALTKDSQFAQ	DQ	PDLSLR	GRDEETQDYQ	QPEPKRDYVL	Snail
	MDKLKYSRC	PLKKRP	IMVE	ESSPEDHLSH	D-----		EPVDLSVAS	AAVPMEPHWM	AKSEPEPQPV	Worniu
	MHTVEDM	LVEKNYSKC	PLKKRP	VNYQ	FEAPQNSNT	P-----	NEPDLCVVK	MEILEENPSE	ELINVSDCCE	Escargot
69	NLSKTPERNS	SSSSNSCLLS	PPVETQDYLP	TEIHMRLTA	GTTGYTTATP	TTINPFQSAF	VMAAGCNPI	ALWSSYQPHL		Snail
	PTELRRRFD	AMNQTKQLA	RRIWEETREI	ARAFPDVFT	EEIAKSLARL	GYGEFELPPE	EEVMEPEPEP	EQHLPLRYTR		Worniu
	DEGVDVDHTD	DEHIEEDED	VDVDVSDPN	QTQAAAAA	AAVAAAAA	VVVPTPTYK	YPWNNFHMSP	YTAEFYRTIN		Escargot
149	AFFSPASSM	ASPQSVYSYQ	QMTFPSSPGS	DLETGS----						Snail
	DASPTIIKAE	PSDEEQFLR	NYNNLLKSI	AEYEDCMKM	NIKEEIPPI	SPQLFYPPPT	PLA-----			Worniu
	QPGHQILPLR	GDLIAPSSPS	DSLGLSPPP	HHYHLGRASS	VSPPMRSEMI	HRPIGVRQHR	FLPYQPMPGY	PSLGGYTHTH		Escargot
185	-----	-----	-----	EPEDLSVRND	IPLPALFHLF	DEAKSSSSGA	SVSSSSGYSY	TPAMSASSAS		Snail
	-----	-----	-----	EPEDLSVTQR	RVLSENNMLQ	NVARALLSMQ	HMAPQHAPP	IDMEEDQENQ		Worniu
	HHHAPISPAY	SENSYYSMRS	MTPESSC	SSSLPEDLSLKH	NLNLNLNTSQ	PGEQAAAKTG	DMSPETMPNA	SAKDKNQPP		Escargot
235	VAANHAKNYR	FK-----								Snail
	DINQLKIKSS	NDLYY	QQQC	NKCYATYAGL	VKHQQTAAVE	STEYKIIRSQ	PGSGAIVDQ	TEFCTVQASA	LIQAANVASA	Worniu
	RYQ-----									Escargot
247	-----	I	-----	II	-----	III				
	QSMQKPVGVP	RYHCQDCGKS	YSTYSGLSKH	QQFHCP	SAEG	NQVKKVFSCK	NCDKTYVSLG	ALKMHIRTHT	LPCCKPICGK	Worniu
	-----	CPDCQKS	YSTFSGLT	TKH	QQFHCP	AAEG	NQVKKVFSCK	DCDKTYVSLG	ALKMHIRTHT	Escargot
315	AFSRPWLLQG	HIRTHTEGK	FQCPDCPRSF	ADRSNLRAHQ	QTHVDVKKYA	CQVCHKSF	MSLLNKHSSS	NCITITIA*		Snail
	AFSRPWLLQG	HIRTHTEGK	FSCQHCNRAF	ADRSNLRAHM	QTHSDVKKY	CPTCTKSF	MSLLAKHLQS	GCQTEQSGGP		Worniu
	AFSRPWLLQG	HIRTHTEGK	FSCQCHRAF	ADRSNLRAHL	QTHSDIKKY	CTSCSKTF	MSLLTKHSEG	GCPPGSAGSS		Escargot
	SGSGGGFDQ	QLQQHLQVYE	EGHNPHQLYY	AGSVGSSNGE	EEEGGEYQM	PPAIY*				Worniu
	SSSELNYAGY	AEF*								Escargot

Fig. 1. The Wor protein contains a zinc-finger domain homologous to those of Sna and Esg. The amino acid sequence alignment is shown. The identity in the zinc-finger domains (boxed) is 85% (118/139 amino acids) between Esg and Wor and 74% (103/139 amino acids) between Sna and Wor. Unlike Sna and Esg, which have five zinc fingers (indicated as I–V), Wor has a sixth one (boxed) located immediately N-terminal to the conserved region. The three proteins also have two conserved P-DLS-K motifs (shaded boxes) located in the N-termini. This motif has been shown to interact with the dCtBP co-repressor. There is also a conserved domain (shaded), which is highly basic and has unknown function, located in the N-termini. The dashes ‘-’ represent gaps introduced to maximize the alignment. The dots (·) indicate identical residues between Sna and Wor, or between Wor and Esg. The identity between Sna and Esg is not shown specifically.

dynamic during embryonic development. The gene is expressed in the epidermis, neuroectoderm and imaginal precursor cells. Previous findings (Hayashi *et al.*, 1993; Fuse *et al.*, 1994, 1996) demonstrated that the Esg protein probably acts through the cdc2 kinase to maintain the proper cell cycle in larval imaginal disc cells; in *esg* mutant larvae the imaginal disc cells lose their diploidy as they re-enter the S phase without going through mitosis. Moreover, *esg* and *sna* are both expressed in the embryonic wing imaginal disc primordia and the two genes have redundant functions in this tissue; the *vestigial* marker gene expression in the disc is lost in *esg sna* double mutants. Despite a clear demonstration of the redundant requirements of *sna* and *esg* in the wing disc, the double mutant was reported to have no significant embryonic CNS phenotype (Fuse *et al.*, 1996). Thus, the function of *sna* in nervous system development has remained a mystery.

