SUPPLEMENTAL DATA

Astrocytes Assemble Thalamocortical Synapses by Bridging Nrx1α and NL1 via Hevin

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

All experiments were conducted in accordance with the institutional animal care and use committee guidelines (IACUC Protocol Numbers A-185-11-08 and A173-14-07)

RGC Culture System to Study Synapse Formation *In Vitro*

Neuronal culture systems that are traditionally used to study synapse formation, such as hippocampal or cortical neurons, are usually obtained from late embryonic or neonatal rodent brains (Banker, 1980; Craig and Banker, 1994). Therefore, dissecting out the roles of astrocytes in synapse formation from their strong influence on neuronal maturation and survival in these systems has been difficult. To circumvent this limitation, a purified retinal ganglion cell (RGC) culture system was developed that utilizes an immunopanning strategy to isolate RGCs from postnatal rodent retina (Meyer-Franke et al., 1995). This culture system is particularly suitable to test the effects of astrocyte-secreted factors in synapse development because: **1)** Fully differentiated RGCs can be purified from seven-day-old (P7) rodent retinas. **2)** Purified RGCs can be cultured in the absence of any other cell type in serum-free media of known composition. **3)** RGCs cultured under these conditions form extensive axonal and dendritic processes but do not form many synapses. **4)** Excitatory synapse formation can be robustly stimulated by addition of astrocyte-feeder layers, astrocyte-conditioned media, or purified synaptogenic proteins such as thrombospondins (TSPs) or hevin. **5)** Changes in synapse number, size, and ultrastructure can be determined by established methods such as the quantification of the increase in the number of co-localized pre and postsynaptic markers by immunocytochemistry. **6)** All the synaptic contacts between RGCs are a uniform class of excitatory synapses (i.e. they contain vesicular glutamate transporter 2 in their presynaptic vesicles). Thus the changes observed in excitatory synapse

number and morphology can be directly attributed to the tested condition and not to an indirect effect of the conditions on inhibitory connectivity. **7)** Gene expression in RGCs can be modified at any stage during culturing by transfecting with DNA or shRNA constructs (Meyer-Franke et al., 1995).

RGCs were purified by sequential immunopanning to greater than 99.5% purity from P7 Sprague-Dawley rats (Charles River) and cultured in neurobasal medium with B27 supplement, BDNF, CNTF, and forskolin on laminin-coated coverslips, as previously described (Christopherson et al., 2005; Meyer-Franke et al., 1995; Ullian et al., 2004). RCGs were cultured for 3–4 days to allow robust process outgrowth and then cultured with hevin, truncated hevin (Hevin-N, Hevin-C, Hevin-N/FS+ or Hevin-N/ΔFS) or hevin deletion mutants (Hevin-**ΔA**, Hevin-**ΔDE** or Hevin-**ΔD**) for an additional 6 days. In other experiments, hevin was applied with either recombinant purified Fc, or Nrx1α-ECD-Fc for 6 days. In antibody blocking experiments, hevin was preincubated with either control Rat IgG, Rat IgG-12:54 (anti-Hevin) or Rat IgG-12:155 (anti-Hevin) for 1 hour at 37^{o}C at 1:1 molar ratio of Hevin:IgG.

Synapse Assay on RGCs

For synapse quantification in RGC cultures, cells were fixed for 7 minutes with 4% warm paraformaldehyde (PFA), washed three times in Dulbecco's phosphate-buffered saline (DPBS), and blocked in 200µl of a blocking buffer containing 50% normal goat serum and 0.2% Triton X-100 for 30 minutes. After blocking, coverslips were washed three times in DPBS, and 200µl of primary antibody solution consisting of rabbit anti-homer-1 (1:500, 160 002, Synaptic Systems) and mouse anti-bassoon (1:500, SAP7F07/VAM-PS003F, Enzo/Assay Designs) was added to each coverslip. Coverslips were incubated overnight at 4°C, washed three times in

DPBS, and incubated with 200µl of Alexa-594 (for presynaptic proteins) and Alexa-488 (for postsynaptic proteins) conjugated 2^o antibodies (Invitrogen, diluted 1:1000) in antibody buffer. Following incubation for 2 hours at room temperature (RT), coverslips were washed 3-4 times in DPBS and mounted in vectashield mounting medium with DAPI (Vector Laboratories Inc) on glass slides (VWR Scientific). Secondary-only controls were routinely performed and revealed no significant background staining.

Mounted coverslips were imaged using Zeiss AxioImager M1 (Zeiss) fluorescence microscope. Healthy cells that were at least two cell diameters from their nearest neighbor were identified and selected at random using DAPI fluorescence. 8-bit digital images of the fluorescence emission in red, green and DAPI channel were recorded for each selected cell using a CCD camera. Merged images of red (bassoon) and green (homer-1) channel were analyzed for co-localized puncta by using a custom-written plug-in for NIH software ImageJ, Puncta analyzer (written by Bary Wark, available upon request, cagla.eroglu@dm.duke.edu). Details of the assay can be found in (Ippolito and Eroglu, 2010). This assay takes advantage of the fact that pre and postsynaptic proteins appear co-localized at synaptic junctions due to their close proximity. We verified numerous times that this analysis generates counts that are similar to the numbers we obtain counting by eye. We have previously shown that the increase in co-localized puncta that is induced by hevin corresponds to an increase in the number of synaptic structures, counted by electron microscopy (Kucukdereli et al., 2011). Hevin-induced synapses in RGC cultures are ultrastructurally identical to the synapses that are induced by astrocytes (determined by EM). However, hevin induces postsynaptically silent synapses (Kucukdereli et al., 2011).

