Roles of DSCAM in axonal decussation and fasciculation of chick spinal interneurons

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ABSTRACT The ventral midline of the embryonic neural tube, the floor plate, has a profound role in guiding axons during embryonic development. Floor plate-derived guidance cues attract or repel axons, depending on the neuronal subtype and developmental stage. Netrin-1 and its receptor, Deleted in Colon Carcinoma (DCC), are the key constituents of commissural axons guidance cues toward the floor plate. Recent studies have implicated Down Syndrome Cell Adhesion Molecule (Dscam) as an additional Netrin-1 receptor. In this study, we examined the role of Dscam in guiding defined spinal dorsal interneuron populations. *In vivo* knockdown and ectopic expression of Dscam were performed in the dorsal dl1, dl2 and dl3 interneurons of chick embryos, by separately increasing or decreasing Dscam expression in each of these three specific interneuronal populations. Neuron-specific gain and loss of function of Dscam had no effect on the axonal trajectories of dl1-3 neurons. The commissural neurons, dl1c and dl2, crossed the midline, and the ipsilaterally projecting neurons, dl1i and dl3, projected ipsilaterally. However, the fasciculation of dl1 axons was diminished when Dscam expression was attenuated. Dscam is not required for either attraction to or repulsion from the floor plate. In contrast, Dscam is required for the fasciculation of axons, probably via homophilic interaction.

KEY WORDS: *spinal cord, axon guidance, floor plate, DSCAM, fasciculation*

Introduction

In bilaterally symmetric vertebrates, the floor plate at the ventral midline plays a crucial role in directing axon growth during the formation of neuronal circuits within the central nervous system. Floor plate cells provide a variety of guidance cues for growing axons, that elicit divergent effects depending on the neuronal cell type or its developmental stage (Dickson and Zou, 2010, Evans and Bashaw, 2010). Biochemical, *in vitro* and genetic evidence has demonstrated that the floor plate protein, Netrin-1, and its axonal receptor, Fra/DCC, are a key ligand-receptor pair that guides axons toward the floor plate (Dickson and Zou, 2010; Evans and Bashaw, 2010; Kennedy *et al.,* 1994; Serafini *et al.,* 1996; Serafini *et al.,* 1994). In vertebrates, Shh and VEGF, which are expressed in the floor plate, and their axonal receptors, Boc, Ptc and Flk1, are additional attractive cues that synergize with netrin-1 in directing commissural growth cones toward the floor plate (Charron *et al.,* 2003, Okada *et al.,* 2006, Ruiz de Almodovar *et al.,* 2011).

The incomplete failure of commissural midline crossing in *DCC* mutant mice suggests that other receptors may convey the Netrin-1 signal (Fazeli *et al.,* 1997). Several biochemical and genetic results implicated the Down Syndrome Cell Adhesion Molecule (Dscam) as a receptor for Netrin-1 that participates in regulation of midline crossing: 1) Netrin-1 binds to Dscam and DCC at similar affinities (Andrews *et al.,* 2008, Liu *et al.,* 2009, Ly *et al.,* 2008). 2) Knockdown of *Dscam* by siRNA in mouse and chick embryonic neural tubes causes a substantial reduction in the number of commissural axons that cross the midline (Liu *et al.,* 2009, Ly *et al.,* 2008). 3) In an *in vitro* turning assay using rat neural tube explants, blocking either DCC or Dscam activity does not alter the turning of commissural axons within the neuroepithelium toward an exogenous Netrin-1 source, whereas blockade of both receptors prevents turning (Ly *et al.,* 2008). 4) In a similar assay using chick neural tube explants,

Abbreviations used in this paper: DCC, deleted in colon carcinoma; Dscam, Down Syndrome cell adhesion molecule.

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inhibition of Dscam by expression of siRNA or a dominant negative form of Dscam is sufficient to block the turning (Liu *et al.*, 2009). 5) Expression of *Dscam* in *Xenopus* neurons mediates turning toward a Netrin-1 gradient in the absence of DCC signaling (Ly *et al.*, 2008). 6) In flies, *Netrin* mutants have a similar phenotype to *Dscam* mutants. Additionally, overexpression of *Dscam* in axons that do not normally cross the midline is able to induce ectopic midline crossing (Andrews *et al.*, 2008).

All these data are consistent with Dscam as an attractive receptor for Netrin-1. However, genetic analysis of *Dscam* mutants in mice has provided only partial support for this idea. In one Dscam allele, *Dscam²ᴶ*, increased number of spinal commissural interneurons was apparent, and mutant mice displayed motor hypertonia and lack of locomotor coordination (Lemieux *et al.*, 2016, Thiry *et al.*, 2016). In another allele, commissural axons reach and cross the floor plate and *in vitro* they show normal outgrowth in response to Netrin-1 (Palmesino *et al.*, 2012). One difference is that the *Dscam²ᴶ* allele may not be a null: it contains a four base pair duplication in exon 19, leading to a frameshift and truncation of the open reading frame. Hence, it is not clear whether the *Dscam²ᴶ* is

a hypomorph or a neomorph.

