Synaptic Gain Control at a Visual Synapse: Gated by Competition
And Constrained Homeostatically

by

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By

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Abstract

Visual information is relayed from retina to the brain at first order synapses within the lateral geniculate nucleus (dLGN). During development, activity-dependent synaptic competition drives the segregation of retinal ganglion cell terminals into eye-specific zones. It has been assumed that the gain of synaptic transmission within these eye-specific zones is equivalent, providing uniform information transfer from the periphery to the CNS. Here, we revise this understanding. First, we demonstrate that anatomical segregation of retinal axons triggers a profound (200-300%) potentiation of neurotransmitter release selectively within the projection zone of the ipsilateral eye. Second, optogenetic recruitment of genetically defined axons within the ipsilateral projection zone provides evidence that functional synaptic connectivity is sub-stratified within the ipsilateral dLGN. Thus, we define a new functional organization within the dLGN and propose that synaptic competition acts as a developmental timer that triggers respecification of set point synaptic gain within the ipsilateral dLGN.
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Chapter 1 - Introduction

The development of complex neural circuitry occurs in stages. Molecular cues govern the guidance of axons to the correct termination zone (Tessier-Lavigne & Goodman, 1996; Crair & Mason, 2016). Motile growth cones are transformed into immature synaptic connections, establishing a crude wiring diagram. Activity-dependent processes, including synaptic competition, subsequently refine the wiring diagram (Huberman, et al., 2008; Cang & Feldheim, 2013). Finally, synaptic connections mature both pre- and postsynaptically. Presynaptic maturation can include a change in presynaptic calcium channel subtypes (Nishimune, et al., 2004; Gundelfinger, et al., 2016), altered release probability (Branco & Staras, 2009), increased size of the readily releasable vesicle pool (Mozhayeva, et al., 2002) and increased coupling of vesicles to sites of presynaptic calcium influx (Chen, et al., 2015; Catterall & Few, 2008). Postsynaptic maturation includes regulated changes to neurotransmitter receptor subunit number and composition (Gu & Hall, 1988; Hall & Sanes, 1993; Hooks & Chen, 2006; Kumar, et al., 2002; Louros, et al., 2014; Hauser, et al., 2014). Ultimately, point-to-point connectivity is achieved, and synaptic connections function with highly reproducible properties that are sustained throughout life by potent homeostatic mechanisms (Davis, 2013; Maffei & Fontanini, 2009; Turrigiano, 2012).

One of the least well-understood stages of circuit development is specification of synaptic function, including synaptic gain and neurotransmitter release dynamics. It is clear that synaptic connections are constructed with a high degree of functional specificity throughout the developing brain. For example, cerebellar granule cells receive input from different sensory modalities, each with characteristic gains and
dynamical properties (Chabrol, et al., 2015). A similar degree of specification is observed at inhibitory connections in neocortex (Bartley, et al., 2008). The specification of synapse-specific gain can be driven by target-derived signals (Davis & Murphey, 1993; Scanziani, et al., 1998). And, synapses on different domains of a single target neuron can be specified to have region-specific gain and dynamical properties (Linhoff, et al., 2009; Jia, et al., 2010; Williams, et al., 2010; de Wit, et al., 2011).

Importantly, synaptic connection strengths and dynamics must be specified at the correct time during circuit development. For example, recent theoretical work argues that the progression of neural circuitry from an initially chaotic state to a state characterized by realistic patterns of neural activity requires that synaptic gain be specified early in the process and constrained within a relatively narrow range (Sussillo & Abbott, 2009; Sussillo, et al., 2015). As another example, the ‘balance’ of excitatory and inhibitory synaptic gain (E-I balance) is considered a fundamental, emergent property of neural circuit development. Delayed acquisition of appropriate E-I balance is hypothesized to contribute to childhood epilepsy (Williamson, et al., 1995; Bozzi, et al., 2012) and, during adolescent life, has been proposed as a contributing factor in schizophrenia (Faludia & Mirnics, 2011). Further, the developmental emergence of E-I balance is thought to be dysregulated in diverse models of neurological disease including autism spectrum disorder (Nelson & Valakh, 2015; Rubenstein & Merzenich, 2003). Finally, it is well-established that homeostatic signaling systems exist to maintain highly characteristic synaptic strengths throughout the nervous system, preserving both relative synaptic weights on individual postsynaptic target cells (Turrigiano, et al., 1998; Turrigiano, 2008) and preserving characteristic presynaptic release properties of
individual synapses (Davis & Goodman, 1998; Davis, 2013). Yet, the mechanisms that define 'set point' synaptic gain remain poorly understood.

This dissertation will examine the retinogeniculate synapse, the first central nervous system visual synapse between retinal ganglion cells and the visual thalamus. The development of this circuit involves the attainment of each of the aforementioned milestones. I will review the current understanding of the mechanisms underpinning the progression of this synaptic connection from its early imprecise and unruly state to the mature, exquisitely precise circuit that underlies vision. I will then present data revealing a powerful and previously unappreciated process of visual system maturation. These results identify a new role for competition-driven circuit refinement in establishing the timing of synaptic maturation, and suggest that the stereotyped timelines observed in the development of many neural circuits may be emergent properties of the interaction of developmental processes, rather than hard-wired sequences.

Initial circuit patterning

We have used as a model system the first central synapse of the mammalian visual system, the connection of retinal ganglion cells (RGCs) to thalamic relay neurons (RNs) in the dorsal lateral geniculate nucleus (dLGN). Relay neurons in turn project primarily to layer IV of the visual cortex, and cortical neurons from layer VI supply excitatory feedback to the dLGN. The development of the RGC to RN (retinogeniculate) synapse has been the focus of much research (Cramer & Sur, 1995; Huberman, et al., 2008; Crair & Mason, 2016; Kerschensteiner & Guido, 2017; Litvina & Chen, 2017; Bickford, et al., 2010; Budisantoso, et al., 2012; Linden, et al., 1981; Shatz, 1983; Rakic,
One striking feature of retinogeniculate development is the segregation of eye-specific inputs into discrete anatomical zones (Jaubert-Miazza, et al., 2005; Huberman, et al., 2002; Sretavan & Shatz, 1986; Reese, 1988; Muir-Robinson, et al., 2002). At birth, retinal inputs from the two eyes are intermixed in the mouse dLGN, but within the first two postnatal weeks the dLGN resolves well-segregated zones: a large zone that receives input specifically from the contralateral eye, and a smaller region that receives input predominantly from the ipsilateral eye.

**Chronological development**

The initially-overlapping termination zones of retinal axons from both eyes, though imprecise, are not established randomly. Rather, they are the consequence of anatomical organizational programs involving molecular guidance factors and differential developmental timelines. Mouse retinal ganglion cells project from the eyes to the optic chiasm, where the vast majority of them cross to innervate the contralateral hemisphere of the brain (Dräger & Olsen, 1980). The remaining RGCs project to the ipsilateral hemisphere, traveling adjacent to the contralaterally-projecting RGCs in a compact white matter tract called the optic tract. Fibers within the optic tract are sorted into subregions preserving multiple retinotopic maps and separating projections from the two eyes (Torrealba, et al., 1981; Chan & Guillery, 1994; Plas, et al., 2005). This organization is thought to be established partly by sorting RGCs based on their time of arrival at the optic chiasm (Torrealba, et al., 1982), although molecular cues are also at work (Hörnberg, et al., 2016; Leung, et al., 2013).
Differential timing of innervation by the two eyes also plays a role in organizing the dLGN (Godement, et al., 1984; Sretavan & Shatz, 1987). Contralaterally-projecting RGCs (C-RGCs) innervate the dLGN of C57BL/6 mice by embryonic day 16 (E16), occupying the entire nucleus (Godement, et al., 1984). Ipsilaterally-projecting RGCs (I-RGCs) arrive at the border of the dLGN by E16, but remain in the optic tract until roughly two days later, at E18 (mice are born at approximately E18.5). By postnatal day 2 (p2), the initial I-RGC projections occupy much of the medio-dorsal dLGN, the region to which their mature axon termination zones will be restricted, but also reach the ventral dLGN (Godement, et al., 1984). It is interesting to note that this pattern of early domination by the contralateral eye is recapitulated in the connections from the dLGN to the visual cortex (Crair, et al., 1998), and thus appears to be a general mechanism of visual circuit development.

Molecular guidance cues

The decision of whether to cross the optic chiasm, and where to target the zone of RGC projection within the dLGN, are further specified by attractive and repulsive molecular guidance factors. Contralaterally- and ipsilaterally-projecting RGCs differentially express hundreds of genes during their embryonic development (Wang, et al., 2016). Thus, RGCs have laterality-specific identities, resulting in differential responses to molecular cues (Williams, et al., 2003; Williams, et al., 2006; Land & Shamalla-Hannah, 2001). The best understood of these is the Eph/ephrin system. Ephrin-B2 is expressed by radial glia in the developing mouse optic chiasm, while its receptor EphB1 is expressed in the region of retina that contains RGCs fated to project
ipsilaterally (Williams, et al., 2003). The interaction of EphB1 and ephrin-B2 strongly inhibits outgrowth in I-RGCs, such that they are repulsed at midline of the optic chiasm, and fail to cross (Williams, et al., 2003; Petros, et al., 2010).

Once retinal ganglion cells arrive at the dLGN, the Eph/ephrin system acts again to sculpt their pattern of innervation (Huberman, et al., 2005). EphA5 is expressed in a gradient across the mouse retina, with highest expression in the region containing ipsilaterally-projecting RGCs (Feldheim, et al., 1998). The EphA5 ligands ephrin-A2 and ephrin-a5 are present in the dLGN from E14 to at least p4, oriented in a gradient with highest expression in the latero-ventral dLGN, where I-RGCs are never found in mature mice. Interactions with both ephrin-A2 and ephrin-A5 are repellant to EphA5-expressing RGCs (Feldheim, et al., 1998). This suggests a mechanism for the initial thalamic targeting of I-RGCs whereby they reach the border of the dLGN and, upon innervation, are induced by the presence of repulsive cues to project deeply into the medio-dorsal region of the nucleus. Indeed, mice lacking ephrin-A2, ephrin-A3 and ephrin-A5 have severely mistargeted ipsilateral retinogeniculate projections (Pfeiffenberger, et al., 2005). Interestingly, eye-specific segregation within these misplaced patches is unaffected; see below.

The efficacy of Eph/ephrin-mediated guidance cues is modulated by retinal ganglion cell activity in vitro (Nicol, et al., 2007) and in vivo (Pfeiffenberger, et al., 2005; Pfeiffenberger, et al., 2006). This is perhaps unsurprising, as the presence and activity of retinal cells play a fundamental role in many phases of dLGN development. The timing of the innervation of the dLGN by feedback neurons projecting from the visual cortex (the corticogeniculate connection) is regulated by retinogeniculate afferents. Mice
in which retinogeniculate input is surgically or genetically ablated exhibit early
corticogeniculate innervation (Seabrook, et al., 2013). Retinal inputs influence the
temporal expression pattern of an extracellular matrix protein that is inhibitory to
corticogeniculate afferents, such that the presence of RGCs sets the timing of the
development of the cortical feedback circuit (Seabrook, et al., 2013; Brooks, et al.,
2013). Conversely, retinogeniculate innervation is severely impaired in mice lacking
visual cortex (Shanks, et al., 2016), suggesting a complex interdependence between
the two major sources of excitatory input to the dLGN. After relay neurons, the most
abundant cell type in the dLGN is local inhibitory interneurons, which comprise ~20% of
cells. These interneurons migrate into the dLGN during the first postnatal week. This
process depends on the presence and activity of retinal ganglion cells: interneurons fail
to migrate deeply into the dLGN if retinal input is surgically removed, or if spontaneous
retinal activity is disrupted by genetic or pharmacological means (Golding, et al., 2014).
Notably, retinal activity-dependent competition underlies the precise segregation of
retinogeniculate input from the two eyes into laterality-specific layers (see below).
Together, this work suggests that a detailed understanding of the regulation of
retinogeniculate synaptic connectivity is a prerequisite for explaining the timing and
developmental patterning of the major components of the first central visual synapse.

**Eye-specific segregation and refinement**

Retinal axons from both eyes project to overlapping territories within the dLGN by
p2 (Godement, et al., 1984). During the following week, I-RGCs contract their projection
zones to a small region in the dorso-medial dLGN, referred to here as the ipsilaterally-
innervated dLGN (I-dLGN). At the same time, C-RGC axons withdraw from the I-dLGN while maintaining their presence in the larger, surrounding contralaterally-innervated region of the dLGN (C-dLGN), resulting in precise segregation of the inputs originating from each eye (Godement, et al., 1984; Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006; Sretavan & Shatz, 1986). At the level of individual RGCs, this process involves the elaboration of axon branches that initially reach the eye-appropriate region of the dLGN, and the elimination of incorrectly targeted branches (Sretavan & Shatz, 1984; Dhande, et al., 2011).

**Activity-dependent competition**

The process of eye-specific segregation in a variety of model organisms is thought to be driven by activity-dependent competition. Specifically, spontaneous patterned activity called “retinal waves,” during which cholinergic synaptic activation propagates a wave of RGC action potentials across the developing retina, is required for the formation of eye-specific RGC innervation zones (Feller, et al., 1996; Penn, et al., 1998; Mooney, et al., 1996; Shatz & Stryker, 1988; Huberman, et al., 2002; Stellwagen & Shatz, 2002; Butts, et al., 2007). When tetrodotoxin is delivered to the developing kitten dLGN in order to prevent relay neuron activity, both the contraction of I-RGCs and the removal of C-RGCs from the ipsilaterally-innervated region of the dLGN fail (Shatz & Stryker, 1988). Similar results obtain when retinal wave activity is specifically blocked by binocular injection of epibatidine in the ferret (Penn, et al., 1998) or disrupted by retina-specific knockout of an acetylcholine receptor subunit in the mouse (Xu, et al., 2015). Furthermore, monocular retinal wave blockade results in an
expansion of the synaptic territory claimed by the unblocked eye, and a loss of territory by the blocked eye, suggesting that relative activity levels between the eyes may dictate the outcome of binocular competition in the dLGN (Penn, et al., 1998).