Thalamic and Cortical Neuron Co-culture and Synapse Assay

Purified (glia-free) thalamic and cortical neuron co-cultures (Th/Cx) were prepared as described in (Risher et al., 2014). Briefly, thalami and cortices of P1 mouse pups were dissected out and processed for cell isolation separately. Tissues were papain digested followed by trituration in low ovomucoid and high ovomucoid solutions. Triturated cells were applied to negative panning dishes with BSL coatings to discard unwanted cells and cell debris. Cell suspensions were added to the positive panning dishes (coated with anti-L1, Millipore) to obtain pure populations of thalamic or cortical neurons (>95% neuron). Thalamic and cortical neurons were co-cultured at a 1:1 ratio in growth media described above. To stop the growth of proliferating cells such as astrocytes, two days post plating, AraC was added to the mixed cultures at a final concentration of 2μM. 36-40 hours later, AraC containing media was completely removed and replaced by normal RGC growth media. Cultures were fed every 3 days. On DIV7/8 the cultures were incubated with Hevin or Hevin-**ΔDE** proteins for the next 6 days with another feed in between. On DIV13/14 cells were fixed and stained as described for RGCs except: guinea pig anti-VGluT2 (1:1000, Millipore) and mouse anti-PSD95 (1:500, Neuromab, CA) antibodies were used to visualize pre and postsynaptic sites, respectively. Only cortical neurons were imaged and analyzed. Thalamic neurons were avoided by the appearance of bright VGluT2 staining within the cell soma.

Synaptic Vesicle Recycling/Release Assay in RGCs

RGCs were cultured and treated with either growth media (GM) only, hevin or astrocyte conditioned medium (ACM) as described above. On final day, RGCs were depolarized in a solution containing 47mM KCl and guinea pig anti-luminal synaptotagmin (1:800, #105105, Synaptic Systems, Germany) antibody for 5 minutes in the incubator at 37°C. Depolarization solution was removed and cells were washed once with warm DPBS for 10 minutes, followed by

4% PFA fixation and staining for mouse anti-bassoon, rabbit anti-homer-1 as mentioned above. Images were taken in Zeiss 710 confocal microscope in Z-stacks of 5-8 sections per image. Z-project images were compressed to generate maximum intensity projection images. Presynaptically active synapses were analyzed as triple colocalization of the signals for luminal synaptotagmin, bassoon and homer-1 using puncta analyzer as described above.

Whole-cell Patch Clamp Recordings in Autaptic RGC Cultures

RGCs were purified as mentioned above and were plated at a low density (2500 cells per coverslip) on plastic Aclar 22C (Allied Signal) coverslips that were coated with droplets of PDL and laminin (1:1). Purified hevin or astrocyte-conditioned media (ACM) were applied to autaptic RGC cultures on DIV6 and 9. NMDA-induced currents in individual autaptic RGCs were recorded between DIV12-14.

Whole-cell voltage recordings were performed at room temperature (28°C) to measure NMDA-induced currents. The data were acquired at a rate of 10kHz and filtered at 3kHz using an EPC-10 amplifier (HEKA, Germany) and Patchmaster software (HEKA, Germany). The patch pipettes were pulled from borosilicate capillaries (World Precision Instruments, Inc.). When filled with the pipette solution, the resistance of the pipettes was 4-5MΩ. The recording chamber was continuously superfused (3-4ml/min). For NMDA-induced current recording, pipette solution contained (in mM): CsCl 130, NaCl 9, $MgCl_2$ 1, EGTA 10, HEPES 10, adjusted to pH 7.4 with CsOH. The external solution was composed of (in mM): NaCl 140, $CaCl_2$ 2, HEPES 10, glucose 10, with 1μM glycine, 100μM picrotoxin, 2μM strychnine and 0.5μM TTX, adjusted to pH 7.4 with NaOH. NR2B component and NR2B current density were obtained by subtracting the ifenprodil-insensitive NMDA-induced currents from the total NMDA-induced currents.

COS7 and HEK293 Cell Cultures

COS7 cells and HEK293 cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1% Pen/Strep, 1% Glutamine, and 1% sodium pyruvate.

DNA Constructs and Protein Purification

Full-length cDNA of hevin (a.a. 17-650) and its truncation constructs hevin-C (a.a. 351-650), hevin-N (a.a. 17-350) were cloned into the pAPtag5 vector (GeneHunter) between SfiI and XhoI sites. Various constructs expressing deletion mutants of hevin (Hevin-**ΔA** (lacking a.a. 88-115), Hevin-**ΔDE** (lacking a.a. 351-440) and Hevin-**ΔD** (lacking a.a. 351-372)) were generated by site directed mutagenesis reaction using specific primers (mentioned in oligonucleotides list at the end) and QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). This vector already possesses a secretion sequence 5' of the SfiI site which enabled secretion of all hevin fragments that were cloned into it. The above constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Recombinant proteins were purified from conditioned culture media by Ni-chelating chromatography using Ni-NTA resin (Qiagen) according to the manufacturer's instructions.

Plasmid constructs overexpressing HA-NL1B, HA-Nrx1α, HA-Nrx1β and shRNAs against NL1, NL2, and NL3 are used from (Chih et al., 2005; Chih et al., 2006). Control shRNA (shControl) was made by randomly rearranging the original NL1 shRNA sequence and cloning it back into the original plasmid vector pLenLox3.7 beta actin-EGFP with U6 promoter. Plasmids expressing HA-NL3 (human), HA-NL4 (human), HA-LRRTM2, Nrx1β-CFP, Nrx1α-CFP, Nrx2α-CFP, Nrx3α-CFP, YFP-NL2, were kind gifts from Dr. Ann Marie Craig (University of British Columbia) (Graf et al., 2004; Siddiqui et al., 2010). Nrx2α-GFP was made by subcloning Nrx2α

cDNA into pEGFP-N1 vector between HindIII and ApaI sites. HA-NL2 was made by cloning NL2 cDNA into pCAG-HA-NL1 between BspEI and XmaI sites.

TM fusion constructs: cDNA encoding the transmembrane region of LDL-TM and the cytoplasmic tail of CD46 was cloned into AP, Hevin, Hevin-N,and Hevin-C containing pAPtag5 plasmids between XhoI and XbaI sites. HA-tag was added at the N-terminus after the secretion signal by site directed mutagenesis to generate HA-tagged TM constructs.

