The C Domain of Netrin UNC-6 Silences Calcium/Calmodulin-Dependent Protein Kinase- and Diacylglycerol-Dependent Axon Branching in Caenorhabditis elegans

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Second messenger systems mediate neuronal responses to extracellular factors that elicit axon branching, turning, and guidance. We found that mutations in Caenorhabditis elegans that affect components of second messenger systems, a G-protein subunit, phospholipase Cβ, diacylglycerol (DAG) kinase, and calcium/calmodulin-dependent protein kinase (CaMKII), have no obvious effect on axon responses to UNC-6 except in animals in which the N-terminal fragment, UNC-6ΔC, is expressed. In these animals, the mutations enhance or suppress ectopic branching of certain axons. Netrin UNC-6 is an extracellular protein that guides circumferential migrations, and UNC-6ΔC has UNC-6 guidance activity. We propose that the guidance response elicited by the UNC-6 N-terminal domains involves mechanisms that can induce branching that is sensitive to CaMKII- and DAG-dependent signaling, and that the UNC-6 C domain is required in cis to the N-terminal domains to silence the branching and to maintain proper axon morphology.

Key words: netrin; UNC-6; Caenorhabditis elegans; guidance; axon branching; genetics; G-protein subunit; Qα; phospholipase Cβ; PLCβ; diacylglycerol kinase; DAK; calcium/calmodulin-dependent protein kinase; CaMKII; neuropeptide Y receptor

The regulation of axon guidance and branching is critical for the proper development of the nervous system. Recent studies suggest that guidance and branching share common mechanisms (Brose et al., 1999; Lim et al., 1999; Kalil et al., 2000). For example, fragments of proteins known to mediate axon guidance can promote axon branching. In Caenorhabditis elegans, netrin UNC-6 guides circumferential migrations, and the expression of an N-terminal fragment has been shown to cause additional axon branches from ventral cord motor neurons (Lim et al., 1999). In vertebrates, Slit2 has been implicated in axon guidance and the branches from ventral cord motor neurons (Lim et al., 1999). In C. elegans, netrin UNC-6 guides circumferential cell and axon migrations (Hedgecock et al., 1990; Ishii et al., 1992; Wadsworth et al., 1996). Axons that express the UNC-5 and UNC-40 netrin receptors migrate away from UNC-6 sources; axons that express UNC-40 migrate toward UNC-6 sources. UNC-6 is expressed in changing patterns by 12 types of cells and is predicted to create a stable global cue peaking near the ventral midline and to create local cues on cell surfaces (Wadsworth and Hedgecock, 1996; Wadsworth et al., 1996).

UNC-6 was the first characterized member of the netrin family, and residues 1–437 were designated domains VI, V-1, V-2, and V-3 based on the similarity of the domains to the N-terminal domains of laminin subunits (Ishii et al., 1992). Residues 438–591 were designated domain C, and it was observed that the same motif is found in C3, C4, and C5 complement proteins but not in a paralogous protein, α2 macroglobulin (Ishii et al., 1992). Recently, a modified domain C (C') was found in a functionally divergent form of vertebrate netrin designated netrin-G1 (Nakashiba et al., 2000). These observations indicate that UNC-6 C is a conserved structural module; they suggest that UNC-6 C has a biological function.

We have shown previously that in unc-6ΔC transgenic animals, which express UNC-6 without UNC-6 C, the ventral nerve cord motor neurons extend additional processes circumferentially (see Fig. 3B) (Lim et al., 1999). This activity requires the netrin receptors UNC-5 and UNC-40 (Lim et al., 1999). The expression of UNC-6ΔC provides a means to explore the mechanisms by which axons respond to secreted factors. We have examined the morphology of neurons in unc-6ΔC animals and have uncovered mutations that modulate these morphologies. From these results, we are able to make predictions regarding the mechanisms by which morphological changes to axons might be controlled.

