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Calmodulin and Son of sevenless dependent signaling pathways regulate midline crossing of axons in the *Drosophila* CNS

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SUMMARY

The establishment of axon trajectories is ultimately determined by the integration of intracellular signaling pathways. Here, a genetic approach in *Drosophila* has demonstrated that both Calmodulin and Son of sevenless signaling pathways are used to regulate which axons cross the midline. A loss in either signaling pathway leads to abnormal projection of axons across the midline and these increase with *roundabout* or *slit* mutations. When both Calmodulin and Son of sevenless are disrupted, the midline crossing of axons mimics that seen in *roundabout* mutants, although Roundabout remains expressed on crossing axons. Calmodulin and Son of sevenless also regulate axon crossing in a *commissureless* mutant. These data suggest that Calmodulin and Son of sevenless signaling pathways function to interpret midline repulsive cues which prevent axons crossing the midline.

Key words: Calmodulin, Sos, Axon guidance, *Drosophila*, Repulsion, CNS

INTRODUCTION

During axon guidance, receptors on the surface of an extending axon's growth cone detect extracellular guidance cues and initiate intracellular signaling cascades which ultimately govern pathway selection and the target choices of the growth cone (Cook et al., 1998; Gallo and Letourneau, 1999; Suter and Forscher, 1998; Tessier-Lavigne and Goodman, 1996). Biochemical and genetic studies have helped identify many of the key components used in the decision to cross or not to cross the midline of an organism (Flanagan and Van Vactor, 1998; Thomas, 1998). Commissural axons are steered to the midline by chemoattractive gradients of Netrin, a secreted protein found in several organisms (Culotti and Merz, 1998; Kennedy et al., 1994; Serafini et al., 1994). Mutations in the two *Drosophila netrin* genes cause severe defects in anterior and posterior commissure formation (Harris et al., 1996; Mitchell et al., 1996). As Netrins guide axons to the midline, Slit, a secreted protein at the midline, acts as a repulsive guidance cue preventing certain axons from crossing or re-crossing the midline (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen et al., 1999; Wang et al., 1999).

Drosophila neurons interpret the Slit repulsive signal through the action of a pair of cell surface proteins, Commissureless (Comm), and Roundabout (Robo) (Kidd et al., 1998a,b; Seeger et al., 1993). Robo is a receptor for Slit and is expressed on most CNS axons. In *robo* mutants, too many crossovers occur as commissures form and axons which should remain on one side now meander back and forth over the midline (Kidd et al., 1998a,b). The *comm* gene product is a transmembrane protein expressed on the surface of midline glia which apparently functions to remove Robo from commissural axons to allow them to traverse the midline (Kidd et al., 1998a,b; Tear et al., 1996). Once axons have crossed the midline, Robo expression is up-regulated, presumably to prevent axons from re-crossing the midline. In *comm* mutants, all axons are prevented from crossing the midline and no commissures form (Seeger et al., 1993; Tear et al., 1996). In *robo comm* double mutants, the *robo* phenotype prevails; since there is no Robo to detect Slit, Comm is not required (Kidd et al., 1998a,b; Seeger et al., 1993). Evidence for a second receptor system is also accumulating but has not yet been characterized (see Hummel et al., 1999; Kidd et al., 1999).

Components of the intracellular machinery used to transduce these attractive and repulsive cues are also being identified (e.g. Song and Poo, 1999). Netrin-mediated attraction or repulsion is regulated by cyclic AMP levels (Ming et al., 1997, 1999; Song et al., 1997), cytoskeletal connection (Hong et al., 1999), and certain adhesion molecules (Hopker et al., 1999). Calcium also regulates many aspects of growth cone behavior (e.g. Goldberg and Grabham, 1999; Gomez and Spitzer, 1999) and calcium-Calmodulin (CaM) signaling is required for proper midline guidance (VanBerkum and Goodman, 1995). In the latter work, a novel CaM inhibitor, called kinesin-antagonist (KA), was expressed using the neurogenic enhancer element of the *fushi tarazu* gene (ftzng) in a subset of CNS neurons that normally do not cross the midline. KA expression decreases endogenous CaM activation of target proteins in the growth cone and this leads to specific axon guidance defects including stalls at selected choice points, failure to fasciculate properly and abnormal crossing of the midline (VanBerkum and Goodman, 1995).

This work further characterizes the role of CaM in midline

guidance. *robo, slit* and KA mutations interact synergistically to increase the number of axon bundles abnormally crossing the midline. KA also induces axon bundles to cross the midline in the absence of Comm protein. In addition, loss-of-function mutations in *Son of sevenless* (*Sos*; Bonfini et al., 1992) causes specific axon crossover defects without detectable alterations in cell differentiation. *Sos*-dependent crossovers are enhanced by KA or *slit* mutations. In fact, when both CaM and Sos signaling pathways are disrupted simultaneously, midline guidance errors are synergistically increased and embryos display a phenotype remarkably similar to that observed in *robo* mutants, without a decrease in Robo expression. KA and Sos also interact to increase the number of axon bundles crossing in a *comm* mutant. Thus, our data demonstrate that both CaM and Sos signaling pathways are required to prevent certain axons crossing the midline.

MATERIALS AND METHODS

Stocks and breeding

Stocks were raised on conventional cornmeal and molasses based medium at room temperature or 25°C. Conventional breeding strategies and/or recombination experiments were used to combine gene mutations in the same fly. Map and chromosome locations of all named mutations in this study are available from FlyBase. KA^{26} is a viable KA P-element insertion at position 25C10 of chromosome II; KA^{16B1} has two KA insertions at positions 25C and 46F of chromosome II. KA^{16c1} is a third chromosome insertion at position 67B13. $comm^{KA93A}$ is a KA P-element insertion into the *comm* gene and is also referred to as $comm^{6}$ (Tear et al., 1996). We retain the KA93A allele designation to emphasize the presence of the KA minigene. All stocks carried a $white^{1118}$ mutation on the first chromosome to identify the mini-*white* gene present on P elements. Complementation analysis confirmed the presence of the other lethal mutations. To identify the genotypes of stained embryos, β-galactosidase (β-gal) marked balancer chromosomes were present in final stocks (see below).

Histology

Eggs were collected overnight at 25°C on apple juice agar plates smeared with yeast paste. With the exception of the analysis with anti-Robo, embryos were processed for immunocytochemistry using standard methods (see VanBerkum and Goodman, 1995). The modifications described by Kidd et al. (1998a) were utilized for anti-Robo (mAb13C9) staining. Rabbit polyclonal anti-Sos (provided by U. Banerjee) was used at a 1:500 dilution and embryos photographed and the images digitally adjusted simultaneously to facilitate comparison of staining intensity. Axons pathways were visualized using HRP-linked secondary antibodies with 3,3′-diaminobenzidine (DAB) as a substrate. Genotypes of embryos were established using a limited X-gal [5-bromo-4-chloro-3-indolyl β-D-galactopyranoside] reaction for detection of β-gal expression (0.05% X-gal and 2-3 hours at room temperature). The pattern of β-gal enzyme activity was dictated by the presence of either an *elav*-β-gal mini-gene on CyO (designated CyOβe), which expresses β-gal in all neurons, or an actin-β-gal minigene on TM3 (designated TM3Aβ), which expresses β-gal in muscle tissue. The X-gal staining pattern indicates which balancer chromosome(s) is present in the embryo and therefore the genotype of the embryo.

RESULTS

In wild-type stage 16 embryos, the anti-Fasciclin II monoclonal antibody (mAb 1D4) reveals three longitudinal fascicles running the length of the nerve cord (Fig. 1A). Based on identification of the neurons pioneering these pathways, the medial and middle fascicles on each side of the midline are now referred to as the pCC/MP2 and the MP1 pathways respectively, while the third lateral pathway remains unnamed (Hidalgo and Brand, 1997; Lin et al., 1994). The striking feature of Fasciclin II (FasII) expression at this stage is the absence of staining in any axon bundle crossing the midline, even though the vast majority of axons (approx. 90%) must cross the midline. However, in every segment of *robo* mutants, Fas II-positive axons of the medial pCC/MP2 pathway meander back and forth across the midline and 'whirl' between commissures (Fig. 1C; Kidd et al., 1998a). These neurons abnormally cross the midline because they fail to properly detect and respond to midline repulsive cues (Kidd et al., 1998a,b, 1999).

