RNA interference screen to identify genes required for *Drosophila* embryonic nervous system development

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RNA interference (RNAi) has been shown to be a powerful method to study the function of genes *in vivo* by silencing endogenous mRNA with double-stranded (ds) RNA. Previously, we performed *in vivo* RNAi screening and identified 43 *Drosophila* genes, including 18 novel genes required for the development of the embryonic nervous system. In the present study, 22 additional genes affecting embryonic nervous system development were found. Novel RNAi-induced phenotypes affecting nervous system development were found for 16 of the 22 genes. Seven of the genes have unknown functions. Other genes found encode transcription factors, a chromatin-remodeling protein, membrane receptors, signaling molecules, and proteins involved in cell adhesion, RNA binding, and ion transport. Human orthologs were identified for proteins encoded by 16 of the genes. The total number of dsRNAs that we have tested for an RNAi-induced phenotype affecting the embryonic nervous system, including our previous study, is 7,312, which corresponds to ≈50% of the genes in the *Drosophila* genome.

high-throughput screen | neural development | neural mutants

Several genetic screenings using lethal or semilethal mutations generated by P-element insertions or chemical mutagens have led to the identification of genes involved in the development of the nervous system in the *Drosophila* embryo. Although such strategies generally have been successful, some genes may have escaped detection because cold spots in DNA that are somewhat refractory to P-element insertion and loci that are less susceptible to ethyl methane sulfonate-induced mutations are present in the genome. In addition, it is difficult to analyze the function of a gene during early embryonic development because maternally stored transcripts can compensate for the absence of zygotic expression. Therefore, RNA interference (RNAi) can be a useful tool to overcome hurdles of conventional genetic screens.

In RNAi, a 21-nucleotide-long dsRNA molecule, small interfering RNA (siRNA), that can be delivered either directly to the cell or produced from the processing of a longer precursor by an endonuclease, Dicer, binds to a multiprotein complex (RISC). The activation of RISC leads to complementary base pairing between the antisense strand of the siRNA and the target mRNA species; this process ultimately triggers the degradation of the target mRNA molecule (1, 2). RNAi has been used successfully to screen the genomes of various species to identify genes involved in biological processes (3). Previously, we used an RNAi-based *in vivo* screen to identify genes required for the development of the embryonic nervous system in *Drosophila* (4). From a library of double-stranded (ds) RNAs corresponding to approximately one-fourth of the fruit fly genome, we identified 43 genes including 18 novel genes whose roles in embryonic nervous system development had not been described. In the present work, we report an additional 22 genes involved in embryonic nervous system development.

Results and Discussion

RNAi Screen. Genes involved in the embryonic development of the *Drosophila* nervous system were identified by RNAi, which extends the results of our previous study (4). Double-stranded RNA was injected into preblastoderm embryos, and the embryos were incubated to allow development to proceed to about stage 15–16. Nervous system development was examined by staining with mAb 22C10, a monoclonal antibody that recognizes the microtubule-associated protein futsch (5). mAb 22C10 stains cell bodies and axons of all neurons in the peripheral nervous system (PNS) and a subset of neurons in the ventral nerve cord (VNC) of the central nervous system (CNS) (6). Therefore, RNAi-induced mutant phenotypes, such as disruption of the nervous system, collapse of axon tracts, fasciculation defects, or loss or gain of neurons, often can be distinguished from wild-type embryos. The staining patterns of mAb 22C10 in the VNC and the PNS of wild-type embryos are shown in Fig. 1 *A* and *B*, respectively.

Throughout the screening procedure, we adopted highly conservative criteria of evaluation to eliminate false-positives. First, to identify candidate genes affecting nervous system development, each dsRNA was injected into at least 50–100 embryos, and only when RNAi phenotypes were found in 50% or more of the injected embryos and when results from multiple experiments by different investigators were consistent were the results considered positive. Next, to confirm the effect of RNAi on the nervous system, dsRNAs were synthesized from different regions of the candidate genes and cross-checked by injection into embryos in more than three experiments by independent investigators. From 3,998 dsRNAs, we identified 22 genes affecting the embryonic nervous system (Table 1). The effects of 16 of the genes on the embryonic nervous system have not been described previously (sections A and B in Table 1). Loss-of-function mutant phenotypes of the remaining genes were reported previously to affect the embryonic nervous system (section C in Table 1).

