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FORMATION, ORGANIZATION, AND SYNAPTIC PROPERTIES OF CIRCUITS
BETWEEN THALAMIC RETICULAR NUCLEUS AND DORSAL LATERAL
GENICULATE NUCLEUS

By

Peter W Campbell

B.A., Grinnell College, 2006

A dissertation submitted to the Faculty of the
School of Medicine of the University of Louisville in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy in Anatomical Sciences and Neurobiology

Department of Anatomical Sciences and Neurobiology
University of Louisville
Louisville, Kentucky

May 2020

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A Dissertation Approved on

February 4th 2020

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DEDICATION

This dissertation is dedicated to all my family and friends who have supported me throughout this long venture. Particularly my wife, Meagen, our daughter, Rowan, and my parents.

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ABSTRACT

FORMATION, ORGANIZATION, AND SYNAPTIC PROPERTIES OF CIRCUITS BETWEEN THALAMIC RETICULAR NUCLEUS AND DORSAL LATERAL GENICULATE NUCLEUS

Peter W Campbell

February 4th 2020

The thalamic reticular nucleus (TRN) is an interface between thalamus and cortex that regulates thalamocortical rhythms and modulates sensory processing. Thalamocortical axon collaterals provide feedforward excitatory input onto GABAergic TRN neurons, which in turn convey feedback inhibition to dorsal thalamus. Here we used the mouse visual system to study the organization, pattern of innervation and functional responses between TRN and the dorsal lateral geniculate nucleus (dLGN). We used genetically modified mice to target components of this feedback loop (feedforward: CRH-Cre; feedback: GAD65 or SST-Cre) and we created Math 5^{-/-} strains to examine the impact of visual deafferentation in these circuits. We conducted both anterograde and retrograde neural tracing to assess the organization of sensory feedforward projections in TRN and established that the configuration we observed in adults is present at perinatal ages in both WT and Math 5^{-/-} mice. To examine the formation and

functional maturation of intrathalamic circuits, we used confocal microscopy to examine when terminals arrive, and electrophysiologic recordings during optogenetic stimulation in acute thalamic slice preparations to assess the maturation of functional responses. Although thalamocortical projections were present in TRN at birth, feedforward circuits emerged during the second postnatal week and continued to mature through the third week. Feedback projections were established during the first postnatal week but functional responses continued to develop through the fourth postnatal week. In *Math 5^{-/-}* mice, feedforward projections were unchanged compared to WT but feedback terminals arrived at earlier ages and exhibited more synaptic suppression at higher rates of stimulation. In adults, ultrastructural analysis of feedback projections revealed that TRN terminals only form synapses with relay neurons, a result confirmed by electrophysiological recordings. Recordings in acute slices established that tonic TRN activation hyperpolarized and suppressed relay neuron firing in a frequency-dependent manner. The impact of frequency was confirmed in vivo in an awake behaving head fixed preparation. Together, these experiments advance our understanding of the synaptic nature of TRN inhibition of dLGN. They also establish the developmental pattern of connectivity between dLGN and TRN, both in the presence and absence of retinal signaling.

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INTRODUCTION

The thalamic reticular nucleus (TRN) occupies a key position for modulating thalamocortical (TC) and corticothalamic (CT) activity since it is located at the interface between the thalamus and the internal capsule where TC and CT projections intersect (Crick, 1984; Guillery et al., 1998; Pinault, 2004). TRN is comprised entirely of GABAergic neurons that form an inhibitory shell across the lateral border of dorsal thalamus (Fogerson and Huguenard, 2016; Halassa and Acsády, 2016; Houser et al., 1980; McCormick and Bal, 1997). The major inputs to these neurons arise from axon collaterals off TC and CT projections, which drive TRN activity (figure 1). These collaterals converge into modality-specific domains (figure 2) of which there are at least seven: five sensory (visual, auditory, somatosensory, gustatory and visceral), one motor and one limbic (Crabtree, 2018; Guillery and Harting, 2003; Kimura, 2017; Pinault, 2004; Shosaku et al., 1989). Axons from TRN project back to dorsal thalamus where they inhibit TC activity (figure 1). Thus, the connections between dorsal thalamus and TRN form an intrathalamic feedback loop.

TRN neurons inhibit dorsal thalamus by one of two modes of activity: tonic and burst (Crunelli et al., 2018; Huguenard and McCormick, 2007; Huguenard

and Prince, 1992; Lee et al., 2007; McCormick et al., 1994). Tonic firing consists of single action potentials mediated sodium channels whereas burst firing is comprised of a calcium conductance that often has up to 15 sodium-mediated action potentials riding the crest of this potential. The interplay between tonic and burst firing in TRN contributes to the generation of some rhythms in cortex and underlies many of the functional properties attributed to TRN (Halassa and Kastner, 2017; Llinás and Steriade, 2006; McCormick et al., 2015).

In wakeful states most TRN neurons exhibit tonic firing with rates that vary widely based on behavioral state (Chen et al., 2016b; Contreras et al., 1993; Domich et al., 1986; Hartings et al., 2003; Lymer et al., 2019; McCafferty et al., 2018; Pinault et al., 2001; Steriade et al., 1986). As the brain transitions to sleep, TRN tonic activity decreases and burst firing predominates during non-REM sleep states (Fernandez et al., 2018; Llinás and Steriade, 2006; Marczyński et al., 1984; Marks and Roffwarg, 1993; McCormick and Bal, 1997; Steriade, 2000; Vantomme et al., 2019). Sleep spindles emerge during early stages of sleep and are an example of a cortical rhythm that originates in TRN (Buzsáki, 1991; Contreras et al., 1997; Crunelli et al., 2018; Fuentealba and Steriade, 2005; Halassa and Acsády, 2016; Vantomme et al., 2019).

In addition to alterations in TRN activity due to sleep-wake states, TRN firing is also modulated by attentional processes (Crabtree, 2018; Halassa and Kastner, 2017; McAlonan et al., 2006, 2008). Specifically, TRN activity decreases within a sensory sector when an animal is cued to a stimulus of the same modality, but activity increases when a non-congruent modality is cued

(McAlonan et al., 2006, 2008; Wimmer et al., 2015) (McAlonan et al., 2008; Wimmer et al., 2015). The decrease in TRN activity disinhibits TC neurons and facilitates TC transmission. Similarly, activation of TRN neurons within a sensory sector inhibit TC transmission of the same modality but not other senses (Mushiake et al., 1984; Shosaku et al., 1984, 1989). These results support Crick's hypothesis (1984) that the TRN operates as an attentional searchlight sampling activity in thalamus and cortex to selectively modulate thalamocortical transmission.

While the TRN can mediate physiological processes such as setting cortical rhythms or gating thalamocortical transmission based on attentional demands, other work has implicated TRN in pathological processes – specifically epilepsy, attentional disorders and autism spectrum disorder (ASD). Of the neurological disorders that involve TRN, the best studied is absence epilepsy. This form of epilepsy occurs in children 3-10 years of age and is characterized by brief episodes of loss of consciousness. Typical absence spells end abruptly with resumption of previous activity and intra-ictal EEG classically shows a 3Hz spike and wave pattern for 5-10s (a spike-wave discharge; Crunelli and Leresche, 2002; Major and Thiele, 2007; McCormick et al., 2015; Paz and Huguenard, 2015; Steriade, 2005). Two rat models of this disease exist, the Genetic Absence Epilepsy Rats from Strasbourg (GAERS; Vergnes et al., 1982) and Wistar Albino Glaxo rats originating from Rijswijk (WAG/Rij; Luijtelaar and Coenen, 1986) but other rodent lines have been noted to have spike-wave discharges as well (Crunelli and Leresche, 2002; Letts et al., 2014; Luijtelaar et al., 2014). These

models have demonstrated that epileptic spells are mediated by oscillatory burst firing in TRN (Crunelli and Leresche, 2002; McCafferty et al., 2018; McCormick et al., 2015; Paz and Huguenard, 2015; Steriade, 2005). In addition to epileptic spells, there is some evidence that children with absence epilepsy also have executive function impairments including attentional deficits (Fastenau et al., 2009; Gencpinar et al., 2016; Hessen et al., 2018; Høie et al., 2006). Moreover, in addition to typical absence epilepsy, atypical forms exist that occur in the setting of more severe disorders (e.g. Lennox–Gastaut). In addition to absence seizures, these disorders involve cognitive impairment as well as intellectual disability and are refractory to treatments (Crunelli and Leresche, 2002; Luijtelaar et al., 2014). Thus, while TRN clearly contributes to the spike-wave discharges seen in absence epilepsy, it is unclear whether associated impairments executive function are also attributable to TRN dysfunction.

In addition to epilepsy, TRN is also implicated in disorders of attention (e.g. ADHD) and sensory processing (e.g. ASD). A family with ASD, intellectual disability and ADHD was found to have a genetic deletion of a protein, PTCHD-1 (Nakajima et al., 2019; Torrico et al., 2015; Wells et al., 2016). Studies in mice revealed that this protein was localized to TRN and mice lacking this protein exhibited reduced burst firing in TRN (Wells et al., 2016). Additionally, patients with ASD exhibit sleep impairments and fewer sleep spindles (Limoges et al., 2005; Tessier et al., 2015). Patients with ASD can exhibit hypersensitivity to some sensory stimuli and MRI studies have found primary sensory cortices are overactive in ASD patients compared to controls (reviewed by Lainhart, 2015, but

see also Nakajima et al., 2019). Together, these findings implicate TRN dysfunction in multiple neurological disorders that arise in childhood. However, we currently lack a fundamental understanding of how TRN develops, specifically the formation of intrathalamic circuit connecting TRN with TC neurons.

The mouse visual system has become a preeminent model system to examine the formation and function of circuits (Cox and Beatty, 2017; Guido, 2018; Huberman, 2007; Huberman and Niell, 2011; Kerschensteiner and Guido, 2017; Litvina and Chen, 2017; Morgan et al., 2016; Niell, 2015; Priebe et al., 2014; Seabrook et al., 2017). Mice permit numerous genetic manipulations that enable the dissection and interrogation of circuits that is not possible in other species. For example, specific neuronal populations can be visualized and/or stimulated by using the targeted expression of fluorescent or optogenetic proteins (Adamantidis et al., 2015; Deisseroth, 2015; Luo et al., 2018; Madisen et al., 2009, 2012; Zhao et al., 2011). Additionally, these genetic manipulations can be combined with knock-out mouse lines for loss-of-function studies. These tools have been utilized within the mouse visual thalamus, the dorsal lateral geniculate nucleus (dLGN), to examine how non-retinal inputs gate the transmission of visual information to cortex as well as how such circuits develop (Guido, 2018; Kerschensteiner and Guido, 2017). Since TRN is one of the main non-retinal projections to dLGN, here we use this system to examine the formation of intrathalamic circuits between TRN and dLGN as well as to interrogate the synaptic basis of TRN-mediated inhibition in dLGN.

The principal cell type in dLGN is the thalamocortical relay neuron which transmits visual information from retina to primary visual cortex (figures 1 and 3). However, most of the synapses in dLGN are non-retinal in origin (Bickford et al., 2010; Guido, 2018; Morgan et al., 2016). In addition to TRN, non-retinal inputs arise from cortex, brainstem cholinergic nuclei and intrinsic interneurons (figure 3). While it is unclear when TRN projections arrive in dLGN, examination of other non-retinal inputs suggest that the formation of dLGN circuitry follows a conserved developmental plan whereby retinal terminals arrive first and coordinate non-retinal innervation (Bickford et al., 2010; Brooks et al., 2013; Charalambakis et al., 2019; Godement et al., 1984; Jaubert-Miazza et al., 2005; Seabrook et al., 2013a; Sokhadze et al., 2018a). Retinal signaling is also required for cortical map formation and may contribute to the overall maturation of TC activity (Ackman and Crair, 2014; Ackman et al., 2012; Colonnese et al., 2010; Kirkby et al., 2013; Murata and Colonnese, 2018a).

Experiments on animals that lack retinal inputs confirm the importance of retinal signaling for development of the visual system. Using a knockout mouse that lacks any retinal input to the brain (Brown et al., 1998, 2001; Wang et al., 2001b), the Guido lab has shown that in *Math 5^{-/-}* mice non-retinal innervation is disrupted: CT innervation is accelerated (Brooks et al., 2013; Seabrook et al., 2013a), brainstem cholinergic fibers are mis-routed and delayed (Sokhadze et al., 2018a), while intrinsic interneurons exhibit dystrophic growth and reduced synaptic connectivity (Charalambakis et al., 2019). Further work on CT innervation identified a chondroitin sulfate proteoglycan, aggrecan, as a repulsive

molecule that prevented CT innervation and that was degraded after retinal innervation (Brooks et al., 2013). Work in enucleated animals has also demonstrated that the size and connections of primary sensory cortices changes in the absence of sensory inputs (Chabot et al., 2007; Charbonneau et al., 2012; Dooley and Krubitzer, 2018; Henschke et al., 2017; Kahn and Krubitzer, 2002; Karlen et al., 2006; Kupers and Ptito, 2014; Négyessy et al., 2000). However, it is unclear what effect, if any, visual deafferentation has on the formation of the intrathalamic circuits connecting TRN and dLGN. It is also unknown if the lack of retinal inputs disrupts the arrangement of sensory sectors in TRN, as is the case in cortex.

Thus, TRN inhibition of dorsal thalamus is thought to act as a searchlight but it is unclear how that searchlight forms. Yet even in adult animals the synaptic basis of TRN-mediated inhibition is poorly understood. While CT terminals form synapses on both relay neurons and intrinsic interneurons, it remains unknown whether TRN terminals contact both cell types (Cox and Beatty, 2017; Li et al., 2003; Murata and Colonnese, 2018a; Sherman and Guillery, 1996; Wang et al., 2001a). Moreover, while it is clear that TRN activity can suppress retinogeniculate transmission, it is unclear what role the frequency of inhibition has on relay neuron firing.

In this dissertation, we explore the interconnections between dLGN and TRN. In Chapter I, we perform retrograde tracing experiments to establish whether TRN sectors are present at young ages. Having identified the location of visual TRN at perinatal ages, we then examined the formation of feedforward

(dLGN to TRN) and feedback (TRN to dLGN) circuits. These experiments were performed by utilizing different lines of transgenic mice that enabled selective visualization or stimulation of feedforward or feedback projections. We also repeated these experiments in Math 5^{-/-} mice to examine what impact the absence of retinal signaling had on both the arrangement of TRN sectors as well as the development of feedforward and feedback circuits. In Chapter II we assessed TRN synapses in dLGN at the ultrastructural level. We also used an in vitro thalamic slice preparation as well as an in vivo head-fixed awake behaving mouse to determine the nature of TRN inhibition on relay neuron activity. Together, these experiments provide a fundamental understanding of the formation and function of thalamic inhibition in mice.

Figure 1. Simplified schematic of feedback loops connecting thalamus, TRN and cortex. TC neurons of thalamus project to layer IV of cortex (solid lines), and receive projections from layer VI (dotted lines). TRN neurons (black) receive input from axon collaterals of TC and CT neurons. These projections are arranged in a segregated manner such that TC and CT axons of the same modality travel together as they course through TRN and the internal capsule.

Figure 2. Cartoon illustrating sectorial arrangement of thalamic reticular nucleus (TRN). Sensory TRN is divided into modality-specific sectors that are circumscribed by modality-specific thalamic and cortical inputs. Ascending thalamocortical projections from three first order nuclei (dLGN, blue; MGN, red; and VB, green) give off collaterals in TRN that terminate in non-overlapping regions. Descending corticothalamic projections from primary visual (V1, blue), auditory (A1, red) or somatosensory (S1, green) cortex give off collaterals in TRN that converge in a modality-specific manner. Sectors are arranged in both dorsal-ventral and rostral-caudal (not shown) planes.

Figure 3. Wiring diagram illustrating the pattern of connectivity within the visual thalamus. Retinal projections (blue) drive activity in dorsal lateral geniculate (dLGN) intrinsic interneurons (black) and relay neurons (R, purple). Visual information is transferred from relay neurons to visual cortex (yellow) by thalamocortical axons, which pass through the visual sector of the thalamic reticular nucleus (TRN, green). Within the TRN thalamocortical axons give off collaterals that excite TRN neurons (feedforward excitation), which project back to dLGN relay neurons to suppress thalamocortical transmission (feedback

inhibition). Together, feedforward excitation and feedback inhibition between TRN and dLGN comprise a thalamic inhibitory feedback loop.

CHAPTER I

FORMATION OF INTRATHALAMIC CIRCUITS BETWEEN THALAMIC RETICULAR NUCLEUS AND DORSAL LATERAL GENICULATE NUCLEUS

Introduction

The thalamic reticular nucleus (TRN) is a shell-like structure that surrounds many of the primary sensory nuclei of the dorsal thalamus (Guillery and Sherman, 2002; Halassa and Sherman, 2019; Jones, 2007; Mitrofanis and Guillery, 1993; Pinault, 2004). Comprised entirely of GABAergic neurons, the TRN serves as an important interface between thalamus and cortex (Crick, 1984; Houser et al., 1980; McCormick et al., 2015; Steriade, 2005). Primary excitatory drive to TRN arises from thalamus and cortex while the TRN is the primary source of inhibition to sensory nuclei of the thalamus (Deleuze and Huguenard, 2016; Guillery and Harting, 2003; Guillery and Sherman, 2002; Guillery et al., 1998; Jones, 1975, 2007; Liu and Jones, 1999). These ascending glutamatergic and descending GABAergic projections form an intrathalamic feedback loop between TRN and dorsal thalamus, which is thought to modulate sensory

transmission in a state dependent manner, mediate some aspects of attention, and set cortical rhythms (Cox et al., 1997a; Crick, 1984; Fiebelkorn and Kastner, 2019; Halassa and Kastner, 2017; Jones, 2002; Llinás and Steriade, 2006; McCormick and Bal, 1997; O'Connor et al., 2002; Pinault, 2004; Sherman and Guillery, 2002; Sillito and Jones, 2007; Zikopoulos and Barbas, 2012). This intrathalamic circuit also contributes to multiple neurodevelopmental diseases including epilepsy, autism spectrum disorder and attention hyperactivity disorder (Cheong and Shin, 2013; Crunelli and Leresche, 2002; Doyle-Thomas et al., 2014; Huguenard and McCormick, 2007; van Luijtelaar and Sitnikova, 2006; McCormick et al., 2015; Nair et al., 2015; Normand et al., 2013; Paz and Huguenard, 2015; Ritter-Makinson et al., 2019; Sitnikova et al., 2016; Steriade, 2005; Thatcher et al., 2009; Wells et al., 2016). Currently, we lack a fundamental understanding of the underlying circuitry in these neurological disorders as well as how and when this inhibitory feedback loop is formed. In order to develop therapies and treatments for these neuropathological conditions, especially those that emerge during early childhood, it is critical to understand the normal development of these networks.

The mouse visual system has become a prominent model system to assess thalamic circuit development (Chen et al., 2016a; Danaei and Huberman, 2012; Fox and Guido, 2011; Guido, 2018; Huberman, 2007; Huberman and Niell, 2011; Kerschensteiner and Guido, 2017; Priebe et al., 2014; Seabrook et al., 2017). The thalamocortical (TC) relay neurons of the dorsal lateral geniculate nucleus (dLGN) are the exclusive relay of retinal information to visual cortex

(figure 1). In mice, TC axons reach the cortical subplate by E14.5 and normal pathfinding is influenced by scaffolding provided by TRN and the adjacent perithalamic reticular nucleus (Agmon et al., 1993; Feldheim et al., 2010; Hevner et al., 2002; Inan and Crair, 2007; López-Bendito, 2018; Mitrofanis and Baker, 1993; Molnár et al., 1998, 2012; Schiff et al., 2011). Upon reaching visual cortex, TC axons shape many aspects of cortical development including architecture, size and inputs from other areas (Catalano and Shatz, 1998; Espinosa and Stryker, 2012; Feldheim et al., 2010; Ghosh and Shatz, 1992; Miller et al., 1993; Niell, 2015; Priebe et al., 2014; Thompson et al., 2017; Vue et al., 2013). However, little is known about how these TC axon collaterals innervate TRN. Importantly, TRN is arranged in a sectorial manner such that thalamocortical and corticothalamic axons pass through and innervate specific regions of TRN (see figure 2; Coleman and Mitrofanis, 1996; Conley and Diamond, 1990; Fitzgibbon, 2007; Jones, 2007; Mitrofanis and Guillery, 1993; Ohara et al., 1980). There are at least seven sectors: five sensory, one motor and one limbic (Guillery et al., 1998; Pinault, 2004). Yet it is unclear when these sectors emerge and how modality-specific domains arise. Additionally, it remains unknown when feedforward projections from dLGN relay neurons innervate visual TRN due to technical challenges.