CaM and Robo signaling pathways prevent abnormal crossovers

In KA mutants, axons of the pCC/MP2 pathway also cross the midline in several locations but rarely 'whirl' around commissures (Fig. 1B; VanBerkum and Goodman, 1995). Similar crossovers are observed in *robo* mutants rescued by expression of Robo protein using the ftzng promoter (Kidd et al., 1998a). Here, the ability of KA and *robo* mutations to interact genetically was determined by quantifying the number of FasII-positive axons crossing the midline in a number of KA and *robo* double mutants (Table 1A,B). KA *robo* double mutants were created using two *robo* alleles, $robo^{1}$ and $robo^{8}$, and two KA insertions, KA^{26} in the second chromosome and KA^{16c1} in the third chromosome. Approximately 53% of heterozygous $robo^{1}$ mutants exhibit periodic crossovers when stained with mAb 1D4. Only 5% of embryos carrying one copy of KA^{26} exhibit crossovers and this increases to 51% in embryos expressing two copies of KA^{26}. However, when one copy of KA^{26} is introduced into heterozygous $robo^{1}$ embryos the penetrance of crossovers increases to 72% and with two copies of KA^{26}, 80-100% of embryos heterozygous for either $robo^{1}$ or $robo^{8}$ have crossovers (Fig. 1D). Indeed, with the stronger $robo^{1}$ allele, 70% of embryos exhibited at least 5 crossovers. Similar interactions were observed between $robo^{1}$ and the KA^{16c1} insertion (Table 1A,B). In double homozygous KA *robo* embryos, the characteristic *robo* phenotype of axon crossing and whirling around the commissures is clearly evident in most segments (Fig. 1E). However, gaps in the longitudinal connectives are also observed and in severe mutants, the continuity of the longitudinal connectives is lost (Fig. 1F). These results indicate that mutations in the CaM- and Robo-mediated signaling pathways interact to increase the number of Fas II-positive crossovers observed in embryos. A similar result is observed if one copy of *slit*, the ligand for Robo is mutated (Table 1E). Compared to 24% of heterozygous $slit^{2}$, or 5% of heterozygous KA^{26} embryos exhibiting crossovers, 64% of transheterozygous $slit^{2}$/KA embryos exhibit crossovers. Therefore, the ability of pCC/MP2 axons to correctly interpret midline repulsive cues and stay on the ipsilateral side is dependent on the strength of both Slit/Robo- and CaM-mediated signaling.

The axon trajectories observed in stage 16 embryos with mAb 1D4 arise from a series of pioneering events occurring during earlier stages of embryonic development. In wild-type embryos, pCC and vMP2 neurons (orange in Fig. 2A) extend axons

anteriorly to fasciculate with the posteriorly projecting axons of MP1 and dMP2 neurons (blue in Fig. 2A) of the adjacent segment (Hidalgo and Brand, 1997; Lin et al., 1994). When pioneering events are completed by stage 14, two distinct portions of the pathway are observed when embryos are stained with mAb 1D4: a common pathway between segments where the axons of pCC, vMP2, MP1 and dMP2 are tightly fasciculated (black arrow in Fig. 2A,B), and two separate pathways within each segment called the pCC/MP2 and the MP1 pathways (orange and blue arrows in Fig. 2A,B). During the remainder of development, pioneer axons re-shuffle and follower axons join them to form the mature pCC/MP2 and MP1 pathways seen at stage 16 (Fig. 1A). A hallmark of the *robo* phenotype is the

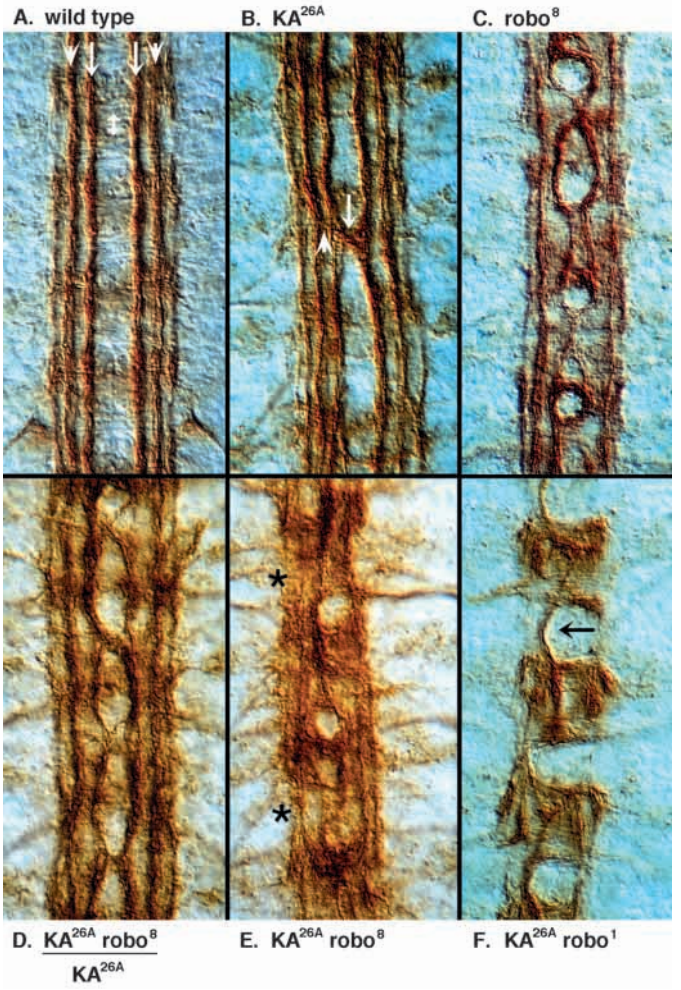

Fig. 1. KA and *robo* mutations interact to regulate axon crossing of the midline. Stage 16 embryos were stained with mAb 1D4 to detect Fasciclin II expression. (A) Wild-type embryos exhibit three longitudinal fascicles on each side of the midline but no commissural axons are stained (double-headed arrow). The pCC/MP2 pathway (arrow) and MP1 pathway (arrowhead) are indicated. (B) Homozygous KA^{26} embryos exhibit occasional crossovers of the pCC/MP2 pathway (arrow) and/or the MP1 pathway (arrowhead). (C) In *robo* mutants, the pCC/MP2 pathway crosses back and forth across the midline in every segment. (D) In KA^{26} embryos with one copy of *robo* mutated, axons cross the midline in almost every segment. (E) In double homozygous KA^{26} $robo^{8}$ embryos, the *robo* phenotype is evident and, in addition, small gaps in the longitudinal connective start to appear (asterisk). (F) With the more severe $robo^{1}$ allele, co-expression of KA^{26} causes a loss in the integrity of the longitudinal connectives and only remnants of the pCC/MP2 pathway are evident (arrow).