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Abbreviations: NB, neuroblast; PNS, peripheral nervous system; VNC, ventral nerve cord.

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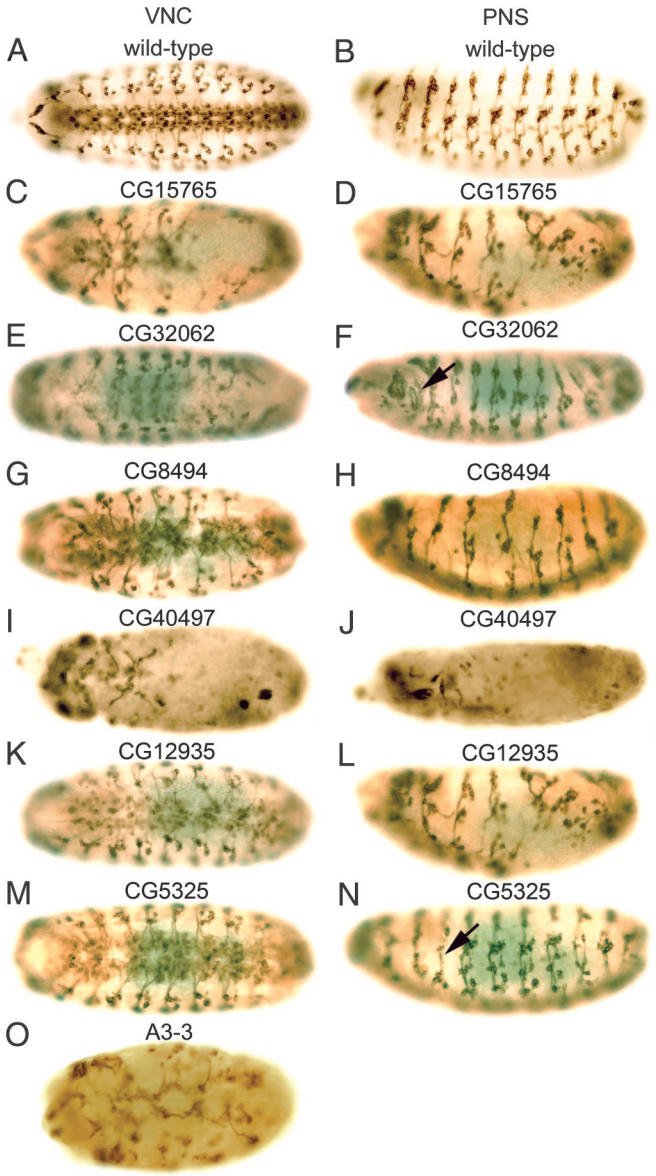

Fig. 1. Wild-type and RNAi-induced phenotypes of novel genes with unknown functions. (*A* and *B*) Wild-type patterns of the VNC and PNS, respectively, of *Drosophila* embryos stained with mAb 22C10. (*C–N*) RNAi mutant phenotypes after injection of dsRNAs synthesized from *CG15765* (*C* and *D*), *CG32062* (*E* and *F*), *CG8494* (*G* and *H*), *CG40497* (*I* and *J*), *CG12935* (*K* and *L*), *CG5325* (*M* and *N*), and *A3-3* (*O*). Ventral (*A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*) and lateral (*B*, *D*, *F*, *H*, *J*, *L*, and *N*) views of stage 15/16 embryos are shown. In all images, anterior is to the left, and in lateral views of embryos, dorsal is up.

Genes with Previously Uncharacterized Nervous System Phenotypes. Among the 16 genes with RNAi-induced nervous system phenotypes are seven genes, *CG15765*, *CG32062*, *CG8494*, *CG40497*, *CG12935*, *CG5325*, *XNP*, and *A3-3*, whose functions are uncharacterized, and nine genes, such as *Mediator complex subunit 25* (*MED25*), *Tetraspanin 86D* (*Tsp86D*), *XNP pimples* (*pim*), *ATP-dependent chromatin assembly factor* large subunit (*Acf1*), *cactus* (*cact*), *lame duck* (*lmd*), *olf186-F*, and *IGF-II mRNA-binding protein* (*Imp*), whose functions have been reported; however, no roles have been reported for these genes in the development of the embryonic nervous system. RNAi-induced mutant phenotypes for these genes are shown in Figs. 1 and 2.