Fc tagged fusion constructs: cDNA encoding Fc protein was first cloned into the pAPtag5 vector between BglII and XhoI sites. The C-terminal-tagged Fc expressing constructs were made by cloning cDNA of hevin, Hevin-N, Nrx1α-ECD (a.a. 31-1354) and Nrx1β (a.a. 5-262) between HindIII and BglII sites. The N-terminal-tagged Fc-expressing construct was made by cloning cDNA of Hevin-C between XhoI and XbaI sites. Transfections were performed as previously mentioned. Recombinant Fc-tagged proteins were purified from conditioned culture media of HEK293 cells by affinity purification using HiTRAP protein G affinity columns (GE Healthcare) following the manufacturer's instructions. Purity of recombinant proteins was verified on SDS-PAGE gel with GelCode blue stain (Pierce). The optical-density of pure proteins was determined at 280nm wavelength using a NanoDrop 2000 instrument (ThermoScientific). The concentration of pure proteins was calculated entering the exact amino acid sequence of each protein in Richard's Protein Calculator program (http://www.mrc-lmb.cam.ac.uk/ms/methods/proteincalculator.html).

RGC Transfections

RGCs were transfected using the Lipofectamine LTX (Invitrogen) reagent on DIV4-5. Briefly, 300µl conditioned culture medium was removed from the cells and saved in another tissue

culture plate at 37°C in 10% CO_2 incubator. The cells were then fed with 200µl fresh media. The transfection mix was made as per manufacturer's protocol with 1:1.25 DNA to LTX ratio. Briefly, 0.25µg of each shRNA construct or rescue hNLG3 construct (total 1µg DNA) and 1.25µl of Lipofectamine LTX was used in each well (in a 24 well plate). After 2-2.5 hours cells were washed twice with warm DPBS and were fed with 200µl of fresh RGC growth media and 300µl of the saved conditioned media. Hevin treatment was started 2 days post-transfection for shRNA constructs. EGFP was present in each shRNA construct to enable us to identify transfected cells. Typical transfection efficiencies ranged between 10% and 15%. Cells were stained for synapses after 6 days of hevin treatment as described before in the synapse assay section. In this experiment, Rb-homer-1 was detected by using a secondary goat anti-rabbit antibody conjugated to Alexa 647. Images of transfected cells were taken in three channels (green for EGFP (pseudo colored blue)), red for bassoon, and far-red for homer-1 (pseudo colored green). The number of synapses on EGFP positive cells was quantified as follows. Briefly, Puncta Analyzer plugin for ImageJ was utilized to identify and mark the co-localized synaptic puncta in the entire image as described above. Co-localized puncta that are localized to the EGFP-transfected dendrites and soma were counted using cell-counter option in ImageJ. Transfected cell areas (soma and dendrites) were measured for each cell using the Image J measure option on the EGFP-channel image. The synaptic density was calculated by dividing the number of synapses counted per cell by the total area of the cell.

Presynaptic Organization "Hemisynapse" Assay

RGCs were cultured for DIV5-6 at 15K per well density in a 24-well plate as mentioned above. HEK293cells, at 90% confluency, were transfected with plasmids expressing mCherry, HA-AP-TM, HA-hevin-TM, HA-hevin-N-TM, HA-hevin-C-TM, or HA-NL1 at 1µg per well

concentration in a 6 well plate using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. One day post transfections, HEK293 cells were quickly trypsinized, counted and plated at a 20-25K density onto cultured RGCs (DIV5-6) in the presence of 1μM Ara-C (cytosine arabinoside, C6645 Sigma-Aldrich). Three days later, cells were washed, fixed and stained. Briefly, transfected HEK293 cells were stained with rat anti-HA (1:2000, 1867423, Roche) in the red channel, and presynaptic specializations of the RGCs were stained with rabbit anti-synapsin-1 (1:1000, 1925-SYNP, Phosphosolutions) in the green channel. Mounted coverslips were imaged using a Zeiss 710 inverted confocal microscope (Carl Zeiss). Healthy and well-attached HEK293 cells were identified and selected at random using red fluorescence. Z-sections were imaged at 0.5μm intervals for a total of 20-25 stacks (covering the entire height of the HEK293 cells). 8-bit digital images of the fluorescence emission in red and green channels were recorded for each selected cell. Using ZEN2009 light edition software, each image was processed to produce maximum intensity projections (MIP) images and orthogonal sections of a single slice. These MIP images were then analyzed for co-localized puncta per HEK293 cell using ImageJ Puncta analyzer mentioned in the synapse assay on RGCs section. To quantify the % area of synapsin-1 (green) onto HA-NL1B-expressing HEK293 (red) cells, NIH software ImageJ was used. Briefly, total area of a transfected HEK293 (red) cell was measured by obtaining the perimeter of the same HEK293 cell with the help of a binary conversion tool in the NIH ImageJ software and using the area measurement option. The total area of synapsin-1 within the same HEK293 cell's perimeter was measured. Data is represented as % coverage of HEK293 cell by synapsin-1. All the images were thresholded in exactly the same way to analyze synapsin-1 area size in the green channel. Synapsin-1 signal smaller than 8 pixels in size was excluded in the data analysis as background.