Indeed, monocular pharmacological manipulations designed to increase retinal wave activity in a single eye enlarge both the ipsilateral and contralateral projection zones from that eye (Stellwagen & Shatz, 2002). The specific mechanisms by which activity directs the maintenance of one population of RGC axons and the elimination of another are unknown. However, recent work demonstrated that glutamatergic release from I-RGCs is required for the elimination of C-RGCs from the ipsilaterally-innervated dLGN (Koch, et al., 2011). In contrast, glutamate is dispensable for the maintenance of ipsilaterally-projecting RGCs terminals within the I-dLGN, suggesting that activity sculpts eye-specific patterning in part through mechanisms that do not rely on excitatory synaptic transmission (Koch, et al., 2011).

It is worth noting that these results are based on anatomically-defined eye-specific segregation, in which axon terminals from one or both eyes are labeled and the extent of their projections is visually assessed. By this metric, anatomical eye-specific segregation is complete in mice by approximately p8 (Godement, et al., 1984). However, electrophysiological observations demonstrate that individual relay cells retain functional binocular innervation until the following week, placing the completion of functional eye-specific segregation at roughly p15 (Žiburkus & Guido, 2006). Furthermore, a recent study using microelectrode arrays to sample the responses of dLGN relay neurons to optical stimuli in mice aged p50-100 found no cells that were driven solely by ipsilaterally-projecting RGCs (Howarth, et al., 2014). Rather, they
observed that relay neurons were either driven exclusively by C-RGCs, or by both C-RGCs and I-RGCs, suggesting that relay cells innervated by ipsilaterally-projecting RGCs do not lose their functional synapses originating from C-RGCs by adulthood. The relative synaptic efficacy of the two eyes differed broadly among binocular cells, suggesting a complex and variable pattern of innervation of the I-dLGN and challenging the notion that competition establishes monocular retinogeniculate innervation through the application of straightforward activity-dependent rules.

**Retinogeniculate connectivity**

A consensus is emerging that the retinogeniculate synapse represents a far more complex and sophisticated visual information processing center than was previously appreciated (Weyand, 2016; Litvina & Chen, 2017). Among the strongest evidence for this shift in perspective are new connectomic studies, which make use of ultrastructural and modern circuit tracing techniques to describe the connectivity of the retinogeniculate synapse with unprecedented precision (Hammer, et al., 2015; Morgan, et al., 2016; Rompani, et al., 2017). Until recently, it has been assumed on the basis of strong and well-replicated electrophysiological evidence that the development of retinogeniculate circuit was marked by a progression from extreme polyinnervation (>20 inputs per relay cell) to innervation by one or a few RGC inputs, all originating in the same eye (Chen & Regehr, 2000; Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006; Sincich, et al., 2007; Mastronarde, 1992; Cleland, et al., 1971; Hooks & Chen, 2006). Whether recording in vivo or in different ex vivo brain tissue preparations, these studies have consistently observed that the majority of the retinal synaptic drive to a given
mature relay cell comes from a small number of presynaptic RGCs. Recent anatomical work suggested a plausible mechanism for this reduction in retinogeniculate convergence: presynaptic boutons of individual RGCs appear to increase in size and to cluster during development from p8 to p20, perhaps reflecting the concentration of increasingly strong synaptic contacts onto progressively fewer relay neurons (Hong, et al., 2014).

It is therefore surprising that the recent connectomic analyses have found evidence for a high degree of convergence of RGC inputs onto mature dLGN relay neurons located within the C-dLGN. An ultrastructural study reconstructed a portion of all presynaptic axons innervating a number of relay cells in an adult (aged p32) mouse (Morgan, et al., 2016). They identified 30-40 axon segments forming structural synapses with each relay cell. Although they could not be certain that these axon segments did not originate from a smaller number of common RGCs that branched outside the small reconstructed volume of dLGN, they speculate that this explanation is not consistent with the pattern of RGC axon branching typically observed at the light level (Morgan, et al., 2016). Another recent article used stochastic fluorescent reporter expression to uniquely label a small population of individual RGC axon terminals, and observed that clusters of retinal terminals generally included axons from multiple inputs (Hammer, et al., 2015). Given the sparseness of labeled RGCs, their convergence on common postsynaptic targets suggests a substantial amount of polyinnervation. Further electron microscopy and reconstruction of the synapse revealed clusters of terminal boutons containing between 3 and 12 or more presynaptic axons (Hammer, et al., 2015). Although this result is limited by the same caveat discussed above concerning
the possibility of RGC branching outside the reconstructed volume, it is consistent with the authors’ finding that at the optical level, mature retinal bouton clusters reflect a high degree of convergence.

The clearest insight into the pattern of retinal innervation to dLGN relay neurons comes from recent work that painstakingly combined viral, genetic and surgical techniques to identify the presynaptic retinal inputs to single relay cells (Rompani, et al., 2017). The authors used multiple levels of control to fluorescently label the RGCs innervating single dLGN relay neurons in mice aged p32-p52. In contrast to the long-held understanding of retinogeniculate segregation, but consistent with the recent electrophysiological report discussed above (Howarth, et al., 2014), the authors found relay cells that were innervated either by C-RGCs only, or by both C-RGCs and I-RGCs, but none that received inputs solely from the ipsilateral eye (Rompani, et al., 2017). Approximately 50% of the monocularly-innervated relay cells exhibited RGC connectivity of the type that would be predicted based on the classic interpretation of electrophysiological evidence: innervation by a small number of spatially proximate RGCs. The remaining half of monocularly-innervated relay neurons had a relatively large number of presynaptic inputs (6-36 per relay cell). This result is in line with the recent ultrastructural data discussed above (Hammer, et al., 2015; Morgan, et al., 2016).

Binocularly-innervated dLGN cells displayed an even greater degree of RGC convergence, receiving input from up to 91 retinal ganglion cells (Rompani, et al., 2017). Approximately half of the binocularly-innervated relay cells received a greater number of inputs from the ipsilateral eye, and half from the contralateral eye; these inputs tended
to include greater diversity in RGC subtype than did monocularly-innervated cells. Thus, the connectomic development of the portion of the dLGN receiving ipsilateral retinal input proceeds along a different trajectory than the region innervated solely by contralateral RGCs. It is important to note that neither this experiment nor the previously discussed ultrastructural assays provide information about the synaptic efficacy of ipsilateral or contralateral retinogeniculate connections. Indeed, a recent study assayed binocular retinogeniculate input by Ca$^{2+}$ imaging dLGN axon terminals in the visual cortex, and found that 21% of geniculate terminals responded only to ipsilateral stimuli, while only 14% reflected functional binocular innervation (Jaepel, et al., 2017). It remains possible that while ultrastructural evidence suggests massively convergent RGC innervation and stable binocular input to mature relay cells, relatively few of these inputs form synaptic connections of sufficient strength to reliably transfer visual information (Chen, et al., 2016). This would reconcile most of the anatomical and functional data; unfortunately, it is difficult to design an experiment capable of bridging these two modes of inquiry. Nevertheless, these studies highlight the need for further investigation into the wiring of the retinogeniculate synapse, with a particular emphasis on understanding the development of the ipsilaterally-innervated region of the dLGN.

**Neuronal identity and biased competition**

The previously discussed investigations of the role of activity-dependent competition have firmly established that the relative activity of the two eyes plays a crucial role in the establishment of properly sized (and, at least at the level of optical analysis, segregated) eye-specific termination zones. Yet, there is also evidence that
the rules governing binocular competition are not so straightforward as to simply reflect activity levels. Contralaterally- and ipsilaterally-projecting RGCs display striking asymmetries in their development and projection patterning, suggesting that the outcome of synaptic competition may be instructed or biased based on the identities of the competing neurons, and not merely their activity.

First, it is notable that the position and spatial extent of the ipsilateral RGC termination zone is highly stereotyped within the dLGN, indicating that at a gross level, the outcome of binocular competition is non-stochastic (Godement, et al., 1984; Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006; Coleman, et al., 2009; Reese, 1988). Furthermore, ipsilaterally-projecting RGCs can be forced to innervate portions of the dLGN that normally receive only contralateral input, by disrupting molecular guidance cues (Pfeiffenberger, et al., 2006) or retinal waves (Muir-Robinson, et al., 2002). In both cases, despite their localization to small, misplaced patches, I-RGCs successfully compete for postsynaptic territory, resulting in the withdrawal of C-RGCs. This suggests that the rules of binocular competition carry a strong bias towards I-RGCs, allowing them to outcompete numerically superior C-RGCs regardless of their position within the dLGN, and strongly argues against a simple Hebbian mechanism of eye-specific segregation. Rather, it would appear that I-RGCs occupy a privileged role in binocular competition, and may follow a different developmental trajectory than the better-studied population of C-RGCs. Finally, it bears emphasizing that while it is unknown how these analyses of eye-specific segregation relate to the recent connectomic work discussed above, the sorting of retinal inputs into monocularly innervated regions in the dLGN is well-supported by functional and anatomical data in a
variety of organisms, and remains likely to reflect a crucial, if not entirely understood, property of retinogeniculate development (Jaubert-Miazza, et al., 2005; Huberman, et al., 2002; Sretavan & Shatz, 1986).

**Strengthening and maturation**

Although functional assays consistently estimate a reduction in the number of presynaptic RGC inputs per relay cell during early development, the total retinal synaptic drive through this synapse is stable or increases over this interval (Chen & Regehr, 2000; Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006; Hooks & Chen, 2006; Hooks & Chen, 2008; Hohnke, et al., 2000). This suggests that as synaptic inputs are eliminated during circuit refinement, the remaining afferents strengthen to preserve or enhance transmission (Chen & Regehr, 2000; Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006; Hooks & Chen, 2006). Input strengthening is hypothesized to reflect an increase in presynaptic neurotransmitter release, driven by an increase in the number of release sites or available vesicles, based on the relative stability of other synaptic parameters and the observation that presynaptic terminals are enlarged and concentrated on postsynaptic targets during the period of input strengthening (Chen & Regehr, 2000; Hong, et al., 2014). The blockade of presynaptic activity by intraocular tetrodotoxin administration from p10-15 impairs input refinement and strengthening, preserving a large number of weak RGC inputs per relay neuron, and resulting in a reduction of total AMPAR-mediated synaptic drive (Hooks & Chen, 2006). In contrast, dark rearing mouse pups from p10-15 has no effect on refinement and strengthening, indicating that spontaneous retinal activity, rather than visual experience, drives these
processes (Hooks & Chen, 2006). Beyond its activity-dependence, the extent and mechanism of input strengthening remains little studied, despite its critical role in stabilizing information transfer through the retinogeniculate synapse during developmental remodeling.

The maturation of the RGC to dLGN synapse is also associated with a number of postsynaptic changes. This includes a robust increase in the ratio of total AMPAR-mediated to NMDAR-mediated synaptic current (Chen & Regehr, 2000; Hooks & Chen, 2006; Koch & Ullian, 2010; Litvina & Chen, 2017). This increase is unaffected by dark rearing from p10-15, but is impaired by retinal activity blockade through the same time period, suggesting that spontaneous retinal activity is required for this aspect of circuit maturation (Hooks & Chen, 2006). Silencing retinal input prevents the developmental strengthening of total AMPAR-mediated synaptic drive, but not NMDAR-mediated drive, suggesting a specific role for retinal activity in promoting transmission through AMPA receptors (Hooks & Chen, 2006). Previous work has established the presence of silent synapses in the developing retinogeniculate circuit (Chen & Regehr, 2000; Liu & Chen, 2008; Koch & Ullian, 2010). It is therefore possible that the normal developmental increase in the AMPA:NMDA current ratio is driven in part by the conversion of silent synapses to functional ones, by the insertion of AMPA receptors. Together with the previously discussed literature, this may suggest a mechanism for retinogeniculate input strengthening in which maturing RGCs expand their presynaptic release capacity by adding silent release sites, apposed only by postsynaptic NMDA receptors. These silent synapses would then undergo maturation through the insertion of AMPA receptors, in a process that depends on spontaneous retinal activity (Mooney, et al., 1993).
The subunit composition of NMDA receptors is also developmentally regulated at the retinogeniculate synapse. The decay of NMDA receptor-mediated current accelerates during synapse maturation (Chen & Regehr, 2000; Ramoa & Prusky, 1997; Hooks & Chen, 2006). This reflects a transition from NMDA receptors containing the NR2B subunit to those containing the NR2A subunit, a developmental switch that occurs in other central nervous system synapses and can be associated with the attainment of mature and stable neurotransmission (Cho, et al., 2009; Erisir & Harris, 2003; Williams, et al., 1993; Carmignoto & Vicini, 1992; Monyer, et al., 1994). The acceleration of NMDAR-mediated current is prevented in mice undergoing retinal activity blockade from p10-15, but is unaffected by dark rearing (Hooks & Chen, 2006). Therefore NMDAR subunit switching, like input number refinement and AMPAR-mediated strengthening, depends on spontaneous retinal activity rather than visual experience at the retinogeniculate synapse. It remains unknown whether these processes share common mechanisms or, rather, are independently regulated downstream of retinal activity.

