GENOMIC STRATEGIES REVEAL A TRANSCRIPTIONAL CASCADE THAT

CONTROLS SYNAPTIC SPECIFICITY IN *CAENORHABDITIS ELEGANS*

By

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To my fiancée
and parents

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MOTOR NEURON SPECIFICATION AND MECHANISMS OF SYNAPTIC
SPECIFICITY

Introduction

As single-celled organisms evolved into more complex, multicellular animals, the
need to coordinate the functions of distinct body parts arose. For example, movement is
essential to the success of most organisms; without coordinated locomotion capturing
prey or evading predators would be impossible. Additionally, muscles for eating and
breathing must be stimulated in order to maintain life. Thus, centralized nervous systems
were formed as a command center to transmit signals to the body. These initially arose as
simple clusters of neurons sending processes to peripheral organs and evolved into
complex circuits composed of billions of cells. Regardless of the complexity, in order to
function properly neurons must extend processes and connect to the correct target cells.
Advances in neurobiology have fueled the discoveries of genes involved in neural
specification and axon guidance; however, how neurons recognize their cognate partners
is poorly understood.

This introduction will review the transcriptional control of motor neuron
development in vertebrates and invertebrates, as well as discuss the known molecules
involved in synaptic target recognition. The last section will provide an in depth
discussion about two transcriptional co-repressor proteins, UNC-4 and UNC-37, which
function together in the *C. elegans* motor circuit to control synaptic specificity.

Vertebrate motor neuron specification

The developing vertebrate spinal cord contains distinct progenitor domains for interneurons and motor neurons

The ventral spinal cord is composed of interneurons and motor neurons that function to transmit impulses to muscle. Progenitor domains situated in the medial portion of the spinal cord produce post-mitotic neurons that then migrate to the outer layers of the neural tube (Figure 1.1) (Lee and Pfaff 2001). The five defined progenitor domains, p0-p3 and pMN, give rise to interneurons V0-V3 and motor neurons (MNs). Motor neurons can be categorized based on migration patterns and axonal trajectories (Figure 1.2) (reviewed in Shirisaki and Pfaff, 2002). For example, MMC neurons reside in the medial motor column and LMC cells migrate to the lateral motor column. These two motor columns are clearly separated in the brachial and lumbar regions of the spinal cord. Motor columns are correlated with muscle target specificity. LMC neurons innervate limb muscle; MNs in the lateral half of the LMC (LMCl) synapse onto dorsal limb muscle while those in the median half (LMCm) target ventral limb muscle. MMC neurons synapse onto either dermomyotome or body wall musculature, depending on whether they are in the median or lateral MMC, respectively.

A gradient of Sonic Hedgehog establishes neuronal progenitor domains in the vertebrate spinal cord

The ventral spinal cord progenitor domains are specified by a gradient of the morphogen Sonic Hedgehog (Shh), which is secreted from the notochord and floorplate (Chiang et al. 1996). Progenitor cells in the ventral spinal cord translate the Shh gradient
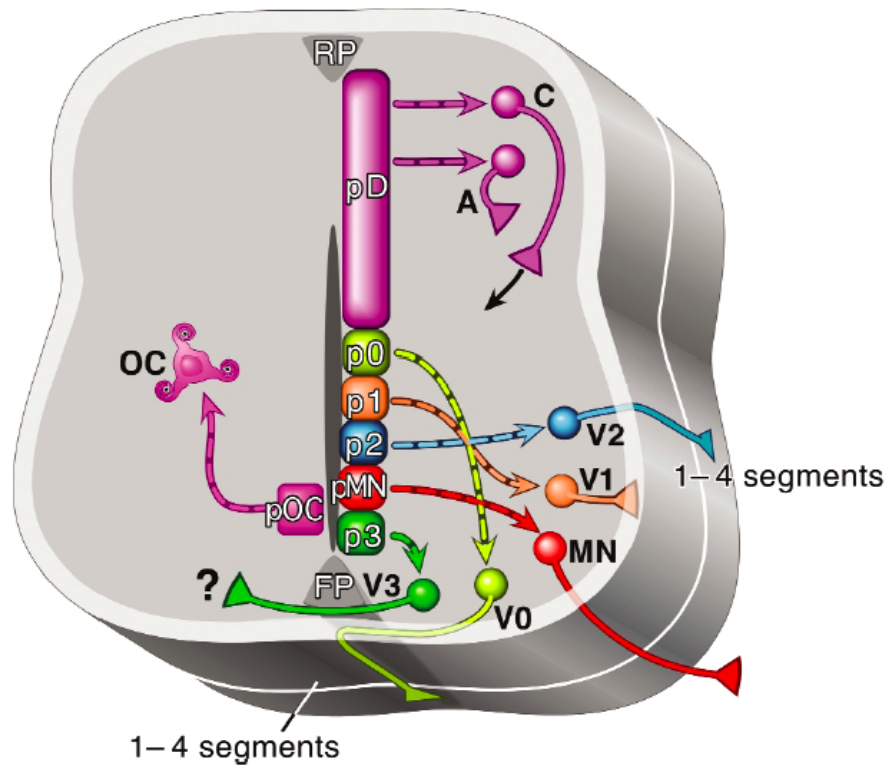
Figure 1.1 The developing vertebrate spinal cord.
Progenitor domains (pD, p0-3, pMN) in the medial portion of the spinal cord give rise to post-mitotic neurons (A, C, V0-V3, MN) which migrate to outer layers. These post-mitotic neurons display stereotypical migration and axonal patterns. For example, the pMN domain produces motor neurons (MN) which send axons to peripheral muscle. During development, the p3 and pMN domains switch to pOC to make oligo-dendrocytes (OC). RP - roofplate, FP - floorplate. Reprinted from Lee and Pfaff, 2001.
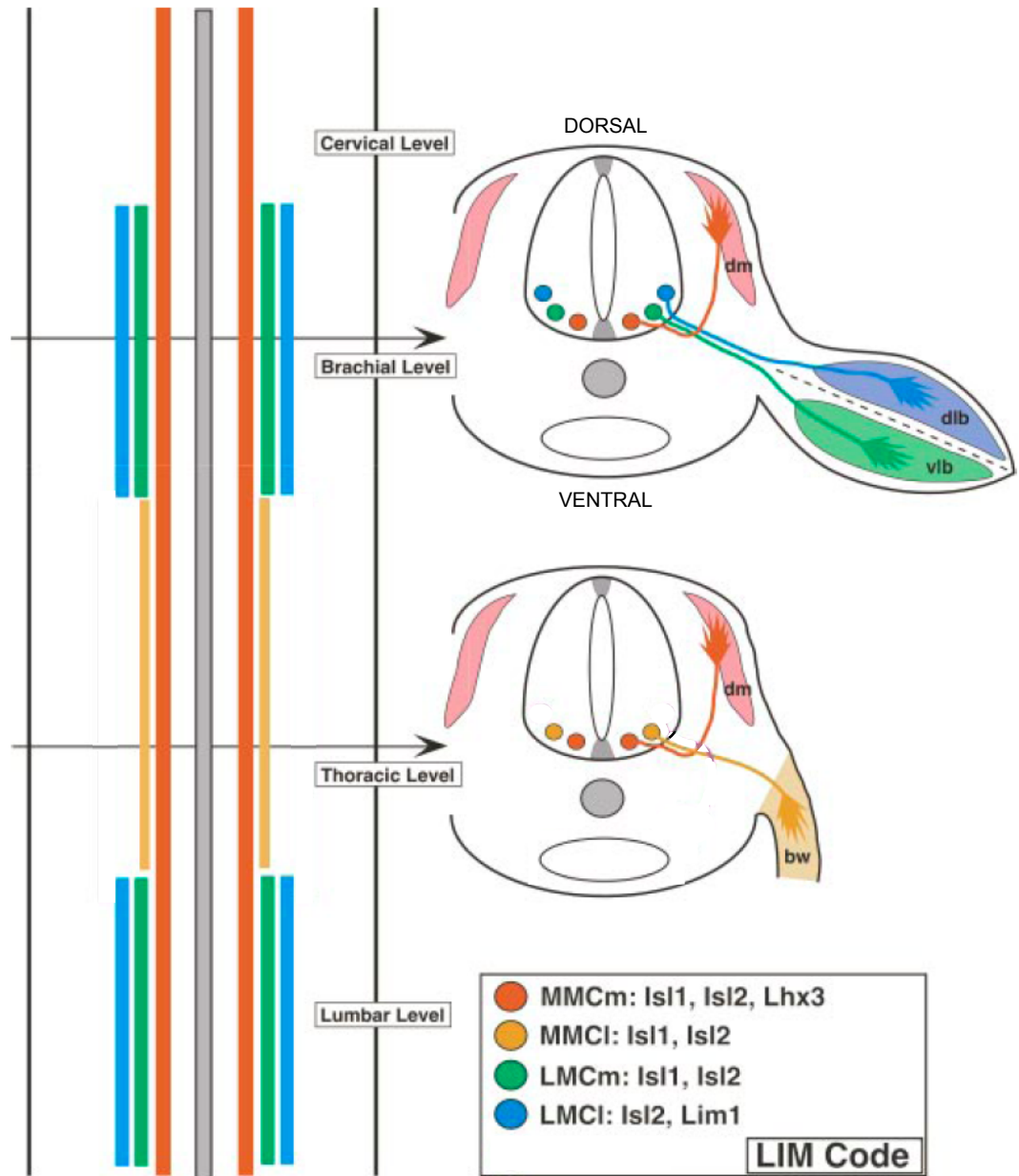
Figure 1.2 Subtypes of motor neurons in the ventral spinal cord.
An open-book whole-mount view (left) shows motor neurons positioned in clearly
defined columns. Specific subtypes of neurons are identified by unique axonal projec-
tions and synaptic targets. For example, neurons in the medial half of the medial motor
column (MMCm) innervate dermomyotome (dm) while those in the lateral half
(MMCl) connect with bodywall muscle (bw). These MN subtypes are specified by the
combinatorial expression of LIM-HD proteins (the LIM Code). For example, MMCm
neurons express Lhx3 as well as Isl1 and Isl2, whicle MMCl neurons express only
Isl1/2. LMC -lateral motor column. dlb - dorsal limb bud. vlb - ventral limb bud. Gray
areas, from dorsal to ventral, are the roof plate, floor plate, and notochord. Adapted
from Shirasaki and Pfaff, 2002.

into a patterned expression of transcription factors. These transcriptional regulators fall into two classes; Class I genes are repressed by Shh while Class II genes are activated by Shh (Figure 1.3). The opposing action of Class I and Class II factors further refine the progenitor domain boundaries (Briscoe et al. 2000). For example, in the motor neuron progenitor domain (pMN), the Class I gene Pax6 restricts the expression of the Class II genes Nkx2.2/2.6 to the ventral P3 domain (Figure 1.3) (Ericson et al. 1997). Conversely, the ventral boundary of Pax6 expression is defined by the reciprocal inhibition by Nkx2.2/2.9.

This cross-inhibitory mechanism is largely dependent on physical interactions of these transcription factors with Groucho/TLE proteins. All but one (the bHLH protein Olig2) of the identified Class I and Class II factors are eh1-containing homeodomains. The eh1 motif has been shown mediate direct interactions with Groucho, a co-repressor protein (Smith and Jaynes 1996; Jimenez et al. 1997; Tolkunova et al. 1998; Winnier et al. 1999). And in fact, this interaction with Groucho is necessary for the Class I and Class II factors to cross-repress each other (Muhr et al. 2001). As a result, a de-repression model for the specification of the ventral spinal cord has been established in which cell specification is achieved by actively suppressing other fates (Muhr et al. 2001). In other words, repressing a transcriptional repressor, in concert with general activators, can effectively activate (or de-repress) downstream target genes (see Chapter VI for more details).

Motor neurons arise from a progenitor domain which co-expresses Olig2, Nkx6.1, Nkx6.2, and Pax6. These factors are essential for promoting motor neuron fate by restricting the expression of other homeodomain proteins. For example, in *Nkx6.1* mutant

mice motor neurons and V2 interneurons are replaced with ectopic V1 interneurons (Sander et al. 2000). These fate changes are correlated with a ventral expansion of Dbx2 expression, which is normally repressed in the pMN and p2 domains by Nkx6.1 (Sander et al. 2000).

