CHAPTER II

SYNAPTIC SPECIFICITY DEPENDS ON A SUBSET OF UNC-4 REGULATED GENES

Introduction

The UNC-4 homeodomain protein specifies presynaptic inputs to VA motor neurons. In *unc-4* mutants, VAs are miswired with inputs from interneurons normally reserved for VB sister cells. As a result, *unc-4* mutants are incapable of crawling backward. Our evidence indicates that UNC-4 functions in VAs in concert with the UNC-37/Groucho co-repressor protein to inhibit the expression of VB-specific genes (Figure 1.14). This model, then, predicts that these VB genes induce connections with B-type command interneurons AVB and PVC. Thus, loss of these VB-genes would cause a forward movement defect, as inputs to VBs would be perturbed. Furthermore, loss of such genes in *unc-4* and *unc-37* mutants are expected to suppress the backward movement defect by eliminating these ectopic VB-like inputs.

A major goal of my project was to identify *unc-4* regulated B-motor neuron specific genes with roles in the UNC-4 pathway. Previous work had identified two B-motor neuron specific genes, *acr-5* and *del-1,* that are negatively regulated by UNC-4 and UNC-37 (Figure 1.15) (Winnier et al. 1999). *acr-5* encodes an α-type nicotinic acetylcholine receptor (nAChR) subunit. A transgenic line expressing 4 kb of upstream *acr-5* promoter sequence displays GFP in unidentified head and tail neurons, as well as in both VB and DB motor neurons (Winnier et al. 1999). When this reporter is placed in an *unc-4* or *unc-37* background, ectopic expression is seen in both DA and VA motor

neurons. *del-1* encodes a subunit of a DEG/ENaC channel. In L2 animals, *del-1*::GFP is observed in VB motor neurons. Furthermore, *del-1* is negatively regulated by UNC-4 and UNC-37, as this reporter comes on in VA motor neurons in *unc-4* and *unc-37* mutants. Thus, *del-1* and *acr-5* fit the first part of our model: they are B-specific genes that are regulated by the UNC-4/UNC-37 complex. *glr-4* is a glutamate receptor subunit, and it was reportedly expressed in DB motor neurons (Brockie et. al., 2001). We obtained this GFP reporter and tested it for UNC-4/UNC-37 regulation. This chapter describes my genetic experiments to determine if ACR-5, DEL-1, and GLR-4 are mediators of synaptic specificity.

Materials and Methods

Nematode Strains

Nematodes were grown as previously described (Brenner 1974). All genetics were performed at 25ºC. The wild-type strain is N2 (Bristol). *unc-4* mutant alleles used in this work are: *wd1*, *e120, e2320, e887* (Miller et al. 1992). The following deletion alleles were obtained from the Oklahoma Medical Research Foundation *C. elegans* Gene Knockout Consortium: *acr-5* (*ok180), acr-5 (ok182), acr-5 (ok205*), and *del-1 (ok150)*. *del-1 (bz33)* was a gift from Monica Driscoll (Rutgers University). *glr-4 (ak78)* was a gift from A. Villu Maricq. All deletion alleles were outcrossed at least 4X (*acr-5* against *unc-32*, *del-1* against N2).

glr-4::GFP animals (Brockie et al. 2001) were scored for expression in the ventral nerve cord. This transgene was crossed into an *unc-4 (e120); lin-15 (n729ts)* animal to test for regulation.