We have identified from the Berkeley *Drosophila*

Genome Project database a novel gene that encodes a protein with a zinc-finger domain highly homologous to those of Sna and Esg. Based on the sequence homology and the functional relationship to *sna* and *esg*, we have named this gene *worniu* (pronounced war-niu, Chinese for snail). RNA *in situ* hybridization reveals extensive expression of *worniu* (*wor*) in the developing nervous system. *wor* is located between *esg* and *sna*, ~100 kb apart, in the 35D region of the second chromosome. Deletions that uncover all three genes exhibit severe reduction of the expression of CNS markers, including *fushi tarazu* (*ftz*), POU domain protein-2 gene (*pdm-2*) and *even-skipped* (*eve*). At later stages the mutant embryos also have highly reduced axonal structure. Transgenic expression of Sna, Wor or Esg can each rescue efficiently the early CNS defects, particularly the *ftz* pattern, and partially the late defects. Since many early neuroblast marker expressions are normal in the deletion mutants, the Sna family proteins probably function around the stage

Fig. 2. *wor* is expressed in the developing nervous system. (A–F) The RNA *in situ* hybridization of *wor* during neurogenesis in wild-type embryos. The orientation of the embryos is anterior to the left. (A) and (G) are dorsal views, (B), (C) and (E) are ventral views, and (D), (F) and (H) are sagittal views. (A) A late stage 8 embryo showing the earliest detectable staining seen in the primordial brain region. (B) An early stage 9 embryo showing *wor* expression in two columns of neuroblasts along either side of the ventral midline, which is indicated by a horizontal line at the cephalic region. (C) A mid-stage 9 embryo showing all three columns of delaminated neuroblasts expressing *wor*. (D) The sagittal view of a stage 9 embryo showing the expression of *wor* in the delaminating neuroblasts. (E) A stage 10 embryo showing that most of the neuroblasts express *wor*. (F) Expression of *wor* is seen in the ventral nerve cord and the developing brain in a germ band-retracted embryo. (G) The dorsal view of an embryo stained with antisense *sna* RNA probe, showing expression in most of the neuroblasts at stage 10. The arrow indicates the centrally located neuroblasts in each hemisegment which have a lower level of RNA. (H) Expression of *esg* at stage 10, showing expression in the neuroectoderm and in some CNS precursors, indicated by the arrowheads.

of GMC formation. Sna homologs have been identified in many different species (Sargent and Bennett, 1990; Nieto *et al.*, 1992, 1994; Smith *et al.*, 1992; Hammerschmidt and Nusslein-Volhard, 1993; Jiang *et al.*, 1998; Sefton *et al.*, 1998). The demonstration of an essential function of Sna family proteins in *Drosophila* nervous system development may also provide a novel avenue to study neurogenesis in vertebrates.

Results

Worniu is a novel member of the *Snail* family proteins

We searched for genes that had sequence homology to Sna in the Berkeley *Drosophila* Genome Project database. The search resulted in the identification of one such gene (in P1 clone DS03023), which we named *worniu* (*wor*). Using the genomic sequence information, we isolated full-length cDNA from early embryonic libraries. Sequence comparison between the genomic and complementary DNA (DDBJ/EMBL/GenBank accession No. AF118857) reveals that the gene contains only one small intron located in the 5' end of the protein coding region. The putative Wor protein sequence contains a C-terminal domain with

six zinc fingers that are very similar to those of Sna and Esg, even though those proteins contain only five fingers (Figure 1). The N-terminal halves of these proteins have rather divergent sequences, except that they all contain a conserved basic motif (shaded) very close to the N-termini. The function of this motif is not known. Moreover, the proteins contain two P-DLS-K motifs (shaded boxed in Figure 1). The P-DLS-K domains in Sna have been shown to interact with the *Drosophila* C-terminal binding protein (dCtBP) and to play important roles in transcriptional repression (Nibu *et al.*, 1998a,b; Poortinga *et al.*, 1998). Since all three Sna family proteins contain highly homologous corepressor-interacting and DNA-binding domains, and can bind to similar DNA sequences (data not shown; Ip *et al.*, 1992a; Kasai *et al.*, 1992; Mauhin *et al.*, 1993; Fuse *et al.*, 1994), it is possible that they bind to promoters of overlapping sets of target genes and repress transcription.

wor expresses extensively in neuroblasts, similar to *sna*

The *wor* cDNA was used to prepare an antisense RNA probe for embryo *in situ* hybridization in order to study the spatial and temporal expression patterns. While there