CG15765 is a gene of unknown function encoding a protein that contains a C-type lectin domain expressed in the nervous system at late embryonic stages and is highly enriched in the adult fly brain (www.flyatlas.org). Suppression of *CG15765* gene expression by RNAi resulted in a phenotype characterized by hypoplasia and disorganization of both the VNC and the PNS (Fig. 1 *C* and *D*). The level of *CG15765* RNA increases markedly in 12- to 16-h-old embryos and then decreases during later embryonic development [supporting information (SI) Fig. 4*A*]. *CG32062* encodes a protein containing a ribonucleoprotein 1 RNA recognition motif. The molecular function of this gene has not been reported; however, treatment of *Drosophila* cell lines with *CG32062* dsRNA greatly reduced the number of cells and impaired secretion from the Golgi apparatus and Ca^{2+} transport (www.flyrnai.org). An enhancer trap fly line bearing an insertion within *CG32062* exhibits a segmentally modulated expression pattern in the larval CNS (7). *CG32062* mRNA also is expressed in the embryonic nervous system (7). Injection of *CG32062* dsRNA in early embryos resulted in a decrease in neural cells in the medial region of the VNC (Fig. 1*E*), and some misrouted axons in the PNS (arrow in Fig. 1*F*). RT-PCR results show that *CG32062* is zygotically expressed (SI Fig. 4*B*). *CG8494* is a gene of unknown function encoding a protein that exhibits limited sequence homology to *fat facets* (*faf*), a *Drosophila* deubiquitinating enzyme that is strongly expressed in the developing CNS. Overexpression of *faf* in the nervous system leads to an increase in the number of synaptic boutons and synaptic branches at neuromuscular junctions (8). The human homolog of *CG8494* encodes ubiquitin-specific processing protease 20 (Table 1). The neural phenotype observed after injection of *CG8494* dsRNA consists of hypoplasia and disorganization of both the VNC and the PNS (Fig. 1 *G* and *H*, respectively). *CG40497* encodes a protein of unknown function. Suppression of *CG40497* gene expression by RNAi resulted in global hypoplasia of epidermal cells (Fig. 1 *I* and *J*). The embryos did not develop beyond stage 14, and they showed severe loss of neurons and abnormal segmental structure of the nervous system. *CG12935* is a gene of unknown function. Silencing of *CG12935* expression by RNAi resulted in a decrease in the number of neuromeres and severe disruption of both the VNC and the PNS (Fig. 1 *K* and *L*). RT-PCR analysis showed that *CG12935* is expressed at the highest level in 4- to 8-h embryos, and expression decreases rapidly after germ band elongation (SI Fig. 4*D*). *CG5325* encodes a protein of unknown function that is an ortholog of *Caenorhabditis elegans Prx-19* and *Homo sapiens Pex19*, both encoding proteins essential for peroxisome biogenesis (9). Pex19 is involved in CNS development and neuron migration (10), and mutation of *Pex19* results in Zellweger syndrome, which includes mental retardation (11). Down-regulation of *CG5325* by injection of dsRNA into embryos resulted in disruption of the VNC (Fig. 1*M*), misrouting of axons (arrow in Fig. 1*N*), and disorganization of dorsal clusters of cells in the PNS (Fig. 1*N*). *A3-3* encodes a protein containing a basic region-leucine zipper (bZIP) motif, similar to the *fos* oncogene. bZIP transcription factors bind to DNA as dimers and regulate gene expression (12). The human ortholog of *A3-3* is FOSB. Suppression of *A3-3* expression by RNAi resulted in severe disruption of the VNC and hypoplasia (Fig. 1*O*).

MED25 encodes a protein possessing RNA polymerase II transcription mediator activity. In *Drosophila*, 30 MED molecules have been identified by biochemical and genomic analyses (13). Although the target of MED25 has not been identified, *MED25* has been shown to be required for lipopolysaccharide- or heat shock-induced gene expression (14). Treatment of *Drosophila* S2 cells with *MED25* dsRNA resulted in a decrease in cell viability (www.flyrnai.org). The RNAi-induced *MED25* phenotype includes the failure of extension of some axons from the VNC (arrowhead in Fig. 2*A*). All dorsal cell clusters in the PNS are missing (arrow in Fig. 2*B*). Tsp86D is a member of the tetraspanin superfamily, i.e., integral membrane proteins with four transmembrane domains involved in signal transduction, cell adhesion, and regulation of cellular development and proliferation (15). In *Drosophila*,