Single-worm PCR

Single-worm PCR was used to genotype animals to test for the presence of *acr-5*, *del-1,* and *glr-4* deletion alleles. 10-20 adult animals were placed on individual NGM plates and allowed to self overnight. Each animal was then placed in 3 µl of freshly prepared lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.5% Tween-20, 0.5% NP40, 0.01% gelatin, 200 µg/ml proteinase K) in a sterile 0.5 ml eppendorf tube. A drop of mineral oil was added, and then the tubes were placed on a block of dry ice until the liquid was frozen. Tubes were immediately placed in a PCR machine (MJ Research) running the ONEWORM program (60ºC 1 hr, 99ºC 15 min). 22.5 µl PCR mix (1X: 15.5 µl dH2O, 2.5 µl Promega Buffer B, 1.5 µl Promega 25 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix, 0.5 µl Promega Taq, 2 µl primer mix, 12.5 µM each) containing the appropriate primers (listed below) was added to each tube. PCR reactions were run using the SV2 program (95ºC, 45 s; 55ºC, 45 s;72ºC, 4 m 10 s; 30 cycles) for *acr-5* and *del-1*. For *glr-4*, the GLR-4 KO program was used (94ºC, 30 s; 55ºC, 30 s; 72ºC, 1 min; 30 cycles). 15 µl of each reaction was run on a 1% agarose gel to analyze the genotype. Three controls were used in each experiment: N2 lysate, homozygous mutant lysate, and N2 genomic DNA (100 ng). Primers used to detect *acr-5* deletions: Forward primer K03F8.2 IR1, 5' - ATCACCGGCTACAACGTCTT - 3'; Reverse primer K03F8.2 IL1, 5' - TGTAGAGTG TCGCTGTTCGG - 3'. Primers used to detect *del-1 (ok150)* deletion: Forward primer E02H4.1 ER1, 5' - AGTGCTGTCACACCAAGCAC - 3'; Reverse primer E02H4.1 EL1, 5' - GAAACGGTGAGTGCCAA TTT - 3'. Primers used to detect *del-1 (bz33)* deletion: Forward primer del1p10, 5' - GAC GACAGGAGTGCATAGGT - 3'; Reverse primer del-1p16, 5' - GTATTGATCTTGCTG GCATGGC - 3'. Primers used to detect *glr-4 (ak78)*

deletion: Forward primer glr4p1, 5' - ATGACGTGGTCATCCATAAACATG - 3'; Reverse primer glr4p2, 5' - TTCTCCACAAGCAAGTAAGAATGC - 3'; Poison primer (reverse) glr4p3, 5' - CAGTCAGTAGAGATACACCTAATG - 3'. Table 2.1 lists the expected sizes of the wildtype and deletion mutant PCR fragments.

Movement assays to phenotypically characterize the deletion alleles

The locomotion assay(Mendel et al. 1995; Segalat et al. 1995; Lackner et al. 1999) and the thrashing assay(Miller et al. 1996) were used to quantify the movement rates of N2, *acr-5 (ok180), del-1 (ok150),* and the double mutant *acr-5(ok180); del-1(ok150)*. For the locomotion assay, 60 mM NGM plates were inoculated with 100 µl of OP50-1 and spread over the entire surface of the plate. Plates were incubated overnight at 37ºC, and then stored at 4ºC until ready to use. For each experiment, 10 L4 animals for each genotype were picked to individual plates and grown for 20 hours at 20ºC. Animals were then scored for the number of body bends (defined as a complete sinusoidal wave, ~) in a 3 minute period. Results were averaged and plotted with the standard deviation in Excel. For the thrashing assay, worms were grown at 25ºC. L4 animals were picked to unseeded plates, then transferred into one well of a 96 well plate containing 2 drops (~50 µl) M9 Buffer. Worms were allowed to equilibrate for 2 minutes and then were analyzed for thrashing. A thrash is defined as the complete movement of the head from side to side (i.e. ⊃--⊂--⊃). Thrashes were counted during a 2 minute period for 10 individual worms. The thrashes/minute were averaged and plotted with standard deviations in Excel.

Table 2.1

Expected sizes of wildtype and deletion mutant PCR fragments

Allele	**Primers used**	**Expected size (kb)**
acr-5 (+)	K03F8.2 IR1 + IL1	~3.5
acr-5 (ok180)	K03F8.2 IR1 + IL1	~1.5
acr-5 (ok182)	K03F8.2 IR1 + IL1	~2
acr-5 (ok205)	K03F8.2 IR1 + IL1	~1.5
del-1 (+)	E02H4.1 ER1 +EL1	~3.3
del-1 (ok150)	E02H4.1 ER1 +EL1	~1.2
del-1 (+)	del1p10 + p16	~2.8
del-1 (bz33)	del1p10 + p16	~1.4
glr-4 (+)	glr1p1 +p2 + p3	0.442
glr-4 (ak78)	glr1p1 +p2 + p3	0.600

