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NMDA Receptor Expression by Retinal Ganglion Cells Is Not Required for Retinofugal Map Formation Nor Eye-specific Segregation In The Mouse

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Abstract

Retinal ganglion cells (RGCs) project topographically to the superior colliculus (SC) and dorsal lateral geniculate nucleus (dLGN). Spontaneous activity plays a critical role in retinotopic mapping in both regions; however, the molecular mechanisms underlying activity-dependent refinement remain unclear. Previous pharmacologic studies implicate NMDA receptors (NMDARs) in the establishment of retinotopy. In other brain regions, NMDARs are expressed on both the pre- and post-synaptic side of the synapse, and recent work suggests that pre-synaptic and post-synaptic NMDARs play distinct roles in retinotectal developmental dynamics. To directly test the role of NMDARs expressed by RGCs in retinofugal map formation, we took a conditional genetic knockout approach to delete the obligate GluN1 subunit of NMDARs in RGCs. Here, we demonstrate reduced GluN1 expression in the retina of Chrnb3-Cre;GluN1$^{flox/flox}$ (pre-cKO) mice without altered expression in the SC. Anatomical tracing experiments revealed no **significant** changes in termination zone size in the SC and dLGN of pre-cKO mice, suggesting NMDAR function in RGCs is not **an absolute requirement** for topographic refinement. Further, we observed no change in the eye-specific organization of retinal inputs to the SC nor dLGN. To verify that NMDA induces activity in RGC terminals, we restricted GCaMP5 expression to RGCs and confirmed induction of calcium transients in RGC terminals. Together, these findings demonstrate that NMDARs expressed by RGCs are not required for retinofugal **topographic** map formation nor eye-specific segregation in the mouse.

Significance Statement

Topographic organization of retinal inputs in the brain is **thought to be** critical for the efficient relay of spatial information in the visual scene. Previous studies suggest NMDARs play a crucial role in establishing topography in the superior colliculus; however, these studies could not distinguish between potential pre- or post-synaptic roles. Here, we show NMDAR function in retinal ganglion cells (RGCs) is not required for the establishment of topography. Further, we find RGC NMDARs are not required to establish or maintain eye-specific laminae in retinorecipient regions.

Introduction

Retinal ganglion cells (RGCs) project to two **main** image forming regions, the superior colliculus (SC) and the dorsal lateral geniculate nucleus (dLGN), where their axon terminals are organized topographically. The establishment of topography occurs in a protracted process during the first week of postnatal life in the mouse (Johnson & Triplett, 2021). Initially, diffuse terminations are refined to topographically appropriate locations in a manner dependent on a combination of molecular cues (Feldheim & O'Leary, 2010), axon-axon competition (Triplett et al., 2011), and neuronal activity (McLaughlin et al., 2003; Pfeiffenberger et al., 2006).

The activity driving retinofugal projection refinement is spontaneous, consisting of highly correlated bursts of action potentials, termed retinal waves, that propagate across the retina (Meister et al., 1991; Wong et al., 1993) and are transferred to downstream areas (Ackman et al., 2012). Retinal waves progress through three stages based on their mode of propagation, mediated first by gap junctions, then acetylcholine, and finally glutamate. Disruption of cholinergic waves perturbs retinotopic map formation in the SC and dLGN (McLaughlin et al., 2003; Chandrasekaran et al., 2007; Cang et al., 2008; Xu et al., 2015). While it is clear the normal pattern of retinal waves is critical for topographic map formation in the SC and dLGN, the molecular mechanisms by which activity mediates these processes remain unclear.

N-methyl-*D*-aspartate receptors (NMDARs) are ionotropic glutamate receptors widely expressed throughout the brain and play a critical role in activity-dependent synaptic strengthening (Nicoll & Malenka, 1999). Previous studies suggest a critical role for NMDARs in the establishment of retinocollicular connectivity. Indeed, pharmacological blockade showed a disruption in retinocollicular map organization when applied locally to the SC (Simon et al., 1992) or tectum (Cline & Constantine-Paton, 1989). Further, the receptive field size of SC neurons was increased upon chronic blockade, and lesion-induced plasticity was disrupted (Huang & Pallas, 2001). Intriguingly, NMDAR blockade resulted in dramatic changes to retinal arborization dynamics (Rajan et al., 1999; Ruthazer et al., 2003; Munz et al., 2014), suggesting

a potential role for NMDARs localized to RGC terminals. However, pharmacologic studies of retinotopy could not elucidate the neuronal populations in which NMDAR activity was required.

While best studied for their function at the post-synaptic side of the synapse, accumulating evidence suggests that NMDARs may also be expressed pre-synaptically in many brain regions (Pittaluga & Raiteri, 1990; Aoki et al., 1994; and Paoletti et al., 2013; Bouvier et al., 2015; Bouvier et al., 2018), including visual cortical circuits where they mediate spike-timing-dependent plasticity of connections between neurons in layer 4 (L4) and L2/3 (Corlew et al., 2007; Bouvier et al., 2018). Previous studies suggest that developing RGCs express NMDARs raising the possibility they could be localized pre-synaptically in retinorecipient regions (Massey & Miller, 1990; Mittman et al., 1990; Watanabe et al., 1994). Indeed, recent work demonstrated that pre- and post-synaptic NMDARs have distinct but complementary roles in developmental plasticity in the retinotectal system (Kesner et al., 2020). However, whether NMDARs expressed by RGCs are required for topographic refinement remains unclear.

To address this, we took a conditional genetic approach to delete the obligate GluN1 subunit of NMDARs in RGCs without altering its expression in retinorecipient regions, allowing us to determine the potential role of pre-synaptic NMDARs in retinocollicular and retinogeniculate circuit formation. We confirmed a reduction of GluN1 expression in the retina of Chrnb3-Cre;GluN1$^{flox/flox}$ (pre-cKO) mice by *in situ* hybridization and quantitative PCR. Surprisingly, neither the topographic refinement nor the eye-specific segregation of retinal inputs in the SC and dLGN were altered in pre-cKO mice, suggesting a minimal role for NMDAR function in RGCs in these processes. To probe whether NMDARs might be activated pre-synaptically, we prepared slices of the SC from mice expressing a genetically-encoded GCaMP5 restricted primarily to RGCs. Indeed, administration of NMDA induced modest Ca^{2+} transients, suggesting that NMDARs may be localized pre-synaptically in the mouse SC. Together, these data suggest a limited role for NMDARs expressed by RGCs in the development and maintenance of ordered projections to image-forming retinorecipient regions.

Materials and Methods

Mice

Adult and juvenile mice of either sex were used. Their ages ranged between postnatal day (P) 2-12 or P25-60. The Chrnb3-Cre transgenic mouse line, described previously (Drayson & Triplett, 2019), was obtained (MMRRC 036469-UCD) and genotyped with two primers against Cre (GTC-CAA-TTT-ACT-GAC-CGT-ACA-CC and GTT-ATT-CGG-ATC-ATC-AGC-TAC-ACC). Mice harboring a floxed allele of the Grin1 gene ($GluN1^{flox}$) and GCaMP5-IRES-tdTomato reporter mice were generated and genotyped as described previously (Tsien et al., 1996; and Gee et al., 2014). All animals were housed in the research animal facility at Children's National Research Institute, and all experimental procedures were approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Mice were anesthetized on ice (< P8) or with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) (>P9) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (pH 7.4). Brains and eyes were dissected and post-fixed in 4% PFA at 4°C overnight or 30 min, respectively. After post-fixation, brains and eyes were briefly washed in PBS before cryoprotection in 30% (brains) or 10% (eyes) sucrose at 4°C for 24-48 hrs. Tissues were then embedded in O.C.T. compound (Tissue-Tek 4583) and cooled in a -80°C freezer prior to being sectioned with ThermoScientific Micron HM 525 cryostat. Sections of 20 µm (brain) and 14 µm (eye) were collected directly onto SuperFrost Gold Plus microscopy slides (Fisher Scientific) and dried overnight at room temperature. Sections were incubated in blocking solution (1% serum, 0.25% Triton X-100) for 1 hour at room temperature and then incubated with primary antibodies diluted in blocking solution at 4°C overnight. The following primary antibodies were used: Anti-Beta 3 (Santa Cruz SC-6045, RRID: AB_2065343), Anti-Brn-3a (Santa Cruz SC-31984, RRID: AB_2167511), Anti-Calretinin (Millipore AB1550,

