CHAPTER VI

GENERAL DISCUSSION AND FUTURE DIRECTIONS

It is clear that transcriptional repression plays a pivotal role in neuronal differentiation. In this work, I have investigated the mechanism by which the transcriptional repressor complex, UNC-4 and UNC-37/Groucho, acts in VA motor neurons to control synaptic specificity in *C. elegans*. To this end, I employed powerful new methods to generate cell-specific transcriptional profiles of post-embryonic cells. Using these strategies I queried the genome for *unc-4* regulated genes. My search identified CEH-12/HB9, an evolutionarily conserved transcription factor that specifies motor neuron differentiation in diverse species. This discussion will reiterate the importance of transcriptional repression in the nervous system and propose future directions to identify the *unc-4* regulated molecules that directly control synaptic choice.

The de-repression model of neural induction

As described in Chapter I, non-overlapping homeodomain proteins delineate interneuron and motor neuron progenitor domains in the developing vertebrate ventral spinal cord (Figure 1.3) (reviewed in Shirasaki and Pfaff, 2002). For example, motor neuron progenitors co-express Nkx6.1, Pax6, and Olig2 while the adjacent P2 interneuron progenitor domain is labeled by Nkx6.1, Pax6, and Irx3. These progenitor boundaries are sharpened by the Groucho/TLE-dependent cross-repressive interactions of these transcription factors; for example, Irx3 restricts Olig2 to more ventral domains (Muhr et

al. 2001). These data suggest a model in which neural fates are induced indirectly by a "de-repression" mechanism: genes that specify neuron identity are activated by negatively regulating repressor proteins. In other words, the combinatorial expression of unique transcription factors effectively "turns off the wrong genes and allows expression of the right ones". For instance, in motor neuron progenitors, Nkx6.1, Pax6, and Olig2 negatively regulate genes expressed in interneuron progenitors (e.g. Irx3, Dbx2) and thereby indirectly promote the expression of motor neuron (MN) determinants (i.e. HB9). This model implies, therefore, that general activators are present in all neural progenitors to promote the expression of these motor neuron determinants. Recently, Sam Pfaff's group provided direct evidence that supports this idea (Lee et al. 2004). The general activators Sp1 and E2F activate a low level of HB9 expression in all spinal cord neurons. Transcriptional repressors (i.e. Irx3 in P2 progenitors) utilize distinct modules in the HB9 promoter to repress expression in non-MN domains. These repressor factors are excluded from MN progenitors by the combined repressive actions of Nkx6.1, Pax6, and Olig2. Additionally, MNs express transcription factors (i.e. Isl1) that enhance the expression of HB9. In this manner, inducers of neural fate are de-repressed in the correct progenitor domains.

In invertebrate ventral nerve cords, a similar de-repression model of neural fate is beginning to emerge. In *Drosophila*, the cross-repressive relationship in motor neurons between Even-skipped (Eve) and dHb9 delineates dorsal vs ventral projections, respectively (Broihier and Skeath 2002; Fujioka et al. 2003). In *C. elegans*, the identity of a subset of ventral cord motor neurons is determined by repressor proteins (Figure 6.1).

