The NCLX-type Na⁺/Ca²⁺ Exchanger NCX-9 Is Required for Patterning of Neural Circuits in Caenorhabditis elegans*

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NCLX is a Na⁺/Ca²⁺ exchanger that uses energy stored in the transmembrane sodium gradient to facilitate the exchange of sodium ions for ionic calcium. Mammals have a single NCLX, which has been shown to function primarily at the mitochondrion and is an important regulator of neuronal physiology by contributing to neurotransmission and synaptic plasticity. The role of NCLX in developmental cell patterning (e.g. in neural circuits) is largely unknown. Here we describe a novel role for the Caenorhabditis elegans NCLX-type protein, NCX-9, in neural circuit formation. NCX-9 functions in hypodermal seam cells that secrete the axon guidance cue UNC-129/BMP, and our data revealed that ncx-9 alleles mutant animals exhibit developmental defects in stereotyped left/right axon guidance choices within the GABAergic motor neuron circuit. Our data also implicate NCX-9 in a LON-2/heparan sulfate and UNC-6/netrin-mediated, RAC-dependent signaling pathway to guide left/right patterning within this circuit. Finally, we also provide in vitro physiology data supporting the role for NCX-9 in handling calcium exchange at the mitochondrion. Taken together, our work reveals the specificity by which the handling by NCLX of calcium exchange can map to neural circuit patterning and axon guidance decisions during development.

Na⁺/Ca²⁺ exchanger proteins utilize the electrochemical gradient of sodium ions to extrude or introduce calcium ions into the cell (1–3). In animals, Na⁺/Ca²⁺ exchangers are divided into three groups based upon substrate specificity and stoichiometry: Na⁺/Ca²⁺ exchangers (NCX)⁵ exchange sodium for calcium, Na⁺/Ca²⁺/K⁺ exchangers exchange sodium for potassium and calcium, and Na⁺, Li⁺/Ca²⁺ exchangers (NCLX; also referred to as CCX, for calcium cation exchanger) exchange sodium or lithium for calcium (1, 3, 4). Na⁺/Ca²⁺ exchangers have been shown to regulate calcium exchange at the cell membrane, endoplasmic reticulum, mitochondrion (in the case of NCLX), and nucleus (3, 5). Because Na⁺/Ca²⁺ exchangers are low affinity/high capacity transporters, they are most effective at regulating calcium homeostasis during transient calcium fluxes, commonly found in excitatory cells like neurons and muscles (1–3). Hence, they are predominantly expressed in skeletal muscles, cardiac muscle, and the brain (6 – 8).

Calcium has well defined roles in secretion of hormones and neurotransmitters, and recently NCLX was implicated in secretion of insulin (9). However, not much is known about secretion of axon guidance cues and whether calcium plays a role in the establishment of stereotyped gradients of axon guidance cues in vivo. The focus of our research described here is on the NCLX-type exchanger, NCX-9, in Caenorhabditis elegans, for which we identify a role in the development of D-type motor neurons that form a GABAergic commissural motor neuron circuit. NCX-9 is expressed in hypodermal seam cells that secrete UNC-129/BMP, which is a commissural guidance cue for the GABAergic motor neuron circuit. The DD and VD neurons that comprise this GABAergic circuit are spatially positioned along the ventral nerve cord of the animal (10). During development, each D-type neuron first extends an anterior process, which then makes a left or right directional choice for dorsal circumferential guidance. Once the commissure reaches its dorsal target, it bifurcates and innervates dorsal muscles (10). There are 6 DD neurons and 13 VD neurons, of which only commissures for DD1 and VD2 fasciculate and extend circumferentially along the left side of the worm, whereas the rest migrate circumferentially along the right side. The mechanism by which the commissures in this circuit make the left/right guidance choices is not well understood, but some evidence suggests that interactions between growth cones and extracellular guidance signals may regulate this process (11, 12).

Here we describe novel roles for the C. elegans NCLX-type protein NCX-9 in patterning of the GABAergic WD/DD circuit. NCX-9 functions in the hypodermal seam cells that secrete the axon guidance cue UNC-129/BMP and ncx-9 alleles mutants exhibit defects in the stereotyped patterning scheme of the DD/DD circuit. Through genetic approaches, we place NCX-9 into a signaling framework with the UNC-6/netrin RAC-depen-
dent pathway and a LON-2/heparan sulfate (HS) pathway. Finally, we provide in vitro evidence supporting the role for NCX-9 in handling calcium exchange at the mitochondrion. These data provide the first description of Na\(^{+}/\)H\(^{+}\)/Ca\(^{2+}/\)H\(^{+}\) exchange in circuit development in C. elegans and a robust signaling framework for left/right patterning of VD/DD commissures.

**Results**

**ncx-9 Is Expressed in the Seam Cells**—To examine the expression pattern of ncx-9, we generated a reporter GFP fusion by fusing the ncx-9 promoter sequence to a GFP gene (Fig. 1A). Stable transgenic lines expressing this reporter were examined microscopically to identify the cells in which ncx-9 was expressed. We detected ncx-9 expression in the seam cells of the organism. Seam cells are neuroectodermal cells that are laterally positioned and embedded within the major hypodermis, hyp7, and run longitudinally across both sides of the animal’s body (Fig. 1, B and C). Expression of ncx-9 is visible in the seam cells across all larval stages, and expression persists into the adult stage of the organism (Fig. 1B).

**Left/Right Projection Patterning of GABAergic Motor Neuron Commissures Is Defective in ncx-9\(^{-/-}\) Mutants**—We analyzed two mutant strains with lesions in the ncx-9 gene; the lesion in the first allele, gk234237, is defined by a premature stop codon, and the second allele, gk773708, results in a glycine to glutamic acid residue change (Fig. 2A). Both lesions occur in close proximity to the \(\alpha_{1}\)-repeat domain that facilitates ion exchange (Fig. 2B). The GABAergic commissural circuit in C. elegans is composed of two classes of neurons: the DDs and the VDs. There are a total of 19 DD/VD neurons; 13 of these are of the VD class, and 6 are of the DD class. Within this circuit, there exists a left/right patterning scheme where 17 commissures extend along the right side of the worm, and only two (DD1 and VD2) fasciculate and extend along the left side of the animal’s body (Fig. 2C; shown is a simplified schematic of the circuit because the DD and VD commissures first extend an anterior process that subsequently bifurcates and migrates circumferentially). The left/right asymmetric VD/DD commissural pattern is highly stereotyped in wild type organisms. A comprehensive molecular mechanism for asymmetrical patterning within the