Another possible explanation for the discrepancies is that specific subpopulations of spinal interneurons might differ in their dependence on Dscam, with alterations in one group masked by dispensability of Dscam for other groups. To explore this possibility, we selectively marked and manipulated three separate populations of dorsal spinal interneurons in chick: dI1, dI2 and dI3. The recent use of neuron-specific enhancer elements, which enables tracking of defined interneuron populations, has revealed that the dorsal interneurons dI1-3 have a different laterality preference (Avraham *et al.*, 2010a, Avraham *et al.*, 2009, Gowan *et al.*, 2001, Helms and Johnson, 1998, Reeber *et al.*, 2008). dI1 neurons project axons both ipsilaterally and contralaterally (Avraham *et al.*, 2009, Wilson *et al.*, 2008), dI2 have mostly commissural axons, while dI3 project their axons ipsilaterally (Avraham *et al.*, 2010a, Avraham *et al.*, 2009). In each case, we performed both loss- and gain-of-function tests, by either knock-down or elevation of Dscam levels in an interneuron-specific manner. We found that dI1-, dI2- and dI3-specific manipulations of *Dscam* expression did not change the tra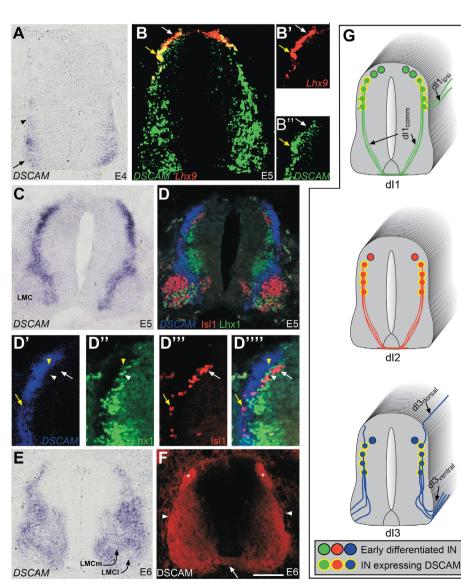jectory choice of each of the dorsal interneuron populations. However, Dscam was required for proper axon fasciculation.

Results

Expression pattern of Dscam in the embryonic chick spinal cord

To explore whether Dscam contributes to axon growth and guidance in the chick

Fig. 1. Expression pattern of *Down Syndrome cell adhesion molecule (Dscam)*. In situ *hybridization* **(A-E)** *and immunohistochemistry* **(F)** *were performed on cross sections of chick embryos.* **(A)** *At E4* Dscam *is expressed in motor neurons (arrow) and in ventral interneurons (arrowhead).* **(B)** *Double* in situ *hybridization with* Dscam *(green) and Lhx9 (red).* Dscam *is not expressed in the early-differentiated dI1 neurons (white arrow), but is expressed in the differentiated dI1 neurons (yellow arrow).* **(C)** In situ *hybridization in the lumbar level of an E5 embryo.* **(D)** *The section in (C) was stained with antibodies against Lhx1/5 and Isl1. The bright-field image of (C) was photo-converted to a dark-field image. The early-differentiated dI2 neurons (white arrowhead) are Lhx1/5+/ Dscam-, while the differentiated dI2 neurons (yellow arrowhead) are Lhx1/5+/ Dscam+* **(D', D")**. *The early-differentiated dI3 neurons (white arrow) are Isl1+/ Dscam-, while the differentiated dI3 neurons (yellow arrow) are Isl1+/ Dscam+* **(D''', D'''')**. **(E)** *At E6,* Dscam *is expressed at low levels in LMCl neurons and high levels in LMCm neurons.* **(F)** *Dscam protein is detected on axons that cross the floor plate (arrow), project ipsilaterally (arrowheads) and at the dorsal root entry zone (asterisks).* **(G)** *Scheme summarizing the projection pattern of dI1-3 neurons and the expression of* Dscam *in those neurons. Scale bar in (F) equivalent to 100 μm for (D), 60 μm for (D'-D''''), 200 μm for (A), 125 μm for (B) and 75 μm for (E,F).*
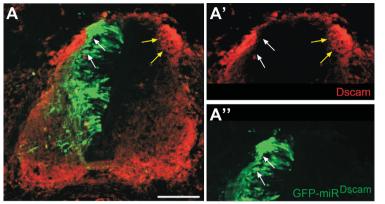
Fig. 2. The effect of miR^Dscam^ on Down Syndrome cell adhesion molecule (Dscam) expression. *GFP-miR^Dscam^ cassette was expressed in the chick hemi-tube. A cross section stained with anti-Dscam is shown. At the dorsal spinal cord, neurons expressing miR^Dscam^ do not express Dscam (white arrows), while neurons at the dorsal part of the un-electroporated side express Dscam (yellow arrows). Scale bar in (A), 100 μm.*

spinal cord, we first characterized its spatial and temporal expression. *In situ* mRNA hybridization, combined with antibody staining for cell fate markers, was performed on E4-E6 chick embryo cross sections. At thoracic level of E4, *Dscam* mRNA is expressed in motor neurons and in ventral interneurons (Fig. 1A). Expression in motor neurons is observed in the thoracic-level motor neurons at E5 (Fig. 1B) and E6 (data not shown). *Dscam* is not expressed at E5 in the lateral motor column (LMC) neurons (Fig. 1 C,D). At E6, LMCl neurons express low levels, while LMCm neurons express high levels of *Dscam* (Fig. 1E). Expression in the dorsal interneurons is apparent at E5 (Fig. 1 C,D). Notably, at E4 the dorsal commissural neurons, whose axons reach the floor plate at this developmental stage, do not express detectable levels of *Dscam* (Fig. 1A).