Fig. 3. Early CNS developmental defects associated with deletion of *esg*, *wor* and *sna*. Embryos from wild type (left panels) and the *Df(2L)osp29* strain (right panels) were hybridized with different Dig-U-labeled antisense RNA probes and the expression pattern developed using the anti-Dig-U antibodies and alkaline phosphatase reaction. The *ac*, *hb*, *dpn* and *scrt* patterns (A–H) indicate that the early phase of neuroblast formation is largely normal. The mutant embryos shown in the right panels all have a gap in the midline region, showing that they have a gastrulation defect that is due to the deletion of the *sna* gene in *Df(2L)osp29*. The staining of *ftz*, *pdm-2* and *eve* in GMC and neurons shows that the deletion embryos are highly defective in the expression of these CNS markers (I–N). The posterior staining of *eve* (M, N), which is not under the regulation of *Sna*, *Wor* or *Esg*, serves as an internal control for the staining.

is no maternal RNA deposition, zygotic expression can be detected first at the onset of neurogenesis. At late stage 8, *wor* transcript can be observed in two small patches of cells in the dorsal head region anterior to the cephalic furrow, representing precursor cells of the developing brain (Figure 2A). At stage 9, *wor* expresses in the first wave of delaminating neuroblasts along either side of the midline (Campos-Ortega, 1993; Goodman and

Doe, 1993), as well as in cells in the head region (Figure 2B–D). Later in the germ band-extended embryo, most of the neuroblasts contain *wor* RNA (Figure 2E). This pattern greatly resembles that of *sna* at this stage of development (Figure 2G), except that *sna* expression in some of the centrally located neuroblasts in each hemisegment is at lower levels (Figure 2G, arrow). In later stages, *wor* continues to express in the brain and part

Fig. 4. The CNS morphology is highly defective in late stage embryos of the *Df(2L)osp29* mutants. Embryos from wild type (left panels) and the *Df(2L)osp29* strain (right panels) were stained with monoclonal antibodies to examine the morphology of the axon scaffold (BP102), the ventral nerve cord (BP106), and the cell bodies and axons of a subset of CNS and PNS (22C10). (A and B) Sagittal views of stage 13 embryos, showing that the homozygous mutant has little BP102 antigen staining remaining. (C and D) Ventral views of stage 14 embryos stained with BP106. The *Df(2L)osp29* mutant embryo has very little expression of the antigen and very poorly developed ventral nerve cord. (E and F) Ventral views of stage 15 embryos stained with 22C10, showing that both CNS and PNS are very poorly differentiated in the mutant.

of the ventral nerve cord (Figure 2F). We did not detect expression of *wor* in any other embryonic tissue.

There is no extensive expression of *esg* in the neuroblasts similar to that shown for *wor* or *sna*. However, it has been demonstrated that *esg* RNA is expressed in the ventral neuroectoderm (Whiteley *et al.*, 1992; Yagi *et al.*, 1998). Careful examination of the expression reveals that *esg* transcript is probably present in the CNS, albeit at variable levels (Figure 2H, arrowheads). Based on the expression analyses, we hypothesized that the newly identified *wor* might serve a redundant function with that of *sna* or *esg* during neural development. This would explain why neither single nor double mutants of *sna* and *esg* showed severe defects in the nervous system.

Deletion of the *sna* family genes correlates with CNS developmental defects

To test the hypothesis that the Sna family proteins function redundantly in the developing nervous system, we analyzed the neural phenotype associated with a deletion that uncovers all three genes. *wor* is located between *esg* and *sna*, ~100 kb apart, in the 35D region of the second chromosome [Berkeley *Drosophila* Genome Project (FlyBase, 1999)]. We took advantage of the close proximity of these genes and examined the phenotypes in the *Df(2L)osp29* mutant (Df: 35B1–35E6). Since high levels of Sna and Wor are present in the neuroblasts, we first analyzed the expression of the proneural gene *achaete*, which marks a subset of early delaminating neuroblasts.

This expression is not affected in the *osp29* deletion mutants (Figure 3A and B). The expression patterns of additional neuroblast markers including *hunchback* (*hb*), *dpm*, *scrt* (Figure 3C–H) and *lethal of scute* (data not shown) also are similar in wild-type and mutant embryos. The slight abnormality of the expression patterns is probably due to the gastrulation defect associated with the *sna* mutation. Therefore, the early waves of neuroblast delamination are normal in the absence of the Snail family proteins.

We then examined the CNS patterns of GMC markers *ftz*, *pdm-2* and *eve*. *ftz* is expressed in a number of midline precursor cells and extensively in GMC (Doe *et al.*, 1988b). In contrast to the neuroblast markers, the *ftz* expression is almost abolished in the mutant embryo (Figure 3I and J). The *pdm-2* gene is also expressed in some neuroblasts and GMC. The early neuroblast expression of *pdm-2* in the mutant is rather normal (data not shown), while the expression in later staged embryos is highly defective (Figure 3K and L). *eve* gene products are present in a number of GMC and postmitotic neurons during normal development. We find that all the *eve* CNS expression is absent in homozygous *osp29* deletion mutant embryos (Figure 3M and N). Taken together, the deletion mutant that uncovers the three *sna* family genes shows severe defects in CNS development.

The nervous system phenotype of the deletion mutant at late stages of embryogenesis was evaluated by staining of different neural markers as illustrated in Figure 4.

