Axon Response to Guidance Cues Is Stimulated by Acetylcholine in *Caenorhabditis elegans*

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ABSTRACT Gradients of acetylcholine can stimulate growth cone turning when applied to neurons grown in culture, and it has been suggested that acetylcholine could act as a guidance cue. However, the role acetylcholine plays in directing axon migrations *in vivo* is not clear. Here, we show that acetylcholine positively regulates signaling pathways that mediate axon responses to guidance cues in *Caenorhabditis elegans*. Mutations that disrupt acetylcholine synthesis, transportation, and secretion affect circumferential axon guidance of the AVM neuron and in these mutants exogenously supplied acetylcholine improves AVM circumferential axon guidance. These effects are not observed for the circumferential guidance of the DD and VD motor neuron axons, which are neighbors of the AVM axon. Circumferential guidance is directed by the UNC-6 (netrin) and SLT-1 (slit) extracellular cues, and exogenously supplied acetylcholine can improve AVM axon guidance in mutants when either UNC-6– or SLT-1–induced signaling is disrupted, but not when both signaling pathways are perturbed. Not in any of the mutants does exogenously supplied acetylcholine improve DD and VD axon guidance. The ability of acetylcholine to enhance AVM axon guidance only in the presence of either UNC-6 or SLT-1 indicates that acetylcholine potentiates UNC-6 and SLT-1 guidance activity, rather than acting itself as a guidance cue. Together, our results show that for specific neurons acetylcholine plays an important role *in vivo* as a modulator of axon responses to guidance cues.

CELLS secrete molecules that help guide axon growth cone migrations. In *Caenorhabditis elegans*, UNC-6 (netrin) and SLT-1 (slit) are secreted guidance cues and are ligands for the UNC-40 (DCC) and SAX-3 (Robo) receptors, which are present at the surface of the migrating axons (Hedgecock *et al.* 1990; Serafini *et al.* 1994; Chan *et al.* 1996; Keino-Masu *et al.* 1996; Wadsworth *et al.* 1996; Zallen *et al.* 1998; Brose *et al.* 1999; Hao *et al.* 2001). During nervous system development, different cells can spatially and temporally express a guidance cue to create dynamic patterns (Wadsworth *et al.* 1996). This expression can provide pathway and long-range signals, allowing for complex axon trajectories during the formation of neural circuits (Wadsworth and Hedgecock 1996). Additional extracellular guidance cues and other factors can further modify growth cone responses and alter trajectories. How multiple extracellular molecules together direct growth cone migrations is still not well understood.

Acetylcholine is best known as a molecule secreted at synapses, where it acts as a neurotransmitter. However, there is evidence to suggest that during early development acetylcholine has other roles, including the role of an axon guidance cue (Ruediger and Bolz 2007). Studies using chick, *Xenopus*, and *Drospophila* embryonic neurons indicate that acetylcholine is also secreted before synapses form (Hume *et al.* 1983; Young and Poo 1983; Yao *et al.* 2000). During this time, acetylcholine might influence different aspects of nervous system development, including the process of axon guidance. Moreover, under cell culture conditions, defined extracellular gradients of acetylcholine elicit turning responses from neuronal growth cones (Zheng *et al.* 1994; Kuffler 1996). A developmental role for acetylcholine in axon pathfinding *in vivo* was revealed when it was shown that *Drosophila* photoreceptor axons do not properly project to their targets when acetylcholine synthesis or metabolism is altered or eliminated (Yang and Kunes 2004).

In this article, we present evidence that extracellular acetylcholine is required for a migrating axon to properly respond to guidance cues *in vivo*. In *C. elegans*, the ventral

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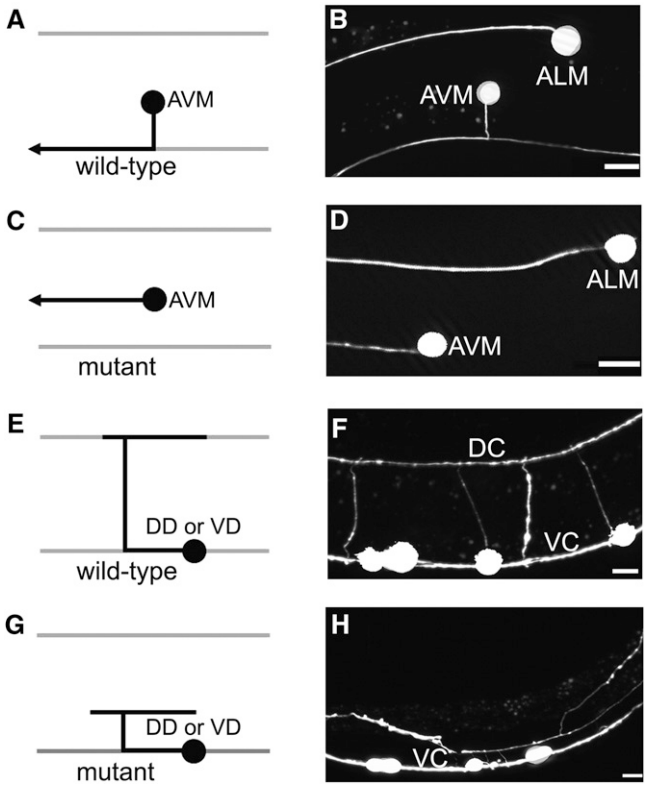