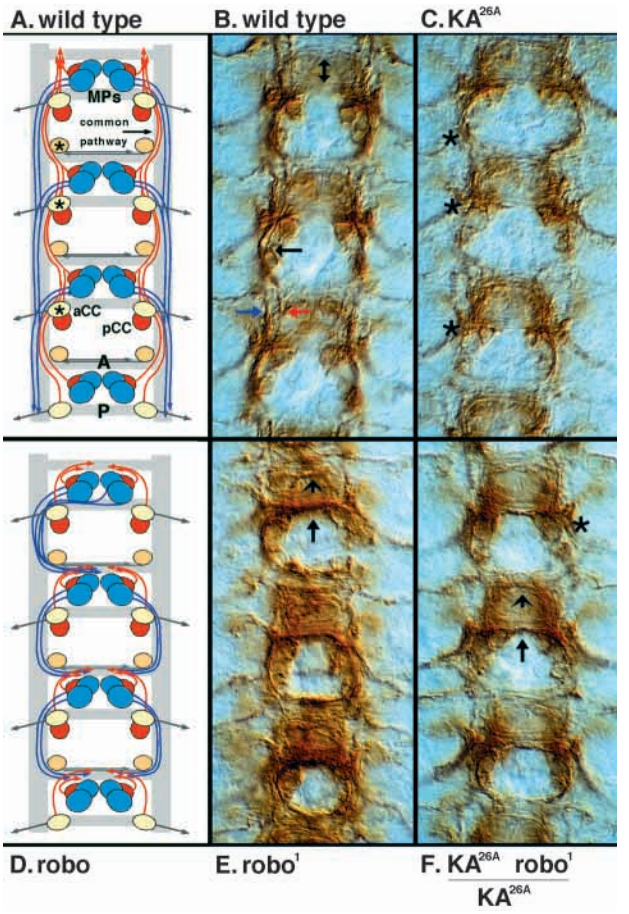

Fig. 2. *robo* mutations increase crossovers of pioneer neurons expressing KA. (A,B) The final trajectories of pioneering events at stage 14 (illustrated in A) are directly comparable with the mAb 1D4 stained embryo depicted in B. The MP1 and dMP2 neurons (blue) extend posteriorly to meet and fasciculate with anteriorly extending vMP2 and pCC neurons (orange) in a common pathway (black arrow) between segments. These pioneers form separate MP1 (blue, and blue arrow in B) and pCC/vMP2 (orange, and orange arrow in B) pathways within each segment. The position of emerging commissures in B is indicated by a double headed arrow. (C) In KA^{26} mutants, pioneer neurons stall at specific locations (asterisks in A and C) and the common MP pathway does not form. (D) A diagram of the axon trajectories observed in each of the three segments of (E) a stained *robo* mutant is depicted. For clarity, in D axons are shown nearing the midline, but not crossing; by this stage, these axons have crossed the midline and are extending along the contralateral connective. In the bottom segments, dMP2 and MP1 neurons (blue in D) extend along the ipsilateral connective and cross the midline at the anterior commissure, while pCC and vMP2 neurons (orange in D) project anteriorly before crossing the midline. In the top segment, axons from the MP1 cluster immediately cross the midline (arrow in E), join their sister neurons on the contralateral side and re-cross the midline at the anterior commissure. Also in the top segment, pCC neurons immediately extend axons across the midline (arrowhead in E). (F) In KA^{26} mutants with one copy of *robo* mutated, stalls are evident (star), and pCC (arrowhead) and axons in the MP cluster (arrow) now extend axons across the midline as observed in homozygous *robo* embryos.

Table 1. Quantitation of abnormal midline crossing in various mutant combinations

Genotype*	*n*	% Embryos with crossovers of: 0	1-2	3-4	5+	P(%)‡	E‡
A. KA alone							
KA^{26}/C	21	95	5	0	0	5	1.0
KA^{26}	92	49	37	9	5	51	2.1
KA^{16c1}	30	34	54	11	2	66	1.8
B. KA and robo§							
$robo^{1}$/+	55	47	51	2	0	53	1.4
KA^{26} $robo^{1}$/+	61	28	59	11	2	72	1.2
KA^{26} $robo^{1}$/KA^{26}	33	0	12	18	70	100	5.7
KA^{26} $robo^{8}$/KA^{26}	30	20	33	27	20	80	3.4
$robo^{1}$/C ; KA^{16c1}	26	0	15	38	46	100	5.0
C. Sos loss of function§							
Sos^{e49}/C	51	100	0	0	0	0	0.0
Sos^{e49}	43	61	23	16	0	39	2.1
KA^{26} Sos^{e49}/KA^{26}	52	42	42	10	6	58	2.2
Sos^{e49}/C ; KA^{16c1}	16	38	31	0	31	63	4.2
Sos^{e49}/$robo^{1}$	42	74	26	0	0	26	1.2
D. Sos gain of function							
KA^{26} Sos^{JC2}/KA^{26}	96	68	32	0	0	32	1.2
KA^{26} Sos^{JC2}/KA^{26} Sos^{JC2}	43	86	14	0	0	14	1.0
Sos^{JC2}/$robo^{1}$	44	71	29	0	0	29	1.2
Sos^{JC2}/KA^{26} $robo^{1}$	54	78	22	0	0	22	1.4
KA^{26} Sos^{JC2}/KA^{26} $robo^{1}$	34	3	15	38	44	97	4.5
KA^{26} Sos^{Jc2}/KA^{26} $robo^{8}$	35	9	31	43	17	91	3.3
E. KA, Sos, and slit							
$slit^{2}$/+	37	76	24	0	0	24	1.2
KA^{26}/$slit^{2}$	36	36	53	6	6	64	2.0
Sos^{e49}/$slit^{2}$	52	37	56	6	2	64	1.8
$KA^{26}Sos^{e49}$/$slit^{2}$	29	0	17	52	31	100	4.0
$robo^{1}$/$slit^{2}$	51	2	27	25	45	98	4.4
F. KA, Sos, and drk							
drk^{eoa}	20	100	0	0	0	0	0.0
KA^{26} drk^{eoa}/KA^{26}	24	42	38	21	0	58	2.0
KA^{26} drk^{eoa}	49	33	51	10	6	67	2.1
Sos^{e49}/drk^{eoa}	16	100	0	0	0	0	0.0
$KA^{26}Sos^{e49}$/KA^{26} drk^{eoa}	25	20	52	24	4	80	2.1
G. KA, Sos, and comm							
$comm^{KA93A}$	74	47	38	14	1	53	1.9
KA^{16c1} $comm^{KA93A}$	71	11	55	27	7	89	2.4
Sos^{e49} ; $comm^{KA93A}$	14	64	38	0	0	36	1.0
KA^{26} Sos^{e49}/C ; $comm^{KA93A}$	22	0	45	50	5	100	2.9
KA^{26} Sos^{e49} ; $comm^{KA93A}$	18	0	6	44	50	100	4.7

*The absence of a designation for the sister chromosome indicates homozygosity. C, a CyO balancer expressing β-galactosidase under the *elav* promoter. +, wild-type parental chromosome.

‡P, penetrance, the percentage of embryos that exhibited at least one abnormal crossover. E, expressivity, the total number of crossovers scored in all embryos divided by the number of embryos with at least one crossover.

§robo, KA^{26} Sos^{e49}, and Sos^{e49} ; KA^{16c1} homozygous embryos exhibit crossovers in every segment with 100% penetrance: counting individual crossovers is not possible in these cases.

crossover of pioneer neurons of the pCC/MP2 and MP1 pathways as they reach the anterior commissure (Fig. 2D,E; Kidd et al., 1998a). In KA mutants, pioneer neurons are not observed to cross the midline by stage 14 but instead stall at or near the aCC motorneuron or the SP1 interneuron (Fig. 2C; VanBerkum and Goodman, 1995). However, in stage 14 embryos heterozygous for *robo* and expressing two copies of KA^{26} (genotype KA^{26} $robo^{1}$/KA^{26}), pioneer neurons will periodically cross the midline (Fig. 2F). In these embryos, KA-mediated stalls are still evident, but in addition, pCC will occasionally cross the midline and pioneer neurons within the MP cluster extend axons directly across the midline as observed in *robo* mutants (Fig. 2D,E). Similar results are observed in heterozygous *robo* embryos with two copies of KA^{16c1}. The frequency of crossovers in heterozygous KA *robo* embryos at stage 14 is consistently lower then expected from the number of crossovers observed in stage 16 embryos, but these early crossovers may potentiate the ability of later axons to cross.

A Sos-mediated signaling pathway also regulates axon guidance at the midline

Son of sevenless (Sos), a guanine nucleotide exchange protein critical to Ras signal activation (Bonfini et al., 1992), also plays a role in regulating midline guidance of axons. In homozygous Sos^{e49} mutants, the longitudinal connectives are often spaced closer together than in wild-type embryos, and 39% of embryos exhibit axons crossing the midline in a manner similar to that observed in KA mutants (Table 1C; Fig. 3A,C). Loss-of-function mutations in one copy of *Sos* do not significantly enhance KA induced crossovers. However, in double homozygous KA^{26} Sos^{e49} embryos, the pCC/MP2 pathway freely crosses the midline in almost every segment to produce a phenotype remarkably similar to that observed in *robo* mutants (Fig. 3B,D). In fact, both KA^{26} Sos^{e49} and Sos^{e49} KA^{16c1} double mutants phenocopy a wide spectrum of defects observed in *robo* mutants at stage 16, though crossovers have not been observed at stage 14.