Summary

Taken together, the work discussed above suggests that retinogeniculate development involves a complex and highly regulated network of developmental processes. The circuit is wired in stages: first molecular guidance cues guide retinal axons to their appropriate initial termination zones. Next, activity-dependent competition refines axonal projection and connectivity. Finally, the synapse undergoes pre- and postsynaptic maturation to achieve its characteristic pattern of information transfer.
Each of these steps has been the focus of extensive investigation, and yet our understanding of the circuit remains incomplete limited by reliance on a number of key assumptions. Notably, synapse development has been studied nearly exclusively within the contralaterally-innervated zone of the dLGN and it is generally assumed that synapses within the ipsilaterally-innervated zone develop with similar properties (Bickford, et al., 2010; Chen & Regehr, 2000; Koch & Ullian, 2010; Liu & Chen, 2008; Žiburkus & Guido, 2006). However, recent work has described fundamental differences in the connectivity and developmental remodeling of the ipsilaterally- and contralaterally-projecting RGC to dLGN circuit.

Contralaterally-projecting RGCs express different, and more mature, molecular profiles than do I-RGCs during development (Wang, et al., 2016). Innervation occurs earlier and to a greater extent in the contralateral visual pathway at both the dLGN and the visual cortex than in ipsilateral projections (Godement, et al., 1984; Crair, et al., 1998). Axon terminal extension and morphology is shaped by differential responses to molecular guidance cues in C-RGCs and I-RGCs (Feldheim, et al., 1998; Pfeiffenberger, et al., 2006). Binocularly-innervated relay cells receive different innervation patterns than do those innervated solely by C-RGCs (Rompani, et al., 2017), suggesting differential activity patterns in the subregions of the dLGN, and the outcome of activity-dependent competition appears biased in favor of I-RGCs (Muir-Robinson, et al., 2002; Pfeiffenberger, et al., 2005). In sum, these results argue for fundamental differences between the ipsilaterally- and contralaterally-projecting components of the retinogeniculate pathway, which must be understood functionally in order to interpret previous work characterizing the development of the visual circuit.
In this dissertation, we demonstrate that synapses formed by ipsilaterally-projecting RGCs develop along a fundamentally different trajectory than those in the contralaterally-innervated dLGN. Synapses within ipsilaterally-innervated thalamus undergo a dramatic increase in synaptic gain, ultimately becoming more than twofold stronger than the synapses formed in the nearby contralateral zone of the dLGN. We then demonstrate that this program of synaptic gain control is gated by the resolution of binocular competition. Surgical and genetic manipulations to alter the duration of competition between the two eyes result in commensurate shifts in the timing of synapse strengthening within ipsilateral dLGN. We propose a model whereby activity-dependent synaptic competition not only refines anatomical connectivity in the developing dLGN, it also functions as a developmental timer to gate the subsequent establishment of eye-specific homeostatic set points for synaptic gain by allowing a circuit-specific increase in the number of available release sites. We speculate that the precise transition from activity-dependent synaptic competition to region-specific synaptic gain control will influence the subsequent refinement of downstream cortical circuits.
Chapter 2 – Synaptic Gain Control at a Visual Synapse: Gated by Competition and Constrained Homeostatically

Results

We have characterized synaptic transmission at the synapse between retinal ganglion cells (RGC) and relay neurons (RN) within the dorsal lateral geniculate nucleus (dLGN). We chose to assess transmission at two time points. The first, postnatal day 12-15 (p12-15), coincides with complete anatomical and functional segregation of RGC axons into eye-specific termination zones in the dLGN (Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006). The second time point, postnatal day 21-26 (p21-26), allows for ~10 days of synapse maturation in the presence of visual experience following eye opening at p12 ± 1 day. At each time point, we compare retinogeniculate synaptic transmission to RNs that reside either within the dLGN zone innervated by the contralateral eye (C-dLGN) or ipsilateral eye (I-dLGN). To target recording electrodes to RNs within either C-dLGN or I-dLGN, we prepared parasagittal slices through the visual thalamus that contained several mm of the innervating optic tract. We labeled a large subset of ipsilaterally-projecting RGCs by expressing Cre recombinase under the serotonin transporter promoter (ET33; referred to hereafter as SERT-Cre), which is used to drive a Cre-dependent fluorescent reporter (TdTomato, mGFP, or a syp-TdTomato fusion protein) (Koch, et al., 2011). Reporter expression permitted visualization of axon terminals originating from the ipsilateral eye (I-RGCs), allowing us to target our recordings to either the I-dLGN or the surrounding C-dLGN (Figure 1A; Supplemental Figure S1).
We analyzed retinogeniculate synaptic transmission by stimulating the axon tract harboring RGC axons originating from both ipsilateral and contralateral eyes (Turner & Salt, 1998; Chen & Regehr, 2000). Stimulation was begun at sub-threshold levels and was then gradually increased until excitatory postsynaptic current (EPSC) amplitudes reached a maximal amplitude that remained unchanged despite further increases in stimulus intensity, referred to hereafter as the maximal EPSC (EPSC\text{max}). We quantified EPSC\text{max} and the stepwise increase in EPSC amplitudes that occurred as stimulus intensity was gradually increased (Figure 1B). Quantification of the stepwise increases in EPSC amplitudes allowed for estimation of the number and strength of inputs received by a given postsynaptic relay neuron (see Methods). We assayed AMPAR- and NMDAR-mediated currents at membrane potentials of -70 mV and +40 mV, respectively.

Selective Strengthening of Synapses in the Ipsilateral Projection Zone of the dLGN

We first assessed transmission in the C-dLGN at p12-15 and compared this to transmission at p21-26, an analysis that is directly comparable to previously published work from other laboratories (Turner & Salt, 1998; Chen & Regehr, 2000; Litvina & Chen, 2017). We document results that are quantitatively similar to previous findings. AMPAR-mediated EPSC\text{max} amplitudes did not change between p12-15 and p21-26 (median: 0.64 nA, n=14; p21-26 median: 1.0 nA, n=12; p>0.4, Wilcoxon Rank-Sum Test; Figure 1C). NMDAR-mediated EPSC\text{max} amplitude decreased from p12-15 to p21-26 (p12-15 median: 0.91 nA, n=14; p21-26 median: 0.51 nA, n=12; p<0.05, Wilcoxon Rank-Sum Test). These results were also reflected in the strengths of individual inputs
(Figure 1D, see Methods) (NMDA: p12-15 median: 0.34 nA, n=26; p21-26 median: 0.23 nA, n=18; p<0.05; AMPA: p12-15 median: 0.39 nA, n=23; p21-26 median: 0.55 nA, n=19, p>0.3, Wilcoxon Rank-Sum Test). The number of presynaptic inputs per relay cell, estimated by counting the discrete EPSC amplitudes evoked by varying optic tract stimulation, did not change (p12-15 median: 2.0 inputs, n=14; p21-26 median: 2.5 inputs, n=12; p>0.4, Wilcoxon Rank-Sum Test) (Figure 1E). Another method for estimating input number, the fiber fraction, revealed no difference between the two time points (see Figure S2A and Methods). Finally, we found that the time constant of decay for NMDAR-mediated currents significantly decreased between p12-15 and p21-26, consistent with a normal developmental switch from receptors that incorporate NR2B to receptor subunits to receptors that incorporate NR2A subunits (p12-15 median: 91.4 msec, n=14; p21-26 median: 78.2 msec, n=12; p<0.01, Wilcoxon Rank-Sum Test) (Figure 1F). These data are consistent with a program of postsynaptic maturation, consistent with prior observations in this system (Liu & Chen, 2008).

We observed a strikingly different pattern of functional maturation when we recorded within the ipsilateral projection zone of the dLGN. First, at p12-15, AMPAR- and NMDAR-mediated EPSCs\textsubscript{max} were statistically identical to synapses to the C-dLGN (AMPA: C-dLGN median: 0.64 nA, n=14; I-dLGN median: 0.67 nA, n=12, p>0.8; NMDA: C-dLGN median: 0.91 nA, n=14; I-dLGN median: 0.64 nA, n=12; p>0.3; Wilcoxon Rank-Sum Test). However, when we assessed transmission at p21-26, we found that maximal AMPAR and NMDAR EPSCs within the I-dLGN were dramatically increased compared to both the earlier time point (AMPA: p12-15 median: 0.67 nA, n=12; p21-26 median: 2.3 nA, n=15, p<0.05; NMDA: p12-15 median: 0.64 nA, n=12; p21-26 median:
1.4 nA, n=15; p<0.05, Wilcoxon Rank-Sum Test) (Figure 1G) and to age-matched synapses in C-LGN (AMPA: C-dLGN median: 1.0 nA, n=12; I-dLGN median: 2.3 nA, n=15, p<0.05; NMDA: C-dLGN median: 0.51 nA, n=12; I-dLGN median: 1.4 nA, n=15; p<0.01, Wilcoxon Rank-Sum Test). Evidence of significantly increased synaptic strength was also seen when we assessed the distribution of individual input sizes within the I-dLGN (NMDA: p12-15 median: 0.27 nA, n=14; p21-26 median: 0.62 nA, n=21; p<0.05; AMPA: p12-15 median: 0.44 nA, n=14; p21-26 median: 1.2 nA, n=21, p<0.05, Wilcoxon Rank-Sum Test) (Figure 1H). The same trend was observed when minimal stimulation was used to recruit and estimate the amplitude of single inputs (see Figure S3 and Methods). However, there was no change in estimated input number (p12-15 median: 2.5 inputs, n=12; p21-26 median: 2.0 inputs, n=15; p>0.3, Wilcoxon Rank-Sum Test) (Figure 1I) or fiber fraction (Figure S2B). Finally, we observed a decrease in the NMDA-mediated current time constant of decay indicative of a normal postsynaptic maturation process from p12-15 to p21-26 (p12-15 median: 104.1 msec, n=12; p21-26 median: 83.1 msec, n=15; p<0.05, Wilcoxon Rank-Sum Test) (Figure 1J). The striking increase in both the AMPAR EPSC$_{\text{max}}$ and the NMDAR EPSC$_{\text{max}}$ without a change in the estimated input number suggest that synaptic transmission matures along a fundamentally different trajectory within I-dLGN compared to C-dLGN. This finding may have significant implications for activity-dependent wiring of downstream cortical circuitry (see Discussion).

*Synaptic gain doubles without a change in quantal size*
The observed doubling of EPSC$_{\text{max}}$ within I-dLGN, occurring without an apparent change in input number, could be driven by changes that are either presynaptic, postsynaptic, or a combination of the two. To examine a possible postsynaptic contribution, we estimated the mean spontaneous excitatory postsynaptic current amplitude (sEPSCs). Consistent with previous work (Chen & Regehr, 2000), the mean AMPAR-mediated sEPSC amplitude increased slightly from p12-15 to p21-26 in the C-dLGN (p12-15 mean +/- SEM: 16.4 +/- 0.70 pA, n=13; p21-26 mean +/- SEM: 19.1 +/- 0.80 pA, n=10; p<0.05, unpaired t-test). The same was true for recordings within I-dLGN (p12-15 mean +/- SEM: 13.4 +/- 0.49 pA, n=11; p21-26 mean +/- SEM: 16.2 +/- 1.1 pA, n=10; p<0.05, unpaired t-test). However, quantal size at p21-26 did not differ when comparing C-dLGN and I-dLGN recording sites (contralateral zone mean +/- SEM: 19.1 +/- 0.80 pA, n=10; ipsilateral zone mean +/- SEM: 16.2 +/- 1.1 pA, n=10; p>0.05, unpaired t-test). Thus, a change in quantal size cannot account for the dramatic increase in EPSC$_{\text{max}}$ that is selectively observed in the I-dLGN (Figure 2A, 2C).

We note that the frequency of sEPSCs increased from p12-15 to p21-26 in the I-dLGN (p12-15 mean +/- SEM: 1.3 +/- 0.3 Hz, n=11; p21-26 mean +/- SEM: 2.9 +/- 0.4 Hz, n=10; p<0.01, unpaired t-test), while it was statistically unchanged in the C-dLGN (p12-15 mean +/- SEM: 2.6 +/- 0.3 Hz, n=13; p21-26 mean +/- SEM: 3.9 +/- 0.8 Hz, n=10; p>0.1, unpaired t-test) (Figure 2B). Nonetheless, the frequencies at p21-26 are statistically identical comparing C-dLGN and I-dLGN (contralateral zone mean +/- SEM: 2.9 +/- 0.4 Hz, n=10; ipsilateral zone mean +/- SEM: 3.9 +/- 0.8 Hz, n=10; p>0.2, unpaired t-test). These data are consistent with a developmental expansion of the number of presynaptic release sites, originating from a constant number of RGC axons,
for the I-dLGN, as previously suggested for synapses within the C-dLGN (Chen & Regehr, 2000; Hong, et al., 2014).

Thalamic dLGN relay neurons receive input not only from RGCs, but also from excitatory neurons originating in the cortex (Jacobs, et al., 2007). Although RGC axons are formed in close proximity to the relay neuron cell body (Wilson, et al., 1984; Liu, et al., 1998), allowing good electrophysiological access, it remains formally possible that our estimates of quantal size are confounded by the inclusion of quantal events originating from both RGC and cortical inputs. To address this potential issue, we attempted to isolate spontaneous miniature release events that originate from the retinogeniculate synapse. To do so, we substituted extracellular calcium with strontium (3 mM) to desynchronize stimulus-dependent synaptic vesicle release (Figure 2D). Release events that occur within 500ms of nerve stimulation are biased toward asynchronous EPSCs (aEPSCs) that originate from stimulated retinal axons, as opposed to spontaneous release originating from cortical inputs that reside in a different, un-stimulated, axon tract (Chen & Regehr, 2000; Dodge, et al., 1969; Koch, et al., 2011). First, we demonstrated that the mean aEPSC amplitude recorded in I-dLGN at p12-15 was statistically identical to the mean sEPSC recorded at the same age in the same termination zone (sEPSC mean +/- SEM: 13.4 +/- 0.49 pA, n=11; aEPSC mean +/- SEM: 14.5 +/- 1.04 pA, n=4; p>0.2, unpaired t-test). This confirmed the reliability of this technique. Next, we demonstrated that the mean aEPSC amplitude recorded in I-dLGN did not change as a function of development from p12 to p26 (p12-15 mean +/- SEM: 14.5 +/- 1.04 pA, n=4; p21-26 mean +/- SEM: 14.8 +/- 0.80 pA, n=6; p>0.8, unpaired t-test) (Figure 2E). Taken together, our data argue that the developmental
doubling of \( \text{EPSC}_{\text{max}} \) observed in I-dLGN cannot be accounted for by a change in quantal size.