Genetics to test for suppression of the Unc-4 phenotype

Deletion alleles were crossed into *unc-4* backgrounds to determine if *del-1* and/or *acr-5* could suppress the backward movement defect. *acr-5 (ok180*) males were generated by heat-shocking L4 hermaphrodites at 30ºC for 5 hours. *ok180* males were crossed into *unc-4* mutants *(wd1*, *e120, e2320, e887*). Non-Unc F1 progeny were selfed to generate Unc animals. Single-worm PCR was used (see above) to homozygose *acr-5 (ok180)*. Since *del*-1 is on the X chromosome, *del-1* males were generated by crossing N2 males with *del-1* hermaphrodites. Crosses, as above, were performed to introduce the deletion into the *unc-4* background. To check for redundancy between ACR-5 and DEL-1, a triple mutant was generated: *unc-4 (e120); acr-5 (ok180); del-1 (ok150)*. For this, *ok180* males were crossed into *acr-5 (ok180); del-1 (ok150)* to create *ok180; ok150* males. The double mutant males were crossed into *unc-4 (e120); acr-5 (ok180)* hermaphrodites. Again, non-Unc F1 progeny were picked and allowed to self. Unc F2 progeny were screened for the presence of both deletions initially (using both primer sets in the mix). Those animals that had at least one copy of each deletion allele were selfed and homozygosed for one deletion and then the other. PCR with both primer sets on 10 animals confirmed that both deletion alleles were homozygous. Thrashing assays (described above) were used to quantitate movement of N2, *unc-4 (e120), e120; acr-5(ok180), e120; del-1(ok150),* and *e120; ok180; ok150* animals.

To test if *glr-4 (ak78)* could suppress the *unc-4* defect, I created a recombinant chromosome containing both the *glr-4* deletion as well as the *unc-4 (e120)* allele. I crossed *glr-4 (ak78)* homozygous males into *unc-4 (e120)* hermaphrodites. Three non-Unc hermaphrodites were picked from this cross to self. Ten Unc F2 progeny were

screened using single worm PCR for the *glr-4* deletion. One animal contained the recombinant chromosome and was allowed to self to homozygose the *glr-4* deletion. To make the quadruple mutant, *glr-4 (ak78) unc-4 (e120); acr-5 (ok180); del-1 (ok150)*, I mated *ok180* males into *ok180; ok150* hermaphrodites. The males from this cross (*ok180*/*ok180; ok150*/0) were crossed into *glr-4 (ak78) unc-4 (e120)* animals. Non-unc F1 progeny were allowed to self. Unc F2 progeny were screened for the presence of both *acr-5 (ok180* and *del-1 (ok150)* deletions. Deletions were homozygosed as described above.

Results

glr-4, an AMPA glutamate receptor subunit, is regulated by UNC-4

A. Villu Maricq's lab showed that *glr-4*::GFP is expressed in DB motor neurons in the ventral cord (Brockie et al. 2001). We obtained this reporter and confirmed expression in DBs. We also noted consistent expression in VB and AS motor neurons, and mosaic expression in D-class motor neurons (Figure 2.1A). I crossed *glr-4*::GFP into *unc-4 (e120)* animals and observed ectopic expression of *glr-4*::GFP in DA and VA motor neurons (Figure 2.1B,C). Thus, I concluded that *glr-4* is negatively regulated by UNC-4.

acr-5, *del-1*, and *glr-4* deletion mutants have no movement defects

Because *acr-5*, *del-1,* and *glr-4* are cell-surface molecules, ion channel subunits, and are B-class genes that are regulated by UNC-4, they are strong candidates for

