Reelin is required for class-specific retinogeniculate targeting

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Abstract

Development of visual system circuitry requires the formation of precise synaptic connections between neurons in the retina and brain. For example, retinal ganglion cells (RGCs) form synapses onto neurons within subnuclei of the lateral geniculate nucleus (LGN) – i.e. the dorsal LGN (dLGN), ventral LGN (vLGN) and intergeniculate leaflet (IGL). Distinct classes of RGCs project to these subnuclei: the dLGN is innervated by image-forming RGCs, while the vLGN and IGL are innervated by non-image-forming RGCs. To explore potential mechanisms regulating class-specific LGN targeting we sought to identify differentially expressed targeting molecules in these LGN subnuclei. One candidate targeting molecule enriched in the vLGN and IGL during retinogeniculate circuit formation was the extracellular matrix molecule reelin. Anterograde labeling of RGC axons in mutant mice lacking functional reelin (relnrl/rl) revealed reduced patterns of vLGN and IGL innervation and misrouted RGC axons in adjacent non-retino-recipient thalamic nuclei. Using genetic reporter mice, we further demonstrated that mistargeted axons were from non-image-forming, intrinsically-photosensitive RGCs (ipRGCs). In contrast to mistargeted ipRGC axons, axons arising from image-forming RGCs and layer VI cortical neurons correctly targeted the dLGN in relnrl/rl mutants. Taken together, these data reveal reelin is essential for the targeting of LGN subnuclei by functionally distinct classes of RGCs.

Keywords

synaptic targeting; axon guidance; retinal ganglion cell; thalamus; extracellular matrix; melanopsin

Introduction

Retinal ganglion cells (RGCs), the output neurons of the retina, project axons to over twenty distinct CNS nuclei. Due to the complexity of visual information conveyed by retinal inputs (eg. color, contrast, light intensity and object movement) RGC axons are targeted to domains within retino-recipient nuclei by at least three distinct mechanisms. The best characterized of these targeting mechanisms involves the sorting of RGC axon terminals into topographically arranged maps within a retino-recipient nucleus. Such maps convey the position of information in the visual field to a spatially correlated region of a retina-recipient nucleus. Wnts, BMPs, ephrins/Ephs, ALCAM, and L1 each have well documented roles in the targeting of RGC axons into these retinotopic maps in mouse (Demyanenko and Maness...
In addition to being retinotopically mapped, retinal inputs are sorted into eye-specific domains within retino-recipient nuclei (Muscat et al. 2003; Huberman et al. 2008a; Sanes and Yamagata 2009). In mouse lateral geniculate nucleus (LGN), ephrins/Ephs and teneurins contribute to the initial patterning of eye-specific retinal input (Pfeiffenberger et al. 2005; Leamey et al. 2007; Rebsam et al. 2009). Subsequently, MHC1, C1q, and neuronal pentraxins – in combination with neuronal activity – contribute to the refinement of these inputs into non-overlapping eye-specific domains (Huh et al. 2000; Bjartmar et al. 2006; Stevens et al. 2007; Datwani et al. 2009; Feller 2009).

Lastly, RGCs are grouped into functional and morphological classes and axons from each class target specific retino-recipient nuclei or specific domains within retino-recipient nuclei (Volgyi et al. 2009; Masland 2001; Sanes and Zipursky 2010). While projections patterns of several classes of mouse RGCs have recently been reported (eg. Hattar et al. 2006; Kim et al. 2008, 2010, Huberman et al. 2008b, 2009), the mechanisms regulating class-specific targeting remain unclear. To address this issue, we sought to identify molecular cues responsible for class-specific targeting of RGC axons in mouse LGN – a retino-recipient nucleus where class-specific targeting is most evident. The LGN is comprised of 3 functionally distinct subnuclei – the dorsal LGN (dLGN), the ventral LGN (vLGN) and the intergeniculate leaflet (IGL). While the dLGN receives image-processing retinal input (Hale and Sefton 1978; Luth et al. 1993; Kim et al. 2008, Huberman et al. 2008b, 2009), the vLGN and IGL receive non-image forming input from intrinsically photosensitive RGCs (ipRGCs) (Morin et al. 2003; Hattar et al. 2002, 2006)(Figure 1A,B). By screening for differentially expressed targeting cues within these LGN subnuclei we identified several candidates that may contribute to class-specific retinogeniculate targeting. Here, we provide compelling evidence that one candidate – the extracellular matrix (ECM) molecule reelin – is essential for LGN subnuclei-specific targeting by ipRGC axons, but not by axons originating from image-forming RGCs.

**Materials and methods**

**Antibodies**

A rabbit polyclonal antibody direct against melanopsin was kindly provided by Dr. C.K.Chen (VCU; diluted 1:1000 for whole-mounts and 1:2500 for cryosections). A rabbit polyclonal antibody directed against β-galactosidase (LacZ) was kindly provided by Dr. J.R.Sanes (Harvard University; diluted 1:500). Antibodies for the following antigens were purchased; rabbit anti-calretinin (Millipore; diluted 1:1000); rabbit anti-VGLUT2 (Synaptic Systems; diluted 1:500); rabbit anti-GFP (Invitrogen; diluted 1:250); mouse anti-reelin (Abcam, Inc.; diluted 1:1000); rabbit anti-NPY (ImmunoStar Inc.; diluted 1:500); mouse anti-NeuN (Millipore; 1:200); rabbit anti-Gad65/67 (Chemicon; 1:500); mouse anti-SMI32 (Covance Inc.; 1:500); rabbit anti-Dab1 (Abnova Inc.; diluted 1:500). Fluorescently conjugated secondary antibodies were purchased from Invitrogen or Jackson Immunoresearch (diluted 1:1000).

**Mice**

Wild-type C57 and CD1 mice were from Charles River Inc. Reeler mutant mice (reln<sup>rl/rl</sup>) and scrambler mutants (dab<sup>1scm/scm</sup>) were obtained from Jackson Laboratories. The generation of opn4-tau-LacZ and golli-tau-GFP mice were described previously (Hattar et al. 2002; Jacobs et al. 2007). Genomic DNA was isolated and genotyping performed as previously described (Su et al. 2010). The following primer pairs were used: reln-TTA ATC
TGT CCT CAC TCT GCC CTC T and GCA GAC TCT CTT ATT GTC TCT AC; mutant retn- TTA ATC TGT CCT CAC TCT GCC CTC T and TTC CTC TCT TGC ATC CTG TTT TG (Yamamoto et al. 2003); lacZ-TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA and ATG TGA GCG AGT AAC AAC CGG TCG GAT TCT; gfp- AAG TTC ATC TGC ACC ACC G and TTC TTG AAG AAG ATG GTG CG. Scrambler mutants were identified by their ataxic gait or their malformed CNS. All analyses conformed to NIH guidelines and protocols approved by the VCU Institutional Animal Care and Use Committee.