**Ipsilateral-specific enhancement of presynaptic vesicle release**

The \( \text{EPSC}_{\text{max}} \) doubles at RGC synapses in I-dLGN without a change in quantal size and without a change in the estimated number of axons innervating each relay cell. Thus, the change in synaptic gain must be due to either an increase in the probability of vesicle release (PVR), or a change in the number of presynaptic release sites. An increase in PVR that occurs without a change in the number of release sites will enhance synaptic depression during paired-pulse stimulation. Given that synaptic gain doubles in the ipsilaterally-innervated dLGN, a substantial shift in the PVR and thus the paired-pulse ratio (PPR) would be required. However, the PPR, assayed over a range of inter-stimulus intervals, did not change as a function of age in either the C-dLGN or I-dLGN (Figure 3E, H). Note that these data were collected in the presence of the AMPAR desensitization inhibitor cyclothiazide. These results argue the I-dLGN-specific increase in synaptic gain is not driven by a developmental change in PVR.

We next examined estimated the number of presynaptic release sites (N) by calculating the size of the readily releasable vesicle pool (RRP) according to established methods (Schneggenburger, et al., 1999; Müller, et al., 2012). Again, recordings were made in the presence of the desensitization inhibitor cyclothiazide. Retinal axons were stimulated at 20 Hz (20-25 pulses) (Figure 3A). Back extrapolation of the cumulative EPSC from steady state, and dividing by the mean sEPSC amplitude, allows estimation of the RRP (Figure 3B). As a control, we examined synapses in C-dLGN and found no
change in RRP as a function of developmental age (p12-15 median: 199 vesicles, n=10; p21-26 median: 165 vesicles, n=10; p>0.9, Wilcoxon Rank-Sum Test) (Figure 3C). This analysis also allows an alternate method for the calculation of release probability. By dividing an estimate of the number of vesicles released during the initial EPSC of the stimulus train by the calculated RRP, one arrives at an approximation of PVR, referred to here as P_{\text{train}} (Schneggenburger, et al., 1999). We find that P_{\text{train}} is also unchanged as a function of development in the C-dLGN (p12-15 median: 0.36, n=10; p21-26 median: 0.39, n=10; p>0.7, Wilcoxon Rank-Sum Test) (Figure 3D), consistent with the PPR results reported above. Thus, all synaptic parameters are unchanged at the C-dLGN synapse between p12 and p16, as predicted by the stable EPSC_{\text{max}}.

When these experiments were repeated in I-dLGN, profound differences were observed. We found a 1.5-fold increase in RRP as a function of age (p12-15 median: 289 vesicles, n=7; p21-26 median: 429 vesicles, n=13; p<0.05, Wilcoxon Rank-Sum Test) (Figure 3F). This change in RRP occurs without a change in P_{\text{train}} (p12-15 median: 0.36, n=7; p21-26 median: 0.33, n=13; p>0.5, Wilcoxon Rank-Sum Test) (Figure 3G) and without a change in PVR (Figure 3H). It has been established that multi-vesicular release can occur at the retinogeniculate synapse (Budisantoso, et al., 2012). Therefore, the large increase in RRP could be achieved by either an expansion of the RRP at a fixed number of release sites, or an increase in the number of release sites, or a combination of the both processes. One interpretation of our data is that a new set point for synaptic gain is established and achieved within the I-dLGN between p12 and p26. Since EPSC_{\text{max}} doubles without a change in input number, the enhanced transmission could reflect a set point for EPSC_{\text{max}}, or a set point that controls the gain of
individual presynaptic RGC inputs. These data raise the following question: what triggers the development change in synaptic set point specifically within the I-dLGN?

Premature strengthening of ipsilateral RGC synapses following premature removal of competing retinal axons

The onset of ipsilateral-specific synaptic strengthening occurs after postnatal day 15. This time point coincides with the resolution of binocular competition within I-dLGN, assessed both anatomically and functionally (Žiburkus & Guido, 2006; Jaubert-Miazza, et al., 2005; but see Howarth, et al., 2014). Thus, we hypothesized that the elimination of contralateral fibers from the ipsilateral projection zone could be a developmental event that triggers the subsequent potentiation of EPSC$_{\text{max}}$, measured at RGC terminals in I-dLGN. To test this hypothesis, we surgically eliminated contralateral RGCs during postnatal development, thereby removing the source of competing synaptic inputs to the ipsilateral projection zone of the dLGN. We then asked whether this manipulation lead to precocious strengthening of the ipsilateral RGC projections to the dLGN. We performed neonatal (p0-2) monocular enucleation to eliminate one set of RGC inputs to the visual thalamus. At the time of enucleation, RGC axons from both eyes have innervated the dLGN and the process of eye-specific segregation is underway. Monocular enucleation eliminates RGC axons from the dLGN over a period of 2 days (Upton, et al., 1999), resulting in monoinnervation of the I-dLGN beginning by approximately postnatal day 4 (Figure 4A). Importantly, the ipsilaterally projecting RGCs from the remaining eye remain focused in a compact zone within the dLGN, although
the size of this ipsilateral projection zone is expanded compared to non-enucleated controls (Godement, et al., 1980; Hayakawa & Kawasaki, 2010).

We recorded from the ipsilaterally-innervated dLGN at p12-15 in mice that underwent early monocular enucleation (Figure 4B, left). We found that EPSC\textsubscript{max} was significantly greater than in non-enucleated controls, consistent with premature synapse strengthening triggered by the removal of competing contralateral inputs (Figure 4C, left). Indeed, AMPAR-mediated EPSC\textsubscript{max} was strengthened more than 3-fold compared to non-enucleated controls (non-enucleated median: 0.67 nA, n=12; enucleated median: 2.2 nA, n=12, p<0.05, Wilcoxon Rank-Sum Test) (Figure 4D, left). The NMDAR-mediated EPSC\textsubscript{max} was similarly strengthened (non-enucleated median: 0.64 nA, n=12; enucleated median: 2.2 nA, n=12; p<0.0001, Wilcoxon Rank-Sum Test) (Figure 4B-D, right). Finally, we note that the absolute amplitude of AMPAR-mediated EPSC\textsubscript{max} recorded in enucleated I-dLGN at p12-15 was statistically identical to EPSC\textsubscript{max} recorded in non-enucleated I-dLGN at p21-26.

To determine if increased synaptic drive is maintained after premature strengthening, we recorded in enucleated animals at p21-26. We found that AMPAR-mediated synaptic strength was sustained through p21-26, where it matched the normal developmental program of non-enucleated controls (non-enucleated median: 2.3 nA, n=15; enucleated median: 2.7 nA, n=9; p>0.5, Wilcoxon Rank-Sum Test) (Figure 4D). Remarkably, the level of NMDAR-mediated synaptic transmission attenuated in enucleated mice by p21-26, to precisely converge on the level of NMDAR drive in non-enucleated controls (non-enucleated median: 1.4 nA, n=15; enucleated median: 1.4 nA, n=9; p>0.5, Wilcoxon Rank-Sum Test) (Figure 4C). Previous work has noted a
decrease in the ratio of NMDAR to AMPAR-mediated current as the retinogeniculate synapse matures (Chen & Regehr, 2000; Hooks & Chen, 2006). We propose, therefore, that the slight reduction of NMDAR-mediated drive in enucleated mice reflects the maturation of the precociously potentiated synapse between p12 and p21. This proposal is consistent with another observation. We find that the developmental change in the rate of NMDA current decay is unaltered by monocular enucleation, suggesting that the program of postsynaptic receptor maturation is unaffected by the enucleation of competing contralateral axons (Figure 4E) (p12-15 median: 115 msec, n=12; p21-26 median: 80 msec, n=9; p<0.0001, Wilcoxon Rank-Sum Test).

Finally, we collected additional control information. Monocular enucleation did not alter the number of presynaptic inputs per relay cell recorded at p12-15 or p21-26 (p12-15 median: 2.0 inputs, n=12; p21-26 median: 2.0 inputs, n=9; p>0.7, Wilcoxon Rank-Sum Test) (Figure 4F). Similarly, the amplitude of spontaneous EPSCs was stable through development in enucleated mice (p12-15 mean +/- SEM: 12.8 +/- 0.37 pA, n=11; p21-26 mean +/- SEM: 12.8 +/- 1.2 pA, n=6; p>0.9, unpaired t-test) (Figure 4G). Thus, the precocious enhancement of EPSC_{\text{max}} in I-dLGN appears to be achieved by the same mechanisms by which EPSC_{\text{max}} is normally enhanced during development of control mice. It should be emphasized that this increase cannot be accomplished by I-RGC axons simply occupying dendritic territory that was previously occupied by competing C-RGC axons. Our results are based on stimulation of the entire optic tract, such that the EPSC_{\text{max}} reflects the recruitment of all RGC inputs. The total number of presynaptic release sites is increased, as shown above (Figures 1 and 3). Taken together, our data argue that the developmental program of synaptic strengthening is
triggered by the loss of competing contralateral RGC axons in I-dLGN. Our data also argue that EPSC_{max} amplitude is somehow constrained from exceeding its mature level, even when the time window for synapse strengthening is increased by nearly two weeks following enucleation. Again, we propose that potentiation of EPSC_{max} is due to the developmental establishment of a new homeostatic set point for EPSC_{max}, and the expression of this set point is triggered by the removal of competing contralateral RGC axons.

**Evidence for Functionally Biased Target Selection in the I-dLGN**

If elimination of competing contralateral axons drives the developmental increase in EPSC_{max} within I-dLGN, then preventing the removal of contralateral axons should prevent this from occurring. Therefore, we sought a method to block the elimination of contralateral RGC axons from the ipsilateral dLGN. The ideal manipulation would allow the ipsilateral projection zone to form within the dLGN, would allow early synapse development to proceed such that each relay cell receives the correct number of RGC inputs, and would cause the contralateral axons to persist within the ipsilateral dLGN between p12-26. Blocking retinal waves or blocking RGC activity with TTX does not suit our purposes because, under these conditions, ipsilateral RGCs fail to coalesce into a specific region of the dLGN, RGC axons fail to properly refine their termination zones, and in some instances, ipsilateral RGC terminals are eliminated from the dLGN (Penn, et al., 1998; Huberman, et al., 2002; Shatz & Stryker, 1988; Xu, et al., 2011).

We took advantage of a previously published paradigm in which the SERT-Cre driver mouse line was used to selectively excise the vesicular glutamate transporter
(VGLUT2) from a subset of ipsilaterally-projecting RGCs. This manipulation (VGLUT2 conditional knockout, referred to hereafter as VGLUT2-cKO) prevents the loading of synaptic vesicles with glutamate and, ultimately, leads to a block in glutamatergic transmission at ipsilateral RGC terminals (Koch, et al., 2011). In VGLUT2-cKO mice, ipsilaterally-projecting RGC terminals coalesce into a well-defined ipsilateral projection zone within the dLGN, originally assayed at p10 (Koch, et al., 2011) and repeated here at p12 (Figure S1). Contralateral RGC axons persist within the ipsilateral domain until at least p21 (data not shown). Anatomically, this manipulation is precisely what we hoped to achieve. However, the functional consequences of this manipulation remained largely undefined. Therefore, we first examined synapse development within the ipsilateral dLGN of VGLUT2-cKO mice.

First, we compared synaptic transmission between SERT-Cre(+) VGLUT2 knockout mice (homozygous for the floxed VGLUT2 allele) and control mice (see Methods). All mice expressed a Channelrhodopsin-2-TdTomato fusion protein in a SERT-Cre-dependent manner, permitting the visualization and optical excitation of Cre-expressing ipsilateral RGCs (the same axons that lack VGLUT2 in the VGLUT2-cKO mice). We recorded within I-dLGN and compared synaptic transmission evoked by electrical stimulation of the optic tract to synaptic transmission recorded following optical stimulation of the presynaptic terminals (Figure 5A). Electrical stimulation recruits both ipsilaterally- and contralaterally-projecting RGCs, while optical stimulation can only recruit SERT-Cre(+) ipsilateral RGCs. Thus, optical stimulation allows us to directly assess transmission from VGLUT2-cKO terminals.
We compared optical versus electrical stimulation in control mice, thereby assessing the fraction of I-dLGN neurons that receive input from SERT-Cre(+) I-RGC axons. In control mice (p21-26), 6/11 cells (55.5%) with detectable electrically-evoked synaptic input also displayed strong EPSCs in response to optogenetic stimulation, indicating innervation by SERT-Cre(+) ipsilateral RGCs (Figure 5B, top left; Figure 5C). The remaining 5/11 cells exhibited no optically-evoked responses, and presumably were innervated solely by SERT-Cre(-) ipsilateral RGCs (Figure 5B, bottom left). Note that for cells that respond to both optical and electrical stimulation, the maximal synaptic currents elicited by electrical vs. optical stimulation were not statistically different by Wilcoxon Rank-Sum Test (electrical stimulation median: 1.3 nA, n=6; optical stimulation median: 0.80 nA, n=6; p>0.8) or paired t-test (mean of differences (electrical-optical stimulation): -0.1 pA; SEM of differences: 0.2 pA; p>0.6). This observation is consistent with previously published reports highlighting similar axonal and ‘over-bouton’ stimulation (Jackman, et al., 2014; but see Litvina & Chen, 2017, and associated Discussion). Our data further demonstrate that the SERT-Cre(+) subpopulation of ipsilateral RGCs is relatively large, comprising approximately half of all ipsilateral RGCs. Taken together, these observations demonstrate that individual relay cells in the ipsilaterally-innervated dLGN receive primarily SERT-Cre(+) RGC input, or SERT-Cre(-) RGC input, but rarely a combination of the two. This suggests a previously unappreciated level of precision for synaptic connectivity in ipsilateral dLGN.

