Brief Communications

Genetic Analysis of DSCAM's Role as a Netrin-1 Receptor in Vertebrates

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Down syndrome cell adhesion molecule (DSCAM) has mainly been characterized for its function as an adhesion molecule in axon growth and in self-recognition between dendrites of the same neuron. Recently, it has been shown that DSCAM can bind to Netrin-1 and that downregulation of DSCAM expression by siRNAs in chick and rodent spinal cords leads to impaired growth and turning response of commissural axons to Netrin-1. To investigate the effect of complete genetic ablation of DSCAM on Netrin-1-induced axon guidance, we analyzed spinal commissural neurons in *DSCAM*-null mice and found that they extend axons that reach and cross the floor plate and express apparently normal levels of the Netrin receptors DCC (deleted in colorectal carcinoma) and Neogenin. *In vitro*, commissural neurons in dorsal spinal cord explants of *DSCAM*-null embryos show normal outgrowth in response to Netrin-1. We therefore conclude that DSCAM is not required for Netrin-induced commissural axon outgrowth and guidance in mice.

Introduction

Netrin-1 and its receptor DCC (deleted in colorectal carcinoma) play an essential role in directing axons toward the midline in bilateral animals (Evans and Bashaw, 2010). Nevertheless, several lines of evidence suggest that not all of Netrin's function in axon attraction is mediated by DCC (Serafini et al., 1994; Keino-Masu et al., 1996; Fazeli et al., 1997), and it was recently reported that DSCAM (Down's syndrome cell adhesion molecule) also acts as a Netrin-1 receptor (Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009); however, genetic evidence implicating DSCAM in Netrin-mediated axon guidance in vertebrates is still lacking.

Vertebrate commissural (C) neurons are located in the dorsal spinal cord and project their axons toward and across the floor plate located at the ventral midline (Tessier-Lavigne et al., 1988). Netrin-1 secreted from the floor plate binds to DCC expressed on C axons, promoting their outgrowth and attraction to the midline. Upon midline crossing by C growth cones, DCC signaling is silenced and cues distinct from Netrin-1 instruct C axon exit from the midline, prevent midline reentry and guide them in a rostral direction (Evans and Bashaw, 2010). Biochemical and functional experiments in the context of C neurons have provided evidence that there are additional Netrin-1 receptors such as Neogenin and DSCAM, but the genetic evidence in support of such function is still lacking.

DSCAM is a conserved Ig superfamily transmembrane protein which has been implicated as a Netrin-1 receptor in both *Drosophila* and vertebrates (Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009; Schmucker and Chen, 2009). DSCAM overexpression in *Drosophila* CNS neurons forces axons to cross the nervous system midline, consistent with an attractive Netrin receptor function (Andrews et al., 2008). Moreover, *Drosophila* triple knock-out of DSCAM, its paralog DSCAM3 and DCC homolog Frazzled results in stronger midline crossing defects than the removal of both *Drosophila* Netrins, suggesting that DSCAM also functions in a Netrin-independent fashion (Andrews et al., 2008).

The main evidence implicating DSCAM in Netrin signaling in vertebrates comes from *in vitro* experiments in which DSCAM function is blocked in cultured chick, and rodent embryonic spinal cords, resulting in impaired C axon extension and guidance (Ly et al., 2008; Liu et al., 2009). *In vitro* turning responses to Netrin-1 were also affected by DSCAM loss of function (Ly et al., 2008; Liu et al., 2009). Although these experiments include rescues of siRNA knockdown phenotypes by wild-type cDNAs, the effects of complete loss of DSCAM function in C axon outgrowth and guidance are ideally addressed by analysis of genetic null mutations.