While *Sos* loss-of-function mutations enhance crossovers, a Sos^{JC2} gain-of-function allele (Karlovich et al., 1995) suppresses KA-dependent axon crossovers (Table 1D). Compared to 51% of KA^{26} homozygous embryos exhibiting crossovers, only 32% of homozygous KA^{26} embryos exhibit crossovers when one copy of Sos^{JC2} is present and penetrance decreases to 14% when two copies of Sos^{JC2} are present. One copy of Sos^{JC2} will decrease the frequency of crossovers in heterozygous $robo^{1}$ mutants and also partially suppresses the interaction between KA and *robo*; crossovers drop from 72% in KA^{26} $robo^{1}$ heterozygous embryos, to 22% when one copy of Sos^{JC2} is also present. However, if two copies of KA^{26} are present in heterozygous $robo^{1}$ or $robo^{8}$ mutants, one copy of Sos^{JC2} is not sufficient to suppress the almost complete penetrance of crossovers.

To further investigate the role of Sos in midline guidance, we asked whether Sos^{e49} loss-of-function mutations interact with *robo* or *slit* (Table 1C,E). Heterozygous Sos^{e49} embryos do not exhibit crossovers nor does a mutation in one copy of *Sos* increase the midline crossing errors of heterozygous $robo^{1}$ mutants. However, if Slit is mutated, a significant increase in crossovers is observed. Compared to only 24% of heterozygous $slit^{2}$ mutants exhibiting crossovers, 64% of transheterozygous Sos^{e49}/$slit^{2}$ embryos exhibit axon crossing defects.

Consistent with a role for Sos in midline guidance, mutations in *drk*, an adapter protein which brings Sos to membrane receptors (Raabe et al., 1995), also enhance KA induced midline crossing (Table 1F). Homozygous drk^{e0A} embryos do not exhibit crossovers, but 67% of double homozygous KA^{26} drk^{e0A} embryos show at least one crossover compared to 51% in KA^{26} embryos alone. Many homozygous KA^{26} drk^{e0A} mutants also exhibit the *robo*-like phenotypes observed in KA^{26} Sos^{e49} embryos, although additional axon scaffold defects are often present (data not shown). Most (80%) transheterozygous KA^{26} drk^{e0A}/KA^{26} Sos^{e49} embryos also display several abnormal crossovers.

Sos expression is reduced in *Sos* mutants, but cell differentiation is normal

Since Sos signaling has primarily been associated with cell differentiation (e.g. Karlovich et al., 1995; Lu et al., 1993), it is important to determine if the axon guidance defects observed in Sos mutants resulted from defects in neuron or glia differentiation. However, KA^{26}, Sos^{e49} and KA^{26} Sos^{e49} double homozygous embryos stained with anti-Engrailed, anti-Even-skipped or anti-Wrapper (Noordermeer et al., 1998) exhibit normal expression patterns for these antigens (data not shown). Therefore, gross alteration in neuronal or midline glial differentiation do not appear to occur in these mutants. This may be attributed to the level of Sos protein which remains present in the nerve cords of Sos^{e49} mutants (Fig. 4). In wild-type embryos, antibody staining indicates that Sos is expressed in most cells and is enriched in the ventral nerve cord, including most axon tracts. In homozygous Sos^{e49} mutants, Sos protein levels are reduced, but significant amounts remain present in the nerve cord, including the axon tracts, and at the midline. Taken together, these results suggest that Sos has a specific role in the growth cone to translate guidance information at the midline. Moreover, the combinatorial interactions between KA, *Sos* and *slit* mutations and the ability of KA *Sos* mutants to phenocopy *robo* suggest that Sos functions with these signaling pathways to interpret midline repulsive cues.

Abnormally crossing axons express Robo

The axon crossovers observed in KA and KA *Sos* mutants would also occur if these signaling pathways decreased Robo expression or interfered with the ability to maintain Robo at the cell surface. To assess Robo expression, we stained our mutants with mAb 13C9 raised against Robo (Kidd et al., 1998a). In earlier stages (12-14), Robo expression in KA^{26}, KA^{16B1} (which has four copies of KA), and KA^{26} Sos^{e49} double mutants is indistinguishable from that in wild-type embryos. Robo is expressed on many neurons and is especially high on the pioneer neurons of the pCC/MP2 pathway (Kidd et al., 1998a). However, in stage 16 embryos, the Robo expression pattern is altered (Fig. 5). In wild-type stage 16 embryos, Robo is expressed at high levels in the two longitudinal connectives and only very weakly in the commissures (Kidd et al., 1998a). In our KA and KA *Sos* mutants, Robo expression in the longitudinal connectives appears normal, but surprisingly, Robo expression remains high on many axons crossing the midline of KA^{26} or KA^{16B1} embryos and in almost all commissures of the KA^{26} Sos^{e49} double mutants (Fig. 5). Therefore, Robo expression is certainly not decreased in KA or KA *Sos* mutants; indeed, at least some axons are crossing the midline despite the continued expression of Robo.

KA and *Sos* induce crossovers in a *comm* background

Although Comm is usually necessary to allow axons to cross the midline, axons will cross the midline in the absence of Comm protein if a midline repellent is not detected, as is observed in in *robo comm* double mutants (Kidd et al., 1998a,b; Seeger et al., 1993). Since our data suggest that CaM and Sos may function in the transduction of midline repulsion factor(s), we examined whether a reduction in CaM or Sos signaling would induce axons to cross in a *comm* mutant (Table 1G). KA^{16B1} $comm^{8}$ double homozygous mutant embryos express four copies of KA and are null for Comm protein. As observed using mAb 1D4 (Fig. 5A,E) or BP102, which recognizes a surface antigen on CNS neurons (not shown), KA expression induces a number of axon bundles to cross the midline despite the continued absence of commissures in these mutants. Improper crossovers are also observed in $comm^{KA93A}$ mutants, where a fortuitous KA P-element insertion into the *comm* gene provides simultaneous loss of Comm protein and KA expression in the ftzng pattern (also called $comm^{6}$; Tear et al., 1996). The number of crossovers increase as more KA is expressed: 38% of homozygous $comm^{KA93A}$ embryos exhibit FasII-positive axon bundles crossing the midline and this increases to 55% when KA is doubled in KA^{16c1} $comm^{KA93A}$ double mutants (Fig. 6B,C; Table 1G). When stained with mAb BP102, it is apparent that these mutants clearly lack commissures but a few isolated axon bundles cross the midline (Fig. 6E,F). The similarity of axon tract morphology in both 1D4 and BP102 stained embryos suggests that the vast majority of axons crossing the midline are Fas II positive.

Mutations in *Sos* also increase the number of axons crossing the midline in a *comm* mutant as long as multiple copies of KA are expressed (Table 1G). In the Sos^{e49} $comm^{KA93A}$ double mutant, which expresses only two copies of KA, the number of crossovers does not increase compared to that observed in $comm^{KA93A}$ embryos. However, as the concentration of KA increases to four copies, as occurs in a KA^{26} Sos^{e49} $comm^{KA93A}$ triple mutant, many axon bundles cross the midline in almost every segment, although proper commissures do not form as evidenced by the wide spacing of the longitudinal connectives (Fig. 6D,H). Again comparing BP102 and 1D4 staining, most of the midline crossing axons appear to be FasII positive. Gaps in the longitudinal connectives are also evident as KA copy number increases (Fig. 6D,H). Similar results are observed in triple mutant Sos^{e49} KA^{16c1} $comm^{KA93A}$ embryos (data not shown).