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**FIGURE 1.** **Expression pattern of ncx-9.** A, promoter element used in the GFP reporter fusion for ncx-9. Genomic loci were downloaded from Wormbase. The promoter sequence for ncx-9 that was PCR-amplified is represented in beige, and the GFP coding sequence is indicated in green. B, expression of the ncx-9 GFP reporter fusion (ncx-9p::gfp) is observed in the seam cells of animals across all larval stages and persists into adulthood (scale bar, 25 \(\mu\)m). White arrowheads indicate seam cells. By the L4 and adult stage, the seam cells fuse together to form one longitudinal syncytium. C, schematic of seam cells embedded in the major hypodermis, hyp7, in adult hermaphrodites. There are 16 seam cells positioned laterally on the left and right side of the adult animal’s body by the L4 stage.
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VD/DD circuit has not been fully established, although a role for various different effectors has been described: the pioneering AVG interneuron, which expresses UNC-6/netrin (12), the ADAM (a disintegrin and metalloprotease) protein UNC-71 (11), and the cadherin HMR-1 (13). We tested whether NCX-9, a hypodermal protein, might have a role in patterning the...
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VD/DD circuit during development. We found that each ncx-9⁻/⁻ mutant line displayed a significant increase in the ectopic left-sided commissures of the GABAergic VD/DD motor neurons on day 1 of adulthood (Fig. 2, D–F). A representative image of the ectopic left-sided commissural defect is shown in Fig. 2D, which shows multiple ectopic left-sided VD/DD commissures at the posterior of the animal. We found that in ncx-9(gk234237) and ncx-9(k773708) mutant animals, there is a 3-fold increase in the frequency of left-sided commissures (excluding DD1/VD2) per animal, in comparison with wild type animals that robustly retain the canonical asymmetrical patterning scheme of this circuit (Fig. 2F; p < 0.005 for wild type versus ncx-9(gk234237) mutants; p < 0.005 for wild type versus ncx-9(gk773708) mutants; n = 100 for all strains examined). This phenotype is highly penetrant at 55 and 53% in ncx-9(gk234237) and ncx-9(k773708), respectively, in comparison with 20% penetrance in wild type animals (Fig. 2G; n = 100 for all strains examined). Furthermore, we generated a transheterozygous animal carrying both ncx-9(gk773708) and ncx-9(gk234237) lesions and observed ectopic left-sided commissures in 47% of the animals and a nearly 3-fold increase in the frequency of ectopic left-sided commissures per animal (Fig. 2, F and G; p < 0.005 for wild type versus ncx-9(gk234237)/ncx-9(gk773708) mutants). To confirm that the patterning defect observed in ncx-9 mutants is developmental, we examined patterning of VD/DD commissures in late L2 stage animals and found significant enhancement of defect in ncx-9(gk234237) mutants in comparison with wild type (Fig. 2H; p < 0.05 for wild type versus ncx-9(gk234237) mutants; n = 50 for all strains examined). We next chose to examine whether NCX-9 functions in the hypodermal seam cells to regulate left/right patterning of VD/DD commissures. To examine the functional site of activity of NCX-9 for its role in VD/DD circuit patterning, we used RNAi to knockdown expression of NCX-9 in the strain DMH106 that has RNAi restricted to the hypodermis only. We observed significant enhancement of defect for DMH106 animals injected with dsRNA targeting ncx-9 when compared with DMH106 animals that were not given dsRNA injections targeting ncx-9, showing that ncx-9 is required in the hypodermis to regulate left/right patterning of VD/DD commissures through a cell non-autonomous mechanism (Fig. 2I; n = 100 for each experimental condition). We also used RNAi to knockdown NCX-9 in the strain XE1375, in which RNAi knockdown is restricted to GABAergic neurons (14). We observed significant defects in XE1375 animals in which RNAi was used to knock down NCX-9 when compared with control, suggesting that NCX-9 does not function within the GABAergic motor neurons to regulate VD/DD circuit patterning (p = 0.20 versus control). NCX-9 is a homolog of the mammalian mitochondrial Na⁺/Ca²⁺ exchanger NCLX, and we chose to examine whether pharmacological inhibition of mitochondrial Na⁺/Ca²⁺ exchange using the NCLX-selective antagonist CGP37157 would disrupt mitochondrial Na⁺/Ca²⁺ exchange. Wild type animals were fed Escherichia coli strain OP50 mixed with the drug CGP37157 or with DMSO. Animals fed OP50 mixed with the drug showed significant enhancement of defect when compared with animals fed OP50 + DMSO or OP50 alone, suggesting that mitochondrial Na⁺/Ca²⁺ exchange plays a significant role in VD/DD left/right patterning (Fig. 2I; n = 100 for each experimental condition). NCX-9 Works through the RAC-dependent netrin Signaling Pathway to Regulate VD/DD Commissural Patterning—To further investigate the mechanism for left/right patterning within the VD/DD motor neuron circuit, we examined mutants of the UNC-6/netrin signaling pathway. We chose members of the UNC-6/netrin signaling pathway because they have been shown to function during development for circumferential guidance of DD/VD commissures. The signaling pathway downstream of the UNC-6/netrin receptors UNC-40/DCC and UNC-5 bifurcates into two parallel pathways: RAC-dependent signaling and RAC-independent signaling. We examined left/right patterning within the DD/VD circuit in mutants of each pathway. These mutants included ced-10(n1993) (CED-10 is RAC-type GTPase), pak-1(ok488) (PAK-1 is a p21-activated kinase), mig-2(mu28) and mig-2(gm103) (MIG-2 is a RHO-type GTPase), max-2(cy2) (MAX-2 is a p21-activated kinase), unc-40(n324) (UNC-40/DCC is a netrin receptor), and unc-129(ev557) (UNC-129 is a BMP-like member of the TGF-β superfamily). MAX-2 has been implicated in both RAC-dependent and RAC-independent branches of the UNC-6/netrin signaling pathway (15). We observed significant defects in left/right patterning for each mutant examined within the UNC-6/netrin signaling pathway as compared with wild type animals (Fig. 3A; *, p < 0.005 for all bars versus wild type; n = 100 for all strains examined), revealing for the first time a role for RAC-dependent and RAC-independent signaling members in left/right patterning within the VD/DD circuit. Next, we investigated whether NCX-9 functions in the RAC-dependent or
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FIGURE 3. NCX-9 works through the RAC-dependent netrin signaling pathway to regulate VD/DD commissural patterning. A, box plot plotting the average number of ectopic left-sided VD/DD commissures for wild type animals and the following mutant genotypes: nce-9(gk234237); unc-129(ev557), nce-9(gk234237);unc-129(ev557), mig-2(mu28), nce-9(gk234237);mig-2(mu28), mig-2(gm103), nce-9(gk234237);mig-2(gm103), pak-1(0k488), nce-9(gk234237); pak-1(0k488), ced-10(n1993), nce-9(gk234237);ced-10(n1993), max-2(cy2), nce-9(gk234237);max-2(cy2), max-2(nv162), nce-9(gk234237);max-2(nv162), unc-40(n324), nce-9(gk234237);unc-40(n324), unc-40(c.a.), and nce-9(gk234237);unc-40(c.a.) (*, p < 0.05 for all strains versus wild type; n.s., not significant). Sample size for each genotype, including wild type, was 100. None of the mutant genotypes representing RAC-dependent effectors displayed enhancement in the background of nce-9(gk234237) as compared with either single mutant genotype alone (p > 0.05); however, the RAC-independent effector max-2(cy2) and max-2(nv162) and unc-40(c.a.) displayed significant enhancement in the background of nce-9(gk234237) when compared with either single variant alone (p < 0.005). B, bar chart plotting the numbers of normal (two) plus ectopic (anything more than than two) left-sided VD/DD commissures for each of the genotypes from A. Sample size for each genotype, including wild type, was 100. C, bar chart plotting the penetrance of embryonic lethality for wild type, nce-9(gk234237), max-2(cy2), and nce-9(gk234237);max-2(cy2). D, box plot plotting the average percentage of VD/DD motor neuron commissures failing to reach the dorsal cord per animal for each of the genotypes from C (*, p < 0.005 versus wild type). Sample size for each genotype, including wild type, was 50. The nce-9(gk234237);max-2(cy2) double mutant displays significant enhancement of defect as compared with wild type, nce-9(gk234237), and max-2(cy2). E, bar chart plotting the anterior/posterior biasing of ectopic left-sided VD/DD commissures across a population of animals (n = 50). Error bars, S.D.