Figure 1 Mutations cause axon migration phenotypes. Left column, schematic diagram of the AVM neuron (A and C) and the DD and VD neurons (E and G); right column, fluorescence photomicrographs of larvae bearing a *mec-4*::*gfp* transgene that expressed GFP in the AVM neuron (B and D) or a *unc-47*::*gfp* transgene that expressed GFP in the DD and VD motor neurons (F and H). In each panel, left is anterior and up is dorsal. Bars, 10 μm. (A and B) In wild-type larvae the AVM neuron is located on the lateral right side of the *C. elegans* body wall. During the L2 stage, the AVM axon is guided ventrally to the ventral nerve cord where it turns and migrates anteriorly to the nerve ring. (C and D) In mutant larvae that have axon migration defects caused by loss of UNC-6 or SLT-1 guidance signaling, the AVM axon often fails to migrate ventrally and instead travels anteriorly. (E and F) In wild-type larvae the DD and VD motor neurons send processes along the ventral nerve cord and circumferentially along the body wall to the dorsal midline. DC, dorsal nerve cord; VC, ventral nerve cord. (G and H) In mutant larvae that have axon migration defects caused by loss of UNC-6 guidance signaling the DD and VD axons wander across the body wall and rarely reach the dorsal midline.

axon migration of the AVM neuron is directed by the UNC-6 and SLT-1 guidance cues through signaling mediated by the UNC-40 and SAX-3 receptors (Hedgecock *et al.* 1990; Wadsworth *et al.* 1996; Hao *et al.* 2001; Yu *et al.* 2002; Gitai *et al.* 2003). At the beginning of the L2 larval stage, the AVM axon migrates ventrally. When the growth cone reaches the ventral nerve cord it turns anteriorly, eventually reaching the nerve ring in the head where it makes the majority of its synapses (Figure 1). We show that extracellular acetylcholine potentiates the response of the AVM axon to the UNC-6 and SLT-1 guidance cues, indicating that acetylcholine can play an important role in patterning neural connections during nervous system development.

Materials and Methods

Strains

Animals were cultivated and maintained according to standard techniques at 20° or 25° (Brenner 1974). Bristol strain N2 was used as wild-type genetic background. All genetic lesions used for this study are strong loss-of-function or null alleles unless otherwise indicated. Strains used in study as follows: *IM1147 unc-40(e1430)I*; *oxIs12X*, *IM936 unc-104(e1265)II*; *zdIs5I*, *IM941 unc-40(e1430)I*, *zdIs5I*; *unc-104(e1265)II*, *IM939 rpm-1(ur299)V*; *unc-104 (e1265)II*; *zdIs5I*, *IM945 unc-104(e1265)II*; *clec-38(ur280) V*; *zdIs5I*, *IM940 unc-104(e1265)II*; *sax-3(ky123)X*; *zdIs5I*, *IM942 unc-104(e1265)II*; *unc-6(ev400)X*; *zdIs5I*, *IM938 unc-104(e1265)IV*; *slt-1(eh15)X*; *zdIs5I*, *IM1148 unc-104 (e1265)II*; *oxIs12X*, *IM946 rpm-1(ur299)V*; *unc-104(e1265) II*; *sax-3(ky123)X*; *zdIs5I*, *IM948 unc-104(e1265)II*; *clec-38 (ur280)V*; *unc-40(e1430)I*; *zdIs5I*, *IM1053 unc-17(e245)IV*; *zdIs5I*, *IM944 unc-40(e1430)I*; *unc-17(e245)IV*; *zdIs5I*, *IM945 unc-17(e245)IV*; *sax-3(ky123)X*; *zdIs5I*, *IM1054 unc-17(e245)*; *unc-6(ev400)X*; *zdIs5I*, *IM1043 unc-17 (e245)IV*; *slt-1(eh15)X*; *zdIs5I*, *IM1149 unc-17(e245)IV*; *oxIs12 X*, *IM937 cha-1(p1152)IV*; *zdIs5I*, *IM943 cha-1 (p1152)IV*; *slt-1(eh15)X*; *zdIs5I*, *IM1150 cha-1(p1152)IV*; *oxIs12 X*, *IM1151 unc-5(e53)IV*; *oxIs12 X*, *IM947 deg-3 (u701)V*; *slt-1(eh15)X*; *zdIs5I*, *IM648 unc-40(e1430)I*; *zdIs5I*, *IM712 sax-3(ky123)X*; *zdIs5I*, *IM650 unc-6(ev400)X*; *zdIs5I*, *IM1152 unc-6(ev400)*, *oxIs12 X*, *IM647 slt-1(eh15)X*; *zdIs5I*, *IM950 deg-3(u701)V*; *zdIs5I*, *IM649 unc-6(ev400)*, *slt-1(eh15)X*; *zdIs5I*, *IM1112 urEx386* [*mec-4*::*unc-104*, *odr-1*::*dsred*], *IM1113 unc-104(e1265)*; *zdIs5I*; *urEx386* [*mec-4*::*unc-104*, *odr-1*::*dsred*], *IM1110 urEx385* [*unc-3*:: *unc-104*, *odr-1*::*dsred*], *IM1111 unc-104(e1265)*; *zdIs5I*; *urEx385* [*unc-3*::*unc-104*, *odr-1*::*dsred*], *IM1098 urEx380* [*mec-4*::*unc-17*, *odr-1*::*dsred*], *IM1105 unc-17(e245)I*; *slt-1 (eh15)X*; *zdIs5I*; *urEx380* [*mec-4*::*unc-17*, *odr-1*::*dsred*], *IM1097 urEx379* [*unc-3*::*unc-17*, *odr-1*::*dsred*], *IM1106 unc-17(e245)I*; *slt-1(eh15)X*; *zdIs5I*; *urEx379* [*unc-3*::*unc-17*, *odr-1*::*dsred*], and *AGC11 deg-3(u701)V*; *slt-1(eh15)X*; *zdIs5*; *cueEx6* [*mec-4*::*deg-3*, *odr-1*::*dsred*].