Next, we repeated optical versus electrical stimulation in the SERT-Cre(+) VGLUT2-cKO animals. In cells that responded electrically, none showed an optically driven synaptic response (0/6) (Figure 5B, right; Figure 5F). Thus, SERT-Cre(+)
ipsilateral RGCs that lack expression of VGLUT2 fail to release glutamate at p21-26. This result also demonstrates that these terminals, which persist anatomically (data not shown), have not compensated for early loss of VGLUT2 by expressing a different glutamate transporter. The SERT-Cre(-) axons that were electrically recruited could represent either SERT-Cre(-) RGCs projecting from the ipsilateral eye, or they could represent persistent contralateral fibers, or a combination of the two. The amplitudes of electrically- and optically-driven synaptic responses are plotted for each cell (Figure 5D-F). From these data, we can conclude that the VGLUT2-cKO mouse represents a system in which the ipsilateral projection zone coalesces, contralateral fibers persist, and input number refines correctly compared to controls (see below). We could next test whether the persistent presence of contralateral fibers prevented strengthening of the RGC synapse in the ipsilaterally-innervated zone of the dLGN.

**Failure of ipsilateral synapse strengthening following failure of input segregation**

To determine whether persistent contralateral innervation of the I-dLGN suppresses the normal program of synaptic strengthening, we quantified synaptic transmission within the ipsilateral zone of the dLGN in VGLUT2-cKO mice (Figure 6A). We electrically stimulated the optic tract while recording from the ipsilaterally-innervated zone of the dLGN. First, at p12-15, synaptic transmission is indistinguishable from that of controls. Both maximal synaptic drive (Figure 6B-D, left) and the estimated number of axonal inputs (Figure 6E, left) are in agreement with control values presented earlier. Thus, despite the fact that ~50% of ipsilateral RGC innervation is rendered mute, the system adapts to this perturbation and achieves the correct input number and the
correct maximal synaptic drive. This is consistent with a homeostatic set point for maximal synaptic gain and a compensatory program that re-establishes correct synaptic drive. This conclusion is supported by previous observations regarding the homeostatic capacity of the developing visual system (Chandrasekaran, et al., 2007; Shah & Crair, 2008).

When we examined synaptic transmission in the ipsilaterally-innervated dLGN at p21-26, the normal program of strengthening that occurs in control animals was completely blocked. Instead, maximal synaptic drive remained unchanged compared to the earlier (p12-p15) time point (Figure 6B-D, right). It is notable that synaptic maturation, as reflected in the decreased time constant for the decay of NMDAR-mediated EPSCs, proceeded normally in VGLUT2-cKO mice (p12-15 median: 110 msec, n=11; p21-26 median: 90 msec, n=12; p<0.01, Wilcoxon Rank-Sum Test) (Figure 6F). Thus, postsynaptic maturation proceeded, even though the presynaptic strengthening program was halted.

We next characterized synaptic transmission in greater depth, attempting to identify the basis for failed enhancement of EPSC$_{max}$ in the VGLUT2-cKO mice. The sEPSC amplitude did not differ between control and VGLUT2-cKO mice at p21-26 (control mean +/- SEM: 16.2 +/- 1.1 pA, n=10; VGLUT2-cKO mean +/- SEM: 15.1 +/- 0.97 pA, n=10; p>0.4, unpaired t-test) (Figure 6G). Further, there was no developmental change in quantal size (p12-15 mean +/- SEM: 13.3 +/- 0.69 pA, n=7; p21-26 mean +/- SEM: 15.1 +/- 0.97 pA, n=10; p>0.05, unpaired t-test) (Figure 6G). We performed high frequency optic tract stimulation as previously described to estimate the size of the readily releasable vesicle pool and release probability in VGLUT2-cKO mice at p21-26.
We observed a significantly smaller RRP in VGLUT2-cKO mice compared to age-matched controls (control median: 429 vesicles, n=13; VGLUT2-cKO median: 143 vesicles, n=10; p<0.05, Wilcoxon Rank-Sum Test) (Figure 6H). But, release probability estimated using the RRP amplitude was no different in control and VGLUT2-cKO mice (control median: 0.33, n=13; VGLUT2-cKO median: 0.34, n=10; p>0.7, Wilcoxon Rank-Sum Test) (Figure 6I). Finally, we carried out paired stimulation protocols to assay release probability at various interstimulus intervals, and found that control and VGLUT2-cKO mice exhibited indistinguishable paired pulse ratios (Figure 6J). Thus, failure to increase EPSC_{max} within the ipsilaterally-innervated dLGN in VGLUT2-cKO mice is best explained as a failure to increase the number of presynaptic release sites, as defined by the readily releasable vesicle pool.

These results demonstrate that when both contralateral and ipsilateral fibers persist within the I-dLGN, the normal program of developmental synaptic strengthening fails (Figure 6K-L). If synaptic drive is dominated by contralateral fibers in the VGLUT2-cKO, it is possible that the failure to strengthen reflects the normal synapse development profile of contralaterally projecting RGCs, which normally do not express a strengthening program (Figure 1). However, since ~50% of relay cells within the ipsilaterally-innervated dLGN of control mice receive SERT-Cre(-) input from the ipsilateral eye (Figure 5C), we would have expected to observe strengthening in a subset of our recorded cells. A population of large amplitude responses was not observed, arguing that the presence of contralateral innervation precludes the ipsilateral strengthening program. This is consistent with the demonstration that early removal of contralateral fibers accelerates the ipsilateral strengthening program (Figure 4).
However, these results do not completely untangle the role of contralateral fibers and the muted VGLUT2-lacking ipsilateral inputs in the failure to strengthen synaptic gain. To address this, we examined the consequences of early contralateral input removal in the VGLUT2-cKO mouse.

**Partial restoration of ipsilateral input strengthening in VGLUT2-cKO mice**

If the presence of contralateral fibers actively prevents the synapse strengthening program within the ipsilateral dLGN of the VGLUT2-cKO mice, then surgical removal of the persistent contralateral fibers should restore the strengthening program, even in the presence of muted I-RGC fibers. Thus, we performed neonatal monocular enucleations in VGLUT2-cKO mice, and recorded from relay cells within the dLGN ipsilateral to the remaining eye. In this paradigm, the only remaining source of functional retinal input to the I-dLGN is from SERT-Cre(-) ipsilateral RGCs (Figure 7A).

We recorded from the I-dLGN at p12-15 and p21-26. In enucleated VGLUT2-cKO mice at p12-15, we observed precocious strengthening of the NMDA current to a significantly greater amplitude than age-matched non-enucleated control mice (control median: 0.64 nA, n=12; enucleated VGLUT2-cKO median: 1.6 nA, n=14; p<0.01, Wilcoxon Rank-Sum Test) (Figure 7B-C, left). The median response (1.6 nA) did not differ from the level of synaptic drive that control synapses achieved (1.4 nA) during the late-strengthening program. Two conclusions can be drawn. First, this finding demonstrates that the population of SERT-Cre(-) ipsilateral RGCs can respond to the resolution of binocular competition by augmenting NMDAR-mediated synaptic transmission. Second, the degree of precision with which NMDA currents reach control levels in the face of both genetic and surgical perturbation argues, once again, that the
level of synaptic drive is homeostatically constrained to achieve a specific set point level of synaptic drive.

In contrast, AMPAR-mediated currents remained statistically unchanged in the enucleated VGLUT2-cKO mice at p12-15 (control median: 0.67 nA, n=12; enucleated VGLUT2-cKO median: 1.2 nA, n=14; p>0.5, Wilcoxon Rank-Sum Test) (Figure 7D, left). The deficit in AMPAR-mediated transmission suggests that enucleation spurs an increase in the prevalence of silent synapses in VGLUT2-cKO mice, presumably representing immature synapses that have yet to achieve significant AMPA receptor insertion (Koch & Ullian, 2010). It seems plausible that the precocious strengthening of synaptic drive in the enucleated VGLUT2-cKO mice might be somewhat delayed, given the severity of the perturbation to the system. Nonetheless, the data are consistent with the restoration of synapse strengthening by the removal of contralateral innervation within I-dLGN. These results also suggest that late strengthening of ipsilateral synaptic transmission may be a two-step process that parallels the process of synapse development in this and other circuits, including within the visual system (Wu, et al., 1996; Cline & Haas, 2008). First, the elimination of contralateral inputs drives the strengthening of NMDAR transmission, representing an increase in silent synapses. Second, the silent synapses are converted to fully functional, AMPA receptor-containing synapses through a process that depends on the identity or activity of the I-RGCs themselves.

Finally, we asked whether the potentiated NMDAR-mediated synaptic transmission was maintained through p21-26. We found that the amplitude of NMDAR-mediated current declined (p12-15 median: 1.6 nA, n=14; p21-26 median: 0.82 nA,
n=11; p<0.01, Wilcoxon Rank-Sum Test), reverting to the baseline amplitude observed at p12-15 in controls, and to levels observed in the VGLUT2-cKO at both p12-15 and p21-26 (Figure 7B-D, right). AMPAR-mediated transmission did not change by p21-26. Interestingly, the time constant for NMDAR current decay decreased as expected through late development (Figure 7E), and input number was stable (Figure 7F) and similar to that observed in controls. It remains unclear why precocious synapse strengthening is not maintained. Nonetheless, several conclusions are possible. First, the data are consistent with an emerging model in which the presence of contralateral axons actively prevents the initiation of a synapse strengthening program expressed by ipsilaterally-projecting RGC axons within the ipsilateral dLGN. Second, at p12-15, monocularly enucleated VGLUT2-cKO mice exhibited AMPAR currents that were precisely the same amplitude as observed in wild type controls, despite a ~50% reduction in ipsilateral RGC inputs that could release glutamate, and despite the absence of compensatory innervation from the contralateral eye. Therefore, some homeostatic process must be engaged to ensure that the correct number of axons target each relay cell in the ipsilateral dLGN, and that maximal drive is normal. Ultimately, we observe a failure to sustain the new homeostatic set point for synaptic transmission to the ipsilaterally-innervated dLGN at p21-p26 in VGLUT2-cKO mice. We cannot rule out the possibility that SERT-Cre(+) ipsilaterally-projecting RGCs are the only subpopulation capable of carrying out the newly identified program of late strengthening .. Regardless, when taken together, our data support the conclusion that there is a re-specification of synaptic gain selectively within I-LGN that is consistent with a new homeostatic set point.
Figure 1. Selective and Potent Strengthening of Ipsilateral Synaptic Transmission in the dLGN

(A) Schematic of retinogeniculate circuit wiring. Retinal ganglion cells (RGCs) project from the right (red) and left (blue) eyes to the dorsal lateral geniculate nuclei (dLGN). Most RGC project contralaterally, while a small fraction project to a confined patch in the ipsilateral dLGN. Example placements of recording electrodes within the dLGN are shown. (B) Sample traces of cells recorded in the contralaterally- (blue) or ipsilaterally-innervated (red) dLGN. Negative-going AMPAR currents and positive-going NMDAR currents evoked by optic tract stimulation at varying intensities are overlaid. The recruitment of individual inputs can be observed as the discrete EPSC amplitudes visible in each example. Stimulus artifacts are blanked. (C,G) Maximal NMDAR- and AMPAR-mediated EPSCs. (D,H) Cumulative distribution function of the amplitudes of
individual retinal inputs of >100 pA. (E,I) Estimated number of retinal inputs per thalamic relay cell. (F,J) Time constant for the decay of NMDAR-mediated current evoked by saturating stimulus intensity. All significance tests performed using the Wilcoxon Rank-Sum test.
Figure 2. Postsynaptic Quantal Amplitude Cannot Account for the Strengthening of Ipsilateral Synaptic Transmission

(A) Mean amplitude and (B) frequency of spontaneous quantal EPSCs. (C) Example traces of spontaneous quantal EPSCs. (D) Example traces showing evoked asynchronous EPSCs. The first large negative deflection represents synchronous release following optic nerve stimulation, and is clipped to allow the following asynchronous release to be shown clearly. Stimulus artifacts are blanked. (E) Mean amplitude of asynchronous EPSCs in the ipsilaterally-innervated thalamus. All significance tests performed using the unpaired t-test.
Figure 3. Evidence that Enhanced Presynaptic Release Drives Ipsilateral Strengthening

(A) Example trace (top) with quantification (bottom) showing the diminishing response of a postsynaptic relay cell to 20 Hz presynaptic input stimulation. Stimulus artifacts are blanked. (B) The cumulative EPSC (black) is calculated by adding the amplitude of each response to the sum of the previous responses. The final five cumulative EPSC amplitudes, primarily comprising recycled vesicles, are fit with a linear regression (gray). The y-intercept of this line is an estimate of the total current evoked by readily-releasable vesicles, after subtracting the contribution of vesicular recycling. (C, F) The number of vesicles in the readily releasable pool (RRP). The cumulative EPSC intercept values calculated as in (B) are divided by the corresponding spontaneous EPSC amplitude to estimate the size of the RRP. (D, G) Synaptic release probability, calculated as the following ratio: the amplitude of the response to the first stimulus in the train, divided by the cumulative EPSC intercept value representing the theoretical current evoked by release of all readily releasable vesicles. (E, H) Paired pulse ratios comparing the amplitudes of two EPSCs evoked by stimulation at a variety of latencies. All significance tests performed using the Wilcoxon Rank-Sum test.
Figure 4. Eliminating Contralateral Inputs Drives Precocious Strengthening of Ipsilateral Synaptic Transmission