We used cell type specific markers to verify the profile of *Dscam* expression in dI1-3 neurons. *In situ* double fluorescence hybridization to Lhx9 and *Dscam* reveals that the most dorsal dI1 neurons, the early-differentiated dI1 neurons, do not express *Dscam*, while the lateral and more ventral differentiated dI1 neurons express *Dscam* (Fig. 1B). The expression of *Dscam* in dI2 neurons was studied using Lhx1/5 antibody. The early-differentiated, dorsally/medially located dI2 neurons, do not express *Dscam*, while the more

lateral and ventral dI2 neurons express *Dscam* (Fig. 1D). Isl1 was used as a dI3 neuronal marker. Consistently, expression of *Dscam* is restricted to the ventro/laterally migrating differentiated dI3 neurons (Fig. 1D). Hence, *Dscam* is expressed in the late differentiated dI1-3 neurons.

The dorsal interneurons vary in their axonal projection pattern. Along the dorsal/ventral axis, dI1c and dI2 project their axons contralaterally, while dI1i and dI3 neurons project ipsilaterally (Fig. 1G). The expression of Dscam protein along the axons was studied utilizing Dscam-specific antibody (Yamagata and Sanes, 2008). Dscam protein is present in the dorsal root entry zone, consistent with its expression in the DRG, in the white matter, at the lateral and ventral funiculi, where the longitudinal ipsilateral axons project, and at the floor plate, the crossing zone of the commissural axons (Fig. 1F). Thus, Dscam is expressed in both commissural and ipsilaterally projecting neurons and along their axons.

Strategies for loss and gain of function of Dscam *in the chick spinal cord*

We previously used a plasmid-based electroporation approach for knock-down and ectopic expression of *Dscam* in the chick retina (Yamagata and Sanes, 2008). Here, we used micro-RNA targeting Dscam (miR^Dscam^) and and a *Dscam* cDNA, both under the regulation of the CAGG enhancer/promoter or interneuron-specific enhancers, for loss and gain of function (Table 1). To assess our ability to down-regulate *Dscam* expression in the spinal cord, spinal cords of chick embryos were electroporated at Hamburger-Hamilton Stage 18. At E6, embryos were removed, fixed and analyzed for Dscam protein expression. Electroporation of spinal neurons with miR^Dscam^ decreased the expression of the Dscam in the soma and axons (Fig. 2A).

Dscam is not required or sufficient for the selection of axonal trajectories by dI1 neurons

To manipulate specific interneurons, we subcloned the miR^Dscam^ cassette in a Cre-dependent plasmid or a Cre/Gal4 double conditional plasmid (Avraham *et al.*, 2009), using the approach of Wilson and Stoeckli (2011). For dI1-specific perturbations, the Cre-conditional miR^Dscam^ plasmid was electroporated along with a EdI1::Cre plasmid (Avraham *et al.*, 2009) (Table 1, Fig. 3E). The size of the spinal cords of miR^Dscam^ and control GFP-electroporated embryos was comparable, indicating no general effect on develop-

TABLE 1

PLASMIDS USED FOR KNOCK-DOWN AND ECTOPIC EXPRESSION OF DSCAM

Neuron	Enhancers	Target plasmids for loss of function	Target plasmids for gain of function	Fig.
dI1	EdI1::Cre	CAG::GFP-miR^Dscam^ CAG::STOP^LoxP^-taumyc		2B,D
			CAG::Dscam-IRES-GFP	2C
		CAG::STOP^LoxP^-GFP-miR^Dscam^		3B,F
	EdI1::Gal4		UAS::mCherry-2A-Dscam	3D,G
dI1	EdI1::Cre EdI1::Gal4	UAS::taumyc^LoxP^-GFP-miR^Dscam^		6B
dI2	EdI1/2::Cre EdI2/V1::Gal4	UAS::STOP^LoxP^-GFP-miR^Dscam^		4B,E
			UAS::STOP^LoxP^- mCherry-2A- Dscam	4C,E
dI3	215::Gal4 242::Cre	UAS::STOP^LoxP^-GFP-miR^Dscam^		5B,E
			UAS::STOP^LoxP^- mCherry-2A- Dscam	5C,E

ment (Fig. 3 A-D). The ratio of ipsilaterally projecting axons from the total longitudinal axons did not differ between miR^{Dscam} and control GFP-electroporated embryos (Fig. 3A,B,F, Fig. S1A, Table 2). Likewise, the ectopic expression of *Dscam* in dl1 neurons did not alter the ipsi/contralateral ratio of dl1 axons. The ratio of ipsi/ total-projecting dl1 axons was similar between the *Dscam* over-expressing dl1 neurons and control dl1 neurons (Fig. 3 C,D,G, Fig. S1B, Table 2). Thus, neither down- nor up-regulation of *Dscam* in dl1 neurons significantly affects the ipsi- versus contralateral choice of dl1 neurons.

Fig. 3. Loss and gain of function of *Dscam* in dl1 neurons. *Using the plasmids illustrated in* **(E)**, Dscam *levels were down-* **(B)** *and up-* **(D)** *regulated. GFP expressed by the same drivers (Cre for A,B and Gal4 for C,D) was used as a control. Three representative images from each group are shown. White arrows point to the contralaterally projecting axons. Arrowheads point to the ipsilaterally projecting axons.* **(E)** *Schemes showing the molecular strategies for manipulating* Dscam *levels in dl1 neurons.* **(F)** *Quantification of the ipsilaterally projecting dl1 axons following knockdown of* Dscam *in dl1 neurons. A t-test was performed to compare the adjusted %ipsi of the miR^{Dscam} to the Control group. The two groups do not appear to be significantly different for alpha=0.05 (p-value=0.074).* **(G)** *Quantification of the ipsilaterally projecting dl1 axons following ectopic expression of* Dscam *in dl1 neurons. A t-test was performed to compare the adjusted %ipsi of the* Dscam *to the GFP group. The groups do not appear to be statistically different (p-value=0.226). Box plot charts* **(G,H)**: *The boxes, enclose 50% of the results; the horizontal lines above and under the box enclose 90% of the results; the black and gray horizontal lines within the box are the average and the median, respectively. Scale bar in (D), 125 μm.*