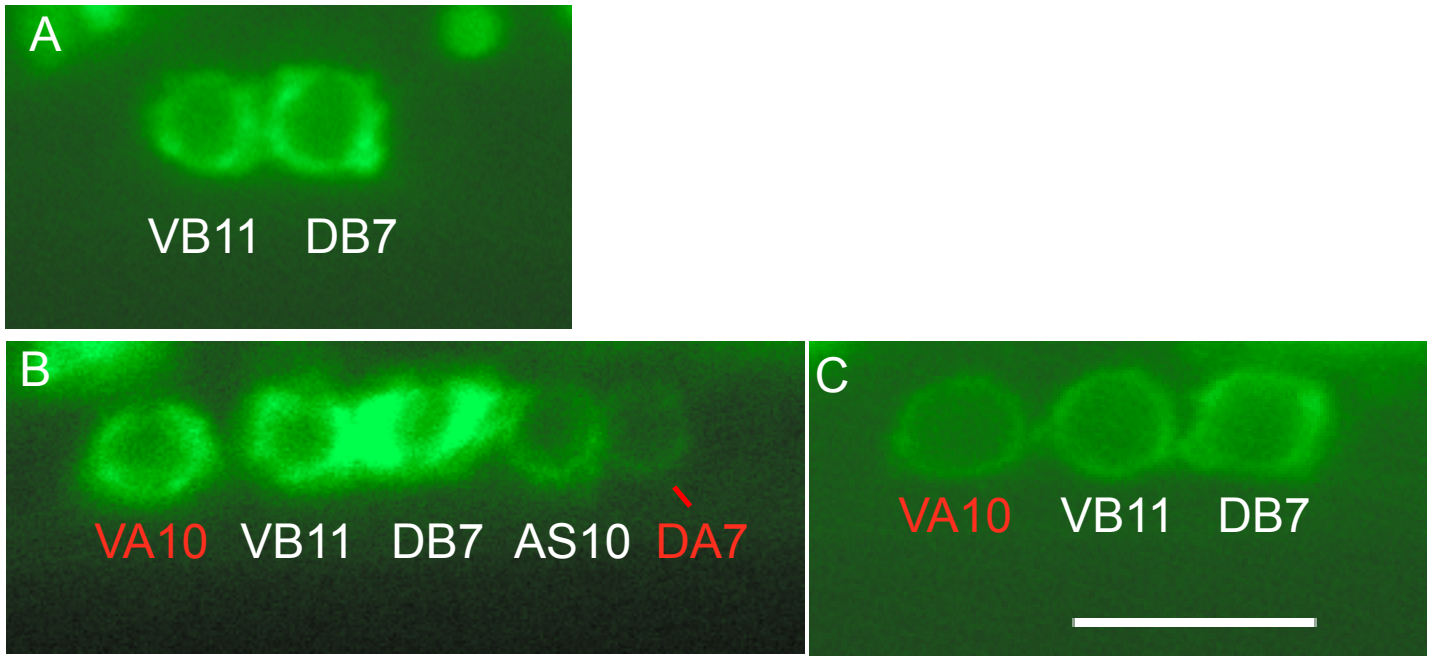

Figure 2.1 *glr-4*::GFP is negatively regulated by *unc-4*.
A. *glr-4*::GFP is expressed in VB, DB, and AS (not shown) motor neurons in wildtype L2 larvae.
B, C. *glr-4*::GFP is ectopically expressed in VA and DA motor neurons (red lettering) in *unc-4 (e120)* L2 larvae.

scale bar = 5 μm

mediators of synaptic specificity. In that event, our model predicts that the removal of ACR-5, DEL-1 and/or GLR-4 function would disrupt B-type (AVB, PVC) inputs to VB motor neurons and therefore result in a forward movement defect. To test this idea, we obtained the *acr-5 (ok180)* and *del-1 (ok150)* deletion alleles from the *C. elegans* knockout consortium (Oklahoma Medical Research Foundation) and the *glr-4 (ak78)* deletion allele from A. Villu Maricq (Utah). All of these deletion alleles are putative nulls (Figure 2.2). The *acr-5* deletion removes 2 kb of sequence between exon 6 and intron 9 encoding for 3 of the 4 transmembrane domains; since acetylcholine receptors are known to be degraded if not properly folded, it is likely that no ACR-5 protein reaches the membrane {Wanamaker, 2003 #2773}. The *del-1* deletion removes 2 kb of sequence between intron 5 and intron 11, removing one of the two transmembrane domains. The resulting *del-1* transcript is in frame, suggesting that some mutant protein may exist; however, it is likely that the removal of one transmembrane domain disrupts trafficking and DEL-1 protein stability. The *glr-4 (ak78)* allele contains a 1.8 kb deletion between intron 9 and exon 15.

All *acr-5, del-1,* and *glr-4* deletion mutants display normal movement when observed crawling on a plate (data not shown). Quantitative assays were used to confirm normal motility for *acr-5* and *del-1* deletion alleles (see Materials and Methods). Both *acr-5 (ok180), del-1 (ok150)* and the double mutant (*acr-5; del-1*) show locomotory activity that is indistinguishable from wildtype (Figure 2.3A). I utilized a thrashing assay to detect subtle movement defects. When nematodes are placed in liquid, they constantly swim or "thrash". As shown in Figure 2.3B, *acr-5(0)* and *del-1(0)* exhibit normal thrashing rates. The double mutant, *acr-5(0); del-1(0)* also shows wildtype motility in