Table 1. Genes identified by RNA interference

Gene	Molecular function	Human ortholog*
A. With unknown functions		
CG15765	C-type lectin, sugar binding	No homolog
CG32062	RNP-1 RNA-binding region	*Ataxin 2-binding protein 1*
CG8494	Unknown	*Ubiquitin-specific protease 20*
CG40497	Unknown	No homolog
CG12935	Unknown	No homolog
CG5325	Unknown	*Pex19*
A3-3	DNA binding, basic leucine zipper	*FOSB*
B. With known functions		
MED25	RNA polymerase II transcription mediator	*ARC92 (MED25)*
Tsp86D	Cell–cell adhesion, signal transduction	*TSPAN33*
XNP	ATP-dependent helicase	*ATR-X*
pim	Sister chromatid segregation	No homolog
Acf1	Chromatin assembly and remodeling	*BAZ 1A*
cact	Establishing dorsoventral polarity	*NFKBIA*
lmd	Putative zinc finger transcription factor	No homolog
olf186-F	Calcium release-activated calcium channel	*TMEM142A*
Imp	Regulating translation, mRNA binding	*IGF2BP1*
C. With known nervous system development phenotypes		
dmt	Nuclear localization motifs, PNS development	No homolog
pbl	GTP exchange factor, initiating cytokinesis	*ECT2*
pros	Homeodomain, neuronal cell fate	*PROX1*
crb	Adherens junctions, epithelial cell polarity	*CRB1*
arm	β-Catenin, epithelial cell polarity	*CTNB1* (catenin β1)
arr	Low-density lipoprotein receptor	*LRP6*

*Human orthologs shown are as reported (http://inparanoid.cgb.ki.se).

functional redundancy or compensatory mechanisms have been observed for some of the 35 members of the tetraspanin family (16). *Tsp86D* is expressed in the VNC and the hypocerebral ganglion (16). Suppression of *Tsp86D* gene expression by RNAi resulted in abnormal formation of the VNC (Fig. 2*C*) and axon pathfinding defects (arrowhead in Fig. 2*D*) in the PNS. *XNP* encodes a protein containing an SNF2-related helicase domain, similar to human ATR-X, encoded by *α-Thallassemia/mental retardation, X-linked ATR-X* gene. Mutations of *ATR-X* result in mental retardation with hematologic, skeletal, and genital defects (17). Although the molecular function of XNP has not been studied, a gain-of-function screen to identify genes involved in the development of the mushroom bodies showed that overexpression of *XNP* resulted in fewer neurons in the mushroom bodies (18). Embryos injected with *XNP* dsRNA exhibited disruption of the VNC (Fig. 2*E*) and reduction of the intersegmental and segmental nerves (Fig. 2*F*). pim is a protein involved in sister chromatid segregation during mitosis (19, 20). A high level of maternal *pim* transcript is present in the early embryo with mitotically synchronized cell division before cellularization; after that, *pim* transcripts remain distributed throughout the embryo and become restricted to the developing nervous system where cells proliferate in late embryos (20), consistent with the RT-PCR results showing that *pim* is highly expressed during early stages of development and decreases rapidly at later stages (SI Fig. 4*H*). Injection of *pim* dsRNA in early embryos resulted in a loss of neurons in the VNC and a loss of neurons and severe disruption of the PNS (Fig. 2 *G* and *H*). We observed a similar RNAi-induced mutant phenotype by injection of dsRNA corresponding to *three rows* (4), which also is required for sister chromatid separation (21). pim protein associates *in vivo* with three rows forming a complex (21). The *Acf1* gene encodes a large subunit of the ATP-using chromatin assembly and remodeling factor (ACF) required for the assembly of histone–DNA complexes into periodic nucleosome arrays (22). Acf1 contains two PHD fingers and a bromodomain, and it associates with Imitation of Switch (ISWI), a homolog of yeast SWI2/SNF2 and a small subunit of ACF (22, 23). The expression of *Acf1* was observed in a subset of cells in the midline primordium region of the CNS (24). Embryos injected with *Acf1* dsRNA exhibited missing segments (Fig. 2 *I* and *J*), loss of neurons, and disruption of the architecture of the VNC (Fig. 2*I*) and severe hypoplasia and disorganization of the PNS (Fig. 2*J*). Although *Acf1* genetic mutants develop normally during embryogenesis (22), the severe RNAi phenotype we observed can be explained by the depletion of maternally stored *Acf1* mRNA by RNAi. *cactus*, a homolog of vertebrate NFkBIA, contains seven ankyrin repeats and possesses transcription regulator activity (25). *cactus* is involved in the innate immune response through the Toll signaling pathway (26) and also is required for the establishment of dorsal-ventral polarity by direct interaction with Dorsal, a homolog of vertebrate NFκB (27, 28). A loss-of-function mutation of *cactus* leads to ventralized embryos lacking dorsal cuticle and embryonic lethality (27). *cactus* is necessary for the normal function of the larval neuromuscular system, and loss-of-function cactus mutants have altered bouton numbers and neurotransmitter release at neuromuscular junctions (29). Reduction of *cactus* gene expression in embryos by RNAi resulted in severe hypoplasia and overall disruption of both the VNC and the PNS (Fig. 2 *K* and *L*). *lmd* encodes a putative zinc finger transcription factor related to the vertebrate GLI superfamily (30). *lmd* is expressed exclusively in mesodermal cells in a Wingless- and Notch-dependent manner and acts as a key regulator of myogenesis (30). The earliest expression of *lmd* is observed in the primordium of the caudal visceral mesoderm at stage 10, and strong expression is predominantly in fusion-competent myoblasts at stage 12 (30), consistent with our RT-PCR result showing that the highest expression of *lmd* is in 4- to 12-h-old embryos (SI Fig. 4*I*). A loss-of-function mutation of *lmd* leads to a complete lack of myoblast fusion