Postsynaptic Organization "Bead Clustering" Assay

RGCs (DIV6-7) were cultured at 45-50K density in a 24 well plate as described above. Fc-tagged proteins were bound on protein G coated 2.8µm diameter magnetic beads (Dynabeads® Protein G, Life Technologies) as follows. Briefly, a master mix of beads was washed 3-4 times with DPBS (GIBCO). Beads were incubated with respective Fc-tagged proteins in DPBS with 0.5% BSA for 6-8 hours at 4^{o}C. A surplus of Fc-tagged proteins was used to ensure complete saturation of the protein G beads (i.e. 0.3µg protein per 1µg of beads). Protein-bound beads were then washed 3-4 times with DPBS and re-suspended in RGC growth medium. These beads were added to the RGCs with a final amount of 50-60µg beads per well. After 3 days of RGC-bead co-culture, cells were gently washed, fixed and stained as per the synapse staining protocol described above. Postsynaptic clustering was detected by rabbit anti-homer-1 and goat-anti-rabbit-Alexa-488 (green), and beads were detected by Alexa Fluor 594 conjugated goat anti-human IgG (H+L) that recognized the Fc-tag (1:1000, Jackson Labs). Confocal images of cells and their processes were taken at 40x magnification using Zeiss 710 inverted confocal microscope with Z-series steps of 0.2µm. Each image contained 1-2 neuronal cell bodies and numerous dendrites. Total number of beads per imaging field was counted to measure adhesiveness of the Fc-tagged proteins. The homer-1 clustering efficiency of the coated beads was measured by counting the total number of beads with at least one homer-1 puncta (a puncta was defined as homer-1 signal that was more than 16 pixels in size).

For experiments to test the effect of hevin on the ability of Nrx1α-Fc to cluster homer-1, the beads were prepared as described above and were further incubated with soluble hevin or BSA (100nM final concentration in 1ml per condition) for 2 hours at 4^{o}C. Beads were then washed and resuspended in RGC growth medium to be added to RGCs. The homer-1 clustering on the

beads was quantified as the total area of homer-1 signal within the total bead area (red) per field using ImageJ. Homer-1 signal smaller than 16 pixels in size was excluded in the data analysis as background. To calculate the homer-1 puncta area per bead, average total homer-1 area per field was divided by the number of total beads in that field. Data presented is from 10-15 fields per condition. To determine the role of NLs, beads were prepared in the same fashion but were co-cultured with RGCs transfected with shNL constructs with GFP reporters. For quantification of homer-1 clustering, first we calculated the total bead area that contacted GFP-filled dendrites. Then we measured the area of homer-1 within those beads per image to calculate homer-1 area/bead. The final data is presented as fold change in homer-1 puncta area/GFP colocalized bead area compared to shCon transfected RGCs in GM only condition (n=10 cells/condition).

Anti-Neurexin-1 Antibody Blocking Experiments

RGCs were isolated and plated as described above. DIV3 RGCs were treated with the following antibodies (final concentration at 1.45µg antibody per well): rabbit polyclonal anti-Nrx1α (Alomone labs Cat# ANR-031, Jerusalem, Israel), mouse monoclonal anti-Nrx1β (Millipore Cat# MABN607, Temecula, CA), mouse monoclonal anti-Nrx1 (both α and β, BD Transduction Labs, USA) in the presence or absence of hevin (80nM) for 6 days. As controls isotype- and species-matched mouse monoclonal IgG or rabbit polyclonal IgG antibodies were used. Synaptic staining was performed as described above.

RGC-COS7 "Nrx-NL Co-clustering" Assay

RGCs were cultured at 45-50K density and transfected on DIV4-5 as mentioned above. Briefly, 0.5µg of HA-NL1B-expressing plasmids were transfected in each well using Lipofectamine-LTX. The same day, COS7 cells were transfected with Nrx1β-CFP and Nrx1α-CFP-expressing

plasmids as mentioned above in the hemisynapse assay section. Two days post transfection, COS7 cells were trypsinized quickly, counted and resuspended in RGC growth medium and added at a density of 20-25K to the respective RGCs in the presence or absence of purified recombinant Hevin, Hevin-**ΔA, Hevin-ΔD** and Hevin-**ΔDE** (final concentration of 100 nM each). After 3 days of co-culture, cells were fixed and stained. Briefly, anti-HA antibody was used to detect NL1B transfected RGCs in red channel, and transfected COS7 cells were detected by anti-GFP (1:2000, Novus Biologicals) antibody in the green channel. For analysis, transfected COS7 cells that were approximately 2-3 cells away from a transfected RGC were chosen for imaging. Confocal images of COS7 cells were taken in the Zeiss 710 inverted microscope as mentioned above in Z-stacks of 0.5μm steps with a total of 25-30 steps. MIP images were generated as mentioned above. To quantify the NL1B (red) clustering onto Nrx-expressing COS7 (green) cells, NIH software ImageJ was used. Briefly, the total area of a Nrx-transfected COS7 (green) cell was measured using the binary conversion tool in the NIH ImageJ software and applying the area measurement option. Then the total area of NL1B within the same COS7 cell's perimeter was measured. The ratio of the total area of red (NL1B) over total area of green (Nrx-CFP expressing COS7) per each cell was obtained (Fig. 4D.). All the images were thresholded in exactly the same way to analyze NL1B cluster size, in the red channel.

As a control to confirm that the CFP-tagged Nrx1α was properly expressed and trafficked to the plasma membrane, we also tested whether Nrx1α is capable of clustering LRRTM2, a high-affinity postsynaptic interaction partner for Nrx1α (Linhoff et al., 2009; Siddiqui et al., 2010). We found that Nrx1α-CFP was able to strongly co-cluster LRRTM2 that was expressed in RGCs (Figure S4H).