(A) Schematic illustrating the result of neonatal monocular enucleation. The remaining eye develops in the absence of a binocular competitor. Recordings were performed in the ipsilaterally-innervated region of the dLGN receiving input from the remaining eye (gold). (B) Example average traces of the maximal AMPAR and NMDAR responses to optic tract stimulation. Stimulus artifacts are blanked. (C, D) Maximal NMDAR and AMPAR-mediated EPSCs recorded in enucleated mice (gold), compared to the previously presented values from control mice (red). (E) Time constant for the decay of NMDAR-mediated current evoked by saturating stimulus intensity. (F) Estimated number of retinal inputs per thalamic relay cell. (G) Mean amplitude of spontaneous quantal EPSCs. (H-I) Proposed models for the development of the ipsilateral retinogeniculate synapse. Under normal conditions (top), binocular competition occurs during the first approximately eight postnatal days, resulting in monocular innervation at p12-15. Late ipsilateral presynaptic strengthening and postsynaptic maturation then occur, resulting in markedly increased ipsilateral synaptic transmission by p21-26. In mice undergoing neonatal monocular enucleation (bottom), monocular innervation is achieved early, potentially resulting in precocious synaptic strengthening and/or
maturation. Significance tests for (C-F) performed using the Wilcoxon Rank-Sum test; for (G) using the unpaired t-test.
Figure 5. VGLUT2 Knockout from SERT-Cre(+) Ipsilateral Retinal Ganglion Cells Eliminates Glutamate Release

(A) Schematic illustrating the experimental paradigm. Electrical stimulation of the optic nerve drove firing of retinal inputs from both the ipsilateral and contralateral eyes, while optical stimulation recruited only SERT-Cre(+) ipsilateral RGCs. Recordings were made in the ipsilaterally-innervated patch of the dLGN. (B) Example average traces comparing electrically- and optically-evoked postsynaptic currents. Two control cells are shown: one with a strong electrically-evoked (black) response and a similarly sized optically-evoked (red) response (top left), and one with no optically-evoked current (bottom left). In contrast, no cells in VGLUT2-cKO mice exhibited detectable optically-evoked inputs (right). Stimulus artifacts are blanked. (C) The percentage of relay cells with detectable electrically-evoked EPSCs that also displayed optically-evoked EPSCs. (D) Scatter plot comparing EPSCs evoked by electrical and optical stimulation for all cells. The dashed gray line indicates unity. (E-F) Paired observation plots showing the same data.
presented in (D), to better illustrate the relative amplitudes of electrically and optically evoked responses. Significance test in (C) performed using the Chi-square test.
Figure 6. Failure of Ipsilateral Synapse Strengthening in the Absence of Normal Input Segregation

(A) Schematic illustrating the effect of VGLUT2 knockout in a subset of ipsilaterally-projecting RGCs. Recordings were made in the ipsilaterally-innervated zone of the dLGN, which in VGLUT2-cKO mice also contained axon terminals from the contralateral
eye. (B) Example average traces of the maximal AMPAR and NMDAR responses to optic tract stimulation. Stimulus artifacts are blanked. (C, D) Maximal NMDAR and AMPAR-mediated EPSCs recorded in VGLUT2-cKO mice (purple), compared to the previously presented values from control mice (red). (D) Estimated number of retinal inputs per thalamic relay cell. (F) Time constant for the decay of NMDAR-mediated current evoked by saturating stimulus intensity. (G) Mean amplitude of spontaneous quantal EPSCs. (H) Estimated number of vesicles in the readily releasable pool size. (I) Presynaptic release probability, estimated through high-frequency stimulation as previously described. (J) Paired pulse ratios comparing the amplitudes of two EPSCs evoked by stimulation at a variety of latencies. (K-L) Hypothesized models for the impact of impaired eye-specific segregation. In contrast to control mice (left), VGLUT2-cKO mice fail to segregate retinal inputs from the two eyes. If such segregation is a prerequisite for the strengthening of ipsilateral synaptic transmission, then these mice would exhibit no late increase in ipsilateral synaptic gain. All significance tests were performed using the Wilcoxon Rank-Sum test except for (H), for which the unpaired t-test was used.
Figure 7. Eliminating Contralateral Inputs in Animals Lacking Normal Input Segregation Triggers Precocious but Transient Ipsilateral Synaptic Strengthening

(A) Schematic illustrating the experimental paradigm. Recordings were made in the ipsilaterally-innervated region of the right hemisphere dLGN in VGLUT2-cKO mice. The contralateral eye was neonatally enucleated, leaving only retinal input of ipsilateral origin. Inputs expressing SERT-Cre were muted as a result of Cre-mediated excision of the VGLUT2 allele, and so all observed glutamatergic EPSCs originated from the remaining population of SERT-Cre(-) ipsilateral RGCs. (B) Example average traces of the maximal AMPAR and NMDAR responses to saturating optic tract stimulation. Stimulus artifacts are blanked. (C,D) Maximal NMDAR and AMPAR-mediated EPSCs recorded in enucleated VGLUT2-cKO mice (gold), compared to the previously presented values from control mice (red). (E) Time constant for the decay of NMDAR-mediated current evoked by saturating stimulus intensity. All significance tests were performed using the Wilcoxon Rank-Sum test.
Figure 8. Model for a Change in Set Point for Ipsilateral Synaptic Transmission, Gated by Competition and Constrained Homeostatically

(A) At p0, relay neurons in the ipsilaterally-innervated dLGN (I-dLGN) receive synaptic input from both the contralateral (blue) and ipsilateral (red) eyes. Each circle is representative of synaptic inputs to a relay neuron in the dLGN. Based on the data presented in our study, we propose that the contralateral fibers release a signal that initially prevents ipsilateral inputs from strengthening (blue repressors). This negative regulation could act presynaptically to inhibit strengthening of the presynaptic terminal, or it could act postsynaptically to prevent the relay cell from fully expressing a new ‘set point’ for synaptic gain. During the time from p0 to p12, contralateral inputs to the I-dLGN are gradually eliminated, thereby relieving the repression of the ipsilateral strengthening program. (B). Once contralateral fibers are eliminated, the ipsilateral inputs to I-dLGN neurons strengthen dramatically from p12 to p26, targeting a new set point for synaptic gain (C).
Figure S1. Eye-Specific Segregation Fails in VGLUT2-cKO Mice

(A-B) The ipsilaterally-innervated dLGN (I-dLGN, outlined with dashed line) of a control (left) and a VGLUT2-cKO (right) p12 mouse are shown. SERT-Cre(+) RGCs from the ipsilateral eye (I-RGCs) are labeled by Cre-dependent expression of tdTomato. Contralateral fibers are labeled by CTB-488 (see Methods). (C-D) In control mice, contralaterally-projecting RGCs are seldom found within the I-dLGN. In VGLUT2-cKO mice, the I-dLGN shows substantial innervation by contralateral RGCs (C-RGCs), demonstrating a failure of eye-specific segregation. (E-F) SERT-Cre(+) RGCs in control mice show extensive overlap with VGLUT2 (antibody label, see Methods). In contrast, VGLUT2 label within the I-dLGN of VGLUT2-cKO mice mostly does not colocalize with SERT-Cre(+) RGCs, demonstrating the efficacy of the conditional knockout. Images are a single confocal plane. Scale bars are 50 um.
Figure S2. Fiber Fraction is Unchanged from p12 to p26

Fiber fraction (see Methods) is reported at each time point for various genetic and surgical conditions. No condition exhibited a significant difference from p12-15 to p21-26. (A-B) Fiber fraction for relay cells located in the C-dLGN and I-dLGN. Supplements Figure 1. (C) Fiber fraction for relay cells located in the I-dLGN, following neonatal enucleation of the contralateral eye. Supplements Figure 4. (D) Fiber fraction for relay cells located in the I-dLGN of VGLUT2-cKO mice. Supplements Figure 6. (E) Fiber fraction for relay cells located in the I-dLGN of VGLUT2-cKO mice, following neonatal enucleation of the contralateral eye. Supplements Figure 7. All significance tests were performed using the Wilcoxon Rank-Sum test.
Figure S3. Ipsilateral RGC Single Fiber AMPA Strength Selectively Increases
Single fiber strength (see Methods) is reported at each time point for various genetic and surgical conditions. (A-B) Single fiber strength for relay cells located in the C-dLGN (A) and I-dLGN (B). Supplements Figure 1. (C) Single fiber strength for relay cells located in the I-dLGN, following neonatal enucleation of the contralateral eye. Supplements Figure 4. (D) Single fiber strength for relay cells located in the I-dLGN of VGLUT2-cKO mice. Supplements Figure 6. (E) Single fiber strength for relay cells located in the I-dLGN of VGLUT2-cKO mice, following neonatal enucleation of the contralateral eye. Supplements Figure 7. All significance tests were performed using the Wilcoxon Rank-Sum test.
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**Oligonucleotides**

Primers for genotyping, see Table 2 | This paper | N/A

**Software and Algorithms**

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Chapter 3 – Conclusions and Future Directions

Here we define a new process of circuit maturation in the developing mouse visual system (Figure 8). The segregation of retinal inputs to the dLGN into eye-specific zones is a fundamental organizing principle of binocular visual system development in mammals. It has been generally assumed that synaptic transmission at the synapse between retinal ganglion cells and thalamic relay neurons are uniform in both the contralateral and ipsilateral fields of the mature dLGN. This assumption, while never directly tested, is important because it underlies our understanding of how information flows from the retina through the thalamus to the cortex (Coleman, et al., 2009). We now reveal that relay cells in the ipsilateral dLGN acquire a new set point for synaptic excitation during postnatal development, more than doubling synaptic drive selectively to the ipsilaterally- versus contralaterally- innervated relay neurons. Thus, in addition to the anatomical segregation of eye-specific inputs to the dLGN, we now demonstrate functional divergence of synaptic gain in an eye-specific manner. The onset of this divergence occurs after p15, following the refinement of RGC axon terminals of eye-specific zones. This developmental sequence might ensure that the zone-specific strengthening of synaptic gain does not interfere with the mechanisms of synaptic competition underlying anatomical segregation.

It is interesting to speculate how synapse strengthening within the I-LGN might influence cortical development. For example, in both cat and rodent, thalamic input to the cortex is initially dominated by the contralateral eye. But, around 10 days after eye-opening, this dominance wanes as input from the ipsilateral eye gains strength (Crair, et al., 1998; Smith & Trachtenberg, 2007; Trachtenberg, 2015). It has been speculated
that the strengthening of initially weak ipsilateral input to the cortex requires a non-Hebbian mechanism of plasticity. The 2-3 fold strengthening of synaptic transmission in the I-dLGN that we observe occurs at approximately the same developmental time as the equilibration of ipsilateral and contralateral cortical input observed in mice and other systems, and could reasonably contribute to this cortical plasticity. Thus, this newly identified program of selective ipsilateral synaptic potentiation could provide an underlying mechanism for the proposed non-Hebbian strengthening of ipsilateral cortical input, as postulated in a recent study examining corticogeniculate transmission (Jaepel, et al., 2017).

Finally, we propose that the process of synaptic competition serves a new purpose during the development of the dLGN, acting not only to refine anatomical connectivity, but to initiate a process of functional synapse maturation specifically within the I-dLGN. We provide evidence that the process of synapse strengthening within the ipsilateral dLGN is triggered by the anatomical removal of fibers originating from the contralateral eye. First, we demonstrate that early removal of contralateral fibers causes premature ipsilateral strengthening. Conversely, a manipulation that precludes removal of contralateral fibers from the ipsilateral dLGN cause a block in the strengthening program, at least over the time period examined in this study. By way of explanation, we propose that contralateral axons emit a signal that actively suppresses a developmental program that re-specified the set point for synaptic gain within the ipsilateral zone of the thalamus (Figure 8B). This implies a new role for input elimination, acting as a timer that gates the expression of subsequent phases of neural development and circuit maturation.
**Input takeover versus re-specification of set point synaptic gain**

Synaptic competition is an organizing principle of neural development that applies to the refinement of synaptic connectivity throughout the nervous system, including the neuromuscular junction, cerebellum, cortex, and thalamus. Competition allows initially diffuse patterns of innervation to be refined such that one or only a few inputs contact each postsynaptic target cell. In general, competition is waged over a constant field of postsynaptic neurotransmitter receptors. Therefore, as synapses are eliminated, the remaining (or winning) inputs are strengthened because they ‘take over’ the postsynaptic receptors previously occupied by the eliminated synapses. As a consequence, the presynaptic neuron adds new release sites and increases its relative strength, but the postsynaptic cell ‘holds’ synaptic gain constant by defining the number of receptor fields. Classical synaptic takeover cannot fully account for the increase in total synaptic gain that we observe within the ipsilateral dLGN. We demonstrate that maximal drive is increased by up to 300%. Furthermore, this effect is accounted for by an expansion in the readily releasable vesicle pool without a change in mEPSP amplitude or presynaptic release probability. The most parsimonious explanation is that new release sites have been added to increase synaptic gain between p15 and p26. This would most likely be achieved by the addition of new active zones, either to pre-existing synaptic boutons or through the addition of new synaptic boutons. In either case, it requires coordinated changes in both the presynaptic and postsynaptic cells to create new release sites (Figure 8A). Previous work has revealed that synaptic boutons are rearranged in the contralateral zone of the thalamus between p12 and p20 (Hong, et
al., 2014). However, this study found no change in bouton number, consistent with our observation that synaptic gain remains constant in contralaterally-innervated thalamus. It is interesting to speculate that a similar process of bouton reorganization within ipsilaterally-innervated thalamus, coupled to a switch in the homeostatic set point for synaptic transmission, could drive and maintain the increase in synaptic gain that we observe.