RRID: AB_90764), Anti-RBPMS (PhosphoSolutions 1830-RBPMS, RRID: AB_2492225), and Anti-Satb2 (ABcam AB34735, RRID: AB_2301417). Sections were washed thoroughly in PBS and then incubated with appropriate secondary antibodies (Biotium, RRIDs: AB-2534102, AB-162543) and DAPI diluted in blocking solution for 1 hour at room temperature. Confocal images were acquired at 20X magnification with an Olympus FV1000 microscope, with an Olympus DP71 digital camera attached. Images were analyzed and processed with FIJI (Schindelin et al., 2012). For each genotype, we averaged the number of counted cells over three different retinal sections from each of three animals. Data were analyzed, and graphs were constructed with GraphPad Prism8. All error bars represent the standard error of the mean (SEM), and statistical analysis was determined using the Mann-Whitney Rank Sum test.

In situ *hybridization*

Tissue was collected and fixed as described above. Complementary DNA for GluN1 [containing nucleotides 25296659-25298429 of the open reading frame (ORF)] was used to make antisense and sense digoxigenin-labeled RNA probes and recognize exons 11 to 16. Slides were pretreated with PBS at room temperature for 5 minutes to rehydrate the slides prior to being fixed with 4% PFA (pH 7.4) for 15 min. Slides were also pretreated with proteinase K (1 µg/mL) to increase hybridization efficiency. Prior to being treated with the RNA probe, slides had 300 µL of hybridization buffer (50% formamide, 5X SSC pH: 4.5, 1% SDS, 50 µg/mL yeast tRNA, 50 µg/mL heparin) covered with parafilm, and incubated at 70°C for 1 hour. RNA probes were diluted 1:200 in hybridization buffer and placed onto the slides, covered with parafilm, and incubated at 70°C overnight. Slides were washed and blocked with TBST/HISS (1X TBS, 1% Tween-20, and 5% HISS) for 1 hour at RT. Antibodies against DIG were diluted (1:2000) in TBST/HISS, and 200µL was placed on each slide for 4°C overnight. Slides were then washed four times for 15 minutes with TBST and then washed with NTMT 3 times for 5 minutes. Slides

were then treated with 200µL of BMPurple (Roche) and developed for 12 hours at RT. Images were acquired at 4X, 10X, and 20X magnifications with a brightfield Olympus BX61 microscope.

Quantitative PCR

Total RNA was isolated from micro-dissected SC and the whole retina using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad #7326830). Synthesis of cDNA was carried out using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). qPCR was performed on a CFX96 real-time system (Bio-Rad #1708890) in a 20-µl reaction mixture using SsoAdvanced Universal SYBR Green PCR master mix (Bio-Rad). Cycle parameters were 3 s at 95 °C and 30 s at 60 °C. Data were normalized to housekeeping gene18S. GluN1 primers: 5′-CCAGATGTCCACCAGACTAAA -3′ and 5′-CCATTGACTGTGAACTCCTCTT-3′ (Set 1), 5′-AAGGAGTGGAACGGAATGATG-3′ and 5′-GGCTTGGAGAACTCTATGTACTG-3′ (Set 4), 5′-GTAGCTGGGATCTTCCTCATTT-3′ and 5′-TTCTTCCTCCACACGTTCAC-3′ (Set 5). 18S primers: 5′-CTTTGTCAAGCTCATTTCCTGG-3′ and 5′-TCTTGCTCAGTGTCCTTGC-3′. Data were analyzed using the comparative CT method, and graphs were constructed with GraphPad Prism8 (Schmittgen & Livak, 2008). All error bars represent the SEM, and statistical analysis was determined using an unpaired student t-test.

Anterograde RGC axon labeling

Adult mice (P25-60) were anesthetized by intraperitoneal injection with a ketamine/xylazine cocktail (100/10 mg/kg). Additionally, adult mice were given Buprenex (0.3 mg/kg) for analgesia. Pups (P0-P10) were anesthetized on ice. For focal or bulk labeling of RGCs, a 10% solution of lipophilic dye DiI (1,1'-dioctodecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate) in dimethyleformamide (DMF) or a 2% solution of cholera toxin subunit B (CTB) 488 and 555 in PBS, respectively, was injected using a pulled-glass micropipette attached to a Picospritzer III (Parker-Hannifin). The glass micropipette was

inserted into the retina of the anesthetized animal, and ~100 nL of DiI solution was injected into one eye or ~500 nL CTB-488 was injected into the left eye, and CTB-555 was injected into the right eye. Animals recovered for 1 week (adults) or 2 days (pups) before being euthanized and their brains post-fixed in 4% PFA overnight as described above. The termination zone (TZ) of DiI-labeled RGCs was visualized in whole mount via epifluorescent microscopy. Brains were then embedded in 2-3% agarose and sectioned coronally at 150 microns on a vibratome. The dLGN and SC were visualized at 1.25X magnification via epifluorescent microscopy and analyzed using FIJI.

Image analysis

To determine topographic refinement of TZs, we calculated a termination zone index (TZI), for which the size of the TZ in the SC or dLGN, expressed as a percent of the target area, was divided the injection size, expressed as a percent of the flat-mounted retina. For the dLGN, we quantified the TZI for all sections containing the TZ and determined an average across sections for each animal. The TZIs were statistically analyzed by running a student's t-test with GraphPad Prism8. To assess eye-specific segregation, each coronal section that contained labeled retinal terminals **was** assessed independently using FIJI, and the average across all sections was used. The boundaries of the dLGN/SC were outlined on a grayscale 8-bit image, and the background was cleared before measuring the size of the dLGN/SC. The areas of ipsilateral and contralateral retinal innervation were determined independently, and the overlapping co-localized pixels were then analyzed using the 'AND' function of FIJI's image calculator. The measurement of the overlap**/ipsi area, overlap/total dLGN, and ipsi patch length** was then statistically analyzed by running a student's t-test or 2-way ANOVA with GraphPad Prism8 for adult and developmental ages, respectively. All error bars represent SEM. **Outliers were identified by running a Grubb's test (outlier test) with GraphPad's Outlier Calculator and removed from analysis.**

Acute brain slice preparation

Chrnb3-Cre;GCaMP5-tdTomato pups aged P2-6 of either sex were used. Pups were decapitated, and the brains were rapidly removed and immersed in ice-cold cutting solution (230 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.04 mM Na-Ascorbate, and 10 mM glucose, pH 7.2 to 7.4). Coronal and sagittal slices (300 μm) were cut with a vibratome (Leica VT1000S) and transferred to artificial cerebral spinal fluid (aCSF) (126 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.04 mM Na-Ascorbate, and 10 mM glucose, pH 7.2 to 7.4, osmolarity = 310 mOsm/L) bubbled with 95% O_2 and 5% CO_2 or into $MgCl_2$-free aCSF (126 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.04 mM Na-Ascorbate, and 10 mM glucose, pH 7.2 to 7.4, osmolarity = 310 mOsm/L) bubbled with 95% O_2 and 5% CO_2). Slices recovered in oxygenated aCSF for 1 hour at room temperature (21° to 25°C) before acute slice imaging. During recordings, slices were placed in a perfusion chamber and superfused with oxygenated aCSF at room temperature for the duration of the experiment. The cells were visualized with a 20X immersion objective (Olympus Optical, New York, NY) and epifluorescence.