Cell Aggregation Assay

HEK293 cells were transfected with vectors co-expressing either GFP with a Nrx isoform or RFP with a NL1 splice variant as indicated in Figures S4E in 6 well plates. After 2 days, medium was supplemented with 1μM AraC (to stop overgrowth) and cells were allowed 2 more days to grow. After 4 days of transfection, cells were washed with PBS followed by incubation with PBS containing 2mM EDTA at room temperature for 2min. Gently cells were triturated to obtained single cell suspension followed by mixing at a density of $3.0x10^6$ cells/ml. Cells were incubated in 1.7-ml eppendorf tubes at room temperature with rotation in total volume of 500μl of Neurobasal medium without phenol red (GIBCO) supplemented with 1x glutamine in the presence or absence of hevin or hevin deletion mutants at a final concentration of 200nM. After 1hr cells were plated onto coverslips and fluorescence images were taken at 5x magnification with a Zeiss AxioImager M1 (Zeiss). The quantification in Figure S4F and S4G was performed by analyzing the cell aggregate size using Image J. Briefly, each image was thresholded equally and masked to create binary image. The area of particles greater than the area of an average single cell was measured from the binary image using the analyze particles tab. The data is represented as cumulative probability of clustered cell size (a.u.). (n=27 (for S4F) and n=18 (for S4G) random fields per conditions, at least two independent experiment was performed). The quantification in Figure S4J was performed by analyzing total area of colocalized signal between green (GFP) and red (RFP) using Image J with Puncta analyzer plugin. The data is represented as fold change in the colocalized area of GFP and RFP compared to the GFP only and RFP only mixed cells condition (n=18 random fields/conditions, at least 2 independent experiment was performed and similar results were obtained).

Immunoprecipitations and Western Blotting

Hevin, Hevin-C, Hevin-N, Hevin-N/FS+, Hevin-N/ΔFS (his-myc tagged), Nrx1α, Nrx1β, and NL-1,-2,-3, and -4 (HA-tagged) were expressed in HEK293 cells by transient transfection using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Two days post transfections, HEK293 cell lysate was prepared for co-immunoprecipitations. Cells were washed with cold 1xTBS (with 1mM Ca^{++} and 1mM Mg^{++}) 3 times and scraped from the tissue culture plates. The cells were pelleted and resuspended in ice-cold solubilization buffer (25mM Tris pH 7.2, 150mM NaCl, 1mM $CaCl_2$ and 1mM $MgCl_2$) with protease inhibitors (Complete EDTA-free, Roche) and 0.5% Surfact-Amps NP-40 (Pierce) and were incubated at 4°C for 15min to allow for solubilization. The insoluble debris was removed by centrifugation (20,000g for 10 minutes). The supernatant was then diluted two-fold by adding equal volume of solubilization buffer without NP-40, thus making a final concentration of 0.25% NP-40. To pull down HA-tagged Nrxs and NLs, the diluted lysate was then incubated with anti-HA antibody conjugated red affinity beads (Sigma-Aldrich) overnight at 4°C while rotating. After binding was completed the beads were washed 3 times with the solubilization buffer containing 0.25% NP-40 and protease inhibitors. The bound proteins were eluted by addition of non-reducing SDS-PAGE buffer (2X, Pierce) and 15min incubation at 37°C. The eluate was then transferred to a clean tube and β-mercaptoethanol was added. The samples were denatured at 40°C for 20min and loaded on SDS-PAGE gels (4-15%, BioRad). After SDS-PAGE electrophoresis, proteins were transferred onto PVDF membranes and were blotted for target proteins. Immunoprecipitated NLs were detected by using rat anti-HA antibody (1:2500, Roche). Nrxs were detected by either rat anti-HA or rabbit anti-Neurexin1 antibody (1:1000, Synaptic Systems). Hevin, Hevin-N, Hevin-N/FS+, and Hevin-N/ΔFS were detected by rat anti-hevin (12:155 at 1μg/ml concentration) and Hevin-C was detected by rat anti hevin (12:54 at 1μg/ml concentration). Horseradish peroxidase

conjugated anti-rat, anti-rabbit, (1:5000, Jackson Laboratories) and anti-goat (1:5000, R&D systems) were used as secondary antibodies and the detection was performed with an ECL kit (Amersham).

Triple co-immunoprecipitation: For detecting triple complex formation between Nrx1α-Hevin-NL1B, HEK293 cells were transfected with either Nrx1α-CFP, Nrx2α-GFP, HA-NL1B or HA-NL1A. Two days later cells expressing a Nrx-α isoform were mixed at 1:1 ratio with cells expressing a NL1 splice variant (Figure 4A). Next day cells were washed with 1x PBS followed by addition of neuron growth media with or without various concentrations of Hevin or Hevin-**ΔDE**. 24 hours later cells were lysed and incubated with GFP-Trap®_A beads (chromotek, Germany) overnight. Immunoprecipitated complexes were washed once followed by elution and SDS-PAGE. Anti-GFP, Anti-HA and Anti-Hevin (12:155) antibodies were used to detect Nrx-α, NL1 and Hevin or Hevin-**ΔDE** respectively, in western blots following triple Co-IPs.

RNA Isolation and RT-PCR

Various brain regions (cortex (C), hippocampus (H), and superior colliculus (SC)) were dissected out from postnatal day 7 (P7) rat brain. Immediately after dissections, brain regions were flash frozen in liquid nitrogen. Flash frozen tissues were crushed into powder using mortal and pestle in the presence of liquid nitrogen. The powder was then directly lysed in TRIzol reagent (Invitrogen) to isolate total RNA following manufacturer's protocol. For RNA isolation from cultured neurons (RGCs or Thalamic only (Th), cortical only (Cx) or thalamo-cortical mixed (Th/Cx)), cells were cultured in 6-well plates and at DIV6, washed once with 1x PBS and directly lysed in TRIzol reagent. 1μg of total RNA was reverse-transcribed using the high capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Isoform specific primers

were designed to amplify various isoforms of NRXNs and NLGNs from cDNA (see the list below). Semiquantitative RT-PCR was performed and PCR products were run by agarose gel electrophoresis. DNA fragments were imaged using ethidium bromide fluorescence. Actin or GAPDH expression was used as a loading control for cDNA.

Mice

The Hevin KO mice used in these studies are on a SVE/129 background as previously published (Kucukdereli et al., 2011; Risher et al., 2014). The Nrx1α KO mouse line was acquired from Dr. Catherine Fernandes (King's College London) and was crossed into a mixed background upon receipt (Grayton et al., 2013). The NL1 mouse line was a gift from Dr. Nils Brose and is in a C57Bl/6 background (Varoqueaux et al., 2006). All animals were used in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Duke Division of Laboratory Animal Resources (DLAR) oversight.