RAC-independent signaling pathways by testing for enhancement in double mutant animals of each arm of the pathway. We found significant enhancement of left/right patterning defects in max-2(cy2);nce-9(gk234237) double mutants compared with nce-9(gk234237) and max-2(cy2) single mutants and failed to observe any significant enhancement in the nce-9(gk234237); unc-129(ev557), nce-9(gk234237);ced-10(n1993), nce-9(gk234237); pak-1(0k488), nce-9(gk234237);mig-2(mu28), or nce-9(gk234237); unc-40(n324) double mutants as compared with the single mutant in each case (Fig. 3A; p < 0.005 for max-2(cy2) versus max-2(cy2);nce-9(gk234237);Unc-129(ev557) versus nce-9(gk234237);unc-129(ev557); p = 0.4 for mig-2(mu28) versus mig-2(mu28);nce-9(gk234237); p = 0.6 for pak-1(0k448) versus pak-1(0k448);nce-9(gk234237); p = 0.7 for ced-
10(n1993) versus ced-10(n1993); ncx-9(gk234237); p = 0.57 for unc-40(n324) versus unc-40(n324); ncx-9(gk234237); n = 100 for all strains examined). We also examined mig-2(gm103) mutants that carry a Mig-2 gain of function allele and observed significant defects compared with wild type (Fig. 3A; p < 0.005 versus wild type; n = 100). To confirm the enhancement of defects observed in VD/DD left/right patterning in ncx-9(gk234237); max-2(cy2) when compared with max-2(cy2) alone, we examined a second max-2 allele max-2(nv162) and the double mutant ncx-9(gk234237); max-2(nv162). We see enhancement of VD/DD left/right patterning in ncx-9(gk234237); max-2(nv162) in comparison with max-2(nv162), confirming our observation that NCX-9 functions parallel to MAX-2 and netrin RAC-independent signaling (Fig. 3A; p < 0.005 for max-2(nv162) versus ncx-9(gk234237); max-2(nv162); n = 100 for all strains examined). We examined mig-2(gm103) in an ncx-9(gk234237) background and observed no difference in defect when compared with mig-2(gm103) alone (Fig. 3A; p = 0.09 for mig-2(gm103) versus ncx-9(gk234237); mig-2(gm103); n = 100). We also examined the strain LE2336, which carries a constitutively active variant of the netrin receptor UNC-40/DCC (UNC-40(c.a.)) and also generated a strain carrying the constitutively active netrin receptor UNC-40/DCC in an ncx-9(gk234237) background. We do not see VD/DD left/right patterning defects in UNC-40(c.a.) when compared with wild type (Fig. 3A; n = 100 for all strains examined). Interestingly, this strain shows a high degree of circumferential axon guidance defects in VD/DD neurons, suggesting that constitutively active UNC-40/DCC disrupts circumferential axon guidance but does not disrupt left/right-stereotyped patterning. The strain carrying constitutively active UNC-40/DCC in an ncx-9(gk234237) background shows enhancement of defect when compared with wild type and UNC-40(c.a.) (Fig. 3A; p < 0.005 for UNC-40(c.a.) versus UNC-40(c.a.); ncx-9(gk234237); n = 100 for all strains examined). The left/right commissural patterning defect is penetrant in >50% of the population in single mutants of all examined genes within the RAC-dependent and RAC-independent arms of the UNC-6/netrin signaling pathway (Fig. 3B; n = 100 for all strains examined). These mutants also exhibit an increase in the frequency of worms with more severe patterning defects where as many as six commissures are ectopically routed along the left side in some animals examined (Fig. 3B). We observed a major increase in the penetrance of commissural patterning defect in only ncx-9(gk234237); max-2(cy2) double mutants when compared with max-2(cy2) and ncx-9(gk234237) single mutants (Fig. 3B; max-2(cy2) animals exhibit 52% penetrance, whereas ncx-9(gk234237); max-2(cy2) double mutants exhibit 70% penetrance). We also observed several additional defects in ncx-9(gk234237); max-2(cy2) double mutants. Approximately 95% of the progeny for ncx-9(gk234237); max-2(cy2) double mutant animals were embryonically lethal as compared with ~2% for wild type, ~2% for ncx-9(gk234237), and ~4% for max-2(cy2), which further validates a parallel role for NCX-9 and MAX-2 during development (Fig. 3C). Furthermore, ncx-9(gk234237); max-2(cy2) double mutants show a mild but significant enhancement in the average number of VD/DD commissures per animal that fail to reach the dorsal nerve cord when compared with wild type or the single mutants of ncx-9(gk234237) and max-2(cy2) (Fig. 3D; *, p < 0.005 versus wild type; n = 50 for all strains examined). This indicates that NCX-9 may have a role in commissural guidance of VD/DD motor neurons. Together, these data show that NCX-9 functions in the same pathway as RAC-dependent effectors of the UNC-6/netrin signaling pathway to set left/right patterning of the VD/DD GABAergic circuit.

We next chose to examine whether disruption of left/right patterning in wild type and mutants is biased across the anterior/posterior axis of the animal. To quantify whether there is a positional anterior or posterior bias toward misrouted VD/DD commissures along the left side of the animal, we used the vulva as a centering reference to divide the anterior/posterior of the worm. Commissures for DD1–4 and VD1–8 generally lie anterior of the vulva, whereas commissures for DD5–6 and VD9–13 lie posterior of the vulva. In wild type and mutants of ncx-9(gk234237), max-2(cy2), and ced-10(n1993), left/right patterning defects are biased toward more posterior VD/DD commissures (Fig. 3E; n = 50 for all strains examined). Interestingly, misexpression of HST-6 in hypodermal cells has been shown to predominantly disrupt the patterning scheme of posterior DB motor neurons (16). Collectively, this indicates that projection schemes within circuits may be regulated in a spatially restricted manner.