Here we provide evidence that C neurons of mice lacking DSCAM do not exhibit outgrowth or guidance defects in response to Netrin-1. Furthermore, we report that compound mutants for *DCC* and *DSCAM* exhibit a commissure deficiency comparable to *DCC* single mutants, and therefore conclude that

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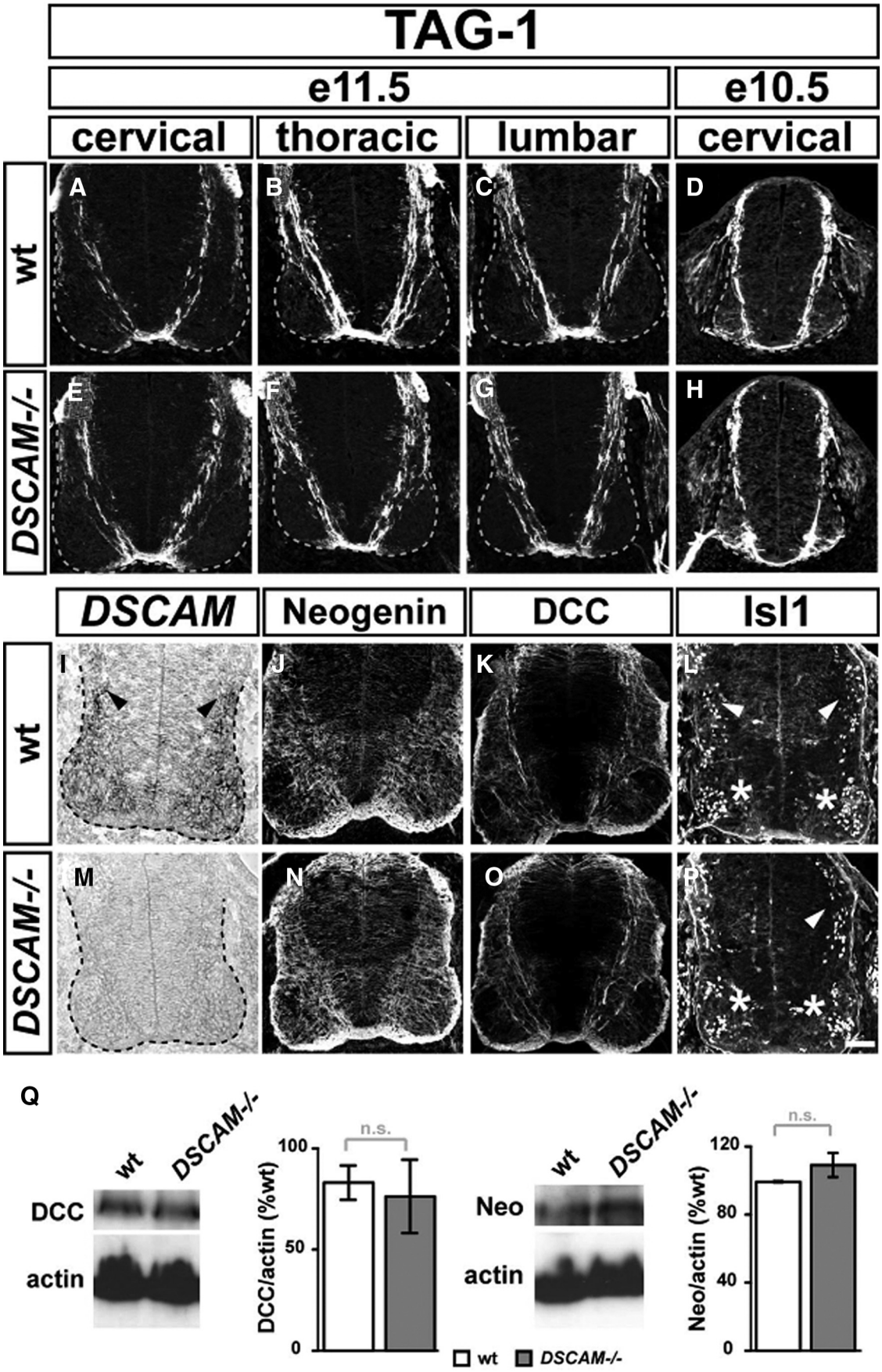

Figure 1. Normal commissural axon trajectory in *DSCAM* mutant spinal cord. ***A–H***, TAG-1 immunostaining revealed that commissural neurons of *DSCAM* $^{-/-}$ embryos (***E–H***) reached and crossed the floor plate as in control littermates (***A–D***). $n = 4$ embryos per genotype. ***I–P***, While *DSCAM* mRNA (***I***, ***M***) was not detected in *DSCAM* $^{-/-}$ e11.5 embryos (***M***), (*Figure legend continues.*)