DISCUSSION

Intracellular signaling pathways are ultimately responsible for dictating how extracellular guidance cues are used by a growth cone to arrive at a guidance decision. This work has identified two major intracellular signaling pathways, CaM and Sos, which are essential for correctly deciphering the guidance information used to prevent some axons from crossing the midline. When CaM and Sos signaling pathways are perturbed,

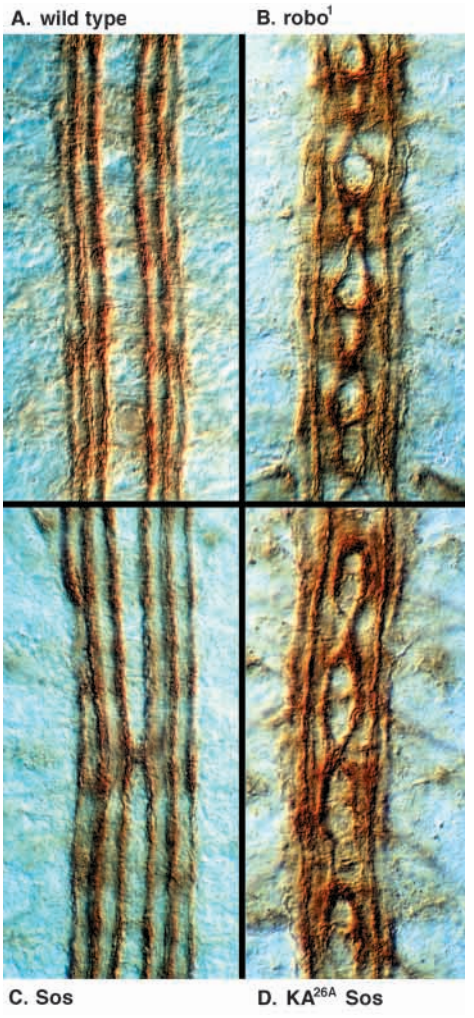

Fig. 3. KA and *Sos* function together with Robo to regulate midline guidance. (A) In wild-type embryos, no mAb 1D4 stained axons cross the midline. (B) In homozygous *robo* ^{1} embryos, axons of the pCC/MP2 pathway freely cross and re-cross the midline. (C) Homozygous *Sos*^{e49} mutants display occasional axon crossovers and the longitudinal connectives are spaced closer together than observed in the wild-type embryo in A. (D) In homozygous KA^{26} *Sos*^{e49} mutants, a phenotype strikingly similar to *robo* mutants (B) is observed.

singularly or in combination, axons in the pCC/MP2 pathway abnormally cross the midline much like those observed in *robo* mutants. The penetrance and frequency of crossovers increase with the addition of either *robo* or *slit* mutations, and crossovers occur even in the absence of Comm protein. Together, our data suggest that the CaM, Sos and Robo signaling pathways are all used to interpret repulsive information at the midline which prevents the neurons in the pCC/MP2 pathway from projecting axons across the midline.

The pCC/MP2 pathway neurons are an ideal system for understanding the signaling mechanisms underlying midline guidance events because the axon trajectory of these neurons is easily assessed, and gene expression in these neurons is readily altered using the ftzng promoter element. Pioneer neurons of the pCC/MP2 pathway extend axons immediately adjacent to the midline but never cross (see Fig. 2), in part due to the expression of Robo from the onset of axon extension (Kidd et al., 1998a,b). Robo detects the midline repellent Slit and prevents these neurons from crossing the midline (Kidd et al., 1999). In *robo* mutants, the loss of Robo causes pCC/MP2 axons to meander back and forth over the midline and this phenotype is rescued by re-expressing Robo in these neurons using the ftzng promoter (Kidd et al., 1998a). CaM-dependent signaling in the growth cone of pCC/MP2 neurons is also required to prevent these axons from crossing. If CaM signaling is reduced in pCC/MP2 neurons by expressing KA under the regulation of the ftzng promoter, their axons project abnormally across the midline (VanBerkum and Goodman, 1995). Thus both CaM and Robo signaling are used in these neurons to prevent crossovers.

Here, we demonstrate that KA, *robo* and *slit* mutations interact synergistically to increase the number of pCC/MP2 pathway axons crossing the midline (Table 1). Thus, a reduction in either CaM or Robo signaling sensitizes the neuron to a decrease in the other pathway and causes more axons to cross the midline inappropriately. Similar interactions are observed between *robo* and *slit* mutations (Table 1E; Kidd et al., 1999). A loss in Sos-dependent signaling also increases the likelihood that pCC/MP2 axons cross the midline. This appears to be a specific function for Sos in axon guidance, as opposed to its more typical role in cell differentiation (e.g. Karlovich et al., 1995; Lu et al., 1993), since no alterations in Engrailed, Even Skipped or Wrapper staining patterns were observed in *Sos* or KA *Sos* mutants. Similar results have been observed previously for KA mutants aone (VanBerkum and Goodman, 1995). Our analysis of Sos expression also indicates that axon crossing defects are quite sensitive to the levels of Sos protein since a substantial amount of Sos protein remains

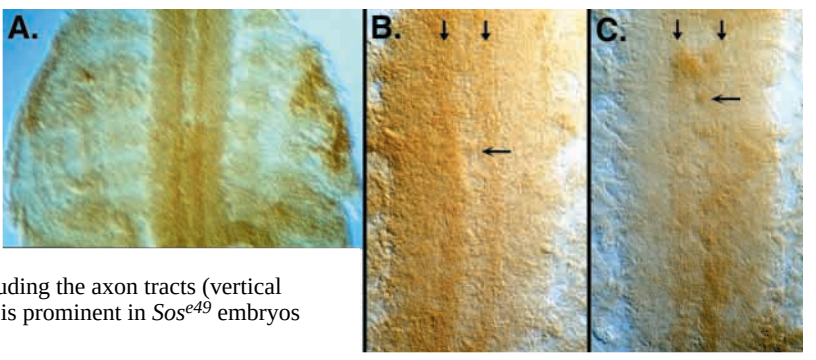

Fig. 4. Sos is expressed in the nerve cord of both wild-type and *Sos*^{e49} mutants. Wild-type (A,B) or homozygous *Sos*^{e49} (C) embryos are stained with a rabbit anti-Sos polyclonal antibody. (A) A low magnification (25×) view of a wild-type embryo illustrates Sos expression in most cells of the embryo and especially the ventral nerve cord. (B) At higher magnification (100×), Sos expression in all cells of the nerve cord as well as in axons of the commissures and longitudinal connectives (vertical arrows) is evident. (C) In *Sos*^{e49} embryos, Sos expression is reduced but considerable amounts remain present in the nerve cord, including the axon tracts (vertical arrows). The expression of Sos in unidentified midline cells is prominent in *Sos*^{e49} embryos but also occurs in wild-type embryos (horizontal arrows).

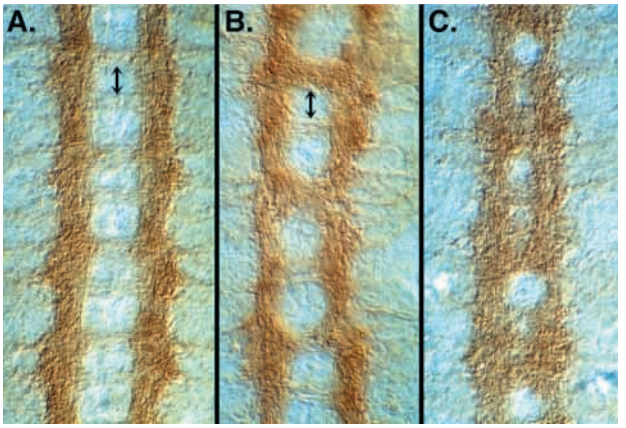

Fig. 5. Axons crossing the midline in KA^{26} and KA^{26} *Sos^{e49}* double mutants express Robo. Expression of Robo protein in stage 16 embryos is revealed using mAb 13C9. (A) In wild-type embryos, Robo protein is expressed at high levels in the longitudinal connectives and only very weakly in anterior and posterior commissures (double-headed arrow). (B) In KA^{16B1} embryos expressing four copies of KA, and (C) in KA^{26} *Sos^{e49}* double mutants, commissures form in all segments and Robo is clearly expressed on axon bundles crossing the midline.

in these embryos, most likely from maternal loading of Sos messenger RNA (Lu et al., 1993).