Ca^{2+} imaging and analysis

Ca^{2+} imaging was performed with an Olympus (Tokyo, Japan) FluoView FVMPE-RS Multiphoton Microscope imaging system using FluoView software and a Ti:Sapphire laser source emitting 140 fs pulses at an 80 MHz repetition rate with a wavelength adjustable for 690-1040 nm (Maitai DeepSee pulsed, infrared laser). Full-field of view images **were** acquired with XY raster scanning using the 20X 0.95 NA water-immersion objective. Changes in fluorescence (ΔF) was quantified using ImageJ (NIH) software and expressed as a percentage of baseline (%ΔF/F). Time-lapse images of neuron Ca^{2+} signaling were recorded at a frame rate of 1Hz. ROIs will be selected based on the appearance of GCaMP5G Ca^{2+} transients in the time-lapse

images. To trigger Ca^{2+} transients, the agonist NMDA (50 µM) and control K^+ (mM) were dissolved in aCSF and delivered locally by a pressure pulse (10 psi; 100-500 ms) using a Picospritzer III (Parker Instrumentation, Chicago, IL) while the antagonist MK-801 (10 µM) was delivered via bath perfusion. To avoid potential artifacts due to K^+ administration, it was always the last agent tested in any given slice. Data were analyzed, and graphs were constructed with GraphPad Prism8. All error bars represent the SEM, and statistical analysis was determined using a one-way ANOVA followed by Tukey's multiple comparisons test, as indicated in the figure legend. $^{**}p < 0.01$, $^{****}p < 0.0001$, Not Significant (N.S.) $p > 0.05$.

Results

RGC-specific loss of GluN1 expression in Chrnb3-Cre;GluN1$^{flox/flox}$ mice

To study the RGC-specific role of NMDAR function during topographic map formation, we took a conditional genetic approach. We crossed the Chrnb3-Cre line, in which Cre recombinase is expressed broadly by RGCs but not in retinorecipient areas such as the SC or dLGN (Drayson & Triplett, 2019), with a line harboring a floxed allele of the *Grin1* gene coding for the GluN1 subunit of the NMDAR (GluN1^{flox}), in which exon 11 through the 3' end are flanked by loxP sites (Tsien et al., 1996) to generate pre-cKO mice. Previous reports suggest that recombination of this locus results in the complete loss of NMDAR function (Tsien et al., 1996).

In the transgenic Chrnb3-Cre mouse line, the majority of expression is observed in the ganglion cell layer (GCL) and distributed broadly across the retina (Drayson & Triplett, 2019). Indeed, we observed that when Chrnb3-Cre mice are crossed with β-galactosidase (LacZ) and tdTomato (tdTom) reporter lines, LacZ expression is observed throughout the retina (Fig. 1A), and tdTom expression is restricted to the GCL (Fig. 1B). In order to determine if our genetic strategy was valid, we performed RNA *in situ* hybridization for *GluN1* mRNA in P4 retinal tissue. In control retinas (from mice that were genotyped as Cre^{-} and either GluN1$^{fl/+}$ or GluN1$^{fl/fl}$, [Ctl]), the *GluN1* anti-sense probe produced a strong signal in cell bodies throughout the GCL and INL (Fig. 1C). These findings are consistent with previous reports in which nearly all RGCs expressed the GluN1 subunit (Brandstätter et al., 1994). Strikingly, little to no *GluN1* mRNA expression was detected in the retinas of P4 pre-cKO mice (Fig. 1D), suggesting we were able to successfully ablate GluN1 from the GCL in our experimental animals during the first postnatal week. However, some GluN1 expressing cells in the GCL and IPL were observed (Fig. 1D arrowhead); these cells may be the small population of RGCs not targeted in the Chrnb3-Cre line or displaced amacrine cells (Drayson and Triplett, 2019). Notably, signal from the *GluN1* anti-sense mRNA probe did label cell bodies throughout the SC of both Ctl and pre-cKO brains (Fig. 1E & F), suggesting NMDAR function remains intact in this region.

297 To further demonstrate that *GluN1* expression was reduced in pre-cKO retinas, we
298 performed RT-qPCR comparing the SC and retina of Ctl and pre-cKO animals at P0 using three
299 primer sets (Fig. 1H & I). Primer set 1 is located at exon 9, upstream of the flanking site of the
300 GluN1 gene. Primer set 4 is located near exon 12, just after the flanking site. Primer set 5 is
301 located near exon 19, towards the end of the GluN1 gene (Fig. 1G). This analysis confirmed
302 that our pre-cKO animal had a knockdown of GluN1 expression in the retina with both set 4 and
303 5 (Set 4 Ctl: 1.020 ± 0.09136, n = 6; Set 4 pre-cKO: 0.4604 ± 0.03565, n = 8; $p < 0.001$,
304 student's t-test; Set 5 Ctl: 1.007 ± 0.05547, n = 6; Set 5 pre-cKO: 0.1029 ± 0.04445, n = 8; $p <$
305 0.001, student's t-test) (Fig. 1H). As expected, we saw no change in relative expression when
306 analyzing primer set 1, since the region amplified by this set was not ablated (Set 1 Ctl: 0.8573
307 ± 0.03889, n = 6; Set 1 pre-cKO: 0.9283 ± 0.04024, n = 8; $p = 0.2134$, student's t-test) (Fig. 1I).
308 Additionally, the qPCR analysis with all three primer sets confirmed that GluN1 expression was
309 unchanged in the SC (Set 1 Ctl: 0.8259 ± 0.05876, n = 6; Set 1 pre-cKO: 0.7911 ± 0.07140, n =
310 8; $p = 0.8876$, student's t-test; Set 4 Ctl: 0.7815 ± 0.07300, n = 6; Set 4 pre-cKO: 0.8136 ±
311 0.04889, n = 8; $p = 0.9100$, student's t-test; Set 5 Ctl: 0.8128 ± 0.08384, n = 6; Set 5 pre-cKO:
312 0.6149 ± 0.08005, n = 8; $p = 0.0954$, student's t-test) (Fig. 1H). Together, these data indicate
313 neurons in the GCL of the retina express *GluN1* during developmental stages and demonstrate
314 this expression is dramatically reduced in pre-cKO retinas, but not the SC.
315
316 *Cytoarchitecture of the retina is unaffected in the absence of GluN1 expression in RGCs*
317 Previous studies suggest a critical role for NMDA signaling in the survival of RGCs
318 (Shen et al., 2006). Thus, we wanted to determine if RGC-specific deletion of GluN1 altered the
319 number of RGCs or morphological organization of the retina. To begin, we performed
320 immunohistochemistry for markers of different retinal cell types. First, we analyzed the RGC
321 markers RBMPS and Brn3a, since a substantial proportion of Chrnb3-Cre-tagged RGCs
322 expresses Brn3a (Drayson & Triplett, 2019) (Fig. 2A-B & F-G). The number of RBPMS labeled

cells in a 500 x 500 μm field of the retina for pre-cKO animals (258.7 ± 10.48, n = 3) was not significantly different from controls (263.0 ± 9.644, n = 3) ($p > 0.9999$, Mann-Whitney test) (Fig. 2K). The number of Brn3a labeled cells in a 500 x 500 μm field of the retina for pre-cKO animals (122.7 ± 5.239, n = 3) was not significantly different from controls (119.3 ± 5.783, n = 3) ($p > 0.9999$, Mann-Whitney test) (Fig. 2L). Similarly, we detected no significant difference in the number of cells labeled with SatB2, a putative marker of direction-selective RGCs (Sweeney et al., 2019) (Ctl: 68.0 ± 1.732, n = 3; pre-cKO: 73.0 ± 2.082, n = 3; $p = 0.2000$, Mann-Whitney test) (Fig. 2M). In order to further examine the morphology of the retina, we stained for Beta3, a marker for Off bipolar cells, and calretinin, which labels a variety of amacrine cells and RGCs, including their processes in the inner plexiform layer. As with RBPMS, Brn3a and SatB2, we found no significant difference in number of cells expressing Beta3 nor calretinin between control and pre-cKO retinas (Beta 3 Ctl: 163.0 ± 7.371, n = 3; Beta3 pre-cKO: 155.3 ± 15.24, n = 3; $p > 0.9999$, Mann-Whitney test; calretinin Ctl: 134.7 ± 7.767, n = 3; calretinin pre-cKO: 134.7 ± 3.512, n =3; $p > 0.9999$, Mann-Whitney test) (Fig. 2D-E, I-J, & N-O). Further, the organization of Beta3 and calretinin-stained processes in the inner plexiform layer in pre-cKO retinas were grossly organized similarly to those in Ctl retinas. Overall, these data suggest **that neither** the population of RGCs nor cytoarchitecture of the retina is adversely impacted due to a loss of NMDAR function in RGCs.