**Immunohistochemistry (IHC)**

IHC was performed on 16 µm coronal cryo-sectioned tissues as described previously (Su et al. 2010) or on whole-mount retinal preparations. For whole-mount staining, retinas were dissected and post-fixed in 4% paraformaldehyde (PFA) in PBS. Retinas were washed, incubated in blocking buffer (2.5% bovine serum albumin [BSA], 5% normal goat serum [NGS]), 0.1% tritonX100 in PBS), and subsequently incubated with antibodies diluted in blocking buffer for 24–48 hours at 4°C. Following washing in PBS and incubation with secondary antibodies (diluted in blocking buffer), retinas were mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) with VectaSheild (Vector Laboratories, Burlingame, CA). Co-labeling with antibodies from the same host species was performed with a Zenon labeling kit (Invitrogen). Images were acquired on a Zeiss Axiolmager A1 fluorescent microscope or a Leica SP2 confocal microscope. When comparing different ages of tissues or between genotypes, images were acquired with identical parameters. For quantifying numbers of ipRGCs in retinal whole-mounts 5 to 10 random images (875 µm × 650 µm) were acquired on a Zeiss Axiolmager A1 from control or retnrl/rl mutant retinas. All melanopsin-expressing ipRGCs in these images were counted manually. A total of 11 control retinas and 12 mutants retinas were analyzed. For quantifying the spatial extent of M1 ipRGC arborization into mutant (retnrl/rl and dab1scm/scm) dLGN, images of LacZ-immunoreactivity were acquired from P21 opn4-tau-lacZ+/+ mice, retnrl/rl;opn4-tau-lacZ+/+ mutants, and dab1scm/scm;opn4-tau-lacZ+/− mice. The total area of dLGN and the area of dLGN containing LacZ-immunoreactivity were measured with AxioVision software (Carl Zeiss Imaging Systems, Thornwood, NY). A minimum of 3 animals (per genotype and per age) were compared in all IHC experiments.

**In situ hybridization (ISH)**

ISH was performed on 10–16 µm coronal cryosectioned tissues as described previously (Su et al. 2010). The generation of syt1 and syt2 riboprobes was previously described (Fox and Sanes 2007). Using a similar protocol riboprobes were generated from adams15, vldlr, and apoer2 IMAGE clones (Clone IDs 30619053, 3968213, 40109899, respectively) (OpenBiosystems Inc.; Huntsville, Al). A minimum of 3 animals per genotype and age were compared in ISH experiments.

**Microarray analysis**

LGN subnuclei were isolated from postnatal day 3 (P3) vLGN and IGL (vLGN/IGL) or dLGN. Mice were decapitated, brains were removed and 300 µm coronal sections were cut in ice-cold DEPC-PBS with a vibratome. vLGN/IGL or dLGN were micro-dissected and tissues from at least 5 littersmates were pooled per sample. RNA was isolated using the BioRad Total RNA Extraction from Fibrous and Fatty Tissue kit (BioRad, Hercules, CA). RNA purity assessment, first and second strand cDNAs preparation, cRNAs generation, hybridization to Agilent Whole Genome 44k×4 mouse arrays, and data analysis with Agilent Feature extraction and GeneSpring GX v7.3.1 software packages were performed by GenUs Biosystems (Northbrook, IL). To be considered differentially expressed genes must have
been 2-fold higher in the averaged sample sets (n=3, p<0.05). 3 samples were analyzed per region.

Quantitative PCR (qPCR)

RNA was purified from pooled samples isolated from P3, P6, P8, P10, and P14 vLGN/IGL or dLGN as described above. cDNAs were generated with Superscript II Reverse Transcriptase First Strand cDNA Synthesis kit (Invitrogen, La Jolla, CA). qPCR was performed on a Chromo 4 Four Color Real-time system (BioRad) using iQ SYBRGreen Supermix (BioRad) as described previously (Su et al. 2010). The following primer pairs were used: actin – TTC TTT GCA GCT CCT TCG TT and ATG GAG GGG AAT ACA GCC C; reln – CTT CTC AGA GCA TTG GAG GC and ACA TGA GAG GCC ACC ACA CT; slit2 – TTC AGT TGT TTC CTG AGC TGC and CCT TCC TTG GAA TTG CTT GA; thbs4 – AAT TCA CTG TGA TGG GAC GG and CAG CCA GCT GCA AGT TGT T; sema3c – TGT ACG AGG ATC TTC CCA GC and CTG CTG GTG GGA CAG ACT AA. A minimum of 4 experiments (each in triplicate) was run for each gene, at each age examined. Each individual run on the Chromo 4 Four Color Real-time system included separate actin controls.

Intraocular injections of anterograde tracers

Intraocular injection of cholera toxin subunit B (CTB) conjugated to AlexaFluor488 or AlexaFluor 594 (Invitrogen) was performed as described previously (Jaubert-Miazza et al. 2005). After 1–2 days, mice were euthanized and brains fixed in 4% paraformaldehyde. 80–100µm coronal sections were sectioned on a vibratome and mounted in ProLong Gold (Invitrogen). Retinal projections were analyzed from at least 5 animals for each age and genotype. Images were acquired on a Leica SP2 confocal microscope. To quantify the spatial extent of vLGN and IGL innervation by retinal axons, serial coronal sections encompassing the entire LGN (~14–18 80 µm sections) were obtained and imaged from 6 P12 reln<sup>rl/rl</sup> mutants and 6 littermate controls (for example see serial sections shown in Supplemental Figure S4). Measurements of the entire LGN area and the area of retinal innervation to vLGN and IGL in mutants and controls were obtained using AxioVision software.

Pupillary light reflexes (PLRs)

After 1 hour of dark adaptation, mice (n=3 per genotype) were restrained and one eye monitored under infrared light with a Sony DCR-HC96 camera. PLRs were evoked by 30 seconds of high intensity light (1.7mW/cm<sup>2</sup>) from a 473 nm light-emitting diode. Video frames were captured for 20 seconds prior to the application of light and during the 30-second burst of low intensity light. Pupil size was measured from video images prior to the onset of light and at the end of the 30-second burst of light.