Determinants of post-mitotic motor neuron fate

Post-mitotic neural fate is specified by a separate group of downstream transcription factors. Here, the unique combinations of progenitor factors define the expression domains of distinct post-mitotic transcription factors. One of these, the HB9 homeodomain protein is a determinant of motor neurons (MNs) (Arber et al. 1999). HB9 is expressed at a low level throughout the neural tube; HB9 expression is upregulated in MNs by the active repression of *Nkx2.2* and *Irx3* by Pax6 and Olig2 (Figure 1.4) (Lee et al. 2004). HB9 specifies motor neuron fate by repressing expression of post-mitotic interneuron determinants, such as the V1 gene *Chx10* (Thaler et al. 1999). HB9 proteins are sufficient to induce motor neuron traits when ectopically expressed in non-MN domains in the chick spinal cord (Tanabe et al. 1998). HB9 null mice show defects in motor neuron migration that effectively blurs the usually distinct delineation of MMC and LMC columns. Additionally, axonal outgrowth defects and a complete loss of the phrenic nerve (innervates the diaphragm) are also observed (Arber et al. 1999; Thaler et al. 1999). Together, these results are indicative of a critical role for HB9 proteins in consolidating motor neuron fate in the vertebrate spinal cord.

The LIM-HD factor (see below) Islet-1 (Isl1) is essential for motor neuron development. Isl1 is expressed in all post-mitotic motor neurons before it is restricted to
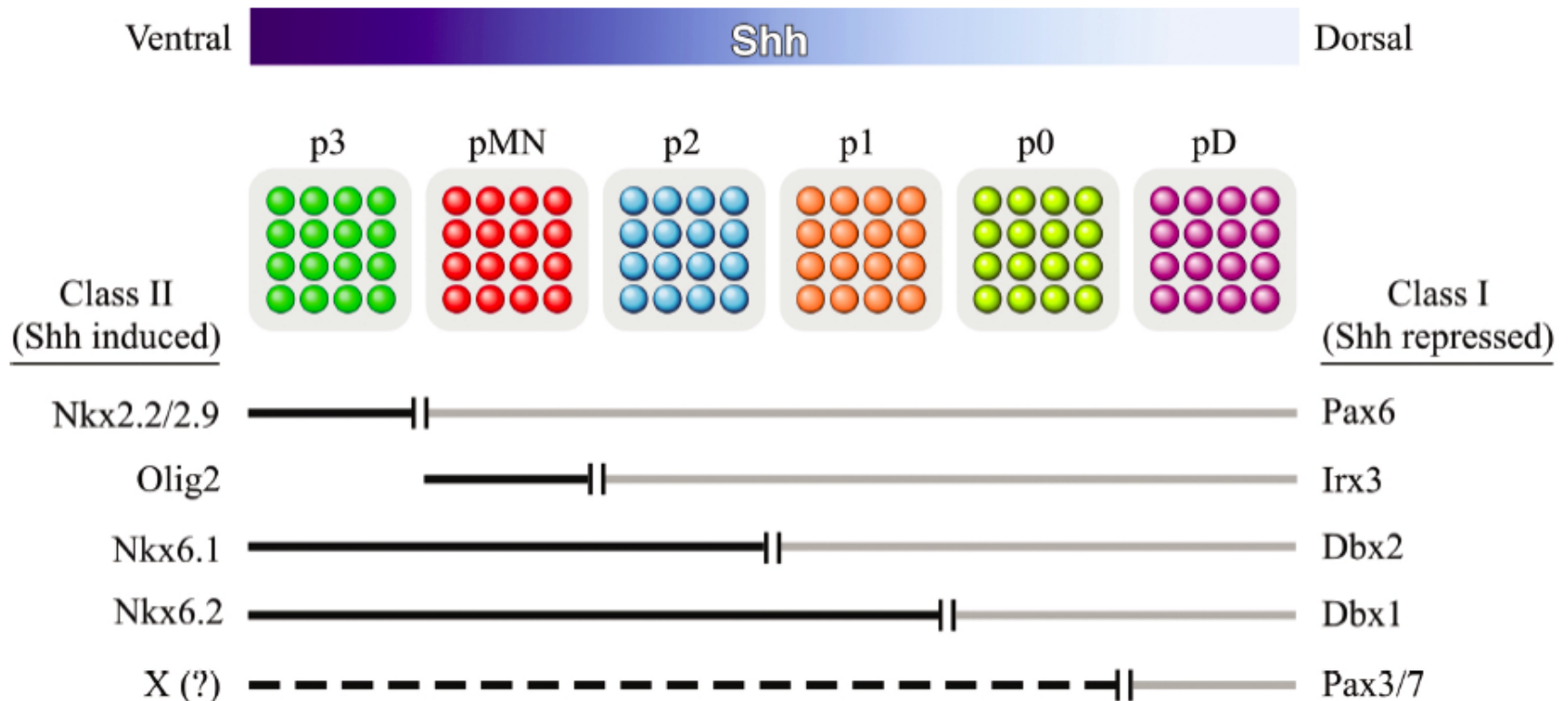
Figure 1.3 Progenitor domains in the spinal cord are established by a Sonic Hedgehog (Shh) gradient.
Shh forms a high-ventral to low-dorsal gradient which activates Class II genes and represses Class I genes. These two classes of transcription factors then establish progenitor domain boundaries via Groucho-mediated cross repression. p0-p1 - ventral interneuron progenitor domains, pMN - motor neuron progenitor domain, pD - simplified dorsal spinal cord progenitor domain. Reprinted from Lee and Pfaff, 2001.
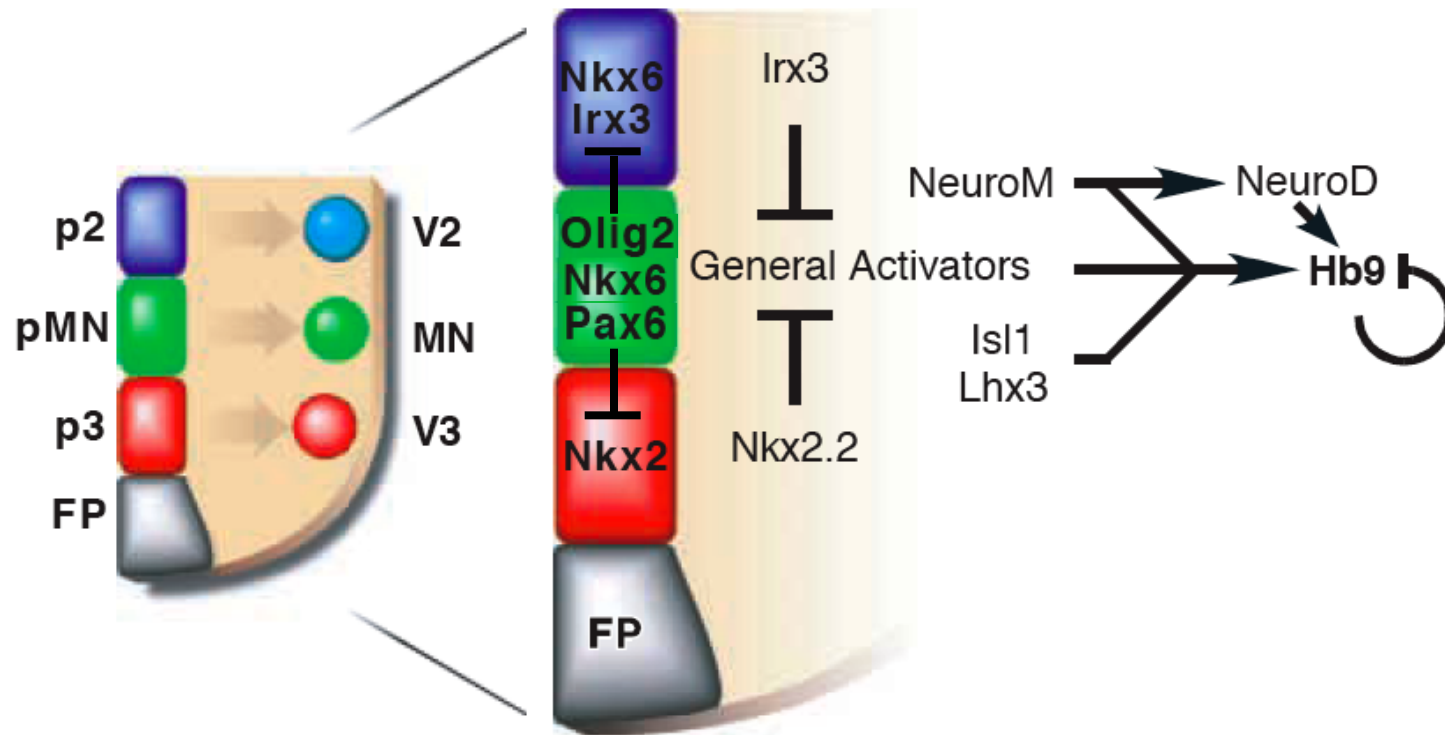
Figure 1.4 HB9 expression in motor neurons is upregulated by active repression of Nkx2.2 and Irx3.
The motor neuron progenitor domain (pMN, green) is surrounded by two interneuron progenitor domains (p2, blue and p3, red). These domains express unique combinations of eh1-containing homeodomain proteins (close-up on right). In motor neurons (green), Nkx2 and Irx3 are not expressed in motor neurons via the repressive actions of Pax6 and Olig2, respectively, which allows for a low-level expression of HB9 by general activators. In post-mitiotic motor neurons (far right), HB9 expression is enhanced by the bHLH factors NeuroM and NeuroD as well as the LIM-HD factors Isl1 and Lhx3. Adapted from Lee, et al., 2004.

specific subtypes. Motor neurons are absent in *Isl1* mutant mice as evidenced by the lack of known motor neuron marker expression (Pfaff et al. 1996). It appears that this early role of Isl1 in motor neurons is important for survival rather than directly influencing fate. If the latter were true, more interneurons would be expected in the ventral spinal cord in the *Isl1* mutant. Instead, there is an increased level of apoptosis, thus suggesting that Isl1 is essential for motor neuron survival.

Subtypes of motor neurons are defined, in part, by the combinatorial expression of LIM-HD transcription factors (Figure 1.2). This specific molecular strategy of motor neuron fate determination, called the LIM Code, is widely employed by many vertebrate and invertebrate species (Hobert and Westphal 2000; Shirasaki and Pfaff 2002). LIM-HD proteins contain a homeodomain and two N-terminal zinc-finger motifs called the LIM domain (after the *C. elegans* proteins LIN-11 and MEC-3 and the vertebrate factor Isl1). The LIM domain is thought to mediate protein-protein interactions. In this manner, homo- and heterodimer of LIM-HD proteins can increase the complexity of this transcriptional mechanism. Thus, unique combinations of LIM-HD proteins interact to promote specific motor neuron fates.

Invertebrate motor neuron specification

Drosophila homologs of vertebrate neural proteins also specify motor neuron identity

During *Drosophila* embryogenesis, ~40 motor neurons found in stereotypical positions in each abdominal hemisegment innervate a collection of 30 body wall muscles (Landgraf et al. 1997). It is striking that these motor neuron pools are specified by

homologs of transcription factors with known roles in vertebrate motor neuron development. For example, *islet* (Isl1/2 homolog) is expressed in motor neurons which innervate ventral body wall muscle via the transverse nerve (TN) and two branches of the intersegmental nerve (ISNb and ISNd) (Figure 1.5). In *islet* mutants, these ventrally-projecting motor neurons exhibit defects in axon fasciculation and target selection (Thor and Thomas 1997). Similarly, Lim3 (Lhx3/4 homolog) is co-expressed with Islet in TN and ISNb projecting motor neurons (Thor et al. 1999). Loss of *lim3* activity results in ISNb motor neurons projecting into the ISNd target area. Conversely, ectopic expression of Lim3 in ISNd motor neurons causes projections onto muscles innervated by ISNb motor neurons. Thus, Islet and Lim3 act to specify ISNb vs. ISNd fates, respectively. However, these phenotypes do not explain the function of Islet/Lim3 co-expression in TN motor neurons. Recently, Certel and Thor (2004) found that a POU homeodomain protein, Drifter/Ventral veinless (Dfr), acts with Lim3 and Islet in ISNb motor neurons to distinguish between ISNb and TN fates. Consistent with this idea, ectopic expression of Dfr in TN motor neurons is sufficient to induce ISNb-like projections (Certel and Thor 2004). The interaction of POU and LIM-HD proteins in neural fate is conserved; for example, the POU protein UNC-86 and the MEC-3 LIM-HD protein function together to define touch neuron identity in *C. elegans* (Duggan et al. 1998).