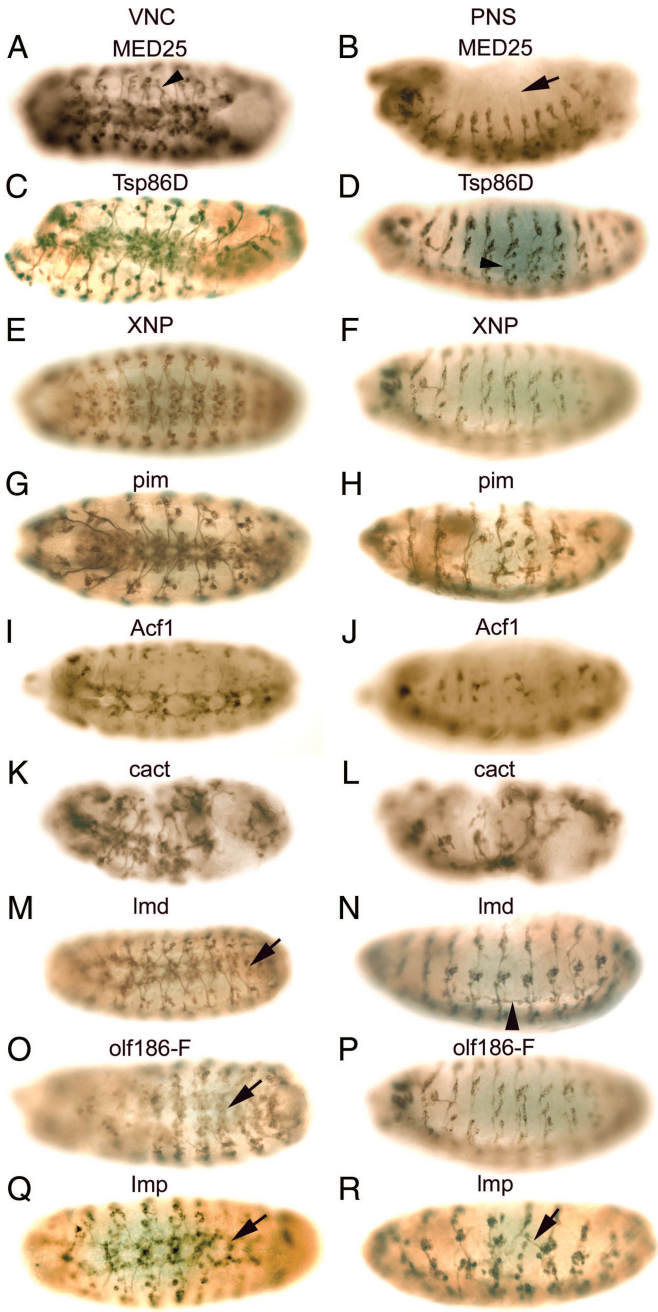

Fig. 2. RNAi-induced phenotypes of novel genes with known functions. Embryos were injected with dsRNAs from *MED25* (*A* and *B*), *Tsp86D* (*C* and *D*), *XNP* (*E* and *F*), *pim* (*G* and *H*), *Acf1* (*I* and *J*), *cact* (*K* and *L*), *lmd* (*M* and *N*), *olf186-F* (*O* and *P*), and *Imp* (*Q* and *R*). After staining with mAb 22C10, ventral (*A*, *C*, *E*, *G*, *I*, *K*, *M*, *O*, and *Q*) and lateral (*B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*, and *R*) views are shown.

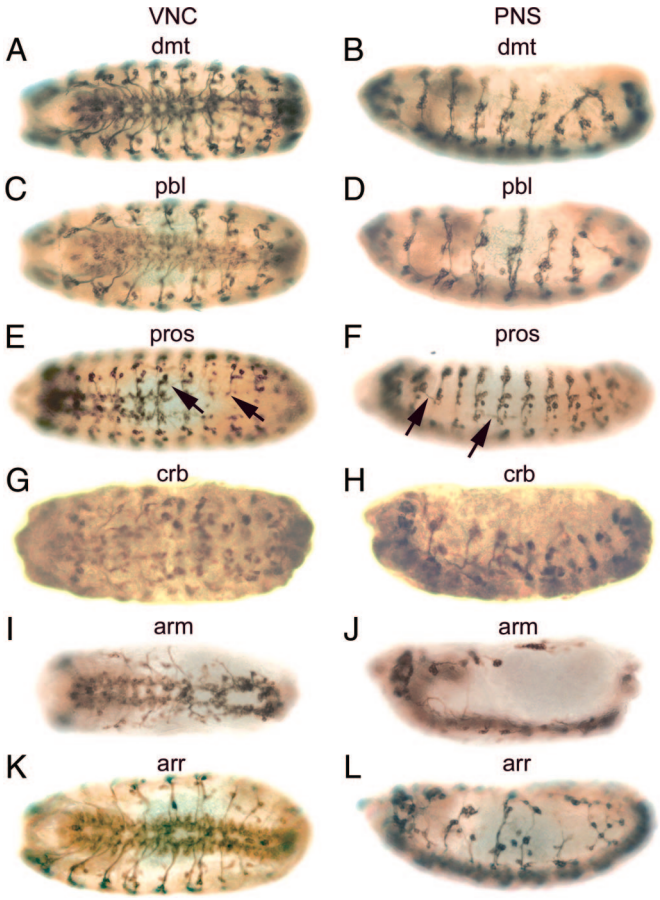

Fig. 3. RNAi-induced phenotypes of known genes whose functions have been described in the development of the embryonic nervous system. Embryos were injected with dsRNAs from *dmt* (*A* and *B*), *pbl* (*C* and *D*), *pros* (*E* and *F*), *crb* (*G* and *H*), *arm* (*I* and *J*), and *arr* (*K* and *L*) and were stained with mAb 22C10. The photographs were taken from ventral (*A*, *C*, *E*, *G*, *I*, and *K*) and lateral (*B*, *D*, *F*, *H*, *J*, and *L*) views.

and to embryonic lethality (30, 31). We observed that many embryos injected with *lmd* dsRNA exhibited relatively normal development of the CNS; however, some embryos had abnormal VNC morphology (arrow in Fig. 2*M*). Injected embryos exhibited a more severe altered phenotype in the PNS, such as