Results

Identification of nuclei-specific candidate targeting cues

To address how functionally distinct classes of RGC axons target distinct domains within retino-recipient nuclei, we sought to identify the mechanisms regulating class-specific targeting of the mouse LGN – a thalamic nucleus in which classes of image-forming and non-image-forming retinal inputs are segregated into distinct subnuclei. The segregation of class-specific retinal inputs appeared to occur as retinal axons first enter the LGN (Supplemental Figure S1; see also Kim et al. 2010), suggesting that molecular cues within LGN subnuclei pattern class-specific retinogeniculate axon targeting. Since there is a paucity of information regarding the factors regulating class-specific targeting of axons in...
LGN subnuclei, we screened differentially expressed guidance and targeting molecules in either vLGN and IGL (vLGN/IGL) or dLGN. vLGN and IGL were pooled since they receive innervation from similar classes of RGCs (Hattar et al. 2006). RNA was isolated from LGN subnuclei at ages corresponding to the arrival of retinal axons (i.e. P3) (Godement et al. 1984; Jaubert-Miazza et al. 2005) and differentially expressed genes were identified by Agilent microarrays. Numerous genes coding for extracellular matrix molecules and soluble morphogens known to guide axonal growth and targeting were identified as being over two-fold enriched in either vLGN/IGL or dLGN (Figure 1C). Furthermore, microarray analysis confirmed the differential expression of known transcription factors in either vLGN or dLGN and identified a novel set of LGN subnuclei-specific transcription factors (Supplemental Figure S2) (Nakagawa and O’Leary 2001; Jones and Rubenstein 2004).

In the present study, we focus on molecular cues enriched in and necessary for retinal innervation of vLGN/IGL since all retinogeniculate axons encounter these subnuclei (Figure 1A,B). To narrow our list of vLGN/IGL-enriched targeting genes to those likely to contribute specifically to retinogeniculate targeting, we examined the developmental expression of candidate genes at ages corresponding to RGC axon arrival (i.e. P3), arborization and refinement (P6–14) (Jaubert-Miazza et al 2005). The peak expression of one gene – *relin* – coincided with the arrival of RGC axons (Figure 1D). *Reln* encodes the large glycoprotein reelin, which has previously been shown to contribute to both the regulation of neuronal migration (D’Arcangelo et al. 1995) and axonal growth and targeting (Borrell et al. 1999, 2007; Wu et al. 2008). It is noteworthy that microarray analysis not only revealed a ~7 fold enrichment in *relin* mRNA in vLGN/IGL (compared with dLGN), but also identified an alternatively spliced form of *relin* mRNA that was >6 fold enriched in the more ventral LGN subnuclei (data not shown). At P3, *relin* expression (at both the mRNA and protein level) was highly enriched in vLGN/IGL compared with the dLGN and other retino-recipient nuclei, including the olivary pretectal nucleus (OPN) and suprachiasmatic nucleus (SCN) (Figure 1D–F and S3). The specificity of reelin-IHC at these ages was confirmed by demonstrating a lack of immunoreactivity in LGN of mutant mice lacking reelin (i.e. *relin* /mutants) (D’Arcangelo et al. 1995) (Supplemental Figure S3). In contrast to its high level of expression from P0 to P3, reelin expression in the vLGN/IGL was markedly decreased when RGC axons were no longer entering the LGN (Figure 1D,G). Based upon its developmental expression and previously documented functions, reelin was a prime candidate to contribute to class-specific targeting of RGC axons in the LGN.

Most functions of reelin can be attributed to its binding of very low density lipoprotein receptor (VLDLR) or apo-lipoprotein E receptor 2 (ApoER2) (Benhanyon et al. 2003). For reelin to directly influence retinogeniculate targeting, we hypothesized that classes of RGCs must express reelin receptors. RGCs reside within the inner most layer of the retina, the ganglion cell layer (GCL) (Figure 2A). *In situ* hybridization revealed that a small subset of cells within this layer expressed mRNAs encoding either VLDLR or ApoER2 (Figure 2B,C).

Following binding to VLDLR or ApoER2, reelin induces the activation of disabled-1 (Dab1), a cytoplasmic molecule essential for reelin function (Howell et al. 1997; Sheldon et al. 1997). To assess whether Dab1 was also expressed in subsets of RGCs, IHC was performed on P13 wild-type retinal cross-sections. It is noteworthy that previous reports have documented Dab1 expression in AII amacrine cells within mouse retina (Rice and Curran 2000; Rice et al. 2001). Our analysis confirmed expression of Dab1 in amacrine cells within the inner nuclear layer (INL) (arrows in Figure 2D,E’), but also revealed a small subset of cells that expressed Dab1 within the GCL (arrowhead in Figure 2D,E’). To determine whether these Dab1-expressing cells were RGCs whose axons target vLGN and IGL, i.e. intrinsically-photosensitive RGCs (ipRGCs), we co-labeled retinal sections for
Dab1 and melanopsin (Meln), the functional photo-pigment expressed by ipRGCs. Indeed, these analyses showed that some ipRGCs expressed Dab1 (Figure 2E).

As previous reports also suggested that Dab1 and reelin were expressed by mutually exclusive cell types within the retina (Rice and Curran 2000; Rice et al. 2001), we next examined reelin expression in the GCL of P14 wild-type retina. Reelin expression was detected in many, but not all, cells within the GCL (Figure 2F). IHC for both reelin and Meln demonstrated that most ipRGCs lacked significant reelin expression (87.8% ± 3.2% [SD] ipRGCs lacked reelin; N=3 mice, n=452 ipRGCs)(Figure 2G,H). Melanopsin-IHC labeled several classes of ipRGCs (Figure 2G,H; see Ecker et al. 2010). Classes of ipRGCs whose dendrites stratified in the OFF-sublaminae of the inner plexiform layer (IPL)(Figure 2G) and those whose dendrites stratified in the ON- sublaminae of the IPL (Figure 2H) both lacked reelin expression. To specifically address reelin expression in M1 ipRGCs, a class of ipRGC that projects to the vLGN and IGL (Hattar et al. 2006), reelin-IHC was performed on retinal sections from transgenic mice in which M1 ipRGCs were selectively labeled with LacZ (opn4-tau-LacZ mice; Hattar et al. 2002, 2006). Nearly all M1 ipRGCs lacked significant reelin expression (97.9% ± 0.2 [SD] M1 ipRGCs lacked reelin expression; N=2 mice, n=194 RGCs)(Figure 2I). Therefore, our data show that classes of ipRGCs express Dab1 but not reelin. Together, these studies reveal that some RGCs have the machinery to respond to LGN-derived reelin.

Reelin and its canonical signaling pathway are necessary for RGC targeting of the vLGN and IGL—To assess the role of reelin in retinogeniculate targeting, we analyzed retinal projections in reln$^{rl/rl}$ mutants. Intraocular injections of fluorescently conjugated cholera toxin subunit B (CTB) were used to label all RGC axons (Figure 3A)(Muscat et al. 2003; Jaubert-Miazza et al. 2005). Several striking defects were observed in RGC projections in reln$^{rl/rl}$ mutants. First, the spatial extent of retinal innervation to IGL and vLGN, but not dLGN, was markedly reduced in mutants (45.1% ± 6.5% [SEM] reduced extent of innervation in reln$^{rl/rl}$ IGL, n=6 mice, p<0.05 by Student’s t-test; 16.4% ± 3.4% [SEM] reduced extent of innervation in reln$^{rl/rl}$ vLGN, n=6 mice, p<0.05 by Student’s t-test) (Figure 3A,B and Supplemental Figure S4). Second, numerous misrouted RGC axons were observed in non-retino-recipient thalamic nuclei adjacent to LGN (Figure 3B,D). These misrouted axons originated from both contra- and ipsi-lateral eyes and exited mutant LGN at either the IGL-dLGN border (Figure 3B,D) or from the vLGN (data not shown). Reduced innervation and misrouting of retinal axons were observed as early as P1 (Figure 3E,F), suggesting that reelin was necessary for the initial targeting of retinal axons to the vLGN and IGL.