Of particular interest is the strong interaction between KA and *Sos* which causes axons to mimic the crossovers observed in *robo* mutants. It is noteworthy that in these mutants neither Sos- nor CaM- (see Heiman et al., 1996; Kovalick et al., 1992) dependent signaling pathways are completely inhibited yet axons in the pCC/MP2 pathway are free to cross the midline. This suggests that the combined loss in Sos and CaM signaling reduces the ability of pCC/MP2 axons to interpret midline repulsive cues. This is supported by our observation that mutations in one copy of the *slit* gene interact with one copy of KA, heterozygous *Sos*, or heterozygous KA *Sos* mutations to induce several axons to abnormally cross the midline. Further evidence that the CaM and Sos signaling pathways work together to prevent axon crossing is the suppression of KA induced crossovers by the hyperactivation of the Sos pathway with one or two copies of the *Sos^{JC2}* allele (Avery and Wasserman, 1992). Cross talk between calcium- and Sos-dependent signaling pathways has been documented in other systems, including neurite outgrowth of PC12 cells (Finkbeiner and Greenberg, 1996; Lev et al., 1995; Rusanescu et al., 1995). Together these data suggest that all three pathways, CaM, Sos and Robo, must function in the growth cone of pCC/MP2 neurons to force them to remain on the ipsilateral side.

Our supposition that CaM and Sos signaling pathways are involved in the transduction of midline repulsive cues is further supported by the ability of KA and Sos mutations to induce crossovers in *comm* mutants. It is known from *robo comm* double mutants that a failure to detect the midline repellent Slit will cause axons to cross the midline in the absence of Comm protein (Kidd et al., 1998a; Seeger et al., 1993). Similarly, in our KA *comm* and KA *Sos comm* mutants, axon bundles cross

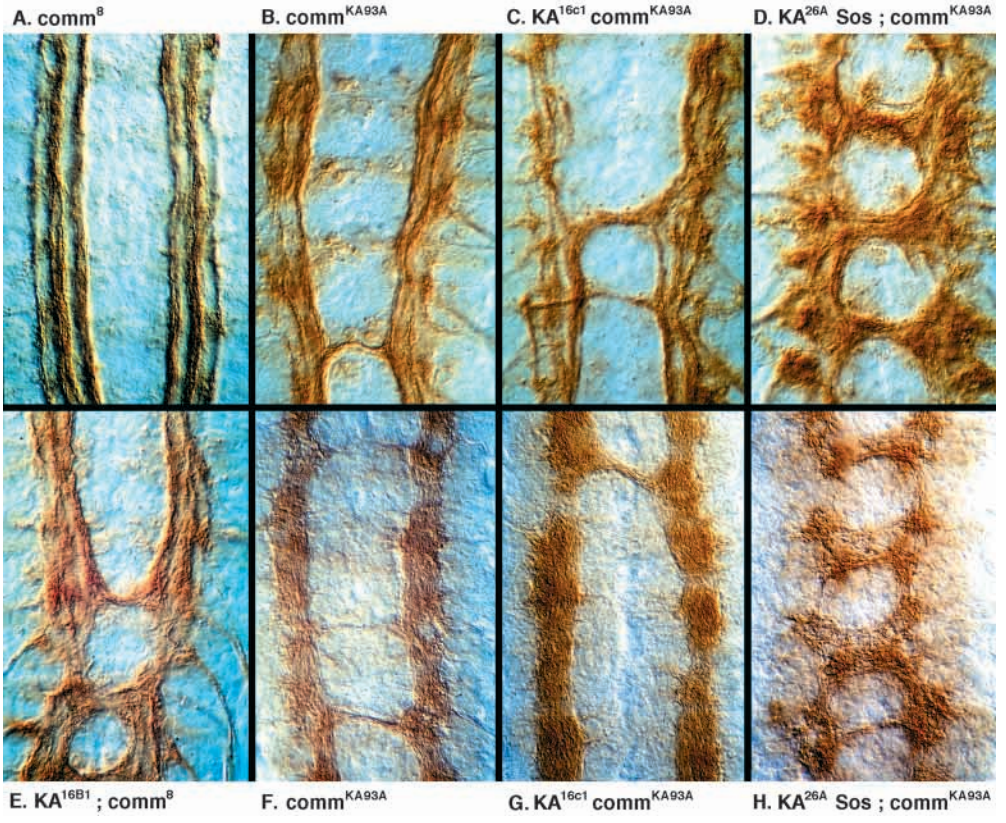

Fig. 6. KA and *Sos* mutations act together to increase axon crossing in *comm* mutants. Stage 16 embryos are stained with either mAb 1D4 (A-E) or mAb BP102, which recognizes a surface antigen present on all CNS neurons (F-H). (A,E) In *comm^{8}* (A) mutants no commissures form and although the longitudinal connectives separate further then normal, they remain reasonably well organized (see Fig. 1A for wild type). Expression of four copies of KA in a *comm^{8}* mutant (E) is sufficient to induce several FasII-positive axon bundles to abnormally cross the midline, but commissures do not form and the connectives become slightly disorganized. (B,F) The hypomorphic *comm^{KA93A}* allele also exhibits a few axon bundles crossing the midline when stained with either mAb 1D4 (B) or mAb BP102 (F). (C,G) When two additional copies of KA^{16c1} are introduced into the *comm^{KA93A}* mutant, significantly more axons will cross the midline (G) and most express FasII (C). (D,H) In KA^{26} *Sos^{e49} comm^{KA93A}* triple mutants, a disruption in both CaM and Sos signaling causes thick axon bundles to cross the midline in almost every segment. A comparison of mAb 1D4 (D) or mAb BP102 (H) stained embryos suggests that most axons crossing the midline are FasII positive. While large axon bundles are crossing, proper commissures still do not form and gaps are evident in the longitudinal connectives (H).

the midline, suggesting that these signaling pathways transduce repulsive information (Fig. 6). While commissures reform in *robo comm* double mutants (Kidd et al., 1998a,b; Seeger et al., 1993), the failure to form commissures in our mutants is consistent with the restricted expression pattern of KA. A comparison of BP102 and 1D4 staining patterns (Fig. 6), suggests that most axons crossing the midline in KA *comm* and KA *Sos comm* mutants are FasII positive. Using the data presented by Lin et al. (1994) which compares Fas II expression with Tau-lacZ expression under the ftzng promoter, we estimate that 30-40% of FasII axons, and especially those of the pCC/MP2 pathway, also express KA and could account for the axons we observe crossing in our mutants. It remains possible that some non-KA-expressing neurons also cross the midline in these mutants, perhaps by fasciculating with KA-expressing axons. However, the ability of FasII-positive axons to cross the midline in a *comm* mutant is dependent on the expression of multiple copies of KA, even when Sos is mutated. Only a few crossovers are observed in *Sos* $comm^{KA93A}$ homozygous mutants expressing two copies of KA, but several large bundles cross in KA^{26} *Sos* $comm^{KA93A}$ triple mutants, which have four copies of KA expressed. Thus, it seems that growth cones are able to ignore midline repulsive cues and cross the midline in a *comm* mutant when at least CaM or, preferably, CaM and Sos signaling pathways are disrupted in a neuron.

KA and KA Sos mutants phenocopy *robo* mutations without decreasing Robo expression. Surprisingly, Robo is still present on some axons crossing the midline (Fig. 5). Since crossovers occur sometime after stage 14 in KA and KA *Sos* mutants, we could not observe neurons in the act of crossing the midline while still expressing Robo. It is possible, therefore, that Robo is transiently cleared from the membrane while axons are actually crossing the midline and up-regulated later when the remaining Comm, expressed at the midline, is not sufficient to clear Robo from the membrane (Tear et al., 1996). A second explanation is also possible, and points to a critical role for CaM and Sos in midline guidance. KA is expressed in neurons that should never cross the midline and that express high levels of Robo from the start of axon extension. The machinery to remove Robo from the membrane is present in these neurons, since Robo is cleared if Comm is artificially expressed in these neurons (Kidd et al., 1998a,b). However, there is no evidence that Comm is normally present in these neurons, and this would seem unlikely since expression of Comm would remove Robo from the membrane and cause these axons to cross the midline. Other neurons are thought to respond to Comm at the midline using an unidentified receptor (Tear et al., 1996); since KA expressing neurons do not cross the midline they may not express a Comm receptor and would be unable to respond to Comm at the midline to clear Robo from the membrane. In this case, the ability of axons to cross the midline while maintaining Robo expression (and presumably activity) is strong evidence that a disruption in the CaM and Sos signaling pathways blocks some critical components of the machinery used by Robo to transduce Slit signals from the midline. Clearly, identification of the Comm receptor and its expression pattern will help address this issue.