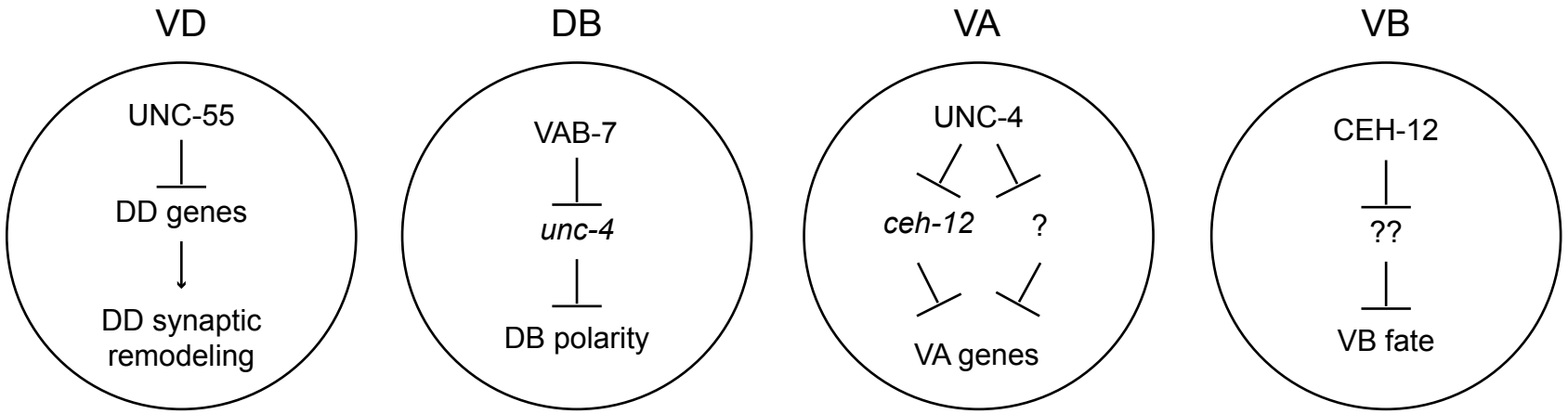

Figure 6.1 Model of repressor protein function in the specification of *C. elegans* ventral cord motor neurons. In VD motor neurons, the nuclear hormone receptor UNC-55 inhibits VDs from adopting the synaptic output of DDs by preventing the expression a DD genetic program. The VAB-7/Eve factor functions in DB motor neurons to repress the A-class specifier *unc-4* to properly specify DB axonal polarity. In VA motor neurons, UNC-4 represses at least two factors, including *ceh-12*/*HB9*, to specify A-type synaptic inputs. In VB motor neurons, it is reasonable to assume that CEH-12/HB9 negatively regulates unknown genes to induce VB fate.

For example, UNC-55 acts in larval VD motor neurons to repress an embryonic DD genetic program (Shan et al. 2005). DB motor neurons are specified, in part, by the VAB-7/Eve repression of the DA-gene *unc-4* (Esmaeili et al. 2002) whereas UNC-4 acts in A-class motor neurons to repress the VB-gene *ceh-12/HB9* (Chapter V). Since HB9 proteins are likely transcriptional repressors (Thaler et al. 1999; Broihier and Skeath 2002; William et al. 2003), CEH-12 could function in VB motor neurons to repress a transcription factor that acts in a different motor neuron (e.g. the DB gene *vab-7*). The de-repression of these downstream genes profoundly affects cell fate. For example, in *unc-55* mutants, VD motor neurons adopt the synaptic pattern of the DDs (Walthall 1990). Loss of *vab-7* activity in DB motor neurons results in axonal polarity reversal (Figure 1.11, Chapter I) (Esmaeili et al. 2002). In VA motor neurons, UNC-4 activity is required to specify pre-synaptic inputs; in *unc-4* mutants a subset of VAs are miswired with inputs reserved for their sisters, the VBs, and as a consequence these animals cannot crawl backward (Figure 1.10, Chapter I) (White et al. 1992). In contrast, loss of *ceh-12* does not greatly perturb VB fate; *ceh-12* mutants do not have an obvious forward movement defect, and known VB markers (e.g. *acr-5, del-1*) are expressed normally. It is possible that CEH-12 acts redundantly with another gene to specify VB fate.

In summary, it is clear that the induction of neural fates, axonal trajectories, and synaptic specificities is regulated by transcriptional repression. Continued studies in vertebrates and invertebrates should uncover the downstream genes that function in these pathways.

Gene expression profiling the nervous system

As described in this work, the isolation of cell-specific mRNAs is a powerful strategy to determine the 'fingerprint' of a cell-type. By using mRNA-tagging, we identified ~1600 genes that are enriched in the larval *C. elegans* nervous system (Chapter IV). These data are remarkably robust, detecting the presence of genes expressed in as few as two neurons. Over half the genes (52%) in the pan-neural enriched dataset have human homologs, suggesting that this dataset contains many genes with functions in the nervous system of diverse species.