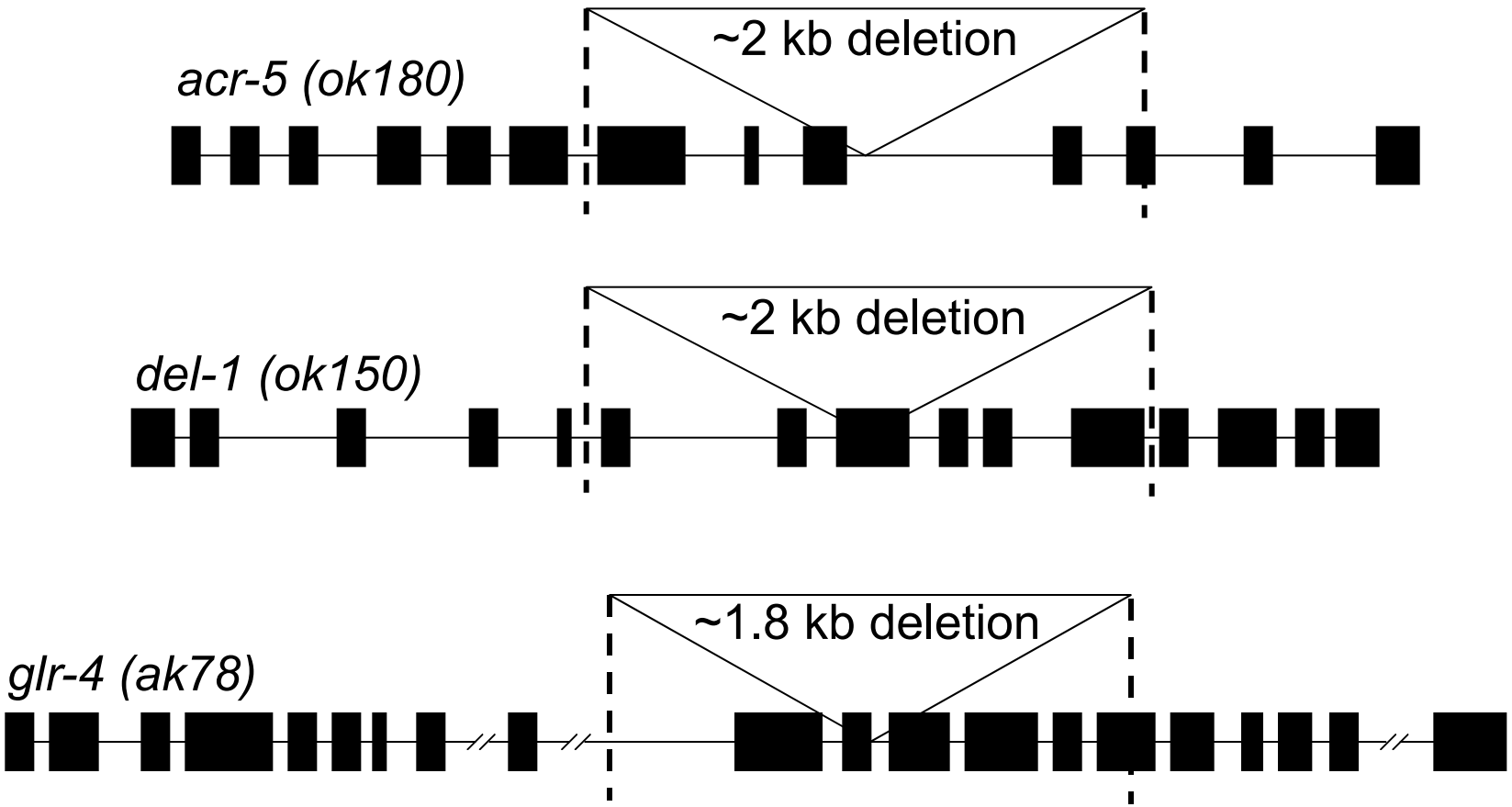

Figure 2.2 *acr-5*, *del-1*, and *glr-4* deletion mutants.
The *acr-5 (ok180)* deletion removes 2 kb of sequence between intron 6 and exon 9. The *del-1 (ok150)* mutant has 2 kb of sequence deleted between introns 5 and 11. The *glr-4 (ak78)* mutant contains a 1.8 kb deletion between intron 9 and exon 15.
Black boxes represent exons, thin black lines depict introns.

this assay, which rules out the possibility of redundancy. Although quantitative locomotion and thrashing assays were not performed for *glr-4 (ak78)*, the absence of an obvious effect of this mutation on normal movement argues against the possibility that *glr-4* is required for normal inputs to VB motor neurons. Furthermore, our lab has found that the predominant *glr-4* transcript contains in frame stop codons, suggesting that this is actually a pseudogene (K. Watkins, D. Miller, unpublished data). Together, these results indicate that ACR-5, DEL-1, and GLR-4 are not **necessary** in B-type neurons to promote B-type inputs.