MATERIALS AND METHODS

Strains. The following mutations and strains were used for mapping and double-mutant constructions: N2; RW7000; IM145  urb771  IM#183

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IM175 rfp4 II; IM222 npr-1(ur89); CX4056 npr-1(ad609)lon-2(e678) X; CX3048 npr-1(ky13) X; DJA658 npr-1(n1355)lon-2(e678) X; and MT1434 egl-30(n686) I. The following strains were generated in this study: IM342 lon-2(e678)unc-6(e400) X; IM290 npr-1(ur89)lon-2(e678) X; IM337 npr-1(ky13)lon-2(e678) X; IM234 lon-2(e678)unc-6AC (ur757); IM289 npr-1(ky13)lon-2(e678)unc-6AC(ur757); IM288 npr-1(ad609)lon-2(e678)unc-6AC(ur757); IM341 npr-1(ky13)lon-2(e678)unc-6AC(ur757); IM235 npr-1(ky13)lon-2(e678)unc-6AC(ur757); IM338 egl-30(n686)npr-1(u989)lon-2(e678); IM339 egl-30(n686)npr-1(u989)lon-2(e678); IM340 egl-30(n686)unc-6AC(ur757); IM357 ur191s

Genetic screen. A second filial generation (F2) screen was performed using egl-30 as a phenotypic marker. The strain IM145, which carries a transgene, ur757, which encodes hemagglutinin (HA) epitope-tagged UNC-6 that does not include domain C (Lim et al., 1999), the ur757 transgene was made by removing the sequence that encodes for domain C from an unc-6::HA clone that had been shown to rescue all mutant phenotypes in the unc-6 null genetic background (Wadsworth et al., 1996). In unc-6 null mutants, expression of ur757 exhibits rescue of the unc-6 mutant phenotype, which includes an uncoordinated (u224) behavior. In unc-6(u224) animals, unc-6(u224) caused a slightly uncoordinated phenotype. The transgene also expresses green fluorescent protein (GFP) throughout the nervous system and confers a rolling phenotype to the animals (Lim et al., 1999). F2 animals were screened for wild-type movement; the selected mutants were examined for wild-type axon branching by epifluorescence microscopy. The isolated mutant strains were outcrossed against wild-type N2 six times to remove other possible contaminations. Expression of the transgene was confirmed by the rolling phenotype as well as by GFP expression; the absence of the UNC-6 protein was further confirmed by Western blot analysis using monoclonal antibody 12CA5 (Boehringer Mannheim, Mannheim, Germany) to detect the HA epitope.

npr-1 analysis. ur89 was mapped to the npr-1 region. From the cross ur89 X unc-6(hu1643)unc-6(e400) X, 1/50 Lon non-unc recombinants were cloning. Noncomplementation between ur89 and other npr-1 alleles was tested by scoring for clumping of trans heterozygotes (non-Lon progeny) from a cross such as ur89 X n1353 lon-2. To characterize npr-1(ur89) animals, the allele was linked with lon-2(e678) on linkage group X by crossing ur89 X lon-2(e678)unc-6(e400) X, 1/50 Lon non-unc recombinants segregated clumping of npr-1(ur89)lon-2(e678). Transgenic npr-1 strains were generated by standard methods (Mello and Fire, 1995). npr-1(ur89) animals were injected with pm4, a plasmid that contains an insert of npr-1 genomic DNA derived from the solitary strain n2 (de Bono and Bargmann, 1998). The npr-1 insert contains a 7.4 kb promoter region and 2.3 kb of the coding region. pm4 was coinjected with the markers rol-6(4ts1006) and causes a dominant rolling phenotype, and pPD18.33, which expresses GFP under the myo-2 promoter, to label motor neurons (Mello and Fire, 1995). Independently derived strains were found to rescue the ur89 phenotype in the clumping assay. To test whether the results of the assay were affected by the rolling phenotype conferred by prf4, the assay was repeated using animals that also carried the dip-11(e224) allele, which suppresses the rol-6(4ts1006) rolling phenotype. npr-1(ur89);dip-11(e224) males were crossed with npr-1(ur89);ex162;p4;pm4;prf4[pD18.33] hermaphrodites; ur89;ur89;dp-11/+; progeny that carried the ex162 transgene were chosen. Dpy non-Rol F2 progeny with pharyngeal-muscle GFP expression were selected and the assay was repeated. There was no significant difference in the assay results. To identify the molecular lesion in npr-1(ur89), PCR fragments including the entire coding sequence and intron region were amplified from the genomic DNA of npr-1(ur89) animals. PCR products were cloned into a Bluescript SK+ vector (Stratagene, La Jolla, CA), and both PCR fragments and the subclones were sequenced by an automated sequencer. The mutation was confirmed by sequencing two independent PCR fragments.