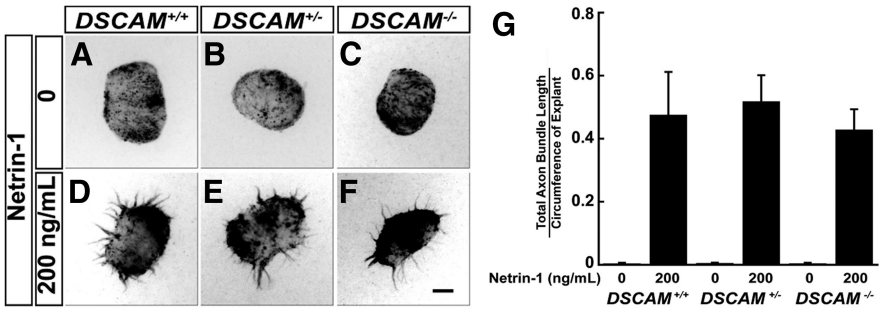

Figure 2. Netrin-induced axon outgrowth is unaffected by *DSCAM* mutation. ***A–F***, Dorsal spinal cord explants of e11.5 *DSCAM* mutant (***C***, ***F***), $DSCAM^{+/-}$ (***B***, ***E***) and control littermates (***A***, ***D***) were cultured in the presence or absence of 200 ng/ml Netrin-1. ***G***, Quantification of axon outgrowth. $N > 6$ explants per condition; $n = 4$ wild-type, 7 $DSCAM^{+/-}$ and 4 $DSCAM^{-/-}$ embryos. Scale bar (in ***F***), 20 μm in all panels.

DSCAM is not required for Netrin signaling in C axon guidance in the murine spinal cord *in vivo*.

Materials and Methods

Animals. All mice were maintained on C57BL/6 background and genotyped by PCR as previously described (Fazeli et al., 1997; Amano et al., 2009). Embryos analyzed were of either sex.

Immunostaining and in situ *mRNA detection.* Immunofluorescence stainings were performed on 12 μm cryosections as described previously (Palmesino et al., 2010). Mouse anti TAG-1 (4D7, Developmental Studies Hybridoma Bank) was used at a dilution of 1:25, goat anti-DCC (Santa Cruz Biotechnology) was used at 1:1000, rabbit anti-Neogenin (R&D Systems) was used at 1:100, and rabbit anti-Isl1 (kind gift from T. Jessell, Columbia University, New York, NY) was used 1:20,000 (Tsuchida et al., 1994).

In situ mRNA detections were performed as previously described (Kao et al., 2009; Palmesino et al., 2010). Probe sequence details are available upon request.

Western immunoblot. Spinal cords of e11.5 mouse embryos were lysed in 1% Triton X-100 buffer containing (in mM): 50 HEPES, pH 7.4, 150 NaCl, 10% glycerol, 1.5 MgCl, 1 EDTA, protease inhibitors (Complete, Roche). Proteins were then separated by SDS-PAGE and transferred to Immobilon membrane (Millipore) followed by ECL-Western blot analysis with the following antibodies: DCC monoclonal (BD Biosciences), polyclonal Neogenin (R&D Systems), and monoclonal β-actin (Sigma).

Dorsal spinal cord explants. The dorsal spinal cord explant assay was performed as previously described (Serafini et al., 1994) on e11.5 mouse embryos using recombinant mouse Netrin-1 (R&D Systems).

DiI injections. Spinal cord open-book preparations from e12.5 embryos were fixed overnight in 4% paraformaldehyde and washed in PBS. The lipophilic dye CM-DiI (Invitrogen) was injected dorsally at multiple sites along the rostro-caudal axis on one side of the open-book preparation and allowed to diffuse for 1 d before mounting with Mowiol and immediately followed by imaging.

Image quantification. Images were acquired using a Zeiss LSM confocal microscope or a Leica DM6000 microscope with Improvision Volocity software. Axon outgrowth and explant circumference of dorsal spinal cord explants were measured using the NeuronJ plugin for NIH ImageJ. Western blots were quantified by digitizing the exposed x-ray film, measuring the number of signal pixels in the band and subtracting the background. Ratio of DCC or Neogenin to actin expression was then normalized to the highest wild-type value.