Expression of the GluN1 subunit of the NMDAR in RGCs is not required in topographic refinement

We next tested our hypothesis that NMDAR expression in RGCs is required for the establishment of retinofugal topography utilizing the focal DiI tracing technique in adult animals (P25-60), as previously described (Kay et al., 2018). In every animal, the axonal projections from the focal DiI injection into the retina showed a topographically appropriate termination zone

(TZ) in the SC of adult Ctl and pre-cKO mice (Fig. 3A & B). Previous studies in which NMDAR function was disrupted pharmacologically suggested that while labeled RGCs terminate in roughly the appropriate topographic zone, the size of the termination field was increased (Simon et al., 1992). To determine if TZ size was altered in pre-cKO mice, we calculated the termination zone index (TZI) by normalizing the TZ size by the injection site size. We observed no change in TZI in mice lacking GluN1 in RGCs (TZI: Ctl: 8.806 ± 2.738, n = 6; pre-cKO: 11.13 ± 3.597, n = 9; p = 0.6481, student's t-test) (Fig. 3C). Further, we did not observe stray arbors that might indicate subtle deficits not detectable by quantification of TZI (Fig. 3A' & B'). These data suggest NMDAR function in RGCs is not required for retinocollicular refinement.

In addition to the SC, RGCs project topographically to the dLGN, where spontaneous activity plays a critical role in establishing topography, **along with** molecular cues (Pfeiffenberger et al., 2006). Therefore, in addition to testing the retinocollicular refinement of our transgenic animals, we observed the retinogeniculate refinement within the same DiI injected animals. **Topographic refinement was not significantly altered, though there appeared to be a trend toward a reduction in TZI in pre-cKO animals** (TZI: Ctl: 6.229 ±1.308; pre-cKO: 3.141 ± 0.6921; p = 0.0520, student's t-test) (Fig. 3D-F). Together, these data suggest expression of GluN1 in RGCs is not required for the development of retinogeniculate map refinement.

Eye-specific segregation in the SC and dLGN

While retinofugal topography appeared unchanged in the absence of GluN1 expression in RGCs, we reasoned that other developmental processes in visual circuit development that are more reliant on activity-dependent mechanisms may be altered. The segregation of eye-specific inputs in visual areas has served as a classical model to demonstrate the role of both spontaneous activity and visual experience in circuit development and plasticity (Feller, 2009). Indeed, it has been established that during eye-specific segregation in the LGN, large-scale

refinement takes place. Glutamatergic waves generated in the retina are critical **for** maintenance of segregation (Demas et al., 2006), and NMDAR blockade in *in vitro* preparations alters the frequency of glutamatergic waves, but not other attributes, such as velocity (Blankenship et al., 2009). Further, NMDARs have been implicated in the segregation of artificially-induced eye-specific inputs in the frog tectum (Cline & Constantine-Paton, 1990).

In order to determine if the expression of GluN1 in RGCs is critical for appropriate eye-specific segregation, we intraocularly injected fluorescently-labeled cholera toxin B subunit (CTB)-488 in the left eye and CTB-555 in the right eye and observed their terminations in the dLGN. As expected, in both control and pre-cKO adults, the majority of the dLGN was occupied by contralateral projections except for the dorsomedial region where the ipsilateral projections terminate (Fig. 4A-F). We analyzed eye-specific segregation by calculating the area of overlap between contralateral and ipsilateral projections in relation to the area occupied by ipsilateral projections and found no significant difference between control and pre-cKO adult mice (Overlap/Ipsi: control: 34.18 ± 2.958, n=11; pre-cKO : 38.08 ± 2.172, n=10; p = 0.3090, student's t-test) (Fig. 4G).

Next, we analyzed the overlap in the SC of these animals and found that eye-specific segregation was not significantly different between the SCs of pre-cKO and control adult animals (Overlap/Ipsi: Ctl: 2.382 ± 0.2595, n=11; pre-cKO: 2.004 ± 0.2784, n=10; p = 0.3320, student's t-test) (Fig. 5A-C). Although eye-specific segregation is heavily dependent on activity, these data suggest GluN1 expression in RGCs is not required in order to achieve the mature segregation of eye-specific inputs in the LGN, consistent with previous studies (Hahm et al., 1991; Smetters et al., 1994).

We next wondered if the developmental trajectory of eye-specific segregation might be altered in the absence of GluN1 expression in RGCs. We tested this possibility by analyzing the overlap **of contralateral and** ipsilateral projections **in** pups (P2-P12) (Fig. 6). At **P4**, the retinogeniculate axons from the two eyes are not well separated, as expected (Fig. 6A & D).

However, over time, eye-specific segregation became more and more refined by the end of the second post-natal week (Fig. 6B-C & E-F). As expected, we found a main effect of age in the amount of overlap between ipsi- and contra-RGC terminals in the dLGN**, when calculated as a proportion of the ipsi-RGC domain** ($p < 0.0001$, 2-way ANOVA). Interestingly, we found that overlap was different between each age group ($p < 0.05$, Tukey's multiple comparisons test) except for between P6 and P8 ($p = 0.7511$). These data suggest paradoxical increases in overlap from P2 to P4 and P10 to P12 in our dataset, but the general trend is a decrease in overlap over time, consistent with previous data (Pfeiffenberger et al., 2005). However, we did not find any effect of genotype ($p = 0.1657$, 2-way ANOVA) nor any interaction between age and genotype ($p = 0.1516$, 2-way ANOVA) **(Fig. 6G). We next wondered if the increase in overlap was due to changes in the size of the ipsi-RGC domain in the dLGN. To do so, we calculated the amount of overlap of ipsi- and contra-RGC terminals as a proportion of the total size of the dLGN. Again, we observed a main effect of age ($p < 0.0001$, 2-way ANOVA), but found no effect of genotype ($p = 0.4183$) nor any interaction between age and genotype ($p = 0.1981$) (Fig. 6H). Interestingly, we found significant differences between all age groups ($p < 0.0001$, Tukey's multiple comparisons test) except when comparing P6 to P8 ($p = 0.6827$) and P10 to P12 ($p = 0.1773$). Lastly, we analyzed the ipsilateral patch length over the length of the dLGN (Fig. 6I). Similar to our analyses of overlap, we found a main effect of age ($p < 0.0001$, 2-way ANOVA), but not genotype ($p = 0.8871$) nor any interaction the two ($p = 0.6740$). For this metric, we found significant differences when comparing P2 or P4 to all ages ($p < 0.05$), when comparing P6 to P10 and P12 ($p < 0.05$), and a trend towards a difference when comparing P6 to P8 ($p = 0.0740$).** Altogether, these data suggest NMDAR activity in RGCs is not required for the development or maintenance of eye-specific segregation**, but reveal age-dependent effects on eye-specific segregation.**