The npr-1 lon-2unc-6AC(ur757) animals were constructed by crossing npr-1 lon-2 males with unc-6AC(ur757) hermaphrodites and selecting Lon Rol F2 progeny. The ur757 transgene maps to LGH and is followed by the Rol phenotype and by the pan-neuronal expression of unc-19::GFP. For the unc-6AC(ur757) transgene was crossed into ex162 animals by standard methods for unlinked genes. Transgenic animals expressing an Npr-1::GFP reporter were obtained by microinjection of plM#200, a plasmid constructed by inserting 7 kb of upstream regulatory sequence and 1 kb of npr-1 coding sequence from

pm4 in frame with the GFP coding sequence of vector pPD95.79 (supplied by A. Fire, Carnegie Institution of Washington, Baltimore, MD). Transgenes were integrated by γ-ray irradiation and four independent lines were established. To express npr-1(ur89) ectopically in DD and VD motor neurons, the unc-129 motor neuron-specific promoter (Colavita et al., 1998) was placed upstream of the npr-1 coding sequence, the npr-1 sequence was altered to encode the C178Y change, and the GFP coding sequence was inserted in frame immediately before the stop codon sequence. The pIM201 plasmid was used to create integrated transgenic strains. Expression of the transgene was monitored by the motor neuron expression of GFP.

Axon outgrowth and aldicarb sensitivity assays. For the outgrowth assay, animals were scored by epifluorescence microscopy. Living animals were mounted on a slide in a small drop of M9 buffer on a 5% agar pad. L4 larvae or young adults were randomly picked and scored for the presence of ectopic processes at the ventral sublateral–lateral boundary on the right side of the body wall between the vulva and the retrovesicular ganglion (Lim et al., 1999). The unc-6AC(ur757) animals were picked at random from plates in this study, whereas in the study by Lim et al. (1999), animals with the strongest roller phenotype were selected. Animals with a strong roller phenotype have a slightly lower percentage of ectopic axons.

For scoring the DD and VD motor neurons, immunofluorescence histochemistry was used to stain animals for GABA as described by McIntire et al. (1992) using rabbit anti-GABA antisera (Sigma, St. Louis, MO) and Cy3-conjugated AffiniPure goat anti-rabbit antisera (Jackson ImmunoResearch, West Grove, PA). The sensitivity to aldicarb (Chem Services Inc., West Chester, PA) was determined as described previously (Lackner et al., 1999). Briefly, in each experiment, 20 L4 worms were placed on 1 mm Aldicarb plates and prodded every 10 min over a 2 hr period to determine whether they retained the ability to move; worms that failed to respond to this harsh touch were classified as paralyzed. Each experiment was repeated a minimum of three times.

RESULTS

Netrin UNC-6 and UNC-6ΔC affect axon guidance and branching of the DD and VD motor neurons

Netrin UNC-6 is required to guide circumferential cell and axon migrations (Hedgecock et al., 1990; Ishii et al., 1992). To investigate the relationship between axon guidance and axon branching that is influenced by UNC-6, we examined the DD and VD motor neurons (Fig. 1). The guidance of the circumferential axons of these particular neurons was shown by McIntire et al. (1992) to require UNC-6. We reasoned that the branching of these axons might be especially susceptible to the effects of UNC-6, because each neuron extends circumferentially an UNC-6 responsive axon from an UNC-6 nonresponsive axon that runs longitudinally in the ventral nerve cord. The DD axons develop in the embryo and the VD axons develop in the larva. Together these neurons circumferentially extend 17 axons along the right body wall and 2 axons along the left body wall in wild-type animals (White et al., 1986). The axons are GABAergic and can be specifically visualized using anti-GABA antibodies (McIntire et al., 1992).

We scored the total number of DD and VD motor neuron axons leaving from the right side of ventral nerve cord in unc-6 null mutants. We found that an average of 16.3 ± 0.7 (n = 23) axons leave in wild-type animals, whereas an average of 8.0 ± 1.8 (n = 33) axons leave in unc-6 null mutants (Fig. 2B). This raises the possibility that DD/VD neurons may fail to extend circumferential axons in the unc-6 null mutant. It is also possible that these branches form but simply stay within the ventral nerve cord, however, analyses of the ventral nerve cord of unc-6 null mutants. We found that an average of 16.3 ± 0.7 (n = 23) axons leave in wild-type animals, whereas an average of 8.0 ± 1.8 (n = 33) axons leave in unc-6 null mutants (Fig. 2B).
circumferential axons from the ventral nerve cord and in preventing the ectopic branching of the axons once they leave the cord.