disorganization and misrouting with axons crossing segmental boundaries in the ventrolateral region (arrowhead in Fig. 2*N*). olf186-F is a protein encoding Ca^{2+} release-activated Ca^{2+} channel modulator-1 (CRACM1) identified by a genome-wide RNAi-based screen in *Drosophila* cells for genes affecting calcium influx across the plasma membrane (32). RNAi phenotypes of *Drosophila* embryos injected with *olf186-F* dsRNA exhibit defects in the organization of the VNC (arrow in Fig. 2*O*) and severe disruption and hypoplasia of the PNS (Fig. 2*P*). *Imp* is an IGF-II mRNA-binding protein similar to zipcode-binding protein (ZBP-1). A gain-of-function screen showed that *Imp* is involved in axon pathfinding and synapse formation in the neuromuscular system of *Drosophila* larva (33). Norga *et al.* (34) also reported that a P-element insertion in *Imp* results in an increase in the number of adult sensory bristles. Maternally derived *Imp* mRNA is distributed evenly in the whole embryo at early stages, and in later stages, *Imp* is expressed exclusively in the developing CNS (35). We confirmed the maternal expression of *Imp* by RT-PCR (SI Fig. 4*K*). Injection of *Imp* dsRNA in early embryos resulted in loss of some segments, loss of neurons, and axon pathfinding defects in both the VNC and the PNS (arrows in Fig. 2 *Q* and *R*).