Results

Normal commissural axon trajectory in *DSCAM* mutant spinal cord

The recent generation of *DSCAM*-null mice afforded us the opportunity to test genetically whether DSCAM is required for normal C axon guidance. DSCAM deletion of exon 1 results in complete ablation of *DSCAM* mRNA as assessed by *in situ* hybridization and Northern blotting, providing evidence that this allele is a null mutation of *DSCAM* ($DSCAM^{-/-}$) (Amano et al., 2009). When maintained on a Bl6 background the majority of $DSCAM^{-/-}$ animals were reported to die within 48 h of birth due to respiratory deficiencies (Amano et al., 2009) which we also observed (9 litters analyzed, 15 *DSCAM* mutants; data not shown). To investigate the effect of complete *DSCAM* ablation on C axon guidance, we monitored C axon projections by TAG-1 immunostaining in lumbar, thoracic and cervical spinal cord sections of embryonic day 11.5 (e11.5) $DSCAM^{-/-}$ mutants and control wild-type littermates (Dodd et al., 1988; Amano et al., 2009). In *DSCAM*-null spinal cords, TAG-1 protein expression was detected in a similar way in C neurons, when compared with control littermates, and, surprisingly, TAG-1-expressing C axons reached and crossed the floor plate as in control littermates (Fig. 1*A–C*,*E–G*; $n = 4$ embryos per genotype). To ascertain whether an earlier C axon projection phenotype might be corrected by e11.5, we also examined e10.5 TAG-1-expressing C axons in spinal cord sections but found no differences between wild-type and $DSCAM^{-/-}$ embryos (Fig. 1*D*,*H*; $n = 4$ embryos per genotype). We also examined the development of corpus callosum, hippocampal, anterior and posterior commissures whose formation is netrin-dependent (Serafini, 1996). We did not observe any obvious defects in the development of these structures in e18.5 *DSCAM*-null mutant embryos (data not shown).

To rule out the possibility of compensation by increased expression of other Netrin-1 receptors, we examined the expression of DCC and Neogenin in $DSCAM^{-/-}$ and control embryos at e10.5 and e11.5, when C axons are reaching and crossing the floor plate (Dodd et al., 1988). Analysis of $DSCAM^{-/-}$ e11.5 spinal cords revealed the absence of *DSCAM* mRNA (Fig. 1*M*) and expression of DCC and Neogenin mRNA and protein at levels comparable to wild-type littermate (Fig. 1*J*,*K*,*N*,*O*,*Q*; data not shown; 76.2 $\pm$ 18.5 vs 83 $\pm$ 8.5 arbitrary units wt vs $DSCAM^{-/-}$ DCC protein, respectively; $p > 0.5$ Student's *t* test; 109.1 $\pm$ 7 vs 99.23 $\pm$ 0.4 arbitrary units wt vs $DSCAM^{-/-}$ Neogenin protein, respectively; $p = 0.237$ Student's *t* test).

Netrin-1-induced axon outgrowth is unaffected by *DSCAM* mutation

We reasoned that the lack of C axon projection phenotypes in $DSCAM^{-/-}$ spinal cords could be because of partial compensation by cues other than Netrin-1 (Charron et al., 2003; Evans and Bashaw, 2010) or because of a very high endogenous Netrin-1 concentration (Serafini et al., 1994; Moore and Kennedy, 2006). To isolate the effect of Netrin-1 in C axon outgrowth and to better control its concentration, dorsal spinal cords explants of e11.5 $DSCAM^{-/-}$ mutants and control littermates were cultured in the presence or absence of Netrin-1 at a concentration previously shown to be sensitive to the loss of Ne-

←

(*Figure legend continued.*) DCC (***O***) and Neogenin (***N***) expression was maintained at levels comparable to wild-type (***J***, ***K***) and position and numbers of motor neurons was not affected as revealed by Isl1 immunostaining (***L***, ***P***). $n = 4$ embryos per genotype. Arrowheads, commissural neurons; asterisks, motor neurons. Scale bar (in ***P***), 65 μm in all panels. ***Q***, Western blot analysis of e11.5 $DSCAM^{-/-}$ spinal cords reveal that DCC and Neogenin expression was maintained at levels comparable to wild-type ($n = 3$ per genotype).

