Figures and figure supplements

Dystroglycan is a scaffold for extracellular axon guidance decisions

L Bailey Lindenmaier et al
**Figure 1.** Dystroglycan functions non-cell autonomously to guide spinal commissural axons. (A) Immunostaining of E12.5 spinal cord shows Dystroglycan protein (magenta, left panel) expression in commissural axons (L1, green, middle panel). In the high magnification insets, arrows indicate Dystroglycan (DG) protein localization. (B) TUJ1 (green, middle panel) labels neuronal cell bodies and commissural axons. Merge (right panel) shows DG protein expression in commissural axons. (C) **Dag1**\(^{+/+}\), (D) Dag1\(^{lox/-}\); Sox2\(^{Cre}\), (E) Dag1\(^{cyto/-}\), and (F) Dag1\(^{lox/-}\); Wnt1\(^{Cre}\) genotypes. (G) Percent correct turn for each genotype: DG\(^{+/+}\), DG\(^{lox/-}\); Sox2\(^{Cre}\), DG\(^{cyto/-}\), and DG\(^{lox/-}\); Wnt1\(^{Cre}\). *p < 0.05.

<table>
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<th>Genotype</th>
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<th># injection sites</th>
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<td>18</td>
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<td>stalling; AP randomization</td>
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<tr>
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<td>8</td>
<td>59</td>
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Figure 1 continued on next page.
the enriched expression of Dystroglycan in the basement membrane of the spinal cord proximal to the axons. (B) Commisural neurons from E12 dorsal spinal cord cultured for two days in vitro (2DIV) were stained with antibodies to Dystroglycan (magenta, left panel), TUJ1 (green, middle panel). Dystroglycan is present throughout the cell body, axon and growth cone (arrow). (C–F) Dil injections in open-book preparations of E12 spinal cords were used to examine the trajectory of commissural axons. In controls (C), axons extend through the floor plate, then execute an anterior turn (n=6 animals, 49 total injection sites). In Dag1F/;Sox2Cre mice (D), axons stall within the floor plate and post-crossing axons exhibit anterior-posterior randomization (n=3 animals, 18 total injection sites). (E) Commisural axons in mice lacking the intracellular domain of Dystroglycan (Dag1bcyto/−) show normal crossing and anterior turning (n=3 animals, 34 total injection sites). Conditional deletion of Dystroglycan from commissural neurons in Dag1F/; Wnt1Cre mice (F) did not affect floor plate crossing or anterior turning (n=8 animals, 59 total injection sites). Higher magnification insets for each image show the anterior (top) and posterior (bottom) trajectories of post-crossing commissural axons. (G) Quantification of open book preparations. On average, 97.62 ± 3.39% of controls, 3.03 ± 4.80% of Dag1F/;Sox2Cre mutants, 89.52 ± 4.80% of Dag1bcyto/− mutants, and 95.31 ± 2.94% of Dag1F/; Wnt1Cre mutants showed normal crossing and anterior turning. All of the Dag1F/;Sox2Cre mutants with turning defects also showed stalling within the floor plate. *p< 0.001, one-way ANOVA, Tukey’s post hoc test. Scale bar = 100µm (A), 10µm (B) and 50µm (F–H).

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Figure 1—figure supplement 1. Analysis of Dystroglycan expression and commissural axon phenotypes in spinal cord sections. (A) An antibody raised against the intracellular domain of Dystroglycan shows staining in the basement membrane and in both pre-crossing and post-crossing commissural axons. (B) In Dag1<sup>−/−</sup> mutants, staining is significantly decreased.