TABLE 2

SUMMARY OF THE STATISTICAL ANALYSIS

Fig.	Statistical test	Dependent Variable	Significance	Summary statistics*			
				Group	N	Mean	Std Dev
3F	Dunnett's test	%ipsi	No (P=0.074)	GFP	38	50.66	6.67
				miR^{Dscam}	39	53.70	7.94
3G	Dunnett's test	%ipsi	No (P=0.226)	DSCAM	23	73.18	9.28
				GFP	41	76.07	8.99
4	Dunnett's test	%comm	Yes, for both (multiple comparisons, controls for the overall alpha level of 0.05. see fig 4E)	GFP	33	92.10	3.73
				ectopic Dscam	25	88.43	5.44
				miR^{Dscam}	53	89.30	5.85
5	Dunnett's test	%ipsi	miR^{DSCAM} is different and ectopic DSCAM is not significantly different from the control (multiple comparisons, controls for the overall alpha level of 0.05. see fig 5E)	GFP	21	97.44	0.99
				ectopic Dscam	16	97.13	1.52
				miR^{Dscam}	35	98.19	0.84
6	paired T-Test	The width of miR^{Dscam} and taumyc axonal bundle	Yes (P<0.0001)	miR^{Dscam} minus taumyc	14	34.29	19.10

*For Figures 3-5, after adjustment for embryo effect

Dscam *is not required or sufficient for the commissural axon projection of dI2 neurons*

To direct dI2-specific knockdown in dI2 neurons, the miRDscam cassette was subcloned into a Gal4/Cre dual conditional vector. The dual conditional miR^{Dscam} plasmid was co-electroporated with the dI2 enhancers plasmid-mix (Avraham *et al.*, 2009) (Table 1, Fig. 4D). A robust commissural projection following *Dscam* knockdown was observed in dI2 neurons (Fig. 4A,B). The majority of dI2 axons projected contralaterally following dI2-specific expression of either GFP, miR$^{Dsc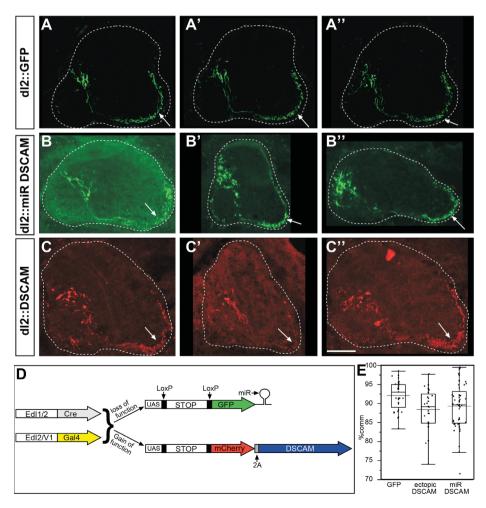am}$, or *Dscam* (92.2+/-3.7%, 89.3+/-5.9% and 88.4%+/-5.4%, respectively; Fig. 4 C,E, Fig. S2, Table 2). Statistical comparisons with a control using Dunnett's Method demonstrated a minor, though statistically significant, difference between the control and the manipulated dI2 axons. However, we observed no qualitative alteration in the trajectory of dI2 axons toward and across the floor plate. nor was there a significant difference in the commissural ratio between the gain and loss of function groups.

Dscam *is not required or sufficient for the ipsilateral axonal projection of dI3 neurons*

The dual conditional miR^{Dscam} plasmid and the ectopic *Dscam* were co-electroporated with the dI3 enhancer plasmid mix (Table 1, Fig. 5D). A robust ipsilateral preference was observed for dI3 axons in both control and manipulated embryos (Fig. 5A-C). The percentage of ipsilaterally projecting axons were: 97.44+/-0.99% in the control embryos, 98.19+/-0.84% in the miR^{Dscam} expressing
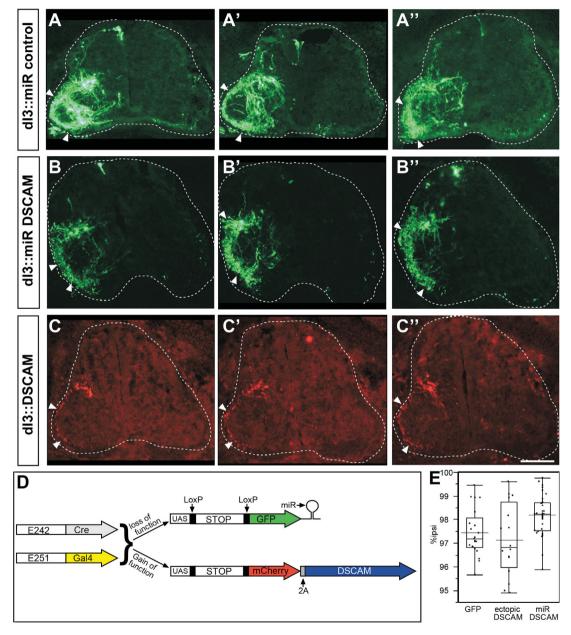
Fig. 4. Loss and gain of function of *Dscam* in dI2 neurons. *Using the plasmids illustrated in* **(D)**, *Dscam levels were down-* **(B)** *and up-* **(C)** *regulated in dI2 neurons. GFP expressed by the same drivers was used as a control* **(A)***. Three representative images from each group are shown. White arrows point to the contralaterally projecting axons.* **(D)** *Schemes showing the molecular strategies for manipulating Dscam levels in dI2 neurons.* **(E)** *Quantification of the contralaterally projecting dI2 axons following knockdown and ectopic expression of Dscam. The difference between the ectopic and the miR^{DSCAM} groups and the control group was assessed using the Dunnett's test (multiple comparisons). The test compares each level to the control level and controls for the overall alpha level (with an alpha of 0.05). The ectopic and the miR^{DSCAM} groups are significantly different from the control group (p-value=0.017 and 0.032 respectively). Box plot chart* **(E)***: The boxes, enclose 50% of the results; the horizontal lines above and under the box enclose 90% of the results; the black and gray horizontal lines within the box are the average and the median, respectively. Scale bar in (C), 125 μM.*