In vertebrates, HB9 is expressed in all motor neurons, where it functions in part to repress genetic programs initiated in adjacent interneuron progenitor domains (see above) (Arber et al. 1999; Thaler et al. 1999). In contrast, *Drosophila Hb9 (dHb9)* is expressed in only a subset of motor neurons and interneurons (Broihier and Skeath 2002; Odden et al. 2002). Broihier and Skeath (2002) discovered that dHb9 expression in motor neurons
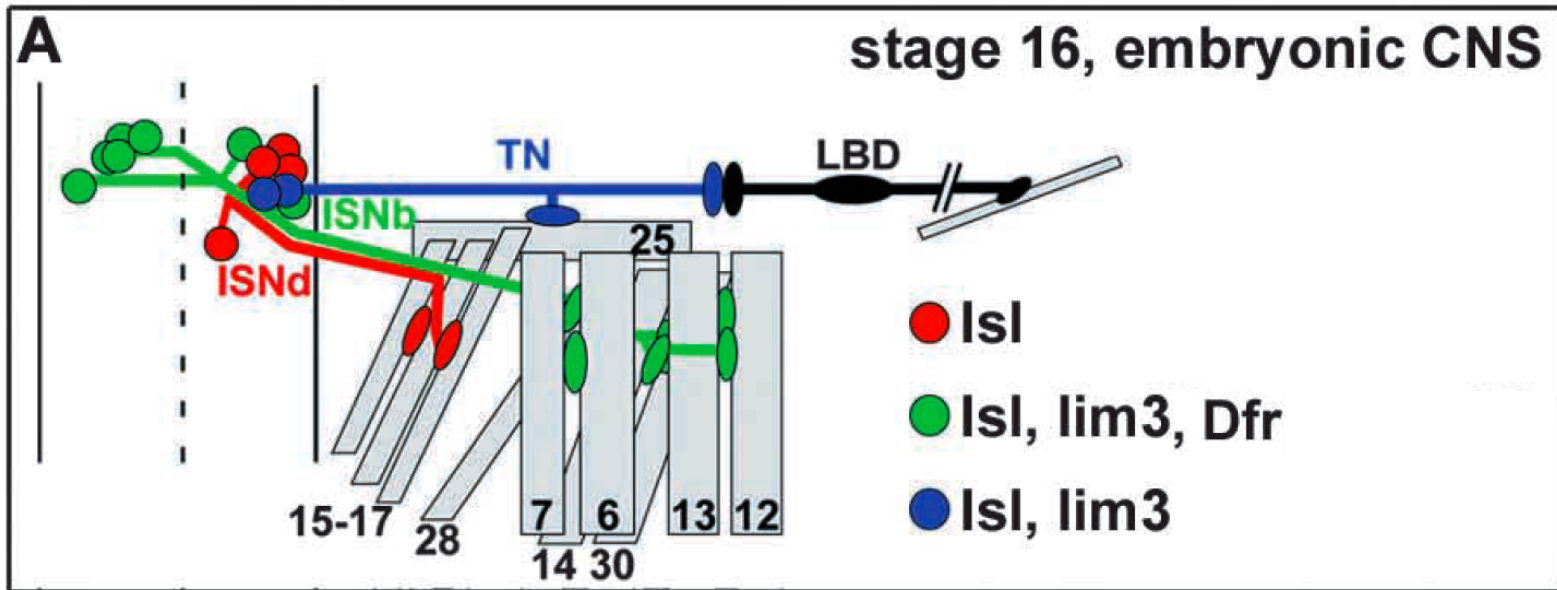
Figure 1.5 A transcription code specifies motor neuron subtypes in *Drosophila*.
In each abdominal hemisegment in the *Drosophila* embryo, ~40 motor neurons send projections from the ventral nerve cord to synapse onto 30 muscle fibers. Shown here is the ventral muscle field at embryonic stage 16, innervated by motor neurons which exit the ventral cord and fasciculate with the intersegmental nerve (ISN) or the transverse nerve (TN). Unique combinations of transcription factors specify these three motor neuron types. For example, islet (Isl) is expressed in all ventral-projecting motor neurons. The combined actions of Lim3 and Drifter (Dfr) act with Isl to specify ISNb fate while Lim3 and Isl1 synergize in TN motor neurons. (LBD - lateral bipolar dendrite neuron).
Adapted from Certel and Thor, 2004.

is limited to those with ventral projections. In *dHb9* mutants, a fraction (~20%) of ISNb

motor neurons fail to defasiculate from the ISN and instead project to dorsal muscle

fields (Broihier and Skeath 2002). dHb9 expression overlaps substantially with Islet and

Lim3, suggesting that these factors work in parallel to specify ventral fate. Consistent

with this idea, the penetrance of the ISNb defect is substantially higher in *isl; dHb9*

double mutants than in either single mutant alone. dHb9 promotes ventral fates in part by

repressing the dorsal specifier Even-skipped (see below). In *dHb9* mutants ectopic Eve+

cells arise in neurons that normally express dHb9. Furthermore, ectopic expression of

dHb9 in all neurons is sufficient to repress Eve expression in all Eve+ motor neurons

(Broihier and Skeath 2002).

In contrast to dHb9 and Islet, which specify ventrally-projecting motor neurons,

Even-skipped (Eve) specifies dorsally-projecting motor neurons (Landgraf et al. 1999).

Eve is both necessary and sufficient to induce dorsal motor axon outgrowth. In *eve*

mutants ISN fails to migrate to the dorsal muscle field, instead arresting in the ventral or

dorsolateral fields. In contrast, ectopic Eve expression in non-ISN neurons switches their

axonal fasciculation and guidance to that of ISN (i.e. onto dorsal muscles). Eve and dHb9

act in a cross-repressive manner to specify dorsal and ventral fates, respectively. In *eve*

mutants dHb9 is ectopically expressed in dorsally-projecting motor neurons (Broihier and

Skeath 2002; Fujioka et al. 2003)..

Recently, Broihier and colleagues (2004) demonstrated that the fly Nkx6.1/6.2

homolog, Nkx6, acts in parallel with dHb9 to promote ventral projections in a subset of

motor neurons (the RPs) by repressing Eve expression and promoting Islet and Lim3

expression. Additionally, Nkx6 acts independently to promote axonogenesis in Nkx6+

neurons; for example, ISNb and ISNd branches are absent in a significant portion of *Nkx6* mutant hemisegments (Broihier et al. 2004).

In summary, *Drosophila* homologs of vertebrate proteins that function in motor neuron specification play a similar role in flies.

The *C. elegans* motor circuit

The *C. elegans* nervous system is composed of exactly 302 neurons; over 1/3 (113/302) of these are motor neurons. A majority of motor neurons (80) lie in the ventral nerve cord (VNC) and in ganglia at either end. A subset of these motor neurons extends commissures circumferentially to establish the dorsal nerve cord whereas others innervate ventral muscles adjacent to the VNC. These motor neurons receive inputs from command interneurons located in head and tail ganglia. Together they form a motor circuit that controls the dorsal/ventral contractions that propel the animal forward or backward (Figure 1.6).

C. elegans **ventral cord motor neurons and the control of coordinated movement**

There are 8 classes of motor neurons in the ventral nerve cord (DA, VA, DB, VB, VC, DD, VD, AS). These classifications are defined, in part, by stereotypical morphologies and shared presynaptic inputs (Figure 1.7) (White et al. 1976). For example, VA motor neurons extend anteriorly directed axonal projections and receive inputs from AVA (chemical and electrical), AVD and AVE (chemical) command interneurons to control backward movement (Figure 1.8) (White et al. 1986). In contrast, the lineal sisters of VA motor neurons, the VBs, project posteriorly-directed axons and
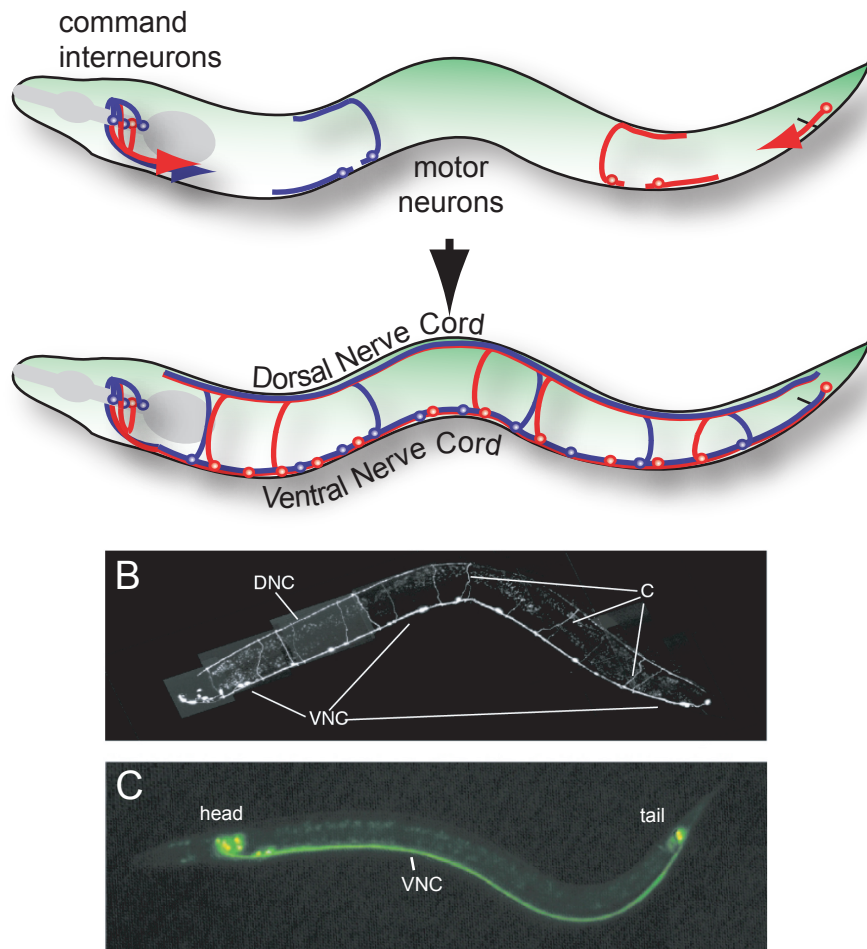
Figure 1.6 The *C. elegans* motor circuit.
A. Command interneurons in the head and tail extend processes into the ventral nerve cord to synapse with specific classes of motor neurons. "Red" and "Blue" circuits denote separate sets of command interneurons and motor neuron partners. Selected motor neurons shown here are presynaptic to adjacent body muscle cells in either the ventral or dorsal nerve cords.
B. GFP driven by the *unc-47* promoter labels the GABA circuit with motor neurons extending processes in the ventral nerve cord (VNC) and to the dorsal nerve cord (DNC) via commissures (C). (reprinted from Francis 2003)
C. A subset of neurons, including the command interneurons AVA, AVB, AVD, AVE (head) and PVC (tail) are marked with the *nmr-1*::GFP reporter to show projections into the VNC (adapted from Francis 2003).
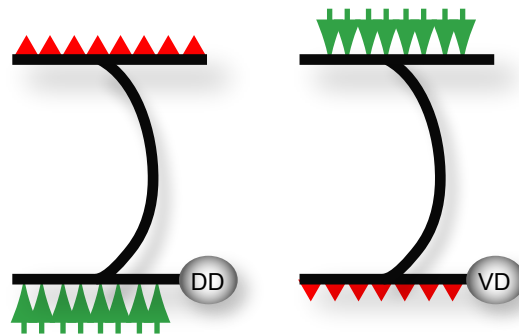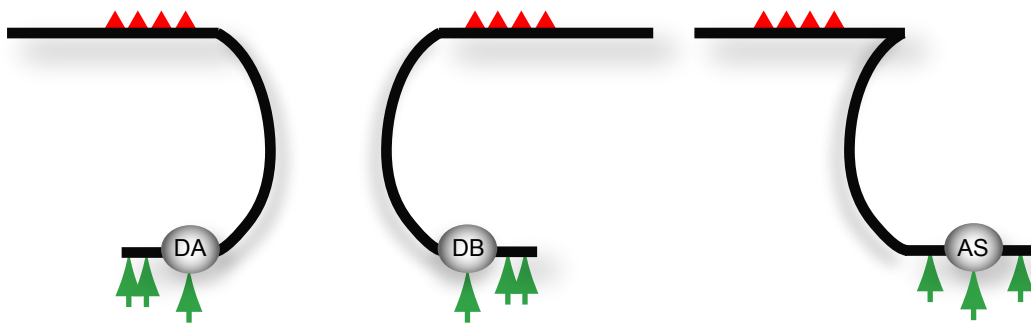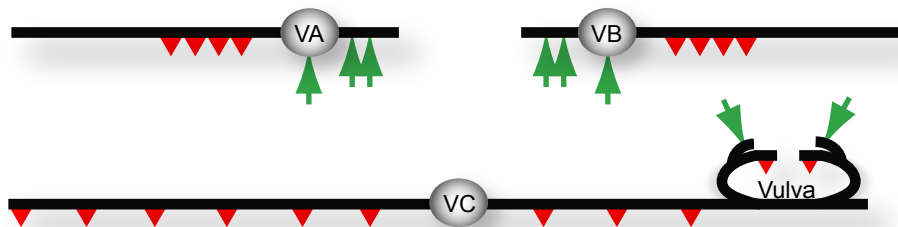
Figure 1.7 Morphology of adult ventral cord motor neurons.
Representative members of each motor neuron class are shown here. Note that five of the motor neuron classes (DA, DB, DD, AS, VD) extend commissures to the DNC whereas three (VA, VB, VC) are restricted to the VNC. Each motor neuron is representative of its class although minor differences in morphology do occur within each class. For example, in contrast to other VC class motor neurons, VC4 and VC5 do not make NMJs with body wall muscle as indicated in this diagram.
Green arrows depict input from other motor neurons. Red triangles denote NMJs with body wall muscle cells.