DNA constructs

pIM 218 encodes *mec-4*::*unc-104* and was made by PCR amplifying *unc-104 cDNA* sequence from a *C. elegans* cDNA library (Invitrogen, Carlsbad, CA) using primers: forward, GATCGCATCCTAGGATGTCATCGGTTAAAGTAGCTGT and reverse, GATCGCATGGTACCTTATGAAGCAATTGAAGATGAT GTT. The PCR product was digested with *Avr*II and *Kpn*I restriction enzymes and was cloned into the *Nhe*I and *Kpn*I sites behind the *mec-4* promoter sequence of plasmid pIM 207 (Quinn *et al.* 2006). pIM 219 is a plasmid with an *unc-3* promoter. The *unc-3* promoter is amplified from plasmid pBP6-1 using primers: forward, CCTGCAGGAAGCTTGAT CAAACCGTGA and reverse, CTGTCAACCCCGGGCCA CAGTTTT. The PCR product was digested with *Hind*III and *Sma*I and was cloned into pPD52-102 to replace the

mec-7 promoter. pIM 220 encodes *unc-3*::*unc-104*. It was made by cloning the *Avr*II and *Kpn*I digested *unc-104 cDNA* into the *Nhe*I and *Kpn*I sites behind the *unc-3* promoter sequence of pIM 219. pIM 221 encodes *mec-4*::*unc-17* and pIM222 encodes *unc-3*::*unc-17*. pAGC1 encodes *mec-4*::*deg-3* and was made by PCR amplifying *deg-3 cDNA* sequence from a *C. elegans* cDNA library.

Transgenes

Transgenic strains were created by injecting DNA into N2 hermaphrodites using described methods (Mello and Fire 1995). A total of 5 ng/μl of pIM220 was injected into N2 animals along with 50 ng/μl of *odr-1*::*dsred* co-injection marker. The transgenic lines were maintained as extrachromosomal arrays by following the red fluorescent protein (RFP). Three independent lines were established. The array of one line, *IM1110*, was crossed into *unc-104 (e1265)*; *zdIs5I* to generate strain *IM1111*. The strain *IM1112* and *IM1113* were similarly made. The strain *IM1097* was generated by injecting 5 ng/μl of pIM222 along with 50 ng/μl of *odr-1*::*dsred* into the N2 strain. The *IM1097* was then crossed into *unc-17(e245)IV*; *slt-1(eh15)X*; *zdIsI* animals to generate strain *IM1106*. The strains *IM1098* and *IM1105* were made similarly. The *cueEx6* transgene was generated by injecting 5 ng/μl of pAGC1 along with 50 ng/μl of *odr-1*::*dsred* into the N2 strain. The *cueEx6* transgene was then crossed into *deg-3(u701)V*; *slt-1(eh15)X*; *zdIs5* animals to generate strain *AGC11*.

Fluorescence microscopy

Animals were mounted on 5% agarose pads in M9 buffer containing 10 mM levamisole and observed with ×40 as well as ×63 objectives on a Carl Zeiss Axio-Imager Z1 microscope. Touch receptor neuron AVM was visualized using a *mec-4*::*gfp* marker in wild-type or mutant backgrounds. The axon guidance defect was scored as failure of the axon to reach the ventral nerve cord. The DD and VD motor neurons were visualized using an *unc-47*::*gfp* marker in wild-type or mutant backgrounds. The axon guidance defect was scored as failure of the axons to reach the dorsal nerve cord.

Exogenous acetylcholine treatment

Embryos were placed on the plates containing 1 mg/ml acetylcholine. After the animals hatched and grew to the L3 stage, the axon guidance defects of the AVM or the DD/VD neurons were scored under fluorescence microscopy.

Results

Exogenously supplied acetylcholine can modulate UNC-6– and SLT-1–mediated AVM axon guidance

Acetylcholine can influence growth cone turning in cell cultures, so we wondered whether acetylcholine could influence AVM axon guidance if it was simply added to the growth medium. Although the exogenous acetylcholine is noxious under these experimental conditions, it does not cause AVM axon guidance defects. We find that exogenous acetylcholine rescues AVM ventral axon guidance defects in *unc-6(ev400)*, *slt-1(eh15)*, *unc-40(e1430)* and *sax-3(ky123)* mutants. However, it could not rescue the AVM axon guidance defects of the double *unc-6(ev400)*, *slt-1(eh15)* mutants, in which both of the ventral axon guidance cues are disrupted (Figure 2A). These results indicate that acetylcholine can modulate the responsiveness of AVM to the UNC-6 and SLT-1 guidance cues.