Preparation of Synaptic Fractions

The cortices were rapidly dissected from the brains of P25 hevin WT or KO littermate animals (n=6 each genotype from 4 different litters) and homogenized with 16 strokes using a Teflon-glass homogenizer in 1.3ml ice-cold homogenization buffer (HB) containing 320mM sucrose, 4mM HEPES (pH 7.4), 1mM EGTA, phosphate inhibitors (5mM NaF and 1mM Na_3VO_4) and protease inhibitor mix Complete (Roche). The homogenate was centrifuged at 700g for 10min at 4^oC. The supernatant (S1) was centrifuged again at 12,000g for 15min to obtain synaptosome fraction (P2). For separating synaptic cytosol containing vesicles (LS1) and synaptic membrane (LP1), the pellet (P2) was lysed hypo-osmotically and centrifuged at 35,000g for 20min. Pellet LP1 was resuspended in HB containing 1% TritonX-100. Protein concentration was determined

by Micro BCA protein assay kit (Pierce-Thermo Scientific). Equal amounts of protein (10μg) were loaded in each lane with 5x pink sample buffer (Thermo Scientific) and 5% 2-mercaptoethanol. Following primary antibodies were used for western blotting: Rb anti-NL1 (1:1000,Synaptic Systems), Rb anti-NL2 (1:1000, Synaptic Systems), Rb anti-PSD95 (1:250, Zymed), Ms anti-homer-1(1:1000, Synaptic Systems), Ms anti-SAP102 (1:5 Tissue supernatant, Neuromab), Rb anti-NR2A (1:3000, Millipore), Ms anti-NR1 (1:500, Neuromab), Ms anti-NR2B (1:3 TC, Neuromab), Rb anti-GluR1 (1:400, abcam), Rb anti-GluR2 (1:350, abcam), Gt anti-hevin (1:2000, R&D systems), Ms anti-synaptophysin (1:1000, Synaptic systems) and Ms anti-beta actin (1:5000, abcam). Proteins were detected by Near Infra-red & Chemiluminescent Imaging Technology (LI-COR biosciences) and imaged by Odyssey infrared imaging system. Protein bands in western were quantified by using ImageJ software. Each sample was run 3 times and density was averaged then normalized with their averaged actin intensity. Data is shown as % of WT littermates (Figure S5A).

Peptide Array Synthesis, Fc-tagged Protein Overlay, and Rat anti-Hevin Antibody (12:155) Epitope Identification

To better understand the interactions of hevin with Nrx1α and NL1 and to determine the importance of these interactions in the synaptogenic function of hevin, we next mapped putative Nrx1α and NL1B interacting sites within hevin by using a hevin peptide array. Peptide arrays were constructed as follows: peptides (15-mer) of hevin corresponding to amino acids 17-559 were synthesized and immobilized onto PVDF membranes as previously described (Soderling et al., 2002) with Auto Spot Robot ASP 222 (Intavis AG). Peptide array blots were incubated with Fc-tagged Nrx1α (ECD) at 200nM or NL1B (ECD) at 100nM. Fc-only protein overlays at same

concentrations were used as control. HRP-conjugated anti-human IgG was used for detecting interacting peptide spots. HRP signal was developed by ECL reagent and detected on X-ray films.

Using the same arrays we determined the epitopes for rat anti-hevin monoclonal antibody 12:155. To do so we incubated the peptide array with rat anti-hevin 12:155 at 1µg/ml overnight followed by HRP-tagged goat anti-rat secondary. The peptides that bound 12:155 were detected on X-Ray films following ECL reagent application.

Protein Isolation from Brain Tissue and Western Blotting

Three hevin WT and KO littermates were perfused of blood with TBS at P25. The brains were removed and the V1 visual cortices were dissected while observing under a dissection scope. V1 cortices were isolated from P25 hevin WT and KO littermate mice and flash frozen in liquid nitrogen (n=3 each genotype). To prepare protein lysates for Western blot analyses, the frozen brains were crushed with mortal and pestle in liquid nitrogen. The resulting powder was incubated with 500µl of Tissue Protein Extraction Reagent (TPER, Thermo 78510) plus protease inhibitors at 4^{o}C while rocking for 30 minutes. The lysate was then spun down at 20,000g on a bench top centrifuge for 20 minutes at 4^{o}C to pellet the cell debris. The protein supernatant was collected and quantified using a micro BCA protein assay kit (Thermo 23235), and stored at -20^{o}C. 5µg of protein lysate from each sample was loaded and blotted as described above using the following antibodies: NL1 (rabbit anti-NL1; Synaptic System 129 013), VGluT1 (guinea pig anti-VGluT1; Millipore AB5905), VGluT2 (guinea pig anti-VGluT2; Millipore AB2251) and Tubulin (mouse anti-α-tubulin; Sigma T9026).

***In Vivo* V1 Visual Cortex Protein Injections**

Hevin protein rescue injections were performed as in (Risher et al.) with modifications. Briefly, P13 Hevin KO, and NL1 HET or KO animals were deeply sedated with 200µl of ketamine (150mg/kg)/xylazine (15mg/kg) administered via intraperitoneal injection. Following sedation, the animal was securely situated in a mouse and neonatal rat stereotaxic instrument adaptor (Stoeling). A pulled 30-gauge glass needle was positioned over V1 visual cortex (AP: -2.1mm, ML: -0.35mm, DV: -0.25mm, relative to bregma) using a stereotaxic frame (David Kopf Instruments). 200ng of protein (either Hevin or Hevin-**ΔDE**) was infused slowly over 1.5-2 minutes using a Nanoject (Drummond). Following injections, the skin was sutured shut and the pups were allowed to recover. Pups were taken three days later at P16 for brain harvesting and analysis.