accept inputs from the forward circuit command interneurons AVB (electrical) and PVC (chemical) (White et al. 1986).

The wiring diagram provides a model for *C. elegans* locomotion. The cholinergic VA and VB motor neurons provide excitatory output to ventral muscle while DA and DB motor neurons excite dorsal muscle. The GABAergic motor neurons, DD and VD, provide antagonistic activity for these ventral and dorsal excitatory neurons by 'intercepting' cholinergic neuromuscular junctions (NMJs). For example, VDs send dendrites to the dorsal cord where they are post-synaptic to DA and DB motor neurons. In this manner, cholinergic excitation of muscle on one side of the animal triggers GABAergic relaxation on the other side (Figure 1.8).

Embryonic vs. larval motor neuron differentiation

DA, DB, and DD motor neurons are born during embryonic development (Sulston et al. 1983). All of these motor neurons send commissures to the dorsal cord (Figure 1.7). The DA axons turn anteriorly upon reaching the dorsal side, the DBs turn posteriorly, and the DDs send out projections in both directions. At hatching, DA and DB neurons innervate dorsal muscle whereas the DDs synapse with ventral muscle (White et al. 1978; White et al. 1986). (DD motor neurons reverse polarity to innervate dorsal muscles after hatching, see below). DA and DB motor neurons are excitatory and express acetylcholine while the DDs are inhibitory and express GABA (McIntire et al. 1993; Rand and Nonet 1997)

At the end of the L1 stage, ectodermal Progenitor cells ("P-cells") migrate from
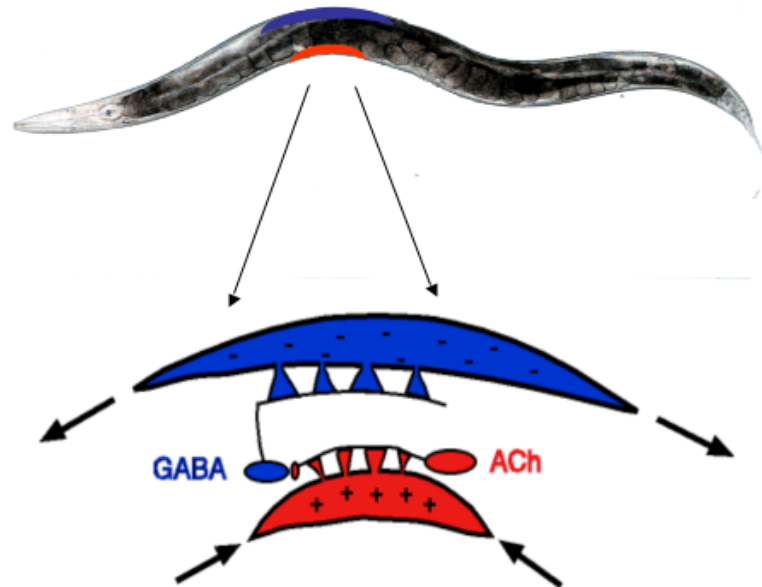
**Figure 1.8 The opposing actions of GABAergic and cholinergic motor neurons
drive dorsal/ventral contractions.**
Nematodes crawl on their sides in a sinusoidal fashion. These waves of dorsal/ventral
contraction are established by the cross-talk between GABAergic and cholinergic motor
neurons. As such, when cholinergic neurons contract muscle on one side (e.g. ventral),
GABAergic neurons inhibit muscle excitation on the opposing side (e.g. dorsal).
Figure courtesy of Janet Richmond.

lateral positions to the ventral side of the animal. Here the anterior daughters of the P-cells (P.a) undergo a stereotypical series of cell divisions to generate the 53 post-embryonic ventral cord motor neurons (Figure 1.9) These motor neurons fall into five classes: VA, VB, VC, VD, and AS. With the exception of the AS neurons, all of these classes innervate ventral muscle (Figure 1.7).

Genetic control of motor neuron differentiation in *C. elegans*

Since *C. elegans* is so amenable to genetic manipulation of the nervous system (a nematode does not need to crawl much in order to live in laboratory conditions), many mutants affecting differentiation and function of specific classes of motor neurons have been isolated. Below I will describe transcription factors involved in *C. elegans* motor neuron specification that have been largely revealed by these genetic screens.

Transcription factors affecting GABAergic motor neurons

There are 19 motor neurons in the *C. elegans* ventral nerve cord that express the neurotransmitter GABA, 13 VD and 6 DD. Described below are two transcription factors which specify combined D-class traits (UNC-30) as well as regulate a key morphological distinction between the two classes (UNC-55).

UNC-30

Mutations in *unc-30* display a "shrinker" phenotype in which muscle on the dorsal and ventral sides contract simultaneously rather than alternately as in normal movement (Jin et al. 1994). This phenotype is consistent with the observation that *unc-30* mutants

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Figure 1.9 Specific blast cells (P-cells) give rise to larval motor neurons in the ventral nerve cord.
All animals are viewed from the ventral side.
A. L1 larvae. P-cells migrate ventrally from lateral locations in an anterior-to-posterior wave in the early L1. (adapted from Sulston 1976).
B. The anterior P cell daughter (Pn.a) divides in the late L1 to generate larval motor neurons. Note deviations from stereotypical lineage of P3.a-P8.a for Pn.a cells located at anterior and posterior ends of the VNC (e.g. P1.a). "x" denotes a cell death (adapted from Chalfie and White 1988).
C. L2 larva showing the full complement of ventral cord motor neurons (small circles). Pn.p ectodermal blast cells display prominent nucleoli and are reliable landmarks in the queue of ventral cord motor neuron nuclei. (adapted from Sulston and Horvitz 1977). Ganglia at either end of the VNC are rvg (retrovesicular ganglion) and pag (preanal ganglion).

A

W

P1,P2   P3,P4   P5,P6   P7,P8   P9,P10   P11,P12

rvg

pag =

early L1

ventral cord motor neurons

rvg

pag =

mid L1

B

P cell

Pn.a   Pn.p

hypodermoblast

Neuroblast					
W	AVF	VB	X	VA	VD
P1.a	AVF	VB	X	AS	VD
P2.a	VA	VB	X	AS	VD
P3-8.a	VA	VB	VC	AS	VD
P9-10.a	VA	VB	X	AS	VD
P11.a	VA	X	X	AS	VD
P12.a	VA	X	X	PDB	VD