426 *Activation of NMDA-mediated response in RGC terminals in retinorecipient areas*

427 Thus far, our data suggest that retinofugal development is not dependent on NMDAR
428 expression in RGCs. One possibility for this could be that pre-synaptic NMDARs may not be
429 located in the terminals of RGCs in these retinorecipient centers, contrary to what has been
430 suggested in the frog tectum (Kesner et al., 2020). To test this, we crossed the Chrnb3-Cre line
431 with Cre-dependent GCaMP5G::tdTom reporter mice (Gee et al., 2014) and performed Ca^{2+}
432 imaging in retinorecipient areas, where only RGC terminals would be labeled. We began by
433 testing the activity of RGC axons in the SC with NMDA at 50mM and 100mM, and found that an
434 elicited calcium response could be visualized in Mg^{2+}-free aCSF, but not in aCSF (Fig. 7A-C &
435 E) (NMDA in Mg^{2+}-free aCSF: 28.48 ± 3.962, n = 6; NMDA in aCSF: -3.907 ± 0.9898, n = 5, p <
436 0.0001, Tukey's *post hoc* test). Importantly, this response was significantly smaller than that
437 observed when we administered 5mM K^+ stimulation as a positive control (Fig. 7D & E) (K^+
438 stimulation in aCSF: 44.10 ± 1.482, n = 3, p = 0.0060, Tukey's *post hoc* test), suggesting that
439 NMDA application was not driving wholesale activation of RGCs, but rather specific activation of
440 NMDARs on RGC terminals. Indeed, when we administered 50mM and 100mM of NMDA in the
441 presence of the specific NMDAR antagonist, MK-801, the calcium response was ablated (Fig.
442 7C & E) (NMDA in Mg^{2+}-free aCSF + MK-801: 3.057 ± 0.3887, n = 6, p < 0.0001, Tukey's *post*
443 *hoc* test). We observed a similar pattern of activation of RGC terminals in the dLGN (data not
444 shown), consistent with previous data demonstrating that most RGCs that project to the dLGN
445 also project to the SC (Dhande et al., 2011). Overall, these data suggest that NMDA-mediated
446 activity is elicited by direct stimulation of RGC terminals in the SC, supporting the presence of
447 pre-synaptic NMDARs at retinocollicular synapses.

448 **Discussion**

449 During the development of the visual system, RGCs undergo extensive remodeling
450 mediated by a combination of molecular cues, axon-axon competition and neuronal activity in
451 order to develop precise terminations in retinorecipient regions. Spontaneous activity in the form
452 of retinal waves helps ensure retinocollicular and retinogeniculate refinement; however, the
453 specific mechanisms by which activity mediates these processes remain unclear. Here, we
454 tested the role of NMDARs expressed by RGCs in the development of precise retinofugal
455 projection organization in the mouse. We found that our novel conditional genetic approach
456 successfully ablated NMDARs from RGCs without altering gross retinal organization or
457 expression in the SC. Anatomical tracing experiments revealed no changes in topographic
458 refinement nor eye-specific segregation in either the SC or dLGN. This is the case despite the
459 fact that we observed NMDA-elicited Ca^{2+} transients in RGC terminals in retinorecipient regions.
460 Together, these data demonstrate that NMDARs expressed by RGCs are not required for
461 topographic refinement nor eye-specific segregation in image-forming retinorecipient regions
462
463 *NMDARs expressed by RGCs are not required for retinotopy*
464 Previous studies demonstrated that neuronal activity mediated through NMDARs plays
465 an important role in establishing the topography of retinocollicular projections. When NMDARs
466 were chronically blocked during SC development, mRNA levels of GluN1 are decreased (Hofer
467 et al., 1994); additionally, proper activity level of NMDARs is required for the appropriate
468 development and refinement of the retinofugal map (Cline & Constantine-Paton, 1989; Simon et
469 al., 1992; King et al., 1996; and Mize & Butler, 2000). Further, terminal arbors of RGCs in the
470 optic tectum exhibit robust dynamics, which are disrupted when NMDAR function is blocked
471 (Rajan et al., 1999; Ruthazer et al., 2003; Munz et al., 2014). And NMDARs play a critical role in
472 the plasticity required for synaptic convergence following map compression in the SC (Huang &
473 Pallas, 2001). However, these pharmacological studies could not distinguish between potential

474 contributions of pre-synaptic or post-synaptic NMDAR activity in these processes, each of which
475 have been implicated in plasticity in other brain regions (Paoletti et al., 2013).
476 To overcome this limitation, we developed a conditional knockout model to directly
477 determine if NMDARs expressed by RGCs play a role in map formation. In the SC of pre-cKO
478 mice, we did not observe alterations in the size of TZs from labeled RGCs, demonstrating that
479 NMDARs expressed by these neurons are not required for topographic refinement in this region.
480 Interestingly, these data are somewhat inconsistent with recent work in which sparser
481 retinotectal terminals were observed when GluN1 expression was knocked down specifically in
482 RGCs (Kesner et al., 2020). However, the total size of the terminal arbor may have been less
483 dramatically impacted, as no change was observed in total terminal branch length. This result is
484 consistent with previous studies leveraging pharmacologic NMDAR blockade, in which
485 disruptions of RGC axonal arbor dynamics were observed (Rajan et al., 1999; Ruthazer et al.,
486 2003; Munz et al., 2014), but the overall mature organization of arborizations was not
487 dramatically altered (Cline & Constantine-Paton, 1989). Indeed, functional analyses revealed no
488 changes in overall receptive field size when GluN1 expression was knocked down in RGCs,
489 consistent with minimal change in topography (Kesner et al., 2020). One possible reason for a
490 lack of phenotype in terms of topographic refinement may be that the Cre line chosen is
491 expressed in only **~65%** of RGCs (Drayson & Triplett, 2019). However, our qPCR data suggest
492 a substantial knockdown of all retinal expression of *GluN1*, suggesting that the vast majority of
493 RGCs lack expression in this model. **Intriguingly, we did observe a trend towards a**
494 **decreased TZI for retinogeniculate projections, which would be consistent with sparser**
495 **terminals reported for retinotectal projections lacking NMDARs in RGCs (Kesner et al.,**
496 **2020). This raises the intriguing possibility that pre-synaptic NMDARs may play distinct**
497 **roles in circuit formation in the SC and dLGN. However, more sophisticated analyses of**
498 **retinal convergence in the SC and dLGN are needed to draw firm conclusions regarding**
499 **the context-dependent roles of pre-synaptic NMDARs.**

NMDARs in RGCs are not required for eye-specific segregation

The establishment of eye-specific organization in the visual system has served as a model to understand the mechanisms underlying circuit development and plasticity (Hensch & Quinlan, 2018; and Arroyo & Feller, 2016). Intriguingly, previous studies in which eye-specific segregation was induced in the context of retinotectal projections in frogs (Constantine-Paton & Law, 1978) suggested activation or inhibition of NMDAR function could enhance or disrupt segregation, respectively (Cline & Constantine-Paton, 1990). Here, we found no changes in the mature organization of eye-specific lamina in the SC nor dLGN when NMDAR function was disrupted in RGCs. **Although, as noted for the lack of phenotype observed for retinocollicular projections, the fact that not all RGCs are targeted in Chrnb3-Cre mice could mask a potential role for pre-synaptic NMDARs in eye-specific segregation.** Further, we did not observe alterations in the developmental trajectory of segregation in the dLGN between genotypes **in any of our analyzed parameters**. Interestingly, we did observe increases in overlap **as a percent of the ipsi-RGC domain** from P2 to P4 and P10 to P12. **The increased overlap from P2 to P4 may be driven by the fact that ipsi-RGC innervation of the dLGN is not complete until P4 (Godement et al., 1984). One explanation for the increase overlap observed between P10 to P12 could be a reduction in the number ipsi-RGC prior to eye opening. Indeed, when we analyzed overlap as a proportion of the size of the dLGN, we did not observe significant differences between P10 and P12. However, we found that the size of the ipsi-RGC patch, as measured by its length, decreased until P8, but not thereafter. Together, these data support a slight reversal of eye-specific segregation just before eye-opening, which could be masked depending on the method of quantification.** Of note, many investigations of eye-specific segregation in the mouse dLGN did not sample with the frequency that we did (every two days) (Jaubert-Miazza et al., 2005; Muir-Robinson et al., 2002; Demas et al., 2006). One study that sampled with the same

frequency did not report overlap at P2 or P12 (Pfeiffenberger et al., 2005). Thus, the changes we observe may reflect a high degree of dynamics in eye-specific sorting in the mouse dLGN. Though it is important to note that the changes we observed were small, and the general trend was consistent with these previous studies.