Immunofluorescence and Imaging

Hevin WT/KO, NL1 WT/KO, and Nrx1α WT/KO littermate brain pairs were harvested at P25 following Heparin/TBS and 4% PFA perfusion. Protein injected brains were harvested at P16 and similarly perfused. Virus infected animals were perfused at P30. Brains were submerged in 4% PFA overnight, followed by 30% sucrose/TBS immersion for cryo-protection and were later embedded in 2:1 30% sucrose/OCT. 20µm sections were obtained using a cryostat (Leica). Sections were washed twice in 1x TBS buffer with 0.02% Triton (TBST), then blocked for 1 hour with 5% goat serum in TBST. The sections were then incubated overnight with the following antibodies: NL1 (rabbit anti-NL1; Synaptic System 129 013), hevin (rat anti-hevin monoclonal 12:155) VGluT1 (guinea pig anti-VGluT1; Millipore AB5905), VGluT2 (guinea pig anti-VGluT2; Millipore AB2251), PSD95 (rabbit anti-PSD95; Life Technologies 516900), NeuN (mouse anti-NeuN; Millipore MAB377), MAP2 (rabbit anti-MAP2; Synaptic Systems 188-002), and GFAP (rabbit anti-GFAP; Dako Z0334). Species-specific secondary antibodies were then

incubated with the sections for 2 hours followed by three washes of TBST and finally mounted with DAPI containing mounting media. The hevin antibody has been characterized previously (Kucukdereli et al., 2011) and does not give staining signal in hevin KO brains. Images were acquired using the Leica SP5 or Zeiss 780 confocal microscopes.

Image Analysis

Image analyses to determine co-localization and/or close association of NL1 with hevin or VGluTs were performed in FIJI. Littermate P25 hevin WT and KO mice were stained with NL1 and hevin and VGluT1 or VGluT2. Images corresponding to two-three 3.25μm Z-stacks of 25 optical sections per animal, 3 animals per genotype were imaged for NL1, hevin and VGluT1 or VGluT2. For analysis, each channel (VGluTs, hevin, or NL1) was isolated and divided to create maximum projection images (MPI) of three consecutive optical sections for a total of 5 MPIs per image stack. Every channel of each image was then equally thresholded between genotypes and masked to create a binary image. Background staining particles smaller than 0.035μm^2 were then excluded from each image. The binary image was smoothed and remasked. The number of puncta in each channel was counted and masked onto the other channels. Double and triple overlaying masks were also quantified. To analyze NL1 and MAP2 staining across all cortical layers and compare the changes between hevin WT and KO animals, line scan intensities were obtained using FIJI. Line scans began at the pia and extended 950μm into the corpus callosum. The data were binned at 50μm intervals, averaged, and plotted on a line graph.

Analyses of Intracortical and Thalamocortical Synapses by Immunostaining

For analyses of intracortical and thalamocortical synapse numbers in NL1 WT/KO pairs, Nrx1α WT/KO pairs and hevin protein injected mouse brains, mouse brain sections containing V1

visual cortex were stained with antibodies against VGluT1 or VGluT2 and PSD95 using techniques described above. 5μm-thick Z-stacks images were acquired with Leica SP5 confocal microscope with the 63x-objective. Each Z-stack was converted into 5 MPI by condensing 3 consecutive optical sections using ImageJ. Colocalized puncta counts of intracortical VGluT1/PSD95 and thalamocortical VGluT2/PSD95 were obtained using the ImageJ plugin Puncta Analyzer. For all *in vivo* analyses, 45 MPIs (5 MPI/tissue section, 3 tissue sections/animal, 3 animals/genotype or condition) were analyzed. To determine if the colocalization is above random chance, same analyses were conducted on images where we rotated VGluT1, or VGluT2 channels 90^{o} clockwise (Figure S6A-D). Random chance analysis was similarly performed and verified for stained protein injected experiments (from Figure 6) and demonstrate that VGluT2/PSD95 colocalization is not due to random overlap of puncta (data not shown).

Thalamocortical Axon Tracing

Circuit tracing procedure was performed as previously described (Kim et al., 2015; Risher et al., 2014). NL1 HET/KO and Nrx1α WT/KO P20 mice were deeply anesthetized with intraperitoneal (i.p.) injection of ketamine (150mg/kg) / xylazine (15mg/kg). Using Nanoject (Drummond), mice were stereotactically injected (David Kopf Instruments) with 50nl of EF-1a promoter-driven Flex-AAV-GFP within the dLGN [AP: -2.0mm; ML: 2.0mm; DV: 2.3mm (from brain surface)]. To visualize specific neurons in dLGN that project to visual cortex, 100nl of rabies virus glycoprotein-coated Lenti-FuGB2-Cre (synapsin promoter) was infected into the V1 region of visual cortex [AP: -3.5mm; ML: 2.5mm; DV: 0.3mm (from brain surface)] for retrograde expression of Cre recombinase. Ten days after infection (P30), mice were transcardially perfused with 4% PFA in PBS. Brains were removed, postfixed overnight at 4°C,

and then cryo-protected with 30% sucrose in PBS. Coronal sections were cut (50µm thick) by cryostat (Leica CM 3000) and counterstained with a DAPI (Sigma-Aldrich). After washing three times, the sections were coverslipped with FluorSave (CalBioChem) aqueous mounting medium. For the axonal fiber tracing, images were taken by tile scan imaging using LSM 710 confocal microscope (Zeiss) with a 10x objective under control of Zen software (Zeiss). High magnification images were taken by 20x objective lens (Zeiss).

Generation of AAV virus expressing hevin

AAV expressing hevin plasmid (AAV-gfaABC1D-HevinMycHis) was generated by cloning hevin cDNA into pZac2.1gfaABC1D-cyto-GCaMP3 vector (Addgene Plasmid #44331) between NheI and NotI sites. Hevin cDNA also included IgKappa chain secretion signal at the N-terminus and Myc-His tag at the C-terminus (from pAPtag5 vector). The plasmid was packaged in AAV2/5 virus by Penn Vector Core facility (University of Pennsylvania). AAV2/5 virus together with human GFAP promoter (gfaABC1D) is highly selective for astrocyte infection and transgene expression (Shigetomi et al., 2013).