Legend

- A-class
- B-class
- D-class
- ASn
- VCn

C

1   2   3   4   5   6   7   8   9   10

rvg

pag =

Pn.p cell

ventral cord motor neurons

L2

20
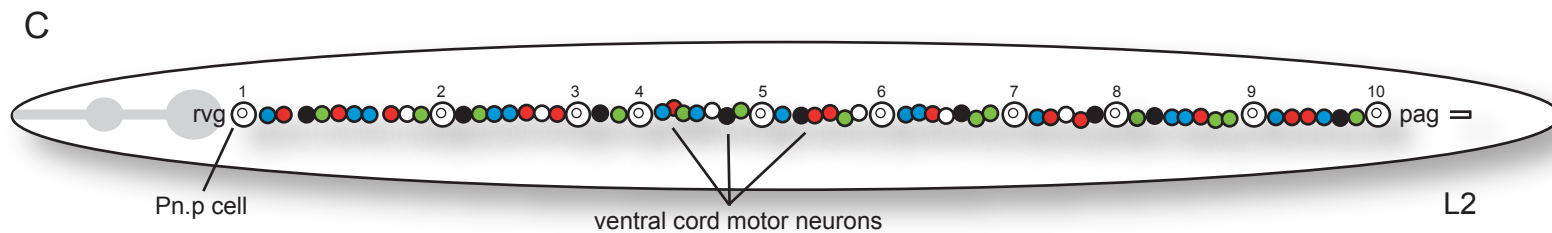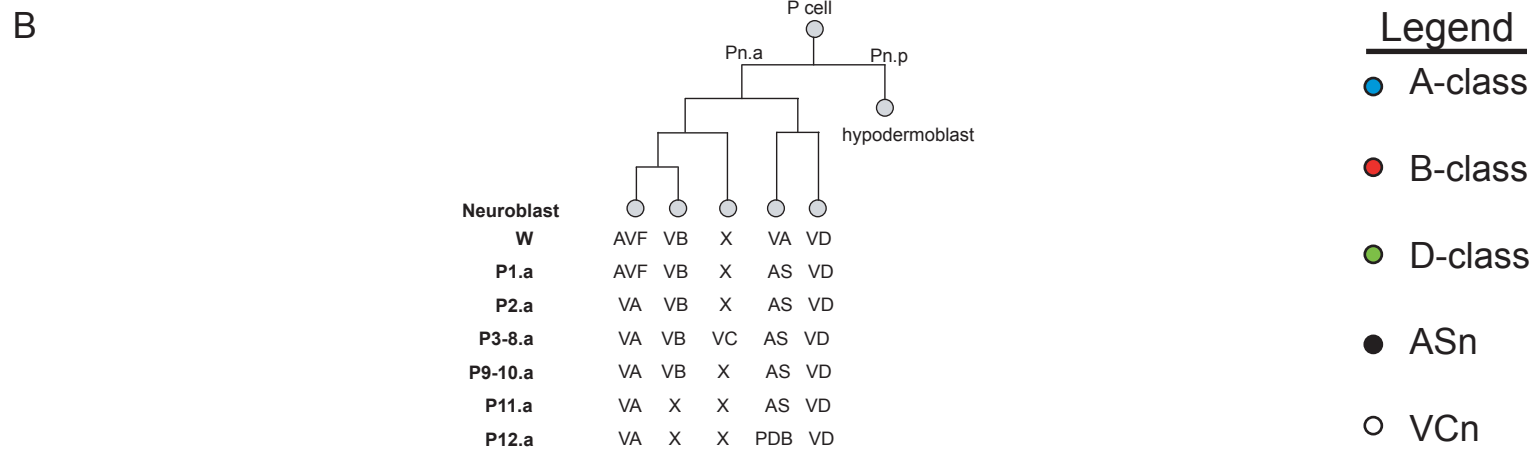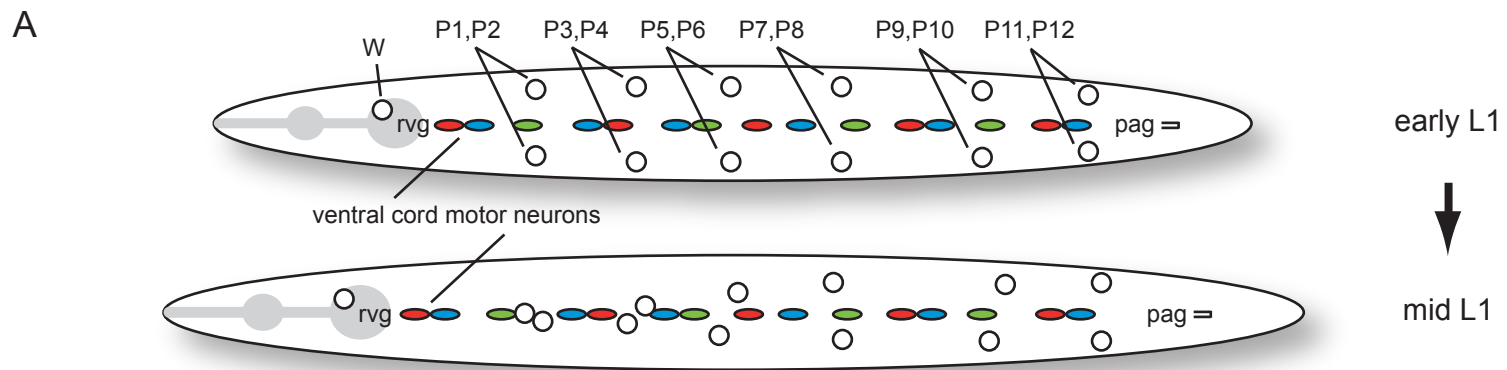
lack GABA expression in D-type motor neurons (McIntire et al. 1993). UNC-30 is the founding member of a class of homeodomain proteins, which include vertebrate Pitx genes (Jin et al. 1994; Westmoreland et al. 2001). UNC-30 is necessary in D-type motor neurons to activate expression of *unc-25*/GAD (GABA synthetic enzyme) and *unc-47*/VGAT (vesicular GABA transporter) (Eastman et al. 1999), two hallmarks of GABAergic identity. A vertebrate homolog of UNC-30, Pitx2, rescues the *unc-25* expression defect in *C. elegans*, thereby demonstrating conservation of function between these widely divergent species (Westmoreland et al. 2001). *unc-30* mutants also display some D-type axon guidance and synaptic defects (Jin et al. 1994).

UNC-55

DD motor neurons initially form NMJs with ventral muscle in the embryo. Later, after hatching, DD motor neurons remodel to innervate dorsal muscle (White et al. 1978). This polarity reversal is correlated with the birth and differentiation of VD motor neurons in the L1 that innervate ventral muscle (Walthall 1990). In *unc-55* mutants, VD motor neurons innervate dorsal muscles, similar to the DD motor neurons (Walthall and Plunkett 1995). Molecular cloning revealed that *unc-55* encodes a member of the large, diverse family of COUP nuclear hormone receptors and is expressed in VD (and AS) motor neurons shortly after their birth (Zhou and Walthall 1998). These results suggest a model in which UNC-55 blocks expression of DD genes that specify dorsal output. This idea is substantiated by an elegant experiment in which ectopic UNC-55 expression in the DDs inhibits the redeployment of DD synapses to the dorsal side (Shan et al. 2005). Concomitant with this result, a known DD gene, *flp-13*, is turned off in these neurons. It

would be worthwhile to identify the UNC-55 target genes that function in synaptic remodeling.

Transcription factors involved in specifying cholinergic motor neurons

The cholinergic neurons comprise the largest group of motor neurons in the animal, as acetylcholine is the excitatory neurotransmitter used to stimulate muscle contraction. Ablation experiments, coupled with the wiring diagram, were used to assign functions to various neurons. For example, A-class ventral cord neurons function in backward locomotion whereas B-class ventral cord neurons promote forward movement (Chalfie et al. 1985). Several genes that perturb forward and backward locomotion have been isolated and found to affect cholinergic fate. These include *unc-17*/VAChT (vesicular acetylcholine transporter) and *cha-1*/ChAT (choline acetyltransferase). This section will summarize the transcription factors that affect cholinergic motor neurons.

UNC-3

unc-3 animals are severely uncoordinated, unable to propagate body dorsoventral contractions to propel the worm forward or backward (Brenner 1974; Herman 1984). Head movements are unaffected, indicating aberrant functioning of the ventral cord circuit. In *unc-3 (e151)* mutant animals the ventral cord is highly disorganized (Durbin 1987). UNC-3 is an Olf-1/EBF transcription factor family member that is expressed in all cholinergic ventral cord motor neurons (Prasad et al. 1998). Thus, *unc-3* may be a key transcription factor necessary for defining the cholinergic fate. Consistent with this notion, UNC-17 expression is absent in ventral cord motor neurons in *unc-3* mutants, but

head expression appears unaffected (K. Lickteig, D. Frisby, J. Duerr, DMM, and J. Rand, personal communication). Current evidence suggests that UNC-3 directly binds the *unc-17* promoter to drive expression. *unc-17* β mutants contain mutations in the promoter region, specifically in a sequence which is an exact match for the O/E consensus binding site. In addition, *unc-3* mutants have improper expression of other cholinergic genes (i.e. *unc-4, acr-2*). Thus, *unc-3* appears to define both a general cholinergic fate as well as define specific cell fates.

UNC-4

  unc-4 mutant animals have a highly recognizable phenotype, the inability to crawl backward (Brenner 1974). When tapped on the head, *unc-4* mutants coil dorsally, usually adopting an inverted omega (ʊ) shape (White et al. 1992). EM reconstruction of *unc-4 (e120)* mutants revealed that a subset of VA motor neurons were miswired with connections reserved for their lineal sisters, the VBs (White et al. 1992). VA neurons normally receive chemical and electrical inputs from AVA interneurons, but in *unc-4* mutants the VAs instead receive electrical inputs from AVB interneurons and chemical synapses from PVC interneurons (Figure 1.10) (White et al. 1986, DMM unpublished results; White et al. 1992). Intriguingly, *unc-4* mutants display normal ventral cord morphology and process placement in the ventral cord, suggesting that UNC-4 controls the specificity of synaptic inputs but not other VA traits. *unc-4* encodes a Prd-like homeodomain protein that is expressed in VA motor neurons but not in presynaptic command interneurons (Miller et al. 1992) (Miller and Niemeyer 1995). An *unc-4* suppressor screen uncovered a rare, dominant mutation in *unc-37*, the nematode Groucho
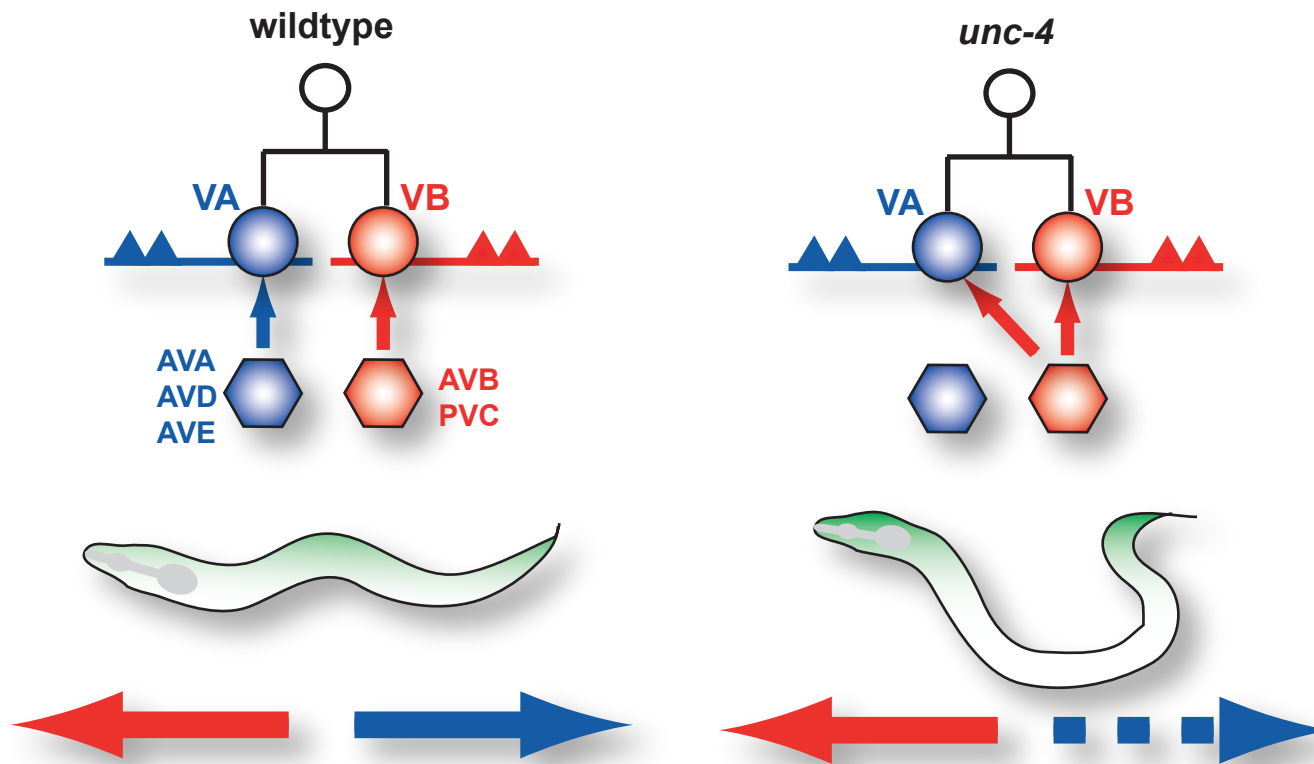
Figure 1.10 VA motor neurons are miswired with VB-type inputs in *unc-4* mutants.
Most VA and VB motor neurons arise from a common precursor and synapse with independent sets of command interneurons to control backward (blue) and forward (red) locomotion, respectively. Inputs to VA motor neurons are from AVA (gap junction, chemical synapse) and from AVD, AVE (chemical synapses). Command interneuron inputs to VBs are from AVB (gap junction) and PVC (chemical synapse). In *unc-4* mutants, VA motor neurons are miswired with inputs normally reserved for VB sisters. As a consequence, *unc-4* mutants are incapable of backward movement (dashed arrow).

co-repressor homolog (Miller et al. 1993; Pflugrad et al. 1997). Genetic and biochemical evidence suggest that the engrailed homology 1 (eh1) domain of UNC-4 and the WD repeat of UNC-37 physically interact (Winnier et al. 1999). These findings are consistent with a model in which UNC-4 and UNC-37 interact in VA motor neurons to repress VB-specific genes. This model was substantiated by the observation that GFP reporters for two VB genes, *acr-5* (acetylcholine receptor subunit) and *del-1* (DEG/ENaC subunit) are ectopically expressed in VAs in *unc-4* and *unc-37* mutants (Figure 1.15).

unc-4 is also expressed in DAs, SABs, VCs, and the pharyngeal neuron I5. These motor neurons, however, are not miswired in *unc-4* mutants (Miller and Niemeyer 1995). What does *unc-4* do in these cells? Close examination of EM sections of *unc-4* mutants revealed a 40% reduction in the number of synaptic vesicles in *unc-4* neurons (Lickteig et al. 2001). Antibody staining of synaptic vesicle proteins (such as UNC-17/VAChT) corroborated this finding (Lickteig et al. 2001). Thus UNC-4 and UNC-37 act to specify both input and output in A-class neurons. It is unclear if these two defects are linked in VA motor neurons. Evidence from vertebrate systems indicates that neuronal activity may be important in synaptic stabilization (reviewed in (Waites et al. 2005). Because UNC-4 activity is required between the L2 and L3 stages to maintain proper synaptic inputs onto VA motor neurons well after synapses have been formed, it is plausible that the lack of synaptic output leads to the improper synaptic input (Miller et al. 1992; Lickteig et al. 2001). A mechanistic explanation of these two *unc-4* synaptic defects remains to be established, however.