To determine whether reelin required Dab1 function for its role in retinogeniculate targeting we examined retinal innervation of the LGN in scrambler mutants (dab1$^{scm/scm}$), which lack functional Dab1 (Howell et al. 1997; Sheldon et al. 1997). In all dab1$^{scm/scm}$ mutants examined (n=10) misrouted RGC axons were observed exiting the LGN medially to enter non-retino-recipient thalamic nuclei (Figure 4A–C). In contrast to reln$^{rl/rl}$ mutants, however, only 50% of dab1$^{scm/scm}$ mutants displayed sparse retinal arbors in the IGL (Figure 4B). In mutants where a striking avoidance of the IGL was not observed, retinal arbors in the mutant IGL appeared disorganized compared with controls (Figure 4A,C). As was observed in reln$^{rl/rl}$ mutants, defects in the pattern of IGL innervation were observed as early as P2 (Figure 4D–F). Taken together these findings suggest that reelin signals, at least in part, through Dab1 to pattern the initial formation of retinogeniculate circuits.

In addition to its role in regulating axonal growth and targeting, the reelin-Dab1 signaling pathway has well documented roles in neuronal migration and lamination (D’Arcangelo et al. 1995; Howell et al. 1997; Sheldon et al. 1997). In contrast to other regions of the reln$^{rl/rl}$...
mutant brain, few significant alterations in the cytoarchitecture of mutant LGN have been documented. Our analysis confirmed a relatively normal distribution of neurons (by NeuN IHC) in mutant LGN (Figure 5A,B). A reduction in the size of the LGN in retn<sup>rl/rl</sup> mutants was observed, but this difference was not restricted to the LGN and was presumably due to mutants being considerably smaller than littermate controls (Falconer 1951). One defect that has been reported in retn<sup>rl/rl</sup> mutant LGN, is the presence of aberrant axonal tracts coursing through mutant dLGN (Frost et al. 1986). Axon tracing studies revealed that such misrouted axon tracts did not arise from the retinal axons but instead were formed by corticothalamic axons originating from layer VI cortical neurons (Figure S5). The location of these corticothalamic axons in dLGN and the non-retino-recipient portion of vLGN suggest they are unlikely to contribute to the mis-targeting of retinal axons in retn<sup>rl/rl</sup> mutant LGN.

We next explored whether defects in retinogeniculate targeting resulted indirectly from an abnormal formation of the three LGN subnuclei in absence of reelin. LGN subnuclei are each derived from different embryonic origins (Altman and Bayer 1989; Botchkina and Morin 1995), therefore to examine whether each were properly generated in the absence of functional reelin we assayed the distribution of several known subnuclei-specific markers in retn<sup>rl/rl</sup> mutant LGN. The IGL, but not the adjacent vLGN or dLGN, contain an abundance of neuropeptide Y (NPY)-expressing relay neurons, as well as glial fibrillary acidic protein (GFAP)-expressing astrocytes (Botchkina and Morin 1995)(Figure 5A,C). Many interneurons and projection neurons within the vLGN and IGL express glutamate decarboxylase 65 (Gad65)(Moore and Speh 1993; Morin et al. 1992)(Figure 5E). Relay neurons within the vLGN and dLGN, but not IGL, contain an isoform of neurofilament recognized by the antibody SMI32 (Figure 5I)(see also Jaubert-Miazza et al. 2005). In addition to these known markers, we identified two novel subnuclei-specific markers. Neurons within the vLGN, but not the IGL or dLGN, express synaptotagmin 2 (syt2) mRNA (Figure 5G), whereas, only neurons within the dLGN express ‘a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 15’ (adamts15) mRNA (Figure 3K). Immunostaining and in situ hybridization for each of these markers revealed that the 3 LGN subnuclei form appropriately and remain distinct in retn<sup>rl/rl</sup> mutants (Figure 5). Importantly, NPY-expressing IGL relay neurons, which are the targets of RGC axons (Thankachan and Rusak 2005), remain confined to the IGL and were not dispersed into inappropriate thalamic regions (Figure 5A,B,K,L). Together, these data suggest that misrouted axons in retn<sup>rl/rl</sup> mutants are in fact mistargeted rather than correctly targeted to neurons that have migrated into inappropriate regions of thalamus.

Reelin is required for the targeting of ipRGCs in the LGN

Defects in the pattern of retinal innervation to retn<sup>rl/rl</sup> vLGN and IGL led us to hypothesize that reelin was essential for retinogeniculate targeting by ipRGCs. To ensure that defects in vLGN and IGL targeting were not consequences of intra-retinal defects we examined the distribution and morphology of ipRGCs in retn<sup>rl/rl</sup> mutant retina. Whole-mount Meln-IHC revealed that the distribution and morphology of ipRGCs were indistinguishable between mutant or control retinas (Figure 6A,B and Supplemental Figure S5A). Additionally, numbers of Meln-immunoreactive ipRGCs appeared similar in whole-mount preparations of P12 mutant and control retinas (controls contained 42.4 ± 7.9 [SD] ipRGCs per field, N=11 retinas, n=86 fields; mutants contained 41.3 ± 9.6 [SD] ipRGCs per field, N=12 retinas, n=101 fields). Importantly, the observed defects in retinogeniculate targeting were also not due to an inability of mutant ipRGCs to endocytose or anterogradely transport CTB (Figure 4A',B',C–F). Thus, defects in RGC innervation of vLGN and IGL did not appear to arise from intraretinal defects in ipRGCs.

We also assessed whether ipRGC axons correctly targeted other appropriate retino-recipient nuclei in retn<sup>rl/rl</sup> mutants. Classes of ipRGCs innervate the OPN and SCN (Muscat et al.
CTB labeling in re\textsuperscript{rl/rl} mutants revealed normal patterns of retinal innervation to these nuclei (Figure 6C–F). Furthermore, re\textsuperscript{rl/rl} mutants retained rudimentary pupillary light reflexes (PLRs), behaviors that depend upon ipRGC innervation of the OPN (Figure 6G,H)(Guler et al. 2008). Therefore, in the absence of reelin ipRGCs were not only morphologically normal, but generated axons capable of targeting some appropriate retino-recipient nuclei and retained rudimentary function.