*Homeostasis in the developing dLGN*

We hypothesize that the strengthening of synaptic gain specifically within the ipsilateral zone of the thalamus is due to a change in the homeostatic set point for synaptic transmission. A homeostatic set point can be inferred if a cell responds to a perturbation and maintains a constant functionality (Davis, 2006). There is prior evidence for set point synaptic gain specification in the developing mouse visual system. Desynchronization of retinal waves impairs the refinement of RGC connectivity in the superior colliculus. As a consequence, postsynaptic neurons at postnatal day 6-7 receive a large number of weak synaptic inputs that combine to achieve the correct maximal synaptic drive. Thus, although input number is perturbed, synaptic gain remains correct, suggesting the existence of a set point for synaptic gain. Furthermore, input number and strength are restored to control levels by p21-25, demonstrating the use of multiple homeostatic systems to achieve a synaptic set point through development (Chandrasekaran, et al., 2007; Shah & Crair, 2008). Our data include perturbations that further imply the existence of a homeostatic set point. First, when contralateral innervation is surgically removed, premature ipsilateral strengthening
occurs, reaching the precise level that is normally achieved later in development, as if a set point for synaptic gain has been prematurely targeted (Figure 4). Second, in the VGLUT2-cKO animals, approximately 50% of ipsilaterally projecting RGCs fail to release glutamate but anatomically persist within the ipsilateral zone (Figure 5). In this situation, synaptic gain, measured at p12-15, is precisely normal (Figure 6). It remains uncertain what compensatory mechanism is engaged, but the correct synaptic gain and input number are achieved in the face of this profound perturbation, arguing once again for a homeostatic set point that specifies synaptic gain at the retinogeniculate synapse. Finally, if synaptic gain is specified by a homeostatic set point, our data argue that this set point changes within the I-dLGN, a shift that is caused by the removal of contralateral innervation. While changes in synaptic gain are common in neural development, evidence for age-dependent re-specification of a homeostatic set point for presynaptic neurotransmitter release has rarely been defined.

The molecular basis of a homeostatic set point remains completely unknown. It is has been proposed that the set point is genomically defined and closely associated with the specification of cell identity during development (Davis, 2006; Davis, 2013). In other biological systems, it is determined that the set point is ‘evolutionarily defined’ (Giordano, 2013). Nonetheless, there is evidence in other systems that a homeostatic set point for synaptic transmission can be re-specified at a specific time point during animal life-span (Mahoney, et al., 2014). More specifically, at 42-44 days of adult life, set point synaptic gain is increased at the Drosophila adult NMJ. The specific age at which this homeostatic re-specification occurs is not dissimilar to the developmental switch in a homeostatic set point that we, and others, propose in the mammalian visual
system. It is interesting to speculate how ipsilaterally-innervated relay neurons might adopt a different set point compared to relay neurons in the contralateral zone. One possibility is that all relay neurons are initially equivalent. The specification of the ipsilateral zone is driven by molecular events including an Eph/Ephrin gradient that could drive the establishment of ipsilateral relay cell identity and homeostatic signaling. This is an intriguing idea, in part, because Eph/Ephrin signaling has been implicated in homeostatic synaptic plasticity in a very different system, the Drosophila NMJ (Frank, et al., 2009).

*Implications for Dark-Rearing Dependent Plasticity*

It was recently demonstrated that visual deprivation at late stages of thalamic development (p20-27) alters RGC connectivity within the contralateral dLGN. Specifically, late dark rearing induces an approximately 3 fold increase in the number of RGC synaptic inputs (Hooks & Chen, 2006; Hooks & Chen, 2008). These studies have been conducted in contralaterally-innervated dLGN. If the timing of late dark rearing plasticity is conserved in I-dLGN, then this type of plasticity cannot account for the observed synapse strengthening that we observe. First, the strengthening that we observe is not accompanied by a change in input number. Second, the timing is incorrect. We document strengthening in the ipsilaterally-innervated dLGN between p15 and p21. Dark rearing that begins at birth and extends into this time period does not alter RGC connectivity (Hooks & Chen, 2006). Likewise, if dark rearing begins at p15, retinal input and strength is not abnormal at p22-26 (Hooks & Chen, 2008). The authors conclude that an extended period of visual experience is necessary prior to visual
deprivation in order for late dark rearing plasticity to be expressed. This plasticity could be considered a type of homeostatic response. Homeostatic plasticity would require a well-defined set point, which we argue is determined in the interval of p15-p21. Thus, our data could provide one explanation for the time dependence of late dark rearing induced plasticity.

**A Comparison with dLGN Connectomics**

Recent anatomical studies have provided unprecedented visualization of synapse organization within the dLGN (Hammer, et al., 2015; Morgan, et al., 2016; Rompani, et al., 2017). This analysis has revealed previously unappreciated complexity. At present, there is no simple way to move between an electrophysiological analysis of synaptic gain and connectomic information. One conclusion derived from these datasets is that each relay neuron may be contacted by many more axons that predicted from electrophysiological assays. However, this has no bearing on the concept that synaptic drive is homeostatically constrained as long as the homeostatic signaling system is assessing and regulating synaptic function as opposed to input number. Indeed, our manipulations of RGC input number, and previously published data (Chandrasekaran, Shah, & Crair, 2007; Shah & Crair, 2008), argue in favor of functional homeostatic control.

However, in keeping with the proposed complexity of synapse organization based on EM reconstructions, our data may imply a new level of molecular specificity governing synapse development in the thalamus. In our experiments, we express ChR2 exclusively in the SERT-Cre(+) RGCs. We then compare maximal EPSC amplitudes
derived from optogenetic stimulation versus electrical stimulation at single relay neurons within the ipsilaterally-innervated zone. Optogenetic stimulation occurs over the synaptic boutons, activating all potential SERT-Cre(+) terminals contacting an individual relay neuron. Electrical stimulation is initiated in the optic tract. In contrast to recently published work in the contralaterally-innervated dLGN, the average EPSC amplitude elicited optogenetically is no different when compared to electrical stimulation (p>0.8) of inputs to the l-dLGN. We conclude that ex vivo slices that precisely bisect the ipsilateral innervation zone also preserve the ipsilaterally projecting axon tract.

When we compare optical versus electrical stimulation, we find evidence that individual relay neurons are preferentially driven by either SERT-Cre(+) RGCs (selectively optically driven) or SERT-Cre(-) RGCs (recruited electrically). For example, when a thalamic relay cell receives SERT-Cre(+) innervation, based on optogenetic stimulation, electrical stimulation produces a very similar EPSC amplitude. This is true across a range of EPSC$_{\text{max}}$ amplitudes, from 0.1 nA to 3.7 nA. Therefore, the SERT-Cre(+) axons, when present, are the major contributor to the evoked EPSC. The converse is also true. We find 5 of 11 cells that can only be driven by electrical stimulation, suggesting selective innervation by SERT-Cre(-) axons. This type of segregation would be expected if each relay neuron is strongly innervated by one or at most two RGC axons, as predicted by electrophysiological estimation of input number (here and in prior work; Chen & Regehr, 2000; Jaubert-Miazza, et al., 2005; Žiburkus & Guido, 2006). However, if one takes into account new connectomic information, then each relay neuron may receive input from as many as 40-90 different RGC axons. If so, then 40-90 SERT-Cre(+) axons functionally segregate from a similar number of SERT-
Cre(-) axons to innervate single relay neurons, implying functional input segregation during thalamic development. Indeed, this is consistent with observations from recent connectomic reconstructions of the dLGN demonstrating that different anatomically-defined bouton subtypes preferentially innervate individual relay cells (Morgan, et al., 2016). The unlikely alternative is that SERT-Cre(+) and SERT-Cre(-) axons remain intermingled at each target, but only one type of axon terminal remains functional. Consistent with recent anatomical observations (Rompani, et al., 2017), our findings suggest that synaptic organization is fundamentally different within ipsilaterally- vs. contralaterally-innervated thalamus.

Future work will be required to elucidate the molecular mechanisms and functional consequences of the ipsilateral-specific retinogeniculate strengthening program reported here. Ultrastructural observations comparing the developing C-dLGN and I-dLGN would shed light on differences in their structural synaptic organization, and could reveal the anatomical correlate for the increase in readily releasable vesicle pool size we observe. We demonstrate that I-RGC strengthening fails in VGLUT2-cKO mice in which eye-specific segregation is impaired, consistent with our hypothesis that binocular segregation acts as a gate for this later developmental program. However, our conclusions from this data are limited by at least two caveats specific to the VGLUT2-cKO mouse: first, the population of I-RGCs that lack glutamatergic release may be the population that normally undergoes strengthening; second, synapses from muted VGLUT2-lacking I-RGCs may form a structural barrier to the expansion of synaptic connectivity from the remaining population of I-RGCs. Future work should disrupt eye-specific segregation using other means, such as disrupting or eliminating retinal waves.
(Penn, et al., 1998; Xu, et al., 2015) or blocking ERK signaling (Naska, et al., 2004), and examine the effect on ipsilateral RGC strengthening. Together, these experiments would more clearly define the relationship between binocular competition and ipsilateral RGC synaptic development.

Finally, one of the most intriguing aspects of the present work is the possibility that ipsilateral retinogeniculate strengthening underlies the balancing of ipsilateral and contralateral input to the visual cortex. If this is true, then uncovering the rules governing the development of eye-specific visual pathways may improve our understanding of binocular vision impairments such as amblyopia and strabismus. Future work should characterize the causal relationship, if any, between ipsilateral retinogeniculate strengthening and the increased ipsilateral geniculocortical drive that establishes the mature cortical retinotopic map (Crair, et al., 1998; Smith & Trachtenberg, 2007; Trachtenberg, 2015).

In summary, we define a new functional organization within the dLGN and propose that synaptic competition acts as a developmental timer that triggers re-specification of set point synaptic gain within the ipsilateral dLGN. If this mechanism is generalizable to neural circuit development in other areas of the central nervous system, then these findings could be relevant to the onset and expression of neurological and psychiatric disease. Inherited or de novo mutations that alter circuit connectivity could reasonably impair that ability to attain subsequent homeostatic set points, further altering the flow of information through complex neural circuitry.
Methods

Experimental Model and Subject Details

All animal use was in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the UCSF Institutional Animal Care and Use Committee, in compliance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care. All strains were maintained on the C57BL/6J background. Mice were raised in 12 hour light/dark cycles, with food and water provided ad libitum. Within each genetic and surgical condition, mice of either sex were used. Mice were sacrificed and tissue collected at either 12-15 postnatal days or 21-26 postnatal days. None of the genetic conditions induced lethality or increased the probability of adverse outcomes.

All mice in the study carried one or two copies of the SERT-Cre allele (Tg(Slc6a4-cre)ET33Gsat/Mmucd). This Cre driver selectively labels a subset of ipsilaterally-projecting RGCs, without substantial expression in contralaterally-projecting RGCs or in the dLGN (Koch, et al., 2011). Cre recombinase was used to induce expression of one of four Cre-dependent fluorescent reporters: TdTomato (B6.Cg-Gt(Rosa)26Sor\textsuperscript{tm9(CAG-tdTomato)Hze/J}), membrane-bound GFP (B6;129P2-Mapt\textsuperscript{tm2Arbr/J}), synaptophysin-TdTomato fusion protein (B6;129S-Gt(Rosa)26Sor\textsuperscript{tm34.1(CAG-Syp/tdTomato)Hze/J}), or channelrhodopsin-2-TdTomato fusion protein (B6.Cg-Gt(Rosa)26Sor\textsuperscript{tm27.1(CAG-COP4*H134R/tdTomato)Hze/J}). The combination of the SERT-Cre allele and a reporter allele permitted the visualization of the ipsilaterally-innervated dLGN. Additionally, VGLUT2-cKO mice were homozygous for the floxed VGLUT2 allele
(B6.129S4-Slc17a6<sup>tm1Rpa</sup>/J), while genetic control mice carried one or no copies of the floxed VGLUT2 allele. Mice were genotyped using the primers listed in Table 2.

**Method Details**

*Slice Preparation*

Acute brain slices bisecting the dorsal lateral geniculate nucleus (dLGN) and containing several millimeters of its innervating optic tract were prepared as previously described (Turner & Salt, 1998; Chen & Regehr, 2000; Koch, et al., 2011). Mice at either p12-15 or p21-26 were anesthetized through isoflurane inhalation, then decapitated. Their brains were rapidly removed and submerged for one minute in ice-cold cutting solution consisting of (in mM): 78.3 NaCl, 23 Dextrose, 33.8 Choline Chloride, 2.3 KCl, 1.1 NaH<sub>2</sub>PO<sub>4</sub>, 6.4 MgCl<sub>2</sub>, 0.45 CaCl<sub>2</sub>, and 23 NaHCO<sub>3</sub>, continuously oxygenated by bubbling with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Brains were removed and rapidly dissected on an ice-cold cutting surface. A parasagittal cut was made at approximately 15° relative to the cerebral longitudinal fissure, such that the right hemisphere and a small portion of the left hemisphere were preserved. Two additional cuts were made to remove the olfactory bulb and the cerebellum. The brain was then glued onto a chilled cutting platform with the medial face downwards. The cutting platform was angled at approximately 20°, elevating the dorsal portion of the brain. The cutting platform was submerged in ice-cold oxygenated cutting solution, and sections were cut using a Vibratome (Leica VT1200S). This cutting geometry placed the sections in the same plane as the optic tract as it reaches the dLGN, permitting the preservation and stimulation of retinal inputs to the thalamus. As progressively more medial sections were taken, anatomical landmarks were carefully observed and section thickness
adjusted, such that the final slice was of 300-325 um in thickness, with its medial face placed to bisect the ipsilaterally-innervated dLGN. Only one such slice could be prepared per animal. The following section was also kept, and fluorescent reporter expression in both slices was used to verify proper placement within the I-dLGN. Slices were immediately transferred from the Vibratome to a bath containing oxygenated cutting solution at 30°C, where they recovered for 25 minutes. They were then transferred to oxygenated 30°C artificial cerebrospinal fluid (ACSF), containing (in mM): 125 NaCl, 10 Dextrose, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, and 25 NaHCO₃ (305 mOsm) and recovered for another 25 minutes. Finally, these baths were allowed to come to room temperature.