Genes with Known Nervous System Phenotypes. RNAi-induced phenotypes for six genes, *dalmatian* (*dmt*), *pebble* (*pbl*), *prospero* (*pros*), *crumbs* (*crb*), *armadillo* (*arm*), and *arrow* (*arr*), whose roles in the development of the embryonic nervous system have been described previously, are shown in Fig. 3. *dmt* encodes a putative nuclear protein that is not related to any known protein. Expression of *dmt* increases during germ band

extension and becomes more restricted to the PNS, VNC, and embryonic brain during stage 12 (36). Embryos carrying hypomorphic mutant alleles of *dmt* display a mild phenotype, such as infrequent loss of neurons and occasional pathfinding defects in the PNS (37); whereas more severe alleles exhibit a general disorganization of the PNS (34). RNAi-induced phenotypes exhibited disruption of the VNC (Fig. 3*A*) and severe hypoplasia and misrouting of neurons in the PNS (Fig. 3*B*), consistent with genetic mutant phenotypes. *pbl* encodes a GTP-exchange factor for Rho 1 GTPase that is an essential regulator of cytokinesis (38). High levels of maternally provided *pbl* transcripts are present in preblastoderm embryos, and the highest protein levels are in nuclei of dividing cells, such as embryonic neuroblasts (39). These results are consistent with our RT-PCR analysis showing the highest level of expression of *pbl* during very early embryogenesis (SI Fig. 4*M*). Embryos homozygous for *pbl* alleles display gross defects in axon trajectories, loss of neurons, and fasciculation defects in the PNS, leading to embryonic lethality (40). A gain-of-function screen also revealed that *pbl* is required for controlling motor axon guidance and synaptogenesis (33). Injection of *pbl* dsRNA in embryos resulted in an abnormal VNC (Fig. 3*C*), loss of neurons, misrouting of axons, and fasciculation defects in the PNS (Fig. 3*D*), in agreement with genetic mutant phenotypes. pros is a transcription factor containing a homeodomain and is involved in neuronal fate determination and the formation of axons (41). During neuroblast (NB) division in early embryogenesis, pros is localized basally in NBs and is segregated asymmetrically to the smaller daughter cell [ganglion mother cell (GMC)] (42, 43). *pros* is expressed in a subset of NBs and GMCs but not in VNC neurons (41, 44). Homozygous mutant alleles display defects in axon pathfinding in both the CNS and PNS, leading to aberrant neuronal connections (41, 45). We observed a similar RNAi-induced phenotype after injection of *pros* dsRNA into embryos. Intersegmental and segmental nerves often are missing or are not properly extended in the VNC (arrows in Fig. 3*E*), and sensory axons exhibit aberrant connections or pathfinding defects in the PNS (arrows in Fig. 3*F*). crb is an integral membrane protein containing EGF-like repeats and is localized exclusively in the apical membranes of epithelial cells during cellularization and also is expressed in many tissues, including NBs and the embryonic PNS (46). crb is involved in the biogenesis of adherens junctions composed of cadherin–catenin complexes (47, 48) and is required for the establishment of epithelial cell polarity (49). The RNAi-induced mutant phenotype of *crb* exhibits global hypoplasia of the VNC and severe disruption of the PNS including defects in neuromere organization and axon genesis (Fig. 3 *G* and *H*). arm is a homolog of vertebrate catenin β-1 and is involved in many biological processes during embryogenesis, such as cell–cell adhesion, establishment/maintenance of cell polarity, and NB fate determination depending on the Wg/Wnt signaling pathway (for a review, see ref. 50). arm binds to α-catenin, E-cadherin, and shotgun (51) at adherens junctions in the apical membrane of the epidermis, and it regulates cell polarity. arm is expressed in many tissues, including the embryonic CNS and larval brain where arm is enriched in fiber tracks of axons (52). Homozygous *arm* mutant alleles exhibit strong segment polarity defects and the absence of embryonic cuticle, whereas hypomorphic mutant alleles display defects in NB fate determination and axon development (53). Embryos injected with *arm* dsRNA exhibit severe RNAi-induced phenotypes, such as hypoplasia and overall disruption both of the CNS and the PNS (Fig. 3 *I* and *J*). Epidermal cells are not formed (Fig. 3*I*). We also observed decreases in the size of embryos resulting from segment polarity defects (data not shown). This phenotype resembles the *shotgun* RNAi-dependent phenotype shown in our previous study (4). *arr* is an essential factor in the Wg/Wnt signaling pathway functioning in embryonic segmentation, limb development, and CNS organization (54). Loss of *arr* expression disrupts midgut morphogenesis and the nervous system (55). The mutant embryos exhibit disorganized neuropile, gaps in the longitudinal nerves, tangled and misguided commissural nerves in the CNS, and reduced and mispositioned sensory neurons in the PNS (55). RNAi-dependent suppression of *arr* gene expression resulted in disorganization in the VNC (Fig. 3*K*) and severe hypoplasia and pathfinding defects in the PNS (Fig. 3*L*), which are similar to the genetic mutant phenotypes.