trin receptor function (Fig. 2) (Serafini et al., 1994; Moore and Kennedy, 2006). In *DSCAM*$^{-/-}$ and control explants, axonal outgrowth was only detected in the presence of 200 ng/ml Netrin-1 (Fig. 2*D–F*). Moreover, in *DSCAM* mutants the mean length of axon bundles was 42.8% of explant circumference and was not significantly different from that observed in *DSCAM* heterozygous or wild-type littermates (Fig. 2*G*; 51.8% and 47.6% respectively, $p = 0.48$ *DSCAM*$^{+/-}$ vs *DSCAM*$^{-/-}$, $p = 0.76$ wild-type vs *DSCAM*$^{-/-}$, Student's *t* test). These data demonstrate that DSCAM is dispensable for the *in vitro* outgrowth of C axons in response to Netrin-1.

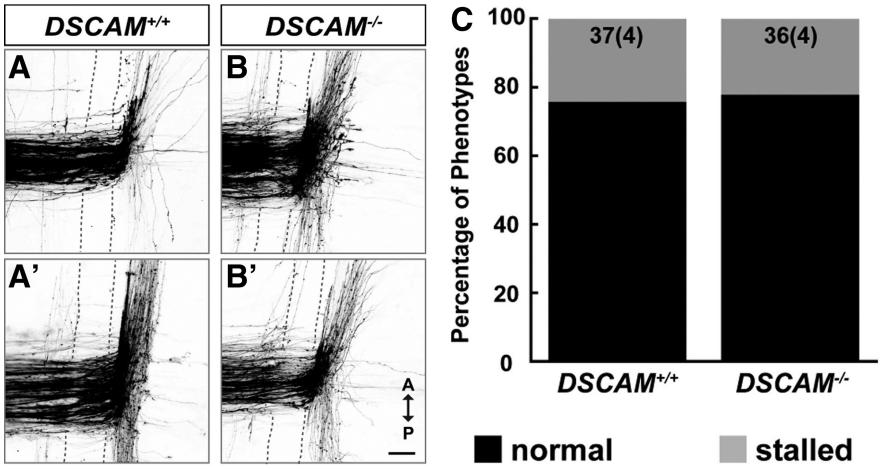

Figure 3. Normal commissural axon floor plate crossing in *DSCAM*-null mutants. ***A–B***, DiI injections into e12.5 dorsal spinal cords (on the left side of the shown "open book") of control littermates (***A***, ***A′***) and *DSCAM mutants* (***B***, ***B′***) show most commissural axons crossed the floor plate (dashed line) and turned anteriorly. ***C***, Quantification of DiI labeled axonal projections. $N = 36$ injections in *DSCAM*$^{-/-}$ and 37 in wild-type embryos; $n = 4$ embryos per genotype. Scale bar (in ***B′***), 50 μm in all panels.