Mutations that disrupt acetylcholine synthesis, transportation, and secretion affect AVM ventral guidance

To study the acetylcholine effect, we used mutants that should have a deficiency of extracellular acetylcholine. We found that mutations of *cha-1*, *unc-17*, and *unc-104* that cause a reduction of function have AVM ventral axon guidance defects (Figure 2B). CHA-1 is a choline acetyltransferase and is expressed in ventral nerve cord cholinergic neurons (Alfonso *et al.* 1994). UNC-17 is a vesicular acetylcholine transporter that loads acetylcholine into synaptic vesicles (Alfonso *et al.* 1993) and UNC-104 is a kinesin that transports vesicles (Otsuka *et al.* 1991). In *unc-104* mutants, vesicles accumulate in neuronal cell bodies and consequently VAChT and ChAT immunoreactivity are abnormally concentrated in the cell bodies; thus *unc-104* mutations likely also disrupt CHA-1 and UNC-17 activities (Hall and Hedgecock 1991; Duerr *et al.* 2008). We note that the alleles used are not null and that the null alleles of these genes are lethal. Therefore it is difficult to make inferences about the relative contribution of each gene for guidance on the basis of the severity of the guidance defect caused by these partial loss-of-function alleles.

To experimentally test whether the mutations are affecting the ability of acetylcholine to influence AVM axon guidance, we grew *unc-104(e1265)* mutants on the plates containing acetylcholine. We find when exogenous acetylcholine is supplied, the AVM ventral axon guidance defect is suppressed in the mutant (Figure 2B). This further supports that the mutations disrupt AVM axon guidance by limiting the availability of extracellular acetylcholine.

The acetylcholine effect is specific to certain neurons

It is possible that exogenous acetylcholine improves the AVM guidance in the *unc-104(e1265)* mutants by stimulating UNC-6 processing and increasing the availability of extracellular UNC-6. In fact, a recent study provides evidence that UNC-104 might be required for UNC-6 secretion (Asakura *et al.* 2010). In this study, a Venus-tagged UNC-6 showed a punctate distribution pattern throughout the cytoplasm and axons of wild-type neurons, but in *unc-104 (e1265)* mutants the UNC-6 was observed evenly distributed in the cell body with little detected in the axon. If this mislocalization prohibited UNC-6 secretion, other axons that require UNC-6 for guidance should likewise be affected by