The expression of UNC-6ΔC was shown to have partial UNC-6 guidance activity and to cause axon branching of ventral nerve cord motor neurons (Lim et al., 1999). Here the morphology of the DD and VD neurons was specifically examined. We find that the number of DD and VD axons that extend from the ventral nerve cord in animals that express unc-6ΔC in the unc-6 wild-type and unc-6(−) background is nearly normal (Fig. 2B). Furthermore, the percentage of circumferential axons that reach the dorsal midline is greater in these animals than in unc-6 null animals (48% in unc-6(+);unc-6ΔC, 42% in unc-6(−);unc-6ΔC, and 1% in unc-6(−) animals) (Fig. 2B). However, despite the improved guidance of the axons, ectopic branching is not reduced in the unc-6ΔC animals (Fig. 2C). These results, together with the branching observed in unc-40 mutants, indicate that the ectopic branching is not simply the result of the failure of dorsal guidance; they raise the possibility that UNC-6, but not UNC-6ΔC, mediates another response that prevents inappropriate branching of the circumferential axons.

**Alleles of npr-1 suppress the branching of the circumferential DD and VD axons in unc-6ΔC animals**

The above model predicts separate UNC-6 activities that modulate the axon guidance and branching responses. Therefore, we reasoned that it might be possible to isolate mutations that affect the ectopic branching but not the guidance response to UNC-6ΔC. A genetic screen was performed to isolate mutations that suppress the branching of additional processes from ventral nerve cord motor neurons in unc-6ΔC animals (Fig. 3A,B). This screen took advantage of the observation that the additional motor neuron branches caused by the expression of an unc-6ΔC transgene, urIs77, in an otherwise wild-type animal result in an uncoordinated phenotype (Lim et al., 1999). We reasoned that mutations that suppress the additional branching might restore wild-type movement. Because in general axon guidance mutants have an uncoordinated phenotype, selecting mutant animals with wild-type movement should isolate mutations that affect the ability to induce the extra motor neuron branches but not axon guidance. Thus, in principal, this screen isolates new mutations only if the axon branching response is separable from the axon guidance response. From a screen of 40,000 haploid genomes, we isolated six mutations that partly suppress the urIs77-induced motor neuron processes.

We have identified one suppressor, ur89, as an allele of npr-1. Without unc-6ΔC expression, the ur89 allele causes a social behavior phenotype; that is, the mutants aggregate together on food to form clumps. The same phenotype was described for npr-1, a gene that encodes a predicted seven-transmembrane receptor of the neuropeptide Y receptor family (de Bono and Bargmann, 1998). We examined previously identified alleles of npr-1 and found that npr-1(ad609) also suppresses all additional motor neuron processes in a fraction of unc-6ΔC animals; however, npr-1(ky13) and npr-1(n1353) do not (Fig. 3C). The expression of unc-6ΔC partially suppresses the clumping phenotype conferred by any of the npr-1 mutations (data not shown). Genetically, the ur89 allele fails to complement npr-1(n1353) or npr-1(ky13), and the expression of an npr-1(+);transgene rescues the clumping phenotype of ur89 animals and the ur89 suppression of extra motor neuron processes in unc-6ΔC;ur89 animals. By DNA sequence analysis, we determined that the ur89 mutation changes residue 178 from a cysteine to a tyrosine. Cysteine 178 is con-

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**Figure 1.** The morphology of DD and VD motor neurons is shown. **A.** The morphology of the DD and VD neurons. The circumferential axons extend from the distal end of the longitudinal axon in the ventral nerve cord and migrate dorsally to the dorsal nerve cord. **B.** Schematic transverse section of the adult hermaphrodite body wall. The DD and VD circumferential axons migrate between the basal surface of the epidermis and the basement membranes. Axon morphology was scored at the different dorsoventral positions indicated. **C.** The DD and VD neurons were visualized using anti-GABA immunocytochemistry. In unc-6 null mutants, the circumferential axons wander laterally. Some axons terminate without branching (asterisk), whereas others produce ectopic branches before terminating (arrow).
npr-1 is expressed in ventral nerve cord motor neurons that produce additional branches in response to UNC-6ΔC

The DA, DB, DD, and VD motor neurons have been observed to have additional ventral nerve cord motor neuron branches in unc-6ΔC animals (Lim et al., 1999). To determine whether the npr-1 suppressors might act within any of these ventral nerve cord motor neurons, we made a reporter construct by fusing the sequence coding for GFP after the npr-1 sequence that encodes to the 10th amino acid of the fourth predicted transmembrane domain. We observed strong expression in DD and VD motor neurons (Fig. 4). In unc-6ΔC animals, ectopic DD and VD branches are induced; these are completely suppressed in a large fraction of the npr-1(ad609);unc-6ΔC and npr-1(ur89);unc-6ΔC animals (Fig. 3D). Expression in DA and DB neurons was weak or absent. Note that rather than scoring the number of processes directly, which is difficult because unambiguously identifying a branch can be difficult (Fig. 3B), we measured the proportion of animals that have ectopic processes. This was done by scoring for the presence of any processes at the sublateral–lateral boundary in a region of the animal in which normally no processes are observed (see Materials and Methods) (Lim et al., 1999) (Fig. 3A,B).