acr-5 and *del-1* do not suppress the Unc-4 backward movement defect

While ACR-5 and DEL-1 are not necessary to impose B-type inputs, it remains plausible that they are **sufficient**, such that ectopic expression in VA motor neurons in *unc-4* and *unc-37* mutants could still impose B-type connections (Figure 2.4). This model predicts that the removal of ACR-5, DEL-1, and/or GLR-4 function from VAs in *unc-4* mutants should suppress the Unc-4 backward movement defect. To test for this possibility, I crossed the *acr-5*, *del-1,* and *glr-4* deletions into *unc-4 (e120)* mutant animals and assayed backward movement. By a simple 'tap' test all three double mutants (*unc-4; acr-5, unc-4; del-1,* and *glr-4 unc-4*) coil dorsally instead of moving backward, similar to *unc-4 (e120)* alone. I made the triple (*unc-4; acr-5; del-1)* and quadruple mutants *(glr-4 unc-4; acr-5; del-1)*; these also failed to crawl backward, thus ruling out the possibility that these proteins act redundantly. The results of a thrashing assay also failed to reveal any effects of *acr-5, del-1,* and *glr-4* on *unc-4* movement (Figure 2.3B).

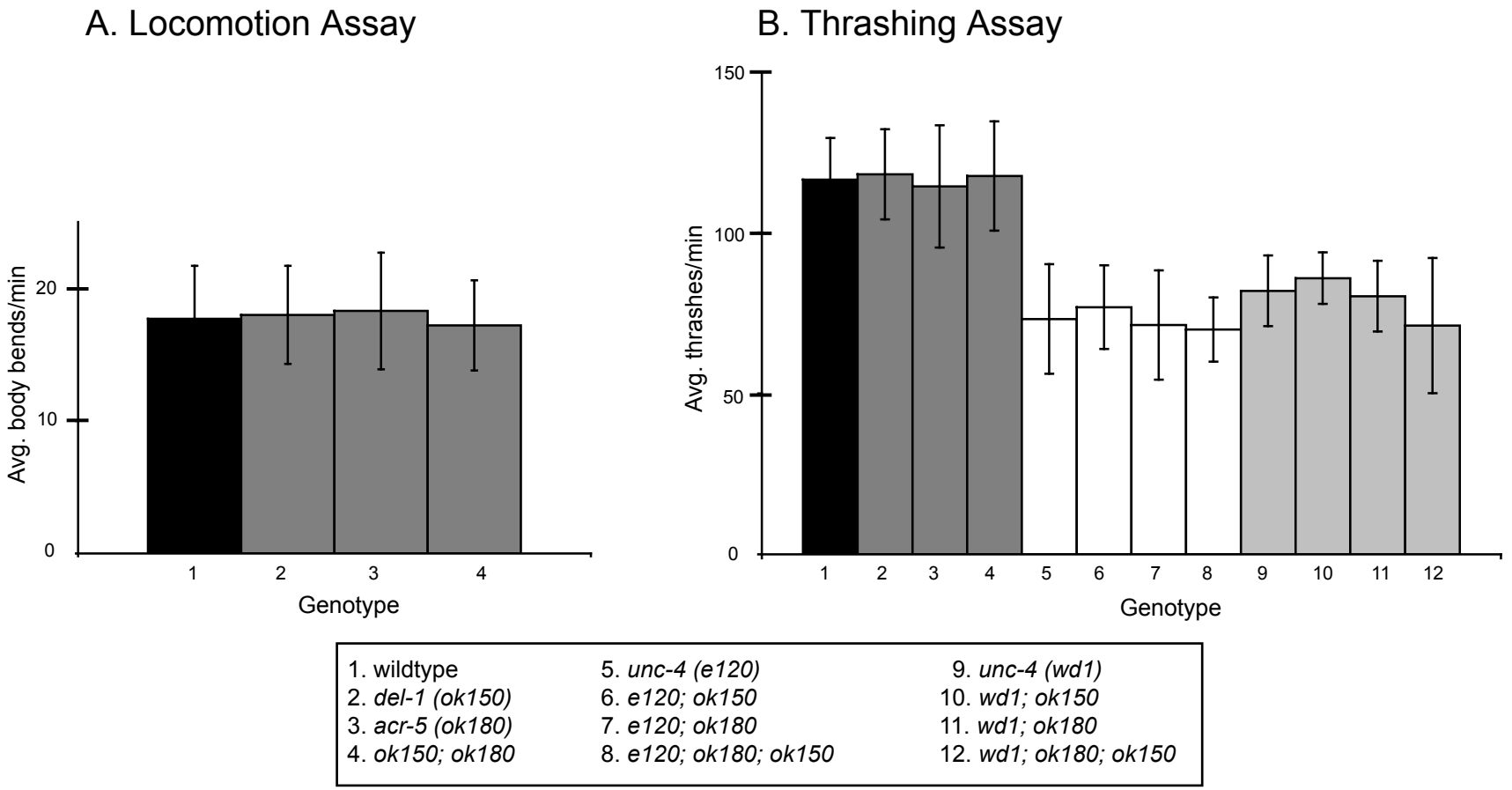

Figure 2.3 Behavioral assays with *acr-5* and *del-1* deletion mutants.