Fig. 5. Genetic analysis reveals a linkage of CNS defects with the *esg-wor-sna* loci. The chromosomal division 35A–35F is represented by the bold line on top of the left panel. The approximate locations of *outspreed* (*osp*), *Suppressor of Hairless* [*Su(H)*] and *lace* are shown as reference points. The lines underneath represent the regions that are uncovered in each of the deletions indicated to the right. The break points are based on previous genetic data (see Materials and methods) and RNA *in situ* hybridization. The expression is based on RNA *in situ* hybridization or BP102 antibody staining assays using whole-mount embryos collected from each of the fly lines. ‘+’ indicates RNA expression or BP102 staining not worse than that shown in *sna* mutants (Figure 6A and B). ‘-’ represents no detectable RNA expression (see Figure 3N), or almost total loss of BP102 staining (as shown in Figure 4B). ‘-/+’ assignment for BP102 has a disrupted pattern similar to those shown in Figure 6D or H; for *eve* it is similar to that in Figure 6C. ‘+/-’ assignment for *eve* is similar to that in Figure 6G. The *R11* deletion has a hatched line at the distal end, representing some uncertainty of the break point. The *do-1/TE35D-22* transheterozygote also has the deletion represented by a hatched line, because it is a prediction from the break points of the two parental deficiencies. ND, not determined. ‘x’ at the bottom of the left panel represents point mutation.

Staining with BP102, a monoclonal antibody recognizing all CNS axon scaffold and commissures (Seeger *et al.*, 1993), reveals that *Df(2L)osp29* homozygous embryos have a severe disruption in CNS formation (Figure 4A and B). Similar results can be observed with the BP106 antibody (Figure 4C and D), which recognizes the CNS transmembrane glycoprotein neurotactin (Hortsch *et al.*, 1990), and the 22C10 antibody (Figure 4E and F), which stains a subset of cell bodies and axons in the CNS and peripheral nervous system (PNS) (Fujita *et al.*, 1982). It is worth noting that *sna*⁻ embryos exhibit both gastrulation and germ band retraction phenotypes. Such morphological abnormality renders some difficulty in the analysis of the CNS phenotype in late stage embryos. However, the CNS shows only minor defects in embryos that are mutated for *sna* alone (see Figure 6B), demonstrating that the CNS phenotype in *Df(2L)osp29* homozygotes is not solely due to the gastrulation defect. Rather, it is because of the deletion of genes other than or in addition to *sna*.

Sna-related proteins have collaborative functions in CNS development

Since the *osp29* deletion uncovered a large region, we tested whether the observed CNS defect was a result of deleting the zinc-finger genes or other unrelated genes. We first performed RNA *in situ* hybridization on embryos obtained from deficiency strains with break points in the division 35 region (Ashburner *et al.*, 1990) to assay for the expression of *esg*, *wor* and *sna* (Figure 5). We then studied the correlation of CNS phenotype with various deletions by examining the expression of both *eve* RNA

and BP102 antigen. Deletions that remove distally located genes including *esg*, or those that remove proximally located genes including *sna* do not exhibit CNS phenotype stronger than those of *sna* mutants. On the other hand, four deletions [*Df(2L)osp29*, *Df(2L)do-1*, *Df(2L)A48* and *Df(2L)TE35BC-24*] that all uncover the three genes exhibit the severe neural defects already described for *Df(2L)osp29*. These results show a clear correlation of the CNS phenotype with the deletion of the region between *esg* and *sna*.

The *Df(2L)noc^{4L}Sco^{rv9R}* extends from the distal direction (Ashburner *et al.*, 1990) and deletes both *esg* and *wor* but not *sna* (Figure 5). The expression of CNS markers is normal in this mutant (Figure 6E and F), demonstrating that the function of *wor* by itself is not essential for CNS development. The deletion *Df(2L)TE35D-22* extends from the proximal direction and uncovers both *sna* and *wor* but not *esg*. These mutant embryos, however, have much reduced *eve* expression and CNS axonal structure (Figure 6G and H). This phenotype was less severe than that seen in *esg*⁻ *wor*⁻ *sna*⁻ embryos (compare with Figures 3N and 4B). Another line of evidence that supports the involvement of Sna family proteins is the *R11* deletion. This mutant exhibits slightly reduced RNA expression of both *sna* and *wor* at late stages, while *esg* expression is undisturbed. This reduced expression of *wor* and *sna* correlates with a slightly abnormal *eve* CNS expression (Figure 5), again supporting the idea that reducing both *wor* and *sna* activities can lead to abnormal development of the nervous system.