Within dLGN, non-retinal inputs comprise 90% of synapses within the nucleus and the developmental time course of such modulatory projections have been described utilizing many of the sophisticated genetic tools available in mice (Bickford et al., 2010; Guido, 2018). Despite these recent advances, it is still

unclear when TRN feedback inhibitory projections to dLGN form and how they mature (figure 1). Early reports using lipophilic dyes described TRN innervation occurring at embryonic ages (Grant et al., 2012; Mitrofanis and Baker, 1993; Molnár et al., 1998) but more recent work found that inhibitory synaptic profiles emerge in dLGN well after retinal ones, which arise at perinatal ages (Bickford et al., 2010).

Retinal signaling guides a number of developmental events in dLGN. In the absence of retinal input the size of dLGN is decreased but the overall density of neurons is increased (Crair and Mason, 2016; Cullen and Kaiserman-Abramof, 1976; El-Danaf et al., 2015; Guido, 2018; Heumann and Rabinowicz, 1980; Williams et al., 2002). Furthermore, the maturation of dendritic morphology of TC relay neurons is altered and their soma size is reduced (El-Danaf et al. 2015). Intrinsic interneurons exhibit an altered dendritic morphology and their targeting and migration to and within dLGN is disrupted (Charalambakis et al., 2019; Golding et al., 2014; Jager et al., 2016). Furthermore, retinal signaling coordinates the timing and nature of circuit assembly within dLGN (Guido, 2018; Kerschensteiner and Guido, 2017). For example, the removal of retinal inputs disrupts feedforward inhibitory circuits between intrinsic interneurons and TC relay neurons (Charalambakis et al., 2019). Non-retinal input from brainstem cholinergic nuclei is delayed in the absence of signaling, likely due to an inability to follow retinal axons to dLGN (Sokhadze et al., 2018a). However, corticogeniculate fibers arrive at earlier postnatal ages due to the absence of a repellent cue that arises from retinal signaling (Brooks et al., 2013; Seabrook et

al., 2013a). TRN is the main inhibitory projection to dLGN yet it is unclear whether the formation of this circuit is similarly coordinated by retinal signaling.

In addition to coordinating the developmental time course of dLGN circuitry, retinal signaling also impacts the maturation of visual cortices. In visually deafferented animals, primary visual cortex retains the cytoarchitecture of higher visual areas, is decreased in overall size, and receives inputs from non-visual thalamic nuclei (Chabot et al., 2008; Charbonneau et al., 2012; Dooley and Krubitzer, 2018; Henschke et al., 2017; Kahn and Krubitzer, 2002; Karlen et al., 2006; Kupers and Ptito, 2014). Thus, in the absence of retinal inputs to dLGN both the morphology of TC relay neurons as well as the architecture of their axonal targets in cortex exhibit dystrophic maturation. However, it is unclear whether the absence of retinal signaling has any effect on visual TRN – either the sectorial arrangement of the nucleus or on the time course of feedforward innervation from dLGN to visual TRN.

Here, we examined the formation of the inhibitory feedback loop connecting dLGN and TRN both in the presence and absence of retinal input. Additionally, we determined if the sectorial arrangement of TRN was present at early postnatal ages and whether this arrangement was altered by visual deafferentation. To study the impact of retinal signaling we took a loss of function approach using the Math 5^{-/-} mouse. This well-characterized mutant mouse provides a genetic form of deafferentation as mice that lack the transcription factor Math 5 fail to form an optic nerve and are devoid of any retinal input to the brain (Brown et al., 1998, 2001; El-Danaf et al., 2015). Using both WT and Math 5^{-/-}

mice, we visualized thalamic input to TRN using both anterograde and retrograde tracing techniques. We also employed transgenic mouse lines that enabled the visualization or manipulation of either feedforward (dLGN to TRN) or feedback (TRN to dLGN) circuits. We used the corticotropin releasing hormone – Cre (CRH-Cre) line to target TC neurons in dLGN as well as other first-order thalamic relays (Monavarfeshani et al., 2018; Sokhadze et al., 2018b). TRN neurons were visualized using the GAD65 EGFP mouse (López-Bendito et al., 2004). Channelrhodopsin 2 (ChR2) was targeted to either TC or TRN neurons by crossing the Ai32 reporter line (Adamantidis et al., 2015; Madisen et al., 2009, 2012; Zhao et al., 2011) with either CRH-Cre or somatostatin-cre (SST-Cre) mice (Ahrens et al., 2014; Clemente-Perez et al., 2017; Klug et al., 2018; Wells et al., 2016). These crosses enabled the evaluation of synaptic maturation using optogenetic activation of presynaptic terminals within an acute thalamic slice preparation during electrophysiological patch clamp recordings. Using these techniques we assessed the developmental time course of both structural and functional innervation between TRN and dLGN as well as the impact retinal signaling has on the formation of these circuits.

Materials and Methods

Subjects

Experiments were conducted in mice P0-P46 of either sex. We used a transgenic strain that expresses enhanced green fluorescent protein (EGFP) in TRN GABAergic neurons: GAD65 EGFP (Jurgens et al., 2012; López-Bendito et al., 2004). We also utilized two Cre-driver lines, somatostatin-Cre (SST-Cre; Jax stock no. 013044, RRID: IMSR_JAX:013044) and corticotropin releasing hormone-Cre (CRH-Cre, MMRRC no. 030850-UCD, RRID: MMRRC_030850-UCD), to target reporter expression within TRN (SST-Cre) or thalamocortical neurons (CRH-Cre). The Cre-drivers were crossed with reporters to enable Cre-dependent expression of either tdTomato (Ai9; Jax stock no. 007909) or channelrhodopsin 2 – EYFP (ChR2; Ai32; Jax stock no. 012569). All lines were also crossed onto a *Math 5^{-/-}* background in order to study circuits in the absence of retinal inputs. All breeding and experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

Cholera toxin subunit B injections

To label thalamocortical neurons and their projections in TRN via retrograde transport, P1 CRH-Cre or CRH-Cre x *Math 5^{-/-}* mice were deeply anesthetized by cryoanesthesia (>7min on a paper-lined sealed container of crushed ice until reflexes were absent) and the skull was pierced by a sterile needle. A glass pipette (10-20um tip diameter) was filled with a 1% solution of Cholera toxin subunit B (CTB) conjugated to different Alexa Fluors (488, 546 or 647; Invitrogen) dissolved in distilled water. The pipette containing CTB was

attached to a picospritzer and 1-4uL was injected into the cortex. Cortical targets were identified in relation to the transverse sinus, superior sagittal sinus and central sulcus. Following surgery, pups were warmed on a heating pad and returned to the dam once they were warm, pink, and capable of spontaneous movement. After a 24-72hr survival period, animals were deeply anesthetized by cryoanesthesia and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (4% PFA). Brains were removed and post-fixed for 24hr in 4% PFA and then transferred to phosphate buffered saline [PBS: 0.01 M phosphate buffer (PB) with 0.9% NaCl]. Prior to sectioning (see histology, below), whole brains were imaged using an Olympus SZX2-ILLB stereomicroscope with Prior Scientific Lumen 200 fluorescence illumination system to determine cortical injection sites.

Histology

Mice were deeply anesthetized (<P5: cryoanesthesia, described above; >P5: isoflurane vapors) and transcardially perfused with PBS followed by 4% PFA. Prior to sectioning, brains were post-fixed in 4% PFA overnight then transferred to PBS. A vibrotome (Leica VT1000S) was used to make coronal sections that were typically 70um thick (immunohistochemistry was performed on 35um – thick sections, below). Since intrinsic fluorescent protein expression was sufficient for visualization, sections were directly mounted onto gelatin-subbed slides using ProLong mounting medium containing DAPI (Life Technologies P36931).

Immunohistochemistry

To visualize thalamocortical axon collateral terminals in TRN we used vesicular glutamate transporter 2 (vGluT2) labeling. At least three coronal sections (35um) containing the visual sector of TRN from at least two different animals were collected from CRH-Cre and CRH-Cre x Math 5^{-/-} animals from P5-P28. Immunolabeling was performed in cohorts such that WT and Math 5^{-/-} tissue were run in conjunction (<P14: on the same slide, >P14: in the same jar). Sections were blocked (10% normal goat serum [NGS; Vector Labs, S-1000, RRID: AB_2336615] and 0.3% Triton X-100 in PBS) for 1hr and then incubated overnight in rabbit anti-vGluT2 (SysSys, AB135403 1:500, RRID: AB_887883; diluted with 10% NGS in PBS). After a wash in PBS, a secondary antibody, goat anti-rabbit 488 (ThermoFisher, A11034, RRID: AB_2576217) was applied for 1hr. Unless otherwise noted, all antibodies were diluted 1:100 in PBS with 1% NGS. Sections were washed and then immunolabeled to visualize neurons using the same blocking procedure (above) and incubated overnight in mouse NeuN (Milipore, MAB377, RRID: AB_2298772). After a wash, sections were incubated for 1hr with biotinylated goat anti-mouse (Vector Labs, BA9200, RRID: AB_2336171) and then labeled with streptavidin-AF647 (ThermoFisher, S21374, RRID: AB_2336066, 1:100 diluted in PBS).

Image Acquisition and analysis

Middle sections of either visual TRN (feedforward) or dLGN (feedback) were selected for imaging using a multiphoton laser scanning confocal microscope (Olympus, model no. FV1200BX61) equipped with a 20x (0.75 NA)

objective. Fluorophores were excited using Ar (488nm) and HeNe (635nm) lasers, and Z-stacked images (1.26um optical sections) were acquired using Fluoview software at a scanning resolution of 1600 x 1600 pixels. Acquisition parameters were kept consistent across sections when imaging for quantitative analysis (figures 10 and 11) but channels used to identify regions of interest were optimized for each section (figure 8).

To quantify the spatial extent of innervation at different postnatal ages we analyzed at least three coronal sections through the middle of either dLGN (AP: -2.35) or visual TRN (AP: -1.70). Z-stacks of each image were collapsed into a two-dimensional image and imported into Photoshop (Adobe). The target nucleus of innervation (feedforward: visual TRN; feedback: dLGN) was delineated by combining anatomical features, the location of labeled cell bodies (feedforward: VB; feedback: vLGN), and cellular organization revealed by NeuN staining (above), however the ventral border of visual TRN was defined as a horizontal line from the lateral apex of TRN to the medial border with VB based on tracing experiments in adult animals (figure 5). Once a nucleus was isolated, images were binarized using a threshold that clearly distinguished signal and background fluorescence. Binarized images were imported into ImageJ (NIH, RRID: SCR_003070) in order to count both the number of fluorescent pixels as well as the total number of pixels within a nucleus. These values were used to calculate the percent area that contained fluorescent pixels. At each postnatal age, percentages from >3 hemispheres taken from >2 animals were combined to determine the median percent area.

In Vitro Slice Preparation and Fixation

Brain slices were made from deeply anesthetized mice (<P5: cryoanesthesia, described above; >P5: isoflurane vapors) and rapidly decapitated as described previously (refs). Briefly, the brain was removed from decapitated mice and placed into 4°C oxygenated cutting solution (in mM): Sucrose 234, glucose 11, NaHCO₃ 26, MgSO₄ 10, KCl 2.5, CaCl₂ 0.5, NaH₂PO₄ 1.25. A vibratome (Leica VT1200S) was used to make 270µm thick thalamic slices. Some slices were checked for Chr2-EYFP expression using a stereomicroscope (Olympus SZX16) equipped with an LED for illumination (Prior Lumen 200; images from this preparation are shown in figure 12). Thalamic slices were incubated in warm (32°C) oxygenated aCSF for 30min (in mM: NaCl 126, NaHCO₃ 26, glucose 10, KCl 2.5, MgCl₂ 2, CaCl₂ 2, NaH₂PO₄ 1.25). For experiments, slices were transferred to a recording chamber and continuously perfused (2-3mL/min) with 32°C aCSF.

Using an upright microscope (Olympus BX51WI), thalamic nuclei were visualized with DIC optics and fluorescent filters (GFP: Chroma 49002) using a 10x or 60x water immersion objective. While dLGN and TRN were readily identifiable at 10x based on anatomical landmarks, we identified visual TRN by targeting dorsal TRN in sections that contained little, if any, of the rostral pole of dLGN. Patch electrodes were made from borosilicate glass using a vertical puller (Narashige PC-10). For both voltage clamp recordings in TRN as well as current clamp recordings in dLGN, the electrode internal solution contained (in mM) 117 K-gluconate, 13 KCl, 1.0 MgCl₂, 0.07 CaCl₂, 0.1 EGTA, 0.1, 10 HEPES.

Voltage clamp recordings in dLGN utilized a cesium-based internal solution (in mM: 117 Cs-gluconate, 11.0 CsCl, 1.0 MgCl₂, 1.0 CaCl₂, 0.1 EGTA, 10.0 HEPES, 2 Na₂-ATP, 0.4 Na₂-GTP). The final tip resistance of all electrodes was 4-7 MΩ. For all recordings, biocytin (0.5%, Sigma) was included in the internal solution for intracellular filling and to enable confirmation of recording location as well as 3-D neuron reconstruction using confocal microscopy (Charalambakis et al., 2019; El-Danaf et al., 2015; Krahe et al., 2011).

Following recording, slices were fixed overnight in 4% PFA and then washed with PBS. Later, the tissue was incubated overnight with AlexaFluor 647 conjugated streptavidin (ThermoFisher, S21374, RRID: AB_2336066, 1:100 diluted in PBS with 0.1% Triton X-100). Slices were mounted on glass slides using Prolong Gold with DAPI (Life Technologies P36931) and coverslipped. Neuron location was confirmed on a epifluorescent microscope (Olympus BX43 with Chroma 3900, 39002, 39004 and 39007 filters for 350, 488, 546 and 647nm fluorescence) using a LED (X-Cite 120 Lumen Dynamics) 635nm and CellSense software. Cells were also imaged using a confocal microscope (described above) to create three-dimensional neuron reconstructions.

Optogenetic Stimulation and Electrophysiological Recording

Whole cell recordings were made using an amplifier (Multiclamp 700B, Molecular Devices), filtered at 3-10kHz and digitized (Digidata 1440A). Recordings were conducted in voltage and current clamp. Pipette capacitance, series resistance, input resistance, and whole-cell capacitance were monitored throughout the recording session.

Light gated responses were evoked using a light emitting diode (LED, Prizmatix) that delivered blue light through the 60x objective. Blue light pulses were 0.3mm in diameter with a light power of 525mW/mm² and each pulse lasted for 1ms. During voltage clamp recordings, inhibitory currents were measured by holding the neuron at 0mV using a cesium-containing electrode (above) while neurons were held at -70mV to measure excitatory currents using a potassium-containing electrode (above). Current clamp recordings also used a potassium-containing electrode and holding current was injected to adjust the membrane potential to -70mV before stimulation. During voltage clamp recordings, trains of 10 blue light pulses were presented at different temporal frequencies (0.25, 0.5, 1, 5, 10 and 50 Hz). The incidence of response was assessed by determining if the peak amplitude was $>2 \times \text{RMS}$ (root mean squared) of baseline. While the RMS was calculated from individual trials during a stimulus, all other measurements were averages of 3-6 trials. The amplitude of response was measured in the initial recording by comparing the peak amplitude to baseline levels obtained for 2s just prior to photostimulation. Synaptic suppression was quantified by dividing the amplitude of the 10th response by the amplitude of the 1st response and then multiplied by 100. Current clamp recordings were used to assess how TRN stimulation affects spiking activity in dLGN relay neurons. A 1.5s square wave depolarizing current pulse was injected to evoke reliable spike firing at rates $>5\text{Hz}$. While the amount of current injected to evoke firing was greater in WT than in *Math 5^{-/-}* animals (two-way ANOVA, $F_{\text{genotype}(1,94)}=278.0$, $p<0.0001$), the baseline firing rates were not significantly

different between genotypes or frequencies (two-way ANOVA, $F_{\text{genotype-frequency}(5, 260)}=1.862$, $p=0.1013$). The percent change in spike firing was calculated by comparing equivalent periods (0.5s) of activity in the presence and absence of blue light stimulation (10, 20 and 50Hz; presented for 0.5s). In order to minimize the effects of frequency accommodation or burst firing, measures of spike firing were obtained 0.5s after the onset of the 1.5s current pulse (see figure 17B and C). Typically, the measurements described above were based on an average of 8 stimulus presentations.

Statistical Analyses

All statistical tests were performed using Prism 8.0 (Graphpad Software, La Jolla, CA). The majority of our data were comprised of percentages, which normally require non-parametric statistical tests but because we were comparing across multiple variables, we were often limited to parametric tests and consequently set alpha to <0.01 in those cases. Anatomical data was calculated as the percent area covered by labeled terminals and analyzed by a two-way ANOVA (Sidak multiple comparison tests; figures 10 and 11). Significant differences ($p<0.01$) in feedback innervation (P0 – P3) were confirmed by repeated Mann-Whitney tests comparing genotype within a single age. The percent area of both feedback and feedforward innervation was also fit to a one-phase association non-linear curve, of which the plateau was constrained to be less than 100. We determined whether a model of a single shared curve for both WT and Math 5^{-/-} datasets was preferable to a model of multiple curves using Akaike's informative criteria. For physiological data, we used a two-sided Fisher's

exact test ($p < 0.05$) to compare the incidence of responding cells within a single age (figures 14A and 16A). We used two-way ANOVAs to test for significant differences in amplitude (using Sidak's multiple comparison tests; figures 14B and 16B). However, the synaptic suppression data was comprised of multiple variables: age, genotype, stimulation frequency and stimulus number. Therefore, we ran multiple two-way ANOVAs to isolate one variable while comparing the other two. Because this method could not account for the multiple comparisons, and because we were using incomplete data sets (e.g. cells from young animals could not follow stimulation at rates of 50Hz) we again set alpha at $p < 0.01$. Initially, we compared genotype by stimulus number at 1Hz within each postnatal week using two-way ANOVAs with a Geisser-Greenhouse correction for sphericity (figures 14C and 16C). However, because synaptic suppression stabilized by the 10th pulse, we calculated the paired pulse ratio (N_{10}/N_1) at each postnatal week, for each stimulation rate and in both genotypes (figures 14D and 16D). These values were then compared by multiple two-way ANOVAs and the results detailed in tables 1 and 2. For the suppression of dLGN spike firing (figure 19) we ran three-way ANOVAs (using Tukey multiple comparison tests, $p < 0.01$) but were required to run three different tests since only two stimulus frequencies could be compared within one test (see table 3). Within-cell analyses (figure 19B) were analyzed with two-way ANOVAs with repeated measures as well as a Geisser-Greenhouse correction for sphericity.

Results

Visualization of Feedback and Feedforward Projections

To examine feedforward projections from dLGN to TRN, we crossed corticotropin releasing hormone-Cre (CRH-Cre) mice with Ai9 reporter strain (Monavarfeshani et al., 2018; Sokhadze et al., 2018b). This cross expressed tdTomato in thalamocortical neurons from primary sensory nuclei (figure 4, left, purple and figure 5). In dLGN this expression is apparent as early as P0 and restricted exclusively to relay neurons with axons that pass through TRN and terminate in visual cortex. It is important to note that the projections from dLGN to TRN involve fine caliber axon collaterals while thalamocortical axons are of much larger caliber and bundled into fascicles (Pinault, 2004). Therefore, at the light microscopic level, it is not possible to differentiate axon collaterals from axons of passage. Consequently, vesicular glutamate transporter 2 (vGluT2) immunohistochemistry (Fremeau et al., 2001, 2004; Groh et al., 2013; Herzog et al., 2001; Takamori et al., 2001) was used to visualize terminal boutons of thalamocortical axon collaterals in TRN (figure 4, left, blue; figure 8, blue).

To visualize feedback projections from TRN to dLGN we used GAD65-EGFP mice (Jurgens et al., 2012; López-Bendito et al., 2004), which labels TRN neurons with EGFP as early as P0 (figure 4, right, green). Importantly, other sources of thalamic inhibition, including neurons of the pretectum and intrinsic interneurons of dLGN do not express EGFP, indicating that the exclusive source of EGFP labeling in dLGN arises from TRN.

We also asked whether the removal of retinal signaling influenced the development of feedforward and feedback projections by utilizing a mutant mouse that lacked retinal ganglion cells, the Math 5^{-/-} mouse (Brown et al., 1998, 2001; El-Danaf et al., 2015; Wang et al., 2001b). Math 5^{-/-} mutants of CRH-Cre x Ai9 and GAD65-EGFP were created and compared to WT mice from each line in order to assess the impact of retinal signaling on the formation of feedforward (CRH-Cre x Ai9 x Math 5^{-/-}) or feedback (GAD65-EGFP x Math 5^{-/-}) circuitry.