NCX-9 Works with Heparan Sulfate Proteoglycan Modifiers to Regulate VD/DD Commissural Patterning—The DA/DB motor neuron circuit in C. elegans also develops a highly stereotyped left/right commissural patterning scheme. A molecular mechanism for left/right-handedness within the cholinergic DA/DB motor neuron circuit of C. elegans has been described (16). Patterning of the DA/DB motor neurons is established by interactions between axon guidance cues, receptors present on the neuronal growth cone membrane and the extracellular matrix in the surrounding hypodermal cells. We chose to investigate whether ncx-9 and unc-129 regulate commissural patterning schemes beyond the VD/DD circuit and examined stereotyped patterning of the DA/DB commissures in ncx-9(gk234237) and unc-129(ev557) mutants. We found that ncx-9(gk234237) mutants maintain stereotyped left/right patterning of DA/DB commissures, suggesting that ncx-9 does not globally regulate patterning in various classes of motor neurons (Fig. 4A, n = 50 for all strains examined). However, we observed a mild but significant defect in DA/DB commissural patterning in unc-129 mutants, suggesting that unc-129 may regulate patterning in the DA/DB motor neuron circuit as well (Fig. 4A). Heparan sulfate modifiers have been shown to play a role in asymmetric patterning of DA/DB motor neurons in C. elegans, and we chose to examine whether the HS pathway regulates commissural patterning in motor neuron circuits other than DA/DB and examined the VD/DD patterning scheme in mutants of HS pathway components (16). These mutants included lon-2(e678) (LON-2 is a hypodermally expressed heparan sulfate core protein), sdn-1(zh20) (SDN-1 is a neuronally expressed heparan sulfate core protein), hst-2(ok595) (HST-2 is a 2-O-sulfotransferase), and hst-6(ok273) (HST-6 is a 6-O-sulfotransferase). In each case, we observed significant defects in left/right patterning for each mutant as compared with wild type animals (Fig. 4B; *, p < 0.005 versus wild type; n = 50 for all strains examined).
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wild type; \( n = 100 \) for all strains examined), revealing that HS core proteins and their modifiers regulate patterning of VD/DD motor neuron commissures. Mutants of \( hst-2(ok595) \) are 100% penetrant for defective left/right VD/DD commissural patterning and exhibit the most severe defects, with several mutants exhibiting as many as eight ectopic left-sided VD/DD commissures (Fig. 4C; \( n = 100 \) for all strains examined). Next, we investigated whether NCX-9 functions with the HS pathway for left/right VD/DD patterning by testing for enhancement in double mutants. We failed to observe any significant enhancement in the \( ncx-9(gk234237); lon-2(e678) \), \( ncx-9(gk234237); hst-2(ok595) \), and \( ncx-9(gk234237); hst-6(ok273) \) double mutants as compared with the single mutant in each case (Fig. 4B; \( p = 0.16 \) for \( lon-2(e678) \) versus \( ncx-9(gk234237); lon-2(e678); p = 0.21 \) for \( hst-2(ok595) \) versus \( ncx-9(gk234237); hst-2(ok595); p = 0.49 \) for \( hst-6(ok273) \) versus \( ncx-9(gk234237); hst-6(ak273) \)). However, we observed significant enhancement of defect in \( ncx-9(gk234237); sdn-1(zh20) \) double mutants as compared with the single mutant, suggesting that \( ncx-9 \) functions in a pathway parallel to \( sdn-1 \) (Fig. 4B; \( p = 0.02 \) for \( sdn-1(zh20) \) versus \( ncx-9(gk234237); sdn-1(zh20) \)). We also examined VD/DD commissural guidance in \( hst-2 \) and \( hst-6 \) mutant animals and only observed significant defects in commissural guidance for \( hst-2(ok595) \) mutant animals as compared with wild type animals (Fig. 4D; \( \ast, p < 0.005; n = 50 \) for all strains examined). Finally, we examined the genetic relationship between HS modifier \( HST-6 \) and the UNC-6/netrin receptor UNC-40/DCC for left/right patterning and commissural guidance of VD/DD motor neurons. We observed significant enhancement of left/right patterning in \( hst-6(ok273); unc-40(n324) \) double mutants when compared with wild type or single mutants of \( hst-6(ok273) \) and \( unc-40(n324) \) (Fig. 4E; \( \ast, p < 0.005 \) versus wild type; \( n = 100 \) for all strains examined). We also observed significant enhancement of commissural guidance defects in \( hst-6(ok273); unc-40(n324) \) double mutants when compared with wild type or single mutants of \( hst-6(ok273) \) and \( unc-40(n324) \) (Fig. 4F; \( \ast, p < 0.005 \) versus wild type; \( n = 100 \) for all strains examined). These data show that \( NCX-9 \) functions in the same pathway as \( LON-2/\gamma\text{lypican and HS modifiers HST-2 and HST-6 and works parallel to SDN-1/syndecan to set left/right patterning of the VD/DD GABAergic circuit. Furthermore, the HS modifier HST-6 functions parallel to the netrin receptor UNC-40 for left/right patterning and commissural guidance of VD/DD motor neurons, suggesting that NCX-9 facilitates VD/DD circuit development through two divergent signaling mechanisms.

NCX-9 Facilitates Sodium Calcium Exchange at the Mitochondrion in Vitro—The mammalian counterpart of the \( C. \) elegans \( NCX-9 \) protein in humans and mice, called NCLX, has previously been shown to function primarily as a mitochondrial sodium calcium exchanger (5). To examine the subcellular site of \( Ca^{2+} \) exchange activity for NCX-9, we co-transfected HEK-293T cells with either NCX-9 or NCLX and shNCLX plasmids, which reduces endogenous NCLX but not the transfected NCLX, and monitored \( Ca^{2+} \) exchange at the plasma membrane and mitochondria (5). To measure plasma membrane \( Ca^{2+} \) exchange, we preloaded cells with the cytosolic \( Ca^{2+} \)-sensitive dye Fura-2. In each case, we used the human NCLX as a positive control. NCLX, when ectopically expressed in HEK-293T cells, primarily functions at the mitochondria, but some of it still reaches the plasma membrane (3, 5). We first measured cytosolic \( Ca^{2+} \) fluxes in HEK-293T cells that express NCX-9 and compared the \( Ca^{2+} \) exchange activity with that of cells expressing human NCLX or negative control (not expressing any NCX-9 or human NCLX) that were preloaded with Fura-2/AM. From these sets of experiments, we found that after replacing extracellular \( Na^{+} \) with NMDG, there was a rapid increase in cytosolic \( Ca^{2+} \) influx in NCLX-expressing cells (Fig. 5A), consistent with the reported plasma membrane residual activity of human NCLX (3). In contrast, \( Ca^{2+} \) influx observed in NCX-9-expressing cells was not significantly different from our non-transfected negative control cells (Fig. 5A). The subsequent addition of \( Na^{+} \) induced a strong \( Ca^{2+} \) efflux in cells expressing human NCLX (Fig. 5A). The rate of \( Ca^{2+} \) influx in NCLX-expressing cells was found to be significantly higher \((10 \pm 2\text{-fold}) \) compared with negative control cells (Fig. 5B), whereas no \( Ca^{2+} \) influx was monitored in NCX-9-expressing or control cells. Cells transfected with NCLX also showed a higher \((12 \pm 3\text{-fold}) \) \( Ca^{2+} \) efflux rate compared with the negative control cells (Fig. 5C), whereas NCX-9-expressing cells showed only residual activity. This suggests that NCX-9 is not functional at the plasma membrane.