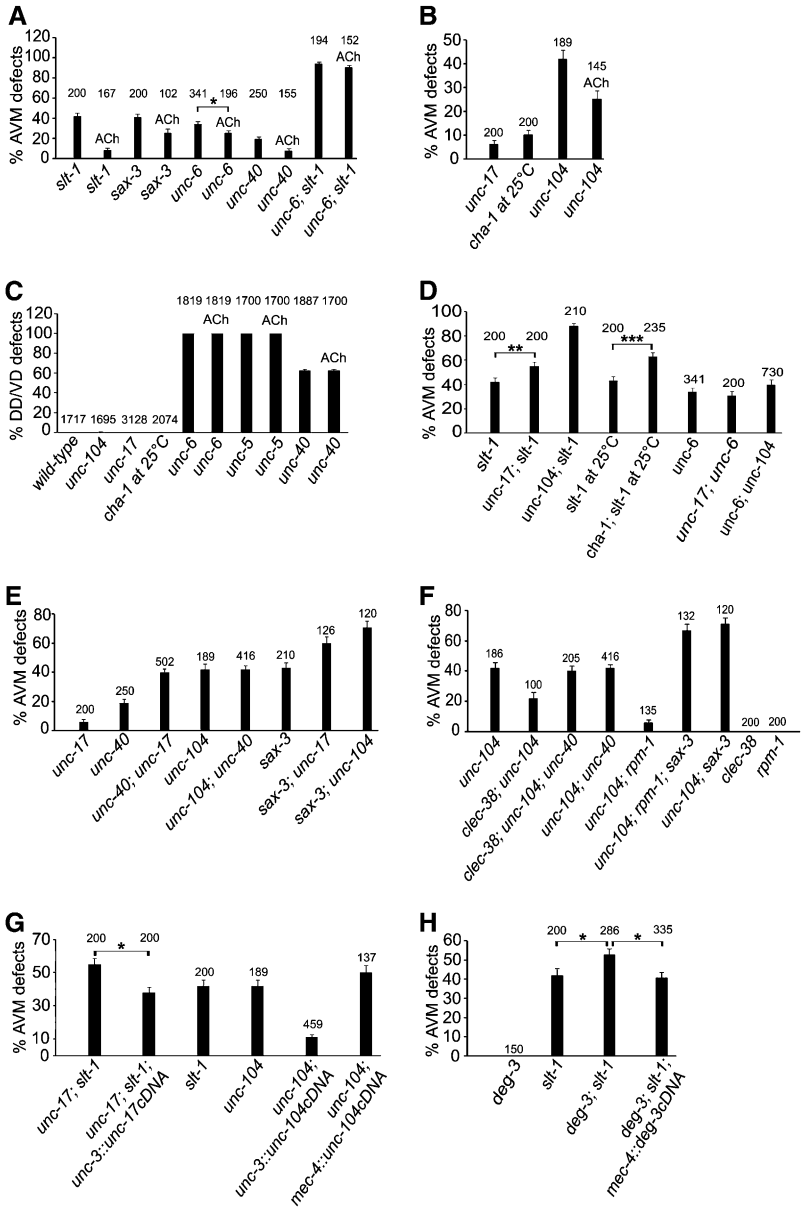

Figure 2 Acetylcholine enhances UNC-6 and SLT-1 guidance signaling for AVM, but not for DD and VD neurons. Quantification of axon guidance defects is shown in each panel. Number above bar indicates number of animals scored (A, B, D–H) or total number of axons scored (C). Error bars represent standard error of proportions (A–H). (A) Exogenously supplied acetylcholine rescues AVM axon guidance defects in *unc-6(ev400)*, *slt-1(eh15)*, *unc-40 (e1430)*, or *sax-3(ky123)* mutant background but not in *unc-6(ev400)*, *slt-1(eh15)* mutants. Asterisks indicate statistically significant difference (*$P < 0.05$, *z*-test for two proportions). (B) Strong loss-of-function mutation in *unc-17*, *unc-104*, or *cha-1* causes AVM ventral axon guidance defect, while exogenously supplied acetylcholine rescues the AVM axonal guidance defect caused by *unc-104 (e1265)*. (C) Strong loss-of-function mutation in *unc-17*, *unc-104*, or *cha-1* does not cause DD or VD axon guidance defect. Exogenously supplied acetylcholine has no effect on DD/VD axon guidance in the *unc-6(ev400)*, *unc-40 (e1430)*, and *unc-5(e53)* mutant backgrounds. (D) Strong loss of function of *unc-17*, *unc-104*, or *cha-1* enhances AVM axon guidance defects caused by *slt-1(eh15)*. Asterisks indicate statistically significant difference (**$P <$ 0.005, ***$P <$ 0.0000, *z*-test for two proportions). (E) Strong loss of function in *unc-17* and *unc-104* enhance AVM axon guidance defects caused by *unc-40(e1430)* as well as *sax-3(ky123)*, respectively. (F) Either *rpm-1(ur299)* or *clec-38(ur280)* suppresses the AVM axon guidance defects in the *unc-104(e1265)* mutants. However, the axon guidance defects are not suppressed in either *rpm-1(ur299)*; *unc-104(e1265)*; *sax-3(ky123)* or *clec-38(ur280)*; *unc-104(e1265)*; *unc-40(e1430)* mutants. (G) *unc-17 cDNA* expression, which is driven by *unc-3* promoter but not by *mec-4* promoter, rescues the axon guidance defects in *unc-17(e245)*; *slt-1(eh15)* mutants. Similarly, *unc-104 cDNA* expression, which is driven by *unc-3* promoter but not by *mec-4* promoter, rescues the axon guidance defects in *unc-104(e1265)* mutants. Asterisks indicate statistically significant difference (**$P < 0.005$, *z*-test for two proportions). (H) The *deg-3(u701)* mutation enhances the AVM ventral axon guidance defect caused by *slt-1(eh15)*. When *deg-3* cDNA is expressed in AVM by using the *mec-4* promoter to drive expression in touch receptor neurons, the enhancement is suppressed. Asterisks indicate statistically significant difference (*$P < 0.05$, *z*-test for two proportions).

the *unc-104(e1265)* mutation. We therefore examined the guidance of the neighboring DD and VD motor neuron axons, which also require UNC-6–mediated guidance. We find that unlike AVM axon guidance, the DD and VD axons migrate normally in the *unc-17(e245)*, *unc-104(e1265)*, and *cha-1(p1152)* mutants (Figure 2C). Because the DD and VD axons are guided normally, we conclude that the AVM guidance defects observed in *unc-17*, *unc-104*, and *cha-1* mutants are not explained by a deficiency in the synthesis, transportation, or secretion of UNC-6. We also note that neither the addition of exogenous acetylcholine, which presumably causes higher levels of acetylcholine, nor the mutations, which presumably cause lower levels of acetylcholine, affect the DD and VD axon migrations (Figure 2C). Together, these observations suggest that acetylcholine does not indirectly affect the AVM axon migration, as an indirect mechanism would likely affect all nearby axon migrations.