A. *acr-5 (ok180), del-1 (ok150),* and *ok180;ok150* deletion mutants move at the same rate as wildtype worms on solid media.

B. *acr-5 (ok180), del-1 (ok150)*, and *ok180;ok150* deletion mutants thrash at the same rate as wildtype worms in liquid media. In addition, these deletion mutants neither enhance nor suppress the thrashing rates of two *unc-4* mutants (*e120* and *wd1*).

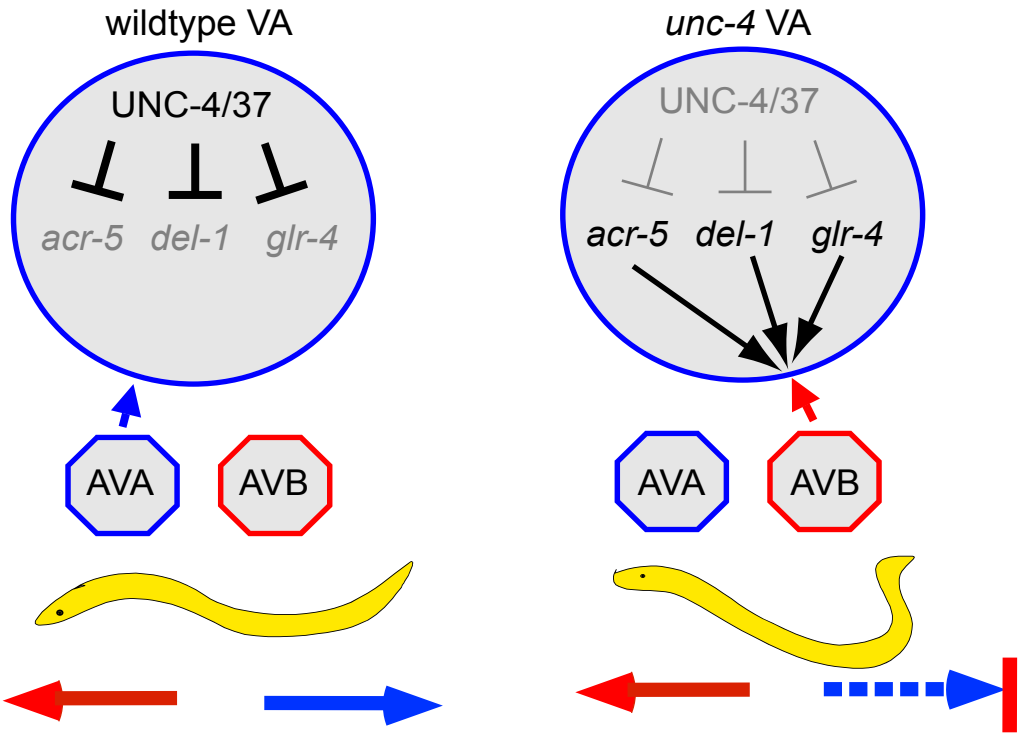

Figure 2.4 Hypothetical model for synaptic specificity in VA motor neurons.

(Left) In the wildtype VA, UNC-4 and UNC-37 act as corepressors to inhibit expression of the ion channel components *acr-5*, *del-1*, and *glr-4*. This repression allows for proper specification of inputs by the AVA command interneuron (small blue arrow) and normal coordinated locomotion (large red and blue arrows).

(Right) In *unc-4* mutants, loss of UNC-4/UNC-37 activity leads to depression of *acr-5*, *del-1*, and *glr-4,* which then direct inputs from AVB (small red arrow) onto VA motor neurons and thereby disrupt backward locomotion (dashed blue arrow).

This model is ruled out by genetic experiments (described in the text) with deletion alleles of *acr-5, del-1*, and *glr-4*, which do not perturb normal movement or suppress the *unc-4* movement defect.

These data suggest that ACR-5, DEL-1, and GLR-4 are not **sufficient** to impose B-type inputs.