We also compared in detail the neural phenotypes of

Fig. 6. Varying degrees of CNS defect in different combinations of *sna* family mutants. BP102 and *eve* staining of different mutant embryos are shown. (A and B) The *sna*⁻ embryos show nearly normal *eve* expression. The BP102 staining reveals some defects in the CNS axons. However, this may be a result of morphogenetic defects associated with *sna* mutant embryos. (C and D) The *esg*⁻ *sna*⁻ double mutant embryos appear to have *eve* expression similar to the *sna*⁻ embryos, but the BP102 staining reveals a more severe CNS phenotype at late stages. (E and F) The embryos obtained from the *noc*^{4L} *Sco*^{rv9R} deletion, which uncovers *esg* and *wor* but not *sna*, have *eve* expression and BP102 staining indistinguishable from that of wild type. (G and H) The *TE35D-22* mutants, which are deleted for *wor* and *sna* but not *esg*, exhibit a very significant loss of *eve*-expressing neurons. The developing CNS revealed by BP102 staining also shows severe defects.

esg and *sna* point mutants. The *esg* mutant embryos have CNS morphology indistinguishable from that of wild type (data not shown) (Fuse *et al.*, 1996). The *sna* mutant has almost normal *eve* expression and slightly defective ventral nerve cord formation (Figure 6A and B). This mild phenotype may be an indirect result of the gastrulation defects associated with *sna* mutants. In the double mutant, *eve* expression (Figure 6C) appears to be similar to that of *sna*⁻ (Fuse *et al.*, 1996), but the BP102 staining reveals a more severe morphological disruption of the nervous system (Figure 6D). Point mutation of *wor* has not been identified, thus the phenotypes of other double mutants await future investigation. Taken together, the results are consistent with the hypothesis that the CNS functions of individual *sna* family genes can be compensated by the other two. Deleting two of the three genes, particularly *wor* and *sna*, at the same time can lead to a rather severe neural phenotype.

Transgenic expression of *Sna*, *Wor* or *Esg* rescues early CNS defects

To confirm the function of these three proteins in neural development, we constructed transgenic rescue plasmids

in which individual genes (*esg*, *wor* or *sna*) were placed under the control of a 2.8 kb *sna* promoter, which contains an enhancer element that directs expression in the neuroblasts (Ip *et al.*, 1992b, 1994b). The transgenic flies obtained were then crossed with the *osp29* strain and analyzed for CNS development. In the presence of any one of the three constructs the *ftz* expression is restored significantly (Figure 7D–F), in contrast to the almost complete loss of expression in embryos from the parental mutant line (Figure 7B and C). Analysis of the rescued pattern under higher magnification reveals that part of the *ftz* staining is clearly absent. However, more detailed analysis is required to pinpoint the exact cell lineages that are missing. Nevertheless, the results demonstrate that each of the three *sna* family genes can perform essential functions in the CNS in the absence of the other two.

The rescue of the expression of *pdm-2* and *eve*, both of which were defective in the *osp29* mutant, by the transgenes was also examined. While all three *sna* family genes clearly can rescue the expression of *pdm-2* (Figure 8C–E), the effect is not as extensive compared with that of *ftz*. For *eve* RNA, the transgenes rescue the

Fig. 7. Transgenic rescue of *ftz* CNS expression by the *sna*-related genes. The transgenic lines, *P[sna]*, *P[wor]* and *P[esg]*, expressing one of the three genes under the control of the 2.8 kb *sna* promoter were constructed as described in Materials and methods. These flies were crossed together with the *osp29* deficiency. Embryos collected from parents that carry homozygous transgene on the third chromosome and heterozygous deletion on the second chromosome were used for staining. All panels show ventral views of the embryos, except (C) shows the side view. The genotypes of the embryos are indicated in the bottom right corners of the panels. Each transgene shows significant rescue of *ftz* expression (D, E, F) when compared with the almost undetectable expression in the *osp29* embryo (B, C). However, the rescue is not complete, as comparison under higher magnifications (G, H) reveals that some staining of *ftz* is missing in the clusters of GMC in the rescued lines. The presence of two rescue transgenes (I and J, low and high magnification, respectively) exhibits slightly better rescue. The *P[sna] P[wor]* and *P[sna] P[esg]* combinations are not shown because most rescued mutant embryos show staining that is almost indistinguishable from that of wild type.

expression in a significant number of cells when compared with the total loss of expression in the parental *osp29* mutant (Figure 8G–J). The rescue of *eve*, again, is not as extensive as that of *ftz*. Later stage CNS morphology in the rescued embryos was also monitored by BP102 staining. The embryos carrying the transgenes have slightly better overall CNS axonal morphology (Figure 8M and N), but they are still highly abnormal when compared with the wild type.

We also performed pairwise recombination of the transgenes and tested whether they could achieve better rescue. By staining embryos obtained from stable lines that are homozygous for two transgenes, we found that the constructs direct the expression of *ftz* slightly better (compare Figure 7I with E and F). Meanwhile, the *eve*

and BP102 antigen expression in the presence of two transgenes reveals only minor improvement of the axonal morphology (Figure 8K, L and N). These results suggest that the three proteins may have some collaborative function. It is also possible that the promoter used has limitation in driving the rescue transgenes or that there are additional genes involved for the severe CNS phenotype.