Acetylcholine modulates the AVM signaling response to UNC-6 and SLT-1

We found that exogenous acetylcholine can rescue AVM ventral axon guidance defects in *unc-6(ev400)*, *slt-1(eh15)*, *unc-40(e1430)* and *sax-3(ky123)* mutants, but could not rescue the AVM axon guidance defects of the double *unc-6 (ev400)*, *slt-1(eh15)* mutants (Figure 2A). Since these results suggest that acetylcholine can modulate the responsiveness of AVM to UNC-6 and SLT-1, we examined the requirements of acetylcholine for the guidance signaling pathways by using the *unc-17(e245)*, *unc-104(e1265)*, and *cha-1(p1152)* mutations. The AVM axon migrates toward

the UNC-6 sources, which are ventral nerve cord neurons, and it migrates away from the SLT-1 sources, which are the dorsal muscles (Wadsworth *et al.* 1996; Hao *et al.* 2001). In the *slt-1(eh15)*, *unc-6(ev400)* mutants that lose both SLT-1 and UNC-6, 94% of the AVM axons fail to migrate ventrally. Either *slt-1(eh15)* or *unc-6(ev400)* causes ~40% of AVM ventral guidance defects (Figure 2A). We found that *unc-17(e245)*, *unc-104(e1265)*, or *cha-1(p1152)* enhances the AVM ventral axon guidance defect caused by *slt-1(eh15)*, which suggests that acetylcholine potentiates UNC-6–mediated axon guidance (Figure 2D). Our results are similar to those previously reported for *unc-104(e1265)* (Asakura *et al.* 2010).

We found that *unc-17(e245)* or *unc-104(e1265)* does not enhance the AVM ventral axon guidance defect caused by *unc-6(ev400)* (Figure 2B). However, these results do not indicate that acetylcholine has no effect on SLT-1 signaling in AVM. Because the alleles used in these experiments are not null, they might not be strong enough to enhance the axon guidance defects in *unc-6(ev400)* mutants. Furthermore, our results indicate that exogenously supplied acetylcholine improves guidance in response to the SLT-1 guidance cue, albeit not as strongly as for the response to the UNC-6 guidance cue (Figure 2A). Consistent with the idea that acetylcholine affects both UNC-6 and SLT-1 signaling, there is evidence that the UNC-6 and SLT-1 signaling pathways in AVM act synergistically, rather than independently (Quinn *et al.* 2006). Thus, acetylcholine might affect both UNC-6 and SLT-1 signaling and it has stronger effect on UNC-6 signaling.

Since UNC-6 and SLT-1 guidance is mediated by the UNC-40 and SAX-3 receptors (Hedgecock *et al.* 1990; Wadsworth *et al.* 1996; Hao *et al.* 2001; Yu *et al.* 2002; Gitai *et al.* 2003), we also examined *unc-17(e245)* and *unc-104(e1265)* mutants with *unc-40(e1430)* or *sax-3(ky123)* loss-of-function mutations. We observe that the *unc-40(e1430)* and *sax-3 (ky123)* loss-of-function AVM axon guidance phenotypes are enhanced by *unc-17(e245)* and *unc-104(e1265)*, respectively (Figure 2E). This supports the hypothesis that acetylcholine affects both UNC-6 and SLT-1 signaling mediated by the receptors.

If disrupting acetylcholine secretion inhibits UNC-40– and SAX-3–mediated signaling, then increasing the activity of these receptors might suppress the AVM axon guidance defects observed in the *unc-104(e1265)* mutants. To test this idea, we used strong loss-of-function mutations in *rpm-1* and *clec-38*. RPM-1 is an E3-ubiquitin ligase that influences axon outgrowth by negatively regulating SAX-3 and UNC-5 (Li *et al.* 2008). CLEC-38 is a transmembrane protein with C-type lectin-like domains that regulates axon outgrowth by negatively regulating UNC-40 activity (Kulkarni *et al.* 2008). We find that *rpm-1* or *clec-38* loss-of-function mutations can suppress AVM ventral axon guidance defects caused by *unc-104(e1265)* (Figure 2F). Furthermore, there is no suppression in either *unc-104(e1265)*; *rpm-1(ur299)*; *sax-3(ky123)* or in *unc-104(e1265)*; *clec-38(ur280)*; *unc-40(e1430)* mutants. These results are consistent with the idea that acetylcholine modulates the UNC-40– and SAX-3–mediated signaling responses to UNC-6 and SLT-1 in AVM.

Acetylcholine does not modulate the DD and VD signaling response to UNC-6

Our results indicate that acetylcholine can modulate UNC-40–mediated signaling to improve the ability of UNC-6 to guide the AVM axon ventrally. We also presented evidence that loss of extracellular acetylcholine affects AVM axon guidance but not the guidance of the neighboring DD or VD axons. On the basis of these results, we hypothesized that in *unc-40(e1430)* and *unc-5(e53)* mutants, exogenous acetylcholine would not suppress the guidance defects of the DD or VD axons, which require UNC-6 and the UNC-6 receptors, UNC-40 and UNC-5. We found that this is the case (Figure 2C), further indicating that the DD and VD neurons respond differently than AVM to acetylcholine. The different responses also suggest that the enhancement of AVM axon guidance by exogenous acetylcholine is not the result of improving the extracellular environment for the migrating AVM axon, since this would likely also improve the ability of the DD and VD axons to reach their targets.