In addition to roles in synaptic plasticity, axonal refinement, and arbor stabilization, NMDARs have also been implicated in the generation of glutamatergic waves, which play a critical role in the maintenance of eye-specific segregation (Cline & Constantine-Paton, 1989; Iwasato et al., 1997; Ruthazer & Cline, 2004; Hu et al., 2005; Demas et al., 2006; Munz et al., 2014). Indeed, NMDAR blockade in *in vitro* preparations alters the frequency of glutamatergic waves, but not other attributes, such as velocity (Blankenship et al., 2009). These results raise the possibility that disruption of NMDAR expression in RGCs might alter the pattern of glutamatergic waves. While we did not monitor these waves directly, the lack of an eye-specific segregation phenotype observed in pre-cKO mice, both in the mature and developing state, suggests NMDARs in RGCs are dispensable for the wave-dependent information mediating maintenance of segregation. Indeed, these findings are consistent with recent work elucidating a role for NMDARs on the pre-synaptic side of bipolar cell terminals in the initiation and propagation of glutamatergic waves (Zhang, et al., 2016a).

NMDARs may be present on developing RGC terminals

The lack of disruptions in topography and eye-specific segregation we observed in pre-cKO mice raised the question of whether, in fact, NMDARs are localized and functional in terminals of developing RGCs. The expression pattern of NMDARs in the developing brain has been difficult to examine due to a lack of suitable antibodies for immunolocalization of the obligate GluN1 subunit. While, recent studies suggest NMDARs are expressed in both the cell bodies and dendrites of RGCs (Zhang, et al., 2016b), the successful labeling of NMDARs on axons or at terminals has not been reported in murine models. To explore the possibility of

NMDARs located pre-synaptically in murine RGC terminals, we used combination Chrnb3-Cre;GCaMP5::TdTom mice. This animal model allows us to not only visualize RGC terminals in slices through retinorecipient regions, but it also restricts Ca^{2+} indicator expression to RGC terminals. Using this methodology, we observed Ca^{2+} transients in RGC terminals in both the dLGN and the SC, which were ablated in the presence of the NMDAR-specific antagonist, MK-801. These data suggest that NMDARs are localized pre-synaptically. However, the possibility that indirect activation of RGC terminals occurs via administration of NMDA cannot be ruled out, as the temporal dynamics of GCaMP are too slow to resolve this. However, the presence of axo-axonal synapses onto RGCs that could lead to such a result have not been reported to our knowledge.

Conclusion

Here, we have utilized a conditional genetic knockout method to probe the role of NMDARs expressed by RGCs in the development of ordered connectivity in image-forming retino-recipient nuclei. We did not observe alterations in either topography or eye-specific segregation in the SC or dLGN in pre-cKO mice, demonstrating that NMDARs in RGCs play a minimal role in these processes. Further, using conditional expression of genetically-encoded Ca^{2+} indicators in RGCs, we present evidence that NMDARs may be present in developing RGC terminals in the mouse.

571 References

572 Ackman, J. B., et al. (2012). Retinal waves coordinate patterned activity throughout the
573 developing visual system. *Nature, 490*(7419), 219-225. doi:10.1038/nature11529

574 Aoki, C., et al. (1994). Cellular and subcellular localization of NMDA-R1 subunit
575 immunoreactivity in the visual cortex of adult and neonatal rats. *Journal of Neuroscience,*
576 *14*(9), 5202-5222. doi:10.1523/JNEUROSCI.14-09-05202.1994

577 Arroyo, D. A., & Feller, M. B. (2016). Spatiotemporal features of retinal waves instruct the wiring
578 of the visual circuitry. *Frontiers in Neural Circuits, 10*, 54. doi:10.3389/fncir.2016.00054

579 Blankenship, A. G., et al. (2009). Synaptic and extrasynaptic factors governing glutamatergic
580 retinal waves. *Neuron, 62*(2), 230-241. doi:10.1016/j.neuron.2009.03.015

581 Bouvier, G., et al. (2015). Presynaptic NMDA receptors: Roles and rules. *Neuroscience, 311*,
582 322-340. doi:10.1016/j.neuroscience.2015.10.033

583 Bouvier, G., et al. (2018). Towards resolving the presynaptic NMDA receptor debate. *Current*
584 *Opinion in Neurobiology, 51*, 1-7. doi:10.1016/j.conb.2017.12.020

585 Brandstätter, J. H., et al. (1994). *Expression of NMDA and high-affinity kainate receptor subunit*
586 *mRNAs in the adult rat retina*. Oxford] : Blackwell Science. doi:10.1111/j.1460-
587 9568.1994.tb00607.x

588 Cang, J., et al. (2008). Selective disruption of one cartesian axis of cortical maps and receptive
589 fields by deficiency in Ephrin-as and structured activity. *Neuron, 57*(4), 511-523.
590 doi:10.1016/j.neuron.2007.12.025

591 Chandrasekaran, A. R., et al. (2007). Developmental homeostasis of mouse retinocollicular
592 synapses. *Journal of Neuroscience, 27*(7), 1746-1755. doi:10.1523/JNEUROSCI.4383-
593 06.2007

594 Cline, H. T., & Constantine-Paton, M. (1990). NMDA receptor agonist and antagonists alter
595 retinal ganglion cell arbor structure in the developing frog retinotectal projection. *Journal of*
596 *Neuroscience, 10*(4), 1197-1216. doi:10.1523/JNEUROSCI.10-04-01197.1990

597 Cline, H. T., & Constantine-Paton, M. (1989). NMDA receptor antagonists disrupt the
598 retinotectal topographic map. *Neuron, 3*(4), 413-426. doi:10.1016/0896-6273(89)90201-8

599 Constantine-Paton, M., & Law, M. I. (1978). Eye-specific termination bands in tecta of three-
600 eyed frogs. *Science, 202*(4368), 639-641. doi:10.1126/science.309179

601 Corlew, R., et al. (2007). Developmental switch in the contribution of presynaptic and
602 postsynaptic NMDA receptors to long-term depression. *Journal of Neuroscience, 27*(37),
603 9835-9845. doi:10.1523/JNEUROSCI.5494-06.2007

604 Demas, J., et al. (2006). Failure to maintain eye-specific segregation in nob, a mutant with
605 abnormally patterned retinal activity. *Neuron, 50*(2), 247-259.
606 doi:10.1016/j.neuron.2006.03.033

607 Dhande, O. S., et al. (2011). Development of single retinofugal axon arbors in normal and β2
608 knock-out mice. *The Journal of Neuroscience, 31*(9), 3384-3399.
609 doi:10.1523/JNEUROSCI.4899-10.2011

610 Drayson, L. E., & Triplett, J. W. (2019). A Chrnb3-Cre BAC transgenic mouse line for
611 manipulation of gene expression in retinal ganglion cells. *Genesis, 57*(9), e23305-n/a.
612 doi:10.1002/dvg.23305