To directly assess whether ipRGC axons were misrouted in the re\textsuperscript{rl/rl} LGN, mutants were crossed with opn\textsuperscript{4-tau-LacZ} mice, since M1 ipRGCs are labeled in this line and M1 ipRGCs densely innervate vLGN and IGL (Figure 7A)(Hattar et al. 2006; Ecker et al. 2010). In P11 re\textsuperscript{rl/rl};opn\textsuperscript{4-tau-LacZ} mutants several defects were observed in the pattern of ipRGC projections to vLGN and IGL. First, M1 ipRGC projections avoided central regions of mutant IGL and instead formed a dense, narrow network of fibers adjacent to the ventral aspect of dLGN (Figure 7B). Second, a large cohort of ipRGC axons invaded ventro-medial regions of dLGN (see arrows in Figures 7B’,D’). The area of dLGN occupied by misrouted M1 ipRGC axons were measured in P21 mutants and controls. While few M1 ipRGC arbors were observed in control dLGN, nearly 20% of the area of the re\textsuperscript{rl/rl} mutant dLGN contained LacZ-expressing ipRGC arbors (2.9% ± 1.1% [SEM] of the area of control dLGN contained M1 ipRGC arbors versus 19.2% ± 2.9% [SEM] in re\textsuperscript{rl/rl} mutant dLGN; N=6, p<0.05 by Student’s t-test). Lastly, the majority of retinal axons misrouted out of the IGL or vLGN contained LacZ, revealing they originated from M1 ipRGCs (Figure 7BC).

We next examined the role of Dab1 in the correct targeting of M1 ipRGC axons. Scrambler mutants (dab\textsuperscript{1scm/scm}) were crossed to the opn\textsuperscript{4-tau-LacZ} mice described above. In the absence of Dab1, M1 ipRGCs invaded non-retino-recipient thalamic regions and occupied a statistically significant area of mutant dLGN (13.0% ± 1.5% [SEM] of dab\textsuperscript{1scm/scm} mutant dLGN contained M1 ipRGC arbors, N=6, p<0.05 by Student’s t-test). Analyses in both re\textsuperscript{rl/rl};opn\textsuperscript{4-tau-LacZ} and in dab\textsuperscript{1scm/scm};opn\textsuperscript{4-tau-LacZ} mice also demonstrated that M1 ipRGC projections to SCN and OPN were unaffected by the absence of functional reelin (Figure S6 and data not shown)(see Hattar et al. 2006; Ecker et al. 2010). These findings identify, for the first time, that at least one class of non-image-forming retinal axons require reelin and Dab1 for targeting appropriate LGN subnuclei.

Reelin is not required for subnuclei-specific targeting of dLGN-projecting retinal axons

We next tested whether image-forming classes of RGCs require reelin for appropriately targeting dLGN. For this we used three approaches – labeling retinal terminals, labeling dLGN-projecting axons and co-labeling misrouted retinal axons with CTB and cell-specific markers of dLGN-projecting axons. To label axon terminals from image-forming RGCs we examined the distribution of vesicular glutamate transporter 2 (VGLUT2), a synaptic component enriched in dLGN-projecting retinal axons (Figure 8A; Land et al. 2004). In re\textsuperscript{rl/rl} mutant LGN the density and distribution of VGLUT2-immunoreactivity appeared remarkably similar to controls, suggesting that image-forming, dLGN-projecting RGC axons were not misrouted into vLGN or IGL in the absence of functional reelin (Figure 8B). Second, calretinin-expressing dLGN-projecting retinal axons (Luth et al. 1993; Huberman et al 2008b) were labeled in mutants and controls by IHC. As in control LGN, calretinin-containing retinal axons were excluded from vLGN and IGL in re\textsuperscript{rl/rl} mutant LGN (Figure 8C,D). VGLUT2- or calretinin-immunoreactivity alone could not reveal whether axons misrouted into non-retino-recipient thalamic nuclei in re\textsuperscript{rl/rl} mutants originated from image-forming classes of RGC. To test whether any mistargeted RGC axons belonged to image-forming classes of RGCs, axons in re\textsuperscript{rl/rl} mutant thalamus were co-labeled by calretinin-IHC and CTB. No misrouted CTB-labeled RGC axons were observed to contain calretinin in mutant thalamus (Figure 8E,F). Together, these experiments demonstrate that
classes of image-forming RGC axons correctly target dLGN in the absence of functional reelin.

Reelin is not required for subnuclei-specific targeting of layer VI corticothalamic axons

In addition to retinal axons, cortical axons differentially target LGN subnuclei (Jacobs et al. 2007). Cortical neurons in layer VI project axons that course through the internal capsule and around vLGN and IGL to selectively innervate dLGN (Figure 9A). Despite coursing around vLGN and IGL, these corticothalamic axons do not enter or arborize in ventral LGN subnuclei. To address whether cortical axons required reelin for normal LGN targeting, reln<sup>rl/rl</sup> mutants were crossed to golli-tau-GFP transgenic mice, in which layer VI neurons were selectively labeled with GFP (Jacobs et al. 2007). Corticothalamic axons from layer VI neurons avoided vLGN and IGL and selectively innervate dLGN in controls (Figure 9B,D). As expected, disruptions in cortical layering were observed in reln<sup>rl/rl</sup>;golli-tau-GFP mutant mice (Figure 9C). As described above, axons from layer VI cortical neurons also displayed fasciculation defects and took altered courses through the internal division of the vLGN, a region that does not receive retinal input (Harrington 1997). Yet despite defects in neuronal position and axonal trajectory, layer VI corticothalamic axons correctly avoided the IGL and retino-recipient portion of the vLGN and arborized only in the dLGN of reln<sup>rl/rl</sup>;golli-tau-GFP mutants (Figure 9E). We draw two important conclusions from these findings: neither reelin, nor neuronal position, are critical determinants for layer VI corticothalamic axons to correctly avoid vLGN and IGL and specifically target dLGN.

Discussion

Identifying the mechanisms that underlie class-specific targeting of axons to appropriate regions of the brain is critical for our understanding of how complex neural circuits -- and their associated behaviors -- are established. While the visual system has long been a useful model system in studying the initial establishment and subsequent refinement of neural circuits, the vast number of RGCs and the difficulty in labeling individual classes of RGCs have hampered investigations aimed at elucidating the mechanisms of class-specific targeting. Here, rather than focusing on identifying the mechanisms that drive the targeting of a single class of RGCs, we focused on identifying cues that may drive the segregation of RGC axons that convey either image-forming visual input or non-image forming visual input. In doing so, we identified a plethora of candidate targeting molecules differentially expressed in LGN subnuclei. Analysis of one of these cues – reelin – revealed for the first time a molecule necessary for class-specific retinogeniculate targeting.