Sectorial Arrangement of TRN

The TRN is comprised of at least seven modality-specific sectors: a limbic sector, a motor sector and five sensory sectors (visual, somatosensory, auditory, gustatory and visceral; (Crabtree, 2018; Guillery et al., 1998; Jones, 1975; Pinault, 2004). These sectors are defined by the source of their excitatory inputs, which they receive from ascending thalamocortical and descending corticothalamic inputs, and arranged in non-overlapping, modality-specific manner. In mammals sensory sectors occupy caudal regions of TRN such that the visual sector is located in dorsal-lateral aspect of TRN. Auditory and somatosensory sectors are both located in caudal ventral-lateral regions but the auditory sector is more caudal than the somatosensory sector (Coleman and Mitrofanis, 1996; Conley and Diamond, 1990; Crabtree, 2018; Fitzgibbon, 2007; Guillery and Harting, 2003; Harting et al., 1991; Kimura, 2017; Ohara and Lieberman, 1985). An example of this organization for mouse is shown in figure 5. As described above, in the CRH-Cre line Cre is expressed in thalamocortical neurons of first-order sensory thalamic nuclei such as the ventral medial

geniculate nucleus (vMGN, figure 5A), dLGN (figure 5B) and the ventrobasal complex (VB, figures 5B and C). Terminals from thalamocortical neurons can be seen in primary auditory cortex (figures 5A and B), primary visual cortex (figures 5A and B), primary somatosensory cortex (figure 5C) as well as TRN (figure 5C). In the coronal plane, caudal sections of TRN are comprised of a dorsal head, a lateral apex and a ventral tail (figure 5D; refs). We injected the Cre-dependent anterograde viral tracer FLEX-AAV-ChIEF-tdT into vMGN (figure 5E, top), VB (figure 5E, middle) and dLGN (figure 5E, bottom). The corresponding tdT-labeled axonal projections within TRN (figure 5F) demonstrate that thalamocortical axons are segregated into non-overlapping sectors: the auditory sector is in the caudal tail of TRN (figure 5F, top), the somatosensory sector is also in the tail of TRN but is rostral to the auditory sector (figure 5F, middle) and the visual sector is in the head of the caudal TRN, dorsal to the apex (figure 5F, bottom). Thus, the visual sector of TRN is restricted to the head (dorso-caudal) region above the apex while non-visual sectors (auditory and somatosensory) are ventral to the apex (figure 5G).

To address whether this arrangement is present at early postnatal ages, we made injections of a retrograde tracer (CTB conjugated to different Alexa dyes) into visual (figure 6, blue) or somatosensory cortex (figure 6, yellow) in WT mice at postnatal day 1 (n=6). As early as P2, CTB injections into visual cortex (figure 6A) resulted in retrograde labeling of thalamocortical neurons in dLGN as well as LP, but not in VB (figure 6B). In all six cases, the labeled axons within TRN were restricted to the head (i.e., dorsal-caudal region) of the nucleus (figure

6C). Moreover, we failed to detect any axonal labeling ventral to the apex of TRN, a boundary that delineates visual and nonvisual sectors of TRN (above). A similar modality specific patterning was evident following CTB injections into somatosensory cortex (figure 6D). These injections labeled TC neurons that were restricted exclusively somatosensory thalamic nuclei such as VPM and VPL of the ventrobasal complex (VB; figure 6E). In TRN, the axons from somatosensory were restricted to the ventro-medial region at or below the apex (figure 6F). Thus, these tracing experiments demonstrate that the sectorial arrangement of sensory TRN is established as early as P2.

While these sectors are established at early ages, it is unclear whether this arrangement is preserved in the absence of sensory signaling. To test for this possibility, we examined the visual and somatosensory sectors of TRN by performing CTB injections in *Math 5^{-/-}* mice (figure 7). Similar to WT, injections into V1 (figure 7A) and S1 (figure 7D) at P1 labeled axons in a sensory specific manner by P2 (figures 7B and E). V1 injections led to axonal labeling in the head of TRN (n=3, figure 7C, blue) while S1 injections labeled axons in ventromedial regions, at or below the apex (n=2, figure 7F, yellow).

Thalamic Innervation of TRN

We used CRH-Cre x Ai9 mice to examine how and when thalamocortical (TC) axons course through the visual sector of TRN. We also employed vesicular glutamate transporter 2 (vGluT2) immunohistochemistry, which labels synaptic terminals of TC cells (Fremeau et al., 2001, 2004; Groh et al., 2013; Herzog et al., 2001; Takamori et al., 2001), to label the synaptic terminals of TC axon

collaterals in TRN. Figure 8 illustrates the punctate vGluT2 labeling of TC axon collaterals (blue) in relation to both TC axonal labeling (purple, visualized as Cre-dependent tdTomato expression in CRH-Cre x Ai9 mice) and somatic thalamic labeling (yellow, via NeuN labeling). Note the complementary patterning of vGluT2 labeling in relation to the large caliber axonal fascicles (tdTomato) as well as TRN somata (NeuN). It is also important to note that vGluT2 puncta within TRN are much smaller compared to puncta in VB (figure 8, blue). This difference is consistent with reports that TC terminals within TRN are much smaller than lemniscal terminals in VB (Pinault, 2004).

Figure 9 provides examples of coronal sections of labeled TC axons at different postnatal ages. As early as P0 TC axons course through TRN on a trajectory from the medial aspect of the nucleus to the lateral edge where they aggregate to form the internal capsule (figure 9). At later ages, axons within TRN are arranged into fascicles forming a reticular structure. This pattern emerges as early as P7 and is readily apparent within the head of TRN by P14.

To assess whether the absence of retinal inputs disrupted the maturation of TC projections to TRN, we crossed CRH-Cre x Ai9 mice onto a Math 5^{-/-} background. Similar to WT animals, at P0 in Math 5^{-/-} mice, TC axons were present throughout TRN (figure 9, bottom) with reticular architecture arising between P7 and P14.

To determine when TC axon collateral terminals appear in visual TRN and whether they are affected by the removal of retinal signaling we examined vGluT2 labeling in coronal sections from age-matched WT (top) and Math 5^{-/-}

(bottom) mice (figure 10A). In WT animals, there was a progressive increase in vGluT2 labeling with age. The visual TRN was largely devoid of vGluT2 puncta at P7 (figure 10A) but then encompassed the entire visual sector by P21. To analyze the rate of TRN innervation by TC axon collaterals, we quantified the percentage of visual TRN area covered by vGluT2 puncta across several different postnatal ages and plotted the median percentage at each age (figure 10B; Sokhadze et al., 2018b). In WT animals, the area covered by vGluT2 puncta significantly increased with age (filled circles, $n=104$, Kruskal-Wallis: $p<0.0001$). During the first postnatal week (P5-P7) vGluT2 labeling was sparse but showed a progressive increase to cover nearly half of visual TRN by P10 (P5 and P7 vs P10, Dunn's multiple comparison test: $p<0.01$) and all of the visual sector by P21 (all comparisons between P10, P14 and P21, $p<0.01$). At P21, thalamic innervation of visual TRN plateaued and did not differ from P28 ($p=0.3063$). Thus, innervation of visual TRN by TC axon collaterals dramatically increases during the second postnatal week (P7-P14) and is complete by the end of the third postnatal week (P21).

Similar to WT, there was a similar age related increase in vGluT2 puncta labeling in the visual sector of TRN of Math 5^{-/-} mice (figure 10B, open circles, $n=72$, Kruskal-Wallis: $p<0.0001$). Moreover, the age-related increase in vGluT2 labeling had a similar trajectory (a single shared non-linear model was 90.08% more probable than a model using two separate curves for WT and Math 5^{-/-} data, Akaike's informative criteria, $R^2=0.8948$, $W=0.9590$, $p<0.0001$), showing that the density of labeling at each of the ages tested were virtually identical (two-

way ANOVA: $F_{\text{genotype}(1,164)}=0.5503$, $p=0.4593$; Tukey's multiple comparison test: $p>0.05$ at each age). Together, these data demonstrate that while TC axons course through TRN at birth, the appearance of axon collaterals increases during the second postnatal week and reaches an adult-like state by the end of the third postnatal week (P21). Furthermore, the absence of retinal input does not alter the time course of TC innervation of visual TRN.

Feedback Innervation from TRN to dLGN

To assess when feedback inhibitory projections innervate dLGN and test whether the arrival of this input is disrupted in the absence of retinal signaling, we analyzed coronal sections of dLGN obtained from early postnatal GAD65 and GAD65 x Math 5^{-/-} mice (P0-P18, figure 10A). In WT animals, TRN fibers are absent from dLGN at birth, begin to innervate the nucleus at P2 from the medioventral border of the nucleus, and cover dLGN in a dense plexus of terminals by the end of the first postnatal week (figure 11A, top). We analyzed the rate of feedback innervation by quantifying the spatial extent of TRN terminals expressed as a percentage of dLGN area (Seabrook et al., 2013a; Sokhadze et al., 2018a) and plotted the median percent area covered by TRN terminals at each age (figure 11B). In WT animals (filled circles, $n=47$), TRN terminals arrived in dLGN at P2 and showed a progressive increase with age (Kruskal-Wallis: $p<0.0001$). Most of the projections arrived between P2-4 and showed nearly a 3-fold increase over this period (median values: P2=22.40, P4=61.34). By P6, TRN fibers and their terminals formed a dense plexus that spanned the entire nucleus and reached adult like values (median values:

P6=83.87, P9=82.67, P18=86.08). Thus, TRN feedback innervation of dLGN is complete by the end of the first postnatal week.

In the absence of retinal signaling TRN projections seemed to arrive sooner but reached adult like levels of innervation at ages similar to WT (figure 11B open circles, n=59; a non-linear model comprised of two distinct curves was >99.99% more probable than a model with a single shared curve for WT and Math 5^{-/-} data, Akaike's informative criteria, R²: WT=0.8619, Math 5^{-/-}: 0.8997; Shapiro-Wilk normality test WT: W=0.9574, p=0.1043, Math 5^{-/-} W=0.9694, p=0.1431). In Math 5^{-/-} mice, TRN terminals were present at birth and showed a two-fold increase in innervation between P0 and P2, but between P2 and P4 increased by only 16.7% (figure 11A, bottom; median spatial extent: P0=26.18, P2=51.02, P4=61.28). This pattern of innervation was significantly different from WT animals (two-way ANOVA, F_{genotype(1,89)}=37.02, p<0.0001, F_{age x genotype(6,89)}=30.56, p<0.0001) showing a greater rate of innervation between P0-3 compared to WT (Sidak's multiple comparison test, p<0.0001 for each age) but was similar to WT by P4 (p=0.9989). Together, these data demonstrate that TRN projections arrive in dLGN during the first postnatal week and that loss of retinal signaling simply accelerates innervation.

Using Optogenetics to Examine the Functional State of Developing Connections Between TRN and dLGN

We adopted an optogenetic approach to assess the development of TRN and dLGN connections by conducting visually-guided whole cell electrophysiological recordings from acute thalamic slices obtained from mice

that underwent genetic insertion of channelrhodopsin 2 (ChR2) into dLGN terminals of axon collaterals in TRN or TRN terminals within dLGN. To target feedforward inputs to TRN we utilized crosses of CRH-Cre x Ai32 (CRH-Cre x ChR2; figure 12A) and to isolate feedback connections to dLGN, we created somatostatin-Cre x Ai32 (SST-Cre x ChR2) mice (figure 12D) on WT and Math 5^{-/-} backgrounds. These strains express a channelrhodopsin 2 – EYFP fusion protein in Cre-expressing cells (Deisseroth, 2015; Lin, 2010; Madisen et al., 2012; Zhao et al., 2011). To confirm that we could evoke responses in early ages, we recorded directly from neurons that are targeted for ChR2 expression. Indeed, we found that EYFP was expressed in targeted neurons at early ages (P2-5) and was activated by blue light stimulation. For example, at P5 EYFP was expressed in both dLGN TC neurons (figure 12B, left) as well as their projections in TRN (figure 12B, right). Moreover, voltage clamp recordings from ChR2-expressing dLGN TC neurons demonstrated that as early as P6 neurons could follow a train of brief blue light pulses (1ms each) at high temporal frequencies (50Hz; figure 12C, top) while a single prolonged light pulse evoked a sustained inward current that showed little attenuation (500ms, figure 12C, bottom). Similarly, in the feedback projection (figure 12D) imaging of SST-Cre x ChR2 animals showed robust opsin expression in TRN soma at P2 (figure 12E, left) as well as fibers in dLGN at P6 (figure 12E, right). During current-clamp recordings in TRN neurons from SST-Cre x ChR2 animals brief (1ms) flashes of blue light evoked depolarizations as early as P2 (figure 12F, top) while longer light pulses (15ms) evoked a train of action potentials (figure 12F, bottom). Importantly, in the

feedforward circuit postsynaptic responses were recorded no earlier than P5 while feedback postsynaptic responses were recorded no earlier than P4 (see below). Therefore, these recordings demonstrate robust light-evoked somatic responses in both feedforward and feedback circuits at the postnatal age that postsynaptic responses emerge. Thus, these examples demonstrate that ChR2 is expressed, functional, and capable of producing robust activity at early ages in both strains of transgenic mice.

Development of feedforward synaptic connections between dLGN and TRN

To assess when functional feedforward connections between dLGN and TRN neurons emerge and to test whether this time course is influenced by the loss of retinal signaling, we conducted whole-cell patch clamp recordings from P5 – P35 CRH-Cre x ChR2 WT and Math 5^{-/-} mice (figure 13A). Postsynaptic activity evoked by repetitive blue light stimulation was measured during voltage clamp recordings using a potassium-based internal solution while holding TRN neurons at -70mV (Jurgens et al., 2012; Seabrook et al., 2013a; Sokhadze et al., 2018b). Using DIC optics, we targeted 186 WT and 114 Math 5^{-/-} neurons in the visual sector of TRN. Of these neurons, 182 WT and 104 Math 5^{-/-} were filled with biocytin during recording and based on their confocal reconstruction, were confirmed to be in visual TRN. During recording, trains of brief (1ms) blue light flashes (10 in total) were administered at temporal frequencies of 0.25Hz, 0.5Hz, 1Hz, 5Hz, 10Hz and 50Hz to evoke excitatory postsynaptic currents (EPSCs examples of 0.5Hz, 5Hz and 50Hz are shown in figure 13B). In WT animals, activation of CRH-containing TC axon collaterals in visual TRN evoked little

activity during the first postnatal week. Responses at this age were rare (3/18 responded), of low amplitude and could only follow lower rates of stimulation (for example, 0.5Hz but not 5Hz or 50Hz as shown in figure 13B). Between the second and third postnatal weeks excitatory responses became more prevalent and increased in amplitude. Additionally, synapses developed the ability to follow higher rates of repetitive stimulation during these ages (as high as 50Hz by week 3), but responses were suppressed by repeated stimuli to levels less than half the initial amplitude after only a few stimuli (figure 13B).

By the fourth postnatal week (P22-P28), peak amplitudes were large and resembled mature responses. A similar pattern of responses were found in Math 5^{-/-} mice (figure 13B).

A summary of these events is shown in figure 14 where we plot the incidence of responsive cells (figure 14A), the amplitude of the initial response (figure 14B), the emergence of paired pulse suppression to a 1Hz stimulus train (figure 14C), as well as the extent of synaptic suppression by repeated stimuli at different rates of stimulation across postnatal weeks 2-4 (figure 14D). In WT, blue light stimulation evoked EPSC activity in 116/186 neurons (figure 14A, filled circles). During the first postnatal week, only a few neurons showed weak light evoked responses (WT: 3/18, 17%; Math 5^{-/-}: 0/8, 0%). The incidence of light evoked responses showed a progressive increase between postnatal weeks 2-3 so that by the end of week 3 nearly all neurons showed responses (P19-21, WT: 28/31, 90%; Math 5^{-/-}: 16/18, 89%). This progression was unaffected by the loss of retinal signaling. Recordings in Math 5^{-/-} mice showed a similar trajectory as

WT (Fisher's exact test, $p > 0.05$ at all ages tested). Consistent with the increase in incidence, we also noted an increase in peak amplitude and paired pulse suppression for both WT and Math $5^{-/-}$ mice.

Overall, amplitude increased with age (figure 14B, two-way ANOVA, $F_{\text{age}(2,138)} = 5.743$, $p = 0.0040$) but was unaffected by the loss of retinal signaling ($F_{\text{genotype}(1,138)} = 0.2572$, $p = 0.6128$). In WT and Math $5^{-/-}$ mice, peak amplitude increased during the second postnatal week but was stable between weeks 3 and 4 (Tukey's multiple comparison test, week 2 vs week 3: $p = 0.0174$; week 2 vs week 4: $p = 0.0096$; week 3 vs week 4: $p = 0.7982$).

We also assessed the emergence of synaptic suppression by repetitive stimulation by presenting trains of blue light pulses (20 pulses, 1ms duration) at multiple temporal frequencies (0.25Hz, 0.5Hz, 1Hz, 5Hz, 10Hz, and 50Hz) during weeks 2-4 for both WT and Math $5^{-/-}$ mice. At all temporal frequencies, the initial response produced a maximal EPSC that exhibited a progressive decline that stabilized by the 5th pulse (except for 50Hz trains that stabilized by the 7th). An example of synaptic suppression by repeated stimuli is shown for responses evoked by a 1Hz stimulus train (figure 14C). At each postnatal week responses from WT (solid symbols) and Math $5^{-/-}$ (open symbols) were indistinguishable (two-way ANOVAs, week 2: $F_{\text{genotype}(1,37)} = 0.06785$, $p = 0.7959$; week 3: $F_{\text{genotype}(1,57)} = 1.896$, $p = 0.1739$; week 4 $F_{\text{genotype}(1,24)} = 1.074$, $p = 0.3103$). However, similar to peak amplitude (above), significantly more suppression was observed during the second postnatal week (red circles) than either the third (green squares) or fourth (blue triangles) weeks (two-way ANOVAs, WT: $F_{\text{age}(2,79)} = 13.63$,

$p < 0.0001$, Math $5^{-/-}$: $F_{\text{age}(2,39)} = 11.98$, $p < 0.0001$; Tukey's multiple comparison tests: week 2 vs week 3 or 4: $p < 0.0001$ in both WT and Math $5^{-/-}$). To quantify synaptic suppression at all temporal frequencies we calculated the paired pulse ratio between the 10th and 1st response for each stimulus. Overall, the extent of suppression decreased with age, increased with higher rates of stimulation and was unaffected by the loss of retinal signaling (table 1, figures 14D). A summary of these interactions is shown in figure 14D, which demonstrates that there was no difference between WT (solid symbols) and Math $5^{-/-}$ mice (open symbols) at any age (x-axis) or at any stimulation rate (color-coded; also see table 1). Within either WT or Math $5^{-/-}$ mice, there was a significant decrease in suppression with age but this effect was inconsistent within a single stimulation rate (table 1). The lack of an effect at higher temporal frequency stimulation is likely due to the relative immature state of the synapse, which at younger ages could not follow higher rates of stimulation (see figure 13B). Additionally, we observed an expected increase in suppression with higher temporal frequency stimulation (table 1), which was likely due to depletion of neurotransmitter (Jackman and Regehr, 2017; O'Donovan and Rinzel, 1997; Regehr, 2012; Sherman, 2012; Thomson and Thomson, 2000; Zucker and Regehr, 2002).

Development of Feedback Synaptic Connections Between TRN and dLGN

To examine the maturation of postsynaptic activity in feedback projections from TRN to dLGN both in the presence and absence of retinal inputs, we conducted whole-cell patch clamp recordings from WT and Math $5^{-/-}$ P2 – P35 SST-Cre x ChR2 mice (figure 15A). Postsynaptic activity evoked by repetitive

blue light stimulation was measured voltage clamp recordings using a cesium-based internal solution while holding relay neurons at 0mV (Charalambakis et al., 2019; Govindaiah and Cox, 2004). We used DIC optics to target 352 WT and 195 Math 5^{-/-} neurons in dLGN. Figure 15B provides examples from each postnatal week (week 1 – week 5) of light-evoked inhibitory postsynaptic currents (IPSCs) evoked by 0.5Hz, 5Hz and 50Hz stimulus trains (20 pulses each of 1ms duration). In WT animals, blue light stimulation of SST-containing fibers in dLGN evoked infrequent inhibitory activity early in the first postnatal week (5/14 responded at P4) but most relay neurons exhibited activity by P7 (18/21). Synaptic activity at this age was of low amplitude and could only follow slower rates of stimulation (for example, 0.5Hz but not 5Hz or 50Hz as shown in figure 15B). Although all relay neurons responded to blue light stimulation by P12 (P10-12: 18/18), responses continued to progressively increase in amplitude between the second and third postnatal weeks. Synaptic activity was suppressed by repetitive stimulation but progressively developed the ability to follow higher temporal frequencies of stimulation such that synapses could follow rates as high as 50Hz by the third postnatal week. By the fourth postnatal week evoked IPSCs were of large amplitude and exhibited less paired pulse suppression than earlier ages and this pattern of response was maintained through the fifth week. A similar pattern of responses was observed in the absence of retinal signaling (figure 15B).