The analysis of npr-1 provides evidence that signaling pathways within the DD and VD motor neurons mediate the development of the additional processes. For each strain, transcription of the unc-6ΔC transgene was confirmed by the expression of the GFP marker; the presence of the UNC-6ΔC protein, which contains an HA epitope tag, was confirmed by Western blot analysis (data not shown). Our results indicate that different cells express NPR-1 and UNC-6ΔC. To further examine the cell-autonomous suppression of branching by npr-1(ur89), we ectopically expressed the mutant receptor NPR-1(C178Y) in the cholinergic DA and DB ventral nerve cord motor neurons, which are the other subset of motor neurons affected by unc-6ΔC expression. We observed that when the receptor was expressed in unc-6ΔC animals in the npr-1 null background the combined ectopic branching of DD, VD, DA, and DB neurons was reduced, whereas the ectopic branching of DD and VD neurons alone was unchanged (Fig. 3C,D). By inference, we conclude that the mutant receptor can function cell autonomously even in the DA and DB motor neurons to suppress ectopic branching.

The npr-1(ur89) and npr-1(ad609) mutations may specifically affect the activity of components involved in regulation of ectopic branching in unc-6ΔC animals. Aside from suppressing the branching response and causing the npr-1 phenotype of mutant social behavior, the ur89 and ad609 alleles do not have other obvious phenotypes. Interestingly, other alleles of npr-1, including the predicted loss-of-function (lf) alleles, affect the social behavior but do not affect the number of motor neuron branches. The ur89 mutation changes one of the two cysteine residues that are thought to form a disulfide bond that governs the topology of the extracellular loops of G-protein-coupled receptors. This topology is predicted to be critical for receptor activation (Perlman et al., 1995; Le Gouill et al., 1997; Zhang et al., 1999). We speculate that the ur89 and ad609 alleles produce an altered NPR-1 protein that inactivates downstream effectors required to mediate the ectopic branching, perhaps by sequestering a factor.
transmitter release (Lackner et al., 1999; Miller et al., 1999). EGL-8, a homolog of phospholipase Cβ (PLCβ), is predicted to be a downstream effector of Gqα EGL-30 (Lackner et al., 1999; Miller et al., 1999); we found that egl-8(n488), a loss-of-function allele, also suppresses all motor neuron processes induced by unc-6ΔC in a significant fraction of egl-8(n488);unc-6ΔC animals (Fig. 5A). Activated PLCβ cleaves phosphatidylinositol 4,5-biphosphate to produce inositol 1,4,5-triphosphate and diacylglycerol (DAG). In turn, these two second messengers may modulate intracellular events through their respective regulation of intracellular free Ca²⁺ and protein kinase C isozymes (Singer et al., 1997). In addition, DAG binds the presynaptic protein UNC-13 and recruits it to release sites (Nurish et al., 1999), DGK-1, a diacylglycerol kinase (DAK), acts antagonistically to EGL-30 and EGL-8, presumably by converting DAG to phosphatic acid and thereby reducing DAG levels (Miller et al., 1999). Consistent with this antagonistic role, dkg-1(n62), a loss-of-function allele (Nurish et al., 1999), increases the proportion of unc-6ΔC animals with extra ventral nerve cord motor neuron branches (Fig. 5A).

Calcium/calmodulin-dependent protein kinase (CaMKII) is an enzyme that is thought to be critical for regulating synaptic strength and other neural functions. In C. elegans, there is one CaMKII gene, unc-43, and mutations affect neuronal gene expression and the density of ventral nerve cord synapses (Reiner et al., 1999; Rongo and Kaplan, 1999; Troemel et al., 1999). CaMKII activity is reduced by the loss-of-function mutation unc-43(n498(n1186)), whereas constitutive calcium-independent CaMKII activity is caused by the gain-of-function (gf) mutation unc-43(n498). Compared with unc-6ΔC animals, a greater proportion of animals with additional ventral nerve cord motor neuron branches is observed in the unc-43(n1186);unc-6ΔC strain and all of the additional motor neuron branches are suppressed in a significant fraction of unc-43(n498);unc-6ΔC animals (Fig. 5A). These results suggest that the level of UNC-43 CaMKII activity is important for regulating the branching response to UNC-6ΔC.