ACR-5 does not act downstream of VAB-7/Eve in DB motor neurons to control axonal polarity

Since *acr-5* does not have a role in UNC-4 mediated synaptic specificity, I asked a different question: Does ACR-5 act downstream of the Even-skipped homeodomain transcription factor VAB-7 in DB motor neurons to control axonal polarity? As described in the introduction, DB motor neurons extend posteriorly directed axons in the dorsal nerve cord. In *vab-7/eve* mutants, DB axons reverse polarity and now project anteriorly, like the DA motor neurons (Figure 1.11) (Esmaeili et al. 2002). This disruption of the neuronal circuitry is correlated with a distinctive forward movement defect. *unc-4*::GFP is ectopically expressed in DBs in *vab-7* mutants, suggesting that UNC-4 is acting to turn off DB genes and thereby promote a DA fate. In support of this model, *acr-5*::GFP expression in DBs is abolished in *vab-7* mutants. These defects are suppressed in *unc-4; vab-7* double mutants; DBs project posteriorly, *unc-4*::GFP is turned off and *acr-5*::GFP expression is restored. These results beg the question: Is *acr-5* required for posterior polarity in DBs? ACR-5 is an attractive candidate for this role because it is a nictotinic acetylcholine receptor (nAChR). Mu-Ming Poo's group has shown that cultured *Xenopus* spinal neuron growth cones turn in response to an ACh gradient. Further, the nAChR antagonist D-turbocurarine inhibits this effect, thus suggesting that ACh acts through nAChR to induce growth cone turning (Zheng et al. 1994).

To determine if ACR-5 functions to direct DB axons, I crossed the DA/DB marker *unc-129*::GFP into *acr-5(ok180)* animals and assayed the polarity of DA/DB motor neurons. In this experiment, all DB motor neuron projections show normal posterior polarity in *acr-5* mutant (data not shown). Thus, ACR-5 does not function independently downstream of VAB-7 in the DB axonal polarity pathway. It remains possible, however, that ACR-5 works redundantly with another nAChR subunit. *acr-16* is an α7-like subunit that has been recently shown to be involved in ACh signaling at the neuromuscular junction (Touroutine et al. 2005). *acr-16*::GFP is normally expressed in body wall muscle and in DB motor neurons (Touroutine et al. 2005). We have recently shown that *acr-16*::GFP expression in DBs is ablated in *vab-7* mutants (R. Fox, SEV, DMM, unpublished data). Furthermore, UNC-4 negatively regulates *acr-16* expression in DA motor neurons (R. Fox, SEV, DMM, unpublished data). It is therefore a formally possible that ACR-5 and ACR-16 work together to control DB motor neuron polarity. Rebecca Fox, a fellow student in the lab, is generating the *acr-5; acr-16* double mutant to test this idea.

Discussion

unc-4 mutants, with their specific miswiring defect, provide an excellent opportunity to uncover the mechanisms of synaptic specificity. We have proposed that UNC-4 regulated VB genes induce inputs from AVB and PVC (B-type). The finding that three VB-expressed ion channel subunits (ACR-5, DEL-1, GLR-4) are regulated by UNC-4 was therefore intriguing; it seemed likely that these cell-surface molecules could act to direct B-type inputs. My genetic experiments, however, have now revealed that

these genes are neither **necessary** nor **sufficient** to specify inputs from AVB and PVC. I have also ruled out the possibility that these genes act redundantly in the *unc-4* synaptic specificity pathway; the failure of the combined *glr-4, acr-5,* and *del-1* deletions to suppress the Unc-4 phenotype suggests that they do not act in parallel pathways to direct B-type inputs. In addition, when these deletion mutants are placed in an *unc-4* background, they do not enhance the forward movement defect, which should happen if inputs to VBs are perturbed. Thus, it appears that ACR-5, DEL-1, and GLR-4 are not involved in synaptic choice, or that they act in a pathway that is redundant to another UNC-4 target gene.

It seems plausible that mechanisms of synaptic specificity would be robust and potentially include redundant pathways; essential neural functions are fundamentally dependent on correct wiring patterns. The results of my studies of the UNC-4 pathway are consistent with this idea. Genetic analysis is also consistent with the hypothesis that UNC-4 regulates more than one downstream target in the synaptic specificity pathway. For example, if *unc-4* regulated VA inputs via a single target gene, then loss-of-function mutations in this hypothetical gene should suppress the Unc-4 phenotype. However, despite extensive genetic screens, *unc-4* suppressor mutations of this type have never been uncovered. Therefore, we concluded that other strategies needed to be employed to identify the elusive UNC-4 target genes.

Upon the completion of the *C. elegans* genome in 1999, it became possible to query all the genes at once using DNA microarray technology; this approach became the focus of my next experiments (see Chapter III).