A summary of this pattern of synapse formation is shown in figure 16 where we plot the incidence of responsive cells (figure 16A), the amplitude of the

initial evoked IPSC (figure 16B), the emergence of paired pulse suppression to a 1Hz stimulus train (figure 16C) as well as the extent of synaptic suppression by repeated stimulation (figure 16D). In WT, blue light stimulation evoked inhibitory activity in 302/352 relay neurons (figure 16A, solid circles). Functional synapses emerged between P2 and P4 (P2: 0/9, 0% responded, P4: 5/14, 36%) but the majority of relay neurons responded by the end of the first postnatal week (P7: 18/21, 86%). All neurons responded by eye opening (P10-12: 18/18, 100%) and this level of activity was maintained at later ages (>P12: 198/198, 100%). The developmental timing of when synapses emerged was unaffected by the absence of retinal signaling as recordings in Math 5^{-/-} showed a similar trajectory (Fisher's exact test, $p > 0.05$ at all ages tested). While all relay neurons responded to blue light stimulation during the second postnatal week, peak amplitude and paired pulse suppression did not stabilize until later ages.

Overall, the amplitude of inhibitory responses in relay neurons exhibited a progressive increase with age (figure 16B, two-way ANOVA $F_{\text{age}(4,156)} = 30.26$, $p < 0.0001$) but was not affected by the absence of retinal signaling ($F_{\text{genotype}(1,156)} = 0.1095$, $p = 0.7412$). IPSCs were significantly smaller at early ages (postnatal weeks 1 and 2) compared to later ages (weeks 4 and 5; Tukey's multiple comparisons test WT or Math 5^{-/-}: week 1 vs week 3, 4 or 5: $p < 0.01$, week 2 vs week 4 or 5: $p < 0.05$) but were unchanged after the third postnatal week (WT or Math 5^{-/-}: week 3 vs week 4 or 5: $p > 0.05$).

Inhibitory activity was suppressed by repetitive stimulation, which was evoked by presenting trains of blue light pulses (20 pulses, 1ms duration) at

multiple temporal frequencies (0.25Hz, 0.5Hz, 1Hz, 5Hz, 10Hz, and 50Hz) during postnatal weeks 1-5 in both WT and Math 5^{-/-} mice. At each rate of stimulation, the initial response produced a maximal IPSC that exhibited a progressive decline that stabilized by the 6th pulse (except for 50Hz trains that stabilized by the 9th pulse). An example of synaptic suppression evoked by a 1Hz stimulus train (figure 16C) demonstrates that at each postnatal week WT (solid symbols) responses were similar to those from Math 5^{-/-} animals (open symbols; two-way ANOVAs, week 2: $F_{\text{genotype (1,41)}}=0.0003$, $p=0.9866$; week3: $F_{\text{genotype (1,32)}}=0.0620$, $p=0.8049$; week 4: $F_{\text{genotype (1,26)}}=2.911$, $p=0.0999$; week 5: $F_{\text{genotype (1,16)}}=0.0048$, $p=0.9455$). However, there was a progressive reduction in synaptic suppression as age increased (two-way ANOVA, $F_{\text{age(7,115)}}=11.31$, $p<0.0001$, Tukey's multiple comparison test, within either WT or Math 5^{-/-}, week 2 vs week 3, 4 or 5: $p<0.0001$, week 3 vs week 5: $p<0.05$). To quantify synaptic suppression at all temporal frequencies we calculated the paired pulse ratio between the 10th and 1st response for each stimulus. Overall, paired pulse suppression was reduced at older ages or at lower temporal frequency stimulation while the absence of retinal signaling lead to increased suppression at higher rates of stimulation (table 2, figure 16D). These interactions are summarized in figure 16D, which illustrates that, as expected, higher stimulation rates produced more suppression than lower rates (color-coded, but see table 2). Within each stimulation rate, there was a progressive decrease in paired pulse suppression as age increased (figure 16D, x-axis; table 2). However, at higher stimulation rates (5Hz and 10Hz) there

was also significantly more suppression in Math 5^{-/-} mice compared to WT, an effect that also trended toward significance at 50Hz (table 2).

Development of Feedback Suppression of Relay Neuron Firing

Finally, we assessed how the emergence of inhibitory feedback from TRN to dLGN influences retinogeniculate signal transmission during development. To address this, we performed current clamp recordings of dLGN relay neurons in both WT (n=77) and Math 5^{-/-} (n=49) SST-Cre x ChR2 mice, using potassium-containing electrodes (figure 17A). To evoke a steady train of action potentials, neurons at baseline (-70mV) were injected with a square pulse of depolarizing current (1500ms, mean $\pm$ SEM: 161.6 $\pm$ 4.8pA) that triggered a steady train of firing (at least 5Hz, mean $\pm$ SEM: 13.3 $\pm$ 0.3Hz), which was similar across genotypes and stimulation frequencies (two-way ANOVA, $F_{\text{genotype-frequency}(5,260)}=1.862$, $p=0.1013$). Figure 17B demonstrates two examples from two postnatal ages (left: postnatal week 2; right: postnatal week 4) in which a current pulse (gray) evokes tonic firing before (control, left) and during (right) blue light stimulation of TRN terminals (blue). An expanded view of these responses (figure 17C) illustrates the epoch of activity that was analyzed: the percent change in firing rate for each cell was calculated by comparing matching periods (500ms each, horizontal black line) in the middle of a depolarization in the absence (left) and presence (right) of blue light stimulation.

We explored the nature of TRN inhibition of relay neuron firing by analyzing the effects of three different stimulation rates (10Hz, 20Hz and 50Hz) across postnatal weeks 1-6 both in the presence and absence of retinal signaling

by using WT and Math 5^{-/-} mice. Figure 18 provides examples of relay neuron firing suppressed by TRN activity at two temporal frequencies (10Hz, left and 50Hz, right) in both WT (figure 18A) and Math 5^{-/-} (figure 18B) animals. These examples suggest that during the first two postnatal weeks in both WT and Math 5^{-/-} mice, TRN mediated inhibition had little impact on spike firing of relay neurons. Instead, suppression emerged during the third postnatal week and had a maximal impact by the fourth week, which was maintained throughout the sixth postnatal week. These data are summarized in figure 19A where percent change in spike firing is plotted by postnatal week. In WT and Math 5^{-/-} mice, during the first two postnatal weeks, photostimulation of TRN terminals had no impact on relay neuron firing (all stimulation frequencies combined, mean percent of control firing $\pm$ SEM, WT week 1: 100.02 ± 3.14 , week 2: 85.28 ± 1.68 ; Math 5^{-/-}: week 1: 101.27 ± 3.16 , week 2: 92.44 ± 1.83). However, by week three all rates of stimulation led to a significant decrease in activity (figure 19A, two-way repeated measures ANOVAs, WT: $F_{\text{stimulation}(1,25)}=41.59$, $p<0.0001$; Math 5^{-/-}: $F_{\text{stimulation}(1,26)}=39.19$, $p<0.0001$). By the fourth postnatal week TRN activation greatly reduced relay neuron firing (WT: 11.26 ± 5.13 , Math 5^{-/-}: 25.42 ± 7.68), which was maintained throughout the sixth week (WT: 14.00 ± 4.81 , Math 5^{-/-}: 8.20 ± 3.35). Additionally, during the sixth postnatal week the difference between control and stimulation firing rates (WT: $12.62 \pm 1.01\text{Hz}$, Math 5^{-/-}: $15.91 \pm 1.21\text{Hz}$) approximated the mean control firing rate ($13.3 \pm 0.3\text{Hz}$), which suggests that TRN activation completely suppressed relay neuron activity.

While age had a large impact on TRN suppression of relay neurons, the stimulation rates utilized in this study had little impact on spike firing across all ages (table 3). Additionally, the absence of retinal inputs had no effect on TRN suppression of thalamocortical transmission (table 3). These findings are highlighted in figure 19B, which depicts the firing frequency for all individual neurons from the second postnatal week or the fourth postnatal week that received all three rates of blue light stimulation. This within-cell data illustrates a clear distinction between responses from week 2 (solid symbols) and week 4 (open symbols) in both WT (blue, n=13) and Math 5^{-/-} (red, n=15) neurons (two-way repeated measures ANOVAs, WT: $F_{\text{age}(1,11)}=85.27$, $p<0.0001$; Math 5^{-/-}: $F_{\text{age}(1,13)}=97.14$, $p<0.0001$). Additionally, there was no significant difference in firing rate between WT and Math 5^{-/-} animals either within the second postnatal week (two-way repeated measures ANOVA, $F_{\text{genotype}(1,11)}=0.4323$, $p=0.5244$) or the fourth postnatal week (two-way repeated measures ANOVA, $F_{\text{genotype}(1,13)}=2.433$, $p=0.1428$). However, while the temporal frequency of TRN activity had no effect on relay neuron firing during the second postnatal week (two-way repeated measures ANOVA, $F_{\text{frequency}(1.446, 15.91)}=0.5806$, $p=0.5178$, Geisser-Greenhouse's $\epsilon=0.7230$), frequency significantly impacted relay neuron activity during the fourth postnatal week (two-way repeated measures ANOVA, $F_{\text{frequency}(1.118, 14.54)}=19.65$, $p=0.0004$, Geisser-Greenhouse's $\epsilon=0.5591$). This suggests that TRN may inhibit thalamocortical transmission in a frequency-dependent manner but only in adult animals ($\geq P28$). Thus, the data from within-

cell measures demonstrates that the efficacy of TRN mediated inhibition increases with age.

Figure 4. Examples of mice used to investigate the assembly of feedforward excitatory (left, purple) and feedback inhibitory (right, green) circuits. In CRH-Cre x Ai9 mice (left), fluorescent labeling of dLGN thalamocortical neurons was apparent at birth (top) and thalamocortical axons coursed through TRN at this age. At later ages (P28, middle), thalamocortical projections in TRN gave off many axon collaterals with terminals that contain vesicular glutamate transporter 2 (vGluT2; blue). A high magnification of TRN at this age (bottom) highlights that vGluT2 puncta in TRN are smaller and more diffuse than labeled trigeminal

inputs to the ventrobasal complex (large blue puncta surrounding labeled neurons in bottom left). Fluorescent labeling of TRN neurons was observed at birth in GAD65 EGFP mice (right, top) and terminals were present just ventral and medial to dLGN. At later ages (P28, middle) TRN continued to exhibit dense somatic labeling (left) while labeled terminals filled dLGN. A high magnification image of dLGN at this age demonstrates the dense plexus of terminals covering the nucleus as well as the absence of any somatic labeling (bottom).

Figure 5. Characterization of sensory sectors of TRN in adult mice. Coronal sections from CRH-Cre x Ai9 mice depict thalamocortical (TC) labeling in first order sensory thalamic relay nuclei. **A-C.** tdTomato expression is shown in TC projections originating from ventral medial geniculate nucleus (vMGN, A), dLGN (B), and the ventrobasal complex (VB, B and C). **D.** NeuN staining in a coronal section of TRN illustrates cytoarchitectural borders and landmarks of the nucleus including its head, apex and tail. **E.** Representative coronal sections from three cases of Cre-dependent anterograde viral tracer (FLEX-AAV-ChIEF-tdTomato) injections into adult CRH-Cre mice. Viral tracer specifically labeled TC neurons in

either vMGN (top), VB (middle) or dLGN (bottom). **F.** Coronal sections of TRN from the same cases as in E, which illustrate the sensory-specific sectorial arrangement of TC projections to TRN. Injections into vMGN (top) and VB (middle) both labeled the tail of TRN below the apex but these projections were segregated in the rostral-caudal plane such that vMGN axons were caudal to those from VB. TC projections from dLGN (bottom) were only found in the head of TRN above the apex. **G.** Summary diagram illustrating the location of visual and non-visual sectors in the coronal plane of TRN. Since dLGN projections were restricted to the head of TRN, a horizontal line through the apex of TRN was used to identify visual TRN.

Figure 6. Identification of TRN sensory sectors in P2 mice. **A.** An image of the cortical surface of a P2 brain prior to sectioning demonstrates the specificity of CTB (blue) injected into visual cortex. Coronal sections from this case are shown in B and C. **B.** Retrograde labeling was observed in most thalamocortical (TC) neurons in dLGN (outlined). Some TC neurons in pulvinar were also labeled but no CTB was observed in VB (ventral to dLGN) or vMGN (not shown). **C.** In TRN, CTB-labeled projections from visual thalamus were restricted to the head of TRN. No CTB-labeling was observed below the apex. **D.** Prior to sectioning, the cortical surface of a P2 brain detailed the specificity of a CTB injection into somatosensory cortex (yellow). Coronal sections from this case are shown in E and F. **E.** Retrograde labeling of TC neurons was seen throughout much of VB, but no CTB was observed in dLGN (outlined). **F.** CTB-labeled TC projections

from VB were observed at and below the apex of TRN. Because labeled TC neurons in visual (B and C) and somatosensory (E and F) thalamus occupied distinct sectors of TRN, these tracing studies show that modality-specific sectors are present as early as P2.

Figure 7. Establishment of TRN sectors at P2 in the absence of retinal signaling.

A. The cortical surface of a P2 Math 5^{-/-} brain was imaged to assess the spread of a CTB injection (blue) into visual cortex. Coronal sections from this case are shown in B and C. **B.** Retrograde labeling of thalamocortical (TC) neurons was prevalent in dLGN (outlined) but absent in VB (ventral to dLGN) or vMGN (not shown). **C.** TC neurons from visual thalamus were segregated in the head of TRN, dorsal to the apex. **D.** The cortical surface of a P2 Math 5^{-/-} brain demonstrates a precise CTB injection (yellow) into somatosensory cortex. Coronal sections from this case are shown in E and F. **E.** TC neurons retrogradely labeled by CTB restricted to VB and no labeled neurons were observed in dLGN (outlined). **F.** In TRN, TC neurons from VB were confined to

territory at or ventral to the apex. No labeled fibers were observed dorsal to the apex or in the head of TRN.

Figure 8. Visualization of TC terminals in visual TRN using vesicular glutamate transporter 2 (vGluT2) immunohistochemistry. Example confocal images of a coronal section containing visual TRN from a P28 CRH-Cre x Ai9 mouse depict the pattern of immunohistochemical labeling of vGluT2 (blue) and NeuN (yellow).

Individual channels are isolated (top) and a combined image is shown (bottom). Cre-dependent tdTomato (purple) labeled TC neurons and their axons coursing through TRN (outlined). vGluT2 immunohistochemistry (blue) specifically labels TC terminals in TRN but also labeled trigeminal inputs to VB (refs). As expected, the vGluT2-positive terminals on TC axon collaterals in TRN appeared smaller than the large puncta observed on trigeminal terminals in VB. NeuN (yellow) is a marker of neuronal nuclei and was used to delineate the lateral boundary of TRN.

Figure 9. Formation of TC projections to TRN in the presence and absence of retinal input. Confocal images of coronal sections of tdTomato expression in age-matched WT (top) and Math 5^{-/-} (bottom) CRH-Cre x Ai9 mice. tdTomato-labeled TC axons were present within TRN at birth (P0, left), however a reticular organization was not apparent in TRN until P7 and emerged within the head of TRN by P14 (middle).

Figure 10. Emergence of TC axon collateral terminals in visual TRN in WT and Math 5^{-/-} mice. **A.** Example confocal images of coronal sections depict a progressive increase in vGluT2-positive TC terminals within visual TRN (black outline) in WT (top) and Math 5^{-/-} (bottom) mice between P7 and P21. In both WT and Math 5^{-/-}, at P7 vGluT2 labeling was absent (left) but puncta were apparent by P10 and increased in density so that the head of TRN was enveloped in TC terminals by P21. **B.** Summary plot depicting the spatial extent of TC axon collateral terminals in visual TRN as a function of postnatal age. Each data point represents the median ($\pm$ 95% confidence interval) percent of visual TRN area

covered by vGluT2 labeling in both WT (solid symbols, n=104 hemispheres) and Math 5^{-/-} (open symbols, n=72 hemispheres) mice. Values were derived from ≥ 8 hemispheres from ≥ 2 animals using 2-3 successive sections of visual TRN. The area covered by vGluT2 labeling increased significantly with age in both WT (Kruskal-Wallis: $p < 0.0001$) and Math 5^{-/-} (Kruskal-Wallis: $p < 0.0001$) mice but did not differ between genotypes (two-way ANOVA: $F_{\text{genotype}(1,164)} = 0.5503$, $p = 0.4593$; Tukey's multiple comparison test: $p > 0.05$ at each age). Moreover, a comparison of nonlinear fits determined that a model using a shared nonlinear curve was 90.08% more probable compared to a model using two separate nonlinear curves (Akaike's informative criteria, R^2 of single curve: 0.8948; Shapiro-Wilk normality test: $W = 0.9590$, $p < 0.0001$; homoscedasticity test: $p = 0.1846$).

Figure 11. Formation of TRN projections to dLGN in the presence and absence of retinal input. **A.** Coronal sections from early postnatal GAD65 mice containing middle regions of dLGN. Examples show the developmental time course of TRN innervation of dLGN both in the presence (WT, top) and absence (Math 5^{-/-}, bottom) of retinal input. **B.** Summary plot showing the percentage of dLGN area covered in fluorescent fibers as a function of postnatal age for both WT (solid symbols) and Math 5^{-/-} (open symbols). Each data point (green) is the median value of ≥ 3 hemispheres from ≥ 2 animals with error bars representing 95% confidence intervals. The median percent area of dLGN covered by TRN

terminals was significantly different from P0 – P3 (two-way ANOVA, $p < 0.0001$ at each age) but not at P4 ($p = 0.9989$). Nonlinear fits of WT and Math 5^{-/-} data determined that a model of two different curves for each data set was >99.99% more probable compared to a model of a single shared curve (Akaike's informative criteria, R^2 WT: 0.9274, Math 5^{-/-}: 0.7976; Shapiro-Wilk normality test WT: $p = 0.207$, Math 5^{-/-}: $p = 0.4040$; homoscedasticity test WT: $p = 0.0032$, Math 5^{-/-}: $p = 0.2015$).

Figure 12. Characterization of functional ChR2 expression at perinatal ages in feedforward and feedback circuits. **A.** Condensed wiring diagram illustrating somatic blue light stimulation of dLGN relay neurons. **B.** Examples of ChR2-EYFP expression at P5 in both TC soma (left) and terminals (right) of feedforward projections. **C.** Voltage clamp recordings from P6 ChR2-expressing dLGN neurons demonstrating light-evoked currents to both high rates of brief light pulses (50Hz, 1ms each, 20 pulses total) and a prolonged blue light flash (250ms). **D.** Condensed wiring diagram depicting somatic stimulation of TRN neurons by blue light. **E.** Examples of ChR2-EYFP expression in TRN soma (at

P2, left) and terminals within dLGN (at P6, right). **E.** Current clamp recording from P2 SST-Cre x ChR2 TRN neuron showing light-evoked depolarizations (top) and action potentials (bottom) evoked by brief flashes of blue light (1ms pulses, top; 5ms pulses, bottom).

Figure 13. Example voltage clamp traces from visual TRN neurons in WT and Math 5^{-/-} during whole cell patch clamp recordings. **A.** Condensed wiring diagram demonstrating blue-light stimulation of TC terminals within TRN. **B.** Example

traces increase in frequency from left to right (0.5Hz, 5Hz and 50Hz) and increase in age from top (P1-P7) to bottom (P22-P28). Within each age, examples are shown from both WT (top) and Math 5^{-/-} (bottom) mice. Cells were held at -70mV and presented with trains of blue light pulses (1ms each, n=10 for 0.5Hz, n=20 for 5Hz and 50Hz). Blank areas indicate that no cell at that age could follow a stimulus train at the given frequency (e.g. 5Hz in week 1).

Figure 14. Summary plots demonstrating the maturation of EPSC incidence, amplitude and paired pulse suppression in visual TRN neurons. **A.** Plot of the percent of responsive cells across early postnatal life in both WT (solid symbols, n=186) and Math 5^{-/-} (open symbols, n=114) animals. Each point represents data

from a minimum of 4 and a maximum of 39 cells (median=15). At no age was there a significant difference in the percentage of responsive cells between WT and Math 5^{-/-} (Fisher exact test, $p>0.05$). **B.** The amplitude of initial EPSC is plotted by postnatal week in WT (solid symbols, $n=96$) and Math 5^{-/-} (open symbols, $n=50$). Bars represent the mean amplitude ($\pm$ SEM) within each postnatal week. There was a significant increase in amplitude with age (two-way ANOVA, $F_{\text{age}(2,138)}=5.743$, $p=0.0040$) but no difference between WT and Math 5^{-/-} ($F_{\text{genotype}(1,138)}=0.2572$, $p=0.6128$). **C.** Synaptic suppression of light evoked EPSCs as a function of stimulus number. Percent EPSC amplitude to 1Hz stimulus train was calculated by dividing the n^{th} response by the initial response and multiplying by 100. Each point represents the mean and SEM of at least 12 neurons. Paired pulse suppression increased across the first few pulses (two-way ANOVA, $F_{\text{stimulus}(1.415, 167.0)}=637.8$, $p<0.0001$, Geisser-Greenhouse $\varepsilon=0.1572$) but stabilized by at least the 5th pulse ($p>0.05$). While suppression did not differ between WT (solid symbols) and Math 5^{-/-} (open symbols) at any age ($p>0.05$), synapses were more suppressed during the second postnatal week (red circles, $F_{\text{age}(2,39)}=11.98$, $p<0.0001$) than either week 3 (green squares, $p<0.0001$) or 4 (blue triangles, $p<0.0001$). **D.** Summary plot of synaptic suppression across postnatal weeks 2-4 within WT and Math 5^{-/-} mice. Trains of repeated blue light stimuli were presented to TRN neurons in either WT (solid symbols) or Math 5^{-/-} (open symbols) mice and the paired pulse ratio was calculated by dividing the 10th response by the 1st response. Six temporal frequencies of stimulation (0.25Hz – 50Hz) were tested and are grouped by color (lower frequencies = reds,

higher frequencies = purples). Each data point depicts the mean and SEM of at least 5 neurons. Both age and stimulus frequency had significant impacts on paired pulse suppression but there was no difference between WT and Math 5^{-/-} (see table 1).