Our work has also identified ~415 genes that are enriched in larval A-class motor neurons (Chapter IV). In this mid-L2, only ~20 neurons express the homeodomain gene *unc-4*. By optimizing the mRNA-tagging method, we were able to 'push the boundary' to obtain a rich, cell-specific profile from this limited number of cells. The specificity of these data is confirmed in part by the observation that *unc-4* is the most highly enriched transcript in this list. Furthermore, several genes with known functions in A-class neurons were enriched, such as the acetylcholine synthetic enzyme choline acetyltransferase (*cha-1*) and the vesicular acetylcholine transporter (*unc-17).*

The main motivating factor for developing and optimizing these cell-specific profiling techniques was to find candidate UNC-4 target genes. UNC-4 is a fascinating transcription factor because it controls one aspect of VA fate, synaptic specificity. In *unc-4 (e120)* mutants, VA motor neurons are miswired with presynaptic inputs from interneurons that innervate their sister cells, the VBs; VA process placement and morphology appear wildtype. We have proposed that the VA wiring defect arises from ectopic expression of the VB-specific transcription factor CEH-12. CEH-12 in turn may

repress VA genes to block the creation of normal inputs. Thus, our expression profile of VA motor neurons may include genes that are involved in pre-synaptic target selection. As mentioned in Chapter I, several cell adhesion proteins have been implicated in synaptogenesis. For example, the Ig domain protein SYG-1 functions with its partner SYG-2 to define specific connections in the *C. elegans* egg laying circuit (Shen and Bargmann 2003; Shen et al. 2004). Two genes encoding Ig proteins, *syg-1* and *rig-6*. are enriched in larval A-class neurons. We have obtained mutants for both genes, and neither mutant has a backward movement defect. This observation suggests that neither Ig protein is necessary for proper wiring of VA motor neurons. Alternatively, these genes could act redundantly. This idea could be tested by generating the double mutant (*syg-1; rig-6*) and determine if it phenocopies the Unc-4 defect. If so, then profiling A-type command interneurons (AVA, AVD, AVE) could identify a partner gene that functions with RIG-6 and SYG-1 in VA synaptogenesis (see below).

Using cell-specific profiling techniques to find transcription factor target genes

The UNC-4 homeodomain protein functions with its co-repressor protein UNC-37/Groucho in VA motor neurons to specify presynaptic inputs (Pflugrad et al. 1997). My genetic experiments have determined that this transcriptional complex regulates at least partially redundant genes (Chapter II). In fact, Unc-4 suppressor screens have failed to identify downstream targets. Thus, we set out to identify the full complement of UNC-4 regulated genes by querying the genome with cell-specific mRNA. Our mRNA-tagging studies revealed ~280 *unc-37* regulated genes, a subset of which are likely also regulated by *unc-4*. As described in Chapter IV, we determined that one of these genes, *ceh-12*, the

nematode HB9 homolog, is an authentic target. *ceh-12*::GFP is expressed in VB motor neurons and negatively regulated by UNC-4 and UNC-37 in the VAs (Figure 5.2). Furthermore, our genetic experiments place CEH-12 downstream of UNC-4 in the synaptic specificity pathway (Chapter IV). Thus, this work has demonstrated that cell-specific profiling strategies can identify bona fide transcription factor target genes.

mRNA-tagging vs. MAPCeL

Our lab has utilized two cell-specific profiling methods for gene expression profiling in *C. elegans*. Micro Array Profiling C. elegans celLs (MAPCeL) uses fluorescence-activated cell sorting to enrich for specific GFP-tagged embryonic cells (Fox et al. 2005). This technique is quite robust and easily implemented. Generation of GFP-expressing transgenics is facile and well-established. RNA extraction and amplification methods are improving dramatically. The availability of a FACS machine and skilled technician is essential and should be available at most major research universities. However, MAPCeL has limitations. For example, post-embryonic cell types do not arise in the cultures. Furthermore, the enrichment of a limited number of GFP-expressing cells by FACS is difficult. Also, exact staging of cell age is not possible. Thus, MAPCeL is a robust technique for profiling subsets of embryonic cells but is not applicable to postembryonically derived cells and may be insensitive to developmentally regulated or context dependent gene expression.