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axons (A'). (B) Lack of staining in spinal cord sections from Dag1b<sup>Cyto</sup>-mutants verifies the specificity of the Dystroglycan antibody. (C–F) L1, Robo1 and Robo2 antibodies were used to label commissural axons in E12 spinal cord sections from Dag1<sup>F/+</sup> (C–C’), Dag1<sup>F/-</sup>;Sox2<sup>Cre</sup> (D–D’), Dag1b<sup>Cyto</sup>- (E–E’), and Dag1<sup>F/-</sup>;Wnt1<sup>Cox</sup> (F–F’) mutants. Post-crossing axons are disorganized and the ventrolateral funiculus appears fragmented in Sox2<sup>Cre</sup> mutants (D–D’), but appears normal in Dag1b<sup>Cyto</sup>- (E–E’), and Dag1<sup>F/-</sup>;Wnt1<sup>Cox</sup> (F–F’) mutants. Scale bar = 100 μm.

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Figure 2. Dystroglycan is required for axon tract formation in the forebrain. (A) L1 immunohistochemistry on P0 brain sections from Dag1$^{F/+}$;Sox2$^{Cre}$ controls (n = 3 animals) labels descending CTAs and ascending TCAs in the internal capsule. In Dag1$^{F/-}$;Sox2$^{Cre}$ (n = 4 animals) (B) and Ispd$^{79P/L79P}$ (n = 5 animals) (C) mutants, the internal capsule is highly disorganized, with axons projecting into the upper layers of the cortex (red arrows), forming ectopic bundles in the ventral telencephalon (red asterisks), and abnormal projections extending ventrally (red arrowheads). High magnification insets show L1 + axons in the intermediate zone of the cortex of controls (A’) and ectopic axonal projections into the upper cortical layers in Dag1$^{F/-}$;Sox2$^{Cre}$ (B’) and Ispd$^{79P/L79P}$ (C’) mutants. Dil injection in the thalamus of Dag1$^{F/Cre}$;Sox2$^{Cre}$ controls (n = 4 animals) labels TCAs as they cross the DTB, extend through the ventral telencephalon, across the PSPB, and into the intermediate zone of the cortex. In Dag1$^{F/-}$;Sox2$^{Cre}$ (n = 4 animals) (E) and Ispd$^{79P/L79P}$ (n = 4 animals) (F) mutants, TCAs fail to cross the DTB, and instead project ventrally out of the diencephalon. High magnification insets show Dil-labeled TCAs extending into the intermediate zone of the cortex of controls (D’), and a lack of labeled TCAs in the cortex of Dag1$^{F/-}$;Sox2$^{Cre}$ (E’) and Ispd$^{79P/L79P}$ (F’) mutants. Dil injection in the cortex of Dag1$^{F/Cre}$;Sox2$^{Cre}$ controls (n = 3 animals) labels CTAs as they extend across the PSPB, through the ventral telencephalon, and across the DTB into the thalamus. CTAs in Dag1$^{F/-}$;Sox2$^{Cre}$ (n = 4 animals) (H) and Ispd$^{79P/L79P}$ (n = 5 animals) (I) mutants fail to cross the PSPB or take abnormal trajectories through the ventral telencephalon. High magnification insets show Dil-labeled CTAs extending into the thalamus in controls (G’), and a lack of labeled CTAs in the thalamus of Dag1$^{F/-}$;Sox2$^{Cre}$ (I’) and Ispd$^{79P/L79P}$ (F’) mutants. (J–L) Schematic summarizing CTA (brown) and TCA (blue) axon trajectories in controls (J), Dag1$^{F/-}$;Sox2$^{Cre}$ (K) and Ispd$^{79P/L79P}$ (L). Scale bar = 500 μm.

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Figure 2—figure supplement 1. Anterior commissure, lateral olfactory tract and corpus callosum phenotypes in Ispd^{L79*/L79*} mutants. (A) Dystroglycan is expressed in the basement membrane surrounding the brain at E14.5. Dystroglycan is also expressed in the developing thalamus and in the axons that form the internal capsule (A, A’ inset). (B) Lack of staining in sections from Dag1^{b/cytol-} mutants verifies the specificity of Dystroglycan staining in the cortex. (C–F) L1 staining was used to label forebrain axon tracts in P0 wildtype (n = 3 animals) (C,E) and Ispd^{L79*/L79*} mutants (n = 3 animals) (D,F). The corpus callosum (red asterisk) in Ispd^{L79*/L79*} mutants appears largely normal compared to controls. In contrast, the anterior commissure (red arrows) is thinner and disorganized in Ispd^{L79*/L79*} mutants (D). The lateral olfactory tract (red arrowheads) extends along the pial surface of the ventrolateral
telencephalon in controls (C,E), whereas it appears hyperfasciculated and projects deeper into the piriform cortex as a disorganized bundle in Ispd<sup>L79<sup>−</sup></sup> <sub>L79</sub> mutants (D,F). Scale bar = 500 μm.