Next, we measured mitochondrial \( Ca^{2+} \) fluxes evoked by ATP-dependent purinergic activation in mitochondria using mitoPericam in cells expressing NCX-9 or NCLX while knocking down the endogenous NCLX using a short hairpin RNA (shNCLX) (Fig. 5D). Consistent with previous studies (5),

FIGURE 4. NCX-9 works with heparan sulfate proteoglycan modifiers to regulate VD/DD commissural patterning. A, box plot plotting the average number of ectopic DA/DB commissures for wild type animals and the following mutant genotypes: \( ncx-9(gk234237) \) and \( unc-129(ev557) \). B, box plot plotting the average number of ectopic left-sided VD/DD commissures for wild type animals and the following mutant genotypes: \( ncx-9(gk234237); lon-2(e678) \), \( ncx-9(gk234237); hst-2(ok595) \), and \( ncx-9(gk234237); hst-6(ak273) \). C, bar chart plotting the number of ectopic left-sided VD/DD commissures for each of the genotypes from B. D, box plot plotting the average percentage of VD/DD motor neuron commissures failing to reach the dorsal cord per animal for \( ncx-9(gk234237); hst-2(ok595) \), \( ncx-9(gk234237); hst-6(ak273) \), and \( ncx-9(gk234237); hst-6(ak273); p < 0.005 versus wild type). Sample size for each genotype, including wild type, was 50. None of the mutant genotypes representing HS modifiers displayed enhancement in the background of \( ncx-9(gk234237) \) as compared with either single mutant genotype alone \((p > 0.05) \). However, a mutant of the core HS protein \( sdn-1(zh20) \) displayed significant enhancement in the background of \( ncx-9(gk234237) \) when compared with either single mutant alone \((p = 0.02 \) for \( sdn-1(zh20) \) versus \( ncx-9(gk234237); sdn-1(zh20) \)). E, bar chart plotting the number of ectopic left-sided VD/DD commissures for each of the genotypes from B. F, box plot plotting the average percentage of VD/DD motor neuron commissures failing to reach the dorsal cord per animal for \( ncx-9(gk234237); hst-2(ok595) \), \( ncx-9(gk234237); hst-6(ak273) \), and \( ncx-9(gk234237); hst-6(ak273); p < 0.005 versus wild type). Sample size for each genotype, including wild type, was 50. None of the mutant genotypes representing HS modifiers displayed enhancement in the background of \( ncx-9(gk234237) \) as compared with either single mutant genotype alone \((p > 0.05) \). However, a mutant of the core HS protein \( sdn-1(zh20) \) displayed significant enhancement in the background of \( ncx-9(gk234237) \) when compared with either single mutant alone \((p = 0.02 \) for \( sdn-1(zh20) \) versus \( ncx-9(gk234237); sdn-1(zh20) \)).
endogenous NCLX activity in cells transfected with shNCLX was reduced by ~3-fold (Fig. 5E). Notably, however, Ca\(^{2+}\) efflux in cells expressing either NCX-9 or NCLX was similarly enhanced by ~5-fold compared with shNCLX-transfected cells and thus fully rescued (Fig. 5E). Together, these in vitro experiments demonstrate that NCX-9, like NCLX (5), is localized at the mitochondria and regulates sodium calcium exchange in these organelles.

Discussion

Here we provide the first detailed analysis of a Na\(^{+}\)/Ca\(^{2+}\) exchanger family in C. elegans categorized NCX-9 as a candidate mitochondrial exchanger based upon predicted domains and sequence similarity to the mammalian mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger, NCLX (17). Here we further tested this hypothesis by first using a pharmacological inhibitor of mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchange in C. elegans and were able to induce patterning defects in the DD/VD circuit of wild type animals. Next, we examined calcium exchange of NCX-9 at the mitochondrial membrane and cytosolic plasma membrane in vitro and determined that NCX-9 handles exchange at the mitochondrion in HEK-293T cells.

Our work presents a model in which the function of NCX-9 in seam cells influences left/right projection choices made by DD/VD neurons at the ventral midline. We show that NCX-9 functions in the RAC-dependent arm of the UNC-6/netrin signaling pathway and also in a heparan sulfate pathway through the core protein LON-2/glypican and HS modifiers HST-2 and HST-6. Globally diffused secretion of UNC-6/netrin from ventral hypodermal cells P1/2, P3/4, P5/6, P6/7, P8/9, P10/11, and P11/12 has been shown to regulate circumferential guidance of

FIGURE 5. In vitro characterization of NCX-9 exchange activity. A, representative traces of cytosolic Ca\(^{2+}\) transport in NCLX- and NCX-9-expressing HEK-293T cells preloaded with Fura-2/AM superfused in the presence or absence (replaced by NMDG) of Na\(^{+}\) in Ca\(^{2+}\)-containing Ringer’s solution (see “Experimental Procedures”). Cells not expressing any NCX-9 or human NCLX are considered as control. B, average initial rates of cytosolic Ca\(^{2+}\) influx from A after the addition of NMDG Ringer’s solution (n = 3) (*, p < 0.005 versus control). C, average initial rates of cytosolic Ca\(^{2+}\) efflux from A after the addition of NMDG Ringer’s solution (n = 3) (*, p < 0.005 versus control; n.s., not significant). D, representative traces of mitochondrial Ca\(^{2+}\) transport in HEK-293T cells expressing human NCLX or NCX-9 and cotransfected with mitoPericam (a genetically encoded mitochondrial Ca\(^{2+}\) sensor) and shNCLX. Mitochondrial Ca\(^{2+}\) transients were evoked in cells superfused with ATP (100 μM at the indicated time). Cells transfected with vector are termed as control. E, average initial rates of mitochondrial Ca\(^{2+}\) efflux taken from D (n = 10). *, p < 0.005 versus control; **, p < 0.005 versus shNCLX. For B, C, and E, data are mean ± S.D. (error bars).
DD/VD commissures (18). Hypodermal cells also secrete the heparan sulfate LON-2/glypican, which works with UNC-6/netrin to facilitate dorsal guidance of DD/VD commissures (19). Another secreted molecule, UNC-129, is expressed in seam cells and dorsal body wall muscles and plays a significant role in commissural guidance of D-type motor neurons (20–22). Previously, UNC-129 had been described as a regulator of long-range guidance of motor neuron commissures because it boosts UNC-5 + UNC-40/DCC signaling to enhance the response of dorsally migrating growth cones (21). Our work shows that UNC-129 also plays a significant role in establishing the left/right patterning scheme of DD/VD neurons, suggesting that its circuit development roles are not spatially limited to the dorsal region. It is possible that the NCX-9 regulates secretion of UNC-129 from seam cells and thus contributes to the left/right patterning scheme of DD/VD commissures. A role for mitochondrial Na+/Ca2+ exchangers in secretion has been demonstrated in the human NCLX, the vertebrate homolog of NCX-9 (9). NCLX regulates secretion of insulin in β-cells by triggering efflux of mitochondrial Ca2+. Glucose uptake and its oxidation in β-cells are succeeded by an increase in intracellular Ca2+ and subsequent uptake of cytosolic Ca2+ into mitochondria for boosting ATP production (23). NCLX modulates mitochondrial Ca2+ levels by stimulating Ca2+ efflux and thus functions as a rate-limiting step for mitochondrial Ca2+ signaling and ATP production induced by cellular glucose (9). Furthermore, Ca2+ clearance from mitochondria via NCLX also shapes the cytosolic Ca2+ response that regulates insulin secretion (9). Interestingly, the severity of patterning defects observed in both mutant alleles of ncx-9 and unc-129(ev557) mutants is remarkably similar, which may hint toward a closely linked relationship between the two (Fig. 3, A and B).