Calcium influx through voltage-gated calcium channels appears to be one means by which UNC-43 CaMKII is activated (Troemel et al., 1995; Rongo and Kaplan, 1999). Moreover, cytoplasmic Ca²⁺ levels affect growth cone extensions and can regulate the turning response of cultured Xenopus axons to netrin-1 (Gomez and Spitzer, 1999; Hong et al., 2000; Zheng, 2000). Therefore, we examined whether unc-2 and unc-36, genes that encode

Figure 3. Alleles of npr-1 suppress the UNC-6ΔC-elicted outgrowth of ventral nerve cord motor neuron processes. A–D, The percentage of animals that had any additional processes induced by unc-6ΔC expression was measured by scoring for displaced axons at the anterior midbody right ventral sublateral–lateral boundary using pan-neural expression of GFP (Lim et al., 1999) (A–C) or for all DD and VD axons using anti-GABA antibodies (D). A, In the wild type, ventral nerve cord motor neuron processes migrate longitudinally in the ventral nerve cord (vc) and circumferentially (asterisks) past the ventral sublateral nerve (vsl), the lateral canal-associated nerve (can), and toward the dorsal midline. The AVM axon migrates to the vc. B, Expression of unc-6ΔC induces the outgrowth of additional processes that migrate from the ventral nerve cord motor neurons, past the vsl, and to the boundary (arrowheads) between the ventral sublateral and lateral epidermal cells (Lim et al., 1999). No axons are present in wild-type animals at this position. As in wild-type animals, the AVM axon migrates ventrally and circumferential motor neuron axons (asterisks) migrate to the dorsal midline (out of the plane of view). Scale bar: A, B, 25 μm. C, npr-1 alleles were examined for the suppression of all additional outgrowth induced by unc-6ΔC expression. npr-1(ky13) introduces a stop codon after the first of the seven transmembrane domains of the neuropeptide Y receptor homolog and is most likely a loss-of-function allele (de Bono and Bargmann, 1998). urs231 is an integrated transgene that expresses the npr-1(urs89) sequence under a promoter that drives expression in the DA and DB neurons. These animals were scored in the npr-1(ky13) background. D, Expression of npr-1(ad609) and npr-1(urs89) was tested for the ability to suppress the UNC-6ΔC-elicted outgrowth of DD and VD motor neuron processes. Asterisks indicate values that differ from control unc-6ΔC animals at p < 0.001; error bars indicate the SE of proportion.

Mutations that affect second messenger systems enhance or suppress ectopic branching in unc-6ΔC animals

The evidence that signaling pathways within the motor neurons influence the branching of additional motor neuron processes led us to examine whether known neuromodulatory pathway mutations also have an effect. We first tested whether altering G-protein signaling could affect the induction of ectopic axon branches, because neuropeptide Y receptors are G-protein-coupled and because it was reported that adenosine A2b receptor, also a G-protein-coupled receptor, is a netrin-1 receptor (Corset et al., 2000). We found that egl-30(n686), which affects one of the G-protein α-subunits (Gqα) (Brundage et al., 1996), suppresses all induced motor neuron processes in a significant fraction of egl-30(n686);unc-6ΔC animals (Fig. 5A). Whereas EGL-30 is required for viability, egl-30(n686) is a splice acceptor site mutation that causes reduced copies of full-length EGL-30 and is not lethal (Brundage et al., 1996).

EGL-30 is a component of a signaling network that is present in most, if not all, neurons in C. elegans. In the ventral nerve cord motor neurons, activation of the Gqα pathway stimulates neuro-

Figure 4. npr-1 is expressed in DD and VD ventral nerve cord motor neurons. A, B, Immunofluorescence micrographs of a larva expressing a npr-1::GFP reporter. The larva was stained with anti-GFP antibodies (A) and anti-GABA antibodies (B). NPR-1::GFP is coincident with the GABAAergic motor neurons DD and VD of the ventral nerve cord (arrowheads). Scale bar, 25 μm.
$\alpha_1$- and $\alpha_2$-subunits of voltage-dependent calcium channels, respectively (Schafer and Kenyon, 1995; Lee et al., 1997), influence the branching. We found that the loss-of-function alleles unc-2(e55) and unc-36(e251) do not affect the number of ventral nerve cord motor neuron branches (Fig. 5A), suggesting that the branching response to UNC-6 is independent of calcium influx through these channels.