It is becoming increasingly clear that neural fate is specified via repressor factors. As stated above, Groucho-mediated repression, interacting via eh1-containing

homeodomains, also define specific neural fates in the mammalian spinal cord (see above) (Muhr et al. 2001). Thus, identifying the genes that UNC-4 regulates could elucidate a mechanism for neural development in vertebrates. However, the downstream targets of this repressor complex have remained elusive. Our lab has now optimized cell-specific profiling methods and uncovered a bona fide UNC-4 target gene (Chapters IV-V).

VAB-7/Evenskipped

vab-7 encodes the *C. elegans* Even-skipped homolog and functions in patterning embryonic mesoderm and in motor neuron differentiation (Ahringer 1996; Esmaeili et al. 2002). *vab-7* mutants exhibit a distinctive forward Unc phenotype, suggesting dysfunction of the B-type circuit. This model is substantiated by VAB-7 antibody staining of DB motor neuron nuclei. Esmaeili and colleagues (2002) discovered that DB motor neuron axonal polarity is reversed in *vab-7* mutants; instead of turning posteriorly upon reaching the dorsal cord, DB axons project anteriorly (Figure 1.11) (Esmaeili et al. 2002). Additionally, *unc-4*::GFP is ectopically expressed and *acr-5*::GFP is turned off in DB motor neurons in *vab-7* mutants (Esmaeili et al. 2002). Since *unc-4* is necessary for A-class fate (see above), it was predicted that the ectopic expression of UNC-4 was inducing the DB polarity switch as well as the extinction of *acr-5*::GFP expression. In *unc-4*;*vab-7* double mutants DB axons turn posterior in the dorsal cord, thereby confirming that UNC-4 is required for the Vab-7 axonal polarity defect (Figure 1.11) (Esmaeili et al. 2002). In addition, *acr-5*::GFP expression is also restored to *vab-7* mutant DB neurons by the *unc-4* mutation. The correlation of *acr-5* expression with posterior
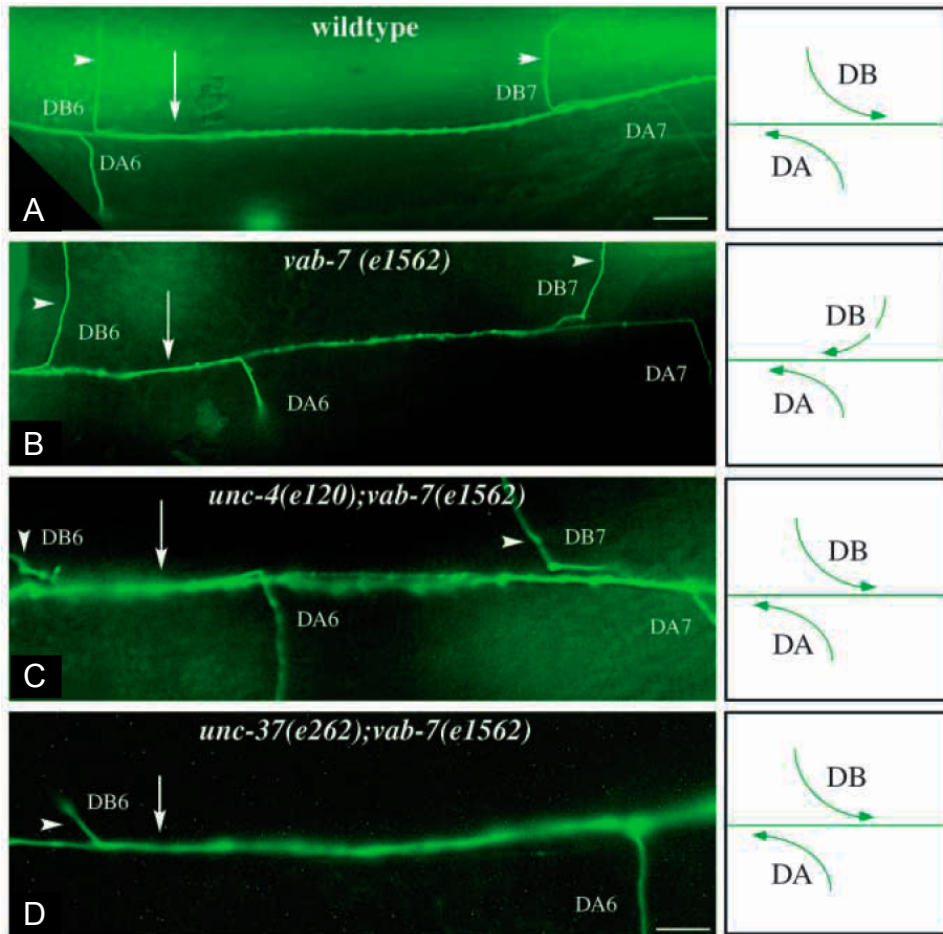
Figure 1.11 Axonal polarity of DB motor neurons is reversed in *vab-7* mutants but restored in *unc-4;vab-7* double mutants.
All panels are viewed from the dorsal side, anterior to the left. DA motor neurons turn anteriorly upon reaching the dorsal cord while DB motor neurons turn posteriorly in wildtype animals (A). In *vab-7* mutants, the polarity of DB motor neurons reverse, now resembling the anterior-projecting DA motor neurons. This reversal defect is suppressed in *unc-4; vab-7* (C) and *unc-37; vab-7* (D) double mutants.
Reprinted from Esmaeili, et al., 2002.

DB polarity could be indicative of a mechanism in which acetylcholine signaling directs DB axon outgrowth. Chapter II below outlines genetic experiments designed to test this idea.

Strikingly, both fly and vertebrate Eve proteins promote proper axonal polarity in the nervous system (Figure 1.12). As described above, in flies lacking Eve activity dorsally-projecting motor neurons are mis-specified (Landgraf et al. 1999). In the vertebrate spinal cord, in *Evx1* mutant mice V0 interneurons adopt a V1-like fate, as evidenced by a change in their axonal trajectory (Moran-Rivard et al. 2001). Thus, Eve proteins act as transcriptional repressors in diverse species to promote proper axonal outgrowth.

LIN-11

LIN-11/Lhx1, one of seven *C. elegans* LIM-HD transcription factors, is a founding member of the LIM-HD family. It is the only *C. elegans* LIM-HD transcription factor that is known to be expressed in ventral cord motor neurons. A *lin-11*::GFP reporter is expressed in head and tail neurons, the vulva and spermatheca, and VC motor neurons (Hobert et al. 1998). VC neurons are unique among the ventral cord motor neurons, in that their axons exit the cord to innervate the vulval muscle to control egg-laying (Figure 1.7). VC motor neurons are generated normally in *lin-11* mutants but display defasciculated axons (Hobert et al. 1998). This is in contrast to LIM-HD function in flies and vertebrates, where loss of a LIM-HD results in a homeotic change in cell fate. This result could mean that the role of LIM-HD proteins has evolved as neural circuits became more complex.
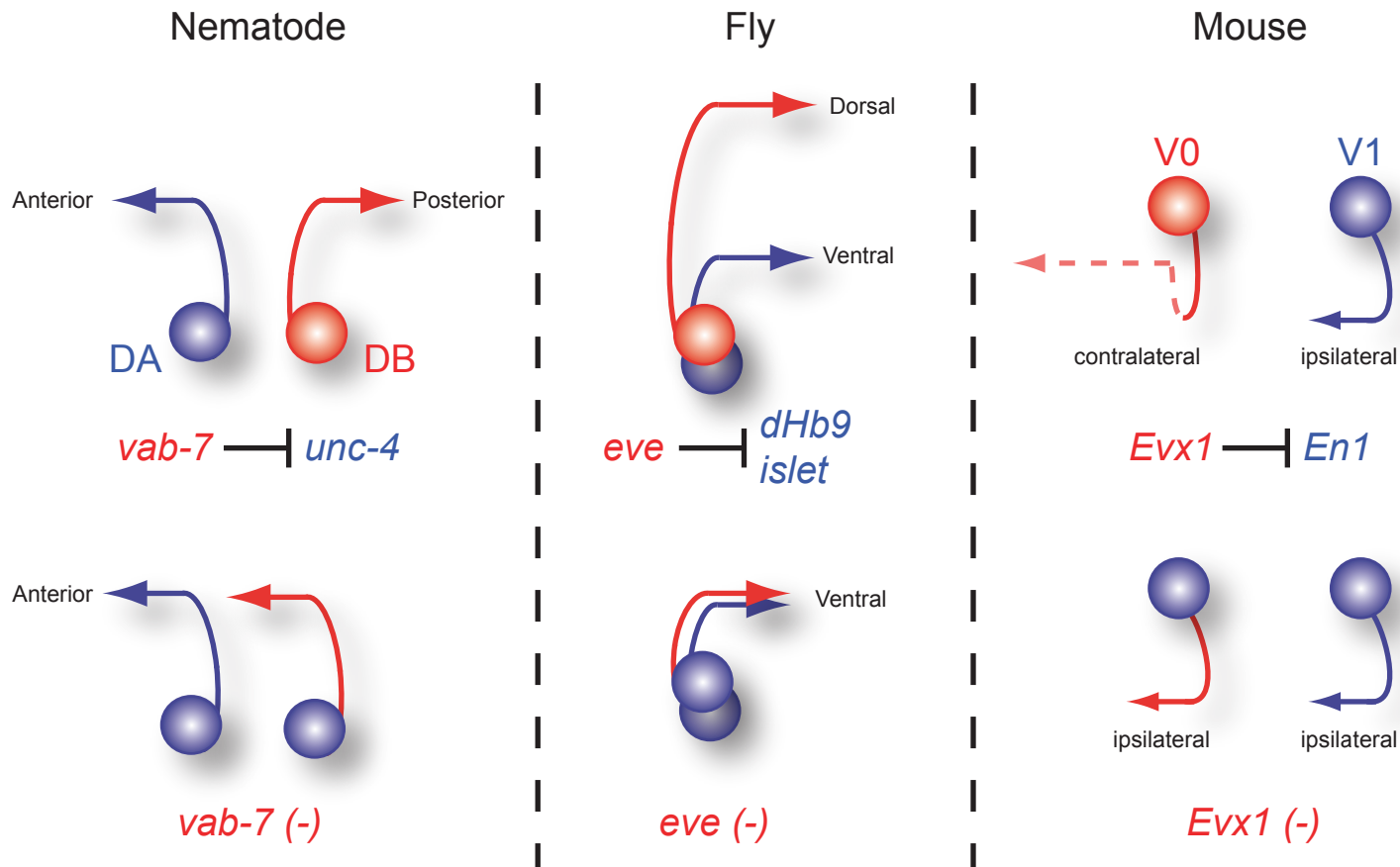
Figure 1.12 Even-skipped (Eve) transcriptional repressor activity defines neuron identity and axonal trajectory in the axial motor circuits of diverse species.