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Figure 3. Dystroglycan is required in ventral telencephalon neuroepithelial cells to guide corticothalamic and thalamocortical axons. L1 staining of P0 brain sections from Dag1<sup>Flox/+</sup> controls (n = 3 animals) (A, F), Dag1<sup>Flox/-</sup>;Foxg1<sup>Cre</sup> (n = 4 animals) (B), Dag1<sup>Flox/-</sup>;Gbx2<sup>CreaERT2</sup> (n = 4 animals) (C), Dag1<sup>Flox/-</sup>;Emx1<sup>Cre</sup> (n = 3 animals) (D), Dag1<sup>Flox/-</sup>;Dlx5/6<sup>Cre</sup> (n = 3 animals) (E), and Dag1<sup>b<sub>cyto</sub>/WT</sup> (n = 5 animals) (G). A’-E’ illustrate the recombination patterns in each Cre/CreERT2 line in the blue shaded area. Deletion of Dystroglycan throughout the neuroepithelium of the dorsal and ventral telencephalon in Dag1<sup>Flox/-</sup>;Foxg1<sup>Cre</sup> mutants (B, B’) results in abnormal projections in the internal capsule (red arrowheads) and abnormal axonal projections into the upper layers of the cortex (red arrows). Deletion of Dystroglycan from the neuroepithelium of the dorsal telencephalon with Emx1<sup>Cre</sup> mutants (D) results in abnormal axonal projections into the upper layers of the cortex (red arrow), but normal internal capsule formation. Deletion of Dystroglycan from the thalamus with Gbx2<sup>CreaERT2</sup> (C) or ‘corridor’ cells with Dlx5/6<sup>Cre</sup> (E) did not affect axon guidance. Deletion of the intracellular domain of Dystroglycan in Dag1<sup>b<sub>cyto</sub>/</sup> mutants (G) did not affect formation of the internal capsule compared to control littermates (F). A-G Scale bar = 500 μm.

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Figure 3—figure supplement 1. Dil labeling of CTAs and TCAs in Dystroglycan conditional mutants. (**A**–**E**) Dil injections into the cortex (top row) or thalamus (bottom row) of Dystroglycan conditional mutants labels CTAs and TCAs, respectively. CTAs (**B**) in Dag1^{Flox/−}; Foxg1^{Cre} mutants (n = 3 animals) take an abnormal trajectory through the ventral telencephalon, and TCAs (n = 4 animals) (**B’**) fail to cross the DTB and instead extend ventrally out of the diencephalon. CTAs in Dag1^{Flox/−}; Gbx2^{CreERT2} (n = 3 animals) (**C**), Dag1^{Flox/−}; Emx1^{Cre} (n = 5 animals) (**D**) and Dag1^{Flox/−}; Dlx5/6^{Cre} (n = 3 animals) (**E**) mutants are normal, as are TCAs in Dag1^{Flox/−}; Gbx2^{CreERT2} (n = 4 animals) (**C’**), and Dag1^{Flox/−}; Dlx5/6^{Cre} (n = 3 animals) (**E’**) mutants. TCAs in Dag1^{Flox/−}; Emx1^{Cre} (n = 4 animals) (**D’**) mutants project through the internal capsule normally, but project into the upper layers prematurely upon entering the cortex (red arrows). Scale bar = 500 μm.