Figure 15. Example voltage clamp traces from dLGN relay neurons in WT and Math 5^{-/-} during whole cell patch clamp recordings. **A.** Condensed wiring diagram depicting blue light photoactivation of TRN terminals within dLGN. **B.** Example traces increase in frequency from left to right (0.5Hz, 5Hz and 50Hz) and increase in age from top (P1-P7) to bottom (P29-P35). Within each age, examples are shown from both WT (top) and Math 5^{-/-} (bottom) mice. Cells were held at 0mV and presented with trains of blue light pulses (1ms each, n=10 for 0.5Hz, n=20 for 5Hz and 50Hz). Blank areas indicate that no cell at that age could follow a stimulus train at the given frequency (e.g. 5Hz in week 1).

Figure 16. Summary plots demonstrating the maturation of IPSC incidence, amplitude and paired pulse suppression in relay neurons. **A.** Plot of the percent of responsive cells across early postnatal life in both WT (solid symbols, n=352) and Math 5^{-/-} (open symbols, n=114) mice. Each point represents data from a

minimum of 7 and a maximum of 66 cells (median = 25). There was not a significant difference in the percentage of responsive cells at any age (Fisher exact test, $p > 0.05$). **B.** The amplitude of initial IPSC is plotted by postnatal week in WT (solid bars $n=64$) and Math 5^{-/-} (open bars, $n=102$). Bars represent mean amplitude ($\pm$ SEM) within each postnatal week. There was a significant increase in amplitude with age (two-way ANOVA, $F_{\text{age}(4,156)}=30.26$, $p < 0.0001$) but no difference between WT and Math 5^{-/-} ($F_{\text{genotype}(1,156)}=0.1095$, $p=0.7412$). **C.** Synaptic suppression of light evoked IPSCs as a function of stimulus number. Percent IPSC amplitude to 1Hz stimulus train was calculated by dividing the n^{th} response by the 1st response and multiplying by 100. Each point depicts the mean ($\pm$ SEM) of at least 7 neurons. Paired pulse suppression initially increased within a train of stimuli (two-way ANOVA, $F_{\text{stimulus}(1.103, 126.9)}=939.7$, $p < 0.0001$, Geisser-Greenhouse $\epsilon=0.1226$) but stabilized by the 3rd pulse (Tukey's multiple comparison test, 3rd pulse vs later pulses: $p > 0.05$). While suppression did not differ between WT (solid symbols) and Math 5^{-/-} (open symbols) at any age ($p > 0.05$), synapses were more suppressed during the second postnatal week (red circles, $F_{\text{age}(7,115)}=11.31$, $p < 0.0001$) compared to older ages (week 2 vs week 3, 4 or 5, $p < 0.0001$). **D.** Summary plot of synaptic suppression across postnatal weeks 1-5 within WT and Math 5^{-/-} mice. Relay neurons from either WT (solid symbols) or Math 5^{-/-} (open symbols) mice were stimulated with trains of blue light stimuli and the paired pulse ratio was calculated by dividing the 10th IPSC by the 1st IPSC. Trains at six temporal frequencies (0.25Hz – 50Hz) were used (plotted by color: low frequencies in reds, high frequencies in purples) and data

points represent the mean ($\pm$ SEM) from at least 4 neurons. Paired pulse suppression decreased with age, increased with higher temporal frequencies of stimulation, and was increased in Math 5^{-/-} mice but only with 5Hz and 10Hz stimulation (see table 2).

Figure 17. Examples of TRN-mediated feedback suppression of dLGN relay neuron firing. **A.** Condensed wiring diagram illustrating blue light stimulation of TRN terminals in dLGN. **B.** Examples of entire stimulation protocol at two ages (week 2: P14, and week 4: P28). The voltage responses of relay neurons (black) were measured during two injections of current (gray) that evoked a train of action potentials for 1500ms. During the second current injection a train of blue light stimuli (1ms pulses, blue) was applied for 500ms. **C.** Magnification of current injections from B. The rate of relay neuron firing in the absence of blue light

stimulation (control, left) was compared to the rate of firing during TRN photoactivation (stimulation, right). The firing frequency was measured during the middle 500ms of a 1500ms current injection (black bar, top) in order to calculate the percent change in firing frequency (stimulation / control * 100). The current injected was adjusted to evoke a control firing rate ≥ 5 Hz (mean current injected $\pm$ SEM: 161.6 ± 4.8 pA; mean control firing frequency $\pm$ SEM: 13.3 ± 0.3 Hz). Examples in B and C utilized a 50Hz temporal frequency stimulus in WT animals.

Figure 18. Examples demonstrating the maturation of TRN-mediated suppression of dLGN relay neuron firing in WT and Math 5^{-/-} mice. **A.** A train of action potentials (black) were evoked from dLGN relay neurons by a 1500ms current injection (gray). In the middle of this depolarization, a train of blue light pulses (1ms each, blue) at either 10Hz (left), 20Hz (not shown) or 50Hz (right) photoactivated TRN terminals. Examples from WT mice at early postnatal ages (weeks 1-6) depict that TRN activity during the first two postnatal weeks minimally impacted relay neuron firing whereas TRN suppressed firing at later ages (weeks 4-6). **B.** Similar to WT (above), examples from Math 5^{-/-} mice demonstrate that TRN had little influence on relay neuron firing at early ages but could suppress activity in older animals.

Figure 19. Summary plots of the suppression of relay neuron firing by TRN activation in both WT and Math 5^{-/-} mice. **A.** The percent change in firing frequency was calculated by comparing the firing rate of a relay neuron in the absence and presence of blue light and is plotted across postnatal age (weeks 1-6). Three different temporal frequencies of blue light stimulation were used: 10Hz

(WT: blue, Math 5^{-/-}: red), 20Hz (WT: cyan, Math 5^{-/-}: orange) and 50Hz (WT: green, Math 5^{-/-}: purple). TRN activation had little impact on relay neuron firing during the first two postnatal weeks but dramatically suppressed firing during the last two postnatal weeks (table 3). **B.** Similar plot for 28 neurons that were presented with all three stimulation rates (10Hz, 20Hz and 50Hz). This within-cell data illustrate that there was no difference between WT (blue circles) and Math 5^{-/-} (red squares) mice during either week 2 (solid symbols; two-way repeated measures ANOVA, $F_{\text{genotype}(1,11)}=0.4323$, $p=0.5244$) or week 4 (open symbols; two-way repeated measures ANOVA, $F_{\text{genotype}(1,13)}=2.433$, $p=0.1428$). Additionally, TRN photoactivation did not decrease relay neuron activity during week 2 (solid symbols and lines) but greatly reduced relay neuron firing during week 4 (open symbols and dashed lines; two-way repeated measures ANOVAs, WT: $F_{\text{age}(1,11)}=85.27$, $p<0.0001$; Math 5^{-/-}: $F_{\text{age}(1,13)}=97.14$, $p<0.0001$). The decrease in firing frequency during week 4 was dependent on stimulation frequency (two-way repeated measures ANOVA, $F_{\text{frequency}(1.118, 14.54)}=19.65$, $p=0.0004$, Geisser-Greenhouse's $\epsilon=0.5591$) as 10Hz photoactivation suppressed firing less than higher rates of activation (Tukey's multiple comparison test: $p<0.05$).

Figure 20. Summary timeline illustrating maturation of innervation between TRN and dLGN. **A.** Maturation of feedforward projections from dLGN to TRN. Top: Thalamocortical projections (purple) are present in TRN at birth but terminals on axon collaterals (blue) do not emerge until the second postnatal week. The number of axon collateral terminals gradually increases to a maximal state by the

fourth postnatal week. Bottom: Excitatory responses are absent during the first postnatal week and highly immature during the second postnatal week such that EPSCs are of low amplitude and are completely suppressed by high temporal frequency activity. By the fourth postnatal week evoked activity is of large amplitude and can follow stimulus trains as high as 50Hz. **B.** Formation of TRN feedback projections to dLGN. Top: TRN fibers (green) are present at the ventral-medial edge of dLGN at birth. During the first postnatal week fibers innervate dLGN and cover much of the nucleus. Middle: functional responses arise during the first postnatal week but are of low amplitude and cannot follow trains of stimuli greater than 0.5Hz. Responses continued to mature during postnatal weeks 2 and 3 so that by the 4th postnatal week evoked IPSCs were of a maximal amplitude and able to follow high temporal frequency activity (at least 50Hz). Evoked synaptic activity during the 4th postnatal week is indistinguishable from later ages. Bottom: TRN inhibition has no effect on retinogeniculate transmission during the first two postnatal weeks. During the third postnatal week, activation of TRN terminals decreases relay neuron firing but TRN cannot suppress retinogeniculate transmission until the fourth postnatal week.

A	0.25 Hz		0.5 Hz		1 Hz	
	F	p	F	p	F	p
Age	F (2, 109) = 2.630	P=0.0766	F (2, 134) = 25.26	P<0.0001	F (2, 118) = 10.67	P<0.0001
Genotype	F (1, 109) = 1.382	P=0.2423	F (1, 134) = 0.3646	P=0.5470	F (1, 118) = 0.02304	P=0.8796
Interaction	F (2, 109) = 0.2524	P=0.7774	F (2, 134) = 0.2786	P=0.7573	F (2, 118) = 2.056	P=0.1326

	5 Hz		10 Hz		50 Hz	
	F	p	F	p	F	p
Age	F (2, 106) = 4.608	P=0.0120	F (2, 80) = 1.638	P=0.2008	F (2, 71) = 0.6395	P=0.5306
Genotype	F (1, 106) = 0.4610	P=0.4986	F (1, 80) = 3.878	P=0.0524	F (1, 71) = 3.752	P=0.0567
Interaction	F (2, 106) = 1.186	P=0.3095	F (2, 80) = 1.850	P=0.1639	F (2, 71) = 6.308	P=0.0030

B	WT		Math 5^{-/-}	
	F	p	F	p
Age	F (2, 410) = 6.131	P=0.0024	F (2, 208) = 21.26	P<0.0001
Frequency	F (5, 410) = 92.47	P<0.0001	F (5, 208) = 115.1	P<0.0001
Interaction	F (10, 410) = 3.389	P=0.0003	F (10, 208) = 1.451	P=0.1600

Table 1. Analysis of synaptic suppression of feedforward thalamocortical synapses within TRN. **A.** Two-way ANOVAs were run to compare the effects of age and the presence of retinal signaling (genotype: WT vs Math 5^{-/-}) on synaptic suppression. Six separate analyses were run that compared the effects of postnatal age and genotype within one of six stimulation frequencies (0.25Hz, 0.5Hz, 1Hz, 5Hz, 10Hz, and 50Hz). Alpha was set to $p \leq 0.001$ to account for running multiple comparisons. These analyses show that postnatal age significantly influences synaptic suppression but there was no difference between WT and Math 5^{-/-} mice. **B.** Two additional two-way ANOVAs were run to compare all stimulation frequencies with age within either WT (left) or Math 5^{-/-} (right) mice.

A	0.25 Hz		0.5 Hz		1 Hz	
	F	p	F	p	F	p
Age	F (4, 86) = 15.10	P<0.0001	F (4, 151) = 15.82	P<0.0001	F (3, 116) = 30.18	P<0.0001
Genotype	F (1, 86) = 1.740	P=0.1906	F (1, 151) = 2.001	P=0.1592	F (1, 116) = 0.0737	P=0.7865
Interaction	F (4, 86) = 2.804	P=0.0306	F (4, 151) = 4.235	P=0.0028	F (3, 116) = 0.2631	P=0.8518

	5 Hz		10 Hz		50 Hz	
	F	p	F	p	F	p
Age	F (3, 96) = 14.87	P<0.0001	F (3, 89) = 11.90	P<0.0001	F (2, 48) = 14.86	P<0.0001
Genotype	F (1, 96) = 6.946	P=0.0098	F (1, 89) = 13.17	P=0.0005	F (1, 48) = 6.452	P=0.0144
Interaction	F (3, 96) = 1.838	P=0.1453	F (3, 89) = 0.9779	P=0.4069	F (2, 48) = 0.2813	P=0.7561

B	WT		Math 5^{-/-}	
	F	p	F	p
Age	F (3, 181) = 31.95	P<0.0001	F (3, 320) = 40.03	P<0.0001
Frequency	F (4, 181) = 87.98	P<0.0001	F (4, 320) = 181.3	P<0.0001
Interaction	F (12, 181) = 0.8933	P=0.5549	F (12, 320) = 1.900	P=0.0336

Table 2. Analysis of synaptic suppression of feedback TRN synapses within dLGN. **A.** The effects of postnatal age and retinal signaling (genotype: WT vs Math 5^{-/-}) on the paired pulse ratio were assessed by two-way ANOVAs. Six separate analyses were run that compared the effects of postnatal age and genotype within one of six stimulation frequencies (0.25Hz, 0.5Hz, 1Hz, 5Hz, 10Hz, and 50Hz). To account for running multiple comparisons, alpha was set to $p \leq 0.001$. Within each rate of stimulation, postnatal age significantly impacted synaptic suppression. However, at higher temporal frequency stimulation (5Hz and 10Hz), suppression was also significantly affected by the absence of retinal signaling. **B.** Two additional two-way ANOVAs were run to compare all stimulation frequencies with age within either WT (left) or Math 5^{-/-} (right) mice.

	10Hz and 20Hz		10Hz and 50Hz		20Hz and 50Hz	
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
Age	F (4, 155) = 72.49	P<0.0001	F (4, 178) = 63.23	P<0.0001	F (4, 153) = 39.33	P<0.0001
Genotype	F (1, 155) = 0.6766	P=0.4120	F (1, 178) = 0.1161	P=0.7337	F (1, 153) = 0.7014	P=0.4036
Frequency	F (1, 155) = 4.173	P=0.0428	F (1, 178) = 1.294	P=0.2569	F (1, 153) = 0.005789	P=0.9395
Age x Genotype	F (4, 155) = 4.379	P=0.0022	F (4, 178) = 2.550	P=0.0408	F (4, 153) = 1.008	P=0.4050
Age x Frequency	F (4, 155) = 0.6934	P=0.5976	F (4, 178) = 0.4126	P=0.7994	F (4, 153) = 0.4684	P=0.7589
Genotype x Frequency	F (1, 155) = 0.002810	P=0.9578	F (1, 178) = 1.416	P=0.2357	F (1, 153) = 0.06702	P=0.7961
Age x Genotype x Frequency	F (4, 155) = 0.2717	P=0.8959	F (4, 178) = 0.8946	P=0.4684	F (4, 153) = 0.1643	P=0.9561

Table 3. Analysis of TRN suppression of dLGN spike firing. Three-way ANOVAs were run to compare the effects of age, stimulation rates and the presence of retinal inputs (genotype: WT vs Math 5^{-/-}) on the suppression of relay neuron firing by TRN activation. TRN terminals were activated at three temporal frequencies: 10Hz, 20Hz and 50Hz. These stimulation rates were compared as pairs in three separate statistical tests: 10Hz vs 20Hz (left), 10Hz vs 50Hz (middle), and 20Hz vs 50Hz (right). Alpha was set to $p \leq 0.001$ to account for running multiple comparisons. These analyses show that only age, not stimulation rate nor the presence of retinal inputs, significantly influences relay neuron firing.

Discussion

Our retrograde tracing experiments reveal that thalamocortical axons pass through TRN in an orderly, modality-specific manner. As early as P2 there was a well-defined and non-overlapping arrangement that was similar to the organization we noted in adult mice, which is consistent with reports from other mammals (Crabtree, 2018; Guillery and Harting, 2003; Pinault, 2004). We found that the visual sector of TRN was dorsal to the apex of TRN and rostral to the majority of dLGN. The somatosensory sector was restricted to the body and tail of TRN at, or below, the apex. These results are consistent with previous studies using lipophilic dyes that showed TC and CT axons are topographically organized as they pass through TRN at embryonic ages, meet in the internal capsule at E14, and use each other for axonal guidance (Carlos and O'Leary, 1992; Garel et al., 2002; Grant et al., 2012; Hevner et al., 2002; López-Bendito, 2018; Molnár and Blakemore, 1995). Since TC axons pass through the TRN sectors that correspond to their sensory modality and because CT axons use TC projections for guidance (Grant et al., 2012; López-Bendito, 2018; Molnár and Blakemore, 1995), it is likely that CT projections are arranged in a similar manner in TRN but this has yet to be shown conclusively.

We also found that the absence of retinal input to the brain did not alter the sectorial arrangement of ascending TC projections through TRN. This finding is in agreement with work by Huffman and colleagues (Dye et al., 2012; Kozanian et al., 2015) showing that binocular enucleation failed to disrupt the sensory-specific modular organization of TRN. Together, these studies suggest

that the establishment of TRN organization is determined by intrinsic guidance cues that are unaffected by signaling from the periphery (López-Bendito, 2018; Montiel et al., 2011).

Our anatomical and electrophysiological experiments in strains that isolate the reciprocal connections between TRN and dLGN reveal that feedback projections from TRN to dLGN are established before feedforward thalamic projections to TRN. This sequence is summarized in figure 20. TRN terminals were present at the ventral-medial border of dLGN at birth and began to innervate the nucleus at P2 (figure 20B, green). While TRN terminals covered dLGN in a dense plexus of terminals by P7, functional synapses were immature at this age as evoked responses were of low amplitude and could not follow trains of stimuli at rates greater than 0.5Hz (figure 20B, upper traces). Synaptic responses continued to mature through the second and third postnatal week but by the fourth postnatal week evoked IPSCs were of large amplitude, exhibited less synaptic suppression at higher rates of stimulation (>10Hz; figure 20B, upper traces) and could completely suppress relay neuron firing (figure 20B, lower traces). Thus, feedback projections formed during the first postnatal week but functional synapses continued to mature until the fourth postnatal week.

In contrast, while TC axons passed through visual TRN at birth, we did not observe terminals from axon collaterals until P10 (figure 20A, purple). However, roughly half of visual TRN was covered by feedforward terminals by P14 and the entire area was innervated by P21. The formation of functional synapses followed a similar time course: synaptic responses were rare before P10 but

evoked activity was observed in over half of TRN neurons by P14 (figure 20A, traces). While peak amplitudes at P14 were small, both amplitude and the incidence of responses reached maximums by the third postnatal week (P21) and were consistent with values obtained at later ages.

Consequently, while feedforward innervation began after feedback, feedforward projections matured more quickly and stabilized earlier than those from TRN. Work by Colonnese and colleagues (Colonnese and Phillips, 2018; Murata and Colonnese, 2018a) suggests that just before eye opening a critical change in thalamocortical processing occurs as there is a shift in cortical activity from discontinuous to continuous. Moreover, they suggest that this inflection point is dependent upon the emergence of dLGN state-dependent activity. Our results indicate that the intrathalamic circuit between dLGN and TRN forms just before eye opening, which is contemporaneous to this developmental switch in cortical activity. Therefore, the establishment of intrathalamic circuits at early postnatal ages could mediate, at least in part, this developmental change in cortical activity.

While TRN feedback inhibition of dLGN is established prior to eye opening, other non-retinal inputs develop along a similar time course. Corticogeniculate axons reach dLGN between P3-4 and cover the nucleus by P12 while functional responses are present at P7 but are immature in that they exhibit little paired pulse facilitation (Seabrook et al., 2013a). Intrinsic interneurons also appear to migrate into dLGN during the first postnatal week (Charalambakis et al., 2019; Golding et al., 2014). However, in contrast to

feedback inhibition from TRN that emerges during the first postnatal week, interneuron-mediated feedforward inhibition is largely absent until well into the second postnatal week (Bickford et al., 2010). Terminals from brainstem cholinergic projections also arrive during the first postnatal week yet they emerge over a slower time course: they triple in density during the second postnatal week but do not attain a maximal level of innervation until the fourth postnatal week (Sokhadze et al., 2018a). Thus, all non-retinal inputs to dLGN emerge after retinal innervation, but TRN inputs are the first non-retinal terminals to arrive and the only ones studied to date that completely cover dLGN by the end of the first postnatal week.