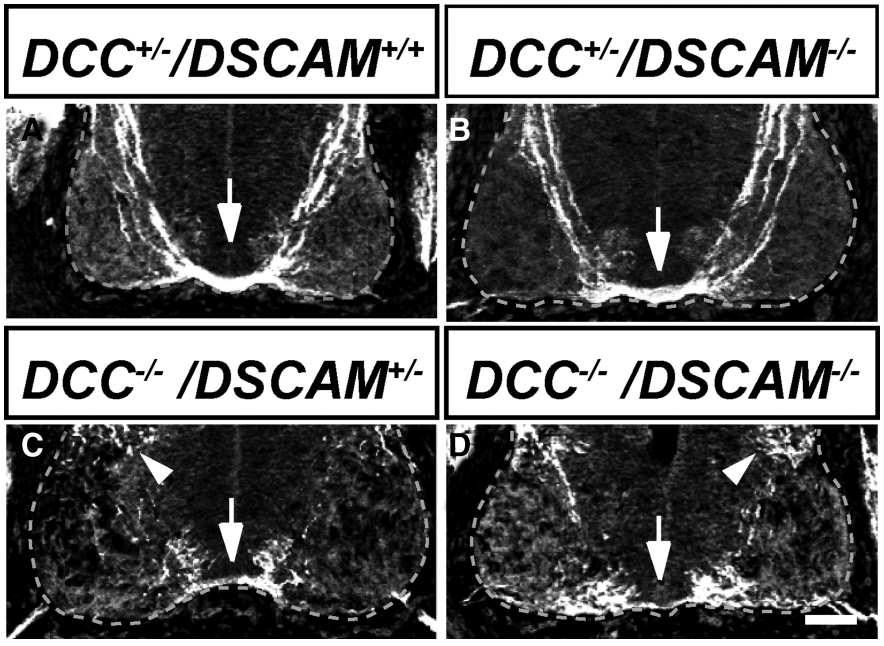

Figure 4. DCC/DSCAM compound mutants have commissural axon trajectory similar to *DCC* mutants. ***A–D***, TAG-1 immunostaining at e11.5 brachial spinal cord embryos. Commissural neurons of *DCC*$^{+/-}$*; DSCAM* $^{-/-}$ embryos (***B***) reached and crossed the floor plate as in control littermates (***A***). Like *DCC* mutants (***C***), a small percentage of TAG-1 commissural neurons of e11.5 *DCC/DSCAM* compound mutant embryos (***D***) reached and crossed the floor plate (arrows) while the majority were not able to reach the floor plate (arrowheads). $N = 3$ *DCC*$^{-/-}$*; DSCAM*$^{-/-}$, 3 *DCC*$^{-/-}$, 3 *DCC*$^{+/-}$*; DSCAM*$^{-/-}$, and 3 control embryos. Scale bar, 50 μm in all panels.

Normal commissural axon floor plate crossing in *DSCAM*-null mutants

Once C axons reach the floor plate, they cross it, enter one of the longitudinal fascicles on the contralateral side and project rostrally. To determine whether any of these stereotyped events were affected in *DSCAM* mutants we labeled C axons by injecting DiI into the dorsal spinal cord of fixed e12.5 embryos at several rostrocaudal locations and, to allow the dye time to diffuse, we analyzed the location of C axons 24 h later. In wild-type embryos, DiI-labeled C axons reached the floor plate, where only 24% of them were stalled, while a majority of them were observed to project rostrally (76%). Analysis of a similar number of injection sites in *DSCAM*$^{-/-}$ mutants showed 22% of C axons were stalled at the floor plate while 78% of them were found to project anteriorly after crossing the floor plate, indicating normal projections of commissural neurons in the absence of DSCAM ($p = 0.82$, χ^2 test for independence; Fig. 3*C*).

Absence of DSCAM does not enhance the commissural axon trajectory defects in *DCC* mutants

DSCAM was shown to interact with the Netrin-1 receptor DCC (Ly et al., 2008; Liu et al., 2009). Since in *DCC* mutants a small percentage of C axons is able to reach and cross the floor plate (Keino-Masu et al., 1996) we reasoned that in this C axon subpopulation, DSCAM might be required for axonal attraction toward the floor plate. We therefore examined C axon trajectory by TAG-1 immunostaining in e11.5 brachial spinal cords of *DCC*$^{-/-}$ *DSCAM*$^{-/-}$ compound mutants, *DCC*$^{-/-}$ mutants and control littermates. In *DCC*$^{-/-}$*; DSCAM*$^{-/-}$ compound mutants the majority of TAG-1 C axons were not able to reach the floor plate, and surprisingly, similar number of C axons reached and crossed the floor plate as observed in *DCC* mutants (Fig. 4*C*,*D*; $n = 3$ *DCC*$^{-/-}$; *DSCAM*$^{-/-}$ and 3 *DCC*$^{-/-}$ embryos), indicating that the lack of DSCAM expression does not exacerbate the *DCC*$^{-/-}$ C axon phenotype. We next asked whether DSCAM loss could be exacerbated by the loss of one allele of DCC and examined C axon trajectories in e11.5 brachial spinal cords of *DCC*$^{+/-}$*; DSCAM*$^{-/-}$ mutants. In these, we observed a similar number of C axons reaching and crossing the floor plate as in control animals (Fig. 4*A*,*B*; $n = 3$ per genotype).