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Figure 3—figure supplement 2. Recombination pattern in Gbx2CreERT2 and Emx1Cre mice. (A) Gbx2CreERT2 mice crossed to the A19; Rosa26lox-stop-lox-tdTomato reporter (n = 2 animals) were dosed with 2.5 mg tamoxifen at e10.5. Analysis of brains at E16 showed recombination of the tdTomato reporter (green) in thalamic neurons/axons. (B) Emx1Cre mice crossed to the A19; Rosa26lox-stop-lox-tdTomato reporter (n = 2 animals) showed recombination of the tdTomato reporter (green) in cortical neurons/axons at P0. Scale bar = 500 μm.

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Figure 4. Dystroglycan interacts with the LG1 domain of Celsr3. (A) Schematic of Celsr3 protein structure, highlighting the location of Cadherin, Laminin G (LG), EGF, Hormone Receptor Domain (HRM) and GPCR Proteolytic Site (GPS) domains. (B) Fc-tagged α-Dystroglycan (Fc-DG) secreted from 293 T cells was incubated with Alkaline Phosphatase (AP)-tagged Celsr3-LG1, Celsr3-LG2, or AP-tag alone, and complexes were isolated with Protein A/G beads. DG-Fc interacts selectively with Celsr3-LG1, but not Celsr3-LG2. (C) AP-Celsr3-L1, AP-Celsr3-LG2, or AP-tag alone were incubated with WGA enriched brain lysate, and complexes were purified with Ni-NTA beads. AP-Celsr3-LG1 binds endogenous glycosylated Dystroglycan, whereas AP-Celsr3-LG2 and AP-tag do not. (D) COS7 cells transfected with full-length Dystroglycan were incubated with 5 nM AP-tag, AP-Celsr3-LG1, AP-Celsr3-LG2, or AP-Slit-Cterm. Both AP-Celsr3-LG1 and AP-Slit-Cterm exhibited selective binding. Scale bar = 50 μm.

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**Figure 5.** Dystroglycan binding requires specific motifs in Celsr3 LG1. (A) Top: schematic showing the structure of the LG4 domain of Laminin-α2 (PDB:1OKQ), highlighting conserved residues critical for Dystroglycan binding: Arginine2803 (blue), Aspartate2808 (red) and Glycine2826 (purple). Bottom: Partial sequence alignment of murine Celsr3-LG1 (amino acids 1540–1574) with murine Laminin-α2-LG4 (amino acids 2795–2828) shows conservation at Arginine1548 (blue), Aspartate1564 (red) and Glycine1572 (purple) of Celsr3. (B–C) 293 T cells transfected with Celsr3-GFP or mutant Celsr3R1548Q-GFP showed no differences in expression levels or cell surface localization by immunocytochemistry (B) or western blotting (C). (D) Figure 5 continued on next page
Mutation of Celsr3-LG1 at Arginine1548 (AP-LG1\textsuperscript{R1548Q}) results in loss of binding to FC-tagged Dystroglycan. (E–J) Section binding assay with 5 nM AP alone (E–F), AP-Celsr3-LG1 (G–H), or AP-Celsr3-LG1\textsuperscript{R1548Q} (I–J). Inset panels show higher magnification of the ventrolateral funiculus (top panels E', G', I') and the internal capsule (bottom panels, F', H', J'). AP-Celsr3-LG1 binds to commissural axons in the ventrolateral funiculus (arrow, G,G') and the internal capsule (arrow, H,H'). AP-Celsr3-LG1\textsuperscript{R1548Q} shows minimal binding in either region and is almost indistinguishable from AP alone. Scale bar = 10 μm (B), 100 μm (E,G,I), 500 μm (F,H,J).