Acetylcholine from cholinergic neurons modulates AVM ventral axon migration

To determine a source of the acetylcholine that influences AVM ventral axon guidance, we expressed *unc-17* cDNA and *unc-104* cDNA using the *unc-3* promoter to drive expression in ventral midline cholinergic motor neurons (Prasad *et al.* 1998). We also expressed *unc-17* cDNA and *unc-104* cDNA in touch receptor neurons, including AVM, by using the *mec-4* promoter (Lai *et al.* 1996). We found that expression of *unc-17* cDNA in cholinergic motor neurons could rescue AVM ventral axon guidance defects caused by the *unc-17 (e245)* mutation in the *slt-1(eh15)* background (Figure 2G). We did not observe rescue in the *mec-4* promoter experiments. We also found that expression of *unc-104* cDNA in the ventral midline cholinergic motor neurons could rescue the AVM ventral axon guidance defects caused by the *unc-104(e1265)* mutation (Figure 2G). Again, we did not observe rescue using the *mec-4* promoter. These results indicate that release of acetylcholine from cholinergic neurons is sufficient to influence AVM axon guidance. We further note that since these motor neurons send processes to the dorsal midline, the source of acetylcholine may not be well localized in the animals, and, therefore, similar to the interpretation of the exogenous acetylcholine experiments, we conclude the acetylcholine effect does not require gradients to form.

Acetylcholine nicotinic receptor functions cell autonomously to influence the AVM axon guidance response to UNC-6

The turning responses of growth cones in culture are dependent on the activation of neuronal nicotinic acetylcholine

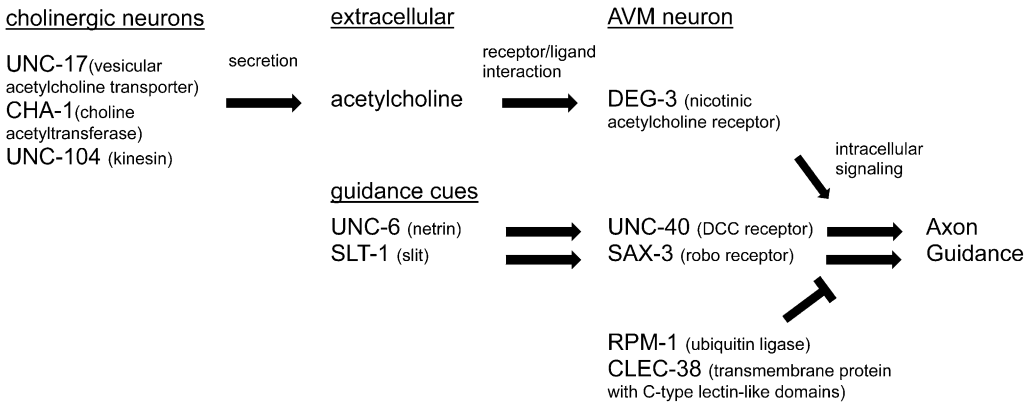

Figure 3 Acetylcholine potentiates AVM axon guidance through UNC-6– and SLT-1–induced signaling. Diagram showing the sites of action of the molecules described. Acetylcholine secreted by ventral nerve cord cholinergic neurons is sufficient to enhance the AVM response to UNC-6 and SLT-1 guidance cues through its interaction with AVM acetylcholine receptors. RPM-1 and CLEC-38 act within AVM to negatively regulate the guidance signaling and have the opposite effect of acetylcholine.

receptors (Zheng *et al.* 1994). We therefore examined whether the effects of acetylcholine on AVM axon guidance might involve such receptors in *C. elegans*. The nicotinic acetylcholine receptor DEG-3/DES-2 is a heteromeric receptor formed by DEG-3 and DES-2; it is expressed in the touch receptor neurons and is localized in the cell body and neuronal processes but not at the synapse (Treinin and Chalfie 1995; Treinin *et al.* 1998; Yassin *et al.* 2001). We found that the null mutation *deg-3(u701)* enhances the AVM ventral axon guidance defect caused by *slt-1(eh15)*, which suggests that the response to the UNC-6 guidance cue is inhibited by disrupting the acetylcholine receptor. Furthermore, expression of DEG-3 in AVM by using the *mec-4* promoter to drive expression in touch receptor neurons rescues the axon guidance defects caused by *deg-3(u701)* in the *slt-1(eh15)* background (Figure 2H). Together these observations indicate that the activity of DEG-3/DES-2 receptors expressed by AVM can regulate guidance responses to UNC-6.

Discussion

Although it acts as a neurotransmitter, acetylcholine may also have other conserved roles that help regulate the development of nervous systems. One of these may be the ability to influence axon guidance. We found that mutations that should reduce extracellular acetylcholine levels cause AVM axon guidance defects and that these defects can be rescued by exogenous acetylcholine. The AVM axon is guided by the UNC-6 and SLT-1 cues and when either UNC-6– or SLT-1–mediated signaling is disrupted, exogenous acetylcholine can improve AVM axon guidance. However, if both UNC-6 and SLT-1 signaling pathways are deficient, exogenous acetylcholine has no effect. Together these results suggest that acetylcholine has the ability to potentiate AVM axon guidance through UNC-6– and SLT-1–induced signaling.