The *C. elegans eve* homolog, *vab-7*, negatively regulates *unc-4* to prevent DB motor neurons from adopting the anterior trajectory of DA motor neurons. In flies, dorsal motor neurons adopt a ventral trajectory when *islet* and *dHB9* are derepressed in *eve* mutants. The mouse *eve* homolog, *Evx1*, represses *engrailed* (*en*) to maintain the medial location and contralateral axonal trajectory of V0 interneurons in the spinal cord.

29

Mechanisms of synaptic specificity

After specification, neurons must extend processes to establish connections with target cells. Key extracellular cues and guidance receptors that govern this process have been identified. Much less is known, however, about the molecular mechanisms that control the next step - the creation of a synapse with a specific cell. Studies of the visual system of lower vertebrates led Roger Sperry to propose a "chemo-affinity" model in which each neuron displays a unique label for recognition by its cognate partner (Sperry 1963). While this model is generally accepted, the mechanisms controlling target recognition and synapse formation remain poorly understood. The aim of this section is to discuss proteins with known roles in synapse formation, such as cell adhesion molecules, and to suggest that these proteins are under genetic control.

Cadherins

Cadherins and their partner catenins have well-established roles in the formation of cell-cell junctions (reviewed in (Patel et al. 2003). There are at least five classes of cadherins: classical (type I), atypical (type II), desmosomal, protocadherins, and orphan. Classical cadherins usually form homophilic complexes between adjacent cells, although heterophilic interactions with other cadherin family members have been observed (Shimoyama et al. 2000).Thus, one can imagine a model in which neurons and their target cells express the same cadherin, which would bind the two cells together and initiate synapse formation. This model is supported by the findings that cadherins are found in a region next to the active zone at developing synapses in the vertebrate brain (Fannon and Colman 1996; Uchida et al. 1996). In the chick spinal cord, motor neuron

pools and their partner proprioceptive sensory neurons express matching type II cadherins, thereby suggesting the basis of selectivity for monosynaptic connections in this circuit (Price et al. 2002). Additional evidence suggests that cadherin-catenin complexes are involved in pre- and post-synaptic development. Expression of a dominant-negative N-cadherin in hippocampal neurons disrupts dendritic spine morphology, resulting in a loss of post-synaptic density 95 (PSD-95) puncta (Togashi et al. 2002). Additionally, β-catenin activity is needed to recruit a uncharacterized protein complex to regulate pre-synaptic development (Bamji et al. 2003). In *Drosophila,* N-Cadherin functions in the visual system to correctly target R-cells to their appropriate layer in the brain(Lee et al. 2001). Loss of N-Cadherin disrupts the topographic map in both the lamina and the medulla. Specific loss of N-Cadherin in the R7 cells results in improper termination of axons in the layer normally reserved for R8. In summary, the roles of cadherin/catenin complexes in synaptogenesis and synaptic specificity are just beginning to be elucidated. Further experiments should provide clues as to whether the adhesive role of cadherins, their signaling through catenins, or both are necessary for synapse formation. For example, one could imagine a scenario in which cadherins act to keep two neurons attached in order for other signals to specify pre- and post-synaptic specializations. Conversely, it is possible that cadherin/catenin signaling initiates a program that results in the formation of a synapse. Thus, while cadherins have important roles in synapse formation, the molecular mechanisms they employ are not well-understood.

Neurexins/Neuroligins

The neurexin transmembrane proteins and their ligands the neuroligins are highly expressed in the vertebrate brain during the time of peak synaptogenesis (Ruegg 2001). Neuroligins-1, -3, and -4 localize to the postsynaptic density of excitatory glutamatergic synapses while neuroligin-2 is primarily found at inhibitory GABA synapses (Song et al. 1999; Graf et al. 2004). Intriguingly, non-neuronal cells induced to express neuroligins cluster synaptic vesicle proteins to the sites where axonal growth cones contact them, a striking example of the synapse-forming potential of these molecules (Scheiffele et al. 2000). In cultured hippocampal cells, oligomerized neuroligin-1 can induce β-neurexin clustering in the pre-synaptic cell, which then recruits synaptic vesicles (Dean et al. 2003).These data are consistent with a model in which post-synaptic neuroligin induces pre-synaptic specialization on target neurons via its interactions with neurexin. Furthermore, neuroligins may be recruited to the synapse by a post-synaptic mechanism independent of its association with neurexin and post-synaptic density protein 95 (PSD-95) (Dresbach et al. 2004). These data suggest that neuroligin targeting to the synapse may be an early step in the assembly of the synaptic scaffold (Dresbach et al. 2004).

As mentioned above, distinct neuroligin isoforms are expressed in excitatory and inhibitory neurons. It was recently shown that neurexin at glutamatergic synapses signals via neuroligin-1 to promote glutamate receptor aggregation and other glutamate post-synaptic scaffolding proteins. Additionally, neurexin-neurligin-2 signaling at GABAergic synapses recruits the GABA synthetic enzyme glutamic acid decarboxylase (GAD) and the vesicular GABA transporter (VGAT) to the pre-synaptic area and induces clustering of $GABA_A$ receptors at the post-synaptic specialization (Graf et al. 2004). So what

defines the type of synapse that will be made? It appears to depend upon the levels of PSD-95 expressed in the post-synaptic cell. Increased expression of PSD-95 results in the sequestration of neuroligin-2 in excitatory synapses (Levinson et al. 2005). In summary, neuroligins and neurexins are sufficient to induce pre- and post-synaptic development in excitatory and inhibitory synapses.

Ig domain proteins

Some members of the neural CAM family contain extracellular Ig domains. It is becoming increasingly apparent that Ig domain proteins have important roles in synapse formation and specificity. For example, in *Drosophila* Fasciclin II is expressed in motor neurons and target muscle fibers, where it is required for synapse stabilization (Schuster et al. 1996). Two fly sidekick proteins, *sdk-1* and *sdk-2*, are expressed in non-overlapping retinal neurons that project to specific sublaminae in the inner plexiform layer (IPL). These neurons are directed to stop in specific sublaminae by expression of the same SDK in the cognate partner neurons (Yamagata et al. 2002). Furthermore, ectopic expression of SDK1 in non-SDK+ neurons is sufficient to direct projections to SDK1+ sublaminae (Yamagata et al. 2002). In vertebrates, SynCAM is a brain-specific Ig protein that is localized to synapses (Biederer et al. 2002). Expression of SynCAM in non-neuronal cells is sufficient to induce functional synapses when co-cultured with hippocampal neurons (Biederer et al. 2002). In *C. elegans*, the Ig proteins SYG-1 and SYG-2 form a heterophilic adhesion complex that acts as a guidepost to direct HSN neurons to synapse onto VC motor neurons and vulval muscles (Shen and Bargmann 2003; Shen et al. 2004). Intriguingly, SYG-2 does not function in neurons, but instead in the adjacent vulval

epithelium. SYG-2 is expressed in vulval epithelium at the site of the future synapses and induces SYG-1 clustering in HSN axons, which then recruits synaptic vesicles and presumably other pre-synaptic machinery. There are many uncharacterized Ig superfamily proteins in *C. elegans*; it would be worthwhile to determine if any others are involved in a similar manner to direct synaptic specificity.

One of the most intriguing Ig domain proteins is DSCAM (Down's Syndrome Cell Adhesion Molecule). In *Drosophila, Dscam* encodes an adhesive protein with ten Ig domains and six fibronectin repeats. Remarkably, *Dscam* has ~38,000 potential splice isoforms by alternatively splicing variable exons in a mechanism reminiscent of the generation of antibody receptor diversity (Figure 1.13) (Schmucker et al. 2000). Current evidence suggests that many of these isoforms are indeed expressed *in vivo* (Schmucker et al. 2000; Hummel et al. 2003; Neves et al. 2004; Zhan et al. 2004), suggesting a model in which this extensive diversity of Dscam is utilized by the nervous system as a recognition code for synaptic specificity (Schmucker et al. 2000). Consistent with this idea, a single Dscam molecule binds homophilically and not heterophilically with other Dscam isoforms (Wojtowicz et al. 2004). *Dscam* mutant flies exhibit a wide variety of neuronal phenotypes, including axon branching defects in the mushroom body (MB) lobes and incorrect glomerulus targeting in the olfactory system (Wang et al. 2002; Hummel et al. 2003). However, the MB defect can be rescued by isoform specific expression, thus indicating that distinct Dscam isoforms are not necessary for proper MB morphology (Zhan et al. 2004). Thus, a direct role for Dscam in the specification of synaptic targets has not been determined.

**Transcriptional control of synaptic specificity**

As described in detail above, transcription factors are essential for delineating motor neurons from other neurons in the spinal cord, as well as for specifying subtypes of motor neurons. It seems likely that synaptic connectivity is also under genetic control. In vertebrates, ETS family transcription factors, such as Er81 and PEA3, are strong candidates for this role, as they are expressed in motor neurons innervating a single muscle (Lin et al. 1998). In addition, these factors are expressed early in the development of motor and sensory neurons, suggesting that they could be critical determinants of identity. In support of this idea, *Er81* mutant mice have defects in synaptic connectivity leading to severe motor discoordination (Arber et al. 2000). Surprisingly, however, this defect does not affect the specification formation of neuromuscular junctions between motor neurons and muscle. Instead, distinct sensory neurons that function in the stretch reflex circuit fail to terminate in the ventral spinal cord, thus reducing the number of direct connections onto motor neurons. Since these sensory neurons appear to be generated normally, these data suggest that Er81 acts in these cells post-mitotically to influence synaptic connectivity. In support of this idea, neurotrophin-3 (NT3) mutant mice display a synaptic defect similar to *Er81* mutants; in *NT3* mutants, sensory neurons fail to express Er81, thus explaining the phenotype (Patel et al. 2003). In contrast to Er81, PEA3 is necessary in motor neurons for proper muscle targeting and arborization (Ladle and Frank 2002; Livet et al. 2002).