613 Feldheim, D. A., & O'Leary, D. D. M. (2010). Visual map development: Bidirectional signaling,
614 bifunctional guidance molecules, and competition. *Cold Spring Harbor Perspectives in*
615 *Biology, 2*(11), a001768. doi:10.1101/cshperspect.a001768

616 Feller, M. B. (2009). Retinal waves are likely to instruct the formation of eye-specific
617 retinogeniculate projections. *Neural Development, 4*(1), 24. doi:10.1186/1749-8104-4-24

618 Gee, J. M., et al. (2014). Imaging activity in neurons and glia with a Polr2a-based and cre-
619 dependent GCaMP5G-IRES-tdTomato reporter mouse. *Neuron, 83*(5), 1058-1072.
620 doi:10.1016/j.neuron.2014.07.024

621 Godement, P., et al. (1984). Prenatal and postnatal development of retinogeniculate and
622 retinocollicular projections in the mouse. *Journal of Comparative Neurology (1911), 230*(4),
623 552-575. doi:10.1002/cne.902300406

624 Hahm, J. O., et al. (1991). *Disruption of retinogeniculate afferent segregation by antagonists to*
625 *NMDA receptors*. London] : Nature Pub Group. doi:10.1038/351568a0

626 Hensch, T. K., & Quinlan, E. M. (2018). Critical periods in amblyopia. *Visual Neuroscience, 35*,
627 E014. doi:10.1017/S0952523817000219

628 Hofer, M., et al. (1994). Regulation of NMDA receptor mRNA during visual map formation and
629 after receptor blockade. *Journal of Neurochemistry, 62*(6), 2300-2307. doi:10.1046/j.1471-
630 4159.1994.62062300.x

631 Hu, B., et al. (2005). BDNF stabilizes synapses and maintains the structural complexity of optic
632 axons in vivo. *Development (Cambridge), 132*(19), 4285-4298. doi:10.1242/dev.02017

633 Huang, L., & Pallas, S. L. (2001). NMDA antagonists in the superior colliculus prevent
634 developmental plasticity but not visual transmission or map compression. *Journal of*
635 *Neurophysiology, 86*(3), 1179-1194. doi:10.1152/jn.2001.86.3.1179

636 Iwasato, T., et al. (1997). NMDA receptor-dependent refinement of somatotopic maps. *Neuron*
637 *(Cambridge, Mass.), 19*(6), 1201-1210. doi:10.1016/S0896-6273(00)80412-2

638 Jaubert-Miazza, L., et al. (2005). Structural and functional composition of the developing
639 retinogeniculate pathway in the mouse. *Visual Neuroscience, 22*(5), 661-676.
640 doi:10.1017/S0952523805225154

641 Johnson, K. O., & Triplett, J. W. (2021). Wiring subcortical image-forming centers: Topography,
642 laminar targeting, and map alignment. *Current Topics in Developmental Biology, 142*, 283-
643 317. doi:10.1016/bs.ctdb.2020.10.004

644 Kay, R. B., et al. (2018). Visual subcircuit-specific dysfunction and input-specific mispatterning
645 in the superior colliculus of fragile X mice. *Journal of Neurodevelopmental Disorders, 10*(1),
646 23. doi:10.1186/s11689-018-9241-1

647 Kesner, P., et al. (2020). Postsynaptic and presynaptic NMDARs have distinct roles in visual
648 circuit development. *Cell Reports (Cambridge), 32*(4), 107955.
649 doi:10.1016/j.celrep.2020.107955

650 King, A. J., et al. (1996). The development of topographically-aligned maps of visual and
651 auditory space in the superior colliculus. *Progress in Brain Research, 112*, 335-350.
652 doi:10.1016/s0079-6123(08)63340-3

653 Massey, S. C., & Miller, R. F. (1990). N-methyl-D-aspartate receptors of ganglion cells in rabbit
654 retina. *Journal of Neurophysiology, 63*(1), 16-30. doi:10.1152/jn.1990.63.1.16

655 McLaughlin, T., et al. (2003). Retinotopic map refinement requires spontaneous retinal waves
656 during a brief critical period of development. *Neuron, 40*(6), 1147-1160. doi:10.1016/S0896-
657 6273(03)00790-6

658 Meister, M., et al. (1991). Synchronous bursts of action potentials in ganglion cells of the
659 developing mammalian retina. *Science, 252*(5008), 939-943. doi:10.1126/science.2035024

660 Mittman, S., et al. (1990). Concomitant activation of two types of glutamate receptor mediates
661 excitation of salamander retinal ganglion cells. *The Journal of Physiology, 428*(1), 175-197.
662 doi:10.1113/jphysiol.1990.sp018206

663 Mize, R. R., & Butler, G. D. (2000). The NMDAR1 subunit of the N-methyl-D-aspartate receptor
664 is localized at postsynaptic sites opposite both retinal and cortical terminals in the cat
665 superior colliculus. *Visual Neuroscience, 17*(1), 41-53. doi:10.1017/s0952523800171044

666 Muir-Robinson, G., et al. (2002). Retinogeniculate axons undergo eye-specific segregation in
667 the absence of eye-specific layers. *The Journal of Neuroscience, 22*(13), 5259-5264.
668 doi:10.1523/JNEUROSCI.22-13-05259.2002

669 Munz, M., et al. (2014). Rapid hebbian axonal remodeling mediated by visual stimulation.
670 *Science, 344*(6186), 904-909. doi:10.1126/science.1251593

671 Nicoll, R. A., & Malenka, R. C. (1999). Expression mechanisms underlying NMDA receptor-
672 dependent long-term potentiation. *Annals of the New York Academy of Sciences, 868*, 515-
673 525. doi:10.1111/j.1749-6632.1999.tb11320.x

674 Paoletti, P., et al. (2013). NMDA receptor subunit diversity: Impact on receptor properties,
675 synaptic plasticity and disease. *Nature Reviews. Neuroscience, 14*(6), 383-400.
676 doi:10.1038/nrn3504

677 Pfeiffenberger, C., et al. (2005). Ephrin-as and neural activity are required for eye-specific
678 patterning during retinogeniculate mapping. *Nature Neuroscience, 8*(8), 1022-1027.
679 doi:10.1038/nn1508

680 Pfeiffenberger, C., et al. (2006). *Ephrin-as and patterned retinal activity act together in the*
681 *development of topographic maps in the primary visual system*. Washington, D.C. : Society
682 for Neuroscience. doi:10.1523/JNEUROSCI.3595-06.2006

683 Pittaluga, A., & Raiteri, M. (1990). Release-enhancing glycine-dependent presynaptic NMDA
684 receptors exist on noradrenergic terminals of hippocampus. *European Journal of*
685 *Pharmacology, 191*(2), 231-234. doi:10.1016/0014-2999(90)94153-O

686 Rajan, I., et al. (1999). NMDA receptor activity stabilizes presynaptic retinotectal axons and
687 postsynaptic optic tectal cell dendrites in vivo. *Journal of Neurobiology, 38*(3), 357-368.
688 doi:10.1002/(SICI)1097-4695(19990215)38:33.0.CO;2-#

689 Ruthazer, E. S., et al. (2003). Control of axon branch dynamics by correlated activity in vivo.
690 *Science, 301*(5629), 66-70. doi:10.1126/science.1082545

691 Ruthazer, E. S., & Cline, H. T. (2004). Insights into activity-dependent map formation from the
692 retinotectal system: A middle-of-the-brain perspective. *Journal of Neurobiology, 59*(1), 134-
693 146. doi:10.1002/neu.10344

694 Schindelin, J., et al. (2012). Fiji: An open-source platform for biological-image analysis. *Nature*
695 *Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019

696 Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C T
697 method. *Nature Protocols, 3*(6), 1101-1108. doi:10.1038/nprot.2008.73