We examined a constitutively active variant of UNC-40/DCC in an ncx-9(gk234237) background and saw enhancement of defect compared with the strain with constitutively active UNC-40/DCC alone. This suggests that NCX-9 probably functions downstream of UNC-40/DCC. Interestingly, we did not observe DD/VD left/right patterning defects in the strain carrying the constitutively active variant of UNC-40/DCC, although it is defective for dorsal circumferential axon guidance (24). We also examined ncx-9(gk234237) in the background of mig-2(gm103), which is a constitutively active variant of the MIG-2 RAC protein that functions downstream of UNC-40/DCC. We observed no significant enhancement in ncx-9(gk234237);mig-2(gm103) double mutants compared with mig-2(gm103) alone, suggesting that NCX-9 probably functions upstream of MIG-2. Thus, our analysis indicates a role for NCX-9 that is likely to be downstream of UNC-40/DCC but upstream of the RAC proteins (MIG-2 and CED-10) and also the PAK proteins (PAK-1 and MAX-2), which function downstream of the RACs. UNC-40/DCC is membrane-bound and functional at the growth cones of DD/VD neurons, and this raises an interesting question of how NCX-9 might be functioning downstream of UNC-40/DCC, despite being functional in the seam cells rather than the growth cone, as our data indicate. A role downstream of UNC-40/DCC for NCX-9 could be linked to UNC-129/BMP, which is also functional in seam cells. Previous work on this constitutively active variant of UNC-40/DCC has shown that it works independently of UNC-6/netrin, but its relationship with UNC-129/BMP remains unknown (24). As mentioned previously, UNC-129/BMP enhances the signaling strength of the UNC-40/DCC-UNC-5 complex (21). NCX-9, although functioning downstream of UNC-40, might be required to facilitate interactions between UNC-40 in the growth cone and the extracellularly secreted UNC-129/BMP. Supporting this hypothesis is our observation that the phenotype observed in DD/VD left/right patterning in unc-129 mutants is highly similar to ncx-9 mutants, and the UNC-40/DCC constitutively active strain in a background of ncx-9(gk234237) is phenotypically similar to ncx-9(gk234237) alone.

Commissures of DD/VD neurons rely on Wnt signaling for anterior/posterior guidance and on UNC-6/netrin for circumferential dorsal guidance (25, 26). The basal lamina surrounding the DD/VD neurons has a globally diffused UNC-6/netrin gradient, yet 17 of 19 commissures originating from these neurons predominantly show an affinity toward right-sided circumferential guidance. Our data show that DD/VD commissures are unable to make correct left/right projection choices when genes within the UNC-6/netrin signaling pathway are disrupted, suggesting that UNC-6/netrin and its downstream effectors play a critical role in establishing a left/right directional bias for circumferential guidance. We also show that components of the UNC-6/netrin signaling pathway have an anterior/posterior spatial bias toward exerting a stronger patterning influence in the posterior commissures of this circuit. Our data raise the question of how a globally diffused UNC-6/netrin gradient could create patterning specificity and directional bias for circumferential guidance within this circuit. It is likely that UNC-6/netrin interacts with other guidance-related components of the extracellular matrix to generate stereotyped patterning schemes. Indeed, UNC-6/netrin has been genetically and biochemically implicated in a pathway with the hypodermal HS LON-2/glypican to influence dorsal guidance of DD/VD commissures (19). Another HS, SDN-1/syndecan, and its modifier HST-6 regulate DD/VD commissural guidance but do so in a pathway that is parallel to UNC-6/netrin (19, 22). Our work shows that HST-6 acts parallel to UNC-40/DCC in regulating the patterning scheme and dorsal guidance of DD/VD commissures. It is likely that parallel functions of HST-6 with UNC-40/DCC are through its modifications on the side chains of SDN-1, which acts parallel to UNC-6/netrin, unlike LON-2/glypican, which acts within the same pathway as UNC-6/netrin. Furthermore, we show that NCX-9 functions in a pathway with LON-2/glypican but has functions parallel to SDN-1/syndecan. LON-2/glypican is a hypodermally functioning HS core protein, whereas SDN-1/syndecan functions neurally, suggesting that NCX-9 may function and interact primarily through cell-non-autonomous mechanisms to regulate circuit patterning (27, 28). It is possible that NCX-9 functions through the heparan sulfate pathway by regulating the HS side chain biosynthesis machinery. Several components of the HS side chain biosynthesis machinery are expressed and functional in the seam cells, in which NCX-9 functions. The rib-1 gene is required for elongation of HS side chains and, similar to NCX-9, is expressed in the seam cells (29). Furthermore, rib-1 regulates the left/right asymmetric patterning of DD/VD neu-
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Two genes that are critical for the sulfation of HS side chains, *pst-1* and *pps-1*, are also expressed and functional in the seam cells (30, 31). PST-1 is a transporter of the *C. elegans* high energy sulfate donor 3’-phosphoadenosine 5’-phosphosulfate (PAPS), which is encoded by *pps-1*. PAPS (PAPS-1) donates a sulfate group to HS sulfotransferases, such as HST-2 and HST-6, that are required for adding sulfate groups to HS side chains (30). Interestingly, *pps-1* shows a highly restricted expression pattern that is strongest in the seam cells and weakly observed in other neighboring hypodermal cells (30). This is rather interesting, because the process of HS side chain sulfation also takes place in muscles and neurons, suggesting that PPS-1 is probably secreted from the seam cells and the hypoderms and transported to other cell types where HS side chain sulfation takes place. The mechanism by which PPS-1 facilitates HS side chain sulfation in cells in which it is not expressed is still unknown. Interestingly, PST-1, the transporter of PPS-1, is also found to function in the same pathway as HST-2 and HST-6, similar to NCX-9, although a role for PST-1 in VD/DD patterning is unclear (31). It may be the case that NCX-9 mechanistically functions alongside the HS biosynthesis machinery that is present in the seam cells. Future work could explore the contributions of seam cells to HS biosynthesis machinery and focus on investigating a role for NCX-9 in this process.