**Physiological state of the neurons potentiate branching in unc-6ΔC but not unc-6 wild-type animals**

Our genetic analyses indicate that the branching of the ventral nerve cord motor neuron elicited by UNC-6ΔC can be enhanced or suppressed by certain neuromodulatory mutations. However, by themselves these mutations do not induce additional motor neuron branches (0%, n = 100 for each). Although altering $\text{Ca}^{2+}$- and cAMP levels in vitro affects axon responses to netrin (Ming et al., 1997; Song and Poo, 1999; Hong et al., 2000), axon guidance defects are not observed in the mutants we tested. However, in some cases there are defects in the positioning of neuronal cell bodies (Tam et al., 2000) (our unpublished observations). To verify that the physiological state of the neurons is altered by the mutations and to establish the relationship between the branching response and second messenger signaling activity, we determined the sensitivity of animals to the acetylcholinesterase inhibitor aldicarb. In this assay, sensitivity is a measure of acetylcholine release and the sensitivity of animals to the acetylcholinesterase inhibitor aldicarb. The synaptic release of acetylcholine was assayed by the degree of paralysis induced by the cholinesterase inhibitor aldicarb. Expression of unc-6ΔC, egl-8(sa47), egl-30(n686);unc-6ΔC, egl-8(sa47); unc-6ΔC, egl-30(n686);unc-6ΔC, and unc-43(n498) gf decreases the release of acetylcholine, making animals more resistant to aldicarb. Expression of dgk-1(nu62) enhances the release of acetylcholine and makes the animals less resistant. Data points are the mean ± SEM of at least three trials.

**Guidance mediated by UNC-6ΔC is affected by ectopic branching**

Several of the mutations inhibit the ectopic branching of the DD and VD circumferential axons. We investigated whether this inhibition affects the dorsal guidance of the axons (Fig. 6). In unc-6ΔC animals, which also express endogenous UNC-6, ectopic
axon branches are observed across all lateral regions, with nearly one-half of the axons reaching the dorsal midline. In comparison, unc-6ΔC animals with the npr-1(ur89), egl-8(n488), or unc-43(gf) mutation have fewer ectopic axon branches across all lateral regions and the axons extend further dorsally, with a majority reaching the dorsal midline (Fig. 6B,C). Thus, inhibiting ectopic branching improves the directed axon extension. However, stimulation of ectopic branching by the unc-43(lf) mutation does not alter the ability of the axons to be dorsally guided relative to the expression of unc-6ΔC alone (Fig. 6B,C). Taken together, these results suggest that the ability of UNC-6ΔC to mediate dorsal guidance is affected by the mutations, but dorsal guidance mediated by the endogenous UNC-6 is not. This further supports the idea that the ectopic branching is a direct consequence of UNC-6ΔC rather than a consequence of disrupting endogenous UNC-6 functions.

**DISCUSSION**

Both UNC-6 and UNC-6ΔC can guide circumferential DD and VD axons. Compared with unc-6(–) animals, unc-6(–)/unc-6ΔC or unc-6(–)/junce-6ΔC animals have better extension of circumferential axons from the nerve cord and better dorsal guidance of the circumferential axons; however, the penetrance of the ectopic branching phenotype is not reduced. In comparison with unc-6(–)/unc-6ΔC animals, unc-6(lf)/unc-6ΔC animals show slightly improved dorsal guidance and less ectopic branching. These results suggest that UNC-6ΔC competes with the endogenous UNC-6, which can suppress the ectopic branching. This was also suggested from the results of the expression of different unc-6ΔC transgenes in unc-6(–) and unc-6(lf) backgrounds (Lim et al., 1999). We also show that mutations that affect CaMKII- and DAG-dependent signaling modulate the ectopic branching phenotype in unc-6ΔC animals but do not affect axon morphology in UNC-6 wild-type animals. Our interpretation of these results is that UNC-6 C is responsible for inhibiting the effects of CaMKII- and DAG-dependent signaling, which, if not silenced, can modulate axon morphology.