Feedforward innervation of TRN is also established prior to eye opening but is still immature at this age. That innervation of TRN occurred after P7 was surprising as the same TC axons innervate L4 pyramidal neurons during the first postnatal week, express vGluT2 at early postnatal ages, and can propagate retinal waves to cortex as early as P6 (Ackman et al., 2012; Kast and Levitt, 2019; Louderback et al., 2006; Schlaggar and O'Leary, 1994). However, the time course of feedforward innervation was similar to the establishment of cholinergic projections to visual TRN (Sokhadze et al., 2018b). However, cholinergic innervation follows a ventral-dorsal pattern as terminals arrive in somatosensory TRN prior to visual sectors. Cortical innervation of dorsal thalamus follows a similar pattern where somatosensory nuclei are innervated prior to dLGN (Grant et al., 2012; Jacobs et al., 2007; López-Bendito, 2018; Montiel et al., 2011), but it

is still unknown when CT collaterals innervate TRN and whether they follow a pattern similar to cholinergic or thalamic inputs.

We also investigated what impact the absence of retinal signaling had on intrathalamic circuit formation. We show that the loss of retinal inputs leads to acceleration in dLGN innervation by TRN projections. While the innervation of dLGN by other non-retinal inputs is also disrupted by the absence of retinal signaling (interneurons: Charalambakis et al., 2019; Golding et al., 2014; cholinergic: Sokhadze et al., 2018a), our results are most similar to those from cortex. In *Math 5^{-/-}* mice, corticogeniculate innervation is accelerated such that CT terminals arrive two days earlier than in WT (Seabrook et al., 2013a). This acceleration was found to be mediated by the early degradation of aggrecan (Brooks et al., 2013), which normally repels CT innervation of dLGN. It is unclear if aggrecan also repels TRN terminals or the timing of feedback innervation is controlled by another molecule. However, as both TRN and CT terminals are accelerated in the absence of retinal signaling, and since both inputs are accelerated to a similar degree, it is likely that a similar mechanism regulates the timing of innervation for both inputs.

In *Math 5^{-/-}* mice, we also observed greater paired pulse suppression at higher rates of stimulation and in older animals (>10Hz, >P21). Previous work has shown that the intrinsic membrane properties of relay neurons are altered in the absence of retinal signaling as *Math 5^{-/-}* mice exhibit higher input resistance and lower decay times compared to WT (El-Danaf et al., 2015). However, while only the first two postnatal weeks were compared, corticogeniculate synapses in

Math 5^{-/-} mice did not differ in paired pulse facilitation (Seabrook et al., 2013a). Thus, further studies are needed to determine if and how TRN and CT feedback is balanced.

In contrast to the organization of dLGN, the absence of retinal signaling had no effect on either the arrangement of TRN sectors or the innervation by TC axon collaterals. Previous studies using lipophilic dyes suggested that TC and CT projections were unaltered in enucleated mice but had not investigated these projections in TRN (Dye et al., 2012; Kozanian et al., 2015). Although the organization of thalamic input to cortex is disrupted in the absence of peripheral signaling (Chabot et al., 2007; Dooley and Krubitzer, 2018; Karlen and Krubitzer, 2009; Kupers and Ptito, 2014), we likely did not observe a similar change in TRN because TC and CT axons pass through TRN well before retinal axons arrive in dLGN (Grant et al., 2012; Guido, 2018; Jaubert-Miazza et al., 2005; Molnár and Blakemore, 1995; Molnár et al., 2012). Thus, the loss of sensory input affects TC organization in cortex but not necessarily the arrangement of TRN axon collaterals or their function.

In conclusion, the experiments presented here detail the formation of the intra-thalamic circuit connecting dLGN and TRN. We demonstrate that the visual sector of TRN is anatomically distinct just after birth and that feedforward innervation from dLGN to TRN begins at the end of the first postnatal week, reaching a mature state two weeks later. Feedback inhibition from TRN to dLGN begins at P2 and although terminals cover dLGN by the end of the first postnatal week, functional responses do not mature for three more weeks (P28) when TRN

was first able to completely suppress relay neuron firing. We also show that in the absence of retinal signaling, the formation of much of this circuitry follows a developmental time course that is not altered from WT. Although TRN terminals arrived in dLGN at earlier postnatal ages and exhibited more paired pulse suppression at higher rates of stimulation, no other component of the entire intrathalamic loop was disrupted by the removal of retinal inputs. Together these studies provide a foundation that can be used to investigate whether neurodevelopmental diseases that recruit this network alter the normal developmental pattern that is established here.

CHAPTER II

SYNAPTIC PROPERTIES OF THE FEEDBACK CONNECTIONS BETWEEN THE THALAMIC RETICULAR NUCLEUS AND THE DORSAL LATERAL GENICULATE NUCLEUS

Introduction

The thalamic reticular nucleus (TRN) is an important interface between thalamus and cortex, playing a key role in the attentional modulation of sensory processing, the generation and propagation of thalamocortical rhythms during sleep and wakefulness, and the prevention of hypersynchronous oscillations associated with certain disease states (Crick, 1984; Fogerson and Huguenard, 2016; Halassa and Acsády, 2016; McCormick and Bal, 1997; Pinault, 2004; Steriade, 2005). Comprised entirely of GABAergic neurons, the TRN surrounds much of the dorsal thalamus (Houser et al., 1980; Jones, 2007). This nucleus receives excitatory drive from both thalamocortical and corticothalamic axon collaterals and in return, TRN neurons project to thalamic nuclei to inhibit activity in a state dependent manner (Figure 21; Guillery and Harting, 2003; Guillery et al., 1998; Halassa and Acsády, 2016; Halassa and Kastner, 2017; Pinault, 2004).

TRN mediated inhibition and its impact on sensory processing is perhaps best understood by examining the connections between TRN and the dorsal lateral geniculate nucleus (dLGN), the thalamic relay of retinal signals to the visual cortex (Cox and Beatty, 2017; Guillery and Sherman, 2002; Hirsch et al., 2015; Usrey and Alitto, 2015). Although the circuits linking TRN to dLGN has been the subject of intense investigation, what remains unclear is how variable rates of TRN activity affect retinogeniculate signal transmission. While many studies have focused on oscillatory activity and burst firing in TRN (Fogerson and Huguenard, 2016; McCormick and Bal, 1997; McCormick et al., 2015; Pinault, 2004; Steriade, 2005), the majority of neurons within the visual sector of TRN do not fire in bursts (Huh and Cho, 2016; Lee et al., 2007). Moreover, sensory transmission occurs in the awake state, when burst firing is rare, and tonic firing prevails (Bezudnaya et al., 2006; Ramcharan et al., 2000, 2005; Swadlow and Gusev, 2001; Weyand et al., 2001). Indeed, tonic firing rates of TRN neurons vary widely during wakefulness (Chen et al., 2016b; Contreras et al., 1993; Domich et al., 1986; Hartings et al., 2003; Lymer et al., 2019; Pinault et al., 2001). Nonetheless, TRN-mediated inhibition is generally thought to operate in a binary manner, selectively gating thalamocortical transmission in an all-or-none fashion (Crick, 1984; Guillery and Sherman, 2002; Halassa and Acsády, 2016; Llinás and Steriade, 2006; Pinault, 2004). While it is clear that TRN activation can lead to a complete suppression of thalamocortical transmission, the bulk of the evidence supporting this view is restricted to manipulations that involve a single, or a limited range of repetitive electrical stimulation (Crandall et al., 2015;

Kayama, 1985; Kim et al., 1997; Olsen et al., 2012; Shosaku and Sumitomo, 1983; Shosaku et al., 1984, 1989; Thomson, 1988; Yingling and Skinner, 1976; but see Herd et al., 2013). Thus, while different behavioral states alter both TRN and dLGN activity, it is currently unclear how different rates of TRN firing impact retinogeniculate transmission.

The dLGN of the mouse offers a unique opportunity to explore this issue since the circuitry linking TRN to dLGN is highly conserved and it contains both thalamocortical neurons as well as intrinsic interneurons (Guido, 2018). Here we addressed how variable rates of TRN activation influence the degree of dLGN activity by utilizing a variety of transgenic mouse models that allow for the visualization and manipulation of TRN input to dLGN. We made use of a transgenic line that expresses GFP in TRN neurons to delineate TRN synaptic profiles in dLGN (GAD65; Jurgens et al., 2012; López-Bendito et al., 2004). Additionally, we adopted an optogenetic approach by expressing channelrhodopsin 2 (ChR2), or one of its variants (ChIEF), into TRN terminals, in order to selectively activate postsynaptic inhibitory activity in dLGN neurons. Finally, the nature of TRN inhibitory activity was assessed in vitro by using an acute thalamic slice preparation, as well as in vivo by utilizing an awake, behaving head fixed mouse preparation.

Materials and Methods

Subjects

Experiments were conducted in adult mice (>P21) of either sex. We used C57BL/6J (Jax stock no. 00064) mice as well as two transgenic strains that express green fluorescent protein (GFP) in GABAergic neurons. The GAD67-GFP (line G42; Jax stock no. 007677) labels dLGN interneurons (Charalambakis et al., 2019; Golding et al., 2014; Jager et al., 2016; Leist et al., 2016; Seabrook et al., 2013b) and a GAD65-GFP strain that labels TRN neurons (Jurgens et al., 2012; López-Bendito et al., 2004). We also crossed somatostatin-cre (SST-Cre knock-in mouse; Jax stock no. 013044) with Ai32 mice (floxed-ChR2 and EYFP; Jax stock no. 012569) and used the offspring to examine the postsynaptic responses of dLGN neurons evoked by blue light stimulation of TRN terminals (Ahrens et al., 2014; Clemente-Perez et al., 2017; Klug et al., 2018; Wells et al., 2016). All breeding and experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

Electron Microscopy

We used GAD65-GFP mice to study the ultrastructure of TRN terminals in dLGN. Adult mice were deeply anesthetized with Avertin (0.5mg/g) and perfused transcardially with a fixative solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (PB). The brain was removed from the skull and 70 μ m thick coronal sections were cut using a vibratome (Leica Microsystems, Buffalo Grove, IL). Selected sections that contained GFP were

incubated overnight in a 1:1000 dilution (0.1µg/ml) of a rabbit anti-GFP antibody in PB with 1% normal goat serum (Millipore, Billerica, MA, catalog #AB3080, RRID:AB 91337). The following day, the sections were incubated in a 1:100 dilution of a biotinylated goat-anti-rabbit antibody (Vector Laboratories, Burlingame, CA, 1 hour), followed by incubation in a solution of avidin and biotinylated horseradish peroxidase (ABC solution, Vector Laboratories, 1 hour), and reacted with nickel-enhanced diaminobenzidine (DAB).

Sections that contained terminals labeled with the GFP antibody were postfixed in 2% osmium tetroxide, dehydrated in an ethyl alcohol series, and flat embedded in Durcupan resin between two sheets of Aclar plastic (Ladd Research, Williston, VT). Durcupan-embedded sections were first examined with a light microscope to select areas for electron microscopic analysis. Selected areas were mounted on blocks, ultrathin sections (70-80nm, silver-gray interference color) were cut using a diamond knife, and sections were collected on Formvar-coated nickel slot grids. Selected sections were stained for the presence of gamma amino butyric acid (GABA) by utilizing a postembedding immunocytochemical protocol described previously (Bickford et al., 2010, 2015). Briefly, we used a 0.25µg/ml concentration of a rabbit polyclonal antibody against GABA (Sigma-Aldrich, St. Louis, MO, catalog #A2052, RRID:AB_477652) and the GABA antibody was tagged with a goat-anti-rabbit antibody conjugated to 15-nm gold particles (BBI Solutions USA, Madison, WI). The sections were air dried and stained with a 10% solution of uranyl acetate in methanol for 30 minutes. Ultrathin sections were examined with a transmission electron microscope

(Hitachi HT7700, Hitachi High Technologies, Clarksburg, MD) and the synaptic profiles containing labeled terminals were imaged. The diameters of pre- and postsynaptic profiles were measured (Image J, RRID: SCR_003070) and gold particles were counted to calculate the gold particle density overlying each profile.

AAV Injections

An adeno-associated virus (AAV) containing a ChIEF-tdTomato cassette (Jurgens et al., 2012) was injected into the TRN of adult C57BL/6J or GAD67 mice using methods described previously (Bickford et al., 2015; Jurgens et al., 2012; Sokhadze et al., 2018b). Adult mice were deeply anesthetized with a ketamine/xylazine (8mg/0.6mg/mL; 0.01mL/g) and 50nL of AAV-ChIEF-tdTomato was injected into TRN (10nL/min) using a Hamilton syringe attached to a nanopump. After surgery, animals were closely monitored for three days to assess wound healing and given carprofen (1.25mg/mL; 0.1mL/25g SC) if they displayed signs of pain or distress. Between 10-14 days after injection, mice were euthanized, and brain tissue was harvested for in vitro slice recordings.

In Vitro Slice Preparation And Optogenetic Stimulation

Brain slices were made from mice that were deeply anesthetized with isoflurane and rapidly decapitated using methods described previously (Charalambakis et al., 2019; Seabrook et al., 2013a). The brain was rapidly removed and placed into 4°C oxygenated cutting solution (in mM): Sucrose 234, glucose 11, NaHCO₃ 26, MgSO₄ 10, KCl 2.5, CaCl₂ 0.5, NaH₂PO₄ 1.25.

270 μ m-thick thalamic slices were made using a vibratome (Leica). For 30 minutes, slices were incubated in warm (32°C) oxygenated aCSF (in mM): NaCl 126, NaHCO₃ 26, glucose 10, KCl 2.5, MgCl₂ 2, CaCl₂ 2, NaH₂PO₄ 1.25. Recordings were conducted in a chamber perfused continuously with 32°C ACSF at a rate of 2-3mL/min.

Thalamic nuclei were visualized on an upright microscope (Olympus BX51WI) with DIC optics and fluorescent filters (GFP: Chroma 49002; tdTomato: Chroma 49005) using a 10x or 60x water immersion objective. A vertical puller (Narashige PC-10) was used to pull patch electrodes from borosilicate glass. For current clamp recordings, the electrode internal solution contained (in mM) 117 K-gluconate, 13 KCl, 1.0 MgCl₂, 0.07 CaCl₂, 0.1 EGTA, 0.1, 10 HEPES. For voltage clamp recordings, the electrode solution contained (in mM) 117 Cs-gluconate, 11.0 CsCl, 1.0 MgCl₂, 1.0 CaCl₂, 0.1 EGTA, 10.0 HEPES, 2 Na₂-ATP, 0.4 Na₂-GTP. The final tip resistance of all electrodes was 4-7 M Ω .

Whole cell recordings were made in current and voltage clamp using an amplifier (Multiclamp 700B, Molecular Devices), filtered at 3-10kHz, and digitized (Digidata 1440A) at 20kHz. Pipette capacitance, series resistance, input resistance, and whole-cell capacitance were monitored throughout the recording session.

The light-gated responses were activated using a light emitting diode (LED, Prizmatix) that delivered blue light through the 60x objective. Blue light pulses of varying duration were 0.3mm in diameter with a light power of

525mW/mm². During some of the recordings, antagonists for AMPA (DNQX, 20μM, Tocris) and NMDA (CPP, 10mM Tocris) or GABA_A receptors (SR95531, 20μM tocris) were bath applied to block postsynaptic activity.

For all recordings, biocytin (0.5%, Sigma) was included in the internal solution for intracellular filling and 3-D neuron reconstruction using confocal microscopy (Charalambakis et al., 2019; El-Danaf et al., 2015; Krahe et al., 2011). Following the completion of the recording session, slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), then washed with PBS and incubated overnight with AlexaFluor 647 conjugated streptavidin (Invitrogen, S21374) in a PBS solution containing Triton X-100 (0.1%). Slices were subsequently mounted with Prolong Gold with DAPI (Invitrogen, P36931) and cover slipped. Digital images of biocytin filled neurons were acquired using a multiphoton laser scanning confocal microscope (Olympus FV1200BX61). A HeNe laser (635nm) was used to excite far-red, biocytin fluorescence. Neurons were imaged using a 10x (0.3 NA) or 20x (0.75 NA) objective lens at a scanning resolution of 1600x1600 pixels. A sequential series of optical slices with an optimal step size through the Z-axis of 1.26μm (20x/0.75 NA lens) created a 3-D, Z-stacked dataset. Three-dimensional neuron reconstructions were compiled, and dendritic architecture was assessed to determine whether the labeled neuron was a relay cell or interneuron (Charalambakis et al., 2019; El-Danaf et al., 2015; Krahe et al., 2011).

In Vitro Assessment Of Postsynaptic Activity In dLGN

We measured the inhibitory postsynaptic activity of dLGN neurons evoked by blue light stimulation of TRN terminals. For inhibitory postsynaptic currents (IPSCs), neurons were held at 0mV using a cesium-based electrode and presented with a train of twenty pulses of blue light (1ms each) at different temporal frequencies (0.2, 1, 5, 20 and 50Hz). The amplitude of IPSCs was measured from baseline levels obtained 1s prior to photostimulation. To quantify any changes in IPSC activity evoked by repetitive stimulation, the amplitude of the n^{th} response was divided by the amplitude of the initial response, and then multiplied by 100 to calculate the percent change (Jurgens et al., 2012). To measure the voltage changes associated with repetitive stimulation of TRN terminals, dLGN neurons were recorded at -55mV in current clamp using potassium-based electrodes. Stimulus trains consisted of 25 pulses of blue light (1ms duration) presented at different temporal frequencies (2, 5, 10, 20, and 50Hz). The mean voltage change was calculated during blue light stimulation and compared to a 1s baseline period. To examine how TRN stimulation affects spiking activity, dLGN neurons were held at -70mV and given a 1.5s square wave depolarizing current pulse (mean $\pm$ SEM = 175.8 ± 9.4 pA) of sufficient strength to evoke a steady train of spike firing > 5Hz. Changes in spike firing were then calculated by comparing equivalent periods (0.5s) of activity in the presence or absence of blue light stimulation (2, 5, 10, 20 and 50Hz). In order to minimize the effects of frequency accommodation or burst firing, measures of spike firing were obtained 0.5s after the onset of the 1.5s current pulse (Figure

27A). Typically, the measurements described above were based on the average of 4 stimulus presentations.

In Vivo Recordings In Awake Head Fixed Mice And Optogenetic Stimulation

SST-Cre x ChR2 mice were prepared for in vivo recordings using methods similar to Tschetter et al. (2018). Mice were initially anesthetized and maintained with an IP injection of ketamine (150mg/kg) and xylazine (15mg/kg), and if needed, supplemented with 25% of the initial dose. The mice were then fitted with a custom head plate that was used to stabilize the head in a stereotaxic device, and a craniotomy was made to permit subsequent electrode penetration through dLGN. The craniotomy was sealed with silicone (Kwik-Cast) and mice were allowed to recover. After surgery, mice were given an analgesic (meloxicam, 5mg/kg) and monitored for 48hrs for any signs of discomfort or distress.

Two to four days after the initial surgery, mice were secured to a head fixed frame and acclimated to run on a Styrofoam ball. The silicone plug was removed, and the cortical surface was covered in a thin layer of agarose/ACSF to prevent drying. An optrode consisting of an insulated tungsten microelectrode (2-4M Ω) glued to a fiber optic cable (100 μ m diameter) was lowered into the brain using coordinates that target dLGN (bregma -2.25 ap, 2.25 ml). At the end of a recording session (45-90 min), the mice were deeply anesthetized, perfused transcardially with a fixative solution, and the brain excised for electrode penetration reconstruction. The fiber optic was connected to a 473nm blue-light

laser (Laserglow). The light intensity was 20mW/mm², and the duration and frequency of blue light pulses were under computer control.

During a recording session, multiunit activity was acquired using an extracellular amplifier (TDT BioAmp). The signal was amplified by 2000X, bandpass filtered at 300-2000Hz, and sampled at 20kHz. Single units were identified by spike waveform analysis using conventional protocols developed by Spike2 software. Units were determined to be in dLGN based on their dorsal-ventral coordinates and electrode tract reconstruction.

In Vivo Assessment Of dLGN Unit Activity

To examine how TRN stimulation affects dLGN spiking activity, units that were spontaneously active and stable were stimulated with blue light pulses (5ms) presented at different temporal frequencies (5, 20 and 50Hz). Changes in unit activity were then calculated by comparing the activity during equivalent periods just before (baseline), and during blue light stimulation (5Hz: 2 sec; 20 and 50Hz: 0.5 sec). Baseline unit activity was not significantly different between the three temporal stimulus frequencies used (mean $\pm$ SEM = 30.6 $\pm$ 4.4Hz, one-way ANOVA, $F_{(2,52)}=2.588$, $p=0.0849$). Typically, the measurements described above were based on the average of 5 stimulus presentations.