In contrast, mRNA-tagging can be used to isolate transcripts from post-embryonic cells, and because this method is applied to intact animals, it should be capable of providing authentic temporal profiles of cell-specific gene expression. Although not

tested, mRNA-tagging should work to profile embryonic as well. Thus, stage-specific profiling is possible using this method. As demonstrated in Chapter IV, mRNA-tagging generates rich profiles of specific cell-types. However, it is difficult to obtain cell-specific 3XFLAG::PAB-1 expressing transgenic animals; our empirical data suggest that the only solution is to create several transformed lines and screen them for the expected expression pattern. While we have succeeded in improving the method, the remaining background RNA could hinder attempts to generate deeper profiles of single-cells. This technique will detect the highest enriched genes in a given cell-type, though, which should aid in the understanding of that cell type. For our future experiments, we will continue to use mRNA-tagging because our interests lie in post-embryonic synaptic specification.

Future Directions

Our genomic strategies have revealed an authentic UNC-4 target gene, CEH-12. Our genetic experiments have determined that CEH-12 acts redundantly with at least one other gene to control synaptic specificity in VA motor neurons. It is reasonable to conclude, therefore, that our datasets contains other bona fide regulated genes. In *C. elegans*, RNAi is a powerful method for functional screens of large numbers of genes (Kamath and Ahringer 2003; Kamath et al. 2003). However, the current evidence indicates that neurons are largely refractory to the effects of systemic RNAi. We have confirmed this observation; feeding wildtype nematodes bacteria expressing *unc-4* dsRNA produces an incompletely penetrant, weak Unc-4 defect (R. Fox, SEV, DMM, unpublished data). In contrast, nematodes fed with the muscle-specific *unc-15* dsRNA

produces a highly penetrant, strong Unc defect (R. Fox, SEV, DMM, unpublished data). Several researchers have identified mutants that are super-sensitive to RNAi (Simmer et al. 2002; Kennedy et al. 2004). For example, the exonuclease ERI-1 functions as a negative regulator of RNAi by degrading small, interfering RNAs (siRNAs) (Kennedy et al. 2004). Recently, the Ruvkun lab found that members of the Rb pathway function in parallel with ERI-1 to negatively regulate RNAi and that the combination of *eri-1 (0)* with mutants in the Rb pathway (e.g. *lin-15b*) results in a strain in which RNAi against neuronal genes is highly effective (Sieburth et al. 2005; Wang et al. 2005). Thus, we could utilize this strain (*eri-1; lin-15b*) to identify genes in our *unc-37* regulated dataset that may function in parallel with CEH-12 to control VA synaptic specificity. Since *ceh-12 (0)* can suppress the weak *unc-4 (ts)* allele, the creation of an *unc-4 (ts); eri-1 (0); lin-15b (0)* will allow us to detect other genes that can also restore backward locomotion. Additionally, generating a *ceh-12 (0); unc-4 (0); eri-1 (0); lin-15b (0)* strain allows us to identify *unc-37* regulated genes that interact with *ceh-12* to fully suppress strong *unc-4* alleles. Positive genes for which mutants are available will be re-tested for *unc-4* suppression, and GFP reporters will be assayed for regulation by *unc-4* and *unc-37*.

We are also taking a candidate approach to identify the other presumptive UNC-4 target genes. The interaction of Nkx6 and HB9 proteins in specifying motor neuron fate in vertebrates and flies is well-established. Since the nematode Nkx6 homolog, *cog-1*, is normally expressed in VB (and VA) motor neurons and is upregulated in my *unc-37* dataset and, it is a strong candidate UNC-4 target gene. A new student in the lab, Judsen Schneider, is now testing this idea by determining if *cog-1* alleles enhance *ceh-12* suppression of *unc-4* mutants.