We considered several models that could explain our observations. We favor a model that predicts acetylcholine directly affects guidance signaling pathways within AVM (Figure 3). When strong loss-of-function mutations in *unc-17*, *unc-104*, or *cha-1* cause lower extracellular levels of acetylcholine, AVM axon guidance is defective because the responsiveness of AVM to the UNC-6 and SLT-1 guidance cues is reduced. We also considered a model where acetylcholine affects the secretion of the guidance cues. In this case, lower extracellular levels of acetylcholine caused by the *unc-17*, *unc-104*, or *cha-1* mutations result in lower levels of extracellular UNC-6 and SLT-1, which in turn causes AVM axon guidance defects because of a loss of guidance information. Exogenous acetylcholine might be able to rescue the guidance defects in the *unc-6* or *slt-1* loss-of-function mutants because it somehow stimulates the secretion of the guidance cue. We also considered models whereby the many developmental and morphological defects caused by *unc-17*, *unc-104*, or *cha-1* mutations physically alter the extracellular distribution of guidance cues or other pathway components used by the axon. These conditions would alter the ability of the axon to interpret guidance cues or even physically block the AVM axon from reaching its target. Again, exogenous acetylcholine would somehow reverse these conditions. We do not favor these latter models because in these situations all axon migrations that depend on the guidance cues would likely be affected. This is not the case, however, since we observe that neighboring DD and VD axons are correctly guided in the mutants, indicating that the UNC-6 guidance cue, which is required for DD and VD axon guidance, is present and can guide these axons to their targets despite any changes to the axon's environment. Furthermore, we have found that DEG-3 functions cell autonomously in the AVM neuron to mediate the influence of acetylcholine on the AVM's response to UNC-6. This observation supports the idea that acetylcholine functions directly on the AVM neuron and is inconsistent with the idea that it might function indirectly by regulating other cells.

How could acetylcholine enhance SLT-1– and UNC-6–induced signaling? We suggest that during AVM axon outgrowth, AVM acetylcholine-activated receptors regulate calcium influx in response to extracellular acetylcholine levels. Cytosolic Ca^{2+} is one of the key regulators of growth cone motility and it helps mediate both attractive and repulsive responses to many extracellular guidance cues (see review in Zheng and Poo 2007). In support of this idea, the

turning response of growth cones in culture to netrin-1 gradients depends on Ca^{2+} influx through plasma membrane Ca^{2+} channels (Hong *et al.* 2000) and there is an increase in cytosolic Ca^{2+} on the side of the growth cone facing the source, which is a micropipette delivering the netrin (Henley and Poo 2004).

Significantly, we present evidence that extracellular levels of acetylcholine can influence guidance cue signaling *in vivo*. If this is due to the ability of acetylcholine to alter cytosolic Ca^{2+} levels within a migrating growth cone, then it raises the possibility that localized sources of acetylcholine *in vivo* could alter the type of response that a growth cone has to guidance cues at specific sites. This idea is based on the observations that in culture, different patterns of Ca^{2+} elevation can trigger attractive and repulsive turning responses to netrin-1 (Hong *et al.* 2000). Thus, depending on how cytosolic Ca^{2+} levels are altered, acetylcholine sources could promote, inhibit, or even alter the direction of an axon's outgrowth. Furthermore, we have also shown genetic interactions between genes that could regulate extracellular acetylcholine levels and *clec-38* or *rpm-1*. CLEC-38 and RPM-1 not only influence axon guidance receptor activity but they also affect the ability of neurons to form presynaptic structures (Li *et al.* 2008). An intriguing possibility is that acetylcholine and netrin secretion by intermediate target neurons could be important signals that coordinate axon outgrowth responses to guidance cues and synaptogenesis. In the case of the AVM axon, acetylcholine and UNC-6 are secreted by the target neurons of the ventral nerve cord. Apparently, the responsiveness of AVM to UNC-6 changes at the ventral nerve cord as the axon turns and migrates anteriorly. AVM also makes a choice of producing only a few synapses within the ventral nerve cord, instead making the majority of its synapses in the nerve ring within the head (White *et al.* 1986).

Although the same guidance cue is used by several neurons, the effects can be regulated to produce unique responses. Acetylcholine has an effect on UNC-6 guidance for the AVM axon, but not for the DD and VD axons. However, acetylcholine levels do have an effect on an UNC-6–mediated activity that regulates DD and VD axon branching (Wang and Wadsworth 2002). In *unc-6* null mutants, DD and VD axons often fail to branch and extend processes dorsally (Wang and Wadsworth 2002). Experiments using the expression of an UNC-6 protein that lacks the C domain suggest that a branching activity is controlled by the activity of the C domain (Lim *et al.* 1999). It is proposed that UNC-6 becomes associated with receptor complexes on the surface of the motor neurons and domain C silences a branching outgrowth activity induced by the N-terminal domains. These motor neurons might normally branch at specific locations where the domain C-mediated inhibition becomes repressed. It was shown that the N-terminal–mediated branching outgrowth activity is sensitive to acetylcholine release (Wang and Wadsworth 2002). Like the guidance response, this branching activity also requires the UNC-6 receptors. Furthermore, loss-of-function and gain-of-function alleles of *unc-43* enhance or suppress, respectively, the DD and VD branching induced by the N-terminal domains of UNC-6. UNC-43 is a calcium/calmodulin-dependent protein kinase (CaMKII). These observations again support an idea that acetylcholine can influence UNC-6–induced signaling pathways that control axon outgrowth by influencing cytosolic Ca^{2+} levels.

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