by LoxP sites and proceeded by EGFP (EGFP-miR^{Dscam}) was cloned downstream to UAS promoter (Fig. 6C). The WT/KD plasmid was co-electroporated with EdI1-driven Cre and Gal4 plasmids to drive expression in dI1 neurons. Neurons that incorporate all the three plasmids will express EGFP-miR^{Dscam} and consequently will down regulate *Dscam* expression, while neurons that incorporate only the EdI1::Gal4 and WT/KD plasmids will express *taumyc*. Other combination of plasmids will not lead to expression of either the reporter or the miR^{Dscam} cassette. We excluded axons that expressed both EGFP and taumyc; they likely initiated expression of taumyc early, and only subsequently activated the miR^{Dscam} via late expression of Cre.

The main result was that the Dscam-deficient (EGFP-positive) axons were spread over a broader area than the control (taumyc-positive) axons (Fig. 6A,B). To quantify the difference, we measured the width (in pixels) of the dI1 axon bundle at the circumferential axis (Fig. 6D). The average width of miRDscam dI1 bundle was 2.05+/- 0.86 times greater than the width of the control bundle.

Due to the fact that the wild type and mutated axons differ from each other regarding their fascicule width, the statistical analysis was preformed in the following manner: the width of miR^{Dscam} and the control (taumyc expressing axons) were scored per section.
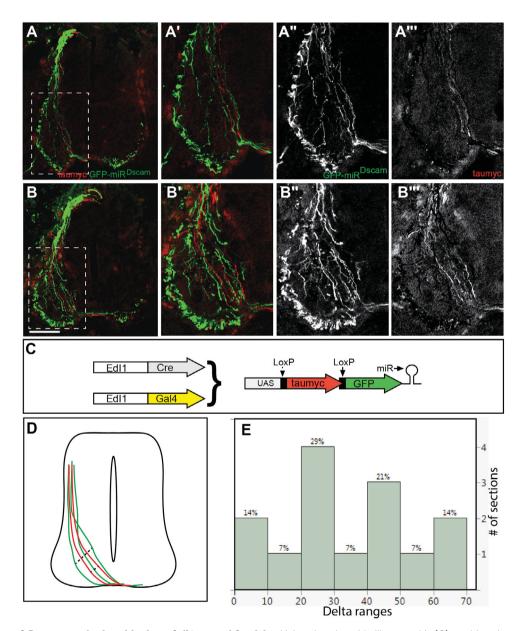

Fig. 6. Knockdown of *Dscam* results in widening of dI1 axonal fascicle. *Using the plasmids illustrated in* **(C)**, *a chimeric axonal genotype of dI1 neurons was obtained: Wild type dI1 neurons expressing miR^{DSCAM} and mutated dI1 neurons expressing GFP-miR^{DSCAM}. The wide fanning of GFP-miR^{DSCAM} axons* **(A,A',A'',B,B'B'')** *versus the tight bundle of the wild type, taumyc-expressing axons* **(A''',B''')**, *is evident. The width of the bundles was measured as illustrated* **(D)**. *The chart* **(E)** *shows the histogram of the differences. The X axis shows delta ranges - the width of the miR^{Dscam} fascicule minus the width of taumyc fascicule (width measured as pixels). The Y axis shows the the counts (number of samples). Above each bar the percent of sections is specified. Scale bar in (A), 100μm for (A) and 60 μm for (A'-A''').*

Deltas (the width of miR[Dscam] fascicle minus taumyc fascicle) were evaluated per image. Thus, if the two groups widths were similar, these deltas would tend to be distributed around zero, while larger deltas would indicate larger miR[Dscam] widths compared to the control. Deltas evaluated from the 14 sections, range between 7 and 32 with an average of 34.3 +/- 19.1. A T-Test was used for testing the null hypothesis that the deltas are zero. The test showed a statistically significant result, rejecting the null hypothesis (P<0.0001). (Fig. 6E, table 2). Therefore, we concluded that the width of miR[Dscam] dl1 axons was significantly larger than that of control axons. Thus, *Dscam* is required for the fasciculation of dl1 axons as they project toward the floor plate.

Discussion

Genetic evidence in the fly supports the role of Dscam in the guidance of embryonic commissural axons by Netrin-1 dependent and independent modes (Andrews *et al.*, 2008). In vertebrates, down-regulation of *Dscam*, via siRNA treatment, was shown to inhibit the axonal growth of dorsal interneurons toward the floor plate in mouse and chick (Liu *et al.*, 2009, Ly *et al.*, 2008). Conversely, the *in vivo* requirement of Dscam for commissural guidance in vertebrates was contested in a recent paper that used a null allele, which reported a normal projection pattern of spinal commissural axons in the (Palmesino *et al.*, 2012).