In the *Drosophila* olfactory system, the lineage of Projection Neurons (PN) and birth order is correlated with specific glomerular targets (Jefferis et al. 2001). Transcription factors have been shown to interpret this lineage to specify synaptic
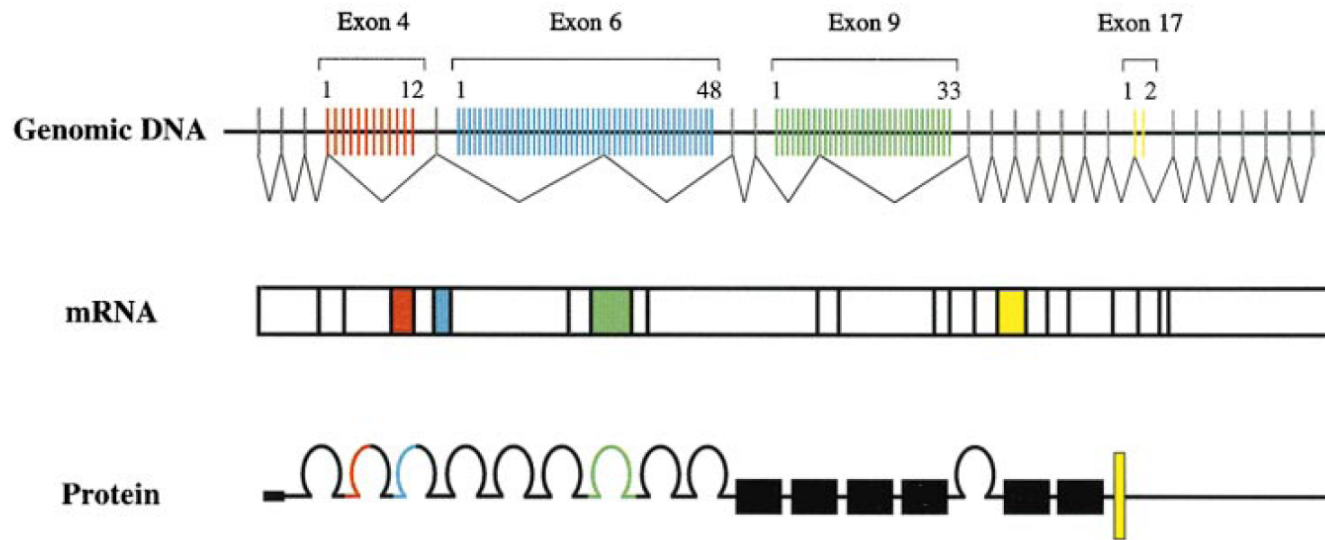
Figure 1.13 Alternative splicing of *Drosophila* Dscam can potentially generate ~38,000 isoforms.
Dscam contains four variable exons, three of which encode for portions of the extracellular Ig domains while the other specifes the transmembrane domains.
Reprinted from Schmucker, et al., 2000.
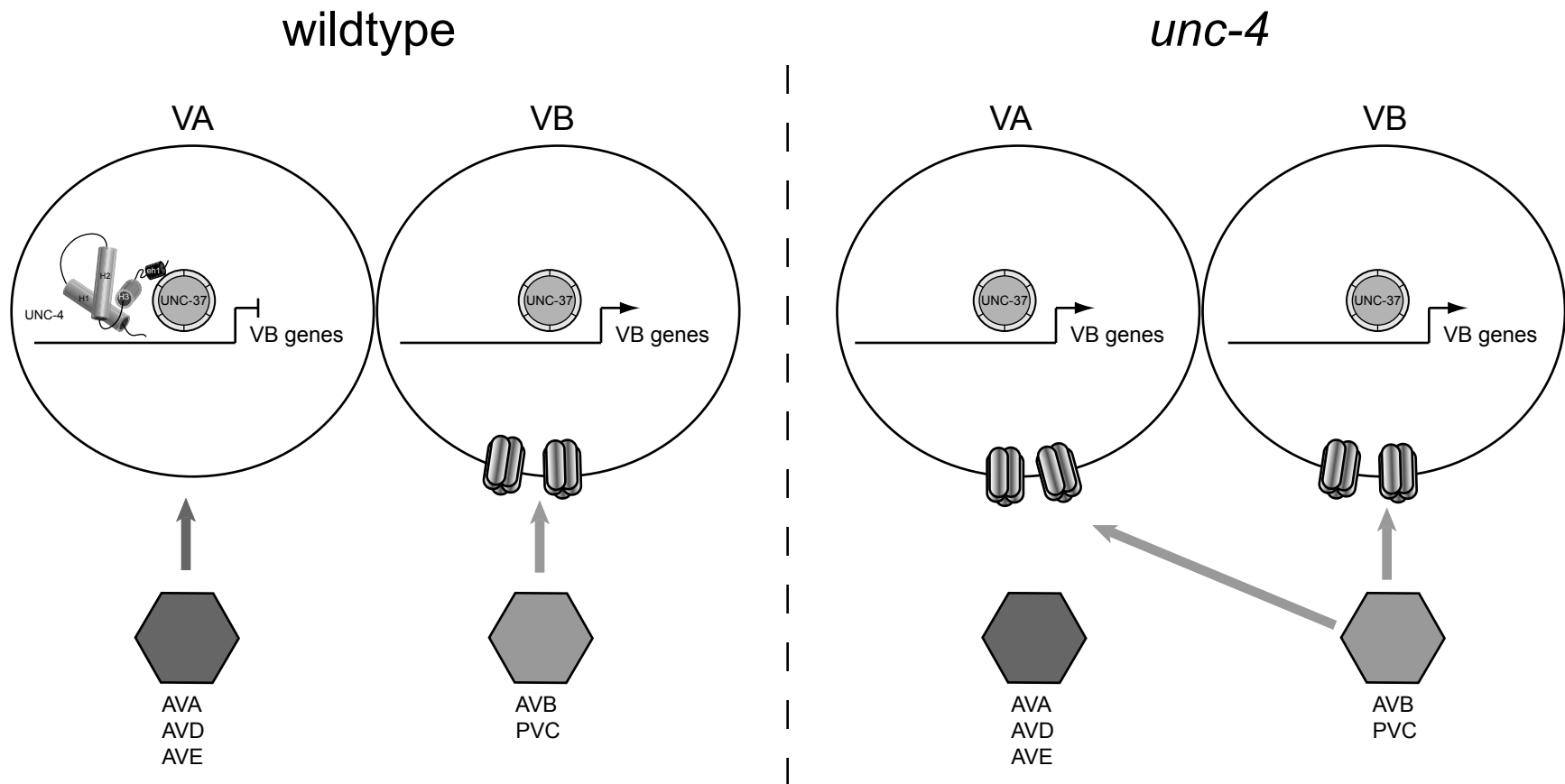
Figure 1.14 Model of UNC-4 regulated synaptic specificity.
In wildtype VA motor neurons, UNC-4 and its corepressor UNC-37/Groucho repress VB specific genes to specify pre-synaptic inputs from AVA, AVD, and AVE interneurons. In VB motor neurons, VB genes are not repressed and thereby mark these neurons for inputs from AVB and PVC interneurons. In *unc-4* mutant VA motor neurons, derepression of VB genes labels VAs as "VB-like" such that they now receive inputs from AVB and PVC.
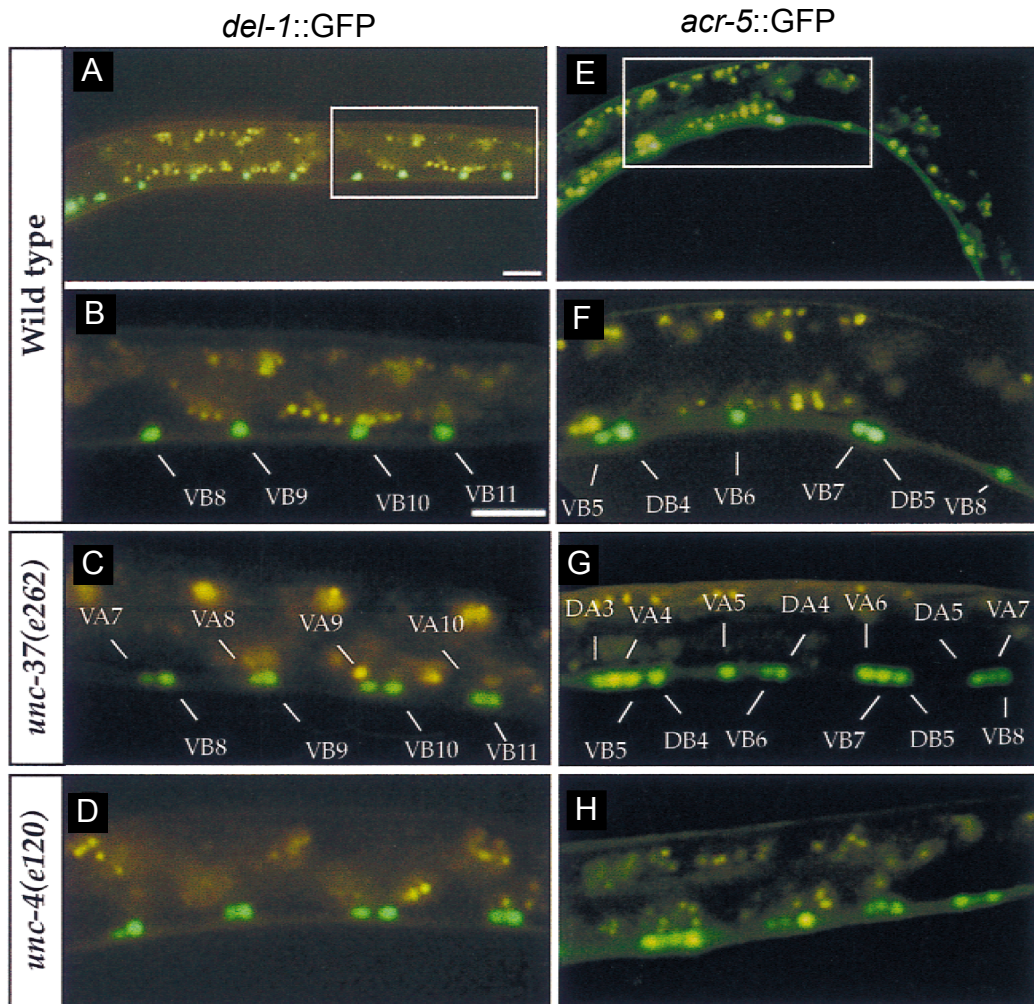
Figure 1.15 *del-1*::GFP and *acr-5*::GFP are negatively regulated by UNC-4 and UNC-37 in A-class neurons.

del-1::GFP is expressed in VB motor neurons in the ventral nerve cord (VNC) in wildtype animals (A,B). In *unc-4 (e120)* and *unc-37 (e262)* mutants, ectopic expression is detected in VA motor neurons (C,D). Similarly, *acr-5*::GFP is expressed in DB and VB motor neurons in wildtype animals (E,F) and is depressed in DA and VA motor neurons in *unc-4* and *unc-37* mutants (G,H). Scale bars = 20 μm.

Scale bar in A applies to A and E, scale bar in B applies to B-D and F-H. Adapted from Winnier, et al., 1999.

connectivity. The POU domain proteins Acj6 and Drifter act in non-overlapping PNs to correctly target PN axons onto their partner glomeruli (Komiyama et al. 2003). A similar transcriptional mechanism acts in the *Drosophila* visual system to correctly target photoreceptor axons to the proper ganglia (reviewed in (Tayler and Garrity 2003). Normally the R1-R6 photoreceptor neurons project to the lamina while R7 and R8 axons migrate past the lamina to synapse in two distinct layers of the medulla. In *brakeless* mutants, a majority of R1-R6 axons fail to terminate in the lamina and instead continue into the medulla (Rao et al. 2000; Senti et al. 2000). Brakeless encodes a novel nuclear protein with two isoforms, one of which contains a putative zinc-finger domain, suggesting that it may be involved in regulating gene expression. In support of this idea, the transcription factor Runt is de-repressed in R2 and R5 cells in *brakeless* mutants; in addition, ectopic expression of Runt in R2 and R5 phenocopies *brakeless* mutants (Kaminker et al. 2002). Together, these data indicate the importance of transcriptional repression in the olfactory and visual systems in flies.

As described above, in *C. elegans* the UNC-4 homeodomain transcription factor, along with its co-repressor UNC-37/Groucho, act in VA motor neurons to control synaptic specificity. Most VA motor neurons arise from a common lineage with VB motor neurons. The VAs adopt inputs from AVA, AVD, and AVE command interneurons to control backward movement, while the forward circuit interneurons AVB and PVC synapse onto VBs (Figure 1.10). In *unc-4* mutants, a subset of VA motor neurons are miswired, now accepting inputs from the B-type interneurons. As a consequence, *unc-4* mutants cannot crawl backward.

UNC-4 has been shown to physically interact with UNC-37/Groucho via its eh1 domain. These data suggest that UNC-4 acts as a repressor. Our model proposes that UNC-4 and UNC-37 act in VA motor neurons to repress VB-specific genes; loss of UNC-4 activity results in de-repression of these VB genes that control the miswiring event (Figure 1.14). This model was substantiated by the identification of *unc-4* regulated genes (Figure 1.15). *acr-5* encodes an acetylcholine receptor subunit; an *acr-5*::GFP reporter is expressed in VB and DB ventral cord motor neurons (Winnier et al. 1999). When *acr-5*::GFP is crossed into *unc-4* and *unc-37*, ectopic expression is seen in VA and DA motor neurons. Similarly, in L2 animals, a reporter gene for the VB-specific *del-1* DEG/ENaC subunit is ectopically expressed in VA motor neurons in *unc-4* and *unc-37* mutants.

Given the fact that several membrane proteins have been associated with synaptic connectivity, as outlined above, the ion channel subunits ACR-5 and DEL-1 are attractive candidates to mediate synaptic specificity in the *C. elegans* motor circuit. Genetic experiments described in Chapter II, however, rule out a role for these proteins in synaptic choice. Querying the whole genome in search of UNC-4 regulated transcripts revealed that CEH-12, the nematode HB9 gene, is an authentic UNC-4 target gene that likely acts downstream to control inputs to VA motor neurons (Chapters III, IV, V).