Differential expression of targeting cues in LGN subnuclei

To elucidate some of the mechanisms contributing to class-specific axonal targeting, we examined the development of specific connections between functionally distinct classes of RGCs and different LGN subnuclei. Two broad mechanisms have been proposed to generate such specificity: 1) Class-specific retinal arbors could initially be diffusely spread throughout the entire LGN and then be refined to appropriate subnuclei based upon functional activity. 2) The initial projections of axons originating from a single class of RGCs could be programmed to innervate only appropriate target regions. To distinguish between these possibilities in the LGN, we examined the development of a set of class-specific retinal projections that express Calr and whose arbors are confined to the dLGN of adult rodents (Luth et al. 1993; Huberman et al. 2008b). As Calr-containing RGC axons initially entered the LGN, they bypassed the vLGN and IGL and arborized selectively in dLGN (Figure S1; see also Kim et al. 2010). This suggested that the targeting of class-specific retinal axons to LGN subnuclei was driven by specific targeting cues and not by activity-dependent refinement. By comparing mRNA transcripts in either vLGN/IGL or
dLGN we identified numerous families of molecules known to direct the growth, guidance and targeting of axons that were differentially expressed in LGN subnuclei. These included, but were not limited to, semaphorins, neurotrophins, growth factors, slits, Wnts, and ECM molecules (i.e. reelin, thrombospondin 4, collagen IX, and several members of ADAMTS family)(Hamel et al. 2008; Sanes and Yamagata 2009, Huber et al. 2003, Fox 2008, Huberman et al. 2010).

Based upon its high enrichment in perinatal vLGN/IGL (~7 fold enriched in vLGN/IGL compared to dLGN by microarray; Figure 1C) and its developmental regulation (Figure 1D–G), we focused here on the role of reelin in class-specific retinogeniculate targeting. Our results demonstrate that reelin and its canonical signaling pathway are critical for the targeting of ipRGC axons to the vLGN and IGL. However, other aspects of class-specific retinogeniculate (and corticothalamic) targeting were largely unaffected by the absence of functional reelin. For example, dLGN-projecting retinal and cortical axons did not require reelin to correctly avoid vLGN or IGL as they coursed around these regions. Moreover, based upon the abundance of RGC axons in \(reln^{ulk}\) mutant vLGN, it appears likely that classes of vLGN-projecting RGC axons exist that do not require reelin for targeting. Here we have demonstrated that M1 ipRGCs require reelin, but several other classes of ipRGCs exist that target vLGN (Ecker et al. 2010); it remains unclear whether these classes require reelin. Since reelin-independent aspects of class-specific retinogeniculate targeting exist, it is possible -- perhaps likely -- that other guidance and targeting cues identified in our screens may contribute to these aspects of retinogeniculate targeting. Not only have many of the identified families of candidate cues been demonstrated to contribute to axonal guidance and targeting, but many have known affects on RGC axons. Neurotrophins and FGFs affect the terminal branching and targeting of retinal axons (Inoue and Sanes 1997; Cohen-Cory and Fraser 1994,1995; McFarlane et al. 1995,1996; Webber et al. 2003, 2005). Wnts mediate medial-lateral topographic mapping of retinal projections to the chick tectum (Schmitt et al. 2006). Slits and semaphorins both inhibit or repel the growth of retinal axons (Steinbach et al. 2002; Niclou et al. 2000). Analysis of these molecules in class-specific targeting of the LGN clearly warrants future attention.

Reelin’s role in class-specific retinogeniculate targeting

In the present study we specifically explored what roles the large extracellular glycoprotein reelin may contribute to retinogeniculate targeting. Previous studies have defined roles for reelin in the targeting of specific classes of axons in the mammalian hippocampus. Layerspecific hippocampal targeting of axons from the entorhinal cortex (EC), but not targeting of commissural axons, requires target-derived reelin (Del Rio et al. 1997; Borrell et al. 1999). In vitro assays further demonstrated that such roles for reelin in entorhino-hippocampal connections are due, at least in part, to direct roles of reelin on neurons (Borrell et al. 2007). Classes of neurons that are responsive to reelin express Dab1 (Borrell et al. 2007) – an adaptor molecule that is central to the canonical reelin signaling cascade (Rice et al. 1998; Sheldon et al. 1997; Howell et al. 1997). Like reelin, Dab1 expression is essential for layer-specific entorhino-hippocampal connections (Borrell et al. 2007). However, since both reelin and Dab1 are necessary for cortical and hippocampal lamination (Sheldon et al. 1997; Howell et al. 1997), defects observed in hippocampal targeting in mutants lacking either reelin or Dab1 could arise from the correct targeting of cells that have misplaced. But, organotypic co-cultures of Dab1-deficient EC with wild-type hippocampi demonstrated that axons from EC neurons lacking Dab1 are indeed mistargeted regardless of the position of appropriate partner neurons (Borrell et al. 2007). Taken together, these studies demonstrate that reelin and its canonical signaling pathway are directly necessary for the formation of class-specific hippocampal connections. Likewise, it has also been reported that reelin and Dab1 are required for synaptic targeting in the retina (Rice et al. 2001).
On the basis of the above described studies and the spatio-temporal expression of reelin in the LGN, reelin initially appeared to be a prime candidate capable of influencing class-specific retinogeniculate targeting. Our data now provides compelling evidence supporting this: reelin is necessary for the targeting of at least one class of RGC axons (M1 ipRGC axons) to vLGN and IGL. In addition to those defects we report here for M1 ipRGCs, it is noteworthy that defects in the targeting of retinal axons to superior colliculus (SC) have also been described previously in relnrl/rl mutants (Frost et al. 1986; Baba et al. 2007). However, our data suggest that the mechanisms responsible for retinogeniculate and retinocollicular targeting defects are different. Defects in neuronal lamination precede the arrival of RGC axons in mutant SC (Baba et al. 2007), suggesting axonal targeting defects arise (at least in part) as a result of abnormal SC cytoarchitecture. In contrast, our data strongly suggest that defects in retinogeniculate targeting in relnrl/rl mutants do not arise from LGN disorganization or an absence of appropriate target neurons in IGL or vLGN. Based on the presence of mistargeted M1 ipRGC axons in mutant dLGN and non-retino-recipient thalamus -- regions lacking their appropriate target neurons (eg. NPY-expressing neurons) in both controls and mutants -- our data suggests that these ipRGC axons are indeed mistargeted and not merely targeting correct partners that are misplaced into inappropriate LGN subnuclei in the absence of functional reelin. In support of this hypothesis, we found that like EC neurons (Borrell et al. 2007) some ipRGCs express Dab1 and may therefore be capable of directly responding to LGN-derived reelin. We further discovered that similar to layer-specific entorhino-hippocampal connections, the formation of retinogeniculate connections required Dab1. As our findings strongly parallel those supporting reelin’s role in entorhino-hippocampal targeting, we suspect a more direct role for reelin in class-specific retinogeniculate targeting. Of course it remains possible that multiple mechanisms are responsible for the defects observed in the targeting of relnrl/rl LGN. This possibility may shed light on why misrouted RGC axons were observed in all of the dab1scm/scm mutant LGN examined, but only 50% of these exhibited a lack of RGC axons within the putative IGL.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We thank Dr. J.R. Sanes for comments on the manuscript and for providing anti-β-galactosidase antibodies. We thank Drs. S.Hattar and A.T.Campagnoni for providing opn4-tau-LacZ and golli-tau-GFP mice, respectively, and Dr. C.K.Chen for providing anti-melanopsin antibodies. This work was supported by the Thomas F. Jeffress and Kate Miller Jeffress Memorial Trust (M.A.F.) and an NIH/NEI grant (EY012716)(W.G.). Microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility supported, in part, with funding from NIH-NINDS Center Core grant (5P30NS047463-02).