In summary, our work provides the first evidence of a role for Na⁺/Ca²⁺ exchangers in neural circuit development, and we provide a signaling framework for the regulation of patterning schemes within neural circuits. We show that reduction of NCX-9 function in seam cells perturbs left/right projection choices of DD/VD commissures through a RAC-dependent and heparan sulfate pathway. Our work raises several important questions that require further investigation. Primarily, the precise role of NCX-9 within seam cells needs to be understood. Calcium is as an essential component of several signaling pathways, and seam cells are major contributors to the morphogenesis of the animal (32). Thus, calcium handling via NCX-9 in seam cells could contribute to several important developmental pathways. Indeed, we find that embryonic lethality is highly penetrant in double mutants of NCX-9 and MAX-2, suggesting that calcium handling via NCX-9 is critical for development (Fig. 3C). Our work provides a genetic framework for the stereotyped patterning scheme of the DD/VD commissures. How the growth cone integrates information from multiple guidance cues to generate a directional bias in circumferential guidance remains an attractive question for future investigation.

**Experimental Procedures**

**Animals and Maintenance**

The following animals were used in our study: Bristol N2 (wild type), CZ1200 julS76 II[unc-25p::GFP + lin-15(+)]; *lin-15B(n765) X*, DMH65 julS76 II[unc-25p::GFP + lin-15(+)]; *unc-9(gk234237) V*, DMH32 julS76 II[unc-25p::GFP + lin-15(+)]; *unc-9(gk773708) V*, DMH53 julS76 II[unc-25p::GFP + lin-15(+)]; *pak-1(ok448) X, LE478 julS6 II[unc-25p::GFP + lin-15(+)]; *pig-2(mu28) X, HJ1 max-2(cy2) II; otxS12 X[unc-47p::GFP + lin-15(+)], HJ154 max-2(nv162) II; otxS12 X[unc-47p::GFP + lin-15(+)], LE2336 lqsS128 [unc-25:: myr::unc-40], LE2562 juls76 II[unc-25p::GFP + lin-15(+)]; *unc-73(rh40) I, LE2531 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-40(n324) I, LE847 julS76 II[unc-25p::GFP + lin-15(+)]; *ced-10(n1993) IV, DMH55 julS76 II[unc-25p::GFP + lin-15(+)]; *ncx-9(gk234237) V; pak-1(ok448) X, DMH70 julS6 II[unc-25p::GFP + lin-15(+)]; *ncx-9(gk234237) V; mig-2(mu28) X, DMH71 otxS12 X[unc-47p::GFP + lin-15(+)]; *ncx-9(gk234237) V; max-2(cy2) II, DMH72 julS6 II[unc-25p::GFP + lin-15(+)]; *ncx-9(gk234237) V; unc-40(n324) I, DMH77 [unc-129p::GFP]; *ncx-9(gk234237) V, DMH80 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-9(gk234237) V; hst-2(ok595) X, DMH81 julS6 II[unc-25p::GFP + lin-15(+)]; *hst-2(ok595) X, DMH82 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-9(gk234237) V, DMH80 julS6 II[unc-25p::GFP + lin-15(+)]; *hst-6(ok273) X, DMH83 julS6 II[unc-25p::GFP + lin-15(+)]; *hst-6(ok273) X, DMH86 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-40(n324) I, DMH87 banEx11 [unc-9p::GFP; pmyo-2p:: mCherry], DMH90 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-129(ev557) IV, DMH96 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-129(ev557) IV; *ncx-9(gk234237) V, DMH99 julS6 II[unc-25p::GFP + lin-15(+)]; *sdn-1(2)xh20 X, DMH100 julS6 II[unc-25p::GFP + lin-15(+)]; *sdn-1(2)xh20 X, *ncx-9(gk234237) V, DMH101 julS6 II[unc-25p::GFP + lin-15(+)]; *lon-2(e678) X, DMH102 julS6 II[unc-25p::GFP + lin-15(+)]; *lon-2(e678) X, *ncx-9(gk234237) V, DMH103 [unc-129p::GFP]; *unc-129(ev557) IV, DMH104 julS6 II[unc-25p::GFP + lin-15(+)]; *mig-2(gm103) X; DMH105 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-9(gk234237) V; *mig-2(gm103) X; DMH106 rde-1(ne1219) V; [pKK1260(lin-26p:: nls::GFP); pkI1253(lin-26p:: rde-1) + prF6(rol-6(su1006))]; julS6 II[unc-25p::GFP + lin-15(+)]; DMH120 julS6 II[unc-25p::GFP + lin-15(+)]; *unc-9(gk234237) V; *lqS128 [unc-25p:: myr::unc-40], DMH121 julS6 II[unc-25p::GFP + lin-15(+)]; *ncx-9(gk234237) V; *mig-2(mu162) II, XE1375 (lin-15B(n744) IV; *eri-1(mg366) IV; *rde-1(ne1219) V; *wpSi1[unc-47p::rde-1:SL2:sid-1,Cbunc-119(+)] II; *wpS1[unc-47p::mCherry] I).

Homozygous mutants were selected by PCR and sequencing of the lesion. Genomic DNA was isolated by harvesting animals and collecting in a 1.5-ml tube. 200 μl of lysis buffer (60 μg/ml proteinase K, 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.45% IGEPAL, 0.45% Tween 20, 0.01% gelatin) was added to the tube and then frozen at −80°C for 10 min, followed by incubation at 60°C for 1 h, followed by 95°C for 15 min. The following primers were used for genotyping: for *ncx-9(gk773708), ncx-9(gk773708)F* (gtctccttttaatcattcgtcctcaaa and *ncx-9(gk773708)R* (gaactactggtcctagttgctgc) to identity a G to A base pair change; for *ncx-9(gk234237), ncx-9(gk234237)F* (cgtctgctgctagtaatgcttcag) and *ncx-9(gk234237)R* (tgtagtgcggtagatgctgc) to identify a C to T base pair change; for *ced-10(n1993), ced-10(n1993)F* (ccctaaaaagctttacttcagc) and *ced-10(n1993)R* (ggcaagaaatatcagctggtc) to identify an A to C base pair change; for *pig-2(mu28), pig-2(mu28)F* (gtggctgagttccatgagagttg) and *pig-2(mu28)R* (gacagagcagagagagacacag) to identify a G to A base pair change; for *pak-1(ok448), pak-1(ok448)F* (aatctctcctcgctcgtgctg) and *pak-1(ok448)R* (acactctgctcgtgc) to identify a 1425-bp deletion; for *max-2(cy2), max-
2(cy2)F (cgatactgctctaaactctacg) and max-2(cy2)R (ctcattcttgcttttacgct) to identify a C to T base pair change; for hst-2(ok595)R (cgctcttgctgactcagttgat) to identify a 1335-bp deletion; for hst-6(ok273), hst-6(ok733)F (atgttgctttgctgctgactcagttgat) and hst-6(ok273)R (gtctcttgctgactcagttgat) to identify a 1258-bp deletion; for rde-1(ne219), rde-1(ne219)F (gggtctctttagtctgagttgat) and rde-1(ne219)R (agaagttttggttaacgaaatgttag) to identify a C to T base pair change; and T7 (cgtaatacgactcactatag). Mutants of hst-2(ok595)R (ggtgctctaggacattttgatagtct) to identify a 1063-bp deletion; for sdn-1(zh20), sdn-1(zh20)F (gtgacctacaaatgtctgctg) and sdn-1(zh20)R (gtctctgctgactcagttgat) to identify a 1258-bp deletion; for unc-54(n324), unc-54(n324)F (gtagctacaacaataggtcagtagt) and unc-54(n324)R (tcaagtatgtacaggttcctgatga) to identify a C to T base pair change; and T7 (cgtaatacgactcactatag). Mutants of unc-40(n324), mig-2(gm103), mig-2(nv162), and unc-129(ev557) and strains carrying lls128 [unc-25::myr::unc-40] were identified by visibly severe DD/VD motor neuron axon guidance defects and uncoordinated locomotion. Mutants of lon-2(e678) were identified by their long body size phenotype. NGM plates were seeded with E. coli strain OP50 and maintained according to a standard protocol (33). All animals examined were hermaphrodites (XX) unless otherwise stated.