Statistical Analyses

All statistical tests were performed using Prism 8.0 (Graphpad Software, La Jolla, CA). Parametric statistical tests were used to analyze gold particle density, IPSC amplitude, and mean voltage. We used both Tukey's (one-way

ANOVA) or Dunnett's (two-way ANOVA) methods for correcting for multiple comparisons during post-hoc tests. Non-parametric tests (Kruskal-Wallis and Friedman) were used to compare terminal size and percent changes in spike firing. Post-hoc tests for these analyses employed a Dunn's correction for multiple comparisons.

Results

TRN terminals in the dLGN

To examine the projections from TRN to dLGN, we utilized GAD65-GFP mice (Jurgens et al., 2012; López-Bendito et al., 2004). The GAD65-GFP mouse line exhibits robust labeling of TRN neurons (Figure 22A) and their terminals in the dLGN (Figure 22B). There is no evidence of somatic labeling in the dLGN (Figure 22B) or pretectum (not shown), indicating that in this transgenic strain, the GFP terminal labeling in dLGN arises from the TRN.

To examine the nature of TRN contacts within the dLGN, we prepared GAD65-GFP tissue for electron microscopy, which was conducted by the Bickford lab. Figure 22C illustrates a TRN terminal (green), labeled with a GFP antibody (DAB reaction) as well as a GABA antibody (gold particles). This terminal forms a synapse (arrows) with a non-GABAergic (presumed relay neuron) dendrite (blue). We sampled 10 ultrathin sections through the dLGN of an adult GAD65-GFP mouse and detected a total of 72 TRN terminals that were labeled with DAB and numerous gold particles (≥ 40 particles per μm^2 ; mean density $\pm$ SEM: 105.9 ± 3.60). All sampled TRN terminals formed synapses with non-GABAergic postsynaptic profiles (mean density of overlying gold particles $\pm$ SEM: 4.42 ± 0.36 particles/ μm^2). The profiles postsynaptic to TRN terminals were large dendrites or somata (range: $0.18 - 12.35\mu\text{m}^2$; mean: $1.56 \pm 0.31\mu\text{m}^2$). Thus, GABAergic TRN terminals formed synapses with the somata and proximal dendrites of non-GABAergic relay neurons.

To compare the size and dendritic location of TRN terminals relative to other presynaptic terminals in the dLGN, we used data from a previous study (Bickford et al., 2015) in which we quantified the size of retinogeniculate (n = 179), corticogeniculate (n = 87) and tectogeniculate terminals (n = 108) along with the size of their postsynaptic profiles. Figure 22D shows that TRN terminals (n = 72, green) were significantly larger than corticogeniculate (CG, blue) and tectogeniculate (TG, black) terminals (Kruskal-Wallis, CG: $p < 0.0001$; TG: $p = 0.0009$), but smaller than retinogeniculate terminals (RG, red; $p < 0.0001$). Figure 22E demonstrates that the diameter of profiles postsynaptic to TRN terminals (n = 72) was similar to the diameter of profiles postsynaptic to retinogeniculate (n = 179) and tectogeniculate (n = 108) terminals (Kruskal-Wallis, RG: $p = 0.6902$; TG $p = 0.1818$) and larger than profiles postsynaptic to corticogeniculate terminals ($p = 0.0003$). Thus, TRN terminals contact large diameter postsynaptic elements, suggesting they innervate more proximal regions of dLGN relay neurons (Bickford et al., 2010, 2015).

Expression of Opsins in TRN Neurons

To examine the nature of postsynaptic activity evoked by the TRN, we adopted an optogenetic approach. First, we (B. Osteen and W. Guido) injected an AAV viral vector into the TRN (Figure 23A) to express the channelrhodopsin variant CHIEF-tdTomato in TRN neurons (Bickford et al., 2015; Jurgens et al., 2012). In a second approach, we crossed somatostatin-Cre mice with the Ai32 line in order to target ChR2-EYFP in Cre-expressing neurons within TRN (Figure 23D; Ahrens et al., 2014; Clemente-Perez et al., 2017; Klug et al., 2018; Wells et

al., 2016). To confirm that both approaches could activate TRN neurons, we (G. Govindaiah) conducted whole cell recordings using biocytin-filled pipettes in the TRN. A representative TRN neuron is reconstructed from a ChIEF-injected (Figure 23B) and SST-Cre x ChR2 mouse (Figure 23E). In ChIEF-injected animals, long single pulses (500ms) of blue light evoked a sustained depolarization that led to spike firing (Figure 23C). Furthermore, in both mice, repetitive stimulation with brief pulses (1ms) were sufficient to evoke a train of depolarizations with spikes riding the peaks (Figure 3C: 10Hz; Figure 23F: 20 and 50Hz). Figure 23C illustrates that this light-evoked activity was unaffected by the bath application of ionotropic glutamate blockers (20 μ M DNQX and 10mM CPP). Thus, when activated both opsins reliably evoked spike firing in TRN neurons.

Optogenetic Activation Of TRN Terminals In dLGN

Visualized, in vitro whole cell recordings were obtained from 60 relay neurons and 7 interneurons of dLGN. Relay neurons were identified by their large round somata, voltage responses to brief injections of current, and their dendritic architecture based on biocytin-filled reconstructions (n = 40, figure 24A; Krahe et al., 2011). Interneurons were targeted using the GAD67-GFP transgenic mouse, which labels intrinsic interneurons (figure 24D; Charalambakis et al., 2019; Golding et al., 2014; Jager et al., 2016; Leist et al., 2016; Seabrook et al., 2013b). These had fusiform-shaped somata labeled with GFP and highly expansive dendritic trees that emanated from opposite poles of their cell bodies (Charalambakis et al., 2019; Seabrook et al., 2013b). To investigate TRN-

mediated inhibition of dLGN neurons, we (G. Govindaiah) conducted voltage clamp recordings using a cesium-based internal solution while holding the neuron at 0mV (Charalambakis et al., 2019; Govindaiah and Cox, 2004, 2006). As shown in figure 24B, repetitive blue light stimulation of TRN terminals evoked large inhibitory postsynaptic currents (IPSCs) in relay neurons ($n = 8$), and when temporal rates ≥ 5 Hz were used, the amplitude of IPSCs began to attenuate after the initial pulse (i.e., synaptic depression). These IPSCs were unaffected by bath application of glutamate blockers (20 μ M DNQX and 10mM CPP) but were inhibited by the GABA_A antagonist SR95531 (20 μ M figure 24C). By contrast, similar patterns of stimulation failed to evoke any postsynaptic activity in interneurons ($n = 7$, figure 24E). Together, these data demonstrate that TRN terminal activation inhibits relay neurons but not intrinsic interneurons of dLGN.

To quantify the degree of synaptic depression noted among relay neurons, we presented trains of blue light pulses (1ms) at different temporal frequencies (0.2, 1, 5, 20 and 50Hz). An example of light evoked responses to repetitive stimulation is shown in figure 25A. At low temporal frequencies (0.2 and 1Hz) TRN terminal activation triggered large and relatively stable IPSCs for each pulse of the stimulus train. However, higher rates of stimulation (≥ 5 Hz) led to a form of synaptic depression, with the initial response evoking the maximal amplitude and subsequent responses showing a progressive decrease in amplitude that stabilized after the 5th pulse. A summary of these interactions is shown in figure 25B ($n = 8$). The initial response to all stimulus trains produced large IPSC activity ($n = 40$ IPSCs, mean $\pm$ SEM: 736.6 ± 62.0 pA). At low rates of stimulation

(0.2 and 1Hz) little if any change in IPSC amplitude occurred throughout the entire stimulus train. However, frequencies of 5, 20 and 50Hz showed a progressive decline in IPSC amplitude between the 2-5th pulse (two-way repeated measures ANOVA, $F_{(19,285)} = 98.67$, $p < 0.0001$). These changes stabilized by the 5th pulse whereby the overall magnitude of inhibition was on average 47-56% of the maximal response evoked by the initial pulse. Thus, TRN terminal activation led to large amplitude IPSC activity, that began to attenuate in response to repetitive stimulation at temporal frequencies ≥ 5 Hz.

To further explore the effects of repetitive TRN stimulation on dLGN relay neuron activity we recorded synaptic responses in current clamp mode using potassium-based electrodes and measured the mean change in the membrane potential. An example of TRN mediated inhibitory postsynaptic potentials (IPSPs) is shown in figure 26A. Blue light stimulation evoked large IPSPs that diminished in amplitude when repeated stimuli were presented at rates ≥ 5 Hz. As expected, these responses were blocked during the bath application of the GABA_A antagonist, SR95531 (20 μ M). Nonetheless, high rates of stimulation (10, 20 and 50Hz) still resulted in larger and more sustained levels of hyperpolarization compared to lower rates of stimulation (2 and 5 Hz). Figure 26B summarizes these interactions for 35 dLGN relay neurons. For each neuron the mean hyperpolarizing voltage response evoked by a stimulus train is plotted as a function of the temporal frequency. We found that a progressive increase in temporal frequency led to significantly higher levels of membrane hyperpolarization (one-way ANOVA $F_{(4, 71)} = 19.50$, $p < 0.0001$). Stimulation at 20

and 50Hz led to larger changes than 2 or 5Hz (mean $\pm$ SEM = 2Hz: 0.52 ± 0.15 mV; 5Hz: 1.09 ± 0.09 ; 20Hz: 2.39 ± 0.24 mV; 50Hz: 4.03 ± 0.39 mV; $p < 0.01$).

Overall, these data suggest that the TRN mediated inhibition exhibits a form of synaptic depression at high rates of stimulation (figures 25-26), it remains sufficient to strongly hyperpolarize dLGN relay neurons. Moreover, the overall impact of TRN inhibition is far greater at higher stimulation rates (figure 26).

Next, we explored how different rates of TRN stimulation affect the spike activity of dLGN neurons. To address this, we conducted in vitro dLGN recordings in current clamp mode, and stimulated TRN terminals at different rates while injecting a depolarizing current pulse that triggered a steady train of spike firing (mean $\pm$ SEM $17.5 \text{ Hz} \pm 0.72\text{Hz}$). An example is shown in figure 27A in which a square wave current pulse evoked tonic firing before (control, left) and during (right) blue light TRN terminal stimulation. While both low and high rates of TRN stimulation led to decreased firing, stimulation rates of 20 and 50Hz often led to a complete suppression of spike firing (in 9/29 neurons). This pattern is summarized in figure 27B for 29 relay neurons which plots firing frequency as a percent change of control recordings. TRN stimulation at all temporal frequencies led to a significant decline in spike firing (two-way repeated measures ANOVA $F_{(1, 86)} = 311.8$, $p < 0.0001$; vs control firing: 2Hz: $p = 0.0126$, all other rates: $p < 0.0001$). Moreover, an increase in temporal frequency stimulation led to a progressive decline in spike firing (Kruskal-Wallis test, $p < 0.0001$; vs 2Hz: 10Hz: $p = 0.0353$, 20Hz: $p = 0.0003$, 50Hz: $p < 0.0001$; vs 5Hz: 20Hz: $p = 0.0205$, 50Hz:

$p=0.0012$), so that stimulation rates of 20 and 50Hz produced firing levels that were $\leq 12\%$ of control values.

The relationship between dLGN spike firing and the frequency of TRN stimulation was also examined for 7 neurons that were stimulated at 2, 5, 10, 20 and 50Hz (figure 27C). Again, TRN stimulation significantly decreased spike firing (Friedman test, $p<0.0001$). Within each of the 7 neurons, compared to control, only rates of 10, 20 and 50Hz significantly reduced cell activity (2Hz: $p>0.999$, 5Hz: $p=0.4819$, 10Hz: $p=0.0091$; 20Hz: $p=0.0010$; 50Hz: $p=0.0001$). Together, these data show that TRN terminal stimulation decreases the firing rate of relay neurons in a frequency-dependent manner, with rates of ≥ 10 Hz having the greatest impact on spike firing.

Finally, we tested whether the frequency dependent effects observed in vitro also occur in vivo. To accomplish this, we adopted an optogenetic approach to stimulate TRN terminals, and record unit activity in dLGN of an awake, head-fixed SST-Cre x ChR2 mouse (figure 28A). By targeting dLGN neurons with a custom-designed optrode consisting of a tungsten electrode attached to a fiber optic cable, we could stimulate TRN terminals in dLGN while simultaneously recording unit activity. We recorded from a total of 37 dLGN neurons that responded to TRN stimulation. An example of a dLGN recording in which 3 neurons were isolated, before, during, and after blue light stimulation of nearby TRN terminals is shown in figure 28B. The raster plots for the 3 isolated neurons are shown above the multi-unit recording. Repetitive blue light stimulation at 5 Hz diminished activity while stimulation at 50Hz completely suppressed spike firing.

To further confirm this frequency-dependent suppression, we (S. Masterson) examined the extent to which 3 different rates of stimulation (5Hz, 20Hz and 50Hz) affect the spontaneous activity of dLGN neurons. Figure 29A provides examples of the spontaneous activity for 3 dLGN neurons recorded before, during and after repetitive stimulation of TRN terminals at 5Hz, 20Hz and 50Hz. Indeed, an increase in temporal frequency stimulation led to a progressive decrease in unit activity. These interactions are summarized in figure 29B for 37 dLGN neurons. For each neuron, the effects of TRN stimulation are expressed as a percent change in firing compared to baseline activity. Similar to in vitro experiments, in vivo TRN stimulation reduced dLGN activity in a frequency-dependent manner (Kruskal-Wallis test, $p=0.0400$). Stimulation rates of 50Hz reduced activity by twice as much as 5Hz (median percent control, 5Hz: 29.4%, 50Hz: 12.6%; $p=0.0416$). Furthermore, spike activity was completely abolished in 10/34 (29%) neurons stimulated at either 20 or 50Hz. A within-cell comparison further illustrates these interactions (Figure 9C). For 11 neurons that were stimulated with all three frequencies, (5, 20 and 50Hz) their spontaneous activity was significantly decreased by 20 and 50Hz, but not by 5Hz (Friedman test, $p<0.0001$; vs control: 5Hz: $p=0.1908$, 20Hz: $p=0.0004$, 50Hz: $p=0.0002$). Together, the data show that dLGN cells are suppressed by TRN in a frequency-dependent manner and that activating TRN at higher temporal frequencies can reduce dLGN firing to levels that are $<20\%$ of baseline.

Figure 21. Wiring diagram depicting the pattern of connectivity between the dorsal lateral geniculate nucleus (dLGN), retina, thalamic reticular nucleus (TRN), and visual cortex (VC). TRN receives excitatory input (red) from layer VI of visual cortex and axon collaterals of dLGN relay neurons (R). Inhibitory elements and TRN feedback inhibition to dLGN are shown in blue.

Figure 22. Feedback projections from TRN to dLGN. **A-B.** Coronal sections through dorsal thalamus of the GAD65-GFP mouse illustrating GFP+ neurons (green) in TRN (**A**) and their projections to dLGN (**B**). Note the presence GFP+

neurons in ventral LGN (vLGN) but absence in dLGN. Scale bar, 100 μ m. **C.** Electron microscopic image of a TRN terminal (green overlay) in the dLGN labeled with an antibody against GFP (dark reaction product) and an antibody against GABA (high density of gold particles). The terminal synapses (arrows) on a non-GABAergic dendrite (low density of gold particles). Scale bar, 600nm. **D.** A cumulative distribution showing the diameter of different presynaptic terminals in dLGN. **E.** A cumulative distribution showing the diameter of different postsynaptic profiles. TRN terminals and postsynaptic profiles in green, all others measured from a previous study (Bickford et al., 2015; red: retinogeniculate (RLP), black: tectogeniculate (TG), blue: corticogeniculate (CG)).

Figure 23. Opsin expression and blue light activation of TRN neurons. **A.** Expression of tdTomato (red) in a coronal section through TRN, 8 days after injection of an adeno-associated virus (AAV) containing a ChIEF-tdTomato cassette. **B.** Confocal reconstruction of biocytin filled TRN neuron (white) in a tdTomato labeled section. **C.** Whole cell *in vitro* recordings of the same neuron showing the voltage responses evoked by blue light stimulation. *Left:* A single 500ms pulse of blue light produces a prolonged depolarization and train of action potentials. *Right top:* A 10Hz (1ms, 20 pulse) train of blue light generates a succession of unitary depolarizations and spike firing. *Right bottom:* Light evoked activity persists during bath application of glutamate antagonists (DNQX 20 μ M and CPP 10mM). **D.** Expression of EYFP in TRN of a somatostatin-Cre x Ai32 (ChR2-EYFP) mouse. **E.** Confocal reconstruction of biocytin filled TRN neuron in ChR2-EYFP mouse. **F.** Corresponding recordings showing that high rates of stimulation (*left:* 20Hz, *right* 50Hz) evokes reliable voltage responses and spike firing. In **C** and **F**, blue traces below the voltage responses depict the pattern of TRN photostimulation.

Figure 24. Opsin expression in TRN terminals and light evoked synaptic responses in dLGN neurons. **A.** Reconstruction of a biocytin filled relay neuron in

dLGN containing ChIEF-tdTomato labeling of TRN terminals. **B.** Whole cell voltage clamp recording of the same neuron showing that repetitive blue light stimulation evokes a train of inhibitory postsynaptic currents (IPSCs). **C.** Similar recording showing that bath application of glutamate antagonists (DNQX and CPP 20 μ M) has no effect on light evoked IPSCs. However, bath application of the GABA_A antagonist SR95531 (20 μ M) blocks light evoked IPSCs. **D.** Reconstruction of a biocytin filled GFP+ interneuron in dLGN containing ChIEF-tdTomato labeled TRN terminals from a GAD-67 mouse. **E.** Recording from neuron in **D** showing that photostimulation of TRN terminals fails to evoke any postsynaptic activity. In **B**, **C** and **E** blue traces below the synaptic responses depict the pattern TRN photostimulation. All recordings are done with cesium-based electrodes and at a holding potential of 0mV.

Figure 25. Optically evoked TRN IPSCs in dLGN relay neurons recorded *in vitro*.

A. *Left:* Examples of IPSCs of a relay neuron evoked by stimulating TRN terminals at different temporal frequencies (0.2, 1, 5, 20 and 50Hz). *Right:* Corresponding responses depicting an expanded time scale of the initial responses shown in left panel. Blue traces below synaptic responses depict the pattern of TRN photostimulation. All recordings are done with cesium-based electrodes and at a holding potential of 0mV. **B.** Summary plot showing the degree of synaptic suppression of light-evoked TRN IPSCs as a function of stimulus number for each temporal frequency tested. To calculate the percent change in IPSC amplitude, the n^{th} response was divided by the amplitude of the initial response and multiplied by 100. High rates ($\geq 5\text{Hz}$) of stimulation lead to a synaptic depression of IPSCs ($p < 0.0001$). Each point represents the mean and SEM from a total of 8 neurons.

Figure 26. Optically evoked TRN IPSPs in dLGN relay neurons recorded *in vitro*.

A. Examples of IPSPs recorded from a relay neuron evoked by stimulation of TRN terminals at different temporal frequencies (2, 5, 10, 20, and 50Hz). The bottom voltage recording shows that bath application of the GABA_A antagonist SR95531 (20 μ M) blocks light evoked IPSP activity. Blue traces below synaptic responses depict the pattern TRN photostimulation. All responses recorded at a holding potential of -55mV. **B.** Summary graph plotting the mean voltage response as a function of stimulus frequency for a total of 35 neurons. Each point represents a separate response from a different neuron. The mean voltage response was calculated by measuring the average voltage response across the entire train of stimulus presentations (25 pulses). For each frequency tested, the individual data is plotted along with the mean (horizontal line $\pm$ SEM). High rates of stimulation lead to a greater degree of hyperpolarization ($p < 0.01$ vs 2Hz or 5Hz).

Figure 27. TRN stimulation suppresses spike firing of dLGN relay neurons recorded *in vitro*. **A.** Examples of voltages responses from a dLGN relay neuron showing a train of spikes evoked by current pulse injection (black). Responses in the left column show spikes in the absence of TRN stimulation, while those on the right depict the suppression of spike firing brought about by TRN stimulation (blue) at different temporal rates (2, 5, 10, 20 and 50Hz). **B.** Summary plot showing the changes in spike firing brought about by TRN stimulation. The firing rate is plotted as a percent control and based on matching periods (0.5s, black horizontal line in **A**) of dLGN activity. For each cell ($n = 29$), the percent of control firing frequency is plotted along with the group mean (horizontal line $\pm$ SEM). **C.** Similar plot for 7 neurons in which all five frequencies were tested. Each color represents a different neuron. The between- (**B**, compared to 2Hz, $*p < 0.05$, $***p < 0.001$) and within-cell analyses (**C**, compared to control, $**p < 0.01$, $***p < 0.001$) indicate that high rates of stimulation lead to a greater suppression of spike firing.