We have reexamined the role of Dscam in the guidance of chick spinal interneurons using cell-type specific knock-down and ectopic expression strategies. The loss and gain of function tools used in this study were previously utilized to demonstrate the requirement of Dscam for directing the lamina-specific synaptic connection of Dscam-expressing retinal ganglion cells (Yamagata and Sanes, 2008). A Knock-down of *Dscam* in the commissural interneurons, dl1c and dl2, did not prevent their extension toward and across the floor plate. Complementary, ectopic expression of *Dscam* in the ipsilaterally projecting populations, dl1i and dl3, did not alter their ipsilateral projection. Though, we argue that as in the mouse, also in the chick spinal cord Dscam is not involved in attraction to the floor plate. In addition, we have demonstrated that the ipsilateral projection of dl1c and dl3 neurons is not altered following *Dscam* knockdown, and the commissural projection of dl1c and dl2 is not affected succeeding ectopic expression of *Dscam*. Providing evidence for the dispensability of *Dscam* for midline repulsion of ipsilateral projecting neurons, which was not addressed in the mouse genetic model (Palmesino *et al.*, 2012). Our results do not provide substantial support for the idea that Dscam serves as a neuronal receptor for cues that promote midline crossing of spinal interneurons. In this respect, our conclusions are consistent with those of Palmesino *et al.*, (2008). On the other hand, we did observe small but statistically significant decreases or increase in crossing when Dscam levels were decreased in dl2 and dl3 neurons, respectively. We were unable to obtain a quantitative measure of the extent of Dscam attenuation in these neurons. Therefore, we cannot formally exclude the possibility that more complete inhibition might have led to a more dramatic effect. New gene editing tools will provide a means of testing this possibility in the future.

The binding of netrin-1 to Dscam was shown using diverse biochemical and cell biology means (Andrews *et al.*, 2008, Liu *et al.*, 2009, Ly *et al.*, 2008). Genetic evidence in flies supports the role of Dscam in mediating attraction toward the midline-derived Netrin molecules (Andrews *et al.*, 2008). An additional interpretation for Netrin/Dscam interaction is suggested: Dscam1 functions to counter *Drosophila* sensory neuron dendritic targeting signals provided by secreted Netrin-B and Frazzled. Loss of Dscam1 function resulted in Frazzled-dependent aberrant dendrite accumulation at a Netrin-B-expressing target (Matthews and Grueber, 2011). The counter-netrin activity of Dscam may reside from competition on Netrin binding between Dscam and Fra/DCC. Since the affinity to Netrin of both receptors is similar, higher levels of Dscam will sequester Netrin from binding the Fra/DCC. Alternatively, Dscam-mediated self-avoidance that is triggered by homophilic binding of Dscam, may initiate a signaling cascade that inhibits the Netrin/DCC signaling pathway. The relative balance between the levels of Dscam and DCC can shift the equilibrium between attraction and repulsion in this model as well.

Netrin-1/Dscam interaction in vertebrates may modulate Netrin-1 activity at other, post floor plate, choice points along the pathway. The possible role of Dscam in the guidance of the axons along the late longitudinal segments rather than the early circumferential segment of their projection, relies on the temporal profile of *Dscam* expression. At E4, when commissural axons cross the floor plate, *Dscam* is not expressed in the dorsal interneurons (Fig. 1A). At E5, *Dscam* expression is absent in the early-differentiated commissural interneurons that project toward the floor plate, while its expression is up-regulated in the presumed post-crossing late-differentiated neurons (Fig. 1 B-D). *Dscam* may participate in silencing netrin-1 responsiveness in post-floor plate-crossing commissural axons.

Homophilic interaction between Dscam expressing neurons was demonstrated in the chick and mouse retina. Our results highlight the homophilic activity also in the wiring of spinal interneurons. The novel wild type/knockdown chimeric system (Fig. 6) that we have developed assisted in revealing the requirement of Dscam for axonal fasciculation of dl1 axons. Scoring the phenotype of wild type and mutated neurons, within the same specimen, is advantageous over using control and manipulated chick embryos, since the efficiency of gene delivery may vary significantly between embryos. Applying this method is currently limited to neurons that can be targeted by a single enhancer. Hence, the possible role of Dscam in the fasciculation of dl2 and dl3, as well as in other spinal interneurons was not addressed in the current study. Therefore, we cannot exclude a dl1-specific role of Dscam in axonal fasciculation. Notably, no fasciculation defects, in TAG expressing commissural axons, were reported in the Dscam null mouse (Palmesino *et al.*, 2012). The discrepancy may arise from the fact that dl1 are only a fraction of commissural axons, and their projection is obscure by other commissural axons.

The responsiveness of growth cones to guidance cues is context-dependent. Repulsion from- versus attraction to- a defined guidance molecule can be gated by different receptor complexes or different intracellular signaling pathways (Dickson and Zou, 2010, Evans and Bashaw, 2010). Similarly, adhesive and repellent interactions between axons, that leads to either fasciculation or defasciculation, respectively, are also context-dependent. Role for Dscam in adhesive interaction in the retina (Yamagata and Sanes, 2008), as well as a repulsive molecule that prevents fasciculation of RGC's dendrites (Fuerst *et al.*, 2009), was demonstrared. Likewise, in *Drosophila*, Dscam is implicated in dendrites avoidance

(Matthews *et al.*, 2007) as well as attraction (Tadros *et al.*, 2016). It is not yet clear what controls the opposing activities of Dscam.