DOI: https://doi.org/10.7554/eLife.42143.010
Figure 6. Dystroglycan:Celsr3 interactions are required for spinal commissural axon guidance. (A) Western blotting of brain lysates from Celsr3<sup>R1548Q/R1548Q</sup> mutants and wildtype littermates show no difference in size or expression level of Celsr3 or Celsr1 protein. Brain lysate from Celsr3<sup>-/-</sup> mutants is
include as a control for antibody specificity. (B, C). In Celsr3<sup>WT/R1548Q</sup> heterozygous controls (B), Dil labeling of open book preparations shows that commissural axons extend through the floor plate, then execute an anterior turn in 86.5 ± 2.52% of injection sites (n = 7 animals, 49 total injection sites). In contrast, only 22.32 ± 6.35% of injection sites in Celsr3<sup>R1548Q/R1548Q</sup> mutants (n = 6 animals, 48 total injection sites) (C) show normal anterior turning, with the remaining 77.68% exhibiting AP randomization after crossing the floor plate, similar to Dag1<sup>F/−</sup>;Sox2<sup>C−/−</sup>, Ispdc<sup>799V/L799</sup>, and Celsr3<sup>C−/−</sup> mice. Higher magnification insets for each image show the anterior (top) and posterior (bottom) trajectories of post-crossing commissural axons. (D) Quantification of open book preparations, *p<0.001, Student’s T-test. (E–J) L1 immunohistochemistry (E, H) and Dil labeling of thalamocortical (F, I) and corticothalamic (G, J) axons show no defects in internal capsule formation in Celsr3<sup>R1548Q</sup> mutants. High magnification insets show Dil-labeled thalamocortical axons extending into the intermediate zone of the cortex (F′, I′) and Dil-labeled corticothalamic axons entering the thalamus (G′, J′). (K) Proposed model for Dystroglycan:Celsr3 interactions in guiding commissural axons. Scale bar = 50 μm (B, C), 500 μm (E–J). DOI: https://doi.org/10.7554/eLife.42143.011
Figure 6—figure supplement 1. Analysis of Celsr\textsuperscript{R1548Q} mutants. (A) Evolutionary conservation of Celsr3-LG1 region that forms a putative binding interface with Dystroglycan. The conserved Arginine (blue), Aspartic Acid (red) and Glycine (purple) residues critical for LG domain binding are

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\caption{Analysis of Celsr\textsuperscript{R1548Q} mutants. (A) Evolutionary conservation of Celsr3-LG1 region that forms a putative binding interface with Dystroglycan. The conserved Arginine (blue), Aspartic Acid (red) and Glycine (purple) residues critical for LG domain binding are}
\end{figure}

CAG(Q)

M. musculus SGLLFYNGRLNEKHDPLALELVAQVRLTYSTGES
H. sapiens SGLLFYNGRLNEKHDPLALELVAQVRLTYSTGES
D. rerio NGLLFYNGRNEKHLPEAILDGQKLYSTGES
D. melanogaster NGLLLYNRGYELHIPEAFIELHSGVSFSFSLGDH
C. elegans NGVLVFRTG--DKRSPFVEVSVDRLQKVSLEDGE

CELSR3: GGGCTACTCTTCTACAACGGGCCTGAACGAGAAGCATGAC
GLFLYNGRLNEKHLPEAILDGQKLYSTGES

A

B

C

WT HET MUT

Celsr\textsuperscript{R1548Q/WT} Celsr\textsuperscript{R1548Q} L1

L1/Robo1+Robo2

Robo1+Robo2

D

E

D'

E'

D''

E''

Figure 6—figure supplement 1 continued on next page
highlighted. (B) Schematic of the Celsr3 nucleotide and amino acid sequence highlighting the specific sequence targeted to generate CelsrR1548Q knock-in mice. (C) Genotyping of wildtype, CelsrR1548Q/+ , and CelsrR1548Q/R1548Q mutants. (D–E) L1, Robo1 and Robo2 antibodies were used to label commissural axons in E12 spinal cord sections from CelsrR1548Q/+ (D–D") and CelsrR1548Q/R1548Q (E–E"), mutants. Post-crossing axons appear normal in CelsrR1548Q/R1548Q mutants. Scale bar = 100 μm (D,E).

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