Discussion

We have identified in *Drosophila* a gene encoding a zinc-finger protein highly homologous to Sna. This gene is also closely linked to *sna* and *esg* on the left arm of the second chromosome. We have named this gene *worniu*, based on the functional and structural relationship to both

Fig. 8. Transgenic rescue of the expression of CNS markers. (A–E) Expression patterns of *pdm-2*, (F–L) expression of *eve*, and (M, N) expression of BP102 antigen. All panels are ventral or ventral–lateral views of the embryos. The patch of staining in the middle of the *pdm-2*-hybridized embryos [most clearly in (B)] is the out of focus, midgut staining. The *P[sna]*-containing mutant embryo (C) has clearly better staining compared with the parental mutant embryo (B). *P[wor]* and *P[esg]* (D, E) can also rescue, but slightly weaker than that of *P[sna]*. Similar results were obtained for the rescue of *eve* expression by the transgenes (H–J). The rescue is weak but significant. The presence of two rescue transgenes together does not improve the *eve* expression significantly (K, L), when compared with that of single transgenes. The axonal structure revealed by BP102 staining is also weakly rescued by the transgenes (M, compare with the parental mutant in Figure 4B). Rescue by the two transgenes is only marginally better (N).

sna and *esg*. Experimental results demonstrate that *wor*, *sna* and *esg* participate in neurogenesis. The simultaneous loss of function of all three genes correlates with severe defects in neural development. By transgenic rescue assays, we show that this family of proteins act early to determine neural cell fate, around the time of GMC formation.

The identification of the novel gene *wor* was a critical step that led to the elucidation of the function of the *sna* family in CNS development. Not only does *wor* code for a protein that contains a homologous zinc-finger domain,

it also expresses in the developing CNS. The expression pattern greatly resembles that of *sna*, suggesting a possible functional redundancy. A series of experiments designed to test this hypothesis demonstrated that these genes indeed are important during neurogenesis. First, deletions that simultaneously uncover all three genes exhibit severe CNS developmental defects, and these CNS defects map to within a 200 kb region containing these three genes. Secondly, while *sna* or *esg* mutation does not show a severe neural phenotype, deleting two out of the three genes could result in varying degrees of CNS develop-

Fig. 9. A model for the function of Sna family proteins in *Drosophila* neurogenesis. The Sna family proteins are not required for early neuroblast formation, since in the deletion mutant embryos all neuroblast markers tested have staining similar to that in the wild type. However, GMC and neuronal markers including *ftz*, *pdm-2* and *eve* are severely affected. The rescue experiments demonstrate that the *ftz* expression is rescued extensively. Therefore, the temporal requirement of Snail family proteins is likely to be around the time of GMC formation. The arrows indicate genetic hierarchy, not necessarily direct regulation. Since the genetic rescue is weak at late stages, we do not know whether the Sna family proteins are solely responsible for the highly disrupted CNS morphology observed in the *Df(2L)osp29* mutant. Moreover, further investigation is required to determine whether the Sna family can regulate other neural determinants.

mental defects. Thirdly, the expression in transgenic flies of the three genes individually is sufficient to rescue at least part of the CNS defects.

While the CNS defects exhibited in the deletion mutants can be restored by the transgenes, the rescue, particularly the late phenotypes such as the axonal structures, is not complete. These results are consistent with the idea that Sna family proteins function at an early stage of neural development and that late patterns are farther downstream, and therefore less efficiently rescued. Previous genetic analyses demonstrated that at least in the RP2 neuronal lineage *ftz* acts upstream of *pdm-2* and *eve* (Doe *et al.*, 1988a,b; Bhat and Schedl, 1994; Bhat *et al.*, 1995; Chu-LaGriff *et al.*, 1995; Yeo *et al.*, 1995). Therefore, *ftz* is probably a proximal genetic target in the hierarchy downstream of the Sna family proteins, and it is possible that *ftz* functions upstream of other genes including *pdm-2* and *eve* to control the development of multiple neuronal lineages. Such a hypothesis does not exclude the possibility that the Sna family also regulates other neural determinants in addition to *ftz* at the stage of GMC formation (Figure 9).

The lack of complete rescue by the transgenes may also be due to the limitation of the *sna* promoter used. The 2.8 kb promoter, although it has been shown to direct the expression of *lacZ* (Ip *et al.*, 1992b, 1994b) and the transgenes (data not shown) in many neuroblasts, may not have driven the transgenic protein products at sufficiently high levels or at later stages required for late gene expression. A third possibility is that there may be additional genes responsible for the neural phenotypes observed in the deletion mutants. If these additional genes exist, they must be located within the region uncovered by the *do-1/TE35D-22* transheterozygote (Figure 5) and probably between *esg* and *sna*.

Although individual expression of the three genes can

rescue the CNS phenotype, we suggest collaborative, but not strictly redundant, functions of the three genes. This is because if they are redundant, the presence of any one of them should be equally efficient to direct CNS development. However, the expressivity of the different mutant combinations is rather variable (Figure 5). The rescue experiments also reveal that the transgenic *sna* functions a little better than the transgenic *wor* and *esg*. Furthermore, the expression of endogenous *esg* in the CNS is not as extensive as the other two genes. Therefore, *sna* is playing a more significant role, while *wor* is somewhat less efficient and *esg* the least important in CNS development. Nonetheless, the genetic rescue experiments demonstrate that all three can control neural differentiation. A similar collaborative role of several proteins in CNS development has precedence in the *achaete-scute* complex function (Jimenez and Campos-Ortega, 1990; Parras *et al.*, 1996; Skeath and Doe, 1996), in *pdm-1*- and *pdm-2*-dependent regulation (Bhat and Schedl, 1994; Bhat *et al.*, 1995; Yeo *et al.*, 1995), and in the *frizzled* receptors' mediation of *wg* signaling (Bhat, 1998b).