AAV2/5 Administration

Hevin KO pups at P3 were anesthetized by hypothermia (7min on ice), and 50-100nl of an Adeno Associated Viral vector 2/5 (AAV2/5) expressing the full length Hevin protein was injected into the visual cortex using a Hamilton neurosyringe. After recovery, the pups were returned to their dam and home cage.

Ocular Dominance Plasticity Recordings

At P23-25, Hevin WT, HET, KO and Hevin KO virus-injected mice were anesthetized with isoflurane, and Marcaine (Hospira, Inc. Lake Forest, IL) was topically applied to the scalp incision site. Two burr holes were drilled in each hemisphere of the skull (for the recording electrode and the reference electrode), and tungsten electrodes (FHC, Inc. Bowdoin, ME) were implanted at stereotaxical coordinates corresponding to layer 4 of the binocular zone of the visual cortex (0.5mm rostral from lambda, 3.1mm lateral to the midline, at a depth of 480μm). Reference electrodes were also implanted, at a more rostral location to the recording site (2mm caudal to bregma and lateral to the midline). A nail was secured on the skull, rostral to all electrodes, to immobilize the animal's head during visually evoked potential (VEP) recordings. The nail and electrodes were stabilized on the skull with cyanoacrylate glue. The animal was then allowed to recover on a 37°C heating pad until it could ambulate voluntarily, and then singly housed until the terminal experiment. 0.05mg/kg Buprenorphine (Reckitt Benckiser Healthcare, Ltd. UK) was administered as post-operative analgesia, twice a day for 48 hours. At P26-28, animals were habituated to the VEP equipment and head-fixed position for 30-60 minutes, to avoid movement during VEP recording. The mice were then presented with a visual stimulus to each eye independently, during which VEPs were recorded. Recordings were conducted using XCell-3 amplifiers (FHC, Inc.), a 1401 digitizer (CED, Cambridge, UK), and Spike 2 software (Cambridge Electronics Design, Cambridge, UK). XCell-3 amplifiers were set at a low cutoff frequency of 10Hz and a high cutoff frequency of 100Hz. The visual stimulus consisted of a full-field phase-reversing ordinal sine grating at 0.5Hz with 100% contrast, was controlled by a custom program written in MATLAB (MathWorks, Natick, MA), and was presented at a distance of 21cm from the animal. The angle of the stimulus was changed (45° to 135°) before and after monocular deprivation, to avoid any confounds with respect to stimulus-

selective response potentiation (Cooke and Bear, 2010). Recordings were made of at least 100 stimulus presentations, and peak to trough amplitudes were measured and averaged. Contralateral Bias Indices (CBI) was calculated as the ratio of the contralateral VEP over the ipsilateral VEP response for each animal. This same recording procedure was repeated after 7 days in order to again measure VEPs after monocular deprivation. ODP experiments were performed with a minimum of 5 animals/genotype.

Monocular Deprivation

Soon after the baseline recording was made, animals were anesthetized with isoflurane, and the edges of the upper and lower eyelids were cut. A few drops of proparacine (Akorn, Inc; Lake Forest, IL) were applied as a topical analgesic. 7-0 prolene surgical suture (Ethicon, Inc.) was used to stitch the lids together, and tissue glue (Abbott Laboratories; Chicago, IL) was used to bind the edges together. A layer of cyanoacrylate glue was used to cover the sutures, to prevent the mice from scratching the wound and reopening the eye. Animals were then allowed to recover on a 37° heating pad until able to ambulate voluntarily, after which they were returned to their home cage, where they remained monocularly deprived for a period of 7 days.

Oligonucleotides for RT PCR:

Nrx1α_F: 5`-TGAGAGAGGATGCGAAGGG-3`

Nrx1β_F: 5`-TCCAGTTTGGGAGCGCACC-3`

Nrx1_R: 5`-GAAGGGCTGGCCCTGCTC-3`

NL1A_F: 5`-CTATACTTAAACATCTATGTCCCA-3`

NL1A_R: 5`-GTAGCTTGCCAACACACTCCCATCAT-3`

NL1B_F: 5`-GCTGACTTTATCCCATTATTCTGAA-3`

NL1B_R: 5`-GCTGGAAAGGGCTGTTCCACTCTGA-3`

NL2A_F: 5`-CTGTACCTCAACCTCTACGTGCCC-3`

NL2A_R: 5`-ATAGGCAGCCAGGACTGAGCCGTC-3`

NL3A_F: 5`-TGCCAACTTGGATATCGTCG-3`

NL3A_R: 5`-GTTTAGCACCACTGTCTCGG-3`

Actin_F: 5`-GGGTATGGGTCAGAAGGAC-3`

Actin_R: 5`-TGAAGCTGTAGCCACGCTCG-3`

Oligonucleotides for Site-Directed Mutagenesis:

HevinΔA_F:_5`GACAAAACCTACAGCTTCGAAGGCACAAGTGAGCCTCAACAGAAAA GTCTCCCGGAG-3`

HevinΔA_R:_5`CTGTTGAGGCTCACTTGTGCCTTCGAAGCTGTAGGTTTTGTCCTGCTC TGTTGACTGTTC-3`

HevinΔDE_F:_5`CAGGCGATGACTTCGGTTCTTGCACGAACTTCCAATGTAAAAGGGG ACACATTTGC-3`

HevinΔDE_R:_5`ATTGGAAGTTCGTGCAAGAACCGAAGTAGTCATCGCCTGCACCATG CTTAGAGTCATCC-3`

HevinΔD_F:_5`GCAGGCGATGACTACTTCGGCGGCGAGGAGACAACGACTGGCGAGA

GTGAGAACCGG-3`

HevinΔD_F:_5`GTTGTCTCCTCGCCGCCGAAGTAGTCATCGCCTGCACCATGCTTAGAG

TCATCCCCG-3`

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