**Electrophysiology**

Whole-cell voltage clamp recordings of dLGN relay cells were made at room temperature, in oxygenated ACSF constantly perfused at approximately 1 mL/min. Data were acquired using pClamp 10 (Molecular Devices), at a sampling rate of 10 kHz. All recordings were made in the presence of the GABAₐ receptor antagonist bicuculline (20 uM) to block inhibitory disynaptic connections from local interneurons and the nucleus reticularis. The cortex was severed from the slice to eliminate excitatory disynaptic connections from corticothalamic inputs. Glass electrodes of 1.5-3 MOhm resistance were filled with internal solution containing (in mM): 100 CsCl, 35 CsF, 10 EGTA, 10 HEPES, 8 Sucrose, and 0.1 Methoxyverapamil HCl, (295 mOsm, pH adjusted to 7.25 with CsOH). Relay cells were patched, and their input resistances (R_{inp}) were monitored for stability and compensated at 20-35%. Cells whose R_{inp} exceeded 20 MOhm were
discarded. The ipsilaterally-innervated and contralaterally-innervated dLGN were identified based on the presence or absence of a Cre-dependent fluorescent reporter, respectively (see above).

A parallel bipolar stimulating electrode consisting of platinum iridium wires spaced at 300 um (FHC, Cat#: PBSA0375) was placed touching the surface of the slice, with one wire on each side of the optic tract next to the ventral LGN. The optic tract was stimulated for 1 msec, and excitatory postsynaptic currents (EPSCs) were recorded in relay cells. This process was repeated every 20 seconds. We began by stimulating at subthreshold intensities which evoked no postsynaptic response. We gradually increased stimulation intensity, resulting in increased EPSCs, until further increases in stimulation no longer resulted in larger responses. The maximal EPSC amplitude is referred to as EPSC$_{\text{max}}$. Generally, EPSCs increased in stepwise changes consistent with the recruitment of individual axons. These stimulation protocols were repeated as relay cells were held at membrane potentials of -70 mV and +40 mV to assay AMPAR-mediated and NMDAR-mediated EPSCs, respectively. The time constant for the decay of NMDAR-mediated current was calculated by fitting each trace containing the EPSC$_{\text{max}}$ with a single exponential, then averaging to find an ensemble time constant.

**Individual Input Strength and Number**

All recordings were conducted in mice aged p12 or older, when the retinogeniculate synapse has undergone substantial refinement to achieve a small number of retinal inputs per relay cell (Žiburkus & Guido, 2006). We were therefore able to clearly resolve stepwise increases in AMPAR and NMDAR EPSCs in all but two cells,
enabling us to estimate the number and synaptic strengths of individual inputs, by calculating the amplitude of sequential steps in the evoked EPSC. Another method of estimating the strength of individual inputs is single fiber stimulation (Hooks & Chen, 2008). We began stimulation at subthreshold levels, then gradually increased stimulation until an EPSC was observed. This protocol permits the recruitment and analysis of a single RGC input. The amplitude of this first detectible synaptic input is referred to as the “single fiber amplitude.” The NMDAR-mediated single fiber amplitude was also used to compute an estimate of the degree of polyinnervation of each relay cell. This method, called the fiber fraction, does not depend on accurately distinguishing stepwise changes in EPSC amplitude (Chen & Regehr, 2000; Hooks & Chen, 2008). The fiber fraction is calculated by dividing the single fiber amplitude by the EPSC_{max} for each relay cell. The result reflects the proportion of total innervation provided by the single fiber. Cells with fiber fractions near one receive most of their synaptic drive from a single input, while those with fiber fractions near zero may be innervated by a larger number of weaker inputs.

Quantal Size and Frequency

Spontaneous EPSCs (sEPSCs) were collected for all relay cells by recording at a sampling rate of 10 kHz and a holding potential of -70 mV. sEPSC amplitudes and kinetics were determined through semi-automated threshold detection and fitting by MiniAnalysis (Synaptosoft), after a 1 kHz low pass filter was applied. Thalamic relay cells receive excitatory input from corticothalamic neurons in addition to retinal ganglion cells, and so these events cannot all be assumed to be retinal in origin. To minimize the
contribution of sEPSCs from cortical fibers, we took advantage of a structural asymmetry in the organization of retinal and cortical inputs. Corticothalamic synapses to relay cells are typically placed more distally than retinal ones, and are thought to undergo more severe dendritic filtering as a result (Wilson, et al., 1984; Liu, et al., 1998; Golshani, et al., 1998; Krahe & Guido, 2011). We therefore examined the distribution of 10-90% rise times within our sEPSC dataset, and applied cutoffs across each age group (2.3 msec for p12-15 mice; 1.5 msec for p21-26 mice) to remove the sEPSCs that exhibited the slowest kinetics, and were thus likeliest to arise from corticothalamic inputs. The median remaining sEPSC for each cell was found, then cells within each condition were averaged to find the population mean. sEPSC frequency was determined by dividing the number of sEPSCs observed (after the cutoffs discussed above were applied) by the duration of sEPSC recordings.

We observed a striking developmental increase in the strength of ipsilateral synaptic transmission, and so we were especially interested in the development of quantal size in the ipsilaterally-projecting retinogeniculate synapse. We therefore collected a sample of quantal events enriched for those of retinal origin by inducing asynchronous vesicular release at the retinogeniculate synapse. We prepared slices through the I-dLGN as described above, except that calcium was omitted from the ACSF during recovery. We patch-clamped relay cells within the I-dLGN, and determined the amplitude of electrical stimulation required to reliably evoke an EPSC_{max}. We then perfused the recording chamber with a modified strontium-containing ACSF containing the following changes from the recipe used elsewhere in this work (in mM): 2 MgCl₂, 0 CaCl₂, and 3 SrCl₂. Strontium acts as an inefficient substitute for calcium, such that
stimulation of retinal inputs results in the slow, desynchronized release of vesicles (Chen & Regehr, 2000; Dodge, et al., 1969; Koch, et al., 2011). This asynchronous release was observable as a dramatically increased frequency of quantal events (termed asynchronous EPSCs, or aEPSCs) within 500 msec of stimulation to evoke an EPSC_{max}. aEPSC amplitudes were determined through semi-automated threshold detection and fitting by MiniAnalysis (Synaptosoft), analyzing all events falling within 500 msec of optic tract simulation, after a 1 kHz low pass filter was applied.

**Vesicle Pool Size and Release Probability**

Readily releasable vesicle pool size was estimated using the established method of cumulative EPSC amplitudes (Schneggenburger, et al., 1999; Müller, et al., 2012). Relay cells were held at a command potential of -70 mV while retinal axons were stimulated at 20 Hz (20-25 pulses), depleting the vesicular pool until the evoked EPSC reached a steady state reflecting only vesicle recycling. All successful trials within a cell were averaged before analysis. Recordings were made in the presence of the AMPAR desensitization inhibitor cyclothiazide (75 uM), which slows the decay of AMPAR-mediated current, and so EPSCs after the first pulse generally rode on top of the decay phase of the preceding EPSC. For this reason, the baseline for EPSCs after the first pulse was determined by fitting the decay phase of the previous response with a double exponential function, and extrapolating it forward. EPSC amplitudes were calculated as the difference between the peak of the EPSC and the value of the extrapolated decay phase of the preceding EPSC at the same time as the peak being measured. A line was fit to the linear phase of the cumulative EPSC plot (the final 2-7 pulses) and back-
extrapolated to the time of the first response, to estimate the current evoked by the readily releasable vesicle pool. This value was divided by the mean sEPSC value for corresponding age and genotype condition, yielding an approximation of the number of vesicles contained in the readily releasable pool. Note that because application of cyclothiazide potentiates AMPAR-mediated current, this calculation likely overestimates the absolute size of the readily releasable pool.

This result can also be used to estimate the probability of vesicular release. We have described above our method for estimating the total current evoked by release of the readily releasable vesicle pool ($I_{RRP}$). By dividing the amplitude of the average EPSC$_{max}$ for each cell by its estimated $I_{RRP}$, we arrive at an approximation of the fraction of vesicles released during the EPSC$_{max}$ (Schneggenburger, et al., 1999). This estimate of release probability is referred to here as $P_{train}$.

Another method for estimating synaptic release probability is to deliver pairs of stimuli at a short latency, and to measure the paired-pulse ratio (PPR). We elicited AMPAR EPSC$_{max}$ at interstimulus intervals of 50, 100, 200, or 500 msec, with trials separated by 20 seconds. Recordings were performed in the presence of cyclothiazide (75 μM). All trials (3-5) within a cell were averaged before analysis. The amplitude of the second peak was measured from the peak of the EPSC to the baseline found by fitting the decay phase of the first peak with a double exponential and extrapolating it forward, as described above.

Optical Stimulation
Optically-induced EPSCs were evoked in mice expressing ChR2 in SERT-Cre(+) RGCs. First, relay cells were patched and their EPSC$_{\text{max}}$ were determined as discussed above using electrical stimulation of the optic tract. Next, the water-immersion lens (Nikon Fluor 40x/0.80W) of the patching microscope was focused and centered on the cell being recorded. Full-field illumination of the axon terminals innervating the cell was achieved by directing 470 nm light (Thorlabs M470F1 LED) through the objective for 1 ms. The light formed a circle of approximately 1 mm$^2$ on the slice, with intensity of roughly 1 mW/mm$^2$ (LED intensity in the fiber prior to entering the microscope optics was approximately 25 mW).

**Enucleation**

Monocular enucleations were performed on postnatal day 0-2. Mouse pups were deeply anesthetized through hypothermia. The analgesic proparacaine hydrochloride (Bausch & Lomb, 0.5%) was applied to the palpebral fissure, which was then gently opened using sharp forceps. The eyeball was numbed with further administration of proparacaine, displaced using curved forceps, then the optic nerve was cut and the eye removed. Bleeding was halted with gentle pressure by a silver nitrate applicator stick (StyptStix; Butler Schein) into the orbit. Pups were recovered on a heating pad.

**Dye-labeling RGCs and Immunohistochemistry**

The right hemisphere of control and VGLTU2-cKO mice were evaluated for the distribution of contralateral RGCs and VGLUT2 antibody labeling within the I-dLGN (Figure S1). Retinal ganglion cells in the left eye were labeled by intravitreal injection of
Alexa Fluor® 647 Cholera Toxin Subunit B (CTb-647; Thermo Fisher). On p10, pups were deeply anesthetized through isoflurane administration (3% isoflurane in 2 L O₂/min). The analgesic proparacaine hydrochloride (0.5%; Akorn, NDC: 17478-263-12) was applied to the palpebral fissure, which was then gently opened using sharp forceps. The eye was numbed with proparacaine and gently displaced using curved forceps. A sharp glass pipette was used to slowly inject 1.5 uL of CTb-647 (1.25 mg/mL in PBS). The injector was retracted and triple antibiotic ophthalmic ointment (Bausch and Lomb; NDC: 24208-780-55) was applied to the eye. Pups were recovered on a heating pad. Mice were sacrificed two days after CTb-647 injection (p12). Pups were deeply anesthetized by an overdose of ketamine and xylazine (150/10 mg/kg), and perfused transcardially with paraformaldehyde (PFA; 4% in PBS). Brains were fixed overnight in 4% PFA, then cryoprotected for 2 days in 30% sucrose in PBS. Sections were frozen in Optimal Cutting Temperature compound (Tissue-Tek) then cryosectioned at 30 um. Sections were rehydrated in PBS, then blocked for one hour at room temperature in blocking solution consisting of PBS with 10% goat serum and 0.2% Triton-X100. Sections were then incubated for 18 hours in primary antibody solution consisting of PBS with 1% goat serum, 0.2% Triton-X100, and guinea pig polyclonal anti-VGLUT2 antibody (diluted 1:1000; Millipore). Sections were washed in PBS, then incubated for 1 hour in secondary antibody solution consisting of PBS with 1% goat serum, 0.2% Triton-X100, and goat anti-guinea pig antibody conjugated to Alexa Fluor 488 (diluted 1:1000; Thermo Fisher). Sections were washed in PBS, then mounted with VECTASHIELD Hardset Mounting Medium (Vector Laboratories).
Data Analysis and Statistics

Data was acquired using Clampex 10 (Molecular Devices), sampling at 10 kHz. Analysis was performed offline using custom software written in MATLAB (Mathworks) and Excel (Microsoft). Statistical tests and N’s for each experiment are reported in the text. All data presented in box-and-whisker plots are tested using the Mann-Whitney Rank-Sum unpaired nonparametric test. All data presented in bar graphs with SEM bars are tested using the unpaired t-test. For all figures, p values are indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001.
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