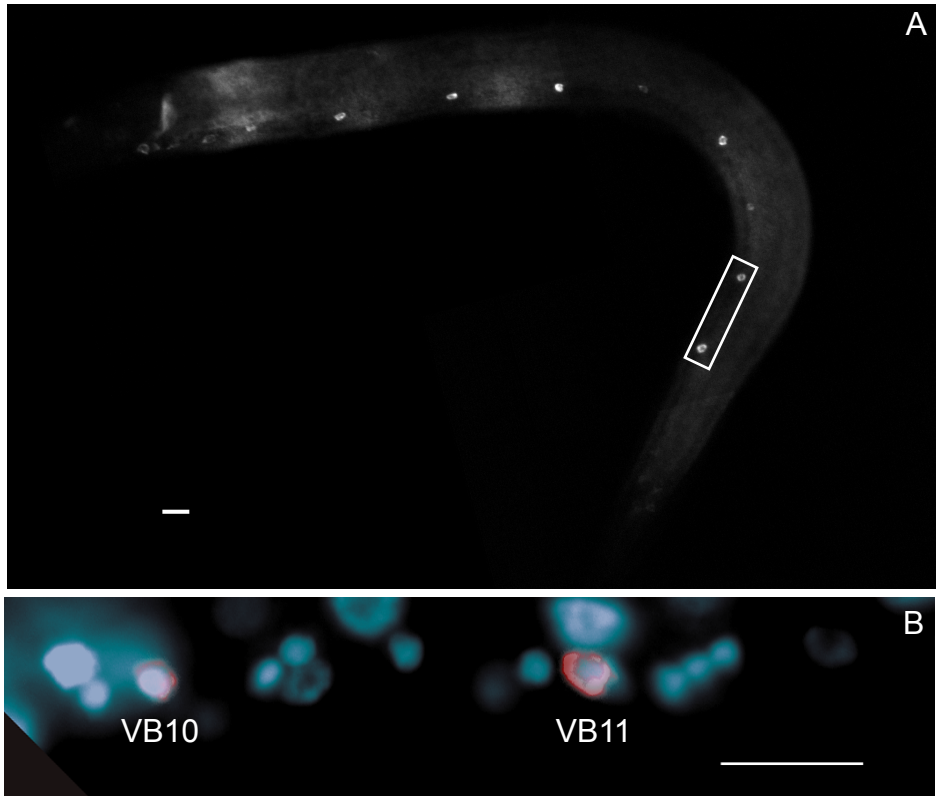

Figure 6.2 *ceh-12*::3XFLAG::PAB-1 expression in L2 larvae.

A. Antibody staining detects FLAG::PAB-1 expressing VB neurons (white circles).

B. Close-up of ventral cord (boxed image in A), showing anti-FLAG staining (red) in cytoplasm surrounding only VB nuclei (DAPI, blue).

Anterior is left, ventral is down. Scale bars = 10 µm

Application of the mRNA-tagging strategy to define other neural specificity genes

We will profile wildtype VB motor neurons in L2 larvae using a *ceh-12*::3XFLAG::PAB-1 transgene (Figure 6.2). This dataset will be extremely valuable as it will enable us to 1) determine the genetic similarities and differences between two sister cells and 2) identify the subset of our *unc-37* regulated genes that are enriched in VB motor neurons.

Command interneurons must express specific determinants to pair them with their target motor neurons. Thus, generating a profile the A-type and B-type command interneurons at the mid-L2 stage should identify genes that could function in this role. To perform these experiments, we need promoters which express only in A-type and B-type command interneurons. Clay Spencer, a former RAII in our lab, identified a fragment of the *nmr-1* promoter that drives expression in AVA, AVD, AVE, and PVC interneurons. Crossing this transgene into *ceh-14* mutants ablates expression in PVC neurons, thus creating an A-type interneuron-specific driver. Furthermore, at the recent International *C. elegans* meeting we learned of an AVB specific promoter (Denise Walker, personal communication). Thus, we can begin to assemble the genes that function in the A-type motor circuit vs the B-type motor neurons and identify genes with differential expression in either circuit, such as cell adhesion molecules. For example, if different neurexin/neuroligin pairs were found in the two circuits we could test them for roles in defining synaptic specificity.

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