We have attempted to test the role of the pan-neural gene *scrt* by crossing the *scrt* mutant to the *osp29* deletion background. Unfortunately, no stable fly line could be established. Based on the sequence homology, however, *scrt* is less closely related to the three *sna* family genes. Furthermore, in the deletion mutants where *scrt* is intact, the expression patterns of *ftz*, *pdm-2* and *eve* are mostly, if not totally, abolished. This shows that *scrt* alone cannot direct the expression of these markers in the absence of the other three genes. If *Scrt* collaborates with the other zinc-finger proteins in neurogenesis, the effect should be subtle.

Increasing numbers of *sna*-related genes have been identified in diverse species (Sargent and Bennett, 1990; Nieto *et al.*, 1992, 1994; Smith *et al.*, 1992; Hammerschmidt and Nusslein-Volhard, 1993; Jiang *et al.*, 1998; Sefton *et al.*, 1998). These proteins have been assigned to the Sna family based mostly on the similarity of the sequences in the zinc-finger domains. The expression patterns and some functional studies of the vertebrate proteins suggest a role in regulating cell movement (Hammerschmidt and Nusslein-Volhard, 1993; Nieto *et al.*, 1994; Savagner *et al.*, 1997; Sefton *et al.*, 1998). However, gene knock-out experiments demonstrated that mutating a mouse *Slug* homolog did not lead to a detectable cell movement defect (Jiang *et al.*, 1998). Such a result suggests a possible redundant function provided by other genes, similar to our report here. If the vertebrate homologs do have a function in controlling cell movement, it would be reminiscent of the control of cell movement during gastrulation by the *Drosophila* Sna. The expression of vertebrate Sna proteins in developing CNS, however, has not been demonstrated. A careful examination of the expression and function in the CNS is needed to reveal the importance. The analysis of the functions of Sna, Esg and Wor in *Drosophila* CNS development will certainly provide a foundation for similar analysis in other species.

Materials and methods

Drosophila stocks and genetics

Fly stocks were maintained at 24°C using standard cornmeal–yeast–agar medium. The *y w⁶⁷* stock was used for all P element transformation.

Microinjection was performed using the $\Delta 2$ -3 transposase helper plasmid, and the genetic crosses that established the rescue lines were as described previously (Ip et al., 1994a; Hemavathy et al., 1997). The recombination of the transgenic chromosomes was carried out by crossing the transgenic $y w^{67}$ stocks pairwise. Female flies that were transheterozygous for the two transgenes were collected and mated with third-chromosome balancer male flies. Male offspring that had a dark red eye color were collected and mated with balancer stocks. These potential recombinants were confirmed by *in situ* hybridization analysis of the expression patterns ectopically in the mesoderm for *wor* and *esg*, or by genetic rescue for *sna*. The genetic analysis of *wor*, *sna* and *esg* utilized the lines shown in Table I. In addition, the *sna*^{llg05}, *esg*^{35Ce} single mutants and *sna*^{HG31} *esg*^{G66B} double mutant were used in the experiments. The details of the mutations are available on FlyBase (FlyBase, 1999), and in Fuse et al. (1996), Lindsley and Zimm (1992) and Ashburner et al. (1990).

Plasmids and cloning of *worniu* cDNA

A DNA fragment of *wor* was obtained by PCR amplification of an embryonic cDNA library (Brown and Kafatos, 1988) using primers based on the genomic sequence that contains the putative coding region of *wor* (P1 clone DS03023). The fragment was sequenced to confirm its identity and used as a probe for cloning cDNA from the library. The cDNA was sequenced completely and one intron was found in the 5' coding region when compared with the genomic sequence. The cDNA sequence has been deposited in DDBJ/EMBL/GenBank under the accession No. AF118857.

Plasmids for transgenic rescue were constructed by sub-cloning the coding regions of *wor*, *sna*, or *esg* into the pCaSpeR vector. This vector had been modified such that a 2.8 kb *sna* promoter region was inserted into the *Bam*HI and *Kpn*I sites of the pCaSpeR-AUG- β -gal vector (Thummel and Pirrotta, 1992) and this is called pCaSpeR-Snailp (Ip et al., 1992b). The cDNA fragments were then cloned into the pCaSpeR-Snailp to replace the *lacZ* gene coding sequence. The *sna* cDNA fragment was isolated from a pAct5C-*sna* cDNA by digesting with *Kpn*I and *Bgl*II (blunted), and cloned into the *Kpn*I and *Xba*I (blunted) sites of the transformation vector. For *esg*, plasmid pPAesg (kindly provided by S.Hayashi) was cut with *Xba*I, filled in to blunt and then cut with *Eco*RV. The 1.6 kb fragment carrying the *esg* gene was then cloned into the *Asp*718- and *Xba*I-digested, and T4 polymerase-blunted pCaSpeR-Snailp vector. The *wor* gene fragment was obtained from the pG3Act5C-*worniu*, which was cut with *Bgl*II, filled in to blunt and then digested with *Kpn*I. The 1.6 kb fragment was then cloned into the *Kpn*I and *Xba*I (blunted) sites of pCaSpeR-Snailp.