698 Shen, Y., et al. (2006). N-methyl-d-aspartate receptors in the retina. *Molecular Neurobiology,*
699 *34*(3), 163-179. doi:10.1385/MN:34:3:163

700 Simon, D. K., et al. (1992). N-methyl-D-aspartate receptor antagonists disrupt the formation of a
701 mammalian neural map. *Proceedings of the National Academy of Sciences of the United*
702 *States of America, 89*(22), 10593-10597. doi:10.1073/pnas.89.22.10593

703 Smetters, D. K., et al. (1994). An N-methyl- D-aspartate receptor antagonist does not prevent
704 eye-specific segregation in the ferret retinogeniculate pathway. *Brain Research, 658*(1),
705 168-178. doi:10.1016/S0006-8993(09)90023-3

706 Sweeney, N. T., et al. (2019). Expression of transcription factors divides retinal ganglion cells
707 into distinct classes. *Journal of Comparative Neurology (1911), 527*(1), 225-235.
708 doi:10.1002/cne.24172

709 Triplett, J. W., et al. (2011). Competition is a driving force in topographic mapping. *Proceedings*
710 *of the National Academy of Sciences of the United States of America, 108*(47), 19060-
711 19065. doi:10.1073/pnas.1102834108

712 Tsien, J. Z., et al. (1996). The essential role of hippocampal CA1 NMDA Receptor–Dependent
713 synaptic plasticity in spatial memory. *Cell, 87*(7), 1327-1338. doi:10.1016/S0092-
714 8674(00)81827-9

715 Watanabe, M., et al. (1994). Differential distributions of the NMDA receptor channel subunit
716 mRNAs in the mouse retina. *Brain Research, 634*(2), 328-332. doi:10.1016/0006-
717 8993(94)91938-0

718 Wong, R. O. L., et al. (1993). Transient period of correlated bursting activity during development
719 of the mammalian retina. *Neuron, 11*(5), 923-938. doi:10.1016/0896-6273(93)90122-8

720 Xu, H., et al. (2015). Spatial pattern of spontaneous retinal waves instructs retinotopic map
721 refinement more than activity frequency. *Developmental Neurobiology, 75*(6), 621-640.
722 doi:10.1002/dneu.22288

723 Zhang, J., et al. (2016). (b). High-resolution quantitative immunogold analysis of membrane
724 receptors at retinal ribbon synapses. *Journal of Visualized Experiments,* (108), 53547.
725 doi:10.3791/53547

726 Zhang, R., et al. (2016). (a). Stereotyped initiation of retinal waves by bipolar cells via
727 presynaptic NMDA autoreceptors. *Nature Communications, 7*(1), 12650.
728 doi:10.1038/ncomms12650

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731 **Figure Legends**

732 **Figure 1. Retina-specific knockout of GluN1 in Chrnb3-Cre;GluN1$^{flox/flox}$ mice.** (A) Flat-
733 mounted retina of a Chrnb3-Cre;Rosa^{LacZ} reporter mouse line. (B) Section through the retina of
734 a P8 Chrnb3-Cre;Rosa^{TdTom} reporter mouse reveal cells labelled in the ganglion cell layer
735 (green). (C & D) Sections through the retina of P4 Ctl (C) and pre-cKO (D) mice labeled with
736 GluN1 antisense probe (arrowheads). *GCL, ganglion cell layer; IPL, inner plexiform layer; INL,*
737 *inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer* (E & F) Sagittal
738 sections through the midbrain of P4 Ctl (E) and pre-cKO (F) mice labeled with GluN1 antisense
739 probe and higher magnification of GluN1 of the superficial and deep layers of the Ctl (E') and
740 pre-cKO (F') SCs. (G) Schematic of the GluN1 allele and approximate locations of the different
741 primer sets for qPCR and LoxP sites. (H & I) qPCR data for P0 micro-dissected retina (H) and
742 P0 SC (I) between Ctl and pre-cKO mice.
743
744 **Figure 2. Cytoarchitecture of retina unchanged in Chrbn3-Cre;GluN1$^{flox/flox}$ mice.** (A-J)
745 Sections through the retinas of P4 Ctl (A-E) and pre-cKO (F-J) mice stained for RBPMS (A & F),
746 Brn3a (B & G), SatB2 (C & H), Beta3 (D & I), and calretinin (E & J). (K-O) Quantification of the
747 density of cells labeled with markers used in (A-J).
748
749 **Figure 3. Retinofugal topography is unaltered in Chrnb3-Cre;GluN1$^{flox/flox}$ mice.** (A & B)
750 Whole-mount fluorescent images of the termination zones (TZs) of labeled RGCs observed in
751 the SC (dashed area) for Ctl (A) and pre-cKO (B) mice and coronal sections through the
752 corresponding TZs (A' & B'). *P, posterior; L, lateral; D, dorsal* (C) Quantification of the
753 termination zone indices (TZIs) in the SC. (D & E) Coronal sections through the dLGN of Ctl (D)
754 and pre-cKO (E) mice reveal the TZs of labeled RGCs. (F) Quantification of the termination
755 zone indices (TZIs) in the dLGN.
756

Figure 4. Mature organization of eye-specific segregation in the dLGN is unaltered in Chrnb3-Cre;GluN1$^{flox/flox}$ mice. (A-F) Coronal sections through the dLGN of Ctl (A-C) and pre-cKO (D-F) reveal the terminals of bulk labelled RGCs originating from the contralateral (A & D) or ipsilateral (B & E) eye, as well as the degree of overlap (C & F). *D, dorsal; M, medial* (G) Quantification of the amount of overlapping contralateral and ipsilateral inputs to the dLGN.

Figure 5. Mature organization of eye-specific segregation in the SC is unaltered in Chrnb3-Cre;GluN1$^{flox/flox}$ mice. (A-F) Coronal sections through the SC of Ctl (A-C) and pre-cKO (D-F) reveal the terminals of bulk labelled RGCs originating from the contralateral (A & D) or ipsilateral (B & E) eye, as well as the degree of overlap (C & F). *D, dorsal; M, medial* (G) Quantification of the amount of overlapping contralateral and ipsilateral inputs to the SC.

Figure 6. Developmental trajectory of eye-specific segregation in the SC is unaltered in Chrnb3-Cre;GluN1$^{flox/flox}$ mice. (A-F) Coronal sections through the SC of Ctl (A-C) and pre-cKO (D-F) reveal the terminals of bulk labelled RGCs originating from the contralateral (green) and ipsilateral (magenta) eyes at postnatal day 4 (P4) (A & D), P8 (B & E), and P12 (C & F). *D, dorsal; M, medial* (G) Quantification of the amount of overlapping contralateral and ipsilateral inputs to the dLGN over the ipsilateral area at the indicated ages in Ctl and pre-cKO mice. (H) Quantification of the amount of overlapping contralateral and ipsilateral inputs to the dLGN of Ctl and pre-cKO mice at indicated ages, presented as the percent of the total dLGN area. (I) Quantitative comparison of the ipsilateral patch length between Ctl and pre-cKO at indicated ages, expressed as the percentage of the dLGN length covered by the ipsilateral patch along the DM-VL axis.

Figure 7. NMDA elicited Ca^{2+} transients in the terminals of developing RGCs. (A-D) Time series fluorescent imaging of Ca^{2+} response in RGC terminals elicited in acute brain slices from

P6 Chrnb3-Cre;GCaMP5::TdTom mice by focal application of NMDA in the SC in Mg^{2+}-free aCSF (A), NMDA stimulation in the SC in aCSF (B), NMDA stimulation in the SC in Mg^{2+}-free aCSF + MK-801 (C), and K^{+} stimulation in the SC in aCSF (D) from a glass pipette (white dashes in center panel). Graphs of change in fluorescence over time at four different regions of interest (dashed colored circles) reveal the response elicited under each condition (right panels). (E) Quantification of the peak ΔF/F (%) in the indicated conditions.