Dscam is expressed in numerous interneurons in the spinal cord, which raises the question whether Dscam can serve as a cell type specific fasciculation factor. Expression of other Ig-super family molecules that share the homophilic interaction activity: Sidekicks and Contactins, is more restricted in the spinal cord. Hence, a combinatorial expression of DSCAM/Sdk and Contactins may serve as homophilic code for self fasciculation of axonal bundle in the spinal cord, as it was shown in the retina (Yamagata and Sanes, 2012).

Materials and Methods

DNA

The GFP-miR-Dscam-b cassette (Yamagata and Sanes, 2008), was amplified by PCR using forward [ATGCTAGCAAAACCATGGTGAG-CAAGGG] and reverse [ATGCGGCCGCTAGATATCTCGAGTGCGGCC] primers. PCR product was digested with NheI and NotI and subcloned into a Cre or Gal4/Cre dependent plasmids (Avraham *et al.*, 2009, Hadas *et al.*, 2014). The mCherry-2A-Dscam cassette (Yamagata and Sanes, 2008) was excised by EcoRI+NotI digest and subcloned downstream to the UAS promoter. Plasmids containing dI1, dI2 and dI3 enhancers driving Cre and Gal4 were described previously (Avraham *et al.*, 2010a, Avraham *et al.*, 2009, Hadas *et al.*, 2014).

In ovo *electroporations*

Fertilized White Leghorn chicken eggs were incubated at 38.5-39°C. A DNA solution of 5 mg/ml was injected into the lumen of the neural tube stage 17-18. Electroporation was performed using 3 x 50 ms pulses at 30V, applied across the embryo using a 0.5 μm Tungsten wire and a BTX electroporator (ECM 830). Embryos were incubated for 3 days prior to analysis (Avraham *et al.*, 2010b).

Immunohistochemistry

Embryos were fixed overnight at 4°C in 4% paraformaldehyde/0.1 M phosphate buffer, washed twice with PBS, incubated in 30% sucrose/PBS for 24 hours, and embedded in OCT. Cryostat sections (14 μm) were collected on Superfrost Plus slides and kept at −70°C. The following antibodies were used: rabbit polyclonal GFP antibody (Molecular Probes), Lhx2/9, Lhx1/5, Isl1 (provided by T. Jessell, Columbia University, New York, NY) Dscam (Medimabs, Montreal). Cy2, RRX and cy5 were used as fluorochromes. Images were captured under a microscope (Axioscope 2; Zeiss) with a digital camera (DP70; Olympus) or confocal microscope (FV1000; Olympus).

For *in situ* double mRNA fluorescence the Tyramide Signal Amplification (TSA) kit (Perkin Elmer) was used. For double: mRNA *in situ* + immunohistochemistry, the traditional dig-AP (Roche) protocol was used. Following the AP color reaction, slides were incubated with the appropriate antibody as described above.

Quantification

Each experiment included both manipulated and control groups, which were electroporated within two hours of each other. Comparisons were made between manipulated and control embryos within an experiment. For each group 2-5 embryos were electroporated. At E6 embryos were removed from the eggs, fixed and sectioned. In each cross section the number of axons projecting longitudinally was scored. The number of pixels in the white matter at the ipsilateral and contralateral was calculated using ImageJ tools.

Analysis methodology: In some instances, there was a large embryo to embryo variability within groups. Thus, the analyses were performed on adjusted data, after removing the embryo effect. T-test was applied on the adjusted values to compare between two groups. In the cases of three groups, where each level is compared to a control, the multiple comparisons Dunnet's test was used. The charts show the adjusted values. In the supplementary figures, both the original values and the embryo data are presented.

References

ANDREWS, G.L., TANGLAO, S., FARMER, W.T., MORIN, S., BROTMAN, S., BERBEROGLU, M.A., PRICE, H., FERNANDEZ, G.C., MASTICK, G.S., CHARRON, F. *et al.,* (2008). Dscam guides embryonic axons by Netrin-dependent and -independent functions. *Development* 135: 3839-3848.

AVRAHAM, O., HADAS, Y., VALD, L., HONG, S., SONG, M.R. and KLAR, A. (2010a). Motor and dorsal root ganglia axons serve as choice-points for the ipsi-lateral turning of dI3 axons. *J. Neurosci.* 30: 15546-15557.

AVRAHAM, O., HADAS, Y., VALD, L., ZISMAN, S., SCHEJTER, A., VISEL, A. and KLAR, A. (2009). Transcriptional control of axonal guidance and sorting in dorsal interneurons by the Lim-HD proteins Lhx9 and Lhx1. *Neural Dev* 4: 21.

AVRAHAM, O., S. ZISMAN, Y. HADAS, L. VALD and A. KLAR (2010). Deciphering axonal pathways of genetically defined groups of neurons in the chick neural tube utilizing in ovo electroporation. *J Vis Exp* 39: 1792. (doi: 10.3791/1792)

CHARRON, F., STEIN, E., JEONG, J., MCMAHON, A.P. and TESSIER-LAVIGNE, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* 113: 11-23.

DICKSON, B.J. and ZOU, Y. (2010). Navigating intermediate targets: the nervous system midline. *Cold Spring Harb Perspect Biol* 2: a002055.

EVANS, T.A. and BASHAW, G.J. (2010). Axon guidance at the midline: of mice and flies. *Curr Opin Neurobiol* 20: 79-85.

FAZELI, A., DICKINSON, S.L., HERMISTON, M.L., TIGHE, R.V., STEEN, R.G., SMALL, C.G., STOECKLI, E.T., KEINO-MASU, K., MASU, M., RAYBURN, H. *et al.,* (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* 386: 796-804.