Figure 28. Photostimulation of TRN terminals in dLGN during in vivo recordings.

A. Coronal section through dLGN of an SST-Cre xAi32 mouse used during in vivo recording, showing the expression of EYFP (green) in TRN terminals. **B.**

Extracellular unit recordings in dLGN of an awake head fixed mouse (black trace) from which single units can be isolated (3 cells, raster plot above). Unit activity is diminished during photostimulation of TRN terminals. Blue traces below unit activity depicts the pattern of blue light stimulation (left: 5Hz, right: 50Hz). Inset shows an expanded view of activity just prior to stimulation.

Figure 29. TRN stimulation suppresses LGN unit activity *in vivo*. **A.** Raster plots and corresponding unit recordings for 3 dLGN neurons showing the degree of suppression associated with stimulating TRN terminals at different rates (5, 20 and 50Hz). Blue traces below unit activity depicts the pattern of blue light stimulation. **B.** Summary plot showing the changes in spike firing brought about by TRN stimulation. The firing rate is plotted as a percent control and based on matching periods of control, pre-stimulation activity. Each point represents a neuron and the percent of control firing frequency is plotted along with the group mean (horizontal line $\pm$ SEM). **C.** Similar plot for 11 neurons in which 3 rates were tested (5, 20 and 50Hz). Each color represents a different neuron. The between- (**B**, compared to 5Hz, $*p<0.05$), and within-cell analyses (**C**, compared to control, $***p<0.001$) indicate that high rates of stimulation lead to a greater suppression of spike firing.

Discussion

Our results indicate that the photostimulation of TRN terminals in dLGN led to a frequency dependent inhibition of dLGN activity, with higher rates of stimulation resulting in increasing levels of inhibition and suppression of spike firing. Moreover, TRN – mediated inhibition of dLGN was restricted to relay neurons. We found no evidence, at the ultrastructural or functional level, of TRN input onto intrinsic interneurons.

The absence of TRN input onto dLGN interneurons may be a unique feature of rodent TRN feedback connections, but additional studies in higher mammals (e.g., cat and galago) lend support to the view that overall, thalamocortical relay neurons of first order, sensory nuclei are the primary recipient of TRN input (Liu et al., 1995; Uhlich et al., 2003; Wang et al., 2001a). By contrast, connections between TRN and interneurons seem more prevalent among non-sensory (e.g., anterior and motor) thalamic nuclei (Ilinsky et al., 1999; Kultas-Ilinsky et al., 1995; Tai et al., 1995). Such differences suggest that the TRN is organized into subnetworks that are defined by their projection patterns onto specific cell types belonging to different thalamic nuclei (Halassa and Acsády, 2016).

As expected, TRN stimulation evoked large inhibitory currents in dLGN relay neurons (Cox et al., 1997b; Crandall et al., 2015; Herd et al., 2013), a property that is consistent with their ultrastructural synaptic profile. For example, we found TRN terminals to be relatively large, making contacts primarily on proximal dendritic regions of relay neurons. Moreover, repetitive stimulation,

especially at high rates led to a form of synaptic depression with the initial response being the largest and subsequent ones showing a ~50% decline in amplitude by the 5th pulse in the train. Such depression is similar to that reported between TRN and thalamocortical neurons of the ventrobasal complex (Ahrens et al., 2014; Bessaïh et al., 2006; Cox et al., 1997a, 1997b; Crandall et al., 2015; Evrard and Ropert, 2009; Herd et al., 2013; Ulrich and Huguenard, 1996). Together, these observations suggest that TRN projections, at least those to first order thalamic nuclei, show a limited degree of convergence (Pinault, 2004; Pinault and Deschênes, 1998; Pinault et al., 1995) and a high probability of transmitter release (Zucker and Regehr, 2002).

It is important to note that even though high rates of repetitive stimulation led to synaptic depression, it also produced a greater degree of hyperpolarization. For example, 20-50Hz rates of stimulation led to a 4-8 fold increase in membrane hyperpolarization when compared to 2-5Hz. While these observations are consistent with the reported decay tau of TRN responses in thalamus (~55ms), other factors, such as the activity dependent recruitment of extrasynaptic GABA receptors could further potentiate postsynaptic inhibition during high rates of stimulation (Herd et al., 2013).

These frequency dependent effects on membrane hyperpolarization also had a corresponding impact on dLGN spike firing. For neurons recorded in vitro, increasing stimulus frequency between 2-50Hz resulted in a progressive decrease in spike firing that ranged from 10-70% of control values. Although more variable, a similar trend was observed during in vivo recordings from awake

head fixed mice, suggesting that even modest changes in TRN activity can have a dynamic impact on retinogeniculate signal transmission. It is important to note that not all neurons showed such graded effects, for many (9/29 in vitro; 10/34 in vivo) higher rates of stimulation ($\geq 20\text{Hz}$) often led to a complete suppression of activity. Thus while, TRN stimulation at rates of $\geq 20\text{Hz}$ can sometimes lead to a total suppression of dLGN activity, lower rates of TRN stimulation simply diminish relay neuron firing. This inflection point is intriguing and seems to correspond to the state-dependent changes in TRN activity that occur during sleep-wake transitions (Steriade et al., 1986) or from switching attentional states from one sensory modality to another (Chen et al., 2016b; Wimmer et al., 2015). Overall, these data support the view that TRN inhibition allows for a more graded control of thalamic output, one that operates on a variable temporal scale to modulate thalamocortical transmission in a state-dependent manner (Halassa and Acsády, 2016; Halassa and Kastner, 2017). Thus, while the TRN is often viewed as a searchlight that turns thalamic activity on and off in a spatially discrete manner (Crick, 1984; Soto-Sánchez et al., 2017), it appears to function more like a “dimmer switch” when gating thalamocortical transmission.

SUMMARY AND CONCLUSIONS

In this dissertation we examined the formation, maturation and synaptic nature of the intrathalamic feedback loop that connects TRN and dLGN. In chapter I, we utilized neural tracers to demonstrate that the sectorial arrangement of TRN is assembled at perinatal ages. We used this map to determine that feedforward innervation of visual TRN begins during the second postnatal week and is established by the third postnatal week. Feedback projections from TRN to dLGN form earlier, during the first postnatal week, but take longer to mature as functional responses develop through the first four postnatal weeks. Finally, we determined that this time course of innervation is largely unaffected by the absence of retinal signaling. Specifically, Math 5^{-/-} mice (which are devoid of retinal input to the brain) were different from WT in only two measures: TRN terminals arrived at earlier ages and exhibited more synaptic suppression. In chapter II, we demonstrated that TRN formed large synaptic terminals exclusively on dLGN relay neurons. We also optogenetically activated these terminals with trains of repetitive stimuli at a wide range of temporal frequencies, both in vitro and in vivo. We found that although higher rates of stimulation suppressed synaptic activity, stimulation at higher temporal frequencies also hyperpolarized relay neurons more than lower frequencies. Moreover, we observed that stimulation rates $\geq 20\text{Hz}$ can completely suppress

retinogeniculate transmission while lower rates only diminish relay neuronal activity.

Although the sectorial arrangement of TRN had been established in rats (Coleman and Mitrofanis, 1996; Guillery et al., 1998; Kimura, 2017; Ohara and Lieberman, 1985; Pinault, 2004), it was unclear whether this organizational structure existed in mice. To confirm this, we performed Cre-dependent anterograde viral tracing using the CRH-Cre mouse (Monavarfeshani et al., 2018; Sokhadze et al., 2018b). We labeled TC neurons from visual, somatosensory and auditory primary thalamic relay nuclei and found labeled axons in caudal TRN in each case. TC axons were segregated into distinct sectors such that projections from dLGN occupied regions dorsal to the apex of TRN, primarily in the head of the nucleus. Fibers carrying somatosensory or auditory signals were restricted to regions at or below the apex, with auditory fibers covering TRN regions more caudal compared to somatosensory fibers.

Having established the location of visual TRN in mouse, we used retrograde labeling of TC axons in perinatal mice to determine whether this arrangement is present at early ages. We found that TC axons from visual and somatosensory thalamus are segregated as early as P2. Moreover, these axons are found in the same regions as the corresponding modality-specific sectors we identified in adults. While previous studies in rodents had established that TC and CT axons traverse TRN at embryonic ages (Grant et al., 2012; López-Bendito and Molnár, 2003; Molnár and Blakemore, 1995), it was unclear whether sensory sectors were established at these early ages. Our results demonstrate

that the modality-specific sectors of sensory TRN are established at perinatal ages.

Although TC fibers pass through TRN at embryonic ages (López-Bendito, 2018; Molnár et al., 2012; Nakagawa, 2019; Schiff et al., 2011), it was unclear when axon collaterals from these projections innervated TRN neurons, or when TRN feedback inhibition formed in thalamus. Using transgenic mice that specifically labeled either feedforward or feedback projections, as well as immunohistochemistry, we established the developmental time course for both projections within the visual system. We found that TRN projections to dLGN were established first, during the first postnatal week, but that functional responses continued to mature until the fourth postnatal week. Feedforward projections formed later, during the second postnatal week, but stabilized during the third postnatal week. Previous work has suggested an embryonic, or perinatal, time course for the development of this intrathalamic feedback loop but these studies relied on methods that were unable to target TRN or dLGN with the specificity of the current study (Mitrofanis and Baker, 1993; Molnár et al., 1998; Warren and Jones, 1997). Additionally, some of these studies focused on the somatosensory system, which tends to develop earlier in rodents (Feldheim et al., 2010; Grant et al., 2012; López-Bendito, 2018; Molnár et al., 2012; Schlaggar and O'Leary, 1994). Thus, our results establish that the intrathalamic feedback loop within the visual system forms at postnatal ages and is functional just before eye opening. This developmental time course could provide a mechanism for the emergence of dLGN state-dependent activity since TRN inhibition is thought to

mediate shifts in thalamic firing modes based on behavioral states (Colonnese and Phillips, 2018; Fiebelkorn and Kastner, 2019; Fogerson and Huguenard, 2016; Halassa and Kastner, 2017; Llinás and Steriade, 2006; McCormick and Bal, 1997; Murata and Colonnese, 2018a). Moreover, such state-dependent activity in dLGN is thought to be necessary to mediate the developmental switch in cortical activity observed just before eye opening by Colonnese and colleagues (Murata and Colonnese, 2018b). Thus, the formation of intrathalamic circuits may have important consequences in the maturation of thalamocortical signaling that have yet to be explored.

We also examined the formation and maturation of intrathalamic circuits in the absence of retinal signaling using a mouse devoid of retinal inputs, the Math 5^{-/-} mouse (Brown et al., 1998, 2001; El-Danaf et al., 2015; Wang et al., 2001b). Previous work demonstrated that in the absence of retinal signaling, dLGN relay neurons exhibited dystrophic growth but that visual TC axons remained segregated from somatosensory projections within thalamus (Dye et al., 2012; El-Danaf et al., 2015; Kozanian et al., 2015). We repeated retrograde tracing studies (described above) in Math 5^{-/-} mice and found that the sectorial arrangement of TRN is not disrupted by the absence of retinal signaling. Moreover, examination of feedforward projections established that thalamic input to visual TRN is unchanged in Math 5^{-/-} mice. Thus, the anatomical arrangement of TRN, as well as the formation and maturation of feedforward inputs do not depend on peripheral signaling.

However, in dLGN we found that TRN innervation was accelerated in the absence of retinal signaling. While all non-retinal projections to dLGN studied to date are disrupted by the absence of retinal inputs, our results most closely resemble those of CT innervation (Brooks et al., 2013; Seabrook et al., 2013a). Similar to our findings for TRN inputs, in Math 5^{-/-} mice CT projections arrive at earlier ages but short term synaptic plasticity (paired pulse facilitation) is unaffected $\leq$ P14. This acceleration of CT innervation is mediated by the early degradation of aggrecan, a repulsive chondroitin sulfate proteoglycan (Brooks et al., 2013). CT projections wait in TRN at embryonic ages and appear to follow TRN projections to reach their targets (Grant et al., 2012; Jacobs et al., 2007; Montiel et al., 2011). Thus it is likely that there is a shared mechanism regulating the timing of innervation of both inputs, which may be aggrecan or a similar molecule.

We also examined the nature of TRN suppression of relay neuron activity. In chapter I, we determined that activation of TRN terminals did not alter relay neuron activity before the third postnatal week, and did not suppress firing until at least the fourth postnatal week. This time course was similar in both WT and Math 5^{-/-} animals.

In chapter II, we established that the rate of TRN activity suppresses relay neuron spiking in a frequency-dependent manner. Although TRN synapses are suppressed by repeated stimulation, higher rates of stimulation hyperpolarized relay neurons and suppressed firing more than lower rates. Both in vitro and in vivo, rates $\geq$ 10Hz had the greatest impact on suppressing relay neuron firing.

Moreover, TRN stimulation rates $\geq 20\text{Hz}$ often completely suppressed dLGN spikes while lower rates attenuated activity. The different effects observed with higher and lower stimulation rates correspond to the rates of TRN activity reported during different behavioral states (Chen et al., 2016b; Steriade et al., 1986; Wimmer et al., 2015). This graded control of sensory transmission expands our understanding of how TRN modulates thalamocortical transmission.

In chapter II, we also determined that TRN terminals form synapses with dLGN relay neurons but not intrinsic interneurons. Studies in the sensory thalamus of other mammals had established that TRN primarily forms synapses with thalamocortical neurons but often observed a small number of synapses onto interneurons (Gentet and Ulrich, 2003; Liu et al., 1995; Wang et al., 2001a). However, we found no evidence of any TRN synapses onto interneurons – either physiologically or at the ultrastructural level. In anterior and motor thalamus of higher mammals, these synapses are more prevalent, which supports the hypothesis that TRN projections are organized into subnetworks defined, in part, by their projection patterns within different thalamic nuclei (Crabtree, 2018; Halassa and Acsády, 2016).

The existence of multiple subnetworks within TRN has been the subject of recent reviews (Crabtree, 2018; Halassa and Acsády, 2016). Subnetworks have been identified based on activity during different behavioral states (Chen et al., 2016b; Halassa et al., 2014), protein expression (e.g. SST, parvalbumin or calbindin; Clemente-Perez et al., 2017), organization of gap-junction coupling (Connors, 2017; Haas et al., 2011; Lee et al., 2014), as well as thalamic

projections (Crabtree, 2018; Pinault, 2004). Additionally, rodent studies of genetic models of either autism spectrum disorder or absence epilepsy have identified subsets of TRN neurons that contribute to these disease states (McCafferty et al., 2018; Nakajima et al., 2019; Wells et al., 2016). However, the existence and organization of such subnetworks within the visual TRN remains largely unexplored.

Overall, our studies show the organizational pattern, developmental time course and synaptic nature of the intrathalamic feedback loop connecting dLGN and TRN. This work establishes a foundation that can be used to examine the role of TRN inhibition in neurodevelopmental disorders. A particularly appealing focus is the development of TRN inhibition in a rodent models of absence epilepsy as genetic rat models only exhibit seizures after postnatal day 30 (Crunelli and Leresche, 2002; Crunelli et al., 2018; Luijtelaa et al., 2014), which, based on our data, suggests that seizures arise just after the maturation of TRN-dLGN circuitry. Another avenue for future research is to contrast cortical and TRN innervation of dLGN. Both inputs are regulated by retinal inputs and follow a similar trajectory to innervate dLGN but CT terminals innervate both relay neurons and intrinsic interneurons whereas TRN terminals form synapses only with relay neurons. Finally, neurons within the visual system exhibit specific visual response properties but to date, no study has employed these characteristics to interrogate visual TRN and its inhibition of dLGN. Thus, it is unclear whether TRN inhibits relay neurons with identical response properties, antithetical responses or whether such organization exists at all. Furthermore, if

such subnetworks existed, it is unknown how they modulate retinogeniculate transmission in a state-dependent manner.

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APPENDIX

LIST OF ABBREVIATIONS

A1: Primary auditory cortex	AAV: Adeno-associated virus
ACh: acetylcholine	ACSF: Artificial cerebrospinal fluid
AF: Alexa Fluor	ANOVA: Analysis of variance
AP: Antero-posterior	ChR2: channelrhodopsin-2
CRH: Corticotropin releasing hormone	CT: Corticothalamic
CTB: Cholera toxin subunit B	dLGN: Dorsal lateral geniculate nucleus
DV: Dorso-ventral	eYFP: enhanced yellow fluorescent protein
IPSC: Inhibitory postsynaptic current	LED: Light-emitting diode
ML: Medio-lateral	NGS: Normal goat serum
P: Postnatal day	PBS: Phosphate-buffered saline
PCR: polymerase chain reaction	PFA: Paraformaldehyde
Po: Posterior nucleus	RGC: Retinal ganglion cell
RMS: Root mean squared	S1: Primary somatosensory cortex
SA: Streptavidin	SC: Superior colliculus
TC: thalamocortical	tdT: tdTomato
TRN: Thalamic reticular nucleus	V1: Primary visual cortex
VB: Ventrobasal complex	vLGN: Ventral lateral geniculate nucleus
vMGN: ventral medial geniculate nucleus	VPM: ventral posteromedial nucleus
VPL: Ventral posterolateral nucleus	WT: Wild type

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PUBLICATIONS

- Campbell, P.W., Govindaiah, G., Masterson, S., Bickford, M., & Guido, W. (2020). Synaptic properties of the feedback connections between the thalamic reticular nucleus and the dorsal lateral geniculate nucleus. *Journal of Neurophysiology*, in revision.
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PROFESSIONAL SOCIETIES

2005- present	Member, Society for Neuroscience (SfN)
2013- present	Member, American Academy of Neurology
2013- present	Member, American Physician Scientist Association

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2017- present	Member, Child Neurology Society
2017- 2019	Member, American Physician Scientist Association Policy Committee
2017	Association for Research in Vision and Ophthalmology
2017	American Physician Scientist Association Southeast Meeting Planning Committee

SELECTED ABSTRACTS

Gordon, T., Whitley, J., Campbell P.W., Whyland, K., Masterson, S., Zhou, N., & Bickford, M. (2018). GABAergic synaptic connections within the visual sector of the mouse thalamic reticular nucleus: Extrinsic projections from the pretectum. Society for Neuroscience annual meeting, poster number 059.16.

Fligor, C., Campbell, P.W., Langer, K., Zhang, C., Suter, D., Guido, W., & Meyer, J. (2018). Human pluripotent stem cell-derived retinal ganglion cells display extensive neurite outgrowth in response to intrinsic and extrinsic signals. Society for Neuroscience annual meeting, poster number 196.15.

Campbell, P.W. & Guido, W. (2018). Formation and Maturation of Intrathalamic Connections Between TRN and dLGN. Gordon Research Conference, Barga, IT.

Charlambakis, N., Campbell, P.W., Govindaiah, G. & Guido, W. (2018). The absence of retinal input affects the targeting, dendritic architecture, and synaptic connectivity of intrinsic interneurons in mouse dLGN. Gordon Research Conference: Thalamocortical Interactions. Barga IT

Campbell, P.W. & Guido, W. (2017). Formation and Maturation of Feedback Inhibition to the Visual Thalamus. 32nd Annual MD/PhD National Student Conference.

Campbell, P.W. & Guido, W. (2017). Development of Intrathalamic Connections Between dLGN and TRN. Association for Research in Vision and Ophthalmology annual meeting, poster number A0184.

Campbell, P.W. & Guido, W. (2016). Development of feed-forward and feedback connections between dLGN and TRN. Society for Neuroscience annual meeting, poster number 529.21.

Charlambakis, N., Govindaiah, G., Campbell, P.W., & Guido, W. (2016). The absence of retinal input affects the targeting and morphological development of interneurons of dLGN. Society for Neuroscience annual meeting, poster number 529.23.

Campbell, P.W., Seabrook, T.A., Govindaiah, G., & Guido, W. (2015). Early maturation of TRN GABAergic feedback inhibition to dLGN. Society for Neuroscience annual meeting, poster number 148.03.

INVITED PRESENTATIONS

- 2019 Inhibition of Visual Thalamus by the Reticular Nucleus. Society for Neuroscience Louisville Chapter Meeting, Louisville, KY
- 2018 Formation and Maturation of Intrathalamic Connections Between TRN and dLGN. Gordon Research Seminar, Barga, IT.
- 2017 Formation of Intrathalamic Connections Between Visual Thalamus and TRN. Southeastern Medical Science Symposium, Atlanta, GA.
- 2017 Development of Feedback Inhibition to Dorsal Lateral Geniculate Nucleus. Association for Research in Vision and Ophthalmology annual meeting, abstract number 3410
- 2016 Formation and Maturation of TRN Innervation of the Dorsal Lateral Geniculate Nucleus. Society for Neuroscience Louisville Chapter Meeting, Louisville, KY.
- 2005 Upregulation of nAChRs in a model of nicotine addiction. PEW Undergraduate Research Symposium in the Biological Sciences and Psychology, Washington University, St. Louis, MO.