In summary, genes found in this work encode transcription factors, a chromatin remodeling factor, membrane receptors, signaling molecules, and other proteins involved in various biological processes. We observed similar RNAi-dependent phenotypes in genes involved in the same pathway, such as *arr*, a receptor of Wnt/Wg, and *arm*, a key regulator of the Wnt/Wg signaling pathway. Because of RNAi, both phenotypes display severe hypoplasia and axon pathfinding defects during the development of the embryonic nervous system. In addition, down-regulation by RNAi of *CG8494*, which encodes a putative deubiquitinating protease, and *pbl*, encoding a substrate for ubiquitin E3 ligase (56), resulted in disruption of the nervous system, emphasizing the importance of ubiquitin-dependent protein degradation in the development of the nervous system. Comparison of the phenotypes identified from our RNAi screen with the corresponding mutant phenotypes obtained in genetic screens showed that RNAi-induced mutant phenotypes resemble genetic mutant phenotypes, indicating that RNAi can be used efficiently to identify genes that are involved in the development of the embryonic nervous system of *Drosophila*.

Materials and Methods

dsRNA Synthesis. For the primary RNAi screen to select candidate genes required for embryonic nervous system development, we synthesized dsRNAs from the Unigene 1.0 cDNA library as described previously (4). After selection of candidate genes, dsRNA again was synthesized but from a different region of the gene, to eliminate false-positives and to determine whether RNAi-induced mutant phenotypes were reproducible. For this second dsRNA synthesis, two rounds of PCR amplifications were performed. The primers were designed by Primer3 Input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The specificity of the amplified fragments was confirmed by NCBI Blast Searches (www.ncbi.nlm.nih.gov/BLAST), and the primer sequences, size of dsRNAs, and the cDNA or genomic regions used for template are shown in SI Table 2. For the first PCR amplification, either genomic DNA or cDNA synthesized from total RNA from wild-type adult flies was used. For the second PCR step, primers were used containing 18 bp of nested inner sequences of the first amplified fragment and a T7 RNA polymerase promoter sequence at the 5′ end. After PCR, *in vitro* transcription was catalyzed by T7 RNA polymerase (Takara Bio, Otsu, Shiga, Japan). The RNA products were treated with DNase I (Takara Bio) and then purified with mini-Quick Spin RNA columns (Roche Applied Science, Indianapolis, IN). After annealing sense and antisense RNA, the concentration of dsRNA was measured and adjusted to 1 μg/μl in 5 mM KCl/0.1 mM NaH_2PO_4, pH 6.8, containing 5% food color (green; McCormick, Baltimore, MD).

Embryo Injection and Immunohistochemistry. *Drosophila melanogaster*, Oregon R strain (wild type) was used for dsRNA injection. Embryo collection, injection, and immunostaining were performed as described previously (4).

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