The molecular mechanism by which UNC-6ΔC triggers ectopic branching is unknown. Netrin is thought to induce receptor complexes that can trigger different types of axon responses depending on the components they contain (Hong et al., 1999; Stein and Tessier-Lavigne, 2001). UNC-6ΔC may allow the formation of UNC-6 receptor complexes that can promote directed extension of growth cones but cannot inhibit responses that lead to branching. This inhibition requires the UNC-6 C domain working in cis to the N-terminal domains within the receptor complex. It is interesting that the UNC-6 C module has been found in a number of proteins, including the complement C345 protein family, frizzled related proteins, type I C-proteinase enhancer proteins (PCOLCEs), and tissue inhibitors of metalloproteinases (TIMPs) (Ishii et al., 1992; Leyns et al., 1997; Banyai and Patthy, 1999). In PCOLCE and TIMP proteins, the UNC-6 C module is involved in the regulation of metalloproteinase activity (Murphy et al., 1991; Hulmes et al., 1997; Langton et al., 1999). This raises the possibility that without UNC-6 C the UNC-6ΔC-containing complexes are more susceptible to regulation by proteases. It has been found that chemical inhibitors of metalloproteinases potentiate netrin-mediated axon outgrowth in vitro and that the netrin receptor homolog of UNC-40, deleted in colorectal cancer (DCC), is a substrate for metalloproteinase-dependent ectodomain shedding (Galko and Tessier-Lavigne, 2000).

Models of UNC-6/netrin guidance predict axon responses to gradients of the molecule. Our results indicate that the ectopic branching in unc-6ΔC animals is caused by a separate branching mechanism that is sensitive to UNC-6 C function, rather than by guidance errors caused by a novel distribution of UNC-6ΔC.
First, expression of UNC-6ΔC causes the ectopic branching in only a subset of UNC-6-responsive neurons that extends along the entire body wall (Lim et al., 1999). A novel distribution of UNC-6ΔC would be expected to affect all of the UNC-6-responsive axons that are present along the body wall. Second, when UNC-6 is ectopically expressed, the branching phenotype is not observed, although the guidance of axons is severely disrupted in such animals (Ren et al., 1999). This indicates that a novel distribution of UNC-6 is not sufficient to cause the ectopic branching phenotype. Third, circumferential axon migrations are partially rescued when unc-6ΔC is expressed in unc-6 null animals, indicating that the proposed gradient and guidance information of UNC-6ΔC is not significantly different from that of UNC-6 in wild-type animals (Lim et al., 1999). Finally, we have uncovered mutations in genes that enhance or suppress the ectopic branching by acting within the branching neurons themselves. It is more likely that the mutations affect the cellular machinery that mediates an axon branching response than the extracellular distribution of UNC-6ΔC.

Second messenger signaling pathways, particularly cyclic nucleotides and Ca2+, are thought to play an important role in the regulation of axon responses to extracellular guidance molecules (for review, see Song and Poo, 1999; Gomez et al., 2001). For example, in vitro culture assays using Xenopus spinal neurons have shown that intracellular Ca2+ and cAMP levels are involved in dictating growth cone behavior in response to netrin-1 (Ming et al., 1997; Hong et al., 2000). Moreover, CaMKII, acting in a Ca2+-dependent manner, can mediate growth cone turning in response to acetylcholine (Zheng et al., 1994), and antagonist blocking of the acetylcholine receptor can inhibit the attractive response to netrin-1 (K. Hong, personal communication). This is interesting because the Gqα-EGL-30–PLCβ EGL-8 pathway produces DAG in response to acetylcholine in C. elegans (Brandt et al., 1996; Lackner et al., 1999). Thus, observations in culture and in C. elegans are consistent with the notion that the CaMKII- and DAG-dependent signaling cascades are linked in the control of UNC-6/netrin responses.

CaMKII- and DAG-dependent signaling, which can modulate DD and VD axon morphology and cause ectopic branching, must be silenced during the dorsally directed migrations. In the unc-6 wild-type background, CaMKII- and DAG-dependent signaling are blocked by the activity mediated by UNC-6 C. In unc-6ΔC animals, the ability of CaMKII- and DAG-dependent signaling to alter axon morphology is not inhibited, and mutations such as dgk-1(II) and unc-43(II), which stimulate the signaling activity (as judged by their ability to elevate acetylcholine release), increase branching activity. Conversely, other mutations that inhibit signaling activity (by decreasing acetylcholine release), such as egl-8, egl-30, and unc-43(gf), diminish branching activity. The silencing effect of UNC-6 C is physiologically significant, because the suppression is required to prevent inappropriate responses that would cause erroneous morphological changes. Extracellular guidance molecules may have evolved strategies to counteract some process of the guidance machinery that tends to introduce branching. Although our results do not directly address what causes axons to branch at their normal stereotyped positions, they suggest that any mechanism that releases the inhibition mediated by UNC-6 C could trigger branching or turning responses.

REFERENCES