Discussion

Our observations indicate that in mice, DSCAM is not required for Netrin-induced axon outgrowth *ex-vivo* or for guidance to and at the floor plate *in vivo*. Here we discuss the differences between genetic

and RNA interference-mediated loss of DSCAM function and possible compensatory mechanisms in Netrin signaling.

The main published evidence for DSCAM function as Netrin receptor in vertebrates comes from acute downregulation of DSCAM using specific siRNAs, resulting in defects of Netrin-induced commissural axon outgrowth and guidance to the midline (Ly et al., 2008; Liu et al., 2009). These experiments also included well controlled rescue experiments arguing that the effects of DSCAM loss are specific. Generally, siRNAs constitute a useful tool for functional analysis and their effects recapitulate phenotypes observed in mice null mutants (Helmbacher et al., 2000; Luria et al., 2008; Kao et al., 2009; Palmesino et al., 2010). However, this is not always the case as it was previously reported that some siRNA phenotypes were not confirmed by genetic analyses in mice (Wu et al., 2006; Moore et al., 2008). These and our observations suggest that acute loss of gene function may differ in its effects from a deletion of function over a long term, such as a null genetic mutation.

The divergences in phenotype between DSCAM downregulation through siRNA and complete genetic ablation can also be explained by compensatory effects, in which acute downregulation of DSCAM does not allow sufficient time for other components of Netrin-1 signaling to compensate for the lack of DSCAM expression. However, in *DSCAM* mutants, expression of other known Netrin-1 receptors was unaffected, suggesting that, if it does occur, the lack of DSCAM expression may be corrected in more subtle ways. Possible compensatory mechanisms may occur in the post-translational regulation of other Netrin receptors or in the regulation of downstream signaling such as DCC phosphorylation (Meriane et al., 2004). Though we cannot exclude this possibility completely, the observation that mice lacking both DCC and DSCAM have C axon guidance errors similar to what is observed in mice lacking only DCC (Keino-Masu et al., 1996) argue against it.

Netrin-1 is secreted by floor plate cells and forms a gradient in the spinal cord (Kennedy et al., 2006) and thereby induces first, axon outgrowth, and then guidance of the axons to the midline (Serafini et al., 1994; Ming et al., 2002). Maximal outgrowth of dorsal spinal cord explants happens in the presence of 200 ng/ml Netrin-1 (Serafini et al., 1994; Moore and Kennedy, 2006). Our results show that in the presence of this optimal concentration of Netrin, C axons of *DSCAM* mutants extend their axons to levels comparable to wild-type embryos. It cannot be excluded that DSCAM may determine the sensitivity at which C axons are able to respond to Netrin-1, but, if so, this clearly is not sufficient to produce *in vivo* defects.

Could other axonal projections be more sensitive to loss of DSCAM than commissural neurons? In addition to C neurons, Netrin-1 plays a role in axon guidance for many other neuronal types and where examined, these neurons are known to require DCC to respond to Netrin-1 (Moore, 2007). We cannot, however, exclude the possibility that DSCAM is required for responses of some neuronal populations to Netrin-1. In this context, it is interesting to note that DSCAM mutants display abnormal Botzinger complex rhythmicity (Amano et al., 2009), which is dependent on normal hindbrain commissure development. In addition, DSCAM might have Netrin-1 independent functions, as suggested by the observation that DSCAM is required for retinal ganglion cell dendrite fasciculation (data not shown; Fuerst et al., 2009).

In summary, based on the absence of axon outgrowth and axon guidance defects in *DSCAM*-null mice, the lack of compensation by expression of other Netrin receptors and the lack of enhanced guidance defects in *DCC/DSCAM* mutants compared with *DCC*-null mice, we conclude that DSCAM is not required for Netrin-1-mediated C axon guidance in vertebrates.

Note added in proof

Using the described conditions, we could not see any difference in DSCAM expression in spinal cord sections of wild-type and DSCAM mutants using five different commercial antibodies: mouse anti-DSCAM (Millipore), rabbit anti-DSCAM (Novus), and goat anti-DSCAM (R&D Systems) used at a 1:100 dilution.

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