Embryo *in situ* hybridization

RNA *in situ* hybridization was performed as described previously (Hemavathy et al., 1997). Briefly, *in vitro* transcription was performed in the presence of digoxigenin-UTP (Dig-U) to synthesize Dig-U-labeled RNA from a linearized plasmid template. Fixed embryos were hybridized with Dig-U-labeled RNA overnight, and then incubated with alkaline

phosphatase-conjugated anti-digoxigenin antibodies. The signal was developed using the alkaline phosphatase reaction.

Immunohistochemical staining of embryos

Localization of expressed proteins or CNS antigens was monitored by staining embryos with monoclonal antibodies that are specific for their corresponding epitopes. Embryos collected on apple/grape juice plates were dechorionated in fresh Chlorox for 3 min and washed thoroughly with water and Triton X-100/NaCl (0.1%/0.1 mM). Embryos were then transferred to 3.6 ml of buffer B (10 mM KPO₄ pH 6.8, 15 mM NaCl, 45 mM KCl, 2 mM MgCl₂). After addition of 0.4 ml formaldehyde (37%) and 4 ml of heptane, the embryos were vigorously shaken for 12 min. The bottom layer of buffer and formaldehyde was then removed. Eight milliliters of methanol were added and the embryos were once again shaken vigorously for 1 min. Then the top heptane layer was removed and the devitelinized embryos that settled to the bottom were washed with methanol four times and stored at 4°C until staining.

Antibody staining was performed by first blocking the embryos in 5% milk in 1× phosphate-buffered saline (PBS) for ~3 h. Primary antibody was diluted in 1× PNMT (1× PBS, 500 mM NaCl, 0.5% milk, 0.1% Tween) and embryos incubated in 1:50 primary antibody overnight at 4°C. The next morning, the embryos were washed with 1× PNMT by nutating at room temperature four times each for ~30 min. Biotinylated anti-mouse secondary antibody was diluted 1:400 in PNMT and added to the embryos for overnight incubation at 4°C. Embryos were then washed six times in 1× PNMT each for ~30 min. Biotin-streptavidin-horseradish peroxidase complex (Vectastain ABC kit, Elite; Vector Laboratories, Inc., CA) was first formed by adding the individual solutions together ~30 min prior to mixing with the embryos. The complex containing solution was incubated with the embryos at room temperature for 1 h. Embryos were then washed eight times in PBT (1× PBS, 0.1% Tween) each for 5 min. The signal was developed by incubation with DAB in the presence of H₂O₂. The reaction was terminated by washing the embryos with PBT and then with ethanol. The embryos were rinsed with ethanol and xylene, and then mounted in Permount (Fisher) for microscopy.

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Table I. Lines used for genetic analysis of *wor*, *sna* and *esg*

Deletion	Break points	Genetic information
<i>Df(2L)b87e25</i>	34C1; 35C1-2	<i>kuz⁻-vas⁻</i>
<i>Df(2L)osp18</i>	35B1-2; 35C4-5	<i>osp⁻-esg⁻</i>
<i>Df(2L)noc^{4L}Sco^{9R}</i>	35B1-2; 35D1	<i>noc⁻-l(2)35Da⁻</i>
<i>Df(2L)do-1</i>	35B1; 35D2	<i>ms(2)34Fe⁻-sna⁻</i>
<i>Df(2L)osp29</i>	35B1-3; 35E6	<i>osp⁻-beat⁻</i>
<i>Df(2L)TE35BC-24</i>	35B4-6; 35E1-2	<i>l(2)35Bg⁻-Ca-αI⁻</i>
<i>Df(2L)A48</i>	35B2-3; 35D5-7	<i>osp⁻-lac⁻</i>
<i>Df(2L)TE35D-22</i>	35D3; 35D7	<i>l(2)35Da⁻-ms(2)35Eb⁻</i>
<i>Df(2L)RII</i>	35D3; 36A2	<i>sna⁻-dac⁻</i>
<i>Df(2L)Sco^{9R}18</i>	35B1-2; 35B2-4;	<i>noc⁻-Adh⁻; sna⁻-beat⁻</i>
	35D1-2; 35E2	
<i>Df(2L)Sco^{9R}14</i>	35B1-2; 35B2-4;	<i>noc⁻-Adh⁻; sna⁻-</i>
	35D1-2; 35E2	<i>l(2)35Dh⁻</i>
<i>Df(2L)TE35D-14</i>	35D1-2; 35E1-2	<i>sna⁻-ms(2)35Eb⁻</i>
<i>Df(2L)Sco^{9R}16</i>	35D1-2; 35E2	<i>sna⁻-Ca-αI⁻</i>
<i>Df(2L)TE35D-1</i>	not visible	<i>sna⁻-CycE⁻</i>
<i>Df(2L)TE35D-11</i>	35D3-4	<i>sna⁻-l(2)35Dh⁻</i>
<i>Df(2L)r10</i>	35E1-2; 36A6-7	<i>sna⁻-dac⁻</i>
<i>Df(2L)RM5</i>	35E1-2; 36A4-5	<i>BicC⁻-dac⁻</i>

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