**Generation of GFP Reporter Gene Fusions**

The transcriptional GFP reporter fusions were made by PCR fusion as described previously (34). The promoter sequence was amplified by PCR using High Fidelity polymerase (Roche Applied Science). A GFP coding region that contained the unc-54 3′-UTR was PCR-amplified from the plasmid pPD95.75 (available from AddGene) using the primers GFP-F (AGCTT-GCATGCTGCACTGCACTG) and GFP-R (AAGGCCC-GTACGCCGACTAGTGG). Following PCR amplification and purification of the promoter and GFP fragment, a fusion PCR step was performed using 10 ng of each amplicon with a promoter-specific primer and the GFP nested primer: ncx-9 promoter-specific primer, TATGGGTTTCCATCAATCCATTTT; GFP nested primer, GGAAACAGTTATGTTTGGTATAATTGGG. The resulting PCR fusion was microinjected into the germ line of wild type (N2) animals.

**Reporter Gene Expression Analysis**

The transcriptional GFP reporter fusions were injected into wild type (N2) animals, and stable lines were selected and maintained. Stable transgenic lines were imaged using a Zeiss Axio Imager M2 under ×40 magnification. Seam cells were identified by their lateral positioning along the body wall and the distinct longitudinal syncytium they form upon fusion in adults.

**Phenotype Analysis**

Left/right patterning defects were characterized as the presence of more than one (VD1 and DD2 seen normally as a single process on the left side) commissures running along one side of the animal’s body. Adult animals were staged on plates for 3 h and subsequently removed. Their progeny were examined at the day 1 adult stage for defects in VD/DD left/right patterning. The number of ectopic left-sided commissures was counted per animal, and penetrance was examined as the percentage of worms in the total population displaying a left/right choice defect. To assess whether the defect observed in adults was developmental, we examined VD/DD left/right patterning in late L2 stage animals. Animals were mounted on 2% agarose pads with 5 mM sodium azide and imaged using a Zeiss LSM 710 upright confocal system.

DD and VD motor neuron commissural guidance defects were quantified as described previously (15) by counting the number of normal and defective commissures from populations from each genotype and calculating the percentage defect. Commissures were visualized by crossing animals into the background of transgenes jul576[unc-25(prom)::GFP + lin-15(+)] and oxIs12[unc-47(prom)::GFP + lin-15(+)]. The number of normal or defective commissures from a population was counted, and percentage defects were determined by summing all of the defective commissures and dividing this by the total number of commissures. A defect was scored if the commissure could not be seen to connect with any part of the dorsal nerve cord. Penetrance was scored by calculating the percentage of animals across a population exhibiting at least one commissure connectivity defect.

To quantify embryonic lethality, adult animals were transferred onto staging plates seeded with E. coli strain OP50 for 4 h and subsequently removed. Later, from these staged plates, an individual day 1 adult animal was staged on a seeded plate for 3 h at room temperature and subsequently removed. The number of eggs laid during this time was counted by examining the plate under a dissection microscope. The plates were examined on the second day (24 h from day 1 adult parent removal), and the number of non-hatched eggs on plates was counted under a dissection microscope. The number of non-hatched eggs after 24 h was divided by the total number of eggs present on the first day to assess the penetrance of embryonic lethality. This was repeated five times for each genotype.

To examine whether ncx-9 functions in the hypodermal seam cells to regulate DD/DD left/right patterning, we performed a hypodermis specific knockdown of ncx-9 using dsRNA in the strain DMH106 that has RNAi restricted to the hypodermis only. The dsRNA sequence was amplified from the L4440 vector containing a dsRNA sequence targeting the ncx-9 gene (CUUkp3304C014Q clone, SourceBioscience). The amplified sequence was subsequently used to make dsRNA via an in vitro transcription reaction. DMH106 animals were then injected with dsRNA targeting ncx-9. The injected animals were cloned 24 h postinjection, and their progeny were scored for VD/DD left/right patterning at the day 1 adult life stage. DMH106 animals that were not injected with dsRNA targeting ncx-9 were used as control and also examined at the day 1 adult life stage for VD/DD left/right patterning.

To examine whether mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange regulates VD/DD left/right patterning, animals were fed E. coli strain OP50 mixed with the drug CGP37157 (Tocris), a selective antagonist of the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger NCLX. CGP37157 was dissolved in DMSO at a working concentration of 100 mM. 100 μl of the drug was mixed with 900 μl of OP50, and regular NGM plates were subsequently seeded with this OP50-drug mix. Positive control plates were each seeded with a mixture of 900 μl of OP50 and 100 μl of DMSO. Negative control plates were seeded with only OP50. Adults
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were staged on plates for 4 h and subsequently removed. Once their progeny reached day 1 adulthood, they were examined for defects in VD/DD left/right patterning.

Physiology

Cell Culture and Transfection—HEK-293T cells were cultured in DMEM with 10% FBS. For the Fura-2 experiment, cells grown on glass coverslips were co-transfected with 8 μg of either NCLX or NCX-9 and with 2 μg of shNCLX, which reduces endogenous (Sigma (Mission TRC shRNA Target Set TRCN-5045)) but not ectopic NCLX expression using standard calcium phosphate precipitation as described previously (3).

For measurement of mitochondrial Ca$^{2+}$ levels, cells were transiently cotransfected with mitoPericam (a genetically encoded mitochondrial Ca$^{2+}$ sensor) along with the plasmids as described in the legend to Fig. 5.

Fluorescent Cytosolic and Mitochondrial Ca$^{2+}$ Imaging—Cytosolic Ca$^{2+}$ imaging with Fura-2 was performed as described previously (3). Mitochondrial Ca$^{2+}$ levels were monitored using transiently transfected mitochondria targeted mitoPericam as described previously (5).

Statistics

All box plots were constructed using BoxPlotR. The following description uniformly applies to all box plots in each figure. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the 5th and 95th percentiles, outliers are represented by dots; crosses represent sample means; gray boxes around crosses indicate 95% confidence intervals of the means. Sample size (n) is listed above strain names. Statistical analysis was performed with Student’s two-tailed t test using SPSS Statistics version 22, and p ≤ 0.05 was considered statistically significant.

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