Whether CaM and Sos-mediated signaling is working directly downstream of Robo or in closely associated, but parallel signaling pathways to prevent axons from crossing is difficult to ascertain from this genetic data alone. If these signaling pathways lie downstream of Robo, our data suggest that both CaM and Sos are activated upon Slit binding to Robo, and result in growth cone repulsion. Interestingly, increased levels of calcium have been implicated in growth cone retraction and growth cone collapse, two ways in which a growth cone may respond to a repulsive agent (e.g. Bandtlow et al., 1993; Bandtlow and Loschinger, 1997; Loschinger et al., 1997; Murray and Whitington, 1999; Takei et al., 1998). In addition, retrograde actin flow, which leads to filopodial retraction, is stimulated by CaM activation of myosin light chain kinase (Lin et al, 1996; Welnhofer et al., 1999). Two other CaM target proteins, cAMP adenylyl cyclase and phosphodiesterase (e.g. Xia and Storm, 1997), regulate cAMP concentrations in a cell which can alter a neuron's response to Netrin 1 and other guidance cues (Loschinger et al., 1997; Ming et al., 1997; Song et al., 1997; Wang and Zheng, 1998).

Activation of a Sos signaling pathway can affect cytoskeletal dynamics by activating various GTPases known to regulate growth cone behavior and axon guidance (Luo et al., 1994; Kaufman et al., 1998; Nimnual et al., 1998). Moreover, the cytoplasmic tail of Robo, known to be essential for signaling function (Bashaw and Goodman, 1999), has a tyrosine residue which could recruit Sos via Drk (which interacts with KA; Table 1) or *dreadlocks* (*dock*), another SH2-SH3 adapter protein which affects axon guidance (Garrity et al., 1996; Desai et al., 1999). Alternatively, Robo may bind Enabled (Ena; Kidd et al., 1998a), a known substrate for Abelson tyrosine kinase (Abl; Gertler et al., 1995; Wills et al., 1999), which has been implicated in commissure formation (Elkins et al., 1990; Hu et al., 1998). If Sos binds to phosphotyrosine residues on Ena (also via an adapter protein) it could be indirectly recruited to Robo.

Another possibility is that a disruption in both the CaM and Sos signaling pathways indirectly causes abnormal crossovers (Song and Poo, 1999). CaM has been identified as a player downstream of several guidance molecules (e.g. Archer et al., 1999; Doherty and Walsh, 1996; Zheng et al., 1996). Indeed, the gaps in the longitudinal connectives observed with increasing copies of KA in a *comm* mutant (Fig. 6) or in KA *robo* mutants, which are not seen in *robo* mutants alone, suggest CaM may function downstream of other guidance cue receptors to allow extension through the connective. Once these signals are attenuated by expression of KA, axons may inadvertently cross the midline. However, if CaM only functions in cell adhesive mechanisms within the connectives, it is difficult to explain why axons cross the midline in *comm* mutants when no other axons cross and the presence of Slit is still being read by Robo.

Since CaM and Sos appear to interpret a midline repulsive cue, the existence of an additional midline repulsion system working in parallel to Robo represents an interesting possibility. In *robo* mutants, axons cross the midline but then move to the longitudinal connective, instead of collapsing at the midline as observed in *slit* mutants (Kidd et al., 1999). It has been suggested that this occurs because the continued presence of Slit at the midline is detected by a second receptor system, and candidate genes include a second *robo* gene (Kidd et al., 1999) or *karussel* (Hummel et al., 1999). As our data shows, heterozygous *slit* mutations interact very strongly with single copies of KA, *Sos* or KA *Sos* together, to force axons

across the midline. The interaction between Sos and *slit* mutations, especially when compared to the lack of *Sos* and *robo* interaction, is particularly striking. It seems that if the activity of both repulsion systems is decreased due to the reduction of a common ligand (Slit), a disruption in CaM and/or Sos signaling dramatically increases midline crossing errors. Most of our results, including the synergistic effects of KA, *Sos*, *robo* and *slit* mutations, the *robo*-like phenotype of KA *Sos* mutants, and the enhancement of crossovers in *comm* mutants can be explained by a parallel decrease in both midline repulsive systems upon disruption of the CaM and Sos signaling pathways.

Thus while the mechanisms by which CaM and Sos contribute to an axon guidance decision at the midline remain unclear, our data clearly indicate that CaM and Sos signaling pathways are critical to the transduction of repulsive information at the midline.

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REFERENCES

Archer, F. R., Doherty, P., Collins, D. and Bolsover, S. R. (1999). CAMs and FGF cause a local submembrane calcium signal promoting axon outgrowth without a rise in bulk calcium concentration. *Eur. J. Neurosci.* **11**, 3565-3573.

Avery, L. and Wasserman, S. (1992). Ordering gene function: the interpretation of epistasis in regulatory hierarchies. *Trends Genet.* **8**, 312-316.

Bandtlow, C. E. and Loschinger, J. (1997). Developmental changes in neuronal responsiveness to the CNS myelin-associated neurite growth inhibitor NI-35/250. *Eur. J. Neurosci.* **9**, 2743-2752.

Bandtlow, C. E., Schmidt, M. F., Hassinger, T. D., Schwab, M. E. and Kater, S. B. (1993). Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. *Science* **259**, 80-83.

Bashaw, G. J. and Goodman, C. S. (1999). Chimeric axon guidance receptors: the cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. *Cell* **97**, 917-926.

Bonfini, L., Karlovich, C. A., Dasgupta, C. and Banerjee, U. (1992). The Son of sevenless gene product: a putative activator of Ras. *Science* **255**, 603-606.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96**, 795-806.

Cook, G., Tannahill, D. and Keynes, R. (1998). Axon guidance to and from choice points. *Curr. Opin. Neurobiol.* **8**, 64-72.

Culotti, J. G. and Merz, D. C. (1998). DCC and netrins. *Curr. Opin. Cell Biol.* **10**, 609-613.

Desai, C. J., Garrity, P. A., Keshishian, H., Zipursky, S. L. and Zinn, K. (1999). The *Drosophila* SH2-SH3 adapter protein Dock is expressed in embryonic axons and facilitates synapse formation by the RP3 motoneuron. *Development* **126**, 1527-1535.

Doherty, P. and Walsh, F. S. (1996). CAM-FGF receptor iInteractions: A model for axonal growth. *Mol. Cell Neurosci.* **8**, 99-111.

Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M. and Goodman, C. S. (1990). Genetic analysis of a Drosophila neural cell adhesion molecule: interaction of fasciclin I and Abelson tyrosine kinase mutations. *Cell* **60**, 565-575.

Finkbeiner, S. and Greenberg, M. E. (1996). Ca(2+)-dependent routes to Ras: mechanisms for neuronal survival, differentiation, and plasticity? *Neuron* **16**, 233-236.

Flanagan, J. G. and Van Vactor, D. (1998). Through the looking glass: axon guidance at the midline choice point. *Cell* **92**, 429-432.

Gallo, G. and Letourneau, P. C. (1999). Axon guidance: A balance of signals sets axons on the right track. *Curr. Biol.* **9**, R490-492.

Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S. L. (1996). Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* **85**, 639-650.

Gertler, F. B., Comer, A. R., Juang, J. L., Ahern, S. M., Clark, M. J., Liebl, E. C. and Hoffmann, F. M. (1995). enabled, a dosage-sensitive suppressor of mutations in the Drosophila Abl tyrosine kinase, encodes an Abl substrate with SH3 domain-binding properties. *Genes Dev.* **9**, 521-533.