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J Neurosci. Author manuscript; available in PMC 2012 January 12.


J Neurosci. Author manuscript; available in PMC 2012 January 12.


Figure 1. Reelin is selectively expressed in vLGN and IGL

A, B. Schematic representation of non-image-forming RGC (A) and image-forming RGC (B) axons in LGN subnuclei. C. Differentially expressed guidance and targeting cues in either vLGN/IGL or dLGN, displayed in a heat plot and normalized to the mean intensity of each gene. Color scale depicts fold difference between vLGN/IGL and dLGN. RNA was purified from 3 sets of vLGN/IGL and dLGN samples, each of which was analyzed by Agilent microarray and is represented by a column in the heat plot. Each samples contained tissue pooled from at least 3 mice. D. Developmental regulation of differential gene expression in vLGN/IGL compared with dLGN (± S.D.) assessed by qPCR. RNA was isolated from 5 ages. In contrast to other genes, the highest levels of reln mRNA enrichment in vLGN/IGL coincided with the arrival of RGC axons. E–G. Immunohistochemistry (IHC) for reelin on coronal sections of mouse thalamus. IHC confirmed the developmental regulation of reelin in vLGN and IGL during periods of retinogeniculate targeting. Dotted green and white lines outline vLGN and dLGN, respectively. Scale bar in G = 300 µm for E–G. d – dLGN; i – IGL; v – vLGN; OT – optic tract.
Figure 2. Reelin-signaling components are expressed in the GCL of mouse retina

A. ISH for synaptotagmin 1 (syt1) mRNA in P13 wild-type retinal cross-sections demonstrated the distribution of neurons within the GCL. B,C. In contrast to the wide distribution of syt1 mRNA, ISH revealed that apoer2 (B) and vldlr (C) mRNAs were expressed by small subsets of cells within the GCL (see arrowheads in B,C). D. Dab1-IHC in P13 wild-type retinal cross-sections confirmed expression of Dab1 by amacrine cells in the INL (arrows)(see Rice and Curran 2000). Sparse labeling of Dab1 was also observed in a subset of cells in the GCL (arrowhead). E. Co-labeling of Meln and Dab1 in P13 wild-type retinal cross section. Arrowhead depicts Dab1-expressing ipRGC. Arrows indicate Dab1-expressing amacrine cells. Asterisk denotes non-specific immunoreactivity. F. Reelin-IHC
in P13 wild-type retinal cross-sections. Many cells within the GCL express reelin, however, arrowheads indicate cells with no significant reelin expression. G,H. Co-labeling of P13 wild-type retinal cross-sections with Meln and reelin antibodies revealed that most ipRGCs do not express reelin (see arrowheads). Meln-expressing RGCs in G and H presumably represent different classes of ipRGCs since there dendrites stratify in different sublaminae of the IPL (see arrows). Both classes of ipRGCs appear to lack reelin expression. I. Reelin-IHC was performed on P13 retinal cross sections from opn4-tau-LacZ transgenic mice. M1 ipRGCs were labeled by LacZ-IHC. Arrowhead highlights a M1 ipRGC that lacks reelin expression. For all images, nuclei were labeled by DAPI staining. INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer. Scale bar = 15 µm for A–C,E and 20 µm for D,F–I.
Figure 3. Reelin is necessary for retinogeniculate targeting

A,B. Retinogeniculate projections in P14 control (A) or reln\textsuperscript{1/1} mutant mice (B) assessed by labeling of RGC axons with CTB. Left eyes were injected with AlexaFluor 594-CTB and right eyes with AlexaFluor 488-CTB. LGN from right hemispheres are shown. ‘Contra’ denotes retinal arbors originating from cells in the contralateral eye and ‘ipsi’ denotes those originating from ipsilateral eye. In mutant LGN (B), note the near absence of retinal projections in IGL (arrows), the reduced pattern of vLGN innervation, and the presence of mistargeted axons (asterisk). C,D show high magnification images of areas depicted by asterisks in A,B, respectively. Projections from both eyes have been merged into a grayscale image for improved visualization (CTB[contra+ipsi]). E,F. Retinogeniculate projections in

*J Neurosci. Author manuscript; available in PMC 2012 January 12.*
P1 control (+/+; E) or reln

mutant mice (rl/rl; F). RGC axons from both eyes were labeled with the same fluorescently conjugated form of CTB (CTB[b]). Arrowhead in F highlights mistargeted RGC axons. G. IHC for Dab1 and DAPI in P14 wildtype retinal cross sections. Arrows in indicate IGL. d – dLGN; v – vLGN. Scale bar in A = 400 µm for A,B; in C = 200 µm for C,D; in E = 300 µm for E,F.
Figure 4. Dab1 is necessary for retinogeniculate targeting

A,B. Retinogeniculate projections in P14 control (A) or dab1<sup>scm/scm</sup> mutant mice (B,C) assessed by labeling of RGC axons with CTB. Left eyes were injected with AlexaFluor 594-CTB and right eyes with AlexaFluor 488-CTB. LGN from right hemispheres are shown. ‘Contra’ denotes retinal arbors originating from cells in the contralateral eye and ‘ispi’ denotes those originating from ipsilateral eye. In mutants two different retinogeniculate phenotypes were observed. In one set of <sup>dab1</sup><sup>scm/scm</sup> mutants (B) a near absence of retinal projections were observed in IGL (arrows), whereas in another set, retinal projections were observed in the IGL (C). In both cases (B,C), mutants exhibited a reduced pattern of vLGN innervation and mistargeted axons were observed exiting the LGN (asterisks). A’,B’,C’ show high magnification images of areas depicted by asterisks in A,B,C respectively.