FUERST, P.G., BRUCE, F., TIAN, M., WEI, W., ELSTROTT, J., FELLER, M.B., ERSKINE, L., SINGER, J.H. and BURGESS, R.W. (2009). DSCAM and DSCAML1 function in self-avoidance in multiple cell types in the developing mouse retina. *Neuron* 64: 484-497.

GOWAN, K., HELMS, A.W., HUNSAKER, T.L., COLLISSON, T., EBERT, P.J., ODOM, R. and JOHNSON, J.E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* 31: 219-232.

HADAS, Y., ETLIN, A., FALK, H., AVRAHAM, O., KOBILER, O., PANET, A., LEVTOV, A. and KLAR, A. (2014). A 'tool box' for deciphering neuronal circuits in the developing chick spinal cord. *Nucleic Acids Res.* 42: e148.

HELMS, A.W. and JOHNSON, J.E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* 125: 919-928.

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.

LEMIEUX, M., O, D.L., THIRY, L., BOULANGER-PIETTE, A., FRENETTE, J. and BRETZNER, F. (2016). Motor hypertonia and lack of locomotor coordination in mutant mice lacking DSCAM. *J Neurophysiol* 115: 1355-1371.

LIU, G., LI, W., WANG, L., KAR, A., GUAN, K.L., RAO, Y. and WU, J.Y. (2009). DSCAM functions as a netrin receptor in commissural axon pathfinding. *Proc Natl Acad Sci USA* 106: 2951-2956.

LY, A., NIKOLAEV, A., SURESH, G., ZHENG, Y., TESSIER-LAVIGNE, M. and STEIN, E. (2008). DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. *Cell* 133: 1241-1254.

MATTHEWS, B.J. and GRUEBER, W.B. (2011). Dscam1-Mediated Self-Avoidance Counters Netrin-Dependent Targeting of Dendrites in *Drosophila. Curr Biol* 21: 1480-1487.

MATTHEWS, B.J., KIM, M.E., FLANAGAN, J.J., HATTORI, D., CLEMENS, J.C., ZI-PURSKY, S.L. and GRUEBER, W.B. (2007). Dendrite self-avoidance is controlled by Dscam. *Cell* 129: 593-604.

OKADA, A., CHARRON, F., MORIN, S., SHIN, D.S., WONG, K., FABRE, P.J., TESSIER-LAVIGNE, M. and MCCONNELL, S.K. (2006). Boc is a receptor for sonic hedgehog in the guidance of commissural axons. *Nature* 444: 369-373.

PALMESINO, E., HADDICK, P.C., TESSIER-LAVIGNE, M. and KANIA, A. (2012). Genetic analysis of DSCAM's role as a Netrin-1 receptor in vertebrates. *J Neurosci* 32: 411-416.

REEBER, S.L., SAKAI, N., NAKADA, Y., DUMAS, J., DOBRENIS, K., JOHNSON, J.E. and KAPRIELIAN, Z. (2008). Manipulating Robo expression *in vivo* perturbs commissural axon pathfinding in the chick spinal cord. *J Neurosci* 28: 8698-8708.

RUIZ DE ALMODOVAR, C., FABRE, P.J., KNEVELS, E., COULON, C., SEGURA, I., HADDICK, P.C., AERTS, L., DELATTIN, N., STRASSER, G., OH, W.J. *et al.,* (2011). VEGF mediates commissural axon chemoattraction through its receptor Flk1. *Neuron* 70: 966-978.

SERAFINI, T., COLAMARINO, S.A., LEONARDO, E.D., WANG, H., BEDDINGTON, R., SKARNES, W.C. and TESSIER LAVIGNE, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87: 1001-1014.

SERAFINI, T., KENNEDY, T.E., GALKO, M., 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.

TADROS, W., XU, S., AKIN, O., YI, C.H., SHIN, G.J., MILLARD, S.S. and ZIPUR-SKY, S.L. (2016). Dscam Proteins Direct Dendritic Targeting through Adhesion. *Neuron* 89: 480-493.

THIRY, L., LEMIEUX, M., O, D.L. and BRETZNER, F. (2016). Role of DSCAM in the development of the spinal locomotor and sensorimotor circuits. *J Neurophysiol* 115: 1338-1354.

WILSON, N.H. and STOECKLI, E.T. (2011). Cell type specific, traceable gene silencing for functional gene analysis during vertebrate neural development. *Nucleic Acids Res.*

WILSON, S.I., SHAFER, B., LEE, K.J. and DODD, J. (2008). A molecular program for contralateral trajectory: Rig-1 control by LIM homeodomain transcription factors. *Neuron* 59: 413-424.

YAMAGATA, M. and SANES, J.R. (2008). Dscam and Sidekick proteins direct lamina-specific synaptic connections in vertebrate retina. *Nature* 451: 465-469.

YAMAGATA, M. and SANES, J.R. (2012). Expanding the Ig superfamily code for laminar specificity in retina: expression and role of contactins. *J Neurosci* 32: 14402-14414.

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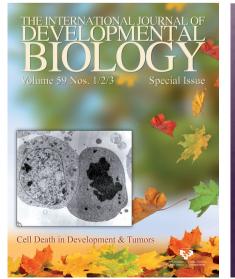Int. J. Dev. Biol. (2008) 52: 481-487

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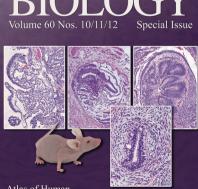Int. J. Dev. Biol. (2007) 51: 609-631

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