Goldberg, D. J. and Grabham, P. W. (1999). Braking news: calcium in the growth cone. *Neuron* **22**, 423-425.

Gomez, T. M. and Spitzer, N. C. (1999). In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* **397**, 350-355.

Harris, R., Sabatelli, L. M. and Seeger, M. A. (1996). Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs. *Neuron* **17**, 217-228.

Heiman, R. G., Atkinson, R. C., Andruss, B. F., Bolduc, C., Kovalick, G. E. and Beckingham, K. (1996). Spontaneous avoidance behavior in Drosophila null for calmodulin expression. *Proc. Natl. Acad. Sci. USA* **93**, 2420-2425.

Hidalgo, A. and Brand, A. H. (1997). Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*. *Development* **124**, 3253-3262.

Hong, K., Hinck, L., Nishiyama, M., Poo, M. M., Tessier-Lavigne, M. and Stein, E. (1999). A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell* **97**, 927-941.

Hopker, V. H., Shewan, D., Tessier-Lavigne, M., Poo, M. and Holt, C. (1999). Growth-cone attraction to netrin-1 is converted to repulsion by laminin- 1. *Nature* **401**, 69-73.

Hu, S., Sonnenfeld, M., Stahl, S. and Crews, S. T. (1998). Midline Fasciclin: a Drosophila Fasciclin-I-related membrane protein localized to the CNS midline cells and trachea. *J. Neurobiol.* **35**, 77-93.

Hummel, T., Schimmelpfeng, K. and Klambt, C. (1999). Commissure formation in the embryonic CNS of *Drosophila*. *Development* **126**, 771-779.

Karlovich, C. A., Bonfini, L., McCollam, L., Rogge, R. D., Daga, A., Czech, M. P. and Banerjee, U. (1995). In vivo functional analysis of the Ras exchange factor son of sevenless. *Science* **268**, 576-579.

Kaufmann, N., Wills, Z. P. and Van Vactor, D. (1998). *Drosophila* Rac1 controls motor axon guidance. *Development* **125**, 453-461.

Kennedy, T. E., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425-435.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. *Cell* **96**, 785-794.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S. and Tear, G. (1998a). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* **92**, 205-215.

Kidd, T., Russell, C., Goodman, C. S. and Tear, G. (1998b). Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. *Neuron* **20**, 25-33.

Kovalick, G. E. and Beckingham, K. (1992). Calmodulin transcription is limited to the nervous system during Drosophila embryogenesis. *Dev. Biol.* **150**, 33-46.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B. and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* **376**, 737-745.

Li, H. S., Chen, J. H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z. H., Nash, W., Gick, C. et al. (1999). Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. *Cell* **96**, 807-818.

Lin, C. H., Espreafico, E. M., Mooseker, M. S. and Forscher, P. (1996). Myosin drives retrograde F-actin flow in neuronal growth cones. *Neuron* **16**, 769-782.

Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G. and Goodman, C. S. (1994). Genetic analysis of Fasciclin II in Drosophila: defasciculation, refasciculation, and altered fasciculation. *Neuron* **13**, 1055-1069.

Loschinger, J., Bandtlow, C. E., Jung, J., Klostermann, S., Schwab, M. E.,

Bonhoeffer, F. and Kater, S. B. (1997). Retinal axon growth cone responses to different environmental cues are mediated by different second-messenger systems. *J. Neurobiol.* **33**, 825-834.

Lu, X., Chou, T. B., Williams, N. G., Roberts, T. and Perrimon, N. (1993). Control of cell fate determination by p21ras/Ras1, an essential component of torso signaling in Drosophila. *Genes Dev.* **7**, 621-632.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.

Ming, G., Song, H., Berninger, B., Inagaki, N., Tessier-Lavigne, M. and Poo, M. (1999). Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. *Neuron* **23**, 139-148.

Ming, G. L., Song, H. J., Berninger, B., Holt, C. E., Tessier-Lavigne, M. and Poo, M. M. (1997). cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225-1235.

Mitchell, K. J., Doyle, J. L., Serafini, T., Kennedy, T. E., Tessier-Lavigne, M., Goodman, C. S. and Dickson, B. J. (1996). Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* **17**, 203-215.

Murray, M. J. and Whitington, P. M. (1999). Effects of roundabout on growth cone dynamics, filopodial length, and growth cone morphology at the midline and throughout the neuropile. *J. Neurosci.* **19**, 7901-7912.

Nguyen Ba-Charvet, K. T., Brose, K., Marillat, V., Kidd, T., Goodman, C. S., Tessier-Lavigne, M., Sotelo, C. and Chedotal, A. (1999). Slit2-mediated chemorepulsion and collapse of developing forebrain axons. *Neuron* **22**, 463-473.

Nimnual, A. S., Yatsula, B. A. and Bar-Sagi, D. (1998). Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. *Science* **279**, 560-563.

Noordermeer, J. N., Kopczynski, C. C., Fetter, R. D., Bland, K. S., Chen, W. Y. and Goodman, C. S. (1998). Wrapper, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheath commissural axons in Drosophila. *Neuron* **21**, 991-1001.

Raabe, T., Olivier, J. P., Dickson, B., Liu, X., Gish, G. D., Pawson, T. and Hafen, E. (1995). Biochemical and genetic analysis of the Drk SH2/SH3 adaptor protein of Drosophila. *EMBO J.* **14**, 2509-2518.

Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S. and Halegoua, S. (1995). Calcium influx induces neurite growth through a Src-Ras signaling cassette. *Neuron* **15**, 1415-1425.

Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C. S. (1993). Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron* **10**, 409-426.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* **78**, 409-424.

Song, H. J., Ming, G. L. and Poo, M. M. (1997). cAMP-induced switching in turning direction of nerve growth cones. *Nature* **388**, 275-279.

Song, H. J. and Poo, M. M. (1999). Signal transduction underlying growth cone guidance by diffusible factors. *Curr. Opin. Neurobiol.* **9**, 355-363.

Suter, D. M. and Forscher, P. (1998). An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance. *Curr. Opin. Neurobiol.* **8**, 106-116.

Takei, K., Shin, R. M., Inoue, T., Kato, K. and Mikoshiba, K. (1998). Regulation of nerve growth mediated by inositol 1,4,5-trisphosphate receptors in growth cones. *Science* **282**, 1705-1708.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C. S. and Seeger, M. A. (1996). commissureless controls growth cone guidance across the CNS midline in Drosophila and encodes a novel membrane protein. *Neuron* **16**, 501-514.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

Thomas, J. B. (1998). Axon guidance: crossing the midline. *Curr. Biol.* **8**, R102-104.

VanBerkum, M. F. and Goodman, C. S. (1995). Targeted disruption of Ca(2+)-calmodulin signaling in Drosophila growth cones leads to stalls in axon extension and errors in axon guidance. *Neuron* **14**, 43-56.

Wang, K. H., Brose, K., Arnott, D., Kidd, T., Goodman, C. S., Henzel, W. and Tessier-Lavigne, M. (1999). Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell* **96**, 771-784.

Wang, Q. and Zheng, J. Q. (1998). cAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. *J. Neurosci.* **18**, 4973-4984.

Welnhofer, E. A., Zhao, L. and Cohan, C. S. (1999). Calcium influx alters actin bundle dynamics and retrograde flow in Helisoma growth cones. *J. Neurosci.* **19**, 7971-7982.

Wills, Z., Bateman, J., Korey, C. A., Comer, A. and Van Vactor, D. (1999). The tyrosine kinase Abl and its substrate enabled collaborate with the receptor phosphatase Dlar to control motor axon guidance. *Neuron* **22**, 301-312.

Xia, Z. and Storm, D. R. (1997). Calmodulin-regulated adenylyl cyclases and neuromodulation. *Curr. Opin. Neurobiol.* **7**, 391-396.

Zheng, J. Q., Poo, M. M. and Connor, J. A. (1996). Calcium and chemotropic turning of nerve growth cones. *Perspect. Dev. Neurobiol.* **4**, 205-213.