D–F. Retinogeniculate projections in P2 control (D) or <sup>dab1</sup><sup>scm/scm</sup> mutant mice (E,F). RGC axons from both eyes were labeled with the same fluorescently conjugated form of CTB (CTB[b]). Similar to analysis at P14, 2 distinct phenotypes were observed in the pattern of IGL innervation by RGC axons in P2 <sup>dab1</sup><sup>scm/scm</sup> mutants (see arrows in E,F). In both cases, misrouted axons were observed exiting mutant LGN and entering non-retino-recipient thalamic regions (see E’,F’). D’,E’,F’ show high magnification images of areas depicted by asterisks in D,E,F respectively. Arrows indicate IGL. d – dLGN; v – vLGN. Scale bar = 150 µm.
Figure 5. LGN subnuclei are present, remain distinct, and contain appropriate neural populations in the absence of functional reelin.
IHC and ISH were performed on coronal sections of P14–21 relnrl/rl mutant or littermate control brains with subnuclei- or neuron-specific markers. A,B. NeuN and NPY IHC in P14 relnrl/rl mutant or control LGN. NPY remained properly restricted to the IGL in the absence of reelin. C,D. GFAP-IHC demonstrated the presence and confinement of astrocytes in P21 control and mutant IGL. E,F. IHC revealed a normal distribution of Gad65 in P21 relnrl/rl mutant vLGN and IGL. G,H. ISH revealed a confinement of syt2-expressing neurons in vLGN of P21 mutants and controls. I,J. SMI32-immunoreactivity was appropriately enriched in vLGN and dLGN, but absent from IGL in P21 mutants and controls. K,L.
Adams15 mRNA, assessed by ISH, was correctly localized in P14 mutant and control dLGN. Sections were co-stained with NPY antibodies to demonstrate the location of the IGL. Note that NPY-positive cells did not invade dLGN in the absence of functional reelin. In C–J, retinal axons were anterogradely labeled with CTB (CTB[b]). In each image the IGL is encircled by white dots. d – dLGN; v – vLGN; \(v_r\) – retino-recipient portion of vLGN; \(v_{nr}\) – non-retino-recipient portion of vLGN. Asterisks denote corticothalamic axon tracts. Single color panels for each merged image can be found in Supplemental Figure 6. Scale bar is 400 µm.
Figure 6. Reelin is not required for the generation or development of non-image-forming RGCs, nor their projections to non-LGN retino-recipient nuclei.

A,B. Non-image-forming ipRGCs were labeled by melanopsin (Meln) IHC in P12 control (+/+) and \( reln^{rl/rl} \) mutant (rl/rl) retinal whole-mounts. The distribution and morphology of ipRGCs appeared similar in controls and mutants. A’,B’ Dendritic stratification of melanopsin-immunolabeled ipRGCs (green) in OFF- and ON-sublaminae of the IPL (arrows) appeared similar in cross sections of P12 \( reln^{rl/rl} \) mutant and littermate control retinas. Moreover, mutant and control ipRGCs (green) demonstrated equal capacities to endocytose CTB (red).

C,D. Retinal projection to other retino-recipient nuclei innervated by ipRGC appear normal in the absence of functional reelin. RGC projections to the OPN...
(C, D) and SCN (E, F) were assessed by injections of different fluorescently conjugated CTB into each eye of P12 controls and mutants. The patterns of innervation of these nuclei were indistinguishable in mutants or controls. G–H. Pupillary light reflexes (PLRs) remained present in reln<sup>rl/rl</sup> mutants. P14 mutant and control mice (n=3 each) were dark adapted for >1hr and then exposed to a 30s 1.7mW/cm<sup>2</sup> light. Pupil constriction was captured on video and images were used to measure pupil size (white circles in G) before the onset of light and after 30s of light. In H, the percent pupil constriction following light exposure was calculated by comparing to pupil size during dark adaptation. Scale bar in A = 100 µm for A, B; in A' = 25 µm for A', B'; in C = 200 µm for C–F.
Figure 7. Non-image-forming ipRGCs require reelin and Dab1 for retinogeniculate targeting

A–E. IpRGC projections in P11 controls (A), reln+/−;opn4-tau-LacZ+/+ mutants (B) and 
dab1+/−;opn4-tau-LacZ+/− mutants (D). M1 ipRGCs were labeled by LacZ-IHC (green). All RGC axons were labeled by binocular injection of CTB (CTB[b]; red). Arrowheads in B–E indicate mistargeted RGC axons containing LacZ. Arrow in B′,D′ highlight misrouted LacZ-containing M1 ipRGC axons invading dLGN in mutants (compare with arrow in A′). C–C″ and E–E″ are high magnification images of mistargeted axons depicted by asterisks in B and D. Note that LacZ-immunoreactivity is reduced in dab1+/−;opn4-tau-LacZ+/− mutants (D) compared with images in A,B since opn4-tau-LacZ heterozygotes were analyzed. F–H. By P21 mistargeted ipRGC axons have extensive arbors within dLGN in

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renh^{rl/rl}\cdot opn4-tau-LacZ and dab1^{scm/scm}\cdot opn4-tau-LacZ mutants, but not controls (compare arrows in F–H). Scale bar in A = 300 µm for A,B,D,F–H; in C = 25 µm for C,E.
Figure 8. Reelin is dispensable for dLGN targeting by axons derived from image-forming RGCs 

A,B. VGLUT2-immunolabeled dLGN-projecting retinal terminals appear similar in P12 controls (+/+, A) and reln\textsuperscript{1l/1l} mutants (rl/rl, B). C,D. Calretinin (Calr)-immunolabeled RGC axons appeared similarly confined to dLGN in P12 control (+/+, C) and reln\textsuperscript{1l/1l} mutant (rl/rl, D) LGN. Arrows indicate abrupt border of Calr-containing arbors at the dLGN-IGL border. 

E,F. Misrouted CTB-labeled RGC axons in adjacent non-retino-recipient thalamic nuclei do not contain Calr in P14 reln\textsuperscript{1l/1l} mutants (see arrowheads). Scale bar in A = 300 µm for A–D; in E = 15 µm for E,F.
Figure 9. Reelin is dispensable for dLGN-targeting by corticothalamic axons
A. Schematic demonstrating layer VI cortical axons pass through the internal capsule (IC), bypass vLGN/IGL, and selectively innervate dLGN. B,C. Layer VI cortical neurons were labeled by GFP-IHC in P12 reln$^{+/+}$;golli-tau-GFP controls (B) or reln$^{rl/rl}$;golli-tau-GFP mutants (C). Normal cortical layering and the location of white matter tracts (WM) are depicted in A. Layer VI cortical neurons and their projections in transgenic mice were labeled by GFP-IHC. D,E. Although corticothalamic axons have an altered course in reln$^{rl/rl}$;golli-tau-GFP mutants (see asterisks in E), they selectively target dLGN as in reln$^{+/+}$;golli-tau-GFP controls. d – dLGN; v – vLGN; i – IGL; IC – internal capsule. Scale bar in = 300 µm.